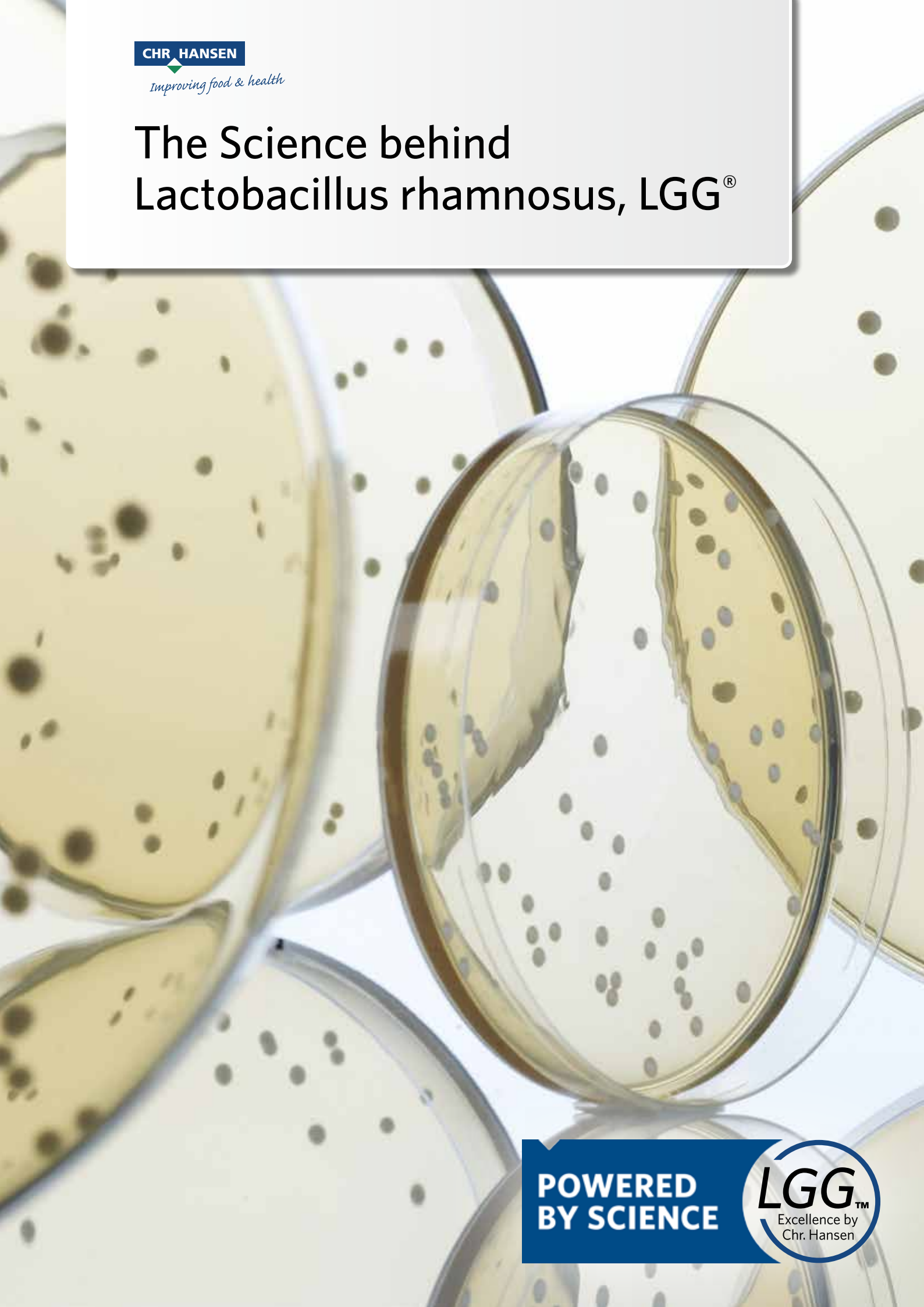
CHR. HANSEN

Improving food & health

The Science behind Lactobacillus rhamnosus, LGG®



POWERED BY SCIENCE

LGG™ Excellence by Chr. Hansen

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Summary

A Documented Solution

Lactobacillus rhamnosus, LGG®[1] (hereafter referred to by use of the trademark LGG®) is the world's best documented probiotic strain. After extensive research of naturally occurring lactobacilli, it was isolated by Gorbach and Goldin in 1985 (Goldin et al. 1992; Gorbach 1996). Since then the LGG® strain has been comprehensively studied *in vitro, in vivo* and in humans and is now described in more than 1100 scientific publications, out of which more than 300 are reports of studies in humans. LGG® has shown its beneficial health effect in numerous clinical studies, primarily within immune function and gastrointestinal health in children and adults.

Backed by Science

Strain characteristics and mechanisms of the LGG® strain have been established through extensive *in vitro* testing. These studies show that LGG® exhibits acid and bile tolerance and has excellent adherence properties to the intestinal mucosa, both important characteristics to increase persistence of the bacteria in the gastrointestinal (GI) tract. Clinical studies show that LGG® survives through the GI tract and can help restore and maintain the natural balance of good bacteria in the gut. LGG® shows good pathogen inhibition *in vitro* as well as enhancement of the gut barrier function. Furthermore, LGG® demonstrates strong immune interactions and stimulatory effects on immune cells.

These important characteristics of a probiotic strain translate into a number of beneficial health effects as demonstrated in numerous published clinical studies. LGG® has been tested most intensely in relation to immune defense against pathogens in the GI tract in children and antibiotic-associated side effects in adults. Moreover, the immune modulatory effects of LGG® have been studied intensely, mainly in relation to respiratory health and beneficial change in response to allergens.

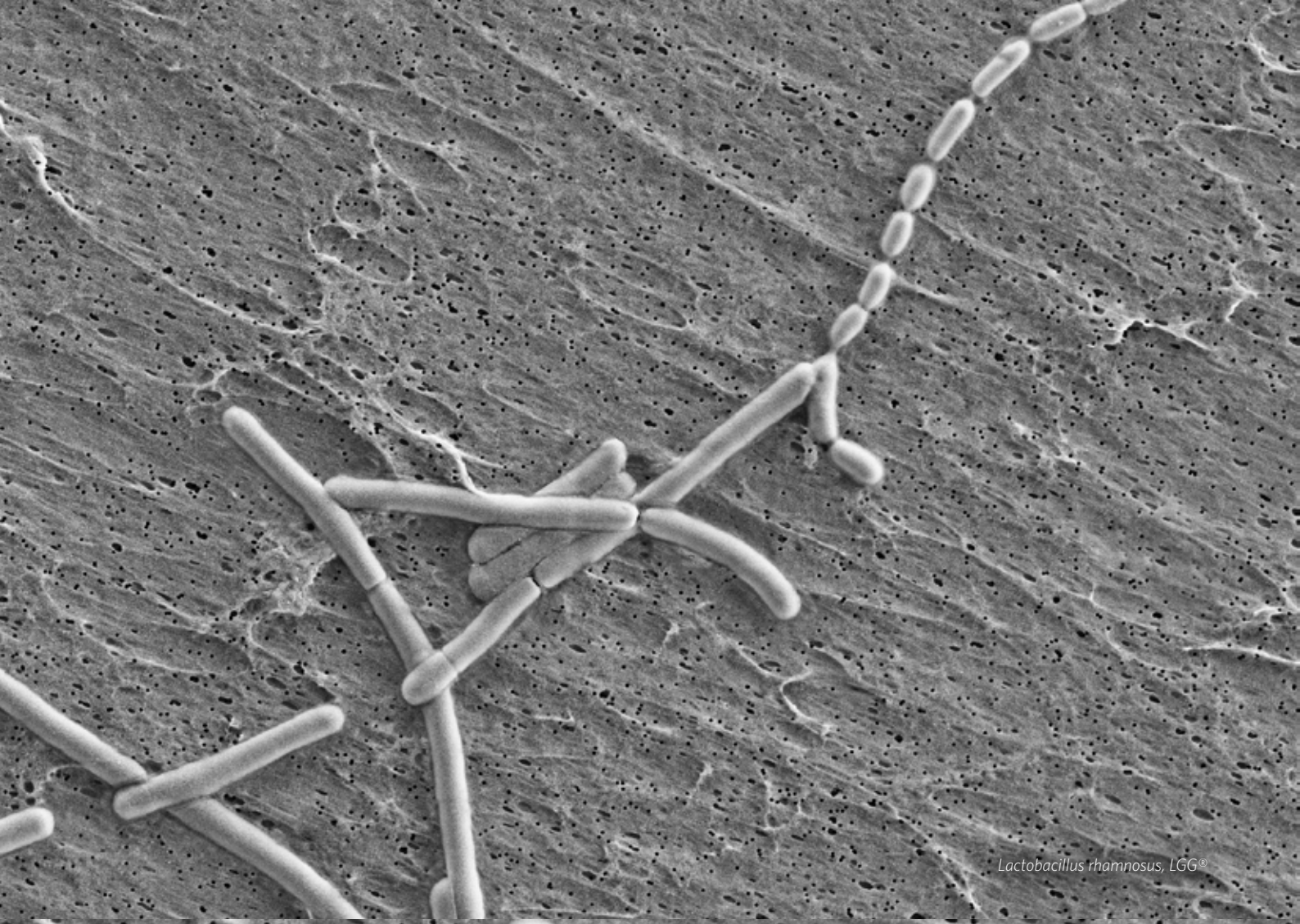
The safety of long term use of LGG® has been demonstrated in several studies. LGG® is considered safe for its intended use as an ingredient in food and dietary supplements to be consumed by a healthy population including infants.

The complete genome sequence of LGG® has been mapped and published (Kankainen et al. 2009).

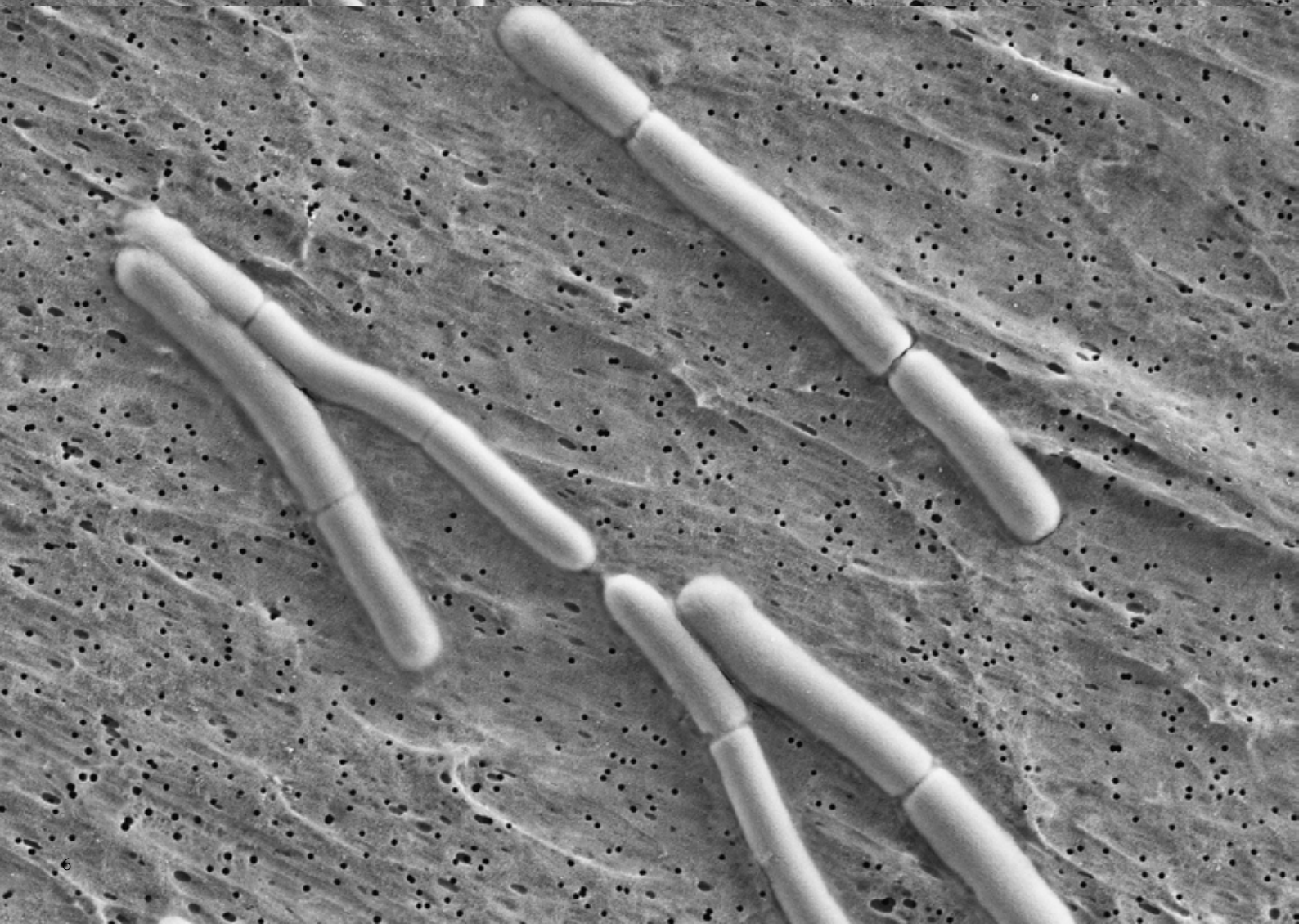
Frozen culture

[1] *LGG® is a registered trademark of Chr. Hansen A/S*

Test tubes for in-vitro testing



Lactobacillus rhamnosus, LGG®



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 10 million different non-human genes (Nielsen et al. 2014). Not surprisingly, the human microbiome plays a major role in human health through intimate interaction with our 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 confer a benefit 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, the ingested live microorganisms need to provide a beneficial effect on the host in order to be a probiotic. Thirdly, probiotics 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.

Strain Level

Probiotic properties are mainly strain specific (e.g. FAO/WHO 2001) and cannot be regarded as general for the entire species. The consensus of strain specificity is based on scientific research showing that different strains within the same species may display different probiotic effects. It is therefore essential to document characteristics, safety and efficacy of a probiotic at strain level (e.g. *Lactobacillus rhamnosus*, LGG®) and not only at species level (e.g. *Lactobacillus rhamnosus*).

The following text provides a review of the scientific documentation on the LGG® probiotic strain. It is not a complete listing of all available data on LGG® but rather a review of selected key data.

1. Taxonomy & Characterization

1.1 Taxonomy

Anette Wind, MSc
Senior Principal Scientist
Identification, R&D Microbial Platform

Lactobacillus is a genus of lactic acid producing, Gram-positive, non-spore forming, non-motile, and facultative anaerobic bacteria. *Lactobacillus* constitutes the major part of the lactic acid bacteria group. They are common constituents of the indigenous microbiota both in the human intestinal tract as well as in the vaginal tract.

The LGG® trademark applies to a catalase-negative, rod-shaped bacterium. The strain was originally thought to be a *Lactobacillus acidophilus* strain and was named Lactobacillus GG after the researchers that isolated the strain (SL Gorbach and BR Goldin). Later, as taxonomic methods improved, the strain was reclassified as *Lactobacillus rhamnosus*. Following current taxonomic opinion, LGG® (registered trademark of Chr. Hansen A/S) is identified as *Lactobacillus rhamnosus*. It has been deposited in the American Type Culture Collection as ATCC 53103.

"The strain was specifically selected to have optimal characteristics for a Lactobacillus dairy strain to benefit human health"

1.2 Origin & Selection

LGG® was isolated from a fecal sample from a healthy human adult in 1985 (Gorbach 1996). The strain was specifically selected to have optimal characteristics for a *Lactobacillus* dairy strain to benefit human health (Box 1, Gorbach 1996; Doron et al. 2005).

LGG® is technologically well suited, expressing fermentation activity, good stability and acid and bile tolerance, also as freeze-dried products in dietary supplements. Furthermore, the LGG® strain 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. LGG® has a history of safe use in food products since 1990 (Salminen et al. 2002; Doron and Snydman 2015) and has been used in infant formula, dietary supplements and fermented milk products worldwide.

Box 1:
Criteria for an Ideal Probiotic Strain

Resistance to acid and bile
Attachment to human epithelial cells
Colonization of the human intestine
Production of antimicrobial substances
Good growth characteristics
Beneficial effects of human health

Strain selection

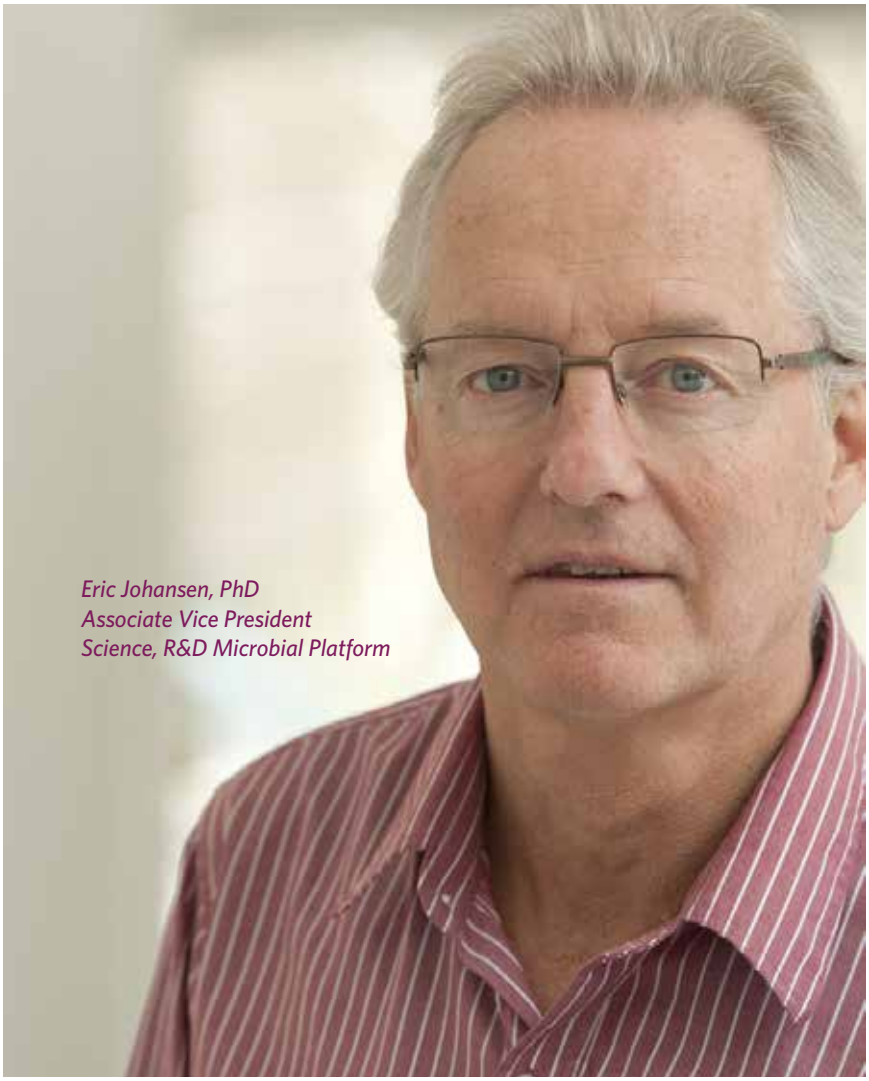
1.3 The Genome

Eric Johansen, PhD
Associate Vice President
Science, R&D Microbial Platform

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 bacterial strain is fundamental for full characterization of the strain and for thorough explorations of its mechanisms and potential as a probiotic.

The complete genome sequence of Lactobacillus GG (now referred to by the LGG® trademark) was made publically available in 2009 (Kankainen et al. 2009) and consists of a single circular chromosome of 3.0 million base pairs (Figure 1). Analysis of this sequence revealed a number of genes which are suggested to be critical to the probiotic capabilities of this strain (Kankainen et al. 2009). Differences to other *L. rhamnosus* strains were subsequently identified by Douillard et al. (2013).

One important use of a genome sequence analysis is to confirm the absence of sequences such as antibiotic resistance genes and virulence factors which are considered to be undesirable in probiotic products. The genome sequence of LGG® has been analyzed by the method of Bennedsen et al. (2011) confirming the absence of genes belonging to either of these undesirable categories. Possession of the complete genome sequence facilitates a number of other technologies for characterizing a strain. This includes gene expression studies, comparative genomics 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 LGG® (Danielsen and Johansen 2009; Garrigues et al. 2013).



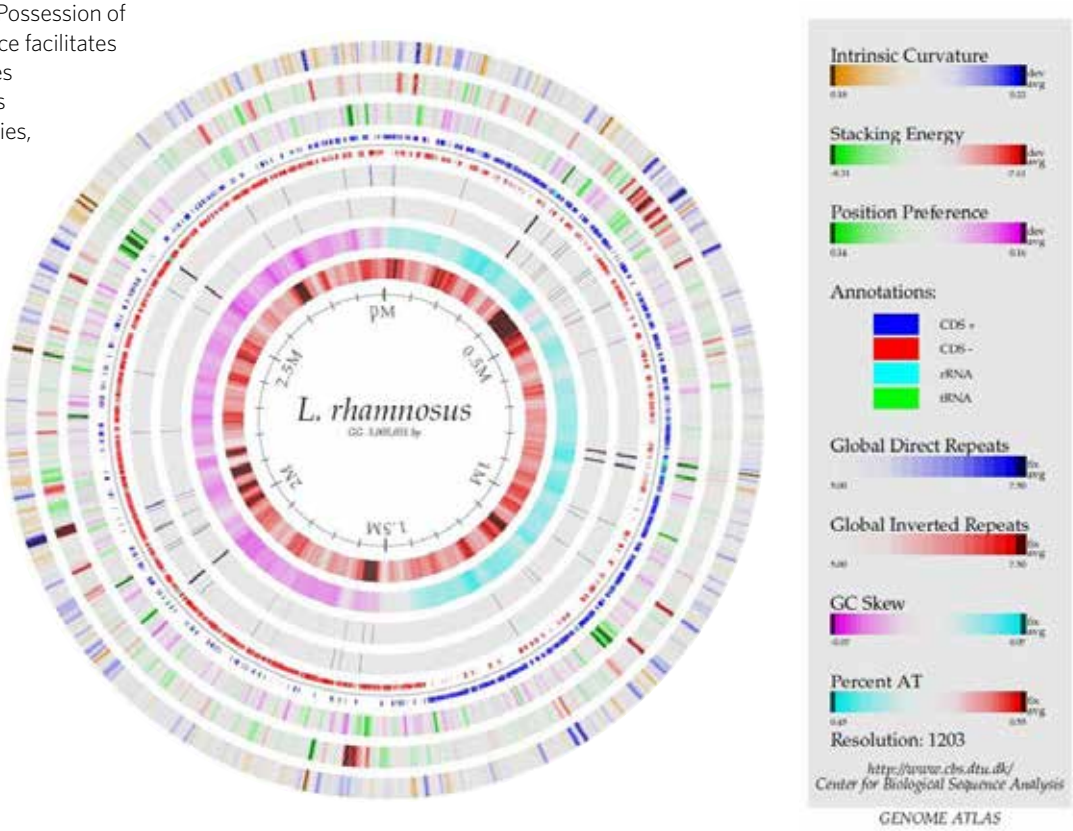


Figure 1. *The circular chromosome of Lactobacillus rhamnosus, LGG® as represented by a genome atlas diagram (Jensen et al. 1999)*

2. Strain Characteristics & Mechanisms

Anita Wichmann, PhD
Senior Scientist
Microbiome and Human Health Innovation

"LGG® exhibits acid and bile tolerance, and it shows excellent adherence properties to the intestinal mucosa"

The LGG® probiotic strain interacts with various components of the GI tract to influence host health. Strain characteristics and mechanisms of LGG® have been established through extensive *in vitro* testing. LGG® exhibits acid and bile tolerance, and it shows excellent adherence properties to the intestinal mucosa, both important characteristics to increase persistence of the bacteria in the GI tract. Furthermore, LGG® shows good pathogen inhibition, enhancement of gut barrier function, as well as strong immune interactions and stimulatory effects on immune cells; all very important characteristics of a probiotic strain (Figure 2).

2.1 Acid & Bile Tolerance

Two important factors in the body's defense against ingested microorganisms are gastric acid and bile, which protect against invading pathogens. However, acid and bile can also kill potentially beneficial probiotic bacteria. For probiotic effects that are dependent on viability and physiological activity in the intestine, the ability of a probiotic strain to survive in the presence of acid and bile is an important trait. An *in vitro* study found that LGG® survived at a rate of 73% after a two hour incubation at pH 2.5 and at a rate of 81% after a two hour incubation in either 0.3% or 1% bile (Mandal et al. 2016). These pH and bile concentrations are representative of physiological conditions in the stomach and small intestine, respectively, and the results indicate that LGG® has high acid and bile tolerance.

2.2 Bile Salt Hydrolase

Secretion of bile salts into the small intestine is important for emulsification of fats and proper digestion, but bile salts also pose a challenge to survival of ingested probiotic bacteria. The LGG® genome contains a gene encoding bile salt hydrolase (BSH), an enzyme that is important for coping with the high bile salt concentrations in the small intestine. A study characterizing the bile stress response of LGG® found that expression of BSH was strongly upregulated within 10 minutes of exposure to 0.2% bile (Koskenniemi et al. 2011), confirming that LGG® is well-equipped to survive upon encountering bile in the small intestine.

2.3 Adhesion

The ability of a probiotic strain to adhere to the intestinal mucosa is important for transient colonization. LGG® exhibits a strong capacity to adhere to mucus and to epithelial cells *in vitro* (Tuomola et al. 1999; Lebeer et al. 2012). LGG® has hair-like

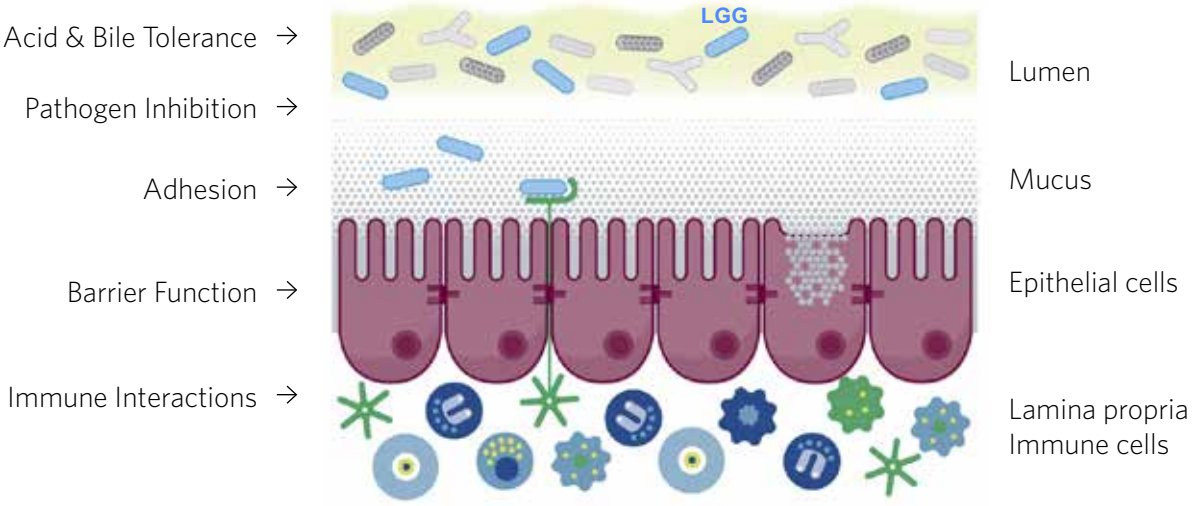


Figure 2. *LGG® interacts with various components of the GI tract to influence host health*

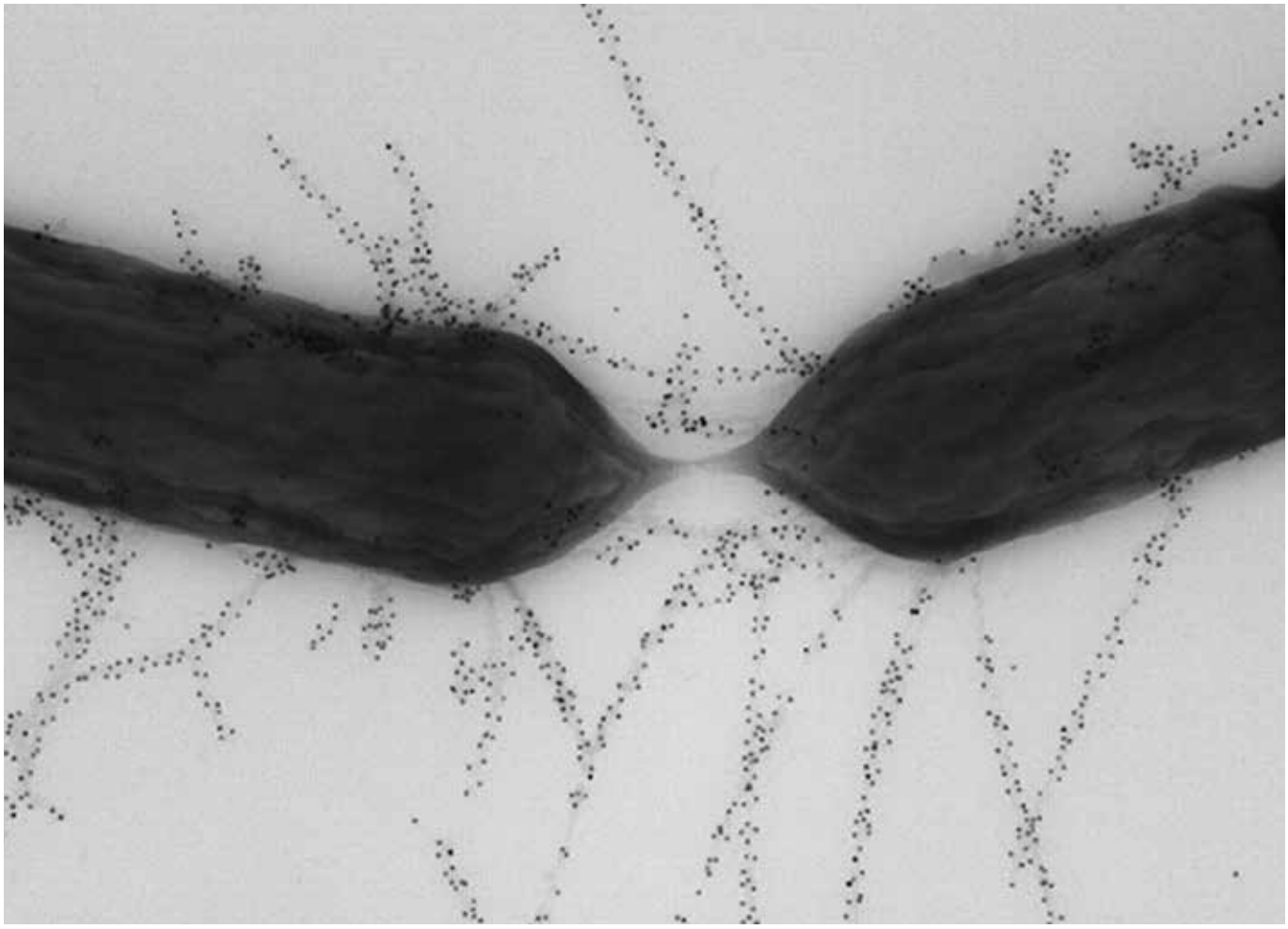


Figure 3. *TEM image of Lactobacillus rhamnosus GG* cells labeled with SpaA antiserum and 10-nm protein A gold particles.*
*Source: Justus Reunanen et al. Appl. Environ. Microbiol. 2012; 78:2337-2344. Reprinted with permission. (*LGG®)*

appendages on its cell surface called pili, which have been shown to be important for adhering to mucus *in vitro* (Figure 3).

A closely related strain that naturally lacks pili, *L. rhamnosus* Lc705, as well as mutants of LGG® that specifically lack pili adhere poorly to mucus *in vitro* compared with LGG® (Rasinkangas et al. 2014). In a human intervention study, LGG® was also shown to persist longer and at higher levels than *L. rhamnosus* Lc705 in the intestine (Kankainen et al. 2009).

A gene cluster encoding the pili subunits, spaCBA, has been identified in the LGG® genome, and as shown in Figure 3 the physical presence of pili on the cell surface has been visualized by transmission electron microscopy (TEM) (Kankainen et al. 2009; Reunanen et al. 2012). Deletions of the spaCBA genes have been reported for *L. rhamnosus* GG isolates from some commercial products[2] (Sybesma et al. 2013), implying a loss of pili and a reduced colonization potential. Importantly, genomic analysis of seven different Chr. Hansen production batches of LGG® confirmed genomic integrity and retention of the spaCBA genes (Chr. Hansen data on file), demonstrating the value of the highly controlled production and quality processes at Chr. Hansen A/S.

In summary, the pili on LGG® are important for mucus adhesion and persistence in the GI tract. Since adhesion facilitates a close interaction with the intestinal epithelium, it is expected to promote beneficial effects of LGG® on other mechanisms such as pathogen inhibition, barrier function and immune interactions as described in the following sections.

2.4 Pathogen Inhibition

The ability to inhibit disease-causing pathogens is an important mechanism by which probiotics may reduce the frequency, severity and/or duration of gastrointestinal infections. Several mechanisms by which probiotics may inhibit pathogens have been proposed, including production of inhibitory substances (lactic acid, bacteriocins), competition for nutrients or sites of adherence (mucus, cell receptors), toxin degradation, and induction of host immune responses.

Early work in the Gorbach lab, which isolated the LGG® strain, showed that concentrated LGG® supernatant was capable of inhibiting of a number of pathogens *in vitro*, including multiple strains of *E. coli, Streptococcus, Pseudomonas, Salmonella, Bacteroides fragilis*, and *Clostridium* (Silva et al. 1987). Later work showed that LGG® could inhibit *Salmonella enterica* subsp. *enterica* serovar Typhimurium invasion into Caco-2 cells (a human intestinal epithelial cell line) and reduce infection parameters in mouse models (Hudault et al. 1997).

The molecules responsible for these antimicrobial effects of LGG® are still not fully understood. While some evidence points to lactic acid, (De Keersmaecker et al. 2006), other experiments have shown that lactic acid alone cannot account for the full effect and suggest that other molecules are involved (Silva et al. 1987; Marianelli et al. 2010). For example, a short peptide with a strong antimicrobial activity against the Gram-negative pathogens *E. coli* EAEC 042 and *Salmonella typhi* has been isolated from LGG® conditioned medium (Lu et al. 2009). Since lactic acid can permeabilize the

[2] *L. rhamnosus GG is sold as LGG without trademark in some commercial probiotic products from suppliers other than Chr. Hansen A/S.*

"The pili on LGG® are important for mucus adhesion and persistence in the GI tract"

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Senior Scientist
Microbiome and Human Health Innovation

outer membrane of Gram-negative bacteria (Alakomi et al. 2000), lactic acid production by LGG® might work in conjunction with another antimicrobial molecule to exert its pathogen inhibition effects.

Another example of pathogen inhibition by LGG®, where a competitive exclusion mechanism involving the pili has been proposed, is in elimination of vancomycin-resistant enterococci (VRE). Hospital-acquired infections caused by VRE are a growing threat for weak and immunocompromised individuals, as VRE are resistant to many antibiotics and efficient at acquiring new antibiotic resistance genes. Thus, new approaches are needed. Several clinical studies have shown a beneficial effect of LGG® supplementation on eliminating colonization of VRE in hospitalized patients (Manley et al. 2007; Szachta et al. 2011). Genomic analysis of a clinical isolate of VRE, *Enterococcus faecium* E1165, revealed that it has a pili-encoding gene cluster PGC-3 that exhibits high sequence similarity to the spaCBA pili-encoding gene cluster of LGG® (Tytgat et al. 2016a). *In vitro* studies suggest that LGG® pili and *E. faecium* E1165 pili compete for the same mucus binding sites (Tytgat et al. 2016a), indicating that LGG® may competitively exclude VRE in the GI tract. Altogether, these studies indicate that probiotic administration with LGG® shows promise for elimination of VRE.

2.5 Barrier Function Enhancement

Maintenance of an intact mucus layer and epithelial cell layer in the GI tract is critical for maintenance of health. Enhancement of gut barrier function is one of the central and generally accepted mechanisms of probiotics as the ability to enhance intestinal barrier function may reduce the passage of pathogens and antigens across the gut barrier.

"LGG® is one of our best strains for enhancing gut barrier function"

Research at Chr. Hansen using Caco-2 cell monolayers, a well-established model of the intestinal epithelium, indicates that *L. rhamnosus*, LGG® is one of our best strains for enhancing gut barrier function (Chr. Hansen data on file).The LGG® strain has two major secreted proteins, Msp1/p75 and Msp2/p40, which have been shown to protect Caco-2 monolayers against hydrogen peroxide-induced epithelial barrier disruption (Seth et al. 2008). These purified secreted proteins limit cytokine-induced cell death and stimulate cell proliferation in *in vitro* models (Yan et al. 2007). Furthermore, oral administration of purified Msp2/p40 from LGG® showed a protective effect against chemically-induced colitis and epithelial barrier disruption in an experimental model in mice (Yan et al. 2011). Altogether, these mechanisms indicate how LGG® may protect and promote repair of the intestinal barrier.

2.6 Immune Interactions

Interaction with the immune system is another important mechanism of probiotics (Bron et al. 2011). Seventy to eighty percent of the body's immune cells are located in the gastrointestinal tract (Vighi et al. 2008), and gut microbes, including transiently colonizing probiotic bacteria, play a significant role in shaping immune responses (Macpherson et al. 2004). The ability to interact with immune cells and modulate immune function in the gut may increase resistance to infections and increase tolerance, potentially decreasing allergic conditions. Various components of LGG® can modulate immune responses of both epithelial and immune cells, and three key examples will be described here.

"...LGG® may induce host immune responses in the intestine that are beneficial for fighting infections"

First, lipoteichoic acid (LTA) is a major component of the cell wall of Gram-positive bacteria, and the specific structure of LTA varies among different strains of bacteria. The LTA of LGG® has been shown to interact with the Toll-like receptors TLR2-6 on the surface of Caco-2 intestinal epithelial cells and to stimulate expression of IL-8, a chemokine that plays a key role in recruiting neutrophils to the site of infection (Claes et al. 2012). Neutrophils are a type of white blood cells that fight infections by engulfing microbes, secreting antimicrobial substances and generating extracellular traps that bind and kill microbes. Thus, the ability of LGG® to stimulate IL-8 from epithelial cells *in vitro* suggests that LGG® may induce host immune responses in the intestine that are beneficial for fighting infections (Figure 4).

Second, purified pili from LGG® can interact with a specific motif on dendritic cells (DC-SIGN) to increase expression of cytokines such as IL-12 (Tytgat et al. 2016b). Research at Chr. Hansen has also shown that whole LGG® cells can stimulate secretion of IL-12 from dendritic cells *in vitro* (Chr. Hansen data on file). The capacity of a probiotic strain or purified bacterial component to stimulate IL-12 is an indication of their potential to stimulate Type 1 T helper (Th1) cells. Dendritic cells are used as a model for understanding how ingested probiotic bacteria could affect T cell populations, since dendritic cells in the lamina propria of the intestine can sample bacterial antigens and secrete various cytokines that drive differentiation of naïve T cells into different T cell subsets, such as Th1 cells. An increase in Th1 responses is important for the immune defense against pathogens, and a balance of Th1/Th2 cells is important for attenuation of hypersensitivity to allergens. Thus, the ability of LGG® to stimulate IL-12 *in vitro* supports a model in which LGG stimulates immune modulatory effects in the intestine that are beneficial for maintaining health.

Third, if some probiotic bacteria are lysed in the intestine, they can release specific DNA motifs that have immune modulating potential. Bacterial DNA is different from human DNA in that unmethylated cytosine-guanine dinucleotide (CpG) motifs are frequent. The LGG® genome contains a specific CpG motif, named ID35, which can stimulate Th1 responses *in vitro* as well as in an ovalbumin-sensitized mouse model of allergy (Iliev et al. 2005; Iliev et al. 2008). Although LGG® exhibits good acid and bile tolerance, it is likely that some fraction of ingested bacteria will not survive passage through the intestine. The immune modulating potential of the ID35 DNA motif provides an example of how LGG® may exert beneficial health effects even if it is not viable.

In conclusion, the immune modulatory mechanisms described here provide plausible explanations for some of the clinically documented effects of LGG®, for example the positive effects shown in clinical studies of on GI and respiratory health (Vlasova et al. 2016; Hojsak et al. 2010a), skin health (Kalliomäki et al. 2001) and attenuation of hypersensitivity to allergens (Berni Canani et al. 2013).

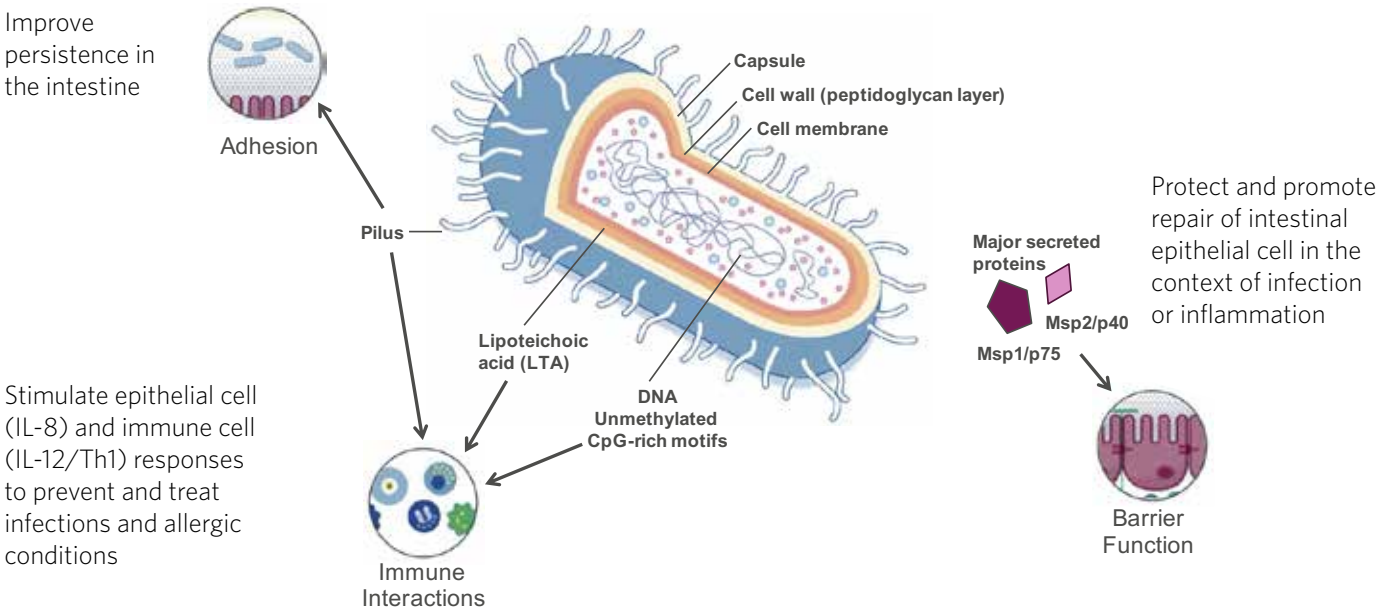

Figure 4. *The molecular modes-of-action of LGG® support the clinically documented benefits of LGG® in the defense against pathogens and attenuation of hypersensitivity to allergens.*

3. Clinical Efficacy
– Survival and Modulation of Microbiota

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Human Health

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3.1 Documented Efficacy

Lactobacillus rhamnosus (LGG®) is the world's most documented probiotic strain. The LGG® strain has been comprehensively studied *in vitro*, *in vivo* and in humans and is now described in more than 1100 scientific publications out of which more than 300 are reports of studies in humans.

"LGG® has shown beneficial effects within various health areas in newborn, preterm infants, children, pregnant women, adults and elderly"

It is a prerequisite for a probiotic to have a documented beneficial effect in clinical studies. Probiotic properties are mainly strain specific (e.g. FAO/WHO 2001) and cannot be regarded as general for the entire species. Thus, the clinical or laboratory effects documented for one probiotic strain cannot be assumed for another probiotic strain, not even strains within the same species (Fuchs-Tarlowsky et al. 2016). Clinical studies and systematic reviews within different indication areas have highlighted that different probiotic species and strains can have very different effects both *in vivo* and *in vitro* (e.g. Fuchs-Tarlowsky et al. 2016; Hungin et al. 2013; Mantegazza et al. 2017). Dating back to 1987, LGG® has been tested in clinical trials for over 30 years (Gorbach et al. 1987). LGG® has shown beneficial effects within various health areas in newborn, preterm infants, children, pregnant women, adults and elderly in dosages ranging from $1x10^8$ (Kumpu et al. 2013) to $2x10^{12}$ CFU/day (Basu et al. 2009) with the majority of the studies within the areas of immune function and gastrointestinal function.

3.2 Survival in the Gastrointestinal Tract

Some probiotic mechanisms assume viability and physiological activity of the probiotic at the target site. Biopsies or samples from the relevant target site can confirm presence and viability of the ingested bacteria, but since the target site may not always be well-defined, fecal recovery, although less sensitive, is often used to confirm viability of probiotics in the GI tract.

Several clinical studies have confirmed *in vitro* findings of LGG® being able to survive through the GI tract and adhere to the intestinal mucus and epithelial cells. Alander at al. (1999) compared recovery of viable cell in colonic biopsy samples and feces taken from adults after consumption of a fermented drink with LGG® for 12 days. Interestingly, LGG® was recovered in the majority of the biopsies (87.5%) for up to 21 days after LGG® intake was stopped, while viable LGG® was recovered in all feces samples after 14 days, but only in 25% of the feces samples after 21 days. After 28 days, no LGG® was recovered from fecal samples, but in 28.6% of the colonic biopsies. Similarly, in a recent study (Poutsiaka et al. 2017) using PCR techniques, viable LGG® was identified in feces of 89.5% of adult volunteers after 21 days of intake of powder sachets containing LGG® but not 28 days after termination of the intake.

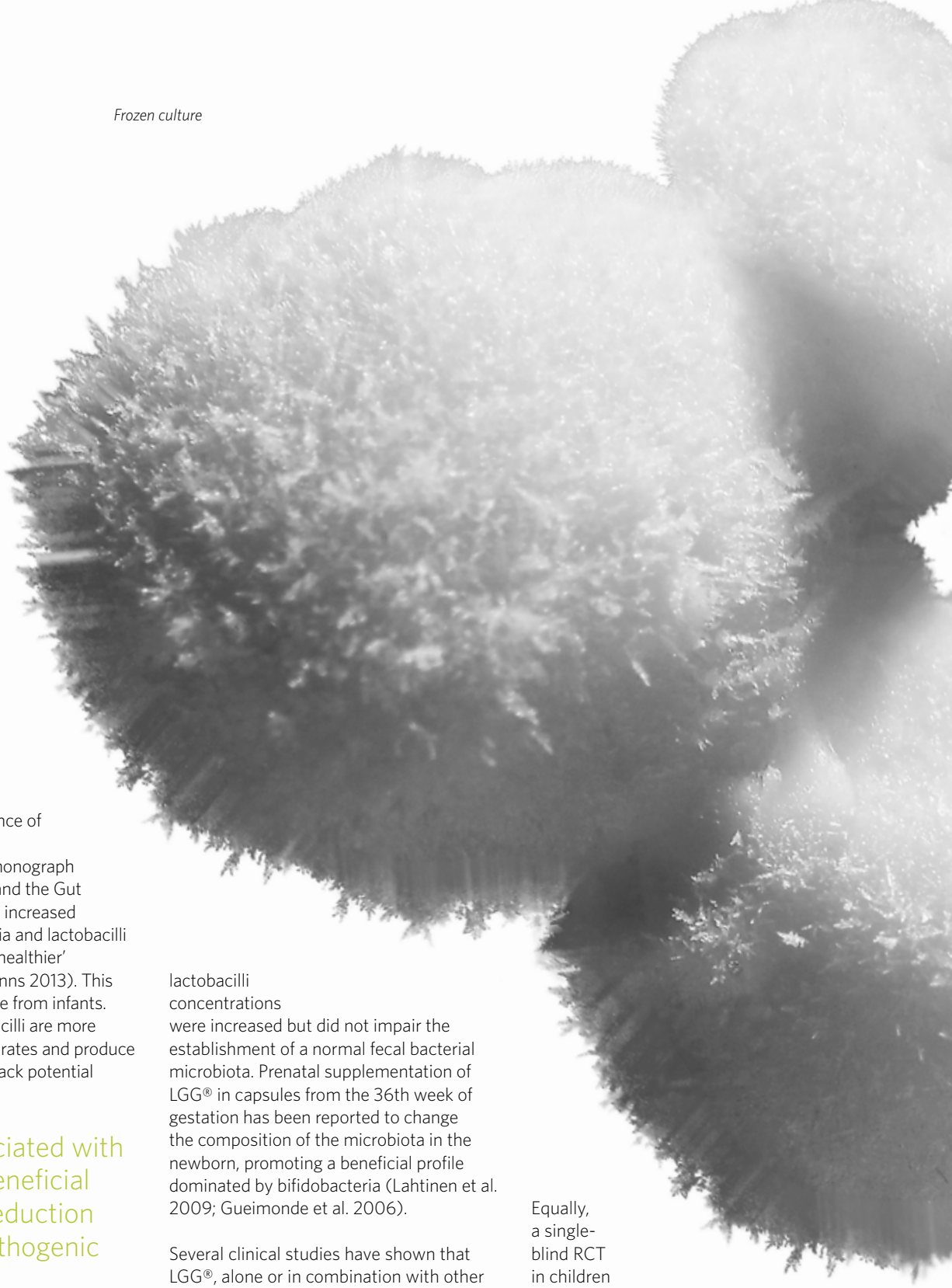
"...LGG® transiently colonizes the human colonic mucosa"

Studies have investigated fecal recovery of LGG® after ingestion in various types of products. In a study performed by Goldin et al (1992) on 76 healthy adult volunteers, LGG® was recovered in the feces of all subjects receiving fermented drinks and in 86% of all subjects receiving frozen concentrate when tested up to 7 days after discontinuation of LGG® intake. Subsequently, it has been demonstrated, that LGG® can be recovered in fecal samples from children and adults when consumed in various types of products such as yoghurt, cheese and capsules (Saxelin et al. 2010) as well as infant formula (Vendt et al. 2006).

Taken together, these data show that not only does LGG® survive well during the passage through the GI tract, particularly when ingested with food or dairy products (Goldin et al. 1992); it also transiently colonizes the human colonic mucosa.

3.3 Modulation of the Intestinal Microbiota

The human large intestine is host to a wide variety of bacteria, with lactobacilli being prominent members of this complex ecosystem. The intestinal microbiota serves an important function in maintaining health. A healthy human microbiota is metabolically active and acts as a defense mechanism for our body. Deviations in its composition are related to multiple disease states within and beyond the GI tract (Salminen and Gueimonde 2005). A symbiotic relationship exists between the gastrointestinal microbiota and the host, with the host providing a stable environment and nutrients for the microbiota, while the microbiota has a significant role in maturation of the GI



tract, processing nutrition and protecting the host from harmful microbes. Furthermore, the gastrointestinal microbiota is the largest immunological organ of the body playing an important role in the maturation and maintenance of the immune system.
The ILSI Europe concise monograph on 'Probiotics, Prebiotics and the Gut Microbiota' stated that an increased proportion of bifidobacteria and lactobacilli is thought to represent a 'healthier' microbial composition (Binns 2013). This is partly based on evidence from infants. Bifidobacteria and lactobacilli are more likely to ferment carbohydrates and produce acids, and they generally lack potential toxicity (Binns 2013).

"...LGG® is associated with an increase in beneficial bacteria and a reduction in potentially pathogenic bacteria"

Some studies have investigated fecal species diversity after postnatal ingestion of LGG®. While one study found that LGG® seemed to affect neonatal intestinal colonization causing a higher species diversity compared to placebo (Agarwal et al. 2003), others found that the overall microbial diversity did not seem to change (Ismail et al. 2012). In a small study where 15 newborns were administered LGG® for two weeks (Sepp et al. 1993), 67% excreted LGG® and in eight cases (53%) LGG® was found in feces two weeks after administration was stopped. The intestinal lactobacilli concentrations were increased but did not impair the establishment of a normal fecal bacterial microbiota. Prenatal supplementation of LGG® in capsules from the 36th week of gestation has been reported to change the composition of the microbiota in the newborn, promoting a beneficial profile dominated by bifidobacteria (Lahtinen et al. 2009; Gueimonde et al. 2006).

Several clinical studies have shown that LGG®, alone or in combination with other probiotics or ingredients, is associated with an increase in beneficial bacteria and a reduction in potentially pathogenic bacteria (e.g. Benno et al. 1996; Manley et al. 2007; Szachta et al. 2011). Notably, some studies have shown a beneficial effect of LGG® supplementation on eliminating colonization of vancomycin-resistant enterococci (VRE) in hospitalized patients (Manley et al. 2007; Szachta et al. 2011). In a double-blind, randomized, placebo-controlled trial (RCT) in which yoghurt with LGG® was given to renal patients for eight weeks, VRE was cleared in all patients in the LGG® group but only in 8.3% in the control group (Manley et al. 2007).

Equally, a single-blind RCT in children showed a significant higher VRE clearance in the probiotic group compared with control groups after three weeks (Szachta et al. 2011). Furthermore, some smaller studies indicate that LGG® may be able to reduce re-occurrence of proliferation of Clostridium difficile in patients with relapsing C difficile-induced diarrhea (Doron et al. 2005).

In conclusion, clinical studies indicate that LGG® can improve the balance of the intestinal microflora in children and adults favoring growth of beneficial bacteria and reducing potentially pathogenic bacteria.

4. Clinical Efficacy - Health Benefits

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Scientific Advisor
Human Health

Probiotics have been defined as *'Live microorganisms which when administered in adequate amounts confer a health benefit on the host'* by FAO/WHO (2001), a definition that was later been confirmed by an international consensus group (Hill et al. 2014). As the definition implies, probiotics should convey health benefits. They can do so by interacting with commensal bacteria but can also have a direct impact on the host. Some of the key challenges are to understand the mechanisms of action of probiotics and to elucidate more specifically which probiotic strains can offer which health benefits (Binns 2013).

"...results with one specific *Lactobacillus* strain cannot be generalized"

Probiotic bacteria are proposed to benefit human health mainly by three general mechanisms of action. First, certain probiotics can exclude or inhibit pathogens, either through direct action or through influence on the commensal microbiota. A second mechanism is the capacity of certain probiotic strains to enhance the epithelial barrier function by modulating signaling pathways or increase tight junction functioning. Third, most probiotic strains can also modulate host immune responses, exerting strain-specific local and systemic effects (Segers and Lebeer 2014).

As described in Section 2 of this booklet, the LGG® strain has shown strong results on all of these important mechanisms *in vitro* and has furthermore been shown to have a variety of beneficial effects in humans. Given the complexity of these three main functions, it is evident that different strains must evoke different responses in the host. Therefore, results with one specific *Lactobacillus* strain cannot be generalized (Lebeer et al. 2008). Molecular research on probiotics should carefully pay attention to these strain-specific properties. Different probiotic *Lactobacillus* strains have been associated with different effects related to their specific capacities to express particular surface molecules or to secrete proteins and metabolites directly interacting with host cells (Lebeer et al. 2008).

During 30 years of research, a high number of *in vitro* studies, experimental animal models and clinical interventions have explored the potential benefits of LGG® on human health, mainly in the areas of immune and gastrointestinal function. Ultimately only clinical studies can document efficacy in human populations, while *in vitro* studies as well experimental animal studies may help to explain the mechanisms by which the probiotic strain exerts its effect. Since the first clinical publication in 1987 (Gorbach et al. 1987) the LGG® strain has been extensively studied in humans and there are now more than 300 reports of studies in humans with LGG® alone or in combination with other probiotics, where LGG® has proven its beneficial health effect in immune and gastrointestinal health in children and adults. The following sections will present results from the most important clinical studies documenting the health benefits of the LGG® probiotic strain.

[3] *Bifidobacterium, BB-12® is the Bifidobacterium animalis subsp. lactis strain and BB-12® is a registered trademark of Chr. Hansen A/S*

4.1 Gastrointestinal Health

Given its excellent intestinal mucus adherence capacities, LGG® is often selected as candidate probiotic in studies of defense against pathogens in the GI tract (Segers and Lebeer 2014). Most of these studies have been conducted in children and the data has consistently shown a statistically significant benefit of LGG® alone or in combination with other probiotics (e.g. *Bifidobacterium*, BB-12®[3]) in supporting the immune defense against pathogens in the GI tract.

4.1.1 Infants & Children

Infectious diarrhea is a major world health problem, responsible for several million deaths each year (FAO/WHO 2001). Although the majority of deaths occur amongst children in developing countries, it is estimated that up to 30% of the population even in developed countries are affected by foodborne diarrhea each year. Some of the strongest evidence for a beneficial effect of defined probiotic strains on defense against pathogens in the GI tract has been established using LGG®, mainly in children affected by rotaviruses (e.g. FAO/WHO 2001).

Nosocomial (hospital- or healthcare acquired) infections may prolong hospital stay, worsen treatment outcome, and increase resistance of microorganisms to antimicrobials thereby increasing the cost of health care (Hojsak et al. 2017). The incidence of nosocomial infections in children in developed countries is 5.1% to 11.6% (Hojsak et al. 2017). Gastrointestinal infections account for the majority of nosocomial infections with rotavirus as a major pathogen (Hojsak et al. 2017).

A double-blind RCT including 90 hospitalized children (6 mo-5 yrs) looked at the effects of supplementation with $6x10^9$ CFU/day LGG® and micronutrients (vitamin B, C and zinc) (Bruzzese et al. 2016). The results of the study showed that fewer children in the LGG® group than in the control group contracted nosocomial infections during their hospital stay (p=0.008) with a reduced incidence of nosocomial gastrointestinal infection (p=0.016). A reduced duration of hospital stay was observed in the LGG® group (p=0.003) as well as a reduced incidence of infections in the three months follow-up period (p=0.02). The mean duration of infection symptoms during the follow-up period was significantly shorter in the LGG® group than the control group (p=0.03). Cox regression analysis showed a 56% reduction in the risk of infections in the LGG® group compared with the control group (HR=0.44; 95% CI: 0.22–0.89; p=0.023)

Another double-blind RCT including 742 hospitalized children (> 1 year) investigated the effect of $1x10^9$ CFU/day LGG® in fermented milk (Hojsak et al. 2010a). The results showed that the risk of both respiratory- and gastrointestinal infections was significantly reduced in the LGG® group compared with the control group. Furthermore, there was a significant reduction in number of gastrointestinal infections lasting >2 days.

An earlier double-blind RCT included 81 infants of 1-36 months of age (Szajewska et al. 2001). The children received LGG® ($6x10^9$ CFU twice daily) or placebo for the duration of their hospital stay. In the LGG® group the risk of nosocomial diarrhea was significantly reduced compared with the control group (6.7% vs. 33.3%, p=0.002). This was also the case for rotavirus gastroenteritis (2.2% vs. 16.7%, p=0.02), while there was no significant difference in duration of diarrhea between the groups.

Another RCT (n=71) looked at 4-45 months old children hospitalized with acute diarrhea from rotavirus (Isolauri et al. 1991). They were given an LGG®-fermented milk product, LGG® powder (both in doses of 1010-1011 CFU twice daily) or pasteurized yoghurt (control) for 5 days after oral rehydration. The duration of diarrhea was observed to be significantly shorter in the LGG® groups (1.4 days in both) compared with the control group (2.4 days) ($p<0.001$).
The described studies were all performed in Europe, but other studies have looked into population groups from developing countries. One RCT randomized 204 undernourished Peruvian children (6 to 24 months) to receive either LGG® (3.7×10^{10} CFU/day) or placebo once daily, 6 days a week for 15 months (Oberhelman et al. 1999). A significantly lower incidence of diarrhea was observed in the LGG® group compared with the control group (5.21 vs. 6.02, $p=0.028$) and significantly less adenovirus infections were observed in the LGG® group compared with the control group (8 vs. 19, $p=0.03$). The effects were the largest in non-breastfed children.

Another study performed in north India (Aggarwal et al. 2014) was an open-labeled RCT including 200 children (6 mo – 5 yrs) with acute watery diarrhea. The children received either LGG® (1×10^{10} CFU/day) or no probiotic for five days in addition to standard WHO management of diarrhea. Median duration of diarrhea was significantly shorter in children in LGG® group ($p<0.001$). Also, there was

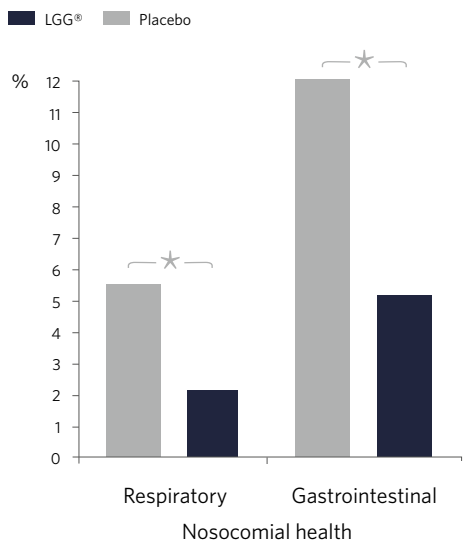

Figure 5: *In a randomized, double-blind, placebo-controlled study by Hojsak et al. 2010, LGG® was found to improve nosocomial health.*

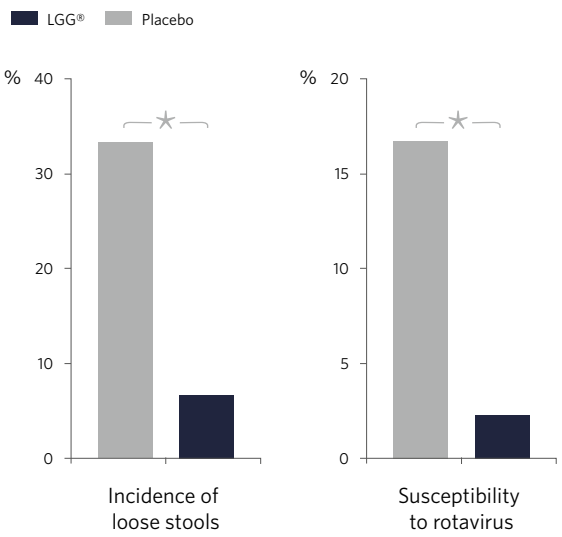

Figure 6: *LGG® reduced the susceptibility of loose stools and rotavirus in a study by Szajewska et al. 2001.*

significantly faster improvement in stool consistency in children receiving LGG® than in the control group (p<0.001) and there was significant reduction in average number of stools per day in the LGG® group (p<0.001) compared with the control group.

Another study performed in India (Sindhu et al. 2014) enrolled 124 children (6 mo-5 yr) with gastroenteritis, testing positive for either rotavirus or Cryptosporidium species in stool. One-third of the children had severe diarrhea. The children were randomized to receive LGG® or placebo once daily for 4 weeks. At the end of follow-up, fewer children with rotavirus diarrhea in the LGG® group had repeated diarrheal episodes compared with the control group (25% vs. 46%; p=0.048) and fewer had impaired intestinal function (48% vs. 72%; p=0.027). A significant increase in immunoglobulin G (IgG) levels post intervention was observed in children with rotavirus diarrhea receiving LGG® (p=0.003). Among children with *Cryptosporidium* diarrhea, those receiving LGG® showed significant improvement in intestinal permeability as measured by lactulose to mannitol ratio for intestinal permeability.

Finally, a recent study assessed the effect of a combination of LGG® and *Bifidobacterium*, BB-12®, on diarrhea in children with severe acute malnutrition (SAM) in Uganda. The study was a double-blind RCT enrolling 400 children (6-59 months) with SAM. Each day, the children received a stick with $5x10^9$ CFU of LGG® and $5x10^9$ CFU of BB-12®. Duration of the study was on average 18 days (inpatient period) followed by an 8-12 weeks outpatient period. The primary endpoint was days with diarrhea during inpatient treatment while days with diarrhea during outpatient treatment was a secondary endpoint. Although there was no difference between the control and probiotic groups on the primary endpoint, a 26% reduction in duration of diarrhea was observed in the probiotic group during outpatient treatment (p=0.025).

A meta-analysis (Szajewska et al. 2013) combined data from 11 RCTs (n = 2444) and concluded that LGG® significantly reduced the duration of diarrhea compared with placebo or no treatment. Another meta-analysis (Szajewska et al. 2011) looked at three RCTs involving 1092 children. It was concluded, that LGG® administration for the duration of hospital stay was associated with significantly lower rates of diarrhea and symptomatic rotavirus gastroenteritis.

A guideline based on a systematic review of evidence and performed by a working group for ESPGHAN on probiotics for the prevention of nosocomial diarrhea in children was published in 2017 (Hojsak et al 2017). Based on the level of evidence for LGG®, the working group specifically recommends using LGG® if the use of probiotics for preventing nosocomial infections in children is considered.

Taken together, these studies indicate that LGG® may have a beneficial effect on the immune defense against pathogens in the GI tract in infants and children.

4.1.2 Adult Travelers

Two studies investigated the effect of LGG® in adult travelers. In one study, 820 volunteer tourists were given $2x10^9$ CFU/day LGG® or placebo in sachets before their trip to a destination with high risk of diarrhea (Oksanen et al. 1990). The data was collected by a questionnaire that was returned during the home flight. Probiotic administration showed a reduction in the incidence of acute diarrhea in one of the two destinations (24% vs. 40%, p=0.04 for one week's trip), but not in the total population (41% vs. 46%, ns.). In the second study (Hilton et al. 1997) adult participants (n=245) were randomized to LGG® ($2x10^9$ CFU/day) or placebo and the results showed a significantly lower incidence of acute diarrhea in the LGG® group compared with the control group (3.9% vs. 7.4%, p=0.05).

In summary, LGG® may help maintain gastrointestinal health and a healthy stool consistency while travelling.

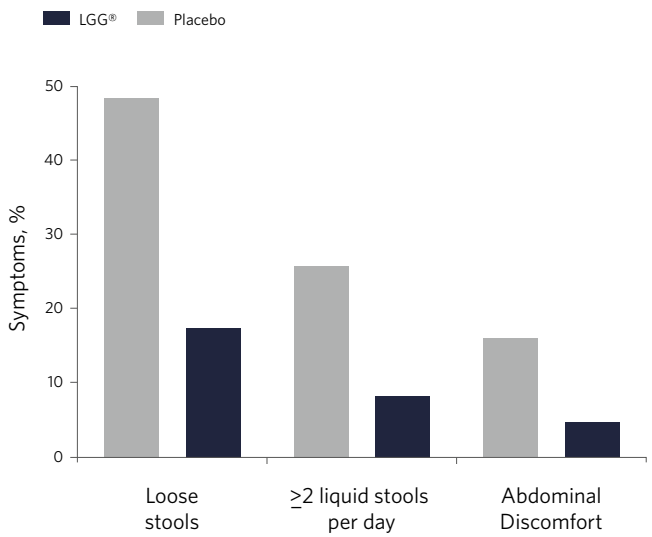

Figure 7: *Effect of L. rhamnosus GG on intestinal symptoms caused by antibiotics in children (Vanderhoof et al 1999)*

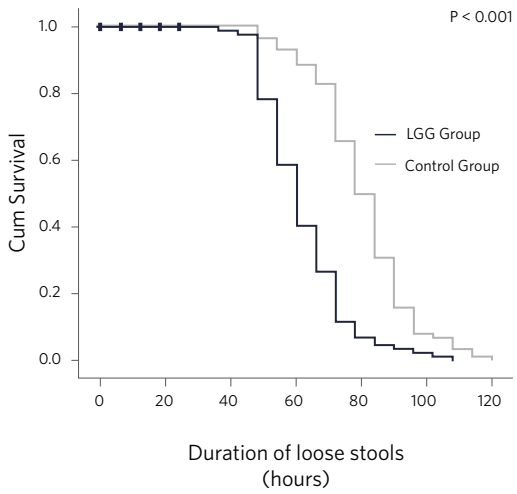

Figure 8: *From Aggarwal et al. 2014*

4.1.3 Antibiotic-Associated Loose Stools

The administration of antimicrobial agents disturbs the ecological balance between the host and the microbiota (Sullivan et al. 2001). Therefore administration of antibiotics may cause side-effects such as abdominal pain and diarrhea. One of the most common uses for probiotics is to decrease gastrointestinal discomfort as well as the increased frequency and liquidity of stools in relation to antibiotic treatment. Ultimately, this may increase compliance to the antibiotic treatment and furthermore, probiotics may accelerate recovery of normal gastrointestinal comfort and bowel habits after antibiotic treatment.

In a double-blind RCT, common acute infections in 188 children were treated by commonly used antibiotics (Vanderhoof et al. 1999). Half of the children received LGG® capsules (1-2x10^{10} CFU/day); the other half received identical placebo capsules. Significantly fewer daily defecations were reported in the LGG® group than in the control group. Furthermore, the stools were more solid and the LGG® group had less abdominal pain than the placebo group. LGG® did not cause any side effects.

In another study, children were prescribed oral antibiotics for the treatment of acute respiratory infections (Arvola et al. 1999). The children were randomized to receive either one LGG® capsule twice a day (2x10^{10} CFU) or a placebo capsule. Within two weeks of antimicrobial treatment the incidence of diarrhea was observed to be significantly lower in the LGG® group (5%) compared with the probiotic control group (16%, p=0.05).

In an early study in healthy adult volunteers, reduced diarrhea, abdominal distress, stomach pain and flatulence were observed when LGG® but not placebo was supplemented with erythromycin (Siitonen et al. 1990).

A recent review looked at studies on probiotics for antibiotic associated diarrhea in children (Mantegazza et al. 2017). The authors considered that probiotics have strain-specific effects and thus focused on individual probiotic strains and not on probiotics in general. After reviewing the literature, they made specific recommendation for the use of LGG® (Mantegazza et al. 2017).

4.1.3.1 Helicobactor pylori

In adults, probiotics have been given parallel with Helicobacter pylori triple therapy in several studies to investigate the ability of probiotics to support the host defense against gastrointestinal discomfort and increased frequency and liquidity of stools in relation to the treatment.

Three studies where LGG® is given over the same period as H. Pylori triple therapy in adults have been published. In a pilot study (Armuzzi et al. 2001a), 120 asymptomatic volunteers carrying H. pylori were randomized to triple therapy for one week or the same regimen supplemented with LGG® (6x10^9 CFU/sachet) for two weeks. Bloating, diarrhea and taste disturbances were the most frequent side effects during the eradication week and were observed to be significantly reduced in the LGG® group compared with the non-probiotic group (bloating: p=0.014; diarrhea: p=0.023; taste disturbances: p=0.007). In a subsequent double-blind RCT 60 healthy asymptomatic H. pylori positive volunteers were randomized to one week eradication therapy with parallel supplementation of LGG® (109 CFU/sachet) or probiotic placebo for two weeks (Armuzzi et al. 2001b). Again, diarrhea, nausea and taste disturbances were observed to be significantly reduced in the LGG® group compared to the control group. An overall assessment of treatment tolerability showed a significant difference in favor of the LGG® group (p=0.04). There was no difference between the groups in the success of the eradication of H. pylori which was about 80%.

A study using a very similar design (Cremonini et al. 2002) also observed reduction of incidence of antibiotic-associated diarrhea after supplementation with LGG® in comparison with the probiotic placebo group (5% vs. 30% in week 1, p=0.018).

A recent study investigated LGG® in combination with Bifidobacterium, BB-12® in a double-blind RCT with 804 subjects with H. pylori infection (Hauser et al. 2015). All subjects received standard H. pylori triple therapy eradication. LGG® + BB-12® were supplemented in capsules of 0.2-20x10^9 CFU/day. Probiotics were ingested twice daily for two weeks. What was described as an increased cure rate was observed in the LGG® + BB-12® group (p<0.001). Furthermore, compliance to treatment was higher in the probiotic group and significantly reduced incidences of several gastrointestinal side effects such as diarrhea (p<0.001), nausea (p<0.001), bloating (p=0.022), and flatulence (p<0.001) were observed in the probiotic group.

In conclusion, clinical studies using probiotic doses ranging from 1-2x10^{10} CFU/day in children and a dose of 6x10^9 CFU/day in adults (in one study undefined between 0.2-20x10^9) have shown that LGG® may reduce gastrointestinal discomfort as well as frequent and loose stools related to antibiotic therapy in children and adults.

Although LGG® is susceptible to the most common antibiotics it has been shown to be able to survive in the intestines during antibiotic treatment. The survival of LGG® may be explained by the antibiotic and bacterial preparations being taken at different times, and possibly by the lower antibiotic level in the bowel than in the blood stream.

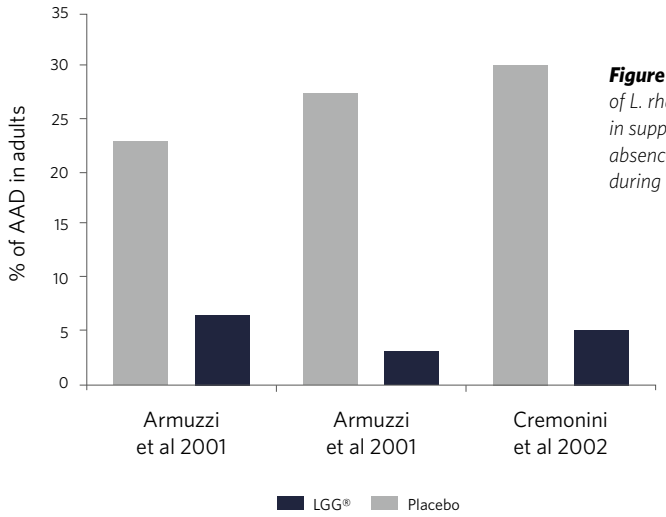

Figure 9: *Efficacy of L. rhamnosus GG in supporting the absence of loose stools during antibiotic use.*

4.1.4 Gastrointestinal Discomfort

Occasional episodes of abdominal pain or discomfort (e.g. bloating, abdominal pain/cramps, straining and rumbling), in the absence of organic diseases or biochemical abnormalities, are commonly associated with food or drug intake or with alterations of bowel habits and vary between individuals in frequency and severity. Reducing gastrointestinal discomfort is considered an indicator of improved gastrointestinal function, which is a beneficial physiological effect for the general population (EFSA NDA Panel 2016).

Functional gastrointestinal disorders such as irritable bowel syndrome (IBS), often includes symptoms of abdominal pain or discomfort, constipation and/or diarrhea and abdominal bloating/distention (Drossman 2016; Lacy et al. 2016). The world-wide prevalence of IBS is 11.2% based on a meta-analysis of 80 studies involving 260,960 subjects. Prevalence rates are higher for women than for men and younger people are more likely to be affected than those older than age 50 years (Lovell and Ford 2012; Lacy et al 2016).

A growing number of studies indicate that the diversity, stability and metabolic activity of the gut microbiota may be altered in patients with irritable bowel syndrome (IBS) compared with healthy individuals (Collins 2014). Likewise, animal studies have demonstrated that changes in the gut microbiota can result in altered host function, in domains relevant to IBS, e.g. gut motility, visceral pain responses, intestinal permeability, and brain function and behavior (Collins 2014). Colonization with gut microbiota from patients with IBS can also induce gut dysfunction in mice reminiscent of that seen in IBS. Together these findings strongly suggest that the microbiota contributes to the expression of IBS (Collins 2014).

Two studies looked at the effect of LGG® supplementation in children with functional abdominal pain disorders (Gawronska et al. 2007; Francavilla et al. 2010). One double-blind RCT enrolled 104 children (6-16 years) with functional dyspepsia (FD), IBS or functional abdominal pain (FAP) according to the Rome II criteria (Gawronska et al. 2007). LGG® ($3x10^9$ CFU) or matching placebo was given twice daily for 4 weeks. The primary outcome measure was no pain at the end of the study. The results showed that more subjects in the LGG® group than in the placebo group had no pain at the end of the study (25% vs. 9.6%). This result was reflected in the IBS group where 33% in the LGG® group had no pain vs. 5% in the control group. Here, also a reduced frequency of pain was observed in the LGG® group (P=0.02). For the FD and FAP groups no differences were found.

In the other double-blind RCT, 141 children (5-14 years) with IBS or FP according to the Rome II criteria received LGG® ($3x10^9$ CFU) or placebo twice daily for 8 weeks, followed by an 8 weeks follow-up period (Francavilla et al. 2010). The primary outcome was overall pain at the end of the intervention period. At entry and at the end of the trial, children underwent a double-sugar intestinal permeability test. There was a significant reduction of both frequency (P<0.01) and severity (P<0.01) of abdominal pain in the LGG® group but not the control group compared with baseline. These differences were still observed at the end of the follow-up period (P<0.02 and P<0.001, respectively). A significant decrease in the number of children with abnormal results from the intestinal permeability test was observed in the LGG® group (P<0.03) but not the control group. These effects were mainly observed in children with IBS

suggesting that the effect on abdominal pain in this group may be secondary to improvement of the gut barrier.
Excessive crying in an otherwise healthy infant coincides with several maturational processes taking place in the gastrointestinal tract in response to massive antigen challenges by microbial colonization and food intake. The principal attempts to control excessive crying have focused on various dietary regimens and modulation of the gut microbiota (Pärtty et al. 2012). Results from a study on 89 infants, 7-12 weeks old, showed a link between the composition of the gut microbiota and fussing and crying (Pärtty et al. 2012). In a double-blind RCT by the same research group, LGG® was supplemented during the first 2 months of life (109 CFU/day from day 1-30 and $2x10^9$ CFU/day from day 31-60) and the infants were followed up for 1 year (Pärtty et al. 2013). Infants classified as excessive criers were significantly less frequent in the LGG® group than in the placebo group. The control group had a higher percentage of *Clostridium histolyticum* bacteria in their stools than did the LGG® group (P=0.05) (Pärtty et al. 2013).

In summary, these clinical studies, using probiotic doses ranging from $1x10^9$ to $6x10^9$ CFU/day, showed beneficial effects of LGG® on abdominal pain or discomfort in infants and children.

4.2 Immune Health

There is evidence that some probiotics support the host immune defense against pathogens in the respiratory tract. The effect of LGG® on pathogens in the respiratory tract has been evaluated in children. Furthermore, LGG® in combination with Bifidobacterium, BB-12® has been tested in college students living in dormitories.

In a study where 513 healthy children attending daycare centers were randomized to consume milk enriched with LGG® ($1-2x10^8$ CFU/day) or standard milk at their daycare meals five days a week, for seven months, absence from daycare due to illness was less frequent in the LGG® group than the control group (4.9 vs. 5.8 days/child, p=0.03) (Hatakka et al. 2001). Also, children in the LGG® group had one week more without respiratory symptoms than children in the control group during the study (5 vs. 4 weeks, p=0.03). Children in the LGG® group had fewer respiratory infections with complications (e.g. otitis media) than the control group as diagnosed by physicians and children in the LGG® group needed fewer antibiotics to treat respiratory tract infections (Hatakka et al. 2001).

A double-blind RCT included 281 healthy children of >1 years of age attending daycare centers (Hojsak et al. 2010b). The children received 100 ml fermented milk with LGG® ($1x10^9$ CFU/day) or placebo for 3 months. The results showed that the LGG® group had significantly fewer respiratory tract infections than the control group (43.2% vs. 67.6%, p<0.001). Furthermore, there was a reduced number of respiratory tract infections lasting longer than 3 days (p<0.001) and fewer days of absence from daycare (p<0.001) in the LGG® group (Hojsak et al. 2010b).

Another double-blind RCT by Hojsak et al (2010a) showed a significantly reduced the risk of acquiring nosocomial infections in the LGG® group when LGG® ($1x10^9$ CFU/day in fermented milk) or placebo was administered daily in 742 hospitalized children (> 1 year). LGG® reduced risk of respiratory infections significantly and there was a significant reduction in number of respiratory infections lasting >3 days.

Test tubes for in-vitro testing

College students may be at increased risk for upper respiratory infections compared with the general adult population due to a multi-stressor environment, characterized by inadequate sleep and psychological stress. Additionally, living in residence halls facilitates the transmission of viruses from one student to another. A 12-weeks double-blind RCT included 231 college students (18-25 years) living in campus residence halls (Smith et al. 2012). LGG® and Bifidobacterium, BB-12® ($1x10^9$ CFU of each strain/day) were delivered in stick packs for daily supplementation. Study endpoints were health related quality of life during upper respiratory tract infection, duration of illness, and severity of illness. The duration of upper respiratory tract infections were reduced with 33% in the probiotic group (p=0.001) and severity was reduced with 34% (p=0.0003) compared with the control group. Furthermore, students in the probiotic group missed significantly fewer school days compared with the control group (p=0.002).

In conclusion, the ability of LGG® to support immune defense against pathogens in the respiratory tract has been investigated in infants and children in doses of $1\text{-}6x10^9$ CFU/day and in adults in combination with *Bifidobacterium*, BB-12® in a dose of $1x10^9$ CFU of each strain/day.

4.2.1 Immune Response to Vaccine

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. 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 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.

LGG® has been tested in two vaccine studies in adults, one in combination with polio vaccine (de Vrese et al. 2005) and one in combination with influenza vaccine (Davidson et al. 2011). A 5 week RCT included 64 healthy adults, ranging in age from 20-30 years. The subjects were given $1x10^{10}$ CFU/day of LGG® one week prior to poliovirus vaccination and four weeks after. Endpoints were effect on neutralizing antibodies and vaccine-specific immunoglobulins, IgA, IgG and IgM. LGG® enhanced systemic protection by a significant increase in IgA titers (p=0.036) and there was an increase in polio-virus specific neutralizing antibodies ($p<0.05$) and in vaccine-specific IgA and IgG relative to baseline. In conclusion LGG® induced an immunological response that may enhance systemic protection of cells from virus infection by increasing production of virus neutralizing antibodies.

Davidson et al. (2011) performed an RCT on LGG® as adjuvant supplement for influenza vaccine. 42 healthy adults (18-49 years) were enrolled and randomized to receive $2x10^{10}$ CFU/day of LGG® in capsules or placebo capsules for four weeks, starting when influenza vaccine was given. The primary endpoint was level of protective antibody titers at day 28. The results showed a significant increase of 29% in protection against the H3N2 (A-) strain at day 28 with LGG® vs. control (p=0.048). No difference was observed between LGG® and control for the H1N1 (B-) strain.

An early study in infants (Isolauri et al. 1995) found that infants (2-5 months) who received LGG® showed an increased response with regard to rotavirus-specific IgM secreting cells after LGG® was given in conjunction with D x RRV rhesus-human reassortant live oral rotavirus vaccine. The observed response rates in infants receiving LGG® were 96% for IgM (vs. 85% in the control group) and 93% for IgA (vs. 74% in the control group).

In conclusion, studies in adults and infants have shown that LGG® can enhance antibody production as well as increase vaccine-specific antibodies after vaccination.

4.2.2 Immune Response to Allergens

Factors contributing to atopic diseases are aberrant barrier functions of the skin epithelium and gut mucosa and dysregulation of the immune response to environmental antigens (Isolauri et al. 2008). To evaluate if the development of allergic diseases, such as atopic dermatitis (AD) can be prevented in early infancy by modulating the intestinal microflora with probiotic bacteria, a group of families at high risk of allergy was selected and 159 mothers were randomized to receive two LGG® (1010 CFU) or placebo capsules daily for 2-4 weeks before the expected date of the birth (Kalliomäki et al. 2001). After the birth, either the breastfeeding mother or the infant consumed the bacteria for six months. The children were clinically examined at the age of two and prevalence of AD was 23% in the LGG® group and 46% in the control group (p=0.008). This result was confirmed in a 4-years as well as a 7-years follow-up (Kalliomäki et al. 2003; Kalliomäki et al. 2007). In the 7-years follow-up, in accordance with Cox regression the risk of AD was significantly reduced in the LGG® group compared with the control group (odds ratio,0.58; 95% CI, 0.35-0.94; p=0.027) (Kalliomäki et al. 2007).

Petri dishes

In another study LGG® was given to infants that manifested AD during exclusive breastfeeding and had no exposure to any infant food or substitute formula (Isolauri et al. 2000). They were weaned to a probiotic supplemented whey protein formula (LGG® or bifidobacteria) or placebo formula. After two months, AD was significantly improved in the probiotic groups compared with the control group (p=0.002).

Some studies have investigated whether probiotic bacteria can induce beneficial change in response to allergens in food-allergic infants.

In one RCT, 31 infants with AD and cow's milk allergy (CMA) were randomized to receive infant formula with LGG® ($5x10^8$ colony-forming units/g formula) or placebo (Majamaa and Isolauri 1997). There was a significant decrease in mean Severity Scoring of Atopic Dermatitis (SCORAD) in the LGG® group (p=0.008) but not in control group. A significant decrease in SCORAD (p=0.007) was also seen in a small group of breast-fed infants, where LGG® was given to their nursing mothers (Majamaa and Isolauri 1997).

In another double-blind RCT, 230 infants with AD and symptoms suggestive of CMA were randomized to receive supplementation with either LGG®, a mixture of LGG® and 3 other probiotic strains, or placebo for four weeks (Viljanen et al. 2005). In the whole group, the SCORAD decreased by 65%. In IgE-sensitized infants, the LGG® group showed a greater reduction in SCORAD than did the control group (p=0.036).

Looking into acquisition of tolerance to cow's milk, Berni Canani and coworkers performed two RCTs (Berni Canani et al. 2012; 2013), both showing that addition of LGG® to an extensively hydrolyzed casein formula (EHCF) accelerates acquisition of tolerance to cow's milk in infants with CMA compared with patients receiving EHCF alone.

In 2013-14, Lundelin et al (2017) performed a prospective long-term follow-up on children who had received LGG® alone or in combination with other probiotic strains perinatally in four separate studies performed between 1997 and 2012. The analysis showed that children given LGG® alone or in combination with other defined probiotics had a lower risk of developing allergic disease (allergic rhinitis, eczema, food allergy, or asthma) in long-term follow-up (Lundelin et al. 2017).

Taken together, these studies suggest that LGG® can induce a beneficial physiological change in response to allergens (e.g. in AD) which may lead to a lower the risk of developing allergic diseases such as food allergy and asthma.

4.3 Oral Health

In this booklet we will not go into details with research into other health areas than mentioned above. It should however be mentioned, that there is growing evidence that LGG® may a have potential within oralw health. In a double-blind RCT in 594 children (1-6 years), it was examined whether milk containing LGG® ($0.1\text{-}0.3x10^9$ CFU/day) had an effect on caries and the risk of caries in children when given 5 days a week for 7 months (Näse et al. 2001). Caries risk was calculated based on clinical and microbiological data, comprising *Streptococcus mutans* levels from dental plaque and saliva. The results showed less dental caries as well as lower *Streptococcus mutans* counts in the LGG® group. Also the risk of caries was significantly reduced in the LGG® group (OR=0.56, p=0.01; controlled for age and gender: OR=0.51, p=0.004). Similar results were found in another study where an inhibitory effect on *Streptococcus mutans* was observed when LGG® was administered in yoghurt (Glavina et al. 2012). Hatakka et al. (2007) showed that $0.5x10^9$ CFU/day of LGG® added to cheese could reduce the risk of high oral yeast counts by 75% and hypersalivation by 56% in an elderly population.

5. Safety

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5.1 Evaluation by Authorities

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 was addressed in the following way: *"Information acquired to date shows that lactobacilli 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".*

"...the LGG® strain is 'Generally Regarded as Safe' (GRAS) by the Food and Drug Administration (FDA)"

"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 probiotic 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.

In Europe, strains belonging to the species *Lactobacillus rhamnosus* have been granted Qualified Presumption of Safety (QPS) status by the European Food Safety Authority (EFSA Panel on Biological Hazards (BIOHAZ) 2017).

In the USA, the LGG® strain is 'Generally Regarded as Safe' (GRAS) by the Food and Drug Administration (FDA) as an ingredient in infant formula powder intended for consumption by term infants from the time of birth (GRN No. 231).

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.

5.2 Antibiotic Susceptibility

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. 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 (EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2012) and performs the tests according to internationally recognized methods published by ISO and 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 LGG®. LGG® is sensitive to most antibiotics in clinical use. All L. rhamnosus strains, including LGG®, are resistant to vancomycin due to the structure of their cell wall (inherent factor) (Klein et al 2000).

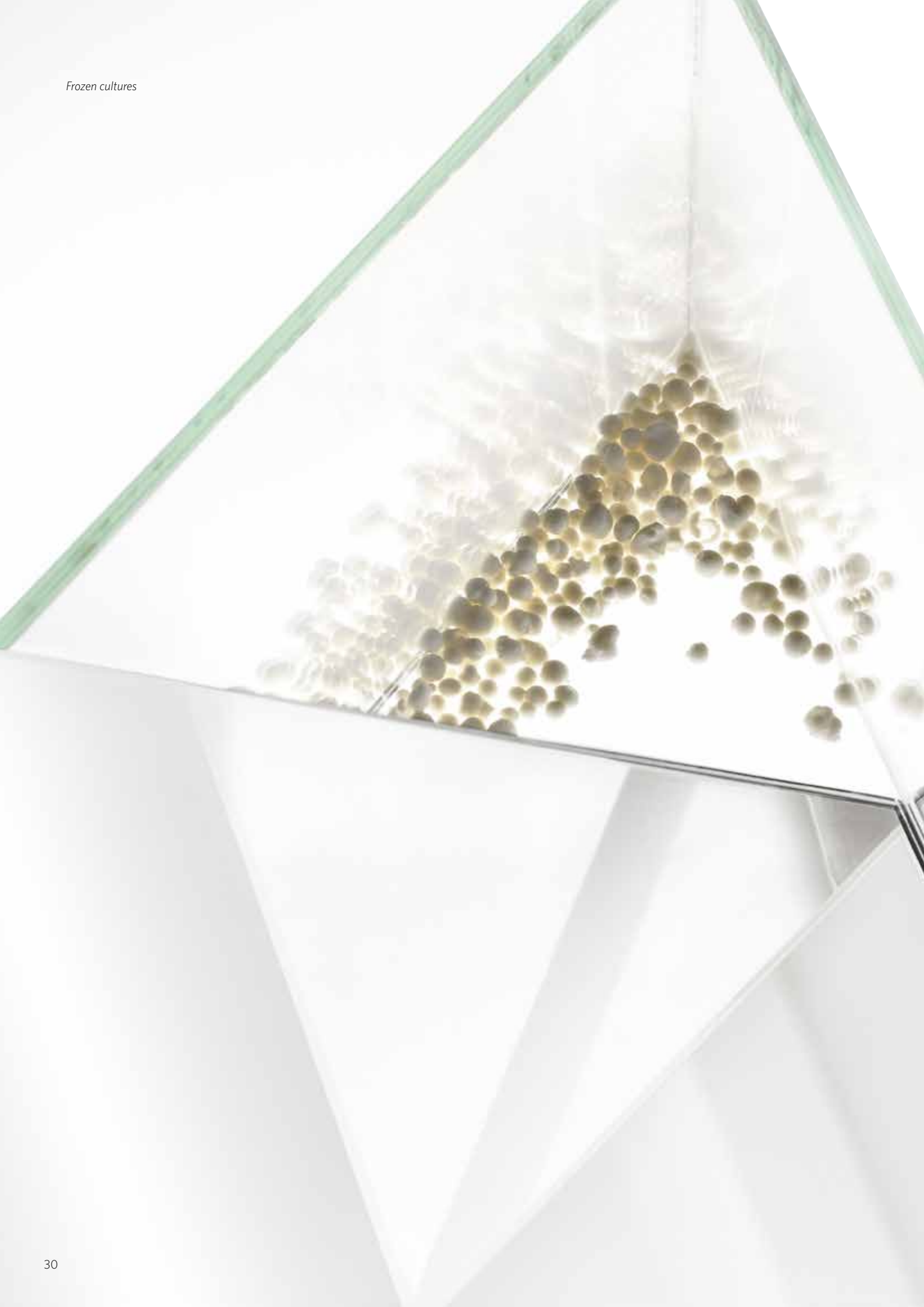
5.3 Studies in Humans

LGG® is considered safe due to the long history of safe human exposure to species of Lactobacillus, to strains of subspecies of *Lactobacillus rhamnosus*, and to *Lactobacillus rhamnosus*, LGG® in particular.

"Besides the long history of safe use worldwide, LGG® has been tested in numerous clinical studies"

LGG® has been used worldwide since 1990 as an ingredient in food and dietary supplements.

Besides the long history of safe use worldwide, LGG® has been tested in numerous clinical studies within various health areas in newborn, preterm infants, children, pregnant women, adults and elderly in dosages ranging from $1x10^8$ (Kumpu et al. 2013) to $2x10^{12}$ CFU/day (Basu et al. 2009). Supplementation periods have ranged from one week to at least one year. The dosage forms have been milk powder, dairy products or dietary supplements in the form of capsules, tablets or powder sticks / sachets.



The vast majority of publications do not describe any serious adverse events related to the use of LGG®. Large epidemiological studies have shown that rapidly increasing consumption of the LGG® probiotic strain did not increase the incidence of *Lactobacillus* or *L. rhamnosus* isolates in blood culture samples (Salminen et al. 2002) and no risk groups of immune compromised patients could be identified (Salminen et al. 2004). In Finland the increase in consumption of the probiotic strain LGG® did not lead to an increase in the incidence of bacteremia (Boriello et al. 2003). Rare cases of bacteremia (due to lactobacilli) have been reported (Saxelin et al. 1996). However, infections associated with probiotic strains of lactobacilli are rare (Land et al. 2005) and there are no safety concerns with the use of LGG® in a healthy population.

There have been a few, rare case reports of serious adverse events following LGG® consumption. These include some case reports of sepsis in infants or children (Dani et al. 2016; Barraud et al. 2010; Land et al. 2005) and a few case reports of sepsis (Zein et al. 2008; Meini et al. 2015; Vahabnezhad et al. 2013), or other serious infections (Mackay et al. 1999; Rautio et al. 1999; Ishihara et al. 2014) in adults. These findings have mainly been described in pre-term infants or in subjects who had a compromised immune system. In a large epidemiological study the pathogenic potential of LGG® has been described as very low (Saxelin et al. 1996). In a retrospective study, 743 preterm neonates with very low birth weight were routinely administered LGG® for 4-6 weeks. Surveillance cultures were taken from a variety of sites from each infant and no LGG® ever grew in any culture. Also no clinical sepsis episode was attributable to LGG® (Manzoni et al. 2011).

In most cases, the bacteria isolated from the blood of patients with bacteremia were not investigated at a genomic level. Recent studies underline that even though bacteremia or sepsis has been sporadically observed after probiotic supplementation, the bacteria isolated from the patient need to be investigated at the genomic level by modern techniques such as DNA fingerprinting or whole genome sequencing (WGS) to clarify whether the probiotic strain is the same as the strain isolated from the blood of the patient (Aroutcheva et al. 2016; Nissilä et al 2017). In one study, DNA fingerprinting showed a clear difference between the probiotic strain and the strain isolated from the blood of a patient that developed bacteremia after probiotic supplementation (Aroutcheva et al. 2016). Another recent study showed that 16 *L. rhamnosus* strains collected in blood cultures from bacteremic patients in Finland, where consumption of LGG® is high, were clearly different from LGG® when analyzed at a genomic as well as a phenotypic level.

As a consequence of all the above, we do not expect any increased risk of bacteremia due to the use of LGG®.

Two recent studies have looked at the long-term effect of LGG® exposure in children (Lundelin et al. 2017; Scalabrin et al. 2017). Scalabrin et al. (2017) looked at the long-term safety data during the first five years of life on the use of LGG® containing formulas for one year. As a continuation of a previous 120 days study (Scalabrin et al. 2009), 183 of the participants continued to receive the LGG® formula for one year. Body weight, height, behavioral development and adverse events were recorded during the study period, whereas specific adverse events (allergy and infection related events) as well as serious adverse events were recorded for five years. LGG® was associated with normal growth and development through five years of age, as well as absence of relevant infections, allergic events or serious adverse events that could be attributed to early consumption of LGG®. In 2013-14, Lundelin et al (2017) performed a prospective long-term follow-up on children who had received LGG® alone or in combination with other probiotic strains in four separate studies performed between 1997 and 2012. The study found no differences in growth patterns or non-communicable disease prevalence between children who had received probiotics or placebo perinatally.

In conclusion, there are a large number of products containing LGG® and a large exposure of the general population to LGG® with many million subjects exposed over a period of more than 30 years. The very few occasional reports on any safety issues do not give cause for safety concerns about the continued safe use of LGG®.

Based on the above information, it can be concluded that *lactobacillus rhamnosus*, LGG® is safe for its intended use as a dietary ingredient in food and dietary supplements to be consumed by a healthy population, including newborn infants.

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