Yoghurts & fermented milks

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SCIENTIFIC SURVEY . LACTIC ACID BACTERIA . PROBIOTICS

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What can be said about the bioactive peptides produced when milk is fermented by probiotic lactic acid bacteria?

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Research carried out today into the beneficial action of probiotics provides a solid scientific basis for the development of functional fermented milks. But the data acquired still needs to be supported by a better understanding of the action mechanisms (*cf*. S. Salminen's editorial in *Yoghurts & fermented milks* N°25); mechanisms that take into account both the bacteria themselves and the products of their metabolism. Many studies have focused on the bacteria; however far fewer have looked into the potential action of products coming from the metabolism of the bacteria and in particular from protein degradation - peptides and free amino acids.

Thanks to their enzymatic baggage made up of proteases and peptidases, lactic acid bacteria release peptides during the fermentation of milk (Pritchard & Coolbear, 1993). The development of analysis methods such as mass spectrometry together with classic separation techniques are helping make clearer the complexity of the composition of these fermentation mediums to help characterize their component parts. Numerous peptides have been identified in this way in fermented milk. Recent work by Schieber & Brücker (2000) count no less than thirty peptides in yoghurt, which increases greatly when highly proteolytic lactobacilli such as *Lactobacillus helveticus* are added to these fermented milks (Smacchi & Gobbetti, 2000).

Among the peptides identified, the presence of numerous peptides or peptide precursors with biological activity have been noted in the milk protein sequence (Maubois et Léonil, 1989) and are released by enzymatic hydrolysis. The activities of these peptides that have been recognized to date are numerous and include anti-hypertensive, immunomodulatory, cholesterol-lowering, antimicrobial, opioid, antithrombotic and antioxidant activities. It should be noted that the peptides the most currently present in fermented milk have immunomodulating, opiatic, antihypertensive and cholesterol-lowering activity. Some of these activities are also those claimed by probiotics. The dividing line between activities linked intrinsically to bacteria and those linked to their metabolism is based on a (not always convincing) thesis on the effects observed between live and dead bacteria. Recent literature has shown that there are still difficulties to explain these mechanisms fully. Bacterial metabolism leads to fermentation products that may have a direct effect on certain functions associated with the digestive tract such as immunity or an indirect effect via modifications to the intestinal flora. The complexity of the system means that it is difficult to know whether a probiotic effect is only due to the bacterium itself, to a synergy between several probiotics or to the bacterium and its fermentation products. To this complexity can also be added the strain-dependant and multi-factor character of the probiotic effects. For these, we now have a major asset to help understand these mechanisms – almost twenty sequenced genomes (Saxelin *et al.*, 2005).

While focusing on bioactive peptides, we should not forget other questions:

- Since there is little proteolysis in yoghurt, are there enough released peptides to provoke the effects in question? How does bacterial lysis act to modulate the degradation of proteins *in situ* when probiotics are added?
- How can the action associated with the degradation of milk proteins by digestive enzymes in the tract during the ingestion of fermented milks be integrated? Research targeting only bacteria must consider the matrix in which they are transported.
- What is the future of these peptides in the digestive tract? Are they protected by the complexity and the interactions within the food matrix?

Although the discussion here is focused on peptides, the same is true for other products coming from bacterial metabolism such as fatty acids. So no hasty conclusions are drawn, caution should encourage us not to forget the complexity of the mechanisms involved.

Pritchard GG, Coolbear T (1993). The physiology and biochemistry of the proteolytic system in lactic acid bacteria. Microbiol Rev. 12(1-3):179-206.
Maubois JL, Léonil J (1989). Peptides du lait à activité biologique. Lait 69, 245-269.
Saxelin M, Tynkkynen S, Mattila-Sandholm T, de Vos WM (2005). Probiotic and other functional microbes: from markets to mechanisms. Curr Opin Biotechnol 16, 204-211.
Schieber A, Brückner H (2000). Characterization of oligo- and polypeptides isolated from yoghurt. Eur Food Technol 210, 310-313.
Smacchi E, Gobbetti M (2000). Bioactive peptides in dairy products: synthesis and interaction with proteolytic enzymes. Food Microbiol 17, 129-141.

Probiotics strengthen the immune system barrier in mice

The interaction of probiotics with the host is complex and the consequences vary according to the physiological state of the host. Where there is inflammation, some probiotics tend to attenuate the inflammation whereas, in healthy individuals, other probiotics may stimulate the immune response (1). A study in mice has tested this hypothesis and offers a new argument on how to interpret the mechanisms involved (2).

The authors used a model of colitis induced by *Helicobacter hepaticus* in IL-10-deficient mice (mouse model predisposed to colitis). The impact of two probiotic species was studied: *Bifidobacterium breve* and *Streptococcus thermophilus*.

48 animals were divided into 4 groups and treated according to the protocol described in the table below.

Cytological observations of the colon and the measurement of the spontaneous production of pro-inflammatory cytokines showed that, compared to the control group, all the groups infected with *H. hepaticus* were suffering from moderate inflammation. When compared to the *H. hepaticus* group, the probiotics were not beneficial to the histological appearance of the colon.

Compared to all the other groups, the non-specific stimulation of the immune cells of the MC group with *Escherichia coli* resulted in a significantly greater production of pro-inflammatory cytokines (IFNγ, TNFα, IL12).

The number of CD4+ et CD8+ T cells secreting IFNγ was significantly greater in the MC group to that observed in the control and *H. hepaticus* groups. For the LAB group, only the number of CD4+ cells was greater than that of the control group.

Group name	***Infected with H. hepaticus?***	***Product administered orally for 10 weeks***
Control	No	Placebo
H. hepaticus	Yes	Placebo
MC	Yes	An equivalent-volume mixture of the supernatant fluids of the two probiotics. This mixture has no bacteria but contains the bacterial secretion products corresponding to 5x10^5 CFU.
LAB	Yes	Bacterial suspension of the two live bacteria (50% of each); dose of 5x10^5 CFU administered to each animal at each feeding time.

In the colon, transcellular permeability to macromolecules and paracellular diffusion of small molecules were reduced in the MC group compared to the control and *H. hepaticus* groups.

In a context of moderate inflammation, administering the supernatant fluid of the two probiotics stimulated a Th1 type immune response and strengthened the intestinal barrier. Consuming the probiotics themselves had a more moderate effect, affecting only the immune response. It should, however, be noted that the dose of *B. breve* and *S. thermophilus* administered (5x10^5 CFU) was much smaller than the doses generally considered as effective.

In mice suffering from moderate inflammation, a mixture of the supernatant fluids of *B. breve* and *S. thermophilus* strengthened the immune response and the intestinal barrier. This impact on immunity would have been caused by metabolites produced by the probiotic in the culture medium.

1• **Heyman M, Terpend K, Menard S (2005).** Effects of specific lactic acid bacteria on the intestinal permeability to macromolecules and the inflammatory condition. Acta Paediatr Suppl. 94(449):34-6.
2• **Menard S, Laharie D, Asensio C, Vidal-Martinez T, Candalh C, Rullier A, Zerbib F, Megraud F, Matysiak-Budnik T, Heyman M (2005).** Bifidobacterium breve and Streptococcus thermophilus secretion products enhance T helper 1 immune response and intestinal barrier in mice. Exp Biol Med (Maywood). 230(10):749-56.

Can a fermented, heat-treated milk improve the symptoms of allergies in adults?

In terms of preventing allergies, several studies show the beneficial effects of probiotics in children, in particular those resulting from *Lactobacillus rhamnosus* GG on atopic eczema (3). In adults, using probiotics to treat allergies is an area of research that is still wide open.

A clinical trial was conducted in 2002 and 2003, during the Spring which is the peak period for cedar pollen in Japan (4). All the subjects enrolled were specifically allergic to this pollen. In the first year, 23 patients were enrolled; for 6 weeks each one received randomly and double blind either a placebo, or a heat-treated, fermented milk (fermented with *Lactobacillus acidophilus* L-92). In the second year, the group studied (with different subjects to the first year) included 20 patients treated according to the same protocol but for a 10-week period. The parameters analyzed during the study were:

- the quantity of allergens in the atmosphere
- the subjective "symptom - medication" score*, reflecting the patient's opinion
- the allergic symptoms score evaluated by a doctor
- the concentration of specific IgE in the blood and the Th1/Th2 ratio.

For each of the two periods, only one of the parameters, that giving the subjective score, showed a significant improvement of the severity of the allergy during the intake of the heat-treated, fermented milk. For the first period, it was the score for the symptoms reported for the eyes. For the second period, the authors evoked a reduction in the *"distress in the life of the subjects"*, a subjective concept that was not defined by the authors and for which the experimental assessment was not described either. It therefore appears difficult to take this second period into account when interpreting the results. None of the other parameters measured in the tested subjects showed any significant differences from the placebo group.

With no clear conclusions, this clinical study shows that it is difficult to define subjective scores and to analyze them, even more so since they are not correlated with objective parameters. As the authors themselves suggest, such a study would have been improved by involving more subjects. Furthermore, a comparison of the effects of a fermented milk and the same heat-treated fermented milk would be relevant, given the high stakes regarding the viability of the probiotics present in the products.

* This score, defined by the Japanese allergology society, includes a nasal and ocular evaluation score for each patient and a score representing the type of medication (antihistamine or steroid) taken to relieve the symptoms.

3• **Salminen SJ, Gueimonde M, Isolauri E (2005).** Probiotics that modify disease risk. J Nutr. 135(5):1294-8. Review.
4• **Ishida Y, Nakamura F, Kanzato H, Sawada D, Yamamoto N, Kagata H, Oh-Ida M, Takeuchi H, Fujiwara S (2005).** Effect of milk fermented with Lactobacillus acidophilus strain L-92 on symptoms of Japanese cedar pollen allergy: a randomized placebo-controlled trial. Biosci Biotechnol Biochem. 69(9):1652-60.

Probiotics improve the symptoms related to irritable bowel disease

Irritable bowel syndrome is a digestive disorder with no morphological or histological lesions. Its etiology is still unexplained and treatment is limited. With no reliable cure available, consumption of probiotics gives hope of being able to improve patient comfort by relieving symptoms (5). Two recent studies, carried out randomized, double blind and placebo controlled, present results supporting this hope.

The first study included 48 subjects suffering from irritable bowel disease (6). The clinical protocol included a two-week observation period then a 4-week period when the tested product was administered. The product was a mixture of the VSL#3 strains ($9x10^{11}$ CFU per day), consumed mixed with yoghurt. Throughout the study, including during the observation period, the patients filled out a daily questionnaire giving details of the abdominal symptoms and characteristics of the feces. At the end of each week of treatment, the subjects noted whether or not they had felt a lessening of the feeling of distention; a subject was considered to be responding to treatment if she/he felt an improvement for at least half the treatment period. Finally scintigraphy was used to take a colic transit measurement before and after the administration period.

No side effects were noted relating to the consumption of probiotics. After 4 weeks of treatment, the flatulence score was significantly reduced ($p=0.01$) for those taking probiotics as compared to the placebo. Despite a tendency towards reduced flatulence, the other symptoms studied were not significantly modified. The feeling of distention was reduced in 46% of the subjects receiving the probiotics as opposed to 33% of the control subjects (a reduction that was not statistically significant). Intestinal transit was significantly delayed by consuming VSL#3. No links between this effect and a reduction in flatulence were highlighted.

Administering probiotics mixed with yoghurt raises questions about the independence of the tested product's effect, compared to the bacteria contained in the yoghurt. The authors, aware of this possible bias, explain that giving the probiotic in this way makes it more palatable. They argue that some results show that consuming yoghurt together with VSL#3 hardly changes the quantity of bacteria found in the feces. However, the doubt remains - without calling into question the beneficial effects of the treatment, it causes incertitude as to the strains and doses that are really effective.

The second study included 86 subjects suffering from irritable bowel disease (7). In capsule form, these patients received daily and for 6 months either a placebo or a probiotic mix (*Lactobacillus rhamnosus* GG, *Lactobacillus rhamnosus* LC705, *Bifidobacterium breve* Bb99, *Propionibacterium freudenreichii shermanii* JS; total of 8 to $9 x10^9$ CFU/day with identical quantities for each strain).

The symptoms were evaluated from a questionnaire filled out monthly by the patients. The primary symptoms included abdominal pain, distension, flatulence and borborygmus; secondary symptoms were also studied as well as the frequency and form of the feces and the use of medication for this disorder. Quality of life and diet were reported at the start, middle and end of the study.

Between the start and the end of the study, the mean score for primary symptoms fell by 42% in the group receiving the probiotics as opposed to 6% in the control group ($p=0.015$ for the variation). Taken individually, each of the scores for the primary symptoms was lower in the group receiving the probiotics (after adjustment of the initial value) compared to the control group, but the difference was not statistically significant except for borborygmus. Two of the secondary symptoms concerning defecation, were significantly improved by probiotics compared to the placebo.

In this medium term study, consumption of a probiotic mix significantly reduced all the symptoms felt by patients suffering from irritable bowel syndrome. Associated with an absence of side effects, these results mean that use of this probiotic mix can be envisaged to improve the comfort of patients suffering from irritable bowel syndrome.

5• **Verdu EF, Collins SM (2005).** Irritable bowel syndrome and probiotics: from rationale to clinical use. Curr Opin Gastroenterol.;21(6):697-701.
6• **Kim HJ, Vazquez Roque MI, Camilleri M, Stephens D, Burton DD, Baxter K, Thomforde G, Zinsmeister AR (2005).** A randomized controlled trial of a probiotic combination VSL# 3 and placebo in irritable bowel syndrome with bloating. Neurogastroenterol Motil. ;17(5):687-96.
7• **Kajander K, Hatakka K, Poussa T, Farkkila M, Korpela R (2005).** A probiotic mixture alleviates symptoms in irritable bowel syndrome patients: a controlled 6-month intervention. Aliment Pharmacol Ther. 22(5):387-94.

Lactobacillus GG allegedly does not strengthen the intestinal barrier

A particular target was chosen - children at risk from tropical enteropathy. This asymptomatic pathology is common among children from poorer families in developing countries. It consists of atrophy of the small intestine and could be linked to colonization by pathogens that damage intestinal integrity *via* an immune system process. The probiotic *Lactobacillus* GG has already shown it can be effective in counter-balancing colonization by pathogens (8) and to strengthen the intestinal barrier in children (9). It was selected to attempt to restore intestinal integrity in children at risk from tropical enteropathy.

161 children, aged between 3 and 5 years old, received randomly and double blind either a placebo, or *Lactobacillus* GG ($1x10^{11}$ CFU/day) for 30 days (10). Each product was mixed into the child's diet. Intestinal integrity was evaluated at the start and end of the study in a protocol that included the consumption of sugars not metabolized by the body (lactulose, mannitol and sucrose). The ratios in which these sugars were excreted in the urine was used to measure the integrity of the intestinal barrier.

Values for the lactulose / mannitol ratio upon enrolment showed that 73% of the children were suffering from tropical enteropathy. The probiotic caused no side effects whatsoever. In comparison with the placebo, consumption of *Lactobacillus* GG did not modify the excretion of the sugars used as markers.

In this study of children, of whom 73% were suffering from tropical enteropathy, consumption of *Lactobacillus* GG did not improve the integrity of the intestinal barrier. However, before concluding that there is absolutely no effect, the authors propose using other ways of measuring intestinal permeability. Other probiotics could also be tested.

8• **Vanderhoof JA, Young RJ, Murray N, Kaufman SS (1998).** Treatment strategies for small bowel bacterial overgrowth in short bowel syndrome. J Pediatr Gastroenterol Nutr. 27(2):155-60.
9• **Kalliomaki M, Salminen S, Arvilommi H, Kero P, Koskinen P, Isolauri E. Related (2001).** Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. Lancet. 357(9262):1076-9.
10• **Galpin L, Manary MJ, Fleming K, Ou CN, Ashorn P, Shulman RJ (2005).** Effect of Lactobacillus GG on intestinal integrity in Malawian children at risk of tropical enteropathy. Am J Clin Nutr. 82(5):1040-5.

Resolving diarrhoea in neonates - testing the theory of an association between probiotics and zinc

In children, it has already been shown that the symptoms of diarrhoea can be improved by consuming probiotics (11). Zinc is another nutritional method of relieving the symptoms of children suffering from diarrhoea. A recent study has evaluated the impact of combining probiotics and zinc on diarrhoea in non-hospitalized children (12).

In this study, carried out double-blind, 65 children seeing the doctor for severe diarrhoea were divided randomly between two groups receiving standard infant formula or a formula containing a probiotic supplement until the diarrhoea had cleared up. The supplement consisted of a probiotic mix (*Streptococcus thermophilus*, *Bifidobacterium lactis*, *Lactobacillus acidophilus*; $2x10^9$ CFU of each strain being given daily), 0.3 g/day of fructo-oligosaccharides (FOS) and 10 mg/day of zinc. The subjects were examined daily to see the degree of dehydration, the presence of fever or vomiting and the frequency and consistency of the feces.

In the children receiving the supplement, the diarrhoea cleared after 1.43 days as opposed to 1.96 days for the control group (p=0.017). In the sub-group of subjects with vomiting, the vomiting tended to clear more quickly in the group receiving the supplement, but the difference was not significant (0.27 days as against 0.81 days, p=0.06).

In this study, the joint administration of a probiotic mix, FOS and zinc shortened the duration of diarrhoea in infants of less than one year of age. At this stage, the beneficial effect cannot be attributed to one or other of the components of the nutritional supplement. The study is however interesting from a therapeutic viewpoint since it raises the possibility of using this type of supplement effectively in young children.

11• **Szajewska H, Mrukowicz JZ (2001).** Probiotics in the treatment and prevention of acute infectious diarrhea in infants and children: a systematic review of published randomized, double-blind, placebo-controlled trials. J Pediatr Gastroenterol Nutr. 33 Suppl 2:S17-25.
12• **Shamir R, Makhoul IR, Etzioni A, Shehadeh N (2005).** Evaluation of a diet containing probiotics and zinc for the treatment of mild diarrheal illness in children younger than one year of age. J Am Coll Nutr. 24(5):370-5.

A lactobacillus against articular pain

Irritable bowel syndrome is often accompanied by arthralgia, expressed as pain in the joints with no apparent lesions. In patients suffering from Crohn's disease and ulcerous colitis and also suffering from arthralgia, consumption of the VSL#3 probiotic mix for three months in an open study with no control group reduced the articular pain experienced. This pilot study brings with it the hope that the probiotic can improve the lives of these patients. However, the result still needs to be confirmed by randomized, double bind studies.

13• **Karimi O, Pena AS, van Bodegraven AA (2005).** Probiotics (VSL#3) in arthralgia in patients with ulcerative colitis and Crohn's disease: a pilot study. Drugs Today (Barc). 41(7):453-9.

Can probiotics be used in major bruns' cases?

Some probiotic strains have shown a capacity to strengthen the intestinal barrier in weakened individuals; in this vein, the possible effect of probiotics on skin burn cases has been evaluated in rats. In animals with 30% severe burns, consuming a mixture of probiotics reduced bacterial translocation and reduced intestinal atrophy (histological score on a numbered scale) in the 24 hours following the burns. This result highlights a new potential therapeutic application for probiotic bacteria - treating major burns' cases.

14• **Gun F, Salman T, Gurler N, Olgac V (2005).** Effect of probiotic supplementation on bacterial translocation in thermal injury. Surg Today. 35(9):760-4.

Report on the activity of lactobacillus and bifidobacteria on pathogenic micro-organisms

A review has reported on current knowledge as regards the anti-microbial activity of lactobacillus and bifidobacteria. The author summarizes results obtained *in vitro* and *in vivo* in animals and humans. Anti-microbial activity has been highlighted fighting the majority of pathogens that are clinically important for man. The different mechanisms involved are also reviewed. Finally, the author reminds us that the effects of probiotics on pathogenic micro-organisms are specific to each strain and that their action mechanism remains largely unexplored.

15• **Servin AL (2004).** Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. FEMS Microbiol Rev. 28(4):405-40.

A probiotic has a cariogenic effect on rats

That probiotics are harmless for humans is generally well-accepted and confirmed by the absence of side effects. However, a study on rats has shown that the *Lactobacillus salivarius* LS 1952R strain may well be propitious to tooth decay. This conclusion suggests the need to study any appearance of this side effect in humans more carefully and to promote caution when consuming this probiotic.

16• **Matsumoto M, Tsuji M, Sasaki H, Fujita K, Nomura R, Nakano K, Shintani S, Ooshima T (2005).** Cariogenicity of the probiotic bacterium Lactobacillus salivarius in rats. Caries Res. 39(6):479-83.

A fermented milk modulates the immunity of the cancerous mouse

Probiotics are seen as agents capable of modulating the immune response. Some probiotics could in particular exert an action on cancer. A study of mice has shown that the oral consumption of milk fermented with *Lactobacillus helveticus* R389 regulates the immune response in the mammary glands of animals with mammary tumors (increase in IgA and CD4+ cells). However, additional studies will need to investigate whether consumption of fermented milk can reduce the size of the tumor.

17• **de Moreno de Leblanc A, Matar C, Thériault C, Perdigon G (2005).** Effects of milk fermented by Latobacillus helveticus R389 on immune cells associated to mammary glands in normal and a breast cancer model. Immunobiology. 210:349-358.

18• Collado MC, Gueimonde M, Hernandez M, Sanz Y, Salminen S (2005).
Adhesion of selected Bifidobacterium strains to human intestinal mucus and the role of adhesion in enteropathogen exclusion. J Food Prot. 68(12):2672-8.

19• Picard C, Fioramonti J, Francois A, Robinson T, Neant F, Matuchansky C (2005).
Review article: bifidobacteria as probiotic agents - physiological effects and clinical benefits. Aliment Pharmacol Ther. 22(6):495-512.

20• Henriksson A, Borody T, Clancy R (2005).
Probiotics under the regulatory microscope. Expert Opin Drug Saf. 4(6):1135-43.

21• Bjorksten B (2005).
Evidence of probiotics in prevention of allergy and asthma. Curr Drug Targets Inflamm Allergy. 4(5): 599-604.

22• Prakash S, Bhathena J (2005).
Live bacterial cells as orally delivered therapeutics. Expert Opin Biol Ther. 5(10):1281-301.

23• Snelling AM (2005).
Effects of probiotics on the gastrointestinal tract. Curr Opin Infect Dis. 18(5):420-6.

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