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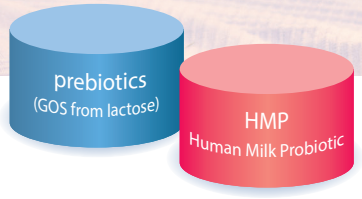
Scientific dossier



L. fermentum CECT5716 and Galacto-oligosaccharides in HiPP ORGANIC COMBIOTIC® formulae

HMP – originally isolated from breast milk

Information for health care professionals

Content

1. The infant microbiota

The number of human-associated microorganisms is tremendous, and estimated at 10^{14} to 10^{15} bacterial cells. These bacteria thus outnumber human somatic cells by approximately a factor of ten. Strictly speaking, only 10 % of our cells is human and 90 % is microbes. As for the number of different species, conservative sources speak of 400 to 500, other authors of at least a 1,000. Exact numbers are difficult to determine because many microorganisms cannot be bred. However, recent studies make it seemingly clear that there is no absolutely sterile region in our body. Apart from such densely populated regions as the urogenital tract, digestive tract and the oral cavity, bacteria or rather their DNA, have also been identified in the brain and in the placenta (Reid G et al. 2015).

Yet, undoubtedly, the by far largest concentration of bacteria (just under 99 %) is found in the intestine. This makes sense for several reasons. For one, the food residues in the intestine are an optimum source of nutrition for microorganisms. Secondly, the huge surface area of the intestine is a prerequisite for the effective absorption of nutrients. If the intestine with its many folds and villi were spread out flat, the result would be an area covering approx. 400 m^2 (as a comparison, the lungs are about 100 m^2, and the skin about 2 m^2). At the same time, every day our body encounters antigens and alien microbes which we take up with our food, and the intestinal immune system (GALT – gut-associated lymphatic tissue) has to differentiate which to classify as pathogenic and which as non-pathogenic. More than two thirds of all immunocompetent cells are found in the intestine. As we will see later, our immune system and the intestinal microbiota work together closely. These microorganisms are crucial, especially at the beginning of life, since they "train" the infant's still immature immune system.

The right bacterial balance is an important basis for human health. The availability of adequate physiological bacterial colonisation is therefore necessary to oust intruding extraneous pathogens. In addition, microorganisms produce numerous substances that affect the intestinal environment and thus the metabolism as a whole.

Characteristically, our endogenous intestinal microbiota prevents the growth and proliferation of new bacteria in the intestine. As a result, the dissemination of enteropathogenic bacteria is inhibited or impeded (colonisation resistance). The best weapon against the incursion of viruses and bacteria is therefore a well-developed endogenous intestinal microbiota.

1.1. Initial colonisation

For a long time, it was assumed that birth is the starting signal for the initial colonisation of the infant organism with bacteria. Newer research data suggest, however, that the first contact with bacteria already takes place in the womb. In various studies bacteria, or rather their DNA, could be detected in the amniotic fluid, the placenta, in umbilical cord blood, in the meconium, and even in follicular fluid (DiGiulio DB et al. 2008; Jiménez E et al. 2005 and 2008; Satokari R et al. 2009; Pelzer ES et al. 2013). Based on our knowledge today, we must assume that we are confronted with bacteria from the point of conception. It is well-known that the infant is inoculated with the mother's vaginal and rectal microbes as the infant passes through the birth canal. These microbes are later also found in the infant's intestines.

Over time, the infant's microbiota undergoes a change. Though the details are not yet completely known, it is assumed today that colonisation follows a clear pattern. First to settle are the so called facultative anaerobes or aerotolerant microbes as the "pioneer" microbes. Among these first colonisers of the intestine are the species enterobacteria, lactobacilli (notably L. fermentum, L. acidophilus and L. salivarius), and several enterococci and staphylococci (Bischoff SC 2009). These bacteria tolerate or deplete the oxygen present in the intestine and so form the environment for the second genus, the strictly anaerobic microbes, of which bacteroides and bifidobacteria are important representatives (**figure 1**). In the course of colonisation, the anaerobic microbes represent by far the largest number

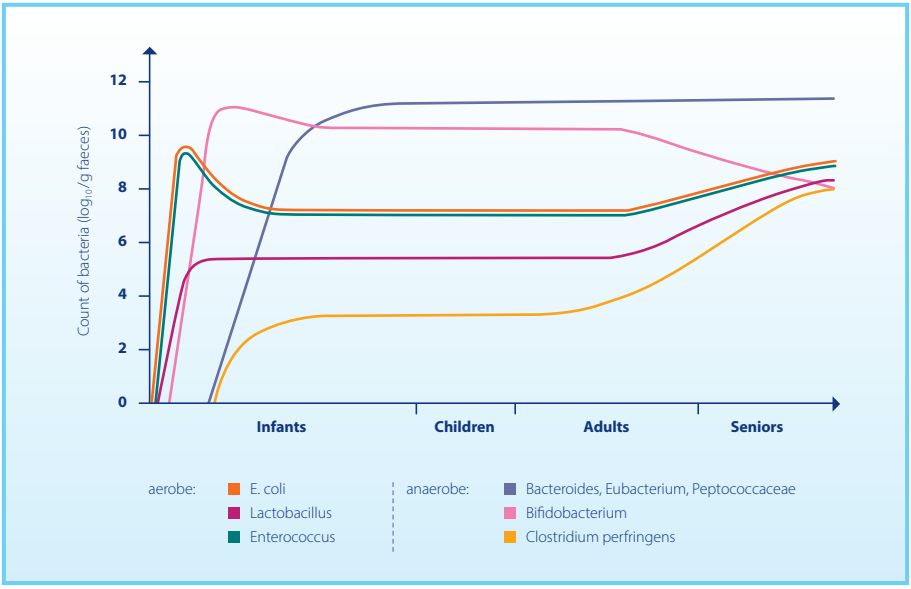


Fig. 1: Changes of gut microbiata composition in the course of a life (modified acc. to Schulze J et al. 2008)

of bacteria in the intestine. This, however, does not necessarily mean that they are also the most important group. Ultimately, what matters for "healthy" intestinal microbiota is the synergy between various microorganisms.

The transition of the infant microbiota to that of an adult starts as early as at the age of about five days and gathers pace with the introduction of complementary food (Palmer C et al. 2007). Yet, when exactly the intestinal composition stabilises and achieves biodiversity as in the adult is still contested. It is currently believed that this happens between the age of two and five years (Rodríguez JM et al. 2015). The intestinal microbiota then hardly changes until later, senior life (**figure 1**).

Each individual has a unique and highly variable composition of intestinal microorganisms, though a core combination of microorganisms is common to all humans (Tremaroli V, Bäckhed F 2012). According to recent studies, it seems that three so called enterotypes exist depending on the predominant bacterial strain (bacteroides, prevotella and ruminococcus) found (Arumugam M et al. 2011). What significance this has for the individual (advantages, risks), is the focus of ongoing studies.

The composition of the intestinal microbiota has an impact on the entire organism since it influences many intestinal functions and the immune system.

1.2. The intestinal microbiota – critical for the immune system

At the time of birth, many organ systems are immature. This applies to the immune system as well, which has to be "trained" in order to reach its full potential. Precisely this task is taken up by the intestinal microbiota. By means of various mechanisms, it acts directly against extraneous microbes and so helps prevent infections. This way, the "positive" bacteria in the intestine form a sort of natural barrier against extraneous microbes, i.e. they block receptors on the cells of the intestinal epithelium making them inaccessible to pathogens.

Furthermore, the positive bacteria produce substances that have a bacteriostatic and/or bactericidal effect. Some strains, like lactobacilli, produce lactic acid during the degradation of undigested food residues. This lactic acid lowers the pH value in the intestine, creating an environment that is hostile to many pathogens. Just as important as their direct action against pathogens is the modulation of the immune system by intestinal bacteria. These support the maturation of the gut-associated lymphatic tissue (GALT) and ensure that the immune cells in the intestine multiply in number and vary in character. Very importantly, they promote the tolerance development of the immune system against commensal bacteria, i.e. the "good" bacteria are tolerated while the "bad" ones are combated. Such defence mechanisms are controlled both by the congenital as well as the acquired immune system.

How important this process is, could be demonstrated emphatically in an animal study. When germ-free mice were transferred into a normal open-cage environment and thus exposed for the first time

to microbe-contaminated surroundings, they died shortly thereafter from simple infections. The reason why, was that mice raised germ-free only possess congenital unspecific and almost no acquired specific intestinal immune defences. However, when the germ-free mice were implanted with low-dose mixtures of species-specific intestinal flora, they survived in the open-cage environment. By rapidly developing a functional GALT, they became protected against infections by environmental microbes (Schulz J et al. 2008).

Consequently, it is decisive for immune system development and protection against infections to have a healthy intestinal microbiota, since gastrointestinal bacteria are considered the earliest and strongest stimulus for the development of the gut-associated lymphatic tissue. Researchers agree that close attention should be paid to a well and appropriately developing intestinal microbiota, as it plays an important role in the maturation of the immune system and protection against infections.

Evidence has been growing that dysbiosis, an imbalance of the intestinal microbiota, can have a negative impact on health in the long term. Higher risks of obesity, allergies, diabetes, intestinal disorders, infections and even anxiety disorders and depression have all been inferred (Collado MC et al. 2012; Houghteling PD, Walker WA 2015).

1.3. What factors affect the intestinal microbiota?

As previously touched on, it is the early influencing factors which are most particularly able to lastingly impact the development of the physiological intestinal microbiota. Yet, it is the maternal microbiota and the mother's health status and lifestyle that will influence as early as in pregnancy which microbes she will pass on to her child. Of further relevance, moreover, are the nature and timing of birth, host genetics, diet and environmental factors such as siblings, time spent at nurseries, medication (antibiotics) and contact with household pets (**figure 2**).

1.3.1. The role of the birth process

The birth process strongly impacts the colonisation of the intestine of the newborn with bacteria. During a vaginal birth, the child comes in contact with the vaginal and faecal flora of the mother. Children born by vaginal delivery have a markedly more physiological intestinal microbiota than children born by primary caesarean delivery. While the microbiota after vaginal delivery is more like in the mother's intestine, skin flora and hospital bacteria are predominantly found in children born by caesarean delivery (**figure 3**).

Caesarean-born children are therefore considered to be at a higher risk of developing diseases later in life (Cho CE, Norman M 2013). A further adverse influence on the microbial flora of mother and child is the usual treatment of the mother with prophylactic antibiotics after a caesarean.

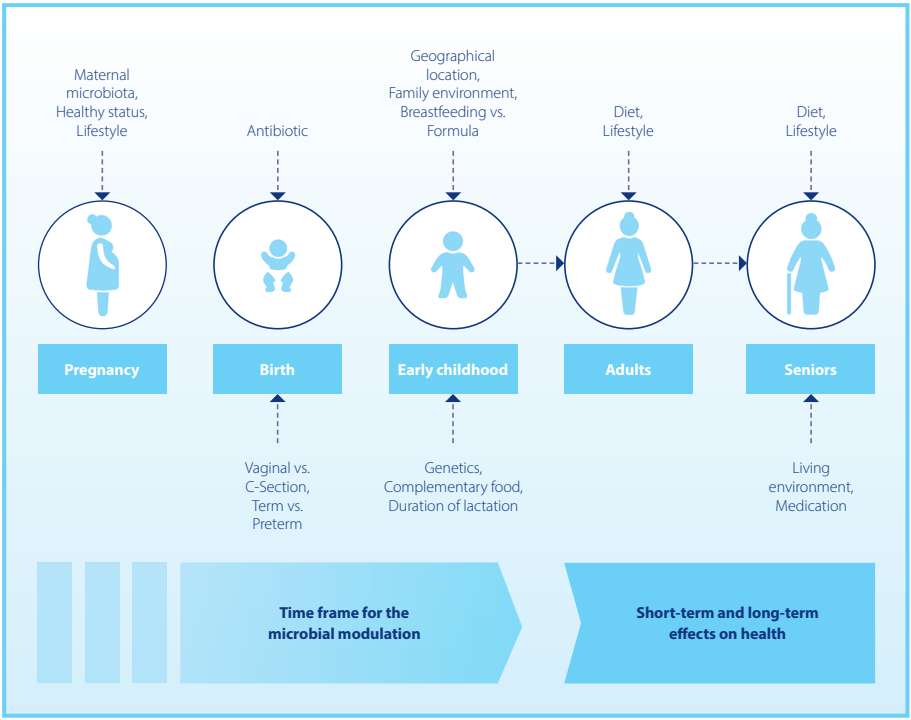


Fig. 2: Factors influencing the gut microbiota (Rodríguez JM et al. 2015)

These differences may persist until the age of seven (Renz-Polster H et al. 2005; Cho CE, Norman M 2013), and there is now ample recorded evidence that a caesarean delivery is associated with later health risks such as allergy, asthma, type I diabetes and also coeliac disease due to an imbalanced intestinal microbiota (Cho CE, Norman M 2013; Thavaganam S et al. 2008; Laubereau B et al. 2004).

1.3.2. The role of the diet

Several studies indicate that bottle-fed children have a different intestinal microbiota to breastfed children. The microbiota of breastfed children is largely dominated by bifidobacteria and lactobacilli. Compared to bottle-fed children, it shows a lower counts of the Bacteroides, Clostridium coccoides group, Staphylococcius and Enterobacteriaceae (Matamoros S et al. 2013). Children receiving an industrially manufactured formula have also been shown to have a broader range of intestinal bacteria.

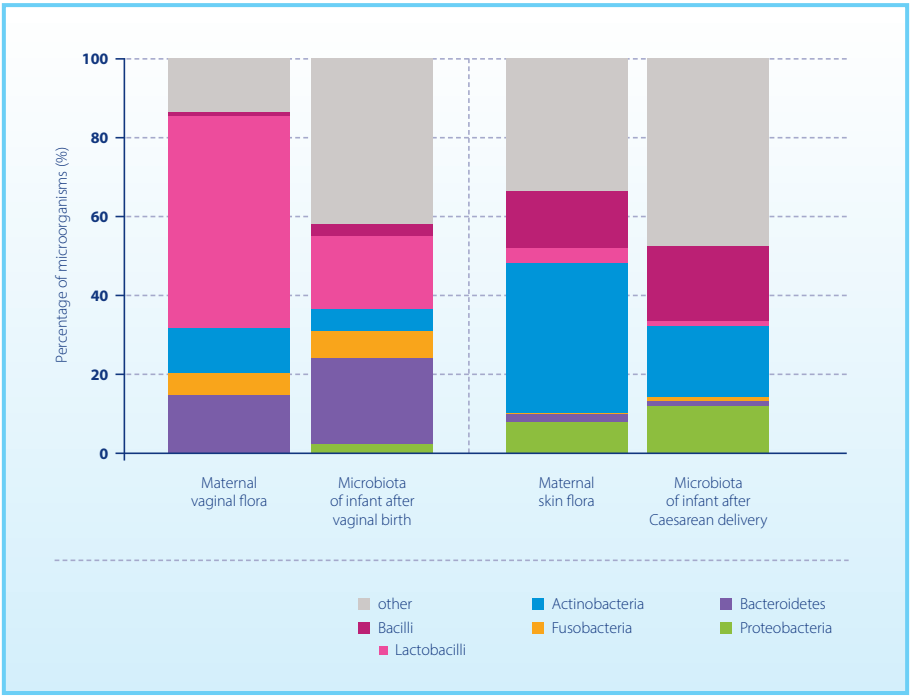


Fig. 3: Effects of the type of birth on the microbiota of the infant (modified acc. to Dominguez-Bello MB et al. 2010)

1.4. Pro-, pre-, and synbiotics – their influence on the intestinal microbiota

Acquired microorganisms as well as special dietary ingredients can influence the endogenous intestinal microbiota. This specifically concerns the pro- and prebiotics or rather a combination of them, the synbiotics.

Probiotics are live, non-pathogenic microorganisms which – when administered in sufficient numbers – have a preventive or therapeutic effect on the macroorganism, that is to say that they provide it with a health benefit (FAO/WHO 2001). Lactic acid bacteria are among the best-known probiotics, and specifically are certain lactobacillus and bifidus strains.

Prebiotics, however, are special substrates that can neither be cleaved enzymatically, nor absorbed in the upper part of the digestive tract. They reach the colon in undigested form, where they serve as a substrate for bacterial fermentation and stimulate the reproduction of non-pathogenic intestinal bacteria (Gibson GR, Roberfroid MD 1995). This process quickens the growth of bacteria that are capable of degrading prebiotics. These are mainly bifidobacteria and lactobacilli. Their growth and metabolic

activities allow potentially pathogenic agents to settle to a lesser extent. The bacteria in the intestine, for example, produce short-chain fatty acids through carbohydrate degradation which then lower the pH value in the intestine and so create an unfavourable environment for many pathogens. Examples of active prebiotic substances are the galacto-oligosaccharides, fructo-oligosaccharides and inulin.
If a food product contains both probiotics and prebiotics, the combination is also referred to as a **synbiotic** (Bischoff SC 2009).

1.5. Influence of breast milk on the intestinal microbiota of the infant

The child's diet is an important factor in influencing the microbiota of the intestine. In this instance too, breast milk can be considered as the gold standard because it contains, among other things, probiotic bacteria with positive effects and breast milk oligosaccharides with prebiotic properties. Since breast milk combines both components (pre- and probiotics), it is also referred to as a natural synbiotic foodstuff.

1.5.1. Breast milk is not sterile

The understanding that breast milk contains a multitude of bacteria is still relatively new. Breast milk has long been considered sterile. Most recently though, breast milk has been shown to contain bacteria which pass on to the baby during breastfeeding (Martín R et al. 2003; Heikkila MP et al. 2003). Besides the essential nutrients, it contains between 10^3 and 10^5 bacterial CFU per millilitre which means an average drinking amount of 800 mL approximately 10^5 to 10^7 CFU per day for an infant (Martín et al. 2005; Martín et al. 2007; Gueimonde M et al. 2007). Therefore, not surprisingly, the infant's intestinal microbiota mirrors the microbiota of breast milk (Heikkila MP et al. 2003; Martín R et al. 2004).

Some 200 different types of breast milk bacteria (around 50 genera) have already been identified. These include not only lactic acid bacteria, such as lactobacilli or bifidobacteria, but also streptococci, pseudomonads or staphylococci, which are pathogenic microorganisms but in breast milk apparently have no adverse effects (Martín R et al. 2007; Collado MC et al. 2009; Martín R et al. 2009). The main lactobacilli are L. fermentum, L. gasseri, L. rhamnosus, L. acidophilus, L. salivarius, L. lactis and L. reuteri (Martín R et al. 2003 and 2007; Sinkiewicz G et al. 2008). In samples of breast milk from German and Austrian mothers, lactobacilli were found more frequently than bifidobacteria. The most common type of bacteria found was L. fermentum (25 %), beside L. salivarius (35 %). Individual bifidobacteria species were noticeably less frequent: B. breve (14 %), B. lactis and B. longum (4 % respectively) (**figure 4**).

Large regional and local differences have also been found between breast milk microbiotas apart from the individual bacterial diversity in breast milk (Sinkiewicz G et al. 2008).

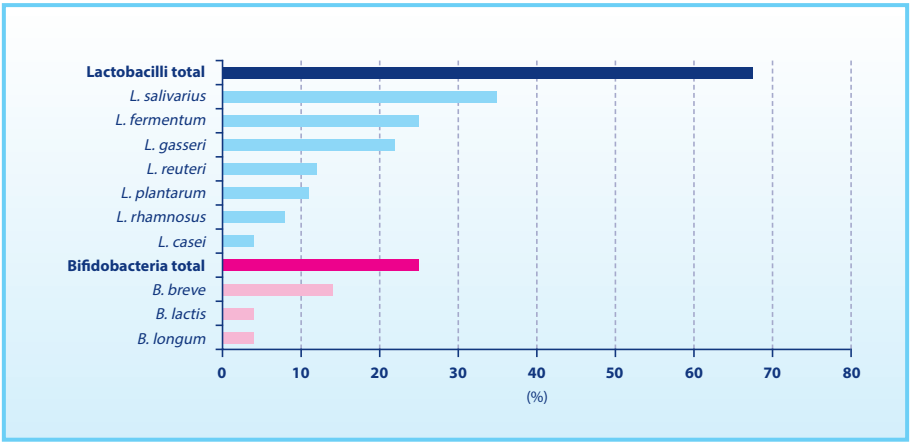


Fig. 4: Frequency (%) of lactobacilli and bifidobacteria in 160 breast milk samples (Soto A et al. 2014)

Of all the bacteria identified in breast milk, the lactobacilli attract the most attention due to their probiotic potential. Still, it must be noted that not all lactobacilli can be regarded as being probiotic. Under a comprehensive selection process, three bacterial strains of the lactobacillus group with probiotic properties were isolated from 1,500 breast milk lactobacillus cultures: L. gasseri CECT5714, L. salivarius CECT5713 and *L. fermentum CECT5716* (Martín et al. 2003). Numerous preclinical and clinical studies have been undertaken on these bacteria to prove their safety and efficiency (Lara-Villoslada F et al. 2007). HiPP's infant formulae contain the probiotic strain *L. fermentum CECT5716* and this is the focus of the subsequent appraisal (chapter 3).

For the composition of breast milk, it is crucial how the mother's intestinal microbiota is composed, because bacteria are believed to be able to pass from the mother's intestine to breast milk via the vascular-lymphatic system (**figure 5**) (Perez PF et al. 2007; Fernández L et al. 2013; Jost T et al. 2014; Rodríguez JM 2014). Dendritic cells play a key role in this "entero-mammary pathway". They can open the "tight junctions" between the epithelial cells, take up bacteria from the intestinal lumen with their extensions (dendrites) and subsequently close the "tight junctions" again (Rescigno M et al. 2001; Uhlig HH, Powrie F 2003). The bacteria then reach the breast mucosa and thus finally the milk via the mesenteric lymph nodes and the bloodstream (Martín R et al. 2004). This process of bacterial translocation is a physiological mechanism and is augmented during pregnancy and lactation (Perez PF et al. 2007). Bacteria can be identified in the mammary glands from the last trimester of pregnancy onwards. At birth and in the time soon after, the concentrations of bacteria in breast milk reach a maximum and then during lactation decrease slowly and steadily. Once the child has been weaned, bacteria can no longer be found in the mammary glands (Langa S 2006).

Of additional interest is the fact that the microbiota undergoes change during lactation and that there are also differences between overweight mothers and those of a normal weight (Cabrera-Rubio R et al. 2012).

Further likely sources of those bacteria found in breast milk are the maternal skin (migration from the mammary ducts) and the microflora from the mouth cavity of a breastfed child (**figure 5**).

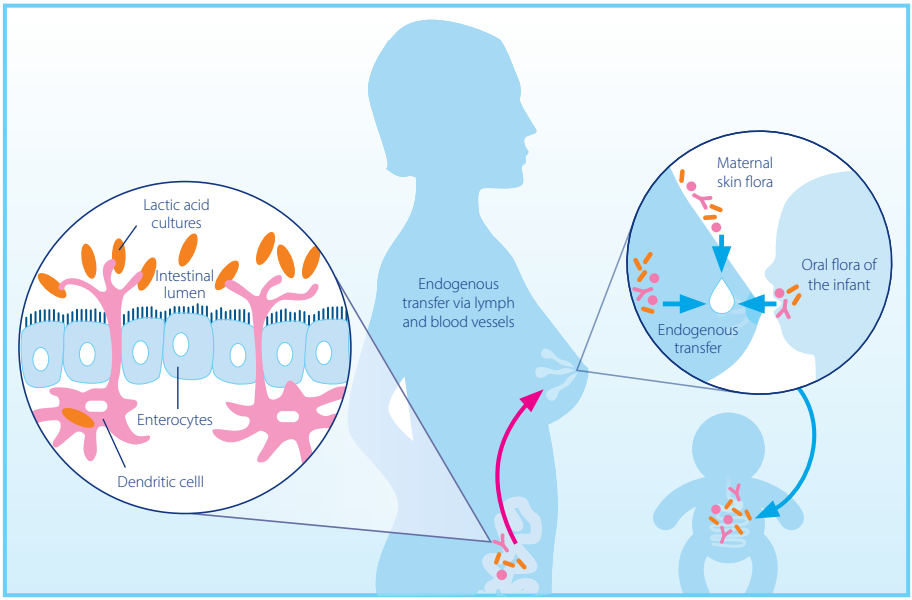


Fig. 5: Possible origin of bacteria in breast milk (Fernandez L et al. 2013)

1.5.2. Maternal microbiota can colonise the intestine of the infant

This raises the question whether the bacteria present in breast milk are capable of settling in the intestine of the child. It has recently been confirmed that they indeed can. Lactic acid bacteria originating from breast milk can be identified in stool samples from infants (Martín R et al. 2003; Martín V et al. 2012). Maternal bacteria can consequently significantly contribute to protective barrier formation in the intestine of the infant and to a favourable influence on the immune system. The formation of a physiological intestinal microbiota assists the infant's immune system. The bacteria in breast milk probably also afford a passive immunisation that protects the breastfed infant from infections (Howie PW et al. 1990; López-Alarón M et al. 1997; Wright AL et al. 1998). This effect is also supported due to

the combined action of several components present in breast milk (immunoglobulins, immunocompetent cells and various antimicrobial compounds) (Lara-Villoslada F et al. 2007). The low susceptibility of breastfed infants to infections is also closely correlated to the prebiotic property of the oligosaccharides in breast milk, which selectively stimulate the growth of intestinal bacteria that exert a positive effect in the infant gut (Kunz C et al. 2000).

1.5.3. Oligosaccharides in breast milk

Human milk oligosaccharides (HMOs) are the main constituents of breast milk besides lactose and fats (Rudloff S, Kunz C 2012). Unlike cow's milk, mature breast milk at 10 to 20 g/L contains large amounts of milk oligosaccharides. Five monosaccharides are the constituents of the oligosaccharide structures in breast milk: glucose, galactose, fucose, N-acetylglucosamine and sialic acid (N-acetylneuraminic acid). The composition of human milk oligosaccharides (HMOs) is very complex; 200 different multiple sugar compounds have been identified so far (Rudloff S, Kunz C 2015).

One of the essential functions of HMOs is that they serve as a source of nutrition for certain bacteria and thus influence the microbial colonisation of the intestine and the development of the intestinal immune system. Breast milk oligosaccharides are also being investigated regarding their inhibition of pathogenic microorganisms from attaching to intestinal epithelial cells. More on these substances can be found in chapters 2 and 4.

For more detailed information on probiotics and prebiotics in breast milk, we would like to refer you to the book "Prebiotics and Probiotics in Human Milk" (McGuire M, McGuire M, Bode L 2017, Elsevier Verlag).

2. Requirements on probiotics and prebiotics in infant formulae

As already described in chapter 1.5, the bacterial environment of the digestive tract is of great importance. It seems that since the digestive tract of the infant initially contains few bacteria[1] but then is colonised by bacteria during birth and later through the intake of food, the composition of breast milk or the infant formula used is decisive for the further development of the intestinal microbiota. Breast milk contains both probiotics and prebiotics. Modern infant formulae, given the positive properties of the symbiotically composed breast milk, are enriched with pro- and prebiotics, so that children, who cannot be breastfed, can roughly benefit from the positive effects of this natural example.

The main probiotics used in infant milk formulae are lactobacilli and bifidobacteria. Galacto-oligosaccharides (GOS), fructo-oligosaccharides (FOS), acidic oligosaccharides (AOS), polydextrose (PDX) or blends thereof are often used as prebiotic substances (Braegger C et al. 2011).

Probiotics and prebiotics have a strong influence on infant microbiota formation, on the immune system and thus on the entire metabolism. For this reason, specific safety recommendations apply regarding the use of pro- and prebiotics in infant formulae. The European panel of specialists ESPGHAN, for example, requires that the **safety and benefit** is established individually in scientific studies for each prebiotic and probiotic substance and for any combination of the two. Furthermore, long-term data are required that will show what influence the early administration of probiotics may have on later health (Braegger C et al. 2011).

Additionally, the probiotics and prebiotics used must be shown to have certain technological properties, to ensure that they can be produced in flawless quality and that their properties remain stable under application.

These requirements were implemented in the product development of a synbiotic formula with the probiotic L. fermentum CECT5716 and the prebiotic GOS (galacto-oligosaccharides obtained from lactose) (chapters 3 and 4).

[1] The earlier assumption that the intestine of the foetus is sterile has been discredited – bacteria can already reach the foetal intestine through the placenta

3. *L. fermentum CECT5716* – a unique probiotic

Although intestinal colonisation starts as early as pregnancy, breast milk is the most important source of commensal bacteria for the breastfed child. Breast milk is not sterile but rather contains a multitude of bacteria (**table 1**). In particular the lactic acid bacteria are relevant, as they can have a positive influence on the environment of the intestine. From over one thousand species of lactic acid bacteria, among others, the lactobacillus strain Lactobacillus fermentum hereditum CECT5716 with probiotic properties could be isolated from breast milk (Martín R et al. 2003; Martín R et al. 2005). This lactic acid bacterium was selected by HiPP for use in infant and follow-on formulae. The choice was based on the facts that it was **originally isolated from breast milk**, belonged to the primary colonisers of the intestine and is considered a safe and efficient probiotic.

Tab. 1: Common bacterial groups and genera from breast milk samples (modified acc. to Martín R et al. 2004)

Bacterial group	Main species	Bacterial group	Main species
Staphylococcus sp.	*S. epidermis*	***Lactobacillus sp.***	*L. gasseri*
	S. hominis		*L. rhamnosus*
	S. capitis		*L. acidophilus*
	S. aureus		*L. plantarum*
			L. fermentum
Streptococcus sp.	*S. salivarius*		
	S. mitis	***Enterococcus sp.***	*E. faecium*
	S. parasanguis		*E. faecalis*
	S. peroris		

3.1. Selection criteria for *L. fermentum CECT5716*

L. fermentum CECT5716 followed a meticulous selection process. Safety, probiotic properties and technological aspects were the most important areas of the selection process. **Table 2** shows a brief overview of the different steps in selecting *L. fermentum CECT5716.*

Tab. 2: Selection criteria for the use of *L. fermentum CECT5716*

A. Safety criteria	• Biochemical and genetic taxonomy, held on file in an international data base • Production of biogenic amines • Enzymatic activities • Exclusion of a transfer of antibiotic resistance genes
B. Functional criteria	• Resistance to digestive secretions (gastric acid, bile acid) • Fermentative abilities • Intestinal colonisation • Production of metabolites • Production of antimicrobial substances • Immunological parameters
C. Technological criteria	• Growth and stability

3.2. Safety studies with *L. fermentum CECT5716*

L. fermentum CECT5716 was investigated in numerous cell culture and animal studies, followed by studies in humans, first in adults, and later in infants. The study results indicate that *L. fermentum CECT5716* is not only a safe probiotic strain but also survives the gastrointestinal tract (Martín R et al. 2005, Lara-Villoslada F et al. 2009) and has a positive influence on the immune system (Peran L et al. 2006, Olivares M et al. 2006, Arribas B et al. 2009, Peran L et al. 2007). Studies in adults (Olivares M et al. 2007, Arroyo R et al. 2010) and in children (Maldonado J et al. 2008, Maldonado J et al. 2012, Gil-Campos M et al. 2012; Maldonado-Lobón JA et al. 2015) have confirmed the safety and efficacy of *L. fermentum CECT5716*.

3.2.1. Safety of *L. fermentum CECT5716* in animals

A safety evaluation of *L. fermentum CECT5716* showed that an administration in doses 10,000 times higher than those normally taken in by adults (based on body weight) was safe in mice (Lara-Villoslada F et al. 2009). The treatment of Balb/C mice with 10^{10} CFU per day over a period of 28 days showed no negative effects. Body weight, tissue weights, including that of liver, spleen, thymus, heart and kidneys, as well as the biochemical and haematological parameters and food intake were similar in

the study and control group. Furthermore, no bacteriaemia or bacterial translocation to the liver or spleen as a cause of the treatment could be observed. The results also indicate that the strain does not transmit any antibiotic resistance to other bacteria.

3.2.2. Safety of *L. fermentum CECT5716* in humans

As to the safety in humans, a study conducted with healthy **adults** has been completed. This study has revealed that the daily intake of *L. fermentum CECT5716* at a dose of 10^{10} CFU over a period of 28 days is safe and tolerated well without any observed side effects which could be associated with the administration of the probiotic (Olivares M et al. 2007).

A further four clinical studies conducted with **infants** additionally investigated the safety of *L. fermentum CECT5716*. The first two studies investigated the safety of *L. fermentum CECT5716* in infants older than six months (Maldonado J et al. 2008; Maldonado J et al. 2012). The third and fourth studies investigated the safety of *L. fermentum CECT5716* in infants from birth (Gil-Campos M et al. 2012; Maldonado-Lobón JA et al. 2015).

One double-blind, randomised placebo-controlled study investigated the safety of *L. fermentum CECT5716* (2 x 10^8 CFU/day) in infants aged between six and twelve months (n = 80). Both groups received a follow-on formula (control group without *L. fermentum*, study group with *L. fermentum*) for a period of three months. The results revealed no significant differences in growth between the two groups (**table 3**). Moreover, no side effects associated with consumption of the formula were reported.

Tab. 3: Clinical parameters of infants (six to twelve months old) according to the formula used at 0 and 3 months after intervention (Maldonado J et al. 2008)

	Control group (follow-on formula without *L. fermentum*)		**Study group** follow-on formula with *L. fermentum*)	
	0	**3 months**	**0**	**3 months**
Weight (g)	7579 ± 870	9002 ± 1011	7101 ± 957	8574 ± 1150
Length (cm)	65.8 ± 2.3	71.1 ± 2.3	69.8 ± 3.2	69.8 ± 2.3
Head Circumference (cm)	43.7 ± 1.3	45.8 ± 1.3	43.0 ± 1.3	45.3 ± 1.3

The other studies in the target infant group are presented in chapter 5.

3.2.3. Further *L. fermentum CECT5716* safety parameters

- The *L. fermentum CECT5716* genome has been identified and fully sequenced. The sequencing of the genome has provided additional information on the safety and on the probiotic property of the germ (Jiménez E et al. 2010).

- EFSA (European Food Safety Association) in 2007 granted the lactic acid bacterium *L. fermentum CECT5716* qualified presumption of safety (QPS) status (EFSA 2007). QPS evaluation is a safety assessment conducted by EFSA on microorganisms that are added to food- and feedstuffs. Their use must not pose a health risk.

- *L. fermentum CECT5716* received GRAS status in 2015 (Substances Generally Recognized As Safe), Notice No. GRN 000531 (FDA 2015). This status is an American Food and Drug Administration (FDA) safety designation and is only granted if safety-relevant studies have been presented.

- The WHO has also attested the safe use of lactobacilli as a probiotic, thus also regarding *L. fermentum CECT5716* (FAO/WHO Expert Consultation 2001).

3.3. Efficacy studies with *L. fermentum CECT5716*

L. fermentum CECT5716 was identified and characterised using comprehensive biochemical, molecular and genetic techniques. These procedures are essential prerequisites for the implementation of an adequate safety evaluation and a safe use in humans. Many safety studies and investigations of the functional and technological properties have been completed. The most important results from these studies, in particular those regarding the probiotic properties and safe use, are listed below.

3.3.1. *L. fermentum CECT5716* can colonise the human intestine

Compared to other lactobacilli, *L. fermentum CECT5716* survives the gastrointestinal passage in high numbers (74 % survival rate) (**figure 6**). Surviving gastrointestinal conditions is a major requirement of a probiotic.

After oral intake, *L. fermentum CECT5716* is found in the faecal microbiota of healthy adults. *L. fermentum CECT5716* was present in 92 % of the faecal samples compared to only in 12 % of those from the control group without *L. fermentum CECT5716* (Olivares M et al. 2007).

L. fermentum CECT5716 moreover has demonstrated high adhesion to intestinal cells (Martín R et al. 2005). As a result, this can impede pathogenic bacteria from adhering to the intestinal cells (Severin AL et al. 2004).

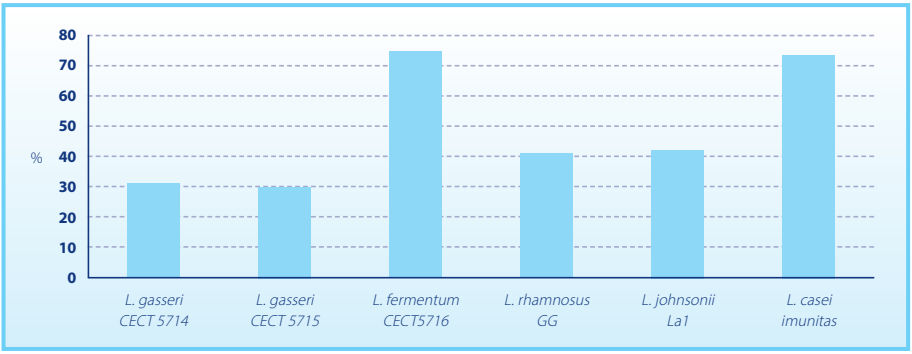


Fig. 6: Percentage (%) of the Lactobacilli survived to conditions simulating those of the human gastrointestinal tract (modified acc. to Martín R et al. 2005)

3.3.2. *L. fermentum CECT5716* supports the integrity of the intestinal mucosa

L. fermentum CECT5716 is capable of fermenting a variety of carbohydrates in the intestine and producing **short-chain fatty acids (SCFAs)**, which are an important source of energy for intestinal cells (Peran L et al. 2006). SCFAs increase the absorption of water and salt in the intestine, participate actively in colon epithelial cell metabolisation and reduce the pH value, thereby supporting the growth of healthy bacteria and inhibiting the growth of pathogenic microorganisms. Furthermore, the SCFAs propionic acid and acetic acid are transported to various organs such as the muscles, brain and heart, where they serve as an energy source.

L. fermentum CECT5716 induces goblet cells to produce **mucins** (glycoproteins) and so protects the intestinal epithelium from physical, chemical and bacteriological damage (Olivares M et al. 2006).
L. fermentum CECT5716 produces **glutathione**, a natural antioxidant, which e.g. protects the intestine from oxidative damage (Peran L et al. 2006).

3.3.3. *L. fermentum CECT5716* protects against intestinal infections

L. fermentum CECT5716 has demonstrated in vitro protection against gastrointestinal infections, as found in two experimental models of the enteropathogenic strain Salmonella choleraesuis. The addition of *L. fermentum CECT5716* to a nutrient solution that contains S. choleraesuis led to a significant decrease in pathogen growth compared to the control media.
In a study assessing the in vivo antibacterial effect of L. fermentum CECT5716 in a mouse model infected with S. choleraesuis, significantly more animals receiving *L. fermentum CECT5716* survived (60 %) compared to the control animals (10 %). This suggests that *L. fermentum CECT5716* can exercise **antagonistic effects** against salmonella infections in vivo and in vitro (Olivares M et al. 2006).

L. fermentum CECT5716 produces **antimicrobial substances** that inhibit the growth of pathogenic strains like E. coli 433, Salmonella choleraesuis spp., Listeria Scott A and Staphylococcus aureus. One possible mechanism is the production of lactic acids by *L. fermentum CECT5716* in copious amounts which leads to a decrease in pH value in the intestine by which pathogenic microorganism growth is impaired. *L. fermentum CECT5716* also increases the membrane permeability of Gram-negative bacteria, thereby reducing their viability and enhancing their exposure to bactericidal compounds (Olivares M et al. 2006).

L. fermentum CECT5716 induces the **production of mucins** which constitute the initial barrier of the intestinal epithelium as protection against infections. Of the several classes of mucins, *L. fermentum CECT5716* specifically increases MUC2 and MUC5B gene expression. Thus, *L. fermentum CECT5716* improves the protective layer of the intestinal epithelium against infections (Olivares M et al. 2006).

L. fermentum CECT5716 **inhibits the adhesion of pathogens** to the intestinal mucosa and so promotes the elimination of pathogens with the faeces. As soon as a pathogen reaches the intestinal mucosa, it is prerequisite for bacterial colonisation and invasion for it to adhere to the components of the extracellular matrix of the host. Due to this epithelial adhesion the pathogen cannot be eliminated from the intestine. *L. fermentum CECT5716* is able to bind to the intestinal pathogens and thus prevents them from reaching the intestinal mucosa and attaching to it and being taken up by the epithelial cells (Olivares M et al. 2006).

3.3.4. *L. fermentum CECT5716* contributes to a modulation of immune response

It has been shown that *L. fermentum CECT5716* can exhibit either a reinforcing or anti-inflammatory effect, depending on the immune status.

L. fermentum CECT5716 reinforces the immune response after influenza vaccination. After a vaccination, it was shown that the NK cells (first barrier against pathogens) and the antibody IgA increased – an effect that did not occur in the placebo group (Olivares M et al. 2007).

L. fermentum CECT5716 supports the natural and acquired immune response which could be shown by demonstrating, besides an activation of the NK cells, also an effect on various T-cells (Perez-Cano FJ et al. 2010).

3.3.5. *L. fermentum CECT5716* has an influence on maternal mastitis treatment

Administrating *L. fermentum CECT5716* during lactation as treatment for infectious mastitis seems to be an effective alternative to the commonly prescribed antibiotics. While being treated with *L. fermentum CECT5716*, women suffering from infectious mastitis had significantly fewer pathogenic bacteria (staphylococci, streptococci, corynebacteria) in their milk samples than those under a treatment of antibiotics. They also recovered faster, experienced less pain and had rarer relapses (**figure 7**).

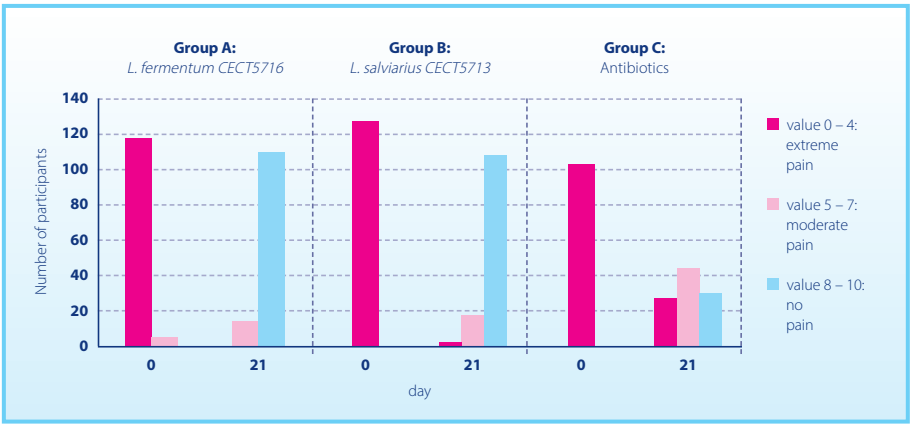


Fig. 7: Reported pain by women with infectious mastitis at the start (day 0) and end (day 21) of the study (Arroyo R et al. 2010)

4. Galacto-oligosaccharides – a tried and tested prebiotic

Galacto-oligosaccharides (GOS) belong to carbohydrates which are not degradable by human digestive enzymes. They are also called prebiotics. They pass through to the lower regions of the intestine, to the colon, where they can be metabolised by the "good" bacteria (lactobacilli and bifidobacteria).

Human milk oligosaccharides have a very complex structure. Thanks to advances in the fields of biotechnology and chemical synthesis, a few specific structures can already be investigated in vivo and added to infant milk formulae. Current oligosaccharides for industrial use mostly have a simpler structure and differ in regard to their base substance. Fructose, which is only present in vegetable foodstuffs such as chicory, leek, onions and bananas, serves as the base substance for fructo-oligosaccharides (FOS) and inulin. Galacto-oligosaccharides (GOS) are gained from milk sugar (lactose) and have been used in the food industry for over 30 years. So far, galactose has been the only monosaccharide to be found both in human and in industrial oligosaccharides (GOS) (**table 4**).

The decision in favour of the galacto-oligosaccharides as a prebiotic substance to be used in the HiPP infant and follow-on formulae was made because these substances are more closely related to the

Tab. 4: Occurrence of monosaccharides in breast milk (Kunz C, Rudloff S 2006)

Monosaccharide	Breast milk	Prebiotic
Glucose	traces	+
Galactose	+	+
N-acetylglucosamine	+	–
Fucose	+	–
N-acetylneuraminic acid	+	–
Fructose	–	+
Xylose	–	+
Arabinose	–	+
Other	–	+

+ occurs / **–** does not occurr

milk oligosaccharides that are present in breast milk than the vegetal fructo-oligosaccharides (FOS), whose base structure fructose is not contained in breast milk.

GOS are synthesised enzymatically from lactose. GOS consist of a chain of galactose monomers, usually with glucose at the reducing end. The chain length shows up to seven molecular building blocks.

Needless to say that GOS fulfil the required scientific proof of the safety of their use required by ESPGHAN.

Studies with infants fed with GOS-enriched infant formulae show that the formulae are well-tolerated, the administration is safe and that lactobacilli and bifidobacterial growth is promoted (bifidogenic effect). The galacto-oligosaccharides were tested in the concentrations 0.24 g/100 mL (Ben XM et al. 2004 and 2008), 0.4 g/100 mL (Ashley C et al. 2012, Giovannini M et al. 2013), 0.44 g/100 ml (Sierra C et al. 2015) and 0.5 g/100 mL (Fanaro S et al. 2009, Sierra C et al. 2015).

Important results evaluating safety and effectiveness are summarised below.

4.1. GOS are safe, well-tolerated and have prebiotic effects for infants

Full-term, healthy infants were fed over a period of six months either with an **infant formula** not containing any prebiotic additive (n=52) or with an infant formula containing GOS (0.24 g/100 mL) (n=69) (Ben XM et al. 2004). A reference group was comprised of breastfed children. After a three- and six-month intervention, a significant increase in bifidobacteria and lactobacilli in faeces was seen in the GOS-supplemented group compared to the group receiving an infant milk formula without GOS. There were no significant differences between the GOS group and the group of breastfed children. The increase in bifidobacteria was similar to those observed in breastfed, full-term infants. Furthermore, the GOS group showed a significant increase in short-chain fatty acids (SCFAs) and a lower pH value in the faecal than the control group.

A further publication by Ben XM et al. 2008 has supported the results of the study referenced above (**figure 8**).

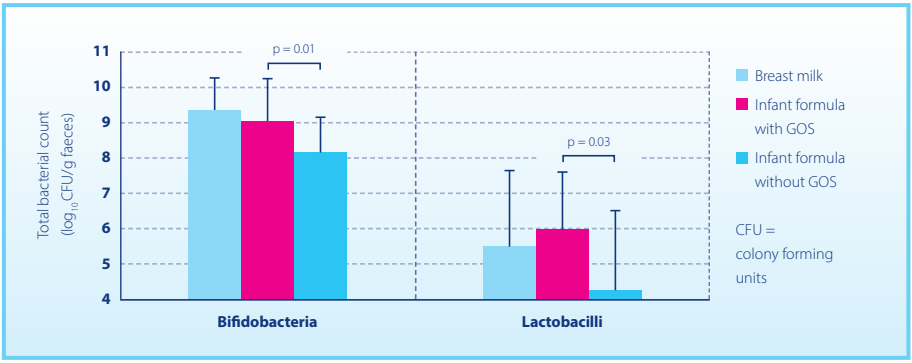


Fig. 8: Lactobacilli and bifidobacterial count in the faeces of infants after three months of feeding in relation to nutrition (Ben XM et al. 2008)

The supplementation of a **follow-on formula** with GOS (0.5 g/100 ml) led to improved bifidus growth during the intervention period. In a multi-centre, randomised study, four to six months old, healthy children (n=159) were fed either a control formula or a GOS-enriched follow-on formula over an 18-week period. A complementary diet was introduced during the intervention period. After the six-week intervention, the faecal bifidobacterial count in the active treatment group was significantly higher than in the control group. The increase remained stable until the end of the study, 12 weeks later. The study shows that even under complementary diet introduction, a bifidogenic effect can be achieved by administering GOS (**figure 9**).

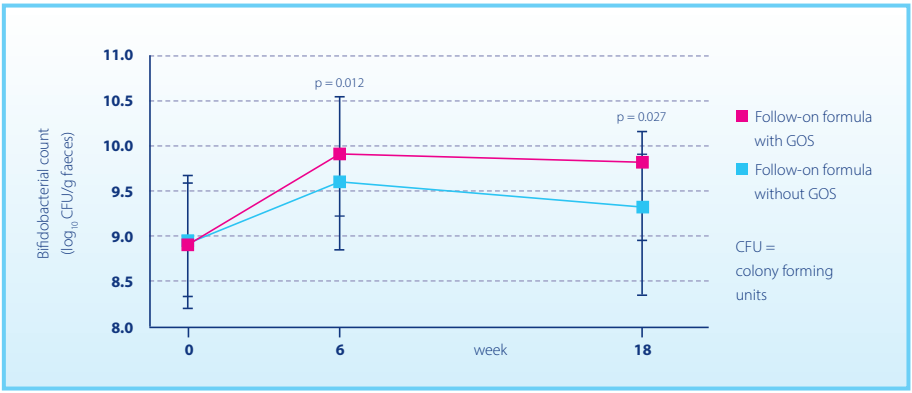


Fig. 9: Bifidobacterial count in the faeces of infants in relation to nutrition at the time points 0, 6, 18 weeks of intervention (Fanaro S et al. 2009)

In a multicentre, double-blind, placebo-controlled prospective study, the prebiotic effect of GOS in healthy infants (n=365) in the first year of life has been confirmed. GOS-enriched **infant formula** (0.44 g/100 mL) and **follow-on formula** (0.5 g/100 mL) were used. Children in the study group showed an increased number of bifidobacteria, a lower pH value in stool, increased stool frequency and softer stool consistency (Sierra C et al. 2015).

Ashley et al. 2012 studied the safety and tolerability of prebiotic galacto-oligosaccharides (with and without polydextrose (PDX)) in a multi-centre, double-blind prospective study with 419 children from birth. The infants were either fed with an **infant formula** without prebiotics (n=142), an infant formula with 0.4 g/100 mL GOS (n=138) or a 1:1 mixture of 0.4 g/100 mL GOS/PDX (n=139). Anthropometric data were measured after 14, 30, 60, 90, and 120 days. There were no differences between the groups, either in terms of growth or with regard to food intake, tolerance and the occurrence of side effects.

4.2. GOS affect stool characteristics

The addition of GOS led to softer stools (Ben XM et al. 2008, Fanaro S et al. 2009, Sierra C et al. 2015, Ashley C et al. 2012) (**figure 10**) and increased stool frequency (Ben XM et al. 2008, Sierra C et al. 2015). The stool properties thus better resemble that of the breastfed infant.

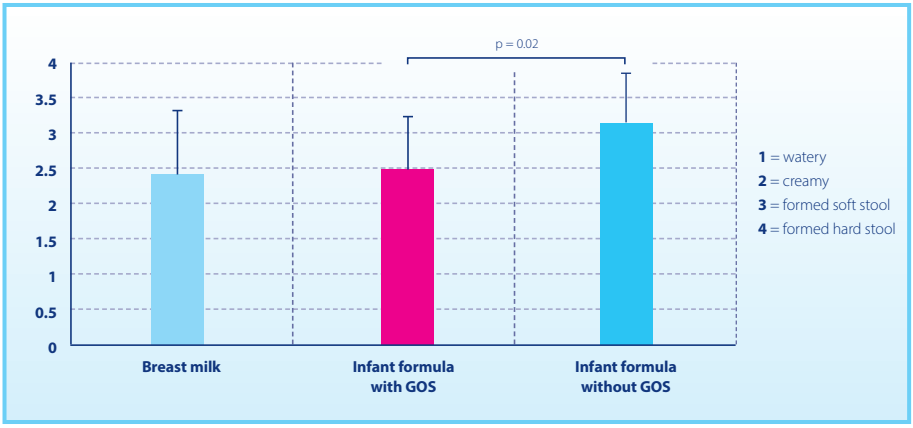


Fig. 10: Infant stool consistency after three months of feeding in relation to nutrition (Ben XM et al. 2008)

4.3. GOS show positive effect in infantile colic

A multi-centre, double-blind, controlled study (Giovannini M et al. 2014) showed that a GOS-enriched infant formula (0.4 g/100 mL) positively influenced the occurrence of infantile colic. The promotion of bifidobacteria and lactobacilli growth in the study group (n=83) together with lower clostridia counts in faecs significantly reduced the occurrence of infantile colic compared to the control group (n=80) (infant formula without GOS). Infantile colic thus seems to be related to the production of intestinal gas induced by clostridium growth.

4.4. Further safety aspects of GOS

In 2007, the GOS mixture used by HiPP received GRAS status (Substances Generally Recognized As Safe), Notice No. GRN 000236 (FDA 2008). This status is an American Food and Drug Administration (FDA) safety designation and is only granted if safety-relevant studies have been presented.

5. Studies investigating the safety and benefit of the combination of *L. fermentum CECT5716* and GOS in infants

The European panel of specialists ESPGHAN requires both safety and benefit to be established individually in scientific studies for each prebiotic and probiotic substance and for any combination of the two. In addition, long-term data are required that show the influence of early administered probiotics on future health (Braegger C et al. 2011).

This requirement has successfully been fulfilled in clinical studies for the combination of the prebiotic galacto-oligosaccharides (GOS) and the probiotic *L. fermentum CECT5716* in both infant and follow-on formulae (**table 5**).

Tab. 5: Overview of clinical studies on the use of galacto-oligosaccharides (GOS) and *L. fermentum CECT5716* in infants

GOLF* 1: Safety and benefit in infants 6 to 12 months of age	
Design	Double-blind, randomised, placebo-controlled
Number of participants	188 infants aged 6 months
Intervention period	6 months
Control group	Follow-on formula with GOS (0.4 g/100 mL)
Study group	Follow-on formula with GOS (0.4 g/100 mL) and *L. fermentum* ($2x10^8$ CFU/day)
Primary outcome	Incidence of infections
Secondary outcomes	Growth and development, febrile episodes, administration of antibiotics, faecal parameters

(Maldonado J et al. 2012)

*GOLF = abbreviation for the combination of GOS and *L. fermentum*

GOLF* 2: Safety and benefit in infants between birth and 6 months of age	
Design	Double-blind, randomised, placebo-controlled
Number of participants	121 infants aged between 21 days and the end of 1st month of life
Intervention period	6 months
Control group	Infant formula with GOS (0.3 g/100 mL)
Study group	Infant formula with GOS (0.3 g/100 mL) and *L. fermentum* (10^7 CFU/g)
Primary outcome	Average weight gain from the start of the study until the completed 4th month of life
Secondary outcomes	Height/head circumference, faecal parameters, incidence of infections, acceptance and tolerance

(Gil-Campos M et al. 2012)

GOLF* 2 Follow-up study on the long-term safety of infant formula	
Number of participants	91 children
Age of children at final measurement	3 years
Data collection	0.5, 1, 2, 3 years of life
Endpoints	**Primary:** Growth based on body weight, body length and head circumference at 3 years **Secondary:** Frequency of infections, allergies and non-infectious diseases, hospitalisations, surgical interventions, stool parameters (microbiota, SCFA, IgA), digestion (bowel movement, flatulence, abdominal pains)

(Maldonado-Lobón JA et al. 2015)

*GOLF = abbreviation for the combination of GOS und *L. fermentum*

5.1. The combination of *L. fermentum CECT5716* and GOS is safe

Both double-blind, randomised, placebo-controlled studies on infant and follow-on formulae revealed a normal range in terms of infant growth and development.

At the time of completion of **GOLF 1 study (follow-on formula)**, there were no significant differences between the groups in terms of the growth parameters body weight, body height and head circumference (Maldonado J et al. 2012) (**figure 11**).

In the **GOLF 2 study (infant formula)** as well, there were no significant differences observed in terms of weight and head circumference. Likewise, at the age of four months, there were no significant differences between the groups with regard to body height. However, at the end of the study (at the age of six months), the children in the study group were significantly bigger compared to the control group. The children's increase in height (cm/day) was similar, however, and no significant differences were found (Gil-Campos M et al. 2012) (**figure 11**).

In the **GOLF 2 Follow-up study (long-term safety of infant formula)** there were no significant differences observed between both the control and the study group, as well. At the age of three years, the mean values of weight, height, and head circumference were similar in the children of both groups (**figure 11**). No differences were found in the incidence of infections, non-infectious diseases and intestinal problems. The intestinal bacteria found in the stool were also comparable in the respective groups. The follow-up of the clinical study with infant formula confirmed the long-term safety of an early use of *L. fermentum CECT5716* (Maldonado-Lobón JA et al. 2015).

5.2. The combination of *L. fermentum CECT5716* and GOS is well-tolerated

GOLF 2 study (infant formula): Tolerance and compliance (use of formula according to study protocol) were good. The behaviour patterns due to the formula, such as stool colour, stool frequency, stool consistency, flatulence, regurgitation, sleep behaviour and mood, were comparable in both groups (Gil-Campos M et al. 2012).

GOLF 1 (follow-on formula): Tolerance and compliance were good. There were no reports of adverse events (e.g. spewing out food). No differences were seen in the amounts drunk per day in both groups (Maldonado J et al. 2012).

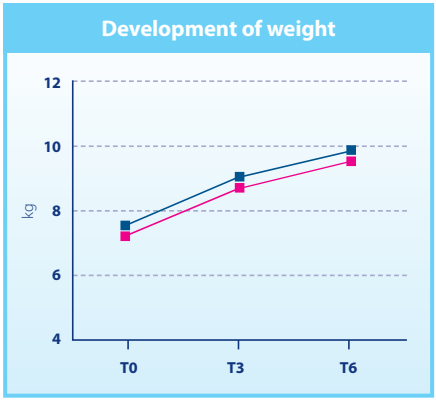
Safety
Follow-on formula (GOLF 1)

(Maldonado J et al. 2012)

- Control group: only prebiotics (GOS)
- Study group: prebiotics + probiotics (GOS +*L. fermentum*)

T0: start of study
T3: after 3 months
T6: after 6 months

*p<0.05



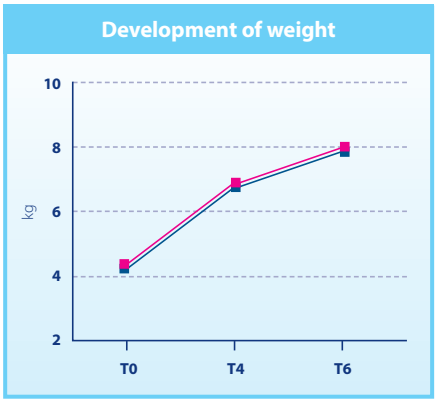
Safety
Infant formula (GOLF 2)

(Gil-Campos M et al. 2012)

- Control group: only prebiotics (GOS)
- Study group: prebiotics + probiotics (GOS +*L. fermentum*)

T0: start of study
T4: after 4 months
T6: after 6 months

*p<0.05



Long-term safety
of infant formula
(GOLF 2 Follow-up study)

(Maldonado-Lobón JA et al. 2015)

- Control group: only prebiotics (GOS)
- Study group: prebiotics + probiotics (GOS +*L. fermentum*)

*p<0.05

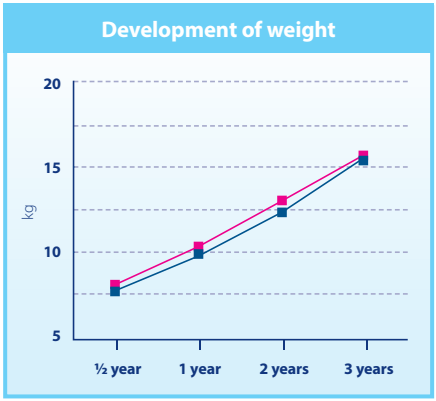
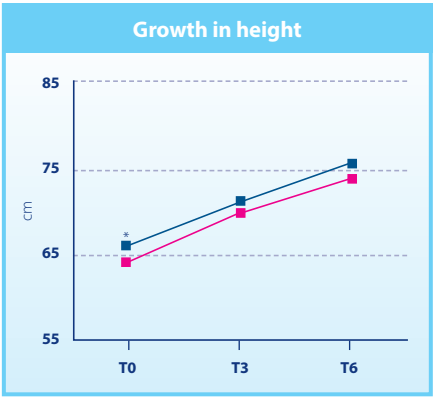
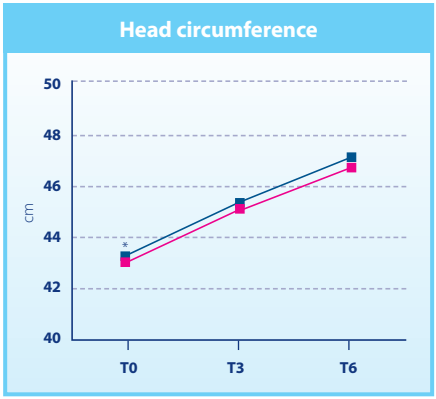
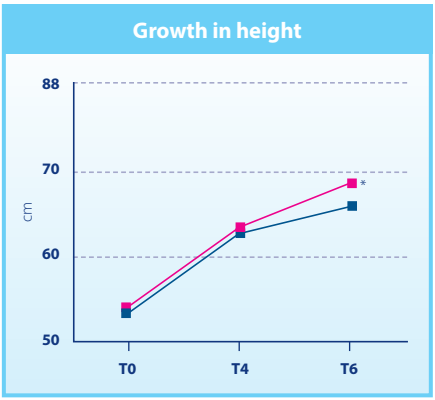
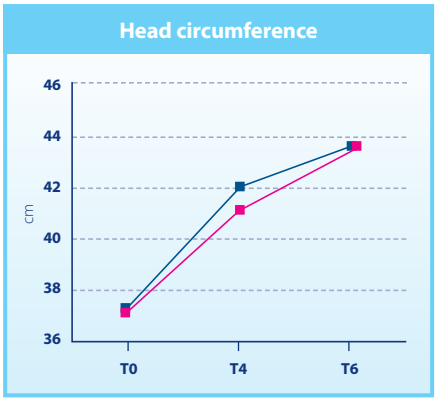


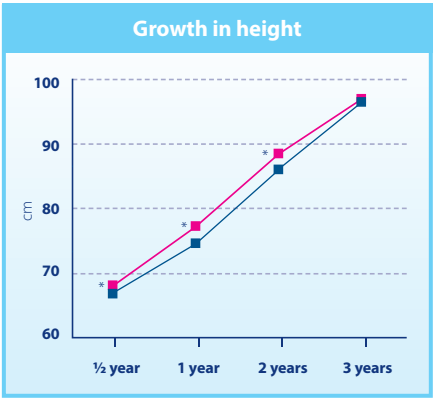
Fig. 11: Anthropometric data for body weight, body height, head circumference in relation to nutrition

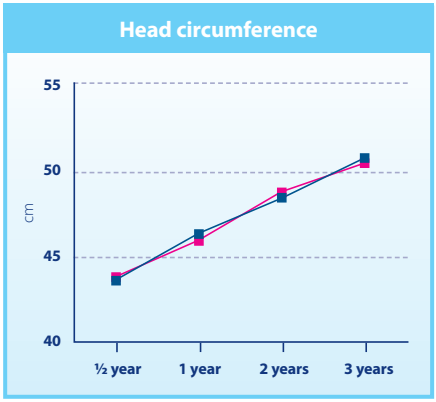












(values are mean values)

5.3. The combination of *L. fermentum CECT5716* and GOS changes the microbiota

A stool sample analysis at the end of the study showed a significant increase in lactobacilli and bifidobacteria for both the infant formula study (GOLF 2) and the follow-on formula study (GOLF 1). A 78 % increase of lactobacilli as well as a 70 % increase of bifidobacteria was measured in the stool of infants who received follow-on formula.

These changes (multiplication of the positive bacteria) as a result of the combination of probiotic and prebiotic may perhaps explain the lower incidence of gastrointestinal infections (Gil-Campos M et al. 2012; Maldonado J et al. 2012).

5.4. The combination of *L. fermentum CECT5716* and GOS leads to reduced infection rates

GOLF 2 study (infant formula): During the study period of six months, the occurrence of gastrointestinal infections decreased significantly by 71 % (**figure 12**).

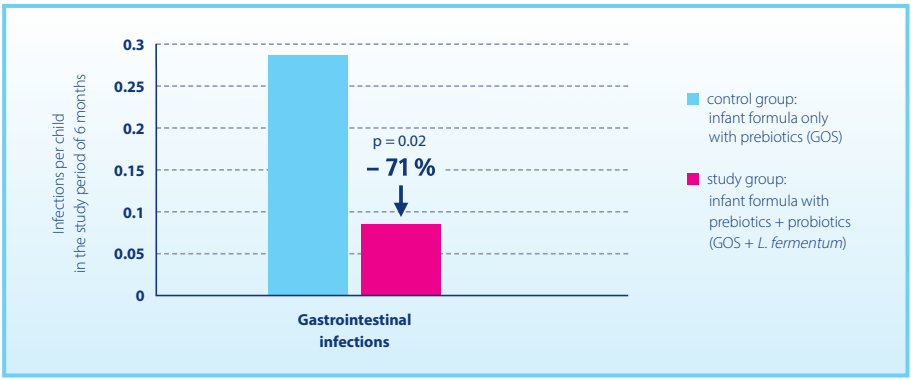


Fig. 12: Incidence of infection in relation to used infant formula (Gil-Campos M et al. 2012)

GOLF 1 study (follow-on formula): During the study period of six months, the incidence of respiratory tract infections as well as gastrointestinal infections was observed to decrease significantly by 26 % and 46 %, respectively (**figure 13**). Viewing all occurring infections together, the study group observed a 30 % reduction in the incidence of infections.

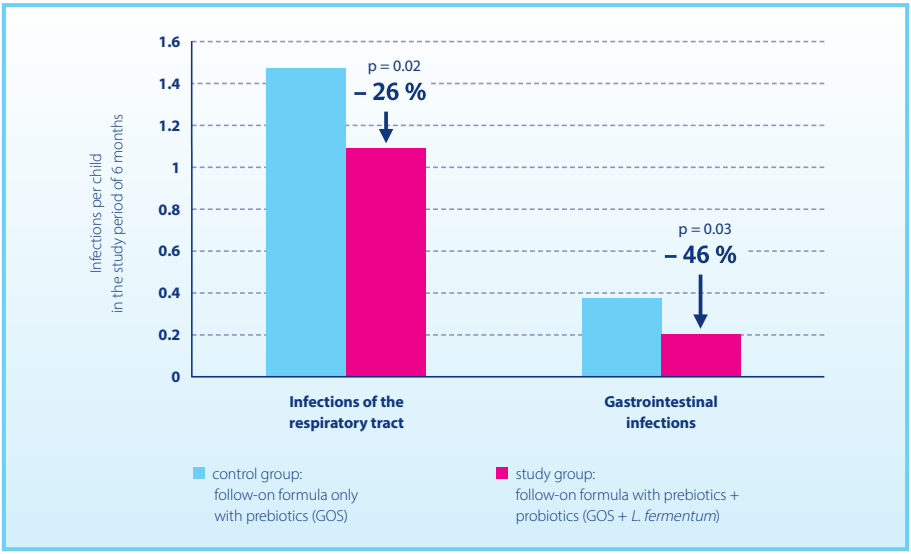


Fig. 13: Incidence of infection in relation to used follow-on formula (Maldonado J et al. 2012)

6. Summary and conclusion

The intestine and in particular the intestinal microbiota are key prerequisites for a functioning immune system. Therefore, the formation of a balanced and stable intestinal microbial colonisation is of crucial importance to human health. Formation starts as early as in the womb during foetal development which then later, in the first few months of life, takes on its special imprint. Birth type, the environment, illnesses and treatments, all play an important role.

Obviously, nutrition, and therefore the composition of breast milk or of the infant formula fed, is of immense significance. Depending on the composition of the food, the contained substances pass through to the lower intestinal regions and can affect the microorganisms present there in terms of their quality and quantity. An influence is exerted mainly by bacteria whose intake was exogenous, as well as by specific substances that serve as an energy source for the bacteria and stimulate their growth to a greater or lesser extent.

Thanks to its composition, breast milk ideally promotes the formation of a positive intestinal microbiota in infants. Two building blocks are of crucial importance for the formation of the "basic microbial environment": the bacteria in breast milk as well as specific carbohydrates called human milk oligosaccharides (HMOs). In this context, they are referred to as prebiotics and probiotics.

Of those bacteria present, the lactic acid bacteria are particularly significant, as they can favourably influence the intestinal environment. As one of over a thousand colonies of lactic acid bacteria, the lactobacillus strain Lactobacillus fermentum CECT5716 with probiotic properties could be isolated from breast milk (Martin R et al. 2003; Martin R et al. 2005).

This dossier put special emphasis on describing the properties and the safe and efficient use of *L. fermentum CECT5716*, a bacterium that belongs to the first colonisers of the intestine.

L. fermentum CECT5716 has been classified by EFSA as safe for use (QPS, qualified presumption of safety) in food and feedstuffs and scientific evidence was provided for the following properties:

- ***L. fermentum CECT5716* survives the passage through the digestive tract in high proportion and displays high adhesion to intestinal cells**;
- **supports the integrity of the intestinal mucosa;**
- **protects against intestinal infections;**
- **contributes to immune system modulation;**
- **influences maternal mastitis;**
- **is safe and effective in infants;**
- **has a well-characterised and fully sequenced genome.**

Besides probiotic lactic acid bacteria, the availability of prebiotic substances as the second important building block is significant for the intestinal microbiota imprint. Originating from the human milk oligosaccharides (HMOs) occurring in breast milk, galacto-oligosaccharides (GOS) are well-suited for being used in infant and follow-on formulae. Galacto-oligosaccharides (GOS) are gained from milk sugar (lactose) and have been used in the food industry for over 30 years. They are closer to the oligosaccharides in breast milk because their basic building block galactose is also found in breast milk. Fructose, the basic building block for vegetal fructo-oligosaccharides (FOS), by contrast, is not contained in breast milk (Kunz C, Rudloff S 2006). GOS have been approved for use in food (GRAS status of the FDA) and the following properties are scientifically proven:

- **beneficial influence on microbiota and stool habits**
- **prebiotic effect**
- **good tolerance and safe for use in infants**

The safe use and good tolerance of the combination of GOS and *L. fermentum CECT5716* both in follow-on formula and infant formula could be proven in two randomised, controlled clinical studies. The long-term safety of an early administration of *L. fermentum CECT5716* was confirmed in a 3-year follow-up to the clinical study with infant formula.

In addition, there was a positive change observed in the microbiota (a 78 % increase in lactobacilli, a 70 % increase in bifidobacteria) and a significant reduction in the incidence of infections. A decrease in diarrhoeal diseases by 71 % was achieved in the infant formula study, a decrease in infections of the respiratory tract by 26 % and gastrointestinal infections by 46 % was observed in the follow-on formula study.

In accordance with the requirement for the use of probiotics and prebiotics in infant formulae by the European Society for Paediatric Gastroenterology Hepatology and Nutrition, ESPGHAN, the safety and benefit of *L. fermentum CECT5716* and GOS in infants was demonstrated in three studies (Maldonado J et al. 2012; Gil-Campos M et al. 2012; Maldonado-Lobón JA et al 2015). Safety of the individual substances has been confirmed by the EFSA and the FDA by granting both QPS status and GRAS status to *L. fermentum CECT5716* and GRAS status to GOS.

7. Selection of abstracts

7.1. *L. fermentum CECT5716*

In vitro immunomodulatory activity of Lactobacillus fermentum CECT5716 and Lactobacillus salivarius CECT5713: two probiotic strains isolated from human breast milk.

Immunobiology. 2010 Dec;215(12):996-1004.

Pérez-Cano FJ, Dong H, Yaqoob P.

Commensal bacteria, including some species of lactobacilli commonly present in human breast milk, appear to colonize the neonatal gut and contribute to protection against infant infections, suggesting that lactobacilli could potentially modulate immunity. In this study, we evaluated the potential of two Lactobacillus strains isolated from human milk to modulate the activation and cytokine profile of peripheral blood mononuclear cell (PBMC) subsets in vitro. Moreover, these effects were compared to the same probiotic species of non-milk origin. Lactobacillus salivarius CECT5713 and Lactobacillus fermentum CECT5716 at 10^5, 10^6 and 10^7 bacteria/mL were co-cultured with PBMC (10^6/mL) from 8 healthy donors for 24 h. Activation status (CD69 and CD25 expressions) of natural killer (NK) cells (CD56+), total T cells (CD3+), cytotoxic T cells (CD8+) and CD4+ T cells was determined by flow cytometry. Regulatory T cells (Treg) were also quantified by intracellular Foxp3 evaluation. Regarding innate immunity, NK cells were activated by addition of both Lactobacillus strains, and in particular, the CD8+ NK subset was preferentially induced to highly express CD69 (~90%, $p<0.05$). With respect to acquired immunity, approximately 9% of CD8+ T cells became activated after co-cultivation with L. fermentum or L salivarius. Although CD4+ T cells demonstrated a weaker response, there was a preferential activation of Treg cells (CD4+CD25+Foxp3+) after exposure to both milk probiotic bacteria ($p<0.05$). Both strains significantly induced the production of a number of cytokines and chemokines, including TNFa, IL-1β, IL-8, MIP-1a, MIP-1ß, and GM-CSF, but some strain-specific effects were apparent. This work demonstrates that L. salivarius CECT5713 and *L. fermentum CECT5716* enhanced both natural and acquired immune responses, as evidenced by the activation of NK and T cell subsets and the expansion of Treg cells, as well as the induction of a broad array of cytokines.

Complete genome sequence of Lactobacillus fermentum CECT5716, a probiotic strain isolated from human milk.

J Bacteriol. 2010 Sep;192(18):4800.

Jiménez E, Langa S, Martín V, Arroyo R, Martín R, Fernández L, Rodríguez JM.

Lactobacillus fermentum is a heterofermentative lactic acid bacterium and is frequently isolated from mucosal surfaces of healthy humans. Lactobacillus fermentum CECT 5716 is a well-characterized probiotic strain isolated from human milk and, at present, is used in commercial infant formulas. Here, we report the complete and annotated genome sequence of this strain.

Treatment of infectious mastitis during lactation: antibiotics versus oral administration of Lactobacilli isolated from breast milk.

Clin Infect Dis. 2010 Jun 15;50(12):1551-8.

Arroyo R, Martín V, Maldonado A, Jiménez E, Fernández L, Rodríguez JM.

Background: Mastitis is a common infectious disease during lactation, and the main etiological agents are staphylococci, streptococci, and/or corynebacteria. The efficacy of oral administration of Lactobacillus fermentum CECT5716 or Lactobacillus salivarius CECT5713, two lactobacilli strains isolated from breast milk, to treat lactational mastitis was evaluated and was compared with the efficacy of antibiotic therapy.

Methods: In this study, 352 women with infectious mastitis were randomly assigned to 3 groups. Women in groups A (n = 124) and B (n = 127]) ingested daily 9 log(10) colony-forming units (CFU) of *L. fermentum CECT5716* or L. salivarius CECT5713, respectively, for 3 weeks, whereas those in group C (n =101) received the antibiotic therapy prescribed in their respective primary care centers. Results. On day 0, the mean bacterial counts in milk samples of the 3 groups were similar (4.35-4.47 log(10) CFU/mL), and lactobacilli could not be detected. On day 21, the mean bacterial counts in the probiotic groups (2.61 and 2.33 log(10) CFU/mL) were lower than that of the control group (3.28 log(10) CFU/mL). *L. fermentum CECT5716* and L. salivarius CECT5713 were isolated from the milk samples of women in the probiotic groups A and B, respectively. Women assigned to the probiotic groups improved more and had lower recurrence of mastitis than those assigned to the antibiotic group.

Conclusions: The use of *L. fermentum CECT5716* or L. salivarius CECT5713 appears to be an efficient alternative to the use of commonly prescribed antibiotics for the treatment of infectious mastitis during lactation.

Safety assessment of Lactobacillus fermentum CECT5716, a probiotic strain isolated from human milk.

J Dairy Res. 2009 May;76(2):216-21.

Lara-Villoslada F, Sierra S, Díaz-Ropero MP, Rodríguez JM, Xaus J, Olivares M.

Lactobacillus fermentum CECT5716, a probiotic strain isolated from human milk, was characterized in a previous study. The objective of this study was to evaluate its sensitivity to antibiotics and its potential toxicity and translocation ability after oral administration to mice. For this purpose, 40 Balb/C mice were divided in two groups (n=20 per group). One group was treated orally with 10(10) colony forming units (cfu)/mouse/day of Lb. fermentum CECT5716 during 28 d. The other group only received the excipient and was used as control. Food intake, body weight, bacterial translocation and different biochemical and haematological parameters were analysed. Oral administration of Lb. fermentum CECT5716 to mice had no adverse effects on mice. There were no significant differences in body weight or food intake between control and probiotic-treated mice. No bacteraemia was observed and there was no treatment-associated bacterial translocation to liver or spleen. Stress oxidative markers were not different in control and probiotic-treated mice. These results suggest that the strain Lb. fermentum CECT5716 is non-pathogenic for mice even in doses 10,000 times higher (expressed per kg of body weight) than those normally consumed by humans.

Evaluation of the preventative effects exerted by Lactobacillus fermentum in an experimental model of septic shock induced in mice.

Br J Nutr. 2009 Jan;101(1):51-8.

Arribas B, Rodríguez-Cabezas ME, Comalada M, Bailón E, Camuesco D, Olivares M, Xaus J, Zarzuelo A, Gálvez J.

The preventative effects of the probiotic Lactobacillus fermentum CECT5716 were evaluated in the lipopolysaccharide (LPS) model of septic shock in mice. The probiotic was administered suspended in drinking water at the final concentration of 108 colony-forming units/ml for 2 weeks before the induction of an endotoxic shock by an intra-peritoneal injection of LPS (400 microg/200 microl per mouse). Blood and different organs were collected after 24 h to evaluate the severity of the endotoxic shock and the preventative effects of the probiotic. L. fermentum reduced TNF-alpha levels in blood, which promotes the major alterations observed during septic shock, as well as the infiltration of activated neutrophils into the lungs. Furthermore, free radical overproduction

and oxidative stress were associated with a significant decrease in hepatic glutathione levels in septic mice, and with an excessive NO production attributed to the induction of the inducible isoform of NO synthase (iNOS). In fact, hepatic glutathione levels were signi-ficantly increased in the group of mice receiving the probiotic, and the increased iNOS expression both in the colon and lungs was down-regulated in those mice treated with L. fermentum. Finally, pre-treatment with L. fermentum may also exert its protective action modulating the expression of different cytokines in splenocyte-derived T cells such us IL-2, IL-5, IL-6 or IL-10. In conclusion, pre-treatment with L. fermentum may exert its protective action against LPS-induced organ damage in mice by a combination of several actions including its antioxidant properties and by reduction of the synthesis of the pro-inflammatory TNF-alpha and IL-6.

Oral tolerance studies of the human milk probiotic Lactobacillus fermentum CECT5716.

WCPGHAN 3-World Congress of Pediatric Gastroenterology, Hepatology and Nutrition.
p 163-167. Editors Fagundes-Neto U and Uauay R. Medimond International Proceedings 2008.

Maldonado J, Narbona E, Sempere L, Boza J, Olivares M, Lara-Villoslada F.

It has been suggested that human milk probiotics could be involved in the beneficial effects observed in breast-fed children. Thus, there is an increasing interest in the addition of probiotics to infant formula in order to mimic these functional effects. The objective of the present work is to evaluate the safety of the human milk probiotic Lactobacillus fermentum CECT5716. The administration of this strain to Balb/c mice in a dose 10000 times higher than that normally consumed by humans did not cause any adverse effect and there was no traslocation of the strain from gut to other tissues. In a clinical trial in adult volunteers (22-35y), L. fermentum (10^{10}cfu/day) was well tolerated and no deleterious effects were reported. Finally an infant formula containing L. fermentum ($2x10^{8}$cfu/day) was well tolerated by 6 months old children. Thus, it can be concluded that L. fermentum is safe for human consumption.

Oral intake of Lactobacillus fermentum CECT5716 enhances the effects of influenza vaccination.

Nutrition. 2007 Mar;23(3):254-60.

Olivares M, Díaz-Ropero MP, Sierra S, Lara-Villoslada F, Fonollá J, Navas M, Rodríguez JM, Xaus J.

Objective: We studied the coadjuvant capability of oral consumption of the breast-milk-isolated strain Lactobacillus fermentum (CECT5716) for an anti-influenza vaccine.

Methods: A randomized, double-blinded, placebo-controlled human clinical trial including 50 volunteers (31 male and 19 female) was performed to address the immunologic effects of an intramuscular anti-influenza vaccine in adults (33.0 +/- 7.7 y old). Fifty percent of volunteers received an oral daily dose of methylcellulose (placebo) or probiotic bacteria (1 x 10(10) colony-forming units/d) 2 wk before vaccination and 2 wk after vaccination.

Results: Two weeks after vaccination there was an increase in the proportion of natural killer cells in the probiotic group but not in the placebo group. The vaccination induced an increase in T-helper type 1 cytokine concentrations and in T-helper and T-cytotoxic proportions in both groups; however, the probiotic group showed a significant higher induction in some of these parameters. Regarding the humoral effects, induction of antibody res-ponse in the placebo group could not be detected. In the case of the probiotic group, a significant increase in antigen specific immunoglobulin A was detected. Although an increase in total immunoglobulin M was observed, changes in anti-influenza antigen specific immunoglobulin M were not observed. The incidence of an influenza-like illness during 5 mo after vaccination (October to February) was lower in the group consuming the probiotic bacteria.

Conclusion: Oral administration of the strain *L. fermentum CECT5716* potentates the immunologic response of an anti-influenza vaccine and may provide enhanced systemic protection from infection by increasing the T-helper type 1 response and virus-neutralizing antibodies.

A comparative study of the preventative effects exerted by two probiotics, Lactobacillus reuteri and Lactobacillus fermentum, in the trinitrobenzenesulfonic acid model of rat colitis.

Br J Nutr. 2007 Jan;97(1):96-103.

Peran L, Sierra S, Comalada M, Lara-Villoslada F, Bailón E, Nieto A, Concha A, Olivares M, Zarzuelo A, Xaus J, Gálvez J.

The intestinal anti-inflammatory effects of two probiotics isolated from breast milk, Lactobacillus reuteri and L. fermentum, were evaluated and compared in the trinitrobenzenesulfonic acid (TNBS) model of rat colitis. Colitis was induced in rats by intracolonic administration of 10 mg TNBS dissolved in 50% ethanol (0.25 ml). Either L. reuteri or L. fermentum was daily administered orally (5 x 10(8) colony-forming units suspended in 0.5 ml skimmed milk) to each group of rats (n 10) for 3 weeks, starting 2 weeks before colitis induction. Colonic damage was evaluated histologically and bio-chemically, and the colonic luminal contents were used for bacterial studies and for SCFA production. Both probiotics showed intestinal anti-inflammatory effects in this model of experimental colitis, as

evidenced histologically and by a significant reduction of colonic myeloperoxidase activity (P<0.05). L. fermentum significantly counteracted the colonic glutathione depletion induced by the inflammatory process. In addition, both probiotics lowered colonic TNFalpha levels (P<0.01) and inducible NO synthase expression when compared with non-treated rats; however, the decrease in colonic cyclo-oxygenase-2 expression was only achieved with L. fermentum administration. Finally, the two probiotics induced the growth of Lactobacilli species in comparison with control colitic rats, but the production of SCFA in colonic contents was only increased when L. fermentum was given. In conclusion, L. fermentum can exert beneficial immunomodulatory properties in inflammatory bowel disease, being more effective than L. reuteri, a probiotic with reputed efficacy in promoting beneficial effects on human health.

Lactobacillus fermentum, a probiotic capable to release glutathione, prevents colonic inflammation in the TNBS model of rat colitis.

Int J Colorectal Dis. 2006 Dec;21(8):737-46.

Peran L, Camuesco D, Comalada M, Nieto A, Concha A, Adrio JL, Olivares M, Xaus J, Zarzuelo A, Galvez J.

Background and aims: Inflammatory bowel disease is associated with intestinal oxidative stress. In the present study we test the preventative effect of Lactobacillus fermentum, a probiotic that produces per se glutathione, in the trinitrobenzenesulphonic acid (TNBS) model of rat colitis.

Methods: Colitis was induced in rats by intracolonic administration of 10 mg of TNBS dissolved in 0.25 ml of 50% ethanol. L. fermentum was administered orally (5x10(8) CFU suspended in 0.5 ml of skim milk) to a group of rats for 3 weeks, starting 2 weeks before colitis induction. Colonic damage was evaluated both histologically and biochemically, and the colonic luminal contents were used for bacterial studies as well as for short chain fatty acid (SCFA) production.

Results: L. fermentum treatment resulted in an amelioration of the inflammatory response in colitic rats as evidenced histologically and by a significant reduction of colonic MPO activity (P<0.05). The probiotic partially counteracted the colonic glutathione depletion induced by the inflammatory process. In addition, probiotic-treated colitic rats showed significant lower colonic tumour necrosis factor (TNF) alpha levels (P<0.01) and inducible nitric oxide synthase (iNOS) expression when compared to non-treated rats. Finally, the probiotic induced growth of Lactobacilli species and production of SCFA in colonic contents in comparison with control colitic rats.

Conclusions: Administration of the probiotic L. fermentum facilitates the recovery of the inflamed tissue in the TNBS model of rat colitis, an effect associated with increased levels of glutathione as well as with amelioration of the production of some of the mediators involved in the inflammatory response of the intestine, such as TNFalpha and NO.

Antimicrobial potential of four Lactobacillus strains isolated from breast milk.

J Appl Microbiol. 2006 Jul;101(1):72-9.

Olivares M, Díaz-Ropero MP, Martín R, Rodríguez JM, Xaus J.

Aims: The antimicrobial potential of four lactobacilli (Lactobacillus salivarius CECT5713, Lactobacillus gasseri CECT5714, L. gasseri CECT5715 and Lactobacillus fermentum CECT5716), isolated from fresh human breast milk, was evaluated in this study and compared with Lactobacillus coryniformis CECT5711, a reuterin-producing strain isolated from an artisan goat's cheese.

Methods and results: Agar diffusion tests, competitive adhesion assays and mucin expression assays were carried out in order to value the antibacterial properties of the lactobacilli strains. The antibacterial capability of the strains was tested in vivo by using a murine infection model with Salmonella choleraesuis. The results revealed that all the strains studied, displayed antibacterial properties against pathogenic bacteria. However, the antibacterial potential varied among the lactobacilli tested and, in fact, L. salivarius CECT5713 showed not only the best in vitro antibacterial activity, but also the highest protective effect against a Salmonella strain in the murine infection model.

Conclusion: The four breast-milk lactobacilli, and particularly L. salivarius CECT5713, possess potent antibacterial activities that result in a higher protection against S. choleraesuis CECT4155 in a mouse infection model.

Significance and impact of the study: These results suggest that lactobacilli from breast milk could contribute to an anti-infective protection in neonates and would be excellent candidates for the development of infant probiotic products.

Isolation of commensal bacteria from umbilical cord blood of healthy neonates born by cesarean section.

Curr Microbiol. 2005 Oct;51(4):270-4.

Jiménez E, Fernández L, Marín ML, Martín R, Odriozola JM, Nueno-Palop C, Narbad A, Olivares M, Xaus J, Rodríguez JM.

In a previous study, lactic acid bacteria were isolated from meconium obtained from healthy neonates born by cesarean section. Such a finding suggested that term fetuses are not completely sterile, and that a mother-to-child efflux of commensal bacteria may exist. Therefore, presence of such bacteria in umbilical cord blood of healthy neonates born by elective cesarean section was investigated. The blood samples were submitted to an enrichment step and then in-oculated onto agar plates. All the identified isolates belonged to the genus Enterococcus, Streptococcus, Staphylococcus, or Propionibacterium. Later, a group of pregnant mice were orally inoculated with a genetically labeled E. faecium strain previously isolated from breast milk of a healthy woman. The labeled strain could be isolated and polymerase chain reaction detected from the amniotic fluid of the inoculated animals. In contrast, it could not be detected in the samples obtained from a noninoculated control group.

Probiotic potential of 3 Lactobacilli strains isolated from breast milk.

J Hum Lact. 2005 Feb;21(1):8-17; quiz 18-21, 41.

Martín R, Olivares M, Marín ML, Fernández L, Xaus J, Rodríguez JM.

Breast milk is an important factor in the initiation, development, and composition of the neonatal gut microbiota. In a previous study, the authors isolated lactic acid bacteria from milk of healthy mothers. Since some of the identified isolates belonged to the genus Lactobacillus, the objective of this work was to evaluate the probiotic potential of 2 Lactobacillus gasseri and 1 Lactobacillus fermentum strains. Different assays, including survival to conditions simulating those existing in the gastrointestinal tract, production of antimicrobial compounds, adherence to intestinal cells, production of biogenic amines, degradation of mucin, enzymatic profile, and pattern of antibiotic resistance, were per-formed. Globally, the results showed that the probiotic potential of lactobacilli isolated from milk of healthy mothers is, at least, similar to that of the strains commonly used in commercial probiotic products. This fact, together with the presence of prebiotic substances, indicates that breast milk is a natural synbiotic food.

Human milk is a source of lactic acid bacteria for the infant gut.

J Pediatr. 2003 Dec;143(6):754-8.

Martín R, Langa S, Reviriego C, Jimínez E, Marín ML, Xaus J, Fernández L, Rodríguez JM.

Objectives: To investigate whether human breast milk contains potentially probiotic lactic acid bacteria, and therefore, whether it can be considered a synbiotic food. Study design Lactic acid bacteria were isolated from milk, mammary areola, and breast skin of eight healthy mothers and oral swabs and feces of their respective breastfed infants. Some isolates (178 from each mother and newborn pair) were randomly selected and submitted to randomly amplified polymorphic DNA (RAPD) polymerase chain reaction analysis, and those that displayed identical RAPD patterns were identified by 16S rDNA sequencing.

Results: Within each mother and newborn pair, some rod-shaped lactic acid bacteria isolated from mammary areola, breast milk, and infant oral swabs and feces displayed identical RAPD profiles. All of them, independently from the mother and child pair, were identified as Lactobacillus gasseri. Similarly, among coccoid lactic acid bacteria from these different sources, some shared an identical RAPD pattern and were identified as Enterococcus faecium. In contrast, none of the lactic acid bacteria isolated from breast skin shared RAPD profiles with lactic acid bacteria of the other sources.

Conclusions: Breast-feeding can be a significant source of lactic acid bacteria to the infant gut. Lactic acid bacteria present in milk may have an endogenous origin and may not be the result of contamination from the surrounding breast skin.

7.2. Galacto-oligosaccharides (GOS)

Prebiotic effect of an infant formula supplemented with galacto-oligosaccharides: randomized multicenter trial.

J Am Coll Nutr. 2014; 33(5): 385–393.

Giovannini M, Verduci E, Gregori D, Ballali S, Soldi S, Ghisleni D, Riva E.

Objective: The objective of the study was to investigate the effects of a galacto-oligosaccharides (GOS)-supplemented formula on the intestinal microbiota in healthy term infants, with a specific consideration for gastrointestinal symptoms as colic, stool frequency and consistency, regurgitation.

Methods: This was a randomized, double-blind, controlled, parallel-group clinical trial performed simultaneously by 6 centers in Italy. Three groups were considered: breastfed, formula-fed, and GOS-supplemented formula-fed infants. Formula-fed infants were randomized to receive either the control or the study formula and consume the assigned formula exclusively until the introduction of complementary feeding. The nutritional composition of the 2 formulas were identical, apart from the supplemented GOS (0.4 g/100 mL) in the study formula. Four different types of bacteria were evaluated in order to assess the efficacy of GOS-supplemented formula on infants: Bifidobacterium, Lactobacillus, and Clostridium, Escherichia coli.

Results: A total of 199 breastfed infants and 163 formula-fed infants were recruited. When considering stool frequency and consistency, GOS-supplemented formula presented normal and soft stools in the majority of episodes (89%). In the supplemented group the incidence of colic was lower with respect to the control group. A significantly lower count of Clostridium and a higher count of Bifidobacterium were found when comparing study formula and control formula in infants with colic. In children with colic the ratio between Clostridium count and Bifidobacterium and Lactobacillus count was in favor of the latter two when considering the GOS-supplemented formula group with respect to the control one.

Conclusions: The prebiotic-supplemented formula mimicked the effect of human milk in promoting Bifidobacterium and Lactobacillus growth and in inhibiting Clostridium growth, resulting in a significantly lower presence of colic.

Prebiotic effect during the first year of life in healthy infants fed formula containing GOS as the only prebiotic: a multicentre, randomised, double-blind and placebo-controlled trial.

Eur J Nutr. 2015 Feb;54(1): 89-99.

Sierra C, Bernal MJ, Blasco J, Martínez R, Dalmau J, Ortuño I, Espín B, Vasallo MI, Gil D, Vidal ML, Infante D, Leis R, Maldonado J, Moreno JM, Román E.

Purpose: Currently, there is no consensus concerning the possible beneficial colonic and systemic effects of prebiotic-containing infant formula. This study assesses whether the feeding of a galactooligosaccharides (GOS)-containing infant formula (0.44 g/dl of GOS) and the subsequent feeding of a GOS-containing follow-on formula (0.50 g/dl of GOS) have a prebiotic effect on intestinal microbiota that helps to decrease infections and allergy manifestations in healthy infants during the first year of life.

Methods: A multicentre, randomised, double-blind and placebo-controlled trial was carried out on 365 healthy term infants enrolled before 8 weeks of age and randomly assigned to a formula with or without GOS, until 12 months of age. The incidence of infections and allergy manifestations, the antibiotics prescribed and faecal characteristics were recorded up to 12 months of age, while faecal samples were collected up to 4 months for the measurement of secretory immunoglobulin A, short-chain fatty acids and microbiota.

Results: A prebiotic effect on the faecal analysis was observed at 4 months of life. The GOS group showed a lower faecal pH ($P = 0.019$), a lower decreasing trend in secretory immunoglobulin A ($P = 0.078$), lower butyric acid concentration ($P = 0.040$) and an increase in Bifidobacterium counts ($P = 0.010$). Changes in faecal characteristics involved greater frequency ($P < 0.001$) and softer consistency ($P < 0.05$). The incidence of infections or allergic manifestations during the first year of life was similar in both groups, with no statistical differences ($P > 0.05$).

Conclusions: The feeding of GOS-containing infant formula produced a definite prebiotic effect consisting of changes in faecal composition and microbiota, and in faecal consistency and the frequency of defaecation. No changes in the incidence of infection or allergic manifestation during the first year of life were observed.

Galactooligosaccharides are bifidogenic and safe at weaning: a double-blind randomized multicenter study.

J Pediatr Gastroenterol Nutr. 2009 Jan;48(1):82-8.

Fanaro S, Marten B, Bagna R, Vigi V, Fabris C, Peña-Quintana L, Argüelles F,
Scholz-Ahrens KE, Sawatzki G, Zelenka R, Schrezenmeir J, de Vrese M, Bertino E.

Objectives: The primary objective of this study was to determine the bifidogenic effect of galactooligosaccharides (GOS) in a follow-on formula and the effects on other intestinal bacteria. Secondary objectives were the effects on stool characteristics, growth, and general well-being.

Participants and methods: In a multicenter, double-blind study, 159 healthy infants, formula-fed at enrollment (at 4-6 months), were randomized to an experimental follow-on formula supplemented with 5 g/L (GOS) (77 infants), or to a standard follow-on formula (control, 82 infants). Infants were evaluated at enrollment (study day 1 = sd1), after 6 weeks (study day 2 = sd2), and after an additional 12 weeks (study day 3 = sd3). At each study day, a fresh stool sample for the bacterial counts was collected, and the growth parameters were measured. At sd2, urinary specimens were collected for the evaluation of urinary osmolarity.

Results: At sd2 and sd3, the GOS group had a higher median number (colony-forming units per gram of stool) of bifidobacteria than did the control group (sd2 GOS 9.2 x 10(9) vs control 4.4 x 10(9), P = 0.012); (sd3 GOS 7.2 x 10(9) vs control 2.4 x 10(9), P = 0.027). Other bacteria did not show any significant differences between the 2 groups at all study days. The GOS produced softer stools but had no effect on stool frequency. The urinary osmolarity (mOsm/L) at sd2 was comparable in both groups. Supplementation had no influence on the incidence of gastrointestinal side effects or on the growth of the infants.

Conclusions: These data indicate that the addition of GOS (5 g/L) to a follow-on formula positively influences the bifidobacteria flora and the stool consistency in infants during the supplementation period at weaning. No local or systemic side effects were recorded.

Low level of galactooligosaccharide in infant formula stimulates growth of intestinal Bifidobacteria and Lactobacilli.

World J Gastroenterol. 2008 Nov 14;14(42):6564-8.

Ben XM, Li J, Feng ZT, Shi SY, Lu YD, Chen R, Zhou XY.

Aim: To investigate the effect of a new infant formula supplemented with a low level (0.24 g/100 mL) of galactooligosaccharide (GOS) on intestinal micro-flora (Bifidobacteria, Lactobacilli and E. coli) and fermentation characteristics in term infants, compared with human milk and a standard infant formula without GOS.

Methods: Term infants (n = 371) were approached in this study in three hospitals of China. All infants started breast-feeding. Those who changed to formula-feeding within 4 wk after birth were randomly assigned to one of the two formula groups. Growth and stool characteristics, and side effects that occurred in recruited infants were recorded in a 3-mo follow-up period. Fecal samples were collected from a subpopulation of recruited infants for analysis of intestinal bacteria (culture technique), acetic acid (gas chroma-tography) and pH (indicator strip).

Results: After 3 mo, the intestinal Bifidobacteria, Lactobacilli, acetic acid and stool frequency were significantly increased, and fecal pH was decreased in infants fed with the GOS-formula or human milk, compared with those fed with the formula without GOS. No significant differences were observed between the GOS formula and human milk groups. Supplementation with GOS did not influence the incidence of crying, regurgitation and vomiting.

Conclusion: A low level of GOS (0.24 g/100 mL) in infant formula can improve stool frequency, decrease fecal pH, and stimulate intestinal Bifidobacteria and Lactobacilli as in those fed with human milk.

Supplementation of milk formula with galactooligosaccharides improves intestinal micro-flora and fermentation in term infants.

Chin Med J (Engl). 2004 Jun;117(6):927-31.

Ben XM, Zhou XY, Zhao WH, Yu WL, Pan W, Zhang WL, Wu SM, Van Beusekom CM, Schaafsma A.

Background: Oligosaccharides in human milk may protect infants by improving the intes-tinal micro-flora and fermentation. This study was to investigate effects of infant formula milk consisting of galactooligosaccharide (GOS) on intestinal microbial populations and the fermentation characteristics in term infants in comparison with that of human milk.

Methods: The test formula (Frisolac H, Friesland, Netherland) was supplemented with GOS at a concentration of 0.24 g/dl. Human milk and another formula without oligo-saccharides (Frisolac H, Friesland, Netherland) were used as positive and negative control respectively. Growth, stool characteristics, and side effects of the recruited infants were recorded after 3 and 6 months' follow-up, and the fecal species were collected for the analysis of intestinal micro-flora, short chain fatty acid (SCFA) and pH.

Results: At the end of 3- and 6-month feeding period, intestinal Bifidobacteria and Lacto-bacilli were significantly increased in infants fed with GOS supplemented formula and human milk when compared with infants fed with negative control formula; however, there was no statistically significant difference between GOS supplemented formula and human milk groups. Stool characteristics were influenced by the supplement and main fecal SCFA (acetic), and stool frequency were significantly increased in infants fed with GOS supplemented formula and human milk, while the fecal pH was significantly decreased as compared with that of negative control ($P < 0.05$). Supplementation had no influence on incidence of side effects (including crying, regurgitation and vomiting).

Conclusions: Supplementing infant formula with GOS at a concentration of 0.24 g/dl stimulates the growth of Bifidobacteria and Lactobacilli in the intestine and stool characteristics are similar to in term infants fed with human milk.

7.3. Combination *L. fermentum CECT5716* and GOS

Long-term safety of early consumption of Lactobacillus fermentum CECT5716: A 3-year follow-up of a randomized controlled trial.

Pharmacol Res. 2015 May-Jun;95-96:12-9.

Maldonado-Lobón JA, Gil-Campos M, Maldonado J, López-Huertas E, Flores-Rojas K, Valero AD, Rodríguez-Benítez MV, Bañuelos O, Lara-Villoslada F, Fonollá J, Olivares M.

Lactobacillus fermentum CECT5716 is a probiotic strain originally isolated from human breast milk. Previous clinical studies in infants showed that the early administration of a milk formula containing this probiotic strain was safe and may be useful for the prevention of community-acquired infections. This is a 3-year follow-up study aimed at evaluating the long-term effects produced by the early consumption of an infant formula supplemented with L. fermentum CECT5716 (experimental group, EG) compared with a control formula without the probiotic (control group, CG). The infants included in this follow-up study had previously completed a 5-month randomized double-blind controlled trial (from 1 to 6 months of age), where the safety and tolerance of the probiotic formula was evaluated. The main outcome of the follow-up study was the growth of the children. The secondary outcomes included the incidence of infectious and non-infectious diseases, parameters related with intestinal function and faecal microbiota. At 3 years, the mean values of weight, length and head circumference were similar in children of the EG compared with those of the CG. No differences were observed in the incidence of infectious and non-infectious diseases or disorders related with intestinal function. The pattern of faecal microbiota was also similar between both groups. In conclusion, this 3-year study shows that the early administration of the probiotic of *L. fermentum CECT5716* in an infant formula is safe and it does not produce measurable differences in children compared with a control formula.

Lactobacillus fermentum CECT 5716 is safe and well tolerated in infants of 1-6 months of age: a randomized controlled trial.

Pharmacol Res. 2012 Feb;65(2):231-8.

Gil-Campos M, López MÁ, Rodriguez-Benítez MV, Romero J, Roncero I, Linares MD, Maldonado J, López-Huertas E, Berwind R, Ritzenthaler KL, Navas V, Sierra C, Sempere L, Geerlings A, Maldonado-Lobón JA, Valero AD, Lara-Villoslada F, Olivares M.

The objective of the study was to evaluate the safety and tolerance of an infant formula supplemented with Lactobacillus fermentum CECT5716, a probiotic strain isolated from breast milk, in infants of 1-6 months of age. A randomized double blinded controlled study including healthy infants was conducted. One month aged infants received a prebiotic infant formula supplemented with L. fermentum (experimental group) or the same formula without the probiotic strain (control group) for 5 months. The primary outcome of the study was average daily weight gain between baseline and 4 months of age. Secondary outcomes were other anthropometric data (length and head circumference), formula consumption, and tolerance. Incidence of infections was also recorded by pediatricians. No significant differences in weight gain were observed between both groups, neither at 4 months of age (29.0±7.8 vs 28.9±5.7g/day) nor at 6 months (25.1±6.1 vs 24.7±5.2g/day). There were no statistically significant differences in the consumption of the formulae or symptoms related to the tolerance of the formula. The incidence rate of gastrointestinal infections in infants of the control group was 3 times higher than in the probiotic group (p=0.018). Therefore, consumption of a prebiotic infant formula enriched with the human milk probiotic strain *L. fermentum CECT5716* from 1 to 6 months of life is well tolerated and safe. Furthermore, the consumption of this formula may improve the health of the infants by reducing the incidence of gastrointestinal infections.

Human milk probiotic Lactobacillus fermentum CECT5716 reduces the incidence of gastrointestinal and upper respiratory tract infections in infants.

J Pediatr Gastroenterol Nutr. 2012 Jan;54(1):55-61.

Maldonado J, Cañabate F, Sempere L, Vela F, Sánchez AR, Narbona E, López-Huertas E, Geerlings A, Valero AD, Olivares M, Lara-Villoslada F.

Objectives: The aim of the study was to examine the effects of a follow-on formula con-taining Lactobacillus fermentum CECT5716 (L. fermentum) on the incidence of infections in infants between the ages of 6 and 12 months.

Patients and methods: A randomized double-blinded controlled study including infants at the age of 6 months was conducted. Infants were assigned randomly to either follow-on formula supplemented with L. fermentum plus galactooligosaccharide (experimental group, EG), or the same formula supplemented with only galactooligosaccharide (control group, CG). The main outcome was the incidence of infections for the 6-month duration of the study.

Results: The EG showed a significant 46% reduction in the incidence rate (IR) of gastro-intestinal infections (EG: 0.196±0.51, CG: 0.363±0.53, IR ratio 0.54, 95% confidence interval [CI] 0.307-0.950, P=0.032), 27% reduction in the incidence of upper respiratory tract infections (EG:

0.969±0.96, CG: 1.330±1.23, IR ratio 0.729, 95% CI 0.46-1.38, P=0.026), and 30% reduction in the total number of infections (EG: 1.464±1.15, CG: 2.077±1.59, IR ratio 0.70, 95% CI 0.46-1.38, P=0.003), at the end of the study period compared with CG.

Conclusions: Administration of a follow-on formula with *L. fermentum CECT5716* may be useful for the prevention of community-acquired gastrointestinal and upper respiratory infections.

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List of abbreviations

fig.	figure
AOS	acidic oligosaccharides
CECT	Colección Española de Cultivos Tipo
DNA	deoxyribonucleic acid
EFSA	European Food Safety Authority
ESPGHAN	European Society for Paediatric Gastroenterology, Hepatology and Nutrition
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FOS	fructo-oligosaccharides
GALT	gut-associated lymphatic tissue
GRAS	Generally Recognized As Safe
GOLF	combination of GOS and L. fermentum
GOS	galacto-oligosaccharides
HMOs	human milk oligosaccharides
IgA	immunglubuline A
CFU	colony forming units
SCFAs	short-chain fatty acids
NK cells	natural killer cells
PDX	polydextrose
QPS	Qualified Presumption of Safety
Tab.	table
WHO	World Health Organisation

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