Universitat Autònoma de Barcelona

GENETIC DETERMINISM OF MEAT RABBIT CECAL MICROBIOTA AND ITS ROLE IN THE HOST'S FEED EFFICIENCY

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Doctoral thesis to obtain the PhD degree in Animal Production of the Universitat Autònoma de Barcelona, December 2021.

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Dr. Juan Pablo Sánchez Serrano, researcher at the Animal Breeding and Genetics Program of Institut de Recerca i Tecnologia Agroalimentàries, states:

that the research work and drafting of doctoral thesis entitled

“Genetic determinism of meat rabbit cecal microbiota and its role in the host’s feed efficiency”

have been performed, under his supervision, by

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and certifies:

that the present study has been conducted at the Animal Breeding and Genetics Program of Institut de Recerca i Tecnologia Agroalimentàries, and the resulting doctoral thesis fulfils the requirements for opting for the PhD degree in Animal Production of the Universitat Autònoma de Barcelona.

And to place that on the records, the present document is signed in December 2021.

Dr. Juan Pablo Sánchez Serrano María Velasco Galilea

The cover of this thesis was designed by Nerea Rogido, @seikacela.

The present PhD thesis was framed within the Spanish projects RTA2011–00064–00-00 and RTI2018-097610R-I00 funded by the *Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria* (INIA), and the Feed-a-Gene project that received funding from the European Union's H2020 program under grant agreement no. 633531. The PhD candidate, María Velasco Galilea, was a recipient of a *Formación de Personal Investigador* (FPI) pre-doctoral fellowship from INIA, associated with the research project RTA2014–00015-C2–01.

C´est le temps que tu as perdu pour ta rose

qui fait ta rose si importante.

Le Petit Prince

- Antoine de Saint-Exupéry -

All disease begins in the gut.

- Hippocrates -

El verdadero tesoro del hombre

es el tesoro de sus errores.

- José Ortega y Gasset -

A mis padres, por hacerlo posible.

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Summary

One of the biggest challenges of this century is to develop strategies that satisfy the increasing demand for animal protein resulting from the rapidly growing world population. Such strategies must focus on producing more protein-rich food while using fewer inputs and minimizing environmental impact. Feed efficiency is a crucial phenotype to address this challenge since food expenses represent the most significant proportion of the total costs in the meat rabbit industry.

The articles included in the present thesis have generated knowledge about the meat rabbit intestinal microbiota: a new phenotype related to individual variation in feed efficiency. The study of the influence of different environmental factors and the genetic determinism of this phenotype has allowed investigating its role in the host's feed efficiency, unraveling its underlying biological processes, and exploring the possibility of predicting and improving such a complex phenotype.

In the first study, an assessment of the microbial populations inhabiting the rabbit cecum and hard feces of 21 young specimens subjected to two different feeding regimes was performed through sequencing of 16S rRNA gene amplicons. The results of such analysis revealed a predominant presence of bacterial phyla *Firmicutes* (76%), *Tenericutes* (8%), and *Bacteroidetes* (7%). Although no variations in terms of microbial richness and diversity were found between both sampling origins, compositional differences for the relative abundance of a large number of taxa were revealed.

The second study evaluated the influence of the environment offered by two different facilities, the administration of antibiotics and the level of feeding on the cecal microbial diversity and composition of 425 kits. Although results determined that the farm environment exerted the largest impact, the other analyzed factors shaped the relative abundances of certain microorganisms. This study highlights the importance of offering a controlled breeding environment that minimizes cecal microbiota alterations that could potentially affect animal performance.

Summary

In the third study, original approaches based on the traditional animal mixed model and alternative definitions and expansions of the microbial relationship matrix were proposed. Such approaches enabled us to determine that an important proportion of the phenotypic variance of traits related to feed efficiency is attributable to cecal microbiota. Moreover, the inclusion of microbial information significantly increased the predictive capacity of the models, even for cage-average phenotypes on which cecal microbiota had not been characterized in all the individuals involved in the record.

In the fourth study, the use of the Bayes factor as a measure of statistical relevance demonstrated that rabbit cecal microbiota is under host genetic control. The evidence of such control was decisive for genera *Bacteroides*, *Parabacteroides*, *Dehalobacterium*, and *Butyricimonas* for which heritability estimates were 0.27-0.35.

In the last study, a simulation highlighted the limited statistical power of the available data (412 kits) to detect genomic regions responsible for the variation of rabbit cecal microbiota through genome-wide association studies. Despite these limitations, more than 300 variants spread across 10 chromosomes were cautiously declared to be associated with the variation of 19 microbial traits. Given their implication in metabolic and immunological processes, 44 genes within these regions were proposed as candidates for the modulation of cecal microbiota.

These findings demonstrate the contribution of the holobiont system (host-gut microbiota-environment) to rabbit feed efficiency and lay the foundations for a new line of research of scientific and practical interest for the meat rabbit industry.

Resumen

Uno de los mayores desafíos del presente siglo es el desarrollo de estrategias que permitan satisfacer la creciente demanda de proteína animal derivada del acelerado crecimiento de la población mundial. Dichas estrategias deben enfocarse en la producción de mayor cantidad de proteína minimizando los insumos y el impacto ambiental. La eficiencia alimentaria es un fenotipo fundamental para abordar este desafío dado que los gastos de alimentación suponen el porcentaje más importante de los costos totales de la producción cunícola.

Los trabajos incluidos en la presente tesis han generado conocimiento sobre la microbiota intestinal del conejo de carne: un nuevo fenotipo relacionado con la variación individual de la eficiencia alimentaria. El estudio de la influencia de diferentes factores ambientales y de la base genética de este fenotipo ha permitido, además de explorar su papel en la eficiencia alimentaria del hospedador, ahondar en la comprensión de sus mecanismos biológicos subyacentes y explorar la posibilidad de predecir y/o mejorar este fenotipo tan complejo.

En el primer trabajo, mediante la amplificación y secuenciación del gen 16S rRNA, se caracterizaron las comunidades microbianas presentes en el ciego y las heces de 21 gazapos sometidos a dos regímenes alimentarios diferentes. Dicho análisis reveló la predominancia de los filos bacterianos *Firmicutes* (76%), *Tenericutes* (8%) y *Bacteroidetes* (7%). Aunque no se hallaron variaciones en términos de riqueza ni diversidad microbianas entre ambos orígenes muestrales, sí se encontraron diferencias composicionales en un elevado número de taxones microbianos.

En el segundo trabajo se evaluó la influencia del ambiente ofrecido por dos granjas distintas, la administración de antibióticos y el nivel de ingesta sobre la composición y diversidad de las comunidades microbianas cecales de 425 gazapos. Aunque los resultados determinaron que el mayor impacto es ejercido por el entorno ambiental, se evidenciaron diferencias composicionales en la abundancia de ciertos microorganismos debidas al efecto de los otros factores estudiados. Este estudio destaca la importancia de ofrecer un ambiente controlado que minimice alteraciones en la microbiota cecal que potencialmente pudieran afectar el rendimiento productivo.

Resumen

En el tercer trabajo se plantearon nuevos enfoques basados en el modelo animal y definiciones originales de la matriz de parecido basada en relaciones microbianas que permitieron determinar que una proporción importante de la varianza fenotípica de distintos caracteres relacionados con la eficiencia alimentaria es atribuible a la microbiota cecal. Además, considerar la información microbiana supuso una mejora significativa de la capacidad predictiva de los modelos, incluso para fenotipos colectivos en los que la microbiota cecal no había sido caracterizada en todos los individuos implicados en el registro.

En el cuarto trabajo se evidenció, usando el factor de Bayes como una medida de relevancia estadística, que la microbiota cecal del conejo está bajo control genético del hospedador. La evidencia de dicho control fue decisiva para los géneros *Bacteroides*, *Parabacteroides*, *Dehalobacterium* y *Butyricimonas* para los que se estimaron heredabilidades de 0,27-0,35.

En el último trabajo se recurrió a un estudio de simulación que evidenció el limitado poder estadístico de los datos disponibles (412 gazapos) para la detección de regiones genómicas implicadas en la variación de la microbiota cecal del conejo usando estudios de asociación de genoma completo. Pese a estas limitaciones, cautamente se declararon más de 300 variantes distribuidas a lo largo de 10 cromosomas como asociadas con la variación de 19 caracteres microbianos. Se propusieron, dada su implicación en procesos metabólicos e inmunológicos, 44 genes dentro de estas regiones como candidatos en la modulación de la microbiota cecal.

Estos hallazgos demuestran la contribución del sistema hospedador-microbiota-ambiente sobre la eficiencia alimentaria y sientan las bases de una novedosa línea de investigación de interés científico y práctico para la industria cunícola.

Resum

Un dels majors reptes del segle present és el desenvolupament d'estratègies que permetin satisfer la creixent demanda de proteïna animal derivada de l'accelerat creixement de la població mundial. Aquestes estratègies han d'enfocar-se en la producció de major quantitat de proteïna minimitzant els inputs i l'impacte ambiental. L'eficiència alimentària és un fenotip fonamental per abordar aquest repte donat que les despeses de l'alimentació suposen el percentatge més important dels costos totals de la producció cunícola.

Els treballs inclosos en la present tesi han generat coneixement sobre la microbiota intestinal del conill de carn: un nou fenotip relacionat amb la variació individual de l'eficiència alimentària. L'estudi de la influència de diferents factors ambientals i de la base genètica d'aquest fenotip ha permès, a més d'explorar el paper de l'eficiència alimentària de l'hoste, aprofundir en la compressió dels seus mecanismes biològics subjacents i explorar la possibilitat de predir i/o millorar aquest fenotip tan complex.

En el primer treball, mitjançant l'amplificació i seqüenciació del gen 16S rRNA, es van caracteritzar les comunitats microbianes presents en el cec i les femtes de 21 llorigons sotmesos a dos règims alimentaris diferents. Aquesta anàlisi va revelar la predominança dels fílums bacterians *Firmicutes* (76%), *Tenericutes* (8%) i *Bacteroidetes* (7%). Malgrat no es van trobar variacions en termes de riquesa ni diversitat microbiana entre ambdós orígens mostrals, si es van trobar diferències composicionals en un elevat nombre de taxons microbians.

En el segon treball es va avaluar la influència de l'ambient ofert per dues granges diferents, l'administració d'antibiòtics i el nivell d'ingesta sobre la composició i diversitat de les comunitats microbianes cecals de 425 llorigons. Tot i que els resultats van determinar que el major impacte és exercit per l'entorn ambiental, es van evidenciar diferències composicionals en l'abundància de certs microorganismes degut a l'efecte dels altres factors estudiats. Aquest estudi destaca la importància d'oferir un ambient controlat que minimitzi alteracions en la microbiota cecal que potencialment poguessin afectar el rendiment productiu.

Resum

En el tercer treball es van plantejar nous enfocaments basats en el model animal i definicions originals de la matriu de semblança basada en relacions microbianes que van permetre determinar que una proporció important de la variància fenotípica de diferents caràcters relacionats amb l'eficiència alimentària és atribuïble a la microbiota cecal. A més a més, considerar la informació microbiana va suposar una millora significativa de la capacitat predictiva dels models, inclús per a fenotips col·lectius en els que la microbiota cecal no havia estat caracteritzada en tots els individus implicats en el registre.

En el quart treball es va evidenciar, utilitzant el factor de Bayes com a mesura de rellevància estadística, que la microbiota cecal del conill està sota control genètic de l'hoste. L'evidència d'aquest control fou decisiva pels gèneres *Bacteroides*, *Parabacteroides*, *Dehalobacterium* i *Butyricimonas* per als que es van estimar heretabilitats de 0,27-0,35.

En l'últim treball es va recórrer a un estudi de simulació que va evidenciar el limitat poder estadístic de les dades disponibles (412 llorigons) per a la detecció de regions genòmiques implicades en la variació de la microbiota cecal del conill emprant estudis d'associació de genoma complet. Malgrat aquestes limitacions, cautament es van declarar més de 300 variants distribuïdes al llarg de 10 cromosomes com associades a la variació de 19 caràcters microbians. Es van proposar, donada la seva implicació en processos metabòlics i immunològics, 44 gens dins d'aquestes regions com a candidats en la modulació de la microbiota cecal.

Aquestes troballes demostren la contribució del sistema hoste-microbiota-ambient sobre l'eficiència alimentària i estableixen les bases d'una nova línia d'investigació d'interès científic i pràctic per a la indústria cunícola.

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List of publications

The present thesis is based on the following articles:

M. Velasco-Galilea, M. Piles, M. Viñas, O. Rafel, O. González-Rodríguez, M. Guivernau & J.P. Sánchez
Rabbit microbiota changes throughout the intestinal tract.
Frontiers in Microbiology, 2018, *9*, p. 2144.
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M. Velasco-Galilea, M. Guivernau, M. Piles, M. Viñas, O. Rafel, A. Sánchez, Y. Ramayo-Caldas, O. González-Rodríguez & J.P. Sánchez
Breeding farm, level of feeding and presence of antibiotics in the feed influence rabbit cecal microbiota.
Animal Microbiome, 2020, *2*(1), pp. 1-16.
https://doi.org/10.1186/s42523-020-00059-z

M. Velasco-Galilea, M. Piles, Y. Ramayo-Caldas & J.P. Sánchez
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M. Velasco-Galilea, M. Piles, Y. Ramayo-Caldas, L. Varona & J.P. Sánchez
Bayes factor for elucidating the influence of host genetics, litter, and cage effects on rabbit cecal microbiota through linear and zero-inflated Poisson mixed models.
Genetics Selection Evolution, (under review)

M. Velasco-Galilea, B. Buitenhuis, Y. Ramayo-Caldas, M. Ballester, M. Piles & J.P. Sánchez
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Networks of inbreeding coefficients in a selected population of rabbits.
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https://doi.org/10.1111/jbg.12500

J.P. Sánchez, A. Legarra, **M. Velasco-Galilea**, M. Piles, A. Sánchez, O. Rafel, O. González-Rodríguez & M. Ballester
Genome-wide association study for feed efficiency in collective cage-raised rabbits under full and restricted feeding.
Animal Genetics, 2020, *51*(5), pp. 799-810.
https://doi.org/10.1111/age.12988

Contributions to congresses and conferences

M. Velasco-Galilea, M. Piles, M. Viñas, O. Rafel, Y. Ramayo-Caldas, O. González-Rodríguez, M. Guivernau & J.P. Sánchez
Prediction of growth and feed efficiency performances in growing rabbits from their gut microbiota
12th World Rabbit Congress. 2021. 3 November to 5 November. Nantes (France)
Oral communication

M. Piles, L. Tusell, **M. Velasco-Galilea**, M. Ballester & J.P. Sánchez
A comparative study of machine learning methods to predict average daily gain from single nucleotide polymorphisms
12th World Rabbit Congress. 2021. 3 November to 5 November. Nantes (France)
Oral communication

M. Piles, L. Tusell, **M. Velasco-Galilea**, V. Helies, L. Drouilhet, O. Zemb, J.P. Sánchez & H. Garreau
Machine learning algorithms for the prediction of feed efficiency based on cecal microbiota
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Oral communication

M. Velasco-Galilea, M. Piles, Y. Ramayo-Caldas, L. Varona & J.P. Sánchez
Bayes factor for elucidating the genetic determinism of rabbit cecal microbiota through mixed normal and zero-inflated Poisson models
12th International Symposium on Gut Microbiology. 2021. 13 October to 15 October. Virtual
Poster
FEMS Young Scientists grant for a full registration

M. Velasco-Galilea, M. Piles, M. Viñas, O. Rafel, Y. Ramayo-Caldas, O. González-Rodríguez, M. Guivernau & J.P. Sánchez
Does rabbits' cecal microbial information improve prediction of individual growth, and cage feed intake and feed conversion ratio?
6th International Conference of Quantitative Genetics (ICQG6). 2020. 2 November to 12 November.
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US-Based Early Career Researcher Scholarship for a full registration.

M. Velasco-Galilea, M. Piles, M. Viñas, O. Rafel, Y. Ramayo-Caldas, O. González-Rodríguez, M. Guivernau & J.P. Sánchez
Genetic determinism of rabbits' cecal microbiota
6th International Conference of Quantitative Genetics (ICQG6). 2020. 2 November to 12 November.
Poster
US-Based Early Career Researcher Scholarship for a full registration.

Contributions to congresses and conferences

S.T. Rodríguez-Ramilo, A. Reverter, J.P. Sánchez, **M. Velasco-Galilea**, O. González & M. Pile**s**
Assessing inbreeding networks from partial correlations and information theory in rabbits.
37th International Society for Animal Genetics (ISAG) Conference. 2019. 7 July - 12 July. Lleida (Spain)
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J.P. Sánchez, A. Legarra, **M. Velasco-Galilea**., M. Piles, O. Rafel, O. González-Rodríguez & M. Ballester
Genomic regions associated with individual growth and cage feed efficiency in rabbits under two feeding regimes.
37th International Society for Animal Genetics (ISAG) Conference. 2019. 7 July - 12 July. Lleida (Spain)
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J.P. Sánchez, A. Legarra, **M. Velasco-Galilea**., M. Piles, O. Rafel, O. González-Rodríguez & M. Ballester
Genome wide association study of individual growth and cage feed efficiency in rabbits under two feeding regimes.
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Oral communication

S.T. Rodríguez-Ramilo, A. Reverter, J.P. Sánchez, **M. Velasco-Galilea**, O. González & M. Pile**s**
Application of partial correlations and information theory to estimates of inbreeding networks in rabbits.
XVIII Jornadas sobre Producción Animal, ITEA. 2019. 7 May to 8 May. Zaragoza (Spain)
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Y. Ramayo-Caldas, **M. Velasco-Galilea**, M. Piles, O. Rafel, O. González-Rodríguez, M. Guivernau & J.P. Sánchez
Enterotypes, network and functional profiling of rabbit cecal microbiota.
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Poster

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M. Piles, C. Fernández-Lozano, **M. Velasco-Galilea**, O. González, J.P. Sánchez, R. Quintanilla & M. Ballester
Machine learning transcriptome analysis to identify genes associated with feed efficiency in pig.
69th Annual Meeting - European Association for Animal Production (EAAP). 2018. 27 August to 31 August. Dubrovnik (Croatia)
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M. Velasco-Galilea, M. Piles, O. Rafel, O. González-Rodríguez, M. Guivernau & J.P. Sánchez
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Oral communication

M. Velasco-Galilea, M. Piles, O. Rafel, O. González-Rodríguez, M. Guivernau & J.P. Sánchez
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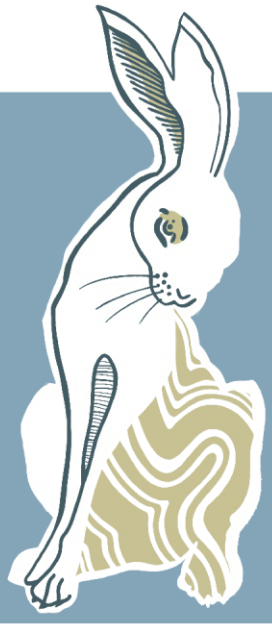
Poster

CHAPTER 1

GENERAL INTRODUCTION



1.1. Evolution of rabbit meat production and consumption

Cuniculture can be defined as the agricultural practice of domestic rabbits breeding for their meat, fur, or wool. In Europe, this practice dates back to at least the 5th century A.D., when complete domestication of the wild *Oryctolagus cuniculus* probably took place in the French medieval monasteries (Sandford, 1992). Rabbits were typically kept as part of the household livestock and husbandry fell to the children. They were principally bred for their meat, but their fur also added an important economic value. European sailors took domestic rabbits to different ports around the world and brought new varieties back to Europe with them. Their international commercial use started in the late 18th century together with the rise of scientific animal breeding (Dunlop and Williams, 1996). These principles were also applied in rabbits whose reproductive cycle allowed for fast selection progress in a short period of time. Thus, individuals, cooperatives, and national breeding centers developed different rabbit breeds.

The New Zealand and the Californian, along with crossbreds, are the meat breeds more frequently used for commercial purposes that have given rise to specialized lines. A three-line crossbreeding system is widely used: two lines are selected for litter size obtaining crossbred does, and another line is selected for growth rate. The crossbred females, which have a good reproduction ability, are mated with a male of the third line. Therefore, their offspring will have a high growth rate transmitted by the sire line (Baselga and Blasco, 1989). Rabbits have been raised for their meat in a wide range of places around the world, but their large-scale commercialization has been focused on Asia and Europe over the last three decades (**Figure 1.1**).

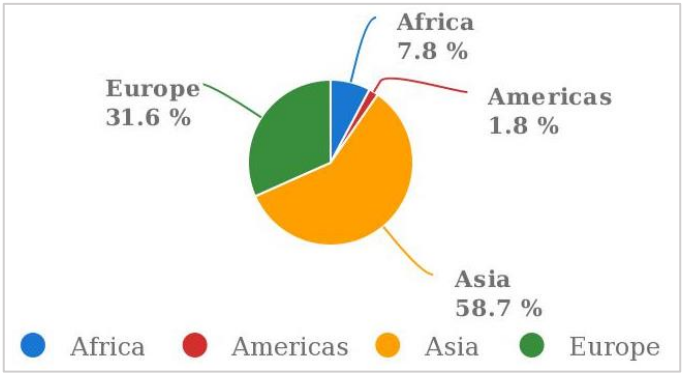


Figure 1.1| Average worldwide production of rabbit meat by region from 1993 to 2019 (FAOSTAT, 2021).

Intensive rabbit breeding for meat production was developed in Europe during the 1970s (Lebas *et al*., 1997). Together with Italy and France, Spain is one of the European Union's leading rabbit meat producers (**Figure 1.2**). Spanish cuniculture has experienced significant economic and productive changes in recent years. A decline in local production, a fall in the number of farms, and the professionalization of rabbit breeding have conditioned the productive evolution of the sector. The difficulties the rabbit industry is facing are compounded by a progressive reduction in consumption, and raising criticism related to animal welfare and ethical concerns.

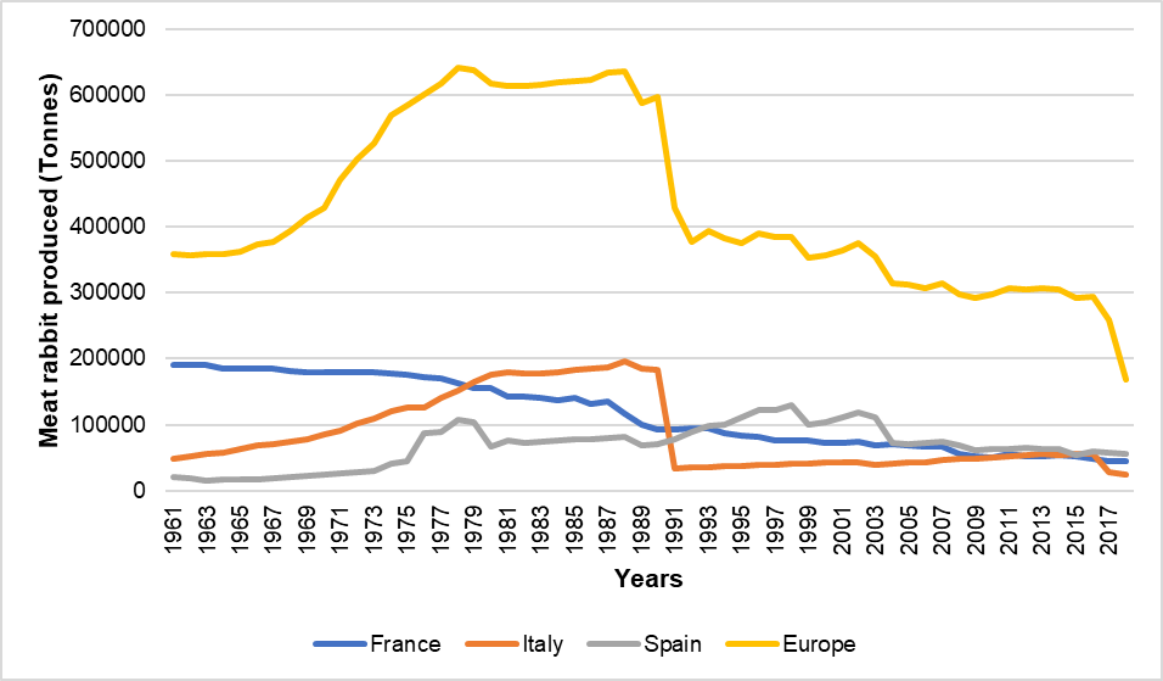


Figure 1.2| Evolution of production of rabbit meat by European leading rabbit meat producers from 1961 to 2017 (FAOSTAT, 2021).

Rabbit meat is not popular worldwide; however, it is considered a traditional meat species in Mediterranean countries like Spain. Although rabbit meat is the fifth type of meat most popular in Spain, its consumption has been reduced by 28% over the last five years (Montero-De Vicente and López-Navarro, 2020). The number of meat rabbits has also been reduced by 12% during that period except in some autonomous communities like País Vasco and Castilla y León whose number of meat rabbits increased by 49% and 29%, respectively (Montero-De Vicente and López-Navarro, 2020).

Castilla y León, followed by Cataluña, is the current leader in the Spanish market concerning the concentration of production. But in these regions, and also at the national level, the number of farms and the production of rabbit meat have both experienced a significant reduction.

There is no denying that cuniculture is facing a challenging period characterized by a reduction in production and consumption in Spain and the rest of Europe. It will therefore be necessary to develop sustainable and welfare production systems to ensure the survival of the industry. Keeping concerns of traditional consumers in mind together with the development of new rabbit meat products and commercial strategies to attract new potential consumers.

1.2. Feed efficiency in rabbit as a monogastric livestock species

Feed conversion ratio (FCR) is a measure of the efficiency with which the body transforms feed into the desired animal output (e.g., eggs or meat). Depending on the investment costs, food expenses can represent up to 70% of the total cost of main monogastric livestock production species (e.g., poultry, pork, or rabbit) in current intensive systems (Cartuche *et al*., 2014; Whittemore and Kyriazakis, 2006). For this reason, feed efficiency (FE) is a key trait in the rabbit meat industry that breeders have always been trying to improve to enhance profitability and the environmental sustainability of the farm.

In this context, a reduction of the feeding costs is of vital importance to optimize FE. In rabbit meat production, FCR is the measure most often used to study FE and can be defined as the ratio between the kilograms of feed consumed and the kilograms of weight gain. From a productive perspective, this ratio is defined at the farm level for given periods of time (e.g., years). The current European intensive production systems have a farm FCR of 3.63 in Spain (Rosell and González, 2009), 3.60 in France (Lebas, 2007), and 3.82 in Italy (Xiccato *et al*., 2007). These studies highlight a large variation in FCR among farms in the three countries, but a clear improvement tendency of this ratio over the past few years.

In rabbit farms, depending on weaning date and slaughter weight, about 50-60% of the feed is consumed in the fattening unit and 40-50% in the reproduction unit (Maertens, 2009). Both units may strongly influence the overall farm FCR, so appropriate actions to reduce FCR must be taken for both does and fatteners. Farm FCR is influenced by several factors on which actions are possible to reduce such ratio. These factors are discussed below and can be classified into three main pillars: farm management, nutrition, and genetics.

The number of rabbits weaned per doe and the FCR of the reproduction unit are highly influenced by fertility rate, litter size, and pre-weaning mortality. Gidenne *et al*. (2017) reported farm FCR variations according to productivity rate and slaughter weights. At a slaughter weight of 2.50 kg, they calculated a farm FCR > 4.00 when the number of rabbits weaned per doe in a year is lower than 40. However, with 50 rabbits weaned per doe, the farm FCR decreased 0.64 points. On the other hand, at smaller slaughter weight, they estimated a farm FCR drop of 0.39 points with 40 rabbits weaned per doe at a slaughter weight of 2.00 kg in comparison with the same number of rabbits weaned per doe but at a slaughter weight of 2.50 kg. This is because maintenance requirements raise gradually with age and a rapid increase in the FCR of fatteners is observed above 2.00 kg of live weight. With regard to the sex, Trocino *et al*. (2015) did not report significant differences in FCR between males and females before reaching the common slaughter weight. However, after this age, a higher adipose tissue deposition in females results in a worse FCR.

The farm FCR is also highly influenced by the health status of the animals. Both mortality and morbidity impact on FCR of fatteners, either by feed consumption without a final meat production output or by a deterioration of the animal performance. The effect of increasing mortality and the stage in which the loss occurs was studied by Maertens (2010). As would be expected, mortalities in late fattening stages have the worst effect on the farm FCR. For example, a mortality rate of 20% was estimated to increase the FCR at the end of the growing period by 26%, while a mortality rate of 10% would increase FCR by 11%. It is important to note that mortalities during the fattening period also impact the FCR of the reproduction unit since these animals consumed feed before weaning and the

consumption of the mother is consequently divided over less weaned kits. Fortunately, several actions related to management or nutrition can be carried out to curb mortality in rabbits. These strategies are discussed in detail in section 1.3. of this general introduction.

Nutrition is another major pillar for FE of the growing rabbit closely linked to the dietary digestible energy (DE) content of the feed (Lebas, 1975). Animals regulate the amount of food consumed to control their DE intake. Blood sugar levels are fundamental to the regulation of feed intake (FI) in monogastric species. Given that rabbits are monogastric herbivores, Gidenne and Lebas (2006) hypothesized that the blood glucose level is probably the principal blood component that regulates the FI. However, FCR is more correlated with the acid detergent fiber (ADF) than with the DE. Energy-dense diets can improve FCR, but the rabbit cannot ingest enough DE to keep an optimal growth rate above 25% dietary ADF. The energy content of rabbit diets is lower than that for pigs or chickens because of fiber requirements (Gidenne, 2003). Low fiber rabbit diets, although maintain or improve FCR, increase the risk of digestive disorders and diarrheas (Gidenne *et al*., 2000; Bennegadi *et al*., 2001). De Blas *et al*. (1995) pointed that a dietary concentration of 16 to 17% of non-digestible fibers led to an optimal FCR.

Furthermore, high-energy diets can be an interesting alternative since a replacement of starch by lipids in the feed, which contain more than twice DE, suppose an increase of the rabbits' DE intake to the same FI. The administration of such diets could be especially beneficial during the finishing phase, when most of the feed is consumed and the risk of the emergence of enteric disorders has fallen (Corrent *et al*., 2007). After weaning, feed restriction or high-fiber diets are often administrated to curb mortality caused by digestive diseases. In this regard, the application of feed restriction for two or three weeks after weaning followed by a period of *ad libitum* FI reduces the risk of enteric disorders at the same time as FCR improves (Gidenne *et al*., 2009; Romero *et al*., 2010; Gidenne *et al*., 2012).

Genetics is another major pillar on which actions can be taken to improve FE. This action is particularly focused on the growing kits' FCR (i.e., the efficiency of the

transforming energy and nutrients in the feed into meat in the slaughter animals). It was first indirectly improved by selection for growth rate (Larzul and De Rochambeau, 2004; Orengo *et al*., 2009). Unfortunately, however, such indirect selection is sub-optimal since Piles *et al*. (2004) demonstrated that the genetic correlation between FE and rabbits' growth is lower than initially expected. Another criterion that allows an improvement of FE is the residual FI (RFI) that represents the fraction of total FI that is not explained by maintenance requirements or growth. RFI is computed as the residual of multiple regression of the FI on metabolic weight and on the average daily gain (ADG).

Some recent studies have reported heritability estimates for growth and FE traits. Heritability estimates for ADG recorded in animals fed *ad libitum* (ADG_{AL}) were moderate and ranged from 0.21 (Piles and Sánchez, 2019) to 0.31 (Piles and Blasco, 2003). However, Piles and Sánchez (2019) reported a lower heritability estimate for ADG recorded in animals fed under restriction (ADG_R), which implies difficulties in achieving a response to selection for growth or indirectly for FE. Heritability estimate for RFI was 0.16 (Drouilhet *et al*., 2013), and ranged from 0.19 (Drouilhet *et al*., 2013) to 0.48 (Moura *et al*., 1997) for FCR.

At present, selection of rabbits for FE improvements is being conducted in France (Drouilhet *et al*., 2013) and Spain (Piles and Sánchez, 2019). Two principal selection strategies are followed to improve FCR. The first strategy aims at increasing ADG for the same amount of FI, thus, selecting those animals that express the highest growth capacity. The application of feed restriction is necessary to ensure that all the animals have the same FI and guarantee that the difference in growth is a consequence of a difference in FE. The second strategy aims at reducing the FI for the same body weight, thus, selecting animals based on their RFI. Selection for ADG would lead to heavier animals without a decrease in food expenses, while selection for RFI would lead to lower feed costs (Drouilhet *et al*., 2016).

It is worth stressing that improving FE is essential to increase the competitiveness of the rabbit industry but also to reduce animal excretion and decrease the environmental impact of the production. In this regard, Gidenne *et al*. (2017)

reported that selection for FE reduced by 13% nitrogen excretion. Therefore, selecting for FE through a lower FI seems an appropriate strategy to reduce the environmental impact. Nevertheless, private breeding companies only select for growth rate because the measurement of individual FI is difficult, especially when animals are group-raised.

In summary, although literature is still scarce, rabbit production has a large potential to improve FE and reduce the environmental impact. The Feed-a-Gene European project enabled the exploration of this potential.

1.2.1. Rabbit within the Feed-a-Gene project

The development of the present thesis has been framed within the Feed-a-Gene European project (Horizon 2020, grant agreement no: 633531, https://www.feed-a-gene.eu/). The global and main objective of the project was to better adapt different components of monogastric livestock species (i.e., rabbit, pig, and chicken) to improve the overall FE and reduce the environmental impact. It involved the development of new and alternative feed resources and feed technologies, the identification and selection of robust animals that are better adapted to fluctuating conditions, and the development of feeding techniques that allow optimizing the potential of the feed and the animal. The search for these new solutions is necessary to increase the efficiency and sustainability of livestock production systems, but also to face new challenges related to animal health and welfare, product quality and security, or environmental impact.

This project worked with the three main species used in monogastric livestock production, which are responsible for about 13% of nitrogen excretion and 18% of phosphate excretion from livestock in Europe (Velthof *et al*., 2015). Moreover, the production of these species is highly concentrated, and major concentrations of such excretions in water are found in main monogastric livestock production areas (the North-West of France, Denmark, the Netherlands, or the North-East of Spain). The existing diversity in the current livestock production systems, together with the emerging new real-time phenotyping of animals (i.e., precision feeding and high-

throughput molecular technologies), offer a high potential for an efficiency improvement through a better adaptation of the nutrient supply to the individual or group animal requirements.

The Feed-a-Gene project had a duration of 60 months and was composed of eight work packages that shared common experiments and facilities among project partners allowing to limit animal experimentation. Rabbit trials have mainly involved different tasks encompassed by work packages one, two, and five.

The aim of work package one was to develop novel high-quality European-based feed protein ingredients and develop methodologies for characterization of chemical and nutritional properties of feed in real-time. The objective of one task of this work package that involved growing rabbits was to study the impact of residual biomass pulp obtained after the extraction of protein from green biomass on the nutritional value of conventional and upgraded rapeseed meals for growth performance, FI, FCR, and digestibility. This trial revealed a strong affection in nutrient digestibility by the type and the level of inclusion, but no effect on rabbit growth and FE performances (from Deliverable D1.5 of Feed-a-Gene project, 2019).

Work package two aimed at exploring and identifying new traits related to individual animal's response variation to FE under different environmental conditions. One of the tasks encompassed by this work package was focused on the individual FI and feeding behavior in rabbits as new phenotypes to improve FE. To this aim, a feeding device (**Figure 1.3**) for the control of individual FI of rabbits raised in collective cages was developed by the Institute of Agrifood Research and Technology (IRTA) in collaboration with the technology-driven company CLAITEC. The development of this device was inspired by the necessity of having individual measurements of FI in rabbits raised in groups on commercial farms. Such records are of vital importance for selection strategies using individual information since the genetic correlation between FE and rabbits' growth is relatively low (Piles *et al*., 2004).

Figure 1.3| Feeding devices installed in the rabbit experimental farm at Torre Marimon, Caldes de Montbui (from Juan Pablo Sánchez Serrano, 2020).

These feeding devices work as scanners that send the status of all the sensors to a server each second. Thus, an internal software and a website interface that allow the interaction with the device were developed for appropriate management and records storage. Apart from this software, a second one was created for the daily automatic processing of FI records (from Deliverable D2.2 of Feed-a-Gene project, 2019).

The use of feeding devices is a promising strategy to improve animal nutrition, management, FE, and the overall sustainability of rabbit production. Moreover, their use allowed the definition of new phenotypes (i.e., daily FI, feeding rate, daily feeding duration, number of visits to the feeder, or the duration of the visits) whose potential as new selection criteria was investigated in work package 5 of the project (from Deliverable 2.3 of Feed-a-Gene project, 2019).

Despite the aforementioned advantages offered by feeding devices, their use modified animal performances compared to conventional feeders. A large number of animals per feeder or the presence of a tunnel to access the food could be responsible for these modifications (Sánchez *et al*., 2018). Further research is needed to disentangle the nature of the interaction of the rabbits with the electronic feeding device and to improve its use.

Finally, work package five benefited from the knowledge and data generated in other work packages to explore new traits and models for estimating breeding values for FE. Its main aim was to identify new strategies to select animals within breeding programs without impairing product quality, welfare, and robustness by considering the diversity of production environments and feed resources in the European Union and anticipating the effects of climate change on production systems. One of the tasks encompassed by this work package was focused on new traits to select for FE. It is driven by concern over FE recording and its associated costs and difficulties of FI measuring in rabbit farms that still rely on measurements made in individual cages. The problem is that this type of measurement is questioned in terms of welfare, but even more importantly, it is not representative of the performance of animals raised in groups. Thus, direct measurements of FI and FE recorded with electronic feeders developed in work package two were tested for genetic designs. These records were used to compute heritability estimates for individual average daily feed intake (ADFI; $h^2 = 0.29$) and ADG ($h^2 = 0.47$), which open the doors to a breeding program directly considering both traits to improve FE (from Deliverable 2.3 of Feed-a-Gene project, 2019).

A further aim of this task was to identify biological markers associated with FE that can be potentially measured on a large number of individuals at production farms and improve selection accuracy. Hence, genome-wide association studies (GWAS) were conducted with the Affymetrix Axiom Orcun SNP Array (Thermo Fisher Scientific), which includes 199,692 variants, to find single nucleotide polymorphisms (SNPs) associated with phenotypes related to FE in rabbits. Such GWAS are the first association studies reported in this species, and they were performed with two experimental designs. The first experimental design was performed at the French National Institute for Agricultural Research (INRA) and involved about 300 animals from a line selected for low RFI and 300 more from a non-selected line. A linear mixed model including the SNP effect as a regression on the allelic dose was applied to different traits related to FE. Some significant signals were found at a chromosome-wide level for all the traits analyzed (**Table 1.1**). However, no clear candidate gene was encompassed by quantitative trait locus (QTL) regions, so that further research will be necessary to identify causative variants.

Table 1.1| QTL regions and candidate genes detected for ADG, FI, RFI and FCR.

Trait	Chromosome	N of SNPs	Candidate genes
ADG	5	17	*PLA2G15*, *SLC7A6*, *PRMT7*, *SMPD3*, *ZFP90*, *CDH3*, *DH1*, *HAS3*, *UTP4*, *SNTB2*, *IP7*, *NFAT5NQO1*, *NOB1*, *WP2*, *PSMD7*
FCR	7	3	*CCDC192*, *SLC12A*, *FBN2*, *SLC27A6*, *ISOC1*, *ADAMTS19*, *MINAR2*
FCR	18	1	-
FI	6	2	*OTX2*, *ZP3*, *SSC4D*, *YWHAG*, *MDH2*, *STYXL1*, *POlow*, *RFIRHBDD2*, *EPHB4*, *ZAN*, *EPO*, *GNB2*, *GIGYF1*, *FBXO24*, *POP7*, *ACTL6B*, *TFR2*, *SAP25*, *LRCH4*, *AGFG2*, *NYAP1*, *TSC22D4*, *PPPR35*, *MEPCE*, *ZCWPW1*, *STAG3*
RFI	18	20	*HPSE2*, *CNNM1*, *GOT1*, *ABCC2*, *ANTPD7*, *COX15*, *PKD2L1*, *DNMPB*, *CPN1*, *ERLIN1*, *CHUK*, *BLOC1S2*, *WINT8B*, *SEC31B*, *1F1AN*, *PAX2*

(Adapted from Deliverable 2.3 of Feed-a-Gene project, 2019).

The second experimental design was performed at the Institute of Agrifood Research and Technology (IRTA) by Sánchez *et al.* (2020) and involved 438 animals from the Caldes line (Gómez *et al*., 2002) under two feeding regimes. An animal model including the SNP effect as a regression on the allelic dose was applied for ADG_{AL} and ADG_{R}, and a two-trait animal model that jointly fitted the performance trait and the SNP allele content was implemented for FE traits collected from cage groups. One hundred and eighty-nine significant signals were found at the chromosome-wide level for all the traits analyzed. Twenty candidate genes located in twelve different QTL regions were proposed to explain the variation of the analyzed growth and FE traits (**Table 1.2**). *FTO*, *NDUFAF6*, and *CEBPA* genes were previously reported as associated with the phenotypes of interest in monogastric species.

Table 1.2| QTL regions and candidate genes detected for ADG, FI, RFI and FCR.

Trait	Chromosome	N of SNPs	Candidate genes
ADG_{AL}	3	3	*CA2*
ADG_{AL}	3	11	-
ADG_{AL}	3	1	-
ADG_{AL}	3	111	*NDUFAF6*, *TP53INP1*
ADG_{AL}	3	1	-
ADG_{AL}	5	1	*FTO*, *AKTIP*
ADG_{AL}	5	2	-
ADG_{AL}, RFI	21	96	*ATXN2*, *ACAD10*, *TRAFD1*, *PTPN11*
ADG_{R}	9	66	*FEZF2*, *PTPRG*
ADG_{R}	12	0	-
ADG_{R}	13	90	*RC3H1*, *TNFSF18*
ADG_{R}	17	29	*LGALS3*, *TMEM260*
FCR	6	16	*SIK1B*
FCR	16	16	*PLA2G4A*
FI	5	13	*CEBPA*, *KCTD15*
RFI	21	26	*SELENOM*

(Adapted from Sánchez *et al*., 2020).

No QTL regions were found in common between GWAS conducted with the two different experimental designs. This is a clear indication of the lack of statistical power of both experiments. Indeed, the sample sizes used for these studies only allow for the proper detection of strong effects (see Chapter seven). Nevertheless, these are the first GWAS for growth and FE traits performed in rabbits with a dense SNP chip panel. In addition, Sánchez *et al*. (2020) proposed a new modeling approach that allows GWAS for the traits recorded as group averages and even when genotypes are not available for all the individuals.

Another task of this work package was largely conducted to develop statistical models and procedures for selection on FE that account for indirect genetic and social interaction effects. Such effects are the ones that an individual exerts on the phenotype of its group mates on animal welfare, productivity, and health. Response to selection depends on the genetic parameter for both direct and social genetic effects, so ignoring these latter for traits that could be affected by the interactions

between cage mates could lead to wrong estimates. The importance of social effects in rabbits was evaluated within the Feed-a-Gene project with the development and implementation of models for estimating genetic and environmental parameters of direct and social effects. Piles *et al*. (2017) suggested that the selection of rabbits for ADG_{AL} might fail to improve ADG_R in rabbits that are fed under restriction since the contribution of social effects to the estimation of total breeding values of rabbits under restriction is important, but not for that estimation of rabbits fed *ad libitum*. Moreover, the genetic correlation between direct and social genetic effects for animals is negative and moderate on restricted feeding but null on *ad libitum* feeding. It is, therefore, of great importance that selection for ADG is performed under production conditions regarding the feeding regime and accounting for social effects when feed restriction is applied. Besides these aspects, David *et al*. (2018) investigated the variation of social effects for ADG_R over time with structured antedependence models. The main conclusion of this research was that social effects are larger after mixing animals at weaning than later in the growing period, probably because of the establishment of social hierarchy that is generally observed at that time. Therefore, accounting for social effects in the selection criterion maximizes genetic progress.

Finally, the key task as concerns the present thesis was the one which aimed at evaluating the effect of genetics and different environmental factors (i.e., maternal transmission, feeding regime, breeding farm, or administration of antibiotics) on rabbit gut microbiota and at proposing new methodologies to explode gut microbial variability as a heritable phenotype affecting growth and FE. Microbial diversity and composition of rabbits from experiments above described were characterized through Illumina sequencing of 16S rRNA gene amplicons in a MiSeq platform. No more details about the results of this task are presented in this section since they all were objectives of the present thesis and will be developed in the following chapters.

1.3. Management strategies to curb mortality in rabbits

The implementation of appropriate management strategies is vital to curb mortality and morbidity in rabbits since, as previously mentioned in section 1.2. of this general introduction, the health status of the animals is of great relevance for the farm FE from a productive and economic point of view (Rosell and González, 2009). The emergence of different diseases that affect meat rabbits is handled with progress and knowledge in the fields of nutrition, genetics but also animal management (Morton *et al*., 2005).

Mortality may depend on the genotype of the animal and aspects related to the mother's performance like litter size, birth weight, order of parturition (Harris *et al*., 1982), but also on the farm management and environmental factors. Therefore, it is crucial to know about and study those external factors affecting mortality to enhance prevention and reduce it through adequate management strategies. Comprehensive published compilations on rabbit diagnostic cases are scarce, but Rosell and de la Fuente (2016) described the principal causes of mortality in breeding rabbits on Spanish commercial farms, which were respiratory diseases and digestive disorders. A more recent study carried out in 2019 indicates that parasitic diseases, particularly those causing digestive disorders and affecting young animals, were the principal cause of rabbit mortality in northern Spain between 2000 and 2018 (Espinosa *et al*., 2020). The prevalence of digestive diseases such as coccidiosis or encephalitozoonosis was higher in animals raised in traditionally managed farms with poor hygienic-sanitary conditions, inadequate management systems, and poor health prophylactic protocols. These inadequate farm conditions together with immature immune systems of young rabbits may easily promote digestive disease outbreaks (Gomez-Bautista *et al*., 1987; Pakandl, 2013). Early detection of encephalitozoonosis outbreaks is vital from a clinical and public health point of view because of its zoonotic potential (Mathis *et al*., 2005).

Epizootic rabbit enteropathy (ERE) is a severe digestive disorder that appeared in France in 1996 and spread very rapidly to the rest of Europe, becoming the main cause of mortality in rabbit breeding. This pathology mainly affects young rabbits

after weaning and can be responsible for very high mortality rates up to 80%. It is characterized by a distended abdomen, emissions of small quantities of watery diarrhea, and a decrease in FI (Licois *et al*., 2005). The etiology of ERE is still unknown, but a pathogenic agent is thought to be involved since it is contagious and the administration of antimicrobials prevents its emergence (Maertens *et al*., 2005). At present, management strategies that guarantee proper environmental hygiene minimizing the proliferation of pathogens, feed restriction, or the administration of antibiotics are employed to prevent the emergence of ERE and other pathologies.

Therefore, management strategies related to housing, feeding, and the use of antimicrobials to improve breeding rabbit health are presented in the following sub-sections.

1.3.1. Biosecurity of the breeding farm

The term biosecurity refers to those management strategies and measures that aim at preventing the introduction and/or spread in the farm of harmful microorganisms to minimize the risk of transmission of infectious diseases (World Health Organization, 2006). In a rabbit facility, attention to biosecurity is the most effective tool to reduce and prevent the introduction of diseases since an incident from just one animal could have adverse effects on the entire farm. Failure to implement biosecurity strategies and practices involves a greater risk of disease introduction and the consequent facing the accompanying economic losses.

Potential sources of health threats to a rabbit farm are physical transference from visitors, mechanical transference from equipment or supplies brought and, what is thought to be the major cause of biosecurity problems on farms, biological transference from the introduction of new and sick animals into the facility (Waage and Mumford, 2008). The best way to prevent these health threats is to implement a health management program based on three key management principles (i.e., farm visitors access, animal health, and operation) discussed below (Kylie *et al*., 2017).

Access management refers to farm visitors as well as their movement between areas and access to other animal species. External visitors can potentially harbor pathogens on or under their shoes, hands, clothing, or hair. Thus, it is important to ensure visitors wear protective clothing and foot and hair covering. It is also highly recommended to limit traffic near rabbit facilities and keep visitor vehicles out of the areas accessible to the animals. Vehicles used for animal transportations between farms and/or the slaughterhouse must always be clean and corrected disinfected.

Animal health management implies the monitoring and treatment of diseases and the establishment of protocols for animal movement and quarantines. New animals introduced into the farm should be kept in a separate area during a quarantine period to facilitate the monitoring of their health status and prevent the spread of potential diseases, especially those without the exhibition of clinical signs, to the other animals. The facility must always meet the minimum standards for animal cages and dispose of an isolation area for sick animals. Cared should be provided for the quarantined animals after having handled the rest, and workers should never wear clothing that has been used in the isolation facility. Animals should be monitored every day for signs of illness to isolate those displaying disease symptoms. Regarding viral diseases (i.e., rabbit hemorrhagic disease and myxomatosis), the establishment of vaccination programs is extremely effective against the emergence and spread of these pathologies on the farm (Dalton *et al*., 2012; Bertagnoli and Marchandeau, 2015).

Finally, operation management includes measures to correctly store and process food and water and keep facilities clean and in good repair. It includes the practice of routine cleaning, disinfection, and preventative maintenance of nests, cages, watering, and feeding devices. It is important to encourage employees to wash their hands before and after working with the animals, to frequently wash the farm tools and equipment, and to take special care of young animals which are more susceptible to diseases because of the immaturity of their immune system. On the other hand, the facility should be maintained under good ventilation conditions and ensure that other animal species which can be carriers of diseases never enter the

farm and remove any standing water that could turn into a breeding ground for mosquitoes.

A biosecurity program can be accomplished in all rabbit farms if common sense and science are employed. An effective biosecurity protocol can prevent significant economic losses and ensure that final products are safe for human consumption.

1.3.2. Feed restriction

The reduction of the quantity of food administrated to the animal (i.e., feed restriction) decreases the incidence of digestive disorders affecting rabbits described above (Gidenne *et al*., 2012). Moreover, Gidenne *et al*. (2009) showed that the application of feed restriction improves rabbit FE during the whole growing period if, after a restricted period, animals are fed *ad libitum*. Other studies (Boisot *et al*., 2003; Bovera *et al*., 2013) also observed a reduction in mortality and morbidity without impacting the slaughter weight when growing rabbits received a restricted diet (Meo *et al*., 2007). This latter faster growth observed when rabbits are fed again *ad libitum* does not occur at the expense of food overconsumption, so a positive impact on FE is reached. Such compensatory growth after a period of feed restriction has been reported by Ledin (1984) and Romero *et al*. (2010). Feed restriction is a widely applied commercial practice (Tudela, 2008) to improve FE while reducing mortality due to enteric diseases. This practice is common in France since their larger commercial weight allows a longer fattening period so that there is room for both phases: restricted feeding until 63-70 days of age and one extra week under full feeding before slaughtering.

The underlying mechanisms of the benefits (i.e., mortality reduction and FE improvement) achieved with feed restriction are still unclear. Gidenne (2003) reported a lower mortality rate and a higher transit speed of particles and liquids in rabbits fed with diets rich in fiber. It has been hypothesized that the speed of transit of food could affect the digestive health of young rabbits, but this theory was discarded because the mean retention time increases in restricted rabbits. However, this greater retention of food in the gastrointestinal tract (GIT) of restricted animals

could be responsible for a greater nutrient digestibility reported by Ledin (1984) and Xicatto *et al*. (1992). This fact could explain the FE improvement also sometimes observed during the restriction period, although it is also highly dependent on the diet (Gidenne *et al*., 2012).

Regarding disease tolerance, Gidenne and Feugier (2009) demonstrated that feed restriction modifies ingestion and food fermentation patterns producing an acidity peak in the cecum that could confer protection to the animal against digestive diseases by impairing the growth of pathogenic microorganisms (Boisot *et al*., 2003). This peak of acidity disappears when the animals are fed again *ad libitum*, which would imply the loss of the protective effect against enteropathies (Gidenne *et al*., 2009; Birolo *et al*., 2020).

Another hypothesis is that feed restriction could reduce mortality rate and improve FE by shaping the gut microbial composition and fermentative activity. This assumption would be supported by the major role that microbial communities that inhabit the GIT have on the host's immunity (Belkaid and Hand, 2014; Mazmanian *et al*., 2008) and nutrient absorption (Hooper *et al*., 2001). Drouilhet *et al*. (2016) found different fermentation patterns and microbial phylotypes between rabbits selected for RFI and non-selected animals, suggesting a key role of intestinal microorganisms in FE. The fast development of sequencing techniques and the reduction of their costs allow studying the contribution of gastrointestinal microbiota to rabbit FE, which is one of the main objectives of the present thesis.

1.3.3. Administration of antimicrobials

An antimicrobial is an agent that kills microorganisms or impairs their growth. These agents can be grouped according to the microorganisms they act primarily against (e.g., antibiotics and antifungals are used against bacteria and fungi, respectively) or according to their function (e.g., microbicides kill microorganisms while bacteriostatic only inhibit the growth of bacteria).

Different antimicrobial molecules have been widely administered in rabbit meat production, especially after weaning, to curb mortality peaks caused by the onset of gastrointestinal symptoms (Gidenne *et al*., 2010). It has raised a global concern for the emergence of antibiotic-resistant bacteria, and European Union needed to ban the use of antibiotics in animal feeds as growth promoters in 2006 (EC 1831/2003). At the time the experiments for the present thesis were conducted, from 2014 to 2016, the administration of a mix of up to four antibiotics was permitted to prevent or treat the emergence of potential infectious diseases on farms. Nonetheless, nowadays, only one antibiotic molecule can be administered, and substantial efforts are being conducted towards searching for efficient alternatives which allow for a complete withdrawal of antimicrobials in animal feeds. Furthermore, a withdrawal period is required from the time antibiotics are administered until slaughter allowing, time for removing residues from the animal's system.

Multiple studies have shown alterations caused in rabbit gut microbiota by the administration of antibiotics in the feed (Abecia *et al*., 2007; Eshar and Weese, 2014; Zou *et al*., 2016). Some antibiotics can adversely affect the intestinal microbiota of growing rabbits, killing beneficial bacteria, and allowing pathogens to grow. Thus, it is important to restrict its use to avoid altering the normal development of the animal gut microbiota.

1.4. Microbial communities inhabiting the gastrointestinal tract

The field of microbiome research has experienced rapid growth over the past two decades becoming a topic of great scientific and public interest. So much so that the mammal microbiome is considered to be the “last organ” (Baquero and Nombela, 2012). The fast development of next-generation sequencing (NGS) technologies (Rogers and Venter, 2005) has significantly reduced the time and cost of studying the microbiome. Nevertheless, despite the rapid growth in microbiome research interest from a wide range of research fields, there is a lack of consensus on the definition of the term microbiome (Marchesi and Ravel, 2015) and on other related terms employed to describe different aspects of microbial communities and the environments they inhabit. In this editorial article published in the journal

Microbiome, Marchesi and Ravel proposed clear definitions of each of these terms to avoid misunderstanding of results provided by the scientific community.

Konopka (2009) defined microbial communities as multi-species assemblages in which organisms live together in a contiguous environment and interact between them. The word microbiome is of Ancient Greek origin: “micro” (μικρος) means small and “biome” is derived from the Greek word “bíos” (βιος, life). The first definition for this term was proposed by Whipps *et al*., (1988), who described it as a characteristic microbial community in a reasonably well-defined habitat that has distinct physicochemical properties as their “theatre of activity” (i.e., the whole spectrum of molecules produced by the microorganisms) (**Figure 1.4**). But during the last few decades, many other definitions for the term microbiome have been published (Lederberg and McCray, 2001; Prosser *et al*., 2007). Nevertheless, some of these definitions only describe the terms as encompassing the genomes of microorganisms. Thus, the complexity of the diverse hierarchies of interactions established between microorganisms with one another and with their host biotic and abiotic environments is not completely captured (Berg *et al*., 2020). This variety of definitions for the term microbiome were discussed by a panel of international experts from diverse microbiome fields within the European MicrobiomeSupport project (www.microbiomesupport.eu/), concluding that the first definition (Whipps *et al*., 1988) is nowadays still the most comprehensive one.

The term microbiota is also of Ancient Greek origin. It is the combination of the words “micro” (μικρος, small) and “biota” (βιοτα, the living organisms of an ecosystem). The first definition for this term was proposed in a study that emphasized the importance of the microorganisms inhabiting the human body in healthy and sick individuals (Lederberg and McCray, 2001). The microbiota encompasses all living members forming the microbiome (i.e., bacteria, archaea, fungi, algae, and small protists). The inclusion of phages, viruses, plasmids, and mobile genetic elements is controversial. And so is relic DNA (i.e., extracellular DNA derived from dead cells), which can comprise up to 40% of the sequenced microbial DNA in soil (Carini *et al*., 2016). Interestingly enough, despite the abundance of relic DNA, it has a minimal effect on taxonomic and phylogenetic diversity estimates (Lennon *et al*., 2018).

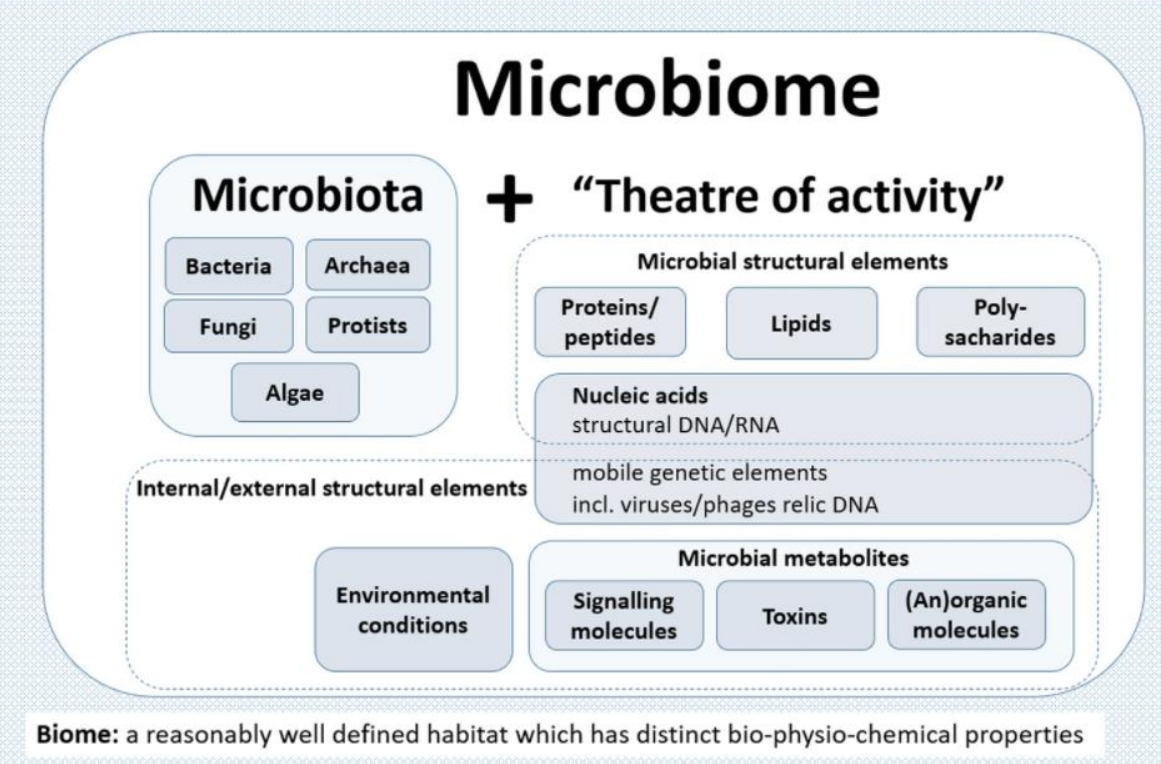


Figure 1.4| Scheme of the term microbiome containing the microbiota (community of microorganisms) and their structural elements, metabolites, and the surrounding environment (Berg *et al*., 2020).

As mentioned in the previous paragraph, the term microbiota is defined as the assemblage of living microorganisms inhabiting a specific environment. Given that phages, viruses, plasmids, mobile genetic elements, and free DNA are usually not considered living organisms (Dupré and O'Malley, 2013), they would not form part of the microbiota. Nevertheless, the term microbiome proposed by Whipps *et al*., (1988) encompassed the community of the microorganisms (i.e., microbiota), but also their "theatre of activity" that involves all the molecules generated by the microorganisms, their host and structured by the surrounding environment (**Figure 1.4**). This is the reason why all mobile genetic elements and relic DNA should be encompassed by the term microbiome but not by the microbiota.

It is noteworthy to mention that the term microbiome is frequently confused with the term metagenome, which is the collection of genomes and genes from the members of a microbiota (Marchesi and Ravel, 2015). Such collection is obtained and characterized through shotgun sequencing of DNA (i.e., metagenomics).

The microbes that inhabit a specific niche interact between them affecting fitness, functional capacities, and dynamics of the microbiome (Banerjee *et al*., 2018). The stability of the ecosystem relies on these interactions that can be positive, negative, or neutral (**Figure 1.5**). Mutualism is the best-studied interaction in the microbiology field in which all members of the community benefit from the others' activity. The host-mammals gut microbiome interaction is a good example of beneficial mutualism in which the host obtains energy from short-chain fatty acids produced by bacteria through fermentation of the glycans provided by the host (Backhed *et al*., 2005).

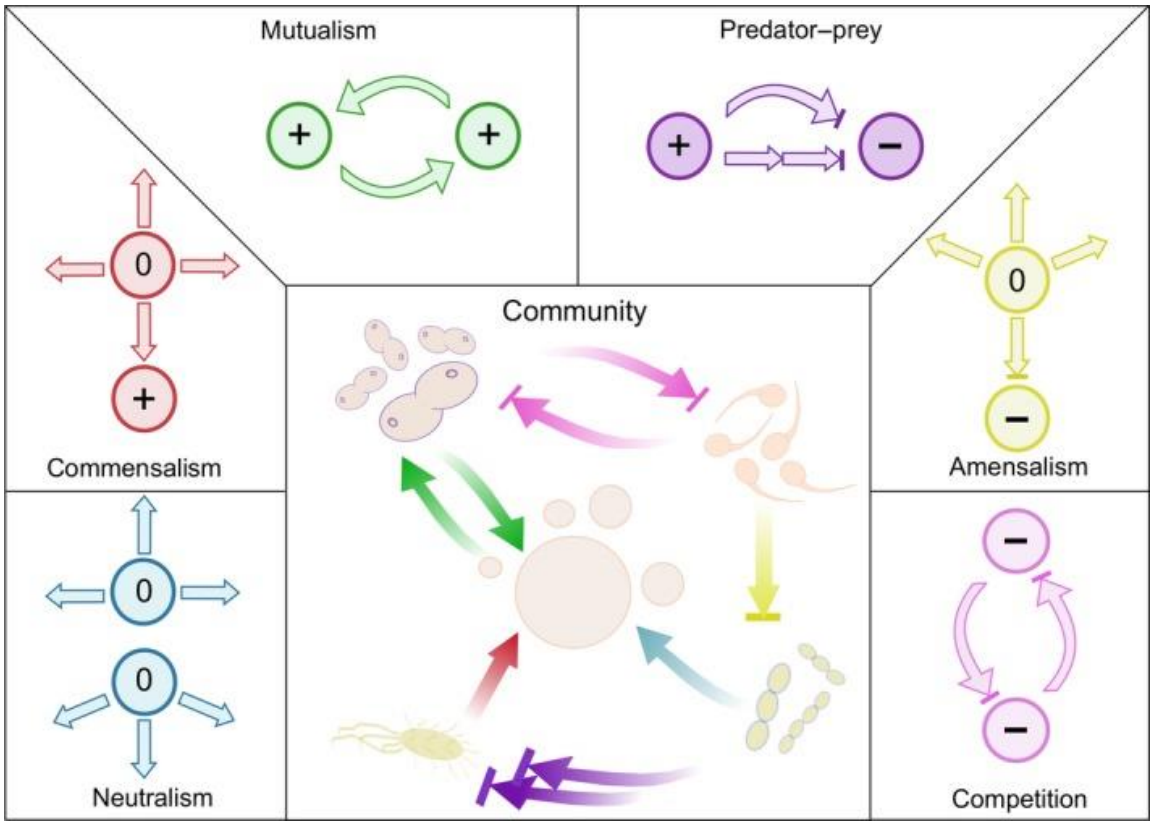


Figure 1.5| Interactions between microorganisms (Zuñiga *et al*., 2017).

Commensalism is another positive type of interaction in which only one member benefits without affecting the rest, such as happens in nitrification or methanogenesis processes (Allison *et al*., 1993). Neutralism occurs when microorganisms have no significant effect on each other. In an interaction of amensalism, one member is negatively affected while the rest neither benefit nor are harmed. However, the interaction that occurs when one member is

disadvantaged and another obtains benefits is named parasitism. Finally, competition is the interaction in which all members are negatively affected by the presence of the rest. An example of this type of interaction is the one established between autotrophic and heterotrophic bacterial populations competing for the oxygen of the environment (Tsuno *et al*., 2002).

In the following sub-section, the reader will find a historical overview of the evolution experienced by the microbiome research field from its microbiology origins until its establishment as a discipline itself.

1.4.1. Evolution of microbial profiling technologies

The study of microbial communities has largely evolved along with the development of new technologies and inventions that boosted their research (**Figure 1.6**). The first report of microorganisms by Antonie Leeuwenhoek dates back to 1676. Thanks to the development of the first microscopes, he investigated unknown bacteria, fungi, and protozoa from water, mud, and oral samples (Hamarneh, 1960). Antonie Leeuwenhoek is considered the "Father of Microbiology" and he also described the first interaction between microorganisms within complex communities with the discovery of biofilms (Høiby, 2017).

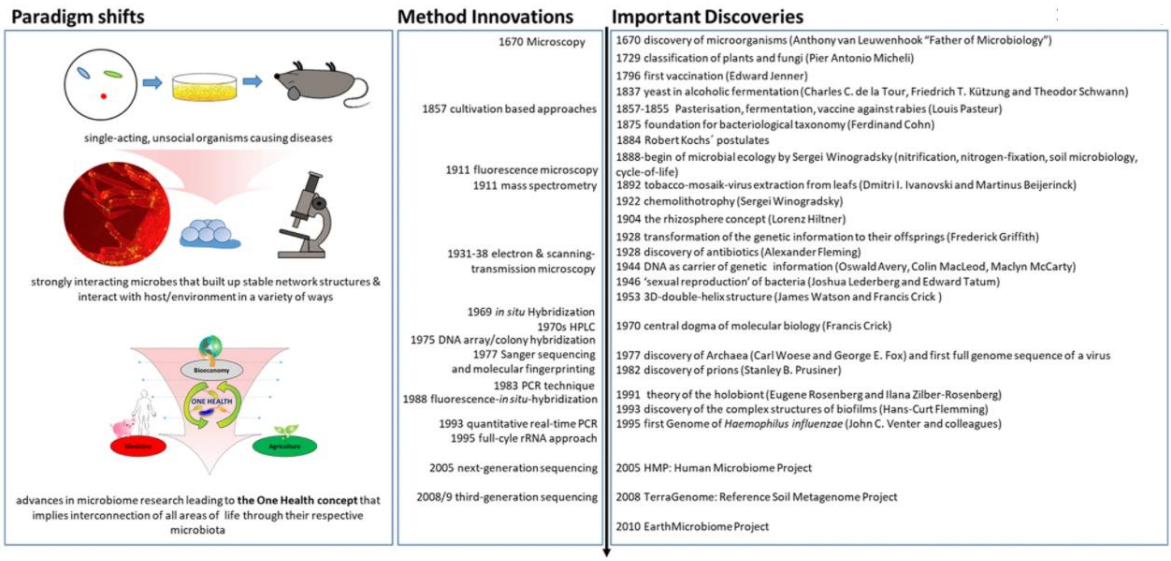


Figure 1.6| The evolution of microbiome research from the 17th and 21st centuries (Berg *et al*., 2020).

In 1884, Robert Kochs' postulates of the origin of animal diseases caused by microbial infections laid the foundation for the pathogenicity concept. This also shifted the focus of the microbiology research on the role of microorganisms as pathogenic agents that should be eliminated (Evans, 1976). However, posterior research demonstrated that only some microorganisms are responsible for animal disease while the presence and role of most microbes are necessary for ecosystem homeostasis.

The new field of microbial ecology emerged with the study by Sergei Winogradsky on bacterial nitrification in 1888, who is considered the founder of modern microbiology (Dworkin and Gutnick, 2012). Winogradsky isolated the first pure cultures of nitrifying bacteria and confirmed these bacteria carry out the steps of conversion of ammonia to nitrite and of nitrite to nitrate. This discovery led to the concept of the cycles of sulfur and nitrogen in nature, resulting in another paradigm shift: microbiologists became aware of the microorganisms' ubiquity in all natural environments (Podolsky, 2012), and that their interactions with hosts are vital for population dynamics (Bassler, 2002).

During the 17th and 19th centuries, the study of microorganisms was limited to the study of their morphological characteristics (i.e., those which were visible through a microscope) and cultivation-based approaches. But large and rapid advances started with the discovery of DNA as the hereditary material (Avery *et al*., 1944), the development of PCR (Mullis *et al*., 1986), and sequencing technologies that made possible the investigation of microbial communities with cultivation-independent approaches. Another significant milestone for the analysis of microbial communities was the use of phylogenetic markers (e.g., 16S and 18S rRNA genes, or internal transcribed spacer regions) introduced by Woese and Fox in 1977 (Woese and Fox, 1977) that allow to barcode bacteria, archaea, fungi, algae, and protists. At this historical turning point, it is meaningful to introduce the 16S ribosomal gene, in the analysis of which has been based on the study of microbial communities inhabiting the cecum of rabbits involved in this thesis.

National Institutes of Health (NIH) defines ribosomes as cellular particles made of RNA and protein that serve as the site for protein synthesis in the cell. The ribosome reads the sequence of the messenger RNA (mRNA) and, using the genetic code, translates the sequence of RNA bases into a sequence of amino acids. In bacteria, ribosomes are scattered throughout the cytoplasm, and their sedimentation velocity in an ultracentrifuge is 70S. These structures can dissociate into big (50S) and small (30S) subunits. In turn, the 50S subunit is primarily compound by 31 different proteins and two small molecules of ribosomal RNA (rRNA 23S and rRNA 5S), while the 30S subunit is primarily compound by 21 different proteins and the 16S rRNA (**Figure 1.7**). These molecules of rRNA are ubiquitous and easy to detect since they are present in a high number of copies.

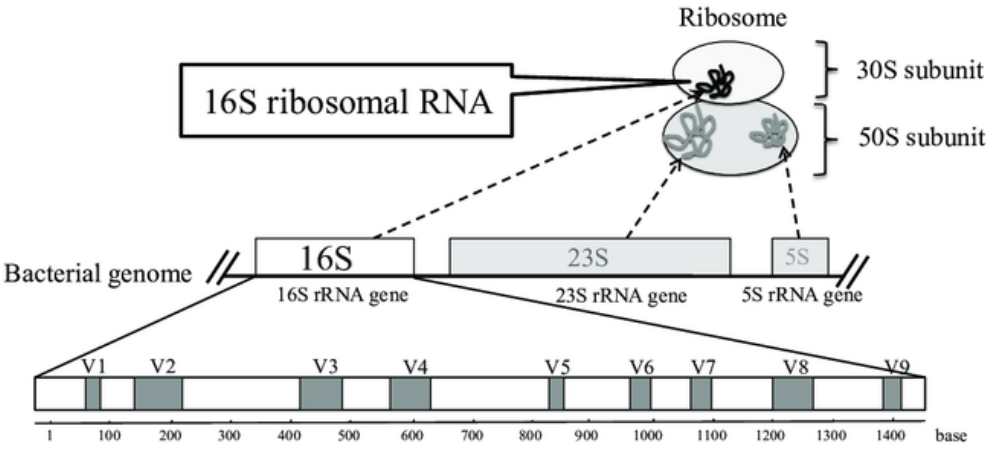


Figure 1.7| Schema of a ribosome and the 16S rRNA gene. The white and grey boxes indicate conserved regions and hypervariable regions (V1-V9), respectively (Fukuda *et al*., 2016).

The 16S rRNA gene encodes a rRNA molecule of the 30S ribosomal subunit present in all prokaryotic cells, including bacteria and archaea. This gene is commonly used for identifying bacteria and is preferable over 5S and 23S genes for several reasons (Clarridge, 2004; Rajendhran and Gunasekaran, 2011). The first is that it is a relatively short gene of approximately 1,500 base pairs (bp). The second reason is that the 16S rRNA is composed of ten regions that are common among most bacteria (conserved regions) and are combined with nine hypervariable regions (**Figure 1.7**). This combination of conserved and hypervariable regions is optimal for the design of primers for the conserved regions (Lane, 1991) while the diversity of the hypervariable ones allows for phylogenetic assignment (Gray *et al*., 1984). Thus, due to its extensive usage, the number of 16S rRNA gene sequences stored

in public databases is constantly increasing, facilitating the identification and classification of microorganisms.

The principal molecular approaches targeting the 16S rRNA gene can be grouped in quantitative PCR (qPCR), hybridization of probes, fingerprinting, and sequencing. A brief overview of them is presented below (**Figure 1.8**).

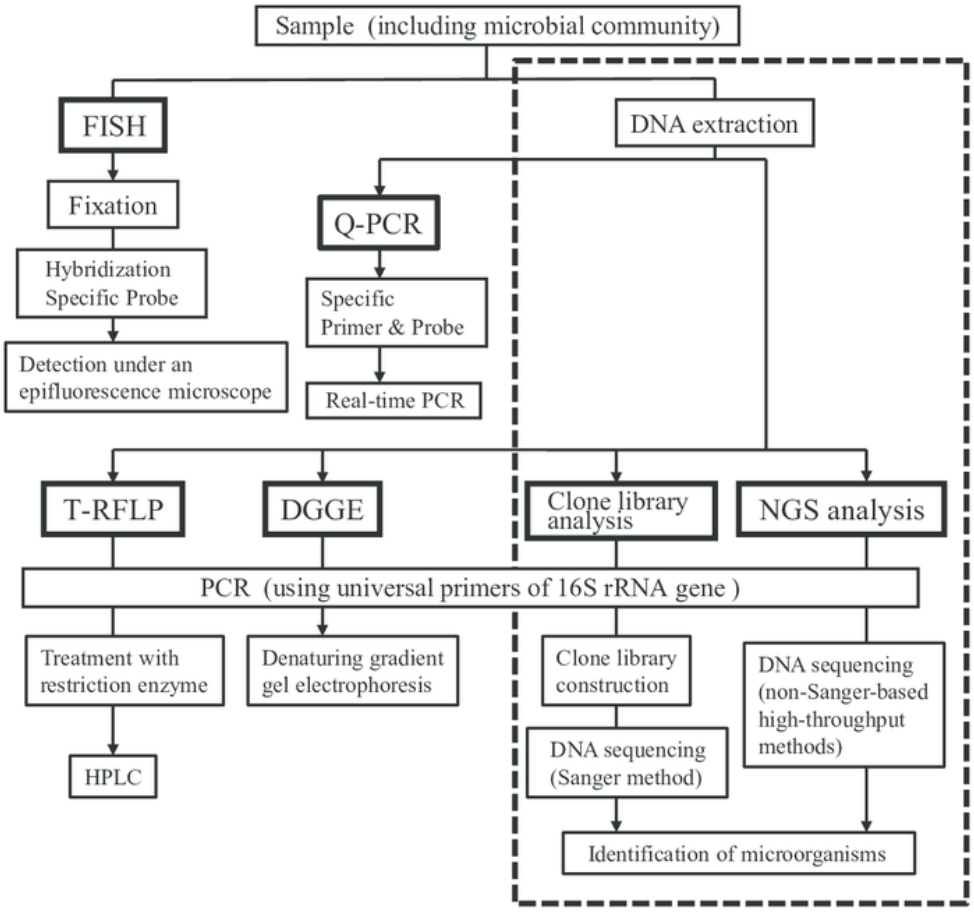


Figure 1.8| Workflow of molecular methods targeting 16S rRNA gene (Fukuda *et al*., 2016).

The qPCR allows measuring the abundance of the 16S rRNA genes of a target bacteria (Bustin *et al*., 2005). The accumulation of amplicons is measured in real-time during each cycle of the PCR by using fluorescent dyes (e.g., SYBR Green) or fluorescent probes. Methods based on the hybridization probes use short monocatenary sequences of oligonucleotides that are complementary to specific sequences of microbial DNA, thus allowing the phylogenetic identification and quantification of specific microbial species present in a sample (Amann *et al*., 1995). Fluorescence *in situ* hybridization (FISH) enables phylogenetic identification by the

hybridization of fluorescence-labeled oligonucleotide probes. FISH requires neither a previous PCR process nor the extraction of DNA, thus avoiding the bias associated with the amplification of DNA. However, this approach does not allow the identification of unknown species, and it has a low resolution to identify different phylogenetic groups present in a sample.

Fingerprinting techniques allow separating mixed 16S rRNA genes even though they have the same size. This is possible because the differences between their sequences lead to different molecular weights that will generate different patterns in a gel subjected to electrophoresis. Terminal restriction fragment length polymorphism (T-RFLP) is based on variations present in the sequence of amplicons of 16S rRNA genes (Kitts, 2001). In T-RFLP, amplicons labeled with a fluorescent primer in their terminal region are fragmented using restriction enzymes that are separated into different fragment patterns whose size and peak height are analyzed by high-performance liquid chromatography (HPLC) or a DNA sequencer. Thus, T-RFLP is useful to quickly measure the microbial diversity of a sample, but it does not allow for phylogenetic identification. Another fingerprinting approach is denaturing-gradient gel electrophoresis (DGGE), in which PCR amplicons of 16S rRNA gene are loaded in a polyacrylamide gel containing a linear gradient of DNA denaturant and subjected to electrophoresis (Muyzer *et al*., 1993). The melting behavior is determined by variations present in the sequence of amplicons that end their migration in the gel at different positions. DGGE is a rapid semi-quantitative approach, but like T-RFLP, it has the bias associated with the PCR process and the impossibility of direct phylogenetic identification.

The clone library analysis involves the cloning of PCR amplicons of the 16S rRNA gene into *Escherichia coli* using a plasmid vector. Then, the transformed clones are sequenced using the Sanger method (Sanger *et al*., 1977), and the sequences are compared to 16S rRNA gene sequences stored in databases by the basic local alignment search tool (BLAST) algorithm. Given the high quality of the sequences achieved with these methods, clone libraries of 16S rRNA genes are appropriate for the identification of unknown species. Sanger sequencing method was widely used until the 1990s when rapid development of NGS began.

NGS technologies filled the Sanger method's gaps providing their high-throughput, low-cost, and rapid DNA sequencing (Metzker, 2010). The principal sequencers are 454 GS FLX (Roche), MiSeq/HiSeq (Illumina), and Ion PGM (Ion Torrent), which can sequence millions of DNA fragments in a few days. Like the clone library analysis, NGS technologies also require the previous amplification of the 16S rRNA gene but not the construction of a clone library using *Escherichia coli*. Moreover, the number of reads obtained with any of the NGS technologies is much larger than the achieved with the Sanger sequencing method. These technologies are powerful and have allowed the discovery of novel microbes and the exploration of new environments. In the 454 GS FLX technology, after individually fixing each amplicon to a microbead, the DNA fragments are amplified in an emulsion PCR. The resulting beads are put into a microwell that is filled with a sequencing reaction mixture. This technology is based on pyrosequencing chemistry. Therefore, when an oligonucleotide is added during PCR, pyrophosphate is released and a burst of light is detected by the system, and this information is translated to nucleotide sequences with an associated base quality value (Ronaghi *et al*., 1998). This technology provides a higher yield than Sanger sequencing at a lower cost but with shorter read lengths.

The Ion PGM is a small potentiometer that detects the changes in hydrogen potential generated by the release of a proton when a nucleotide is added in a sequencing reaction (Rothberg *et al*., 2011). The yield and cost of this technology outperform the 454 GS FLX technology, although reads generated by Ion PGM are shorter (Whiteley *et al*., 2012). Nonetheless, this reduction of length reads implies higher sequencing error rates making it necessary to sequence with higher coverage. In this regard, MiSeq and HiSeq Illumina platforms have become very popular technologies due to their high yield at low cost. The foundation of Illumina technologies is the reversible termination sequencing by synthesis with nucleotides fluorescently labeled. When these nucleotides are incorporated in the sequencing reaction, the fluorescence is registered, and the fluorophore is removed and allows the incorporation of the next nucleotide (Bentley *et al*., 2008).

These technologies are currently the most used for microbiome research, but new sequencing approaches (e.g., PacBio RS from Pacific Bioscience or Oxford Nanopore) are being developed to reduce costs, fill the gaps, and improve the yield of the existing ones. Reads obtained from NGS technologies have demanding computational requirements for their storage and bioinformatics analysis. Finally, to taxonomically annotate the reads generated with these approaches, they must be compared against a database such as The Ribosomal Database Project (Wang *et al*., 2014), Silva (Quast *et al*., 2012), or Greengenes (DeSantis *et al*., 2006), that store sequences specific of different ribosomal genes belonging to a growing number of microorganisms.

It is also worth mentioning that the metabolic potential of a microbial community can be deciphered by a whole metagenome approach where the total DNA from all microorganisms is extracted to prepare and sequence whole shotgun libraries (Tyson *et al*., 2004; Venter *et al*., 2004). This approach is the most advanced technology to describe microbial variability of samples as well as its physiological potential. The sequencing is performed from little fragments of DNA, randomly obtained with restriction enzymes, that will finally be assembled to reconstruct the original sequence using a reference. A first reference gene catalog of the rabbit gut microbiome through a whole metagenome shotgun sequencing approach is being constructed (Achard *et al*., 2016).

The rapid development of the sequencing technologies described in this sub-section and the increasing number of microbial ecology studies during this century have revolutionized the field and highlighted the ubiquity of microbial communities in association with a higher organism and their fundamental role in mammals physiological and immunological processes (Belkaid and Hand, 2014). The analysis of genomes and metagenomes in a high-throughput way opens the doors to characterize and unravel the functional potential of individual microorganisms and the whole community within their host. The present thesis, which aims at describing the microbial communities inhabiting the meat rabbit cecum and their potential role in FE, is a practical example of this.

1.4.2. Anatomy and functions of the rabbit gastrointestinal tract

It is important to make a brief overview of the anatomy and the particularities of the rabbit GIT that make it an ideal setting for the growth of certain microorganisms. As a monogastric herbivore, the rabbit GIT is adapted to process large amounts of fiber-rich feed, including specific adaptations, from teeth to an enlarged hindgut for fermentation, and the separation of cecal digesta particles allowing for cecotrophy (Fortun-Lamothe and Gidenne, 2006).

The total length of the adult rabbit GIT is 4.5-5 m (Lebas *et al*., 1997). The organization and principal characteristics of the different segments that comprise the rabbit GIT are shown in **Figure 1.9**. Rabbits have 28 teeth (2/1 0/0 3/2 3/3) that grow continuously during their whole life. Salivary glands produce saliva with low amylase concentration, and the esophagus is short merely to transport food from the mouth to the stomach. Thus, feed eaten by the rabbit quickly reaches the stomach that, contrary to other mammals, is characterized by a very acid (1.5 to 2.0) pH that varies along the day mainly in the fundus (i.e., the blind part) in relation to the storage of soft feces (Lebas *et al*., 1997). The glands included in the stomach wall secrete hydrochloric acid, pepsin, and some minerals (Ca, K, Mg, and Na). The feed remains in the rabbit's stomach for 2-4 hours, and then it is gradually moved through the pylorus into the small intestine (Lebas *et al*., 1997).

The length of rabbit's small intestine is about 3 m and 0.8-1 cm in diameter, and it is divided into three parts: duodenum, jejunum, and ileum. As the contents reach the upper part, whose pH is slightly basic (7.2-7.5), they are diluted by the flow of bile and the first intestinal secretions. The pH of the rabbit ileum is more acidic (6.2-6.5) at the end of the small intestine, where enzymes of the pancreatic juice break down the feed contents and, as occur in other monogastric species, pass through the intestinal wall to be transported to the cells through the blood (Lebas *et al*., 1997).

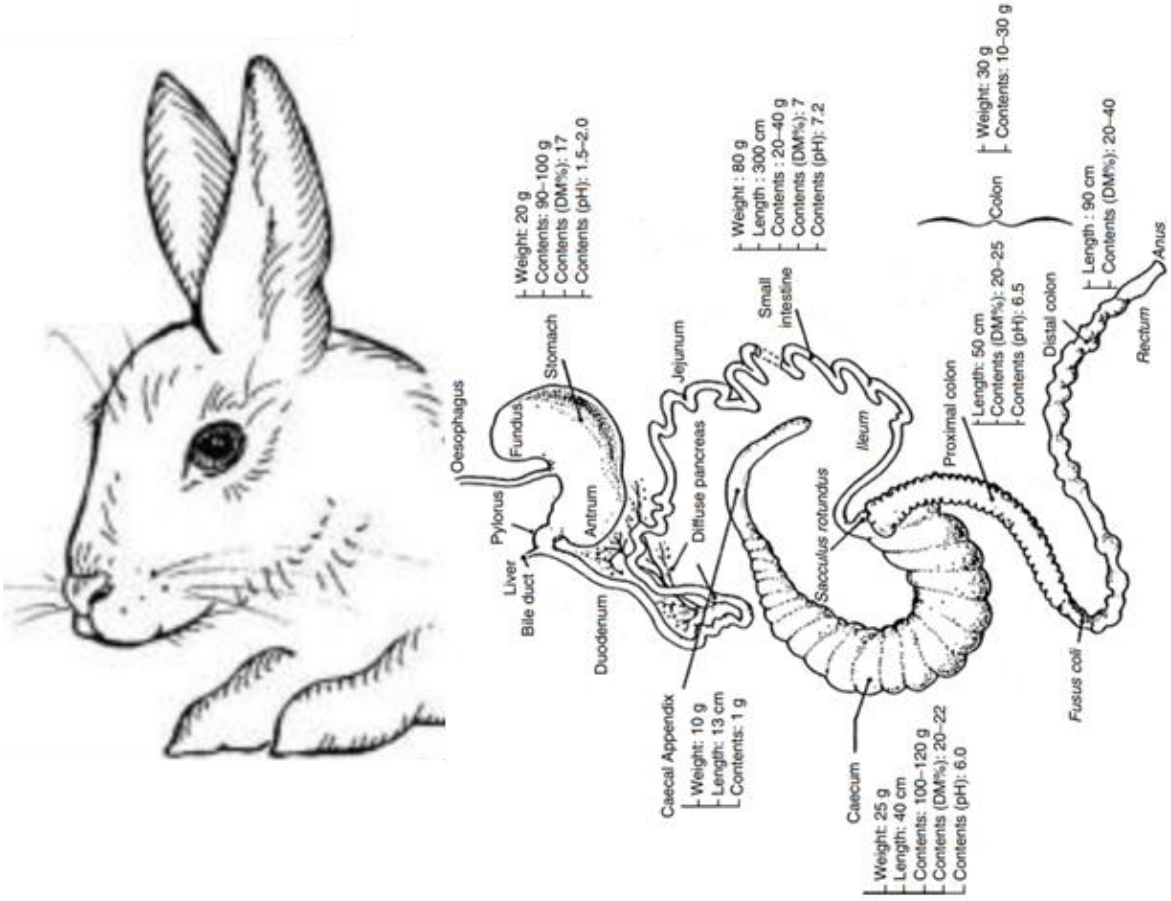


Figure 1.9| The gastrointestinal tract of a rabbit. Numerical values are those observed in the New Zealand breed fed *ad libitum* with a pelleted feed at twelve weeks of age (adapted from Lebas *et al*., 1997).

The cecum starts in the ileocaecal valve (*sacculus rotundus*), and it stores about 40% of the whole digestive content. Its length is about 40-45 cm and its diameter of 3-4 cm. The pH of this segment of the rabbit GIT varies, depending on microbial activity and feeding pattern, around 6.0. The feed particles that are not broken down enter the cecum, where they remain for 2 to 18 h under the action of bacterial enzymes (Lebas *et al*., 1997). During this period, cecal bacteria break down the remaining particles that are freed and pass through the GIT walls into the bloodstream. Finally, the cecal content is evacuated into the colon.

The cecal appendix is located at the end of the cecum, and its walls are composed of lymphoid tissues. The colon begins at the base of the cecum and lengths 1.5 m. This segment is divided into two parts: proximal (50 cm long, 2-3 cm of diameter) and distal colon (1 m, 1 cm of diameter) that end with the rectum and the anus (Lebas *et al*., 1997). The peculiarities of rabbits, and the rest of *Lagomorpha*, rely

on the dual function of the proximal colon. If the cecal content reaches the colon in the early morning, it undergoes a few biochemical changes: its wall secretes mucus that envelops the soft pellets (i.e., cecotrophes) generated by the wall contractions. However, if the cecal content enters the colon at any other time of the day, the proximal colon activity is completely different: successive waves of contractions in alternating directions begin. The first wave acts to evacuate the content while the second one pushes it back into the cecum. Under the varying pressure and rhythm of these contractions, the contents are squeezed. Most of the liquid part, containing soluble products and small particles of less than 0.1 mm, is forced back into the cecum (Björnhag, 1972). The solid part, containing mainly large particles over 0.3 mm, forms hard feces that are then expelled through the anus. Therefore, this dual action results in the generation of two types of excrement by the colon: the hard feces and the cecotrophes.

Cecotrophy is a particular herbivorous nutritional strategy to benefit from the microbial protein and to obtain vitamins necessary for the rabbit. Rabbit cecotrophes are rich in protein (half of bacterial origin and half of imperfectly broken-down feed particles and intestinal secretions) and water-soluble vitamins B and C. As opposed to the hard feces that are excreted, the cecotrophes are ingested back by the rabbit directly after being expelled through the anus. Then, these soft pellets stay in the stomach for 4-6 h, where their envelope structure is broken, and follow the same digestive process as normal feed.

The rabbit GIT is not only involved in nutrient digestion but protects against pathogens. After birth, it experiences a gradual maturation influenced by ontogenic factors (i.e., related to the age and the growth of the individual), diet, and interactions between microorganisms. The final anatomy of the GIT is stabilized at nine weeks of age, except the cecal appendix that does not finish its maturation until eleven weeks. The composition of the rabbit GIT mucosa is shown in **Figure 1.10**. The morphology of the intestinal epithelium experiences deep changes during the weeks after birth. The intestinal villa, which were thin and lengthened, become broader (Yu and Chiou, 1997), and the crypts deepened. The maturation of the intestinal mucosa follows a proximo-distal gradient (Toofanian and Targowski, 1982) and does not

complete until day 20 of age. Ridges begin to emerge in the walls of cecal and colon mucosa from day 16 of age (Yu and Chiou, 1997), together with the establishment of intestinal microbiota.

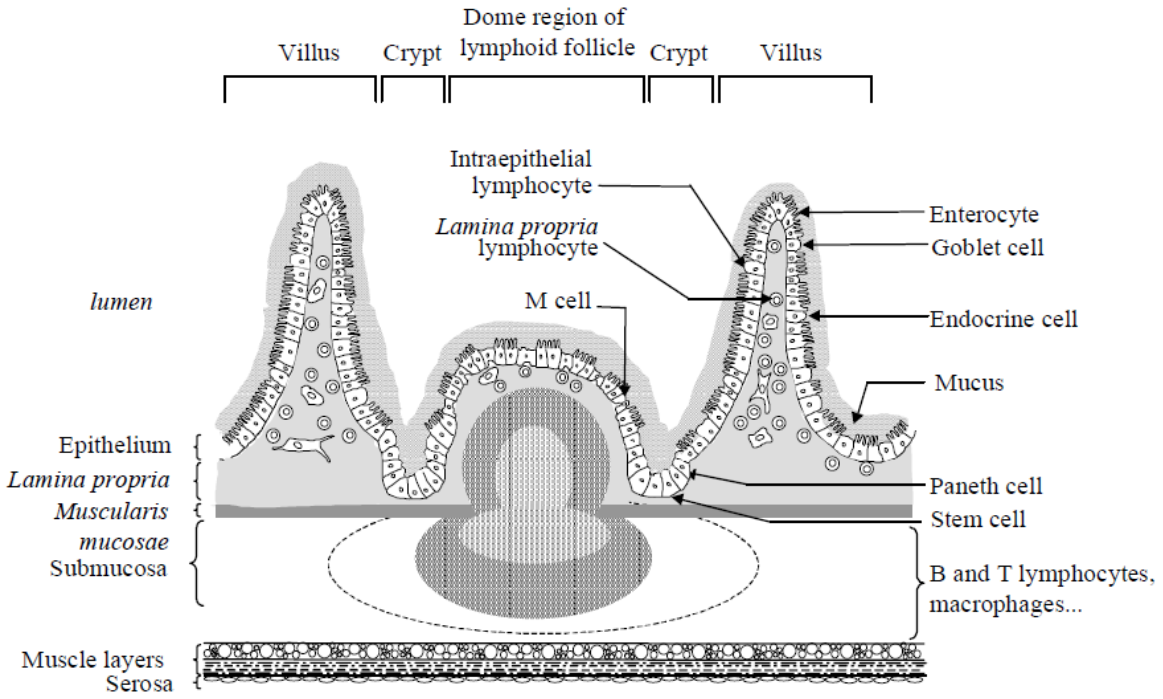


Figure 1.10| Composition of the rabbit gastrointestinal mucosa, including the digestive epithelium (enterocytes, Goblet cells and Paneth cells) and the gut-associated lymphoid tissue (Fortun-Lamothe and Gidenne, 2006).

As mentioned in previous paragraphs, nutrient digestion takes place in the stomach and small intestine under the action of enzymes secreted by the rabbit, but also of bacterial enzymes that hydrolyze those nutrients escaping the intestinal absorption. While the development of the rabbit enzymatic system depends mainly on ontogenic factors (Gallois *et al*., 2005), the development of the bacterial enzymatic activity depends mainly on the nutrients arriving the cecum and diet digestibility (Gidenne and Fortun-Lamothe, 2002). Nutrient degradation by microorganisms in the cecum results in the production of volatile fatty acids (VFA), ammonia, carbon dioxide, methane, and hydrogen. The cecal fibrolytic activity is not detectable in young rabbits until they reach two weeks of age. Bellier *et al*. (1995) demonstrated that the fermentation pattern changes with age by decreasing ammonia concentration (and the pH as a result) and increasing VFA (Gidenne and Fortun-Lamothe, 2002).

At the same time that the maturation of the digestive function takes place, the digestive immune system develops to ensure the defense of the host gut mucosa against pathogens. However, gut mucosa also has non-immunological mechanisms (e.g., peristalsis, permanent renewal of digestive epithelium, bacteriolytic and bacteriostatic capacity of mucus). The immune system is activated when these mechanisms cannot eliminate the pathogenic agent. The digestive mucosa is associated with the gut-associated lymphoid tissue (GALT) that neutralizes pathogens and protects mucosa by controlling the inflammatory response. The rabbit lymphoid system is similar to that of other mammals except because it possesses the *sacculus rotundus*, an additional structure located at the ileocecal junction (Mage, 1998).

Another very important function of the GIT is its role in the immune response. The innate primary immune response occurs along the GIT and is the first line of defense, while the adaptive immune response acts against a specific foreign element in the GIT. The latter is played by the induction sites that identify the agent and activate the cell reaction against antigens, and the effector sites that eliminate the foreign agent (Drouet-Viard and Fortun-Lamothe, 2002). In the rabbit, the induction sites are plenty of lymphoid organized in ten Peyers' patches along the small intestine (Mage, 1998) that are composed of many dome-follicles that extend into the lumen (**Figure 1.10**). The dome-follicles contain B cells producing IgM, macrophages, and CD4-T cells (Ermak *et al*., 1994), and the interfollicular regions contain T cells (Hein, 1999). The antibody repertoire of the rabbit is generated in three stages (Knight and Crane, 1994). Before three weeks of age, the neonatal antibody repertoire is established by B cells generated during B lymphopoiesis. Between four and eight weeks of age, the primary antibody repertoire develops in the GALT and provides the unique reservoir of B cells for the whole life of the rabbit. After that, this repertoire is modified during antigen-specific immune responses during adulthood, thus generating the secondary repertoire under the influence of GIT microbiota.

The previous anatomical description has introduced the regions hosting the microbial populations inhabiting the GIT. In this respect, the rabbit GIT microbiota

consists of about 100 to 1000 billion microorganisms per gram of digesta (Savage, 1987) and harbors a complex and diverse community. In rabbits, an abundant microbiota (10^{10} to 10^{12} bacteria / g) is present throughout the cecum-colon and in hard and soft feces and has also been studied in the ileum where its abundance (10^{6} to 10^{8} bacteria / g) is lower (Combes *et al*., 2012). Despite the demonstrated existence of active microbial populations in proximal and distal segments of rabbit GIT, the cecum is the main fermenter organ. Thus, it is not surprising that it hosts the richest and the most diverse microbial community of its GIT (Gouet and Fonty, 1979). Kingdom *Bacteria* dominate the rabbit GIT (Gouet and Fonty, 1979; Forsythe and Parker, 1985), while the archaeal population is estimated at 10^{7} archaea per g of content (Combes *et al*., 2011). Regarding eukaryotes, the rabbit GIT seems to lack anaerobic fungi and protozoa (Bennegadi *et al*., 2003), except in animals affected by coccidiosis (Lelkes and Chang, 1987).

According to the first studies that aimed at characterizing the taxonomic composition of microbial communities inhabiting the growing rabbit intestinal microbiota using 16S rRNA, most of the bacteria belong to phylum *Firmicutes* (90%) and only 4% of the species to phylum *Bacteroidetes*, followed by phyla *Actinobacteria* and *Proteobacteria* (Abecia *et al*., 2005; Monteils *et al*., 2008; Massip *et al*., 2012). Within phylum *Firmicutes*, *Clostridia* is the predominant class, and the principal families are *Ruminococcaceae* and *Lachnospiraceae* (Massip *et al*., 2012; Zou *et al*., 2016). According to Zou *et al*. (2016), the most abundant genera in rabbit cecum are *Ruminococcus*, *Oscillospira*, *Coprococcus*, and *Bacteroides*.

Despite the bacteria predominance, an archaeal population dominated by genus *Methanobrevibacter* (Kušar and Avguštin, 2010) also inhabits the rabbit cecum (10^{7} archaea / g). A particular interest linked to environmental impact has been focused in recent years on the strictly anaerobic methanogenic *Archaea* residing in the GIT. These species are integrated at the end of the food chain and allow the elimination of hydrogen from fermentation to provide methane (Jones *et al*., 1987), which is a greenhouse gas that represents a loss of 7% of the energy and carbon ingested by the animal (Boadi *et al*., 2004).

One of the principal objectives of the present thesis has been the characterization of the microbial composition of hard feces and cecum content of growing meat rabbits (see Chapter three). Although rabbit microbiota is very homogenous between adult individuals (Combes *et al*., 2011), external factors can disturb this stability and modify the overall microbial diversity or the relative abundances of specific taxa. The potential effect on GIT microbial communities of external factors will be discussed in the following sub-section.

1.4.3. External factors shaping microbial diversity and composition

An overview of biological intrinsic (i.e., those related to the host) and environmental factors potentially influencing rabbit microbiota described in the literature is shown in **Figure 1.11**.

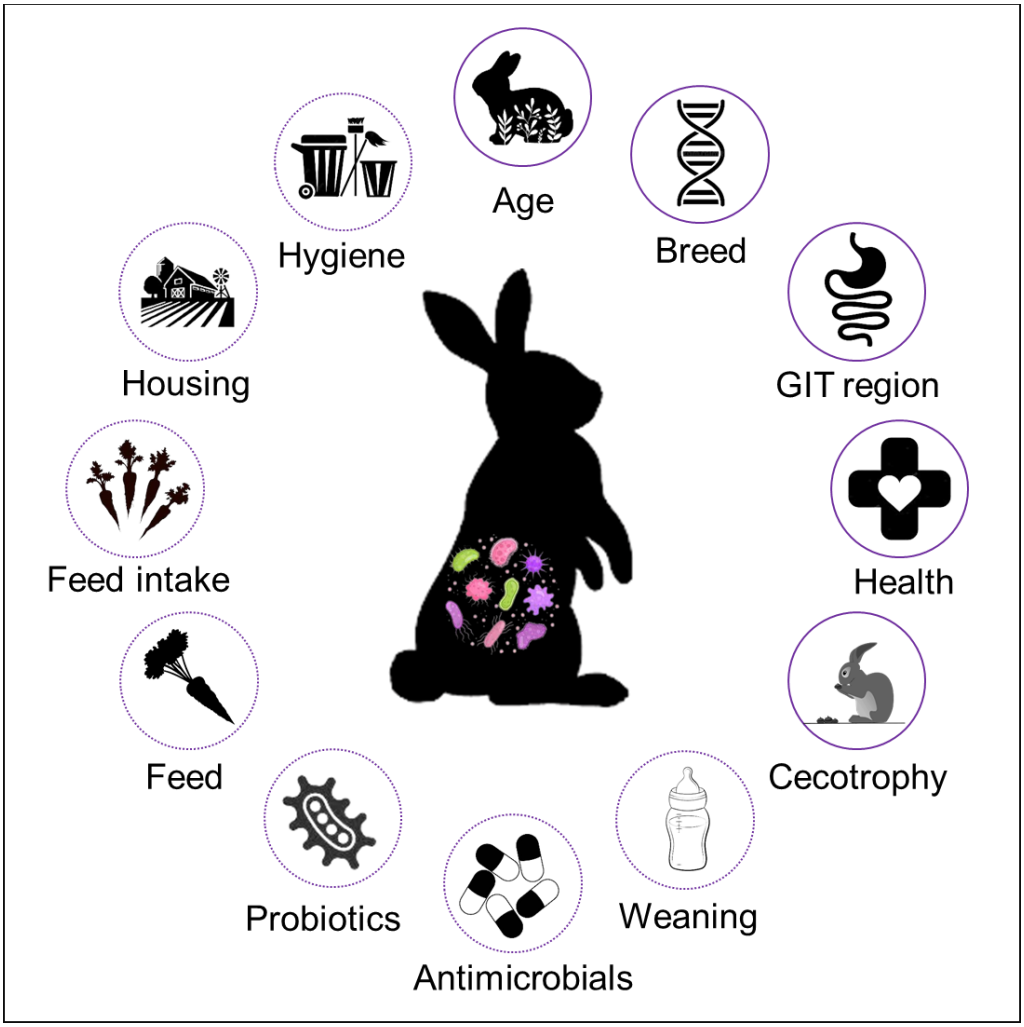


Figure 1.11| Host (solid lines) and external (dashed lines) factors that affect the rabbit gut microbiota.

Jiménez *et al*. (2008) demonstrated that mammals' GIT is not completely sterile *in utero*. Nonetheless, it is considered that microbial colonization starts at birth when the individual passes through the birth canal and enters in contact with the immediate environment (Berg, 1996). From this moment on, an organized colonization is produced by the introduction of ecological succession of species. This gradual establishment of an increasingly diverse community reaches its climax at 70 days of age (Combes *et al*., 2011). During the first weeks of the rabbit life, its cecal microbiota is composed of the same proportions of strict anaerobes and facultative anaerobes microorganisms. But later, the abundance of the latter falls and disappears in some individuals after weaning (Gouet and Fonty, 1979). A recent study investigated the dynamic distribution of gut microbiota in commercial meat rabbits from weaning to finishing (28-72 days of age) through 16S rRNA gene sequencing (Fang *et al*., 2020a). This study observed significant differences in gut microbial structure and increased microbial richness and diversity with age. These findings are in complete agreement with those of Combes *et al*. (2011) outlined above.

The genetic background of the host has been recognized as a factor that could influence GIT microbial composition in humans (Benson *et al*., 2010), mice (Org *et al*., 2015), chickens (Schokker *et al*., 2015), or pigs (Xiao *et al*., 2017). In meat rabbits, few studies have investigated the effects of host breed on the GIT microbiota. Recently research by Ye *et al*. (2021) evaluated whether breed factors could alter the gut microbial community structure by comparing two commercial meat rabbit breeds. These authors found that host breeds exerted a greater effect on gut microbial diversity structure than age. Different breeds or lines of the same animal species could have their own GIT microbial composition originated by differences in intestinal physiology and immune system development. The role of the host's genetic on GIT microbiota will be further discussed in the following sub-section since the main objective of the present thesis is to study this relationship.

Cecotrophy is a particular behavior of rabbits and an important intrinsic factor affecting cecal microbiota. As previously explained, rabbits produce two types of excrements: soft feces covered with a layer of mucus (i.e., cecotrophes) and hard

feces. The fecal pellets left by the doe are eaten by the kits during nursing, being one of the first reservoirs for colonization of their GIT (Moncombe *et al*., 2004). Thus, this cecotrophagic behavior has an essential contribution to the early implantation of the microbiota in offspring (Combes *et al*., 2012). The impairment of this behavior reduced growth, altered lipid metabolism (Wang *et al*., 2019), and delayed the implantation of *Bacteroides* (Kovács *et al*., 2006). Combes *et al*. (2014) confirmed that preventing kits from ingesting doe's feces delayed the microbial ecological succession, especially of families *Bacteroidaceae* and *Ruminococcaceae*.

The rabbit GIT regions consist of the stomach, duodenum, jejunum, ileum, caecum, and colon. These regions have different functions that impact the dynamics of the harboring microbial communities, so it should be considered when choosing the sampling protocol. Given that cecum is the principal site for bacterial fermentation of indigestible dietary fibers and the production of VFA in rabbits, it is not surprising that most of the microbiome studies focused on characterizing rabbit cecal and fecal microbial communities (Monteils *et al*., 2008; Kušar and Avguštin, 2010; Zeng *et al*., 2015). Indeed, this has been one of the main objectives of the present thesis (see Chapter three). There is evidence that fecal samples are pretty similar regarding the large intestine microbiota, but their adequacy for other GIT regions is doubtful (Fang *et al*., 2019). Cotozzolo *et al*. (2020) characterized the microbial composition and diversity across the GIT in New Zealand White rabbits. In this study, four clusters were identified: stomach, small intestine (duodenum and jejunum), ileum, and large intestine (cecum and colon). The differences in the microbial composition across the GIT could be explained by the anatomy, the environmental conditions (pH 1.5-2 in the stomach and 7.5 and 6.5 in the small and large intestine, respectively), and the different physiological functions in the digestion of feed. These existing differences across sections of the rabbit GIT suggest different requirements for the types of microbial communities that need to be present in each part. Cotozzolo *et al*. (2020) reported an increased microbial diversity towards the foregut and the large intestine. The cecum showed the highest microbial complexity and is mainly colonized by anaerobic microorganisms, which is linked to cecotrophy and its fermentative function. Thus, the rabbit GIT is composed of four microbial niches characterized by different physicochemical conditions that force the adaptation of microorganisms

suggesting a co-evolution of microbiota and the GIT of the host. Consequently, to avoid misinterpretation of results comparisons between different groups or studies, the section from which the intestinal samples are collected must be taken into account.

The health status of the animal is another intrinsic factor that clearly influences the composition and diversity of microbial communities inhabiting the GIT. ERE, a severe disease of unknown etiology that mainly affects post-weaning animals, is a good illustration of this in rabbits. Given the severity of this disease, large efforts have been made to unravel its etiology, and bacterial involvement is now accepted since antibiotic treatments are effective in ERE prevention (Licois *et al*., 2005). Nevertheless, although clostridial species and coliforms are frequently present in sick animals, the specific microorganisms involved in its onset have not been identified (Lelkes and Chang, 1987; Huybens *et al*., 2009). Bäuerl *et al*. (2014) compared the cecal microbiota of healthy rabbits and rabbits affected by ERE. This study revealed that a remarkable dysbiosis accompanied by a reduced microbial diversity was the most relevant feature of ERE rabbits cecal microbiota. It is, however, noteworthy that dysbiosis may not be the cause of ERE but rather a consequence of the disease. While cecal microbiota of healthy animals contains high proportions of *Ruminococcus*, ERE rabbits cecal microbiota is rich in opportunistic and pathogen bacteria such as *Akkermansia muciniphila*, *Clostridium*, *Lysinibacillus*, *Bacteroides*, or *Escherichia* species (Bäuerl *et al*., 2014). A more recent study that examined the microbial variations caused by ERE in the stomach, small intestine, and cecum confirmed the reduced microbial richness in affected animals accompanied by a decrease in the abundances of phylum *Firmicutes* and an increase of phylum *Proteobacteria* in the stomach and cecum, and also of phyla *Bacteroidetes* and *Verrucomicrobia* in the small intestine (Jin *et al*., 2018). Hence, opportunistic pathogens are often found in the cecal microbiota of affected animals, although it may not be the origin of ERE or other diseases but notably contribute to dysbiosis.

On the other hand, a growing number of studies have analyzed the impact of different environmental factors on rabbit GIT microbiota. Nutrition is one of the most studied since food is a key factor affecting the balance of GIT microbial communities since it conditions their supply of nutrients and energy. In rabbits, the transition to solid food is gradual while the proportion of doe's milk ingested decreases. Weaning has beneficial effects on the maturation of the cecum and colon: increase of the organs (Gallois *et al*., 2005), fermentation activity stimulation (Kovács *et al*., 2012), and maturation of GALT (Carabaño *et al*., 2010). By contrast, the development of cecum is slower when rabbits are prevented from eating solid food (Combes *et al*., 2008), and the colonization by cellulolytic bacteria is delayed (Padilha *et al*., 1999). Read *et al*. (2019) observed a large shift in the structure and composition of cecal microbial communities at weaning mainly characterized by an increase in diversity and a decline of facultative anaerobes. However, consistent interactions between different species do not occur until the solid feed intake is well established. Moreover, the alteration of the microbiota at the onset of solid food ingestion is associated with a major shift in the production of bacterial metabolites, especially butyrate, coinciding with the transcriptomic regulation of key components of both, the immune and physical gut barrier (Beaumont *et al*., 2020).

A few weeks after weaning, when microbial communities inhabiting the rabbit GIT are stable, the dietary composition can still alter this microbial fitness. A glaring example of this is the fiber-deficient diets that lead to changes in the microbial composition and alterations in the fermentation profile (Michelland *et al*., 2011), which often results in a higher incidence of enteropathy (Gidenne *et al*., 2004). On the other hand, the administration of dehydrated alfalfa could improve the health status by favoring an appropriate digestive microbiota (Mattioli *et al*., 2019). Moreover, the alfalfa particle size affects methane production. A finer particle size favors the growth of the methane-producer genus *Methanobrevibacter* and the growth performance of rabbits (Liu *et al*., 2018). Besides, the protein concentration of the food has an important effect on rabbit GIT health (Carabaño *et al*., 2009). Nutritional studies have shown that a reduction of protein content (Chamorro *et al*., 2007) and arginine supplementation (Chamorro *et al*., 2010) reduced mortality and the abundance of *Helicobacter* and *Clostridium* species.

The use of diets supplemented with antibiotics has generated substantial controversy in recent years because of the risks associated with the presence of residues in animal products. On one hand, the presence of antibiotics in the animal GIT could select resistant bacteria, which could be transferred to other animals or even humans (Barton, 2000). On the other hand, the administration of antibiotics before eight weeks of age may alter the rabbit GIT and the normal development of its immune system. As different studies reported, the effect of antibiotics on rabbit microbiota depends on the molecule used. The administration of bacitracin reduced the fermentation activity of the lactating female (Abecia *et al*., 2007), while the administration of apramycin and tylosin reduced mortality of rabbits after weaning, but also their microbial diversity (Chamorro *et al*., 2007). The administration of deoxynivalenol also reduced the microbial diversity of ileum, cecum, and colon in weaned rabbits (Wang *et al*., 2020).

As it has been already indicated, feed restriction is an effective way to protect the rabbit against enteropathy as an alternative to antibiotics. Other non-drug options include the use of prebiotics and/or probiotics. A prebiotic is a non-digestible food ingredient that positively affects the host by selectively stimulating the growth and/or activity of one or a limited number of intestinal bacteria (Gibson and Roberfroid, 1995). Most prebiotics are short-chain carbohydrates that cannot be hydrolyzed in the small intestine. Prebiotics are a fermentable substrate that led to the production of lactic acid and VFA by different modes of action: i) stimulating the growth of beneficial bacteria for the host, ii) masking the binding sites of pathogens to the mucosa, or iii) binding to pathogens (Combes *et al*., 2012). Studies assessing the influence of prebiotics in rabbits often show contradictory results for the same prebiotics (Falcão-e-Cunha *et al*., 2007).

The Food and Agriculture Organization of the United Nations defines a probiotic as a living microorganism that, when administered in adequate amounts, confers a health benefit on the host. The effect of probiotics on rabbit microbiota depends on the microorganism strains used and their capacity to maintain their metabolic activity in the GIT (Fonty and Gouet, 1989). The administration of *Lactobacillus acidophilus* (Amber *et al*., 2004) and *Clostridium butyricum* (Liu *et al*., 2019) increases the

abundance of cellulolytic bacteria. Dietary supplementation with *Bacillus subtilis* may improve rabbit growth, intestinal homeostasis, and strength innate immune response by enhancing the expression of β-defensin (Guo *et al*., 2017).

Finally, extreme housing hygiene conditions are suspected to be a risk factor for animal health (Madec *et al*., 1998) due to a delayed exposure to microorganisms (Bailey, 2010). Studies conducted in pigs have revealed that high hygiene environments have a negative effect on the normal succession of the GIT microbiota and immune system (Mulder *et al*., 2011; Inman *et al*., 2010; Le Floc'h *et al*., 2014). Conversely, low hygiene conditions failed to induce an inflammatory response in rabbits and affected their cecal microbiota, particularly genera belonging to family *Ruminococcaceae* (Combes *et al*., 2017). In spite of this, keeping a certain degree of cleanness in the barns is mandatory to keep a high biosecurity level (Kylie *et al*., 2017).

Chapter four explores the effect of the production environment and different management practices on the cecal microbiota of growing rabbits.

1.4.4. Genetic determinism of gastrointestinal microbiota

Heritability is the extent to which the total phenotypic variation for a trait is attributable to genetic rather than environmental factors. A fundamental question is how strongly the microbiota is genetically inherited as opposed to being shaped by the environment. In the previous sub-section, the large role played by environmental factors on GIT microbial composition and diversity has been proved. However, the role of host genetics is still a source of debate since several studies have reported evidence of a certain effect of host genetics on the observed variation of the humans and mice microbiomes. Other studies, however, did not report such host genetic effects on the variation of the microbiomes (Turnbaugh *et al*., 2009; Yatsunenko *et al*., 2012). However, it is worth mentioning the low sample sizes of these studies and the fact that they considered broad microbial measures instead of individual taxa.

More recent studies have attempted to estimate the heritability for microbiomes of different species and in different sampling regions of the GIT using different methods. Goodrich *et al*. (2014) estimated heritabilities for individual taxa using fecal samples from more than 400 pairs of monozygotic and dizygotic human twins, demonstrating that the overall microbial composition was more similar between monozygotic twins than dizygotic twins. Moreover, many taxa mainly belonging to phylum *Firmicutes* were heritable. Another study conducted in the Hutterites, which are a human religious population that lives in North America and eats communally, identified 15 heritable taxa mainly encompassed by phyla *Firmicutes* and *Proteobacteria* (Davenport *et al*., 2015). Most of these taxa were common to those reported as heritable in the twins study previously conducted by Goodrich *et al*. (2014), demonstrating that certain taxa are consistently heritable irrespective of cultural and environmental differences between human populations (Davenport, 2016). A more recent re-analysis of the twins study with a larger number of individuals reported average heritabilities for bacterial taxa ranging between 0.02 and 0.08 (Rothschild *et al*., 2018). These results suggest that although certain taxa would be under host genetic control, the overall microbiota heritability is relatively low.

Some studies have also attempted to estimate the microbiota heritability in livestock. In dairy cattle, Sasson *et al*. (2017) suggested that certain taxa inhabiting the rumen would be highly heritable. Further, Roehe *et al*. (2016) reported that the archaeal abundance in ruminal digesta would also be under host genetic control. Nevertheless, Difford *et al*. (2018) indicated that host genetics influencing the rumen microbiota and methane emission would be independent of each other, so breeding for low methane emitting cows is unlikely to result in unfavorable changes in the rumen microbiome. In pigs, few studies have reported heritabilities for different microbial taxa with estimates ranging from low to moderate (Camarinha-Silva *et al*., 2017; Yang *et al*., 2016), suggesting a partial genetic control of the microbial populations inhabiting the pig gut. A more recent study attempted to summarize the overall microbial composition of individuals through alpha-diversity indexes (Lu *et al*., 2018). This study reported moderate heritability estimates for alpha diversity (0.10-0.40). In rabbits, the influence of host genetics on microbial diversity and

specific microbial taxa remains unknown, so the present thesis has aimed at shedding light on this regard (see Chapter six).

Once the heritability of a trait is reported, the genuine next step is to identify the genomic regions and candidate genes involved in the variation of the phenotype. In this regard, several GWAS studies have been conducted to identify host genetic variants associated with gut microbiota. The first GWAS with this aim were conducted in mice and identified several QTLs associated with the abundances of certain taxa present in the stool (Benson *et al*., 2010; Leamy *et al*., 2014; Org *et al*., 2015). It is worth mentioning that genera for which these studies reported significant associations were taxa reported as heritable (e.g., members of phylum *Firmicutes*) by human gut microbiota studies (Goodrich *et al*., 2014; Davenport *et al*., 2015). In humans, the first microbial GWAS of the microbiota from 15 different body sites (Blekhman *et al*., 2015) reported associations with genes involved in immune and signaling functions. This suggests that cellular mechanisms, such as immune response and cell-to-cell signaling, may play a role in the heritability of gut microbiota. In pigs, Cheng *et al*. (2018) reported two QTL regions that could potentially control the abundance of particular taxa, while Crespo-Piazuelo *et al*. (2019) identified 17 genomic regions associated with the abundance of genera *CF231*, *Phascolarctobacterium*, *Prevotella*, *Streptococcus, Akkermansia*, *and SMB53*.

In rabbits, no GWAS to identify the genomic regions involved in the host genetic control of gut microbiota has been reported in the literature to this day. The first microbial GWAS of the cecal rabbit microbiota is presented in this thesis (see Chapter seven) thanks to the current availability of an array commercialized by Affymetrix that contains almost 200,000 SNPs and an improved version of the OryCun2.0 reference assembly of the rabbit genome (Carneiro *et al*., 2014). Besides that, microbial GWAS are also being conducted at INRA using rabbits from a factorial design aiming at disentangling the maternal transmission of gut microbiota from the direct genetic effect of the animal in a cross-fostering trial between and within rabbit lines selected or not for FE.

1.4.5. Relationship between microbiota and feed efficiency

The existing link between GIT microbiota and complex phenotypes, principally related to health, has a rich body of literature in humans (Cho and Blaser, 2012; Clemente *et al*., 2012; Henry *et al*., 2021). In the field of livestock production, a growing number of studies have hypothesized that the GIT microbiota could be associated with growth, complex traits related to FE, immunocompetence, or methane, nitrogen, and phosphorous emissions.

In ruminants, recent findings have emphasized the association of the composition and function of the rumen microbiome with traits of economic interest such as methane emission and FE. Methane is an outstanding greenhouse gut associated with ruminant production involved in global warming (Wallace *et al*., 2017). Besides its negative environmental impact, methanogenesis implies a loss of 2-12% of dietary energy responsible for a reduction of the host FE (Johnson and Johnson, 1995). Given the close link between methanogenesis and cattle rumen, research on their microbial communities has been conducted for the last years to improve cattle FE and reduce environmental impact (Myer *et al*., 2015). One can imagine that the rumen microbiome may have an important role in FE since rumen microorganisms actively participate in the conversion of feed into energy. In fact, recent studies in dairy and beef cattle reported lower rumen microbial diversity and richness in efficient animals (Shabat *et al*., 2016; Li and Guan, 2017). On the other hand, bacterial families *Lachnospiraceae* and *Veillonellaceae* (Li and Guan, 2017; Myer *et al*., 2015) and archaeal taxa like *Methanomassiliicoccale* and *Methanobrevibacter* (Carberry *et al*., 2014a; Carberry *et al*., 2014b) have been reported to be associated with FE. However, some inconsistencies have been reported between studies that may be due to the influence of dietary composition (Durunna *et al*., 2011). These inconsistencies suggest that the association between the rumen microbiota and host FE may be partially driven by diet. Nevertheless, other studies reported diet-independent effects of the rumen microbiota on FE (Hernandez-Sanabria *et al*., 2012; Ellison *et al*., 2017; Carberry *et al*., 2012). Thus, it could be hypothesized that core microorganisms would be associated with variation in FE irrespective of diet.

With regard to monogastric species, the main interest lies in growth and phenotypes related to FE. It is reasonable to suggest that GIT microbiota is likely to impact these traits. If it was the case, potential manipulation of microbiota to improve animal growth and FE could have economic and environmental benefits. In pigs, recent studies have investigated the early establishment of gut microbial communities (Mach *et al*., 2015) and identified enterotypes related to growth (Ramayo-Caldas *et al*., 2016). Moreover, Lu *et al*. (2018) reported an association between growth and specific microbial taxa as well as alpha-diversity. Despite, the challenges in identifying reliable associations, several studies reported an association of genera *Treponema*, *Methanobrevibacter*, and *Lactobacillus* with FE (Yang *et al*., 2016; Valeriano *et al*., 2017; Bergamaschi *et al*., 2020; McCormack *et al*., 2017; Quan *et al*., 2018). With regard to rabbits, recent studies reported associations of gut microbiota with growth (Zeng *et al*., 2015; Fang *et al*., 2020b) and FE (Drouilhet *et al*., 2016).

In this context, some studies have gone one step further by exploring the contribution of microbiota to the phenotypic variation of complex traits. In this regard, Difford *et al*. (2018) introduced the concept of microbiability to account for the overall microbial composition as part of phenotypic variation. To model the microbial effect with a linear mixed model is necessary to define a microbial relationship matrix. Briefly, Difford *et al*. (2018) proposed a variance-covariance matrix as $\mathbf{M} = \frac{\mathbf{XX}'}{\mathrm{n}}$, where **X** is the matrix of the log-transformed microbial relative abundance for all animals and n is the number of taxa within the population. Thus, the predictions of individual effects would represent the overall microbial effect for each animal, and the ratio of variance explained by the microbial effect over the total phenotypic variance is the microbiability of the trait. It is nevertheless important to bear in mind that microbiability reflects an environmental component of the total phenotypic variation of a trait, so it does not have a genetic interpretation. Motivated by Difford *et al*. (2018), other studies have estimated microbiabilities for traits related to growth and FE traits in pigs (Camarinha-Silva *et al*., 2017) and Japanese quails (Vollmar *et al*., 2020). The microbiability estimates for growth and FE traits reported by these studies ranged from 0.09 to 0.28.

Furthermore, the microbiota can be seen as a potential source of information of animal performance. The literature on the power of microbial information to predict complex phenotypes in livestock is scarce and non-existent in rabbits. The role and the predictive value of rabbit cecal microbiota on growth and FE are discussed in Chapter five.

1.4.6. Microbiome data peculiarities and analytics challenges

It is undeniable that the possibility of analyzing the microbiome of diverse organisms and environments offered by NGS techniques has enhanced the understanding of the metabolic and physiological roles of microorganisms. Such advances have transformed the scientific capacity to investigate the composition and diversity of complex microbial communities that inhabit mammals GIT. It has resulted in a boom of studies, but the interpretation across studies is hindered by the lack of standardization in the laboratory protocols, bioinformatics, and statistical procedures followed by different research groups. This lack of a standardized analytical approach has led to concerns about reproducibility and reliable comparisons across studies.

16S rRNA gene amplicon sequencing has been the technique most used to study complex microbiomes. This approach relies on PCR amplification, and it is necessary to consider that this step can introduce bias related to the pair of primers (Klindworth *et al*., 2013), target region (Woo *et al*., 2008; Yu *et al*., 2008), GC content (Aird *et al*., 2011; Benjamini and Speed, 2012), or the input DNA concentration (Rintala *et al*., 2017). Despite the enormous advancements in sequencing and computational analyses, many factors can origin biases and errors. This section shows an overview of experimental, sequencing, computational, and analytics challenges in the microbiome field.

An appropriate study design could help to reduce confounding effects and to improve data processing. A rationalized study implies a sufficiently large sample size, the use of controls to identify real signals, and the generation of complete metadata containing details of all the samples used for the experiment (Martin *et al*.,

2018). On the other hand, the handling of samples once collected is also important because it can become a confounding factor that might affect the results and interpretations of the study (Thomas *et al*., 2012). In this regard, it is very important to avoid external contaminations during sample collection using aseptic laboratory tools (Salter *et al*., 2014). It is crucial to freeze the sample immediately after its collection to preserve the quantity and quality of microbial DNA (Cuthbertson *et al*., 2014) and maintain proper storage conditions (Choo *et al*., 2015; Tap *et al*., 2019). The choice of the method for DNA extraction is crucial to capture the largest number of different microorganisms. In this regard, mechanical lysis (i.e., bead beating) leads to better yields, so its application is recommendable before standard extraction (Albertsen *et al*., 2015). Amplicon-based NGS approaches rely on amplification with barcode primer pairs, purification, and generation of libraries before sequencing. Naturally, these steps are also potential sources of variation and bias. Furthermore, the different sequencers used for microbiome studies display different kinds of sequencing errors (Minoche *et al*., 2011) that, for instance, can lead to the underrepresentation or absence of some bacteria. The use of a positive control could help to prevent this issue.

The bioinformatic pipeline employed to process the sequencing data can impact the characterization of microbial diversity and composition. There are no agreed standards for raw reads processing, and the default parameters of sequence analysis software often need to be tuned. One of the main sequencing challenges is to discriminate between sequencing errors from real sequences. Two principal methods are used for this purpose: operational taxonomic unit (OTU)-based tools such as QIIME or Mothur (Caporaso *et al*., 2010; Schloss *et al*., 2009), and amplicon sequence variant (ASV)-based tools such as DADA2 (Callahan *et al*., 2016). The first strategy resolves sequencing errors by clustering the reads based on a similarity threshold, commonly 97%, into OTUs (Westcott and Schloss, 2015), while the ASV-based method uses a denoising approach that exploits the predictable structure of certain error types to attempt to reassign or eliminate noisy reads (Tikhonov *et al*., 2015). A comparative study between both approaches pointed out that OTUs provide lower taxonomic resolution (Callahan *et al*., 2017). During the development of the present thesis, both strategies were applied to rabbit

microbiome data. The comparison of results obtained with both approaches showed that the OTU-based method worked better for this data, which might be due to high variability in the number of reads between samples. Another issue regarding the taxonomic assignment is the errors, including contaminants, contained in the reference sequence databases (Sheik *et al*., 2018).

The particularities of microbiome data raise serious challenges for statistical analysis. For instance, the large inter-individual variability, heteroscedastic variation (i.e., variance increasing with mean abundance), and large biological and technical variations are often not properly approximated by classical Gaussian or log-normal models, requiring customized analytical approaches (Moreno-Indias *et al*., 2021). Microbiome data are skewed and sparse (i.e., only a few taxa are common to most samples, whereas the rest of taxa are rare, and zeros dominate all other values). But there is an additional challenge to distinguish whether these zeros are structural (i.e., the microorganism is absent in most samples) or sampling (i.e., the microorganism is present, but the sequencing depth is insufficient to detect it). In many cases, zero inflation can entail biased estimates for some available statistical methods and modeling approaches. Appropriate modeling of high proportions of zeros is an active area of research since it plays an important role concerning sensitivity, specificity, and accuracy of differential abundance analysis depending on normalization and statistical methods (Pan, 2021).

A critical issue inherent to microbiome data is that sequencing read depths are not uniform across samples due to the experimental and sequencing factors above mentioned (Nayfach and Pollard, 2016). In order to be able to conduct meaningful comparisons across samples and studies, accounting for such sequencing differences through a proper normalization is essential (Badri *et al*., 2020). Hence, the purpose of the normalization is to correct for sampling bias and library size variability. Total sum scaling (TSS) is a user-friendly normalization for count data consisting of dividing each read count by the total number of reads, transforming the counts into proportions. The problem is that this transformation produces relative abundances that are compositional and, thus, subsequent statistical analysis can lead to spurious results and interpretations (Silverman *et al*., 2017). Another widely

used normalization strategy is rarefaction that consists of a random subsampling so that all samples have the same number of reads (Hughes and Hellmann, 2005). The main problem of this approximation is the loss of valid information due to the need to discard valid reads to standardize the library to a constant size across samples (McMurdie and Holmes, 2014). In this case, although the records are numerical counts, all sum up to a defined constant. Thus, rarefaction does not solve the compositional problem either. On the other hand, cumulative sum scaling (CSS) is a normalization method based on the division of raw counts by the cumulative sum of counts up to a given percentile determined by a data-driven approach (Paulson *et al*., 2013). CSS was designed specifically for microbiome sequencing data. However, the determination of the percentiles can fail due to the high-count variability, so normalization techniques that are robust to sparsity need to be explored.

Other transformations like the centered log-ratio (CLR) seem to be more appropriate to deal with compositional data (Aitchison, 1982). However, the large sparsity of microbiome data prevents the log transformation of zero denominators. A trick to perform log transformation is to replace the zeros by an arbitrary pseudo-count (i.e., a small value), but this means assuming that all zeros are caused by undersampling (McMurdie and Holmes, 2014). Moreover, the choice of the pseudo-count is not based on any rigorous statistical foundation, and several studies have shown that this strategy introduces substantial biases and that different pseudo-counts can generate very different results (Silverman *et al*., 2020; Costea *et al*., 2014). Another issue related to these transformations is that the interpretation of the results could be difficult if they must be linked to the original scales.

In summary, recent advances in sequencing technologies to explore microbiome data have motivated the development of new methods, algorithms, and computational tools. Nevertheless, the complexities inherent to microbiome data together with the lack of standardized experimental, sequencing, and analytical approaches hampers the interpretation and comparison between results from different studies. There is an imperative need for comprehensive discussion among the scientific community to standardize sample collection, storage, processing,

sequencing, and data analyses protocols. The statistical treatment of microbiome data throughout the whole thesis has intended to be as rigorous as possible.

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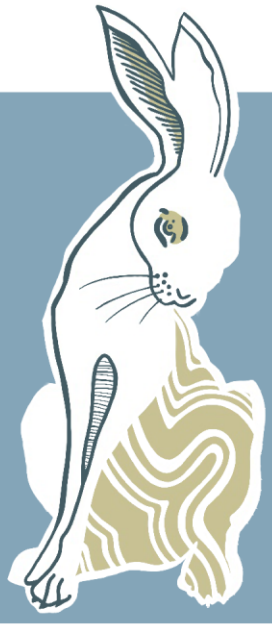
1.6. List of abbreviations

ADF	acid detergent fiber
ADFI	individual average daily feed intake
ADG	average daily gain
ADG_{AL}	average daily gain recorded in animals fed *ad libitum*
ADG_{R}	average daily gain recorded in animals fed under restriction
ASV	amplicon sequence variant
BLAST	basic local alignment search tool
bp	base pair
CSS	cumulative sum scaling
CLR	centered log-ratio
DE	digestible energy
DGGE	denaturing-gradient gel electrophoresis
ERE	epizootic rabbit enteropathy
FCR	feed conversion ratio
FE	feed efficiency
FI	feed intake
FISH	fluorescence *in situ* hybridization
GALT	gut-associated lymphoid tissue
GIT	gastrointestinal tract
GWAS	genome-wide association study
HPLC	high-performance liquid chromatography
NGS	next-generation sequencing
OTU	operational taxonomic unit
qPCR	quantitative polymerase chain reaction
QTL	quantitative trait locus
RFI	residual feed intake
SNP	single nucleotide polymorphism
T-RFLP	terminal restriction fragment length polymorphism
TSS	total sum scaling
VFA	volatile fatty acids

CHAPTER 2

OBJECTIVES



The main objective of this PhD thesis was to generate knowledge about the influence of meat rabbit cecal microbiota on the host's feed efficiency and unravel the environmental and genetic bases of composition and diversity of microbial communities inhabiting the rabbit cecum. The specific aims were:

I. To characterize and compare the microbial diversity and composition of hard feces and cecum content of individuals from a paternal rabbit line fed with different intake levels.

II. To describe the influence of environmental factors (i.e., breeding farm, level of feeding, and administration of antibiotics) on diversity and composition of rabbit cecal microbial communities.

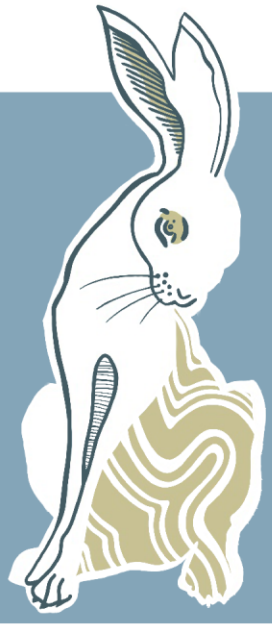
III. To gain insight into the role of rabbit cecal microbiota on complex phenotypes of economic interest and to assess its value to predict cage feed efficiency and individual growth performances.

IV. To evaluate the influence of genetic, litter, and cage effects on different microbial traits representing rabbit cecal microbiota at different levels of depth using Bayesian linear and zero-inflated Poisson mixed models.

V. To identify the host genomic regions involved in the control of rabbit cecal microbial composition and diversity.

CHAPTER 3

RABBIT MICROBIOTA CHANGES THROUGHOUT THE INTESTINAL TRACT



Explanatory note

This article is somehow related to the Master's thesis "Caracterización del microbioma digestivo de una línea de conejo sometida a dos tratamientos alimentarios diferentes" developed by María Velasco Galilea during 2016 (http://hdl.handle.net/10251/74486) since the main objective of both was the characterization of the microbial communities of the rabbit cecum and feces.

There are, however, clear and important differences between them. In the Master's thesis, differences regarding the animals categorized by the combination of sample origin (i.e., feces or cecum) and feeding regime were studied through alpha-diversity indexes and principal coordinate analysis. Thus, a preliminary study of the effect of the level of feeding and the origin of the samples on the rabbit microbiota was conducted. Moreover, the work developed during the Master's thesis was useful to fine-tune the MiSeq technology and a bioinformatics pipeline for sequence processing.

In the article presented in this chapter, the bioinformatics pipeline tuned during the Master's thesis was used to characterize the microbiota of feces and cecum from 21 rabbits, but the statistics and methodology behind this study are very different. On one hand, paired analyses of variance (i.e., to account for the paired structure of the data given two types of samples were collected from each individual) with bootstrap were conducted at different taxonomic levels to detect differences in taxonomic compositions between sample origins.

On the other hand, different multivariate techniques (PCA, PCoA, and sPLS-DA) were explored to assess the existence of differences between cecal and fecal microbial communities as a whole, taking into account the dependency between taxa.

Article I

frontiers in Microbiology

ORIGINAL RESEARCH
published: 13 September 2018
doi: 10.3389/fmicb.2018.02144

Rabbit microbiota changes throughout the intestinal tract

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Frontiers in Microbiology (2018), *9*, p. 2144

Rabbit microbiota changes throughout the intestinal tract

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3.1. Abstract

To gain insight into the importance of carefully selecting the sampling area for intestinal microbiota studies, cecal and fecal microbial communities of Caldes meat rabbit were characterized. The animals involved in the study were divided in two groups according to the feed intake level they received during the fattening period; *ad libitum* (n = 10) or restricted to 75% of *ad libitum* intake (n = 11). Cecum and internal hard feces were sampled from sacrificed animals. Assessment of bacterial and archaeal populations was performed by means of Illumina sequencing of 16S rRNA gene amplicons in a MiSeq platform. A total of 596 OTUs were detected using QIIME software. Taxonomic assignment revealed that microbial diversity was dominated by phyla *Firmicutes* (76.42%), *Tenericutes* (7.83%) and *Bacteroidetes* (7.42%); kingdom *Archaea* was presented at low percentage (0.61‰). No significant differences were detected between sampling origins in microbial diversity or richness assessed using two alpha-diversity indexes: Shannon and the observed number of OTUs. However, the analysis of variance at genus level revealed a higher presence of genera *Clostridium*, *Anaerofustis*, *Blautia*, *Akkermansia*, *rc4-4* and *Bacteroides* in cecal samples. By contrast, genera *Oscillospira* and *Coprococcus* were found to be overrepresented in feces, suggesting that bacterial species of these genera would act as fermenters at the end of feed digestion process. At the lowest taxonomic level, 83 and 97 OTUs in feces and cecum, respectively, were differentially represented. Multivariate statistical assessment revealed that sparse partial least squares discriminant analysis (sPLS-DA) was the best approach for this purpose. Interestingly, the majority of the most discriminative OTUs selected by sPLS-DA were found to be differentially represented between sampling origins in univariate analysis. Our study provides evidence that the choice of intestinal sampling area is relevant due to important differences in some taxa's relative abundance that have been revealed between rabbits' cecal and fecal microbiota. An appropriate sampling intestinal area should be chosen in each microbiota assessment.

Keywords: gut microbiota, fecal microbiota, cecal microbiota, feed restriction, meat rabbit, paired analysis, multivariate approaches, 16S Illumina sequencing.

3.2. Introduction

Microbial populations that inhabit animals' gastrointestinal tract constitute their microbiota: a complex ecosystem, able to autoregulate its own homeostasis. It is well known that a mammal's intestinal microbiome plays a very important role in metabolic, nutritional, physiological and immunological processes (Flint *et al*., 2012) but also in farm animal's productivity (Heinrichs and Lesmeister, 2005; Drouilhet *et al*., 2016). A symbiotic relationship is therefore established between the host and its intestinal microbiota. The emergence of next generation sequencing (NGS) techniques together with an increasing reliability of reference taxonomic databases such as SILVA (Yilmaz *et al*., 2013), RDP (Wang *et al*., 2007) or Greengenes (McDonald *et al*., 2012) have allowed a deeper knowledge of the influence that intestinal microbiome exerts on host animals.

In the case of the rabbit, the physicochemical properties of its gastrointestinal tract (near neutral pH, high humidity and stable temperature around 35-40 ºC) promote the rapid growth of mutualistic microbiota while the animal gets the bacterial fermentation end-products of some materials that cannot be degraded by the host on its own (Mackie, 2002). In these conditions, rabbit intestinal microbiota contains 100-1000 billions of microorganisms per gram covering over 1000 different species, predominating kingdom *Bacteria* over archaeal populations (Combes *et al*., 2011). Despite the demonstrated existence of active microbial populations in proximal and distal segments of rabbit gastrointestinal tract (Gouet and Fonty, 1979), cecum is the main fermenter organ. For this reason, most studies that aimed to study rabbit's intestinal microbiota have been focused on the characterization of cecal microbial communities (Abecia *et al*., 2005; Bäuerl *et al*., 2014; Kušar and Avguštin 2010). Cecal microbiota of rabbit and other lagomorph species is dominated by phylum *Firmicutes* while cecal microbiota of rodents, a relatively close mammalian order, is dominated by phylum *Bacteroidetes* (Li *et al*., 2017).

In other monogastric livestock species, such as chicken and pig, previous studies have characterized the differences between their cecal and fecal microbiotas (Oakley and Kogut, 2016; Fang *et al*., 2017; He *et al*., 2016). Crowley *et al*. (2017)

compared the microbial composition from different organs of the digestive tract (stomach, jejunum, cecum, appendicular cecum, proximal colon, distal colon and rectum) in wild rabbits and they found that the different physicochemical properties of each compartment restrict or promote the growth of specific microbial populations. However, little is known about the differences in the composition of the microbial communities that inhabit the domesticated rabbit cecum and feces.

The objective of this study was to characterize and compare the microbial communities of hard feces and cecum content collected from two groups of animals from a meat rabbit line fed with different intake levels. Our results will help establish whether feces could be considered a proxy indicator to assess composition and diversity of intestinal microbiota. This will be particularly important for those studies that require a monitoring of the microbiota over time in order to avoid the manipulation of the animal's gastrointestinal tract that could alter its microbial composition.

3.3. Materials and Methods

3.3.1. Experimental design and sampling

The sampling materials from animals used in this work came from an experiment conducted at the Institute for Food and Agriculture Research and Technology (IRTA) between July 2012 and July 2014. This experiment was developed to estimate the effect of the interaction between the genotype and the feeding regime on growth, feed efficiency, carcass characteristics and health status of the animals. Towards this aim, 7,864 animals from Caldes line (Gómez *et al*., 2002), selected since the 1980's to increase the average daily gain during the fattening period (32-66 days of age), were controlled since weaning. Animals were housed in 969 collective cages, with a surface of 0.38 m^2, containing eight rabbits each one. All animals in this experiment were bred under the same management conditions and fed with the same standard pellet diet supplemented with antibiotics (oxytetracycline, valnemulin and colistin), except during the last fattening week, when an antibiotic free food was provided. During the 5 weeks that the fattening period lasted, food was supplied

once per day in a feeder with three places. Details of food composition can be found in **Table 3.1**. Water was also provided *ad libitum* during the whole fattening period.

Table 3.1| Feed composition on a wet basis.

Component	Amount
Crude fiber (%)	18.70
Crude protein (%)	15.02
Ashes (%)	8.97
Ether extract (%)	3.28
Oxytetracycline (mg/kg)	400
Valnemulin (mg/kg)	30
Colistin (mg/kg)	100

The average daily feed intake in one *ad libitum* cage was 0.17 kg/day/rabbit which implies 66.48 mg/rabbit of oxytetracycline, 4.99 mg/rabbit of valnemulin and 16.62 mg/rabbit of colistin. The average daily feed intake in one restricted cage was 0.13 kg/day/rabbit which implies 49.86 mg/rabbit of oxytetracycline, 3.74 mg/rabbit of valnemulin and 12.47 mg/rabbit of colistin.

The animals were under two different feeding regimes: 1) *ad libitum* (V) or 2) restricted (R) feeding to 75% of the *ad libitum* feed intake. The amount of food provided to the animals under R feeding regime in a given week for each batch was obtained as 0.75 times the average feed intake of kits on V from the same batch during the previous week, plus 10% corresponding to the estimated increase of feed intake as the animal grows.

Kits were randomly assigned to one of these two feeding regimes after weaning (32 days of age). They were categorized into two groups according to their size (“big” if body weight at weaning was greater than 700 g or “small” otherwise) in order to obtain homogenous groups regarding animal size within each feeding regime. A maximum of two kits of the same litter were assigned to the same cage, aiming to remove the possible association between cage and maternal effects on animal growth during the fattening period. For this particular study 23 rabbits from the aforementioned experiment were randomly selected. Their distribution across the different levels of factors is shown in **Table 3.2**.

Table 3.2| Distribution of animals in groups according to feeding regime and size.

Feeding regime	[a]Size	Number of animals
Restricted	Small	4
	Big	9
Ad libitum	Small	1
	Big	9

[a]Animals classified according to their size at weaning: "big" if body weight was greater than 700 g or "small" otherwise.

At slaughtering (66 days of age) hard feces and cecum samples were collected from each animal, kept cold in the laboratory (4ºC) and immediately stored at -80°C until total genomic DNA extraction.

3.3.2. DNA extraction, library generation and sequencing

The extraction of total genomic DNA was performed by means of a bead-beating protocol (kit ZR Soil Microbe DNA MiniPrep™-ZymoResearch, Freiburg, Germany) following manufacturer's recommendations. A total of 250 mg of each cecal and fecal samples was submitted to a mechanical lysis in a FastPrep-24™ Homogenizer (MP Biomedicals, LLC, Santa Ana, USA) at a speed of 1x6 m/s for 60 seconds allowing an efficient lysis of archaea and Gram-positive and negative bacteria species. Purity and integrity of total DNA from each sample was checked in a Nanodrop ND-1000 spectrophotometer equipment (NanoDrop products; Wilmington, USA) following the protocol described by Desjardins and Conklin (2010). All extracts had a proper purity (> 1.6, according to absorbance ratio 260 nm/280 nm) to avoid polymerase chain reaction (PCR) inhibition issues during downstream PCR and sequencing steps.

The V4-V5 hypervariable region of total genomic DNA was amplified with specific primers and then re-amplified in a limited-cycle PCR reaction to add sequencing adaptors and 8 nt dual-indexed barcodes of multiplex Nextera® XT kit (Illumina, Inc., San Diego CA, USA) according to manufacturer's instructions. The initial PCR reactions were performed for each sample (23 cecal and 23 fecal) using 12.5 µl of 2x KAPA HiFi HotStart Ready Mix, 5 µl of each PCR primer: forward universal primer

515Y (5'-GTGYCAGCMGCCGCGGTAA-3') and reverse universal primer 926: (5'-CCGYCAATTYMTTTRAGTTT-3') (Parada *et al*., 2016) and 2.5 µl of microbial DNA (5 ng/µl). The initial thermal cycling procedure consisted of an initial denaturation step at 95ºC for 3 min, followed by 25 cycles of 95ºC for 30 s, 55ºC for 30 s and 72ºC for 30 s, and a final extension of 72ºC for 5 min. The second thermal cycling procedure added the indexes and sequencing adaptors to both ends of the amplified regions by using 25 µl of 2x KAPA HiFi HotStart Ready Mix, 5 µl of each index (i7 and i5), 10 µl of PCR Grade water and 5 µl of the first PCR product. The procedure consisted of an initial denaturation step at 95ºC for 3 min, followed by 8 cycles of 95ºC for 30 s, 55ºC for 30 s and 72ºC for 30 s, and a final extension of 72ºC for 5 min. Final libraries were cleaned up with AMPure XP beads, validated by running 1 µl of a 1:50 dilution on a Bioanalyzer DNA 1000 chip (Agilent Technologies, Inc., Santa Clara CA, USA) to verify its size, quantified by fluorometry with PicoGreen dsDNA quantification kit (Invitrogen, Life Technologies, Carlsbad, CA, USA), pooled at equimolar concentrations and paired-end sequenced in parallel in a MiSeq Illumina 2x250 platform at the Genomics and Bioinformatics Service (SGB) of the Autonomous University of Barcelona.

3.3.3. Bioinformatics - sequence processing

The resulting paired-ended V4-V5 16S rRNA gene reads were assembled into contigs with the python script *multiple_join_paired_ends.py* by using QIIME software (version 1.9.0) (Caporaso *et al*., 2010). Then the contigs were curated using the QIIME script *split_libraries.py* with default parameters in order to assign contigs to samples and to remove low-quality (minimum quality score < Q19) contigs. UCHIME algorithm (Edgar *et al*., 2011) was used to remove chimeric sequences generated during the process of DNA amplification. The totality of filtered contigs were clustered into operational taxonomic units (OTUs) with a 97% similarity threshold using the QIIME script *pick_open_reference_otus.py* with default parameters (Rideout *et al*., 2014) that grouped, through UCLUST algorithm (Edgar, 2010), sequences against Greengenes reference database (version gg_13_5_otus) and also made a *de novo* clustering of those that did not match the database. The generated OTU table was filtered at: 1) sample level by discarding samples with

less than 5,000 final contigs and at 2) OTU level by removing OTUs with less than 0.01% counts across samples. Finally, OTU table was normalized using the Cumulative Sum Scaling (CSS) method proposed by Paulson *et al*. (2013) yielding the normalized abundances of 596 OTUs for 43 samples. Note that three samples (cecal and fecal collected from one rabbit of size class “big” fed under restriction and cecal from another rabbit also of size class “big” and fed under restriction) did not pass the established threshold defined during the edition and quality control processes. In addition to this, in order to always keep parity between samples, i.e., for each animal to have both cecal and fecal samples, one fecal sample (from a rabbit of size class “big” fed under restriction) passing quality control was finally discarded for the next statistical analyses. Therefore, final analyses comprised of both types of samples (hard feces and cecum) from 21 animals. Taxonomic assignment of representative sequences of each OTU defined (596) was conducted by mapping them to the Greengenes reference database gg_13_5_otus with the UCLUST consensus taxonomy assigner (QIIME default parameters). The raw sequence data were deposited in the sequence read archive of NCBI under accession no (SRP149070).

3.3.4. Statistical analysis

3.3.4.1. Alpha-diversity and univariate statistical analysis

In order to compare diversity and richness between fecal and cecal communities, the Shannon and the observed number of OTUs (the count of unique OTUs found in a sample) indexes were computed after OTUs normalization at 15,000 contigs. The statistical method used for the communities’ comparison was a paired samples analysis of variance that included the following factors: sampling origin (feces/cecum), feeding regime (*ad libitum*/restricted), the interaction between them and the animal from which the samples were collected. The significance threshold was set at 0.05 type I error.

Differences in OTUs composition between cecal and fecal samples were estimated for those OTUs detected in at least 5% of the samples. For this purpose, analyses of variance were implemented by fitting a model defined by the factors sampling

origin (feces/cecum), feeding regime (*ad libitum*/restricted) and the animal from which the samples were collected. Consideration of the animal effect into the model allowed for accounting for the paired structure of the data. The effect of the sampling origin was assessed as the differences between the expected OTUs counts in both cecum and feces. Significance of the sampling origin was based on the F statistic, but instead of defining the threshold for declaring significance based on the theoretical F distribution, empirical bootstrap p-values were computed after 1,000 resamples. The use of bootstrapping allowed inferences to be made from the results obtained without the need for assuming that data are normally distributed. In this case, the p-value was defined as the proportion of bootstrap rounds showing an F statistic value equal or greater than that obtained with the original data set. P-values were corrected defining a false discovery rate (FDR) of 0.05 (Benjamini and Hochberg, 1995).

This bootstrap analysis of variance approach was also implemented to study the effect of the sampling origin on the relative abundance of bacteria at phylum and genus levels.

3.3.4.2. Multivariate statistical analysis

In addition to the univariate paired analysis of variance, three multivariate analyses were performed to assess whether there were differences between cecal and fecal communities as a whole, taking into account the dependency between OTUs. The first one was a descriptive analysis using principal coordinate analysis (PCoA) (Gower, 1966) on weighted Unifrac phylogenetic distance matrix (Lozupone and Knight, 2005). The second analysis was also a descriptive technique, principal component analysis (PCA) (Hotelling, 1933), but it was performed considering the paired structure of the data (Liquet *et al*., 2012). This was achieved by subtracting from the OTU count of a given sample the mean of the two samples belonging to the animal from which they were taken. The last multivariate method implemented was the sparse partial least squares discriminant analysis (sPLS-DA) which is a method based on partial least squares regression applied for classification. PLS consists in a multivariate regression which allows for the correlation of the

information contained in a predicting matrix to the information contained in a response matrix or vector (Burnham *et al*., 1996). In this case, the response was a vector which encoded the sampling origin that we aimed to predict from OTUs content. Moreover, sPLS includes a LASSO penalization to select the most informative predictors. sPLS-DA can simultaneously find, by maximizing the covariance between the predicting and the response matrices, the combination of OTUs which best discriminate samples according to their sampling origin and integrate both data sets in a one-step procedure (Lê Cao *et al*., 2008). In order to account for individual variation in the data, OTUs content was defined as deviations from individual means, as it was done for PCA. Unlike PCoA or PCA, sPLS-DA is not only a descriptive approach since it can infer which OTUs should be selected to perform the best discrimination of samples according to a given factor; the sampling origin in this study.

R packages “phyloseq”, “mixOmics” and “ggplot2” were employed for statistical analysis and plotting as elsewhere described (McMurdie and Holmes, 2013; Lê Cao *et al*., 2018; Wickham, 2010).

3.4. Results

3.4.1. Sequencing and processing

The sequencing process generated a total of 5,337,066 reads which, after different filtering steps and chimera removal, resulted in a total of 1,707,620 valid contigs. These final sequences were clustered into 596 non-singleton containing OTUs. Each sample had on average 40,657 final contigs (range: 16415-68080) and 482 OTUs (range: 411-541) (**Table 3.S1**).

3.4.2. Differences in diversity and richness between sampling origins

In this study, we found an average of 428 observed OTUs in cecum samples and 433 in feces samples. The estimated Shannon indexes were 4.66 and 4.67 in cecum and feces samples respectively (**Table 3.3**). The comparison of alpha diversities between fecal and cecal samples did not reveal any significant difference in

microbial diversity or richness at 15,000 contigs normalization (**Figure 3.1A**, *P* > 0.05) nor when both sampling origins were compared within feeding regime (**Figure 3.1B**, **Table 3.3**, *P* > 0.05). In contrast, the observed number of OTUs index showed significant differences between feeding regimes as the means estimated were 425 in restricted animals and 437 in *ad libitum* animals (**Table 3.3**, *P* = 0.03, p-value is not shown in table nor figures).

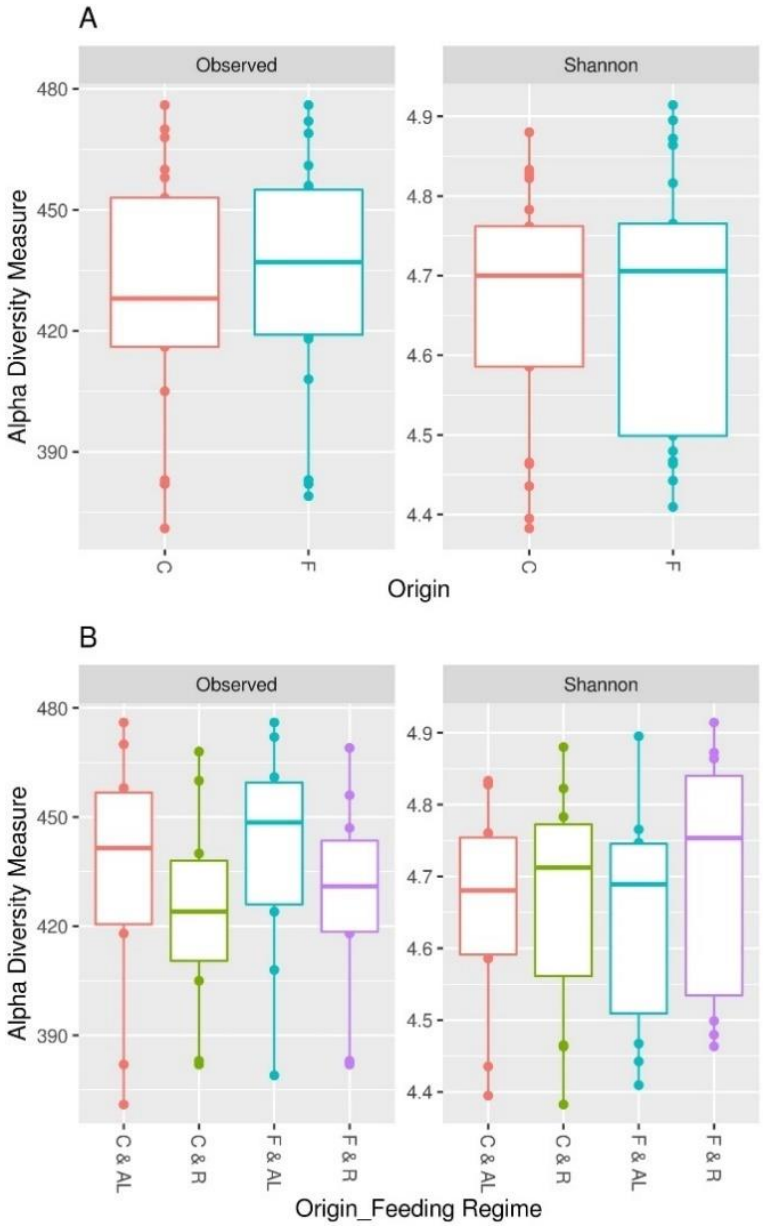

Figure 3.1| Microbial richness and diversity between cecum and feces samples. The intestinal microbial richness was estimated by the observed number of OTUs index, and the microbial diversity was studied by Shannon index. **(A)** Significant differences in microbial richness and diversity between cecum and feces samples were not identified (*P* > 0.05; paired samples analysis of variance). **(B)** Significant differences in microbial richness and diversity between cecum and feces samples in *ad libitum* or restricted rabbits were not identified (*P* > 0.05; paired samples analysis of variance).

Table 3.3| Estimated mean and standard deviation of observed number of OTUs and Shannon α-diversity indexes calculated in cecum and feces samples.

Feeding Regime	Index	Cecum samples	Feces samples	*P*
Restricted	Observed OTUs	423.91 (27.40)	427.91 (27.19)	0.73
	Shannon	4.67 (0.16)	4.69 (0.17)	0.70
Ad libitum	Observed OTUs	433.90 (35.08)	440.40 (30.25)	0.66
	Shannon	4.65 (0.15)	4.64 (0.16)	0.93
Average	Observed OTUs	428.67 (30.91)	433.86 (28.67)	0.58
	Shannon	4.66 (0.15)	4.67 (0.16)	0.81

3.4.3. Taxonomic characterization of cecum and feces microbial communities

The final OTU table encompassed 596 OTUs of which 307 were annotated in Greengenes database gg_13_5_otus and 289 corresponded to new reference OTUs constructed from a random sampling of sequences that did not map against the reference. 580 out of the 596 declared OTUs could be taxonomically assigned at kingdom level. All of them could be assigned at phylum and class levels, belonging to 8 and 12 different taxa, respectively. 577 OTUs could be assigned at order level to 13 different taxa. At family level, 308 OTUs could be assigned to 22 different taxa. 118 OTUs could be assigned at genus level to 23 different taxa while only 10 OTUs were taxonomically assigned at species level. It is important to stress that resolution of MiSeq technology in this study impaired taxonomic assignment capacity at family level since it was only possible in 51% of OTUs and, more drastically, at genus level allowing the assignment of only 20% of them. Nevertheless, given the large importance of functional roles played by bacteria that can be assigned at genus level, the analysis of differential representation of genera between the two sampling origins was conducted for those in which taxonomic assignment at this level was possible.

The two types of samples showed similar relative abundances for taxa and the predominant phyla were, in both cases, *Firmicutes* (present in an average percentage of 76.28 in feces and 76.55 in cecum), followed by *Tenericutes* (8.17 in feces and 7.48 in cecum) and *Bacteroidetes* (7.37 in feces and 7.46 in cecum) (**Table 3.4**). In spite of the small magnitude of the differences they reached significance in some cases ($P < 0.05$). As it can be observed in **Table 3.4**, phyla *Actinobacteria* and *Verrumicrobia* were found to be overrepresented in cecum samples, while *Cyanobacteria* and *Tenericutes* were overrepresented in feces. The only phylum belonging to kingdom *Archaea* that could be identified was *Euryarchaeota* which was presented in an average percentage of 0.61‰ in both sampling origins. All species of this phylum were taxonomically assigned to the methanogenic genus *Methanobrevibacter*.

Table 3.4| Microbial composition at phylum level in cecum and feces.

Phylum	Mean relative abundance in cecum (%) (SD)	Mean relative abundance in feces (%) (SD)	Difference Cecum-Feces ± SE	P_{FDR}
Actinobacteria	0.729 (0.097)	0.617 (0.119)	0.110 ± 0.023	0.000
Bacteroidetes	7.458 (1.243)	7.367 (1.263)	0.092 ± 0.090	0.473
Cyanobacteria	0.873 (0.440)	1.399 (0.670)	-0.514 ± 0.072	0.000
Euryarchaeota	0.061 (0.096)	0.062 (0.095)	-0.001 ± 0.011	0.928
Firmicutes	76.546 (1.733)	76.276 (1.809)	0.253 ± 0.170	0.215
Proteobacteria	1.613 (0.363)	1.634 (0.312)	-0.016 ± 0.043	0.783
Tenericutes	7.484 (0.899)	8.172 (1.057)	-0.681 ± 0.169	0.000
Verrucomicrobia	1.810 (0.378)	1.651 (0.300)	0.158 ± 0.034	0.000
Unknown	3.427 (0.433)	2.822 (0.674)	0.599 ± 0.092	0.000

The predominant classes in both sampling origins were *Clostridia* (76.14%), *Mollicutes* (7.54%) and *Bacteroidia* (7.41%). At family level, the predominant taxa were *Ruminococcaceae* (44.37%) and *Lachnospiraceae* (36.51%), both belonging to phylum *Firmicutes*. Finally, results contained in **Table 3.5** show that predominant genera were *Ruminococcus* (5.13%), *Oscillospira* (2.47%), *Bacteroides* (2.36%) and *Blautia* (2.10%). Paired samples analysis of variance implemented to study the effect of the sampling origin on the relative abundance of species at genus level revealed that 8 genera, out of the 23 in which taxonomic assignment was possible,

were differentially represented between feces and cecum. Genera *Clostridium*, *Anaerofustis*, *Blautia*, *Akkermansia*, *rc4-4* and *Bacteroides* were overrepresented in cecum while feces showed a higher relative abundance of genera *Oscillospira* and *Coprococcus* (**Table 3.5**). Genera *Anaerofustis* and *rc4-4* showed the smallest relative abundances (0.14 and 0.19% respectively) while the rest of the genera ranged between 1.21 and 2.48 %.

Paired bootstrap analysis of variance revealed that 180 OTUs showed abundances significantly different between sampling origins: 83 and 97 OTUs were overrepresented in fecal and cecal samples, respectively (**Table 3.S2** and **Table 3.6,** this last shows the 10 OTUs showing the strongest overrepresentation). In fecal samples these 83 overrepresented OTUs were assigned, at the lowest taxonomic level, to the candidate species *Eutactus* (1 OTU) and *Flavefaciens* (2 OTUs); candidate genera *Coprococcus* (3 OTUs), *Oscillospira* (7 OTUs) and *Ruminococcus* (3 OTUs); candidate families *Ruminococcaceae* (10 OTUs) and *S24-7* (4 OTUs); candidate orders *Clostridiales* (34 OTUs), *RF32* (1 OTU), *RF39* (10 OTUs) and *YS2* (7 OTUs); and candidate class *Alphaproteobacteria* (1 OTU). On the other hand, the 97 OTUs overrepresented in cecal samples were assigned to the candidate genera *Akkermansia* (4 OTUs), *Anaerofustis* (1 OTUs), *Blautia* (10 OTUs), *Clostridium* (4 OTUs), *Oscillospira* (1 OTU), *Phascolarctobacterium* (1 OTU) and *Ruminococcus* (2 OTUs); candidate families *Mogibacteriaceae* (2 OTUs), *Christensenellaceae* (1 OTU), *Clostridiaceae* (1 OTU), *Coriobacteriaceae* (2 OTUs), *Lachnospiraceae* (16 OTUs), *Rikenellaceae* (1 OTU) and *Ruminococcaceae* (8 OTUs); candidate orders *Bacteroidales* (1 OTU), *Clostridiales* (30 OTUs) and *ML615J-28* (1 OTU); and candidate class *Betaproteobacteria* (1 OTU) while 10 OTUs could not be assigned to any taxonomic level. These results at OTU level show remarkable coincidences with the analyses directly performed on the relative abundance of taxa at phylum and genera levels. This is consistent with two possibilities: a case of phylum encompassing one or a reduced number of genera, (like *Verrucomicrobia* and *Akkermansia*) or when all the OTUs in a given taxa show an effect on the same direction (for example an overrepresentation of the 10 OTUs assigned to genus *Blautia* in cecal samples).

Table 3.5| Microbial composition at genus level, grouped by phylum, in cecum and feces.

Phylum Genus	Mean relative abundance in cecum (%) (SD)	Mean relative abundance in feces (%) (SD)	Difference Cecum-Feces ± SE	P_{FDR}
Actinobacteria				
Adlercreutzia	0.175 (0.038)	0.149 (0.043)	0.023 ± 0.012	0.092
Bacteroidetes				
Bacteroides	2.436 (0.571)	2.358 (0.562)	0.079 ± 0.028	0.023
Butyricimonas	0.160 (0.173)	0.158 (0.163)	0.001 ± 0.013	0.959
Odoribacter	0.164 (0.091)	0.166 (0.077)	-0.001 ± 0.011	0.959
Parabacteroides	0.212 (0.217)	0.204 (0.210)	0.008 ± 0.006	0.233
Rikenella	0.475 (0.264)	0.457 (0.261)	0.020 ± 0.020	0.421
Euryarchaeota				
Methanobrevibacter	0.061 (0.096)	0.061 (0.095)	-0.001 ± 0.011	0.959
Firmicutes				
Anaerofustis	0.148 (0.070)	0.124 (0.057)	0.024 ± 0.008	0.024
Anaerostipes	0.302 (0.141)	0. 360 (0.137)	-0.059 ± 0.028	0.083
Blautia	2.532 (0.351)	2.086 (0.285)	0.444 ± 0.058	0.000
Clostridium	1.585 (0.221)	1.437 (0.221)	0.148 ± 0.029	0.000
Coprobacillus	0.173 (0.113)	0.164 (0.119)	0.009 ± 0.014	0.583
Coprococcus	1.163 (0.300)	1.295 (0.318)	-0.130 ± 0.028	0.000
Epulopiscium	0.210 (0.130)	0.194 (0.114)	0.017 ± 0.027	0.583
Oscillospira	2.345 (0.420)	2.598 (0.355)	-0.255 ± 0.058	0.000
Phascolarctobacterium	0.307 (0.240)	0.311 (0.248)	-0.007 ± 0.034	0.959
rc4-4	0.198 (0.040)	0.173 (0.043)	0.026 ± 0.010	0.034
Roseburia	0.056 (0.072)	0.078 (0.069)	-0.022 ± 0.012	0.123
Ruminococcus	5.070 (0.736)	5.197 (0.814)	-0.124 ± 0.091	0.233
Proteobacteria				
Desulfovibrio	0.507 (0.140)	0.493 (0.114)	0.014 ± 0.013	0.390
Oxalobacter	0.125 (0.067)	0.104 (0.070)	0.022 ± 0.011	0.083
Tenericutes				
Anaeroplasma	0.263 (0.162)	0.229 (0.162)	0.033 ± 0.014	0.054
Verrucomicrobia				
Akkermansia	1.810 (0.378)	1.651 (0.300)	0.158 ± 0.034	0.000
Unknown	79.523 (1.509)	79.954 (1.461)	0.599 ± 0.092	0.000

Table 3.6| OTUs most differentially represented between fecal and cecal samples.

OUT ID and taxonomical assignment	Mean abundance (CSS OTU units) (SD)	Difference Cecum-feces ± SE	P_{FDR}	[a]Discriminant sPLS-DA
NR57, Firmicutes; *Clostridia*; *Clostridiales*; *Ruminococcaceae*	3.603 (2.298)	−2.503 ± 0.311	0.000	NO
NR60, Firmicutes; *Clostridia*; *Clostridiales*	2.944 (2.283)	−2.247 ± 0.201	0.000	NO
581388, Cyanobacteria; *4C0d-2*; *YS2*	3.847 (1.935)	−2.034 ± 0.173	0.000	YES
NR28, Firmicutes; *Clostridia*; *Clostridiales*; *Ruminococcaceae*	7.464 (1.893)	−1.860 ± 0.180	0.000	YES
550894, Cyanobacteria; *4C0d-2*; *YS2*	3.651 (1.871)	−1.713 ± 0.219	0.000	YES
NR12, Firmicutes; *Clostridia*; *Clostridiales*	4.650 (1.058)	1.706 ± 0.125	0.000	YES
589410, Cyanobacteria; *4C0d-2*; *YS2*	1.649 (1.468)	−1.544 ± 0.190	0.000	YES
542830, Cyanobacteria; 4C0d-2; YS2	2.340 (1.800)	−1.313 ± 0.309	0.011	NO
NR411, Proteobacteria; *Alphaproteobacteria*; *RF32*	1.990 (1.551)	−1.214 ± 0.255	0.000	YES
197832, Firmicutes; *Clostridia*; *Clostridiales*; *Lachnospiraceae*; *Coprococcus*	3.295 (3.191)	−1.208 ± 0.357	0.011	NO

[a]*Last column indicates whether the OTU belongs to component 1 of sPLS-DA.*

3.4.4. Clusterization of samples according to their origin with different multivariate methods

First, a principal coordinate analyses (PCoA) from weighted Unifrac phylogenetic distance matrix calculated from the final OTU table was performed. In **Figure 3.2**, each sample is located in a specific position of a bidimensional chart in function of its microbiota composition. No clear pattern of separation of samples by their sampling origin could be appreciated.

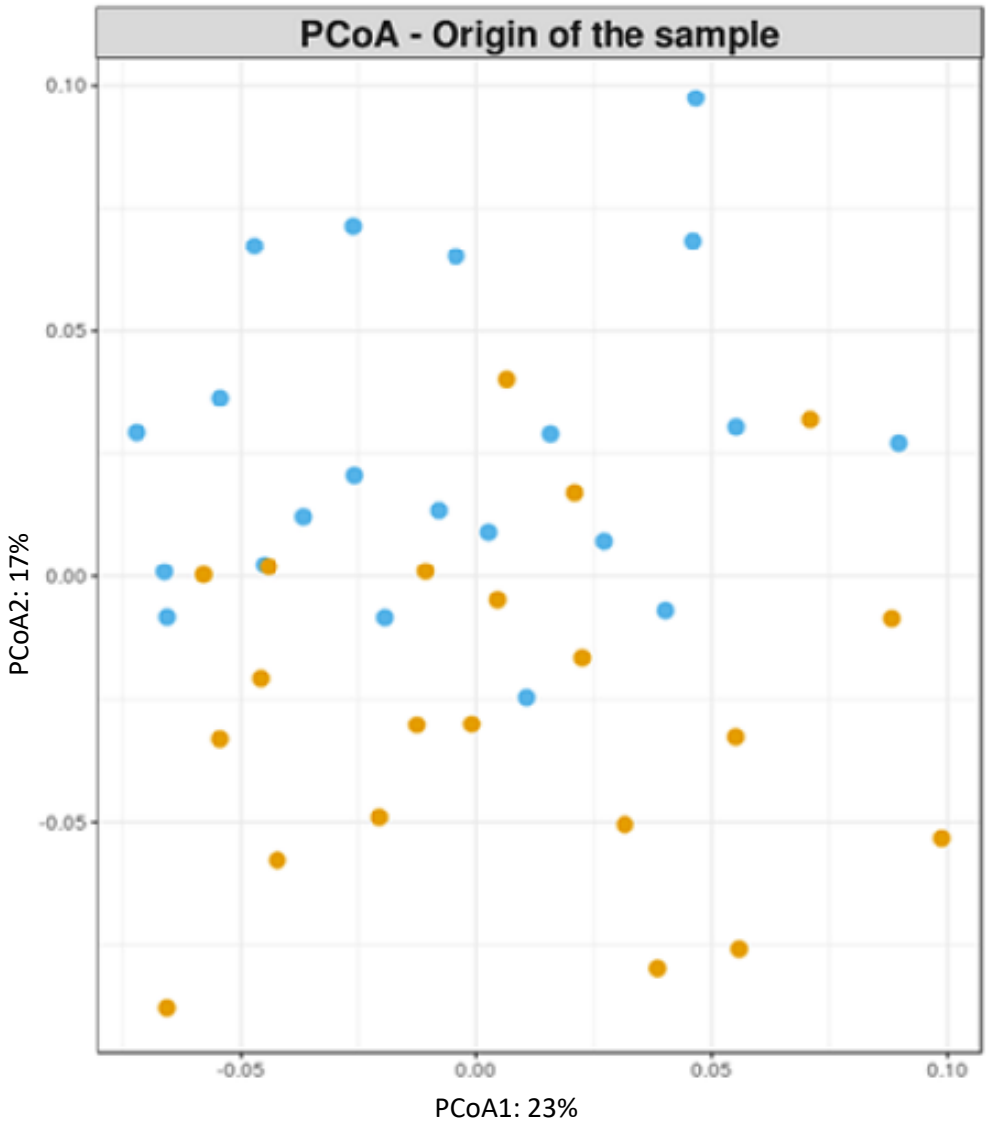

Figure 3.2| Principal coordinate analysis of weighted Unifrac phylogenetic distance matrix. Cecal and fecal samples are blue and orange colored, respectively.

The paired principal component analysis (PCA) was implemented in order to take into account the fact that each pair of cecal and fecal samples which belonged to the same rabbit showed a better separation pattern than PCoA. Components 1 and 2 explained 18% and 17% of variance respectively (**Figure 3.3A**).

But the multivariate method that best clustered the samples according to their sampling origin was the paired sparse partial least squares discriminant analysis (sPLS-DA) which took into account the fact that two different samples were collected from the same animal and indeed it was only conducted with the OTUs that best discriminated samples by their sampling origin (70 and 50 for components 1 and 2, respectively) (**Figure 3.3B**). The seventy OTUs that were part of the component 1 explained 17% of total variance. Forty of them were found to be overrepresented in cecum and 30 in feces (**Figure 3.4**). It should be noted that 66 OTUs declared as differentially represented between cecum and feces by sPLS-DA were also declared as differentially represented between sampling origins by the univariate bootstrap analyses of variance previously performed. The 10 OTUs most differentially represented between sampling origins (according to univariate analyses of variance) can be found in **Table 3.6** with an indication of whether the OTU belonged to the first component of the sPLS-DA analysis. The representative sequences of these OTUs are showed in **3.S3**.

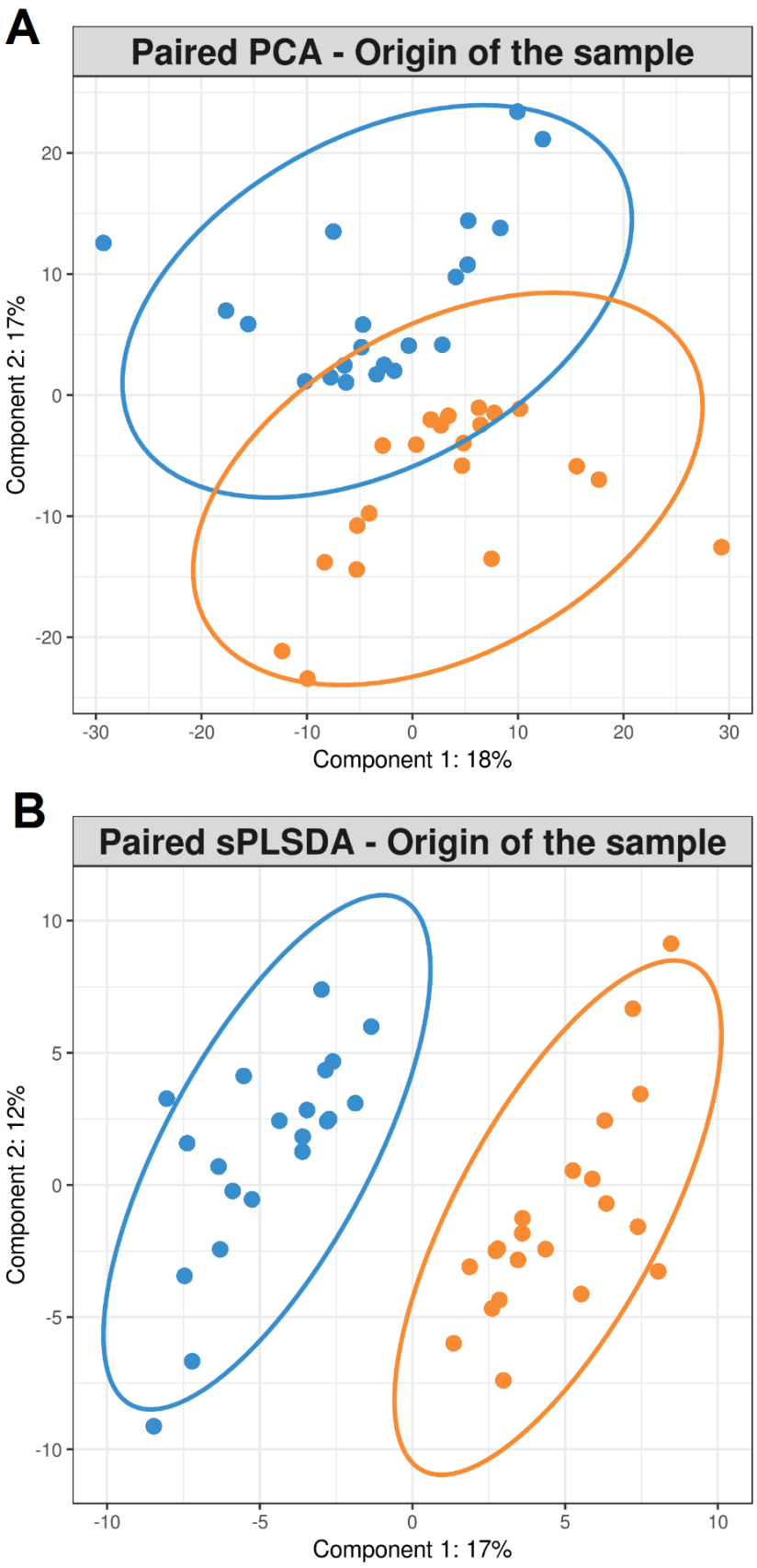

Figure 3.3| (A) Paired samples principal component analysis **(B)** Paired samples sparse partial least squares discriminant analysis representing 21 cecum (blue) and 21 feces (orange) samples.

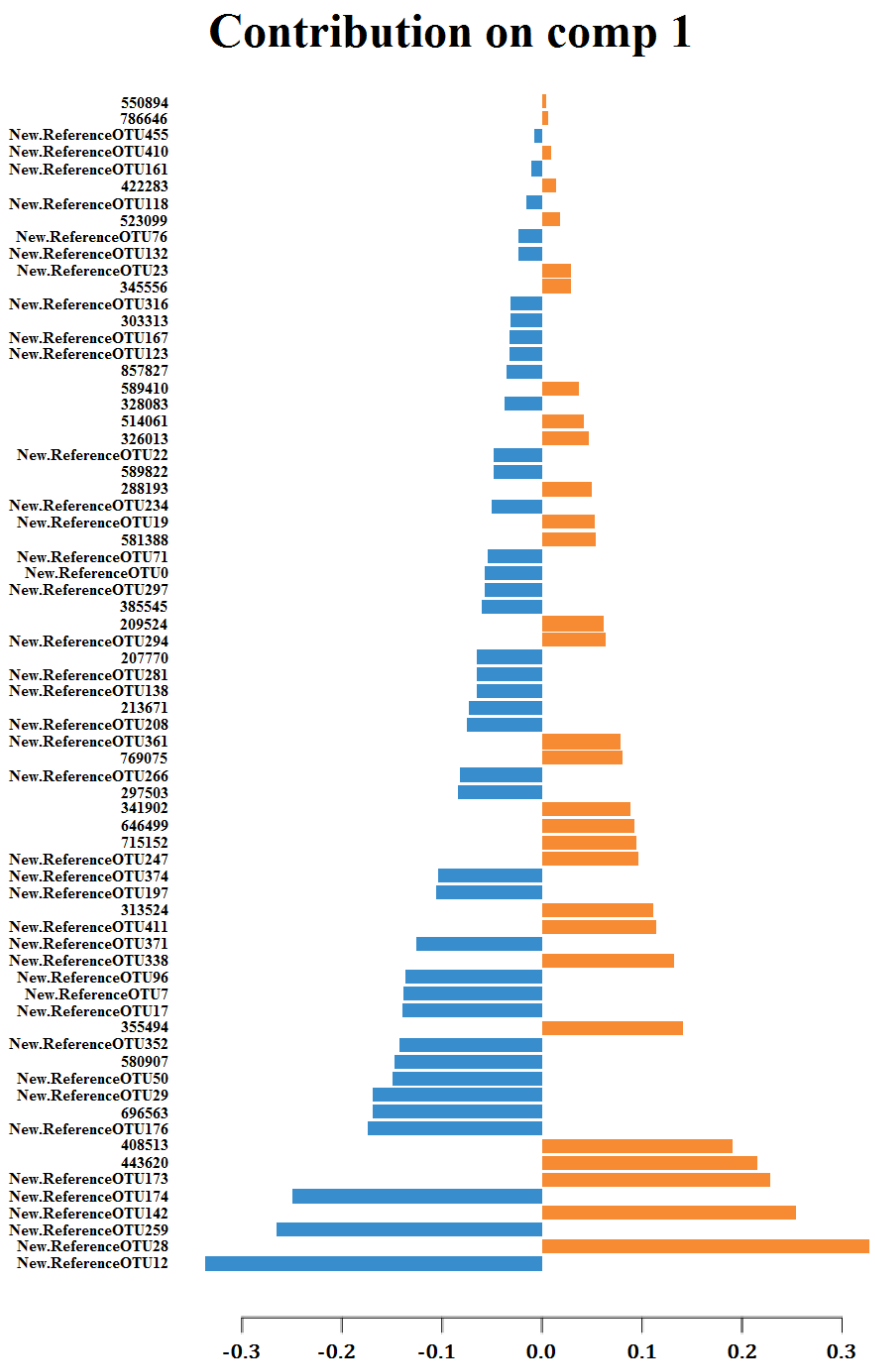

Figure 3.4| Contribution of each OTU, on component 1 of sPLS-DA, to the discrimination of samples regarding to their sampling origin: cecum (blue) or feces (orange).

3.5. Discussion

In this study, we aimed to evaluate the importance of selecting a proper sampling intestinal area for rabbit microbiota studies. To give an answer to this question, 16S rRNA gene amplicons from cecal and fecal samples collected from 21 meat rabbits randomly distributed in two feeding groups with different intake levels were sequenced in an Illumina MiSeq platform.

Similar to our work, sequencing results from other studies performed on gastrointestinal microbial populations of caecotrophagic animals (rabbit and guinea pig) (Zeng *et al*., 2015; Crowley *et al*., 2017), hare and pika (which, like rabbit, are members of the clade Glires) (Li *et al*., 2017), other livestock species like broiler chicken (Han *et al*., 2016) and on other environments, such as goats' rumen (Wang *et al*., 2016) or sheeps' lung (Glendinning *et al*., 2016), showed variable results in the average number of final contigs per samples. Our results are in accordance with the well-known fact that sequencing of the 16S rRNA gene could be strongly influenced by different factors like the storage of the sample, the method used for DNA extraction and library generation or the sequencing platform (Pollock *et al*., 2018). In addition, the variance found in the average final number of contigs and OTUs per sample can be accentuated by the software used, the parameters chosen for sequence filtering or the strategy followed for OTU picking (Allali *et al*., 2017). As in the present study in which the number of final contigs per sample ranged from 16,415 to 68,080, the study performed by Correa-Fiz *et al*. (2016) also showed a large variation (ranging from a minimum of 7,338 to a maximum of 844,521 final contigs per sample). On the other hand, the fact that some studies (Zeng *et al*., 2015, Wang *et al*., 2016) presented a larger number of OTUs per sample (range: 1,600-6,900) than the present one (range: 411-541) would be due to the fact that they used a different strategy for OTU picking by including an additional de novo clusterization step of sequences which did not match against the reference database.

Our estimates of alpha-diversity with Shannon and the observed number of OTUs indexes did not reveal significant differences between sampling origins. The fact that

fecal samples were collected directly from rectum at slaughter could reduce the chances of environmental contamination, which contributed to reduce differences in terms of diversity or richness between sampling origins. Similarly, Zeng *et al*. (2015) who characterized the cecal and fecal microbiota of two groups of rex rabbits with high or low body weight did not observe differences either in diversity or richness when they compared alpha-diversity indexes between both sampling origins. However, in the study performed by He *et al*. (2016) in which they compared microbial diversity and richness between cecum and feces samples collected from pigs, they found that fecal samples had a significantly higher alpha-diversity than cecal samples.

With regard to the taxonomic characterization of microbial diversity of cecum and feces, our results are consistent with previous studies on growing rabbit intestinal microbiota (Massip *et al*., 2012; Monteils *et al*., 2008; Combes *et al*., 2017). Nevertheless, relative abundances of the main phyla were different between studies. A quantitative comparison of our study with the first two, shows that they found a higher percentage of *Firmicutes* (90%) and approximately half the amount of *Bacteroidetes* (4%). Differences between these phyla could be related to sample storage conditions, as Bahl *et al*. (2012) demonstrated their importance in *Firmicutes* to *Bacteroidetes* 16S rRNA ratio in human fecal samples. Another putative explanation for these discrepancies could be related to updates and changes to the reference databases. For example, it is noteworthy that the presence of phylum *Tenericutes* was revealed in our study, which had not been reported in previous studies in rabbits. The fact that the only class that phylum *Tenericutes* contains, *Mollicutes*, was previously classified within phylum *Firmicutes* is the most plausible hypothesis to explain the differences in the relative abundance of phylum *Firmicutes* found between our study and previous ones (Massip *et al*., 2012; Monteils *et al*., 2008; Combes *et al*., 2017). The relative abundance of this phylum in our study was situated in the same range as phylum *Bacteroidetes.* In previous studies it was usual to find phylum *Actinobacteria* as the third most abundant. Other putative reason for explaining differences could be due to the fact that different 16S rRNA gene regions were sequenced: V3-V4 hypervariable regions in Massip *et al*. (2012) and Combes *et al*. (2016), the whole gene in Monteils' study and V4-V5

hypervariable regions in our study. Another hypothesis could be that the pair of primers employed in our study hybridized better for the sequences belonging to this phylum than primers used in previous studies.

Similar to the cecal microbial characterization at class level of rex rabbits performed by Zou *et al*. (2016), our results revealed that the predominant class was *Clostridia*. But in contrast, they found *Bacteroidia* as the second predominant class while it was the third, followed by *Mollicutes*, according to our results. Our study revealed that the predominant families within phylum *Firmicutes* were *Ruminococcaceae* and *Lachnospiroceae* in agreement with the results of Massip *et al*. (2012). As with cecal microbial characterization at genus level of rex rabbits performed by Zou *et al*. (2016), our results revealed that the predominant genera were *Ruminococcus* and *Oscillospira*. But in contrast, we found *Bacteroides* and *Blautia* to be the following predominant genera while they reported that *Coprococcus* and *Bacteroides* were the next more abundant.

Note that all *Archaea* species detected in our study belonged to genus *Methanobrevibacter* which encompasses different hydrogenotrophic methane-producing species. The presence of this genus in rumen microbial communities is well known (Henderson *et al*., 2013; Patra *et al*., 2017). Moreover, previous studies have also described its presence in the gastrointestinal tract of humans (Thomas *et al*., 2017) and monogastric animals (Luo *et al*., 2017; Hou *et al*., 2016); including rabbit as Kušar and Avguštin (2010) reported in their study. Nonetheless, Mi *et al*. (2018) revealed the low presence of methanogenic archaea compared to *Bacteria* domain in rabbit cecum, due to its acidic pH (≈ 5.8) which does not favor methanogenic archaea. It is noteworthy to mention that Mi *et al*. (2018) found *Methanobrevibacter* as the main archaeal population. The small ratio between archaea/bacteria of cecal and fecal samples affected the archea sequence detection, resulting in the archaeal biodiversity being very low.

Although we observed similar microbial diversity and richness between feces and cecum samples, both multivariate and bootstrap univariate analysis revealed that community structures were significantly different in both types of samples. Our

results revealed an enrichment of 6 known genera in cecal samples and 2 genera in fecal samples considered in detail below.

Despite the fact that the overall relative abundance of phylum *Firmicutes* did not show differences between sampling origins, most of the genera differentially represented in both type of samples belong to this phylum. This is not surprising because three quarters of bacteria belong to this phylum, which encompasses a large number of lower taxonomic groups. All genera differentially represented within this phylum belong to different families of class *Clostridia*. Genus *Clostridium* (family *Clostridiaceae*) is an anaerobic Gram-positive bacteria whose presence in intestinal microbiota has been reported in human (Lloyd-Price *et al*., 2016) and many animal species like mouse (Uebanso *et al*., 2017), chicken (Han *et al*., 2016; Oakley *et al*., 2016) or pig (Fang *et al*., 2017). Bäuerl *et al*. (2014) reported a greater presence of this genus in cecal microbiota of rabbits affected by epizootic rabbit enteropathy (ERE) than in healthy animals. But not all *Clostridium* species are pathogenic and it is possible to find this genus in normal microbiota as Oakley *et al*. (2016) reported its presence in the cecum of 6-week healthy broiler chickens. Probably, the majoritiy of *Clostridum* species that inhabit rabbit cecum are cellulose-degrading symbiotic microorganisms that help the host in digestion of plant materials. Little is known about the presence of *Anaerofustis* (family *Eubacteriaceae*) in intestinal communities. Arrazuria *et al*. (2016) found an association between the presence of this genus in cecal samples collected from female rabbits and *Mycobacterium avium paratuberculosis* infection. Some *Anaerofustis* species could be involved in the fermentation of carbohydrates and glucose metabolism in the cecum (Lawson, 2015), which could be compatible with the overrepresentation we observed for this genus in cecum which is well known to be the main fermenting organ in rabbits. Within *Ruminococcaceae*, the most abundant family of phylum *Firmicutes*, the genus *Oscillospira* was overrepresented in fecal samples. This genus has been proved to be one of the core genera of some herbivore's rumen microbiota like cattle or sheep (Mackie *et al*., 2003) and horse's fecal microbiota (O' Donnell *et al*., 2013). It is a non-cultured anaerobic bacteria but now, thanks to next generation sequencing, we can detect it. Zeng *et al*. (2015) also reported an overrepresentation of *Oscillospira* in soft feces, which indicates that

species of this genus could be involved in fermentation processes as Gophna *et al*. (2017) inferred that some *Oscillospira* species are butyrate producers. Within the second most abundant family, *Lachnospiraceae*, we found an overrepresentation of genera *Blautia* and *Coprococcus* in cecum and feces, respectively. *Blautia* is an important member of animal intestinal microbiota, especially after weaning as Chen *et al*. (2017) reported in their study with piglets during the weaning transition. Park *et al*. (2012 & 2013) isolated two *Blautia* species in human feces able to ferment carbohydrates and degrade glucose producing acetate and lactate. Consistent with these previous studies and with the one done by Zeng *et al*. (2015) in which they found a higher representation of *Blautia* in soft feces than in hard feces, the relative enrichment of this genus in cecum versus feces observed in our study could imply that it plays an important role in carbohydrate and glucose digestion in rabbit cecum. On the other hand, *Coprococcus* is an anaerobic Gram-positive bacteria that actively ferments carbohydrates, producing butyric and acetic acids with formic or propionic acids (Holdeman and Moore, 1974). Some studies have previously described the presence of this genus in human (Canani *et al*., 2016) and horse (Mach *et al*., 2017) feces. An overrepresentation of *Coprococcus* in rabbit feces could be due to the fact that members of this genus actively participate in fermentation processes in the cecum and after having played their role they cannot be fixed to intestinal walls again and they are expelled with the feces. It is thought that these bacteria found in the final product of feed digestion could be dead bacteria (Fu *et al*., 2018).

Within the phylum *Bacteroidetes*, the only genus differentially represented between sampling origins was *Bacteroides* (family *Bacteroidaceae*). *Bacteroides* is an anaerobic Gram-negative bacteria that constitutes an important portion of the mammalian gastrointestinal microbiota (Jandhyala *et al*., 2015; Rodríguez *et al*., 2015). This genus has an important role in the degradation of vegetal polysaccharides (Fang *et al*., 2017) and in amino acid fermentation (Dai *et al*., 2011) which could be the reason for its overrepresentation in cecum where it is supposed to play an active role.

Finally, *Akkermansia* is also a well-known genus of phylum *Verrumicrobia* that inhabits intestinal microbiota of mammals (Derrien *et al*., 2004; Borton *et al*., 2017) and recently found in reptiles (Rawski *et al*., 2016; Ouwerkerk *et al*., 2017). Several studies have demonstrated that some *Akkermansia* species are mucin degraders (Belzer and De Vos, 2012) related with gut inflammation. However, current studies have elucidated that these species also contribute to the reparation of mucosal wounds (Alam *et al*., 2016) and they could be employed as probiotics (Gómez-Gallego *et al*., 2016). Previous studies that have characterized microbial communities of different sections across rabbit and chicken gastrointestinal tracts have also found a significant overrepresentation of this genus in cecum with respect to other sections (Zeng *et al*., 2015; Han *et al*., 2016). Moreover, Borton *et al*. (2017) reported an increase in the relative abundance of these bacteria in mouse gut as a consequence of low levels of inflammation. For all of this, we hypothesize that the presence of *Akkermansia* species in cecum could be involved in the formation of a protective mucosa layer that would help rabbits to deal with inflammatory processes.

It is important to note that different studies have identified genera *Bacteroides*, *Akkermansia* and *Oscillospira* as obesity-associated intestinal microbial species (Zhang *et al*., 2017; Zhao *et al*., 2017; de la Cuesta-Zuluaga *et al*., 2017) as well as Tan *et al*. (2018) have found an association between particular species of genera *Akkermansia* and *Clostridium* with psoriasis in humans. We think that careful consideration of the sampling area in this kind of studies is important to ensure reliable detection of these genera. Monitoring these genera as plausible obesity indicators could be considered in future association studies in order to link intestinal microbiota and particular production traits, such as growth or feed efficiency in livestock animals.

Furthermore, in this study different multivariate approaches to group samples by their origin were performed and different results were obtained due to the fact that the principles on which they are based are different. PCA transformed the 596 potentially correlated variables (OTUs) into a smaller number of uncorrelated variables, or principal components, so that the first component captured as much of the existing variability in the data set. On the contrary, PCoA was based on the

Unifrac dissimilarity matrix containing distances between samples in function of their microbiota composition in order to represent these phylogenetic distances, with the lowest possible dimensional coordinates. The paired PCA, although it captures the maximum possible variability, did not necessarily capture the part that explains the most important variation according to the categorical variable for which we wanted to classify our samples; the sampling origin in this case (James *et al*., 2013). According to our results, the approach that best discriminated samples according to their sampling origin was the paired sPLS-DA. It took into account the complex structure of the experimental design in which two different samples were collected from two different "compartments" of the same individual at the same time. This multivariate method allowed for the capture of the sampling origin effect within the animal separately from the variation between animals. Decomposing the within variance from the between variance (Liquet *et al*., 2012) enables the finding of those OTUs differentially represented between origins which best discriminate both type of samples.

The results of our study show that, overall, the microbial structures of rabbit feces and cecum are similar in terms of richness and diversity, since it should be remembered that we have compared biological samples belonging to locations closely situated throughout the animal intestinal tract that share similar physicochemical conditions. Furthermore, fecal samples were collected from the rectum avoiding the contact of microorganisms with the natural environment and, consequently, with the oxygen that would cause oxidative stress and more drastic changes in some bacterial populations. Nevertheless, it is important to bear in mind the existence of compositional differences in the relative abundance of an important number of taxa and OTUs. Both sampling origins contained the same 8 phyla but the relative abundances of half of them were differentially represented between origins. Similarly, at genus level, we found an overrepresentation of some genera such as *Blautia* or *Akkermansia* in cecal samples which would be involved in carbohydrate digestion and in immune protection against inflammation. On the other hand, an overrepresentation of genera *Oscillospira* and *Coprococcus* in fecal samples could indicate an active participation of these bacteria in fermentation at the end of the feed digestion process or correspond to dead species that were

excreted once they have played their main role in the cecum. Finally, at OTU level we found, with both univariate and multivariate approaches, 66 were differentially represented between origins in all analyses performed. According to our results, we propose the collection of feces in those studies aiming for a shallow characterization of the intestinal microbiota. On the contrary, for those studies interested in a specific characterization of the composition of microbial communities, it is necessary to consider the fact that important differences in the relative abundance of some taxa, even at phylum level, between cecum and feces have been reported. The decision as to which area of the intestinal tract should be sampled will therefore depend on the objectives of each study.

To sum up, the existence of diversity and compositional differences between rabbit cecum content and internal hard feces microbial communities has been revealed in the present study. In future studies, cecal microbiota of a larger number of rabbits bred under different management conditions, such as feeding regime or the presence of antibiotics in the feed, need to be analyzed to gain insight into the effect of these conditions on rabbit intestinal microbiota and the effect of microbial diversity and composition on animal performance.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethics

The research protocol was approved by the animal care and use committee of the Institute for Food and Agriculture Research and Technology (IRTA).

Author contribution

JS, MP and OR conceived the experimental design. JS, OR, MP and MVG collected biological samples. MVG, OG, MP and MG processed the samples in the laboratory. MVG processed and analyzed the sequencing data, interpreted data, prepared figures and tables, and wrote the manuscript. JS and MV helped analyzing the sequencing data. JS, MP, MV and MG helped interpreting the data, and wrote and revised the manuscript. All authors read and approved the final manuscript.

Funding

The experimental design of this work was conducted thanks to funding from INIA project RTA2011-00064-00-00. This study was part of the Feed-a-Gene project and received funding from the European Union's H2020 program under grant agreement no 633531. MVG is a recipient of a ‘Formación de Personal Investigador (FPI)’ pre-doctoral fellowship from INIA, associated with the research project RTA2014-00015-C2-01.

Acknowledgements

The authors are grateful to the staff of Unitat de Cunicultura, IRTA (Oscar Perucho and Carmen Requena) for their contribution to data recording and animal care during the experiment. The authors also thank to Genomics and NGS Unit, CRAG (Armand Sánchez, Nicolas Boulanger and Joana Ribes) for assistance in massive libraries preparation. Useful interactions with Yuliaxis Ramayo-Caldas (IRTA) in relation to the bioinformatics processing of samples are deeply acknowledged.

3.6. Supplementary material

The Supplementary Material for this article can be found in the Annexes section.

3.7. References

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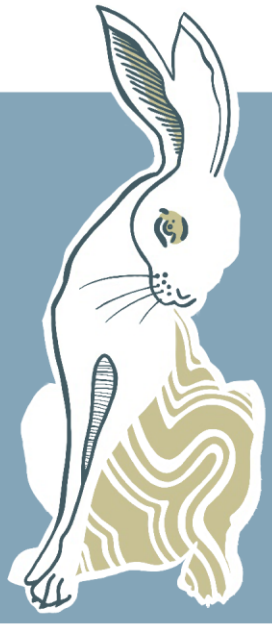
CHAPTER 4

BREEDING FARM, LEVEL OF FEEDING AND PRESENCE OF ANTIBIOTICS IN THE FEED INFLUENCE RABBIT CECAL MICROBIOTA



Article II

Velasco-Galilea *et al. Animal Microbiome* (2020) 2:40
https://doi.org/10.1186/s42523-020-00059-z

Animal Microbiome

RESEARCH ARTICLE Open Access

Breeding farm, level of feeding and presence of antibiotics in the feed influence rabbit cecal microbiota

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Animal Microbiome (2020), *2*(1), pp. 1-16

Breeding farm, level of feeding and presence of antibiotics in the feed influence rabbit cecal microbiota

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4.1. Abstract

Background: the effect of the production environment and different management practices in rabbit cecal microbiota remains poorly understood. While previous studies have proved the impact of the age or the feed composition, research in the breeding farm and other animal management aspects, such as the presence of antibiotics in the feed or the level of feeding, is still needed. Characterization of microbial diversity and composition of growing rabbits raised under different conditions could help better understand the role these practices play in cecal microbial communities and how it may result in different animal performance. **Results:** four hundred twenty-five meat rabbits raised in two different facilities, fed under two feeding regimes (*ad libitum* or restricted) with feed supplemented or free of antibiotics, were selected for this study. A 16S rRNA gene-based assessment through the MiSeq Illumina sequencing platform was performed on cecal samples collected from these individuals at slaughter. Different univariate and multivariate approaches were conducted to unravel the influence of the different factors on microbial alpha diversity and composition at phylum, genus and OTU taxonomic levels. The animals raised in the facility harboring the most stable environmental conditions had greater, and less variable, microbial richness and diversity. Bootstrap univariate analyses of variance and sparse partial least squares-discriminant analyses endorsed that farm conditions exerted an important influence on rabbit microbiota since the relative abundances of many taxa were found differentially represented between both facilities at all taxonomic levels characterized. Furthermore, only five OTUs were needed to achieve a perfect classification of samples according to the facility where animals were raised. The level of feeding and the presence of antibiotics did not modify the global alpha diversity but had an impact on some bacteria relative abundances, albeit in a small number of taxa compared with farm, which is consistent with the lower sample classification power according to these factors achieved using microbial information. **Conclusions:** this study reveals that factors associated with the farm effect and other management factors, such as the presence of antibiotics in the diet or the feeding level, modify cecal microbial communities. It highlights the importance of offering a controlled breeding environment that reduces differences in microbial cecal composition that could be responsible for different animal performance.

Keywords: cecal microbiota, meat rabbit, breeding farm, feed restriction, antibiotics, 16S MiSeq Illumina sequencing, analysis of variance, multivariate approach.

4.2. Background

Microbial communities that inhabit the gastrointestinal tract (GIT) of animals constitute a complex ecosystem whose members constantly interact between themselves and with their host (Gaskins, 1997). These interactions ensure homeostatic balance maintenance since GIT ecosystem components are involved in many physiological and immunological processes (Belkaid and Hand, 2014). In the case of the domestic meat rabbit (*Oryctolagus cuniculus*), a small herbivorous mammalian belonging to the family *Leporidae*, cecum is the main organ for microbial fermentation. Thus, it is not surprising that the rabbit cecum hosts the richest and the most diverse microbial community of its GIT (Gouet and Fonty, 1979). For this reason, the cecum has been the organ preferably chosen in previous rabbit gut microbiota assessments (Abecia *et al*., 2007; Zou *et al*., 2016; Zhu *et al*., 2017; Chen *et al*., 2019).

Thanks to the development of next generation sequencing (NGS) technologies, and their rapidly decreasing costs, it is currently possible to characterize the gut microbiota of a large number of animals. This characterization allows a deeper comprehension of the differences between animals concerning their microbial composition and diversity. It is hypothesized that the production environment could partially mediate these differences. Our general aim is to provide further evidence of the effect of different management and environmental factors on cecal microbial composition and diversity. In relation to this topic, there is a certain amount of information already published. A growing number of studies have revealed changes in rabbit cecal microbial communities exerted by age (Combes *et al*., 2011) or the type of feed provided to the kits after weaning (Zhu *et al*., 2017; Chen *et al*., 2019). Another factor that causes variation is the administration of antibiotics in the feed. Different molecules have been widely administered in rabbit meat production, especially after weaning, to curb mortality peaks (sometimes over 20%) as a result

of the onset of gastrointestinal symptoms (Gidenne *et al*., 2010). Multiple studies have shown alterations caused in gut microbiota by the administration of antibiotics in the feed (Zou *et al*., 2016; Eshar and Weese, 2014). Despite the European Union having banned the use of antibiotics in animal feeds as growth promoters since 2006 (EC 1831/2003), at the time this experiment was conducted, the administration of a mix of up to four antibiotics was permitted to prevent or treat the emergence of potential infectious diseases on farms. Nowadays, the administration of only one antibiotic molecule is allowed and substantial efforts are being made towards searching for efficient alternatives which allow for a complete withdrawal of antibiotics in animal feeds. In this context, the application of feed restriction during the growing period was proposed as an interesting alternative to the use of antibiotics. Quantitative feed restriction is a widely applied commercial practice which consists of reducing the amount of feed the animal would consume by a certain percentage when the food is provided *ad libitum*. Gidenne *et al*. (2009) demonstrated that feed restriction, despite penalizing animal growth, improves feed efficiency and reduces mortality due to enteric disorders. It is hypothesized that these positive effects could be partially explained by changes in gut microbial composition or activity originated by the application of feed restriction. However, techniques used so far to study this possible association have found no evidence of it (Gidenne *et al*., 2009).

This study, which comprises a large number of animals in an experimental design involving different management and environmental factors, is intended to unravel changes in diversity and composition of rabbit cecal microbial communities associated with these factors. It will allow for a better understanding of how the farm where the animal was raised, the presence of antibiotics in the feed, and feed restriction shape the cecal microbiota of growing rabbits.

4.3. Results

4.3.1. Sequence processing

After the removal of doubletons and samples with low sequence counts, 425 rabbit cecal samples (**Additional file 4.1**) were represented on 14,928,203 sequence counts clustered into 963 different OTUs. Each sample had on average 35,125 final sequences (range: 10,157-678,798) and 677 OTUs (range: 197-841) (**Additional files 4.2** and **4.3**). **Figure 4.1** shows two histograms representing the sample richness and the proportion of OTUs present across samples. Most of the samples had more than 700 different OTUs (mode = 748) and nearly 140 OTUs were present in all the samples.

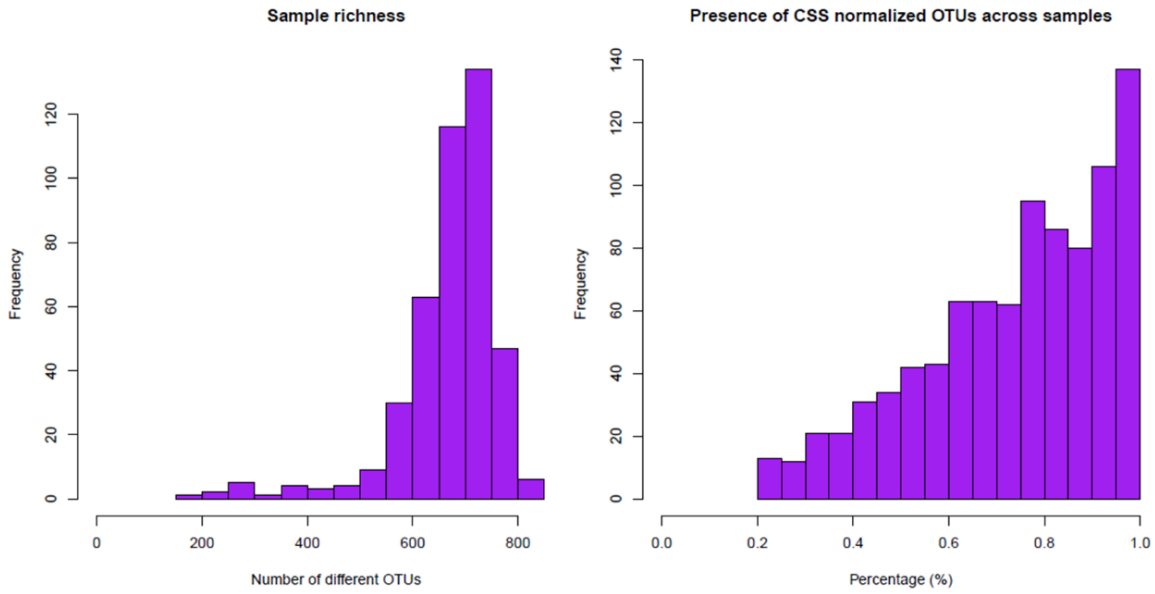

Figure 4.1| Sample richness and presence of CSS-normalized OTUs across samples.

Taxonomic assignment of representative OTUs against the Greengenes reference database gg_13_5_otus (**Additional file 4.4**) revealed the presence of 8 different known phyla with an average of 8 phyla per sample (range: 7-8) (**Additional file 4.5**), and 28 different known genera with an average of 24 genera per sample (range: 17-28) (**Additional file 4.6**).

4.3.2. Animal management and farm environment shaping cecal microbial alpha diversity

The study of alpha diversity was performed after rarefying the prefiltered and unnormalized OTU table to 10,000 sequences per sample. Rarefaction generated a table which contained the sequence counts of 963 different OTUs for 425 samples. The average (standard deviation) number of observed OTUs within animal was 560.52 (75.03) and the average Shannon index within animal was 5.09 (0.26). The comparison of alpha diversities revealed that the group of animals raised in farm B had greater alpha diversity than the group of animals raised in farm A (estimated differences of 40.20 (9.83) observed OTUs and 0.17 (0.03) Shannon indexes; P_{FDR} < 0.001). Furthermore, larger variability in both indexes was observed in farm A than in farm B. No significant differences for the two alpha diversity indexes were found between feeding regimes within both farms (**Figure 4.2**, P_{FDR} > 0.05), nor between the presence and the absence of antibiotics in the feed within farm B (**Figure 4.2**, P_{FDR} > 0.05).

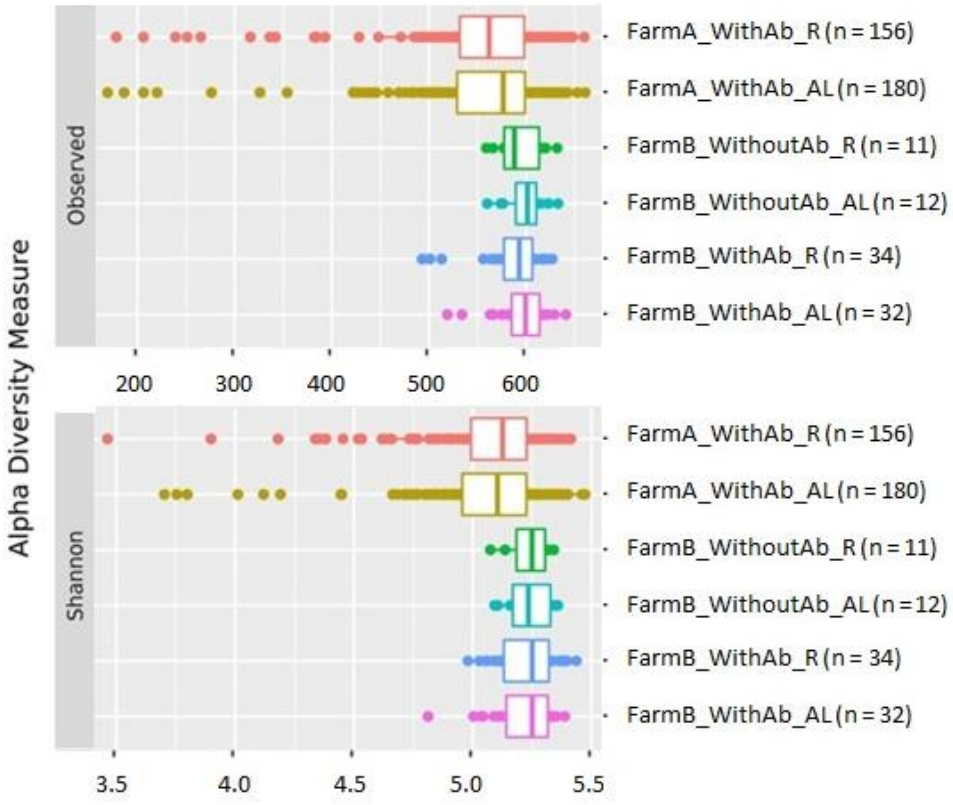

Figure 4.2| Microbial richness and diversity between samples grouped according to management that animals received. The cecal microbial richness and diversity were estimated by the observed number of different OTUs and the Shannon indexes, respectively.

4.3.3. Animal management and farm environment shaping cecal microbial composition

According to the taxonomic assignment of representative sequences (**Additional file 4.4**) performed with the UCLUST consensus taxonomy assigner on the Greengenes reference database gg_13_5_otus, *Firmicutes* (76.74%), *Tenericutes* (7.22%) and *Bacteroidetes* (6.26%) were the predominant phyla, accounting for more than 90% of the microbial diversity, in the rabbit cecal samples studied (**Figure 4.3**).

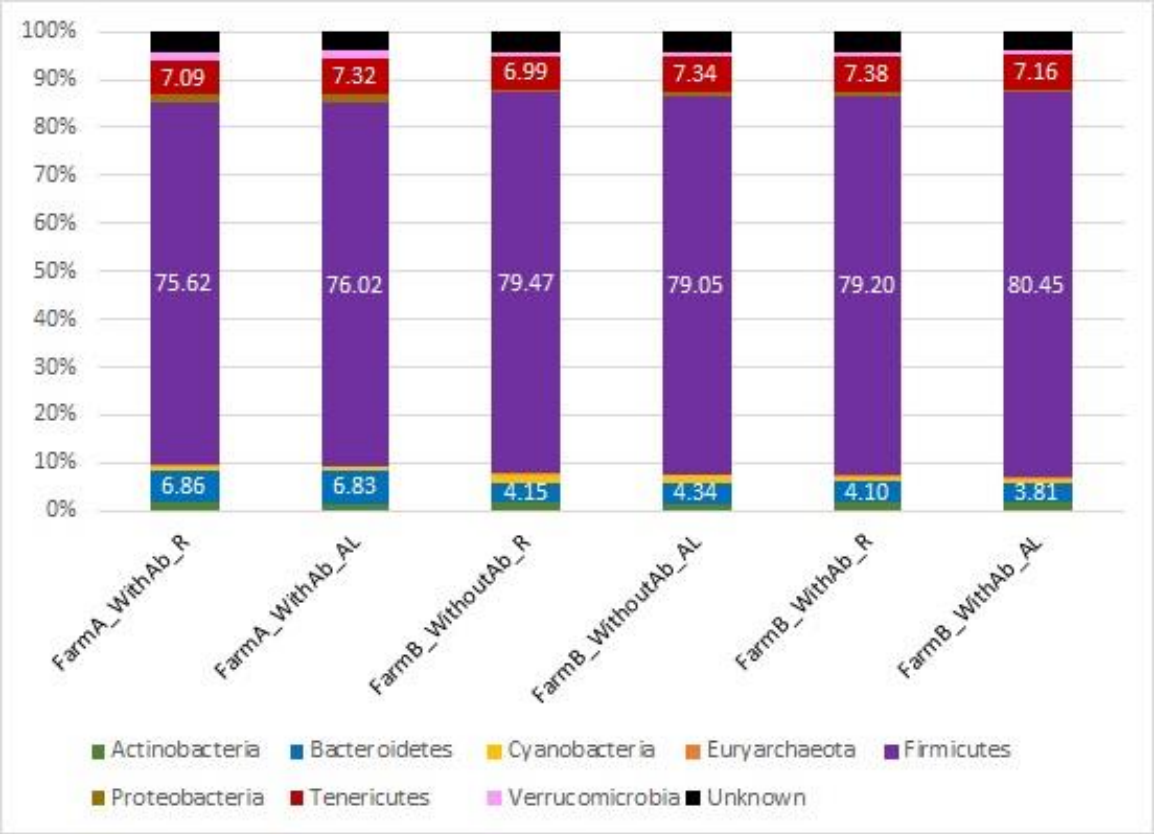

Figure 4.3| Phyla relative abundances of samples grouped according to farm, level of feeding and presence of antibiotics in the feed.

4.3.3.1. Differential growth and cecal microbial composition across farms

The facility where the animals were raised affected their growth performance. Animals raised in farm B exhibited a faster growth (47.11 grams/day) than those raised in farm A (44.19 grams/day). The estimated average daily gain difference between farm B and farm A was 2.92 ± 0.94 grams per day ($P < 0.05$). Cecal

samples of rabbits raised in farm A showed an overrepresentation of phyla *Bacteroidetes*, *Proteobacteria* and *Verrucomicrobia* while phyla *Euryarchaeota*, *Cyanobacteria* and *Firmicutes* were found to be overrepresented in cecal samples of rabbits raised in farm B (**Table 4.1**).

Table 4.1| Microbial composition at phylum level in cecal samples of rabbits grouped by farm.

Phylum	Mean relative abundance in farm A (%) (SD)	Mean relative abundance in farm B (%) (SD)	Estimated difference farm A - farm B ± SE	P_{FDR}
Actinobacteria	1.62 (0.67)	1.84 (0.33)	-0.14 ± 0.08	0.09
Bacteroidetes	6.84 (1.81)	4.03 (0.70)	2.74 ± 0.22	0.00
Cyanobacteria	0.77 (0.40)	1.05 (0.36)	-0.39 ± 0.05	0.00
Euryarchaeota	0.13 (0.19)	0.44 (0.17)	-0.28 ± 0.02	0.00
Firmicutes	75.83 (3.34)	79.66 (1.53)	-3.78 ± 0.41	0.00
Proteobacteria	1.83 (0.62)	0.66 (0.12)	1.14 ± 0.07	0.00
Tenericutes	7.21 (1.47)	7.25 (0.93)	0.00 ± 0.18	0.99
Verrucomicrobia	1.62 (0.45)	0.91 (0.24)	0.68 ± 0.05	0.00

Genera *Ruminococcus* (4.32%), *Blautia* (2.96%) and *Oscillospira* (2.37%) dominated the meat rabbit cecal microbiota. Most of the relative abundance differences at genus level were found differentially represented between animals raised in the different farms: genera *Bacteroides*, *Parabacteroides*, *Rikenella*, *Anaerofustis*, *Anaerostipes*, *Clostridium*, *Coprobacillus*, *Anaeroplasma* and *Akkermansia* were overrepresented in cecal samples of rabbits raised in farm A while genera *Adlercreutzia*, *Butyricimonas*, *Odoribacter*, *Methanobrevibacter*, *Blautia*, *Butyrivibrio*, *Coprococcus*, *Dehalobacterium*, *Dorea*, *Oscillospira*, *rc4-4* and *Oxalabacter* were overrepresented in cecal samples of rabbits raised in farm B. Interestingly, genera *Epulopiscium*, *p-75-a5*, *Phascolarctobacterium*, *Campylobacter* and *Desulfovibrio* were only found in samples collected from farm A (**Table 4.2**).

Table 4.2| Relative abundances of genera, grouped by phylum, differentially represented between farms ($P_{FDR} < 0.05$).

Genus	Mean relative abundance in farm A (%) (SD)	Mean relative abundance in farm B (%) (SD)	Estimated difference farm A - farm B ± SE
Actinobacteria			
Adlercreutzia	0.89 (0.47)	1.14 (0.23)	-0.19 ± 0.06
Bacteroidetes			
Bacteroides	1.88 (0.67)	0.80 (0.35)	1.10 ± 0.08
Butyricimonas	0.16 (0.19)	0.35 (0.17)	-0.19 ± 0.02
Odoribacter	0.23 (0.21)	0.44 (0.20)	-0.21 ± 0.03
Parabacteroides	0.25 (0.18)	0.07 (0.07)	0.18 ± 0.02
Rikenella	0.39 (0.24)	0.18 (0.13)	0.25 ± 0.03
Euryarchaeota			
Methanobrevibacter	0.13 (0.19)	0.44 (0.17)	-0.28 ± 0.02
Firmicutes			
Anaerofustis	0.12 (0.08)	0.08 (0.04)	0.03 ± 0.01
Anaerostipes	0.17 (0.08)	0.12 (0.04)	0.06 ± 0.01
Blautia	2.86 (0.67)	3.22 (0.46)	-0.36 ± 0.08
Butyrivibrio	0.10 (0.07)	0.13 (0.06)	-0.03 ± 0.01
Clostridium	1.09 (0.26)	0.87 (0.13)	0.21 ± 0.03
Coprobacillus	0.20 (0.27)	0.14 (0.08)	0.08 ± 0.03
Coprococcus	1.96 (0.42)	2.26 (0.29)	-0.28 ± 0.05
Dehalobacterium	0.05 (0.08)	0.18 (0.03)	-0.13 ± 0.01
Dorea	0.46 (0.12)	0.51 (0.09)	-0.05 ± 0.02
Epulopiscium	0.14 (0.11)	0.00 (0.00)	0.15 ± 0.01
Oscillospira	2.11 (0.53)	2.85 (0.31)	-0.79 ± 0.07
p-75-a5	0.13 (0.06)	0.00 (0.00)	0.13 ± 0.01
Phascolarctobacterium	0.27 (0.24)	0.00 (0.00)	0.26 ± 0.03
rc4-4	0.13 (0.06)	0.23 (0.03)	-0.10 ± 0.01
Proteobacteria			
Campylobacter	0.08 (0.08)	0.00 (0.00)	0.08 ± 0.01
Desulfovibrio	0.58 (0.22)	0.00 (0.00)	0.57 ± 0.03
Oxalabacter	0.10 (0.06)	0.13 (0.03)	-0.03 ± 0.01
Tenericutes			
Anaeroplasma	0.23 (0.18)	0.10 (0.09)	0.12 ± 0.02
Verrucomicrobia			
Akkermansia	1.62 (0.45)	0.91 (0.23)	0.68 ± 0.05

The analyses on the CSS-normalized OTUs revealed that 648 out of the 946 OTUs showed signatures significantly different between farms. Out of these, 276 were overrepresented in farm A, while 372 were overrepresented in farm B. **Table 4.S1** shows the estimated difference between farms for these OTUs, their sequences and their assignment at the lowest taxonomic level. Only 9 of them could be assigned at species level and 129 were assigned to known genera.

These results showed remarkable coincidences with those obtained from the analyses directly performed on the relative abundance of taxa at phylum and genera levels. An example that illustrates this match is the overrepresentation of genus *Akkermansia* in farm A. This genus is encompassed by phylum *Verrucomicrobia* that was also overrepresented in rabbits raised in farm A, as well as 6 out of the 7 OTUs assigned to this phylum.

4.3.3.2. Differential growth and cecal microbial composition across feeding regimes

The feeding regime affected the rabbits' growth performance in both facilities. Animals fed AL had a higher growth (48.74 and 55.77 grams/day in farms A and B, respectively) than those fed R (38.95 and 38.65 grams/day in farms A and B, respectively). The estimated average daily gain difference between AL and R groups was 9.79 ± 0.58 and 17.12 ± 1.08 grams per day in farms A and B, respectively ($P < 0.001$). An overrepresentation of phyla *Cyanobacteria* (estimated difference R - AL = 0.11 ± 0.04; $P_{FDR} = 0.04$) and *Verrucomicrobia* (estimated difference R - AL = 0.11 ± 0.05; $P_{FDR} = 0.04$) was found in cecal samples of rabbits fed R and raised in farm A. On the other hand, phylum *Euryarchaeota* was overrepresented in animals fed R and raised in farm B (estimated difference R - AL = 0.14 ± 0.04; $P_{FDR} < 0.001$). At genus level, the only significant contrast was observed for *rc4-4* which resulted overrepresented in samples from animals fed AL in farm A (estimated difference R - AL = -0.03 ± 0.01; $P_{FDR} < 0.001$) while in farm B none of the genera resulted differentially represented ($P_{FDR} > 0.05$) between feeding regimes. The contrasts based on the CSS-normalized OTUs revealed 51 and 9 OTUs differentially represented between feeding regimes within farms A and B, respectively. Within farm A, 32 OTUs were overrepresented in cecal samples of rabbits that were fed AL and 19 OTUs in the samples from rabbits fed R. Within farm B, 7 OTUs were overrepresented in cecal samples of rabbits that were fed AL and 2 OTUs were overrepresented in rabbits that were fed R. **Table 4.S2** shows the estimated difference between feeding regime within farm of these OTUs, their sequences and their assignment at the lowest taxonomic level. The analyses based on the CSS-normalized OTUs within farm A were in full accordance with the

analyses performed at genus level given that all OTUs assigned to genus *rc4-4* (phylum *Firmicutes*) were overrepresented in cecal samples of rabbits fed AL.

4.3.3.3. Effect of the presence of antibiotics in the feed

The effect of the presence of antibiotics in the feed could only be assessed within farm B given that all rabbits raised in farm A received feed supplemented with antibiotics. Animals that received antibiotics had a slightly higher growth (47.29 grams/day) than those that did not (46.59 grams/day). The estimated average daily gain difference between groups was not significant (0.69 ± 2.43 grams per day; P = 0.78). Cecal samples of rabbits that received feed free of antibiotics showed an overrepresentation of phyla *Cyanobacteria* compared to those that received feed supplemented with antibiotics (estimated difference without antibiotics - with antibiotics = 0.49 ± 0.09; $P_{FDR} < 0.001$). In addition, the analyses on the CSS-normalized OTUs revealed an overrepresentation of 15 and 29 OTUs in cecal samples of rabbits that received a feed supplemented or free of antibiotics; respectively. **Table 4.S3** shows the estimated difference between the presence and the absence of antibiotics in the feed for the OTUs in which the differences reached the significance threshold. The OTU sequences as well as their assignment at the lowest taxonomic level are also shown in **Table 4.S3**. Only 1 of these OTUs could be assigned at species level (*Bacteroides fragilis*) and 2 OTUs at genus level (*Oscillospira* and *Coprococcus*).

4.3.4. Microbial information as a classifier of cecal samples according to farm environment and animal management

Sparse partial least squares-discriminant analyses (sPLS-DA) on the CSS-normalized OTUs were conducted to discriminate samples according to the factors considered in this study (i.e., the farm where the animal was raised, the presence or the absence of antibiotics in the feed and the feeding regime). The tuning process of the sPLS-DA conducted to discriminate samples according to the farm where the rabbits were raised selected 5 OTUs for component 1 and 1 OTU for component 2 (**Figure 4.4**). Component 1 explained 7.00% of the total variance while component

2 explained 0.67%. The classification performance of this sPLS-DA could be said to be perfect since its overall and balanced error rate (BER) per class across 1000 replicates of 5-folds cross-validation runs was 0.00 (0.00). Furthermore, two OTUs of component 1 had a stability higher than 0.9.

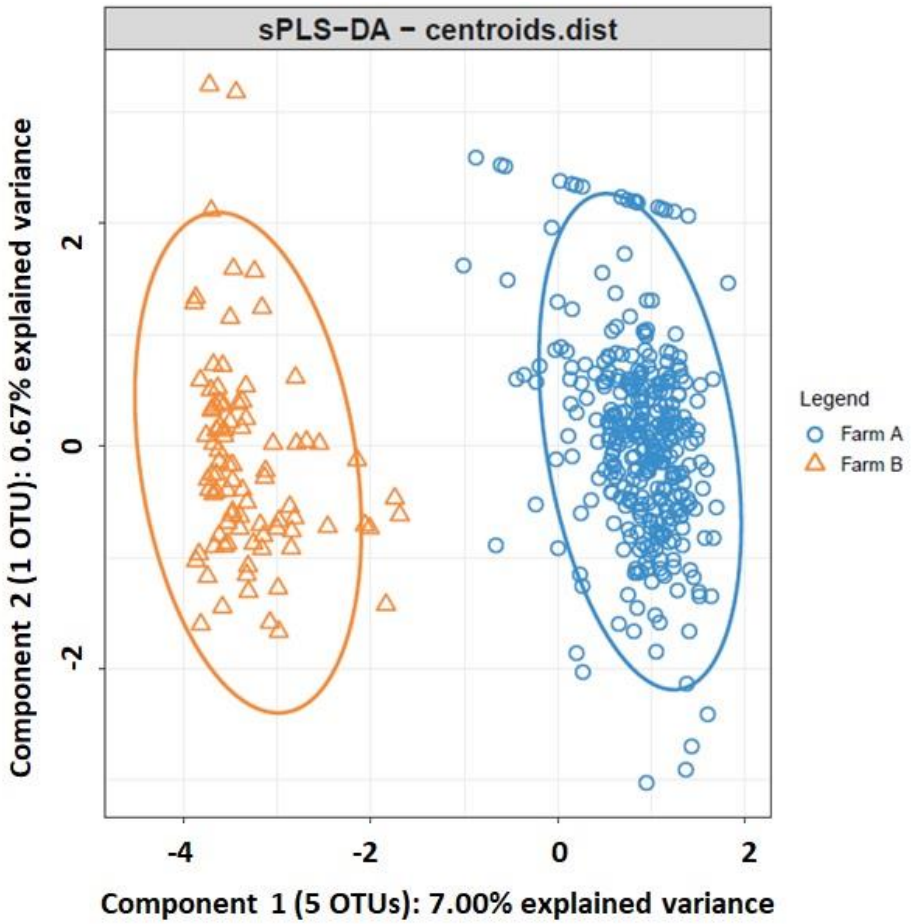

Figure 4.4| Sparse partial least squares discriminant analysis representing cecal samples of rabbits raised in farm A (blue) and in farm B (orange).

The sPLS-DA performed to discriminate samples across feeding regimes within farm A selected 70 OTUs for component 1 and 65 OTUs for component 2 (**Figure 4.5**). Component 1 explained 2.34% of the total variance while component 2 explained 5.58%. The cross-validation assessment of the classification performance of this sPLS-DA showed an overall and BER per class of 0.27 (0.02). The stability of 18 and 5 OTUs selected in components 1 and 2, respectively, across the different cross-validation folds was higher than 0.9.

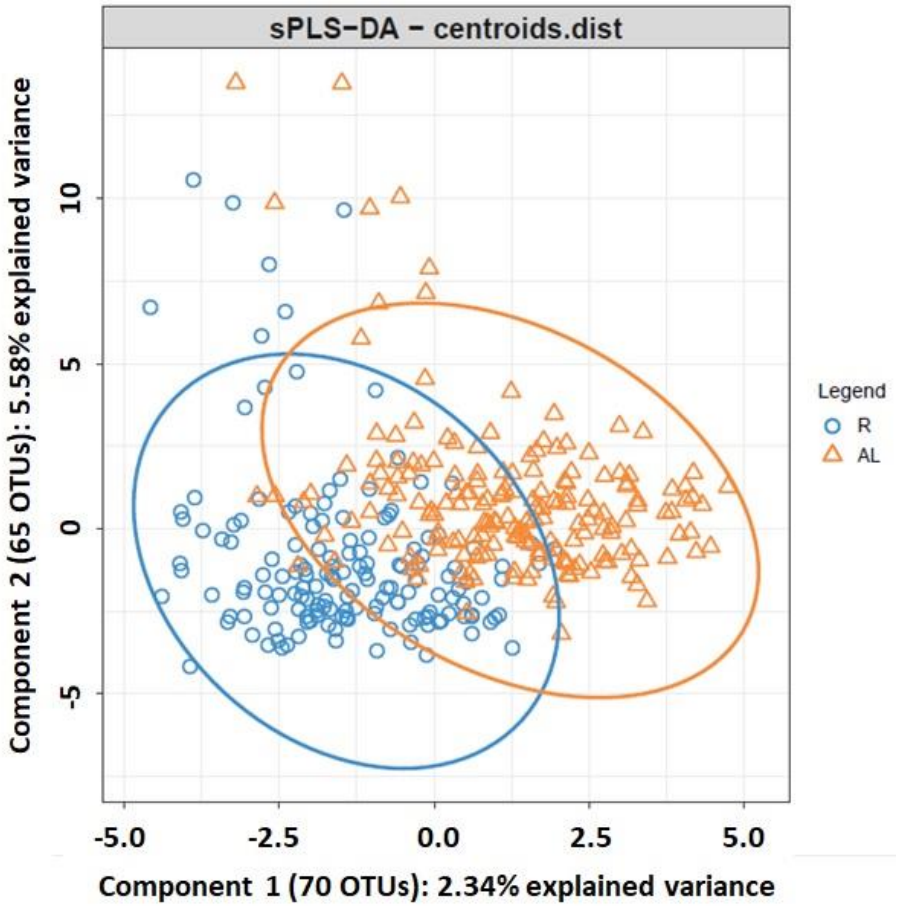

Figure 4.5| Sparse partial least squares discriminant analysis representing cecal samples of rabbits raised in farm A and fed R (blue) or AL (orange).

Finally, the sPLS-DA conducted to discriminate samples of animals raised within farm B according to the combination of the presence or not of antibiotics in the feed and the feeding regime selected 9 OTUs for component 1 and 70 OTUs for component 2 (**Figure 4.6**). Component 1 explained 3.05% of total variance and defined the discrimination between samples from animals fed with antibiotics and those fed without antibiotics. On the other hand, component 2 explained 3.05% of total variance and defined the discrimination between samples from animals fed R and those belonging to animals fed AL. The cross-validation assessment of the classification performance of this sPLS-DA showed an overall BER of 0.32 (0.15). The BER per class was 0.34 (0.12) for samples fed R without antibiotics, 0.46 (0.14) for samples fed AL without antibiotics, 0.29 (0.11) for samples fed R with antibiotics, and 0.20 (0.07) for samples fed AL with antibiotics. The stability of 3 and 11 OTUs selected in components 1 and 2, respectively, across the different cross-validation folds was higher than 0.9.

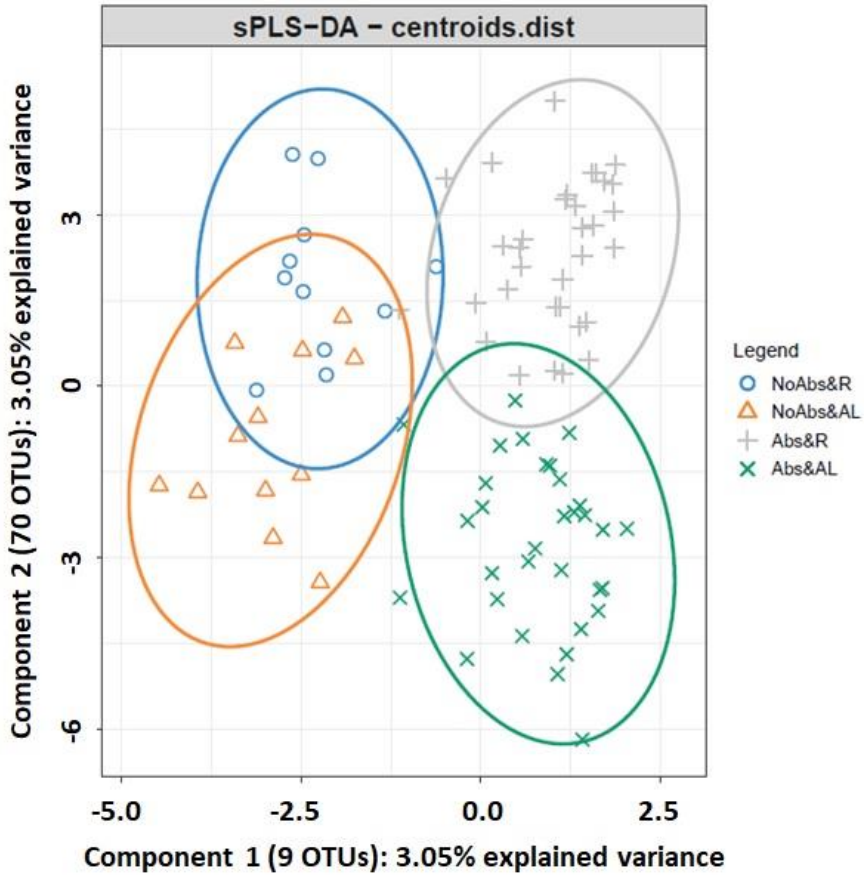

Figure 4.6| Sparse partial least squares discriminant analysis representing cecal samples of rabbits raised in farm B and fed R without antibiotics (blue), fed AL without antibiotics (orange), fed R with antibiotics (gray), and fed AL with antibiotics (green).

4.4. Discussion

The influence of farm environment and common commercial practices of animal management on their gut microbiota are not yet well known in many livestock species. In this study, we have aimed to disentangle potential changes in microbial diversity and composition of meat rabbit cecal communities as a result of being raised in different farms and subjected to different handling during their growing period. To shed light on this matter, we conducted a microbiota comparison of a large number of rabbits raised in different farms, feeding regimes, and fed with feed supplemented or free of antibiotics.

4.4.1. 16S rRNA gene-based characterization of meat rabbit cecal microbiota

The Illumina MiSeq sequence processing of samples collected from these animals revealed that phyla *Firmicutes*, *Tenericutes* and *Bacteroidetes* dominate the growing meat rabbit cecal ecosystem representing more than 90% of its entire microbial composition. This fact is in accordance with previous studies that have characterized the rabbit cecal microbiota (Zou *et al*., 2016; Chen *et al*., 2019; Velasco-Galilea *et al*., 2018) and reported *Firmicutes* as the predominant phylum. However, there are discrepancies between studies in establishing which other phyla are also prevalent in this ecosystem. Whereas we found phyla *Tenericutes* and *Bacteroidetes* representing 7.22% and 5.93% of the cecal microbial composition, respectively, Chen *et al*. 2019 and Zou *et al*. (2016) reported *Bacteroidetes* as the second predominant phylum representing 18% and 20% of New Zealand White and Rex rabbit cecal microbial composition, respectively. Conversely, other studies that have previously characterized meat rabbit fecal microbiota identified higher relative abundances of phyla *Proteobacteria* and *Verrucomicrobia* (Kylie *et al*., 2018; Eshar and Weese, 2014). Velasco-Galilea *et al*. (2018) reported *Firmicutes* (76.42%), *Tenericutes* (7.83%) and *Bacteroidetes* (7.42%) as the predominant phyla of meat rabbit fecal and cecal microbial communities. These discrepancies found across studies could be attributed to technical issues (e.g., pair of primers, sequencing platform, bioinformatic pipeline employed to process raw sequences or reference database used for the taxonomic assignment of the representative sequences) or to purely biological reasons (e.g., breed, age or section of the GIT sampled). Nonetheless, Kylie *et al*. (2018) depicted that the relative increase in less beneficial phyla, such as *Proteobacteria*, could be related to seasonal climate changes that directly impact rabbits' health. This impact affects the susceptibility to enteritis and possibly feed conversion efficiency. In any case, this phylum was more prevalent in farm A where the animals were more exposed to changes in climate conditions.

4.4.2. Farm environment modify alpha diversity

Regarding the alpha diversity assessment, Shannon and the observed number of OTUs indexes revealed the existence of significant differences between the experimental farm where the rabbits were raised. Cecal samples collected from rabbits raised in farm B had greater richness and diversity than those belonging to animals raised in farm A. This could be explained by more stable environmental conditions in farm B (i.e., facility better insulated) than in farm A. It has been already shown that intestinal health is positively associated with microbial diversity (Larsen and Claassen, 2018). In our case, this better health could be said to be granted by the more stable environmental conditions offered by farm B. The most exposed environmental conditions of farm A, combined with the fact that samples of animals raised in this facility were collected from rabbits produced in 4 different batches, could also explain the larger variability in both indexes observed in this farm (Kylie *et al*., 2018). Despite not having observed significant differences between the presence or not of antibiotic in the feed, nor between feeding regimes, it is noteworthy to mention that samples collected from animals fed AL in both farms had a greater, although not significant, richness than those fed R. This fact is consistent with previous studies in mice that observed a lower alpha diversity in animals with a restricted level of feeding (O'Neil *et al*., 2017; Chen *et al*., 2016; Zarrinpar *et al*., 2014). Surprisingly, but in agreement with our results, studies performed in pigs (Soler *et al*., 2018), chicken (Kumar *et al*., 2018) and Rex rabbits (Zou *et al*., 2016) also did not show clear significant differences on alpha diversity indexes between animals fed on diets with antibiotics with respect to those on diets free of antibiotics. Nevertheless, these studies were able to detect differences in the relative abundances of some specific species between diets. For example, Kumar *et al*. (2018) found that the inclusion of bacitracin in the feed did not affect the chicken bacterial phyla. However, they observed differences between the control and the bacitracin-fed group in the ileal and cecal bacterial populations at lower taxonomic levels. It is worth noting that the antibiotic withdrawal at the beginning of the last week of the rabbits' lives equalized the diets of both groups and possibly their microbial populations, which may explain some lack of differences between them.

4.4.3. Farm environment has a large impact on rabbit cecal microbiota

Despite the lack of differences in microbial diversity and richness across management factors; univariate studies revealed differential microbial composition across the studied factors. In addition, the performed multivariate analysis evidenced a certain classification power of the samples on the different levels of management and environment factors based on the microbial composition of the samples.

As it might be expected, analyses of variance confirmed that the breeding farm strongly impacts meat rabbit cecal microbial composition. Our results revealed that the relative abundances of 6 out of 8 phyla are differentially represented between both farms. At genus level, we detected significant differences in the relative abundances of almost all of them. Genera *Bacteroides*, *Parabacteroides*, *Rikenella*, *Anaerofustis*, *Anaerostipes*, *Clostridium*, *Coprobacillus*, *Anaeroplasma* and *Akkermansia* were enriched in cecal samples of rabbits housed in farm A. The first three belong to phylum *Bacteroidetes* and genus *Bacteroides* is the most abundant of them in meat rabbit cecum. Species of this genus are anaerobic Gram-negative members of the family *Bacteroidaceae* that play an important role in the degradation of vegetal polysaccharides and amino acid fermentation in the mammal GIT (Fang *et al*., 2017; Dai *et al*., 2011). Moreover, this genus is involved in propionic acid and lactate formation depending on nitrogen organic availability. Nonetheless, some authors showed that great amounts of *Bacteroides* could predict obesity tendency. *Parabacteroides* is also an anaerobic Gram-negative bacterium (family *Porphyromonadaceae*) involved in amino acid transport and metabolism, energy production and conversion, lipid transport and metabolism, recombination and repair, cell cycle control, cell division, and cell motility in the intestinal microbiota of the growing rabbit (Sun *et al*., 2020). This genus was specifically found in the cecal microbiota of mice raised in conventional conditions and absent in those raised in pathogen-free facilities in a study performed under different housing conditions (Müller *et al*., 2016).

Within the phylum *Firmicutes*, genus *Clostridium* (family *Clostridiaceae*) is an anaerobic Gram-positive bacterium that inhabits the GIT of many mammals where

it acts by degrading cellulose. However, some *Clostridium* species (e.g., *C. perfringens* and *C. difficile*) are pathogenic, and an enrichment of this genus has previously been described in rabbits affected by epizootic rabbit enteropathy (Bäuerl *et al*., 2014). This genus, together with genus *Bacteroides*, was found enriched in the cecal microbiota of mice housed in open cages compared with those kept in individual ventilated cages (Thoene-Reineke *et al*., 2014). Both genera have been associated with an exacerbation of the intestinal inflammatory response in mammals (Terán-Ventura *et al*., 2010). Genus *Anaerofustis* (family *Eubacteriaceae*) has been found enriched in cecal samples of rabbits affected by paratuberculosis infection (*Mycobacterium avium*) (Arrazuria *et al*., 2016).

Within the phylum *Verrucomicrobia*, genus *Akkermansia* is an anaerobic Gram-negative bacterium that encompasses mucin degrader species (Belzer and De Vos, 2012). In the cecum, a proper enrichment of this genus could maintain a suitable mucosal turn-over, thus exerting a protective effect that could help the animal to deal with inflammatory processes.

It is worth mentioning that we have detected genera *Epulopiscium*, *p-75-a5*, *Phascolarctobacterium*, *Campylobacter* and *Desulfovibrio* only in the cecal samples of rabbits housed in farm A. The first three are encompassed within the phylum *Firmicutes.* Genus *Epulopiscium* is a large size Gram-positive bacterium that has a nutritional symbiotic relationship with surgeonfish that eats algae and detritus. This bacterium is physically similar to the phylogenetically related *Metabacterium polyspora* which is an endospore-producing bacterium isolated from the cecum of guinea pigs (Angert *et al*., 1996). On the other hand, genera *Campylobacter* and *Desulfovibrio* are Gram-negative bacteria that belong to phylum *Proteobacteria*. Some species of these genera are pathogens responsible for infections and diarrheas in mammals. The exclusive presence of these genera in farm A could indicate the existence of a potential dysbiosis of the animals raised in that facility that could affect their sanitary status and growth. While farm A was a semi-open-air facility, farm B was artificially ventilated and offered more controlled environmental conditions that favor animal growth. Moreover, the presence of sulfate-reducing bacteria (SRB) such as *Desulfovibrio* could be enhanced by sulfate-secreting

bacteria (SSB) such as *Rikenella* in farm A where this genus is significantly more predominant. It is noteworthy to mention that SRB could also obtain sulfate via *cross-feeding* mediated by *Bacteroides*-encoded sulfatases (Rey *et al*., 2013), and interestingly, this phylum is more prevalent in farm A.

Regarding sample classification based on the sPLS-DA study, given the important differences in gut microbial composition found between farms, a perfect classification of the samples can be achieved with only 5 OTUs. One of these 5 OTUs was overrepresented in farm B and belonged to family *S24-7* (phylum *Bacteroidetes*). The remaining 4 were overrepresented in farm A and belonged to family *Barnesiellaceae* (phylum *Bacteroidetes*), order *Bacteroidales* (phylum *Bacteroidetes*), and genera *Desulfovibrio* (phylum *Proteobacteria*) and *Bacteroides* (phylum *Bacteroidetes*). It is worth mentioning that these 5 OTUs were also declared as differentially represented between farms by the univariate analyses.

4.4.4. Administration of antibiotics impact on some taxa relative abundances

Within farm B, the effect of the presence of antibiotics in the feed was assessed by comparing the microbial cecal composition of rabbits fed with antibiotics with that of some animals that received feed without antibiotics. As stated above, we did not detect significant differences in alpha diversity, nor in genera relative abundances, between both groups. However, some significant differences were observed at phylum and OTU levels. An overrepresentation of phylum *Cyanobacteria* was found in rabbits fed without antibiotics. The detection of this bacterial phylotype, commonly assigned to photosynthetic activity, in the rabbit cecum could suggest contamination during the GIT sampling. However, Zeng *et al*. (2015) previously reported its presence in rabbit feces. In the present study, all OTUs taxonomically assigned to phylum *Cyanobacteria* are as well encompassed in the order *YS2*. Interestingly, it was demonstrated that this order does not really have photosynthetic capacity and it is currently classified within the candidate phylum *Melainabacteria* (Di Rienzi *et al*., 2013). The non-photosynthetic cyanobacteria *YS2*, now named

Gastranaerophilales, is a fermenter gut-associated order present in humans and other animals such as squirrels, where its exact role is unknown but it has the capacity to produce hydrogen, fix nitrogen and synthesize vitamins B and K (Di Rienzi *et al*., 2013; Monchamp *et al*., 2019; Liu *et al*., 2020). Our results, in accordance with Kylie *et al*. (2018), revealed that rabbits fed without antibiotics exhibited higher abundances of OTUs assigned to phylum *Bacteroidetes* than those fed with antibiotics. In addition, samples of rabbits that received antibiotics had a significant increase of an OTU taxonomically assigned to genus *Coprococcus.* Interestingly, a study that evaluated the differences in bacterial communities of Rex rabbits fed with different antibiotics also found an overrepresentation of this bacterium in animals treated with zinc bacitracin (Zou *et al*., 2016). *Coprococcus* is an anaerobic bacterium that may protect against colon cancer in humans by producing butyric acid (Ai *et al*., 2019). We hypothesized that the administration of antibiotics could modulate the abundance of some *Coprococcus* species to provide intestinal protection on meat rabbits. However, it is important to recognize that the reduced sample size of the group of rabbits fed without antibiotics may have limited the statistical power to detect microbial composition differences associated with this factor.

4.4.5. Feed restriction modifies *Euryarchaeota* and some bacteria relative abundances

Within farm B, the effect of the feeding regime in microbial composition was also assessed by comparing samples of animals fed R with those fed AL. The main difference found was for phylum *Euryarchaeota* which was overrepresented in animals fed R in farm B. All *Euryarchaeota* species found in the rabbit cecum belong to genus *Methanobrevibacter* that encompasses different hydrogenotrophic methane-producing species. Previous studies in humans (Shen and Maitin, 2015) and cattle (McCabe *et al*., 2015; McGovern *et al*., 2017) found an overrepresentation of *Methanobrevibacter* species in individuals submitted to feed restriction and a negative correlation between the abundance of this bacterium and body mass index. A prevalence of *Methanobrevibacter* species could be a positive indicator of a healthy microbiota since restricted animals showed an overrepresentation of this

genus. The main purpose of applying feed restriction is to improve intestinal health, reducing weaning mortality. The growth of *Methanobrevibacter* is supported by fermenters such as *Gastranaerophilales* and butyrate-producing bacteria such as *Anaereostipes* via interspecies formate/hydrogen transfer (Bui *et al*., 2019). A study in mice determined that *Methanobrevibacter smithii* facilitates *Bacteroides thetaiotaomicron* capacity to digest glycans resulting in increased production of short-chain fatty acids (Samuel and Gordon, 2006). The same study defined *M. smithii* as a "*power broker*" that regulates polysaccharide fermentation efficiency that influences the fat stores. The lower prevalence of methanogenic archaea in farm A could be explained by the high presence of SRB that outcompete with methanogens for hydrogen consumption. This fact could favor hydrogen sulfide production and compromise the rabbits' health.

Regarding the sample classification based on the sPLS-DA study conducted within farm B, component 1 and component 2 discriminated between animals that did or did not received antibiotics in the feed and between feeding regimes, respectively. It is worth mentioning that 8 out of 9 OTUs selected in component 1 were also declared as differentially represented between the presence or the absence of antibiotics in the feed by the univariate analyses. Within farm A, an sPLS-DA was also performed to classify samples according to the feeding regime using microbial information. Although a large number of OTUs were selected as classifier variables in the tuning process of this sPLS-DA, the classification error rate was high. It implied a poor discrimination capacity of samples according to the feeding regime the animal received. Nevertheless, bootstrap univariate analyses of variance detected some significant differences at all taxonomic levels analyzed between feeding regimes within farm A. At genus level, *rc4-4* was overrepresented in animals fed AL. This genus belongs to phylum *Firmicutes* and it is known as an obesity-associated bacterium (Ziętak *et al*., 2016) and as a pathogenic candidate identified in mice with multiple sclerosis (Gandy *et al*., 2019). A potential pro-inflammatory role has been proposed for this genus (Gandy *et al*., 2019) what could be related to a reduced incidence of enteric disorders when feed restriction is applied. It is worth mentioning that family *Peptococcaceae*, which encompasses genus *rc4-4*, is strongly related to total rabbit weight gain from weaning to 12-week-old (North *et al*.,

2019). Although in our study this genus was prevalent in animals fed AL, its association with weight gain is not clear since the greater growth exhibited by these animals was consequence of higher feed intake.

4.4.6. Rabbit cecal microbiota is shaped by farm environment and animal management

Different approaches have been applied in this study to evaluate the effect of different environments and management practices, commonly used in rabbit production, in their cecal microbial composition and diversity. Those animals raised in the best insulated facility (farm B) appear to have a microbiota characteristic of healthier animals than those raised in the open-air facility (farm A). It is worth mentioning that the rabbits were housed in cages interspersed with feeding regime. This fact could make possible the exchange of microorganisms between animals of different feeding regimes and therefore have reduced the differences observed between regimes. However, the joint consideration of 70 OTUs in the sPLS-DA made possible a certain discrimination power of samples according to the level of feeding received by each animal raised in farm A. It implies the existence of cecal microbiota content patterns characteristic of each regime which could be revealed thanks to the univariate analyses conducted at different taxonomic levels. Similarly, the sPLS-DA performed within farm B also involved the consideration of 70 OTUs to discriminate samples according to the amount of feed consumed. Within this farm, the classification of samples regarding the presence or the absence of antibiotics in the feed needed a smaller number of OTUs than the feeding regime but greater than the farm. This suggests that the effect of the presence of antibiotic in feed is stronger than the feeding level. The lack of a group of samples collected from animals that did not receive antibiotics precluded the evaluation of the magnitude of importance of this effect over the feeding level on the cecal microbiota of animals raised in farm A. It might have been possible that the magnitude of the effect of the presence of antibiotics in the feed was larger in farm A than that observed in farm B. The experimental design of this study prevented the comparison of the effect of antibiotic treatments across farms on rabbits' microbial communities. The implication of the discussed microbial composition and diversity differences originated by the studied

management and environmental factors on the animals' performance still needs to be investigated. In future studies the role of specific groups of bacteria in rabbit growth and feed efficiency will be analyzed.

4.5. Conclusions

The analysis of a large number of animals from a paternal rabbit line has allowed a deeper comprehension of the role played by different management and environmental factors shaping the composition and diversity of cecal microbial communities. It reveals that the farm environment offered to the rabbits during their growth play a key role that can result in different microbial alpha diversity and composition of almost all species that inhabit the rabbit GIT. This highlights the importance that a stable and controlled environment could have in the intestinal health and, consequently, in animal performance. It seems clear that the better insulated conditions of farm B favored the presence of a gut microbiota characteristic of healthier animals. Although the level of feeding and the presence of antibiotics in the feed did not modify the global diversity of cecal microbial communities, these factors can increase or decrease the prevalence of specific bacteria which could lead to a microbial composition potentially beneficial for the animal or, at the other extreme, to an origin of future intestinal dysbiosis.

4.6. Methods

4.6.1. Animals and experimental design

All biological samples used in the study were collected from animals of an experiment conducted at the Institute of Agrifood Research and Technology (IRTA) in different periods and involving two different farms. The objective of that experiment was to estimate the effect of the interaction between the genotype and the feeding regime (i.e., the amount of feed provided during fattening) on growth, feed efficiency, carcass characteristics, and health status of the animals (Piles and Sánchez, 2019). For this particular study, 425 meat rabbits from Caldes line (Gómez *et al*., 2002) of that experiment were randomly selected. Most of them (336) were raised in 4 different batches in a semi-open-air facility (farm A). The remaining

animals (89) were produced in a single batch in another facility under better controlled environmental conditions (farm B). Rabbits raised in farm A were housed in collective cages containing 8 kits each one while those raised in farm B were housed in cages with 6 kits each one. All animals were raised under the same management conditions and received the same standard pelleted diet. Twenty-three rabbits raised in farm B received a diet free of antibiotics and the remaining sixty-six received the same diet but supplemented with antibiotics. Those raised in farm A received oxytetracycline, valnemulin, and colistin while those in farm B received oxytetracycline, valnemulin and neomycin. At the time this experiment was conducted, it was possible to use up to four types of molecules to prevent or treat the emergence of potential infectious diseases on farms. However, nowadays, only one antibiotic molecule is allowed. During the last fattening week all the animals received an antibiotic free diet. Feed was supplied once per day in a feeder with three places for the 4-5 weeks that the fattening lasted. Water was provided *ad libitum* during the whole fattening period. The animals were under two different feeding regimes: (1) *ad libitum* (AL) or (2) restricted (R) to 75% of the AL feed intake. The amount of feed supplied to the animals under R feeding regime in a given week for each batch was computed as 0.75 times the average feed intake of kits on AL from the same batch during the previous week, plus 10% to account for a feed intake increase as the animal grows. Kits were randomly assigned to one of these two feeding regimes after weaning (32 days of age). They were categorized into two groups according to their size at weaning (big if their body weight was greater than 700 g or small otherwise) aiming to obtain homogenous groups regarding animal size within feeding regime. A maximum of two kits of the same litter were assigned to the same cage in order to remove the possible association between cage and maternal effects on animal growth during the fattening period. The distribution of these animals across the different levels of management factors is shown in **Table 4.3**. The body weight of each animal was weekly recorded. The individual average daily gain was computed as the slope of the within animal regression of all body weight measurements recorded during the growing period.

Table 4.3| Distribution of rabbits in groups according to different management factors.

Farm	Batch	Feed	Feeding regime	Number of rabbits
A	1	With antibiotics	Ad libitum	27
A	1	With antibiotics	Restricted	30
A	2	With antibiotics	*Ad libitum*	35
A	2	With antibiotics	Restricted	41
A	3	With antibiotics	*Ad libitum*	61
A	3	With antibiotics	Restricted	53
A	4	With antibiotics	*Ad libitum*	57
A	4	With antibiotics	Restricted	32
B	5	With antibiotics	*Ad libitum*	32
B	5	With antibiotics	Restricted	34
B	5	Without antibiotics	*Ad libitum*	12
B	5	Without antibiotics	Restricted	11

4.6.2. Sample processing, DNA extraction and sequencing

Animals were slaughtered (at 66 and 60 days of age in farm A and farm B, respectively) and cecal samples of each rabbit were collected in a sterile tube, kept cold in the laboratory (4ºC) and stored at -80ºC. DNA extraction, amplification, Illumina library preparation and sequencing followed methods described previously (Velasco-Galilea *et al*., 2018). Whole genomic DNA was extracted from 250 mg of each cecal sample using ZR Soil Microbe DNA MiniPrep™ kit (ZymoResearch, Freiburg, Germany) according to manufacturer's instructions with the following modification: cecal samples were mechanically lysed in a FastPrep-24™ Homogenizer (MP Biomedicals, LLC, Santa Ana, CA, United States) at a speed of 1 x 6 m/s for 60 s facilitating an efficient lysis of archaea and bacteria species. Integrity and purity of DNA extracts were measured with Nanodrop ND-1000 spectrophotometer equipment (NanoDrop products; Wilmington, DE, United States) according to Desjardins and Conklin's protocol (Desjardins and Conklin, 2010). All DNA extracts had adequate integrity and purity (absorbance ratio 260 nm/280 nm > 1.6) to avoid PCR inhibition issues.

A fragment of the 16S rRNA gene including the V4-V5 hypervariable regions was amplified with F515Y/R926 primer combination (5'-GTGYCAGCMGCCGCGGTAA-3', 5'-CCGYCAATTYMTTTRAGTTT-3') (Parada *et al*., 2016) and then re-amplified in a limited-cycle PCR reaction to add sequencing adaptors and 8 nucleotide dual-indexed barcodes of multiplex Nextera® XT kit (Illumina, Inc., San Diego CA, United States) following manufacturer's instructions. The initial PCR reactions were performed for each sample using 12.5 µl 2x KAPA HiFi HotStart Ready Mix, 5 µl forward primer, 5 µl reverse primer and 2.5 µl template DNA (5 ng/ µl). The initial PCR conditions were as follows: initial denaturation for 3 minutes at 95 ºC, 25 cycles of 30 seconds at 95 ºC, 30 seconds at 55 ºC and 30 seconds at 72 ºC; and final extension for 2 minutes at 72 ºC. The addition of indexes and sequencing adaptors to both ends of the amplified regions took place in a second PCR by using 25 µl 2x KAPA HiFi HotStart Ready Mix, 5 µl index i7, 5 µl index i5, 10 µl PCR Grade water and 5 µl concentrated amplicons of initial PCR. The second PCR conditions were as follows: initial denaturation for 3 minutes at 95 ºC, 8 cycles of 30 seconds at 95 ºC, 30 seconds at 55 ºC and 30 seconds at 72 ºC; and final extension for 5 minutes at 72 ºC. Final libraries were cleaned up with AMPure XP beads, validated by running 1 µl of a 1:50 dilution on a Bioanalyzer DNA 1000 chip (Agilent Technologies, Inc., Santa Clara, CA, United States) to verify their size, quantified by fluorometry with PicoGreen dsDNA quantification kit (Invitrogen, Life Technologies, Carlsbad, CA, United States), pooled at equimolar concentrations and paired-end sequenced in 5 parallel plates in an Illumina MiSeq 2 x 250 platform at the Genomics and Bioinformatics Service (SGB) of the Autonomous University of Barcelona (UAB).

4.6.3. Bioinformatic pipeline for OTU calling

Sequence processing was performed using QIIME software (version 1.9.0) (Caporaso *et al*., 2010). In a first step, the resulting paired-ended V4-V5 16S rRNA gene reads were assembled into contigs with the python script *multiple_join_paired_ends.py*. Then the contigs were curated using the script *split_libraries.py* with default parameters in order to assign them to samples and to discard those with a low-quality (Q19 was the minimum acceptable quality score).

Chimeric sequences generated during the process of DNA amplification were detected with a UCHIME algorithm (Edgar *et al*., 2011) and removed. The totality of filtered contigs were clustered into operational taxonomic units (OTUs) with a 97% similarity threshold using the script *pick_open_reference_otus.py* with default parameters (Rideout *et al*., 2014) that grouped, through a UCLUST algorithm (Edgar, 2010), the sequences against Greengenes reference database (version gg_13_5_otus) and also made a *de novo* clustering of those that did not match the database. The generated OTU table was filtered at: (1) sample level: by discarding samples with less than 5,000 final sequence counts and at (2) OTU level: by removing the doubleton ones. The filtered OTU table contained the sequence counts of 963 OTUs for 425 samples. Taxonomic assignment of representative sequences of each OTU defined (963) was conducted by mapping them to the Greengenes reference database gg_13_5_otus with the UCLUST consensus taxonomy assigner (QIIME default parameters). The raw sequence data were deposited in the sequence read archive of NCBI under the BioProject accession number PRJNA524130. Metadata, the prefiltered and normalized OTU tables, and corresponding taxonomic classifications are also included as **Additional files 4.1, 4.2, 4.3** and **4.4**, respectively.

4.6.4. Models and statistical methods

In order to study differences in diversity and richness between rabbits grouped according to farm environment and management that they received, two alpha diversity indexes (Shannon and the observed number of OTUs) were computed from the OTU table rarified to 10,000 sequences per sample with “phyloseq” R package (McMurdie and Holmes, 2013). The statistical method chosen to assess alpha diversity differences between these groups of animals was an analysis of variance that included a factor resulting from the combination of four factors (the farm where the animal was raised, the batch, the presence or the absence of antibiotics in the feed and the feeding regime). The significance threshold was set at 0.05 for type I error.

Different approaches were considered to assess the influence of the environments and management factors on microbial composition. A bootstrap analysis of variance was individually implemented for each OTU to test whether it was differentially represented between the different categories of the factors studied. This univariate analysis was conducted by normalizing the OTU table with the cumulative sum scaling (CSS) method (Paulson *et al*., 2013) and only for those OTUs which were detected in at least 5% of the samples and had a sum of its counts resulting in a frequency greater than 0.01% of the total sum of all OTUs counts across all samples. It was implemented by fitting a model defined by the combination of the four aforementioned factors by using *lm()* function in R (R Development Core Team, 2010). Then, the differences between the CSS-normalized OTUs counts in the different levels of the studied factors were tested. The significance between the levels of the main factors: farm, presence of antibiotics in the feed and feeding regime was assessed using an F statistic. When the involved interaction terms were significant, the contrasts of interest were studied nested within the levels of other interacting factors, i.e., feeding regime was studied within farm levels. When the interaction terms were not significant, the effects of the different levels were averaged, i.e., the effects of the levels of the batches within farm A were averaged to present the effect associated with this farm. In the performed F tests, instead of relying on the theoretical distribution of the statistic under the null hypothesis to define the p-values, they were empirically computed using bootstrap after 1,000 permutations of the dependent variable with respect to the design matrix of factors in the model. The use of bootstrapping enabled the hypothesis test to be done without the necessity of assuming that data are normally distributed, which is an assumption that fails for OTUs counts. *P*-value was defined as the proportion of bootstrap rounds having an F statistic value equal to or greater than that obtained with the original dataset. *P*-values were corrected defining a false discovery rate (FDR) of 0.05 (Benjamini and Hochberg, 1995). This bootstrap analysis of variance approach was also implemented in order to study the effect of the management factors on the relative abundance of bacteria at phylum and genus levels.

The value of the microbial information to classify samples into the three factors considered in our study was explored using multivariate techniques. In particular,

sparse partial least squares-discriminant analysis (sPLS-DA) (Lê Cao *et al*., 2008) was used to find the combination of OTUs that allowed the best classification of cecal samples according to: (1) the farm where the animals were raised, (2) the feeding regime within farm A and (3) the combination of feeding regime and the presence or absence of antibiotics in the feed for the animals raised in farm B. This approach was implemented through the R package "mixOmics" (Rohart *et al*., 2017). In a first step, the function *tune.splsda()* was used to select the optimal sparsity parameters of the sPLS-DA model: the number of components and the number of variables (OTUs) per component. For the tuning process, a 5-fold cross-validation repeated 10 times was performed one component at a time, with a maximum of 4 components, on an input grid of values that indicate the number of variables to select on each component. The sparsity parameters were defined, based on the BER and centroids distance, and then included in the final sPLS-DA model. Samples were represented on the first two components and colored according to their class (e.g., R or AL in the case of the feeding regime) in a sample plot with the function *plotIndiv()*. The performance of the sPLS-DA model was assessed with a 5-fold cross-validation repeated 1,000 times that randomly split the data in training and validation sets. In this data partition, it was ensured that 20% of the samples within each level of the discriminant factor were assigned to the validation set. Five different partitions were performed for each replicate to guarantee a different sample distribution in each validation set. The sPLS-DA model with the sparsity parameters previously defined was adjusted in the training set and its classification performance was assessed in the validation set using the overall and BER per class as criteria. The stability of the OTUs selected on each component was also assessed in the cross-validation by computing the selection frequency of each variable across the replicates.

4.7. List of abbreviations

AL *ad libitum*

BER balanced error rate

CSS cumulative sum scaling

FDR false discovery rate

GIT	gastrointestinal tract
NGS	next generation sequencing
OTU	operational taxonomic unit
PCR	polymerase chain reaction
R	restricted
sPLS-DA	sparse partial least squares-discriminant analysis
SRB	sulfate-reducing bacteria (SRB)
SSB	sulfate-secreting bacteria (SRB)

Declarations

Ethics approval and consent to participate

This study was carried out in accordance with the recommendations of the animal care and use committee of the Institute for Food and Agriculture Research and Technology (IRTA). The protocol was approved by the committee of the Institute for Food and Agriculture Research and Technology (IRTA).

Consent for publication

Not applicable.

Availability of data and materials

The raw sequence data were deposited in the sequence read archive of NCBI under the accession number SRP186982 (BioProject PRJNA524130). Metadata, the prefiltered and unnormalized OTU table, the filtered and CSS-normalized OTU table and corresponding taxonomic assignments have all been included as **Additional files 4.1**, **4.2**, **4.3** and **4.4**, respectively. Relative abundances phyla and genera tables have also been included as **Additional files 4.5** and **4.6**, respectively. OTUs differentially represented between the studied factors, their sequences and their assignment at the lowest taxonomic level have been included as **Additional files 4.7**, **4.8** and **4.9**. The Additional information for this article can be found in the Annexes section.

Competing interests

The authors declare that they have no competing interests.

Funding

The experimental design of this work was conducted thanks to funding from INIA project RTA2011-00064-00-00. This study was part of the Feed-a-Gene project that received funding from the European Union's H2020 program under grant agreement no. 633531, and the Spanish project RTI2018-097610R-I00. MVG is a recipient of a "Formación de Personal Investigador (FPI)" pre-doctoral fellowship from INIA, associated with the research project RTA2014-00015-C2-01. YRC was funded by Marie Skłodowska-Curie grant (P-Sphere) agreement no. 6655919 (EU).

Authors' contributions

JS, MP and OR conceived the experimental design. JS, OR, MP and MVG collected biological samples. MVG, OGR, MP, MG and AS processed the samples in the laboratory. MVG processed and analyzed the sequencing data, interpreted data, prepared figures and tables, and wrote the manuscript. JS and YRC helped analyzing the sequencing data. JS, MG, MP, MV and YRC helped interpreting the data, and wrote and revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We would like to thank Oscar Perucho, Josep Ramon and Carmen Requena (staff of Unitat de Cunicultura, IRTA) for their contribution to data recording and animal care during the experiment. We also acknowledge Nicolas Boulanger and Joana Ribes (Genomics and NGS Unit, CRAG) for their assistance in massive libraries preparation. The English revision of the manuscript conducted by Mr. Roderick Cantlay-Hollis is also acknowledged.

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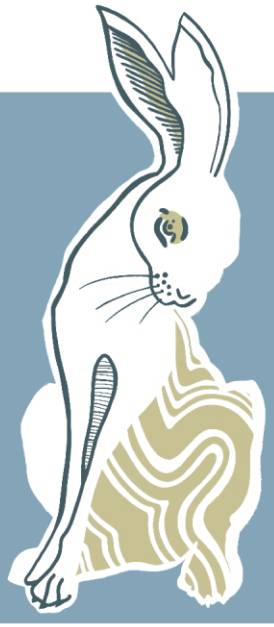
CHAPTER 5

THE VALUE OF GUT MICROBIOTA TO PREDICT FEED EFFICIENCY AND GROWTH OF RABBITS UNDER DIFFERENT FEEDING REGIMES



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Scientific Reports (2021), *11*, p. 19495

The value of gut microbiota to predict feed efficiency and growth of rabbits under different feeding regimes

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5.1. Abstract

Gut microbiota plays an important role in nutrient absorption and could impact rabbit feed efficiency. This study aims at investigating such impact by evaluating the value added by microbial information for predicting individual growth and cage phenotypes related to feed efficiency. The dataset comprised individual average daily gain and cage-average daily feed intake from 425 meat rabbits, in which cecal microbiota was assessed, and their cage mates. Despite microbiota was not measured in all animals, consideration of pedigree relationships with mixed models allowed the study of cage-average traits. The inclusion of microbial information into certain mixed models increased their predictive ability up to 20% and 46% for cage-average feed efficiency and individual growth traits, respectively. These gains were associated with large microbiability estimates and with reductions in the heritability estimates. However, large microbiabililty estimates were also obtained with certain models but without any improvement in their predictive ability. A large proportion of OTUs seems to be responsible for the prediction improvement in growth and feed efficiency traits, although specific OTUs taxonomically assigned to 5 different phyla have a higher weight. Rabbit growth and feed efficiency are influenced by host cecal microbiota, thus considering microbial information in models improves the prediction of these complex phenotypes.

5.2. Introduction

Feed efficiency (FE) is a fundamental trait in rabbit breeding since food expenses often represent up to 70% of the production costs (Cartuche *et al*., 2014). The difficulties entailed in measuring the individual animals' feed intake (FI) are the main reason why most programs do not perform a direct selection for FE. An alternative commonly used to improve FE is the indirect selection for average daily gain (ADG) or body weight (BW) at the end of the growing period (Estany *et al*., 1992). Nevertheless, the genetic correlation between these growth traits and FE may be not high enough to result in an optimal selection response (Piles *et al*., 2004). Therefore, it would be worth exploring new traits allowing alternative selection strategies such as FE definitions based on cage-average FI records. In this regard,

the present study uses cage-average records of FI and individual records of BW collected from animals raised in groups, thus reflecting the reality of commercial farms where animals are raised in groups.

The cecum is the main organ harboring the microbial fermentation processes in the domestic meat rabbit, *Oryctolagus cuniculus*. This organ hosts a complex microbial ecosystem dominated by bacterial phyla *Firmicutes*, *Tenericutes*, and *Bacteroidetes* (Velasco-Galilea *et al*., 2018a). The interactions that are continuously taking place between bacteria and their host ensure the homeostatic balance maintenance of the cecum ecosystem. Previous studies revealed that relative abundances of these, and other less abundant taxa, vary between individuals and are affected by external factors such as the breeding farm, the level of feeding, or the administration of antibiotics (Velasco-Galilea *et al*., 2020).

In the field of livestock production, certain studies have hypothesized that the rabbit gut microbiota could be associated with growth (Zeng *et al*., 2015) and FE (Drouilhet *et al*., 2016). Furthermore, a recent study has identified several operational taxonomic units (OTUs) and KEEG pathways associated with ADG in commercial meat rabbits (Fang *et al*., 2020a). Nonetheless, a fact that should not be overlooked is the strong impact on the animals' growth and FE exerted by the breeding environment or common rabbit breeding strategies such as feed restriction (Gidenne *et al*., 2012), thus when considering the role of gut microbiota on performance traits these management and environmental effects must not be ignored. Studies are necessary to investigate the connection between the gut microbiota and animal performance together with these external factors that also affect growth and FE while shaping microbial communities (Velasco-Galilea *et al*., 2020). Moreover, the existing collinearity between microbiota and management effects difficult the finding of real associations of the animal growth with specific taxa abundances.

This study aims at understanding the role of microbial communities inhabiting the cecum on the FE and the growth of rabbits raised in collective cages under different feeding regimes. The use of sparse partial least squares regression (sPLSR) and mixed models in cross-validation schema will allow unraveling the value of cecal

microbiota to predict cage FE and individual growth performances in a rabbit line selected for post-weaning growth.

5.3. Results

5.3.1. Influence of genetics and cecal microbiota on rabbit growth and FE

Table 5.1 includes statistics of marginal posterior distributions for heritabilities (h^2), microbiabilities (m^2), and phenotypic variances for individually recorded traits (ADG_{AL} and ADG_R) obtained with the dataset including only records of animals in which microbiota was assessed (mDataset). Similarly, **Table 5.2** and **Table 5.3** include estimates for the same parameters referring both to individual growth and cage-average traits ($\overline{\mathrm{ADFI}}_{\mathrm{AL}}$, $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$ and $\overline{\mathrm{ADFCR}}_{\mathrm{AL}}$). In these latter two cases, the estimates were computed with the dataset including records of animals in which microbiota was assessed as well as of their cage mates (fullDataset). Statistics were obtained with the model not including the microbial effect (M1) and with the models fitting the microbial effect (M2) by considering different prior assumptions. Trace plots and histograms of Markov chains from the posterior distribution of the parameters of these models using different prior assumptions and datasets are included as **Additional file 5.4**.

The heritabilities (h^2) obtained with M1 and the mDataset were 0.21 and 0.29 for ADG_{AL} and ADG_R, respectively (**Table 5.1**). The posterior means of h^2 obtained with M1 and the fullDataset were markedly lower, 0.15 and 0.09 for ADG_{AL} and ADG_R, respectively (**Table 5.2** and **Table 5.3**). However, estimates cannot be considered significantly different between datasets. The h^2 estimates with M2 models including the microbial effect ranged, depending on the prior assumption for the microbial effects and the dataset used for the analysis, from 0.05 to 0.15 for ADG_{AL} and from 0.07 to 0.09 for ADG_R. These ranges for m^2 varied from 0.00 to 0.79 for ADG_{AL} and from 0.00 to 0.77 for ADG_R. In general, it was observed that the higher the magnitude of m^2, the higher the changes in the h^2 estimates from M1 to M2. It is important to note that the lowest estimates of m^2 for both traits were obtained in the

analyses in which all the individual records were considered for the study and the elements of the covariance matrices for animals without microbial composition were generated considering cage-average CSS OTU counts ($\mathbf{M}_{\bar{\mathbf{O}}}, \mathbf{M}_{\bar{\mathbf{B}}}$ or $\mathbf{M}_{\bar{\mathbf{U}}}$) (**Table 5.3**). The posterior means of m^2 for both traits were almost null for nearly all the cases studied with these covariance matrices, except for ADG_{AL} when the covariance matrix was defined from the Bray-Curtis distance matrix ($\mathbf{M}_{\bar{\mathbf{B}}}$) and for ADG_R when the covariance matrix was defined from the weighted Unifrac distance matrix ($\mathbf{M}_{\bar{\mathbf{U}}}$) . Note that large estimation errors were observed in both cases. These errors can also be linked with the poor mixing of the sampling processes that are evidenced in the trace plots provided in the **Additional file 5.4**.

Regarding cage-average traits, the posterior means of h^2 obtained with M1 were medium-high ranging from 0.26 ($\overline{\mathrm{ADFI}}_{\mathrm{AL}}$) to 0.49 ($\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$) (**Table 5.2** and **Table 5.3**). When the microbial effect was included, these posterior means tended to decrease. The h^2 obtained with M2 models ranged, depending on the prior assumption for the microbial effects, from 0.11 to 0.24 for $\overline{\mathrm{ADFI}}_{\mathrm{AL}}$, from 0.12 to 0.44 for $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$, and from 0.08 to 0.30 for $\overline{\mathrm{ADFCR}}_{\mathrm{AL}}$. The posterior means of m^2 ranged from 0.03 to 0.58 for $\overline{\mathrm{ADFI}}_{\mathrm{AL}}$, from 0.10 to 0.76 for $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$, and from 0.16 to 0.78 for $\overline{\mathrm{ADFCR}}_{\mathrm{AL}}$. Note that for all cage-average traits the highest posterior mean of h^2 and the lowest posterior mean of m^2 were obtained when the microbial covariance matrix was expanded using cage-average CSS OTU counts and then computing their cross-product ($\mathbf{M}_{\bar{\mathbf{O}}}$). The lowest posterior means of h^2 and the highest posterior means of m^2 were obtained with the microbial covariance matrix $\mathbf{M}_{\bar{\mathbf{U}}}$ (i.e., expanding the OTU table using cage-average CSS OTU counts and then computing the weighted Unifrac distance matrix). It is worth mentioning that, similarly to growth traits, the posterior means of the parameters obtained with M2 models based on expanding the CSS OTU table by cage-average before computing the respective distance matrices ($\mathbf{M}_{\bar{\mathbf{O}}}, \mathbf{M}_{\bar{\mathbf{B}}}$ or $\mathbf{M}_{\bar{\mathbf{U}}}$) (**Table 5.3**) are associated with large posterior standard errors. For these analyses, poor mixing was also observed (**Additional file 5.4**). Given our dataset size, the covariance structure generated with this expansion procedure seems not suitable to properly identify the covariance between animals due to sharing cecal microbial composition. The posterior means of h^2 and m^2 for these traits seem to be more consistent when they were obtained with the M2

models based on the expansion of the microbial relationship matrices that just included ones in the diagonal and zeros outside the diagonal for the animals without microbial information (**Table 5.2**). In this case, a similar pattern was obtained with $\mathbf{M_{O,0}}$, $\mathbf{M_{B,0}}$ and $\mathbf{M_{U,0}}$: h^2 decrease from 0.26 (M1) to 0.19 for $\overline{\mathrm{ADFI}}_{\mathrm{AL}}$, from 0.49 (M1) to 0.32 for $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$, and from 0.34 (M1) to 0.21 for $\overline{\mathrm{ADFCR}}_{\mathrm{AL}}$ while m^2 ranged from 0.45 to 0.49 for $\overline{\mathrm{ADFI}}_{\mathrm{AL}}$, from 0.38 to 0.42 for $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$, and from 0.45 to 0.49 for $\overline{\mathrm{ADFCR}}_{\mathrm{AL}}$.

Table 5.1| Means (SD) of marginal posterior distributions of the heritability (h^2), microbiability (m^2) and phenotypic variance (Phe. Var.) for ADG_{AL} and ADG_R obtained with the mDataset.

Parameter	Model	Microbial matrix	ADG_{AL}	ADG_R
h^2	M1	--	0.21(0.14)	0.29(0.19)
Phe. Var.	M1	--	41.20(4.37)	32.80(3.93)
h^2	M2	$\mathbf{M_O}$	0.07(0.07)	0.13(0.09)
m^2	M2	$\mathbf{M_O}$	0.67(0.15)	0.56(0.12)
Phe. Var.	M2	$\mathbf{M_O}$	93.08(26.03)	57.90(12.51)
h^2	M2	$\mathbf{M_B}$	0.05(0.05)	0.07(0.06)
m^2	M2	$\mathbf{M_B}$	0.79(0.12)	0.77(0.10)
Phe. Var.	M2	$\mathbf{M_B}$	193.85(83.54)	129.08(46.78)
h^2	M2	$\mathbf{M_U}$	0.08(0.09)	0.14(0.13)
m^2	M2	$\mathbf{M_U}$	0.60(0.26)	0.49(0.26)
Phe. Var.	M2	$\mathbf{M_U}$	174.85(168.52)	91.03(72.38)

ADG_{AL}: average daily gain in rabbits fed *ad libitum*; ADG_R: average daily gain in rabbits fed under restriction; SD: standard deviation; M1: model without microbial effects; M2: model fitting the microbial effects; $\mathbf{M_O}$: microbial relationship covariance matrix defined from CSS normalized OTU counts, $\mathbf{M_B}$: microbial relationship covariance matrix defined from Bray-Curtis distance matrix; $\mathbf{M_U}$: microbial relationship covariance matrix defined from weighted Unifrac distance matrix.

Table 5.2| Means (SD) of marginal posterior distributions of the heritability (h^2), microbiability (m^2) and phenotypic variance (Phe. Var.) for individual traits (ADG_{AL} and ADG_R) and cage-average traits ($\overline{\mathbf{ADFI}}_{\mathbf{AL}}$, $\overline{\mathbf{ADRFI}}_{\mathbf{AL}}$ and $\overline{\mathbf{ADFCRI}}_{\mathbf{AL}}$) obtained with the fullDataset by expanding the corresponding microbial relationship matrix with ones in the diagonal and zeros outside.

Parameter	Model	Microbial matrix[1]	ADG_{AL}	ADG_R	$\overline{\mathbf{ADFI}}_{\mathbf{AL}}$	$\overline{\mathbf{ADRFI}}_{\mathbf{AL}}$	$\overline{\mathbf{ADFCR}}_{\mathbf{AL}}$
h^2	M1	--	0.15(0.09)	0.09(0.07)	0.26(0.18)	0.49(0.20)	0.34(0.20)
Phe. Var.	M1	--	79.79(4.67)	57.02(3.40)	635.14(102.99)	206.59(33.06)	0.20(0.03)
h^2	M2	$\mathbf{M_{O,0}}$	0.11(0.06)	0.08(0.05)	0.19(0.13)	0.33(0.15)	0.22(0.14)
m^2	M2	$\mathbf{M_{O,0}}$	0.63(0.06)	0.66(0.05)	0.48(0.18)	0.38(0.17)	0.47(0.18)
Phe. Var.	M2	$\mathbf{M_{O,0}}$	90.54(5.47)	66.50(4.13)	676.55(118.29)	219.47(37.77)	0.21(0.04)
h^2	M2	$\mathbf{M_{B,0}}$	0.12(0.07)	0.07(0.06)	0.19(0.13)	0.31(0.15)	0.22(0.14)
m^2	M2	$\mathbf{M_{B,0}}$	0.56(0.06)	0.61(0.05)	0.49(0.18)	0.42(0.17)	0.49(0.17)
Phe. Var.	M2	$\mathbf{M_{B,0}}$	92.04(5.67)	68.13(4.38)	711.55(128.31)	227.88(40.04)	0.22(0.04)
h^2	M2	$\mathbf{M_{U,0}}$	0.13(0.07)	0.07(0.06)	0.19(0.13)	0.32(0.15)	0.22(0.15)
m^2	M2	$\mathbf{M_{U,0}}$	0.52(0.06)	0.58(0.05)	0.45(0.19)	0.40(0.17)	0.45(0.18)
Phe. Var.	M2	$\mathbf{M_{U,0}}$	92.11(5.78)	68.26(4.43)	711.42(128.01)	226.68(39.58)	0.22(0.04)

ADG_{AL}: average daily gain in rabbits fed *ad libitum*; ADG_R: average daily gain in rabbits fed under restriction; $\overline{\mathrm{ADFI}}_{\mathrm{AL}}$: average daily feed intake in rabbits fed *ad libitum*; $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$: average daily residual feed intake in rabbits fed *ad libitum*; $\overline{\mathrm{ADFCR}}_{\mathrm{AL}}$: average daily feed conversion ratio in rabbits fed *ad libitum*; SD: standard deviation; M1: model without microbial effects; M2: model fitting the microbial effects.

[1]The expansion of the microbial relationship matrix ($\mathbf{M_O}, \mathbf{M_B}$ or $\mathbf{M_U}$) was done by including ones in the diagonal and zeros outside the diagonal for the animals without microbial information.

Table 5.3| Means (SD) of marginal posterior distributions of the heritability (h^2), microbiability (m^2) and phenotypic variance (Phe. Var.) for individual traits (ADG_{AL} and ADG_R) and cage-average traits ($\overline{\mathrm{ADFI}}_{\mathrm{AL}}$, $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$ and $\overline{\mathrm{ADFCRI}}_{\mathrm{AL}}$) obtained with the fullDataset by expanding the OTU matrix with the cage-average counts.

Parameter	Model	Microbial matrix[1]	ADG_{AL}	ADG_R	$\overline{\mathbf{ADFI}}_{\mathbf{AL}}$	$\overline{\mathbf{ADRFI}}_{\mathbf{AL}}$	$\overline{\mathbf{ADFCR}}_{\mathbf{AL}}$
h^2	M1	--	0.15(0.09)	0.09(0.07)	0.26(0.18)	0.49(0.20)	0.34(0.20)
Phe. Var.	M1	--	79.79(4.67)	57.02(3.40)	635.14(102.99)	206.59(33.06)	0.20(0.03)
h^2	M2	$\mathbf{M}_{\bar{\mathbf{O}}}$	0.14(0.09)	0.09(0.07)	0.24(0.17)	0.44(0.19)	0.30(0.18)
m^2	M2	$\mathbf{M}_{\bar{\mathbf{O}}}$	0.08(0.05)	0.00(0.00)	0.03(0.06)	0.10(0.12)	0.16(0.09)
Phe. Var.	M2	$\mathbf{M}_{\bar{\mathbf{O}}}$	85.71(6.42)	57.08(3.40)	635.52(102.28)	209.30(34.46)	0.21(0.03)
h^2	M2	$\mathbf{M}_{\bar{\mathbf{B}}}$	0.09(0.06)	0.09(0.07)	0.16(0.12)	0.23(0.13)	0.20(0.14)
m^2	M2	$\mathbf{M}_{\bar{\mathbf{B}}}$	0.39(0.13)	0.06(0.03)	0.44(0.19)	0.56(0.17)	0.44(0.16)
Phe. Var.	M2	$\mathbf{M}_{\bar{\mathbf{B}}}$	133.31(32.36)	61.00(6.57)	1059.88(359.15)	407.68(135.59)	0.32(0.09)
h^2	M2	$\mathbf{M}_{\bar{\mathbf{U}}}$	0.15(0.09)	0.07(0.06)	0.11(0.10)	0.12(0.12)	0.08(0.08)
m^2	M2	$\mathbf{M}_{\bar{\mathbf{U}}}$	0.00(0.00)	0.25(0.23)	0.58(0.24)	0.76(0.20)	0.78(0.17)
Phe. Var.	M2	$\mathbf{M}_{\bar{\mathbf{U}}}$	79.83(4.67)	88.33(43.15)	2106.33(1622.31)	1284.29(948.14)	1.20(0.80)

ADG_{AL}: average daily gain in rabbits fed *ad libitum*; ADG_R: average daily gain in rabbits fed under restriction; $\overline{\mathrm{ADFI}}_{\mathrm{AL}}$: average daily feed intake in rabbits fed *ad libitum*; $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$: average daily residual feed intake in rabbits fed *ad libitum*; $\overline{\mathrm{ADFCR}}_{\mathrm{AL}}$: average daily feed conversion ratio in rabbits fed *ad libitum*; SD: standard deviation; M1: model without microbial effects; M2: model fitting the microbial effects.

[1]The expansion of the microbial relationship matrix ($\mathbf{M}_{\mathbf{O}}, \mathbf{M}_{\mathbf{B}}$ or $\mathbf{M}_{\mathbf{U}}$) was done before computing the respective distance matrices, assigning to the animals without microbial information the cage-average of the CSS normalized OTU counts.

5.3.2. Predictive ability of individual growth and cage FE from microbial information

Table 5.4 shows the correlation coefficient between observed and predicted records of individual traits (ADG_{AL} and ADG_R) in the validation set reached with the different tested models and the mDataset. It was observed that the consideration of microbial information resulted in a significant prediction improvement of the individually measured growth traits only when $\mathbf{M_O}$ or $\mathbf{M_B}$ were used as covariance matrix between individual microbial effects. The consideration of microbial information in M2 models improved the predictive capacity of ADG_{AL} and ADG_R by 25% and 46%, respectively.

Table 5.4| Across 100 replicates average (SD) correlation coefficient between observed and predicted ADG_{AL} and ADG_R records with sPLSR and mixed models using the mDataset.

Model	Microbial matrix	ADG_{AL}	ADG_R
M1	--	0.30(0.15)	0.39(013)
M2	$\mathbf{M_O}$	0.36(0.13)*[a]	0.56(0.11)*[a]
M2	$\mathbf{M_B}$	0.38(0.13)*[a]	0.57(0.12)*[a]
M2	$\mathbf{M_U}$	0.30(0.14)	0.39(0.13)
sPLSR1	--	0.50(0.11)	0.28(0.14)
sPLSR2	--	0.51(0.11)	0.19(0.16)

ADG_{AL}: average daily gain in rabbits fed *ad libitum*; ADG_R: average daily gain in rabbits fed under restriction; SD: standard deviation; M1: mixed model without microbial effects; M2: mixed model fitting the microbial effects; $\mathbf{M_O}$: microbial relationship covariance matrix defined from CSS normalized OTU counts, $\mathbf{M_B}$: microbial relationship covariance matrix defined from Bray-Curtis distance matrix; $\mathbf{M_U}$: microbial relationship covariance matrix defined from weighted Unifrac distance matrix; sPLS1: sparse Partial Least Squares Regression model with systematic effects as predictors; sPLS2: sparse Partial Least Squares Regression model with systematic effects and CSS OTU counts as predictors.
*M2 or sPLSR2 correlation between observed and predicted records significantly higher (bootstrapped paired t test) than M1 or sPLSR1 correlation after Bonferroni correction for multiple testing at the $P < 0.05$ level.
[a]M2 or sPLSR2 correlation between observed and predicted records higher than M1 or sPLSR1 correlation in at least 80% of the replicates.

When $\mathbf{M_U}$ was used as covariance matrix between individual microbial effects no improvement of the predictive capacity was observed for any trait. The same was observed when microbial information was included in sPLSR2 models fitting systematic effects and CSS OTU counts. sPLSR2 models did not exhibit better predictive ability than those models just fitting the systematic effects (sPLSR1).

Table 5.5 shows the correlation coefficient between observed and predicted records of individual growth traits (ADG_{AL} and ADG_{R}) in the validation set when different mixed models and microbial covariance matrices were used. In this case, the analyses were conducted using the fullDataset. Here the correlation coefficient between observed and predicted records of each trait in the validation set was computed separately for the animals with microbial information and for the animals without this information. The only consistent improvement in the predictive ability was observed on animals in which cecal microbiota was assessed for ADG_{R} using M2 models based on the expansion of the microbial relationship matrices including ones in the diagonal and zeros outside the diagonal. The predictive capacity of ADG_{R} with these M2 models increased by 17% with respect to M1.

Finally, **Table 5.6** shows the correlation coefficient between observed and predicted records of cage-average traits ($\overline{\mathrm{ADFI}}_{\mathrm{AL}}$, $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$ and $\overline{\mathrm{ADFCR}}_{\mathrm{AL}}$) in the validation set reached with the different mixed and sPLSR models under study using the fullDataset.

Table 5.5| Across 100 replicates average (SD) correlation coefficient between observed and mixed model predicted ADG_{AL} and ADG_R records using the fullDataset by expanding the microbial relationship covariance matrix in different ways.

		Animals with microbial information		Animals without microbial information	
Model	**Microbial matrix**	**ADG_{AL}**	**ADG_R**	**ADG_{AL}**	**ADG_R**
M1	--	0.46(0.15)	0.48(0.15)	0.39(0.11)	0.42(0.14)
M2	$\mathbf{M_{O,0}}^1$	0.47(0.14)	0.56(0.14)*[a]	0.37(0.10)	0.42(0.14)
M2	$\mathbf{M_{B,0}}^1$	0.46(0.15)	0.57(0.15)*[a]	0.37(0.10)	0.43(0.14)
M2	$\mathbf{M_{U,0}}^1$	0.45(0.15)	0.55(0.14)*[a]	0.37(0.10)	0.43(0.14)
M2	$\mathbf{M_{\bar{O}}}^2$	0.47(0.14)*	0.48(0.15)	0.39(0.10)	0.42(0.14)
M2	$\mathbf{M_{\bar{B}}}^2$	0.47(0.15)*	0.48(0.15)	0.39(0.10)*	0.42(0.14)
M2	$\mathbf{M_{\bar{U}}}^2$	0.45(0.15)	0.48(0.15)	0.39(0.10)	0.42(0.14)

ADG_{AL}: average daily gain in rabbits fed *ad libitum*; ADG_R: average daily gain in rabbits fed under restriction; SD: standard deviation; M1: mixed model without microbial effects; M2: mixed model fitting the microbial effects; $\mathbf{M_O}$: microbial relationship covariance matrix defined from CSS normalized OTU counts, $\mathbf{M_B}$: microbial relationship covariance matrix defined from Bray-Curtis distance matrix; $\mathbf{M_U}$: microbial relationship covariance matrix defined from weighted Unifrac distance matrix.

[1]The expansion of the microbial relationship matrix ($\mathbf{M_O}$, $\mathbf{M_B}$ or $\mathbf{M_U}$) was done by including ones in the diagonal and zeros outside the diagonal for the animals without microbial information.

[2]The expansion of the microbial relationship matrix ($\mathbf{M_O}$, $\mathbf{M_B}$ or $\mathbf{M_U}$) was done before computing the respective distance matrices, assigning to the animals without microbial information the cage-average of the CSS normalized OTU counts.

*M2 correlation between observed and predicted records significantly higher (bootstrapped paired t test) than M1 correlation after Bonferroni correction for multiple testing at the $P < 0.05$ level.

[a]M2 correlation between observed and predicted records higher than M1 correlation in at least 80% of the replicates.

Table 5.6| Across 100 replicates average (SD) correlation coefficient between observed and predicted individual cage-average $\overline{\mathbf{ADFI}}_{\mathbf{AL}}$, $\overline{\mathbf{ADRFI}}_{\mathbf{AL}}$ and $\overline{\mathbf{ADFCRI}}_{\mathbf{AL}}$ records with sPLSR and mixed models using the fullDataset.

Model	Microbial matrix	$\overline{\mathbf{ADFI}}_{\mathbf{AL}}$	$\overline{\mathbf{ADRFI}}_{\mathbf{AL}}$	$\overline{\mathbf{ADFCR}}_{\mathbf{AL}}$
M1	--	0.79(0.11)	0.42(0.21)	0.61(0.16)
M2	$\mathbf{M}_{\mathbf{O},\mathbf{0}}$[1]	0.83(0.08)*[a]	0.50(0.19)*[a]	0.69(0.12)*[a]
M2	$\mathbf{M}_{\mathbf{B},\mathbf{0}}$[1]	0.83(0.08)*[a]	0.50(0.19)*[a]	0.69(0.12)*[a]
M2	$\mathbf{M}_{\mathbf{U},\mathbf{0}}$[1]	0.82(0.08)*[a]	0.50(0.18)*[a]	0.69(0.12)*[a]
M2	$\mathbf{M}_{\bar{\mathbf{O}}}$[2]	0.79(0.11)	0.41(0.21)	0.61(0.16)
M2	$\mathbf{M}_{\bar{\mathbf{B}}}$[2]	0.79(0.11)	0.41(0.21)	0.61(0.16)
M2	$\mathbf{M}_{\bar{\mathbf{U}}}$[2]	0.79(0.11)	0.42(0.21)	0.61(0.15)
sPLSR1	--	0.79(0.08)	-0.31(0.14)	0.65(0.15)
sPLSR2	--	0.73(0.09)	0.17(0.21)*[a]	0.39(0.18)

$\overline{\mathrm{ADFI}}_{\mathrm{AL}}$: average daily feed intake in rabbits fed *ad libitum*; $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$: average daily residual feed intake in rabbits fed *ad libitum*; $\overline{\mathrm{ADFCR}}_{\mathrm{AL}}$: average daily feed conversion ratio in rabbits fed *ad libitum*; SD: standard deviation; M1: mixed model without microbial effects; M2: mixed model fitting the microbial effects; $\mathbf{M}_{\mathbf{O}}$: microbial relationship covariance matrix defined from CSS normalized OTU counts, $\mathbf{M}_{\mathbf{B}}$: microbial relationship covariance matrix defined from Bray-Curtis distance matrix; $\mathbf{M}_{\mathbf{U}}$: microbial relationship covariance matrix defined from weighted Unifrac distance matrix; sPLS1: sparse Partial Least Squares Regression model with systematic effects as predictors; sPLS2: sparse Partial Least Squares Regression model with systematic effects and cage-average CSS OTU counts as predictors.

[1]The expansion of the microbial relationship matrix ($\mathbf{M}_{\mathbf{O}}$, $\mathbf{M}_{\mathbf{B}}$ or $\mathbf{M}_{\mathbf{U}}$) was done by including ones in the diagonal and zeros outside the diagonal for the animals without microbial information.

[2]The expansion of the microbial relationship matrix ($\mathbf{M}_{\mathbf{O}}$, $\mathbf{M}_{\mathbf{B}}$ or $\mathbf{M}_{\mathbf{U}}$) was done before computing the respective distance matrices, assigning to the animals without microbial information the cage-average of the CSS normalized OTU counts.

*M2 or sPLSR2 correlation between observed and predicted records significantly higher (bootstrapped paired t test) than M1 or sPLSR1 correlation after Bonferroni correction for multiple testing at the $P < 0.05$ level.

[a]M2 or sPLSR2 correlation between observed and predicted records higher than M1 or sPLSR1 correlation in at least 80% of the replicates.

The M2 mixed models in which the elements of the covariance matrices for animals without microbial information were generated from cage-average CSS OTU counts did not add any predictive value for any trait. On the contrary, the consideration of microbial information resulted in a significant improvement of the predictive ability of all traits with all M2 mixed models based on microbial relationship matrices expanded with ones in the diagonal and zeros outside the diagonal for the animals without microbial information. When these models are used, the predictive ability increased by 5%, 20% and 14% for $\overline{\mathrm{ADFI}}_{\mathrm{AL}}$, $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$ and $\overline{\mathrm{ADFCR}}_{\mathrm{AL}}$, respectively, over M1. These improvements were nearly the same irrespectively the covariance matrix considered: $\mathbf{M}_{\mathbf{O},\mathbf{0}}$, $\mathbf{M}_{\mathbf{B},\mathbf{0}}$ or $\mathbf{M}_{\mathbf{U},\mathbf{0}}$.

Regarding the sPLSR multivariate approach, the correlation coefficient between observed and predicted records reached in the validation set with the model that

only included the systematic effects as predictors (sPLSR1) was pretty high and in most cases better than that achieved with the sPLSR2 models (i.e., also including the cage-average CSS OTU counts as predictors). The only exception was observed for $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$ what could be said to be expected since a correction by batch effect is implicit in its definition. Thus, the systematic effects considered do not play any role in the prediction of the observations, indeed, an average negative correlation associated with large dispersion was observed. This average correlation turned positive (although of low magnitude: 0.17) when CSS OTU counts were considered, resulting in a significant improvement of the predictive capacity of the model for this cage-average phenotype.

5.3.3. Identification of relevant OTUs for the prediction of rabbit growth and FE

The observed improvement in the predictive ability of the sPLSR2 model for $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$ could be explained by the systematic selection of 7 OTUs in more than 80 out of the 100 replicates conducted. **Table 5.7** shows the taxonomic assignment with the RDP classifier of the selected OTUs, and their representative sequences can be found in **Additional file 5.5**. Out of these OTUs, 5 belong to family *Lachnospiraceae* and 2 are unclassified bacteria. The Pearson's correlations between these OTUs and $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$ were computed to quantify the degree of association. These correlations ranged from -0.33 to 0.31 (**Table 5.7**).

Table 5.7| Taxonomic assignment of the OTUs selected in the sPLSR analysis for $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$.

OTU ID and taxonomical assignment	Pearson's correlation
874627 Unclassified *Bacteria*	0.31*
NR1922 Unclassified *Lachnospiraceae*	-0.27*
NR2153 Unclassified *Lachnospiraceae*	0.31*
NR3628 Unclassified *Lachnospiraceae*	-0.33*
NR381 Unclassified *Lachnospiraceae*	-0.31*
NR4083 Unclassified *Lachnospiraceae*	0.32*
NR768 Unclassified *Bacteria*	-0.27*

$\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$: average daily residual feed intake in rabbits fed *ad libitum*.
**P* for Pearson's correlation t-test between the relevant OTU and $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$ lower than 0.05.

On the other hand, sPLSR models were used to fit the posterior means of the individual microbial effects predicted for growth and FE traits with M2 models and microbial covariance matrices $\mathbf{M_{O,0}}, \mathbf{M_{B,0}}$ or $\mathbf{M_{U,0}}$ to identify the most relevant OTUs for the prediction of such phenotypes. **Table 5.8** shows, for each trait and covariance matrix, the number of OTUs selected from a total of 946 in at least 80 out of the 100 replicates conducted.

Table 5.8| Number of OTUs selected in at least 80 out of the 100 sPLSR replicates conducted for microbial effects predicted with covariance matrices $\mathbf{M_{O,0}}, \mathbf{M_{B,0}}$ **and** $\mathbf{M_{U,0}}$ for growth and FE traits.

Trait	$\mathbf{M_{O,0}}$	$\mathbf{M_{B,0}}$	$\mathbf{M_{U,0}}$	Most relevant[1]
ADG_{AL}	911	931	673	16
ADG_{R}	887	874	621	13
$\overline{\mathrm{ADFI}}_{\mathrm{AL}}$	850	785	490	25
$\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$	600	793	480	16
$\overline{\mathrm{ADFCR}}_{\mathrm{AL}}$	824	832	877	13

ADG_{AL}: average daily gain in rabbits fed *ad libitum*; ADG_{R}: average daily gain in rabbits fed under restriction; $\overline{\mathrm{ADFI}}_{\mathrm{AL}}$: average daily feed intake in rabbits fed *ad libitum*; $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$: average daily residual feed intake in rabbits fed *ad libitum*; $\overline{\mathrm{ADFCR}}_{\mathrm{AL}}$: average daily feed conversion ratio in rabbits fed *ad libitum*; $\mathbf{M_{O,0}}$: microbial relationship covariance matrix defined from CSS normalized OTU counts and expanded by including ones in the diagonal and zeros outside the diagonal for the animals without microbial information, $\mathbf{M_{B,0}}$: microbial relationship covariance matrix defined from Bray-Curtis distance matrix and expanded by including ones in the diagonal and zeros outside the diagonal for the animals without microbial information; $\mathbf{M_{U,0}}$: microbial relationship covariance matrix defined from weighted Unifrac distance matrix and expanded by including ones in the diagonal and zeros outside the diagonal for the animals without microbial information.
[1]The most relevant OTUs were those with the greatest loading weights and that were selected with $\mathbf{M_{O,0}}, \mathbf{M_{B,0}}$ and $\mathbf{M_{U,0}}$.

Additionally, **Table 5.S1** shows the taxonomy of the most relevant OTUs (i.e., those having the greatest loading weights and selected with the three M2 models) for the prediction of growth and FE traits based on the individual microbial effects predicted with the linear mixed models. The Pearson's correlations between each OTU and the traits are also shown in **Table 5.S1** while their representative sequences can be found in **Additional file 5.7**. Sixteen OTUs seemed to have an important weight for the prediction improvement of ADG_{AL}. Ten of them belong to phylum *Firmicutes*, 2 to phylum *Euryarchaeota*, and 4 OTUs are unclassified *Bacteria*. Thirteen OTUs were found to be relevant to improve the predictive ability of mixed models for ADG_{R}. Of these OTUs, 10 belong to phylum *Firmicutes*, 2 to phylum *Euryarchaeota* and 1

to phylum *Bacteroidetes*. Twenty-five OTUs were found to be involved in the improvement of the predictive ability of mixed models for $\overline{\mathrm{ADFI}}_{\mathrm{AL}}$. Most of them (20 OTUs) belong to phylum *Firmicutes*, 1 to phylum *Bacteroidetes*, 1 to phylum *Actinobacteria*, 1 to phylum *Proteobacteria*, and 2 OTUs are unclassified *Bacteria*. Sixteen OTUs were found to be relevant to improve the predictive ability of mixed models for $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$. Out of these OTUs, 8 belong to phylum *Firmicutes*, 3 to phylum *Bacteroidetes*, 1 to phylum *Proteobacteria*, and 4 OTUs are unclassified *Bacteria*. Finally, 13 OTUs were responsible for the prediction improvement of $\overline{\mathrm{ADFCR}}_{\mathrm{AL}}$ when microbial information was fitted in the proposed mixed models. Most of them (8 OTUs) belong to phylum *Firmicutes*, 2 to phylum *Bacteroidetes*, and 3 OTUs are unclassified *Bacteria*. It is worth mentioning that some OTUs were found to be relevant for the prediction of more than one trait. In this regard, two OTUs belonging to genus *Methanobrevibacter* and one to order *Clostridiales* were found to be relevant for the prediction of both growth traits, i.e., $\mathrm{ADG_R}$ and $\mathrm{ADG_{AL}}$. One OTU taxonomically assigned to family *Lachnospiraceae* was found to be relevant for the prediction of both $\mathrm{ADG_{AL}}$ and $\overline{\mathrm{ADFI}}_{\mathrm{AL}}$. Seven OTUs (2 belonging to genus *Eisenbergiella*, 1 to class *Alphaproteobacteria*, 1 to genus *Longibaculum*, 1 to family *Erysipelotrichaceae*, 1 to family *Lachnospiraceae*, and 1 unclassified *Bacteria*) were found to be relevant for the prediction of both $\overline{\mathrm{ADFI}}_{\mathrm{AL}}$ and $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$. Three OTUs (1 belonging to genus *Ruminococcus*, 1 to genus *Blautia*, and 1 to family *Lachnospiraceae*) were found to be relevant for the prediction of both $\mathrm{ADG_R}$ and $\overline{\mathrm{ADFI}}_{\mathrm{AL}}$. Two OTUs (1 belonging to genus *Butyricimonas*, and 1 unclassified *Bacteria*) were found to be relevant for the prediction of both $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$ and $\overline{\mathrm{ADFCR}}_{\mathrm{AL}}$. One OTU belonging to genus *Butyricicoccus* was found to be relevant for the prediction of $\mathrm{ADG_R}$, $\mathrm{ADG_{AL}}$ and $\overline{\mathrm{ADFI}}_{\mathrm{AL}}$. Finally, one OTU belonging to family *Lachnospiraceae* was found to be relevant for the prediction of $\mathrm{ADG_R}$, $\overline{\mathrm{ADFI}}_{\mathrm{AL}}$ and $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$ (**Table 5.S1**). In **Figure 5.1**, a Venn diagram shows the degree of overlap between traits regarding the most relevant OTUs for their prediction. In general, this degree of overlap was small, but it responds to the nature of traits. For example, $\overline{\mathrm{ADFCR}}_{\mathrm{AL}}$ has only relevant OTUs in common with $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$, being both feed efficiency traits. On the other hand, $\overline{\mathrm{ADFI}}_{\mathrm{AL}}$ has the largest amount of OTUs in

common with other traits: $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$ and both growth traits (i.e., ADG_R and ADG_{AL}) that are strongly influenced by the animal's intake.

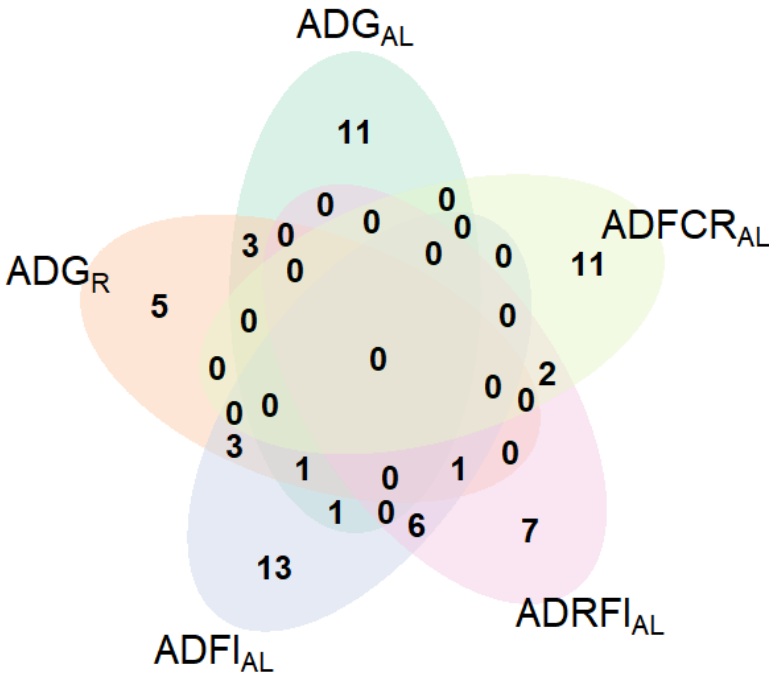

Figure 5.1| Venn diagram showing the numbers and overlap of most relevant OTUs for the prediction of the 5 traits analyzed. ADG_{AL}: average daily gain in rabbits fed *ad libitum*; ADG_R: average daily gain in rabbits fed under restriction; $ADFI_{AL}$: average daily feed intake in rabbits fed *ad libitum*; $ADRFI_{AL}$: average daily residual feed intake in rabbits fed *ad libitum*; $ADFCR_{AL}$: average daily feed conversion ratio in rabbits fed *ad libitum*.

5.4. Discussion

The role of microbial communities inhabiting the rabbit cecum on key breeding traits related to FE remains unknown. To shed light on this matter, we have reported heritabilities and microbiabilities of ADG under different feeding regimes commonly used in meat rabbit commercial farms. We have also computed such ratios for cage-average traits related to FI and FE in animals fed AL. Dealing with such cage-average performances, while having only measured cecal microbial information in a few animals per cage, is a statistical modeling challenge. We have faced it using different approaches, with the final objective of evaluating the predictive value of microbial information for both individual growth and cage-average FE phenotypes.

The study of ADG has particular significance for rabbit breeding programs since this trait is commonly selected to indirectly improve FE. Apart from that, the commercial application of feed restriction (i.e., a reduction in the amount of the feed provided to

the animal) is common since it improves FE and reduces mortality and morbidity caused by enteric disorders (Gidenne *et al*., 2009). Piles and Sánchez (2019) estimated a low genetic correlation between ADG_{AL} and ADG_R, and the genome-wide association study conducted by Sánchez *et al*. (2020) identified different QTL regions for both traits. Such findings support the existence of different genetic backgrounds for these traits. Thus, in this study, we reported the posterior means of the heritability (h^2) for ADG_{AL} and ADG_R separately. In line with previous results (Piles and Sánchez, 2019), we have found a lower h^2 for ADG_R, which implies difficulties to achieve a response to selection for growth or indirectly for FE.

In this context, one can understand the relevance of exploring whether microbiota explains a significant percentage of the phenotypic variance of these traits as well as the value of microbial information to predict such complex traits as tools to define the degree of influence of microbial information on the traits of interest. A clear effect of microbial composition on the traits of interest would open the door to search and select for taxa positively associated with them. Ross *et al*. (2013), motivated by the existence of numerous exploratory studies in humans and other animals aiming at relating the microbiome to a complex trait, tested a method to predict body mass index in humans and methane production phenotypes in cattle. Their results showed that microbial information could be useful to predict complex host phenotypes, and even suggested that it could exceed prediction accuracies based on the host genome for traits largely influenced by the gut microbiota. Following that study, others have been conducted in an attempt to evaluate the utility of microbial information to predict complex phenotypes in different livestock species. However, to date, there is a lack of knowledge about the value of microbial information to predict phenotypes related to growth in rabbits. This is the first study to assess the value of cecal microbiota to predict individual growth traits in meat rabbits using different modeling approaches. What is more, this is the first time that the predictive value of microbial information is evaluated when this information has not been measured in all the individuals contributing to the phenotype. The first challenge we faced was to properly define a between-animals relationship matrix due to microbial effects (**M**). Thus, we replicated each analysis with three alternative definitions of **M**: one defined from CSS normalized OTU counts ($\mathbf{M_0}$) and two defined from two

classical measures of distance; Bray-Curtis ($\mathbf{M_B}$) and weighted Unifrac ($\mathbf{M_U}$). A second challenge was to define an appropriate way to expand **M** for those animals in which cecal microbiota was not assessed. These developments are strongly linked with several prediction tools based on kernel methods already proposed (Ramon *et al*, 2021). In our study, we have derived kernel matrices by implementing an ad-hoc solution to transform distance matrices into proper covariance matrices, while Ramon *et al*. (2021) directly derived the kernel matrices associated with distance metrics from raw information. Not having microbial information for all the animals under study would request, anyhow, some heuristics to generate valid covariance matrices to be included in the mixed models.

Despite the difficulties mentioned above and the fact that, in general, a low predictive ability for growth traits was observed (the correlation coefficient between observed and predicted records in the validation set with M1 was not higher than 0.4), we have been able to detect a certain predictive ability improvement by considering microbial information. Such consideration improved the predictive capacity of mixed models for ADG_{AL} and ADG_{R} by 25% and 46%, respectively, in the dataset comprised of only the rabbits in which cecal microbiota was assessed (mDataset). When the role of the microbial information was assessed by inspecting the percentage of phenotypic variance explained by the bacterial effect, a large proportion was attributed to the bacterial effect, being this large proportion of the phenotypic variance accompanied by a sharp reduction of the h^2 which is probably related to a certain degree of association between cecal microbiota and host genotype. This was even observed for the case in which the definition of the **M** covariance matrix was based on the weighted Unifrac distance matrix. However, for this particular case, we did not see any improvement when considering microbial information for predicting ADG_{AL} or ADG_{R}. This result highlights the need to accompany any assessment of the proportion of the phenotypic variance attributed to the microbial effect (i.e., microbiability) by validation of its actual predictive value.

The predictive value of models not including the microbial effect for growth traits was slightly higher (up to 0.46-0.48) with the fullDataset (i.e., that comprised of records from rabbits in which cecal microbiota was assessed and from their cage

mates without such microbial information) than with the mDataset. In this case, however, the predictive value added by microbial information was more limited, being only significant for ADG_R of animals in which microbiota was assessed, and exclusively when the expansion of **M** for those animals without microbial information was based on the identity matrix. Despite this limited predictive value of the microbial information when the fullDataset was studied, and similar to that observed in some cases when the mDataset was considered, a very large percentage of the phenotypic variation of ADG_{AL} was estimated to be explained by cecal microbiota when the covariance matrix **M** was expanded using the identity matrix. The large estimates of m^2 for this trait can be said to be artifacts given that they are not accompanied by an improvement in the predictive capacity of the model, and they seem to be associated with an increase of the phenotypic variance estimates regarding M1. Such increase could be associated with an increment of the residual variance in the model, probably linked with the existence of a certain degree of collinearity between the covariance matrices of the different factors in the model. In this regard, the results obtained using covariance matrixes **M** expanded with cage-average CSS OTU counts could be said to be more coherent, since the null microbiability estimates are associated with a null improvement of the prediction of both growth traits (ADG_{AL} or ADG_R).

Fang *et al*. (2020b) found that only 10% of the phenotypic variance of finishing weight in commercial meat rabbits was explained by the gut microbiome. Besides that, previous studies in Japanese quails (Vollmar *et al*., 2020) and pigs (Camarinha-Silva *et al*., 2017) estimated m^2 for body weight gain of 0.18 and 0.28, respectively. These large differences between our current results for growth traits and the previous ones could be simply due to the study of different definitions of these traits in different species or to the use of different approaches and definitions of **M** to estimate m^2. We report a predictive value of cecal microbiota for ADG_{AL}, in line with that reported for daily gain in pigs by Camarinha-Silva et al. (2017) applying microbial best linear unbiased prediction (M-BLUP) and by Maltecca *et al*. (2019) using Bayesian models, machine learning approaches and semi-parametric kernel model. In our study, another important point to note is that the predictive value of cecal microbiota was higher for ADG_R than for ADG_{AL}. This result suggests that

ADG_R is more strongly influenced by gut microbial composition than ADG_{AL}, which is more affected by host genetics as Piles and Sánchez (2019) previously evidenced.

Regarding the study of cage-average phenotypes, the current difficulties in individually recording FI of rabbits bred in group suppose the major limitation to conduct a direct selection for FE. Therefore, definitions of FE in this study rely on group records of FI and individual records of growth. In addition to this constraint, in the current study, we have also faced the challenge that supposes not having microbial information for all the individuals of a cage. Our modeling approaches allow including the phenotypic information of cage mates on which cecal microbiota was not assessed. Thus, we present the first study to predict cage-average FI and FE traits in a rabbit sire line with a mixed model approach using microbial information although it was only measured in approximately 30% of the individuals within cage. To deal with this limitation, we tested two different expansions of three microbial covariance matrices for the animals in which microbiota was not assessed to be able to consider the contributions of all individuals to the cage performance traits.

Our modeling approaches exhibited moderate predictive abilities for the cage-average phenotypes, higher than those obtained for the individually measured growth traits. This result was not surprising since the prediction of individual measures is more challenging than averages. Moreover, the inclusion of microbial information increased the predictive ability of mixed models by 5%, for $\overline{\mathrm{ADFI}}_{\mathrm{AL}}$, 20% for $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$ and 14% $\overline{\mathrm{ADFCR}}_{\mathrm{AL}}$ over the model not considering a microbial effect. It is worth mentioning that this improvement was only achieved when the expansion of the microbial relationship matrix for those animals without microbial information was based on the identity matrix (i.e., for those animals without microbial information the diagonal elements of the covariance matrix were set to one and elements outside the diagonal were fixed to zero). These improvements in the prediction were accompanied by large microbiability estimates, which in turn were associated with a reduction of heritability estimates. Clear evidence of ill-conditioned models was observed for those cases in which the expansion of the covariance matrices was based on cage-average CSS OTU counts given that large microbiabilities were

estimated but they were not associated with improvements in the prediction, but with increased phenotypic variance estimates. The consideration of cage-average CSS counts to expand the covariance matrix could have increased the collinearity between the individual microbial and the cage effects, deteriorated the parameters identification, and altered convergence properties (**Additional file 5.4**).

Previous studies have evaluated the value of gut microbiota to predict complex traits related to FE in other livestock species. In cattle, Delgado *et al*. (2019) found a set of microbial contigs obtained from a *de novo* metagenome assembly that allowed high classification power for samples with extreme values of FE and FI traits. They found that these microbial contigs had a certain predictive ability for such traits in an independent cattle population. In pigs, Camarinha-Silva *et al*. (2017) achieved higher prediction accuracies for FI and feed conversion with M-BLUP method than with the same method but employing the genomic relationship matrix (G-BLUP). They quantified that 21% of the phenotypic variance of feed conversion in pigs is explained by the gut microbiome. In Japanese quails (Vollmar *et al*., 2020) and pigs (Camarinha-Silva *et al*., 2017), 9% and 16% of the phenotypic variance of FI, respectively, seem to be explained by the gut microbiome. In line with these studies estimating microbiabilities of traits related to FI and FE, we have also reported that a large percentage of the phenotypic variance of these phenotypes can be explained by the cecal microbiota. Such percentage was, in most cases, larger than that explained by the additive genetic effects. Nonetheless, as we have previously indicated, large microbiability estimates are not always associated with improvements in the predictive capacity of the models. Thus, such estimates should be interpreted with caution.

What seems clear from our results is that in those cases in which an improvement in the predictive ability of the model was evidenced, the estimated high microbiability was accompanied by a reduction in the heritability estimates with respect to those obtained in models not fitting the microbial effect. We interpret this as indirect evidence of certain host genetic control over the gut microbial composition. Several studies have already reported the existence of moderate heritability for certain microbial taxa and diversity indexes in humans (Goodrich *et al*., 2014; Goodrich *et*

al., 2016), pigs (Lu *et al*., 2018; Cheng *et al*., 2018; Crespo-Piazuelo *et al*., 2018; Reverter *et al*., 2021) or cattle (Sasson *et al*., 2017). A preliminary study in the same meat rabbit population used in the current study has also directly shown that cecal microbiota is under genetic control (Velasco-Galilea *et al*., 2018b). These results are relevant from a biological perspective to better understand the symbiotic relationship between host and gut microbial communities, but also from an applied perspective. In the case we confirm that relevant OTUs (i.e., associated with performance traits of interest) have a clear host genetic control, selective breeding could be considered as an additional tool to promote the presence of such favorable microbial taxa in the gut of a given livestock population.

The predictive ability of multivariate sPLSR models for the traits under study did not improve by considering microbial information, except for $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$. This result was discouraging since with this approach we had hoped to identify the group of OTUs responsible of an improvement in the predictive ability. The unique case in which we identified a group of OTUs that appears to confer a predictive value was for $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$. We detected some unclassified OTUs belonging to family *Lachnospiraceae* moderately correlated with this trait, some of them positively and others negatively. This is not surprising given this is a big family encompassing numerous different genera. Siegerstetter *et al*. (2017) found different *Lachnospiraceae* genera enriched in both low or high residual feed intake chickens and suggested that these bacteria could promote the host FE by stimulating fatty acid, amino acid, and vitamin synthesis. In short, with sPLSR we have not been able to detect the improvement in the predictive ability observed with mixed models, suggesting the existence of an added value of microbial information that cannot be captured by all predictive machineries when the amount of data and microbial information are limited.

Our implemented mixed model approach integrates all the available pedigree information in the analysis. Such information is particularly relevant for the analysis of cage-average traits since it allows to share information between cages according to the additive genetic relationships. This way, predictions of individual phenotypes

include variability between cage mates. However, the same cage-average measurement was assigned to all cage mates in the sPLSR model approach.

We have thus tried an alternative application of sPLSR models by fitting the posterior means of individual microbial effects estimated with M2 mixed models for each trait to identify the most relevant OTUs contributing to the improvement of the model predictive ability. This approximation has allowed us to identify for each trait a number of OTUs that are systematically chosen by the sPLSR models fitted with the three different matrices based on the identity matrix (i.e., those that we have found associated with gains in the predictive ability of the model) having the greatest loading weights.

We have detected four unclassified OTUs belonging to family *Lachnospiraceae* moderately correlated with growth traits: one positively and other negatively with ADG_R, and two positively with ADG_{AL}. This is not surprising given this is a big family encompassing numerous different genera. Fang *et al*. (2020b) identified a positive association between members of this family and ADG of commercial meat rabbits. Another study in the same population of rabbits reported a positive association between members of family *Lachnospiraceae* and finishing BW (Fang *et al*., 2020a). Interestingly, we have found two different OTUs belonging to genus *Methanobrevibacter* positively associated with ADG_{AL} and negatively with ADG_R. Kušar and Avguštin (2010) suggested that methanogenic microorganisms inhabiting the rabbit cecum are predominantly *Methanobrevibacter* species. This result was supported by the study conducted by Velasco-Galilea *et al*. (2018a) in which all archaeal species identified in the rabbit cecum and feces belonged to such methanogenic genus that encompasses different hydrogenotrophic methane-producing species. Conversely, McGovern *et al*. (2017) and McCabe *et al*. (2015) reported a negative correlation between the abundance of this genus and body mass index, as well as an overrepresentation of this genus in cattle under fed restriction.

We have identified a positive association between an unclassified member of family *Ruminococcaceae* and ADG_R. This result is in agreement with the above-mentioned

studies in meat rabbits that also identified a positive association of this family with ADG and finishing BW (Fang *et al*., 2020a; Fang *et al*., 2020b). Interestingly, we have found a negative association between genus *Bacteroides* and ADG_R and $\overline{ADFI}_{AL}$, as well as between genus *Butyricicoccus* and ADG_R. Genus *Bacteroides* has been associated with obesity in humans (de la Cuesta-Zuluaga *et al*., 2018). However, it is worth mentioning that this genus encompasses pathogenic species, such as *Bacteroides fragilis* (Yekani *et al*., 2020), that could lead to a diversion of nutrients from growth towards immune response. Previous studies have hypothetized that an overgrowth of *Bacteroides* species in the rabbit gut could lead to a decrease of butyrate yield and, consequently, to the incidence of epizootic rabbit enteropathy (Jin *et al*., 2018). Several studies have demonstrated that the application of feed restriction after weaning reduces the risk of enteric disorders in rabbits (Gidenne *et al*., 2009; Romero *et al*., 2010; Gidenne *et al*., 2012). In this regard, a lighter presence of genus *Bacteroides* in restricted animals could be associated with the benefits conferred by this feeding strategy. Previous studies, indeed, have found a negative correlation between this genus and pig BW (Mach *et al*., 2015; Yang *et al*., 2016).

It is also noteworthy that we have identified three different OTUs taxonomically assigned to genus *Neglecta* that are negatively associated with $\overline{ADFI}_{AL}$. This genus encompasses pathogenic bacterial species, and it has been associated positively with pig ADG in a previous study conducted by Tran *et al*. (2018). On the other hand, we have identified two and five unclassified OTUs belonging to family *Lachnospiraceae* positively correlated with $\overline{ADRFI}_{AL}$ and $\overline{ADFI}_{AL}$, respectively. In cattle, in accordance with our results, Li and Guan (2017) and Shabat *et al*. (2016) found an overrepresentation of family *Lachnospiraceae* in less efficient animals (greater RFI). High relative abundances of members belonging to this family could suggest a more active cecum fermentation, which leads to increased butyrate short-chain fatty acid that is a nutrient for the gut of the animal. Besides that, we have found one OTU taxonomically assigned to genus *Olsenella* that seems to be relevant for the prediction of $\overline{ADRFI}_{AL}$, and that is positively associated with this trait. Members of this genus ferment starch and glycogen substrates to produce lactic, acetic, and formic acid (Göker *et al*., 2010). In line with our results, Ellison *et al*.

(2017) and Kubasova *et al*. (2018) reported higher abundances of *Olsenella* in the rumen of low feed efficient lambs and piglets, respectively.

On another note, we have found several OTUs relevant for the prediction of traits related to FE analyzed in this study, i.e., $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$ and $\overline{\mathrm{ADFCR}}_{\mathrm{AL}}$. Two OTUs taxonomically assigned to genus *Paramuribaculum* were found negatively correlated with $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$. Members of this genus are involved in the metabolism of carbohydrates, lipids, vitamins, and amino acids as well as in glycan biosynthesis (Lagkouvardos *et al*., 2019). On the other hand, we have identified OTUs belonging to class *Acidaminococcaceae* and genus *Negativibacillus* positively correlated with $\overline{\mathrm{ADFCR}}_{\mathrm{AL}}$. Zhang *et al*. (2021) suggested a role of genus *Negativibacillus* in sheep feed efficiency throughout the fermentation of complex carbohydrates. Conversely, Elolimy *et al*. (2020) identified an enrichment of class *Acidaminococcaceae* and genus *Negativibacillus* in the most efficient Holstein heifer calves.

Finally, we want to highlight that, in line with previous studies, we have observed that bacterial members assigned to the same taxonomic group can either be positively or negatively associated with a given phenotype. The observed heterogeneity in this study includes members of family *Lachnospiraceae* and genera *Rumminoccocus*, *Butyricicoccus*, and *Bacteroides*. This suggests that these OTUs belong to functionally and/or physiologically different species encompassed within the same taxa. Our experimental design faithfully represents rearing conditions of most commercial farms in which kits are bred in collective cages, however, it does not grant the optimal statistical power to unravel the foundations behind these biological processes. For future studies with this purpose, an experimental design based on individual measures could be, although costly, more appropriate.

5.5. Conclusions

Significant improvements in the prediction of individual growth and cage-average traits related to FE were observed when cecal microbial information was fitted into the models. However, these improvements are not general and depend to a large extent on the prediction method used as well as on the prior information considered

to define the covariance matrix between animals due to their cecal microbial effect. We have introduced a novel modeling approach based on the traditional mixed animal model that, relying on the pedigree information, enables the estimation of variance components and the evaluation of the predictive value of microbial information for cage-average performances even when microbiota was not assessed in all individuals of the cage. Caution must be taken, however, to interpret the magnitude of the proportion of the phenotypic variance explained by the individual gut microbial effect since large microbiabilities estimates are not necessarily associated with gains in the predictive ability of the model. In general, a certain drop in heritability estimates was observed when both additive genetic and individual microbial effects were fitted at the time. This suggests that part of the effect associated with the prediction improvement by considering cecal microbial information partially has a genetic origin. We are in the process of assessing this host genetic determinism. Cecal microbiota seems to have a polibacterial role in growth and FE traits since, although we have identified certain OTUs with a relevant weight, a large proportion of OTUs are responsible for the prediction improvement achieved with mixed models.

5.6. Methods

5.6.1. Animals

All animals involved in the study were raised at the rabbit facilities of the Institute of Agrifood Research and Technology (IRTA) in two different periods. The animals come from the Caldes line (Gómez *et al*., 2002) that has been selected for post-weaning growth since 1983, and it is commonly used as a terminal sire line within the three-ways crossbreeding schema for rabbit meat production in Spain. The animals used in this study were randomly selected from 5 batches of a larger experiment conducted to estimate the effect of the interaction between the genotype and the feeding regime on growth, feed efficiency, carcass characteristics, and health status of the animals (Piles and Sánchez, 2019).

Most of the animals were produced in 4 batches in a semi-open-air facility during the first semester of 2014, and the remaining were produced in a single batch in

another facility under better controlled environmental conditions in spring 2016. The animals bred in the first facility were housed in collective cages, containing 8 kits each one, from weaning (32 days of age) until the end of the fattening period (66 days of age). On the other hand, the kits raised in the second facility were housed in cages of 6 kits each one and their growing period was slightly shorter (32 - 60 days of age).

Beyond these differences, all animals received the same management and were fed with a standard pelleted diet. Water was provided *ad libitum* and feed was supplied once per day in a feeder with three places for the 4-5 weeks that the fattening lasted. At weaning, the animals were randomly assigned to one of the two different feeding regimes under assessment: (1) *ad libitum* (AL) or (2) restricted (R) to 75% of the AL FI. The amount of feed supplied to the animals under R in each week for each batch was computed as 0.75 times the average FI of kits on AL from the same batch during the previous week, plus 10% to account for a FI increase as the animals grow. Kits under both feeding regimes were categorized into two groups according to their BW at weaning (big if their BW was greater than 700 g or small otherwise) to generate homogeneous groups regarding animal size within feeding regime. A maximum of two kits from the same litter were assigned to a single cage to avoid confounding between cage and maternal effects.

The individual BW was weekly recorded for all animals in both feeding regimes, and the cage FI was also weekly recorded in AL cages. From BW raw records, individual ADG was computed as the slope of the within animal regression of all BW measurements on their respective ages in days. This trait was individually computed for each feeding regime, thus obtaining ADG on AL (ADG_{AL}) or under R (ADG_{R}). For the AL cages, three additional traits were computed. The individual average daily feed intake ($\overline{\mathrm{ADFI}}_{\mathrm{AL}}$) was computed as the total FI of the cage during the whole growing period divided by the number of days and the number of kits that each cage contained. The individual average daily residual feed intake ($\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$) was obtained as the residual of a batch-nested multiple regression of $\overline{\mathrm{ADFI}}_{\mathrm{AL}}$ on the $\overline{\mathrm{ADG}}_{\mathrm{AL}}$ and the cage-average mid-growing-period day metabolic weight ($\overline{\mathrm{MW}}_{\mathrm{AL}}$). Finally, the

individual average daily feed conversion ratio ($\overline{\mathrm{ADFCR}}_{\mathrm{AL}}$) was computed as the ratio between $\overline{\mathrm{ADFI}}_{\mathrm{AL}}$ and the $\mathrm{ADG}_{\mathrm{AL}}$ cage-average ($\overline{\mathrm{ADG}}_{\mathrm{AL}}$).

Two different datasets were considered for the analyses performed in this study. The mDataset was represented by the 425 kits from which cecal samples were collected at the end of their growing period for microbiota assessment, and the fullDataset included these 425 kits and their cage mates. On average, cecal microbiota was assessed in 2 kits by cage. The number of animals and cages within feeding regime and batch are shown in **Table 5.9**, and the descriptive statistics of the traits under study are presented in **Table 5.10**.

Table 5.9| Number of individual and cages within feeding regime and batch. Animals with microbiota assessed and non-assessed are distinguished for the individual records.

	Individuals				Cages	
	With microbiota		W/o microbiota			
Batch	**R**	**AL**	**R**	**AL**	**R**	**AL**
1	45	44	51	52	16	16
2	30	27	66	61	12	11
3	41	35	103	84	18	15
4	53	61	195	211	31	34
5	32	57	96	126	16	23

R: Animals under restriction; AL: animals fed *ad libitum*.

Table 5.10| Descriptive statistics of growth and FE traits.

Trait	Dataset	N	Mean	SD	IQR
$\mathrm{ADG}_{\mathrm{AL}}$ (g/day)[1]	mDataset	224	55.12	6.52	7.30
$\mathrm{ADG}_{\mathrm{AL}}$ (g/day)[1]	fullDataset	758	53.21	9.42	8.49
$\mathrm{ADG}_{\mathrm{R}}$ (g/day)[1]	mDataset	201	36.35	5.85	7.56
$\mathrm{ADG}_{\mathrm{R}}$ (g/day)[1]	fullDataset	712	35.35	7.99	8.27
$\mathrm{ADFI}_{\mathrm{AL}}$ (g/day)[2]	fullDataset	99	151.37	17.01	20.93
$\mathrm{ADRFI}_{\mathrm{AL}}$ (g/day)[2]	fullDataset	99	0.00	5.92	6.66
$\mathrm{ADFCR}_{\mathrm{AL}}$ (g/day)[2]	fullDataset	99	2.84	0.24	0.33

$\mathrm{ADG}_{\mathrm{AL}}$: average daily gain in rabbits fed *ad libitum*; $\mathrm{ADG}_{\mathrm{R}}$: average daily gain in rabbits fed under restriction; $\overline{\mathrm{ADFI}}_{\mathrm{AL}}$: average daily feed intake in rabbits fed *ad libitum*; $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$: average daily residual feed intake in rabbits fed *ad libitum*; $\overline{\mathrm{ADFCR}}_{\mathrm{AL}}$: average daily feed conversion ratio in rabbits fed *ad libitum*; SD: standard deviation; IQR: interquartile range; mDataset: dataset including only records of animals in which microbiota was assessed; fullDataset: dataset including records of animals in which microbiota was assessed as well as of their cage mates.
[1]Refers to individual traits.
[2]Refers to cage traits.

5.6.2. Sample processing, DNA extraction and sequencing

Animals were slaughtered at morning after fasting (at 66 and 60 days of age in first and second facility, respectively) and cecal samples of 425 rabbits were collected in a sterile tube, kept cold in the laboratory (4ºC), and stored at -80ºC. DNA extraction, amplification, Illumina library preparation and sequencing followed methods described in previous studies (Velasco-Galilea *et al*., 2018a; Velasco-Galilea *et al*., 2020). Whole genomic DNA was extracted from 250 mg of each biological sample according to manufacturer's instructions of kit ZR Soil Microbe DNA MiniPrep Kit (Zymo Research, Freiburg, Germany). Cecal samples were mechanically lysed in a FastPrep-24 homogenizer (MP Biomedicals, LLC, Santa Ana, CA, United States) at a speed of 6 m/s for 60 s, thus facilitating an efficient lysis of archaeal and bacterial species. Integrity and purity of DNA extracts were measured with NanoDrop ND-1000 spectrophotometer equipment (NanoDrop products; Wilmington, DE, United States) following Desjardins and Conklin's protocol (Desjardins and Conklin, 2010). All DNA extracts showed adequate integrity and purity (absorbance ratio 260 nm/280 nm > 1.6) to avoid PCR inhibition issues. A fragment of the 16S rRNA gene that included the V4-V5 hypervariable regions was amplified with the F515Y/R926 pair of primers (5'-GTGYCAGCMGCCGCGGTAA-3', 5'-CCGYCAATTYMTTTRAGTTT-3') (Parada *et al*., 2016). The initial polymerase chain reaction (PCR) was conducted for each sample using 12.5 µl 2x KAPA HiFi HotStart Ready Mix, 5 µl forward primer, 5 µl reverse primer and 2.5 µl template DNA (5 ng/ µl). The PCR conditions were the following: initial denaturation for 3 minutes at 95 ºC, 25 cycles of 30 seconds at 95 ºC, 30 seconds at 55 ºC and 30 seconds at 72 ºC; and final extension for 2 minutes at 72 ºC. The fragment was then re-amplified in a limited-cycle PCR reaction to add sequencing adaptors and 8 nucleotide dual-indexed barcodes of the multiplex Nextera XT kit (Illumina, Inc., San Diego CA, United States) according to manufacturer's instructions. The adaptors and barcodes were added to both ends of the fragment in a second PCR by using 25 µl 2x KAPA HiFi HotStart Ready Mix, 5 µl index i7, 5 µl index i5, 10 µl PCR Grade water and 5 µl concentrated amplicons of the initial PCR. The second PCR conditions were the following: initial denaturation for 3 minutes at 95 ºC, 8 cycles of 30 seconds at 95 ºC, 30 seconds at 55 ºC and 30 seconds at 72 ºC; and final extension for 5 minutes at 72 ºC. Final libraries were

cleaned up with AMPure XP beads, validated by running 1 µl of a 1:50 dilution on a Bioanalyzer DNA 1000 chip (Agilent Technologies, Inc., Santa Clara, CA, United States) to verify their size, quantified by fluorometry with PicoGreen dsDNA quantification kit (Invitrogen, Life Technologies, Carlsbad, CA, United States), pooled at equimolar concentrations and paired-end sequenced in 5 parallel plates in an Illumina MiSeq 2 x 250 platform at the Genomics and Bioinformatics Service (SGB) of the Autonomous University of Barcelona (UAB).

5.6.3. Bioinformatic pipeline for OTU calling

Sequence processing was performed using QIIME software version 1.9.0 (https://github.com/biocore/qiime/releases/tag/1.9.0) (Caporaso *et al*., 2010) as described in Velasco-Galilea *et al*. (2020). The first step consists of assembling the paired-ended V4-V5 16S rRNA gene reads into contigs with the python script *multiple_join_paired_ends.py*. The resulting contigs were filtered (those with a quality score smaller than Q19 were discarded) and assigned to samples using the python script *split_libraries.py* with default parameters. Chimeric sequences generated in the PCR were detected with UCHIME algorithm (Edgar *et al*., 2011) and removed. The filtered contigs were clustered into operational taxonomic units (OTUs) with a 97% similarity threshold using the script *pick_open_reference_otus.py* with default parameters (Rideout *et al*., 2014). This script uses the UCLUST algorithm (Edgar, 2010), to first align the sequences against Greengenes reference database (version gg_13_5_otus) (McDonald *et al*., 2012), and then to make a *de novo* clustering of those contigs that did not match the database. After doubletons removal, the filtered OTU table contained the sequence counts of 963 OTUs for 425 samples. Finally, the OTU table was normalized with the cumulative sum scaling (CSS) method (Paulson *et al*., 2013). **Figure 5.2** provides a graphical summary of the present experimental design and the phenotypes analyzed together with microbiota assessment of cecal samples and the bioinformatic pipeline used for OTU calling. Taxonomic assignment of representative sequences of each OTU was conducted with the QIIME default parameters of the UCLUST consensus taxonomy assigner by mapping the sequences against the Greengenes reference database gg_13_5_otus. The raw

sequence data were deposited in the sequence read archive of NCBI under the BioProject accession number PRJNA524130. Metadata, OTU table, and corresponding taxonomic assignments are also included as **Additional files 5.1**, **5.2** and **5.3**, respectively. In summary, after executing the bioinformatic processing, 14,928,203 filtered sequences clustered into 963 different OTUs were obtained for 425 cecal rabbit samples. Most of these OTUs were assigned to phyla *Firmicutes* (76.74%), *Tenericutes* (7.22%) and *Bacteroidetes* (6.26%). Details on the taxonomic assignment can be found at Velasco-Galilea et al. (2020).

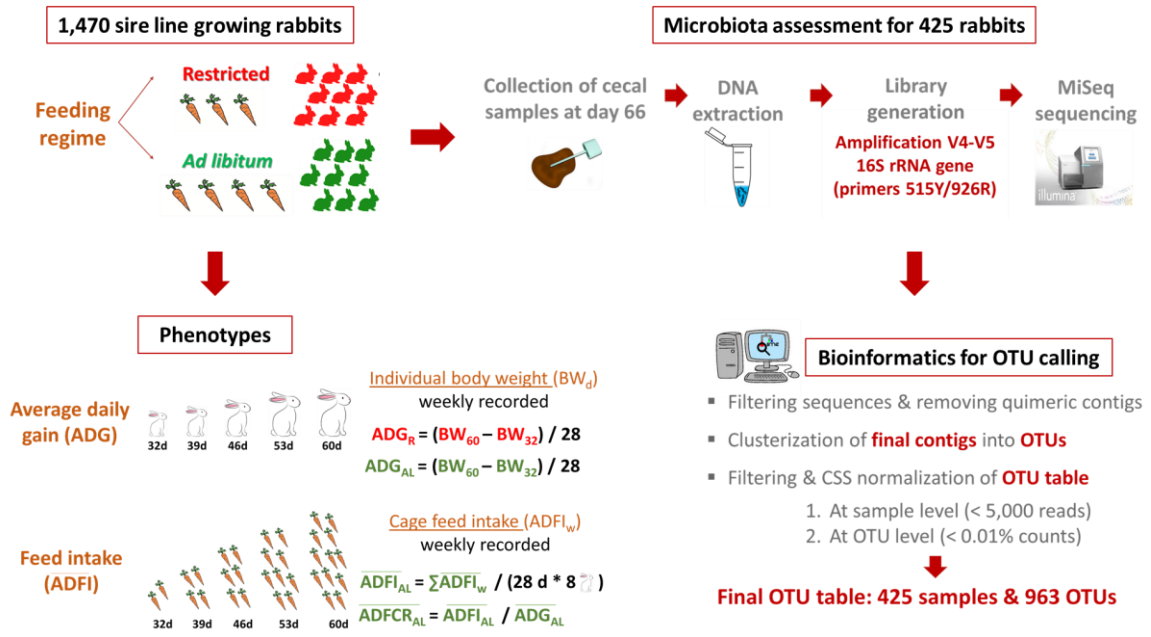

Figure 5.2| Graphical summary of the experimental design, phenotypes analyzed, microbiota assessment of cecal samples and bioinformatic pipeline for OTU calling.

5.6.4. Statistical analyses: mixed models

5.6.4.1. Parameter estimation

The following univariate microbial mixed linear model was fitted to estimate the marginal posterior distributions of additive, litter, cage, and microbial effects of the individual growth traits ADG_{AL} and ADG_R with the mDataset:

$$\mathbf{y = X\beta + Z_A a + Z_L l + Z_C c + Z_M m + e,}$$

where **y** is a vector containing the phenotypes (ADG_{AL} or ADG_{R}); **β** is a vector of the systematic effects of batch (5 levels) and of BW at weaning (2 levels: big and small) with its corresponding incidence matrix **X**; **a** is a vector including the additive genetic effects with the corresponding incidence matrix $\mathbf{Z_A}$; **l** is a vector with litter birth effects with the corresponding incidence matrix $\mathbf{Z_L}$; **c** is a vector including cage effects with the corresponding incidence matrix $\mathbf{Z_C}$; **m** is a vector having the animal microbial effects with the corresponding incidence matrix $\mathbf{Z_M}$; finally **e** is a vector of residuals. The mDataset used in these analyses included phenotypic information of 425 rabbits born from 318 litters and housed in 192 cages, while the pedigree included relationships of 2,547 individuals.

The fullDataset was used to estimate the marginal posterior distributions of additive, litter, and microbial effects of $\overline{\mathrm{ADFCR}}_{\mathrm{AL}}$, $\overline{\mathrm{ADFI}}_{\mathrm{AL}}$ and $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$ from records on the 99 AL cages available. The following univariate microbial mixed linear was fitted:

$$\mathbf{y = X\beta + Z_A a + Z_L l + Z_M m + e,}$$

where **y** is a vector containing cage trait phenotypes ($\overline{\mathrm{ADFCR}}_{\mathrm{AL}}$, $\overline{\mathrm{ADFI}}_{\mathrm{AL}}$ or $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$); **β** is a vector including the systematic effects of batch (5 levels) and of BW at weaning (2 levels: big and small) with its corresponding incidence matrix **X**. As described above, vectors **a**, **l**, **m** and **e** correspond to additive genetic, litter birth, animal microbial and residual effects, respectively. However, the corresponding incidence matrices $\mathbf{Z_A}$, $\mathbf{Z_L}$ and $\mathbf{Z_M}$ are not composed by zeros and ones but by real numbers representing the proportions of the different levels of the factor contributing to the cage-average.

In both models, the same sets of prior distributions were considered for the different factors. The systematic effects (**β**) were *a priori* assumed to follow uniform distributions. The assumed prior distribution for the additive genetic effects was $\mathbf{a} \sim NMV(\mathbf{0}, \mathbf{A}\sigma^2_{\mathrm{A}})$, with **A** being the numerator relationship matrix (Henderson, 1973) and σ^2_{A} being the additive genetic variance. The prior distribution assumed for the litter effects was $\mathbf{l} \sim NMV(\mathbf{0}, \mathbf{I}\sigma^2_{\mathrm{L}})$, with **I** being an identity matrix of appropriate dimension, and σ^2_{L} being the litter birth variance. The prior distribution for the cage

effects was **c** ~ *NMV* (**0**, **I**σ_C^2), with **I** also being an identity matrix of appropriate dimension, and σ_C^2 being the cage variance. In different analyses, alternative prior distributions were assumed for the vector of animal-specific microbial effects, being its general form **m** ~ *NMV* (**0**, **M**σ_M^2), with **M** being a between-animals relationship matrix due to microbial effects, and σ_M^2 being the animal microbial variance. Three alternative definitions of **M** were considered in three separate analyses: i) $\mathbf{M_O} = \mathbf{OO'}$, with **O** being the row-normalized CSS OTU count matrix, [n (animals) x m (OTUs)]; the **O** matrix was row-wise normalized by dividing the row vector elements by the row norms ensuring that $\mathbf{M_O}$ had ones in its diagonal (this definition is fairly similar to that previously proposed by Difford *et al*. (2018); ii) $\mathbf{M_B} = 1 - \frac{\mathbf{B^2}}{2}$; with **B** being the Bray-Curtis distance matrix (Bray and Curtis, 1957) computed from the CSS OTU count matrix; and iii) $\mathbf{M_U} = 1 - \frac{\mathbf{U^2}}{2}$; with **U** being the weighted Unifrac distance matrix (Lozupone and Knight, 2005) computed from the CSS OTU count matrix. Both distance matrices (**B** and **U**) were computed using the “phyloseq” R package (McMurdie and Holmes, 2013).

To deal with the fact that microbial information was only available for some of the rabbits within a cage, it was necessary to generate the rows and columns of the between-animal covariance matrices due to the cecal microbial content for the animals not having microbial information assessed. This approach allows to consider the contributions of all individuals to the cage-average performance traits. Two different expansion strategies were adopted: i) assigning to the animals without microbial information the within cage-average of each CSS OTU count, and then computing $\mathbf{M_{\bar{O}}}$, $\mathbf{M_{\bar{B}}}$ and $\mathbf{M_{\bar{U}}}$ between the 1,470 animals under study (425 having microbial information plus their cage mates without microbial information); ii) first computing $\mathbf{M_O}$, $\mathbf{M_B}$ and $\mathbf{M_U}$ from the 425 animals with microbial information and then expanding with ones in the diagonal and zeros out of the diagonal the rows and columns corresponding to animals not having microbial information, thus obtaining $\mathbf{M_{O,0}}$, $\mathbf{M_{B,0}}$ and $\mathbf{M_{U,0}}$. The resulting covariance matrices were forced to be positive definite by conducting an eigen-value decomposition, saving all the positive eigen-values and their associated eigen-vectors, and finally reconstructing the covariance matrices from these elements. Note that the original (obtained between the 425

animals having microbial composition) Bray-Curtis or unweighted Unifrac distance matrices could be undefined matrices, i.e., mixing positive and negative eigen values, since distance matrices are pairwise constructed. Thus, certain incongruities could exist when the distances are studied beyond pairs of individuals, which translate into non-positive definition of the whole distance matrix. These incongruities must be corrected if the distance matrix is going to be used as a covariance matrix.

The MCMC Bayesian estimation procedure was conducted using gibbsf90test program (Misztal *et al*., 2015). Chains of 2,000,000 samples were run discarding the first 500,000 to allow the algorithm to reach convergence to the marginal posterior distributions. Finally, one in every 10 samples was saved. Trace plots and histograms of Markov chains from the posterior distribution of the parameters of Bayesian models fitted for the individual growth traits and for the cage FE traits are included as **Additional file 5.4**.

The fractions of the phenotypic variance of ADG_{AL} and ADG_{R} explained by σ^2_A (heritability), σ^2_L (litter variance ratio), σ^2_C (cage variance ratio), and σ^2_M (microbiability; Difford *et al*., 2018) were calculated as:

$$h^2 = \frac{\sigma^2_A}{\sigma^2_P};\ l^2 = \frac{\sigma^2_L}{\sigma^2_P};\ c^2 = \frac{\sigma^2_C}{\sigma^2_P};\ m^2 = \frac{\sigma^2_M}{\sigma^2_P},$$

where $\sigma^2_P = \sigma^2_A + \sigma^2_L + \sigma^2_C + \sigma^2_M + \sigma^2_e$ is the phenotypic variance.

Similarly, for the cage traits ($\overline{ADFCR}_{AL}$, $\overline{ADFI}_{AL}$ and $\overline{ADRFI}_{AL}$), the fractions of the phenotypic variance explained by σ^2_A (heritability), σ^2_L (litter variance ratio), and σ^2_M (microbiability) were calculated as:

$$h^2 = \frac{\sigma^2_A}{\sigma^2_P};\ l^2 = \frac{\sigma^2_L}{\sigma^2_P};\ \ m^2 = \frac{\sigma^2_M}{\sigma^2_P},$$

where $\sigma^2_P = \sigma^2_A + \sigma^2_L + \sigma^2_M + 7\sigma^2_e$ is the phenotypic variance. Given that σ^2_e represents the cage residual mean, it is necessary to multiply it by 7 (the average number of animals within cage in this study), thus obtaining an individual residual variance estimate referred to individual records. Note that l^2 and c^2 were defined but related results are not presented in this study.

5.6.4.2. Predictive ability assessment

For each trait, two cross-validations assessments were conducted to evaluate whether including microbial information in the model improves its predictive ability. The first one was based on the above-described mixed model whose predictive performance was compared with that of the same model but without considering the microbial effect. Cross-validations were replicated 100 times. In each of them, the dataset for the individually measured traits (ADG_{AL} and ADG_{R}) was randomly split into training and validation sets with probabilities 0.9 and 0.1, respectively. This partition was done in a manner that ensured all litters and cages of the animals in the validation set were also represented in the training set. For the cage traits ($\overline{\mathrm{ADFCR}}_{\mathrm{AL}}$, $\overline{\mathrm{ADFI}}_{\mathrm{AL}}$ and $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$), the dataset was randomly split in a way that cages within a given batch were assigned to the training or the testing set with probabilities 0.8 and 0.2, respectively. The predictive ability of each model was defined as the average, across 100 replicates, correlation coefficient between predicted and observed phenotypes in the validation set. In this cross-validation assessment, the training step of the model was conducted using the expectation-maximization residual maximum likelihood (EM-REML) algorithm as implemented in the program remlf90 (Misztal *et al*., 2015). Paired t test (R Development Core Team, 2010) was applied to compare the across replicates mean correlations obtained with the model considering microbial effect to that from the model that ignored this information. The tests were assumed paired because the same dataset was used in each replicate of both analyses (i.e., with and without bacterial effect). Empirical bootstrap p-values for the paired t test were computed after generating 1,000 bootstrap samples under the null hypothesis of the correlation coefficients from both models across the 100 replicates. The bootstrap p-value was defined as the proportion of bootstrap rounds having an estimated difference equal to or greater than that obtained with the original dataset. A p-value lower than 0.05, after Bonferroni correction (Bonferroni, 1936), was considered to support the rejection of the hypothesis of both models having the same predictive ability. In those cases where the null hypothesis was rejected, the percentage of times across the 100 replicates that the correlation coefficient obtained with the model considering microbial information was higher than that obtained with the model that ignored such information was computed.

5.6.5. Statistical analyses: multivariate models

5.6.5.1. Predictive ability assessment

Another predictive performance assessment was conducted using a multivariate approach. Individual (ADG_{AL} and ADG_{R}) and cage traits ($\overline{\mathrm{ADFCR}}_{AL}$, $\overline{\mathrm{ADFI}}_{AL}$ and $\overline{\mathrm{ADRFI}}_{AL}$) were fitted with sparse Partial Least Squares Regression (sPLSR) models. The predictors of the first sPLSR model where the columns of the design matrix obtained with the *model.matrix()* R function (R Development Core Team, 2010) after fitting for each trait a linear model defined by the same systematic effects as those used in the mixed model approach (i.e., batch and body size at weaning). The second sPLSR model fitted for each trait include as predictors the abovementioned systematic effects together with the 946 CSS OTU counts which were detected in at least 5% of the samples and had a sum of its counts resulting in a frequency greater than 0.01% of the total sum of all OTUs counts across all samples. CSS OTU counts on the 425 rabbits having measures of gut microbial composition were directly used for the analysis of the individual growth records. For the cage-average traits, it was needed to associate these cage-average performances to the cage-average CSS OTU counts. For each trait, the corresponding dataset was randomly divided into 5 folds, 4 of which constituted the learning dataset, and the remaining was used as the validation dataset. Before fitting the sPLSR on the learning dataset, optimal tuning parameters sparsity and number of latent components were chosen by an internal 5-fold cross-validation using *cv.spls()* function of the "spls" R package (Chung *et al*., 2019) within ranges (0.01-0.99) and (1-20) for sparsity and number of latent components, respectively. With the tuning parameters returned by the *cv.spls()* function, the combination that resulted in the minimum mean squared prediction error (MSPE) was used to finally fit the sPLSR to the learning dataset by the function *spls()*. Then, the fitted sPLSR model was used to predict the host trait performances of the validation dataset. This process was replicated 20 times with different seeds, thus obtaining 100 replicates for each trait and model tested. The predictive ability of each model was defined as the average, across 100 replicates, correlation coefficient between predicted and observed host trait phenotypes in the validation dataset. The significance of the differences in the correlation coefficient between observed and predicted records across these 100 replicates was tested

using the bootstrap paired t tests previously described for the mixed model analysis. In this case, the comparison involved the correlations between observed and predicted records obtained with a model just fitting the systematic effects and with other model fitting both systematic effects and CSS OTU counts. Additionally, when the predictive ability of the model including the microbial information was declared as better than that obtained with that of the model only including the systematic effects as predictors, the taxonomy of those OTUs selected in more than 80% of the sPLSR replicates was studied with the reference taxonomic database RDP (Wang *et al*., 2007). Finally, the Pearson's correlation was computed to quantify the degree of association between selected OTUs and the trait of interest.

5.6.5.2. Identification of relevant OTUs

Multivariate sPLSR models were also used to fit the posterior means of the individual microbial effects predicted with the univariate microbial mixed linear models that led to a significant prediction improvement of growth and FE traits. This approach was conducted in an attempt to identify the most relevant OTUs for the prediction of such phenotypes. In each case, the microbial composition records associated with the animals that conformed the mDataset were randomly divided into 5 folds (1 and 4 folds constituted the validation and the learning dataset, respectively). Before fitting the sPLSR on the learning dataset, optimal tuning parameters sparsity and number of latent components were chosen by an internal 5-fold cross-validation using *cv.spls()* function of the "spls" R package as described above. A sPLSR model was then fitted to the learning dataset by the function *spls()* with the tuning parameters returned by the *cv.spls()* function using the 946 CSS OTU counts as predictors. This process was replicated 20 times with different seeds for each trait and model tested to select those OTUs chosen in at least 80 out of the 100 replicates conducted. The OTUs considered as relevant for the prediction of a given trait were those having the greatest loading weights (i.e., below 5th and above 95th percentile values) and that were selected with all the models tested. The taxonomy of the relevant OTUs was studied with the reference taxonomic database RDP and the Pearson's correlation was computed to quantify the degree of association between each OTU and the trait of interest.

5.7. Additional information

The Additional information for this article can be found in the Annexes section.

5.8. References

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5.9. List of abbreviations

$\overline{\mathbf{ADFCR}}_{\mathbf{AL}}$	average daily feed conversion ratio on *ad libitum* feeding regime
$\overline{\mathbf{ADFI}}_{\mathbf{AL}}$	average daily feed intake on *ad libitum* feeding regime
ADG	average daily gain
ADG$_{AL}$	average daily gain on *ad libitum* feeding regime
$\overline{\mathbf{ADG}}_{\mathbf{AL}}$	cage-average daily gain on *ad libitum* feeding regime
ADG$_{R}$	average daily gain on restricted feeding regime
$\overline{\mathbf{ADRFI}}_{\mathbf{AL}}$	average daily residual feed intake on *ad libitum* feeding regime
AL	*ad libitum* feeding regime
BW	body weight
CSS	cumulative sum scaling

FE	feed efficiency
FI	feed intake
fullDataset	dataset including records of animals in which microbiota was assessed as well as of their cage mates
mDataset	dataset including only records of animals in which microbiota was assessed
M-BLUP	microbial best linear unbiased prediction
MSPE	mean squared prediction error
$\overline{\mathbf{MW}}_{\mathbf{AL}}$	cage-average mid growing period day metabolic weight ($BW^{0.75}$)
OTU	operational taxonomic unit
PCR	polymerase chain reaction
R	restricted feeding regime
EM-REML	expectation-maximization residual maximum likelihood
sPLSR	sparse partial least squares regression

Author contributions

JS and MP conceived the experimental design. JS, MP and MVG collected biological samples. MVG and MP processed the samples in the laboratory. MVG processed and analyzed the sequencing data, interpreted data, prepared figures and tables, and wrote the manuscript. JS and YRC helped analyzing the sequencing data. JS, MP and YRC helped interpreting the data, and revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We would like to thank Oscar Perucho, Josep Ramon and Carmen Requena (staff of Unitat de Cunicultura, IRTA) for their contribution to data recording and animal care during the experiment. We also want to thank Oriol Rafel, Marc Viñas, Miriam Guivernau and Olga González for their help collecting and processing the biological samples. We acknowledge Armand Sánchez, Nicolas Boulanger and Joana Ribes (Genomics and NGS Unit, CRAG) for their assistance in massive libraries preparation.

Declarations

Ethics approval and consent to participate

This study was carried out in compliance with the ARRIVE guidelines. This study was carried out in accordance with the relevant guidelines and regulations of the animal care and use committee of the Institute for Food and Agriculture Research and Technology (IRTA) which adopts “The European Code of Conduct for Research Integrity”. The protocol was approved by the committee of the Institute for Food and Agriculture Research and Technology (IRTA).

Availability of data and materials

The raw sequence data were deposited in the sequence read archive of NCBI under the accession number SRP186982 (BioProject PRJNA524130). Metadata, the filtered and CSS-normalized OTU table and corresponding taxonomic assignments have all been included as **Additional files 5.1**, **5.2** and **5.3**, respectively.

Competing interests

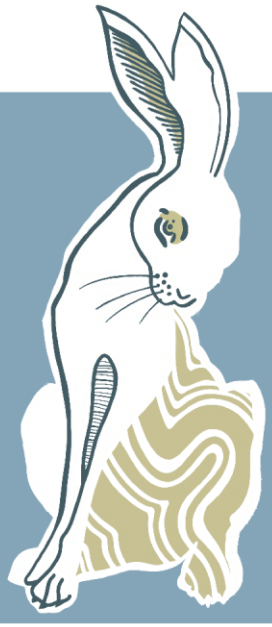
The authors declare that they have no competing interests.

Funding

The experimental design of this work was conducted thanks to funding from INIA project RTA2011-00064-00-00. This study was part of the Feed-a-Gene project that received funding from the European Union’s H2020 program under grant agreement no. 633531, and the Spanish project RTI2018-097610R-I00. MVG is a recipient of a “Formación de Personal Investigador (FPI)” pre-doctoral fellowship from INIA, associated with the research project RTA2014-00015-C2-01. YRC is recipient of a Ramon y Cajal post-doctoral fellowship (RYC2019-027244-I) from the Spanish Ministry of Science and Innovation.

CHAPTER 6

BAYES FACTOR FOR ELUCIDATING THE INFLUENCE OF HOST GENETICS, LITTER, AND CAGE EFFECTS ON RABBIT CECAL MICROBIOTA THROUGH LINEAR AND ZERO-INFLATED POISSON MIXED MODELS



Article IV

Bayes factor for elucidating the influence of host genetics, litter and cage effects on rabbit cecal microbiota through linear and zero-inflated Poisson mixed models

María Velasco-Galilea, Miriam Piles, Yuliaxis Ramayo-Caldas, Luis Varona and Juan P. Sánchez

Genetics Selection Evolution (under review)

Bayes factor for elucidating the influence of host genetics, litter and cage effects on rabbit cecal microbiota through linear and zero-inflated Poisson mixed models

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6.1. Abstract

Background

Rabbit cecum hosts and interacts with a complex microbial ecosystem that contributes to the variation of traits of economic interest. Although the influence of host genetics on microbial diversity and specific microbial taxa has been studied in humans, pigs, or cattle, it remains unknown in rabbit. This study aims to disentangle through a Bayes factor approach the relevance of genetic, litter and cage effects on a set of 989 microbial traits representative of rabbit cecal microbiota.

Results

Sequence processing of 16S rRNA-based analysis of the cecal microbiota of 425 rabbits resulted in the relative abundances of 29 genera, 951 OTUs, four microbial alpha-diversity indexes, and the first five principal components calculated from the OTU table. Each microbial trait was adjusted with mixed linear and zero-inflated Poisson (ZIP) models. All models included additive genetic, litter and cage effects, as well as body weight at weaning and batch as systematic factors. The marginal posterior distributions of model parameters were estimated using MCMC Bayesian procedures. Deviance information criterion was used for model comparison concerning the statistical distribution of the data (Normal or ZIP), while the Bayes factor was computed as a measure of the strength of evidence in favor of the genetic, litter, and cage influence on microbial traits. All microbial traits were better adjusted with the linear model except all OTUs present in less than 10% of the animals, and 25 out of 43 whose frequency of presence ranged between 10 and 25%. On a global scale, there is substantial evidence of genetic control for three principal components, number of OTUs observed and Shannon indexes. At the taxa-specific level, a significant proportion of OTUs and genera relative abundances are influenced by additive genetics, litter, and cage effects. An important influence of host genetics and the nursing environment has been found for members of genera *Bacteroides* and *Parabacteroides*, while family *S24-7* and genus *Ruminococcus* are highly influenced by cage effects.

Conclusions

This study demonstrates that host genetics shapes the overall rabbit cecal microbial diversity and that a significant proportion of taxa are either influenced by genetics and environmental factors like litter and/or cage.

6.2. Background

The bacterial communities that inhabit rabbits' gastrointestinal tract play a key role in animal metabolism, nutrition, and state of the immune system (Flint *et al*., 2012). In the particular case of this herbivorous mammalian, the richest and the most diverse microbial community lies in its cecum (Gouet and Fonty, 1979). Rabbit cecal microbial composition and diversity evolve from a simple and unstable community at birth into a complex and more homogeneous one in adult individuals (Combes *et al*., 2011). Despite this stability reached in the adulthood, previous studies have revealed the effect of external factors, such as feed composition (Zhu *et al*., 2017; Chen *et al*., 2019), level of feeding (Abecia *et al*., 2007; Velasco-Galilea *et al*., 2020) or the administration of antibiotics (Abecia *et al*., 2007; Zou *et al*., 2016; Velasco-Galilea *et al*., 2020), to shape gut microbial composition and diversity.

Beside the aforementioned influence of environmental factors on rabbit cecal microbiota, host genetics could also potentially play an important role. Several studies in humans (Goodrich *et al*., 2017; Cahana and Iraqi, 2020), cattle (Difford *et al*., 2018; Wallace *et al*., 2019; Li *et al*., 2019; Saborío-Montero *et al*., 2020; Zhang *et al*., 2020), pigs (Camarinha-Silva *et al*., 2017; Chen *et al*., 2018; Lu *et al*., 2018; Ramayo-Caldas *et al*., 2020) or mice (Campbell *et al*., 2012) have investigated the role of host genetics on gut microbiota and have reported moderate heritabilities for certain microbial taxa and diversity indexes. Thus, the interest in the interplay between host genetics and the gut microbiota with an impact on many complex traits like human diseases, feed efficiency, or methane emissions in cattle is steadily growing.

In rabbit breeding, feed efficiency and growth are key productive traits for economic profit (Cartuche *et al*., 2014). Studies that attempt to unravel the existence of a

potential link between those traits, host genetics and microbiota are of great relevance for the rabbit industry to define effective genetic selection and production strategies leading to sustainable production and animal well-being. In this respect, previous studies have reported association between gut microbiota and growth (Zeng *et al*., 2015) or feed efficiency in rabbits (Drouilhet *et al*., 2016). What is more, an important percentage of phenotypic variance of growth, feed intake and feed efficiency in growing rabbits has been attributed to cecal microbiota (Velasco-Galilea *et al*., 2021). However, there is still a need to disentangle the genetic background of rabbit cecal microbiota, which might open the doors for selective breeding for the presence of microbial taxa positively associated with relevant traits. In this regard, Velasco-Galilea *et al*. (2021) provided some indirect evidence of host genetic control over rabbit cecal microbiota since part of the predictive value of microbial information for feed efficiency and other performance traits can be partially explained by the host additive genetic effect. Nonetheless, it is necessary to explicitly assess whether it exists an overall host genetic control over microbiota or whether, on the contrary, only certain taxa or operational taxonomic units (OTUs) are influenced by genetic effects. Moreover, to design effective breeding programs based on microbial information, it would be necessary to know whether the heritable taxa are associated with relevant production traits.

Many OTUs are only present in a small percentage of the samples, which implies overdispersion due to an excessive number of zero counts that are not appropriately adjusted with linear model. Thus, a zero-inflated Poisson (ZIP) model could be suitable to estimate heritability for these traits (Xu *et al*., 2015). In a ZIP model, a given OTU is not observed (zero count) with probability p or it is observed with a number of counts coming from a Poisson distribution with parameter λ (the mean number of observations) with probability $1 - p$.

Therefore, the objective of the present study was to unravel the influence of genetic, litter and cage effects on a set of 989 microbial traits (i.e., the relative abundances of 29 genera, 951 normalized OTUs, four microbial alpha-diversity indexes, and five principal components) in a meat rabbit population raised under standard commercial conditions. These traits were analyzed using Bayesian linear and ZIP mixed models,

and the statistical relevance of ratios of the different variance components to the phenotypic variance estimates was evaluated through Bayes factor (BF).

6.3. Methods

6.3.1. Animals

Four hundred twenty-five meat rabbits from the Caldes line (Gómez *et al*., 2002) were involved in this study conducted at the Institute of Agrifood Research and Technology (IRTA). Three hundred thirty-six were produced in four batches and housed in collective cages, each containing eight kits, in a semi-open-air facility during the first semester of 2014. Additionally, eighty-nine were produced in a single batch and housed in collective cages, each containing six kits, in another facility under better controlled environmental conditions in spring 2016. Since weaning (32 days of age), all the animals received the same management and were fed with a standard pelleted diet supplemented with antibiotics except twenty-three rabbits raised in the second facility which received a diet free of antibiotics. The fattening period lasted five and four weeks for the animals raised in the first and the second facility, respectively, and during the last fattening week all the animals received an antibiotic free diet. Water was supplied *ad libitum* and feed once per day in a feeder with three places. After weaning, kits were classified into two groups according to their size ("big" if their body weight was greater than 700 g or "small" otherwise) and randomly assigned to feeding regime *ad libitum* (AL) or restricted (R) to 75% of the AL feed intake. The amount of feed supplied to the animals under R in each week for each batch was computed as 0.75 times the average feed intake of kits on AL from the same batch during the previous week, plus 10% to account for a feed intake increase as the animal grows. To prevent from a possible association between cage and maternal effects, a maximum of two kits belonging to the same litter were assigned to the same cage.

6.3.2. Sample collection, DNA extraction and sequencing

Cecal samples were collected from each animal on slaughter in a sterile tube, kept cold in the laboratory (4ºC), and stored at -80ºC. Extraction and amplification of

DNA, Illumina library preparation and sequencing were described in Velasco-Galilea *et al*., 2020. To facilitate an efficient lysis, two hundred fifty mg of each sample were mechanically lysed in a FastPrep-24TM Homogenizer (MP Biomedicals, LLC, Santa Ana, CA, United States) at a speed of 6 m/s for 60 s. Kit ZR Soil Microbe DNA MiniPrepTM (ZymoResearch, Freiburg, Germany) was used to extract the whole genomic DNA. The integrity and purity of the DNA were measured with Nanodrop ND-1000 spectrophotometer equipment (NanoDrop products; Wilmington, DE, United States) following Desjardins and Conklin's protocol (Desjardins and Conklin, 2010). The F515Y/R926 pair of primers (5'-GTGYCAGCMGCCGCGGTAA-3', 5'-CCGYCAATTYMTTTRAGTTT-3') (Parada *et al*., 2016) was used to amplify a fragment of the 16S rRNA gene that included the V4-V5 hypervariable regions. An initial polymerase chain reaction (PCR) was conducted for each sample with 12.5 µl 2x KAPA HiFi HotStart Ready Mix, 5 µl forward primer, 5 µl reverse primer and 2.5 µl template DNA (5 ng/ µl) under the following conditions: initial denaturation for 3 minutes at 95 ºC, 25 cycles of 30 seconds at 95 ºC, 30 seconds at 55 ºC and 30 seconds at 72 ºC; and final extension for 2 minutes at 72 ºC. Afterwards, sequencing adaptors and eight nucleotide dual-indexed barcodes of the multiplex Nextera® XT kit (Illumina, Inc., San Diego CA, United States) were added in a second PCR reaction with 25 µl 2x KAPA HiFi HotStart Ready Mix, 5 µl index i7, 5 µl index i5, 10 µl PCR Grade water and 5 µl concentrated amplicons of the initial PCR. The conditions applied during this second reaction were the following: initial denaturation for 3 minutes at 95 ºC, 8 cycles of 30 seconds at 95 ºC, 30 seconds at 55 ºC and 30 seconds at 72 ºC; and final extension for 5 minutes at 72 ºC. The libraries obtained were cleaned up with AMPure XP beads, and then validated by running 1 µl of a 1:50 dilution on a Bioanalyzer DNA 1000 chip (Agilent Technologies, Inc., Santa Clara, CA, United States) to verify their size, quantified by fluorometry with PicoGreen dsDNA quantification kit (Invitrogen, Life Technologies, Carlsbad, CA, United States). After size verification, libraries were pooled at equimolar concentrations and paired-end sequenced in 5 parallel plates in an Illumina MiSeq 2 x 250 platform at the Genomics and Bioinformatics Service of the Autonomous University of Barcelona.

6.3.3. Bioinformatics processing of microbial traits

A detailed description of the QIIME software (version 1.9.0) (Caporaso *et al*., 2010) pipeline followed for sequence processing can be found at Velasco-Galilea et al., 2020. Briefly, paired-ended reads were assembled into contigs using the python script *multiple_join_paired_ends.py* with default parameters. Then those contigs with a quality score smaller than Q19 were discarded, and the remaining ones were assigned to samples using the python script *split_libraries.py* with default parameters. The UCHIME algorithm (Edgar *et al*., 2011) was used to detect and remove the chimeric sequences generated during the PCR reactions. The filtered contigs were clustered into OTUs with a 97% similarity threshold using the script *pick_open_reference_otus.py* with default parameters (Rideout *et al*., 2014) and Greengenes reference database (version gg_13_5_otus) (McDonald *et al*., 2012). The OTU table obtained was normalized with the cumulative sum scaling (CSS) method (Paulson *et al*., 2013). Finally, the UCLUST consensus taxonomy assigner was used to conduct the taxonomic assignment of representative sequences of each OTU by mapping the sequences against the Greengenes reference database gg_13_5_otus. The raw sequence data were deposited in the sequence read archive of NCBI under the BioProject accession number PRJNA524130. Metadata, OTU table, and corresponding taxonomic assignments can be found at **Additional files 6.1**, **6.2** and **6.3**, respectively. After the bioinformatic processing, 989 representative traits of the rabbit intestinal microbiota were defined and analyzed in the present study. These microbial traits can be categorized into four different groups: the relative abundances of 29 genera, 951 CSS-normalized OTUs, four microbial alpha-diversity indexes computed at 10,000 contigs (total number of OTUs observed, Chao1, Shannon and Simpson's inverse), and the first five principal components (PC) computed from the OTU table. Genera relative abundances, microbial alpha-diversity indexes and PCs were standardized subtracting their mean and dividing by their standard deviation. Finally, these standardized microbial traits and CSS-normalized OTUs were multiplied by 100 and subsequently rounded to the nearest integer.

6.3.4. Statistical models

6.3.4.1. Zero-Inflated Poisson (ZIP) mixed model

Let $\mathbf{y} = (y_1, y_2, \ldots, y_n)'$ be the vector of records of some specific microbial trait on n individuals. For each record, the probability of having a zero count under the ZIP model is $p(y_i = 0) = p + (1-p)e^{-\lambda_i}$, and it happens with probability p in the sample set. Therefore, p is a population parameter. On the other hand, the probability of having k counts (k = 1, 2, …, ∞) in the sample set is $p(y_i = k) = \frac{(1-p)e^{-\lambda_i}\lambda_i^k}{k!}$. It occurs with probability $(1-p)$, and it is the probability function of a Poisson distribution with parameter λ_i, with p as defined before. This λ_i is a specific parameter of the individual. Conditioning on both p and $\boldsymbol{\lambda}$, the vector including all individuals λ_i, the likelihood function can be expressed as follows.

$$p(\mathbf{y}|\boldsymbol{\lambda}, p) = \prod_{y_i=0}\left[p + (1-p)e^{-\lambda_i}\right]\prod_{y_i>0}\left[\frac{(1-p)e^{-\lambda_i}\lambda_i^{y_i}}{y_i!}\right]$$

Considering these two re-parameterizations:

$$\lambda_i^* = \log(\lambda_i)$$
$$p^* = \log\left(\frac{p}{1-p}\right)$$

The previous conditional likelihood can be expressed as:

$$p(\mathbf{y}|\boldsymbol{\lambda}^*, p^*) = \prod_{y_i=0}\left[\left(\frac{1}{(1+e^{p^*})}\right)\left[e^{p^*} + e^{-\exp(\lambda_i^*)}\right]\right]\prod_{y_i>0}\left[\left(\frac{1}{(1+e^{p^*})}\right)\frac{e^{-\exp(\lambda_i^*)+\lambda_i^* y_i}}{y_i!}\right]$$

since $\lambda_i = \exp(\lambda_i^*)$ and $p = \frac{\exp(p^*)}{1+\exp(p^*)}$.

In a subsequent hierarchical level, different factors can be included as a linear model to explain the vector $\boldsymbol{\lambda}^*$, thus, the assumed distribution for $\boldsymbol{\lambda}^*$ was the following normal density:

$$p(\boldsymbol{\lambda}^*|\mathbf{V},\boldsymbol{\beta}) \sim \mathrm{MVN}(\mathbf{X}\boldsymbol{\beta},\mathbf{V}),$$

where $\boldsymbol{\beta}$ is a vector of systematic factors including the effects of the different categories of the combination between breeding farm, diet, and feeding regime (6 levels), of the batch (5 levels) and of the body weight at weaning (2 levels). $\mathbf{X}$ is a design matrix that relates the observations to the systematic effects, and $\mathbf{V}$ is the covariance matrix between the elements of $\boldsymbol{\lambda}^*$. The structure of $\mathbf{V}$ was not diagonal, and it was defined as follows:

$$\mathbf{V} = \sigma_P^2[\mathbf{Z_A A Z_A'} h^2 + \mathbf{Z_L Z_L'} l^2 + \mathbf{Z_C Z_C'} c^2 + \mathbf{I}(1 - h^2 - l^2 - c^2)],$$

where σ_P^2 is the phenotypic variance and the scalars h^2, l^2 and c^2 represent the ratios of additive genetic, litter and cage variances over the phenotypic variance. The assumed *prior* distribution of these ratios was uniform in the space [0,1], with the constraint that the sum of them must be lower than one:

$$p(h^2) = p(l^2) = p(c^2) = U(0,1), \text{and } h^2 + l^2 + c^2 \in [0,1].$$

Similarly, a uniform distribution along the positive real numbers was assumed for σ_P^2. $\mathbf{Z_A}$, $\mathbf{Z_L}$ and $\mathbf{Z_C}$, are design matrices relating the observations with animals in the pedigree, litters and cages, respectively; and matrix $\mathbf{A}$ is the numerator relationship matrix (Henderson, 1973). Uniform priors were also assumed for the elements of $\boldsymbol{\beta}$ and p^*, in this last case bounded between -5 and +5.

The posterior density can be written as:

$$p(\boldsymbol{\lambda}^*, p^*, \mathbf{V}, \boldsymbol{\beta}|\mathbf{y}) \propto p(\mathbf{y}|\boldsymbol{\lambda}^*, p^*)p(\boldsymbol{\lambda}^*|\mathbf{V},\boldsymbol{\beta})p(p^*)p(\mathbf{V})p(\boldsymbol{\beta})$$

$$p(\boldsymbol{\lambda}^*, p^*, \mathbf{V}, \boldsymbol{\beta}|\mathbf{y}) \propto \prod_{y_i=0}\left[\left(\frac{1}{(1+e^{p^*})}\right)\left[e^{p^*} + e^{-\exp(\lambda_i^*)}\right]\right]\prod_{y_i>0}\left[\left(\frac{1}{(1+e^{p^*})}\right)\frac{e^{-\exp(\lambda_i^*)+\lambda_i^* y_i}}{y_i!}\right] \times$$
$$|\mathbf{V}|^{n/2}\exp\left\{-\frac{1}{2}(\boldsymbol{\lambda}^* - \mathbf{X}\boldsymbol{\beta})'(\mathbf{VI})^{-1}(\boldsymbol{\lambda}^* - \mathbf{X}\boldsymbol{\beta})\right\}$$

This model specification is pretty similar to that previously proposed for studying mastitis cases in dairy cows (Rodrigues-Motta *et al*., 2007). The differences introduced in this study refer to the specifications for $\boldsymbol{\lambda}^{*}$: we assume a model in which a number of factors have been absorbed into the residual, while in the study by Rodrigues-Motta *et al*. (2007), these factors are explicitly fitted into the model being part of the vector of means. The two models are equivalent (beyond differences on the prior assumptions) but, the parameterization used here is the one that allows the computation of the BF for the ratio of variances in a parametric space defined between zero and one, including both limits (Varona *et al*., 2001).

This parameterization has, however, much higher computational demands than that of Rodrigues-Motta *et al*. (2007). First, because $\mathbf{V}$ must be updated and inverted repeatedly; and second because Metropolis-Hasting steps are needed to update the conditional posterior distribution of the ratios. In contrast to the case when the effects are explicitly considered into the model (Rodrigues-Motta *et al*., 2007), the BF can be computed for testing whether the additive genetic, litter, and cage effects are null or not since this model parameterization allows a null value of the ratio. The derivation of the conditional posterior distributions can be followed in the studies in which our model is based on: Rodrigues-Motta *et al*. (2007) and Varona *et al*. (2001).

6.3.4.2. Linear mixed model (LMM)

This model can be considered a simplification of the previous one since the generation process assumed for all data was the same as that assumed for the logarithm of the vector of λ parameters of the individual Poisson distributions ($\boldsymbol{\lambda}^{*}$) corresponding to those records with non-zero counts for each trait (transformed CSS-normalized OTU counts, transformed relative abundances of genera, transformed PCs, and transformed alpha-diversity indexes). Thus, the distribution of the data given the model parameters can be written as:

$$\mathrm{p}(\mathbf{y}|\mathbf{V},\boldsymbol{\beta})\sim\mathrm{MVN}(\mathbf{X}\boldsymbol{\beta},\mathbf{V}).$$

Accordingly, same model specifications including both the structure of $\mathbf{V}$ and the *prior* definitions, were defined. For the implementation, we used the conditional posterior distributions of this LMM derived by Varona *et al*. (2001), since we assumed the same *prior* distributions as they did.

6.3.4.3. Criteria for model comparison

Two model choice criteria were applied for each of the 989 microbial traits analyzed in this study. First, it was evaluated whether the trait was better adjusted with the LMM or the ZIP model. For this purpose, we used the deviance information criterion (DIC) that favored that model with the lowest value (Spiegelhalter *et al*., 2002). The statistical relevance of additive genetic, litter and cage effects was evaluated in both cases (LMM and ZIP model) using the BF. Thus, for each model (LMM and ZIP), three BFs were computed to assess the null hypotheses of whether additive genetic, litter or cage effects have null effect versus the alternative hypothesis that assumed that these factors have a non-null effect. These three hypotheses were independently tested by computing the BFs of $h^2 = 0$ against $h^2 \neq 0$ (BF_{h^2}), $l^2 = 0$ against $l^2 \neq 0$ (BF_{l^2}), and $c^2 = 0$ against $c^2 \neq 0$ (BF_{c^2}).

$$BF_{h^2} = \frac{3}{p(h^2=0\,|\mathbf{y})},\ BF_{l^2} = \frac{3}{p(l^2=0\,|\mathbf{y})},\ \text{and}\ BF_{c^2} = \frac{3}{p(c^2=0\,|\mathbf{y})}$$

The derivations of these definitions of the BF can be found in Varona *et al*. (2001). The evaluation of the marginal posterior of the ratios at zero implies, since these marginal posterior are only defined up to a proportionally constant, the computation of this proportionality constant: $\int_{h^2=0}^{h^2=1} p(h^2\,|\mathbf{y}) \times \partial h^2$. This integral can be solved numerically in each iteration. The different BFs can be computed as follows from the Markov chain Monte Carlo (MCMC) output:

$$BF_{h^2} = \frac{3}{\sum_{j=1}^{N} \frac{p(h^2 = 0\,|\mathbf{y})_j}{N}}$$

Where N is the number of MCMC iterations and $p(h^2 = 0 \mid \mathbf{y})_j$ is the evaluation of the marginal posterior density of h^2 at zero at each iteration j of the sampling procedure, which is computed as stated above:

$$\frac{p(h^2 = 0 \mid \mathbf{y})_j}{\int_{h^2=0}^{h^2=1} p(h^2 \mid \mathbf{y})_j \times \partial h^2}$$

All the operations were done on the logarithmic scale and after having saved the evaluations of the marginal posterior at zero along the MCMC chain to avoid numerical instabilities during their computation. In this way, it was possible to adjust the evaluations of the marginal posterior at zero for their maximum, thus reducing the needed numerical accuracy:

$$\sum_{j=1}^{N} \frac{p(h^2 = 0 \mid \mathbf{y})_j}{N} = \exp\left\{\log\left(\frac{\sum_{j=1}^{N} \exp\{\log(p(h^2 = 0 \mid \mathbf{y})_j) - m\}}{N}\right) + m\right\}$$

Where m is the maximum value of the vector composed of the N evaluations of $p(h^2 = 0 \mid \mathbf{y})$ on the logarithmic scale. See Sorensen and Gianola (2002) for further details.

BF values were classified according to four levels of evidence (Jeffreys, 1922): BF < 3.2: denominator model supported; 3.2 <= BF < 10: substantial evidence favoring the numerator model; 10 <= BF < 100: strong evidence favoring the numerator model; and BF >= 100: decisive evidence favoring the numerator model.

6.3.4.4. MCMC Bayesian implementation

MCMC Bayesian procedures were used to obtain samples from the marginal posterior distributions. This algorithm was implemented in a Fortran 90 software which is available in our GitHub repository (https://github.com/juanpablo-sanchez/BF-ZIP). For both, LMM and ZIP model, chains of 10,000 samples were run discarding the first 1,000 to allow the algorithm to reach convergence to the

marginal posterior distributions. Convergence diagnostics of the Markov chains was performed by the Geweke test function with coda R package (Plummer *et al*., 2006). Although the parameterization on the variance ratios has high computational demand, it allows for a good mixing. Thus, a reduced number of iterations is needed to properly reach convergence and characterize the marginal posterior distributions.

6.4. Results

6.4.1. Cecal microbial composition and diversity

After bioinformatic sequence processing we identified 951 different OTUs present in at least 5% of the animals. **Table 6.1** shows OTUs' frequency of presence across rabbit samples.

Table 6.1| OTUs' frequency of presence across rabbit cecal samples.

Frequency of presence (%)	Number of OTUs
≥ 5 to ≤ 10	13
> 10 to ≤ 25	43
> 25 to ≤ 50	121
> 50 to ≤ 75	277
> 75 to ≤ 100	497

In **Figure 6.1**, an iris plot illustrates the composition of the 425 samples analyzed. The taxonomic assignment of representative sequences of such OTUs against the Greengenes reference database gg_13_5_otus (see **Additional file 6.3**) revealed the presence of 29 different known genera. Of them, 4 were present in 50-75% of the rabbit samples and 25 in a minimum of 75% of the animals. Table 2 shows a phenotypic summary of the 29 genera relative abundances together with the four microbial alpha-diversity indexes and the first five principal components retained from the OTU table.

Table 6.2| Phenotypic summary of genera, alpha diversity indexes and first five principal components.

Trait	Mean	SD
Genus *Methanobrevibacter*, %	0.19	0.23
Genus *Adlercreutzia*, %	0.95	0.43
Genus *Bacteroides*, %	1.65	0.76
Genus *Parabacteroides*, %	0.21	0.18
Genus *Rikenella*, %	0.35	0.24
Genus *Butyricimonas*, %	0.20	0.19
Genus *Odoribacter*, %	0.27	0.22
Genus *Clostridium*, %	1.05	0.26
Genus *Dehalobacterium*, %	0.08	0.09
Genus *Anaerofustis*, %	0.11	0.07
Genus *Anaerostipes*, %	0.16	0.08
Genus *Blautia*, %	2.94	0.65
Genus *Butyrivibrio*, %	0.11	0.07
Genus *Coprococcus*, %	2.02	0.42
Genus *Dorea*, %	0.47	0.12
Genus *Epulopiscium*, %	0.11	0.11
Genus *Ruminococcus*, %	0.16	0.07
Genus *rc4-4*, %	0.15	0.07
Genus *Faecalibacterium*, %	0.20	0.05
Genus *Oscillospira*, %	2.26	0.58
Genus *Phascolarctobacterium*, %	0.21	0.24
Genus *Coprobacillus*, %	0.19	0.24
Genus *p-75-a5*, %	0.10	0.07
Genus *Oxalobacter*, %	0.11	0.06
Genus *Desulfovibrio*, %	0.46	0.31
Genus *Campylobacter*, %	0.07	0.08
Genus *Ruminococcus*, %	4.32	0.85
Genus *Anaeroplasma*, %	0.20	0.17
Genus *Akkermansia*, %	1.47	0.50
Principal component 1	0.00	17.08
Principal component 2	0.00	15.68
Principal component 3	0.00	9.33
Principal component 4	0.00	7.15
Principal component 5	0.00	6.63
Number of OTUs observed	551.05	91.94
Shannon	5.07	0.30
Simpson	0.98	0.01
Simpson’s inverse	71.01	20.20

SD: standard deviation.

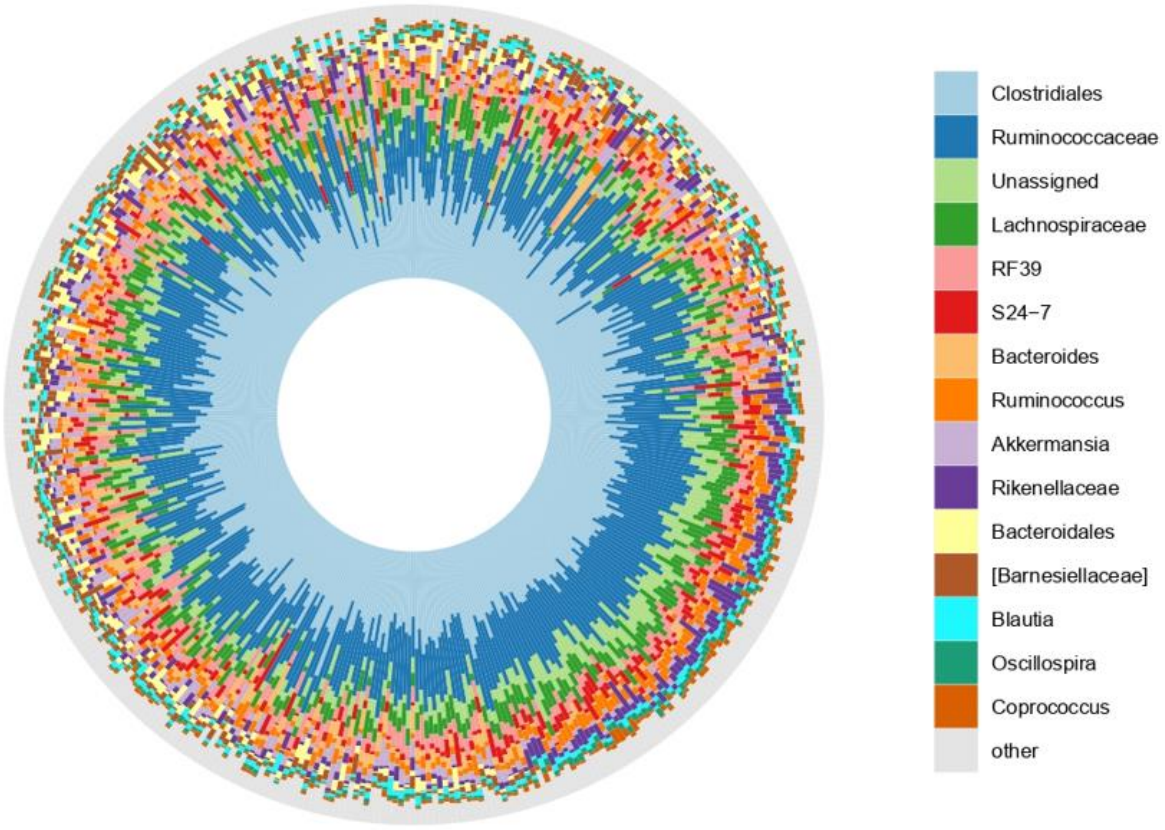

Figure 6.1| Iris plot illustrating the composition of the 425 samples analyzed.

6.4.2. LMM versus ZIP model adjustment of microbial traits

The adjustment of all genera, microbial alpha-diversity indexes, and principal components analyzed was better with the LMM (lower DIC values) than with the ZIP model. Regarding the 951 CSS-normalized OTUs also analyzed in this study, those having a frequency of presence > 25% were better adjusted with the LMM while all those with a frequency of presence < 10% were better adjusted with the ZIP model. Of the 43 OTUs having a frequency of presence between [10-25%), 18 and 25 OTUs were better adjusted with the LMM and the ZIP model, respectively.

6.4.3. Influence of genetic, litter and cage effects on rabbit cecal microbiota

Box and whisker plots of estimated marginal posterior means of the heritability, and litter and cage variance ratios for those OTUs that, according to DIC, are better adjusted with the LMM, and for those for which the ZIP model is preferable are shown in **Figure 6.2** and **Figure 6.3**, respectively. Same plots corresponding to genera relative abundances, microbial alpha-diversity indexes and the first five principal components are shown in **Figure 6.4**, **Figure 6.5** and **Figure 6.6**, respectively. In all of them, microbial traits are categorized by their frequency of presence across rabbit samples and by BFs' levels of evidence favoring the model that included additive genetic **(a)**, litter **(b)**, or cage **(c)** effects. The results summarized in these five figures are insightfully presented in the following paragraphs of the Results section. Generally speaking, these figures show that the BF did not provide evidence of genetic, litter, or cage effects for an important percentage of the microbial traits analyzed. However, for those traits declared to be affected for the host genetics, the litter or the cage, the magnitude of variance ratios estimates was moderate to high with minimum values of 0.15-0.20.

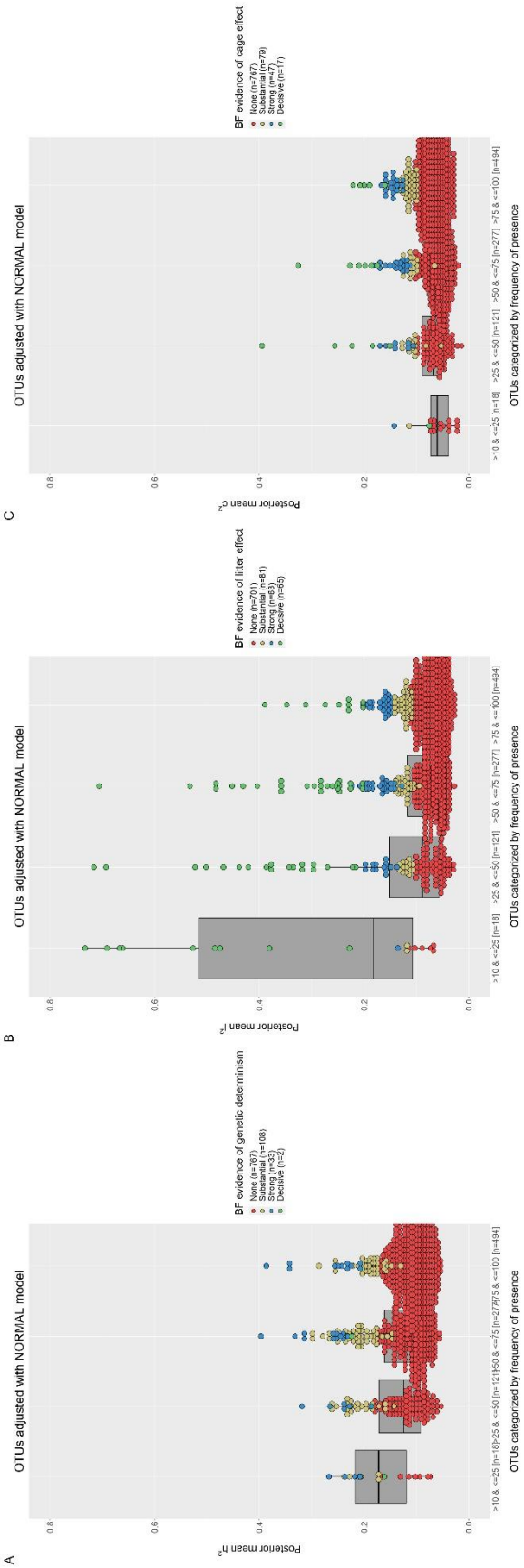

Figure 6.2| Marginal posterior of heritability **(a)**, litter **(b)** and cage **(c)** variance ratios for OTUs adjusted with the normal linear mixed model. OTUs are categorized by their frequency of presence across rabbit samples. Furthermore, each OTU is represented by a dot colored in red, yellow, blue or green when Bayes factor evidenced none, substantial, strong or decisive evidence of additive genetic **(a)**, litter **(b)** or cage**(c)** effect.

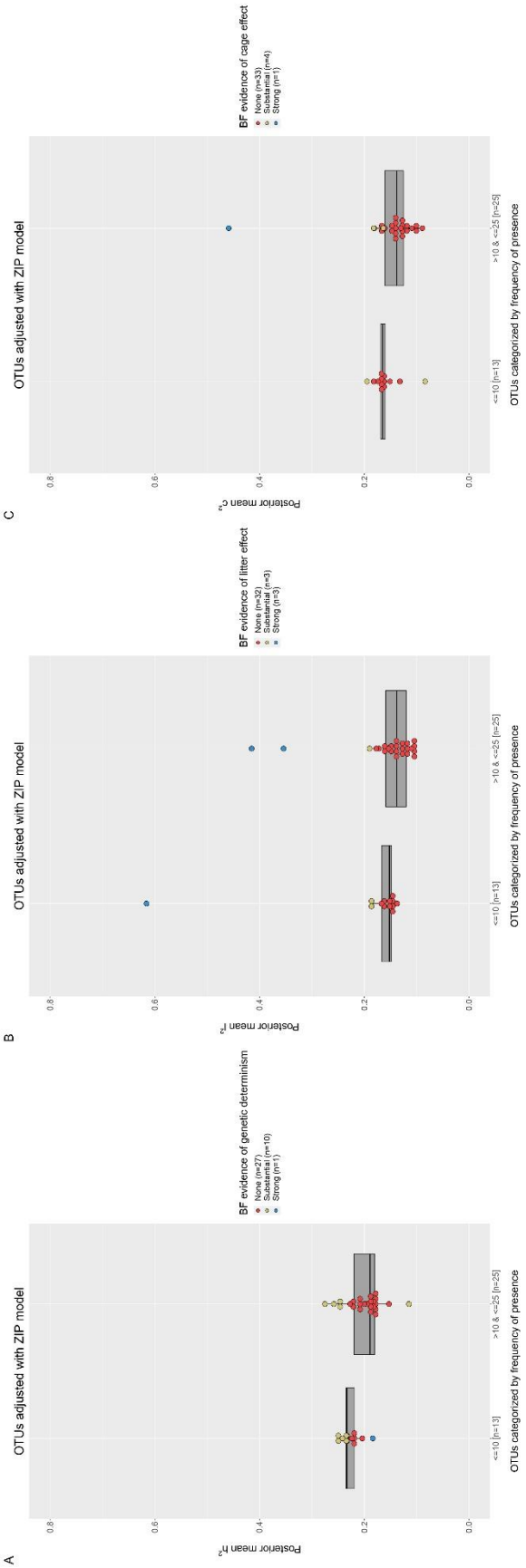

Figure 6.3| Marginal posterior of heritability **(a)**, litter **(b)** and cage **(c)** variance ratios for OTUs adjusted with the zero-inflated Poisson model. OTUs are categorized by their frequency of presence across rabbit samples. Furthermore, each OTU is represented by a dot colored in red, yellow, blue or green when Bayes factor evidenced none, substantial, strong or decisive evidence of additive genetic **(a)**, litter **(b)** or cage**(c)** effect.

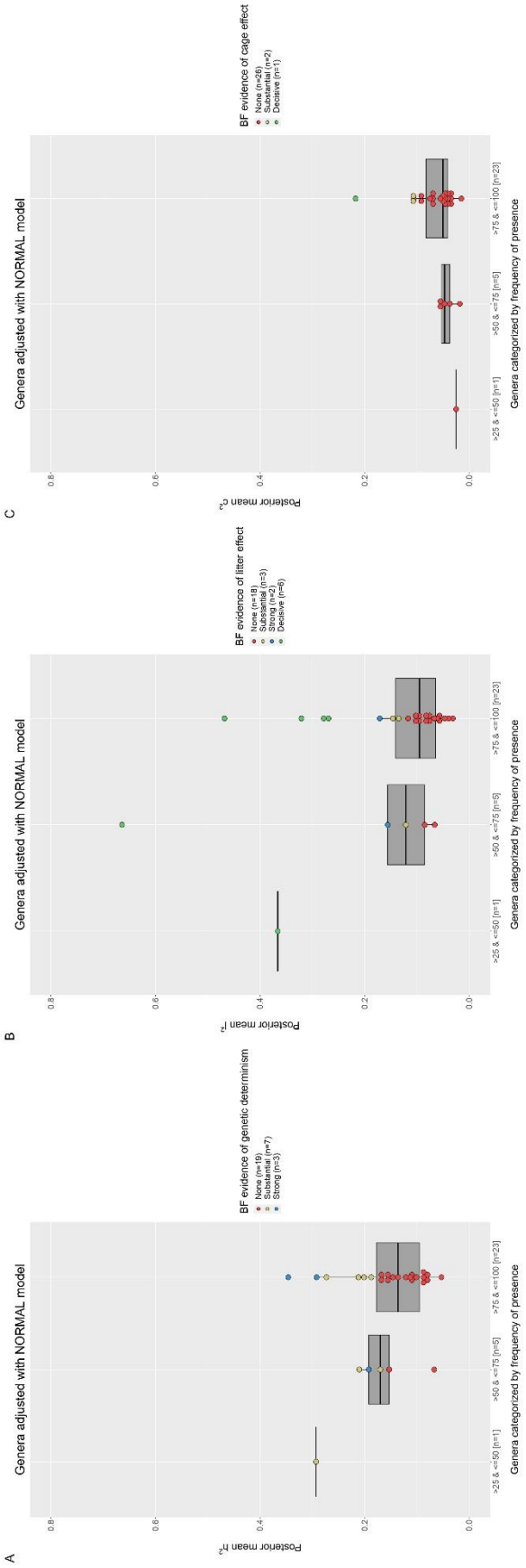

Figure 6.4] Marginal posterior of heritability **(a)**, litter **(b)** and cage **(c)** variance ratios for genera relative abundances adjusted with the normal linear mixed model. Genera are categorized by their frequency of presence across rabbit samples. Furthermore, each genus is represented by a dot colored in red, yellow, blue or green when Bayes factor evidenced none, substantial, strong or decisive evidence of additive genetic **(a)**, litter **(b)** or cage**(c)** effect.

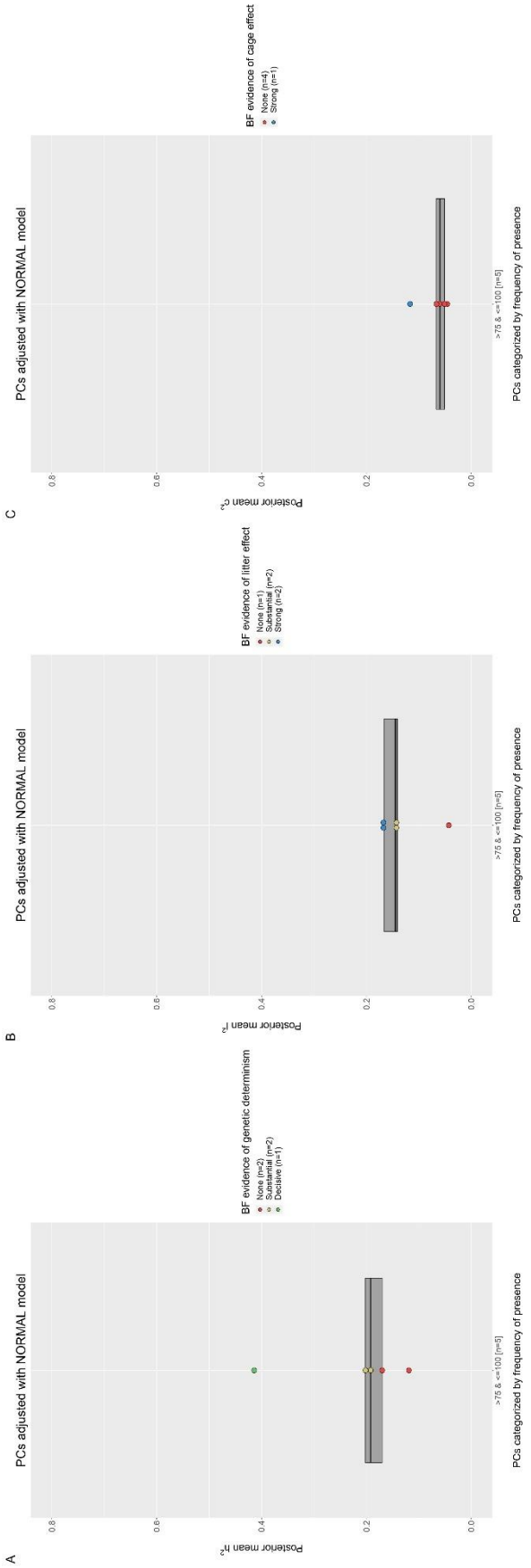

Figure 6.5| Marginal posterior of heritability **(a)**, litter **(b)** and cage **(c)** variance ratios for microbial alpha-diversity indexes adjusted with the normal linear mixed model. Each index is represented by a dot colored in red, yellow, blue or green when Bayes factor evidenced none, substantial, strong or decisive evidence of additive genetic **(a)**, litter **(b)** or cage **(c)** effect.

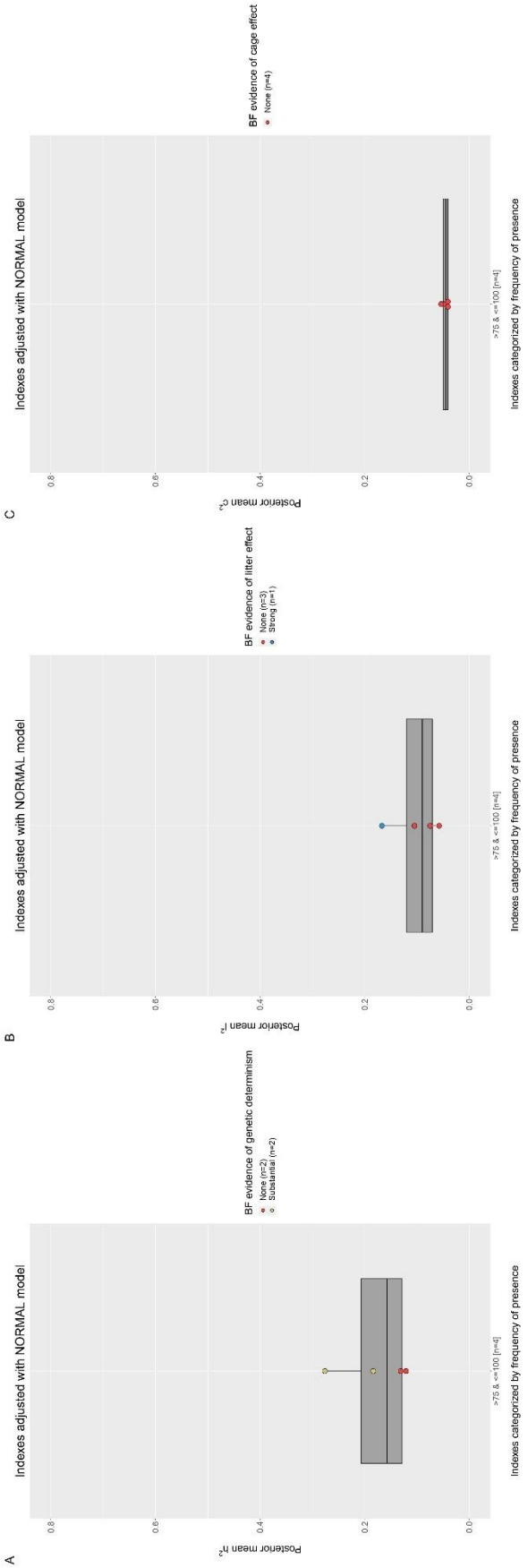

Figure 6.6| Marginal posterior of heritability **(a)**, litter **(b)** and cage **(c)** variance ratios for principal components adjusted with the normal linear mixed model. Each principal component is represented by a dot colored in red, yellow, blue or green when Bayes factor evidenced none, substantial, strong or decisive evidence of additive genetic **(a)**, litter **(b)** or cage**(c)** effect.

6.4.3.1. Microbial traits under genetic control

Table 6.S1 includes BFs, marginal posterior means, and standard deviations of heritability for OTUs for which the BF declared to be influenced by genetic effects together with the associated probability of these estimates being greater than 0.10. The taxonomic assignment of the representative sequences of such OTUs and their frequency of presence are also shown in **Table 6.S1**. **Table 6.3** summarizes marginal posterior means of heritability for OTUs, categorized by frequency of presence, better adjusted with the normal LMM and for which the BF evidenced genetic control. Similarly, **Table 6.4** summarizes marginal posterior means of heritability for OTUs, categorized by frequency of presence, better adjusted with the ZIP model and for which the BF evidenced genetic control. The BF provided some type of evidence of genetic control for 154 OTUs out of 951 total OTUs analyzed. The BFs between models with and without additive genetic effect evidenced a substantial (BF_{h^2} >= 3.2) genetic control for 108 and 10 OTUs better adjusted with the normal LMM and the ZIP model, respectively. A strong (10 <= BF_{h^2} < 100) genetic control evidence was found for 33 and one OTUs better adjusted with the normal LMM and the ZIP model, respectively. Finally, a decisive (BF_{h^2} >= 100) evidence of genetic control was found for two OTUs that were better adjusted with the normal LMM. The taxonomic assignment of these two OTUs revealed that one of them belongs to genus *Bacteroides* and the other to genus *Parabacteroides*, and their marginal posterior means (standard deviations) of heritability were 0.16 (0.07) and 0.22 (0.08), respectively (see **Table 6.S1**). Overall, estimates of heritability for these OTUs reflected medium values (from 0.12 to 0.40). It should be, however, recognized that such estimates are accompanied by large standard deviations as a consequence of our limited sample size. Nevertheless, it is worth stressing that 51 out of the 154 OTUs identified as being under genetic control had a probability equal or greater than 0.80 that their heritability has a value greater than 0.10.

The genetic determinism of the 38 remaining microbial traits (genera relative abundances, alpha-diversity indexes and principal components computed from the OTU table) was also assessed. The BF provided some type of evidence, which reached a decisive level in some cases, of genetic control for the relative

abundances of 10 genera, 3 principal components, and number of OTUs observed and Shannon indexes. Marginal posterior means and standard deviations of heritability, together with the associated probability of these estimates being greater than 0.10, of these traits are shown in **Table 6.5**.

The BF evidenced a substantial (BF_{h^2} >= 3.2) genetic control for genera *Dehalobacterium*, *Epulopiscium*, *Methanobrevibacter*, *Butyricimonas*, *Odoribacter*, *Blautia* and *Oxalobacter*. A strong (10 <= BF_{h^2} < 100) genetic control evidence was found for genera *Phascolarctobacteirum*, *Bacteroides* and *Parabacteroides*. The estimates (marginal posterior means) of the heritability for these genera ranged from 0.17 to 0.35. The greatest heritability estimates, accompanied by high BF values, were found for genera *Bacteroides*, *Parabacteroides* and *Dehalobacterium*. These three genera had a probability greater than 0.80 that their heritabilities are greater than 0.10. Although a strong evidence of genetic control was reached for genus *Phascolarctobacterium*, its heritability (0.19) estimate was not one of the highest ($P(h^2 > 0.1) = 0.73$). On the other hand, $1/BF_{h^2}$ values were greater than 3.2 only for ten OTUs and genus *Coprococcus*, which are clearly not heritable.

Finally, regarding traits that globally integrate rabbit cecal microbiota, a substantial evidence of genetic control was found for the number of OTUs observed and Shannon indexes, and for principal components two and five. The highest heritability estimates and BF values were reached for the number of OTUs observed index (h^2 = 0.28; BF_{h^2} = 7.30) and the principal component 4 (h^2 = 0.41; BF_{h^2} = 143.80). The additive genetic background for both traits was clearly demonstrated with a probability greater than 0.80 that their heritabilities are greater than 0.10

Table 6.3| Mean (standard deviation) of Bayes factor and heritability estimates for OTUs under genetic control adjusted with the normal LMM.

Frequency of presence (%)	Substantial genetic evidence ($3.2 \leq BF_{h^2} < 10$)			Strong genetic evidence ($10 \leq BF_{h^2} < 100$)			Decisive genetic evidence ($BF_{h^2} \geq 100$)		
	BF_{h^2}	h^2	n	BF_{h^2}	h^2	n	BF_{h^2}	h^2	n
All	5.31 (1.62)	0.21 (0.03)	108	24.83 (18.09)	0.26 (0.05)	33	159.38 (15.27)	0.19 (0.04)	2
>10 to ≤ 25	5.03 (1.32)	0.20 (0.03)	6	26.92 (12.50)	0.23 (0.03)	4	170.18 (-)	0.16 (-)	1
>25 to ≤ 50	5.49 (1.79)	0.21 (0.03)	23	36.19 (31.80)	0.25 (0.04)	6	-	-	0
>50 to ≤ 75	5.46 (1.70)	0.21 (0.04)	44	21.23 (12.57)	0.28 (0.05)	12	148.58 (-)	0.22 (-)	1
>75 to ≤ 100	5.07 (1.48)	0.19 (0.03)	35	21.82 (14.73)	0.27 (0.07)	11	-	-	0

BF_{h^2} = Bayes factor of the model with additive genetic effects against the same model without additive genetic effects

Table 6.4| Mean (standard deviation) of Bayes factor and heritability estimates for OTUs under genetic control adjusted with the ZIP model.

Frequency of presence (%)	Substantial genetic evidence ($3.2 \leq BF_{h^2} < 10$)			Strong genetic evidence ($10 \leq BF_{h^2} < 100$)		
	BF_{h^2}	h^2	n	BF_{h^2}	h^2	n
All	4.49 (1.73)	0.24 (0.04)	10	11.37 (-)	0.18 (-)	1
≥ 5 to ≤ 10	3.62 (0.35)	0.24 (0.01)	5	11.37 (-)	0.18 (-)	1
> 10 to ≤ 25	5.37 (2.16)	0.23 (0.06)	5	-	-	0

BF_{h^2}= Bayes factor of the model with additive genetic effects against the same model without additive genetic effects.

Table 6.5| Bayes factors, marginal posterior means (standard deviations) of heritability for genera, principal components and alpha-diversity indexes influenced by genetic effects.

Trait	Mean (SD) h^2	P(h^2 > 0.1)	BF_{h^2}
Genus *Methanobrevibacter*	0.21 (0.13)	0.79	7.75
Genus *Butyricimonas*	0.27 (0.19)	0.79	4.39
Genus *Odoribacter*	0.19 (0.13)	0.71	3.50
Genus *Bacteroides*	0.29 (0.17)	0.87	13.88
Genus *Parabacteroides*	0.35 (0.17)	0.91	31.15
Genus *Dehalobacterium*	0.29 (0.19)	0.83	8.62
Genus *Blautia*	0.20 (0.12)	0.78	7.01
Genus *Epulopiscium*	0.17 (0.11)	0.70	5.85
Genus *Phascolarctobacterium*	0.19 (0.12)	0.73	10.22
Genus *Oxalobacter*	0.21 (0.13)	0.78	6.12
Principal component 2	0.20 (0.14)	0.73	3.78
Principal component 4	0.41 (0.17)	0.97	143.80
Principal component 5	0.19 (0.13)	0.71	3.37
Number of OTUs observed	0.28 (0.17)	0.84	7.30
Shannon	0.18 (0.13)	0.70	3.41

SD: standard deviation; BF_{h^2}: Bayes factor of the model with additive genetic effects against the same model without additive genetic effects.

6.4.3.2. Microbial traits influenced by the litter

Table 6.S2 includes BFs, marginal posterior means and standard deviations of litter variance ratio for those OTUs the BF declared to be influenced by litter effects together with the associated probability of these ratios being greater than 0.10. The taxonomic assignment of the representative sequences of such OTUs and their frequency of presence are also shown in **Table 6.S2**. Marginal posterior means of litter variance ratio for OTUs, categorized by frequency of presence, better adjusted with the normal LMM and for which the BF evidenced litter influence are summarized in **Table 6.6**. Additionally, the same information for OTUs better adjusted with the ZIP model and for which the BF evidenced litter influence is shown in **Table 6.7**.

The BF provided some type of evidence of litter effect for 215 OTUs out of 951 total OTUs analyzed. Six of them showed a better adjustment with the ZIP model and the remaining 209 were better adjusted with the LMM. BF values between models with and without litter effects evidenced a substantial (BF_{l^2} >= 3.2) litter influence for 81 and three OTUs better adjusted with the normal LMM and the ZIP model,

respectively. A strong (10 <= BF_{l^2} < 100) litter influence evidence was found for 63 and three OTUs better adjusted with the normal LMM and the ZIP model, respectively. Finally, a decisive (BF_{l^2} >= 100) evidence of litter influence was found for 65 OTUs that were better adjusted with the normal LMM. The taxonomic assignment of these OTUs revealed that most of them belong to genera *Parabacteroides*, *Phascolarctobacterium*, and species *eggerthii* and *fragilis* of genus *Bacteroides*. Overall, marginal posterior means of the litter variance ratio ranged from 0.12 to 0.19 (**Table 6.6** and **Table 6.7**), but litter variance ratio estimates reached values from 0.37 to 0.54 for the aforementioned OTUs for which large BF values were observed (see **Table 6.S2**). Eighty-nine OTUs of the 215 declared to be influenced by litter effects had a probability equal or greater than 0.80 that their litter variance ratio is greater than 0.10. It is noteworthy that marginal posterior means of litter variance ratio were greater than 0.50 for 12 OTUs of which six belong to genus *Bacteroides*, four to genus *Phascolarctobacterium*, one to genus *Parabacteroides* and the other to genus *Rikenella* (see **Table 6.S2**). It should be mentioned that $1/BF_{l^2}$ values were greater than 3.2 for 107 OTUs, which are not influenced by litter.

Marginal posterior means of litter variance ratio, together with the associated probability of these ratios being greater than 0.10, for genera relative abundances and the traits defined in an attempt to globally integrate rabbit cecal microbiota can be found in **Table 6.8**. Evidence of litter influence was revealed for ten genera, four principal components and number of OTUs observed. An undeniable litter influence was shown for genera *Butyricimonas* (l^2 = 0.28), *Bacteroides* (l^2 = 0.27), *Parabacteroides* (l^2 = 0.47), *Rikenella*(l^2 = 0.32), *Dehalobacterium* (l^2 = 0.37) and *Phascolarctobacterium* (l^2 = 0.66) with decisive BF values (BF_{l^2} >= 100) and P(l^2 > 0.1) = 0.96. On the other hand, genera *Coprococcus*, *rc4-4* and *Faecalibacterium* are not influenced by litter ($1/BF_{l^2}$ > 3.2).

Finally, the number of OTUs observed and all the principal components, except the third one, were found to be influenced by litter effects, with marginal posterior means of litter variance ratio between 0.14 and 0.17 (**Table 6.8**).

Table 6.6| Mean (standard deviation) of Bayes factor and litter variance ratio estimates for OTUs influenced by litter adjusted with the normal LMM.

Frequency of presence (%)	**Substantial genetic evidence ($3.2 \leq BF_{l^2} < 10$)**			**Strong genetic evidence ($10 \leq BF_{l^2} < 100$)**			**Decisive genetic evidence ($BF_{l^2} \geq 100$)**		
	BF_{l^2}	l^2	**n**	BF_{l^2}	l^2	**n**	BF_{l^2}	l^2	**n**
All	5.29 (1.79)	0.12 (0.01)	81	29.46 (22.86)	0.17 (0.02)	63	∞ (∞)	0.37 (0.15)	65
>10 to ≤ 25	4.08 (0.80)	0.12 (0.00)	2	10.09 (-)	0.14 (-)	1	∞ (∞)	0.54 (0.17)	9
>25 to ≤ 50	5.45(1.73)	0.12 (0.01)	16	25.23 (16.74)	0.17 (0.02)	13	∞ (∞)	0.40 (0.14)	19
>50 to ≤ 75	5.49 (1.90)	0.12 (0.01)	28	34.90 (26.65)	0.17 (0.02)	23	∞ (∞)	0.33 (0.12)	27
>75 to ≤ 100	5.12 (1.77)	0.12 (0.01)	35	27.51 (21.94)	0.17 (0.02)	26	7.98E4 (2.33E5)	0.27 (0.06)	10

BF_{l^2}= Bayes factor of the model with litter effects against the same model without litter effects

Table 6.7| Mean (standard deviation) of Bayes factor and litter variance ratio estimates for OTUs influenced by litter adjusted with the ZIP model.

Frequency of presence (%)	Substantial genetic evidence ($3.2 \leq BF_{l^2} < 10$)			Strong genetic evidence ($10 \leq BF_{l^2} < 100$)		
	BF_{l^2}	l^2	N	BF_{l^2}	l^2	n
All	3.77 (0.46)	0.19 (0.00)	3	36.69 (23.57)	0.46 (0.14)	3
≥ 5 to ≤ 10	3.80 (0.64)	0.19 (0.00)	2	59.05 (-)	0.62 (-)	1
> 10 to ≤ 25	3.70 (-)	0.19 (-)	1	25.51 (19.01)	0.38 (0.04)	2

BF_{l^2}= Bayes factor of the model with litter effects against the same model without litter effects

Table 6.8| Bayes factors, marginal posterior means (standard deviations) of litter variance ratio for genera, principal components and alpha-diversity indexes influenced by litter effects.

Trait	Mean (SD) l^2	P(l^2 > 0.1)	BF_{l^2}
Genus *Butyricimonas*	0.28 (0.10)	0.96	728.23
Genus *Odoribacter*	0.14 (0.08)	0.64	7.21
Genus *Bacteroides*	0.27 (0.09)	0.97	809.53
Genus *Parabacteroides*	0.47 (0.10)	1.00	1.50E11
Genus *Rikenella*	0.32 (0.08)	1.00	3.67E4
Genus *Dehalobacterium*	0.37 (0.10)	1.00	9.66E4
Genus *Anaerofustis*	0.15 (0.08)	0.68	8.80
Genus *Epulopiscium*	0.12 (0.07)	0.58	4.52
Genus *Phascolarctobacterium*	0.66 (0.07)	1.00	∞
Genus *Desulfovibrio*	0.17 (0.09)	0.78	16.53
Genus *Campylobacter*	0.16 (0.08)	0.73	11.91
Principal component 1	0.17 (0.08)	0.77	16.98
Principal component 2	0.14 (0.08)	0.65	6.33
Principal component 4	0.17 (0.09)	0.73	24.47
Principal component 5	0.15 (0.08)	0.68	9.62
Number of OTUs observed	0.17 (0.09)	0.75	15.55

SD: standard deviation; BF_{l^2}: Bayes factor of the model with litter effects against the same model without litter effects.

6.4.3.3. Microbial traits influenced by the cage

Table 6.S3 includes BFs, marginal posterior means and standard deviations of cage variance ratio for those OTUs the BF declared to be influenced by cage effects together with the probability of these parameters being greater than 0.10. The taxonomic assignment of the representative sequences of such OTUs and their frequency of presence are also shown in this file. **Table 6.9** shows the marginal posterior means of cage variance ratio for OTUs, categorized by frequency of presence, better adjusted with the normal LMM and for which BF evidenced cage influence. Similarly, **Table 6.10** includes the same information for OTUs better adjusted with the ZIP model and for which BF declared cage influence.

Cage effect was found for 143 OTUs better adjusted with the normal LMM of which 79, 47 and 17 showed substantial, strong and decisive, respectively, evidence. While four and one OTUs better adjusted with the ZIP model showed substantial and strong, respectively, evidence of cage effect. The taxonomic assignment of these OTUs revealed that many of them belong to families *S24-7* and

Ruminococcaceae (see **Table 6.S3**). Overall, marginal posterior mean cage variance ratio ranged from 0.11 to 0.24 (**Table 6.9** and **Table 6.10**), but three OTUs for which large BF values were calculated reached cage variance estimates up to 0.46 (see **Table 6.S3**). Two of these OTUs were assigned to family *S24-7* (see **Table 6.S3**). It should be noted that $1/\mathrm{BF}_{c^2}$ values were greater than 3.2 for 130 OTUs, which are not influenced by cage.

Finally, marginal posterior means of cage variance ratio, together with the associated probability of this ratio being greater than 0.10, for genera relative abundances and the traits defined to globally integrate rabbit cecal microbiota can be found in **Table 6.11**. Evidence of cage influence was revealed for three genera and principal component four whose marginal posterior means of cage variance ratio ranged from 0.11 to 0.22. Although these estimates are accompanied by large standard deviations as a consequence of our limited sample size, a patent cage influence was demonstrated for genus *Ruminococcus* ($c^2 = 0.22$; BF = 648.80; $P(c^2 > 0.1) = 0.95$).

Table 6.9| Mean (standard deviation) of Bayes factor and cage variance ratio estimates for OTUs influenced by cage adjusted with the normal LMM.

Frequency of presence (%)	Substantial genetic evidence ($3.2 \leq BF_{c^2} < 10$)			Strong genetic evidence ($10 \leq BF_{c^2} < 100$)			Decisive genetic evidence ($BF_{c^2} \geq 100$)		
	BF_{c^2}	c^2	n	BF_{c^2}	c^2	n	BF_{c^2}	c^2	n
All	5.68 (1.94)	0.11 (0.01)	79	27.92 (22.14)	0.14 (0.02)	47	6.93E7 (2.81E8)	0.21 (0.07)	17
> 10 to ≤ 25	3.34 (-)	0.11 (-)	1	14.79 (-)	0.14 (-)	1	497.12 (-)	0.08 (-)	1
> 25 to ≤ 50	5.82 (1.33)	0.10 (0.02)	14	36.98 (22.55)	0.14 (0.02)	7	2.32E8 (5.19E8)	0.24 (0.09)	5
> 50 to ≤ 75	5.62 (2.13)	0.11 (0.01)	21	23.82 (21.38)	0.14 (0.02)	17	2.71E6 (6.63E6)	0.22 (0.05)	6
> 75 to ≤ 100	5.72 (2.04)	0.11 (0.01)	43	28.80 (23.04)	0.15 (0.01)	22	700.53 (684.16)	0.20 (0.02)	5

BF_{c^2}: Bayes factor of the model with cage effects against the same model without cage effects

Table 6.10| Mean (standard deviation) of Bayes factor and cage variance ratio estimates for OTUs influenced by cage adjusted with the ZIP model.

Frequency of presence (%)	Substantial genetic evidence ($3.2 \leq BF_{c^2} < 10$)			Strong genetic evidence ($10 \leq BF_{c^2} < 100$)		
	BF_{c^2}	c^2	n	BF_{c^2}	c^2	n
All	3.93 (0.70)	0.16 (0.05)	4	37.39 (-)	0.46 (-)	1
≥5 to ≤10	4.53 (0.04)	0.14 (0.08)	2	-	-	0
>10 to ≤25	3.33 (0.16)	0.17 (0.01)	2	37.39 (-)	0.46 (-)	1

BF_{c^2}: Bayes factor of the model with cage effects against the same model without cage effects.

Table 6.11| Bayes factors, marginal posterior means (standard deviations) of cage variance ratio for genera, principal components and alpha-diversity indexes influenced by cage effects.

Trait	Mean (SD) c^2	P(c^2 > 0.1)	BF_{c^2}
Genus *Ruminococcus*	0.22 (0.07)	0.95	648.80
Genus *Dorea*	0.11 (0.07)	0.52	3.23
Genus *Faecalibacterium*	0.11 (0.06)	0.50	3.31
Principal component 4	0.12 (0.06)	0.58	19.69

SD: standard deviation; BF_{c^2}: Bayes factor of the model with cage effects against the same model without cage effects.

6.5. Discussion

The influence of many external factors on rabbit cecal microbial composition and diversity is unquestionable (Abecia *et* al., 2007; Zou *et* al., 2016; Zhu *et al*., 2017; Chen *et al*., 2019; Velasco-Galilea *et al*., 2020). However, the potential existence of host genetic determinism remains unknown in this species. To shed light on this matter, we have reported heritabilities, together with litter and cage variance ratios estimates, for microbial traits on which a LMM and ZIP mixed models were fitted. Moreover, in this study, we have assessed the statistical relevance of such estimates through BF.

Previous studies in humans and different livestock species have pointed out the existence of host genetic determinism of gut microbiota, but there is no study in rabbits. For the first time, we have evaluated the host genetics, litter, and cage effects on the microbial composition of the cecum, which is the organ that contains the greatest microbial diversity and complexity (Goeut and Fonty, 1979). In this study, we have defined a set of 989 microbial traits that claim to represent cecal microbial composition and diversity with different levels of complexity. The CSS-normalized abundances of 951 OTUs can be considered the most specific level of defining a microbial community. Such traits show the particular feature of having a very variable frequency of presence across samples. This means that while some OTUs are present in all or almost all the animals (core OTUs), others are only detected in some animals. The distribution of those OTUs which are only present in a small percentage of the animals analyzed is clearly far from normality and, not

surprisingly, are better adjusted with the ZIP model. Despite this, all the microbial traits analyzed in this study whose frequency of presence was higher than 25% were better adjusted with the normal LMM model according to DIC. For those traits showing a clear excess of zeros (i.e., having a frequency of presence across samples lower than 15%) DIC clearly favored the ZIP model. Previous microbiome studies have also modeled microbiome data with the ZIP model to account for the excess of zeros of many taxa that are rare and only detected in a small proportion of samples (Lee *et* al., 2020). Such studies argue that the application of a conventional linear model is inappropriate for zero-inflated data. However, in this study, the ZIP model only overcame the LMM for those microbial traits with a very marked excess of zeros.

The BF evidenced genetic control for 34% and 16% of the genera and OTUs, respectively, analyzed in this study that inhabit the rabbit cecum. These results are in line with the heritability analysis conducted by Goodrich *et al*. (2014) in humans that found evidence of genetic control for 10% of the 945 taxa identified in that study, and with an assessment of the host genetics influence on the rumen microbiota (Li *et al*., 2019) which found that 34% of the microbial taxa analyzed (from genus to phylum levels) were heritable. Our heritability estimates for the relative abundances of those genera and OTUs declared to be under host genetic control by the BF reflected medium values. This is also in agreement with earlier studies in humans and other livestock species. However, it is noteworthy that these studies suggested that the main heritable bacteria belong to phylum *Firmicutes*, whereas taxa encompassed by phylum *Bacteroidetes* are generally not heritable (Goodrich *et* al., 2016; Li *et al*., 2019). A discussion of the results regarding the influence of the host genetics, litter, and cage effects on taxa encompassed by phyla *Bacteroidetes* and *Firmicutes* will be presented below. After that, the influence of such effects on microbial alpha-diversity indexes and principal components will be also debated.

In our study, according to the BF, the strongest evidence of genetic control was found for two OTUs taxonomically assigned to genera *Bacteroides* and *Parabacteroides* which are both encompassed by phylum *Bacteroidetes*. Moreover, the greatest heritability estimates were found for these two genera (h^2

Parabacteroides = 0.35; h^2 *Bacteroides* = 0.29). Chen *et al*. (2018) and Bergamaschi *et al*. (2020) also reported some heritable taxa encompassed by phylum *Bacteroidetes* in pigs. Species belonging to genera *Bacteroides* and *Parabacteroides* are anaerobic Gram-negative bacterium involved in the degradation of vegetal polysaccharides and amino acid fermentation, amino acid transport, and cell motility in the gastrointestinal microbiota of the growing rabbit (Dai *et al*., 2011; Sun *et al*., 2020). Although the BF and our heritability estimates for genera *Bacteroides* and *Parabacteroides* clearly reveal the existence of host genetic determinism, the environmental effect of litter has a profound impact on the relative abundances of both genera (l^2 *Parabacteroides* = 0.47; l^2 *Bacteroides* = 0.27). The nursing environment provided by the mother and siblings also has an important impact on the relative abundance of genus *Rikenella* (l^2 *Rikenella* = 0.32) which is also encompassed by phylum *Bacteroidetes*. Litter effects play an important role on phenotypic traits related to rabbit growth and feed efficiency (Piles and Sánchez, 2019). Microbial colonization of rabbits and mammals' gastrointestinal tract is considered to occur immediately after birth when newborns acquired their immature microbiota from a combination of maternal and external microbes (Combes *et* al., 2011; de Agüero *et al*., 2016). The impact of the nursing environment on the relative abundances of these genera still prevails at the slaughter age when cecal samples were collected from animals analyzed in this study. Remarkably, the ratio of phenotypic variance due to litter effects overcomes the value of 0.50 for six OTUs belonging to genus *Bacteroides* and for one OTU taxonomically assigned to genus *Parabacteroides*. It is also worth noting that the cage seems to play an important effect in the relative abundances of members of family *S24-7*. Bacteria within this family, encompassed by the order *Bacteroidales*, are dominant in the mouse gut microbiota and have been detected in the gastrointestinal tract of different mammals. The classification of this family was ambiguous because it had not been cultured, but the functional analysis conducted by Lagkouvardos *et al*. (2019) renamed it as family *Muribaculaceae*. In a recent study on mice, members of the family *Muribaculaceae* were shown to be major mucin monosaccharide foragers in the gut (Pererira *et al*., 2020).

High heritability values, accompanied by a strong evidence of genetic determinism provided by the BF, were also estimated for genera *Dehalobacterium* (h^2 = 0.29) and *Butyricimonas* (h^2 = 0.27). Both genera belong to phylum *Firmicutes* and have been previously reported as heritable in humans (Goodrich *et* al., 2014; Goodrich *et al*., 2016). Such studies reported a module of co-occurring heritable families within which family *Christensenellaceae* was the hub (i.e., the node connected to most other nodes) connected to heritable families *Methanobacteriaceae* and *Dehalobacteriaceae*. Interestingly, we have also found substantial evidence of genetic control for genus *Methanobrevibacter* which is encompassed by family *Methanobacteriaceae*. Genus *Methanobrevibacter* is the single genus belonging to phylum *Euryarchaeota* detected in rabbit cecum. It encompasses different hydrogenotrophic methane-producing species whose abundance has been associated with single-nucleotide polymorphisms located within a long noncoding RNA, however, this link remains uncertain (Bonder *et al*., 2016). Besides, taxa belonging to family *Methanobacteriaceae* were reported to have heritability estimates greater than 0.50 in a beef cattle population (Abbas *et al*., 2020). It is worth emphasizing that our results also show heritability estimates statistically greater than zero for genera *Blautia* and *Odoribacter*, which is consistent with previous results in humans (Le Roy *et al*., 2018; Xu *et al*., 2020).

Our results also revealed an important impact of the litter effects on the relative abundances of genera *Butyricimonas* (l^2 = 0.28), *Dehalobacterium* (l^2 = 0.37) and *Phascolarctobacterium* (l^2 = 0.66). BF and heritability estimates also suggested a genetic determinism for these three genera, but the effect exerted by the nursing environment seems to be of greater magnitude. On the other hand, the role played by cage environmental effects was found to be strong for some species encompassed by genus *Ruminococcus*. Genetic and litter effects, in the contrary, do not seem to have any relevant influence on such genus. However, La Reau *et al*. (2016) and Li *et al*. (2019) found that the abundance of genus *Ruminococcus* was influenced by host genetics. Remarkably, this genus displays large diversity and, in this study, we have reported four OTUs taxonomically assigned as *Ruminococcus* that showed a clear genetic determinism.

On a global scale, our results suggest that a substantial part of the cecal microbial variability is under host genetic control since the BF pointed out evidence of genetic determinism for three principal components, number of OTUs observed and Shannon indexes. We have found a clear genetic background for number of OTUs observed index ($h^2 = 0.28$) and principal component 4 ($h^2 = 0.41$) this is in line with previous heritability assessments of alpha-diversity in pigs (Lu *et* al., 2018; Bergamaschi *et al*., 2020), humans (Goodrich *et al*., 2016), and in the study conducted by Saborío-Montero *et al*. (2021) in cattle that overall estimated the heritability of rumen microbiota by the aggregation of the OTU table into principal components. Microbial complexity can be summarized into principal component and alpha-diversity indexes, which are heritable traits that could be incorporated into breeding programs. Nonetheless, it is important to bear in mind that alpha-diversity at weaning might not be an accurate predictor of diversity at later stages in rabbit life. Rabbit cecum hosts a rich and complex microbial ecosystem that is shaped by many non-genetic factors, however, a significant proportion of the microbial traits analyzed in this study showed moderate heritabilities. Although cecal samples analyzed in the present study were collected from nearly adult rabbits, these estimates should be interpreted with caution since microbial composition varies over time and does not stabilize until the animal reaches adulthood. As we have stated, recent studies in different livestock species have attempted to disentangle the genetic determinism of gut microbiota but had not paid enough attention to non-genetic factors, such as litter or cage effects, whose influence is even more relevant than the additive genetic effects.

For the first time, in this study, we have evaluated the role played by host genetics, litter and cage effects on a set of traits that attempt to represent rabbit cecal microbiota at different levels of depth. We think that understanding the effect of host genetics, litter or cage found for certain microbial traits could be more relevant from a biological knowledge perspective than from a practical point of view. An example of this would be the genus *Methanobrevibacter* which is clearly heritable and seems to be linked to methane emissions. The genetic determinism in the host for methane emissions and the relative abundance of this genus would offer the possibility to alter microbial composition through selection and to breed for rabbits that reduce

climate impact. Although a selection to reduce this genus could be postulated, it would only account for a certain part of such emissions. Moreover, members of this genus could be beneficial for other relevant traits, thus selecting a given trait through microbiota might be risky since negative responses for the other traits of interest could be also obtained. In addition, a direct selection somehow guarantees a balanced modification of all the elements involved in the metabolic pathway of the trait.

Finally, we want to highlight that subjacent mechanisms involved in host genetic determinism on cecal microbiota remain still unknown. Future genome-wide association studies with large datasets are necessary to identify the host genomic regions involved in the control of overall microbial diversity and abundances of specific taxa.

6.6. Conclusions

The Bayesian analysis of a set of 989 microbial traits conducted in this study with LMM and ZIP mixed models has allowed disentangling the influence of additive genetic, litter and cage effects on different levels of complexity of rabbit cecal microbiota through BF. Fitting these microbial traits with a LMM model was preferable except for the analyses of CSS-normalized abundances of rare OTUs characterized by a marked excess of zeros that led to a better adjustment with the ZIP model. The calculation of BF as an assessment tool of the statistical relevance of heritability, litter and cage variance ratios estimates has allowed us to unravel different levels of influence of such effects on global cecal microbial composition and on an important proportion of OTUs and genera relative abundances. It is worth mentioning the important influence of host genetics and the nursing environment found for members of genera *Bacteroides* and *Parabacteroides*, while family *S24-7* and genus *Ruminococcus* are highly affected by cage effects. The findings of this study support that host genetics, cage and nursing environment contribute to the variation of rabbit cecal microbial composition, but functional and genome-wide association studies are needed to advance knowledge of the underlying mechanisms.

6.7. List of abbreviations

AL *ad libitum* feeding regime
BF Bayes factor
CSS cumulative sum scaling
DIC deviance information criterion
LMM linear mixed model
MCMC Markov chain Monte Carlo
OTU operational taxonomic unit
PCR polymerase chain reaction
R restricted feeding regime
ZIP zero-inflated Poisson

Declarations

Ethics approval and consent to participate

This study was carried out in accordance with the recommendations of the animal care and use committee of the Institute for Food and Agriculture Research and Technology (IRTA). The protocol was approved by the committee of the Institute for Food and Agriculture Research and Technology (IRTA).

Consent for publication

Not applicable.

Availability of data and materials

The raw sequence data were deposited in the sequence read archive of NCBI under the accession number SRP186982 (BioProject PRJNA524130). Metadata, the filtered and CSS-normalized OTU table and corresponding taxonomic assignments have all been included as **Additional files 6.1**, **6.2** and **6.3**, respectively. The Additional information for this article can be found in the Annexes section.

Competing interests

The authors declare that they have no competing interests.

Funding

The experimental design of this work was conducted thanks to funding from INIA project RTA2011-00064-00-00. This study was part of the Feed-a-Gene project that received funding from the European Union's H2020 program under grant agreement no. 633531, and the Spanish project RTI2018-097610R-I00. MVG is a recipient of a "Formación de Personal Investigador (FPI)" pre-doctoral fellowship from INIA, associated with the research project RTA2014-00015-C2-01. YRC is recipient of a Ramon y Cajal post-doctoral fellowship (RYC2019-027244-I) from the Spanish Ministry of Science and Innovation.

Authors' contributions

JPS and MP conceived the experimental design. MVG, JPS and MP collected biological samples. MVG and MP processed the samples in the laboratory. MVG processed and analyzed the sequencing data, interpreted data, and prepared figures and tables. JPS and LV contributed analyzing the sequencing data. MVG and JPS wrote the manuscript. JPS, MP, YRC and LV helped interpreting the data, and revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We would like to thank Oscar Perucho, Josep Ramon and Carmen Requena (staff of Unitat de Cunicultura, IRTA) for animal care and their contribution to data recording. We acknowledge Oriol Rafel, Marc Viñas, Miriam Guivernau and Olga González for their help collecting and processing the biological samples. We also acknowledge Armand Sánchez, Nicolas Boulanger and Joana Ribes (Genomics and NGS Unit, CRAG) for their assistance in massive libraries preparation.

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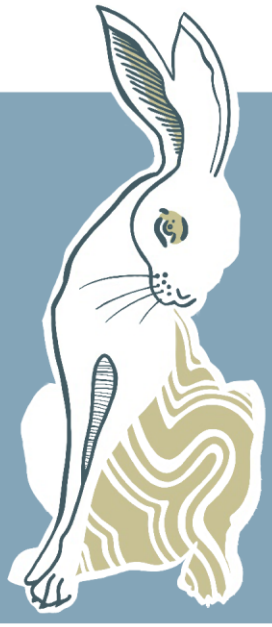
CHAPTER 7

IDENTIFICATION OF GENOMIC REGIONS INVOLVED IN THE GENETIC CONTROL OF THE MEAT RABBIT CECAL MICROBIOTA AND ASSESSMENT OF MICROBIAL GWAS DETECTION POWER



Article V

Identification of genomic regions involved in the genetic control of the meat rabbit cecal microbiota and assessment of microbial GWAS detection power

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(In preparation)

Identification of genomic regions involved in the genetic control of the meat rabbit cecal microbiota and assessment of microbial GWAS detection power

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7.1. Abstract

Background

The present study intends to identify genomic regions involved in the host genetic control of cecal microbiota by performing a genome-wide association study (GWAS) using 412 rabbits genotyped with a high-density chip containing almost 200,000 single nucleotide polymorphisms (SNPs) and an improved version of the OryCun2.0 reference assembly of the rabbit genome. For this purpose, the cecal microbial community of these 412 animals was phenotyped by sampling and characterizing the V4-V5 hypervariable regions of the 16S rRNA gene and defining a set of microbial traits representative of the cecal microbiota at different levels of depth. Two different approaches were applied to identify host genomic regions associated with the microbial traits under study: mixed model regression at each SNP position (MIX-GWAS) and BayesC. A simulation study was also conducted to assess the statistical power of both approaches to identify host genomic regions associated with a simulated normally distributed phenotype under alternative heritabilities scenarios.

Results

Our simulation assessment clearly showed the rather limited power of the data structure and sample size regardless of the analysis method considered. A power of detection greater than 75% was only achieved for those windows containing a QTN with a strong effect that explained at least 50% of the phenotypic variance. Moreover, the simulation assessment revealed that the positive predictive value rate of MIX-GWAS was about one-third of that of BayesC. Despite this limited statistical power, the MIX-GWAS analysis declared 334 signals spread across 10 chromosomes as significantly associated with 19 microbial traits. Our previous knowledge about the genetic background of these traits accompanied by a deep analysis of the genes annotated on the regions harboring these signals led us to prudently propose QTL regions on OCCs 1, 6, 8, 15, and 19 involved in the host genetic control of the rabbit gut microbiome. These regions include genes like *SLC12A9*, *ABCA5*, *ADH4*, *DLAT*, *CSF2*, *GNB2*, *GABRA1*, or *TNFSF13B* implicated in homeostatic, metabolic, or immune system processes.

Conclusions

Despite the limited statistical power of our data structure, we have identified different genomic regions in ten chromosomes that we prudently declare as associated with the variation of rabbit cecal microbiota, particularly one on OCC 12 that is associated with the variation of one OTU assigned to genus *Butyricimonas*. Nonetheless, this knowledge has more relevance from a biological perspective than from an applied point of view, given that the link between this genetic control and that for traits of interest, such as growth or diseases resistance, is not evident.

7.2. Background

The rabbit gastrointestinal tract (GIT) harbors a complex and diverse microbial community of about 100 to 1,000 billion microorganisms per gram of digesta (Savage, 1987), covering over 1,000 different species, predominating the kingdom *Bacteria* over archaeal populations (Combes *et al*., 2011). The cecum is the main organ for microbial fermentation in the domestic meat rabbit (*Oryctolagus cuniculus*). Therefore, despite the presence of active microbial populations throughout the whole GIT, the cecum hosts the most diverse and richest microbial community (Gouet and Fonty, 1979). A symbiotic relationship is established between the host and its GIT microbiota (Gaskins, 1997). Such a relationship has co-evolved promoting the growth of mutualistic microorganisms that facilitate the degradation of nutrients and ensure proper homeostatic balance maintenance (Flint *et al*., 2012). The composition of this complex microbial community is shaped by the dynamically changing physical and chemical conditions within the cecum. At the same time, the bacterial and archaeal communities contribute to the cecal environmental conditions and the host’s nutrient availability (Mackie, 2002).

A growing number of studies have characterized the microbial communities inhabiting the rabbit GIT, especially those present in the cecum (Abecia *et al*., 2007; Zou *et al*., 2016; Zhu *et al*., 2017; Chen *et al*., 2017). The establishment of a homogeneous and stable cecal microbiota is achieved when the rabbit reaches adulthood (Combes *et al*., 2011). However, such stability may be altered over the life of the animal by multiple factors, including the diet (Gidenne *et al*., 2004;

Carabaño *et al*., 2009; Chamorro *et al*., 2010), level of feeding (Abecia *et al*., 2007; Velasco-Galilea *et al*., 2020), and the administration of antibiotics (Abecia *et al*., 2007; Zou *et al*., 2016; Velasco-Galilea *et al*., 2020).

Indeed, as reported by previous studies, the cecal microbiota is closely related to growth and feed efficiency in rabbits (Zeng *et al*., 2015; Drouilhet *et al*., 2016; Fang *et al*., 2020, Velasco-Galilea *et al*., 2021a). Unfortunately, the potential association between host genetics and gut microbiota in such traits of economic interest is relatively unexplored. Unraveling such potential association is highly recommended to know whether host genetics influences the relative abundances of specific taxa related to traits of economic interest, identify host genetic markers involved in this control, and potentially manipulate gut microbiota through selection. In this connection, Li *et al*. (2019) and Wen *et al*. (2021) have identified some heritable microorganisms in the cattle rumen and the chicken gut, respectively, that are in addition associated with feed efficiency. A partial genetic control has been suggested for the pig gut microbiota by low to moderate heritability estimates reported for different microbial taxa and alpha-diversity (Camarinha-Silva *et al*., 2017; Yang *et al*., 2016; Lu *et al*., 2018; Ramayo-Caldas *et al*., 2020). In rabbits, Velasco-Galilea *et al*. (2021b, see Chapter six of the present thesis) reported non-null heritability estimates for a large proportion of microbial traits, and Ye *et al*. (2021) also evidenced variability across breeds for their microbial gut composition.

Once the heritability of microbial taxa is reported, the genuine next step is to identify the genomic regions and candidate genes involved in their variation. Likewise, genome-wide association studies (GWAS) have identified host genetic variants associated with mice (Benson *et al*., 2010; Org *et al*., 2015), humans (Goodrich *et al*., 2014; Blekhman *et al*., 2015; Davenport *et al*., 2015), cattle (Li *et al*., 2019), pigs (Cheng *et al*., 2018; Crespo-Piazuelo *et al*., 2019; Bergamaschi *et al*., 2020), or chickens (Wen *et al*., 2021) gut microbiota.

The present study intends to identify rabbit genomic regions involved in the host genetic control of cecal microbiota at different levels of depth using two approaches: mixed model regression at each SNP position (MIX-GWAS) and BayesC. Although

many GWAS have successfully detected QTL regions associated with microbial traits, these studies are very underpowered due to sample size limitations (Klein, 2007; Hong and Park, 2012). For this reason, we accompanied ours with a simulation assessment of the statistical power of the two different approaches to identify host genomic regions associated with a normally distributed phenotype simulated given the available pedigree and data structure, as well as a sample of the recorded genotypes that was assigned to the base population.

7.3. Methods

7.3.1. Animals

The present study was conducted at the Institute of Agrifood Research and Technology (IRTA) and involved 412 rabbits from a paternal line (the Caldes line, Gómez *et al*., 2002). Three hundred twenty-four of these were raised in four batches and housed in collective cages, each containing eight kits, in a semi-open-air facility during the first semester of 2014. In addition, eighty-eight kits were raised in another facility under better controlled environmental conditions in spring 2016. These kits were produced in a single batch and housed in collective cages, each containing six kits. All the animals received the same management and were fed with a standard pelleted food supplemented with antibiotics, except 23 kits raised in the second facility that received an antibiotic-free diet. Rabbits were weaned at the age of 32 days. The fattening period lasted five weeks for the animals raised in the first facility and four weeks for those raised in the second facility. All the animals received food free of antibiotics during their last fattening week. The feed was supplied once per day in a feeder with three places, and water was provided *ad libitum*. Kits were classified into two groups according to their size (“big” if their body weight was greater than 700 g or “small” otherwise), and they were randomly assigned to feeding regime *ad libitum* (AL) or restricted (R) to 75% of the AL feed intake after weaning. The amount of feed supplied to the animals under R in each week for each batch was computed as 0.75 times the average feed intake of kits on AL from the same batch during the previous week, plus 10% to account for a feed intake increase as the animal grows. A maximum of two kits belonging to the same litter

was assigned to the same cage to prevent a possible association between the cage and maternal effects.

7.3.2. Collection of cecal samples, microbial DNA extraction and 16rRNA gene sequencing

At slaughter, cecal samples from each animal were collected in a sterile tube. The samples were kept cold in the laboratory at 4ºC and were stored at -80ºC. Details of the processes regarding DNA extraction and amplification, Illumina library preparation, and sequencing can be found at Velasco-Galilea *et al*., 2020. Briefly, 250 mg of each cecal sample were mechanically lysed in a FastPrep-24TM Homogenizer (MP Biomedicals, LLC, Santa Ana, CA, United States) at a speed of 6 m/s for 60 s. Genomic DNA was extracted with kit ZR Soil Microbe DNA MiniPrepTM (ZymoResearch, Freiburg, Germany), and then, its integrity and purity were measured with Nanodrop ND-1000 spectrophotometer equipment (NanoDrop products; Wilmington, DE, United States) following Desjardins and Conklin's protocol (Desjardins and Conklin, 2010). The pair of primers F515Y/R926 (5'-GTGYCAGCMGCCGCGGTAA-3', 5'-CCGYCAATTYMTTTRAGTTT-3') (Parada *et al*., 2016) was used to amplify a fragment of the 16S rRNA gene including the V4-V5 hypervariable regions. For each cecal sample, an initial polymerase chain reaction (PCR) was carried out with 12.5 µl 2x KAPA HiFi HotStart Ready Mix, 5 µl forward primer, 5 µl reverse primer, and 2.5 µl template DNA (5 ng/ µl) under the following conditions: initial denaturation for 3 minutes at 95 ºC, 25 cycles of 30 seconds at 95 ºC, 30 seconds at 55 ºC and 30 seconds at 72 ºC; and final extension for 2 minutes at 72 ºC. Then, sequencing adaptors and 8 nucleotide dual-indexed barcodes of the multiplex Nextera® XT kit (Illumina, Inc., San Diego CA, United States) were added in a second PCR with 25 µl 2x KAPA HiFi HotStart Ready Mix, 5 µl index i7, 5 µl index i5, 10 µl PCR Grade water and 5 µl concentrated amplicons of the first PCR under the following conditions: initial denaturation for 3 minutes at 95 ºC, 8 cycles of 30 seconds at 95 ºC, 30 seconds at 55 ºC and 30 seconds at 72 ºC; and final extension for 5 minutes at 72 ºC. The libraries obtained were cleaned up with AMPure XP beads. The final libraries were validated by running 1 µl of a 1:50 dilution on a Bioanalyzer DNA 1000 chip (Agilent Technologies, Inc., Santa

Clara, CA, United States) to verify their size, quantified by fluorometry with the PicoGreen dsDNA quantification kit (Invitrogen, Life Technologies, Carlsbad, CA, United States). Finally, libraries were pooled at equimolar concentrations and paired-end sequenced in five parallel plates in an Illumina MiSeq 2 x 250 platform at the Genomics and Bioinformatics Service of the Autonomous University of Barcelona.

7.3.3. Bioinformatics processing of microbial traits

Bioinformatics processing of the raw reads obtained from MiSeq sequencer was performed with the QIIME software (version 1.9.0) (Caporaso *et al*., 2010). Details of the pipeline followed for sequence processing can be found at Velasco-Galilea et al., 2020. Briefly, the python script *multiple_join_paired_ends.py* with default parameters was used to assemble the paired-end reads into contigs. The python script *split_libraries.py* with default parameters was applied to assign the resulting contigs to their samples and to discard those contigs with a quality score smaller than Q19. The UCHIME algorithm (Edgar *et al*., 2011) was used to detect and remove the chimeric sequences associated with PCR amplification. The python script *pick_open_reference_otus.py* with default parameters (Rideout *et al*., 2014) and the Greengenes reference database (version gg_13_5_otus) (McDonald *et al*., 2012) were used to cluster the filtered contigs into Operational Taxonomic Units (OTUs) with a 97% similarity threshold. The resulting OTU table was normalized with the cumulative sum scaling (CSS) method (Paulson *et al*., 2013). The taxonomic assignment of the representative sequences of each OTU was performed using the UCLUST consensus taxonomy assigner by mapping the sequences against the Greengenes reference database gg_13_5_otus. The raw sequence data were deposited in the sequence read archive of NCBI under the BioProject accession number PRJNA524130. Metadata, OTU table, and corresponding taxonomic assignments can be found at **Additional files 7.1**, **7.2** and **7.3**, respectively. After bioinformatic sequence processing, 951 different OTUs present in at least 5% of the animals were identified. Further details on the bacterial and archaeal populations present within the cecum of this rabbit population can be found at Velasco-Galilea *et al*. (2020). On the basis of these 951 OTUs, a set of microbial

traits representative of the rabbit cecal microbiota were defined and analyzed in this study: the relative abundances of 8 phyla and 29 genera, 951 CSS-normalized OTUs, four microbial alpha-diversity indexes computed at 10,000 contigs (total number of OTUs observed, Chao1, Shannon and Simpson's inverse), and the first five principal components (PC) computed from the OTU table. Phyla and genera relative abundances, microbial alpha-diversity indexes, and PCs were standardized subtracting their mean and dividing by their standard deviation. Finally, these standardized microbial traits and CSS-normalized OTUs were multiplied by 100 and subsequently rounded to the nearest integer.

7.3.4. Collection of liver samples, host DNA extraction and SNP genotyping

Rabbit genomic DNA was extracted from liver samples collected at slaughter with the kit MN Nucleospin Tissue (Macherey-Nagel, Germany). Afterward, the DNA integrity and purity were measured with Nanodrop ND-1000 spectrophotometer equipment (NanoDrop products; Wilmington, DE, United States) following Desjardins and Conklin's protocol (Desjardins and Conklin, 2010). Four hundred twelve rabbits were genotyped with the Affymetrix Axiom OrcunSNP Array (Affymetrix, Inc. Santa Clara, CA, USA), which includes 199,692 SNPs. Of these variants, 161,830 were segregating in the rabbit population involved in the present study (Sánchez *et al*., 2020). Quality control of the SNPs was performed with the PLINK software (version 1.9) (Chang *et al*., 2015) and included the following criteria: (i) individual call rate > 0.90; (ii) SNP call rate > 0.95; (iii) SNP minor allele frequency (MAF) > 0.05; (iv) and only autosomal SNPs with known positions in the OryCun2.0 assembly (Carneiro *et al*., 2014) were used. The final dataset consisted of 114,604 genotyped SNPs from 412 rabbits.

7.3.5. Statistical analyses

7.3.5.1. Simulation assessment of the statistical power of the data structure available

As an initial step, a simulation study using the SBVB program (Pérez-Enciso *et al*., 2017) was conducted to assess the statistical power of the real data structure employed in this study. A set of phased genotypes for the animals in the base population of the pedigree animals for which we aim to simulate the genotypes is requested as input by SBVB. Such genotypes of the base population are dropped down throughout the pedigree generating genotype information for all the descendants. In addition, the program requests a list containing the positions and effects of the QTNs responsible for the variation of the trait to be simulated. Finally, a heritability value must be provided to scale the actual SNPs effects so that the normally distributed resulting trait has the desired heritability. In our pedigree, 82 animals constituted the base population. The phased genotypes for these animals were sampled from the 412 real genotypes analyzed for this study. Phasing was done using the Phasebook package (Druet and Georges, 2010), and only considering the first 14 chromosomes to simplify the calculations in the simulation assessment. The same set of 20 SNPs positions and effects were assumed across 50 replicates. The positions and respective effects of these QTNs are shown in (**Table 7.1**). As output, the program generates the genotypes and breeding values for all the animals in the pedigree, and a vector of phenotypes compatible with the initially desired heritability. In a second step of the simulation, the generated breeding values of the 412 individuals with actual microbial information were retained and standardized to have a variance of 1 ($\mathbf{a_0}$). These values were used for the generation of the phenotypic records using the following model:

$$y_{ijkhl} = B_k + S_h + l_l + c_j + a_i + e_{ijkhl}$$

Where y_{ijkhl} corresponds to the phenotype of the i-th individual, born in the l-th litter, and raised in the j-th cage, during the k-th batch (5 levels), and belonging to the h-th class of size at weaning (2 levels: above or below the average). Thus, the phenotypes are explained by the sum of the respective levels of the fixed factors

associated with batch ($\mathbf{B}$) and size at weaning ($\mathbf{S}$), and those of the random factors associated with litter ($\mathbf{l}$), cage ($\mathbf{c}$) and additive genetic ($\mathbf{a}$) effects. During the data generation, the fixed effect vectors were arbitrarily set to $\mathbf{B}' = [4.5 \quad 4.5 \quad 5.5 \quad 3.5 \quad 4.0]$ and $\mathbf{S}' = [0.0 \quad 1.0]$. The litter and cage effects were sampled from the following normal distributions:

$$\mathbf{l} \sim \mathrm{MVN}\left(\mathbf{0}, \mathbf{I}\sigma_{\mathrm{l}}^2\right)$$

$$\mathbf{c} \sim \mathrm{MVN}(\mathbf{0}, \mathbf{I}\sigma_{\mathrm{c}}^2)$$

Similarly, the residual terms were sampled from the following normal distribution:

$$\mathbf{e} \sim \mathrm{MVN}(\mathbf{0}, \mathbf{I}\sigma_{\mathrm{e}}^2)$$

As previously stated, the vector containing the additive genetic effects ($\mathbf{a}$) will be a function of the vector ($\mathbf{a_0}$) previously generated with SBVB program.

The same phenotypic variance was assumed for the three heritability scenarios assessed:

$$\sigma_{\mathrm{P}}^2 = \sigma_{\mathrm{l}}^2 + \sigma_{\mathrm{c}}^2 + \sigma_{\mathrm{a}}^2 + \sigma_{\mathrm{e}}^2 = 10$$

The magnitude of the litter and cage effects was also assumed to be always the same. Thus, the ratios of their variances over the phenotypic variance were 0.10 and 0.15 for the litter and the cage effects, respectively:

$$\mathrm{l}^2 = \frac{\sigma_{\mathrm{l}}^2}{\sigma_{\mathrm{P}}^2} = 0.10$$

$$\mathrm{c}^2 = \frac{\sigma_{\mathrm{c}}^2}{\sigma_{\mathrm{P}}^2} = 0.15$$

The three scenarios under study were characterized by heritabilities equal to 0.10, 0.30 and 0.50. Therefore, $\sqrt[2]{1}$, $\sqrt[2]{3}$ and $\sqrt[2]{5}$ factors were applied to the vector containing the standardized breeding values ($\mathbf{a_0}$) to obtain the breeding value that will be used for data generation in each scenario (i.e., heritabilities of 0.10, 0.30 and 0.50). For the data generation process, the same structure as that of the real data was considered regarding the design of the pedigree, and fixed and random factors. The output of each simulation was a vector containing the phenotypes, the design matrices (that were constant across replicates), and the genotype matrix. Note that the 20 SNPs declared to be responsible for the genetic variation of the simulated trait were removed from the genotype matrix.

Fifty replicates were conducted for each of the three scenarios. For each replicate, the two methods considered for the analysis of the real data (MIX-GWAS and BayesC, see below) were used to detect the simulated QTL regions. The same fixed effects that were used for the simulation were considered in both models, however, litter and cage effects were only considered in the analysis using the BayesC approach. These two factors were not considered in the analysis with the MIX-GWAS model, but a polygenic effect was considered for this approach. In each approach, the SNPs effects were considered in different ways: fitting a mixed model regression at each SNP position in the MIX-GWAS and fitting all the SNPs at a time in the BayesC (see below for further details).

As the positions and effects of the SNPs were constant across all the replicates of each scenario, the number of times that 1 Mb window containing a QTN is declared as a QTL region out of the total number of replicates can be counted. Similarly, the number of times that QTLs are declared within 1 Mb window that does not contain the actual SNPs responsible for the genetic variation of the trait (i.e., false positive) can also be counted. For the BayesC analysis, a given 1 Mb window was declared to be a QTL region when its window posterior probability of association (WPPA) was greater than 0.90. In the case of MIX-GWAS analysis, a 1 Mb window was considered to be a QTL region when at least one SNP encompassed by this window was declared to be associated with the trait after a genome-wide adjustment of the *P*-value to a false discovery rate (FDR) of 0.05.

7.3.5.2. Real data analysis

Two alternative statistical methods were used in order to identity host genomic regions associated with the microbial traits under study.

7.3.5.2.1. Mixed model regression at each SNP position (MIX-GWAS)

This procedure relies on the consideration of a mixed linear model at each position of the genome to be tested. The software GCTA (Yang *et al*., 2011), which allows fitting this type of model, was used. For each tested position (p), the model was the following:

$$y_{ikht} = B_k + S_h + x_{pi}a_p + u_i + e_{ikht}$$

Where y_{ikhj} corresponds to the t-th microbial phenotype, recorded on the i-th animal, with genotype vector $\mathbf{x_i}$, raised in the k-th batch and belonging to the h-th class of size at weaning (below or above the average weaning weight of the batch). B_k and S_h correspond to the effects of the k-th batch (5 levels) and the h-th class of size at weaning (2 levels). u_i is the random additive genetic effect of the i-th individual. The assumed distribution of the vector of additive genetic effects was the following multivariate normal distribution: $\mathbf{u} \sim \mathrm{MVN}(\mathbf{0}, \mathbf{G}\sigma_u^2)$, where σ_u^2 is the additive genetic variance and $\mathbf{G}$ is the genomic relationship matrix calculated using the filtered autosomal SNPs based on the methodology of Yang *et al*. (2011). The SNP effect at the p-th genomic position was fitted as the linear regression (a_p) of the trait of interest on the allele count in that particular position (x_{pi}) coded as 0, 1 or 2.

The statistical significance of the regression coefficient on the allele count in a given position is assessed with GCTA using a chi-squared test, assuming that the ratio $\frac{\widehat{a_p}}{\widehat{\sigma_{a_p}}}$ (i.e., the estimated effect of the SNP over its standard error) follows under the null hypothesis a chi-squared distribution with one degree of freedom. Afterward, the raw *P*-values associated with the different chi-squared tests were genome-wide adjusted to a FDR of 0.05.

7.3.5.2.2. BayesC for genome signal detection

This procedure fits all the SNP genotypes at a time, and it was originally proposed as a genomic selection tool (Habier *et al*., 2011). As a consequence of the assumed prior distribution for the SNPs effects, it allows for variable selection and has also been used as a method to pinpoint the genomic regions harboring genes involved in the control of the traits of interest (Fernando and Garrick, 2013). An important advantage of these methods is their Bayesian nature, meaning that uncertainty is expressed in probabilistic terms, and further corrections for multiple tests are not needed (Fernando *et al*., 2017).

The BayesC model employed was the following:

$$y_{ijkhlt} = B_k + S_h + l_l + c_j + \sum_{p=1}^{N_{SNPs}} x_{pi} a_p + e_{ijkhlt}$$

All the terms of this model equation have been previously described either in the model equation used for the simulation or in the model equation describing the MIX-GWAS analysis approach. Nonetheless, as for BayesC a full Bayesian approach is adopted, it is needed to specify the prior assumptions for each term. In this regard, uniform priors were considered for the systematic effects of batch (B_k) and size at weaning (S_h). For litter (l_l), cage (c_j) and residual terms (e_{ijkhlt}), multivariate normal distributions were considered:

$$\mathbf{l} \sim \text{MVN}\left(\mathbf{0}, \mathbf{I}\sigma_l^2\right), \mathbf{c} \sim \text{MVN}(\mathbf{0}, \mathbf{I}\sigma_c^2), \text{ and } \mathbf{e} \sim \text{MVN}(\mathbf{0}, \mathbf{I}\sigma_e^2)$$

The assumed prior distribution for the SNPs effects (a_p) was a mixture with the following specifications:

$$a_p|\pi, \sigma_p^2 = \begin{Bmatrix} 0 \text{ , with probability } \pi \\ \sim N\left(0, \sigma_p^2\right), \text{with probability } (1-\pi) \end{Bmatrix}$$

In a second hierarchical level, the prior distribution for the variance hyperparameters must be specified. In our implementation, they were assumed to follow unbounded uniform distributions along the positive values. The mixture parameter π was assumed to be known and equal to 0.01, meaning that only about 1,146 SNPs were allowed to have a non-null effect. π has been estimated in other implementations, yielding the method known as BayesCπ.

In the present study, this model was implemented with the program GS3 (Legarra *et al*., 2014). Given that the Fortran code of this software is publicly available, it was possible to include the possibility of fitting zero-inflated Poisson (ZIP) records in the code. The Bayesian derivation by Rodrigues-Motta *et al*. (2007) for this type of models was implemented. Under this derivation, the same assumptions about the BayesC model described above are valid for the random variable representing the logarithm of the individual's Poisson distribution parameters. The criterion to declare regions as associated with the microbial traits of interest was a WPPA greater than 0.90 (Fernando *et al*., 2017) in both implementations of the BayesC model, either assuming ZIP or normally distributed records. In this study, the windows were defined by non-overlapping regions of 1 million base pairs. Following the definition by Fernando *et al*. (2017), the WPPA was the proportion of times, along the Gibbs sampling algorithm, that at least one SNP within that particular window was declared to have a non-null effect on the trait of interest.

7.3.6. Gene annotation and functional prediction

Windows containing a significantly associated QTN were annotated ±1 Mb around. This cut-off was set in accordance with the linkage disequilibrium (LD) pattern decay described by Sánchez *et al*. (2020). Gene annotations were retrieved from the Ensembl Genes 104 Database with the BIOMART software (Smedley *et al*., 2015) using the OryCun2.0 reference assembly. Functional classification and pathway analyses of the annotated candidate genes were carried out using ClueGO version 2.5.8 plug-in of Cytoscape (Bindea *et al*., 2009). Orthologous human gene names were retrieved from the Ensembl Genes 104 Database for functional categorization when a rabbit gene name was not assigned to the gene stable id.

7.4. Results

7.4.1. Power of MIX-GWAS and BayesC approaches for detecting causal variants

The statistical power of MIX-GWAS and BayesC approaches for the data structure employed in this study to identify host genomic regions associated with the composition and diversity of cecal microbial communities was evaluated through a simulation. The simulation consisted of 50 replicates for each of the three scenarios of heritability for the simulated microbial trait (i.e., $h^2 = 0.10$, $h^2 = 0.30$, and $h^2 = 0.50$). The same set of 20 SNPs positions and effects was used across the different replicates in the three scenarios. **Table 7.1** shows the positions of these simulated quantitative trait nucleotides (QTNs) and their respective effects.

Table 7.1| Simulated QTNs.

QTN	OCC[1]	Position (Mb)	Window	Effect[2] ($h^2 = 0.10$)	Effect[2] ($h^2 = 0.30$)	Effect[2] ($h^2 = 0.50$)
1	1	14.85	12	-0.06	-0.10	-0.12
2	2	19.17	61	0.27	0.46	0.59
3	2	86.59	123	0.05	0.09	0.12
4	3	49.26	168	-0.21	-0.37	-0.48
5	3	77.63	195	-0.17	-0.29	-0.37
6	4	0.17	225	-0.10	-0.17	-0.22
7	4	24.45	246	0.05	0.09	0.12
8	4	36.91	256	-0.04	-0.07	-0.09
9	5	39.90	339	0.04	0.08	0.10
10	5	42.17	342	0.27	0.47	0.61
11	6	34.13	412	0.07	0.11	0.15
12	8	3.84	499	-0.01	-0.01	-0.01
13	9	14.68	539	-0.27	-0.47	-0.61
14	10	55.62	592	-0.06	-0.10	-0.12
15	13	35.59	715	0.08	0.14	0.18
16	13	75.02	750	0.08	0.14	0.19
17	13	77.15	752	-0.18	-0.31	-0.40
18	14	61.92	843	-0.10	-0.18	-0.23
19	14	77.69	857	-0.08	-0.14	-0.18
20	14	91.62	870	0.17	0.29	0.37

[1]*Oryctolagus cuniculus* chromosome.
[2]QTN effect expressed relative to phenotypic standard deviations of the trait ($\sqrt[2]{10}$).

Table 7.2 shows the statistical power of both approaches under three scenarios of heritability to declare the windows containing the 20 simulated QTNs as significantly

associated with the simulated microbial phenotype. This power of detection is expressed as the percentage of times across 50 replicates that a given 1 Mb window was declared to contain a SNP significantly associated with the simulated trait by a WPPA greater than 0.90 (BayesC) or a genome-wide P_{FDR} lower than 0.05 (MIX-GWAS). QTNs in **Table 7.2** are placed in descending order according to their absolute value of effect expressed relative to the raw phenotypic standard deviations of the trait.

Table 7.2| Statistical power of MIX-GWAS and BayesC to detect simulated QTNs under different scenarios of heritability.

QTN	Window	h^2 = 0.10		h^2 = 0.30		h^2 = 0.50	
		BayesC[1]	MIX-GWAS[2]	BayesC[1]	MIX-GWAS[2]	BayesC[1]	MIX-GWAS[2]
10	342	0.00	0.02	0.08	0.44	0.78	0.94
13	539	0.00	0.02	0.10	0.46	0.80	0.88
2	61	0.00	0.00	0.12	0.42	0.78	0.84
4	168	0.00	0.00	0.00	0.06	0.22	0.44
17	752	0.00	0.00	0.00	0.06	0.06	0.10
20	870	0.00	0.00	0.00	0.08	0.14	0.24
5	195	0.00	0.00	0.00	0.00	0.00	0.10
18	843	0.00	0.00	0.00	0.00	0.02	0.02
6	225	0.00	0.00	0.00	0.02	0.06	0.10
16	750	0.00	0.00	0.00	0.00	0.02	0.02
19	857	0.00	0.00	0.00	0.00	0.00	0.02
15	715	0.00	0.00	0.00	0.00	0.00	0.00
11	412	0.00	0.00	0.00	0.00	0.00	0.02
14	592	0.00	0.00	0.00	0.00	0.00	0.00
1	12	0.00	0.00	0.00	0.00	0.00	0.00
3	123	0.00	0.00	0.00	0.00	0.00	0.00
7	246	0.00	0.00	0.00	0.00	0.00	0.00
9	339	0.00	0.00	0.00	0.00	0.00	0.08
8	256	0.00	0.00	0.00	0.00	0.00	0.00
12	499	0.00	0.00	0.00	0.00	0.00	0.00

[1]BayesC threshold for declaring a QTN within a given 1 Mb window was WPPA >0.90.
[2]MIX-GWAS threshold for declaring a QTN within a given 1 Mb window was $P_{FDR} < 0.05$.

The simulation study revealed that the MIX-GWAS approach has a greater power of detection to declare a QTL region than BayesC. Though perhaps not surprising given the limited sample size and data structure, power of detection greater than

75% was only achieved when h^2 was 0.50, and only for those windows containing a QTN with an effect greater than 0.59 phenotypic standard deviations (**Table 7.2**). For these three windows containing the QTNs with the strongest effect (10, 13, and 2), the MIX-GWAS returned strongly up-biased estimates (up to 50%) of the most strongly associated SNPs within these windows.

Table 7.3 shows a comparison of detection statistics for 20 windows containing a QTN of a total of 894 windows between MIX-GWAS and BayesC approaches under three simulated scenarios of heritability. A false positive (FP) is an outcome where the method incorrectly declares a window as containing a QTN, while a false negative (FN) is an outcome where the method does not declare as significantly associated with the phenotype a window that actually contains a true QTN. In this regard, the MIX-GWAS approach overcame BayesC since it exhibited lower values of FNs across all three scenarios. A decrease in the number of FNs was observed for both methods as the heritability of the phenotype increased. On the other hand, the BayesC approach had a lower number of FPs values than MIX-GWAS. It is, however, noteworthy that an important percentage of FPs was declared for windows adjacent to another window that contained a QTN.

A true positive (TP) is an outcome where the approach correctly detects a window that contains a QTN. Similarly, a true negative (TN) is an outcome where the method does not declare that a given window contains a QTN when it does not contain a real signal. In accordance with the statistical power of both methods to detect simulated QTNs under different scenarios of heritability (**Table 7.2**), greater number of TPs were found for both methods as the heritability increased. Although the MIX-GWAS approach overcame BayesC at detecting windows containing a QTN across the three scenarios, these values were pretty low. Regarding the number of TNs, both methods exhibited high values, although those for the BayesC were slightly better.

Table 7.3| False positive, false negative, true positive, and true negative values of MIX-GWAS and BayesC to detect simulated QTNs under different scenarios of heritability.

	False positives (FP)				False negatives (FN)		True positives (TP)		True negatives (TN)	
	BayesC		MIX-GWAS							
	Adjacents	Non-adjacents	Adjacents	Non-adjacents	BayesC	MIX-GWAS	BayesC	MIX-GWAS	BayesC	MIX-GWAS
$h^2 = 0.10$										
Nº windows	0	0	1	0	20	18	0	2	874	873
Total windows	874	874	874	874	20	20	20	20	874	874
Percentage	0.00%	0.00%	0.11%	0.00%	100.00%	90.00%	0.00%	10.00%	100.00%	99.89%
$h^2 = 0.30$										
Nº windows	1	0	10	12	17	13	3	7	873	852
Total windows	874	874	874	874	20	20	20	20	874	874
Percentage	0.11%	0.00%	1.14%	1.37%	85.00%	65.00%	15.00%	35.00%	99.89%	97.48%
$h^2 = 0.50$										
Nº windows	4	1	17	33	11	7	9	13	9	13
Total windows	874	874	874	874	20	20	20	20	874	874
Percentage	0.46%	0.11%	1.95%	3.78%	55.00%	35.00%	45.00%	65.00%	99.42%	94.28%

These detection statistics are accompanied by the sensibility, specificity, positive predictive value (PPV), and negative predictive value (NPV) of both methods (**Table 7.4**). In this case, the sensitivity (i.e., true positive rate) would reflect the ability to correctly identify those windows containing a QTN of each method, whereas the specificity (i.e., true negative rate) corresponds to the capacity of the method to do not declare a window as containing a QTN when any real signal is present within this window. Both approaches showed high specificity rates, although the BayesC approach slightly overcame MIX-GWAS. Nevertheless, low sensitivity rates were reported for both approaches, especially for BayesC.

Table 7.4| Sensibility, specificity, and positive and negative predictive values of MIX-GWAS and BayesC to detect simulated QTNs under different scenarios of heritability.

	$h^2 = 0.10$		$h^2 = 0.30$		$h^2 = 0.50$	
	BayesC	**MIX-GWAS**	**BayesC**	**MIX-GWAS**	**BayesC**	**MIX-GWAS**
Sensitivity[1]	0.00%	10.00%	15.00%	35.00%	45.00%	65.00%
Specifity[2]	100.00%	99.89%	99.89%	97.48%	99.43%	94.28%
PPV[3]	-	66.67%	75.00%	24.14%	64.29%	20.63%
NPV[4]	97.76%	97.98%	98.09%	98.50%	98.75%	99.16%

[1]Sensitivity = TP / (TP + FN)
[2]Specifity = TN / (TN + FP)
[3]Positive predictive value (PPV) = TP / (TP + FP)
[4]Negative predictive value (NPV) = TN (TN + FN)

The PPV reflects the probability that a window with a positive test truly contains a QTN (i.e., the proportion of true positive cases among all the positive cases declared by the test). Similarly, the NPV captures the probability that a window with a negative test truly does not contain any QTN (i.e., the proportion of true negative cases among all the negative cases declared by the test). Both approaches declared accurately (NPV rates > 97%) that a window truly did not contain any SNP. However, windows declared by the BayesC to contain a SNP significantly associated with the phenotype were really true positives in a percentage three times greater than the MIX-GWAS approach.

7.4.2. Host genomic regions involved in the control of composition and diversity of cecal microbial communities

In the light of the results obtained in the simulation study, the sample size and data structure available to conduct microbial GWAS (mGWAS) with the real phenotypes would only allow us to detect some QTNs with an effect of at least 0.60 phenotypic standard deviations. Indeed, estimates of such effects must be interpreted with caution since an overestimation of them is expected with the MIX-GWAS approach. Without forgetting the reality of this latter, we proceed to detail the significant associations found for the microbial traits analyzed and discuss the potential biological bases underneath these signals.

No significant association was returned by the BayesC (WPPA > 0.90) approach for any of the microbial traits analyzed since the maximum WPPA estimated value was 0.70 in a window located on *Oryctolagus cuniculus* chromosome (OCC) 19 for an OTU taxonomically assigned to genus *Phascolarctobacterium* with the analysis that assumed a normal distribution of the phenotypic records. Nor was a clear association declared when the analysis based on BayesC assumed a ZIP distribution for the phenotypic records. In this case, the maximum WPPA was 0.67, and it was associated with a window located on OCC 12 for an OTU taxonomically assigned to order *Bacteroidales*.

Despite the negative results obtained with the BayesC approach, and although the statistical power of our data seems rather limited, the MIX-GWAS approach declared 334 SNPs (**Table 7.S1**) located on 10 OCCs as significantly associated with 19 microbial traits. **Table 7.5** summarizes the windows significantly associated with the traits of interest after multiple testing correction at the genome-wide level. Graphical representation of the results obtained is presented in Manhattan plots for the 19 microbial traits (**Figure 7.S1**).

Table 7.5| Windows containing SNPs significantly associated with the microbial traits analyzed with the MIX-GWAS approach.

Trait	Window	OCC[1]	Position (Mb; start-end)	SNPs in the window	Significant SNPs in the window[2]	SNP name[3]	MAF[4]	Effect[5]	P_{FDR}[6]
124470	172	1	180.09-181.09	77	1	AX-147160810	0.06	-0.29	2.15E-02
124470	1147	12	7.49-8.48	55	18	AX-147128657	0.11	-0.27	2.89E-04
124470	1148	12	8.49-9.09	49	19	AX-147107505	0.11	-0.27	2.89E-04
124470	1619	15	49.45-50.45	70	1	AX-147088033	0.16	-0.20	2.91E-02
124470	1626	15	56.51-57.51	61	1	AX-147112983	0.06	-0.28	3.65E-02
157802	478	3	133.96-134.95	60	2	AX-147139981	0.08	0.68	2.16E-02
314029	116	1	121.58-122.57	70	1	AX-147148999	0.19	0.36	4.24E-02
314029	117	1	122.59-123.57	70	11	AX-147066191	0.19	0.38	4.24E-02
346794	906	8	108.91-109.89	71	5	AX-147062936	0.36	-0.38	1.63E-02
346794	907	8	109.91-110.89	75	2	AX-147177630	0.42	0.38	1.51E-02
524842	185	1	193.23-194.21	52	9	AX-147110928	0.18	0.45	2.41E-02
578960	639	6	25.49-26.49	96	3	AX-147150052	0.13	0.52	2.57E-02
578960	1034	10	22.26-23.24	65	2	AX-147171352	0.14	0.49	4.48E-02
NR1121	1944	19	52.01-53.00	62	14	AX-147086137	0.42	0.35	2.64E-02
NR1121	1945	19	53.02-54.00	75	4	AX-147109903	0.41	0.34	2.64E-02
NR1391	156	1	162.97-163.97	57	9	AX-147091835	0.14	0.55	4.84E-02
NR1794	99	1	103.53-104.52	56	2	AX-147089616	0.23	0.46	2.46E-03
NR1794	100	1	104.59-105.59	71	9	AX-147110873	0.22	0.46	2.46E-03
NR1794	105	1	109.63-110.3	74	11	AX-147050877	0.07	0.64	1.76E-02
NR1794	108	1	112.67-113.66	60	1	AX-147099894	0.27	0.39	7.91E-03
Actinobacteria	109	1	113.69-114.69	49	20	AX-147140420	0.06	0.81	2.29E-03

NR1794	110	1	114.73-115.72	50	25	AX-146986692	0.07	0.79	2.29E-03
NR1794	111	1	115.74-116.72	79	8	AX-147117376	0.07	0.78	2.29E-03
NR1794	114	1	119.42-120.31	46	6	AX-147017556	0.07	0.72	4.66E-03
NR2147	569	4	75.58-76.57	74	4	AX-147067709	0.06	-0.79	4.21E-02
NR2269	388	3	38.53-39.53	83	1	AX-147145011	0.43	0.35	1.51E-02
NR2269	392	3	42.56-43.51	61	13	AX-147106496	0.15	0.49	1.34E-02
NR2269	393	3	43.57-44.56	68	30	AX-147092933	0.15	0.50	1.34E-02
NR2269	394	3	44.57-45.56	64	8	AX-147145803	0.14	0.50	1.34E-02
NR2723	370	3	20.29-21.26	37	2	AX-147029851	0.07	0.52	3.50E-02
NR2745	364	3	14.20-15.19	48	11	AX-147079557	0.35	-0.37	3.02E-02
NR276	483	3	139.00-139.99	73	3	AX-147170597	0.30	0.32	3.93E-02
NR276	484	3	140.01-141.0	78	33	AX-147034740	0.25	0.35	3.93E-02
NR276	485	3	141.01-141.94	47	15	AX-147167299	0.24	0.37	3.93E-02
NR276	1725	16	55.22-56.22	54	1	AX-147096626	0.29	0.33	4.31E-02
NR276	1740	16	70.64-71.64	74	2	AX-147054373	0.08	0.54	3.93E-02
NR276	1744	16	74.76-75.75	60	12	AX-147022482	0.28	0.37	3.93E-02
NR276	1745	16	75.76-76.75	52	7	AX-147104300	0.32	0.34	3.93E-02
NR3356	907	8	109.91-110.89	75	4	AX-147161100	0.23	0.40	3.54E-02
NR4269	1264	12	134.41-135.41	52	2	AX-147085506	0.10	0.41	2.72E-02
NR741	1266	12	136.86-137.84	42	1	AX-147043571	0.16	0.44	7.06E-03
Actinobacteria	639	6	25.49-26.49	96	4	AX-147166320	0.13	0.53	3.12E-02
PC2	639	6	25.49-26.49	96	3	AX-147150052	0.13	-0.34	4.36E-02

[1]*Oryctolagus cuniculus* chromosome.
[2]Genome-wide $P_{FDR} < 0.05$.
[3]Name of the most significant SNP within the window.
[4]Minor allele frequency of the most significant SNP.
[5]Effect of the most significant SNP expressed in phenotypic standard deviations.
[6]P_{FDR} value of the most significant SNP within the window.

One window (639: 25.49-26.49 Mb) located on OCC 6 was declared to contain 4 and 3 SNPs significantly associated with the relative abundance of phylum *Actinobacteria* and PC2, respectively. The estimated effects, expressed as raw phenotypic standard deviations, of the SNPs with the strongest association within this window were 0.53 and -0.34 for the relative abundance of phylum *Actinobacteria* and PC2, respectively (**Table 7.5**).

The remaining 17 microbial traits, for which the MIX-GWAS approach declared some SNP significantly associated with, correspond to CSS-normalized OTUs. Fifteen of them were taxonomically assigned to phylum *Firmicutes* and the remaining two to phylum *Bacteroidetes* (**Table 7.5**).

Within the phylum *Firmicutes*, two OTUs belong to genus *Ruminococcus*, two to family *Ruminococcaceae*, three to family *Lachnospiraceae*, one to family *Clostridiaceae*, and the remaining seven to order *Clostridiales*. OTUs NR2269 and NR2745, belonging to genus *Ruminococcus*, showed significant associations with 52 and 11 SNPs encompassed by 4 and 1 windows, respectively, located on OCC 3 (**Table 7.S1**). The estimated effects of the SNPs with the strongest association within each window ranged from 0.35 (for a SNP on the window 388: 38.53-39.53 Mb) to 0.50 (for a SNP on the window 394: 44.57-45.56 Mb) raw phenotypic standard deviations (**Table 7.5**).

One window located on OCC 8 contained 4 SNPs significantly associated with one OTU taxonomically assigned to family *Ruminococcaceae* (NR3356). Within this window, AX-147161100, the SNP with the strongest association had an estimated effect of 0.40 phenotypic standard deviations. Another OTU also assigned to this family (NR1121) showed significant associations with 14 and 4 SNPs encompassed by two adjacent windows of OCC 19. The estimated effect of the SNP showing the strongest association within these windows was 0.35 phenotypic standard deviations (**Table 7.5**). Sixteen SNPs located in four windows (639: 2 SNPs, 1034: 2 SNPs, 156: 9 SNPs, and 1264: 2 SNPs) were declared to be significantly associated with any of the three OTUs taxonomically assigned to family *Lachnospiraceae* (578960, NR1391, and NR4269). As noted above, window 639

(OCC 6) also contained SNPs significantly associated with the relative abundance of phylum *Actinobacteria* and PC2. The estimated effects of the SNPs with the strongest association within each window ranged from 0.41 (for a SNP on the window 1264: 134.41-135.41 Mb) to 0.55 (for a SNP on the window 156: 162.97-163.97 Mb) raw phenotypic standard deviations (**Table 7.5**). One window located on OCC 1 contained 9 SNPs significantly associated with one OTU taxonomically assigned to family *Clostridiaceae* (524842). Within this window, AX-147110928, the SNP with the strongest association had an estimated effect of 0.45 phenotypic standard deviations (**Table 7.5**).

One hundred eighty-one SNPs located in 22 windows located on OCCs 1, 3, 4, 8, 12 and 16, were declared as significantly associated with any of the seven OTUs taxonomically assigned to order *Clostridiales* (157802, 314029, 346794, NR1794, NR2147, NR276 and NR741). AX-147140420 was the SNP (OCC 1, window 109: 113.69-114.69 Mb) most strongly associated with NR1794, and its estimated effect was 0.81 phenotypic standard deviations (**Table 7.5**). The SNP most strongly associated with OTU 314029 had an estimated effect of 0.38 phenotypic standard deviations. This SNP is encompassed by window 117 located on OCC 1. On OCC 3, four windows (478, 483, 484, and 485) encompassed SNPs significantly associated with OTUs 157802 and NR276. The estimated effects of the SNPs with the strongest association within each window ranged from 0.32 phenotypic standard deviations (for a SNP on 139282325 bp significantly associated with OTU NR276) to 0.68 phenotypic standard deviations (for a SNP on 133998414 bp significantly associated with OTU 157802) (**Table 7.5; Table 7.S1**). The OTU NR276 was also significantly associated with 22 SNPs encompassed by four windows located on OCC 16. Within this OCC, the estimated SNP effect having the strongest association with OTU NR276 was 0.54 phenotypic standard deviations (**Table 7.5**). 346794 showed significant associations with 5 and 2 SNPs encompassed by two adjacent windows of OCC 8. One of these windows (907) also contained SNPs significantly associated with OTU NR3356 (assigned to family *Ruminococcaceae*) (**Table 7.5**). One window (569: 75.58-76.57 Mb) located on OCC 4 was declared to contain 4 SNPs significantly associated with OTU NR2147 (**Table 7.5**). The estimated effect of the SNP showing the strongest association within this window

was -0.79 phenotypic standard deviations. A single SNP in 137077726 bp on OCC 12 was significantly associated with OTU NR741, and it showed an estimated effect of 0.44 phenotypic standard deviations (**Table 7.5; Table 7.S1**).

Within the phylum *Bacteroidetes*, two SNPs encompassed by window 370 located on OCC 3 were declared to be significantly associated with OTU NR2723 that belongs to order *Bacteroidales*. The estimated SNP having the strongest association was 0.52 phenotypic standard deviations (**Table 7.5**). Finally, five windows located on OCCs 1, 12 and 15, contained 40 SNPs declared to be associated with an OTU taxonomically assigned to genus *Butyricimonas* (124470). The estimated effects of the SNPs with the strongest association within each window ranged from -0.20 (for a SNP on the window 1619 of OCC 15: 49.45-50.45 Mb) to -0.29 (for a SNP on the window 172 of OCC 1: 180.09-181.09 Mb) phenotypic standard deviations (**Table 7.5**).

7.4.3. Candidate genes and pathways associated with rabbit cecal microbiota

A total of 426 protein-coding, 32 snRNA, 20 snoRNA, 3 miRNAs, 1 miscRNA, and 1 vault RNA were annotated ± 1 Mb around the windows that MIX-GWAS declared to contain SNPs significantly associated with any of 19 microbial traits at the genome-wide level (**Table 7.S2**). After a detailed exploration of the annotated genes functions, 44 candidate genes located on 6 OCCs were proposed to explain the phenotypic variation of 11 microbial traits (**Table 7.6**).

On OCC 1, genes related to the pyruvate metabolism (*DLAT*), collagen degradation (*MMP1*, *MMP3*, *MMP7*, *MMP8*, *MMP10*, *MMP12*, *MMP13*, and *MMP20*), and the immune system (*IL18*, *BIRC2*, *BIRC3*, and *RACK1*) were annotated within the chromosomal interval 103.53-120.31 Mb (windows 99-114, **Table 7.6**). MIX-GWAS declared that 62 variants within this interval are significantly associated with an OTU belonging to order *Clostridiales* (NR1794, **Table 7.5**).

Table 7.6| Candidate genes for windows containing SNPs significantly associated with the microbial traits.

Window	OCC[1]	Position (Mb; start-end)	Trait	Gene
99-100	1	103.53-105.59	NR1794	*DLAT*, *IL18*,
108-111	1	112.67-116.72	NR1794	*BIRC2*, *BIRC3*, *MMP1*, *MMP3*, *MMP7*, *MMP8*, *MMP10*, *MMP12*, *MMP13*, *MMP20*
114	1	119.42-120.31	NR1794	*RACK1*
185	1	193.23-194.21	524842	*FOLS1*, *MS4A2*
364	3	14.20-15.19	NR2745	*ACSL6*, *CSF2*, *IL4*, *IL5*, *IL13*
392-394	3	42.56-45.56	NR2269	*GABRA1*, *GABRA6*, *GABRB2*, *GABRG2*
639	6	25.49-26.49	578960, PC2, *Actinobacteria*	*CCL24*, *MDH2*, *SLC12A9*, *GPC2*, *GNB2*, *HSPB1*, *SIK1*, *SIK1B*, *EPO*, *POR*
907	8	109.91-110.89	346794, NR3356	*TNFSF13B*
1619	15	49.45-50.45	124470	*ADH4*, *ADH5*, *ADH6*
1944-1945	19	52.01-54.00	NR1121	*ABCA5*, *ABCA6*, *ABCA9*, *KCNJ2*, *KCNJ16*, *MAP2K6*

[1]*Oryctolagus cuniculus* chromosome.

Nine variants within the chromosomal interval 193.23-194.21 Mb (window 185), also located on OCC 1, were declared to be significantly associated with the phenotypic variation of an OTU belonging to family *Clostridiaceae* (524842, **Table 7.5**). Genes related to the immune system were annotated within this interval (*FOLS1* and *MS4A2*, **Table 7.6**).

On OCC 3, genes related to fatty acid degradation (*CSF2*) and the immune system (*CSF2*, *IL4*, *IL5,* and *IL13*) were annotated within the chromosomal interval 193.23-194.21 Mb (window 364, **Table 7.6**). MIX-GWAS declared that this chromosomal interval contains eleven variants significantly associated with an OTU belonging to genus *Ruminococcus* (NR2745, **Table 7.5**). Fifty-one variants located on OCC 3 (42.56-45.56 Mb, windows 392-394) were declared to be significantly associated with the phenotypic variation of another OTU also belonging to genus *Ruminococcus* (NR2269, **Table 7.5**). Genes annotated within this interval are related to the activation of gamma aminobutyric acid (GABA) receptors (*GABRA1*, *GABRA6*, *GABRB2*, *GABRG2*).

On OCC 6, three variants within the interval 25.49-56.49 Mb (window 639) were significantly associated with the variation of three different microbial traits (PC2, phylum *Actinobacteria*, and OTU 578960 taxonomically assigned to family *Lachnospiraceae*, **Table 7.5**). Genes related to the immune system (*CCL24*), metabolism (*MDH2*, *GPC2*, *SIK1*, *SIK1B*, and *POR*), GABA receptors (*GNB2*), and homeostasis (*SLC12A9* and *EPO*) were annotated within this chromosomal interval (**Table 7.6**).

On OCC 8, within the interval 109.91-110.89 Mb (window 907), four variants were significantly associated with the variation of OTU NR3356 belonging to family *Ruminococcaceae*. Within the same chromosomal interval, two variants were associated with OTU 346794 belonging to order *Clostridiales* (**Table 7.5**). The tumor necrosis factor ligand superfamily member 13B protein coding gene (*TNFSF13B*) related to immune functions was annotated within this chromosomal interval.

On OCC 15, genes related to the pyruvate metabolism (*ADH4*, *ADH5*, and *ADH6*), were annotated within the chromosomal interval 49.45-50.45Mb (window 1619, **Table 7.6**). Within this interval, the MIX-GWAS declared the existence of two variants significantly associated with an OTU belonging to genus *Butyricimonas* (124470, **Table 7.5**).

Finally, on OCC 19, eighteen variants within the interval 52.01-54.00 Mb (windows 1944-1945) were significantly associated with the variation of an OTU taxonomically assigned to family *Ruminococcaceae* (NR1121, **Table 7.5**). Genes related to lipid homeostasis (*ABCA5*, *ABCA6*, and *ABCA9*), activation of GABA receptors (*KCNJ2* and *KCNJ16*), and signaling (*MAP2K6*) were annotated within this chromosomal interval (**Table 7.6**).

7.5. Discussion

The present study provides the first mGWAS conducted in a rabbit population using a highly dense SNP array for a set of microbial traits representative of the cecal microbiota at different levels of depth. In addition, our study is accompanied by a simulation assessment that has allowed us to get an overview of the statistical power of our dataset to identify the positions on the genome and effects of the SNPs associated with the variation of the microbial traits studied.

Recent studies in humans (Goodrich *et al*., 2014; Davenport *et al*., 2015; Rothschild *et al*., 2018), mice (Campbell *et al*., 2012), chickens (Wen *et al*., 2021), or pigs (Cheng *et al*., 2018; Bergamaschi *et al*., 2020; Ramayo-Caldas *et al*., 2020) suggested that the gut microbial composition and diversity is partially heritable. Our recent study in rabbits also suggested that some microbial taxa are under host genetic control (Velasco-Galilea *et al*., 2021b). Despite all these studies point out to low heritability of the overall gut microbiota, the fact that some microbial taxa seem to be under a clear host genetic control has motivated the investigation of microbiome-host genome associations through mGWAS. This kind of analysis has attempted to not only identify heritable taxa but also to find the host genetic variants that underlie such heritability, with the final aim of, for example, conducting a

genomic or marked assisted selection aiming to breed animals with an optimal gut microbial composition regarding the traits of interest.

In this study, we have applied two alternative approaches to identify host genomic regions and propose candidate genes associated with rabbit cecal microbiome: MIX-GWAS and BayesC. The first approach tests each marker for association with the phenotype of interest, whereas the Bayesian GWAS simultaneously fits all markers; being able to account for most of the genetic variance (Fernando and Garrick, 2013). Before applying and comparing both methodologies we challenged ourselves with the following question: how powerful are our data to accurately detect a QTL region containing a SNP responsible for the variation of a microbial trait?

Our simulation assessment has allowed us to test the ability of both approaches to detect a set of simulated QTNs spread across the genome with different effects on a simulated microbial phenotype normally distributed. We found that both methods only declared three of the twenty windows containing a QTN as QTL regions in the maximum heritability scenario. The average power of detection of the MIX-GWAS was slightly better and overcame that of BayesC by 12%. The statistical power to detect associations between SNP variants and a phenotype largely depends on the experimental sample size or the distribution of effect sizes of causal genetic variants that are segregating in the population (Visscher *et al*., 2017). Therefore, the low detection power of both approaches is not surprising given our limited sample size and confirmed that we will only be able to detect strong signals for QTNs responsible for an important part of the variation of our microbial phenotypes. In addition, in the MIX-GWAS case, we confirmed strongly up-biased estimates of the SNPs effects with the simulation performed. The inflated estimates of QTL effect sizes were also expected since this issue has already been reported in the literature (Göring *et al*., 2001). This issue is probably due to the Beavis effect (Beavis, 1994), which is a well-known phenomenon by which overestimated QTL effect sizes tend to reach statistical significance. Its main consequence is that we can expect an upward bias in the estimated effects of the QTNs declared by the MIX-GWAS approach.

But to what extent can we expect that our signals detected will be FPs or real QTNs with inflated effects? Our simulation study has shed some light on the severity of this matter. The MIX-GWAS returned a larger number of FPs than BayesC. However, the Bayesian approach detected a lower number of windows containing a simulated QTN (i.e., this method is associated with a larger number of FNs). Indeed, as indicated above, both methods presented low sensitivity rates since repeatedly declared as QTLs only those windows containing SNPs with relatively large effect sizes. Thus, although a QTL with a small effect is present in our population, it will be rarely detected with our limited sample size.

In summary, for our purpose, both approaches have their own advantages and weaknesses. The MIX-GWAS presents a greater capacity to effectively declare true associations between SNP variants and the trait of interest than BayesC. Nevertheless, the probability that a QTL region declared by the Bayesian approach really contains a SNP variant associated with the phenotype is greater. Therefore, a BayesC signal will be more reliable than one declared by the MIX-GWAS. However, with this latter we expect to catch more real variants but also false signals even if a multiple testing correction at the genome-wide level is applied. Hence further biological analysis of the QTL regions declared by any GWAS approach is fundamental to discriminate FPs from true associations and identify candidate genes associated with the phenotypic variation of the traits of interest.

From this point, we will proceed to discuss the results obtained by the mGWAS conducted on the real genotypes and microbial traits. The Bayesian approaches did not return any significant association. We want to highlight a very important point that has not been mentioned in the discussion, and it is the fact that the litter and cage effects were not adjusted with the MIX-GWAS. Not including such effects implies ignoring the existing data covariance between records belonging to animals of the same litter or cage. Adequately considering such covariance means recognizing that our data will be less informative than other datasets including all animals from different litters and cages. This covariance structure has been properly modeled with BayesC but not with MIX-GWAS, which may have important consequences on reaching statistical significance.

Conversely, the MIX-GWAS revealed more than 300 variants spread across 10 OCCs associated at genome-wide level with 19 microbial traits. Before discussing these genetic variants, we have to highlight that multiple testing correction has been applied accounting for the number of tested SNPs, but not by the number of tested traits. That is not strictly speaking a proper correction since it should have been applied to the number of tested hypotheses (i.e., the number of tested SNPs x the number of tested microbial traits). We should like to add here that we calculated the effective number of independent tests by principal components decomposition of the whole dataset including the centered and scaled microbial traits, and 355 independent traits were suggested to explain the 98% of the whole microbial variation. Thus, the suggestive genome-wide significance was set at 1.41×10^{-4} (0.05/355). After this strict correction, only six variants located on OCC 12 were declared as significantly associated with the variation of OTU 124470 (genus *Butyricimonas*). Velasco-Galilea *et al*. (2021b, see Chapter six of the present thesis) reported substantial evidence of genetic control for this trait. Given the lower frequency of presence of this OTU in the rabbits' cecum (this trait was only detected in 20% of the animals), it was better adjusted with a ZIP model and its heritability estimate was 0.26. Therefore, we hypothesized that a variant in this chromosome could favor the presence of genus *Butyricimonas* in the rabbit cecum. We have, however, deliberately limited the correction at the genome-wide level within the trait to allow greater signal detection. We are aware that an important number of these 300 variants and their estimated effects could be spurious and up-biased. However, we consider it highly relevant to analyze their biological foundations and propose several candidate genes. These candidates may be confirmed in the future if the same variants are consistently reported by other GWAS in different populations.

Given the current state of the art, the diversity and composition of gut microbial communities are predominantly shaped by external factors. The overall genetic determinism is low, except for certain microbial taxa whose variation is associated with different regions spread across the host genome. In this regard, we previously observed substantial evidence of a non-null heritability (Velasco-Galilea *et al*., 2021b, see Chapter six of this thesis) for three traits that the MIX-GWAS has declared to be associated with certain genomic regions. It is particularly relevant to

highlight the above-mentioned OTU 124470, whose heritability estimate was 0.26, which seems to be associated with different variants spread across regions located on OCCs 1, 12, and 15. Similarly, the heritability estimates for PC2 and OTU 346974 (order *Clostridiales*) were 0.20 and 0.23, respectively, and seem to be associated with genomic regions on OCC 6 and OCC 8, respectively. It is, however, equally important to note that no previous evidence of genetic determinism was reported for the remaining 16 microbial traits for which the MIX-GWAS declared to be associated with different genomic regions. We interpret this lack of consistency as a piece of evidence pointing to many of the signals detected by the MIX-GWAS are FPs.

Despite a growing number of studies in humans and livestock having started to conduct mGWAS to pinpoint the host genomic regions that may be involved in the determination of microbial diversity and composition, all of them are very underpowered (Davenport *et al*., 2015). Consequently, most findings do not reach statistical significance after multiple testing correction. Rothschild *et al*. (2018) demonstrated that there is almost no overlap between the QTL regions reported in different studies, even when allowing associations with different microbial taxa to be considered as an overlap. It is, however, worth mentioning that different mGWAS signals reported in this and previous studies are close to host genes involved in immune-related, signaling and metabolic pathways (Benson *et al*., 2010; Goodrich *et al*., 2014; Leamy *et al*., 2014; Blekhman *et al*., 2015; Davenport *et al*., 2015; Org *et al*., 2015; Cheng *et al*., 2018; Crespo-Piazuelo *et al*., 2019; Bergamaschi *et al*., 2020). Genes involved in these pathways deserve a particular focus since microorganisms inhabiting the mammals' gut confers benefits to the host regarding digestion of complex polysaccharides or preventing the growth of pathogens (Flint *et al*., 2012). Similarly, the host immune response could modulate the microbial composition to keep a proper homeostatic balance (Belkaid and Hand, 2014).

As an overall point to address, we can indicate that all mGWAS approaches will require several orders of magnitude larger sample sizes to confidently declare underpin variants. It goes without saying that independent mGWAS in different populations will be crucial to discriminate between FPs and signals which are biologically meaningful.

7.6. Conclusions

The simulation assessment has revealed that the sample size and structure of our dataset are underpowered to confidently identify host genomic regions linked to the variation of rabbit cecal microbiota through mGWAS. Despite such limited statistical power, we have been able to identify some QTL regions spread across ten chromosomes that, of course, prudently, can be declared as associated with the variation of rabbit cecal microbiota. Remarkably, we have proposed genes involved in homeostatic, metabolic, or immune system processes as candidates for the variation of different microbial traits. Our results lay an important foundation for future mGWAS, which will hopefully be conducted with larger sample sizes in other populations, to validate these genes and their underlying biological role.

7.7. List of abbreviations

AL *ad libitum*
CSS cumulative sum scaling
FDR false discovery rate
FN false negative
FP false positive
GIT gastrointestinal tract
GWAS genome-wide association study
LD linkage disequilibrium
MAF minor allele frequency
mGWAS microbial genome-wide association study
NPV negative predictive value
OTU operational taxonomic unit
QTL quantitative trait loci
QTN quantitative trait nucleotide
OCC *Oryctolagus cuniculus* chromosome
PC principal component
PCR polymerase chain reaction
PPV positive predictive value

R	restricted
SNP	single nucleotide polymorphism
TN	true negative
TP	true positive
WPPA	window posterior probability of association
ZIP	zero-inflated Poisson

Declarations

Ethics approval and consent to participate

This study was carried out in accordance with the recommendations of the animal care and use committee of the Institute for Food and Agriculture Research and Technology (IRTA). The protocol was approved by the committee of the Institute for Food and Agriculture Research and Technology (IRTA).

Consent for publication

Not applicable.

Availability of data and materials

The raw sequence data were deposited in the sequence read archive of NCBI under the accession number SRP186982 (BioProject PRJNA524130). Metadata, the filtered and CSS-normalized OTU table and corresponding taxonomic assignments have all been included as **Additional files 7.1**, **7.2** and **7.3**, respectively. A description of the genetic variants declared as significantly associated with the variation of 19 microbial traits by the MIX-GWAS after multiple testing correction at the genome-wide level can be found in **Additional file 7.4: Table 7.S1**. The Manhattan plots for the 19 microbial traits are graphically represented in **Additional file 7.5: Figure 7.S1**. A list containing the genes annotated around the windows that MIX-GWAS declared to contain variants significantly associated with any of 19 microbial traits at the genome-wide level can be found in **Additional file 7.6: Table 7.S2**). The Additional information for this article can be found in the Annexes section.

Competing interests

The authors declare that they have no competing interests.

Funding

The experimental design of this work was conducted thanks to funding from INIA project RTA2011-00064-00-00. This study was part of the Feed-a-Gene project that received funding from the European Union's H2020 program under grant agreement no. 633531, and the Spanish project RTI2018-097610R-I00. MVG is a recipient of a "Formación de Personal Investigador (FPI)" pre-doctoral fellowship from INIA, associated with the research project RTA2014-00015-C2-01. YRC is recipient of a Ramon y Cajal post-doctoral fellowship (RYC2019-027244-I) from the Spanish Ministry of Science and Innovation.

Authors' contributions

JPS and MP conceived the experimental design. MVG, JPS, and MP collected the biological samples. MVG and MP processed the samples in the laboratory. MVG processed and analyzed the sequencing data, interpreted data, prepared figures and tables, and wrote the manuscript. JPS and YRC contributed to the analysis of sequencing data. JPS helped write the manuscript. JPS, BB, YRC, MB, and MP helped interpret the data and revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We would like to thank Oscar Perucho, Josep Ramon and Carmen Requena (staff of Unitat de Cunicultura, IRTA) for animal care and their contribution to data recording. We acknowledge Oriol Rafel, Marc Viñas, Miriam Guivernau and Olga González for their help collecting and processing the biological samples. We also acknowledge Armand Sánchez, Nicolas Boulanger and Joana Ribes (Genomics and NGS Unit, CRAG) for their assistance in massive libraries preparation.

7.8. References

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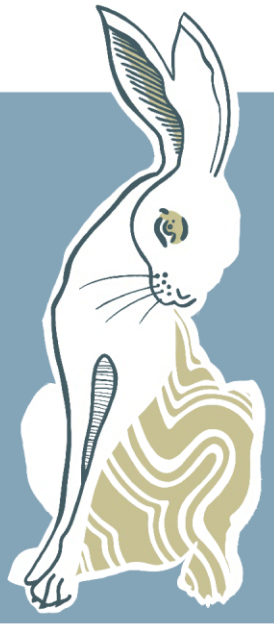
CHAPTER 8

GENERAL DISCUSSION



The steady global population growth experienced in recent decades has resulted in the apparition of a new challenge for the livestock industry: to meet the increased demand for animal protein. This challenge implies searching for efficient methods to produce more food while using fewer inputs and minimizing environmental impact. Feed efficiency (FE) is a crucial phenotype for the meat rabbit industry since food expenses can represent up to 70% of the total costs (Cartuche *et al*., 2014). Therefore, a reduction of feeding costs is key to optimizing FE and reducing the environmental impact. In recent years, large efforts have been made to improve the ratio between the kilograms of feed consumed and the kilograms of weight gain in the current European intensive production systems through farm management, nutrition, and genetics selection (Gidenne *et al*., 2017). Given the difficulties of individually measuring feed intake (FI) of rabbits raised in groups, breeding programs have traditionally improved FE through indirect selection for growth rate or body weight at slaughter (Estany *et al*., 1992), which are highly heritable phenotypes moderately correlated with direct measures of FE that can easily be measured individually.

The identification of new traits related to individual animal variation in FE, together with the understanding of its underlying biological processes, could help in improving this complex phenotype in rabbits. In this respect, the present thesis has aimed at exploring the effect of host genetics and different environmental factors on rabbit gut microbiota as a potential new phenotype affecting growth and FE performances. The main results obtained in the previous chapters will be discussed in this section, highlighting the contribution of gut microbiota to rabbit FE and the potential of this new phenotype to be included in a selection index.

The rabbit gastrointestinal tract (GIT) harbors a complex ecosystem of microorganisms whose members are constantly interacting between them and the immediate environment (Gouet and Fonty, 1979). Thus, the presence of different bacterial and archaeal species is conditioned by these interactions and the physicochemical conditions of the environment that force their adaptation (Whipps *et al*., 1988). The starting point was the characterization of microbial communities present in the cecum and hard feces of the meat rabbit population studied in the

present thesis (Chapter three). The assessment of bacterial and archaeal populations by means of 16S rRNA gene amplicons in an Illumina MiSeq platform revealed that the rabbit cecum, as well as expelled hard feces, are dominated by three bacterial phyla: *Firmicutes* (76%), *Tenericutes* (8%), and *Bacteroidetes* (7%, Velasco-Galilea *et al*., 2018). This finding is in agreement with previous studies that also reported an overwhelming presence of phylum *Firmicutes* in the cecum microbiome of growing meat rabbits (Massip *et al*., 2012; Combes *et al*., 2017; Monteils *et al*., 2008). It is noteworthy that our study, for the first time, reported the presence of phylum *Tenericutes* in the rabbit gut. A plausible explanation for the absence of this phylum in previous research is that it encompasses class *Mollicutes*, which was previously classified within phylum *Firmicutes* (Brown *et al*., 2007). Subsequent studies have also reported these bacteria phyla in different sections of the GIT in rabbits (Cotozzolo *et al*., 2020; Hu *et al*., 2021). Kingdom *Archaea* is also present in cecum and hard feces, but in a very low percentage (0.61‰) and limited to genus *Methanobrevibacter*. The low prevalence of methanogenic archaea can be explained by an acidic pH that hinders their growth (Mi *et al*., 2018).

The most recent research confirms that the cecum, which is the main organ for microbial fermentation, is the richest and most diverse section along the GIT (Cotozzolo *et al*., 2020; Hu *et al*., 2021). No significant differences in microbial richness and diversity were found between the cecum and hard feces in our study. However, univariate and multivariate analytical approaches revealed compositional differences in the relative abundance of an important number of taxa, even at the phylum level, between sampling origins. These differences suggest different requirements for the types of microbial communities that need to be present in each part. For instance, a higher presence of genera *Blautia* or *Akkermansia* in cecal samples is explained by their implication in the degradation of glucose and carbohydrates or immune protection against inflammatory processes, respectively. Whereas an overrepresentation of genera *Oscillospira* and *Coprococcus* in the hard feces would explain their active participation in fermentation at the end of the feed digestion process. Therefore, it is necessary to carefully consider the existence of these differences when deciding the area of the GIT to be sampled according to the objectives and possibilities of each study.

Once the microbial communities inhabiting the meat rabbit cecum were taxonomically identified, the next step was to study the influence of environmental and host-associated factors on their composition and diversity. Microbial colonization is considered to begin at birth when the animal passes through the birth canal and enters in contact with the immediate environment (Berg, 1996). From this moment on, a gradual and organized colonization by different species takes place until stability of the ecosystem is reached at 70 days of age in rabbits (Combes *et al*., 2011). Despite this stability reached in adulthood, previous studies provided undeniable evidence of external factors, such as diet, hygiene conditions, or the administration of antimicrobials, shaping the composition and diversity of rabbit cecal microbial communities at different extents (Abecia *et al*., 2007; Zou *et al*., 2016; Zhu *et al*., 2017; Chen *et al*., 2019).

The experimental design of the dataset employed for the research work of the present thesis motivated the study of potential changes in diversity and composition of rabbit cecal microbial communities exerted by the breeding farm, the level of feeding, and the administration of antibiotics (Chapter four). The different approaches applied to evaluate the impact of these factors revealed a large effect of the farm environment offered to the rabbits during their growth on microbial diversity and composition at all taxonomic levels analyzed. Such strong impact was confirmed by significant differences found between breeding farms in the relative abundances of almost all phyla and genera. Moreover, the exclusive presence of genera *Campylobacter* and *Desulfovibrio* in the semi-open-air facility, both belonging to phylum *Proteobacteria* and encompassing different pathogenic species responsible for infections and diarrheas in mammals, suggested signs of a possible dysbiosis in these animals. These bacteria could be considered biomarkers of a potential GIT dysbiosis, highlighting the importance of offering a close and controlled breeding environment to ensure adequate animal growth and intestinal health.

Despite the results of this study did not point to overall modifications of the cecal microbial diversity by the level of feeding or the administration of antibiotics, these factors can impact the relative abundances of certain microorganisms. Particularly, and in accordance with a previous study in rabbits (Kylie *et al*., 2018), the animals

that did not receive antibiotics showed higher abundances of taxa belonging to phylum *Bacteroidetes*. On the other hand, the administration of antibiotics led to an increase of genus *Coprococcus*. Such bacterium was also found to be overrepresented in a previous study in which Rex rabbits received different molecules of antibiotics (Zou *et al*., 2016). Therefore, the administration of antibiotics could modulate the abundance of some *Coprococcus* species and offer intestinal protection.

Regarding feed restriction, which is a management strategy commonly applied in commercial facilities to prevent the onset of intestinal disorders, we found that a prevalence of *Methanobrevibacter* species may be a positive indicator of a healthy gut microbiota since restricted animals showed an overrepresentation of this genus. Furthermore, a lower prevalence of methanogenic archaea in the cecum of animals raised in the semi-open-air facility could be explained by a high presence of sulfate-reducing bacteria like *Desulfovibrio* that outcompete with methanogens for hydrogen consumption. This competition could favor the production of hydrogen sulfide, compromising the rabbits' intestinal health. This is a good example of how an external factor can increase or decrease the prevalence of specific species and lead to a dysbiosis or, by contrast, to a microbial composition potentially beneficial for the health status of the animal.

The relationship between gut microbiota and complex phenotypes, mainly related to health, has been deeply explored in humans (Cho and Blaser, 2012; Clemente *et al*., 2012; Henry *et al*., 2021). The field of livestock production is still developing its knowledge of the interplay between the gut microbiome and host performance. Some studies in other monogastric species (i.e., pigs and chickens) have reported an association between growth and specific microbial taxa as well as alpha-diversity indexes (Lu *et al*., 2018; Siegerstetter *et al*., 2017). In rabbits, fewer studies have attempted to characterize the association of cecal microbiota with growth (Zeng *et al*., 2015; Fang *et al*., 2020) and FE (Drouilhet *et al*., 2016) performances.

In this regard, some studies have started to explore the contribution of microbial composition to the phenotypic variances of complex traits (i.e., microbiability) as well

as the predictive power of the overall microbial profile in different livestock species (Camarinha-Silva *et al*., 2017; Maltecca *et al*., 2018; Difford *et al*., 2018; Delgado *et al*., 2019; Vollmar *et al*., 2020). The literature on the role and the phenotypic predictive power of microbial information for growth and FE performances in rabbits is virtually non-existent. Chapter five of the present thesis has aimed to address this gap of knowledge.

In this study, we reported heritabilities and microbiabilities for average daily gain (ADG) under different feeding regimes and cage-average traits related to FI and FE. Moreover, original approaches based on the traditional animal mixed model and alternative definitions of expansion of the microbial relationship matrix were proposed to deal with cage-average records and the fact that cecal microbial information was only available in a few animals within a cage. In line with previous estimates for rabbit ADG under different feeding regimes, we found a lower heritability for ADG under restriction (Piles and Sánchez, 2019). However, a large proportion of the phenotypic variance of both growth traits was attributed to the bacterial effect and including microbial information significantly increased the model predictive ability, especially for ADG under restriction. This result suggests that this trait is more strongly influenced by gut microbiota than ADG of animals fed *ad libitum*.

Similarly, large microbiability estimates associated with reduced heritabilities were found for cage-average phenotypes related to FI and FE. Our modeling approaches exhibited moderate predictive abilities for these phenotypes, which significantly improved with the inclusion of microbial information when the expansion of the microbial relationship matrix for animals without such information was based on the identity matrix. Therefore, an important take-home message is that large microbiability estimates must be interpreted with caution since they are not always translated into improvements in the predictive capacity of the models.

All in all, results presented in Chapter five led us to conclude that a certain degree of association exists between the rabbit cecal microbiota and host genotype since a large proportion of the phenotypic variance accompanied by a sharp reduction of

the heritability was found for growth, FI and traits related to FE when microbial information was accounted in the models. In addition, a large proportion of microorganisms seems to be responsible for the prediction improvement observed in growth and FE traits, suggesting a polibacterial role of cecal microbiota in these complex phenotypes. Selective breeding for operational taxonomic units (OTUs) associated with FE phenotypes could only be considered as an additional tool to promote the presence of certain microorganisms in the gut of a rabbit populations if those relevant OTUs are under genetic control. In this connection, the further evaluation of the genetic determinism of the OTUs most relevant for the prediction of growth and FE traits revealed that about one third of them would be under host genetic control (**Table 8.1**).

Table 8.1| Mean (standard deviation) of heritability estimates for the most relevant OTUs for the prediction of individual traits (ADG_{AL} and ADG_{R}) and cage-average traits ($\overline{ADFI}_{AL}$, $\overline{ADRFI}_{AL}$ and $\overline{ADFCR}_{AL}$) declared to be under genetic control.

OTU ID and taxonomical assignment	Trait	h^2
NR768 Unclassified *Bacteria*	$\overline{ADFCR}_{AL}$	0.16 (0.10)
NR2626 Unclassified *Bacteria*	$\overline{ADFCR}_{AL}$ $\overline{ADRFI}_{AL}$	0.27 (0.16)
988375 Genus *Butyricimonas*	$\overline{ADRFI}_{AL}$ $\overline{ADFCR}_{AL}$	0.25 (0.19)
NR570 Unclassified *Acidaminococcaceae*	$\overline{ADFCR}_{AL}$	0.19 (0.10)
356011 Genus *Ruminococcus*	ADG_{R} $\overline{ADFI}_{AL}$	0.23 (0.17)
NR3985 Unclassified *Bacteria*	$\overline{ADRFI}_{AL}$	0.21 (0.14)
332732 Genus *Bacteroides*	$\overline{ADFI}_{AL}$	0.22 (0.14)
NR4624 Genus *Butyricicoccus*	ADG_{R} $\overline{ADFI}_{AL}$ ADG_{AL}	0.25 (0.16)
798164 Unclassified *Firmicutes*	$\overline{ADRFI}_{AL}$	0.23 (0.13)
NR2377 Unclassified *Bacteria*	$\overline{ADRFI}_{AL}$	0.25 (0.17)

NR669 Genus *Methanobrevibacter*	ADG_R ADG_{AL}	0.17 (0.11)
NR2465 Genus *Coprobacter*	$\overline{ADFCR}_{AL}$	0.18 (0.13)
NR733 Genus *Paramuribaculum*	$\overline{ADRFI}_{AL}$	0.18 (0.12)
339013 Genus *Bacteroides*	ADG_R	0.26 (0.16)
849440 Genus *Methanobrevibacter*	ADG_R ADG_{AL}	0.25 (0.14)
NR2019 Genus *Neglecta*	$\overline{ADFI}_{AL}$	0.21 (0.13)
1110378 Unclassified *Ruminococcaceae*	$\overline{ADFCR}_{AL}$	0.19 (0.12)
NR2545 Genus *Neglecta*	$\overline{ADFI}_{AL}$	0.22 (0.15)
NR3011 Unclassified *Bacteria*	ADG_{AL}	0.21 (0.16)
4299126 Unclassified *Alphaproteobacteria*	$\overline{ADFI}_{AL}$ $\overline{ADRFI}_{AL}$	0.23 (0.14)
581388 Unclassified *Bacteria*	ADG_{AL}	0.16 (0.10)
297503 Unclassified *Bacteria*	ADG_{AL}	0.26 (0.17)

ADG_{AL}: average daily gain in rabbits fed *ad libitum*; ADG_R: average daily gain in rabbits fed under restriction; $\overline{ADFI}_{AL}$: average daily feed intake in rabbits fed *ad libitum*; $\overline{ADRFI}_{AL}$: average daily residual feed intake in rabbits fed *ad libitum*; $\overline{ADFCR}_{AL}$: average daily feed conversion ratio in rabbits fed *ad libitum*.

While the influence of external factors on mammals' gut microbiota is undeniable, the existence of a host genetic background responsible for gut microbial variations is still a source of debate. Chapters six and seven of the present thesis have aimed to shed light on this matter. In Chapter six, Bayesian linear and zero-inflated Poisson (ZIP) mixed models were used to assess through Bayes factor (BF) the statistical relevance of host genetics, litter, and cage effects for a set of microbial traits representative of the cecal microbiota at different levels of depth. All the microbial traits analyzed were better adjusted with a linear mixed model except those OTUs whose frequency of presence across samples was lower than 15%. Therefore, the ZIP model only overcame the linear mixed model for the adjustment of traits with a very marked excess of zeros.

The findings of this study revealed different levels of influence of host genetics, litter, and cage effects on global cecal microbial composition and an important proportion of OTUs and genera relative abundances. In line with the existing literature in

humans and cattle (Goodrich *et al*., 2014; Li *et al*., 2019), our study evidenced genetic control for 34% and 16% of the genera and OTUs inhabiting the rabbit cecum, respectively. The heritability estimates for such traits were moderate, ranging from 0.12 to 0.40, strongly suggesting a genetic control of the rabbit cecal microbiota. Such affirmation can be further corroborated by the clear genetic determinism observed for global microbial traits (number of observed OTUs and one principal component), which is also in line with previous heritability assessments of alpha-diversity in humans (Goodrich *et al*., 2016), pigs (Lu *et al*., 2018; Bergamaschi *et al*., 2020), and cattle (Saborío-Montero *et al*., 2021).

Contrary to previous studies that suggested bacteria encompassed by phylum *Firmicutes* are the most heritable (Goodrich *et al*., 2016; Li *et al*., 2019), the strongest evidence of genetic determinism was found for two OTUs taxonomically assigned to genera *Bacteroides* and *Parabacteroides* (phylum *Bacteroidetes*) present in the cecum of the rabbit population analyzed. This evidence of genetic control was supported by the fact that the greatest heritability estimates at the genus level were found for these two genera (h^2 *Parabacteroides* = 0.35; h^2 *Bacteroides* = 0.29), which are involved in the degradation of vegetal polysaccharides and amino acid fermentation, amino acid transport, and cell motility in the gastrointestinal microbiota of the growing rabbit (Dai *et al*., 2011; Sun *et al*., 2020). It is worth mentioning that the nursing environment also seems to exert an important influence on members belonging to these two genera. The impact of the nursing environment, evaluated as the litter effect, on the relative abundances of genera *Bacteroides* and *Parabacteroides* still prevails at the slaughter age when cecal samples were collected.

Within *Firmicutes*, the predominant phylum of rabbit cecum microbiome, our results also provided strong evidence of genetic determinism for genera *Dehalobacterium* (h^2 = 0.29) and *Butyricimonas* (h^2 = 0.27), which had been previously reported as heritable in humans (Goodrich *et al*., 2014; Goodrich *et al*., 2016). Nevertheless, the environmental effect of litter also seems to have a profound impact on the relative abundances of both genera (l^2 *Dehalobacterium* = 0.37; l^2 *Butyricimonas* = 0.28).

Finally, the cage seems to play an important effect in the relative abundance of members of family *S24-7* and genus *Ruminococcus*.

The findings presented in Chapter six support that host genetics, cage, and nursing environment contribute to the variation of rabbit cecal microbial composition. Regarding the number of traits influenced by such factors, the nursing environment would have a significant effect on a higher number of traits (231 microbial traits) than host genetics (169 microbial traits) and cage (147 microbial traits). The next and final step consisted of an attempt to identify the genomic regions and candidate genes involved in the variation of rabbit cecal microbiota using genome-wide association studies (GWAS).

In Chapter seven, we have presented the results of the first microbial GWAS (mGWAS) conducted using two alternative approaches (i.e., MIX-GWAS and BayesC) in a rabbit population using a highly dense SNP array for a set of microbial traits representative of the cecal microbiota at different levels of depth. Moreover, our study was accompanied by a simulation assessment that allowed us to get an overview of the statistical power of our dataset to identify the positions on the genome and effects of the SNPs associated with the variation of the microbial traits analyzed. Unfortunately, with this simulation, we confirmed the limited power of both approaches to detect QTL regions given our data structure and limited sample size. Therefore, we could only expect to capture strong signals related to QTNs responsible for an important part of the variation of our microbial phenotypes. The probability of capturing a signal corresponding to a real QTN is higher with the MIX-GWAS. However, the rate of false positive signals is also higher with this approach. On the contrary, the detection power of BayesC is lower, but the probability that a QTL region declared by this approach really contains a SNP variant associated with the phenotype is greater. This underlines the necessity to perform further biological analyses of the QTL regions declared by any GWAS approach that helps to discriminate false positives from true associations and identify candidate genes associated with the phenotypic variation of the traits of interest.

A growing number of studies have attempted to identify genomic regions controlling microbial composition through GWAS on relatively small populations in humans, mice, or pigs (Benson *et al*., 2010; Goodrich *et al*., 2014; Leamy *et al*., 2014; Blekhman *et al*., 2015; Davenport *et al*., 2015; Org *et al*., 2015; Cheng *et al*., 2018; Crespo-Piazuelo *et al*., 2019; Bergamaschi *et al*., 2020). The limited sample sizes employed in such studies translated into a lack of statistical significance after multiple testing correction. Thus, these studies tended to be less strict in these corrections to allow for some signal detection (e.g., most mGWAS applied chromosome-wide multiple testing correction instead of genome-wide). The final consequence of this loose significance threshold definition is that, even though QTL regions and candidate genes have been proposed, there is almost no overlap between them (Rothschild *et al*., 2018). Therefore, it confirms the necessity of several orders of magnitude larger sample sizes to confidently declare QTLs regions with mGWAS.

In our study, the MIX-GWAS declared more than 300 variants spread across ten chromosomes associated with 19 microbial traits at the genome-wide level. After a more stringent correction by the effective number of independent tests, only six variants located on chromosome 12 were declared as significantly associated with the variation of an OTU taxonomically assigned to genus *Butyricimonas*. Interestingly, the Bayes factor declared substantial evidence of genetic control for this trait (Chapter six), and its heritability estimate was 0.26. *Butyricimonas* is a butyrate-producing bacteria with anti-inflammatory properties that help maintain a healthy gut (Yang *et al*., 2017). Butyrate is a major source of energy for cells that cover the epithelial surface of the large intestine (Honda and Littman, 2012), which suggests that a variant on chromosome 12 could favor the presence of genus *Butyricimonas* in the rabbit cecum that will help restore the epithelial barrier in times of challenge and inflammation (Hamilton *et al*., 2015).

The other variants associated with different microbial traits were less powerful, so we prudently propose several QTL regions on different chromosomes involved in the host genetic control of the rabbit cecal microbiota. These regions include genes involved in homeostatic, metabolic, or immune system processes that will deserve

special attention in future studies that need to be conducted in independent populations with larger sample sizes.

The studies included in the present thesis have characterized, as rigorously as possible, the influence of meat rabbit cecal microbiota on the host's feed efficiency and unraveled the environmental and genetic bases of composition and diversity of microbial communities inhabiting the rabbit cecum. Nevertheless, to increase the sample size of studies, design well-balanced experiments, standardize the analyses protocols, and improve the quality of datasets are imperative necessities to unravel and establish causal relationships among the holobiont system (host-gut microbiota-environment). In **Figure 8.1**, the direct effect of G on P (α) determines the proportion of phenotypic variability attributable to the host (heritability). The effect of G on M (β) determines what can then be interpreted as the heritable portion of M. The joint effect of G on M and P represent the genetic correlation between the microbiome composition and the phenotype. The effect of M on P (γ) determines the microbiability. Finally, the effect of E on P (ε_p) and the effect of E on M (ε_m) can be considered external effects such as management and diet, respectively.

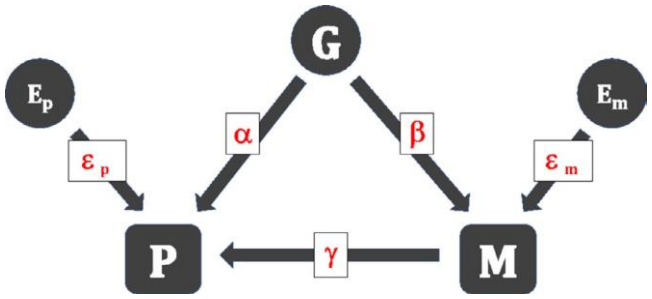


Figure 8.1| Graphic picturing the potential interplay between the host genotype (G), the gut microbiome (M), the environmental components (E_p and E_m), and the phenotype (P) in an animal breeding context (Maltecca *et al*., 2019).

Future multivariate models that allow considering host-gut microbiota-environment relationships as a whole are paramount to inference causality and globally interpret the contribution of microbiota and host genetics to complex phenotypes related to FE. On the other hand, in the light of recent results and ours, understanding this interplay seems more relevant from a biological knowledge perspective than from a practical breeding point of view. Moreover, selecting for FE through microbiota might be risky since negative responses could consequently be obtained for other traits of

interest. In addition, direct selection for the phenotype of interest somehow guarantees a balanced modification of all the elements involved in its metabolic pathway, being the gut microbiota one of them that might be affected.

8.1. List of abbreviations

ADG average daily gain
BF Bayes factor
FE feed efficiency
FI feed intake
GIT gastrointestinal tract
GWAS genome-wide association study
mGWAS microbial genome-wide association study
OTU operational taxonomic unit
ZIP zero-inflated Poisson

8.2. References

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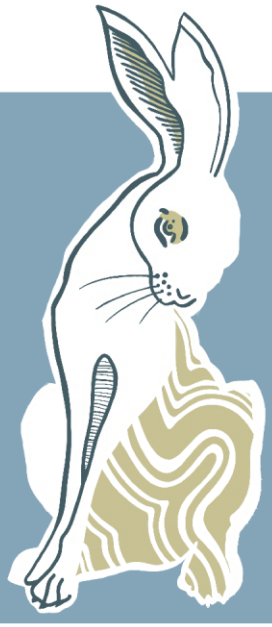
CHAPTER 9

CONCLUSIONS



I. The assessment of bacterial and archaeal populations inhabiting the meat rabbit cecum and expelled hard feces through 16S rRNA gene amplicon sequencing revealed a predominant presence of phylum *Firmicutes*, followed by phyla *Tenericutes* and *Bacteroidetes*, accounting these three bacterial phyla for 90% of the total microbial composition. Acidic pH could hinder the growth of archaeal species whose presence was one order of magnitude lower than *Bacteria* and limited to the genus *Methanobrevibacter*.

II. Although no significant differences in microbial richness and diversity were found between the cecum and hard feces, univariate and multivariate analytical approaches revealed compositional differences in the relative abundance of a large number of taxa, even at the phylum level. These findings suggest different functional requirements for the specific microbial communities that need to be present in each section of the rabbit gastrointestinal tract.

III. The stability of cecal microbial communities reached in adulthood is shaped, at different extents, by the breeding farm and commonly applied management practices. Different analytical approaches determined that the farm environment offered to the growing rabbits exerts the largest impact on their cecal microbial diversity and composition. The exclusive presence of potentially pathogenic *Campylobacter* and *Desulfovibrio* species in the facility most exposed to changes in climate conditions is a prospective biomarker for the risk of future gastrointestinal dysbiosis outbreaks.

IV. The high prevalence of *Methanobrevibacter* species in the cecum of rabbits submitted to feed restriction can be interpreted as an indicator of good intestinal health since it is a management strategy commonly applied in commercial farms as an alternative to antimicrobials given its proven preventive effectiveness against the onset of intestinal disorders. Further research is needed to confirm this finding, but it supports the hypothesis that the benefits of applying feed restriction may be due to changes in gut microbial composition and activity.

V. Original approaches based on the traditional animal mixed model and alternative definitions and expansions of the microbial relationship matrix have been proposed to deal with cage-average feed intake records and the lack of microbial information for most animals within cages. Such approaches have enabled to determining that a large proportion of the phenotypic variance of complex traits related to growth and feed efficiency is attributable to microbial effect. Furthermore, sharp reductions of the heritability observed for all traits when including the microbial effect in the model hint at the existence of some degree of association between the rabbit cecal microbiota and the host genotype.

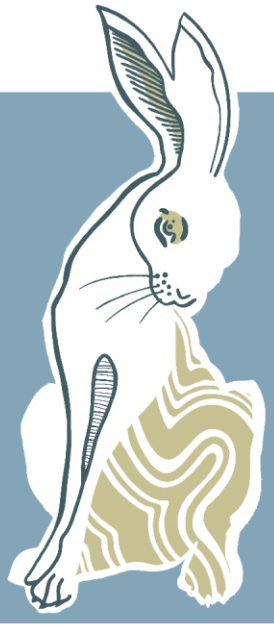
VI. The inclusion of microbial information through certain microbial relationship matrixes significantly increased the capacity of the models to predict animal performances. A polibacterial role of cecal microbiota in these complex traits is suggested by the large proportion of microorganisms that seem to be responsible for these predictive improvements. Nevertheless, the interpretation of microbiability must be taken with care since large estimates are not always translated into improvements in the predictive ability of the models.

VII. The statistical relevance of host genetics on microbial traits representative of rabbit cecal microbiota, assessed through the Bayes factor, confirmed an overall genetic determinism. Particularly, clear genetic control was evidenced for approximately one-fifth of the operational taxonomic units and one-third of the genera present in the rabbit cecum. Moderate heritabilities, ranging from 0.12 to 0.40, were estimated for these traits and the most heritable taxa belong to genera *Bacteroides*, *Parabacteroides*, *Dehalobacterium*, and *Butyricimonas*. Additionally, a profound impact of the nursing environment was also found on the relative abundances of the latter two genera. However, members of family *S24-7* and genus *Ruminococcus* are highly influenced by cage effects.

VIII. Simulation assessment has revealed a very limited power of our data, given its structure and limited sample size, to detect quantitative trait loci regions responsible for the variation of rabbit cecal microbiota through genome-wide association studies. Only strong signals related to variants responsible for an important variation of the microbial trait are expected to be captured. Furthermore, biological analyses of the regions declared to be associated with the phenotype of interest by this approach are mandatory to discriminate between false positives and true associations.

IX. Despite the mentioned limitations of our data, the MIX-GWAS approach declared more than 300 variants, spread across ten *Oryctolagus cuniculus* chromosomes, associated with 19 microbial traits at the genome-wide level. The annotation of the regions containing these variants led us to carefully propose 44 candidate genes involved in homeostatic, metabolic, and immune system processes. Future research, desirably conducted with larger sample sizes in independent populations, will be needed to confirm these candidates proposed to explain the variation of the meat rabbit cecal microbiota.

ANNEXES



Supplementary material of chapter 3

Table 3.S1| Summary of metadata and OTU tables.

Rabbit ID	Feeding regime	Origin	Number initial sequences	Number final sequences	OTU number
113061	*Ad libitum*	Cecum	189,825	66,886	458
113061	*Ad libitum*	Feces	159,674	58,402	471
113101	*Ad libitum*	Cecum	173,618	58,805	517
113101	*Ad libitum*	Feces	120,847	41,672	507
113064	Restricted	Cecum	65 274	22,024	459
113064	Restricted	Feces	110,027	38,949	482
113087	Restricted	Cecum	119,866	38,489	514
113087	Restricted	Feces	189,836	68,080	523
115804	Restricted	Cecum	89,808	30,558	451
115804	Restricted	Feces	110,928	38,186	462
115231	Restricted	Cecum	80,883	25,563	411
115231	Restricted	Feces	157,615	41,635	422
115263	Restricted	Cecum	62 ,14	20,347	414
115263	Restricted	Feces	79,270	28,610	433
113210	Restricted	Cecum	54,571	16,415	462
113210	Restricted	Feces	73,208	25,905	482
115040	*Ad libitum*	Cecum	125,282	42,774	485
115040	*Ad libitum*	Feces	143,182	50,473	482
115776	Restricted	Cecum	62,347	22,035	445
115776	Restricted	Feces	113,805	32,274	469
113133	*Ad libitum*	Cecum	79,711	24,641	417
113133	*Ad libitum*	Feces	106,305	31,062	424
113150	*Ad libitum*	Cecum	91,105	28,390	500
113150	*Ad libitum*	Feces	85,920	27,021	489
115240	Restricted	Cecum	91,364	22,548	468
115240	Restricted	Feces	65,777	23,168	469
115162	Restricted	Cecum	192,857	60,444	507
115162	Restricted	Feces	78,863	24,470	485
115124	*Ad libitum*	Cecum	159,913	49,757	488
115124	*Ad libitum*	Feces	98,913	32,880	486
115279	*Ad libitum*	Cecum	195,975	60,580	498
115279	*Ad libitum*	Feces	156,229	50,705	510
115280	*Ad libitum*	Cecum	174,784	56,390	506
115280	*Ad libitum*	Feces	148,880	47,751	523
115379	*Ad libitum*	Cecum	240,333	66,847	532
115379	*Ad libitum*	Feces	201,265	57,850	541
113238	*Ad libitum*	Cecum	217,076	67,179	532
113238	*Ad libitum*	Feces	186,912	60,045	526
113115	Restricted	Cecum	156,466	43,654	479
113115	Restricted	Feces	117,032	40,182	487
113198	Restricted	Cecum	102,019	29,623	525
113198	Restricted	Feces	106,587	34,351	519

Table 3.S2| OTUs differentially represented between fecal and cecal samples ($P_{FDR} < 0.05$).

OUT ID and taxonomical assignment	Mean abundance (CSS OTU units) (SD)	Difference Cecum-Feces ± SE	P_{FDR}	[a]Discriminant sPLS-DA
1110378,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae* ;*Oscillospira*	2.648 (1.349)	0.865 ± 0.198	0.000	NO
178839,Firmicutes;*Clostridia* ;*Clostridiales* ;*Mogibacteriaceae*	4.035 (0.744)	0.387 ± 0.100	0.006	NO
207770,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae* ;*Ruminococcus*	5.577 (1.869)	0.755 ± 0.181	0.000	YES
210945,Firmicutes;*Clostridia* ;*Clostridiales* ;*Clostridiaceae*	3.761 (1.456)	0.343 ± 0.123	0.050	NO
213671,Bacteroidetes;*Bacteroidia* ;*Bacteroidales* ;*Rikenellaceae*	1.317 (1.520)	0.583 ± 0.166	0.006	YES
216941,Verrucomicrobia;*Verrucomicrobiae* ;*Verrucomicrobiales* ;*Verrucomicrobiaceae* ;*Akkermansia*	7.113 (1.340)	0.418 ± 0.110	0.006	NO
279340,Firmicutes;*Clostridia* ;*Clostridiales*	1.374 (1.821)	0.402 ± 0.173	0.017	NO
290079,Firmicutes;*Clostridia* ;*Clostridiales* ;*Lachnospiraceae*	1.759 (0.984)	0.528 ± 0.201	0.021	NO
297503,Firmicutes;*Clostridia* ;*Clostridiales*	3.852 (2.250)	0.970 ± 0.201	0.000	YES
299422,Verrucomicrobia;*Verrucomicrobiae* ;*Verrucomicrobiales* ;*Verrucomicrobiaceae* ;*Akkermansia*	5.210 (2.163)	0.831 ± 0.179	0.000	NO
299902,Firmicutes;*Clostridia* ;*Clostridiales* ;*Lachnospiraceae* ;*Blautia*	4.381 (1.781)	0.852 ± 0.139	0.000	NO
303313,Firmicutes;*Clostridia* ;*Clostridiales* ;*Lachnospiraceae* ;*Blautia*	1.608 (1.040)	0.661 ± 0.187	0.000	YES
314586,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae*	3.710 (1.039)	0.498 ± 0.146	0.021	NO
321135,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae*	2.505 (1.513)	0.432 ± 0.145	0.015	NO
322258,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae*	2.917 (1.067)	0.573 ± 0.162	0.006	NO
328083,Firmicutes;*Clostridia* ;*Clostridiales* ;*Clostridiaceae* ;*Clostridium*	6.330 (1.059)	0.576 ± 0.086	0.000	YES
330792,Firmicutes;*Clostridia* ;*Clostridiales*	2.468 (1.296)	0.313 ± 0.108	0.037	NO
342182,Firmicutes;*Clostridia* ;*Clostridiales*	2.253 (2.401)	0.514 ± 0.086	0.000	NO
352489,Firmicutes;*Clostridia* ;*Clostridiales* ;*Lachnospiraceae*	2.718 (1.055)	0.734 ± 0.187	0.000	NO
385545,Firmicutes;*Clostridia* ;*Clostridiales*	1.785 (1.709)	0.588 ± 0.181	0.017	YES
423830,Firmicutes;*Clostridia* ;*Clostridiales*	4.192 (1.568)	0.770 ± 0.201	0.000	NO
527988,Firmicutes;*Clostridia* ;*Clostridiales* ;*Eubacteriaceae* ;*Anaerofustis*	2.460 (1.108)	0.409 ± 0.150	0.050	NO
576853,Firmicutes;*Clostridia* ;*Clostridiales*	6.528 (1.110)	0.424 ± 0.094	0.000	NO
580907,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae*	5.687 (0.945)	0.648 ± 0.106	0.000	YES
589822,Firmicutes;*Clostridia* ;*Clostridiales* ;*Lachnospiraceae* ;*Blautia*	2.533 (1.373)	1.037 ± 0.169	0.000	YES
590015,Firmicutes;*Clostridia* ;*Clostridiales*	3.221 (1.458)	0.596 ± 0.143	0.006	NO
621649,Firmicutes;*Clostridia* ;*Clostridiales*	9.088 (0.927)	0.430 ± 0.086	0.000	NO
696563,Firmicutes;*Clostridia* ;*Clostridiales* ;*Lachnospiraceae*	3.773 (1.197)	0.823 ± 0.142	0.000	YES

OUT ID and taxonomical assignment	Mean abundance (CSS OTU units) (SD)	Difference Cecum-Feces ± SE	P_{FDR}	[a]Discriminant sPLS-DA
798164,Firmicutes;*Clostridia* ;*Clostridiales*	3.114 (2.058)	0.599 ± 0.188	0.021	NO
857827,Bacteroidetes;*Bacteroidia* ;*Bacteroidales*	7.327 (2.595)	0.503 ± 0.190	0.043	YES
NR0,Firmicutes;*Clostridia* ;*Clostridiales* ;*Lachnospiraceae*	5.491 (1.434)	0.904 ± 0.114	0.000	YES
NR1,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae* ;*Ruminococcus*	6.505 (0.789)	0.336 ± 0.101	0.011	NO
NR104,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae*	8.199 (0.795)	0.301 ± 0.094	0.011	NO
NR118,Unknown	4.575 (1.058)	0.801 ± 0.167	0.000	YES
NR12,Firmicutes;*Clostridia* ;*Clostridiales*	4.650 (1.058)	1.706 ± 0.125	0.000	YES
NR123,Firmicutes;*Clostridia* ;*Clostridiales* ;*Lachnospiraceae* ;*Blautia*	2.658 (1.735)	0.658 ± 0.193	0.017	YES
NR128,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae*	1.514 (2.122)	0.310 ± 0.108	0.017	NO
NR132,Firmicutes;*Clostridia* ;*Clostridiales* ;*Lachnospiraceae*	3.352 (2.047)	0.728 ± 0.170	0.000	YES
NR138,Unknown	1.822 (1.066)	0.716 ± 0.215	0.015	YES
NR152,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae*	10.895 (0.384)	0.286 ± 0.080	0.000	NO
NR16,Firmicutes;*Clostridia* ;*Clostridiales*	1.856 (1.948)	0.531 ± 0.153	0.006	NO
NR167,Firmicutes;*Clostridia* ;*Clostridiales*	2.074 (1.096)	0.782 ± 0.216	0.000	YES
NR17,Firmicutes;*Clostridia* ;*Clostridiales*	5.571 (0.897)	0.776 ± 0.140	0.000	YES
NR174,Firmicutes;*Clostridia* ;*Clostridiales* ;*Lachnospiraceae* ;*Blautia*	5.810 (1.084)	1.010 ± 0.108	0.000	YES
NR176,Unknown	8.681 (0.978)	0.948 ± 0.127	0.000	YES
NR181,Firmicutes;*Clostridia* ;*Clostridiales* ;*Lachnospiraceae* ;*Blautia*	2.054 (1.498)	0.720 ± 0.156	0.000	YES
NR197,Firmicutes;*Clostridia* ;*Clostridiales* ;*Lachnospiraceae* ;*Blautia*	9.075 (1.003)	0.583 ± 0.098	0.000	YES
NR199,Firmicutes;*Clostridia* ;*Clostridiales*	2.258 (1.788)	0.943 ± 0.314	0.017	NO
NR208,Proteobacteria;*Betaproteobacteria*	5.274 (0.831)	0.801 ± 0.198	0.000	YES
NR212,Firmicutes;*Clostridia* ;*Clostridiales*	4.328 (2.081)	0.742 ± 0.198	0.006	NO
NR215,Firmicutes;*Clostridia* ;*Clostridiales* ;*Veillonellaceae* ;*Phascolarctobacterium*	3.914 (3.498)	0.550 ± 0.201	0.048	NO
NR218,Firmicutes;*Clostridia* ;*Clostridiales*	2.083 (1.434)	0.896 ± 0.163	0.000	NO
NR22,Unknown	2.966 (1.209)	0.862 ± 0.228	0.017	YES
NR220,Firmicutes;*Clostridia* ;*Clostridiales*	2.658 (2.171)	0.985 ± 0.146	0.000	NO
NR224,Firmicutes;*Clostridia* ;*Clostridiales*	4.542 (1.522)	0.580 ± 0.117	0.000	NO
NR231,Firmicutes;*Clostridia* ;*Clostridiales* ;*Lachnospiraceae*	2.849 (2.131)	0.423 ± 0.160	0.048	NO
NR234,Firmicutes;*Clostridia* ;*Clostridiales* ;*Lachnospiraceae*	3.051 (1.517)	1.144 ± 0.147	0.000	YES
NR237,Firmicutes;*Clostridia* ;*Clostridiales* ;*Clostridiaceae* ;*Clostridium*	1.640 (1.567)	0.507 ± 0.171	0.021	NO
NR244,Firmicutes;*Clostridia* ;*Clostridiales* ;*Lachnospiraceae* ;*Blautia*	2.894 (1.183)	0.527 ± 0.197	0.048	NO
NR25,Firmicutes;*Clostridia* ;*Clostridiales* ;*Lachnospiraceae*	5.812 (1.026)	0.411 ± 0.093	0.000	NO
NR259,Unknown	7.230 (2.010)	1.038 ± 0.165	0.000	YES

OUT ID and taxonomical assignment	Mean abundance (CSS OTU units) (SD)	Difference Cecum-Feces ± SE	P_{FDR}	[a]Discriminant sPLS-DA
NR274,Firmicutes;*Clostridia* ;*Clostridiales*	3.637 (1.643)	0.516 ± 0.169	0.028	NO
NR281,Firmicutes;*Clostridia* ;*Clostridiales* ;*Lachnospiraceae*	3.367 (1.217)	0.448 ± 0.147	0.015	YES
NR286,Firmicutes;*Clostridia* ;*Clostridiales*	4.013 (1.738)	0.611 ± 0.156	0.011	YES
NR29,Firmicutes;*Clostridia* ;*Clostridiales*	2.556 (3.770)	0.731 ± 0.133	0.000	YES
NR297,Unknown	3.292 (1.028)	0.794 ± 0.206	0.011	YES
NR308,Firmicutes;*Clostridia* ;*Clostridiales* ;*Christensenellaceae*	2.648 (1.459)	0.407 ± 0.122	0.006	NO
NR316,Firmicutes;*Clostridia* ;*Clostridiales*	2.251 (1.421)	0.933 ± 0.288	0.031	YES
NR322,Firmicutes;*Clostridia* ;*Clostridiales* ;*Mogibacteriaceae*	3.246 (1.532)	0.701 ± 0.130	0.000	NO
NR323,Firmicutes;*Clostridia* ;*Clostridiales* ;*Lachnospiraceae* ;*Blautia*	1.991 (1.133)	0.481 ± 0.146	0.017	NO
NR345,Firmicutes;*Clostridia* ;*Clostridiales* ;*Lachnospiraceae*	3.073 (1.222)	0.415 ± 0.161	0.050	NO
NR352,Firmicutes;*Clostridia* ;*Clostridiales* ;*Lachnospiraceae*	6.028 (1.028)	0.768 ± 0.108	0.000	YES
NR360,Unknown	2.963 (2.737)	0.960 ± 0.189	0.000	NO
NR371,Unknown	5.485 (1.880)	0.856 ± 0.203	0.006	YES
NR374,Firmicutes;*Clostridia* ;*Clostridiales* ;*Lachnospiraceae* ;*Blautia*	1.912 (1.415)	0.539 ± 0.164	0.024	YES
NR382,Firmicutes;*Clostridia* ;*Clostridiales*	4.964 (2.243)	0.513 ± 0.133	0.011	NO
NR383,Firmicutes;*Clostridia* ;*Clostridiales* ;*Lachnospiraceae*	3.189 (0.913)	0.541 ± 0.175	0.017	NO
NR397,Firmicutes;*Clostridia* ;*Clostridiales*	4.352 (1.673)	0.381 ± 0.132	0.037	NO
NR4,Firmicutes;*Clostridia* ;*Clostridiales*	2.970 (1.872)	0.997 ± 0.262	0.011	NO
NR407,Verrucomicrobia;*Verrucomicrobiae* ;*Verrucomicrobiales* ;*Verrucomicrobiaceae* ;*Akkermansia*	5.349 (1.766)	0.635 ± 0.167	0.011	NO
NR417,Firmicutes;*Clostridia* ;*Clostridiales* ;*Lachnospiraceae*	4.225 (1.236)	0.441 ± 0.156	0.043	NO
NR42,Firmicutes;*Clostridia* ;*Clostridiales* ;*Clostridiaceae* ;*Clostridium*	6.506 (0.813)	0.431 ± 0.097	0.000	NO
NR437,Firmicutes;*Clostridia* ;*Clostridiales*	2.646 (1.755)	0.566 ± 0.154	0.011	NO
NR443,Firmicutes;*Clostridia* ;*Clostridiales* ;*Lachnospiraceae*	3.385 (1.226)	0.516 ± 0.167	0.017	NO
NR449,Firmicutes;*Clostridia* ;*Clostridiales* ;*Lachnospiraceae*	4.955 (1.343)	0.588 ± 0.181	0.015	NO
NR451,Tenericutes;*RF3* ;*ML615J-28*	2.079 (2.481)	0.306 ± 0.123	0.039	NO
NR453,Firmicutes;*Clostridia* ;*Clostridiales* ;*Lachnospiraceae*	4.415 (0.856)	0.545 ± 0.154	0.011	NO
NR455,Actinobacteria;*Coriobacteriia* ;*Coriobacteriales*	3.385 (1.272)	0.617 ± 0.135	0.000	YES
NR457,Firmicutes;*Clostridia* ;*Clostridiales*	2.558 (2.209)	0.571 ± 0.153	0.000	NO
NR50,Actinobacteria;*Coriobacteriia* ;*Coriobacteriales* ;*Coriobacteriaceae*	5.889 (0.823)	0.887 ± 0.132	0.000	YES
NR54,Firmicutes;*Clostridia* ;*Clostridiales*	2.457 (1.575)	0.380 ± 0.145	0.048	NO
NR6,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae*	3.433 (1.406)	0.475 ± 0.154	0.037	NO
NR7,Firmicutes;*Clostridia* ;*Clostridiales* ;*Clostridiaceae* ;*Clostridium*	4.916 (1.050)	0.594 ± 0.097	0.000	YES
NR71,Unknown	2.842 (1.566)	1.075 ± 0.258	0.000	YES

OUT ID and taxonomical assignment	Mean abundance (CSS OTU units) (SD)	Difference Cecum-Feces ± SE	P_{FDR}	[a]Discriminant sPLS-DA
NR76,Firmicutes;*Clostridia* ;*Clostridiales*	2.242 (1.293)	0.404 ± 0.150	0.028	YES
NR86,Unknown	5.277 (1.211)	0.664 ± 0.236	0.031	NO
NR96,Verrucomicrobia;*Verrucomicrobiae* ;*Verrucomicrobiales* ;*Verrucomicrobiaceae* ;*Akkermansia*	3.982 (1.742)	0.728 ± 0.173	0.006	YES
1108356,Tenericutes;*Mollicutes* ;*RF39*	4.885 (0.863)	-0.520 ± 0.157	0.017	NO
157802,Firmicutes;*Clostridia* ;*Clostridiales*	3.029 (1.465)	-0.536 ± 0.111	0.000	NO
173245,Firmicutes;*Clostridia* ;*Clostridiales*	5.881 (1.618)	-0.748 ± 0.166	0.006	NO
197832,Firmicutes;*Clostridia* ;*Clostridiales* ;*Lachnospiraceae* ;*Coprococcus*	3.295 (3.191)	-1.208 ± 0.357	0.011	NO
206151,Firmicutes;*Clostridia* ;*Clostridiales* ;*Lachnospiraceae* ;*Coprococcus*	5.146 (1.754)	-1.092 ± 0.266	0.000	NO
208769,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae*	4.523 (1.031)	-0.630 ± 0.183	0.015	NO
209492,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae*	4.083 (0.941)	-0.442 ± 0.161	0.050	NO
209524,Firmicutes;*Clostridia* ;*Clostridiales* ;*Lachnospiraceae* ;*Coprococcus*	4.180 (2.710)	-0.815 ± 0.206	0.000	YES
210867,Firmicutes;*Clostridia* ;*Clostridiales*	1.571 (2.383)	-0.664 ± 0.252	0.039	NO
210895,Firmicutes;*Clostridia* ;*Clostridiales*	5.284 (1.511)	-0.514 ± 0.105	0.000	NO
258404,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae* ;*Ruminococcus*	1.934 (1.883)	-0.541 ± 0.185	0.039	NO
258980,Firmicutes;*Clostridia* ;*Clostridiales*	2.062 (1.775)	-0.441 ± 0.148	0.024	NO
261966,Firmicutes;*Clostridia* ;*Clostridiales*	4.416 (1.878)	-0.815 ± 0.274	0.034	NO
267220,Bacteroidetes;*Bacteroidia* ;*Bacteroidales* ;*S24-7*	3.001 (2.285)	-0.828 ± 0.190	0.000	NO
269386,Bacteroidetes;*Cyanobacteria* ;*4C0d-2* ;*YS2*	1.453 (1.619)	-0.948 ± 0.281	0.011	NO
275194,Firmicutes;*Clostridia* ;*Clostridiales*	3.675 (1.664)	-0.597 ± 0.143	0.000	NO
279048,Cyanobacteria;*4C0d-2* ;*YS2*	1.617 (1.714)	-1.115 ± 0.282	0.006	NO
279179,Tenericutes;*Mollicutes* ;*RF39*	2.974 (1.906)	-0.704 ± 0.195	0.015	NO
288193,Firmicutes;*Clostridia* ;*Clostridiales*	4.272 (1.229)	-0.712 ± 0.157	0.000	YES
289538,Firmicutes;*Clostridia* ;*Clostridiales*	2.647 (1.282)	-0.904 ± 0.194	0.006	NO
313524,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae*	2.081 (1.101)	-0.815 ± 0.216	0.011	YES
314029,Firmicutes;*Clostridia* ;*Clostridiales*	2.874 (2.558)	-0.370 ± 0.133	0.034	NO
326013,Firmicutes;*Clostridia* ;*Clostridiales*	5.019 (1.803)	-0.875 ± 0.156	0.000	YES
333768,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae* ;*Oscillospira*	3.239 (0.993)	-0.687 ± 0.195	0.006	NO
337724,Bacteroidetes;*Bacteroidia* ;*Bacteroidales* ;*S24-7*	1.560 (2.540)	-0.240 ± 0.085	0.021	NO
341902,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae* ;*Oscillospira*	1.960 (0.851)	-0.807 ± 0.216	0.000	YES
345556,Firmicutes;*Clostridia* ;*Clostridiales*	9.442 (0.849)	-0.503 ± 0.201	0.020	YES
355312,Firmicutes;*Clostridia* ;*Clostridiales*	2.019 (1.751)	-0.730 ± 0.210	0.011	NO
355494,Firmicutes;*Clostridia* ;*Clostridiales*	3.751 (1.175)	-0.733 ± 0.151	0.000	YES
408513,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae* ;*Oscillospira*	2.535 (0.938)	-0.952 ± 0.187	0.000	YES

OUT ID and taxonomical assignment	Mean abundance (CSS OTU units) (SD)	Difference Cecum-Feces ± SE	P_{FDR}	[a]Discriminant sPLS-DA
422283,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae* ;*Oscillospira*	6.000 (0.675)	-0.467 ± 0.119	0.017	YES
4343981,Tenericutes;*Mollicutes* ;*RF39*	4.024 (0.901)	-0.384 ± 0.128	0.037	NO
443620,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae* ;*Oscillospira*	3.316 (1.081)	-0.766 ± 0.195	0.000	YES
4443094,Firmicutes;*Clostridia* ;*Clostridiales*	2.057 (1.167)	-0.603 ± 0.224	0.050	NO
514061,Tenericutes;*Mollicutes* ;*RF39*	5.081 (1.022)	-0.596 ± 0.122	0.000	YES
523099,Firmicutes;*Clostridia* ;*Clostridiales*	2.510 (1.035)	-0.478 ± 0.222	0.032	YES
528071,Firmicutes;*Clostridia* ;*Clostridiales*	1.551 (0.981)	-0.630 ± 0.205	0.017	NO
542830,Cyanobacteria;*4C0d-2* ;*YS2*	2.340 (1.800)	-1.313 ± 0.309	0.011	NO
550894,Cyanobacteria;*4C0d-2* ;*YS2*	3.651 (1.871)	-1.713 ± 0.219	0.000	YES
559149,Cyanobacteria;*4C0d-2* ;*YS2*	2.575 (1.424)	-0.652 ± 0.223	0.028	NO
571111,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae* ;*Ruminococcus*	2.314 (1.773)	-0.675 ± 0.199	0.006	NO
581388,Cyanobacteria;*4C0d-2* ;*YS2*	3.847 (1.935)	-2.034 ± 0.173	0.000	YES
584263,Firmicutes;*Clostridia* ;*Clostridiales*	4.379 (1.568)	-0.541 ± 0.174	0.015	NO
589410,Cyanobacteria;*4C0d-2* ;*YS2*	1.649 (1.468)	-1.544 ± 0.190	0.000	YES
640999,Firmicutes;*Clostridia* ;*Clostridiales*	5.065 (1.527)	-0.576 ± 0.171	0.015	NO
646499,Firmicutes;*Clostridia* ;*Clostridiales*	3.368 (1.567)	-1.009 ± 0.224	0.006	YES
715152,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae*	3.609 (1.625)	-0.418 ± 0.204	0.063	YES
769075,Firmicutes;*Clostridia* ;*Clostridiales*	2.980 (1.151)	-0.830 ± 0.202	0.000	NO
772972,Firmicutes;*Clostridia* ;*Clostridiales*	4.768 (1.378)	-0.563 ± 0.163	0.011	NO
786646,Firmicutes;*Clostridia* ;*Clostridiales*	1.614 (1.073)	-0.545 ± 0.206	0.034	YES
NR102,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae*	8.501 (0.671)	-0.302 ± 0.126	0.050	NO
NR108,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae*	6.741 (2.712)	-0.382 ± 0.168	0.050	NO
NR116,Firmicutes;*Clostridia* ;*Clostridiales*	3.599 (2.915)	-0.608 ± 0.175	0.000	NO
NR117,Firmicutes;*Clostridia* ;*Clostridiales*	4.705 (1.478)	-0.845 ± 0.169	0.000	NO
NR142,Proteobacteria;*Alphaproteobacteria*	3.271 (1.553)	-1.075 ± 0.220	0.000	YES
NR163,Tenericutes;*Mollicutes* ;*RF39*	3.366 (1.355)	-0.540 ± 0.176	0.024	NO
NR173,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae*	4.582 (0.864)	-0.798 ± 0.135	0.000	YES
NR177,Bacteroidetes;*Bacteroidia* ;*Bacteroidales* ;*S24-7*	2.379 (3.298)	-0.416 ± 0.139	0.006	NO
NR19,Firmicutes;*Clostridia* ;*Clostridiales*	4.534 (1.063)	-0.585 ± 0.142	0.000	YES
NR194,Tenericutes;*Mollicutes* ;*RF39*	5.479 (0.968)	-0.513 ± 0.152	0.017	NO
NR211,Tenericutes;*Mollicutes* ;*RF39*	2.331 (1.948)	-0.343 ± 0.119	0.017	NO
NR23,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae*	4.874 (0.777)	-0.526 ± 0.167	0.031	YES
NR242,Firmicutes;*Clostridia* ;*Clostridiales*	4.016 (2.260)	-0.677 ± 0.183	0.015	NO

OUT ID and taxonomical assignment	Mean abundance (CSS OTU units) (SD)	Difference Cecum-Feces ± SE	P_{FDR}	[a]Discriminant sPLS-DA
NR247,Firmicutes;*Clostridia* ;*Clostridiales*	1.782 (1.195)	-0.788 ± 0.191	0.006	YES
NR256,Tenericutes;*Mollicutes* ;*RF39*	2.871 (1.866)	-0.653 ± 0.247	0.037	NO
NR28,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae*	7.464 (1.893)	-1.860 ± 0.180	0.000	YES
NR294,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae* ;*Oscillospira*	6.143 (1.036)	-0.953 ± 0.199	0.000	YES
NR295,Bacteroidetes;*Bacteroidia* ;*Bacteroidales* ;*S24-7*	2.556 (3.770)	-0.267 ± 0.097	0.037	NO
NR307,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae*	5.790 (0.696)	-0.428 ± 0.131	0.011	NO
NR310,Firmicutes;*Clostridia* ;*Clostridiales*	4.732 (2.305)	-0.957 ± 0.167	0.000	NO
NR32,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae* ;*Ruminococcus*	2.656 (1.504)	-0.571 ± 0.230	0.039	NO
NR338,Firmicutes;*Clostridia* ;*Clostridiales*	4.746 (1.384)	-0.825 ± 0.149	0.000	YES
NR339,Firmicutes;*Clostridia* ;*Clostridiales*	4.439 (1.403)	-0.721 ± 0.182	0.000	NO
NR349,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae* ;*Ruminococcus*	3.281 (1.246)	-0.829 ± 0.259	0.021	NO
NR361,Firmicutes;*Clostridia* ;*Clostridiales*	3.909 (1.022)	-0.767 ± 0.150	0.000	YES
NR37,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae* ;*Oscillospira*	2.407 (1.276)	-0.853 ± 0.267	0.006	NO
NR395,Firmicutes;*Clostridia* ;*Clostridiales*	6.412 (1.790)	-0.901 ± 0.243	0.006	NO
NR410,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae*	3.770 (1.750)	-0.363 ± 0.131	0.021	YES
NR411,Proteobacteria;*Alphaproteobacteria* ;*RF32*	1.990 (1.551)	-1.214 ± 0.255	0.000	YES
NR414,Firmicutes;*Clostridia* ;*Clostridiales*	4.655 (2.255)	-0.628 ± 0.222	0.048	NO
NR423,Firmicutes;*Clostridia* ;*Clostridiales*	3.846 (1.439)	-0.921 ± 0.175	0.000	NO
NR448,Tenericutes;*Mollicutes* ;*RF39*	4.128 (0.975)	-0.542 ± 0.141	0.006	NO
NR456,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae* ;*Ruminococcus*	1.022 (1.316)	-0.784 ± 0.192	0.011	NO
NR461,Tenericutes;*Mollicutes* ;*RF39*	2.820 (1.325)	-0.384 ± 0.110	0.017	NO
NR57,Firmicutes;*Clostridia* ;*Clostridiales* ;*Ruminococcaceae*	3.603 (2.298)	-2.503 ± 0.311	0.000	NO
NR60,Firmicutes;*Clostridia* ;*Clostridiales*	2.944 (2.283)	-2.247 ± 0.201	0.000	NO
NR87,Firmicutes;*Clostridia* ;*Clostridiales* ;*Lachnospiraceae* ;*Coprococcus*	3.547 (2.668)	-0.480 ± 0.129	0.021	NO

[a]Last column indicates whether the OTU belongs to component 1 of sPLS-DA.

Supplementary material 3.S3| Representative sequences of the 10 OTUs most differentially represented between fecal and cecal samples.

>**NR57**

GTGTCAGCCGCCGCGGTAATACGTAGGGAGCGAGCGTTGTCCGGAATTACTGGGTGTAAAGGGAGTGT
AGGCGGGACTGTAAGTCAGATGTGAAATGTAGGGGCTCAACCCCTGCCCTGCATTTGAAACTGTAGTTCT
TGAGTGAAGTAGAGGTAAGCGGAATTCCTAGTGTAGCGGTGAAATGCGTAGATATTAGGAGGAACATCA
GTGGCGAAGGCGGCTTACTGGGCTTTTACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATT
AGATACCCTGGTAGTCCACGCTGTAAACGATGATCACTAGGTGTGGGGGGACTGACCCCTTCCGTGCCG
CAGTTAACACAATAAGTGATCCACCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGAAATTGACGG

>NR60

GTGTCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTATCCGGATTTACTGGGTGTAAAGGGAGCGTA
GGTGGCGGTGCAAGTCAGAAGTGAAATGCCGGGGCTCAACCCCGGAGCTGCTTTTGTAACTGCACAGCT
GGAGTGCAGGAGGGGTAAGCGGAATTCCTAGTGTAGCGGTGAAATGCGTAGATATTAGGAGGAACACCG
GTGGCGAAGGCGGCTTACTGGACTGTAACTGACACTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATT
AGATACCCTGGTAGTCCACGCCGTAAACGATGAATACTAGGTGTCGGGGAGCATCAGCTCTTCGGTGCC
GCAGCCAACGCAATAAGTATTCCACCTGGGGAGTACGTTCGCAAGAATGAAACTCAAATGAATTGGCGG

>581388

GTGTCAGCAGCCGCGGTAATACGGGGGGTGCAAGCGTTGTCCGGAATCATTGGGCGTAAAGCGTTCGT
AGGCGGCATGCCAAGTCTGGTGTTAAATCCCGGGGCTCAACTCCGGTCAAGCATTGGATACTGGTAAGC
TAGAATGTGGTAGAGGTTAAGGGAATTCCTGGTGTAGCGGTGAAATGCGTAGATATCAGGAGGAACACC
GGTGGCGTAAGCGCTTAACTGGGCCATAATTGACGCTGAGGAACGAAAGCCGGGGTAGCAAATGGGATT
AGATACCCCAGTAGTCCCGGCTGTAAACGATGGATACTAGGTGTTGCGGGTATCGACCCCTGCAGTGCC
GCAGCCAACGCGATAAGTATCCCGCCTGGGGAGTACGCACGCAAGTGTGAAACTCAAAGAAATTGACGG

>NR28

GTGTCAGCAGCCGCGGTAATACGTAGGGAGCGAGCGTTGTCCGGAATTACTGGGTGTAAAGGGAGCGT
AGGCGGGGTTGCAAGTCAGATGTGAAAAGTAGGGGCTTAACCCCTGAACTGCATTTGAAACTGTAATTCT
TGAGTGAAGTAGAGGTAAGCGGAATTCCTAGTGTAGCGGTGAAATGCGTAGATATTAGGAGGAACATCA
GTGGCGAAGGCGGCTTACTGGGCTTTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATT
AGATACCCTGGTAGTCCACGCTGTAAACGATGATCACTAGGTGTGGGGGGATAAGGACCCTTCCGTGCC
GCAGTTAACACAATAAGTGATCCACCTGGGGAGTACGGTCGCAAGGCTGAAACTCAAATAAATTGACGG

>550894

GTGTCAGCAGCCGCGGTAATACGGGGGGTGCAAGCGTTGTCCGGAATCATTGGGCGTAAAGCGTTCGT
AGGCGGCATGCCAAGTCTGGTGTTAAATCCCGGGGCTCAACTCCGGTCAAGCATTGGATACTGGTAAGC
TAGAATGTGGTAGAGGTTAAGGGAATTCCTGGTGTAGCGGTGAAATGCGTAGATATCAGGAGGAACACC
GGTGGCGTAAGCGCTTAACTGGGCCATAATTGACGCTGAGGAACGAAAGCCGGGGTAGCAAATGGGATT
AGATACCCCAGTAGTCCCGGCTGTAAACGATGGATACTAGGTGTTGCGGGTATCGACCCCTGCAGTGCC
GCAGCCAACGCGATAAGTATCCCGCCTGGGGAGTACGCACGCAAGTGTGAAACTCAAATAAATTGACGG

>NR12

GTGTCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTATCCGGATTTACTGGGTGTAATGGGAGCGCA
GGCGGCGATGCAAGCCAGAAGTGAAAACCCGGGGCCCAACCCCGCGGATTGCTTTTGGAACTGTGTTG
CTGGAGTGCAGGAGAGGCAAGCGGAATTCCTGGTGTAGCGGTGAAATGCGTAGATATCAGGAGGAACA
CCGGTGGTGAAGGCGGCTTGCTGGACTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGG
ATTAGATACCCTGGTAGTCCACGCGGTAAACGATGAATACTAGGTGTCGGTGTGCAAAGCGCATCGGTG
CCGCAGCTAACGCAGTAAGTATTCCACCTGGGGAGTACGTTCGCAAGAATGAAACTCAAATAAATTGACG
G

>589410

GTGTCAGCAGCCGCGGTAATACGGGGGGTGCAAGCGTTGTCCGGAATCATTGGGCGTAAAGCGTTCGT
AGGCGGTATGTCAAGTCTGGTGTTAAATCCCGGGGCTTAACTCCGGTCCAGCATTGGATACTGGCAAACT
AGAATGTGGTAGAGGTAAAGGGAATTCCTGGTGTAGCGGTGAAATGCGTAGATATCAGGAGGAACACCG
GTGGCGTAAGCGCTTTACTGGGCCATAATTGACGCTGAGGAACGAAAGCCGGGGGAGCAAATGGGATTA
GATACCCCAGTAGTCCCGGCCGTAAACGATGGATACTAGGTGTTGCGGGTATCGACCCCTGCAGTGCCG
CAGCCAACGCGATAAGTATCCCGCCTGGGGAGTACGCACGCAAGTGTGAAACTCAAAGAAATTGACGG

>542830

GTGTCAGCAGCCGCGGTAATACGGGGGGTGCAAGCGTTGTCCGGAATCATTGGGCGTAAAGCGTTCGT
AGGCGGCATGCCAAGTCTGGTGTTAAATCCCGGGGCTCAACTCCGGTCAAGCATTGGATACTGGTAAGC
TAGAATGTGGTAGAGGTTAAGGGAATTCCTGGTGTAGCGGTGAAATGCGTAGATATCAGGAGGAACACC
GGTGGCGTAAGCGCTTAACTGGGCCATAATTGACGCTGAGGAACGAAAGCCGGGGTAGCAAATGGGATT
AGATCCCCCAGTAGTCCCGGCTGTAAACGATGGATACTAGGTGTTGCGGGTATCGACCCCTGCAGTGCC
GCAGCCAACGCGATAAGTATCCCGCCTGGGGAGTACGCACGCAAGTGTGAAACTCAAATAAATTGGCGG

>NR411

GTGCCAGCAGCCGCGGTAATACGAAGGGTGCGAGCGTTGTTCGGAATTACTGGGCGTAAAGGGTGAGT
AGGCGGTTTAGTAAGATAGCGGTGAAATGCCAGAGCTTAACTTTGGAATTGCCGTTATAACTATTAAGCTA
GAGTGACAGAGAGGATATTGGAATACCCAGTGTAGAGGTGAAATTCGTAGATATTGGGTAGAACACCGGT
GGCGAAGGCGAGTATCTGGCTGTAGACTGACGCTGAGGCACGAAAGCATGGGGATCAAACAGGATTAG
ATACCCTGGTAGTCCATGCTGTAAACGATGAATGCTAGTTGTTGGTAGGGATCAGTGACGAAGCAAACGC
GATAAGCATTCCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATAAATTGACGG

>197832

GTGTCAGCAGCCGCGGTAATACGTATGGTGCAAGCGTTATCCGGATTTACTGGGTGTAAAGGGTGCGTA
GGTGGTGAGACAAGTCTGAAGTGAAAATCCGGGGCTCAACCCCGGAACTGCTTTGGAAACTGCCTGACT
GGAGTACAGGAGAGGTAAGTGGAATTCCTAGTGTAGCGGTGAAATGCGTAGATATTAGGAGGAACACCA
GTGGCGAAGGCGACTTACTGGACTGTAACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTA
GATACCCTGGTAGTCCACGCCGTAAACGATGAATACTAGGTGTCGGGGCCCAAAGGGCTTCGGTGCCGC
AGCAAACGCAATAAGTATTCCACCTGGGGAGTACGTTCGCAAGAATGAAACTCAAAGAAATTGACGG

Supplementary material of chapter 4

Additional file 4.1| Metadata associated with the 425 rabbit cecal samples analyzed in this study. Open access file available in:
<https://static-content.springer.com/esm/art%3A10.1186%2Fs42523-020-00059-z/MediaObjects/42523_2020_59_MOESM1_ESM.txt>

Additional file 4.2| Prefiltered and unnormalized OTU table used for statistical analyses in this study. Open access file available in:
<https://static-content.springer.com/esm/art%3A10.1186%2Fs42523-020-00059-z/MediaObjects/42523_2020_59_MOESM2_ESM.txt>

Additional file 4.3| Filtered and CSS-normalized OTU table used for statistical analyses in this study. Open access file available in:
<https://static-content.springer.com/esm/art%3A10.1186%2Fs42523-020-00059-z/MediaObjects/42523_2020_59_MOESM3_ESM.txt>

Additional file 4.4| Taxonomic assignments for all OTUs in **Additional file 4.2**. Open access file available in:
<https://static-content.springer.com/esm/art%3A10.1186%2Fs42523-020-00059-z/MediaObjects/42523_2020_59_MOESM4_ESM.txt>

Additional file 4.5| Relative abundances phyla table built from the collapse of the filtered and CSS-normalized OTU table at phylum level. Open access file available in:
<https://static-content.springer.com/esm/art%3A10.1186%2Fs42523-020-00059-z/MediaObjects/42523_2020_59_MOESM5_ESM.txt>

Additional file 4.6| Relative abundances genera table built from the collapse of the filtered and CSS-normalized OTU table at genus level. Open access file available in:
<https://static-content.springer.com/esm/art%3A10.1186%2Fs42523-020-00059-z/MediaObjects/42523_2020_59_MOESM6_ESM.txt>

Additional file 4.7: Table 4.S1| OTUs differentially represented between farms. Open access file available in:
<https://static-content.springer.com/esm/art%3A10.1186%2Fs42523-020-00059-z/MediaObjects/42523_2020_59_MOESM7_ESM.xlsx>

Additional file 4.8: Table 4.S2| OTUs differentially represented between feeding regimes within farms. Open access file available in:
<https://static-content.springer.com/esm/art%3A10.1186%2Fs42523-020-00059-z/MediaObjects/42523_2020_59_MOESM8_ESM.xlsx>

Additional file 4.9: Table 4.S3| OTUs differentially represented between the presence and the absence of antibiotics in the feed within farm B. Open access file available in:
<https://static-content.springer.com/esm/art%3A10.1186%2Fs42523-020-00059-z/MediaObjects/42523_2020_59_MOESM9_ESM.xlsx>

Supplementary material of chapter 5

Additional file 5.1| Metadata associated with the 425 rabbit cecal samples analyzed in this study. Open access file available in:
<https://assets.researchsquare.com/files/rs-441480/v2/81c124f00fef556f3e406320.txt>

Additional file 5.2| Filtered and CSS-normalized OTU table used for statistical analyses in this study. Open access file available in:
<https://assets.researchsquare.com/files/rs-441480/v2/f309430619736a45512248e4.txt>

Additional file 5.3| Taxonomic assignments for all OTUs in **Additional file 5.2**. Open access file available in:
<https://assets.researchsquare.com/files/rs-441480/v2/a1652edb8b43b05687e633a3.txt>

Additional file 5.4| Trace plots and histograms of Markov chains from the posterior distribution of the parameters of Bayesian models. Open access file available in:
<https://assets.researchsquare.com/files/rs-441480/v2/65b733c3435e47d6a382835b.rar>

Additional file 5.5| Representative sequences of the OTUs selected in the sPLSR analysis for $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$. Open access file available in:
<https://assets.researchsquare.com/files/rs-441480/v2/7d9ff4471e5f333b41caa905.txt>

Additional file 5.6: Table 5.S1| Relevant OTUs for the prediction of individual traits ($\mathrm{ADG}_{\mathrm{AL}}$ and $\mathrm{ADG}_{\mathrm{R}}$) and cage-average traits ($\overline{\mathrm{ADFI}}_{\mathrm{AL}}$, $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$ and $\overline{\mathrm{ADFCRI}}_{\mathrm{AL}}$). Open access file available in:
<https://assets.researchsquare.com/files/rs-441480/v2/c32a6505951c3f305e5c12e7.docx>

Additional file 5.7| Representative sequences of the OTUs relevant OTUs for the prediction of individual traits ($\mathrm{ADG}_{\mathrm{AL}}$ and $\mathrm{ADG}_{\mathrm{R}}$) and cage-average traits ($\overline{\mathrm{ADFI}}_{\mathrm{AL}}$, $\overline{\mathrm{ADRFI}}_{\mathrm{AL}}$ and $\overline{\mathrm{ADFCRI}}_{\mathrm{AL}}$) in **Additional file 5.6**. Open access file available in:
<https://assets.researchsquare.com/files/rs-441480/v2/31214be05c28cbb66d9bca89.txt>

Supplementary material of chapter 6

Additional file 6.1| Metadata associated with the 425 rabbit cecal samples analyzed in this study. Open access file available in:
<https://assets.researchsquare.com/files/rs-441480/v2/81c124f00fef556f3e406320.txt>

Additional file 6.2| Filtered and CSS-normalized OTU table. Open access file available in:
<https://assets.researchsquare.com/files/rs-441480/v2/f309430619736a45512248e4.txt>

Additional file 6.3| Taxonomic assignment of representative sequences of each OTU in **Additional file 6.2**. Open access file available in:
<https://assets.researchsquare.com/files/rs-441480/v2/a1652edb8b43b05687e633a3.txt>

Additional file 6.4: Table 6.S1| Bayes factors, marginal posterior means and standard deviations of heritability for OTUs under genetic control together with the associated probability of these estimates being greater than 0.10.

Adjusted with the ZIP model

OTU ID	Mean h^2	Standard deviation h^2	$P(h^2 > 0.1)$	Bayes factor[1]	Frequency of presence (%)	Kingdom	Phylum	Class	Order	Family	Genus	Species
278912	0.24	0.18	0.76	3.21	11	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7		
356011	0.23	0.17	0.74	3.30	7	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus	
339336	0.24	0.17	0.74	3.43	6	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU4438	0.24	0.18	0.75	3.49	6	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7		
190844	0.25	0.18	0.76	3.50	11	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae		
New.ReferenceOTU3820	0.25	0.18	0.76	3.67	6	Bacteria	Firmicutes	Clostridia	Clostridiales			
278675	0.25	0.18	0.76	4.19	10	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7		
1517779	0.28	0.19	0.79	4.88	16	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae		
New.ReferenceOTU1306	0.12	0.10	0.47	7.45	22	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7		
124470	0.26	0.18	0.79	7.81	20	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Odoribacteraceae	Butyricimonas	
1105984	0.18	0.15	0.65	11.37	7	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	

[1]Bayes factor of the model with additive genetic effects against the same model without additive genetic effects.

Adjusted with the LMM model

OTU ID	Mean h^2	Standard deviation h^2	$P(h^2 > 0.1)$	Bayes factor[1]	Frequency of presence (%)	Kingdom	Phylum	Class	Order	Family	Genus	Species
New.ReferenceOTU501	0.16	0.11	0.65	3.23	42	Unassigned						
New.ReferenceOTU2836	0.16	0.11	0.66	3.25	85	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU782	0.18	0.12	0.69	3.26	53	Unassigned						
204542	0.21	0.15	0.72	3.26	22	Bacteria	Firmicutes	Clostridia	Clostridiales			
361679	0.19	0.14	0.71	3.28	60	Bacteria	Firmicutes	Clostridia	Clostridiales			
351253	0.19	0.13	0.70	3.28	89	Bacteria	Firmicutes	Clostridia	Clostridiales			
207713	0.17	0.12	0.66	3.37	66	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU1282	0.19	0.13	0.71	3.37	48	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	
291090	0.21	0.15	0.74	3.43	60	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides	distasonis
New.ReferenceOTU1848	0.19	0.13	0.71	3.51	97	Unassigned						
New.ReferenceOTU4284	0.19	0.14	0.70	3.54	83	Bacteria	Firmicutes	Clostridia	Clostridiales			
349892	0.16	0.11	0.66	3.54	84	Bacteria	Tenericutes	Mollicutes	RF39			
New.ReferenceOTU2040	0.15	0.10	0.64	3.58	78	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		
352533	0.18	0.12	0.71	3.59	51	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU1251	0.15	0.09	0.64	3.60	53	Bacteria	Firmicutes	Clostridia	Clostridiales			
328083	0.20	0.14	0.71	3.64	98	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	
192364	0.20	0.14	0.73	3.67	53	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU3211	0.19	0.13	0.72	3.69	88	Unassigned						
New.ReferenceOTU3011	0.21	0.16	0.72	3.70	46	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU696	0.16	0.10	0.66	3.71	90	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU3003	0.13	0.08	0.59	3.76	92	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU1474	0.15	0.09	0.64	3.79	75	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	
New.ReferenceOTU1727	0.18	0.12	0.71	3.80	97	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	
New.ReferenceOTU4513	0.21	0.14	0.74	3.81	56	Bacteria	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Akkermansia	
New.ReferenceOTU2138	0.18	0.13	0.70	3.82	94	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		
577562	0.20	0.14	0.72	3.89	94	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU2476	0.16	0.11	0.66	3.92	46	Bacteria	Tenericutes	Mollicutes	RF39			
New.ReferenceOTU733	0.18	0.12	0.69	3.97	64	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7		
449833	0.19	0.13	0.72	4.03	72	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7		
988375	0.25	0.19	0.74	4.04	60	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Odoribacteraceae	Butyricimonas	
New.ReferenceOTU2545	0.22	0.15	0.77	4.05	60	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus	
New.ReferenceOTU1309	0.22	0.14	0.76	4.10	39	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	
New.ReferenceOTU3985	0.21	0.14	0.73	4.12	55	Unassigned						
New.ReferenceOTU146	0.23	0.15	0.78	4.13	43	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU4073	0.23	0.16	0.75	4.16	69	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU2650	0.18	0.12	0.72	4.17	44	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		
348602	0.21	0.14	0.74	4.18	44	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU2932	0.18	0.11	0.71	4.27	24	Bacteria	Firmicutes	Clostridia	Clostridiales			
720944	0.17	0.11	0.70	4.28	72	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira	
New.ReferenceOTU2465	0.18	0.13	0.68	4.36	54	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Barnesiellaceae		
New.ReferenceOTU1405	0.21	0.14	0.75	4.40	25	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		
New.ReferenceOTU3430	0.20	0.13	0.74	4.43	31	Bacteria	Firmicutes	Clostridia	Clostridiales			
332732	0.22	0.14	0.76	4.52	66	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	
New.ReferenceOTU606	0.22	0.15	0.76	4.53	65	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira	
1110378	0.19	0.12	0.73	4.59	93	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira	
874627	0.18	0.12	0.72	4.60	80	Bacteria	Tenericutes	Mollicutes	RF39			
New.ReferenceOTU1396	0.18	0.11	0.71	4.70	91	Unassigned						
New.ReferenceOTU768	0.16	0.10	0.67	4.71	79	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU4565	0.20	0.13	0.76	4.72	84	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU3530	0.24	0.15	0.79	4.78	70	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU1836	0.22	0.14	0.78	4.84	53	Bacteria	Firmicutes	Clostridia	Clostridiales	Mogibacteriaceae		
105659	0.19	0.12	0.74	4.97	85	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		
569030	0.23	0.16	0.74	4.98	49	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Rikenella	
New.ReferenceOTU3424	0.16	0.10	0.67	4.99	68	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Epulopiscium	
589277	0.14	0.11	0.57	5.03	37	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	
New.ReferenceOTU2377	0.25	0.17	0.80	5.15	96	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU4593	0.23	0.16	0.75	5.21	25	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU4338	0.23	0.16	0.75	5.29	48	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Rikenella	
575041	0.23	0.16	0.77	5.30	74	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae		
New.ReferenceOTU370	0.22	0.14	0.77	5.34	98	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU4624	0.25	0.16	0.80	5.37	33	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU2439	0.20	0.13	0.76	5.44	94	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		
New.ReferenceOTU331	0.18	0.11	0.71	5.45	55	Bacteria	Proteobacteria	Alphaproteobacteria				
New.ReferenceOTU4158	0.21	0.15	0.72	5.46	30	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Rikenella	
New.ReferenceOTU2607	0.19	0.12	0.74	5.54	93	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		
New.ReferenceOTU3465	0.23	0.14	0.78	5.56	47	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU2285	0.20	0.12	0.75	5.61	61	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		
533198	0.20	0.13	0.76	5.63	94	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Oxalobacter	
New.ReferenceOTU669	0.17	0.11	0.70	5.74	43	Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter	
208479	0.28	0.19	0.79	5.75	57	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Odoribacteraceae	Butyricimonas	
New.ReferenceOTU1425	0.20	0.12	0.76	5.76	99	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		
New.ReferenceOTU4299	0.21	0.13	0.76	5.76	75	Bacteria	Firmicutes	Clostridia	Clostridiales			
581388	0.16	0.10	0.69	5.77	91	Bacteria	Cyanobacteria	4C0d-2	YS2			
New.ReferenceOTU2816	0.18	0.11	0.72	5.77	83	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU1951	0.20	0.12	0.76	5.80	75	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU4080	0.18	0.11	0.72	5.86	85	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU761	0.23	0.15	0.79	5.91	50	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus	
New.ReferenceOTU3591	0.21	0.15	0.72	5.95	63	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	
449353	0.25	0.17	0.78	6.08	51	Bacteria	Firmicutes	Clostridia	Clostridiales	Dehalobacteriaceae	Dehalobacterium	
New.ReferenceOTU2222	0.23	0.15	0.79	6.28	54	Unassigned						
157017	0.19	0.12	0.75	6.29	49	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU1618	0.24	0.16	0.76	6.36	20	Unassigned						
New.ReferenceOTU2019	0.21	0.13	0.77	6.54	81	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus	
351272	0.21	0.14	0.74	6.57	75	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	
524318	0.20	0.13	0.73	6.63	32	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	
New.ReferenceOTU1639	0.17	0.11	0.71	6.71	22	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
216710	0.21	0.13	0.77	6.81	98	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU3176	0.29	0.18	0.83	6.89	92	Unassigned						
297503	0.26	0.17	0.80	6.94	79	Bacteria	Firmicutes	Clostridia	Clostridiales			
288379	0.27	0.16	0.83	6.97	62	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU381	0.30	0.19	0.84	7.03	52	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		
556126	0.21	0.14	0.74	7.13	66	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	
New.ReferenceOTU465	0.25	0.15	0.82	7.16	75	Bacteria	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae		
364179	0.26	0.17	0.80	7.33	84	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	
New.ReferenceOTU181	0.30	0.19	0.84	7.41	52	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU2839	0.24	0.15	0.81	7.48	65	Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae		
342182	0.24	0.15	0.81	7.52	60	Bacteria	Firmicutes	Clostridia	Clostridiales			
4299126	0.23	0.14	0.80	7.56	34	Bacteria	Proteobacteria	Alphaproteobacteria	RF32			
New.ReferenceOTU719	0.21	0.13	0.79	7.65	62	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU1081	0.23	0.14	0.81	7.97	43	Unassigned						
New.ReferenceOTU1033	0.17	0.10	0.73	8.09	81	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	
293097	0.18	0.11	0.76	8.38	70	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU447	0.21	0.13	0.80	8.62	60	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU1725	0.20	0.12	0.78	8.94	64	Bacteria	Tenericutes	Mollicutes	RF39			
New.ReferenceOTU2626	0.27	0.16	0.84	8.98	56	Unassigned						
New.ReferenceOTU1922	0.18	0.11	0.76	9.16	77	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		
351231	0.26	0.17	0.81	9.57	31	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	fragilis
234488	0.26	0.17	0.81	9.62	49	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	
New.ReferenceOTU4137	0.23	0.14	0.81	10.05	35	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU2893	0.26	0.15	0.84	10.14	96	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU4373	0.32	0.20	0.85	10.20	61	Unassigned						
New.ReferenceOTU2585	0.21	0.12	0.80	11.57	92	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU1611	0.27	0.16	0.82	11.62	20	Unassigned						
New.ReferenceOTU3833	0.25	0.14	0.85	11.73	91	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		
339013	0.26	0.16	0.84	12.44	63	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	
208769	0.23	0.13	0.83	12.56	100	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU787	0.24	0.13	0.84	12.63	61	Bacteria	Proteobacteria	Alphaproteobacteria	RF32			
New.ReferenceOTU1514	0.23	0.13	0.84	14.27	90	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus	
849440	0.25	0.14	0.85	14.79	63	Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter	
New.ReferenceOTU3868	0.21	0.11	0.81	15.33	96	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
346794	0.23	0.12	0.84	16.61	80	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU369	0.31	0.18	0.89	16.67	66	Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio	
New.ReferenceOTU3327	0.24	0.13	0.85	16.97	28	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae		
New.ReferenceOTU1353	0.26	0.14	0.86	17.80	67	Unassigned						
New.ReferenceOTU3047	0.26	0.14	0.86	17.82	63	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7		
New.ReferenceOTU3335	0.25	0.14	0.87	19.11	66	Bacteria	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae		
798164	0.23	0.13	0.84	19.83	73	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU570	0.19	0.10	0.77	21.33	41	Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Phascolarctobacterium	
385545	0.24	0.13	0.86	22.63	39	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU866	0.24	0.12	0.88	23.55	91	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU1234	0.39	0.20	0.93	25.84	80	Bacteria	Firmicutes	Clostridia	Clostridiales			
334383	0.22	0.11	0.82	26.21	16	Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Phascolarctobacterium	
585989	0.21	0.10	0.82	27.62	17	Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Phascolarctobacterium	
New.ReferenceOTU2680	0.33	0.17	0.91	27.88	74	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU3143	0.24	0.12	0.88	28.65	71	Bacteria	Firmicutes	Clostridia	Clostridiales			
305608	0.24	0.11	0.87	42.21	16	Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Phascolarctobacterium	
New.ReferenceOTU1774	0.35	0.17	0.94	42.42	83	Unassigned						
New.ReferenceOTU1188	0.27	0.13	0.90	51.88	33	Unassigned						
953855	0.34	0.16	0.94	56.02	99	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae		
New.ReferenceOTU1150	0.40	0.18	0.95	56.93	68	Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae		
349809	0.32	0.13	0.94	94.09	29	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	eggerthii
851323	0.22	0.08	0.92	148.58	54	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides	
577377	0.16	0.07	0.83	170.18	23	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	

[1]Bayes factor of the model with additive genetic effects against the same model without additive genetic effects.

Additional file 6.5: Table 6.S2| Bayes factors, marginal posterior means and standard deviations of litter variance ratio for OTUs influenced by litter effects together with the associated probability of these estimates being greater than 0.10.

Adjusted with the ZIP model

OTU ID	Mean l^2	Standard deviation l^2	$P(l^2 > 0.1)$	Bayes factor[1]	Frequency of presence (%)	Kingdom	Phylum	Class	Order	Family	Genus	Species
New.ReferenceOTU4438	0.18	0.14	0.66	3.35	6	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7		
1517779	0.19	0.14	0.69	3.70	16	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae		
278675	0.19	0.13	0.69	4.26	10	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7		
New.ReferenceOTU1306	0.35	0.27	0.73	12.07	22	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7		
124470	0.42	0.19	0.94	38.95	20	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Odoribacteraceae	Butyricimonas	
1105984	0.62	0.23	0.97	59.05	7	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	

[1]Bayes factor of the model with litter effects against the same model without litter effects.

Adjusted with the LMM model

OTU ID	Mean l^2	Standard deviation l^2	$P(l^2 > 0.1)$	Bayes factor[1]	Frequency of presence (%)	Kingdom	Phylum	Class	Order	Family	Genus	Species
New.ReferenceOTU4214	0.12	0.07	0.55	3.21	53	Bacteria	Firmicutes	Clostridia	Clostridiales			
297502	0.11	0.07	0.49	3.21	65	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		
New.ReferenceOTU1350	0.11	0.07	0.51	3.22	97	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU945	0.11	0.07	0.48	3.23	35	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU3714	0.12	0.07	0.55	3.25	78	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	
754283	0.11	0.07	0.52	3.27	83	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU3370	0.11	0.07	0.49	3.34	97	Bacteria	Tenericutes	Mollicutes	RF39			
New.ReferenceOTU1848	0.12	0.08	0.55	3.37	97	Unassigned						
New.ReferenceOTU2864	0.12	0.07	0.54	3.39	66	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU1188	0.11	0.07	0.51	3.42	33	Unassigned						
New.ReferenceOTU3047	0.10	0.06	0.42	3.48	63	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7		
New.ReferenceOTU4121	0.11	0.07	0.52	3.49	88	Unassigned						
New.ReferenceOTU1907	0.12	0.07	0.55	3.51	21	Bacteria	Firmicutes	Clostridia	Clostridiales			
531052	0.11	0.07	0.51	3.59	84	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Coprococcus	
New.ReferenceOTU4276	0.11	0.07	0.52	3.60	77	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Coprococcus	
New.ReferenceOTU2545	0.13	0.08	0.58	3.62	60	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus	
New.ReferenceOTU328	0.12	0.07	0.55	3.73	67	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU1282	0.11	0.07	0.52	3.75	48	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	
New.ReferenceOTU1336	0.12	0.07	0.56	3.77	80	Bacteria	Tenericutes	Mollicutes	Anaeroplasmatales	Anaeroplasmataceae	Anaeroplasma	
New.ReferenceOTU4083	0.11	0.07	0.52	3.78	65	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		
299422	0.12	0.07	0.56	3.79	88	Bacteria	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Akkermansia	
346794	0.12	0.08	0.56	3.86	80	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU2285	0.12	0.07	0.56	3.87	61	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		
279340	0.12	0.07	0.58	3.90	36	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU2264	0.10	0.06	0.49	3.93	74	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU2035	0.12	0.07	0.55	4.01	96	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU951	0.12	0.07	0.57	4.03	87	Bacteria	Firmicutes	Clostridia	Clostridiales			
4331760	0.12	0.07	0.54	4.06	75	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae		
New.ReferenceOTU2893	0.11	0.07	0.51	4.07	96	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU1631	0.11	0.07	0.53	4.15	60	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus	
585480	0.13	0.07	0.59	4.26	90	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Anaerostipes	
New.ReferenceOTU241	0.12	0.07	0.57	4.31	93	Unassigned						
New.ReferenceOTU362	0.12	0.07	0.54	4.46	55	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU3245	0.12	0.07	0.55	4.47	47	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus	
New.ReferenceOTU799	0.11	0.06	0.52	4.50	27	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU1266	0.12	0.07	0.55	4.50	80	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU1309	0.13	0.07	0.59	4.51	39	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	
New.ReferenceOTU2943	0.12	0.07	0.57	4.64	22	Bacteria	Firmicutes	Clostridia	Clostridiales			
590015	0.12	0.07	0.56	4.66	92	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU4255	0.11	0.07	0.53	4.69	86	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		
New.ReferenceOTU3530	0.12	0.08	0.57	4.77	70	Bacteria	Firmicutes	Clostridia	Clostridiales			
575101	0.13	0.08	0.60	4.83	88	Bacteria	Firmicutes	Clostridia	Clostridiales	Dehalobacteriaceae		
New.ReferenceOTU1557	0.12	0.07	0.59	4.83	94	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus	
New.ReferenceOTU1646	0.12	0.07	0.58	4.94	93	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU4534	0.13	0.08	0.60	4.94	95	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU95	0.12	0.07	0.57	4.94	65	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus	
New.ReferenceOTU2683	0.12	0.07	0.56	5.07	56	Bacteria	Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	Adlercreutzia	
563490	0.13	0.07	0.60	5.10	99	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU2797	0.13	0.07	0.60	5.15	54	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU4290	0.12	0.07	0.59	5.25	59	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira	
New.ReferenceOTU2303	0.12	0.07	0.57	5.29	41	Bacteria	Firmicutes	Clostridia	Clostridiales			
210867	0.12	0.07	0.59	5.37	26	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU501	0.13	0.07	0.61	5.60	42	Unassigned						
New.ReferenceOTU3810	0.12	0.07	0.56	5.95	49	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira	
New.ReferenceOTU3424	0.12	0.07	0.59	6.02	68	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Epulopiscium	
New.ReferenceOTU3628	0.13	0.07	0.61	6.12	82	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU1081	0.13	0.08	0.61	6.14	43	Unassigned						
New.ReferenceOTU4465	0.14	0.08	0.66	6.25	84	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU276	0.12	0.06	0.56	6.25	39	Bacteria	Firmicutes	Clostridia	Clostridiales			
352533	0.14	0.08	0.65	6.32	51	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU747	0.12	0.07	0.59	6.37	88	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	
336627	0.13	0.07	0.61	6.62	80	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU1100	0.13	0.07	0.62	6.78	77	Bacteria	Firmicutes	Clostridia	Clostridiales			
527988	0.14	0.08	0.64	7.02	93	Bacteria	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	Anaerofustis	
New.ReferenceOTU1883	0.13	0.08	0.62	7.05	37	Unassigned						
New.ReferenceOTU352	0.13	0.07	0.62	7.16	61	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU4513	0.14	0.08	0.65	7.23	56	Bacteria	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Akkermansia	
291090	0.14	0.08	0.65	7.31	60	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides	distasonis
New.ReferenceOTU2138	0.14	0.08	0.66	7.55	94	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		
New.ReferenceOTU107	0.13	0.07	0.64	7.65	74	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		
New.ReferenceOTU2222	0.13	0.07	0.61	7.70	54	Unassigned						
New.ReferenceOTU4299	0.14	0.08	0.65	8.02	75	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU1597	0.14	0.07	0.65	8.28	81	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Butyrivibrio	
537219	0.14	0.08	0.67	8.28	78	Bacteria	Firmicutes	Clostridia	Clostridiales			
292871	0.14	0.08	0.66	8.55	76	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU2233	0.14	0.07	0.64	8.58	68	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	
New.ReferenceOTU369	0.15	0.09	0.68	8.68	66	Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio	
New.ReferenceOTU3332	0.14	0.07	0.66	8.71	35	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7		
304037	0.14	0.07	0.65	9.05	73	Bacteria	Firmicutes	Clostridia	Clostridiales			

214031	0.15	0.08	0.70	9.12	38	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae		
New.ReferenceOTU3728	0.14	0.08	0.67	9.74	87	Unassigned						
New.ReferenceOTU3368	0.14	0.07	0.66	10.09	21	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU761	0.15	0.08	0.69	10.20	50	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus	
299902	0.15	0.08	0.70	10.38	89	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	
New.ReferenceOTU432	0.15	0.08	0.71	10.62	97	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Coprococcus	
288379	0.17	0.09	0.73	11.50	62	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU4349	0.14	0.07	0.67	11.52	43	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU3465	0.16	0.09	0.71	11.69	47	Bacteria	Firmicutes	Clostridia	Clostridiales			
328083	0.15	0.08	0.72	11.72	98	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	
New.ReferenceOTU2377	0.16	0.09	0.71	11.96	96	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU4651	0.16	0.08	0.74	12.55	38	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus	
New.ReferenceOTU763	0.13	0.07	0.64	12.81	52	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira	
New.ReferenceOTU1695	0.16	0.08	0.73	12.85	80	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU344	0.16	0.08	0.73	12.92	74	Bacteria	Firmicutes	Clostridia	Clostridiales			
524842	0.15	0.07	0.72	13.62	73	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae		
450576	0.14	0.07	0.71	13.78	72	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU3316	0.15	0.08	0.73	14.17	81	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
348609	0.15	0.08	0.72	14.30	61	Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae		
New.ReferenceOTU892	0.16	0.08	0.76	14.31	76	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU4624	0.16	0.08	0.74	14.74	33	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
321135	0.15	0.08	0.73	14.78	87	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU1678	0.16	0.08	0.75	15.05	97	Unassigned						
342182	0.16	0.08	0.75	15.24	60	Bacteria	Firmicutes	Clostridia	Clostridiales			
348602	0.16	0.08	0.76	15.62	44	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU3054	0.16	0.08	0.73	15.96	91	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU3444	0.14	0.07	0.69	16.05	72	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU1383	0.16	0.08	0.76	16.17	98	Unassigned						
953855	0.15	0.08	0.70	16.45	99	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae		
New.ReferenceOTU2121	0.16	0.08	0.74	16.59	60	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU2626	0.17	0.09	0.75	16.68	56	Unassigned						
New.ReferenceOTU3063	0.16	0.08	0.74	16.78	50	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU1836	0.16	0.08	0.74	16.98	53	Bacteria	Firmicutes	Clostridia	Clostridiales	Mogibacteriaceae		
73753	0.16	0.08	0.75	17.03	86	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Odoribacteraceae	Odoribacter	
New.ReferenceOTU3739	0.17	0.08	0.78	17.54	72	Bacteria	Firmicutes	Clostridia	Clostridiales			
3579707	0.16	0.08	0.76	19.93	69	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Odoribacteraceae	Odoribacter	
346669	0.16	0.08	0.77	20.00	97	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU1490	0.16	0.08	0.76	20.98	91	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU146	0.18	0.09	0.80	21.63	43	Bacteria	Firmicutes	Clostridia	Clostridiales			
351253	0.17	0.08	0.78	24.18	89	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU4661	0.16	0.08	0.77	24.64	83	Bacteria	Firmicutes	Clostridia	Clostridiales			
207340	0.17	0.08	0.80	27.99	77	Bacteria	Firmicutes	Clostridia	Clostridiales	Mogibacteriaceae		
New.ReferenceOTU605	0.17	0.08	0.78	28.39	59	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU1979	0.18	0.09	0.82	28.89	78	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		
New.ReferenceOTU2627	0.18	0.08	0.81	29.65	42	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU3932	0.18	0.08	0.83	33.22	55	Bacteria	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Akkermansia	
New.ReferenceOTU3326	0.18	0.08	0.82	33.96	46	Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio	
New.ReferenceOTU1428	0.16	0.07	0.79	34.20	78	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU3176	0.20	0.09	0.83	34.79	92	Unassigned						
New.ReferenceOTU1824	0.16	0.07	0.78	34.87	84	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU3993	0.18	0.08	0.84	37.86	40	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
355312	0.17	0.07	0.81	42.24	68	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU2693	0.20	0.09	0.86	46.43	47	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU1234	0.19	0.10	0.79	49.03	80	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU1411	0.19	0.08	0.86	49.97	86	Bacteria	Firmicutes	Clostridia	Clostridiales			
268538	0.19	0.08	0.86	54.61	67	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		
301464	0.19	0.08	0.86	57.98	64	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU3555	0.20	0.09	0.86	65.39	36	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Rikenella	
New.ReferenceOTU4514	0.20	0.08	0.88	66.98	53	Unassigned						
192364	0.20	0.08	0.87	72.78	53	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU465	0.19	0.08	0.86	73.02	75	Bacteria	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae		
361679	0.21	0.09	0.89	87.47	60	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU2839	0.22	0.09	0.91	88.01	65	Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae		
577562	0.20	0.08	0.89	90.55	94	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU1343	0.19	0.08	0.88	93.62	99	Bacteria	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Akkermansia	
New.ReferenceOTU703	0.21	0.08	0.90	105.89	61	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU1363	0.20	0.08	0.90	123.25	59	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		
New.ReferenceOTU1196	0.23	0.09	0.93	139.96	90	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU1009	0.20	0.08	0.90	152.77	97	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU606	0.22	0.09	0.92	160.32	65	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira	
New.ReferenceOTU277	0.22	0.08	0.93	182.09	44	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU4073	0.23	0.09	0.93	192.17	69	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU3581	0.20	0.07	0.92	224.04	80	Bacteria	Firmicutes	Clostridia	Clostridiales			
337724	0.22	0.08	0.94	243.63	40	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7		
204542	0.23	0.08	0.94	260.10	22	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU1986	0.23	0.08	0.95	294.76	79	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU381	0.25	0.10	0.94	356.92	52	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		
350277	0.25	0.08	0.96	426.54	97	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	uniformis
New.ReferenceOTU1154	0.23	0.08	0.95	479.56	81	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		
New.ReferenceOTU181	0.25	0.09	0.94	489.48	52	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU4269	0.25	0.08	0.96	528.02	72	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		
New.ReferenceOTU1182	0.26	0.08	0.97	621.62	56	Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio	
New.ReferenceOTU2680	0.25	0.09	0.95	724.90	74	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
207713	0.25	0.08	0.97	1066.11	66	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU162	0.27	0.08	0.98	1269.21	48	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7		
New.ReferenceOTU4284	0.27	0.09	0.98	1320.53	83	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU3252	0.30	0.09	0.98	1402.68	75	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales			
New.ReferenceOTU3985	0.26	0.08	0.97	1697.22	55	Unassigned						
New.ReferenceOTU2875	0.30	0.09	0.99	1880.47	26	Bacteria	Firmicutes	Clostridia	Clostridiales			
339013	0.28	0.09	0.98	2492.78	63	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	
New.ReferenceOTU3837	0.28	0.08	0.99	2537.51	66	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae		
213671	0.32	0.09	1.00	5164.61	44	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae		
295427	0.29	0.08	0.99	7374.08	66	Bacteria	Firmicutes	Clostridia	Clostridiales			
364179	0.31	0.09	0.99	7998.31	84	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	
New.ReferenceOTU3468	0.27	0.08	0.99	8706.89	59	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		
New.ReferenceOTU3891	0.32	0.09	1.00	9251.03	34	Bacteria	Firmicutes	Clostridia	Clostridiales			
575041	0.31	0.09	0.99	9290.98	74	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae		
New.ReferenceOTU3011	0.34	0.09	1.00	19552.73	46	Bacteria	Firmicutes	Clostridia	Clostridiales			
208479	0.36	0.10	1.00	34701.55	57	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Odoribacteraceae	Butyricimonas	
988375	0.36	0.10	0.99	40595.52	60	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Odoribacteraceae	Butyricimonas	
297503	0.35	0.09	1.00	43638.28	79	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU4373	0.36	0.10	1.00	52325.23	61	Unassigned						
New.ReferenceOTU3880	0.34	0.08	1.00	62039.17	46	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU2892	0.30	0.08	1.00	76476.60	50	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU4338	0.38	0.09	1.00	455667.50	48	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Rikenella	
857827	0.39	0.08	1.00	742899.04	78	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales			
569030	0.39	0.09	1.00	942970.28	49	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Rikenella	
New.ReferenceOTU4130	0.38	0.08	1.00	1516668.39	26	Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae		
New.ReferenceOTU4593	0.38	0.09	1.00	2669459.42	25	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU2465	0.43	0.08	1.00	10673931.54	54	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Barnesiellaceae		
New.ReferenceOTU1150	0.40	0.10	1.00	33650498.63	68	Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae		
449353	0.45	0.10	1.00	144504396.64	51	Bacteria	Firmicutes	Clostridia	Clostridiales	Dehalobacteriaceae	Dehalobacterium	
524318	0.42	0.08	1.00	463294561.76	32	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	
351231	0.44	0.09	1.00	1100850370	31	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	fragilis
New.ReferenceOTU1611	0.48	0.09	1.00	3127524825	20	Unassigned						
234488	0.47	0.09	1.00	15425354140	49	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	
New.ReferenceOTU1618	0.49	0.09	1.00	1.34807E+11	20	Unassigned						
351272	0.48	0.08	1.00	3.16289E+11	75	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	
556126	0.49	0.08	1.00	4.51264E+11	66	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	
New.ReferenceOTU4158	0.52	0.08	1.00	2.30769E+13	30	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Rikenella	
New.ReferenceOTU3591	0.53	0.08	1.00	2E+14	63	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	
349809	0.50	0.08	1.00	1E+15	29	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	eggerthii
198530	0.53	0.08	1.00	∞	18	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	
305608	0.66	0.07	1.00	∞	16	Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Phascolarctobacterium	
334383	0.67	0.07	1.00	∞	16	Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Phascolarctobacterium	
577377	0.73	0.05	1.00	∞	23	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	
585989	0.69	0.07	1.00	∞	17	Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Phascolarctobacterium	
589277	0.69	0.06	1.00	∞	37	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	
851323	0.71	0.06	1.00	∞	54	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides	
New.ReferenceOTU570	0.72	0.06	1.00	∞	41	Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Phascolarctobacterium	

[1]Bayes factor of the model with litter effects against the same model without litter effects.

Additional file 6.6: Table 6.S3| Bayes factors, marginal posterior means and standard deviations of cage variance ratio for OTUs influenced by cage effects together with the associated probability of these estimates being greater than 0.10.

Adjusted with the ZIP model

OTU ID	Mean c^2	Standard deviation c^2	$P(c^2 > 0.1)$	Bayes factor[1]	Frequency of presence (%)	Kingdom	Phylum	Class	Order	Family	Genus	Species
1517779	0.16	0.12	0.63	3.21	16	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae		
New.ReferenceOTU4059	0.18	0.12	0.70	3.44	13	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7		
1105984	0.08	0.09	0.29	4.50	7	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	
278675	0.20	0.14	0.71	4.55	10	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7		
New.ReferenceOTU1306	0.46	0.30	0.83	37.39	22	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7		

[1]Bayes factor of the model with cage effects against the same model without cage effects.

Adjusted with the LMM model

OTU ID	Mean c^2	Standard deviation c^2	$P(c^2 > 0.1)$	Bayes factor[1]	Frequency of presence (%)	Kingdom	Phylum	Class	Order	Family	Genus	Species
349892	0.11	0.07	0.52	3.25	84	Bacteria	Tenericutes	Mollicutes	RF39			
New.ReferenceOTU3032	0.10	0.06	0.48	3.33	95	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira	
New.ReferenceOTU1639	0.11	0.07	0.53	3.34	22	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU1056	0.11	0.06	0.50	3.35	52	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU3632	0.11	0.06	0.50	3.35	85	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU2010	0.11	0.07	0.54	3.38	95	Bacteria	Tenericutes	Mollicutes	RF39			
4402042	0.11	0.07	0.53	3.41	70	Bacteria	Firmicutes	Clostridia	Clostridiales			
208479	0.07	0.04	0.20	3.46	57	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Odoribacteraceae	Butyricimonas	
New.ReferenceOTU1263	0.11	0.06	0.50	3.53	80	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira	
New.ReferenceOTU575	0.10	0.06	0.45	3.56	58	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	
48899	0.10	0.06	0.47	3.57	27	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU3393	0.10	0.06	0.44	3.60	93	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU4513	0.10	0.06	0.49	3.66	56	Bacteria	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Akkermansia	
565357	0.11	0.06	0.50	3.71	70	Bacteria	Firmicutes	Clostridia	Clostridiales			
210945	0.10	0.06	0.47	3.72	96	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae		
New.ReferenceOTU4209	0.11	0.06	0.51	3.74	70	Bacteria	Firmicutes	Clostridia	Clostridiales			
1110378	0.10	0.06	0.45	3.78	93	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira	
New.ReferenceOTU3362	0.11	0.07	0.54	3.85	80	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU1673	0.12	0.07	0.56	3.86	85	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU1554	0.10	0.06	0.48	3.92	99	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae		
New.ReferenceOTU1281	0.10	0.06	0.49	3.99	83	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU2520	0.11	0.07	0.54	4.03	91	Bacteria	Firmicutes	Clostridia	Clostridiales	Mogibacteriaceae		
205846	0.12	0.07	0.55	4.07	98	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU3465	0.10	0.06	0.47	4.22	47	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU1379	0.11	0.06	0.52	4.22	81	Bacteria	Tenericutes	Mollicutes	RF39			
New.ReferenceOTU4080	0.11	0.06	0.52	4.26	85	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU708	0.11	0.06	0.53	4.40	64	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae		
New.ReferenceOTU1824	0.11	0.06	0.53	4.44	84	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU1730	0.11	0.06	0.55	4.62	73	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	
New.ReferenceOTU2996	0.10	0.06	0.47	4.64	42	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU2509	0.12	0.07	0.58	4.81	95	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU2864	0.13	0.07	0.60	4.81	66	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU4349	0.11	0.06	0.50	4.82	43	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU2264	0.11	0.06	0.54	4.86	74	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU4130	0.08	0.05	0.34	4.87	26	Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae		
New.ReferenceOTU4073	0.10	0.06	0.46	5.17	69	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU4439	0.11	0.06	0.54	5.22	76	Bacteria	Firmicutes	Clostridia	Clostridiales			
355534	0.11	0.06	0.52	5.26	41	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU370	0.12	0.07	0.54	5.32	98	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU3728	0.10	0.06	0.49	5.34	87	Unassigned						
New.ReferenceOTU4516	0.12	0.07	0.56	5.40	82	Bacteria	Tenericutes	Mollicutes	RF39			
New.ReferenceOTU3153	0.11	0.06	0.52	5.42	76	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU591	0.12	0.07	0.58	5.60	92	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	rc4-4	
New.ReferenceOTU3467	0.11	0.06	0.54	5.63	59	Bacteria	Tenericutes	RF3	ML615J-28			
New.ReferenceOTU1188	0.11	0.07	0.53	5.75	33	Unassigned						
New.ReferenceOTU3699	0.12	0.07	0.58	5.77	98	Bacteria	Tenericutes	Mollicutes	RF39			
295339	0.13	0.07	0.61	5.99	99	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus	
New.ReferenceOTU1289	0.13	0.07	0.63	6.08	94	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	
New.ReferenceOTU4284	0.10	0.06	0.45	6.09	83	Bacteria	Firmicutes	Clostridia	Clostridiales			
350438	0.12	0.06	0.58	6.13	98	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU501	0.11	0.06	0.51	6.17	42	Unassigned						
New.ReferenceOTU4057	0.13	0.07	0.62	6.36	32	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU3436	0.12	0.07	0.59	6.39	100	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
New.ReferenceOTU3301	0.10	0.06	0.48	6.40	88	Bacteria	Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	Adlercreutzia	
511724	0.12	0.06	0.58	6.44	74	Bacteria	Tenericutes	Mollicutes	RF39			
New.ReferenceOTU3517	0.13	0.07	0.61	6.52	37	Bacteria	Firmicutes	Clostridia	Clostridiales			
New.ReferenceOTU2277	0.13	0.07	0.62	6.55	74	Bacteria	Firmicutes	Clostridia	Clostridiales			
613697	0.12	0.07	0.58	6.58	90	Bacteria	Tenericutes	Mollicutes	RF39			
337724	0.10	0.06	0.48	6.68	40	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7		

New.ReferenceOTU1917	0.11	0.06	0.55	6.76	41	Bacteria	Tenericutes	Mollicutes	RF39				
New.ReferenceOTU2776	0.13	0.07	0.61	6.86	60	Bacteria	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae			
New.ReferenceOTU747	0.11	0.06	0.55	6.98	88	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia		
294923	0.11	0.06	0.56	6.98	98	Bacteria	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Akkermansia		
New.ReferenceOTU3633	0.12	0.06	0.58	7.05	89	Bacteria	Firmicutes	Clostridia	Clostridiales				
New.ReferenceOTU1698	0.12	0.07	0.60	7.21	57	Bacteria	Firmicutes	Clostridia	Clostridiales				
New.ReferenceOTU669	0.12	0.06	0.57	7.53	43	Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter		
New.ReferenceOTU4395	0.11	0.06	0.56	8.18	55	Bacteria	Firmicutes	Clostridia	Clostridiales				
349809	0.05	0.03	0.09	8.33	29	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	eggerthii	
New.ReferenceOTU1762	0.13	0.07	0.65	8.69	90	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae			
New.ReferenceOTU1449	0.14	0.07	0.66	8.76	76	Bacteria	Firmicutes	Clostridia	Clostridiales				
New.ReferenceOTU3869	0.13	0.07	0.62	8.84	64	Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae			
New.ReferenceOTU3422	0.13	0.07	0.64	9.04	93	Bacteria	Firmicutes	Clostridia	Clostridiales				
New.ReferenceOTU1234	0.10	0.06	0.49	9.32	80	Bacteria	Firmicutes	Clostridia	Clostridiales				
New.ReferenceOTU4255	0.13	0.07	0.62	9.46	86	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae			
New.ReferenceOTU3581	0.11	0.06	0.56	9.51	80	Bacteria	Firmicutes	Clostridia	Clostridiales				
New.ReferenceOTU1239	0.13	0.07	0.63	9.53	94	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae			
New.ReferenceOTU3985	0.10	0.06	0.48	9.57	55	Unassigned							
New.ReferenceOTU2836	0.12	0.07	0.61	9.69	85	Bacteria	Firmicutes	Clostridia	Clostridiales				
New.ReferenceOTU3999	0.13	0.07	0.63	9.94	62	Bacteria	Firmicutes	Clostridia	Clostridiales				
301109	0.12	0.06	0.58	10.05	57	Bacteria	Tenericutes	Mollicutes	RF39				
New.ReferenceOTU605	0.13	0.07	0.61	10.14	59	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae			
New.ReferenceOTU1966	0.13	0.07	0.64	10.20	74	Bacteria	Firmicutes	Clostridia	Clostridiales				
422283	0.13	0.07	0.64	10.36	100	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira		
720944	0.12	0.06	0.59	10.39	72	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira		
New.ReferenceOTU940	0.13	0.07	0.67	10.47	72	Bacteria	Firmicutes	Clostridia	Clostridiales				
New.ReferenceOTU733	0.14	0.07	0.67	10.49	64	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7			
798164	0.12	0.06	0.59	10.66	73	Bacteria	Firmicutes	Clostridia	Clostridiales				
361679	0.11	0.06	0.56	10.96	60	Bacteria	Firmicutes	Clostridia	Clostridiales				
New.ReferenceOTU1771	0.13	0.07	0.64	11.44	76	Bacteria	Tenericutes	Mollicutes	RF39				
799034	0.13	0.07	0.63	12.38	97	Bacteria	Firmicutes	Clostridia	Clostridiales				
New.ReferenceOTU3555	0.14	0.07	0.68	12.70	36	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Rikenella		
550894	0.14	0.07	0.66	12.80	92	Bacteria	Cyanobacteria	4C0d-2	YS2				
New.ReferenceOTU4152	0.13	0.07	0.65	12.92	70	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae			
New.ReferenceOTU741	0.12	0.06	0.62	13.16	87	Bacteria	Firmicutes	Clostridia	Clostridiales				
New.ReferenceOTU1521	0.14	0.07	0.68	13.42	93	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae			
New.ReferenceOTU1231	0.14	0.07	0.70	13.47	91	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae			
New.ReferenceOTU2745	0.14	0.07	0.68	13.51	99	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus		
New.ReferenceOTU352	0.13	0.06	0.63	14.67	61	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae			
593733	0.14	0.07	0.70	14.79	22	Bacteria	Firmicutes	Clostridia	Clostridiales				
New.ReferenceOTU4280	0.15	0.07	0.72	17.25	97	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Ruminococcus	gnavus	
New.ReferenceOTU4135	0.14	0.07	0.71	18.18	97	Bacteria	Firmicutes	Clostridia	Clostridiales				
New.ReferenceOTU2222	0.14	0.07	0.69	18.54	54	Unassigned							
661055	0.14	0.07	0.72	18.95	92	Bacteria	Firmicutes	Clostridia	Clostridiales				
New.ReferenceOTU3855	0.14	0.07	0.72	21.32	66	Bacteria	Firmicutes	Clostridia	Clostridiales				
New.ReferenceOTU4525	0.16	0.08	0.77	21.57	81	Bacteria	Firmicutes	Clostridia	Clostridiales				
New.ReferenceOTU6	0.15	0.07	0.73	21.97	82	Bacteria	Firmicutes	Clostridia	Clostridiales				
347523	0.16	0.08	0.77	22.77	100	Bacteria	Firmicutes	Clostridia	Clostridiales				
New.ReferenceOTU2121	0.17	0.08	0.79	24.42	60	Bacteria	Firmicutes	Clostridia	Clostridiales				
New.ReferenceOTU2402	0.12	0.06	0.59	24.81	31	Bacteria	Firmicutes	Clostridia	Clostridiales				
4359749	0.15	0.07	0.73	26.26	81	Bacteria	Firmicutes	Clostridia	Clostridiales				
New.ReferenceOTU3327	0.14	0.07	0.70	27.87	28	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae			
561607	0.17	0.08	0.80	30.12	38	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae			
New.ReferenceOTU4291	0.15	0.07	0.74	31.43	86	Bacteria	Firmicutes	Clostridia	Clostridiales				
New.ReferenceOTU69	0.16	0.07	0.77	33.43	85	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae			
New.ReferenceOTU4338	0.11	0.05	0.52	37.65	48	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Rikenella		
837859	0.16	0.07	0.78	40.70	94	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae			
New.ReferenceOTU2476	0.16	0.07	0.78	42.28	46	Bacteria	Tenericutes	Mollicutes	RF39				
1108356	0.15	0.07	0.75	43.77	97	Bacteria	Tenericutes	Mollicutes	RF39				
New.ReferenceOTU763	0.15	0.07	0.76	46.49	52	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira		
356180	0.16	0.07	0.79	48.05	58	Bacteria	Firmicutes	Clostridia	Clostridiales				
New.ReferenceOTU3104	0.17	0.07	0.81	48.65	68	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7			
New.ReferenceOTU4661	0.16	0.07	0.79	63.40	83	Bacteria	Firmicutes	Clostridia	Clostridiales				
New.ReferenceOTU4546	0.17	0.07	0.82	75.84	89	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae			
569030	0.11	0.05	0.58	83.39	49	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Rikenella		
New.ReferenceOTU4449	0.18	0.08	0.85	86.43	53	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7			
New.ReferenceOTU1266	0.16	0.07	0.80	97.64	80	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae			
New.ReferenceOTU276	0.15	0.06	0.79	111.80	39	Bacteria	Firmicutes	Clostridia	Clostridiales				
821538	0.19	0.07	0.87	122.03	57	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus		
New.ReferenceOTU4137	0.18	0.08	0.86	129.64	35	Bacteria	Firmicutes	Clostridia	Clostridiales				
New.ReferenceOTU2893	0.16	0.06	0.82	148.24	96	Bacteria	Firmicutes	Clostridia	Clostridiales				
359950	0.18	0.07	0.87	227.61	51	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus		
449833	0.20	0.07	0.91	330.32	72	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7			
587510	0.19	0.07	0.90	332.26	86	Bacteria	Tenericutes	Mollicutes	RF39				
New.ReferenceOTU1689	0.20	0.07	0.92	361.18	77	Bacteria	Tenericutes	RF3	ML615J-28				
New.ReferenceOTU331	0.21	0.08	0.93	378.04	55	Bacteria	Proteobacteria	Alphaproteobacteria					
577377	0.08	0.04	0.25	497.12	23	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides		
New.ReferenceOTU1063	0.21	0.07	0.94	819.23	77	Bacteria	Firmicutes	Clostridia	Clostridiales				
New.ReferenceOTU759	0.22	0.07	0.96	1841.76	97	Unassigned							
New.ReferenceOTU46	0.26	0.08	0.98	2132.76	30	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7			
New.ReferenceOTU1883	0.22	0.07	0.97	3833.23	37	Unassigned							
New.ReferenceOTU3047	0.23	0.07	0.97	6423.64	63	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7			
New.ReferenceOTU3355	0.33	0.07	1.00	16239091.39	57	Unassigned							
1108422	0.40	0.07	1.00	1161041934.51	33	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7			

[1]Bayes factor of the model with cage effects against the same model without cage effects.

Supplementary material of chapter 7

Additional file 7.1| Metadata associated with the 425 rabbit cecal samples analyzed in this study. Open access file available in:
<https://assets.researchsquare.com/files/rs-441480/v2/81c124f00fef556f3e406320.txt>

Additional file 7.2| Filtered and CSS-normalized OTU table. Open access file available in:
<https://assets.researchsquare.com/files/rs-441480/v2/f309430619736a45512248e4.txt>

Additional file 7.3| Taxonomic assignment of representative sequences of each OTU in **Additional file 7.2**. Open access file available in:
<https://assets.researchsquare.com/files/rs-441480/v2/a1652edb8b43b05687e633a3.txt>

Additional file 7.4: Table 7.S1| Genetic variants declared significantly associated with the variation of 19 microbial traits by the MIX-GWAS after multiple testing correction at the genome-wide level.

Trait	Taxonomy	Frequency of presence	Mean	Standard deviation	OCC[1]	Window	SNP	bp	A1	A2	Minor allele frequency	Effect (phenotypic standard deviations)	*P* value	P_{FDR}[2]
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	99	AX-147127291	104234624	1	2	0.23	0.45	8.13E-07	2.46E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	99	AX-147089616	104254701	1	2	0.23	0.46	7.35E-07	2.46E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	100	AX-147110873	104925811	1	2	0.22	0.46	8.17E-07	2.46E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	100	AX-147008130	104943353	1	2	0.22	0.46	8.81E-07	2.46E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	100	AX-147138560	104949323	1	2	0.22	0.46	8.81E-07	2.46E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	100	AX-147045738	104955898	1	2	0.22	0.46	8.70E-07	2.46E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	100	AX-147121846	105048817	1	2	0.27	0.34	3.83E-06	7.83E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	100	AX-146992105	105140315	1	2	0.20	0.41	2.08E-05	3.41E-02
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	100	AX-147168513	105151723	1	2	0.38	0.23	3.37E-05	4.77E-02
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	100	AX-147107333	105166505	1	2	0.20	0.41	3.15E-05	4.63E-02
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	100	AX-147121847	105180770	1	2	0.20	0.41	2.74E-05	4.24E-02
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	105	AX-147142159	109708080	1	2	0.37	0.32	3.28E-05	4.70E-02
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	105	AX-147117370	109743155	1	2	0.38	0.32	3.23E-05	4.69E-02
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	105	AX-147066175	109832474	1	2	0.37	0.32	3.15E-05	4.63E-02
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	105	AX-147038170	109946942	1	2	0.37	0.32	2.95E-05	4.51E-02
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	105	AX-147068628	109975042	1	2	0.37	0.33	2.22E-05	3.59E-02
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	105	AX-146986386	109982313	1	2	0.37	0.34	1.07E-05	1.84E-02
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	105	AX-147071132	109996357	1	2	0.37	0.32	3.15E-05	4.63E-02
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	105	AX-147050877	110049023	1	2	0.07	0.64	9.98E-06	1.76E-02
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	105	AX-146997986	110095894	1	2	0.16	0.49	2.56E-06	5.64E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	105	AX-147110876	110134396	1	2	0.28	0.45	1.70E-07	2.29E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	105	AX-147115821	110218599	1	2	0.16	0.50	1.83E-06	4.47E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	108	AX-147099894	113530088	1	2	0.27	0.39	3.93E-06	7.91E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	109	AX-147135464	114189674	1	2	0.12	0.33	3.42E-05	4.78E-02
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	109	AX-147017548	114199124	1	2	0.06	0.73	2.90E-06	6.27E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	109	AX-147134363	114208695	1	2	0.06	0.71	6.03E-06	1.15E-02
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	109	AX-147066183	114222336	1	2	0.07	0.70	3.09E-06	6.55E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	109	AX-147140420	114226990	1	2	0.06	0.81	1.72E-07	2.29E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	109	AX-146989357	114230779	1	2	0.06	0.77	5.86E-07	2.29E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	109	AX-147146163	114237183	1	2	0.06	0.77	5.86E-07	2.29E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	109	AX-146987028	114240384	1	2	0.06	0.73	2.55E-06	5.64E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	109	AX-147154000	114283427	1	2	0.07	0.76	7.13E-07	2.46E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	109	AX-147159788	114495230	1	2	0.07	0.80	9.78E-08	2.29E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	109	AX-147073552	114501676	1	2	0.06	0.77	5.22E-07	2.29E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	109	AX-147083001	114526042	1	2	0.06	0.77	6.18E-07	2.29E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	109	AX-147028586	114539497	1	2	0.07	0.74	1.23E-06	3.19E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	109	AX-147011682	114583315	1	2	0.06	0.80	1.67E-07	2.29E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	109	AX-147101814	114610455	1	2	0.06	0.70	9.31E-06	1.67E-02
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	109	AX-146985625	114619333	1	2	0.06	0.77	6.37E-07	2.29E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	109	AX-146985842	114631525	1	2	0.06	0.81	1.04E-06	2.84E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	109	AX-147115824	114645484	1	2	0.06	0.77	6.40E-07	2.29E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	109	AX-146993804	114678097	1	2	0.07	0.76	8.41E-07	2.46E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	109	AX-147053449	114687052	1	2	0.06	0.77	5.86E-07	2.29E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	110	AX-147066184	114733752	1	2	0.06	0.77	5.95E-07	2.29E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	110	AX-147048315	114752330	1	2	0.06	0.77	5.86E-07	2.29E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	110	AX-147141311	114757655	1	2	0.06	0.77	6.22E-07	2.29E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	110	AX-147061000	114776435	1	2	0.06	0.77	5.86E-07	2.29E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	110	AX-147063607	114815246	1	2	0.12	0.35	1.54E-05	2.60E-02
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	110	AX-147097976	114824436	1	2	0.06	0.76	1.79E-06	4.47E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	110	AX-146986692	114852990	1	2	0.07	0.79	2.59E-07	2.29E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	110	AX-147098896	114861743	1	2	0.06	0.77	6.24E-07	2.29E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	110	AX-147056004	114915678	1	2	0.07	0.69	1.03E-05	1.79E-02
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	110	AX-147127299	114949927	1	2	0.06	0.77	5.86E-07	2.29E-03

[1]*Oryctolagus cuniculus* chromosome.
[2]Genome-wide $P_{FDR} < 0.05$.

Trait	Taxonomy	Frequency of presence	Mean	Standard deviation	OCC[1]	Window	SNP	bp	A1	A2	Minor allele frequency	Effect (phenotypic standard deviations)	*P* value	P_{FDR}[2]
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	110	AX-147068636	114983521	1	2	0.06	0.77	5.86E-07	2.29E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	110	AX-147121858	114991002	1	2	0.06	0.77	5.86E-07	2.29E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	110	AX-147117375	114997116	1	2	0.06	0.74	6.42E-06	1.21E-02
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	110	AX-147132088	115008542	1	2	0.06	0.77	6.19E-07	2.29E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	110	AX-146995813	115019830	1	2	0.06	0.77	5.86E-07	2.29E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	110	AX-147142994	115290637	1	2	0.06	0.73	5.37E-06	1.04E-02
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	110	AX-147155705	115298213	1	2	0.06	0.77	6.14E-07	2.29E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	110	AX-147004896	115303073	1	2	0.07	0.78	3.37E-07	2.29E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	110	AX-147019584	115349501	1	2	0.06	0.77	5.86E-07	2.29E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	110	AX-147085208	115369875	1	2	0.07	0.68	8.88E-06	1.62E-02
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	110	AX-146997991	115380970	1	2	0.07	0.74	1.15E-06	3.07E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	110	AX-146997992	115412921	1	2	0.06	0.77	5.86E-07	2.29E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	110	AX-147136514	115476306	1	2	0.06	0.77	4.99E-07	2.29E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	110	AX-147120400	115600334	1	2	0.06	0.77	5.86E-07	2.29E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	110	AX-147001864	115662418	1	2	0.07	0.75	7.55E-07	2.46E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	111	AX-147040699	115834034	1	2	0.06	0.69	1.69E-05	2.81E-02
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	111	AX-147117376	116105130	1	2	0.07	0.78	3.98E-07	2.29E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	111	AX-147048318	116114808	1	2	0.06	0.77	5.86E-07	2.29E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	111	AX-146989358	116138866	1	2	0.06	0.77	5.86E-07	2.29E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	111	AX-147127302	116144216	1	2	0.13	0.35	8.20E-06	1.52E-02
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	111	AX-147125983	116238203	1	2	0.06	0.77	5.86E-07	2.29E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	111	AX-146993808	116521512	1	2	0.33	0.34	2.30E-05	3.62E-02
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	111	AX-147006463	116562711	1	2	0.33	0.34	2.30E-05	3.62E-02
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	114	AX-147058529	119572632	1	2	0.07	0.72	2.14E-06	4.90E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	114	AX-147040702	119597991	1	2	0.07	0.70	3.44E-06	7.17E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	114	AX-147011686	119623887	1	2	0.07	0.69	4.41E-06	8.71E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	114	AX-147017556	119664643	1	2	0.07	0.72	1.99E-06	4.66E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	114	AX-147156235	119674009	1	2	0.07	0.72	1.99E-06	4.66E-03
NR1794	Order *Clostridiales* (*Firmicutes*)	0.47	99.53	153.03	1	114	AX-147015524	119719156	1	2	0.07	0.73	1.79E-06	4.47E-03
314029	Order *Clostridiales* (*Firmicutes*)	0.60	182.72	186.20	1	116	AX-147148999	122535511	1	2	0.19	0.36	3.40E-06	4.24E-02
314029	Order *Clostridiales* (*Firmicutes*)	0.60	182.72	186.20	1	117	AX-147066191	122657633	1	2	0.19	0.38	1.04E-06	4.24E-02
314029	Order *Clostridiales* (*Firmicutes*)	0.60	182.72	186.20	1	117	AX-147136516	122690442	1	2	0.19	0.36	4.87E-06	4.65E-02
314029	Order *Clostridiales* (*Firmicutes*)	0.60	182.72	186.20	1	117	AX-147157239	122719727	1	2	0.20	0.36	3.70E-06	4.24E-02
314029	Order *Clostridiales* (*Firmicutes*)	0.60	182.72	186.20	1	117	AX-147008153	122726498	1	2	0.19	0.36	3.20E-06	4.24E-02
314029	Order *Clostridiales* (*Firmicutes*)	0.60	182.72	186.20	1	117	AX-147129724	122737363	1	2	0.19	0.36	3.40E-06	4.24E-02
314029	Order *Clostridiales* (*Firmicutes*)	0.60	182.72	186.20	1	117	AX-147171781	122743206	1	2	0.19	0.36	3.40E-06	4.24E-02
314029	Order *Clostridiales* (*Firmicutes*)	0.60	182.72	186.20	1	117	AX-147158252	122750773	1	2	0.19	0.36	3.65E-06	4.24E-02
314029	Order *Clostridiales* (*Firmicutes*)	0.60	182.72	186.20	1	117	AX-147015528	122763043	1	2	0.19	0.36	3.40E-06	4.24E-02
314029	Order *Clostridiales* (*Firmicutes*)	0.60	182.72	186.20	1	117	AX-147133219	122769881	1	2	0.19	0.36	3.40E-06	4.24E-02
314029	Order *Clostridiales* (*Firmicutes*)	0.60	182.72	186.20	1	117	AX-147143834	122779726	1	2	0.19	0.36	3.40E-06	4.24E-02
314029	Order *Clostridiales* (*Firmicutes*)	0.60	182.72	186.20	1	117	AX-146992119	122790362	1	2	0.19	0.36	4.75E-06	4.65E-02
NR1391	Family *Lachnospiraceae* (*Firmicutes*)	0.76	144.94	107.31	1	156	AX-147091835	162978181	1	2	0.14	0.55	1.84E-06	4.84E-02
NR1391	Family *Lachnospiraceae* (*Firmicutes*)	0.76	144.94	107.31	1	156	AX-147061053	163288462	1	2	0.14	0.54	3.17E-06	4.84E-02
NR1391	Family *Lachnospiraceae* (*Firmicutes*)	0.76	144.94	107.31	1	156	AX-147107372	163296620	1	2	0.14	0.54	3.17E-06	4.84E-02
NR1391	Family *Lachnospiraceae* (*Firmicutes*)	0.76	144.94	107.31	1	156	AX-147073593	163334497	1	2	0.14	0.54	3.17E-06	4.84E-02
NR1391	Family *Lachnospiraceae* (*Firmicutes*)	0.76	144.94	107.31	1	156	AX-147166574	163375046	1	2	0.14	0.54	3.52E-06	4.84E-02
NR1391	Family *Lachnospiraceae* (*Firmicutes*)	0.76	144.94	107.31	1	156	AX-147093949	163393018	1	2	0.14	0.53	3.80E-06	4.84E-02
NR1391	Family *Lachnospiraceae* (*Firmicutes*)	0.76	144.94	107.31	1	156	AX-147101850	163400640	1	2	0.14	0.54	3.14E-06	4.84E-02
NR1391	Family *Lachnospiraceae* (*Firmicutes*)	0.76	144.94	107.31	1	156	AX-147031034	163442998	1	2	0.14	0.54	3.17E-06	4.84E-02
NR1391	Family *Lachnospiraceae* (*Firmicutes*)	0.76	144.94	107.31	1	156	AX-147058566	163460760	1	2	0.14	0.54	3.17E-06	4.84E-02
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	1	172	AX-147160810	180644975	1	2	0.06	-0.29	4.24E-06	2.15E-02
524842	Family *Clostridiaceae* (*Firmicutes*)	0.72	136.95	114.58	1	185	AX-147109218	193736542	1	2	0.18	0.45	1.45E-06	2.41E-02
524842	Family *Clostridiaceae* (*Firmicutes*)	0.72	136.95	114.58	1	185	AX-146983662	193801151	1	2	0.18	0.44	2.14E-06	3.06E-02

[1] *Oryctolagus cuniculus* chromosome.

[2] Genome-wide $P_{FDR} < 0.05$.

Trait	Taxonomy	Frequency of presence	Mean	Standard deviation	OCC[1]	Window	SNP	bp	A1	A2	Minor allele frequency	Effect (phenotypic standard deviations)	*P* value	P_{FDR}[2]
524842	Family *Clostridiaceae* (*Firmicutes*)	0.72	136.95	114.58	1	185	AX-147110928	193846874	1	2	0.18	0.45	1.47E-06	2.41E-02
524842	Family *Clostridiaceae* (*Firmicutes*)	0.72	136.95	114.58	1	185	AX-147103743	193864887	1	2	0.33	0.26	9.24E-07	2.41E-02
524842	Family *Clostridiaceae* (*Firmicutes*)	0.72	136.95	114.58	1	185	AX-147136540	193923215	1	2	0.18	0.45	1.45E-06	2.41E-02
524842	Family *Clostridiaceae* (*Firmicutes*)	0.72	136.95	114.58	1	185	AX-146986397	193933714	1	2	0.18	0.45	1.45E-06	2.41E-02
524842	Family *Clostridiaceae* (*Firmicutes*)	0.72	136.95	114.58	1	185	AX-147181465	193942449	1	2	0.18	0.45	1.45E-06	2.41E-02
524842	Family *Clostridiaceae* (*Firmicutes*)	0.72	136.95	114.58	1	185	AX-147168051	193965562	1	2	0.18	0.45	1.45E-06	2.41E-02
524842	Family *Clostridiaceae* (*Firmicutes*)	0.72	136.95	114.58	1	185	AX-147176519	193977844	1	2	0.18	0.44	2.49E-06	3.17E-02
NR2745	Genus *Ruminococcus* (*Firmicutes*)	0.99	565.14	187.76	3	364	AX-147034603	14589674	1	2	0.34	-0.35	2.62E-06	3.02E-02
NR2745	Genus *Ruminococcus* (*Firmicutes*)	0.99	565.14	187.76	3	364	AX-147005748	14597802	1	2	0.34	-0.36	1.35E-06	3.02E-02
NR2745	Genus *Ruminococcus* (*Firmicutes*)	0.99	565.14	187.76	3	364	AX-146998867	14637175	1	2	0.34	-0.35	2.73E-06	3.02E-02
NR2745	Genus *Ruminococcus* (*Firmicutes*)	0.99	565.14	187.76	3	364	AX-147079557	14651308	1	2	0.35	-0.37	7.29E-07	3.02E-02
NR2745	Genus *Ruminococcus* (*Firmicutes*)	0.99	565.14	187.76	3	364	AX-147049643	14658091	1	2	0.34	-0.35	1.99E-06	3.02E-02
NR2745	Genus *Ruminococcus* (*Firmicutes*)	0.99	565.14	187.76	3	364	AX-147090742	14668612	1	2	0.34	-0.35	2.62E-06	3.02E-02
NR2745	Genus *Ruminococcus* (*Firmicutes*)	0.99	565.14	187.76	3	364	AX-147088349	14675763	1	2	0.34	-0.35	1.99E-06	3.02E-02
NR2745	Genus *Ruminococcus* (*Firmicutes*)	0.99	565.14	187.76	3	364	AX-147042019	14684246	1	2	0.34	-0.35	2.90E-06	3.02E-02
NR2745	Genus *Ruminococcus* (*Firmicutes*)	0.99	565.14	187.76	3	364	AX-147047114	14706591	1	2	0.34	-0.36	1.17E-06	3.02E-02
NR2745	Genus *Ruminococcus* (*Firmicutes*)	0.99	565.14	187.76	3	364	AX-147148668	14746670	1	2	0.34	-0.35	1.99E-06	3.02E-02
NR2745	Genus *Ruminococcus* (*Firmicutes*)	0.99	565.14	187.76	3	364	AX-147108266	14785915	1	2	0.34	-0.36	1.55E-06	3.02E-02
NR2723	Order *Bacteroidales* (*Bacteroidetes*)	0.08	25.79	112.50	3	370	AX-147029851	20570716	1	2	0.07	0.52	4.40E-07	3.50E-02
NR2723	Order *Bacteroidales* (*Bacteroidetes*)	0.08	25.79	112.50	3	370	AX-147106473	20697579	1	2	0.07	0.51	6.11E-07	3.50E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	388	AX-147145011	38880467	1	2	0.43	0.35	5.40E-06	1.51E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	392	AX-147138054	43187174	1	2	0.15	0.48	3.56E-06	1.34E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	392	AX-147014608	43192938	1	2	0.16	0.47	6.97E-06	1.74E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	392	AX-146989081	43200772	1	2	0.15	0.47	8.56E-06	2.01E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	392	AX-146984058	43216796	1	2	0.16	0.45	6.44E-06	1.64E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	392	AX-146986548	43223764	1	2	0.15	0.48	3.56E-06	1.34E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	392	AX-147106496	43235437	1	2	0.15	0.49	2.24E-06	1.34E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	392	AX-147007387	43241942	1	2	0.15	0.48	3.70E-06	1.34E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	392	AX-147090765	43262850	1	2	0.16	0.46	8.86E-06	2.03E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	392	AX-147039526	43449018	1	2	0.15	0.48	3.57E-06	1.34E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	392	AX-147149988	43473887	1	2	0.16	0.47	4.94E-06	1.42E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	392	AX-147127926	43483950	1	2	0.16	0.48	3.54E-06	1.34E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	392	AX-147090766	43489650	1	2	0.16	0.47	4.94E-06	1.42E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	392	AX-147018622	43508370	1	2	0.16	0.47	4.94E-06	1.42E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	393	AX-147009069	43706436	1	2	0.16	0.47	4.94E-06	1.42E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	393	AX-147010823	43714861	1	2	0.16	0.44	2.07E-05	4.47E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	393	AX-147059833	43737010	1	2	0.16	0.47	4.94E-06	1.42E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	393	AX-146989669	43755764	1	2	0.15	0.48	3.83E-06	1.34E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	393	AX-147037076	43761668	1	2	0.15	0.48	3.69E-06	1.34E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	393	AX-147069949	43774490	1	2	0.15	0.47	6.19E-06	1.64E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	393	AX-147042044	43785591	1	2	0.15	0.49	2.55E-06	1.34E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	393	AX-147090767	43791783	1	2	0.15	0.48	3.56E-06	1.34E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	393	AX-147039528	43813240	1	2	0.15	0.48	3.11E-06	1.34E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	393	AX-147092933	43828748	1	2	0.15	0.50	1.82E-06	1.34E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	393	AX-147052248	43839699	1	2	0.16	0.49	2.20E-06	1.34E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	393	AX-147039529	43903680	1	2	0.15	0.48	3.89E-06	1.34E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	393	AX-147130308	43950378	1	2	0.15	0.49	3.00E-06	1.34E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	393	AX-147147951	43977942	1	2	0.15	0.48	3.56E-06	1.34E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	393	AX-147084167	44172962	1	2	0.16	0.45	1.11E-05	2.48E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	393	AX-147027453	44238605	1	2	0.15	0.49	2.67E-06	1.34E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	393	AX-146996394	44277885	1	2	0.16	0.44	1.97E-05	4.35E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	393	AX-147108299	44351288	1	2	0.15	0.47	6.31E-06	1.64E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	393	AX-147044521	44389088	1	2	0.15	0.48	3.56E-06	1.34E-02

[1]*Oryctolagus cuniculus* chromosome.
[2]Genome-wide $P_{FDR} < 0.05$.

Trait	Taxonomy	Frequency of presence	Mean	Standard deviation	OCC[1]	Window	SNP	bp	A1	A2	Minor allele frequency	Effect (phenotypic standard deviations)	*P* value	P_{FDR}[2]
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	393	AX-147072390	44399878	1	2	0.16	0.47	4.66E-06	1.42E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	393	AX-147010824	44441989	1	2	0.15	0.47	8.61E-06	2.01E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	393	AX-147062400	44449243	1	2	0.15	0.48	3.35E-06	1.34E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	393	AX-147074772	44455495	1	2	0.15	0.48	3.97E-06	1.34E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	393	AX-146985531	44461497	1	2	0.16	0.48	3.01E-06	1.34E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	393	AX-146995323	44470911	1	2	0.15	0.48	3.56E-06	1.34E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	393	AX-147143393	44512002	1	2	0.16	0.49	2.30E-06	1.34E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	393	AX-147111761	44522524	1	2	0.15	0.48	7.15E-06	1.74E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	393	AX-147044522	44531000	1	2	0.15	0.48	3.02E-06	1.34E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	393	AX-146997482	44537843	1	2	0.15	0.48	3.83E-06	1.34E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	393	AX-147081900	44557479	1	2	0.16	0.49	1.91E-06	1.34E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	394	AX-147104662	44574454	1	2	0.15	0.48	3.56E-06	1.34E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	394	AX-147079582	44623567	1	2	0.15	0.48	3.56E-06	1.34E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	394	AX-147113381	44644931	1	2	0.15	0.48	3.56E-06	1.34E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	394	AX-147007390	44774996	1	2	0.15	0.47	5.58E-06	1.52E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	394	AX-147092934	44888297	1	2	0.15	0.48	3.38E-06	1.34E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	394	AX-147153698	44896906	1	2	0.15	0.50	2.44E-06	1.34E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	394	AX-147002690	44918466	1	2	0.15	0.48	3.69E-06	1.34E-02
NR2269	Genus *Ruminococcus* (*Firmicutes*)	0.41	101.97	148.64	3	394	AX-147145803	44925634	1	2	0.14	0.50	2.76E-06	1.34E-02
157802	Order *Clostridiales* (*Firmicutes*)	0.61	152.41	157.51	3	478	AX-147139981	133998414	1	2	0.08	0.68	2.75E-07	2.16E-02
157802	Order *Clostridiales* (*Firmicutes*)	0.61	152.41	157.51	3	478	AX-147049761	134014991	1	2	0.08	0.65	3.77E-07	2.16E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	483	AX-147171954	139052204	1	2	0.30	0.30	2.20E-05	4.12E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	483	AX-147148695	139267954	1	2	0.31	0.32	8.30E-06	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	483	AX-147170597	139282325	1	2	0.30	0.32	1.12E-05	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	484	AX-147102865	140316754	1	2	0.35	0.29	2.98E-05	4.74E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	484	AX-147121201	140328307	1	2	0.35	0.29	2.85E-05	4.60E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	484	AX-147084237	140333428	1	2	0.35	0.29	2.85E-05	4.60E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	484	AX-147047259	140476782	1	2	0.37	0.28	1.08E-05	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	484	AX-147167298	140572188	1	2	0.34	0.30	2.48E-05	4.33E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	484	AX-147044623	140599855	1	2	0.32	0.30	2.49E-05	4.33E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	484	AX-147059940	140608277	1	2	0.33	0.30	3.04E-05	4.78E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	484	AX-146999926	140618346	1	2	0.24	0.35	9.37E-06	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	484	AX-147062482	140626565	1	2	0.24	0.34	2.55E-05	4.36E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	484	AX-147173291	140634946	1	2	0.26	0.34	1.24E-05	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	484	AX-147086470	140648675	1	2	0.24	0.34	1.87E-05	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	484	AX-147099047	140681362	1	2	0.24	0.35	1.09E-05	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	484	AX-147047260	140693613	1	2	0.24	0.35	1.22E-05	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	484	AX-147057465	140736337	1	2	0.25	0.34	2.23E-05	4.12E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	484	AX-147034740	140802576	1	2	0.25	0.35	8.57E-06	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	484	AX-147059942	140878856	1	2	0.24	0.34	2.02E-05	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	484	AX-147022970	140883848	1	2	0.24	0.35	1.22E-05	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	484	AX-147039650	140894605	1	2	0.24	0.34	2.59E-05	4.36E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	484	AX-147175400	140897911	1	2	0.24	0.35	9.18E-06	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	484	AX-147032394	140902660	1	2	0.24	0.34	1.73E-05	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	484	AX-147044625	140906299	1	2	0.24	0.34	2.27E-05	4.13E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	484	AX-147002755	140910730	1	2	0.24	0.35	9.16E-06	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	484	AX-147132699	140914805	1	2	0.24	0.34	2.02E-05	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	484	AX-147129182	140918127	1	2	0.24	0.35	9.37E-06	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	484	AX-147074865	140926266	1	2	0.24	0.35	9.37E-06	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	484	AX-147097058	140930151	1	2	0.24	0.34	2.02E-05	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	484	AX-147150649	140937191	1	2	0.24	0.34	2.21E-05	4.12E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	484	AX-147039651	140956345	1	2	0.24	0.35	1.00E-05	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	484	AX-147020843	140960803	1	2	0.24	0.34	2.02E-05	3.93E-02

[1] *Oryctolagus cuniculus* chromosome.

[2] Genome-wide $P_{FDR} < 0.05$.

Trait	Taxonomy	Frequency of presence	Mean	Standard deviation	OCC[1]	Window	SNP	bp	A1	A2	Minor allele frequency	Effect (phenotypic standard deviations)	*P* value	P_{FDR}[2]
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	484	AX-147022971	140965631	1	2	0.24	0.36	7.80E-06	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	484	AX-147047261	140974884	1	2	0.24	0.34	2.02E-05	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	484	AX-147108368	140979258	1	2	0.24	0.34	1.88E-05	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	484	AX-147052344	140988757	1	2	0.24	0.34	1.75E-05	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	485	AX-147065092	141009123	1	2	0.24	0.36	5.02E-06	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	485	AX-147159533	141027935	1	2	0.24	0.34	1.85E-05	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	485	AX-146982674	141035036	1	2	0.24	0.35	9.37E-06	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	485	AX-147161982	141035308	1	2	0.24	0.35	9.36E-06	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	485	AX-147134955	141038393	1	2	0.24	0.35	9.23E-06	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	485	AX-147084239	141041725	1	2	0.24	0.35	9.37E-06	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	485	AX-147095050	141049911	1	2	0.24	0.36	5.55E-06	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	485	AX-147044626	141053320	1	2	0.24	0.37	5.20E-06	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	485	AX-147155430	141056530	1	2	0.24	0.34	1.75E-05	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	485	AX-147074866	141063953	1	2	0.24	0.35	9.94E-06	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	485	AX-147082002	141067000	1	2	0.24	0.34	2.02E-05	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	485	AX-147133836	141071501	1	2	0.24	0.35	9.29E-06	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	485	AX-147077259	141074980	1	2	0.24	0.36	6.55E-06	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	485	AX-146983582	141102273	1	2	0.25	0.35	1.34E-05	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	3	485	AX-147167299	141102288	1	2	0.24	0.37	4.73E-06	3.93E-02
NR2147	Order *Clostridiales* (*Firmicutes*)	0.83	155.44	107.41	4	569	AX-147067709	76049913	1	2	0.06	-0.79	1.22E-06	4.21E-02
NR2147	Order *Clostridiales* (*Firmicutes*)	0.83	155.44	107.41	4	569	AX-147034830	76402026	1	2	0.11	-0.39	1.47E-06	4.21E-02
NR2147	Order *Clostridiales* (*Firmicutes*)	0.83	155.44	107.41	4	569	AX-146989129	76429281	1	2	0.11	-0.40	1.15E-06	4.21E-02
NR2147	Order *Clostridiales* (*Firmicutes*)	0.83	155.44	107.41	4	569	AX-147090937	76466847	1	2	0.11	-0.40	1.22E-06	4.21E-02
578960	Family *Lachnospiraceae* (*Firmicutes*)	0.08	25.78	122.15	6	639	AX-147166320	27375857	1	2	0.13	0.51	6.73E-07	2.57E-02
578960	Family *Lachnospiraceae* (*Firmicutes*)	0.08	25.78	122.15	6	639	AX-147150052	27391308	1	2	0.13	0.52	5.90E-07	2.57E-02
578960	Family *Lachnospiraceae* (*Firmicutes*)	0.08	25.78	122.15	6	639	AX-147128033	27396979	1	2	0.13	0.51	6.73E-07	2.57E-02
Phylum *Actinobacteria*	Phylum *Actinobacteria*	1.00	171.32	86.18	6	639	AX-147166320	27375857	1	2	0.13	0.53	5.45E-07	3.12E-02
Phylum *Actinobacteria*	Phylum *Actinobacteria*	1.00	171.32	86.18	6	639	AX-147150052	27391308	1	2	0.13	0.51	1.26E-06	4.56E-02
Phylum *Actinobacteria*	Phylum *Actinobacteria*	1.00	171.32	86.18	6	639	AX-147128033	27396979	1	2	0.13	0.53	5.45E-07	3.12E-02
Phylum *Actinobacteria*	Phylum *Actinobacteria*	1.00	171.32	86.18	6	639	AX-147171102	27498929	1	2	0.34	0.36	1.59E-06	4.56E-02
Principal component 2	Principal component 2	1.00	336.19	91.63	6	639	AX-147166320	27375857	1	2	0.13	-0.34	1.14E-06	4.36E-02
Principal component 2	Principal component 2	1.00	336.19	91.63	6	639	AX-147150052	27391308	1	2	0.13	-0.34	8.74E-07	4.36E-02
Principal component 2	Principal component 2	1.00	336.19	91.63	6	639	AX-147128033	27396979	1	2	0.13	-0.34	1.14E-06	4.36E-02
346794	Order *Clostridiales* (*Firmicutes*)	0.79	358.56	228.50	8	906	AX-147103165	109647907	1	2	0.36	-0.36	2.11E-06	3.46E-02
346794	Order *Clostridiales* (*Firmicutes*)	0.79	358.56	228.50	8	906	AX-147032790	109657886	1	2	0.36	-0.37	1.23E-06	2.36E-02
346794	Order *Clostridiales* (*Firmicutes*)	0.79	358.56	228.50	8	906	AX-147062936	109664594	1	2	0.36	-0.38	7.09E-07	1.63E-02
346794	Order *Clostridiales* (*Firmicutes*)	0.79	358.56	228.50	8	906	AX-147138276	109671467	1	2	0.43	-0.38	2.59E-07	1.51E-02
346794	Order *Clostridiales* (*Firmicutes*)	0.79	358.56	228.50	8	906	AX-147023409	109682053	1	2	0.36	-0.38	7.09E-07	1.63E-02
346794	Order *Clostridiales* (*Firmicutes*)	0.79	358.56	228.50	8	907	AX-147156566	109919140	1	2	0.41	0.38	3.94E-07	1.51E-02
346794	Order *Clostridiales* (*Firmicutes*)	0.79	358.56	228.50	8	907	AX-147177630	109933946	1	2	0.42	0.38	3.32E-07	1.51E-02
NR3356	Family *Ruminococcaceae* (*Firmicutes*)	0.58	152.90	177.82	8	907	AX-147162587	110219579	1	2	0.23	0.40	9.27E-07	3.54E-02
NR3356	Family *Ruminococcaceae* (*Firmicutes*)	0.58	152.90	177.82	8	907	AX-147128158	110253632	1	2	0.23	0.40	8.48E-07	3.54E-02
NR3356	Family *Ruminococcaceae* (*Firmicutes*)	0.58	152.90	177.82	8	907	AX-147161100	110258677	1	2	0.23	0.40	8.48E-07	3.54E-02
NR3356	Family *Ruminococcaceae* (*Firmicutes*)	0.58	152.90	177.82	8	907	AX-147124221	110269174	1	2	0.23	0.39	1.73E-06	4.96E-02
578960	Family *Lachnospiraceae* (*Firmicutes*)	0.08	25.78	122.15	10	1034	AX-147094002	22286919	1	2	0.14	0.49	1.96E-06	4.48E-02
578960	Family *Lachnospiraceae* (*Firmicutes*)	0.08	25.78	122.15	10	1034	AX-147171352	22278542	1	2	0.14	0.49	1.66E-06	4.48E-02
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1147	AX-147053694	8291471	1	2	0.20	-0.12	8.53E-06	2.74E-02
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1147	AX-147013730	8298701	1	2	0.10	-0.22	1.67E-05	4.79E-02
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1147	AX-147123355	8333724	1	2	0.20	-0.12	8.61E-06	2.74E-02
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1147	AX-147019806	8339885	1	2	0.20	-0.12	8.61E-06	2.74E-02
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1147	AX-147003491	8350452	1	2	0.11	-0.27	4.14E-08	3.17E-04
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1147	AX-147006654	8368196	1	2	0.11	-0.27	3.35E-08	2.89E-04
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1147	AX-147156775	8375286	1	2	0.11	-0.26	3.52E-08	2.89E-04

[1]*Oryctolagus cuniculus* chromosome.
[2]Genome-wide $P_{FDR} < 0.05$.

Trait	Taxonomy	Frequency of presence	Mean	Standard deviation	OCC[1]	Window	SNP	bp	A1	A2	Minor allele frequency	Effect (phenotypic standard deviations)	*P* value	P_{FDR}[2]
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1147	AX-147043434	8380457	1	2	0.22	-0.14	7.08E-09	1.41E-04
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1147	AX-147028828	8394768	1	2	0.22	-0.14	7.36E-09	1.41E-04
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1147	AX-147026398	8400102	1	2	0.11	-0.27	3.35E-08	2.89E-04
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1147	AX-147128657	8405997	1	2	0.11	-0.27	3.11E-08	2.89E-04
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1147	AX-147045992	8425364	1	2	0.11	-0.26	7.45E-08	5.34E-04
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1147	AX-147008301	8434548	1	2	0.22	-0.14	6.95E-09	1.41E-04
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1147	AX-147033599	8440906	1	2	0.11	-0.27	3.06E-08	2.89E-04
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1147	AX-147000581	8447314	1	2	0.11	-0.27	3.38E-08	2.89E-04
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1147	AX-147094079	8452984	1	2	0.12	-0.25	1.79E-07	1.21E-03
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1147	AX-147152854	8468526	1	2	0.22	-0.14	7.28E-09	1.41E-04
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1147	AX-147048554	8481914	1	2	0.21	-0.15	4.30E-09	1.41E-04
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1148	AX-147109315	8491088	1	2	0.11	-0.27	3.53E-08	2.89E-04
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1148	AX-147107505	8497940	1	2	0.11	-0.27	3.35E-08	2.89E-04
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1148	AX-147098143	8508765	1	2	0.22	-0.14	6.83E-09	1.41E-04
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1148	AX-147063854	8607736	1	2	0.15	-0.20	4.78E-06	2.15E-02
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1148	AX-147127397	8640285	1	2	0.15	-0.20	5.02E-06	2.15E-02
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1148	AX-147045994	8649573	1	2	0.10	-0.22	1.59E-05	4.68E-02
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1148	AX-147124740	8666656	1	2	0.10	-0.24	5.63E-06	2.15E-02
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1148	AX-147111026	8702979	1	2	0.10	-0.24	5.63E-06	2.15E-02
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1148	AX-147151591	8709965	1	2	0.10	-0.23	8.05E-06	2.74E-02
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1148	AX-147152855	8714173	1	2	0.09	-0.24	4.07E-06	2.15E-02
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1148	AX-147011859	8724557	1	2	0.10	-0.24	5.63E-06	2.15E-02
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1148	AX-147142243	8730926	1	2	0.10	-0.23	6.48E-06	2.39E-02
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1148	AX-147056244	8741155	1	2	0.10	-0.24	5.63E-06	2.15E-02
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1148	AX-147115954	8750337	1	2	0.10	-0.24	5.63E-06	2.15E-02
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1148	AX-147010013	8759524	1	2	0.10	-0.24	5.63E-06	2.15E-02
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1148	AX-147163760	8764892	1	2	0.10	-0.24	5.45E-06	2.15E-02
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1148	AX-147156264	8772388	1	2	0.10	-0.23	6.87E-06	2.46E-02
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1148	AX-147006655	8792687	1	2	0.10	-0.24	5.63E-06	2.15E-02
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	12	1148	AX-147085400	8798582	1	2	0.10	-0.24	5.63E-06	2.15E-02
NR4269	Family *Lachnospiraceae* (*Firmicutes*)	0.73	174.80	145.96	12	1264	AX-146984838	134860063	1	2	0.05	0.82	4.40E-07	2.72E-02
NR4269	Family *Lachnospiraceae* (*Firmicutes*)	0.73	174.80	145.96	12	1264	AX-147085506	134924891	1	2	0.10	0.41	4.75E-07	2.72E-02
NR741	Order *Clostridiales* (*Firmicutes*)	0.88	216.59	129.82	12	1266	AX-147043571	137077726	1	2	0.16	0.44	6.16E-08	7.06E-03
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	15	1619	AX-147088033	50200724	1	2	0.16	-0.20	9.41E-06	2.91E-02
124470	Genus *Butyricimonas* (*Bacteroidetes*)	0.20	100.72	207.79	15	1626	AX-147112983	56819962	1	2	0.06	-0.28	1.21E-05	3.65E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	16	1725	AX-147096626	56220110	1	2	0.29	0.33	2.40E-05	4.31E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	16	1740	AX-147054373	71256711	1	2	0.08	0.54	1.54E-05	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	16	1740	AX-147125064	71355648	1	2	0.08	0.53	2.77E-05	4.60E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	16	1744	AX-147029466	75469305	1	2	0.32	0.34	1.48E-05	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	16	1744	AX-147020387	75493263	1	2	0.32	0.34	1.35E-05	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	16	1744	AX-147027001	75506451	1	2	0.32	0.34	1.54E-05	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	16	1744	AX-147132445	75513366	1	2	0.32	0.33	1.80E-05	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	16	1744	AX-147083769	75557999	1	2	0.32	0.34	1.35E-05	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	16	1744	AX-147022482	75565825	1	2	0.28	0.37	6.35E-06	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	16	1744	AX-147049225	75611265	1	2	0.32	0.33	1.65E-05	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	16	1744	AX-147076735	75640073	1	2	0.32	0.34	1.48E-05	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	16	1744	AX-147051774	75684064	1	2	0.32	0.34	1.27E-05	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	16	1744	AX-147076736	75704609	1	2	0.32	0.33	1.96E-05	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	16	1744	AX-147027002	75745708	1	2	0.32	0.34	1.34E-05	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	16	1744	AX-147022483	75753323	1	2	0.32	0.33	1.62E-05	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	16	1745	AX-147176620	75759302	1	2	0.32	0.34	1.35E-05	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	16	1745	AX-147029467	75776145	1	2	0.32	0.34	1.36E-05	3.93E-02
NR276	Order *Clostridiales* (*Firmicutes*)	0.39	91.33	147.99	16	1745	AX-147029468	75807218	1	2	0.32	0.34	1.35E-05	3.93E-02

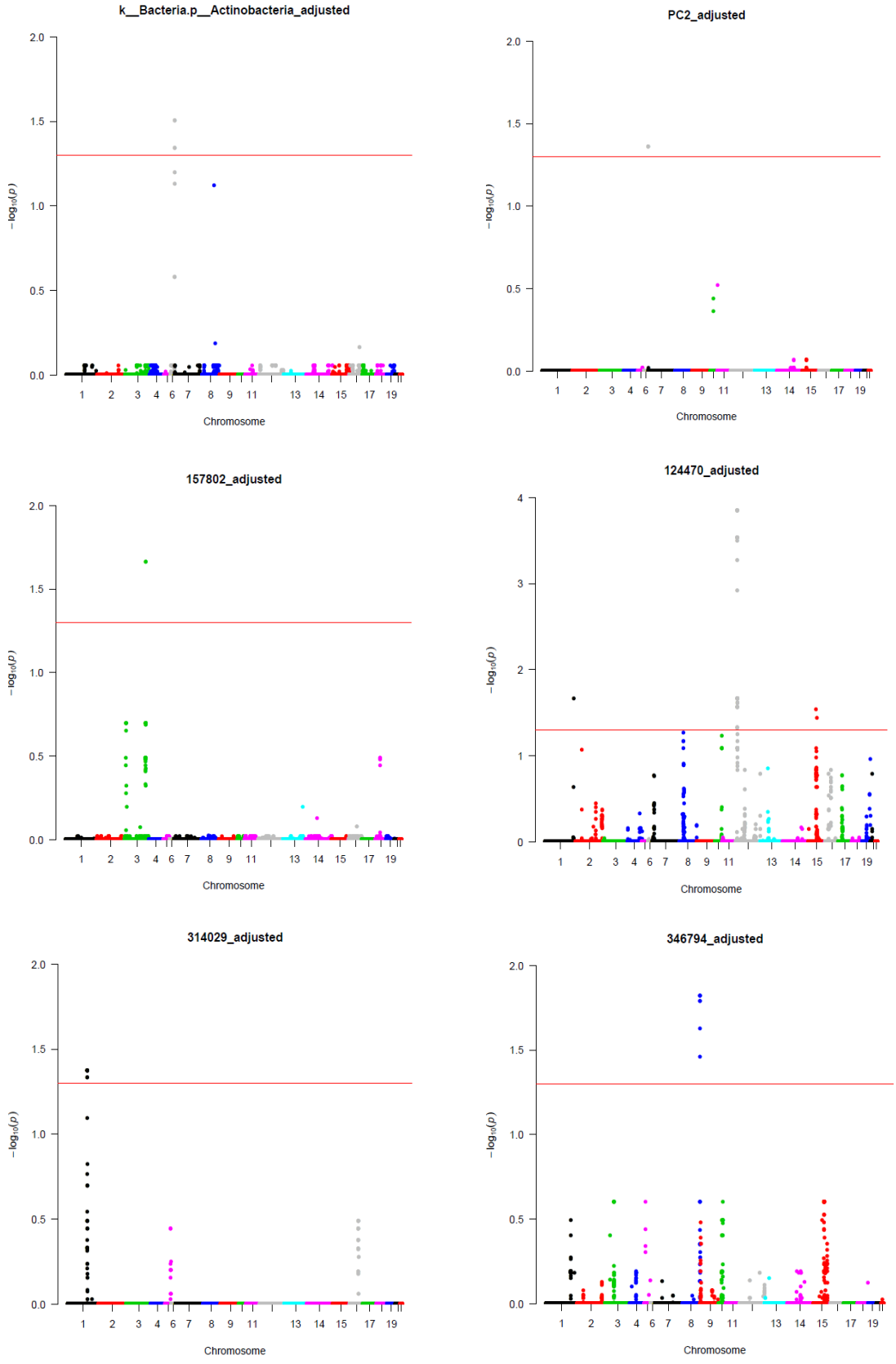
[1] *Oryctolagus cuniculus* chromosome.

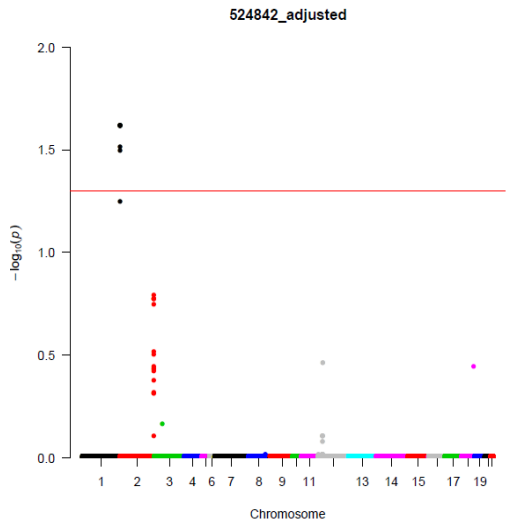
[2] Genome-wide $P_{FDR} < 0.05$.

Trait	Taxonomy	Frequency of presence	Mean	Standard deviation	OCC[1]	Window	SNP	bp	A1	A2	Minor allele frequency	Effect (phenotypic standard deviations)	*P* value	P_{FDR}[2]
NR276	Order *Clostridiales (Firmicutes)*	0.39	91.33	147.99	16	1745	AX-147002435	75818806	1	2	0.32	0.33	1.74E-05	3.93E-02
NR276	Order *Clostridiales (Firmicutes)*	0.39	91.33	147.99	16	1745	AX-147096636	75843118	1	2	0.32	0.34	1.48E-05	3.93E-02
NR276	Order *Clostridiales (Firmicutes)*	0.39	91.33	147.99	16	1745	AX-147104300	75855166	1	2	0.32	0.34	1.37E-05	3.93E-02
NR276	Order *Clostridiales (Firmicutes)*	0.39	91.33	147.99	16	1745	AX-147074337	75861690	1	2	0.32	0.34	1.40E-05	3.93E-02
NR1121	Family *Ruminococcaceae (Firmicutes)*	0.84	173.37	114.07	19	1944	AX-147016425	52466562	1	2	0.45	0.34	8.05E-07	2.64E-02
NR1121	Family *Ruminococcaceae (Firmicutes)*	0.84	173.37	114.07	19	1944	AX-147147148	52503140	1	2	0.41	0.34	1.46E-06	2.64E-02
NR1121	Family *Ruminococcaceae (Firmicutes)*	0.84	173.37	114.07	19	1944	AX-147086137	52517853	1	2	0.42	0.35	6.80E-07	2.64E-02
NR1121	Family *Ruminococcaceae (Firmicutes)*	0.84	173.37	114.07	19	1944	AX-147178374	52549938	1	2	0.42	0.34	1.42E-06	2.64E-02
NR1121	Family *Ruminococcaceae (Firmicutes)*	0.84	173.37	114.07	19	1944	AX-146985505	52594128	1	2	0.42	0.32	4.97E-06	3.87E-02
NR1121	Family *Ruminococcaceae (Firmicutes)*	0.84	173.37	114.07	19	1944	AX-147004008	52603232	1	2	0.46	0.32	2.01E-06	2.70E-02
NR1121	Family *Ruminococcaceae (Firmicutes)*	0.84	173.37	114.07	19	1944	AX-147181591	52623370	1	2	0.43	0.33	3.48E-06	3.63E-02
NR1121	Family *Ruminococcaceae (Firmicutes)*	0.84	173.37	114.07	19	1944	AX-147051965	52639502	1	2	0.42	0.32	4.81E-06	3.87E-02
NR1121	Family *Ruminococcaceae (Firmicutes)*	0.84	173.37	114.07	19	1944	AX-147072132	52674126	1	2	0.42	0.32	5.30E-06	3.87E-02
NR1121	Family *Ruminococcaceae (Firmicutes)*	0.84	173.37	114.07	19	1944	AX-147138894	52689352	1	2	0.42	0.33	2.59E-06	2.97E-02
NR1121	Family *Ruminococcaceae (Firmicutes)*	0.84	173.37	114.07	19	1944	AX-147034383	52774117	1	2	0.41	0.32	5.87E-06	3.95E-02
NR1121	Family *Ruminococcaceae (Firmicutes)*	0.84	173.37	114.07	19	1944	AX-146993339	52825472	1	2	0.41	0.33	2.12E-06	2.70E-02
NR1121	Family *Ruminococcaceae (Firmicutes)*	0.84	173.37	114.07	19	1944	AX-146988051	52831808	1	2	0.45	0.33	1.23E-06	2.64E-02
NR1121	Family *Ruminococcaceae (Firmicutes)*	0.84	173.37	114.07	19	1944	AX-147018404	52850444	1	2	0.45	0.33	1.61E-06	2.64E-02
NR1121	Family *Ruminococcaceae (Firmicutes)*	0.84	173.37	114.07	19	1945	AX-147109903	53185957	1	2	0.41	0.34	1.37E-06	2.64E-02
NR1121	Family *Ruminococcaceae (Firmicutes)*	0.84	173.37	114.07	19	1945	AX-147024906	53240715	1	2	0.42	-0.31	5.40E-06	3.87E-02
NR1121	Family *Ruminococcaceae (Firmicutes)*	0.84	173.37	114.07	19	1945	AX-147016428	53245716	1	2	0.42	-0.31	5.40E-06	3.87E-02
NR1121	Family *Ruminococcaceae (Firmicutes)*	0.84	173.37	114.07	19	1945	AX-147051966	53255837	1	2	0.43	-0.31	7.61E-06	4.84E-02

[1]*Oryctolagus cuniculus* chromosome.
[2]Genome-wide $P_{FDR} < 0.05$.

Additional file 7.5: Figure 7.S1| Manhattan plots for 19 microbial traits.

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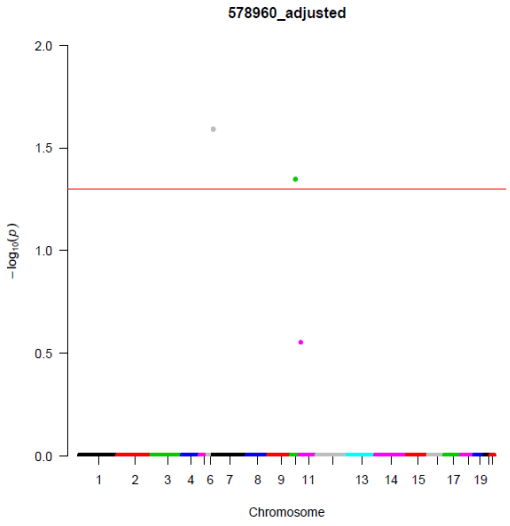
Chromosome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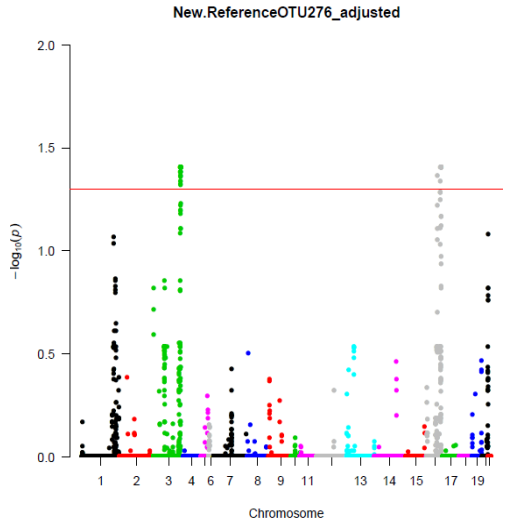
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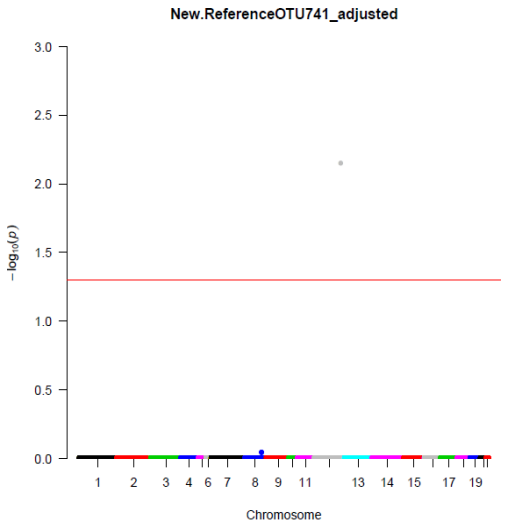
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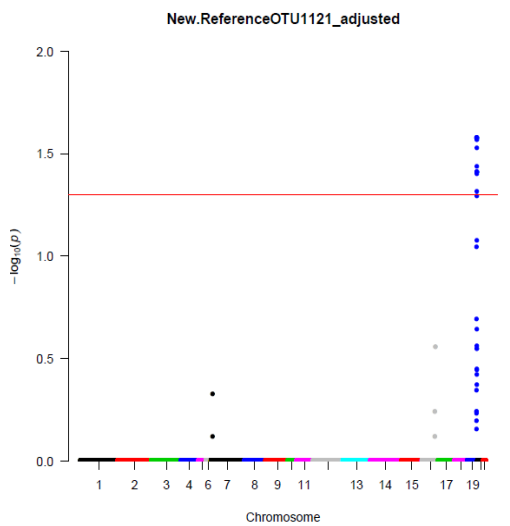
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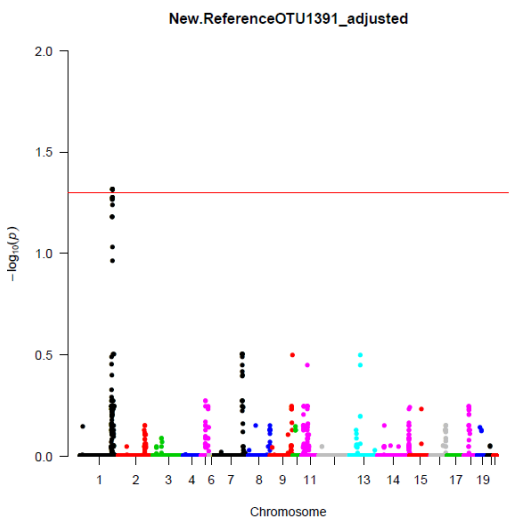
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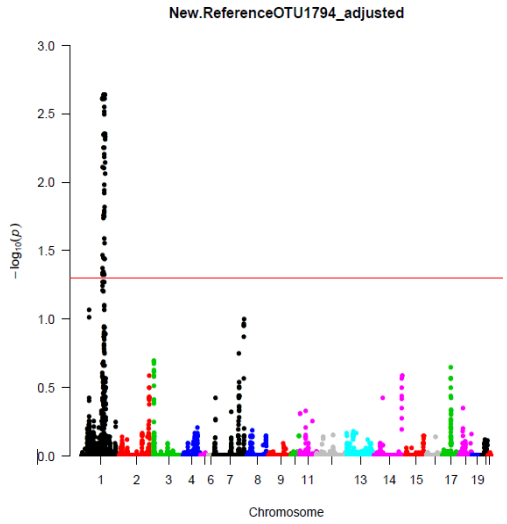
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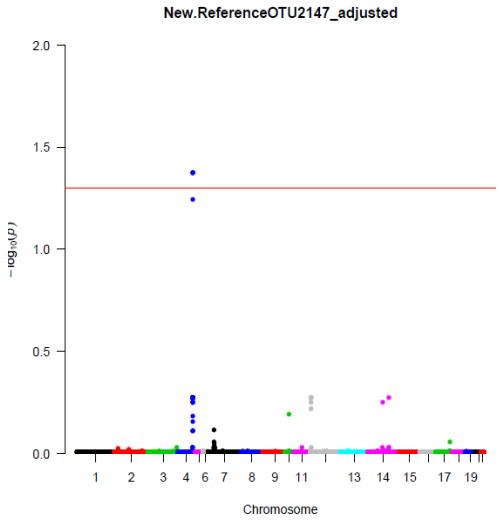
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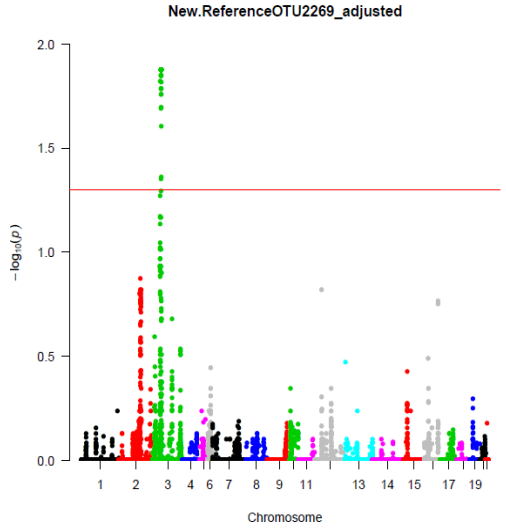
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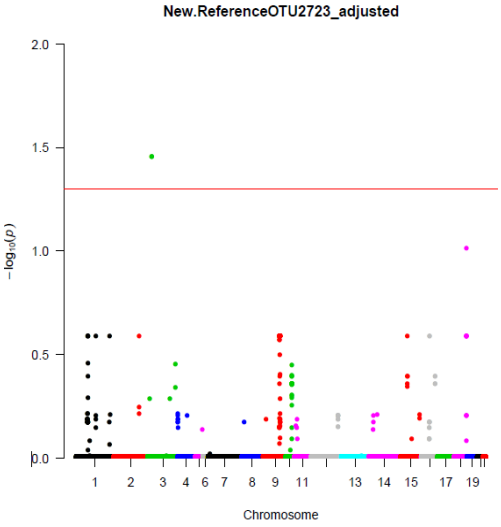
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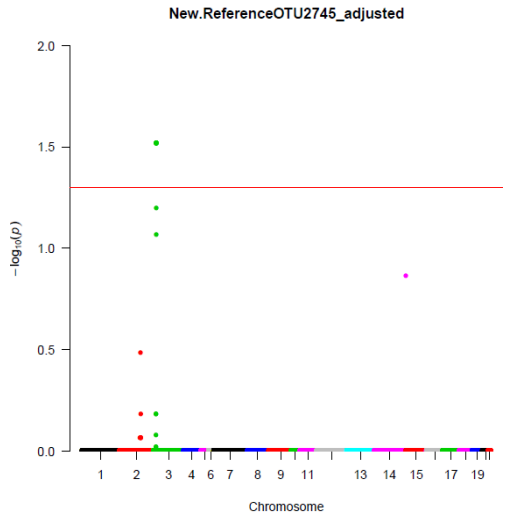
Chromosome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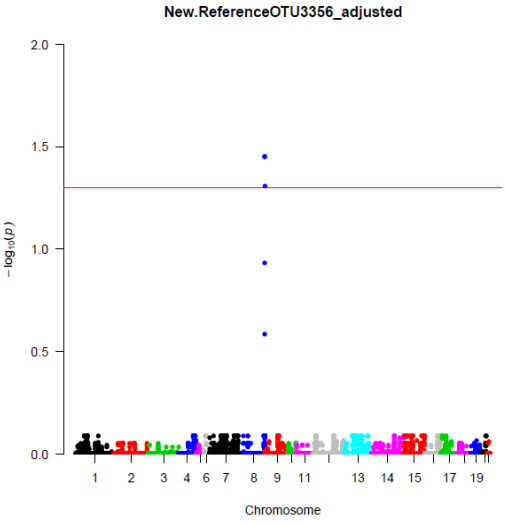
Chromosome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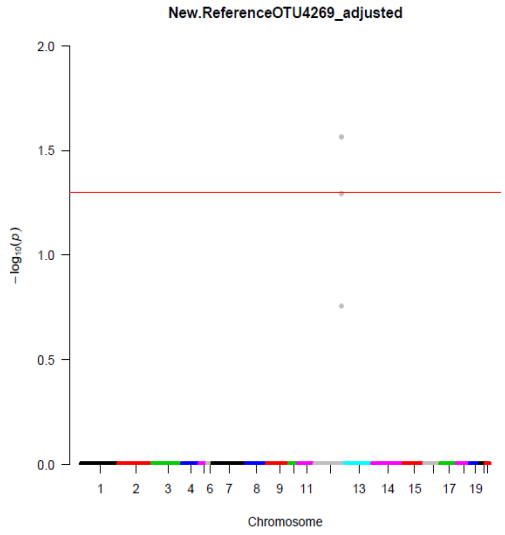
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1.0
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$-\log_{10}(p)$
1
2
3
4
6
7
8
9
11
13
14
15
17
19
Chromosome

Additional file 7.6: Table 7.S2| Genes annotated around the windows that the MIX-GWAS declared to contain variants significantly associated with any of 19 microbial traits at the genome-wide level.

OCC[1]	Gene start (bp)	Gene end (bp)	Gene stable ID	Gene name	Gene type	Human Gene name
1	103076973	103403200	ENSOCUG00000006190	NCAM1	protein_coding	NCAM1
1	104116161	104127484	ENSOCUG00000009672		protein_coding	PLET1
1	104144624	104151577	ENSOCUG00000007360	PTS	protein_coding	PTS
1	104153714	104288640	ENSOCUG00000009662	BCO2	protein_coding	BCO2
1	104208433	104234438	ENSOCUG00000005255		protein_coding	IL18
1	104276207	104276335	ENSOCUG00000025177		snoRNA	SNORA11
1	104288605	104290455	ENSOCUG00000017358	TIMM8B	protein_coding	TIMM8B
1	104291152	104306137	ENSOCUG00000017354	NKAPD1	protein_coding	NKAPD1
1	104308328	104316435	ENSOCUG00000028144	PIH1D2	protein_coding	PIH1D2
1	104315403	104353713	ENSOCUG00000015590	DLAT	protein_coding	DLAT
1	104359957	104448028	ENSOCUG00000012919	DIXDC1	protein_coding	DIXDC1
1	104450633	104456800	ENSOCUG00000012977	C11orf52	protein_coding	C11orf52
1	104460634	104461649	ENSOCUG00000012976		protein_coding	HSPB2
1	104460634	104461649	ENSOCUG00000012976		protein_coding	HSPB2-C11orf52
1	104462664	104465787	ENSOCUG00000012972	CRYAB	protein_coding	CRYAB
1	104470817	104475531	ENSOCUG00000029240	C11orf1	protein_coding	C11orf1
1	104475628	104480018	ENSOCUG00000034621	FDXACB1	protein_coding	FDXACB1
1	104481916	104645120	ENSOCUG00000012930	PPP2R1B	protein_coding	PPP2R1B
1	104646449	104793737	ENSOCUG00000012915	SIK2	protein_coding	SIK2
1	104844504	104864855	ENSOCUG00000012904	LAYN	protein_coding	LAYN
1	104868697	104892936	ENSOCUG00000012891	HOATZ	protein_coding	HOATZ
1	104894553	104921531	ENSOCUG00000027498		protein_coding	BTG4
1	105028595	105052636	ENSOCUG00000012566	POU2AF1	protein_coding	POU2AF1
1	105100758	105110386	ENSOCUG00000022422	COLCA2	protein_coding	COLCA2
1	105119303	105157796	ENSOCUG00000009285	C11orf53	protein_coding	C11orf53
1	105325533	105326573	ENSOCUG00000017811		protein_coding	CTDSP2
1	105411910	105413162	ENSOCUG00000039274		protein_coding	PSMC4
1	105770187	105902069	ENSOCUG00000013948	ARHGAP20	protein_coding	ARHGAP20
1	106089401	106158812	ENSOCUG00000017826		protein_coding	FDX1
1	108746324	108761554	ENSOCUG00000011878	SLC35F2	protein_coding	SLC35F2
1	108870382	108938953	ENSOCUG00000011869	ELMOD1	protein_coding	ELMOD1
1	108966739	109370463	ENSOCUG00000025398	CWF19L2	protein_coding	CWF19L2
1	109064046	109064825	ENSOCUG00000038405		protein_coding	PPIL4
1	109603363	109941502	ENSOCUG00000004490	GUCY1A2	protein_coding	GUCY1A2
1	110542467	110543042	ENSOCUG00000023190		protein_coding	AKIRIN1
1	110573411	110597042	ENSOCUG00000000231	AASDHPPT	protein_coding	AASDHPPT
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1	112364735	112638071	ENSOCUG00000015312	PDGFD	protein_coding	PDGFD
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1	113476884	113507326	ENSOCUG00000006512	DCUN1D5	protein_coding	DCUN1D5
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1	114085284	114180830	ENSOCUG00000006165		protein_coding	TMEM123
1	114161365	114183218	ENSOCUG00000013890	BIRC2	protein_coding	BIRC2
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1	114274384	114276187	ENSOCUG00000034499		protein_coding	SIK1
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1	114547352	114692292	ENSOCUG00000025834	YAP1	protein_coding	YAP1

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1	114734361	114734657	ENSOCUG00000035862		protein_coding	MT-ND4L
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1	114893812	114893922	ENSOCUG00000028894	U2	snRNA	RNU2-38P
1	114897411	114912402	ENSOCUG00000029200	ANGPTL5	protein_coding	ANGPTL5
1	115005605	115006961	ENSOCUG00000026522		protein_coding	RGS19
1	115173391	115311739	ENSOCUG00000017832	TRPC6	protein_coding	TRPC6
1	115601359	115672617	ENSOCUG00000014693	PGR	protein_coding	PGR
1	115716354	116020191	ENSOCUG00000006927	ARHGAP42	protein_coding	ARHGAP42
1	116108943	116109045	ENSOCUG00000024451	Vault	vault_RNA	VTRNA1-1
1	116342857	117128194	ENSOCUG00000015047	CNTN5	protein_coding	CNTN5
1	116666921	116667033	ENSOCUG00000020328	U2	snRNA	U2
1	117357429	117357573	ENSOCUG00000030649	U2	snRNA	U2
1	119788902	119789008	ENSOCUG00000026620	U6	snRNA	RNU6-86P
1	119873354	119874262	ENSOCUG00000026047		protein_coding	RACK1
1	120385369	120386943	ENSOCUG00000024211	JRKL	protein_coding	JRKL
1	120397369	120437458	ENSOCUG00000007312	CCDC82	protein_coding	CCDC82
1	120444237	120817155	ENSOCUG00000010820	MAML2	protein_coding	MAML2
1	121624473	121710677	ENSOCUG00000026640	SESN3	protein_coding	SESN3
1	121748371	121799205	ENSOCUG00000014040	ENDOD1	protein_coding	ENDOD1
1	121826338	121828614	ENSOCUG00000038702		protein_coding	KDM4D
1	121836191	121838602	ENSOCUG00000035381		protein_coding	KDM4D
1	121842034	121844469	ENSOCUG00000031183		protein_coding	KDM4D
1	121856602	121859891	ENSOCUG00000032220		protein_coding	KDM4D
1	121868368	121869852	ENSOCUG00000001631		protein_coding	KDM4D
1	121904820	121918615	ENSOCUG00000001629	CWC15	protein_coding	CWC15
1	121989693	122169562	ENSOCUG00000001078	AMOTL1	protein_coding	AMOTL1
1	122255495	122322730	ENSOCUG00000002860	PIWIL4	protein_coding	PIWIL4
1	122320980	122322352	ENSOCUG00000014204	FUT4	protein_coding	FUT4
1	122334463	122355258	ENSOCUG00000034472	C11orf97	protein_coding	C11orf97
1	122375716	122377685	ENSOCUG00000014912	ANKRD49	protein_coding	ANKRD49
1	122380603	122462103	ENSOCUG00000014899	MRE11	protein_coding	MRE11
1	122479098	122491139	ENSOCUG00000015502	GPR83	protein_coding	GPR83
1	122542062	122545546	ENSOCUG00000013431	IZUMO1R	protein_coding	IZUMO1R
1	122739267	122790959	ENSOCUG00000009473	PANX1	protein_coding	PANX1
1	122790845	122873124	ENSOCUG00000009466	HEPHL1	protein_coding	HEPHL1
1	122834615	122835099	ENSOCUG00000029520		protein_coding	UBE2I
1	123084924	123115553	ENSOCUG00000026554	VSTM5	protein_coding	VSTM5
1	123122562	123146995	ENSOCUG00000012818		protein_coding	
1	123122562	123146995	ENSOCUG00000012818		protein_coding	MED17
1	123155483	123174270	ENSOCUG00000012810	C11orf54	protein_coding	C11orf54
1	123182085	123183767	ENSOCUG00000001096		protein_coding	TAF1D
1	123187721	123187852	ENSOCUG00000019024		snoRNA	SNORA18
1	123187998	123188070	ENSOCUG00000023335		snoRNA	SNORD5
1	123188669	123188807	ENSOCUG00000018616		snoRNA	
1	123188669	123188807	ENSOCUG00000018616		snoRNA	SNORA8
1	123188669	123188807	ENSOCUG00000018616		snoRNA	
1	123188669	123188807	ENSOCUG00000018616		snoRNA	
1	123189544	123189616	ENSOCUG00000018187		snoRNA	SNORD6
1	123190358	123248675	ENSOCUG00000029678	CEP295	protein_coding	CEP295
1	123202169	123202238	ENSOCUG00000025554		snoRNA	
1	123202436	123202516	ENSOCUG00000025409		snoRNA	
1	123202436	123202516	ENSOCUG00000025409		snoRNA	
1	123202436	123202516	ENSOCUG00000025409		snoRNA	
1	123202436	123202516	ENSOCUG00000025409		snoRNA	
1	123448210	123560885	ENSOCUG00000000199	DEUP1	protein_coding	DEUP1
1	123723183	123786844	ENSOCUG00000000649	SLC36A4	protein_coding	SLC36A4
1	163455024	163456054	ENSOCUG00000028087		protein_coding	TAF9B
1	163681593	164228640	ENSOCUG00000026462	LUZP2	protein_coding	LUZP2
1	180046179	180046325	ENSOCUG00000033509	U2	snRNA	U2
1	181040738	181041079	ENSOCUG00000027982		protein_coding	MRPS14
1	181107743	181108069	ENSOCUG00000022894		protein_coding	ATP5PF
1	181466694	181466855	ENSOCUG00000037141	U2	snRNA	U2
1	192759737	192769432	ENSOCUG00000039555	OOSP4A	protein_coding	OOSP4A
1	192804408	192813908	ENSOCUG00000035279	OOSP4B	protein_coding	OOSP4B
1	192825924	192833226	ENSOCUG00000002420	MS4A3	protein_coding	MS4A3
1	192840013	192846803	ENSOCUG00000009077	MS4A2	protein_coding	MS4A2
1	192879178	192888794	ENSOCUG00000009084		protein_coding	MS4A6A
1	192879178	192888794	ENSOCUG00000009084		protein_coding	MS4A6E
1	192904458	193017292	ENSOCUG00000010079		protein_coding	MS4A4E

1	192904458	193017292	ENSOCUG00000010079		protein_coding	MS4A4A
1	192964382	192992566	ENSOCUG00000012559		protein_coding	MS4A14
1	193022391	193037127	ENSOCUG00000033235	MS4A5	protein_coding	MS4A5
1	193048544	193054455	ENSOCUG00000007941	MS4A1	protein_coding	MS4A1
1	193072961	193092144	ENSOCUG00000007947	MS4A12	protein_coding	MS4A12
1	193107065	193130760	ENSOCUG00000015915	MS4A13	protein_coding	MS4A13
1	193315453	193327931	ENSOCUG00000000079	MS4A8	protein_coding	MS4A8
1	193412807	193430923	ENSOCUG00000026537		protein_coding	MS4A18
1	193435444	193446667	ENSOCUG00000013273	MS4A15	protein_coding	MS4A15
1	193455420	193462267	ENSOCUG00000015310		protein_coding	MS4A10
1	193487131	193491041	ENSOCUG00000026432		protein_coding	CCDC86
1	193492255	193495917	ENSOCUG00000025455	PTGDR2	protein_coding	PTGDR2
1	193505780	193512590	ENSOCUG00000015673	ZP1	protein_coding	ZP1
1	193523793	193533396	ENSOCUG00000015689	PRPF19	protein_coding	PRPF19
1	193539339	193549353	ENSOCUG00000015708	TMEM109	protein_coding	TMEM109
1	193550488	193561477	ENSOCUG00000015713	TMEM132A	protein_coding	TMEM132A
1	193560546	193575083	ENSOCUG00000015723	SLC15A3	protein_coding	SLC15A3
1	193592727	193631909	ENSOCUG00000012464	CD6	protein_coding	CD6
1	193696883	193703300	ENSOCUG00000011143	FOSL1	protein_coding	FOSL1
1	193718930	193724033	ENSOCUG00000020976		protein_coding	DRAP1
1	193719308	193720721	ENSOCUG00000010947	C11orf68	protein_coding	C11orf68
1	193772735	193774646	ENSOCUG00000021968	EIF1AD	protein_coding	EIF1AD
1	193776718	193777943	ENSOCUG00000015741		protein_coding	BANF1
1	193784733	193827889	ENSOCUG00000015722	SF3B2	protein_coding	SF3B2
1	193788022	193794059	ENSOCUG00000016490		protein_coding	CATSPER1
1	193799472	193803646	ENSOCUG00000015102	GAL3ST3	protein_coding	GAL3ST3
1	193828403	193983871	ENSOCUG00000025097	PACS1	protein_coding	PACS1
1	194032136	194032242	ENSOCUG00000025475	U6	snRNA	RNU6-329P
1	194060913	194067063	ENSOCUG00000000905	NPAS4	protein_coding	NPAS4
1	194078352	194080481	ENSOCUG00000020926	MRPL11	protein_coding	MRPL11
1	194101623	194134151	ENSOCUG00000010915	DPP3	protein_coding	DPP3
1	194101623	194109424	ENSOCUG00000010911	PELI3	protein_coding	PELI3
1	194134304	194143072	ENSOCUG00000027758		protein_coding	
1	194134304	194143072	ENSOCUG00000027758		protein_coding	BBS1
1	194144761	194150464	ENSOCUG00000039117		protein_coding	
1	194144761	194150464	ENSOCUG00000039117		protein_coding	BBS1
1	194167898	194180590	ENSOCUG00000023317	ACTN3	protein_coding	ACTN3
1	194180781	194185579	ENSOCUG00000013700	CTSF	protein_coding	CTSF
1	194197173	194199758	ENSOCUG00000022515	CCDC87	protein_coding	CCDC87
1	194200010	194209291	ENSOCUG00000013823	CCS	protein_coding	CCS
1	194227852	194235841	ENSOCUG00000022838		protein_coding	RBM14
1	194244876	194278252	ENSOCUG00000004731	RBM4	protein_coding	RBM4
1	194277710	194328817	ENSOCUG00000014485	SPTBN2	protein_coding	SPTBN2
1	194340816	194444031	ENSOCUG00000009821	RCE1	protein_coding	RCE1
1	194414706	194438187	ENSOCUG00000003485		protein_coding	C11orf80
1	194422395	194422525	ENSOCUG00000024662	SNORA63	snoRNA	SNORA63
1	194422395	194422525	ENSOCUG00000024662	SNORA63	snoRNA	SNORA63C
1	194599865	194731670	ENSOCUG00000009988	KDM2A	protein_coding	KDM2A
1	194743802	194746827	ENSOCUG00000038516		protein_coding	GRK2
1	194754486	194756335	ENSOCUG00000029982		protein_coding	ANKRD13D
1	194758430	194759825	ENSOCUG00000006388		protein_coding	ANKRD13D
1	194761747	194767569	ENSOCUG00000006393	SSH3	protein_coding	SSH3
1	194770434	194835787	ENSOCUG00000024015	RAD9A	protein_coding	RAD9A
1	194802505	194804152	ENSOCUG00000004005		protein_coding	POLD4
1	194802505	194804152	ENSOCUG00000004005		protein_coding	
1	194811735	194819288	ENSOCUG00000003943	CLCF1	protein_coding	CLCF1
1	194835165	194837449	ENSOCUG00000027936	PPP1CA	protein_coding	PPP1CA
1	194839289	194843634	ENSOCUG00000007702	TBC1D10C	protein_coding	TBC1D10C
3	13348977	13620338	ENSOCUG00000003824	CHSY3	protein_coding	CHSY3
3	13998551	13998657	ENSOCUG00000020281	U6	snRNA	RNU6-708P
3	13998551	13998657	ENSOCUG00000020281	U6	snRNA	RNU6-17P
3	13998551	13998657	ENSOCUG00000020281	U6	snRNA	RNU6-18P
3	13998551	13998657	ENSOCUG00000020281	U6	snRNA	RNU6-13P
3	13998551	13998657	ENSOCUG00000020281	U6	snRNA	RNU6-12P
3	13998551	13998657	ENSOCUG00000020281	U6	snRNA	RNU6-32P
3	14082048	14082153	ENSOCUG00000018309	U5	snRNA	RNU5E-8P
3	14082048	14082153	ENSOCUG00000018309	U5	snRNA	RNU5E-10P
3	14082048	14082153	ENSOCUG00000018309	U5	snRNA	RNU5E-9P
3	14082048	14082153	ENSOCUG00000018309	U5	snRNA	RNU5F-1
3	14082048	14082153	ENSOCUG00000018309	U5	snRNA	RNU5E-6P
3	14380902	14393488	ENSOCUG00000006607		protein_coding	HINT1

3	14399882	14423644	ENSOCUG00000000166	LYRM7	protein_coding	LYRM7
3	14503201	14572136	ENSOCUG00000003842	CDC42SE2	protein_coding	CDC42SE2
3	14591772	14790284	ENSOCUG00000003847		protein_coding	
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3	14797746	14928623	ENSOCUG00000024304	FNIP1	protein_coding	FNIP1
3	14940026	15026594	ENSOCUG00000029222	MEIKIN	protein_coding	MEIKIN
3	15033887	15092849	ENSOCUG00000013062	ACSL6	protein_coding	
3	15033887	15092849	ENSOCUG00000013062	ACSL6	protein_coding	ACSL6
3	15144158	15146030	ENSOCUG00000003433	CSF2	protein_coding	CSF2
3	15256646	15305094	ENSOCUG00000008966	P4HA2	protein_coding	P4HA2
3	15322522	15336779	ENSOCUG00000023147	PDLIM4	protein_coding	PDLIM4
3	15351327	15442156	ENSOCUG00000004158	SLC22A5	protein_coding	SLC22A5
3	15531526	15536131	ENSOCUG00000004165	IRF1	protein_coding	IRF1
3	15573697	15575837	ENSOCUG00000004171	IL5	protein_coding	IL5
3	15593649	15672969	ENSOCUG00000025001		protein_coding	RAD50
3	15593649	15672969	ENSOCUG00000025001		protein_coding	
3	15690430	15692138	ENSOCUG00000000154	IL13	protein_coding	IL13
3	15702738	15711514	ENSOCUG00000011943	IL-4	protein_coding	IL4
3	15713102	15713221	ENSOCUG00000020212		snoRNA	
3	15722727	15804489	ENSOCUG00000011761	KIF3A	protein_coding	KIF3A
3	19338232	19824470	ENSOCUG00000009843	SPOCK1	protein_coding	SPOCK1
3	19929843	20086686	ENSOCUG00000007560	KLHL3	protein_coding	KLHL3
3	19951768	19951827	ENSOCUG00000019996	ocu-mir-874	miRNA	MIR874
3	20189716	20227222	ENSOCUG00000014414	MYOT	protein_coding	MYOT
3	20229453	20271062	ENSOCUG00000014419	PKD2L2	protein_coding	PKD2L2
3	20272381	20338593	ENSOCUG00000014431	FAM13B	protein_coding	FAM13B
3	20391489	20396619	ENSOCUG00000005191	WNT8A	protein_coding	WNT8A
3	20412976	20439137	ENSOCUG00000023232	NME5	protein_coding	NME5
3	20438715	20476648	ENSOCUG00000008362	BRD8	protein_coding	BRD8
3	20477190	20485828	ENSOCUG00000015790	KIF20A	protein_coding	KIF20A
3	20486702	20513737	ENSOCUG00000008382	CDC23	protein_coding	CDC23
3	20538736	20555487	ENSOCUG00000008391	GFRA3	protein_coding	GFRA3
3	20579803	20612804	ENSOCUG00000008399	CDC25C	protein_coding	CDC25C
3	20622105	20628617	ENSOCUG00000008410	FAM53C	protein_coding	FAM53C
3	20633646	20717771	ENSOCUG00000008421	KDM3B	protein_coding	KDM3B
3	20719556	20724776	ENSOCUG00000008433	REEP2	protein_coding	REEP2
3	20738524	20741362	ENSOCUG00000008439	EGR1	protein_coding	EGR1
3	20770475	20803894	ENSOCUG00000006650	ETF1	protein_coding	ETF1
3	20812819	20831385	ENSOCUG00000006655	HSPA9	protein_coding	HSPA9
3	20818048	20818122	ENSOCUG00000019434	SNORD63	snoRNA	SNORD63
3	20876951	21173275	ENSOCUG00000007744	CTNNA1	protein_coding	CTNNA1
3	21121140	21123018	ENSOCUG00000007751	LRRTM2	protein_coding	LRRTM2
3	21169006	21456118	ENSOCUG00000007752	SIL1	protein_coding	SIL1
3	21481750	21481908	ENSOCUG00000019330	U1	snRNA	RNVU1-4
3	21481750	21481908	ENSOCUG00000019330	U1	snRNA	RNVU1-3
3	21481750	21481908	ENSOCUG00000019330	U1	snRNA	RNVU1-30
3	21543198	21543399	ENSOCUG00000019393	SNORA74	snoRNA	SNORA74A
3	21550215	21581826	ENSOCUG00000026311		protein_coding	MATR3
3	21550215	21581826	ENSOCUG00000026311		protein_coding	MATR3
3	21593574	21619608	ENSOCUG00000006932	PAIP2	protein_coding	PAIP2
3	21622315	21632165	ENSOCUG00000006934	SLC23A1	protein_coding	SLC23A1
3	21632426	21634352	ENSOCUG00000006942	MZB1	protein_coding	MZB1
3	21636159	21639200	ENSOCUG00000006947	PROB1	protein_coding	PROB1
3	21640852	21647584	ENSOCUG00000006952	SPATA24	protein_coding	SPATA24
3	21657088	21683033	ENSOCUG00000006955	DNAJC18	protein_coding	DNAJC18
3	21690379	21702175	ENSOCUG00000023976		protein_coding	
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3	37839069	38236239	ENSOCUG00000002000	SGCD	protein_coding	SGCD
3	38409832	38454696	ENSOCUG00000011915	TIMD4	protein_coding	TIMD4
3	38520056	38609318	ENSOCUG00000000575		protein_coding	HAVCR1
3	38629480	38632741	ENSOCUG00000002365	FAM71B	protein_coding	FAM71B
3	38648074	38713129	ENSOCUG00000002369	ITK	protein_coding	ITK
3	38752689	38856653	ENSOCUG00000011780	CYFIP2	protein_coding	CYFIP2
3	38812026	38812703	ENSOCUG00000025063		protein_coding	
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3	38856000	38913494	ENSOCUG00000001234	NIPAL4	protein_coding	NIPAL4
3	38918211	39113810	ENSOCUG00000001235	ADAM19	protein_coding	ADAM19
3	39140001	39147379	ENSOCUG00000001053	THG1L	protein_coding	THG1L
3	39155253	39158162	ENSOCUG00000011672	LSM11	protein_coding	LSM11
3	39191568	39260318	ENSOCUG00000014232	CLINT1	protein_coding	CLINT1
3	39216523	39216628	ENSOCUG00000028257	U6	snRNA	RNU6-835P

3	42027802	42318730	ENSOCUG00000006800	ATP10B	protein_coding	ATP10B
3	42698620	42996012	ENSOCUG00000010098	GABRB2	protein_coding	GABRB2
3	43123963	43146818	ENSOCUG00000014884	GABRA6	protein_coding	GABRA6
3	43282583	43336936	ENSOCUG00000016109	GABRA1	protein_coding	GABRA1
3	43463208	43564143	ENSOCUG00000016742	GABRG2	protein_coding	GABRG2
3	44665155	44671337	ENSOCUG00000002129		protein_coding	CCNG1
3	44683151	44689533	ENSOCUG00000005783	NUDCD2	protein_coding	NUDCD2
3	44689554	44723956	ENSOCUG00000022126	HMMR	protein_coding	HMMR
3	44737303	44755320	ENSOCUG00000008074	MAT2B	protein_coding	MAT2B
3	45430711	45432261	ENSOCUG00000031379		protein_coding	GLA
3	133295358	133322238	ENSOCUG00000011890	MED30	protein_coding	MED30
3	133351189	133351812	ENSOCUG00000038310		protein_coding	RAB7A
3	133644809	133954815	ENSOCUG00000015191	EXT1	protein_coding	EXT1
3	134047653	134535595	ENSOCUG00000009066	SAMD12	protein_coding	SAMD12
3	134829082	134858124	ENSOCUG00000011150	TNFRSF11B	protein_coding	TNFRSF11B
3	134967647	135021503	ENSOCUG00000024595	COLEC10	protein_coding	COLEC10
3	138395252	138395415	ENSOCUG00000023261	U1	snRNA	RNU1-77P
3	138395252	138395415	ENSOCUG00000023261	U1	snRNA	RNVU1-32
3	138826966	138829517	ENSOCUG00000008550	ZHX2	protein_coding	ZHX2
3	138874995	138910831	ENSOCUG00000009112	DERL1	protein_coding	DERL1
3	138933561	138995376	ENSOCUG00000009119	TBC1D31	protein_coding	TBC1D31
3	139011170	139028867	ENSOCUG00000015368	FAM83A	protein_coding	FAM83A
3	139038174	139062857	ENSOCUG00000009772		protein_coding	ZHX1-C8orf76
3	139038174	139062857	ENSOCUG00000009772		protein_coding	C8orf76
3	139078878	139081502	ENSOCUG00000009776	ZHX1	protein_coding	ZHX1
3	139210581	139238252	ENSOCUG00000014201		protein_coding	NTAQ1
3	139306981	139358152	ENSOCUG00000005112	FBXO32	protein_coding	FBXO32
3	139445409	139452352	ENSOCUG00000011785	KLHL38	protein_coding	KLHL38
3	139477693	139540254	ENSOCUG00000011787	ANXA13	protein_coding	ANXA13
3	139571237	139618661	ENSOCUG00000011804	FAM91A1	protein_coding	FAM91A1
3	139710979	139904183	ENSOCUG00000026113	FER1L6	protein_coding	FER1L6
3	140194108	140198725	ENSOCUG00000026379	TRMT12	protein_coding	TRMT12
3	140215774	140229652	ENSOCUG00000017399	RNF139	protein_coding	RNF139
3	140230199	140275934	ENSOCUG00000017403	TATDN1	protein_coding	TATDN1
3	140273518	140279290	ENSOCUG00000025074	NDUFB9	protein_coding	NDUFB9
3	140280678	140448335	ENSOCUG00000008102	MTSS1	protein_coding	MTSS1
3	140549025	140549152	ENSOCUG00000020979		snoRNA	SNORA31
3	140689416	140695085	ENSOCUG00000039324	ZNF572	protein_coding	ZNF572
3	140710644	140735727	ENSOCUG00000015771	SQLE	protein_coding	SQLE
3	140737573	140805745	ENSOCUG00000015785	WASHC5	protein_coding	WASHC5
3	140811891	141088037	ENSOCUG00000008539	NSMCE2	protein_coding	NSMCE2
3	140891744	140891986	ENSOCUG00000030414		protein_coding	COX7B
3	141104856	141105154	ENSOCUG00000038943	Metazoa_SRP	misc_RNA	RN7SL178P
3	141117788	141122075	ENSOCUG00000024295	TRIB1	protein_coding	TRIB1
3	141610868	141610972	ENSOCUG00000021693	U6	snRNA	RNU6-365P
4	75069167	75121504	ENSOCUG00000010931	CCDC38	protein_coding	CCDC38
4	75137656	75163689	ENSOCUG00000005523	AMDHD1	protein_coding	AMDHD1
4	75167385	75193674	ENSOCUG00000005528	HAL	protein_coding	HAL
4	75201595	75245579	ENSOCUG00000005535	LTA4H	protein_coding	LTA4H
4	75371042	75464437	ENSOCUG00000034370	ELK3	protein_coding	ELK3
4	75474542	75511662	ENSOCUG00000013583	CDK17	protein_coding	CDK17
4	75661682	76016541	ENSOCUG00000013917	CFAP54	protein_coding	CFAP54
4	76053626	76094569	ENSOCUG00000010944	NEDD1	protein_coding	NEDD1
4	76103176	76103282	ENSOCUG00000025761	U6	snRNA	U6
4	76714478	76714578	ENSOCUG00000020134		miRNA	MIR1251
4	76791364	76791463	ENSOCUG00000018977		miRNA	MIR135A2
6	26473329	26473730	ENSOCUG00000032548		protein_coding	SZRD1
6	26611331	26615730	ENSOCUG00000012402	UPK3B	protein_coding	UPK3B
6	26618144	26650582	ENSOCUG00000000565	DTX2	protein_coding	DTX2
6	26662111	26671566	ENSOCUG00000015762		protein_coding	ZP3
6	26662111	26671566	ENSOCUG00000015762		protein_coding	POMZP3
6	26668043	26687303	ENSOCUG00000016629	SSC4D	protein_coding	SSC4D
6	26713722	26738069	ENSOCUG00000008521	YWHAG	protein_coding	YWHAG
6	26753104	26754383	ENSOCUG00000012690	HSPB1	protein_coding	HSPB1
6	26791443	26855912	ENSOCUG00000014322	MDH2	protein_coding	MDH2
6	26847287	26894306	ENSOCUG00000027186	STYXL1	protein_coding	STYXL1
6	26900350	26913491	ENSOCUG00000021696	POR	protein_coding	POR
6	26962102	26971780	ENSOCUG00000013803	RHBDD2	protein_coding	RHBDD2
6	26989695	26992144	ENSOCUG00000025061		protein_coding	CCL24
6	26994696	26995939	ENSOCUG00000027199		protein_coding	SIK1B
6	26994696	26995939	ENSOCUG00000027199		protein_coding	SIK1

6	27001595	27010068	ENSOCUG00000000382	SLC12A9	protein_coding	SLC12A9
6	27012763	27181342	ENSOCUG00000016902	EPHB4	protein_coding	EPHB4
6	27045103	27084325	ENSOCUG00000005589	ZAN	protein_coding	ZAN
6	27102172	27105135	ENSOCUG00000005399	EPO	protein_coding	EPO
6	27121461	27121883	ENSOCUG00000007818	POP7	protein_coding	POP7
6	27132958	27143372	ENSOCUG00000002663	GIGYF1	protein_coding	GIGYF1
6	27144075	27147121	ENSOCUG00000002657	GNB2	protein_coding	GNB2
6	27158292	27170777	ENSOCUG00000014727	ACTL6B	protein_coding	ACTL6B
6	27184381	27186764	ENSOCUG00000005694		protein_coding	MOSPD3
6	27188476	27193370	ENSOCUG00000031409	PCOLCE	protein_coding	PCOLCE
6	27194545	27203106	ENSOCUG00000022861	FBXO24	protein_coding	FBXO24
6	27203015	27216747	ENSOCUG00000002528	LRCH4	protein_coding	LRCH4
6	27217841	27218847	ENSOCUG00000027938		protein_coding	SAP25
6	27226410	27248928	ENSOCUG00000008631	AGFG2	protein_coding	AGFG2
6	27292276	27292908	ENSOCUG00000025881		protein_coding	APOO
6	27300464	27308085	ENSOCUG00000003810	NYAP1	protein_coding	NYAP1
6	27317232	27325789	ENSOCUG00000021916	TSC22D4	protein_coding	TSC22D4
6	27327603	27331787	ENSOCUG00000024411	C7orf61	protein_coding	C7orf61
6	27356172	27357426	ENSOCUG00000025401	PPP1R35	protein_coding	PPP1R35
6	27358469	27364084	ENSOCUG00000029324	MEPCE	protein_coding	MEPCE
6	27371673	27396772	ENSOCUG00000014388	ZCWPW1	protein_coding	ZCWPW1
6	27470609	27498626	ENSOCUG00000013531	STAG3	protein_coding	STAG3
6	27499999	27502385	ENSOCUG00000017594		protein_coding	GPC2
8	108850027	108851052	ENSOCUG00000026894		protein_coding	RAMACL
8	108850027	108851052	ENSOCUG00000026894		protein_coding	RAMAC
8	109650039	109692581	ENSOCUG00000009763	FAM155A	protein_coding	FAM155A
8	109976266	109979001	ENSOCUG00000015734	LIG4	protein_coding	LIG4
8	109997646	109998659	ENSOCUG00000015739	ABHD13	protein_coding	ABHD13
8	110029565	110062140	ENSOCUG00000012271	TNLG7A	protein_coding	TNFSF13B
8	110241791	110790467	ENSOCUG00000034453		protein_coding	MYO16
10	21247907	21270997	ENSOCUG00000015882	EPDR1	protein_coding	EPDR1
10	21444902	21465126	ENSOCUG00000008939	STARD3NL	protein_coding	STARD3NL
10	21517642	21519191	ENSOCUG00000039334		protein_coding	TRGV4
10	21517642	21519191	ENSOCUG00000039334		protein_coding	TRGV2
10	21517642	21519191	ENSOCUG00000039334		protein_coding	TRGV8
10	21517642	21519191	ENSOCUG00000039334		protein_coding	TRGV5
10	21521543	21522132	ENSOCUG00000025628		protein_coding	TRGV3
10	21521543	21522132	ENSOCUG00000025628		protein_coding	TRGV1
10	21525490	21525991	ENSOCUG00000031806		protein_coding	TRGV4
10	21525490	21525991	ENSOCUG00000031806		protein_coding	TRGV2
10	21525490	21525991	ENSOCUG00000031806		protein_coding	TRGV8
10	21525490	21525991	ENSOCUG00000031806		protein_coding	TRGV5
10	21532549	21533136	ENSOCUG00000032571		protein_coding	TRGV4
10	21532549	21533136	ENSOCUG00000032571		protein_coding	TRGV2
10	21532549	21533136	ENSOCUG00000032571		protein_coding	TRGV8
10	21532549	21533136	ENSOCUG00000032571		protein_coding	TRGV5
10	21545417	21669110	ENSOCUG00000002244	AMPH	protein_coding	AMPH
10	21833127	22021136	ENSOCUG00000000676	VPS41	protein_coding	VPS41
10	22075563	22571587	ENSOCUG00000013896	POU6F2	protein_coding	POU6F2
10	22714677	22720791	ENSOCUG00000011496	YAE1	protein_coding	YAE1
10	22812538	22845149	ENSOCUG00000016581		protein_coding	RALA
10	22955775	23667786	ENSOCUG00000016583		protein_coding	CDK13
10	23081093	23083417	ENSOCUG00000003189	MPLKIP	protein_coding	MPLKIP
10	23083417	23874940	ENSOCUG00000003191	SUGCT	protein_coding	SUGCT
12	7279646	7304216	ENSOCUG00000007245	SIRT5	protein_coding	SIRT5
12	7310898	7409156	ENSOCUG00000007255	RANBP9	protein_coding	RANBP9
12	7469893	7485001	ENSOCUG00000007261	MCUR1	protein_coding	MCUR1
12	7654520	7655263	ENSOCUG00000003841	RNF182	protein_coding	RNF182
12	7726245	7820986	ENSOCUG00000003303	CD83	protein_coding	CD83
12	8847136	9102849	ENSOCUG00000009755	JARID2	protein_coding	JARID2
12	9024833	9024941	ENSOCUG00000019819	U6	snRNA	RNU6-716P
12	9103934	9247152	ENSOCUG00000009767	DTNBP1	protein_coding	DTNBP1
12	9267478	9388134	ENSOCUG00000033525		protein_coding	ANKRD62
12	9303819	9335406	ENSOCUG00000024832		protein_coding	ASNSD1
12	9303819	9335406	ENSOCUG00000024832		protein_coding	
12	9667772	9669668	ENSOCUG00000033985		protein_coding	OR2B3
12	9787294	9788253	ENSOCUG00000022638		protein_coding	OR2W1
12	133666032	134003397	ENSOCUG00000003607	AIG1	protein_coding	AIG1
12	134133999	134150196	ENSOCUG00000008925	ADAT2	protein_coding	ADAT2
12	134150217	134191359	ENSOCUG00000008931	PEX3	protein_coding	PEX3
12	134212085	134264062	ENSOCUG00000033709	FUCA2	protein_coding	FUCA2

12	134263354	134581457	ENSOCUG00000009614	PHACTR2	protein_coding	PHACTR2
12	134595377	134678738	ENSOCUG00000009623		protein_coding	
12	134595377	134678738	ENSOCUG00000009623		protein_coding	LTV1
12	134682475	134709723	ENSOCUG00000009505	PLAGL1	protein_coding	PLAGL1
12	134817249	134817509	ENSOCUG00000008752	SF3B5	protein_coding	SF3B5
12	134906249	134907109	ENSOCUG00000022748	STX11	protein_coding	STX11
12	134984662	135600002	ENSOCUG00000015080	UTRN	protein_coding	UTRN
12	136485519	136698573	ENSOCUG00000010589	FBXO30	protein_coding	FBXO30
12	136799075	136883132	ENSOCUG00000010978	SHPRH	protein_coding	SHPRH
12	136958118	137396799	ENSOCUG00000006898	GRM1	protein_coding	GRM1
12	137499957	137514737	ENSOCUG00000026403		protein_coding	RAB32
12	137583429	137796723	ENSOCUG00000021202	ADGB	protein_coding	ADGB
15	49347866	49458652	ENSOCUG00000007231	EMCN	protein_coding	EMCN
15	49644147	49646977	ENSOCUG00000016259	DDIT4L	protein_coding	DDIT4L
15	49913458	49915074	ENSOCUG00000001888		protein_coding	H2AZ1
15	49916485	49967946	ENSOCUG00000006552	DNAJB14	protein_coding	DNAJB14
15	49977243	49993809	ENSOCUG00000001152	LAMTOR3	protein_coding	LAMTOR3
15	50001714	50057743	ENSOCUG00000001151	DAPP1	protein_coding	DAPP1
15	50176043	50192203	ENSOCUG00000033265	C4orf54	protein_coding	C4orf54
15	50209672	50271910	ENSOCUG00000013288	MTTP	protein_coding	MTTP
15	50283885	50302041	ENSOCUG00000015213		protein_coding	TRMT10A
15	50312406	50334125	ENSOCUG00000005979	C4orf17	protein_coding	C4orf17
15	50425785	50592158	ENSOCUG00000033073	ADH6	protein_coding	ADH6
15	50651157	50749123	ENSOCUG00000023401	ADH4	protein_coding	ADH4
15	50798231	50813150	ENSOCUG00000004263	ADH5	protein_coding	ADH5
15	50824472	50875940	ENSOCUG00000004258	METAP1	protein_coding	METAP1
15	51118531	51119782	ENSOCUG00000011845		protein_coding	MOSPD3
15	51172355	51172825	ENSOCUG00000014372		protein_coding	NCBP2
15	56065344	56066423	ENSOCUG00000013154	ATOH1	protein_coding	ATOH1
15	56125675	57692564	ENSOCUG00000011440	GRID2	protein_coding	GRID2
15	57621100	57621255	ENSOCUG00000038538		protein_coding	RPL39
16	55321885	55628418	ENSOCUG00000014523	SPATA17	protein_coding	SPATA17
16	55398040	55398175	ENSOCUG00000026785		snoRNA	
16	55628243	55821665	ENSOCUG00000014512	GPATCH2	protein_coding	GPATCH2
16	56371207	56371306	ENSOCUG00000020572	U6	snRNA	RNU6-169P
16	56423452	56747063	ENSOCUG00000000984	ESRRG	protein_coding	ESRRG
16	56773722	57100615	ENSOCUG00000014710		protein_coding	USH2A
16	56988591	56988761	ENSOCUG00000023834	U1	snRNA	RNU1-132P
16	70169506	70277901	ENSOCUG00000021242	PKP1	protein_coding	PKP1
16	70293168	70308113	ENSOCUG00000005561	TMEM9	protein_coding	TMEM9
16	70324757	70381515	ENSOCUG00000003619	CACNA1S	protein_coding	CACNA1S
16	70396858	70440912	ENSOCUG00000003606	KIF21B	protein_coding	KIF21B
16	70468773	70484695	ENSOCUG00000021508	INAVA	protein_coding	INAVA
16	70524011	70640878	ENSOCUG00000007754	CAMSAP2	protein_coding	CAMSAP2
16	70719761	70739784	ENSOCUG00000016514	DDX59	protein_coding	DDX59
16	70760848	70828467	ENSOCUG00000016491	KIF14	protein_coding	KIF14
16	70948498	70950219	ENSOCUG00000010120	ZNF281	protein_coding	ZNF281
16	71151805	71278604	ENSOCUG00000006240		protein_coding	NR5A2
16	74392390	74850110	ENSOCUG00000013965	KCNT2	protein_coding	KCNT2
16	74844044	74844179	ENSOCUG00000032066	U2	snRNA	RNU2-35P
16	74844044	74844179	ENSOCUG00000032066	U2	snRNA	RNU2-16P
19	51109794	51659246	ENSOCUG00000008454	CEP112	protein_coding	CEP112
19	51719224	51747920	ENSOCUG00000012115	AXIN2	protein_coding	AXIN2
19	51999202	52088717	ENSOCUG00000003079	RGS9	protein_coding	RGS9
19	52119221	52160069	ENSOCUG00000007640	GNA13	protein_coding	GNA13
19	52193781	52203777	ENSOCUG00000014789	AMZ2	protein_coding	AMZ2
19	52215848	52227671	ENSOCUG00000001594	SLC16A6	protein_coding	SLC16A6
19	52250502	52337251	ENSOCUG00000017402	ARSG	protein_coding	ARSG
19	52338689	52375375	ENSOCUG00000017409	WIPI1	protein_coding	WIPI1
19	52421754	52436752	ENSOCUG00000017419	PRKAR1A	protein_coding	PRKAR1A
19	52443755	52497803	ENSOCUG00000013725	FAM20A	protein_coding	FAM20A
19	52754375	52828097	ENSOCUG00000008011	ABCA8	protein_coding	ABCA8
19	52910594	52915597	ENSOCUG00000033508		protein_coding	ABCA9
19	52930414	52999575	ENSOCUG00000014380	ABCA6	protein_coding	ABCA6
19	53049593	53144677	ENSOCUG00000014338	ABCA5	protein_coding	ABCA5
19	53155605	53155991	ENSOCUG00000021439		protein_coding	RPL22
19	53200639	53328302	ENSOCUG00000007606	MAP2K6	protein_coding	MAP2K6
19	53882319	53883578	ENSOCUG00000026552	KCNJ16	protein_coding	KCNJ16
19	53916674	53923901	ENSOCUG00000010416	KCNJ2	protein_coding	KCNJ2

[1] *Oryctolagus cuniculus* chromosome.