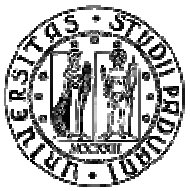

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SCUOLA DI DOTTORATO DI RICERCA IN SCIENZE VETERINARIE

INDIRIZZO SCIENZE BIOMEDICHE VETERINARIE E COMPARATE

CICLO XXII

TESI DI DOTTORATO DI RICERCA:

BIOACTIVE PEPTIDES FROM MILK PROTEINS: FOCUSING ON PEPTIDES DISPLAYING IMMUNOMODULATORY ACTIVITY

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INDEX

ABBREVIATIONS LIST

ACE	Angiotensin converting enzyme
A. oryzae	*Aspergillus oryzae*
AUC	Area Under the Curve
B. lactis	*Bifidobacterium lactis*
BALT	Bronchus-Associated Lymphoid Tissue
BBMV	Brush border membrane vesicles
BPBL	Bovine peripheral blood lymphocytes
C. cardunculus	*Cynara cardunculus*
α-CN, β-CN, κ-CN	α-casein, β-casein, κ-casein
conA	Concanavalin A
E. faecalis	*Enterococcus faecalis*
DBP	Diastolic blood pressure
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
FAAs	Free Amino Acids
FCS	Fetal Calf Serum
FOSHU	Food Specified Health Use
g	Gravity acceleration (9.8 m/s^2)
GALT	Gut-Associated Lymphoid Tissue
GI	Gastrointestinal
L-Glu	L-Glutamine
HA	Hippuric Acid
HBSS	Hank's Buffered Salt Solution
HEPES	Hydroxyethyl Piperazine Ethane Sulphonic Acid

HHL	Hippuryl-Histidyl-Leucine (Hip-His-Leu)
HL	Histidyl-Leucine
HPLC	High Performance Liquid Chromatography
IC_{50}	Inhibitory concentration 50%
IPP	Ile-Pro-Pro
K. marxianus marxianus	*Kluyeromyces marxianus marxianus*
LAB	Lactic acid bacteria
α-LA	α-lactoalbumin
L. acidophilus	*Lactobacillus acidophilus*
L. delb. bulgaricus	*Lactobacillus delbrueckii bulgaricus*
L. casei	*Lactobacillus casei*
L. casei GG	*Lactobacillus casei GG*
L. helveticus	*Lactobacillus helveticus*
L. paracasei	*Lactobacillus paracasei*
L. plantarum	*Lactobacillus plantarum*
L. lactis	*Lactococcus lactis*
L. lactis cremoris	*Lactococcus lactis cremoris*
LC-MS	Liquid Chromatography-Mass Spectrometry
LF	β-lactoglobulin
β-LG	β-lactoglobulin
MALT	Mucosa-Associated Lymphoid Tissue
β_2-MG	β_2-Microglobulin
NCS	Newborn Calf Serum
NEAA	Non Essential Amino acids
NR	Neutral red
PBS	Phosphate buffered saline
P-gp	P-glycoprotein

PS	Penicillin-streptomycin
QSAR	Quantitative Structure-Activity Relationship
RP-HPLC-ESI/MS	Reverse Phase High Performance Liquid Chromatography ElectroSpray Ionization Mass Spectrometry
S. cerevisiae	*Saccharomyces cerevisiae*
SBP	Systolic blood pressure
SD	Standard deviation
SEM	Standard Error of the Mean
SHR	Spontaneous Hypertensive Rat
S. thermophilus	*Streptococcus thermophilus*
TEER	TransEpithelial Electrical Resistance
TFA	Trifluoroacetic acid
TIC	Total Ionization Current
TM	Transport medium
TNBS	Trinitrobenzenesulfonic acid
TJ	Tight junction
TSI	TJ-stabilizing index
UV	Ultraviolet
VPP	Val-Pro-Pro
% v/v	% volume/volume
% w/v	% weight/volume
YGG	Tyr-Gly-Gly

SOMMARIO

I peptidi bioattivi derivati dal latte costituiscono una parte importante del latte, in grado di influenzare lo stato di salute. Attualmente nel latte e nei suoi derivati sono stati identificati e caratterizzati peptidi ad azione oppioide, anti-trombotica, anti-ipertensiva, immunomodulatoria, antiossidante, antimicrobica, anticancro, stimolanti l'assorbimento di minerali e la crescita. In questa tesi particolare attenzione è stata rivolta ai peptidi bioattivi ad attività ACE-inibitoria e immunomodulatoria.

Nell'Esperimento 1 *Enterococcus faecalis* TH563 (*E. faecalis* TH563) e *Lactobacillus delbrueckii* subsp. *bulgaricus* LA2 (*L. delb. bulgaricus* LA2), due ceppi batterici isolati da formaggi tradizionali del Nord Italia, sono stati caratterizzati per la loro capacità di produrre latti fermentati arricchiti in attività ACE-inibitoria e immunomodulatoria. I risultati preliminari hanno dimostrato che il ceppo *E. faecalis* TH563 è in grado di produrre un latte fermentato con elevata attività ACE-inibitoria mentre il ceppo *L. delb. bulgaricus* LA2 produce un latte fermentato con attività immunomodulatoria su linfociti bovini.

Per meglio comprendere i meccanismi che regolano l'attività immunomodulatoria manifestata dal latte fermentato, nell'Esperimento 2 sono stati riportati i risultati di un esperimento atto a valutare gli effetti immunomodulatori del peptide bioattivo YGG. Tale tripeptide può essere generato durante il processo di fermentazione del latte dalla proteina α–lattoalbumina mediante l'azione proteolitica degli enzimi batterici, e quindi anche durante la fermentazione operata dai ceppi *E. faecalis* TH563 e *L. delb. bulgaricus* LA2. YGG è stato somministrato a linfociti isolati da sangue bovino e ne è stata studiata la capacità di modulare la proliferazione dei linfociti e l'espressione (RNA) di due citochine (IL2 e INFγ) in diverse condizioni di coltura (presenza/assenza di attivatori della proliferazione, diverse concentrazioni di siero bovino). Lo studio ha dimostrato che il peptide YGG è in grado di modulare la

proliferazione delle cellule e che tale modulazione è influenzata dalle condizioni di coltura ma non sembra essere mediata dalle citochine oggetto di studio.

Un fattore importante che limita l'impiego su larga scala di alimenti con proprietà bioattive è la biodisponibilità dei peptidi portatori di tali bioattività. I fattori che maggiormente influenzano la biodisponibilità dei peptidi sono la resistenza alla digestione operata dagli enzimi gastrointestinali e la possibilità che tali peptidi possano essere assorbiti dall'epitelio intestinale. A questo scopo, negli Esperimenti 3 e 4 sono stati esaminati il profilo di digestione e i meccanismi di assorbimento del peptide β-CN (193-209). β-CN (193-209) è un peptide bioattivo lungo e idrofobico, derivato dalla β-caseina ed è già stato isolato e identificato in diversi prodotti derivati dal latte come yogurt e latte fermentati. Tale peptide possiede inoltre diverse attività immunomodulatorie. Il profilo di digestione di tale peptide e i meccanismi di assorbimento intestinale sono stati studiati in modelli *in vitro* adatti a rappresentare la mucosa intestinale, come le vescicole della membrana a orletto a spazzola (BBMV) e la linea cellulare Caco-2. Tali esperimenti hanno dimostrato che il peptide viene assorbito intatto dalle cellule Caco-2, probabilmente attraverso un trasporto mediato da vescicole.

In conclusione, il contributo principale di questa tesi di dottorato è stato il fornire nuova conoscenza sui prodotti derivati dal latte ad azione bioattiva. Più specificatamente, questa tesi ha permesso di ottenere nuove informazioni sui meccanismi di produzione dei peptidi bioattivi derivati dal latte, sul loro meccanismo d'azione e sulla loro stabilità nel sistema gastrointestinale. Infine, i risultati ottenuti hanno contribuito a generare nuove idee che potranno costituire nuovi spunti per futuri progetti di ricerca.

SUMMARY

Milk-derived peptides are milk components able to influence specific physiological functions, finally acting on body health condition. At present, the bioactivities described for milk-derived peptides include opiate, antithrombotic, antihypertensive, immunomodulating, antioxidative, antimicrobial, anticancer, mineral-carrying and growth-promoting activities. In this thesis, special attention has been given to bioactive peptides with ACE-inhibitory and immunomodulatory activities.

In the Experiment 1 *Enterococcus faecalis* TH563 (*E. faecalis* TH563) and *Lactobacillus delbrueckii* subsp. *bulgaricus* LA2 (*L. delb. bulgaricus* LA2), two bacterial strains isolated from traditional North Eastern Italy dairy products, have been evaluated for their ability to produce fermented milk rich in ACE-inhibitory and immunomodulatory activities. The preliminary results obtained from this experiment demonstrated that *E. faecalis* TH563 produced a fermented milk with high ACE-inhibitory activity while *L. delb. bulgaricus* LA2 showed an immunomodulatory activity on bovine lymphocytes.

To better understand the mechanisms underlying the immunomodulatory activity of fermented milks, in the Experiment 2 the immunomodulatory effects of the milk-derived bioactive tri-peptide YGG have been examined. YGG could be generated during milk fermentation from α–lactalbumin hydrolysis operated by bacterial enzymes, so it could be present in milk fermented by *L. delb. bulgaricus* LA2. YGG has been administered to purified peripheral blood lymphocytes in different culture conditions (presence/absence of activators of lymphocyte proliferation, different concentration of newborn calf serum) and its effects on lymphocyte proliferation and cytokine RNA expression (IL2 and INFγ) have been analyzed. YGG modulated lymphocytes proliferation, in a manner dependent from culture conditions but its effects did not seem mediated by the modulation of IL2 or INFγ RNA expression.

SUMMARY

An important limiting factor of the large-scale diffusion of food carrying potential bioactivities is the bioavailability of the peptides responsible of such bioactivities. The main factors influencing the bioavailability of peptides are the resistance to digestion enzymes of and the absorption by the intestinal epithelium. In the Experiments 3 and 4 the sensitivity to gastrointestinal enzymes and the mechanisms of absorption of the peptide β-CN (193-209) have been evaluated. β-CN (193-209) is a long hydrophobic peptide derived from β-casein that has been already isolated and identified from fermented milks and yogurt and displayed immunomodulatory properties. The pattern of digestion and the mechanisms of absorption have been evaluated in well-known *in vitro* models for the intestinal epithelium, as the brush border membrane vesicles (BBMV) and the Caco-2 cell line. The results of these studies demonstrated that the β-CN (193-209) peptide is absorbed intact by the Caco-2 monolayer, probably via a vesicles-mediated mechanism.

In conclusion, the main contribution of this PhD thesis was to provide new knowledge about milk-derived products with bioactivities. In particular, original contributions are in relation to the mechanisms by which milk-derived bioactive peptides are generated, express their bioactivities, and their fate in the gastrointestinal tract. As a result, new questions have arisen on this area that could constitute the objective of further research programs in the future.

1. AIM OF THE RESEARCH

The present thesis aimed to elucidate the function and the bioavailability of bioactive peptides present in milk or in milk-derived products, with the purpose to identify the crucial aspects that have to be taken into consideration for an efficient production of bioactive peptides from milk proteins. In this context, special attention has been given to the immunomodulatory activity and to specific milk-derived peptides associated to this bioactivity.

The critical aspects that have to be considered for the production of bioactive peptides from milk proteins are various, as the mechanisms of release of bioactive peptides from milk proteins and the bioavailability of the peptides in the body and in this PhD thesis the more relevant have been evaluated.

First, the mechanisms of generation of the bioactivities from the raw milk, notably the effect of the bacterial strain on the digestive phenomena intervening in the production of fermented milks rich in ACE-inhibitory and immunomodulatory activities, have been studied.

Secondly, the factors involved in the bioavailability of bioactive peptides, as the resistance to digestion and the mechanism of absorption by the intestinal epithelium, have been assessed in two well established *in vitro* models for the intestinal epithelium, as brush border membrane vesicles and Caco-2 cell line.

Finally, the mechanisms of action by which the immunomodulatory peptides manifest their activity once into the target organism have been characterized in bovine peripheral blood lymphocytes.

2. REVIEW OF LITERATURE

2.1. Milk and milk-derived products

Milk is the secretion of the mammary gland, containing approximately 5% lactose, 3.1 protein, 4% lipid and 0.7% minerals. The components of milk provide critical nutritive elements, immunological protection, and biologically active substances to both neonates and adults. It is not surprising, therefore, that the nutritional value of milk is high.

From an objective viewpoint, it seems logical that a lactating animal, as well as providing vital early nutrition, would also protect the health of its offspring via the biochemical influences of its milk. In particular, the notion that components within milk can influence and direct the physiological development of the offspring, as its environmental exposure increases, is now widely accepted [1]. The concept of bovine milk as a biologically active fluid is therefore not new [2], but the identification of factors within bovine milk that may be relevant to improving human health, and the potential development of bovine milk-containing preparations into products with proven health-promoting properties, certainly is [1].

Milk is not only consumed as a raw material but it is transformed in a variety of products to preserve its nutrients. Figure 2.1.1. shows an overview of the range of dairy products deriving from milk processing.

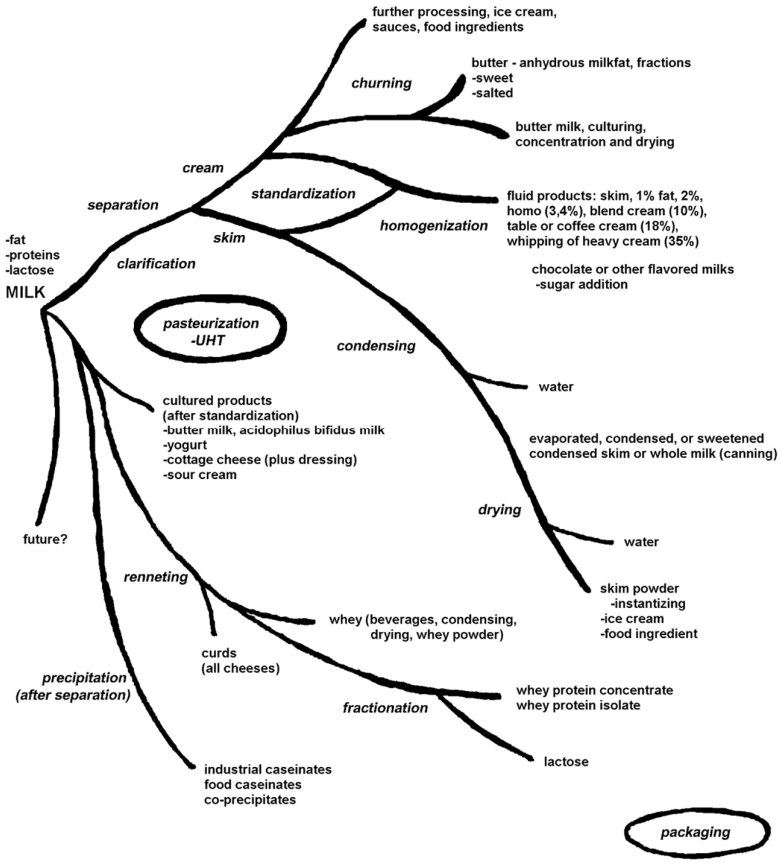

Fig 2.1.1. Overview of the range of dairy products deriving from milk processing. From: http://www.foodsci.uoguelph.ca/dairyedu/home.html.

Among all the dairy products, milk fermentation and cheese making are the oldest methods used to extend the shelf-life of milk, and they have been practiced by human beings for thousands of years [3]. Recently, numerous scientific works [4-7] have demonstrated and confirmed that the consumption of fermented milk and cheeses manifests health beneficial effects that go beyond the nutritional value. Indeed, fermented milk consumption has been associated with reduction of serum cholesterol [8], antihypertensive [5] and osteoprotective [9] effects. The mechanisms of action responsible of these properties have been investigated and have been attributed to the numerous bioactive peptides contained in milk and/or released during milk processing.

It is not surprising that in recent years intense research interest has been focused on identifying biologically active components within bovine milk and milk-derived products, and characterising the way by which mammalian physiological function is modulated by these components. Not surprisingly, a significant proportion of this research has sought to characterise the potential of bovine milk, milk products or milk components to influence some of the most important body physiological functions, as blood pressure [10-12], the immune system [13-15], and the resistance to the infections [16]. For example, there is now a substantial body of evidence to suggest that major components of bovine milk, as well as several constituents or even yogurt and cheese, can regulate blood pressure in humans [5, 17]. The most significant advances in this field have been made over the last five to ten years, and this review will focus primarily on the recent advances and current knowledge in this rapidly expanding field. Moreover, particular attention is given to the milk-derived bioactive peptides responsible of some important health properties.

2.2. Bioactive peptides

2.2.1. Definition

Accordingly to a widely shared definition [18], a bioactive dietary substance is “a food component that can affect biological processes or substrates and, hence, have an impact on body function or condition and ultimately health”. In addition, dietary substances should give a measurable biological effect in the range of doses it is usually assumed in the food and this bioactivity should be measured at a physiologically realistic level [9].

Following this definition, milk-derived bioactive peptides are milk components able to influence some physiological functions, finally acting on body health condition.

Moreover, among the numerous bioactive substances studied up to now, increasing interest is focused on milk-derived bioactive peptides because at present, bovine

milk, cheese and dairy products seem to be extremely important sources of bioactive peptides derived from food.

2.2.2. Mechanisms of production of bioactive peptides

Milk-derived bioactive peptides, and more generally food bioactive peptides, are usually composed of 2-20 amino acids and become active only when they are released from the precursor protein where they are encrypted (Fig. 2.2.2.1.).

Different mechanisms can release the encrypted bioactive peptides from the precursor proteins [19, 20]:

1. *In vivo*, during gastrointestinal digestion trough the action of digestive enzymes or of the microbial enzymes of the intestinal *flora*;
2. During milk processing (e. g. milk fermentation, cheese production) trough the action of microbial enzymes expressed by the microorganisms used as starter;
3. During milk processing trough the action of a single purified enzyme or a combination of selected enzymes;

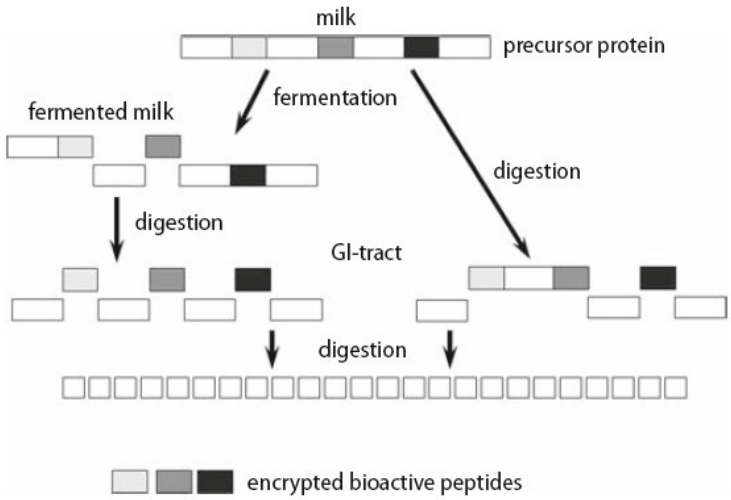

Fig 2.2.2.1. Summarizing scheme of the possible mechanisms by which bioactive peptides can be released from the precursor proteins by microbial fermentation and/or gastrointestinal digestion, from Möller at al., 2008 [9].

2.2.2.1. Bioactive peptide release during gastrointestinal digestion through the action of digestive enzymes or microbial enzymes of the intestinal flora

Bioactive peptides may be released *in vivo* during gastrointestinal digestion. These bioactive peptides are mostly the result of the degradation of casein with several proteases such as pepsin, trypsin or chymotrypsin. At present, despite some experimental works on the stimulation of gastrointestinal digestion of eggs and meat proteins [21, 22], the production of milk-derived bioactive peptides *in vivo* during digestion remains unclear. Before dietary proteins can be cleaved by pancreatic proteases in the intestine, they pass through the stomach, in which food can remain for up to several hours depending on its composition and degree of particle reduction during mastication. In the gastric juice, the proteins undergo degradation by HCl and pepsin. While the peptide products resulting from milk proteins digestion with site-specific pancreatic proteases, such as trypsin or chymotrypsin are well investigated [23, 24], there are only few papers regarding this primary step of human digestion of milk proteins [25, 26].

During gastrointestinal digestion, bioactive peptides may be released from the precursor protein throughout the whole intestine. In fact, proteins contained in food matrices enter the stomach through the cardiac orifice and they are further denatured and partially degraded by the combined action of HCl and pepsin. This first digestion step operated on proteins in the stomach permits the consequent action of the enzymes present in the small intestine, which are the main responsible of protein hydrolysis. Thus, bioactive peptides are predominantly released in this portion of the gastrointestinal tract. Microbial enzymes of the resident gut *flora* can act only on milk proteins that reach the large intestine intact or only partially degraded [9]. Compared to the gastrointestinal enzymes, microbial enzymes, either

in the intestine or used as starter during milk processing, use different cleavage sites. Thus, bioactive peptides liberated by microbial enzymes may differ from those released by digestive enzymes. It remains to be elucidated if these bioactive peptides released by the resident flora of the large intestine could be absorbed and in which extent. In addition, when the bioactive peptides are released by bacterial enzymes during milk fermentation, they could be the target of the action of gastrointestinal enzymes and they may release other bioactive peptides [9].

Moreover, it has been demonstrated that the peptidic profile of milk proteins is significantly different after microbial fermentation, suggesting that microbial proteolysis can be a potential source of bioactive peptides during milk processing [27].

2.2.2.2. Bioactive peptide release during milk processing trough the action of microbial enzymes

Many industrially utilized dairy starter cultures are highly proteolytic. Bioactive peptides can, thus, be generated by the starter and non-starter bacteria used in the manufacture of fermented dairy products. The proteolytic system of lactic acid bacteria (LAB), e.g. *Lactococcus lactis*, *Lactobacillus helveticus* and *L. delb. bulgaricus*, is already well characterized. This system consists of a cell wall-bound proteinase and a number of distinct intracellular peptidases, including endopeptidases, aminopeptidases, tripeptidases and dipeptidases [28]. Rapid progress has been made in recent years to elucidate the biochemical and genetic characterization of these enzymes.

Many recent articles and book chapters have reviewed the release of various bioactive peptides from milk proteins through microbial proteolysis [27, 29, 30]. In addition, a number of studies have demonstrated that hydrolysis of milk proteins by

digestive and/or microbial enzymes may produce peptides with immunomodulatory activities [31].

2.2.2.3. Bioactive peptide release during milk processing trough the action of a single purified enzyme or a combination of selected enzymes

The most common way to produce bioactive peptides is through enzymatic hydrolysis of whole protein molecules. ACE-inhibitory peptides and calcium-binding phosphopeptides, for example, are most commonly produced by trypsin [32-35].

Moreover, ACE-inhibitory peptides have recently been identified in the tryptic hydrolysates of bovine α_{s2}-casein [36] and in bovine, ovine and caprine k-casein macropeptides [37]. Other digestive enzymes and different enzyme combinations of proteinases - including alcalase, chymotrypsin, pepsin and thermolysin as well as enzymes from bacterial and fungal sources - have also been utilized to generate bioactive peptides from various proteins [19, 38].

Proteolytic enzymes isolated from LAB have been successfully employed to release bioactive peptides from milk proteins. Yamamoto and colleagues [39] reported that casein hydrolyzed by the cell wall-associated proteinase from *L. helveticus* CP790 showed antihypertensive activity in spontaneously hypertensive rats. Several ACE-inhibitory peptides and one antihypertensive peptide were isolated from the hydrolysate. Maeno *et al.* [40] hydrolyzed casein using the same proteinase and identified a β-casein-derived antihypertensive peptide, the fragment β-CN (169-175), whose amino acidic sequence is KVLPVPQ. In a recent study, Mizuno and colleagues [41] measured the ACE-inhibitory activity of casein hydrolysates upon treatment with nine different commercially available proteolytic enzymes. Among these enzymes, a protease isolated from *Aspergillus oryzae* showed the highest ACE-inhibitory activity *in vitro* per peptide.

2.2.3. Mechanisms of action of bioactive peptides

It has been already demonstrated that milk-derived peptides show biological effects and are able to influence some specific body function. At present, the bioactivities described for milk-derived peptides includes opiate [42], antithrombotic [43], antihypertensive [5], immunomodulating [15], antioxidative [44], antimicrobial [45], anticancer [46], mineral carrying [34] and growth-promoting properties [47]. In Table 2.2.3.1. a brief summary of bioactive peptides from milk proteins is given.

Bioactive peptide	**Precursor protein**	**Bioactivity**
Casomorphins	α-CN, β-CN	Opioid agonist
a-lactorphin	α-LA	Opioid agonist
b-lactorphin	β-LG	Opioid agonist
Lactoferroxins	LF	Opioid antagonist
Casoxins	K-CN	Opioid antagonist
Casokinins	α-CN, β-CN	ACE-inhibitory
Lactokinins	α-LA, β-LG	ACE-inhibitory
Immunopeptides	α-CN, β-CN	Immunomodulatory
Lactoferricin	LF	Antimicrobial
Casoplatelins	K-CN, Transferrin	Antitrombotic
Phosphopeptides	α-CN, β-CN	Mineral binding, anticariogenic

Table 2.2.3.1. Bioactive peptides derived from milk proteins, from Meisel, 2005 [48].

Bioactive milk peptides could express their function in the intestinal tract [49-53] or inside the body after being absorbed. In any case, it is necessary to demonstrate that the bioactivity of interest is retained *in vivo*.

Therefore, to exert physiological effects *in vivo* after oral ingestion, it is of crucial importance that milk-derived bioactive peptides remain active during gastrointestinal digestion and absorption and reach intact the target site. This signifies that milk-derived bioactive peptides have to be resistant to gastrointestinal, brush border intracellular and serum peptidases [54].

For this reason, scientific works aiming to evaluate the bioavailability of bioactive peptides *in vivo* are gaining of importance [5, 6].

2.2.4. Commercial dairy products and ingredients with health or function claims based on bioactive peptides

It is now well documented that bioactive peptides can be generated during milk fermentation with the starter cultures traditionally employed by the dairy industry. As a result, peptides with various bioactivities can be found in the end-products, such as various cheese varieties and fermented milks. These traditional dairy products may, under certain conditions, carry specific health effects when ingested as part of the daily diet. Table 2.2.4.1. lists a number of studies which have established the occurrence of peptides in various fermented milk products.

Product	**Example of identified peptide**	**Bioactivity**	**Reference**
Cheese type			
Parmigiano-Reggiano	β-CN (8–16), β-CN (58–77), α_{s2}-CN(83–33)	Phosphopeptides, precursor of β-casomorphin	[55]
Cheddar	α_{s1}-CN fragments β-CN fragments	Several phosphopeptides	[56]
Italian varieties: Mozzarella, Crescenza, Gogonzola, Italico	β-CN (58–72)	ACE-inhibitory	[57]
Gouda	α_{s1}-CN (1–9), β-CN (60–68)	ACE-inhibitory	[58]
Festivo	α_{s1}-CN (1–9), α_{s1}-CN (1–7), α_{s1}-CN (1–6)	ACE-inhibitory	[59]
Emmental	α_{s1}-CN fragments β-CN fragments	Immunostimulatory, several phosphopeptides, antimicrobial	[60]
Manchengo	Ovine α_{s1}-CN, α_{s2}-CN, β-CN fragments	ACE-inhibitory	[61]
Fermented milks			
Sour milk	β-CN (74–76), β-CN (84–86), κ-CN (108–111)	Antihypertensive	[12]
Yogurt	Active peptides not identified	Weak ACE-inhibitory	[62]
Dahi	SKVYP	ACE-inhibitory	[63]

Table 2.2.4.1. Bioactive peptides identified in fermented milk products, from Korhonen, 2006 [64].

An increasing number of ingredients containing specific bioactive peptides based on casein or whey protein hydrolysates have been launched on the market within the past few years or are currently under development by international food companies. Such peptides possess, e.g., anticariogenic, antihypertensive, mineral-binding and stress-relieving properties. A few examples of these commercial ingredients and their applications are listed in Table 2.2.4.2.

Brand name	Type of product	Claimed functional bioactive peptide	Health/function claims	Producer
Calpis	Sour milk	VPP, IPP from β-CN and κ-CN	Blood pressure reduction	Calpis Co., Japan
Evolus	Calcium enriched fermented milk drink	VPP, IPP from β-CN and κ-CN	Blood pressure reduction	Valio Oy, Finland
BioZate	Hydrolyzed whey protein isolate	β-LG fragments	Blood pressure reduction	Davisco, USA
BioPURE-GMP	Whey protein isolate	k-CN f(106–169)	Prevention of dental caries, influence the clotting of blood, protection against viruses and bacteria	Davisco, USA
PRODIET F200/Lactium	Flavored milk drink, confectionery, capsules	α_{s1}-CN (91–100)	Reduction of stress effects	Ingredia, France
Festivo	Fermented low-fat hard cheese	α_{s1}-CN (1–9), α_{s1}-CN (1–7), α_{s1}-CN (1–6)	No health claim as yet	MTT Agrifood Research, Finland
Cysteine Peptide	Ingredient-hydrolysate	Milk protein derived peptide	Aids to raise energy level and sleep	DMV International, Netherlands
C12 peptide	Ingredient-hydrolysate	Casein derived peptide	Reduction of blood pressure	DMV International, Netherlands
Capolac	Ingredient	Casein derived peptide	Helps mineral absorption	Arla Foods Ingredients, Sweden
PeptoPro	Ingredient-hydrolysate	Casein derived peptide	Improves athletic performance and muscle recovery	DMV International, Netherlands
Vivinal Alpha	Ingredient-hydrolysate	Whey derived peptide	Aids relaxation and sleep	Borculo Domo Ingredients (BDI), the Netherlands

Table 2.2.4.2. Commercial dairy products and ingredients with health or function claims based on bioactive peptides, from Korhonen, 2006 [64].

2.3. Bioactivities of interest

As already introduced in Paragraph 2.2.3., milk-derived bioactive peptides are potential modulators of various regulatory processes in the body, and they can express hormone-like activities.

Moreover, the primary sequence of some specific bovine proteins, as caseins, contains overlapping regions, partially protected from proteolytic breakdown, that manifest multifunctional properties and influence different biological functions [48]. In particular, ACE-inhibitory and immunomodulatory properties seem to be associated, possibly because both are correlated to the presence of short chain peptides such as VPP and IPP formed during milk fermentation with selected bacterial stains [65].

Therefore, as the present thesis mainly focuses on milk-derived peptides displaying immunomodulatory activity in the following paragraphs, immunomodulatory property is described in more details together with the milk-derived peptides responsible for these bioactivities. In addition, special attention is also given to ACE-inhibitory activity and to the related bioactive peptides, because this activity can be associated to immunomodulatory activity and because it has been the object of the Experiment 1 of this thesis.

2.3.1. ACE-inhibition

The inhibition of the Angiotensin-I-Converting Enzyme (ACE) is a key point in the treatment of the hypertension. ACE is carboxypeptidase (E.C. 3.4.15.1) and catalyzes the cleavage of dipeptides [66]. ACE is responsible for the conversion of angiotensin I, a decapeptide generated by the action of rennin on the substrate angiotensinogen, to the vasoconstrictor octapeptide angiotensin II. Angiotensin II directly acts on blood vessels increasing blood pressure, but it also stimulates the release of aldosterone from the adrenal cortex. Aldosterone increases the reabsorption of sodium and water and the secretion of potassium by the kidney, so

the overall effect is an increased blood pressure (see Fig. 2.3.1.1.). In addition, ACE hydrolyzes the vasodilatator bradykinin, inactivating its lowering pressure effects.

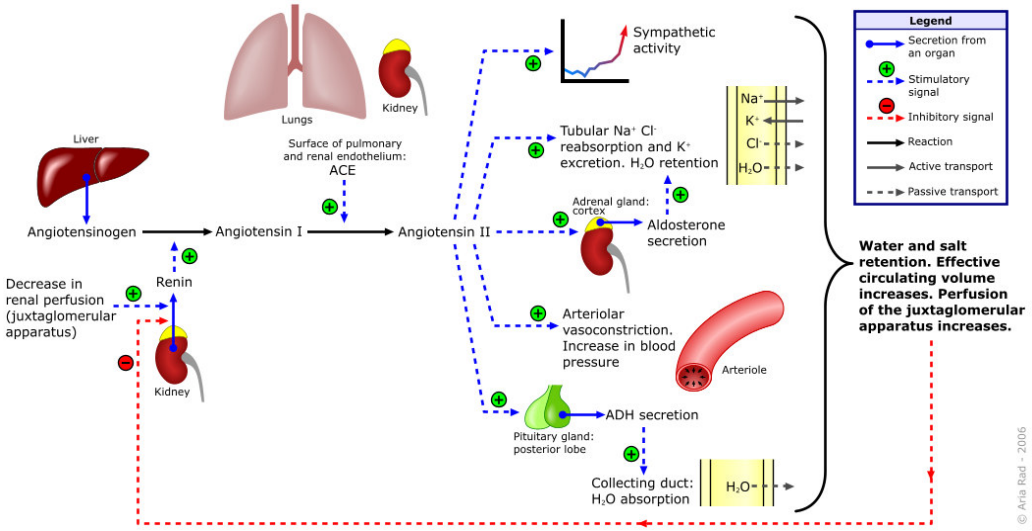

Fig. 2.3.1.1. Summarizing scheme of the effects of the rennin-angiotensin-aldosterone system, from http://en.wikipedia.org/wiki//File:Renin-angiotensin-aldosterone_system.png.

Human ACE is present into two isoforms, somatic ACE and germinal/testicular ACE. Both isoforms are encoded by a single gene located on chromosome 17. The somatic ACE is a membrane-bound protein expressed on the surface of the vascular endothelial cells of the lungs and of the epithelial cells the kidney [67], but it is widely distributes also in many other tissues as thymus and small intestine [68].

In some of these tissues the rennin–angiotensin-aldosterone system is not present: this reinforces the idea that ACE has probably other roles in addition to the production of angiotensin II and the inactivation of bradykinin.

2.3.1.1. Physiology of ACE-inhibition

Exogenous ACE-inhibitors having an antihypertensive effect *in vivo* were first discovered in snake venom [69] and they are thought to be competitive substrates of ACE. Indeed, the first ACE-inhibitor developed for the pharmacological treatment of

hypertension, Captopril, has been obtained modifying a peptide contained in the venom of the a Brazilian snake [70] and designing it upon a hypothetical model of the binding site on the enzyme [71]. Since then, synthetic ACE inhibitors such as captopril, enalapril, alecepril and lisinopril are used extensively in the treatment of essential hypertension despite their undesirable side effects, such as hypotension, cough, increased potassium levels, reduced renal function, angioedema, etc. [33]. ACE-inhibitory peptides derived from milk proteins inhibit ACE as Captopril, thus acting as competitive substrate of this enzyme, but they do not manifest the correlated side effects [72].

Although the structure-activity relationship of ACE-inhibitory peptide has not been fully elucidated, these peptides share common characteristics [73-75]:

- Short chain peptides (2-9 residues);
- Presence of hydrophobic residues in the sequence (aromatic or branched side chains)
- Presence of proline, lysine or arginine residue at the C-terminal end of the bioactive peptide
- Resistance to hydrolysis by digestive enzymes

Pripp and colleagues [76] established quantitative structure–activity relationships (QSAR) for ACE-inhibitory peptides derived from milk proteins. For peptides up to six amino acids, a relationship was found between the ACE-inhibitory activity and some of the peptide characteristics (hydrophobicity and a positively charged amino acid at the C-terminal position). No relationship was found between the N-terminal structure and the ACE-inhibitory activity.

2.3.1.2. ACE-inhibitory peptides derived from milk

ACE-inhibitory peptides derived from milk proteins are released from caseins (casokinins) or from whey proteins (lactokinins) [20, 48]. Casokinins and lactokinins have been identified in fermented milks [77-79] or in milk proteins hydrolysates with selected enzymes, such as pepsin, trypsin and chymotrypsin [40, 80-82].

Usually, the potency of an ACE-inhibitory peptide is its IC_{50} value, which is equivalent to the concentration of the peptide generating a 50% inhibition of ACE activity. IC_{50} value can be obtained by an *in vitro* ACE-inhibition assay. Table 2.3.1.2.1. shows some examples of ACE-inhibitory peptides derived from milk proteins, with the correspondent IC_{50} value.

Peptide sequence	Fragment	IC_{50} (µmol/L)	Reference
VAP	α_{s1}-CN (25-27)	2	[83]
FFVAP	α_{s1}-CN (23-27)	6	[84]
FFVAPPFPEVFGK	α_{s1}-CN (23-34)	77	[84]
FPEVFGK	α_{s1}-CN (28-34)	140	[83]
FGK	α_{s1}-CN (32-24)	160	[83]
YKVLPQL	α_{s1}-CN (104-109)	22	[83]
LAYFYP	α_{s1}-CN (142-147)	65	[83]
DAYPSGAW	α_{s1}-CN (157-164)	98	[30]
TTMPLW	α_{s1}-CN (194-199)	16	[85]
SLVLPVPE	β-CN (57-64)	39	[39]
IPP	β-CN (74-76) κ-CN (108-110)	5	[86]
VPP	β-CN (84-86)	9	[86]
KVLPVPQ	β-CN (169-175)	1000	[40]
KVLPVP	β-CN (169-174)	5	[40]
AVPYPQR	β-CN (177-183)	15	[84]
YQQPVLGPVR	β-CN (193-202)	300	[87]
YPFPGPI	β-CN (60-66)	500	[87]
YGLF	α-LA (50-53)	733	[88]
ALPMHIR	β-LG (142-148)	43	[89]
YL	β-LG (102-103)	122	[62,88]
YLLF	β-LG (102-105)	172	[62,88]
ALKAWSVAR	BSA (208-216)	3	[90]

Table 2.3.1.2.1. Some examples of ACE-inhibitory peptides derived from milk.

There are spectrophotometric, fluorimetric, radiochemical, HPLC and capillary electrophoresis methods to measure IC_{50}. The spectrophotometric method of

Cushman and Cheung [91] is most commonly utilized. It is based on the hydrolysis of hippuryl-His-Leu (HHL) by ACE to hippuric acid (HA) and HL. The extent of HA release from HHL is measured after it is extracted with ethyl acetate. Direct, extraction-free method has been published recently [92, 93]. Another broadly used spectrophotometric method is based on the hydrolysis of a furanocryloyl tripeptide (FA–Phe–Gly–Gly, FAPGG) to FAP and the dipeptide GG [94-96]. However, the observation that the ACE-inhibitory activity differed with the method employed creates a need to standardize the methodologies to evaluate *in vitro* ACE-inhibitory activity [94, 95]. In practice, differences may arise among the results of different assays due to the use of different substrates or, within the same assay, due to the use of different test conditions or ACE from different origins. In particular, ACE activity levels need to be carefully controlled to obtain comparable and reproducible values [94, 96].

It has also to be considered that the IC_{50} value is not always directly related to the *in vivo* hypotensive effects. Hypotensive effects can be measured in spontaneous hypertensive rats (SHR), which are genetically predisposed to have a high blood pressure and constitute an accepted model for human primary hypertension [72, 78, 79, 97-103] and in clinical trial with hypertensive patients [11, 82, 104, 105]. The parameter monitored to assess the hypotensive effects of these products normally is blood pressure in normal subjects or in subjects affected by hypertension [5], as depicted in Figure 2.3.1.2.1..

Some peptides that manifest a reduced ACE-inhibitory activity *in vitro* express a significant hypotensive effect when administered *in vivo,* confirming that the *in vitro* ACE-inhibitory activity is not always directly related to the *in vivo* hypotensive effects.

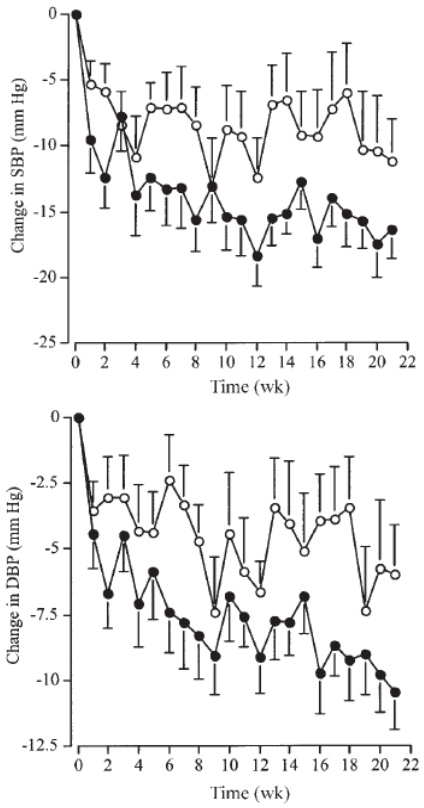

Fig. 2.3.1.2.1. Mean (±SEM) change in systolic blood pressure (SBP) and diastolic blood pressure (DBP) from baseline during the 21 weeks of treatment in the test product (●; n = 19) and control (○; n = 17) groups, from Seppo *et al.*, 2003 [5].

For example, some milk-derived peptides have lower ACE-inhibitory activity *in vitro* than the synthetic ACE inhibitor Captopril, but they usually display higher *in vivo* activities than the efficacy levels extrapolated from the *in vitro* activities. This fact has been attributed to a higher affinity to the tissues and a slower elimination [106], but it may also be an indication of the existence of an additional mode of action than the inhibition of ACE [54]. It could be possible that the peptides with a low *in vitro* ACE-inhibitory activity could act as pro-drugs, releasing the active fragment by the action of digestive or serum peptidases [97].

Conversely, some other ACE-inhibitory peptides manifest a high *in vitro* activity but have no hypotensive effects *in vivo.* For example, the peptide FFVAP derived from

α_{s1}-CN (23-27) is a potent ACE-inhibitory peptide *in vitro* (IC_{50} = 6 µmol/L) [84], but it has not hypotensive effect *in vivo.*

The difficulty to establish a direct relationship between ACE-inhibitory activity *in vitro* and antihypertensive activity *in vivo* may depend upon different reasons but peptide bioavailability after oral administration plays a major role. As already introduced in Paragraph 2.2.2., ACE-inhibitory peptides have to remain active during gastrointestinal digestion and absorption and reach intact the target site.

As marked before, the evaluation of real hypotensive efficacy of peptides with high *in vitro* ACE-inhibitory activity is further complicated by the different ACE-inhibition assays that can be applied for the calculation of the IC_{50} [71, 94, 107]. In addition, in *in vivo* experiment and clinical trials, different experimental designs (measurement of arterial blood pressure at different points, different administration routes, or doses) and the use of the animal model vs human experimentation make difficult to examine the antihypertensive effects of ACE-inhibitory peptides.

However, testing the *in vitro* ACE-inhibitory activity could be still a necessary first screening step, because it is based on a biological mechanism and the *in vitro* assays are relatively easy and do not require expensive laboratory equipments. Nevertheless, *in vivo* experiments and clinical trials are needed to demonstrate if the hypotensive effect of these bioactive peptides is retained at physiological level.

Moreover, *in vivo* studies would permit to clarify the physiological mechanisms and the targets of ACE-inhibitory peptides, once absorbed and circulating in the blood.

The main hypothesis on the mechanism of action of ACE-inhibitory milk peptides assumes that absorbed peptides enter the blood circulation, concentrate in the aorta where they exert their activity on the ACE expressed on the surface of endothelial cells (Fig 2.3.1.2.2.) [5, 100].

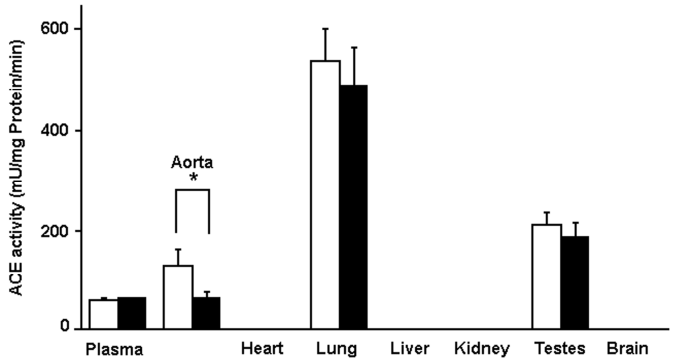

Fig. 2.3.1.2.2. The activity of ACE in various tissues from spontaneously hypertensive rats administered with saline (□) or sour milk (■), from Takano, 2002 [108].

However, mechanisms other than ACE-inhibition cannot be excluded. Indeed, Hirota and colleagues proposed that ACE-inhibitory peptides from caseins may improve the vascular endothelial dysfunction in subjects with mild hypertension [82], possibly inhibiting the release of the vasoactive substances such as the vasoconstrictor endothelin-1, eicosanoids and nitric oxide [109]. For example, the lactokinin ALPMHIR was found to inhibit the release of ET-1, an endothelial peptide that evokes contractions in smooth muscle cells, an effect that might be dependent or independent of ACE-inhibition [109].

It has to be added that, conversely to the purified ACE-inhibitory peptides, fermented milks manifesting ACE-inhibitory activity also contain live starter bacteria and other components, as calcium, that could contribute to the overall *in vivo* hypotensive effect manifested during the studies [5, 103].

As shown by Nurminen *et al*. [110], the peptide YGLF, formed by *in vitro* proteolysis of α-lactalbumin (fragment 50-53) with pepsin and trypsin, lowered blood pressure after subcutaneous administration to SHR and this was abolished by the opioid receptor antagonist naloxone. Therefore, a mechanism of action driven by the stimulation of peripheral opioid receptors and subsequent nitric oxide release that causes vasodilatation was proposed. Other studies have highlighted the existence

of vasorelaxant opioid peptides arising from β-Lactoglobulin such as β-LG (102-105) [102] and from human casein, as YVPFPPF and YPFPPL [111].

2.3.1.3. Microorganisms and enzymes for the production of fermented milk with ACE-inhibitory activity

At present, the *in vivo* hypotensive activity has been demonstrated for fermented milks, milk proteins hydrolysates and purified ACE-inhibitory milk-derived peptides.

In vivo and *in vitro* studies have also confirmed that the microorganisms or the peptidases used to obtain milk-derived products rich in ACE-inhibitory activity are of extreme importance in influencing the quality and the quantity of ACE-inhibitory peptides.

At the moment, the microorganisms used for the production of fermented milk with ACE-inhibitory effects are selected for their high proteolytic activity and their food safety, thus proteolytic LAB becoming the most used microorganisms. Nakamura and colleagues [12] first selected a strain of *L. helveticus* together with *Saccharomyces cerevisiae* to produce a fermented milk containing potent ACE-inhibitory peptides, as IPP and VPP. Then *L. helveticus* has been preferred for the purpose, although other LAB have shown good performance. Yogurt-type products fermented with *L. delb. bulgaricus* and *Lactobacillus lactis* subsp. *cremoris* were also shown to contain ACE-inhibitory peptides [29].

Recently, Muguerza *et al.* [112] assayed the ACE-inhibitory activity of fermented milk samples produced with 231 microorganisms isolated from raw cow's milk samples. Among them, four *E. faecalis* strains resulted in the production of fermented milk with potent ACE-inhibitory activity (IC_{50} = 34–59 μg/mL) and antihypertensive activity in SHR.

Thus, strain selection is one of the main factors that influence the release of ACE-inhibitory peptides. Tables 2.3.1.3.1a. and 2.3.1.3.1b. summarize the microorganisms whose ability to produce fermented milks with high ACE-inhibitory activity has been tested. However, further progress in this area may come from elucidation of the specificity of microbial proteolytic systems in the integrated environments prevailing in dairy products.

Microorganism	Identified peptides	Protein source	Reference
L. helveticus CP790, and *S. cerevisiae*	Nd	β-CN, κ-CN	[11, 39, 86]
L. helveticus LBK16H	β-CN (74-76) κ-CN (108-110)	β-CN, κ-CN	[5, 10, 103]
L. helveticus CPN4	β-CN (84-86)	Whey proteins	[113]
L. helveticus NCC2765	β-CN (62-67) β-CN (75-83) β-CN (149-153) β-CN (155-158) β-CN (183-190) β-CN (198-205) β-CN (208-213) β-CN (208-224) α_{s2}-CN (205-212)	α_{s1}-CN, β-CN	[114]
L. helveticus CHCC637	Nd	Milk	[78]
L. helveticus CHCC641	Nd	Milk	[78]
Starter composed by a mix of *S. thermophilus* CR12, *L. casei* LC01, *L. helveticus* PR4, *L. plantarum* 1288	Nd	Goat milk	[115]
L. delb. bulgaricus SS1	β-CN (6-14) β-CN (7-14) β-CN (73-82) β-CN (74-82) β-CN (75-82)	β-CN, κ-CN	[29]
L. lactis cremoris FT4	β-CN (6-14) β-CN (7-14) β-CN (47-52) β-CN (169-175) κ-CN (152-160) κ-CN (155-160)	β-CN, κ-CN	[29]
K. marxianus marxianus		β-LG	[116]
E. faecalis CECT5728	nd	Bovine milk	[79]
E. faecalis CECT5727	nd	Bovine milk	[112]
E. faecalis CECT5826	nd	Bovine milk	[112]
E. faecalis CECT5827	nd	Bovine milk	[112]

Table 2.3.1.3.1a. Summary of microorganisms whose ability to produce fermented milk with high ACE-inhibitory activity has been tested. Nd: not determined.

Microorganism	Identified peptides	Protein source	Reference
Starter for kefir	β-CN (48-56) β-CN (94-105) β-CN (94-106) β-CN (203-209) β-CN (50-54) β-CN (58-68) α_{s1}-CN (97-102) α_{s2}-CN (174-179) α_{s1}-CN (18-23) α_{s2}-CN (203-208) κ-CN (119-123)	Milk	[117, 118]

Table 2.3.1.3.1b. Summary of microorganisms whose ability to produce fermented milk with high ACE-inhibitory activity has been tested.

Hydrolysis with gastrointestinal proteinases has also been used to examine the effect of digestion on the release and the breakdown of ACE-inhibitory peptides from intact milk proteins (see Table 2.3.1.3.2a. and Table 2.3.1.3.2b.) [81, 83, 85, 89, 119]. Plant proteinases can also be used to release ACE-inhibitory peptides (see Table 2.3.1.3.2a. and Table 2.3.1.3.2b.) [41, 72, 82]. In addition, cell-wall proteases from LAB have been used to hydrolyze milk proteins [33, 39, 40, 81, 120].

Enzymes	Protein source	Identified peptides	Reference
Proteinase K	Cheese whey proteins	β-CN (59-64) β_2-MG (18-20) β-LG (78-80) BSA (221-222) β-CN (62-63) β-CN (157-158) β-CN (205-206)	[81]
Trypsin	Caseins	α_{s1}-CN (23-27) α_{s1}-CN (23-34) β-CN (177-183) α_{s1}-CN (194-199)	[84, 85]
Trypsin	Bovine β-LG	β-LG (142-148)	[89]
Trypsin	α_{s2}-CN	α_{s2}-CN (25-32) α_{s2}-CN (81-89) α_{s2}-CN (81-91) α_{s2}-CN (92-98) α_{s2}-CN (174-179) α_{s2}-CN (174-181) α_{s2}-CN (182-184) α_{s2}-CN (206-207)	[36]

Table 2.3.1.3.2a. Summary of enzymes whose ability to produce fermented milks with high ACE-inhibitory activity has been tested.

Enzymes	Protein source	Identified peptides	Reference
Proteases from *C. cardunculus*	Ovine/caprine cheese-like systems	β-CN (95-99) β-CN (191-194) β-CN (191-198)	[121]
Proteinase from *L. helveticus* PR4	Caseins of different species	Bovine β-CN (58-76) Bovine α_{s1}-CN (24-47) Bovine α_{s1}-CN (169-193) Ovine α_{s1}-CN (1-6) Ovine α_{s2}-CN (182-185) Ovine α_{s2}-CN (186-188) Caprine β-CN (58-65) Caprine α_{s2}-CN (182-187) Buffalo β-CN (58-66) Human β-CN (58-66)	[122]
L. helveticus CP790	β-CN, α-CN	β-CN (169-175) β-CN (140-143) α_{s2}-CN (198-202) α_{s2}-CN (189-202) α_{s1}-CN (104-109) α_{s2}-CN (190-197) α_{s2}-CN (189-197)	[40, 86]
Proteases and peptidases from *A. oryzae*	Caseins	β-CN (74-76) β-CN (84-86)	[41, 72, 82, 105]

Table 2.3.1.3.2b. Summary of enzymes whose ability to produce fermented milks with high ACE-inhibitory activity has been tested.

In the late 1990s, many publications were devoted to food products with bioactive properties. In addition, research in this field was showing promising prospects for the use of such products or ingredients in food market, thereby creating added value for manufacturers and benefits for consumer health. There was –and still there is- an urgent call for legislation, which would make possible new array of foods [123].

As consumption of products enriched with ACE-inhibitory peptides has risen slowly since their introduction into the Japanese market in 1997, in Japan the commercial diffusion of bioactive food ingredients is regulated by FOSHU system (Food for Specified Health Use) [124]. This is a list of foods or food ingredients approved by the Japanese Department of Health because they have demonstrated their bioactivity and their safety with enough scientific evidence to support health claims. Even if blood pressure-lowering products containing ACE-inhibitory tripeptides are currently on the market in the USA and in Europe (Spain, UK, Finland, Switzerland,

Italy, Iceland and Portugal), there is not a regulatory framework for bioactive foods or bioactive food ingredients. The rules to be applied are numerous and they depend on the nature of the foodstuff. Actually, in Europe, the General Food Law regulations definitively are applicable to regulate the use of bioactive peptides in the food marked and the associate use of health claims [123].

The first beverage with ACE-inhibitory peptides was commercialized in Japan, with the name Amiru S Calpis® (Calpis Co. Ltd., Japan). This fermented milk is produced by fermenting milk with *L. helveticus* CP790 and *S. cerevisiae*. Nakamura *et al*. [12, 86], identified the peptides VPP and IPP and this beverage revealed a significant decrease in systolic blood pressure when ingested by hypertensive men [10, 125].

A new milk drink launched by Unilever under the Flora/Becel pro-active® also contains VPP and IPP. This product is the first European fermented milk drink designed to help lowering blood pressure and it contains a casein hydrolysate produced by *A. oryzae* protease and it has been marketed by Calpis as AmealPeptide. Recently, a study [105] was conducted among patients with high-normal blood pressure and mild hypertension, who took different doses of this beverage and a significant difference in systolic blood pressure between the placebo group and the group receiving the beverage was observed. In both cases, a higher dose of VPP is necessary due to the lower potency of this peptide compared to IPP [64, 125].

Another available commercial product is named Evolus® (Valio Ltd, Finland or Kaiku Vitabrands, Spain), a fermented milk with *L. helveticus* LBK-16H, which also exerts a significant antihypertensive effect in humans [5, 125, 126].

Other *L. helveticus* strains used in the production of antihypertensive fermented milk foods are *L. helveticus* R211, R389 [127] and LMG 11474 [128], as well as CHCC641 and CCCH637 from Chr. Hansen A/S [97].

Two other commercial products, a casein hydrolysate containing the peptide FFVAPFEVFGK (a_{s1}-CN (23-34)) named Casein DP (Kanebo, Ltd, Japan), and C12 peptide (DMV, The Netherlands), and a whey protein hydrolysate (BioZate, Davisco, US) were also claimed to lower blood pressure in humans [33, 129, 130].

2.3.2. Immunomodulation

The immune response can be influenced by various factors. Numerous reports demonstrate that milk bioactive peptides can interact with the immune system at different levels. The next paragraphs provide a brief overview of the immune system and of the effects of the milk-derived peptides implicated in the modulation of immune responses.

2.3.2.1. Overview of the physiology of the immune system

The immune system is a body wide network of cells, tissues, and organs that has evolved to defend the body against pathogens and foreign material, generally called as “non-self”. Pathogens include infectious organisms as bacteria, viruses and parasites and foreign material include for example toxins. All the non-self substances capable of triggering an immune response are known as antigens (from the National Cancer Institute of USA, www.cancer.gov/cancertopics/understandingcancer/immunesystem/).

The organs of the immune system are positioned throughout your body and include the bone marrow that is involved in the production of the immune cells. The thymus, where T lymphocytes mature; the spleen and the lymph nodes that contain specialized compartments where immune cells gather and confront antigens.

In addition to these organs, clumps of lymphoid tissue are found in many parts of the body, especially in the linings of the digestive tract (GALT), the airways (BALT) and the various mucosal compartments of the body (MALT) (from the National Cancer Institute of USA).

Cells of the immune system are of various nature and each population has a particular role. Neutrophils are particularly active against bacteria. Monocytes circulate in the bloodstream for about one to three days and then typically move into tissues throughout the body, where they differentiate into tissue resident macrophages or dendritic cells. Circulating monocytes are responsible for phagocytosis of antigens. Basophils are granulocytic cells that release granules containing histamine and they play a role in both parasitic infections and allergies. Mast cells are very similar in morphology and function to basophils but they resident cells of several types of tissues. Eosinophils are granulocites with the main role of combating multicellular parasites and some infections. Finally, Natural killer cells (or NK cells) are a type of cytotoxic lymphocyte. NK cells play a major role in the rejection of tumors and cells infected by viruses. The cells kill by releasing the proteins called perforin and granzyme that cause the target cell to die by apoptosis (from http://en.wikipedia.org/wiki/Immune_system).

In particular, these cells populations constitute the first line defense against antigens. In fact they are involved in the recruitment of the immune cells to sites of infection, through the production of chemical factors. In addition they promote the clearance of dead cell and they activate the process of inflammation that is one of the first responses of the immune system to infection or irritation. The first response to an antigen is rapid and important but it is not selective against the antigen and it is called innate immune response. This means that the cells of the innate system

recognize and respond to pathogens in a generic way, not conferring long-lasting or protective immunity to the host (from http://en.wikipedia.org/wiki/Immune_system).

Cooperating with the innate immune system to eliminate pathogens, the other part of the immune system is the adaptative immune system that is composed of highly specialized, systemic cells and processes that recognize and "remember" specific pathogens. In this way the response to the pathogen is more selective and efficient each time the pathogen is encountered. The most important cells intervening in this system are lymphocytes (see Fig. 2.3.2.1.1.).

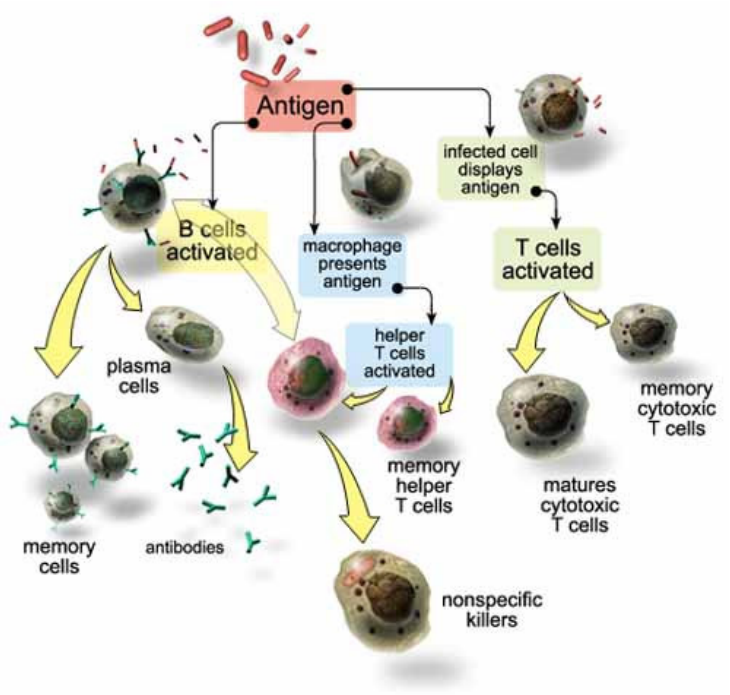

Fig. 2.3.2.1.1. Overview of the human immune response system. From http://www.uta.edu/chagas/html/bioIlmS1.html.

These cells attack the pathogens after antigen-presenting cells such as dendritic cells (or macrophages) display the foreign substance in the form of antigen fragments. Lymphocytes can be divided in different subgroups, called T lymphocytes (or T cells) and B lymphocytes (or B cells).

The B cell turns into a plasma cell that produces and releases into the bloodstream thousands of specific antibodies. Antibodies are large soluble proteins used to recognize, identify and neutralize specific antigens. There are different types of antibody, differing in biological properties, each has evolved to handle different kinds of antigens (from http://en.wikipedia.org/wiki/Immune_system).

The T cells coordinate the entire immune response and eliminate the viruses hiding in infected cells and contribute to the immune defenses in a cell-mediated way and can be sub-grouped as follow. T helper cells (T_H cells) assist other white blood cells in immunologic processes, including maturation of B cells into plasma cells and activation of cytotoxic T cells and macrophages, among other functions. Cytotoxic T cells (T_C cells, or CTLs) destroy virally infected cells and tumor cells, and are also implicated in transplant rejection. After the infection has resolved, another subset of antigen-specific T cells persist and they are called Memory T cells. They quickly expand to large numbers of effector T cells upon re-exposure to their cognate antigen, thus providing the immune system with "memory" against past infections. Memory T cells comprise two subtypes: central memory T cells (T_{CM} cells) and effector memory T cells (T_{EM} cells). Finally, Regulatory T cells (T_{reg} cells), formerly known as suppressor T cells, are crucial for the maintenance of immunological tolerance. Their major role is to shut down T cell-mediated immunity toward the end of an immune reaction and to suppress auto-reactive T cells that escaped the process of negative selection in the thymus (from http://en.wikipedia.org/wiki/T_cell).

The efficient components of the immune system act cooperatively to eliminate the infection. The “communication” between the different parts is mediated by specialized chemical mediators, called cytokines. Cytokines are diverse and potent chemical messengers secreted by the cells of the immune system. Cytokines include interleukins, growth factors, and interferons.

Lymphocytes, including both T cells and B cells, secrete cytokines called lymphokines, while the cytokines of monocytes and macrophages are called monokines.

Many of these cytokines are also known as interleukins because they serve as a messenger between leukocytes. Binding to specific receptors on target cells, cytokines recruit many the different subsets of the immune system. In addition, cytokines encourage cell growth, promote cell activation, direct cellular traffic, and destroy target cells--including cancer cells. Moreover, it is common for different cell types to secrete the same cytokine or for a single cytokine to act on several cell types. Cytokines are redundant in their activity, meaning that the same function can be stimulated by different cytokines.

Tables 2.3.2.1.1a. and 2.3.2.1.1b. depict the function of the most important cytokines involved in lymphocyte activation and proliferation (from http://en.wikipedia.org/wiki/Cytokine).

Cytokine	Producing Cell	Target Cell	Function
IL1α, IL1β	Macrophages, Monocytes, B cells, Dendritic Cells	T cells	Co-stimulation
		B cells	Maturation and proliferation
		NK cells	activation
		Various cell types	Inflammation, acute phase response, fever
IL2	T helper 1 cells	Activated T and B cells, NK cells	Growth, proliferation, activation
IL4	T helper 2 cells	Activated B cells	Proliferation and differentiation IgG_1 and IgE synthesis
		Macrophages	MHC Class II
		T cells	Proliferation
IL5	T helper 2 cells	Activated B cells	Proliferation and differentiation IgA synthesis
IL10	T helper 2 cells	Macrophages	*Cytokine production*
		B cells	Activation

Fig. 2.3.2.1.1a. Some of the cytokines produced by lymphocytes and their activities. *Italicized* activities are inhibited. Ig: Immunoglobulin. From http://microvet.arizona.edu/courses/MIC419/Tutorials/cytokines.html.

Cytokine	Producing Cell	Target Cell	Function
IL12	Macrophages B cells	Activated T cytotoxic cells	Differentiation into CTL (with IL2)
		NK cells	Activation
INFγ	T helper 1 cells, T cytotoxic cells, NK cells	Various cell types	*Viral replication*
		Macrophages	MHC expression
		Activated B cells	Ig class switch to IgG_{2a}
		T helper 2 cells	*Proliferation*
		Macrophages	Pathogen elimination
TNFβ	T helper 1 cells and T cytotoxic cells	Phagocytes	Phagocytosis, NO production
		Tumor cells	Cell death

Fig. 2.3.2.1.1b. Some of the cytokines produced by lymphocytes and their activities. *Italicized* activities are inhibited. Ig: Immunoglobulin.
From http://microvet.arizona.edu/courses/MIC419/Tutorials/cytokines.html.

In particular, two important cytokines involved in lymphocytes proliferation and activation are IL2 and INFγ. IL2 is a T cell growth factor produced by T helper 1 (T_{H1}) and NK cells. As an autocrine and paracrine growth factor, IL2 induces proliferation and differentiation of T and B cells. IL2 is responsible for the progress of T lymphocytes from the G1 to the S phase in the cell cycle and also for stimulation of B cells for antibody synthesis. IL2 stimulates the growth of NK cells and enhances the cytolytic function of these cells, producing lymphokine-activated killer cells. IL2 can also induce interferon INFγ secretion by NK cells.

INFγ is an important macrophage-activating lymphokine and it is involved in the induction of other cytokines, particularly T Helper 2 cytokines, such as IL4, IL5, and IL10. Because of its role in mediating macrophages and NK cell activation, INFγ is important in host defense against intracellular pathogens and viruses and against tumors responses thus influencing downstream immunological responses (From http://microvet.arizona.edu/courses/MIC419/Tutorials/cytokines.html).

2.3.2.2. Immunomodulatory peptides derived from milk

Immunomodulatory milk peptides act on the immune system and cell proliferation responses thus influencing downstream immunological responses and cellular functions.

Indeed, in 1981 Jollés and colleagues [131] discovered that a tryptic hydrolysate of human milk possessed *in vitro* immunostimulatory activity (more specifically, stimulation of phagocytosis of sheep red blood cells and production of hemolytic antibodies against the same cells).

In the following years, a number of potentially immunoregulatory peptides were identified encrypted in bovine caseins [132-136] and whey proteins [137, 138], which can manifest different effects (see Table 2.3.2.2.1a. and Table 2.3.2.2.1b.). Some casein-derived peptides (residues 54-59 of human β-casein and residues 194-199 of α_{s1}-casein) can stimulate phagocytosis of sheep red blood cells by murine peritoneal macrophages [135, 139], exert a protective effect against *Klebsiella pneumoniae* [140] or modulate proliferative responses and immunoglobulin production in mouse spleen cell cultures (fragment 1-28 of bovine β-casein, [132, 141].

More recently, lactoferricin B, obtained by hydrolysis of lactoferricin by pepsin, was found to promote phagocytic activity of human neutrophils [142]. Others fragments (fragment 18-20 of κ-casein, fragment 90-96 of α_{s1}-casein) can either stimulate or inhibit lymphocyte proliferation depending upon the concentration used [134, 143], while some whey-derived peptides can affect cytokine production from leucocytes [137, 138].

Protein sequence	Fragment	Activity	Reference
Bovine α_{s1}-CN	α_{s1}-CN (1-23)	Stimulation of phagocytosis and immune responses against bacterial infections	[144]
Bovine α_{s1}-CN	α_{s1}-CN (23-34)	Stimulation of phagocytosis and immune responses against bacterial infections	[85, 135]
Bovine α_{s1}-CN	α_{s1}-CN (90-96)	Stimulation effect on lymphocytes proliferation, NK activity and neutrophil locomotion	[140, 145]
Bovine α_{s1}-CN	α_{s1}-CN (90-95)	Stimulation effect on lymphocytes proliferation, NK activity and neutrophil locomotion	[140, 145]
Bovine α_{s1}-CN	α_{s1}-CN (194-199)	Stimulation of phagocytosis and immune responses against bacterial infections	[146]
Bovine α_{s2}-CN	α_{s2}-CN (1-32)	Stimulatory effect on spleen cells	[147]
Bovine β-CN	β-CN (1-28)	Stimulatory effect on spleen cells	[132, 141, 147]
Bovine β-CN	β-CN (63-68)	Stimulatory effect on spleen cells	[147]
Bovine β-CN	β-CN (191-193)	Stimulatory effect on spleen cells	[146, 147]
Bovine β-CN	β-CN (191-209)	Stimulation of phagocytosis of sheep red blood cells by murine peritoneal macrophages	[139, 147]
Bovine β-CN	β-CN (60-66)	Modulation of lymphocytes proliferation	[134]
Bovine β-CN	β-CN (193-202)	Modulation of lymphocytes proliferation	[134]
Bovine β-CN	β-CN (193-209)	Induction of proliferative response in rat lymphocytes; modulation of cytokine production by murine macrophages	[147, 148]
Human β-CN	β-CN (54-59)	Stimulation of phagocytosis of sheep red blood cells by murine peritoneal macrophages	[139, 147]
Bovine κ-CN	κ –CN (106-169)	Depression of lymphocytes proliferation	[133]
Hydrolyzed α-Lactalbumin	Nd	Enhancement of immune response of mitogen stimulated B lymphocytes	[20, 57, 149]
Hydrolyzed β-Lactoglobulin	Nd	Enhancement of immune response of mitogen stimulated B lymphocytes	[16, 57, 149]

Table 2.3.2.2.1a. Immunomodulatory peptides derived from milk proteins.

Protein sequence	Fragment	Activity	Reference
Bovine Lactoferrin	LF (17-41)	Antiviral action against the human immunodeficiency virus and the human cytomegalovirus, cytokine release modulation from leucocytes.	[137, 138, 150, 151]
Lactoferrin	Lactoferrin peptic hydrolysate	Stimulation of proliferation and antibody production in murine splenocytes and Peyer's patch cells	[138, 152]
Ovine colostral whey (proline rich peptides)	VESYVPLFP (peptide sequence)	Stimulatory effect on spleen cells	[147, 153]
Bovine κ-CN, α_{s1}-LA	κ-CN(38-40), α_{s1}-LA (18-20), κ-CN(38-39), α_{s1}-LA (18-19), α_{s1}-LA (50-51)	Modulation of lymphocytes proliferation, protection against malaria infection	[134, 154-157]

Table 2.3.2.2.1b. Immunomodulatory peptides derived from milk proteins.

It seems, therefore, that the immunomodulatory potential of bovine milk and bovine milk bioactive peptides is not restricted to the cells of bovine derivation, although the precise effects of these milk components may be different on target cells of different species.

However, the mechanisms by which these milk-derived peptides exert their immunomodulatory effects or influence cell proliferation are not currently fully elucidated. Some immunomodulatory peptides are multifunctional peptides and may modulate cell proliferation by interacting with opioid receptors. This is the case of the opioid peptide β-casomorphin derived from human β-casein that *in vitro* inhibits the proliferation of human lamina propria lymphocytes via opiate receptor [145]. Indeed, immune system and opioid peptides are related and it has already been demonstrated that opioid receptors are expressed on T lymphocytes [158, 159].

Other milk-derived peptides with immunomodulatory activity belong to the caseinophosphopeptides class. For example, the commercially available caseinophosphopeptide preparation CPP-III, consisting mainly of the fragments α_{s2}-CN (1-32) and β-CN (1–28) from bovine caseins, enhances the proliferative response induced by lipopolysaccharide, phytohaemagglutinin and concanavalin A

(conA) stimulation, and immunoglobulin production in mouse spleen cell cultures [14, 160]; this immunostimulating activity was attributed to the o-phospho-L-serine residue, hence suggesting that such a bioactivity is relatively stable to proteinase action in the intestinal tract [161]. The study of Otani *et al*. [162] focusing on the effects of CPP-III on serum and intestinal immunoglobulin G and immunoglobulin A secretion in mice proved that oral use of caseinophosphopeptide is beneficial toward enhancement of the mucosal immunity.

In addition, an alternative hypothesis involves a possible immunomodulatory action via ACE-inhibitory mechanism. ACE-inhibitory peptides are well known for their antihypertensive properties because they inhibit the conversion of angiotensin I to angiotensin II, but have also been found to prevent cleavage of bradykinin, that is mediated by ACE [135]. Bradykinin acts as a mediator of the acute inflammatory process and is thus able to stimulate macrophages, enhance lymphocyte migration and induce the secretion of cytokines from lymphocyte in culture. It should be noted that a common structural feature of several ACE-inhibitory peptides and some immunomodulatory peptides is the presence of arginine as the C-terminal residue [163].

Immunomodulatory milk-derived peptides may contribute to the overall immune response and may ameliorate immune system function. Migliore-Samour [140] suggested that casein derived peptides are involved in the stimulation of the newborn's immune system. It cannot be excluded that the immunostimulating activities may also have a direct effect on the resistance to bacterial and viral infection of adult humans.

2.3.2.3. Microorganisms for the production of fermented milk with immunomodulatory activity

Also in the case of immunomodulatory peptides, milk fermentation contributes to the generation of fermented milk with potential immunological activity (Table 2.3.2.3.1.).

Laffineur and colleagues [15] demonstrated that milk fermented with *L. helveticus* modulates lymphocyte proliferation *in vitro*. The same bacterial species selected for the ability to produce fermented milk with high ACE-inhibitory activity produces a fermented milk with immunomodulatory properties. It would be interesting to establish whatever the peptides released during milk fermentation responsible of ACE-inhibitory are also implicated in immunomodulatory activity.

In general, *L. helveticus* is known to have high proteolytic activity, causing the release of oligopeptides from digestion of milk proteins. Rachid *et al*. [4] demonstrated that the administration of *L. helveticus* decreases the growth rate of tumors in a murine model for mammary carcinoma.

In addition, LeBlanc and colleagues [164] used the strain *L. helveticus* R389 to ferment milk. The fermented milk was administered to mice with fibrosarcoma, resulting in a decrease of tumor size.

Milk fermented by *L. helveticus* not only manifested anti-tumoral properties but also induced the total antibody production against *E. coli* O157:H7 in mice infected by this pathogen [53, 164].

Microorganism	Protein source	Reference
L. helveticus 5089	Caseins	[15]
L. helveticus R389	Milk	[4, 52, 164]
L. paracasei NCC2461	Tryptic-chymotryptic hydrolysate of β-LG	[165]
L. casei GG (ATCC 53103)	Caseins	[166, 167]
L. casei GG	Milk	[168]
L. acidophilus	Milk	[168, 169]
L. casei rhamnosus GG	Caseins	[169, 170]
L. delb. bulgaricus ATCC11842	Milk	[169]
B. lactis BB12	Milk	[169]
S. thermophilus DSM4022	Milk	[169]

Table 2.3.2.2.1. Summary of microorganisms whose ability to produce fermented milks with immunomodulatory activity.

Fermented milks with immunomodulatory properties are not produced exclusively by *L. helveticus* (Table 2.3.2.2.1.). Milk fermented by *L. paracasei* [165] was shown to produce peptides from β-lactoglobulin that stimulate IL10 production and depress lymphocyte proliferation. Additionally, *L. casei* GG was used to produce a casein hydrolysate that suppresses human T cell activation, modulating IL2 expression [166, 167, 170].

The immunomodulatory activity is independent from the presence of living microorganisms, as evidenced by Perdigon [168] and by Vinderola [52] who reported that the supernatant of fermented milk cultured with *L. casei*, *L. acidophilus* and *L. helveticus* strains increased the immune response independently from the presence of lactobacilli. This result was obtained also by De Simone [13] that tested the INFγ production of human peripheral blood lymphocytes in response to filtered yoghurt devoid of microorganisms. More recently LeBlanc examined the antibody production following *E. coli* O157:H7 infection following the administration of a cell-free supernatant from *L. helveticus* fermented milk and found that the increased antibody production is not related to viable microorganism [53].

Microorganisms other that bacteria, as a cell-free extract obtained from the yeast *S. cerevisiae* can be used for milk fermentation, producing a milk hydrolysate with potential apoptosis-inducing effect in human leukemia HL-60 cells, as observed by Roy *et al*. [171].

In addition, as already demonstrated for milk proteins [172, 173], bioactive peptides present in yoghurt actually decreased cell proliferation with IEC-6 or Caco-2 cells, which may explain, at least partially, why consumption of yoghurt has been associated with a reduced incidence of colon cancer [174, 175].

The molecular mechanism by which the previous mentioned microorganisms enhance the immune system is not yet clear but the previously discussed reports

strongly support the fact that immunomodulatory peptides released in fermented milk contribute to the immunoenhancing and antitumor properties of dairy products.

It should be stressed that the extreme difficulty to establish how immunomodulatory peptides and fermented milks influence the immune function is strictly linked to the immune system complexity. Paragraph 2.3.2.1. already demonstrated that this system comprises a complex interplay between different cell populations and molecules. Thus, when the immunomodulatory activity of a bioactive peptide is assessed *in vitro*, the single experimental result could demonstrate the specific involvement of a particular milk-derived peptide in an immune mechanism but this result is not conclusive in determining if this peptide its effects would be significant for the whole immune system. For this reason the preferred term to describe the influence of milk on the immune system is modulation, because the potential for enhancement and suppression depends also on the target cells chosen to test the immunomodulatory activity of the bioactive peptide [1].

2.3.2.4. Two examples of immunomodulatory peptides derived from milk proteins

At present, most attention on immunomodulatory peptides has been focused on lactoferricin, a pepsin-derived peptide from lactoferrin [132, 176-178], and on glycomacropeptide, a k-casein-derived peptide (κ-CN (106–169)) [179, 180] present in appreciable amounts in some whey protein concentrates and whey protein isolates. But, as already revised before, during milk fermentation a number of bioactive peptides are formed by enzymatic hydrolysis of milk proteins with immunomodulatory potential. Particular attention will be given in this PhD thesis to the fragment α-LA (18-20) (a tri-peptide named YGG) and to the long fragment β-CN (193-209) because they have been chosen as model peptides to study the immunomodulatory activity and the absorption mechanism of bioactive peptides derived from milk proteins.

2.3.2.4.1. YGG peptide

The peptide YGG (Tyr-Gly-Gly) represents an interesting example of cryptic peptide with putative immunomodulating effects, as it can originate from at least two different sources. First, it is the product of the hydrolysis of Leu-enkephalin and Met-enkephalin [181, 182], and thus it is an endogenous peptide. In addition, it can be considered as a potential nutraceutical, because it is also encrypted in milk proteins and can be released during the digestion of bovine milk, in particular from α-lactalbumin (fragment 18-20) [31, 134].

It is known that Met-enkephalins, the YGG endogenous progenitor, can enhance human T cell proliferation and IL2 production *in vitro* in the absence of mitogens, possibly through the activation of opioid receptors present on the cell surface [183]. The enhancement of human peripheral blood lymphocytes proliferation and protein synthesis *in vitro* was obtained also with YGG administration in presence of conA [134, 184]. In addition, it was observed that YGG can affect INFγ and IL2 secretion in murine splenocytes stimulated with suboptimal concentration of conA in serum-free medium [157].

Stimulatory effects on cell proliferation were observed also in leukocytes obtained from mice administrated *in vivo* with either Met-enkephalin or YGG, suggesting that Met-enkephalin effects on the immune cells are mediated by YGG [185]. More recently, the immunomodulatory effect of YGG was confirmed *in vivo* by the observation that the peptide administration modulated the delayed-type hypersensitivity responses to tuberculin derivatives in hairless guinea pigs [154]. It is noteworthy to observe that YGG seems to have a biphasic effect on the parameters studied so far, as it showed an enhancing effect at low doses and an inhibitory effect at higher doses [154, 157].

It should be noted that YGG is contained several times in the primary structure of bovine κ-casein and α-lactalbumin and it could be released during milk fermentation or gastrointestinal digestion from the precursor proteins. In addition, it is a tripeptide

and, as already demonstrated for other milk-derived bioactive peptides [186], it can be assumed that it can pass across the intestine by a carrier-mediated peptide transport system in quantitatively significant amounts and, hence, may reach peripheral target sites.

2.3.2.4.2. β-CN (193-209) peptide

The β-CN (193–209) peptide is released from the C-terminal end of β-casein by hydrolysis with pepsin-chymosin. It is a 17 residues long peptide with the amino acid sequence Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val.

This peptide was isolated and identified from yoghurt and fermented milks as well as several types of cheese including Feta and Camembert studies [187, 188].

This peptide displays immunomodulatory properties and shows mitogenic activity on primed lymph node cells and unprimed rat spleen cells [147], it manifests chemotactive activity on L14 lymphoblastoid cell line [189], and enhances phagocytosis in rat macrophages [148, 190].

In addition, a smaller fragment of β-CN (193–209), corresponding to the amino acid sequence Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile, displayed ACE-inhibitory activity, further supporting the concept that ACE-inhibitors may also act as immunomodulatory peptides by acting as bradykinin-potentiating peptides [37].

Interestingly, the presence of 4 proline residues within the sequence can protect the long peptide β-CN (193–209) from the action of peptidases. So it could be possible that this peptide can cross the intestinal barrier in an intact bioactive form.

2.4. Bioactive peptide digestion

Some bioactive peptides can express their activity directly on the gastrointestinal tract but the majority of them has to reach their target site inside the body. They have to remain stable during the digestion process and cross the gastrointestinal

barrier maintaining their biological activities. It is thus important to know the physiology of digestion of proteins and peptides in the gastrointestinal tract, more specifically the human GI system, to understand the mechanisms determining the bioavailability of bioactive peptides *in vivo*.

2.4.1. Physiology of the digestion of proteins and peptides

In humans, the most important sites for the digestion of proteins and peptides are the stomach and the small intestine. The stomach is the portion of the GI tract that is located between the *cardia* and *pylorus* valves (see Fig. 2.4.1.1.). It can be divided in different regions which differ for the structure and functionality of the glands distributed in the gastric mucosa.

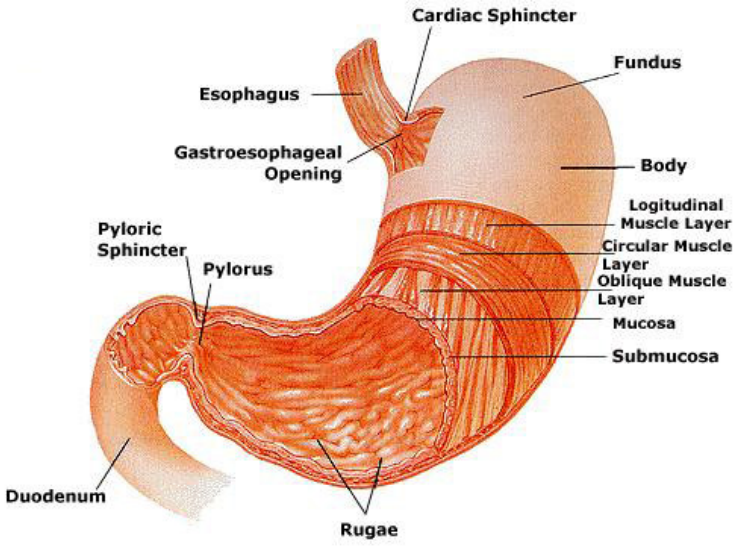

Fig. 2.4.1.1. The anatomic structure of the human stomach, from www.acm.uiuc.edu/sigbio/project/digestive/middle/stomach2.jpg

The gastric glands are composed by different types of cells, as HCl-secreting parietal cells, pepsinogen-secreting cells, mucous-secreting cells, and endocrine cells (see Fig. 2.4.1.2.).

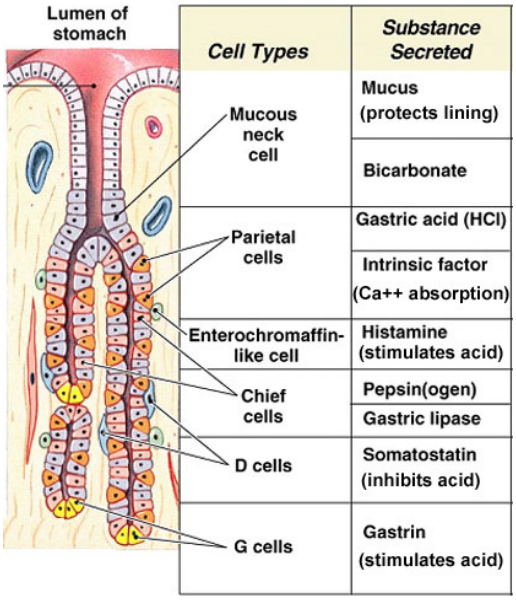

Fig. 2.4.1.2. Structure and function of a gastric gland, from www.colorado.edo/intphys/class/IPHY3430-200/image/21-25.jpg

The human small intestine is 2–6 m in length and is loosely divided into three sections – duodenum, jejunum and ileum – which comprise 5%, 50% and 45% of the length, respectively (see Fig. 2.4.1.3.).

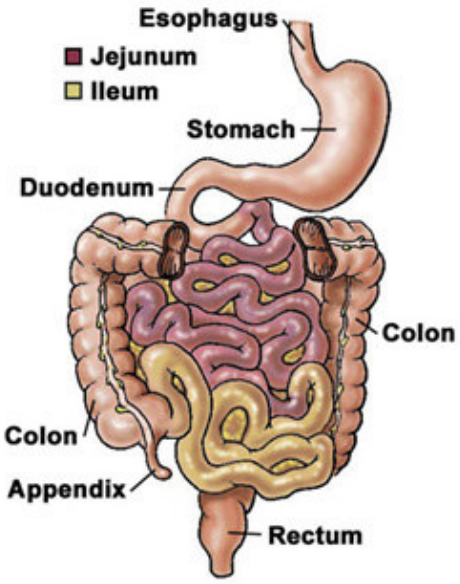

Fig. 2.4.1.3. The three sections of the small intestine, that is duodenum, jejunum (rose) and ileum (yellow), from www.yoursurgery.com/procedures/intussusception/images/SmBowelAnat.jpg.

The surface of the small intestinal region has various unique projections that significantly increase the potential surface area available for digestion and absorption. Macroscopic valve-like folds encircle the inside of the intestinal lumen and increase the surface area of the small intestine threefold. In addition, the presence of villi and microvilli increases the surface area by 30-fold and 600-fold, respectively. In particular, brush border membrane is the highly folded membrane that covers the entire surface of the small intestine and constitutes the massive surface area cited earlier. It is highly developed as a metabolically functional membrane, incorporating a selection of enzymes, transporters and receptors [191]. The key function of the small intestine is the selective absorption of major nutrients. In addition, it serves as a barrier to digestive enzymes and ingested foreign substances.

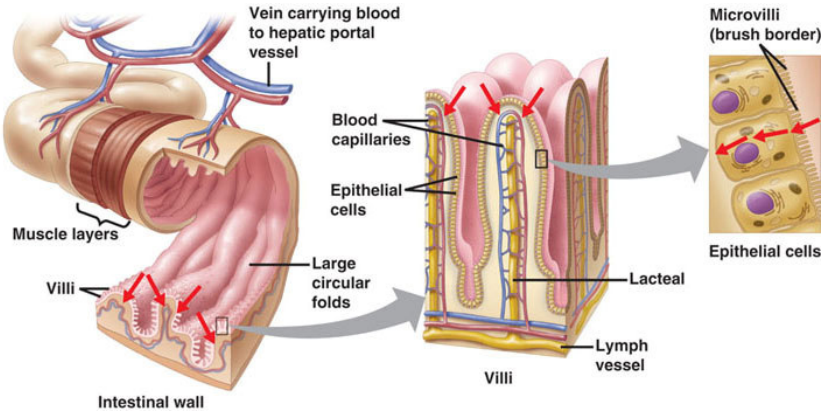

Fig. 2.4.1.4. The structure of lumen and of the epithelium of the small intestine, from http://kvhs.nbed.nb.ca/gallant/biology/small_intestine.jpg

The epithelial cells in the intestinal region are of heterogeneous nature, and they include enterocytes or absorptive cells, goblet cells (that secrete mucin), endocrine cells, Paneth cells, M cells and tuft and cup cells (see Fig. 2.4.1.4.). Enterocytes are the most common epithelial cells and they are thus responsible for the majority of the absorption of nutrients and drugs in the small intestine. The enterocytes are

polarized, and have distinct apical and basolateral membranes that are separated by tight junctions (TJ).

From a physiological point of view, the gastrointestinal tract is designed to break down dietary proteins and peptides into subunits sufficiently small to be absorbed [191, 192]. Digestive processes for proteins and peptides are catalyzed by a variety of enzymes specialized in the hydrolysis of peptide bonds, called peptidases. These peptidases have the wide substrate specificity and so they are considered the most important barrier limiting the absorption of bioactive peptides [191].

Peptidases are divided into 2 classes: endopeptidases, which hydrolyze peptide bonds interior to the terminal bonds of the peptide chain, and exopeptidases, which hydrolyze the bond linking N-terminal or C-terminal amino acid of the peptide chain. The most important endopeptidases are of pancreatic origin and are trypsin, chymotrypsin and elastases; the carboxy-peptidases A and B belong instead to the group of C-terminal exopeptidases [191, 192].

Peptide degradation is mediated by the small intestine but the first step of the degradation of proteins and peptides is mediated by the stomach. Denaturation of protein in the acid environment of the stomach by various pepsins represents the first step in protein digestion. This process is quantitatively of minor importance because only tiny amounts of amino acids are released whereas the bulk of predominantly large polypeptides appears in the duodenum [193]. In the stomach the main peptidase is pepsin, an endopeptidase secreted by stomach mucosa. Pepsin normally reduces proteins and large peptides into big oligopeptides. The generated peptides and the intact proteins pass in the intestinal lumen and undergo the action of pancreatic peptidases, which is the main event of intraluminal digestion. The set of pancreas peptidases is various and it permits to degrade the

majority of large peptides. Most of these enzymes are secreted by the pancreas in an inactive form (trypsinogen, chymotrysinogen, proelastase, procarboxypeptidase). Activation of trypsinogen requires enterokinase, a small intestinal mucosal enzyme. Activation of the other precursors requires trypsin. Intraluminal hydrolysis of large polypeptides results in oligopeptides composed of 2-8 amino acids. The luminal phase of protein digestion leads therefore mainly to appearance of oligopeptides, but only to small amounts of free amino acids [193].

The relative importance of this luminal hydrolysis in the overall degradation is dependent on the size and the respective amino acid composition of the peptide [194]. However, even when luminal peptide degradation occurs, it constitutes at best the 20% of the total degradation in a given intestinal segment. This implies that significant degradation of the peptide requires at least the contact with brush border membrane or uptake into the enterocytes [192]. Indeed, peptidases in the brush border membrane are probably the biggest deterrent to the absorption of small peptides across the intestinal mucosa [194] and the mucosa of small intestine expresses at least 15 peptidases (see Table 2.4.1.1a. and Table 2.4.1.1b.).

Enzyme	Substrates and Properties	Products
Endopeptidases:	**Hydrolysis of internal peptide bonds:**	
Enterokinase	- Of trypsinogen (initiation of luminal digestion)	Trypsin
Neutral endopeptidase (EC 3.4.24.11) PABA peptide hydrolase	- At hydrophobic amino acids of α-casein, insulin etc.	Peptides
Dipeptidases:	**Hydrolysis of dipeptides into AA**	
Zn-stable Asp-Leu peptidase	Dipeptides, esp. Asp-Leu	Amino acids
Gly-Leu peptidase	Dipeptides, esp. Gly-Leu metalloenzyme (Zn)	Amino acids
Membrane dipeptidases	Dipeptides (glutathione conjugate)	Amino acids

Table 2.4.1.1a. Intestinal brush border associated proteases and peptidases, from Hartmann *et al.,* 2007 [195].

Enzyme	Substrates and Properties	Products
Oligopeptidases:	**Hydrolysis of N-terminal peptide bonds:**	
Aminopeptidases (AP)	AP are metalloenzymes (Zn, Ca, Co).	Amino acids di-, tripeptides
AP N (EC 3.4.11.2)	AA-oligopeptide of neutral amino acids	
AP A (EC 3.4.11.7)	AA-oligopeptide of acidic amino acids	
AP P (EC 3.4.11.9)	Pro-oligopeptide	
AP W (EC 3.4.11.16)	X-Trp, X-Trp-oligopeptide	
Dipeptidyl-AP IV (DPP IV; EC 3.4.14.5)	X-Pro-oligopeptide X-Ala-oligopeptide X-Lys-oligopeptide DPP IV is a serine protease.	X-Pro, X-Ala, X-Lys, oligo-, dipeptides
Carboxypeptidases (CP):	**Hydrolysis of C-terminal peptide bonds:**	
dipeptidyl-CP I; EC 3.4.15.1	Angiotensin, Oligopeptide-Pro Synonym: angiotensin-converting enzyme, peptidase P	Peptides
γ-glutamyl-transpeptidase EC 2.3.2.2	Peptides with bound γ-glutamyl, e.g. gluthation	Peptides, γ -glutamyl-AA
Folate conjugase; EC 3.4.19.9	Polyglutamyl folate	Folic acid
CP M; EC 3.4.17.12	Peptide-Lys, peptide-Arg	Basic amino acids

Table 2.4.1.1b. Intestinal brush border associated proteases and peptidases, from Hartmann *et al.,* 2007 [195].

In general it appears that brush-border peptidases are active mainly against tri-, tetra, and higher peptides reducing them to the amino acid residues [192, 196] (see Fig. 2.4.1.5.). The cytosol indeed contains a set of peptidases particularly active against di and tri peptides. So, after the action of brush border peptidases, there is only little possibility that large peptides could be absorbed.

Regardless of the mechanisms of absorption, the bioactive peptides that enter the enterocyte undergo the action of the peptidases of the cytosol or the cellular organelles. Indeed, the lysosome contains a massive array of enzymes, estimated over 60 in number, which are capable of degrading any biological macromolecule, including peptides and proteins [194, 197].

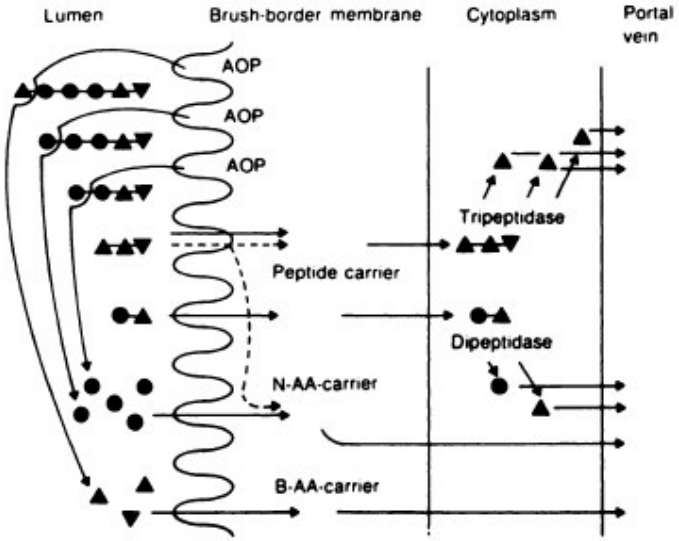

Fig. 2.4.1.5. Schematic representation of the brush border membrane and cytoplasmic phase of intestinal protein assimilation. The dual mechanism of peptide absorption is indicated in the dashed line. AOP = amino-oligopeptidase, N-AA-carrier = carrier for neutral amino acids, B-AA-carrier = carrier for basic amino acids. From Caspary, 1992 [193].

2.4.1.1. The digestion of bioactive peptides derived from milk proteins

The release of ACE-inhibitory peptides upon gastrointestinal digestion of milk proteins or protein fragments, as well as the resistance to digestion of known ACE-inhibitory sequences has been tested in several *in vitro* studies where the gastrointestinal process was mimicked by the sequential hydrolysis with pepsin and pancreatic enzymes (trypsin, chymotrypsin, carboxy and aminopeptidases). These studies showed that gastrointestinal digestion is an essential factor in determining ACE-inhibitory activity [188, 198]. The conditions of the simulated gastrointestinal digestion (enzyme preparation, temperature, pH and incubation time) greatly influence the degree of proteolysis and the resultant ACE-inhibitory activity [198]. The action of brush-border peptidases, the recognition by intestinal peptide transporters and the subsequent susceptibility to plasma peptidases also determine the physiological effect [54].

2.4.2. Digestion Models

Resistance to hydrolysis is one of the main factors influencing the bioavailability of a bioactive peptide. However, few *in vitro* and *ex vivo* models are available about the simulation of the digestion of bioactive peptides. In addition, the majority of the reports are not specifically realized to study the digestion of bioactive peptides but their main purpose is to develop digestion models for the evaluation of potential allergenicity of food proteins [199], in particular milk proteins [200]. On the contrary, a consistent body of literature exists about *in vitro* and *ex vivo* models for the absorption of bioactive peptides, as Caco-2 cell lines [186, 201, 202].

The effects of digestive enzymes on bioactive peptides, in particular ACE-inhibitory peptides derived from different food matrices, have been evaluated in *in vitro* gastrointestinal simulated systems [203]. The first methods were developed by Garrett and colleagues on soy proteins [204] and then slightly modified by Hernandez-Ledesma and colleagues [188], Picariello and colleagues [205] and Lo and colleagues [206] that applied the protocol on milk proteins. The common purpose of these experiments was to assess the effects of the peptidases of the stomach and the pancreas on the preservation of the ACE-inhibitory activity of different hydrolysates. Other authors [207, 208] preferred to use the Corolase PP® instead of the pancreatin. The Corolase PP® is a proteolytic enzyme preparation from pig pancreas glands that contains, in addition to trypsin and chymotrypsin, numerous amino-acid- and carboxy-peptidases.

However, these models are not completely predictive of the resistance of the bioactive peptides because they do not mimic all the physiological factors affecting food digestion, as pH variations, the relative amounts of the enzymes, the interactions with other molecules, and the *ratio* peptidase/tested compound. These variations may affect the rate of enzymatic degradation of the bioactive peptides

under study, therefore affecting the estimated bioavailability of these bioactive peptides.

In general, the bioavailability studies that are the most adherent to the reality are *in vivo* measurement in humans with or without using a labeling technique.

Investigation of the human digestive process normally involves a feeding study and the acquisition of serial samples of *digesta* from the stomach and upper small intestine via naso-gastric/naso-duodenal aspiration, the rest of the small intestine being inaccessible [209]. Human *in vivo* studies are, however, time-consuming, very expensive, complicated, and produce variable results.

In vivo studies on laboratory animal are also available to evaluated the digestion of milk-derived bioactive peptides and have the advantage to be less expensive but the majority is effectuated on rodents [210], in which differences between the metabolism of this species and human make it difficult to extrapolate the human situation.

Thus, there is an increasing need to develop *in vitro* gastrointestinal digestion models that could mimic the human digestion processes. *In vitro* methods therefore offer an appealing alternative to human and animal studies. They can be simple, rapid, and low in cost and may provide insights not achievable in whole animal studies.

In fact, in the last years new *in vitro* gastrointestinal digestion models incorporating the multi-phase nature of the digestive processes, to mimic the passage the food into the stomach and then into the gut, have been developed or adapted for assessing digestibility of food allergens [211, 212], but a potential application on the study of physiology of the digestion of bioactive peptides could be feasible.

Such models have to be sufficiently refined to allow the process of digestion to be followed in some detail and have to be validated against *in vivo* data. Ideally, an *in*

vitro model should offer the advantages of rapid representative sampling at any time point, testing the whole food matrix (or diet) instead of the isolated protein precursor of the bioactive peptide and be capable of handling solid foods which cannot easily be tested *in vivo*. Moreover, *in vitro* digestion models should consider three main stages: (i) processing in the mouth, (ii) processing in the stomach (cumulative to the mouth) and (iii) processing in the duodenum (cumulative of mouth and stomach). These three phases can be considered separately or in combination depending on the purpose of the study [209].

The development of some of these multi-phase digestion models has provided useful information, demonstrating the importance of using a physiologically relevant *in vitro* digestion system. These systems can be grouped into different class, that is static and dynamic *in vitro* digestion models.

Static models (also known as biochemical models) are defined as models where the products of digestion are not removed during the digestion process (i. e. no absorption) and which do not mimic the physical processes that occur *in vivo* (e. g. shear, mixing, hydration, changing conditions over time, etc). Good static models are particularly useful where there is limited digestion, e. g. stomach, but are less applicable for total digestion studies. These types of models are predominately used for digestion studies on simple foods and isolated or purified nutrients [209].

Many of these models are quite crude, and simply involve homogenization of food, acidification with hydrochloric acid, addition of gastric enzymes followed by a varying delay simulating gastric residence time, neutralization with sodium carbonate or sodium hydroxide and the addition of pancreatic enzymes and bile salts whilst stirring at 37℃. The rate of loss of a component or the appearance of a component is used to measure the progress of the reactions, but normally the system is allowed to run to completion to simulate total digestion. Frequently, the ratios of surfactants,

enzymes and substrates are not physiological because the model is intended to cause exhaustive digestion in the belief that this is what occurs *in vivo* [209].

Despite the valuable information that can be obtained from the static multiphase digestion models, these systems only consider the biochemistry of the digestion but they do not take into account several factors that could play an important role in the digestibility of proteins such as the gastrointestinal transit or the appropriate mixing at each stage of digestion (peristalsis). This means that, to address correctly all these issues, the application of dynamic models should be preferred.

It is becoming increasingly clear that in order to understand the digestion of structured foods, it is insufficient to simply consider the biochemistry of the gut, as the gastrointestinal processing plays an equally important role. This more holistic view of digestion will allow to move away from the static models of digestion, which are only able to process simple meals and isolated nutrients, to dynamic models, incorporating the physical processing of the gut, which can be used during digestibility studies on “structured” meals (i.e. real foods or food materials) [209]. Dynamic models may or may not remove the products of digestion but have the advantage that they include the physical processing and temporal changes in luminal conditions that mimic conditions *in vivo*. This is particularly useful where the physical condition of the *digesta* changes over time, e.g., viscosity, particle size reduction, and takes into account some temporal effects not otherwise considered, e. g., unstirred layers, diffusion, creation of colloidal phases, partitioning of nutrient between phases, etc [209]. As a result, some dynamic *in vitro* models (Fig. 2.4.2.1. and Fig. 2.4.2.2.) have been described [213, 214].

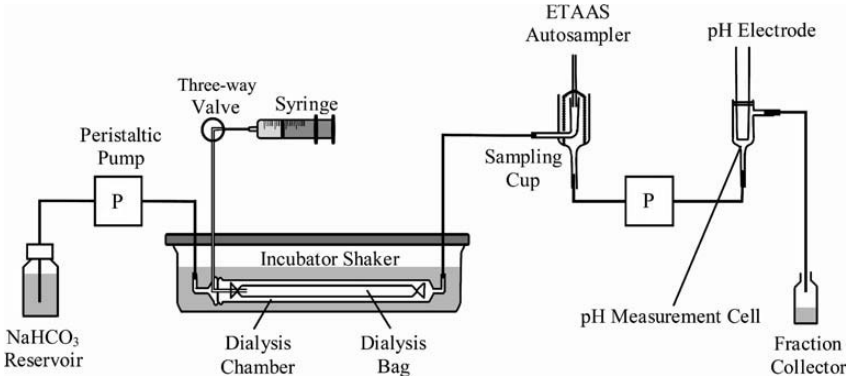

Fig. 2.4.2.1. Diagram of the continuous flow dialysis system coupled with on-line electrothermal atomic-absorption spectrometry and pH measurement, from Promchan *et al.*, 2005 [213].

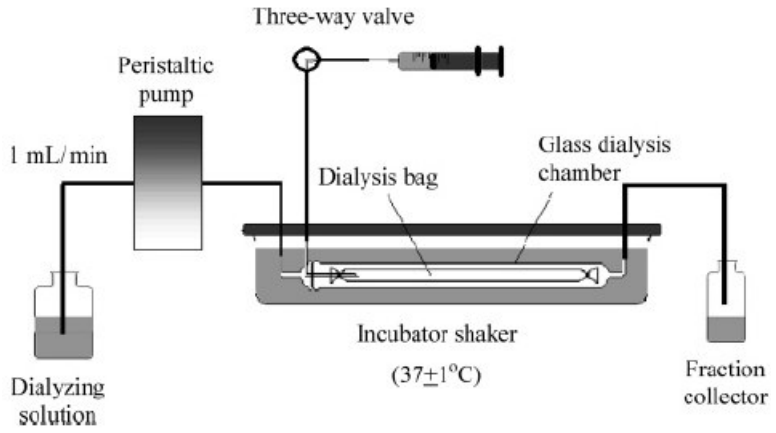

Fig. 2.4.2.2. Diagram of the proposed continuous flow *in vitro* dialysis system, from Shiowatana *et al.*, 2006 [214].

An intermediated model for the evaluation of the release of ACE-inhibitory activity during the digestion has been realized by Vermeirssen and colleagues [198]. This is a semi-continuous model based on the batch physiological digestion (Fig. 2.4.2.3.). In this reactor, the influence of temperature and incubation time in the stomach and small intestine phase on the formation of ACE-inhibitory activity and the degree of proteolysis is investigated.

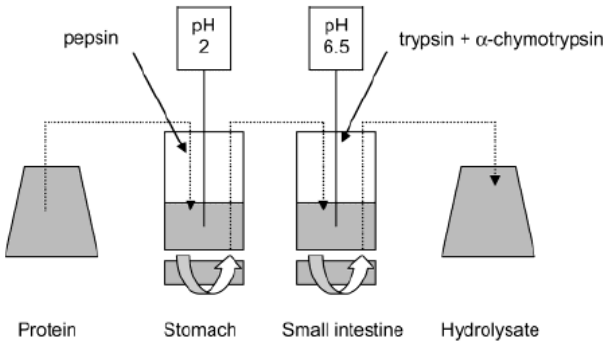

Fig. 2.4.2.3. Experimental setup for the semi-continuous digestion, from Vermeirssen *et al*., 2003 [198].

More recently, Bastianelli and colleagues [215] explored the mathematical modelization of the nutrient digestion in pig. This four-compartments model (see Fig. 2.4.2.4.) permitted to integrate various factors that normally affect food digestion. This approach could be applied to evaluate the digestion of bioactive peptides, because the porcine system has been shown to be a valid approximation of the analogous systems in humans and has been used extensively to model human digestion [216-218].

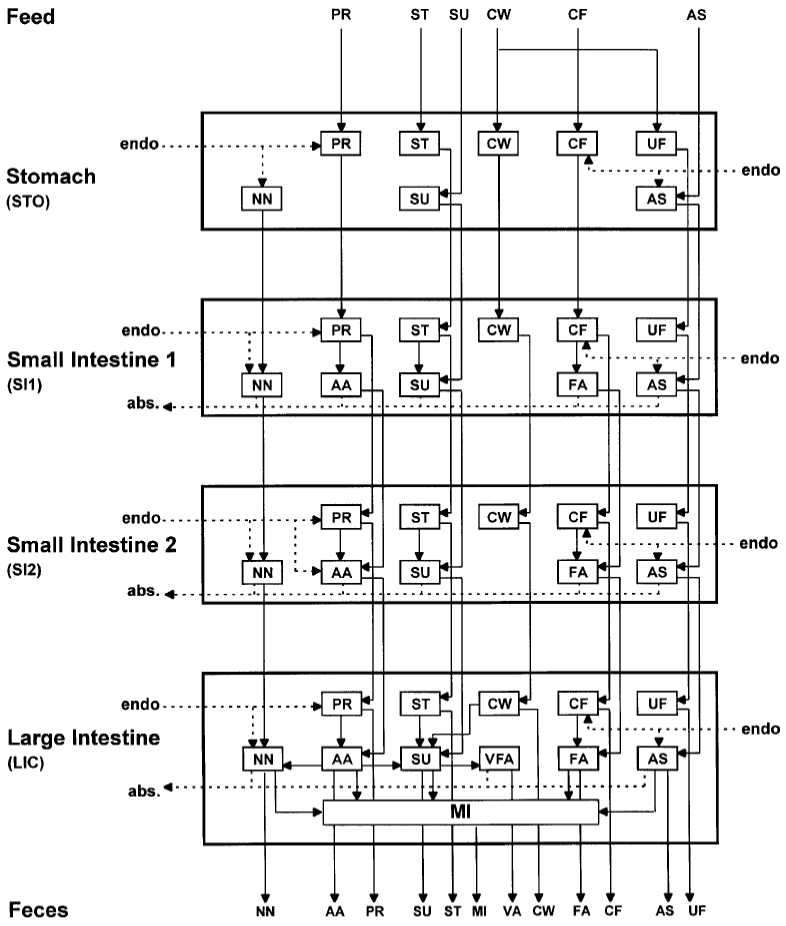

Fig. 2.4.2.4. Diagram of the model developed by Bastianelli *et al.*, 1996 [215]. The model is developed with four compartments (AC); stomach (STO); two parts of the small intestine (SI1 and SI2), and large intestine (LIC). Biochemical subcompartments (BSC) are non-protein nitrogen (NN), protein (PR), pool of amino acids (AA), starch (ST), sugars (SU), digestible cell walls (CW), lipids (CF), volatile fatty acids (VFA), undigestible cell walls (UF), and minerals (AS). In addition, there is a microbial subcompartment in LIC (MI). Flows between compartments are represented (solid lines). Other flows are endogenous secretions (endo) and absorption (abs), represented in broken lines.

At the moment, for human studies, a dynamic computer–controlled *in vitro* system that mimic the human physiological condition in the stomach and in the intestine has been realized [219], but with the main purpose to investigate the fate of food mutagens (Fig. 2.4.2.5.).

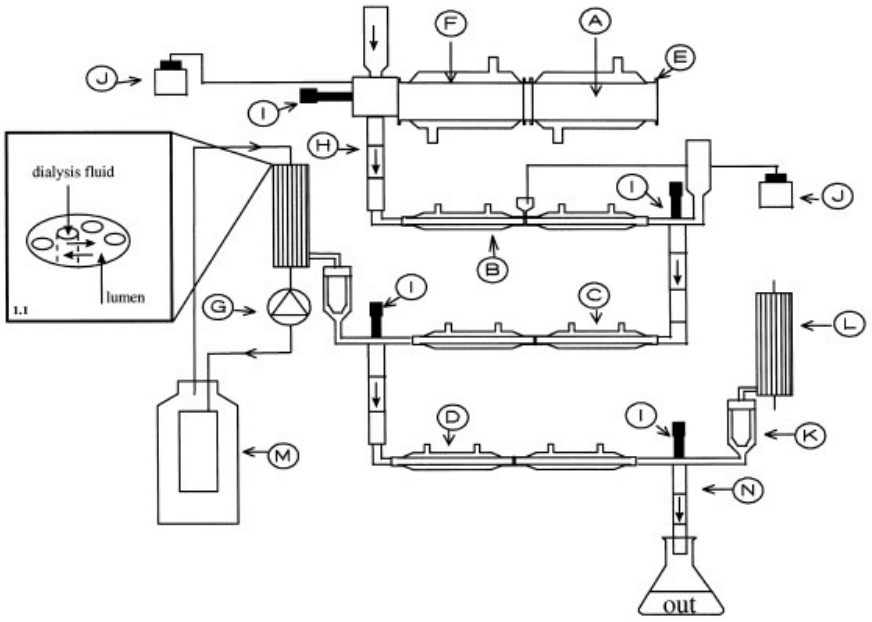

Fig. 2.4.2.5. Schematic diagram of the dynamic *in vitro* model of the stomach and the small intestine (TIM): (A) gastric compartment; (B) duodenal compartment; (C) jejunal compartment; (D) ileal compartment; (E) glass jacket; (F) flexible wall; (G) rotary pump; (H) pyloric valve; (I) pH electrodes; (J) secretion pump; (K) pre-filter; (L) hollow fiber membrane; (M) dialysis system; (N) ileal delivery valve; 1.1 detail of the hollow fiber membrane system, from Krul *et al*., 2000 [219].

An innovative approach to predict the uptake of iron in humans has been developed by Glahn and colleagues [220]. This *in vitro* model (Fig. 2.4.2.6.) combines Caco-2 cell line in conjunction with *in vitro* digestion techniques and develops a model whereby foods undergo simulated peptic digestion followed by intestinal digestion in the presence of Caco-2 cell monolayers. The conditions of this model have been designed to simulate the gastrointestinal environment while still maintaining a rapid and inexpensive system. This model system is unique among applications of Caco-2 cells and *in vitro* digestion techniques in that it allows uptake to occur simultaneously with food digestion under pH conditions similar to those found along the absorptive surface of the intestinal mucosa. Furthermore, the addition of the human-derived component, i.e., Caco-2 cells, transforms this model system into a tool capable of conducting experiments that might not be feasible or practical to

conduct *in vivo* [220]. In recent years, this system has been widely used to test iron bioavailability studies from different food matrices [221-223].

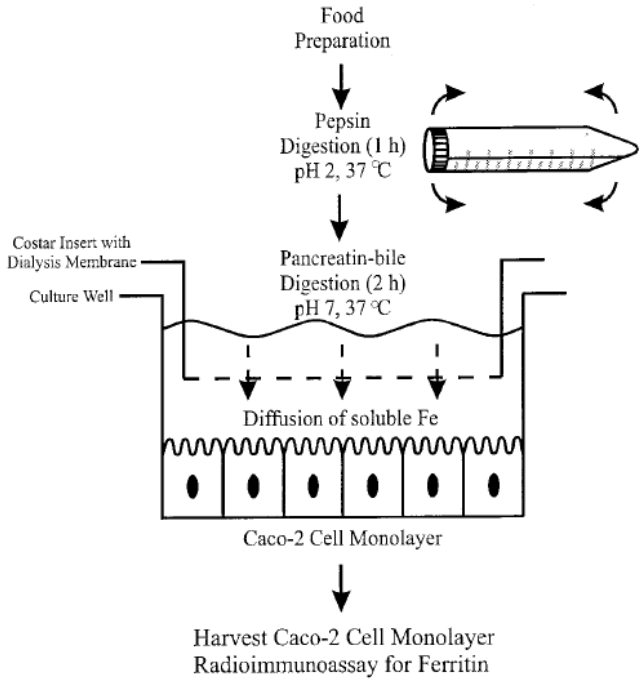

Fig. 2.4.2.6. Diagram of *in vitro* Caco-2 cell culture model developed by Glahn and colleagues [220].

2.4.2.1. The brush-border membrane vesicles

Whereas more complex models can simulate many aspects of the human physiology, the simple models are easy to perform and allow simultaneous determination of a large number of samples. For example, isolated brush border membrane vesicles (BBMV) are a useful and widely accepted model to study *in vitro* the interactions of food proteins with the apical membrane of small intestine epithelial cell. Indeed, this methodology emerged in the late 1960s and was perhaps the most influential technique in membrane transport until the cloning and electrophysiology era. BBMV greatly facilitated the study of the uptake of solutes in intestinal epithelia, in particular peptide transport [224, 225].

BBMV are the result of the polarization of the epithelial cells. Small intestine cells have two cell membrane domains, the apical and basolateral part. By calcium

precipitation it is possible to isolate brush border membrane-enriched fractions. This method was originally described for BBMV isolation of human small intestine [226].

It is possible to verify the effective enrichment and isolation of brush border membrane during the BBMV preparation measuring the activity of some enzymes exclusively located in the brush border membrane, as alkaline phosphatase, sucrase-isomaltase and dipeptidylpeptidase IV. These enzymes are also expressed in the apical membrane of differentiated Caco-2 and T84 cells [227-229].

The isolation of BBMV permits that the most important digestive enzymes expressed by the intestinal brush border membrane are concentrated in these vesicles that are quite stable and easy to handle. So the potential application of BBMV in the evaluation of the specific effect of brush border peptidases on bioactive peptides could be explored, without the presence of the pancreatic enzymes. This methodology could contemporaneously permit the evaluation of the absorption of the bioactive peptide.

2.5. Bioactive peptide absorption

After digestion, di- and tri-peptides can be easily absorbed in the intestine, but it is not clear if larger bioactive peptides can be absorbed from the intestine and reach the target organs. Some bioactive peptides, in particular C-terminal proline containing peptides, are resistant to proteolysis [41], suggesting that this class of peptides have a better chance to be absorbed in their active form.

To better understand the fate of a bioactive peptide in the gut the following paragraphs show a brief overview of the physiology of peptide absorption.

2.5.1. Physiology of the absorption of proteins and peptides

Approximately 90% of the absorption in the gastrointestinal tract occurs in the small intestinal region. The specialized epithelial barriers of the gastrointestinal tract

separate fluid-filled compartments from each other. They restrict and regulate the flux of substances in both directions. In general, the transfer of all substances, from H^+ ions to the largest proteins, across these barriers can occur via paracellular or transcellular routes (Fig. 2.5.1.1.).

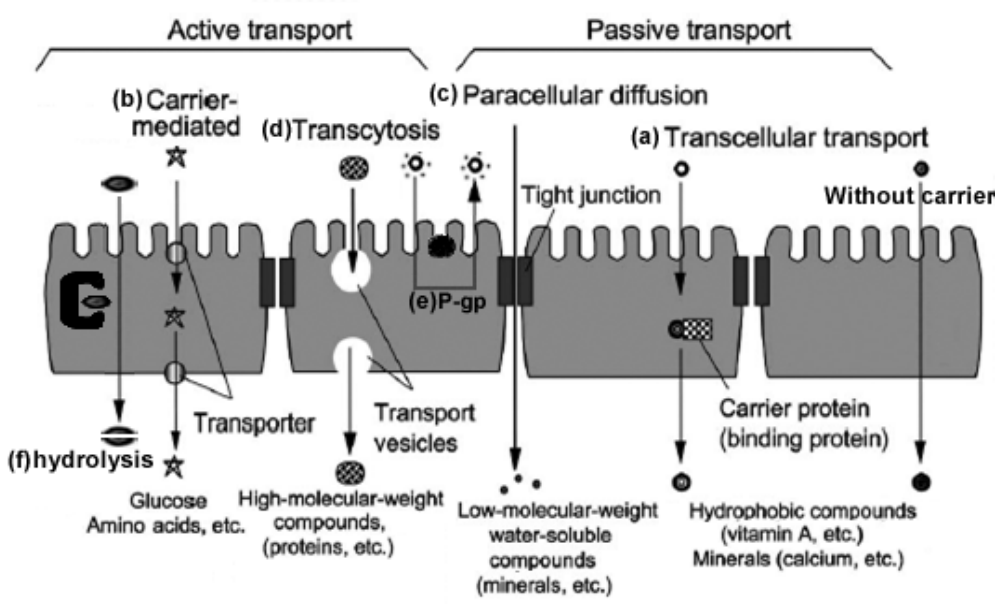

Fig. 2.5.1.1. Different pathways for intestinal absorption of a compound. The intestinal absorption of a compound can occur via several pathways: (a) transcellular passive permeability; (b) carrier-mediated transport; (c) paracellular passive permeability, and (d) transcytosis. However, there are also mechanisms that can prevent absorption: (e) intestinal absorption can be limited by P-gp, which is an ATP-dependent efflux transporter; and (f) metabolic enzymes in the cells might metabolize the bioactive peptide, from Shimizu, 2007 [230] .

The transcellular route (see Fig. 2.5.1.1.(a)) requires the transport of the solute across two morphologically and functionally different cell membranes (e.g. the apical and the basolateral membrane), by either active or passive processes. The extent of simple passive diffusion of substances across the membranes depends on their size, charge and lipophilicity and could be facilitated by a carrier system and has been observed for most smaller inorganic and organic solutes [231].

Among the active systems for transcellular transport of peptides the most important is the Peptide Transporter 1 (PepT1) (see Fig. 2.5.1.1.(b)). Human PepT1 is a 729-

residues transmembrane protein complex with 12 transmembrane domains (see Fig. 2.5.1.2.) belonging to the proton-dependent oligopeptide transporter (POT) family. This transporter is mostly a degradative way because cytosolic peptidases rapidly hydrolyze most of the di- and tripeptides transported by PepT1. One of the most commonly used and best known reference ways to study the peptide transport mediated by of PepT1 is the substrate [^{14}C]glycylsarcosine (Gly-Sar). Gly-Sar is relatively stable against intra- and extracellular enzymatic hydrolysis and it acts as competitive substrate of PepT1. Other labeled reference substrates quite often used to study peptide transport by PepT1 are [^{3}H]D-Phe-L-Ala, [^{3}H]D-Phe-L-Gln and D-Ala-L-Lys-Ne-7-amino-4-methylcoumarin-3-acetic acid [232].

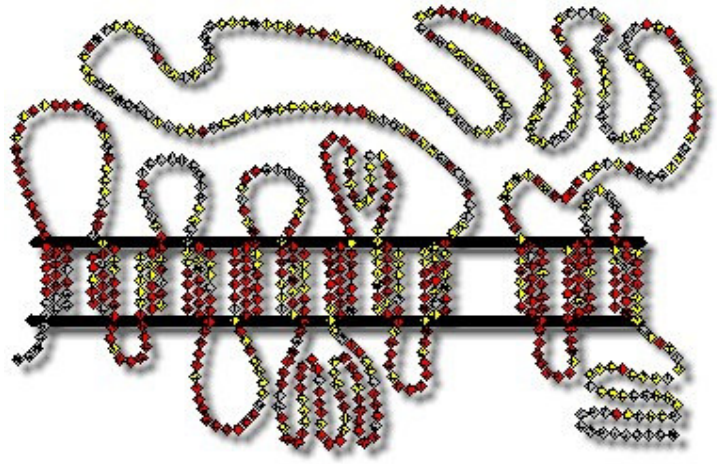

Fig. 2.5.1.2. Membrane topology of human PepT1, from http://www.wzw.tum.de/nutrition/index.php?id=31.

The paracellular pathway (see Fig. 2.5.1.1.(c)) is very often restricted by tight junctions (TJ), and the ability of substances to cross epithelia between the cells by simple passive diffusion depends mainly on their size. TJ between intestinal epithelial cells play an important role in the regulation of transport of organic and inorganic compounds from the gut lumen towards blood circulation [233]. TJ maintain the specificity of apical and basolateral domains and form a fence to prevent mixing of apical and basolateral membrane components as well as an

occluding barrier between neighboring cells [234]. TJ membrane proteins interact with scaffold proteins to connect them with various signal transduction and transcriptional pathways involved in the regulation of TJ function [235]. Although the permeability of TJ varies significantly within different epithelia, TJ are generally reported to be impermeable to molecules with *radii* larger than 11-15 Å [236]. Paracellular passive diffusion process is applicable for a wide variety of low-molecular weight compounds including peptides [186, 237, 238]. Moreover, it has to be considered that TJ are affected by several extracellular stimuli, as nutrients, INFγ and cytokines [239-242]. Thus the paracellular transport of small compounds, as bioactive peptides, could be different *in vivo* than that predicted from the *in vitro* approaches [243].

The potent mycotoxin cytochalasin D could be used to perturb the TJ. The administration of cytochalasin D could be useful to study the mechanism of absorption of those food-derived compounds whose transport could be mediated by tight junction, as small peptides. Cytochalasin D acts as an inhibitor of actin polymerization and it disrupts actin microfilaments. Several studies aiming to elucidate the mechanism of absorption of bioactive peptides used Cytochalasin D to increase the permeability of paracellular passive transport [201, 202, 244].

Large proteins or peptides that cannot be absorbed by PepT1 are translocated across cell layers mainly by specialized transcytotic processes involving membrane invagination and vesicle internalization (Fig. 2.5.1.1.(d)). Cellular internalization via vesicles could be divided in fluid-phase endocytosis, that does not require any interaction between the peptide and the apical membrane [245], and in receptor-mediated absorptive endocytosis [246] that involves a binding with the plasma membrane before being incorporated into endocytotic vesicles.

Once internalized inside the vesicles, the proteins or peptides are recycled back to the plasma membrane or processed in the course of a multistep transport sequence through various intracellular organelles, such as endosomes, prelysosomes and lysosomes [247]. If the fusion with lysosomes does not completely disrupt the endocytosed molecules, they could also be transported to the opposite cell surface completing the transcytotic process [248].

Transcytosis could be studied by selective inhibitors of this pathway, as wortmannin. Wortmannin was first discovered in 1957 in the broth of the fungi *Penicillium wortmannin* Klocker [249] and it is a specific covalent inhibitor of phosphoinoside 3-kinase and it is also involved in the inhibition of receptor-mediated endocytosis [250]. Cardone and Mostov observed that wortmannin inhibits transcytosis in epithelial cells, more specifically in those related to the mucosal immunity [251].

At present, numerous data demonstrate that dipeptides and tripeptides are transported intact from the lumen into the enterocytes by the H+/peptide transporter PepT1 [5, 6, 11]. Peptides resistant to cytosolic peptidases may be transported intact across the basolateral membrane of intestinal cells by a peptide transport system that has been characterized so far only at the functional level.

The mechanisms involved in the transfer of peptides across the intestinal basolateral membrane to the blood side are still under debate. The investigation by Dyer *et al.* [252] using rabbit enterocyte basolateral membrane vesicles was the first to study basolateral peptide transport. This report described a system relatively specific for small peptides that, just as PepT1 in the apical membrane, is stimulated by an inwardly directed H^+ gradient. Yet, the H^+ gradient across the basolateral membrane is expected to be very small. This might provide the basis for transcellular movement of small peptides across the enterocyte despite the fact that the peptide transport systems in both poles of the cell are H^+ dependent [253]. Terada *et al.* [254] reported that uptake of [^{14}C]Gly-Sar across the basolateral

membrane in Caco-2 cells cultured on filters was less sensitive to extracellular pH than uptake across the apical membrane by PepT1. Importantly, the uptake did not proceed against a concentration gradient. This result led to the conclusion that the basolateral system is a facilitative peptide transporter whereas PepT1 is an active transporter [254]. If few information is available on basolateral transport of small peptides, still less is known on the basolateral transport of large peptides, that is mainly mediated by an esocytotic vesicles.

In conclusion, the intestinal transport of peptides is not fully elucidated and many questions remain open. For example, the mechanism by which the transport systems for peptides are differentially sorted in the enterocyte to be inserted into the brush border and/or the basolateral membrane is still controversial. Another serious lack of knowledge exists about the number of carriers per cell and the substrate turnover rates. The identity of the postulated basolateral peptide transporters remains to be elucidated. The intestinal absorption of ACE inhibitors needs to be re-evaluated. Furthermore, inter-individual differences in peptide transport should be one of the priorities of future research in this area [231].

2.5.2. Physical and chemical characteristics of potentially absorbable bioactive peptides

To exert physiological effects after oral ingestion, it is of crucial importance that milk-derived bioactive peptides remain active during gastrointestinal digestion and absorption and reach the circulation. The bioavailability of peptides depends on a variety of structural and chemical properties, i.e. resistance to proteases, charge, molecular weight, hydrogen bonding potential, hydrophobicity and the presence of specific residues [192, 253, 255]. Indeed, proline- and hydroxyproline-containing peptides are relatively resistant to degradation by digestive enzymes [41, 256, 257].

Furthermore, tripeptides containing the C-terminal proline–proline are reported to be resistant to proline-specific peptidases [73] and have been shown to be stable under simulated gastrointestinal digestion conditions [41]. As already explained in paragraph 2.5.1., peptides consisting of two or three amino acids can be absorbed intact from the intestinal lumen into the blood circulation via different mechanisms for intestinal transport [54]. The presence of the milk-derived ACE-inhibitory peptide IPP was recently demonstrated in measurable amounts in the circulation of volunteers that consumed a drink enriched in IPP and VPP [6].

Other characteristics contribute to the resistance to hydrolysis. For example, when isolated, some casein-derived peptides tend to be highly negatively charged and phosphorylated, making them resistant to further proteolysis [258]. Thus, some of the bioactive peptides could be absorbed across the intestinal mucosa to enter the circulation or be retained in the lumen and pass into the colon. The latter is likely based on evidence that ingested casein-derived phosphopeptides can be isolated from rat feces [259].

2.5.2.1. The absorption of bioactive peptides derived from milk proteins

For some bioactive tripeptides the intestinal absorption has been already demonstrated. For example, VPP was detected in the abdominal aorta of SHR 6 hours after its administration in sour milk, which strongly suggests that it is transepithelially transported [260]; more recently the absorption was observed also in humans [6]. Paracellular transport, through the intercellular junctions, was suggested as the main mechanism, since the transport via the short-peptide carrier, PepT1, led to a quick hydrolysis of the internalized peptide [186]. In the case of larger sequences, the susceptibility to brush border peptidases is the primary factor that decides the transport rate [244]. For example, the heptapeptide lactokinins (ALPMHIR) was transported intact, although in concentrations too low to exert an ACE-inhibitory activity, which suggests cleavage by aminopeptidases [261].

2.5.3. Absorption models

Early studies on peptide transport were performed in feeding or perfusion experiments *in vivo* and *in situ*. Tissue, cell and membrane preparations, such as the Ussing chamber technique (see Fig. 2.5.3.1.), the everted gut sac or ring technique (see Fig. 2.5.3.2.), and brush-border membrane vesicles, have been used for at least 50 years and are still being used today. During times, other methods have been realized to study peptide absorption and the variety of the systems can be grouped into three main groups: *in vivo*, *ex vivo* and *in vitro*. None of them is completely exhaustive and so a combination of some of them is necessary to understand if a peptide of interest can be absorbed.

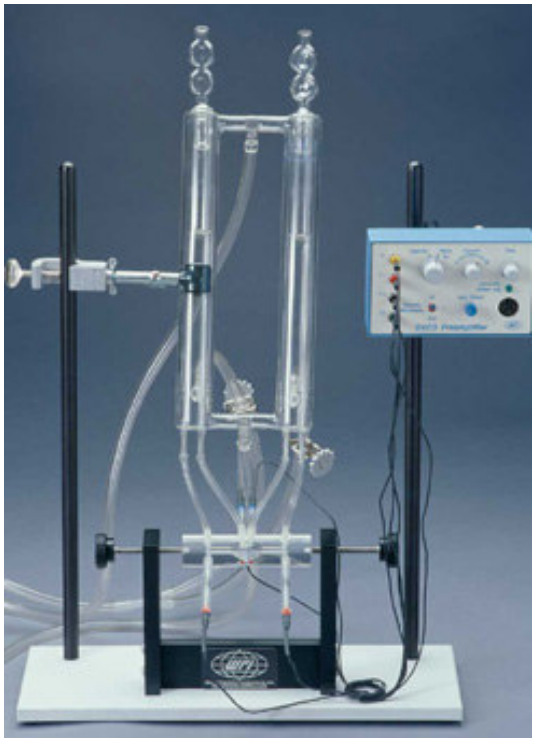

Fig. 2.5.3.1. Representation of the Ussing chamber technique, from http://www.warneronline.com/product_info.cfm?name=Introduction to Ussing Chamber and System from Warner&id=1401.

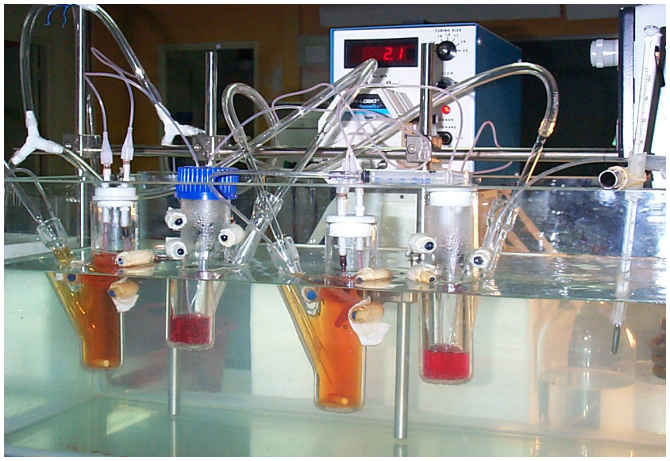

Fig. 2.5.3.2. Representation of the everted gut sac technique.

The *in vivo* methods usually evaluated the concentration of the labeled bioactive peptide of interest in the circulation and thus they permitted to understand if a peptide crosses the intestinal barrier and if it distributes in blood in enough amount to reach the target site, exhibiting its bioactivity. *In vivo* studies have been performed in humans [11, 82, 104, 105] or animals [72, 78, 79, 97-103] with a GI system supposed to be comparable to human GI system. However, only little information can be acquired about the transport mechanism at molecular level in the intestinal epithelium.

The *ex vivo* methods are a good compromise between the *in vivo* and the *in vitro* systems because they take into consideration the intestinal tissue complexity and organization, and some information on transport mechanism at molecular level can be acquired. In fact, these methodologies evaluate the absorption of the molecule of interest sampling a part of the intestinal tube. However, compared to the *in vivo* systems, they give insufficient information on the fate and the stability of the bioactive peptide, once absorbed and in the blood. In addition, they are not always so easy to perform. The *ex vivo* methods include various techniques, briefly explained here. For example, the everted gut sac (see Fig. 2.5.3.2.), that is a simple and useful model first employed to study drug transport [262]. This methodology

consists of a freshly excised small intestine incubated in appropriate tissue medium and the content of the molecule of interest is tested in the intestinal sac. The everted gut sac has been used to study the uptake of lipid vesicles [263], proteins and macromolecules with oral drug delivery potential. It is useful because it provides quantitative information on the uptake and absorption of the tested compound [264]. Another *ex vivo* technique is the *in situ* perfusion system that monitor the disappearance from the lumen of the gut segment and the measurement of plasma concentrations of the molecule following perfusion [265]. Although disappearance from the lumen in many cases may provide an adequate estimation of absorption, caution in interpretation of results from studies with this technique is warranted since an overestimation of absorption due to biotransformation, binding, and/or partitioning can occur [266].

Isolated intestinal tissues have been employed to determine uptake of oligopeptides across the apical membrane and transepithelial transport [267-270]. The major issues associated with the use of isolated tissues are the life-span of preparations and the metabolic activity of enterocytes, which often precludes transepithelial transport studies due to intracellular hydrolysis [270]. In this class of *ex vivo* absorption models The Ussing chamber technique (see Fig. 2.5.3.1.) has been applied to the study of transepithelial transport mechanisms of various compounds. Tissue preparations in Ussing chambers have been demonstrated to be viable (from both electrical measurements and transepithelial flux studies) and to maintain their integrity (based on electrical measurements and flux studies with passive permeability markers) for several hours *in vitro*. So tissue studies provide a convenient and rapid method for assessing mechanisms involved in transepithelial transport and segmental differences in these transport processes [264].

Among the *in vitro* models, the cell cultures provide a useful system for the rapid assessment of the intestinal absorption of various substances because cells are able to express the typical features of mature enterocyte.

Cell line	Species of origin	Special characteristics
Caco-2	Human colon adenocarcinoma, several clones	Most well-established cell model Differentiates and expresses some relevant transport system and enzymes Expression of enzymes and transport is variable
MDCK	MDCK epithelial cells	Polarized cells ideal for transfection
LLC-PK1	Pig kidney epithelial cells	Polarized cells with low intrinsic transporter expression, ideal for transfection
2/4/A1	Rat fetal intestine epithelial cells	Temperature-sensitive Ideal for paracellularly absorbed compounds
TC-7	Caco-2 sub clone	Similar to Caco-2
HT-29	Human colon	Contains mucus-producing goblet cells
IEC-18	Rat small intestine cell line	Provides a size-selective barrier for paracellularly transported compounds

Table 2.5.2.1. Cell culture models currently used for absorption assessment, from Balimane and Chong, 2005 [271].

Even if freshly isolated epithelial cells provide a convenient method for evaluating uptake [272, 273], they could result in loss of polarity and viability and so varieties of cell monolayer models (see Table 2.5.2.1.) that mimic *in vivo* intestinal epithelium in humans have been developed and currently enjoy widespread popularity, because of the several advantages:

- they can serve as a rapid screening tool for the absorption studies,
- they are simpler than the vascularly perfused intestinal model,
- they provide information on the absorption and transport of molecules across intestinal mucosa, an advantage over the intestinal loops and everted sacs which are more suitable for the study of molecule uptake into the mucosal cells,
- they replace other intestinal absorption models which use animals,
- they provide information on the intestinal absorption and metabolism at cellular level,

- they provide information on the possible mucosal toxicity caused by the substance of interest,
- most of them do not need interspecies correlation because of human origin.

Thus, for the peptides whose *in vitro* permeability reflects *in vivo* permeability, cell culture can be effectively used a first evaluation step.

Among the various cell lines, colon carcinoma cell lines grow relatively rapidly into confluent monolayers and exhibit a differentiated absorptive phenotype under certain culture conditions. Therefore, they have been used as a tool for studying enterocytic differentiation and function, including cell structure [274], brush border morphogenesis [275], synthesis and localization of brush border enzymes [229, 276], electrolyte transport [277] and amino acid/protein uptake [227, 278]. The most employed colon carcinoma cell lines are mostly three; HT-29, which is undifferentiated when grown under standard culture conditions and expresses enterocytic differentiation only after deprivation of glucose from the culture media [279] or addition of certain inducers [280], then the cell lines Caco-2 and T84 that spontaneously differentiate at confluence and show features of small intestinal enterocytes [274, 277, 281, 282].

Despite the advantages, a clear limitation of these systems is that intestinal segmental differences in transport cannot be discerned. In addition, the cell model composed of solely absorptive cells may be a oversimplified system, because the intestinal epithelium is a conglomerate of absorptive enterocytes and other cells such as mucous-secreting cells (the second most frequent cell type), endocrine cells, and M cells [283].

Another tool to evaluate *in vitro* the absorption of different molecules is constituted by the vesicles isolated from brush border membrane. At present, results from

membrane vesicle studies have not been consistent because of the extreme variability during vesicles preparation and the inability to monitor the internal milieu prior to experiment. For example, this experimental issue was observed for the pH dependent overshoot phenomena of oligopeptide transport that has been demonstrated in some but not all studies [284]. The “leakiness” of vesicle preparations or lack of appropriate conditions at the time of the experiments may also account in part for the differences observed.

2.5.3.1. The Caco-2 cell line model

An accepted model system for the enterocyte of the human small intestine is the Caco-2 cell line (Fig. 2.5.3.1.1.), as - with ongoing differentiation - this colon carcinoma cells exhibit morphological and functional similarities to non-malignant human enterocytes (as cell polarization, expression of brush border enzymes, formation of tight junctions (TJ), the microvillous structure, the carrier-mediated transport system for di- and tri-peptides and amino acids PepT1) [285].

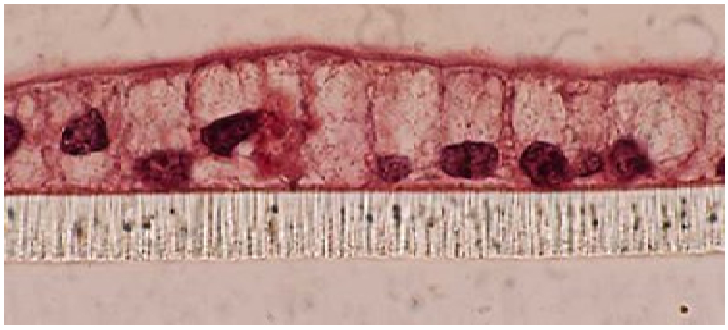

Fig. 2.5.3.1.1. Caco-2 cell monolayer, from www.fi.cnr.it/r&f/n4/images/spadoni.jpg

The TJ which regulates the paracellular transport of the cell monolayer has also been expressed in Caco-2 cell monolayers cultured on a semipermeable filter. In addition, transcytotic activity has also been observed in Caco-2 cells.

TJ function of Caco-2 cells can be determined and monitored by the measurement of TransEpithelial Electrical Resistance (TEER) using a two compartment cell culture system, separating the upper (apical) part of the epithelium from a

basolateral compartment mimicking the cellular sites facing blood circulation [195]. One main advantage of the TEER assay is that it is non-destructive and changes in TEER and consequently TJ permeability can be monitored over a long period of time [286]. Fig. 2.5.2.1.2. describes the measurement in Caco-2 cell.

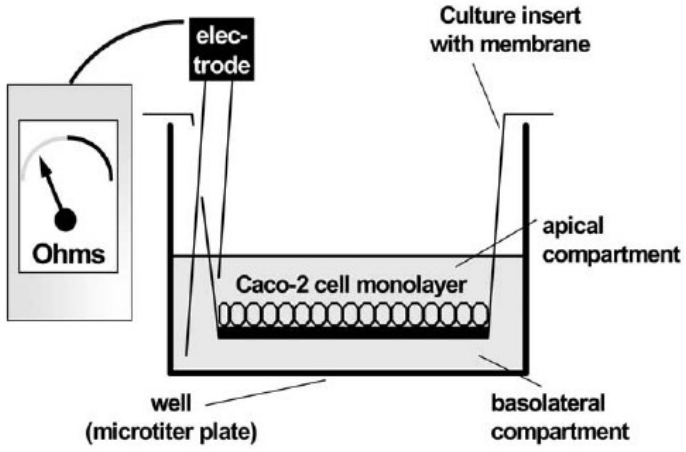

Fig. 2.5.2.1.2. Transepithelial electrical resistance principle of measurement, from Hartmann et al, 2007 [195].

Integrity of cultured monolayers is also detectable by carrying out transport studies using water-soluble reference compounds that can be absorbed by TJ channels between the cells (e.g. radio-labeled mannitol, phenol red, Lucifer Yellow, or flourescein; Mr: 182, 354, 57, 332 Da, respectively). Quantification is then made by detecting the reference substance in the basolateral compartment [287].

Pure Caco-2 cell system shows some limitations, as the potential overexpression of the P-glycoprotein (P-gp), which may lead to higher excretion rates of the tested molecules (i. e. the bioactive peptide of interest) and consequently lower permeabilities in the absorptive direction [288]. In addition, because of the absence of a prominent mucus layer on the surface of Caco-2 cell monolayers produced i*n vivo* by goblet cells, the apical pH will mainly be determined by the culture medium [289], normally fixed at pH 7.4.

A better prediction of the absorption could be gained, if the apical pH is 5.5–6.5 and this can be achieved without compromising the integrity of Caco-2 cell monolayers, as demonstrated by Palm and colleagues [289] and Yamashita and colleagues [290]. The change in pH has been evaluated in permeability studies for passively permeated drugs [291] and the authors found that Caco-2 cells better mimicked the *in vivo* conditions and gave more reliable information about the absorption of drugs across the enterocytic membrane.

Finally, it is well known [292] that permeabilities of compounds that are transported via carrier-mediated absorption are lower in the Caco-2 cell system as compared to the human small intestine, probably also reflecting the colonic origin of this cell line. In recent years several mucus-producing goblet cell sublines have been established from human intestinal HT29 cells, as HT29-MTX [293-295], a cell population that consists exclusively of differentiated, gastric-like mucus secreting, goblet-type cells that retain their differentiated phenotype after reversion to a methotrexate (MTX)-free medium and they also can be grown in monolayers.

EXPERIMENT 1: Fermented milk from *Enterococcus faecalis* TH563 or *Lactobacillus delbrueckii bulgaricus* LA2 manifests different degrees of ACE-inhibitory and immunomodulatory activities

3.1. Introduction

There is evidence that several food or food ingredients provide a benefit beyond the nutrients they contain. These substances are defined as functional food and their putative biological effects have been extensively studied. To date, antihypertensive and immunomodulatory bioactivities are frequently exploited in the production of foodstuffs formulated to provide putative health benefits [9, 296].

The bioactive properties of fermented milks are often correlated to the generation of specific peptides from milk proteins. The bioactive peptides are inactive when encripted in the sequence of the precursor proteins but can be released by enzymatic proteolysis during intestinal digestion or food processing [7]. Interestingly, Angiotensin-I Converting Enzyme (ACE) inhibitory and immunomodulatory properties seem to be associated, possibly because both are correlated to the presence of short chain peptides [65].

So far, lactic acid bacteria have been preferred to others microorganisms to produce fermented milks rich in ACE-inhibitory activity [40], in particular *Lactobacillus helveticus* (*L. helveticus*) [12, 86], *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. delb. bulgaricus*) and *Lactococcus lactis* subsp. *cremoris* (*L. lactis cremoris*) [29]. Moreover, some bacterial strains, mostly lactic acid bacteria, release components during fermentation that possess immunomodulatory activity [4, 7]. Lactic acid bacteria fermentation products potentiate the cell-mediated immune response by increasing the proliferative response of lymphocytes to concanavalin A (conA), a known activator of lymphocyte proliferation [297]. In addition, some findings suggest that milk fermented by *Lactobacillus* strains can modulate the immune response

against breast cancer cells in mice [4] and improve innate-defense capacity in human [7].

However, species other than those belonging to *Lactobacillus* genus are often isolated from dairy products, which may possess interesting properties [41, 188]

The aim of our study was to measure the ACE-inhibitory and immunomodulatory bioactivities in milk fermented with *Enterococcus faecalis* TH563 and compare them to those generated by *L. delb. bulgaricus* LA2. These strains belong to a panel of 14 bacterial strains (7 *L. delb. lactis*, 2 *L. delb. bulgaricus*, 1 *L. helveticus*, 2 *L. paracasei* and 2 *E. faecalis*) representing species that are frequently isolated from traditional dairy products of North Eastern Italy and showing different degrees of proteolytic activity. The focus of the present study in *E. faecalis* was because it is an enterococcal species frequently found in dairy products, traditional cheeses in particular, where it may play an important role in determining chese taste and texture [298, 299]. Altough *E. faecalis* is reported to generate fermented milk with ACE-inhibitory activity [79, 112, 300, 301] few information about its ability to generate immunomodulatory activity is available. On the contrary, *L. delb. bulgaricus* is commonly used as the starter culture for the production of yogurt and fermented milks, and it may represent a fairly well known control.

3.2. Materials and Methods

3.2.1. Chemicals and Reagents

Hank's balanced salt solution (HBSS) was purchased from Lonza, Switzerland. Gibco-Invitrogen (United Kindom) supplied L-Glutamine (L-Glu). Lymphoprep was purchased from Axis-Shield, Norway. Sigma–Aldrich (Italy) supplied Angiotensin-converting enzyme (ACE), concanavalin A (conA), ethyl acetate, hippuryl-histidyl-leucine (HHL), HCl, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) powder, new-born calf serum (NCS), NaOH, penicillin-streptomycin solution,

RPMI-1640 medium, and Triton X100. Na borate buffer and NaCl were obtained from Carlo Erba, Italy. MRS broth and sterilized skim milk were supplied by Biolife, Italy and M17 broth by Difco Laboratories, Michigan, USA.

3.2.2. Bacteria culture

E. faecalis TH563 and *L. delb. bulgaricus* LA2 were evaluated for their proteolytic activity as described by Hull [302] and in accordance with International Dairy Federation (IDF) standard 149A (1997) [303].

Lactobacilli were propagated in MRS broth for 24 h at 44 °C, while enterococci were propagated in M17 broth for 24 h at 37 °C. Revitalized microorganisms were used to inoculate (1%, v/v) 10 mL of sterilised skim milk, which was incubated for 24 h at 44°C (lactobacilli) and 37 °C (enterococci). One mL of these milk pre-cultures was used to inoculate 100 mL of skim milk. Incubation was carried out under sterile conditions at 44 °C (lactobacilli) and 37 °C (enterococci). Fermented milk was produced with skim milk under sterile conditions in order to avoid the presence of enzyme interference by contaminating microorganisms.

3.2.3. Separation of the peptide fraction

Fermented milk samples were centrifuged at 20000 × *g* for 15 min at 15 °C (J2-21 Beckman Coulter centrifuge, JA 20 rotor, Fullerton, California, USA) to remove bacteria debris. The supernatant was filtered with Amicon Centricon Ultra15 (molecular weight cut-off 5000 Da; Millipore, Billerica, Massachusetts, USA) by centrifugation at 3200 × *g* for 40 min at 15° C. The fraction with molecular weight lower than 5000 Da (5000 Da fraction) was stored at -20 °C and used for further analyses. The concentration of peptides in the 5000 Da fractions was spectrophotometrically determined by the method of Layne [304].

3.2.4. ACE-inhibitory activity

The ACE-inhibitory activity of the 5000 Da fractions was measured by the method of Cushman and Cheung [91], as modified by Nakamura and colleagues [86]. An Ultrospec 3000 spectrophotometer (Amersham Pharmacia Biotech, New Jersey, USA) was used to measure the optical density of each 5000 Da fraction.

Each test was performed in triplicates and the measured absorbance was used for the calculation of the percentage of ACE inhibition (% ACE-I) as follows:

$$\% \text{ ACE-I} = 100 \cdot (B - A) \div (B - C),$$

where A is the optical density of the samples in the presence of ACE, B is the optical density of the total activity, and C is the optical density of the blank. Data underwent analysis of variance and differences between mean values were analysed by the test of Duncan (SPSS Inc., Chicago, Illinois, USA).

3.2.5. Bovine peripheral blood lymphocytes proliferation

Ten mL of 5000 Da fraction of fermented milk by *E. faecalis* TH563 and 30 mL of 5000 Da fraction of fermented milk by *L. delb. bulgaricus* LA2 were dried under vacuum and the obtained powders were dissolved in 5 mL of complete medium prepared as follow: RPMI-1640 medium containing 10 % of NCS, 2 mmol/L of L-Glu, 100 µg/mL of streptomycin and of 100 U/mL of penicillin. The concentration of peptides in the 5000 Da fraction for the proliferation test was determined spectrophotometrically as described by Layne [304]. The 5000 Da fractions were sterilized by filtration (0.22 µm filters) and stored at -20 °C until use.

Bovine peripheral blood lymphocytes (BPBL) were isolated from whole heparin-anticoagulated blood of nine non-pregnant, non-lactating dairy cows without clinical symptoms by density gradient centrifugation using the Lymphoprep reagent. Cells were suspended in completed medium in the presence of 2 µg/mL of conA as mitogen and were incubated at 37 °C in 5% CO_2. After 24 h of differentiation, non

adherent BPBL were separated from adherent leukocytes and tested for viability with Trypan blue staining. Viable BPBL were adjusted at density of $3 \cdot 10^6$ cells/mL in complete medium and incubated for 48 h in a 96-well microplate (100 µL cell suspension per well) with or without conA (2 µg/mL, positive control) and in presence of increasing concentrations (from 0 µg/mL to 100 µg/mL) of each fermented milk. At the end of the incubation period, proliferation test was assessed by 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) proliferation test, following the manufacturer's instructions. Briefly, MTT powder was dissolved in Hanks' balanced salt solution (5 mg/mL), added to the cells (15 µL per well) and incubated for 3 h to allow the reductases of living cells to convert the MTT into the insoluble formazan. The formazan was then eluted with 10% (v/v) Triton X100 and the absorbance was measured at a wavelength of 570 nm with background subtraction at 630 nm using a microplate reader (Spectra Count, Packard Bioscience).

Each cell proliferation test was performed in triplicates. The results were expressed as the percentage of the optical density observed in the conA-treated BPBL (% conA). Relative variations of cellular proliferation produced by each fermented milk were analysed using a Generalised Linear Model (GLM, SPSS Inc.). Differences between mean values were analysed by the test of Dunnett (SPSS Inc.).

3.3. Results

E. faecalis TH563 and *L. delb. bulgaricus* LA2 showed a proteolytic activity of 0.292 and 0.100 mg of tyrosine/mL, respectively. The peptide concentration in the 5000 Da fraction was greater in milk fermented by *E. faecalis* TH563 than in milk fermented by *L. delb. bulgaricus* LA2 (14.78 mg/mL and 4.89 mg/mL, respectively).

Milk fermented by *E. faecalis* TH563 showed a significantly ($P < 0.05$) higher ACE-inhibitory activity (69.43 % ± 3.12) than *L. delb. bulgaricus* LA2 (60.86 % ± 1.01).

The persistency of high ACE-inhibitory values up to 1:50 dilution for *E. faecalis* TH563 indicated an enzyme saturation effect that disappeared at 1:100 dilution. On the contrary, ACE-inhibitory activity in milk fermented by *L. delb. bulgaricus* LA2 was significantly reduced to very low levels when the 5000 Da fraction was diluted 10-folds ($P < 0.05$) (Fig. 3.3.1.).

The peptide concentration in the samples for MTT was 30.43 mg/mL and 37.72 mg/mL for *E. faecalis* TH563 and *L. delb. bulgaricus* LA2, respectively.

The 5000 Da fraction obtained from the milk fermented by *E. faecalis* TH563 did not significantly affect BPBL proliferation either with or without the mitogen conA (Fig. 3.3.2A.). The 5000 Da fraction obtained from the milk fermented by *L. delb. bulgaricus* LA2 was able to decrease the conA-induced BPBL proliferation when added at 5 µg/mL ($P < 0.001$), and at 25 µg/mL and at 50 µg/mL ($P < 0.01$) peptide concentration (Fig. 3.3.2B.), but not at 100 µg/mL. At this concentration other factors might be present in a sufficent concentration to counteract the inhibitory effect on BPBL proliferation. Moreover, this fermented milk administered without conA did not significantly influence BPBL proliferation, even if a slight increase in BPBL proliferation was observed at peptide concentration of 5 µg/mL (Fig. 3.3.2B.).

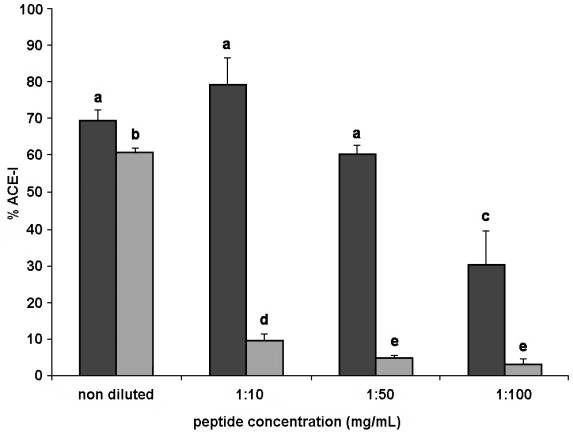

Fig. 3.3.1. ACE-inhibitory activity of the 5000 Da fraction obtained after Amicon Ultra15 filtration of fermented milks. ACE-inhibitory activity was expressed as the percentage of ACE inhibition (% ACE-I). Milk fermented by *E. faecalis* TH563 (dark grey bars) showed a higher ACE-inhibitory activity if compared to *L. delb. bulgaricus* LA2 (light grey bars). Results are presented as means ± SEM of 3 independent experiments. Different superscripts indicate statistically different means ($P < 0.05$; Duncan test).

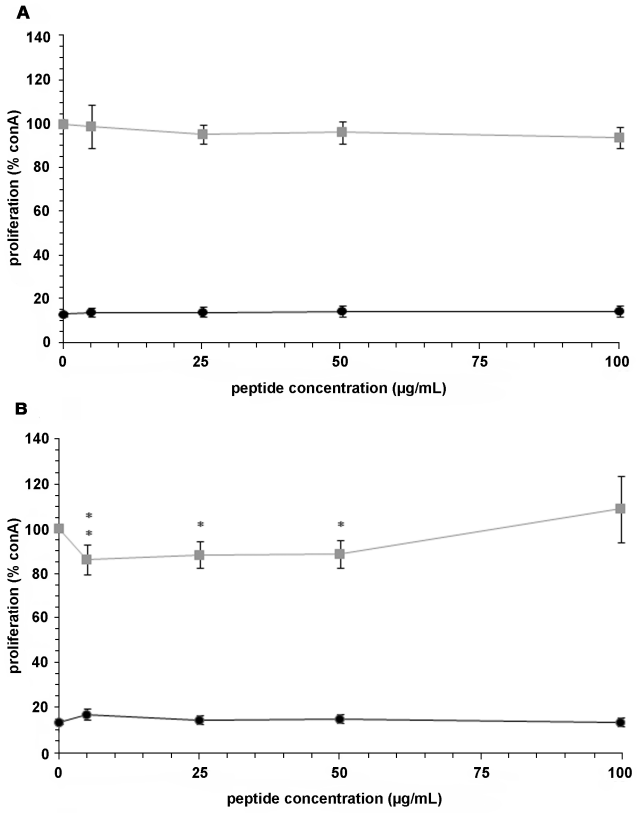

Fig. 3.3.2. Dose-response effect of 5000 Da fraction obtained from milk fermented by *Enterococcus faecalis* TH563 (A) or *Lactobacillus delbrueckii bulgaricus* LA2 (B) on cellular proliferation assayed by 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT) proliferation test, in presence (■) or absence (●) of the mitogen conA. The data were expressed as the percentage of the optical density observed in conA-treated bovine peripheral blood lymphocytes cultured without fermented milk but in presence of conA (positive control). Results are presented as means ±SEM of 9 independent experiments for each strain. Asterisks indicate means significantly different from the positive control (* $P > 0.01$; ** $P < 0.001$; Dunnett test).

3.4. Discussion

In the present study, ACE-inhibitory and immunomodulatory activities of milk fermented by two different bacterial strains, *E. faecalis* TH563 or *L. delb. bulgaricus* LA2, were compared.

A different ACE-inhibitory activity was observed between the two bacterial strains, and the highest value was measured in milk fermented by *E. faecalis* TH563. *E. faecalis* is not usually employed in the production of dairy food, since some strains can harbour potential virulence factors or antibiotic resistance [305]. However, it is

frequently found in traditional cheeses, where it plays an important role in determining cheese taste and texture [298]. Even if strains of *E. faecalis* have been reported to possess high proteolytic activity [299], the ability to produce fermented milks with ACE-inhibitory activity has been scarcerly documented [112, 301]. In the present experiment, ACE inhibitory activity seemed to be positively related to the proteolytic activity of the strain of interest. In fact, *E. faecalis* TH563 showed the highest proteolytic activity and the highest peptide concentration in the 5000 Da fraction, suggesting potentially greater ability to produce small peptides, which are the main responsible of ACE inhibitory activity [306] .

In this experiment, ACE-inhibitory and immunomodulatory activities were not associated, differently from the assumption of Narva and colleagues [65]. In fact, *E. faecalis* TH563 did not alter BPBL proliferation, while *L. delb. bulgaricus* LA2 slightly but significantly inhibited BPBL proliferation at low concentrations in presence of conA. Both bacterial strains could not affect proliferation of BPBL keep in culture without conA. This result supports the hypothesis of Fujiwara and colleagues [307] suggesting that immunomodulatory activity is essentially expressed by strains of lactobacilli.

It is difficult to explain how fermented milks could modulate the cells of the immune system and it is even more complicated to identify specific components produced during milk fermentation responsible for these immunomodulatory activities. Fermented milks are complex matrices, rich not only in proteins and peptides but also in sugars, fat, minerals and polysaccharides of the bacterial membrane that can contribute to the whole immunomodulatory effect. On this regard, it was demonstrated that milk fatty acids produced during fermentation affect cellular proliferation [308].

The preliminary results of our work suggest the possibility to use *E. faecalis* strains to produce fermented milk with ACE-inhibitory activity. However, it would be necessary to evaluate *E. faecalis* strains for safety aspects because their presence

in food system is still a matter of controversy due to their pathogenic potential [309]. *E. faecalis* TH563 does not carry vanA or vanB genetic determinants for vancomycin transferable antibiotic resistance [298], but in order to completely assess its safety as adjunct culture in fermented milk, the strain should be tested for the absence of other potential virulence factors such as haemolysin, aggregation substances, surface proteins ace and esp [298]. Finally, it would be interestingly to evaluate if milk fermented with both *E. faecalis* TH563 and *L. delb. bulgaricus* LA2 as mixed culture could generate a fermented milk showing both ACE-inhibitory and immunomodulatory activities.

3.5. Take-home message

Enterococci are a widely distributed group of bacteria belonging to LAB. The present work demonstrated that the strain *E. faecalis* TH563 produced a fermented milk enriched in ACE-inhibitory activity. In addition, this work demonstrated that *E. faecalis* TH563 manifested an elevate proteolytic activity. It is thus possible to hypothetize a relation between the ACE-inhibitory activity and the ability of *E. faecalis* TH563 strain to efficiently convert proteins into peptides.

The relation between ACE-inhibitory activity and the production of peptides during milk fermentation has been already explored by Nielsen and colleagues [300] on 13 strains belonging to the *genus Lactobacillus*, the *genus Lactococcus* and the *genus Streptococcus*. The authors demonstrated that the highest ACE-inhibitory activity value was obtained by the most proteolytic strains evaluated in the study.

Even if the link between the proteolytic activity and ACE-inhibitory activity has been investigated, little is known for other bioactivities carried by peptides. In the present work the preliminary results obtained from *E. faecalis* TH563 and *L. delb. bulgaricus* LA2 did not seem to highlight that immunomodulatory activity on BPBL is related to proteolytic activity.

At present, scarce attempt has been made to study this relation. It would thus be interesting to extend the investigation of the proteolytic and imunomodulatory activities on a large panel of bacterial strains isolated from dairy products. This investigation would also be helpful to explore the correlation between ACE-inhibitory and immunomodulatory activities.

At the moment, only Narva and colleagues [65] and Huttunen and colleagues [310] studied the multifunctional properties of the bioactive peptides IPP and VPP, two well characterized peptides derived from milk proteins, in particular on bone cells *in vitro*, but no data is available on immunomodulatory activity of these peptides that could be potentially produced during milk fermentation, in particular by the strains *E. faecalis* TH563 and *L. delb. bulgaricus* LA2.

EXPERIMENT 2: Effects of YGG on (concanavalin a-induced) proliferation and IL2 and INFγ expression of bovine peripheral blood lymphocytes

4.1. Introduction

There is increasing evidence that proteolytic cleavage gives rise to hidden peptides with bioactive properties that often cannot be predicted and are totally distinct from the parent protein. The liberation of these protein fragments has been shown to be prevalent in proteins associated with endocrine signaling, the extracellular matrix, the complement cascade and milk. This phenomenon may represent an important mechanism for increasing diversity of protein function [311].

A number of potentially immunoregulatory peptides are encrypted in bovine caseins [132-136], and whey proteins [137, 138], which can manifest different effects. Some casein-derived peptides (residues 54-59 of human β-casein and residues 194-199 of α_{s1}-casein) can stimulate phagocytosis of sheep red blood cells by murine peritoneal macrophages [135, 139]. Other fragments (fragment 18-20 of κ-casein, fragment 90-96 of α_{s1}-casein) can either stimulate or inhibit lymphocyte proliferation depending upon their concentration [134, 143], while some whey-derived peptides can affect cytokine production from leucocytes [137, 138].

The peptide YGG represents an interesting example of cryptic peptide with putative immuno-modulating effects, as it can originate from at least two different sources. First of all, it originates from the hydrolysis of Leu-enkephalin and Met-enkephalin [181], and thus it is an endogenous peptide. In addition, it can be considered as a potential nutraceutical, because it is also encrypted in milk proteins and can be released during the digestion of bovine milk, in particular from α-lactalbumin [31, 134].

It is known that Met-enkephalins, the YGG endogenous progenitor, can enhance human T cell proliferation and IL2 production *in vitro* in the absence of mitogen,

possibly through the activation of opioid receptors present on the cell surface [183]. The enhancement of human peripheral blood lymphocytes proliferation and protein synthesis *in vitro* was obtained also with YGG administration in presence of conA [134]. In addition, it was observed that YGG can affect INFγ and IL2 secretion in murine splenocytes stimulated with suboptimal concentration of conA in serum-free medium [157].

Stimulatory effects on cell proliferation were observed also in leukocytes obtained from mice administrated *in vivo* with either Met-enkephalin or YGG, suggesting that Met-enkephalin effects on the immune cells are mediated by YGG [185]. More recently, the immunomodulatory effect of YGG was confirmed *in vivo* by the observation that the peptide administration modulated the delayed-type hypersensitivity responses to tuberculin derivatives in hairless guinea pigs [154]. It is noteworthy to observe that YGG seems to have a biphasic effect on the parameters studied so far, as it showed an enhancing effect at low doses and an inhibitory effect at higher doses [154, 157].

It is important to consider that the experimental conditions can affect the immune-response to the peptides. In particular, when lymphocytes are stimulated *in vitro*, the culture conditions may significantly affect the cellular response [312].

The aim of this work was to use bovine peripheral blood lymphocytes (BPBL) to study the effects of the peptide YGG on lymphocyte proliferation and the quantitative expression of IL2 and INFγ. In particular, this work aimed to study the effects of the concentration of newborn calf serum (NCS). NCS is currently used for lymphocyte culture, but it is rich in growth factors of various nature and its use could influence the cellular biology [312, 313] masking the effects of the immunomodulatory peptides under study.

4.2. Materials and Methods

4.2.1. Chemicals and Reagents

Hank's balanced salt solution (HBSS) was purchased from Lonza, Switzerland. Gibco-Invitrogen (United Kindom) supplied all the RNA-extraction reagents, the pCR 2.1 plasmid TA cloning kit, L-Glutamine (L-Glu), and all the PCR reagents, with the exception of the Power SyBRGreen PCR Master Mix that was obtained from Applied Biosystems (California, USA) and reverse and forward primers for PCR amplification of IL2 and INFγ that were supplied from Eurofins MWG Operon, Germany. QIApre Spin Miniprep kit was obtained from QIAGEN GmbH, Germany.

Lymphoprep was purchased from Axis-Shield, Norway. Sigma–Aldrich, (Italy) supplied concanavalin A (conA), HCl, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) powder, new-born calf serum (NCS), penicillin-streptomycin solution, RPMI-1640 medium, and Triton X100. Synthetic Tyr-Gly-Gly (YGG) peptide was produced by GenScript Corp., New York, USA.

4.2.2. BPBL Harvesting and Propagation

The procedure for BPBL harvesting and propagation used for the present study followed the protocol already described at the beginning of the Paragraph 3.2.5. “Bovine Peripheral Blood Lymphocytes proliferation”.

After the separation of non-adherent BPBL from adherent leucocytes the viable cells were re-suspended in essential medium with either 10% NCS or 2.5% NCS and used as described below.

4.2.3. Part 1: BPBL proliferation

This experiment was performed to study the effects of YGG on conA-stimulated BPBL proliferation at different NCS concentrations.

Viable BPBL were divided in two aliquots and adjusted at concentration of $3*10^6$ cell/mL in either essential medium with either 2.5% NCS or 10% NCS. The cells (100 µL cell suspension/well) were incubated for 48 h in a 96-well microplate (Corning Incorporated, New York, USA) with increasing concentrations (0 – 1 mmol/L) of synthetic YGG with or without conA (2 µg/mL). The incubation in essential medium with 10% NCS and conA, and without YGG was considered as the positive control for BPBL proliferation. At the end of the incubation period, cell proliferation was measured by MTT assay, as previously described at the end of Paragraph 3.2.5. "Bovine Peripheral Blood Lymphocytes proliferation".

The experiment was independently repeated using BPBL obtained from 6 animals, and each assay was performed in triplicate.

4.2.4. Part 2: IL2 and INFγ gene expression

This experiment was performed to compare the effects of YGG and conA on the expression of INFγ and IL2 genes at different NCS concentrations.

The experiment was independently repeated using BPBL obtained from 6 animals and each assay was performed in triplicates.

BPBL were re-suspended at $3*10^6$ cell/mL in essential medium either in 2.5 % NCS or 10% NCS, and dispersed in a 6-well plate (3 mL/well, Corning Incorporated). Then BPBL were incubated for 48 h at 37 °C in 5% CO_2 in essential medium added with NCS either alone, or with 2 µg/mL conA, or with YGG (0.1 mmol/L). BPBL cultured in essential medium with 10% NCS and without YGG and conA were considered as the reference culture conditions.

At the end of the incubation period, total RNA was extracted from the cultured cells using 1 mL Trizol Reagent®, according to the manufacturer's instructions. RNA concentration was determined measuring the absorbance at 260 nm in Ultrospec 3000 spectrophotometer (Amersham Pharmacia Biotech, New Jersey, USA). The integrity of the RNA was evaluated by electrophoresis in 2% agarose-gel stained

with ethidium bromide (0.2 µg/mL). Two µg of RNA were treated with DNAse I and reverse transcribed into cDNA with Superscript II enzyme, according to the manufacturer's instructions. The obtained cDNAs was used in absolute and relative quantitative PCR, using an ABI 7500 Real-Time PCR System (Applied Biosystems, California, USA). Absolute quantification of IL2 and INFγ transcripts was calculated within basal condition samples. Standard curves were created by amplification of serial dilutions (from $1:10^{-1}$ to $1:10^{-6}$) of IL2 and INFγ plasmids. cDNAs were amplified in 20 µL PCR mixtures containing the following final concentrations: 1X Taq Polymerase buffer, 1.5 mmol/L $MgCl_2$, dNTPs mixture 0.2 mmol/L each, 500 nmol/L forward and reverse primer (Table 4.2.4.1.), and 0.5 U of Taq DNA polymerase. Amplifications were performed in a Eppendorf Mastercycler Personal Thermal cycler (Eppendorf, New York, USA) by 32 cycles of denaturation at 94 ℃ for 45 seconds, annealing at 52 ℃ (IL2) or 57 ℃ (INFγ) for 45 seconds, extension at 72 ℃ for 45 seconds. The PCR products were cloned in pCR 2.1 plasmid under TA cloning kit conditions. The two plasmids were purified by QIApre Spin Miniprep kit and quantified at 260 nm with in Ultrospec 3000 spectrophotometer (Amersham Pharmacia Biotech). Absolute and relative Real Time PCR were performed in a mixture containing: 1X Power SyBRGreen PCR Master Mix, 300 nmol/L forward and reverse primer, and under the following PCR conditions: 2 minutes at 50 ℃, 10 minutes at 95 ℃, and 40 cycles with 95 ℃ for 15 seconds and 60 ℃ for 1 minute. The absolute quantity of unknown samples (x) was calculated with the equation y = bx+c, where y is the Ct value (threshold cycle), c is the y-axis intercept and b the slope of standard curve. Relative mRNA expression of target genes INFγ and IL2 was calculated with the comparative CT method ($2^{-\Delta\Delta CT}$) [314, 315]. The amount of target genes were normalized to the β-actin gene, chosen as endogenous control (primers sequence shown in Table 4.2.4.1.). Quantitative analysis of IL2 and INFγ expression was done in triplicates. For both IL2 and INFγ, the relative expression analysis was normalized in that measured in the BPBL sample obtained from one of

the six cows and cultured in the essential medium alone, considered as expression level 1.

Gene	PCR product (bp)	Primers
IL2	100	For 5' TGCTGCTGGAATTTACAGTTGCT 3' Rev 5' TTAACCTTGGGCGCGTAAAA 3'
INFγ	103	For 5' CTGCTCTGTGGGCTTTTGG 3' Rev 5' CATCTGGGCTACTTGCATTAAAATAC 3'
β-actin	183	For 5' CCATCTATGAGGGTCACGCGC 3' Rev 5' TTCTCAAAGTCCAAGGCCACGTA 3'

Table 4.2.4.1. Primers used for the qualitative PCR and the real-time RT-PCR; bp: basepairs.

4.2.5. Data analysis

Within each cow, BPBL proliferation obtained in the different culture conditions was expressed as the percentage of the maximum absorbance observed in the positive control (essential medium with 10% NCS and 2 µg/mL conA). Data recorded at each culture condition were compared with the positive control by the test of Mann-Whitney.

The test of Mann-Whitney was used also to compare the quantitative cytokine expression in BPBL in the different culture conditions with the reference culture condition (essential medium with 10% NCS).

As the cytokine expression in the BPBL obtained from the 6 animals was greatly variable in quantity, the deviations of cytokine expression from the reference culture condition were analyzed within each animal. Responses were classified as "increased" (if the expression of the cytokine was 50% greater than the reference condition), "unchanged" (if the expression of the cytokine varied less than ±50% than the reference condition), and "decreased" (if the expression of the cytokine was 50% lower than the reference condition). The distribution of the responses was studied by the Pearson's χ^2-test.

All data were analyzed by SPSS 15.0 (SPSS Inc.), and the level of statistical significance was set at $P < 0.05$.

4.3. Results

4.3.1. BPBL proliferation

BPBL proliferation measured by the MTT proliferation assay is shown in Fig. 4.3.1.1.. The maximum proliferation was observed when cells were cultured in presence of conA without YGG (Fig. 4.3.1.1., A & C). In this condition, cell proliferation was not affected by the NCS concentration in the culture medium. Conversely, when cell culture was carried out without both conA and YGG (Fig. 4.3.1.1., B & D), proliferation was significantly lower in 10% NCS than 2.5% NCS ($P < 0.05$). An inhibitory effect of YGG on BPBL proliferation was observed only in presence of conA, and the response was affected also by the NCS concentration in the culture medium. If BPBL were cultured in 10% NCS, a significant decrease in proliferation was observed at YGG concentration of 1 mmol/L ($P < 0.05$; Fig. 4.3.1.1. A). On the other hand, when YGG was administered in presence of 2.5% NCS, a significant inhibitory effect was already present at YGG concentration of 10^{-12} mol/L ($P < 0.05$; Fig. 4.3.1.1. C).

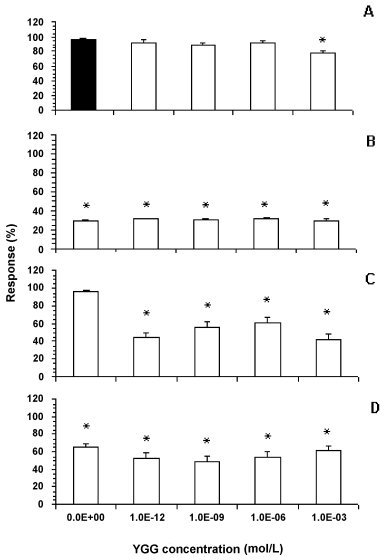

Fig. 4.3.1.1. Effect of synthetic YGG on BPBL proliferation in different culture conditions (A: 10% NCS with conA; B: 10% NCS without conA; C: 2.5% NCS with conA; D: 2.5% NCS without conA). Data (mean ±SEM) are expressed as the percentage of the maximum absorbance observed and compared with the positive control (black box) by the test of Mann-Whitney (SPSS Inc.; * $P < 0.05$).

4.3.2. IL2 and INFγ gene expression

Quantitative cytokine expression is shown in Fig. 4.3.2.1.. The administration of conA significantly enhanced IL2 expression in comparison with the reference condition ($P < 0.05$) in BPBL cultured in either 2.5% and 10% NCS. The administration of YGG did not affect IL2 mRNA concentration. INFγ expression showed the same pattern of response, even though no significant differences in comparison with the reference culture condition could be observed, possibly due to the great variability of mRNA concentration observed between animals, which was particularly high when lymphocytes were cultured in 10% NCS and conA. The similar trend of IL2 and INFγ expression in response to the culture conditions was confirmed by the significant correlation observed between the mRNA concentration of the two cytokines ($r^2 = 0.953$, $P < 0.01$). INFγ expression was approximately 100-folds greater than IL2 expression, and a great between animal variability was observed in the response in both cytokines.

As mRNA concentration was very variable between animals, the responses of BPBL within each cow to the different culture conditions were compared with the response to the reference condition (essential medium with 10% NCS), and results are reported in Table 4.3.2.1.. The administration of both YGG and conA in presence of 10% NCS induced an IL2 mRNA increase in 5 cows, while the cytokine expression decreased in one cow in both culture conditions ($P < 0.05$). Conversely, the distribution was random when cells were cultured in 2.5% NCS. Although the different culture conditions altered INFγ expression in comparison with the reference condition, the response was highly variable between individual cows.

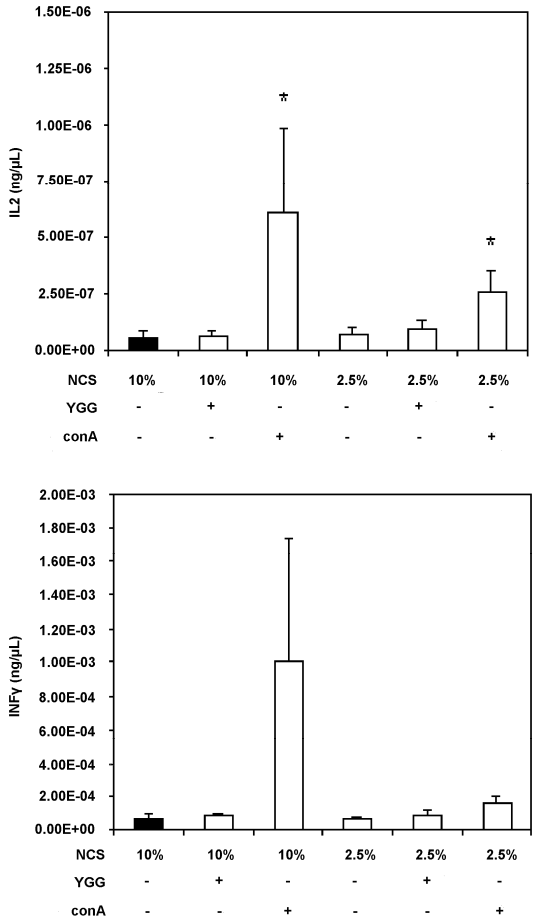

Fig. 4.3.2.1. Quantitative expression (mean ± SEM) of IL2 and INFγ mRNA in response to YGG (0.1 mmol/L) or conA (2 µg/mL). Asterisks indicate means significantly different from the reference culture condition (black box; Mann-Whitney test; SPSS Inc.; $P < 0.05$).

	IL2 response (N)				INFγ response (N)			
Culture conditions	**Incr**	**Unch**	**Decr**	**P**	**Incr**	**Unch**	**Decr**	**P**
NCS 10% (RC)	0	6	0	--	0	6	0	--
NCS 10% + YGG	5	0	1		2	3	1	
NCS 10% + conA	5	0	1		4	2	0	
NCS 2.5%	2	3	1		1	5	0	
NCS 2.5% + YGG	4	1	1		3	1	2	
NCS 2.5% + conA	4	0	2		4	0	2	

Table 4.3.2.1. Effects of the culture conditions on IL2 and INFγ expression in the individual cows in comparison with the reference culture condition (NCS 10%, no conA or YGG added). YGG and conA were used at concentrations of 0.1 mol/L and 2 µg/mL, respectively. When the Pearson's $\chi 2$ test in a row was significant, the observed frequencies were not casually distributed and an effect attributable to the culture condition could be postulated. RC: reference condition; Incr: increased; Unch: unchanged; Decr: decreased.

4.4. Discussion

In this work, the possibility to use the bovine as an alternative source of lymphocytes has been explored to develop a bioassay to test immunoactive peptides, as blood samples from animals slaughtered for commercial meat production are available in practically unlimited amounts. This would allow avoiding the use of laboratory animals and overcoming the need to recruit human volunteers.

As YGG and YG are immunoactive peptides resulting from both enkephalin [154, 185] and milk protein [134] cleavage, they may represent an interesting model for functional immunoactive peptides of food origin, providing that they can be released during digestion and cross the intestinal barrier. The release of YGG from enkephalins can be achieved by the action of enzymes such as aminopeptidase N (EC 3.4.11.2), peptidyl-dipeptidase A (EC 3.4.15.1) and endopeptidase 24.11 (EC 3.4.24.11), which can be secreted by or are associated to immune cells and other tissues [316, 317]. Interestingly, those enzymes are expressed in the brush border surface of the human and rat enterocytes [191, 318], suggesting that YGG encrypted in milk proteins could be released. Furthermore, several studies revealed optimistic perspectives about the intestinal absorption of small peptides, in particular di- and tri-peptides, as there are indications that they can escape from the action of brush-border and cytoplasmic peptidases [186, 201, 244, 319-321], and their transepithelial transport may be achieved by carrier-mediated transport through PepT1 [322] or by paracellular route [186, 321].

As YG seems to be the most active form [134], while YGG is thought to be the main product of enkephalin degradation and less susceptible to protease attack [323, 324], the latter has been used as a model of potentially absorbable immunoactive peptide. If the peptide reached the circulation it could interact with the cells of the immune system and exert its function(s), although plasma/serum components could affect YGG activity.

To highlight the effect of serum on YGG activity, BPBL has been cultured in presence of two concentrations of NCS, and it has been observed that higher NCS concentrations showed an inhibitory effect on cell proliferation *per se*, which was always abolished by the addition of conA in the culture medium. Conversely, no effect of serum concentration on quantitative cytokine expression was detectable, even though other works indicated that variations in NCS concentration could affect cytokine expression in lymphocytic leukemia cells [325] and in a macrophagic cell line, where it could depress TNFα production [326].

Although the effects of YGG on the cells of the immune system has been proven also *in vivo* [154, 185], its mechanism of action is far from being fully elucidated. At present, only few papers [134, 155, 157] examined the effects of YGG *in vitro* on lymphocytes using different experimental approaches. Sizemore and colleagues [155] studied the effects of YG and YGG on conA-induced regulatory T cell activity on cell proliferation. They found that both peptides increased *in vitro* proliferation of T cells stimulated by conA, and YG showed the greatest biological activity. Moreover, those authors observed a biphasic effect as YGG stimulated proliferation at lower concentrations (10^{-13} – 10^{-14} mol/L) and inhibited proliferation at higher concentrations.

More recently, Kayser and Meisel [134] stimulated with YGG human peripheral blood lymphocytes previously activated with conA and estimated cell proliferation by BrdU incorporation. Proliferation was only slightly stimulated by YGG in comparison with the dipeptide YG, and the maximum increase in lymphocyte proliferation induced by YGG was about 20-30% of the control (culture medium only). In addition, the stimulatory effect of YGG on cell proliferation was abolished at higher concentrations (10^{-4} - 10^{-5} mol/L) of peptide added. Unfortunately, that paper did not give information about serum effects on YGG activity.

In the present work, YGG did show neither mitogenic activity, as it did not alter cell proliferation if added alone to the cell culture, nor additive action to the pro-

proliferative effect of conA over the concentration interval under examination. Likely, no pro-proliferative effect of YGG could be detected because peptide concentrations lower than 10^{-12} mol/L were not tested, which likely are pro-proliferative [327]. However, an inhibitory effect of YGG on conA-induced BPBL proliferation was observed, as the peptide decreased proliferation by 20-60%, depending on the concentration used. In addition, NCS concentrations clearly modulated the BPBL proliferative response to YGG. In fact, lymphocytes were more sensitive to YGG at lower NCS concentration, indicating an inhibitory action of serum on the peptide activity. This is in agreement with previous observations that enkephalins and YGG can be rapidly hydrolyzed/inactivated in human plasma [328-331], and plasma proteins can bind enkephalins and their related peptides [327, 332] rendering them unable to exert their effects.

The majority of T cells responds to and produces IL2 upon activation. Piva *et al*. [157] reported that both YG and YGG could affect the expression of IL2, IL4 and INFγ in murine splenocytes activated with suboptimal concentration of conA in a serum-free culture system. Those authors observed that both peptides stimulated INFγ protein production at very low concentrations (10^{-13} mol/L) and inhibited both INFγ and IL2 at higher concentrations (10^{-7} - 10^{-3} mol/L), while no enhancing effect on IL2 secretion could be detected.

In the present study, the ability of YGG to affect IL2 and INFγ expression was studied using 10^{-3} mol/L YGG, because an effect on BPBL proliferation could always be observed at that peptide concentration. To summarize, when BPBL were activated with conA, they responded increasing both proliferation and cytokine expression, IL2 in particular. On the contrary, no significant YGG effect on IL2 and INFγ mRNA concentrations could be seen, although a slight IL2 mRNA increase in 5 out 6 animals was detected if the peptide was administered in presence of 10% NCS. It is possible that the inhibitory YGG action on cytokine expression was

suppressed/masked by the presence of NCS in the culture medium, and it might be observed only in a serum-free culture system.

It was hypothesized that YGG and YG could exert their suppressive effects through a yet unidentified binding site that selectively binds those peptides with low affinity [157].

In conclusion, the anti-proliferative effect of YGG could be observed *in vitro* also in BPBL, despite the presence of serum in the culture medium. However, serum concentration significantly influenced the assay outcome, as proliferation of conA-activated BPBL was inhibited in a manner inversely related to NCS concentration. On the other hand, high YGG concentrations did not inhibit the synthesis of IL2 and INFγ mRNA. The use of bovine lymphocyte culture as a bioassay to evaluate the action of immunomodulatory substances needs to be further validated examining more culture conditions and, perhaps, selecting different lymphocyte populations. In this respect, it is important to consider that peripheral lymphocytes may not be the target for immunoactive peptides introduced by the diet. In fact, YGG present in the gut can have a good chance to exert its biological activity without reaching the circulation, as it may be hypothesized that the peptide can be transferred to the gastrointestinal-associated lymphoid tissue (GALT) at the level of Payer's patches by Antigen-Presenting Cells, where it may exert its putative effects at the serosa level by influencing cytokine release [333].

4.5. Take-home message

The present work demonstrated that the bioactive peptide YGG had an immunomodulatory activity and, in particular, it modulated the proliferation of mitogen-activated bovine lymphocytes. Kayser and Meisel [134] previously demonstrated that YGG peptide modulated the proliferation of lymphocytes of other species, more specifically human lymphocytes. In both cases, the effects of YGG

were evaluated on isolated lymphocytes maintained in culture. It is possible that in the serum added to the culture medium some unidentified factors affect the activity of YGG. In the present work, to acquire better knowledge of the role of NCS, the effect of YGG on lymphocytes proliferation has been evaluated at two specific serum concentrations in the culture medium. The next step would be to characterize YGG effects when serum is not present in the culture medium.

Another important information that can be acquired from the present study is about the stability of YGG to serum peptidases obtained incubating the peptide with medium added with serum. In fact, serum peptidases may influence the effects of YGG during proliferation test but also may contribute to determine the possibility of the peptide to reach intact its target site, once absorbed and circulating into the body.

The present work explored also the possibility to use the bovine lymphocytes to test immunomodulatory peptides. The preliminary results obtained are encouraging but a deeper characterization of the isolated lymphocytes. In particular, it would be useful to characterize the cytokines that they express when they are activated, and the response to the different concentrations of serum in the culture medium, because it would help to understand the molecular mechanisms of these bioactive peptides.

EXPERIMENT 3: Study of the bioactive properties and the transport of the peptide β-CN (193-209), a 17-residues peptide of bovine β-casein, through Caco-2 monolayers [1]

5.1. Introduction

Milk proteins are a source of peptides that exhibit numerous bioactivities including antihypertensive, opiate, immunomodulatory, antimicrobial, antioxidant or mineral-binding activities [15, 48, 77, 334, 335]. Among those, the β-CN (193–209) peptide is released from the C-terminal end of β-casein by hydrolysis with pepsin. This peptide was isolated and identified from yoghurt and fermented milks as well as several types of cheese [60, 187, 188]. It is a 17 residues long peptide with the amino acid sequence Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val. This peptide displays immunomodulatory properties and shows mitogenic activity on primed lymph node cells and unprimed rat spleen cells [147]. It manifests chemotactic activity on L14 lymphoblastoid cell line [189], and enhances phagocytosis in rat macrophages [148, 190].

To exert their biological activity, some peptides have to cross the gastrointestinal barrier, and reach the circulation and target sites in an active form [261]. Resistance to enzymatic degradation and transport through intestinal cells are the two important factors influencing the bioavailability of orally ingested peptides. There are some distinctive features determining the possibility of a peptide to be absorbed intact through the intestinal epithelium, such as its molecular mass, hydrophobicity, charge or tendency to aggregate [336, 337]. Interestingly, the presence of 4 proline residues within the sequence can protect the long β-CN (193–209) peptide from the action of peptidases. As a consequence, this peptide appears as a good candidate for crossing the intestinal barrier in an intact bioactive form. The main routes

[1] Part of this experiment has been accepted for publication by Molecular Nutrition & Food Research with the following title "The (109-203) 17-residues of β-casein is transported through Caco-2 monolayer", written by Regazzo D., Mollé D., Gabai G., Tomé D., Dupont D., Léonil J. and Boutrou R.

recognized for transepithelial absorption of peptide in the gut include the PepT1 transporter-mediated transport for di- and tri-peptides, the paracellular passive transport via tight junctions, the transcellular passive diffusion and transcytosis that is a transcellular route involving endocytotic uptake, intracellular transport via transcytotic vesicles and basolateral secretion [338].

The aim of this study was to determine the sensitivity of the β-CN (193–209) peptide to hydrolysis by brush border enzymes and its transepithelial transport across Caco-2 cell monolayer as a model of intestinal epithelium. The pathway of transepithelial transport was investigated by using selective inhibitors of the different routes, including the dipeptide Gly-Pro that competitively inhibits the peptide transporter PepT1 [336], cytochalasin D that opens tight junctions by altering the cytoskeletal structure [339] and increasing the passive paracellular route, and wortmannin as an inhibitor of transcytosis [340]. In addition the effects of this immunomodulatory peptide on the viability and tight junction stability of Caco-2 cells was investigated to better characterize its biological activities.

5.2. Materials and Methods

5.2.1. Chemicals and Reagents

Dulbecco's modified Eagle's medium (DMEM), non-essential amino acids (NEAA), gentamycin sulphate, Hank's balanced salt solution (HBSS) and phosphate buffered saline (PBS) were purchased from Lonza, Switzerland. Gibco-Invitrogen (United Kindom) supplied L-Glutamine (L-Glu) and trypsine-EDTA. Fetal calf serum (FCS) was purchased from Dutscher, France. Sigma–Aldrich, (France) supplied cytochalasin D, dimetilsulfoxide (DMSO), Glycil-Proline dipeptide (Gly-Pro), glycine, glucose, HCl, hydroxyethyl piperazine ethane sulphonic acid (HEPES), mannitol, neutral red powder, para-nitrophenyl phosphate, trinitrobenzenesulfonic acid (TNBS) and Triton X100. Wortmannin was obtained from LC Laboratories, Massachusetts,

USA. Acetonitrile, acetic acid ethanol, 2-Amino-(hydroxymethyl)-1,3-propanediol (TRIS) and trifluoroacetic acid (TFA) were purchased from Fluka, France.

5.2.2. Preparation of β-CN (193-209)

The peptide was obtained in a purified form as previously described [341]. Conditions for hydrolyzing β-CN were slightly modified for β-CN concentration (5 g/L), molar ratio chymosin/β-CN (1/8000) and duration of hydrolysis (150 min). Then, the reaction was stopped by heat inactivation of the enzyme (80°C, 15 min). The pH of the mixture was subsequently adjusted to 4.6 with 1 mol/L HCl to precipitate and remove by centrifugation (7000 x *g* for 20 min) whole casein and its large fragments. After readjusting the pH to 6.5, the supernatant was ultra filtered (Spiral-wound UF cartridge S10T3 MWCO 3 KDa; Amicon, Lexington, Massachusetts, USA) and the ultra filtrate was concentrated with a membrane (Filtron membrane 1 KDa) and then freeze-dried. The peptide, identified by electrospray mass spectrometry (ESI/MS), was obtained with a purity of 98% estimated by RP-HPLC-ESI/MS as described in Paragraph 5.2.7.

5.2.3. Cell Culture

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, Maryland, USA). Cells were cultured in DMEM supplemented with 20% FCS, 1% NEAA, 2 mmol/L L-Glu and 25 µg/mL gentamycin sulphate. They were incubated at 37°C in humidified atmosphere containing 5% CO_2. The monolayer became confluent 4-5 days after seeding $3 \cdot 10^6$ cells/flasks (75 cm^2 flasks, Greiner Bio-one, France), and the cells were subcultured at split *ratio* of 1:5 by trypsinization (0.5% trypsin and 0.05% EDTA). The medium was changed every second day. The cells used in this study were at passages 35-45. For transport studies, cells were seeded in cell culture inserts with Anopore® membranes (0.2 µm pore sizes; 25 mm

diameter; 4.7 cm^2 grown surface, from Nunc, Denmark) at $4.5 \cdot 10^5$ cells/cm^2 density and incubated in six-well culture plates (Nunk). The medium was changed every 2 days. The monolayer became confluent after 4 days, and the cells differentiated for another 21 days before performing transepithelial transport experiments. The integrity of the cell layer was evaluated by TransEpithelial Electrical Resistance (TEER) measurement with EVOM epithelial volt-ohm meter (World Precision Instruments, Florida, USA). Only Caco-2 monolayers showing TEER higher than 300 Ω/cm^2 were used for the experiments.

The integrity of the monolayers was checked before, during and after the experiment, TEER values remained stable around at 300 Ω/cm^2 and no significant reduction was observed following the incubation with the peptide in comparison with cells that were not incubated with the peptide. Neither β-CN (193-209) administration nor incubation with inhibitors affected cellular viability that at the end of the experiments was not significantly different from the viability of the control (cell monolayers without β-CN (193-209)), assessed at the beginning of the experiments.

5.2.4. Transepithelial transport studies

After TEER measurement, Caco-2 cells monolayers were gently rinsed twice with PBS, and transport medium (TM, HBSS supplemented with 25 mmol/L glucose and 10 mmol/L HEPES) was added to the apical (2 mL) and to the basolateral (2 mL) compartments. After 30 minutes of incubation, medium was replaced with fresh TM containing 0, 0.1, 0.5, 1, 2 or 4 mmol/L of β-CN (193-209) peptide. The inserts were incubated at 37°C for 120 min and the apical and basolateral solutions were sampled at the beginning and at the end of incubation period for RP-HPLC-ESI/MS analyses to measure β-CN (193-209) concentration in both compartments. For inhibition experiments, Gly-Pro (5, 10, 20 mmol/L) was dissolved in TM, and wortmannin (0.25, 0.5, 1 µmol/L) and cytochalasin D (0.25, 0.5, 1 µg/mL) were dissolved in DMSO and immediately diluted in TM (0.05% DMSO final

concentration). The cell monolayers were incubated 30 min with the inhibitors or with 0.05% DMSO, as a control, before the peptide transport experiments. During and at the end of the experiments, TEER was measured and data were recorded only from experiments in which TEER was higher than 250 Ω/cm^2. To exclude that addition of β-CN (193-209) and/or inhibitors could be toxic for the cells, cellular viability was assessed at the end of each experiment using the vital dye neutral red, as described in the Paragraph 5.2.5.

5.2.5. Effects of β-CN (193-209) on cellular viability

To assess if peptide addition could be toxic for the cells, cellular viability was evaluated at the end of each experiment using the vital dye neutral red (NR). NR is a weak cationic dye that diffuses readily across plasma and organelle membranes, accumulating in the lysosomes. The principle of the assay is based on the fact that the loss of membrane integrity induced by test agents results in decreased retention of NR (quantification of NR at 540 nm). Damaged or dead cells thus appear unstained in comparison with healthy control cells. Similarly, lower absorbance after NR extraction is an indication of reduced cellular viability [342]. The applied protocol is based on the NR uptake assay first described by Borenfreund and Puerner [343].

Briefly, TM used for the incubation was removed, the cells were washed twice with sterile PBS and fresh TM was replaced. Then Neutral Red Solution (0.33% in PBS, w/v) was added to the medium in an amount equal to 10% of the medium volume and cells were allowed to incubate for 120 min at 37 °C. At the end of the incubation period, the medium was carefully removed, the cells quickly rinsed twice with sterile PBS. The incorporated dye was then solubilized in a volume of Solubilization Solution (1% acetic acid, 50% ethanol, 49% milliQ water) equal to the original volume of culture medium. The cultures were allowed to stand for 10 minutes at room temperature, enhancing the dye solubilization by gentle stirring in a rotatory shaker. The absorbance was measured at a wavelength of 540 nm with background

subtraction at 690 nm using a microplate reader (Spectramax M2, Molecular Devices, France).

5.2.6. Effects of β-CN (193-209) on tight junctions: TJ-stabilizing activity

The transepithelial transport studies with increasing concentrations of β-CN (193-209) were also used to study the characteristics of the tight junction-stabilizing activity of β-CN (193-209) on Caco-2 monolayers. At the end of incubation, before testing cellular viability, the TEER values were measured and the relative TEER values to the non treated monolayers were calculated. These relative values were therefore designated as the TJ-stabilizing activity index (TSI), and expressed the stabilizing activity of β-CN (193-209) by using the TSI value. The TSI value was defined as:

$$TSI = \frac{\text{TEER value of the treated cells}}{\text{TEER value of the untreated cells}}$$

5.2.7. RP-HPLC-ESI/MS analyses

Analytical RP-HPLC was carried out using Agilent HP1100 chromatographic system (Agilent Technologies, Massy, France). Separations were performed on a narrow-bore Symmetry C_{18} column (5 µm particle size, 2.1 × 150 mm, Waters, WAT 056975, Milford, Massachusetts, USA), equipped with a C_{18} cartridge guard. The elution was run at 0.25 mL/min and 40°C by a binary gradient with acetonitrile as an organic modifier. Solvent A contained 0.106% TFA in MilliQ water (v/v) and solvent B contained 0.1% TFA in acetonitrile-MilliQ water (80:20, v/v). Samples were analyzed by on-line RP-HPLC-ESI/MS. The column was initially equilibrated with 10% of solvent B. Samples were applied to the column and eluted by a linear gradient of solvent B performed as follows: 0-25 min, 10-70%; 25-27 min 100%, the column was held at 100% during 3 min and then equilibrated at 10% during 10 min. Throughout on-line coupling, splitting of chromatographic flow was achieved by a

low dead volume tee with 85% of the flow directed to the UV detector and 15% to the mass spectrometer. This split allows a perfect superposition between UV and TIC (Total Ionization Current) detection. Peaks were detected both by UV absorbance at 214 nm and peptide mass spectrometry by TIC.

The β-CN (193–209) peptide was quantified in accordance to a standard curve, established with chosen quantities (from 0.053 nmol to 1.063 nmol) of purified peptide β-CN (193–209). β-CN (193–209) quantity (x) in apical and basolateral solutions was calculated with the equation y=bx+c, where y is the UV absorbance at 214 nm, c is the y-axis intercept and b the slope of a standard curve.

The proteolysis of the peptide was analyzed by LC-MS. The mass spectrometer (API III+ SCIEX, Thornhill, Ontario, Canada) comprises a triple quadrupole equipped with an atmospheric pressure ionization source. Analysis was carried out in positive detection mode. A 75 µm sprayer was usually set at 4800 V for generated multiply-charged ions and orifice set between 60 to 90 V depending on experiments. The nebulizer pressure was set around 0.5 MPa and the curtain gas set to 1.2 L/min. The instrument mass-to-charge (m/z) scale was calibrated with polypropylene glycols. All peptide mass spectra were obtained from the average signal of multiple scans. Each scan was acquired over the range of m/z values from 500 to 2000 using a step size of 0.5 Da and a dwell time of 0.5 ms. The measured masses were matched with predicted enzymatic fragments by using the software BioMultiview 1.3.1 (MDS Perkin Elmer Sciex, Thornhill, Canada).

5.2.8. Assessment of β-CN (193-209) hydrolysis

Hydrolysis of β-CN (193-209) was determined by measuring free amino groups (-NH_2 groups) with trinitrobenzenesulfonic acid (TNBS) as described by Boutrou *et al.* [344], following 1:4 dilution in distilled water. Briefly, the supernatant (10 µL) was added to 100 µL of potassium borate (1 mol/L, pH 9.2) and 40 µL of TNBS (1.2 g/L). After incubation (1 h, 37°C), the absorbance was measured at 405 nm using a

multiplate reader (Spectramax M2, Molecular Devices, France). Free amino groups were quantified with glycine as the standard.

5.2.9. Data analysis

The results were expressed as the mean values of at least three independent experiments. The β-CN (193-209) basolateral concentration in function of its administered apical concentration was subjected to regression analysis using the Logistic Dose-Response interpolation equation (4 parameters) provided by Table-Curve2D software program (Jandel Scientific, San Rafael, California, USA). The effect of the inhibitors on β-CN (193-209) flux was evaluated by analysis of variance. The differences between each experimental condition and the control were analyzed by the Dunnett test (Statgraphics Plus 4; Manugistics, Inc, Maryland, USA). Differences with P-values < 0.05 were considered as significant.

The analysis of variance and the Dunnett test for the post-hoc was also applied to assess the effects of β-CN (193-209) addition on TEER values and cellular viability.

5.3. Results

5.3.1. Transepithelial transport of β-CN (193-209) across the Caco-2 cells

The RP-HPLC-ESI/MS analysis and the standard curve generated using pure β-CN (193–209) peptide permitted the quantification of this peptide in apical and basolateral solutions (Fig. 5.3.1.1.). It has been verified using LC-MS/MS that the β-CN (193-209) peptide was the sole one present in the apical solution at the beginning of the incubation. After 120 minutes of incubation, the peptide was not significantly hydrolyzed by the brush border exopeptidases, and the products of hydrolysis were the peptides β-CN (194-209) and β-CN (193-208) (Fig. 5.3.1.2.). The hydrolysis of the β-CN (193-209) peptide in apical solution was quantitatively limited over the experimental duration and regardless of the peptide concentration

(Fig. 5.3.1.3A. and Fig. 5.3.1.3B.). When the peptide was added at 2 mmol/L, a 10% decrease in its concentration in the apical compartment was observed after 2 h incubation, and the peptide left remained intact. For higher concentrations, the hydrolysis was less than 10%. RP-HPLC-ESI/MS analysis of basolateral solution showed that the β-CN (193-209) peptide and its two derived fragments were absorbed intact through Caco-2 monolayer. After 120 min incubation in the apical compartment at the milli molar range, the β-CN (193-209) peptide appeared in basolateral compartment at the micro molar range with concentration values following a saturable pattern (Fig. 5.3.1.4.), described by a sigmoidal curve.

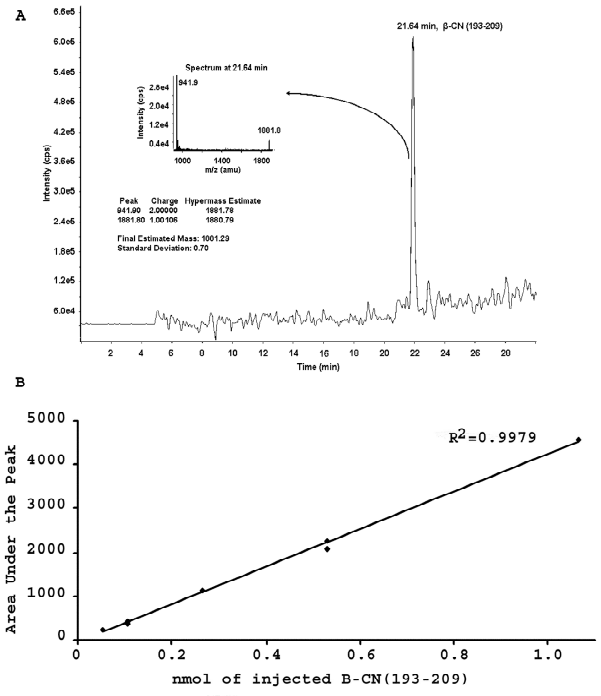

Fig. 5.3.1.1. Identification and quantification of β-CN (193-209) by RP-HPLC-ESI/MS analysis. A. Identification and estimation of β-CN (193-209) purity level are shown on the spectrum and on the TIC graph. β-CN (193-209) was added in the apical compartment at 2 mmol/L and apical solution immediately analyzed. B. Quantification of β-CN (193-209) in apical and basolateral solutions using a five-point calibration curve of pure β-CN (193-209) as standard analyzed by RP-HPLC-ESI/MS (see Paragraph 5.2.7).

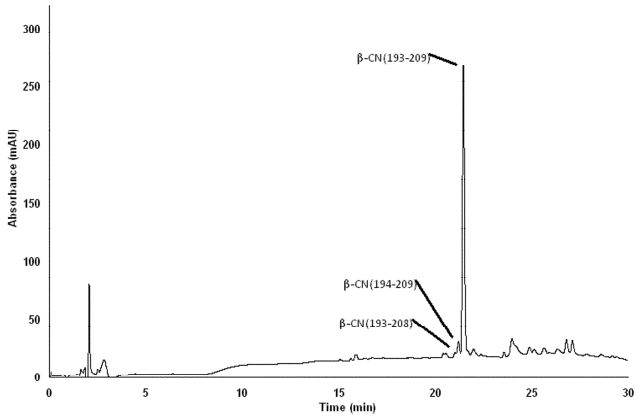

Fig. 5.3.1.2. RP-HPLC-ESI/MS analysis of the apical solution after 120 minutes of incubation. β-CN (193-209) was previously added (2 mmol/L) in the apical compartment at time 0. During the incubation with Caco-2 monolayer, β-CN (194-209) and β-CN (193-208) peptides were generated from β-CN (193-209) hydrolysis.

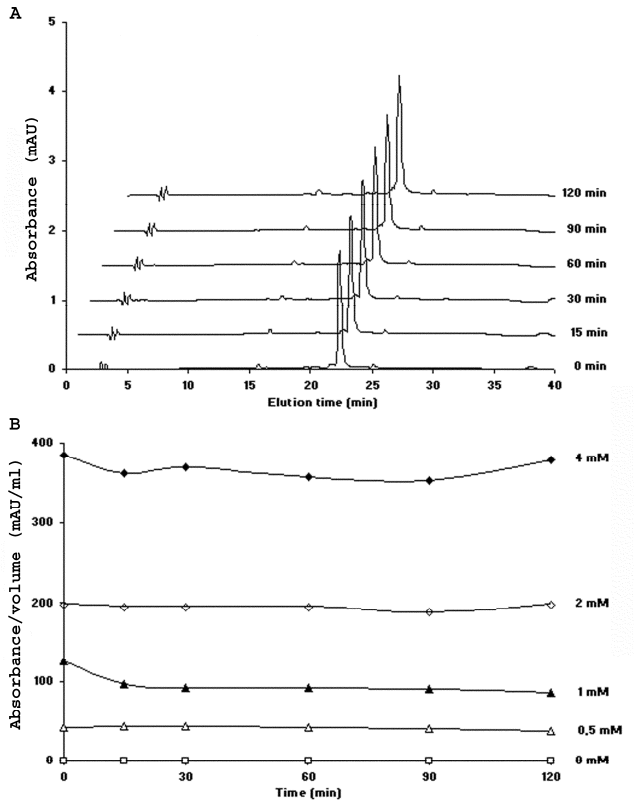

Fig. 5.3.1.3. Stability of the peptide β-CN (193-209) at the apical compartment of Caco-2 cell monolayer. A. LC-chromatograms obtained from RP-HPLC-ESI/MS analysis of the apical solution in the presence of 2 mmol/L β-CN (193-209) from 0 to 120 minutes. The peak eluted at 22 minutes corresponds to the peptide. B. Change in peak height of β-CN (193-209) introduced at different concentrations in the apical compartment of Caco-2 cell monolayer, as determined from LC chromatograms.

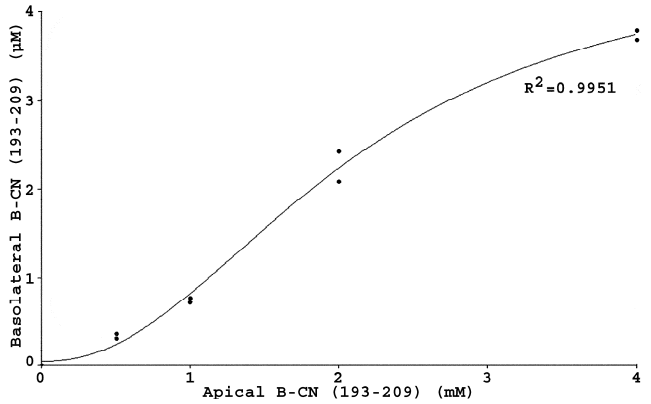

Fig. 5.3.1.4. Concentration dependence transport of β-CN (193-209) across Caco-2 monolayer. Different amounts of peptide were incubated in apical compartment and β-CN (193-209) was evaluated in basolateral solution after 120 minutes incubation at 37°C. Quantification was obtained as described in Paragraph 5.2.7.

5.3.2. Influence of Gly-Pro, Cytochalasin D and wortmannin on β-CN (193-209) transport

To evaluate the pathway of the transepithelial transport of β-CN (193-209), the effect of some inhibitors on the apical to basolateral flux of β-CN (193-209) was tested (Fig. 5.3.2.1.). The transport of β-CN (193-209) was not significantly decreased by Gly-Pro (applied from 5 to 20 mmol/L) that competitively inhibits the peptide transporter PepT1. In the range from 0.25 to 1 µg/mL, the treatment with cytochalasin D, a tight junctions disruptor, reduced TEER values approximately of 20%, indicating that paracellular route was similarly expanded regardless the concentration of the β-CN (193-209). Nevertheless, the presence of cytochalasin D at 0.25, 0.5 and 1 µg/mL did not significantly alter apical to basolateral flux at any concentration used. On the contrary, the addition of the inhibitor of transcytosis wortmannin in the range from 0.25 to 1 µmol/L significantly ($P < 0.05$) reduced the flux of β-CN (193-209) through the Caco-2 monolayer and an average 53% decrease of transport was determined (Fig. 5.3.2.1.).

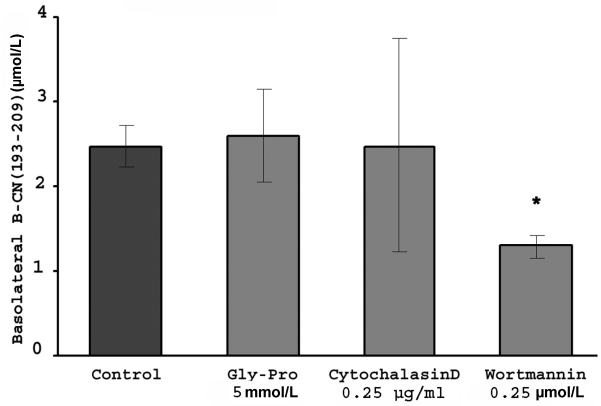

Fig. 5.3.2.1. Effects of Gly-Pro (a competitive inhibitor of peptide transporter PepT1), cytochalasin D (a disrupter of tight junction) and wortmannin (an inhibitor of transcytosis) on transport of 2 mmol/L of β-CN (193-209) peptide across the Caco-2 cell monolayers. Results are expressed as the mean ±SEM (n=3). Means were compared to the control using the Dunnett test (* $P < 0.05$).

5.3.3. Influence of β-CN (193-209) on Caco-2 TJ stability and permeability

To assess if the addition of β-CN (193-209) could influence the tight-junction stability of the Caco-2 monolayer, TEER of the monolayers was measured at the beginning and at the end of the transepithelial transport experiments and the TEER values of the monolayers in contact with the peptide were compared to those non treated, chosen as control. In control cells, TEER values significantly decreased ($P < 0.05$) during the 120 min of incubation (Fig. 5.3.3.1). The addition of β-CN (193-209) at concentrations of 0.5, 1, 2 or 4 mmol/L significantly reduced this phenomenon and TEER values measured at the end of incubation in monolayer incubated with the peptide were not significantly decreased from the values measured at the beginning of the experiments (Fig. 5.3.3.1). This result was confirmed by the calculated TSI values, as indicated in Table 5.3.3.1. All TSI calculated from β-CN (193-209) at concentrations higher than 0.1 mmol/L were higher than 1.

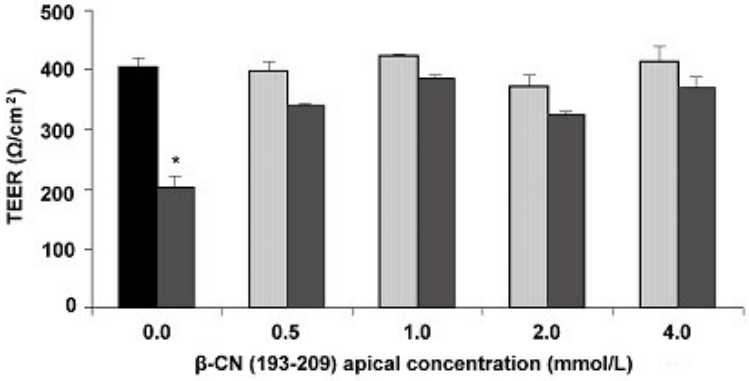

Fig. 5.3.3.1. TEER values measured during transepithelial transport experiments. β-CN (193-209) peptide was added to the apical compartment at different concentrations, TEER was measured immediately after peptide addition (light grey bars) and at the end of the experiment (dark grey bars). Results are expressed as the mean ±SEM (n=3). Means were compared to the control (black bar) using the Dunnett test. * Significantly higher ($P < 0.05$) than the control.

β-CN (193-209) apical concentration (mmol/L)	TSI value
0	1.00
0.5	1.66*
1	1.89*
2	1.58*
4	1.80*

Table 5.3.3.1. TSI values calculated for the transepithelial transport experiments. β-CN (193-209) peptide was added to the apical compartment at different concentrations, TEER was measured immediately after peptide addition and at the end of the experiment. TSI was calculated as indicated in Materials & Methods section, Paragraph 5.2.6. TSI values were compared to the control (Caco-2 monolayers not incubated with the peptide) using the Dunnett test.
* Significantly higher ($P < 0.05$) than the control.

5.3.4. Influence of β-CN (193-209) on Caco-2 viability

To assess if the addition of β-CN (193-209) could influence the viability of the Caco-2 monolayer, the cells were subjected to the vital dye neutral red assay. The viability of the monolayers in contact with the peptide was compared to those shown by the non treated cells, that were chosen as control. In control cells, incubation time significantly decreased viability values ($P < 0.001$) (Fig. 5.3.4.1). The addition of β-CN (193-209) at concentrations or 0.5, 1, 2 or 4 mmol/L significantly reduced this

phenomenon and viability values measured after 120 min of incubation in monolayer incubated with the peptide were not significantly decreased from the viability values measured at the beginning of the experiments (Fig. 5.3.4.1.).

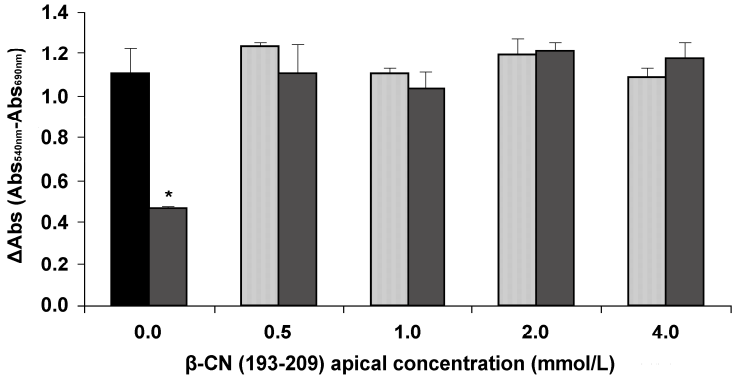

Fig. 5.3.4.1. Viability values measured during transepithelial transport experiments. β-CN (193-209) peptide was added to the apical compartment at different concentrations, viability was measured immediately after peptide addition (light grey bars) and at the end of the experiment (dark grey bars). Results are expressed as the mean ±SEM (n=3). Means were compared to the control using the Dunnett test (* $P < 0.001$).

5.4. Discussion

The hypothesis that peptides escape digestion and are transported from the intestinal lumen into blood circulation is gaining acceptance for small peptides, mainly due to the growing number of studies describing the *in vitro* transepithelial transport of bioactive peptides [186, 201, 202, 244, 261, 320, 321, 345-348]. In the present study we demonstrate that the 17 residue β-CN (193-209) peptide and the derived β-CN (193-208) and β-CN (194-209) peptides are transported across Caco-2 cell monolayer, with the major contribution of the transcytosis mechanisms.

To exert its biological effects an ingested peptide must first resist intestinal hydrolysis. To study the resistance of the β-CN (193-209) peptide to brush-border membrane peptidases, Caco-2 cell monolayer has been used because, under specific culture conditions, Caco-2 cells undergo a process of differentiation leading to the expression of several morphological and functional characteristics of the

enterocyte including the microvillus structure and of brush-border enzymes in the apical membrane [227, 276]. About 10% of the β-CN (193-209) peptide were hydrolyzed through the action of amino- and carboxy-peptidases present on the apical membrane, but the β-CN (193-209) peptide and the two derived peptides, β-CN (193-208) and β-CN (194-209), were not further hydrolyzed by brush-border membrane exopeptidases. Moreover, the three peptides resisted the action of intracellular peptidases. In general, due to their rapid hydrolysis by the brush border or cytoplasmic peptidases, the bioavailability of 2 to 9 residues-peptides is extremely low [186, 202, 244, 246, 261, 321, 346, 348]. The resistance of the β-CN (193-209) peptide to the action of Caco-2 brush-border peptidases is possibly related to its proline-rich sequence (4 proline residues on 17 residues), and other proline containing peptides were found to be resistant to intestinal proteolysis [41, 257]. This finding is further confirmed by Savoie and colleagues [256], who observed that peptides rich in proline and glutamic acid are more resistant to pepsin and pancreatin activity, suggesting that the β-CN (193-209) peptide would resist to gastric and duodenal digestion. This hypothesis was affirmed by a regular appearance of the β-CN (193-209) peptide among the main peptides released from the stomach of milk-fed calf [349]. Thereafter this peptide appears in the intestinal lumen where it can be absorbed. To our knowledge no data exist on the hydrolysis of the C-terminal end of β-casein in human fed a milk diet.

Caco-2 cells cultured on a semi permeable filter were used to demonstrate that the β-CN (193-209) peptide could be transported through the intestinal barrier. Moreover, additional experiments using selective inhibitors of the different routes for the transepithelial transport of the β-CN (193-209) peptide suggested the involvement of transcytosis among the different transport pathways. Caco-2 cells have been used for the present study because they express the carrier-mediated transport systems for amino acids and di- and tri-peptides [350, 351], show a transcytotic activity [245], and develop tight junctions that are involved in the

paracellular route [227, 352]. Our results did not permit to totally exclude a possible involvement of the paracellular route in the transport of β-CN (193-209) peptide because of large standard deviation observed when cytochalasin D was administrated to the cells. In addition, the 20% reduction of the TEER might be insufficient to increase the paracellular transport of macromolecules. Nevertheless the fact that the β-CN (193-209) peptide was mainly transported by transcytosis is possibly related to its physico-chemical characteristics: it is a large and hydrophobic peptide, negatively charged under the experimental conditions (neutral pH). So the passive paracellular transport via tight junctions was not the probable route because it is normally applicable to the absorption of water-soluble low-molecular-weight substances [353] and short-chain peptides [186, 237, 238, 244] and, in general, it is specific for positively charged molecules because tight junctions are overall negatively charged [354]. Regarding the transcellular route, the results obtained from the present study showed that the transporter PepT1 was not involved in the transport of the β-CN (193-209) peptide across Caco-2 cell monolayer. This result reinforces the assumption that this large peptide have only little possibility to be transported by the H^+-coupled PepT1 transporter because PepT1 is an active and saturable symporter known for intestinal absorption of di- and tri-peptides [231, 336, 337]. The low level of degradation of the peptide during its transepithelial transfer strongly suggests that passive transcellular diffusion is not the main pathway involved in the transport of the peptide. In contrast, the significant reduction of the transport in the presence of wortmannin indicated transcytosis as a potential candidate for the transepithelial transport of the β-CN (193-209) peptide [340].

Simultaneously to its identification, the β-CN (193-209) peptide was quantified in apical and basolateral compartments using RP-HPLC-ESI/MS analysis. The concentration of peptide absorbed was 0.2-0.3 mmol/L, even if higher concentrations were applied in the apical compartment. From these results obtained via a model approach, it is difficult to evaluate whether the absorption of the peptide

when present in food matrix would be comparable. Nevertheless, the food matrix such as cheese will be extensively disorganized when reaching the small intestine. After 120 min incubation, the β-CN (193-209) peptide appeared in the basolateral compartment at 2 µmol/L. Thus, the actual amount of peptide transepithelially transported was about 1%. This value is similar to the one determined for the antihypertensive tripeptide VPP whom 2% was transported from the apical to the basolateral side [186]. In contrast to our 17-residues peptide, 87% of the tripeptide were absorbed by the cells. Consequently the absorption via endocytosis appears as to be the limiting step in the transepithelial transport of the long peptide. The low amount of β-CN (193-209) peptide absorbed is probably linked to its physico-chemical characteristics, in particular its hydrophobicity [192, 255]. Assuming that the β-CN (193-209) peptide is transported mainly through a transcytosis route, a vesicular-mediated internalization, the mechanism involved is probably absorption by apical cell membrane through hydrophobic interactions [355]. Moreover, considering the presence of arginine residue in its sequence, the β-CN (193-209) peptide could form hydrogen bonds with lipid phosphates of cell membranes thus favoring the translocation process via transcytosis route [356]. A similar mechanism has been described for the absorption of some peptides, as bradykinin that is a 9 residues peptide with 3 proline residues and basic oligopeptides [244, 246, 357].

The present work evaluated also the effects of β-CN (193-209) peptide on Caco-2 viability and tight junction stability and it demonstrated that the peptide added to the Caco-2 monolayers at different concentrations for 2 hours was able to maintain the TEER values and cell viability at high levels, not significantly different from the control cells (cells without the peptide, at time 0). In contrast, the TEER values and the viability of control cells after two hours in simple TM were drastically decreased. A mixture of free amino acids was administered to the monolayers to have an additional control. The amino acids were chosen among the residues that constitute the sequence of the β-CN (193-209) peptide, in the same molar *ratio*. In contrast to

the intact β-CN (193-209) peptide, the mixture of amino acids was no table to maintain the TEER values and the viability at high levels, which were not different from the values of the control cells incubated in TM for 2 hours (data not shown). This result permitted to exclude that the positive effects of the β-CN (193-209) peptide on TEER and viability of Caco-2 monolayers were due to the fact that the addition of the peptide simply represented a possible source of amino acids for the cells, that were not present in TM, a simple medium used to maintain stable the pH of the cells and to give them glucose as a source of energy. It is thus possible to hypothetize that β-CN (193-209) peptide may exert some biological effects also on Caco-2 cells, and in particular, that the modulation of TEER could finally influence the transport of the molecules that are predominantly absorbed *via* paracellular pathway. It remains to establish the mechanism of action by which the β-CN (193-209) peptide acts on these cells. A possible hypothesis is that β-CN (193-209) could be faster absorbable and so nutritionally superior to the mixture of free amino acids of comparable amino acid composition, as already demonstrated for other peptides by [253, 322, 358, 359].

In conclusion, this study evidencied that β-CN (193-209) manifested some biological effects also on Caco-2 cells and it demonstrated the transepithelial transport of the β-CN (193-209) peptide, a long and hydrophobic peptide across a well known *in vitro* model of intestinal epithelium. The significant inhibitory effect of wortmannin on the transepithelial transport of β-CN (193-209) peptide suggests that transcytosis is the most important mechanism of transport for the peptide through the Caco-2 cells monolayer, even if other mechanisms of transport cannot be completely excluded. It remains to elucidate the exact molecular mechanism underlying β-CN (193-209) translocation into the cells to more precisely identify the tissue target of this peptide to exert a regulatory physiological effect. As a consequence, the visualization of the translocation steps would be of crucial importance to better characterize the route for intestinal β-CN (193-209) passage.

5.5. Take-home message

The present work demonstrated that the immunomodulatory peptide β-CN (193-209) is absorbed intact through a Caco-2 cells monolayer, maily by trancytosis. This result is of physiologycal importance because the demonstration of the absorption of an intact 17-residues peptide in a model of the intestinal epithelium confirm the possibility that long peptides could be absorbed intact also *in vivo.*

This results is of extreme importance for the bioavailability of the bioactive peptides contained in food matrices, but also for all the peptides that could be potentially used for the formulation of oral vaccines. Thus, the incouraging results obtained in the present work should be further explored and the mechanisms of absorption of long peptides better investigated, possibly using *in vitro* models other than Caco-2 cell line. In addition, the peptide β-CN (193-209) could be used as a model peptide, to which compare the absorption of other peptides of interest.

The number of studies describing the *in vitro* transepithelial transport of bioactive peptides is growing [186, 201, 202, 244, 261, 320, 321, 345-348]. All the data collected from absorption studies could be extremely useful to establish the essential characteristics that allow a bioactive peptide to be absorbed intact in high quantities across the gut. At the same time, the physiological and molecular characterisation of the intestinal mucosa would permit to identify the systems responsible for the absorption of the intact peptides, and to clarify how the activity of these systems could be modulated.

EXPERIMENT 4: Assessment of digestion of the peptide β-CN (193-209), a 17-residues peptide of bovine β-casein, on brush border membrane vesicles

6.1. Introduction

The β-CN (193–209) peptide derived from bovine β-casein has already been characterized for its immunomodulatory activity [147, 148, 189, 190] and for its absorption and its sensitivity to hydrolysis to brush border enzymes in a Caco-2 monolayer (see Experiment 3 of this thesis).

The preliminary results obtained demonstrated that this peptide resists to hydrolysis of the caco-2 enzymes, thus permitting its absorption across the cell monolayer.

It is important to better characterize how this immunomodulatory peptide is degraded by intestinal enzymes because it would also help to clarify the factors affecting peptide bioavailability.

In this view, *in vitro* models other than Caco-2 cell line are available, such as the brush border membrane vesicles (BBMV) isolated from intestine mucosa. This simple digestion model has already been used to study the digestion profile of other bioactive peptides derived from milk proteins [360]. The advantage of BBMV is that they contain the intestinal enzymes involved in the digestion of nutrients, although BBMV has been originally used to characterize brush border enzymes and to evaluate the transport and the uptake of various molecules.

Moreover, BBMV isolation is simple and already standardized and BBMV can be easily isolated from the intestine of different species, as mouse, pig, rabbit and human [361-366]. In particular, BBMV isolated from the intestine of the adult pig could be very useful as digestion and absorption models, because the pig has been recognized as an excellent model for the human gut [218, 367], due to the similarity of its GI tract physiology, in particular the small intestine, to that of

humans. The same similarity has been found also for the piglet as a model for the human infant GI system [216, 217, 368, 369].

For these reasons the aim of the present study was to determine the digestion profile of the β-CN (193–209) peptide, in particular evaluating its hydrolysis in BBMV isolated from the pig intestine (pBBMV) and comparing it with the digestion profile obtained from the digestion with BBMV isolated from the piglet intestine (wpBBMV), with the purpose to find different hydrolysis patterns between the adult and the infant.

6.2. Materials and Methods

6.2.1. Chemicals and Reagents

$CaCl_2$, KCl and sodium acetate were supplied by Pancreac Quimica Sa, Spain. Sigma–Aldrich (France) supplied the Bradford reagent, Fast Garnet, glycine, glucose, hydroxyethyl piperazine ethane sulphonic acid (HEPES), HCl, mannitol, para-nitrophenyl phosphate, phenylmethylsulphonyl fluoride (PMSF), trinitrobenzenesulfonic acid (TNBS). Phe-Pro β-naphtylamide was supplied by Bachem, Germany. Acetonitrile, acetic acid ethanol, 2-Amino-(hydroxymethyl)-1,3-propanediol (TRIS) and trifluoroacetic acid (TFA) were purchased from Fluka, France. Sodium carbonate was obtained from Prolabo, France and sulfosalicylic acid from Merck, Germany. NaCl were obtained from Carlo Erba, Italy.

6.2.2. Preparation of β-CN (193-209)

β-CN (193–209) peptide was isolated as described in Paragraph 5.2.2.

6.2.3. Preparation of BBMV

Preparation of pig BBMV (pBBMV) and piglet BBMV (wpBBMV) was performed as described by Boutrou and colleagues [360]. Briefly, the jejunum of a freshly killed

pig (male weighing 50 kg) or of a piglet (male ageing 24 days, weighing 15 kg) was removed and rinsed with cold 0.9% NaCl. All subsequent steps were performed on ice or at 4 °C.

The intestinal mucosa was scraped off with a glass slide and homogenized in a Warring blender (Grosseron, Saint Herblain, France) at full speed twice for 30 s in 20 vol (w/v) of homogenate media (50 mmol/L mannitol in 2 mmol/L TRIS-HCl buffer, pH 7.0, and 0.1 mmol/L PMSF). $CaCl_2$ was then added to a final concentration of 12.5 mmol/L, and the suspension was stirred in an ice bath for 1 h. The suspension was centrifuged (5000 x *g*, 15 min at 4 °C). The pellet was discarded, and the supernatant was subjected to a second centrifugation at 12000 x *g* for 30 min at 4 °C. The resulting pellet containing the crude brush border fragments was disrupted into microvillus membrane in homogenate media (1 mL per 4 g of mucosa) using a 2 mL syringe with a 0.5 mm × 16 mm needle. 0.5 mL of the obtained samples were frozen and stored in liquid N_2 until use.

Purification and enrichment of the BBMV were checked by determination of the marker enzymes alkaline phosphatase (EC 3.1.3.1) and dipeptidyl peptidase IV (DPPIV) (EC 3.4.14.5). To measure the alkaline phosphatase activity, samples were diluted 1:100 in 0.1 mol/L sodium carbonate buffer, pH 9.4, and mixed to an equal volume of para-nitrophenyl phosphate. The absorbance at 405 nm was measured each minute for 10 min to determine the activity. To measure the DPPIV activity, samples were diluted 1:20 in 0.02 mol/L TRIS-HCl buffer, pH 7.5. Fifty microliters were incubated with 50 µL of 0.66 mmol/L Phe-Pro β-naphtylamide at 37 °C. The reaction was stopped by adding 50 µL of a mixture containing 1 mg/mL Fast Garnet, 10% (v/v) Triton X100, and 1 mol/L sodium acetate, pH 4.0, after 0, 5, 10, 15, and 20 min, and the absorbance at 550 nm was measured.

The protein concentration was determined by using the Bradford reagent with bovine serum albumin as a standard. The specific alkaline phosphate and DPPIV activities were 10- and 15-fold enriched, respectively, in the final pBBMV fraction and 9 and 150-fold enriched for wpBBMV.

6.2.4. Assessment of B-CN (193-209) digestion by pBBMV and wpBBMV

Digestion of β-CN (193–209) was performed at 37 °C in 35 mmol/L HEPES-TRIS buffer and 0.15 mol/L KCl, pH 7.0. The VMBB/substrate *ratio* was previously evaluated in a preliminary study to monitor the digestion kinetics. Digestion was started by mixing an equal volume of the substrate solution (1 mmol/L) and pBBMV or wpBBMV preparation diluted 1:50 in HEPES-TRIS buffer. At selected times, samples (volume of 0.4 mL) were collected, and the reaction was stopped by centrifuging pBBMV or wpBBMV preparation (2000 x *g* for 1 min). The supernatant was stored at –20 °C until analysis. A blank sample was realized by adding the buffer without β-CN (193–209), and as control, β-CN (193–209) was incubated without pBBMV or wpBBMV.

Total digestion (sum of the peptide fragments and the free amino acids) was determined by measuring free amino groups (-NH_2 groups) with TNBS as described in Paragraph 5.2.8., after 1:4 dilution in distilled water. The free amino acids (FAAs) produced throughout digestion (without including peptide fragments) were determined as described elsewhere [370], after precipitation of peptides with 3% sulfosalicylic acid. The concentration of each of the 20 FAAs analyzed was summed to estimate the total amount of FAAs. The results obtained from the quantification of -NH_2 groups and FAAs were expressed in the same concentration unit (mmol/L) to monitor the amount of the peptide that is degraded to peptide fragments and finally converted in FAAs.

6.2.5. Identification of peptides by RP-HPLC-ESI/MS

The RP-HPLC-ESI/MS identification of β-CN (193–209) fragments generated during the peptide digestion with pBBMV and wpBBMV was carried out as described in Paragraph 5.2.7.

6.2.6. Data analysis

The results of the quantification of the free amino groups (-NH_2) and FAAs and the RP-HPLC-ESI/MS analyses were expressed as the mean value of at least 3 independent experiments.

The Area Under the Curve (AUC) obtained from the LC-MS profiles of the RP-HPLC-ESI/MS analyses of two selected fragments (β-CN (195–202) and β-CN (199–206)) formed during β-CN (193–209) digestion with pBBMV or wpBBMV was used to monitor the digestion progression of β-CN (193–209) peptide in the two models. The appearance and the disappearance of the peptides β-CN (195–202) and β-CN (199–206) were monitored together with the disappearance of β-CN (193–209) because these two fragments were identified along the progression of both digestion of β-CN (193–209) with pBBMV and wpBBMV and because the correspondent picks in the LC profiles were easily distinguishable and identifiable from the other fragments formed during the digestion.

For both fragments β-CN (195–202) and β-CN (199–206), the kinetics was evaluated using AUC as indicator of the amount of the fragment, normalized to 100% of the highest AUC value that each fragment showed during digestion progression.

A plot of the AUC of both β-CN (195-202) and β-CN (199-206) vs digestion time permitted to determine the half-life $t_{½}$ of each fragment. The $t_{½}$ was defined as the time (min) required for the disappearance of the 50% of the fragment of interest, after it has reached the highest value of AUC.

For the calculation of the $t_{½}$ of each fragment, the plot of the AUC vs digestion time was interpolated and the equation that showed the best fitting (in terms of R^2) was chosen. The progression of β-CN (193-209) digestion in function of time was also monitored as the sum of the AUC of all the fragments formed during digestion. This parameter was also normalized to 100% at the highest value of AUC sum and the $t_{½}$ was calculated for the total normalized AUC.

6.3. Results

6.3.1. Assessment of digestion

The measurement of free amino groups ($-NH_2$) permitted the evaluation of the total digestion during time. For both pBBMV and wpBBMV the quantity of free amino groups increased during time. In particular, the release of free amino groups in wpBBMV increased linearly up to 120 min and reached the maximum at 180 min. Thereafter the quantity of free amino groups remained constant till the end of digestion (Fig. 6.3.1.1.). For pBBMV the release of free amino groups was linear up to the end of the digestion. The digestion time course was three times higher for wpBBMV than for pBBMV (see Fig. 6.3.1.1.). The analysis of a control sample, that is BBMV incubated without the substrate β-CN (193-209), allowed to estimate that the free amino groups that could be generated from digestion of endopeptidase was negligible, 0.752 ± 0.147 mmol/L at time 0 and 0.276 ± 0.084 mmol/L at 480 min for pBBMV, and 0.308 ± 0.050 mmol/L at time 0 and 0.781 ± 0.086 mmol/L at 480 min for wpBBMV.

FAAs are generated by the action of aminopeptidases and carboxypeptidases. Regarding the digestion with wpBBMV, FAAs were continuously produced with a pattern similar to the correspondent release of free amino groups (see slopes in Fig. 6.3.1.1.). For wpBBMV the quantity of free amino groups and the amount of FAAs became the same at the end of the digestion, signifying that after 480 min of

incubation with the wpBBMV all the initial amount of β-CN (193-209) was converted in free amino acids. During the digestion with pBBMV FAAs production linearly increased but with a FAAs release rate that was almost two times lower than the corresponding free amino groups production. At the end of digestion, all the initial amount of β-CN (193-209) was converted in free amino acids, even for pBBMV, as at that time the amount of free amino groups and the quantity of FAAs were the same. The control sample of FAAs production was 0.01 mmol/L at time 0 and 0.005 mmol/L at 480 min for pBBMV, and 0.02 mmol/L at time 0 and 0.02 mmol/L at 480 min for wpBBMV, signifying that even in the case of FAAs production, the quantity of FAAs that could be generated without the substrate was negligible.

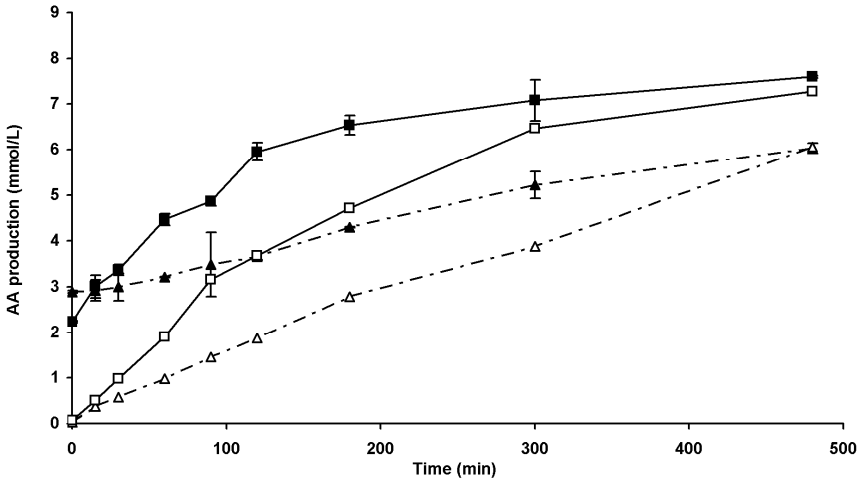

Fig. 6.3.1.1. Determination of free amino groups (-NH_2, expressed in mmol/L eq. Glicine, —■— for wpBBMV and – –▲– – for pBBMV) and FAAs (expressed in mmol/L, —□— for wpBBMV and – –△– – for pBBMV) by the enzymes of wpBBMV and pBBMV. Results are expressed as the mean ±SD (n=3).

6.3.2. Kinetics of digestion

For β-CN (195-202) fragment the AUC increased during the first 90 min in wpBBMV and during the first 180 min in pBBMV, than it decreased for the rest of time. β-CN (195-202) completely disappeared after 300 min of digestion in the case of wpBBMV. Conversely, it was still present at the end of incubation time

(480 min) in the pBBMV digestion. The rate of disappearance of the fragment was higher in wpBBMV than in pBBMV (Fig. 6.3.2.1a.). For β-CN (199-206) fragment the AUC increased during the first 60 min in wpBBMV and during the first 120 min in pBBMV, than it decreased for the rest of time. β-CN (199-206) completely disappeared after 300 min of digestion in the case of wpBBMV. Conversely, it was still present at the end of incubation time (480 min) in the pBBMV digestion. As for the fragment β-CN (195-202), the rate of disappearance of the fragment β-CN (199-206) was higher in wpBBMV than in pBBMV (Fig. 6.3.2.1b.). The plot of the AUC of either β-CN (195-202) and β-CN (199-206) vs digestion time permitted to determine the half-life ($t_{½}$) (Fig. 6.3.2.1a., Fig. 6.3.2.1b. and Table 6.3.2.1.). Both β-CN (195-202) and β-CN (199-206) showed a higher $t_{½}$ value in pBBMV than in wpBBMV, indicating that wpBBMV degraded the fragments more rapidly than pBBMV (Table 6.3.2.2.). The intact β-CN (193-209) peptide is identified till 60 min during wpBBMV digestion and till 90 min during pBBMV. The progression of β-CN (193-209) digestion in function of time is visualized in Figure 6.3.2.2.. The $t_{½}$ resulted 73 min and 169 min for wpBBMV and pBBMV, respectively (Table 6.3.2.2.). The total digestion rate is 2.3 times higher for wpBBMV than for pBBMV.

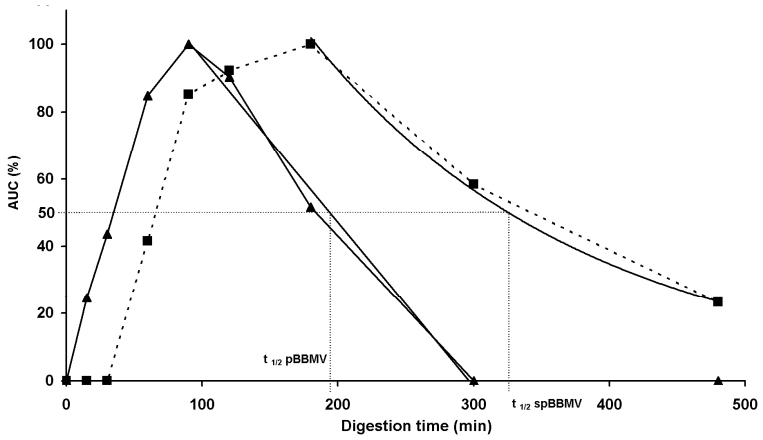

Fig. 6.3.2.1a. Appearance and disappearance of the fragment β-CN (195-202) derived from β-CN (193-209) during digestion with BBMV, presented as a percentage of the relative absorbance of each fragment identified, and normalized to 100% at the maximum value of AUC. The digestion kinetics were used to determine the half-life t ½ for both fragments in pBBMV (– –■– –) and wpBBMV (—▲—).

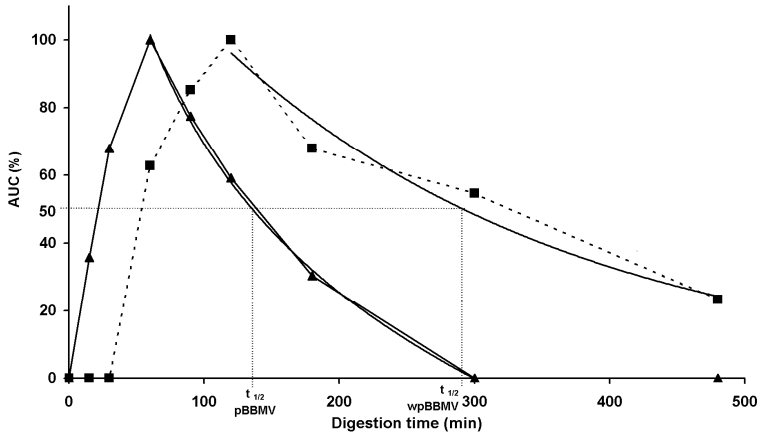

Fig. 6.3.2.1b. Appearance and disappearance of the fragment β-CN (199-206) derived from β-CN (193-209) during digestion with BBMV, presented as a percentage of the relative absorbance of each fragment identified, and normalized to 100% at the maximum value of AUC. The digestion kinetics were used to determine the half-life t ½ for both fragments in pBBMV (– –■– –) and wpBBMV (—▲—).

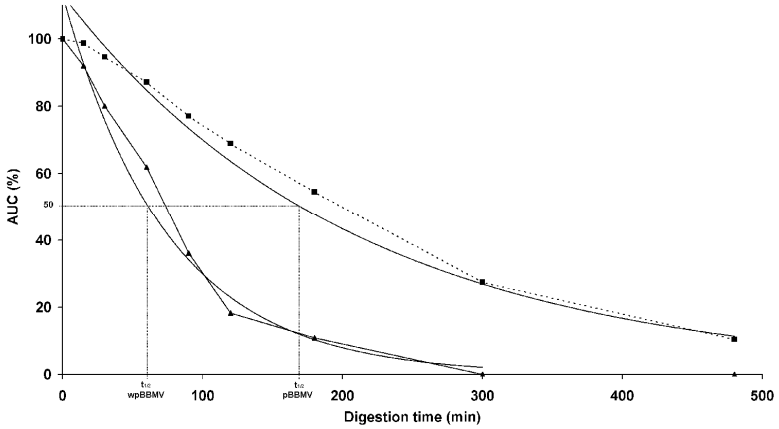

Fig. 6.3.2.2. Progression of β-CN (193-209) digestion in function of time. This parameter was expressed as the sum of the AUC of all the fragments formed during digestion. This parameter was normalized to 100% at the highest value of AUC sum and the t ½ was calculated for the total normalized AUC.
pBBMV (– –■– –) and wpBBMV (—▲—).

	pBBMV		wpBBMV	
	Equation	**R^2**	**Equation**	**R^2**
β-CN (195-202)	$y=245.67e^{-0.0049x}$	0.9987	y=-0.4871x+144.48	0.9921
β-CN (199-206)	$y=152e^{-0.0038x}$	0.9720	y=-63.131ln(x)+359.96	0.9984
Sum of the AUC	$y=112.97e^{-0.0048x}$	0.9880	$y=133e^{-0.133x}$	0.9743

Table 6.3.3.1. List of the equations used to calculate the half life ($t_{½}$) of the fragments β-CN (195-202) and β-CN (199-206) and the progression of β-CN (193-209) digestion in function of time.

$t_{½}$ (min)			
β-CN (195-202)		β-CN (199-206)	
wpBBMV	**pBBMV**	**wpBBMV**	**pBBMV**
103.96	145.72	75.61	172.59

Table 6.3.3.2. Comparison of $t_{½}$ values calculated for β-CN (195-202) and β-CN (199-206) fragments in wpBBMV and pBBMV.

6.3.3. Identification of peptides generated during digestion

All the samples were analyzed by RP-HPLC-ESI/MS to identify the fragments released throughout the digestion time from both wpBBMV and pBBMV, as shown in Figure 6.3.3.1..

Generally, all the digestion products were of low molecular weight and mostly hydrophobic, thus they were eluted between 12-26 min. During pBBMV digestion, as depicted in Figure 6.3.1.2., the number of generated fragments increased up to the maximum after 60 min (11 fragments) and then decreased down to 5 at the end of digestion. During wpBBMV digestion, the number of generated fragments reached the highest value already after 15 min of incubation with the vesicles (12 fragments, Fig. 6.3.1.2.) and then decreased down to 4 fragments at the 90th min of the digestion. RP-HPLC-ESI/MS did not identify any fragment between minute 300 and the end of the experiment of digestion with wpBBMV.

As shown in Figure 6.3.3.1., the generated fragments were the same in both digestion procedures, even if they appeared and disappeared more rapidly in wpBBMV. Indeed, only two fragments were identified selectively in one of the two digestions (grey bars in Fig. 6.3.3.1.). The fragment β-CN (205-209) was identified only in pBBMV digestion; instead the fragment β-CN (207-209) was found only in wpBBMV digestion.

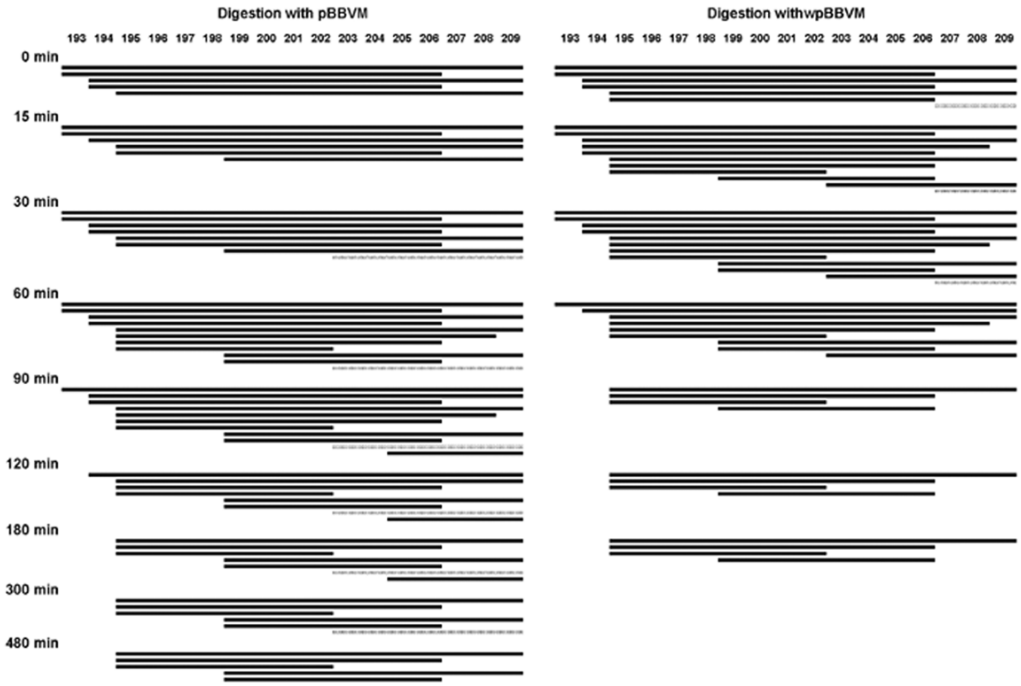

Fig. 6.3.3.1. Comparison of the digestion patterns of β-CN (193-209) during time as identified by RP-HPLC-ESI/MS. Grey lines represent the peptides that appeared only during the digestion with one type of BBMV.

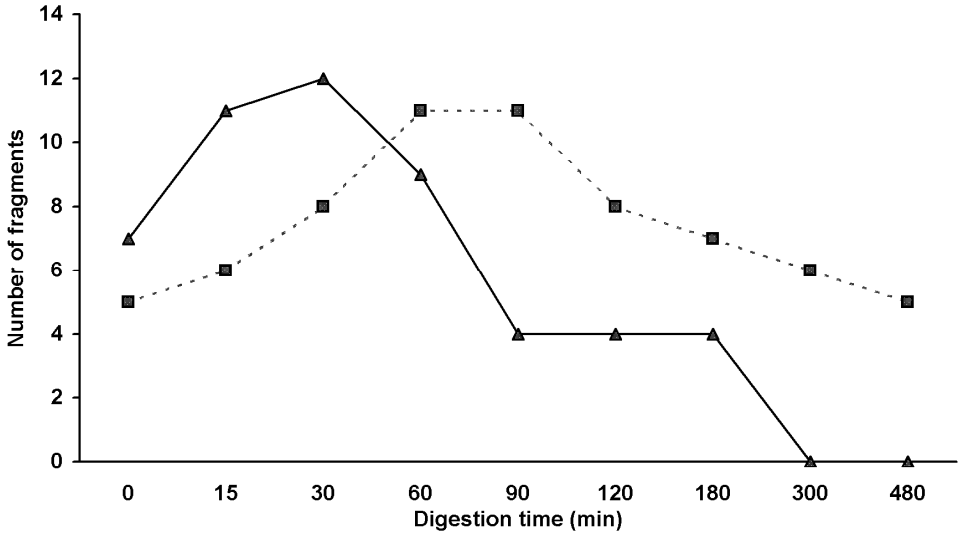

Fig. 6.3.3.2. Comparison of the digestion profile of β-CN (193-209) in function of time. The digestion profile is evaluated as the number of fragments identified during β-CN (193-209) digestion by RP-HPLC-ESI/MS analysis. pBBMV (– –■– –) and wpBBMV (—▲—).

6.4. Discussion

In the present study, pig and piglet BBMVs were used to evaluate the intestinal BBM digestion of the peptide β-CN (193-209), a peptide endowed with numerous biological activities [147, 148, 189, 190]. Although this peptide showed some chemical and physical characteristics that made it resistant to proteolysis by Caco-2 cells brush border enzymes (see Experiment 3 of the present thesis), such as its proline-rich sequence and its hydrophobicity, it was completely hydrolyzed by the enzymes of both pBBMV and wpBBMV. The results of the present work showed for the first time that the peptide β-CN (193-209) could be completely hydrolyzed in the intestinal lumen by porcine BBM enzymes, and the similarity of the porcine GI tract, in particular the small intestine, to the human GI tract make this result possible also in humans. It has to be considered that BBMV are an *in vitro* model that, as Caco-2 cell line, does not represent all the physiological conditions of the human and the porcine GI tract. In particular, the transit time in the small intestine is variable but it is likely that nutrients are not subjected to the action of BBM enzyme for 8 hour, and it could be possible that *in vivo* the peptide β-CN (193-209) would partially resist to the hydrolysis.

Another interesting result was that the pattern of the digestion of the peptide was not different in the two *in vitro* models, because the intermediate fragments formed were identified in both pBBMV and wpBBMV digestions (see Fig.6.3.3.1). The adult and the infant model differed only for the digestion rate of β-CN (193-209) that was faster in wpBBMV than in pBBMV. The difference in the digestion progression could be correlated to the higher DPPIV specific activity shown by wpBBMV, which presented a 10- fold higher value of DPPIV specific activity than pBBMV. All the other digestion conditions for wpBBMV and pBBMV were identical (i.e. same peptide concentration, BBMV concentration, and peptide/BBMV *ratio*), and they did not influence the digestion rate.

The identification of fragments generated by RP-HPLC-ESI/MS allowed making some hypothesis on the enzymes that intervened during the digestions. In both cases, the bond Tyr_{193}-Gln_{194} at the N-terminus could be the first to be cleaved by an exopeptidase, subsequently followed by the bond Gln_{194}-Glu_{195}, always by the action of an exopeptidase. At the C-terminus, the action of exopeptidases could have been less marked than at the N-terminus because of the cleavage of the bond Pro_{206}-Ile_{207} by an endopeptidase that released the fragment β-CN (207-209). The endopeptidases could be responsible also of the cleavage of the bonds Leu_{198}-Gly_{199} and Arg_{202}-Gly_{203}. In particular, the endopeptidases generated the fragment β-CN (195-202) that resisted to the proteolytic action of vesicles enzyme up to the end of digestion for pBBMV and up to 180 min for wpBBMV.

The assessment of digestion by monitoring the free amino groups ($-NH_2$) and FAAs amount in function of time allowed the comparison of the endopeptidases activity to the activity of exopeptidases (Fig. 6.3.1.1.). At the end of the digestion time, the amount of free amino groups overlapped the quantity of FAAs in both wpBBMV and pBBMV, demonstrating that all the generated fragments (quantified as $-NH_2$ groups) were completely hydrolyzed to free amino acids (quantified as FAAs). Thus, the exopeptidases activity mainly contributed to the digestion of the peptide β-CN (193-209).

It is of note that 8 hours of digestion is not necessarily representative of a digestion process *in vivo* [360], because the *ratio* between the concentration of the peptide/ and the concentration of BBMV has been selected to better monitor the digestion kinetics and understand the mechanisms and possible differences in the pattern of digestion between the adult (pBBMV) and the infant models (wpBBMV).

The peptide β-CN (193-209) has to remain intact in the intestine to express its biological activity *in vivo*, while in the present work, the peptide was completely digested by BBMV enzymes. The BBMV represents an *in vitro* model of the BBM of the enterocytes, enriched in proteolytic enzymes in comparison to the intestinal

enterocytes. For this reason, it could be possible that in physiological conditions and transit time, β-CN (193-209) peptide would resist to complete hydrolysis.

A better knowledge of the digestion of bioactive peptides is needed to understand their *in vivo* stability in the intestinal tract, and the mechanisms of interaction with the intestinal mucosa [360]. With this purpose, the present study on the *in vitro* simulated BBM digestion of the immunomodulatory peptide β-CN (193-209) permitted to clarify the enzyme category that mostly intervene in the digestion of this peptide and the most probable cleavage sites in its sequence.

In conclusion, the present work demonstrated that BBMV completely degraded β-CN (193-209) peptide and that the most involved enzymes are exopeptidases. In addition the present work confirmed the usefulness of BBMV as a tool to understand the mechanisms that determine the digestion profile of the bioactive β-CN (193-209). Further work is needed to integrate all the data regarding the stability of this peptide and other similar bioactive peptides in different *in vitro* gastrointestinal models to identify the most important characteristics that contribute to bioactive peptide bioavailability.

6.5. Take-home message

The present study demonstrated that the immunomodulatory peptide β-CN (193-209) obtained from bovine β-casein is completely digested by the BBM enzymes of porcine BBMV, an *in vitro* model for the intestinal epithelium. This is an important result that integrates the data obtained on the digestion of the peptide achieved from the Experiment 3, using Caco-2 monolayers. However, these results should be completed with other *in vitro* models that obviate the limitations of Caco-2 cell line and BBMV, and that better represent the physiological conditions of the digestion process. In this sense, it can be hypothesized that primary cell cultures

from porcine gut mucosa would give helpful data because they are more similar in their phenotype to the mature enterocyte.

GENERAL DISCUSSION

7.1. Studies on the digestion and absorption of bioactive peptides

The Experiments 3 and 4 explored how the immunomodulatory peptide β-CN (193-209) generated from β-casein is digested and absorbed in two models for the human GI tract. In particular, the important result of this work is that this peptide could resist to hydrolysis by intestinal proteases and peptidases and a part of the starting amount given to the cells could by absorbed intact by the cell monolayer. However, a lot of work should be done to better understand the mechanisms that permitted the absorption, and the enzymes that primarily intervene in the digestion of the model peptide β-CN (193-209) should be identified.

At the moment many models for human digestion and absorption of different nutrients in the GI tract are available (see Paragraphs 2.4. and 2.5.), but none of them is specifically made for the evaluation absorption and digestion of bioactive peptides. The main characteristics that should be taken into consideration in the realization of a specific model should be the role of the proteases and peptidases of the GI tract in liberating bioactive peptides that are not already present in a free and active form in food matrices and in hydrolyzing some other bioactive peptides already present in the food.

It may be hypothesized that the already established dynamic models or the computer-controlled system that at present better represent the human GI complex physiology should be first modified for the integrated study of digestion and absorption and also characterized for the most important enzymes involved in the generation and stability of bioactive peptides.

Some useful implementations should be directed also in the field of *in vitro* models based on cell lines. A better prediction of absorption could be gained, if the apical pH is 5.5–6.5 and this can be achieved without compromising the integrity of Caco-2

cell monolayers, as demonstrated by Palm and colleagues [289] and Yamashita and colleagues [290]. The change in pH has been evaluated in n-in-one permeability studies for passively permeated drugs [291] and the authors found that Caco-2 cells better mimicked the *in vivo* conditions and gave more reliable information about the absorption of drugs across the enterocytic membrane, so it could be hypothesized that the same more reliable results could be also obtained in the case of the evaluation of bioactive peptide absorption.

Complementary information on the absorption of bioactive peptides could be gained from cell lines other than Caco-2. It is well known [292] that permeabilities of compounds that are transported via carrier-mediated absorption are lower in the Caco-2 cell system as compared to the human small intestine, probably reflecting the colonic origin of this cell line. In recent years several mucus-producing goblet cell sublines have been established from human intestinal HT29 cells, as HT29-MTX [293-295], a cell population that consists exclusively of differentiated, gastric-like mucus secreting, goblet-type cells that retain their differentiated phenotype after reversion to a methotrexate (MTX)-free medium and they also can be grown in monolayers. At present these cells lines are mostly used for drug absorption studies but it cannot be excluded a potential application in bioactive peptide absorption evaluation. A possible implementation of the Caco-2 cell line model could be the use of cell lines transfected with a specific oligopeptide transporter for the evaluation of the structural features required for interaction and transport. In addition, helpful information could be acquired using primary cell cultures isolated from the intestine mucosa, an expanding research area, as demonstrated by the increasing number of reports focusing in particular on bovine intestine cell culture [371-373].

Some studies on the digestion and absorption of bioactive peptides derived from milk-proteins, with special attention to ACE-inhibitory ones, have already demonstrated that some bioactive tripeptides could be absorbed. For example, VPP was detected in the abdominal aorta of spontaneous hypertensive rats (SHR) 6 hours after its administration in sour milk, which strongly suggests that it is transepithelially transported [260]; more recently the absorption was observed also in humans [6]. Paracellular transport, through the intercellular junctions, was suggested as the main mechanism, since the transport via the short-peptide carrier, PepT1, led to a quick hydrolysis of the internalized peptide [186]. In the case of larger sequences, the susceptibility to brush border peptidases is the primary factor that decides the transport rate [244]. For example, the heptapeptide lactokinins (ALPMHIR) was transported intact, although in concentrations too low to exert an ACE-inhibitory activity, which suggests cleavage by aminopeptidases [261].

The Experiment 3 of the present thesis gave some insight on the absorption of long bioactive peptides. In fact, in the case of the peptide β-CN (193-209), the result obtained leaded to the hypothesis that its transport could be mainly mediated by transcytosis, even if a role of the paracellular transport could not be completely excluded. No data were obtained about a possible energy-dependent transporter for long peptides.

More research is needed in this respect, with the effort being concentrated in elucidating the pharmacokinetics and the distribution profile of milk-derived bioactive peptides in the different tissues.

7.2. The evaluation of the immunomodulatory activity of bioactive peptides

From the present thesis, in particular from Experiments 1 and 2, some questions have risen about the best method that would permit a reliable evaluation of the

effective activity of bioactive peptides and milk-derived products with a potential immunomodulatory action. Indeed, the immunomodulatory effects of yogurt (i. e. against cancer) have been studied, mostly using animal models [72, 78, 79, 97-103]. Conversely, few human studies on the immunostimulatory effects of yogurt and immunomodulatory peptides have been conducted [7]. Although the results of these studies mostly support the notion that yogurt has immunostimulatory effects, poor study design, lack of appropriate controls, and short duration of most of the studies limit the value of the conclusions that can be drawn from them. Most early animal and human studies included too few subjects in each group, and most of them did not include statistical analysis. Even in animal studies, the majority used short-term feeding protocols, which might induce a transient adjuvant effect rather than a long-term stimulation of the immune response. Furthermore, most studies investigated the effect of intravenous or intraperitoneal administration or *in vitro* application of yogurt and immunomodulatory peptides on different variables of the immune response but, as immunomodulatory peptides are consumed orally and they may be altered in the GI tract, the results of these studies may not reflect what would be found if the yogurt had been consumed orally.

The main problem however is that all the studies investigated the effects of immunomodulatory peptides or yogurt on *in vitro* indexes of the immune response (i.e. lymphocytes proliferation by DNA or protein synthesis, or antibody production, or cytotoxicity ability) and these parameters could not represent the complexity of the variables of the whole immune system *in vivo*. As a consequence, the preliminary result obtained on the immunomodulatory activity of the peptide YGG and on the milk fermented by *L. delb. bulgaricus* LA2 should be confirm in an *in vivo* model, taking into account the modification on the bioactive peptide operated by the GI tract and considering also the fact that the gut-associated immune system is increasingly being recognized as playing an important role in host defense. In fact, the M-cells of the Peyer's Patches dispersed in the intestinal mucosa may contribute

to the translocation of intact bioactive peptides across the intestinal epithelium, thus increasing the possibility of these peptides to act on the different subpopulations of intestinal cells {Otani, 1995 111 /id}. This aspect of the immune response is particularly relevant to determining the beneficial effects of bioactive peptides because their systemic effects may depend on the interaction of the peptides with the immune cells of the gut. However, the interactions between immunomodulatory peptides and the gut-associated immune system has been scarcely explored [51, 52, 132, 162] and thus expanding the knowledge in this field would be of extreme importance.

7.3. Future perspectives on the production of dairy food with ACE-inhibitory and immunomodulatory properties

Experiment 1 gave some preliminary and encouraging results about the possibility to generate fermented milk with ACE-inhibitory activity by a bacterial strain belonging to *E. faecalis* species. This result confirms the fact that bioactive peptides and milk-derived products with antihypertensive or immunomodulatory properties can be produced in different ways but fermentation with LAB is the preferred one. Expanding the knowledge about the proteolytic systems of interesting LAB, and their activity under various conditions, more specifically belonging to *E. Faecalis* species, could be a relevant step to improve the amount and the stability of ACE-inhibitory peptides in the dairy products [125]. In addition, further progress in this area might be obtained through genetic engineering, to provide the most suited strain with the desired proteolytic capacity, and also from studies regarding the interaction between strains in environments as those prevailing in fermented milks and cheeses [125].

Moreover, regardless the source containing bioactive peptides and the associated bioactivity, it is important that bioactive peptides must be stable during the final processing, packaging and storage. Furthermore, the hydrolysate should have well-

defined technological functionalities not to impart the required functionality of the carrier food [125].

Hence, more information must be acquired on the influence of food processing, preparation and preservation on the bioactivity of bioactive peptides.

CONCLUSIONS

The review of literature and the results obtained in the present thesis suggest that food microorganisms, isolated from food matrices, in particular of bacterial origin, act on the nutrients contained in the food. These microorganisms could thus generate functional foods enriched in specific components able to influence important physiological processes of the human body, as blood pressure or immune response. In this view, the present work explored the possibility to use *E. faecalis* TH563 to produce fermented milk with ACE-inhibitory activity and *L. delb. bulgaricus* LA2 to obtain fermented milk with immunomodulatory activity, even if it would be necessary to evaluate *E. faecalis* TH563 for safety aspects.

As a consequence, there is an increasing need to select the microorganism present in food matrices for their ability to produce functional food enriched in specific bioactivities on large scale. More research is thus needed to characterize the microorganisms and the associated bioactivities and to develop new methods permitting the unambiguous quantification of the bioactivity in the foodstuff and the identification of the food components responsible of such bioactivity. For example, it would be interesting to identify the presence of the peptides β-CN (193-209) and YGG in milk fermented by *L. delb. bulgaric*us LA2 to acquire better knowledge about the mechanisms determining the associated immunomodulatory activity.

With this purpose, the Experiments 1 and 2 have been realized. They aimed to study the immunomodulatory activity of the milk fermented by two bacterial strains frequently found in dairy products of the North-East of Italy and to clarify the mechanism of action of a milk-derived peptide with already documented immunomodulatory activity on lymphocytes, considered as a model peptide, as the peptide YGG derived from α-lactalbumin. The present work demonstrated that YGG modulated bovine lymphocyte proliferation and that this effect is dependent upon serum concentration and on the presence of lymphocyte activators, such as

concanavalin A, in the culture medium. Nevertheless, it has been observed that the YGG effects on lymphocyte proliferation did not seem to be mediated by a modulation of the RNA expression of IL2 and INFγ, two important cytokines involved in lymphocytes activation and proliferation. The obtained results, together with the Paragraph 2.3.2., demonstrate that the *in vitro* methods manifest some limitations in the characterization of immunomodulatory bioactivity and that an exhaustive view of the action of immunomodulatory peptides could be achieved only by a multi-view approach that should take into account the complexity of the interactions between the bioactive peptide and the different components of the immune system *in vivo*. In fact, the Experiment 1 and the Paragraph 7.1. in the General Discussion section evidence the lack of knowledge about the interaction of the immunomodulatory peptides derived from food and the immune system dispersed along the GI tract (as GALT, Peyer's Patches, antigen-presenting cells) that could represent a potential target of immunomodulatory peptides, even before to be absorbed at gut level and circulate in the body.

At the moment the interactions between food-derived peptides and the gut-associated immune system have been explored to elucidate the mechanisms underlying allergies but it would be interesting to apply the same approach to evaluate the bioactivities, considering both allergies and bioactivities as properties that could be displayed by peptides.

The present thesis focused also on the physiology of absorption of bioactive peptides and demonstrated for the first time that a long hydrophobic bioactive peptide crossed intact a Caco-2 cell monolayer, a well recognized *in vitro* model for the intestinal epithelium. In fact, the milk-derived immunomodulatory peptide β-CN (193-209) was demonstrated to be resistant to the digestion of gastrointestinal peptidases and to pass intact across Caco-2 cells. In addition, the digestion profile of this peptide has been studied in brush border membrane vesicles.

This interesting result permits to suggest that even large peptides could be absorbed in small quantities and that it cannot be excluded that at these concentrations the peptide β-CN (193-209) could interact with the gut-associated immune system, as explained before.

As a consequence, the assessment of the digestion profile and of the mechanism of absorption of β-CN (193-209) could be considered as model studies for the evaluation of the bioavailability of bioactive peptides, such as YG peptide. In fact, it would be helpful to examine the bioavailability of this bioactive peptide, checking for the resistance to gastrointestinal and serum peptidases. For example, it would be interesting to identify the presence of the peptides β-CN (193-209) and YGG in milk fermented by *L. delb. bulgaricus* LA2 to acquire better knowledge about the mechanisms determining the associated bioactivity.

In conclusion, new questions have arisen on the area of bioactive peptides that could constitute the objective of further research studies in the future.

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