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Poultry Industry Waste:
Protein hydrolizates as growth stimulator for microorganisms potentially probiotic.

Ph. D. thesis

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Things need their own time.

When that moment will arrive, you will forget to have waited.

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1 Introduction

The interest of markets for fermented foods has increased in recent years thanks to the positive perception of their impact on consumer health. The production of fermented foods is one of the oldest food preservation technologies known to man. Fermentation not only increases shelf life and microbiological safety of foods but also makes some foods more digestible (Caplice & Fitzgerald, 1999). Lactic acid bacteria (LAB) are mainly involved in the production of fermented foods: they are used as starter cultures for different foods such as dairy, meat, vegetables and cereals (Yamamoto et al., 2003; Caplice & Fitzgerald, 1999). At the beginning of the twentieth century E. Metchnikoff was the first who linked the consumption of fermented milk with health maintenance and improvement. Nowadays consumers and food industry are more conscious about the relationship between health and diet. The result is the increasing expansion of the market of functional foods, dietary supplements and nutraceuticals (Vasiljevic & Shah, 2008). Among these products, probiotics and prebiotics deserve a special attention because of their scientifically supported health promoting properties. They are recognized throughout the developed world (Saad, 2013). The global market for probiotics is in fast growth since the early 2000's: it was estimated to 15.9 billon US$ in 2008 and is forescast to reach US$ 28.8 in 2015 (Granato et al., 2010). Among the countries that have shown growth in the probiotic market, Europe represents the larger and faster market followed by Japan (Granato et al., 2010). Probiotic microorganisms, normally used, are LAB and bifidobacteria (WHO/FAO, 2001; Mercenier et al., 2003). LAB constitute a group of bacteria that comprehend several morphological, metabolic, and physiological characteristics. The general description of the bacteria included in the group is gram-positive, non-sporing, non-respiring cocci or rods, which produce lactic acid as the major end product during the fermentation of carbohydrates. The following genera are considered the principal LAB: *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus,* and *Weissella*. The genus *Bifidobacterium* although is often considered belonging to LAB group and it shares some of their features, is phylogenetically unrelated, it belongs to Actinobacteria, and has a unique mode of sugar fermentation (Ludwig and Klenk, 2001).

In this PhD thesis only *Lactobacillus* and *Bifidobacterium* genera will be considered since these genera are by far the most important with probiotic strains for human use (Saarela et al., 2000; Holzapel et al., 1998; Klein et al., 1998).

1.1 *Lactobacillus* Genus

The genus *Lactobacillus* is very heterogeneous, including several species different in phenotypic, biochemical, and physiological features. The genus *Lactobacillus* belongs to the phylum Firmicutes, Class Bacilli, Order Lactobacillales, Family Lactobacillaceae and its closest relatives, being grouped within the same Family, are the genera *Paralactobacillus* and *Pediococcus* (Garrity et al., 2007). The heterogeneity is reflected by the range of low G+C DNA content (< 50%) of the DNA of the species included in the genus (Schleifer and Stackebrandt, 1983). Lactobacilli are Gram-positive bacteria, unable to sporulate, occurring as rods or cocco-bacilli (Hammes and Vogel, 1995). They are catalase negative, even if pseudocatalase activity can sometimes be present in some strains and in presence of a heme group (Felis and Dellaglio, 2007). They are almost ubiquitous and can be found in almost all the environments where carbohydrates are available, such as food (dairy products, fermented meat, sourdoughs, vegetables, fruits, beverages), respiratory, gastro-intestinal (GI) and genital tracts of humans and animals, sewage and plant material (Felis et al., 2009). Glucose is fermented predominantly to lactic acid in the homofermentative case, or equimolar amounts of lactic acid, CO2 and ethanol (and/or acetic acid) in the heterofermentative counterpart (Gomes & Malcata, 1999).

1.1.1 Carbohydrate metabolism

Table 1 shows how the *Lactobacillus* species can be arranged in three different groups on their metabolic characteristics. The principle for the division is the presence or absence of the key enzymes of homo- and hetero-fermentative sugar metabolism, fructose-1,6-diphosphate (FDP) aldolase and phosphoketolase, respectively (Axelsson, 2004).

Table 1. Arrangement of genus Lactobacillus (modified from Axelsson, 2004)

Characteristic	Group I, Obligately homofermentative	Group II, Facutltatively heterofermentative	Group III, Obligately heterofermentative
Pentose fermetation	-	+	-
CO2 from glucose	-	-	+
CO2 from gluconate	-	+(a)	+(a)
FDP* aldolase present	+	+	-
Phosphoketolase present	-	+(b)	+
	Lb. acidophilus	*Lb. casei*	*Lb. brevis*
	Lb. delbrueckii	*Lb. plantarum*	*Lb. fermentum*
	Lb. helveticus	*Lb. curvatus*	*Lb. reuteri*
	Lb. salivarius	*Lb. sakei*	*Lb. buchneri*

(a) When fermented.
(b) Inducible by pentose.
*fructose di-phosphate.

Regarding sugar fermentation there are two major pathways for hexose (e.g. glucose) fermentation among lactobacilli (Fig. 1). Both require the activation of sugars with a high-energy phosphate bond. Glycolysis (Embden-Meyerhof-Parnas pathway) is the most common pathway among LAB and it is typical of group I and group II of lactobacilli. It is characterized by the formation of fructose-1,6-diphosphate (FDP), which is split by a FDP aldolase into dihydroxyacetonephosphate (DHAP) and glyceraldehyde-3-phosphate (GAP). GAP (and DHAP via GAP) is then converted to pyruvate in a metabolic sequence including substrate-level phosphorylation at two sites.

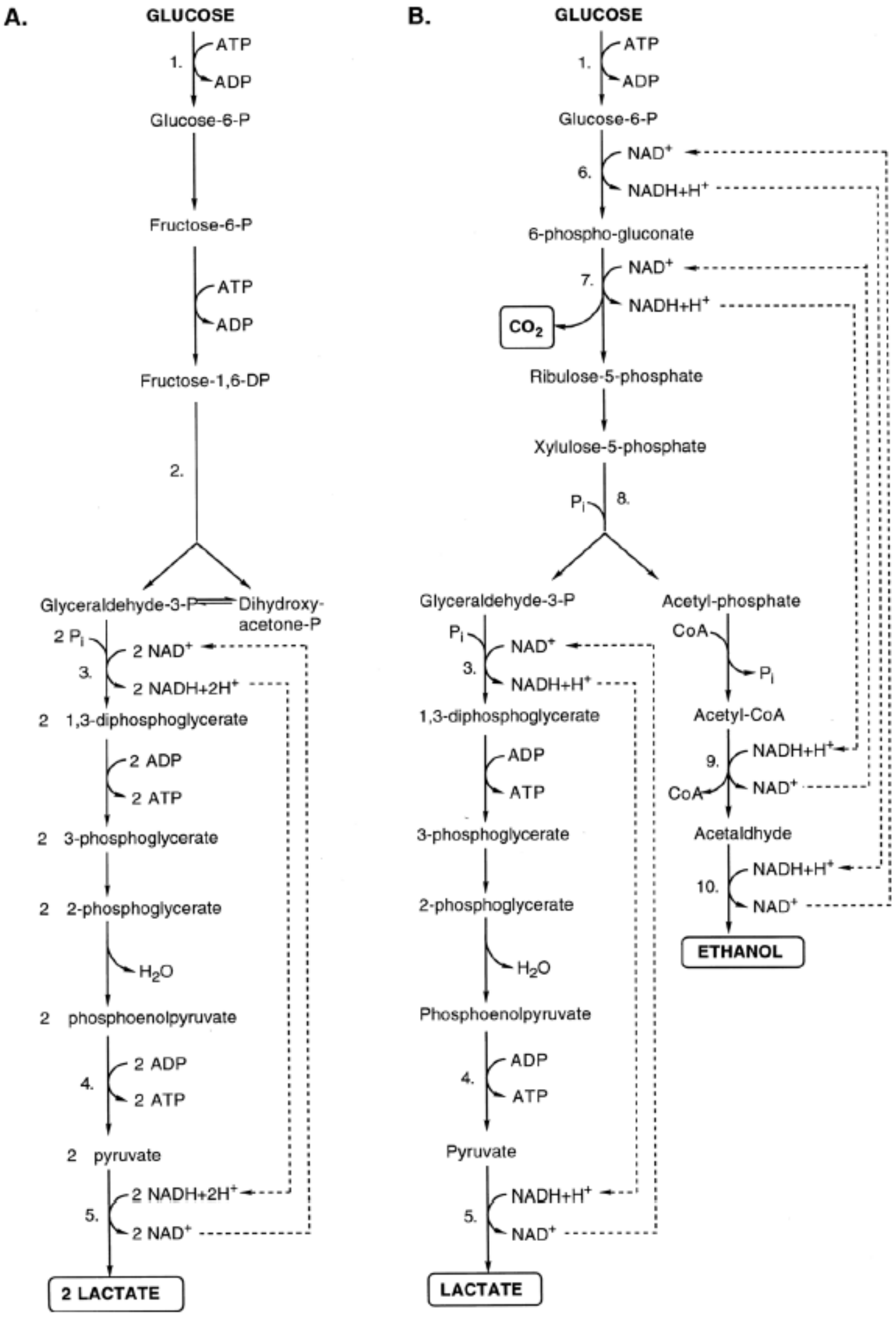

Figure 1 Major fermentation pathways of glucose: (A) homolactic fermentation (glycolysis, Embden-Meyerhof-Parnas pathway); (B) heterolactic fermentation (6-phosphogluconate/phosphoketolase pathway). Selected enzymes are numbered: 1. Glucokinase; 2. fructose-1,6-diphosphate aldolase; 3. glyceradehyde-3-phosphate dehydrogenase; 4. pyruvate kinase; 5. Lactatedehydrogenase (LDH); 6. glucose-6-phosphate dehydrogenase; 7. 6-phosphogluconate dehydrogenase; 8. phosphoketolase; 9. acetaldehyde dehydrogenase; 10. alcohol dehydrogenase (from Axelsson, 2004).

Under normal conditions, i.e., sugar excess and limited access to oxygen, pyruvate is reduced to lactic acid by a NAD$^+$-dependent lactate dehydrogenase (LDH), thereby re-oxidizing the NADH formed during the earlier glycolytic steps. A redox balance is thus obtained, lactic acid is virtually the only end product, and the metabolism is referred to as homolactic fermentation (Fig. 1A). The other main fermentation pathway is the 6-phosphogluconate-phosphoketolase pathway (Kandler and Weiss, 1986). It is characterized by initial dehydrogenation steps (Fig. 1B) with the formation of 6-phosphogluconate, followed by decarboxylation. The remaining pentose-5-phosphate is split by phosphoketolase into GAP and acetyl phosphate. GAP is metabolized in the same way as for the glycolytic pathway, resulting in lactic acid formation. When no additional electron acceptor is available, acetyl phosphate is reduced to ethanol via acetyl CoA and acetaldehyde. Since this metabolism leads to significant amounts of other end products (CO2, ethanol) in addition to lactic acid, it is referred to as heterolactic fermentation. Homolactic fermentation of glucose results in 2 mol of lactic acid and a net gain of 2 ATP per mol glucose consumed. Heterolactic fermentation of glucose through the 6-PG/PK pathway gives 1 mol each of lactic acid, ethanol, and CO2 and 1 mol ATP/mol glucose.

1.1.2 Nitrogen metabolism

In this regard one of the most extensively studied system is the proteolytic system of dairy LAB in particular of *Lactococcus lactis*. The reason is, of course, the technological significance in milk fermentation, as it has been shown that a proteolytic system is necessary for appreciable and rapid growth in milk (Kunji et al., 1996; Christensen et al., 1999). LAB have a very limited capacity to synthesize amino acids using inorganic nitrogen sources therefore they need as a nitrogen source preformed amino acids in the growth medium (Axelsson, 2004). Undoubtedly, the most important application of LAB is their use as starter strains in the manufacture of various fermented dairy products: in particular, *Streptococcus thermophilus* and *Lactococcus lactis* are widely used dairy starters and are of major economic importance (Savijoki et al., 2006) . For what concern lactobacilli several species are used as components of starter cultures for the production of fermented milk and various kinds of cheeses. *Lactobacillus delbrueckii ssp. bulgaricus, Lactobacillus helveticus, Lactobacillus paracasei* are of great importance for the dairy/cheese industry (Ummadi and Curic-Bawden, 2010). Moreover, selected strains of *Lactobacillus acidophilus, Lactobacillus johnsonii* and *Lactobacillus reuteri* are exploited because of their probiotic properties. The requirement for amino acids differs among the species and strain variations exist within species (Morishita et al., 1981). Several studies have been performed in order to understand the general nutritional requirements of *Lactobacillus* spp. Elli et al. (2000) and Chervaux et al. (2000) described the nutrient necessities of 22 *Lactobacillus* strains using chemically defined medium, which contains 21 amino acids and other nutrients. In general, for optimal growth and viability, these lactobacilli require fermentation media supplemented with abundant carbon and nitrogen sources, vitamins, micro and macronutrients and nucleotides bases.

In 2007 Horn and colleagues obtained similar results. They found that *Lactobacillus plantarum* grows better than *Lactobacillus sakei* in a medium containing fish peptones. They assumed that the differences were related to the high nutritional requirements of *Lactobacillus sakei. Lactobacillus plantarum* needs arginine, leucine, isoleucine, tyrosine, valine, and pantothenic acid for growth, while *Lactobacillus sakei* has several additional requirements (lysine, methionine, riboflavin, and nicotinic acid), which may explain why *Lactobacillus sakei* grows less well in a medium containing fish peptone (Møretrø et al. 1998). *Lactobacillus sakei* is known to have the most fastidious nutritional requirements of all the lactobacilli (Lauret et al. 1996). Hence, a medium supporting growth of *Lactobacillus sakei* should permit growth of all other lactobacilli (Safari et al., 2009). The proteolytic system of lactococci is more investigated because of their role in cheese/dairy production and the extent of this knowledge can be transferred in some degree to other LAB (Christensen et al., 1999). The degradation of proteins by LAB, able to do it, starts with the action of extracellular proteases. Cell-envelope proteases (PrtP) are critical for growth of LAB in milk (Romero and Klaenhammer, 1993), because they hydrolyze casein into 100 smaller peptide fragments. PrtP is synthesized as an inactive precursor molecule and requires a membrane-bound lipoprotein (PrtM) for its autocatalytic maturation process (Haandrikman et al., 1991). Altermann (2005) and colleagues found that the silico analyses revealed the presence of both PrtP (La1512) and PrtM (La1588) in *Lactobacillus acidophilus* NCFM, sharing significant similarities to *Lactobacillus gasseri, Lactobacillus johnsonii, Lactobacillus rhamnosus, Lactobacillus plantarum* WCFS1 and *Lactobacillus paracasei*. Five different types of these enzymes were cloned and characterized from LAB, including PrtP from *L. lactis* and *Lactobacillus paracasei*, PrtH from *Lactobacillus helveticus*, PrtR from *Lactobacillus rhamnosus*, PrtS from *Streptococcus thermophilus*, and PrtB from *Lactobacillus bulgaricus* (Kok et al. 1988; Holck and Naes 1992; Gilbert et al. 1996; Pederson et al. 1999; Siezen 1999; Fernandez-Espla et al. 2000; Pastar et al. 2003).

Peptide uptake occurs via oligopeptide transport systems (Opp system), and di-/tri-peptide transporters. In addition, various amino acid transport systems have been identified with a high specificity for structurally similar amino acids (Peltoniemi et al., 2002; Charbonnel et al., 2003). The Opp proteins belong to a superfamily of highly conserved ATP-binding cassette transporters that mediate the uptake of casein-derived peptides (Higgins, 1992). The Opp system of

Lactococcus lactis transports peptides up to at least 18 residues and the nature of these peptides significantly affects the transport kinetics involved (Detmers et al., 1997; Juillard et al., 1998). Opp systems observed for other LAB demonstrate to be similar to that described for *Lactococcus* (Garault et al., 2002; Peltoniemi et al., 2002). Then the cells take up the casein-derived peptides that are degraded by a concerted action of peptidases with differing and partly overlapping specificities (Kunji et al., 1996). The intracellular endopeptidases, general aminopeptidases (PepN and PepC), and the Xprolyl dipeptidyl aminopeptidase (PepX) are the first enzymes to act on oligopeptides. Several endopeptidases were characterized from LAB. They are all metallopeptidases, like PepO and PepF, with the exception of the *Lactobacillus helveticus* PepE, which was shown to exhibit a thiol-dependent activity (Fenster et al., 1997). A common feature of endopeptidases is their ability to hydrolyze internal peptide bonds but they cannot hydrolyze intact proteins, for instance casein. The broad specificity metallopeptidase PepN and cysteine peptidase PepC are capable of acting on oligopeptides and they have been characterized from diverse LAB strains. Collectively, these enzymes can remove the N-terminal amino acids from a peptide: their specificity depending on the peptide length and the nature of the N-terminal amino acid residue (Kunji et al., 1996; Christensen et al., 1999). Di/tripeptides generated by endopeptidases, general aminopeptidases, and PepX are next subjected to additional cleavage by the tripeptidase, PepT, and dipeptidases, PepV and PepD. These enzymes are specific for peptides containing hydrophobic amino acids including leucine, methionine, phenylalanine, or glycine. An enzyme possessing specificity toward di/tripeptides with N-terminal leucine residues and dipeptides containing proline was biochemically characterized from *Lactobacillus delbrueckii* subsp. *bulgaricus* (Klein et al., 1995). Other peptidases with more specific substrate specificities include: PepA, which liberates N-terminal acidic residues (glutammic acid and aspartic acid) from peptides that are three to nine residue long; PepP, which acts tripeptides carrying proline in the middle position; PepR and PepI, which act on dipeptides containing proline in the penultimate position; PepQ, which cleaves dipeptides carrying proline in the second position; and PepS, which shows preference for peptides containing two to five residues with Arg or aromatic amino acid residues in the N-terminal position (Kunji et al., 1996; Christensen et al., 1999; Fernandez-Espla and Rul, 1999).

1.2 *Bifidobacterium* Genus

Bifidobacteria were first isolated and described in 1899-1900 by Tissier, who described rod-shaped, non-gas-producing, anaerobic microorganisms with bifid morphology, present in the faeces of breast-fed infants, which he termed *Bacillus bifidus*. Bifidobacteria are generally characterized as gram-positive, non-spore forming, non-motile and catalase-negative (Sgorbati et al., 1995). Bifidobacteria are heterofermentative and most strains are strictly anaerobic; some bifidobacteria can tolerate oxygen (Shimamura et al., 1992; Nebra and Blanch, 1999). *Bifidobacterium* genus is not included in the traditional LAB group due to its genetic unrelatedness (Klijn et al., 2005), but the bacterium is often found in the same LAB's habitats, and its metabolism produces lactic acid as an end-product. For these reasons bifidobacteria are often included in the LAB family even though they are phylogenetically distinct with a G+C content ranging from 42% to 67% (Biavati et al., 2001). The genus *Bifidobacterium* belongs to the family of *Actinomycetaceae*, which also comprises corynebacteria, mycobacteria and streptomyces. With the exception of species isolated from human dental caries, sewage or insects, the majority of bifidobacteria species are found in the gastrointestinal tract (GIT) of mammals (Klijn et al., 2005). Bifidobacteria constitute the major part of the normal intestinal human microflora. They appear in the stools a few days after birth and increase in number thereafter especially in the breast-fed infants (Ishibashi and Shimamura, 1993; Heinig and Dewey, 1996). They are predominant in the large bowel contributing to 10% of the intestinal microflora in adults (Turroni et al., 2008), but a decrease in these levels of bifidobacteria is showing with age. There are many species of bifidobacteria such as *Bifidobacterium adolescentis, Bifidobacterium angulatum, Bifidobacterium bifidum, Bifidobacterium breve, Bifidobacterium catenulatum, Bifidobacterium denticolens, Bifidobacterium dentium, Bifidobacterium gallicum, Bifidobacterium infantis, Bifidobacterium inopinatum, Bifidobacterium lactis, Bifidobacterium longum, Bifidobacterium pseudocarenulatum* (Gomes and Malcata, 1999).

1.2.1 Carbohydrates metabolism

Bifidobacteria are saccharoclastic organisms that produce acetic acid and lactic acid without generation of carbon dioxide, except during degradation of gluconate (Figure 2). Heterofermentation, by fructose 6-phosphate phosphoketolase (F6PK), pathway is initiated by splitting fructose 6-phosphate into one C2 and one C4 moiety. The conversion of the C2 moiety to acetate is paralleled by the formation of heptose 7-phosphate from the C4 moiety concomitant with the formation of a triose moiety derived from an additional molecule of fructose 6-phosphate. The heptose 7-phosphate is subsequently split into two molecules of acetate and one molecule of pyruvate. The second triose moiety left from fructose 6-phosphate is converted into lactate. Therefore, the fermentation of two moles of hexose results in three moles of acetate and two moles of lactate. The key enzyme in this glycolytic fermentation is fructose 6-phosphate phosphoketolase. Besides glucose, all bifidobacteria from human origin are also able to utilise galactose, lactose and usually, fructose as carbon sources. *Bifidobacterium* spp. are, in some instances, also able to ferment complex carbohydrates (Shah and Iankaputhra, 1997).

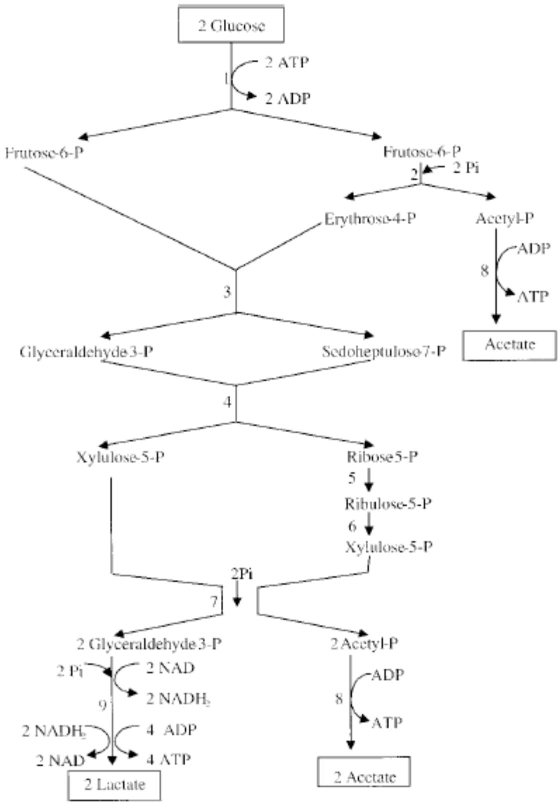

Figure 2. Formation of acetate and lactate from glucose by the bifidum pathway. 1-hexokinase and fructose-6-phosphate isomerase, 2-fructose-6-phosphate phosphoketolase, 3-transaldolase, 4-transketolase, 5-ribose-5-phosphate isomerase, 6-ribulose-5-phosphate-3-epimerase, 7-xylulose-5-phosphoketolase, 8-acetate kinase, 9-enzymes as in homofermentative pathway (adapted from Rasic and Kurmann, 1983).

1.2.2 Nitrogen metabolism and requirements

Bifidobacterial proteolytic systems are somewhat poorly investigated in contrast to the huge research works about LAB proteolytic system. Aminopeptidase and iminopeptidase activities were determined in a cell extract of *Bifidobacterium breve* by Cheng and Nagasawa (1985), Seo et al. (2007) demonstrated that *Bifidobacterium longum* cell extracts possess a dipeptidase, pepD. ElSoda et al. (1992) have reported general caseolityc activity for *Bifidobacterium infantis* and *Bifidobacterium longum*. Moreover *Bifidobacterium longum, Bifidobacterium infantis, Bifidobacterium adolescentis* showed activities referable to a X proyl dipeptidil peptidase, an iminopetidase, di and tri peptidases. Amino, di-, tri-, and carboxypeptidase activities of

Bifidobacterium longum subsp. *Infantis, Bifidobacterium longum* subsp. *longum* and *Bifidobacterium adolescentis* have been demonstrated by Seo et al. (2007). A genomic study reported by Schell et al. (2002) describes that *Bifidobacterium longum* subsp. *longum* NCC2705 genomes encodes more than 20 peptidases as well as oligopeptide transporters. Most strains contain a leucine aminopeptidase, while a few have a valine aminopeptidase (Desjardins et al., 1990). Differently from *Lactobacillus* species, cell wall-associated proteinase such as a PrtP has not been identified in the bifidobacteria (Janer et al., 2004). Accordingly, bifidobacteria often grow poorly in pure milk as they lack extracellular proteolytic capacity exhibited by LAB (Shah, 2000; Seo et al., 2007). Most bifidobacteria can use ammonium salts as their only source of nitrogen (Azaola et al., 1999), but supplementation of peptides and amino acids is considered a requirement for the economical production of these strains (Ummadi and Curic-Bawden, 2010). Nitrogen requirements are known to be usually strain dependent but the typical nitrogen sources are peptides/amino acids, cysteine and ammonium salts. Other nitrogen containing molecule that could be added is N-acetyl-glucosammine which is essential for cell wall production (Exterkate and Veerkamp, 1969).

1.2.3 Promoting factors

Bifidobacteria are fastidious bacteria that require complex and expensive media for propagation, such as Man Rogosa and Sharpe medium (de Man et al., 1960) supplemented with growth-promoting factors (Doleyres et al., 2002). The growth of bifidobacteria seems to be positively affected by the presence of growth promoting factors. Poch and Bezkorovainy (1988) supplemented an entirely synthetic minimum base medium with growth factors (for example bovine casein digest, yeast extract, human milk whey) in order to identify those essential to the development of the various species of *Bifidobacterium*. Only *Bifidobacterium adolescentis* and *Bifidobacterium longum* were able to develop in the unsupplemented medium. All the other species required the presence of growth factors of various types. Most species of the genus *Bifidobacterium* are unable to develop in a totally synthetic medium and require complex biological substances such as bovine casein digestate, lactoserum of bovine milk, porcine gastric mucin, or yeast extract (Poch and Bezkorovainy, 1988; Petschow and Talbott, 1990). Two types of promoting factors are recognised: **growth factors** which are metabolized by the body, or by the microflora, of the upper gastrointestinal tract (for example, threonine, yeast extract, cysteine, peptone, dextrin, maltose, and β-glycerophosphate) and **bifidogenic factors,** which are substances that survive direct metabolism by the host and reach the large intestine (Modler et al., 1994). These factors include lacteal secretions (N-acetylglycosamine-containing saccharides), fructooligosaccharides, lactoferrin, lactulose and lactitol, oligoholosides and polyholosides (raffinose, stachyose, and insulin), xylooligosaccharides (D-xylan), and transgalactosylatedoligosaccharides (isogalactobiose, galsucrose, and lactosucrose) (Ventura et al., 2004). Promoting factors can also be non-glycosylated peptides derived from protein after hydrolysis using proteinase (Tamime et al., 1995; Zhao et al., 1996). A number of studies have reported that proteinaceous compounds found in human milk (Liepke et al., 2002) and cow milk (Petschow and Talbott, 1990) have the ability to promote the growth of bifidobacteria. Molecules that are present in dairy industry effluents such as whey (Mahalakshmi and Murthy, 2000) and by-products of latex rubber production also have this property (Ishizaki 1989; 1995; Oiki et al., 1996; Etoh et al., 2000). The factors with general activity are hydrolysates of bovine casein and yeast extracts rather than human milk lactoserum. The other growth factors, human or bovine milk lactosera, porcine gastric mucin, and bovine albumin serum digestate, are active with regard to certain species only (Poch and Bezkorovainy, 1988). For what concern casein the disulfide/ sulfhydryl residues of k-casein are important biologically active compounds responsible for this phenomenon in *Bifidobacterium bifidum* and *Bifidobacterium longum*. The growth-promoting activity resides in the k-casein portion and not in the carbohydrate portion after trypsin digestion. It appears that the combination of disulfide/sulfhydryl residues with something else is the basis of the microbial growth-promoting activity in hydrolysates of casein, porcine gastric mucin, and yeast extract (Poch and Bezkorovainy, 1988).

1.3 *Lactobacillus* and *Bifidobacterium* cultures: Industrial applications

1.3.1 Probiotic cultures

Metchnikoff is considered to be the inventor of probiotics. It is probably from his work (1908) that the first scientific considerations on probiotics were made. Metchnikoff noticed the longevity of the Caucasian population and its frequent consumption of fermented milks, and he proposed that the acid-producing organisms in fermented dairy products could prevent the proliferation of enteric pathogens, and the subsequent intoxication of the large intestine, thus leading to a prolongation of the consumer's life. In Japan Dr. Minoru Shirota (1899-1982) began investigating LAB. At the beginning of the 20th century the Japanese living conditions were substandard due to malnutrition and poor sanitation, which resulted in a range of digestive disorders and general ill health. During his research, Dr. Shirota became convinced that LAB were the key to a long, healthy life and had the potential to improve health standards. His determination led to a breakthrough in

1930, when he successfully researched a unique lactic acid bacterium with the ability to survive the acidic conditions of the stomach. This enabled the bacterium to reach the intestines alive where it helps to make a positive contribution to the balance of intestinal flora. The bacterium was named the *Lactobacillus casei* Shirota strain. Dr. Shirota developed a milk-based drink to deliver the *Lactobacillus casei* Shirota strain to the gut and named it 'Yakult', creating the world's first commercial probiotic drink (http://www.yakult.co.jp). Probiotic is a relatively new word meaning "for life" and it is generally used to name the bacteria associated with the beneficial effects for the humans and animals. It was probably Vergio (1954) who first introduced the term "probiotic" in his manuscript "Anti- und Probiotika": he compared the detrimental effects of antibiotics and other antimicrobial substances on the gut microbial population, with factors ("Probiotika") favourable to the gut microflora. In the 1965 Lilley and Stillwell used the word "probiotic" referred to something that promotes the growth of other microorganisms in opposition to the term antibiotic. Only at the end of the century, it become clear that intestinal microflora had several important functions (Del Piano et al., 2006). At the end of the 80's Fuller (1989) redefined the meaning of "probiotic" as live microbial feed supplements that beneficially affect the host animal by improving its intestinal microbial balance. Such microorganisms may not necessarily be constant inhabitants of the GIT, but they should have a beneficial effect on the general and health status of man and animal (Fuller, 1989; Havenaar et al., 1992; Salminen et al., 1998). More recently FAO/WHO gave a new meaning to this word, defining "a probiotic as a live microorganisms that when administered in adequate amount confer a health benefit on the host". This definition of probiotic has restricted the use of the word "probiotic" to those products that not only contain live microorganisms but also provide an adequate dose of probiotic bacteria in order to exert the desirable effects (FAO/WHO, 2001). The Japanese Fermented Milks and Lactic Acid Bacteria Beverages Association stated a standard for products to be denominated as a probiotic food. These products have to contain more or equal to 10^7 CFU/ml of probiotic microorganisms at the end of shelf-life (Ishibashi and Shimamur, 1993). Many microorganisms can be used as probiotic such as bacteria, yeasts or moulds (Ouwehand et al., 2002). Currently, probiotic microorganisms normally used are predominantly LAB and bifidobacteria (WHO/FAO, 2001; Mercenier et al., 2003).

1.3.1.1 *Probiotic strain features*

While selecting a probiotic strain several aspects have to be considered:

- Safety features;

- Functionality features;
- Technological aspects.

First of all safety aspects include the following specifications:

(a) Strains for human use are preferably of human origin; (b) they are isolated from healthy human GIT; (c) they have a history of being non-pathogenic; (d) they have no history of association with diseases such as infective endocarditis or gastrointestinal disorders; (e) they do not carry transmissible antibiotic resistance genes (Morgensen et al., 2002).

The functional requirements of probiotics should be considered:

(a) Acid tolerance and tolerance to human gastric juice; (b) bile tolerance (an important property for survival in the small bowel); (c) adherence to epithelial surfaces and persistence in the human GIT; (d) immunostimulation, but no pro-inflammatory effect; (e) antagonistic activity against pathogens such as *Helicobacter pylori, Salmonella* sp*., Listeria monocytogenes* and *Clostridium difficile*; (d) antimutagenic and anticarcinogenic properties (Morgensen et al., 2002).

Even though a probiotic strain fulfils the necessary safety and functional criteria, the aspects related to probiotic production and processing are also of utmost importance. The probiotic strains must have good technological properties so that they can be manufactured and incorporated into food products. Several technological aspects have to be considered in probiotic selection. These include the following: (a) good sensory properties; (b) phage resistance; (c) viability during processing; (d) stability in the product and during storage (Puupponen-Pimia et al., 2002). Good viability and activity of probiotics are considered prerequisites for optimal functionality. However, several studies have shown that non-viable probiotics can have beneficial effects such as immune modulation and carcinogen binding in the host (Ouwehand and Salminen, 1998; Salminen et al., 1999). Thus, for certain probiotic strains it might be sufficient that they grow well during initial production steps (to obtain high enough numbers in the product) but they do not necessarily need to retain good viability during storage (Mattila-Sandholm et al., 2002; Saarela et al., 2000).

1.3.1.2 ***Probiotic Bacteria***

Probiotic strains are generally belonging to the genera *Lactobacillus* and *Bifidobacterium*, and to a lesser extent to *Pediococcus, Propionibacterium, Enterococcus, Bacillus, Streptococcus* and *Saccharomyces* (Champagne & Møllgaard, 2008). The list of more used probiotic microorganisms is reported in table 2. *Lactobacillus* and *Bifidobacterium* strains are the major representatives of probiotics, both in the food and pharmaceutical market (Holzapfel and Schillinger, 2002).

Members of the genera *Lactobacillus* and *Bifidobacterium* have a long and safe history in the manufacture of dairy products and are also found as part of gastrointestinal microflora (Shah, 2007). *Lactobacillus* species have long been associated with the production of fermented foods, including dairy products, vegetables, meat, and sourdough bread. Their desirable rapid acidification also contributes to flavour, texture, and nutrition. Instead, the addition of bifidobacteria to foods has been more recent. They are purposely added because of their reported health benefits. Despite the low dominance of lactobacilli in the GI micro-ecology compared to the colonic associated bifidobacteria, they represent a major component of the microbiota residing in the small intestine (Zilberstein et al., 2007). Consequently, certain strains of *Lactobacillus*, particularly those of human origin, have been exploited as probiotics (O'Flaherty et al., 2009).

Table 2. Probiotic microorganisms (Adapted from Prado et al., 2008)

***Lactobacillus* spp.**	***Bifidobacterium* spp.**	**Others**
Lb. acidophilus	*B. adoslescentis*	*Bacillus cereus*
Lb. amylovorus	*B. animalis*	*E. faecalis*
Lb. brevis	*B. breve*	*E. faecium*
Lb. casei	*B. bifidum*	*Escherichia coli*
Lb. ramnosus	*B. infantis*	*Lc. lactis sp. cremoris*
Lb. crispatus	*B. lactis*	*Lc. lactis sp. lactis*
Lb. delbrueckii sp. *bulgaricus*	*B. longum*	*Leuconostoc mesenteroides* sp. *dextranicum*
Lb. fermentum		*P. acidilactici*
Lb. gasseri		*Propionibacterium freudenreichii*
Lb. helveticus		*Saccaromyces boulardii*
Lb. johnsonii		
Lb. lactis		
Lb. paracasei		
Lb. plantarum		
Lb. reuteri		

1.3.2 Starter cultures

The preservation of foods by fermentation is an ancient technology: it has been an effective form of extending the shelf-life of foods for millennia (Giraffa, 2004). In this way it was possible to preserve different kinds of raw materials: fruits, cereals, honey, vegetables, milk, meat and fish (Hansen, 2002). The old fermentation processes occurred naturally and they could be induced at most with back-slopping. Starter cultures are specific bacteria, usually LAB, that start the fermentation process (Parente and Cogan, 2004). They are added to the raw fermentation substrate in a large number and incubated under specific condition depending on the final desired product and the kind of starter. Nowadays spontaneous fermentations, difficult to control, are typical of small scale for traditional niche fermented products. Conversely controlled fermentation became common in large-scale processes. Even if many Protected Designation of Origin fermented food are produced using natural cultures, modern large-scale production exploits defined strain starter systems to ensure consistency and quality in the final product. LAB are involved in many fermentation processes of milk, meats, cereals and vegetables. Their fermentation metabolism generates first of all lactic acid and a range of products (organic acids, alcohol and carbon dioxide), which have a preservative effect through limiting the growth of spoilage and/or pathogenic flora in the food product.

Moreover, a number of desirable products, which affect the quality of the food may be produced, including flavour compounds, as well as compounds which may have positive implications for texture (exopolysaccharides) or for health such as vitamins, antioxidants and bioactive peptides (Ross, 2002; Messens and De Vuyst, 2002).

1.3.2.1 *Starter culture production*

LAB are widely used in the production of fermented food, and they constitute the majority of the volume and the value of the commercial starter cultures. Today we can divide cultures into two categories as reported the European food and feed cultures association (http://www.effca.com):

1. Starters and bulk starters (also called natural or traditional cultures) in which the use is decreasing in developed countries. For instance they come from milk that has not undergone any sanitation treatment or from back-slopping (the reuse of a fraction of the previous production). Their composition is complex, undefined and varies according to their origin.
2. Selected industrially produced cultures that are composed of pure strains, alone or in combination which are manufactured after a careful selection process under strictly controlled conditions. They are sold in liquid, frozen or freeze-dried formats

LAB are produced in specialised fermenters, under strict hygiene conditions. Typically, the process involves a number of different steps. A typical production process is illustrated in Figure 3, which consists of the following steps: (a) handling of inoculation material, (b) preparation of media, (c) propagation of cultures in fermenters under pH control, (d) concentration, (e) freezing, (f) drying and (g) packaging and storage (Høier et al., 2010;).

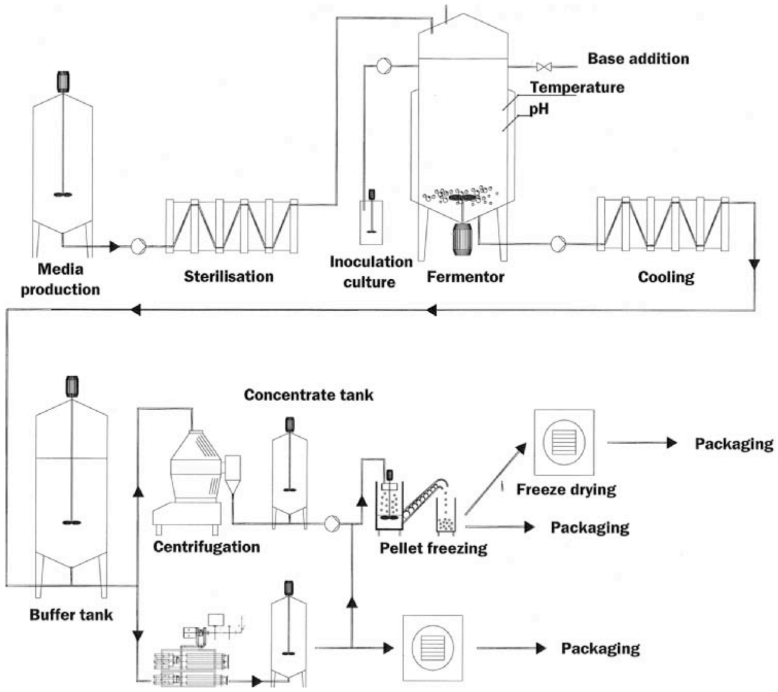

Figure 3 Process flow of typical starter culture production (Adapted from Høier et al., 2010)

Growth media for the production of cultures are composed of selected components and supplemented with various nutrients, such as yeast extract, vitamins and minerals. The culture growth medium is heated to an ultra-high temperature and cooled to a proper temperature for the culture, growth is optimised by maintaining the pH constant by the addition of an alkali, such as NaOH or NH_4OH (Høier et al., 2010). Other critical parameters such as temperature, agitation rate and headspace gases in the fermenters are optimised for each strain. After fermentation, the contents are cooled, and the biomass is harvested by centrifugation or membrane filtration, giving a further 10–20-fold concentration of the cells (http://www.effca.com).

1.4 Biomass Production

1.4.1 Design of fermentation medium

Primarily, fermentation media have to fulfil the complex nutritional requirements of each specific species/strain: provide essential elements as amino acid, peptides, vitamins, minerals, nucleic acid bases and other growth factors.

Table 3 Ingredients used for the preparation of media for commercial starter production (modified from Champagne, 1998).

Type of Nutrient	Ingredient
Sugars	Monosaccharides (glucose, fructose) Disaccharides (sucrose,lactose, maltose) Dextrins and maltodextrins Non fat dry milk (lactose) Whey (lactose)
Proteins	Non fat dry milk Whey Whey protein concentrates
Other nitrogen sources	Peptones Casein hydrolyzates Whey protein hydrolyzates Soy protein hydrolysates Meat protein hydrolyzates Hydrolyzates careal solids Yeast extracts
Vitamins and minerals	Yeast extracts Corn steep liquor
Others	Tween/oleic acid Mineral salts Defoamers Buffer

Table 3 highlights some commonly used raw ingredients. To be used in fermentations, raw ingredients need to be inexpensive, readily available and of reproducible quality (Champagne, 1998). The ingredients listed in table 3 have often a fluctuating composition because of the nature of the raw biological material affected by the season and/or the region where they are produced (Pasupuleti and Demain, 2010). Unfortunately the quality of the final product depends on these variations, resulting in significant production losses. To overcome this problem a strategy is to use blends of the same ingredient-type but from different manufacturers (Pasupuleti and Demain, 2010).

1.4.1.1 *Energy and carbon sources*

LAB and bifidobacteria do not possess a functional tricarboxylic acid cycle (TCA), which makes their energy generating pathways inefficient. Therefore, in order to support anabolic processes and cell growth, energy source has to be supplied in sufficient amounts (Ummadi and Curic-Bawden, 2010). Lactose and glucose represent the preferred energy sources for most industrial lactobacilli (Axelsson, 2004). During growth in a complex medium that contains amino acids and peptides, the main function of the sugar source is to generate energy. However, if the cell wall components (teichoic acid and peptidoglycans) and RNA precursors (purine and pyrimidine bases) are not supplemented into the medium, a small fraction of sugar and a portion of ATP will be used to synthesize these compunds. It is therefore important to supplement adequate forms of nitrogen sources and growth factors (Ummadi and Curic-Bawden, 2010).

1.4.1.2 *Nitrogen sources*

Fermentation medium can represent almost 30% of the cost for the production of microbial cells and bioproducts (Miller and Churchill, 1986), and the nitrogen source tends to be the most expensive medium constituent. Peptones represent not only a source of organic nitrogen but also a source of amino acids or specific peptides. They are defined as protein hydrolysates that are readily soluble in water and they have not precipitate by heat, by alkalis or by saturation with ammonium sulfate (Amezaga and Booth, 1999). Different materials from animal and plant sources are used for the production of peptones, most of them are valuable and relatively expensive (Parrado et al., 1993; Dufosse at al., 2001; Vasileva et al., 2007). Today there are many companies such as Oxoid (England), Merck (Germany), Difco (USA) and Sigma-Aldrich (USA) that produce different products: bacto-peptone, tryptone peptone, fish peptone, meat peptone, neopeptone and protease peptone for microbiological studies. All these products have high price, for istance, nowadays, the price of 1 kg tryptone peptone (Product No. P0556-1KG) (FLUKA) is given as 373,00 € (http://www.sigmaaldrich.com). Also yeast extract is used as the main source of nitrogen and vitamins for lactic acid production by microorganisms, but it is too expensive for large-scale fermentations. Complex media commonly employed for growth of LAB are not economically attractive due to their high amount of expensive nutrients such as yeast extract, peptone, and salts (Mercier et al., 1992).

1.4.1.3 *Alternative Nitrogen sources: Food industry leftovers.*

The expansion of biotechnology created an increasing demand for high-quality microbial growth media and stimulated the search for inexpensive sources. The nitrogen source is usually the most expensive component of bacterial growth substrates (Clausen et al., 1985; De la Broise et al., 1998). In particular protein hydrolysates are widely used in the manufacture of probiotics, starter cultures and fermented products due to their exploitation in microbiological media by fermentation and biotechnology industries (Pasupuleti and Demain, 2010). Moreover a number of studies have reported that proteinaceous compounds found in human milk (Liepke et al., 2002) and cow milk (Petschow and Talbott 1991) have the ability to promote the growth of bifidobacteria. Molecules that are present in dairy industry effluents such as whey (Mahalakshmi and Murthy 2000) and by-products of latex rubber production also have this property (Ishizaki 1989; 1995; Oiki et al., 1996; Etoh et al., 2000). For this reason there is a huge amount of works that studied the exploitation of raw materials as new cheaper ingredients for microbial growth media. Usually food, animal or agricultural wastes are used as resources. Many studies are focused on using different wastes of food industry such as whey (Mahalakshmi and Murthy, 2000), fishery leftovers (Martone et al., 2005), fibrous proteins sources (Kurbanoglu and Algur, 2002) (horns, nails, etc), for production of ingredients intended for formulation of microbiological media. Biological wastes contain several reusable substances of high value such as soluble sugars, fibre or proteins. Direct disposal of such wastes to soil or landfill causes serious environmental problems (Benta-Coker and Ojior, 1995). For instance in Mexico approximately only 15% of the waste bovine blood is used for animal feed (Gomez-Juareza et al., 1999). The remaining blood is disposed by municipal sewers and landfills, causing severe environmental problems due to the associated high organic pollutant (biochemical oxygen demand, BOD) and microbial loads. The most effective way of reducing the negative environmental effects due to the accumulation of animal processing by-products is the incorporation of them into productive processes (Sancho et al., 2004) and gives them a surplus value by innovative technologies. It is an environment friendly method of waste management. Biocatalytic-based or hybrid technologies provide a vital outlet for conversion of collagen and keratin-containing stocks into value-added proteinaceous ingredients for feed, food and microbiological applications (Bashkar et al., 2007; Gupta and Ramnani, 2006).

In the production of meat for human consumption, up to 50% of the animal weight is discharged, becoming a leftover (Arvanitoyannis and Ladas, 2008). In the EU, the total leftover mass from the meat industry amounts to 15 million tons, formally residues, but practically potential raw materials rich in proteins and lipids. In spite of this potentiality, most of this material is incinerated. Only 22% is converted into feed and barely 3% can become food (http://www.prospare.eu).

In Japan 14.5 million head of cattle and swine and 333 million chickens are bred each year. Residual parts, however, such as horn, hoof, skin and bone are wasted. The fishing industry also creates large amounts of scales and bones as waste by-products (Morimura et al., 2002). Probably more than 50% of the remaining material from the total fish capture is not used as food and involves near 32 million tons of waste (Kristinsson and Rasco, 2000). Because this secondary raw material includes head, viscera, skin, bone and some muscle tissue, it is an important protein source. However environmental regulations are becoming stricter, requiring new methods for discarding fish waste. Many authors (Martone et al., 2005; Horn et al., 2007; Safari et al., 2009) proposed an alternative: to hydrolyze waste to obtain fish protein hydrolysates, containing proteins with desirable functional properties (Martone et al., 2005). Research has been carried out in order to develop methods to transform these wastes into useful products (Perea et al., 1993; Kristinsson and Rasco, 2000; Larsen et al., 2000; Guerard et al., 2001; Coello et al., 2002; Laufenberg et al., 2003).

1.4.1.4 *Proteinaceous hydrolysates from poultry waste*

During the last three years a European project named PROSPARE (http://www.prospare.eu) was aimed to convert unmarketable secondary resources from poultry into valuable peptide hydrolyzates that can be exploited in the food, feed, and green chemical sectors.

In particular, it was proposed a new process with mild conditions of short-term hydrothermic and enzymatic hydrolysis steps that ensure high conservancy of the biologically valuable compounds has been recently developed in the framework of the European project PROSPARE (http://www.prospare.eu). Specific methodology, processing equipment and enzyme blends at moderate temperature (about 55 °C) for short times are applied to by-products (poultry bones and meat trimmings and poultry feathers). Two kinds of proteinaceous hydrolysates are obtained: Functional Animal Protein (FAP) and Functional Feather Protein (FFP). FAP was obtained starting from different types of poultry processing by-products (broiler backs, necks) after grinding, enzymatic hydrolysis, refining and clarification of the primarily broth, separation of the fat layer, vacuum-concentration and spray-drying. Enzymatic hydrolysis of poultry meat and bone residues was carried out under mild condition: using multienzyme composition containing four commercially available enzyme preparations namely – Alcalase, Neutrase, Protamex, Flavourzyme (Novozymes, Bagsvaerd, Denmark). Other hydrolysis parameters (duration of hydrolysis, hydromodule - water to raw material ratio) were optimized by multifactor methodology (Nikolaev et al., 2008). The multiphase process yields up to 85% top quality protein concentrate (liquid, concentrated, dry) – more than 42% in comparison with the current technology outputs, and with no fats, low osmosis and high solubility (http://www.prospare.eu). The second innovation obtained is the development of a new hydrothermal process of keratin hydrolysis, starting from feathers, which creates tasteless premium feathers protein mill (FFP). Chicken feathers are composed of over 90% protein, the main component being keratin, a fibrous and insoluble protein highly cross-linked with disulfide and other bonds. The feathers constitute up to 10% of total chicken weight. Feather keratin has high cysteine content. It also shows an elevated content of the amino acids glycine, glutamic acid, proline, alanine, serine and valine, but lower amounts of lysine, methionine and tryptophan (Taskin and Kurbanoglu, 2011). FFP was produced by short-term hydrothermal hydrolysis of the feathers under semi-dry-conditions, followed by an enzymatic treatment with Alkaline protease C isolated from filamentous fungi *Acremonium chrysogenum* (Eremeev et al., 2009). Optimal parameters of enzymatic hydrolysis for FFP production were defined based on the results of multifactor experiment (Eremeev et al., 2009). It was also shown that the optimized conditions for hydrothermal processing of feather raw material allowed a full decontamination of protein concentrate, at the same time providing practically complete preservation of heat-sensitive amino acids (PROSPARE internal report). The product output from the first stage of the process reaches >85% digestibility rate, <2% fat and ash. Almost 100% solubility and >95% digestibility are obtained from the second stage, which consists of an enzymatic treatment of the FFP obtained in the first stage (http://www.prospare.eu).

The reutilisation of biological wastes is of great interest since, due to legislation and environmental reasons, the industry is increasingly being forced to find an alternative use for its residual matter. Moreover, the use of these wastes considerably reduces the production costs. Thus, the development of potential value-added processes for these wastes is highly attractive and allows an environment friendly method of waste management.

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2 Aim of the thesis

One of the main topics of food industry is the waste management. The traditional waste chain usually ends with disposal but nowadays this solution is no more sustainable both economically and from the environmental point of view. Protein hydrolyzates are widely used in the manufacture of probiotics, starter cultures and fermented products due to their exploitation in microbiological media by fermentation and biotechnology industries. Many studies are focused on using of different wastes of food industry such as whey, fishery leftovers, fibrous proteins sources (horns, nails, etc.), for production of ingredients intended for formulation of microbiological media. In fact growth substrate costs often comprise the major part of the production cost of microbial cells and bio-products from the fermentation industry. In this context the formulation of the fermentation medium is undoubtedly the most important step in the production of starter and probiotic cultures.

This research is aimed at investigate the possibility to use proteinaceus hydrolizates obtained from poultry industry waste as nitrogen source in microbiological media for cultures involved in food production such as *Lactobacillus* and *Bifidobacterium* genus: the former as starter and probiotic and the latter as probiotic. These two genera have complex nutritional requirements and they need a complete medium that can support their growth and enhance it indeed. In this way it could be possible give these wastes a new commercial value because growth media represent a very high cost in microbiological propagation for this reason it is economically interesting to find less expensive supplements to replace the common ones.

3 Growth Promotion of Bifidobacterium Species by Poultry Bone and Meat Trimming Hydrolyzate

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Growth Promotion of *Bifidobacterium* Species by Poultry Bone and Meat Trimming Hydrolyzate

Camilla Lazzi, Federica Meli, Arnaldo Dossena, Monica Gatti, and Erasmo Neviani

Abstract: The growth of bifidobacteria that are employed in the production of functional food is often slow or limited, even on synthetic media. In this study, we investigated whether a peptide hydrolyzate (functional animal protein [FAP]), from poultry bones and meat trimmings, could be a potential source of growth stimulators. The bifidogenic activity of FAP on 18 strains of *Bifidobacterium* species was assessed via 2 different techniques: turbidimetric measurements and a direct count by fluorescence microscopy. Growth experiments were performed in B12 broth as the basal medium, B12 broth supplemented with N-acetylglucosamine, and B12 broth supplemented with FAP. FAP supplementation yielded the highest maximum optical density (OD) and count values. The use of the microscopic fluorescence counts allowed for better evaluation of the extent of growth and assessment of the viability of cells. FAP from poultry bones and meat trimmings has potential as a growth stimulator for different bifidobacteria of human origin. FAP is a promising ingredient for inclusion in industrial media that are used to culture probiotic strains, including bifidobacteria, because it supports growth very well and maintains cells at a high level of viability.

Keywords: bifidogenic activity, fluorescence microscope, functional animal protein, growth-promoting factors, peptide hydrolyzate

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Practical Application: Proteinaceous hydrolyzate can be considered a promising ingredient for industrial media that are used to culture probiotic strains, including bifidobacteria, because it improves bacterial growth and maintains cells at a high level of viability.

Introduction

Bifidobacteria are gram-positive, heterofermentative, nonmotile, nonspore forming rods. They can display a range of distinct cell forms, including regular rods and various branched shapes. They were 1st isolated by Tissier (1899) from the feces of breast-fed infants, and their presence in the gut is associated with a healthy microbiota (Klijn and others 2005).

Bifidobacteria are considered to be examples of health-promoting constituents of the microbiota because some species act as probiotics, which are microorganisms that, when administered in adequate amounts, confer health benefits on the host as defined in "Guidelines for the evaluation of probiotic in food" (FAO/WHO 2002). In particular, the use of some *Bifidobacterium* spp. as probiotics is widespread in food industries (Heller 2001). For these reasons, bifidobacteria have become economically important, because they are added in high numbers as live bacteria to numerous food preparations with various health-related claims (Klijn and others 2005).

Although they are commonly employed in the production of functional food, their growth is often slow or limited even on synthetic media because of the absence of a growth-promoting factor (Etoh and others 1999). Bifidobacterial growth is stimulated by the presence of 2 types of promoting factors: growth factors that are metabolized by the body, or by the microbiota, of the upper gastrointestinal tract (for example, threonine, yeast extract, cysteine, peptone, dextrin, maltose, and β-glycerophosphate) and bifidogenic factors, which are substances that survive direct metabolism by the host and reach the large intestine for preferential metabolism by bifidobacteria (Modler 1994). These factors include lacteal secretions (N-acetylglycosamine-containing saccharides), fructooligosaccharides, lactoferrin, lactulose and lactitol, oligoholosides and polyholosides (raffinose, stachyose, and insulin), xylooligosaccharides (D-xylan), and transgalactosylated-oligosaccharides (isogalactobiose, galsucrose, and lactosucrose) (Ventura and others 2004). Promoting factors can also be nonglycosylated peptides derived from protein after hydrolysis using proteinase (Tamime and others 1995; Zhao and others 1996). A number of studies have reported that proteinaceous compounds found in human milk (Liepke and others 2002) and cow milk (Petschow and Talbott 1991) have the ability to promote the growth of bifidobacteria. Molecules that are present in dairy industry effluents such as whey (Mahalakshmi and Murthy 2000) and by-products of latex rubber production also have this property (Ishizaki 1989; 1995; Oiki and others 1996; Etoh and others 2000).

In this study, it was investigated whether a peptide hydrolyzate (functional animal protein [FAP]) from poultry bones and meat trimmings could have a positive effect on the growth of bifidobacteria. FAP samples were provided by the European project Progress in Saving Proteins and Recovering Energy (PROSPARE,

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www.prospare.eu). The aim of PROSPARE is to convert unmarketable secondary resources from poultry into valuable peptide hydrolyzates that can be exploited in the food, feed, and green chemical sectors.

The aim of this work was to explore the features of FAP as a microbial growth promotion factor for its possible use in the production of *Bifidobacterium* biomass with improved viability. Specifically, the performance of FAP as a nitrogen ingredient was evaluated in a minimal basal medium. To achieve this goal, the growth stimulation activity of FAP was assessed through the comparison of 2 different techniques. Turbidimetric measurements and direct counts by fluorescence microscopy allowed for the evaluation of both the growth and the viability of bifidobacteria in the presence of peptide hydrolyzate.

Materials and Methods

Bacterial strains

Eighteen strains belonging to 15 *Bifidobacterium* species were used in this work (Table 1). Ten strains from the microbial collection of the Dept. of Genetics, Biology of Microorganisms, Anthropology, and Evolution, Univ. of Parma, were isolated from human fecal samples and identified as described by Turroni and others (2009). Eight reference strains were purchased from the "American Type Culture Collection" (ATCC, Manassas, Va., U.S.A., http://www.atcc.org/) and the "Belgian coordinated collection of microorganisms" (LMG, Gent, Belgium, http://bccm.belspo.be/). All strains were maintained as culture stocks in 15% glycerol (w/v) at −80 °C and routinely cultured for 48 h under anaerobic conditions at 37 °C in MRS (Man Rogosa Sharpe) broth modified by the addition of cysteine hydrocloride at 0.05% (Oxoid Italia, Milano, Italy) as suggested by Rada (1997).

Peptide hydrolyzate

FAP was provided by the State Institution All-Russian Research Inst. for Poultry Processing Industry of Russian Academy of Agricultural Science within the European project PROSPARE. FAP was obtained starting from leftovers of the avian industry after grinding, enzymatic hydrolysis, separation of the fat layer, filtration, and drying. The processing conditions, in accordance with a multifactorial experimental plan, were the following: neutral pH, concentration of commercial (Novozymes, Bagsvaerd, Denmark) and new enzymes blend of 0.15% to 0.30% (www.prospare.eu), and 50 °C to 65 °C for 3 h. The resulting white powder was very hygroscopic and easily soluble in water and was characterized for its content of free and total amino acids.

Table 1–*Bifidobacterium* strains belonging to 18 different species studied in this work.

Strains	Species	Origin
328	*B. pseudocatenalatum*	Fecal samples
340	*B. pseudolongum*	Fecal samples
342	*B. animalis* subsp. *animalis*	Fecal samples
350	*B. longum 1*	Fecal samples
382	*B. bifidum*	Fecal samples
392	*B. magnum*	Fecal samples
397	*B. angulatum*	Fecal samples
398	*B. asteroides*	Fecal samples
407	*B. longum 2*	Fecal samples
416	*B. catenulatum*	Fecal samples
LMG11597	*B. subtile*	Reference strain
LMG11596	*B. gallicum*	Reference strain
LMG21814	*B. longum 3*	Reference strain
ATCC25527	*B. animalis* subsp. *animalis*	Reference strain
LMG10510	*B. choerinum*	Reference strain
LMG11341	*B. merycicum*	Reference strain
LMG11592	*B. minimum*	Reference strain
LMG21589	*B. scardovii*	Reference strain

Amino acid content

The total amino acid content was determined after hydrolysis in 6N HCl at 110 °C for 24 h. A standard commercial method for the quantification of amino acids was 1st applied. This method is based on a precolumn derivatization procedure with aminoquinolyl-*N*-succinimidyl carbamate AccQ.Tag amino acid analysis (Waters, Saint-Quentin, France) followed by the determination of the amino acid content by reverse-phase-high-performance liquid chromatography (Waters) with fluorescence detection (λ excitation = 250 nm, λ emission = 395 nm). This procedure was also used for the evaluation of free amino acids in the hydrolyzates.

The evaluation of amino acid content in the same samples was also performed, after acidic hydrolysis in 6N HCl for 24 h at 110 °C, in the presence of performic acid, to transform the Cys and Met residues in cysteic acid and methionine sulfone, so as to quantify Cys and Met. For Trp evaluation, a standard spectrophotometric assay was used, based on the recording of the UV absorption in the Trp absorption region (around 280 to 290 nm), followed by the calculation of the 4th derivative of the curve and the measurement of the difference between the minimum at 285 nm and the maximum at 290 nm. D-amino acids were also determined from the total pool after an acidic hydrolysis in 6N HCl at 110 °C under nonoxidizing conditions for a shorter time (15 h instead of 24 h) in order to avoid method-induced racemization. After hydrolysis, the amino acids were derivatized 1st with isopropanol in the presence of HCl and then with trifluoroacetic anhydride in order to obtain the isopropyl esters-trifluoroacetamido derivatives that were analyzed by GC/MS HP-6890N GC-5973MSD (Hewlett Packard, Palo Alto, Calif., U.S.A.) on a Chirasil-Val column.

Molecular composition of peptide hydrolyzate

After dissolution of the protein hydrolyzate in 0.1N HCl, the liquid extract that was obtained was ultrafiltered (step 1: ultrafiltration, 10 kDa) by using Amicon® Ultra Centrifugal Filters, Ultracel 10K (4 mL, nominal cut-off 10 kDa) (Millipore, Milford, Mass., U.S.A.). The retentate was recovered (step 2: dissolution of retentate) after dissolution in water with 0.1% of formic acid; next, the retentate was dried, 1st by rotavapor and then under vacuum, and weighed. The filtrate was collected and divided into 2 parts; one aliquot was ultrafiltered (step 3: ultrafiltration, 3 kDa) by using Amicon® Ultra Centrifugal Filters, Ultracel 3K (4 mL, nominal cut-off 3 kDa), and the 2nd aliquote was dried, 1st by rotavapor and then under vacuum, and weighed. Alternatively, the dissolved protein hydrolyzate was ultrafiltered by using Amicon® Ultra Centrifugal Filters, Ultracel 3K (4 mL, nominal cut-off 3 kDa). The retentate was recovered after dissolution in water with 0.1% formic acid, and dried by rotavapor; the filtrate was dried, 1st by rotavapor and then under vacuum, and weighed. The data obtained in the 2 different experiments were compared to better define the relative content of the fractions with the ranges of molecular weights of <3 kDa, 3 to 10 kDa, >10 kDa.

Growth assays

After incubation for 48 h under anaerobic conditions at 37 °C in MRS broth supplemented with cysteine hydrochloride at 0.05%, each culture was centrifuged at 8000× *g* for 10 min at 4 °C. The harvested cells were washed twice, suspended in Ringer solution

(Oxoid Italia), and used to inoculate (2%, v/v) B12 (Difco Laboratories, Detroit, Mich., U.S.A.) broth, B12 broth supplemented with N-acetylglucosamine (0.02 g/L, 2.20 g/L, 22.00 g/L) (Sigma-Aldrich, Milano, Italy), and B12 broth supplemented with FAP (0.02 g/L, 2.20 g/L, 22.00 g/L). Prior to inoculation, media were filtered through Millex®-GV 0.22 μm filters (Millipore). Microbial growth after incubation at 37 °C under anaerobic conditions was evaluated by both turbidimetric measurement (Golod and others 2009) and microscopic count. A dilution step was performed before measuring the optical density (OD) of solution with an OD value greater than 1.8. The extent of growth was monitored at various time points (0, 22, 44, 66, and 88 h) by determining OD_{650} using a spectrophotometer (Jasco v-530, Tokyo, Japan).

Fluorescence microscopy counts were performed using the LIVE/DEAD® BacLight™ Bacterial Viability Kit for microscopy (Invitrogen Ltd, Paisley, U.K.).

The LIVE/DEAD® BacLight™ bacterial viability kit, used to evaluate the number of viable cells, is formed by a mixture of the green fluorescence nucleic acid stain SYTO® 9, which labels all cells, and the red fluorescence nucleic acid stain propidium iodide, which penetrates only bacteria with damaged membranes and quenches the green stain SYTO® 9. Thus, bacteria with intact cell membranes stain green, whereas bacteria with damaged membranes stain red. Considering only the green cells, it was possible to count the number of viable cells; considering the sum of the green and red cells, it was possible to evaluate the total number of cells.

The 2 BacLight™ stains, SYTO® 9 (0.7 μL) and propidium iodide (1 μL), were added to a 1-mL sample of washed cells. Samples were incubated for 15 min at room temperature in the dark. After incubation, the stained samples were filtered through a 0.2-μm black Isopore™ membrane filter (Millipore). The filter was mounted in BacLight™ mounting oil, as described by the manufacturer.

The count was performed by a Nikon Eclipse 80i epifluorescence microscope (Nikon, Tokyo, Japan) equipped with a C-SHG1 100-W mercury lamp. Nikon filter set B2A FITC was used (excitation wavelength, 450 to 490 nm; emission wavelength, 500 to 520 nm). Pictures of each field were taken and then superimposed through the Nis Elements software (ver. 2.10, Nikon).

The number of bacteria per milliliter of sample was calculated from counts of 5 microscopic fields (at 1000×) with the following formula:

$N = (C \times A/a \times V) \times D$, where N is the number of cells per milliliter, C is the number of cells per observation field, A is the filtration area (mm^2), a is the observation field area (mm^2), V is the volume of the filtered samples (mL), D is the dilution factor (Bottari and others 2010, Gatti and others 2006).

The growth experiments were performed in duplicate, and resulting values were averaged in each experiment.

Results and Discussion

Chemical composition of peptide hydrolyzate

The analysis of the molecular mass distribution revealed 3 main fractions with molecular weights >10 kDa, 3 to 10 kDa, <3 kDa. The results obtained by weighing the ultrafiltered fractions showed a relative percentage of around 16% for the fraction > 10kDa, of around 22% for the medium molecular weight fraction (3 to 10 kDa), and of around 62% for the low molecular weight fraction, which also contained the free amino acids.

The quantities of free and total amino acids in the FAP sample that was used in this study are reported in Table 2. Leu and Lys were the most abundant free amino acid. Glu and Asp were the most abundant amino acids in the total amino acid pool, whereas the sulfurated amino acid Cys was present in the mixture in the lowest amount. Regarding the D-amino acids content, practically no racemization was observed in the sample; traces of the D-enantiomer were found only for Asp, which indicated that the hydrolytic treatment for the preparation of FAP was quite mild and that the sample was free of bacterial contamination (Marchelli and others 2007).

Evaluation of microbial growth

The growth experiments were carried out on 18 *Bifidobacterium* strains (Table 1) by using B12 as the basal culture medium. B12 was also supplemented with either N-acetylglucosamine or FAP for their assessment as growth-promoting factors.

Three different concentrations (0.02 g/L, 2.20 g/L, and 22.00 g/L) of N-acetylglucosamine and FAP were preliminary tested. The highest concentration corresponds to the total content of nitrogen sources in MRS, which is a rich selective medium for lactobacilli and bifidobacteria when supplemented with cysteine hydrochloride at 0.05% (De Man and others 1960; Sykes and Skinner 1973). This highest concentration yielded the best results for both N-acetylglucosamine and FAP (data not shown); therefore, the following experiments were carried out with 22.00 g/L.

The growth of each *Bifidobacterium* species was monitored every 22 h to 88 h via turbidimetric measurement. Every strain displayed a characteristic growth curve, but the highest OD_{650nm} value was obtained after 66 h (data not shown). Therefore, this length of incubation was used for the following experiments.

The level of viability, after 66 h of incubation under anaerobic condition at 37 °C, varied depending on the media and the strains (Figure 1). The percentage of dead cells was, on average, lower in B12 supplemented with FAP (18%) than in B12 (25%) or in B12

Table 2–Total and free amino acids content in the FAP sample.

Amino acids	Free amino acids (mg/g ± SD)	Total amino acids (mg/g ± SD)
Glycine	5.33 ± 0.36	49.87 ± 1.55
Alanine	17.57 ± 0.05	64.54 ± 1.65
Serine	13.81 ± 1.27	38.67 ± 1.95
Threonine	9.23 ± 0.50	27.48 ± 0.43
Cysteine	0.83 ± 0.17	8.22 ± 2.77
Cystine	1.47 ± 0.00	2.93 ± 0.00
Methionine	10.76 ± 0.08	10.47 ± 3.58
Asparagine	16.48 ± 0.26	77.21 ± 0.86
Aspartic acid	22.46 ± 1.13	108.94 ± 0.49
Glutamine	16.97 ± 0.25	88.62 ± 1.60
Glutamic acid	21.96 ± 0.19	119.42 ± 0.16
Proline	9.00 ± 0.02	49.70 ± 0.31
Lysine	28.11 ± 1.41	54.86 ± 0.46
Arginine	15.66 ± 0.09	49.65 ± 0.55
Histidine	6.54 ± 0.39	18.18 ± 0.13
Valine	14.82 ± 0.19	38.69 ± 0.30
Isoleucine	17.93 ± 0.61	39.27 ± 0.40
Leucine	32.93 ± 1.10	56.01 ± 0.21
Tyrosine	11.07 ± 0.40	16.60 ± 0.27
Phenylalanine	15.14 ± 0.59	28.29 ± 0.19
Tryptophan	5.22 ± 0.20	6.58 ± 0.04

Number of replicates: 5
"Free amino acids" refers to the amount of amino acids present in the free form. "Total amino acids" refers to the total content of amino acids after hydrolysis of the FAP sample with 6N HCl at 110 °C for 24 h.

supplemented with N-acetylglucosamine (38%). In particular, no viable cell of *B. minimum* and *B. merycicum* was recovered from B12, and no viable cells of *B. longum* 1 or *B. merycicum* were recovered from B12 supplemented with N-acetylglucosamine. The percentage of dead cells in B12 supplemented with FAP was always less than 45% (Figure 1).

Cells growth results, as determined by turbidimetric analysis, and the number of viable cells after 66 h of incubation at 37 °C are reported in Figure 2A. Based on OD values, B12 medium alone permitted only limited bifidobacterial growth as previously observed (Ibrahim and Bezkorovainy 1994). Furthermore, our results reveal that the known bifidogenic carbohydrate N-acetylglucosamine (Modler 1994) exhibits a low growth-promotion effect. In contrast, FAP provided the best growth support to all the studied strains, although this stimulation appeared to be variable among the species. The greatest stimulatory effect was observed with the strain *B. longum* 1. Based on OD data, *B. subtile* showed a similar capacity to grow in all 3 media, underlying its insensitivity to FAP addition (Figure 2A).

Considering the number of viable cells, the positive effect of FAP addition was confirmed, and this stimulation appeared to have low variability among the species, ranging from 8.07 to 9.16 log cell/mL (Figure 2B). The bifidogenic activities detected in B12 supplemented with FAP were considerable for *B. merycicum, B. longum* 1*,* and *B. pseudolongum*, which reached the highest values in comparison with the other species (9.16, 9.05, and 9.01 log cell/mL, respectively). *B. choerinum* and *B. scardovii* maintained the same viability in all 3 media. The average number of viable cells cultured in the presence of FAP was 8.57 log cell/mL; in the culture media with N-acetylglucosamine and without growth-promoting factors, the averages were 5.57 log cell/mL and 6.42 log cell/mL, respectively (Figure 2B). The correlation between the OD values and the number of viable cells was lower than 50% (data not shown). This result is not in contradiction because the 2 techniques provide different information. For example, for the strain *B. longum* 1, FAP enhanced both the OD value and the viable cells count, whereas the high OD value detected for *B. longum* 2 did not correlate with a high number of viable cells.

In order to highlight the different growth-promotion effects of FAP and N-acetylglucosamine, the increase in microbial growth in B12 supplemented with FAP and N-acetylglucosamine in comparison with the basal culture medium B12 was evaluated. The increase in OD was calculated by subtracting the OD value that was obtained in basal culture medium B12 from the OD value that was obtained in B12 supplemented with either FAP or N-acetylglucosamine (Figure 3A). The increase in the number of viable cells was calculated by subtracting the log number of viable cells counted in basal culture medium B12 from the log number of viable cells counted in B12 supplemented with either FAP or N-acetylglucosamine (Figure 3B).

Despite the fact that N-acetylglucosamine has been identified as a bifidogenic compound, growth in B12 supplemented with FAP yielded the highest maximum OD (Figure 3A) and number of viable cells (Figure 3B). N-acetylglucosamine improved the viability of *B. magnum* and *B. minimum* (Figure 3B).

Considering the 2 techniques used for monitoring the extent of growth in the 2 substrates, it is possible that the evaluation of viable cell number via microscopy provides a better overview compared with photometric absorbance measurements. Only by microscopic counts was it possible to observe an increase in growth of *B. magnum* and *B. minimum* in the medium containing N-acetylglucosamine.

Furthermore, the extent of growth of the 18 *Bifidobacterium* strains in B12 supplemented with FAP was not demonstrated well by turbidimetric analysis.

The mechanism by which FAP increases the growth of bifidobacteria has not been studied yet; nevertheless, we propose the following 3 hypotheses. (1) FAP acts as a simple essential amino acid supplier. Actually, FAP was mainly constituted by the low molecular weight fraction (<3 kDa) that contained both short peptides and free amino acids. In this regard, Etoh and others (1999) stated that the promoting effect on the growth of bifidobacteria is due to a low molecular weight nitrogen fraction (<1 kDa). Nonetheless, experimental results from this study show that FAP stimulates the bifidobacterial growth even in the presence of a very rich growth medium, such as MRS with cysteine (data not shown).

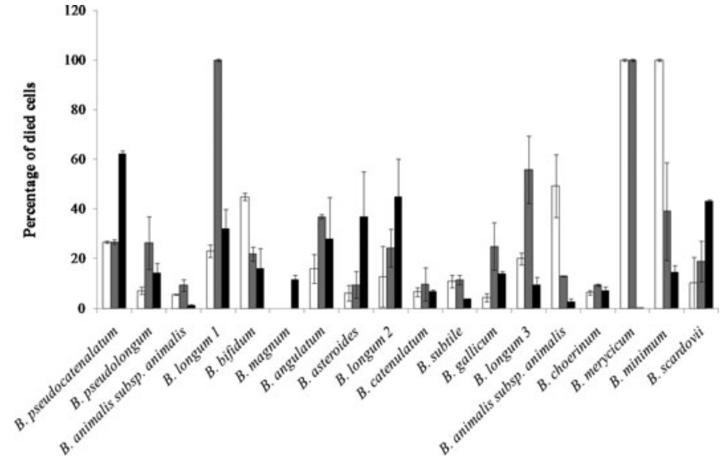

Figure 1–Percentage of nonviable cells of 18 *Bifidobacterium* strains after culture for 66 h at 37 °C under anaerobic condition in B12 assay medium (light gray bars), B12 broth supplemented with N-acetylglucosamine (medium gray bars), and B12 broth supplemented with FAP (dark gray bars).

In particular, for *B. merycicum*, *B. longum* 1, and *B. pseudolongum*, the presence of FAP in MRS cysteine enhanced the growth of viable cells by 1, 0.4, and 0.6 log cell/mL, respectively (data not shown). This result led us to propose that peptide hydrolyzate does not simply act as a nitrogen source for the microorganism. (2) FAP probably contains specific peptides that bifidobacteria, after uptake in the cell by oligopeptide transport systems, can degrade due to the presence of a specific peptidase that is able to hydrolyze peptide bonds within an oligopeptide. Therefore, the growth variability that emerged among the strains could depend on the different abilities of bifidobacteria proteolytic enzymes and peptide transport systems. In this regard, very little is known about the proteolytic enzyme systems of *Bifidobacterium* (Sela and others 2010). The analysis of the *B. longum* NCC2705 genome predicted more than 20 peptidases, including general aminopeptidases, peptidases specific for proline residues, dipeptidases, and endopeptidases, as well as an ABC-type transporter system that is specific for oligopeptides (Schell and others 2002). Janer and others (2005) reported that the increase in the growth of *B. animalis* subsp. *lactis* in milk supplemented with whey peptide fraction is due to the activity of a PepO endopeptidase. (3) FAP may contain, among peptides, a specific growth-promoting factor. Different research has shown the importance of peptide structure in growth-promotion activity. Indeed, the addition of single amino acids that are contained in the active peptide do not stimulate the growth of bifidobacteria strains (Zhao and others 1996; Etho and others 2000; Liepke and others 2002). The work of Liepke and others (2002) highlights that the peptides of human milk, which are known to promote the growth

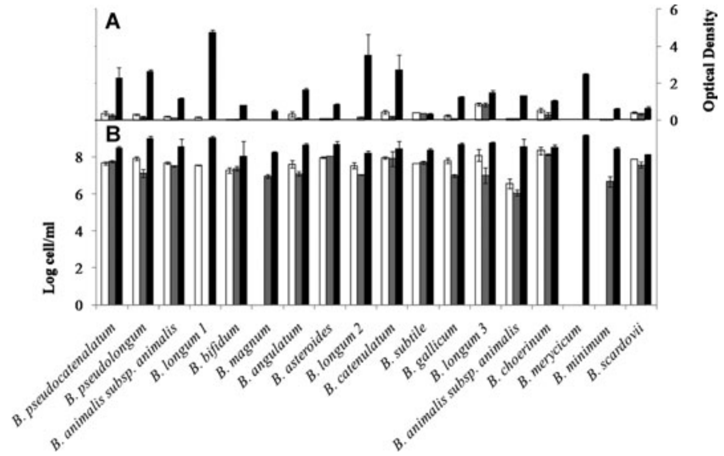

Figure 2–Growth of 18 *Bifidobacterium* strains in B12 assay medium (light gray bars), B12 broth supplemented with N-acetylglucosamine (medium gray bars), and B12 broth supplemented with FAP (dark gray bars), after culture for 66 h at 37 °C under anaerobic condition, as assessed by turbidimetric measurements (OD) (A) and fluorescence microscopic counts (log cells/mL) (B).

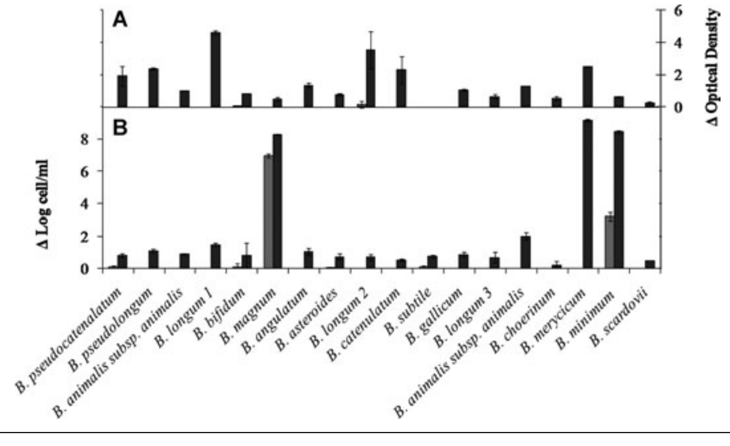

Figure 3–Extent of microbial growth of 18 *Bifidobacterium* strains in FAP (light gray bars) and N-acetylglucosamine (dark gray bars) compared with the basal culture medium B12 determined by turbidimetric measurements (A) and fluorescence microscopic counts (B). The increase in OD (ΔOD) was calculated by subtracting the OD value obtained in basal culture medium B12 from the OD value obtained in medium supplemented with FAP or N-acetylglucosamine. The increase in number of viable cells (Δ log cells/mL) was calculated by subtracting the number of viable cells counted in basal culture medium B12 from the number of viable cells counted in medium supplemented with FAP or N-acetylglucosamine.

of bifidobacteria, are characterized by the presence of one or 2 disulfide bonds within a single oligopeptide chain or between 2 different ones.

Conclusion

This study suggested that the use of by-products from the poultry industry in cultured media can promote the growth of a wide range of *Bifidobacterium* species of human origin. FAP is a promising ingredient for inclusion in industrial media for probiotic strains such as bifidobacteria not only because it improves bacterial growth, which has nutrient-demand requirements, but also because it maintains cells at a high level of viability. Further studies are being performed in order to analyze the molecular composition of the peptide hydrolyzate and to elucidate the mechanism by which the hydrolyzate acts during biomass synthesis.

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4 Growth promotion of Bifidobacterium and Lactobacillus species by proteinaceous hydrolysates derived from poultry processing leftovers.

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Original article

Growth promotion of *Bifidobacterium* and *Lactobacillus* species by proteinaceous hydrolysates derived from poultry processing leftovers

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Summary Bacterial growth media represent a high cost in industrial applications, and for this reason, it is economically important to find less expensive supplements to replace the traditional ones. In the present work, peptide hydrolysates obtained from poultry meat and bone residues (functional animal protein [FAP]) and from feathers (functional feather protein [FFP]) were studied to determine their ability for the production of microbial biomass with improved viability. The results obtained were compared with those obtained with other supplement nutritive compounds used in fermentation growth media. The molecular composition of the hydrolysates in terms of total and soluble nitrogen, molecular weight distribution, total and free amino acids, was determined. The growth and cellular state of *Bifidobacterium* and *Lactobacillus* strains were studied by turbidimetric measurements and direct count by fluorescence microscopy. Overall, this study suggested that by-products from poultry industry provide a good alternative to substitute expensive supplements for growth of *Lactobacillus* and *Bifidobacterium* with a high level of viability.

Keywords *Bifidobacterium*, cell viability, growth-promoting factors, *Lactobacillus*, protein hydrolysate.

Introduction

In the EU, the total leftover mass from the meat industry amounts to 15 million tons, formally residues, but practically potential raw materials rich in proteins and lipids. In spite of this potentiality, most of this material is incinerated. Only 22% is converted into feed, and barely 3% can become food (PROSPARE website).

The most effective way of reducing the negative environmental effects because of the accumulation of animal processing by-products is the incorporation of them into productive processes (Sancho *et al.*, 2004) and gives them a surplus value by innovative technologies. Biocatalytic-based or hybrid technologies provide a vital outlet for conversion of collagen and keratin-containing stocks into value-added proteinaceous ingredients for feed, food and microbiological applications (Gupta & Ramnani, 2006; Bhaskar *et al.*, 2007).

A new process with mild conditions of short-term hydrothermic and enzymatic hydrolysis steps that ensure high conservancy of the biologically valuable compounds has been recently developed in the framework of the European project PROSPARE (www.prospare.eu). The main by-products transformed in this process are poultry bones and meat trimmings and poultry feathers. Two kind of proteinaceous hydrolysates are obtained: Functional Animal Protein (FAP) and Functional Feather Protein (FFP). It is known that protein hydrolysates are used in a wide variety of products in the fermentation and biotechnology industries (Pasupuleti & Demain, 2010). In particular, protein hydrolysates are widely used in the manufacture of probiotics, starter cultures and fermented products because of their exploitation in microbiological media by fermentation and biotechnology industries (Pasupuleti & Demain, 2010). Many studies are focused on the use of different wastes of food industry such as whey (Mahalakshmi & Murthy, 2000), fishery leftovers (Martone *et al.*, 2005) and fibrous proteins sources

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(Kurbanoglu & Algur, 2002) (horns, nails, etc.) for the production of ingredients intended for formulation of microbiological media. In fact, growth substrate costs often comprise the major part of the production cost of microbial cells and bioproducts from the fermentation industry (De la Broise *et al.*, 1998). In this context, the formulation of the fermentation medium is undoubtly the most important step in the production of starter and probiotic cultures. The aim of this work was to explore the features of FAP and FFP for the production of microbial biomass with improved viability and compare them with those of other supplement nutritive compounds used in fermentation growth media. The growth and cellular state of eighteen *Bifidobacterium* and twenty-eight *Lactobacillus* strains were studied by turbidimetric measurements and direct count by fluorescence microscope. Furthermore, the molecular composition of the hydrolysates and microbial growth were correlated.

Materials and methods

Bacterial strains

Eighteen strains belonging to fifteen *Bifidobacterium* species and twenty-eight strains belonging to fourteen *Lactobacillus* species were used in this work (Table S1). Ten *Bifidobacterium* strains of human faecal samples were identified as described by Turroni *et al.* (2009). Eight *Bifidobacterium* strains were purchased from the 'American Type Culture Collection' (ATCC, Manassas, VA, USA, http://www.atcc.org/) and 'Belgian co-ordinated collection of microorganism' (LMG, Gent, Belgium, http://bccm.belspo.be/) (Table S1). Twenty-seven *Lactobacillus* strains were purchased from 'Belgian co-ordinated collection of microorganism' (LMG, Gent, Belgium, http://bccm.belspo.be/) and 'German Collection of Microorganisms and Cell Cultures' (DSMZ, Braunschweig, Germany, http://www.dsmz.de). One strain, L26, belongs to the microbial collection of Department of Genetics, Biology of Microorganisms, Anthropology and Evolution, University of Parma and was isolated from dairy sample (Table S1). All the strains were maintained as culture stocks in 15% glycerol (w/v) at −80 °C. *Bifidobacterium* strains were routinely grown for 48 h in anaerobic conditions at 37 °C in MRS broth modified by the addition of cysteine hydrocloride at 0.05% (Oxoid Italia, Milano, Italy). *Lactobacillus* strains were routinely grown for 24 h in MRS broth (Oxoid Italia) following the best growth condition for each species.

Protein hydrolysates

Eight FAP samples named 58T, 78T, 81T, 82T, 83T, 100T, 100AT, 101T and one FFP named 6L were provided by the State Institution All-Russian Research Institute for Poultry Processing Industry of Russian Academy of Agricultural Science within the European project PROSPARE. Functional animal proteins were obtained starting from different types of poultry processing by-products (broiler backs, necks) after grinding, enzymatic hydrolysis, refining and clarification of the primarily broth, separation of the fat layer, vacuum-concentration and spray-drying. Enzymatic hydrolysis was carried out under mild condition using multienzyme composition containing four commercially available enzyme preparations namely Alcalase 2.4 LFG, Neutrase 0.5 L, Protamex 1.5 MG, Flavourzyme 500 MG (Novozymes, Bagsvaerd, Denmark). The raw material grinned (500 kg) was placed in the reactor and mixed with appropriate amount of water and multienzyme composition. Other hydrolysis parameters (duration of hydrolysis, hydromodule – raw material to water ratio) were optimised by multifactor methodology (Nikolaev *et al.*, 2008). The optimal hydrolysis parameters were temperature 55 ± 2 °C, duration of fermentation 2.5 h, hydromodule 1:2, dosage of multienzyme composition 0.25% of raw material weight (enzymatic activity comprised 1.2 AU per g of raw material). The mixture was continuously stirred by a blade agitator at 24 rpm. Enzymatic hydrolysis was continued for 2.5 h with subsequent exposure of mixture at 90–95 °C for 30 min. During this period, both pasteurisation of the bouillon and inactivation of proteolytic enzymes occurred. A set of FAP samples used for this study was obtained at a slight variation of enzymatic hydrolysis condition (Table S2). Functional feather protein was produced by short-term hydrothermal hydrolysis of the feathers under semi-dry-conditions, followed by an enzymatic treatment with Alkaline protease C isolated from filamentous fungi *Acremonium chrysogenum* (Eremeev *et al.*, 2009). Optimal parameters of enzymatic hydrolysis for FFP production were defined based on the results of multifactor experiment (Eremeev *et al.*, 2009).

Total and soluble nitrogen

For soluble nitrogen, FAP or FFP sample (0.5 g) was weighed by analytical balance (Gibertini, Novate Milanese, Milan, Italy) and dissolved in 180 mL deionised water. The solution was centrifuged at 1790 ***g*** at 5 °C for 40 min, filtered on paper filter. Soluble nitrogen was extracted from 50 mL of the solution through addition of 10% trichloroacetic acid (TCA) and incubated for 30 min at room temperature. The mixture was centrifuged and filtered. 40 mL of previous solution was digested with 96% sulphuric acid (7 mL), phosphosulphuric acid (10 mL), copper (II) oxide, sodium sulphate (antifoam), and potassium sulphate (selenium catalyst) in a block of digester at 420 °C for

1 h. After cooling, the sample was distilled with 32% sodium hydroxide to obtain extraction of ammonia, in presence of 4% boric acid solution containing bromocresol green and methyl red as pH indicator. Soluble nitrogen was determined by titration with 0.1 N HCl, following the equation: % SN = (N*mL HCl*M HCl) *Fc/(g of sample*1000), where 'N' is the nitrogen molecular weight, 'M HCl' is the molarity of HCl, and 'Fc' is the dilution correction factor, calculated taking into account all the dilution performed during the sample preparation.

For total nitrogen, FAP or FFP sample (0.5 g) was weighted, digested and distilled in the same conditions reported for soluble nitrogen. Total nitrogen was determined by titration with 0.1 N HCl.

Free amino acid determination

0.03 g of dried FAP or FFP was dissolved in 10 mL of deionised water and D,L-Norleu (340 μL of a 5 mM solution) was added as internal standard; the mixture was directly analysed by HPLC-ESI/MS/MS system. All amino acids standard stock solutions were prepared at 2.5 mM, in deionised water. Mixed working standard solution, containing all twenty proteic amino acids, cystine and NorLeu was prepared to obtain a final concentration of 0.5 mM of each analyte. To obtain a standard calibration curve, the final solution was diluted two, three, four, five and six times.

All samples and standard solution were analysed using an Alliance 2695 separation system (Waters, Milford, MA, USA) with a Spherisorb (5 μm CN 2.1 × 250 mm) narrow bore column (Waters) followed by a Proteo (Phenomenex, Torrance, Ca, USA) analytical column (4 μm C18, 90 Å, 250 × 2.0 mm). The mobile phase was composed by H_2O + 0.2% CH_3CN + 0.1% HCOOH (eluent A) and CH_3CN + 0.1% HCOOH (eluent B). Gradient elution was performed according to the following steps: isocratic 100% A for 15 min, from 100% A to 30% B by linear gradient in 30 min, 10 min of a washing step with 100% B, plus reconditioning. Flow rate: 0.22 mL min^{-1}; first column temperature: 30 °C; second column temperature: 25 °C; Injection volume: 5 μL. Detection was performed using a triple quadrupole Quattro MicroTM API (Micromass, Manchester, UK) mass spectrometer applying the following conditions: positive ionisation mode, capillary voltage 3.0 kV, cone voltage 25 V, source temperature 130 °C, desolvation temperature 350 °C, cone gas flow (N2): 100 L h^{-1}, desolvation gas flow (N2): 600 L h^{-1}; collision energy 14 eV, dwell time: 0.1 s. Selected Reaction Monitoring (SRM) detection. All data were acquired and processed by the software MASSLYNX 4.0 (Waters).

Quantitative analysis was set up by the internal standard method, with D/L-norleucine as internal standard. Amino acid contents were estimated as follows: AAC = R_f x (A_S/A_{IS}) x nmoles$_{IS}$, where: AAC, amino acid content in nmoles; R_f = relative response factor as determined by the calibration curve; A_S, amino acid area in the sample obtained by the integration of the SRM chromatogram; A_{IS}, internal standard area obtained in the same way.

Total amino acid determination

0.05 mg of dried FAP or FFP was weighted, and 6 mL of 6 N HCl was added and mixed. The tube was flushed with nitrogen for 1 min to remove air. Hydrolysis was then carried out at 110 °C for 23 h. After cooling, the internal standard (7.5 mL of Norleucine 5 mM in water) was added, and the mixture was filtered through paper filter.

Acid hydrolysis was used for the determination of all amino acids except tryptophan (Trp), cysteine (Cys) and methionine (Met). For Cys and Met performic acid, oxidation followed by acid hydrolysis was used. In this case, an amount of 0.05 g of dried FAP or FFP sample was weighed and 2 mL of neat performic acid freshly prepared were added; samples were kept for 16 h at 0 °C. Then 0.3 mL of hydrobromic acid was added to remove excess performic acid. The bromine formed was removed by drying with nitrogen flow. The acid hydrolysis was then performed with 6 N HCl. HPLC-ESI/MS/MS analysis was performed as previously described for free amino acids determination. Met and Cys were determined as methionine sulphone and cysteic acid, respectively. For Trp determination, 15 mg were dissolved in 15 mL of 0.1 M NaOH, just before analysis. 2.5 mL of previous solution was directly analysed by an UV/VIS Spectrometer. The external standard used for the calibration curve was *N*-Acetyl-L-Tryptophanamide in a concentration range between 15 and 185 μM. After the recording of the spectra (between 250 and 350 nm), the fourth derivative, for each sample or standard solution, was evaluated and the absorbance value of the maximum and minimum, respectively at 289 and 286 nm, was obtained. The two values were added, and the result was inserted into the calibration curve previously evaluated. The calibration curve was obtained by plotting the fourth derivative of different solutions of *N*-Acetyl-L-Tryptophanamide, at different dilutions, against concentration to determine the concentration of Trp, and the result was expressed in mg g^{-1}.

Molecular weight distribution determination

Molecular weight distribution of FAPs and FFP was determined by SE-HPLC on BioSep-SEC-S 2000 column (7.8·300 mm) (Phenomenex) installed into ProStar HPLC chromatograph (Varian Inc., USA). Protein and peptide kits from GE Healthcare (USA), Sigma (USA) and Serva (Germany) were used as standards for mass calibration and quantitative calibration. Protein hydrolysates were dissolved (2 mg mL^{-1}) in 50 mM sodium phosphate buffer, pH 6.8, filtered through 0.45 μm PVDF filters (Sartorius, AG, Germany) and injected (10 μL) into the system with subsequent elution with 25 mM sodium phosphate buffer, pH 6.8, at a flow rate of 1 mL min^{-1} and detection at 214 nm.

Growth assays

The growth of *Bifidobacterium* strains was evaluated after 48 h in anaerobic conditions at 37 °C in MRS broth (Oxoid Italia) with cysteine hydrocloride at 0.05%. Each culture was centrifuges at 8000 ***g*** for 10 min at 4 °C. The harvested cells were washed twice, suspended in Ringer solution (Oxoid Italia) and used as inoculum 2% (v/v) in the following media: B12 assay medium (Difco Laboratories, Detroit, MI, USA) broth was used as basal medium (B12) as suggested by Etoh *et al.* (1999), B12 broth supplemented with *N*-acetylglucosammine at 22.00 g L^{-1} (Sigma-Aldrich, Milano, Italy) as control medium (B12-Nag) and B12 broth supplemented with each FAPs (58T, 78T, 81T, 82T, 83T, 100T, 100AT, 101T) and one FFP (6L) at 22.00 g L^{-1} (B12-FAP/FFP) were used to evaluate the hydrolysates' growth promotion. Each medium was filtered by 0.22 μm filters Millex®-GV 0.22 μm (Millipore, Milford, MA, USA). Evaluation of microbial growth after incubation at 37 °C in anaerobic condition was made by turbidimetric measurement by determining OD_{650} using a spectrophotometer (Jasco v-530, Tokyo, Japan).

The growth of *Lactobacillus* strains was evaluated for 24 h in species specific conditions in MRS broth (Oxoid Italia). Each culture was centrifuges at 8000 ***g*** for 10 min at 4 °C. The harvested cells were washed twice, suspended in Ringer solution (Oxoid Italia) and used as inoculum 2% (v/v) in a homemade MRS broth without the major nitrogen sources (MRSN). This medium recipe was based on the MRS medium recipe (Oxoid) (g L^{-1}): glucose, 20.00; dipotassium hydrogen phosphate, 2.00; sodium acetate trihydrate, 5.00; triammonium citrate, 2.00; magnesium sulphate eptahydrate, 0.20; manganese sulphate tetrahydrate, 0.05; complex nitrogen source, 22.0 g L^{-1} (referred to as peptone, yeast extract, 'Lab Lemco' powder); and Tween80, 1 mL L^{-1}. MRSN was used as basal medium while MRSN supplemented with Yeast Extract (Oxoid Italia) at 22.00 g L^{-1} as control medium (MRSN-YE). MRSN supplemented with each FAPs and one FFP (MRSN-FAP/FFP) at 22.00 g L^{-1} was used to evaluate the hydrolysates growth promotion. Each medium was filtered by 0.22 μm filters Millex®-GV 0.22 μm (Millipore). Evaluation of microbial growth after incubation in conditions according to the species was made by turbidimetric measurement by determining OD_{650} using a spectrophotometer (Jasco v-530).

Two series of experiments were carried out for all the strains in each condition tested and resulting values were the average of these two repetition. The Principal Component Analysis (PCA) was performed using STATISTICA 6.1 (StatSoft Italia Srl, Padova, Italy).

Viability assay

The growth and the viability of the cells were evaluated by direct count for both *Bifidobacterium* strains and *Lactobacillus* strains. *Bifidobacterium* strains were grown in B12 broth, B12-Nag (22.00 g L^{-1}) and B12-6L at 22.00 g L^{-1}. While *Lactobacillus* strains were grown in MRSN, MRSN-YE (22.00 g L^{-1}) and MRSN-78T (22.00 g L^{-1}).

Fluorescence microscopy counts was performed using a LIVE/DEAD® BacLightTM Bacterial Viability Kit for microscopy (Invitrogen Ltd, Paisley, UK) as described by Lazzi *et al.* (2011).

Two series of experiments were carried out for all the strains in each condition tested and resulting values were the average of these two repetition.

Results

Molecular composition of the hydrolysates

The molecular composition of the eight FAP (58T, 78T, 81T, 82T, 83T, 100T, 100AT, 101T) and the FFP (6L) samples was determined by total and soluble nitrogen determination and free and total amino acid analysis.

Total and soluble nitrogen for all samples are reported in Table S3. The data clearly indicate that most of the nitrogen fraction is present in all samples in soluble form. Free and total amino acid contents in FAP samples are reported in Fig. S1.

The data indicated, consistently with nitrogen content determinations, that FAP samples are essentially composed of proteinaceous derivatives. Quite interestingly, although soluble proteins are always around 95% of the total protein, free amino acids constitute only about 30% of total amino acids, indicating that most of the soluble proteic fraction is actually composed by peptides. The composition of the free aminoacidic fraction was found to be dominated by Leu, Lys, Glu, Gln, Asp, Asn, Ala, Ile, with a very low content in Cys and Cyss (data not shown). The total aminoacidic fraction was found to be dominated by Glu and Asp, with a very low content of sulphorated amino acids and Trp (data not shown).

FFP sample 6L, obtained through enzymatic hydrolysis after hydrothermal treatment of feathers, although very rich in nitrogen compounds, appeared to be quite poor in free amino acids, which were less than 3% of the total mass and about ten times less than FAP samples (Fig. S1). This indicates that in sample 6L almost the totality of the soluble nitrogen fraction is actually composed by peptides. In this sample, the composition of the free aminoacidic fraction was dominated by Lys and Ser, whereas sulphorated amino acids and Trp were practically absent. Analogously, total aminoacidic fraction in FFP appeared to be dominated by Ser and Glu, whereas Met, His and Trp were present in the lesser amount (data not shown).

Molecular weight distribution of protein hydrolysates was determined by size-exclusion chromatography. Relative content of high (M.w. > 10 kDa), medium (M. w. 3–10 kDa) and low (M.w. < 3 kDa) fractions were estimated using the corresponding calibration curve. The data obtained are listed in Table S4. High molecular weight fraction (M.w. > 10 kDa) of hydrolysates is composed of proteins, whereas the latter two fractions are mainly constituted by oligopeptides (medium molecular weight fraction – M.w. 3–10 kDa) and short peptides with free amino acids (low molecular weight fraction – M.w. < 3 kDa). The data of Table S4 clearly indicate that molecular weight distribution of FAP is closely related to the type of raw material used for its production. Functional animal protein samples obtained from broiler backs (58T, 78T, 83T, 81T and 82T) are predominantly (>50%) composed of low molecular weight compounds (Table S4). In a meantime, FAP derived from necks (100T, 100AT and 101T) was shown to have notably (nearly two times) lower relative content of high molecular weight constituents along with nearly equal relative contributions of medium and low molecular weight fractions. Thus, FAP samples obtained from chicken necks are less intensively hydrolysed as compared to that produced from broiler backs. The latter is in line with data on free and total amino acid composition of FAP (Fig. S1). In contrast to FAP, FFP was characterised by very low content of high molecular weight constituents (<2%) along with overwhelming input (74%) of low molecular weight fraction (M.w. < 3 kDa). Assuming the data on low content of free amino acids in FFP, it could be concluded that it is mainly composed of short peptides.

Growth assay

The growth-promoting activity of the eight FAP (58T, 78T, 81T, 82T, 83T, 100T, 100AT, 101T) and one FFP (6L) samples was tested on eighteen *Bifidobacterium* strains and twenty-eight *Lactobacillus* strains with turbidimetric technique. The growth of bifidobacteria in medium supplemented with FAPs and FFP was compared with that in basal (B12) and control (B12-Nag) media. The growth of lactobacilli in media supplemented with FAPs and FFP was compared with that in basal (MRSN) and control (MRSN-YE) media.

The chart in Fig. S2 shows the average value of the extent of microbial growth of eighteen *Bifidobacterium* strains in B12-FAP or B12-FFP and B12-Nag, compared to the basal medium B12, as determined by turbidimetric measurements. The extent of microbial growth was obtained subtracting the OD value in B12 from the OD value in B12-Nag, B12-FAPs or B12-FFP after 48 h of incubation. The coefficient of variation (CV) expresses the variability of strains responses, among the *genus*, to the supplemented culture media. *Bifidobacterium* strains showed coefficient of variation that ranged from 38% to 453%. The highest variability was observed for B12-83T and B12-Nag, while comparable growth behaviour (low CV) was observed in presence of FAP 81T and FFP 6L. The FAP effect on *Bifidobacterium* growth was different: 58T, 78T, 81T, 82T and 83T enhanced less the growth of all the species as compared to 100T, 100AT and 101T. In the presence of FFP 6L, the highest *Bifidobacterium* growth was observed (Fig. S2). Moreover, the lesser CV (38%), indicating a similar growth behaviour among the genus, was also observed (Fig. S2).

The chart in Fig. S3 represents the average value of the extent of microbial growth of twenty-eight *Lactobacillus* strains in the supplemented media compared to the basal culture medium MRSN, as determined by turbidimetric measurements. The extent of microbial growth was obtained subtracting the OD value in MRSN from the OD value in MRSN-YE, MRSN-FAPs or MRSN-FFP after 24 h of incubation. The CV, expressing the variability of strains responses among the *genus*, ranged from 55% to 73%.

Regardless of the variability of responses among the species, the MRSN-FAPs and FFP positively affected the growth of the strains in comparison with basal medium. Moreover, OD mean values in the FAPs media were comparable with the control medium (MRSN-YE). On the other hand, FFP 6L enhanced less than YE the *Lactobacillus* microbial growth (Fig. S3). It is worthwhile to notice that 78T, the richest in free aminoacids, was the FAP that most positively affected the growth of lactobacilli (Fig. S3).

To evaluate the effect of the different substrates on the growth of *Bifidobacterium* and *Lactobacillus* strains the PCA was performed. The forty-six variables represented the extent of growth of *Bifidobacterium* and *Lactobacillus* strains as determined by turbidimetric analysis, and the nine objects were the FAPs and FFP. As shown in Fig. S4a, the components 1 and 2 explain 62% and 17% of the variance of data set, respectively. This implies that the separation among the data set mainly occurred on the first component axis. As a matter of fact, as clearly shown in Fig. S4a, data points corresponding to the *Bifidobacterium* growth rate are on the left, while those corresponding to the *Lactobacillus* growth rate are on the right of the biplot, with the exception of L10 strain (*Lactobacillus delbrueckii* ssp. *bulgaricus*), that behaved likewise *Bifidobacterium* strains.

The data corresponding to the *Lactobacillus* growth rate are grouped on the right of the biplot, and they are separated also for the component 2. When the objects for the components are examined (Fig. S4b), the first component segregated FFP from FAPs. Moreover, FAPs group together in four clusters considering both the two components (Fig. 4b). It should be noted that clustering of FAP samples into four groups meet well the data on their molecular weight distribution (Table S4) and on their content in free and total aminoacids (Fig. S1). Functional animal proteins 81T, 82T, 83T and 58T were treated under similar process conditions that led to similar free aminoacids content. Instead FAP 78 is characterised by the highest content of free aminoacids (0.362 g g^{-1}). Moreover, FAP 78T has the lowest percentage of the medium (M.w. 3–10 kDa) molecular weight fraction. These characteristics could be due to a high enzyme dosage and a high hydromodule value (Table 2). Increasing multienzyme dosage and water to raw material ratio could result into more pronounced secondary proteolysis of oligopeptides leading to enrichment of FAP in free amino acids and short peptides (di-, tripeptides).

Viability assay

The data obtained from the growth assay suggest that FFP 6L and FAP 78T could be optimal growth promoters for *Bifidobacterium* and *Lactobacillus,* respectively. For this reason, they were chosen to evaluate their effect on cells viability. The viable cells number was evaluated by fluorescence microscopic technique.

Cell viability for *Bifidobacterium* strains in the media B12-6L varied from 6.46 log cell mL^{-1} for B16 strain (*B. pseudocatenalatum*) to 9.86 log cell mL^{-1} for B4 strain (*B. asteroides*) and was always higher than the values observed in basal and control medium (Fig. S5). It is worth noting that *B. merycicum* (B13) was not able to growth in any media. In particular, the initial inoculum, 6.15 log cell mL^{-1} (data not shown) was not able to duplicate and lost viability in B12 assay medium and in B12-6L. Even, in the presence of Nag, the cells' inoculum was autolysed totally (data not shown). B5, B10, B14, B16 and B17 strains kept their viability in basal control medium and in proteinaceous hydrolysate presence. B9, B12 and B6 strains kept their viability only with proteinaceous hydrolysate presence. All of them lost viability, or even they underwent to autolysis, in the other growth condition (data not shown).

Figure S6 showed the level of viability among the *Lactobacillus* strains in the culture media supplemented with 78T. In MRSN only seven strains (L8, L10, L12, L14, L16, L17, L18) were not able to grow and did not keep their viability. When complex nitrogen sources, FAP or YE, were added, values of viable cells resulted high in all the strains. The strain L19 (*L. pentosus*) grew in the same way in MRSN, MRSN-YE, MRSN-78T and seemed to be not affected by the absence of complex nitrogen source. Among the strains different growth capacity and level of viability were observed. The strain that presented the lowest number of viable cells (7.10 log cell mL^{-1}) was L10 (*L. delbrueckii* ssp. *bulgaricus*), while L6 (*L. casei*) evidenced a higher degree of vitality (10.41 log cell mL^{-1}) in MRSN-78T (Fig. S6).

Discussion

The aim of the present work was to explore the ability of proteinaceous hydrolysates as microbial growth promoter, also in consideration for their potential use in the production of microbial biomass with improved viability.

Biomass production of those belonging to *Lactobacillus* and *Bifidobacterium* genus, can be of great importance because of their large implication in functional food (Giraffa *et al*., 2010; Gobbetti *et al*., 2010).

Lactobacillus genus, as all lactic acid bacteria, needs rich growth media containing compounds such as amino acids, peptides, fatty acids, vitamins and nucleic acids. These compounds are usually found in the form of a complex nitrogen source generally provided by MRS medium, the standard laboratory medium for lactobacilli (De Man *et al*., 1960). The selection of the appropriate type of the nitrogen source is very important. For this reason, in the industrial fermentations, supplemental components are usually added to increase the amount of biomass. Yeast extract (YE) is the most commonly used nitrogen supplement in laboratory scale fermentation (Salgado *et al*., 2009). Many studies reported its use as a supplement (Aeshlimann & Von Stockar, 1990; Mehaia & Cheryan, 1991; Nancib *et al*., 2005;), but wide application of YE in industrial biotechnological processes is limited by its relatively high cost. In this work, the growth and viability of lactobacilli in medium supplemented with FAPs and FFP were compared with those obtained in a control medium with addition of yeast extract. Overall, *Lactobacillus* growth in the presence of FAPs was very similar, or even better, than in MRS supplemented with YE. Similar growth behaviour in each FAPs studied, among the *Lactobacillus* species, was observed. *Lactobacillus* growth stimulation by FAP can be related to their high soluble nitrogen content. Actually, among FAPs, 78T, the richest in free amino acids, showed the greatest potential in biomass production. This result is in agreement with multiple amino acids auxotrophies typical of lactobacilli, which can modify their ability to growth depending of amount and quality of amino acids in a medium (Morishita *et al.*, 1981).

As most of the FAPs soluble proteic fraction are composed by peptides, the effect of the different FAPs depends also on the ability of lactobacilli proteolytic enzymes and peptide transport systems (Pritchard & Coolbear, 1993; Kunji *et al.*, 1996; Aspmo *et al.*, 2005). Lactic acid bacteria have three different systems for transport of free amino acids, di- and tri-peptides and oligopeptides in size up to six amino acid residues (Pritchard & Coolbear, 1993). Although, all these systems are involved into the uptake of essential amino acids from the nutritional medium, the highest rate of uptake is provided by the transporting di- and tri-peptides system (Van Niel & Hahn-Hägerdal, 1999). Therefore, it could be concluded that protein hydrolysates with the high content of short peptides (di- and tri-peptides) could be expected to exhibit pronounced effect on growth of lactobacilli. Otherwise when FFP (rich in oligopeptides but poor in free amino acids) is used the activities and/or specificity of extracellular proteases seem to be not enough to allow a great biomass production, because in this medium *Lactobacillus* growth was not enhanced.

Differently from *Lactobacillus*, FFP appeared to be the most stimulating supplementation for bifidobacteria, both in terms of growth rate and viability. Functional feather protein 6L, albeit very rich in nitrogen compounds, appeared to be quite poor in free amino acids, which were <3% of the total mass and about ten times less than FAPs. Thus, in FFP 6L almost the totality of the soluble nitrogen fraction is composed by peptides. This suggests that bifidobacteria are able to hydrolyse the FFP peptides, present in the sample, in a better way as compared to *Lactobacillus*, or that they find, among the peptides, some specific growth-promoting factors. It is known that bifidobacteria growth is stimulated by the presence of different promoting factors (Modler, 1994). Promoting factors can be non-glycosilated peptides derived from protein after hydrolysis using proteinase (Tamime *et al.*, 1995; Zhao *et al.*, 1996). A number of studies reports that proteinaceous compounds found in human milk (Liepke *et al.*, 2002) and in cow milk (Petschow & Talbott, 1991) have the ability to promote growth of bifidobacteria. Also molecules present in dairy industry effluents like whey (Mahalakshmi & Murthy, 2000) or by-product of latex rubber production have this feature (Ishizaki, 1989; Ishizaki, 1995; Oiki *et al.*, 1996; Etoh *et al.*, 2000). The peptides of human milk that are known to exhibit growth-promoting effect on bifidobacteria, are characterised by the presence of one or two disulphide bonds within the single oligopeptide chain or between two different ones (Liepke *et al.*, 2002). Functional feather protein is produced from feather keratin that is known to be quite abundant (up to 4%) in cysteine residues forming a network of disulphide bonds providing 3D structure of keratin filaments (Korner, 2008). Enzymatic hydrolysis of feather keratin could result into formation of cystine-containing bifidogenic oligopeptides which have structures similar to molecular targets with those identified in human milk.

Conclusion

Bacterial growth depends on the biosynthesis and/or uptake of medium components required for the formation of biomass. Growth media represent a very high cost in industrial application, and for this reason, it is economically interesting to find less expensive supplements to replace the common ones. Currently, a great deal of attention is being paid on the biotechnological potential of food-industrial residues. This study suggested that by-products from poultry industries provide a good alternative to substitute expensive nutrient supplements with cheaper renewable low-cost products for growing *Lactobacillus* and *Bifidobacterium*. In food application, the viability of probiotic strains, coupled with the amount of biomass, is used as the measure for probiotic suitability. Functional animal proteins for *Lactobacillus* and FFP for *Bifidobacterium* are promising ingredients of industrial media not only because they support growth very well but also because they allow to maintain an high level of viability.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Total (light grey bars) and free aminoacid (dark grey bars) content in FAP and FFP samples. Error bars refer to the standard deviations obtained by three independent replicates of the determination.

Figure S2. Average Optical Density at 650 nm (Δ $OD_{650\ nm}$) value of the extent of microbial growth of 18 *Bifidobacterium* strains in FAP/FFP and Nag respect to the basal culture medium B12 determined by turbidimetric measurements. Coefficient of variation is reported on top of the bars.

Figure S3. Average Optical Density at 650 nm (Δ $OD_{650\ nm}$) value of the extent of microbial growth of 28 *Lactobacillus* strains in FAP/FFP and YE respect to the basal culture medium MRSN determined by turbidimetric measurements. Coefficient of variation is reported on top of the bars.

Figure S4. PCA biplot of variables: extent of growth of each strains (a); PCA biplot of objects: media supplemented with FAPs or FFP (b).

Figure S5. Number of viable cells (log cell/mL) of 18 *Bifidobacterium* strains after 66 hour at 37°C in anaerobic condition in B12 (dark grey bars), B12-Nag (medium grey bars), and B12-6L (light grey bars).

Figure S6. Number of viable cells (log cell/mL) of 28 *Lactobacillus* strains after 24 hour at best growth condition for each species in MRSN (dark grey bars), MRSN-YE (medium grey bars), and MRSN-78T (light grey bars).

Table S1. Table of strains.

Table S2. Hydrolysis conditions for obtaining of protein hydrolysates from leftovers of poultry processing industry.

Table S3. Total and soluble nitrogen of FAP and FFP.

Table S4. Molecular weight distribution of FAP and FFP.

Table S1. Table of strains

Strain	Species	Origin
B1	*B. angulatum*	Fecal samples
B2	*B. animalis* subsp. *animalis*	Reference strain ATCC25527
B3	*B. animalis* subsp. *animalis*	Fecal samples
B4	*B. asteroides*	Fecal samples
B5	*B. bifidum*	Fecal samples
B6	*B. catenulatum*	Fecal samples
B7	*B. choerinum*	Reference strain LMG10510
B8	*B. gallicum*	Reference strain LMG11596
B9	*B. longum*	Fecal samples
B10	*B. longum*	Fecal samples
B11	*B. longum*	Reference strain LMG21814
B12	*B. magnum*	Fecal samples
B13	*B. merycicum*	Reference strain LMG11341
B14	*B. minimum*	Reference strain LMG11592
B15	*B. pseudolongum*	Fecal samples
B16	*B. pseudocatenalatum*	Fecal samples
B17	*B. scardovii*	Reference strain LMG21589
B18	*B. subtile*	Reference strain LMG11597
L1	*L. acidophilus*	Acidophilus milk LMG8151
L2	*L. acidophilus*	Reference strain LMG9433
L3	*L. brevis*	White stilton cheese LMG11434
L4	*L. brevis*	Reference strain LMG6906
L5	*L. casei*	Human LMG23516
L6	*L. casei*	Reference strain LMG6904
L7	*L. curvatus*	English hard cheese LMG12007
L8	*L. curvatus*	Reference strain LMG9198
L9	*L. delbrueckii bulgaricus*	Homemade yogurt LMG12168
L10	*L. delbrueckii bulgaricus*	Reference strain LMG13551
L11	*L. fermentum*	Fermented beets LMG6902
L12	*L. fermentum*	Human LMG8900
L13	*L. helveticus*	Reference strain LMG13555
L14	*L. helveticus*	Cheese LMG18225
L15	*L. lactis*	Fermented meat LMG18223
L16	*L. lactis*	Reference strain LMG7942
L17	*L. paraplantarum*	Reference strain DSM10667
L18	*L. paraplantarum*	Origin unknown LMG18398
L19	*L. pentosus*	Reference strain DSM20314
L20	*L. pentosus*	Reference strain LMG10755
L21	*L. plantarum*	Reference strain LMG18399
L22	*L. plantarum*	Picklet cabbage LMG6907
L23	*L. reuteri*	Reference strain LMG13557
L24	*L. reuteri*	Reference strain LMG9213
L25	*L. rhamnosus*	Reference strain LMG6400
L26	*L. rhamnosus*	Hard cheese
L27	*L. salivarius*	Human LMG235206
L28	*L. salivarius*	Reference strain LMG9477

Table S2. Hydrolysis conditions for obtaining of protein hydrolysates from leftovers of poultry processing industry.

Sample code	**58 T**	**78 T**	**83 T**	**81 T**	**82 T**	**100 T**	**100 AT**	**101 T**	**6L**
Raw Material	broiller backs	broiller backs	broiller backs	broiller backs	broiller backs	necks	necks	necks	HTST treated feather
[*]**Hydromodule**	1:1.5	1:2.5	1:2.0	1:2.0	1:2.0	1:2.0	1:2.0	1:2.0	1:4.0
Enzyme dosage **(**AU[a] per g of raw material)	0.73	1.21	0.97	0.97	0.97	1.21	1.21	1.21	10.0

[a] – AU Anson units of endoprotease activity determined with sodium caseinate as a substrate at pH 7.0 and 55°C.

[*]Hydromodule value is the raw material to water ratio

Table S3. Total and soluble nitrogen of FAP and FFP

Sample code	**58 T**	**78 T**	**83 T**	**81 T**	**82 T**	**100 T**	**100 AT**	**101 T**	**6L**
Total N %	11.9	12.7	12.4	12.7	13.1	11.4	12.5	12.5	14.5
Soluble N %	11.8	11.8	12.1	11.8	12.7	11.2	11.3	11.4	12.0

Table S4. Molecular weight distribution of FAP and FFP

Sample code	**58 T**	**78 T**	**83 T**	**81 T**	**82 T**	**100 T**	**100 AT**	**101 T**	**6L**
>10 kDa,%	16.1	25.0	20.1	19.2	27.2	11.0	6.7	11.3	1.8
3-10 kDa,%	31.8	17.2	21.1	21.2	20.6	45.7	53.9	44.0	24.4
<3 kDa,%	52.1	57.8	58.7	59.7	52.2	43.3	39.4	44.7	73.8

Legend to figure

Figure S1 Total (light grey bars) and free aminoacid (dark grey bars) content in FAP and FFP samples. Error bars refer to the standard deviations obtained by three independent replicates of the determination.

Figure S2 Average Optical Density at 650 nm (Δ OD$_{650nm}$) value of the extent of microbial growth of 18 *Bifidobacterium* strains in FAP/FFP and Nag respect to the basal culture medium B12 determined by turbidimetric measurements. Coefficient of variation is reported on top of the bars

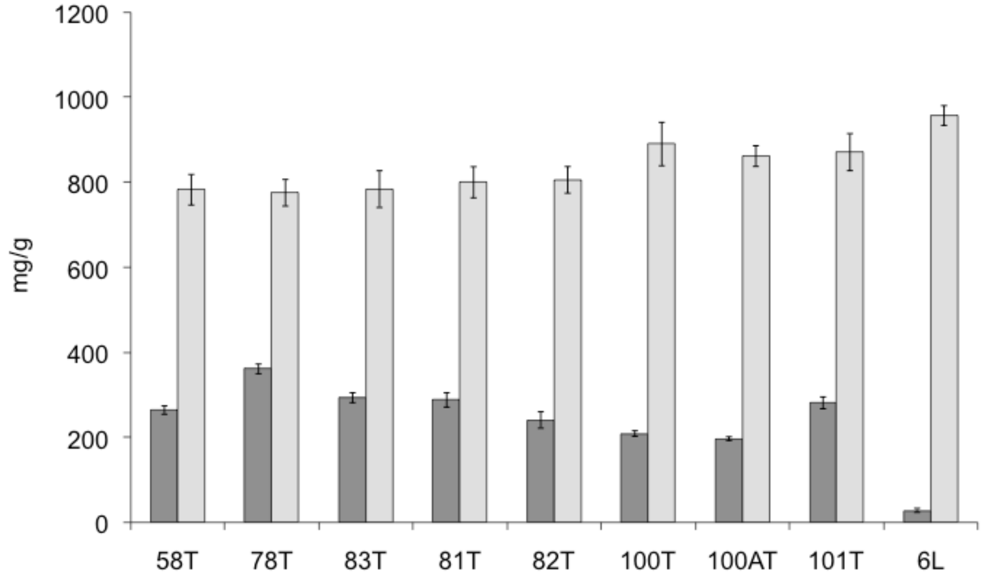
Figure S3 Average Optical Density at 650 nm (Δ OD$_{650nm}$) value of the extent of microbial growth of 28 *Lactobacillus* strains in FAP/FFP and YE respect to the basal culture medium MRSN determined by turbidimetric measurements. Coefficient of variation is reported on top of the bars

Figure S4 PCA biplot of variables: extent of growth of each strains (a); PCA biplot of objects: media supplemented with FAPs or FFP (b)

Figure S5 Number of viable cells (log cell/mL) of 18 *Bifidobacterium* strains after 66 hour at 37°C in anaerobic condition in B12 (dark grey bars), B12-Nag (medium grey bars), and B12-6L (light grey bars)

Figure S6 Number of viable cells (log cell/mL) of 28 *Lactobacillus* strains after 24 hour at best growth condition for each species in MRSN (dark grey bars), MRSN-YE (medium grey bars), and MRSN-78T (light grey bars)

Figure S1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1000
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mg/g
58T
78T
83T
81T
82T
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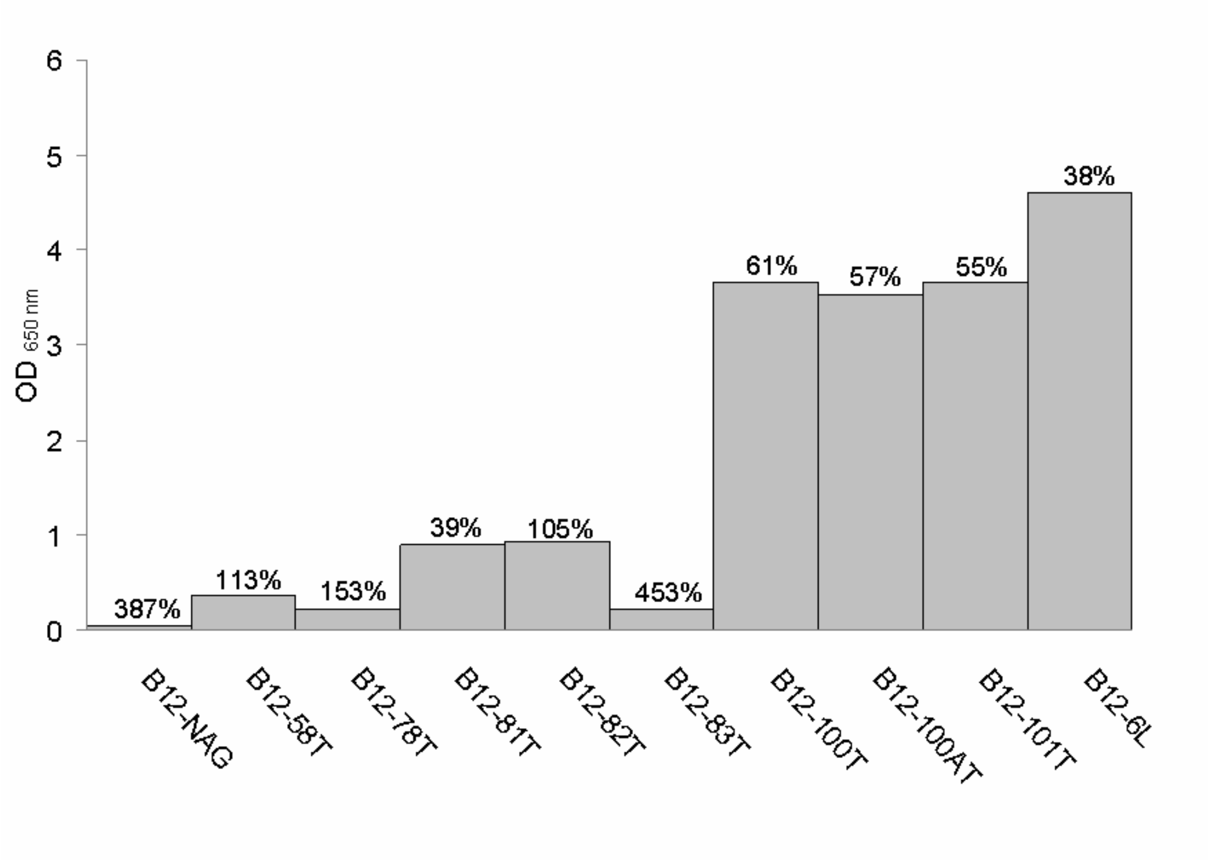
100AT
101T
6L

Figure S2



OD 650 nm
6
5
4
3
2
1
0
387%
113%
153%
39%
105%
453%
61%
57%
55%
38%
B12-NAG
B12-58T
B12-78T
B12-81T
B12-82T
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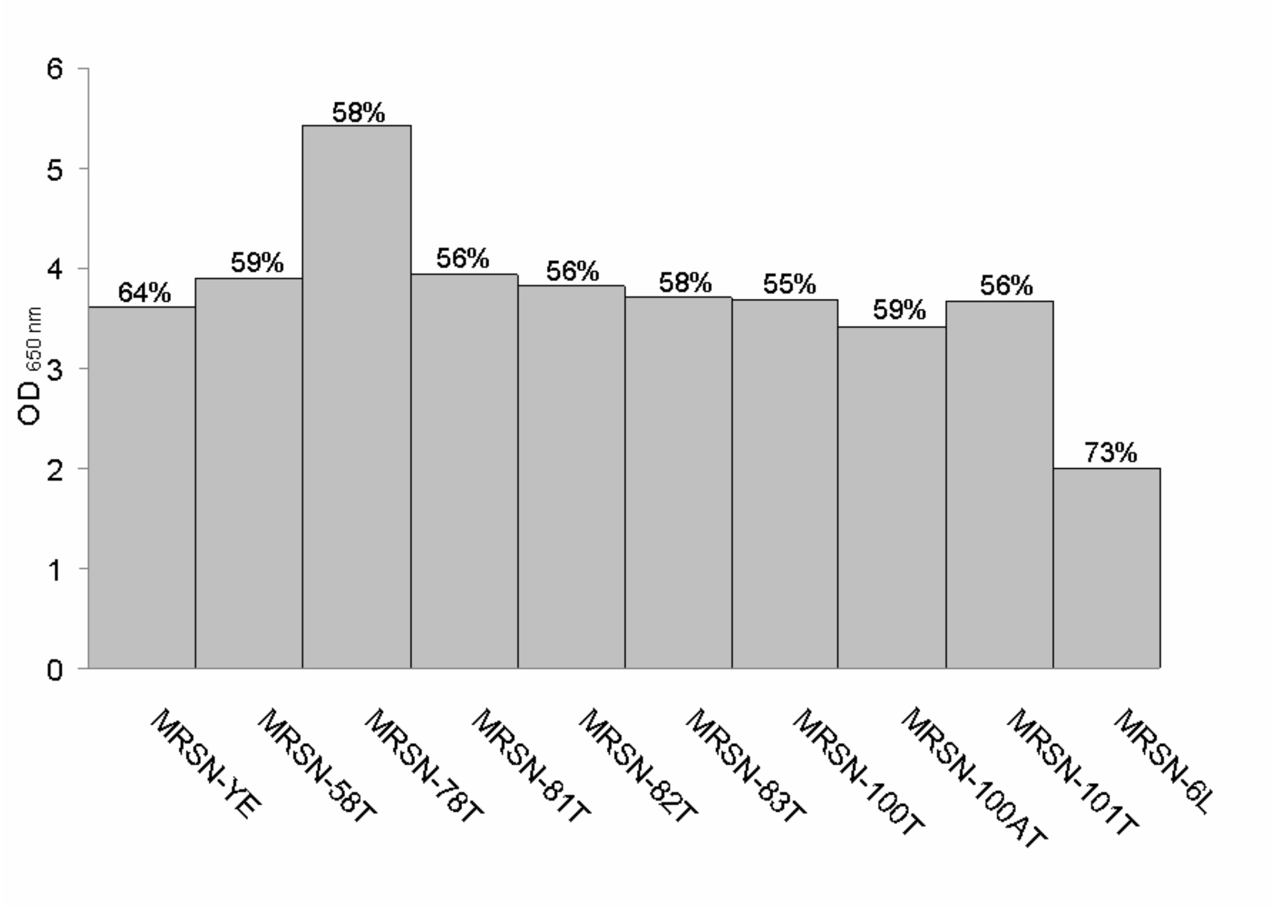
B12-100AT
B12-101T
B12-6L

Figure S3


OD 650 nm
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1
2
3
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64%
59%
58%
56%
56%
58%
55%
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56%
73%
MRSN-YE
MRSN-58T
MRSN-78T
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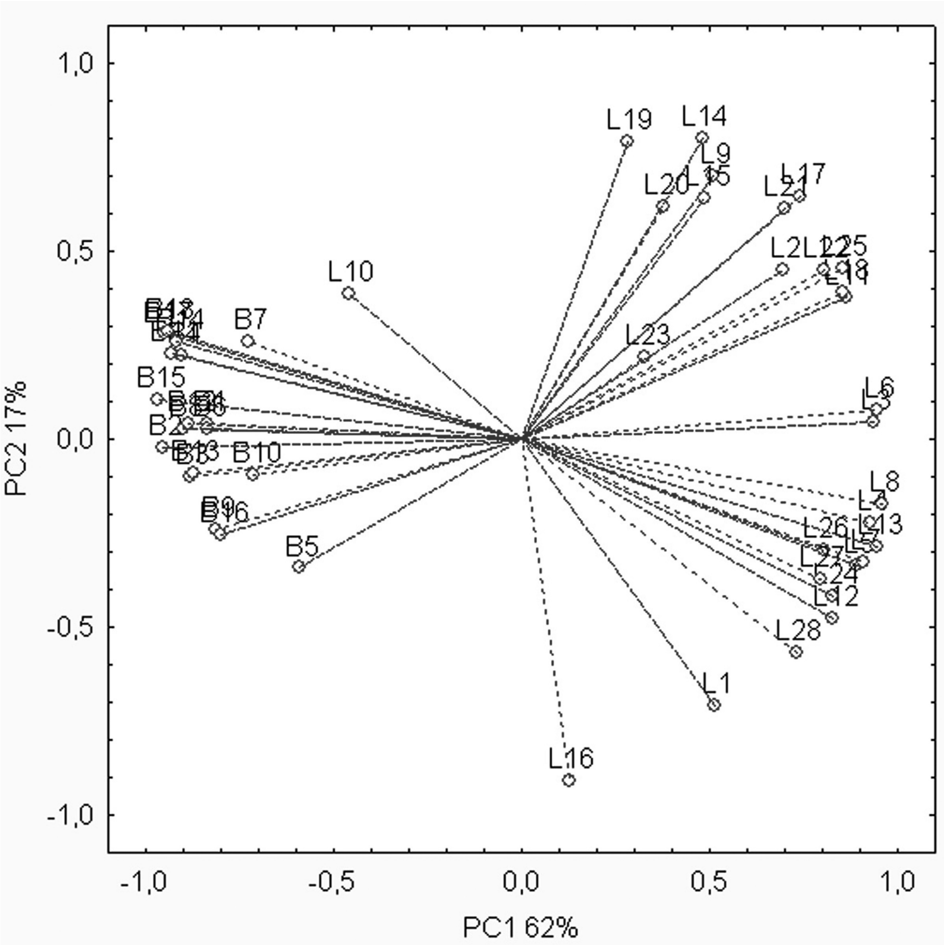
MRSN-6L

Figure 4a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PC2 17%
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PC1 62%
L19
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L2
L25
L11
L10
B7
B15
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B10
B16
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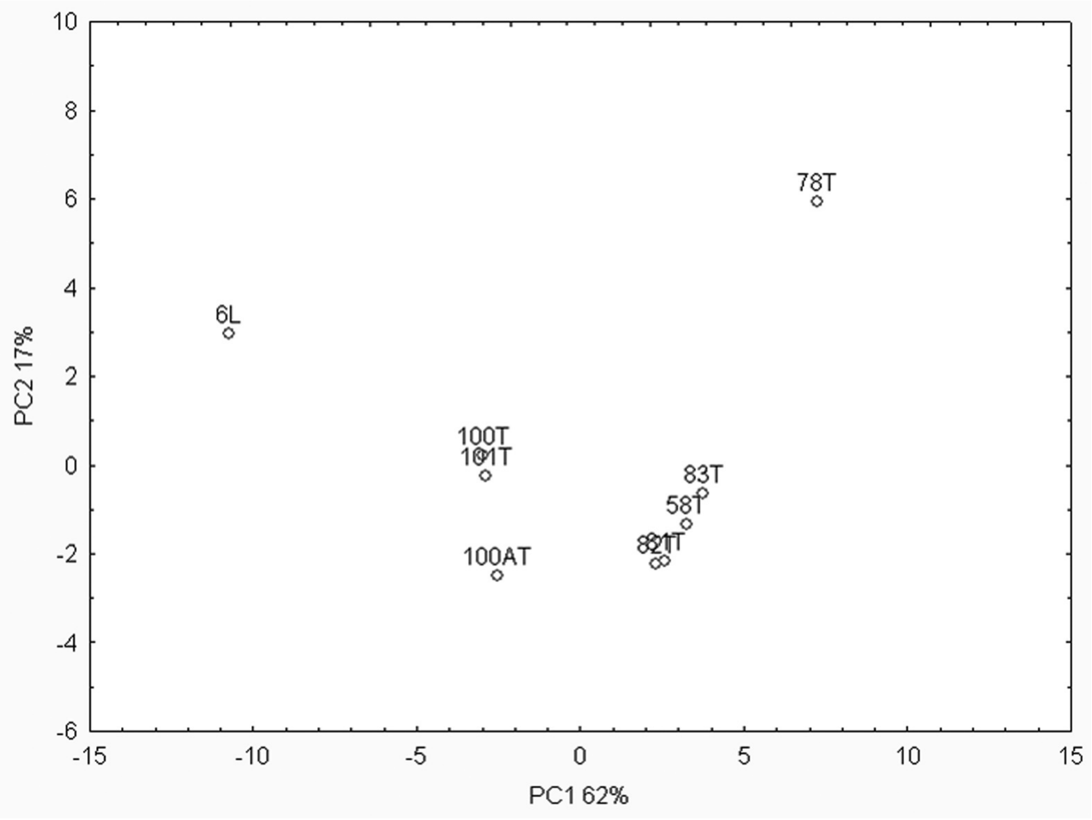
L12
L28
L1
L16

Figure 4b


PC2 17%
PC1 62%
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5
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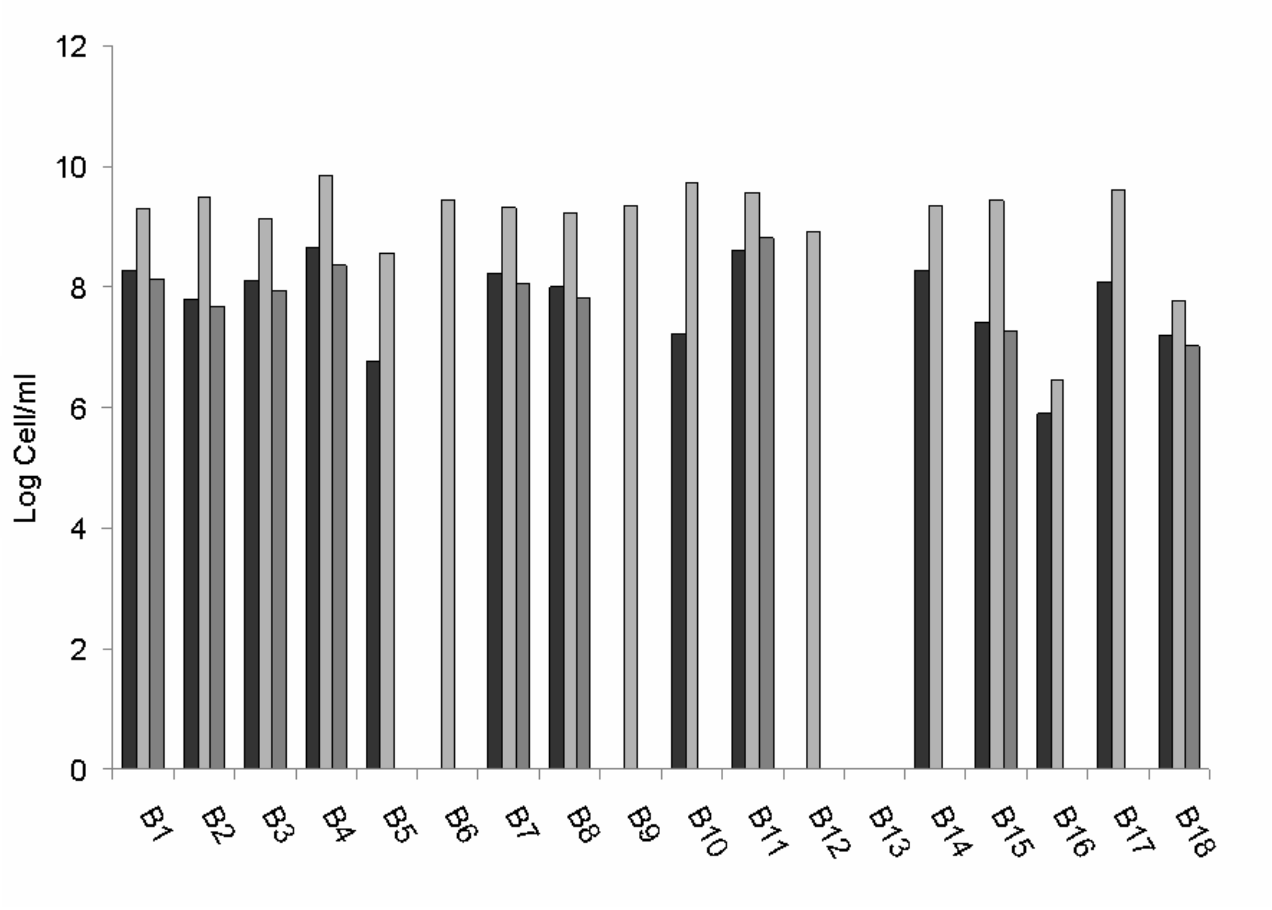
15
78T
6L
100T
101T
83T
58T
81T
82T
100AT

Figure S5



12
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Log Cell/ml
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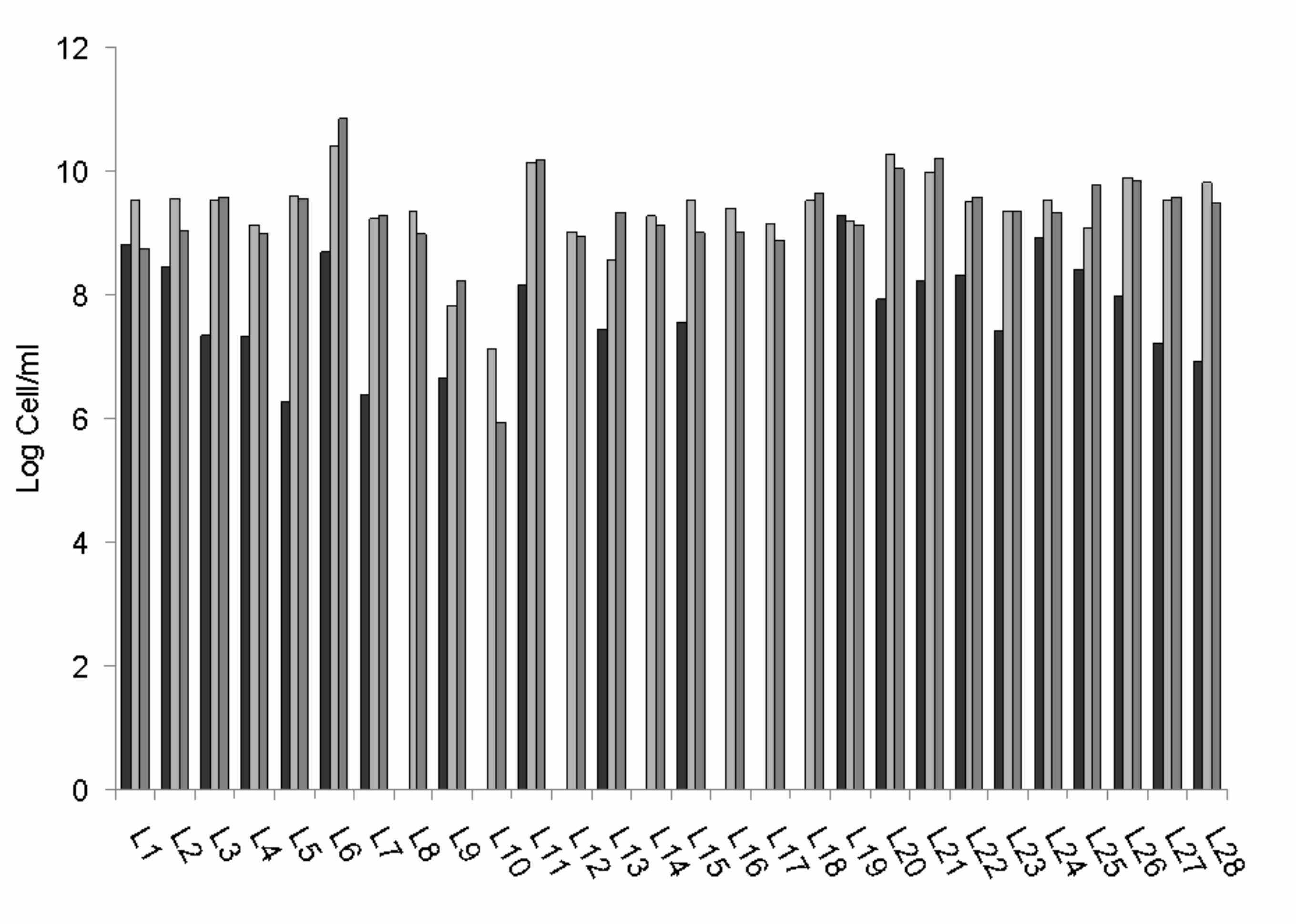
B17
B18

Figure S6


Log Cell/ml
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5 Effect of protein hydrolizates on growth kinetics and aminopeptidase activities of *Lactobacillus.*

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Effect of protein hydrolizates on growth kinetics and aminopeptidase activities of *Lactobacillus*

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Keywords: poultry hydrolizates, *Lactobacillus*, peptone, growth kinetics, aminopeptidase

Running Title: **Effect of protein hydrolizates on *Lactobacillus* strains**

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Abstract

The goal of this study was to evaluate how two new hydrolizates from poultry by products act on ten lactobacilli growth kinetics when supplemented to the growth medium. These effects were compared with ones induced by two most common commercial hydrolizates, i.e. Tryptone and Peptone. Moreover aminopeptidase activities (AA) of each strain were determined to investigate the effect of the growth condition on the modulation of aminopeptidase pattern. Five cell extracts of each strain, obtained from their cultivation in MRS and in presence of the two common and the two new hydrolizates, were considered. AA was investigated against five different chromogenic substrates: β-naphthyl amide (β-NA) derivates of L-anomers of leucine (Leu), lysine (Lys), proline (Pro), glycine-proline (Gly-Pro) and phenilalanine-proline (Phe-Pro). Growth medium, supplemented with one of new hydrolizates, 78T, as only nitrogen source, demonstrated to reach the maximum growth rate and the biomass yield higher than Tryptone and Peptone. A great variability of AA was observed. The modulation of aminopeptidase pattern was affected more by the behaviour of each strain than by the growth medium composition.

1. Introduction

Lactobacillus spp are widely used in the food process industry as starter culture for the manufacture of fermented foods and beverages or as probiotics for incorporation into functional foods (Leroy & De Vuyst, 2004). They are generally know to be nutritionally fastidious bacteria indeed they need complex growth media that have to contain compounds as amino acids, peptides, fatty acids, vitamins and nucleic acid (Stolaki et al., 2011). All these components usually are provided in standard laboratory medium such as Man Rogosa Sharpe (MRS) (De Man et al., 1960).

In the production of microbial cell mass and bioproducts by the fermentation industry, the growth substrate constitutes a major cost and the nitrogen source is usually the most expensive component of bacterial growth substrate (Taskin et al., 2011). Protein hydrolyzates are widely used in the manufacture of probiotics and starter cultures due to their exploitation in microbiological media by fermentation and biotechnology industries (Pasupuleti & Demain, 2010; Sodini et al., 2005).

The recovery of poultry processing leftovers as high quality protein hydrolizates was the subject of the European project PROSPARE “PROgress in Saving Proteins and Recovering Energy” (www.prospare.eu). The Functional Animal Protein (FAP) and Functional Feather Protein (FFP) obtained by a short-term hydrothermic and enzymatic hydrolysis of poultry wastes have shown to promote the microbial growth and to enhance the cell viability for several species of *Lactobacillus* and *Bifidobacterium* (Lazzi et al., 2012; Lazzi et al., 2011). With the aim to propose these by-products as nitrogen source in cultured media instead of expensive nutrient supplements, in the present study we further explored their growth promotion effect in comparison with commercial ones, Peptone and Tryptone. Moreover, to deepen this aspect, we study the expression of aminopeptidase activities as response of the growth in the presence of different hydrolizates.

2. Material and methods

2.1. Bacterial strains

Ten *Lactobacillus* strains were studied: nine of these were purchased from “Belgian co-ordinated collection of microorganism” (LMG, Gent, Belgium, http://bccm.belspo.be/) and one strain, Lr85, belongs to the microbial collection of Department of Food Science, University of Parma (Table 1). All the strains were maintained as culture stocks in 15% glycerol (w/v) at − 80 °C*.* *Lactobacillus* strains were routinely grown overnight in MRS (Oxoid Italia, Milano, Italy) broth following the best growth condition for each species (Table 1).

2.2. Growth Media

Five different growth media were used in this study: MRS (Oxoid), MRS broth without the major nitrogen sources as basal medium (Lazzi et al., 2012) (MRSN) supplemented with 22g/L of different proteinaceus hydrolizates: FAP 78T (78T) from poultry bones and meat trimmings, FFP 6L (6L) from poultry feathers, Tryptone (TRY) (Oxoid) and Peptone (PEP) (Oxoid).

2.3. Growth curves

Ten strains belonging to *Lactobacillus* genus were grown on five different media: MRS, MRSN-78T, MRSN-6L, MRSN-TRY and MRSN-PEP. The growth conditions, i.e. temperature and aerobic/anaerobic environments, were set up according to each species (Table 1). Their growth was monitored during 24 hours by reading turbidity at 600 nm (OD600) to obtain growth curves for each strains in each cultured media. The maximum cell density, OD_{max}, was determined as the maximal OD value reached at the stationary phase. The maximum specific growth rate, μ_{max}, was determined by calculating the slope of the exponential growth phase ($\mu_{max}= \Delta \ln(OD600)/\Delta t$, where *t* is time and expressed as h^{-1}). Each growth curve was carried out in duplicate.

2.4. Aminopeptidase assay

Cell extract of the strains were obtained from their cultivation in MRS, MRSN-6L, MRSN-78T, MRSN-TRY, MRSN-PEP. To allow an adaptation at the culture media and an optimal growth rate, cells extracts (CE) were obtained following the methods of Chen and Steele (1998). Protein concentrations were determined as using the Bradford commercial kit (Bio-Rad Laboratories, Hercules, Calif.) with bovine serum albumin (Sigma-Aldrich) as a standard.

Aminopeptidase activity (AA) was investigated against five different chromogenic substrates: β-naphthyl amide (β-NA) derivates of L-anomers of leucine (Leu), lysine (Lys), proline (Pro), glycine-proline (Gly-Pro) and phenilalanine-proline (Phe-Pro). Each CE was incubated with 0.650 mmol/L solutions of β-naphthyl amide derivates (Bachem Feinchemikalien AG, Switzerland) and 0.05 mol/L phosphate buffer pH 7.0 at 37°C for specific time to maintain the measured in the linear range of the reaction: for Gly-Pro and Leu 30 minutes and for Phe-Pro, Lys and Pro 3 hours. The reaction was stopped adding 250 µL of 2.0 mol/L HCl. The degree of hydrolysis was determined by measuring the colored product of an azocopulation reaction by reading spectrophotometrically at 580 nm according to Bouquien et al. (1998).

The arbitrary unit of enzyme activity (AA) was defined as micromoles of β-naphthylamide released per minute and per gram of proteins presents in each CE. Each assay was carried out in triplicate.

2.5. Statistical analyses

Descriptive statistics, ANOVA ($p<0,05$) and *post hoc* Tuckey test were conducted with SPSS ver. 19 (IBM Software, Milan, Italy).

3. Results and Discussion

3.1. Growth Curves

Ten strains belonging to *Lactobacillus* genus were grown on five different media: MRS, MRSN-78T, MRSN-6L, MRSN-PEP, and MRSN-TRY. Their growth was monitored during 24 hours and their growth curves were obtained. The stationary phase was reached after 12 to 14 hours of incubation.

Analysing the μ_{max} values (Figure 1), there was not significant differences between MRS and the other four media. Otherwise μ_{max} values obtained with MRSN-78T (0,18± 0,07) (mean values ±

standard deviation) are significantly different when compared to MRSN-6L (0,09±0,05), MRSN-PEP (0,09±0,05) and MRSN-TRY (0,10±0,08). In particular MRSN-78T promoted the highest μ_{max} mean values (0,18±0,07) (Figure 1).

Analysing the OD_{max} data there was not a significant difference between MRS and MRSN-78T or MRSN-6L ($p>0,05$). Otherwise MRSN-78T variance was always significantly different from all the other experimental media (MRSN-6L, MRSN-PEP, MRSN-TRY) (Figure 2). This means that the media, supplemented with 78T hydrolizate as only nitrogen source, can sustain beside the maximum growth rate, also the biomass yield (OD_{max}) in the same way of MRS, which contains different nitrogen sources (i.e.: Tryptone, Peptone, Yeast Extract). Moreover MRSN-78T promoted the highest OD_{max} mean values (2,66±0,66, standard error) like MRS (2,40±0,59) (Figure 1). Differently from MRSN-78T, the OD_{max} mean value in MRSN-6L (1,79±0,68) was not significantly different ($p>0,05$) from MRSN-PEP (1,65±0,83) and MRSN-TRY (1,53±0,91) but also from MRS. Therefore the performance of this hydrolizate resulted to be less efficient than the hydrolizates abtained from bone and meat as raw material (Figure 2).

Figure 3 represents how the experimental media could affect each strain's growth density compared to growth density in MRS at the stationary phase: the OD values were calculated by subtracting the OD value obtained in experimental media from the OD value obtained in MRS.

The hydrolizate 78T can fulfil the complex nitrogen requirement of different species of lactobacilli in fact among all the strains only *Lb. fermentum* LMG6902 had not benefit when grown in MRSN-78T and *Lb. plantarum* LMG18399 which grew very well in very similar way in all five media (table 2). In particular, LMG18399 showed high ODmax values ranging from 2,71 ±0,01 in MRSN-TRY to 3,00 ±0,01 in MRS, denoting the skill in adapting to the different media (table 2). The only strain that had benefit also in MRSN-6L was *Lb. curvatus* LMG9198.

3.2. Enzyme assay

After the adaptation to each growth media the CE of each *Lactobacillus* strain was extracted. Five CE for 10 strains against 5 substrates were investigated.

Aminopeptidase activities (AA) of each strain were determined to investigate the effect of the growth condition on the modulation of aminopeptidase pattern. The specific activities of the *Lactobacillus* strains toward the five different chromogenic substrates (Leu β-NA, Lys β-NA, Pro β-NA, Gly-Pro β-NA, Phe-Pro β-NA) substrates were reported in figure 4. AAs are expressed as μmol of β-naphthyl amide released by aminopeptidase per minute and per gram of protein. In the figure the AA of each strain are reported grouped by medium. All the data represent the mean of triplicate experiment ± standard deviation. Generally the variability of AAs is less affected by the growth medium, in particular for the strains that express high AA, such as LR85, LMG6904, and LMG6400. Indeed these strains have high AA against Leu notwithstanding the growth medium. Moreover the CE of *Lactobacillus* spp of this work showed more frequently a higher AA for Leu besides the growth media. As reported by several studies, for instance Gatti et al. (2004), Savijoki et al. (2006), De Dea Lindner et al. (2008), this chromogenic substrate detects the presence of generic aminopeptidases PepC and PepN, often found in lactic acid bacteria (LAB), and these two aminopeptidase can also hydrolyse N-terminal residues of Lys from peptides. In this study it was also observed that the AA for Leu was generally higher than the AA for Lys (figure 4). Therefore AA for Leu could be due also to the presence of another enzyme: PepL, a LAB aminopeptidase that displays high specificity for Leu (Klein et al., 1995). Also the activity of PepX, dipetdidil prolidase, detected by Gly-Pro and Phe-Pro substrates, was often high. This dipetdidil prolidase is often found in species belonging to LAB (Savijoki et al., 2006). For what concern the activity against Pro the strains of this work showed low AA denoting a low activity of the thiol-enzyme prolinase (PepR) and the serine enzyme proline-iminopeptidase (PepI) that can cleave Pro residues in the amino end of peptides (Savijoki et al., 2006). This confirms the result obtained by Herreros and colleagues (2003) with strains belonging to the same species studied in this work.

Overall a great variability was observed among strains: also strains belonging to the same species showed peculiar AA profile. Among the strain studied *Lb. plantarum* (LMG18399), *Lb. fermentum* (LMG6902 and LMG8900) showed poor aminopeptidase activity for each substrate in each growth conditions (Fig 4). For what concern *Lb. rhamnosus* strains (LR85, LMG6400) a high degree of activity was detected toward Leu β-NA, even if the behaviour resulted different between these strains. LR85 reached a maximum value of 200,00±9,43 μmol of β-naphthyl amide in MRS while LMG6400 reached the maximum value of 108,32±2,38 μmol in MRSN-TRY. The AA of *Lb. rhamnosus* strains for Phe-Pro, Gly-Pro, Lys and Pro were present in all the growth conditions but were generally low. In particular the μmol of β-naphthyl amide released did not exceed 50,00±4,72 μmol for LR85 and 23,35±3,21 for LMG6400.

Lb. casei strains (LMG23516 and LMG6904) had AA for Leu, especially LMG6904 showed an high activity as confirmed by Nieto-Arribas and colleagues (2009) that found higher AA for synthetic substrate containing Leu than the one containing Lys. Among all the synthetic substrates AA's LMG23516 were more induced by MRSN-6L and MRSN-PEP than the other ones.

LMG18223, *Lb. delbrueckii lactis* had the high AA for Phe-Pro and Gly-Pro. A poor AA was detected for Lys and Leu, contrary to what was found in several works (Liu et al., 2012, Katsaros et al., 2009; Christensen et al., 1999) that detected the activity of PepC, PepN and PepL on substrates containing Lys and Leu.

The LMG9198, *Lb. curvatus,* showed high activity for Leu in MRSN-6L, MRSN-PEP and MRSN-TRY. The AA for Phe-Pro and Gly-Pro was induced by all the growth conditions and poor AA was detected for Lys and Pro.

Lb. acidophilus, LMG8151, showed AA for Leu, Gly-Pro, Phe-Pro and Lys but not in all growth conditions: only MRS and the media supplemented with the new hydolizates induced these AAs. As reported by Shihata and Shah (2000), *Lb. acidophilus* strains isolated from yogurt showed activities for Leu, Lys and Pro in MRS. *Lb. acidophilus,* LMG8151, isolated from acidophilus milk, showed enzymatic activities in MRS for Leu and Lys too but a very low AA against Pro was detected.

4. Conclusion

Concluding the results about growth kinetics suggest that the experimental hydrolizates from poultry by-products provide a good alternative to substitute expensive nutrient supplements with cheaper renewable low-cost products for growing *Lactobacillus*. The poultry bone and meat hydrolizate, 78T, proposed by Lazzi et al. (2012) as nitrogen source for industrial media, demonstrated not only to well sustain the lactobacilli growth but also to be better than commercial hydrolizates as Tryptone or Peptone. Its ability to sustain the growth, with only two exceptions, is probably due to the high content of free amino acid of this hydrolizate (Lazzi et al., 2012) that could supply the several aminoacids auxotrophies of the studied strains.

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Table 1

Strains studied in this work.

Strain	**Species**	**Growth Condition**
LR85	*Lb. rhamnosus*	30°C, aerobic
LMG18399	*Lb. plantarum*	30°C, aerobic
LMG18223	*Lb. lactis*	30°C, anaerobic
LMG9198	*Lb. curvatus*	30°C, aerobic
LMG8900	*Lb. fermentum*	30°C, aerobic
LMG6904	*Lb. casei*	30°C, aerobic
LMG6400	*Lb. rhamnosus*	30°C, aerobic
LMG23516	*Lb. casei*	30°C, aerobic
LMG6902	*Lb. fermentum*	30°C, aerobic
LMG8151	*Lb. acidophilus*	37°C, anaerobic

Table 2

Values of the maximum specific growth rate (μ_{max}, h^{-1}) and maximum cell density (OD_{max}) of each strain. Standard deviation ranged from 0,002 to 0,06 for μ_{max} and from 0,004 to 0,16 for OD_{max}.

	MRS		**MRSN-6L**		**MRSN-78T**		**MRSN-PEP**		**MRSN-TRY**	
Strain	μ_{max}	OD_{max}	μ_{max}	OD_{max}	μ_{max}	OD_{max}	μ_{max}	OD_{max}	μ_{max}	OD_{max}
LR85	0,14	2,83	0,08	1,82	0,13	3,04	0,07	1,96	0,10	2,36
LMG18399	0,31	3,00	0,21	2,90	0,32	2,96	0,22	2,94	0,26	2,71
LMG18223	0,05	1,34	0,06	1,10	0,08	1,80	0,03	1,13	0,08	1,19
LMG9198	0,11	2,76	0,09	2,93	0,26	3,75	0,07	2,52	0,07	2,45
LMG8900	0,08	2,04	0,06	1,74	0,12	2,42	0,05	1,12	0,06	0,92
LMG6904	0,19	2,37	0,09	1,57	0,26	2,59	0,10	1,64	0,15	1,86
LMG6400	0,14	2,54	0,10	1,74	0,19	2,67	0,09	1,55	0,13	1,80
LMG23516	0,13	3,13	0,08	1,98	0,18	3,45	0,09	2,41	0,17	1,75
LMG6902	0,14	2,41	0,08	1,26	0,14	1,81	0,09	1,15	0,01	0,21
LMG8151	0,12	1,61	0,05	0,87	0,18	2,09	0,05	0,11	0,00	0,06

Legend to the figures

Figure 1 Maximum specific growth rate (μ_{max}, h^{-1}) mean value of the strain considered in this work The bars bearing different letters are significantly different by Tukey's test ($P < 0.05$). All the data represent the means ± standard deviation.

Figure 2 The maximum cell density (OD_{max}) mean value of the strain considered in this work The bars bearing different letters are significantly different by Tukey's test ($P < 0.05$). All the data represent the means ± standard deviation.

Figure 3 Extent of maximum cell density (OD_{max}) of 10 Lactobacillus strains in experimental media: MRSN-6L (Black bars), MRSN-78T (light gray bars), MRSN-PEP (white bars) and MRSN-TRY (dark gray bars) compared with MRS medium determined by turbidimetric measurements. The OD values were calculated by subtracting the OD value obtained in experimental media from the OD value obtained in MRS.

Figure 4 The specific AA of the 10 *Lactobacillus* strains against each chromogenic substrate AAs are expressed as μmol of β-naphthyl amide released by aminopeptidase per minute and per gram of protein. Data are represented with color-coding system where the highest value is black and the lowest is white. All the data represent the mean of triplicate experiment ± standard deviation.

Figure 1

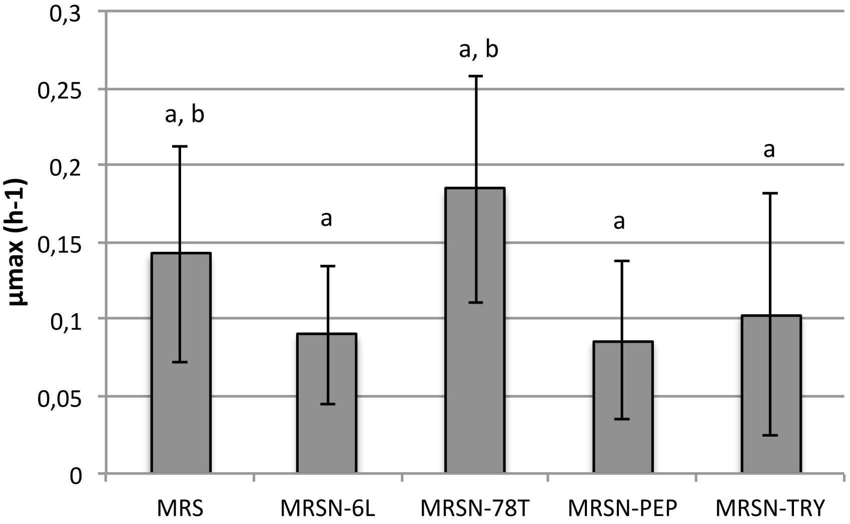
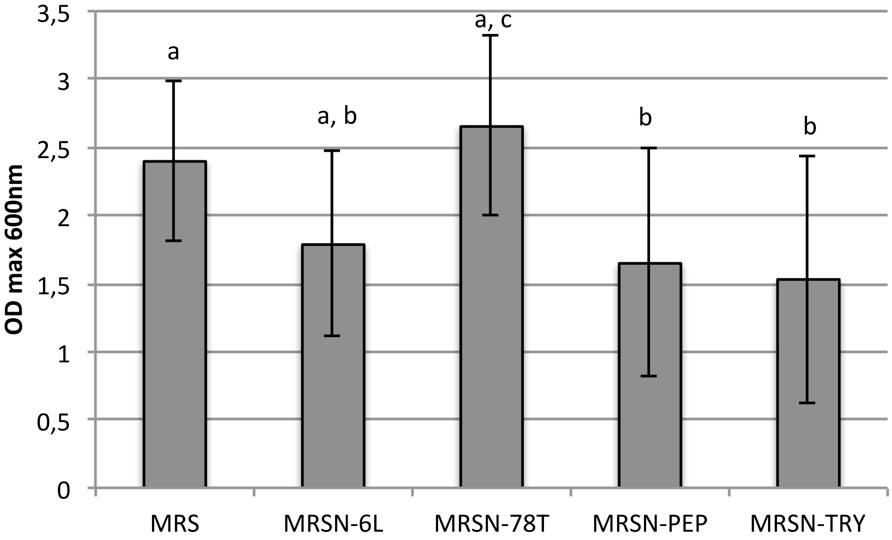

0,3
0,25
0,2
0,15
0,1
0,05
0
µmax (h-1)
a, b
a
a, b
a
a
MRS
MRSN-6L
MRSN-78T
MRSN-PEP
MRSN-TRY

Figure 2



OD max 600nm
3,5
3
2,5
2
1,5
1
0,5
0
a
a, b
a, c
b
b
MRS
MRSN-6L
MRSN-78T
MRSN-PEP
MRSN-TRY

Figure 3

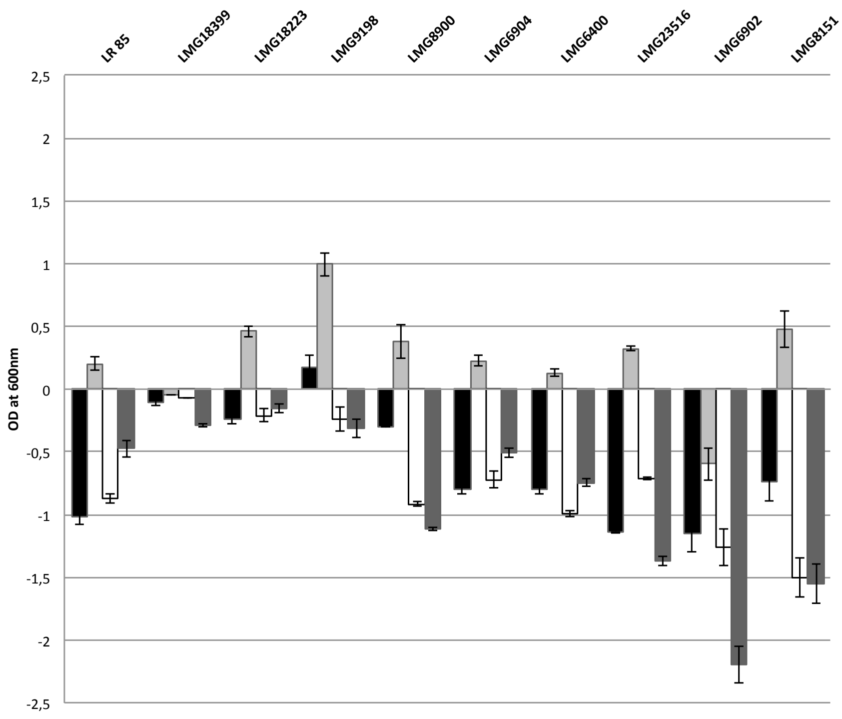

LR 85
LMG18399
LMG18223
LMG9198
LMG8900
LMG6904
LMG6400
LMG23516
LMG6902
LMG8151
OD at 600nm
2,5
2
1,5
1
0,5
0
-0,5
-1
-1,5
-2
-2,5

Figure 4

Growth Media	MRS					MRSN-6L					MRSN-78T					MRSN-PEP					MRSN-TRY				
Substrates / Strains	Gly-Pro	Leu	Phe-Pro	Lys	Pro	Gly-Pro	Leu	Phe-Pro	Lys	Pro	Gly-Pro	Leu	Phe-Pro	Lys	Pro	Gly-Pro	Leu	Phe-Pro	Lys	Pro	Gly-Pro	Leu	Phe-Pro	Lys	Pro
LR 85	20,00 ±0,9	200,00 ±9,43	30,00 ±4,71	22,78 ±0,79	26,67 ±3,01	33,33 ±0,7	190,00 ±4,72	50,00 ±4,72	22,22 ±1,00	33,33 ±5,01	6,67 ±0,9	156,68 ±4,69	13,35 ±3,00	18,3 ±0,79	23,33 ±2,01	30,00 ±4,71	186,68 ±9,43	46,68 ±5,00	22,22 ±1,57	32,22 ±3,00	20,00 ±1,50	186,68±5 ,01	33,33 ±4,01	22,22 ±3,02	30,00 ±1,05
LMG18399	0,83 ±1,18	n.d.	n.d.	n.d.	n.d.	3,33 ±4,71	3,32 ±4,72	5,00 ±2,35	0,83 ±1,18	n.d.	1,67 ±2,36	n.d	n.d.	n.d.	n.d.	7,50 ±1,18	3,33 ±4,71	5,00 ±2,36	2,50 ±0,39	n.d.	9,17 ±3,54	14,17 ±1,18	5,83 ±1,18	12,92 ±8,45	n.d.
LMG18223	16,67 ±0,70	n.d.	66,68 ±3,01	n.d.	n.d.	13,33 ±1,00	3,29 ±0,4	36,68 ±5,01	15,01 ±3,00	n.d.	23,33 ±2,15	n.d.	68,35 ±2,38	3,89 ±2,00	n.d.	40,00 ±1,08	10,00 ±0,51	73,33 ±4,02	13,89 ±3,03	0,56 ±0,05	20,00 ±1,03	1,68 ±0,30	75,00 ±2,36	14,17 ±0,39	n.d.
LMG9198	36,67 ±1,09	6,67 ±0,50	60,00 ±4,03	0,56 ±0,34	0,56 ±0,02	70,00 ±1,00	61,67 ±2,36	65,00 ±2,38	12,22 ±2,01	2,50 ±0,39	26,67 ±1,51	10,00 ±0,5	83,41 ±4,01	n.d.	0,56 ±0,09	75,00 ±2,36	96,63 ±3,3	66,67 ±3,01	13,95 ±3,09	7,50 ±0,39	71,67 ±2,41	51,68 ±2,38	45,00 ±2,36	12,50 ±0,39	1,67 ±0,45
LMG8900	16,67 ±4,71	n.d.	13,34 ±3,02	2,22 ±1,00	n.d.	13,33 ±1,00	n.d.	13,34 ±3,01	1,11 ±0,56	n.d.	20,00 ±1,82	n.d.	23,31 ±4,71	2,22 ±1,00	n.d.	16,67 ±4,71	n.d.	16,67 ±4,72	2,22 ±1,31	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
LMG6904	13,33 ±0,80	156,67 ±4,72	20,00 ±3,04	27,78 ±2,01	24,44 ±3,01	23,35 ±4,72	150,00 ±4,72	33,34 ±3,04	25,00 ±0,79	24,44 ±1,02	10,00 ±4,71	140,00 ±4,01	13,34 ±2,30	24,44 ±2,07	21,11 ±2,03	33,33 ±1,00	166,67 ±3,04	43,34 ±4,71	27,22 ±0,79	27,78 ±2,03	30,00 ±4,70	166,68 ±3,01	40,00 ±5,01	27,22 ±0,79	28,89 ±3,07
LMG6400	6,67 ±0,80	96,80 ±5,3	8,30 ±2,38	14,18 ±0,39	13,61 ±0,39	16,80 ±1,00	101,68 ±2,36	23,35 ±3,21	14,16 ±0,39	15,83 ±0,39	6,67 ±2,00	105,00 ±2,38	10,00 ±0,9	14,17 ±0,39	15,83 ±0,39	10,00 ±2,00	105,00 ±2,36	13,33 ±0,9	12,22 ±1,03	14,17 ±0,39	15,43 ±3,01	108,32 ±2,38	21,68 ±2,36	13,61 ±0,39	15,28 ±0,39
LMG23516	13,33 ±0,08	33,35 ±2,00	20,00 ±5,00	20,56 ±0,79	23,89 ±0,79	73,34 ±9,43	53,00 ±2,01	93,34 ±6,03	41,11 ±1,57	55,55 ±3,01	13,33 ±3,20	33,33 ±2,03	20,00 ±5,00	20,56 ±0,79	22,22 ±2,51	66,67 ±2,00	80,00 ±3,21	80,00 ±3,02	44,44 ±1,78	53,33 ±2,04	20,00 ±3,00	33,34 ±4,01	26,67 ±3,01	20,00 ±1,06	21,67 ±0,79
LMG6902	n.d.	6,67 ±0,50	n.d.	0,56 ±0,79	0,56 ±0,78	n.d.	8,89 ±0,5	8,89 ±2,01	4,44 ±1,01	0,74 ±1,04	14,00 ±1,21	13,34 ±0,54	13,33 ±4,01	20,00 ±1,02	n.d.	n.d.	3,33 ±4,71	3,35 ±2,4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
LMG8151	20,00 ±0,80	46,68 ±5,02	73,34 ±4,00	21,67 ±0,79	2,22 ±0,80	26,68 ±1,00	46,68 ±7,02	80,00 ±4,00	20,56 ±0,79	1,11 ±0,80	6,50 ±4,10	6,68 ±2,00	20,00 ±6,02	22,78 ±0,79	0,56 ±0,79	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

6 Effect of protein hydrolizates on growth kinetics and aminopeptidase activities of *Bifidobacterium.*

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Effect of protein hydrolizates on growth kinetics and aminopeptidase activities of *Bifidobacterium*

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Keywords: poultry hydrolizates, *Bifidobacterium*, peptone, growth kinetics, aminopeptidase, feathers

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Abstract

The goal was to evaluate how a new hydrolizate from poultry feathers acts on eight bifidobacteria growth kinetics and aminopeptidase activities (AA). It was compared with Tryptone and Peptone. The growth kinetics suggest that the experimental hydrolizate could be a cheaper ingredient. Variability of AA was observed. The modulation of AA was not affected by the growth medium composition.

Bifidobacteria are probiotics microorganism widely used in food industries [1]. They are a nutritionally heterogeneous group, whose growth is often slow or limited on synthetic media lacking of a growth-promoting factor [2]. Although Bifidobacteria are able to use ammonium salts as their only source of nitrogen [3], the supplementation of peptides and amino acids are considered requirement for the economical production of these strains [4]. Lazzi and colleagues [5] demonstrated that also poultry feathers enzymatic hydrolizate could be a suitable nitrogen source for the production of microbial biomass with improved viability. Chemical composition of poultry feathers hydrolizate has revealed that it is poor in free amino acids and is mainly composed by peptides [5]. To deepen this aspect, in this work, the growth performances of poultry feathers hydrolizates on eight *Bifidobacterium* strains belonging to different species were compared with two commercial hydrolizates, i.e. Tryptone and Peptone. The maximum cell density (OD_{max}) and the maximum specific growth rate (μ_{max}) were used to determine the nitrogen source preferred by each species tested. Moreover, in order to investigate the effect of the growth condition on the modulation of aminopeptidase pattern, the aminopeptidase activities (AA) of each strain in each condition were studied. Bifidobacterial proteolytic system is poorly investigated in contrast to the huge research works about lactic acid bacteria proteolytic system. Instead this is an important topic because more and more often bifidobacteria are added to several kinds of foods rich in proteins such as acid milk, cheese, soy based food ect. [6, 7, 8]. The knowledge of aminopeptidase activities induced by different growth condition is useful because it could affect the enzymatic activities in the food matrix [4]. Cheng and Nagasawa [9] found that in cell extract of *Bifidobacterium breve* there were aminopeptidase and iminopeptidase, Elsoda and colleagues [10] discovered that *Bifidobacterium infantis*, *Bifidobacterium longum*, and *Bifidobacterium adolescentis* show amino-, di-, tri-, and carboxypeptidase activities. By sequencing the genome of *B. longum NCC2705* more than 20 predicted peptidases were found that could provide amino acids from proteinaceous substrates in the gastrointestinal tract and vagina, where carbohydrates are less abundant [11].

Eight strains of *Bifidobacterium* genus were used in this work. *Bifidobacterium pseudolongum* B340, *B. longum* B350, *Bifidobacterium angulatum* B397, *Bifidobacterium pseudum catenalatum* B328, *Bifidobacterium bifidum* B382 belonging to the collection of Department of Food Science (University of Parma) [12]. Reference strains, *Bifidobacterium animalis* subsp. *animalis* ATCC25527, *Bifidobacterium longum* subsp. *suis* LMG21814, *Bifidobacterium merycicum* LMG11341 were purchased from: the "American Type Culture Collection" (ATCC, Manassas, U.S.A., http://www.atcc.org/) and "Belgian co-ordinated collection of microorganism" (LMG, Gent, Belgium, http://bccm.belspo.be/). All the strains were maintained as culture stocks in 15% glycerol (w/v) at − 80 °C, and routinely grown for 48 hours in anaerobic conditions at 37 °C in MRS (Oxoid Italia, Milano, Italy) with cysteine hydrocloride at 0.05% (Sigma-Aldrich Corporation, St. Louis, Missouri, USA) (MRS).

Five different growth media were used in this study: MRS (Oxoid), MRS without the major nitrogen sources as basal medium [5] (MRSN) supplemented with 22g/L of different proteinaceus hydrolizates: FFP 6L (6L) from poultry feathers, Tryptone (TRY) (Oxoid) and Peptone (PEP) (Oxoid). Their growth was monitored during 66 hours by reading turbidity at 600 nm (OD_{600}) to obtain growth curves for each strain in each cultured medium. The maximum cell density, OD_{max}, was determined as the maximal OD value reached at the stationary phase. The maximum specific growth rate, μ_{max}, was determined by calculating the slope of the exponential growth phase ($\mu_{max} = \Delta \ln(OD600)/\Delta t$, where t is time and expressed as h^{-1}). Each growth curve was carried out in duplicate.

From the culture of each strain in MRS, MRSN-6L, MRSN-TRY, MRSN-PEP cell extract were obtained. After three-repeated subculture in each media, cells extracts (CE) were obtained following the methods of Chen and Steele [13]. Protein concentrations were determined as using the Bradford commercial kit (Bio-Rad Laboratories, Hercules, Calif.) with bovine serum albumin (Sigma-Aldrich) as a standard.

Aminopeptidase activity (AA) was investigated against five different chromogenic substrates: β-naphthyl amide (β-NA) derivates of L-anomers of leucine (Leu), lysine (Lys), proline (Pro), glycine-proline (Gly-Pro) and phenilalanine-proline (Phe-Pro). Each CE was incubated with 0.650 mmol/L solutions of β-naphthyl amide derivates (Bachem Feinchemikalien AG, Switzerland) and phosphate buffer at 37°C for specific time to maintain linear range of the reaction: for Gly-Pro and Leu 30 minutes and for Phe-Pro, Lys and Pro 3 hours. The reaction was stopped adding 250 μL of 2.0 mol/L HCl. The degree of hydrolysis was determined by measuring the colored product of an azocopulation reaction by reading spectrophotometrically at 580 nm [14].

The arbitrary unit of enzyme activity (AA) was defined as micromoles of β-naphthylamide released per minute and per gram of proteins presents in each CE. Each assay was carried out in triplicate.

Descriptive statistics, ANOVA ($p<0{,}05$) and *post hoc* Tuckey test were conducted with SPSS ver. 19 (IBM Software, Milan, Italy).

In the first part of this work the growth kinetics parameters, μ_{max} and OD_{max}, were evaluated (figure 1 and figure 2). They resulted to be very strain dependent and there was not a particular media that affected the growth of all the studied strains in the same way. For what concern OD_{max}, *B. longum* reached the highest OD_{max} in MRSN-6L compared also to MRS. In the cases of two strains (*B. pseudolongum* and *B. pseudocatenolatum*) hydrolizates 6L and TRY induced OD_{max} comparable to MRS ($P>0{,}05$). Three strains (*B. animalis* subsp. *animalis*, *B. angolatum* and *B. bifidum*) obtained better OD_{max} in MRS followed by the other hydrolizates. The strain *B. merycicum* found a better growth media in MRSN-TRY than in the others. Just for one strain, *B. longum* subsp. *suis*, the OD_{max} in MRSN-6L resulted to be lowest. PEP hydrolizate did not allow the growth of *B. bifidum* and *B. angolatum*.

Analysing the maximum specific growth rate values, μ_{max} of two strains (*B. longum* subsp. *suis* and *B. merycicum*) was not affected by different media ($P>0{,}05$). For strains *B. animalis* subsp. *animalis, B. angolatum, B. bifidum* the best μ_{max} was obtained with the complete medium MRS. Strain *B. longum* had the better values of μ_{max} with MRSN-6L or MRSN-TRY ($p<0{,}05$) while *B. pseudocatenolatum* with MRSN-TRY. *B. pseudolongum* obtained similar μ_{max} values in MRS, MRSN-6L and MRSN-TRY while μ_{max} in MRSN-PEP was the lowest.

In the second part of this work aminopeptidase activities (AA) were also examined. In figure 2 are reported the AA of each strain grouped by medium. AA resulted to be strain specific and not correlated with growth media. Generally, among the strains grown in the different media, the activities against substrates containing Phe-Pro and Pro residues were more frequently observed but with different extent of μmol of β-naphthylamide released depending on the strain. Some strains (*B. pseudolongum, B. animalis* subsp*. animalis, B. merycicum, B. angolatum, B. longum*) resulted to have low AA activities. The amount of β-naphthylamide, released by the enzymes present in the CE, did not exceed 50 $\mu mol \times min^{-1} \times g^{-1}$. However, among all the activities, the ones against Phe-Pro and/or Pro were the highest in most cases ($p<0,05$). Some strains showed low AA except for activity against Phe-Pro in some media: *B. pseudocatenolatum* in MRSN-PEP and *B. bifidum* in MRSN-6L and MRSN-TRY. These behaviours observed could denote the presence of an X proyl dipeptidyl peptidase and an iminopetidase whose activity was induced by the media in different ways for each strain. In some cases no AA were detected in some growth conditions despite of a good ODmax value obtained in the same growth media: *B. longum* and *B. longum* subsp. *suis* reached respectively OD_{max} 4,02±0,02 and 2,70± 0,22 in MRSN-6L but any AA was detected from their CE. Probably the among the peptides there are some specific ones that have not a nutritional role but they act as growth stimulator and are not degraded by enzymes.

Concluding, the results about growth kinetics, in particular the maximum cell density, suggest that the experimental hydrolizate from poultry feathers, 6L, could be a starting point for the formulation of cheaper microbiological medium for bifidobacteria. Indeed it was demonstrated that 6L was better than Peptone as only nitrogen source and it often resulted to have performance similar to TRY. Tryptone was, for the strains studied, also a good nitrogen source probably because the trypsin-digested caseinacts as growth enhancer for bifidobacteria [15, 16]. For what concern hydrolizate 6L, very less is know about keratin hydrolizate's microbiological properties. The good performances of the hydrolizate 6L to sustain the growth probably are due to its richness in small peptides [5]. As stated by Proulx and colleagues [17] small peptides are a better amino acid source than free amino acid for bifidobacteria.

Acknowledgment

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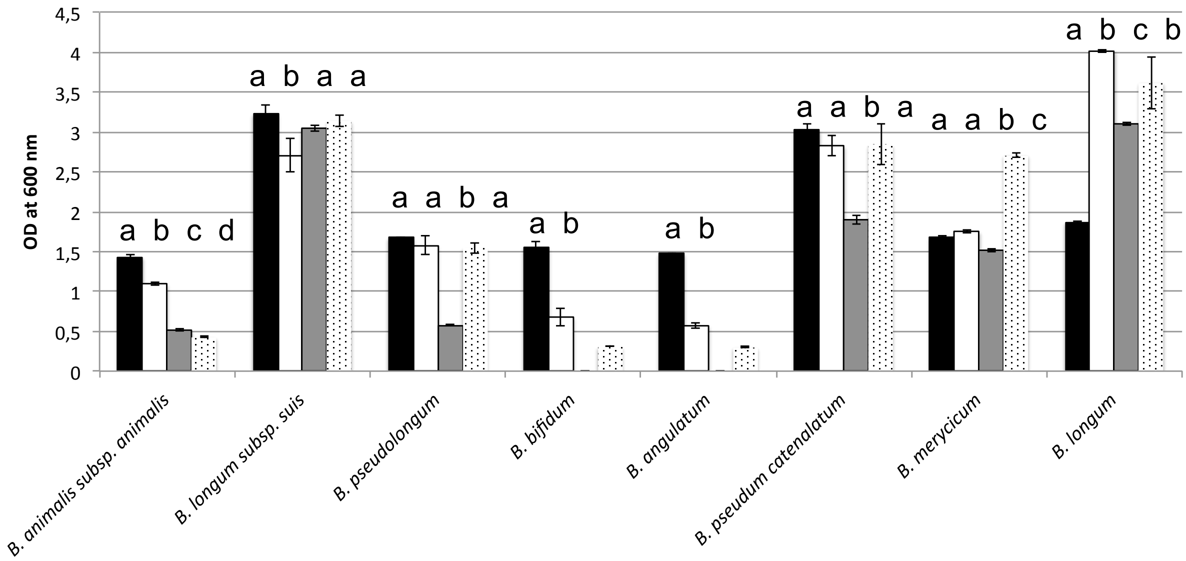
Legend to the figure

Figure 1 **OD$_{\mathrm{max}}$**. The maximum cell density (OD$_{\mathrm{max}}$) of eight strains considered in this work. MRS (Black bars), MRSN-6L (white bars), MRSN-PEP (light gray bars) and MRSN-TRY (dotted bars). The bars bearing different letters are significantly different by Tukey's test ($P<0.05$). All the data represent the means ± standard deviation.

Figure 2 **μ_{max}.** The maximum specific growth rate (μ_{max}, h^{-1}) of eight strains considered in this work. MRS (Black bars), MRSN-6L (white bars), MRSN-PEP (light gray bars) and MRSN-TRY (dotted bars). The bars bearing different letters are significantly different by Tukey's test ($P<0.05$). All the data represent the means ± standard deviation.

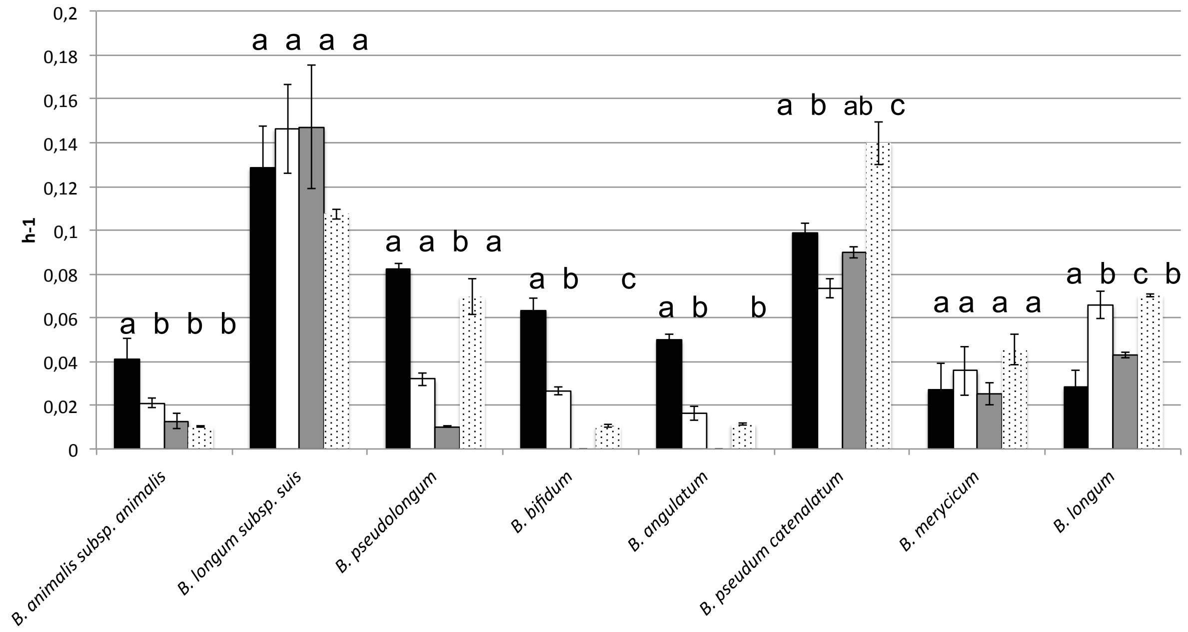
Figure 3 **Aminopeptidase activity**. The specific AA of the 10 *Lactobacillus* strains against each chromogenic substrate are reported.. AAs are expressed as μmol of β-naphthyl amide released by aminopeptidase per minute and per gram of protein. Data are represented with color-coding system where the highest value is black and the lowest is white. All the data represent the mean of triplicate experiment ± standard deviation. In some cases the AA could not be measured because the strains did not grow in that specific media and they are indicated as "not grown" (n. g.). Instead when the CE was obtained but the AA was lower than the limit of detection, "not detected" (n. d.) was used.

Figure 1



OD at 600 nm
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
a b c d
a b a a
a a b a
a b
a b
a a b a
a a b c
a b c b
B. animalis subsp. animalis
B. longum subsp. suis
B. pseudolongum
B. bifidum
B. angulatum
B. pseudum catenalatum
B. merycicum
B. longum

Figure 2


0,2
0,18
0,16
0,14
0,12
0,1
0,08
0,06
0,04
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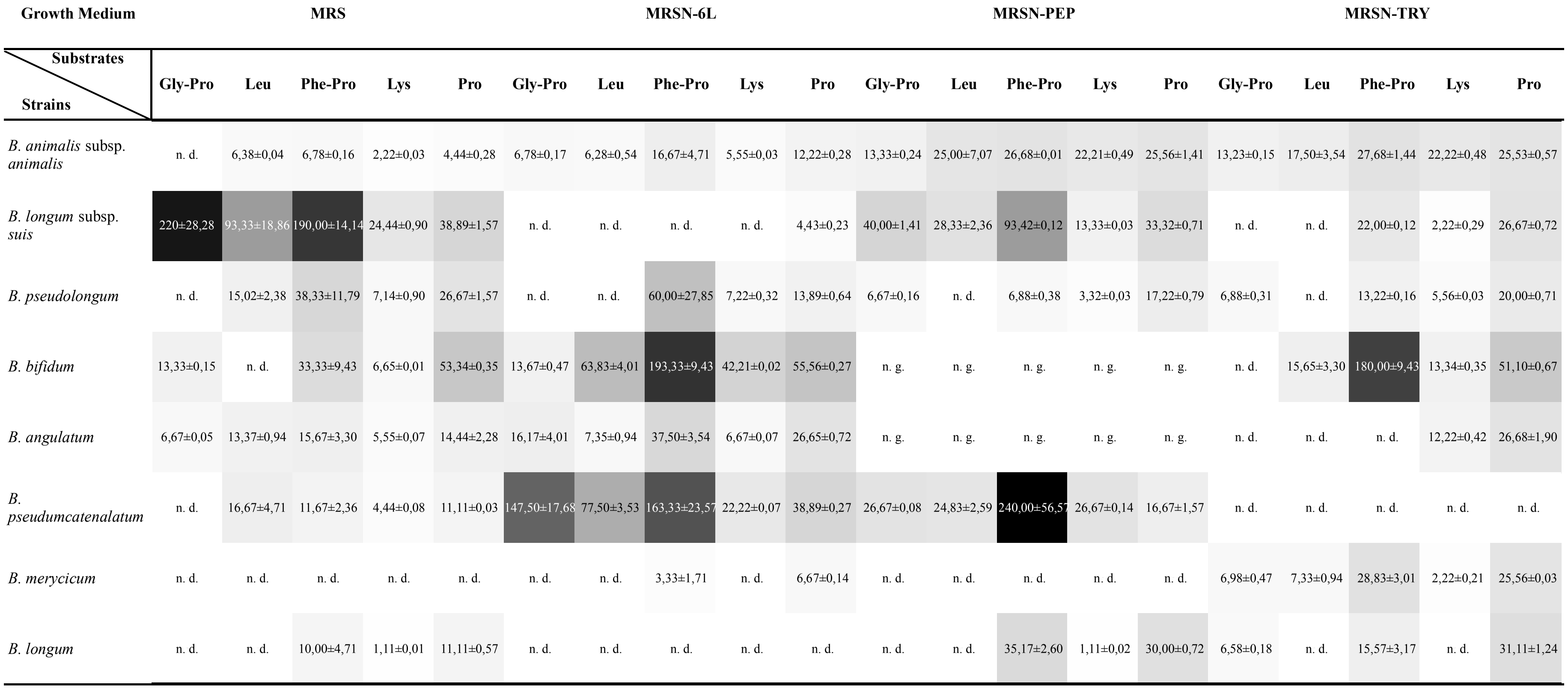
0
h-1
a b b b
a a a a
a a b a
a b c
a b b
a b ab c
a a a a
a b c b
B. animalis subsp. animalis
B. longum subsp. suis
B. pseudolongum
B. bifidum
B. angulatum
B. pseudum catenalatum
B. merycicum
B. longum

Figure 3



Growth Medium	MRS					MRSN-6L					MRSN-PEP					MRSN-TRY				
Substrates / Strains	Gly-Pro	Leu	Phe-Pro	Lys	Pro	Gly-Pro	Leu	Phe-Pro	Lys	Pro	Gly-Pro	Leu	Phe-Pro	Lys	Pro	Gly-Pro	Leu	Phe-Pro	Lys	Pro
B. animalis subsp. *animalis*	n. d.	6,38±0,04	6,78±0,16	2,22±0,03	4,44±0,28	6,78±0,17	6,28±0,54	16,67±4,71	5,55±0,03	12,22±0,28	13,33±0,24	25,00±7,07	26,68±0,01	22,21±0,49	25,56±1,41	13,23±0,15	17,50±3,54	27,68±1,44	22,22±0,48	25,53±0,57
B. longum subsp. *suis*	220±28,28	93,33±18,86	190,00±14,14	24,44±0,90	38,89±1,57	n. d.	n. d.	n. d.	n. d.	4,43±0,23	40,00±1,41	28,33±2,36	93,42±0,12	13,33±0,03	33,32±0,71	n. d.	n. d.	22,00±0,12	2,22±0,29	26,67±0,72
B. pseudolongum	n. d.	15,02±2,38	38,33±11,79	7,14±0,90	26,67±1,57	n. d.	n. d.	60,00±27,85	7,22±0,32	13,89±0,64	6,67±0,16	n. d.	6,88±0,38	3,32±0,03	17,22±0,79	6,88±0,31	n. d.	13,22±0,16	5,56±0,03	20,00±0,71
B. bifidum	13,33±0,15	n. d.	33,33±9,43	6,65±0,01	53,34±0,35	13,67±0,47	63,83±4,01	193,33±9,43	42,21±0,02	55,56±0,27	n. g.	n. g.	n. g.	n. g.	n. g.	n. d.	15,65±3,30	180,00±9,43	13,34±0,35	51,10±0,67
B. angulatum	6,67±0,05	13,37±0,94	15,67±3,30	5,55±0,07	14,44±2,28	16,17±4,01	7,35±0,94	37,50±3,54	6,67±0,07	26,65±0,72	n. g.	n. g.	n. g.	n. g.	n. g.	n. d.	n. d.	n. d.	12,22±0,42	26,68±1,90
B. pseudumcatenalatum	n. d.	16,67±4,71	11,67±2,36	4,44±0,08	11,11±0,03	147,50±17,68	77,50±3,53	163,33±23,57	22,22±0,07	38,89±0,27	26,67±0,08	24,83±2,59	240,00±56,57	26,67±0,14	16,67±1,57	n. d.	n. d.	n. d.	n. d.	n. d.
B. merycicum	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	3,33±1,71	n. d.	6,67±0,14	n. d.	n. d.	n. d.	n. d.	n. d.	6,98±0,47	7,33±0,94	28,83±3,01	2,22±0,21	25,56±0,03
B. longum	n. d.	n. d.	10,00±4,71	1,11±0,01	11,11±0,57	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	35,17±2,60	1,11±0,02	30,00±0,72	6,58±0,18	n. d.	15,57±3,17	n. d.	31,11±1,24

7 Identification, synthesis and evaluation of effect of *Gallus gallus* keratin peptides on growth of *Bifidobacterium longum* subsp. *suis*

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To be submitted

Identification, synthesis and evaluation of effect of *Gallus gallus* keratin peptides on growth of *Bifidobacterium longum* subsp. *suis*

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Keyword: Bifidobacterium longum sbsp. suis, keratin, peptides, solid phase peptide synthesis.

Abbreviations
ESI: Electrospray Ionization
Fmoc: Fluorenylmethyloxycarbonyl
G: Glycine
HPLC: High Performance Liquid Chromatography
I: Isoleucine
L: Leucine
MS: Mass Spectrometry
P: Proline
RP: Reverse Phase
S: Serine
tBu: Tert-butyl
TFA: Trifluoroacetic acid
TIS: Triisopropylsilane
UPLC: Ultra Performance Liquid Chromatography
V: Valine

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Abstract

The hydrolizate obtained from poultry feathers named Functional Feather Protein (FFP) 6L, rich in small peptides (<3KDa), has a strong growth stimulating activity for *Bifidobacterium longum* subsp. *suis* LMG21814. In the present study the changes in the peptides composition of the growth medium containing FFP were analysed before and after the growth of the strain. Depleted peptides after the strain growth were synthetized and used as unique nitrogen source individually and mixed in the culture medium in substitution to the whole FFP 6L; to exclude a simple nutritional effect, other culture mediums were obtained replacing the peptides with an equimolar solution of their constituent free L-aminoacids. These experimental media lost any growth stimulating effect and from the chemical analysis of the broths after the growth, the strain seemed to not use the peptides. This result suggests that the promoting activity of the whole feather hydrolizate is strictly related with complete mixture of the peptides.

1. Introduction

Bifidobacteria, first described by Tissier, are Gram-positive, non-motile, non-spore-forming, anaerobic bacteria with spatulate extremities (Felis and Dellaglio, 2007). They are predominant in the large bowel contributing to 10% of the intestinal microflora in adults (Turroni et al., 2008). Strains of this genus are generally considered beneficial for human health and are for this reason widely used as health-promoting or probiotic components in functional foods (Stanton et al., 2001; Stanton et al., 2005).

Unfortunately bifidobacteria are fastidious microorganisms that require complex and expensive media for propagation supplemented with growth-promoting factors (Doleyres et al., 2002). Most species of the genus *Bifidobacterium* are unable to develop in a totally synthetic medium and require complex biological substances such as bovine casein digestate, lactoserum of bovine milk, porcine gastric mucin, or yeast extract (Poch and Bezkorovainy, 1988; Petschow and Talbott, 1990). Also non-glycosylated peptides, derived from protein after proteinase hydrolysis, have been proposed as promoting factors (Tamime et al., 1995; Zhao et al., 1996). A number of studies have reported that proteinaceous compounds found in human milk (Liepke et al., 2002) and cow milk (Petschow and Talbott, 1990; Etienne et al., 1994) have the ability to promote the growth of bifidobacteria. Molecules that are present in dairy industry effluents such as whey (Mahalakshmi and Murthy, 2000) and by-products of latex rubber production also have this property (Ishizaki 1989; 1995; Oiki et al., 1996; Etoh et al., 2000). Moreover the growth-promoting activity was correlated with the low molecular weight (MW) of peptides, ranging from 1 KDa to 5 KDa (Proulx et al., 1992; Etienne et al, 1994; Etoh et al., 1999)

A recent study (Lazzi et al., 2012) demonstrated that a protein hydrolizate obtained from poultry feathers, named Functional Feather Protein (FFP) 6L, is a better promoting ingredients for the growth of bifidobacteria than other hydrolizates obtained from poultry bones and meats. FFP 6L is quite poor in free amino acids, which were <3% of the total mass and rich in peptides of low MW (MW< 3KDa). Differently other bone and meats hydrolizates, richest in free amino acid and in peptides with MW higher than 10 KDa, did not allow neither the same cell growth nor the same cell viability (Lazzi et al., 2012). This suggests that bifidobacteria, considered in that research, are able to hydrolyse the FFP peptides or that they find, among the peptides, some specific growth-promoting factors.

With the aim to confirm this hypothesis, in the present study the changes in the peptides composition of the growth medium containing FFP were analysed before and after the growth of *Bifidobacterium longum* subsp. *suis* LMG21814. Depleted peptides after the strain growth were synthetized and used as unique nitrogen source in the culture medium in substitution to the whole FFP 6L; to exclude a simple nutritional effect, other culture mediums were obtained replacing the peptides with an equimolar solution of their constituent free L-aminoacids.

2. Materials and methods

2.1 Peptides Synthesis

Reagents and solvents. Fmoc-Leu-Wang resin and Fmoc-Ser(tBu)-Wang resin, Fmoc-Gly-OH, Fmoc-Ser(tBu)-OH, O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate, piperidine, N,N-Diisopropylethylamine, dimethylformamide, dichloromethane, trifluoroacetic acid, triisopropylsilane, diethyl ether and acetonitrile were purchased from Sigma-Aldrich (St. Louis, MO, USA). Formic acid was purchased from ACROS organics (Geel, Belgium). Fmoc-Val-OH was purchased from Advanced Biotech Italia (Monza e Brianza, Italy). Fmoc-Ile-OH was purchased from Fluka (Buchs, Switzerland). Fmoc-Pro-OH was purchased from Novabiochem (Nottingham, UK).

Solid phase peptide synthesis. The peptides VGSIL, GVPIS and SIL were synthesized on solid phase according to Fmoc/t-butyl strategy using a Syro I Fully Automated Peptide Synthesizer (Biotage, Uppsala, Sweden). The peptides were cleaved from the resin using a TFA:TIS:H2O (95:2.5:2.5) solution. The peptide VGSIL was purified using a preparative RP-HPLC-UV (WATERS Delta Prep

4000 coupled with WATERS 2487 Dual λ Absorbance Detector, λ=214 nm). The peptides GVPIS and SIL were purified using a WATERS Sep-pak Classic C18 Cartridges.

2.2 Bacterial strain

The *Bifidobacterium longum* subsp*. suis* LMG21814*,* was purchased from the "Belgian co-ordinated collection of microorganism" (LMG, Gent, Belgium, http://bccm.belspo.be/). The strain was maintained as culture stock in 15% glycerol (w/v) at − 80 °C, and routinely grown for 24 hours in anaerobic conditions at 37 °C in MRS (Oxoid Italia, Milano, Italy) with cysteine hydrocloride at 0.05% (Sigma-Aldrich Corporation, St. Louis, Missouri, USA).

2.3 Feather Hydrolizate

Protein hydrolizate obtained from poultry feathers, named Functional Feather Protein (FFP) 6L was purchased by the European project PROSPARE (Progress in saving proteins and recovering energy, www.prospare.eu)

2.4 Growth Media

In this work experimental media were made with a common base that is MRS broth without the major nitrogen sources as basal medium (Lazzi et al., 2012) (MRSN). This broth was supplemented with 22g L^{-1} of a proteinaceus hydrolizate FFP 6L (6L) from poultry feathers (Lazzi et al., 2012). This medium, named MRSN-6L, was used to adapt the strain and as control. The microorganism was routinely propagated and subcultured three times before experiments in MRSN-6L broth. The bacterial inoculum was prepared from cultures of 24 h at 37°C in anaerobic condition. The culture was centrifuged at 8000 g for 10 min at 4 °C. The harvested cells were washed twice, suspended in Ringer solution (Oxoid Italia) and used as inoculum 3% (v/v) The basal medium MRSN was supplemented with three different peptides (SIL, VGSIL and GVPIS) synthetized ex-novo at 15 mg/ml. Three different broths were obtained: MRSN-SIL, MRSN-VGSIL and MRSN-GVPIS. A fourth broth (MRSN-MIX) was made with the mix of the three peptides at a final concentration of 15 mg ml^{-1}. Media with free amino acids were also used. The basal medium MRSN was supplemented with the equimolar solutions of free L-aminoacids that compose each peptide at 15mg ml^{-1}. Three media were obtained: i) serine, isoleucine and leucine for SIL (MRSN-SILaa), valine, glycine, serine, isoleucine and leucine for VGSIL (MRSN-VGSILaa) and glycine, valine, proline, isoleucine and serine for GVPIS (MRSN-GVPISaa).

2.5 Cell count

The growth and the viability of the cells were evaluated by direct count before and after 60 hours of incubation at 37°C in anaerobic condition. *B. longum* subsp*. suis* LMG21814 was grown in eight different growth media: MRSN-6L, MRSN-SIL, MRSN-VGSIL, MRSN-GVPIS, MRSN-MIX, MRSN-SILaa, MRSN-VGSILaa and MRSN-GVPISaa. Fluorescence microscopy counts was performed using a LIVE/DEAD® BacLightTM Bacterial Viability Kit for microscopy (Invitrogen Ltd, Paisley, UK) as described by Lazzi et al. (2011).

2.6 Chemical analysis

Culture broths of *B. longum* subsp. *suis* LMG21814 were analyzed with RP-UPLC/ESI-MS. The broths were analyzed before (T_0) and after (T_1) 60 hours of incubation. In the case of MRSN-6L, also broth without cell inoculum was analyzed in order to verify that changes in the peptides composition of the media were due only to the bacterial growth. These samples are named: Blank T_0 (broth without cells before incubation) and Blank T_1 (broth without cells after incubation). **HPLC/ESI-MS analysis.** Samples were separated by a RP column (JUPITER 5 µm C18 300 Å 250*2 mm) in an HPLC/ESI-MS/MS (HPLC Waters Alliance 2695 with a triple quadrupole mass spectrometer Waters 4 Micro), using a gradient elution. Eluent A was water with 0.1% formic acid and 0.2% acetonitrile, eluent B was acetonitrile with 0.1% formic acid; gradient: 0-12 min 100% A, 12-77 min from 100% A to 50% A, 77-81 min 50% A, 81-82 min from 50% A to 0% A, 82-90 min 0% A, 90-91 min from 0% A to 100% A, 91-110 min 100% A. The samples were first analysed in Full Scan mode, to identify the characteristic ions and the retention time of the unknown compounds, and then in Daughters Scan modality using a variable collision energy (10, 15 or 20 V). HPLC/ESI-MS/MS parameters were: flow 0.2 ml/min; analysis time 110 min; column temperature 35°C; injection volume 15 µl; acquisition time 7-90 min; ionization type positive ions; scan range 100-2000 m/z; capillary voltage 3.2 kV; cone voltage 30 V; source temperature 100°C; desolvation temperature 200°C; cone gas flow 100 $l\ h^{-1}$; desolvation gas flow 650 l/h. The peptide sequences were assigned on the basis of the mass spectra obtained.

UPLC/ESI-MS analysis. In order to quantify the peptides, the samples were separated by a RP column (ACQUITY UPLC BEH 300 C18 1.7 µm 2.1*150 mm) in an UPLC/ESI-MS system (UPLC Acquity Waters with a single quadrupole mass spectrometer Waters Acquity Ultraperformance) using a gradient elution. Eluent A was water with 0.1% formic acid and 0.2% acetonitrile, eluent B was acetonitrile with 0.1% formic acid; gradient: 0-7 min 100% A, 7-50 min from 100% A to 50% A, 50-52,6 min 50% A, 52,6-53 min from 50% A to 0% A, 53-58,2 min 0% A, 58,2-59 min from 0% A to 100% A, 59-72 min 100% A.

Samples were analysed with UPLC/ESI-MS in the Full Scan mode (flow 0.2 ml/min; analysis time 72 min; column temperature 35°C; sample temperature 18°C; injection volume 5 µl; acquisition time 7-58,2 min; ionization type positive ions; scan range 100-2000 m/z; capillary voltage 3.2 kV; cone voltage 30 V; source temperature 150°C; desolvation temperature 300°C; cone gas flow 100 l/h; desolvation gas flow 650 l/h), the characteristic ions of every peptide were extracted, obtaining eXtract Ion Chromatograms (XICs), in which the identified peptides were integrated with the MassLynx software.

3. Result and discussion

3.1 Peptides Identification

In order to exclude peptides' degradation during the incubation time, a preliminary experiment was carried out. For this purpose the RP-UPLC/ESI-MS analysis was performed on MRSN-6L broth samples, without inoculum, before (Blank T_0) and after (Blank T_1) the incubation period. 60 hours at 37°C did not degrade the peptide mixture of the hydrolizate. From RP-UPLC/ESI-MS chromatogram (Figure 1) the main ions were individuated and listed in table 1.

The same analysis was carried out on MRSN-6L samples inoculated with *B. longum* subsp. *suis* LMG21814 which was able to reach a. cell concentration equal to 8,87 ± 0,48 (standard deviation) log cell ml^{-1} (table 2) after incubation. The characteristic ions of every peptide after the strain's growth were extracted from the Total Ion Chromatogram (TIC), obtaining an eXtract Ion Chromatogram (XIC), and the peaks were integrated, normalizing to 100 the areas of the sample Blank T_0 and expressing in % the others (Figure 2). The RP-UPLC/ESI-MS analysis was performed in triplicate. Different ions shown a significant decrease (one way ANOVA, $p<0.05$) after *B. longum* subsp. *suis* LMG21814 growth: 444, 246, 253, 280, 231, 478, 472, 245, 573, 484, 332, 537, 488, 838, 559, 797, 896, 959 and 710 m/z. The same experiment was repeated to avoid inter-day variability. The peptides that resulted decreased more than the 30% after the growth were 245, 332, 472, 488 m/z and were analyzed with HPLC/ESI-MS/MS. The sequences were IL, SIL, GVPIS and VGSIL respectively. The aminoacidic sequence was assigned on the basis of the mass spectra obtained from the collision induced dissociation (Figure 3). All the peptides identified belong to Feather Keratin I (P02450, *Gallus gallus*). Quite interestingly, the peptides VGSIL, SIL and IL derive from the same region (65-69 aminoacid residue) but are hydrolyzed at different levels. The aminoacidic composition of these peptides is characterized by the presence of a polar amino acid (serine) and other “hydrophobic” residues (glycine, valine and isoleucine).

3.2 *Peptides synthesis and evaluation of effect on the bacterial growth.*

In order to evaluate if each peptide has a proper growth promotion effect the peptides VGSIL, SIL and GVPIS were synthesized according to solid phase peptide synthesis and purified to eliminate interfering compounds. The accuracy of the assigned sequence was confirmed adding to MRSN-6L increasing amounts (1, 5, 10 e 20 µl) of synthetic peptide raw solution (an example is reported in Figure 4). Hereafter the each purified peptide was dissolved in MRSN reaching the final concentration of 15mg/ml. The MRSN-6L broth and MRSN-SIL, MRSN-VGSIL, MRSN-GVPIS, MRSN-MIX broths were inoculated with *B. longum* subsp*. suis* LMG21814 cells. After 60 hours of incubation the strain reached 8,87 ± 0,48 (standard deviation) log cell ml^{-1} from an initial microbial load of 6,65 ± 0,27 log cell ml^{-1} in MRSN-6L in accordance to what was demonstrated in Lazzi et al. (2012): FFP-6L resulted to be a good nitrogen source when added to a minimum medium. Instead when each peptide or the peptides blend were used as only nitrogen source in the growth media the strain was not able to increase cell concentration (table 2). Also the RP-UPLC/ESI-MS analysis did not measure any peptides reduction (data not shown). In order to exclude a simple nutritional effect, other culture media were obtained replacing the peptides with an equimolar solution of their constituent free L-aminoacids (MRSN-SILaa, MRSN-VGSILaa and MRSN-GVPISaa). Also in this experiment any increase in the number of cells was observed (table 2). The lack of growth promoting capacity of experimental broths with free amino acid was also observed by Etienne and colleagues (1994) and by Proulx and colleagues (1994) and it could be explained by possible competition in free amino acid transport systems of the strain. In conclusion, the capacity of the whole feather hydrolizate to sustain the growth of bifidobacteria could be due to a synergic effect of different peptides generated by the action of the proteases. Therefore this effect cannot be reproduced by single peptide alone or by a limited mixture. In this study only the peptides that scored more than the 30% of reduction after the bacterial growth were considered. It has not to be excluded that the promoting activity could be due to peptides that were depleted with a less extent. For this reason a depth study has to be carried out.

4. References

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Table 1 Retention time and characteristic protonated ions of the main peptides present in the MRSN-6L broth.

Rt (min)	ION $[M+H^+]^+$
8.58	219.2
10.23	189.4
12.32	444.4
12.52	246.4
13.04	253.3
13.56	223.3
14.42	280.3
14.74	367.3
15.14	231.4
17.68	478.4
17.94	472.4
18.16	245.4
18.45	573.4
19.31	484.4
20.03	817.5
21.27	332.5
21.54	201.3
22.12	537.4
23.31	488.4
24.34	838.6
24.78	559.5
26.07	959.6
26.54	797.6
27.86	1505.8
28.32	821.5
28.8	896.7
29.61	710.5
30.24	699.6
30.52	799.4
30.54	1256.7
31.77	1094.8
32.1	1181.7
32.54	1195.8
33.56	809.2
34.59	2284.2

Table 2 Cells count in the eight different broths. T0 is the microbial load at the beginning of the incubation time and T1 is the microbial load after 60 hours of incubation.

		log cell/ml		
		Live Cells	Damaged Cells	Total Count
MRSN-6L	T0	6,62	5,50	6,65
	T1	8,82	7,98	8,87
MRSN-SIL	T0	7,19	6,22	7,23
	T1	7,20	6,99	7,41
MRSN-VGSIL	T0	7,31	6,22	7,34
	T1	7,17	7,05	7,41
MRSN-GVPIS	T0	7,24	6,31	7,29
	T1	7,03	7,17	7,40
MRSN-MIX	T0	6,53	5,82	6,61
	T1	6,52	6,19	6,69
MRSN-SILaa	T0	7,50	6,40	7,54
	T1	7,68	7,14	7,79
MRSN-VGSILaa	T0	7,43	6,31	7,46
	T1	7,58	6,40	7,61
MRSN-GVPISaa	T0	7,43	6,47	7,47
	T1	7,53	7,33	7,74

Legend to the figure.

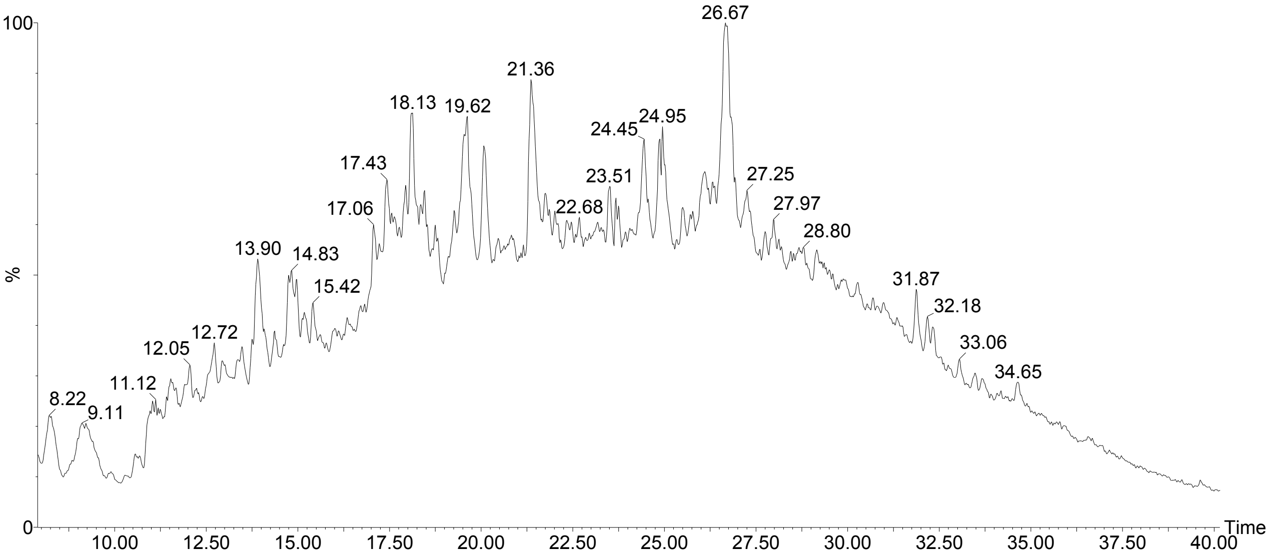
Figure 1 UPLC/ESI-MS chromatogram obtained from MRSN-6L Blank T0 sample broth.

Figure 2 Amount of peptides present in the MRSN-6L broths samples. Blank T0 (broth without cells before incubation, White bars), Blank T1 (broth without cells after incubation, Black bars) broths before and after the strain growth were analysed: MRSN-6L T0 (Black dotted bars) and MRSN-6L T1 (Grey bars).

Figure 3 MS/MS spectra of the identified peptides. The losses from C-term generate Y-series fragments, while the aminoacidic losses from N-term generate B-series fragments.

Figure 4 EXtract Ion Chromatogram of the broth sample spiked with increasing amounts of peptide GVPIS.

Figure 1



26.67
21.36
18.13
19.62
24.45
24.95
17.43
23.51
27.25
17.06
22.68
27.97
28.80
13.90
14.83
15.42
31.87
32.18
12.72
12.05
33.06
11.12
34.65
8.22
9.11
100
%
0
Time
10.00
12.50
15.00
17.50
20.00
22.50
25.00
27.50
30.00
32.50
35.00
37.50
40.00

Figure 2

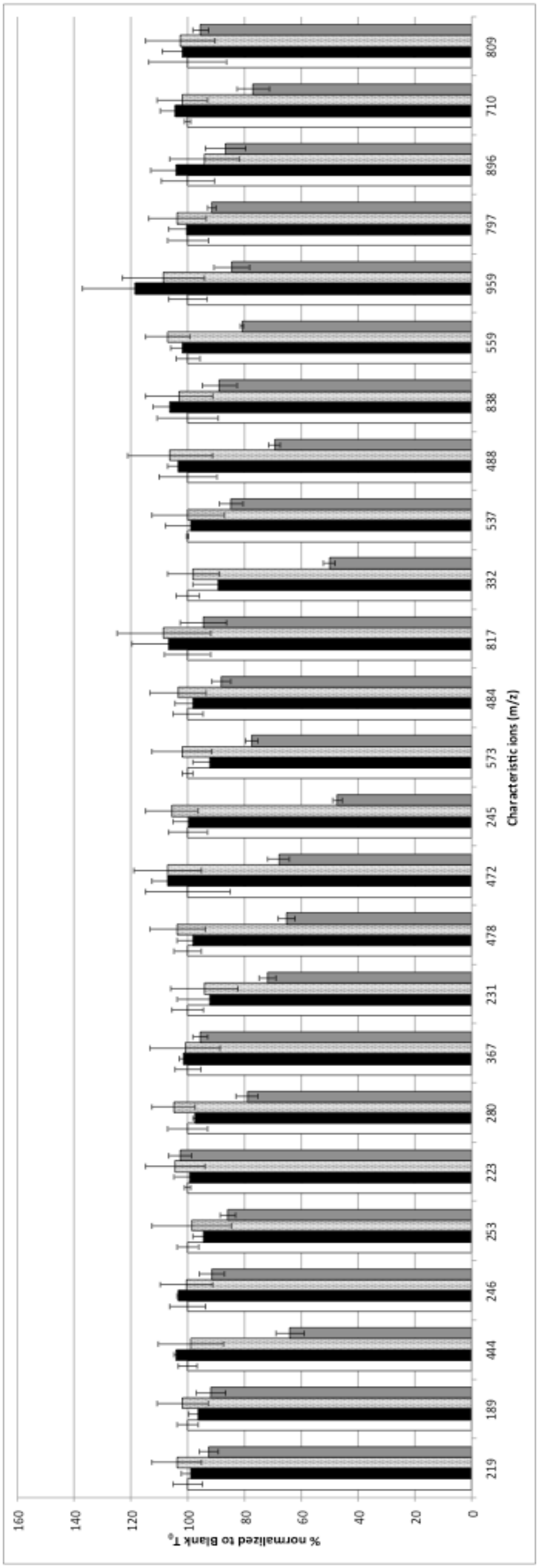

% normalized to Blank T_0
0
20
40
60
80
100
120
140
160
Characteristic ions (m/z)
219
189
444
246
253
223
280
367
231
478
472
245
573
484
817
332
537
488
838
559
959
797
896
710
809

Figure 3

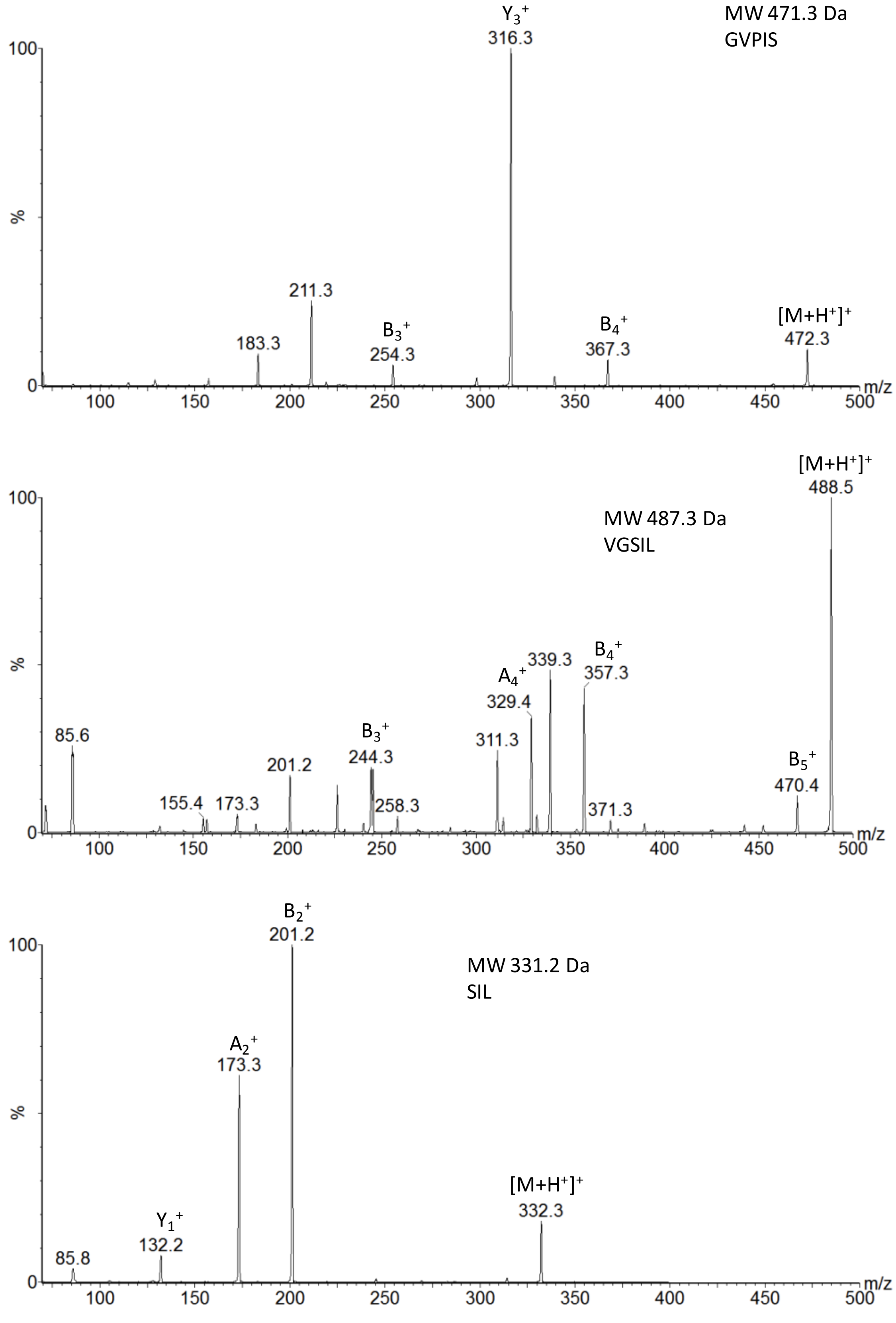
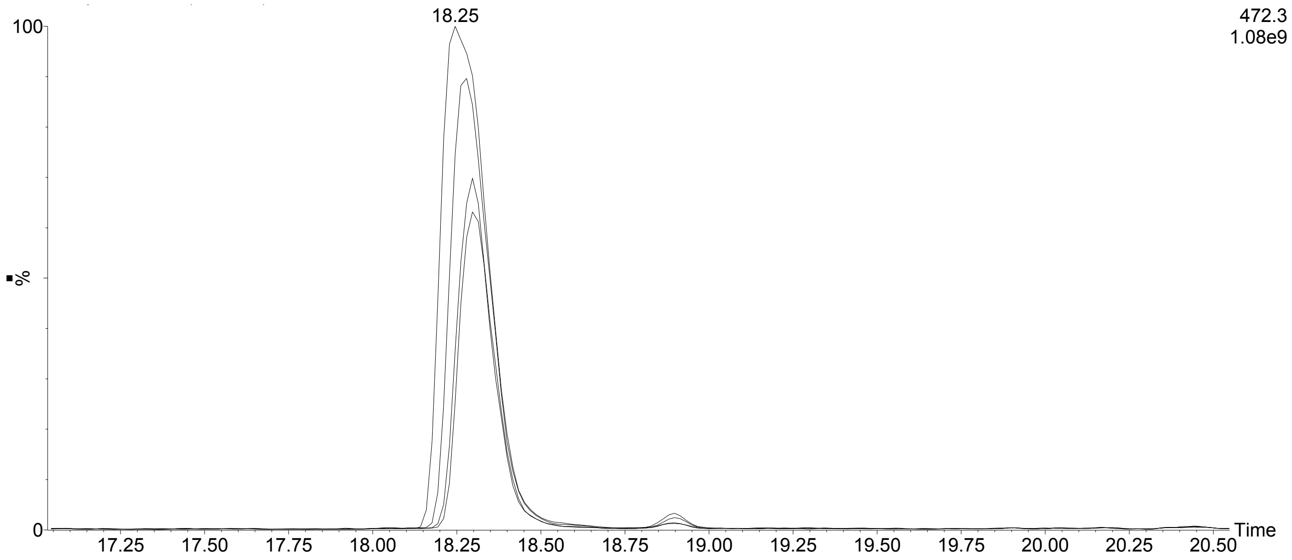

MW 471.3 Da
GVPIS
Y3+ 316.3
211.3
183.3
B3+ 254.3
B4+ 367.3
[M+H+]+ 472.3
100
%
0
100 150 200 250 300 350 400 450 500
m/z
MW 487.3 Da
VGSIL
[M+H+]+ 488.5
85.6
155.4
173.3
201.2
B3+ 244.3
258.3
311.3
A4+ 329.4
339.3
B4+ 357.3
371.3
B5+ 470.4
100
%
0
100 150 200 250 300 350 400 450 500
m/z
MW 331.2 Da
SIL
B2+ 201.2
A2+ 173.3
Y1+ 132.2
85.8
[M+H+]+ 332.3
100
%
0
100 150 200 250 300 350 400 450 500
m/z

Figure 4


18.25
472.3
1.08e9
100
%
0
Time
17.25
17.50
17.75
18.00
18.25
18.50
18.75
19.00
19.25
19.50
19.75
20.00
20.25
20.50

8 Genaral Conclusion

Bacterial growth depends on the biosynthesis and/or uptake of medium components required for the formation of biomass. Microbial culture media represent a very high cost for the production of starter cultures and probiotics especially for those microorganisms, as lactobacilli and bifidobacteria, which have many nutritional requirements. For this reason it is economically interesting to find less expensive supplements, that can stimulate active cultures, to replace the common ones. Currently, a great deal of attention is being paid on the biotechnological potential of food industrial residues. There are many studies about the exploitation of raw materials as new cheaper ingredients for microbial growth media. Usually food, animal or agricultural wastes are used as resources because they contain several reusable substances of high value such as soluble sugars, fiber or proteins. Different wastes of food industry are exploited, such as whey, fishery leftovers, fibrous proteins sources (horns, nails, etc) for production of ingredients intended for formulation of microbiological media. This Ph. D. thesis suggests that by-products from poultry industries, such as bone and meat or feathers, provide a good alternative to substitute expensive nutrient supplements with cheaper renewable low-cost products for growing *Lactobacillus* and *Bifidobacterium* in order to produce high and viable amount of starter or probiotic cultures.

This thesis suggests that hydrolizates from poultry leftovers could be promising ingredient for industrial microbiological media. The reported studies demonstrated that the experimental hydrolizates could improve cell viability and the growth kinetic parameters, as maximum cell density and growth rate. These are important criterions for the selection of growth medium ingredient for the propagation of starter and/or probiotic bacteria. In particular for what concern *Lactobacillus* genus the hydrolizate obtained from meats and bones residues resulted not only to sustain the growth very well but also to be better than commercial hydrolizates as tryptone or peptone. Its capability to sustain the growth is probably due to the high content of free amino acid of this hydrolizate that could supply the several aminoacids auxotrophies of the studied strains.

Instead the hydrolizate obtained from feathers enhanced the bifidobacterial growth in a similar way of commercial peptones. Indeed it was demonstrated that feathers hydrolizate was better than peptone as only nitrogen source and it often resulted to have performance similar to tryptone. Tryptone was, for the strains studied, also a good nitrogen source probably because the trypsin-digested casein acts as growth enhancer for bifidobacteria. For what concern experimental hydrolizate, very less is known about keratin hydrolizate's microbiological properties. The good performances of this hydrolizate to sustain the growth probably are due to its richness in small peptides that are a better amino acid source than free amino acid for bifidobacteria. An additional analysis of mechanisms behind the growth promotion effect of the experimental hydrolisates was preliminary analyzed. A screening of the common aminopeptidase activity was done in order to single out variation in the enzymatic activities of the strains in presence of the different hydrolizates. However the modulation of aminopeptidase pattern was affected more by the peculiar characteristic of each strain that by the growth medium composition.

Moreover for what concern *Bifidobacterium longum* subsp. *suis* LMG21814, the changes in the peptides composition of the growth medium containing feathers hydrolizate were analysed before and after the growth of the strain. The peptides consumed in the highest amount after the strain growth were synthetized and used as unique nitrogen source alone and together in the culture medium in substitution to the whole feathers hydrolizate. In order to exclude a simple nutritional effect, other culture mediums were obtained replacing the peptides with an equimolar solution of their constituent free L-aminoacids. However, when each synthetized peptide or the synthetized peptides blend and free aminoacid solution were used as only nitrogen source in the growth media the strain was not able to grow. In conclusion it can be assumed that the capacity of the whole feather hydrolizate to sustain the growth of bifidobacteria could be due to a synergic effect of different peptides generated by the action of the proteases. Moreover it has not to be excluded that the promoting activity could be due to peptides that did not shown a significant decrease.

9 Curriculum vitae

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Publications list

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