DANISCO

LACTOBACILLUS BULGARICUS LB-87

TM 66-1 US 11.17

Technical Memorandum

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 ingestion of probiotics, friendly bacteria, is beneficial in maintaining the body's delicate microbial balance. This balance is known to enhance intestinal health and the immune system, not to mention other physiological functions, making it a critical factor for general human well-being (de Moreno de LeBlanc and LeBlanc, 2014; Kechagia, 2013; Vandenplas, 2015).

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Hill, 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 DuPont's probiotic research:

- 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 has to fulfil 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 has to be able to resist acid and bile
- The strain must have clinically 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

CHARACTERISTICS OF THE SPECIES

Lactobacillus delbrueckii is a rod shaped, lactic acid-producing, facultative anaerobic bacterium that is non-pathogenic, non-toxigenic, non-motile and non-spore forming. *L. delbrueckii* subspecies *bulgaricus* (herein after referred to as *L. bulgaricus*) has been used historically in the manufacture of dairy products, including yogurt and a number of cheeses. It is established as a widely-used starter culture in the dairy industry, and has traditionally been used together with *Streptococcus thermophilus* in the manufacturing of yogurt (Hao, 2011, Stamatova, 2007). The synergistic relationship between *L. bulgaricus* and *S. thermophilus* as thermophilic starter cultures has been demonstrated historically, and these two bacterial cultures have been recognized as the only required ingredients for yogurt production (ISO 7889:2003 |IDF 117).

In addition to working synergistically with *S. thermophilus* during milk fermentation and yoghurt production, *L. bulgaricus* strains have been used as "adjunct" starter cultures due to their exopolysaccharide production, which is believed to increase moisture retention in certain types of cheese, and also thought to contribute to the positive viscosity and texture of fermented milk products (Teixeira 2014). It has also been demonstrated that genomic variation within strains of *L. bulgaricus* render differences in texture during yogurt production (Hao, 2011).

SELECTION AND TAXONOMY

L. bulgaricus Lb-87 was isolated from a dairy source and was identified according to standard taxonomic guidelines. The taxonomy of strain Lb-87 at the genus and species level has been determined by 16S rRNA sequencing and DNA fingerprinting. *L. bulgaricus* Lb-87 belongs to the *L. Lactobacillus delbrueckii* species and *L. Lactobacillus delbrueckii* subspecies *bulgaricus* (Figure 2). The 500 bp sequence alignment of Lb-87 matched the 16S rRNA region of *L. bulgaricus* with greater than 99% homology. The subspecies was determined by the combined use of the PCR methods described previously (Lick, 2000; Torriani, 1999). According to the taxonomy, *L. bulgaricus* Lb-87 belongs to a species included in the most updated list of Qualified Presumption of Safety (QPS) microorganisms intentionally added to food and feed (EFSA BIOHAZ 2013). DuPont™ Danisco® provides strain Lb-87 as a white to cream-colored freeze-dried probiotic powder for inclusion in food and dietary supplement products globally, and has historically manufactured and commercially offered strains of *L. bulgaricus*.

Figure 1. Scanning electron micrograph of *Lactobacillus*.

GENOMICS

While many strains of *Lactobacillus* have been used within the food industry for a number of years, the advent of whole genome sequencing has brought on the ability to understand these organisms and aspects of safety and evolution within a species over time.

L. delbrueckii subsp. *bulgaricus* is one of the six subspecies of *L. delbrueckii*; the other five subspecies include *L. delbrueckii* subsp. *delbrueckii*, *L. delbrueckii* subsp. *lactis*, and more recently discovered, *L. delbrueckii* subsp. indicus (Dellaglio F, 2005), *L. delbrueckii* subsp. *jakobsenii* (Adimpong, 2013), and *L. delbrueckii* subsp. *sunkii* (Kudo, 2012).

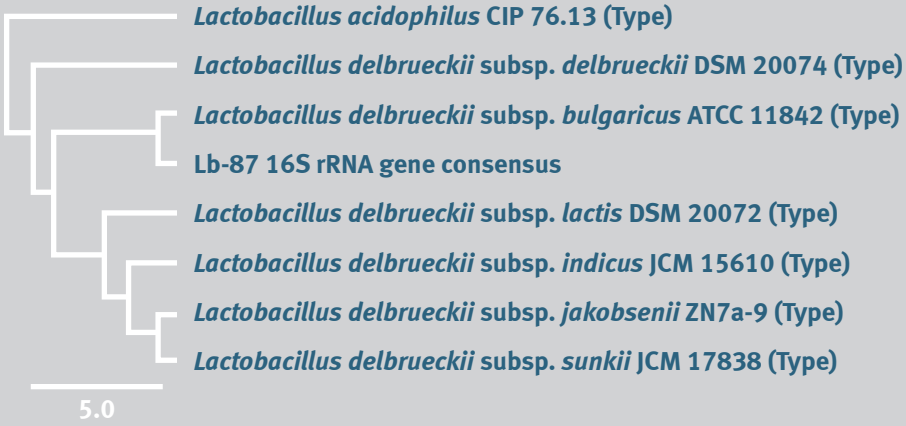

Figure 2. *Lactobacillus delbrueckii* sub-species taxonomy by full-length 16S rRNA gene alignment using Geneious (Biomatters, Auckland, New Zealand). The Lb-87 sequence is a consensus of all 16S genes present in the genome. Other nodes show the type-strain sequences for each known sub-species of *L. delbrueckii*, with *Lactobacillus acidophilus* as the outgroup, obtained from EzTaxon (Kim 2012). Branch lengths represent patristic distances.

L. bulgaricus is closely related to *Lactobacillus lactis*, as both are subspecies of *L. delbrueckii* (El Kafsi, 2014). El Kafsi have shown that both *L. bulgaricus* and *L. lactis* have evolved independently to adapt to the environment specific to milk.

L. bulgaricus, however, has surpassed *L. lactis* in its evolutionary adaptation by progressively losing superfluous functions and acquiring functions that allow an optimization of utilization of resources available in a milk environment. *L. bulgaricus* species have smaller genomes than *L. lactis*, and have lost the ability to metabolize a number of carbohydrates and the capability for amino acid biosynthesis. A complete genome sequence of *Lactobacillus delbrueckii* subsp. *bulgaricus* Lb-87 was obtained using Illumina MiSeq paired-end sequencing. The genome is is as yet unpublished but available by request. The resulting genome was advanced to a single, closed and circular chromosome 1,858,701 total base pairs in length. Comparative genomics to other published sequences revealed 99.93% identity at the DNA level to commercial strain BAA-365. Comparison to the type strain ATCC 11842 indicates a high level of genetic synteny with regions of definition, at a total pairwise identity of 83.2% similarity.

Phylogenetically, *L. bulgaricus* is closely related to other species of *Lactobacillus*, including *L. acidophilus*, *L. helveticus*, *L. johnsonii* and *L. gasseri* (Teixeira 2014). All of which have maintained inclusion on the QPS list (EFSA BIOHAZ 2013) with a recognized history of safe use.

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. In order to maintain the quality, purity and consistency of each production batch of the strain, DuPont makes rigorous use of bacterial frozen 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

L. bulgaricus has been consumed in fermented milks and other food products for decades. *L. bulgaricus* was originally documented in 1930, with reference for food usage dating back to 1979, is listed in the Inventory of Microorganisms With Documented History of Use in Human Food (Bourdichon, 2012). The European Food Safety Authority (EFSA) has also added the species to the QPS list (EFSA 2007, EFSA BIOHAZ 2013). Moreover, *L. bulgaricus* has been listed in the U.S. FDA Generally Recognized as Safe (GRAS) list for use as food additives (U.S. Food and Drug Administration, 2001).

Lactic acid bacteria have long been considered safe and suitable for human consumption. Very few instances of infection have been associated with these bacteria, and those commonly with immunocompromised subjects with underlying health issues (Aguirre and Collins, 1993; Borriello et al, 2003; Gasser, 1994; Gueimonde et al, 2004; Hempel et al, 2011; Salminen et al, 1998). The only documented case of *L. bulgaricus* infection was in 1938, where *L. bulgaricus* was isolated from blood culture of a patient with leukemia. However, the accuracy of identification to the species level can be questioned due to insufficient identification methods. No clinical cases have been reported since (Teixeira 2014).

In vitro, L. bulgaricus has demonstrated very low gelatinase activity, a specific marker reflecting periodontal destruction, indicating that the species is not harmful for gingival health (Stamatova et al, 2007). In order to assess the safety of *L. bulgaricus* Lb-87 further, a fourteen-day acute oral toxicity study was conducted in female rats (DuPont, 2015, Internally generated data). At a dose of 5000 mg/kg (=$3.62x10^{11}$cfu/kg) Lb-87, no mortality, clinical abnormalities, or overall body weight loss was observed. In addition, no gross lesions were present in the rats at necropsy (DuPont, 2015, Internally generated data).

Antibiotic susceptibility patterns

Antibiotic susceptibility profiles are an important means of demonstrating the potential of an organism to be readily inactivated by the antibiotics used in human therapy. Antibiotic resistance 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. 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. 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. Lb-87 has a single multidrug-efflux transporter gene encoded within its genome, which is hypothesized to provide some level of resistance to fluoroquinolones (Neyfakh et al, 1993; Piddock, 2006).

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, especially if it can be passed on to other bacteria; including potentially pathogenic ones. For Lb-87, the antibiotic susceptibility pattern is summarized in Table 1 (DuPont, internally generated data). According to these results, Lb-87 does not display acquired antibiotic resistance.

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 histamine and tyrosine decarboxylase genes has been developed internally in 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 Lb-87 genome. Consequently, Lb-87 is unlikely to produce histamine or tyramine, theoretically decreasing the risk for adverse reactions in those individuals ingesting Lb-87 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. 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. The ratio of D- and L-lactate for Lb-87 is 97.6% D-lactate and 2.4% L-lactate.

In humans, animals, plants and microorganisms, L(+)-lactic acid is a normal intermediate or end product of carbohydrate and amino acid metabolism whereas, D(-)-lactic acid was thought to be "non-physiological" and a possible cause for lactate acidosis. However, mammals (including humans) express D-alpha-hydroxy acid dehydrogenase which is able to metabolise D(-)-lactic acid, albeit at a slower rate then L-lactate dehydrogenase (Ewaschuk et al, 2005)

Although probiotic cultures as nutritional ingredients that produce D(-) lactic acid can be safely administered to infants, the CODEX recommendation not to use D(-) lactic acid producing cultures in food for infants below the age of 12 months, should be followed (Connolly et al, 2005).

L. bulgaricus metabolizes and produces D(-)-lactic acid from four sugars: lactose, glucose, fructose and mannose (Figure 3) (Hao et al, 2011). *L. bulgaricus* strains also have the capability to convert pyruvate into D(-)-lactic acid via a NAD dependent D-lactate dehydrogenase (Razeto et al, 2002).

Table 1. Lb-87 Antibiotic Susceptibility Profiles

Antibiogram of *L. bulgaricus* Lb-87 was established using ISO 10932 IDF223 method and VetMIC Lact-1 and 2 micro-dilution plates that include all antibiotics that are recommended by The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). Recorded Minimum Inhibitory Concentrations (MIC) are displayed. All MIC values are below the Microbial Break Points (MBPs) defined for *L. delbrueckii* subsp. *bulgaricus* (EFSA FEEDAP Panel 2012).

	Gentamycin	Kanamycin	Streptomycin	Tetracycline	Erythromycin	Clindamycin	Chloramphenicol	Ampicillin	Vancomycin	Virginamycin*
	Gm	**Km**	**Sm**	**Tc**	**Em**	**Cl**	**Ch**	**Amp**	**Va**	**Vi***
Lb-87	**Max MIC µg/ml**									
Lactobacillus delbrueckii subsp. bulgaricus Lb-87	**1**	**16**	**8**	**4**	**0.06**	**<0.03**	**4**	**0.12**	**1**	**0.25**
MBP for *Lactobacillus* obligative homofermentative	**16**	**16**	**16**	**4**	**1**	**1**	**4**	**1**	**2**	**4**

*Virginamycin is no longer included in the FEEDAP recommended list of antibiotics

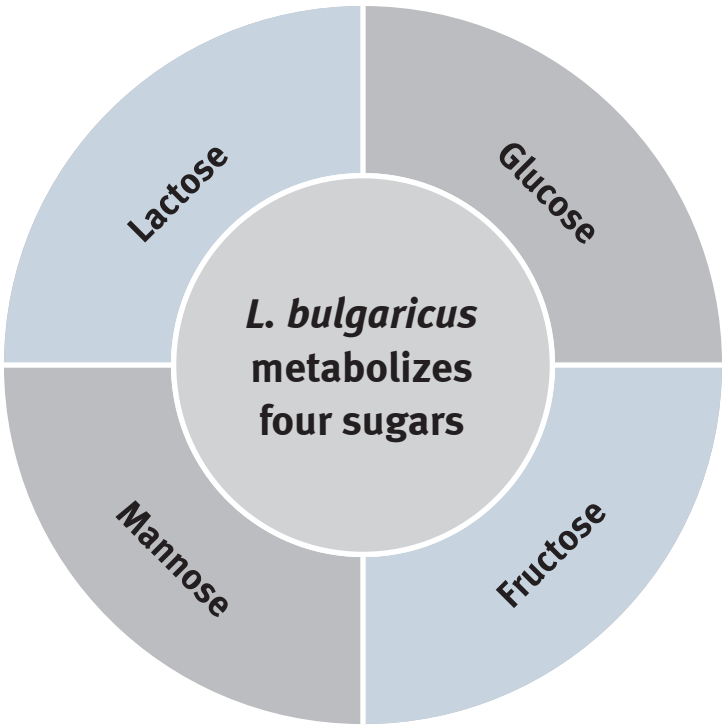

Figure 3. *L. bulgaricus* metabolizes and produces D(-)-lactic acid from four sugars: lactose, glucose, fructose and mannose.

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. Delivering the proper dose of *L. bulgaricus* Lb-87 over the shelf-life of a product can be dependent on many factors such as formulation, processing, packaging and storage temperature and humidity. It is important to consider these factors and run stability trials to develop sound products.

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. Health benefits provided by *L. bulgaricus* were first proposed by Metchnikoff in 1907 and further supported by researchers Douglas and Kopeloff in the 1920s (Teixeira 2014). In general, fermenting of milk, such as in yogurt and cheese making, where lactose is converted into lactate, provides those with lactose intolerance improved lactose digestion. A scientific opinion by the European Food Safety Authority Panel on Dietetic Products, Nutrition and Allergies reported that the claimed benefit of lactose digestion provided by yoghurt cultures, *L. bulgaricus* and *S. thermophilus* in yogurt is a recognized beneficial physiological effect (EFSA Panel on Dietetic Products, 2010).

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 fulfils 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 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 also 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, 2013; Lin et al, 2014; Ouwehand, 2006; Scott et al, 2015).

Resistance to acid and bile and survival in the intestinal passage

According to the generally accepted definition of probiotics, a probiotic

Table 2. Acid and bile tolerance of Lb-87.

	++++ Excellent +++ Very good ++ Good +Fair
Acid tolerance	**+** Fair (‹69% survival in hydrochloric acid and pepsin (1%) at pH 3 for 1 hour at 37°C)
Bile tolerance	**+++** Very Good (80-89% survival in 0.3% bile salt containing medium)

Source: DuPont, internally generated data

Table 3. Adherence of Lb-87 to human colonic mucus *in vitro*.

	+++++ Excellent ++++ Very good +++ Good ++Fair +Poor
Adherence to human colonic mucus	**+++** Good (2 – 3.5% of added bacteria)

Source: DuPont, internally generated data

microorganism should be viable at the time of ingestion to confer health benefit. A variety of traits are believed relevant for surviving passage through the GI tract, the most important of which is tolerance to the highly acidic conditions present in the stomach and to the concentrations of bile salts found in the small intestine. *In vitro* studies conducted by DuPont™ Danisco® have shown that *L. bulgaricus* Lb-87 is fairly resistant to low pH conditions and has a very good survival rate in the presence of bile at the concentrations present in the duodenum (Table 2).

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. *L. bulgaricus* Lb-87 has demonstrated good adhesion to human intestinal mucus in *in vitro* studies (Table 3).

BENEFITS SUMMARY

Based on the data generated, the Lb-87 strain's key attributes can be summarized as follows:

- One of the two bacterial cultures required for yogurt production
- Ability to rapidly convert lactose into lactic acid
- Ability to survive at high temperatures required for production of dairy products
- Long history of documented safe use
- Ability to lend to the positive viscosity and texture of fermented milk products acid and bile resistance
- Ability to convert lactose into lactate, aiding in lactose digestion

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About DuPont™ Danisco®

DuPont™ Danisco® is the brand for a range of products that help provide enhanced bioprotection, an improved nutritional profile, and better taste and texture with greater cost efficiency and lower environmental impact, meeting the needs of manufacturers of food and beverages, dietary supplements and pet food. Through the work of the global network of food scientists and technologists in DuPont™, the Danisco® range is supported by a uniquely broad spectrum of know-how across applications and processing.

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