Chapter 17

Microbial Interactions in the Gut: The Role of Bioactive Components in Milk and Honey

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1. Introduction

The fact that living organisms play a key role on health, was put on a scientific basis at the beginning of the last century by Elie Metchinikoff, when working at the Pasteur Institute in Paris. The findings that Bulgarian peasants, who ingested large amounts of soured milks, also lived to a ripe old age led him to conclude about the beneficial effects of fermented milks.

One of the most convincing demonstrations of the role of the gut microbiota in resistance to disease was provided by Collins and Carter [1]. These authors proved that germ-free guinea–pig was killed by 10 cells of *Salmonella Enteritidis*, but it required 10^9 cells to kill a conventional animal with a complete gut microbiota.

Probiotic was initially defined by Parker [2] as "Organisms and substances which contributes to intestinal microbial balance". Fuller [3] redefined probiotics as "A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". This definition clarifies the need for a probiotic to be viable.

The term prebiotic was subsequently adopted to define "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that improve host health"[4] Modification by prebiotics of the composition of the colonic microbiota leads to the predominance of a few of the potentially health-promoting bacteria, especially, but not exclusively, lactobacilli and bifidobacteria. Much of the work on prebiotics deals with the use of oligosaccharides, although the first demonstration of this type of effect was observed with a disaccharide, lactulose. Gibson and Roberfroid [4] also launched the concept of symbiotic by combining the rationale of pro- and prebiotics, is proposed to characterize some colonic foods with interesting nutritional properties that make these compounds candidates for classification as health-enhancing functional food ingredients.

The bacterial genera most often used as probiotics are lactobacilli and bifidobacteria. At present, probiotics are almost exclusively consumed as fermented dairy products such as yogurt or freeze-dried cultures, but in the future they may also be found in fermented vegetables and meats [5].

The microbial community inhabiting the gastrointestinal tract is characterized by its high population density, wide diversity, and complexity of interactions. Bacteria are predominant but a variety of protozoans, yeasts and bacteriophages are also found. Bacteria are not distributed randomly throughout the gastrointestinal tract but instead are found at population levels and species distributions that are characteristic of specific regions of the tract. The stomach and proximal small intestine contain relatively low numbers of microorganisms. Acid- tolerant lactobacilli and streptococococci predominate in the upper smal intestine. The distal small intestine (ileum) maintains a more diverse microbiota and higher bacterial numbers. The large intestine (colon) is characterized by large numbers of bacteria, low redox potential, and relatively high short-chain fatty acid concentrations. The prominent role played by anaerobic bacteria in this dynamic ecosystem is evident from the finding that more than 99% of the bacteria isolated from human fecal specimens are anaerobic or aerotolerant [6].

The intestinal tract is a dynamic ecosystem that is influenced by host, intrinsic, and environmental factors. Thus, our undestanding of gut microbial interactions and how the gastrointestinal activity is modulated, might help on establishing screening criteria to identify potentially probiotic bacteria suitable for human or animal use.

2. Microbial interactions in the gut

The nature of the microbial interaction can be predominantly by competition or mutualism [7]. In the gut they can affect either the population level of a given strain or the metabolic activity of that strain. In addition, genetic transfers can occur between strains within the gut. The host and the diet cam modulate the expression of the microbial interactions. These interactions involve multiple mechanisms that are poorly understood. Such mechanisms are involved either in the size of subdominant microbial populations or in the metabolic activities of predominant populations. Diet and perhaps other environmental factors, such as stress, can modify their expression.

The gastrointestinal tract of neonates becomes colonized immediately after birth with environmental microorganisms, mainly from the mother by several processes including sucking, kissing, and caressing. The proximity of the birth canal and the anus, as well as parental expression of neonatal care, are effective methods of ensuring transmission of microbes from one generation to the next [6].The pattern and level of exposure during the neonatal period is likely to influence the microbial succession and colonization in the gastrointestinal tract. Infants from developing countries have an early colonization with enterobacteria whereas those born in countries with good obstetric and hygienic procedures, may result in a delayed development pattern or even the absence of certain groups of intestinal bacteria during succession [8].

After the birth process, neonates are continuously exposed to new microbes that enter the gastrointestinal tract with food. This begins with breast milk, which contains up to 10^6 microbes/mL in healthy mothers. The most frequently encountered bacterial groups include staphylococci, streptococci, corynebacteria, lactobacilli, micrococci, propionibacteria and bifidobacteria originated from the nipple and surrounding skin as well as the milk ducts in the breast [6, 9, 10].

A pronounced dominance of bifidobacteria was observed over the entire breast-feeding period, with a corresponding reduction in facultative bacteria [11, 12]. There is a strong evidence suggests that the early composition of the microbiota of neonates plays an important role for the postnatal development of the immune system [13, 14].

Both adults and neonates are regularly exposed to microorganisms via the diet, but are affected differently. The microorganisms entering newborns via milk are more likely to colonize than are those entering healthy adults [6, 15].

Bacterial species or strains that will be established in the infant bowel might be capable to utilize the substrates provided by the diet and the particular human host. *Bifidobacteria*, *E. coli* and enterococci can utilize a wide range of monosaccharides and oligosaccharides which would be provided by the diet. Once established the range of fermentable substrates available to the bacteria changes from mono and oligosaccharides to complex plant polymers (dietary fibre) that pass undigested through to the small bowel. The other major complex carbohydrates is provided by the mucins that are continuously secreted into the bowel by the goblet cells present in the mucosal lining. Strict regulations of catabolic pathways must be an extremely important attribute in a habitat where the nutritional profile will vary from day to day according to the omnivorous and varied dietary preferences of the human host and help [16]

Protection against colonization of the intestinal tract by potentially pathogenic microorganisms, due to the gut microbiota, was called competitive exclusion [17], whose pioneering evidence had been obtained by Nurmi and Rantala [18], with birds. When these, soon after birth, were inoculated with cecal material of an adult bird, the frequency of Salmonella infections was significantly reduced.

Undoubtedly the main benefit attributed to probiotics is the competitive exclusion of pathogens that occurs by different mechanisms including: a) competition for receptors in the intestinal epithelium as occurs with lactobacilli that directly inhibits the binding of *Salmonella*, *E. coli* and other foodborne pathogens b) secretion of factors that inhibit internalization and adhesion of pathogens, as well as increased secretion of mucin as with lactobacilli which stimulate the secretion of MUC2 and MUC3 2 which inhibits the adherence of enteropathogenic *E. coli* c) stimulating the mucosal barrier effect, such as the lactobacilli and bifidobacteria which helps to prevent pathogens from inducing an increase in intestinal permeability; d) production of volatile fatty acids and / or other antibacterial substances, by the anaerobic microbiota besides nutrient competition [19, 20].

Constituents of the normal microbiota and some pathogenic bacteria have the ability to colonize the mucosal surfaces [21] Some microorganisms seem to be able to securely attach to the intestinal epithelium [22], and is thought to be this an important prerequisite for probiotics in a long-term survival during competition against other microorganisms for specific niches and subsequent multiplication. However, no consensus among researchers exists about the fact that a probiotic should or should not adhere to mucosal surfaces, colonize and then exert a probiotic effect, being an alternative its regular consumption to maintain the levels needed to promote the effect, forming a transient microbiota [23].

Another desired effect of a probiotic includes altered metabolism of the intestinal microbiota as the reduction in the synthesis of toxins or carcinogenic substances or an increased production of short-chain fatty acids or other substances that improve the condition of the mucosa. Prebiotics may also be given to augment immune reaction, preferably those that have a protective effect without causing overt inflammation . The ability of lactic bacteria to inactivate mutagenic compounds, such as dyes and N-nitrosamines, has been attributed to cell wall components, such as peptidoglycan and polysaccharides [24].. The lactic acid bacteria also may mediate anticarcinogenic activities by reducing the activity of fecal bacterial enzymes such as nitroredutases, azoredutases and β glucuronidase (EC 3.2.1.31) that convert procarcinogenic to carcinogenic compounds in the colon [14]

The ability to sense other bacteria may have important consequences for competitive and nutritional strategies controlling for example, entry into stationary phase, dispersal and the production of antimicrobial compounds. The ability to interfere with the signalling of bacteria will determine the fitness of the given organism to survive in the gut and may also have therapeutic potential. The study of cell-to-cell communication in gastrointestinal(GI) tract bacteria is not as advanced as it is for bacteria from other ecosystems. In Gram-negative bacteria the best-characterized systems involve *N*-acylhomoserine lactone (acyl-HSL) signals, LuxI family signal synthases and LuxR family response regulators. It appears that Gram-positive bacteria prefer peptide signals, also termed peptide pheromones [25].

Probiotics may play an active role inflammatory bowel diseases by enhancing the intestinal barrier at the mucosal surface. Caballero-Franco et al. [26] investigated whether the clinically tested VSL#3 probiotic formula and/or its secreted components could augment the protective mucus layer in vivo and in vitro. For in vivo studies, Wistar rats were orally administered the probiotic mixture VSL#3 on a daily basis for seven days. After treatment, basal luminal mucin content increased by 60%. In contrast to the animal studies, cultured cells incubated with VSL#3 bacteria did not exhibit increased mucin secretion. However, the bacterial secreted products contained in the conditioned media stimulated a remarkable mucin secretion effect. Among the three bacterial groups (*Lactobacilli*, *Bifidobacteria*, and *Streptococci*) contained in VSL#3, the *Lactobacillus* species were the strongest potentiator of mucin secretion in vitro.

The competitive exclusion of pathogens mediated by lactobacilli is usually performed by two mechanisms: (i) production of antimicrobial substances such as lactic acid and bacteriocins, and (ii) adhesion to the mucosa and coaggregation which can form a barrier which prevents colonization by pathogenic microorganisms [27].

Three mechanisms of aggregation have been reported so far. The first is related to the interaction between the components of the cell surface, as in the oral cavity with *Streptococcus sanguis* and *Prevotella locscheii* in which adhesins are protein-type lectins. Adlerberth et al. [28] observed that the adhesion of *Lactobacillus plantarum* to human colonic cells HT-29 was due to mannose-sensitive attaching mecanism. As the cell walls of the yeast *Saccharomyces cerevisiae* consists polysaccharide containing mannose (mannans), *Escherichia coli* and other enterobacteria containing mannose-specific adhesin receptors agglutinate yeast cells. The ability of binding yeast cells may therefore be an indication of mannose specific activity [29].

Autoaggregation has been correlated with adhesion, which is known to be a prerequisite for colonization and infection of the gastrointestinal tract by many pathogens. Adherence to the epithelium is therefore a prerequisite for enterotoxigenic *Escherichia coli* both to colonize the small intestine and to cause diarrhea, since adherence targets toxins directly onto the epithelial cell [30].

Coaggregation is a process by which genetically distinct bacteria become attached to one another via specific molecules. Cumulative evidence suggests that such adhesion influences the development of complex multi-species biofilms. The coaggregation properties of probiotic strains with pathogens as well as their ability to displace pathogens are of importance for therapeutic manipulation of the aberrant intestinal microbiota. Aggregation abilities of a probiotic with the pathogen strains were strain-specific and dependent on time and incubation conditions [31]

Recently, the complement protein mannose-binding lectin (MBL) has been shown to play a role in the first line of defense against *Candida albicans*. MBL binds to a wide variety of microorganisms through a carbohydrate recognition domain, exhibiting strong binding to *Candida* and other yeast species. The complement system is activated via this lectin pathway, causing opsonization and direct lysis of microorganisms[32]. A number of probiotic bacteria contact recognition proteins, including lectins, enzymes and other factors involved in carbohydrate metablolism , are involved in microbe-microbe host interactions [33].

In other cases, the adhesins are not lectins, such as in the case of *Streptococcus sanguis* and *Streptococccus gordonii* [34].

The second mechanism, described in lactobacilli, is dependent upon secretion of a protein of 32 kDa that promotes aggregation and a high frequency of conjugation [35] According to Collado, Meriluoto and Salminen [31] the ability to autoaggregate, together with cell-surface hydrophobicity and coaggregation abilities with pathogen strains can be used for preliminary screening in order to identify potentially probiotic bacteria suitable for human or animal use.

Finally, in *Enterococcus faecalis*, the ability to promote aggregation is due to secretion of small hydrophobic peptides called sex pheromone with consequent increase of the frequency combination [36, 37]. Pheromones appear to induce the synthesis surface proteins encoded by the plasmid, which mediate cell-cell contact.The sex pheromone system of *Enterococcus*

faecalis is responsible for the clumping response of a plasmid carrying donor strain with a corresponding plasmid free recipient strain due to the production of sex pheromones by the recipient strain. The clumping response is mediated by a surface material (called aggregation substance) which is synthesized upon addition of sex pheromones to the cultures. After induction a dense layer of "hairlike" structures is formed on the cell wall of the bacteria that are responsible for the cell-cell contact which leads to the aggregation of cells [38]

Boris et al. [39] have characterized a peptide produced by *Lactobacillus gasseri* (previously classified as plantarum), which promotes the aggregation of cells of *L. plantarum* and *Enterococcus* spp. The authors hypothesize that these aggregates could mediate protection of the mucosa by the formation of a bacterial film that prevents access of undesirable microorganisms in the vaginal mucosa.

3. Bioactive prebiotic components in milk

Many components of human milk are multifunctional, providing antimicrobial, antiinflammatory, antioxidant effect besides being growth factors [40].

Breast milk not only provides a range of substrates for bacterial growth, but it also appears to be a reservoir for some of the bacteria we inherit, including Lactobacillus sp. and *Bifidobacteria* [41] Breast milk contains viable lactobacilli and bifidobacteria that might contribute to the initial establishment of the microbiota in the new born [10]. Although this needs to be verified and an explanation given with mechanism uncovered as to how lactobacilli reach the mammary gland and if other bacteria do likewise, the end result is that infants are colonized predominantly by lactic acid bacteria [20].

Although it is likely that antimicrobial components in human milk inhibit the growth of pathogenic bacteria, it is also likely that some substances stimulate the growth of beneficial bacteria, *ie*, they have prebiotic activity. This factor, originally called the bifidus factor, may promote the growth of *Lactobacilli* and *Bifidobacteria*, which can limit the growth of several pathogens by decreasing intestinal pH. One possible substance identified was *N*-acetyl-glucosamine [42]. Subsequently, several oligosaccharides have been shown to have this activity, but it is also possible that milk proteins also have such prebiotic activity . Increasing the lactobacilli and bifidobacteria levels is a target for infant formulas and the most common approach to this end has been to include prebiotic compounds [10].

The gut microbiota of breastfed infants is different from that of formula-fed infants. According to Penders [43], exclusively formula-fed infants were more often colonized with *E coli*, *C difficile*, *Bacteroides*, and lactobacilli, compared with breastfed infants. Although Penders et al. [44] showed that formula-fed infants have similar counts of bifidobacteria compared with breast-fed infants, most reports found that breast-fed infants have higher number of bifidobacteria, whereas formula-fed infants develop a mixed flora with a lower level of bifidobacteria [45].

Oliveira [12] studied the influence of diet and type of delivery in 68 neonates aged between seven and 21 days on both composition and evolution of the gut *Bifidobacterium spp*., *Lactobacillus spp*. microbiota. Gut colonization by bifidobacteria was not influenced by the type of delivery but the counts of lactobacilli were higher in those born vaginally as shown in table 1. Lactobacilli numbers in infants fed formula and human milk and born vaginally were significantly higher ($p<0.05$) than those born by caesarean, suggesting a possible microbiota transference from mother to the child. Similar results were reported by Biasucci [46] that demonstrated significant retarded colonization by lactobacilli at 10 days of age in babies delivered by cesarean section. Differently, Martin et al. [47] found that lactic acid bacteria colonization was not significantly related to the delivery method.

Oliveira [12] also found that bifidobacteria numbers in infants born vaginally and fed with breast milk (BM) were higher than the others, while those who received pasteurized human milk from milk banks (HMB) showed a significant lower number of *Bifidobacterium* as compared to other types of feeding (Table 1). No significant differences were observed on infants born by cesarean. These *in vivo* results corroborate with previously, *in vitro* observed data, by Borba and Ferreira [48], who evaluated the effect of human milk pasteurization on growth of different species of *Bifidobacterium*. It was demonstrated that pasteurization of human milk affected the growth of bifidobacteria, indicating that, somehow, the pasteurization process (65°C/30minutos) inhibits bifidogenic factors, or results in the production of inhibitory compounds to this microbial group

The same negative pasteurization effect was observed by Oliveira [12] on the growth of lactobacilli (Table 1). Although breast-milk contains viable lactobacilli and bifidobacteria that might contribute to the initial establishment of the microbiota in the newborn, the negative effect of human milk pasteurization on the lactobacilli and bifidobacteria gut population, cannot be explained solely on the destruction of those bacteria by the pasteurization process. Milk formulas do not contain these bacteria, but favored the development of bifidobacteria and lactobacilli in the intestine reaching a number significantly higher, as compared to the gut microbiota of pasteurized human milk fed infants.

Indeed, the health-promoting effects of breast-milk have been linked partly to the presence of lactobacilli and bifidobacteria in breast-milk [10, 47], but clearly also to different milk bifidogenic components.

Both lactotobacilli and bifidobacteria benefit in environments with low redox potential and the presence of antioxidant compounds present in human milk. Anti-oxidants such as lactoferrin, α-tocopherol, β carotene, cysteine, ascorbic acid, uric acid, catalase and glutathione peroxidase are present in human milk [40]. Most of these compounds are thermo-labile and might have been destroyed during milk pasteurization process. Whey protein is rich in *cysteine*, the thermo-labile amino acid which represents an effective *cysteine* delivery system for the cellular synthesis of glutathione. In addition, the ability of cysteine and cysteine to lower redox potential stimulates de growth of anaerobic or anaero-tolerant bacteria. The repeated processes that donor human milk is submitted before delivery to

newborn infants cause a reduction in the fat and protein concentration. The magnitude of this decrease is higher on the fat concentration and it needs to be considered when this processed milk is used to feed preterm infants [49].

	Cesarean	Vaginally
	Lactobacillus	
HMB	2,4 a A	3,3 b A
FM	2,8 a B	5,7 a A
BM	3,8 a B	5,6 a A
	Bifidobacterium	
HMB	5,6 a A	3,7 b A
FM	5,7 a A	6,5 ab A
BM	6,2 a A	7,4 a A

Treatments with the same small letters in columns and capital letters in rows do not differ significantly by Tukey test (P> 0.05)

Table 1. Averages of the Lactobacilli and Bifidobacteria log numbers, in babies born by cesarean section and vaginally delivery, fed with pasteurized milk from human milk banks (HMB), formula (FM) and breast milk (BM).

3.1. Milk oligosaccharides

For many years, the oligosaccharides were considered for his role in the modulation of intestinal microbiota of infants. Currently, there is strong evidence that free oligosaccharides as well as glycoproteins are potent inhibitors of bacterial adhesion on the surface of the epithelium in the early stages of the infectious process. Therefore, the milk oligosaccharides have two important functions. The first as a source prebiotic stimulating the growth of probiotic bacteria and a second, operating in a non-specific defense mechanism inhibiting pathogens from adhering to the gastrointestinal mucosa. Although the exact pathophysiological mechanism of diarrhea is not yet fully elucidated, it seems that the ability of microorganisms to adhere to the mucosal surface is essential for spreading diarrheagenic bacteria in the duodenum [50].

Concentrations of total oligosaccharides in human milk (HMO) is 5,0-8,0 g per liter whereas just traces are found in cow's milk. In cow's milk, only small amounts of oligosaccharides are detectable, with sialyllactose being the major component [51].

Differences in the qualitative or quantitative aspects of term and preterm milk have not been observed, but compositional changes of oligosaccharides in term milk occurs during lactation with the largest amounts being found at early stages. The highest concentrations of HMOs can be found in colostrum (20 g/L), but even mature milk contains oligosaccharides in concentrations up to 13 g/L [52]. Coppa [11] reported that lactose concentration (±SD) in human milk increased from 56 ± 6.06 g/L on day 4 to 68.9 ± 8.16 g/L on day 120. Oligosaccharide level decreased from 20.9 ± 4.81 g/L to 12.9 ± 3.30 gIL, respectively. Monosaccharides represented only 1.2% of total carbohydrates.

Although intact HMOs may be absorbed, ENGFER et al. [52] postulate that a majority of HOs reach the large intestine, where they serve as substrates for bacterial metabolism. Therefore, HMOs might be considered the soluble fiber fraction of human milk

Human milk compared with other milk species, is considered unique in terms of its complex oligosaccharides content. With few exceptions, HMOs have a core structure consisting of a lactose unit at the reducing end linked to *N*-acetyllactosamine units (type 1 and 2), with branching occurring frequently Residues of L-fucose, sialic acid [N-acetylneuraminic acid (NeuAc), or both can be found linked to the core without further elongation. An elongation is achieved by an enzymatic attachment of GlcNAc residues linked in ß1-3 or in ß1-6 linkage to a Gal residue followed by further addition of Gal in a ß-1-3 or ß-1-4 bond. Thus, a large number of core structures can be formed. Further variations occur due to the attachment of lactosamine, Fuc, and/or NeuAc residues at different positions of the core region and of the core elongation chain (10, 50). The addition of Fuc is dependent on the actions of at least three different fucosyltransferases in a genetically determined process.[51, 52]..

Within human milk oligosaccharides at least 10 containing GlcNAc are known as growth factors for a so-called bifidus biota in breastfed infants. Dietary modulation of the intestinal microflora is today one of the main topics of interest in the nutritional sciences. Fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) are prebiotics whose bifidogenic activity has been proven in adults. Moro and Arslanoglu [19] demonstrated that supplementation of infant formulas with a mixture of GOS and FOS modified the fecal flora of term and preterm infants, stimulating the growth of Bifidobacteria. In the trial with term infants, the bifidogenic effect of the prebiotic mixture was dose dependent and there was also a significant increase in the number of Lactobacilli in the supplemented group.

The similarities between epithelial cell surface carbohydrates and oligosaccharides in human milk strengthen the idea that specific interactions of those oligosaccharides with pathogenic microorganisms do occur preventing the attachment of microbes to epithelial cells. HMOs may act as soluble receptors for different pathogens, thus increasing the resistance of breast-fed infants. Some of the best-characterized adhesins of bacteria are those of *E. coli*, which possesses type 1 fimbriae (mannose sensitive), S fimbriae (sensitive to sialylated galactosides), or colonization factors [a heterogeneous group with various receptor specificities. The various ligand specificities of *E. coli* strains could explain the differences in intestinal colonization of breastfed versus formula-fed newborns: The free oligosaccharides and glycoproteins of human milk, which are present in large amounts and great variety, might prevent intestinal attachment of microorganisms by acting as receptor analogs competing with epithelial ligands for bacterial binding [51]

Rockova et al. [53] reported that two strains of *B. animalis* were unable to grow on a medium containing human oligosaccharides as the sole carbon source in contrast of bifidobacteria from human origin. On the other hand human oligosaccharides seem to be more specific for human origin bifidobacteria compared with fructooligosaccharides. Hence, new prebiotics with similar bifidogenic properties like human oligosaccharides should be developed.

3.2. Milk proteins

Whey proteins constitute about 60-80% of the total protein content of human milk, but only 18% of bovine milk. Furthermore, the composition of whey proteins is different for each of the milks: beta-lactoglobulin, that is not found in human milk, predominates in bovine milk, while alfalactalbumin and lactoferrin predominate in human milk. The alfalactalbumin is necessary for the synthesis of lactose in the mammary gland, through the action of the lactose synthetase enzyme, their concentration in human milk ranges from 0.22 to 0.46 g/dl. The betalactoglobulin has been blamed for allergies to bovine milk [54].

Undenatured whey protein is rich in *cysteine*, the thermo-labile amino acid which represents an effective *cysteine* delivery system for the cellular synthesis of glutathione. Both cysteine and glutamine, along with glycine, are necessary the synthesis of the tri-peptide *glutathione* (GSH), one of the major detoxifiers (Phase II sulfonation) and antioxidants of the body. Enhancing glutathione levels also helps reduce the risk of infections by improving white blood cell functions. However, the unique disulfide cystine bonds of whey are heat sensitive (thermo-labile) so only carefully processed, undenatured whey proteins deliver bioavailable cystine di-peptides for intracellular conversion to cysteine, thus maximizing glutathione levels with its important immune, antioxidant, and detoxification benefits. [55].

3.2.1. Lactoferrin

Whey proteins present in human milk, such as secretory IgA, lactoferrin and lysozyme are very stable in acid medium, and reasonably resistant the action of proteolytic enzymes, it is believed, therefore, that over three quarters of these proteins appear intact in the feces of infants. Approximately 6-10% of lactoferrin is not digested by the intestinal tract, assuming that it can reach the colon and play prebiotic activities [56]

Lactoferrin, a glyco-protein, is a major protein in human milk (1.3-2.8 g/L) while it is present only in traces in cow´s milk. Lactoferrin inhibits the growth of bacteria and fungi due to its ability to bind iron, a function known as *ferro-privation*. Iron is a nutrient usually required for bacterial growth. In this way the effect of lactoferrin can be ascribed to an inhibitory effect against a pathogens rather than a direct stimulus to the development of Bifidobacteria [11].

In addition, lactoferrin also promotes the growth of beneficial bacteria such as *L. bifidus*, helping infants establish good microbial conditions in their intestines, described as "*eubiosis*". It is also an antioxidant that naturally occurs in many body secretions such as tears, blood, breast milk, saliva and mucus. Lactoferrin has anti-viral, anti-tumor activity, anti-infl ammatory / anti-oxidant activity, and immuno-modulating activity [57] Lactoferrin is also a cystine rich sub fraction.

3.2.2. Lisozime

Lysozyme is an antimicrobial enzyme (EC 3.2.1.17) found in tears, saliva, human milk whey, mucus, neutrophil granules and egg- white. It hydrolyses b (1,4) linkage between N acetylglucosamine and N-acetylmuramic acid in bacterial cell wall. Gram positive bacteria

are more susceptible to lysozyme than Gram negative. The enzyme synergistically interacts with other immunoprotective components like IgA, C3 complement components and lactoferin. Human milk contains up to 400 mg/mL of lysozyme, which is a concentration approx. 3000 times higher than in bovine milk.[58]

Resistance to lysozyme and the ability to utilize human milk oligosaccharides (HMOs) were identified as the most important factors affecting the growth of bifidobacteria in human milk. Four out of 5 strains of human origin were resistant to lysozyme and utilized HMOs. In contrast, *B. animalis* was susceptible to lysozyme and did not utilize HMOs [53]

According to Rockova et al. [58] the lysozyme-resistant *Bifidobacterium bifidum* and *Bifidobacterium longum* strains exhibited excellent growth in human milk. In contrast, most of non-indigenous species, such as *C. butyricum*, did not grow in human milk oligosaccharides together with lysozyme may act as prebiotic-bifidogenic compounds inhibiting intestinal clostridia.

3.2.3. Lactoperoxidase

Lactoperoxidase makes up approximately 0.5% of the whey protein. In the presence of hydrogen peroxide (formed in small quantities by cells), catalyzes the oxidation of thiocyanate (part of saliva), forming hypothiocyanate, which can kill both gram-positive and gram-negative bacteria. Thus, lactoperoxidase in human milk may contribute to the defense against infection already in the mouth and upper gastrointestinal tract. Human milk contains active lactoperoxidase, but its physiologic significance is not yet known.[42]

3.2.4. κ-Casein and glycomacropeptide

κ-Casein, a minor casein subunit in human milk, is a glycoprotein with charged sialic acid residues. The heavily glycosylated k-casein molecule has been shown to inhibit the adhesion of *Helicobacter pylori* to human gastric mucosa. K-Casein has been shown to prevent the attachment of bacteria to the mucosal lining by acting as a receptor analogue [42].

Glycomacropeptide is resultant from the tryptic hydrolysis of human k-casein, containing sugars glucosamine and galactosamine. The molecular weight of intact human *k*-casein was estimated to be approximately 33,000. The human *k*-casein contained about 40% carbohydrate (15% galactose, 3% fucose, 15% hexosamines, and 5% sialic acid) and 0.10% (1 mol/mol) phosphorus. Its amino acid composition was similar to that of bovine *k*-casein except for serine, glutamic acid, and lysine contents [59]

Glycomacropeptide helps control appetite and inhibit the formation of dental plaque and dental cavities. It is a growth factor for bifidobacteria (bifidogenic factor 1) Levels of glycomacropeptide may range from 1% to 18% [40]

3.3. Milk fat

The main fatty acids present in human milk are restricted to those with 12-18 carbon atoms chains,namely lauric, myristic, palmitic, palmitoleic, stearic, oleic, linoleic and linolenic. Some of the long chain polyunsaturated acids such as arachidonic and others are derived from essential fatty acids linoleic and linolenic acids, totaling together with their precursors, about 15% of fat of human milk. This percentage is much higher than that found in bovine milk. Palmitic, oleic and linoleic add up together about 70% of total fatty acids of colostrum and 74% of that of mature milk [54]

Corcoran et al. [60] studied the effect of inclusion of various C18 fatty acids with 0–2 double bonds in either *cis* or *trans* configuration on *Lactobacillus rhamnosus* GG survival in simulated gastric juice at pH 2.5. Overall, the data suggest that probiotic lactobacilli can use an exogenous oleic acid source to increase their acid survival and the underlying mechanism most likely involves the ability of increased membrane oleic acid to be reduced by H^+ to stearic acid.

Rosberg-Cody et al. [61] isolate different strains of the genus *Bifidobacterium* from the fecal material of neonates and assessed their ability to produce the cis-9, trans-11 conjugated linoleic acid (CLA) isomer from free linoleic acid. The most efficient producers belonged to the species *Bifidobacterium breve*, of which two different strains converted 29 and 27% of the free linoleic acid to the cis-9, trans-11 isomer per microgram of dry cells, respectively. In addition, a strain of *Bifidobacterium bifidum* showed a conversion rate of 18%/μg dry cells. The ability of some *Bifidobacterium* strains to produce CLA could be another human health-promoting property linked to members of the genus, given that this metabolite has demonstrated anticarcinogenic activity in vitro and in vivo.

4. Bioactive prebiotic components in honey

Most of the honey in the world is produced by bees from the nectar. Nectar is a sugar solution and water, may contain pure sucrose, a mixture of sucrose, glucose and fructose, or glucose and fructose only. The nectar is transported to the combs of the hive, where they will undergo physical and chemical changes responsible for their maturation (Crane, 1983). The chemical composition of honey, as well as aroma, color and medicinal properties, are directly related to the nectar source that originated with the bee species that produced it, with their geographic and climatic conditions. All these factors contribute to the wide variation found in honey [62].

Shin and Ustunol [63] defines honey as natural syrup containing mainly fructose (38.5%) and glucose (31.3%). Other sugars in honey include maltose (7.2%), sucrose (1,5%) and a variety of oligosaccharides (4.2%). In addition to the complex mixture of carbohydrates, are enzymes, minerals, pigments, waxes and pollen. More than one hundred eighty substances have been found in different honey types.

Honey is a complex product of easy digestion and assimilation, constituting a source of energy that contributes to the balance of biological processes in that it contains suitable proportions, enzymes, vitamins, fatty acids, amino acids, phenolic and aromatic substances [64]. In addition contains oligosaccharides which stimulates the growth of probiotic bacteria in the gut [65, 66].

Leite et al. [65], found in various di-and trisaccharides in Brazilian honeys. Maltose showed up in higher levels in honeys surveyed followed by other five disaccharides, turanose, nigerose, melibiose, sucrose, isomaltose and four trisaccharides, maltotriose, panose, melezitose and raffinose..

Cellobiose, gentiobiose, isomaltose, kojibiose, laminaribiose, maltose, maltulose, melibiose, nigerose, palatinose, trehalose, trehalulose, turanose, and sucrose are the main disaccharides found in honey [66, 67]. However, it would be rather difficult to identify the predominant disaccharide or certain combinations in the previously studied honey types. For example, maltulose and turanose were found in many honey samples, however their concentrations varied to a wide extent. Thus, Sanz and others [66] found the highest amounts of maltulose and turanose (0.66 to 3.52 and 0.72 to 2.87 g/100 g of honey, respectively) in 10 samples of honey from different regions of Spain and commercially available nectar and honeydew honeys.

Carbohydrate degradation has been extensively studied in a variety of different *Bifidobacterium* species. Various α- and β-galactosidases, α- and β-glucosidase and β-fructofuranosidases during growth on fructooligosaccharides activities have been characterized in *Bifidobacterium species.* Additionally, starch-, amylopectin-, and pullulan-degrading activities in bifidobacteria have been investigated [68]

Pokusaeva et al. [68] describe the identification of two genes, *agl1* and *agl2*, present in the genome of *B. breve* UCC2003 and responsible for the hydrolysis of α-glycosidic linkages, such as those present in palatinose. The preferred substrates for both enzymes were panose, isomaltose, and trehalulose. The two purified α-1,6-glucosidases were also shown to have transglycosylation activity, synthesizing oligosaccharides from palatinose, trehalulose, trehalose, panose, and isomaltotriose.

Proline is the main amino acid present in honey; it is added by the bee and its amount varies depending on the floral source.[67].

Macedo et al. [69] studied the effect of the *Apis mellifera* honey on growth and viability of commercial strains of lactobacilli and bifidobacteria in fermented milk. Milk was inoculated with 2% of each probiotic separately and added with 3% of honey. After fermentation, were stored at 7 º C for up to 46 days and were evaluated periodically. The honey did not affect the growth or activity of lactobacilli, but exerted significant positive effect ($p<0.05$) on *Bifidobacterium* cultures assisting in maintaining the viability and stimulating metabolic activity of these bacteria, with increased pH reduction.

5. Conclusion

It is well stablished the role of several oligosaccharides as prebiotic substances. The prebiotic effect of human milk, however, is not related to a single growth-promoting substance, but rather to a complex of interacting factors. In particular the prebiotic effect has been ascribed to several oligosaccharides, that is clearly proved. The role and the way milk fat and proteins such as lactoferrin, lysozyme stimulate the growth of probiotic bacteria is not yet clearly defined.

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