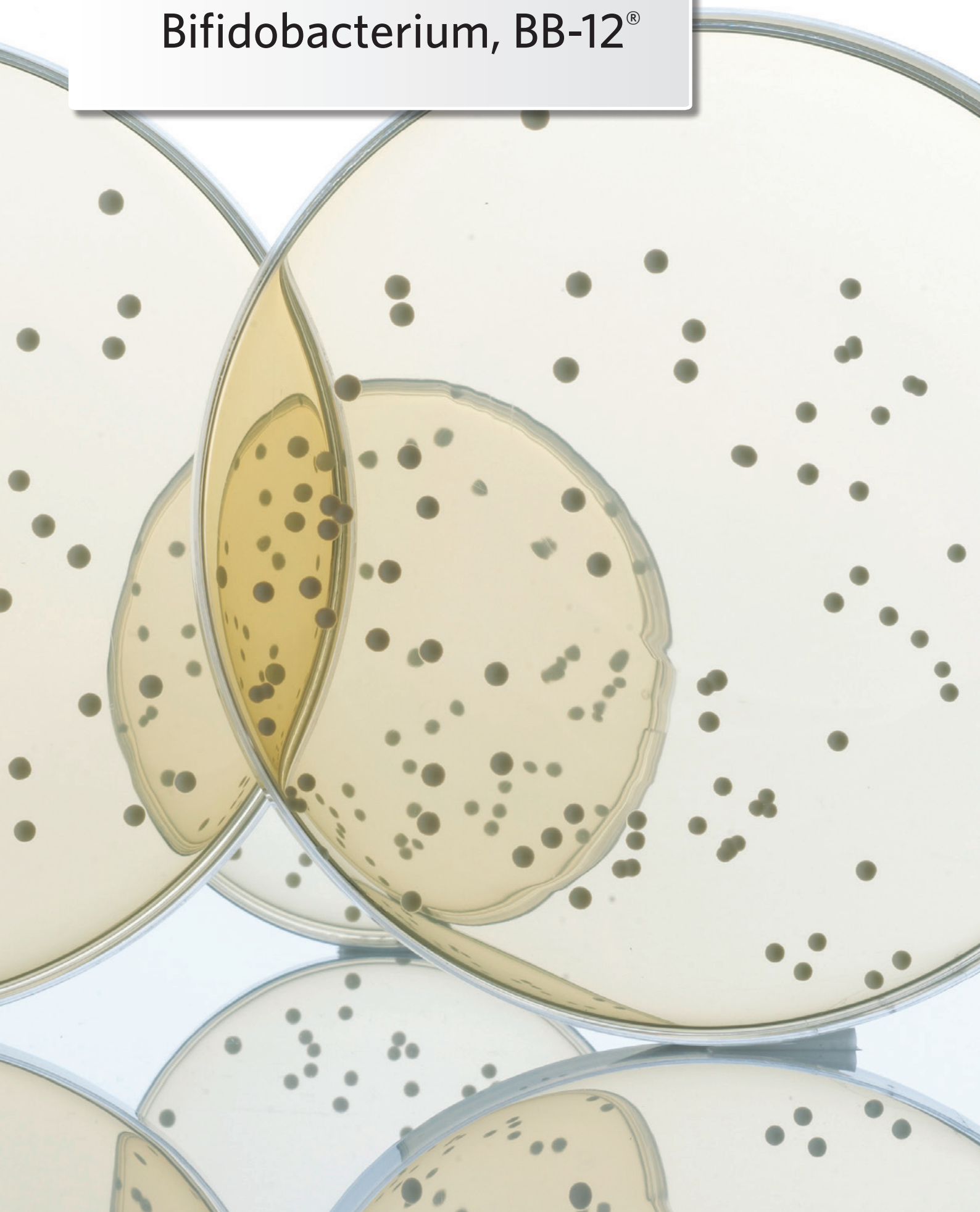
CHR. HANSEN

Improving food & health

The Science behind Bifidobacterium, BB-12®



Editor
Mikkel Jungersen MSc,
Scientific Advisor,
HHN-Scientific Marketing

Published by
Department of Scientific Marketing
Human Health & Nutrition
Chr. Hansen A/S
Bøge Allé 10-12
DK-2970 Hørsholm
Denmark

Cover photo: Petri dishes

Abbreviations
CED Cultures & Enzymes Division
HHN Human Health & Nutrition

Contents

Summary

Leading the Way
Bifidobacterium animalis subsp. *lactis*, BB-12® (hereafter referred to by use of the trademark BB-12®) is the world's best documented probiotic *Bifidobacterium*. It is described in more than 370 scientific publications out of which more than 170 are publications of human clinical studies. BB-12® has proven its beneficial health effect in numerous clinical studies within gastrointestinal health and immune function.

A Proven Solution
Clinical studies have demonstrated survival of BB-12® through the gastrointestinal tract and BB-12® has been shown to support a healthy gastrointestinal microbiota. Furthermore, BB-12® has been shown to improve bowel function, to have a protective effect against diarrhea, and to reduce side effects of antibiotic treatment, such as antibiotic-associated diarrhea. In terms of immune function, clinical studies have shown that BB-12® increases the body's resistance to common respiratory infections as well as reduce the incidence of acute respiratory tract infections.

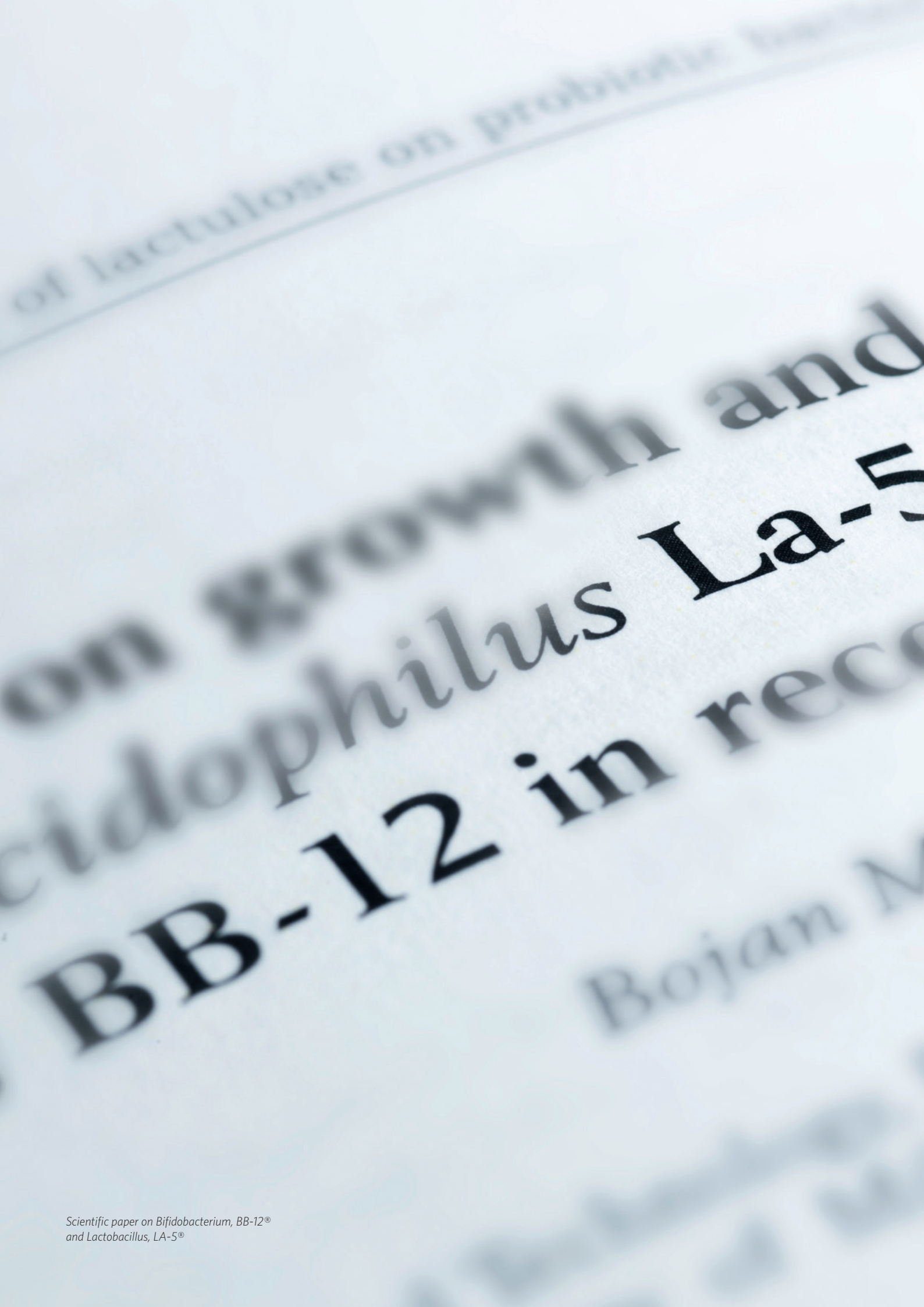
Backed by Science
Strain characteristics and mechanisms of BB-12® have been established through extensive in vitro testing. BB-12® exhibits excellent acid bile tolerance, it contains bile salt hydrolase, and has strong adherence properties, all valuable probiotic characteristics. Pathogen inhibition, barrier function enhancement, and immune interactions are mechanisms that all have been demonstrated for BB-12®.

Following current taxonomy, BB-12® is identified as *Bifidobacterium animalis* subsp. *lactis*. The complete genome sequence of BB-12® has been mapped and published. BB-12® originates from Chr. Hansen's collection of dairy cultures. BB-12® has high stability in foods and as freeze dried powders.

BB-12® is considered safe for its intended use as a dietary ingredient in food and dietary supplements to be consumed by a healthy population including term newborn infants.

Frozen culture.

Test tubes for in-vitro testing.



*Scientific paper on Bifidobacterium, BB-12®
and Lactobacillus, LA-5®*

Introduction

The Microbiota
The human body consists of approximately 37 trillion cells encoded by 23,000 human genes. We are, however, outnumbered by the human microbiome – the bacteria living on and in us. The human microbiome is made up of more than 1500 different species and counts around 100 trillion cells encoded by 3.3 million different non-human genes. Not surprisingly, the human microbiome plays a major role in human health through intimate interaction with our human body. The bacteria living in the intestine – the gastrointestinal microbiota – constitute the largest part of the human microbiome.

Scientific research on the gastrointestinal microbiota as well as of probiotics has increased significantly in the new millennium. The interaction between the gastrointestinal microbiota and probiotics – beneficial bacteria - has gained much awareness. Clinical research has shown that probiotics play a role within various health areas, of which the two main research areas are gastrointestinal health and immune function.

True Probiotics
Probiotics is derived from Greek and means 'for life' as opposed to antibiotics which means 'against life'. Probiotics are defined as '***live microorganisms*** *that, when* ***administered in adequate amounts****, confer a* ***health benefit*** *on the host*' (Hill et al. 2014). From this definition it is evident that a true probiotic requires that some prerequisites must be fulfilled.

First of all, probiotics need to be alive at the time of ingestion and they must be microorganisms. At present, most probiotic organisms are bacteria, belonging to the *Lactobacillus* and *Bifidobacterium* genera.

Secondly, they need to be ingested in a dosage high enough to cause an effect. The recommended, efficacious dosage is closely linked to the clinical documentation, on which it must be based.

Thirdly, the ingested live microorganisms need to pose a beneficial effect on the host in order to be a probiotic. It is important to note, that the beneficial effect of the various probiotics mainly is strain specific and cannot be regarded as general for different species of probiotics.

Strain Level
The consensus of strain specificity is based on scientific research showing that various strains within the same species may display different effects. In order to establish a true probiotic it is therefore essential to document characteristics, safety and efficacy of the specific probiotic strain.

The following text provides a review of the scientific documentation on *Bifidobacterium*, BB-12®. It is not a complete listing of all available data on BB-12®, rather it is a review of selected key data.

Taxonomy & Characterization

1.1 Taxonomy

Anette Wind MSc,
Senior Principal Scientist,
Identification, CED-Innovation

Bifidobacterium is a genus of lactic acid producing, Gram-positive, non-spore forming, non-motile, anaerobic bacteria. Bifidobacteria were discovered and isolated from the feces of a breast fed infant in 1899. They are common constituents of the indigenous microbiota in the human intestinal tract (Reuter 2001).

Bifidobacterium, BB-12® is a catalase-negative, rod-shaped bacterium. It was deposited in the cell culture bank of Chr. Hansen in 1983. At the time of isolation, BB-12® was considered to belong to the species *Bifidobacterium bifidum*. Modern molecular classification techniques reclassified BB-12® as *Bifidobacterium animalis* and later to a *new species Bifidobacterium lactis*. The species *B. lactis* was later shown not to fulfill the criteria for a species and was instead included in *Bifidobacterium animalis* as a subspecies. Today, BB-12® is therefore classified as *Bifidobacterium animalis* subsp. *lactis*. Despite a change in the name over the years, the strain BB-12® has not changed.

"BB-12® is technologically well suited, expressing fermentation activity, a good stability and a high acid and bile tolerance..."

1.2 Origin & Selection

BB-12® originates from Chr. Hansen's collection of dairy cultures. BB-12® is a strain which was specially selected by Chr. Hansen for the production of probiotic dairy products. BB-12® has been used in infant formula, dietary supplements and fermented milk products worldwide. BB-12® is technologically well suited, expressing fermentation activity, good stability and a high acid and bile tolerance, also as freeze-dried products in dietary supplements. Furthermore, BB-12® does not have adverse effects on taste, appearance or on the mouth feel of the food and is able to survive in the probiotic food until consumption.

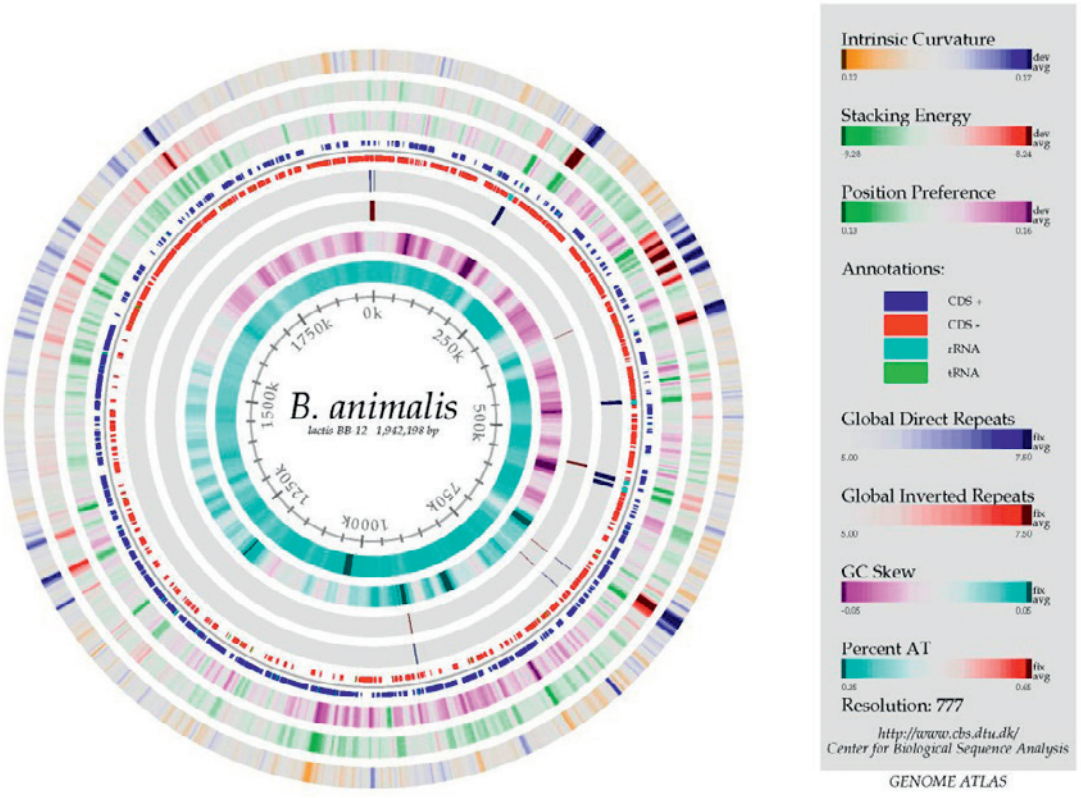
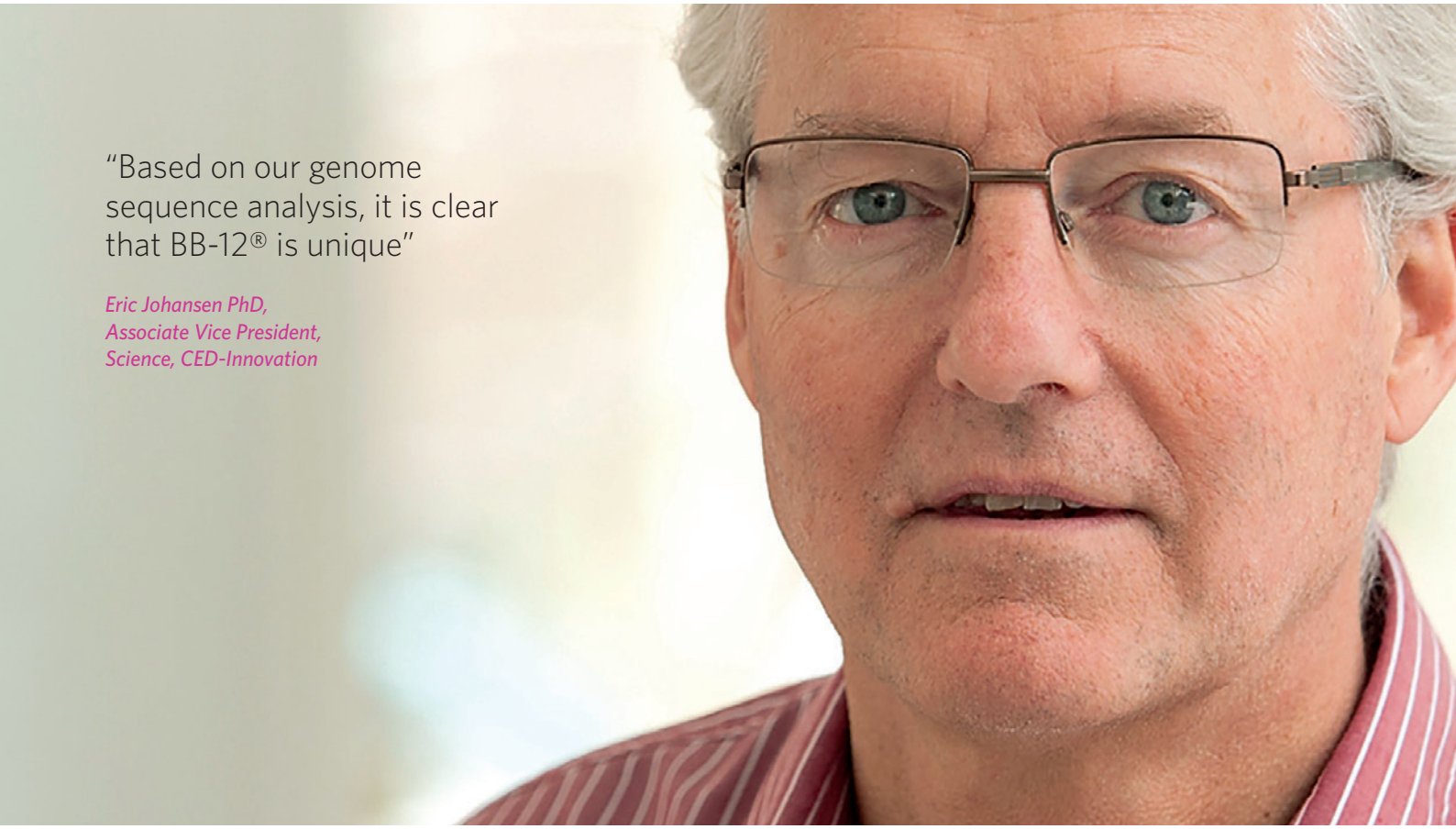


Figure 1. *The BB-12® genome atlas. The physical mapping of the BB-12® chromosome revealed that the genome sequence was correctly assembled. Reproduced from (Garrigues et al. 2013).*



1.3 The Genome

Eric Johansen PhD, Associate Vice President, Science, CED-Innovation

The properties of an organism are encoded in its DNA with the full complement of DNA in an organism being referred to as its genome. DNA sequencing technology has advanced to the point where it is possible to determine the complete genome sequence of any organism. The information hidden in the genome of a bacteria strain is fundamental for full characterization of the strain and for thorough explorations of its mechanisms and potential as a probiotic.

In 2002, a research project was initiated to determine the genome sequence of BB-12® and by 2004 the complete genome of BB-12® was mapped. This was presented at a scientific congress and published in 2005 (Garrigues et al. 2005) and the complete genome sequence was published in 2010 (Garrigues et al. 2010).

The BB-12® genome consists of a single circular chromosome of 1,942,198 base pairs with 1,642 predicted protein-encoding genes, 4 rRNA operons, and 52 tRNA genes. A physical mapping of the BB-12® chromosome revealed that the genome sequence was correctly assembled (Figure 1).

"Possession of the complete genome sequence facilitates a number of other technologies for characterizing a strain"

One important use of genome sequence information is that it allows a comparison to the genomes of other organisms. Based on this, it is clear that BB-12® is a unique strain which can be distinguished from all other strains on the market, including strains which are so closely related that they have nearly identical DNA fingerprints.

Possession of the complete genome sequence facilitates a number of other technologies for characterizing a strain. This includes gene expression studies and comparative genome hybridization using microarrays as well as information required to identify the specific proteins produced by a cell. This information can be used to improve production processes, identify specific compounds which support growth and provide information critical to understanding the mode of action of the probiotic properties of BB-12® (Garrigues et al. 2005, Pedersen et al. 2005).

Strain Characteristics & Mechanisms

2.1 Acid and Bile Tolerance

Thomas Dyrmann Leser PhD,
Senior Principal Scientist,
Discovery, HHN-Innovation

Gastric acid and bile plays an important role in the body's defense against ingested microorganisms, capable of killing and controlling gastrointestinal exposure to many pathogens. However, this same defense mechanism can also disable potentially beneficial microbes. For probiotic effects that are dependent on viability and physiological activity in the intestine, the survival of the probiotic in the presence of gastric acid and bile of the upper gastrointestinal tract is critical.

Several studies have investigated the gastric acid and bile tolerance of BB-12®. An in vitro study assessed five strains of bifidobacteria for acid and bile tolerance, as well as growth on various carbohydrates.

"BB-12® exhibited a very good survival..."

Tolerance to pH 2, pH 3, and pH 4 as well as 1% oxgall was tested. BB-12® exhibited a very good survival at all pH values, and had the best survival compared to the other strains. BB-12® did not grow well at 1% bile but demonstrated high survival rates (Vernazza et al. 2006).

The acid tolerance of 17 strains was compared in an in vitro study exposing them to pH 2-5. BB-12® demonstrated high survival rates. This characteristic was shown to be due in part to the low pH induction of H+-ATPase activity, an enzyme complex involved in maintaining intracellular pH homeostasis in bacteria (Matsumoto et al. 2004).

Twenty-four strains of lactic acid starter bacteria and 24 strains of probiotic bacteria were tested for tolerance to gastric juice

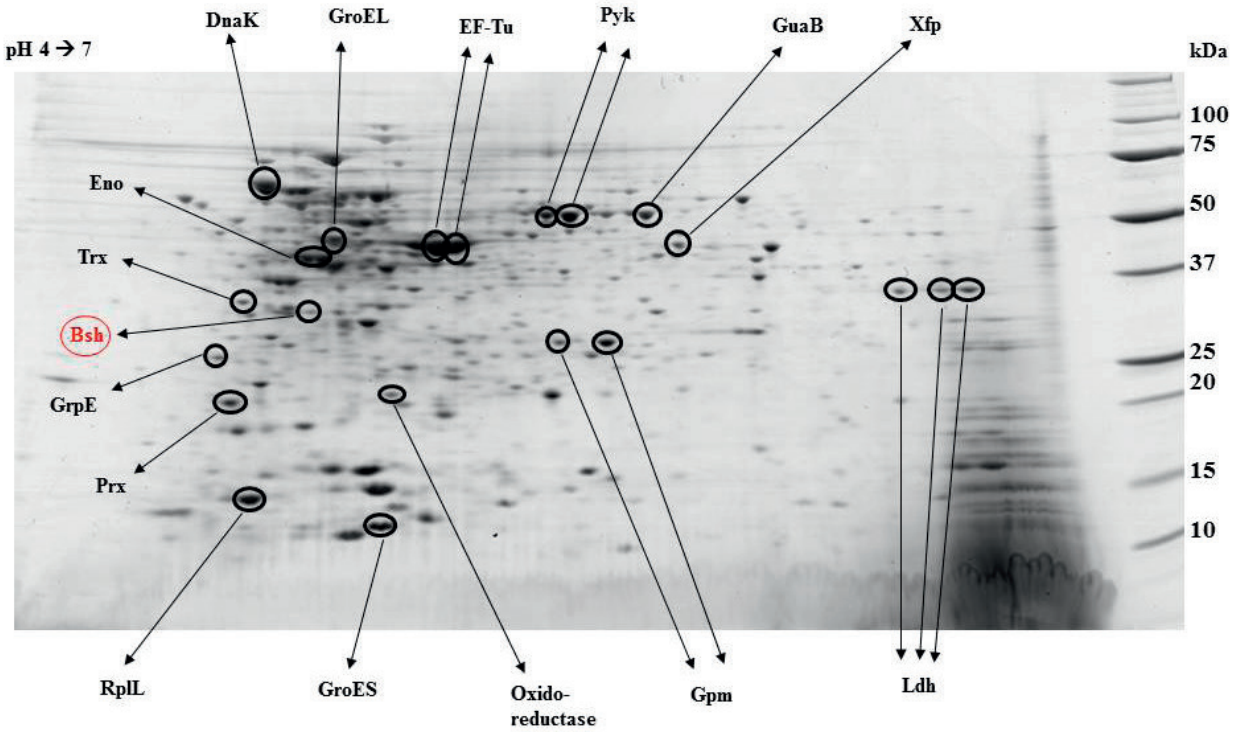


Figure 2. *Protein studies using 2-D gel electro-phoresis has documented the existence and activity of the enzyme responsible for bile salt hydrolase in BB-12®. Marked as* ***Bsh*** *in the picture. Reproduced from (Garrigues et al. 2005).*

"BB-12® is well-equipped to endure this critical passage of the gastrointestinal tract"

Birgitte Stuer-Lauridsen PhD,
Senior Research Scientist,
Identification, CED-Innovation

and bile salts. BB-12® showed high pH tolerance after three hours exposure at pH 3 and pH 2. The bile resistance of BB-12® was moderate showing 24% growth at 1% bile compared to a control. As for deconjugation and growth in the presence of bile salts, BB-12® showed both growth and deconjugation of sodium taurodeoxycholate and sodium glicodeoxycholate, whereas BB-12® grew in the presence of sodium taurocholate and sodium glycocholate without showing any deconjugation (Vinderola 2003).

In a comparative evaluation of 60 human intestinal bifidobacteria isolates of gastric acid and bile survival, BB-12® was demonstrated to survive both conditions just as good as or better than the other tested strains (Chr. Hansen data on file). In an artificial gut model system simulating passage through stomach acid and upper intestinal bile, 60-80% of the BB-12® in a normal capsular dose remains viable (Chr. Hansen data on file).

In conclusion, BB-12® shows high gastric acid and bile tolerance compared to other bifidobacteria. The above data suggests that the majority of BB-12® bacteria may survive gastric acid and bile after consumption by humans. These properties enhance the potential of BB-12® to provide a health benefit to the host.

2.2 Bile Salt Hydrolase

Birgitte Stuer-Lauridsen PhD,
Senior Research Scientist,
Identification, CED-Innovation

The passage through the gastrointestinal tract includes different challenges for live probiotics. Following the harsh and acidic gastric environment, bile salts of the small intestine compose the next challenge. BB-12® contains the gene coding for bile salt hydrolase, an enzyme which is important for coping with the high bile salt concentrations in the small intestine. This enzyme is present and active in BB-12® at all times, a fact which is documented by both microarray analyses and protein studies using 2-D gel electrophoresis (Garrigues et al. 2005)(Figure 2). Having such an enzyme ready for action will provide an advantage for the cell as it allows a quick response to high bile salt concentrations and thus facilitates the safe passage from the small intestine to the large intestine. These data suggest that BB-12® is well-equipped to endure this critical passage in the gastrointestinal tract.

Frozen cultures.

2.3 Adhesion

Thomas Dyrmann Leser PhD,
Senior Principal Scientist,
Discovery, HHN-Innovation

Adhesion of probiotic microorganisms to the intestinal mucosa is considered important for many of the observed probiotic health effects. Adhesion is considered a prerequisite for colonization, pathogen inhibition, immune interactions, and barrier function enhancement. Therefore, adhesion is one of the main selection criteria for probiotic microorganisms.

"...BB-12® displaying the highest level of adhesion ..."

In a study, the adhesion of probiotics, commensal, and potentially pathogenic bacteria was determined in vitro. The adhesion models used were polycarbonate-well plates, with or without mucin, and different configurations of Caco-2 and/or HT29-MTX cell cultures. Adhesion of probiotic strains to untreated wells, as well as mucin-treated wells was high, with BB-12® displaying the highest level of adhesion in both cases. Though to a lower level, BB-12® also adhered to Caco-2 cultures, HT29-MTX cells, and co-cultures of Caco-2:HT29-MTX (Laparra and Sanz 2009).

In another in vitro study, the adhesion properties of BB-12® and other strains to fecal mucus isolated from various species including humans was tested. BB-12® adhered well to all hosts tested with 30% in humans (Rinkinen et al. 2003). Twenty-four strains of bifidobacteria were tested for their ability to bind to immobilized human and bovine intestinal mucus glycoproteins. BB-12® and one other *Bifidobacterium* strain had the highest level of adhesion amongst the tested strains. The adhesion of BB-12® to human mucus was 7.1% (He et al. 2001).

The adhesion of five probiotics and their combinations in the intestinal mucus of infants during and after rotavirus diarrhea and in healthy children was determined in vitro. Mucus was prepared from fecal samples from 20 infants during and after rotavirus diarrhea and from ten healthy age-matched children. BB-12® demonstrated excellent adhesion properties with 31% in healthy children and 26.1% in infants after infection. The distinctive pattern of probiotic adherence was not influenced by rotavirus diarrhea (Juntunen et al. 2001).

Testing of the adherence properties of BB-12® has also been evaluated at the laboratories of Chr. Hansen. In a comparative in vitro test of 60 human intestinal bifidobacteria isolates, BB-12® was demonstrated to adhere to mucus just as good as or better than the other tested strains (Chr. Hansen data on file).

In conclusion, BB-12® has demonstrated high adherence properties in various in vitro settings. This evidence supports that BB-12® possesses the capability to transiently colonize the mucosal surfaces in the intestine, persist at these sites, and thereby increase the possibility for delivering beneficial health effects.

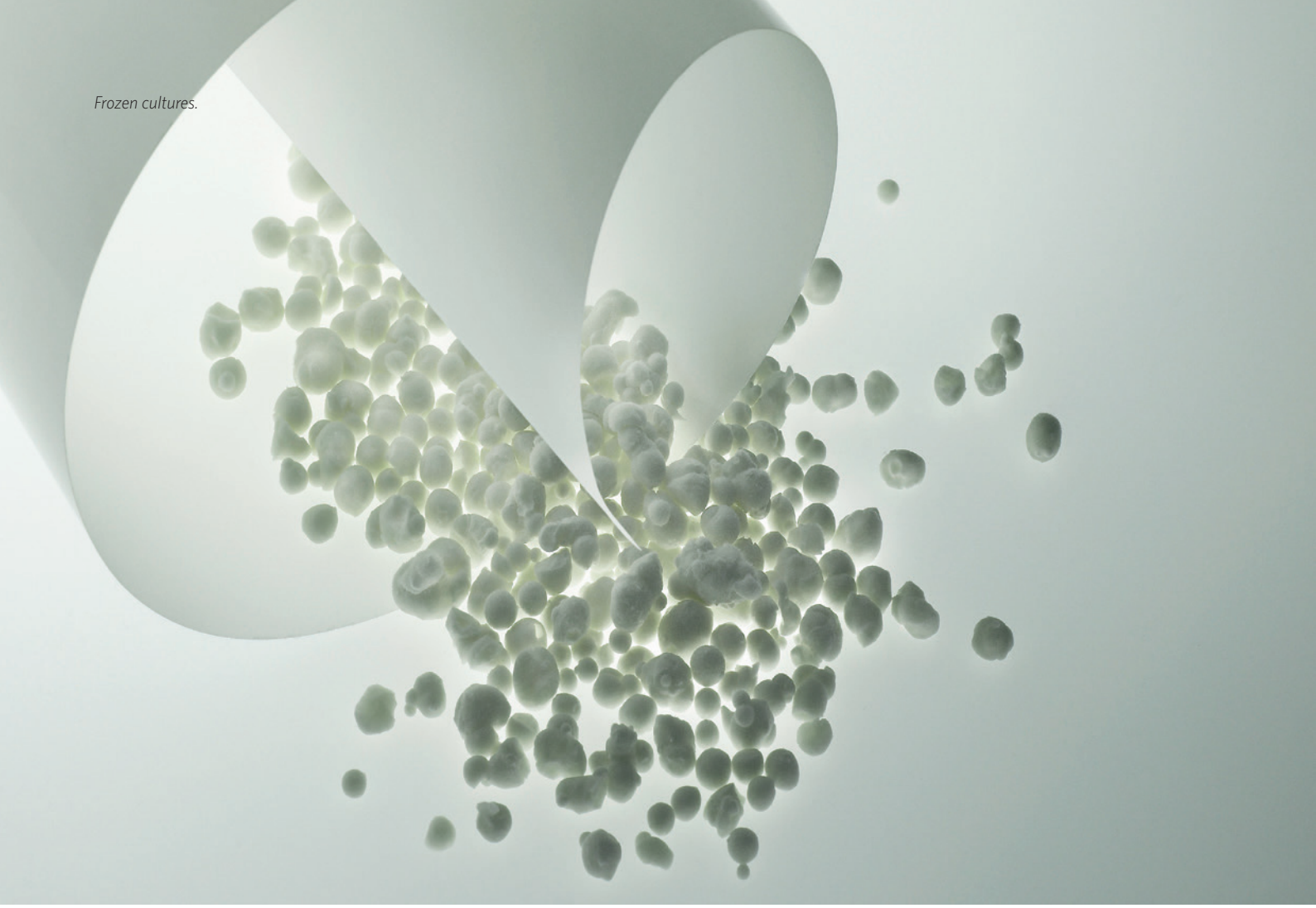
2.4 Pathogen Inhibition

Pathogens are microorganisms that may cause disease in their host. The ability to inhibit pathogens is one of the three main mechanisms of probiotics, barrier function enhancement and immune interactions being the other two. Pathogen inhibition has been proposed to be facilitated through multiple mechanisms including production of inhibitory substances (organic acids, H2O2, bacteriocins), nutrient competition, toxin removal/degradation, competition for sites of adherence (mucus, cell receptors), co-aggregation and virulence modulation, and induction of host immune responses.

"...[BB-12®] significantly reduced adhesion of the tested pathogens"

An *in vitro* study compared four different microorganisms including BB-12® with regards to production of antagonistic substances, amongst others. *Bacillus cereus, Clostridium difficile, Clostridium perfringens* TYPE A, *Escherichia coli* ATCC 4328, *Enterococcus faecalis, Listeria monocytogenes, Pseudomonas aeruginosa, Salmonella enterica subsp enterica serovar Typhimurium, S. enterica subsp. enterica serovar Typhi, Shigella flexneri, Shigella sonnei, and Candida albicans* were used in the antagonism assay. Only BB-12® and one other bacteria strain produced inhibitory zones against the pathogens. BB-12® displayed antagonistic activity against eight out of the twelve tested pathogens and the inhibitory zones produced by BB-12® were in general larger with the only exception of S. *flexneri* (Martins et al. 2009).

Batch and continuous culture anaerobic fermentation systems, inoculated with human feces, were utilized to investigate the antimicrobial actions of two probiotics combined with prebiotics. BB-12® combined with a mixture of oligofructose and xylo-oligosaccharides against *E. coli* and *Campylobacter jejuni* was tested. In batch fermenters, both *E. coli* and *C. jejuni* were inhibited by BB-12® combined with prebiotics. In continuous culture BB-12® and prebiotics inhibited *C. jejuni*. The results suggested that acetate and lactate produced by BB-12® directly were



Frozen cultures.

conferring antagonistic action, rather than as a result of pH lowering.

The ability of commercial probiotic strains currently marketed in European countries, to inhibit, compete with and displace the adhesion of selected potential pathogens to immobilized human mucus was investigated in an in vitro study. Bacterial pathogens were; *Bacteroides vulgatus, Clostridium histolyticum, C. difficile, E. coli* K2, *Enterobacter aerogenes*, *L. monocytogenes, S. enterica serovar Typhimurium*, and *Staphylococcus aureus*. BB-12® was able to adhere to the human mucus and inhibited all pathogens but *E. coli*. BB-12® demonstrated good displacement of *C. difficile, B. vulgatus, E. aerogenes, L. monocytogenes* and to a minor degree *C. histolyticum, S. enterica and S. aureus* (Collado et al. 2007a).

In addition, a competition and exclusion experiment for mucus adherence also demonstrated the ability of BB-12® to reduce binding of pathogens. An in vitro study set out to investigate the protective effect of BB-12® and L. rhamnosus LGG®, alone and in combination, on the adhesion of pathogenic strains to pig intestinal mucus. Pathogens used were S. *enterica* serovar Typhimurium, *C. perfringens, C. difficile*, and *E. coli* K2. BB-12® and LGG® in combination enhanced the adhesion of each other, mainly in large intestinal mucus. Treatment of intestinal mucus with BB-12® and LGG®, alone or in combination significantly reduced adhesion of the tested pathogens (Collado et al. 2007b).

In conclusion, these studies show that BB-12® is capable of inhibiting important gastrointestinal pathogens through production of antimicrobial substance as well as through competition for mucosal adhesion.

2.5 Barrier Function Enhancement

Barrier function enhancement is one of the central and generally accepted mechanisms of probiotics. Maintenance of an intact and functional mucus layer and epithelial cell lining in the gastrointestinal tract is critical in order to stay fit and healthy.

"...BB-12® increased tight junction strength significantly ..."

An in vitro study aimed at testing whether or not fermentation products from probiotics and prebiotics affected tight junction integrity in a Caco-2 cell line model. This was done by measuring the transepithelial electric resistance (TER, ff/cm2) of the Caco-2 cells. Fermentation products from BB-12® increased tight junction strength significantly above that of the untreated control, and in all cases, fermentation products from BB-12® induced the greatest increase in TER compared to other strains tested. These in vitro changes indicate that BB-12® may increase tight junction strength and protect against disruption of the epithelial barrier function (Commane et al. 2005).

2.6 Immune Interactions

Immune interaction is increasingly being acknowledged as a substantial probiotic mechanism. Probiotics are capable of

FACT BOX 1

Strains versus Species

Probiotics are classified by their genus (e.g. *Bifidobacterium*), species (e.g. *animalis*), and strain (e.g. BB-12®). The mammalian equivalent for genus and species could be the wild horse *Equus ferus* (genus, species). A strain can be described as a population of organisms (bacteria) that originates from a single isolate or pure culture. Strains within a species differ from one another genetically and with regard to functional properties. The wild horse equivalent of an isolate would be a single individual of a given species. FAO/WHO has stated that probiotic effects are strain specific[1]. The probiotic characteristics of a particular strain can therefore not be extrapolated to other strains of the same species. Evidence for such as statement lies in research showing that strain-specific proteins responsible for probiotic functional characteristic are expressed in strains with documented clinical effects compared to other strains of the same species[2]. Data also shows that probiotic strains of the same species differ with regard to inhibition of pathogens[3] and adherence to epithelial cells[4]. Therefore, most probiotic characteristics and effects are likely strain rather than species related.

1. FAO/WHO. Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food 2002

2. Kankainen et al. Proceedings of National Academy of Sciences 2009;106:17193-17198

3. Jacobsen et al. Applied and Environmental Microbiology 1999;65:4949-4956

4. Tuomola et al. International Journal of Food Microbiology 2000;60:75-81

communicating with and affect the immune system through immune cells located in the intestine. Seventy to eighty percent of the immune cells are found in the gut.

Several studies have demonstrated the immune modulating effect of BB-12®. The effect of twelve *Bifidobacterium* strains on the maturation process of dendritic cells derived from human monocytes was studied in vitro. Furthermore, proliferation of peripheral blood mononuclear cells and cytokine expression were evaluated. Maturation due to lipopolysaccharide treatment was used as reference. BB-12® was able to induce maturation of dendritic cells to a similar or even higher degree than LPS measured by surface expression markers. Cell-free supernatant only had a weak effect or no effect on maturation of dendritic cells. Expression of cytokines varied to a great extent depending on the strain, however, BB-12® demonstrated induction of IL-12 and TNF-Ð to a high degree and IL-10 to a low degree. In PBMCs, BB-12® induced high levels of IL-10, IFN-Ð and TNF-Ð (Lopez et al. 2010).

The ability of nine different probiotic strains to induce maturation and cytokine/chemokine expression in human dendritic cells at various concentrations was studied. BB-12® was able to induce all cytokines tested (IL-1Ð, IL-6, IL-10, IL-12 and IFN-Ð). The response was dose-dependent and increased with higher dose. With regards to chemokines, BB-12® induced CCL20 in a dose-dependent manner (Latvala et al. 2008).

An in vitro study investigated if fecal precipitates, obtained during consumption of BB-12®, induce an anti-inflammatory response in a murine macrophage-like cell line. Fecal precipitates tended to elicit a higher TNF-Ð response during the period of BB-12® consumption, compared to pre- and post-consumption. No change in response was observed for IL-1Ð and IL-10 (Matsumoto et al. 2007).

"BB-12® was able to induce maturation of dendritic cells..."

In conclusion, these data show that BB-12® is able to interact with the immune cells and demonstrate that BB-12® may have a beneficial impact on the immune function.

Efficacy

Mikkel Jungersen MSc,
Scientific Advisor,
HHN-Scientific Marketing

3.1 Proven Efficiancy

BB-12® is the world's most documented probiotic *Bifidobacterium*. It is described in more than 370 scientific publications out of which more than 170 are publications of clinical studies. Dating back to 1987, BB-12® has been tested in clinical trials for more than 25 years (Black 1987). BB-12® has been tested in clinical trials including subjects from preterm infants to elderly, and it has been administered in dosages up to 100 billion CFU/day.

It is a prerequisite for a probiotic to have documented its beneficial effect on the host in clinical studies. BB-12® has proven its beneficial health effect both within gastrointestinal health and immune function in numerous clinical studies.

3.2 Survival in the Gastrointestinal Tract

Some probiotic mechanisms presuppose viability and physiological activity of the probiotic at the target site. Since the target site may not be well-defined and due to difficulties measuring the viability in situ, fecal recovery is often used to confirm viability of probiotics in the gastrointestinal tract.

In a double-blind, placebo-controlled study, 109 infants were assigned randomly to receive 10 billion CFU/day of BB-12® or placebo in a novel slow releasing tablet inserted in a pacifier. The test tablets were administered twice a day from the age of 1–2 months to 8 months. At the age of 8 months, fecal samples were collected for BB-12® determination and recovery was monitored by quantitative polymerase chain reaction (PCR) method. With a detection limit of log 5, BB-12® was recovered in the feces of 62% of the infants receiving the BB-12® tablet (Taipale et al. 2011).

"...the detected BB-12® in the fecal samples was alive"

A placebo-controlled, cross-over study evaluated the effect of a synbiotic yogurt containing BB-12® and inulin on recovery of BB-12®. Fecal samples were collected from 46 volunteers and recovery, as well as changes in the microbiota were monitored using real-time PCR. BB-12® was recovered in the fecal samples and could be detected in feces up to two weeks after intake. A live/dead PCR procedure indicated that >90% of the detected BB-12® in the fecal samples was alive (Palaria et al. 2012).

In a randomized, placebo-controlled, double-blinded, parallel dose-response study 71 healthy young adults were assigned into five groups. The subjects received either placebo or a mixture of the two probiotics in the four concentrations of 108-1011 CFU/day for three weeks. The fecal recovery of BB-12® increased significantly with increasing dose. In the high dosage group, BB-12® was recovered in 13 out of 15 volunteers (Larsen et al. 2006).

In a study including 14 volunteers the intestinal survival and persistence of BB-12®, F19® and *Lactobacillus acidophilus* NCFB 1748 consumed in fermented milk was tested. Fecal recovery was detected by randomly amplified polymorphic DNA (RAPD) fingerprinting analysis of isolates from lactobacilli-selective media. BB-12® and F19® survived well through the gastrointestinal tract and were detected in 79% and 100%, respectively, of the study subjects after ingestion (Matto et al. 2006).

Another study included 30 healthy adults who were divided into three intervention groups: galacto-oligosaccharide (GOS), or GOS and BB-12®, or BB-12® alone. Fecal samples were collected before the intervention, and after the intervention. BB-12® was recovered from feces using RAPD fingerprinting. Isolates having an identical RAPD fingerprint with BB-12® were detected in high numbers in both the BB-12® group and the GOS + BB-12® group indicating good survival of BB-12® through the gastrointestinal tract. BB-12® was found in 40% of the volunteers from the BB-12® group and the GOS + BB-12® group one week after the intervention and in 20% two weeks after the intervention (Alander et al. 2001).

In conclusion, these data show that not only does BB-12® survive very well during the passage through the gastrointestinal tract, it also transiently colonizes the colon. The data suggest a dose related recovery of BB-12®.

3.3 Modulation of Intestinal Microbiota

The human large intestine is host to a wide variety of bacteria, with bifidobacteria being prominent members of this complex ecosystem. Bifidobacteria and lactobacilli are generally believed to contribute to gastrointestinal health. During old age, bifidobacteria and lactobacilli begin to decline in number, coinciding with proliferation of other bacterial groups, including clostridia and members of the *Enterobacteriaceae* family, which are believed to have adverse effects on gastrointestinal health. Probiotics capable of controlling proliferation of undesirable bacteria and increasing the levels of bifidobacteria and lactobacilli in the colon is considered beneficial.

Several clinical studies have shown that BB-12®, alone or in combination with other probiotics or ingredients, is associated with an increase in beneficial bacteria and a reduction in potentially pathogenic bacteria.

A double-blind, randomized, placebo-controlled dose-response study investigated the impact of a four-week consumption of BB-12®, $8x10^9$ or $38x10^9$ CFU/day, *L. acidophilus*, LA-5®, $1x10^9$ CFU/day, and green tea extract in fermented milk on fecal bacterial counts in 58 healthy adults (Savard et al. 2011). Quantitative PCR results showed significant increases in bifidobacteria counts in the active groups compared to baseline. Numbers of viable fecal lactobacilli were significantly higher and those of enterococci were significantly lower after the intervention when compared to placebo.

"...[BB-12®] is associated with an increase in beneficial bacteria and a reduction in potentially pathogenic bacteria"

In an open-label, single arm study, the effect of two weeks intake of BB-12® (total of 1x1011 CFU/day), *L. paracasei* subsp. *paracasei*, F19®, and L. acidophilus NCFB 1748 in fermented milk on the intestinal microbiota of 15 healthy adults was studied (Matto et al. 2006). The probiotic strains tended to transiently increase the total numbers from baseline of bifidobacteria and lactobacilli in feces. No change in clostridia, enterobacteriacea, or enterococci was detected.

A three arm, single blinded study randomized 30 healthy adults to either $3x10^{10}$ CFU/day of BB-12®, BB-12® in combination with 4 grams GOS, or 4 grams GOS alone for two weeks (Alander et al. 2001). Outcome measures were selected components of the fecal microbiota by culture methods. During the intervention period, the mean numbers of bifidobacteria increased slightly in all study groups. The increase was higher in the BB-12® and GOS group than in the BB-12® group. The increases were statistically significant. No change in lactic acid bacteria, C. *perfringens*, or coliforms were detected during the study.

In conclusion, these studies show that consumption of BB-12® facilitates an increase of the total number of bifidobacteria and may inhibit some undesirable bacteria in the gastrointestinal microbiota. This is believed to support a healthy microbiota in the gut.

Frozen cultures.

3.4 Gastrointestinal Function

3.4.1 Bowel Function

Regular bowel movements, natural transit time and normal stool consistency are part of a well-functioning bowel. However, the boundaries for normal bowel function are wide and vary to a large extent from person to person. The normal range for bowel movements is five to 14 times a week, with outer boundaries of three to 21 times a week. A frequency higher or lower is considered diarrhea or constipation, respectively. The passage time for food through the gastro-intestinal tract is normally within half a day to three days. Lazy tummy or constipation is a widely experienced challenge, especially in the elderly population. Probiotics may support the bowel function in a beneficial way by increasing bowel movement or transit time, or by softening of the stools.

The effect of BB-12® on two primary outcomes – defecation frequency and gastrointestinal well-being - was investigated in a double-blind, randomized, placebo-controlled study (Eskesen et al. 2015). A total of 1248 subjects were randomized after a 2 weeks run-in to $1x10^9$ or 10x109 CFU/day of BB-12® in a capsule or matching placebo for 4 weeks (Figure 3). No significant differences were detected on gastrointestinal well-being between the groups. In a post-hoc analysis on defecation frequency were the criteria for being a responder was tightened, the odds ratio was 1.55 (95% CI 1.22-1.96) with a p-value of P=0.0003 for treatment overall. Also the per-protocol analysis demonstrated BB-12® to improve defecation frequency compared to placebo. Effects on defecation frequency were similar for the two tested dosages, suggesting a ceiling effect of BB-12® which was reached at a dosage of $1x10^9$ CFU/day.

The effect of a fermented oat drink with $1x10^9$ CFU/day of BB-12®, or two B. longum strains, or placebo on bowel movement was tested in 209 elderly nursing home residents in a double-blind, randomized, placebo-controlled study (Pitkala et al. 2007). Residents received the intervention for one to seven months depending on the length of the stay in the nursing home. The group receiving BB-12® had significantly more days with normal bowel movements relative to total number of days with bowel movements: 26.9% for BB-12® and 20% for placebo. The number of subjects experiencing normal bowel movements in more than 30% of the days was increased by 114% in the BB-12® group (Figure 4).

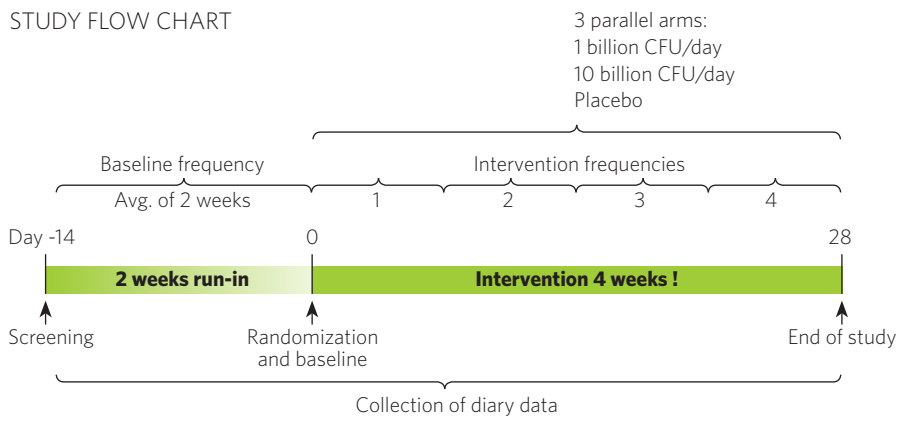


Figure 3. *The randomized, double-blind, placebo-controlled study by Eskesen et al. 2015 resulted in the largest dataset ever published from a randomized, placebo-controlled study on the effect of probiotics on bowel function.*

Studies in healthy adults have demonstrated that BB-12® increases stool frequency and softens the consistency of the stool. In a double-blind, randomized, placebo-controlled, cross-over design, BB-12® in fermented milk was given to healthy females in a dosage of $1x10^9$ CFU/day for two weeks (Uchida et al. 2005). In this study, 41 females had an average stool frequency in the BB-12® period of 8.8 per two weeks compared to 8.0 in the placebo period. When the subjects were divided into constipation (less than eight times per two weeks) and no constipation tendency groups, stool frequency was significantly higher in the BB-12® period compared to the placebo period in the constipation tendency group.

In a similar design, BB-12® in fermented milk at a dosage of $4x10^9$ CFU/day was given for two weeks to 35 healthy females (Nishida et al. 2004). A non-significant increase in stool frequency was found between the BB-12® period and the placebo in all subjects, whereas a statistically significant increase was found between the BB-12® period and the placebo period in constipated subjects with stool frequency of four times per week or less.
In a single-arm placebo-controlled study, 30 healthy adults were given BB-12® in fermented milk at a dosage of $5.2x10^9$ CFU/day for two weeks, followed by a two week period of placebo with a wash-out period in between (Matsumoto et al. 2001). One month after the placebo period, BB-12® in fermented milk at a higher dosage ($15x10^9$

CFU/day) was given for two weeks. The inclusion criteria were a stool frequency of less than four times weekly. The primary outcome measure was stool frequency. Stool frequency was higher in BB-12® low-dosage period compared to the placebo period. Stool frequency was also higher in the high-dosage BB-12® period, compared to the placebo period.

In conclusion, the above studies demonstrate that BB-12® improves bowel function, particularly in subgroups with mild constipation.

3.4.2 Diarrhea in Infants and Children

Diarrhea is a serious cause of infant morbidity and mortality, and the development of preventive measures remains an important goal. Bifidobacteria as well as other lactic acid producing bacteria have been shown to have a protective effect against both acute and persistent diarrhea.

A multicenter, double-blind, placebo controlled study evaluated the efficacy of a milk formula supplemented with BB-12® in the prevention of acute diarrhea in 90 healthy infants younger than eight months living in residential nurseries or foster care centers. There was a tendency toward a decrease in the incidence of diarrhea, with 28.3% of the infants receiving BB-12® experiencing acute diarrhea compared to 38.6% in the placebo group. Number of days with diarrhea was statistically lower in the BB-12® group as well as a lower day-probability of diarrhea. These results suggest that BB-12® have a protective effect against diarrhea (Chouraqui et al. 2004).

In a double-blind, placebo-controlled study, young children who were admitted to a chronic medical care hospital were randomized to receive a standard infant formula or the same formula supplemented with BB-12® and *Streptococcus thermophilus* TH-4® (Saavedra et al. 1994). Children were evaluated daily for occurrence of diarrhea, and fecal samples were analyzed for rotavirus antigen by enzyme immunoassay. Fecal samples were also obtained during an episode of diarrhea for virological and bacteriological analysis. 55 infants were evaluated for a total of 4447 patient-days during 17 months. Eight (31%) of the 26 children who received the control formula and two (7%) of the 29 who received the supplemented formula developed diarrhea during the course of the study. Ten (39%) of the subjects who received the control formula and three (10%) of those who received the supplemented formula shed rotavirus at some time during the study. These results suggest that supplementation of infant formula with BB-12® and S. *thermophilus* TH-4® can reduce the incidence of acute diarrhea and rotavirus shedding in children admitted to hospital.

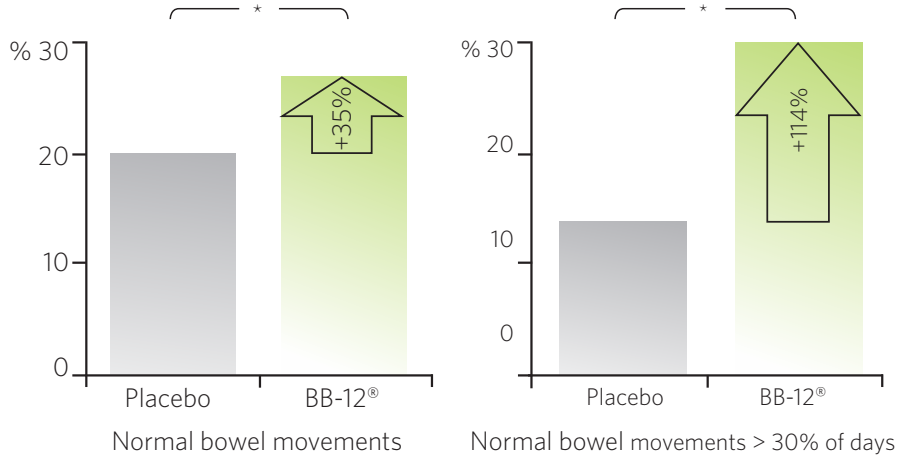
In conclusion, these studies demonstrate that BB-12® may have a beneficial effect on both the incidence and duration of diarrhea in infants and children.

3.4.3 Antibiotic-Associated Diarrhea

Treatment with antibiotics may cause serious side effects. Typically the disturbance of the gastrointestinal microbiota caused by the antibiotics leads to vomiting and diarrhea. Probiotics have shown to be able to reduce the side effects and in addition increase the completion rate of the antibiotic treatment. Furthermore, probiotics may accelerate recovery after the antibiotic treatment.

A randomized, double-blind, placebo-controlled study evaluated the efficacy of BB-12® and LA-5® in the prevention of antibiotic-associated diarrhea (AAD) in 343 patients during a seven day antibiotic treatment. Fourteen days of intervention was evaluated by symptom diary card for AAD assessment. After 14 days treatment, incidence of AAD in the probiotic group was significantly reduced to 10.8% compared to 15.56% in the placebo group. The duration of diarrhea was significantly less (2.32 days) in the probiotic group compared to placebo group (4.58 days). Incidence of severe diarrhea was significantly higher in the placebo

BB-12® IMPROVED BOWEL MOVEMENTS BY 35%



"BB-12® is the best documented Bifidobacterium in the world"

Mikkel Jungersen MSc,
Scientific Advisor,
HHN-Scientific Marketing

group (96%) than the probiotic group (31.6%). These results show that BB-12® and LA-5® can effectively reduce the duration and severity of AAD (Chatterjee et al. 2013).

To investigate matrix-specificity of probiotic effects and particularly the improvement of AAD, a controlled, randomized, double-blind study was performed. Eighty-eight *Helicobacter pylori*-infected but otherwise healthy subjects were given fermented milk containing BB-12® and LA-5®, pasteurized fermented milk with BB-12® and LA-5®, or acidified milk (control) for eight weeks. During week five, a *H. pylori* eradication therapy was performed. *H. pylori* activity was measured via urea breath tests and AAD and other gastrointestinal complaints were recorded by validated questionnaires. Subjects that were given fermented milk with live BB-12® and LA-5® had significantly decreased duration of AAD (four days) compared to subject given pasteurized fermented milk (ten days) or control (ten days). Moreover, probiotics significantly improved gastrointestinal symptoms (de Vrese et al. 2011).

"...BB-12® and LA-5® can reduce the side effects of antibiotic treatment..."

One hundred and sixty *H. pylori* infected patients were randomized into a triple-plus-probiotic-group or a triple-only-group, receiving one week of triple therapy with or without five weeks of supplementation with fermented milk containing BB-12® and LA-5®. Bifidobacteria in anaerobes was tested in stool samples. A significantly higher proportion in the probiotic group completed the seven-day treatment than in the control group (68% vs. 44 %). Common side effects such as vomiting, constipation, diarrhea and metallic taste were significantly decreased in the probiotic group. *H. pylori* eradication rates were significantly higher in the probiotic group. After triple therapy both groups observed depletion of bifidobacteria in stools. However probiotics restored the level of bifidobacteria in four weeks, as opposed to the control group. This shows that BB-12® and LA-5® can reduce the side effects of antibiotic treatment, aid treatment compliance, improve the eradication rates of *H. pylori* and restore the microbiota (Sheu et al. 2002).

The antagonistic effect of BB-12® and LA-5® against *H. pylori* has also been confirmed in other studies (Sheu et al. 2006, Wang et al. 2004).

In conclusion, these studies demonstrate that BB-12® in combination with LA-5® can reduce the incidence and duration of antibiotic-associated diarrhea significantly. In addition BB-12® and LA-5® may suppress *H. pylori* and support restoration of the microbiota in *H. pylori* positive subjects.

3.5 Immune Function

3.5.1 Respiratory Infections

Studying the immune system in healthy humans poses a special challenge. The immune system carries a high degree of buffering capacity of several components which makes it difficult to interpret or predict the exact response at a given time (Albers et al. 2005). The use of a model infection is therefore considered to provide the best method for exploring the function

FACT BOX 2

Facts on Bifidobacterium BB-12®

Documented efficacy

- The world's most well-documented probiotic Bifidobacterium
- Described in more than 300 scientific publications
- Backed by more than 140 clinical studies
- Proven effect within gastrointestinal and immune health
- Tested in newborn preterm infants to elderly within various health areas

Safety & Origin

- Originates from Chr. Hansen's collection of dairy cultures
- Identified as Bifidobacterium animalis subsp. lactis
- Used worldwide since 1985 with no reported adverse events
- Tested in clinical studies with no reported adverse events
- GRAS (Generally Recognized As Safe) notified by FDA
- QPS (Qualified Presumption of Safety) granted by EFSA

and the response of the immune system in healthy humans (Albers et al. 2005, Burleson and Burleson 2007). One of the suggested methods is the use of a vaccine containing killed or attenuated pathogens which will result in a specific immune response. Response to such a challenge can be used as an indicator of an integrated immune response.

Probiotics may interact with the immune system in various ways, e.g. by increasing local and systemic antibody production, by increasing immune cell activity, by modulating signals in epithelial and immune cells, and by induction of phenotypic changes in dendritic cells.

A randomized, placebo-controlled, double-blind human study investigated the impact of BB-12® on the functional capacity of the immune system in healthy humans using a vaccination model. In this study, 54 subjects were given BB-12® and 52 placebo for six weeks. After two weeks subjects received an influenza vaccination. Plasma and saliva samples were collected at baseline and after six weeks for analysis of influenza specific and total antibodies, cytokines IL-2, IL-10 and INF-Ð, and innate immune parameters. BB-12® increased the influenza specific antibody responses compared to placebo (Figure 5) and the number of subjects obtaining a minimum two-fold increase in antibody levels was significantly greater in the probiotic group. No differences were found for cytokines or innate immune parameters. Adverse event incidence and pattern was similar between groups, and tetanus-specific IgG did not change after the intervention indicating that supplementation with BB-12® only elicits specific immune responses (Rizzardini et al. 2012).

A study investigated the effect of BB-12® and LGG® on health-related quality of life during upper respiratory infections. The study assessed how probiotics affects duration of common cold, severity, and the impact of symptoms on daily life. One hundred ninety eight college students were randomized to receive either placebo or BB-12® and LGG® for twelve weeks. Each day, students completed a survey to assess the effect of the probiotic supplementation. The median duration of upper respiratory infections was significantly shorter by two

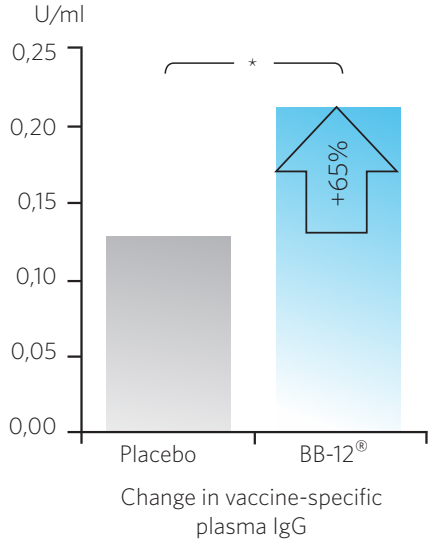


***Figure 5**. In a randomized, double-blind, placebo-controlled study by Rizzardini et al. 2012, BB-12® was shown to improve immune function.*

Label on study product for a randomized, controlled, clinical trial.

FACT BOX 3

Mode of Action

Your gastrointestinal microbiota consists of trillions of bacteria which interact with your body. Already in the beginning of the 20th century the Russian scientist and Nobel laureate Élie Metchnikoff proposed that this gastrointestinal microbiota could be modulated - the harmful pathogenic microbes could be replaced with good bacteria. This was a very important discovery as the interactions between the microbiota and the body are important to your gastrointestinal well-being as well as your immune defense. Actually, three-quarters of the immune cells are located around the gut. Later on, in the middle of the century, the term 'probiotic' was introduced. Since then a lot of research has been carried out seeking to uncover the effect of probiotics and the way they interact with us. We now know that probiotics are able to interact with the microbiota and the immune cells in various ways; probiotics can beneficially affect the intestinal microbiota by inhibiting the pathogenic bacteria and they may also enhance the gut barrier function. In addition to this probiotics can strengthen and support the immune system by increasing antibody production and immune cell activity as well as modulating cytokine production. We have come far in our research on probiotics but we may still be able to uncover new beneficial effects of probiotics and to dig deeper into the way they work.

days, and median severity score was significantly lower by 34% in the probiotic group compared to placebo. Number of missed work days was not different between groups. However, the probiotics group missed 0.2 fewer school days compared to the placebo group. The study shows that BB-12® and LGG® shortens the duration of colds and minimizes the severity for college students, and reduces missed school days (Smith et al. 2013).

"...fewer respiratory infections were reported in the BB-12® group..."

In a randomized, placebo-controlled and double-blind study, the effect of BB-12® on the risk of acute infections in infants was investigated. BB-12® or placebo was administered to 109 infants less than two months of age until the age of eight months. Signs and symptoms of acute infections were registered. In this study there was no effect on gastrointestinal infections or otitis media, however, fewer respiratory infections were reported in the BB-12® group compared to placebo (Figure 6) (Taipale et al. 2011). Another clinical study also showed reduction of acute infections in infancy. In this study 81 formula fed infants less than two months were randomized to either probiotics (BB-12® and LGG®) or placebo for twelve months in a double-blind design. Incidence of early infections and antibiotic use at seven months of age was lower in the group given probiotics compared to placebo group. During the first year of life, infants receiving probiotics also had fewer recurrent respiratory infections (Rautava et al. 2009).

In conclusion, these data demonstrate that supplementation with BB-12® may increase the body's resistance to common infections by strengthening the specific immune response to an immune challenge and that BB-12® can reduce incidence and duration of respiratory infections.

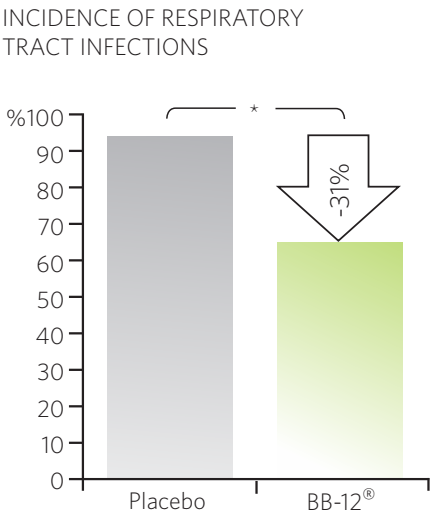


***Figure 6** . BB-12® reduced the risk of respiratory tract infections in a study by Taipale et al. 2011. In this randomized, double-blind, placebo-controlled BB-12® was administered using a slow-release tablet in a pacifier.*

Safety

4.1 Evaluation by Authorities

Mikkel Jungersen MSc,
Scientific Advisor,
HHN-Scientific Marketing

In the FAO/WHO document "Health and Nutritional Properties of Probiotics in Food including Milk Powder with Live Lactic Acid Bacteria" from 2001, safety of probiotics have been addressed in the following way:

"Information acquired to date shows that l actobacilli have a long history of use as probiotics without established risk to humans, and this remains the best proof of their safety. Also, no pathogenic or virulence properties have been found for lactobacilli, bifidobacteria or lactococci. Having stated that, the Consultation acknowledges that under certain conditions, some lactobacilli strains have been associated with adverse effects, such as rare cases of bacteremia. However, a recent epidemiological study of systematically collected lactobacilli bacteremia case reports in one country has shown that there is no increased incidence or frequency of bacteremia with increased usage of probiotic lactobacilli"(FAO/WHO 2001).

This was followed by another report published in 2002 (FAO/WHO 2002), where it was recommended to characterize a pro-biotic strain with regards to antibiotic resistance patterns, metabolic activities, side-effects during human studies, adverse incidents in consumers (post-market), as probiotics theoretically may be responsible for systemic infections, deleterious metabolic activities, excessive immune stimulation in susceptible individuals and gene transfer.

Further it was recommended to make an assessment of a lack of infectivity of the probiotic strain in immunocompromised animals as this would add a measure of confidence in the safety of probiotics. In case the strain belongs to a species known to produce a mammalian toxin or to have a hemolytic potential, it should also be tested for these characteristics.

"...BB-12® is Generally Regarded As Safe (GRAS) by the Food and Drug Administration (FDA)..."

In Europe, strains belonging to the species *Bifidobacterium animalis* have been granted Qualified Presumption of Safety (QPS) status by the European Food Safety Authority (EFSA Panel on Biological Hazards (BIOHAZ) 2015).

In the USA, BB-12® is Generally Regarded As Safe (GRAS) by the Food and Drug Administration (FDA) as an ingredient in milk-based infant formula intended for consumption by infants 4 months and older (Food and Drug Administration 2002).

The Codex Alimentarius standard for fermented milks (Codex Stan 243-2003) allows the use of harmless microorganisms in fermented milk products (FAO/WHO 2003).

The Codex Alimentarius standard for infant formula (Codex Stan 72-1981, Revision 2007) (FAO/WHO 1981) and follow-up formula (Codex Stan 156-1987) (FAO/WHO 1987) allow the addition of L(+) lactic acid producing cultures in milk formula products.

4.2 Antibiotic Susceptibility

Birgitte Stuer-Lauridsen PhD,
Senior Research Scientist,
Identification, CED-Innovation

Testing bacteria for phenotypic susceptibility towards antibiotics is currently the best method to investigate whether a strain is likely to have a transferable antibiotic resistance gene, a trait which would render the strain unsuitable in any product. Strains with a resistance caused by antibiotic resistance genes will deviate from the wild-type population. However, when the wild-type strains all show low susceptibility to an antibiotic, the species can be said to carry intrinsic resistance to that particular antibiotic. Such a resistance caused by inherent factors is not considered to be transferable, and the same is true for resistance caused by mutations in chromosomally located genes.

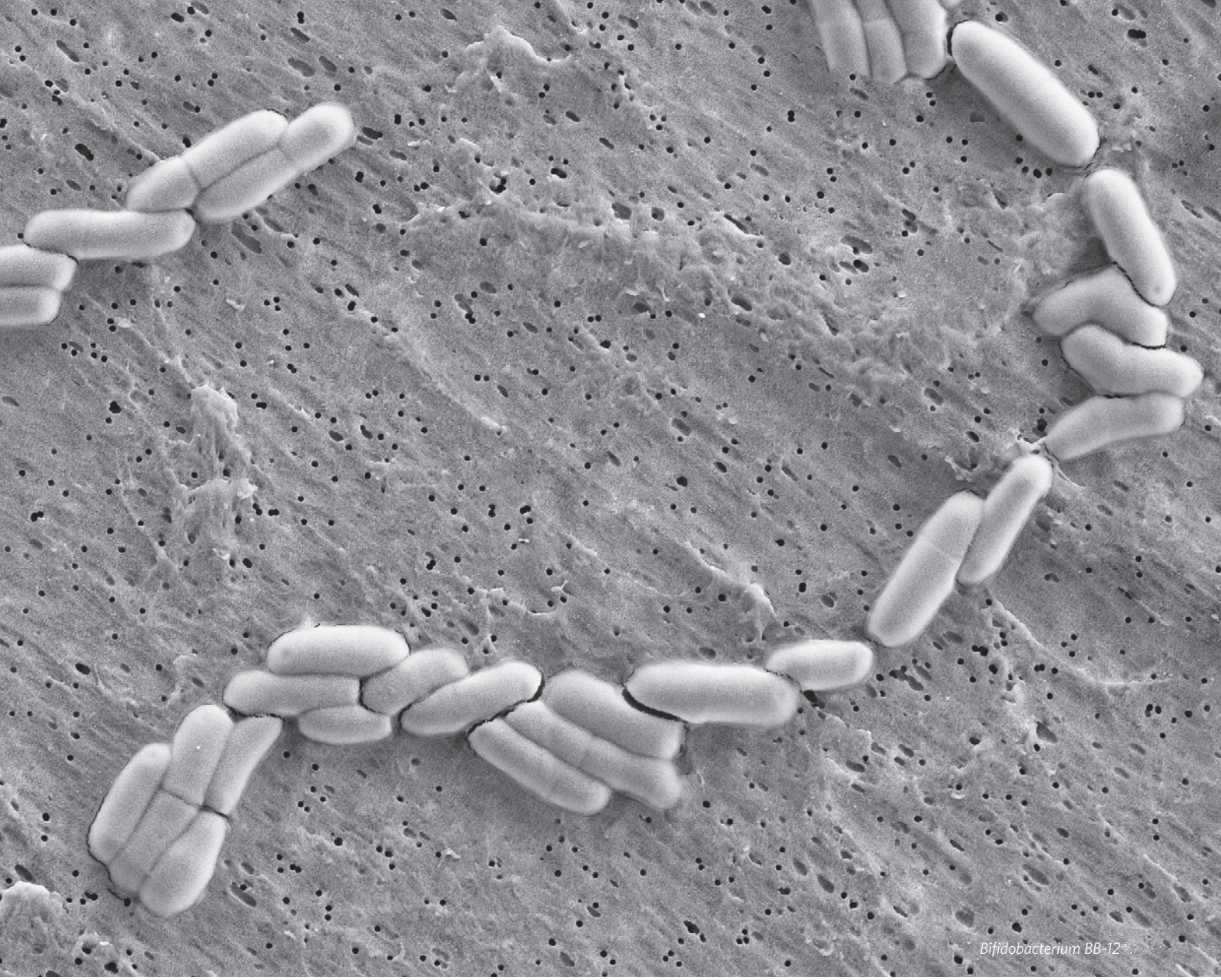
Chr. Hansen follows the guidelines for antibiotic susceptibility testing issued by EFSA and performs the tests according to internationally recognized methods published by ISO or CLSI. Based on the scientific publications, EFSA also publishes breakpoint values for how insensitive a strain may be to a number of antibiotics before it is categorized as resistant, and Chr. Hansen complies with these breakpoints for all strains produced, including BB-12®.

BB-12® has been tested a number of times with different recognized methods and is found to be susceptible to the 13 antibiotics tested.

4.3 Human and Animal Studies

BB-12® is considered safe due to the long history of safe human exposure to species of *Bifidobacterium*, to strains of subspecies of *Bifidobacterium animalis* subsp. *lactis*, and to *Bifidobacterium animalis* subsp. *lactis*, BB-12® in particular.

Beside the long history of use worldwide BB-12® has been tested in numerous human clinical studies and no serious adverse events have been reported.



Bifidobacterium BB-12®.

No adverse events have been reported in vulnerable populations, such as infants, pregnant and lactating women and patient populations. In these populations, BB-12® has been consumed in daily dosages ranging from approximately 0.1 to 400 billion colony forming units (CFU). Supplementation periods have ranged from two weeks to twelve months. The dosage forms have been milk powder, dairy products or dietary supplements in the form of capsules.

No adverse events have been reported in healthy populations. In this population, BB-12® has been consumed in daily dosages ranging from 0.1 billion to 50 billion CFU. Supplementation periods have ranged from one week to seven months. The dosage forms have been milk powder, dairy products or dietary supplements in the form of capsules.

"Beside the long history of use worldwide BB-12® has been tested in numerous human clinical studies..."

In addition to the clinical studies BB-12® has been tested in animals. Administration of BB-12® to immune-compromised animals has shown no adverse immune response.

BB-12® does not permanently colonize the gastrointestinal tract. No deleterious metabolic activities of BB-12® have been described.

Based on the above information, it can be concluded that the use of *Bifidobacterium animalis* subsp. *lactis*, BB-12® is safe for its intended use as a dietary ingredient in food and dietary supplements to be consumed by a healthy population including term newborn infants.

References

Publication on **Bifi dobacterium, BB-12® in bold**

Alander M, Matto J, Kneifel W, Johansson M, Kögler B, Crittenden R, et al. Effect of galacto-oligosaccharide supplementation on human faecal microflora and on survival and persistence of Bifidobacterium lactis Bb-12 in the gastrointestinal tract. Int.Dairy J. 2001;11:817-825.

Albers R, Antoine JM, Bourdet-Sicard R, Calder PC, Gleeson M, Lesourd B, et al. Markers to measure immunomodulation in human nutrition intervention studies. Br.J.Nutr. 2005;94:452-481.

Black FT. Placebo-controlled double-blind trial of 4 lactobacilli strains (HIP) used as prophylactic agent against traveller's diarrhea (2 trials). Report by G.Nirnberger, Bioconsult, GmbH 1987:.

Burleson GR, Burleson FG. Influenza virus host resistance model. Methods 2007;41:31-37.

Chatterjee S, Kar P, Das T, Ray S, Ganguly S, Rajendiran C, et al. Randomised Placebo-controlled Double Blind Multicentric Trial on Efficacy and Safety of Lactobacillus acidophilus LA-5® and Bifidobacterium BB-12® for Prevention of Antibiotic-Associated Diarrhoea. JAPI 2013;61:708-712.

Chouraqui JP, Van Egroo LD, Fichot MC. Acidified milk formula supplemented with bifidobacterium lactis: impact on infant diarrhea in residential care settings. J.Pediatr.Gastroenterol.Nutr. 2004;38:288-292.

Collado MC, Meriluoto J, Salminen S. Role of commercial probiotic strains against human pathogen adhesion to intestinal mucus. Lett. Appl.Microbiol. 2007a;45:454-460.

Collado MC, Grzeskowiak L, Salminen S. Probiotic strains and their combination inhibit in vitro adhesion of pathogens to pig intestinal mucosa. Curr.Microbiol. 2007b;55:260-265.

Commane DM, Shortt CT, Silvi S, Cresci A, Hughes RM, Rowland IR. Effects of fermentation products of pro- and prebiotics on trans-epithelial electrical resistance in an in vitro model of the colon. Nutr.Cancer 2005;51:102-109.

de Vrese M, Kristen H, Rautenberg P, Laue C, Schrezenmeir J. Probiotic lactobacilli and bifidobacteria in a fermented milk product with added fruit preparation reduce antibiotic associated diarrhea and Helicobacter pylori activity. J.Dairy Res. 2011;78:396-403.

EFSA Panel on Biological Hazards (BIOHAZ). Statement on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 3: Suitability of taxonomic units notified to EFSA until September 2015. EFSA Journal 2015;13:4331.

Eskesen D, Jespersen L, Michelsen B, Whorwell PJ, Muller-Lissner S, Morberg CM. Effect of the probiotic strain Bifidobacterium animalis subsp. lactis, BB-12(R), on defecation frequency in healthy subjects with low defecation frequency and abdominal discomfort: a randomised, double-blind, placebo-controlled, parallel-group trial. Br.J.Nutr. 2015:1-9.

FAO/WHO. Guidelines for the Evaluation of Probiotics in Food. Report of a joint FAO/WHO working group on drafting guidelines for the evaluation of probiotics in food. Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food 2002.

FAO/WHO. Health and Nutrition Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria. 2001.

FAO/WHO CA. Codex Standard for Fermented Milks. 2003;CODEX STAN 243.

FAO/WHO CA. Standard for Follow-up formula. 1987.

FAO/WHO CA. Standard For Infant Formula and Formulas For Special Medical Purposes Intended For Infants. 1981.

Food and Drug Administration. GRAS Notice Inventory > Agency Response Letter GRAS Notice No. GRN 000049. 2002.

Garrigues C, Johansen E, Pedersen MB. Complete genome sequence of Bifidobacterium animalis subsp. lactis BB-12, a widely consumed probiotic strain. J.Bacteriol. 2010;192:2467-2468.

Garrigues C, Stuer-Lauridsen B, Johansen E. Characterisation of Bifidobacterium animalis subsp. lactis BB-12 and other probiotic bacteria using genomics, transcriptomics and proteomics. Aust.J.Dairy Technol. 2005;60:84-92.

Garrigues C, Johansen E, Crittenden R. Pangenomics – an avenue to improved industrial starter cultures and probiotics. Curr.Opin. Biotechnol. 2013;24:187-191.

He F, Ouwehan AC, Hashimoto H, Isolauri E, Benno Y, Salminen S. Adhesion of Bifidobacterium spp. to human intestinal mucus. Microbiol.Immunol. 2001;45:259-262.

Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, et al. Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat.Rev.Gastroenterol.Hepatol. 2014;11:506-514.

Juntunen M, Kirjavainen PV, Ouwehand AC, Salminen SJ, Isolauri E. Adherence of probiotic bacteria to human intestinal mucus in healthy infants and during rotavirus infection. Clin. Diagn.Lab.Immunol. 2001:293-296.

Laparra JM, Sanz Y. Comparison of in vitro models to study bacterial adhesion to the intestinal epithelium. Lett.Appl.Microbiol. 2009;49:695-701.

Larsen CN, Nielsen S, Kaestel P, Brockmann

E, Bennedsen M, Christensen HR, et al. Dose-response study of probiotic bacteria Bifidobacterium animalis subsp lactis BB-12 and Lactobacillus paracasei subsp paracasei CRL-341 in healthy young adults. Eur.J.Clin. Nutr. 2006;60:1284-1293.

Latvala S, Pietila TE, Veckman V, Kekkonen RA, Tynkkynen S, Korpela R, et al. Potentially probiotic bacteria induce efficient maturation but differential cytokine production in human monocyte-derived dendritic cells. World J.Gastroenterol. 2008;14:5570-5581.

Lopez P, Gueimonde M, Margolles A, Suarez A. Distinct Bifidobacterium strains drive different immune responses in vitro. Int.J.Food Microbiol. 2010;138:157-165.

Martins FS, Silva AA, Vieira AT, Barbosa FH, Arantes RM, Teixeira MM, et al. Comparative study of Bifidobacterium animalis, Escherichia coli, Lactobacillus casei and Saccharomyces boulardii probiotic properties. Arch.Microbiol. 2009;191:623-630.

Matsumoto M, Hara K, Benno Y. The influence of the immunostimulation by bacterial cell components derived from altered large intestinal microbiota on probiotic anti-inflammatory benefits. FEMS Immunol.Med.Microbiol. 2007;49:387-390.

Matsumoto M, Ohishi H, Benno Y. H+-ATPase activity in Bifidobacterium with special reference to acid tolerance. Int.J.Food Microbiol. 2004;93:109-113.

Matsumoto M, Imai T, Hironaka T, Kume H, Watanabe M, Benno Y. Effect of Yoghurt with Bifidobacterium lactis LKM512 in Improving Fecal Microflora and Defecation of Healthy Volunteers. Journal of Intestinal Microbiology 2001;14:97-102.

Matto J, Fonden R, Tolvanen T, Vonwright A, Vilpponensalmela T, Satokari R, et al. Intestinal survival and persistence of probiotic Lactobacillus and Bifidobacterium strains administered in triple-strain yoghurt. Int.Dairy J. 2006;16:1174-1180.

Nishida S, Gotou M, Akutsu S, Ono M, Hitomi Y, Nakamura T, et al. Effect of Yogurt Containing Bifidobacterium lactis BB-12 on Improvement of Defecation and Fecal Microflora of Healthy Female Adults. Milk Science 2004;53:71-80.

Palaria A, Johnson-Kanda I, O'Sullivan DJ. Effect Of A Synbiotic Yogurt On Levels Of Fecal Bifidobacteria, Clostridia And Enterobacteria. Appl.Environ.Microbiol. 2012;78:933-940.

Pedersen MB, Iversen SL, Sorensen KI, Johansen E. The long and winding road from the research laboratory to industrial applications of lactic acid bacteria. FEMS Microbiol.Rev. 2005;29:611-624.

Pitkala KH, Strandberg TE, Finne Soveri UH, Ouwehand AC, Poussa T, Salminen S. Fermented cereal with specific bifidobacteria normalizes bowel movements in elderly nursing home residents. A randomized, controlled trial. J.Nutr.Health Aging 2007;11:305-311.

Rautava S, Salminen S, Isolauri E. Specific probiotics in reducing the risk of acute infections in infancy--a randomised, double-blind, placebo-controlled study. Br.J.Nutr. 2009;101:1722-1726.

Reuter G. The Lactobacillus and Bifidobacterium microflora of the human intestine: composition and succession. Curr.Issues Intest. Microbiol. 2001;2:43-53.

Rinkinen M, Westermarck E, Salminen S, Ouwehand AC. Absence of host specificity for in vitro adhesion of probiotic lactic acid bacteria to intestinal mucus. Vet.Microbiol. 2003;97:55-61.

Rizzardini G, Eskesen D, Calder PC, Capetti A, Jespersen L, Clerici M. Evaluation of the immune benefits of two probiotic strains Bifidobacterium animalis ssp. lactis, BB-12® and Lactobacillus paracasei ssp. paracasei, L. casei 431® in an influenza vaccination model: a randomised, double-blind, placebo-controlled study. Br.J.Nutr. 2012;107:876-84.

Saavedra JM, Bauman NA, Oung I, Perman JA, Yolken RH. Feeding of Bifidobacterium bifidum and Streptococcus thermophilus to infants in hospital for prevention of diarrhoea and shedding of rotavirus. Lancet 1994;344:1046-1049.

Savard P, Lamarche B, Paradis ME, Thiboutot H, Laurin E, Roy D. Impact of Bifidobacterium animalis subsp. lactis BB-12 and, Lactobacillus acidophilus LA-5-containing yoghurt, on fecal bacterial counts of healthy adults. Int.J.Food Microbiol. 2011;149:50-57.

Sheu BS, Cheng HC, Kao AW, Wang ST, Yang YJ, Yang HB, et al. Pretreatment with Lactobacillus- and Bifidobacterium-containing yogurt can improve the efficacy of quadruple therapy in eradicating residual Helicobacter pylori infection after failed triple therapy. Am.J.Clin. Nutr. 2006;83:864-869.

Sheu BS, Wu JJ, Lo CY, Wu HW, Chen JH, Lin YS, et al. Impact of supplement with Lactobacillus- and Bifidobacterium-containing yogurt on triple therapy for Helicobacter pylori eradication. Aliment.Pharmacol.Ther. 2002;16:1669-1675.

Smith TJ, Rigassio-Radler D, Denmark R, Haley T, Touger-Decker R. Effect of Lactobacillus rhamnosus LGG(R) and Bifidobacterium animalis ssp. lactis BB-12(R) on health-related quality of life in college students affected by upper respiratory infections. Br.J.Nutr. 2013;109:1999-2007.

Taipale T, Pienihakkinen K, Isolauri E, Larsen C, Brockmann E, Alanen P, et al. Bifidobacterium animalis subsp. lactis BB-12 in reducing the risk of infections in infancy. Br.J.Nutr. 2011;105:409-16.

Uchida K, Akashi K, Kusunoki I, Ikeda T, Katano N, Motoshima H, et al. Effect of fermented milk containing Bifidobacterium lactis Bb-12 on stool frequency, defecation, fecal microbiota and safety of excessive ingestion in healthy female students -2nd report. J Nutr Food 2005;8:39-51.

Vernazza CL, Gibson GR, Rastall RA. Carbohydrate preference, acid tolerance and bile tolerance in five strains of Bifidobacterium. J.Appl.Microbiol. 2006;100:846-853.

Vinderola C. Lactic acid starter and probiotic bacteria: a comparative "in vitro" study of probiotic characteristics and biological barrier resistance. Food Res.Int. 2003;36:895-904.

Wang KY, Li SN, Liu CS, Perng DS, Su YC, Wu DC, et al. Effects of ingesting Lactobacillus- and Bifidobacterium-containing yogurt in subjects with colonized Helicobacter pylori. Am.J.Clin.Nutr. 2004;80:737-741.

www.chr-hansen.com