Technical Memorandum

TM 54-2e

Lactobacillus acidophilus NCFM® – a probiotic with proven efficacy

DANISCO

INTRODUCTION

A growing awareness of the relationship between diet and health has led to an increasing demand for products that are able to enhance health beyond providing basic nutrition. Studies have shown that ingestion of probiotics – friendly bacteria – is beneficial in maintaining the body's delicate microbial balance. This balance is known to enhance intestinal health and the immune system, not to mention other physiological functions, making it a critical factor for general human well-being.

> Probiotics are live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host.
>
> *FAO/WHO 2001 [1]*

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

The beneficial effects of probiotics either involve reducing risk factors for a certain disease or improving some of the body's natural functions, thereby helping to maintain the health of the consumer.

So far these effects have been documented primarily in two areas, which are also Danisco's main areas of probiotic study:

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

The suggested health benefits of probiotics are many, and some effects are better established than others. It should, however, be noted that each probiotic strain has its own specific health benefits, and no probiotic 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 product.

- The strain must be safe – this requires identification by appropriate molecular techniques
- The strain has to be able to resist acid and bile
- The strain must have clinically proven health benefits
- The strain should have good technological properties, such as the ability to survive in the final consumer product, whether food or dietary supplements, and either be neutral or contribute favourably to the flavour of the 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 study. In several reviews the scientific evidence for this strain is highly rated [2,3,4,5].

CHARACTERISTICS OF THE SPECIES

Lactobacillus acidophilus is a Gram-positive, non-spore forming, homofermentative, 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 milks (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]. The results of DNA-DNA hybridisation studies have suggested that the previous *L. acidophilus* species was composed of six different species [7], 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 to differentiate by phenotypic methods, so they are still considered 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 hybridisation to a species-specific probe and whole genome sequencing (see figure 2).

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

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 deposit number SD5221.

L. acidophilus NCFM® aliases

Multiple strain designations appear in reference literature for *L. acidophilus* NCFM® or for single colony isolates of the NCFM® parent culture. The designations NCFM®, NCK56, NCK45, N2, RL8KR, RL8KS and RL8K are essentially identical strains, as indicated by their identical chromosomal DNA fragment patterns. N2 was selected by Marschall Products from NCFM® as a smooth, bile-resistant colony. RL8KS was similarly selected in Dr. Todd Klaenhammer's laboratory at North Carolina State University from the parent culture RL8K. NCK45 is a Klaenhammer laboratory designation for NCFM®, and NCK56 is a Klaenhammer laboratory designation for NCFM®/N2. In studies conducted by Simenhoff *et al* [62], *L. acidophilus* NCFM® was abbreviated to LBA.

GENOMICS

L. acidophilus NCFM® is the only 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 [8] Danisco 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 [9], adherence to human cell lines [10], lactose metabolism, and tolerance to physiologically relevant stresses, including acid and bile [8,11,12]. 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 [13,14,15,16]. Genomic information is also used to develop strain identity methods and improve industrial processes.

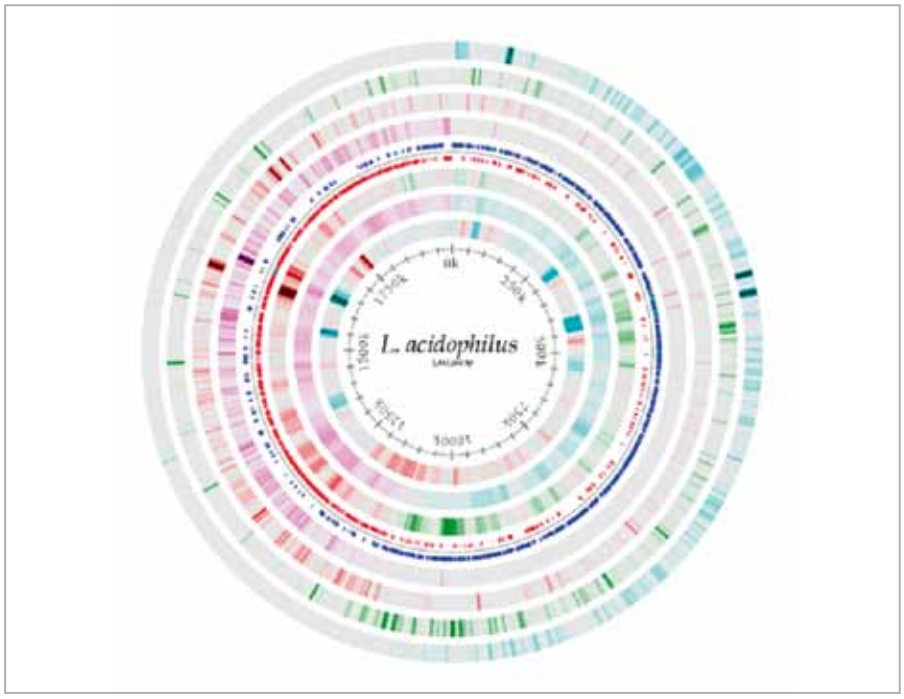

Figure 2. Genome atlas of *L. acidophilus* NCFM® [8].

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, Danisco makes rigorous use of bacterial frozen seed inventories to reduce the risk of genetic drift over time and maintain strain integrity.

Danisco 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 [17,18]. Moreover, no *L. acidophilus* bacteraemia were identified in a 10-year survey in Finland [19].

More specifically, *L. acidophilus* has been consumed in fermented milks and other food products for decades and is listed in the *Inventory of Microorganisms With Documented History of Use in Human Food* [20]. The European Food Safety Authority (EFSA) has also added the species to the Qualified Presumption of Safety list [21].

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

In addition to this long history of safe human consumption, no acquired antibiotic resistance was detected in *L. acidophilus* NCFM® during screening by the EU-funded PROSAFE project. Analysis of the *L. acidophilus* NCFM®

genome sequence has confirmed the absence of known transferable genetic elements related to antibiotic resistance [8].

General safety

In order to assess the safety of *L. acidophilus* NCFM® further, several toxicity studies have been performed in 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 (IG) 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 (10E10 cfu) of *L. acidophilus* NCFM® led to no translocation of the organism or abnormal translocation of the intestinal microbiota. Nor was any significant improvement in colitis observed [22].

Safety was further confirmed in studies with neonatal and adult immune deficient mice, where no mortality was observed among mice fed with *L. acidophilus* NCFM® [23].

HEALTH-RELATED PROPERTIES

L. acidophilus NCFM® has been extensively studied *in vitro*. 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.

In addition to the compelling *in vitro* evidence, strong probiotic benefits have been demonstrated in multiple animal trials and human studies.

These studies have provided extensive insight into the probiotic functionality of the strain. The main outcome of this research is summarised below.

BENEFITS TO INTESTINAL HEALTH AND WELL-BEING

The importance of the intestinal microbiota for health

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

- the GI epithelium
- immune cells
- resident microbiota

The primary function of the human GI tract has long been considered to be the digestion and absorption of nutrients and the excretion of waste end-products. In recent years, however it has become recognised that the gastrointestinal tract fulfils many other functions, which are essential to our well-being.

The GI tract harbours a vast number of microbial cells (10E14), which is 10 times more then the number of cells that make up the human body. The intestinal microbiota are 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 associated with a healthier intestinal tract.

The resident microbes are involved in many metabolic processes, such as the fermentation of undigested carbohydrates into short-chain fatty acids, and also in lipid metabolism and vitamin synthesis.

Another important function of the intestinal microbiota is to stimulate the maturation of the immune system and provide protection against incoming, potentially pathogenic microbes.

When the delicate ecological balance of this highly complex microbial community is disturbed by environmental or physiological factors, predisposition to infectious and immuno-inflammatory diseases is enhanced. It may then become necessary to re-establish a beneficial microbiota.

Research has shown that specific probiotic strains can be used to optimise the composition and activity of the intestinal microbiota and, thus, to reduce the risk for a range of diseases or unfavourable conditions [24,25].

In vitro studies

Resistance to acid and bile

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 colonise 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.

In vitro studies have shown that *L. acidophilus* NCFM® is extremely resistant to low pH conditions and survives

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% 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)

Table 1. Selected characteristics of *L. acidophilus* NCFM® (internally generated data):
++++ Excellent; +++ Very good; ++ Good; + Fair

the presence of bile at concentrations present in the duodenum (table 1).

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 colonise the intestine.

L. acidophilus NCFM®'s ability to adhere to different human epithelial cell lines has been confirmed in several studies [26,27]. It has also been shown that the adhesion property could be further improved by the addition of Ca^{2+} (figure 3) [27].

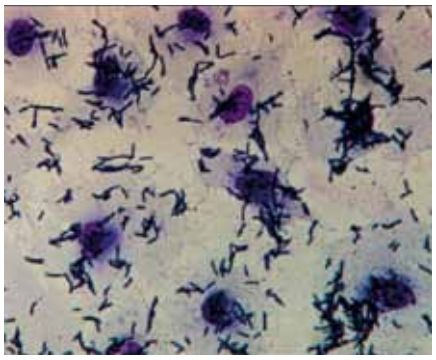

Figure 3. *L. acidophilus* NCFM® shows strong adherence to human fetal intestinal ephithelial cells in a Ca^{2+} mediated system [27].

Genome analysis of *L. acidophilus* NCFM® has indicated the presence of several genes of potential importance to the adherence process, including mucus-binding proteins, fibrinonectin-binding proteins and others. Clusters of genes encoding for exopolysaccharides may also contribute to adherence capabilities [8,28].

Adherence of *L. acidophilus* NCFM® was further measured using two *in vitro* cell lines, Caco-2 and HT-29. 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).

Adherence to human intestinal cells *in vitro*	HT-29: **+++** Caco-2: **+++**

Selected characteristics of *L. acidophilus* NCFM® (internally generated data): ++++ Excellent; +++ Very good; ++ Good; + Fair

To identify putative genes potentially involved in the survival of *L. acidophilus* NCFM® and its colonisation 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 upregulated 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 upregulated during incubation in duodenal juice and bile. These inductions may contribute to the attachment of *L. acidophilus* NCFM® to intestinal epithelial cells [29].

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 minor loss of viability. Combined with its antimicrobial properties, this could indicate that the strain contributes to oral health [30].

Inhibition of pathogens

The protection probiotic bacteria provide against gastrointestinal pathogens is highly important to therapeutic modulation of the enteric microbiota.

Probiotics are able to inhibit, displace and compete with pathogens, although these abilities are strain-dependent.

The probiotic strains' 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 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 the table below is based on such an *in vitro* assay.

Pathogen inhibition *in vitro*	*Salmonella typhimurium:* **+** *Staphylococcus aureus:* **++++** *Escherichia coli:* **+++** *Listeria monocytogenes:* **+**

Selected characteristics of *L. acidophilus* NCFM® (internally generated data): ++++ Excellent; +++ Very good; ++ Good; + Fair

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 [31]. 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 [8].

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 [32].

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 *Enterococcus faecalis*, not against pathogens [33, 34, 35].

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 autoaggregation and high coaggregation, especially with *Clostridium histolyticum* and *Staphylococcus aureus in vitro* [36].

In *in vitro* studies *L. acidophilus* NCFM® showed the 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 [37].

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* [37].

Another *in vitro* study has investigated the growth inhibition of *Bacillus cereus* by different lactobacilli, including *L. acidophilus* NCFM®. In co-culture with the pathogen in a skim milk medium the germination and sporulation of *B. cereus* was inhibited without affecting the growth of *L. acidophilus* NCFM®. The organic acids produced by lactobacilli inhibited the growth of *B. cereus* [38].

The ability of *L. acidophilus* NCFM® and other lactobacilli and bifidobacteria to inhibit the common intestinal pathogens *E. coli* and *S. typhimurium in vitro* was studied using a disc diffusion test and co-culturing *L. acidophilus* NCFM® and the pathogens in a liquid medium. With both methods *L. acidophilus* NCFM® showed a strong growth inhibition against both pathogens. Under these experimental conditions, the antimicrobial activity was strain specific and not due to pH alone [39].

Protozoan parasites of the genus *Cryptosporidium* are a cause of diarrhoea 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® significantly reduced the cell culture infectivity of both pathogens [40].

Animal studies

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.

Immune-compromised mice were protected from experimental *Candida albicans* infection when treated prophylactically with viable *L. acidophilus* NCFM® [41]. More limited protection was demonstrated with heat-killed *L. acidophilus* NCFM® [42].

Another study has evaluated the capacity of *L. acidophilus* NCFM® and another *L. acidophilus* strain to protect immunodeficient mice from orogastric and systemic candidiasis.

Mice diassociated with *C. albicans* and either of the *L. acidophilus* strains had significantly fewer *C. albicans* 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® 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 colonisation with *C. albicans* (compared to *C. albicans*-monoassociated mice). However, the best protection was provided by *L. acidophilus* NCFM®.

The growth of pups born to mice 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 providing varying types and degrees of biotherapeutic effects [43].

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 diarrhoea 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 immuno-stimulation, i.e. enhanced macrophage, lymphocyte, and polymorphonuclear leukocyte responses.

Reduction of cancer-related markers

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

Rat studies have confirmed that faecal azoreductase, β-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 tumours [44,45,46].

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® [47].

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 [48].

Reduction of abdominal pain

Abdominal pain is common in the general population and, in patients with irritable bowel syndrome, is attributed to visceral hypersensitivity. In a rat study it was found that oral administration of *L. acidophilus* NCFM® induced the expression of μ-opioid and cannabinoid receptors in intestinal epithelial cells and 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 – pointing to new approaches in the treatment of abdominal pain and irritable bowel syndrome [49].

Human studies

Survival in intestinal passage

In order to elicit their health benefits, probiotics must generally be able to survive and be active in the GI tract. As discussed above, *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.

The ability to modulate the populations or activity of the human intestinal microbiota is considered an important probiotic characteristic. Human studies have demonstrated that *L. acidophilus* NCFM® survives GI transit and positively influences the microbiota.

During the trials, consumption of 10E10 cfu *L. acidophilus* NCFM® by healthy adults caused the number of faecal lactobacilli to increase [50] (table 1). No change in the levels of bifidobacteria and sulphide-producing bacteria was observed [51].

	Baseline	**NCFM® feeding**	**Washout**
Faecal lactobacilli	*2 weeks*	*2 weeks*	*2 weeks*
	4.6 log cfu/g	4.6 log cfu/g	4.6 log cfu/g
	no *L. acidophilus*	*L. acidophilus* dominant	*L. acidophilus* frequent

Table 1. Influence of *L. acidophilus* NCFM® on faecal lactobacilli [50].

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 faeces of healthy males [52].

A further study evaluated the effect of two probiotic strains, *L. acidophilus* NCFM® and a *B. lactis*, on the composition of faecal microbiota in young children (6-24 months). The results demonstrated a significant increase in *L. acidophilus* and *B. lactis* in faeces after ingestion of the corresponding probiotic strain over a 2-month period, confirming survival of the cultures in the GI tract [53].

Reduction of undesired faecal enzyme activity

Produced by a variety of microbes present in the GI tract, faecal enzymes such as azoreductase, nitroreductase and β-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 faecal enzyme activity [46], while Tannock *et al* provide a more recent reference to the relevance of faecal enzyme activity [54].

Three human intervention trials showed a reduction in azoreductase, β-glucuronidase and nitroreductase [46,55,56]. Subjects consumed 10E9-10E10 cfu *L. acidophilus* NCFM® a day, when added as a concentrate to milk. Figure 4 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

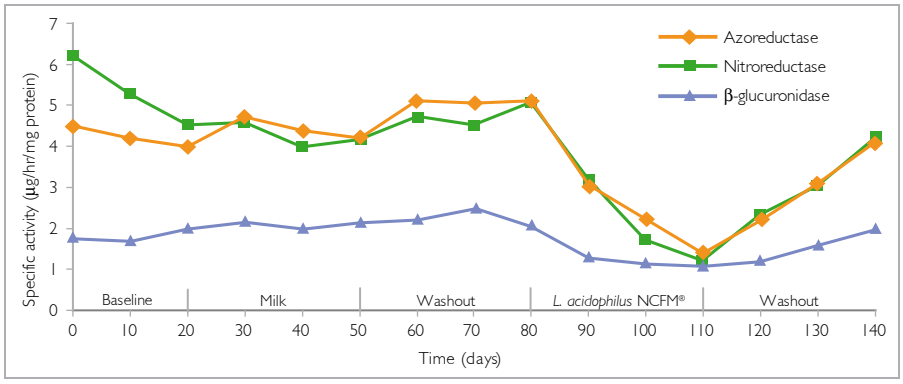

Figure 4. Effect of *L. acidophilus* NCFM® on faecal enzyme activity in humans [56].

north-west Europe. For these people, the consumption of dairy products or other lactose-containing products may lead to gastrointestinal discomfort, such as diarrhoea, 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-intolerants due to the presence of live bacteria with β-galactosidase activity. Other microbes with β-galactosidase, such as *L. acidophilus* [57] 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 [58], indicating improved lactose digestion. Other studies in lactose-intolerant children did not find such a reduction, but did observe a reduction in symptoms [59] (figure 5).

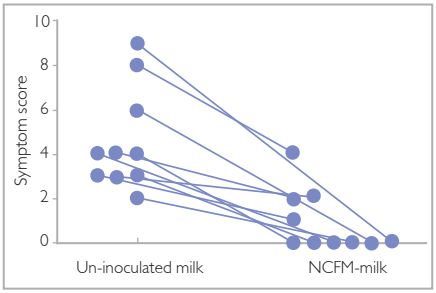

Figure 5. Reduction of lactose-intolerance symptoms [59].

Improvement of SBBO symptoms

The beneficial effects associated with the human intestinal microbial population have been well documented [60]. 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 6) [61], as well as improve nutrition parameters such as body weight gain and caloric intake [61,62,63].

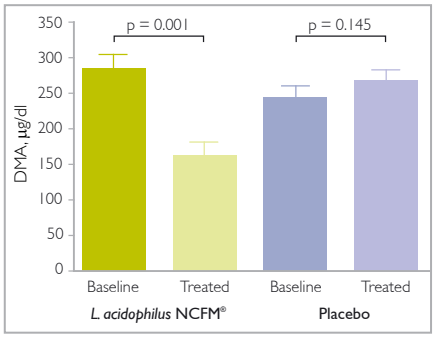

Figure 6. Reduction of serum dimethylamines in SBBO patients [61].

To sum up, *L. acidophilus* NCFM® ingestion 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 diarrhoea

L. acidophilus NCFM® was evaluated in a double-blind, placebo-controlled, randomised 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 [64].

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 diarrhoea [65].

Stabilisation of microbiota during antibiotic treatment

L. acidophilus NCFM® was included in a five-strain formulation, investigated for its ability to stabilise 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 7). 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 8) [66].

Influence of a combination of L. acidophilus NCFM® and lactitol on intestinal and immune parameters

In a study with healthy elderly subjects twice-daily consumption of *L. acidophilus* NCFM® in combination with lactitol was associated with modest improvement

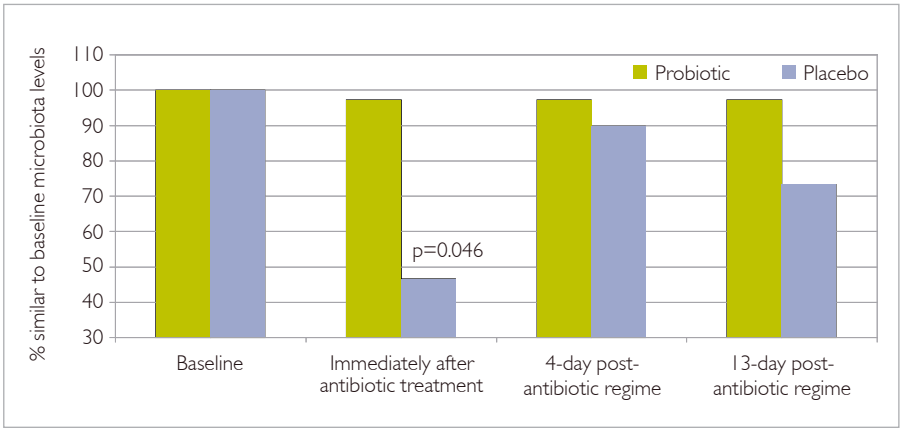

Figure 7. The probiotic mixture containing *L. acidophilus* NCFM® protects the faecal microbiota from disruption by antibiotics, as indicated by the greater dissimilarity of the microbiota of the placebo group compared to the baseline microbiota composition [66].

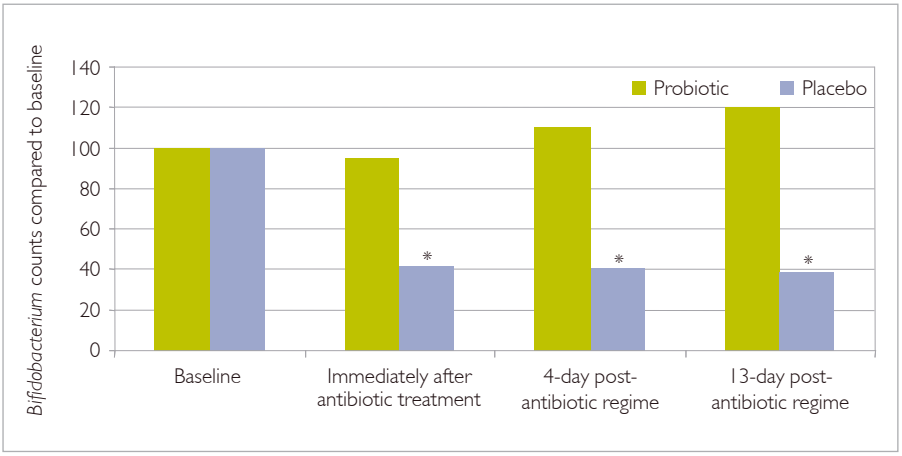

Figure 8. The probiotic mixture containing *L. acidophilus* NCFM® promotes the maintenance of bifidobacteria levels in the faeces of antibiotic-consuming subjects during post-treatment [66].

in stool frequency without any side effects. Furthermore, it increased faecal numbers of *L. acidophilus* NCFM® and bifidobacteria and faecal 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 [67].

Benefits of oral supplementation with synbiotics in young children

Lactobacillus 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 multicentre, open, randomised, 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-flavoured drink (D) with their medication.

Total energy intake, weight gain and faecal lactobacilli levels were significantly greater in the group that consumed the synbiotic 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 faecal 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 maximise energy and fluid intake and to improve the recovery process. The study results suggest that the use of nutritional supplements containing *Lactobacillus acidophilus* NCFM® is beneficial and safe in children undergoing antibiotic treatment [68].

The second study – a double-blind, randomised 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 [69].

BENEFICIAL MODULATION OF THE IMMUNE SYSTEM

The probiotic concept & 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 [70, 71].

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 [70].

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 [70].

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 [70].

Modulation of the immune system is an area of intense study in relation to the Danisco probiotic range. 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 behaviour 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.

In vitro studies

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 mononucleocytes (PBMCs), information is generated that can help determine the ability of each strain to contribute to balanced immune health.

L. acidophilus NCFM® was investigated *in vitro* for its ability to induce the PBMC secretion of selected cytokines: the interleukins (IL) IL-10 and IL-12. 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 *Lc. lactis* and to a lower degree than *E. coli*. IL-12 was induced to a lower degree than *Lc. lactis*,

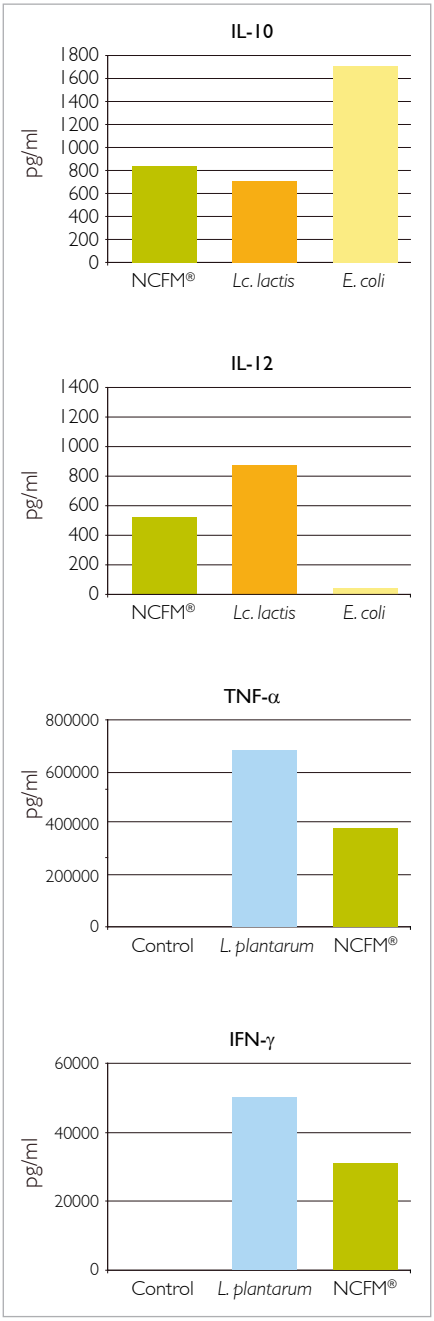

Figure 9. *In vitro* cytokine expression of *L. acidophilus* NCFM® (TNF-α / IF-γ internally generated data, unpublished) [72].

but higher then *E. coli* (figure 9) [72]. This type of cytokine expression is known to shift the immune system towards a so-called Th1 type of response which plays a key role in, for example, warding off tumours and viruses and the anti-allergy response.

In vitro studies have also shown that *L. acidophilus* NCFM® has the ability to up-regulate tumour necrosis factor (TNF)-α and interferon (IF)-γ , cytokines that are involved in cell-mediated im-

munity outside the GI tract. A strain of *L. plantarum* was used as a reference for comparison (unpublished data) (figure 9). In the environment outside the GI tract, TNF-α enhances Th1 cell activity and primes the immune system for "patrol" activities carried out by neutrophils, macrophages and natural killer cells. IF-γ is important in priming macrophages and in antibody production. This data demonstrates that *L. acidophilus* NCFM® may improve the body's defence system by promoting and activating those cytokines important to activating immune responses.

This cytokine data leads to the conclusion that *L. acidophilus* NCFM® promotes more of a Th1 cell-type maturation, due to its up-regulation of TNF-α and IF-γ and moderate expression of IL-10 compared to IL-12.

The immune-modulating properties of *L. acidophilus* NCFM® were further confirmed in *in vitro* studies using bone marrow derived dentritic cells (BMDCs) [73,74].

A study was performed to investigate the role of dentritic 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 own 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 chemokines [73].

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 another experiment the expression of different cytokines and chemokines by activation of BMCDs was investigated. In direct interaction of BMCDs with *L. acidophilus* NCFM® expression of IL-2, IL-6, IL10, IL-12 and TNF-α was observed. There was no activation of BMCDs by *L. acidophilus* NCFM® through the mono-layer of IECs [74].

L. acidophilus, like many other bacteria, 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 [28].

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

An *in vitro* study examined the interactions of *L. acidophilus* NCFM® and its cell surface compounds with DCs. *L. acidophilus* NCFM® 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® lacking the 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® interacts with a major receptor on DCs and regulates DC immune functions [75].

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 cyclo-oxygenases (COX). COX-1 and 2, coded by two different genes, are prostaglandin producing enzymes and important in gastrointestinal health. COX-2 is inducible in most tissues and chronic over-expression being characteristic of inflammation and cancer, while COX-1 is expressed all the time and essential to 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® 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 metabolization 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 kept at basal levels with *L. acidophilus* NCFM®, while in contrast the pathogenic *E. coli* decreased COX-1 and increased COX-2 levels [76].

Animal studies

Effect on chemically induced colitis in a mouse model

Using the cytokine expression data as a predictive test, *L. acidophilus* NCFM® 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® does not significantly reduce intestinal inflammation compared to a control [72].

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® 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® significantly reduced *C. rodentium* infection, inhibited its proliferation, and facilitated its clearance. This effect was found to be more pronounced in mice with preinoculation of *L. acidophilus* NCFM®, indicating a better protection than concurrent administration of the probiotic strain. This preinoculation with *L. acidophilus* NCFM® also prevented mice from 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)-γ), IL-10).

Preinoculation with *L. acidophilus* NCFM® 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 proinflammatory cytokine expression (TNF-α, IL-6, and IL-12) [77].

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 above-mentioned study.

Preinoculation with *L. acidophilus* NCFM® has reduced susceptibility of mice to *Citrobacter* infection, has attenuated colonic pathology and reduced secretion of *Citrobacter* in the feces.

The probiotic treatment has also stimulated the host to produce higher IgA and enhance protective bacterial antigen-specific immune response.

It was demonstrated that when mice were adoptively transferred with *L. acidophilus* NCFM® -primed DCs instead of 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 [78].

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® will stimulate the function of DCs, thereby further increasing the immune response triggered by DCs.

It can be concluded that *L. acidophilus* NCFM® can act as an effective immune modulator and stimulate immune response against enteric bacterial infection.

Stimulation of immunoglobulins in mice

L. acidophilus NCFM® has been further tested in an animal model for its ability to have a positive influence on 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 [79].

Protection from experimental Candida albicans infection

Another study looked at how prior colonisation with *Lactobacillus acidophilus* NCFM® and other probiotic bacteria affected the antibody responses of immunodeficient mice and compared it with the antibody responses produced by alimentary tract colonisation only with *C. albicans*. This study demonstrated that, although the probiotic bacteria did not induce a vigorous antibody response to their own antigens, they altered the antibody responses of mice to *C. albicans*.

The authors have observed mixed immunomodulatory effects of 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®, which effectively prolong survival of immunodeficient mice colonised with *C. albicans* [41], also strongly stimulate the production of antibodies to *C. albicans* antigens in these mice.

This suggests that commensal bacterial flora 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 [80].

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 dendritic 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, neutralising PA antibodies and IgA-expressing cells were all comparable with the subcutaneous administration of standard recombinant PA plus aluminum 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 [81].

Stimulation of natural immunity in mice

Another study investigated the dose-depending effect of a combination of *L. acidophilus* NCFM® and *Bifidobacterium lactis* Bi-07 on specific markers of natural immunity in mice.

Mice were fed with three varying dosages of the probiotic preparation with a total initial cell count of >3.5*10E9/g (0.25; 0.50; 1.50g/kg bodyweight).

At medium and high dose an increased delayed-type hypersensitivity (DTH) was, observed. This is a reaction to an antigen the body has encountered before. Memory T-cells, antigen-specific white blood cells, which provide the immune system with "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 natural killer cell (NK) activity. NK cells belong to the main cellular effectors of natural immunity and are crucial for defence against viral infections and tumour cells.

These results suggest that supplementation with this probiotic combination at certain dosage levels can enhance the natural immunity of mice [82].

Human studies

Stimulation of immunoglobulins

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 10E10 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 (P=0.09) compared to the placebo group. This indicates the stimulation of specific immunity by *L. acidophilus* NCFM® (figure 10) [83].

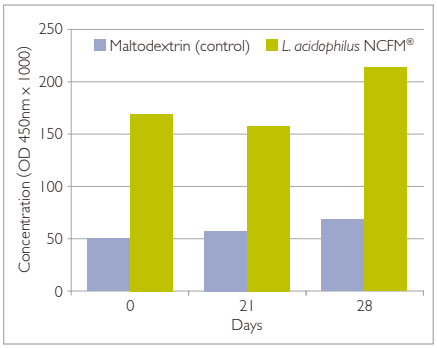

Figure 10. Levels of specific IgA titre in orally vaccinated humans after supplementation with *L. acidophilus* NCFM® compared with placebo [83].

To sum up, *L. acidophilus* NCFM® has been shown to modulate immune response markers in a way that is important to 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 important to protection from infections.

Influence of a probiotic combination on birch pollen allergy

A randomised, 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.

Administration of the probiotic combination also led to a significant increase in the faecal 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 [84].

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.

L/D- LACTIC ACID PRODUCTION

Lactic acid is the most important metabolic end-product of fermentation processes by lactic acid bacteria and other microorganisms.

Due to 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 . L(+) lactic acid is the normal metabolic intermediary in mammalian tissues. D(-) lactic

acid is normally present in the blood of mammals at nanomolar concentrations.

In the past, D(-) lactic acid was thought to be "non-physiological" and, due to the slower metabolism in the human body, the possible cause of lactate acidosis [85,86].

In 1967, this led to a recommendation from WHO/FAO for a maximum D(-) lactic acid intake of 100mg per kg body weight. More recent studies using modern methods have shown that, in fact, the metabolism of D(-) lactic acid in healthy humans is comparable with L-lactate. Due to the scientific evidence, WHO/FAO withdrew this intake recommendation in 1974, maintaining the restriction not to use D(-) lactic acid in food for infants [87].

Special attention has been paid to children below the age of 12 months because their metabolism is premature. The CODEX Standard for Infant Formula for children under 12 months [88] contains the restriction under "Optional Ingredients" – "Only L(+) lactic acid producing cultures may be used". This also applies to the use of cultures as acidity regulators.

The recommendation is based on three studies [89,90,91] in which DL-lactic acid was directly added to infant formulas at concentrations of 0.35 to 0.5%. Some infants in the study could not tolerate lactic acid supplementation. The effects were reversed on withdrawing these high doses of lactic acid from the diet.

In another recent study [92], healthy infants fed a D(-)-lactic acid producing *Lactobacillus* sp. at 10E8 CFU/day from birth to 12 months demonstrated no change in serum D(-) lactic acid levels compared to a placebo-fed control. This study concluded that probiotics producing D(-) lactic acid can be safely fed to infants.

In view of all these results, the use of D(-) lactic acid in infant nutrition is still questioned today.

Nevertheless, these concerns should not be directly applied to the use of probiotic cultures as nutritional ingredients because they do not produce lactic acid in infant formula.

In conclusion, despite the fact that there is no real scientific proof that healthy infants or any healthy human would be affected detrimentally by the addition of lactobacilli that produce D(-) lactic acid, Danisco follows the CODEX recommendation not to use D(-) lactic acid producing cultures in food for infants below the age of 12 months.

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

Internally generated data

ANTIBIOTIC RESISTANCE 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 a bacterium.

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 [8].

The antibiotic susceptibility patterns for *L. acidophilus* NCFM® are summarised in the table below (internally generated data).

***Lactobacillus acidophilus* NCFM antibiogram**	
Amoxicillin	S
Ampicillin	S
Ceftazidime	I
Chloramphenicol	I
Ciprofloxacin	R
Clindamycin	I
Cloxacillin	S
Dicloxacillin	S
Erythromycin	S
Gentamicin	R
Imipenem	R
Kanamycin	R
Neomycin	R
Nitrofurantoin	R
Penicillin G	S
Polymixin B	R
Rifampicin	I
Streptomycin	R
Sulfamethoxazole	R
Tetracycline	I
Trimethoprim	R
Vancomycin	S

S = Susceptible (minimum inhibitory concentration ≤ 4μg/ml)
I = Intermediate (minimum inhibitory concentration = 8 to 32μg/ml)
R = Resistant (minimum inhibitory concentration ≥ 64μg/ml)

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 [94]. 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 [95].

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® [96,97] 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 [98,99]. 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 [100].

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 in helping to prevent urinary and vaginal tract infections [101]. 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, pre-incubation 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 the 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_2O_2, 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.

Reduction of cold and flu symptoms

The impact of *L. acidophilus* NCFM® on respiratory health was investigated in a study involving 326 Chinese children aged between three and five years at day care centres near Shanghai. The 26-week study was conducted from November to May, when children are particularly susceptible to colds and flu. The strain significantly reduced the incidence and duration of fever, upper respiratory infection symptoms and antibiotic use compared to a placebo (figure 11) [102,103].

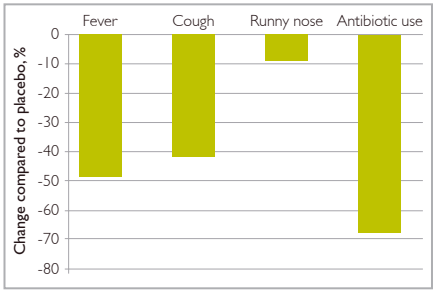

Figure 11. Reduction in incidence of upper respiratory tract infections by *L. acidophilus* NCFM® compared to placebo [103].

UTILISATION OF PREBIOTICS

The ability of gastrointestinal bacteria to utilise 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 [104]. Fructooligosaccharides (FOS) are a well studied family of fructose polymers with beneficial effects on the host microbial flora. FOS are not digested in the upper gastrointestinal tract in humans, but can be degraded by a number of bacteria residing in the lower GI tract.

The ability of *L. acidophilus* NCFM® to utilise various FOS oligomers has been published previously [105] (figure 12). Additional studies have also demon-

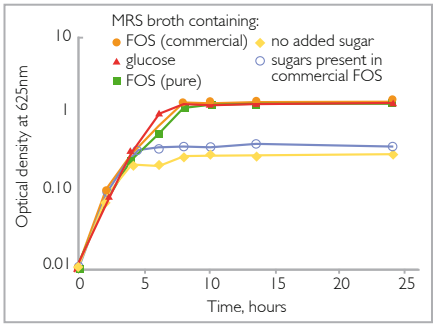

Figure 12. Utilisation of FOS by *L. acidophilus* NCFM® [105].

strated the ability of the strain to utilise hydrolysed inulin [9] and other prebiotics, providing opportunities for formulating synbiotic functional food [106,107,108].

With regard to mechanical properties, *L. acidophilus* NCFM® possesses an efficient and well-characterised FOS transport and catabolic system [9]. This particular system may give *L. acidophilus* NCFM® 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® genome encodes a large variety of genes related to carbohydrate utilisation, including mono-, di-, and polysaccharides [8]. This supports its adaptation to the intestinal environment.

APPLICATIONS & STABILITY

Today there is a general consensus that probiotics have to be consumed in sufficient numbers to provide the desired health benefit.

However, few dose-response studies have been performed, and a true scientific basis for one daily dose is lacking. It is likely that different strains and different effects require different dosages.

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

- proven health benefits
- available as a high-count freeze-dried material
- patented stabilisation 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. [110,111,112,113,114,115,116,117].

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

BENEFIT 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 75 publications, more than 20 of which refer to human studies, in peer-reviewed journals describe the multi-faceted properties of *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 summarised as follows:

- Improves gastrointestinal health and well-being
  - improves the level of natural good bacteria in the body
  - aids digestion and well-being
  - reduces gastro-intestinal discomfort
  - maintains the balance of healthy microflora
  - beneficially affects the intestinal flora
  - 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 a human clinical study
  - may influence immune regulation, as demonstrated by the induction of IL-12 and moderate induction of tumour necrosis factors *in vitro*
  - may reduce symptomes of respiratory tract infections
- Long history of safe use

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