

# LACTOBACILLUS ACIDOPHILUS NCFM® – A PROBIOTIC WITH PROVEN EFFICACY

## 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 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; LeBlanc & LeBlanc 2014, Kechagia et al 2013 ].

*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 possess probiotic properties.

The beneficial effects of probiotics either involve reducing the risk factors for certain diseases 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 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. However, it should be noted that each probiotic strain has its own specific health benefits, and no probiotic elicits all the health benefits proposed. Furthermore, when one probiotic strain has a certain health benefit, it cannot be assumed that another strain, not even when 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 end-product intended for the consumer.

- The strain must be safe – this requires identification by appropriate molecular techniques
- The strain must be resistant to 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, whether food or dietary supplements, and when included in a food either be neutral or contribute favorably to the flavor of that food product

The only certain way to establish the true quality and value of a probiotic strain is by systematic *in vitro* and *in vivo* studies

and, in particular, human clinical trials. *L. acidophilus* NCFM® has been subject to all these types of studies. In several reviews the scientific evidence for this strain is highly rated [Ouwehand et al (eds) 2009, Sanders et al 2001, Reid 1999, Bull et al 2013].

### CHARACTERISTICS OF THE SPECIES

*Lactobacillus acidophilus* is a Gram-positive, non-spore forming, homo-fermentative, catalase-negative rod. It is a common inhabitant of the human intestinal tract, the human mouth and vagina. It is also found in some traditional fermented dairy products (e.g. kefir) and is today widely used in probiotic foods and supplements. Numerous studies have demonstrated the diverse beneficial effects of different strains of *L. acidophilus*, validating its use as a probiotic.

### SELECTION AND TAXONOMY

The group of organisms previously known as *L. acidophilus* was shown to be highly heterogeneous [6] and throughout the development of methods to identify and characterise bacteria it has undergone multiple taxonomic revisions [Bull et al 2013]. The results of DNA-DNA hybridization studies have suggested that the previous *L. acidophilus* species was composed of six different species, which were divided into different DNA homology groups. Homology group A1 was designated as *L. acidophilus*. These six species are quite difficult and sometimes impossible

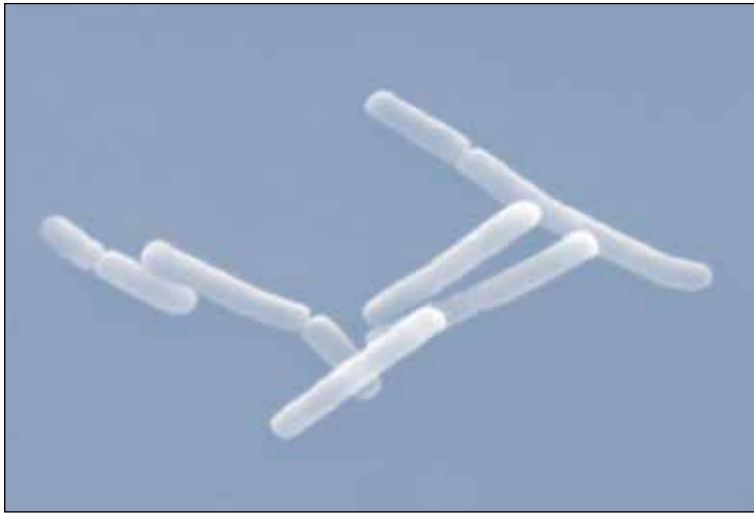


Figure 1. Scanning electron micrograph of *L. acidophilus* NCFM®

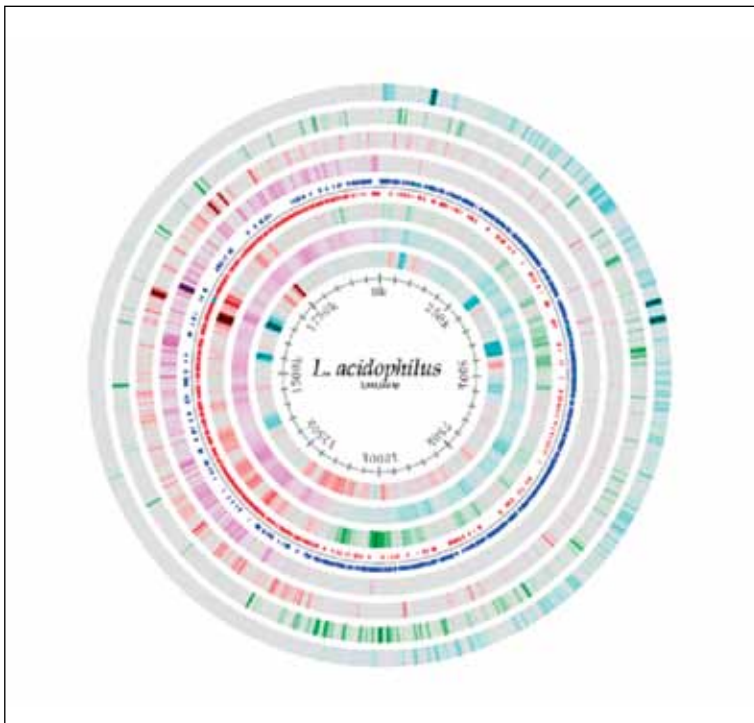


Figure 2. Genome atlas of *L. acidophilus* NCFM® [Altermann et al 2005]

to differentiate by phenotypic methods, so they are still considered members of the “*L. acidophilus* group”.

*L. acidophilus* NCFM® (Figure 1) has been confirmed as a true type A1 *L. acidophilus* using phenotypic and genotypic methods, including 16S rRNA gene sequencing and hybridization to a species-specific probe and whole genome sequencing [Altermann et al 2005] (Figure 2).

*L. acidophilus* NCFM® was first isolated from a human source in the early 1970s. The name NCFM® is derived from “North Carolina Food Microbiology”, the research laboratory at North Carolina State University (NCSU) where this successful isolation took place. The strain has been deposited with the American Type Culture Collection as ATCC 700396 and in the safe deposit of the ATCC as SD5221.

### *L. acidophilus* NCFM® aliases

Multiple strain designations appear in literature for *L. acidophilus* NCFM® or for single colony isolates of the NCFM parent culture. The strains designated NCFM, NCK56, NCK45, N2, RL8KR, RL8KS and RL8K are essentially identical strains, as indicated by their identical chromosomal DNA fragment patterns. In studies conducted by Simenhoff and colleagues (1996), NCFM was abbreviated as LBA [Simenhoff & Dunn, 1996; Simenhoff et al, 1996].

### GENOMICS

*L. acidophilus* NCFM® was the first strain of this species for which the genome has been sequenced and annotated (figure 2). As a partner in the development of the complete *L. acidophilus* NCFM® sequence [Altermann et al 2005, Palomino et al 2015]

DuPont is now expanding this information at many levels. Genomic work has identified several regions important in probiotic functionality and which support the role of *L. acidophilus* NCFM® in maintaining or restoring gastrointestinal well-being. Included in this are genes involved in bacteriocin production, sugar and prebiotic metabolism [Barrangou et al 2003], adherence to human cell lines [Buck et al 2009], lactose metabolism, and tolerance to physiologically relevant stresses including acid and bile [Altermann et al 2005, Azcarate-Peril et al 2004 & 2005]. Genomic reconstruction of metabolic pathways reflects the adaptation of *L. acidophilus* NCFM® to the gastrointestinal environment. Targeted gene disruptions are being evaluated to help establish probiotic functionality in relation to specific gene products and validate and support functional probiotic targets [Goh et al 2009, O’Sullivan et al 2009, Pfeiler et al 2007, Azcarate-Peril et al 2009, Bull et al 2014]. Genomic information is also

used to develop strain identity methods and improve industrial processes.

### 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 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 culture.

### SAFE FOR CONSUMPTION

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 several published studies have addressed their safety [Borriello et al 2003, Hempel 2011]. Moreover, no *L. acidophilus* bacteraemia were identified in a 10-year survey in Finland [Salminen et al 2002]. More specifically, *L. acidophilus* has been consumed in fermented milks and other food products for decades and is listed in the *Inventories of Microorganisms With Documented History of Use in Human Food* [Bourdichon et al 2012]. The European Food Safety Authority (EFSA) has also added the species to the Qualified Presumption of Safety list [EFSA BIOHAZ 2013].

Since its market introduction more than 30 years ago, billions of servings of foods and supplements containing *L. acidophilus* NCFM® have been safely consumed.

### General safety

In order to assess the safety of *L. acidophilus* NCFM® further, several

toxicity studies have been performed on mice. The safety of the strain was evaluated in a colitis mouse model using trinitrobenzenesulphonic acid (TNBS) to induce colitis.

In healthy mice, intra-gastric administration of *L. acidophilus* NCFM® did not show any potential adverse effect on mouse activity, weight and colon inflammation. In TNBS-treated mice (mice with very strong colitis), high doses (10<sup>10</sup> CFU) of *L. acidophilus* NCFM® led to no translocation of the organism nor abnormal translocation of the intestinal microbiota. Nor was any significant improvement in colitis observed [Daniel et al 2006].

Safety was further confirmed in studies with neonatal and adult immune deficient mice, where no mortality was observed amongst mice fed with *L. acidophilus* NCFM® [Wagner et al 1997a].

To investigate the safety and tolerability of *L. acidophilus* NCFM® in conjunction with defined clinical end-points, a cohort of healthy active adults from the trial by West et al 2014 was analyzed for routine hematology and clinical chemistry markers. Supplementation with a combination of *L. acidophilus* NCFM® and *Bifidobacterium lactis* Bi-07™ (5 × 10<sup>9</sup> CFU of each strain) had no impact on these markers [Cox et al 2014]. These data provide evidence supporting the use of this probiotic supplement over a period of 5 months in healthy active adults without obvious safety or tolerability issues.

In summary, NCFM® has been safely investigated in 48 human clinical trials as a single entity and in combination with other probiotics and/or prebiotics. The age of the subjects in these trials has ranged from infants to elderly people

(from 6 months to over 65 years). Most of the studies were conducted in healthy subjects, but some also involved subjects with atopic dermatitis (AD), functional bowel disorders or infections requiring antibiotic treatment. None of the trials have reported any safety concerns related to NCFM®.

### 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 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 or intrinsic*: 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.
- *Acquired*: 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

**Table 1. Antibiotic Susceptibility Profile**

Antibiogram of *L. acidophilus* NCFM® 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 EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). Recorded MICs are displayed in the table below. All MIC values are below or equal to the Microbial Break Points (MBPs) defined for *L. acidophilus* [EFSA FEEDAP Panel 2012].

	Gentamycin	Kanamycin	Streptomycin	Tetracycline	Erythromycin	Clindamycin	Chloramphenicol	Ampicillin	Vancomycin	Virginamycin*
	Gm	Km	Sm	Tc	Em	Cl	Ch	Amp	Va	Vi*
NCFM®	MIC µg/ml									
	Max.	Max.	Max.	Max.	Max.	Max.	Max.	Max.	Max.	Max.
<i>Lactobacillus acidophilus</i>	2	32	2	2	0.06	0.5	4	0.5	<0.25	<0.5
MBP for <i>Lactobacillus acidophilus</i> **	16	64	16	4	1	1	4	1	2	4

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

Of the acquired resistances, the latter is of most concern, as it may also be passed on to other (potentially pathogenic) bacteria. Analysis of the *L. acidophilus* NCFM® genome has confirmed the absence of known transferable genetic elements related to antibiotic resistance [Altermann et al 2005]. The antibiotic susceptibility patterns for *L. acidophilus* NCFM® are summarized in Table 1 [Klare et al 2007].

According to these results, *L. acidophilus* NCFM® does not display acquired antibiotic resistance.

#### L/D-lactic acid production

Lactic acid is the most important metabolic end-product of fermentation processes by lactic acid bacteria and other micro-organisms. Lactic acid fermentation has been used for thousands of years for the production of fermented foods. Due to the 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 micro-organisms, L(+)-lactic acid is a normal

intermediate or end product of the carbohydrate and amino acid metabolic processes. It is important for the generation of energy under anaerobic conditions.

Within the organs of humans and animals, the endogenous synthesis of D(-)-lactic acid is very low in quantity. The isomer is normally present in the blood of mammals at nanomolar concentrations and may be formed from methylglyoxal which derives from lipid or amino acid metabolism. The body also has D-hydroxyacid dehydrogenase activity, but with a much lower activity as for L(+)-lactic acid. *L. acidophilus* NCFM® expresses both L-lactate dehydrogenase and D-lactate dehydrogenase and therefore produces both L(+)- and D(-)-lactic acid (Table 2).

Despite the fact that there is no real scientific proof that healthy infants or any healthy human [Conolly et al 2005] would be affected detrimentally by the addition of lactobacilli that produce D(-)-lactic acid, DuPont follows the CODEX recommendation [CODEX STAN 72-1981 rev 2007] not to use D(-)-lactic acid producing cultures in food for infants below the age of 12 months.

#### 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. *L. acidophilus* NCFM® has demonstrated to be a very versatile strain. Food and supplement

**Table 2.**

<b>L/D lactic acid production</b>	<b>60/40</b>
<b>Molar ratio</b>	<b>Boehringer Mannheim/ R-Biopharm D-lactic acid/ L-lactic acid UV-method</b>

Internally generated data

manufacturers find *L. acidophilus* NCFM® particularly attractive for several reasons, including:

- proven health benefits
- available as a high-count freeze-dried material
- patented stabilization system providing tremendous stability benefits in non-liquid products [109].
- excellent stability in a variety of applications, including milk, yogurt, powder formulations (toddler formulas, powdered beverages, capsules and tablets), chocolate bars, etc. [Gilliland et al 2002, Hughes & Hoover 1995, Sanders et al 1996, Iturrira-Laverty et al 1999, Trahan 2008, Dupont Technical Memorandum 3514, 2093, 2088].

Delivering the proper dose of *L. acidophilus* NCFM® 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.

*L. acidophilus* NCFM® is a safe probiotic with no negative impact on sensory or other product properties under typical usage conditions.

## HEALTH-RELATED PROPERTIES

*L. acidophilus* NCFM® has been extensively studied resulting in the publication of 125 in vitro, 47 animal and 54 clinical trial peer-review studies. These studies have focused on characteristics that indicate beneficial effects such as: acid and bile resistance; adhesion to intestinal and oral surfaces; antimicrobial activity and the ability to bind various environmental toxins; the mechanisms of action in animal models; and efficacy in human clinical

trials. The key findings of this research 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 is comprised of:

- the gastrointestinal 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. However, in recent years 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<sup>14</sup>), which is 10 times more than the number of cells that make up the human body (Luckey, 1972). 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 and potentially pathogenic microbes. When the delicate ecological balance of this highly complex microbial community is disturbed by environmental or physiological factors, the body's 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, reduce the risk of a range of diseases or unfavorable conditions [Guarino et al (eds) 2013, Ouwehand et al (eds) 2006, Scott et al 2015, Lin et al 2014].

### Resistance to acid and bile and survival in the intestinal passage

According to the generally accepted definition of a probiotic, the probiotic microorganism should be viable at the time of ingestion to confer a health benefit. This definition implies that a probiotic must survive GI tract passage and, according to some interpretations, transiently colonize the gut mucosa of the host. A variety of traits are believed to be relevant for surviving passage through the GI tract, the most important of which is tolerance to both the highly acidic conditions present in the stomach and to concentrations of bile salts found in the small intestine (Table 3 shown on page 6).

*In vitro* studies have shown that *L. acidophilus* NCFM® is able to resist low pH conditions similar to those in the stomach. The strain is also able to survive the presence of bile at concentrations present in the duodenum.

**Table 3. Acid and bile tolerance of *L. acidophilus* NCFM®**

			++++ 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	++++	(>90% survival in 0.3% bile salt containing medium)				
Pepsin resistance	+++	(>60% survival in 0.3% pepsin containing medium at pH 2 for 1h)				
Pancreatin resistance	++++	(>60% survival in 0.1% pancreatin containing medium at pH 8 for 2h)				

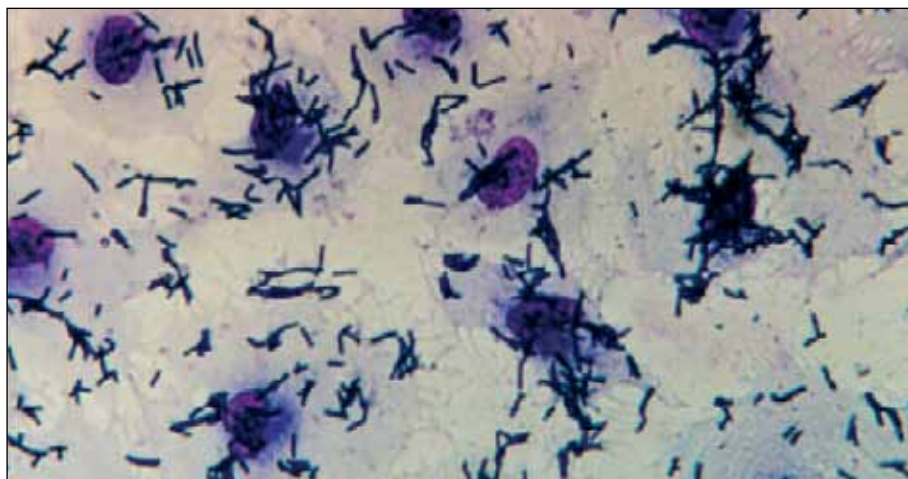
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In another study it was shown that ingestion of *L. acidophilus* NCFM®, consumed in a non-fermented low fat milk, significantly increased the numbers of lactobacilli in the feces of healthy males [Gilliland et al 1978].

A further study evaluated the effect of two probiotic strains, *L. acidophilus* NCFM® and a *B. lactis*, on the composition of fecal microbiota in young children (age 6-24 months). The results demonstrated a significant increase in *L. acidophilus* and *B. lactis* in feces after ingestion of the corresponding probiotic strain over a 2-month period, confirming survival of the cultures in the GI tract [Larsen et al 2008].

#### 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. acidophilus* NCFM®'s ability to adhere to different human epithelial cell lines has been confirmed in several



**Figure 3. *L. acidophilus* NCFM® shows strong adherence to human fetal intestinal epithelial cells [Kleeman & Klaenhammer 1982].**

studies [Kleeman & Klaenhammer 1982, Greene & Klaenhammer 1994].

It has also been shown that the adhesion property could be further improved by the addition of Ca<sup>2+</sup> (Figure 3) [Kleeman & Klaenhammer 1982]. Genome analysis of *L. acidophilus* NCFM® has indicated the presence of several genes of potential importance to the adherence process, including mucus-binding proteins, fibrinectin-binding proteins and others. Clusters of genes encoding for exopolysaccharides may

also contribute to adherence capabilities [Altermann et al 2005, Buck et al 2005]. Adherence of *L. acidophilus* NCFM® was further measured using two *in vitro* cell lines, Caco-2 and HT-29 (Table 4). While this is not a thorough test of the ability of probiotics to adhere to intestinal mucosa in the body, attachment to these cell lines is considered a good indicator of their potential to bind to intestinal tissue. *L. acidophilus* NCFM® shows very good adherence to cultured intestinal cells (HT-29 and/or Caco-2 cells).

**Table 4. Adherence of *L. acidophilus* NCFM® to human intestinal cells *in vitro***

		++++ Excellent	+++ Very Good	++ Good	+ Fair
Adherence to human epithelial cells	HT-29:	+++			
	Caco2:	+++			

(Internally generated data)

To identify putative genes potentially involved in the survival of *L. acidophilus* NCFM® and its colonization of the human digestive system, an *in vitro* human digestion model of the GI tract was used. Specific genes encoding stress-related proteins are strongly up-regulated when exposed to gastric juice, which probably facilitates the survival of *L. acidophilus* NCFM® under the harsh conditions present in the stomach. The genes encoding fibronectin-binding protein and mucin binding protein are up-regulated during incubation in duodenal juice and bile. These inductions may contribute to the attachment of *L. acidophilus* NCFM® to intestinal epithelial cells [Weiss & Jespersen 2008].

### Inhibition of pathogens

The protection that probiotic bacteria provide against gastrointestinal pathogens is highly important to the therapeutic modulation of the enteric microbiota. Probiotics are able to inhibit, displace and compete with pathogens, although these abilities are strain-dependent. The probiotic strain's putative mechanisms of action against pathogenic microorganisms include the production of inhibitory compounds, competition with pathogens for adhesion sites or nutritional sources, inhibition of the production or action of bacterial toxins, ability to coaggregate with pathogens, and the stimulation of the immune system. Several studies have demonstrated the antagonistic activity of *L. acidophilus* NCFM® against common gastrointestinal pathogens and food-borne disease microbes.

*In vitro* inhibition is usually investigated using an agar inhibition assay, where soft agar containing the pathogen is laid over colonies of probiotic cultures, causing the development of inhibition zones around the colonies. This effect

**Table 5. Pathogen inhibition of *L. acidophilus* NCFM® *in vitro***

	++++ Excellent	+++ Very Good	++ Good	+ Fair
<b>Pathogen inhibition <i>in vitro</i></b>	<b><i>Salmonella</i>: +</b>			
	<b><i>Staphylococcus aureus</i>: ++++</b>			
	<b><i>Escherichia coli</i>: +++</b>			
	<b><i>Listeria monocytogenes</i>: +</b>			

(Internally generated data)

may be due to the production of acids, hydrogen peroxide, bacteriocins and other substances that act as antibiotic agents as well as competition for nutrients. It should be pointed out, however, that the extrapolation of such results to the *in vivo* situation is not straightforward. The assessment in Table 5 is based on such an *in vitro* assay.

A study using co-cultures of *L. acidophilus* NCFM® with *Salmonella typhimurium*, *Clostridium perfringens*, *E. coli* or *Staphylococcus aureus* has indicated antimicrobial activity that could not be attributed to a reduction in pH (as this was maintained above 5.7). Hydrogen peroxide formation was responsible for part of the inhibition effect [Gilliland & Speck 1977]. The ability of *L. acidophilus* NCFM® to produce antimicrobial substances was confirmed by the genome sequence of the strain, where 12 putative genes were identified and implicated in the production and processing of antimicrobial substances [Altermann et al 2005]. Lactic acid bacteria have the ability to produce a range of antimicrobial substances of which organic acids, hydrogen peroxide and diacetyl are the most widely known [Ouweland & Vesterlund 2004].

*L. acidophilus* NCFM® was found to produce a bacteriocin, designated lactacin B. *In vitro* tests of inhibition indicated a range of activity only against other *Lactobacillus* strains and *Entero-*

*coccus faecalis*, not against pathogens [Barefoot & Klaenhammer 1983 & 1984, Dobson et al 2007].

The ability to aggregate and coaggregate is desirable for probiotics as this relates to the ability to interact closely with pathogens, perhaps preventing or reducing their adhesion to the mucosa. *L. acidophilus* NCFM® showed auto-aggregation and high coaggregation, especially with *Clostridium histolyticum* and *Staphylococcus aureus in vitro* [Collado et al 2008].

*In vitro* studies of *L. acidophilus* NCFM® demonstrated its ability to inhibit the adhesion of *Bacteroides vulgatus* (by 46.7%), *Clostridium histolyticum* (by 29.1%), *Clostridium difficile* (by 33.5%), *Staphylococcus aureus* (by 45.7%), *Enterobacter aerogenes* (by 41.3%) and *Listeria monocytogenes* (by 15.4%) to intestinal mucus [Collado et al 2007].

The strain was also able to displace *B. vulgatus* (60.6% of adhered bacteria), *C. histolyticum* (61.1% of adhered bacteria), *C. difficile* (52.9% of adhered bacteria), *S. aureus* (20.5% of adhered bacteria), *E. aerogenes* (55.4% of adhered bacteria) and *L. monocytogenes* (51.9% of adhered bacteria) *in vitro* [Collado et al 2007]. Another *in vitro* study has investigated the growth inhibition of *Bacillus cereus* by different lactobacilli, including *L. acidophilus* NCFM®. In co-culture with the patho-

gen in a skim milk medium the germination and sporulation of *B. cereus* was inhibited without affecting the growth of *L. acidophilus* NCFM<sup>®</sup>. The organic acids produced by lactobacilli inhibited the growth of *B. cereus* [Kim et al 2000].

The ability of *L. acidophilus* NCFM<sup>®</sup> and other lactobacilli and bifidobacteria to inhibit the growth of the common intestinal pathogens *E. coli* and *S. typhimurium* *in vitro* was studied using a disc diffusion test as well as co-culturing *L. acidophilus* NCFM<sup>®</sup> and the pathogens in a liquid medium. With both methods, *L. acidophilus* NCFM<sup>®</sup> demonstrated a strong growth inhibition against both pathogens. Under these experimental conditions, the antimicrobial activity was strain specific and not due to pH alone [Kim et al 2002].

Protozoan parasites of the genus *Cryptosporidium* are a cause of diarrhea in domestic livestock and humans worldwide, with bovine *C. parvum* and *C. hominis* being responsible for the vast majority of infections. An *in vitro* study showed that cell-free supernatants of *L. acidophilus* NCFM<sup>®</sup> significantly reduced the cell culture infectivity of both pathogens [Glass et al 2004].

### Prebiotic utilization

The ability of gastrointestinal bacteria to utilize diverse carbohydrates successfully in the intestinal tract may provide a competitive advantage. Prebiotics are non-digestible food ingredients that selectively stimulate the growth and/or activity of beneficial microbial strains residing in the host intestine [Hutkins et al 2015]. Fructooligosaccharides (FOS) are a well-studied family of fructose polymers with beneficial effects on the host microbiota. FOS are not digested in the upper gastrointestinal tract in humans, but can be degraded

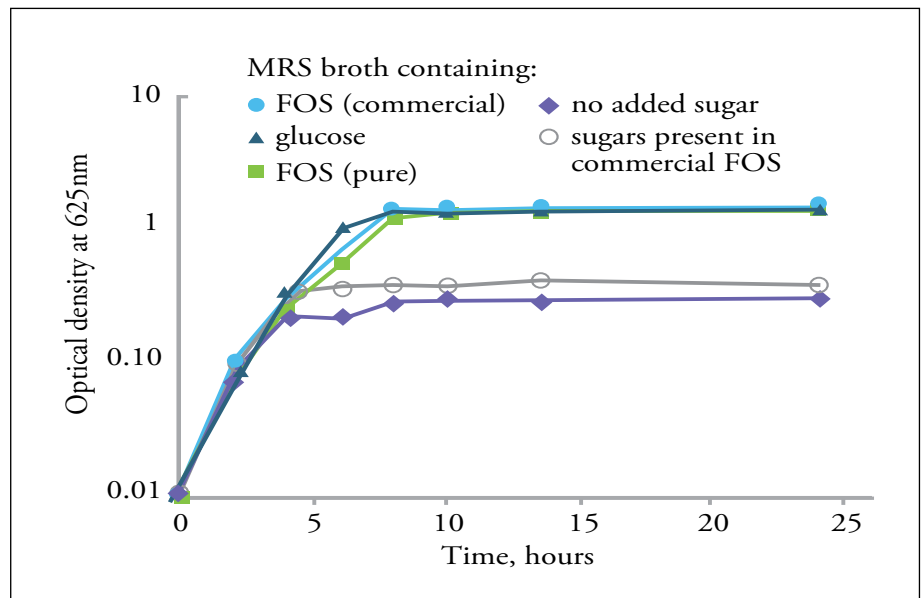


Figure 4. Utilization of FOS by *L. acidophilus* NCFM<sup>®</sup>

by a number of bacteria residing in the lower GI tract.

The ability of *L. acidophilus* NCFM<sup>®</sup> to utilize various FOS oligomers has been published previously [Kaplan & Hutkins 2005] (Figure 4).

Additional studies have also demonstrated the ability of the strain to utilize hydrolysed inulin [Barrangou et al 2003] and other prebiotics, providing opportunities for formulating synbiotic functional food [Barrangou et al 2006, Huebner et al 2007, Swanson et al 2002].

*L. acidophilus* NCFM<sup>®</sup> possesses an efficient and well-characterized FOS transport and catabolic system [Barrangou et al 2003]. This particular system may give *L. acidophilus* NCFM<sup>®</sup> a competitive advantage in the intestinal tract because the substrate is transported into the cell before hydrolysis, reducing the possibility that single carbohydrates become fermentable carbon sources for competing strains in the intestinal environment. Additionally, the *L. acidophilus* NCFM<sup>®</sup> genome encodes a large variety of genes related to carbohydrate utilisation, including

mono-, di-, and polysaccharides [Altermann et al 2005]. This supports its adaptation to the intestinal environment.

To increase the understanding of the metabolic properties of *Lactobacillus acidophilus* NCFM<sup>®</sup> a recent study investigated changes in the abundance of different proteins of the probiotic bacterium when grown on the potential prebiotic cellobiose as compared to glucose. The present findings indicate that cellobiose is able to affect the probiotic activity of *Lactobacillus acidophilus* NCFM<sup>®</sup> with regard to interactions with the host [van Zanten et al 2013].

### Reduction of undesired fecal enzyme activity and other cancer-related markers

Produced by a variety of microbes present in the GI tract, fecal enzymes such as azoreductase, nitroreductase and  $\beta$ -glucuronidase are able to convert procarcinogens present in the digesta into carcinogenic substances. By modulating the activity of these microbes, the level of these enzymes can be reduced. Goldin and Gorbach give a good overview of the significance of fecal enzyme activity [Goldin & Gorbach 1984],

while Uccello et al provide a more recent reference to the relevance of fecal enzyme activity and the potential role of probiotics on colorectal cancer. [Uccello et al 2012].

Several studies have been conducted to evaluate the effect of *L. acidophilus* NCFM® on negative, potentially carcinogenic activities of the animal and human intestinal microbiota.

Rat studies have confirmed that fecal azoreductase,  $\beta$ -glucouronidase, and nitroreductase enzyme activity is reduced in animals fed with *L. acidophilus* NCFM®. In addition they have demonstrated the reduced incidence of 1,2-dimethylhydrazine induced tumors [Goldin & Gorbach 1977, 1980 & 1984]. Aberrant crypt foci (ACF) are lesions in the colon which are putative precursors of colon cancer. ACF in rodents were found to correlate with colon cancer risk and the size and number of adenomas in humans. The ACF system is today widely used to study modulators of carcinogenesis, e.g. to screen for compounds in the diet that might either cause or inhibit colon

cancer. Rat studies have indicated that azoxymethane- induced ACF were reduced when the animals were fed *L. acidophilus* NCFM® [Rao et al 1999].

A mouse study showed that the effects of infective hyperplasia (uncontrolled tissue growth) could be counteracted when *L. acidophilus* NCFM® was administered as a prophylactic, though not when infection with the causative agent (*Citrobacter rodentium*) and administration of *L. acidophilus* NCFM® were simultaneous [Varcoe et al 2002].

Three human intervention trials showed a reduction in azoreductase,  $\beta$ -glucuronidase and nitroreductase [Goldin & Gorbach 1977, Goldin et al 1980, Goldin & Gorbach 1984]. Subjects consumed 10<sup>9</sup>- 10<sup>10</sup> CFU *L. acidophilus* NCFM® a day when added as a concentrate to milk. Figure 5 clearly shows the reduced activity of all three enzymes during *L. acidophilus* NCFM® consumption. Whether *L. acidophilus* NCFM® has any effect on carcinogenesis in humans is speculative as the putative carcinogens that are activated by the enzymes in the human intestine are currently not known.

### Improvement of lactose intolerance

The inability to digest lactose is common in certain populations, mainly outside north-west Europe. For these people, the consumption of dairy products or other lactose-containing products may lead to gastrointestinal discomfort, such as diarrhea, flatulence, abdominal bloating and cramps. This may cause them to eliminate dairy products from their diet and, thereby, an important source of calcium.

Yogurt is usually well tolerated by lactose maldigesters due to the presence of live bacteria with  $\beta$ -galactosidase activity. Other microbes with  $\beta$ -galactosidase, such as *L. acidophilus* [Nielsen & Gilliland 1992] may exhibit similar effects. An objective measure for lactose intolerance is the content of hydrogen in exhaled breath. The hydrogen originates from microbial growth on lactose in the colon. Some studies have shown that the consumption of *L. acidophilus* NCFM® reduces the level of hydrogen in breath [Kim & Gilliland 1983], indicating improved lactose digestion.

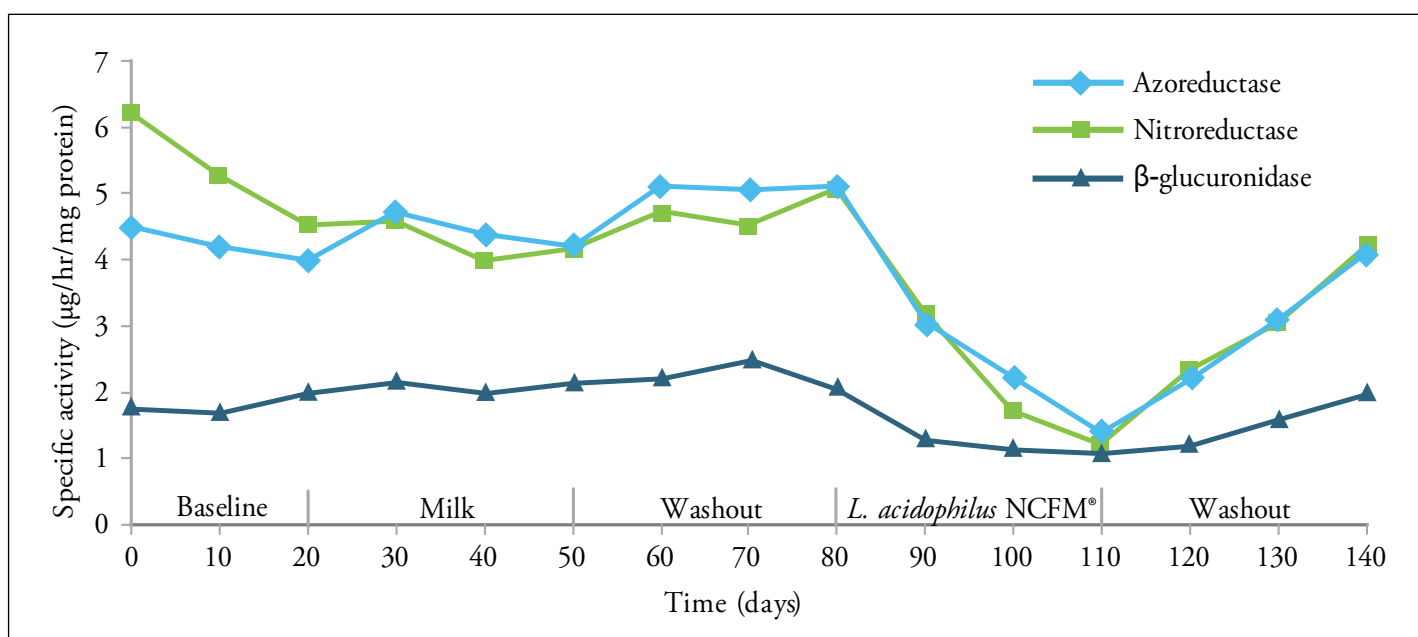


Figure 5. Effect of *L. acidophilus* NCFM® on fecal enzyme activity in humans [Goldin & Grobach 1984]

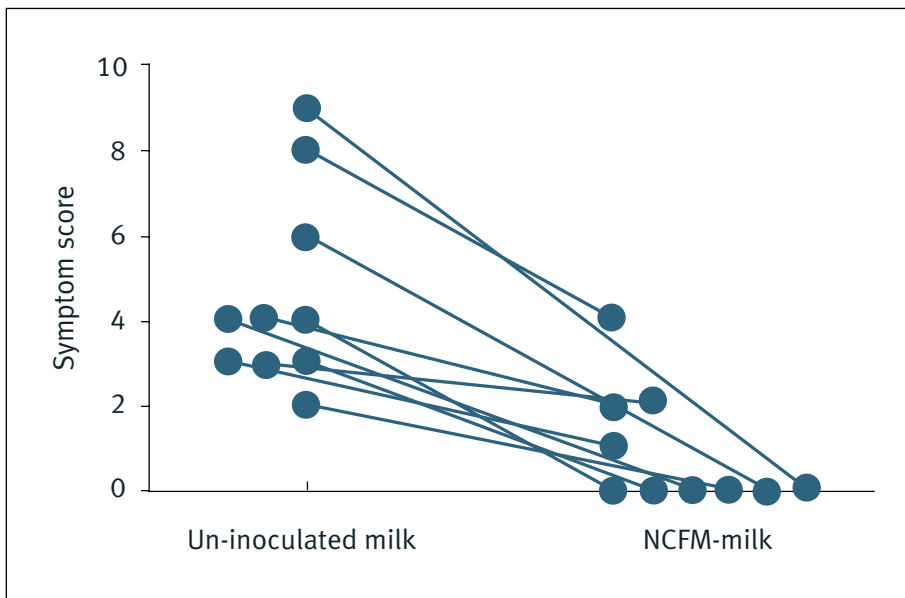


Figure 6. Reduction of lactose-intolerance symptoms [Montes et al 1995]

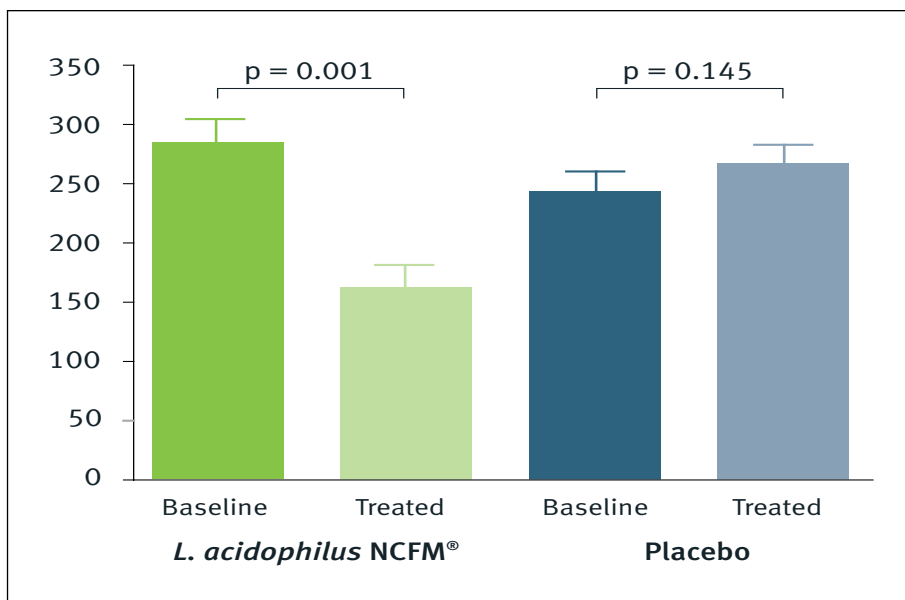


Figure 7. Reduction of serum dimethylamines in SBBO patients [Dunn et al 1998]

Other studies in lactose-intolerant children did not find such a reduction, but did observe a reduction in symptoms [Montes et al 1995] (Figure 6).

#### Improvement of SBBO symptoms

The beneficial effects associated with the human intestinal microbial population have been well documented [Alonso & Guarner 2013]. There are instances, however, where microbiota-associated conditions arise. One of these is small bowel bacterial overgrowth

(SBBO) which can occur in people with renal disease. SBBO is responsible for the production of uremic toxins, including the carcinogens dimethylamine (DMA) and nitrosodimethylamine (NDMA), contributing to decreased nutritional well-being. The administration of *L. acidophilus* NCFM® to humans with end-stage renal failure has been shown to lower serum DMA and NDMA levels significantly (Figure 7) [Dunn et al 1998], as well as improve nutrition parameters such as body

weight gain and caloric intake [Dunn et al 1998, Simenhoff et al 1996, Simenhoff & Dunn 1996].

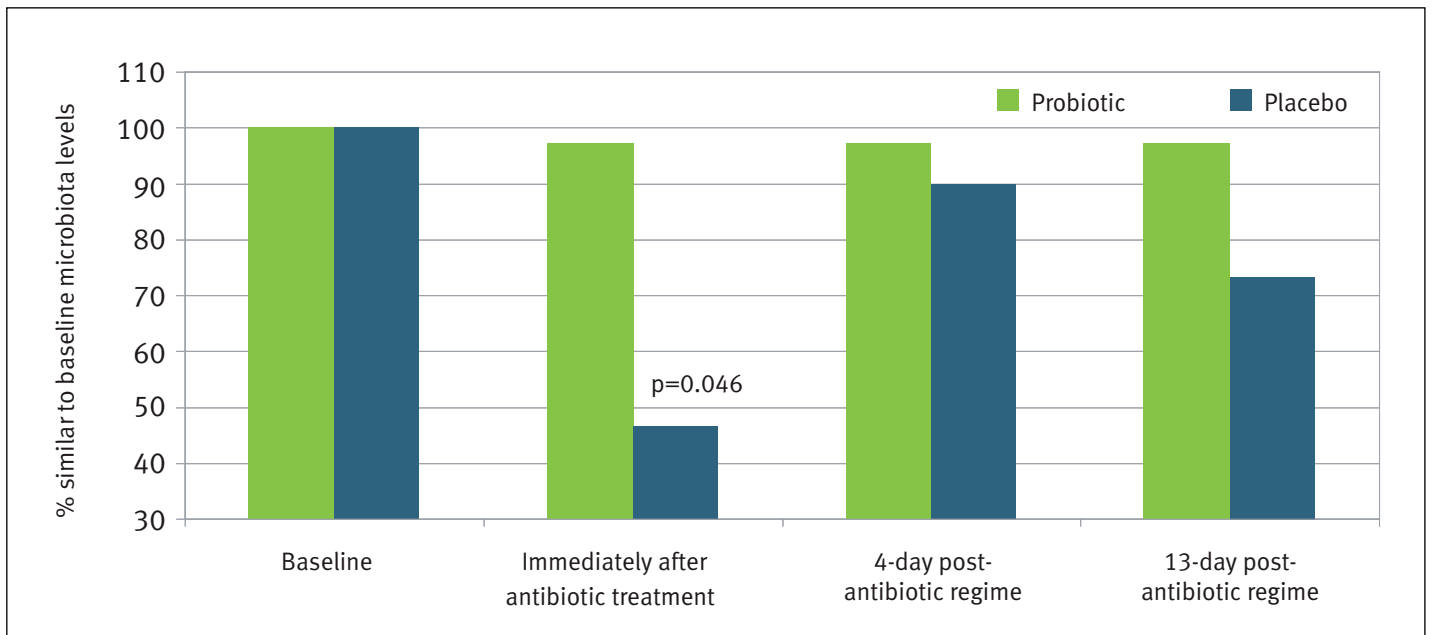
In summary, the ingestion of *L. acidophilus* NCFM® can positively modulate the intestinal microbial population by influencing the pathobiology. It can also significantly reduce the generation of toxic metabolic end-products and promote an improved nutritional status in patients. These findings confirm the health-enhancing attributes associated with *L. acidophilus* NCFM® consumption – specifically its ability to help rebalance intestinal physiology, potentially through multiple modes of action.

#### Reduction of incidence of diarrhea

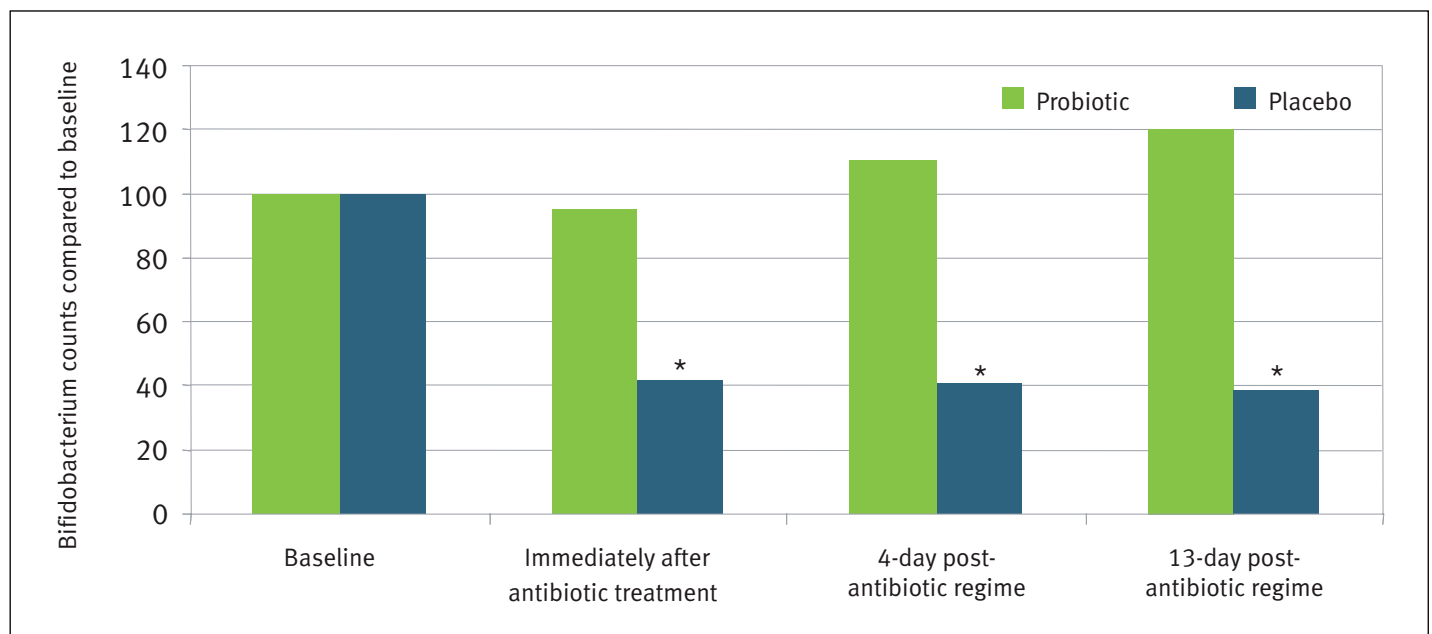
*L. acidophilus* NCFM® was evaluated in a double-blind, placebo-controlled, randomized human clinical study as part of a three-strain formulation (also including *Lactobacillus reuteri* and *B. lactis* Bi-07™). A total of 243 children aged 12-36 months were recruited. During the 14-week intervention period, a statistically significant reduction in the incidence and episodic frequency of diarrhoea was recorded in the probiotic group versus the placebo [Ruiz-Palacios et al 1996]. Furthermore, a combination of *L. acidophilus* NCFM®, *B. lactis* Bi-07™ and soluble fibre was found to give a significant reduction in the number of stools and loperamide use in HIV-positive subjects with diarrhea [Heiser et al 2004].

#### Stabilization of microbiota during antibiotic treatment

Probiotics are known to reduce antibiotic associated diarrhea (AAD) and *Clostridium difficile* associated diarrhea (CDAD) risk in a strain-specific manner [Pattani et al 2013].



**Figure 8.** The probiotic mixture containing *L. acidophilus* NCFM® protects the fecal microbiota from disruption by antibiotics, as indicated by the greater dissimilarity of the microbiota of the placebo group compared to the baseline microbiota composition [Engelbrektson et al 2009].



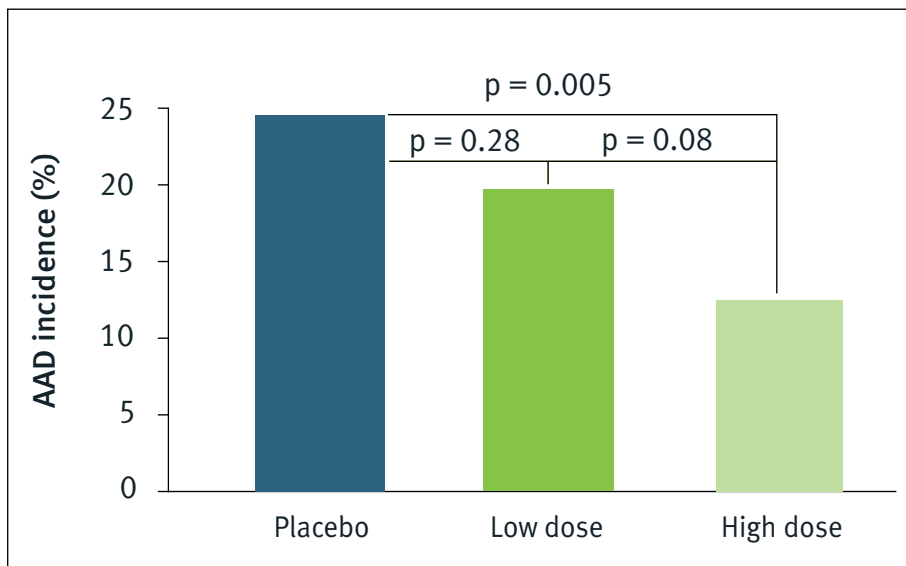
**Figure 9.** The probiotic mixture containing *L. acidophilus* NCFM® promotes the maintenance of bifidobacteria levels on the feces of antibiotic-consuming subjects during post-treatment [Engelbrektson et al 2009].

*L. acidophilus* NCFM® was included in a five-strain 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 8). In addition, the probiotic product still maintained bifidobacteria at

significantly higher levels than that of the placebo group two weeks after the cessation of antibiotic therapy (Figure 9) [Engelbrektson et al 2009].

Another study investigated the effect of a probiotic combination of *L. acidophilus* NCFM® and *B. lactis* Bi-07™ on the fecal microbiota of healthy adults during and after antibiotic exposure.

Subjects given the probiotic combination had significantly higher fecal counts of *L. acidophilus* NCFM® and *B. lactis* at the end of antibiotic treatment period and at the end of the probiotic product study compared to those receiving the placebo. Furthermore, the *B. lactis* levels were restored to base line after completing probiotic consumption and their *Lactobacillus* levels remained



**Figure 10.** Antibiotic-associated diarrhea (AAD) incidence by study group. Low dose:  $4.17 \times 10^9$  colony forming units (CFU), high-dose:  $1.70 \times 10^{10}$  CFU, placebo: micro-crystalline cellulose

stable during the antibiotic exposure. In the placebo group, *B. lactis* levels were still not restored to baseline at the end of the study. Gastrointestinal symptoms were generally mild and did not differ between the treatment groups.

In conclusion, the consumption of the probiotic combination mainly leads to an increase in the fecal levels of the species included in the study product. The study product which included *L. acidophilus* NCFM® may attenuate the undesired side-effects of antibiotic treatment [Forssten et al 2014].

The aim of a further study was to determine the dose-response effect of a four strain probiotic combination on the incidence of AAD and CDAD and the severity of gastrointestinal symptoms in adult in-patients requiring antibiotic therapy. Patients were randomized amongst three study groups: probiotic with  $1.70 \times 10^{10}$  CFU/d (high-dose), probiotic with  $4.17 \times 10^9$  CFU/d (low-dose), or placebo.

The probiotic study products marketed as HOWARU® Restore, consist of equal

amounts of *L. acidophilus* NCFM®, *L. paracasei* Lpc-37™, *B. lactis* Bi-07™, and *B. lactis* BI-04™.

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 10). 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 *L. acidophilus* NCFM®, appears to lower the risk of AAD, CDAD, and gastrointestinal symptoms in a dose-dependent manner in adult in-patients [Ouweland et al 2014].

#### **Beneficial modulation of microbiota**

A study examining the effects of a novel synbiotic consisting of *L. acidophilus* NCFM® (109 CFU/dose) and cellobiose (5 g) on the composition and metabolic activity of human gut microbiota were investigated.

Healthy volunteers were enrolled on a double-blinded, randomized, and placebo-controlled cross-over study and received either the synbiotic product or placebo daily for 3 weeks. The present study confirmed that a synbiotic consisting of *L. acidophilus* NCFM® in combination with cellobiose increase the levels of bifidobacteria and lactobacilli in healthy subjects [van Zanten et al 2014].

Another randomized controlled trial was conducted where fermented milk supplemented with 2 probiotic strains: *B. lactis* Bi-07™ and *L. acidophilus* NCFM® and a prebiotic, isomaltooligosaccharide, which was orally administered to 100 healthy adults for 2 weeks.

The fecal bacterial compositions of these subjects were examined by culturing methods before and after the intervention. Following the intervention, increases in fecal bifidobacteria and lactobacilli and a decrease in fecal enterobacteriaceae were observed amongst the subjects in the test group compared with the control group.

The same fermented milk supplement was also orally fed to mice, and then

immune and fecal bacteria analyses were conducted using the same culturing methods. The same effects on the composition of the intestinal microbiota were observed in mice. Furthermore, the mice in the test group were found to have significantly increased specific immune markers which may indicate a positive effect on the humoral and cell-mediated immunity of host animals. In summary, the synbiotic product significantly increased the populations of lactobacilli and bifidobacteria in both human subjects and mice and may therefore contribute to improve intestinal health [Wang et al 2012].

In a previous human trial it was shown that the consumption of a probiotic cheese containing approximately  $10^9$  CFU/day of *L. acidophilus* NCFM® and *L. rhamnosus* HN001™, significantly increased the natural killer (NK) cell cytotoxicity and phagocytic activity of granulocytes and monocytes [Ibrahim et al 2010].

This study assessed whether the consumption of the same probiotic cheese and the observed systemic immune benefits were associated with changes in the intestinal microbiota and selected fecal immune markers.

It was shown that by administering the probiotic cheese to healthy elderly volunteers, it resulted in modifications of specific components of the intestinal microbiota, but it did not significantly affect the major bacterial groups in the gut. This suggests that the changes in the microbiota induced by the probiotics are specific to certain groups of intestinal bacteria. Specifically, the cheese administration significantly increased the fecal levels of *L. rhamnosus* and *L. acidophilus* NCFM®.

Importantly, probiotic cheese consumption was associated with a trend towards lower counts of *Clostridium difficile* in the elderly, as compared with the run-in period with the control cheese. The effect was statistically significant in the subpopulation of the elderly who harbored *C. difficile* at the start of the study. Due to its role in antibiotic associated diarrhea, a reduction of the basal levels of *C. difficile* may have clinically relevant benefits on the health of the elderly.

Lastly, we did not observe changes in intestinal immune markers during the study, suggesting that the beneficial effects of the probiotic cheese administration are mainly on the systemic innate immunity. Furthermore, the absence of changes in fecal calprotectin and  $\beta$ -defensin concentrations suggests that the probiotic treatment did not cause any adverse inflammatory effects in the intestine [Lahtinen et al 2012].

In addition, the results of both studies demonstrate that *L. acidophilus* NCFM® administered in cheese survives the gastrointestinal transit.

#### **Influence of a combination of *L. acidophilus* NCFM® and lactitol on intestinal and immune parameters in elderly**

There are numerous studies that suggest that aging has a significant effect on the microbiota. Reduced biodiversity, compromised stability and a larger inter-individual variation in the gut microbiota are also commonly reported to be associated with ageing [Rondanelli et al 2015]. Overall, the data support a relationship between diet, microbiota and health status, indicating a role for diet-driven microbiota alterations in varying rates of health decline upon ageing [Claesson 2012]. Probiotics,

prebiotics and synbiotics may improve the health status of elderly individuals by modifying the intestinal environment and microbiota composition and by stimulating the immune system.

A study was conducted to investigate the effect of a probiotic and prebiotic combination (synbiotic) on selected markers of intestinal and immune health, including parameters which are commonly degenerated at old age.

The synbiotic combination used in this study was chosen due to the complementary effects of its components on the gut microbiota. It consisted of the probiotic *L. acidophilus* NCFM® and the prebiotic lactitol and was ingested twice a day with a total daily dose of 10 g lactitol and  $2 \times 10^{10}$  CFU of probiotic bacteria.

Over a two-week period healthy elderly subjects age over 65 were randomized to consume either a placebo or the synbiotic combination twice daily in a double-blind parallel trial.

The consumption of *L. acidophilus* NCFM® in combination with lactitol was associated with modest improvement in stool frequency without any side effects. Furthermore, it increased faecal numbers of *L. acidophilus* NCFM® and bifidobacteria and fecal concentrations of spermidine (an important polyamine in DNA synthesis) and prostaglandin E2 (a potential endogenous anti-inflammatory mediator). The results indicate improved microbiota composition and mucosal functions [Ouwehand et al 2009].

In the framework of the above clinical intervention, another study was conducted to further characterize the impact of the same synbiotic treatment

on microbiota composition in elderly subjects by means of molecular methods. Changes in the major bacterial groups were monitored before, during and after the intervention period using real-time quantitative polymerase chain reaction (qPCR) and group specific primers. Universal changes within the total fecal microbiota were investigated by a non-selective DNA-based method.

The microbiota profiles demonstrated certain relative changes within the microbial community, indicating an increase of bifidobacteria levels during synbiotic supplementation. Quantification by PCR confirmed changes to the microbiota composition; for example increases in total levels of endogenous bifidobacteria and lactobacilli were recorded. The results suggest that the effects of the treatment were specific to certain bacterial groups generally considered to be beneficial for the health. In conclusion, in both studies putatively beneficial changes in the microbiota were observed in the elderly subjects supplemented with the synbiotic product [Björklund et al 2011].

#### **Influence of a combination of *L. acidophilus* NCFM® and prebiotics on chronic constipation**

Functional constipation is a frequent, burdensome gastrointestinal disorder, which greatly affects quality of life. Treatment of this disorder remains challenging. Probiotics have been increasingly investigated for its management. A recent meta-analysis provides evidence that, overall, probiotics improve colonic transit time (CTT), stool frequency, and stool consistency; however, specific probiotics improved only some of these outcomes [Dimidi et al 2014]. The combination of a prebiotic and probiotics could have a potentially synergic effect on the intestinal transit.

Polydextrose has also been reported to positively influence stool consistency and bowel function [Björklund et al 2011]. The aim of the present study therefore was to investigate the combination of polydextrose (Litesse®), *L. acidophilus* NCFM® and *B. lactis* HN019™ in a yogurt on intestinal transit in subjects who suffer from chronic constipation.

The results of this study showed that consumption of yogurt containing polydextrose, *B. lactis* HN019™ and *L. acidophilus* NCFM® significantly shortened CTT after two weeks and may be an option for treatment of constipation [Magro et al 2014].

Similarly, a study with constipated adult women using a combination of fructooligosaccharides and *L. paracasei* Lpc-37™, *L. rhamnosus* HN001™, *L. acidophilus* NCFM® and *B. lactis* HN019™ (10<sup>8</sup>-10<sup>9</sup> CFU) indicated that after 30 days the volunteers had increased frequency of evacuation, as well as improved stool consistency and shape nearer normal parameters than the placebo group, with significant benefits starting during the second and third weeks, respectively. There were no significant differences in abdominal symptoms, but AGACHAN score which is comprised of abdominal pain, bloating and flatulence, was improved in the synbiotic than in the placebo group.

In conclusion, dietary supplementation with a synbiotic containing *L. acidophilus* NCFM® improved evacuation parameters and constipation intensity of chronically constipated women, without influencing abdominal symptoms [Waitzberg et al 2013].

#### **Influence of a combination of *L. acidophilus* NCFM® and *B. lactis* Bi-07™ on functional bowel disorders**

Functional bowel disorders (FBD) are the most common gastrointestinal disorders in the general population. Recent data support a role for the intestinal microbiota in the pathogenesis of functional bowel disorders, suggesting that manipulation of the intestinal microbiota may be beneficial in patients with FBD [Hong & Rhee 2014].

The aim of this study was to investigate the clinical effect of a blend of *L. acidophilus* NCFM® and *B. lactis* Bi-07™, in patients with non-constipation FBD. The study product was provided twice daily for 8 weeks as either a mixture of equivalent amounts of the two probiotic bacteria yielding 10<sup>11</sup> CFU/dose, or a placebo.

The findings showed a significant improvement of bloating symptoms after 4 and 8 weeks in the probiotic group compared to the placebo group (Figure 11 shown on page 15).

The study product was well tolerated. There were no reports of adverse events in either of the groups. Moreover, the lack of increase in fecal markers of intestinal inflammation suggests no inflammatory response to the product.

Importantly, genetic analysis of the fecal microbiota confirmed the presence of the test probiotic strains in the intervention group. Levels of both bacteria were significantly increased in the post-intervention fecal samples taken from patients in the active group, whereas no such increase was found in the samples from patients in the placebo group.

The study findings suggest that the combination of *L. acidophilus* NCFM®

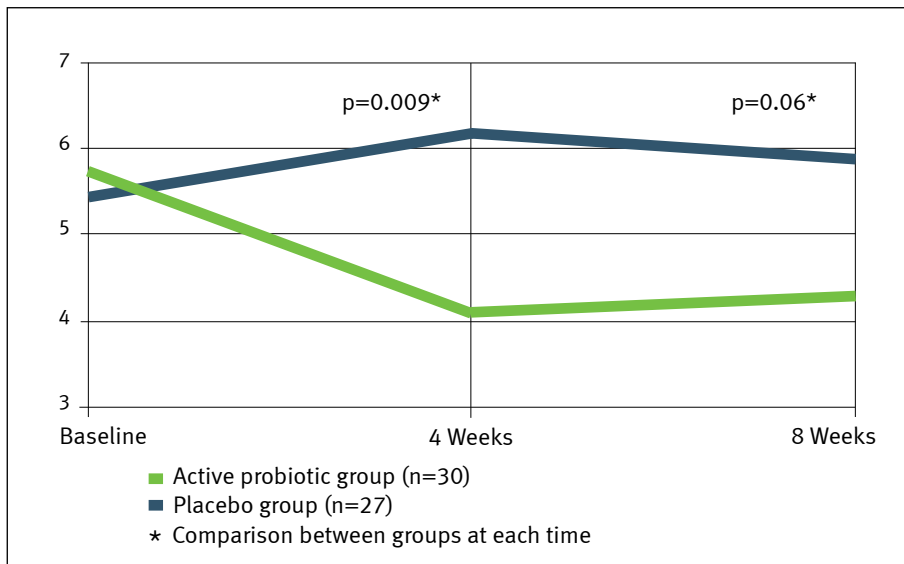


Figure 11. Bloating severity at baseline, 4 and 8 weeks of intervention

and *B. lactis* Bi-07™ may be helpful in alleviating symptoms of bloating in patients with functional GI disorders [Ringel-Kulka et al 2011].

In view of the high prevalence of bloating symptoms in patients with FBD, their overall impact on the patients' health and well-being, and the lack of effective treatment, this probiotic combination may be an important and useful addition to the management of patients with these symptoms.

### Reduction of abdominal pain

Abdominal pain, which is common in the general population and in patients with irritable bowel syndrome, is attributed to visceral hypersensitivity.

A number of *in vitro* and animal studies suggest that there is an interaction between the intestinal microbiome and pathways mediating visceral (originating from inner organs) pain. Therefore it was hypothesized that the administration of specific probiotic bacteria might be beneficial for the treatment of visceral hypersensitivity and abdominal pain caused by IBS.

In a previous animal study [<http://www.ncbi.nlm.nih.gov/pubmed/17159985>] it was found that the oral administration of *L. acidophilus* NCFM® induced the expression of  $\mu$ -opioid and cannabinoid receptors in intestinal epithelial cells, which are responsible for the transmission of nociceptive (pain related) signals to the intestinal nervous system and which have shown to alleviate pain and inflammation [Chichlowski & Rudolph 2015]. *L. acidophilus* NCFM® mediated analgesic functions in the gut – similar to the effects of morphine. These results suggest that the microbiology of the intestinal tract influences our visceral perception. The aim of the present double-blind study with two groups was to determine if *L. acidophilus* NCFM® was the active component in the clinical trial and to investigate the mechanism of action in humans with mild to moderate abdominal pain. Patients were given either *L. acidophilus* NCFM® alone or in combination with *B. lactis* Bi-07™ for 21 days at a total dose of  $2 \times 10^{10}$  CFU/d.

In conclusion it was shown that *L. acidophilus* NCFM® alone, but not the combination of *L. acidophilus* NCFM® and *B. lactis* Bi-07™ modulates

$\mu$ -opioid receptor expression and activity [Ringel-Kulka et al 2014].

A recent study has investigated the effect of a probiotic combination, containing  $2.5 \times 10^{10}$  CFUs of *L. acidophilus* NCFM® and *B. lactis* Bi-07™ on abdominal symptoms; bloating, abdominal pain and altered bowel function, in patients who have undergone colonoscopy.

In conclusion, this study has shown that the probiotic taken daily starting on the night after colonoscopy resulted in a significant reduction in the duration of pain days after colonoscopy compared with placebo [D'Souza et al 2015].

The results of these studies advance the understanding of a possible mechanism for action by which probiotics, and *L. acidophilus* NCFM® in particular, modulate intestinal pain sensation in humans. The studies suggest probiotic intervention as possible new approach in the treatment of abdominal pain and irritable bowel syndrome.

### Benefits of oral supplementation with synbiotics in young children

*L. acidophilus* NCFM® was evaluated in synbiotic nutritional supplements for 1 to 10-year-old children (also including *B. lactis* Bi-07™ and fructo-oligosaccharides) in two human clinical studies. One multicenter, open, randomized, comparative study included acutely ill children aged one to six years who required antibiotic treatment for a bacterial infection. The children received either the synbiotic nutritional supplement (PS), a nutritional supplement without the synbiotic components (P) or a fruit-flavored drink (D) with their medication. Total energy intake, weight gain and fecal lactobacilli levels were significantly greater in the group

that consumed the symbiotic formula (PS). This group also had the lowest rate of relapse or new bacterial infections, though these differences were not statistically significant. There were no significant differences in fecal bifidobacteria levels at the end of antibiotic therapy, although levels were higher in the PS group. There were also no significant differences among the groups in relation to the duration of illness or treatment. All three supplements were generally well tolerated. Appropriate nutrition is particularly important for children during acute phases of illness to maximize energy and fluid intake and to improve the recovery process. The study results suggest that the use of nutritional supplements containing *L. acidophilus* NCFM® is beneficial and safe in children undergoing antibiotic treatment [Schrezenmeir et al 2004].

The second study – a double-blind, randomized 4-month study – was conducted at 13 locations in Brazil, Mexico, Portugal and Spain. The objective was to evaluate the incidence and duration of illness plus anthropometrics in children who received a nutritional supplement with or without synbiotics.

Children recruited for the study were one to six years old and underweight (as defined by a World Health Organization/ National Center for Health Statistics chart (WHO/NCHS)), but otherwise healthy.

Overall, the incidence of sickness, number of sick days and antibiotic use were similar between the two groups. However, in the group consuming the synbiotic formula, subjects aged three to five years, who had at least one episode of illness, experienced significantly fewer sick days. This suggests that the formula may help to reduce the

duration of sickness in some children. The synbiotic group experienced a significant reduction in constipation across all ages. All subjects experienced growth in relation to height, weight and weight/height-ratio.

There were no differences in the growth rate of the synbiotic and control groups. Both supplements used in the study were well tolerated, and the overall incidence of adverse events was very low [[http://www.danisco.com/fileadmin/user\\_upload/healthnutrition/Clinical\\_studies/howaru-duf-synbiotic-fisb.pdf](http://www.danisco.com/fileadmin/user_upload/healthnutrition/Clinical_studies/howaru-duf-synbiotic-fisb.pdf)].

## **BENEFICIAL MODULATION OF THE IMMUNE SYSTEM**

### **The probiotic concept and the immune system**

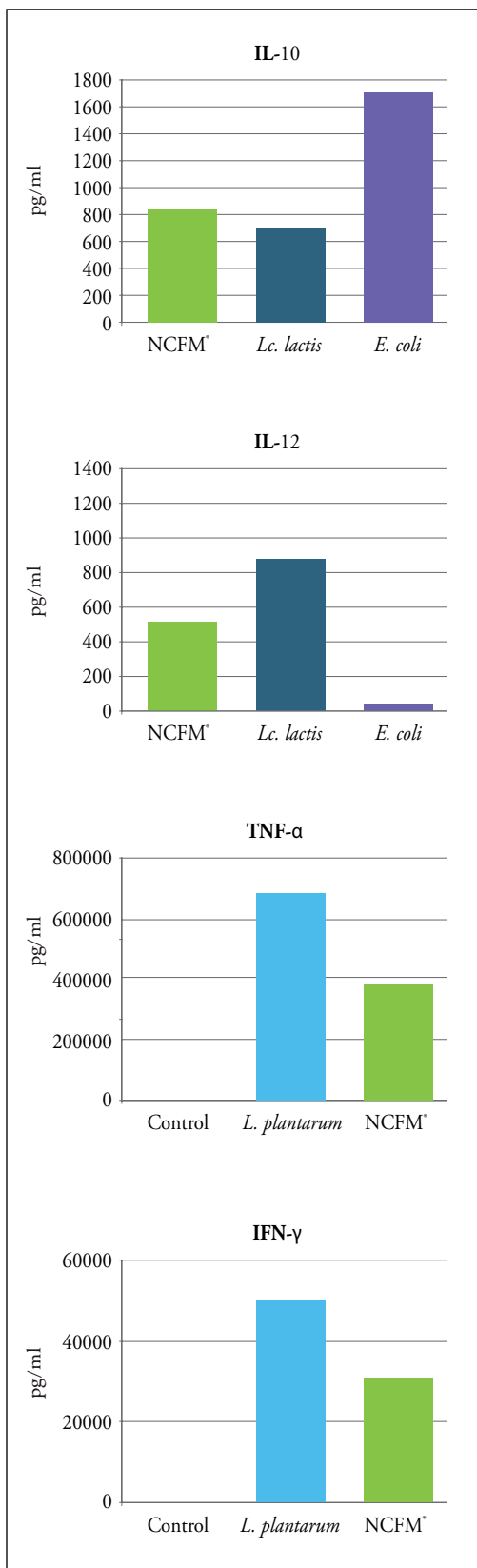
The human immune system is a highly efficient and complex system for defending the body against foreign infectious agents (bacteria, viruses and parasites) as well as from malignant cells and other noxious agents. An immune system that functions optimally is an important safeguard against infectious 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 defence 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]. Modulation of the immune system is an area of intense study in relation to the DuPont™ Danisco® range of probiotics. The goal is to understand how each strain contributes to the maintenance and balance of optimal immune function. The immune system is controlled by compounds known as cytokines. Cytokines are hormone-like proteins made by cells that affect the behavior of other cells and, thereby, play an important role in the regulation of immune system functions. Cytokine expression can be modulated by specific probiotic bacteria. However, interpreting the health relevance of changes in cytokine levels, both from *in vivo* and human studies, remains a challenge.



**Figure 12.** In vitro cytokine expression induced by *L. acidophilus* NCFM® (TNF- $\alpha$  / IFN- $\gamma$  internally generated data, unpublished)

### Expression of cytokines and other immune markers

*In vitro* assays are widely used to define the cytokine expression profiles of probiotics and, thereby, determine their immunological effects. By measuring the impact of probiotic bacteria during interaction with cytokine-expressing peripheral blood mononuclear cells (PBMCs), information is generated that can help determine the ability of each strain to contribute to a balanced immune health. *L. acidophilus* NCFM® was investigated in vitro for its ability to induce the secretion of selected cytokines from PBMCs: the interleukins (IL) IL-10 and IL-12. The IL10/IL-12 ratio is commonly used to distinguish between strains exhibiting pro- or anti-inflammatory profile. The results were compared with a strain of *Lactococcus lactis* and a strain of non-pathogenic *E. coli*. *L. acidophilus* NCFM® was found to induce IL-10 to a slightly higher degree than *L. lactis* and to a lower degree than *E. coli*. IL-12 was induced to a lower degree than *L. lactis*, but higher than *E. coli* (Figure 12) [Foligne et al 2007a]. This type of cytokine expression may shift the immune system towards a so-called Th1 type of response which plays a key role in, for example, warding off tumors, intra-cellular bacteria, viruses and the anti-allergy response.

*In vitro* studies have also shown that *L. acidophilus* NCFM® has the ability to up-regulate tumor necrosis factor (TNF)- $\alpha$  and interferon (IFN)- $\gamma$ , cytokines that are involved in cell-mediated immunity.

A strain of *L. plantarum* were used as a reference for comparison (unpublished data) (Figure 12). IFN- $\gamma$  enhances Th1 cell activity and macrophage functions whereas, TNF- $\alpha$  primes the immune

system for “patrol” activities carried out by neutrophils, macrophages and natural killer cells. This data demonstrates that *L. acidophilus* NCFM® may improve the body’s defence system by promoting the production of those cytokines important to activating immune responses. This cytokine data suggests that *L. acidophilus* NCFM® may promote Th1 cell-type maturation, due to its up-regulation of IFN- $\gamma$  and moderate expression of IL-10 compared to IL-12.

The immune-modulating properties of *L. acidophilus* NCFM® were further investigated in in vitro studies using bone marrow-derived dendritic cells (BMDCs) [Foligne et al 2007b, Zoumpoulou et al 2009]. A study was performed to investigate the role of dendritic cells (DCs) in the anti-inflammatory potential of probiotic bacteria. DCs belong to the group of antigen-presenting cells (APC) that play a central role in regulating immune responses to self and foreign antigens and in inducing and maintaining immunological tolerance. It has been shown that, after activation with various stimuli such as certain probiotic strains, DCs achieve maturation leading to functional changes, e.g. secretion of cytokines and chemokines [Foligne et al 2007b]. Another study investigated the interaction between intestinal epithelial cells (IECs), BMDCs and bacteria in vitro. Here, *L. acidophilus* NCFM® did not induce any stimulation of IECs, either in the presence or absence of BMDCs.

In direct interaction of BMDCs with *L. acidophilus* NCFM® expression of IL-2, IL-6, IL-10, IL-12 and TNF- $\alpha$  was observed. There was no activation of BMDCs by *L. acidophilus* NCFM® through the monolayer of IECs [Zoumpoulou et al 2009].

Like many other bacteria, *L. acidophilus* has a crystalline surface (S) layer consisting of a specific S-layer A protein. This S layer represents the outermost cell wall component and can have many different functions, including responses to specific environmental conditions. It has also been suggested that it is important for *Lactobacillus* adhesion to intestinal epithelial cells and extracellular matrix components [Buck et al 2005].

An *in vitro* study examined the interactions of *L. acidophilus* NCFM<sup>®</sup> and its cell surface components with DCs. *L. acidophilus* NCFM<sup>®</sup> attached to DCs and induced a concentration-dependent activation of DCs. The study further demonstrated that the bacterium binds to a DC-specific receptor. A mutant of *L. acidophilus* NCFM<sup>®</sup> lacking the S-layer A protein, had a significantly reduced ability to bind to this receptor. The study's main conclusion is that the S-layer protein A of *L. acidophilus* NCFM<sup>®</sup> interacts with a major receptor on DCs and regulates DC immune functions [Konstantinov et al 2008].

Although cell surface components of *L. acidophilus* NCFM<sup>®</sup> and other lactobacilli could activate the functions of various antigen-presenting cells such as DCs, the mechanisms of such immune modulations are largely unknown.

Using the cytokine expression data as a predictive test, *L. acidophilus* NCFM<sup>®</sup> is unlikely to be a strong inflammation reducer due to its relatively low induction of IL-10. This has been confirmed in a chemically-induced inflammation animal model. Here, it was shown that *L. acidophilus* NCFM<sup>®</sup> does not significantly reduce intestinal inflammation compared to a control [Foligne et al 2007a].

### Cyclooxygenase expression and intestinal permeability

The gut acts as an internal barrier, preventing pathogenic bacteria and other harmful substances from entering the body. The inner surface of the intestine consists of a layer of cells (epithelium), which are covered by a mucus layer (a visco-elastic layer consisting mainly of protein-linked carbohydrates) which plays a key role in the barrier effect mechanism. Tight junctions are protein structures that link the epithelial cells to one another. These structures control and maintain balanced intestinal permeability. Increased permeability is associated with certain diseases (such as allergies and inflammatory bowel disease), so a proper regulation of the function of tight junctions is important in preventing these diseases. An *in vitro* study measured the effect of cell-free supernatants (CFS) of probiotic strains and a pathogen (*E. coli* O157:H7) on tight junction integrity as well as expression of cyclooxygenases (COX). COX-1 and 2, coded by two different genes, are prostaglandin-producing enzymes and are important for gastrointestinal health. COX-2 is inducible in most tissues and chronic over-expression is a characteristic of inflammation and cancer. COX-1 is permanently expressed and is essential for normal tissue function and repair. To promote intestinal health, it would be desirable to decrease aberrant COX-2 activity while maintaining or even enhancing COX-1 activity. CFS of *L. acidophilus* NCFM<sup>®</sup> did not increase tight junction strength, but decreased it slightly due to high content of lactic acid, an effect not observed *in vivo*, due to rapid metabolism of lactic acid by the microbial community in the human colon. The decrease was significantly less than the decrease caused by the pathogenic

*E. coli* and did not cause any aberrant immunological response when COX-genes were measured. COX-1 and COX-2 were maintained at basal levels with *L. acidophilus* NCFM<sup>®</sup>, while in contrast the pathogenic *E. coli* decreased COX-1 and increased COX-2 levels [Putala et al 2008].

### Effect on *Citrobacter rodentium* colitis in a mouse model

Enteropathogenic *E. coli* (EPEC) is a common pathogen in infantile diarrhea, causing a characteristic histopathologic lesion in the intestinal mucosa. The mouse pathogen *Citrobacter rodentium* causes a similar lesion in the murine intestine and was used as a model in this study. Two-week old BALB/c mice were inoculated with *L. acidophilus* NCFM<sup>®</sup> twice weekly for four weeks before *C. rodentium* infection, or concomitantly with the exposure to *C. rodentium*, at six to eight weeks of age. The probiotics were administered twice weekly thereafter. The main finding of the study was that inoculation with *L. acidophilus* NCFM<sup>®</sup> significantly reduced *C. rodentium* infection, inhibited its proliferation, and facilitated its clearance. This effect was found to be more pronounced in mice with the preinoculation of *L. acidophilus* NCFM<sup>®</sup>, indicating a better protection than with the concurrent administration of the probiotic strain.

This preinoculation with *L. acidophilus* NCFM<sup>®</sup> also prevented mice from the local or systemic spread of infection as indicated by a decrease in bacterial translocation. Probiotic treatment also stimulated regulatory cytokine expression in the colon (transforming growth factor (TGF)- $\beta$ , IL-10). Preinoculation with *L. acidophilus* NCFM<sup>®</sup> was further found to be more effective than concomitant use of the probiotic strain in

the induction of intestinal IgA secretion and in the down-regulation of pro-inflammatory cytokine expression (TNF- $\alpha$ , IL-6, and IL-12) [Chen et al 2005].

The same murine model was used in another study to further evaluate the effect of probiotic treatment on attenuating *Citrobacter*-associated colitis in mice and to explore the role of DCs in the modulation of host response. The results obtained were compatible with those from the above-mentioned study. Preinoculation with *L. acidophilus* NCFM<sup>®</sup> reduced the susceptibility of mice to *Citrobacter* infection, attenuated colonic pathology and reduced the level of secretion of *Citrobacter* in the feces. The probiotic treatment also stimulated the host to produce higher level of IgA and enhance the protective antigen-specific immune response against the bacteria. It was demonstrated that when the *L. acidophilus* NCFM<sup>®</sup>-primed DCs were adoptively transferred to mice, rather than via oral consumption of the strain, there was a similar effect on fecal bacteria counts, IgA levels, colonic histopathology and cytokine levels in mesenteric lymph nodes when there was intestinal bacterial infection [Chen et al 2009].

These findings suggest that DCs play a key role in the ability of probiotics to attenuate *C. rodentium* colitis and that inoculation with *L. acidophilus* NCFM<sup>®</sup> will stimulate the function of DCs, thereby further increasing the immune response triggered by DCs. It can be concluded that *L. acidophilus* NCFM<sup>®</sup> can act as an effective immune modulator and stimulate an immune response against enteric bacterial infection.

### Protection from experimental *Candida albicans* infection

*Candida* yeasts are usually present in most people but uncontrolled overgrowth, for example due to medication or underlying disease can lead to candidiasis, a fungal infection (mycosis), caused by species of the genus *Candida*, predominantly *Candida albicans*. Candidiasis encompasses infections that range from superficial, such as oral thrush and vaginitis, to systemic and potentially severe diseases. The increased incidence of *Candida* infections and their increasing resistance to antifungal antibiotics provides a strong impetus for new research efforts to explore the use of probiotic bacteria for the prophylaxis and therapy of candidiasis [Wagner et al 1997].

A study looked at how prior colonization with *L. acidophilus* NCFM<sup>®</sup> and other probiotic bacteria affected the antibody responses of immunodeficient mice and compared it with the antibody responses produced by the alimentary tract colonized only with *C. albicans* [Wagner et al 2000a]. The study demonstrated that, although the probiotic bacteria did not induce a vigorous antibody response to their own antigens, they altered the mice's antibody response against *C. albicans*. The authors observed mixed immunomodulatory effects of the probiotic bacteria.

The probiotic strains induced antibody responses to some *C. albicans* antigens but inhibited antibody responses to other *C. albicans* antigens. However the data indicate that probiotic bacteria such as *L. acidophilus* NCFM<sup>®</sup>, which effectively prolongs the survival of immunodeficient mice colonized with *C. albicans* [Wagner et al 1997], also strongly stimulate the production of

antibodies to *C. albicans* antigens in these mice.

This suggests that commensal microbiota should be considered an important component of the humoral immune system when protecting against candidiasis. Certain probiotic bacteria can also clearly enhance or suppress antibody responses to antigens administered via the mucosal surfaces of the alimentary tract [Wagner et al 2000a].

More limited protection was demonstrated with heat-killed *L. acidophilus* NCFM<sup>®</sup> [Wagner et al 2000b].

Another study has evaluated the capacity of *L. acidophilus* NCFM<sup>®</sup> and another *L. acidophilus* strain to protect immunodeficient mice from orogastric and systemic candidiasis [Wagner et al 1998]. Mice diassociated with *C. albicans* and either of the *L. acidophilus* strains had significantly lower *C. albicans* count in their stomachs and intestines compared with mice monoassociated with *C. albicans*. The presence of either *L. acidophilus* strain in the alimentary tract reduced the incidence of disseminated candidiasis in mice. *L. acidophilus* NCFM<sup>®</sup> provided better protection against systemic (disseminated) candidiasis of endogenous origin than the other *L. acidophilus* strain. Both *L. acidophilus* strains prolonged the survival of mice after colonization with *C. albicans* (compared to *C. albicans* monoassociated mice). However, the most effective protection was provided by *L. acidophilus* NCFM<sup>®</sup>. The growth of pups born to mice which were diassociated with *C. albicans* and either *L. acidophilus* strain was significantly improved compared to the pups of *C. albicans*-monoassociated mice. Immune responses were evaluated as immunoglobulins in the sera of mice either monoassociated with one of the

*L. acidophilus* strains or *C. albicans* or diassociated with one of the *L. acidophilus* strains and *C. albicans*.

Compared with germ-free mice, mice monoassociated with *L. acidophilus* had increased serum IgG and IgM. *C. albicans*-monoassociated mice had more IgG, IgA and IgM, although the increase in these three immunoglobulins was even higher in mice diassociated with *L. acidophilus* NCFM® and *C. albicans*. The latter data suggests that mice diassociated with either *L. acidophilus* strain and *C. albicans* developed fewer antibodies than *C. albicans*-monoassociated mice. The results show that *L. acidophilus* NCFM® can provide important protection against candidiasis in immunodeficient mice and that different strains of the same species provide varying degrees of biotherapeutic effects [Wagner et al 1998].

In conclusion, due to its antimicrobial activity against common intestinal pathogens and their toxins, *L. acidophilus* NCFM® may improve the composition of the intestinal microbiota, possibly leading to a reduced risk of diarrhea and other intestinal disorders and providing a protective effect against systemic infections. These studies demonstrated that the beneficial effects could also be partially attributed to immunostimulation, i.e. enhanced macrophage, lymphocyte, and polymorphonuclear leukocyte responses.

### Improved effect of vaccination

An animal study was performed to investigate an alternate vaccine strategy for *Bacillus anthracis* infections. A recombinant strain of *L. acidophilus* NCFM® was used to deliver *B. anthracis* protective antigen (PA) via specific dendritic cell-targeting peptides to den-

dritic cells (DCs). Oral vaccination of mice with these PA-peptides induced robust protective immunity against *B. anthracis*. Additionally, the level of serum anti-PA titers, neutralizing PA antibodies and IgA-expressing cells were all comparable with the subcutaneous administration of standard recombinant PA plus aluminium hydroxide. Further development of this strategy for oral delivery of DC-targeted antigens could provide a safe and protective vaccine via a bacterial adjuvant that may potentiate mucosal immune responses against pathogens [Mohamadzadeha et al 2009].

### Enhancement of immune functions in mice

Another study investigated the dose-dependent effect of a combination of *L. acidophilus* NCFM® and *Bifidobacterium lactis* Bi-07™ on specific markers of immunity in mice. Mice were fed with three varying dosages of the probiotic preparation with a total initial cell count of  $>3.5 \times 10^9$  cfu/g (0.25; 0.50; 1.50g/kg bodyweight). At medium and high doses an increased delayed-type hypersensitivity (DTH) was observed. This is a reaction to an antigen that the body has encountered before. Memory T-cells and antigen-specific white blood cells which give the immune system its “memory” against past infections are activated more quickly, providing faster protection against an infection.

Supplementation with the high dose of probiotics also resulted in a significant increase in NK activity. NK cells belong to the main cellular effectors of innate immunity and are crucial for defence against viral infections and tumor cells. These results suggest that supplementation with this probiotic combination at certain dosage levels can enhance immune functions in mice [Cai et al 2008].

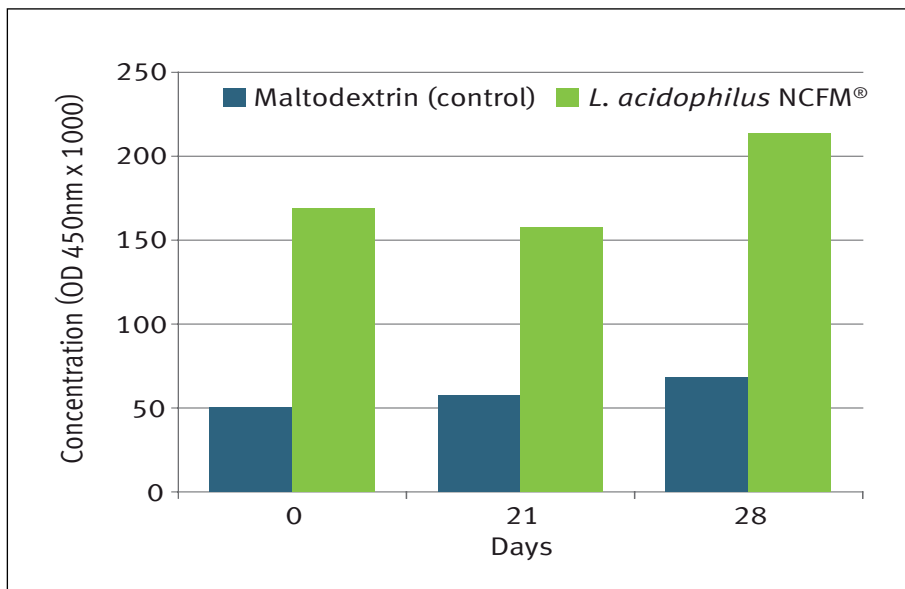
### Stimulation of immunoglobulins

*L. acidophilus* NCFM® has been further tested in an animal model for its ability to positively influence the mucosal and systemic immune response. As a component of yogurt that also contained *S. thermophilus*, *L. bulgaricus* and *B. infantis*, *L. acidophilus* NCFM® has been shown to improve specific intestinal and serum IgA production in mice upon vaccination with cholera toxin [Tejada-Simon et al 1999].

The ability of *L. acidophilus* NCFM® to stimulate specific immunity has been evaluated in a human study measuring primary immune reaction following vaccination. One week prior to oral vaccination with cholera vaccine, healthy volunteers received either a placebo (maltodextrin) or *L. acidophilus* NCFM®. Supplementation with *L. acidophilus* NCFM® or the placebo started on day 0 and continued for 21 days. The subjects consumed two capsules a day with  $10E^{10}$  CFU *L. acidophilus* NCFM® or two capsules a day with maltodextrin (control). On day 7 and 14, the subjects received the oral vaccine. Blood samples were collected on day 0, 21 and 28, and antigen-specific antibodies (immunoglobulins, IgA, IgG, IgM) were determined. These immunoglobulins play different roles in the body's immune defence strategy. Supplementation with *L. acidophilus* NCFM® tended to increase the specific serum IgA for the period D21-D28 compared to the placebo group. This indicates the stimulation of specific immunity by *L. acidophilus* NCFM® (Figure 13 shown on page 21) [Paineau et al 2008].

### Improvement of immune markers

In a randomized, controlled cross-over clinical trial a commercial probiotic cheese was studied for its potential as a



**Figure 13.** Levels of specific IgA titre in orally vaccinated humans after supplementation with *L. acidophilus* NCFM® compared with placebo [Paineau et al 2008].

probiotic food with regard to improvement of selected immune markers in the elderly.

Thirty one healthy elderly volunteers consumed the probiotic cheese containing approximately  $10^9$  CFU/day of *L. rhamnosus* HN001™ and *L. acidophilus* NCFM®. The 4-week probiotic intervention was preceded by a 2-week consumption of probiotic-free cheese (run-in) and followed by a 4-week wash-out period with the same control cheese.

Consumption of the probiotic cheese significantly increased the NK cell ability to kill target tumor cells. A significant increase in phagocytic activity of granulocytes and monocytes was observed for both the control and the probiotic cheese.

Cheese was found to be an effective carrier for the study of probiotics, and it was demonstrated that the regular intake of this probiotic cheese can help to enhance the immune system and that including it in a regular diet may help to improve an elderly person's immune

response against harmful external challenges [Ibrahim et al 2010].

In summary, *L. acidophilus* NCFM® has been shown to modulate immune response markers in a way that is important for priming the cell-mediated immune system outside the intestinal tract. From the data provided by *in vitro*, human and animal studies it can be concluded that *L. acidophilus* NCFM® may improve the body's defence system by promoting and activating immune responses which are important for providing protection against infections.

#### **Impact on respiratory tract illness symptoms:**

##### ***Influence of a probiotic combination 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. The probiotic strains used in this study, a combination of *L. acidophilus* NCFM® and *B. lactis* BI-04™, 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.

In conclusion, the study showed that consumption of this probiotic combination could positively influence nasal eosinophils, which display a strong correlation with clinical and immunological parameters in allergic rhinitis, it also indicated a trend towards reduced nasal symptoms like nasal blocking and runny nose.

The administration of the probiotic combination also led to a significant increase in the fecal numbers of *B. lactis* and *L. acidophilus*, from March to April/May. Numbers remained high until the end of the intervention in June. There was a general change in microbiota during the birch pollen season, with a decrease in the main microbiota groups at the peak of the season. This was observed in both the probiotic and placebo group, indicating that it was not influenced by the intervention. The study results suggest that probiotics may provide an alternative or complementary treatment for pollen allergies [Ouwehand et al 2009]. *L. acidophilus* NCFM® has been shown to exhibit a variety of immune-modulating properties. These provide a mechanistic basis for many of the observed health benefits of the strain and indicate new potential targets for health applications.

##### ***Reduction of cold and flu symptoms***

Common cold is a heterogeneous group of mostly mild upper respiratory tract (URTI) illnesses that have an impact on our daily lives and are considered a major public health issue.

The impact of *L. acidophilus* NCFM® and a combination of *L. acidophilus*

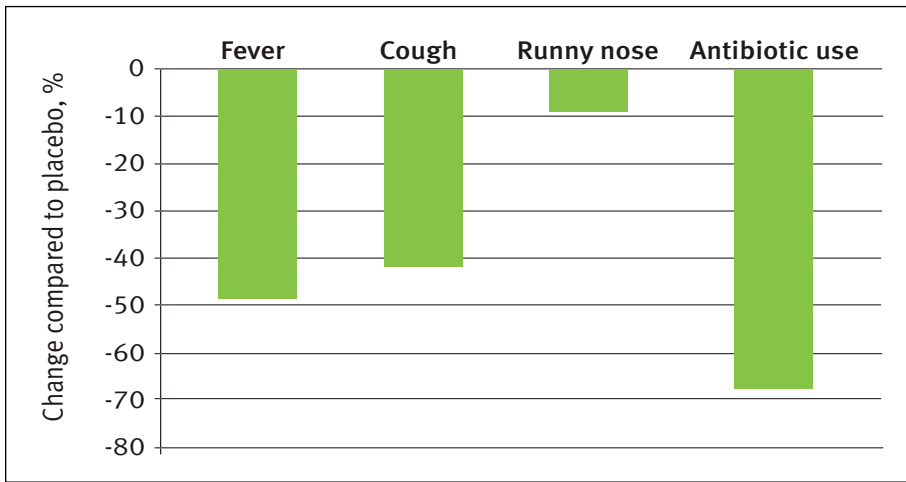


Figure 14. Reduction in incidence of upper respiratory tract infections by *L.acidophilus* NCFM<sup>®</sup> compared to placebo [Leyer et al 2009].

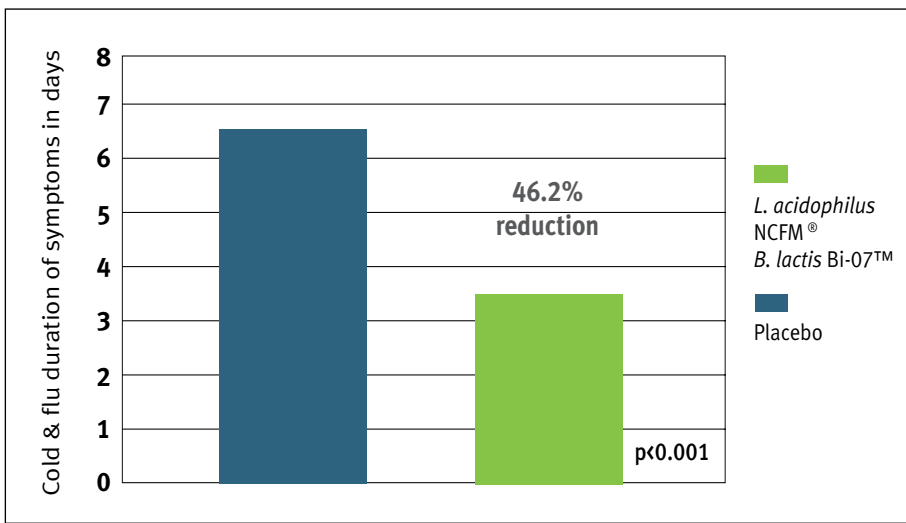


Figure 15. The clinical study showed a reduction in illness duration by nearly half the number of days by *L.acidophilus* NCFM<sup>®</sup> compared to placebo

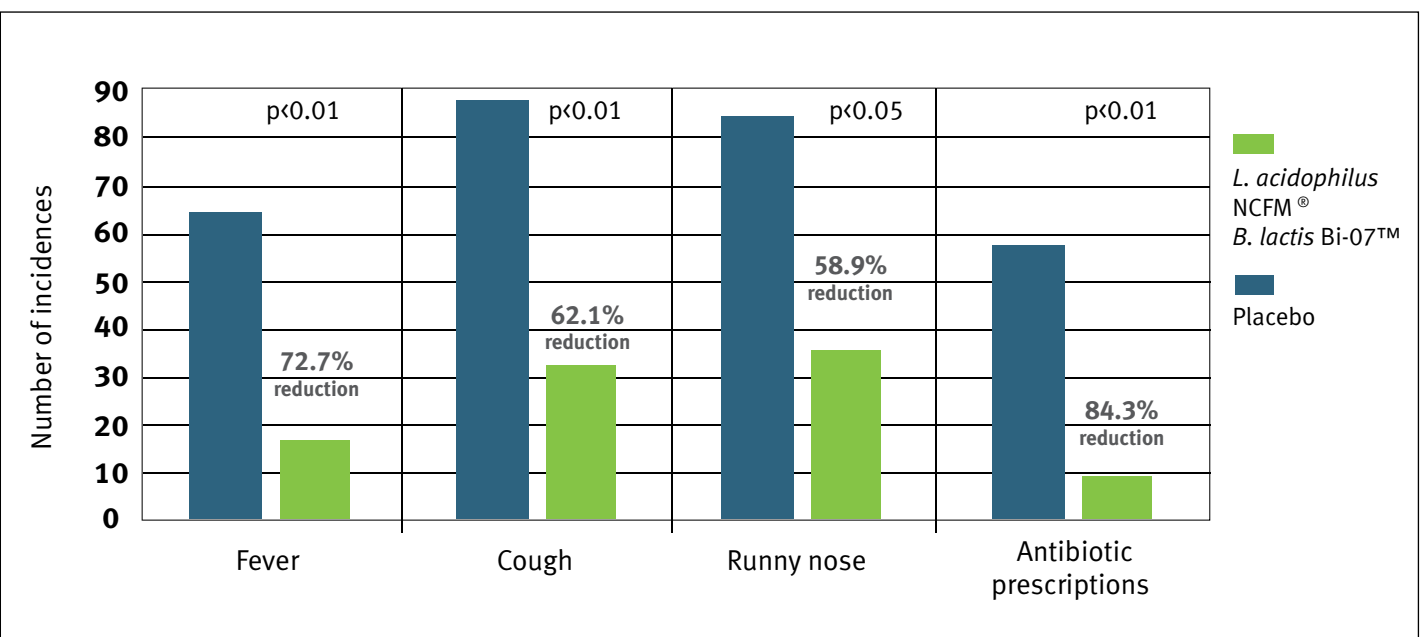


Figure 16. The clinical study showed a considerable reduction of symptoms and an 84.3% reduction in the number of antibiotic prescriptions by the *L. acidophilus* NCFM<sup>®</sup> group compared to the placebo group.

NCFM<sup>®</sup> and *B. lactis* Bi-07<sup>™</sup> on respiratory health was investigated in a study involving more than 300 healthy children between three and five years of age. Both probiotics were administered with a total daily dose of  $1 \times 10^{10}$  CFU. The study was conducted from November to May, when children are particularly vulnerable to seasonal illness because of inclement weather conditions. *L. acidophilus* NCFM<sup>®</sup> significantly reduced the incidence and duration of fever, URTI symptoms and antibiotic use compared to a placebo (Figure 14).

An even more pronounced effect on duration and incidence of cold and flu-like symptoms and a strong reduction of antibiotic use and sick days was observed in the group consuming the probiotic combination of *L. acidophilus* NCFM<sup>®</sup> and *B. lactis* Bi-07<sup>™</sup> (Figure 15 and 16) [Prov Patent Appl 2006, Leyer et al 2009].

Acute URTIs, such as colds and coughs, are one of the main reported causes of illnesses affecting athletes, and may impair

training activity. The effect of consuming a combination of *L. acidophilus* NCFM® and *B. lactis* Bi-07™ with a total daily dose of  $5 \times 10^9$  CFU of each strain, on training performance and incidence of respiratory tract illness symptoms was studied in healthy physically active adults. Probiotics were administered for 5 months throughout the primary cold and flu season and the effect of supplementation on intensity (1-10 scale), frequency (d/wk), duration (h/wk), and training load (product of exercise duration and intensity), were examined. In addition, the participants reported self-assessed symptoms of respiratory and gastrointestinal illness and physical activity.

The study results showed a significant delay to the onset of URT illness among the subjects consuming the probiotic combination (Figure 17). The most notable finding was that the participants in the probiotic group reported a substantially higher exercise duration and exercise training load compared to the placebo group, indicating a greater level of physical activity (Figure 18).

In conclusion, the findings from the above study indicate that *L. acidophilus* NCFM® and *B. lactis* Bi-07™ supplementation may be a useful nutritional adjunct to support a healthy immune system during training [West et al 2014a].

The immune mechanisms by which probiotics reduce susceptibility to upper respiratory tract illness is uncertain. To gain further insight on the mechanisms that might explain the reduction in respiratory illness observed following daily supplementation with a combined *L. acidophilus* NCFM® and *B. lactis* Bi-07™, the peripheral blood of a subset of this cohort was analysed for plasma cytokines, natural killer cell activity and phagocytosis capacity.

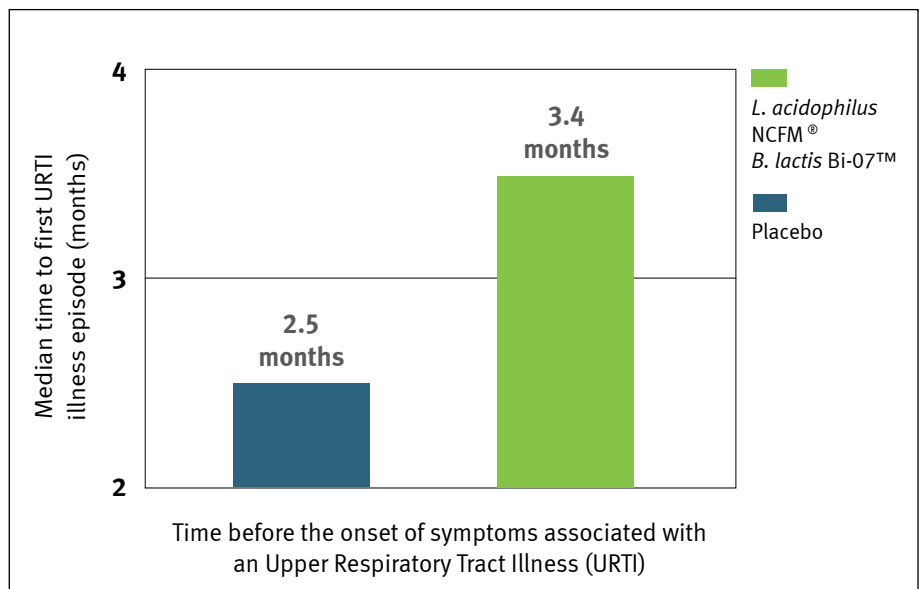


Figure 17. *L. acidophilus* NCFM® delays the time to experiencing URTI illness episodes compared to placebo.

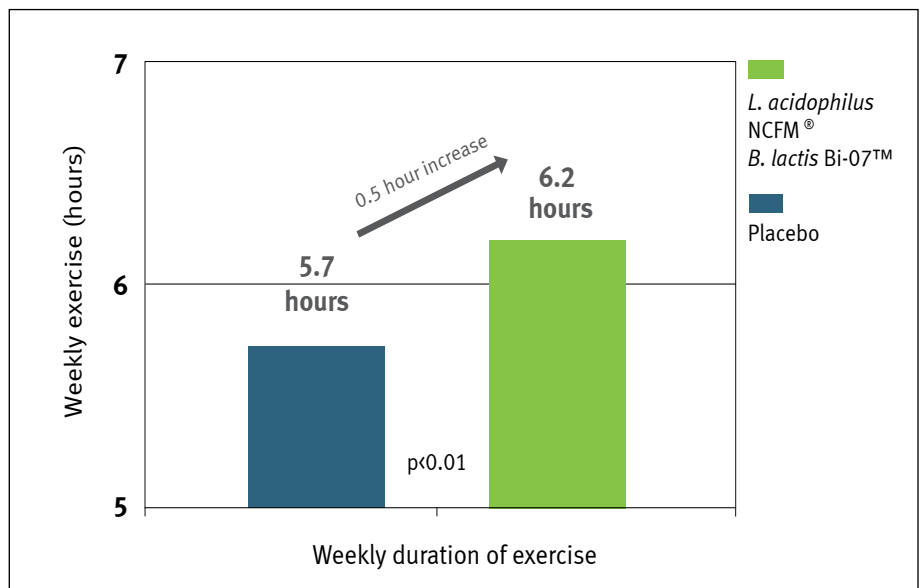


Figure 18. *L. acidophilus* NCFM® increases duration of exercise compared to placebo.

In summary, 150 days of daily supplementation by healthy physically active individuals with the probiotics *L. acidophilus* NCFM® and *B. lactis* Bi-07™ had no significant effect on parameters of the innate immune system. However, it would be premature to conclude that the benefits of supplementation with the combined probiotic on the risk of URTI are not partly related to modulation of the immune system, in particular lymphocyte-mediated function [West et al 2014b].

## OTHER HEALTH-RELATED PROPERTIES

### Oxalate-degrading activity

In humans, an accumulation of oxalic acid can result in a number of pathological conditions, including hyperoxaluria, kidney stones, renal failure, cardiomyopathy and cardiac conductance disorders. A study was undertaken to evaluate the oxalate-degrading activity of 60 Lactobacillus strains, including *L. acidophilus* NCFM®.

The oxalate-degrading activity of *L. acidophilus* NCFM® was found to be 100%, which was as high as the positive control *Oxalobacter formigenes* DSM 4420. The activity of other strains of *L. acidophilus* ranged from 35-100%. This suggests that the use of probiotic strains with oxalate-degrading activity may be of benefit to individuals suffering from oxalate-associated disorders [Turroni et al 2007]. The ability of *L. acidophilus* NCFM® to degrade oxalate was predicted from the presence of the oxalyl-coenzyme A (CoA) decarboxylase and formyl-CoA transferase genes [Azcarate-Peril et al 2006].

### Oral adhesion

*L. acidophilus* NCFM® has been shown to adhere moderately to saliva-coated hydroxyapatite (as a model for teeth) and to survive exposure to saliva for 24 hours with a minor loss of viability. Combined with its antimicrobial properties, this could indicate that the strain contributes to oral health [Haukioja et al 2006].

### Influence on serum cholesterol

Probiotic bacteria have also been reported to lower total cholesterol and LDL cholesterol. However, human studies to date have yielded conflicting results with no clear-cut reduction in cholesterol observed due to probiotic consumption. Studies with *L. acidophilus* NCFM® [Gilliland et al 1985, Gilliland & Walker 1990] have indicated an ability to remove cholesterol from a laboratory growth medium. *L. acidophilus* NCFM® was reported to take up cholesterol in the presence of bile and in the absence of oxygen – both conditions that are present in the intestinal tract. The strain has also been shown to possess genes for bile salt hydrolase, which is involved in bile metabolism [McAuliffe et al 2005, Walker & Gilliland 1993]. The significance of these

in vitro studies has not, however, been confirmed in human studies. Sweet acidophilus milk containing *L. acidophilus* NCFM® was included in a human study on the effects of different dairy products on serum cholesterol. The authors concluded that, when sweet acidophilus milk, yogurt, and buttermilk products were consumed for 3 weeks, none had a significant effect on serum cholesterol [Thompson et al 1982].

### Urogenital applications

Probiotic bacteria in intravaginal applications are widely used to control the incidence of vaginal or urogenital infections. However, only very few clinical studies have been published. Using several laboratory assays, *L. acidophilus* NCFM® was tested for traits thought to be useful to help prevent urinary and vaginal tract infections [Reid 2000]. It was suggested that *L. acidophilus* NCFM® produced a biosurfactant that inhibited the adhesion of *Enterococcus faecalis* 1131 by over 90% in a model system.

*L. acidophilus* NCFM® was also shown to adhere to uroepithelial and vaginal epithelial cells in vitro. Furthermore, preincubation of *L. acidophilus* NCFM® with these same cells followed by subsequent exposure to three uropathogens (*E. coli* Hu734, *K. pneumoniae*, and *P. aeruginosa* AK1) showed that *L. acidophilus* NCFM® competitively excluded these pathogens, inhibiting them by 30, 11 and 30%, respectively. Hydrogen peroxide production may play a role in competitive exclusion of these urogenital pathogens. *L. acidophilus* NCFM® was found to produce H<sub>2</sub>O<sub>2</sub>, but at a low level compared to various *Lactobacillus* strains. The effect of *L. acidophilus* NCFM® suggests a potential application opportunity in the control of vaginal or urogenital infections.

### Impact on insulin sensitivity

Accumulating evidence indicates that the gut microbiota plays a significant role in the development of obesity, obesity-associated inflammation and insulin resistance. This suggests that the gut microbiota may be a target for treating metabolic diseases, the supplementation with probiotics being one approach. Data from animal studies suggest that the intake of probiotic bacteria may improve glucose homeostasis. Inflammatory markers and lipid profile were significantly improved in the animal model, while data from human studies were controversial (Razmpoosh et al 2015).

Suppression of the inflammatory response in human and animal subjects receiving probiotics is considered as a potential mechanism of action of probiotics on glucose homeostasis. (Ruan et al 2015, Firouzi et al 2013, Tremaroli & Bäckhed 2012)

A double-blinded, placebo-controlled, randomised trial was conducted to investigate the effects of oral supplementation with *L. acidophilus* NCFM® on insulin sensitivity and the inflammatory response in subjects with normal or impaired insulin sensitivity.

The key findings were that Insulin sensitivity was preserved among volunteers in the *L. acidophilus* NCFM® group, whereas it decreased in the placebo group. Both baseline inflammatory markers and the systemic inflammatory response were, however, unaffected by the intervention. Therefore it remains unclear if this optimising effect on insulin sensitivity is linked to immune function.

*L. acidophilus* NCFM® was detected in 75% of the faecal samples after treatment with the probiotic. Again, this indicates the ability of *L. acidophilus* NCFM® to survive gastrointestinal passage [Andreasen et al 2010].

## BENEFITS SUMMARY

*L. acidophilus* NCFM® has been commercially available on the North American market for over 30 years and internationally for over 15 years. In recent decades, significant research studies of *L. acidophilus* NCFM® have provided insight into the strain's probiotic functionality. Over 200 publications, more than 50 of which refer to human studies, in peer-reviewed journals describe the multi-faceted properties of *L. acidophilus* NCFM®, probiotic blends and symbiotic products including *L. acidophilus* NCFM® with regard to characterisation, safety and efficacy. Based on this data, it can be concluded that *L. acidophilus* NCFM® has a series of health-related attributes, which can be summarized as follows:

- *Improves gastrointestinal health and well-being*
  - improves the level of natural good bacteria in the body
  - aids digestion and well-being
  - reduces gastrointestinal discomfort
  - maintains the balance of healthy microbiota
  - beneficially affects the intestinal microbiota composition and activity
  - reduces lactose-intolerance symptoms
  - well-suited for intestinal survival
  - high tolerance to gastrointestinal conditions
  - strong adhesion to intestinal cell lines
  - may provide protection against intestinal pathogens as demonstrated in *in vitro* and animal trials
- *Beneficial modulation of immune functions*
  - may improve specific immune response, as demonstrated in human clinical studies
  - may influence immune regulation, as demonstrated by the induction cytokines such as proinflammatory IL-12 and anti-inflammatory IL-10 *in vitro*
  - may reduce symptoms of respiratory tract infections
- *Long history of safe use*

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