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From promise shown *in vitro* to clinical reality - a threshold probiotics still need to cross

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The huge population of bacteria making up the intestinal flora, including lactobacilli and bifidobacteria, form the first line of the host's defences against enteric pathogens (Liévin-Le Moal & Servin, 2006). Although the anti-microbial effects of probiotics (mainly lactobacilli and bifidobacteria) and their repercussions on health are widely claimed today, how legitimate are these claims?

Three groups of experts have recently reaffirmed the need to provide clinical and experimental evidence and have emphasized that proofs must stand up to great scrutiny (FAO/WHO, 2001; Reid et al., 2003a; Aggett et al., 2005). In general terms, the majority of clinical studies do not have the same rigorous protocols as those designed for testing medical drugs, even though great claims are made of their therapeutic effects. Few studies have been carried out randomised, double-blind and placebo-controlled. Only a very few probiotic strains have been the subject of thorough clinical studies (Reid et al., 2003b; Servin, 2004). The most thorough clinical studies, carried out according to the recommendations of the World Health Organization, show that certain probiotics reduce the duration of diarrhoea attacks by half, when added to treatment with an oral rehydration solution. For studies on anti-*Helicobacter pylori* activity, the results show that eradication of the pathogen is improved when the probiotic is used in addition to the classic medical treatment for gastric ulcers. Clinical studies conducted on the preventive effect of probiotics to reinforce the defences of the host have been conducted, in the majority of cases, on an insufficient number of patients, very often recruited according to poorly-defined inclusion and exclusion criteria and for treatment periods that are too short.

Recently, major progress has been made in advancing our knowledge of the anti-microbial activity of certain strains of probiotics. The most firm evidence has been collected by developing a consistent series of studies both *in vitro* and *in vivo*. The most informative *in vitro* studies use the cells of the human intestine that express their structural and functional character as if they were *in situ* and form mono-layers imitating the intestinal barrier. The best translation *in vivo* of the effects observed *in vitro* has been obtained using germ-free and conventional animals. These cell models and animals infected by enteric pathogens have finally made it possible to define the cellular and molecular bases of the anti-microbial properties of selected strains of lactobacilli and bifidobacteria (Servin, 2004). We should therefore remain optimistic and believe that these probing experimental results obtained *in vitro* and in animals will open the way to properly designed clinical studies carried out on a wider scale in order to provide consumers with absolute proof of the benefits of probiotics for our health.

Aggett PJ et al. (2005). PASSCLAIM: consensus on criteria. Eur J Nutr. 44(S1):15-30.
FAO/WHO (2001). Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria. www.who.int/foodsafety/publications/fs_management/en/probiotics.pdf
Liévin-Le Moal V, Servin AL (2006). The front line of enteric host defence against unwelcome intrusion of harmful microorganisms: mucins, antimicrobial peptides and microbiota. Clin Microbiol Rev. 19: sous presse.
Reid G et al. (2003a). New scientific paradigms for probiotics and prebiotics. J Clin Gastroenterol. 37:105-118.
Reid G, Jass J, Sebulsky MT, McCormick JK (2003b). Potential uses of probiotics in clinical practice. Clin Microbiol Rev. 16:658-672.
Servin AL (2004). Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. FEMS Microbiol Rev. 28:405-440.

Lactobacillus acidophilus inhibits inflammation caused by an intestinal pathogen

Several probiotic bacteria have shown a beneficial effect on the inflammation of the gut linked to infection by a pathogen. A study on rats has shown the conditions in which a probiotic can fight infection.

In a murine model of infection by an enteroinvasive pathogen causing lesions in the gut similar to those caused by *Escherichia coli* in humans, researchers analyzed the preventive and/or curative

effect of *Lactobacillus acidophilus* (1). The administration of the probiotic consisted of two weekly doses of 10⁹ CFU. Infection by the pathogen was created by a single oral dose administered within weeks 6 and 8 of the animal's life. Five groups were formed.

• Group A: Probiotic from week 2 until the end of the experiment (no pathogen).

• Group B: Pathogen + probiotic from week 2 until the end of the experiment (pre-inoculation with the probiotic).

• Group C: Pathogen + probiotic from the day of pathogen infection (co-inoculation with probiotic and pathogen).

• Group D: Pathogen only.

• Group E: No specific treatment.

The parameters analyzed and the results observed are described in the tables below:

Macroscopic or histological parameters observed	Statistically significant results	Cytokines analyzed	Statistically significant results
Weight loss	B < D	Total IgA secretion	A > C, D, E B > C, D, E
Inflammation of the colon (histological observation)	B < D	Specific IgA secretion	B > C B > D
Myeloperoxidase activity (inflammatory marker)	B < C B < D	IFN γ , IL10-specific	B > C B > D
Number of pathogens in the faeces	B < D	TNF α , IL6-specific (proinflammatory cytokines)	D > E (the pathogen causes an increase compared to the control) B < D and C < D (the probiotic inhibits an increase due to the pathogen).
Translocation of the pathogen	B < D C < D		
Number of viable pathogens in the gut	B < D C < D		

These results show that administration of the probiotic attenuated the intensity of markers chosen to assess the colitis caused by the pathogen, inoculation with *L. acidophilus* was more effective when started before, rather than simultaneous to, the infection. Also, *L. acidophilus* reduced bacterial translocation and the number of viable pathogens in the gut.

This improvement in the symptoms was accompanied by the inhibition of the inflammatory reaction caused by the pathogen.

Consumption of *L. acidophilus* reduced the symptoms of colitis caused by an enteroinvasive pathogen in mice. The probiotic was more effective when administered both before and after ingestion of the pathogen rather than only from the start of the infection. This experiment confirms the potential benefits of probiotics in infection prevention strategies.

1• Chen CC, Louie S, Shi HN, Walker WA. (2005) Preinoculation with the probiotic *Lactobacillus acidophilus* early in life effectively inhibits murine *Citrobacter rodentium* colitis. *Pediatr Res.* 58(6):1185-91.

Molecular analysis of proteins with a mucus-binding domain found in probiotics

The effect of probiotics on gastro-intestinal pathogens could be based upon competitive inhibition of binding to the gut mucosa. These binding properties are today partly explained by the expression, on their surface, of proteins with a MUB (mucus-binding domain). A genome study of several lactic bacteria has refined our knowledge of these binding domains (2).

By basing themselves on the known sequence of MUB domains for *Lactobacillus reuteri* and *Lactobacillus*

plantarum, the authors sought MUB domains in the genomes of 9 lactic bacteria used in edible fermentations. In all, 48 proteins including at least one MUB domain were identified. The gene groups including these domains vary greatly, even between highly similar species. In the same way, the number of MUB domains in a protein varies within a single species.

These observations explain, at the molecular level, that the ability to bind to the

intestinal mucus varies from one strain to another. In as far as it is highly likely that these binding properties play a role in the action mechanism with the host, these results emphasize that even very similar bacteria may not have the same effects on the host.

2• Boekhorst J, Helmer Q, Kleerebezem M, Siezen RJ. (2006) Comparative analysis of proteins with a mucus-binding domain found exclusively in lactic acid bacteria. *Microbiology.* 152(Pt 1):273-80.

Activating heat shock proteins with *Lactobacillus rhamnosus* GG

a protection mechanism for the gut cells

An expected benefit of probiotics is action against intestinal infections. Several hypotheses have been formulated to explain this mechanism. Probiotics could destroy the pathogens, exert a metabolic competition on the binding sites or encourage the resistance of the intestinal cells to invasion. A recent *in vitro* study has shown that heat shock proteins (HSP) are involved* (3).

A first series of tests showed that the incubation of murine colonocytes with the culture supernatant of *Lactobacillus rhamnosus* GG (LGG) caused the production of two types of HSP (HSP25 and HSP72). A short co-incubation sufficed to activate the mechanism, but protein expression was delayed (from 4 to 18 hours after the contact with the LGG

culture supernatant, depending on the protein). This was a result of transcription induction. Additional experiments showed that the HSP synthesis resulted from the activation of two protein kinases (p38 and JNK) by one or more protein factors produced by LGG.

To verify the protective effect of this mechanism, colonocytes were pre-incubated (or not) with the LGG culture supernatant and exposed to an oxidising agent. Compared to the control, the cells in contact with the culture supernatant suffered less damage. The number of viable cells was increased and the cytoskeleton was preserved. The beneficial effect was lost when the HSP expression was blocked by kinases p38 and JNK inhibitors.

This study highlights the possible action mechanism of LGG on colonocytes: *via* protein kinases, a protein factor may activate HSP transcription. Since these proteins are involved in cell protection during environmental stress, the mechanism could be a means of defence for colonocytes against enteric pathogens. *In vivo* studies will naturally be needed to confirm the relevance of this cascade for the entire body.

* Heat shock proteins, (HSP) are part of the cell defence mechanisms; they are produced in response to environmental stress (temperature, UV, toxins, etc.).

3• Tao Y, Drabik KA, Waypa TS, Musch MW, Alverdy JC, Schneewind O, Chang EB, Petrof EO. (2006) Soluble factors from the probiotic *Lactobacillus* GG activate MAP kinases and induce cytoprotective heat shock proteins in intestinal epithelial cells. *Am J Physiol Cell Physiol.* 290(4):C1018-30.

Recombinant lactic bacteria as vectors of vaccination

against papillomavirus tumours

Harmless and low-cost, probiotics are excellent candidates to be vaccine vectors. In women, infection with type 16 papillomavirus leads to recurring infection and increased risk of cervical cancer. Injectable preventive vaccines have been seen to be effective, but they are costly and require medical intervention. A research team has worked on a vaccination for papillomavirus tumours by the mucous route and has recently shown the efficacy of recombinant lactococcus against this type of cancer in mice (4).

The strategy consisted in administering two strains of recombinant *Lactococcus lactis* by the intra-nasal route: one strain expresses the E7 antigenic determinant of the type 16 human papillomavirus (strain LL E7), the other produces interleukin 12 (strain LL IL12). This latter strain was used because preliminary studies had shown that interleukin 12 potentiates the response of certain antigens. The non-recombinant strain LL was used as a control.

According to the protocol, healthy mice were given firstly LL, LL E7, or LL E7 in association with LL IL12 by the intranasal route (24 animals per group). The probiotics were administered three

times, on day 0, 14 and 28 of the experiment. On day 35, tumorous cells resulting from type 16 human papillomavirus were then injected into the mice by the sub-cutaneous route. The development of the tumour was monitored at the injection site.

All the mice in the group receiving LL bacteria developed lethal tumours. In the animals given LL E7, tumours only appeared in 65% of cases and their size was reduced by at least half compared to the control group. Simultaneous vaccination with two lactococci (LL E7 and LL IL12) was most effective in preventing tumours: 50% of mice were tumour-free and the tumours were 6 times smaller than those observed in the control animals. The protective effect lasted for 3 months after the last administration of the bacteria. In fact, in the mice vaccinated with LL E7 and LL IL12 that were tumour-free after the first administration of tumour cells, a second inoculation of tumorous cells was conducted after three months. None of the mice developed tumours for the next 6 months.

These recombinant lactococci were also assessed as part of a curative protocol

where the bacteria were administered to animals with established tumours (3 administrations, 7 days apart).

As seen previously, the joint administration of LL E7 and LL IL12 was the most effective in treating the disease, leading to a total disappearance of the tumour in 35% of cases.

In these experiments on animals, the contact of recombinant lactococcus with the nasal mucosa eradicated development of a tumoral inoculum in a large percentage of the subjects and, to a lesser extent, eliminated established tumours in certain subjects. If this effect could be reproduced in women, it would make widescale treatment of papillomavirus tumours more accessible, including in developing countries. However, the possibility of using recombinant probiotics in humans will first need to be examined from an ethical, health and regulatory standpoint.

4• Bermúdez-Humarán LG, Cortes-Perez NG, Lefèvre F, Guimarães V, Rabot S, Alcocer-Gonzalez JM, Gratadoux J-J, Rodriguez-Padilla CC, Tamez-Guerra RS, Corthier G, Gruss A, Langella P. (2005) A Novel Mucosal Vaccine Based on Live Lactococci Expressing E7 Antigen and IL-12 Induces Systemic and Mucosal Immune Responses and Protects Mice against Human Papillomavirus Type 16-Induced Tumors. *Immunol.* 175: 7297-7302.

Fermented milk active against arterial pressure in subjects with high blood pressure

The benefits of fermented milks may be based on the presence of live bacteria, but also on the production of functional factors resulting from the metabolism of these bacteria (see the editorial by Joëlle Léonil & Sylvie Lortal in Letter n°26). Among the promising functional factors, we can include tripeptides exerting an inhibiting effect on the angiotensin-converting enzyme (ACE). A study of human patients has produced results showing that a fermented milk containing tripeptides has an active effect on blood pressure (5).

Milk fermented with *Lactobacillus helveticus* LBK-16h, rich in ACE inhibiting peptides (Ile-Pro-Pro 7.5 mg / 100g and Val-Pro-Pro 10 mg / 100g) was administered over 10 weeks, at a rate of 300 ml per day, to 94 subjects with high blood pressure. The study was carried out randomized, double-blind and placebo controlled. The blood pressure was mea-

sured over a 24-hour ambulatory period*, at the start and end of the period and by a practitioner**, 9 times during the study. Weight, blood lipids and ACE activity were also measured.

From the ambulatory measurements, systolic and diastolic pressure was more reduced during the study in the group receiving the fermented milk than in the control group (difference in systolic pressure: $4,1 \pm 0,9$ mm Hg, $p=0,001$; difference in diastolic pressure: $1,8 \pm 0,7$ mm Hg $p=0/048$). The effect was not accompanied by changes to the other parameters, in particular ACE activity.

The authors noted however that the significance of their results was limited by one element of bias. The control product was two to three times less rich in calcium, potassium and magnesium than the tested product. It cannot therefore be excluded that these minerals may have a beneficial effect on blood pressure.

Consumption of milk fermented with *L. helveticus* LBK-16h, rich in hypotensive peptides, reduces blood pressure in hypertensive patients. The issue with the composition of the control product does, however, lead to reservations as to the efficacy of the peptides, especially as no effect was observed on ACE activity. This study reminds us that fermented milks should be considered as a whole, since the milk matrix can play an independent or complementary role to that of probiotics.

* Protocol: 4 measurements per hour during the day, 2 at night, using a Holter-type automatic machine.

** Using a machine that automatically measures blood pressure via the arm.

5• Jauhainen T, Vapaatalo H, Poussa T, Kyronpalo S, Rasmussen M, Korpela R. (2005) *Lactobacillus helveticus* Fermented Milk Lowers Blood Pressure in Hypertensive Subjects in 24-h Ambulatory Blood Pressure Measurement. *Am J Hypertens.* 18(12):1600-5.

A lactobacillus against pain - first results in rats

A pioneering publication cites a hitherto unexplored effect of probiotics - their potential action against pain. The results of an experiment on rats provide elements that may help confirm this hypothesis (6).

The animals were given *Lactobacillus reuteri* (1×10^9 CFU / day) for 9 days, in different forms: viable bacteria, inactivated by heat or gamma rays, culture medium. A control group received a pure culture medium. They were then subjected to painful stimuli. Two types of pain response were considered: the reflex response and the voluntary response. The stimulus used for the reflex response was colorectal distension caused by inflating a plastic balloon in the intestine. Two measurements were taken: heartbeat and nervous discharge relating to a dorsal gland. The conscious response was also estimated through a heat stimulus (the animal's tail was plunged into hot water and the reaction time before the tail was removed was measured) and a mechanical stimulus (pressure

was exerted on the animal's paw and the pressure at which the animal withdrew the paw was noted).

Measuring heartbeat during colorectal distension showed, for the maximum distension tested (80 mm Hg applied in the balloon), significant bradycardia (fall in heartbeat) in the control animals. The bradycardia was not present in animals given the probiotic (whatever form it took: live or inactive) or its culture medium. Two smaller distensions were tested; they did not cause any significant changes in heartbeat. The nervous response caused by colorectal distension increased in relation to the amount of pressure applied. For all the different pressures tested, the response in the control animals was significantly greater to the response seen in each of the groups receiving the probiotic (live or inactive form). Finally, unlike the constitutive response, the conscious response of the animals to the two stimuli tested was not altered by consumption of the probiotic.

Therefore, consumption of *L. reuteri* prior to a painful stimulus appears to reduce the constitutive response in rats, suggesting the existence of an interaction mechanism between the probiotic and the reflex nervous system. This is a health benefit of probiotics examined for the first time. Naturally, many stages are now needed to consolidate the result. In addition, the fact that the probiotic - alive or inactivated - or its culture medium are efficient can let foresee the possibility that various mechanisms may be involved. It is however tempting to hope that, for patients suffering from gastro-intestinal disorders, consumption of probiotics may reduce digestive problems.

6• Kamiya T, Wang L, Forsythe P, Goettsche G, Mao Y, Wang Y, Tougas G, Bienenstock J. (2005) Inhibitory effects of *Lactobacillus reuteri* on visceral pain induced by colorectal distension in Sprague Dawley rats. *Gut.* 55(2):191-6.

Failure of a probiotic in irritable bowel syndrome

In a randomised, double-blind and placebo-controlled study, 39 patients suffering from irritable bowel syndrome (IBS) received daily for 6 months either 2×10^8 CFU of *Lactobacillus reuteri* ATCC 55730 or a placebo. Consumption of the probio-

tic and the placebo both reduced the symptoms of IBS significantly, with no difference between the two effects. As in all therapeutic studies of IBS, this study comes up against the strong placebo effect characterizing this pathology.

7• Niv E, Naftali T, Hallak R, Vaisman N. (2005) The efficacy of *Lactobacillus reuteri* ATCC 55730 in the treatment of patients with irritable bowel syndrome—a double blind, placebo-controlled, randomised study. *Clin Nutr.* 24(6):925-31.

Consuming prebiotics adds to the potential effects of *L. casei*

Prebiotics are substances that stimulate the growth of bacteria; associating them with a probiotic seems to increase the potential effect of the probiotic. In mice and healthy humans, consuming a symbiotic (mixture of prebiotic and probiotic)

that associates dextran with *Lactobacillus casei* ssp *casei* had an immunomodulatory effect not observed with the bacteria taken alone. The effect described is an increase in the natural killer (NK) activity of lymphocytes.

8• Ogawa T, Asai Y, Tamai R, Makimura Y, Sakamoto H, Hashikawa S, Yasuda K. (2005) Natural killer cell activities of synbiotic *Lactobacillus casei* ssp. *casei* in conjunction with dextran. *Clin Exp Immunol.* 2006 Jan;143(1):103-9.

A probiotic effective against a retrovirus

Infection by the HTLV type 1 retrovirus can cause the malfunction of the bone marrow. In a preliminary study without a control product, 10 subjects suffering from myelopathy ingested a fermented

milk, containing *Lactobacillus casei*. After 4 weeks' administration, improvements were observed in the urinary symptoms and muscle spasm.

9• Matsuzaki T, Saito M, Usuku K, Nose H, Izumo S, Arimura K, Osame M. (2005) A prospective uncontrolled trial of fermented milk drink containing viable *Lactobacillus casei* strain Shirota in the treatment of HTLV-1 associated myelopathy/tropical spastic paraparesis. *J Neurol Sci.* 15;237(1-2):75-81.

Production of conjugated fatty acids by probiotics

Some conjugated isomers of linolenic acid (CLA) seem to have nutritional properties with supposed effects against carcinogenesis, atherosclerosis and fatty body mass. As a forerunner to possible development of products containing

these molecules, a synthesis method replacing costly chemical synthesis by biosynthesis via probiotics is under study. Using *Lactobacillus plantarum*, researchers have developed specific production processes for various CLAs from different

sources of fatty acids.

10• Ogawa J, Kishino S, Ando A, Sugimoto S, Mihara K, Shimizu S. (2005) Production of conjugated fatty acids by lactic acid bacteria. *J Biosci Bioeng.*100(4):355-64.

Vaginal pathogens a target for probiotics

Given their harmless nature and their various benefits to health, probiotic bacteria are envisaged for wide-ranging uses. In an *in vitro* study, researchers have shown that *Lactobacillus sporogenes* could be used via the vaginal route, to

fight pathogens *in situ*. The suppository that has been developed releases viable bacteria that produce lactic acid and hydrogen peroxide (two substances that inhibit the growth of vaginal pathogens) and inhibit *in vitro* the growth of a uro-

pathogenic strain of *Escherichia coli*.

11• Kale VV, Trivedi RV, Wate SP, Bhusari KP. (2005) Development and evaluation of a suppository formulation containing *Lactobacillus* and its application in vaginal diseases. *Ann N Y Acad Sci.*1056:359-365.

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