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# Effect of selected probiotics on non-specific cellular and humoral defense mechanisms and protection against salmonellosis – experimental study in broiler chicken

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The stimulation of the nonspecific cellular and humoral defence mechanisms is important for the protection of chickens against pathogens in the gastrointestinal tract. The aim of our study was determine the influence of five strains of probiotics on the nonspecific cellular and humoral defence mechanisms and protection against Salmonella enteritidis after experimental challenge. 1-day-old male broiler chicken of Ross 308 type were used in our experimental study. 230 chicken were rondomly distributed into 6 groups and allocated into two parallel boxes (20 or 15 birds each). The probiotic strains used included Lactobacillus salivarius AWH, L. acidophilus BS, L. helveticus b9, Bifidobacterium longum KNA1 and B. animalis 30. All the strains were deposited in the Culture Collection of the Department of Food Microbiology of IAR&FR PAS in Olsztyn, Poland. They were previously characterised due to their classification, biochemical features, survival in low pH and bile salts, adhesion to the intestinal epithelium, and antagonistic activity against food-borne pathogens. Chickens were receiving active cells of probiotic cultures $2x10^9$ to $2x10^{10}$ cells per bird per day on average, in gradually increasing concentrations, twice a day with 12-hour intervals, in drinking water. On the day 29, ten birds from each group were transferred to the separate boxes and 1 ml of Salmonella enteritidis culture was delivered into the crop each chicken three times (on day 33, 35, and 37) at doses $4.6x10^6$ , $5.7x10^7$ , $5.7x10^8$ cfu per ml, respectively. On the 42nd day of the experimental study, 10 chickens from each group were sacrificed and blood samples were collected for immunological assays. The phagocytic ability of blood leukocytes and proliferative response of blood lymphocytes were examined. Also the lysozyme activity, total □-globulin and IgY levels in serum were determined. The results of our experimental study showed that the selected probiotics activated the nonspecific cell-mediated and humoral-mediated immunity and reduced of mortality after challenge test. In all the tested groups of chickens, the phagocytic ability of blood leukocytes and proliferative response of blood lymphocytes were significantly increased ($P<0.05$), compared to the control group. The highest means of both cell-mediated parameters were observed in groups fed with L acidophilus BS and B. animalis 30. Compared to the control, in all the groups administered with probiotics, the lysozyme activity, total □-globulin and IgY levels in serum were significantly higher ($P<0.05$), whereas total protein level was not significantly different. In the future, we are planing to develop studies into the influence of selected probiotics on the specific cell-mediated and humoral-mediated immunity and protection against other bacterial and viral pathogens in broiler chickens and turkey.

# Prebiotic Effectiveness of Fructooliogosaccharides

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Fructans of inulin type are fructose polymers linked with the characteristic β(2-1) bond which makes them unhydrolysed by human digestive tract enzymes, but available to the bacterial utilisation in the large intestine. Selective stimulation of the colonic *Bifidobacterium* populations by fructans helps to maintain a good intestinal environment, and to enhance some systemic bodily functions. However, the group of the inulin type fructans covers compounds of different degree of polymerisation (DP) of fructosyl chain – low-DP fructooligosaccharides and oligofructose, and long-chain inulin. Are these compounds equally efficient in their prebiotic action? First, the capability of *Bifidobacterium* and *Lactobacillus* for fructan utilisation was examined *in vitro*, followed by studies on prebiotic effectiveness of fructans - in series of *in vivo* experiments on healthy and *Salmonella*-infected rats, using standard or specific (high-protein, high-fat, high-cholesterol) diets. Under *in vitro* conditions, utilisation of fructans in *Bifidobacterium* and *Lactobacillus* depended on the degree of fructose chain polymerisation with a preference for short-chain fructooligosaccharides and oligofructose. The majority of stimulated strains belonged to the species of *B. longum*, *B. animalis*, *L. acidophilus* and *L. rhamnosus*. The selective stimulation of the faecal *Bifidobacterium* populations by short-chain fructans was confirmed *in vivo*. The prebiotic effects of inulins were more diverse, plausibly influenced by the presence of other bacterial strains able to initiate inulin chain degradation. The prebiotic effectiveness of fructans was higher when the overall balance of microflora was disturbed by “unhealthy diet” or pathogen infection, which may be a precious indication for their application in some intestinal disorders and as a component of functional food.

At the end, the current safety considerations will be shortly presented.

# Properties of Potential Probiotic Lactobacillus Strains Isolated from GI Tract

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To be regarded as probiotics bacteria must fulfill several requirements which refer to heath and clinical properties (human origin, resistance to acid and bile, adherence to human intestinal cells, production of antimicrobial substances, antagonism against carcinogenic and pathogenic bacteria, safe for human consumption, clinically validated health effect) and to stability and technological properties (ability to maintain verified viability, maintenance of good flavor and aroma profile after fermentation, maintenance of mild acidity throughout storage, good acidity profile, maintenance of colonizing properties throughout processing and storage, development of good storage stability in fermented products).

The aim of the study was to isolate *Lactobacillus* strains from faeces of 24 healthy infants, identify them and examine their probiotic properties such as: antibacterial activity, antibiotic resistance, survival in presence of bile salts and acidic environment, ability to grow and acidifying of milk, growth in presence of prebiotics (inulin and maltodextrins) in vitro. Resistance to acid and bile of isolated strains was compare to resistance of commercially available *Lactobacillus* and *Bifidobacterium* strains.

Isolated strains belonged to *L. plantarum* – 11 strains, *L. fermentum* - 7 strains, *L. casei* – 1 strains and *L. paracasei* – 1 strains. They showed antibacterial activity against gram-positive and gram-negative test bacteria. Isolated strains inhibited growth of 87 - 100% test bacteria, depends on used isolated strain. Most of the strains were sensitive to rifampicin, nitrofurantion, colistyn, ampicylin and resistant to neomycin, kanamycin, nalidixic acid, gentamycin, oxacyclin, chloramphenicol. Strains showed different resistance to bile salts. The most resistant strain was *L. plantarum* 14, which survived at the population of $10^6$cfu/ml. Nine strains did not survive incubation in presence of bile salts at the population at least 10 cfu/ml. The most resistant commercial strain was *Bifidobacterium lactis*, which remained the viable cells count at the level of $10^3$-$10^5$ cfu/ml at different bile salts concentrations. All of the strains: isolated from GI tract and commercial showed high acid tolerance. Decrease of cells number was not observed during the time of incubation. Isolated strains showed stability during cold storage and fermented milk, but none of them decreased a pH of milk to 4,4 - 4,6. Isolated strains grew well in presence of prebiotics. Their growth in medium containing inulin or dextrins was higher comparing to bulion with and without glucose.

These results indicate that strains isolated from gastrointestinal tract of infants posses same probiotic properties. Nevertheless to regard them as probiotics some additional test should be conducted.

# „Influence of N-nitrosodimethylamine, phenol, p-cresol and indole on growth and survival of intestinal lactic acid bacteria".

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The human gastrointestinal tract is inhabited by a very complex ecosystem of microorganisms. Within the colon the bacterial concentration is between $10^{10}$-$10^{11}$ cells/g intestinal contents comprising at least 500-1000 bacterial species. Intestinal bacteria play a significant role in nutrition and prevention of diseases. They help metabolize undigested polysaccharides, "resistant starch" and fiber, protect from invasion of tissues by pathogenic bacteria – it's the barrier effect, can produce vitamins and affect the host immune response. Intestinal microflora can be divided into two groups - beneficial and harmful. Beneficial bacteria, such as lactic acid bacteria, protect the intestinal tract from proliferation of harmful bacteria. In the healthy subjects, they are well balanced, and beneficial bacteria dominate.

The harmful bacteria possess several enzymes (e.g. *β*-glucuronidse, nitroreductase) responsible for conversion of procarcinogens into carcinogenic substances, such as nitrosamines, heterocyclic aromatic amines, secondary bile acids, indoles, phenols, skatoles, cresols, which can contribute to colon cancer. Beside a variety of different diseases, all the compounds can cause colon cancer by acting like co-carcinogens or promoters.

Intestinal lactic acid bacteria may inhibit colon cancer, but the precise mechanism is unknown. It can include: binding of carcinogens, production of anticarcinogenic metabolites, degradation of potential carcinogens, increase in immune response, alteration of physicochemical conditions in the colon and faecal enzyme activity or stimulation of protective enzymes.

The aim of the study was to estimate the ability of intestinal lactobacilli to growth and survival in the presence of four carcinogens: N-nitrosodimethylamine (NDMA), phenol, p-cresol and indole. Daily NDMA intake and connection with colonic cells is about 10 µg. Physiological level of phenol, p-cresol and indole in colonic contents of healthy humans is about 5,7 - 20,7 µg/ml for phenol, up to 50 µg/ml for p-cresol and up to 7 µg/ml for indole. Above data enabled to select doses appropriate for the research.

Bacterial strains tested were: *Lactobacillus plantarum* WL (LOCK 105), *Lactobacillus casei/paracasei* KNE1. *Lactobacillus casei* J/III, *Lactobacillus casei* BD. All the strains were chosen according to their resistance to acidic pH and bile salts, so as to they could be able to survive in gastrointestinal tract during the transit time.

To define the influence of carcinogens on growth of lactic acid bacteria, the cells were cultivated 24h in MRS medium at $37^0$C in anaerobic conditions in the presence of 5% of $CO_2$, with the addition of final concentrations of NDMA: 2, 20, 100, 500 µg/ml and for phenol, p-cresol and indole: 2, 20 and 100 µg/ml. The control sample for each strain was culture of bacteria without the compounds. To see the influence on growth of bacteria the Koch's plate method was performed and cell number was estimated, at 0h time and after 24h of incubation.

As a result, in case of NDMA, there was no reduction in the number of living bacterial cells as compared with the control. Only 500 µg/ml of NDMA slightly affected the growth of all strains tested. None of the 2, 20 and 100 µg/ml concentrations of phenol and p-cresol as well as 2 and 20 µg/ml of indole influenced the growth of any strain of lactic bacteria, but 100 µg/ml dosage of indole appeared to be slightly toxic for two strains (KNE1 and WL). Thus, all *Lactobacillus* strains were able to grow in the presence of phenol and p-cresol at doses 2, 20 and 100 µg/ml as well as at 2 and 20 µg/ml of indole. Some lactobacilli were resistant even at 100 µg/ml of indole.

To define the influence of the compounds on survival of bacteria, the bacterial biomass achieved after 24h incubation in MRS broth at $37^0$C in anaerobic conditions, was centrifuged (10 000 X g for 10 minutes), and suspended in the phosphate buffer with appropriate concentration of each compound and incubated 168h in $37^0$C in anaerobic conditions. In order to evaluate the survival of lactobacilli, the plate method was used. None of the concentrations of NDMA influenced on the survival of lactic bacteria during 168h of incubation. Phenol, p-cresol and indole, had distinct impact on survival of lactobacilli. In the presence of all concentrations of phenol tested, the number of living cells of strains BD and J/III was changing equally with the control. In case of KNE1 and WL strains, after 120h slight influence of phenol was observed and the amount of living bacteria decreased along with growing concentrations of phenol. For p-cresol - three strains - BD, J/IIII and KNE1 remains no influence in the presence of the compound. Only *L. plantarum* WL – revealed sensibility to concentrations 20 and 100 µg/ml, but just from 120h. For all the strains, there were no changes observed in case of the least concentration of p-cresol – 2 µg/ml. The most toxic agent appeared to be indole. All strains showed reduced ability to survive in the presence of indole after 168h at 100 µg/ml and 20 µg/ml dosage. The most resistant to indole showed to be BD and KNE1 strains. The most sensitive appeared to be J/III and, even 2 µg/ml of indole was toxic for them, although the impact was very slight.

NDMA didn't influence on the survival of intestinal lactic acid bacteria during 168h. Phenol, p-cresol and indole affected the vitality of intestinal lactic bacteria. The impact of the compounds tested depended on dosage (2 µg/ml dose affected only after longer incubation and only in few cases), strain (the most susceptible showed to be *L. plantarum* WL) and the compound itself (the most toxic for all strains appeared to act indole).

## A role of hydrogen peroxide in interactions of lactobacilli with other bacteria and host cells

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Representatives of some Lactobacillus species, similarly to other Gram-positive Streptococcus and Enterococus are known to be effective hydrogen peroxide producers. Although the importance of the activity for microbial systems and bacteria-host interactions is not known, there are scattered data in the literature suggesting that the peroxide of the bacterial origin acts in both ways. Particular attention was given to production of $H_2O_2$ by vaginal lactobacilli which is claimed to be a regulatory factor for vaginal microflora. Our data indicate that, indeed, some vaginal Lactobacillus isolates liberate the peroxide to environment as a response to oxygen and they are able to inhibit in vitro the selected, mostly anaerobic members of the vaginal flora. Hydrogen peroxide in amounts relevant to these accumulated by lactobacilli can trigger apoptosis of epithelial cells and cause cytokine response in immune cells.

On the other hand, most of the Lactobacillus strains produce effective anti-oxydative enzymes, especially Mn-catalase, which can actively inactivate hydrogen peroxide and thus balance oxidative stress caused by this and other reactive oxygen species of both bacterial and host origin.

# Probiotic, fermented beverages based on soy and cereal preparations

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***Abstract***

The aim of the studies was to develop non-milk probiotic beverages containing the selected strains of *Lactobacillus*, *Streptococcus* and *Bifidobacterium* able to create the sensory accepted products under way of fermentation of the selected soybean and cereal preparations.

The materials for fermentation were obtained as follows: *S* (soybean) was the commercial product made under *Polgrunt* brand as *Natural soy beverage*. *R* (rice) was prepared as the water suspension of rice gruel *Nestlé* and *SOR* (soy-oat-rice) – as the suspension of mixed rice gruel *Nestlé*, oatmeal and soymilk *Polgrunt* in water; both comprised the appropriate quantities of saccharose and sodium citrate.

Biological materials for the beverage production were the selected strains belonging to the mentioned above genera: *Lactobacillus plantarum* KKP384 (IAFB), *Streptococcus thermophilus* $T_KM_3$ (IARFR PAS) and *Bifidobacterium infantis* ATCC 15697 (*S*) or *Bifidobacterium breve* ATCC 15700 (*R*, *SOR*). Population numbers of bacteria were determined by plating method using MRS agar (*Lactobacillus*), LAB agar (*Streptococcus*) and modified Garche medium agar (*Bifidobacterium*), and expressed as numbers of cfu·mL$^{-1}$.

The preparations and adequate products were evaluated as for their physicochemical properties, nutritive value and sensory profile. The total solids, crude protein, crude fat, ash content, total acidity and pH were determined by the AOAC methods; mono- and oligosaccharides – by HPLC method; L(+) and D(-) isomers of lactic acid – using Boehringer Mannheim procedure; sensory profile – by the Quality Descriptive Analysis followed by the quantification of the sensory notes.

The two-strain set: *L. plantarum* and *S. thermophilus*, was used for fermentation of the preparations containing 8.64-10.02% of total solids; inocula, as log cfu·mL$^{-1}$, were: 6.26-6.84. The criteria for the assessment of fermentation were the rate of multiplication of bacterial cells during the process and sensory acceptability of properly acidified (pH~4.5) products. Products of 11-19 h fermentation were supplemented with bifidobacterium strain: *B. infantis* (*S*) and *B. breve* (*R, SOR*); inocula, as log cfu·mL$^{-1}$, were 7.15 and 7.49. Final beverages were stored in cold (4$\pm$1$^{o}$C) during four weeks and evaluated according to the basal criteria in week intervals. Probiotic effects of products on the functions of gastrointestinal tract and consumer metabolism were searched in the model experiment with rats of the *Wistar* strain.

Effects of the studies present fermented, probiotic beverages, *S*, *R*, *SOR*, characterized as mild acid in taste and delicate in aroma after two week's storage. The viable cell numbers of bacteria in beverages stored four weeks, as log cfu·mL$^{-1}$, were 7.61-8.34 (*L. plantarum*), 7.34-8.23 (*S. thermophilus*), 8.74$\pm$0.08 (*B. infantis*) and 7.34-8.43 (*B. breve*). Biological study proved the probiotic effects of beverages on rats. These palatable, drinkable products could serve as probiotic beverages for people suffering from milk allergy, lactase deficiency or lactose maldigestion.

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# The Trial of Fermented Carrot Juice Technology Evaluation

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The objective of study was to work out fermented carrot juice technology. Strain of lactic acid bacteria was applied for fermentation. This strains possesses some probiotic properties like resistance to low pH and bile salt.

## Material And Methods

Carrot juice was prepared of carrot purée, water and sugar (7%). Juice was pasteurized (100°C, 15 minutes) then after cooling was inoculed with about 7,32 log cfu/ml$^3$ of lactic acid bacteria and fermented 15 hours in temperature 32°C.

The trial was divided into four stages. **1st Stage:** Selection of strain for fermentation. 8 strains of *Lactobacillus acidophilus* and *Lactobacillus casei*. **2nd Stage:** Fermentation of carrot juice with additive fruit juice. The following fruit juice was applied to prepare mixtures: apple juice, cherry juice and pineapple juice. **3rd Stage:** Choice of fermentation conditions. The carrot and fruit juice was fermented at different temperature and time conditions, as follows: temperature 32°C and 37°C and time 12, 15, 20 hours. **4th Stage:** Determination of inulin addition to fermented carrot juice as a prebiotic additive.

## Results

After first stage, the strain *Lactobacillus acidophilus* CH-2 was chosen to further research, based on sensory analysis. After second stage it was shown that the best mix juice was carrot-apple juice which was used in the next stage. After third stage fermentation conditions were determined. Fermentation time significantly affected pleasantness of carrot-apple juice but fermentation temperature was not significant. After fourth stage carrot juice with 3% inulin and 5% sacharose additives was evaluated as the best.

## Conclusions

The results of this study showed that: pleasentness of fermented carrot juice of *Lactobacillus acidophilus* CH-2 was the best. Apple juice was the best additive to carrot juice. Fermentation time was significant factor in sensory evaluation of fermented apple-carrot juice. 3% inulin and 5% saccharose addition to carrot juice was the best.

# Development of whey cheese - REQUEIJÃO - with added probiotic bacteria

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Strains of *Bifidobacterium lactis* and *Lactobacillus acidophilus* were incorporated into a traditional Portuguese whey cheese (*Requeijão*). Three variants of Requeijão were manufactured: with added salt, with added sugar and without any additives. The viability of said probiotic strains therein was monitored during 28 d of storage at 4 ºC. No significant differences were found in the overall composition between all three types of experimental whey cheeses, which were furthermore comparable to their traditional counterpart. The initial levels of *B. lactis* and *L. acidophilus* were $10^9$ cfu/g; experimental whey cheeses held viable numbers above the recommended threshold of $10^6$ cfu/g by the end of the storage period.