Technical Memorandum

Lactobacillus plantarum **Lp-115**

DANISCO

CHARACTERISTICS OF THE SPECIES

Lactobacillus plantarum is a Gram-positive, non-spore forming, homofermentative rod that frequently occurs spontaneously in high numbers in most lactic acid fermented, plant-based foods including in brined olives, sauerkraut and traditional African ogi and cassava and Asian kimchi. It is also often found on the human gastrointestinal (GI) mucosa (1).

Strains of this species are used as starter cultures in several food products, including sourdough bread, meat products and wine. It is also the most commonly used species in silage production.

SELECTION AND TAXONOMY

L. plantarum Lp-115 has been genetically characterised and properly classified as *L. plantarum* by independent labs using modern genotypic methods including 16S rRNA gene sequence analysis.

L. plantarum Lp-115 is a strain isolated from plant material and has been deposited in the American Type Culture Collection as SD5209.

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 (2-5). Moreover, no *L. plantarum* bacteraemia were identified in a 10-year survey in Finland (6).

The fact that many traditional lactic acid fermented foods spontaneously contain high numbers of *L. plantarum* and that all over the world, these products have a reputation for being safe and wholesome, strongly indicates that *L. plantarum* can be safely consumed. The long tradition for consuming lactic acid fermented foods only strengthens this hypothesis (1).

More specifically, *L. plantarum* is listed in the *Inventory of Microorganisms With Documented History of Use in Human Food* (7). The European Food Safety Authority has also added the species to the Qualified Presumption of Safety list (8).

In addition to a long history of safe human consumption of the species, no acquired antibiotic resistance was detected in *L. plantarum* Lp-115 during screening by the EU-funded PROSAFE project.

The safety of the strain was further evaluated in a colitis mouse model.

High doses (10^{10} CFU) of *L. plantarum* Lp-115 did not result in translocation

of the organism, nor did it induce any potential adverse effect on mouse activity, weight, and colon inflammation or abnormal translocation of members of the intestinal microbiota (9).

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. plantarum* Lp-115 is extremely resistant to low pH conditions and survive the presence of bile at concentrations present in the duodenum.

Acid tolerance	**++++** (>90% survival in hydrochloric acid and pepsin (1%) at pH 3 for 1h at 37°C)
Bile salt tolerance	**++++** (>90% 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. plantarum* Lp-115 (internally generated data): ++++ Excellent; +++ Very good; ++ Good; + Fair

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. plantarum Lp-115 has demonstrated excellent adhesion to human epithelial cell lines (Caco-2) applied in *in vitro* studies.

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

Selected characteristics of *L. plantarum* Lp-115 (internally generated data): ++++ Excellent; +++ Very good; ++ Good; + Fair

Inhibition of pathogens

The protective role of probiotic bacteria 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 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, 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 below table is based on an *in vitro* assay.

L. plantarum Lp-115 displayed *in vitro* inhibition of selected pathogens.

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

Selected characteristics of *L. plantarum* Lp-115 (internally generated data): ++++ Excellent; +++ Very good; ++ Good; + Fair

The ability to aggregate and coaggregate are desirable properties for probiotics as they are related to the ability to interact closely with pathogens and could avoid or reduce their adhesion to the mucosa. *L. plantarum* Lp-115 showed autoaggregation and high coaggregation, especially with *Clostridium histolyticum* and *Staphylococcus aureus in vitro* (10).

L. plantarum Lp-115 also showed the ability to inhibit the adhesion (P < 0.05) of *Bacteroides vulgatus* (30.8%), *Clostridium histolyticum* (20.5%), *Clostridium difficile* (35.7%), *Staphylococcus aureus* (33.4%) and *Enterobacter aerogenes* (30%) *in vitro* (11).

The strain was also able to displace (P < 0.05) *B. vulgatus* (63.1%), *C. histolyticum* (24%), *C. difficile* (54.2%), *St. aureus* (26.8%), *E. aerogenes* (48.9%) and *L. monocytogenes* (36.8%) *in vitro* (11).

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 for lactate acidosis (12, 13). 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, but still with the restriction not to use D(-)-lactic acid in food for infants (14).

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 the age group below 12 months (STAN 72-1981 revision 2007) contains the restriction under "Optional ingredients": "Only L(+)-lactic acid producing cultures may be used" as well as for the use as acidity regulator.

This recommendation is based on three studies (15, 16, 17) in which DL-lactic acid was 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 (18), healthy infants fed a D(-)-lactic acid producing *Lactobacillus* sp. at 10^8 CFU/day from birth to 12 months demonstrated no change in serum D(-)-lactic acid levels compared to placebo-fed control, this study concluded that probiotics producing D(-)-lactic acid can be safely fed to infants.

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

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

In conclusion, despite the fact that there is no real scientific consensus to suggest 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	**45/55**
Molar ratio	Boehringer Mannheim/ R-Biopharm D-lactic acid/ L-lactic acid UV-method

Internally generated data

Oxalate-degrading activity
A study was undertaken to evaluate the oxalate-degrading activity of 60 *Lactobacillus* strains, including *L. plantarum* Lp-115. 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.

The oxalate-degrading activity of *L. plantarum* Lp-115 was found to be 40% compared to a positive control *Oxalobacter formigenes* DSM 4420 (100%) and a negative control *Escherichia coli* ATCC 11105 (0%). The activity of other strains of *L. plantarum* ranged from 0-35%.

The identification of probiotic strains with oxalate-degrading activity may offer the opportunity to relieve individuals suffering from high levels of oxalate in the body and oxalate-associated disorders (13).
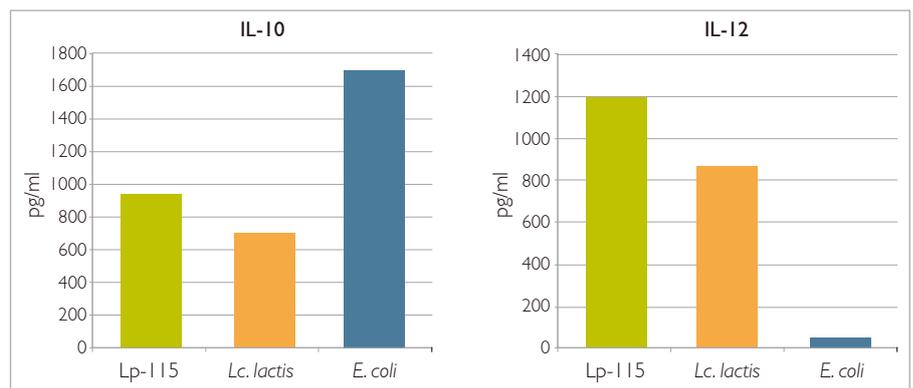

Figure 1. *In vitro* cytokine expression of *L. plantarum* Lp-115 (14).

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. plantarum Lp-115 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 a strain of *Lactococcus lactis* and a strain of non-pathogenic *E. coli*. Similar to *Lc. lactis*, *L. plantarum* Lp-115 induced moderate amounts of IL-10. However, *L. plantarum* Lp-115 induced significantly higher PBMC excretion of IL-12 (figure 1). This 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 (20).

Animal studies
L. plantarum Lp-115 was further evaluated in an inflammation animal model, confirming its ability to contribute to a balanced immune system. Figure 2 demonstrates the percentage of protection from a chemically-induced intestinal inflammation. *L. plantarum* Lp-115 reduces the intestinal inflammation in this model, displaying a capacity to interact with and beneficially modulate or balance the intestinal mucosal immune response (20).

Similar results were obtained in another study using the same model. Here intra-gastric administration of *L. plantarum* Lp-115 in TNBS-treated mice resulted in a moderate, though not significant reduction of the inflammatory score but without any significant reduction of weight loss (9).
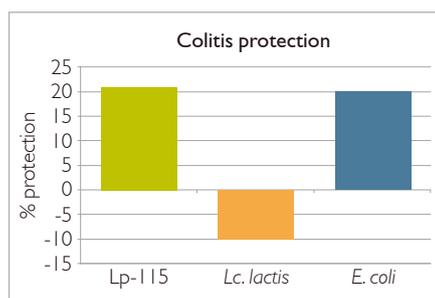
Figure 2. Percentage of protection in an acute murine model of inflammation (TNBS) (20).

Figure 3. Relative change in specific IgG titre in orally vaccinated humans after supplementation with *L. plantarum* Lp-115.

Human studies

The ability of *L. plantarum* Lp-115 to stimulate specific immunity has been evaluated in a human study measuring primary immune reaction following vaccination. Human volunteers were orally vaccinated (using cholera vaccine as the vaccination model) and then received either a placebo (maltodextrin, n=20) or *L. plantarum* Lp-115 (n=9). Supplementation with *L. plantarum* Lp-115 or the placebo started on day 0 and continued for 21 days. The subjects consumed two capsules a day with 10^{10} CFU *L. plantarum* Lp-115 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.

Supplementation with *L. plantarum* Lp-115 resulted in faster IgG induction than in the control group. This indicates stimulation of specific immunity by *L. plantarum* Lp-115 (manuscript submitted, figure 3).

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.

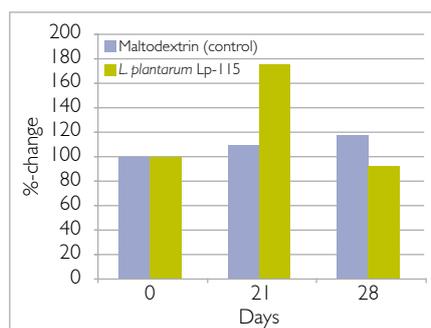Antibiotic resistance is a natural property of microorganisms and existed before antibiotics became used by humans. In many cases, resistance is due to the absence of the specific antibiotic target or is a consequence of natural selection.

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 treatment. 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. plantarum*, pediococci and leuconostoc is due to intrinsic factors related to the composition of their cell wall, and not due to any transmissible elements (21). *L. plantarum* Lp-115 has been confirmed through PCR testing to be free of *Enterococcus*-like vancomycin-resistance genes.

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

The antibiotic susceptibility patterns for *L. plantarum* Lp-115 are summarised in table 1.

Lactobacillus plantarum Lp-115 antibiogram	
Amoxicillin	S
Ampicillin	S
Ceftazidime	R
Chloramphenicol	R
Ciprofloxacin	R
Clindamycin	S
Cloxacillin	R
Dicloxacillin	R
Erythromycin	I
Gentamicin	R
Imipenem	R
Kanamycin	R
Neomycin	R
Nitrofurantoin	R
Penicillin G	R
Polymixin B	R
Rifampicin	S
Streptomycin	R
Sulfamethoxazole	R
Tetracycline	R
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.

BENEFIT SUMMARY

Extensive *in vitro* and *in vivo* studies support the health-enhancing, probiotic properties of *L. plantarum* Lp-115. Following is a summary of these attributes:

- Long history of safe use
- Well-suited for intestinal survival
  - High tolerance to gastrointestinal conditions (acid, bile, pepsin and pancreatin)
  - Strong adhesion to intestinal cell lines
- Ability to inhibit common pathogens
- Beneficial modulation of immune functions
  - May improve specific immune response, as demonstrated in a human clinical study (manuscript submitted)
  - May influence immune regulation, as demonstrated by balancing IL-10/IL-12 *in vitro*

REFERENCES

Publications on *L. plantarum* Lp-115 in bold.

1. Molin, G. The Role of *Lactobacillus plantarum* in Foods and in Human Health In: Farnworth, E.R. (Ed), Handbook of Fermented Functional Foods.

2. Aguirre, M. & Collins, M.D. (1993). Lactic acid bacteria and human clinical infections. J. Appl. Bact. 75:95-107.

3. Gasser, F. (1994). Safety of lactic acid bacteria and their occurrence in human clinical infections. Bull. Inst. Pasteur. 92:45-67.

4. Salminen S., von Wright, A., Morelli, L., Marteau, P., Brassart, D., de Vos, W.M., Fonden, R., Saxelin, M., Collins, K., Mogensen, G., Birkeland, S.-E. & Mattila-Sandholm, T. (1998). Demonstration of safety of probiotics-a review. Int. J. Food Prot. 44:93-106.

5. Borriello, S.P., Hammes, W.P., Holzapfel, W., Marteau, P., Schrezenmeir, J., Vaara, M. & Valtonen, V. (2003). Safety of probiotics that contain lactobacilli or bifidobacteria. Clin. Infect. Dis. 36:775-780.

6. Salminen, M.K., Tynkkynen, S., Rautelin, H., Saxelin, M., Vaara, M., Ruutu, P., Seppo Sarna, S., Valtonen, V. & Järvinen, A. (2002). *Lactobacillus* Bacteremia during a Rapid Increase in Probiotic Use of *Lactobacillus rhamnosus* GG in Finland. Clinical Infectious Diseases. 2002; 35:1155-60.

7. Mogensen, G., Salminen, S., O'Brien, J., Ouwehand, A.C., Holzapfel, W., Shortt, C., Fonden, R., Miller, G.D., Donohue, D., Playne, M., Crittenden, R., Salvadori, B. & Zink, R. (2002). Inventory of microorganisms with a documented history of safe use in food. Bulletin of the International Dairy Federation. 377:10-19.

8. List of taxonomic units proposed for QPS status http://www.efsa.europa.eu/EFSA/Scientific_Opinion/sc_op_ej587_qps_en.pdf.

9. Daniel, C., Poiret, S., Goudercourt, D., Dennin, V., Leyer, G. & Pot, B. (2006). Selecting Lactic Acid Bacteria for Their Safety and Functionality by Use of a Mouse Colitis Model. Applied and Environmental Microbiology. 72(9):5799–5805.

10. Collado, M.C., Meriluoto, J. & Salminen, S. (2007). Adhesion and aggregation properties of probiotic and pathogen strains. Eur Food Res Technol. DOI 10.1007/s00217-007-0632-x.

11. Collado, M.C., Meriluoto, J. & Salminen, S. (2007). Role of commercial probiotic strains against human pathogen adhesion to intestinal mucus. Letters in Applied Microbiology. doi:10.1111/j.1472-765X.2007.02212.x.

12. Cori, C.F. & Cori, G.T. (1929). Glycogen formation in the liver from D- and L-lactic acid. J. Biol. Chem. 81, 389-403.

13. Medzihradsky, F.M. & Lamprecht, W. (1966). Stoffwechseluntersuchungen mit Essig-, Milch- und Zitronensäure. Z. Lebensm.-Unters. -Forschung. 130, 171-181.

14. WHO food additives series no.5, 1974. Available at: http://www.inchem.org/documents/jecfa/jecmono/v05je86.htm.

15. Jacobs, H.M. & Christian, J.R. (1957). Observations on full-term newborn infants receiving an acidified milk formula. Lancet. 77, 157-9.

16. Droese, W. & Stolley, H. (1962). Funktionelle Prüfungen von Säuglingsnahrungen; dargestellt an der Säuremilchernährung junger Säuglinge. Dtsch. med. J. 13, 107-112.

17. Droese, W. & Stolley, H. (1965). Die Wirkung von Milchsäure (D/L), Citronensäure und Protein auf die organischen Säuren im Harn von gesunden Säugligen im 1. Lebensvierteljahr. In: Symp über die Ernährung der Frühgeborenen, Bad Schachen, Mai 1964. Basel/New York: Karger, 63-72.

18. Connolly, E., Abrahamsson, T. & Bjorksten, B. (2005). Safety of D(-)-Lactic Acid Producing Bacteria in the Human Infant. J. Ped. Gastro. Nutr. 41:489-492.

19. Turroni, S., Vitali, B., Bendazzoli, C., Candela, M., Gotti, R., Federici, F., Pirovano, F. & Brigidi, P. (2007). Oxalate consumption by lactobacilli: evaluation of oxalyl-CoA decarboxylase and formyl-CoA transferase activity in *Lactobacillus acidophilus*. Journal of Applied Microbiology (OnlineEarly Articles). doi:10.1111/j.1365-2672.2007.03388.x.

20. Foligne, B., Nutten, S., Grangette, C., Dennin, V., Goudercourt, D., Poiret, S., Dewulf, J., Brassart, D., Mercenier, A. & Pot, B. (2007). Correlation between *in vitro* and *in vivo* immunomodulatory properties of lactic acid bacteria. World Journal of Gastroenterology 13(2):236-243.

21. Delcour, J., Ferain, T., Deghorain, M., Palumbo, E. & Hols, P. (1999). The biosynthesis and functionality of the cell-wall of lactic acid bacteria. Antonie Van Leeuwenhoek. 76(1-4):159-84.

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