Universitat Autònoma de Barcelona

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In Partial Fulfillment of the Requirements for the Degree of

DOCTOR IN MICROBIOLOGY

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FACULTAT DE BIOCIÈNCIES

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**Author** **Director**

**MARTA TERRÉ TRULLÀ**, Investigadora de la Unitat de Remugants de l'Institut de Recerca i Tecnologia Agroalimentària, i

**MONTSERRAT LLAGOSTERA CASAS**, Directora del Departament de Genètica y Microbiologia de la Universitat Autònoma de Barcelona

**Certifiquen:**

Que la memòria titulada **"Characterization of antimicrobial resistance and bacterial diversity in the gastrointestinal and respiratory tract of calves fed different types of milk"**presentada per **Georgina Maynou Lombardo** per optar al grau de Doctor en Microbiologia, ha estat realitzada sota la direcció de la Dra. Marta Terré Trullà i, considerant-la acabada, autoritza la seva presentació perquè sigui jutjada per la comissió corresponent.

I per tal que consti els efectes que corresponen, signa la present a Caldes de Montbui, x de setembre de 2017.

**Marta Terré Trullà** **Montserrat Llagostera Casas**

**Directora** **Tutora**

Per aquests anys tan intensos i bonics. Moltes gràcies Marta. Gràcies per la confiança i l'oportunitat de realitzar el doctorat. Perquè de tu se'n aprèn molt. Perquè has sabut contagiar-me a la perfecció aquesta passió per la ciència. Gràcies per la teva exigència, el teu romanticisme i la teva bogeria. Ha estat un plaer tenir-te com a directora, companya i amiga.

Mr. Bach, infinites gràcies per les correccions, les ensenyances, les crítiques constructives, la teva confiança i el teu suport.

Maria, sense tu no hagués gaudit d'un ADSA tant com ho vaig fer. Moltíssimes gràcies per ensenyar-me com s'expliquen les coses clares i directes. També a no clavar els dents ;-).

Elena i Anna, moltes gràcies per convertir moments d'estrès en pau, pels consells, per l'ajuda incondicional que m'heu ofert sempre. Gràcies per ser tan bones persones. Sou mèlt!

Sector "**RATILLES**", Sandra, Paco, Silvia i Edu. Gràcies per la vostra ajuda durant els meus inicis perillosos al Lab i per tots els moments compartits (halos d'inhibició, dutxes d'aigües netes, amenaces d'autoclau,...). M'ha encantat tenir-vos com a companys. Laieta, Ramón i Àdria, perquè sou genials. Gràcies pel suport d'aquests darrers mesos, per les vaques voladores i per cuidar dels meus mini-cactus. US APRECIUT MOLT! Sector "**RATOLINS**". Marçal infinites gràcies per ser tant bon company. Gràcies per les converses, la teva generositat i ajuda sempre que ho he necessitat. Gemunski, per ser tant divertida i bona persona. T'he trobat a faltar ;-). Brunix, Anna i Montse, gràcies pels dinars i matins de dilluns compartits.

Al meu estimat Jordi. Gràcies per totes les aventures amb els vedells i les ovelles. Per la teva ajuda a l'hora de repartir la llet i fer el manteniment dels corrals. M'enduc records molt bonics amb tu.

Hugh, Annie, Angharad, Berck and Rhonwen. Thank you very much for the opportunity and making me feel like at home.

Dave. Thank you very much for all the moments that we shared and for all your help during my stay at Minnesota. It was a very enriching and fun experience.

Per les germanes que no son de sang. Jess, gràcies per tots aquests anys, per la teva amistat, pel teu suport incondicional, pels consells i els bons moments. Aquest any ja no tenim excusa per un bon viatget.

Susana, Vane, Miriam, Cristina i Eva. Gràcies per la vostra amistat eterna.Vero i Cris, porqué a vuestro lado solo existe felicidad y diversión. Muchas gracias melonas.

Daniel i Txell. Moltes gràcies per la vostra amistat, per tots els cafès compartits, pel suport d'aquests mesos, per suavitzar conjuntament l'estrés d'un doctorat i per les birres que em pagareu ;-).

A l'equip Vether. Moltes gràcies pel suport i paciència d'aquests mesos. Però sobretot, gràcies per l' oportunitat de seguir treballant en allò que més m'apassiona.

Perquè al final de cada discussió sempre arriba la bona entesa. Perquè t'estimo massa. Papa, gràcies pels massatges, les trucades, els sopars entretinguts, per recordar-me que la vida s'ha de prendre amb més calma, però sobretot gràcies per ser-hi sempre. Res de tot això hagués estat possible sense el teu suport.

Perquè sense tu la paraula risc no existiria. Mama, la llista d'agraïments per tu seria infinita però ho resumiré amb un gràcies per ensenyar-me que en aquesta vida només s'aprèn caient 10 mil vegades i que quan un vol pot, per donar suport incondicional a tot el que he volgut fer i per estimar-me tant com jo t'estimo a tu.

Sandreta, per les germanes que som com la nit i el dia però que al final sempre troben un punt mig on trobar-se. Perquè de tu aprenc el significat de les paraules tolerància i respecte. Gràcies per ser-hi sempre, per aquest amor incondicional que ens tenim, per aquesta nena preciosa que tens i que em fa tant feliç.

Avi y iaia gracias por estar en todos los momentos importantes de mi vida. Por vuestro amor y soporte incondicional. Os quiero.

A la meva família, perquè m'encanteu. Gràcies per forma part de la meva vida.

Finalment a la persona que ha fet possible tot això, la persona que m'ensenya cada dia el significat de la paraula amor. Bombi infinites gràcies per fer-me sentir tant gran, per la paciència de tots aquests anys, per la xemeneia que va obrir la porta a aquesta oportunitat, per fer petites les muntanyes grosses, però sobretot per fer-me tant feliç. T'estimo.

L'ús de la llet de rebuig procedent de vaques tractades amb antimicrobians en l'alimentació de vedells lactants és una pràctica habitual entre els ramaders. En aquesta tesi es van realitzar tres estudis per avaluar els efectes de l'alimentació de vedelles lactants amb llet de rebuig sobre el desenvolupament de bacteris resistentsa antimicrobians i la microbiota del tracte gastrointestinal i respiratori. En el primer estudi es van avaluar els patrons de resistència a antimicrobians d'*Escherichia coli* aïllada de mostres fecals i *Pasteurella multocida* de mostres nasals de vedelles Holstein alimentades amb llet de rebuig o amb llet en pols. L'alimentació amb llet de rebuig va augmentar la prevalença d' *E.coli*fecal i de *P. multocida*nasal resistent a alguns de antimicrobians testats. No obstant, patrons de resistència similars entre colònies d'*E. coli* aïllades de mostres ambientals i de vedelles al naixement i a les 6 setmanes d'edat suggereixen l'existència d'altres factors, tals com la contaminació ambiental, en l'adquisició de bacteris resistents. També es van observar diferencies en les probabilitats d'aïllar *E. coli* resistent segons l'edat dels vedells. En el segon i tercer estudi, es va utilitzar llet de rebuig pasteuritzada amb residus d'antibiòtics beta-lactàmics o llet en pols per alimentar vedelles lactants Holstein. La resistència antimicrobiana d'aïllats d'*E. coli* fecal va ser avaluada tant a nivell fenotípic com genotípic a l'inici de l'estudi, i abans i després del deslletament.L'alimentació dels vedells amb llet de rebuig pasteuritzada va augmentar la prevalença d'*E. coli* fenotípicament resistent a diferents antimicrobians beta-lactàmics, així com la probabilitat d'aïllar colònies d'*E. coli* portadores del gen de resistència a cefalosporines $bla_{\text{CMY-2}}$. No obstant, un cop les vedelles eren deslletades, la prevalença d'*E. coli* resistent a beta-lactàmics disminuïa en vedelles alimentades amb llet de rebuig tot i que seguia sent superior que en aquelles alimentades amb llet en pols.

Pel que fa a la microbiota del tracte gastrointestinal i respiratori de les vedelles, ni la diversitat ni la riquesa de les poblacions bacterianes es van veure afectades pel tipus de llet consumida. No obstant, l'anàlisi de distàncies filogenètiques entre comunitats bacterianes va indicar diferències significatives entre règims alimentaris tant en la microbiota nasal com la fecal suggerint un efecte dels residus antimicrobians presents a la llet o d'altres substàncies que no es troben en els lacto-reemplaçants sobre la microbiota de les vedelles. D'altra banda, l'alimentació de vedelles amb llet de rebuig pasteuritzada també va afectar la composició taxonòmica de les poblacions bacterianes del tracte respiratori però no la d'aquelles del tracte gastrointestinal.

En conclusió, l'alimentació de vedelles lactants amb llet de rebuig fomenta la presència de bacteris resistents en el tracte gastrointestinal i nasal del vedells, així com influencia l'estructura de les comunitats bacterianes que colonitzen aquestes parts del cos.

The use of waste milk produced by cows treated with antimicrobials to feed dairy calves is a frequent practice among dairy operators.In this thesis, three studies were conducted to evaluate the effects of feeding waste milk to dairy calves on the development of antimicrobial resistant bacteria and on microbiotaof both, the gastrointestinal and respiratory tract. In the first study antimicrobial resistance profiles in fecal *Escherichia coli* and nasal *Pasteurella multocida* isolated from Holstein dairy calves fed waste milk or milk replacer were compared. Feeding waste milk to dairy calves increased the prevalence of resistant fecal *E. coli* and nasal *P. multocida*to some of the antimicrobials tested. However, similar profiles of antimicrobial resistance among colonies of *E. coli* isolated from environmental samples and calves at birth and at 6 week of age suggested other factors, such as environmental contamination, in the acquisition of resistant bacteria. Furthermore, differences on the probabilities to isolate resistant *E. coli* were also observed depending on calf age. In the second and third study, pasteurized waste milk containing beta-lactam antimicrobial residues was used to feed Holstein dairy calves. Antimicrobial resistance in fecal *E. coli* isolates was evaluated at both, phenotypic and genotypic level, before and after weaning. Feeding pasteurized waste milk to calves increased the prevalence of phenotypic resistant *E. coli* to different beta-lactam antimicrobials, as well as the probability to isolate *E. coli* colonies carrying the cephalosporin resistance gene $bla_{\text{CMY-2}}$. However, once calves were weaned, the prevalence of resistant *E. coli* to beta-lactams decreased in calves fed pasteurized waste milk, although it continued being greater in such calves than in those fed milk replacer.

Regarding microbiota of both, the gastrointestinal and respiratory tract of calves, neither diversity nor richness of bacterial populations was affected by the type of milk ingested. However, the analysis of phylogenetic distances among bacteria communities indicated significant differences between feeding regimes in nasal and fecal microbiota, suggesting an effect of the presence of antimicrobial residues or other substances in waster milk but not in milk replacers. On the other hand, feeding pasteurized waste milk to calves also affected taxonomic composition of nasal bacterial communities but nor those from the gastrointestinal tract.

In conclusion, feeding dairy calves waste milk triggers the presence of resistant bacteria in both, the gastrointestinal and respiratory tract of dairy calves, as well as it influences the structure of bacterial communities colonizing these body regions.

## ABBREVIATIONS USED

**ADG:** average daily gain

**AMC:** amoxicillin-clavulanic acid

**AMP:** ampicillin

**ANOSIM:** one-way analysis of similarity

**BHI:**brain heart infusion broth

**BW:** body weight

**CA:** calving area

**CEFLNI:** cephalonium

**CEFLX:** cephalexin

**CEFP:** cephapirin

**CEFQUI:** cefquinome

**CEFTRI:** cephacetrile

**CLSI:** Clinical and Laboratory Standards Institute

**CP:** crude protein

**CT**: colistin

**DM:** dry matter

**DNA:** deoxyribonucleic acid

**dNTP:** deoxynucleotide

**DO:**doxycycline

**E:**erythromycin

**EDTA:**ethylenediaminetetraacetic acid

**EFT**: ceftiofur

**ENR:** enrofloxacin

**ESBL:** extended-spectrum β-lactamases

**EU:** European Union

**FA:** calf-feeding area

**FDA:** Food and Drug Administration

**FFC:**florfenicol

**FRA:** framycetin

**GEN:** gentamicin

**GIT:** gastrointestinal tract

**HA:** calf-housing area

**Ig:** immunoglobulin

**IMP:**imipenem

**INIA:** Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria

**IRTA:**Institute of Agrifood Research and Technology

**IMP:**imipenem

**K:** kanamycin

**KF:**cephalothin

**LB:** luria-bertani

**MARB:** marbofloxacin

**MIC:** minimal inhibitory concentrations

**MR:**milk replacer

**MY:** lincomycin

**NCCLS:** National Committee for Clinical Laboratory Standards

**NDF:** neutral detergent fiber

**N:** neomycin

**OB:** cloxacillin

**OR:** odds ratio

**OTU:** operational taxonomic unit

**P:** penicillin

**PENET:** penetamato

**PBS:**phosphate-buffered saline

**PCR:** polymerase chain reaction

**PIR:** pirlimycin

**PSF:** pseudo F statistic

**PS2:** pseudo-$T^2$ statistic

**PCoA:** principal coordinate analysis

**pWM:**pasteurized waste milk

**QIIME:** Quantitative Insights Into Microbial Ecology

**rDNA:**ribosomaldeoxyribonucleic acid

**RIFX:** rifaximin

**S:** streptomycin

**SE:** standard error

**SEM:** standard error of the mean

**SPI:** spiramycin

**SROC:** Southern Research and Outreach Center

**STX:** trimethoprim/sulphamethoxazole

**TAE:** tris-acetate-EDTAbuffer

**TE:**tris-EDTA buffer

**TET:** tetracycline

**TYL:** tylosin

**US:**United States

**UV:** ultraviolet

**VFA:** volatile fatty acid

**WM:** waste milk

**pWM:** pasteurized waste milk

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# Chapter 1

# LITERATURE REVIEW

# 1. LITERATURE REVIEW

## Introduction

Globally large amounts of antimicrobials are used in animal food production to treat or prevent infectious diseases as well as for growth promotion. Among these uses, the majority of antimicrobials are orally administrated through animal feed facilitating the intensive production of healthy animals (Grave et al., 2014). Despite the improvements on animal performance and welfare that result from this practice (DuPont and Steele, 1987; Dibner and Richards, 2005), the routine use of antimicrobials in animal feed has been linked to the global health crisis of antimicrobial resistance leading to the ban of antimicrobials for growth promoting purposes in several countries (Marshall and Levy, 2011; Hao et al., 2014).

This thesis will cover different aspects of the current uses of antimicrobials in dairy cattle that influence the emergence of antimicrobial resistances in animals from the early stages of development. Specifically, it will be evaluated the effects of feeding to dairy calves milk from cows treated with antimicrobials, also called waste milk (WM), on the selection of antimicrobial resistant bacteria and resistance genes in both, the gastrointestinal and respiratory tract. Furthermore, the effects of different milk feeding regimes on the composition of calf-gut and -nasal bacterial communities will be evaluated.

## 1. Antimicrobial use in livestock

### *1. 1. Historical background*

Several antimicrobials have been developed since the physician Paul Ehrlich produced the first chemical compound able to cure infectious diseases in humans: arsphenamine. This drug, commercially called Salvarsan or compound 606, was introduced in 1909 to cure syphilis being no toxic to the patients(Thorburn, 1983; Gensini et al., 2007). However, the era of antimicrobials did not begin until 1928 when Alexander Fleming discovered penicillin (P), a compound produced by mold colonies with bactericidal properties(Ligon, 2004). Despite the effectiveness of P in inhibiting bacterial growth, its commercial development for human medicine did not occur until the advent of the Second World War when Ernst Chain and Howard Florey produced sufficient amounts of purified P(Jones and Ricke, 2003). After P, several other

antimicrobial agents such as Prontosil Rubrum, a parent compound of the modern sulfonamide drugs, and streptomycin (S), an antimicrobial produced by certain strains of *Streptomyces griseus*, were also discovered and commercialized to treat infectious diseases in humans(Colebrook and Kenny, 1936; Lederberg, 2000), but also in animals (Abinanti et al., 1960). Nevertheless, the extensive introduction of antimicrobials in veterinary medicine did not begin until the early fifties, when various studies evaluating growth promoting properties of animal protein factors (i. e. vitamin B12), indicated greater growth rates of animals when antimicrobials were added in their diets (Hill and Branion, 1949; Stokstad and Jukes, 1950). Specifically, it was demonstrated that the addition of antimicrobials in animal feedincreased dietary vitamin requirements of the host by disruptions in gut bacterial populations involved in its synthesis (Black et al., 1942). In consequence, feeding to animals purified diets supplemented with an adequate amount of synthetic vitamins along with antimicrobials increased their growth rates over those fed the same diet but without antimicrobials (Moore et al., 1946; Groschke and Evans, 1950; Stokstad and Jukes, 1950).

### *1.1.2. Antimicrobials for growth promotion*

Once growth promoting properties of antimicrobials were proved in different farm animals, the practice of feeding sub-therapeutic doses of antimicrobials became rapidly an integrated part of the majority of developed production systems(Weber et al., 1952). Chronologically, the first antimicrobials for use in animal feed (P and chlortetracycline) were approved in 1951 by the United States (US) - Food and Drug Administration (FDA). Later, in 1953, the same organization approved the use of oxytetracycline as feed additive (Morton, 1989). However, in mid 1960s, experts from United Kingdom first (DuPont and Steele, 1987; Marshall and Levy, 2011), then from US, warned against the implications of feeding sub-therapeutic doses of antimicrobials on the development of food-borne resistant bacteria that could compromise the effectiveness of antimicrobials in combatinginfectious diseases in humans.By this time the transferable nature of antimicrobial resistance by plasmids was also described (DuPont and Steele, 1987). This fact, forced to the United Kingdom government and to the FDA the establishment of independent advisory organizations, known as Swann Committee and FDA Task Force, respectively, which examined the issue of antimicrobials for growth promotion and proposed some recommendations in order to reduce the potentialrisk associatedfor both, human and animal health (Morton, 1989;

Edqvist and Pedersen, 2001).The principal recommendations by Swann Committee were that the use of antimicrobials as feed additive should be restricted only to those antimicrobials that had little or no application as therapeutic agents in both, humanand veterinary medicine as well as to those that cannot confer cross resistance to clinically important antimicrobials for human (Edqvist and Pedersen, 2001). The FDA, for its part, issued a policy statement regarding the use of certain antimicrobials in animal feed being banned those antimicrobials that could compromise the treatment of diseases in humans, unless commercial producers demonstrated that their use did not implicate a potential hazard to public health(Hays et al., 1986). Further on, in 1977 the FDA was recommended to ban the use of P and tetracycline (TET) in animal feed because of their importance in the treatment of human diseases, being only allowed their use to treat active infections (Heinzerling, 2013).

Although most of these recommendations were initially adopted from the respective countries, the increasing opposition and debate from the livestock and pharmaceutical industry together with the limited evidences for zoonotic transmission of resistant bacteria to humans, led to the gradual dissolution of these regulations untiladditional data were gathered and considered(Edqvist and Pedersen, 2001). Only in Sweden, all growth promoting antimicrobials were banned in 1986 (Casewell et al., 2003), while in other countries, antimicrobial agents such as avoparcin, bacitracin, bambermycins, virginiamycin, and tylosin (TYL) gained in popularity as narrower-spectrum substitutes that had a smaller impact on the development of resistance to broad-spectrum antimicrobials (Marshall and Levy, 2011).

### *1.1.3. Termination of growth promoters in Europe*

In middle 1990s, concerns over the emergence of resistant bacteria from livestock arise once again with the isolation of vancomycin-resistant *Enterococcus* in different farm animals. Studies from various European countries (Aarestrup, 1995; Klare et al., 1995) associated the use of avaporacin as feed additive with the detection of *Enterococcus* resistant to vancomycin, which was an important glycopeptide antimicrobial used in humans medicine. Moreover, Simonsen et al.(1998) demonstrated that farm animals exposed to avoparcin could serve as a reservoir for transferable vancomycin resistance to humans by direct contact or through horizontal spread of resistance genes between bacteria. Resistant-vancomycin *Enterococcus* strains were also isolated from clinical samples (Green et al., 1990; Boyce et al., 1994), waste water

(Klare et al., 1993) and foodstuff (Wegener et al., 1997). However, this situation was just the opposite in the US where avaporacin was not used in animal feed and vancomycin-resistant *Enterococcus* strains were only isolated from human clinical samples, but not from animals (Thal et al., 1995; Kühn et al., 2005).

Based on these observations, countries such as Norway, Denmark and Germany suspended voluntarily the use of avaporacin as feed additive between 1995 and 1996. Later, the European Union (EU) banned avoparcin in 1997 and bacitracin, spiramycin, TYL and virginiamycin in 1999(Casewell et al., 2003). Finally, on January 1st 2006, the use of antimicrobials as growth promoters was totally banned in EU, whereas in US the situation again was vastly different being still allowed the use of antimicrobials for these purposes.

The firsts consequence of growth promoter banning inEUwas: an initial decrease in animal performance, that was later partially improved (Wegener, 2003). Furthermore, reductions in antimicrobial usage in agriculture followed by decreases in the occurrence of resistant bacteria in farmed animals were also reported (Aarestrup et al., 2001; Bywater et al., 2005; Bengtsson and Wierup, 2006). Although the termination of growth promoter's usage resulted in lesser occurrence of resistant bacteria in farm animals, there are still present in the farm environment, farm animals and even foodstuff resistant strains that can reach very quickly pre-termination levels of resistance if antimicrobials are readmitted for growth promotion (Wegener, 2003).

### 1.2. Antimicrobial resistance

The evolution of antimicrobial resistance is complex and in most cases involves multiple genetic variations in chromosomal genes by mutations as well as horizontal transference of naturally occurring resistance genes (Davies and Davies, 2010; Chowdhury et al., 2014). Factors such as type of antimicrobial, volume used and treatment duration are the main driving forces behind the selection and dissemination of these resistances (Thomas et al., 1998; Deryke et al., 2006; Olofsson and Cars, 2007). Often, increasing doses of antimicrobials and decreasing treatment durations are proposed to reduce the selection of resistant strains (Guillemot et al., 1998; Deryke et al., 2006; Burgess and Rapp, 2008), especially with those antimicrobials that are clinically important in human medicine. However, antimicrobials for growth promoting purposes are typically administrated in sub-therapeutic doses for long periods of time

and in large number of animals creating optimal conditions for resistance to emergence and spread (APUA, 2010).

### *1.2.1. Association of antimicrobials in animal feed and occurrence of antimicrobial resistance*

The effects of growth promoters on the emergence of antimicrobial resistant bacteria have been extensively evaluated since their introduction in early 1950s (Levy, 1984; Aarestrup, 1999). Relationships between the use of antimicrobials in animal feed and the occurrence of resistance in bacteria of importance in human and animal pathologies have been reported in numerous scientific publications (Bager et al., 1997; Wegener et al., 1999; Inglis et al., 2005). For instance, Shere et al.(1998) evaluated in a 14-month longitudinal study the occurrence of antimicrobials resistant isolates of *Escherichia coli* O157:H7 in dairy farms. They observed that the antimicrobial resistance toTET, chlortetracycline and Pwas related to the use of therapeutic and subtherapeutic doses of antimicrobials in animal feed or water. Similarly, in an epidemiological study on the occurrence of vancomycin-resistant *Enterococci* in different European regions (Spain, United Kingdom, Sweden and Denmark)(Kühn et al., 2005), greater prevalence of vancomycin-resistant *Enterococci* was reported in samples of animal origin from countries where growth promoters were used compared within those where avoparcin was banned (Sweden). Resistant bacteria to growth promoters were equally likely to diminish in prevalence when antimicrobials were removed from animal feed (van Leeuwen et al., 1982; Aarestrup et al., 2001). However, the maintenance of resistance long after the use of antimicrobials was discontinued has also been reported. For instance, in Denmark, where the use of avoparcin as growth promoter was banned in 1995, decreases in the occurrence of glycopeptide-resistant *Enterococcus* spp. during the next three years were observed in broilers, whilst in pigs, it remains at similar levels probably due to co-selection of resistant strains by other antimicrobials widely used in Danish pig herds (Bager et al., 1999).

Co-selection of antimicrobial resistance is a common phenomenon occurring when several resistance genes conferring resistance to different antimicrobials are integrated into mobile genetic elements, such as plasmids or transposons. Co-selection of resistance may also occur when bacteria had single resistance genes or mutations that confer resistance to more than one antimicrobial class, which is called cross-resistance

(Schwarz et al., 2001). This was the case of *E. facium* and *E. faecalis* isolates from broilers and pigs in Denmark, which exhibited cross-resistance between the growth promoter avilamycin and everninomicin, a new antimicrobial for use therapeutically in humans that had a very similar structure to avilamycin (Aarestrup, 1998).Another example is the use of chlortetracycline in growth rations that was also associated with ampicillin (AMP) and TET resistance in fecal *E. coli* isolates from swine farms (Varga et al., 2009) and cattle (Alexander et al., 2010).

### *1.2.2. Dissemination of antimicrobial resistance*

The presence of different resistance genes in mobile genetic elements not only may result in co-selection of antimicrobial resistance, but also in the rapid spread of antimicrobial resistance in different microbial ecosystem. In addition to antimicrobial usage, the ease with which bacteria can acquire resistance genes from other bacteria is considered another major driving force on the occurrence of antimicrobial resistance (Salyers and Amábile-Cuevas, 1997). While resistances developed by chromosomal mutations are only vertically transferred to bacterial progeny (during cell division), resistance mechanisms encoded in mobile genetic elements may be exchanged between bacteria belonging to the same or different bacterial species (Tenover, 2006).Therefore, they may colonize other different habitats where resistance genes could be transferred again. The mechanisms by which bacteria can transfer horizontally resistance genes to other ones are conjugation, transformation and transduction, of which the first one is considered to be the most influential on the dissemination of antimicrobial resistance genes(Von Wintersdorff et al., 2016). Although the spread of antimicrobial resistance by horizontal gene transfer is an ancient naturally occurring mechanism, the extensive use of antimicrobials at both human and animal medicine, has been found to enhance the rate at which these processes occur (Von Wintersdorff et al., 2016). Considering horizontal gene transfer via conjugation, two broad ways by which antimicrobials can promote this process have been described in *in vitro* studies (Lopatkin et al., 2016). First, expose bacteria to sub-lethal doses of antimicrobials enhance conjugation rate by promoting the expression of genes that regulate this process (Stevens et al., 1993; Whittle et al., 2002). Second, the presence of antimicrobials activates the expression of topoisomerases genes for the excision of conjugative transposons from host chromosome and subsequent transfer to other bacteria (Whittle et al., 2002). Associations between antimicrobial treatment and conjugation-mediated transfer of

antimicrobial resistance plasmids have also been suggested in *in vivo* and case studies (Karami et al., 2007; Cavaco et al., 2008). For instance, Jiang et al. (2006)reported that the treatment of calves with ceftiofur (EFT) increased the prevalence of ceftriaxone-resistant bacteria in feces, which may serve as a resistance gene reservoir for horizontal gene transfer to other bacteria. In the same study, *in vitro* conjugation experiments demonstrated the emergence of conjugant bacteria that acquired ceftriaxone-resistance gene blaCMY-2 and class 1 integron by horizontal transfer. However, a high frequency of transfer and horizontal spread of resistance has also been reported in fecal *E. coli* isolated from calves that were never treated with antimicrobials (Yates et al., 2004), demonstrating that selective pressure from antimicrobials is not required for an increase in the occurrence of resistant bacteria.

### *1.2.3. Social and economic implications*

For many years, the use of antimicrobials has allowed great advances in the treatment of infectious diseases both, in human and animal medicine. However, as previously stated, the overuse of antimicrobials in livestock, especially those administered at sub-therapeutic doses in animal feed have led to a significant increase in resistant bacteria to different antimicrobials. One of the most relevant and important consequence has been the emergence of resistance in bacteria that cause zoonotic diseases, which could make difficult the treatment of human infections (Aarestrup, 1999). Bacteria that are usually transferred from animals to people are *Salmonella* spp., *Campylobacter* spp., *E. coli* and *Enterococcus* spp. (WHO, 2011), which have been indicated as important reservoirs of resistance genes that can be easily transferred to other commensal bacteria in the gastrointestinal tract (GIT) of humans (Simonsen et al., 1998; Winokur et al., 2001). In fact, evidences have shown the transfer of resistance genes between *E. coli* and *Salmonella* isolates from both, humans and animals, which expressed an identical plasmid mediating resistance to beta-lactams (Winokur et al., 2001). In addition to resistance genes, the acquisition of resistant bacteria from animals to humans through the food chain has also been reported by several authors (MØlbak et al., 1999; Walker et al., 2000). Also, a greater prevalence of resistant bacteria has been observed in gut microbiota of people that was in direct contact with animals receiving diets supplemented with antimicrobials than in those individuals that were not in contact with them(Hunter et al., 1994). Nevertheless, available data on antimicrobial resistance remains insufficient to determine the overall contribution of antimicrobials in food

producing animals to the resistance problems in humans(Goodyear, 2002; Landers et al., 2012).

Environmental pollution with low doses of antimicrobials and antimicrobial resistant determinants is also a matter of concern. Resistant bacteria that have been selected in the GITof animals are released to the environment via sewage and manure. This fact, not only causes contamination in farm environment with resistant determinants, but also agriculture soil, which is constantly subjected to antimicrobial pressure from animal manures and biosolids (Popowska et al., 2010). The lack of ecological barriers and the growing rate of international food trade also promotes a rapid distribution of resistances to antimicrobials among nations and continents (Acar and Röstel, 2001). Epidemiological studies (Gay et al., 2006; Kim et al., 2008; Shearer et al., 2011) have demonstrated the wide distribution of identical plasmids carrying resistance genes to antimicrobials frequently used in farm animals among various bacterial strains from different geographical regions. This broad distribution of resistant plasmids suggests the important role of mobile genetic elements in bacterial adaptation to different environments by utilizing horizontal gene transfer (Popowska and Krawczyk-Balska, 2013). In addition to resistance genes, virulence factors conferring more pathogenic properties to bacteria has been observed in resistant plasmids (Beceiro et al., 2013). Selection of resistant bacteria carrying these plasmids by antimicrobials may also contribute to serious infectious diseases in both, humans and animals by resistant strains. Indeed,de Verdier et al. (2012)isolated fecal *E. coli* from dairy calves, and they observed more virulence genes in resistant *E. coli* isolates than in those fully susceptible to tested antimicrobials. Furthermore, resistant isolates were more common in calves from herds experiencing episodes of diarrhea than in healthy herds suggesting that the use of antimicrobials may imply co-selection of virulence genes or conversely to the maintenance of resistance in populations of pathogenic bacteria (Boerlin et al., 2005).

Overall, the growing occurrence of bacterial resistance along with the lack of new medicines threat to the modern medicine to go back to a pre-antibiotic era. In this context, important procedures such as major surgery, organ transplantation and cancer chemotherapy could be hazardous without effective antimicrobials to treat and prevent bacterial infections, while increasing treatment costs and mortality(Cars et al., 2008). Similar consequence of antimicrobial resistance could be also found in livestock where

the treatment of infectious diseases in animals also depends on the efficacy of already existing antimicrobials (Bengtsson and Greko, 2014). Moreover, it is probably that the loss of effective treatment in animals leads to decrease welfare and productivity in farm animals resulting in major losses to the global animal food production (Bengtsson and Greko, 2014).

### 1.3. Use of waste milk in calf-feeding programs

Despite the prohibition of antimicrobials as growth promoters, which resulted in marked reductions in the occurrence of resistant bacteria in EU, other livestock operations are involved in the consumption of food containing antimicrobials at sub-therapeutic doses. For instance, the use of antimicrobials in cows during the lactating and dry period, is a frequent practice in dairy industry that results in large amounts of milk containing antimicrobial residues which is known as WM(Duse et al., 2013). This milkcannot be sold for human consumption, and it is oftenused to feed calves to reduce the economic losses of discarding this milk. Calf management surveys done in several countries reported great percentage of farms using WM to fed dairy calves being for example, 83% of the farms in England and Wales(Brunton et al., 2012), 49% in Canada(Vasseur et al., 2010), 36% in US(USDA, 2007)and 56% in Sweden(Duse et al., 2013).

In addition to non-saleable WM, calf feeding programs commonly used on dairy farms also include milk replacer (MR) and with less frequency saleable whole milk. Throughout this section, the positive aspects and risks associated with the use of WM in calf feeding programs instead of MR will be reviewed.

#### *1.3.1. Growth and development of dairy calves*

The use of WM for raising calves is controversial especially due to the risk of pathogenic bacteria that may expose calves to diseases (Selim and Cullor, 1997), as well as antimicrobial residues that may promote the selection of resistant bacteria in calf gut microbiota (Aust et al., 2012)**.**Although WM can be an economical and nutritious source of feed for calves because the greater protein and fat composition of whole milk (25.4 and 30.8%, respectively)(BAMN, 2008) compared with conventional MR (18 to 30% of CP and 18 to 22% of fat)(BAMN, 2014), its quality (especially total solids) may vary greatly from batch to batch of WM leading to calf performance inconsistency (Moore et al., 2009). However, WM, in contrast to MR, can contain a variety of

bioactive substances such as hormones and growth factors (Vasseur et al., 2010) that may influence calf performance and health status. Godden et al. (2005)fed a great number of calves with pasteurized WM(pWM) and a conventional MR (20 and 20% CP and fat, respectively), and they observed greater rates of growth and lower morbidity and mortality rates in calves fed pWM than in those fed the conventional MR. Ziegler et al. (2016) in an attempt to equalize MR and WM nutritional composition, raised calves with pWM (28% CP and 30% fat) and with a 26% CP and a 31% fat MR. They reported an increase in average daily gain (ADG)(Figure 1.1) and greater concentrate intake in pWM compared with MR fed calves.

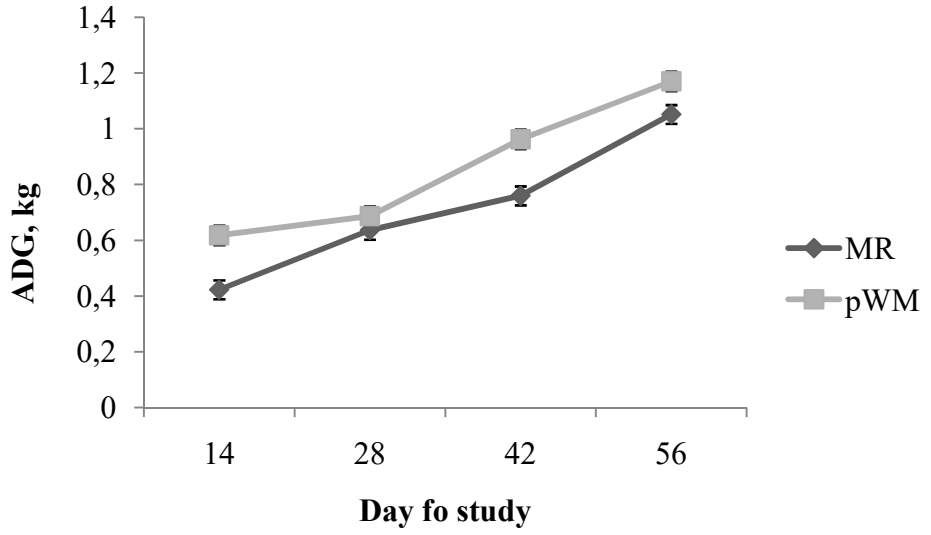


**Figure 1.1.** Evolution of average daily gain (AGD) of calves fed either, milk replacer (MR) or pasteurized waste milk (pWM). Bars denote SE of the mean at each time point. (Ziegler et al., 2016)

*1.3.2. Calf health*

As it was mentioned above, other aspect to consider when feeding WM to calves is the potential to transmit infectious diseases. Milk constitutes a great substrate for microorganism's growth because of its chemical composition and its high content of water. During milking, milk already contains a number of bacteria from the lower parts of the udder that will vary greatly depending on the health status of the mammary gland and management practices in udder pre-milking preparation (Galton et al., 1986). In a cross-sectional prospective study (Selim and Cullor, 1997), the analysis of 189 milk samples from 12 dairy farms indicated that the concentration of bacteria in WM was

greater than in tank milk, MR and colostrum after milking. *Mycobacterium* spp., *Salmonella* spp., *Mycoplasma* spp., *Listeria monocytogenes*, *Campylobacter* spp., *Streptococcus* spp., *Enterobacteriaceae*, *Staphylococcus* spp. and *Escherichia coli* are some of the microorganisms that can be present in milk and cause infections when fed to calves (Selim and Cullor, 1997). In fact, some authors have related feeding WM with the appearance of diseases in calves. This was the case of pre-weaned calves fed WM that were affected with exudative otitis media by *Mycoplasma bovis,*and the infectious agent was also isolated in the tank where WM was stored (Walz et al., 1997). Similarly Butler et al. (2000) related an outbreak of polyarthritis and respiratory disease in a herd of dairy calves with the use of WM that was positive for *Mycoplasma* spp. However, once WM was routinely pasteurized on farm by heating milk to 65ºC for 1 h before feeding, WM samples were negative by culture for *Mycoplasma* and illness in calves was eliminated.

The effectiveness of on-farm WM pasteurization has been evaluated by several authors (Butler et al., 2000; Stabel, 2001; Gao et al., 2002; Stabel et al., 2004) and reductions on the total number of bacteria in WM have been demonstrated without especially altering its nutritional content (Jorgensen et al., 2006). In fact, Jamaluddin et al.(1996)demonstrated greater growth rates in calves fed pWM than in those fed unpasteurized WM. Authors attributed these benefits in part to the lower incidence of diarrhea and pneumonia in calves fed pWM. However, it is important to remember that pasteurizing does not mean sterilization (Moore et al., 2009). Elizondo-Salazar et al.(2010) evaluated six on-farm pasteurization systems, including high-temperature, short-time pasteurizers and low-temperature. They reported a reduction on bacterial countsto the levels specified for pasteurized milk consumption in humans in WM fed to calves in more than 90% of the samples. However, the remaining 10% of samples with greater bacterial counts suggested that on farm-pasteurization systems are likely insufficient to inactivate all potential pathogenic bacteria in WM. Moreover, all measured bacteria counts increased between pasteurization and calf feeding by a rapid growth of bacteria surviving to the process.

Bacterial heat resistance has also been observed from different bacteria, such as *Listeria monocytogenes* and *Mycobacterium paratuberculosis* after survive to different milk pasteurization processes (Lund et al., 2002; Szlachta et al., 2010). This heat tolerance was also observed from bacterial enterotoxins such as those produced by

*Staphylococcus aureus*(Asao et al., 2003), which is one of the main etiologic agent of mastitis in ruminants (Vasudevan et al., 2003).Thus, taken together, these findings suggest that the use of WM in calves feeding programs have to be considered with caution even when pasteurizing, especially in those farms with high incidence of infectious diseases in dairy cows.

### *1.3.3. Antimicrobial resistance*

As discussed earlier in this literature review, the presence of antimicrobials in animal feed also constitutes an important issue on the development of resistance in potential pathogenic bacteria. Resistant bacteria can potentially spread to humans by direct animal contact, food, or the environment (Wegener, 2003). Although antimicrobial levels in WM could be very low and variable depending on the number of treated cows in the farm and the type of treatment applied, feeding WM to preweaning calves could also exert a selective pressure favoring the emergence and dissemination of resistant bacteria in the gut microbiota of calves. It is widely accepted that the selection of antimicrobial resistance occurs when bacterial populations are exposed to antimicrobial agents above the minimal inhibitory concentration (MIC) of the susceptible isolates. However, the selection of resistant bacteria at extremely low antibiotic concentrations has also been demonstrated (Gullberg et al., 2011). In a recent study, Pereira et al. (2014a) collected WM samples from several dairy farms to screen antimicrobial drug residues, and they found antimicrobial residues below the MIC breakpoint for susceptible bacteria in the *Enterobacteriacease* family. Later, Pereira et al. (2014b), fed calves raw milk with added antimicrobial residues at similar concentrations than those found previously in WM (Pereira et al., 2014a), and they demonstrated an increase in the prevalence of antimicrobial resistance in fecal *E. coli* isolates.

The consequences of daily WM intake on the development of resistant bacteria in the gut microbiota of calves have been analyzed in several studies obtaining inconsistent results. Whereas Wray et al. (1990) did not observe differences in the antimicrobial resistance of fecal *E. coli* isolated from calves fed either WM or MR, Aust et al. (2012) reported greater prevalence of resistant fecal *E. coli* in calves fed unpasteurized and pWM compared with those fed unprocessed bulk tank milk. However, the number of animals sampled to evaluate the effect of feeding WM on

antimicrobial resistance of fecal *E. coli* was small. Similarly, Langford et al. (2003) observed a positive relationship between the concentrations of penicillin G in milk fed to calves and the levels of resistance in the lower gut bacterial populations.

Other possibility for antimicrobial resistant bacteria in gut microbiota of calves fed WM is that, in addition to the selective pressure of antimicrobial residues in milk, resistant bacteria already present in WM may directly colonize calf gut microbiota (Geser et al., 2012a). In fact, Donaldson et al.(2006)found that gut microbiota of dairy calves were rapidly colonized by antimicrobial resistant *E. coli* strains shortly after birth, whileKhachatryan et al.(2004) demonstrated greater selective advantage of antimicrobial resistant bacteria in newborn calves than in older.

### *1.3.4. Calf resident microbiota*

The use of antimicrobials in animal feed has also been found to alter the structure of microbial populations colonizing the GITgastrointestinal tract(Looft et al., 2012, 2014; Lin et al., 2013). In ruminants, physiology aspects such as nutrition as well as immune system responses are closely related to the activity of gut microbiota, and diet is one of the main factors influencing on its composition(Yáñez-Ruiz et al., 2015). At birth, calves have an immature and sterile GIT, which is progressively colonized by a high variety of microorganisms (Ziolecki and Briggs, 1961). During the first weeks of life, the main microorganisms colonizing the rumen are anaerobic bacteria that are responsible for the fermentation of indigestible dietary substrates resulting in volatile fatty acids (VFA) neededto stimulate the growth and differentiation of rumen epithelium (Bergman, 1990). The amount of VFA produced in the rumen depends on the type of microorganisms involved in microbial fermentation, and the administration of antimicrobials to animals has been found to alter microbial composition resulting in lesser relative abundance of butyrate-producing bacteria (Yap et al., 2008).

The effects of feeding WM containing antimicrobial residues on gut microbiota of calves have recently been evaluated by several authors (Edrington et al., 2012a; Pereira et al., 2016; Deng et al., 2017), and differences in the composition of microbial populations depending on the type of milk feeding regime has been demonstrated. Feeding WM (either untreated orpasteurized or acidified) instead of untreated bulk milk to calves was suggested to reduce the production of volatile fatty acids (VFA) in calf rumen by lesser relative abundance of bacteria responsible for the degradation of dietary

fiber and polysaccharides in calves fed such kind of milk (Deng et al., 2017). On the other hand, Pereira et al. (2016) demonstrated that feeding milk containing residues of EFT, P, AMP, and oxytetracycline at similar concentrations of those previously reported in WM samples (Pereira et al., 2014a), reduce the prevalence of gram-positive bacteria belonging to the *Clostridium* and *Streptococcus* genera in calf gut microbiota. In other species, the presence of antimicrobials at sub-therapeutic doses in animals feed hasalso been found to promote the growth of certain bacteria that could be pathogenic to the host. Looft et al.(2012) reported increases on the relative abundance of *Escherichia coli* in pigs feed concentrate with chlortetracycline, sulfamethazine, and P, while greater host susceptibility to enteric infections by *Salmonella* serovar *Typhimurium*was observed in mice drinking water with added S and vancomycin antimicrobials(Sekirov et al., 2008). In summary, although alterations in the GIT microbial communities were reported when feeding milk containing antimicrobial residues at sub-MIC doses, the main changes were observed at the genus levels because antimicrobials primarily affect highly sensitive bacteria to these substances (Pereira et al., 2016).

Finally, exposing calf gut microbiota to antimicrobials may also affect nutrient absorption from intestine as well as compromise intestinal integrity by disruptions on commensal bacteria populations. For instance, the activity of resident microbiota contributes to intestinal epithelium development (Malmuthuge et al., 2013, 2015; Sommer and Bäckhed, 2013),and antimicrobial treatment has been found to affect intestinal growth of broilers probably due to reductions in gram-negative bacterial populations (Gunal et al., 2006). Similarly, during the first weeks of life, milk constitutes the sole feeding source for calves, and the intestine is the unique site for liquid feed digestion (Górka et al., 2011), and a reduction ofintestinal epithelial cell proliferation and mucosal atrophy may also allow pathogens in the intestinal lumen to invade it (Gunal et al., 2006).

# Chapter 2

# OBJECTIVES

## OBJECTIVES

The main objective of this thesis was to evaluate the effects of feeding to calves WM produced by cows treated with antimicrobials or with high somatic cell counts on the selection of antimicrobial resistance in bacteria from the GIT and respiratory tract. Furthermore, also, evaluate how different milk feeding regimes influence on the composition of microbial communities colonizing both, the GIT and respiratory tract of dairy calves. The specific objectives were:

1. Evaluating the effects of feeding WM on phenotypicantimicrobial resistance of fecal *Escherichia coli* and nasal *Pasteurella multocida* isolated from pre-weaned dairy calves.
2. Evaluating phenotypically and genotypically antimicrobial resistance from fecal *E. coli* isolated from calves fed either MR or WM at different ages (at birth, before and after weaning).
3. Evaluating other possible sources for acquisition of resistant bacteria different to feeding regime by comparing antimicrobial resistance patterns of environmental *E. coli* with those isolated from calves at different ages, as well as those isolated from calves at birth and their dams.
4. Evaluating the effects of different types of milk feeding regime (MR versus pWM) on bacterial communities from both, the GIT and respiratory tract of dairy calves before weaning.

To achieve these objectives, three studies were conducted:

- Study 1: Feeding of waste milk to Holstein calves affects antimicrobial resistance of *Escherichia coli* and *Pasteurella multocida* isolated from fecal and nasal swabs.
- Study 2: Effects of feeding pasteurized waste milk to dairy calves on phenotypes and genotypes of antimicrobial resistance in fecal *Escherichia coli* isolates before and after weaning.
- Study 3: Influences of milk-feeding programs on fecal and nasal bacterial populations of dairy calves.

# Chapter 3

## FEEDING OF WASTE MILK TO HOLSTEIN CALVES AFFECTS ANTIMICROBIAL RESISTANCE OF *ESCHERICHIA COLI* AND *PASTEURELLA MULTOCIDA* ISOLATED FROM FECAL AND NASAL SWABS

A fraction of this research has been published in:

Journal of Dairy Science, 2017. 100: 1-13

**ABSTRACT**

The objective of this study was to determine the antimicrobial resistance patterns of fecal *E. coli* and nasal *P. multocida* isolates from calves fed either MR or WM in 8 commercial dairy farms (4 farms per feeding program). Fecal and nasal swabs were collected from 20 ± 5 dairy calves at 42 ± 3.2 d of age, and from 10 of these at approximately 1 yr of age in each study farm to isolate the targeted bacteria. Furthermore, resistance of *E. coli* isolates from calf-environment and from 5 calves at birth and their dams was also evaluated in each study farm. Resistances were tested against the following antimicrobial agents: amoxicillin-clavulanic acid (AMC), EFT, colistin(CT), doxycycline (DO), enrofloxacin(ENR), erythromycin(E), florfenicol(FFC), imipenem(IMP), and S.A greater number of fecal *E. coli* resistant to ENR, FFC and S ($P < 0.05$) and more multidrug resistant *E. coli* phenotypes ($P < 0.05$) were isolated in feces of calves fed WM than in those fed MR. However, the prevalence of fecal resistant *E. coli* was also influenced by calf age, as it increased from birth to 6 wk of age for ENR and DO ($P < 0.05$) and decreased from 6 wk to 1 year of age for DO ($P < 0.05$) regardless of the feeding program. From nasal samples, an increase in the prevalence of CT-resistant *P. multocida* ($P < 0.05$) was observed in calves fed WM compared with those fed MR. The resistance patterns of *E. coli* isolates from calves and their dams tended ($P = 0.09$) to differ whereas similar resistance profiles among *E. coli* isolates from farm environment and calves were observed. The findings of this study suggest that feeding calves WM fosters the presence of resistant bacteriain the lower gut and respiratory tracts of dairy calves.

**Key words:** antimicrobial resistance, dairy calf, waste milk

### 3.1. Introduction

In dairy industry, antimicrobial agents are used for the treatment of various diseases such as mastitis, metritis, and dry cow therapy resulting in antimicrobial residues in milk (Bilandžić et al., 2011; De Briyne et al., 2014; Pereira et al., 2014a). To ensure consumer health, milk produced by cows treated with antimicrobials is withdrawn from the dairy industry during both the treatment and milk withdrawal periods (Duse et al., 2013). However, to mitigate milk economic losses, this WM is often used by producers to feed dairy calves (Brunton et al., 2012; Duse et al., 2013).

The use of milk containing antimicrobial residues in calf feeding programs has been shown to select for resistant fecal *E. coli* in dairy calves. For instance, Pereira et al. (2014b), fed calves raw milk with added antimicrobial residues at similar concentrations than those found previously in WM (Pereira et al., 2014a), and they demonstrated an increase in the prevalence of antimicrobial resistance in fecal *E. coli* isolates. However, information about the effects of feeding calves WM on the prevalence of multidrug resistant bacteria is scarce and further research is needed to reach more definitive conclusion about the impact of feeding calves such kind of milk on the selection of resistant bacteria in calf gut microbiota.

Other aspect to consider when feeding calves WM is that antimicrobials ingested through milk could also have an effect to other parts of the body, such as respiratory tract, either by direct contact when drinking or by its absorption in the intestine and later spread in the body. For instance, beta-lactam antimicrobials, both penicillins and cephalosporins, are veterinary drugs commonly used in dairy cows (Kerr and Wellnitz, 2003; De Briyne et al., 2014), and penicillins, especially AMC, are antimicrobial agents extremely well-absorbed in the intestine (Dantzig, 1997; Levison and Levison, 2009). The hypothesis of the present study was that feeding WM to calves will increase the prevalence of gut resistant bacteria, but it also may foster an increase in the incidence of resistant bacteria in the respiratory tract. Thus, the aim of this study was to determine whether there was an increase of antimicrobial resistance patterns to antimicrobials commonly used in dairy farms in *E. coli* from fecal samples and in *P. multocida* from nasal samples of dairy calves fed WM compared with those fed MR.

## 3.2. Material and methods

### *3.2.1. Dairy farms, experimental treatments and sample collection*

Data were collected from eight commercial Holstein dairy farms selected out of 69 farms, for which the calf management was known. These farms were located in the region of Girona (Spain) and sampled from December 2013 to September 2015. Farmswere selected according to herd size and the milk source that dairy producers used to feed their calves. The average number of milking cows in the farms was 117 with a minimum of 70 and a maximum of 230.In four of them, calves were offered MR and in the other four WM from cows either receiving antimicrobial therapy or having with high somatic cell counts. Calf management practices differed among the study farms and were described in Table 3.1.

**Table 3.1**. Milk regime and calf-housing system in each study farm

| | Calf feeding program | | Calf-housing system | |
|---|---|---|---|---|
| Farm | Duration of pre-weaning period (wk) | Milk offered (L/feeding) | Individual housing (d) | Number of calves/pen |
| 1 | 8 | 2.0 | 30 | 5-6 |
| 2 | 13 | 2.0 | 42 | 4-5 |
| 3 | 10 | 2.0 | 30 | 6 |
| 4 | 8 | 1.75 | 14 | 5 or 15 |
| 5 | 6 | 2.0 | 42 | 15 |
| 6 | 8 | 2.0 | 0 | 2-3 |
| 7 | 6 | 2.0 | 60 | 6 |
| 8 | 12 | 2.75 | 21 | 5 or 10 |

Calves were removed from their dams at birth and offered at least 2 feedings of 2-3 L of colostrum. Calves received milk for at least until 6 wk after birth and in some farms calves consumed milk for as long as 12 wk of age. Calves were offered 2 L of milk (MR or WM) twice daily in most of the farms. Generally, calves were housed individually during the first weeks of life, and then moved to group pens before weaning. The antimicrobial agents used on dairy operations, both to treat or prevent diseases, were recorded from each study farm (Table 3.2). The most common antimicrobials used to treat or prevent mastitis, either during dry or lactating periods, were beta-lactams (cephalosporin or not), mainly combined with aminoglycosides. The antimicrobial therapy of infectious diseases such as pneumonia or diarrhea in calves, consisted of a beta-lactam (cephalosporin or not) and fluoroquinolone antimicrobial administrated parentally.

In each farm, fecal and nasal swabs were sampled from 20 ± 5 female calves at 42 ± 3.2 d of age that were enrolled in the study by birth order. The samples were obtained with a sterile transport swab by inserting it into the nasal or rectal cavity and rotating 360º several times. After sampling, each swab was put into a tub with Amies agar gel (VWR International, Llinars del Vallès, Spain), and kept refrigerated during transportation to the laboratory within the following 4 h. Calves that received antimicrobial therapy before 42 d of age were excluded from the study. Following the same sampling procedure, 10 ± 3 calves per farm that were previously sampled and never received antimicrobial therapy were sampled again at 1.3 ± 0.16 yr of age. These samples were obtained from July 2015 to September 2015, and those heifers in the farms that were about 1 yr old at that time were selected.

**Table 3.2.** Antimicrobial agents used in each study farm to treat or prevent diseases distributed by antimicrobial class.

| | | Study farms[1] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | MR farms | | | | WM farms | | | |
| Antimicrobial class | Antimicrobial tested[2] | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Aminoglycosides | S | - | S<br>FRA<br>K[3] | S<br>FRA<br>K | N[3] | - | S<br>FRA | FRA[3]<br>N | GEN[3] |
| Ansamycins | - | - | - | - | - | RIFX[3] | - | - | - |
| β-Lactams Penicillins | AMC | AMC<br>AMP[3]<br>OB[3] | AMC<br>P[3]<br>PENET[3] | AMC<br>AMP<br>OB<br>PENET | AMP | OB | AMC<br>P<br>PENET | P<br>PENET | AMC<br>OB<br>PENET |
| Carbapenem | IMP | - | - | - | - | - | - | - | - |
| Cephalosporins | EFT | - | EFT<br>CEFLX[3] | EFT<br>CEFLX | CEFQUI[3]<br>CEFLNI[3] | CEFTRI[3] | EFT | CEFT[3] | CEFP |
| Fluoroquinolones | ENR | ENR | MARB[3] | ENR<br>MARB | ENR | ENR | ENR | ENR | ENR |
| Lincosamides | | - | - | - | MY[3] | - | - | MY | - |
| Macrolides | E | - | - | - | E<br>TYL[3] | - | - | SPI[3] | E |
| Phenicols | FFC | - | FFC | - | - | - | - | - | - |
| Polypeptides | CT | - | - | - | CT | - | - | - | - |
| Tetracyclines | DO | - | - | - | - | - | - | - | - |

[1]Study farms: MR = milk replacer; WM = waste milk.

[2]Antimicrobial agents tested by disk diffusion method. AMC = amoxicillin; EFT = ceftiofur; ENR = enrofloxacin; E = erythromycin; CT = colistin; DO = doxycyline; FFC = florfenicol; IMP = imipinem; S = streptomycin.

[3]Antimicrobial agents no tested. AMP = ampicillin; CEFLNI = cephalonium; CEFLX = cephalexin; CEFP = cephapirin; CEFQUI = cefquinome; CEFTRI = cephacetrile; FRA = framycetin; GEN = gentamicin; K = kanamycin; MARB = marbofloxacin; MY = lincomycin; N = neomycin; OB = cloxacillin; P = penicillin; PENET = penetamato; RIFX = rifaximin; SPI = spiramycin; TYL = Tylosin.

To evaluate the possibility of acquisition antimicrobial-resistant bacteria from other sources, such as the dam at calving or the farm environment, from June 2014 to June 2015 fecal swabs from 5 ± 1 randomly selected calves were collected at birth and at 6 wk of age and also a fecal swab was obtained from their respective dams after calving. Environmental samples were collected during February and March 2015 once per farm from the calving area (CA), calf-housing area (HA) and calf-feeding area(FA) (Table 3.3). For the CA sampling, 5 sites randomly selected of the floor surface were swabbed using a sterile flocked swab from transport tube (Puritan Medical Products LLC, Guilford, ME) moistened in situ with Nutrient Broth (0.5 g/L beef extract, 1 g/L peptone, 0.5 g/L NaCl). Calf-housing area was sampled following the same procedure for walls and floor. When calves where housed individually, samples were collected from 5 pens randomly selected using the same swab moistened only once with Nutrient Broth. Two randomly selected calf hutches were sampled when calves were housed in groups. Samples from FA were collected swabbing first the water bowls and then the feeding troughs using a dry swab. For individual calf pens, the water and feeding buckets of the 5 selected pens were sampled with the same swab, whereas for grouped calf hutches, only 3 water buckets randomly selected were swabbed along with feeding troughs. For each farm, 3 buckets or feeding bottles used to offer milk to the calves were also swabbed using a dry swab. All environmental samples were maintained on ice until processing samples at the laboratory for bacteriological culture.

**Table 3.3.**Schedule of the different environmental areas sampled for each of the MR and WM farms according to their calf-housing system.

| | Sampling areas[2] | | | | | |
|---|---|---|---|---|---|---|
| | | HA | | FA | | |
| Farm[1] | CA | IP | GP | IP | GP | MB |
| 1 | X | X | X | X | X | X |
| 2 | X | X | - | X | - | X |
| 3 | X | X | X | X | X | X |
| 4 | X | X | X | X | X | X |
| 5 | X | X | - | X | - | X |
| 6 | X | - | X | - | X | X |
| 7 | X | X | - | X | - | X |
| 8 | X | X | X | X | X | X |

[1]Farms from 1 to 4 correspond to those where calves were fed milk replacer and from 5 to 8 where calves were fed waste milk.

[2]Environmental sampling areas: CA = calving area; HA = calf-housing area; FA = calf-feeding area; IP = individual pen; GP = group pen; MB = milk bucket or bottle.

*3.2.2. Bacteria isolation, culture, and identification by PCR*

Fecal and environment swabs were streaked onto Agar McConkey (Oxoid, Madrid, Spain) and incubated for 24 h at 37 ºC. Then, five fecal suspected *E. coli*colonies were isolated from fecal swabs and three from the environmental swabs. Nasal swabs were cultured, following the same procedure and under the same conditions as fecal swabs, on Columbia Agar with 5% sheep blood (Oxoid, Madrid, Spain) for isolation of five suspected *P. multocida* colonies. Suspected colonies were selected by morphologic characteristics after incubation, and subcultured for another 24 h on Columbia Agar with 5% sheep blood to obtain pure bacterial cultures.

For isolates identification, *E. coli* were subjected to specific PCR assays using a set of primers reported by Juck et al. (1996): F (5'-ATC ACC GTG GTG ACG CAT GTC GC-3') and R (5'-CAC CAC GAT GCC ATG TTC ATC TGC-3') (Integrated DNA Technologies, Leuven, Belgium) resulting in a fragment of 486 bp.

The primers set reported by Townsend et al. (1998) were used to identify *P. multocida*: KMT1SP6 (5'-GCT GTA AAC GAA CTC GCC AC-3') and KMT1T7 (5'-ATC CGC TAT TTA CCC AGT GG-3') (Integrated DNA Technologies, Leuven, Belgium) obtaining a product of approximately 460 bp.For DNA amplification, each bacterial pure culture was inoculated into 20µl of MiliQ water, and 2µl of these suspensions was added directly in 23 µl of reaction mixture. Reaction mixtures contained 1X taq buffer with $Mg^{2+}$, 200 µM of dNTPs, 0.5 U of *Taq* DNA polymerase (Biorbyt, Cambridge, UK) and 0.125 µM of each primer. The PCR reactions were conducted using a thermocycler iCycler (Bio-Rad Laboratories, California, USA) and cycled as follows: an initial denaturing step at 95°C for 5 min, followed by 30 cycles at 95°C for 1 min, 55ºC for 1 min, and 72ºC for 1 min, and a final extension of 10 min at 72°C. All PCRs were run with positive controls using *E. coli* ATCC 25922 and *P. Multocida* CECT 962, and negative controls containing MiliQ water in place of the bacterial suspensions. The presence of PCR products was confirmed by electrophoresis on 1.5% agarose gels with SYBR safe DNA gel stain (Thermo Fisher Scientific SL, Barcelona, Spain) in 1 × TAE buffer (Merck KGaA, Darmstadt, Germany) and visualized under UV light. After bacteria identification, each confirmed colony was picked from bacterial pure culture to 1.5 ml tube containing 334 µl of brain heart infusion broth(BHI) (Scharlab SL, Sentmenat, Spain) and 666 µl of 30% glycerol (Panreac Química SLU, Castellar del Vallès, Spain), and frozen at -80ºC.

### *3.2.3. Antimicrobial susceptibility test*

The susceptibility of the confirmed isolates against antimicrobial agents was determined by the disk diffusion method described by the National Committee for Clinical Laboratory Standards (NCCLS) with some modifications on the procedure (CLSI, 2013). Each isolated bacteria from pure culture was suspended into 2 ml of 0.9% NaCl sterile solution and adjusted spectrophotometrically between 0.08 to 0.10 of absorbance at 625 nm (equivalent to 0.5 McFarland standard). After adjusting for turbidity, the sterile cotton swab was dipped in the *E. coli* suspension and then rolled over the dry surface of Mueller-Hinton agar plates (Oxoid, Madrid, Spain) covering it evenly to obtain uniform growth. Mueller-Hinton agar with 5% sheep blood plates (Oxoid, Madrid, Spain) were inoculated following the same procedure with dipped cotton swabs into *P. multocida* suspension. The testedantimicrobial disks were placed

onthesurface of each agar plates using adisk dispenser (Oxoid, Madrid, Spain). Theantimicrobial agentstestedwere: AMC, 20/10 µg; EFT, 30 µg; CT, 10 µg; DO, 30 µg; ENR, 5 µg; E, 15 µg; FFC, 30 µg; IMP, 10 µg and S, 10 µg (Oxoid, Madrid, Spain). The plates were incubated aerobically between 16 and 18 h at 37ºC for *E. coli* susceptibility test and 20 to 24 h for *P. multocida* plates under the same conditions as *E. coli*. After incubation, the zones of inhibition around each disk were measured in millimeters. Obtained values were then classified into 3 categories (resistant, sensitive, or intermediate) following the interpretative criteria adhering to the Clinical Laboratory Standards Institute (CLSI) guidelines as previously described (Benedict et al., 2015; Kar et al., 2015). Because no CLSI breakpoints were available for CT, the interpretative criteria described by Galani et al. (2008) for susceptibility testing in *Enterobacteriacease* were used. Breakpoints used to categorize antimicrobial susceptibility of isolates are specified in Table 3.4. Internal quality control was performed using*E. coli* ATCC 25922 as a reference strain for each batch of isolates tested.

**Table 3.4.**Zone diameters and interpretative criteriaof tested antimicrobial agents used to categorize antimicrobial susceptibility of *Escherichia coli* and *Pasteurella multocida* isolates.

| Antimicrobial agents tested | Disk content (µg) | Resistant | Intermediate | Susceptible | CLSI guideline[1] |
|---|---|---|---|---|---|
| Amoxicillin-Clavulanic acid[2] | 20/10 | ≤ 13 | 14-17 | ≥18 | M100 –S24, 2014 |
| Ceftiofur[3] | 30 | ≤ 17 | 18-20 | ≥21 | VET01-A4, 2013 |
| Colistin[4] | 10 | ≤11 | - | ≥14 | - |
| Doxycycline[2] | 30 | ≤10 | 11-13 | ≥14 | M100 –S24, 2014 |
| Enrofloxacine[3] | 5 | ≤16 | 17-20 | ≥21 | VET01-A4, 2013 |
| Erythromycin[5] | 15 | ≤13 | 14-22 | ≥23 | M100 –S24, 2014 |
| Florfenicol[3] | 30 | ≤14 | 15-18 | ≥19 | VET01-A4, 2013 |
| Imipenem[2] | 10 | ≤19 | 20-22 | ≥23 | M100 –S24, 2014 |
| Streptomycin[2] | 10 | ≤11 | 12-14 | ≥15 | M100-S18, 2008 |

[1]CLSI = Clinical and Laboratory Standards Institute.

[2]Interpretative criteria defined for *Enterobacteriacease* applied in both *Escherichia coli* and *Pasteurella multocida* as CLSI does not define zone diameter breakpoints in *Pasteurella* spp. for these antimicrobial agents.

[3]Interpretative criteria defined for *Pasteurella multocida* in cattleapplied in both *Escherichia coli* and *P. multocida* as CLSI does not define zone diameter breakpoints in *Enterobacteriacease* for these antimicrobial agents.

[4]No zone diameter breakpoints are defined by the CLSI for colistin, and it was followed the ones described by Gallani et al., (2008)

[5]Interpretative criteria defined for *Enterococcus* spp.applied in both *Escherichia coli* and *P. multocida* as CLSI does not define zone diameter breakpoints in both *Enterobacteriacease* and *Pasteurella* spp. for these antimicrobial agents.

*3.2.4. Statistical analyses*

To evaluate the effect of feeding WM or MR on fecal *E. coli* and nasal *P. multocida* resistance to antimicrobial agents tested in calves at 6 wk of age two statistical analyses were performed:

1) a general linear mixed model using the PROC GLIMMIX procedure of SAS (version 9.2, SAS Institute Inc., Cary, NC, USA), in which the proportion of resistant bacteria isolated per calf and for each antimicrobial tested was considered as the dependent variable. Milk feeding practice (MR or WM) was the fixed effect of the model, and farm was considered the random effect. The use within the farm of any of the antimicrobial class tested was used as a block in the statistical model.

2) Based on bacteria resistant profiles represented by the size of the inhibition zones to each of the 9 antimicrobials tested, multidrug resistance phenotypes among *E. coli* and *P. multocida* isolates were described independently by cluster analyses using the Ward's minimum variance method of the PROC CLUSTER procedure. The final number of clusters was determined using the pseudo F statistic (PSF) and pseudo-$T^2$ statistic (PS2) as described by Yoder et al. (2014). A frequency analysis of the cluster distribution between bacteria isolated from calves fed MR and WM was performed by a chi-square test using the PROC FREQ procedure.

Feeding practice (MR or WM) and calf-age (0 d, 42 d or 1 yr) effect on the proportion of resistant fecal *E. coli* isolates were also analyzed using generalized linear mixed models for each antimicrobial tested. Data were split in 2 blocks: from birth to 6 wk of age, and from 6 wk to 1 year of age, considering only the animals that were sampled at both times (36 calves at birth and at 6 wk of age, and 69 calves at 6 wk and 1 yr of age). For this analysis, feeding practice, calf age and its interaction were the fixed effects of the model; farm was the random effect, and the proportion of resistant colonies to each antibiotic tested per calf was the dependent variable. Due to the low isolation of nasal *P. multocida* at 1 yr, this analysis was not performed with data obtained from nasal swabs.

To evaluate the relationship among bacterial antimicrobial resistance in calves, their dams and their environment, *E. coli* colonies isolated from the farm environment, and cows and their calves sampled at birth and at 6 wk of age were grouped at farm level. Then, two fecal *E. coli* clustering analyses were conducted for each farm: 1) *E. coli* isolates from cow and their calves at birth, and 2) *E. coli* isolates from calves (at

birth and at 6 wk of age) and its environment (CA, HA and FA). Then, the most predominant resistant profile among all colonies isolated from each calf, cow, and environmental site was chosen, and they were paired (cow/calf and calf/environment). These data were analyzed by a Wilcoxon signed-rank test using the PROC UNIVARIATE procedure. Finally, antimicrobial resistance clusters were classified as multidrug (≥ 3 antimicrobial resistances) or non-multidrug profiles, and the frequency of these profiles among cows and calves at birth were analyzed with a chi-square test.

## 3.3. Results

### *3.3.1. Descriptive data*

A total of 1,485 *E. coli* colonies were confirmed from fecal swabs, including 785 of 825 from calves at 6 wk of age, 180 of 180 from calves at birth, 176 of 180 from their dams, and 344 of 345 from calves at 1 year old. Among all calves and cows sampled, at least 2 *E. coli* colonies were isolated from each fecal swab. From the 825 colonies isolated from nasal swabs of calves at 6 wk of age, only 301 were confirmed as *P. multocida*, and 5 of 345 colonies from calves at 1 year of age. Among the 165 nasal swabs of calves at 6 wk of age, only 77 provided *P. multocida* colonies ranging from 1 to 5 colonies per calf, whereas among the 69 calves sampled at 1 yr of age, only 3 provided *P. multocida* isolates, ranging from 1 to 2 colonies per calf. In total, 40 environmental samples were collected among all the study farms of which 38 provided *E. coli* colonies ranging from 2 to 3 colonies per sample. The total number of environmental *E. coli* isolates was 102, including 23 from CA, 37 from FA, and 42 from HA.

### ***3.3.2. Effect of calf-feeding practices on antimicrobial resistance***

No isolates of *E. coli* or *P. multocida* resistant to IMP were isolated from calves in this study. Results from the general linear mixed-effects model indicated that in both milk-feeding practices, 90.7 % and 45.3 % of the *E. coli* isolates were resistant to E and DO, respectively, and low percentages of resistant *E. coli* isolates were observed for AMC, EFT, and CT, also being similar in both treatments (Table 3.5). In contrast, percentages of resistant *E. coli* isolates per calf at 6 wk of age for ENR ($P < 0.01$), FFC ($P < 0.05$) and S ($P < 0.05$) were greater in WM compared with MR calves (Table 3.5). The antimicrobial agents used in the study farms to treat or prevent diseases (used as a

block in the statistical model) did not have any effect on the proportion of resistant *E. coli* per calf for any of the antimicrobial agents tested. According to the PSF and PS2 statistics of PROC CLUSTER, the level of clustering was determined and 5 antimicrobial-resistance clusters were created to describe the antimicrobial-resistance profiles in the present study (Table 3.6). Calves fed WM presented a greater ($P < 0.05$) percentage of *E. coli* isolates exhibiting multidrug resistance profiles (≥ 3 antimicrobial agents, clusters D, E) compared with MR-fed calves (Table 3.6). In contrast, greater ($P < 0.01$) percentages of *E. coli* colonies resistant to one or two antimicrobial agents (clusters A, B) were observed in MR- compared with WM-fed calves (Table 3.6).

*Pasteurella multocida* isolates from nasal swabs presented only resistance to CT (24.7% of isolates), E (5.5% of isolates) and S (77.7% of isolates) among the 9 antimicrobials tested. Calves fed WM had a greater proportion of resistant-bacteria to CT ($P = 0.05$) than MR fed calves. Similarly to fecal *E. coli* isolates, the antimicrobial agents used in study farms did not have any effect on the prevalence of resistant *P. multocida* for any of the antimicrobial agents tested. However the cluster analysis indicated that more ($P < 0.001$) calves fed WM had a non-antimicrobial-resistance pattern compared with MR fed calves, and WM calves presented more CT (cluster C) and less S and E (cluster D) ($P < 0.001$) resistance patterns than MR fed calves (Table 3.7).

**Table 3.5.** Percentage, odds ratio (OR), and confidence interval of the general linear mixed-effects model assessing the impact of feeding calves milk replacer (MR) or waste milk (WM) on the presence of resistant fecal *Escherichia coli* to 8 antimicrobials.

| | | | Study farms | | | 95% CI | | |
|---|---|---|---|---|---|---|---|---|
| Antimicrobial class | Antimicrobial agent tested[1] | Class[2] | MR, % (n = 388) | WM, % (n = 397) | OR | Lower | Upper | *P*-value |
| Noncephalosporin β-lactam | Amoxicillin-clavulanic acid | R | 1.7 | 1.1 | 0.7 | 0.1 | 5.9 | 0.702 |
| | | I | 5.3 | 10.7 | 2.1 | 0.6 | 7.4 | |
| Cephalosporin | Ceftiofur | R | 0.8 | 2.7 | 3.5 | 0.4 | 31.4 | 0.266 |
| | | I | 1.8 | 0.4 | 0.2 | 0.0 | 3.4 | |
| Polypeptide | Colistin | R | 8.0 | 6.0 | 0.7 | 0.3 | 2.0 | 0.544 |
| | | I | 84.7 | 85.7 | 1.1 | 0.5 | 2.4 | |
| Tetracycline | Doxycycline | R | 45.4 | 45.2 | 1.0 | 0.5 | 2.0 | 0.984 |
| | | I | 19.1 | 21.4 | 1.2 | 0.5 | 3.0 | |
| Fluoroquinolone | Enrofloxacin | R | 5.6 | 26.6 | 6.1 | 1.8 | 20.8 | 0.004 |
| | | I | 2.3 | 12.3 | 6.0 | 1.5 | 24.8 | |
| Macrolide | Erythromycin | R | 91.1 | 90.2 | 0.9 | 0.2 | 3.8 | 0.877 |
| | | I | 8.8 | 9.8 | 1.1 | 0.3 | 4.7 | |
| Phenicol | Florfenicol | R | 8.9 | 39.4 | 6.6 | 1.5 | 29.8 | 0.014 |
| | | I | 0.0 | 0.0 | - | - | - | |
| Aminoglycoside | Streptomycin | R | 40.5 | 73.5 | 4.1 | 1.3 | 13.2 | 0.019 |
| | | I | 18.9 | 14.4 | 0.7 | 0.4 | 1.2 | |

[1]Imipenem was not included in the analysis since no resistant isolates were detected.
[2]R = resistant; I = intermediate.

**Table 3.6.** Cluster distribution of *Escherichia coli* isolated from calves at 6 wk of age fed milk replacer (MR) or waste milk (WM) during the preweaning period.

| | | Resistant isolates, %[2] | | |
|---|---|---|---|---|
| Cluster | Antimicrobial resistant phenotypes[1] | MR (n=388) | WM (n=397) | *P*-value |
| A | E – CT'[3] | 25.5 | 19.6 | <0.001 |
| B | DO – E – CT'[3] | 30.1 | 11.3 | <0.001 |
| C | E – S – DO'[4]- CT'[3] | 66.0 | 33.0 | 0.548 |
| D | DO – ENR – E – S – CT'[3] | 3.9 | 19.1 | <0.001 |
| E | DO – E – FFC – S – CT'[3] – ENR'[5] | 8.5 | 19.9 | 0.049 |

[1]CT = colistin; DO = doxycycline; ENR = enrofloxacine; E = erythromycin; FFC = florfenicol; S = streptomycin.

[2]Percentatges of resistant isolates by feeding treatment. MR = milk replacer; WM = waste milk.

[3] Intermediate level of resistance to CT according to CLSI breakpoints.

[4] Intermediate level of resistance to DO according to CLSI breakpoints.

[5] Intermediate level of resistance to ENR according to CLSI breakpoints.

**Table 3.7.** Cluster distribution of *Pasteurella multocida* isolated from calves at 6 wk of age fed milk replacer (MR) or waste milk (WM) during the preweaning period.

| | | Resistant isolates, %[2] | | |
|---|---|---|---|---|
| Cluster | Antimicrobial resistant phenotypes[1] | MR (n=194) | WM (n=107) | *P*-value |
| A | - | 6.2 | 25.2 | <0.001 |
| B | S – E'[3] | 62.4 | 0.9 | <0.001 |
| C | CT – E'[3] – S'[4] | 4.1 | 73.0 | <0.001 |
| D | E – S | 27.3 | 0.9 | <0.001 |

[1]CT = colistin; E = erythromycin; S = streptomycin.

[2]Percentatges of resistant isolates by feeding treatment. MR = milk replacer; WM = waste milk.

[3] Intermediate level of resistance to E according to CLSI breakpoints.

[4] Intermediate level of resistance to S according to CLSI breakpoints.

*3.3.3. Effect of calf feeding practices and calf-age on antimicrobial resistance*

No differences in the incidence of *E. coli* resistant to AMC, EFT, CT, DO, FFC and E were found in the isolates from birth to 6 wk of age. In contrast, the proportion of resistant *E. coli* to ENR increased ($P < 0.05$) with calf age (from 0.003 to 0.16 ± 0.057), and decreased for S ($P < 0.05$) (from 0.63 to 0.31 ± 0.258) (Figure 3.1). The evolution of the proportion of *E. coli* resistant to FFC ($P< 0.05$) and S ($P< 0.05$) from birth to 6 wk of age was different depending on the milk feeding practices. The proportion of *E. coli* resistant to FFC increased ($P < 0.05$) with age in calves fed MR (from 0.73 to 0.98 ± 0.883). In contrast, WM-fed calves maintained high proportions of *E. coli* resistant to FFC from birth to 6 wk of age (0.94 ± 0.723 and 0.88 ± 0.664, respectively). Contrary to FFC, MR-fed calves maintained similar proportions of *E. coli* resistant to S (from 0.62 to 0.49 ± 0.352), and WM-fed calves had a decrease ($P < 0.05$) in the proportion of resistant *E. coli* from birth to 6 wk of age (from 0.64 to 0.17 ± 0.376).

The proportion of *E. coli* resistant to AMC, EFT, and CT in calves sampled at both 6 wk and 1 yr of age was in the range of 0 to 5 % of the total *E. coli* isolates and no differences in *E. coli* resistant to these antimicrobials were found from 6 wk to 1 year of age. The proportion of *E. coli* resistant to DO and ENR decreased ($P < 0.05$) and those of E, FFC, and S increased ($P < 0.05$) from 6 wk to 1 yr of age (Figure 3.2). Calves fed MR tended ($P = 0.06$) to have lower and greater proportions of resistant *E. coli* isolates to E and S (E = 91.1%, S = 90.7%), respectively, than WM fed calves (E = 97.6%, S = 57.0%) in calves sampled at 6 wk and 1 year of age. There was no interaction between feeding practices and calf age (6 wk and 1 yr) for any of the antimicrobial agents tested.

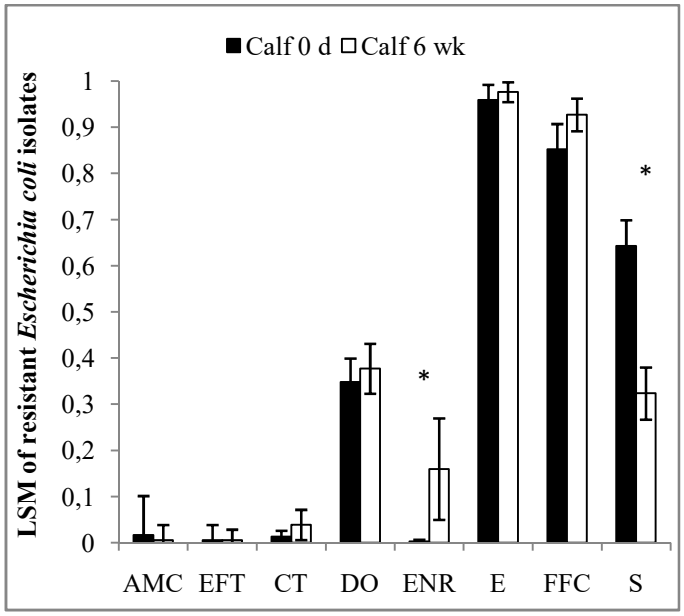


**Figure 3.1.** Proportion of fecal *Escherichia coli* resistant to amoxicillin-clavulanic (AMC), ceftiofur (EFT), colistin (CT), doxycycline (DO), enrofloxacine (ENR), erythromycin (E), florfenicol (FFC) and streptomycin (S) isolated from the same dairy calves at birth and at 6 wk of age.

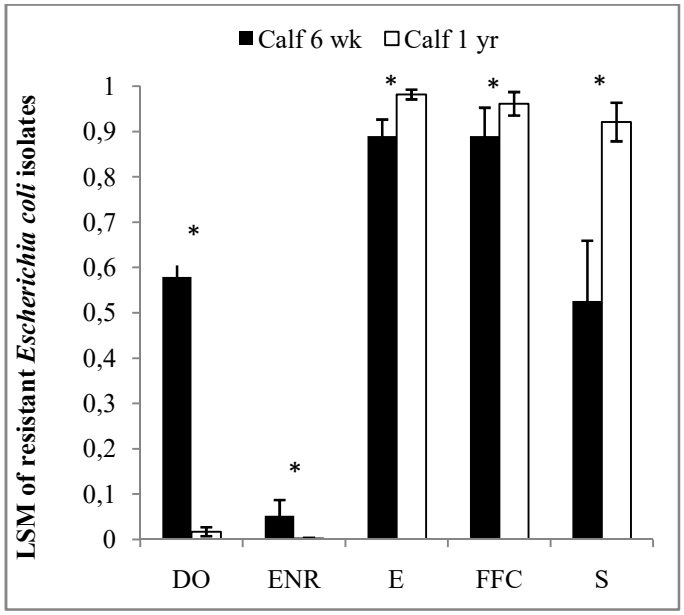


**Figure 3.2.** Proportion of fecal *Escherichia coli* resistant to doxycycline (DO), enrofloxacine (ENR), erytrhromycin (E), florfenicol (FFC) and streptomycin (S), isolated from the same calves at 6 wk (during pre-weaning period) and at 1 year of age.

*3.3.4. Relationship between bacterial antimicrobial resistances*

**Calf and its dam.**The main antimicrobial resistant phenotype of *E. coli* isolated from calves and their dams tended ($P$ = 0.09) to be different. The frequency of multidrug resistant profiles in isolates from calves at birth was greater compared with that of their dams (Table 3.8). Forty-eight multidrug resistant *E. coli* were isolated from cows and calves at birth, from which 81.3 % exhibited resistance to at least DO, E, and S. In the non-multidrug resistant clusters, *E. coli* colonies resistant to E were present in 92.5 % of the colonies.

**Table 3.8.** Percentage of both non-multidrug and multidrug resistant *Escherichia coli* isolated from fecal swabs of newborn calves and their respective dams.

| | Resistant isolates, % | | |
|---|---|---|---|
| | Cow (=176) | Calf (n=180) | P- value[1] |
| Non-multidrug resistant profile | 92.6 | 80.6 | <0.0009 |
| Multidrug resistant profile | 7.4 | 19.4 | <0.0009 |

[1] P-value obtained by chi square test.

**Calf and environment.**There were no differences between paired resistant clusters of calves at birth and CA. Similarly, no differences between paired resistant clusters of calves at 6 wk of age and their HA and FA were observed. Four hundred and sixty-two *E. coli* were isolated from the environment and calves at birth and at 6 wk of age, from which 43.4 % exhibited single resistance to E, and 17.9 % a resistance to both DO and E. The most common multidrug-resistant phenotype (13.6 %) observed in *E. coli* isolates from the environment and calves at birth and at 6 wk of age was against DO, E, and S.

### 3.4. Discussion

In the present study, WM was not subjected to antimicrobial residues analysis. However, greater increases in the prevalence of resistant fecal *E. coli* were observed for ENR, FFC and S in calves fed WM than in those fed MR. Two of these antimicrobials were commonly used in the study farms (ENR, for the treatment of digestive and respiratory infections in calves, and S, for the treatment of intra-mammary infections in lactating cows) (Table 3.2), whereas FFC was only used in one of the MR farms for the treatment of respiratory diseases in both, calves and cows, and the isolation of *E. coli* resistant to this antimicrobial was not expected. Similarly, Berge et al. (2006) reported that feeding calves a MR containing N and TET selected fecal *E. coli* resistant to aminoglycosides other than N, chloramphenicol and sulfonamides, which were not included in the diet of the calves. Gow et al. (2008) observed associations among different resistant genes in fecal *E. coli* isolates in cattle, suggesting that the use of a particular antimicrobial could select for resistance to other antimicrobials within a bacterial population. In particular, resistance to FFC in *E. coli* isolates from cattle was found to be mediated by the gene *floR* in several studies (Cloeckaert et al., 2000; White et al., 2000; Doublet et al., 2002) and this gene has been observed along with other resistant genes in mobile genetic elements, which would increase the risk of co-selection and horizontal dissemination of resistant *E. coli* in animals (Meunier et al., 2010).

Beta-lactams (whether cephalosporins or not) were also antimicrobial agents commonly used in the study farms (Table 3.2) to treat or prevent mastitis in lactating cows and for the treatment of pneumonia or diarrhea in dairy calves. Hence, high levels of resistance in bacterial isolates were expected. However, low percentages of resistance to AMC and EFT were found in *E. coli* from calves fed both types of milk, whereas no resistance to these antimicrobials was detected in *P. multocida*. The most common mechanisms of resistance to beta-lactams are through the expression of different types of beta-lactamases, and in most cases this resistance is acquired by horizontal gene transfer from other bacteria using genetic mobile elements such as conjugative plasmids or transposons (Philippon et al., 2002; Queenan and Bush, 2007; Shaikh et al., 2015). The occurrence of beta-lactam resistant bacteria has been found to vary greatly among different geographical areas and bacterial species (Winokur et al., 2001; Stürenburg and Mack, 2003; Hendriksen et al., 2008) suggesting that resistant bacteria are not likely to arise by antimicrobial pressure alone.

Interestingly, high levels of resistance to DO and E were observed among *E. coli* isolates in calves at birth and at 6 wk of age regardless of feeding treatment and these antimicrobials were either not used (DO) or only used in two of the eight study farms (E) to treat respiratory diseases, mastitis, and metritis (Table 3.2). Several authors (Shoemaker et al., 2001; Bischoff et al., 2005; Yates et al., 2004) have suggested that increases in antimicrobial resistant enteric bacteria may be linked to an efficient horizontal transfer of resistant genes among different species of bacteria even in the absence of antimicrobial pressure. There is also evidence that animal gut microbiota could be colonized by resistant strains from the farm environment or from other animals by direct contact or by consumption of contaminated feed, air or water (Sayah et al., 2005b; Novais et al., 2013). In our study, similar resistance profiles among *E. coli* isolates from farm environment and calves at birth and at 6 wk of age were detected, and in most of cases *E. coli* isolates exhibited resistance to both DO and E. In the present study, some calves were excluded from the study because of antimicrobial therapy, but they remained in contact with the other calves. Therefore, they could also be a focus of horizontal resistance transfer for the non-treated calves sampled herein. Then, the impact of calf-housing system (individual vs group) on antimicrobial resistance in fecal *E. coli* was also evaluated in a model considering the housing system (i.e., calves grouped before or after 42 d of age), the feeding regime, and their interaction as fixed effects. Contrary to the expected results, this analysis indicated that calves that were raised individually had a greater percentage of resistant bacteria to CT compared with calves that were grouped before 42 d of age. No differences were observed for the other antimicrobials analyzed.

*Escherichia coli* isolates from calves sampled at birth also exhibited high levels of resistance to not only DO and E, but also to FFC and S. However, resistance to FFC was not detected and resistance against S was low in *E. coli* isolates from CA. In dairy cattle, it has been suggested that the transmission of resistant bacteria could also occur from cow to calf (Watson et al., 2012), but similarities between resistance patterns of *E. coli* isolates from calves at birth and those found in their dams were not detected herein. It is possible that due to the relatively low paired samples collected from calves and their environment and from calves and their respective dams at birth, this potential association cannot be demonstrated in the current study.

Based on the assumption that resistance mechanisms impose an additional cost to bacteria (Melnyk et al., 2014), a reduction of resistance incidence in older calves

unexposed to antimicrobials was expected. Others authors (Hoyle et al., 2004a,b; Berge et al., 2005) have also indicated an inverse relationship between the occurrence of resistant fecal *E. coli* and age. However, the prevalence of resistant *E. coli* in calves sampled at 6 wk increased at 1 year of age for E, FFC and S. These findings are also in contrast with Khachatryan et al. (2004) who reported a fitness advantage of resistant *E. coli* in the calf enteric environment, but not in older animals.

Regarding the presence of resistant bacteria in the respiratory tract, the isolation rate of *P. multocida* from calves in the present study (36.5% at 6 wk of age, and 1.5% at 1 yr of age) was in the range reported elsewhere. Catry et al. (2006) reported 57.4 % *P. multocida* isolates from 61 healthy calves, whereas Allen et al. (1991) reported a prevalence rate of only 0.5% of 971. Bacteria of the genera *Pasteurella* are considered opportunistic pathogens and their detection has been associated with respiratory disease in cattle (Angen et al., 2009; Garch et al., 2016). In this study, nasal swabs were collected from apparently healthy calves, thus this would likely be the reason for the low rate of *P. multocida* isolation herein. Regarding WM effects on nasal microbiota, an increase of resistant *P. multocida* isolates might have been expected because of a direct contact of nasal mucosa with WM or through antimicrobials residues absorbed in the intestine. However, an increase in the prevalence of resistant *P. multocida* was only observed for CT in calves fed WM and this antimicrobial was only used in one of the MR farms (Table 3.2) to treat both respiratory and digestive infections in calves. Several mechanism of CT resistance have been described: mediated by the addition of modifying proteins to the lipid A moiety of lipopolysaccharide (Yan et al., 2007), involving mutations (Xiao et al., 2015), or plasmid-mediated (Liu et al., 2016). In this context, any of these resistance mechanisms could explain the CT resistance observed herein.

Similar to fecal samples, high levels of resistant *P. multocida* to S were found among all study farms. Kehrenberg et al. (2001) observed that *strA* and *sulII,* genes conferring resistance to S and sulfonamides respectively, were found to be genetically linked on different types of plasmids in bacteria of the genera *Pasteurella* and *Mannheimia*.On the other hand, Guerra et al. (2003) found gene cassettes conferring resistance to S/spectinomycin (*aadA1a*, *aadA2* and *aadA7*) in all *E. coli* isolates from animals carrying class 1 integrons. Resistance to S can also arise through mutations in the 16S rRNA or ribosomal proteins (Connor et al., 1991), but most of the mechanisms of resistance to aminoglycosides are mediated by specific aminoglycoside modifying

enzymes encoded by genes that have been found in mobile genetic elements, such as *aadA* and *strA* genes. This could be one explanation for the remarkable wide occurrence of aminoglycosides resistances observed herein (Sandvang and Aarestrup, 2000; Ramirez and Tolmasky, 2010).

In the present study, feeding WM to calves increased the presence of bacteria with antimicrobial resistances. However, it could not be determined the antimicrobial concentration at which these resistances appeared, because dairy farms used their own WM (different types and concentration of antimicrobials used, and different amount of WM or MR offered), and the exposure of calves to WM and MR differed among farms. Aware of this diversity of the intrinsic factors among farms, farm was included in the statistical model as a random effect.

The lack of treatment interaction with calf-age at 1 yr suggests that feeding WM has a time-limited impact on selecting bacteria with antimicrobial resistances. However, as animals selected at birth were not necessarily sampled at 1 yr, it was not been possible to compare properly the bacteria antimicrobial pattern at birth and 1 yr later when the WM pressure was no longer present.

High levels of resistance to DO and E were observed from environmental and fecal samples of calves fed both types of milk, suggesting an environmental contamination with resistant determinants that could be transferred to calves. However, this hypothesis could not be corroborated herein because resistance was not determined genotypically.

Hence, based on the above limitation of the present study (different WM sources, different sampling periods, different use of antimicrobial and calf management), new experimental approaches under the same conditions and the incorporation of culture-independent techniques to analyze antimicrobial resistance may contribute to a better understanding of the effects of feeding calves WM.

### 3.5. Conclusions

Feeding calves WM fosters the presence of resistant bacteria in the gut and nasal microbiota of calves, with these changes being more evident in the GIT. However, high levels of resistance to E, DO, and S among *E. coli* and to S among *P. multocida* isolates from calves that never received an antimicrobial therapy and were fed either milk replacer or waste milk were also observed. This suggests that, in addition to the type of milk offered to calves, other factors are involved in the presence of resistant bacteria in

calves gut and nasal microbiota such as, horizontal transfer of resistant bacteria from the environment or by direct contact to other animals.

# Chapter 4

# EFFECTS OF FEEDING PASTEURIZED WASTE MILK TO DAIRY CALVES ON PHENOTYPES AND GENOTYPES OF ANTIMICROBIAL RESISTANCE IN FECAL *ESCHERICHIA COLI* ISOLATES BEFORE AND AFTER WEANING

A fraction of this research has been published in:

Journal of Dairy Science, 2017. 100: 1-13

**ABSTRACT**

The aim of this study was to evaluate the effects of feeding pWM to calves on antimicrobial resistance of fecal *E. coli* at both, phenotypic and genotypic levels. Fifty-two Holstein female calves (3 ± 1.3 d of age) were fed one of the two different types of milk: MR without antimicrobials or pWM with beta-lactam residues until weaning at 49 d of age. Fecal swabs of all calves were obtained on d 0, 35, and 56 of the study and three *E. coli* isolates per sample were studied. Phenotypic resistance was tested by disk diffusion method against a panel of 12 antimicrobials. A total of 13 resistance genes comprising beta-lactams, sulfonamides, tetracyclines, and aminoglycosides families were examined by PCR. Feeding pWM to calves increased the presence of phenotypic resistance to AMP, cephalotin (KF), EFT and FFC in fecal *E. coli* compare to MR fed calves. However, the presence of resistance to sulfonamides, tetracyclines, and aminoglycosideswere common in dairy calves independently from their milk-feeding source, suggesting other factors apart of the feeding source are involved in the emergence of antimicrobial resistance.

**Key words:** antimicrobial resistance, calf, pasteurized waste milk

## 4.1. Introduction

Feeding WM to dairy calves increased the prevalence of resistant bacteria to the antimicrobials used in the farm, but also to other antimicrobials that were not used (Aust et al., 2012; Pereira et al., 2014a; Maynou et al., 2017). Cameron-Veas et al. (2015) demonstrated the capacity of resistant strains to transfer beta-lactam resistance genes to other bacteria by conjugation in pigs parentally treated with EFT. In this study, the transconjugants also exhibited resistance to antimicrobial classes other than beta-lactams suggesting co-selection of resistance genes by genetic linkage to others. Furthermore, Mataseje et al. (2010) reported that bacterial isolates from human, animal, and environmental sources had genetically similar plasmids mediating resistance to different antimicrobial classes.

Because of the potential risk of antimicrobial residues in WM, several studies have examined the effect of feeding WM to calves on the occurrence of phenotypic resistant bacteria in the gut microbiota(Langford et al., 2003; Aust et al., 2012; Pereira et al., 2014a). However, only a limited number of studies have focused on the impact of milk feeding practices on antimicrobial resistance at genotypic level (Thames et al., 2012), and even less studies have evaluated shifts on the prevalence of antimicrobial resistance occurring at weaning from non-saleable milk with consistent results (Edrington et al., 2012b; Maynou et al., 2017a). For this reason, the aim of the present study was to assess whether feeding pWM increases the prevalence of resistance in fecal *E. coli* isolates at both, phenotypic and genotypic levels, and whether this potential resistance would decrease after weaning when the antimicrobial pressure from pWM is no longer present. With this objective we have identified the genes involved in the resistance and evaluated the presence of antimicrobial resistance in fecal *E. coli* from calves before and after weaning from both types of milk feeding regimes, pWM and MR.

## 4.2. Material and methods

### *4.2.1. Study farm, experimental treatments, and calf management*

The current study was conducted at the Southern Research and Outreach Center (SROC) of the University of Minnesota from July 2014 to November 2014, and it was approved by the Animal Care Committee in July 2014 under the protocol number 1407-31648A. The enrolled calves were selected from three commercial dairy operations in Minnesota at 3 ± 1.3 d of age. At the farm of origin, calves were offered at least 3 colostrum feedings of 3 to 4 L each within the first 24 h of life and thereafter 2 L of mixed transition milk twice daily. Upon arrival at SROC, calves were weighed and assigned to one of the two milk feeding treatments by farm of origin and body weight (BW): 1) calves fed non medicated all-milk protein MR (26% CP, 31% fat; on a dry matter (DM) basis; Milk Products Inc., Chilton, Wisconsin) at a 12.5% DM concentration; 2) calves fed pWM (28.4% CP, 30.1% fat; DM basis). From arrival to d 56 of study, calves were housed in individual pens (2.3 x 1.2 m) bedded with chopped straw, and separated by panels that avoided a direct contact between animals.

Calves were fed 0.34 kg DM/feeding of their respective milk treatment twice daily from arrival to d 42, and once daily from d 43 to weaning at d 49. Waste milk was collected from a local dairy 2 to 3 times weekly, and stored at 4ºC in a milk tank until pasteurization at 63ºC for 35 min. For each milk load, the solid content of WM was measured using a brix refractometer (Spartan Refractometer, Model A 300 CL, Spartan, Tokyo, Japan) to equalize nutrient intakes between the two feeding treatments. From d 1 to 56, calves had free access to water and textured calf starter (18% CP, 18.5% NDF) supplemented with decoquinate (Elite 18%, Hubbard feeds, Mankato, Minnesota) at 45 g/ton. Health status of the calves was monitored daily, and those that required antimicrobial therapy during the study period were excluded from sampling.

### *4.2.2. Sample collection, bacterial culture and identification*

A total of 52 Holstein female calves (3 ± 1.3 d of age and 38.7 ± 3.27 kg BW) were enrolled in this study, of which 26 were fed MR, and 26 received pWM. Fecal samples of each calf were collected at arrival, at d 35 (pre-weaning), and at d 56 (post-weaning) by inserting a sterile swab (Puritan HydraFlock, Puritan Diagnostics Llc.,

Guilford, Maine) into the rectal cavity and rotating 360º several times. On d 1, only fecal swabs from 10 calves per treatment were collected for bacterial isolation as a control group, while for the last two sampling times (d 35 and 56), all 26 calves per treatment were used. After sampling, each swab was vortered into a 1.5 ml tube containing 334 µl of BHI (Hardy Diagnostics, Santa Maria, California) and 666 µl of 30% glycerol (Alfa Aesar, Ward Hill, Massachusetts, 99 % purity), and frozen at -80ºC for further bacterial culture. At the end of the study, fecal swabs on dry ice were sent for microbiological procedure at IRTA, Caldes de Montbui, Spain. Each fecal sample was then cultured on MacConkey Agar (Oxoid, Madrid, Spain) and incubated for 18-24 h at 37ºC for isolation of *E. coli*. Following the incubation period, three presumptive *E. coli* isolates were selected from each sample based on morphologic characteristics and they were cultured on Columbia Agar with 5% sheep blood (Oxoid, Madrid, Spain) for 18-24 h. Each colony was thereafter identified as *E. coli* by PCR following previously published procedure (Maynou et al., 2017a).

*4.2.3. Antimicrobial susceptibility testing*

Residues of antimicrobials commonly used to treat dairy cows from milk origin farm were screened once for each milk load after pasteurizationusing a commercial enzyme-linked receptor-binding assay test (SNAP beta-lactam test) (Idexx Laboratories Inc., Westbrook, Maine). Most of the antimicrobials used to treat dairy cows in this farm belonged to the beta-lactam family (mainly cephalosporins rather than penicillin), and beta-lactam residues were detected in each load of milk throughout the study. Although, pirlimycin (PIR) hydrochloride is often used for intramammary infusion in lactating cows, antimicrobials belonging to lincosaminide class were not screened from WM loads. In the experimental farm, FFC, ENR, and STX were the most common antimicrobials used to treat calves.

Each confirmed *E. coli* isolate was tested for antimicrobial susceptibility following the standard procedure described by the NCCLS(CLSI, 2014). The antimicrobial agents tested were: AMP, 10 µg; KF, 30 µg; EFT, 30 µg; ENR, 5 µg; FFC, 30 µg; P, 10 units; IMP, 10 µg; PIR, 2µg; trimethoprim/sulphamethoxazole (STX), 25µg; E, 15 µg; TET, 30µg; S, 10 µg(Oxoid, Madrid, Spain). Inhibition halos were interpreted according to the CLSI guidelines as described in Table 4.1.

*4.2.4. PCR amplification of antimicrobial resistance genes*

*Escherichia coli* isolates were investigated for the presence of 13 resistance genes by specific PCR assays (Table 4.2). To obtain DNA templates from each *E. coli* isolates, bacteria were grown on Luria Bertani (LB) Agar plate (Oxoid, Madrid, Spain) at 37ºC for 18 h and suspended on 1 ml of phosphate-buffered saline (PBS) (Oxoid, Madrid, Spain). By centrifuging at 14000 rpm for 5 min, supernatant from each sample were discarded and the pellet resuspended in 100 µl of TE (Tris base 10 mM + EDTA 1 mM) (Sigma-Aldrich, Madrid, Spain). Thereafter, each suspension was heated at 95ºC for 10 min and transferred directly to ice. These DNA templates were maintained as frozen stocks at -80ºC and a 10 fold diluted lysed DNA in TE 10:1 was used as template in the PCRs by adding 2 µl of each suspension to the reaction mixtures.

The PCR assays were performed using single or multiple primer sets within a single reaction mixture depending on the target genes (Table 4.2). The PCRs containing a single set of primers were all performed with GoTaq G2 Flexi DNA polymerase (Promega, Madrid, Spain) using 125 µM of dNTPs (Biorbyt, San Francisco, California), 0.6 µM of primers (Integrated DNA Technologies Inc., Coralville, Iowa), 2 mM of $MgCl_2$, 0.8 X green buffer and 1.25 U of polymerase in a final reaction volume of 25 µl. Single PCR reactions were cycled as follows: an initial denaturing step of 5 min at 94°C, followed by 25 cycles consisting of 1 min at 94°C, 1 min at the corresponding temperature of annealing (Table 4.2), and 1 min at 72ºC, and a final extension step of 7 min at 72°C. For multiplex PCRs, all amplifications were performed using 25 µl of reaction mixture containing 125 µM of dNTPs (Biorbyt, San Francisco, California), 2 mM of $MgCl_2$, 0.8 X green buffer and 2.5 U of polymerase (Promega, Madrid, Spain), whereas the final concentration of each set of primers and cycling conditions differed asdescribed by Kozak et al. (2009). For all PCR amplifications, the products obtained were considered positive based on amplicon size.

**Table 4.1.**Inhibition zone diameters and interpretative criteriaof tested antimicrobial agents used to categorize antimicrobial susceptibility of fecal *Escherichia coli* isolates.

| Antimicrobial agents tested | Disk content (μg) | Resistant | Intermediate | Susceptible | CLSI guideline[1] |
|---|---|---|---|---|---|
| Ampicillin | 10 | ≤13 | 14-16 | ≥17 | (CLSI, 2014) |
| Cephalothin | 30 | ≤14 | 15-17 | ≥18 | (CLSI, 2013) |
| Ceftiofur[2] | 30 | ≤ 17 | 18-20 | ≥21 | (CLSI, 2013) |
| Enrofloxacine[2] | 5 | ≤16 | 17-20 | ≥21 | (CLSI, 2013) |
| Florfenicol[2] | 30 | ≤14 | 15-18 | ≥19 | (CLSI, 2013) |
| Penicillin G[3] | 10 units | ≤14 | - | ≥15 | (CLSI, 2013) |
| Pirlimycin[4] | 2 | ≤12 | - | ≥13 | (CLSI, 2013) |
| Trimethoprim/ sulphamethoxazole | 25 | ≤10 | 11-15 | ≥16 | (CLSI, 2014) |
| Erythromycin[3] | 15 | ≤13 | 14-22 | ≥23 | (CLSI, 2014) |
| Imipenem | 10 | ≤19 | 20-22 | ≥23 | (CLSI, 2014) |
| Streptomycin | 10 | ≤11 | 12-14 | ≥15 | (CLSI, 2008) |
| Tetracycline | 30 | ≤11 | 12-14 | ≥15 | (CLSI, 2013) |

[1]CLSI = Clinical and Laboratory Standards Institute.

[2]Interpretative criteria defined for *Pasteurella multocida* in cattleapplied in *Escherichia coli* as CLSI does not define specific zone diameter breakpoints in *Enterobacteriacease* for these antimicrobial agents.

[3]Interpretative criteria defined for *Enterococcus* spp.applied in *Escherichia coli* as CLSI does not define specific zone diameter breakpoints in *Enterobacteriacease* for these antimicrobial agents.

[4]Interpretative criteria defined for *Staphylococcus* spp. applied in *Escherichia coli* as CLSI does not define specific zone diameter breakpoints in *Enterobacteriacease* for these antimicrobial agents.

**Table 4.2.** Condition of the PCR assays performed for detection of antimicrobial resistance genes in fecal *E. coli* isolates from calves using single or multiple sets of primers.

| Antimicrobial family | PCR[1] | Resistance gene | Primer | | Annenalig T (ºC) | Amplicon size (bp) | Reference |
|---|---|---|---|---|---|---|---|
| | | | Direction | Primer sequence (5' 3') | | | |
| Beta-lactams | 1 | *bla*$_{TEM}$ | F | GCGGAACCCCTATTTG | 61 | 963 | (Olesen et al., 2004) |
| | | | R | ACCAATGCTTAATCAGTGAG | | | |
| | 1 | *bla*$_{CMY-2}$ | F | GCACTTAGCCACCTATACGGCAG | 65 | 758 | (Hasman et al., 2005) |
| | | | R | GCTTTTCAAGAATGCGCCAGG | | | |
| | 1 | *bla*$_{CTX-M}$ | F | ATGTGCAGYACCAGTAARGTKATGGC | 66 | 593 | (Hasman et al., 2005) |
| | | | R | TGGGTRAARTARGTSACCAGAAYCAGCGG | | | |
| | 1 | *bla*$_{SHV}$ | F | TTCGCCTGTGTATTATCTCCCTG | 61 | 854 | (Hasman et al., 2005) |
| | | | R | TTAGCGTTGCCAGTGYTCG | | | |
| Aminoglycosides | 2 | *aadA* | F | GTGGATGGCGGCCTGAAGCC | 63 | 525 | (Kozak et al., 2009) |
| | | | R | AATGCCCAGTCGGCAGCG | | | |
| | 2 | *strA/strB* | F | ATGGTGGACCCTAAAACTCT | 63 | 893 | (Kozak et al., 2009) |
| | | | R | CGTCTAGGATCGAGACAAAG | | | |
| | 2 | *aac(3)IV* | F | TGCTGGTCCACAGCTCCTTC | 63 | 653 | (Kozak et al., 2009) |
| | | | R | CGGATGCAGGAAGATCAA | | | |
| Tetracyclines | 3 | *tetA* | F | GGCGGTCTTCTTCATCATGC | 63 | 502 | (Kozak et al., 2009) |
| | | | R | CGGCAGGCAGAGCAAGTAGA | | | |
| | 3 | *tetB* | F | CGCCCAGTGCTGTTGTTGTC | 63 | 173 | (Kozak et al., 2009) |
| | | | R | CGCGTTGAGAAGCTGAGGTG | | | |
| | 3 | *tetC* | F | GCTGTAGGCATAGGCTTGGT | 63 | 888 | (Kozak et al., 2009) |
| | | | R | GCCGGAAGCGAGAAGAATCA | | | |
| Sulfonamides | 4 | *sul1* | F | CGGCGTGGGCTACCTGAACG | 66 | 433 | (Kozak et al., 2009) |
| | | | R | GCCGATCGCGTGAAGTTCCG | | | |
| | 4 | *sul2* | F | CGGCATCGTCAACATAACCT | 66 | 721 | (Kozak et al., 2009) |
| | | | R | TGTGCGGATGAAGTCAGCTC | | | |
| | 4 | *sul3* | F | CAACGGAAGTGGGCGTTGTGGA | 66 | 244 | (Kozak et al., 2009) |
| | | | R | GCTGCACCAATTCGCTGAACG | | | |

[1]PCR 1 was conducted using single sets of primers for each target gene. PCR 2, 3 and 4 were performed using multiple sets of primers.

### *4.2.5. Statistical analyses*

To evaluate the effect of feeding MR or pWM to calves on the phenotypic and genotypic resistance profile of *E. coli* taking into account the different sampling days, two statistical analyses for each of the antimicrobial agents and resistance genes were performed. First, a generalized linear mixed model using the PROC GLIMMIX procedure of SAS (version 9.2, SAS Institute Inc., Cary, NC, USA), in which milk feeding practice (MR and pWM), sampling times (d 0, 35 and 56), and their interactions were considered as fixed effects. The frequency of resistant bacteria isolated per feeding treatment and sampling day was the dependent variable and calf was considered the random effect. For both phenotypic and genotypic analysis, a binary dependent variable was used (resistant or susceptible), for which both, the isolates with intermediate and susceptible zone diameter, and those that did not carry the resistance gene tested were considered susceptible. When no resistant *E. coli* isolates were detected in calves within some of the groups (feeding treatment x sampling day), the frequency of resistant *E. coli* between feeding treatments and sampling days was analyzed with a Chi-square test.

Second, based on bacteria resistance profiles represented by the size of the inhibition zones to each of the 12 antimicrobial agents tested, phenotypic resistance profiles of the isolates were defined using cluster analysis as described by Yoder et al. (2014). To compare the cluster distribution among bacteria isolated from calves fed MR and pWM, at each sampling day, a Chi-square test was performed. The previous statistical model was also performed for resistance genes.

To analyze which genes may be involved in the phenotypic resistance in *E. coli* isolates to each antimicrobial tested, a generalized linear mixed model with the PROC GLIMMIX procedure of SAS was used. In this model, for each *E. coli* colony the presence or absence of the phenotypic resistance (dependent variable) was correlated with the presence or absence of the tested resistance gene belonging to the same antimicrobial family. To determine the predominant gene within an antimicrobial family involved in resistance, differences among the abundance frequencies of the resistance genes tested in fecal *E. coli* with concurrent phenotypic resistance to the same antimicrobial family were analyzed using a Chi-square test.

## 4.3. Results

### *4.3.1. Descriptive data*

Sixteen calves fed MR and 11 calves fed pWM were treated with antimicrobials for either diarrhea or respiratory diseases and their fecal swabs were excluded. A total of 178 *E. coli* colonies were isolated from fecal swabs, including 28 isolates from calves at d 1 (12 from MR calves, and 16 from pWM), 75 from calves at d 35 and 75 at d 56 (30 isolates from MR calves and 45 from WM calves at each sampling time).

### *4.3.2. Effect of calf feeding practices and calf-age on phenotypic resistance*

Almost all *E. coli* isolates showed resistance to E (98.9%), P (100.0%) and PIR (99.4%) regardless of feeding treatment that were exposed to. However, no resistance to ENR and IMP was detected in *E. coli* isolates from calves fed either type of milk. The prevalence of resistance to AMP and KF did not differ in calves fed MR during the study period, whereas in calves fed pWM, the prevalence increased ($P < 0.05$) from d 1 to 35 (before weaning), and decreased from d 35 to 56 (after weaning) (Table 4.3). No *E. coli* resistant to EFT was isolated at d 1 and 56 in calves fed MR, and no *E. coli* resistant to FFC was isolated in calves fed pWM at d 1. Thus, due to the lack of variability within these treatment groups, the interaction of feeding type with age was not able to be determined for EFT and FFC phenotypes. The percentage of *E. coli* resistant to EFT was greater ($P < 0.05$) and tended ($P \leq 0.10$) to be greater for FFC in calves fed pWM compared with those fed MR (42.2 and 21.5 vs 3.02 and 8.76 % ± 5.42 and 6.69, for EFT and FFC in pWM vs MR fed calves, respectively). Considering the age effect, the prevalence of *E. coli* resistant to EFT increased from day 1 to 35, and decreased again at d 56 (3.0, 47.6 and 10.8 % ± 5.21, at 1, 35 and 56 d, respectively). In contrast to the prevalence of FFC resistances that were greater at 35 and 56 days than at day 1 (26.8 vs 3.1 % ± 6.01, respectively). The prevalence of *E. coli* resistant to TET and S increased ($P < 0.05$) from d 1 to 35 and 56 day (52.5 vs 74.5 and 86.9 % ± 7.02 in TET and 34.0 vs 64.1 and 55.7 % ± 7.36 in S, at 1 vs 35 and 56 d, respectively), regardless of the feeding regime. In contrast, resistance to STX tended ($P = 0.10$) to increase from d 1 to 35 and it was similar to d 1 and 35 at 56 day (9.97, 30.2 and 18.1 % ± 7.11, at day 1, 35 and 56, respectively).

**Table 4.3.** Effect of feeding calves milk replacer (MR) or pasteurized waste milk (pWM) on the prevalence of resistant fecal *E. coli* isolates to several antimicrobials in calves at different ages.

| | MR[2] | | | pWM[3] | | | | *P*-value | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Antimicrobial agent[1] | Day 1, % (n = 12) | Day 35, % (n = 30) | Day 56, % (n = 30) | Day 1, % (n = 16) | Day 35, % (n = 45) | Day 56, % (n = 45) | SEM | Feed | Age | Feed x Age |
| E | 83.3 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | - | - | - | - |
| P | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | - | - | - | - |
| PIR | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 97.8 | - | - | - | - |
| AMP | 40.5 $^{bcd}$ | 18.7 $^{cd}$ | 12.2 $^{d}$ | 45.2 $^{bc}$ | 94.0 $^{a}$ | 55.9 $^{b}$ | 9.93 | <0.01 | 0.01 | <0.01 |
| KF | 35.1 $^{bc}$ | 16.3 $^{bc}$ | 9.6 $^{c}$ | 49.3 $^{b}$ | 90.9 $^{a}$ | 45.0 $^{b}$ | 11.95 | <0.01 | <0.01 | 0.02 |
| EFT[4] | 0.0 | 20.0 | 0.0 | 17.6 | 77.7 | 40.0 | - | <0.01 | <0.01 | - |
| FFC[4] | 8.3 | 13.3 | 23.3 | 0.0 | 46.7 | 35.6 | - | 0.10 | 0.07 | - |
| TET | 61.8 $^{bc}$ | 77.2 $^{ab}$ | 83.8 $^{ab}$ | 43.0 $^{c}$ | 71.7 $^{b}$ | 89.4 $^{a}$ | 9.70 | 0.70 | <0.01 | 0.48 |
| S | 38.4 $^{ab}$ | 63.5 $^{a}$ | 53.4 $^{ab}$ | 30.0 $^{b}$ | 64.7 $^{a}$ | 57.9 $^{ab}$ | 8.13 | 0.91 | 0.05 | 0.84 |
| STX | 5.5 $^{b}$ | 16.4 $^{ab}$ | 16.4 $^{ab}$ | 17.2 $^{ab}$ | 48.8 $^{a}$ | 19.9 $^{b}$ | 9.40 | 0.19 | 0.10 | 0.29 |

$^{abcd}$values with uncommon superscripts within a row differs at $P < 0.05$

[1]Antimicrobial agents tested. AMP = ampicillin; KF = cephalotin; EFT = ceftiofur; FFC = florfenicol; TET = tetracycline; S = streptomycin; STX = trimethoprim/ sulphamethoxazole.

[2]Feeding treatment MR = milk replacer.

[3]Feeding treatment pWM = pasteurized waste milk.

[4]No interaction between feeding regime and calf-age could be statistical proved because of missing resistant isolates at some sampling point.

A total of 7 clusters were constructed to describe the phenotypic resistance profile of fecal *E. coli* isolates (Table 4.4). Because almost all *E. coli* isolates were resistant to at least E, P, and PIR, all defined clusters exhibited multidrug-resistance profiles (three or more different antimicrobial families). Calves fed MR had greater ($P <$ 0.05) percentage of *E. coli* isolates grouped in cluster B and D, which shown resistance to aminoglycosides (S) and tetracyclines (TET), than pWM fed calves (Table 4.4). In contrast, calves fed pWM had more *E. coli* colonies grouped in cluster C, E, and G, with resistance profiles against beta-lactamics (AMP, EFT and KF), than calves fed MR fed (Table 4.4). The presence of resistant colonies of *E. coli* in feces grouped in cluster A decreased with age in both treatments. In contrast, the presence of resistant *E. coli* colonies in feces grouped in cluster D increased with the age. Lastly, presence of resistant *E. coli* colonies grouped into C and G (mainly observed in pWM calves) was high in feces of calves at d 35 than at d 0 or 56 (Table 4.4).

**Table 4.4.** Antimicrobial resistant phenotypecluster distribution of fecal *E. coli* isolated from calves fed MR or pWM at days 1, 35 and 56.

| | | MR, %[3] | | | pWM, %[4] | | | *P*-value[5] | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Cluster[1] | Antimicrobial resistant phenotypes[2] | Day 1 (n = 12) | Day 35 (n = 30) | Day 56 (n = 30) | Day 1 (n = 16) | Day 35 (n = 45) | Day 56 (n = 45) | Feed | Age | Feed x Age |
| A | KF'[5] – S'[7] | 33.3 | 13.3 | 16.7 | 50.0 | 2.2 | 4.4 | 0.14 | <0.01 | 0.12 |
| B | S – TET – KF'[5] | 33.3 | 50.0 | 43.3 | 6.3 | 4.4 | 20.0 | <0.01 | 0.41 | 0.12 |
| C | AMP – EFT – KF | 0.0 | 10.0 | 0.0 | 12.5 | 26.7 | 4.4 | 0.02 | <0.01 | 0.62 |
| D | FFC – S – TET – KF'[6] | 8.3 | 16.7 | 30.0 | 0.0 | 0.0 | 22.2 | 0.03 | <0.01 | 0.07 |
| E | AMP – KF – S – TET –EFT'[8] | 16.8 | 0.0 | 10.0 | 18.7 | 17.8 | 28.9 | <0.01 | 0.20 | 0.17 |
| F | AMP – EFT – KF – STX – TET – S'[7] | 8.3 | 10.0 | 0.0 | 12.5 | 2.2 | 6.8 | 0.98 | 0.42 | 0.13 |
| G | AMP – EFT – FFC – KF – S – STX – TET | 0.0 | 0.0 | 0.0 | 0.0 | 46.7 | 13.3 | <0.01 | <0.01 | - |

[1] All clusters included fecal *E. coli* isolates resistant to erythromycin, penicillin G and pirlimycin.

[2]AMP = ampicillin; EFT = ceftiofur; FFC = florfenicol; KF = cephalothin; S = streptomycin; STX = trimethoprim/ sulphamethoxazole; TET = tetracycline.

[3]Percentatges of resistant isolates in calves fed milk replacer (MR) by calf age.

[4]Percentatges of resistant isolates in calves fed pasteurized waste milk (pWM) by calf age.

[5]*P*- value from Chi-square test.

[6]Intermediate level of resistance to KF according to CLSI breakpoints.

[7]Intermediate level of resistance to S according to CLSI breakpoints.

[8]Intermediate level of resistance to EFT according to CLSI breakpoints.

*4.3.3. Effect of calf feeding practices and calf-age on genotypic resistance*

The beta-lactamase gene $bla_{SHV}$ was not detected in fecal *E. coli* isolates of any of the sampled calves. Differences in the presence of $bla_{TEM}$, $bla_{CTX-M}$ and $bla_{CMY-2}$ could not be observed between feeding treatments and calf-ages because these resistance genes were not detected in some of the feeding treatments and time groups (Figure 4.1). However, the prevalence of resistant *E. coli* harboring $bla_{CMY-2}$ was greater ($P < 0.05$) in calves fed pWM than in those fed MR (40.5 vs 5.5 % ± 4.10, respectively), and it was also greater at d 1 and 35 (before weaning) than at d 56 (35.1 and 35.2 vs 9.27 % ± 8.01, respectively) (after weaning). The proportion of *E. coli* harboring *sul1* at d 35 tended ($P = 0.07$) to be increased in calves fed pWM, whereas a greater proportion of *E. coli* yielding *sul2* ($P = 0.09$) was observed in MR fed calves at the same sampling d (Figure 4.1). Resistant *E. coli* exhibiting *sul3* was greater at d 56 than at d 1 and 35 (21.0 vs 2.8 and 1.0 % ± 3.46, respectively), regardless of the feeding regime. Similarly, the percentage of *E. coli* harboring *tetA* increased with calf age (from 6.0, 41.8 and 67.7 % ± 6.37 at d 0, 35 and 56, respectively), also independently of the feeding regime. The percentage of *E. coli* carrying the *tetB* resistance gene was similar among calves fed both types of milk and in all sampling times. Although *tetC* was not detected in *E. coli* from calves fed pWM at the beginning of the study, its prevalence was 40% at d 35, and decreased to < 10% at d 56. In contrast, the prevalence of *E. coli* isolates containing the *tetC* resistance gene was low in calves fed MR at d 1 and d 56 (<10 %), and it was no detected at d 35. The presence of aminoglycoside resistance genes was observed among *E. coli* isolates from calves fed both types of milk. However, the proportion of *aadA* was greater in *E. coli* of pWM fed calves at d 35 compared with those fed MR.

Five clusters were used to group all *E. coli* isolates according to their genotypic resistance profile (Table 4.5). The percentage of *E. coli* grouped in cluster B that carried resistance genes against tetracyclines, aminoglycosides and sulfonamides, was greater ($P < 0.05$) in MR fed calves than in those fed pWM. In contrast, MR fed calves had a lower percentage ($P < 0.05$) of *E. coli* isolates carrying resistance genes belonging to all of the antimicrobial families tested (cluster E) compared with pWM fed calves. The prevalence of *E. coli* in cluster A, C and D did not differ between feeding regimes. The percentage of *E. coli* isolates in cluster A, which carried only the resistance gene *aadA*,

decreased ($P < 0.05$) with calf-age from 57.1 to 22.7 at d 0 and 56, respectively. Those grouped in cluster C and D, which carried resistance genes from several antimicrobial classes, had greater percentage ($P < 0.05$) at d 56 than at d 1 and 35 (34.7 and 18.7 vs 7.1; and 3.6 vs 20.0 and 1.3 % for C and D clusters at d 56, 1 and 35, respectively). In contrast, the percentage of those isolates grouped in cluster E was greater ($P < 0.05$) at d 35 than at d1 and 56 (25.3 vs 0.0 and 4.0 %, respectively).

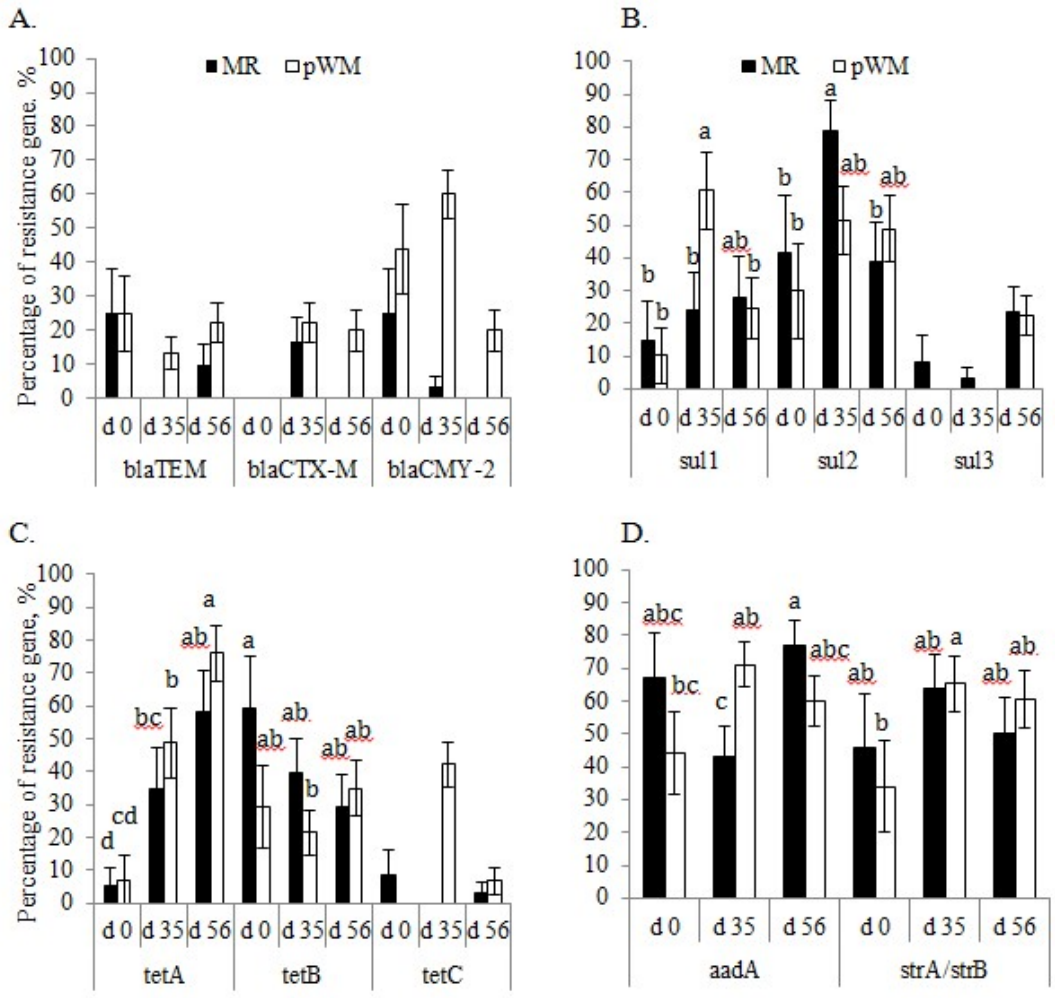


**Figure 4.1**. Effect of feeding calves milk replacer (MR) or pasteurized waste milk (pWM) on the selection of antimicrobial resistance genes in fecal *Escherichia coli* isolates of calves at different ages (1, 35, and 56 d). A) Prevalence of beta-lactam resistance genes. B) Prevalence of sulfonamide resistance genes. C) Prevalence of tetracycline resistance genes. D) Prevalence of aminoglycoside resistance genes. Bars with uncommon letters within gene differ at $P < 0.05$.

**Table 4.5.** Antimicrobial resistant genotypecluster distribution of fecal *E. coli* isolated from calves fed MR or pWM at days 1, 35, and 56.

| | | MR, %[1] | | | pWM, %[2] | | | *P*-value[3] | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Cluster | Antimicrobial resistant genotypes | Day 1 (n = 12) | Day 35 (n = 30) | Day 56 (n = 30) | Day 1 (n = 16) | Day 35 (n = 45) | Day 56 (n = 45) | Feed | Age | Feed x Age |
| A | *aadA* | 41.7 | 16.7 | 26.7 | 68.8 | 37.8 | 37.8 | 0.16 | <0.01 | 0.27 |
| B | *tetB - strA/strB - sul2* | 41.7 | 40.0 | 26.7 | 25.0 | 13.3 | 15.5 | <0.01 | 0.43 | 0.71 |
| C | *tetA - aadA - strA/strB - sul2* | 8.3 | 40.0 | 23.3 | 6.3 | 6.7 | 42.2 | 0.87 | 0.01 | 0.01 |
| D | *tetA - aadA - sul1 - sul3* | 8.3 | 3.3 | 23.3 | 0.0 | 0.0 | 15.6 | 0.18 | <0.01 | 0.41 |
| E | $bla_{CMY-2}$*- tetA - tetC - aadA - strA/strB - sul1 - sul2* | 0.0 | 0.0 | 0.0 | 0.0 | 42.2 | 6.7 | <0.01 | <0.01 | - |

[1]Percentatges of resistant isolates in calves fed milk replacer (MR) by calf age.

[2]Percentatges of resistant isolates in calves fed pasteurized waste milk (pWM) by calf age.

[3]*P*-value from Chi-square test.

*4.3.4. Association between phenotypic and genotypic resistance*

The percentage of *E. coli* carrying one or more of the beta-lactam resistance genes tested was greater in phenotypically resistant AMP colonies than in those that were not phenotypically resistant to AMP (Table 4.6), being the $bla_{\text{CMY-2}}$ the most prevalent ($P < 0.05$) gene in them. Most of the *E. coli* isolates that had the resistance genes $bla_{\text{CTX-M}}$ and $bla_{\text{CMY-2}}$ were also phenotypically resistant to the cephalosporin antimicrobials (KF and EFT) ($P < 0.05$). In contrast, the percentage of *E. coli* isolates that carried the resistant gene $bla_{\text{TEM.}}$ was similar between those that were and those that were not phenotypically resistant to KF and EFT. The most ($P < 0.05$) frequent beta-lactam resistance gene found inKF and EFT resistant *E. coli* was $bla_{\text{CMY-2}}$.

Although some colonies phenotypically not resistant to STX had *sul1, sul2* and *sul3* genes, the percentage of colonies having *sul1* or *sul2* genes was greater in STX phenotypically resistant colonies than in those that were not phenotypically resistant. In contrast to *sul3* gene that was found in both types of colonies independently of their STX resistant phenotype (Table 4.6). Most of the *E. coli* isolates that carried one or more of the *tet* resistance genes were phenotypically resistant to TET, and the most frequent tetracycline resistance gene in TET resistant *E. coli* was *tetA* ($P < 0.05$). Lastly, the majority of S resistant *E. coli* had the resistance gene *strA/strB*, whereas the resistance gen *aadA* was found in both, phenotypic and not phenotypic resistant isolates to S.

**Table 4.6.** Prevalence, odds ratio, and confidence interval of antimicrobial resistance at both genotypical and phenotypical levels in fecal *E. coli* isolated from dairy calves fed pWM or MR (resistant phenotype is the reference value for the OR).

| | | $G^+$ isolates, %[3] | | | | 95% CI | | |
|---|---|---|---|---|---|---|---|---|
| Antimicrobial agent tested[1] | Resistance gene tested[2] | $P^+$ isolates | $P^-$ isolates | SE | OR | Lower | Upper | *P*-value |
| Ampicillin | $bla_{TEM}$ | 29.6 | 1.1 | 0.03 | 37.3 | 4.9 | 286.3 | <0.01 |
| | $bla_{CTX-M}$ | 27.3 | 1.1 | 0.03 | 33.4 | 4.3 | 256.7 | <0.01 |
| | $bla_{CMY-2}$ | 53.4 | 1.1 | 0.03 | 102.0 | 13.4 | 776 | <0.01 |
| | Gene[4] | 95.5 | 1.1 | 0.02 | >999.9 | 201.6 | >999.9 | <0.01 |
| Ceftiofur | $bla_{TEM}$ | 16.1 | 13.4 | 0.04 | 1.2 | 0.5 | 2.9 | 0.62 |
| | $bla_{CTX-M}$ | 29.6 | 1.0 | 0.03 | 40.4 | 5.3 | 311.2 | <0.01 |
| | $bla_{CMY-2}$ | 58.0 | 1.0 | 0.03 | 132.7 | 17.4 | >999.9 | <0.01 |
| | Gene | 85.2 | 13.4 | 0.04 | 37.2 | 15.8 | 87.2 | <0.01 |
| Cephalothin | $bla_{TEM}$ | 11.5 | 16.2 | 0.04 | 0.7 | 0.3 | 1.7 | 0.40 |
| | $bla_{CTX-M}$ | 39.3 | 0.9 | 0.04 | 75.2 | 9.7 | 583.6 | <0.01 |
| | $bla_{CMY-2}$ | 59.0 | 9.4 | 0.04 | 13.9 | 6.2 | 31.2 | <0.01 |
| | Gene | 98.4 | 20.5 | 0.03 | 232.5 | 30.2 | >999.9 | <0.01 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Trimethoprim/ sulphamethoxazole | *sul1* | 97.9 | 10.0 | 0.02 | 423.0 | 53.1 | >999.9 | <0.01 |
| | *sul2* | 87.5 | 36.9 | 0.05 | 12.0 | 4.7 | 30.4 | <0.01 |
| | *sul3* | 16.7 | 8.5 | 0.04 | 2.2 | 0.8 | 5.8 | 0.12 |
| | Gene | 100.0 | 51.5 | 0.02 | >999.9 | 0.0 | >999.9 | 0.96 |
| Tetracycline | *tetA* | 61.9 | 4.5 | 0.04 | 34.2 | 7.9 | 148.8 | <0.01 |
| | *tetB* | 43.3 | 2.3 | 0.03 | 32.8 | 4.3 | 248.8 | <0.01 |
| | *tetC* | 17.2 | 2.3 | 0.03 | 8.9 | 1.2 | 69 | 0.04 |
| | Gene | 100.0 | 6.8 | 0.02 | >999.9 | 0.0 | >999.9 | 0.96 |
| Streptomycin | *aadA* | 62.0 | 61.5 | 0.05 | 1.0 | 0.6 | 1.9 | 0.95 |
| | *strA/strB* | 94.0 | 9.0 | 0.03 | 158.9 | 50.8 | 497.3 | <0.01 |
| | Gene | 98.0 | 65.4 | 0.03 | 25.9 | 5.9 | 114.6 | <0.01 |

[1]Antimicrobial agents tested by the disk diffusion method.

[2]Antimicrobial resistance genes tested by specific PCR assays.

[3]$G^+$ = *Escherichia coli* isolates carrying the antimicrobial resistance genes; $P^+$ = *Escherichia coli* isolates resistant to the antimicrobial agents tested at phenotypical level; $P^-$ = *Escherichia coli* isolates susceptible to the antimicrobials tested at phenotypical level.

[4]*Escherichia coli*isolates carrying any of the antimicrobial resistance genes tested by antimicrobial agent.

## 4.4. Discussion

Although the initial hypothesis was that feeding pWM to calves would increase the prevalence of antimicrobial-resistant *E. coli*, three different antimicrobial resistance patterns were observed among the fecal *E. coli* isolates analyzed herein. In the first place, almost all fecal *E. coli* were resistant to E, whereas 100% of them exhibited resistance to P and PIR. Similarly, Mustika et al. (2015) reported that all *E. coli* O157:H7 isolated from cattle exhibited resistance to P, and Maynou et al. (2017) described a high prevalence of resistance to E among fecal *E. coli* isolated from dairy calves. This may be attributed to the spectrum of activity of the antimicrobials. For example, antimicrobials of lincosamide and macrolide family, such as PIR and E respectively, are mainly active against Gram-positive bacteria (Leclercq, 2002), and have little inhibitory effect against isolates of *Enterobacteriaceae* family. This was also evidenced by Thornsberry et al. (1993) in a study of PIR resistance in pathogens from cows with mastitis. Additionally, the presence of genes that confer resistance to macrolides such as E has been found to be prevalent in Gram-negative bacteria (Ojo et al., 2004), including those that regulate cross-resistance to macrolides, lincosamides, and streptogramins B (Weisblum, 1995; Roberts et al., 1999; Gomes et al., 2016).

A second antimicrobial resistance pattern was influenced by feeding pWM to calves. This was the resistance to the beta-lactams AMP, KF, and EFT, and to lesser extent to FFC. The resistance to beta-lactam antimicrobials increased in calves fed pWM compared with those fed MR. This effect was expected because antimicrobials belonging to this family were commonly used to treat dairy cows for mastitis and other diseases, and residues of beta-lactam antimicrobials were detected within all pWM loads used to feed calves. Similarly, Pereira et al. (2014a) added beta-lactam and tetracycline antimicrobials to the calf MR with at similar concentrations than those found in WM (Pereira et al., 2014b), and the prevalence of fecal *E. coli* resistant to these antimicrobials added in the MR increased in the calf feces. Kaneene et al. (2008) described a decrease of antimicrobial resistance when they shifted from a medicated to a non-medicated milk replacer. Similarly, in the present study once the pressure of pWM disappeared at d 56 after the weaning, phenotypic antimicrobial resistances to AMP, KF, and EFT decreased, but their prevalence immediately after weaning was still greater in calves fed pWM compared with those fed MR. Past studies (Khachatryan et al., 2004; Berge et al., 2005; Edrington et al., 2012b) have reported different other factors beyond milk-feeding practices in young calves that can influence the prevalence of resistant

bacteria in gut microbiota. Age of calves is one of these factors that have mainly been discussed. In some studies the prevalence of resistant *E. coli* to several antimicrobials, including either aminoglycosides, tetracyclines and sulfonamides, decreased with calf-age (Edrington et al., 2012; Maynou et al., 2017), and in others antimicrobial resistance to aminoglycoside, phenicols or tetracycline was similar or increased (Khachatryan et al., 2004; Maynou et al., 2017). This discrepancy may be related to the different sampling season, farm of origin, and animal management.

Contrary to beta-lactams, phenicol antimicrobials such as FFC were never used to treat animals in the experimental farm nor at the farm supplying WM. However, the prevalence of fecal *E. coli* resistant to FFC tended to be greater in calves fed pWM than in those fed MR, suggesting a possible co-selection of FFC resistance by the antimicrobials present in WM. Although molecular basis of bacterial resistance to FFC was not examined in the present study, Meunier et al. (2010) reported plasmid-borne FFC and EFT resistance in *E. coli* isolates from sick cattle, that was encoded by the genes *floR* and $bla_{\text{CMY-2}}$, respectively. In the present study, the cluster distribution of fecal *E. coli* isolates based on their resistance profile and the selection of FFC resistant bacteria demonstrates concurrent resistance to other antimicrobials, including beta-lactams, in calves fed pWM (Table 4.4).

A third resistance pattern was observed for TET and S, with high resistance levels to these antimicrobials in *E. coli* isolated from calves fed both types of milk. Surprisingly, these antimicrobials were not used to treat dairy cows nor to treat calves. This high prevalence might be attributed to the farm environment as a potential source of resistant bacteria or resistance genes that could be transferred among bacterial populations (Sayah et al., 2005a; Kyselková et al., 2015; Maynou et al., 2017a). Similarly, other authors (Guerra et al., 2003; Srinivasan et al., 2007) have detected high levels of TET-resistant isolates with concurrent resistance to S, suggesting a potential plasmid-mediated acquisition of tetracycline and aminoglycoside resistance genes. This is consistent with results from Shin et al. (2015), who reported conjugative transfer of plasmid-mediated TET resistance in almost 83% of the TET-resistant *E. coli* isolated from beef cattle and most of them also exhibited phenotypic resistance to S. In the present study, the transferability of resistance genes was not tested, but several authors (Rosengren et al., 2008, 2009) have indicated increased probability of phenotypic TET resistance when bacterial isolates exhibit S resistance (and vice versa). In fact, TET and

S resistances appeared in all the clusters herein with the exception of Cluster A, which was more frequent in newborn animals (Table 4.4).

Regarding the antimicrobial resistance genes tested herein, the *bla*$_{CMY-2}$ was the most prevalent in *E. coli* isolated from calves fed pWM compared with those fed MR (Figure 4.1). Results from the phenotypic analysis of beta-lactam were not in agreement with the genotypic when evaluating the effect of feeding pWM to calves. This difference might be explained by two factors: 1) the antimicrobial resistance mechanism and 2) the class of beta-lactam antimicrobials exerting the selective pressure. In this study, most of the *E. coli* isolates that were phenotypically resistant to AMP, EFT, and KF carried either or both of the *bla*$_{CTX-M}$ and *bla*$_{CMY-2}$ resistance genes tested. In contrast, the resistance gene *bla*$_{TEM}$ was mostly detected in those isolates resistant to AMP (Table 4.6). This was not surprising since the most common mechanism of resistance to AMP among *E. coli* isolates is the production of TEM beta-lactamases (Livermore, 1995; Briñas et al., 2002; Olesen et al., 2004). In contrast to the CTX-M and CMY-2 betalactamases, the classical TEM enzymes (TEM-1 and TEM-2) are unable to hydrolyze third-generation or oxyimino cephalosporins, but are capable to attach penicillin antimicrobials and first generation of cephalosporins (Bush and Jacoby, 2010; Shaikh et al., 2015). Hence, considering that most of beta-lactam antimicrobials used in dairy farm belong to the third generation of cephalosporins (and therefore the antimicrobial residuals in pWM), a high detection rate of *bla*$_{TEM}$ in beta-lactam resistant isolates was not expected. The production of cefotaximases (CTX-M) has been reported to be the most common cause of resistance to beta-lactam antimicrobials in *Enterobacteriaceae* isolates of cattle (Geser et al., 2012b; Haenni et al., 2014; Gonggrijp et al., 2016). The most prevalent *bla*$_{ESBL}$ gene in fecal *E. coli* isolates of this study was CMY-2. However, the wide variants of extended-spectrum beta-lactamases (ESBLs) identified in *Enterobacteriaceae* isolates (Bonnet et al., 2000; Bonnet, 2004; Gonggrijp et al., 2016) cannot be excluded as a reason for the lesser detection rate of the CTX-M resistance gene observed herein, and in consequence for the lack of pWM effect on its selection. Other possible reason could be variations on the prevalence of ESBL that has previously been reported among different geographical regions (Paterson and Bonomo, 2005; Ghafourian et al., 2015).

Another consideration to the occurrence of beta-lactam resistance genes tested herein was the high prevalence of *E. coli* isolates producing CTX-M and CMY-2. ESBL- and AmpC–producing *E. coli* constitute an important determinant causing of

both, community-onset and nocosomial-onset infections in humans (Pitout et al., 2007, 2009), and farm animals have been recognized as a potential source of acquisition of these organisms via the food chain or by direct contact with humans and animals (Li et al., 2007; Carattoli, 2008; Doi et al., 2010). In a recent study (Randall et al., 2014), the presence of cephalosporin cefquinome residues in WM was associated with the presence of ESBL-producing *Enterobacteriaceae* in dairy farms, and the same CTX-M sequence type and *E. coli* clones were detected in WM, fecal samples, and their environment indicating the potential of ESBL to be transmitted among different ecological niches. In the present study, no *E. coli* colonies from sources other than the enrolled calves were isolated, and either serotyping or genotypic procedures to discriminate among resistant strain of *E. coli* were performed. However, the presence of *E. coli* carrying beta-lactam resistance genes in newborn calves and in those fed MR highlight the rapid spread of resistance genes to beta-lactam, which are important antimicrobials in human medicine (Collignon et al., 2009).

Similarly to the phenotype analysis, a high prevalence of fecal *E. coli* carrying TET and S resistance genes was observed herein, and isolation rates were in the range reported elsewhere. Shin et al. (2015) reported similar prevalence of *tet* genes in beef cattle to those found in the present study. They observed that the majority of fecal *E. coli* isolates from beef cattle carried the *tet*A (46.5%), followed by *tet*B (45.1%), and *tet*C (5.8%) genes. In contrast, others (Gow et al., 2008; Rosengren et al., 2009) have indicated greater prevalence of *tet*B (45.4% and 64.8%) than *tet*A (13.0% and 41.6%) in both cattle and swine, respectively, and detection rate of *tet*C was also very low (8.7% in cattle and 1.6% in swine). Almost all fecal *E. coli* of this study carried an aminoglycoside resistance gene (Table 4.6), being *strA/strB* the most frequent (94% of the isolates). Similar findings were reported in fecal *E. coli* isolates resistant to S from swine, with 62.2% of them carrying the *strA/strB* (Rosengren et al., 2009).Most of the *E. coli* isolates with the resistance gene *strA/strB* carried the genes *sul*2, *tet*A and *tet*B in their genotypic profile. Similarly, Rossengreen et al. (2009) reported a greater probability of detecting *strA/strB* in the presence of *tet*B and *sul*2, while the association between *strA/strB* and *tet*A was less strong. This was also evidenced herein, as *tet*B and *sul*2 were always clustered in resistance profiles carrying the gene *strA/strB*, but *tet*A gene that was not always associated with *strA/strB* in the clusters.

## 4.5. Conclusions

Based on the results of this study, it was concluded that WM produced by cows treated with beta-lactam antimicrobials and used to feed dairy calves contained drug residues in concentrations sufficient to select for resistant *E. coli* in the calf gut. In addition to lincosamide, beta-lactams were the only antimicrobial residues that could be present in WM. However, FFC-resistant *E. coli* were also selected in WM fed calves suggesting the potential of antimicrobial residues in milk to select for resistance to other antimicrobials than those present in milk. Moreover, the high levels of resistance to tetracycline and aminoglycoside antimicrobials found at both phenotypic and genotypic levels, and in calves from both feeding regimens, supported the hypothesis that antimicrobial pressure is not required for the emergence and maintenance of resistance in gut bacterial populations. These findings highlighted the need for further studies evaluating the specific factors involved in the widespread of antimicrobial resistance in bacteria isolated from farm animals, such as genetic linkage of resistance genes in mobile genetic elements and the transfer among different bacterial populations.

## Chapter 5

# IMPACT OF MILK-FEEDING PROGRAMS ON BACTERIAL COMMUNITIES OF THE RESPIRATORY AND THE GASTROINTESTINAL TRACT OF PRE-WEANED DAIRY CALVES

Submitted to Frontiers in Microbiology, October 2017

**ABSTRACT**

The resident microbiota of calves plays an important role on health and nutrition, and it is influenced by extrinsic factors, such as diet. In the present study bacterial communities from both, GIT and respiratory tract of pre-weaned dairy calves fed two different milk feeding programs were characterized using next-generation sequencing Illumina Miseq. Twenty Holstein female dairy calves (38.8 ± 1.40 kg of BW) were fed pWM containing beta-lactam antimicrobials residues, and 20 calves (38.1 ±1.19 kg of BW) were fed MR with similar nutrient composition (27.5% crude protein, 32.1% fat) to waste milk (28.6% crude protein, 30.0% fat) from day 1 to weaning at 49 days of study. Fecal and nasal samples were collected at 42 days of study and DNA extracted for 16S rDNA gene amplification and sequencing. To asses differences on bacterial communities between the 2 milk feeding regimes, sequencing data were examined for alpha and beta diversity, and the relative abundance of OTUs at different taxonomic levels determined for each sample. In general, Chao1, PD Whole Tree, and Shannon alpha diversity indices were similar for fecal and nasal bacterial communities of calves regardless of the feeding regime. However, principal coordinate analysis based on Unweighted Unifrac distances indicated differences in the composition of bacterial communities of calves fed milk replacer compared with those from calves fed pasteurized waste milk. The relative abundance of *Streptococcaceae* family and the genus *Histophylus* was greater ($P< 0.05$) in the nasal microbiota of calves fed MR than in those fed pWM. However, the genus *Prevotella* tended ($P = 0.06$) to be greater in the respiratory tract of calves fed pWM than in those fed MR. Differences in relative abundances of bacterial communities in gut microbiota were only observed at the phylum level, suggesting that antimicrobials present in waste have an un-specified influence at a lower taxonomical level.

**Key words:** bacterial communities, milk feeding regime, resident microbiota, gastrointestinal tract, respiratory tract, 16S rDNA gene sequencing.

## 5.1. Introduction

Growth and development of dairy calves are highly influenced by the activity of resident microbiota (Malmuthuge and Guan, 2017; Malmuthuge et al., 2015). A classic example of microbiota functions is found in the rumen, where fermentation of solid dietary substrates provide the short chain fatty acids necessary for adequate development of the rumen epithelium (Sander et al., 1959; den Besten et al., 2013). Nevertheless, microbial populations colonizing small intestine are also of importance for the development of the host through its influence on protective functions against pathogenic bacteria and its contribution on shaping the immune system (Yu et al., 2012; Sommer and Bäckhed, 2013). Moreover, digestion and nutrition of milk, the sole nutrient source of newborn calves, occurs primarily in the small intestine (Górka et al., 2011) and the activity of resident microbiota is also required for the adequate development of the intestinal epithelium (Sommer and Bäckhed, 2013; Malmuthuge et al., 2015).

The type of feed offered to calves has been demonstrated to influence the structure of the gut microbiota by providing different dietary substrates to bacterial communities (Maslowski and Mackay, 2011; Li et al., 2012; Kasparovska et al., 2016). However, most of the studies evaluating the effects of different dietary regimes on calf gut microbiota have mainly focused on the impact of solid food (Petri et al., 2013; Shanks et al., 2011; Callaway et al., 2010), whereas little information is available about how different milk feeding regimes affect the microbial composition of the gut (Górka et al., 2011; Edrington et al., 2012a; Deng et al., 2017).

On dairy operations, different types of milk can be used to raise calves: MR, saleable whole milk, and un-saleable milk (i.e., containing traces of antimicrobials), also called WM(Brunton et al., 2012). Although, MR has generally been the most common nutrient source in calf feeding programs, the use of non-saleable WM has gained popularity in the last years, in part, due to the large volumes of WM associated with the increasing size of commercial dairies (Edrington et al., 2012a) coupled with its cost advantages (Duse et al., 2013; Brunton et al., 2012). Moreover, the availability of on farm pasteurizers has likely contributed to this trend by reducing bacterial load in WM and in consequence the risk to transmitting infectious diseases to calves. In fact, Godden et al. (2005) demonstrated reduced morbidity and mortality as well as improved growth rates in calves fed pWM than in those fed conventional MR.

Additional concerns related to the use of WM arise from the presence of antimicrobial residues in milk. Previous studies (Maynou et al., 2017a; b) have reported increases in the prevalence of antimicrobial resistant bacteria in the gut and also respiratory microbiota of calves fed WM or pWM compared with those fed MR.

The effects of antimicrobials in animal feed on the gut microbiota have previously been evaluated in the GIT of farm animals (Looft et al., 2012, 2014; Lin et al., 2013) and changes on the taxonomic composition of gut microbiota as well as on the expression of microbial functional genes have been demonstrated. However, there is little information about the effects of liquid feeds containing antimicrobials on the respiratory tract of farm animals. Thus, the aim of the present study was to characterize bacterial populations of the GIT and respiratory tract of preweaned calves fed either, MR and pWM containing antimicrobials residues using next generation sequencing technology.

## 5.2. Material and methods

### *5.2.1. Calf management*

The current study was conducted at the SROC of the University of Minnesota from July to November 2014 and it was approved by the Animal Care Committee under the protocol number 1407-31648A. The SROC Calf and Heifer Facility raised Holstein dairy heifer calves from birth to approximately 6 months of age from three commercial dairy operations in Minnesota. Calves were picked up twice weekly from the respective dairies at 2 to 4 days of age. At the farm of origin, calves were offered at least 3 colostrum feedings of 3 to 4 L each within the first 24 h of life and thereafter 2 L of mixed transition milk twice daily until their transportation to SROC. From arrival to 56 d of age, calves were housed in individual pens (2.3 x 1.2 m) allocated in naturally ventilated calf barns divided into 2 rooms (approximately 40 calves per room). Each individual pen was separated by panels avoiding a direct contact between adjacent animals and bedded with chopped straw. After arrival at SROC and at 56 d old, calves were vaccinated against infectious bovine rhinotracheitis virus, parainfluenza-3 virus, and bovine respiratory syncytial virus (Inforce 3, Zoetis, Florham Park, New Jersey).

*5.2.2. Experimental treatments and sample collection*

Animals of the present study were a subset of calves involved in another study, which performance and intake data were previously reported by Ziegler et al. (2016). A total of forty female Hostelin calves were enrolled in the present study (3.5 ± 1.15 d of age and 39.3 ± 4.25 kg of BW) and were assigned to 1 of 2 milk feeding treatments by farm of origin and BW: 1) a non-medicated all-milk protein MR (26 % crude protein, 31 % fat; on a DM basis; Milk products Inc., Chilton, Wisconsin) at a 12.5% dry matter concentration; or 2) pWM (28.4 % crude protein, 30.1 % fat; dry matter basis) containing traces of antimicrobials. Starting on the day of arrival, calves on each treatment group were fed 0.34 kg of dry matter (either from MR or pWM) per feeding twice daily for 42 d and, in preparation for weaning, once daily from d 43 to weaning at 49 d of age. Waste milk was collected from a local dairy 2 to 3 times weekly and stored at 4ºC in a milk tank until pasteurization at 63ºC for 35 min. For each milk load, the solid content of pWM was measured using a brix refractometer (Spartan Refractometer, Model A 300 CL, Spartan, Tokyo, Japan) to equalize nutrient intakes between the two feeding treatments. For each WM load, a milk sample before pasteurization was analyzed for fat, lactose, protein, total non-fat solids by infrared spectroscopy, and somatic cells by a cell counter (Minnesota DHIA Laboratory, Zumbrota, MN). Total bacteria and coliforms in WM and pWM were also analyzed by plate count for each milk load at the DHIA Laboratory (Zumbrota, Minnesota) to assert that pasteurization treatment decreased 3-log total bacterial and coliform counts. For this analysis, one WM sample was collected before pasteurization and two samples after pasteurization. Residues of antimicrobials commonly used to treat dairy cows from milk origin farm were screened once for each milk load after pasteurizationusing a commercial enzyme-linked receptor-binding assay test (SNAP beta-lactam test) (Idexx Laboratories Inc., Westbrook, Maine). Most of the antimicrobials used to treat dairy cows in this farm belonged to the beta-lactam family (mainly cephalosporins rather than penicillin), and beta-lactam residues were detected in each load of milk throughout the study.

From d 1 to 56 of study, calves had free access to water and textured calf starter (18 % crude protein, 18.5 % neutral detergent fiber) supplemented with decoquinate at 45 g/ton (Elite 18%, Hubbard feeds, Mankato, Minnesota). Individual starter concentrate and milk intake was recorded daily from the beginning of the study until 56

d of study. Body weight of the calves was measured at d 1, 14, 28, 42, 49 and 56 of study. Health of calves was monitored daily and those that requiring antimicrobial therapy before 42 d of study were excluded from the study. Fecal samples were collected via rectal palpation from each calf at d 42 of study and immediately transported to the lab to freeze. Also at 42 d of study, nasal samples were obtained by inserting a sterile swab (Puritan HydraFlock, Puritan Diagnostics Llc., Guilford, Maine) into the nasal cavity, always at the left nostril, and rotating 360º several times. After sampling, each nasal swab was put into a 1.5 ml tube and along with fecal samples was frozen until total acid nucleic extraction.

*5.2.3. DNA extraction, 16S rRNA libraries preparation, and DNA sequencing*

Total nucleic acids were extracted from nasal swabs and 0.3 g of feces mixed with 800 µl and 1000 µl of LB respectively, using the MagMAX Total Nucleic Acid Isolation Kit (Thermo Fisher Scientific, Grand Island, New York). Total nucleic acid extraction was performed according to the manufacturer's instructions. Extracted DNA concentration and quality was determined using a Synergy H1/Take 3 spectrophotometer (BioTek, Winooski, Vermont). All DNA extracts were stored at -20ºC and shipped to the MR DNA laboratory (Shallowater, Texas) for 16S rRNA gene amplification and sequencing on an Illumina MiSeq platform as described below.

The hypervariable regions V1-V4 of the 16S rDNA gene were individually amplified from each sample by PCR using the universal bacterial primers 27Fmod (5'-AGRGTTTGATCMTGGCTCAG-3') and 519Rmodbio (5'-GTNTTACNGCGGCKGCTG-3') with an 8 bp sample-specific barcode on the forward primer. This set of primers produced a fragment of 16S rDNA approximately 500 bp long. PCR reactions were performed using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following thermocycling conditions: 94°C for 3 minuntes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds, 72°C for 1 minute, and a final extension at 72°C for 5 minutes. The resulting PCR products from each sample were visualized by electrophoresis in 2% agarose gels and mixed in equal concentration of DNA for 16S rRNA libraries preparation. The amplicons from pooled samples were then purified using Agencourt AMPure XP beads (Agencourt Bioscience Corporation, Beverly, Massachusetts) and paired-end sequenced (2x300) on an Illumina Miseq platform following the manufacturer's instructions.

### *5.2.4. Sequencing data analysis*

Raw FASTA files containing assembled paired-end reads and quality score files were used to process the reads for quality trimming and taxonomic assignments with the QIIME software pipeline (MacQIIME 1.9.1) (Caporaso et al., 2010). All sequence reads were first decoded based on 8 bp sample-specific barcodes and quality filtered with the following parameters: average quality score < 25 calculated in sliding window of 25 bp; minimum read length: 450; maximum read length: 550; maximum number of mismatches in primer and barcode sequence: 0; maximum number of ambiguous bases: 0 and maximum homopolymer: 8. The remaining sequences were then processed for clustering into operational taxonomic units (OTUs) at a 97% similarity threshold using the UCLUST function, and for the *de novo* and reference-based chimera detection with the intersection method in USEARCH version v5.2.236 (Edgar, 2010). For taxonomic assignment, a representative sequence from each OTU was selected and compared with those in the SILVA reference database version 111 (Quast et al., 2012). Singleton OTUs were removed before conducting further analyses.

### *5.2.5. Statistical analysis*

To assess the effect of feeding regime on calf bacterial communities both, alpha and beta-diversity parameters were computed. To estimate alpha diversity parameters, the observed OTUs, Chao1, PD Whole Tree, and Shannon and Goods coverage indexes were calculated for each sample, and rarefaction curves depicted using QIIME (*alpha_diversity.py* and *alpha_rarefaction.py* scripts). For rarefaction curves, the upper limit of rarefaction depth, which represents the minimal number of reads found within all fecal or nasal samples, was considered. For each alpha diversity indices and type of sample (fecal and nasal), a non-parametric two sample t-test (Monte Carlo with 999 permutations) was performed to assess differences between feeding regimes. For beta-diversity analyses, the Unweighted UniFrac distances were calculated for each type of sample (fecal and nasal), and Principal Coordinate Analysis (PCoA) plots performed based on these distances. Relationships between bacterial communities of calves fed either MR or pWM were tested using a one-way analysis of similarity (ANOSIM) with QIIME python script (*compare_categories.py*). To assess differences in the composition of bacterial communities between feeding regimes, the relative abundance of OTUs from calves fed MR and from those fed pWM was compared at three taxonomic levels.

Furthermore, the core microbiome, defined as those OTUs present in all fecal or nasal samples, was determined for both, calves fed MR and pWM, independently one from the other, and its relative abundance compared between feeding regimes. Estimation of *p*-values was performed through the Kruskal–Wallis test using the PROC NPAR1WAY procedure of SAS (version 9.2, SAS Institute Inc., Cary, NC, USA) with a false detection rate correction for multiple hypotheses testing (PROC MULTTEST procedure of SAS) in both bacterial composition analyses.

## 5.3. Results

Twelve calves fed MR and 9 calves fed pWM were treated with antimicrobials, and their data and samples were excluded of the study. Furthermore, one calf on the pWM treatment consumed much lesser starter than their counterparts (0.71 vs 13 kg of concentrate in 42 days of study, respectively), and it was discarded from the study, because it highly influenced diversity, richness and composition of bacterial communities in both fecal and nasal samples. Therefore, pyrosequencing analysis was performed with 8 calves fed MR and 10 calves fed pWM.

### *5.3.1. Calf performance*

Calves fed pWM weighed 4.8 kg more ($P < 0.05$) than calves fed MR at 42 d of study (Table 5.1). From the beginning of the study to d 42, calves fed MR consumed 11 kg of concentrate and calves fed pWM consumed 15 kg, with the gain to feed ratio being greater ($P < 0.05$) in pWM than in MR fed calves (0.77 and 0.71 ± 0.01, respectively) (Table 5.1).

**Table 5.1.** Effect of milk feeding regime on growth performance and starter feed intake in dairy calves.

| | Feeding regime[1] | | | |
|---|---|---|---|---|
| | MR | pWM | SEM | *P*-value |
| Initial BW, kg | 38.8 | 38.1 | 1.29 | 0.698 |
| BW at 42 d, kg | 65.8 | 70.6 | 1.53 | 0.044 |
| ADG, kg/d | 0.65 | 0.77 | 0.037 | 0.046 |
| Starter intake, kg/d | 0.27 | 0.35 | 0.047 | 0.208 |
| Total DM intake, kg/d | 0.92 | 1.00 | 0.047 | 0.231 |
| Gain to feed ratio | 0.71 | 0.77 | 0.01 | 0.021 |

[1]MR: Milk replacer; pWM: Pasteurized waste milk.

*5.3.2. Descriptive data*

The total number of 16S rRNA inputs generated from both nasal and fecal samples accounted for 2,157,627 sequences with a mean sequence length of 529.6 nucleotides. After removing singletons, and the erroneous and poor quality sequences, 1,159,416 reads remained with an average of 32,206 sequences per sample. Specifically, 26,701 sequences per sample from feces (range 17,327 – 36,598) and 37,711 from nasal swabs (range 18,391 – 39,582). In total, 10,802 OTUs were identified from fecal samples and 7,878 from nasal swabs by clustering sequences at 97% sequence similarity cut-off. The average number of OTUs clustered per calf was 597 in fecal samples (range 479 – 777) and, 438 in nasal swabs (range 251 – 623).

A total of 19 bacterial phyla were identified in the fecal samples. However, the majority of OTUs were clustered in 2 phyla. Globally, an average of 39% of the sequences belonged to the *Bacteroidetes* phylum, 55.7% to *Firmicutes,* and 3.3% to *Proteobacteria*. Almost 2% of the remaining sequences were classified into phyla with lower relative abundances than 1%, leaving only 0.004% of the sequences unclassified. *Lachnospiraceae* was the most abundant family in fecal microbiota representing 31.1%

of the total sequences, and 58.3% within the *Firmicutes* phylum. The second and third most abundant families were *Prevotellaceae* (19.6% of the total sequences) with 51.9% of representation in the *Bacteroidetes* phylum, and *Ruminococcaceae* accounting for 17.8% of the total sequences and 33.2% within the *Firmicutes* phylum. Among *Proteobateria* phylum, *Succinivibrionaceae* was the only family with a relative abundance of more than 1%, representing 2% of the total sequences and 42% of the sequences within the phylum. At the genus level, 124 taxa were identified in fecal microbiota, leaving 45.5% of the total sequences unclassified. *Prevotella* and *Bacteroidetes*, which belong to the *Bacteroidetes* phylum, were the major genera accounting for 15.5% and 9% of all reads, respectively. *Blautia* was the third most abundant genus, which belongs to the *Firmicutes* phylum and represented 8% of the total reads, and 25.7 % of the *Lachnospiraceae* family.

Regarding nasal bacterial communities, a total of 18 bacterial phyla were identified. The majority of sequences (97%) were classified in 6 phyla with an average of relative abundance of more than 1%. The most abundant phyla were *Tenericutes* (29.5 %), *Firmicutes* (19.3%) and *Actinobacteria* (19%), followed by *Proteobacteria*, *Bacteroidetes,* and Fusobacteria (16, 11.5, and 2.5%, respectively). At the family level, only 2 groups of 12 had more than 10% of representation in relation to the total sequences: *Mycoplasmataceae* (29.5%) and *Microbacteriaceae* (13.7%). Within the *Tenericutes* phylum, *Mycoplasmataceae* accounted for 99.9% of the total phylum, whereas *Microbacteriaceae* represented 72.0% of the *Actinobacteria* phylum. *Pasteurellaceae* (9.7%) was also a predominant family within *Proteobacteria* phylum accounting for 60.6% of the total phylum. In contrast to the other phylum, *Bacteroidetes* and *Firmicutes* had more intraspecific diversity being equally represented by at least three different families. At the genus level, 78.3% of the sequences were classified in 259 genera in nasal microbiota, leaving 21.7% without classification. The most abundant genera were *Pseudoclavibacter* and *Mycoplasma* with 13.8 % and 29.5% of the total sequences, respectively. Among the total bacterial genera identified in nasal microbiota, only 11 showed a relative abundance of more than 1%.

### *5.3.3. Effect of feeding regime on alpha diversity indices*

Fecal bacterial communities of dairy calves fed MR did not show differences in the number of observed OTUs, Chao1, PD whole tree, and Shannon index when

compared with those from calves fed pWM (Table 5.2). Similarly, no differences were found for these estimators in the nasal bacterial communities of calves exposed to either of the two feeding regimens (Table 5.2). Rarefaction curves of both fecal and nasal bacterial communities are plotted by feeding regime in Figure 5.1. The Good's coverage was of 0.99 ±0.001 in both, fecal and nasal samples indicating that the sampling effort was sufficient in all cases.

*5.3.4. Effect of feeding regime on fecal and nasal bacterial composition*

Fecal microbiota from calves in the MR treatment tended ($P$ = 0.07) to have greater relative abundance of the *Bacteroidetes* phylum compared with microbiota from the pWM calves, whereas the relative abundance of *Firmicutes* tended ($P$ = 0.07) to be lower in calves fed MR than in those fed pWM (Figura 5.2). No differences in fecal bacterial populations were detected at both, family and genus level between feeding regimes (Figure 5.2).

Feeding regime did not have any effect on bacterial composition of nasal microbiota at any of the taxonomic levels tested (phyla, family, and genus) (Figure 5.3). However, the analysis of Unweighted Unifrac distances, plotted in PCoA graphs (Figure 5.4), indicated phylogenetical differences between bacterial communities of calves fed MR and those fed pWM (ANOSIM test for fecal micobiota; global R = 0.331, $P$< 0.05; ANOSIM for nasal microbiota; global R = 0.135, $P$< 0.05).

Although no differences between feeding regimes were observed in the fecal core microbiome, the nasal core microbiome, defined for both MR and pWM fed calves, revealed differences in relative abundances of bacterial communities (Table 5.3). At the family level, the proportion of *Streptococcaceae* was greater ($P$< 0.05) in calves fed MR than in those fed pWM; whereas, at the genus level (*Streptococcus*) this pattern tended ($P$ = 0.06) to be different. *Histophilus* genus was also more frequent ($P$< 0.05) in nasal microbiota of calves fed MR than in those fed pWM. In contrast, the relative abundance of the genus *Prevotella* tended ($P$ = value) to be greater in pWM fed calves than in those fed MR.

**Table 5.2.** Alpha diversity indices (±SE)for both fecal and nasal bacterial communities from calves fed either milk replacer (MR) or pasteurized waste milk (pWM).

| | Fecal microbiota | | | Nasal micrbiota | | |
|---|---|---|---|---|---|---|
| | MR | pWM | *P*-value | MR | pWM | *P*-value |
| Observed OTUs | 541.0 ± 64.52 | 531.1 ± 56.60 | 0.73 | 335.6 ± 103.00 | 381.5 ± 96.35 | 0.36 |
| Chao1 | 706.5 ± 71.72 | 705.4 ± 63.30 | 0.98 | 466.1 ± 106.60 | 492.5 ± 94.84 | 0.59 |
| PD whole tree | 27.3 ± 2.29 | 26.9 ± 2.28 | 0.74 | 20.4 ± 4.41 | 22.7 ± 3.87 | 0.27 |
| Shannon | 6.1 ± 0.55 | 6.3 ± 0.41 | 0.27 | 3.4 ± 0.94 | 3.5 ± 1.38 | 0.78 |

(A) (B)

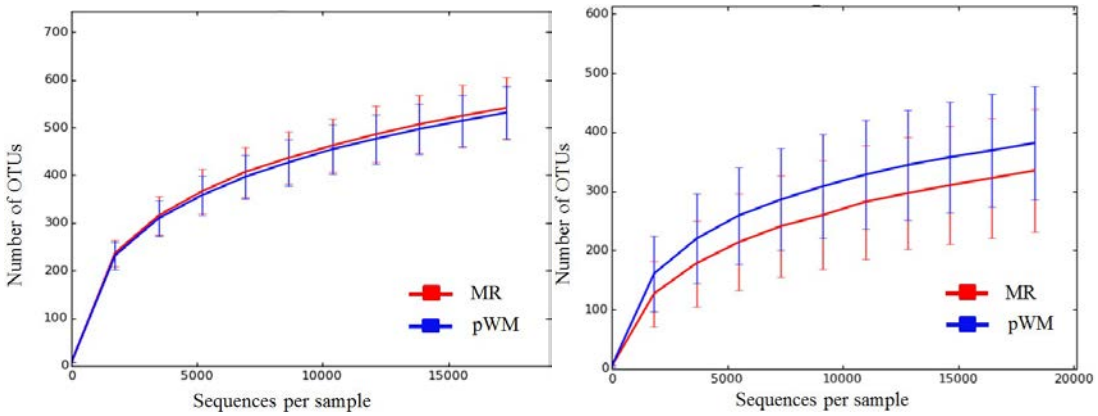


**Figure 5.1.** Rarefaction analyses of 16S rRNA gene sequences from both fecal (A) and nasal (B) bacterial communities of dairy calves. Rarefaction curve of calves fed milk replacer (MR) are depicted in red and those fed pasteurized waste milk (pWM) are depicted in blue. The analysis was performed using a upper limit of rarefaction depth of 17,300 sequences for the fecal microbiota and 18,300 sequences for the nasal microbiota.

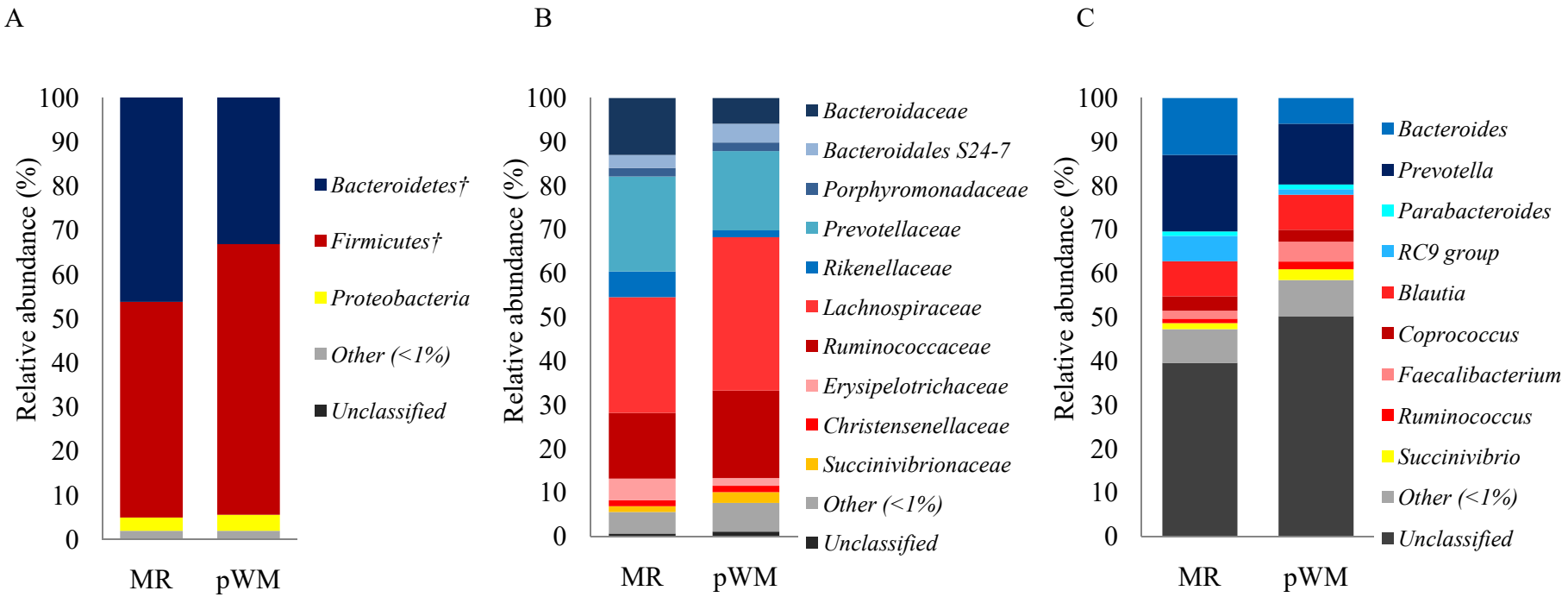


**Figure 5.2.**Mean relative abundance (%) of bacterial phyla (A); family (B), and genus (C), in the fecal microbiota of dairy calves fed either milk replacer (MR) or pasteurized waste milk (pWM). †Indicates that the relative abundances of bacterial divisions tended to be different ($P< 0.10$) between feeding regimes.

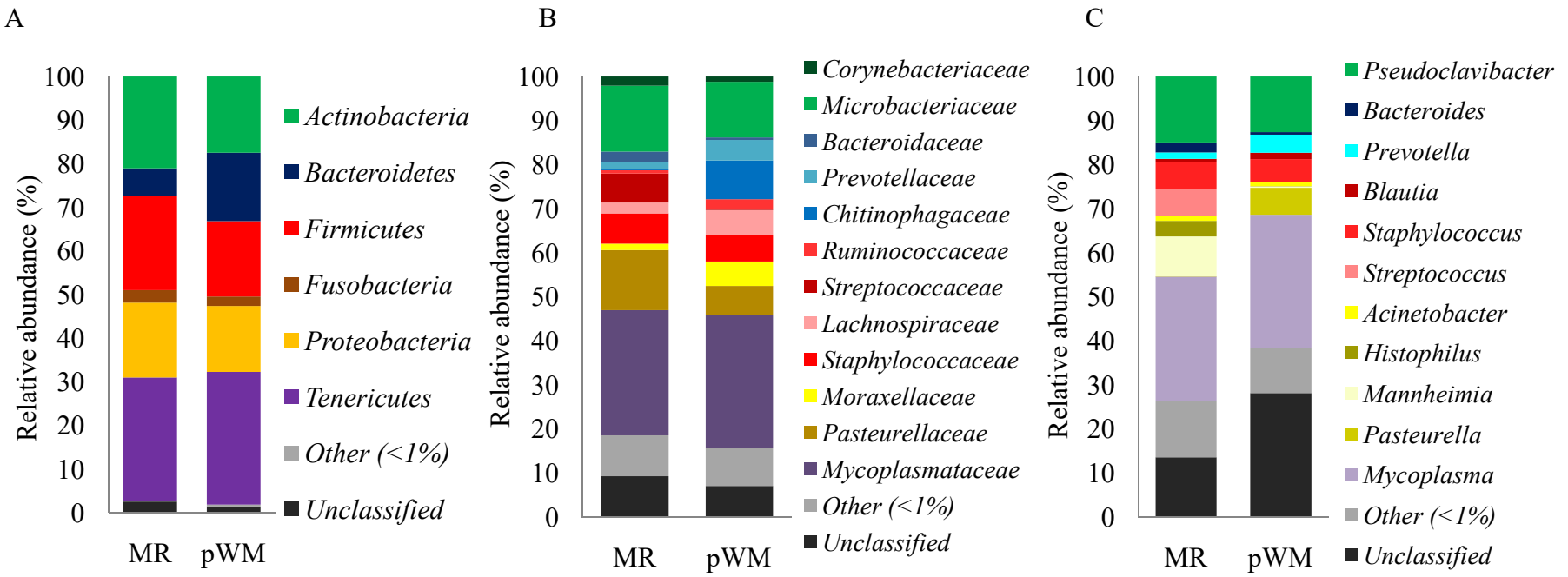


**Figure 5.3.**Mean relative abundance (%) of bacterial phyla (A); family (B), and genus (C), in the nasal microbiota of dairy calves fed either milk replacer (MR) or pasteurized waste milk (pWM).

A B

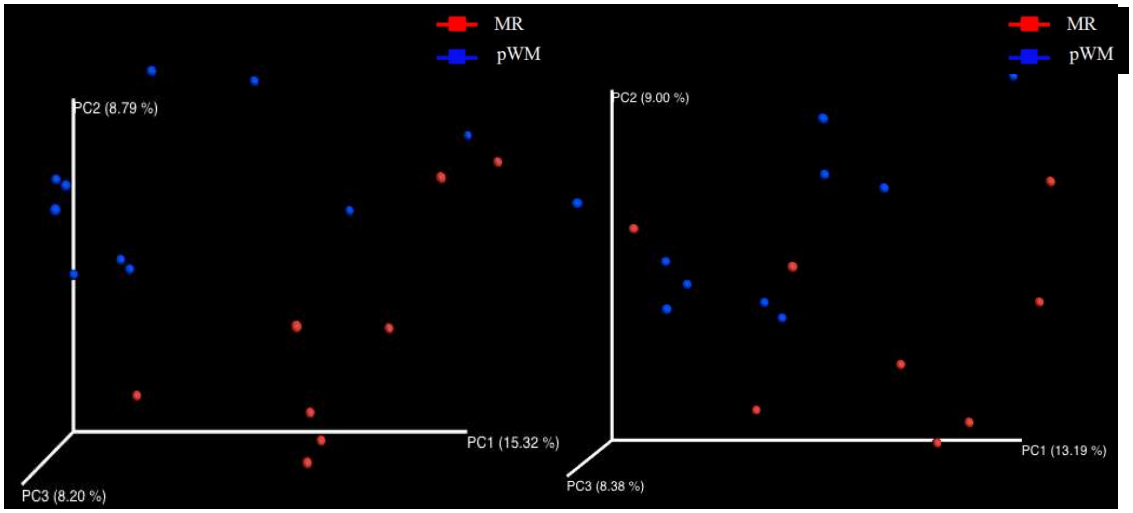


**Figure 5.4.** Principal coordinates analysis (PCoA) representing beta diversity of both fecal (A) and nasal (B) bacterial communities in dairy calves. Figures were computed using unweighted Unifrac distances and a depth coverage of 17,300 sequences per sample. Bacterial communities from calves fed milk replacer (MR) are depicted in red, and those from calves fed pasteurized waste milk (pWM)in blue

**Table 5.3.** Operation taxonomic units (OTUs) displaying different relative abundances in the core nasal microbiota of dairy calves fed either milk replacer (MR) or pasteurized waste milk (pWM).

| OTUs | | | Relative abundance | |
|---|---|---|---|---|
| Phylum | Family | Genus | MR | pWM |
| *Bacteroidetes* | | | | |
| | *Prevotellaceae* | | | |
| | | *Prevotella*** | 0.19 | 1.88 |
| *Firmicutes* | | | | |
| | *Streptococcaceae** | | | |
| | | *Streptococcus*** | 10.7 | 0.06 |
| *Proteobacteria* | | | | |
| | *Pasteurellaceae* | | | |
| | | *Histophilus** | 4.1 | 0.0 |

*Denotes significant differences on the relative abundances of bacterial divisions between feeding regimes.

**Denotes that the relative abundances of bacterial divisions tended to be different between feeding regimes.

**5.4. Discussion**

The predominant phyla in feces from dairy cattle are *Firmicutes*, *Bacteroidetes,* and *Proteobacteria*. Although in the present study *Firmicutes* was the most predominant phylum in both feeding treatments, literature shows inconsistent results with the most representative phylum in preweaned calves. Similar to the present study, Oikonomou et al. (2013) found *Firmicutes* to be the major phylum in feces of preweaning calves, in contrast to Deng et al. (2017) and Malmuthuge et al. (2014) who reported *Bacteroidetes* to be the most dominant phylum in samples from the large-intestine of calves. However, Edrington et al. (2012) reported varying dominant phyla (either *Firmicutes* or *Bacteroidetes*)in preweaning calves at different sampling ages and milk feeding regimes throughout the preweaning period. Furthermore, differential composition between mucosa- and digesta-associated microbiota was also observed throughout the GIT, indicating that the structure of bacterial communities may vary greatly, not only by the region of the GIT sampled, but also depending on the type of sample collected (Malmuthuge et al., 2014; Deng et al., 2017). In the present study, differences in the relative abundance of the phyla *Firmicutes* and *Bacteroidetes* in fecal microbiota might be attributed to three different aspects of the feeding regimes: 1) ingredient composition of MR and pWM, and presence of immune factors (growth factors, cytokines, immunoglobulins...) in pWM but not in MR, 2) differences in starter concentrate intake between feeding regimes, and 3) antimicrobial residues present in pWM.

Calves fed pWM in the present study had an increased feed efficiency, probably because of the greater bioavailability of nutrients in whole milk compared with MR (Lee et al., 2009). Furthermore, calves fed pWM consumed more starter concentrate than calves fed MR. The increase in starter concentrate intake in calves fed pWM together with the greater relative abundance of *Firmicutes* phylum compared with MR fed calves may be responsible for the tendency towards improved feed efficiency of calves fed pWM. Myer et al., (2015) found a great abundance of *Firmicutes* within the rumen microbiota of steers with improved feed efficiency, and Looft et al. (2012) associated improvements of feed efficiency in pigs that received medicated feeds with microbial functional genes related to energy production and conversion, in conjunction with a decrease in fecal *Bacteroidetes*. In studies of distal gut microbiota of mice (Ley et al., 2005; Turnbaugh et al., 2006), changes in the relative abundance of *Bacteroidetes* and *Firmicutes* were found to influence the capacity to harvest energy from the diet.

Specifically, a greater ratio of *Firmicutes* to *Bacteroidetes* was observed in obese mice when compared with their lean counterparts.

In infants, breast-feeding is associated with a decrease in microbiota diversity, a decrease of bacteria from the *Firmicutes* phylum, and some differences in the presence of some *Bifidobactirium* species compared with formula-fed infants (Praveen et al., 2015). However, Carlisle et al., (2013) reported an increase of *Firmicutes* phylumin mice fed maternal milk compared with mice fed a milk substitute, as observed herein. Although a decrease in microbiota diversity has been commonly reported in mammals fed maternal milk (Carlisle et al., 2013; Lee et al., 2015), this effect could not be detected in the present study. Generally, pasteurization reduces some of the growth factors, antimicrobial proteins, and immunoglobulins (Daniels et al., 2017) present in raw milk that might contribute to the reduction in microbial diversity observed in breast-fed infants or animals fed maternal milk. Deng et al. (2017) fed to calves acidified WM, untreated WM, pWM, and untreated bulk milk, and they did not observe differences in alpha diversity in rectal samples. However, greater relative abundances of beneficial bacteria related to the production of short chain fatty acids and involved in important symbiotic host-microbiome relationships were observed in pWM fed calves compared with those fed either untreated or acidified WM. Others (Bach et al., 2017), fed to calves raw milk, pasteurized milk and UHT milk did not found differences in the total counts of Gram positive bacteria between feeding regime. However, bacterial counts of *Lactobacillus*, a gram positive bacterium involved in protective functions by inhibiting the growth of pathogenic bacteria and promoting the immune system response, were found to be lower in both, pasteurized and UHT milk fed calves than in those receiving raw milk. Antimicrobials may have also contributed to the change at the phylum level observed herein, but Pereira et al. (2016) compared the taxonomic levels above the genus of feces from fed either WM containing a low concentration of a combination of the main antimicrobials found in WM (ampicillin, ceftiofur, penicillin G and oxytetracycline) or saleable whole milk and did not observe any disruptions in microbial profiles (above the genus level).

The notion that antimicrobials in feed may also reduce gut bacterial diversity in mammals have also previously demonstrated in pigs feed a starter concentrate supplemented with antimicrobials (Looft et al., 2012). However, in the present study, feeding calves pWM containing beta-lactam antimicrobial residues did not affect diversity estimators, which were represented by the total number of different OTUs,

microbial richness (Chao1), biodiversity (Shannon), and phylogenetic biodiversity (PD whole tree) of bacteria communities. Similar finding were reported by Pereira et al. (2016), who found no differences in alpha diversity estimators between calves fed whole milk with added antimicrobials and those fed whole milk with non-added antimicrobials suggesting that the low antimicrobial drug concentration in milk did not exert sufficient pressure to have a significant effect on gut microbiota.

Although in the current study differences in the composition of fecal microbiota between feeding regimes were only observed at the phylum level, results from PCoA plots and ANOSIM analysis revealed changes in specific structures of bacterial communities from calves depending on the type of milk offered (Figure 4). Differences in bacterial communities could not be evidenced taxonomic division below the phylum, which might be, in part, because calves were sampled at 42 days of age when they consumed 800 g/day of concentrate feed. The relative large consumption of solid feed might contribute to mask the effect of milk-feeding regimes on fecal microbiota at lower taxonomic levels, since feeding starter feed tend to increase the richness of predominant phylotypes along the gastrointestinal tract (Malmuthuge et al., 2013). Similarly, Klein-Jöbstl et al. (2014) also reported a high variability in the composition of bacterial communities among calves during the weeks before weaning than either during the firsts weeks of life or after weaning when gut microbiota became more stabilized.

Regarding taxonomic analysis of the nasal microbiota, the most abundant phyla in the respiratory tract of calves was *Tenericutes* followed by *Firmicutes*, *Actinobacteria*, *Proteobacteria*, and *Fusobacteria*. Additionally, bacterial communities of the respiratory tract had more intra-individual variability than that found in gut microbiota at each of the taxonomical level assessed. These findings were in agreement with those reported by Holman et al. (2015), who reported a large heterogeneityin the relative abundance of several taxa in the nasopharyngeal tract of cattle. Other authors (Holman et al., 2015; Lima et al., 2016) have described *Proteobacteria* and *Firmicutes* in cattleas the most abundant bacterial phyla instead of *Tenericutes* and *Actinobacteria*.

The present study demonstrated changes in nasal bacterial communities in calves fed two different types of milk. Probably, the antimicrobial residues present in pWM might be the main cause behind changes in *Histophilus somni* and *Streptococcus* spp. prevalence. In cattle, antimicrobials such as cephalosporins, tilmicosin, and fluoroquinolones are the first option (Constable et al., 2008) to treat infectious diseases

caused by *Histophilus somni* and *Streptococcus* spp. (Constable et al., 2008), and most of them were used to treat dairy cows in the study farm where WM was collected. Pereira et al. (2016) reported lower abundance of *Streptococcus spp.* and *Clostridium spp.* in fecal microbiota of calves fed whole milk with sub-minimum inhibitory concentration (MIC) of antimicrobials, suggesting these genera as highly sensitive to sub-MIC of antimicrobials. Differences in relative abundances of *Prevotella* herein are difficult to understand. In pigs, the presences of *Prevotella* in the respiratory tract has been associated with farms without respiratory disease (Correa-Fiz et al., 2016).

### 5.5. Conclusions

Overall, results show that feeding pWM with beta-lactam antimicrobial residues affect the composition of bacterial communities from both, fecal and nasal microbiota of pre-weaned calves, with these effects being more evident in the respiratory tract. Furthermore, feeding pWM to calves instead of MR with non-antimicrobial residues and similar nutrient composition to WM, improves feed efficiency and starter concentrate intake inducing changes in the relative abundance of *Firmicutes* and *Bacteroidetes* phyla in the gut microbiota. However, these findings have to be interpreted cautiously since nutrient quality and the amount and type of antimicrobials residues present in WM may vary greatly among dairy farms and over time.

# Chapter 6

# GENERAL DISCUSSION

## 6. GENERAL DISCUSSION

The exposure of bacterial communities at low doses of antimicrobials was demonstrated to allow antimicrobial resistant bacteria to multiply in the absence of susceptible competitors (Schwarz et al., 2001). Therefore, the use of WM in calf feeding programs was suggested to create optimal conditions for antimicrobial resistance emergence in the GIT of calves (Pereira et al., 2014b). The first study of the present thesis (Chapter 3) aimed to determine the effects of feeding WM to dairy calves on the selection of resistant bacteria in calf-gut microbiota, but also in the respiratory tract of calves due to its direct contact to milk when drinking. Because of the possibility of other intrinsic and extrinsic factors influencing on antimicrobial resistance, the effects of calf-age, environment contamination with resistant strains, and transference of resistant bacteria from dam to calves at birth were also evaluated. The results of this study demonstrated an increase on the prevalence of antimicrobial resistant fecal *E. coli* and nasal *P. multocida* in WM fed calves compared with those fed MR without antimicrobials. However, feeding WM to calves not only selected for resistant bacteria to the antimicrobials commonly used in the study farms, but also to others suggesting co-selection of resistance genes by the antimicrobial residues present in milk. An influence of the farm environment on the acquisition of resistant bacteria was also demonstrated, while the analysis of calf-age and dam-calf influences shown inconsistent results. Therefore, we performed a second study (Chapter 4) to evaluate the effects of feeding WM to calves on the selection of resistant fecal *E. coli* at both, phenotypic and genotypic level. In this second study, calves were raised at the same experimental farm with the aim of harmonizing raising conditions and minimizing the effect of external factors influencing on antimicrobial resistance. The results of this study evidenced more clearly the effects of feeding WM to calves on the selection of resistant bacteria to the antimicrobials used in dairy cows, although high levels of resistance regardless of feeding-regime were also demonstrated.

Although the main objective of both studies was to evaluate the effects of feeding WM on the selection of antimicrobial resistant bacteria in calves, not all studies were performed following the same experimental conditions neither at the same geographic region (Table 6.1). Duse et al.(2015) described multiple farm and calf characteristics related with increased shedding of antimicrobial resistant *E. coli*in dairy calves such as, calf-housing system, herd size and geographic location. Thus, in this chapter, factors differing between both studies that could have an effect on the

occurrence of antimicrobial resistant bacteria in dairy calves will be discussed (Table 6.1). To facilitate this purpose, the fecal *E. coli* resistance prevalence for each antimicrobial and study farm was calculated, and this prevalence was classified according to the distribution of their occurrence in 4 classes. Table 6.2 denotes the most prevalent antimicrobial resistances with the darkest color, and the less prevalent with the lightest one. Furthermore, the specific class of antimicrobial used to treat cows and calves in each farm was also considered, and mark with a cross in Table 6.2.

**Table 6.1.** Experimental conditions variables that differed between studies evaluating antimicrobial resistance in calves fed waste milk (WM).

| | **Experimental conditions** | |
|---|---|---|
| **Variable** | **Study 1** | **Study 2** |
| Experimental unit | Farm (n = 8) | Calf (n = 52) |
| Colostrum/transition milk feedings | 3 to 12 | ≥3 |
| WM origin | Several farms | A single farm |
| Type of WM | Unpasteurized | Pasteurized |
| Sampling season | March, 2014 to May, 2015 | August to November, 2014 |
| Geographic location | Girona, Spain | Waseca, US |
| Fecal *E. coli* isolates per sample | 5 per animal | 3 per animal |

**Table 6.2.**A four-degradation colour scale to classify the prevalence of each antimicrobial resistance of fecal *E. coli* isolates from pre-weaned calvesfed with milk replacer (MR) or waste milk (WM) in each study farms according to theiroccurrence. The cross denotes if an antimicrobial of the class tested was used in the study farm.

| | **Study farms**[1] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | |
| **Class of antimicrobials tested** | **MR** | | | | **WM** | | | | **MR** | **pWM** |
| Non-cephalosporins β-lactam | x | x | x | x | x | x | x | x | | x |
| Cephalosporin | | x | x | x | x | x | x | x | | x |
| Polypeptide[2] | | | | x | | | | | | |
| Fluoroquinolone | x | x | x | x | x | x | x | x | x | x |
| Phenicol | | x | | | | | | | x | x |
| Lincosamide[3] | | | | x | | | x | | | x |
| Tetracycline | | | | | | | | | | |
| Sulfonamide[4] | | | | | | | | | x | x |
| Aminoglycoside | | x | x | x | | x | x | x | | |

**0.0 – 3.4 %**
**3.4 – 22.2 %**
**22.2 – 67.5 %**
**67.5 – 100 %**

[1]Farm 1 to 8 denote those farm enrolled in the study from chapter 3. In farms 1 to 4, calves were fed milk replacer (MR) without antimicrobials, and in farms 5 to 8, calves were fed waste milk (WM) from cows treated with antimicrobials. Farm 9 denotes the experimental farm enrolled in chapter 4. In this farm, calves were fed either, MR or pasteurized waste milk (pWM).

[2,3,4]Antimicrobial resistance to polypeptide agents was not tested in the study from chapter 4, whereas antimicrobial resistance to lincosamide and sulfonamide were not tested in the study from chapter 3.

## 6.1. Factors related to the occurrence of antimicrobial resistance

### *6.1.1. Feeding regime*

In chapter 3, antimicrobial resistance was evaluated from dairy calves raised at eight different dairy farms (four farms using MR and four farms using WM to feed calves), whereas, in chapter 4, it was assessed from calves raised at the same experimental farm using either, MR or pWM (Table 6.1). In both studies, feeding WM to calves fostered the presence of resistant fecal *E. coli* to antimicrobials commonly used in dairy cows, which is consistent with other studies reported in the literature (Aust et al., 2012; Duse et al., 2015). However, the selective pressure exerted by antimicrobial residues in WM was more evidenced in chapter 4 than in chapter 3, as it was suggested by the greater prevalence of resistant fecal *E. coli* to those antimicrobials that differed between feeding regimes (Table 3.5 and Table 4.3). Moreover, the different number of isolated colonies differed between studies (Table 6.1), and this also might affect the estimation of the resistance prevalence (Villarroel et al., 2006).

The difference in the occurrence of resistant *E. coli* in pre-weaned calves fed WM in chapter 4 and in Chapter 3 may be explained because WM in chapter 4 was originally from one single farm (Table 6.1). In contrast, animals fed WM in Chapter 3, consumed WM of their own farm, increasing the variability among the study farms in chapter 3. Studies screening drug residues in WM (Bilandžić et al., 2011; Pereira et al., 2014a) have indicated a great variability on antimicrobial concentrations depending on both, the type and administration route of antimicrobials used to treat dairy cows. In the present thesis, most of the antimicrobials used to treat dairy cows belonged to the beta-lactam (either cephalosporin or not) and aminoglycoside class of antimicrobials, which were often administrated through intra-mammary infusions in almost all study farms. The presence of residues of beta-lactam antimicrobials within all pWM loads used to feed calves was demonstrated in chapter 4. Nevertheless, studies (Carmeli et al., 1999; Tam et al., 2005) evaluating pharmacodynamics of various antimicrobials belonging to the same class, specifically beta-lactams, indicated different likelihood of antimicrobial resistance selection among them, probably due to their different bactericidal activity. Thus, the different subclasses of antimicrobials used among dairy farms of chapter 3 (Table 3.2) may also explain the different prevalence of antimicrobial resistance observed in fecal *E. coli* isolates between studies.

In addition to the type of milk offered to the calves, colostrum feedings, the amount of milk (either, WM or MR) and duration of pre-weaning period differed also among study farms (Table 3.1). As it was mentioned in the introduction, the fact that calves follow different milk feeding regimes could modified the composition of gut bacterial communities, which plays an important role in the regulation of the access of exogenous bacteria to the gastrointestinal tract by direct inhibition or by enhancing host immunity (Buffie and Pamer, 2013). Changes on the composition of calf-gut microbiota have also been related to the gradual adaptation of calves to consumption of solid feed (Oikonomou et al., 2013), and the greater the amount of MR offered the lower the solid food intake (Terré et al., 2007). Buffie and Parmer (2013) reported that modifications on the composition of commensal microbiota were related to changes in the immune-mediated colonization resistance of the gut against antimicrobial-resistant bacteria, whereas changes on the structure of gut bacterial communities between calves fed MR and those fed pWM was demonstrated herein (chapter 5). Thus, feeding to calves different types and amounts of milk throughout different periods of time among study farms could result in highly diverse structures of calf-gut microbiota affecting the ability of resistant strains to colonize the GIT of calves.

### *6.1.2. Farm factors*

Although the initial hypothesis was that feeding WM to calves would increase the prevalence of antimicrobial-resistant *E. coli* to antimicrobials commonly used in the study farms, high levels of resistance to other antimicrobials that were not used (tetracyclines) or only used in some study farms (phenicols) were also detected in both, MR and WM fed calves. As mentioned previously, several risk factors at farm level have been found to influence on antimicrobial resistance in fecal *E. coli* isolates from pre-weaned calves. But also, environmental pollution with low doses of antimicrobials and antimicrobial resistant determinants have been considered an important driving force for the occurrence of antimicrobial resistance in farm animals (Sayah et al., 2005b; Novais et al., 2013). In this context and considering that unexpected levels of antimicrobials resistance were reported in both studies from the present thesis, it seems reasonable to consider in more detail those management practices and environment traits that differed among the study farms and that might have an effect on the results. Berge et al. (2005) indicated that the intensity of individual therapeutic treatments

increases the shedding of antimicrobial resistant *E. coli* in dairy calves. Similarly, in the present thesis a great level of phenicol resistance was observed in one MR farm, and this was the only MR farm that used phenicol to treat calves for bovine respiratory diseases (Table 6.2). On the other hand, as it was mentioned in chapter 3 and 4, another possible explanation for the greatest occurrence of resistance to phenicols in WM farms that did not use phenicols to treat animals could be attributed to co-selection of resistance by antimicrobial residues present in milk. Although molecular basis of bacterial resistance to phenicol antimicrobials was not examined in the present thesis, cluster analysis were performed to elucidate possible genetic associations among the resistance phenotypes tested in both, chapter 3 and 4. This analysis allowed us to group all fecal *E. coli* according to their phenotypic resistance profile from the ones with less to those with more multi-resistance phenotypes. As it could be observed in table 3.6 and table 4.4, only the phenotypic profile with more multi-resistance contained the FFC-resistant *E. coli* and it was mainly observed in those calves fed either WM or pWM.

Although tetracycline antimicrobials were not used in any of the study farms, resistance to either, TET and DO was found to be predominant in fecal *E. coli* isolates in calves from both studies regardless of their feeding regimes. This high prevalence was suggested to be attributed to the farm environment in chapter 3 as high levels of this resistance were also observed in *E. coli* isolates from both, calving area and housing area of calves. Other possible explanation for these results was an efficient horizontal transfer of *tetA* and *tetB* resistance genes. These genes were predominant in phenotypic TET-resistant *E. coli* isolates of calves from chapter 4 (Table 4.6), and they have often been found associated to mobile genetic elements (Chopra and Roberts, 2001). Duse et al. (2015) described the influence of several risk factors at farm level such as herd size, housing system and predominant milking system on the occurrence of calf-fecal *E. coli* isolates resistant to TET. Among these factors, only the type of housing system differed among dairy farms participating in the present thesis. However, no significant differences in the occurrence of tetracycline resistant *E. coli* was observed between calves housed in individual pens and those housed in group in chapter 3.

As mentioned previously, the use of beta-lactam antimicrobials in dairy cows was a common practice in all study farms to treat or prevent mastitis as well as for drying dairy cows. However, an increase on the occurrence of fecal *E. coli* resistant to beta-lactam antimicrobials in WM fed calves compared with those fed MR was only observed in chapter 4. Calves in chapter 3 presented a low proportion of resistant *E.*

*coli* to beta-lactams in feces in both feeding regimes and no differences were observed between them. Focusing on the epidemiology of antimicrobial resistance, the occurrence of beta-lactam resistant bacteria has been found to vary greatly among bacterial species and different geographic regions (Winokur et al., 2001; Hendriksen et al., 2008). Thus, it is possible that the different countries where the studies were conducted (Table 6.1) contributed to these differences on the prevalence of beta-lactam resistance. Looking at the literature, it is also evidenced that the effects of antimicrobials on the selection of beta-lactam antimicrobial resistance depends not only on its potential for antimicrobial resistance selection in a bacterial population, but also on the occurrence of resistant determinants in a specific environment that can then be selected (Stürenburg and Mack, 2003). Interestingly, calves in chapter 4 presented high levels of beta-lactam resistant *E. coli* isolates carrying *bla* resistance genes (TEM and CMY-2) during the first days after birth before being fed with pWM or MR (Table 4.3 and Figure 4.1). In contrast, calves in chapter 3 had low levels of resistant *E. coli* to beta-lactam antimicrobials at birth (Figure 3.1). Thus, based on these findings, it seems reasonable that only in calves from chapter 4 the selection of beta-lactam resistant *E. coli* during the pre-weaning period were evidenced, and not from calves in chapter 3. Considering that in chapter 4, bacterial antimicrobial resistance was only evaluated from *E. coli* isolates in calves and not from their environment or dams, elucidates the origin of beta-lactam resistance in calves few days after birth was complicated. However, in a recent study by Cameron-Veas et al. (2016), the presence of cephalosporin resistant *E. coli* in sows was associated with greater probability of shedding resistant *E. coli* to these antimicrobials in 7 day-old piglets that were not treated with antimicrobials. Moreover, as it was suggested from chapter 3 environment contamination with resistant strains could also contribute to the occurrence of beta-lactam resistant *E. coli* in dairy calves.

# Chapter 7

# CONCLUSIONS

The results obtained in this thesis allow concluding that:

1. Feeding waste milk to dairy calves increasesthe presence of resistant *E. coli* in the gastrointestinal tract and resistant *P. multocida* in the respiratory tractof pre-weaned calves, although this effect is more evident in the gastrointestinal tract.

2. The occurrence of resistant fecal *E. coli* in calves is highly influenced by animal age, and its prevalence decreases in weaned calves fed eitherwaste milkor pasteurized waste milkonce the antimicrobial pressure from milk disappeared.

3. Similar phenotypic patterns are observed in *E. coli* isolates from both environment and calvesof the same farm.

4. Feeding waste milk to calves not only selects for resistant fecal *E. coli* to antimicrobials present in the waste milk (and commonly used to treat dairy cows), but also to others not currently used in dairy farms or administrated solely to treat infectious diseases in calves.

5. The presence of multidrug-resistance phenotypes in calves fecal *E. coli*increases when animals are fed with waste milk.

6. Feeding calves waste milk with beta-lactam antimicrobials residues increases the presence of *E. coli* carrying the resistance genes (*bla*$_{CMY-2}$ ,*aadA*, *tetC* and *sul1)* in the dairy calves feces.

7. The presence of *E. coli* carrying *bla*$_{CMY-2}$, *tetC* and *sul1* resistance genes decreases once calves are weaned.

8. Feeding pasteurized waste milk with beta-lactam antimicrobial residues to calves instead of MR with non-antimicrobial residues and similar nutrient composition to WM, induces changes at the taxonomic level in the relative abundance of *Firmicutes* and *Bacteroidetes* phyla in the gut microbiota.

9. Feeding dairy calves pasteurized waste milk with beta-lactam antimicrobial residues instead of milk replacer without antimicrobial residues does not alter alpha-diversity indexes of the gut and nasal microbiota.

10. Feeding pasteurized waste milk with beta-lactam antimicrobial residues to calves instead of MR with non-antimicrobial residues and similar nutrient composition to WM, affects the phylogenetic composition of bacterial communities of fecal and nasal microbiota of pre-weaned calves, being more evident these differences in the respiratory tract.

# Chapter 8

# LITERATURE CITED

## 8. LITERATURE CITED

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