Technical Memorandum

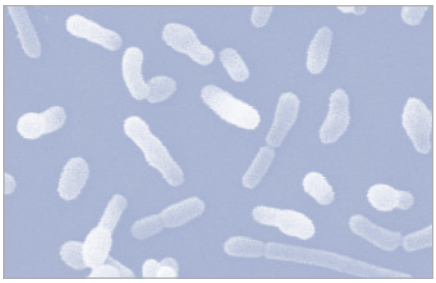
TM 51-1e

Lactobacillus rhamnosus Lr-32

DANISCO

CHARACTERISTICS OF THE GENUS

Lactobacillus rhamnosus has existed in cheese for hundreds of years and is also one of the most common *Lactobacillus* species in breast-fed infants (1). The strain comprises Gram-positive, facultatively anaerobic, non-motile and non-spore-forming, rod-shaped microorganisms.



SELECTION AND TAXONOMY

L. rhamnosus was originally thought to be a sub-species of *L. casei.* Later genetic research found it to be a species in its own right. As a result, in 1989 its taxonomic name was changed from *L. casei* subsp. *rhamnosus* to *L. rhamnosus* (2).

L. rhamnosus Lr-32 has been genetically characterised and properly classified as *L. rhamnosus* by independent labs using modern genotypic methods including 16S rRNA gene sequence analysis. Originally isolated from an unknown source, the strain has been deposited in the American Type Culture Collection as SD5217.

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 (3-7).

More specifically, *L. rhamnosus* is listed in the *Inventory of Microorganisms With Documented History of Use in Human Food* (8) and the Qualified Presumption of Safety list of the European Food Safety Authority (9).

In addition to a long history of safe human consumption, no acquired antibiotic resistance was detected in *L. rhamnosus* Lr-32 during screening by the EU-funded PROSAFE project.

GASTROINTESTINAL PERFORMANCE

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. Although not explicitly stated, this definition implies that a probiotic should survive GI tract passage and, according to some, colonize the host epithelium.

A variety of traits are believed to be relevant for surviving GI tract passage, the most important of which is tolerance both to the highly acidic conditions present in the stomach and to concentrations of bile salts found in the small intestine.

In vitro studies have shown that *L. rhamnosus* Lr-32 is resistant to low pH conditions and survive the presence of bile at concentrations present in the duodenum.

Adhesion to intestinal mucosa

Interaction with the intestinal mucosa is considered important for a number of reasons. Binding to the intestinal mucosa may prolong the time a probiotic strain can reside in the intestine. This interaction with the mucosa brings the probiotic in close contact with the intestinal immune system, giving it a better opportunity to modulate the immune response. It may also protect against enteric pathogens by limiting their ability to colonize the intestine.

Currently, adherence is measured using 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 attach.

L. rhamnosus Lr-32 demonstrated excellent adhesion to human epithelial cell lines applied in *in vitro* studies.

Acid tolerance	**+++** (>70% survival in hydrochloric acid and pepsin (1%) at pH 3 for 1h at 37°C)
Bile salt tolerance	**++++** (>80% survival in 0.3% bile salt containing medium)
Pepsin resistance	**+++** (>40% 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)

Selected characteristics of *L. rhamnosus* Lr-32 (internally generated data):
++++ Excellent; +++ Very good; ++ Good; + Fair

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

Selected characteristics of *L. rhamnosus* Lr-32 (internally generated data): ++++ Excellent; +++ Very good; ++ Good; + Fair

Inhibition of pathogens

The protective role of probiotic bacteria against gastrointestinal pathogens is one of the most important properties for therapeutic modulation of the enteric microbiota. Probiotics are able to inhibit, displace and to compete with pathogens, but the abilities are strain dependent.

The putative mechanisms of action of probiotic strains against pathogenic microorganisms include the production of inhibitory compounds, competition with the pathogen for adhesion sites, or nutritional sources, inhibition of the production or action of bacterial toxins, ability to coaggregate with pathogens and stimulation of immunoglobulin A.

In vitro inhibition is usually investigated using an agar inhibition assay, where soft agar containing the pathogen is laid over colonies of probiotic cultures, and inhibition expressed as the zones of inhibition developing 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 that extending such results to the *in vivo* situation is not straightforward.

The assessment in below table is based on an *in vitro* assay.

L. rhamnosus Lr-32 displayed *in vitro* inhibition of selected pathogens.

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

Selected characteristics of *L. rhamnosus* Lr-32 (internally generated data): ++++ Excellent; +++ Very good; ++ Good; + Fair

L/D- lactic acid production

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

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

In humans, animals, plants, and microorganisms, L(+)-lactic acid is a normal intermediate or end product of the carbohydrate and amino acid metabolisms. It is important for the generation of energy under anaerobic conditions.

In 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, derived from lipid or amino acid metabolism.

L. rhamnosus only produces L(+)-lactic acid.

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

Internally generated data

IMMUNOMODULATION

An immune system that functions optimally is an important safeguard against infectious and non-infectious diseases. The intestinal microbiota represent one of the key elements in the body's immune defence system.

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.

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.

In vitro studies

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 is useful in determining the ability of each strain to contribute to balanced immune health.

L. rhamnosus Lr-32 was investigated *in vitro* for its ability to induce the PBMC secretion of selected cytokines: interleukin IL-10 and IL-12. The results were compared with *Lactococcus lactis*, a starter culture commonly used in the production of various fermented foods, and *Escherichia coli*, a common member of the intestinal microbiota. IL-10 plays a key role in the control of inflammatory responses to intestinal antigens.

L. rhamnosus Lr-32 was found to induce IL-10 to a significantly higher degree than *Lc. lactis* and to a higher degree than *E. coli*. IL-12 was induced to a lower degree than *Lc. lactis*, but higher then *E. coli* (figure 1). This indicates that *L. rhamnosus* Lr-32 has anti-inflammatory properties (10).

Animal studies

In line with the results above, *L. rhamnosus* Lr-32 has further demonstrated an ability to modulate the immune system in an inflammation animal model, validating its ability to

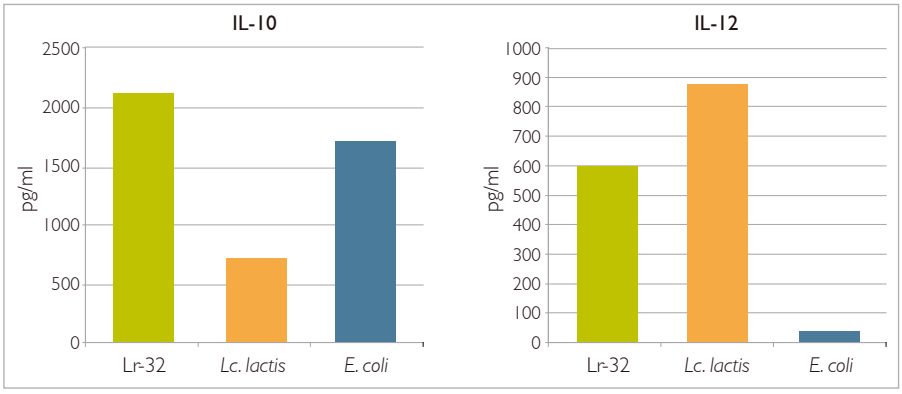


Figure 1. *In vitro* cytokine expression of *L. rhamnosus* Lr-32 (10).

contribute to a balanced immune system. Figure 2 demonstrates the degree of protection from a chemically-induced intestinal inflammation. *L. rhamnosus* Lr-32 has led to a considerable reduction in colitis symptoms and exerts significant protection from intestinal inflammation, demonstrating its ability to interact with and balance the intestinal mucosal immune response (10).

L. rhamnosus Lr-32 was further included in a study 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 orchestrating immune responses to own and foreign antigens. It has been shown that, after activation with different stimuli, DCs achieve maturation, leading to functional and phenotypic changes.

In this study it was demonstrated that probiotic-treated DCs conferred protection against TNBS-induced colitis in mice. While the administration of untreated DCs did not rescue mice from colitis, intra-peritoneal administration of DCs treated with *L. rhamnosus* Lr-32 led to a considerable reduction in the colitis, with reduced weight loss, improved clinical parameters and a significant reduction in macroscopic inflammation scores (figure 3) (11).

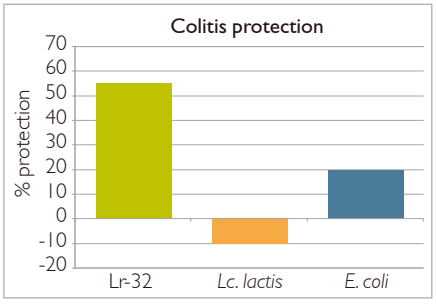


Figure 2. Percentage of protection in an acute murine model of inflammation (TNBS) (9).

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.

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 an 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.

Much concern has arisen in recent years regarding vancomycin resistance, as vancomycin-resistant enterococci are a leading cause of hospital-acquired infections and are refractory to treat-

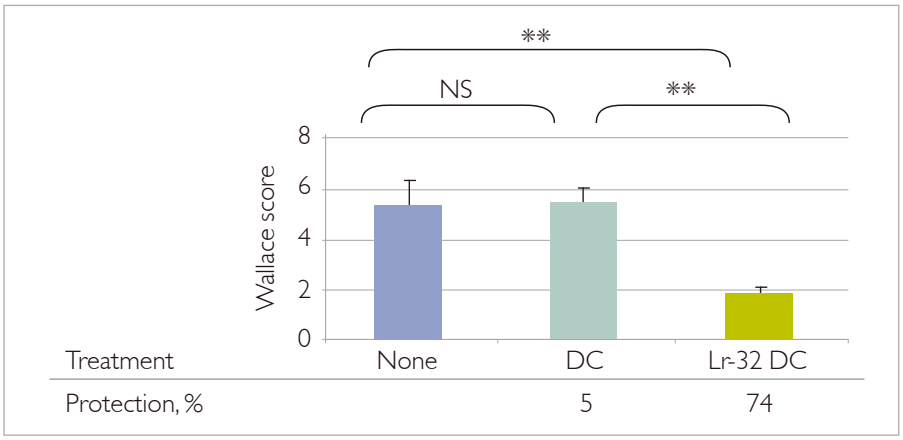


Figure 3. Protective effect of intra-peritoneal administration of LAB-treated DCs on acute TNBS-induced colitis in BALB/c mice. Wallace inflammation scores were calculated after a TNBS challenge in mice either not treated (none) or intra-peritoneally injected with untreated DCs (DC) or DCs treated with *L. rhamnosus* Lr-32 (Lr-32 DC) (11).

ment. The transmissible nature of genetic elements that encode vancomycin resistance in these enterococci is an important mechanism of pathogenicity.

Resistance to vancomycin in certain lactobacilli, including *L. rhamnosus*, pediococci and leuconostoc is due to intrinsic factors related to the composition of their cell wall, and not due to any transmissible elements (12). *L. rhamnosus* Lr-32 has been confirmed through PCR testing to be free of *Enterococcus*-like vancomycin-resistance genes.

As yet no case of antibiotic resistance transfer has ever been identified and reported for lactic acid bacteria used in foods and feed.

***Lactobacillus rhamnosus* Lr-32 antibiogram**	
Amoxicillin	S
Ampicillin	S
Ceftazidime	R
Chloramphenicol	I
Ciprofloxacin	R
Clindamycin	S
Cloxacillin	S
Dicloxacillin	S
Erythromycin	S
Gentamicin	R
Imipenem	R
Kanamycin	R
Neomycin	R
Nitrofurantoin	R
Penicillin G	S
Polymixin B	R
Rifampicin	S
Streptomycin	R
Sulfamethoxazole	R
Tetracycline	I
Trimethoprim	R
Vancomycin	R

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

Table 1.

The antibiotic susceptibility patterns for *L. rhamnosus* Lr-32 are summarised in table 1.

BENEFIT SUMMARY

Based on the data supporting the qualities of the *L. rhamnosus* Lr-32 strain, the health-related attributes can be summarised as follows:

- Well suited to intestinal survival
  - High tolerance of acid and intestinal bile
  - Strong adhesion to intestinal cell lines
- Beneficial modulation of immune functions
  - *L. rhamnosus* Lr-32 may influence immune regulation, as demonstrated by the increased induction of IL-10 *in vitro*
  - *L. rhamnosus* Lr-32 has shown anti-inflammatory properties, as demonstrated through significant protection against TNBS-induced colitis in an animal model

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