UNIVERSITAT ROVIRA i VIRGILI

ACTION OF NATURAL BIOACTIVE COMPOUNDS ON THE ENTEROENDOCRINE SYSTEM

Àngela Casanova Martí

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Action of natural bioactive compounds on the enteroendocrine system

Doctoral Thesis

Thesis supervised by Dr. Monsterrat Pinent Armengol

Biochemistry & Biotechnology Department

MoBioFood Research Group

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FAIG CONSTAR que aquest treball, titulat "Action of natural bioactive compounds on the enteroendocrine system", que presenta Àngela Casanova Martí per a l'obtenció del títol de Doctora, ha estat realitzat sota la meva direcció al Departament de Bioquímica i Biotecnologia d'aquesta universitat i que compleix els requisits per poder optar a la menció internacional de doctorat.

HAGO CONSTAR que el presente trabajo, titulado "Action of natural bioactive compounds on the enteroendocrine system", que presenta Àngela Casanova Martí para la obtención del título de Doctora, ha sido realizado bajo mi dirección en el Departamento de Bioquímica y Biotecnología de esta universidad y que cumple los requisitos para poder optar a la mención internacional de doctorado.

I STATE that the present study, entitled ""Action of natural bioactive compounds on the enteroendocrine system", presented by Àngela Casanova Martí for the award of the degree of Doctor, has been carried out under my supervision at the Department of Biochemistry and Biotechnology of this university and that this thesis is eligible to apply for the international doctoral mention.

Tarragona, 26-6-2017

El/s director/s de la tesi doctoral
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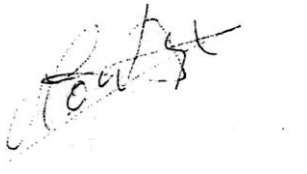

Montserrat Pinent Armengol

Als de casa,

The important thing is to never stop questioning.

Never lose a holy curiosity

Albert Einstein

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SUMMARY

SUMMARY

In the last decade, there has been an increasing prevalence of obesity and metabolic-associated diseases. In view of this fact, finding preventive therapies, as well as treatments for these diseases is of great interest for public health. Gut hormones secreted from enteroendocrine cells (EECs) play a key role in the regulation of food intake and glucose homeostasis. In this context, the research of this thesis has focused on the role of natural bioactive compounds on the enteroendocrine system.

Our research group reported in previous studies that grape seed proanthocyanidin extract (GSPE) increased GLP-1 plasma levels in rats. In this thesis, we elucidated that such increase might be in part explained by the direct action of GSPE on enteroendocrine cells. Moreover, we demonstrate that GSPE also modulates the secretion of the main gut hormones by directly acting on EECs, inducing an increase of GIP and PYY release, while reducing CCK release.

The results obtained in this thesis using organoids culture demonstrated that GSPE up-regulate the main markers of L-cell and modulate transcription factors involved in L-cell differentiation, and thereby point out that the promotion of L-cell differentiation is a mechanism by which GSPE act in prolonged treatments. Moreover, our findings in mid-term treatments revealed that gut microbiota composition is modulated by GSPE and such microbial composition profile correlates with host metabolic parameters, and remarkably with increased active GLP-1 plasma levels.

Furthermore, we found a new source of natural bioactive compounds from chicken feet protein, chicken feet hydrolyzate, and demonstrated that it acts as antihyperglycemic agent in disrupted-glucose homeostasis animals due to the capacity of inhibiting DPP-IV activity and enhancing endogenous GLP-1 release .

In conclusion, the findings obtained in this thesis show that natural bioactive compounds act through different mechanisms on the enteroendocrine system, and thereby could be good therapeutic agents to treat obesity and glucose homeostasis disruption.

RESUM

En els últims anys ha augmentat la presència de l'obesitat i de malalties associades a aquesta. En vista d'aquest fet, la cerca de teràpies preventives així com tractaments per aquestes malalties esdevé de gran interès per la salut pública. Les hormones intestinals secretades per les cèl·lules enteroendocrines juguen un paper clau en la regulació de la ingesta i la homeòstasis de la glucosa. En aquest marc, la recerca d'aquesta tesi doctoral s'ha centrat en l'acció dels compostos naturals bioactius sobre el sistema enteroendocrí.

En estudis previs, el nostre grup de recerca va observar que un extracte de proantocianidines del pinyol del raïm (GSPE) augmentava els nivells plasmàtics de GLP-1 en rates. En aquesta tesi doctoral, s'ha esbrinat que aquest augment podria ser en part explicat per l'acció directa del GSPE sobre les cèl·lules enteroendocrines. D'altra banda, s'ha demostrat que l'acció directa del GSPE també modula la secreció de les principals hormones intestinals, induint un augment de la secreció de GIP i PYY, així com una reducció de la secreció de CCK.

Els resultats obtinguts en el cultiu d'organoids han demostrat que el GSPE incrementa els principals marcadors de les cèl·lules L i modula els factors de transcripció involucrats en la diferenciació d'aquestes cèl·lules. Conseqüentment, aquests resultats senyalen que promoure la diferenciació de les cèl·lules L és un mecanisme d'acció del GSPE en tractament prolongats. D'altra banda, la recerca realitzada en tractaments subcrònics ha revelat que el GSPE modifica la composició de la biota intestinal i que el perfil microbià correlaciona amb els paràmetres metabòlics de l'hoste, del qual destaca la correlació amb els nivells plasmàtics incrementats de la hormona GLP-1 activa.

Altrament, s'ha trobat una nova font de compostos naturals bioactius procedents de la pota de pollastre, l'hidrolitzat de pota de pollastre, la qual s'ha demostrat que actua com un agent antihiperglicèmic en rates que presenten una homeòstasis de la glucosa alterada, degut a la capacitat d'inhibir l'activitat de DPP-IV i d'augmentar la secreció endògena de GLP-1.

En conclusió, els resultats obtinguts en aquesta tesi doctoral mostren que els compostos naturals bioactius actuen en el sistema enteroendocrí per mitjà de diferents mecanismes, i per tant aquests podrien ser uns agents terapèutics adequats per al tractament de la obesitat i la homeòstasis de glucosa alterada.

ABBREVIATIONS

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2-OG 2-oleoylglycerol

Arx Aristaless related homeobox factor

bHLH Basic helix-loop-helix

BMP Bone morphogenetic protein

BP Bioactive peptides

CaSR Calcium-sensing receptor

CBC Crypt base columnar

CCK Cholecystokinin

ChgA Chomogranin A

CNS Central neuronal system

DPP-IV Dipeptidyl peptidase IV enzyme

EEC Enteroencodrine cells

EGC (-)-epigallocatechin

EGCg (-)-epigallocatechin gallate

EGF Epidermal growth factor

Elf3 E47-like factor 3

FFA Fatty acid

FFAR Fatty acid receptor

Foxa ½ Forkhead box A2, transcript variant 1

Gcg Proglucagon

GI Gastrointestinal

GIP Gastric inhibitory polypeptide

GLP-1 Glucagon-like peptide-1

GLP-1R Glucagon-like peptide-1receptor

GPCR G-protein-coupled receptors

GSPE Grape seed proanthocyanidin extract

HES1 hairy/enhancer of split genes

Insm-1 Insulinoma-associated 1, IA-1

Klf4 Kruppel-like factor 4

LCFA Long-chain fatty acids

LPS Lipopolysaccharides

Math1 Mouse atonal homologue 1 (transcription factor)

MCFA Medium- chain fatty acids

Muc2 Mucin 2

NCID Notch intracellular domain

NeuroD1 Neurogenic differentiation 1

Ngn3 Neurogenin 3

NPY Neuropeptide Y

OGTT Oral glucose tolerance test

Pax4 Paired box 4

Pax6 Paired box 6

Pdx1 Pancreas/duodenum homeobox gene-1

PP Pancreatic polypeptide

Ptk6 Protein tyrosine kinase 6

PYY Peptide YY

SCFA Short-chain fatty acid

SLGT-1 Sodium-dependent glucose transporter 1

T2DM Type 2 diabetes mellitus

TA Transit-amplifying

Tgf-βRII Growth factor β type II receptor

I. INTRODUCTION

1. Gastrointestinal tract

The gastrointestinal (GI) tract is an open-ended tube that extends through the body from the mouth to the anus. The lumen, the inside space of this tubular structure, can be defined as a tunnel environment through the body, and the wall of the GI tract as the physical interface between the external environment and the circulation. The main functions of the GI tract are to digest and absorb nutrients and other substances, as well as the propulsion of material through the digestive tract [1].

Food enters the mouth, which is the entrance of the GI tract, and continues through the pharynx and oesophagus to the stomach and intestines and finally into the rectum and anus. The small intestine, the relevant site for absorption, is divided into unequally sized sections: the duodenum, the jejunum and the ileum. The large intestine has two main sections: the cecum (whose size and functions differ between species) and the colon [2].

The intestinal lumen is lined with a single layer of specialized epithelial cells that carry out the main functions of the GI tract, i.e. digestion and absorption of nutrients. It also forms a barrier against luminal pathogens. Moreover, the GI-tract cooperates in controlling the metabolism through hormones secreted from enteroendocrine cells (EEC), which are the largest endocrine organ of the body.

The intestinal surface has an uneven architecture composed of villus and crypt structures. Proliferative cells, stem cells and transit-amplifying (TA) cells reside in the crypts and are triggered to differentiate into one of the four main specialized cells: entrocytes, paneth cells, goblet cells, and EEC [3]. A complex network of molecular signals and pathways regulate the differentiating process of the gut cells in the adult intestine, which will be described in the following sections.

1.1. Differentiation in the intestinal epithelium

The adult intestinal epithelium undergoes rapid renewal to maintain optimal functioning. Regeneration relies on the crypt base, where adult stem cells continually divide to produce highly proliferative progenitors known as TA cells. The nascent TA cells migrate upwards from the depths of the crypt onto the surface of the villi and divide 4-5 times before they commit to the absorptive and secretory cell lineages. Cell proliferation terminates when differentiated cells leave the crypt, where they are exposed to the gut contents and finally sloughed from the villus trips. This renewal cycle operates continually and the cells take 2-7 days to make the journey [4].

1.1.1. Proliferating intestinal cells: Stem cells

Intestinal epithelial homeostasis is based on the balance between self-renewal and differentiation, which is maintained by a complex interplay of multiple regulatory

mechanisms. The intestinal stem cells are cycling, long-lived and multipotent cells. They play an important role in the self-renewal process. Over the past four decades, two models of intestinal stem cell identity have been proposed. The 'stem cell zone model', by Leblond, Cheng and Bjerknes [5], suggests that the slender crypt base columnar (CBC) cells at the crypt bottom and intercalated between Paneth cells are the resident stem cells. The Wnt target gene Lgr5 has been identified as an excellent marker for CBC cells. The Lgr5-CBC cells generally undergo symmetrical division, after which individual daughter cells stochastically adopt a stem cell or TA cell fate, depending on the available niche space [6, 7]. The second model describes an alternative stem cell population that has been postulated to reside at position +4 relative to the crypt bottom, above the Paneth cells [8]. These +4 cells are considered to be relatively quiescent and resistant to acute injury [9]. Therefore, this stem cell pool does indeed play a role in intestinal homeostasis, but can restore the LGR5+ CBC stem cell compartment following injury [10].

The **Wnt pathway** is the first primary force implicated in controlling the maintenance of the crypt cell population in the proliferative state. The central player in the canonical Wnt cascade is β-catenin, which is regulated by a degradation complex consisting of caseine kinase-1 and GSK3-β, both residing in the adenomatous polyposis coli (APC) tumour suppressor. When Wnt ligands are not engaged with their Frizzled and low-density lipoprotein receptor-related protein (LRP) receptor, β-catenin is targeted for ubiquitination and proteasomal degradation. In contrast, the Wnt-Fz-LRP complex transduces a signal into the cell, resulting in unphosphorylated β-catenin translocation into the nucleus and subsequent activation of the T-cell factor/lymphocyte enhancer factor (TCF/LEF) family (**Fig.1**), thus activating a genetic program that supports stemness [11, 12].

Other molecular pathways that play important roles during embryogenesis are involved in the regulation of intestinal epithelium homeostasis in adult organisms. These include the **hedgehog pathway**, which is required for the formation of villi [13] and the repair phase after the injury[14]. **Bone morphogenetic protein (BMP) signalling** mediates the action of the hedgehog pathway, blocking ectopic crypt formation, and the expression of BMP antagonist noggin in the neighbourhood crypts, enabling a crypt-permissive environment[15]. BMP receptors by BMP, which is expressed in the intravillus and intercrypt mesenchymal cells, lead to complexes between Smad1/5/8 and Smad 4 blocking stemness genes in the nucleos (**Fig.1**). Moreover, the BMP pathway antagonizes the Wnt pathway within the differentiated compartment, thereby positioning TA cells in the crypt pockets [16]. **Epidermal growth factor (EGF) signals** induce mitogenic effects in stem cells and TA cells. When EGF is bound to their receptor, the Ras/Raf/Mek/Erk signalling axis is active in crypt epithelium; whereas the inhibition of Mek ablates intestinal stem cells [17] (**Fig.1**). The main protein of the **Hippo pathway** is the Yes-associated protein (YAP), which is found in the crypts and has Wnt antagonizing effects, thereby contributing to the prevention of proliferation. Although under normal conditions the YAP does not

contribute to proliferation, it regulates tissue regeneration caused by injury and tumorigenesis [18, 19]. The **Notch pathway** is also needed to maintain the crypt compartment in its undifferentiated and proliferative state, and is involved in the intestinal cell fate decision. This pathway will be discussed further in the following section.

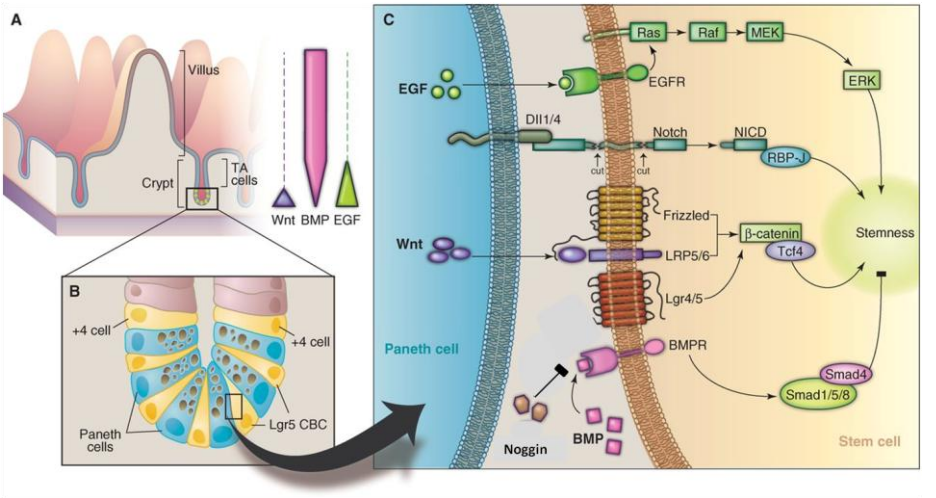

Figure 1. Histological location and biological interaction of intestinal stem cells and their niche, adapted from [20].

1.1.2. Cell lineage specification: Secretory Lineages versus Absorptive Lineage

As mentioned above, TA cells terminally differentiate into the four main epithelial cell linages of the GI tract; that is, the absorptive enterocytes and three other cell types that belong to the secretory lineages: the goblet cells, the Paneth cells, and the enteroendocrine cells. Apart from these four main cell types, there are also some lesser-known cell types, such as M-cells and Tuft cells [21, 22].

The **Notch pathway** plays a central function in the fate decision and differentiation process of the intestinal epithelial cells. The key players of the Notch pathway in mammals are the Notch receptor (Notch 1 though 4) and its ligands of the Delta and Serrate/Jagged subfamilies. These are transmembrane proteins that mediate communication between cells that are in contact (**Fig.1**). Interaction of one of the four Notch receptors with any of five Notch ligands results in activation of proteolytic cleavage and the Notch intracellular domain (NCID) is released from the cell surface [23]. The NICD translocates into the nucleous and binds to the transcription factor RBP-Jk to activate target gene transcriptors [24].

The Wnt and Notch signals cooperate to keep the crypt in the stemness state. Moreover, the Notch pathway in the transit-amplifying compartment controls absorptive versus secretory fate decisions in the intestinal epithelium. Increased Notch

signalling activity through constitutive expression of NICD in the gut epithelium results in a severe reduction of all three secretory cell types [25]. In contrast, inhibition of Notch signalling through pharmacological gamma secretase inhibitors has the opposite effect on intestinal differentiation and results in complete conversion of all epithelial cells into goblet cells [26]. The cells that become secretory are those that escape Notch activation and are also the ones that express Delta proteins, enabling them to activate Notch signalling in their neighbours. Therefore, the Notch pathway controls intestinal fate decisions to mediate lateral inhibition between adjacent cells, preventing neighbouring cells from adopting the same fate [27].

The active Notch pathway results in transcription of the hairy/enhancer of split genes (HES) family, which encodes transcriptional repressors. Hes1 represses the transcription of the basic helix-loop-helix (bHLH) transcription factor Math1 (mouse atonal homologue 1)[28] (**Fig.2**). Lineage tracing studies have shown that all the secretory cell lineages are derived from Math1-expressing precursor cells [29]. While Math1 is required for all three secretory cell lineages, downstream lineage-specific transcription factors, such as Sox9 for Paneth cells [30], Klf4 (Kruppel-like factor 4) for goblet cells [31] and Ngn3/NeuroD1 for EEC [32] (**Fig.2**) are needed for the correct differentiation of these secretory cell lineages.

1.1.2.1. Enterocytes:

The most populous cell on the intestinal villus is the absorptive enterocyte, which represents more than 80 % of all intestinal epithelial cells. Absorptive cells are highly polarized cells joined together by a tight junction. Adequate protein sorting and addressing are necessary for establishing and maintaining cell polarity to maintain the integrity of the epithelial barrier [33]. Their apical surfaces have characteristic microvilli that comprise an elaborate brush border responsible for absorbing and transporting the nutrients across the epithelium. Enterocytes express a high number of genes related to the carbohydrate metabolism (i.e. Phosphofructokinase, Aldolase, Fructose-Bisphosphate B, Glyoxalase), and fatty acid metabolism (i.e. Apolipoprotein A1, Apolipoprotein A4, fatty acid Coenzyme A ligase 5), as well as the dipeptidyl peptidase IV enzyme (DPP-IV), which plays an important role in enterohormone regulation, especially GLP-1[34].

Although the Notch pathway was thought to be needed for absorptive cell specification, recent studies have reported that it is not required, while, in contrast, the repression of Math1 expression and thus secretory cell fate is required [35, 36]. Therefore, deletion of Hes1 results in a decreased number of enterocytes and an increased number of all secretory lineages [28]. Downstream of Hes1, diverse transcription factors are involved in the enterocyte differentiation process, such as Elf3 (E47-like factor 3), Tgf-βRII (growth factor β type II receptor), and Ptk6 (protein tyrosine kinase 6) (**Fig. 2**).

1.1.2.2. Paneth cells:

Paneth cells have a function in innate immunity and contain extensive apical secretory granules that are filled with a variety of antimicrobial proteins, including lysozymes and cryptdins [37]. They are renewed every 3-6 weeks, so they are the exception to the rapid self-renewal in the epithelium intestine [38]. Paneth cells represent the only differentiated cells that escape the upward migration, so they localize to the base of the crypt, where canonical Wnt signalling is active. The Fizzled-5 receptor plays a role in the transduction of Wnt signals in the Paneth cells, which interpret these signals for their specification, differentiation and maturation. The conditional deletion of this receptor results in mispositioned Paneth cells scattered along the villus, due to the lack of expression of the Tcf4 target gene EphB3. Thus, this receptor allows the correct positioning of epithelial cells to be achieved in a Wnt gradient along the crypt-villus axis [39]. Moreover, studies suggest that FGFR-3 (fibroblast growth factor receptor-3) is essential for Paneth cell emergence and lineage allocation [40]. The Wnt transcriptional factor target Sox9, which is expressed in all cells at the crypt bottom, was identified as an essential factor for Paneth cell differentiation [30] (**Fig.2**).

Moreover, Paneth cells have a determinant role in the stem cell niche homeostasis. They are the Wnt source that induces the formation of a new stem cell as well as Paneth cells, and there is a Wnt-driven positive-feedback loop that could lead to ever-expanding crypts [41].

1.1.2.3. Goblet cells:

The goblet cell population is the most abundant secretory lineage among all types of epithelial cells and increases between the duodenum (~4%) and colon (~16%). These cells secrete protective mucus and trefoil proteins that are needed for the effective expulsion of gut contents, and protect against shear stress and chemical damage [42].

As discussed above, Hes1 is expressed in proliferative crypt cells, while Math1 is only expressed in secretory cells. Inhibition of Notch signalling results in a lack of Hes1 expression and the consequent induction of Math1 expression, leading to the conversion of all proliferative cells into goblet cells [26]. Goblet cell differentiation also depends on a zinger-finger transcription factor Kfl4, whose deletion results in the loss of goblet cells [31] (**Fig.2**). Likewise, the inactivation of the goblet cell marker, gastrointestinal mucin 2 (Muc2), leads to the development of adenomas in the small intestine. However, goblet cells of Muc2 -/- mice still express the intestinal trefoil factor, which suggests that some aspects of the differentiation program persist [43].

1.1.2.4. Enteroendocrine cells:

EECs comprise the largest population of hormone-producing cells in the body [44]. They represent approximately 1% of the cells lining the small and large intestinal lumen. Unlike many endocrine glands, gastrointestinal endocrine cells are scattered as individual cells throughout the mucosa and surrounded by non-endocrine cells [5].

Enteroendocrine cells regulate gut functioning through specific hormone secretion. There are more than 15 subtypes of enteroendocrine cells defined according to their morphology and the hormones that they produce.

Three proendocrine bHLH transcription factors (Math1, Neurogenin 3 (Ngn3) and Neurogenic differentiation 1 (NeuroD1)) all target Notch signalling. They function in cascade, which means one factor activates later factors to control both the initial specification of the enteroendocrine cells and their final differentiation. Ngn3 over-expression in the developing intestinal epithelium results in increased numbers of enteroendocrine cells at the expense of goblet cells [45], which demonstrates the importance of Ngn3. The requirement of Ngn3 differs between intestine and stomach. While all intestinal enteroendocrine cells are Ngn3-dependent, its role in the stomach is more limited, where deletion of Ngn3 expression reduces the number of glucagon-, somatostatin- and gastric- secreting cells, but serotonin-, histamine-, and ghrelin-expressing cells are still present [32].

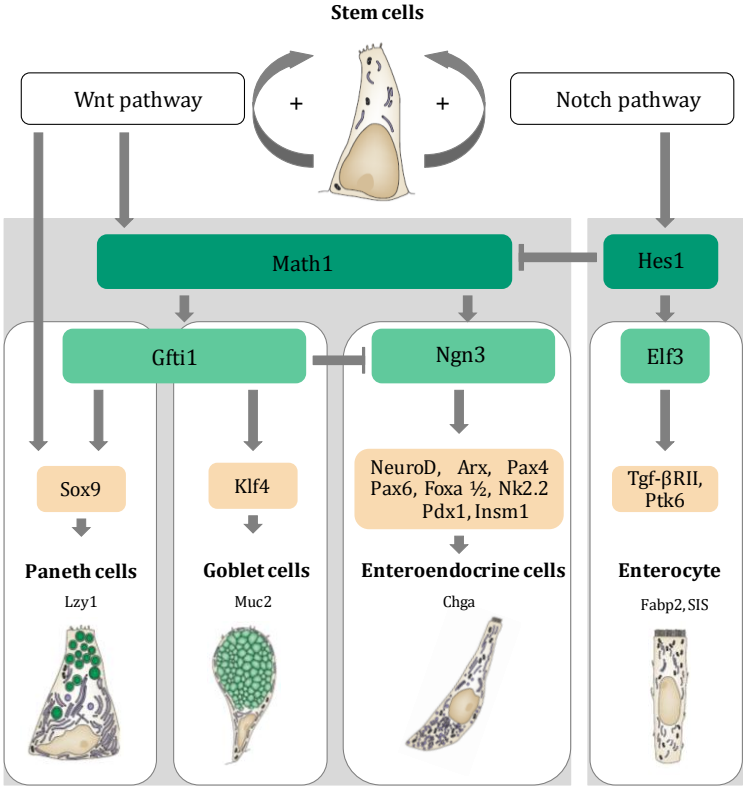

Figure 2. Schematic overview of the genetic hierarchy of epithelial cell lineage commitment in the intestine. Adapted from [42].

A set of transcription factors downstream of Ngn3 have also been shown to play a critical role in the specification of the various subsets of enteroendocrine cells, similarly to pancreatic differentiation. NeuroD1, which acts directly downstream of Ngn3, has been shown to control terminal differentiation of the secretin and

cholecystokinin (CCK) producing subset of enteroendocrine cells [46]. Other factors that have been implicated in enteroendocrine cell fate specification include the zinc-finger transcription factors and homodominan transcription factors, such as Insm-1, Pdx1, Nkx2.2, ARX, Foxa ½, Pax4 and Pax6 [47–51]. Among these, it has been reported that gastric inhibitory polypeptide (GIP) secreting cells are Pax6-dependent [47], while other studies indicate that Pax6 acts together with Foxa1/2 to regulate the transcription of the preglucagon gene [50]. Besides the deletion of Pax4 expression in mice, an increase in glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) transcripts has also been shown, suggesting an increase in the number of L cells. However, deletion of Arx expression resulted in an almost complete loss of GLP1-, GIP- and CCK-expressing cells [51]. Mechanisms for generating the diversity and regional specificity of enteroendocrine cells rely on a complex network of transcription factors and further studies are needed to elucidate the pathways involved in enteroendocrine cell specification.

1.2. Enterohormones: synthesis by EE cells, secretion and function

As mentioned above, the GI tract is the largest endocrine organ of the body. At least 15 types of EECs have been described, which secrete more than 20 different regulatory hormones that are involved in the regulation of a number of physiological processes and exert their effect on tissues including exocrine glands, smooth muscle and the peripheral nervous system [52].

It was generally accepted that EECs could be classified into discrete classes of cells with specific secretory profiles, mainly secreting peptides derived from a single peptide precursor. In this traditional classification, EECs were located in certain segments of the GI tract. Examples of previously described cell family groups include CCK-secreting I-cells located in the duodenum, GLP-1- and PYY-secreting L-cells located in the ileum and colon, among others [53]. However, recent studies have revealed that there are a wide range of EEC types that are capable of expressing a huge combination of different hormone precursors [54]. Based on this knowledge, Engelstoft et al. suggest organising the enteroendocrine system in pan-GI tract enteroendocrine cell types, gastro-selective cell types and a joint multi-capable intestinal-selective cell lineage, which in certain segments of the intestine is divided into specialized cell types that express a range of hormones as previously described in the classical classification [55](**Fig.3**).

The individual hormone gene could be expressed in multiple forms because of tandem genes encoding different hormonal peptides, alternative splicing or differential processing [44, 56]. Some of the gastrointestinal hormones are synthesized from precursor proteins called prehormones, which undergo post-translational processes to produce mature hormones prior to secretion. The post-translational modifications consist in proteolytic cleavage by endopeptidases and exopeptidases and are followed

by the amidation process. The variety of these hormones is derived from a single prehormone that may exhibit different activities like gastrin peptides do (gastrin-6, gastrin-14, gastrin_17, gastrin-34, and gastrin-71) [57].

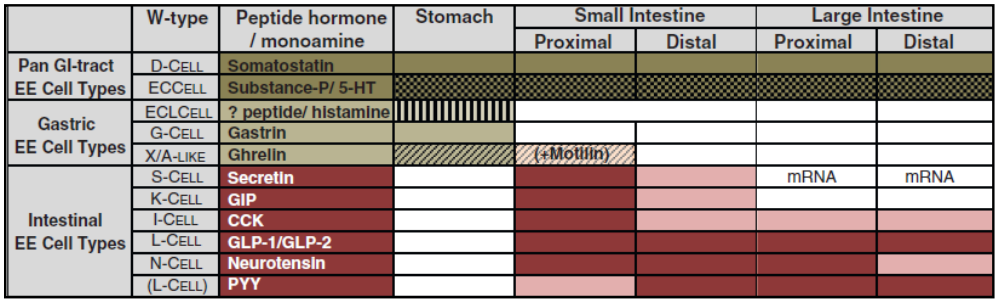

Figure 3. EEC type classification: for each of the cell types the main secretory product and the original Wiesbaden nomenclature (W-type) are indicated. In intestinal EE cell types, the six defined cell types could be considered to be one cell type specialized in various segments or at least one cell lineage, which is indicated by the common maroon colour and the lighter maroon colour indicates that the peptides are less expressed in these segments. The gastric ghrelin cells are significantly different from the ghrelin-motilin cells of the small intestine. Extracted from [55].

EECs are capable of responding to luminal content because their apical side has chemosensing machinery, including taste receptors (TASR), G protein-coupled receptors (GPCR), plus specific transporters and channels, among others. Their secretory products are stored in characterized secretory vesicles, which are the large dense-core type or the smaller synaptic-like type, prior to their secretion through the basolateral membrane by exocytosis [56, 58]. This model is especially appropriate for the open type EECs that reach the luminal surface, whereas the closed type EECs, which do not reach the lumen, can be indirectly regulated by content through neural and humoral mechanisms [59].

When luminal content move through the GI tract, specific macronutrients stimulate the chemosensors machinery. That stimulation leads the modulation of gut hormones release. These hormones, which mainly are GLP-1, CCK, PYY, GIP, and ghrelin, influence the functioning of digestive tract, but also modulate insulin secretion of liver, energy storage of adipose tissue and influence neuronal signaling in appetite centers in the brain to mediate the regulation of food intake, such as the termination of hunger and the induction of satiety. The gut hormones exert their effect via vagal nerve or endocrine signalling, through the interaction of specific receptor expressed in different tissues of the body. In the next section, we focus on describing main gut hormones and their actions.

1.2.1. Glucagon-like peptide 1

Proglucagon is expressed in L-cells of the intestine, pancreatic α-cells and various areas of the central neuronal system (CNS), mainly in the brainstem and hypothalamus. Although a single gene encodes this precursor in all tissue types,

different sets of hormones are synthesized in each tissue due to tissue-specific posttranslational processing. This posttranslational processing produces a number of biologically active peptides, including glucagon, glicentin-related polypeptide (GRPP), intervening peptide-1 (IP-1), GLP-1, and GLP-2, among others. In L-cells, GLP-1 is produced as an inactive 37-amino acid peptide ($GLP\text{-}1_{1\text{-}37}$), which is further cleaved to form active $GLP\text{-}1_{7\text{-}37}$. Both forms can be amidated at the C-terminal residue, resulting in $GLP\text{-}1_{1\text{-}37amide}$ and $GLP\text{-}1_{7\text{-}37amide}$. Although GLP-1 exists in several forms, the most secreted form is $GLP\text{-}1_{7\text{-}37amide}$. Both $GLP\text{-}1_{1\text{-}37amide}$ and $GLP\text{-}1_{7\text{-}37amide}$ are truncated by DPPIV, forming inactive forms [60–62].

DPP-IV is a ubiquitous aminodipeptidase that exists essentially as a membrane-anchored cell-surface enzyme [63]. Its expression is widely distributed along the body tissues, such as kidneys, the GI tract, liver, and pancreas, among others. But DPP-IV is also expressed on endothelial and epithelial cells along the vascular bed. Its soluble form is found in plasma and therefore it is in close proximity with hormones circulating in the blood [64, 65]. The main activity of DPP-IV is to remove N-terminal dipeptides from polypeptides [66], which preferably have a proline or alanine in their second position from the N-terminal. The main DPP-IV substrates include GLP-1, and the other incretin hormone GIP, which are peptides with N-terminal Tyr–Ala and His–Ala respectively [67]. The intact GLP-1 is rapidly hydrolysed by DPP-IV into a shorter and inactive form. Once it reaches the plasma, with a half-life of 1-2 minutes [66]. Only 25% of the active GLP-1 reaches the portal circulation and subsequently the liver, where a further 40-50% is digested by DPP-IV present on hepatocytes. This means that only 15% enters the systemic circulation and may reach other tissues, such as the pancreas or the brain [60]. Therefore, DPP-IV is responsible for inactivating more than 80 % of the secreted GLP-1 [66].

Nutrient ingestion is the primary physiological stimulus for inducing GLP-1 secretion by L-cells. GLP-1 secretion occurs in a biphasic pattern, which consists in a rapid release in 15-30 min after a meal, followed by a second minor peak that occurs in 60-120 min. Both glucose and fat have been reported to be a strong GLP-1-secretagoge after their ingestion [68], after direct administration into intestine [69, 70][69, 70] or into perfused ileal segments [71]. In the murine model, glucose-stimulated GLP-1 release is blocked using sodium-dependent glucose transporter 1 (SLGT-1) knockout mice and SLGT-1 inhibitor [72, 73] , suggesting that glucose metabolism involves glucose transport via SGLT-1 to induce GLP-1 secretion. It has also been proposed that the sweet taste receptors (T1R2, T1R3) are involved in the glucose-sensing mechanism, but there is still controversy about this [74, 75]. On the other hand, G-protein-coupled receptors (GPCRs) have been reported to be activated by dietary fat to stimulate GLP-1 release, including GPR40 and GPR120 by medium- chain fatty acids (MCFAs), long-chain fatty acids (LCFAs) and long-chain unsaturated FAs; and GPR41 and GPR43 by short-chain fatty acids (SCFAs) (reviewed in [76, 77]). Finally, proteins are the macronutrient commonly considered the least effective for stimulating GLP-1 secretion (this will be discussed further in Section 2 (2.2.2.2)).

Although EECs in duodenum can release GLP-1, several authors have suggested that the initial rapid rise in the GLP-1 secretion pattern can also be mediated indirectly by neuro/endocrine pathways (reviewed in [78, 79]). A proximal-distal loop has been described to be GLP-1-release signalling. It has been reported in rats that after a duodenal fat treatment, GIP plasma release is followed by GLP-1 plasma release, and this effect is mediated by the vagus nerve [69, 70]. Some neurotransmitters, including acetylcholine, glycine, GABA and epinephrine, as well as the adrenergic activation of L-cells have been described to be involved in GLP-1 secretion [80–82].

The biological activity of GLP-1 is mediated by its interaction with GLP-1 receptors (GLP-1R), which are expressed in several tissues, including pancreatic islets, kidneys, and lungs, among others and in different sections of the peripheral and central nervous system [78, 83, 84]. GLP-1 together with GIP is responsible for the incretin effect, so it binds to GLP-1R in β-cells in the pancreas leading to an increase in intra-cellular calcium and a subsequent insulin secretion in response to glucose [85]. Moreover, it has been shown that GLP-1 enhances markers of proliferation and differentiation, and decreases markers of apoptosis in the pancreas of Zucker diabetic rats [86, 87]. GLP-1 also improves the glycemic profile by inhibiting glucagon secretion and may also improve glucosal disposal in peripheral tissues [88]. Moreover, like PYY, exogenous GLP-1 administration decreases gastric emptying as well as the speed of intestinal transit (reviewed in [89]). On the other hand, the effects of anoretic GLP-1 on food intake could be mediated via the arcuate nucleus, where proopiomelanocortin (POMC) neurons express the GLP-1R. However, there is a physiological debate about whether GLP-1 can reach central GLP-1R in the hypothalamus through systemic regulation due to their short half-life [90]. Interestingly, Rüttimann et al. [91] reported that intraperitoneal administration of GLP-1 reduces the sensitivity to the anoretic effects of GLP-1 in rats that undergo subdiaphragmatic vagal deafferation, while these effects were not affected when GLP-1 was administered in the vena cava and hepatic portal vein. This suggests that the role of the vagus system in intraperitoneally GLP-1, which acts similarly to endogenous GLP-1 secreted from L-cells, whereas exogenous circulating GLP-1 could bind directly at central receptors.

1.2.2. Cholecystokinin

The I-like cells in mammals and humans are the main CCK-secreting cells, and are located in the upper intestinal tract. Multiple molecular forms have been identified as CCK58 (the most abundant molecular form), CCK33, CCK22, and CCK8 (the most potent molecular form), all of which share the same carboxyl domain and are derived from a 115 amino acid prepropeptide. This carboxyl domain, which has an amidated carboxyl-terminal, is the biologically active portion of the hormone and full potency is not achieved unless the tyrosine residue at position 7 from the carboxyl terminus is sulphated. The CCK length depends on the CCK producing-cell synthesized, EEC of the small intestine and neurons in the GI tract and central nervous system [92, 93].

CCK is secreted from the small intestine in response to the presence of luminal nutrients. Several protein hydrolystates and individual amino acids (phenylalanine, leucine and glutamic acid) have been reported as stimulators of CCK release [94, 95], as well as a LCFA, which also promotes CCK release through LCFA receptors [96, 97]. CCK has different physiological GI functions, including the stimulation of pancreatic secretion, gallbladder contraction, intestinal motility, and inhibition of gastric emptying [98]. These CCK functions are mediated by endocrine, paracrine and/or neurocrine modes of action [99]. Another function attributed to the CCK is the regulation of food intake, which could be mediated by the cholecystokinin 1 (CCK1) receptor on the vagal nerve. It has been described that Otsuka Long-Evans Tokushima Fatty rats, which do not have a CCK1 receptor, are hyperphagic and obese rats and present a lack of normal satiating response to intestinal nutrients [100, 101]. In this line, the administration CCK1 receptor antagonist increase food intake in rodents [102]. However, the CCK satiating property remained unclear for a considerable period of time.

1.2.3. Gastric inhibitory polypeptide

GIP is released from K-like cells that are located in the duodenum. GIP is a 42-residue peptide that is derived by proteolytic processing of a 153-amino acid precursor in humans and 144-aminoacid precursor in rodents [103]. This hormone belongs to the glucagon superfamily that includes secretin, glucagon and the vasoactive intestinal peptide (VIP) [104].

GIP secretion is primarily in response to direct interaction with ingested carbohydrate or fat, and is proportional to the amount of calories ingested and to the glucose absorption rate [105, 106]. The SGLT1 and K_{ATP} channels are involved in glucose-triggered GIP release, while facilitative glucose transporter GLUT5 is involved in fructose-induced GIP release [107, 108]. It has been reported that GIP release depends on the nature of the fatty acid and the monounsaturated FFAs seem to induce a higher GIP secretion than saturated FFAs [109]. Lipids may exert their effect on GIP secretion by activating the G-protein-coupled receptors GPR40, GPR119 and GPR120 [108, 110].

The first physiological role of GIP described was its ability to inhibit gastric acid secretion. Subsequent studies reported that GIP promotes glucose-induced insulin release from β-cells through the activation of adenylyl cyclase and mitogen-activated protein kinase [111, 112]. Following glucose ingestion, GIP and GLP-1 are responsible for 50% of the insulin released, a phenomenon called, as above mentioned, the incretin effect [113]. The two incretin hormones transduce their action by binding G-protein-coupled receptors expressed in pancreatic cells and in peripheral tissues, such as the central nervous system, GI tract, heart, lungs, kidneys, bones and adipose tissue. The two incretin hormones are rapidly cleaved and inactivated by the DPP-IV enzyme. In the pancreas, the incretin engagement also enhances proliferative pathways and has

anti-apoptotic beneficial effects on pancreatic islets cells in both rodents and humans [114, 115].

Moreover, GIP is involved in regulating the lipid metabolism and, unexpectedly, the inhibition of the GIP axis may function as therapeutic treatment against the development of obesity [116]. Miyawaki et al. showed that GIP receptor-deficient mice did not develop insulin resistance or obesity in high-fat-fed and *ob/ob* models, suggesting that GIP stimulated fat deposition into adipocytes [117]. Similar effects were observed in the chronic inhibition of the GIP pathway, including beneficial effects on body weight, insulin sensitivity and glucose tolerance in different obesity models [118–120].

1.2.4. Peptide YY

PYY is secreted by L-like cells that are found along the gut, especially in more distal parts. The structure of PYY has a significant sequence homology with neuropeptide Y (NPY) and pancreatic polypeptide (PP); that is, all peptides have the PP fold structural motif [121–123]. Two endogenous forms, PYY (PYY_{1-36}) and PYY_{3-36}, are released into the circulation. The full-length PYY_{1-36} is derived by proteolytic processing of a large molecule precursor, and PYY_{3-36} is further produced by the digestion of PYY_{1-36} by DPP-IV [124].

PYY is secreted into the circulation after food intake and is reduced by fasting. Like GIP, the magnitude of PYY release is proportional to the calories ingested, and is stimulated by a high fat diet [125]. It has been reported that propionate enhances PYY secretion from rodents *in vivo* and *in vitro* through the FFAR2 receptor [126]. Also in rodents, colonic infusion of SCFAs increases PYY levels *in vivo* [127]. Besides fatty diets, PYY is secreted in response to glucose in humans [128, 129] and Mace et al. showed that PYY is secreted by L-amino acids phenylalanine, tryptophan, asparagine, arginine and glutamine through CaSR activation [130].

PYY, PP and NPY bind to G-protein coupled receptors Y1, Y2, Y4, Y5 and Y6 due to their shared PP motif structure [89]. The affinity between ligand and receptor, and the specific tissue expression of Y receptors determine the different biological activities of each hormone. The Y1 and Y5 receptors are expressed in the hypothalamus and have affinity with NPY and PYY_{1-36}. On the other hand, PYY_{3-36} binds selectively to the Y2 receptor and PP is selective to Y4 [52, 131].

PYY may be involved in the regulation of glucose homeostasis through the coordination of pancreatic islets functions. Peripheral administration of exogenous PYY_{3-36}, together with food or glucose, stimulates insulin secretion in mice through Y2 receptor activation. However, the activation of Y1 receptor by PYY_{1-36} in murine islets inhibits glucose-stimulated insulin secretion [132, 133]. Moreover, both forms of PYY decrease gastric emptying, and PYY_{3-36} is mainly responsible [134].

In addition to pancreatic regulation, PYY also regulates the appetite via a direct central effect and via its effects on gut motility. PYY is involved in the 'ileal brake', decreased gastric emptying and also the speed of intestinal transit (reviewed in [89]). The two PYY forms play opposite roles in the regulation of food intake. The central administration of PYY_{1-36} results in stimulation of feeding, while peripheral administration does not seem to influence appetite, which suggests that the effects are mediated by Y1R located in the hypothalamus (reviewed in [52]). In addition, PYY_{3-36} plays a role as anoretic peptide and the effect of peripheral PYY_{3-36} seems to be mediated by Y2 receptor because the effect is absent when Y2R knockout mice are used and attenuated by Y2 receptor antagonists. Although it has been suggested that peripheral PYY_{3-36} could act through Y2 receptor expressed in vagal afferents of rats, conflicting evidence has been reported about whether PYY_{3-36} induce anorexigenic effects via vagal stimulation [135, 136].

1.2.5. Ghrelin

The X/A-like cells in mammals or D1 cells in humans are the main ghrelin-secreting cells. However, the highest content of ghrelin is in the gastric fundus. Ghrelin-containing cells have also been found in the EEC of rats and humans throughout the GI tract, from duodenum to the colon. They can be classified into open-type cells, which are located in the stomach, and closed-type cells, which gradually increase along the GI tract [137].

Preproghrelin is composed of 117 amino acid peptides in humans and rodents, and after a post-translational process there is a 28-amino acid sequence, which is uniquely modified by the addition of an octanoyl group in third serine residue by O-acyltransferase (GOAT) [138]. This modification is required for ghrelin to bind to the growth-hormone-secretagogue receptor (GHS-R) and to cross the blood-brain barrier [139], where ghrelin can act on growth hormone-releasing hormone (GHRH) neurons and others [140].

The patterns of ghrelin release show that circulating ghrelin levels increase during fasting periods and before meals, and fall after eating. For this reason ghrelin is often defined as the 'hunger hormone'. It has been shown that both the feeling of hunger and food intake increase in humans after intravenous infusion or subcutaneous injection of ghrelin. Different regulatory molecular mechanisms of ghrelin secretion have been described, including, agents released by sympathetic neurons during fasting periods acting directly on β-receptors on the ghrelin-secreting cells of the stomach [141], circadian regulation mechanisms [142], several chemosensory receptors expressed by ghrelin-secreting cells that interact with luminal content [143], and the regulation by other enterohormones (GIP, secretin, somatostatin, and insulin) [144]. Controversial results have been found for nutrient sensing, such as glucose, SCFA and LCFA decrease ghrelin secretion, while tryptophan, peptones and the artificial sweetener sucralose increase ghrelin secretion [143, 145].

Administering peripheral ghrelin results in weight gain by attenuating fat utilization, and chronic central ghrelin infusion enhances the expression of enzymes that promote fat storage in adipose tissue [146]. Therefore, using GHS-R antagonists to block ghrelin signalling is an interesting idea for preventing obesity. Moreover, ghrelin is also involved in regulating gastric motility and the neuronal orexigenic pathway [147, 148].

1.3. The role of gut microbiota in intestinal epithelial cell function

Gut microbiota is described as the microbial community that inhabits the length of the GI tract, and particularly the distal gut. The human intestinal gut hosts trillions of microorganisms, including more than 10^{14} bacteria, which belong to 1000 different bacterial species classified in the bacterial taxonomy, a rank-based classification system [149, 150]. The genome size of the microbiome is 100- to 150-fold more numerous than the human genomes, providing the organisms with additional biological and metabolic functions for maintaining homeostasis in the body [151]. Therefore, the gut microbiota can be considered as an organ that plays a role in controlling energy homeostasis, regulating the immune system, and also in vitamin synthesis and digestion [152–156].

The main taxonomic bacteria in human gut microbiota belong to three major groups: Firmicutes (including *Clostridium*, *Enterococcus*, *Lactobacillus*, *Ruminococcus*, and *Faecalibacterium* genera), Bacteroidetes (including *Bacteroides* and *Prevotella* genera) and Actinobacteria. These are the dominant bacterial phyla that together represent >95% of the total microbiota. The composition of gut microbiota is modulated during a person's lifespan from infancy to old-age by several environmental factors, including life style, use of antibiotics, and dietary pattern [157–159]. Due to several direct and indirect interactions with the host organism, the gut microbiota and the modulation of its composition are closely linked to the health of the host. Hereafter, we focus on the interactions between intestinal microbiome and the GI tract.

There are different mechanisms through which gut microbiota can interact with the GI tract. One of these mechanisms is the fundamental role of the gut microbiota in the fermentation of non-digestible dietary polysaccharides into SCFA. SCFAs produced by gut microbiota fermentation are mainly acetate, propionate and butyrate [160]. SCFAs can act as host signalling molecules by binding to GPR41 and GPR43, expressed by enteroendocrine cells [161]. The activation of GPCR41 by SCFA promotes the secretion of PYY, whereas the activation of the two receptors (GPR41 and GPR43) by SCFA promotes GLP-1 secretion [162, 163]. Moreover, the fermentation of non-digestible carbohydrates can promote differentiation in the proximal colon and consequently increase the number of L-cells in rats [164]. The endocannabinoid-like compound, 2-oleoylglycerol (2-OG), also acts as a key compound linking gut microbiota and the intestine. The 2-OG can bind to GPR119 receptors and trigger GLP-1 secretion. Everard et al.[165] have demonstrated that gut microbiota modulates the tone of the intestinal

endocannabinoid system and that a specific species of bacteria, *Akkermansia muciniphila*, is able to regulate intestine endocannabinoid-like compounds such as 2-OG. Bile acids, which are produced in the liver and are secreted into the duodenum upon ingestion of a meal, also act as molecules linking the intestine and microbiota. TGR5, a bile acid receptor, is expressed by L-cells and its activation increases GLP-1 secretion. Interestingly, the gut microbiota is an important regulator of bile acid metabolism, including the regulation of synthesis and the production of secondary bile acids. Consequently, variations in the composition of microbiota could modulate specific bile acid profiles, thereby their capacity to activate TGR5 and GLP-1 secretion (reviewed in [166]).

Besides mechanisms involving GLP-1 secretion, there are other mechanisms involved in the crosstalk between microbes and host cells. Gut microbiota can increase the absorption of monosaccharides from the gut by facilitating the extraction of calories from ingested dietary substances [167]. In this sense, the carbohydrate response element-binding protein (ChREBP) has been demonstrated to be implicated in the absorption of monosaccharides in the intestine induced by gut microbiota [168]. The intestinal gluconeogenesis (IGN) may be another link between gut microbiota and the intestine. DeVadder et al.[169] reported that the SCFAs butyrate and propionate, which are generated by the fermentation of soluble fibre by gut microbiota, stimulate the expression of the IGN gene via the cAMP-dependent mechanism and gut-brain axis respectively. Finally, another mechanism that has been proposed to link gut microbiota and the intestine is lipopolysaccharides (LPS) of gram-negative bacteria. LPS, together with other molecules, such as peptidoglycan and lipoteichoic acid, originate from gut microbiota and can cause alterations in intestine permeability, immune system activation and related metabolic disorders [170].

Numerous putative mechanisms linking gut microbiota and the GI tract have now been discovered. However, more mechanisms involved in this interaction are still under investigation.

1.4. The enteroendocrine system in disease: obesity and diabetes mellitus

Obesity is a primary risk factor for the most prevalent diseases affecting the worldwide population, including cardiovascular disease, type 2 diabetes mellitus (T2DM) and inflammation [171]. Obesity is regulated by a complex biochemical process, and its pathological mechanisms have been studied widely in adipose tissue, the liver, and muscle [172, 173]. However, the intestine also plays an important role in the pathology of obesity because energy intake from food is involved in the regulation of body weight, and over-nutrition is considered to be the main cause for becoming obese over other environmental factors [174, 175].

The increase in these life-style diseases highlights the need to search for new therapeutic strategies. The GI tract plays a role in controlling the metabolism through peptide hormones secreted from enteroendocrine cells. These hormones from the gut play a central role in nutrient intake signalling, regulating appetite and energy expenditure. As mentioned above, all gut hormones have anoretic effects, they promote satiation (causing the meal to end) and/or satiety (postponing the initiation of the next meal), while ghrelin is the only one that stimulates appetite and food intake. There is evidence that specific enterohormone administered at physiological concentrations can influence the appetite of rodents and humans (reviewed in [174]). Likewise, the effects of gut hormones on food intake and body weight have been observed in bariatric surgery (such as Roux-en-Y gastric bypass), which induces a huge increase in GLP-1 and PYY secretion, and is used to treat obesity. Therefore, the modulation of enterhormone signalling may represent an important target for preventing obesity and related/associated pathologies. Moreover, endogenous gut hormones regulate appetite physiologically, unlike the drugs that are currently available, which are mainly based on influencing the central neurotransmitter systems to reduce appetite. Therefore, gut hormone-based therapies might lead to fewer side effects [174].

Furthermore, the incretin hormones (GLP-1 and GIP) could represent an interesting strategy in the prevention and/or management of T2DM. T2DM is the most common endocrine disorder, characterised by impaired insulin secretion and insulin resistance, and one of the fastest growing non-communicable diseases in the world [176]. The main goal in the treatment of T2DM is to keep blood glucose levels within the normal physiological range. In this sense, GLP-1 and GIP are therapeutically interesting peptides because they are important mediators of glycemic homeostasis, as they are responsible for approximately 50-70% of the total insulin secreted following glucose intake [113]. However, the strategy is mainly focused on increasing GLP-1 levels rather than stimulating the GIP because in patients with T2DM there is a decreased responsiveness of β-cells to GIP action [177]. Accordingly, many incretin-based therapies are focused on using GLP-1 analogues, promoting endogenous GLP-1 secretion or using DPP-IV inhibitors. As mentioned before, DPP-IV is responsible for inactivating more than 80 % of the secreted GLP-1; therefore, DPP-IV inhibitors provide an alternative approach to enhance GLP-1-mediated glucose control and consequently to preventing T2DM.

The response to nutrients differs between obese and non-obese individuals, with changes in digestion, absorption and hormone release. These changes may be a consequence of differences in the intestinal epithelial morphology and function caused by types of diets that leads to obesity. Some studies on diets associated with obesity suggest that these diets might modulate intestinal cell differentiation. A recent study shows that over-nutrition in animals and high glucose or FFA in cultured intestinal epithelial cells prevents β-catenin degradation and thereby intestinal cell proliferation. In contrast, food restriction in *db/db* mice decreased intestinal cell proliferation and

absorption, suggesting that increased food intake induces activation of Wnt signalling and could contribute to enhancing the intestinal absorptive capacity and the development of obesity [178]. While there are a few studies that suggest the EECs number can be changed by nutrient stimulation. Controversial results have recently been reported between high-fat diet and L-cell differentiation. Duca et al. [179] reported that a high-fat diet decreases the L-cell population and GLP-1 secretion through up-regulation of HES gene expression and downregulation of Math1 expression. Similarly, it has been reported that the density of cells staining for chomogranin A (ChgA) and GLP-1 is reduced in high-fat diet fed rodents, together with a reduction of proglucagon (Gcg) and PYY expressions [179–181]. On the contrary, Aranias et al. [182] showed that the GLP-1 cell density increased after high fat consumption in humans. Furthermore, high fiber diet enhances the number of L-cells in rodent [183] and increased L-cell has been observed in intestinal organoids culture after the exposition of SCFA [184]. These findings indicate a potential link between diets that lead to obesity and intestinal differentiation/metabolism. Concomitantly, the results suggest a new strategy in obesity prevention, the possibility to increase L-cell number in order to increase enterohormone release, and thereby improve glucose homeostasis and satiety.

Recent evidence in mice and humans has described that gut microbiota is linked to the development of metabolic diseases, such as obesity and T2DM [185–187]. Studies using germ-free mice demonstrated that gut microbiota plays a causal role in the development of obesity, because germ-free mice transplanted with the microbiome of obese donors gained significantly more weight than germ-free mice transplanted with the microbiome of lean donors [167]**.** Similarly, gut microbiota from obese mice transplanted to germ-free mice leads to a significant increase in body fat content and insulin resistance in recipient mice [167, 188]. These changes were associated with dysbiosis (alteration in the types and numbers of bacteria in the gut). However, controversial patterns in these alterations have been reported. In obese mice and in T2DM patients, an increase in *Firmicutes* and a reduction in *Bacteroidetes* have been observed [189–191]. Similarly, some human studies have documented a reduced representation of *Bacteroidetes* accompanied by a rise in *Lactobacillus* species belonging to the *Firmicutes* phylum in obese subjects [190, 191]. However, some studies have reported other results, such as an increase in species of both *Firmicutes* and *Bacteroidetes* in overweight women or a decrease in *Bacteroidetes* with no differences in *Firmicutes* phylum in obese individuals [192, 193]. Although the alteration of the gut microbiota composition has been associated with obesity and T2DM, further studies are needed to establish specific compositions. Nevertheless, the association between the composition of gut microbiota and the development of metabolic disease has been taken into consideration as a possible novel strategy for preventing and/or managing obesity and T2DM.

The health benefits of dietary components have recently attached interest to disease prevention and health maintenance. Although studies are being carried out on

pharmacological compounds, natural compounds could be used to prevent the development of overweight and obesity-related problems from early preclinical stages. As mentioned above, dietary compounds can modulate enterhormone secretion; for example, glutamine is reported to increase GLP-1 and GIP secretion in humans [194, 195], proanthocyanids are reported to enhance GLP-1 secretion in rats [196], and pea proteins are shown to increase CCK, GLP-1 and PYY levels in humans [197], among others. On the other hand, as mentioned above, SCFAs have been reported to stimulate GLP-1 secretion by promoting L-cell differentiation and increasing the L-cell population, and thus this is a novel strategy for enhancing enterhormone secretion. Moreover, recent studies have shown that specific nutrients could play a potential role in controlling obesity by modulating gut microbiota [198]**.** For these reasons, screening natural compounds to find new therapies could be useful, and it is also recommended to study their action mechanisms to prevent disease in a target population.

Hereafter, we focus on the action of specific bioactive food compounds, polyphenols and peptides, on different elements of GI tract involved in the modulation of the enteroendocrine system and that could have beneficial health implications.

2. Natural compounds with bioactivity in the GI tract

2.1. Polyphenols

Polyphenols are plant secondary metabolites and are generally involved in defence against UV radiation, herbivores, aggression by pathogens, etc. Unlike traditional nutrients, these plant-derived compounds are not essential dietary molecules for short-term animal well-being, but they may exert a positive long-term impact on health through their capacity to modify enzymatic and chemical reactions in animal systems [199, 200].

2.1.1. Structure, classification and bioavailability

These bioactive compounds are present in a wide range of fruits and vegetables, as well as in processed foods and beverages, such as black tea, matured red wine, coffee, and cocoa. Based on their chemical structure [201], they are classified into two main groups: flavonoids and non-flavonoids [199].

Flavonoids are characterized as containing two aromatic rings (A and B) connected by three-carbon atoms that form an oxygenated heterocycle (C). The basic flavonoid skeleton is comprised of 15 carbons and can have numerous substitution patterns resulting in several subgroups. Depending on the hydroxylation pattern, the distribution of the C6-C3-C6 structures, their index of hydrogen deficiency (IHD), and/or their no-hydroxylated functional groups, flavonoids are classified into flavones, flavonols, flavanols, isoflavones, flavanones, and anthocyanidins [202].

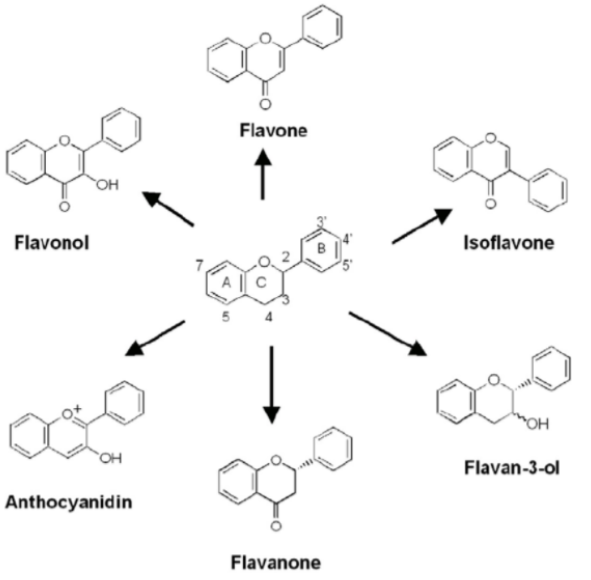

Figure 4. Basic structure of flavonoids and their different type molecules depending on their hydroxylation pattern [203].

Flavanols, also known as flavan-3-ols, are the most structurally complex subclass of flavonoids and represent one of the main phenolic components of the human diet. They can be found in beans, nuts, apples, grapes, cocoa, tea and wine. Unlike other classes of flavonoids, which are usually found in nature in glycoside forms, flavanols are usually present in the aglycone form or esterified with Gallic acid, the commonest non-flavonoid structure [204]. Flavanols exist in the monomer form and the oligomer and polymer form (proanthocyanidins). The two chiral centres, C2 and C3, of flavan-3-ol monomers produce 4 isomers, and the most common structures are (+)-catechin and (-)-epicatechin [201]. Flavan-3-ols monomers can also be found in the gallated form, such as (+)-gallocatechin, (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECg) and (-)-epigallocatechin gallate (EGCg). Conversely, proanthocyanidins (also known as condensed tannins) are oligomeric and polymeric structures of flavanols, which include an additional chiral centre at C4 in the upper and lower units. Dimer (B-type) and trimer (C-type) proanthocyanidins are formed by oxidative coupling between C-4 of the flavan-3-ol upper unit and C-6 or C-8 of the lower unit [201]. Type A proanthocyanidins have an additional ether bond [C-2 (upper unit)–O–C-7 (lower unit)] [199].

Over the years, *in vitro*, *ex vivo* and *in vivo* studies as well as clinical trials have provided ever increasing evidence of the role polyphenols play as potential health compounds [199, 205]. However, the scientific evidence shows that the beneficial effects on health of polyphenols are directly linked to their absorption, distribution, metabolism and excretion. Therefore, considerable attention has been paid to their

bioavailability, as an essential factor for understanding their biological activity, and has been a topic of increasing research in recent years [12, 199, 206].

Polyphenols are recognized as xenobiotics by the organism and are metabolised following the typical detoxification pathway. After ingestion, the small intestine is the first organ relevantly involved in the metabolism and absorption of the polyphenols. It is estimated that only 5%-10% of the total intake of polyphenols, especially those with a low degree of polymerization (monomeric flavan-3-ols and dimer proanthocyanidins), can be absorbed in the small intestine by both passive and facilitated diffusion [207]. Once absorbed, they are extensively metabolized by the action of phase II enzymes (uridine 5′-diphospho-glucuronosyltransferases, sulphotransferases, and catechol-O-methyltransferase), producing their glucuronidated, sulfated, and/or methylated conjugates respectively [206]. This conjugation step first takes place in the small intestine and subsequently occurs in the liver. Next, the conjugates can be returned to the lumen via bile (enterohepatic circulation) or can reach the systemic circulation to be distributed to tissues or excreted by urine [208].

The 90-95% of unmodified polyphenols, mainly oligomers and polymers, are not absorbed by the small intestine and proceed through the gastrointestinal tract. Together with the conjugated metabolites they return through the enteropatic circulation, reach the colon where they are accumulated at high concentrations (up to the mM range) and are exposed to the microbial metabolism [209]. In the colon, favanols can be biotranformed via three metabolic pathways: the production of valeric acid by meta-substitution of the flavanol A ring, the formation of valerolactone compounds by the microbial cleavage of flavanol C- and A- rings, and the breakdown of polymeric flavan-3-ols into their monomers by microbial cleavage of the interflavanic bond C4–C8 [210]. It has been described that gallated flavanols can also reach the colon and are metabolised to the respective flavanol form (monomer or even dimers) and the respective Gallic acid residue by the cleavage of the gallated moiety [211]. In addition, microbial dehydroxylation transforms monomeric forms to propan-2-ol metabolites, which may become valerolactones by microbial A-ring cleavage [210]. The valerolactones can be converted by acidic hydrolysis into valeric acids, which are rapidly transformed into phenylpropionic, phenylacetic, and benzoic acids. Then, microbial metabolites are absorbed and can be metabolized by phase II enzymes, to finally enter the circulation or be eliminated in urine [206].

It has been reported that polyphenol absorption, metabolism and their fate in the organism are affected by external factors, such as polyphenol structure, source, food processing, and the quantity, as well as by internal factors, such as age, sex and health status of the host [212]. Therefore, their bioactivity could be modulated under different situations.

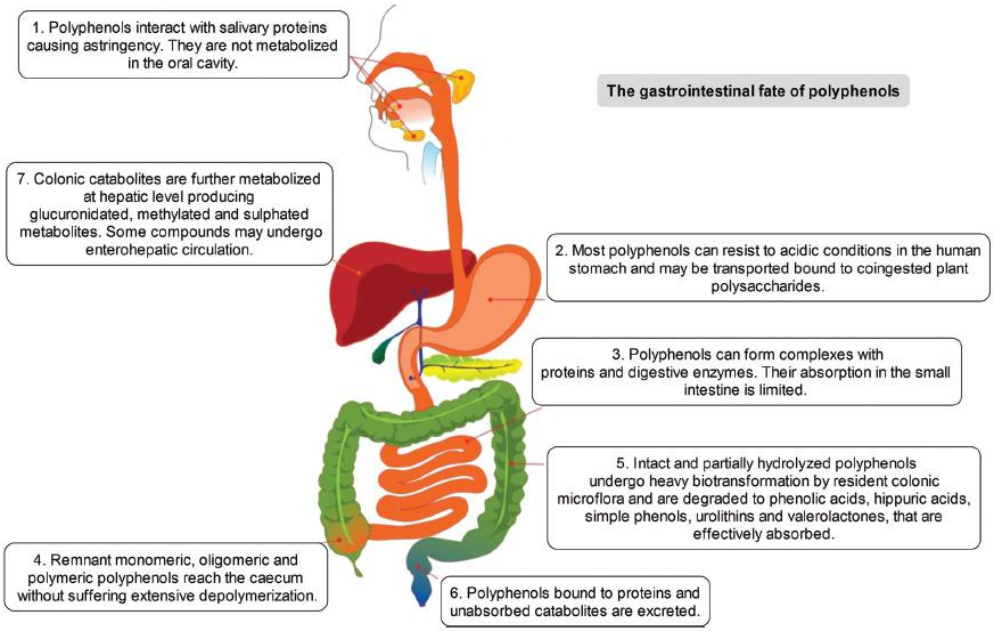

Figure 5. The fate of dietary polyphenols after their ingestion. Extracted from [206].

2.1.2. Flavonoids and the enteroendocrine system

As mentioned before, polyphenols and especially flavonoids have been described as potential bioactive compounds that exhibit a wide array of beneficial effects on health. They can act as antigenotoxic [213], antimicrobial [214], antioxidant [215], antiinflamatori [216, 217] and anti-cancer/anti-proliferative [218, 219] molecules. Among their effects, polyphenols have been reported to have antihypertensive effects [220] and also improve lipid metabolism [221].

Furthermore, flavonoids have been shown to improve glucose homeostasis. However, controversial results have been reported in different glucose-disrupted animal models, because their antihyperglicemic effect seems to be exerted by different mechanisms depending on the molecular origin of the glucose homeostasis disruption**.** Apart from their insulin mimetic effect on liver and peripheral tissues, some mechanisms used by flavonoids are exerted in the intestine, such as the inhibition of the enzymes that participate in carbohydrate digestion and the reduction of glucose absorption by inhibiting the transporters involved in glucose uptake (reviewed in [222]). Moreover, flavonoids have also been reported to have effects on the incretin system, which could be another mechanism for improving glucose homeostasis (reviewed in [223]). Our research group has previously shown that grape seed proanthocyanidin extract (GSPE) (1 mg/kg BW) increases GLP-1 plasma levels in rats, partly due to inhibition of DPP-IV but also due to enhanced secretion [196]. However, the mechanism of action on L-cells of flavonoids, especially GSPE, and whether they could modulate other enterohormones are not yet determined (review [224]).

2.1.2.1. Effects of flavonoids on enterohormone secretion and on EE cells differentiation

It is well known that the secretion of gut hormones is strongly influenced by dietary compounds. Macronutrients are the most marked example, but polyphenols and other micronutrients have also described as important regulators of enterhormones secretion. There are few studies suggesting that flavonoids might modulate enterohormone secretion, some of them reviewed in [224].

Ghrelin is the only enterhormone that exhibits orexigenic effects, inducing the stimulation of appetite and food intake. The effect of isoflavones on ghrelin has been described, whereas there are a few studies using other types of flavonoids. In healthy postmenopausal women, the administration of isoflavones did not modify fasting ghrelin plasma levels, body weight and food intake [225, 226]. In a smaller study, the supplementation of 114 mg/day of isolated isoflavonoids for three months resulted in the inhibition of the age-dependent rise of the fasting plasma ghrelin levels in postmenopausal women with a history of breast cancer [227]. However in that study the lipid profile or insulin sensitivity was not modified, and the body composition and food intake were not assessed. Thus, in human studies clear results are not observed. Instead, animal studies suggest that the isoflavones supplementation can modify the levels of ghrelin and that such modification results in changes of body weight. In ovariectomized rats under a high-fat diet, the administration of different doses (26, 74, 206 mg/kg bw) of soy isoflavones for 4 weeks decreased plasma ghrelin, increased CCK and tended to increase PYY levels. These findings were accompanied with a reduction of body weight and food intake at the higher doses, but with an increased body weight at the lower dose [228]. Similarly, plasma ghrelin levels were reduced by the daily administration of isoflavone genistein (8 mg/kg) for 8 weeks in female mice, but not in males. In this study, the treatment decreased the relative food consumption in female at 1 and 5 weeks and in male at 5 weeks [229]. In studies with other families of flavonoids, an extract rich in naringenin (Citrus grandis) was administered during 12 weeks at different doses (300, 600, 1200 mg/kg bw) in obese zucker rats under a high fat/high cholesterol diet. The results showed that the extract counteracted the reduction of ghrelin induced by the diet. Moreover, the high dose of extract decreased the GLP-1 plasma levels (which were not modified by the diet), while any dose did not change PYY secretion [230]. Finally, a diet supplemented with tea extract rich in monomeric flavanols did not modify fasting ghrelin levels and body weight in T2DM and/or obese humans studies [231–233]. However, Chen et al. increased the dose of phenolic compounds (856.8 mg ECGC) and observed that green tea extract reduced body weight and ghrelin levels in obese women after 12 weeks [234].

There are a few studies that evaluate the action of flavonoids on **CCK** secretion. As mentioned above, increased CCK levels were observed after the administration of soy isoflavones for 4 weeks in ovariectomized rats [228]. *In vitro* assays have shown that hesperetin and naringenin stimulate CCK release in STC-1 cell line, and that the effect

is mediated via the activation of transient receptor potential channels (TRP channels) including TRPA1 and the increase of intracellular calcium levels [235, 236]. Similarly, Al Shukor et al. showed that quercetin, kaempferol and apigenin enhance CCK secretion in STC-1 cell line, but no effect is observed by rutin and baicalein [237].

There are limited studies about the effect of flavonoids on **PYY** and **GIP** secretion. The scarce studies in animals (mentioned above) do not show clear effects on PYY secretion. However, Weickert et al. [226] observed that plasma PYY levels increased after 8 weeks of isoflavonoid treatment in healthy postmenopausal women**.** The effect of red, white and brown-grained sorghum (which contain anthocyanins, condensed tannins and phenolic acids, apart from fiber) was determined in healthy adult volunteers (men and women). The area under the plasma concentration-time curve of postprandial GLP-1 and GIP in both genders and PYY in men was significantly higher in sorghum biscuits groups as compared to the control. The biscuits prepared from red sorghum, which contained the highest polypenol content, exerted the strongest changes on hormone secretion. These results were accompanied by lower satiety ratings after its consumption, although energy intake at a subsequent meal did not differ between treatments [238]. Another research group reported that the ingestion of bilberry extract (36% w/w anthocynins) did not modify GLP-1 and GIP levels in T2DM subjects; although a significant decrease of glucose plasma levels was observed in bilberry extract group [239]. On the other hand, the daily administration of flavonoid glycosides extract from seabuckthorn leaves (SLG) counteracted the increased GIP levels induced by high fat diet in C57BL/6J mice [240]. Our research group have reported that an acute dose of GSPE decreased GIP release after oral glucose load in rats [196].

The **GLP-1** hormone play an important role in glucose homeostasis enhancing insulin secretion; although the incretin effects is its principal action, GLP-1 is also involved in the regulation of food intake. There are some *in vivo* studies that evaluate the action of flavonoids on GLP1 secretion, some of them have been mentioned above. The consumption of green tea extract (1.5 g/day, 856.8 mg EGCg) significantly increased GLP-1 plasma levels after 16 weeks in T2DM subjects [241]. The consumption of berry meal (800 mg polyphenols including bilberries, blackcurrants, cranberries, and strawberries) with 35 g sucrose in healthy subjects tended to increase GLP-1 and decreased glucose concentration, compared with the control meal [242]. Similarly, α-glycosyl-isoquercitrin also enhance GLP-1 secretion when it is simultaneously administered with fructooligosaccharides, but not alone [243]. In the same sense, our research group found that the acute dose of GSPE extract (1g/kg BW) increased GLP-1 plasma levels after an oral glucose load in rats [196]. Also in animal studies, an acute dose of 10 μg/kg BW of cinnamtannin A2 (procyanidin tetramer) increased active GLP-1 and insulin levels in fasted mice [244]. Berberine has been reported to increment portal active GLP-1 levels in healthy and STZ mice and to increase GLP-1 secretion from human enteroendocrine cell line NCI-H716 cells [245, 246]. *In vitro* assays using the same cell line have shown that genistein and daidzen isoflavonoids (derived from

soy fermentation), and glyceollins and phytoalexins (derived from daidzen in soybean with a fung infection) can increase GLP-1 release [247, 248]. Although the direct effect of isoflavonoid family has been reported, such effect of flavanols has not been reported yet. Some studies have shown that flavanols can activate bitter receptors, increase intracellular calcium levels and modulate cellular membrane potential [249–252], but the enterhormone secretion was not assessed in these studies. Regarding bitter receptors, a recent study has reported that Qing-Hua Granule, which is rich in flavonoids, enhance GLP-1 secretion via activation of bitter taste receptor (TAS2R) pathway in the gastrointestinal tract of db/db mice [253].

The increased active GLP-1 levels induced by bioactive compounds can be attributed to several mechanisms. As mentioned above, our research group found that GLP-1 plasma levels are increased by acute dose of GSPE, and such effect might be partly explained for its capacity to inhibit DPP-IV. Gonzalez et al. reported that chronic GSPE treatment decreased DPP-IV activity and gene expression in Caco-2 cell line, and decreased intestinal gene expression in healthy and diet-induced obese rats [254]. Moreover, they assessed that the principal molecules of this extract catechin, epicatechin, B2 dimer and gallic acid, which are absorbed by intestinal CaCo-2 cells, inhibit DPP-IV activity in endothelial HUVEC cells [196]. Cocoa and products derived from it, which contain flavanols, have been shown to inhibit DPP-IV activity *in vitro* [255].

In addition to stimulate secretion and inhibit inactivation, a new strategy to enhance endogenous enterohormone levels might be the promotion of endocrine cell differentiation and thereby the increase of endocrine cell number. Despite there have been no reports of flavonoids and L-cell differentiation, our research group found that chronic GSPE treatment increased ChgA, GLP-1 and PYY, and counteracting the effects induced by cafeteria diet in rats, and suggesting a possible alteration of intestinal differentiation [256]. Moreover, similar extracts have been reported to enhance Muc2 expression together with an increment of the goblet cell density in mice [257]. Besides, flavonoids have been reported to modulate differentiation of other cell lines, such as PC12 nerve cell line [258, 259], Treg cells [260], 3T3-L1 cell line [261], bone marrow stromal cells [262] and calvarial osteoblast-like (ROB) cells [263]. Although these results point that flavonoids could be a good candidate to modulate L-cell differentiation, further studies are required.

2.1.2.2. Effects of flavonoids on gut microbiota

As mentioned above, gut microbiota might be a potential exteriorised organ that can contribute to developing metabolic dysregulation, leading to inflammation in intestinal and peripheral tissues, and alteration of glucose and energy homeostasis [154, 198, 264, 265]. Moreover, gut microbiota might influence directly GI tract trough several mechanisms, the principal mechanism is SCFA produced by gut mircobiota, which enhance the secretion of GLP-1 and PYY. Studies have indicated that the microbiota composition can be influenced by several external disturbances, but dietary changes are one of the most important factors and can lead to 57% of the total structural

variation in gut microbiota [266]. There are some dietary strategies for modulating the composition and metabolic/immunological activity of the gut microbiota: probiotics, which are live microorganisms that its administration in adequate amounts confers a health benefits on the host, and prebiotics, which are an ingredient that fermentation results in specific changes in the composition and/or activity of gut microbiota [267].

In the past few years, dietary polyphenols have been suggested to be potential gut microbial modulators, as they induce oscillations in the composition of the microbiota populations, such as red wine [268, 269], cocoa [270] and promograde polyphenols [271]. It is well known that flavonoids have an antimicrobial effect against pathogenic microorganisms. For example, it has been reported that components of tea (epigallocatechin gallate, epicatechin gallate, epigallocatechin, gallocatechin, epicatechin and catechin) can inhibit the growth of many pathogens, including *Helicobacter pylori* [272], *Staphylococcus aureus, E. coli O157:H7* [273, 274], *Salmonella typhimurium DT104, Listeria monocytogenes, methicillin-resistant S. aureus* [275, 276], and *Pseudomonas aeruginosa* [277], among others. Despite their antimicrobial effect, there are considerably few studies that evaluate the influence of flavonoids on the composition and activity of the non-pathogenic gut microbial community.

Some *in vitro* studies have evaluated the effect of flavonoids on the growth pattern of intestinal bacteria. It was demonstrated that flavonoid aglycone, but not their glycosides, may inhibit the growth of some intestinal bacteria [278]. Similarly, naringenin and hesperetin inhibited the growth of almost all bacteria analysed in the study, whereas their glycoside forms (naringin and hesperidin) had no impact. In the same study, catechin repressed the growth of *Clostridium histolyticum* and increased the growth of *Clostridium coccoides–Eubacterium rectale* group, *Bifidobacterium* spp. and *E. Coli* [279]. Lee et al.[280] evaluated the impact of the phenolic compounds of a tea extract, rich in catechin and epicatechin, and their fecal bacterial metabolites, resulting in a repression in the growth of *Clostridium perfringens*, *Clostridium difficile* and *Bacteroides* spp.. Moreover, flavanoids can also influence the gut microbiota by affecting the adhesion gut bacteria on intestinal cells [281, 282].

The effect of flavonoids on the gut microbiota composition has also been observed in *in vivo* studies, which are summarised in **Table 1**. In human studies, it has been reported that wine polyphenols, cocoa-derived flavanols and isoflavones can modulate the gut microbiota after 4-week treatments [283–285]. The daily consumption of 272 ml of red wine in adult men decreased the plasma levels, TAG, HDL-cholesterol and the C-reactive protein, which is considered to be a blood marker of inflammation. These significant reductions could be linked to changes in the bifidobacteria number [283]. Tzounis et al. [284], who evaluated the effect of cocoa flavonoid in a randomised, double-blind, cross-over intervention study, also observed a reduction in plasma C-reactive protein and TAG, together with an increase in *Lactobacillus* and *Bifidobacterium* populations. These effects on gut microbiota in the dominant bacterial communities were similar to those observed by Clavel et al [285].

Table 1. Summary of studies that evaluate flavonoid effects on gut microbiota composition (adapted from [217]).

Flavonoid	Specie	Dose/Time	Diet	Effects in the microbiota composition	Ref.
Human studies					
Red wine (flavanols, anthocyanins, flavonols, etc)	Adult men	272 ml/ 4 weeks	-	Increase the number of *Enterococcus, Prevotella, Bacteroides, Bifidobacterium, Bacteroides uniformis, Eggerthella lenta,* and *Blautiacoccoides–Eubacterium rectal*	[283]
Cocoa flavonols	Human	494mg/ 4weeks	-	Increase the bifidobacterial and lactobacilli and decreased clostridia populations	[284]
Isoflavones	Postmeno pausal women	100 mg/ 30 days and 60 days	-	Increase microorganisms of *Lactobacillus-Enterococcus* group, *Faecalibacterium prausnitzii* subgroup, and *Bifidobacterium* genus	[285]
Animal studies					
Apple procyanidins (highly polymeric procyanidins (PP))	C57Bl/6J male mice	0.5% PP/ 20weeks	high fat sucrose diet	Decrease the Firmicutes/Bacteroidetes ratio and increase *Akkermansia* genera.	[286]
Cranberry extract (flavanols, phenolic acids, anthocyanins, etc)	C57Bl/6J male mice	200mg/kg BW/8 weeks	high fat/high sucrose diet	Increase the proportion of *Akkermansia* genera.	[287]
Green tea leaves (flavanols)	C57BL/6J female mice	4 % (w/w)/ 22 weeks	high-fat diet	Increase the proportion of *Akkermansia* genera.	[288]
Grape polyphenols (GP) (flavanols, anthocyanins, etc)	C57Bl/6J male mice	1% GP/ 12-13weeks	high-fat diet	Increase the growth of *Akkermansia muciniphila* and decrease the ratio of Firmicutes/Bacteroidetes.	[289]
Grape seed proanthocyanidin extract	C57Bl/6J male mice	300mg/kg BW/7weeks	high-fat diet	Increase the growth of *Clostridium XIVa*, *Roseburia*, and *Prevotella.*	[290]
Quercetin	Wistar rats	30 mg/kg BW/6 weeks	high fat sucrose diet	Decrease Firmicutes populations, Erysipelotrichia class and *Bacillus* genus. Down-regulation of *Erysipelotrichaceae, Bacillus* and *Eubacterium cylindroides* species.	[291]
Red wine polyphenols	F344 male rats	50 mg/kg BW/ 16weeks	-	Increase *Bifidobacterium* and reduce *Clostridium.*	[292]

Concerning animal studies, most of the studies evaluate the potential effect of flavonoids in animals that have a high fat sucrose diet. In order to evaluate whether flavonoids reverse alterations of gut microbial composition associated with diet-induced obesity, Matsomoto et al. [286] assessed, in C57BL/6J mice, the effect of non-absorbable apple procyanidins (PP) during 20 weeks and observed a reduction in endogenous metabolite levels associated with insulin resistance, in weight gain and in inflammatory effects (LPS levels and gut permeability). The administration of PP also decreased the Firmicutes/Bacteroidetes ratio and increased the *Akkermansia* population. Similarly, the daily administration of cranberry extract (200mg/kg) for 8 weeks in mice also increases the proportion of *Akkermansia* [287]. This change in microbial composition was observed in another study, in which the high-fat diet of mice was supplemented with powered green tea and *Lactobacillus plantarum* DSM 15313 [288]. Likewise, the administration of grape polyphenols (GP) during 13 weeks also increased the growth of the *Akkermansia* population and decreased the proportion of Firmicutes to Bacteroidetes in C57BL/6J mice with a high-fat diet. In the same study, Roopchand et al. [289] observed that grape polyphenols (GP) administration increased intestinal gene expression of proglucagon and decreased a gene for glucose absorption (Glut2). Altogether, GP attenuated several effects of a high-fat diet, including weight gain, adiposity, the serum inflammatory markers and glucose intolerance, and the authors suggest that the gut microbiota provides the link in the mechanisms of action in poorly absorbed dietary polyphenols. The administration of a similar extract, grape seed proanthocyanidin extract, during 7 weeks also modulates the gut microbial composition, including *Clostridium XIVa*, *Roseburia* and *Prevotella* in mice with a high-fat diet [290].

In summary, the results of these studies suggest that flavonoids can act as prebiotic and thereby modulate the gut microbiota composition, inducing the growth of beneficial bacteria and reducing the pathogen species population. However, there are few studies in this research field and more studies are needed to confirm the results. Moreover, nowadays the studies that have reported mechanisms driving the crosstalk between gut microbiota and host metabolism in flavonoids field have been focused on intestinal permeability and endotoxemia. Although some prebiotics (i.e inulin, oligofructosa) has been shown to influence the production of gut hormones through the alteration of gut microbial composition (reviewed in [293]), there are scarce studies which evaluate that link after flavonoids administration.

2.2. Bioactive peptides from natural sources

Over the past few decades, there has been a large increase in scientific research into bioactive peptides (BPs) derived from food. These are fragments that are encrypted within the primary protein sequence and must be cleaved to exert their function [294]. These BPs, or cryptides [295], have a broad range of functions, such as inhibiting metabolic enzymes, regulating gene expression and hormonal secretion, and

maintaining physiological homeostasis through physical interaction and direct removal of metabolites. Several *in vitro* studies have demonstrated that BPs can beneficially modulate health markers [296, 297], and some of these BPs have been evaluated *in vivo* in rodents [298–300] and humans [301–303]. Although their effects are less potent than synthetic pharmaceutical drugs, these bioactive peptides are well metabolised and confer less side effects. Furthermore, there is a wide range of available and inexpensive food, processing by-products and under-utilized resources, which can be used as sources to generate these value-added products. These characteristics could potentially lead to their category being expanded in the nutraceutical food sector [294].

The function of bioactive peptides depends substantially on their structure, which in turn depends on the nature of their protein precursor and their production conditions. Therefore, the selection of the parent proteins and the process to liberate the peptide are important. As illustrated in **Figure 6**, the first step in the classical empirical approach involves identifying a suitable protein source, and then releasing bioactive peptide fragments through protein hydrolysis by proteolytic enzymes or via bacterial fermentation under specific process conditions (such as pH, temperature, time) [304]. Subsequent fractionation is necessary to yield enriched bioactive peptide preparation and purification steps to isolate peptides with particular bioactivity. For reference for the research community, the identified peptides can be deposited in web-based open-access databases of bioactive peptides, such as BIOPEP and PepBank.

Recently, *in silico* approaches have been applied to discover bioactive peptides. Examples include computational methods based on knowledge about the structure and activity of the peptides reported in the databases (BIOPEP) to determine the potential biological activity of the protein and the occurrence frequency of bioactive fragments in the protein [305]. Other bioinformatic tools can be used to envisage release of these fragments by specific enzymatic cleavage (such as, ExPASy PeptideCutter, PoPS) [295], while molecular docking has been used to virtually screen peptide sequences [306, 307] or to propose the putative mechanisms of action [308, 309]. Predictive models can also be generated by PeptideRanker server, which is used to identify structural features in large proteins that have previously been associated with known bioactivities [310], or by the quantitative structure activity relationship (QSAR) [311, 312]. These bioinformatics tools could be employed together with the classical approach to improve the generation, discovery and validation of bioactive peptides; however, there are several limitations in the approach to obtain them. Nevertheless, different targeted approaches have attempted to develop alternative strategies to overcome these limitations (such as making the method for identifying peptides more accurate, and improving *in silico* tools) [305, 313].

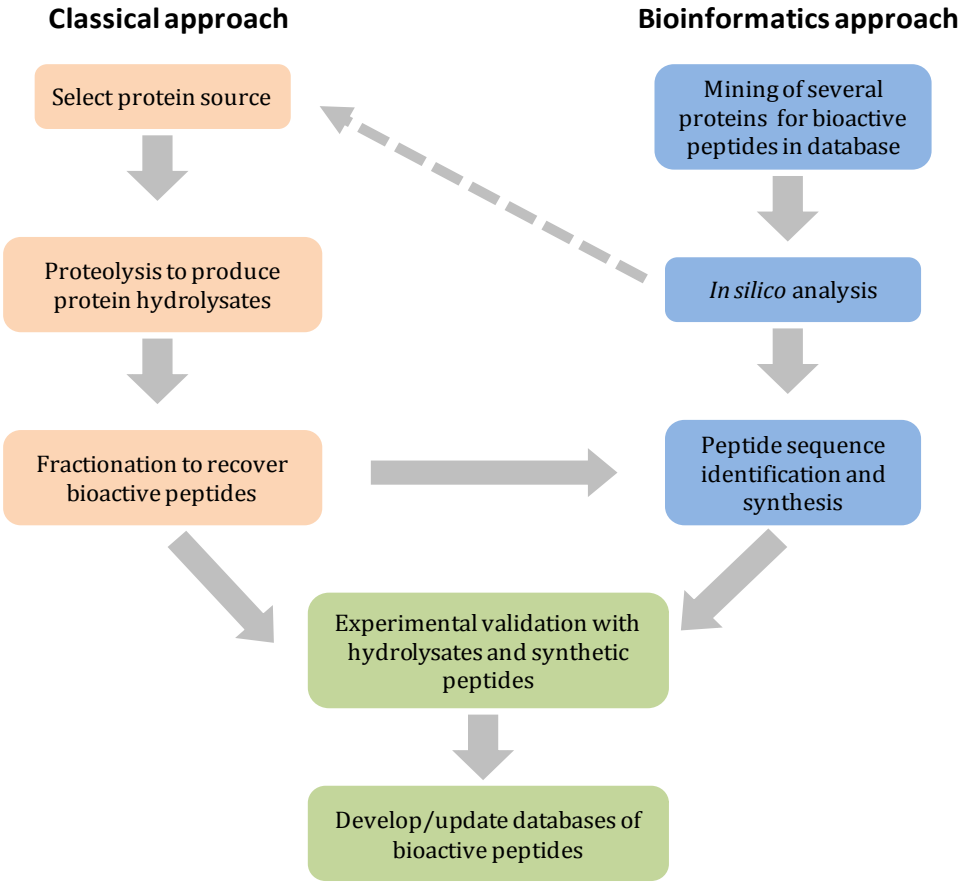

Figure 6. Classical and bioinformatics approaches for the discovery of bioactive peptides in food proteins (adapted from [295, 305])

2.2.1. Bioactive peptides and GLP-1 enterohormone

As mentioned in the section 'GI tract in disease: obesity and diabetes mellitus', dietary compounds are well recognized to play an important role in the prevention and management of T2DM. Many of their effects are involved in the incretin effect, mainly the increase in GLP-1 plasma levels. Accordingly, one of the therapeutic strategies of dietary compounds to enhance insulin secretion is to inhibit DPP-IV in order to increase active GLP-1 levels [314]. It is well known that bioactive peptides act as modulators of enzyme activity, such Angiotensin-converting enzyme inhibitors that have antihypertensive effects [315]. Over the past few years, bioactive peptides have shown their potential to act as DPP-IV inhibitors, which is a research area that is currently expanding.

On the other hand, another point of action to enhance insulin secretion by dietary compounds is the stimulation of endogenous GLP-1 secretion. Some studies have reported that specific amino acids and hydrolysates enhance GLP-1 release [194, 316, 317]. However, the role played by proteins or individual amino acids in triggering incretin secretion remains an area of controversy. Altogether, a few studies have

evaluated the two properties, bioactive peptides as DPP-IV inhibitors and as GLP-1 stimulators, to increase active GLP-1 plasma levels and consequently improve glucose homeostasis.

2.2.1.1. Bioactive peptides as DPP-IV inhibitors

During the past few years, proteins from a variety of food sources have shown their potential to act as DPP-IV inhibitors. As shown in **Table 2**, a variety of food commodities from both animal (such as milk, eggs, fish, meat, etc.) and vegetal (such as oats, quinoa, amaranth, etc.) sources have been studied and shown to be promising sources of DPP-IV inhibitory peptides. As mentioned before, the process to liberate BPs is as important as the protein source. In a hydrolysis process, a range of enzymes are employed, including enzymes obtained from both animal (e.g. pepsin [318], trypsin [319], Corolase PP [320]) and vegetal (e.g. papain [317], protease from pumpkin [321]) sources, as well as food-grade proteinases, such as Alcalase, Flavourzyme, and Protamex derived from microorganisms [322, 323]. Moreover, *in vitro* simulated gastrointestinal digestion has also been reported to produce protein hydrolysates, suggesting that DPP-IV inhibitory peptides could originate during digestion [324, 325].

The effect of DPP-IV-inhibiting protein hydrolysates has been evaluated in numerous *in vitro* studies, which have shown that the half-maximal inhibitory concentration (IC_{50}) values vary widely and range from 0.075 mg/ml to 5.71 mg/ml (**Table 2**). Some studies have reported that the inhibitory activity varies depending on the specificity of the enzyme used, showing differences in the IC_{50} values between 5.0 and 2.7 mg/ml in sodium casein hydrolysates [326], and between 3.71 and 2.21 in barbel hydrolysates [327]. A limited number of studies have identified the responsible DPP-IV inhibitory BPs through fractionation and/or peptide enrichment techniques (based on physicochemical properties such as molecular mass, hydrophobicity or charge), and analysis of the most potent fractions by mass spectrometry. As shown in **Table 2**, these peptides have a wide spectrum of potencies (IC_{50}), ranging from 5 μM to >20,000 μM, amino acids and length compositions (2-17 amino acids long). In addition to these BPs isolated from protein hydrolysates, many other BPs have been identified by *in silico* approaches. Many of the peptides reported are dipeptides and show similar IC_{50} values (4 μM to >20,000 μM) [307, 328–330] to BPs that have been identified in protein hydrolysates (**Table 2**). Although, these BPs have been identified in the sequence of dietary proteins, it is unknown whether BPs could in fact be released from dietary proteins during digestion or enzymatic treatment.

Research has shown that the amino acid sequence plays a predominant role in the DPP-IV inhibitory activity compared to other physicochemical parameters, including length, isoelectric point, hydrophobicity and net charge [307, 331]. DPP-IV preferentially cleaves substrates that bear proline or alanine at their P1 position (Xaa-Pro and Xaa-Ala; where Xaa represents any amino acid) and also acts on substrates that bear other residues, such as glycine, serine, valina and leucine [332]. Hydrophobic

and basic residues at the P_2 position enhance the affinity for cleavage compared with acidic residues. A recent analysis revealed that the presence of tryptophan residue at the N-terminal position increases the susceptibility to cleavage [330]. Although the residues at the N-terminal position may have a major impact by inhibiting DPP-IV, the authors pointed out that the C-terminal amino acid also affects the potency of DPP-IV because it is implicated in the interaction with the enzyme.

To date, a few studies have been carried out on the *in vivo* DPP-IV inhibitory effects of the hydrolysates and peptides from dietary proteins. Peptides derived from milk and bean proteins, which have been shown to inhibit the activity of DPP-IV *in vitro,* were also found to have glycemic effects on mice [333, 334] as plasma glucose levels were found to decrease after an oral glucose tolerance test (OGTT). A β-casein-derived peptide LPQNIPPL found in gouda-type cheese with *in vitro* DPP-IV inhibitory effects has also been tested with animal models. Oral administration of this octapeptide resulted in lower postpandrial glucose under the curve compared to those that did not receive the peptide; however, insulin plasma levels did not differ [335]. In these studies, the authors did not measure plasma DPP-IV activity; therefore, it is unknown whether the effect of lowering the blood glucose observed was caused by inhibition of DPP-IV activity.

As well as hydrolysates from milk and bean protein, hydrolysate produced by Alcalase treatment of the egg protein lysosyme has also been evaluated in *in vivo* models, showing a 25% reduction of blood serum DPP-IV activity and a trend towards higher serum GLP-1 levels after 90 min in diabetic rats undergoing a chronic treatment [336]. The Streptozotocin-induced rats were used to evaluate the effects of hydrolysates of porcine skin gelatin [299], Atlantic salmon skin gelatin [298], and halibut and tilapia skin gelatin [300]. In all studies, diabetic animals showed reduced blood glucose levels during OGTT, increased plasma insulin and active GLP-1 levels, and reduced plasma DPP-IV activity after chronic treatment (42 days with a daily dose of 300 mg/kg of porcine skin gelatin [299]; 35 days with a daily dose of 300 mg/kg of Atlantic salmon skin gelatin hydrolysate [298]; 30 days with a daily dose of 750 mg/kg of halibut and tilapia skin gelatin hydrolysate [300]. Moreover, rodents receiving halibut and tilapia skin gelatin hydrolysates also showed increased total GLP-1 levels. Therefore, the findings of this study suggest that these hydrolysates exert their anti-hyperglycemic effect via dual actions of DPP-IV inhibition and GLP-1 secretion enhancement. Similarly, the ileal administration of zein protein hydrolysate to rats was found to potentiate the incretin effect when administered prior to an intraperitoneal glucose tolerance test, resulting in decreased glucose concentration, increased insulin levels, decreased plasma DPP-IV activity, and increased total and active GLP-1 secretion [337]. Rice-derived peptides were likewise found to act via dual action. The oral administration caused increased plasma GLP-1 levels during intraperitoneal glucose tolerance test, together with reduced plasma DPP-IV activity and higher ratio of active GLP-1 to total GLP-1 following ileal administration [317].

Table 2. Summary of protein hydrolysates, and peptides isolated from them, reported to have in vitro DPP-IV inhibitory activity (adapted from [376]).

Protein source		IC50 (mg/ml)	DPPIV inhibitory peptide identified		Ref.
			Sequence	IC50 (μM)	
Amaranth		1.1	-	-	[308]
Beans	Cowpea	0.58	-	-	[338]
	Navy; Black; Great Northern; Pinto; Red	0.093; ~0.1; ~0.15; ~0.2; ~1	-	-	[325]
Bovine haemoglobin		3.40-0.74	VAAA	141	[339, 340]
Brewers' spent grain		3.57	ILDL; ILLPGAQDGL	1121.1; 145.5	[323]
Camel milk		1.26-0.52	WK; LPVPQ		[341–343]
Cow's milk		1.59-0.68	INNQFLPYPY*	40	[342, 344]
	α-Lactalbumin	0.74	-	-	[345]
		0.036	WLAHKALCSEKLDQ; LAHKALCSEKL; LCSEKLDQ; TKCEVFRE; IVQNNDSTEYGLF; ILDKVGINY; LKPTPEGDL	141; 165; 186; 166; 337; 263; 45	[318, 331]
	β-Lactoglobulin	210 μM	VAGTWY	174	[334]
		1.7	-	-	[345]
		1.28	-	-	[318]
	Lactoferrin	0.38	-	-	[331]
		1.088	-	-	[346]
	Bovine serum albumin	0.51	-	-	[318]
			ER	4480	[347]
	Sodium casein	1.10; 0.88	-	-	[346]

		-	APFPEVF; APFPE; HPIK; GPFPIIV; LPLP; EMPFPK; LPVP; PFP; PQSVLS; YVPEPF; MPLW; LPQYL; LPVPQ; GPFP; PLLQ; VPYPQ; VPLGTQ; LPVPQK; KVLP; LPL; IPI	6%; 6%; 9%; 9%; 11%; 12%; 15%; 20%; 20%; 20%; 23%; 26%; 28%; 34%; 37%; 51%; 55%; 63%; 98%; 100%; 100%[a]	[322]
		5.0-2.7	-	-	[326]
	Whey protein concentrate or isolate	1.51	VAGTWY; TPEVDDEALEK; IPAVF; IPAVFK; VLVLDTDYK	174.0; 319.5; 44.7; 143.0; 424.4	[319]
		0.075	WLAHKAL; WLAHKALCSEKLDQ; LAHKALCSEKL; TKCEVFRE; LKPTPEGDL; LKPTPEGDLEIL; IPAVFKIDA	286; 141; 165; 166; 45; 57; 191	[324, 331]
		0.25; 0.27	IQKVAGTW; LKPTPEGDLEIL; LKPTPEGDLE; VLDTDY; LKALPMH; LKGYGGVSLPE; WLAHKAL	329; 57; 42; 471; 193; 486; 286	[348]
		1.43; 0.99	-	-	[346]
		1.5; 1.1	-	-	[349]
		1.54-0.72	-	-	[350]
Cuttlefish (Sepia officinalis) viscera		1.03	-	-	[351]
Goat's milk	Casein	-	MHQPPQPL; SPTVMFPPQSVL; INNQFLPYPY	350.41; 676.31; 40.08	[344]
Gouda-type cheese		-	VPITPT; LPQNIPP; PQNIPPL; VPITPTL; FPGPIPN; PGPIHNS; IPPLTQTPV; VPPFIQPE; YPFPGPIPN; LPQNIPPL; LPQ	130; 160; 1500; 110; 260; 1000; 1300; 2500; 670; 46; 82	[335]
Hemp		5.71-2.10	-	-	[352]
Palmaria palmata		1.47	ILAP; LLAP; MAGVDHI	43.40; 53.67; 159.37	[353]
Pea		1.08-0.73	-	-	[352]

Protein source		IC50 (mg/ml)	DPPIV inhibitory peptide identified		Ref.
			Sequence	IC50 (μM)	
Lysosyme		1.5-0.4	-	-	[326]
Quinoa		0.98; 0.88	-	-	[354]
Rice	Bran	2.3	IP; LP	410; 2370	[355]
	Brown	1.55-0.91	-	-	[352]
Skin or scale collagen/gelatin	Alaska pollock	2.59; 1.53	-	-	[356]
	Atlantic salmon	-	GPAE; GPGA	49.6; 41.9	[357]
	Barbel fish	3.71-2.21	-	-	[327]
	Deer	-	GPGSPGGPL; GPVGXAGPPGK; GPM(O)GPXGVK; GPVGPSGPXGK; GPAGPXGVXGL1	1638.8; 83.3; 226.9; 93.7; 318.1	[358]
	Halibut, hake, tilapia, milkfish	-	SPGSSGPQGFTG; GPVGPAGNPGANGLN; PPGPTGPRGQPGNIGF; IPGDPGPPGPPGP; LPGERGRPGAPGP; GPKGDRGLPGPPGRDGM	101.6; 81.3; 146.7; 65.4; 76.8; 89.6	[300]
	Pork-skin, cattle-skin, fish-scale, chicken-feet	1.61; 1.65; 3.50; 3.57	GAX; GPA; GPX	>20000; 5030; 2510	[359]
	Pork	1.5	-	-	[360]
Soy		2.21-0.91	-	-	[352]
Spanish dry-cured ham		0.69	AAAAG; AAATP; ALGGA; LVSGM	8130; 6470; >10000; >10000	[361]
Tuna cooking juice		-	PGVGGPLGPIGPCYE; CAYQWQRPVDRIR; PACGGFWISGRPG	116.1; 78.0; 96.4	[362]

[a]Values from [322] are reported in percent relative potency, with 100% and 6% relative potency corresponding to IC50 ~ 5 mM and ~ 120 mM, respectively.

Studies also demonstrate the effect of hydrolysates on GLP-1 action via two different mechanisms in *in vitro* models. The cuttlefish (Sepia officinalis) viscera protein hydrolysate and bovine haemoglobin hydrolysate have been reported to enhance CCK and GLP-1 secretion from STC-1, together with an inhibition of DPP-IV activity [339, 351]. Power-Grant et al. [349] demonstrated that whey proteins hydrolysate inhibit DPP-IV activity *in vitro* and induce an insulin secretion from BRIN BD11β-cells, but only intact whey proteins enhance GLP-1 secretion from STC-1. The action of hydrolysates or peptides on GLP-1 secretion will be detailed in the following section. Another glucoregulatory function of hydrolysates has been reported as the dual action of α-glucosidase and DPP-IV inhibition [318, 321, 323].

2.2.1.2. Biopeptides as GLP-1 stimulators

Although aminoacids, protein and protein hydrolysates have already demonstrated their stimulating effect on gut hormone secretion in *in vitro* and *in vivo* experiments, there is still controversy about their role in triggering incretin secretion.

Peptones have been shown to induce GLP-1 secretion; for instance, they stimulate GLP-1 release and increase proglucagon gene expression from native L-cells in isolated vascularly perfused rat jejuno-ileum, as well as in STC-1 and GLUTag cell lines [71]. Similarly, GLP-1 secretion was triggered by peptones, and less efficiently by di-/tripeptides and non-hydrolysable Gly-Sar in colonic primary cultures. In this study, the authors demonstrated that oligopeptides stimulate GLP-1 secretion through PEPT1-dependent electrogenic uptake and activation of CaSR [316]. Furthermore, Pais et al. [363] reported that GLP-1 secretion from primary L cells was also associated with calcium influx through voltage gate calcium channels (VGCC). They also pointed out that different signalling pathways may be involved in GLP-1 secretion by complex peptide mixtures. Meanwhile, peptones and mixtures of essential amino acids have been reported to activate the ERK1/2 MAPK and p38 MAPK pathway in NCI-H716, and might provide a link to GLP-1 release [364].

Otherwise, various *in vitro* [197, 365–367] and *in vivo* [94, 368, 369] studies have reported controversial responses to GLP-1 secretion, involving different protein sources and hydrolysis rates. In humans, whey protein induces a major increase in postpandrial GLP-1 levels compared to casein protein [368]. In contrast, whey protein and another source, pea protein, were ineffective in altering GLP-1 and PYY secretion in male Wistar rats [369]. In *in vitro* studies, intact proteins and egg-hydrolysates are more potent than other protein hydrolysates (casein, pea and wheat hydrolysates) for increasing GLP-1 and CCK secretion in STC-1 cell line [197]. In contrast, intestinal digests of bovine haemoglobin protein exhibited stronger action on GLP-1 release than partially digested protein (saliva and gastric digest) [340]. Although a low-molecular fraction of wheat protein hydrolysate (LWP) enhances GLP-1 secretion in GLUTag cells, a high-molecular fraction did not. Moreover, the authors found that the LWP-triggered GLP-1 secretion involved activation of the Ca2+/calmodulin-dependent kinase II pathway mediated by G protein-coupled receptor family C group 6 subtype A

(GPRC6A) [367]. Generally, basic L-amino acids (such as L-lysine, L-arginine and L-ornithine) are known to be ligands of GPRC6A [370, 371].

Recently, three peptide sequences, ANVST, TKAVEH and KAAT, were reported to have a GLP-1 enhancing secretion capacity [339]**.** It seems that the incretin effect of proteins is associated with the amino acid profile, but the specific amino acid motif that triggers GLP-1 secretion stimulation has not yet been determined. In the literature, several studies have reported free amino acids as GLP-1 secretion stimulators, such as L-Phenylalanine, L-alanine and L-glutamine [363], L-asparagine [130], and glutamine [372]. The effect of glutamine was confirmed in healthy, obese and diabetic humans [194, 195]. Tolhust et al. [373] demonstrated this effect in isolated mouse L cells and reported that the mechanisms were associated with an increase in cAMP and cytosolic Ca^{2+} levels. Moreover, they found evidence to suggest that electrogenic sodium coupled amino acid uptake is responsible for initiating membrane depolarisation and voltage gated Ca^{2+}, while a second pathway involves increasing intracellular cAMP levels. Young et al. [374] also reported similar results with L-proline, L-serine, L-alanine, L-glycine, L-histidine, L-cysteine and L-methionine in STC-1. A tetra-glycine peptide also led to an increase in intracellular Ca^{2+} levels in NCI-H716, resulting in an increase in GLP-1 release. In contrast to other studies [373, 375], they observed no changes in cAMP. Therefore, the GLP-1 secretion by amino acids, protein and protein hydrolysates and the intracellular signalling pathways underlying stimulus-secretion coupling remain uncertain, but a range of potential signalling pathways have been postulated.

Altogether, these results reveal that bioactive peptides could be a potential therapeutic dietary compound for the prevention and management of T2DM, as they regulate glycemia homeostasis by increasing GLP-1 secretion and inhibiting DPP-IV activity. Since there are few studies that have evaluated the dual action of bioactive peptides, the screening of new hydrolysates for regulating glucose homeostasis is an area that needs to be considered.

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II. HYPOTHESIS AND OBJECTIVES

Hypothesis and objectives

The gastrointestinal tract represents the physical interface between the external environment and the circulation. Consequently, it is the first interaction between dietary components and the host. The interactions of dietary components with receptors and transporters located on the enteroendocrine cells of the intestinal tract can induce the secretion of hormones, which are profoundly involved in the control of metabolism, especially in the control of satiety and glucose homeostasis. Therefore, identifying natural bioactive compounds and the mechanism by which these modulate the secretion of hormones from entereoendocrine cells could lead to the development therapeutic agents for the treatment of obesity and glucose homeostasis disorders.

Flavanols and phenolic acids are considered the most abundant phenolic compounds in the human diet due to their wide distribution in plants and fruits. Their consumption has been associated with beneficial effects in health, and it has been reported to target different tissues of the body. Previous studies performed in our research group reported that grape seed proanthocyanidin extract (GSPE) induces changes at intestinal level, specifically modulating production and cleavage of the anorectic gut hormone glucagon like peptide 1 (GLP-1). These results were linked with glucose homeostasis improvements and thereby indicated that the enteroendocrine system could be an important GSPE target. Therefore, due to its role in body metabolic homeostasis, understanding the potential mechanisms by which GSPE modulates the enteroendocine system is important. Actually, as above mentioned, the enteroendocrine system is not only important in regulation of glucose homeostasis but also in the control of food intake. This thesis has been performed in parallel to another one in which the influence of GSPE on food intake has been studied.

While the beneficial effects of food phenolic compounds have been largely studied, bioactive peptides (BPs) are still a novel field of research. BPs encrypted within dietary protein sequence have been associated to health promoting effects. Different methods have been described to obtain the BPs, which may act upon different parts of the body. Therefore, dietary protein hydrolyzates are a promising source of potential BPs that might interact with the enteroendocrine system at the gastrointestinal level. However few studies have described an effect of BPs on GLP-1 secretion or on the enzyme responsible for GLP-1 inactivation, DPP-IV. Furthermore, very few *in vivo* studies associated the administration of BPs with glucose homeostasis improvement.

On the basis of the information described above, we hypothesized that **bioactive natural compounds might act at different intestinal levels to modulate the amount of the main active enterohormones related to satiety and glucose homeostasis**. Therefore, the main objective of thesis is to elucidate whether bioactive natural compounds, particularly GSPE and bioactive peptides derived from chicken feet, can modulate the levels of the main enterohormones by modulating different intestinal mechanisms.

The **specific objectives** proposed to fulfill the established hypothesis were:

1. To determine whether grape-seed proanthocyanidin extract directly affects the release of the main enterohormones.

Previous results reported that an acute dose of GSPE modulates GLP-1 plasma levels *in vivo*. One potential mechanism by which GSPE could exert this effect, in addition to the previously shown DPP-IV inhibition, is the direct activation of GLP-1 secretion. Hence, we aimed to elucidate if GSPE is able to directly stimulate the release of intestinal GLP-1, and if such action is extended to other enterohormones.

2. To study whether grape-seed proanthocyanidin extract affects intestinal differentiation.

Previous results showed that chronic treatment of GSPE counteract the down-regulation of Chga, GLP-1 and PYY gene expression induced by a cafeteria diet, suggesting that one possible mechanism by which GSPE could act in chronic treatment is the promotion of enteroendocrine cell differentiation. Therefore, we aimed to discover whether GSPE modulates enteroendocrine cell differentiation.

3. To study whether grape-seed proanthocyanidin extract modulates the gut microbiota and if such modulation influences the enteroendocrine system.

In the last few years, the composition of the gut microbiota and its modulation has become of growing interest due to its influence on host metabolism. Several studies reported that other extracts rich in phenolic compounds are able to modulate the composition of the gut microbiota, although none of them evaluated the crosstalk between such modulation and the enteroendocrine system. Therefore, we aimed to explore whether GSPE alters gut microbiota composition and whether such modulation might be linked to the modulation of the enteroendocrine system.

4. To evaluate whether bioactive peptides derived from chicken feet modulate the incretin system through affecting GLP-1 cleavage and secretion.

Previous results showed that GSPE improved glucose homeostasis, in part, through modulating GLP-1 levels. In order to discover new bioactive compounds that could also exert their effects by modulating the incretin system at the intestinal level, we screened protein hydrolysates derived from chicken feet. We searched for bioactive peptides that influence the incretin system, specifically GLP-1, through different mechanisms, and therefore, ameliorate glucose homeostasis.

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Hipòtesi i objectius

La primera interacció entre els components de la dieta i l'hoste la confereix el tracte gastrointestinal, el qual representa la interfície física entre l'ambient extern i la circulació. Les interaccions dels components de la dieta amb els receptors i els transportadors localitzats en les cèl·lules entreoendocrines del tracte intestinal poden induir la secreció d'hormones, les quals estan profundament involucrades amb el control del metabolisme, especialment en el control de la sacietat i de la homeòstasi de glucosa. Per tant, identificar components bioactius alimentaris i els mecanismes pels quals aquests modulen la secreció d'hormones produïdes per les cèl·lules entreoendocrines podria derivar en el desenvolupament d'agents terapèutics per al tractament de l'obesitat i dels trastorns de l'homeòstasi de la glucosa.

Flavanols i àcids fenòlics són considerats els compostos fenòlics més abundants en la dieta humana a causa de la seva àmplia distribució en plantes i fruites. La seva composició s'ha associat amb efectes beneficiosos per a la salut, sent diferents teixits del cos objectiu d'aquests efectes. Estudis previs fets en el nostre grup de recerca van mostrar que l'extracte de proantocianidines de pinyol de raïm (GSPE) indueix canvis a nivell intestinal, específicament modulant la producció i la escissió de la hormona intestinal anorètica, pèptid similar al glucagó tipus 1 (GLP-1). Aquests resultats es van relacionar amb millores en la homeòstasi de la glucosa i conseqüentment van indicar que el sistema entreoendocrí pot ser un objectiu d'acció estratègic per GSPE. Per tant, a causa del seu rol en la homeòstasi del metabolisme de l'organisme, és rellevant entendre els potencials mecanismes pels quals GSPE modula el sistema enteroendocrí. A més a més, tal com s'ha mencionat anteriorment, el sistema enteroendocrí no solament és important en la regulació de l'homeòstasi de la glucosa, sinó que també en el control de la ingesta. Aquesta tesi doctoral s'ha realitzat paral·lelament amb una altra en la que s'ha avaluat la influència del GSPE sobre la ingesta.

Hi ha força estudis que han investigat els efectes beneficiosos dels compostos fenòlics alimentaris, en front això, els pèptids bioactius (BPs) són un camp innovador de recerca. Els BPs encriptats dins de les seqüències de proteïnes dietètiques s'han associat en efectes promotors de la salut. S'han descrit diferents mètodes per obtenir aquests BPs, els quals poden actuar sobre diferents parts de l'organisme. Els hidrolitzats de proteïnes dietètiques són per tant una font prometedora de potencials BPs els quals poden interactuar amb el sistema enteroendocrí a nivell intestinal. Tot i que pocs estudis han descrit l'efecte dels BPs sobre la secreció de GLP-1 o sobre l'enzim responsable de la inactivació de GLP-1. D'altra banda, molts pocs estudis *in vivo* han associat l'administració dels BPs amb la millora de la homeòstasi de la glucosa.

D'acord amb l'explicat anteriorment, es va hipotetitzar que **els compostos naturals bioactius poden actuar a diferents nivells intestinals modulant les quantitats de les principals enterohormones actives relacionades en la sacietat i en la homeòstasi de la glucosa.** Així doncs, el principal objectiu d'aquesta tesi doctoral es determinar si els compostos naturals bioactius, particularment GSPE i els pèptids bioactius procedents de pota de pollastre, poden modular els nivells de les principals enterhormones mitjançant la modulació de diferents mecanismes intestinals.

Els objectius específics proposats per resoldre la hipòtesi establerta són:

1. Determinar si l'extracte de proantocianidines de pinyol de raïm afecta directament la secreció de les principals enterohormones.

Resultats previs van descriure que una dosi aguda de GSPE modula els nivells plasmàtics de GLP-1 *in vivo.* Un mecanisme pel qual GSPE podria exercir aquest efecte, a més a més de la inhibició de DPP-IV que s'ha descrit anteriorment, és la directa activació de la secreció de GLP-1. Per tant, es va voler determinar si GSPE és capaç d'estimular directament la secreció de GLP-1 intestinal, i si aquesta acció s'estén a altres enterohormones.

2. Estudiar si l'extracte de proantocianidines de pinyol de raïm afecta a la diferenciació intestinal.

En estudis previs es van observar que els tractaments crònics de GSPE contrarestaven la disminució de l'expressió gènica de ChgA, GLP-1 i PYY induïda per una dieta de cafeteria, suggerint que un possible mecanisme pel qual GSPE podria actuar en els tractaments crònics és la promoció de la diferenciació de les cèl·lules enteroendocrines. A partir d'aquests resultats, es va voler descobrir si GSPE modifica la diferenciació de les cèl·lules enteroendocrines.

3. Estudiar si l'extracte de proantocianidines de pinyol de raïm modifica la biota intestinal i si aquesta modulació influeix el sistema enteroendocrí.

En els darrers anys, ha augmentat l'interès sobre la composició de la biota intestinal i la seva modulació degut a la seva influència en el metabolisme de l'hoste. Alguns estudis han descrit que altres extractes rics en compostos fenòlics tenen la capacitat de modular la composició de la biota intestinal, encara que cap d'aquests van avaluar la interferència entre aquesta modulació i el sistema enteroendocrí. Partint d'aquesta base, es va voler explorar si GSPE modifica la composició de la biota intestinal i si aquesta modulació pot estar relacionada amb la modulació del sistema enteroendocrí.

4. Avaluar si els pèptids bioactius procedents de pota de pollastre modulen el sistema d'incretines afectant la secreció i escissió de GLP-1.

Resultats previs van descriure que GSPE millora la homeòstasi de glucosa, en part, per mitjà de la modulació dels nivells de GLP-1. Per descobrir nous compostos bioactius els quals puguin també exercir els seus efectes mitjançant la modulació dels sistema d'incretines a nivell intestinal, es van examinar hidrolitzats proteics procedents de pota de pollastre. Es van buscar pèptids bioactius que influeixin a l'hormona incretina GLP-1, mitjançant diferents mecanismes, i conseqüentment, que millorin la homeòstasi de la glucosa.

III. RESULTS

CHAPTER 1

Acute selective bioactivity of grape seed proanthocyanidins on enteroendocrine secretions in the gastrointestinal tract

Àngela Casanova-Martí, Joan Serrano, M Teresa Blay, Ximena Terra, Anna Ardévol and Montserrat Pinent

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PREFACE

In the first manuscript, we focused on studying the acute effect of grape-seed proanthocyanidin extract (GSPE) on the main enterohormones secretion (**objective 1**).

An *ex vivo* model was employed to elucidate if GSPE directly acts on enteroendocrine cells modulating the secretion of enterohormones. The *ex vivo* model enables us to study the specific hormone secretion patterns of different subtypes of enteroendocrine cells distributed along of the gastrointestinal tract. Moreover, to simulate the *in vivo* conditions, the intact extract was employed to treat upper-intestine sections, while GSPE metabolites obtained from caecum extraction after GSPE administration were employed to treat colon segments.

Acute selective bioactivity of grape seed proanthocyanidins on enteroendocrine secretions in the gastrointestinal tract

Àngela Casanova-Martí*, Joan Serrano*, M Teresa Blay, Ximena Terra, Anna Ardévol & Montserrat Pinent

MoBioFood Research Group. Departament de Bioquímica i Biotecnologia, Universitat Rovira i Virgili, c/Marcel·lí Domingo nº1, 43007 Tarragona, Spain

*These authors contributed equally to this work.

ABSTRACT

Background: Intestinal enteroendocrine cells respond to food components by secreting an array of hormones that regulate several functions. We have previously shown that grape seed proanthocyanidins (GSPE) modulate plasma GLP-1 levels.

Objective: This study aimed to deepen on the knowledge of the mechanisms used by GSPE to increase GLP-1, and extend it to its role at modulation of other enterohormones.

Design: We used an *ex vivo* system to test direct modulation of enterohormones; STC-1 cells to test pure phenolic compounds; and rats to test the effects *in vivo* at different gastrointestinal segments.

Results: Our results show that GSPE compounds act at several locations along the gastrointestinal tract modulating enterohormone secretion depending on the feeding condition. GSPE directly promotes GLP-1 secretion in the ileum, while unabsorbed/metabolized forms do so in the colon. Such stimulation requires the presence of glucose. GSPE enhanced GIP and reduced CCK secretion, and gallic acid could be partly responsible for this effect.

Conclusions: the activity of GSPE modulating enterohormone secretion along the gastrointestinal tract may help to explain its effects at regulation of food intake and glucose homeostasis. We show that GSPE acts through several mechanisms; compounds found in GSPE and their metabolites act as GLP-1 secretagogues in ileum and colon, respectively. *In vivo* GLP-1 secretion might also be mediated by indirect pathways involving modulation of other enterohormones that in turn regulate GLP-1 release, such as enhancing GIP and reducing CCK secretion in the duodenum.

Keywords: Phenolic compounds, ileum, colon, enterohormone, GLP-1

INTRODUCTION

The intestinal endocrine cells are the largest organ in the human body. They are responsible for the secretion of enterohormones such as glucagon-like peptide-1 – (GLP-1) and glucose-dependent insulinotropic peptide (GIP) (together called the incretins), peptide tyrosine tyrosine (PYY), cholecystokinin (CCK), and ghrelin, which are involved in the modulation of food intake, digestion, insulin secretion and metabolism. New functions for these hormones are still being studied. The modulation of intestinal enterohormone pathways has attracted increasing interest in the fight against wide spread pathologies such as obesity and type 2 diabetes. Incretin-based therapies have been established for type 2 diabetes and involve the use of GLP-1 analogues to increase GLP-1 receptor agonist concentrations in the pharmacological range and dipeptidyl peptidase-4 (DPP-4) inhibitors to prevent the degradation of endogenous GLP-1 and GIP, which are both substrates for the DPP-4 enzyme, elevating their plasma levels (1). The most effective way to increase enterohormones, especially GLP-1 and PYY, is the bariatric bypass known as Roux-en-Y, which is used to treat obesity and also leads to normalization of glucose homeostasis in diabetic patients. GLP-1 secretagogues are therefore in the spotlight as promising therapies against type 2 diabetes and for weight management.

Although studies are being carried out on pharmacological compounds, there are also natural products with capabilities to enhance enterohormone levels, and these could be used as a complementary therapy or in the area of functional foods. They have been reviewed, and more recently (2,3). Even for these types of product, a complete description of their mechanisms of action is compulsory if their use is to be recommended for a target population. In this regard, grape seed proanthocyanidin extract (GSPE) has been shown to modulate glucose homeostasis and food intake, and this in part is mediated through increases in plasma GLP-1 levels (4,5). GSPE has DPP4 inhibitory properties, but its effects on GLP-1 secretion need further analysis. *In vivo* studies revealed that increases in plasma GLP-1 levels are only observed after a glucose or meal load, which raises the question of whether GSPE compounds might directly stimulate secretion or modulate the interaction of nutrients with the enteroendocrine cells, for instance making it possible for them to reach more distal parts of the intestine. It has been suggested that the monomeric flavanols found in GSPE bind and activate bitter taste receptors, which, in turn, are regarded as interesting targets to modulate enterohormone secretion.

In this study we aim to deepen on the knowledge of the mechanisms used by GSPE to increase GLP-1 levels, and to determine whether it also modulate other enterohormones.

MATERIALS AND METHODS

Materials

GSPE was obtained from Les Dérivés Résiniques et Terpéniques (Dax, France). The same batch (#124029) was used in all the studies. According to the manufacturer, the extract contains monomeric (21.3 %), dimeric (17.4 %), trimeric (16.3 %), tetrameric (13.3 %) and oligomeric (5–13 U; 31.7 %) proanthocyanidins. The small molecules were previously characterized by liquid chromatography-tandem mass spectrometry (6). A detailed phenolic composition of this GPSE is included in Supplemental **Table 1**. (-)-Epicatechin (EC) and gallic acid (GA) were obtained from Sigma (St. Louis, USA). (-)-Epicatechin gallate (ECg) and procyanidin dimer B2 (B2) were obtained from Extrasynthese (Genay, France). The procyanidin dimer B2-gallate (B2g) was obtained from TransMTT (Gieβen, Germany). For all the studies, stocks were prepared in dimethylsulfoxide (DMSO) and further diluted in the specific buffer required for each experiment.

Cell culture STC-1

The STC-1 clonal cell line was accepted as a generous gift from Dr. B. Wice (Washington University of St. Louis) with the permission of Dr. D. Hanahan (University of California, San Francisco, CA). This enteroendocrine cell line was derived from a double-transgenic mouse tumor (7) and cultured in Dulbecco's modified Eagle's medium (DMEM) with GlutaMAX containing 4.5 g/l D-glucose, without sodium pyruvate (Thermo Fisher Scientific, Madrid, Spain), supplemented with 17.5% foetal bovine serum, 100 U/ml penicillin, and 100 mg/l streptomycin (BioWhittaker, Barcelona, Spain), and incubated in a 5% CO2-humidified atmosphere at 37 °C. Cells were used between passage numbers 30-50.

Cellular membrane potential of STC-1

To evaluate membrane potential, STC-1 cells were seeded in a 96-well culture plate at a density of 70,000 cells/well for 2 days until they reached 80-90% confluence. The cellular membrane potential (ΔΨcell) was determined in accordance with the method described by Gonzalez-Aubin et al. (8). Briefly, the ΔΨcell was evaluated using fluorescent probe DIBAC4 diluted in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic

acid (HEPES) with 10 mM glucose, which was monitored with excitation and emission filters set at 493 nm and 516 nm- respectively. Labeled cells were stimulated with pure compounds and added to a final concentration of 1, 10, 100 and 200 μM.

Enterohormone release from STC-1

For secretion studies, 2.0×10^5 cells/well cells were seeded in 24-well culture plates for 2 days to enable 80-90% confluence to be reached. On the day of the experiment, the cells were washed twice with HEPES (20 mM HEPES, 140 mM NaCl, 4.5 mM KCl, 1.2

mM CaCl2, and 1.2 mM MgCl2 at pH 7.4). Pure phenolic compounds dissolved in HEPES buffer with 10 mM glucose were added to each well, using HEPES buffer 0.05% DMSO as vehicle. After an incubation period of 2h at 37ºC, supernatants were collected, centrifuged to remove remaining cells and stored at -80ºC until used to determinate hormone concentration.

Enterohormone release from intestinal segments

Rats were killed and their intestines dissected out. For hormone analysis, samples were collected from three different positions along the GI tract: proximal duodenum, distal ileum and ascending colon. The tissue was rinsed with ice-cold Hank's balanced salt solution (HBSS; Thermo Fisher Scientific, Madrid, Spain) and dissected in segments of tissue (0.75 cm2). After a 10 min washing period, tissue segments were placed in prewarmed (37 ºC) Krebs buffer 0.1% DMSO containing the compounds to be tested with 10 mM glucose or without glucose and 0.1 mM Diprotin A (Enzo Life Sciences International, New York, USA)). Duodenal and ileum segments were treated with GSPE or GA and colonic segments were treated with phenolic metabolites from caecal content for 1h in a humidified incubator at 37ºC and 5% CO2. To obtain the phenolic metabolites, rats (n=7) were administered a GSPE dose of 500mg/kg BW by intragastric gavage 80 min before sacrifice and the caecal content was extracted, with the phenolic content of the caecum of non-treated rats being used as a control. The caecal mass (1 g) was dissolved in 10ml/g PBS (pH 2) and the phenolic compounds were extracted twice with 10ml/g ethyl acetate. The organic fraction was nitrogen-dried overnight and reconstituted in 3ml Krebs buffer 0.1% DMSO for the treatments (9,10).

Tissue viability was checked by the absence of the cytoplasmic marker lactate dehydrogenase (LDH) in the incubated solutions. LDH was analysed using an LDH kit (QCA (Tarragona, Spain)).

Animals and experimental design.

Two sets of female Wistar rats -weighing 180–200 g- were obtained from Harlan (Barcelona, Spain). The subjects were single housed at 22 °C under a 12 h light/dark cycle (lights on at 8 am) with access to standard chow pellets (Teklad Global Diets #2014, Harlan, Barcelona) and tap water ad libitum during a 1 week adaptation period. All procedures were approved by the Experimental Animal Ethics Committee of the Universitat Rovira i Virgili.

For the acute treatment in fasting conditions, overnight fasted female rats (n=5) were treated with 1g/kg GSPE at the end of the dark period by an oral gavage administration. Vehicle (tap water)-treated rats (n=5) were used as a control group. The abdominal cavity was incised and the portal vein catheterized while body temperature was monitored. Portal blood was obtained at 60 minutes under sodium

pentobarbital anaesthesia before the rats were killed by exsanguinations of the aortal vein.

To assess the effects of an acute dose of GSPE in feeding condition, female rats were treated as previously described (11). In brief, animals were fasted from 15.00 h until 18.00h and then treated with 1 g/kg GSPE (*n*=11) or vehicle (*n*=10) (tap water) by an oral gavage administration. The animals were then anaesthetized with 70 mg/kg BW i.p of sodium pentobarbital and the portal vein was catheterized and at 60 min after the dose, 5 ml mash containing 1.5 g of standard chow and 25 mg of xanthan gum as a stabilizer was punctured into the forestomach with an Abbocath-T 18G catheter (Hospira, Lake Forest, IL, USA) at a constant rate of 1 ml/min. Portal blood samples were obtained at 80 min from the beginning. After the 120 min procedure, the animals were sacrificed by exsanguination of the aortal vein.

In both models, intestinal segments from the duodenum, jejunum, ileum and proximal colon were dissected, immediately frozen in liquid nitrogen and then stored at -80º C for further enzyme activity and gene expression analysis.

Enterohormone and glucose quantification

The active GLP-1 concentration from STC-1, intestinal segments and plasma samples was analysed with a GLP-1 3-37 amide ELISA kit (Millipore, Billerica, MA, USA). Total CCK from STC-1 and plasma samples were analysed with a CCK enzyme immunoassay (EIA) kit (Raybiotech, Norcross, GA, USA) and duodenal segments with a CCK8 (desulfated) EIA Kit (Peninsula Laboratories, San Carlos, CA, USA). Total GIP levels from duodenal segments were analysed by a total GIP ELISA kit (Millipore, Billerica, MA, USA). PYY from intestinal segments and plasma samples were measured using a fluorescent immunoassay kit (Phoenix Pharmaceuticals, Burlingame, CA, USA). Glucose plasma levels were analysed with an enzymatic colorimetric kit (glucose oxidase-peroxidase method; QCA, Tarragona, Spain).

Measurement of glucose-6-phosphatase enzyme activity from liver and intestine

Liver and intestinal mucosa activities were determined following a modified version of the previously described protocol (12). Tissues were homogenized in 0.1 M cacodylate buffer (pH 6.5) using a Qiagen Tissuelyser (Qiagen, Hilden, Germany). The suspension was centrifuged and the supernatant incubated in the buffer containing 10 mM glucose-6-phosphate at 37º for 20 min. The reaction was stopped at different times of point by adding 100 g/L trichloroacetic acid. Glucose-6-phosphatase (G6Pase) activity was determined by measuring the amount of glucose release from glucose-6-phosphatase using a glucose oxidase/peroxidase coupling system. To assess G6Pase liver activity, the increase of glucose production was measured using an enzymatic colorimetric kit (glucose oxidase-peroxidase method; QCA, Tarragona, Spain). G6Pase intestinal activity was assayed following Petrolonis et al. (13), using an Amplex® Red Glucose/Glucose Oxidase Assay Kit (Thermo Fischer Scientific, Barcelona, Spain). Both

enzymatic activities were normalized with mg of protein, which was analysed with a BCA Protein Assay Kit (Thermo Fischer Scientific, Barcelona, Spain), using bovine serum albumin as standard.

Quantitative real-time RT-PCR analysis

Total RNA was extracted using Trizol (Thermo Fisher Scientific, Madrid, Spain) and trichloromethane-ethanol (Panreac, Barcelona, Spain), and purified using a Qiagen RNAeasy kit (Qiagen, Hilden, Germany). The complementary DNA (cDNA) was generated using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, USA). Quantitave polymerase chain reaction amplification amplification was performed using specific TaqMan probes (Applied Biosystems, Waltham, USA) and the relative expression of each gene was calculated against the control group using the 2-ΔΔCt method, with cyclophilin A (PPIA) as reference.

Statistical analysis

Results are presented as mean ± SEM. Data were analysed with SPSS (IBM, Chicago, USA). Data from intestinal segments and STC-1 hormones, gene expression, enzyme activity and glucose plasma levels were analysed by Student t-tests. The dose-response effect of pure compounds on cellular membrane potential was analysed by one-way ANOVA. Significance was accepted over 5%.

RESULTS

GSPE stimulates enterohormone secretion *ex vivo*

The effects of GSPE on enterohormone secretion were tested in an *ex vivo* intestine model. Bearing in mind that the enteroendocrine cell type is specialized in expressing different hormones throughout the intestinal tract, GIP and CCK were studied in duodenal segments and GLP-1 and PYY in ileum and colon segments (14). GSPE showed a selective effect at stimulating enterohormone secretion, since in duodenum explants 0.2 mg GSPE/ml (0.17 mg phenolics/ml) increased GIP but decreased CCK secretion (**Table 1**). In ileum explants the same GSPE dose significantly increased secretion of GLP-1 and, to a lower extent, that of PYY (**Table 1**). Lower concentrations of GSPE were also tested and showed no stimulation of GLP-1 secretion (values normalized to those of controls: 1.00 ± 0.08; 1.14 ± 0.12; 1.22 ± 0.32 for control, 0.1 mg GSPE /ml and 0.08 mg GSPE /mL, respectively).

Table 1
Increase in enterohormone concentration in the medium due to GSPE or GA treatment in explants from different intestinal segments.

	Duodenum		Ileum	
	GIP	CCK	GLP1	PYY
GSPE (0.2 mg/mL)	3.32 ± 2.0*	- 0.46 ± 0.0*	2.37 ± 0.6*	1.10 ± 0.3*
GA (31 µg/mL)	0.16 ± 0.2	-0.53 ± 0.1*	2.17 ± 1.2	-

Results are expressed as relative units v. control. * statistically significant differences at p≤0.05, t-student.

In the gastrointestinal tract GSPE is partially absorbed and metabolized, so digested GSPE was used to evaluate the effect at colon. Proximal colonic sections were incubated for 1 hour with non-absorbed phenolic metabolites obtained from the caecal content of animals treated with GSPE and the caecal content of non-treated rats as control. The phenolic concentrations of treated and control colon sections were 188.44 ± 7.02 mg phenolics/l and 88.76 ± 20.64 mg phenolics/l respectively. The caecal content of GSPE-treated rats enhanced GLP-1 secretion and tended to increase PYY levels (**Table 2**).

Table 2
GLP1 and PYY secretion to the medium in colon explants after a 1-hour treatment with digested GSPE

	GLP1	PYY
Control	1.00 ± 0.0	1.00 ± 0.0
Digested GSPE	1.34 ± 0.1*	1.32 ± 0.2#

Results are expressed as relative units v. control. * P<0.05 v. control; # P<0.05 v. control, t-student.

Since GSPE is a complex mixture of several compounds, we aimed to identify those mainly responsible for the modulation of enteroendocrine secretions working with pure flavanol compounds. Given that some of these compounds could only be obtained in very small amounts, we had to use STC-1 cell line which secretes GLP-1 and CCK in a reproducible way (15,16). The *in vitro* results showed an inhibition of GLP-1 and CCK levels by 200 µM of ECG and B2 (**Fig. 1**). Similarly, the same concentration of B2g decreased GLP-1 secretion, respectively (**Fig. 1**). A lower concentration showed no differences among monomers in the CCK secretion, while a decrease was observed using 1 µM of dimer B2 (Fig. 1B). The inhibition of hormone secretion by high (200 µM) doses of compounds was in agreement with a cell membrane hyperpolarization found when cell membrane potential was assessed (**Fig. 1**).

Gallic acid is a non-flavanol phenolic compound found in significant amounts in grape-seed derived extracts (Supplemental **Table 1**). Its effects were assayed in the *ex vivo* explants and in STC-1 cells. In *ex vivo* explants, 6.2 µg/ml of GA caused no significant

effect in stimulating enterohormone secretion and, this dose is within the range of concentration found in 0.2 mg GSPE/ml. A dose five times higher (31 µg/ml of GA) significantly decreased *in vitro* GLP-1 secretion (values normalized to those of the controls: 1.00 ± 0.10 and 0.72 ± 0.07 for control and 31 µg GA /ml, respectively, $p<0.05$). In *ex vivo* explants this dose caused no significant effect on PYY or GLP-1 release, but it inhibited CCK release to the medium by around 50%, similarly to GSPE (**Table 1**).

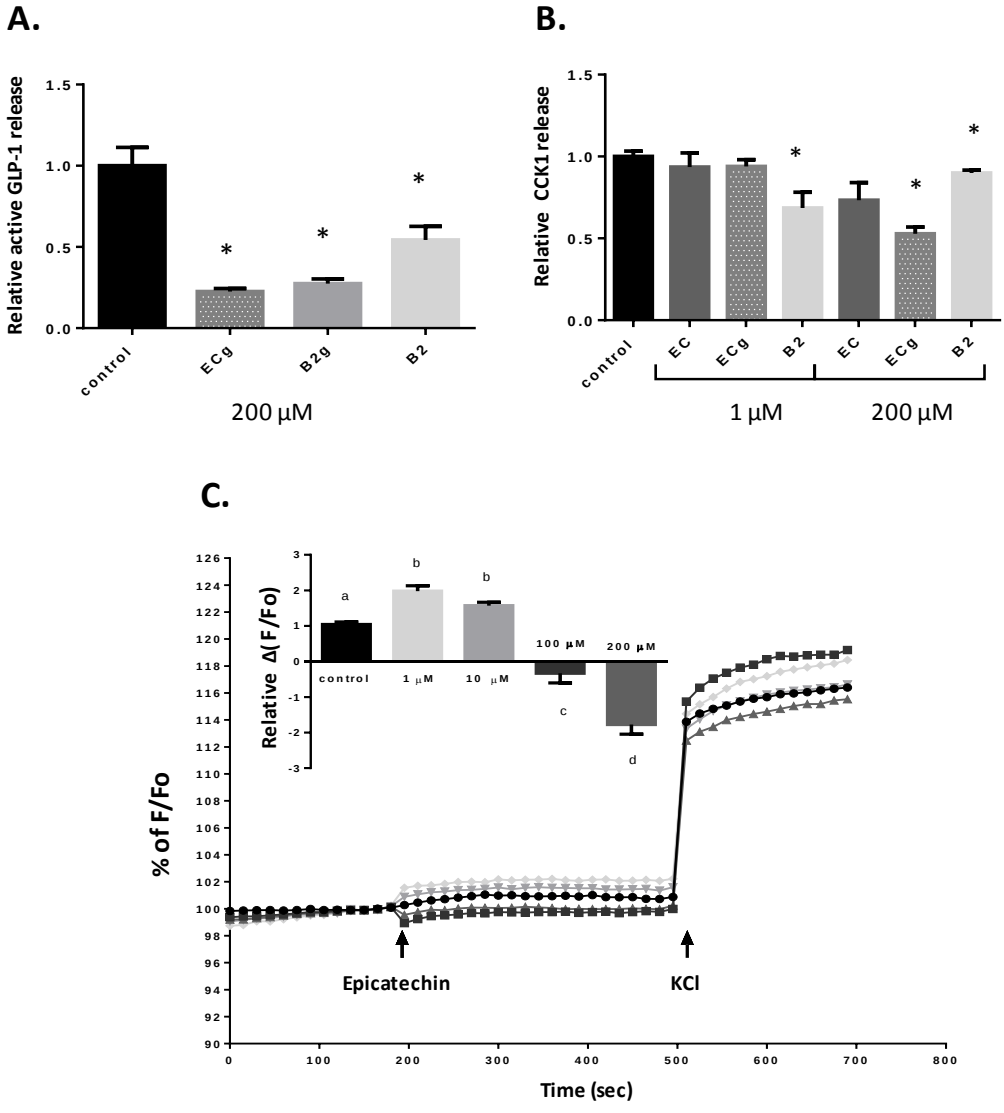

Fig. 1. Effects of flavonols on enterohormone secretion and cellular membrane potential in STC-1 cells. STC-1 cells were treated for 2 hours with 200 µM and 1 µM of different flavanols found in GSPE. GLP-1 (A) and CCK (B) levels were measured in the culture medium. Effects of flavanols on cellular membrane potential after epicatechin stimulation expressed as % F/F_0 and relative $\Delta(F/F_0)$ (normalized to the control cells) (A), where F is fluorescence at 195 s and F0 is basal fluorescence at 180 s. The data are displayed as the mean ± *SEM*. * statistically significant differences versus controls at $P < 0.05$. **a,b,c,d**, statistically significant differences at $P < 0.05$.

GSPE affects colonic enterohormone gene expression depending on the feeding condition

In vivo we previously found that GSPE increases portal active GLP-1 only in the presence of glucose in the intestine (4). We next analysed how the feeding condition affected GSPE action throughout the intestinal tract by comparing overnight fasted animals to fed animals. Table 3 shows that 1g GSPE /kg bw load in fasted animals led to a reduced expression of GLP-1, PYY and CCK in the colon. Conversely, the plasma levels of these enterohormones were not significantly changed (Supplemental **Table 2**).

In animals that after a 1-hour treatment with GSPE were administered food for 1 more hour, we found no differences in enterohormone gene expression. In our experimental conditions, there were also no differences in the gene expression of these hormones between the fed and the fasted control animals. As previously published, these animals showed a modified enterohormone plasma profile (5). We also tested the effects of GSPE in a cell differentiation marker previously shown as a target for GSPE, chromogranin (CgA). **Table 3** shows that a high acute dose, independently of assay conditions (fed or fasted), increases gene expression.

Table 3
Gene expression of the enterohormones from fasted and fed rats.

		conditions	
		fasted	**fed**
GLP-1 colon	Ctrl	1.06 ± 0.2	1.12 ± 0.2
	1g GSPE/Kg bw	0.53 ± 0.0*	1.07 ± 0.2
PYY colon	Ctrl	1.05 ± 0.2	1.13 ± 0.2
	1g GSPE/Kg bw	0.40 ± 0.0*	1.32 ± 0.3
CCK colon	Ctrl	1.01 ± 0.1	1.81 ± 0.8
	1g GSPE/Kg bw	0.36 ± 0.2*	1.09 ± 0.3
Cga colon	Ctrl	1.11 ± 0.3	1.00 ± 0.0
	1g GSPE/Kg bw	2.76 ± 0.5*	2,94 ± 0.5*

Results are expressed as relative units v. control. * statistically significant differences at p≤0.05, t-student

Role of glucose in the effect of GSPE's stimulation of GLP-1 release

Since GSPE increases GLP-1 levels only after feeding (Supplemental **Table 2**) or an oral glucose load, we next used our *ex viv*o model to analyse whether glucose was required for the direct GSPE stimulation of GLP-1 release. The stimulation of GLP-1 secretion achieved by GSPE in medium containing glucose 10 mM (shown in **Table 1**) was not observed in medium without glucose or with only glucose (1.00 ± 0.1, 1.21± 0.1 and 1.17 ± 0.1 in 0 mM glucose, 10 mM glucose and 0.2 mg of GSPE/ml with 0 mM glucose, respectively; data normalized by 0 mM glucose).

In our explant system, glucose can reach the cells either from the apical or the basolateral side. To estimate the possible contribution of basolateral glucose presence, we measured portal glucose levels in the fed and fasted animals. **Fig. 2** shows that portal levels of glucose in overnight-fasted animals treated with GSPE were around 10 mM, while in animals that after 1 hour of GSPE treatment were administered a food load, 20 min after this load glucose levels were around 8 mM (significantly different from the fasted animals p≤0.05). In the fed model the glucose levels of GSPE-treated animals did not differ from the controls, while in the fasted animals GSPE significantly increased portal glucose (**Fig. 2**).

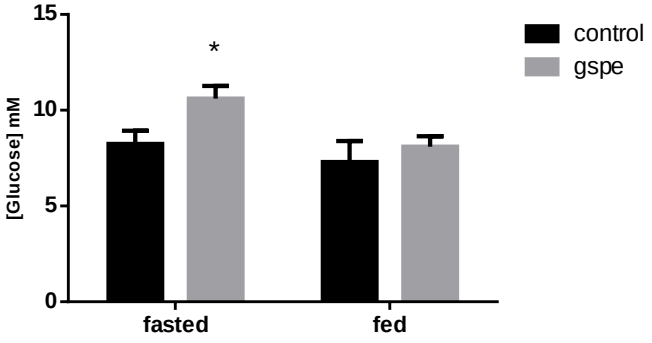

Fig. 2. Effects of GSPE on portal glucose. In the fasted group, portal glucose levels of O/N fasted animals treated with GSPE for 1 hour. In the fed group, 4 hours-fasted animals were treated with GSPE for 1 hour, and then administered a food load. Portal glucose levels were measured 20 minutes after this food load. * statistically significant differences versus controls at $P < 0.05$

Finally we analysed whether GSPE modulation of portal glucose levels involved modulation of intestinal gluconeogenesis. To do this, G6Pase activity was measured in the duodenum and jejunum sections of fasted and fed animals. **Table 4** shows that GSPE inhibits G6Pase activity. In the fasted animals GSPE inhibited around 60% of intestinal activity (in both measured sections), and in the fed animals this inhibition was stronger, reaching 80% (also in both measured sections). We also measured it in the liver of fed animals and found that there was no change in this enzyme's activity due to GSPE treatment (1.00 ± 0.1, 1.02 ± 0.1 in control and treated animals respectively, data normalized by controls).

Table 4
Glucose-6-phospatase intestine activity from fasted and fed rats.

		conditions	
		fasted	fed
G6Pase duodenum	Ctrl	1.00 ± 0.2	1.00 ± 0.2
	1g GSPE/Kg bw	0.31 ± 0.0*	0.24 ± 0.0*
G6Pase jejunum	Ctrl	1.00 ± 0.2	1.00 ± 0.2
	1g GSPE/Kg bw	0.45 ± 0.1*	0.21 ± 0.0*

DISCUSSION

We have previously shown that GSPE increases plasma active GLP-1 levels, that this is in part due to inhibition of DPP4 but also to increased secretion, and that this leads to modulation of glucose homeostasis and satiety (4,5). In this paper, we describe for the first time how GSPE directly modulates enteroendocrine secretions and that it does so differently depending on the intestinal fragment. We also go further into the possible mechanisms for GSPE to exert such modulation of enterohormone secretion and production.

In this study we have used an *ex vivo* system to show that GSPE directly stimulates GLP-1 release in the intestine. Direct stimulation could therefore contribute to the previously reported increase in GLP-1 levels (4). Concerning the mechanisms, we have described that this direct GLP-1 release by GSPE stimulation requires glucose. Furthermore, glucose must be sensed on the luminal side. This agrees with acute *in vivo* results in which, to observe GSPE effects on GLP-1 secretion, either glucose or meal must be added (4,5). Our results suggest that the effects of GSPE are not secondary effects on glucose absorption and/or metabolism, but direct effects on enteroendocrine cells. A possible explanation for the requirement of glucose could involve a 'priming' effect by GSPE of the L-cell in preparation for the subsequent oral glucose-stimulated GLP-1 secretion. This 'priming effect' has previously been shown for ghrelin (17) and insulin (18). Ghrelin pretreatment of GLUTag and NCI-H716 cell lines stimulates GLP-1 release only in a medium containing glucose, similarly to what we observe in GSPE treatments. Furthermore, *in vivo* ghrelin induces GLP-1 release only when an oral glucose load is performed, which is also in concordance with our previous in vivo results (4). Unfortunately, these priming events are still not fully described at the molecular level. Light has only been shed on the requirement of the mitogenactivated protein kinase (MAPK) pathway and MEK-ERK1/2 (17,18), which is a pathway previously shown to be a target for grape seed proanthocyanidins (19).

An unexpected increase in portal glucose levels by GSPE was observed in fasted animals, and we wanted to analyse the role of intestinal gluconeogenesis in this increase. Intestinal gluconeogenesis has been shown as a mechanism used by different food agents to modulate feeding behaviour (20). Our results suggest an inhibition of intestinal gluconeogenesis by acute GSPE treatment. The increased portal glucose may therefore be due to reduced glucose uptake by the liver and pancreas, since polyphenols have been previously shown to inhibit the glucose transporter Glut-2 (21,22) and GSPE down-regulates the glucose transporter Glut-2 and glucokinase expression in liver and pancreas (23). Certainly, considering that glucose may control hunger sensation from the portal vein via signalling to the peripheral neural system (24), increasing portal glucose levels during fasting could, at least in part, contribute to the previously reported (5) satiating effects of GSPE.

GSPE is composed of several different molecules with different bioavailability (25). Polyphenol absorption in the small intestine is in fact relatively low (5%–10%) in comparison to other macronutrients or micronutrients, mainly those with monomeric and dimeric structures (26). The remaining 90%–95% of polyphenols, mainly the polymeric and oligomeric forms, pass through the large intestinal lumen and accumulate in a millimolar range, reaching the colon where they are subjected to microbial catabolism(27). As shown in rats treated with the same extract as ours, some final products of colonic metabolites such as 3-*O*-methylgallic acid and benzoic acids could be detected in the kidneys and liver after 2 hours (25), showing that some GSPE compounds, most likely polymeric forms, circulate through the GI tract and reach the colon. Our results show that GSPE treatment decreased CCK levels in duodenum segments. We had previously shown that *in vivo* GSPE impaired CCK release after food intake (5). Inhibition of CCK *ex vivo* is reproduced by gallic acid, a compound found in the extract mixture. The effects of mainly monomeric and dimeric structures of GSPE were also tested in STC-1 cells, and our data show an inhibition of CCK levels. This suggests that molecules that are well absorbed in the upper intestine could be responsible for this direct inhibition. We also found that this inhibition does not lead to a modulation of CCK basal plasma levels in fasted animals (where CCK release is not stimulated). Regarding GLP-1 secretion, which as previously mentioned, was increased by GSPE in ileum segments, our *in vitro* results in STC-1 cells show that GLP-1 levels are also decreased by a low degree of polymerization structures. Our results also show that metabolites of digested GSPE promote colonic GLP-1 release. In contrast to CCK secretion, these findings could suggest that unabsorbed polyphenols (high degree of polymerization) and microbiota-metabolized polyphenols of GSPE act on endocrine cells to promote GLP-1 secretion. In agreement with this, a previous study demonstrated that a tetrameric procyanidin increases GLP-1 levels in mice and is more effective to insulin stimulation than smaller procyanidins (28). Montagut et al. also demonstrated that oligomers can activate insulin signalling and stimulate glucose uptake (19). Our findings suggest that the absorption and bioavailability of GSPE polyphenols could be involved in enteroendocrine secretion, although further study is needed to understand procyanidin's effects on enterohormone secretion in more detail.

Another function that might be modulated by GSPE is the inhibition of G6Pase activity, since the levels of inhibition are the same in the different parts of the intestine where it has been measured (i.e. duodenum and jejunum). The inhibition of G6Pase was dependent on the feeding condition. We analysed two animal models that received 1h GSPE treatment, one sacrificed in an overnight fasting condition and the other fasted for 3 hours and then administered a food load in the stomach 60 min after the GSPE dose to determine the effect of feeding. Our results show that inhibition of G6Pase was much stronger in the fed animals. In addition, the feeding condition also determined the effects of GSPE at gene expression level. We found a down-regulation of GLP-1, PYY and CCK at colon level in fasted animals treated for 1 hour with GSPE. Modulation of

gene expression of enterohormones has been studied at the level of intestinal stem cell differentiation (29,30). Some studies show that the messenger RNA (mRNA) of enterohormones can also be acutely regulated. In rodents, refeeding after a fasting period (31) or feeding a specific nutrient such as palmitoleic acid (32) modulates the mRNA levels of enterohormones within a short time (1-2 hours). *In vitro* models have also shown acute modulation of enterohormone gene expression by hormones such as insulin (33,34) and nesfatin (35,36). In addition, our results show that when a food load was administered after the GSPE, there were no differences between GSPE and controls. This suggests that the effects of nutrients (either directly or mediated by changes in hormones) counteract the down-regulation of GSPE. It should be noted that the fed study was performed after a shorter (3 hours) fasting period, so we cannot be sure that GSPE had the same effects on gene expression as in our fasting (overnight) experiment. Certainly, the fasted animals where down-regulation was observed showed no differences in GLP-1 and CCK plasma levels. At present we do not know whether such down-regulation at colonic levels might influence enterohormone secretion or if it is involved in the previously mentioned "priming"effect. We also observed that a previously described target of GSPE (37), chromogranin A (CgA), was up-regulated in both conditions. CgA is a marker for enteroendocrine cells (38), but it is unlikely that such short periods of treatment would already affect the number of enteroendocrine cells in the colon. There is a lack of information regarding acute regulation of CgA expression in the intestine. However, future work will focus on the effects of GSPE on the differentiation of enteroendocrine cells. GSPE treatment produced a significant induction of other enterohormone secretion in different parts of the intestine. In the duodenum, GSPE directly enhances GIP secretion, which could contribute to *in vivo* GLP-1 secretion due to the enteroendocrine loop between the duodenal GIP and the ileal GLP-1 (39). In the ileum, GSPE also promotes PYY secretion, to a lesser extent than that of GLP-1, while a trend data was observed in the colon. It was shown that soy isoflavones enhance PYY secretion in humans (40), although there are few data on the effects of polyphenols on PYY secretion. Altogether, these results reinforce the idea that GSPE has effects throughout the gastrointestinal tract, and that a feeding condition modulates the effects. Further studies are needed to go into greater detail regarding the GSPE effects on gastrointestinal tract.

In conclusion, the compounds of GSPE act at several points of the gastrointestinal tract modulating enterohormone secretion, which leads to regulation of food intake and glucose homeostasis. The present results suggest that compounds found in GSPE directly promote GLP-1 secretion in the ileum, and its metabolites do so in the colon. Such direct stimulation requires activation of glucose-induced GLP-1 releasing pathways. *In vivo* GLP-1 secretion may also be mediated by indirect pathways involving modulation of other enterohormones that, in turn, regulate GLP-1 release, such as enhancing GIP and reducing CCK secretion in the duodenum (the latter effect being mediated, at least in part, by gallic acid).

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Disclosure statement

No potential conflict of interest was reported by the authors.

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SUPPLEMENTARY MATERIAL

Table 1
Main phenolic compounds of the grape seed phenolic extract (GSPE) used in this study, adapted from (Margalef et al., 2015).

Compound	Concentration (µmol/g)
Gallic acid	182.64 ±0.47
Protocatechuic acid	8.69 ±0.13
Vanillic acid	4.58 ±0.24
(+)-Catechin	417.96 ±11.75
(-)-Epicatechin	321.91 ±14.71
Epicatechin gallate	47.57 ±2.44
Epigallocatechin	0.88 ±0.10
Epigallocatechin gallate	0.07 ±0.00
Procyanidin dimer B1	153.50 ±5.98
Procyanidin dimer B2	57.46 ±2.40
Procyanidin dimer B3	209.71 ±5.89
Gallated dimers	12.13 ±0.19
Trimers	6.65 ±0.54

Table 2.
Portal hormone levels 60 min after the GSPE gavage

hormone	Control	1g GSPE/Kg bw
GLP-1 (pmol/L)	1.80 ± 0.33	2.72 ± 0.77
PYY (µg/L)	0.14 ± 0.07	0.09 ± 0.04
CCK (µg/L)	0.52 ± 0.05	0.53 ± 0.07

No significant differences, assessed by t-test ($p < 0.05$) were found between control and GSPE.

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CHAPTER 2

Promotion of L-cell differentiation using grape seed proanthocyanidin extract

Àngela Casanova-Martí, Noemi González-Abuín, Joan Serrano, M Teresa Blay, Ximena Terra, Gary Frost, Montserrat Pinent and Anna Ardévol

PREFACE

The second manuscript aimed to evaluate whether GSPE affects the enteroendocrine system modulating intestinal differentiation (**objective 2**).

In the recent years, a three-dimensional intestinal culture model has been developed. In this system, single Lgr5+ stem cells generate spherical structures, so-called organoids, which contain all cell types of the intestinal epithelium comprising multiple crypts and villus structures. Therefore, organoids culture has become a powerful tool to study the intestinal cell differentiation because of their self-renewing capacity and intestinal physiological structure.

Consequently, organoids culture was employed to elucidate the second objective, studying GSPE's effect on gene expression of the main L-cell differentiation markers and transcription factors involves in such differentiation, as well as evaluating the secretion of GLP-1 and PYY in mid-term treatments.

CHAPTER 3

Grape seed proanthocyanidins influence gut microbiota and enteroendocrine secretions in rats

Àngela Casanova-Martí, Joan Serrano, Kevin J. Portune, Yolanda Sanz, M Teresa Blay, Ximena Terra, Anna Ardévol and Montserrat Pinent

Submitted

PREFACE

The third manuscript was aimed to determine the effect of grape-seed proanthocyanidin extract (GSPE) on the gut microbiota composition and to explore whether such effect might be linked to host metabolism and especially to the enteroendocrine system (**objective 3**).

An *in vivo* model was employed because it enabled us to analyze the gut microbiota composition in physiological environment and the crosstalk between host metabolism and the gut microbiota profile. To reach the goal proposed, the gut microbiota composition of different experimental groups was evaluated, as well as different metabolic and physiological parameters. Moreover, correlation analysis was employed to compare metabolic/physiological parameters with the gut microbiota profile.

Grape seed proanthocyanidins influence gut microbiota and enteroendocrine secretions in rats

Àngela Casanova-Martí[1a], Joan Serrano[1a], Kevin J. Portune[b], Yolanda Sanz[b], M Teresa Blay[a], Ximena Terra[a], Anna Ardévol[a] and Montserrat Pinent[a]

[a] MoBioFood Research Group. Departament de Bioquímica i Biotecnologia, Universitat Rovira i Virgili, c/Marcel·lí Domingo nº1, 43007 Tarragona, Spain

[b]Microbial Ecology, Nutrition & Health Research Unit. Institute of Agrochemistry and Food Technology, National Research Council (IATA-CSIC) Av. Agustín Escardino, 7, 46980, Valencia, Spain

[1]These authors contributed equally to this work

ABSTRACT

Grape seed proanthocyanidin extract (GSPE) modulates several parameters involved in metabolic syndrome. GSPE is a mixture of compounds, some which are rapidly absorbed, while others remain in the lumen where they might have effects that are translated to the whole organism. Our aim was to decipher if the 8-day treatment of GSPE, previously shown to reduce food intake, induces changes in the microbiota and enterohormone secretion. The ratio of Firmicutes:Bacteroidetes was lower in the microbiota of GSPE-treated rats compared to controls, and differences in several taxonomic families and genera were observed. Such modulation led to a reduction in cecal butyrate content. GSPE also increased plasma glucagon-like-peptide-1 (GLP-1). Gallic acid did not induce major changes in the microbiota profile nor in GLP-1 secretion. Correlations between several microbiota taxa and plasma triacylglycerol, adiposity, and enterohormones were observed. Modulation of microbiota may be one of the mechanism by which GSPE impacts metabolic health.

Keywords: Proanthocyanidins, microbiota, gastrointestinal tract, enterohormone, GLP-1

INTRODUCTION

Grape seed proanthocyanidins (GSPE) have been shown to possess several beneficial health properties in animals and human studies [1–4]. Some of the effects could directly be mediated by the phenolic compounds of the extract or their metabolites, since derived structural components have been found in several tissues [5] . However, other effects might be mediated through interactions with the gastrointestinal tract where they act by inhibiting enzymes [6], modulating inflammation and/or gut barrier properties [7], or modulating enteroendocrine secretion [8,9]. The effects of flavonoids on gut microbiota have been recently reviewed [7]; but there are few studies analyzing the role of proanthocyanidins on gut microbiota which could also mediate their physiological effects.

Satiety-related enterohormones such as glucagon-like-peptide-1 (GLP-1), peptide YY (PYY), Cholecystokinin (CCK) and ghrelin are signaling molecules that could act through the vagus nerve to signal to the brain. GSPE acutely increases plasma GLP-1 [10], and this has been linked to an amelioration of glucose tolerance [10] and an induction of satiety [11]. Satiating effects have been shown to be maintained during 8 days of treatment with GSPE 0.5 g/kg BW in rats. Interestingly this treatment also increased energy expenditure, which together with reduced food intake led to a lower body weight gain [8]. The increased satiety has been linked to ghrelin secretion modulation, but whether other enterohomones are regulated in this specific treatment has not been shown. These results point out GSPE as an interesting candidate for ameliorating obesity and diabetes. Some authors postulate a key role of microbiota-derived short chain fatty acids (SCFA) [12] on satiety-related mechanisms. In this sense, an emerging role for microbiota and therefore of food that modulate its composition in the control of metabolic homeostasis is being acknowledged. In this context polyphenols, and more specifically flavonoids, have been suggested to act as prebiotic components. In a small human study moderate red wine consumption for 4 weeks increased the phylum Bacteroidetes in humans, and this was correlated with a decrease in plasma triacylglycerol (TAG) and high density lipoprotein (HDL)-cholesterol [13]. In mice, cranberry extract, which is rich in proanthocyanidins and flavanols, modulated gut microbiota by increasing the proportion of the mucin-degrading bacterium *Akkermansia* when administered to mice for 8 weeks undergoing a high-fat sucrose diet [14].

Therefore, in the present study we explore the mechanisms that could help to explain GSPE effects on at the level of the gastrointestinal tract. We hypothesized that modulation of gut microbiota could also be a mechanism for grape seed proanthocyanidins to exert their effects on host metabolic health.

MATERIALS AND METHODS

Materials

GSPE was obtained from Les Dérivés Résiniques et Terpéniques (Dax, France). The same batch (#124029) was used in all studies. According to the manufacturer, the extract contains monomeric (21.3 %), dimeric (17.4 %), trimeric (16.3 %), tetrameric (13.3 %) and oligomeric (5–13 U; 31.7 %) proanthocyanidins. The small molecules were previously characterized by liquid chromatography-tandem mass spectrometry [15]. Gallic acid (GA) was obtained from Sigma (St. Louis, USA).

Animals and experimental design.

Female Wistar rats weighing 180–200 g were obtained from Harlan (Barcelona, Spain). The subjects were single housed at 22°C under a 12 h light/dark cycle (lights on at 8 am) with access to standard chow pellets (Teklad Global Diets #2014, Harlan, Barcelona) and tap water ad libitum during a 1 week adaptation period. All procedures were approved by the Experimental Animal Ethics Committee of the Universitat Rovira i Virgili.

Rats were introduced (1 week) to a daily 4 h fasting and chow was replaced in the dark-onset. After the adaptation, rats were daily treated during 8 days with GSPE or gallic acid 1 h prior to chow replacement by gavage, using tap water as vehicle. A control group (vehicle treated) was performed in parallel. Rats (n = 9 for each group) were sacrificed 80 min after the last dose. Animals were sacrificed by aortal exsanguination under pentobarbital anesthesia, and the heparinized and acidified plasma (0.1 M HCl) was stored at −80ºC. Cecal content together with intestinal segments from the duodenum, jejunum, ileum and proximal colon were immediately frozen in liquid nitrogen and then stored at -80º C for further enzyme activity and gene expression analysis.

Microbiota composition analysis

gDNA from cecal content of rats was extracted using a Fast DNA Stool Mini Kit (Qiagen) according to the manufacturer's instructions with minor variations. 180-220 mg aliquots of cecal content were placed in sterile tubes filled with glass beads and one mL of Inhibitex buffer (Qiagen). Samples were homogenized in a beadbeater for 2 successive rounds for 1 min with intermittent cooling on ice. Samples were then heated to 95°C for 10 min and DNA extraction was carried out according to the manufacturer's standard protocol. Samples were amplified in triplicate via PCR using primers (S-D-Bact-0563-a-S-15 / S-D-Bact-0907-b-A-20) that target the V4-V5 variable regions of the 16S rDNA [16]. Triplicate reactions consisted of final concentrations of Buffer HF (1X), dNTPs (0.11 μM), primers (0.29 μM each) and Taq Phusion High Fidelity (0.007 U/μL) in final volumes of 35 μL. Cycling conditions consisted of 98°C for

3 min, followed by 25 cycles of 95°C for 20 s, 55°C for 20 s, and 72°C for 20 s, followed by a final extension step of 72°C for 5 min. Each sample was tagged with a barcode to allow multiplexing during the sequencing process. Triplicate sample amplicons were combined and purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) according to the manufacturer's instructions and combined in equimolar concentrations before carrying out sequencing on a MiSeq instrument (Illumina). All raw sequence data has been submitted to ENA-EMBL (Accession number: PRJEB21445).

Bioinformatic processing of data was carried out using the software QIIME [17]. Using QIIME, paired-end forward and reverse Illumina reads were joined into contigs, barcodes were extracted, reads were demultiplexed, and then primers were removed using Mothur [18]. Using the UPARSE pipeline for MiSeq amplicon data [19], reads were clustered into OTUs, chimeras were removed and an OTU abundance table was created by remapping reads to representative sequences from OTUs. QIIME was then used for all downstream processing. All samples from the OTU abundance table were rarefied to the sample with the lowest number of reads and singletons were removed. Taxonomy was assigned to representative OTUs using UCLUST [20] against the Greengenes database (version 13.8) [21].

In order to carry out alpha and beta diversity analysis, representative OTU sequences were aligned using PYNAST [22] against the Greengenes' core set alignment. The alignment was filtered using QIIME's default settings and a phylogenetic tree was constructed. Alpha diversity metrics and richness estimators (Shannon's, Simpson's reciprocal, Chao1, Simpson's evenness, Observed OTUs) were then calculated. In order to examine β diversity, distance matrices (using weighted and unweighted UniFrac distances) and Principal Coordinates Analyses (PCoA) were performed using QIIME and PCoAs were visualized using the R package PhyloSeq [23]. A heatplot of correlation analysis was constructed using heatmap2() function in the R package gplots using default parameters.

Short chain fatty acid quantification

Cecal content (~500 mg) was added to 2 ml tubes containing 900 µl QH_2O and H_3PO_4 (0.5% final concentration) and homogenized vigorously followed by centrifugation (15,000 x g, 10 min at 4°C). 350 µl of supernatant was used for the derivatization of SCFAs according to the methods used in Kristensen et al. [24]. Derivatized SCFAs were analyzed using a gas chromatograph-mass spectrometer (Agilent Technologies 5977A MSD) with a low-resolution quadrupole analyzer and gas chromatograph (Agilent 7890B) equipped with an apolar capillary column (30 m, 0.25 mm ID; 0.25 µm film). The carrier gas used was helium. A constant flow mode was used (Split 30: 1; 30 ml / min split flow), with an initial flow of 1 ml/min, initial temperature of 90°C for 2 min, 5°C / min to 222°C for 5 min, followed by 20°C / min to 280°C for 2 minutes, and an

interphase temperature of 280°C. Three ethyl acetate washings were performed prior to injection of samples.

Enterohormone and plasmatic parameters quantification

Enterohormones were analysed using commercial ELISA kits for insulin (Mercodia, Uppsala, Sweden), GLP-1 7-37 amide (Millipore, Billerica, MA, USA), total CCK (Peninsula Laboratories, San Carlos, CA, USA), PYY (Phoenix Pharmaceuticals, Burlingame, CA, USA) and specific octanoyl ghrelin (Phoenix Pharmaceuticals, Burlingame, CA, USA). Glucose and TAG plasma levels were analysed with an enzymatic colorimetric kit (Glucose Oxidase-Peroxidase method from QCA, Tarragona, Spain).

Measurement of DPP-IV activity.

DPP-IV was extracted from rat intestine segments as previously described [6]. Briefly, intestine segments were homogenized using lysis buffer (PBS containing 100 KIU/mL aprotinin and 1% Triton X-100). Then, the obtained samples were centrifuged at 1000 x g at 4ºC for 10 min to eliminate the cellular debris, then centrifuged twice at 20000 x g at 4ºC for 10 min. Supernatants were stored at -80ºC until analysis.

To determine DPP-IV activity, intestinal lysates were incubated with 0.2 mM -chromogenic substrate Gly-Pro-pNA (Bachem, Bubendorf, Switzerland) in Tris–HCl buffer at 37°C. The mixture was measured at 405 nm at 37°C for 30 min in a microplate reader.

Quantitative real-time RT-PCR analysis.

Total RNA was extracted using Trizol (Ambion, USA) and trichloromethane-ethanol (Panreac, Barcelona, Spain), and purified using a Qiagen RNAeasy kit (Qiagen, Hilden, Germany). The cDNA was generated using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, USA). Quantitative PCR amplification was performed using specific TaqMan probes (Applied Biosystems, Waltham, USA): Rn00563215_m1 for CCK, and Rn00562293_m1 for proglucagon. The relative expression of each gene was calculated against the control group using the $2^{-\Delta\Delta Ct}$ method, with cyclophilin A, PPIA (Rn00690933_m1), as reference.

Statistical analysis

Morphometric, plasmatic, food intake parameters, together with enterhormone parameters and SCFAs are presented as mean ± SEM and were analysed with SPSS (IBM, Chicago, USA). These data were analysed by Student t-tests. Differences between means were considered significant when $P<0.05$.

All statistics and data visualization for microbiota data were carried out using the R statistical software and related R packages or QIIME. Comparison of relative

abundances of each phylogenetic group between treatments was carried out for each phylotype (dependent variable) by Kruskal-Wallis tests followed by Wilcoxon rank sum tests to identify significant differences between treatments. Comparison of alpha diversity among dietary treatments was also performed by Kruskal-Wallis and Wilcoxon rank sum tests. To compare bacterial community structure, PERMANOVAs were carried out using the QIIME script compare_categories.py and the adonis method using weighted and unweighted UniFrac distance matrices. Correlation analysis was conducted in order to compare physiological/metabolic parameters with relative abundances of taxonomic groups using cor.test in R. Significance values were corrected for multiple comparisons using false discovery rate (FDR) and a q-value <0.05 was selected as significant.

RESULTS

Enterohormone profile is differentially modulated by GSPE and gallic acid

A GSPE treatment (500 mg GSPE/kg bw for 8 days), previously shown to be effective at inhibiting food intake in male rats [8], was reproduced in female rats in this study. To discriminate between the effect of GSPE versus one of its monomeric components, for which satiating properties have been described [25], we included a group treated with gallic acid at an equivalent amount to that present in the GSPE. Since we aimed to analyze whether there was a modulation of enterohormone secretion, we sacrificed the animals in a condition where anorexigenic hormone secretion is stimulated, by allowing the animals to eat for 20 minutes after a fasting period. At sacrifice, plasma levels of enterohormones were measured. GSPE-treated rats had higher active GLP-1 and active ghrelin levels, while treatment with gallic acid did not significantly change such parameters (**Table 1**). Instead, gallic acid increased CCK levels.

GLP-1 and CCK gene expression was assessed in the ileum and colon of the animals. GSPE caused a significant up-regulation of CCK gene expression in the ileum, while the monomer gallic acid did not induce any change in gene expression compared to the control animals (**Table 2**). In the colon there was no modification either by GSPE or the GA treatment (**Table 2**).

The amount of GLP-1 protein was also measured in intestinal tissue of GSPE-treated and control animals. GSPE did not significantly modify GLP-1 protein either in the ileum or in the colon (**Table 2**).

We previously showed that an increase in GLP-1 secretion by an acute GSPE treatment was in part due to inhibition of DPP-IV activity. Therefore we measured this activity in different intestinal segments. Our results showed no difference in any of the segments due to GSPE treatment. Curiously, gallic acid increased DPP-IV activity in the duodenum (**Table S1**).

Table 1

Enterohormone levels in plasma, morphometric, plasmatic, food intake and SCFA parameters at the sacrifice day for rats in the control, gallic acid and GSPE treatments. Values are represented as mean ± SEM. * P ≤ 0.05 vs. control, # P ≤ 0.1 vs. control, T test. OWAT: ovarian WAT; RWAT: retroperitoneal WAT; MWAT: mesenteric WAT; BAT: Brown adipose tissue.

		Control	Gallic acid	GSPE
Enterohormones	active GLP-1 (pM)	0.59 ± 0.0	0.72 ± 0.1	0.95 ± 0.1 *
	PYY (pg/ml)	105.48 ± 22.9	149.4 ± 28.2	162.75 ± 46.9
	CCK (ng/ml)	1.31 ± 0.2	7.1 ± 1.69 *	6.45 ± 2.89
	active Ghrelin (pg/ml)	303.92 ± 29.8	236.1 ± 29.41	465.61 ± 47.5 *
Plasma parameters	Glucose (mM)	9.86 ± 0.74	9.61 ± 0.83	8.33 ± 0.84
	TAG (mM)	0.21 ± 0.08	0.31 ± 0.08	0.07 ± 0.03
	Insulin (ug/L)	2.31 ± 0.43	2.59 ± 0.36	2.57 ± 0.34
Morphometric parameters	Final weight (g)	263.11 ± 3.40	266.56 ± 5.80	253.44 ± 4.30 #
	MWAT (g)	2.82 ± 0.23	3.65± 0.39	3.33 ± 0.28
	OWAT (g)	3.34 ± 0.35	3.77 ± 0.42	2.67 ± 0.18 #
	RWAT (g)	6.39 ± 0.59	8.76 ± 0.89 *	6.56 ± 0.89
	BAT (g)	0.41 ± 0.03	0.51 ± 0.05	0.36 ± 0.04
	Sum WAT (g)	12.55 ± 0.81	15.77 ± 1.25 *	12.55 ± 1.18
Food intake	kcal (sum total intake)	373.62 ± 6.10	378.15 ± 10.30	342.28 ± 10.90 *
Short chain fatty acids (µmol/g wet intestine content)	Acetic acid	17.639 ± 1.87	17.491 ± 0.97	15.568 ± 1.88
	Propionic	2.815 ± 0.24	2.464 ± 0.08	3.346 ± 0.3
	Butyric acid	9.867 ± 1.02	10.018 ± 0.94	5.595 ± 0.98 *
	Valeric acid	0.149 ± 0.04	0.202 ±0.01	0.143 ± 0.03

In the conditions in which the animals were sacrificed, neither GSPE nor gallic acid significantly changed other biochemical plasmatic parameters, i.e. glucose, insulin and triglycerides (**Table 1**). It was also found that in this model, GSPE but not gallic acid reduced food intake. Actually, gallic acid-treated animals had higher white adipose tissue (sum of different fractions except subcutaneous). Specifically, retroperitoneal white adipose tissue weight was significantly different than the controls. Ovarian WAT of GSPE-treated rats tended to be lower ($p \leq 0.1$) than in the controls, and was significantly lower ($p \leq 0.05$) than that in the gallic acid-treated rats.

GSPE reduces butyrate in cecal content

Short chain fatty acids were measured in the cecal content of treated animals. GSPE led to a reduction in butyrate concentration (**Table 1**), while no differences were observed for acetic, propionic nor valeric acid. This led to significant differences ($p \leq 0.05$, Student T-test) in the ratio of acetate:propionate:butyrate, which was 58.0 ± 2: 9.4 ± 0: 32.6 ± 2 for controls and 63.7 ± 1: 14.4 ± 1: 21.9 ± 2 for GSPE. Gallic acid (GA) did not

induce any change compared to controls in SCFA cecal content (**Table 1**) nor in the ratio acetate:propionate:butyrate (58.6 ± 2: 8.4 ± 1: 32.9 ± 2).

Table 2

Enterohormone gene and protein expression in ileum and colon. Gene expression values are represented as $2^{-\Delta\Delta Ct}$ vs. control rats. * P ≤ 0.05 vs. control, T test. NA: not analyzed.

		ileum			colon		
		Control	**GA**	**GSPE**	**Control**	**GA**	**GSPE**
mRNA	GLP-1	1.12 ± 0.2	0.96 ± 0.2	1.24 ± 0.1	1.11 ± 0.2	0.65 ± 0.2	0.96 ± 0.2
	CCK	0.87 ± 0.1	1.15 ± 0.3	2.25 ± 0.2 *	1.63 ± 0.5	3.03 ± 1.1	0.61 ± 0.1
protein	GLP-1 (pmol/g tissue)	355.36 ± 18.8	NA	365.95 ± 47.6	186.08 ± 59.1	NA	234.64 ± 17.4

GSPE changes microbiota profile, while gallic acid does not

Several diversity indices (Shannon's, Simpson's, Simpson's Reciprocal, Simpson's evenness) and richness estimators (Chao1, observed OTUs) were significantly lower in the GSPE group compared to both Control and Gallic groups, whereas no differences were detected between the control and Gallic groups (**Fig. 1**).

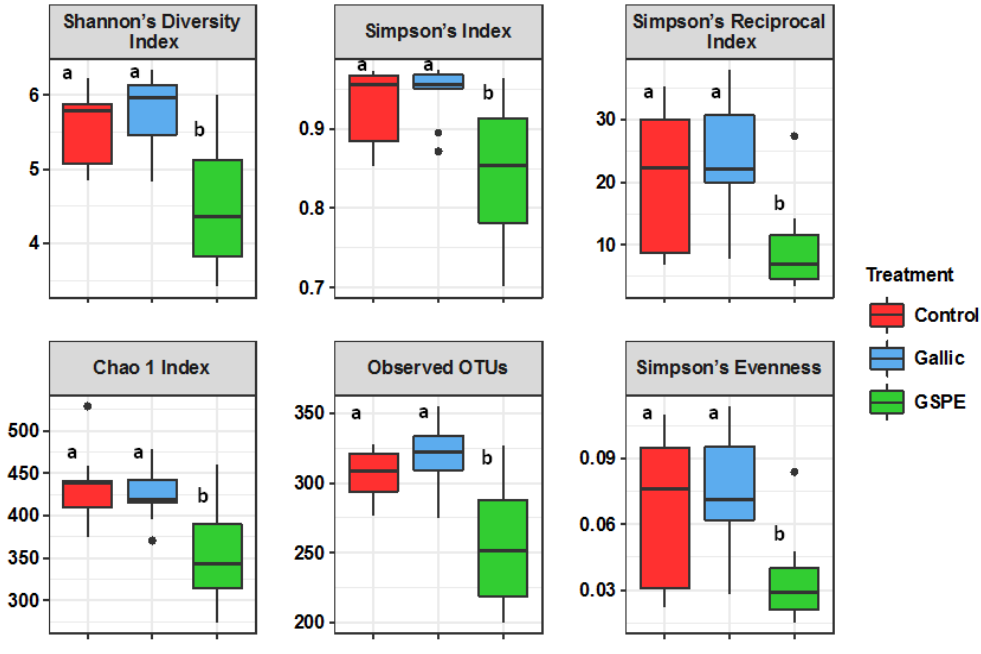

Fig. 1. Alpha diversity indices (Shannon's, Simpson's, Simpson's Reciprocal index, Simpson's evenness) and richness estimators (Chao1 index, observed OTUs) of the gut microbiota from different treated rats, grouped as control, gallic and GSPE, after 8 days of treatment. *Different superscripts* indicate significant differences (P < 0.05) calculated by Wilcoxon rank sum tests.

Bacterial composition in cecal content as revealed by PCoA was significantly different in the GSPE group compared to both Control and Gallic groups using both unweighted (P<0.001) and weighted (P<0.001) UniFrac distances (**Fig. 2 A, B**).

Concerning taxonomic phyla, Firmicutes was significantly higher in the control and Gallic groups compared to the GSPE group, while Bacteroidetes and Proteobacteria were higher in the GSPE group (**Fig. 3**). Primarily, bacteria from the classes Clostridia, Bacteroidia and Alphaproteobacteria and Betaproteobacteria were responsible for these observed patterns (**Fig. 3**). The families *S24-7*, *Bacteroidaceae* and *Porphyromonadaceae* were the notable groups from the class Bacteroidia that were larger in the GSPE treatment compared to the other two treatment groups, while *Alcaligenaceae* (Betaproteobacteria) and *Veillonellaceae* (Clostridia) had similar trends (Fig. 3). In contrast, *Ruminococcacea* and *Dehalobacteriaceae* were larger in the Control and Gallic group compared to the GSPE (**Fig. 3**). Prominent bacterial genera that were significantly higher in the GSPE treatment compared to the Control and Gallic groups include *Bacteroides*, *Parabacteroides*, *Sutterella* and *Phascolarctobacterium*, while *Bilophila* was significantly higher in the GSPE group compared to only the control. [*Ruminococcus*] was significantly lower in the GSPE compared to the other 2 groups and *Oscillospira*, *Coprococcus* and *Dehalobacterium* were significantly lower in the GSPE treatment compared to the Gallic treatment (**Fig. 3**).

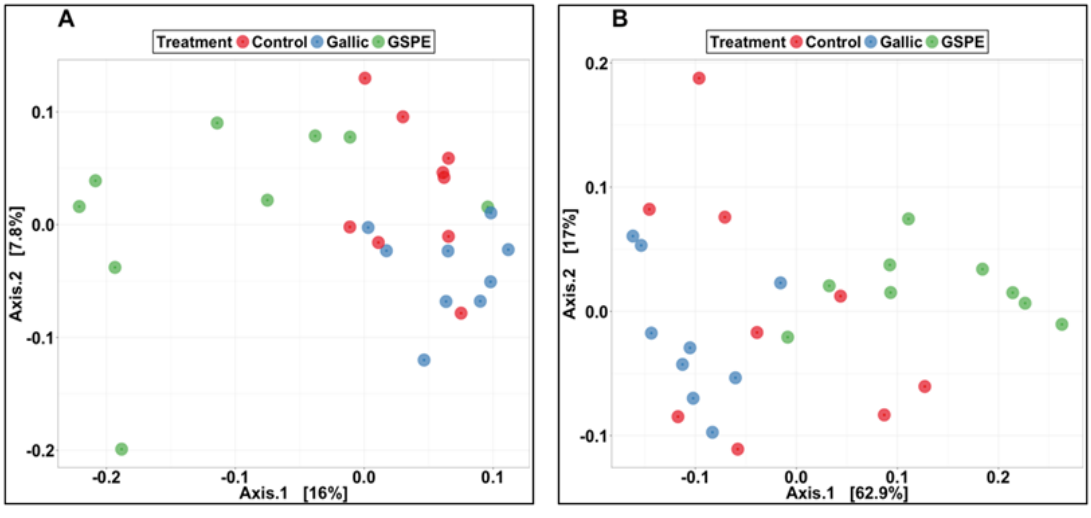

Fig. 2. Principal coordinate analysis (PCoA) illustrating the treatment groups of control gallic acid, and GSPE based on unweighted (A) and weighted (B) UniFrac distances.

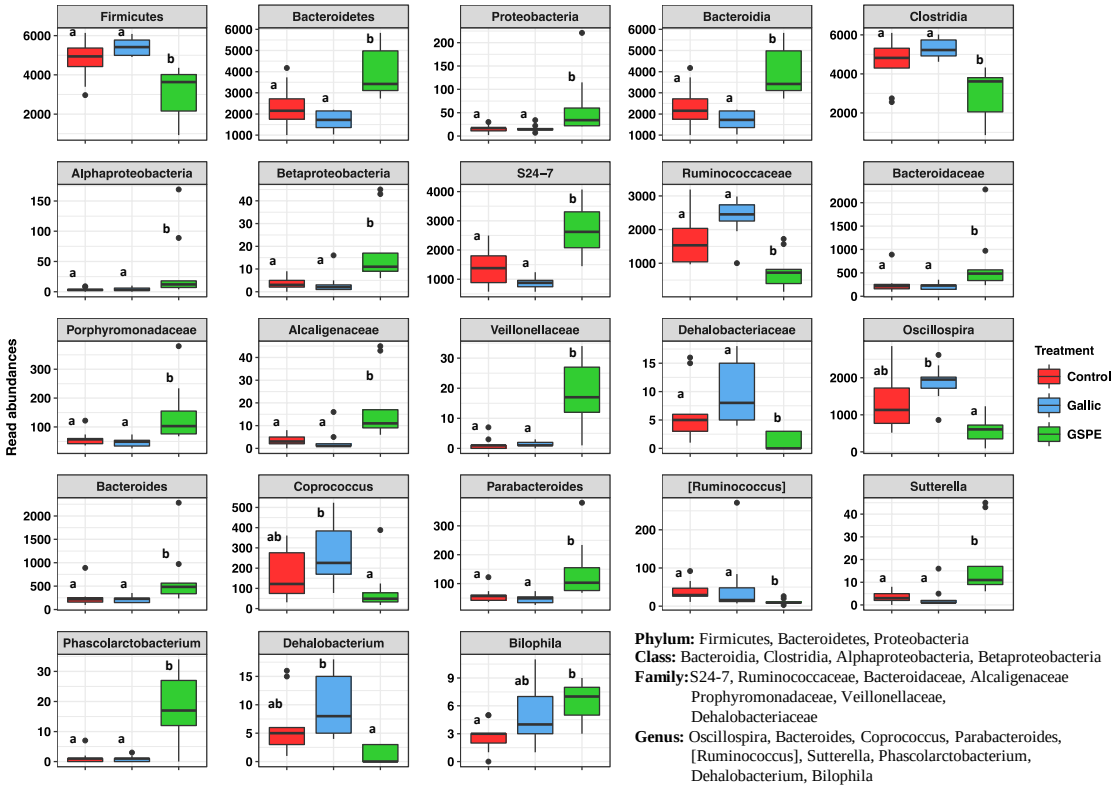

Fig. 3. Abundances of microbiota taxonomic groups which were significantly different among treatments control, gallic acid, GSPE. *Different superscripts* indicate significant differences ($P < 0.05$) calculated by Wilcoxon rank sum tests**.**

Correlations between microbiota and morphometric and metabolic variables

To identify whether bacterial taxa are associated with metabolic and morphometric variables, or with the different parameters that were measured in the intestine, we calculated the Spearman's rank correlation coefficient or Spearman's rho for these parameters. Significant correlations are shown in **Fig. 4**.

We found significant correlations between the levels of short chain fatty acids and multiple bacterial taxa. Acetic acid positively correlated with some families that belong to the Firmicutes phyla, that is the *Clostridiaceae* (and more specifically with the Clostridium genera) and the *Turicibacteraceae* (and more specifically with the *Turicibacter* genera). Positive correlations for acetic acid were also found for the phyla Actinobacteria, and within it for the *Bifidobacteriales*. Butyric acid correlated positively with several genera of the families *Dehalobacteriaceae* and *Ruminococcaaceae* within the Firmicutes phylum, as well as with the *Rikenellaceae* family of the Bacteroidetes phylum. In contrast, butyrate levels negatively correlated with the families *Veillonellaceae* (Firmicutes) and *Porphyromonadaceae* and *Bacteroidaceae*

(Bacteroidetes). Valeric acid also negatively correlated with this last family, and specifically with the *Bacteroides* genera.

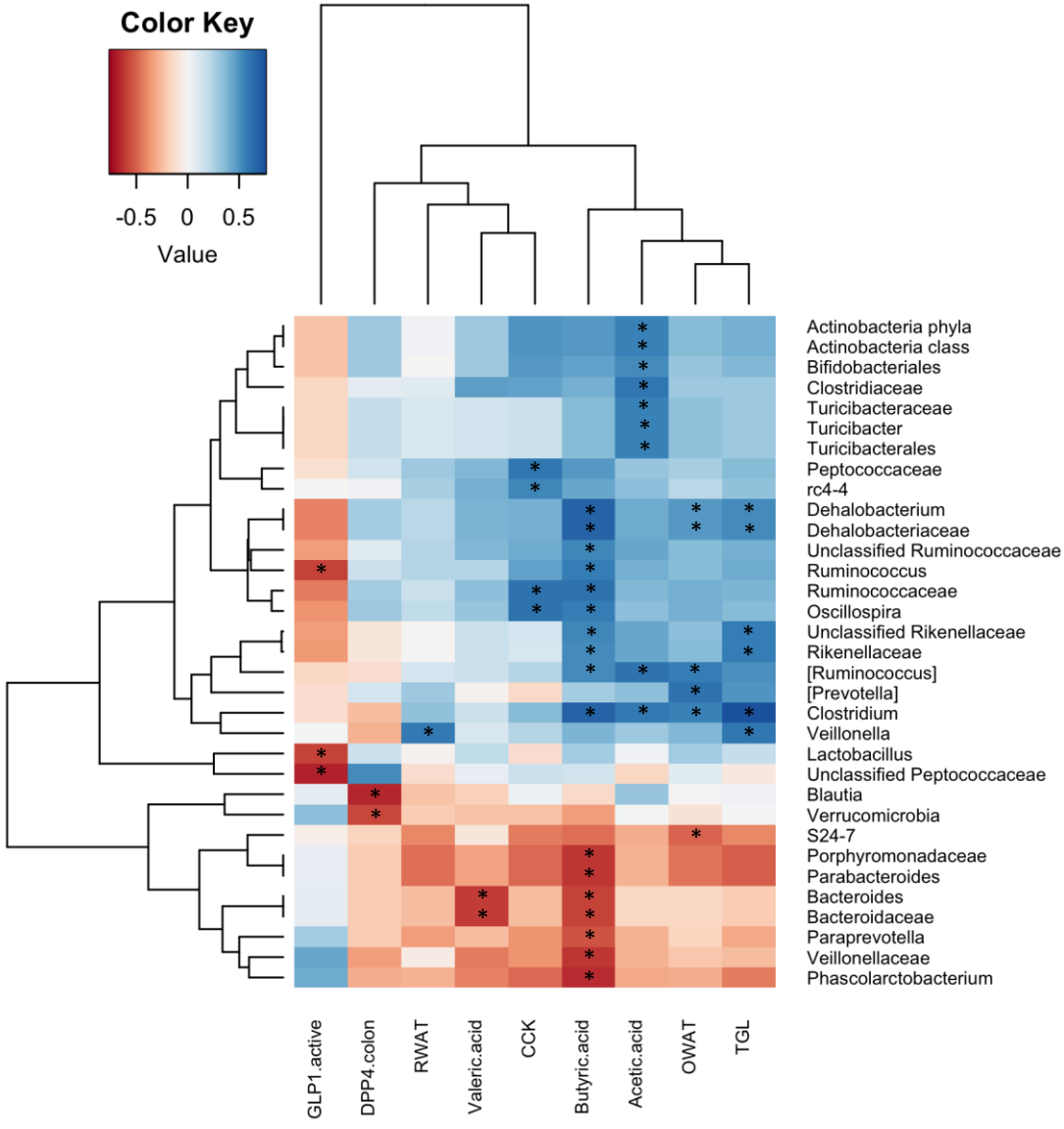

Fig. 4. Heatplot of correlations between the abundance of selected bacterial taxonomic groups with observed concentrations of metabolites, enterohormones and plasmatic parameters. The relative color indicates the value of Spearman's rho. P-values were adjusted with the false discovery rate method for multiple comparisons. *: adjusted P-value < 0.05. Only taxonomic groups that displayed significant correlations with selected parameters are shown.

Fig. 4 also shows significant correlations between some enterohormones and bacterial taxa. Plasma levels of CCK were positively associated with the genera *Oscillospira* (family *Ruminococcaceae*) and the genera rc4-4 (family *Peptococcaceae*) within the Firmicutes phylum. Instead, negative correlations were found between active GLP-1

levels in plasma and the genera *Lactobacillus*, *Ruminococcus*, and the unclassified *Peptococcaceae,* all of them belonging to the Firmicutes phylum.

We also found negative correlations between DPP-IV activity in the colon and the phylum *Verrucomicrobia*, as well as with the genera *Blautia* (phylum Firmicutes).

Ovarian WAT also positively correlated with the genera *Clostridium,* [*Ruminococcus*] and *Dehalobacterium* (Firmicutes phylum), and negatively correlated with the *S24-7* family (within Bacteroidetes phyla). In contrast, retroperitoneal WAT positively correlated with the *Veillonella* genera (Firmicutes).

Finally, the only metabolic parameter that significantly correlated with bacterial taxa was the plasma triglycerides, which showed a strong positive association with the genus *Clostridium*, and also correlated with *Veillonella*, and *Dehalobacterium* (from the Firmicutes phylum), and the family *Rikenellaceae* (from the Bacteroidetes phylum).

DISCUSSION

Grape seed proanthocyanidins have several demonstrated beneficial effects including a potential to alleviate metabolic syndrome parameters. They modulate body weight gain and lipidemia through several different mechanisms [26]. In fact, an 8-day treatment with 500 mg/kg bw of GSPE was demonstrated to reduce body weight gain through inhibition of food intake and activation of energy expenditure [27]. Provided the emerging acknowledged role of microbiota in obesity and the few studies showing that phenolic compounds might have some effect on its composition, in the present paper we analysed whether such GSPE treatment (500 mg/kg bw) during 8 days could modulate microbiota. Concomitantly we investigated other effects at the intestinal level, i.e. regulation of enterohormones, which could help to explain the effects of the GSPE treatment on food intake and body weight gain. We also compared the effects of the whole extract with that of one of its compounds, gallic acid, which is rapidly absorbed in upper intestine to identify the active components in the gut ecosystem.

We found that GSPE changes the microbiota profile, and our results are in agreement with the general view that polyphenols increase Bacteroidetes and decrease Firmicutes phyla [7]. Although there is controversy in regard to whether the Firmicutes/Bacteroidetes ratio is a microbiome-marker of obesity, this ratio has been shown to have some health implications, in particular, higher values of this fraction have been associated with obesity and type 2 diabetes in different human and animal studies [28–30]. The few studies concerning flavanols and microbiota modulation suggest that acting on intestinal flora could be a mechanism for flavonoids to exert beneficial effects, as suggested for red wine [31,32] or by cocoa flavanols [33]. Conclusions of these reviews agree that more evidence is required to define the relationship between flavanols, microbiota and health effects. Our results clearly support the hypothesis that

flavanols act at least partly by modulating gut microbiota composition, and such modulation might contribute to explain the previously shown beneficial effects that exert a similar GSPE treatment [27].

In mice fed a high-fat-sucrose diet, highly polymeric procyanidins (PP) from apple increased the Firmicutes/Bacteroidetes ratio to the level observed in the standard-fed mice after 20 weeks of treatment [34]. In that study, PP increased proportions of sequences assigned to the *Adlerceitzia*, *Roseburia*, *S24-7*, *Bacteroides*, *Anaerovorax*, rc4-4, and *Akkermansia* taxa. In contrast, the proportion of reads assigned to *Clostridium*, *Lachnospiraceae*, and *Bifidobacterium* were reduced by PP administration [34]. Another study showed that grape polyphenols dramatically increased the relative abundance of *A. muciniphila* within the *Verrucomicrobia* phylum, in mice under a high fat diet after 13-week treatment [35]. Accordingly, in mice under a high-fat-sucrose diet, cranberry extract, rich in the flavanols group including procyanidins, increased the proportion of the mucin-degrading bacterium *Akkermansia* when administered to mice for 8 weeks [14]. Recently, it has been shown that a grape seed proanthocyanidin extract administered for 7 weeks together with a high fat diet increased *Clostridium XIVa*, *Roseburia* and *Prevotella* [36]. All of these studies analyzed the effects of treatments that were several-weeks long together with a high fat or high-fat-sucrose diet. Instead, we describe that GSPE changed the microbiota composition after only one week of treatment and in animals fed a standard diet. Indeed the study of Ahne et al. showed that pretreatment with cranberry extract 1 week before high-fat-sucrose feeding was not associated with specific changes in the baseline metagenome [14]. Differences with our results might be attributed to the different compounds of the extract (i.e. A-type procyanidin in cranberry versus B-type in grapes), dose (200 mg cranberry extract/kg bw versus 500 mg GSPE/kg bw) and animal model (mice versus rat). Furthermore, the specific taxonomic families and genera that we find significantly modified by GSPE are mostly different from the above mentioned, with the exception of the *S24-7* family and the *Bacteroides* genera, that were previously described as a targets for polyphenols [34]. Thus, we define several new target taxonomic groups, described at the genera level, influenced by proanthocyanidins, including *Sutterella*, *Pharscolarctobacterium*, *Parabacteroides, Bilophila,* and *Ruminococcus.*

The study of Anhe et al. on cranberry extract, suggested that the related increase in *Akkermansia* population might be sufficient to prevent the negative metabolic phenotype associated with obesity-driven dysbiosis without major modifications in the proportions of Firmicutes and Bacteroidetes [14]. To try to discern whether changes in microbiota were related to modulation of morphometric and metabolic parameters, we performed correlation tests. Our results showed positive correlations between the genera *Clostridium*, and *Dehalobacterium* (both Firmicutes), and negative correlation for *S24-7* with ovarian WAT. Changes in these specific taxa by GSPE could be linked to an impairment of adipose weight increase, despite that in the present conditions (standard diet, 8–day treatment) we do not find significant modulation of total visceral

WAT as expected from this experimental model. Actually, the same GSPE treatment (500 mg/kg bw, 8 days) has previously been shown to increase energy expenditure through increased lipid oxidation in adipose tissue [27]. Furthermore, we found a positive correlation of circulating plasma TAG with *Clostridium*, *Dehalobacterium* and *Veillonella*. GSPE has previously been shown to reduce plasma triglycerides (reviewed in Salvadó et al., 2015) despite that in the present study we do not find statistically significant differences, again probably due to the short-term treatment and standard diet used in the study. Administration of a putative probiotic *Lactobacillus rhamnosus* for 28 days in diet-induced hyperlipidemic rats led to a positive correlation between *Clostridium leptum* and serum triglycerides, which were reduced by the treatment [37]. For other types of putative prebiotics, reduction in *Clostridium* has been observed together with reduction in blood lipids [38]. In our experiment, reduction by GSPE of several taxa that belong to Firmicutes is accompanied with a reduction in butyrate, an energy source for colonocytes. Our results in fact show a positive correlation between butyric acid and several genera, that are reduced by GSPE (e.g. *Dehalobacterium*, *Ruminococcus*), but also negative correlation with other genera within the Bacteroidetes phylum, that are increased by GSPE. In this sense, proanthocyanidins' modulation of the microbiota profile is associated with a reduction of butyrate, that is, of energy harvest from the diet. These results led us to hypothesize that it could be a mechanism to protect from increased TAG accumulation in cases of diet induced-obesity, at least complementary to other well described mechanisms [26]. Further studies on GSPE treatments in high fat diet-fed animals are required to discern whether the genera that we describe as new targets for being modulated by GSPE, are also involved in the beneficial metabolic effects of GSPE. In humans, consumption of red wine polyphenols for 4 weeks significantly increased the number of *Enterococcus, Prevotella, Bacteroides, Bifidobacterium, Bacteroides uniformis, Eggerthella lenta*, and *Blautia coccoides–Eubacterium rectale* groups [13], suggesting that the possible prebiotic effect of proanthocyanidins could be extended to humans.

In the present study, we found increased active GLP-1 in plasma after an 8-day GSPE treatment. Our results of intestinal GLP-1 gene and protein expression suggest that the effect on circulating GLP-1 levels is not due to a modification of GLP-1 production, but of its secretion. Since the last GSPE dose was administered 80 minutes before sacrifice, it could be that the previously shown acute GLP-1-stimulatory GSPE effects [8,10] are maintained after 8 days of treatment, as has been described for ghrelin [27]. In humans, the intake of a specific strain of *Lactobacillus* (i.e. *L. reuteri*) has been shown to increase GLP-1 secretion [39]. However, we found that the levels of active GLP-1 negatively correlated with three genera (*Lactobacillus*, *Ruminococcus* and Unclassified *Peptococcaceaea*) within the Firmicutes phylum. Although the *Lactobacillus* genus identified in our study was not further classified to the species level, it appears that not all strains of *Lactobacillus* demonstrate the same GLP-1 secretion patterns in the host. It has been shown that the gut microbiota fermentation of specific prebiotics or other non-digestible carbohydrates is associated with the secretion of enteroendocrine

peptides produced by L-cells [40]. One possible mechanism is that SCFAs produced by the fermentation of dietary fibers bind to the G-protein-coupled receptors (GPCRs) GPR41 and GPR43, thereby triggering GLP-1 secretion by the L-cells [40]. Our results show that GSPE significantly reduced cecal butyrate content, and modulated the ratio of acetate: propionate: butyrate, although whether these changes could signal to enhance GLP-1 secretion should be further assessed. Alternatively, a direct link between gut microbiota and intestinal bioactive lipids related to the endocannabinoids and involved in enteroendocrine peptide secretion has been suggested as another mechanism for enhanced GLP-1 secretion observed with prebiotics [40]. To our knowledge, there is no information concerning the possible relationship between these three genera and endocannabinoids, thus future studies will reveal whether this may be a mechanism for GSPE.

GSPE is a mixture of different compounds. Matsumoto et al. demonstrated that modification in the proportion of *Akkermansia* in the gut microbiota is due to non-absorbable PPs and that the degree of PP polymerization is an important factor [34]. In the present paper we show that gallic acid, one of the main acids found in the extract does not induce significant modification of microflora or short chain fatty acids, at least in the concentration assayed, which is equivalent to that found in 500 mg GSPE/kg. Moreover we show that gallic acid does not mimic the changes in enterohormone profile induced by GSPE. Furthermore, in this model it does not reduce food intake, and concerning adiposity, it leads to a significant increase in visceral adipose tissue, due to the increased retroperitoneal fraction.

We had previously shown that gallic acid reduced CCK secretion after an acute treatment in a rat duodenum *ex vivo* model [9]. Now we found that an 8-day treatment with gallic acid increased CCK plasma levels. These differences might be due to the different length of time of each treatment (acute vs 8 days). Despite our results showing no significant modulation of CCK gene expression in ileal and colonic tissue of gallic acid-treated rats, suggesting that the effect is not due to an increased production, we also observed a correlation between plasma CCK levels and some microbiota taxa. Plasma CCK positively correlated with the *Oscillospira* genus and the corresponding *Ruminococcae* family. The mechanism and whether there is a relation with CCK secretion remain unresolved, but results confirm that gallic acid is not the main active molecule in the GSPE extract to explain its effects at the gastrointestinal tract.

CONCLUSION

In conclusion, our results show for the first time a clear short-term effectiveness of GSPE at modifying microbiota, increasing the amount of Bacteroidetes and reducing that of Firmicutes, and altering specific genera within these phyla. Modifications in the microbiota led to changes in the short chain fatty acid profile from the cecal content. Our correlation analysis suggests that these changes in the microbiota may be linked to

the modulation of plasma TAG, adiposity, and enterohormone secretion induced by GSPE.

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The authors declare no conflict of interest.

SUPPLEMENTARY MATERIAL

Table 1S. DPP-IV activity/mg protein in different intestinal segments for rats in the control, GSPE and gallic acid treatments. Values are represented as normalized by each control. * P ≤ 0.05 versus control, T test.

	Control	Gallic acid	GSPE
Duodenum	1.00 ± 0,1	1.63 ± 0.3 *	0.91 ± 0.1
Jejunum	1.00 ± 0,1	0.99 ± 0.1	0.97 ± 0.1
Ileum	1.00 ± 0,1	1.21 ± 0.1	1.15 ± 0.1
Distal colon	1.00 ± 0,1	1,01 ± 0.1	0,93 ± 0.05

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CHAPTER 4

Antihyperglycemic effect of chicken feet hydrolyzate via incretin system: DPP4-inhibitory activity and GLP-1 release stimulation.

Àngela Casanova-Martí, Francisca Isabel Bravo, Joan Serrano, Anna Ardévol, Montserrat Pinent and Begoña Muguerza

PREFACE

In the last manuscript, we aimed to discover a new source of natural bioactive compounds that could act at intestinal level. Specifically we analyzed whether chicken feet hydrolyzate modulates the incretin system (**objective 4**).

The *in vitro* evaluation of DPP-IV inhibition enabled us to screen different chicken feet hydrolyzates obtained by enzymatic hydrolysis. Moreover, we used *in vitro* and *ex vivo* models to show that the selected hydrolyzate (with the higher DPP-IV inhibitory activity) was able to modulate the incretin system by another complementary mechanism, the direct effect on enteroendocrine cells, and thereby on GLP-1 secretion.

Finally, we checked whether the selected hydrolyzate had *in vivo* effect improving glucose homeostasis in disrupted- glucose homeostasis rat models.

Results detailed in the manuscript, as well as subsequent studies that have led us also to define the main peptide sequence responsible of DPP-IV inhibition are reported in **related patent**.

Antihyperglycemic effect of chicken feet hydrolyzate via incretin system: DPP-IV-inhibitory activity and GLP-1 release stimulation.

Àngela Casanova-Martí[a1], Francisca Isabel Bravo [b1], Joan Serrano[a], Anna Ardévol[a], Montserrat Pinent[a] and Begoña Muguerza[b]

[a]MoBioFood Research Group. Departament de Bioquímica i Biotecnologia, Universitat Rovira i Virgili

[b]Nutrigenomics Research Group. Departament de Bioquímica i Biotecnologia, Universitat Rovira i Virgili

[1]These authors contributed equally to this work

ABSTRACT

The incretin therapies provide a novel approach to be used in the treatment of type 2 diabetes. In the present study, the potential as natural dipeptidyl-peptidase IV (DPP-IV) inhibitor of 12 hydrolyzates generated from hydrolysis of chicken feet proteins with Neutrase® and Protamex® were investigated. Three hydrolyzates were selected due to their high DPP-IV inhibitory capacity (>80%), showing IC_{50} values around 300 µg/mL. The hydrolyzate obtained from chicken feet proteins treated with a pretreatment (50°C, pH3, 1.5h), followed by a hydrolysis with Neutrase® (25°C, pH7.0, 24h) (which was named p38H) was selected for posterior analysis. Glucose tolerance test was performed on healthy and two types of glucose-intolerance (diet-induced and age-induced) rat models to study the antihyperglycemic effect of the p38H. The dose of 300 mg protein/kg body weight (BW) p38H improves the plasma glucose profile in both glucose-intolerance models. The p38H had no significant effect in rats with normal glycemia. In addition, p38H induced a strong stimulation of active GLP-1 release in enteroendocrine STC-1 cells and in rat ileum tissue. In conclusion, our results point out that proteins of chicken feet treated by Neutrase® and Protamex® are a good source of bioactive peptides as DPP-IV inhibitors. Moreover, our results also highlight the potential of the selected hydrolyzate (p38H) for the management of type 2 diabetes due to the dual function of inhibiting DPP-IV activity and inducing GLP-1 release.

INTRODUCTION

The prevalence of lifestyle-related diseases such as obesity and type 2 diabetes mellitus (T2DM) has become a healthcare problem in developed societies. Therefore, effective strategies are needed to prevent the development of T2DM and the associated pathologies. A recent focus has pointed out to the biology of incretin hormones such as GIP and GLP-1 for glycemic regulation [1].

GLP-1 is an incretin hormone that is released from intestinal L-cells in response to nutrient ingestion that exerts glucoregulatory action through the stimulation of insulin secretion and inhibition of glucagon secretion [2, 3]. In addition to its insulinotropic actions, GLP-1 also improves beta-cell mass, delays gastric emptying, enhances satiety and reduces food intake [4, 5]. Due to their physiological effects, a number of antidiabetic agents targeted towards the so-called incretin therapies have emerged. These therapies are mainly based on the use of GLP-1 mimetics and dipeptidyl peptidase IV (DPP-IV) inhibitors, which protect cleavage of active GLP-1 by DPP-IV [6]. DPP-IV is a serine protease which is widely distributed among many tissues in the body and it is expressed as both membrane and soluble form in a variety of cell types. This enzyme principally cleaves proline or alanine containing dipeptides from the N-terminus of a polypeptide; thereby GLP-1 and GIP are potential DPP-IV targets. The concentration of GLP-1 in plasma rises rapidly after food intake and it is immediately degraded by DPP-IV because of the presence of the enzyme at their site of production. In recent years, DPP-IV inhibitors have been reported to prevent the cleavage of incretins by DPP-IV and to increase the half-life of the active hormones, and hence are available for management of glucose homeostasis [2]. Recently, another potential approach has been postulated, the stimulation of the endogenous secretion of GLP-1 stored in L-cells to improve glycemia [7].

The development of food protein-derived peptides has become a novel strategy for the prevention and management of T2DM. The hydrolyzates rich in bioactive peptides are well metabolized and confer fewer side effects than synthetic pharmaceutical drugs. Furthermore, there is a wide range of available under-utilized resources of the food industry, which could be potential sources of bioactive peptides. Altogether, these characteristics could lead to expand the use of bioactive peptides in the nutraceutical food section [8]. Recent *in silico* analysis revealed that sequences are contained within dietary proteins that present structural features which make them natural precursors for generation of potent inhibitors of DPP-IV [9, 10]. Previous studies have described that hydrolyzates obtained from milk proteins [11, 12], as well as other animal and vegetal sources [13–15] exhibit DPP-IV inhibitory activity *in vitro*. However, only a few studies have evaluated the effects of biopeptides as DPP-IV inhibitors *in vivo*. Acute studies show that biopeptides can decrease plasma glucose concentration relative to the control in an oral glucose tolerance test (OGTT) in rodents [16, 17]. While Mochida

et al. showed that ZeinH (a hydrolyzate prepared from corn zein) administration into the ileal loop produces an inhibition of DPP-IV activity in the ileal vein in rodents [18].

Regarding GLP-1 secretion by dietary proteins, it has been reported that meat hydrolyzates stimulate GLP-1 release in the enteroendocrine cell lines NCI-H716 [19], STC-1 [20], and GLUTag [21] as well as it is a potent secretor of GLP-1 in rodents [22]. Moreover, GLP-1 secretion triggered in response to whey protein has been observed in humans [23]. Even though, human studies have shown that this incretin secretion highly depends on the origin of the protein and its level of digestion [24, 25], as it was also observed in STC-1 cells [26]. It is also interesting the fact that a dual bioactive role has been suggested for protein hydrolyzates, such as DPP-IV inhibitor and α-glucosidase inhibitor [27], or stimulator of GLP-1 secretion [18, 28], highlighting their promising action in the prevention and management of glucose-intolerance pathologies.

Therefore, the aim of this study was to obtain bioactive hydrolyzates that are able to inhibit DPP-IV activity *in vitro* from proteins of chicken feet using *in vitro* enzymatic treatment. Furthermore, we selected a hydrolyzate with high capacity to inhibit DPP-IV activity *in vitro* and evaluated the effect of this hydrolysate on the secretion of GLP-1 in *in vitro* and *ex vivo* models. Finally, we aimed to demonstrate whether this could lead to antihyperglycemic properties in rats.

MATERIALS AND METHODS

Chemicals

Dipeptidyl peptidase IV (from porcine kidney) was purchased from Sigma-Aldrich, Co. (St. Louis, Mo., USA). Gly-Pro-7-amido-4-methylcoumarin hydrobromide (Gly-Pro-AMC) and Pro-p-nitroanilide (Ala-Pro-pNA) were obtained from Bachem AG. Diprotin A (Ile-Pro-Ile) was from Enzo Life Sciences International (New York, USA) and Vildagliptin was form Axon Medchem (Groningen, The Netherlands). Neutrase® 0.8L and Protamex® was received as a kind gift from Novozyme (Copenhagen, Denamark).

Hydrolysis of chicken feet proteins

Chicken feet hydrolyzates were made from chicken feet *from Gallus gallus domesticus as* previously described [29]. Chicken feet were washed, triturated, lyophilized and sieved using 2mm pore size sieve to obtain a powder. This powder was heated in water at different pH (3.0 and 7.5) and temperature (25, 50 and 100°C) for 1.5 hours. After pretreatment, the mixture was hydrolyzed at pH 7.0 and different temperature (25 or 50°C) for 2 hours or 24 hours, and using two different commercial mixes of proteases from *Bacillus amyloliquefaciens* (E.C. 3.4.24), and *Bacillus licheniformis* and *Bacillus amyloliquefaciens* (E.C. 3.4.21.62 and EC 3.4.24.28) so-called as Neutrase® and Protamex®, respectively. In all hydrolysis treatment, the ratio of enzyme:substrate

used was 0.4 Anson Units (AU)/g. To stop the hydrolysis reaction, enzymes were inactivated by heating at 80°C for 10 min in a water bath. Solutions were centrifuged at 10000 x *g* for 24 min at 4°C. The supernatant was collected and was kept at -20°C until further analysis.

Determination of DPP-IV Inhibitory Activity

The inhibition assay was performed using 96-well microplates. The DPP-IV enzyme (diluted with 100 mM Tris HCl buffer ph 8.0 to 0.26 mU per well) and 10 µl of different concentration of test sample was pre-incubated for 10 min at 37°C. The enzymatic assay was initiated by adding the chromogenic substrate Gly-Pro-pNA (final concentration 0.2mM). The mixture was measured at 405 nm at 37°C for 30 min in a microplate reader. DPP-IV inhibition is expressed as a percentage which is the difference of the activity in presence of test peptides versus total activity of the enzyme. The IC_{50} values were calculated for hydrolyzates that achieved the selection criteria: inhibition activity of 80%. Diprotin A (Ile-Pro-Ile), a well-known DPP-IV inhibitor, was used as reference inhibitor and positive control. The protein concentration of the hydrolyzates was assayed by the Kjeldahl method [30].

Animal studies

Female Wistar rats weighing 180-200g and male Wistar rats weighing 450-500g were obtained from Harlan (Barcelona, Spain). The studies in male groups occurred at the facilities of the Technological Center of Nutrition and Health (www.ctns.cat). Upon arrival, the animals were housed singly in animal quarters at 22ºC with a 12-h light/12-h dark cycle and with free access to food and water. After an adaptation period of one week, the animals were used for the experiments.

On the experimental day, the animals were randomized into different groups depending on the treatment received. To study the effect of the selected hydrolyzate p38 (p38H) on a healthy model, female rats were divided into two groups (*n*=7): the control group, treated with the vehicle (tap water), and the group receiving p38H, at 300 mg protein/kg of body weight (BW). To study the effect of the p38H on an obesity model, the rats followed a washing period and then they were fed a cafeteria diet (carrots, bacon, and milk with sugar) plus the standard laboratory chow, as previously described [31, 32]. After ten weeks, the animals were distributed into a cross-over experimental design with two groups (*n*=7), the control group and the p38H group (300 mg protein/kg BW). Finally, p38H was also tested in a model of glucose-intolerance due to age (7-month) [33]. Male Wistar rats were divided into three experimental groups (*n*=6 rats/group): control group, treated with the vehicle (tap water); p38H group, treated with 300 mg protein/kg BW; and a positive control group, treated with the commercial DPP-IV inhibitor vildagliptin (1 mg /kg BW).

For all the studies, the animals were fasted overnight before experimental treatment. At 9 a.m. on the experimental day, the treatment was administered by intragastric

gavage (i.g). After 40 minutes of treatment, animals underwent an intragastric glucose load (2 g of glucose/kg of BW). Tail blood samples were collected into a heparinized capillary tube immediately before and at 15, 30, 60, and 120 minutes after glucose administration. The plasma was immediately separated from the blood by centrifugation (2500 x *g*, 4ºC, 15 min) and stored at -80°C until analysis.

The Animal Ethics Committee of the University Rovira i Virgili (Tarragona, Spain) approved all of the procedures.

Plasma analysis

The glucose plasma levels were assayed using an enzymatic colorimetric kit (Glucose Oxidase-Peroxidase method from QCA, Tarragona, Spain) and DPP-IV plasma activity was measured by a fluorimetric assay following the method as previously described, with some modification based on the fluorimetric substrate and the DPP-IV concentration of the plasma samples.

Cell culture

The STC-1 clonal cell line was received as a kind gift from Dr. B. Wice (Washington University, St. Louis, USA) with the permission of Dr. D. Hanahan (University of California, San Francisco, USA). This enteroendocrine cell line was originated from a double-transgenic mouse tumor [34]. Cells were cultured in DMEM with GlutaMAX containing 4.5 g/l D-glucose, without sodium pyruvate (GIBCO), supplemented with 17.5% fetal bovine serum, 100 U/ml penicillin, and 100 mg/l streptomycin (BioWhittaker) and incubated in a 5% CO_2-humidified atmosphere at 37°C. At least three replicates using different cell passage numbers were performed for each experiment, including at least three wells of each condition in every replicate. Cells were used between passage numbers 30 –50.

***Ex vivo* explants**

Intestinal tissue was obtained from healthy female Wistar rats (n=6). Animals were sacrificed and their intestines were dissected out. To acquire the distal ileum segment, an incision was made in the ileocecal junction and the distal 8 cm of intestine from the ileocecal junction was excised. The ileum was rinsed with ice-cold Hank's balanced salt solution (HBSS; Thermo Fisher Scientific, Madrid, Spain) and was dissected in segments of tissue (0.75 cm2), which were placed on a 24-well plate and kept on ice until the start of secretion studies.

GLP-1 secretion studies

STC-1 cells were seeded in 24-well culture plate at a density of 2.0×10^5 cells/well for 2 days until they reached 80-90% confluency. On the day of the experiment, cells were washed twice with HEPES (20 mM HEPES, 140 mM NaCl, 4.5 mM KCl, 1.2 mM $CaCl_2$, and 1.2 mM $MgCl_2$ at pH 7.4), and p38H dissolved in HEPES buffer with 10 mM glucose

(5 mg/mL) was added to each well. After an incubation period of 2 hours at 37°C, supernatants were collected, centrifuged to remove remaining cells and stored at -80°C until it was used for determination of active GLP-1 concentration.

For the *ex vivo* explant experiments, tissue segments of ileum were placed in prewarmed (37ºC) KRBS buffer (containing 4.5 mmol/L KCl, 138 mmol/L NaCl, 4.2 mmol/L NaHCO3, 1.2 mmol/L NaH2PO4, 2.6 mmol/L CaCl2, 1.2 mmol/L MgCl2 and 10 mmol/L HEPES (adjusted to pH 7.4 with NaOH)) with 10 mM glucose, 0.1 mM diprotin A and supplemented with 15mg/mL p38H. Tissue segments were incubated for 1 hour in a humidified incubator at 37°C and 5% CO_2. After the incubation, the solutions were collected, centrifuged to remove remaining cells and stored at -80°C until it was used for determination of active GLP-1 concentration.

Tissue viability was checked by an absence of the cytoplasmic marker lactate dehydrogenase (LDH) in the incubated solutions. LDH was analyzed using an LDH kit (QCA; Tarragona, Spain).

Active GLP-1 concentration was measured with a GLP-1 7-37 amide ELISA kit (Millipore; Billerica, MA, USA).

Data analyses

The results are expressed as the mean±SEM. The repeated measurements of glucose were performed using a two-way ANOVA test and significant differences among mean values were determined by post hoc Bonferroni. Differences in the glucose area under the curve (AUC) between groups were determined by using one-way ANOVA, followed by a Bonferroni test. GLP-1 measurement was analyzed using a Student's *t*-test. All calculations were performed using SPSS software (SPSS, Chicago, USA). P-values < 0.05 were considered significant in all cases.

RESULTS

DPP-IV inhibitory activity of chicken feet hydrolyzates

Chicken feet were subjected to different conditions (pretreatments at different temperature and pH followed by treatments with different enzymes, temperature and time) to obtain a panel of different hydrolyzates. Then the DPP-IV inhibitory activity of these hydrolyzates was assayed. **Table 1** shows that 3 hydrolyzates out of the 12 tested achieved the selected threshold of 80% inhibition. The hydrolysis treatment was essential to obtain hydrolyzates with DPP-IV inhibition property since the chicken feet powder that was not subjected to hydrolysis (named p86H, Table 1) did not show inhibitory activity. DPP-IV inhibitory activity was dependent on the conditions of pretreatment and hydrolysis treatment. The results showed that the hydrolysis of protein solution subjected a pretreatment in basal conditions (25°C and pH 7.5) led to samples with DPP-IV inhibitory capacity, achieving the threshold of around 40%, in all

the hydrolysis treatment conditions (p103H, p115H, p111H). Modification of pretreatment conditions, by reducing the pH to 3 and increasing temperature (50°C and 100°C) (samples p38H, p16H, p68H in Table 1) exhibit a higher DPP-IV inhibitory activity (80-100%) than the hydrolyzates subjected at pH 7.5 and lower temperatures in the pretreatment.

TABLE 1

Chicken feed hydrolyzates with dipeptidyl peptidase IV inhibitory activity.

Hydrolyzate	Pretreatment (1.5 h)		Treatment (pH 7)			DPP-IV Inhibition (%)[a]
	Temp (°C)	pH	Enzyme	Temp (°C)	Time (h)	
p86H	25	7.5	No			0
p103H	25	7.5	Neutrase®	25	24	48.39
p39H	50	7.5	Neutrase®	25	24	44.40
p102H	25	3	Neutrase®	25	24	60.48
p38H	**50**	**3**	**Neutrase®**	**25**	**24**	**83.22**
p115H	25	7.5	Neutrase®	50	24	40.22
p19H	100	7.5	Neutrase®	50	24	77.19
p114H	25	3	Neutrase®	50	24	55.24
p16H	**100**	**3**	**Neutrase®**	**50**	**24**	**100**
p111H	25	7.5	Protamex®	50	2	44.05
p70H	100	7.5	Protamex®	50	2	60.49
p110H	25	3	Protamex®	50	2	53.30
p68H	**100**	**3**	**Protamex®**	**50**	**2**	**93.30**

[a] Dipeptidyl peptidase IV inhibitory activity of 100% sample fraction under assay conditions

The IC_{50} of the three selected was calculated and expressed in volume (µL) and protein concentration (µg/mL). The IC_{50} given in microlitres measure the hydrolyzate pharmacological potency since this value signifies the volume required to inhibit the enzyme by 50% under the assay condition. The expression of IC_{50} in protein concentration means the specificity of peptide pool mixture against DPP-IV, hence it is an indicator of the pharmacological specificity [35, 36]. All of them showed similar values between 4.03 and 4.82 µL or 297.4 and 302.9 µg protein/mL (**Table 2**). Since the temperature of pretreatment and hydrolysis were lower to obtain p38H, which could imply an industrial production less expensive, this hydrolyzate was selected for posterior experiments.

TABLE 2

Comparison between protein content and DPP-IV Inhibitory Activity of hydrolyzates of chicken feet produced by enzymatic treatments.

hydrolyzate	Protein content (mg/mL) [a]	IC_{50} (µL)[b]	IC_{50} (µg/mL)[b]
p16H	6.83	4.42	297.4
p38H	6.24	4.82	302.9
p68H	6.62	4.45	300.1

[a] Protein was measured by Kjeldahl method.

[b] IC_{50} values are reported as the mean from duplicate assays.

Effects of chicken feet hydrolyzate on plasma glucose

The effect of an acute administration of p38H was tested in two different models of rats with glucose intolerance: diet-induced and age-induced.

In a cafeteria-induced obese rat model, the administration of 300 mg/kg BW p38H together with a glucose load reduced the peak of glucose and tended to normalize the glucose values (**Fig. 1.A**.) Concomitantly, the glucose AUC values after an OGTT was lower in the p38H-treated animals than in the cafeteria group (**Fig. 1.B**). Similarly, aged rats had glucose intolerance (in non-treated animals, values at 120 min did not return to initial levels) and a p38H treatment ameliorated the plasma glucose profile similarly to what the positive control vildagliptin did (**Fig. 2**).

The effects of the hydrolyzate in rats with normal glycemia were also tested. Actually, this was performed in the same group of rats that were subjected to a cafeteria diet, but previously to the cafeteria treatment. In this case, the p38H had no significant effect (Δ Glucose AUC (arbitrary units): 2658.50 ± 902.25 and 3201.28 ± 504.41, control and p38H-treated group, respectively).

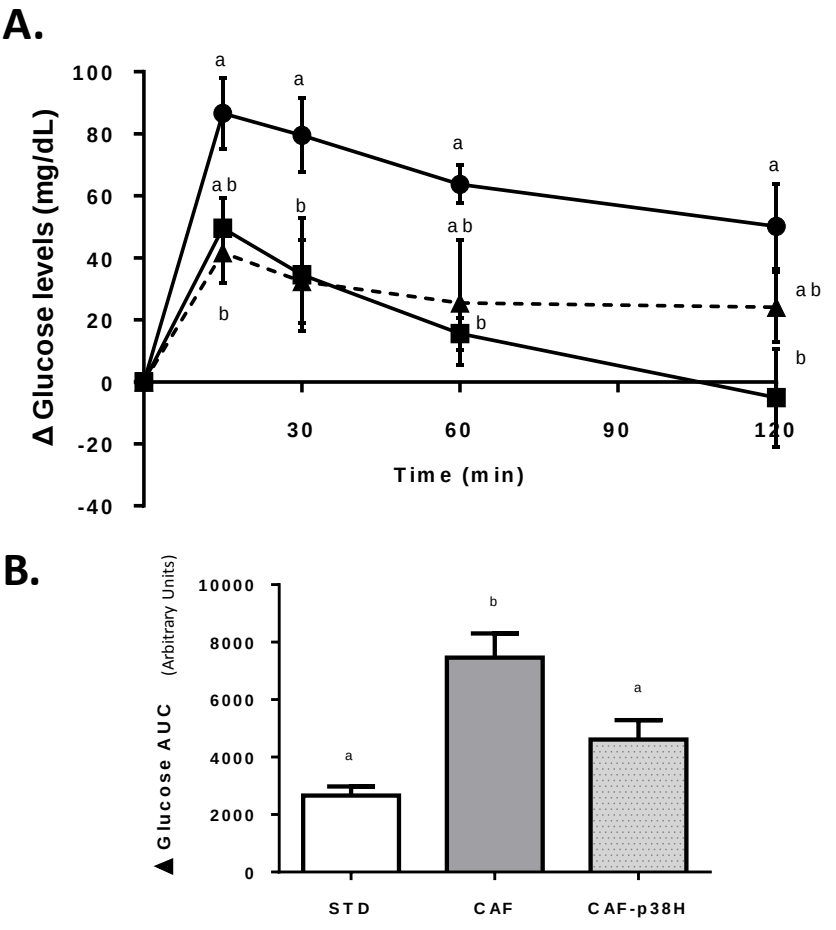

Fig 1. Plasma glucose levels during OGTT after gavage administration of water or p38H (300mg protein/kg BW) (**A**) and evaluation of the post-prandial areas under the plasma glucose curves (**B**) in the standard group (solid square or open bar), cafeteria group (solid circles or grey bar) and p38H-treated cafeteria group (solid triangle or solid and points bar). The data are given as the mean±s.e.m (n=5-7). Mean values with unlike letters were significantly different among groups (**A**, two-way ANOVA and Bonferroni post hoc comparison and **B**, one-way ANOVA and Bonferroni post hoc comparison, $p < 0.05$).

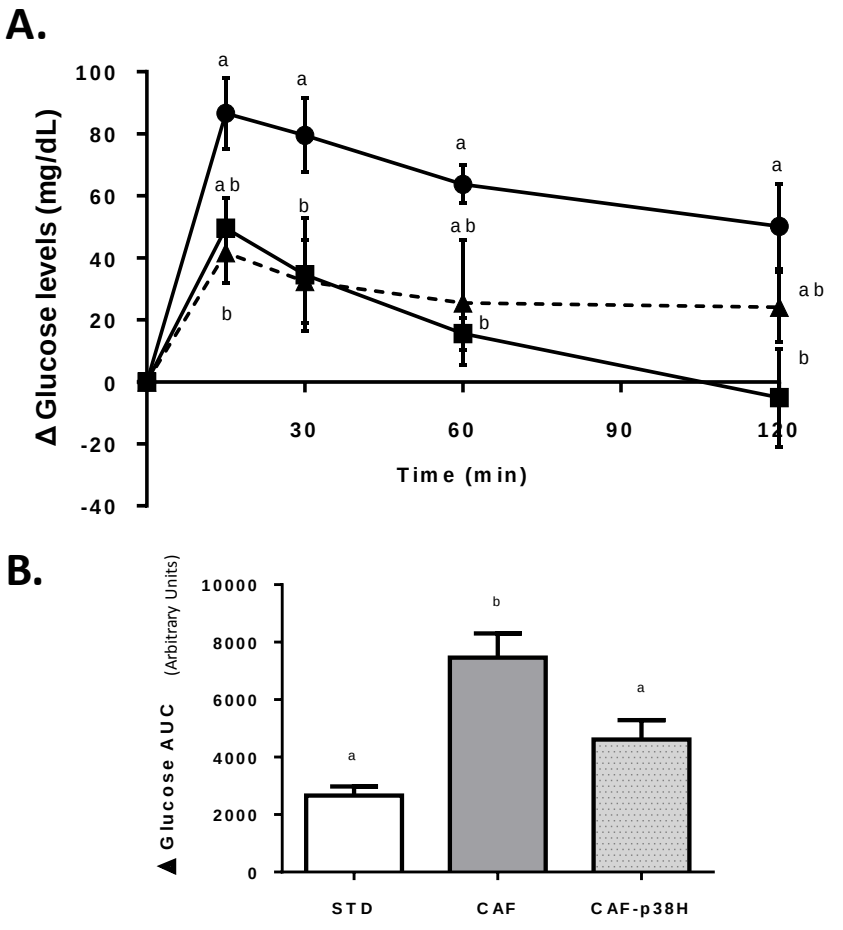

Fig. 2. Plasma glucose levels during OGTT after gavage administration of water, vildagliptin (1 mg /kg BW) or p38H (300mg of protein/kg BW) (**A**) and evaluation of the post-prandial areas under the plasma glucose curves (**B**) in the standard group (white circles or open bar), p38H-treated group (solid triangle or solid and points bar) and vildagliptin-treated group (white squares or grey bar). The data are given as the mean±s.e.m (n=5-7). Mean values with unlike letters were significantly different among groups (**A**, two-way ANOVA and Bonferroni post hoc comparison and **B**, one-way ANOVA and Bonferroni post hoc comparison, $p < 0.05$).

GLP-1 release was stimulated by Chicken hydrolyzate

Finally, we tested *in vitro* and *ex vivo* the capacity of the p38H to stimulate GLP-1 release. In STC-1 enteroendocrine cells, treatments with 5 mg/mL p38H for 2 hours led to a strong (7-fold) increase in GLP-1 levels in the media compared to controls (**Fig. 3.A**). Similarly, GLP-1 secretion from ileum tissue segments was stimulated in response to the 15 mg/mL p38H (**Fig. 3.B**).

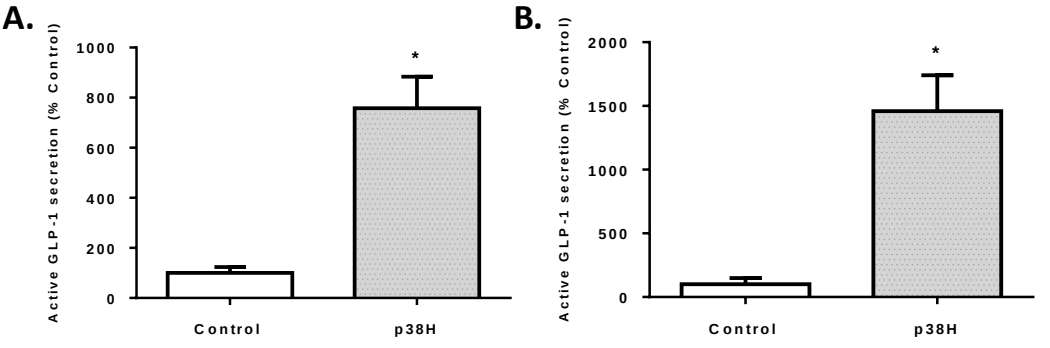

FIG 3. Selected hydrolyzate (p38H) effect on active Glucagon-like peptide-1 (GLP-1) secretion from STC-1 cells (**A**) and ileum tissue segment (**B**). The data are displayed as the means±s.e.m. Stadistically significant difference at $p < 0.05$ calculated using Student t-test.

DISCUSSION

In this paper, we focused on finding natural peptides that act as DPP-IV inhibitors and inductors of the endogenous secretion of GLP-1, which could be a useful strategy against Type 2 diabetes, pathology with a great incidence in developed countries [1, 7].

We define for the first time that chicken feet protein hydrolyzates are able to inhibit DPP-IV activity. Other food proteins had previously been shown to be sources of DPP-IV inhibitors, such as whey [37], milk [38], salmon skin [39] and quinoa [40]. We performed a screening in which chicken feet were subjected to different hydrolysis protocols. Our results show that not all treatments functioned equally, indicating the importance of the treatment conditions in the hydrolyzates behaviour. Three protocols leading to hydrolyzates with DPP-IV inhibitory activity were found. The pretreatment for 1.5 hours at 50°C or 100°C and pH value of 3 was crucial to achieving hydrolyzates with high DPP-IV inhibitory capacity (p38H, p16H, and p68H). In fact, the three selected hydrolyzates presented similar pharmacological potency, since IC_{50} values expressed as microliter are similar (around 4 µL), and also similar pharmacological specificity, shown by similar IC_{50} values expressed as micrograms protein per millilitre (around 300 µg/mL). The IC_{50} that we obtained is 10-fold lower than that found in a previous study which used collagen obtained from chicken-feet and hydrolyzed using *Streptomyces* collagenase (45°C, pH 7.5, 17 hours) [41]. Thus working with the whole

chicken feet source, and using the appropriate conditions (here defined) can lead to solutions with much more DPP-IV inhibitory potency.

The obtained IC_{50} values are within the range of that observed by other food hydrolyzates [13, 42, 43]**.** Lower IC_{50} values of the hydrolyzates compared to purified peptides have been found in other studies [38]. Hence, those results suggest that the concentration of bioactive peptide in the hydrolyzate is low and different bioactive peptides or a different combination of them might be involved in DPP-IV inhibitory activities. Although a further identification of the bioactive peptide would be of interest, we here present important clues to prepare hydrolyzates from chicken feet for a use as antihyperglycemic.

We next selected one of the chicken feet hydrolyzate, p38H, to demonstrate its bioactivity *in vivo*. It must be taken into account that high DPP-IV inhibitory activity *in vitro* could not correspond with high antihyperglycemic effect *in vivo*. This could be because the bioactive peptides contained in the hydrolyzates can be hydrolyzed and transformed into inactive peptides during the gastrointestinal digestion. For this reason, it is essential to validate the hydrolyzate bioactivity *in vivo*. We tested the p38H effects at reducing glycemia in a model of rats fed a cafeteria diet. Our results show that the p38H treatment led to a lower glucose initial peak and a lower glucose curve, with a tendency to reach levels of standard-fed animals. Instead in the healthy rats (assayed previously to the cafeteria treatment), p38H did not modify the AUC of the glucose load. This could be related to the fact that in the cafeteria-fed rats the initial glucose peak was greater (an increase of around 80 mg/dL at 15 min) than before the cafeteria treatment (glucose increase of around 50 mg/dL) because of their disorder in glucose homeostasis. This disorder induced by diet underscored in 120 min after the OGTT when the cafeteria-fed animals glycemia did not return to basal levels, while previously to the cafeteria treatment these rats showed normal OGTT curves. The p38H effects were tested in another model that showed glucose-intolerance, aged rats. In this model, p38H also lowered the plasmatic glucose curve. This is the first trail that acute dose of peptide hydrolyzates influence glucose homeostasis in rat models that present glucose intolerance, while only a few potential DPP-IV inhibitor-peptides have previously been tested *in vivo* for their glucose lowering capacity in healthy models. Uchida et al. tested a ß-lactoglobulin hydrolyzate in mice and observed that glucose levels were reduced at 15 min after an OGTT. It worth mentioning that the glucose dose administered was 10 g/Kg BW, much higher than the one we have tested (2 g/kg BW) and that in agreement led to a great (around 3-fold) increase in glucose peak at this time point [16], reinforcing that the effects are dependent on the amount of circulating glucose. This might be linked to the modest potency of the hydrolyzates compared to commercial purified DPP-IV inhibitors, which we and others [16] observed. Interestingly in aged control animals, the comparison of p38H-treated and vildagliptin-treated animals showed a different glucose pattern: the p38H was not as effective as vildagliptin at reducing the initial glucose peak but normalized its glycemia

at 120 min in a similar way. These results suggest that p38H might act through different mechanisms to ameliorate hyperglycemia.

It must be stated that we measured DPP-IV activity in plasma from the tail and found no effect of p38H in any of the animal experiments (results not shown). However, it has been shown that the main GLP-1 degradation occurs immediately at the site of secretion, and its short half-life generates that only 10-15% active GLP-1 levels remain in the systemic circulation [3, 44]**.** Although various hydrolyzates have been reported to reduce plasma DPP-IV activity after chronic treatments [45–47], few studies have shown that effect in acute treatments. Actually, whey protein has been reported to attenuate hyperglycemia in part by inhibition of DPP-IV activity in the small intestinal tissue, but not in orbital vein [48]. It has also been shown that ileal administration of Zein and rice protein hydrolyzates decreased plasma DPP-IV activity in ileal vein [18, 28]. And in fact, none of the other papers describing *in vivo* antihyperglycemic effects of protein hydrolyzates shows DPP-IV activity in peripheral blood [16, 17]. Therefore, it cannot be excluded that, despite the lack of DPP-IV inhibition in peripheral blood, p38H lead to an amelioration of glucose profile due to the inhibition of local intestinal DPP-IV.

Previously, it has been shown that Zein and rice protein hydrolyzates have antihyperglycemic effects by a dual mechanism of action, both inhibiting DPP-IV activity as well as increasing GLP-1 release [18, 28]. Actually, Mochida et al. showed that Meat hydrolyzate (acquired from Sigma, no description of the meat origin) was also tested and shown to induce intestinal GLP-1 secretion after ileal administration, but it has not the dual effect. Other protein hydrolyzates have been tested as GLP-1 stimulator *in vivo, in situ* and *in vitro* models [19, 23, 49, 26]. We reproduced this approach and tested whether chicken feet hydrolyzate could also modulate GLP-1 release in *in vitro* and *ex vivo* model. Our results show a strong significant increase in GLP-1 levels after p38H treatment in both models. Despite the form in which the hydrolyzate might reach the enteroendocrine L-cells *in vivo* might differ from that used in *in vitro* or *ex vivo*, these results suggest that this might also be a mechanism that helps to reduce glycemia in animals. This additional mechanism could help to explain the differences found between the DPP-IV inhibitor vildagliptin and the p38H in the aged-rats experiment, since it could be that after 120 minutes the p38H reached the intestinal regions where GLP-1 secreting cells are located (mainly ileum and/or colon) and a stimulation of GLP-1 would explain the reduced glucose levels. Further experiments will be required to confirm this hypothesis.

In conclusion, we here report that chicken feet hydrolyzates obtained by Neutrase® treatment reduced glycemia in glucose-intolerant rats and that this might be mediated through their DPP-IV inhibitory capacity. In addition, a stimulation of endogenous GLP-1 secretion could also be involved in the antihyperglycemic effects of the p38H.

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Justificante de presentación electrónica de solicitud de patente

Este documento es un justificante de que se ha recibido una solicitud española de patente por vía electrónica utilizando la conexión segura de la O.E.P.M. De acuerdo con lo dispuesto en el art. 16.1 del Reglamento de ejecución de la Ley 24/2015 de Patentes, se han asignado a su solicitud un número de expediente y una fecha de recepción de forma automática. La fecha de presentación de la solicitud a la que se refiere el art. 24 de la Ley le será comunicada posteriormente.

Número de solicitud:	P201731065	
Fecha de recepción:	04 September 2017, 13:28 (CEST)	
Oficina receptora:	OEPM Madrid	
Su referencia:	143-17	
Solicitante:	Universitat Rovira i Virgili	
Número de solicitantes:	1	
País:	ES	
Título:	Hidrolizados de garras de patas de pollo, sus péptidos y usos de los mismos	
Documentos enviados:	Descripcion-1.pdf (33 p.) Reivindicaciones-1.pdf (4 p.) Resumen-1.pdf (1 p.) Dibujos-1.pdf (10 p.) OLF-ARCHIVE.zip FEERCPT-1.pdf (1 p.) FEERCPT-2.pdf (1 p.) SEQLPDF.pdf (4 p.) SEQLTXT.txt	package-data.xml es-request.xml application-body.xml es-fee-sheet.xml feesheet.pdf request.pdf
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IV. DISCUSSION

1. Thesis summary and general discussion

The increased prevalence of overweight and obese individuals, in both developing and developed societies, highlights the need to find preventative therapies, as well as treatments for obesity and its metabolic-associated diseases. The gastrointestinal tract is the largest endocrine organ in the body producing hormones that act as important mediators of glucose homeostasis and food intake. The focus of type 2 diabetes mellitus (T2DM) treatments is keeping blood glucose levels within the normal physiological range [1]. With regards to body weight management, focusing on the regulation of food intake is one potential strategy as it is well known that the main causes of obesity is increased food intake coupled with decreases energy expenditure [2].

In the last decade, scientific advances have aided the investigation of natural bioactive compounds to reduce obesity and related pathologies, such as cardiovascular disease, T2DM, osteoarthritis and some cancers (colon, endometrial and breast cancers) [3–5]. The use of natural bioactive compounds to modulate gut hormone secretion, and consequently appetite and glucose homeostasis, might be a potential strategy to prevent or/and treat obesity and one of the most associated disease, T2DM. In the field of natural bioactive compounds, the scientific evidence of their action has to be approved as a health claim [6]. Additionally, the complete description of their mechanisms is important if they are to be recommended for the reduction of disease risk in a target population. Thus, this thesis was focused on defining the mechanisms of a known bioactive compound at the intestinal level, i.e. grape seed proanthocyanidin extract (GSPE), specifically on the different mechanisms involved in enteroendocrine system modulation, as well as finding new ones, i.e. bioactive peptides derived from chicken feet, that might act through modulation of these intestinal mechanisms. To achieve our objective different models of study, including *in vitro*, *ex vivo* and *in vivo* models, were used.

The *in vivo* release of enterohormones occurs through direct sensing of luminal components, and also indirectly by vagal and humoral stimulation [7]. Therefore, one important mechanism to understand plasma intestinal hormone levels is the direct interaction of the luminal content with enteroendocine cells (EECs). *In vitro* and ex *vivo* models were used to study the direct effect of compounds on hormone release. In *ex vivo* model, our results showed that GSPE (170 mg phenolics/L) and metabolites of GSPE (~188 mg phenolics/L) increased active GLP-1 levels in ileum and colon segments, respectively. Previous studies done in our research group described that GSPE has antihyperglicemic properties that were attributed to the proanthocyanidins insulin-like effect in adipose tissue [8] and also the proanthocyanidins modulation of insulin secretion and production [9, 10]. However, antihyperglicemic properties are also associated with the incretin effect. Previous studies of our group reported that acute dose of 1g/Kg BW grape seed proanthocyanidin extract (GSPE) increases active

GLP-1 plasma levels after an oral glucose load in healthy rats. Such increase could, in part, explain the increased plasma insulin levels and the decreased plasma glucose levels which were also reported in the same study [11]**.** Therefore, the increased active GLP-1 plasma levels could be derived, in part, due to the direct effect of GSPE on hormone secretion. In contrast to the results that we observed *ex vivo*, previous results done in our research group reported that 50 mg/L of GSPE decreased active GLP-1 secretion in the STC-1 cell line [12]. Different results when using different models were also reported in studies of short chain fatty acids. Valeric acid induced PYY secretion in STC-1 [13], but no changes were observed in ileum and colon intestinal segment after incubation with valeric acid [14]. These controversial results could possibly be explained by the different features that the models used exhibit. The study of the local effect of the compounds on the intestine is a complicated issue due to the complexity of the intestinal architecture. In the intestinal cell line model, the limitations that warrant consideration are the lack of interaction between EEC and other epithelial cells, and the lack of polarity of the cell. On the contrary, the *ex vivo* model, enables us to study the hormone release from enteroendocrine cells that are situated in their normal epithelial environment. Due to their advantages over *in vitro* cell lines, the *ex vivo* model has been optimized at our laboratory to study enterohormone secretion, and the results found are in agreement with previous *in vivo* observations. At this point, it should also be stated that both models present a disadvantage, lack of discrimination between apical and basolateral sides. There are other study models using *ex vivo* samples that overcome this limitation, such as ussing chambers or InTESTine™ [15], but their viability to allow the study of enteroendocrine secretions has not been fully demonstrated yet.

The results obtained *in vitro* (STC-1 cell line) and using *ex vivo* models also elucidated to the direct effect of a selected chicken feet hydrolyzate that is a source of bioactive peptides, on GLP-1 release. It has been suggested that the capacity of protein hydrolysates to enhance GLP-1 secretion depends on the nature of their source precursor [16], their hydrolysis rate [17] and their amino acid profile [18]. Although the specific amino acid motif that triggers GLP-1 secretion stimulation has not yet been determined, it has been reported that peptide transporter-1 (PEPT1) and the calcium-sensing receptor (CaSR) are involved in GLP-1 secretion of di-, tri and oligopeptides [19]**.** Moreover, the apical localisation of PEPT1 emphasises its possible role in the direct effect of bioactive peptide sequence from chicken feet hydrolyzate on GLP-1 release.

While the direct interaction of bioactive compounds with EECs is a possible mechanism to modulate GLP-1 plasma levels, acting on GLP-1 cleavage through inhibiting DPP-IV enzyme is also an important strategy to enhance active GLP-1 plasma levels. Previous studies reported that GSPE inhibits DPP-IV activity *in vitro* assays. In this thesis, we hypothesized that hydrolysates from chicken feet could also be potential DPP-IV inhibitors, due to the structural characteristics of peptides, which can mimetic

the sequence of DPP-IV substrates. The potential capacity of chicken feet as natural source of DPP-IV inhibitor was employed to screen an array of different chicken feet hydrolyzates obtained by different enzymatic treatments. The different hydrolysates were analysed and the most potent was selected. The *in vitro* results determined that chicken feet hydrolyzates inhibit DPP-IV, but not all treatments functioned equally, indicating the importance of production conditions to liberate the bioactive peptides sequence. In the DPP-IV inhibitors classified in peptide-derived peptidomimetic inhibitors, the hydrolysis treatment and the source of the natural precursor used to obtain the hydrolysate are important factors (reviewed in [20]). Given that GLP-1 is an important mediator of glycemic homeostasis, the inhibitory DPP-IV action of chicken feet hydrolyzate as well as the capacity to enhance active GLP-1 secretion, explain the *in vivo* results obtained that show an improvement of glucose levels in disrupted-glucose homeostasis animals. Thus in this thesis we define a novel source of bioactive natural compounds, a novel method to obtain the hydrolysate, and its novel antihyperglicemic property. Actually subsequent studies have led us also to define the main peptide sequence responsible of DPP-IV inhibition (manuscript in preparation).

The *ex vivo* model also confers the possibility to study the region-specific architecture and specific hormone release patterns of different subtypes of EECs, and thereby investigate other enterohormones related with glucose homeostasis and food intake. Our results showed that GSPE treatment directly decreased CCK release in duodenal segments, in agreement with previously observed changes in plasma levels of acute *in vivo* study [21]. GSPE also directly acted on GIP secretion in duodenum segment, inducing an increase of GIP release. This is in contrast with the previously observed reduction in GIP plasma levels in an acute *in vivo* study [11]. The distinct results could be given to other elements of the metabolism, which are not monitored in study models based on local intestinal hormone release, such as *ex vivo* model. These elements could also contribute to gut hormone secretion *in vivo* and thereby the enterohormone levels measured in plasma. Specifically, the feedback regulation between insulin and GIP secretions could be this other signal element [22]. Finally, our results also indicated that GSPE and metabolites of GSPE directly increase PYY secretion in ileum and colon segments, respectively. Only few studies reported the effect of flavonoids on PYY release and all of these studies performed were chronic treatments [23, 24]. Therefore, this is the first time that an acute effect of flavonoids on PYY secretion is reported. Recently, it has been described that ghrelin release was also modulated by GSPE treatment in *ex vivo* and *in vitro* (ghrelinoma cell line) models [25]. The same study reported that monomeric flavanols present in GSPE composition stimulate ghrelin release via activation of bitter taste receptors. Given that monomeric flavanols are reported as agonists of the bitter receptors hTAS2R14 and hTAS2R39 [26–28] and these receptors have been shown to be involved in CCK and ghrelin secretion [25, 29], it is reasonable to postulate that these receptors could be involved in direct effect of GSPE on enterohormone release. However, the heterogenous structural composition of GSPE needs to be considered. For example, it has been

reported that monomeric flavanols enhance ghrelin secretion whereas oligomeric structures inhibited its secretion [25]. Therefore, others receptors and others signals could also be involved in regulating gut hormone release by GSPE.

Once we determined the direct modulation of enterohormone release by an acute dose of bioactive compounds, we wanted to determine which mechanisms could be involved in regulating enterohormone release in mid-term GSPE treatments. In this context, a three dimensional organoids model was used to elucidate whether GSPE affect intestinal differentiation. The organoids model is a self-renewing *in vitro* culture in which single intestinal stem cells generate villus-like epithelial domains in which all differentiated intestinal cell types are present [30, 31]. Therefore, organoids, unlike cell line and *ex vivo* model, enabled us to investigate intestinal cell development in real time and whether GSPE modulates their differentiation in the normal epithelial environment. Moreover, the evaluation of enterohormone secretion in mid-term treatments can also be studied. In addition, we also analyzed the mid-term effects of GSPE on the endocrine system in an 8-day study using healthy rats. The *in vivo* model enabled us to analyse the gut microbiota, which is an important element of the intestinal environment and could be subject to alterations by diet components in a mid or long-term consumption. Furthermore, the crosstalk between the changes in gut microbiota and host metabolism can also be studied in the *in vivo* model.

Chromogranin A (ChgA) is a member of the granin family of acidic secretory glycoproteins that are expressed in endocrine cells and neurons. Chga plays a role in the biogenesis of secretory granules and influences peptide hormone transport into secretory granules [32]. Therefore, the expression of this gene is associated with endocrine cells and it is commonly used as marker of endocrine intestinal cells. Previous results reported that 25 mg/kg BW of GSPE during chronic treatment (12 weeks) counteracts down-regulation of Chga gene levels induced by a cafeteria diet in rats, suggesting a role of GSPE in enteroendocrine cell differentiation [33]. In mid-term treatments, our results showed that 5 mg/L of GSPE up-regulate Chga gene expression in ileum organoids culture. Accordingly, Chga gene expression was also increased by 0.5 g/kg BW of GSPE administered during 8 days in ileum rats. Our results also showed that colonic Chga gene expression is increased after an acute dose of GSPE in *in vivo* studies. Such increase observed in acute treatments could be related with the function of Chga in the biogenesis of secretory granules rather than the promotion of EEC differentation. To further explore the differential effects of GSPE, transcription factors associated with EECs development were studied in ileum organoid culture. Our results suggested that the modulation of the early-transcription factor (Ngn3) and late-transcription factors (Pax4, Pax6, Foxa1/2, Arx) by GSPE leads to L-cell differentiation.

Moreover, we analysed the effect of GSPE on L-cell gene expression markers: GLP-1 and PYY. In accordance with Chga gene expression, an up-regulation of ileal PYY expression was observed in organoids culture and healthy rats. Ileal GLP-1 expression

was also increased during GSPE treatment in organoid culture, but no changes were observed in GLP-1 expression in rats after 8 days of GSPE treatment. It must be considered that the evaluation of intestinal differentiation was mainly performed in an *in vitro* model, and thus extrapolation to *in vivo* effects must be performed carefully. Actually, the organoid cells are constantly interacting with GSPE molecules for the duration of the 72 hours, instead, *in vivo* the molecules of GSPE pass through the GI tract each day when the dose is administered and thereby there is not a constant interaction. Therefore, the length and dose used in the studies may play a main role in the modulation of gene expression. In fact, our results in organoids culture showed that lower doses of GSPE show different effects on the modulation of enteroendocrine cell differentiation depending on the dose. Given that it is difficult to estimate the dose *in vivo* that would be equivalent to the assayed *in vitro*, more studies will be required to fine tune the optimal range of dose-time that reflects in vivo conditions. However, the results obtained in organoids culture suggesting an effect on EE cell differentiation agree with the previous results done in our research group that showed that the down-regulation of Chga, GLP-1 and PYY gene levels, induced by a cafeteria diet, are neutralized by a chronic dose of GSPE [33].

Furthermore, recent studies refute the classical concept of separate cells for separate hormones [34–36]. These studies showed that L-cells from the upper small intestine resemble upper intestine K-cells or I-cells more than colonic L-cells, judging from hormone expression profiling. Therefore, the EECs express an overlap of gut hormones along the gastrointestinal tract. It has been reported that most L-cell populations in the upper small intestine contained CCK, and 10-20 % contained GIP and PYY, together with GLP-1. In the same study, the authors also observed that L-cells of the lower small intestine also co-express GLP-1 together with GIP and CCK genes, but the levels expression are lower compared with proximal small intestine. Moreover, the number of L-cells that co-express PYY are increased progressively between proximal and distal intestine [36]. Altogether, this might be correlated with our findings regarding the ileal GLP-1, CCK and PYY gene expression in rats. However, pools of PYY-, GIP-, or CCK positive cells that do not also express proglucagon have not been reported yet. Therefore, further studies using immunohistochemistry or flow cytometry would be interesting to study the array of hormones that EECs modulated by GSPE express.

GSPE could also affect the entroendocrine system in mid-term treatments through the gut micobiota. Several studies reported that flavanols unabsorbed by the upper intestine pass through the large intestinal lumen reaching the colon where they accumulate at high concentrations and are metabolised by the gut microbiota [37]. There is evidence that the gut microbiota act on flavanols, however, little research has been published about the inverse relation the effect of flavanols on gut microbiota. The results obtained in this thesis revealed that the dose of 0.5g/kg BW of GSPE changed the gut microbial composition after 8-days of treatment in animals fed a standard diet. Only few studies reported the modulation of gut microbiota by flavonoids [38].

Moreover, all of these studies analyse the effects of chronic polyphenol treatment in combination with an energy-dense diet, on the other hand, this thesis found GSPE was able to modulate gut microbiota in only 8 days in rats fed a standard diet. We demonstrated that 0.5g/kg BW of GSPE decreases the ratio of Firmicutes/Bacteroidetes in agreement with the general results obtained with polyphenols [38]. Moreover, our results indicated that GSPE acts on lower levels of microbiome classification, altering specific genera. In this study, new target taxonomic groups influenced by proanthocyanidins were defined, including *Sutterella*, *Pharscolarctobacterium*, *Parabacteroides, Bilophila,* and *Ruminococcus.* In parallel to studying the modulation of gut microbiota, we analysed the crosstalk between the modulation of gut microbiota and host metabolism using correlation analysis. The study of the alteration of the gut microbiota and its relationship with the host represents a real challenge, due to its heterogeneous composition and the complexity in understanding the gut microbiota-host interactions. However, we described some interaction where alterations in gut microbiota induced by GSPE are correlated with some metabolism parameters, especially with the increased active GLP-1 plasma levels. It has been reported that the intake of *Lactobacillus reuteri* results in an increase GLP-1 secretion in humans. It has also been shown that the administration of VSL#3 probiotic promotes GLP-1 secretion through changes in the gut microbiota which were associated to increased levels of short chain fatty acids (SCFA) [39]. SCFAs can bind to GPCR41 and GPR43 receptors expressed by enteroendocrine cells and induce GLP-1 secretion [40, 41]. Our results showed a decrease of butyrate content, although other pathways link gut microbiota and GLP-1 secretion, such as the endocannabinoid system [42] or bile acid metabolism [43]. However, further studies are needed to elucidate whether increased active GLP-1 levels in mid-term treatment is a causal effect of gut microbiota alterations by GSPE.

Recent studies described the gut microbiota's direct influence on the expression of genes involved in intestinal epithelial proliferation and differentiation [44, 45]. Moreover, it has also been shown that the fermentation of non-digestible carbohydrates can promote differentiation in the proximal colon and consequently increase the number of L-cells in rats, suggesting that the fermentation end-products of SCFA could be responsible [46]. This points towards the gut microbiota playing an important role in the regulation of colonic differentiation either directly or indirectly through SCFA. Therefore, in addition to a possible direct effect of GSPE on intestinal differentiation, GSPE could also affect intestinal differentiation through the modulation of microbiota. It must be mentioned that no changes in colonic gene expression were observed after 8-days of treatment in rats, suggesting that further work is required to determine whether GSPE modulates differentiation through changes in the microbiota. Whether this could explain the previously mentioned GSPE effects in long-term treatments, that is counteraction of the down-regulation of Chga, GLP-1 and PYY gene levels induced by a cafeteria diet after 12 weeks, should be further assessed. Moreover, the effects of metabolites of GSPE produced by the microbiota should be considered in

colon tissue. Therefore, further studies are needed to determine the effects of GSPE on colon sections and the mechanisms underlying these effects.

Recent studies done in our group showed that the acute increase of active GLP-1 plasma levels induced by 1g/Kg BW of GSPE is also an effective satiety signal in rats [21]. Importantly, the loss of 0.5 g/Kg BW GSPE's satiating effect following GLP-1 receptor blockade using exendin, suggests that GSPE's satiating effects are mediated by an enhancement in GLP-1 signalling [47]. As above mentioned, such increase in GLP-1 plasma levels is due to a direct effect of GSPE on hormone secretion and also due to the inhibition of DPP-IV enzyme [48]. Moreover, the satiating effect was also observed in rats treated with 0.5 g/Kg BW GSPE for 8 days. These animals also gained less weight and had an increased energy expenditure [49]. An equivalent dose of gallic acid that contains 0.5 g/Kg BW GSPE also showed an acute satiating effect. However, the satiety effect of gallic acid was not extended after 8-day treatment, like with GSPE [47]. As above mentioned, we observed that 0.5 g/Kg BW GSPE modulates the gut microbiota after 8 days, and such modulation correlated with increased active GLP-1 levels. However, our results showed that an equivalent dose of gallic acid doesn't modulate the microbiota composition nor the GLP-1 secretion. Considering these results, we hypothesized that the modulation of the gut microbiota might be a mechanism by which mid-term GSPE treatments have a satiety effect.

Like gallic acid used in *in vivo* treatment, other pure compounds contained in GSPE were also evaluated in an attempt to identify molecules responsible for the acute and mid-term local effect of GSPE on intestinal tissue. Our results showed that monomeric and dimeric structures (epicatechin gallate, dimer B2, dimer B2 gallate, gallic acid) could contribute to the acute CCK secretion induced by GSPE. In mid-term treatments using organoids model, our results showed that epicatechin and gallic acid are not the responsible for all the effects produced by GSPE. However these molecules showed some bioactivity, such as the modulation of the expression of some transcription factors; Pax4 gene expression by gallic acid and FOXA ½ by epicatechin and the modest increase of total GLP-1 secretion after mid-term treatment. These results suggest that gallic acid and epicatechin might be, in part, responsible for the increased GLP-1 secretion observed in mid-term GSPE treatments. The results are obtained using only part of the molecules that form GSPE because some of the other components could only be obtained in very small amounts. However, we conclude that the effects of GSPE are attributed to the complex mixture of compounds present in this extract. In consequence, treatments using pure molecules alone present few effects compared to the effects generated by the whole GSPE. Previous results done in our research group showed that catechin, gallic acid and B2 dimer molecules of GSPE inhibit DPP-IV activity *in vitro*, but no effect was observed in epicatechin treatment. The combination of them potently inhibit DPP-IV [11], suggesting a synergic effect of these molecules. On the other hand, GSPE has been reported to inhibit octanoyl ghrelin in ghrelinoma cell line; in accordance oligomeric molecules also inhibit ghrelin release. In contrast,

monomeric molecules of GSPE enhanced ghrelin secretion, suggesting antagonistic effects of pure molecules on ghrelin secretion [25]. Therefore, each bioactive molecule of GSPE could present different effects and thereby GSPE action could be a result of additive, synergic or antagonistic effects of each molecule on different targets. In accordance, it has been reported that vitamin C in apples contributed to only 0.4% of the total antioxidant activity of the whole apple; most of the antioxidant activity comes from phytochemicals presents in apple skin. Therefore, the whole fruit is more potent than the administration of one pure compound (reviewed in [50]). Moreover, it has been described that the combination of bioactivity of different sources of natural compounds potentiates the effect of each sources administered individually, such as the combination of omega-3 polyunsaturated fatty acids and GSPE [51–54]. Therefore, treatments using mixture of bioactive compounds are more effective than a treatment with one pure molecule.

Taking into account the effects of GSPE and chicken feet hidrolyzates on GLP-1 enterohormone levels and thereby on glucose homeostasis, the study of the effect of the combination of these two natural sources on enteroendocrine system would be interesting. Different studies have reported the use of proteins as carriers of flavonoids, without impairing their bioavailability (milk and catechins) [55], to reduce the bitter taste induced by flavonoids [56, 57]. Therefore, the combination of chicken feet hydrolyzates with GSPE could be a good approach to develop a functional food. However, future studies should evaluate the interaction between proanthocyanidins from GSPE and proteins from chicken feet hydrolyzates and whether such interaction affects the direct action of GSPE on the enteroendocrine system.

Furthermore, the promising results obtained in acute treatment of chicken feet hydrolyzate related with glycemic homeostasis open a new line of research which could be further studied concerning the effect of chicken feet hydrolyzate on other enterohormones related with glucose homeostasis and food intake. It has been reported that pea protein increased fullness and decreased food intake in an ad libitum meal in humans [16], and whey protein is also associated to increased GLP-1 plasma levels and reduced energy intake in humans [58]. Hence, future experiments studying the action of chicken feet hydrolyzate on food intake *in vivo* would be interesting, as well as, evaluating the mid-term and long-term effects of chicken feet hydrolyzate on enteroendocrine system related with glucose homeostasis and food intake.

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CONCLUSIONS

SUMMING UP: MAIN CONCLUSIONS

The main conclusions obtained from this thesis are:

1. **GSPE modulates gut hormone secretion by directly acting on enteroendocrine cells in acute treatments.**

    - An acute dose of GSPE and GSPE metabolites increases active GLP-1 secretion in *ex vivo* ileum and colon segments, respectively.
    - The direct release of GLP-1 following GSPE stimulation requires glucose.
    - GSPE's effect on gut hormone secretion varies along the gastrointestinal tract
        - GSPE increase GIP and decrease CCK release in *ex vivo* duodenum segments.
        - GSPE and GSPE metabolites increase PYY release in *ex vivo* ileum and colon segments, respectively.
    - The reduction of CCK secretion, following GSPE administration, is partly mediated by GA.

2. **The modulation of intestinal differentiation is a mid-term effect of GSPE.**

    - GSPE enhances the gene expression of L-cell differentiation markers Chga, Gcg, and PYY in organoids.
    - GSPE modulates early-transcription factors (Ngn3) and late-transcription factors (Pax4, Pax6, Foxa1/2, Arx) involved in EEC differentiation.
    - GSPE does not only enhance differentiations markers for L-cell, it also up-regulates markers for enterocyte and goblet cells, while reducing the marker for stem cells.

3. **GSPE modulates the composition of the gut microbiota and such modulation is related to host metabolism in rats.**

    - GSPE decrease the ratio of Firmicutes:Bacteroidetes.
    - New target of microbial taxonomic groups influenced by proanthocyanidins are defined: *Sutterella*, *Pharscolarctobacterium*, *Parabacteroides, Bilophila,* and *Ruminococcus*.

- The GSPE-induced changes of the gut microbiota led to a modulation of the acetate:propionate:butyrate ratio.
- The gut microbiota profile, following GPSE treatment, correlates with host metabolic parameters, and notably with increased active GLP-1 plasma levels.

4. **Chicken feet hydrolyzate modulates the incretin system through different mechanisms.**

- An acute dose of chicken feet hydrolyzate stimulates GLP-1 release in *ex vivo* and *in vitro* model.
- DPP-IV activity is inhibited by chicken feet hydrolyzate *in vitro*.
- Chicken feet hydrolyzate improves glucose plasma levels in disrupted-glucose homeostasis rat models.

CONCLUSIONS GENERALS

Les principals conclusions obtingudes en aquesta tesi són:

1. **GSPE modula la secreció de les hormones intestinals mitjançant un efecte directe sobre les cèl·lules enteroendocrines en tractaments aguts.**

    - Una dosi aguda de GSPE i dels seus metabòlits augmenta la secreció de GLP-1 actiu en segments *ex vivo* d'ili i de colon, respectivament.
    - La secreció de GLP-1 produïda per una directa estimulació de GSPE requereix glucosa.
    - L'efecte de GSPE sobre la secreció d'hormones intestinals varia a través del tracte gastrointestinal.
        - GSPE augmenta la secreció de GIP i disminueix la secreció de CCK en segments *ex vivo* de duodè.
        - GSPE i els seus metabòlits augmenten la secreció de PYY en segments *ex vivo* d'ili i de colon, respectivament.
    - La disminució de la secreció de CCK, després de l'administració de GSPE, és causada, en part, per GA.

2. **La modulació de la diferenciació intestinal és un efecte subcrònic del GSPE.**

    - GSPE promou la expressió de gènica dels marcadors de la diferenciació cel·lular de les cèl·lules L en organoids: Chga, Gcg i PYY.
    - GSPE modula els factors de transcripció primerencs (Ngn3) i els factors de transcripció tardans, involucrats en la diferenciació de les EEC.
    - GSPE no solament augmenta els marcadors de diferenciació de les cèl·lules L, sinó que també promou l'increment dels marcadors dels enteròcits i de les cèl·lules calciformes, així com la disminució del marcador de les cèl·lules mare.

3. **GSPE modula la composició de la biota intestinal i aquesta modulació està relacionada amb el metabolisme de l'hoste en rates.**

    - GSPE disminueix el rati Firmicutes:Bacteroidetes.
    - Es defineixen nous grups taxonòmics microbians com objectiu de l'acció de les procianidines: *Sutterella*, *Pharscolarctobacterium, Parabacteroides, Bilophila* i *Ruminococcus*.
    - Els canvis de la biota intestinal induïts per GSPE deriven en la modulació del rati acetat:propionat:butirat.

- El perfil de la biota intestinal, posterior al tractament amb GSPE, correlaciona amb paràmetres metabòlics de l'hoste, del qual destaca la correlació amb l'augment dels nivells de GLP-1 en plasma.

4. **L'hidrolitzat de pota de pollastre modula el sistema d'incretines mitjançant diferents mecanismes.**

    - Una dosi aguda d'hidrolitzat de pota de pollastre estimula la secreció de GLP-1 en els models *ex vivo* i *in vitro*.
    - L'activitat de DPP-IV és inhibida per l'hidrolitzat de pota de pollastre *in vitro*.
    - L'hidrolitzat de pota de pollastre millora els nivells de glucosa en plasma en models de rates que presenten una homeòstasis de la glucosa alterada.

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LIST OF PUBLICATIONS

FULL PAPERS

Casanova-Martí, À., Serrano, J., Blay, M. T., Terra, X., Ardevol, A. and Pinent, M. Acute selective bioactivity of grape seed proanthocyanidins on enteroendocrine secretions in the gastrointestinal tract, *Food Nutr. Res.*, 2017, **61**, 1321347.

Casanova-Martí, À., González-Abuín, N., Serrano, J., Blay, M. T., Terra, X., Pinent, M., Frost, G and Ardevol, A., Promotion of L-cell differentiation using grape seed proanthocyanidins extract. (In preparation)

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PATENTS

Ardévol, A.,Pinent, M., Blay, M. T., Arola, L., **Casanova-Martí, À**., Use of proanthocyanidins for suppressing appetite or inducing satiation. WO/2016/059281. (april 2016)

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ABSTRACTS

Casanova-Martí, À., Serrano, J., Gil-Cardoso, K., Ardevol, A., Terra, X., Blay, M. T., and Pinent, M., Modulation of the enteroendocrine cell functions by flavan-3-ol. Presented at 7-9th September 2015, NUGO week congress, Barcelona, Spain.

Casanova-Martí, À., Serrano, J., Gil-Cardoso, K., Ardevol, A., Terra, X., Blay, M. T., and Pinent, M., Grape-seed proanthocyanidin extract induces Glucagon-like Peptide-1 release in a Rat ex Vivo Intestinal Model. Presented at at 27-30th October 2015, Interantional on Polyphenols and Health, Tours, Franc

In the last decade, there has been an increasing prevalence of obesity and metabolic-associated diseases. In view of this fact, finding preventive therapies, as well as treatments for these diseases is of great interest for public health. Gut hormones secreted from enteroendocrine cells (EECs) play a key role in the regulation of food intake and glucose homeostasis. In this context, the research of this thesis has focused on the role of natural bioactive compounds on the enteroendocrine system.

Our research group reported in previous studies that grape seed proanthocyanidin extract (GSPE) increased GLP-1 plasma levels in rats. In this thesis, we elucidated that such increase might be in part explained by the direct action of GSPE on EECs. Moreover, we demonstrate that GSPE also modulates the secretion of the main gut hormones by directly acting on EECs, inducing an increase of GIP and PYY release, while reducing CCK release.

The results obtained in this thesis using organoids culture demonstrated that GSPE up-regulate the main markers of L-cell and modulate transcription factors involved in L-cell differentiation, and thereby point out that the promotion of L-cell differentiation is a mechanism by which GSPE act in prolonged treatments. Moreover, our findings in mid-term treatments revealed that gut microbiota composition is modulated by GSPE and such composition profile correlates with host metabolic parameters, and remarkably with increased active GLP-1 plasma levels.

Furthermore, we found a new source of natural bioactive compounds, chicken leg hydrolyzate, and demonstrated that it acts as antihyperglycemic agent in disrupted-glucose homeostasis animals due to the capacity of inhibiting DPP-IV activity and enhancing endogenous GLP-1 release.

In conclusion, the findings obtained in this thesis show that natural bioactive compounds act through different mechanisms on the enteroendocrine system, and thereby could be good therapeutic agents to treat obesity and glucose homeostasis disruption.

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