Vitenskapskomiteen for mattrygghet
Norwegian Scientific Committee for Food Safety

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Assessment of infant formula and follow-on formula supplemented with *Lactobacillus fermentum* CECT5716

Opinion of the Panel on (or the Scientific Committee) of the Norwegian Scientific Committee for Food Safety

Assessment of infant formula and follow-on formula supplemented with *Lactobacillus fermentum* CECT5716

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Persons working for VKM, either as appointed members of the Committee or as external experts, do this by virtue of their scientific expertise, not as representatives for their employers. The Civil Services Act instructions on legal competence apply for all work prepared by VKM.

Acknowledgements

The Norwegian Scientific Committee for Food Safety (Vitenskapskomiteen for mattrygghet, VKM) has appointed a working group consisting of both VKM members and external experts to answer the request from the Norwegian Food Safety Authority. The members of the working group are acknowledged for their valuable work on this opinion.

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Assessed by

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Summary

The Norwegian Scientific Commitee for Food Safety (VKM) has appointed a working group of experts to answer a request from the Norwegian Food Safety Authority regarding risk assessment of *Lactobacillus fermentum* CECT5716 in infant formula intended for use from birth (0 month) and in infant formulae and follow-on formulae intended for children after the age of 6 month.

The mandate of this risk assessment was not to evaluate the health claims related to the products as such health claims are assessed by EFSA. However, in the EFSA opinions so far (November 2009), a cause and effect relationship has not been established between the consumption of *L. fermentum* CECT5716 containing products and the claimed effects.

In the assessment of *L. fermentum* CECT5716 by the expert committee in EFSA, the panel considered that the strain is sufficiently characterized.

We are not aware of any data indicating that this strain has been the cause of human disease. Its potential toxicity and ability to translocate has been studied in adult mice after oral administration of doses 10 000 times greater than those normally consumed by humans without observing any bacteremia or translocation to spleen or liver. However, strains of *Lactobacillus* have from time to time been isolated from human blood cultures and may therefore, although seldom, translocate.

L. fermentum CECT5716 is fully susceptible to all antibiotics that are recommended by the Panel on additive and products or substances used in animal feed (FEEDAP) of EFSA and is considered safe with respect to absence of antimicrobial resistance genes. DNA-sequence data do not imply the presence of any gene(s) coding for toxin production, but confirming *in vitro*-studies are missing.

The FBO refers to two randomized clinical studies in *healthy* infants of 1-6months of age with respect to possible safety aspects, concluding that the *L. fermentum* CECT5716 was well tolerated and safe for the groups examined. However, data concerning safety aspects specifically concerned with new-borns, or for immunocompromised infants are lacking. The FBO has submitted some data regarding possible long-term adverse effects of giving the strain daily as a “monoculture” over a prolonged period of time. These data are not sufficient to draw any conclusion regarding long-term safety of the strain.

It is supposed that the early composition of the human gastro-intestinal tract microbiota can have long-lasting functional effects. If that is the case, a daily supply of a “monoculture” of a single, specific strain such as *L. fermentum* CECT 5716, in large quantities over a prolonged period of time to age groups where the intestinal flora is still developing may therefore have unknown, but possible long-lasting adverse effects.

Sammendrag

Vitenskapskomiteen for mattrygghet (VKM) har på oppdrag fra Mattilsynet etablert en ekspertgruppe for å utarbeide en risikovurdering ved bruk av *Lactobacillus fermentum* CECT 5716 som probiotikum i henholdsvis morsmelkerstatning beregnet for nyfødte (0 måneder) og i tilskuddsblanding beregnet for barn fra 6 måneders alderen.

Det har ikke vært vurderingens mandat å evaluere eventuelle helsepåstander relatert til produktene da slike påstander vurderes av EFSA. EFSA har imidlertid så langt (november 2009) konkludert med at det ikke er etablert en årsakssammenheng mellom bruk av produkter som inneholder *L. fermentum* CECT5716 og påståtte helseeffekter.

I en tilsvarende vurdering av et ekspertpanel i EFSA konkluderes det med at stammen *L. fermentum* CECT5716 er tilstrekkelig karakterisert.

VKMs ekspertpanel har ikke funnet data som indikerer at stammen har gitt opphav til infeksjoner hos menneske. Stammens potensielle toksisitet og evne til translokalisasjon har vært undersøkt i dyreforsøk ved bruk av voksne mus og med doser 10.000 ganger høyere enn normalt konsumert av mennesker – uten å påvise bakteriemi eller translokering til milt eller lever. Likevel – enkelte stammer av *Lactobacillus* har fra tid til annen blitt isolert fra humane blodkulturer og kan derfor, om enn sjelden, translokere.

L. fermentum CECT5716 er fullt følsom for alle antibiotika som anbefales av EFSAs «Panel on additive and products or substances used in animal feed (FEEDAP) og kan anses trygg når det gjelder fravær av antibiotika resistensgener. DNA-sekvensdata indikerer at det ikke foreligger gener som koder for toksinproduksjon, men bekreftende *in-vitro*-studier mangler.

Når det gjelder mulige bivirkninger på kort sikt, henviser produsenten til to relativt små randomiserte kliniske studier hos *friske* barn i aldersgruppen 1-6 måneder som konkluderer med at *L. fermentum* CECT5716 ble godt tolerert og var trygg for de undersøkte gruppene. Imidlertid mangler tilsvarende data for nyfødte (fra 0 måneder) og for grupper av *ikke-friske* barn (f. eks. immunkompromitterte). Når det gjelder eventuelle bieffekter på lang sikt, henviser produsenten til et abstrakt av en foreløpig ikke publisert rapport hvor barna i den ene av de ovennevnte studiene er fulgt til 3 årsalderen og hvor den foreløpige konklusjonen er at det ikke er påvist signifikante antropomorfiske eller helseforskjeller etter 3 år. Men utgangspunktet har altså vært *friske* barn som fikk probiotika fra 1 måneders alderen. Dokumentasjonen av sikkerheten for alle grupper av nyfødte (friske og ikke-friske fra 0 måneders alderen) anses derfor for ikke å være adekvat og data fra eldre barn kan ikke ekstrapoleres til denne aldersgruppen.

Det er antatt at den tidligste sammensetningen av den mikrobielle gastro-intestinale floraen kan ha langvarige funksjonelle effekter. Hvis det er tilfelle, kan en daglig tilførsel av en tilnærmet «monokultur» i store mengder av en spesifikk stamme (som *L. fermentum* CECT5716) over en lengre periode til de aldersgruppene hvor den intestinale floraen er i ferd med å etableres, tenkes å ha langvarige, om enn foreløpig ukjente, bieffekter.

Contents

Background

The Norwegian Scientific Committee for Food Safety (VKM) has published several risk- and benefit assessments concerning probiotics in products intended for infants and young children.

The Norwegian Food Safety Authority (NFSA) has, based on these assessments by the VKM, prohibited the sale of a follow-on formula containing probiotics and certain processed cereal-based foods for infants and young children containing probiotics.

The NFSA has received a request from a food business operator (FBO) for an evaluation of two products containing probiotics which they are planning to introduce on the Norwegian market, - an infant formula and a follow-on formula, both supplemented with *Lactobacillus fermentum* CECT5716. The NFSA sent a request (draft sent in December 2012) to the VKM to assess the safety and suitability of the bacterial strain. The assessment should be based on the latest scientific documentation submitted by the FBO. The FBO has provided documentation concerning the bacterial strain, including evidence of the probiotic effect, in addition to documentation on the safety and suitability of the bacterial strain for infants.

The formal request for the assessment of the bacterial strain and the use for infants was sent from the NFSA to the VKM in December 2013. This assessment is requested in order to evaluate whether the requirements of safety and suitability given in the legislation for infant formulae and follow-on formulae and in the legislation for foods for particular nutritional needs are fulfilled.

Terms of reference

Translated from Norwegian terms of reference:

The Norwegian Food Safety Authority has requested the VKM to answer the questions in two steps:

Step 1

1) *Lactobacillus fermentum* CECT5716 in infant formulae:
   a) can *Lactobacillus fermentum* CECT5716 safely be given to infants (from birth)?
   b) are there any contraindications concerning the use of infant formulae supplemented with *Lactobacillus fermentum* CECT5716 (from birth)?

2) *Lactobacillus fermentum* CECT5716 in follow-on formulae:
   a) can *Lactobacillus fermentum* CECT5716 safely be given to infants (from 6 months age)?
   b) are there any contraindications concerning the use of follow-on formulae supplemented with *Lactobacillus fermentum* CECT5716 to infants (from 6 months age)?

Step 2

The term suitability should be evaluated in light of the documentation submitted by the FBO

1) Are infant formulae supplemented with *Lactobacillus fermentum* CECT5716 suitable for infants (from birth)?
2) Are follow-on formulae supplemented with *Lactobacillus fermentum* CECT5716 suitable for infants (from 6 months age)?

Literature

Data sources are articles and reports submitted by the FBO.

The following reports and articles have been provided by the FBO:

- Safety and Efficacy of *L. fermentum* CECT5716: Answer to Questions from VKM
- *L. fermentum* CECT5716 Hipp Infant milk Formula dossier
- Maldonado J, Canabate F, Sempere L, Vela F, Sanchez AR, Narbona E, et al. Human Milk Probiotic *Lactobacillus fermentum* CECT5716 Reduces the Incidence of Gastrointestinal and Upper Respiratory Tract Infections in Infants. J Pediatr Gastroenterol Nutr. 2012; 54(1): 55-61.
- Gil-Campos M LM, Rodriguez-Benitez V, Romera J, Roncero I, Linares D, Maldonado J, et al. *Lactobacillus fermentum* CECT 5716 is safe and well tolerated in infants of 1 to 6 months of age: a randomized controlled trial. Pharmacol Res. 2012 Feb;65(2):231-8

Other relevant background papers used in this assessment are previous opinions on probiotics from VKM:

- The use of probiotics for patients in hospitals. A benefit and risk assessment (Halvorsen *et al.*, 2009).
- Risk assessment on use of *Lactobacillus rhamnosus* (LGG) as an ingredient in infant formula and baby foods (II). VKM 2007.
- Risk assessment on use of *Lactobacillus rhamnosus* (LGG) as an ingredient in infant formula and baby foods. VKM 2005.
- Assessment of benefits and risks of probiotics in processed cereal-based baby foods *Bifidobacterium lactis* Bb12. VKM 2010.
- Nytte- risikovurdering av probiotika i barnemat med fokus på bakterien *Lactobacillus paracasei* ssp. *paracasei* F19

In addition, the following two literature searches were performed in the PUBMED:

1. ("lactobacillus fermentum"[MeSH Terms] OR ("lactobacillus"[All Fields] AND "fermentum"[All Fields]) OR "lactobacillus fermentum"[All Fields]) AND CECT5716[All Fields]

The search returned 10 results.

Relevance screening

The titles of all hits were scanned, and for those that were of potential relevance, the abstracts were also inspected. The relevance screening was performed by the members of the *ad hoc* group, independently. Citations were excluded if they did not relate to the terms of reference. The reference lists in selected citations were scrutinized to identify additional articles or reports, overlooked by the PubMed searches.

2. ("probiotics "[MeSH Terms] AND "gut microbiota "[All Fields]) AND infants [All Fields]

The search returned 114 articles.

Relevance screening

The titles of all hits were scanned, and for those that were of potential relevance, the abstracts were also inspected. The relevance screening was performed by the members of the *ad hoc* group, independently. Citations were excluded if they did not relate to the terms of reference. The reference lists in selected citations were scrutinized to identify additional articles or reports, overlooked by the PubMed searches.

Introduction

There are currently no regulatory guidelines for use of probiotic food in Norway. The strain identification and characterization and safety aspects of *Lactobacillus fermentum* CECT5716 have been evaluated, based on the recommendation from "Guideline for use of probiotics in food" (FAO/WHO 2002) and our previously risk/benefit assessments listed under Section Literature.

In this assessment we have defined newborns as children less than 1 month and infants as children between 1 and 12 months.

This assessment is based on the evaluation of the documentation listed above, under section Literature. The submitted data provided by the FBO give information regarding the probiotic strain, safety and efficacy of their products.

The provided data have been used to evaluate the safety aspects of the probiotic strain *L. fermentum* CECT5716 added to infant formulae and supplements.

It is not in the mandate of this report to evaluate the health claims related to the products as these are assessed by European Food Safety Authority (EFSA). The health claims for *L. fermentum* CECT5716 were evaluated by the EFSA (2010). Notably, the FBO has provided one new randomized clinical trial (Gil-Campos et al. 2012) which was not evaluated by EFSA. The safety aspects of this trial have been evaluated and commented in this current assessment.

Functions of intestinal microbiota of the newborn and infants

The role of human intestinal microbiota during early life stages has been redefined during recent years. It is today considered as being much more important than earlier understood (Collado et al. 2012) and it is now known to have a critical role in the evolution of the intestinal functions and in the overall health-status of the host. The development of the intestinal microflora occurs primarily during infancy, and a distortion in any of the functions of the microbiota could potentially contribute to a wide range of diseases in later life (Vael&Desager 2009).

A normal human intestinal microbiota after the newborn-age is a complex system consisting of a dynamic population of around 500-1000 different microbial species, mainly of bacteria. It is assumed that a fraction (about 25%) of these species is still unknown, and thus not identified. Each individual is estimated to carry at least 160 species at any time (Collado et al. 2012), inhabiting a diversity of environmental niches through the gastrointestinal tract.

The gut microbiota constitutes a critical stimulus for the adequate development and maturation of the immune system, which contributes to reducing infections and aberrant immune response. Exposure to microbes in early life, which largely occurs through the

microbial colonization of the intestine of the **newborn**, has been related to an enhancement of gut barrier function and stimulation of immune development. Intestinal colonization is a substantial antigenic challenge for the newborn and is essential for the maturation of the gut-associated lymphoid tissue and for the regulation of the development of intestinal physiology (Collado et al. 2012).

One of the basic physiological functions of the resident microbiota is a microbial barrier against microbial pathogens. The mechanisms by which the species of the microbiota exert this barrier effect are still largely unknown. There seems to be increasing evidence that lactobacilli and bifidobacteria, which are part of the gastrointestinal microbiota, have antimicrobial activities that participate in the hosts gastrointestinal system of defense (Servin 2004, Olivares et al. 2006).

The development of intestinal microbiota is a complex process. It has traditionally been assumed to start at birth when the infant is exposed to the mothers' microbiota and continues to develop and change during life. At birth the neonate is exposed to a great variety of new microbes and the infant gut becomes gradually colonized by a rapidly diversifying microbiota. The composition of this microbiota depends on several factors, including the maternal microbiota, the diet and lifestyle of the mother, the gestational age, mode of delivery, the diet of the infant, lifestyle and geographical location, possible use of antibiotics, probiotics and prebiotics, or whether the newborn is hospitalized or not. Some publications have reported lactic acid bacteria and bifidobacteria in breast milk (Martin et al. 2003, Martin et al. 2004).

The first microbes to colonize the infant gastro-intestinal tract (GIT) are normally facultative anaerobes such as enterobacteria, coliforms, lactobacilli and streptococci followed on the 2nd-3rd day by anaerobes such as bifidobacteria, bacteroides, and clostridia as the main microbes in the faeces of 1 to 2-week-old newborns (Collado et al. 2012). Bifidobacteria and lactobacilli are Gram-positive lactic acid-producing bacteria which thereafter normally constitute a major part of the microflora in the small intestine.

The microbiota of the infants gut is partly dependent upon the delivery method. Following vaginal delivery, bacteria from the maternal vaginal and intestinal microbiota colonize the infant, whereas following cesarean section, bacteria from the maternal skin surface and from the surrounding environment will colonize the gut of the newborn. Newborns delivered by cesarean section will have a deficiency of strict anaerobes with lower numbers of *E. coli*, *Bacteroides* and bifidobacteria and a higher presence of *Clostridium* species compared with vaginally born infants (Di Mauro et al. 2013). During the early stages of life, the composition of the intestinal microbiota undergoes major modifications, depending on the feeding pattern. Breast-fed infants can have an increased number of *Bifidobacterium* and lactobacilli, whereas formula fed infants can have more enterococci and bacteria belonging to the genus *Enterobacteriaceae* (Di Mauro et al. 2013). However one study concludes that "analysis of infant fecal samples show that the density and distribution of bacterial species are highly variable with no consistent effects of gestational age, delivery mode, diet or probiotic administration, while low bacterial diversity and bacterial overgrowth are commonly associated with necrotizing enterocolitis" (Cilieborg et al. 2012).

The initial bacterial colonization after birth, and its change according to environment, nursing, weaning and drugs, plays a crucial function in the final development of the gut with large shifts in the relative abundances of taxonomic groups. However, the real microbial diversity

and the general composition of the infant gut still remain controversial and insufficiently studied (Di Mauro et al. 2013).

It is considered that a balance of the microbial groups present in the human gut is crucial for maintaining health. When this balance is disturbed, it is assumed that the host-microbe relationship can progress toward a disease state. “Altered intestinal colonization by commensal microorganisms … and reduced microbial diversity has been reported in preterm infants increasing the risk to develop later disease” (Collado et al. 2012). Such diseases may include inflammatory bowel diseases, necrotizing enterocolitis, obesity, various forms of colitis and even autism has been linked to disturbances in human-associated microbiotia. Numerous studies have linked early gut microbiota to the development of atopic diseases and indicated a link between specific bifidobacteria deficiency and increased incidence of atopy. However, no specific microbes have been identified with consistently harmful or protective roles regarding atopic diseases and conflicting results have been obtained regarding the protective role of different *Bifidobacterium* species (Collado et al. 2012).

Possible functions of probiotics

A current level of consensus on probiotic science has been summarized by different international expert panels and bodies, with substantial evidence for beneficial effects and areas with poor or inconsistent effects (Rowland et al. 2010, Braegger et al. 2011, EFSA Panel on Dietetic Products 2014).

The effects of specific probiotic strains are supposed to be mediated by direct interaction with intestinal barrier function or through interactions with immune intestinal cells, especially in the upper part of the gut where probiotics may transiently dominate. Other effects may be mediated indirectly via modulation of gut microbiota, by changing the gut microenvironment, e.g. through bacteriocin production or competitive exclusion. However, “there is a wide range of possible mechanisms which are only just beginning to be unraveled and need further investigations” (many of these mechanisms possibly cannot easily be measured in humans for ethical or feasibility reasons) (Rowland et al. 2010).

There is a general agreement among experts that possible probiotic effects are species and often strain specific. Probiotics can therefore not be properly evaluated as a class but need to be judged on single strain basis.

The intestinal faecal microbiota of the healthy, full-term, vaginally delivered and breast-fed infant is regarded as the “gold standard” for a balanced gut microbiota. However, what exactly constitutes a balanced gut flora has not been defined. Since the intention of probiotic therapy would be to modulate an undefined, but supposedly *unbalanced* indigenous microbiota, the possible effect of deliberately modulating an infant’s gut flora (with probiotic bacteria) should probably be of concern.

In accordance with this, the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) concludes in its “Draft Scientific Opinion on the essential composition of infant and follow-on formula” (2014): “Taking into account the lack of convincing evidence for a benefit of the addition of “probiotics” or “synbiotics” to IF (infant formula) and/or FOF (follow-on formula), the Panel considers that the addition of “probiotics” and/or “synbiotics” to IF or FOF is not necessary”.

Influence of probiotics on the infant gut microbiota

The criteria for selecting articles to evaluate the effects of probiotics on infant's microbiota have been described under Literature search section. Only original studies were included and 10 studies fulfilled our inclusion criteria. A summary of the studies and an evaluation of each study are presented in Appendix I. Data are based on isolation of bacterial species from faeces. Due to great variation in methodology used in these studies (conventional culturing, molecular methods), and also including which probiotic strains and dose, age of the infants, duration of the interventions, delivery of child (vaginal, caesarean), bias caused by different lifestyle of studied populations, diversity of the normal flora of individuals, genetic differences, age and sex, it is difficult to collate the data. One of the limitations of these studies is the use of conventional culturing method. Approximately 60-80% of gut bacteria cannot be cultured using conventional culturing methods and molecular methods like analysis of 16S rRNA are considered more sensitive for quantitative and qualitative analysis of gut microbiota (Panigrahi et al. 2008). This method was not used in all evaluated articles.

The definition of "normal" infant gut microbiota is difficult and almost impossible. The use of probiotics in infants aims to create an intestinal microbiota whose composition is close to that of breastfed term infants. The findings presented in the Appendix I cannot confirm this claim and data are diverging. In some studies the authors concluded that the infant´s microbiota was influenced by probiotics, in other studies not. There is insufficient data in all studies to enable an evaluation of the clinical impacts of various composition of infant´s microbiota, whether dominated by *Lactobacillus*/*Bifidobacterium*, or by *Enterobacteriaceae* /*Clostridium*/ *Bacteriodes*/ *Enterococcus.* We cannot draw any firm conclusion from the data presented in Appendix I, which demonstrates great variation in individual level.

Not all species of *Lactobicillus*/*Bifidobacterium* are necessarily correlated to "healthy" gut. Recent research indicates that "maternal" *Lactobacillus*/*Bifidobacterium* may easily colonize the breastfed infants' gut. Fernandez et al. (2013), proposed that these strains could translocate to the mammary gland through an endogenous route involving maternal dendritic cells (DCs) and macrophages. The *Lactobacillus/Bifidobacterium* presented in breast milk may also contribute to infant digestion through break down of sugars, proteins and other nutrients in breast milk. It can be speculated that these strains are individual-specific based on the composition of the breast milk and may not be used as universal. This may be the reason why commercial probiotic strains are not able to colonize in infants as they are not recovered in faecal samples from children who are been fed with probiotics, after a relatively short time following cessation of consumption.

Hazard identification and characterization

Identification of the bacteria

Lactobacillus fermentum is a Gram-positive, heterofermentative rod-shaped lactic acid bacterium.

The strain *L. fermentum* CECT5716 is marketed as *L. fermentum* heriditum®

L. fermentum CECT5716 is deposited at the Spanish Type Culture Collection (http://www.cect.org/english/). CECT accepts deposits as a restricted-access non-public International Depositary Authority under the Budapest Treaty.

In the assessment of this strain by the expert committee in EFSA, The Panel considers that the food constituent, *Lactobacillus fermentum* CECT5716, which is the subject of the health claims, is sufficiently characterized.

The aspects discussed below are recommended in the guidelines for probiotics (FAO/WHO 2002).

Pathogenic criteria

Translocation

The ability of *L. fermentum* CECT 5716 to translocate from the gut of mice to different tissues was studied by Lara-Villoslada et al. (2009). No bacteraemia was observed in any of the experimental groups. Liver and spleen were aseptically removed and the presence of bacteria was analysed by culture in de Man, Rogosa and Sharpe agar (MRS), a lactic acid bacteria elective medium, and brain heart infusion agar (BHI), a less specific medium. Although translocation of bacteria to liver and spleen was observed, there was no statistically significant difference in the incidence of translocation to liver or spleen between control and treated groups. In addition, colonies found on agar plates were checked by conventional PCR using specific primers and none of the colonies corresponded to the administered strain *L. fermentum* CECT5716.

The experimental study provided by the FBO indicates that *L. fermentum* CECT5716 did not translocate in mice. We are not aware of data which may indicate the translocation of *L. fermentum* CECT5716 in humans.

Platelet aggregation

Platelet aggregation contributes to the pathogenesis of infectious endocarditis and some microorganisms may increase platelet aggregation. So far however, very few studies have been found in which probiotic strains were investigated for their platelet aggregation properties (Halvorsen et al. 2009).

The literature search has not found any data on platelet aggregation by *L. fermentum* CECT5716.

Antimicrobial resistance properties of *L. fermentum* CECT5716

The FBO has submitted a single study (Lara-Villoslada et al. 2009) regarding the antimicrobial susceptibility of *L. fermentum* CECT5716. Minimum Inhibitory Concentration (MICs) of 12 antimicrobial agents was determined by a microdilution method. Nine of the antimicrobial agents tested were those for which EFSA has established microbiological breakpoints (cut-off values) can be used for distinction between *L. fermentum* strains harbouring acquired antimicrobial resistance and susceptible strains (EFSA 2008). These agents included ampicillin, gentamicin, streptomycin, quinpristin/dalfopristin, erythromycin, clindamycin, oxytetracycline, chloramphenicol, kanamycin. MICs of three antimicrobial agents (Linozolid, penicillin G, and fusidic acid) for which tentative cut-off values for *L. fermentum* have been suggested (Klare et al. 2007) were also determined.

L. fermentum CECT5716 was showed to be susceptible to all tested antimicrobial agents.

According to data provided by the FBO, the strain does not harbour plasmid, IS-element(s) and transposon(s) associated with antimicrobial resistance gene(s) (Jimenez et al. 2010).

The sequence data for the *L. fermentum* CECT 5716 genome are available in GenBank/EMBL under accession no. CP002033. In a rapid screening of the sequence data, 54 transposase genes and 4 putative transposase genes were identified in the *L. fermentum* CECT 5716 genome. Transposon is a class of genetic elements that can "jump" to different locations within a genome, either alone or with other genes like antimicrobial resistance genes. Although these elements are frequently called "jumping genes," they are always maintained in an integrated site in the genome. In addition, most transposons eventually become inactive and no longer move.

No antibiotic resistance genes, virulence factors or pathogenic factors were identified in the *L. fermentum* CECT 5716 genome, in association with the transposons or putative transposon genes.

Properties of *L. fermentum* CECT5716 relevant to survival and persistence in the gastrointestinal tract and effect on epithelial cells

Resistance to gastric acidity and bile salts

Following ingestion, the first major potentially inhibitory or fatal hurdle for bacteria in foods is the low pH in the stomach. The pH may be as low as 2 in fasting conditions but higher following ingestion of food. In the duodenum, bile salts are excreted into the lumen and can also exert an antibacterial effect. Bile salts may be inhibitory or destructive to bacterial cells. In vitro testing of tolerance to bile salts is usually carried out by addition of various levels of bile salts to a suitable growth medium (Saarela et al. 2005, Vernazza et al. 2006).

Martin et al. (2005) studied the probiotic potential of several strains, including *L. fermentum* CECT 5716. Survival in a gastrointestinal model, where the bacteria were added in fermented milk to a simulated digestion (both low pH and then bile salts), showed 74% survival of cells. This indicates that the strain could survive to the small intestine.

Adherence to cell lines and human epithelial cells

The ability to adhere to intestinal surfaces is thought to be important for the efficacy of probiotic strains, and is claimed to be one of the main criteria for selecting such strains.

The FBO claims that *L. fermentum* CECT5716 shows "a high rate of adhesion to intestinal cells" (Martin et al. 2005).

Safety aspects

Regarding safety, the following aspects are of great importance:

Antibiotic resistance

The study (Lara-Villoslada et al. 2009), provided by the FBO, showed that *L. fermentum* CECT5716 is susceptible to all tested antimicrobial agents; ampicillin, gentamicin, streptomycin, quinpristin/dalfopristin, erythromycin, clindamycin, oxytetracycline, chloramphenicol, kanamycin, Linozolid, penicillin G, and fusidic acid. Thus, *L. fermentum* CECT5716 fulfils one of the important criterion regarding safety aspects of probiotic strains.

The safety aspect of *L. fermentum* CECT5716 regarding absence of phenotypically antibiotic resistance properties and resistance gene(s) is considered acceptable.

Occurrence of disease

We are not aware of any data which can indicate *L. fermentum* CECT5716 as a cause of disease. However, strains of *Lactobacillus* have been reported to be isolated in blood culture from many patients with bacteraemia (Yazdankhah et al. 2009). In a randomized, placebo-controlled trial, *L. rhamnosus* strain GG was not shown to be effective in reducing the incidence of nosocomial infections. In fact, a statistically *non*significant trend toward an increase in infection was seen (four vs. 11) (Honeycutt et al. 2007). Due to safety concerns regarding the administration of *L. rhamnosus* GG, the study investigators terminated the study.

Mammalian toxin production or hemolytic potential of the probiotic strain

In answer to the question in our documentation checklist, the FBO claims no toxin production or hemolytic potential for *L. fermentum* CECT5716. In the screening of the sequence data (accession no. CP002033), no gene(s) encoding for toxin(s) or hemolysis were identified. However, the FBO has not provided any *in vitro* studies, which may prove the absence of toxin production or haemolytic potential for *L. fermentum* CECT5716. There is probably no reason to believe that *L. fermentum* CECT5716 may produce toxins to mammalian cells or has haemolytic activity.

Possible infectivity of probiotic strain in immunocompromised individuals

The FBO has not provided any data, which can justify the use of *L. fermentum* CECT5716 in immunocompromised individuals, critically ill patients, postoperative and hospitalized patients, neither adults nor children. No contra-indication has been claimed by the FBO for the use of *L. fermentum* CECT5716 in these patient groups.

Due to lack of data, the use of *L. fermentum* CECT5716 in these susceptible groups of patients is not considered acceptable.

Assessment of undesirable short-term side-effects

The FBO has provided the results from two randomized controlled trials in infants, one conducted in the period May 2008-July 2009 (Maldonado et al. 2012) and the other May 2009-September 2010 (Gil-Campos et al. 2012). These studies, seemingly from the same group, are both rather small, including totally 137 and 126 healthy infants of 1-6 months of age, following the children for up to 6 months. New-borns were not included. Both studies concluded that *L. fermentum* CECT5716 was well tolerated and safe for the groups examined. Data regarding safety aspects for new-borns (0-1 month) or for *not-healthy* infants (e.g. critically ill patients, immunocompromised, postoperative or hospitalized patients, preterm) are not presented.

The data from infants aged 1 to 6 month cannot be extrapolated to infants < 1 month. The FBO has applied for marketing authorization for their products for infants from birth (0 month). Thus the short-term side-effects in this age group are not studied and not known.

Assessment of undesirable long-term side-effects

The FBO states that "*L. fermentum* CECT5716 was isolated from human milk (Martin et al. 2003) and as such of human origin. The intake of *L. fermentum* CECT5716, as a natural component of human milk, is the best indicator of its long-term safety". This argument may be contested, as one isolate from one human milk-specimen does not necessarily imply that it is "a natural component of human milk".

The potential toxicity and ability of the strain to translocate has been tested in mice after oral administration of doses 10.000 times higher than those normally consumed by humans (Lara-Villoslada et al. 2009) without observing any bacteraemia or translocation to spleen or liver.

There is a lack of adequate data regarding possible long-term effects of giving the strain daily as a "monoculture" over a prolonged period of time. The FBO has provided the abstract of a hitherto unpublished article where the 126 infants given infant formula supplemented with *L. fermentum* CECT5716 in a previous study (Gil-Campos et al. 2012) were followed for 3 years (Maldonado-Lobon et al. 2014). According to the protocol of this follow-up study, provided by the FBO, the authors had defined the primary response as the children´s weight and growth. The secondary response were defined as; allergies, infections and illness suffered by the child, intestinal microbiota of the child, faecal concentration of short chain fatty acids, faecal concentration of IgA, parameters relating to children´s intestinal function. The abstract provided by the FBO does not give all these data intended to be measured during the follow-up study. At the age 3 years, anthropometric data of 107 children were collected and related to the health of the children. There were no differences in weight values although greater length was observed in children of probiotic group according to the difference already observed at 6 months of age. The authors concluded further that "No significant differences in the incidence of metabolic diseases, allergy or infectious diseases were observed" and that the data therefore support the long-term safety of consumption of infant formula supplemented with *L. fermentum* CECT5716 during the first months of life.

The provided data regarding long-term safety is only an abstract and this is therefore not sufficient for evaluation. In any case, the study includes only healthy children who have got the probiotic-supplemented infant formula only after the age of 1 month. It does therefore not include newborns or special groups of not-healthy children. The aspect of long-term safety may therefore be regarded as inadequately documented.

Influence of *L. fermentum* CECT 5716 on infant gut microbiota

According to the follow-up study protocol, the authors had planned to identify the long-term effect of *L. fermentum* CECT 5716 on infant´s microbiota, but this has not been provided by the FBO. Therefore, this issue cannot be assessed further. The need for data on long-term effects is discussed in "Assessment of undesirable long-term side-effects".

The data presented on Appendix I, for other probiotic strains cannot be extrapolated to *L. fermentum* CECT 5716, since probiotic effect is strain-specific.

Exposure assessment

Information supplied by FBO and label on the product show that the infant formula contains log 7 cfu g^{-1} powder. According to the manufacturer's instructions, for a 2 month infant of 5kg, 5 scoops (1 scoop = 4.3g powder) of powder should be added to 150g (ml) water. The number of viable cells of *L. fermentum* CECT 5716 in the ready to eat serving would then be log 8.33 cfu. With five servings per day, the total number of cells ingested per day would be approximately log 9.

There is a lack of dose-response studies as a basis for supplementation of the amount *L. fermentum* CECT 5716 to infant formula.

As a comparison, a 100g serving of commercial probiotic yoghurt would contain approximately log 9-10 cfu.

Studies of the microflora of human breast milk are sparse. Albesharat et al. (2011) reported lactic acid bacteria in numbers up to log 3 cfu (assume per mL) in the breast milk of lactating Syrian women. Zacarias et al. (2011) reported the presence of bifidobacteria (up to log 2,8 cfu mL^{-1}) in 6 of 22 women in Argentina. Solis et al. (2010) isolated *Streptococcus*, *Enterococcus*, *Bifidobacterium* and *Lactobacillus* in numbers as high as log 5 cfu mL^{-1} in breast milk from lactating women in Spain and also found congruity with the infant´s faecal flora. Fernandez et al. (2013), in a review article, extrapolated results of several authors and estimated that an infant would consume between log 5 and log 7 bacteria daily along with the consumption of 800 ml breast milk. Thus the amount of cells consumed by an infant of *L. fermentum CECT 5716* according to the manufacturer's instructions would to be considerably higher than natural milk levels, in fact up to 10 000 x greater (difference between log 5 and log 9).

Risk characterization

For characterization of risk related to the consumption of *L. fermentum* CECT5716, we have evaluated the following aspects; antibiotic resistance property, occurrence of disease, mammalian toxin production or haemolytic potential of the probiotic strain, possible infectivity of probiotic strain in immunocompromised individuals, assessment of undesirable side-effects (short-term, and long-term).

The FBO has provided no data regarding safety aspects for newborns (0- 1 month), since newborns were not included in the clinical trials. Due to lack of data for children on this age group, the recommendation for use of *L. fermentum* CECT5716 is not acceptable and data from older children cannot be extrapolated to this age group.

The safety aspect of *L. fermentum* CECT5716 regarding absence of acquired antibiotic resistance and resistance genes is considered acceptable. We are not aware of any data indicating that the consumption of this strain has caused human diseases. However, *Lactobacillus* as a genus has, from time to time, been isolated from human blood cultures and may therefore, although seldom, translocate. In the screening of the sequence data no gene(s) encoding for toxin(s) or haemolysins were identified. Theoretically, toxin production should not pose any problem with this strain.

The FBO has not provided any data to support the safe use of *L. fermentum* CECT5716 in immunocompromised individuals, critically ill patients, postoperative and hospitalized patients, neither in adults nor in children. The FBO has not indicated that the *L. fermentum* CECT5716 may be used in these groups of children or whether it is contra-indicated. The potential pathogenicity of *Lactobacillus* is also listed in (Halvorsen et al. 2011).

The FBO has not provided any data regarding effect of *L. fermentum* CECT 5716 on the composition of the infant microbiota, although according the protocol of the follow-up study provided by the FBO, these data may have been collected.

The data provided by the applicant show no short-terms side effect in the submitted clinical studies, however, the long-term safety data is lacking. A daily supply of a "monoculture" of a particular strain in large quantities over a prolonged period of time to an age group lacking an established intestinal flora may have unknown adverse effects. The early microbial

composition of the human gastro-intestinal tract has long-lasting functional effects. If the supply of a "monoculture" leads to an abnormal colonization of the infant gut, the result may be that the postnatal immune system development is affected, that the postnatal maturation of epithelial cell barrier functions is delayed or it can lead to mucosal inflammation that plays a pivotal function in the development of feeding intolerance (Di Mauro et al. 2013). It is not known at what point in life an early change of the GIT microbiota could have a negative effect.

The long-term effects of infant formulas supplemented with probiotic strains have already been discussed for other products supplemented with other probiotic strain (Halvorsen et al. 2011). From birth to 24 months, and especially after weaning, 500-1000 bacterial species are normally established in the intestinal tract. This individual intestinal microbiota is continuously influencing the host and the host's immune system, establishing physiological functions and defense mechanisms. The immaturity and vulnerability of the intestinal microbiota and the immune system makes the two lowest age groups at the highest risk of unwanted health effects of the daily intake of probiotics. A daily intake of probiotics may have negative effect on establishment of intestinal bacterial flora and development of intestinal functions and mucosal immune system in all of the three different age groups. Possible long-term effects of a yearlong monocultural supply in these age groups, have to the best of our knowledge, not been evaluated by the FBO. Our assessment of exposure shows that the amount of cells of *L. fermentum* CECT 5716 consumed by an infant according to the manufacturer's instructions would to be considerably higher than natural levels possibly present in breast milk, in fact up to 10 000 x greater (difference between 10^5 and 10^9).

Due to many uncertainties and lack of knowledge discussed above, the safety of long term use of *L. fermentum* CECT 5716 has not been adequately proved.

The addition of probiotics in infant formula raises several ethical dilemmas where the risks and benefits have to be considered together. For instance, is it acceptable to:

- Apply a potential health-promoting treatment to premature babies when long term negative effects cannot be excluded.
- Not apply a potential health-promoting treatment due to fear of side effects which may be overestimated.
- Carry out tests on babies, both premature and infants, who have no option to choose themselves, when long term side effects cannot be excluded.

The dilemma is how great a risk can be accepted compared to the potential benefits. In life-saving treatments, the acceptable risk is relatively high, as the benefit is potentially high. However the accepted risk is lower when a product is for normal use. Even clinical testing of babies can be questioned which would result in no products of this kind considered safe due to lack of evidence.

Data gaps

- Influence of probiotics on infant gut microbiota
- The metabolic activity of *L. fermentum* CECT5716 on nutrients supplemented to the infant formula
- Dose-response study
- Lack of adequate long-term studies

- Lack of studies for newborns (0-1 month)
- Lack of data on use in immunocompromised children
- Lack of in-depth studies on the microbiology of human breast milk

Answer to the questions

The Norwegian Food Safety Authority has requested the VKM to answer the questions in two steps:

Step 1

1) *Lactobacillus fermentum* CECT5716 in infant formulae:

   a) can *Lactobacillus fermentum* CECT5716 safely be given to infants (from birth)?

The species *L. fermentum* is included in the EFSA-list of bacteria that are presumed to be safe (QPS – qualified presumption of safety). The specific strain *L. fermentum* CECT 5716 has been isolated from normal human milk. It is fully susceptible to all antibiotics recommended by the Panel on additive and products or substances used in animal feed of EFSA and is considered safe regarding absence of antimicrobial resistance genes. We are not aware of any data indicating that this strain has been the cause of human disease. There is therefore no evidence leading to consider the strain *L. fermentum* CECT 5716 as unsafe.

However, there are no systematic studies where this strain has been given as a probiotic supplement to infant formula intended for newborns less than 1 month of age. As the effect and safety of probiotics are considered to be not only species- but also strain-specific, the adequate data for this strain used for this age-group are lacking. Thus the safety for the age group 0-1 months cannot be established.

Two clinical studies conclude that the strain *L. fermentum* CECT 5716 is safe for healthy infants 1-6 months of age.

   b) are there any contraindications concerning the use of infant formulae supplemented with *Lactobacillus fermentum* CECT5716 (from birth)?

There are no specific contraindications other than those following the above-mentioned lack of adequate data concerning effect and safety for this specific strain given to this age-group. In addition, the general considerations mentioned below (point 2) will also apply for this age-group.

Due to lack of data on use in immunocompromised children the use of *L. fermentum* CECT 5716 in these children may be contraindicated.

2) *Lactobacillus fermentum* CECT5716 in follow-on formulae:

   a) can *Lactobacillus fermentum* CECT5716 safely be given to infants (from 6 months age)?

Short-term safety according to the limited number of studies provided by FBO can be considered acceptable. However, the long-term safety for this age group cannot be established.

b) are there any contraindications by use of follow-on formulae supplemented with *Lactobacillus fermentum* CECT5716 to infants (from 6 months age)?

No specific contraindications for use of this probiotic have been mentioned in the assessed literature, but this aspect has apparently also not been considered as only healthy children have been included. Data concerning groups such as preterm children, immunocompromised or otherwise seriously ill children have not been included.

However, especially for the youngest age-groups who still have an immature intestinal microbiota, important general questions remain concerning possible long-term effects and safety of probiotics remains.

Step 2

The term suitability should be evaluated in light of the documentation submitted by the FBO

1) Is infant formulae supplemented with *Lactobacillus fermentum* CECT5716 suitable for infants (from birth)?

2) Is follow-on formulae supplemented with *Lactobacillus fermentum* CECT5716 suitable for infants (from 6 months age)?

As the use of the infant formulae and follow-on formulae supplemented with *Lactobacillus fermentum* CECT5716 was not assessed safe for the relevant age groups VKM concludes that the product is not suitable the same groups and the Panel on Nutrition, Dietetic Products, Novel Food and Allergy (FG7) will not assess the issue further.

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Annex 1

Table 1 - Long term effect of various probiotic strains on gut microbiota in infants

Reference	(Agarwal et al. 2003)
Study design	Prospective, randomized study
No. of subjects	71 preterm infants of less than 2000 g birth weight. Infants less than 1500 g (24 treated, 15 control) received 10(9) LGG orally twice daily for 21 days.
Probiotic(s) strain(s)	*Lactobacillus rhamnosus* GG
Outcome measures	Colonization of Lactobacillus GG(LGG) on the neonatal gut and modification of microbial ecology
Results	Colonization with LGG occurred in 5 of 24 (21%) infants who weighed less than 1500 g versus 11 of 23 (47%) in larger infants. Colonization was limited to infants who were not on antibiotics within 7 days of treatment with LGG. Treatment in infants weighing less than 1500 g resulted in a significant increase in species number by day 7, with further increases by day 21. This increase was mainly the result of increased Gram (+) and anaerobic species. No difference in species number was noted in controls. Mean log CFU of Gram (-) bacteria did not change in treated infants weighing less than 1500 g. However, Gram (+) mean log CFU showed a significant increase on day 21 (6.1 +/- 0.9) compared with day 0 (3.5 +/- 0.9) ($P < 0.05$). No significant changes in species number or quantitative counts were noted after LGG treatment in the infants weighing 1500 to 1999 g LGG was well tolerated in all infants.
Conclusion	LGG is a relatively poor colonizer in infants especially those infants weighing less than 1500 g at birth, it does appear to affect neonatal intestinal colonization patterns.
Evaluation	The divergence in results showed that ”colonization” or ”no-colonization” of LGG in infants gut seemed to be influenced of multifactorial effects in the highly complex intestinal milieu and gut microbiota cannot be controlled only by supplementation of probiotic to the milk/formula milk.

Reference	(Chrzanowska-Liszewska et al. 2012)
Study design	Randomized study; children received probiotics or placebo within 0-3days after birth
No. of subjects	60 patients were initially identified and enrolled but after exclusion criteria were applied, 21 babies were analyzed in the probiotic group and 26 in the placebo group.
Probiotic(s) strain(s)	*Lactobacillus rhamnosus* GG
Outcome measures	Presence of LGG colonization, pathogenic bacteria, somatic growth and length of hospital stay
Results	The number of *Lactobacillus* were significantly higher (p=0.014) on day 7, and 21 (p=0.024) in the study group, and so was the number of *Enterobacteriaceae* on all study days (p=0.004, p=0.000, p=0.000), and *Enterococcus* sp on day 21 (p=0.000). The amount of samples positive for staphylococci was significantly higher in the study group, on days 7 and 42 (p=0.001 and 0.011). We did not show a significant difference in weight gain upon discharge between the groups p=0.567, 95% CI (-168; 305) or mean of hospital stay p=0.421 95% CI (-13.43;5.71).
Conclusion	A preterm infant formula with an addition of probiotics leads to a rapid growth of LGG in the gut of bottle fed infants, but does not decrease the amount of pathogenic organisms, nor increase weight gain during enteral feeding, or decrease length of hospital stay
Evaluation of the study	The authors confirm that the number of pathogenic bacteria is higher in probiotic group than the control group. The reason for this finding has not been discussed.

Reference	(Cox et al. 2010)
Study design	Double blind randomized. Infant feces. Given Log 9 cfu LGG per day, from birth to 6 month old.
No. of subjects	Sixteen 6-month old infants
Probiotic(s) strain(s)	*L. casei* subsp. *rhamnosus* LGG
Outcome measures	DNA from feces -> PCR -> Micro array
Results	Found 1988 taxa (much more diverse than previously reported, method will detect as low as 0.01% of the bacterial community. Per individual 950 – 1333 taxa. Abundance of LGG in samples varied greatly, and was associated with specific increases in other lactobacilli and bifidobacteria (i.e. related).
Conclusion	Increased info about the way that priobitics ”work”. Caused ”dramatic” changes in GIT microbiota (despite *). Promotion of a diverse probiotic flora can be key factor in protection against allergic disease development.
Evaluation	This is an interesting study by using molecular methods for studying the effects of probiotics on infants’ microbiota. However, the number of samples is too few to draw any firm conclusion.

Reference	(Ismail et al. 2012)
Study design	Studied 7-day fecal samples of infants who were deemed high risk for allergic disease. Part of a greater study which was randomized, double blind, placebo-controlled. Probiotics given to mother prenatal.
No. of subjects	98 infants at high risk of allergic disease, whose mothers participated in a pre-natal probiotic eczema prevention study.
Probiotic(s) strain(s)	LGG as tablet/capsule
Outcome measures	T-RFLP analysis of 16S rRNA to assess microbial diversity of day-7 gut microbiota.
Results	Prenatal LGG did not affect microbial diversity. (Previous study showed that LGG was bifidogenic). Also no influence on development of eczema.
Conclusion	Prenatal LGG admin does not affect postnatal flora in infants
Evaluation	Only day-7 microbiota were analysed, although the authors collected faecal samples on day 3, 7, 28, 90 and 180 of life. Data regarding changes in the infants microbiota during of the whole period, in particular in the late period (day 28, 90 and 180) are lacking. There is also lack of data regarding the mode of birth delivery; vaginal vs cesearen.

Reference	(Kirjavainen et al. 2002)
Study design	This randomised study included 21 infants with early onset atopic eczema of whom eight were intolerant (highly sensitised group (HSG)) and 13 tolerant (sensitised group (SG)) to extensively hydrolysed whey formula (EHF). In the SG, six were weaned to EHF without (placebo group (PG)) and seven to EHF with *Bifidobacterium lactis* Bb-12 supplementation (bifidobacteria treated group (BbG)).
No. of subjects	21 infants
Probiotic(s) strain(s)	*Bifidobacterium lactis* Bb-12
Outcome measures	The faecal microflora of infants in the HSG was analysed only before weaning whereas in the SG the faecal microflora was analysed both before and after weaning.
Results	Infants in the HSG had greater numbers of lactobacilli/enterococci than those in the SG. Serum total IgE concentration correlated directly with *Escherichia coli* counts in all infants and with *Bacteroides* counts in the HSG, indicating that the presence of these bacteria is associated with the extent of atopic sensitisation. The effect of supplementation was characterised as a decrease in the numbers of *Escherichia coli* and protection against an increase in *Bacteroides* numbers during weaning.
Conclusion	These data indicate that bifidobacterial supplementation appears to modify the gut microbiota in a manner that may alleviate allergic inflammation. Further studies are needed to confirm this conclusion.
Evaluation of the study	There were too few objects in this study to draw any firm conclusion and the authors have not discussed why the high number of *E. coli* should be associated the extent sensitation in infants. No conclusion can be taken regarding effect of probiotis on gut microbiota when compared with control group.

Reference	(Klewicka et al. 2011)
Study design	Double-blind, randomized, placebo-controlled.
No. of subjects	40 children, aged 6-18 months and suffering from atopic dermatitis before and after 3 month.
Probiotic(s) strain(s)	*Lactobacillus casei* DN—114001
Outcome measures	*Lactobacillus* sp., *Bifidobacterium, Clostridium* sp. *Bacteroides* sp., *Enterococcus* sp. and *Enterobacteriaceae*.
Results	Control group: The total number of fecal *Lactobacillus* sp. decreased from 7.86 Log10 CFU/g to 6.40 Log10 CFU/g. After the next 5 months (without dietary supplementation with the probiotic bacteria) the level of *Lactobacillus* sp. was maintained at the latter value. Probiotic group: During the dietary supplementation with the probiotic strain, the level of *Bifidobacterium* was maintained at 6.15-6.89 Log10 CFU/g while after 5 months it decreased to 5.57 Log10 CFU/g. The population of *Clostridium* sp. was reduced after 3 months of dietary supplementation from 6.49 to 5.83 Log10 CFU/g and was maintained at the latter level during the next 5 months. The dietary supplementation had no effect on populations of *Bacteroides* sp., *Enterococcus* sp. and *Enterobacteriaceae*.
Conclusion	Supplementation of children, with atopic dermatitis, with the preparation of *Lactobacillus casei* DN-114001 positively affected their gut microbiota in terms of *Bifidobacteria* and *Clostridium* populations.
Evaluation of the study	The number of children included in the control and test groups are low to conclude any firm conclusions. Furthermore, there is high range in bacterial cells both in control and test group. The decrease or increase of different bacterial species does not seem to be significant. It is not clear how "*Lactobacillus casei* DN-114001 positively affected their gut microbiota in terms of *Bifidobacteria* and *Clostridium* populations".

Reference	(Loo et al. 2014)
Study design	Follow-up study, analysis of the charts and electronic databases of the PROMPT (Probiotics in Milk for the Prevention of Atopy Trial) study cohort.
No. of subjects	253 infants at risk for allergy who were administered cow's milk supplemented with or without probiotics from the first day of life to the age of 6 months.
Probiotic(s) strain(s)	Different probiotic strains
Outcome measures	The cohort was followed up until the children were 5 years old and clinical outcomes were assessed.
Results	Of the 253 children recruited into the study, 220 (87%) completed the follow-up. At the age of 5 years, there were no significant differences between the groups in the proportion of children who had developed any asthma, allergic rhinitis, eczema, food allergy and sensitization to inhalant allergens.
Conclusion	The supplementation of probiotics in early childhood did not play a role in the prevention of allergic diseases. Clinical/Key Message: Early-life supplementation with probiotics did not change allergic outcomes at 5 years of age.
Evaluation of the study	No information is available regarding effect of probiotics on children´s microbiota. It seems, no matter how the probiotics may influence the gut microbiota, there is no effect on health.

Reference	(Panigrahi et al. 2008)
Study design	Randomized, double-masked, controlled trial
No. of subjects	31 infants; 19 infants in supplements and 12 in placebo group.
Probiotic(s) strain(s)	*Lactobacillus plantarum*
Outcome measures	Colonization of *Lactobacillus plantarum* and other bacterial species
Results	The number of bacterial species was significantly higher on days 21 and 28 in the synbiotic preparation group compared with placebo (P = 0.002 and 0.03, respectively). There was a linear increase in the mean log gram-negative colony counts in the placebo group during the 4-week period that was significantly higher than that in the Lactobacillus group on days 14, 21, and 28 (P < 0.001 for each). In contrast, the supplement group had significantly higher gram-positive colony counts on days 14 (P = 0.002) and 28 (P = 0.04).
Conclusion	There was an increase in bacterial diversity and gram-positive organisms and a reduction of gram-negative bacterial load in the treatment group.
Evaluation	Infants received *Lactobacillus plantarum* and fructooligosaccharides in supplemented group or a dextrose saline placebo. Because a combination preparation was used, it is difficult to specifically attribute the colonization to either the probiotic or prebiotic component in this study, which is not large enough to draw any firm conclusion. The authors have not discussed the biological impact of increase in Gram-negative bacteria in control group and increase of Gram-positive bacteria in supplemented group.

Reference	(Rinne et al. 2005)
Study design	Double blind, placebo-controlled, randomized. Mothers received LGG 4 weeks before expected delivery. Then given to infants for 6 month. Breastfeeding encouraged up to 4 to 6 month.
No. of subjects	96 infants with relative with atopic dermatitis, allergic rhinitis or asthma.
Probiotic(s) strain(s)	LGG
Outcome measures	Fecal *Bifidobacterium* and *Lactobacillus/Enterococcus* counts were higher in breastfed than formula-fed infants at 6 months. Bacterial counts in feces of lactobacilli, enterococci, bifidobacteria assessed by FISH (Fluorescent in situ hybridisation). Also measured circulating Ig(G, A and M) +/- in colostrum at 3, 6 and 12 month.
Results	Levels of all Ig greater in infants given LGG. Greater bifidobacteria in breastfed infants (but not huge differences), effect gone by 12 month.
Conclusion	Probiotics in mothers diet before delivery may promote maturation of the gut immunity.
Evaluation	The authors have not reported the effects of LGG on the composition of infant´s microbiota and the clinical relevance of greater Ig in infants given LGG has not been discussed.

Reference	(Rinne et al. 2006)
Study design	Randomized, double blind, placebo controlled. The treatments of mothers/infants continued for 6 months postnatal.
No. of subjects	32 newborns whose mothers were randomized to receive placebo or *Lactobacillus rhamnosus* GG before delivery.
Probiotic(s) strain(s)	*L. rhamnosus* LGG
Outcome measures	*Bifidobacterium, Lactobacillus/Enterococcus, Bacteroides* and *Clostridium* counts in fecal samples at 6, 12, 18 and 24 months of age, by using fluorescent *in situ* hybridization (FISH).
Results	At 6 months, there were less clostridia in faeces in the placebo compared with the probiotic group (P = 0.026), whereas after long-term follow-up at 2 years, there were less lactobacilli/enterococci and clostridia in faeces in the probiotic group than in the placebo group (P = 0.011 and P = 0.032, respectively).
Conclusion	Administration of LGG in the first months of life was well tolerated and did not significantly interfere with long-term composition or quantity of gut microbiota.
Evaluation of the study	The fluorescent in situ hybridization (FISH) method for the evaluation of the gut microbiota is not optimal. For quantitative and qualitative assessment of infant's microbiota, not only fecal microbiota, but also microbiota in the other parts of gastrointestinal tract is important. Fecal samples do not give direct indication of the mucosal microbiota composition. Furthermore, FISH method recognizes only the number of bacteria is $>10^7$ per gram feces. There is no difference in the composition of infant microbiota in the test group compared to control group, neither in the short-term, nor in the long-term.