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Session 7: Prebiotics and probiotics usefulness against pathologies Immune-stimulating effects of lactic acid bacteria *in vivo* and *in vitro*

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The health-promoting effects of lactic acid bacteria (LAB) are well recognised, making them a popular functional food ingredient. Commercially available probiotic products are often promoted as capable of improving immune defences also in healthy subjects. However, while strain-specific differences exist in the effects of LAB, conventional yoghurt bacteria have proved beneficial as well. For comparing the immunological effects of conventional and probiotic LAB, young healthy women received either a commercially available probiotic fermented milk product or a conventional yoghurt for four weeks. Both treatments showed comparable effects resulting in a stronger immunological reaction to stimuli (natural cytotoxicity against cancer cells, mitogen-induced T-lymphocyte activation and stimulated cytokine production). To study the mechanisms behind these effects, conventional (*Lactobacillus delbrueckii*) and probiotic (*Lactobacillus rhamnosus* GG), LAB were compared *in vitro* at the cellular level. Interestingly, *L. rhamnosus* GG was more potent in inducing maturation of dendritic cells (DC) that play a substantial role in directing the immune response to stimuli. In turn, *L. delbrueckii* provoked a higher secretion of proinflammatory cytokines as well as IL-10. These effects were, however, observed only after direct incubation of DC and LAB, not when both were separated by a layer of enterocyte-like Caco-2 cells. LAB also induced cytokine secretion in peripheral blood mononuclear cells in a similar manner and this effect was reduced in a Caco-2 cell model, suggesting a modulating influence of gut epithelial cells. While both conventional and probiotic strains modulate the immune response, specific properties may offer therapeutic options in the treatment of certain diseases.

Lactic acid bacteria: Prebiotics: Probiotics

With increasing affluence and food abundance, there has been a rising interest in functional food components, offering benefits beyond mere nutrition by beneficially influencing the health of those who ingest them. Among these, lactic acid bacteria (LAB), especially in the form of probiotics, have long been known to enhance the body's ability to ward off infections especially of the gastrointestinal tract. Administration of certain LAB to infants was repeatedly shown to reduce the severity and duration of viral diarrhoea and even to prevent it[1,2]. In this regard, LAB are also relevant for the nutrition in developing countries, where diarrhoea and related diseases are common and contribute particularly to child mortality. However,

there is also evidence for an improved defence against other infections such as influenza and the common cold even though no prevention was achieved[3] LAB have also been suggested as a treatment for atopic diseases, inflammatory bowel diseases and the irritable bowel syndrome and showed beneficial effects in some studies in these conditions[4–7].

The mechanisms behind the immune-modulating effects of LAB are still partly unknown. Peripheral blood mononuclear cells (PBMC) incubated *in vitro* with LAB showed a higher secretion of cytokines, proteins or peptides acting as mediators and regulators of the immune response[8], and the type of cytokines produced was strain dependent.

A higher cytotoxic activity of natural killer cells, involved in the defence against viruses and tumour cells, was shown as well as an increase in phagocytic activity[9].

Direct co-culture of peripheral immune cells with LAB does not reflect the physiological situation especially in healthy subjects. However, PBMC and natural killer cells isolated from the blood of subjects following consumption of LAB also showed increased phagocytic and cytotoxic activity, respectively, after appropriate immunological stimuli[10,11]. This is in accordance with the observed better resistance against various infections.

These immune-modulating properties of LAB have been used as a health claim on foods containing such organisms, particularly probiotic ones. While LAB strains may differ in their suitability for the treatment of some diseases, healthy individuals may benefit just as much from conventional fermented milk as suggested by the long tradition of such foods as health promoting. Indeed, the earliest evidence of the protective effect of LAB came from observations in regular consumers of fermented milk such as yoghurt or kefyr. Furthermore, traditional yoghurt starter cultures are considered probiotic by some experts[7,12].

This led our working group to study the immunological effects of conventional and probiotic LAB strains in more detail. In a first approach, the immunological effects of a probiotic fermented milk product were compared with those of a conventional product *in vivo* in healthy subjects. The mechanisms behind the observed effects were further investigated in an *in vitro* study using cell culture. An overview of both studies is given in the following.

In vivo effects of daily ingestion of fermented milk by young healthy women

Study design, materials and methods

The aim of this study was to compare the immunological effects of a commercially available probiotic fermented milk product with those of a conventional fermented milk. As the main target group for over-the-counter probiotic products is healthy persons, these were chosen as the study population. The study was approved by the Ethical Committee of the City of Vienna (protocol number EK 04-0060204) and the participants provided written informed consent. Results of the study have been published[13,14].

Thirty-three non-smoking young women (aged 22–29 years) were randomly divided into two groups: one received the probiotic product and the other the conventional product. Both products contained the classic yoghurt starter cultures *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* albeit at different concentrations, with additional *Lactobacillus casei* DN114001 in the probiotic product. The bacterial contents were determined as follows: in the conventional product, $6·4 \times 10^7$ colony forming units (CFU) *L. delbrueckii* ssp. *bulgaricus* per ml and $3·9 \times 10^7$ CFU *S. thermophilus* per ml; in the probiotic product, $2·0 \times 10^8$ *L. delbrueckii* ssp. *bulgaricus* per ml, 10^7 CFU *S. thermophilus* per ml and $3·7 \times 10^8$ CFU probiotic *L. casei* DN114001 per ml. Following a one-week equilibrating period during which fermented foods were avoided, participants consumed daily one portion (100 g)

of the respective product for two weeks. The amount was doubled to 200 g/d for another two weeks. A wash-out period with no fermented products lasting two weeks concluded the intervention. Blood samples were taken at the end of each period. Whole blood aliquots were incubated with pokeweed mitogen (40 µg/ml), a stimulant of T-lymphocytes, for 4 h at 37°C and 5 % CO_2. The percentage of helper T cells expressing the activation marker CD69 on their surface was measured by flow cytometry (FACSCalibur dual laser system (Becton Dickinson Biosciences, San Jose, CA, USA)). To assess natural cytotoxicity, PBMC were isolated by gradient centrifugation using Ficoll Paque Plus (Amersham Biosciences AB, Uppsala, Sweden) and co-cultured with myelogenic leukaemic K562 cells as targets in ratios of 25:1, 50:1 and 100:1 (effector to target) for 4 h at 37°C and 5 % CO_2. 7-Aminoactinomycin D (7-AAD, Cell Technology Inc.) was used to stain the lysed target cells and determine their percentage by flow cytometry.

To induce cytokine production, whole blood was incubated separately with phytohaemagglutinin from *Phaseolus vulgaris* (T-lymphocyte stimulant) (5 µg/ml) and lipopolysaccharide from *Escherichia coli* O26:H6 (macrophage and B lymphocyte stimulant; 1 µg/ml) for 24 h at 37°C and 5 % CO_2. Interferon-γ, IL-6 and IL-10 concentrations were measured using the cytometric bead array and flow cytometry, whereas IL-1β and TNFα concentrations were measured with ELISA (both kits from BD Pharmingen, Becton Dickinson Biosciences, San Diego, CA, USA).

Results

After the consumption of both products, the percentage of CD69^{+} helper T cells increased, but this was statistically significant only in the conventional group. After intake cessation, levels decreased again. Likewise, natural cytotoxicity was higher following yoghurt consumption with significant differences in both groups. After the wash-out period, activity rose even further, particularly in the probiotic group, suggesting a longer-lasting effect in this group[13]. Stimulated production of the proinflammatory cytokines interferon-γ, IL-1β and TNFα increased following yoghurt consumption with effects seen in both groups, albeit to varying degrees. Generally, increases were stronger in the conventional group. In contrast, no changes were observed for IL-6, while secretion of IL-10 was slightly but significantly reduced after probiotic yoghurt intake and increased markedly after the wash-out compared with the baseline levels[14]. An overview of the results is given in Table 1. No cytokine production was found in unstimulated blood samples and no marked differences in cell numbers occurred, suggesting that LAB improve the body's reaction to pathogens without modifying the basal cell numbers (in blood) and functions.

In vitro stimulation of human dendritic cells with lactic acid bacteria

To further investigate the mechanisms behind the earlier-described immune stimulation obtained with probiotic and

Table 1. Effects of daily intake of fermented probiotic and conventional yoghurt on markers of immune function in young healthy women

	Baseline		100 g/d		200 g/d		Washout	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Natural cytotoxicity (E:T, 100:1; % lysed target cells)								
Conventional	9	10	24[a]	14	17	20	25[a]	18
Probiotic	19	17	25	18	24	25	35[a,c]	21
Activated (CD69^{+}) CD8^{+} T-lymphocytes (%)								
Conventional	19	6	24[a]	10	24[a,b]	8	17[b]	3
Probiotic	20	10	24	13	23	9	19	9
Activated (CD69^{+}) CD4^{+} T lymphocytes (%)								
Conventional	18	8	18	10	18	9	17	6
Probiotic	17	4	18[a]	6	19[a]	6	16[a]	4
IFN-γ (pg/ml)								
Conventional	418	505	863	749	869	1007	800	781
Probiotic	601	554	1078[a]	1042	1075	999	988[a]	899
IL-1β (pg/ml)								
Conventional	1262	338	1594	524	1761[a]	629	1400	453
Probiotic	1392	283	1594	689	1648	536	1380	528
TNFα (pg/ml)								
Conventional	550	206	698[a]	124	896[a,b]	241	936	773
Probiotic	673	155	706	143	838[a,b]	201	1017[a]	691
IL-6 (pg/ml)								
Conventional	2980	1188	2710	998	2889	1056	3173	1174
Probiotic	3173	939	3101	1161	2931	986	3525	816
IL-10 (pg/ml)								
Conventional	71	50	73	54	81	61	99	76
Probiotic	68	37	50[a]	36	55	27	127[a,b,c]	72

E:T, effector to target ratio; IFN, interferon

Each intervention phase as well as the wash-out period lasted for 2 weeks. Cytokine secretion and expression of CD69 were measured following stimulation with LPS/PHA and pokeweed mitogen, respectively. Data were analysed for statistical differences using repeated measures ANOVA with *post hoc* contrast testing. Values are rounded.

[a] Significantly different from baseline.
[b] Significantly different from 100 g/d.
[c] Significantly different from 200 g/d.
There were no significant differences between the conventional and the probiotic group.
For the conventional group, *n* 16, and for the probiotic group, *n* 17.

conventional LAB *in vivo*, their effects on dentritic cells (DC) were studied at the cellular level. The human epithelial colorectal adenocarcinoma cell line Caco-2 differentiates to an enterocytic phenotype and, hence, has been repeatedly used as a model for the physiological conditions in the gastrointestinal tract.

Study design, materials and methods

The probiotic LAB strain used was *Lactobacillus rhamnosus* GG (ATCC 53103). *L. delbrueckii* ssp. *bulgaricus* (ATCC 11842) was chosen as the conventional strain (LGC Promochem, Wesel, Germany). Caco-2 cells were purchased from DSMZ (Braunschweig, Germany). PBMC were isolated from the blood of four volunteers by gradient centrifugation (Ficoll Paque Plus, GE Healthcare, Vienna, Austria) and monocytes were gained by negative isolation through a magnetic bead system (Dynabeads, MyPure Monocyte Kit 2, Invitrogen, Lofer, Austria). Monocytes were then cultured with granulocyte-macrophage colony-stimulating factor and IL-4 (each at 100 ng/ml) for 7 days to differentiate to immature DC. These cells were incubated separately with the two LAB strains at 1:10 (DC:CFU LAB) for 24 h either directly or separated by a Caco-2 cell monolayer. Analysis of the cytokines interferon-γ, TNFα, IL-1β,

Table 2. Change (%) in expression of CD markers on dendritic cells following direct incubation with lipopolysaccharide (LPS; positive control, 1 µg/ml) or Lactobacilli (1:10) for 24 h compared with the negative control (De Man Rogosa Sharpe broth (MRS) medium only)

CD marker	LPS	*L. delbrueckii*	*L. rhamnosus* GG
CD80	382	141	356[a,b]
CD83	248	97	159
CD86	1195	485[a]	730[c]
CD209	57	26	38
CD54	255	141	190[d]
HLA-DR	199	136	156

Data were analysed for statistical differences using one-way ANOVA and *post hoc* Bonferroni's multiple comparison testing.
[a] $P < 0.01$ compared with the negative control.
[b] $P < 0.05$ compared with *L. delbrueckii*.
[c] $P < 0.001$ compared with the negative control.
[d] $P < 0.05$ compared with the negative control.

IL-10 and IL-12 in the supernatants of DC incubated with LAB was done with a Cytometric Bead Array (Human Soluble Protein Flex Set for CBA, Becton Dickinson Biosciences, San Diego, CA, USA). Cell surface markers on DC were analysed by flow cytometry (FACSCalibur dual laser system).

Results

Expression of the co-stimulatory molecules CD80, CD86 and CD54 as well as the maturation marker CD83 was induced by direct incubation with LAB. However, except for CD86, effects were weaker or absent with *L. delbrueckii* and not statistically significant. Expression of HLA-DR, a molecule of MHC-II involved in antigen presentation, showed a tendency to increase but without reaching statistical significance. On the contrary, CD209 (DC-SIGN), involved in T cell activation and ingestion of antigens by DC, was significantly down-regulated following incubation with both LAB strains with no significant differences between them (see Table 2). The observed modifications are typical for DC maturation. Secretion of IL-1β, IL-10, IL-12 and TNFα increased significantly after direct incubation of DC with *L. delbrueckii*, while a weaker non-significant effect or, in the case of IL-12, no effect could be seen for *L. rhamnosus*. In turn, interferon-γ was not produced (data not shown). When DC were incubated with LAB separated by a Caco-2 monolayer, no effects on cell surface markers or cytokine production were observed (P Klein and I Elmadfa, unpublished results).

Conclusion

The immune-stimulatory effects of LAB are well recognised. While there is much evidence for the influence of various probiotic strains, conventional cultures are also beneficial to health as suggested by the findings described above. Indeed, while the studied strains differed slightly in their effects, all were able to activate immunological functions. Moreover, for most of these latter, the *in vivo* approaches showed no significant differences between the probiotic and the conventional product. Importantly, the response to exogenous stimuli was increased without major changes in the cell numbers.

Under healthy conditions, the gut mucosa presents a natural barrier to ingested exogenous micro-organisms and antigens[15]. Nevertheless, as also suggested by the *in vivo* findings described above, these latter can influence the systemic immune system although direct contact between LAB and the host's cells is normally limited to the gastro-intestinal tract. Cytokines play a central role in cell signalling, transmission of stimuli and directing the immune response. Indeed, the cytokine pattern depends not only upon the producing cell, but also on the stimulus. Thus, the different types of helper T cells that are essential in specific immune defence only differ by their cytokine pattern with helper T cells of type 1 stimulating a cellular proinflammatory response, and T helper type 2 cells a humoral antibody-mediated one[16]. It was shown that LAB have an influence on which cytokines are secreted depending on the particular strain[17]. A number of studies have revealed a role for DC, which are one of the most important antigen-presenting cell populations. Indeed, gastrointestinal DC are able to cross the epithelium and get into direct contact with bacteria in the intestinal lumen[18]. Presentation of bacterial antigens by DC activates T lymphocytes. Furthermore, co-stimulatory molecules on the surface of DC as well as the cytokines they secrete influence their differentiation.

In turn, LAB were shown to have effects on the cytokine pattern and the expression of surface markers by DC[19,20]. Besides, intestinal epithelial cells have an influence on the response to ingested micro-organisms as well through the secretion of cytokines and the expression of co-stimulatory surface molecules. Notably, secretion of proinflammatory cytokines was down-regulated[21,22].

In our *in vitro* study, both, probiotic and conventional LAB, showed effects at the cellular level, albeit in different ways. This is in accordance with the described strain-specific activity of these micro-organisms. Interestingly, in both approaches, *L. delbrueckii* was a more potent inducer of proinflammatory cytokines IL-1β and TNFα. This is possibly reflected in the slightly higher *in vivo* production in the conventional group although a direct comparison is impossible due to the use of different probiotic strains.

The various different effects of single LAB strains on the immune response appear particularly promising with regard to the treatment of some diseases like inflammatory bowel disease or atopic disturbances, but there is still a need for further research.

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