DUPONT

DANISCO

BIFIDOBACTERIUM LACTIS BL-04®

Technical Memorandum

TM 46-5E US 10.18

INTRODUCTION

A growing awareness of the relationship between diet and health has led to an increasing demand for products that are able to enhance health beyond providing basic nutrition. Studies have shown that the ingestion of probiotics, or friendly bacteria, is beneficial for maintaining the body's delicate microbial balance. This balance is known to enhance intestinal health and the immune system, as well as other physiological functions, making it a critical factor for general human well-being (Vandenplas et al, 2015; de Moreno de LeBlanc & LeBlanc 2014, Kechagia et al, 2013, Markowiak & Slizewska 2017).

Definition

Probiotics are live microorganisms that when administered in adequate amounts, confer a health benefit on the host (Hill et al, 2014).

Most probiotics are either lactobacilli or bifidobacteria, although some strains of other microbial genera are also reported to have probiotic properties.

The beneficial effects of probiotics either involve reducing risk factors for a certain disease or improving some of the body's natural functions, thereby helping to maintain the health of the consumer. So far, these effects have been documented primarily in two areas, which are also the main areas of probiotic research for DuPont:

- gastrointestinal well-being
- beneficial modulation of the immune system

The suggested health benefits of probiotics are many, and some effects are better established than others. It should, however, be noted that each probiotic strain has its own specific health benefits, and no probiotic strain elicits all the health benefits that have been proposed for probiotics. Furthermore, when one probiotic strain has a certain health benefit, it cannot be assumed that another strain, not even of the same species, has similar properties.

The origin of a bacterial strain, e.g., the human gastrointestinal tract, is no guarantee or precondition of its performance as a probiotic. For a probiotic strain to be successful, it must fulfill certain requirements. These will improve its functionality in the intestine after consumption and enhance its survival in the product.

- The strain must be safe – this requires identification by appropriate molecular techniques
- The strain should be able to resist acid and bile
- The strain must have scientifically proven health benefits
- The strain should have good technological properties, such as the ability to survive in the final consumer product in sufficient counts until end of shelf-life, whether food or dietary supplements.

The only certain way to establish the true benefit of a probiotic strain is by systematic *in vitro* and *in vivo* studies and, in particular, human clinical trials. *B. lactis* Bl-04 has been subject to several studies.

CHARACTERISTICS OF THE GENUS

Bifidobacterium spp. comprises Gram-positive, non-spore forming, anaerobic, pleomorphic bacteria that were first isolated from the feces of breast-fed infants in 1899 by Tissier.

Bifidobacteria are among the first microbes to colonize the human gastrointestinal (GI) tract. They are one of the most abundant genera present in the healthy infant gut and represent a significant portion of the microbiota throughout a healthy adult life, playing an important role in gut homeostasis and health. During late adulthood and in several diseases, the levels of *Bifidobacterium* and its species diversity decrease. In general, a high proportion of bifidobacteria in the intestinal tract is considered beneficial to health.

Today, bifidobacteria are broadly recognized for their key role in the human intestinal microbiota throughout life. Their actions in the intestine and their impact on the host's immune system and metabolism results in a range of health benefits such as a reduced risk of respiratory tract infections, various gastrointestinal disorders and infections, particularly antibiotic associated diarrhea (Arboleya et al, 2016, O'Callaghan & van Sinderen 2016).

Bifidobacterium animalis subsp. *lactis* is one of the most common *Bifidobacterium* species utilized as a probiotic in commercial products in North America and Europe.

The benefits associated with probiotic strains of *B. animalis* subsp. *lactis* have led to their inclusion in the human diet via formulation into a large array of dietary supplements and foods, including dairy products such as yogurt.

SELECTION AND TAXONOMY/ GENOMICS

B. lactis was originally described by Meile et al, 1997 and was reclassified as *B. animalis* subsp. *lactis* (Masco 2004; Ventura and Zink, 2002). In the interests of simplicity, DuPont refers to strains of this species as *B. lactis*.

B. lactis Bl-04 is a human isolate and has been genetically characterized and classified as *B. lactis* by 16S rRNA gene sequencing and full genome sequence comparison with the type strain DSM 10140 (Barrangou et al, 2009).

B. lactis Bl-04 has been deposited in the American Type Culture collections safe deposit as SD5219. It is also known as DGCC2908 and RB 4825.

Consistent strain identity

For a strain with documented probiotic activity, it is very important that it is not subjected to any genetic or physiological change during processing. To maintain the quality, purity, and consistency of each production batch of the strain, DuPont makes rigorous use of frozen bacterial seed inventories to reduce the risk of genetic drift over time and maintain strain integrity. DuPont also performs bacterial identification based on 16s rRNA gene sequence similarity for every produced batch of probiotics

SAFE FOR CONSUMPTION

General safety

Bifidobacteria have long been considered safe and suitable for human consumption (Saarela 2010).

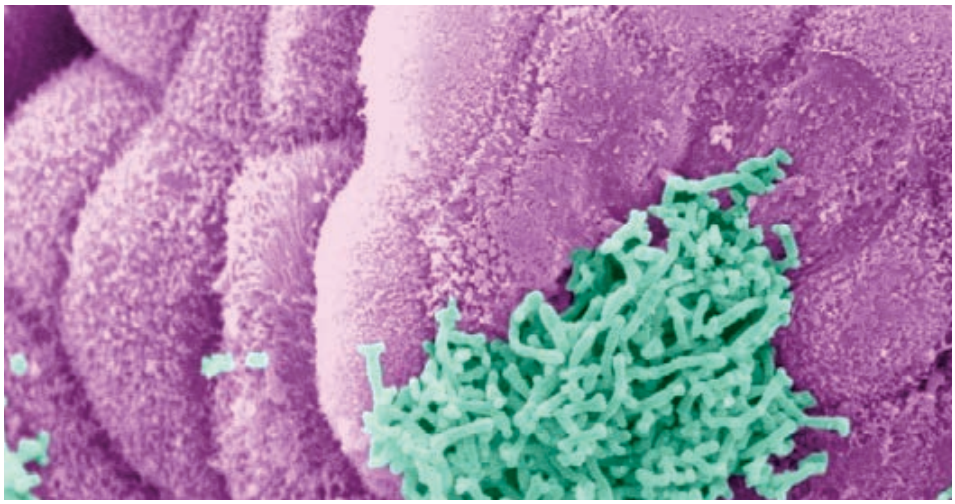


Figure 1. *Bifidobacterium* adhering to a monolayer of Caco-2 intestinal epithelial cells

Furthermore, *B. lactis* has been present in human food for decades and is listed in *Food fermentations: Microorganisms with technological beneficial use* (Bourdichon et al, 2012). The European Food Safety Authority (EFSA) has also added the species to the Qualified Presumption of Safety list (Ricci et al, 2017).

B. lactis Bl-04 has been safely used as a single strain and in combination with other probiotics and/or prebiotics in human clinical trials. These trials have included children, adults, and elderly (Table 1). None of these trials have reported any safety concern related to *B. lactis* Bl-04.

To investigate the safety and tolerability of *B. lactis* Bl-04 in conjunction with defined clinical endpoints, a cohort of healthy subjects from trial by West et al, 2014a were analyzed for routine hematology and clinical chemistry markers (Cox et al, 2014). Supplementation of *B. lactis* Bl-04 over 150 days in healthy adults had no impact on these markers, except plasma urea that was decreased in *B. lactis* Bl-04 group compared to placebo but was still within normal range.

Antibiotic susceptibility patterns

Antibiotic susceptibility patterns are an important means of demonstrating the potential of an organism to be readily inactivated by the antibiotics used in human therapy. Antibiotic resistance can be defined as the ability of some bacteria to survive or even grow in the presence of certain substances that usually inhibit or kill other bacteria. It is a natural property of microorganisms and existed before antibiotics became used by humans. In many cases, resistance is due to the absence of the specific antibiotic target or is a consequence of natural selection. This resistance may be inherent/intrinsic or acquired.

Inherent or intrinsic resistance

Most, if not all, strains of a certain bacterial species are not normally susceptible to a certain antibiotic. The antibiotic has no effect on these cells, being unable to kill or inhibit the bacterium, for example because the target for the antibiotic may be missing. When all strains within a certain taxonomic group show phenotypic resistance to an antimicrobial, such resistance can be considered intrinsic to the taxonomic group.

Table 1. Use of *B. lactis* Bl-04, including probiotic blends, in selected human clinical trials.

Daily dose of Bl-04 (CFU)	Subjects receiving Bl-04 (n)	Age of subjects (yrs)	Duration of supplementation (days)	Reference
2.0×10^9	73	22 (mean)	33	Turner et al 2017
4.0×10^9 (2.0×10^{10} total CFU in blend)	21	20-59	56	Gomes et al 2017
2.0×10^9	161	36 (mean)	150	West et al 2014 a&b West et al 2016
High dose: 4.25×10^9 (1.7×10^{10} total CFU in blend) Low dose: 1.0×10^9 (4.17×10^9 total CFU in blend)	336	50 (mean)	10-21	Ouwehand et al 2014
4.0×10^9 (5.4×10^{10} total CFU in blend)	34	57 (mean)	7	Zhang et al 2013
3.75×10^9	24	9 (mean)	122	Ouwehand et al 2009
2.0×10^{10}	9	38 (mean)	21	Paineau et al 2008
1.0×10^{10} (4.1×10^{10} total CFU in blend)	20	36.5 (mean)	20	Engelbrektson et al 2006 Engelbrektson et al 2009
3.5×10^{10}	9	73 (mean)	28	Bartosch et al 2005

Acquired resistance

Most strains of a bacterial species are usually susceptible to a given antibiotic. However, some strains may be resistant, having adapted to survive antibiotic exposure. Possible explanations for this include:

- a mutation in the gene coding for the antibiotic's target can make the antibiotic less efficient. This type of antibiotic resistance is usually not transferable
- a resistance gene may have been acquired from another bacterium

Of the acquired resistances, the latter is of most concern, as it may also be passed on to other (potentially pathogenic) bacteria. In case such resistance is found, it has to be investigated whether it may be passed on to other (potentially pathogenic) bacteria (D'Aimmo et al, 2007). Analysis of the *B. lactis* Bl-04 genome has confirmed the absence of known transferable genetic elements related to antibiotic resistance (Barrangou et al, 2009). The antibiotic susceptibility patterns for *B. lactis* Bl-04 are summarized in Table 2. According to these results, *B. lactis* Bl-04 does not display acquired antibiotic resistance.

However, *B. lactis* Bl-04 has the same tetracyclin resistance gene *tet*W as the strains evaluated in the study by Gueimonde et al, 2010. Evaluation of all *B. lactis* genomes currently published has revealed that the *tet*W gene is conserved, and therefore likely intrinsic to the species. To date, there has not been any evidence that the conserved *tet*W gene has any ability to transfer resistance, and therefore poses no known risk of transfer. Additionally, the individual sequence composition of the *tet*W gene was analyzed, and no sharp distinction can be made between the overall GC content of the genome and the GC content of the *tet*W gene. This further highlights the likelihood that the gene is intrinsic to *B. lactis* Bl-04, because horizontal gene transfer is often marked with different GC content of the genetic material received than the host genetic material.

Much concern has arisen in recent years regarding vancomycin resistance, as vancomycin-resistant enterococci are a leading cause of hospital-acquired infections and are refractory to treatment. The transmissible nature of genetic elements that encode vancomycin resistance in these enterococci is an important mechanism of pathogenicity.

Table 2. Bl-04 Antibiotic Susceptibility Profiles

Antibiogram of *B. lactis* Bl-04 was established using ISO 10932 IDF223 method and VetMIC Lact-1 and 2 microdilution plates that include all antibiotics that are recommended by the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). Recorded Minimum Inhibitory Concentrations (MICs) are displayed in the table below. All MIC values are below or equal to the Microbial Break Points (MBPs) defined for Bifidobacterium (EFSA Journal 2012; 10(6):2740) except for tetracycline.

APPENDIX: Antibiotic Susceptibility Profile Method used: ISO 10932 IDF 223 with VetMIC Lact 1 and 2 microdilution plates.	Gentamycin	Kanamycin	Streptomycin	Tetracycline	Erythromycin	Clindamycin	Chloramphenicol	Ampicillin	Vancomycin	Virginiamycin*
	Gm	Km	Sm	Tc	Em	Cl	Ch	Amp	Va	Vi*
	Max MIC µg/ml									
Bifidobacterium lactis Bl-04	64	512	64	16	0.06	‹0.03	2	0.5	1	0.25
MBP for *Bifidobacterium***	64	NR***	128	8	1	1	4	2	2	1

* Virginiamycin is no more included in the FEEDAP recommended list of antibiotics.
** EFSA Journal 2012; 10(6):2740
*** NR: not required

Production of biogenic amines

Histamine and tyramine are biogenic amines that occur naturally in a wide range of foods including fermented products. They are formed by the enzymes present in the raw material or are generated by microbial decarboxylation of amino acids. The consumption of food containing large amounts of these amines can induce adverse reactions, such as nausea, headaches, rashes, and changes in blood pressure (Ladero et al, 2010).

In lactic acid bacteria, production of histamine results from the catabolism of histidine by a histidine decarboxylase, and production of tyramine results from the catabolism of tyrosine by tyrosine decarboxylase. A specific detection method for histidine and tyrosine decarboxylase genes has been developed internally to DuPont, based on the scientific literature and on the most updated genomic databases. With this method, no histidine or tyrosine decarboxylase gene was identified in *B. lactis* Bl-04 genome. Consequently, *B. lactis* Bl-04 is unlikely to produce histamine or tyramine, theoretically decreasing the risk for adverse reactions in those individuals ingesting *B. lactis* Bl-04 with sensitivity to either amine.

L/D-lactic acid production

Lactic acid is the most important metabolic end product of fermentation processes by lactic acid bacteria and other microorganisms. For thousands of years, lactic acid fermentation has been used in the production of fermented foods.

Due to its molecular structure, lactic acid has two optical isomers. One is known as L(+)-lactic acid and the other, its mirror image, is D(-)-lactic acid.

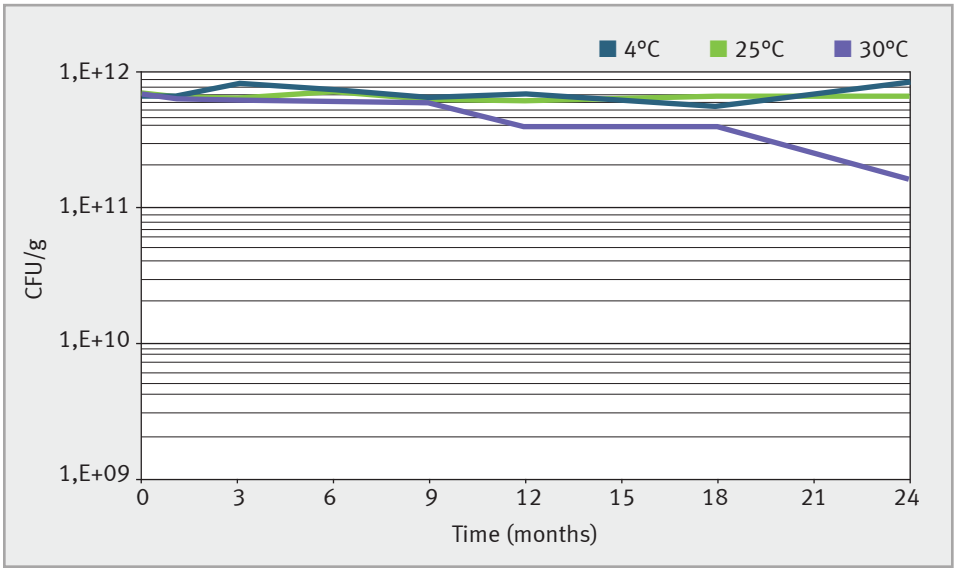
In humans, animals, plants, and microorganisms, L(+)-lactic acid is a normal intermediate or end product of the carbohydrate and amino acid metabolic processes whereas, D(-)-lactic acid was thought to be "non-physiological" and a possible cause for lactate acidosis.

Although some species of probiotic cultures, as nutritional ingredients that may produce D(-)-lactic acid, have been safely administered to infants (Connolly et al., 2005), Codex specifies that only L(+)-lactic acid producing cultures can be used for infant formula and follow up formula. *B. lactis* Bl-04 only produce L(+)-lactic acid (Table 3).

Table 3. L/D Lactic acid production of *B. lactis* Bl-04.

	100/0
L/D lactic acid production molar ratio	Boehringer Mannheim/ R-Biopharm D-lactic acid/ L-lactic acid UV-method

Figure 2. Stability of *B. lactis* Bl-04 culture concentrate



Source: DuPont, internally generated data

PRODUCT STABILITY

Today there is a general consensus that probiotics have to be consumed in sufficient numbers to provide the desired health benefit. It is likely that different strains and different effects require different dosages. Food and supplement manufacturers find the strain particularly attractive for several reasons, including:

- available as a high-count freeze-dried material
- excellent stability in a variety of food and supplement applications

B. lactis Bl-04 has demonstrated to be a very versatile strain. Its survival with and without microencapsulation and with different drying techniques has been studied in several publications (Oliveira et al, 2007a; Oliveira et al, 2007b; Ding and Shah 2009a; Ding and Shah 2009b; Ding and Shah 2009c). In addition, the viability of *B. lactis* Bl-04 in yogurt has been studied (Saccaro et al, 2009).

HEALTH-RELATED PROPERTIES

The health benefits of probiotic bacterial strains have been demonstrated over the years, including a range of health improvement and inhibition of infection. *In vitro*, animal, and human clinical studies have established the efficacy of *B. lactis* Bl-04 as a probiotic with demonstrated health benefits. Research has focused on characteristics that indicate beneficial effects, such as acid and bile resistance, adhesion to intestinal and oral surfaces, antimicrobial activity, and efficacy in human clinical trials. The key findings from research on *B. lactis* Bl-04 are summarized below.

BENEFITS TO INTESTINAL HEALTH AND WELL-BEING

The importance of the intestinal microbiota for health

The human gastrointestinal (GI) tract is an extremely complex ecosystem and represents the host's greatest area of contact with the environment. This ecosystem comprises:

- the GI epithelium
- immune cells
- resident microbiota

The primary function of the human GI tract has long been considered to be the digestion and absorption of nutrients and the excretion of waste end products. In recent years however, it has become recognized that the GI tract fulfills many other functions, which are essential to our well-being. The GI tract harbors a vast number of microbial cells (10^{14}), which may surpass the number of cells that make up the human body (Sender et al, 2016). The intestinal microbiota is estimated to consist of at least 1000 species, although 95-99% of all bacteria belong to just 10 genera. Many members of the intestinal microbiota are beneficial, while others are potentially detrimental or their function not known. A higher concentration of certain genera, including *Lactobacillus* and *Bifidobacterium*, is generally thought to be associated with a healthier GI tract.

The resident microbes are involved in many metabolic processes, such as the fermentation of undigested carbohydrates into short-chain fatty acids, and in lipid metabolism and vitamin synthesis. Another important function of the intestinal microbiota is to stimulate the maturation of the immune system and provide protection against incoming, potentially pathogenic microbes.

When the delicate ecological balance of this highly complex microbial community is disturbed by environmental or physiological factors, predisposition to infectious and immuno-inflammatory diseases is enhanced. It may then become necessary to re-establish a beneficial microbiota. Research has shown that specific probiotic strains can be used to optimize the composition and activity of the intestinal microbiota, and thus to reduce the risk for a range of diseases or unfavorable conditions (Guarino et al, 2013, Scott et al, 2015, Lin et al, 2014).

Resistance to acid and bile and survival in the intestinal passage

A key property for a probiotic is its capability to survive passage through the digestive system. A variety of traits are believed to be relevant for surviving GI tract passage; the most important of which is tolerance both to the highly acidic conditions present in the stomach and to high concentrations of bile salts in the small intestine.

Table 4. Selected characteristics of *B. lactis* Bl-04

++++ Excellent +++ Very good ++ Good + Fair	
Acid tolerance	**++++** (›90% survival in hydrochloric acid and pepsin (1%) at pH 3.5 for 1h at 37°C)
Bile salt tolerance	**++** (70-79% survival in 0.3% bile salt-containing medium)

Table 5. Adhesion properties of *B. lactis* Bl-04

++++ Excellent +++ Very good ++ Good + Fair	
Adherence of B. lactis Bl-04 to human intestinal cells *in vitro*	HT-29: **+++** Caco-2: **+++**
+++++ Excellent ++++ Very good +++Good ++ Fair + Poor	
Adherence of B. lactis Bl-04 to human colonic mucus *in vitro*	**++++** (3.5 - 5% of added bacteria)

Source: DuPont, internally generated data

In vitro studies have shown that *B. lactis* Bl-04 is extremely resistant to low pH conditions (Table 4) and survives the presence of bile at concentrations present in the duodenum (Ding and Shah 2007). In addition, increased amounts of *B. lactis* Bl-04 have been found in feces during clinical trials after feeding *B. lactis* Bl-04 (Bartosch et al, 2005; Ouwehand et al, 2009). In conclusion, these data suggest that *B. lactis* Bl-04 survives passage through the gastrointestinal tract.

Adhesion to intestinal mucosa

While adhesion is not a prerequisite for a strain to elicit probiotic properties, interaction with the intestinal mucosa is considered important for a number of reasons. Binding to the intestinal mucosa may prolong the time a probiotic strain can reside in the intestine. This interaction with the mucosa brings the probiotic in close contact with the intestinal immune system, giving it a better opportunity to modulate the immune response. It may also protect against enteric pathogens by limiting their ability to colonize the intestine.

Currently, adherence is measured using *in vitro* human intestinal epithelial cell lines (Caco-2 and HT-29) and human colonic mucus. While these are not a thorough test of the ability of probiotics to adhere to intestinal mucosa in the body, attachment to these cell lines and mucus *in vitro* is considered a good indicator of their potential to attach. *B. lactis* Bl-04 has demonstrated very good adhesion to human epithelial cell lines (Caco-2 and HT-29) and to human colonic mucus (Table 5) applied in *in vitro* studies.

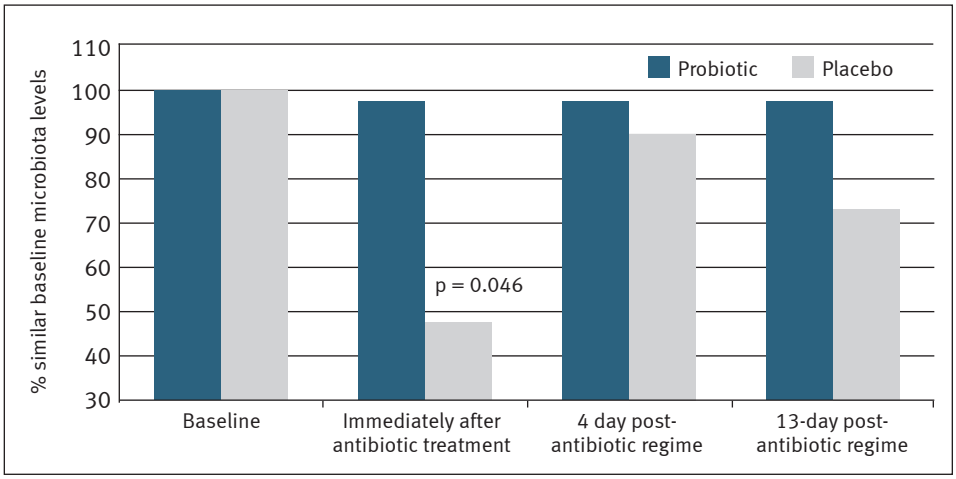
Stabilization of microbiota during antibiotic treatment

B. lactis Bl-04 was included in a five-strain probiotic formulation investigated for its ability to stabilize the intestinal microbiota during and after antibiotic therapy. In this human trial, the probiotic product was found to reduce the antibiotic-induced disturbance of the total microbiota population (Figure 3). In addition, the probiotic product still maintained *Bifidobacterium* at significantly higher levels than that of the placebo group even two weeks after the cessation of antibiotic therapy (Figure 4) (Engelbrektson et al, 2009).

Influence of a probiotic combination including *B. lactis* Bl-04 on diarrhea

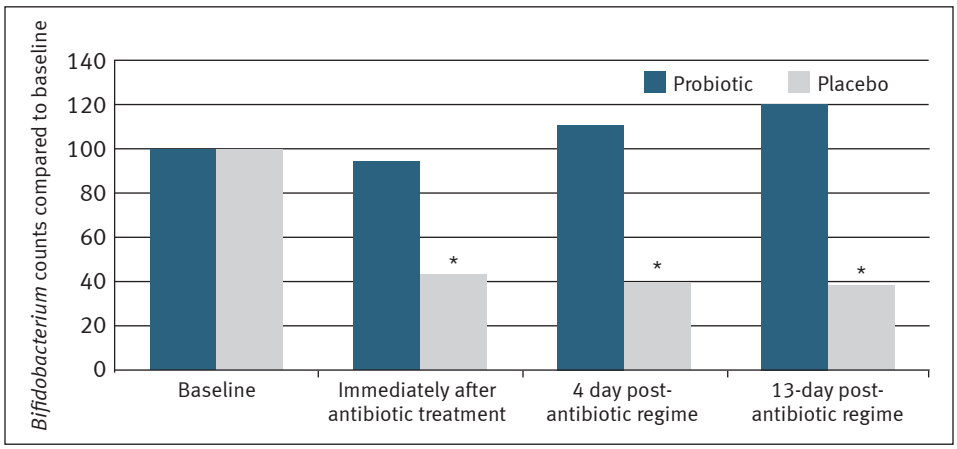
The aim of a further study was to determine the dose-response effect of a four-strain probiotic combination *B. lactis* Bi-07, *L. paracasei* Lpc-37, *L. acidophilus* NCFM, and *B. lactis* Bl-04 on the incidence of antibiotic associated diarrhea (AAD) and *Clostridium difficile* associated diarrhea

Figure 3. The probiotic mixture containing *B. lactis* Bl-04 protects the fecal microbiota from disruption by antibiotics, as indicated by the greater dissimilarity of the microbiota of the placebo group compared to baseline microbiota composition



Source: Engelbrektson et al, 2009

Figure 4. The probiotic mixture containing *B. lactis* Bl-04 promotes the maintenance of bifidobacteria levels in the feces of antibiotic consuming subjects during post-treatment

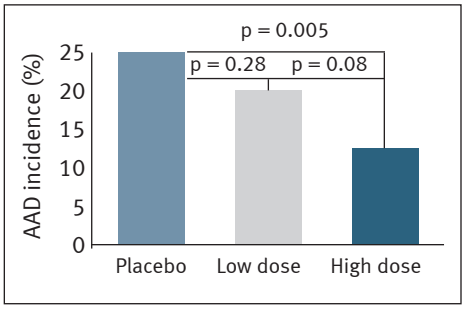


Source: Engelbrektson et al, 2009

(CDAD) and the severity of gastrointestinal symptoms in adult in-patients requiring antibiotic therapy. Patients were randomized amongst three study groups: probiotic with 1.70×10^{10} CFU/d (high dose), probiotic with 4.17×10^{9} CFU/d (low dose), or placebo. The study product was administered daily for 10–21 days, depending on length of antibiotic administration (the study product was consumed until 7 days after the last antibiotic dose was given). The results showed improvements in all measures in both the probiotic groups compared to the placebo group. The lowest AAD incidence was observed in the high-dose group, highest in the placebo group, and intermediate in the low-dose group, (Figure 5). The CDAD incidence was highest in the placebo group and identical with high dose and low-dose groups. Both the average number of liquid stools and the average duration of diarrhea were significantly reduced by both the high and the low dose. Bloating, fever, and abdominal pain were all significantly reduced by the high dose compared to placebo.

In conclusion, the tested four-strain probiotic combination, including *B. lactis* Bl-04 appears to lower the risk of AAD, CDAD, and gastrointestinal symptoms

Figure 5. Incidence of antibiotic associated diarrhea (AAD) by study group



Source: Ouwehand et al, 2014

in a dose-dependent manner in adult in-patients (Ouwehand et al, 2014).

Beneficial modulation of intestinal microbiota

A two-strain formulation, containing *B. lactis* Bl-04 and a *B. bifidum* strain in combination with an inulin-based prebiotic, was also investigated for its ability to beneficially modulate the intestinal microbiota in healthy elderly people. *B. bifidum* and *B. lactis* rRNA genes were measured in fecal samples by real time PCR and in parallel with the cultivation technique. A significant increase in *B. bifidum* and *B. lactis* rRNA was observed while feeding the synbiotic formulation. The synbiotic formulation, including *B. lactis* Bl-04 was shown to modify the composition of intestinal bifidobacterial populations in the healthy elderly volunteers, demonstrating that it may be beneficial to individuals with an unbalanced gut ecosystem that is associated with undesirable bacterial metabolism and higher incidence of infections (Bartosch et al, 2005).

Influence of a probiotic combination including *B. lactis* Bl-04 on modulation of the intestinal microbiota in colon cancer patients

Colorectal cancer (CRC) is one of the most common cancers diagnosed globally each year and the incidence has increased significantly in recent years (Torre et al, 2012).

The majority of the cases are affected by environmental and lifestyle factors, including age, gender, diet, obesity, and levels of physical inactivity. One of the common characteristics of these potential risk factors is that they can cause alterations in the structure of the intestinal microbiota. These alterations (dysbiosis), e.g., reduction of butyrate producing bacteria and increase of opportunistic pathogens, were hypothesized to be closely correlated with the pathogenesis of CRC (Gao et al, 2017, Huxley et al, 2009, Nistal et al, 2015, Qorri et al, 2017).

This study was performed to investigate the microbiota composition of CRC patients compared with controls without neoplastic or inflammatory disease and the potential to modify the colonic microbiota with probiotics (Hibberd et al, 2017).

The probiotic supplementation consisted of two ProBion Clinica (Wasa Medicals AB) tablets daily, totaling a daily dose of 1.4×10^{10} CFU *B. lactis* Bl-04, 7×10^{9} CFU *L. acidophilus* NCFM and 0.63 g inulin.

Biopsy samples were obtained from the normal mucosa and tumor during colonoscopy from 15 patients with colon cancer. Subsequent patient matched samples

were taken at surgery from the tumor and nearby mucosa from the patients with cancer, eight of whom had received the probiotic tablets.

Fecal samples were obtained after colonoscopy at start of the intervention and at surgery. In addition, 21 mucosal biopsies from non-cancer controls were obtained during colonoscopy followed by later fecal samples. The colonic and fecal microbiota was assessed by 16S rRNA gene amplicon sequencing.

The key finding of the study was that the tumor microbiota from CRC patients was characterized by increased microbial diversity of *Fusobacterium*, *Selenomonas,* and *Peptostreptococcus* compared with the control microbiota. This colon cancer-associated microbiota was modified by the probiotic intervention and resulted in an increase of butyrate-producing bacteria and a decrease of colon cancer-associated bacterial genera, such as *Fusobacterium* and *Peptostreptococcus*. The anti-inflammatory benefits of butyrate for colon health are well-documented, and it also has been shown to suppress the growth of colon cancer cells (Louis et al, 2014).

The results of the study show that the colon cancer-associated microbiota can be manipulated by the specific probiotic strains used in this study, resulting in an altered microbiota enriched with beneficial bacteria. Microbiota modulation by probiotics may represent novel prognosis markers and could be considered as part of a therapeutic regime for CRC patients (Gagnière et al, 2016).

Prebiotic utilization

Prebiotics are food ingredients that are nondigestible for humans but are utilized by bacteria in the intestine. Prebiotics have been shown to maintain balance of the gut microbiota by promoting growth of some strains of bifidobacteria and lactobacilli. In general, pathogens have more restricted carbohydrate metabolism. The most commonly used prebiotics are complex oligosaccharides like fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS). Mäkeläinen et al, (2010) showed that *B. lactis* Bl-04 can utilize FOS, GOS, and xylo-oligosaccharides of different lengths, but not xylan, polydextrose, lactitol, gentobiose, or pullulan as a carbon source *in vitro* (Mäkeläinen et al, 2010a; Mäkeläinen et al, 2010b).

BENEFICIAL MODULATION OF THE IMMUNE SYSTEM

The probiotic concept and the immune system

The human immune system is a highly efficient and complex system for defending the body against foreign infectious agents (bacteria, viruses, and parasites) as well as from malignant cells and other noxious agents. An immune system that functions optimally is an important safeguard against infectious and non-infectious diseases. The GI tract is the body's largest immune organ, containing an estimated 80% of all antibody-producing cells. The intestinal microbiota represents one of the key elements in the body's immune defense system (Calder et al, 2013).

The immune system of a newborn is functionally immature. Exposure to antigens during early life is essential to drive the development of the gut mucosal immune system and to maintain immune homeostasis. Microbial antigens derived from the intestinal microbiota and the environment play a crucial role in the maturation of gut-associated lymphoid tissue (GALT) and normal resistance to disease. This has been demonstrated in studies on germ-free mice. Germ-free animals have a poorly developed immune system with fewer IgA plasma cells and intraepithelial lymphocytes in the intestinal mucosa and lower levels of immunoglobulins. Compared to conventionally reared animals, they exhibit increased susceptibility to disease. Reduced microbial exposure in Western societies has also been associated with an increased incidence of atopic and autoimmune disorders (Calder et al, 2013, Versini et al, 2015).

There is a significant amount of evidence to suggest that specific probiotic strains are able to stimulate and regulate several aspects of natural and acquired immune responses. This could either be through stimulation of the gut immune system or modulation of immune cell production and function (Lei et al, 2015).

Probiotic bacteria with the ability to modulate certain immune functions may improve the response to oral vaccination, shorten the duration or reduce the risk of certain types of infection, or reduce the risk of or alleviate the symptoms of allergy and other immune-based conditions (Duerkop et al, 2009, Hardy et al, 2013).

Impact on respiratory tract illness symptoms

Reduction of cold and flu-like symptoms

Common cold is a heterogeneous group of mostly mild upper respiratory tract illnesses (URTI) that have an impact on our daily lives and are considered a major public health issue. Meta-analyses of human studies indicate that the consumption of probiotics may offer a safe and convenient way to reduce the incidence of cold and flu and the severity of symptoms and to have no harmful side effects (Santesso 2015; Kang et al, 2013; Wang et al, 2016).

The impact of *B. lactis* Bl-04 on the incidence of respiratory tract illness episodes in healthy active adults was investigated in a randomized, double-blind, placebo-controlled clinical trial (West et al, 2014a). *B. lactis* Bl-04 was administered with a total daily dose of 2.0×10^9 CFU for five months throughout the primary cold and flu season. The participants reported self-assessed symptoms of respiratory illness – scratchy or sore throat, sneezing, runny or stuffy nose – using a web-based questionnaire. Results showed a 27% reduction of risk of URTI episodes (Figure 6) and a significant

Figure 6. Bl-04 helps to maintain a healthy respiratory function.

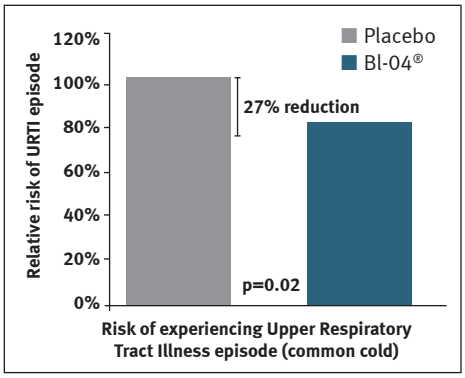
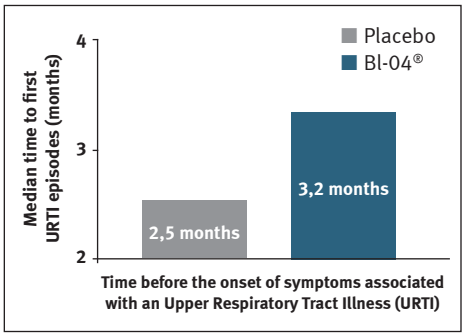


Figure 7. The result of Bl-04 on time before onset of symptoms.



delay in the onset of respiratory tract illness episodes (Figure 7) in the *B. lactis* Bl-04 group compared to the placebo group. Thus, *B. lactis* Bl-04 contributes to the maintenance of respiratory health in the adult population.

Regulatory T-cells (Treg), a subset of lymphocytes, mediate immune suppression and anti-inflammation, and thereby play a pivotal role in the homeostasis of the immune system and in the modulation of the immune response. In this study, it was examined whether the beneficial effects on respiratory illness observed with *B. lactis* Bl-04 were mediated by changes in the frequency of circulating Treg cells.

The main finding of this study was that there was little effect of daily probiotic supplementation on circulating CD41 T-cell or Treg-cell subsets. These results indicate that the reduction in risk of URTI observed following daily supplementation with *B. lactis* Bl-04, in healthy active adults is not associated with changes in the frequency of circulating Treg cells. (West et al, 2016).

The immune mechanisms by which probiotics reduce susceptibility to URTI is uncertain. To gain further insight on the purported cell-mediated immune mechanisms that might explain the reduction in respiratory illness observed following daily supplementation with *B. lactis* Bl-04, the peripheral blood of a subset of the above described study cohort was analyzed for markers including plasma cytokines, natural killer cell activity, and peripheral blood mononuclear cell (PBMC) phagocytosis capacity. No significant effect on parameters of the innate immune system from pre- to post-supplementation was observed; except the concentration of plasma macrophage inflammatory protein (MIP)-1δ which was higher in the *B. lactis* Bl-04 group than the placebo group (West et al, 2014b). The lack of detectable changes in the immune system may be explained by the fact that the volunteers were healthy and blood samples were collected when they were not ill.

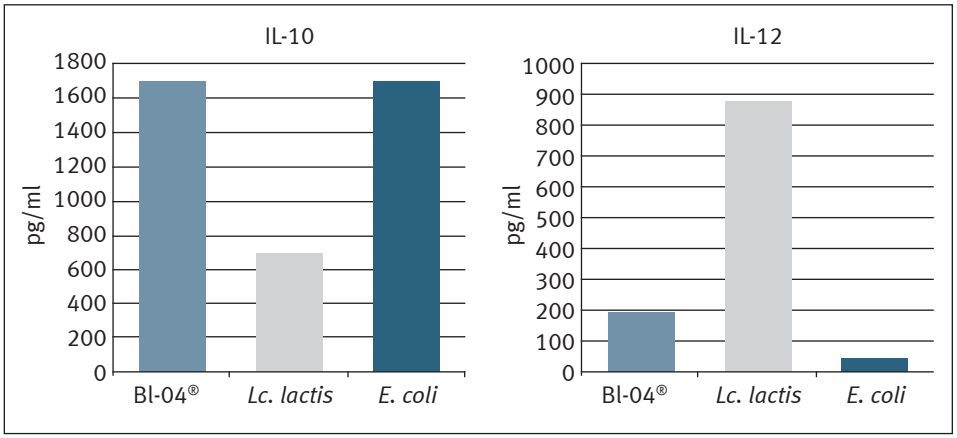
To further investigate how *B. lactis* Bl-04 influences respiratory tract infection risk, a placebo-controlled study was conducted to determine the effect of administration of *B. lactis* Bl-04 on innate and adaptive host responses to experimental rhinovirus (RV) challenge (Turner et al, 2017). Different types of viruses can cause respiratory tract infections, however, rhinoviruses account for most of the common cold episodes (30-50%) (Jacobs et al, 2013). In a randomized, double-blinded, placebo-controlled clinical trial, subjects consumed Bl-04 (n=95) or placebo (n=95) for 28 days before RV type 39 was inoculated into their nose at study day 0. The effect of the supplementation and the course of infection was followed by collecting nasal washes at days -28 and 0 before infection and daily between days 1 and 5 during infection. Viral load and chemokine CXCL8 were analyzed from nasal washes and infection symptoms by Wisconsin Upper Respiratory Symptom Survey -21 questionnaire (WURSS-21). The final analysis population included 115 subjects (after excluding subjects who had previously experienced the cold/infected with the same RV type).

The effect on the response of the chemokine CXCL8 to RV infection was defined as the primary endpoint of the study. CXCL8 is an immune cell-attracting chemokine released constantly in the mucosa to support cell trafficking. During respiratory viral infection, the level of CXCL8 rises and this has been found to modestly correlate with the severity of cold symptoms. The results showed that *B. lactis* Bl-04 maintained CXCL8 level after supplementation for 4 weeks in nasal washes ($p<0.05$). During the infection, CXCL8 concentrations were similar in probiotic and placebo groups, however relative CXCL8 response on days 1-5 (normalized to baseline at day 0) was lower in the probiotic group ($p<0.05$). IP-10, IL-6, and G-CSF showed a similar pattern but did not reach statistical significance. Post-hoc analyses of viral levels in nasal washes showed that during the infection in the *B. lactis* Bl-04 group there were less subjects shedding the virus ($p<0.05$) and increased time-to-shedding of virus compared to placebo group ($p<0.05$). In an ad-hoc analysis, not taking into account multiple corrections, also the viral titer was lower in the *B. lactis* Bl-04 group ($p<0.05$). The probiotic supplementation did not have an effect on symptom scores in the study during the infection (Turner et al, 2017).

Influence of a probiotic combination including *B. lactis* Bl-04 on birch pollen allergy

A randomized, placebo-controlled, double-blind study was performed to investigate whether birch pollen allergy symptoms are linked with gut microbiota changes and whether probiotics have an effect on alleviating symptoms of birch

Figure 8. Induction of IL-10 and IL-12 by *B. lactis* Bl-04 in PBMCs, compared with *Lactococcus lactis* and *Escherichia coli*



Source: Foligne et al, 2007

pollen allergy in children. The probiotic strains used in this study, a combination of *B. lactis* Bl-04 and *L. acidophilus* NCFM were selected on the basis that they had either anti-inflammatory properties or could be expected to induce anti-allergy cytokines, as evaluated in previous *in vitro* and animal trials. Placebo or the probiotic mixture was administered for 4 months before onset of allergy season. Symptoms, blood cytokines, and microbiota were analyzed.

In conclusion, the study showed that consumption of this probiotic combination could reduce nasal eosinophilia – a marker of allergic inflammation, which display a correlation with clinical and immunological parameters in allergic rhinitis, and it also indicated a trend towards reduced nasal symptoms like nasal blocking and runny nose, however the results were not statistically significant.

The administration of the probiotic combination also led to a significant increase in the fecal numbers of *B. lactis* Bl-04 and *L. acidophilus* NCFM from March to April/May, and numbers remained high until the end of the intervention in June. The study results suggest that probiotics may provide an alternative or complementary therapy for alleviating pollen allergy associated inflammation (Ouwehand et al, 2009).

Supporting evidence on the function of *B. lactis* Bl-04 for immune health

B. lactis Bl-04 was investigated *in vitro* for its ability to induce the secretion of anti-inflammatory IL-10 from human PBMCs. It was found that *B. lactis* Bl-04 induced IL-10 to a higher degree and low amount of IL-12 compared to other strains used as a control (Figure 8) (Foligne et al, 2007).

In vivo relevance for IL-10 induction was tested in mice using the trinitrobenzene sulfonic acid (TNBS)-induced colitis model that is characterized by inflammation. The results showed that compared to the placebo group the histological scores for colitis were lower in the *B. lactis* Bl-04 group (Figure 9) (Foligne et al, 2007).

In another study, it was shown that DNA derived from *B. lactis* Bl-04 stimulated secretion of IL-10 with IL-1β and IL-6 from human PBMCs (Lammers et al, 2003).

Overall these studies indicate that *B. lactis* Bl-04 could modulate immune response in humans.

Effect of Bl-04 on oral vaccination

The ability of *B. lactis* Bl-04 to stimulate antibody responses was evaluated in a human study measuring primary immune reaction upon vaccination. Human volunteers were orally vaccinated using cholera vaccine. In addition to this, they received twice daily either a placebo, *B. lactis* Bl-04 (10^{10} CFU/capsule), or one of the other 6 strains in the study. Supplementation with *B. lactis* Bl-04 or the placebo started on day 0 and continued for 21 days. On day 7 and 14, the subjects received the oral vaccine. Blood and saliva samples were collected on day 0, 21, and 28, and cholera antigen-specific IgG (immunoglobulin type G) antibodies were determined.

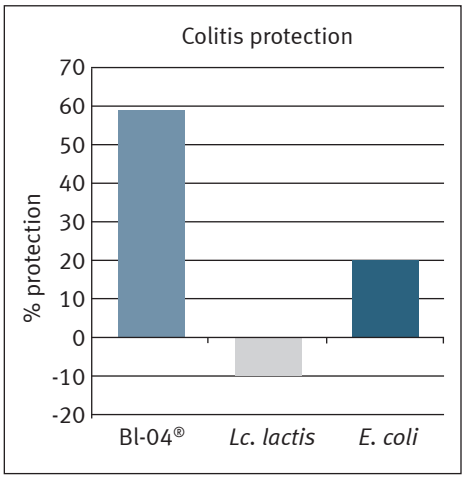
As a result, supplementation with *B. lactis* Bl-04 resulted in faster IgG induction than in the control group indicating stimulation of specific immunity by *B. lactis* Bl-04 (Paineau et al, 2008).

OTHER HEALTH-RELATED PROPERTIES

Influence of a probiotic combination including *B. lactis* Bl-04 on weight management

Recent scientific studies show that gut microbiota may play an important role in the modulation of the body weight of the host, hence modulation of the gut microbiota to prevent or control obesity, including the use of probiotics, has received increasing attention (Kobyliak et al, 2016; Dahiya et al, 2017).

Figure 9. Percentage of protection on an acute murine model of inflammation (TNBS)



Source: Foligne et al, 2007

The objective of this randomized, double-blind trial study was to investigate whether a probiotic mix has additional effects when compared with an isolated dietary intervention on the body composition, lipid profile, endotoxemia, inflammation, and antioxidant profile.

Women who had excess weight or obesity received a probiotic mix including *L. acidophilus, L. casei, Lactococcus lactis*; *B. bifidum,* and *B. lactis* Bl-04, or placebo for 8 weeks. Both groups received a dietary counselling.

In comparison with the placebo group, the probiotic mix group showed a greater reduction in the waist circumference, waist-height ratio, conicity index, plasma polyunsaturated fatty acids, and an increase in the activity of glutathione peroxidase.

As a result, supplementation of a probiotic mix reduced abdominal adiposity and increased antioxidant enzyme activity in a more effective way than an isolated dietary intervention (Gomes et al, 2017).

BENEFITS SUMMARY

B. lactis Bl-04 is a well-characterized strain with documented probiotic effects. Numerous published studies describe the strains properties with regard to characterization, safety and efficacy.

The strains health-related attributes can be summarized as follows:

- Long history of safe use
- Well-suited for intestinal survival
  - High tolerance to gastrointestinal conditions (acid and bile)
- Modulation of immune responses in humans
  - Supports respiratory health in adults
  - Induction of cytokines from human blood immune cells *in vitro*
  - Shown in an animal model to protect against intestinal inflammation and to balance the intestinal mucosal immune response

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