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# Yoghurts & fermented milks

March 2003 - Letter N° 12

Health • Nutrition • Flora

SCIENTIFIC SURVEY . LACTIC ACID BACTERIA . PROBIOTICS

## edito

## Evidence-based review concerning probiotics as therapy for IBD in humans

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Intestinal bacteria play a key role in inflammatory bowel diseases (IBD). Until recently, probiotic therapy has been considered 'folk' medicine. However, there is emerging interest by the general public and scientific communities for the use of probiotics in human health. Evidence-based Medicine refers to practicing clinical medicine according to valid, high-quality experimental studies, as determined by applying specific evaluation criteria. A series of published papers outlines this systematically. It has been widely adopted by clinical reviewers, and serves as a template for this evidence-based review concerning probiotics as therapy for IBD in humans.

There are only six published randomized clinical trials, but some data exist to possibly show efficacy as maintenance therapy in patients with ulcerative colitis, chronic relapsing pouchitis or Crohn's disease.

Three randomised controlled trials (1 in abstract form) have studied *Escherichia coli* strain Nissle 1917 in comparison to mesalamine for maintenance of ulcerative colitis remission (1-2). The review of these studies suggests that 1) there is currently no strong evidence from clinical studies to support the use of the *E.coli* strain Nissle as maintenance therapy for ulcerative colitis; 2) those three studies demonstrating equivalence with mesalamine have similar results to placebo responses seen in previous trials; 3) there are no published trials using probiotics as treatment for active ulcerative colitis.

Pouchitis is a non-specific inflammation of the ileal reservoir following surgical creation of an ileal-anal anastomosis with pouch reservoir, in the setting of ulcerative colitis. Gionchetti et al. published results of two double-blind, randomised, placebo-controlled clinical trials using a probiotic mixture (VSL#3®) in patients with chronic relapsing pouchitis, defined as at least 3 relapses per year (3-4). These two studies support the use of VSL#3® given orally at a dose of 3 g bid, to reduce relapses in the subset of chronic relapsing pouchitis, following induction of remission with a short course of broad-spectrum antibiotics. This therapeutic benefit was not maintained beyond the duration of treatment. These potentially interesting findings should be confirmed by testing the clinical efficacy of probiotic therapy in other clinical settings of pouchitis.

There are very few controlled clinical studies using probiotics in Crohn's disease (5-6). However, the best controlled, randomised, placebo-controlled study using probiotics in Crohn's disease to date has a negative result, albeit in a highly select subgroup of untreated patients having had an ileocelectomy (6).

In conclusion, basic research highlights the importance of bacteria in IBD, and the possibility of probiotics to modify physiological parameters. Obstacles to providing probiotic therapy include selection of appropriate strains, poor product quality standardization, processing and human biologic factors impairing probiotic viability, maintenance of new bacterial populations in the gut, and local product unavailability (7). Studies have focused on specific IBD subgroups, limiting general applicability for the practitioner. Well-designed, randomised clinical studies are still required to define the role of probiotics as therapeutic agents in inflammatory bowel disease.

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## Lactobacillic septicaemia in Finland :

### involvement of probiotics ruled out

In Finland, the probiotic *Lactobacillus rhamnosus GG* was first introduced into dairy products in 1990. Since then, consumption has steadily increased and by 1999 had reached an annual volume of 6 litres of dairy products per person (i.e.  $3.10^{11}$  cfu/person/year). Although the large number of clinical studies all show the harmlessness of this probiotic of human origin, such a relatively recent and massive use raises the question of the influence, if any, of *Lactobacillus GG* on the occurrence of lactobacillic infections.

Salminen's team has analysed samples of lactobacillus taken from blood cells collected by the septicaemia monitoring laboratory at Helsinki hospital and by the Finnish Agency for Recording Infectious Diseases (NIDR), since 1990 and 1995 respectively (8). An assessment of the impact, if any, of increased consumption of *Lactobacillus GG* on the occurrence of septicaemia was conducted, using PCR to characterise the strains of lactobacillus present in these samples.

During the period 1990-2000, lactobacilli were detected in 0.02% of all hemocultures (43/209000) and, of the infected hemocultures, 0.2% contained lacto-bacillus (43/23000). The results recorded by the NIDR on a national scale for the period 1995-2000, revealed 90 cases of lactobacillic septicaemia, i.e. 0.2% of all cases of septicaemia. The annual occurrence of such cases of septicaemia in Finland during this period was estimated at 0.29 cases/10 000 inhabitants.

Of 66 samples (representing 61% of all samples), initially characterised as lactobacillic and useable for species identification by PCR, 48 were lactobacilli belonging to 7 different species : *L. rhamnosus* represented 54% of identified lactobacilli (26 strains), *L. fermentum* and *L. casei* represented 19% and 15% respectively. Of the 26 *L. rhamnosus* isolates, 11 were identified as being of type *L. rhamnosus GG*. However, the techniques used did not make it possible to determine whether

the probiotic *L. rhamnosus GG* was inherent or introduced by consumption.

The study therefore shows that consumption of the probiotic *L. rhamnosus GG* does not increase the number of cases of lactobacillic septicaemia. However, certain cases have been reported in immunodepressed patients (9). It would therefore be interesting to correlate the lactobacillic septicaemia levels with the immune states of the patients.

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9• Husni R. N., Gordon S. M., Washington J. A., Longworth D.L., (1997). *Lactobacillus* bacteremia and endocarditis : review of 45 cases. *Clin. Infect Dis.* 25 : 1048-55.

## Identification of probiotic strains and assessment

### of their sensitivity to antibiotics

Placing at the disposal of consumers a probiotic that is functional, harmless and correctly labelled is extremely important on a market that has increased significantly over the last five years. Initial microbiological analyses have highlighted that the identity and quantity of bacteria found in products does not always correspond to the information given on the packaging (3). Furthermore, the sensitivity to antibiotics of the probiotic strains used has not always been studied. This could restrict their usage considerably.

In the study by Temmerman et al (10), an exhaustive analysis of 55 European probiotic products identified both the nature of the strains present and the correctness of the product label and also characterised the sensitivity of these strains to 6 currently used antibiotics (kanamycin, vancomycin, erythromycin, tetracycline, penicillin and chloramphenicol).

Thirty dehydrated food supplements and 20 dairy products were analysed in this study. The isolates were identified by electrophoresis. Sensitivity to antibiotics

was analysed using the agar disk diffusion technique, the dilution method (determining the minimum inhibiting concentration) or PCR (identification of the vancomycin resistance gene).

268 bacterial isolates were obtained from the tested products. Bacterial cultures were obtained from all the dairy products but only from 63% of the food supplements. The number of bacteria present was  $10^5$  to  $10^9$ cfu/ml and  $10^3$ - $10^6$ cfu/g respectively. Analysis of the protein profiles raised the question of strain identification : for 47% of the food complements and 40% of the dairy products, the strains identified on the product labels were not those identified in this study. The presence of *Enterococcus faecium* was unexpectedly found in 4 food supplements.

A study of sensitivity to antibiotics of 187 isolates showed that some of them were resistant : 79% to kanamycin, 65% to vancomycin, 26% to tetracyclin, 23% to penicillin, 16% to erythromycin and 11% to chloramphenicol. 68.4% were multiresistant. Finally, contradictory results were obtained for the resistance of

*enterococci* to vancomycin, depending on the techniques used. Using the agar diffusion method, 38% were resistant whereas by PCR and the dilution method, no resistance was detected.

This study highlighted the difficulties in identifying probiotic strains leading to labelling errors and emphasised the need to characterise the resistance of probiotics to antibiotics. According to the authors, particular attention should be paid to the possible transfer of this resistance to endogenous flora.

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## Does consumption of synbiotic-enriched yoghurt have an effect on blood cholesterol ?

The hypothesis that the consumption of probiotic-enriched fermented milk can alter blood cholesterol levels is still under study. *In vitro*, some probiotic strains of human origin (*Lactobacillus acidophilus*) were able to eliminate the cholesterol present in the culture medium. In animals, ingestion of oligofructosaccharides causes a suppression of hepatic triglyceride synthesis and VLDL cholesterol leading to a significant reduction in serum triglyceride levels and, to a lesser extent, serum cholesterol (11).

A cross-over clinical study, conducted in three 7-week periods, aimed to determine whether the ingestion of 300g per day of synbiotic-enriched yoghurt, an association of probiotics (*L. acidophilus* 145 :  $10^6 \cdot 10^8$  cfu/g of yoghurt and *B. longum* 913 :  $10^5$  cfu/g) and a prebiotic (oligofructose 1%), can reduce blood lipid levels (12).

In the first period of the study, all subjects (29 women) consumed yoghurt (control). In the second, 11 women continued the same yoghurt consumption while 18 others consumed the same product enriched with synbiotics. At the end of this phase, the two groups' treatments were reversed for a third period. The effect of the pre- and probiotics added to the yoghurt on the blood lipid levels (total cholesterol, HDL, LDL and triglycerides) was estimated during the 3 periods as was the concentration and distribution of short-chain fatty acids in the faeces.

Results showed that the mean concentration of total cholesterol and LDL were not altered by enriching the yoghurt with synbiotics ( $p > 0.05$ ). However, the consumption of both the control yoghurt and the synbiotic yoghurt resulted in a significant increase in HDL concentrations

( $p = 0.002$ ) leading to a reduction in the LDL/HDL ratio ( $p = 0.001$ ).

The presence of synbiotics did not appear to modify either total cholesterol and serum triglyceride levels or the concentration of short-chain fatty acids in the faeces. According to the authors, an increase in HDL levels is attributed to the prolonged consumption of yoghurt, i.e. to the traditional ferments present in yoghurt and not to the presence of synbiotics. This reduction in the LDL/HDL ratio, favourable in the context of coronary disease, will need to be confirmed by other studies.

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## Probiotics improve the tolerance of antibiotics prescribed in *Helicobacter pylori* infections

Today, resistance to antibiotics and the appearance of side effects are the main reasons for abandoning treatment by antibiotics in *Helicobacter pylori* infections (13). The advantages of using probiotics to treat the diarrhoea associated with taking antibiotics has been under study for many years (14). A clinical study was conducted to show the efficacy of associating treatment by antibiotics and probiotics to reduce side effects and to eradicate *H. pylori* (15).

85 patients infected with *H. pylori* but with no apparent symptoms were enrolled in a triple-blind versus placebo controlled clinical trial. The 3 groups of patients were treated with antibiotics (rabeprazol + clarythromicin + tinidazole) for 1 week and the following probiotics, *Lactobacillus GG* ( $12 \cdot 10^9$  cfu/day) or *S. boulardii* ( $10 \cdot 10^9$  cfu/day) or an association of *Lactobacillus acidophilus* and *Bifido-*

*bacterium lactis* ( $10 \cdot 10^9$  cfu/day), for 2 weeks.

The results showed that, in comparison with the placebo, the antibiotic treatment was significantly better tolerated in the 3 groups receiving the probiotics and that side effects (in particular diarrhoea and taste disorders) were considerably reduced. However, the effect of this treatment on the degree of eradication of *H. pylori* could not be established.

These observations must however be tempered by the fact that the tinidazole used in the study does not cause severe diarrhoea, unlike amoxicillin, regularly used to treat *H. pylori*, that would probably increase the incidence of diarrhoea. Furthermore, all the probiotics tested were more effective than the placebo in preventing side effects resulting from

antibiotics, which would appear to indicate that it was the presence of probiotics that was important, rather than the presence of a particular strain.

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## Probiotics and inflammation :

### involvement of NO in the action mechanism

Nitric oxide (NO) is a gaseous mediator released during the conversion of L-arginine into NO and citrullin by nitric-oxide synthases. In inflammatory phenomena, large quantities of NO are produced by inducible NO synthase (iNOS), caused by specific signals such as the release of endotoxins or pro-inflammatory cytokines by some cell types.

It would appear that NO plays a role in resolving intestinal inflammation. Indeed, iNOS deficient mice develop more severe intestinal inflammations than normal mice (16). The release of pro-inflammatory cytokines has been described as possibly being caused by the probiotic *Lactobacillus GG* (17), also capable of causing the production of NO via iNOS in the macrophages and epithelial cells of the human colon (18).

A Finnish team has studied the mechanism for activating iNOS by the probiotic *Lactobacillus GG* compared to its activation by the components of the bacterial wall : lipopolysaccharide (LPS) and lipoteichoic acid (LTA) (19). The mechanism for inducing iNOS in response to LPS or LTA is only partially known. This mechanism seems to involve tyrosine kinase, C protein kinase, mitogen-activated kinases (MAP-kinases) and nuclear factor kappa B (NF-κB).

Cultures of murine J774 macrophage cells were incubated for 24 hours in the presence of INFγ alone or with *Lactobacillus GG*, LPS or LTA. Concentrations of nitrite (the stable NO metabolite) were measured in the supernatant fluids of the cultures. Presence of the iNOS enzyme in the cells was sought.

The results obtained showed identical nitrite concentrations in the supernatant fluids of cultures in the presence of *Lactobacillus GG*, LPS, LTA, or INFγ accompanied by LPS or

LTA (10μM of nitrite); INFγ alone had no effect on the production. The three components tested are all therefore capable of causing nitrite production, the metabolic path used is still to be identified.

In order to identify the metabolic path used by *Lactobacillus GG*, LPS or LTA to produce nitrite, the action of iNOS as a non-specific or specific inhibitor was studied. In both cases, complete inhibition of NO production was observed, suggesting that the nitrites measured did indeed come from the NO produced via iNOS. Furthermore, inhibition of NO production, in the presence of a protein synthesis inhibitor, suggests *de novo* iNOS synthesis. Also, a drastic reduction in the nitrite concentration caused by the probiotic, the LPS or LTA was observed in the presence of a specific tyrosine kinase inhibitor and NF-κB, highlighting the role of this enzyme and transcription factor in NO production.

Varying effects, depending on the inductor used (probiotic, LPS or LTA), were observed when specific p42/44 MAP-kinase or calcium/ calmodulin phosphatase inhibitors were added. Specific PKC and p38 MAP-kinase inhibitors had only a very slight effect to no effect at all.

These results suggest that the mechanism involved in the production of NO caused by *Lactobacillus GG* is very similar to the one used by LPS and LTA. Since the latter is a component of the lactobacillus cell wall, it may be responsible for the effect of *Lactobacillus GG* on NO production.

All the components having an inhibiting effect on the production of NO caused by *Lactobacillus GG* were evaluated for their effect on iNOS protein expression. Very good correlation was observed between

the effect of the inhibitors on both NO production and iNOS enzyme expression.

The results suggest a major role is played by tyrosine kinase and NF-κB, and a partial role by MAP-kinases in the transduction of the cell signal leading to iNOS induction and NO production. This study emphasises that the probiotic *Lactobacillus GG* is capable of stimulating the production of NO, a molecule with numerous effects on the immune and vascular systems via a mechanism that would appear similar to that of LPS. It can therefore also be expected that all lactobacilli containing LTA in their walls can also cause NO production.

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## Probiotics and modulation of

## allergy-type inflammatory responses

Recent results have shown that lactobacilli present in intestinal flora or introduced by fermented products may reduce the risk of allergic disorders (20). Allergic phenomena change the balance of the TH1/TH2 response in favour of a TH2 response, associated with the overproduction of specific interleukins (IL4, IL5, IL9 or IL13). Consequently, measuring the levels of cytokines produced during the consumption of fermented products could provide information on the ability of probiotics to modulate the TH2 response (21).

To do this, cultures of mononuclear cells (PBMC) taken from patients with dust mite allergies or non allergic patients exposed to the same allergen were created in the presence of various probiotics (*L. plantarum* NCIMB8826, *Lactococcus lactis* MG1363, *Lactobacillus casei* ATCC393, *Lactobacillus GG*).

The results obtained show that probiotics inhibit the secretion of IL4 and IL5. This dose-dependant inhibition does not depend on the probiotic used and is seen with live strains or heat-killed strains. It is to be noted that the reduction in cytokine production observed in PBMCs stimulated with an allergen is also observed in the PBMCs of allergic patients restimulated with the specific allergen. The presence of cells presenting the antigen is required to observe the described effect.

In parallel to this inhibition of the TH2 response, stimulation of the production of IL12 and IFN $\gamma$  (cytokines pro-TH1) by probiotics was also observed. Modulation of the TH1/TH2 response by probiotics would therefore appear to occur via a reduction in TH2 cytokines and an increase in the TH1 response.

This observation that probiotics have an effect on the cells of allergic patients

sensitised to an aeroallergen is reported here for the first time and also provides an action mechanism. Nevertheless, the results must now also be seen *in vivo* and it remains to be determined whether these bacteria, that in principle remain intraluminal, can send signals to the immune cells through the epithelium.

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## Probiotics and TH1 type responses :

## stimulation of cytokine production in mice

Numerous works have shown that probiotics are able to modulate the host's immune response. However, the mechanisms involved are still not entirely clear.

Of all the cells of the immune system, activated macrophages devour the micro-organisms, present antigens to T-helper cells and secrete pro-inflammatory cytokines. He et al (22) have studied the effect of probiotics on cytokine production and have shown that the nature of the cytokines produced by the macrophages depends on the strain used.

Eleven strains of heat-killed lactobacilli were incubated for 24 hours with murine macrophages (J774.1), and tested for their ability to cause a secretion of pro-(IL12) and anti-inflammatory (IL10)

cytokines (23). All the strains tested, including *L. casei Shirota* and *L. rhamnosus* used in the dairy industry, stimulated the secretion of IL6, IL12 and IFN $\gamma$ .

This result suggests that the immunomodulator effect of these two probiotics could be linked, at least partially, to the production of IL12, a pro-inflammatory cytokine favouring a TH1 type response. The probiotic *L. acidophilus 0356* also caused the secretion of IL10, a cytokine capable of inhibiting the effect of IL12. The balance between the pro-inflammatory (IL12) and the anti-inflammatory (IL10) cytokine plays an important role in the immuno-regulation of the host.

It would appear that lactobacilli are able to stimulate the macrophages so

they produce both pro- and anti-inflammatory cytokines. This type of heterogeneous regulation suggests that some bacteria present in food may be able to modulate the immune response by either stopping or intensifying inflammation depending on the particular case.

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This scientific letter "Yoghurts & fermented milks" is also available on the following websites :

[www.maison-du-lait.com](http://www.maison-du-lait.com) and [www.syndifrais.org](http://www.syndifrais.org)

## LAB-DOC bibliographic selection

The data base LAB-DOC organised by SYNDIFRAIS, brought together the bibliographic references of the international scientific publications accompanied by the authors' summaries.

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Your suggestions and comments will draw all our attention. Please send them to :

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