

# The Influence of Feeding Diets Containing White Cheese, Produced with Prebiotics and the Potentially Probiotic *Lactobacillus plantarum* Strain, on the Gastrointestinal Microflora of Rats

MONIKA MODZELEWSKA-KAPITUŁA<sup>1</sup>, JAN KŁOBUKOWSKI<sup>2</sup>,  
LUCYNA KŁĘBUKOWSKA<sup>3</sup> and DANUTA WIŚNIEWSKA-PANTAK<sup>2</sup>

<sup>1</sup>Department of Meat Technology and Chemistry, <sup>2</sup>Department of Human Nutrition,  
<sup>3</sup>Department of Industrial and Food Microbiology, Faculty of Food Sciences,  
University of Warmia and Mazury, Olsztyn, Poland

## Abstract

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The effects of inulin HPX and maltodextrins, and also the potentially probiotic *Lactobacillus plantarum* 14 strain, used separately and in combination in white cheese production, on the gastrointestinal microflora of Wistar rats was investigated. The prebiotic addition to the cheese was 2.5%, whereas probiotic and synbiotic cheeses contained at least 10<sup>7</sup> CFU/g of live *L. plantarum* cells. The counts of *Bifidobacterium* sp., *Lactobacillus*, coliforms, and the most probable number of anaerobic proteolytic bacteria were evaluated. After a 10-day feeding experiment, significant changes ( $P < 0.05$ ) were noted in the most probable number of anaerobic proteolytic bacteria spores, which was the highest in the group receiving a diet with the cheese containing the potentially probiotic strain and inulin HPX. A short-time ingestion of low doses of prebiotics or synbiotics did not alter the counts of *Lactobacillus*, *Bifidobacterium*, and coliforms in healthy rats.

**Keywords:** inulin; maltodextrins; rats; microflora; prebiotics; probiotics; synbiotics

For many years, great interest has been observed in view of obtaining new functional products with a marked tendency towards products supplemented with pro- and prebiotics (PUUPPONEN-PIMIA *et al.* 2002). Probiotics are live micro-organisms beneficially affecting the health of consumers by

maintaining or improving their intestinal microbial balance (MATTILA-SANDHOLM *et al.* 1999). Apart from that, they can exert additional positive effects on human health and well-being such as the prevention of gastrointestinal disorders, alleviation of lactose intolerance symptoms, stimulation of

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the immune system, lowering of cholesterol level, enhancement of mineral absorption and vitamin production (KAUR *et al.* 1998; ZIEMER & GIBSON 1998; GOMES & MALCATA 1999; SHORTT 1999). Recently, the application of probiotics in products other than yoghurts or other dairy beverages, e.g., ripened and fresh cheeses, has become widespread (GARDINER 1998; RYHÄNEN *et al.* 2001; KASIMOĞLU *et al.* 2004; BURITI *et al.* 2005).

Prebiotics are defined as food ingredients which are not digested by endogenous enzymes of the host but are transferred in the unchanged form to the large intestine, where they are used by the desired microflora. Prebiotics serve as a substrate for a limited number of bacteria, mainly probiotic *Lactobacillus* sp. and *Bifidobacterium* sp., selectively stimulating their growth and, as a result, beneficially affecting the host's health (CRITTENDEN & PLAYNE 1996; ROBERFROID *et al.* 1998; LOSADA & OLLEROS 2002; MUSSATO & MANCILHA 2007). However, other bacteria, such as clostridia and eubacteria, are also able to utilise some prebiotics as a carbon source (MANNING & GIBSON 2004; RADA *et al.* 2008). The stimulation of the clostridia growth in the large bowel will be not beneficial for the host. A high number of these bacteria may be pathogenic due to their proteolytic capabilities and toxin production (SANTOS *et al.* 2006). Thus, the application in food products of prebiotics, which do not stimulate clostridia, will be desirable.

Such products in which pre- and probiotics are applied together are defined as synbiotics. There are many reports concerning the influence of probiotic (DJOUZI *et al.* 1997; FOOKS 1999; HOLZAPFEL & SCHILLINGER 2002; MEDICI *et al.* 2004) or prebiotic (GIBSON 1999; STRICKLING *et al.* 2000; GRÅSTEN *et al.* 2003; WIELE *et al.* 2004) diet supplementation on the faecal microbiota, but relatively few of them refer to the effect of synbiotic products on gastrointestinal microflora (ALANDER *et al.* 2001; BIELECKA *et al.* 2002a). Still, there is a need to investigate the influence of synbiotics, which are incorporated into real food products, on gastrointestinal microflora.

The aim of the present work was to investigate the influence of feeding white soft cheeses supplemented with prebiotics and potentially probiotic *Lactobacillus plantarum* 14 strain on the intestinal microflora of rats. Usually, in feeding experiments on rats, prebiotics are used in a quantity of 5–10% (ROBERFROID *et al.* 2002; ZAFAR *et al.*

2004; LOBO *et al.* 2006) whereas in dairy products lower amounts of prebiotics are used (DONKOR *et al.* 2007). Therefore, to investigate the influence of pro-, pre-, and synbiotics administered in real food products, white soft cheese was used in the present study as a carrier of a probiotic culture and a relatively low dose of prebiotics. The level of prebiotic addition (2.5%) in cheese was adjusted in the earlier studies (MODZELEWSKA-KAPITUŁA *et al.* 2007) so as to provide good technological and sensory properties of the cheese.

## MATERIAL AND METHODS

**Animals and diets.** The experiment was carried out on standardised white male Wistar rats ( $n = 36$ ) obtained from the Department of Biological Food Evaluation of the Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences in Olsztyn, with the initial body weights ranging from 91 g to 98 g. The animals were housed in individual metabolic metal-free cages in a room at 22°C and 65% relative humidity, with a 12-h light-dark cycle. The cages enabled to collect and separate faeces and urine. The animals were divided into 6 groups of 6 rats each, receiving control and experimental diets. Diets and water were provided *ad libitum*. The animals received diets composed of white soft cheeses with the additives: pro- and prebiotics, and supplements such as vitamins, minerals, potato and corn starches (Table 1). The diets contained 10% of protein ( $N \times 6.38$ ), 1% (w/w) of added vitamins, 3% (w/w) of minerals without Ca, 5% (w/w) of potato starch, and corn starch as a constituent supplementing the composition to 100 g of dry matter of the diet (NRC 1976). In the diet preparation, the fat content in cheeses was considered. Six different diets were prepared: control diet (A) which contained cheese without any prebiotic or probiotic strain addition, diet B – containing the probiotic cheese with *Lactobacillus plantarum* 14, diet C – containing cheese with 2.5% (w/w) of inulin HPX, diet D – with cheese containing *L. plantarum* 14% and 2.5% (w/w) of inulin HPX (polymerisation degree  $DP \geq 23$ , Orafiti, Tienen, Belgium), diet E – with cheese with 2.5% (w/w) of maltodextrin (dextrose equivalent  $DE = 16.2$ , Pepes S.A., Łomża, Poland), and diet F – with cheese containing both the probiotic strain *L. plantarum* 14% and 2.5% (w/w) of maltodextrin. The fat contents in the diets were 15.5%, 14.9%,

Table 1. Diet composition in the feeding experiment on rats

Diet	Constituents (%)				
	cheese	vitamins <sup>a</sup>	mineral salts <sup>b</sup>	potato starch	corn starch
A	61.88	1.00	3.00	5.00	29.12
B	60.75	1.00	3.00	5.00	30.25
C	70.82	1.00	3.00	5.00	20.18
D	81.10	1.00	3.00	5.00	9.90
E	69.30	1.00	3.00	5.00	21.70
F	69.64	1.00	3.00	5.00	21.36

<sup>a</sup>Vitamins per 1 g of the mixture: A 2000 IU, D<sub>3</sub> 200 IU, E 10 UI, K 0.5 mg, choline chloride 200 mg, paraaminobenzoic acid 10.0 mg, inositol 10.0 mg, niacin 4.0 mg, calcium panthothenian 4.0 mg, B<sub>2</sub> 0.8 mg, thiamine 0.5 mg, B<sub>6</sub> 0.5 mg, folic acid 0.2 mg, biotin 0.04 mg, B<sub>12</sub> 0.003 mg, glucose as a constituent supplementing the composition to 1 g (AOAC 1975)

<sup>b</sup>Mineral mixture supplying the following (g/kg): K<sub>2</sub>HPO<sub>4</sub> 81.0, K<sub>2</sub>SO<sub>4</sub> 68.0, NaCl 30.0, CaCO<sub>3</sub> 21.0, NaHPO<sub>4</sub> 21.4, MgO 18.0, corn starch 735.0, microelements 18.0, microelements mixture (g/100 g): ferric citrate (16.7% Fe) 31.0, ZnCO<sub>3</sub> (56% Zn) 4.5, MnCO<sub>3</sub> (44.4% Mn) 23.4, CuCO<sub>3</sub> (55.5% Cu) 1.85, KI 0.04, citric acid 39.21 (modified from mineral mixture – NRC 1978)

16.8%, 20.3%, 17.2%, 18.1% (w/w) in the diets A, B, C, D, E, F, respectively. The number of live *Lactobacillus plantarum* 14, possessing probiotic properties, was at the level of 10<sup>7</sup> CFU/g in every cheese. The cheese used in the study was produced in a dairy plant in Poland according to the manufacture's instruction with commercial mesophilic lactic culture and rennet. Prebiotics and the probiotic strain were added after thermisation (MODZELEWSKA-KAPITUŁA *et al.* 2007).

**Study design.** The feeding experiment lasted 10–5 days of the adaptation period and 5 days of the proper experimental period during which the excrements and uneaten food were collected. In the faeces from the proper experimental period, the numbers of coliforms, *Lactobacillus* sp., *Bifidobacterium* sp., and the most probable number of anaerobic proteolytic bacteria spores were determined. The experiment was approved by the Local Animal Ethics Committee of the University of Warmia and Mazury in Olsztyn, Poland.

Potentially probiotic strain. *Lactobacillus plantarum* 14 was previously isolated from the gastrointestinal tract of a healthy infant and identified using PCR technique. The strain turned out to be acid (pH 2 and 3) and bile resistant (up to 5% of bile salts) and showed antimicrobial activity against Gram-positive and Gram-negative bacteria in agar well-diffusion method (MODZELEWSKA *et al.* 2003; MODZELEWSKA-KAPITUŁA *et al.* 2008a). The strain exhibited desirable technological properties such as: the ability to grow in milk and stability during

the cold storage over 21 days (MODZELEWSKA-KAPITUŁA *et al.* 2008b).

**Microbiological analysis.** Before preparing the diets, the number of live *Lactobacillus plantarum* 14 in soft cheeses was determined. The cheese samples (10-g portions) were blended with 90 ml of peptone water in a laboratory blender (Seward Stomacher 400, Seward Laboratory Systems Inc., Bohemia, USA) and submitted to serial dilutions with the same diluent. The number of potentially probiotic microorganisms was enumerated using MRS with maltose and bromocresol purple solid media after 48 h of anaerobic incubation (Anaerocult C, Merck, Darmstadt, Germany) at 37°C. To assure that apart from *L. plantarum* 14 no other bacteria able to grow on MRS with maltose and bromocresol purple media were present in the cheese, in all cheese versions the numbers of the bacteria on this medium were determined. In the cheese samples without the probiotic strain no colonies grew on this medium.

The faeces from the proper experimental period were collected separately from each rat and weighed. Before performing microbiological analysis, the faeces were stored in sterile containers under refrigeration. The serial decimal dilutions in sterile physiological saline were prepared. The live cell number of *Bifidobacterium* sp. was counted on Garsche's agar medium (BIELECKA *et al.* 2002b) after incubation at 37°C for 48 h in anaerobic conditions (GasPack Plus, BBL, Becton Dickinson, Cockeysville, USA). The identification was based on

the appearance of colonies and cells morphology. The number of *Lactobacillus* sp. was counted on Rogosa agar (Merck, Darmstadt, Germany) after incubation at 37°C for 72 h in anaerobic conditions (Anaerocult C, Merck, Darmstadt, Germany), and that of coliforms on Coliform Chromocult Agar (Merck, Darmstadt, Germany) after incubation at 37°C for 24 hours. The most probable number (MPN) of anaerobic proteolytic bacteria spores was determined after heating the dilutions at 80°C for 20 min and cooling in tubes with Meat Liver Agar (Merck, Darmstadt, Germany), using the three-tube method. The results were read from tables after incubation at 37°C for 72 h under anaerobic conditions. All the media and diluter were prepared with the use of distilled water.

The results were expressed as log colony-forming units (log CFU)/g and colony-forming units (CFU/g). For the statistical analysis, including the comparison between the groups, Duncan's test was applied (Statistica 8.0, StatSoft Inc., Tulsa, USA) at a significance level  $P < 0.05$ .

## RESULTS AND DISCUSSION

In the faeces of rats fed with diets containing probiotic, prebiotic or synbiotic cheeses, the numbers of lactobacilli, coliforms, bifidobacteria, and spores of anaerobic proteolytic bacteria were determined. Lactobacilli were dominating among the groups of microflora determined, reaching  $10^8$  CFU/g, followed by coliforms  $\sim 10^7$  CFU/g and bifidobacteria  $\sim 10^6$  CFU/g. The mean number

of anaerobic proteolytic bacteria spores did not exceed  $3 \times 10^2$  CFU/g (Table 2).

The counts of lactobacilli ranging from 6.72 log CFU/g to 7.69 log CFU/g were not different in the experimental and control groups (Table 2). The highest numbers of bifidobacteria were found in the group of rats receiving diet B containing the cheese with potentially probiotic *L. plantarum* 14 – 6.01 log CFU/g, however, there were no statistically significant differences between this and the other groups in which the numbers of bifidobacteria ranged from 5.36 log CFU/g to 5.69 log CFU/g. The lowest mean number of coliforms was detected in group D (receiving cheese with *L. plantarum* 14 and inulin HPX), i.e. 5.37 log CFU/g compared to 6.33–6.63 log CFU/g in the other experimental groups and 5.97 log CFU/g in the control group, but the results obtained did not differ statistically. The lowest anaerobic proteolytic bacteria spores number (2.36 log CFU/g) was found in the faeces of rats from group F – those fed with cheese containing *L. plantarum* 14 and maltodextrins – whereas in the other groups it ranged from 3.03 log CFU/g to 4.08 log CFU/g in comparison to 2.47 log CFU/g in the control. The highest number of spores was detected in the faeces of rats from group D (the difference of statistical significance,  $P < 0.05$ ), which received cheese with the probiotic strain and inulin HPX.

The study was performed to compare the influence of prebiotic, probiotic or synbiotic cheeses on the microbiology of the gastrointestinal tract of animals. The study using rats showed that the application in the animals diet of cheeses en-

Table 2. Gastrointestinal microflora (count, log CFU/g faeces) of rats ( $n = 36$ ), after 10-day feeding experiment with diets containing probiotic, prebiotic or synbiotic cheese

Diet	Coliforms	<i>Lactobacillus</i> sp.	<i>Bifidobacterium</i> sp.	Spores of anaerobic proteolytic bacteria
A	5.97 ± 1.74 <sup>a</sup>	7.60 ± 0.97 <sup>a</sup>	5.46 ± 0.87 <sup>a</sup>	2.47 ± 0.13 <sup>a</sup>
B	6.44 ± 0.83 <sup>a</sup>	7.69 ± 0.85 <sup>a</sup>	6.01 ± 1.00 <sup>a</sup>	3.03 ± 0.94 <sup>a</sup>
C	6.33 ± 0.90 <sup>a</sup>	7.16 ± 1.28 <sup>a</sup>	5.69 ± 0.99 <sup>a</sup>	3.32 ± 1.34 <sup>a</sup>
D	5.37 ± 0.71 <sup>a</sup>	7.06 ± 0.78 <sup>a</sup>	5.36 ± 1.13 <sup>a</sup>	4.08 ± 1.04 <sup>b</sup>
E	6.63 ± 1.11 <sup>a</sup>	6.72 ± 1.04 <sup>a</sup>	5.51 ± 0.57 <sup>a</sup>	3.06 ± 0.89 <sup>a</sup>
F	6.58 ± 1.33 <sup>a</sup>	6.96 ± 0.69 <sup>a</sup>	5.64 ± 1.18 <sup>a</sup>	2.36 ± 0.00 <sup>a</sup>

<sup>a, b</sup> mean values without common superscripts differ statistically ( $P < 0.05$ )

A – control diet, which contained cheese without any prebiotic or probiotic strain addition; B – F experimental diets, which contained cheese with: B – *Lactobacillus plantarum* 14, C – 2.5% inulin HPX, D – *L. plantarum* 14 and 2.5% inulin HPX, E – 2.5% maltodextrin, F – *L. plantarum* 14 and 2.5% of maltodextrin

riched in prebiotics and/or the potentially probiotic strain did not cause any significant changes in the counts of faecal *Lactobacillus* sp., *Bifidobacterium* sp. or coliforms, however, diet D based on the cheese with inulin HPX and *L. plantarum* 14 increased the number of anaerobic proteolytic bacteria spores.

Different results concerning the coliforms number were obtained by BIELECKA *et al.* (2002b), who investigated the influence of feeding rats with diets containing 10% of fructo-oligosaccharides, lactulose, resistant dextrins or resistant starch. They found that the counts of coliforms were significantly higher ( $P < 0.05$ ,  $P < 0.01$ ) in the groups of animals fed with diets containing these prebiotics. Similar to the current study findings, fructo-oligosaccharides did not stimulate the growth of *Bifidobacterium* sp. whereas other authors (MONTESI *et al.* 2005) reported increased bifidobacteria counts in faeces as a result of fructo-oligosaccharides supplementation to the diet. Although the numbers of proteolytic anaerobic spores in the faeces of rats receiving prebiotics and the control diet did not differ statistically in the study described by BIELECKA *et al.* (2002b), the lowest one was detected in the control group. This was also observed in the present study – out of all experimental groups only in the faeces of rats receiving diet F (maltodextrin and *L. plantarum*) the number of proteolytic spores was lower than in the control, but the difference was not statistically significant.

The lack of *Bifidobacterium* sp. growth stimulation by feeding rats with a diet containing inulin was described by BIEDRZYCKA and BIELECKA (2004). They found that bifidobacteria stimulation by fructo-oligosaccharides depends on their polymerisation degree and is stronger when fructo-oligosaccharides of lower polymerisation degree are used. Highly polymerised (DP  $\geq 22$ ) inulin EXL administered in the amount of 5% over 3 weeks failed to exert any stimulatory effect on the bifidobacteria counts in the faeces of rats. In the present study, similarly polymerised inulin (HPX, DP  $\geq 23$ ) was used. Inulins of high DP are not utilised by *Bifidobacterium* sp., however, other bacteria present in the gastrointestinal tract of humans can initiate inulin degradation to substances which can be used by bifidobacteria (BIEDRZYCKA & BIELECKA 2004).

In the present study, feeding rats with probiotic or synbiotic cheeses with *L. plantarum* did not cause any increase in lactobacilli counts. Differ-

ent findings were described by MINELLI *et al.* (2004) who observed a stimulatory effect of the consumption of milk fermented with probiotic *L. casei* on *Lactobacillus* group in rats. However, CHEN *et al.* (1999) noted an increase in bifidobacteria numbers and no growth stimulation of lactobacilli as a result of probiotic yoghurt (with *L. acidophilus* and *B. bifidum* at least  $10^7$  CFU/ml) consumption by humans, whereas MONTESI *et al.* (2005) did not observe any increase in lactobacilli or bifidobacteria counts after feeding rats a diet containing the probiotic *B. lactis* strain.

## CONCLUSIONS

The application of prebiotic, probiotic, and synbiotic cheeses failed to exert in the present study a significant effect on the gastrointestinal microflora of rats. The statistically insignificant changes observed might be due to the nature of the inulin used in the study or the low dose of prebiotics, which was appropriate from the technological and organoleptic points of view.

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*Corresponding author:*

DR. MONIKA MODZELEWSKA-KAPITUŁA, University of Warmia and Mazury, Faculty of Food Sciences,  
Department of Meat Technology and Chemistry, 10-718 Olsztyn, Poland  
tel.: + 48 895 233 207, e-mail: monika.modzelewska@uwm.edu.pl

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