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RESEARCH FOR CREATION OF FUNCTIONAL FOODS WITH *BIFIDOBACTERIA*

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Twenty-six *Bifidobacterium* strains were isolated from human faeces. Seven strains were identified as *B. bifidum*, 4 strains as *B. breve*, 10 strains as *B. longum*, 2 strains as *B. pseudocatenulatum* and 3 strains as *B. dentium* by 16S rDNA analysis. The isolates from human origin showed strong adherence to the human tissue cultures. Three out of the 12 tested isolates repressed the growth of enteropathogenic bacteria. Utilisation of 9 commercially available oligosaccharides was tested by both *Bifidobacteria* and enteropathogens. Pro-, pre- and synbiotic food was made. Their effect was evaluated in in vivo feeding experiments, where healthy and antibiotic treated mice were used as test animals. During the four-week feeding period the composition of the colonic microbiota of the healthy mice did not change characteristically in any feeding group. However, the microbiota of mice in which it had been killed by antibiotic treatment was recovered by feeding with synbiotic food.

Keywords: *Bifidobacteria*, functional food, identification, probiotic

In the last decades the interest has been increasing for healthy diet. Because of this, food scientists are working on the development of food, which promote host health. A food, which affects the function of the body in a targeted way so as to have positive health effects, is called functional food (GIBSON & ROBERFROID, 1995).

Almost a hundred years have passed since the introduction of the theories on the prolongation of life by the modulation of the intestinal ecosystem. However, only recently has been firmly established the scientific basis of probiotic studies and have sound clinical studies of some strains been published. The physiological and nutritional properties of some probiotic bacteria are now well understood, furthermore, it is possible to verify that some strains are probiotic with documented examples of their ability to maintain and promote the health of the host. It is important that probiotic strains are originated from human source, because some health-promoting effects may be species dependent. Other required properties include acid and bile resistance and the ability to colonise the human intestinal tract. Adherent strains of probiotic bacteria are favoured, because they are more likely to become resident in the intestinal tract, and thus they can express health promoting effects. It is also important for probiotic strains

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to have antagonistic effect on pathogenic bacteria either by production of antimicrobial substance or competitive exclusion. Finally, the strains should be safe for human use (LEE & SALMINEN, 1995; CHARTERIS et al., 1998).

The large intestine is by far the most heavily colonised region of the digestive tract, with up to 10^{12} bacteria for every gram of gut content. Through fermentation colonic bacteria are able to produce a wide range of compounds that have both positive and negative effects on physiology of gut as well as other systematic influences. *Bifidobacteria* produce acetate and lactate as metabolic end products. These lower the pH of the medium and may thus exert an antibacterial effect. An added effect of acid production is the protonation of potentially toxic ammonia to produce NH_4^+ that is non-diffusable and thus lowers the blood ammonia level. *Bifidobacteria* produce vitamins, largely B vitamins. Certain cellular components of *Bifidobacteria* act as immunomodulator, i.e. they promote immunological attack against malignant cells. These bacteria have also been used to restore the normal intestinal flora during and after antibiotic therapy. *Bifidobacteria,* the most predominant bacteria in the intestinal flora of infants, are established shortly after birth. As human ages the numbers of *Bifidobacteria* changes: *Bifidobacteria* decline to the third or fourth largest group (HOOVER, 1993; MODLER, 1994).

In the frame of our work the following aims were fulfilled: Isolation of *Bifidobacteria* from human faeces and foodstuff, study of the adherence of the isolated strain to human tissue cultures, the antagonistic effects on enteropathogens, the metabolism of oligosaccharides and in vivo feeding experiments with healthy and antibiotic treated mice. The final goal of this work was to create functional foods using probiotic *Bifidobacteria* and/or prebiotic oligosaccharide.

1. Materials and methods

1.1. Microorganisms

Lyophilised authentic strains were obtained from DSM (Deutsche Sammlung von Microorganismen und Zellkulturen GmbH, Germany), NCTC (National Collection of Type Cultures, England), NCAIM (National Collection of Agricultural and Industrial Micro-organisms, Hungary) culture collections. Moreover, *Bifidobacterium* strains from human origins were isolated, registered and tested. These strains are introduced in Table 1 of the section of Results and discussion.

Pathogenic microorganisms (*Eschericha coli* 35033, *E. coli* 35034, *E. coli* 30213, *Salmonella typhi* 15001, *Salmonella cholerae-suis* 10051, *Salmonella derby* 10032, *Klebsiella* sp. 52047, *Proteus mirabilis* 60007, *Yersinia enterocolitica* 98001, *Shigella disenteriae* 20001, *Shigella flexnerii* 20015) were obtained from the Hungarian National Collection of Medical Bacteria (HNCMB).

1.2. Cultivation

The microbes were cultivated in non-selective media TPY agar contained (per liter): Tripticase (BBL) 10 g, Phytone (BBL) 5 g, Glucose 5 g, Yeast extract (Difco) 2.5 g, Tween 80 1 ml, L-Cysteine HCl 0.5 g, K_2HPO_4 2 g, $MgCl_2 \cdot 6H_2O$ 0.5 g, $ZnSO_4 \cdot 7H_2O$ 0.25 g, $CaCl_2$ 0.15 g, $FeCl_3$ traces, Agar 15 g, pH adjusted to 6.8 (SCARDOVI, 1986); RCM agar contained (per liter): Yeast extract 3 g, Beef extract 10 g, Peptone 10 g, Glucose 5 g, Soluble starch 1 g, NaCl 5 g, CH_3CO_2Na 3 g, L-cysteine HCl 0.5 g, Agar-agar 15 g, pH adjusted to 6.8 (RASIC, 1990); and in selective media: NPNL agar which can be an RCM agar or MRS agar (after autoclaving) supplemented with Neomycin sulfate 100 mg l^{-1}, Paramomycin sulfate 200 mg l^{-1}, Nalidix acid 15 mg l^{-1}, Lithium chloride 3 g l^{-1}; Galactose (GL) agar contained (per liter): Proteose peptone No. 3 (Difco) 10 g, Trypticase peptone (BBL) 10 g, NaCl 2 g, L-cysteine HCl 0.5 g, K_2HPO_4 1.5 g, $MgSO_4$ 0.2 g, LiCl 0.4 g, Yeast exract (BBL) 10 g, Bacto-agar (Difco) 15 g, after autoclaving add 100 ml of 10% Galactose solution sterilised by filtration (IWANA et al., 1993.); DP agar contained (per liter): Columbia agar base (Merck CM331) 42 g, Glucose 5 g, L-cysteine HCl 0.5 g, Agar 5 g, Propionic acid 5 ml, Dicloxacillin (Sigma 9016) 0.2% solution 1 ml. The propionic acid was added with sterile pipette and the Dicloxacillin was sterile-filtered into the modified Columbia agar after sterilisation, finally the pH value was adjusted to 6.8 by NaOH (10%) (BONAPARTE, 1997). The plates were incubated under anaerobic conditions in Anaerobe Jar + GasPak System (OXOID), or in anaerobic chamber 10% CO_2- 5% H_2- N_2 (Bugbox made by Ruskinn Technology, UK) at 37 °C.

1.3. Isolation

Bifidobacterium strains were isolated from the faeces of breast-fed infants and healthy adults. Twenty-seven infants and 35 young adults gave specimens. The samples were treated within 1 or 2 h to avoid the decay of the anaerobic microbiota. The specimens were spread onto the surface of selective media (NPNL, GL and DP) mentioned before. Colonies were isolated and candidates with bifido-like morphology were identified at genus level by F6PPK test (SCARDOVI, 1986).

1.4. Identification

1.4.1. Fermentation tests: A homemade microplate method with 53 substrates was used. The applied substrates are as follows: glucose, galactose, sorbose, D-glucosamine, D-ribose, D-xylose, L-arabinose, D-arabinose, L-rhamnose, saccharose, maltose, trehalose, α-methyl-D-glucosid, cellobiose, salicin, arbutin, melibiose, lactose, raffinose, melezitose, glycerol, erythritol, ribitol, xylitol, L-arabitol, sorbitol, D-mannitol, dulcitol, m-inositol, D-lyxose, D-turanose, N-acetyl-D-glucosamin, D-arabitol, gluconate, 2-keto-gluconate, amygdalin, methylxylosid, methylmannosid, fructose, mannose, gentibiose, inulin, starch, glycogen, dextrin, aesculin, α-L-fucose, D-glucuronate, arabinogalactan, gastric mucin, guar gum, amylopectin and arginin.

The 48-h-old cells were suspended in RCM medium without carbohydrate (yeast extract 3 g, meat extract 10 g, peptone 10 g, cystein, HCl 0.5 g, NaCl 5 g, Na-acetate 3 g, agar 0.1 g, distilled water 1000 ml; transfer 12 ml in a tube and autoclave at 121 °C for 15 min). The suspension was transferred into the wells of the microplate at 25 µl aliquots, each. The final concentrations of the substrates were adjusted to 0.5% (w/v) in every well. Bromcresol purple (30 mg l^{-1}) was applied as indicator. Evaluation was carried out on the basis of the acid production and the NH_3 formation. The colour changes were recorded after one week of incubation at 37 °C in anaerobic jars. Identification in species level was made by means of software Bifbsp.txt (unpublished). Moreover, complex carbohydrate fermentation test and "key for differentiation of *Bifidobacterium* species of human origin" published by CROCIANI and co-workers (1994) were used also for the identification of *Bifidobacterium* species of human origin.

1.4.2. 16S rDNA sequence analysis: The strains were lysed and the amplification of the almost complete 16S rDNA molecule was performed according to the method elaborated by VON STETTEN and co-workers (1998). The universal 16S rDNA binding primers 5'f [5'-AGAGTTTGATCCTGGCTCA-3'] and 3'r [5'-CGGCTACCTTGTTACGAC-3'] (position 1511-1493 in the *E. coli* numbering system) were used for the PCR. The PCR protocol started with a denaturation step for 5 min at 95 °C, followed by 30 cycles of denaturation for 20 s at 95 °C, primer annealing for 40 s at 55 °C and elongation for 2 min at 72 °C each. A final elongation step was added at 72 °C for 5 min. After PCR amplification, the DNA was purified using the QIAquick PCR Purification Kit (Qiagen) according to the instruction of the manufacturer, followed by a PEG precipitation of the purified product. After purification, the samples were subjected to a cycle-sequencing PCR according to FACIUS and co-workers (1999), using the ThermoSequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech). Eleven percent (v/v) DMSO and 7% formamid were added to facilitate the cycle sequencing PCR in the case of the high G+C Gram-positive bacteria such as *Bifidobacteria*. Universal primer 5'f IRD800 [5'-AGA GTT TGA TCC TGG CTC A-3'], *E. coli* numbering 8–26 was used for the cycle-sequencing PCR. The protocol for the cycle-sequencing PCR started with a denaturation step (for 5 min at 88 °C), followed by 25 cycles of denaturation (for 30 s at 88 °C), primer annealing (for 25 s at 42 °C) and elongation (for 3 min at 50 °C). The process was finished by a final elongation step (at 50 °C for 5 min). Sequencing was performed on a LI-COR sequencer (MWG Biotech), typically yielding sequence length of approximately 800 to 1000 bases per run.

Identification of the strains was carried out by comparison of the partial sequences with sequences in database Genbank (BLAST). According to STACKEBRANDT and GOEBEL (1994), a homology of more than 97% shows the species identity between the relevant sequence and the sequence in the database.

1.5. Tissue adherence of Bifidobacteria

The adherence of *Bifidobacteria* was investigated in human tissue cultures. Three tissue culture lines, HeLa, HT29 and Hep2 were applied. The 24-h monolayer tissue cultures were treated according to the following procedure:

1. Remove the nutrient medium from the tissue culture and add 500 μl of the 6-h bacterium suspension with 10^3 cells ml^{-1} concentration to each of the tissue boxes. Then add 1 ml of nutrient medium to each of the tissue boxes.
2. Incubate at 37 °C for 3 h.
3. Remove the nutrient medium from the tissue culture and wash with phosphate buffer (pH 7). Then add 1 ml of nutrient medium to each of the tissue boxes.
4. Incubate at 37 °C for 2 h.
5. Remove the nutrient medium from the tissue cultures and wash with phosphate buffer (pH 7).
6. Fix with alcohol and stain with 1% methylene blue solution.
7. Adherence was evaluated under a light microscope. With regard to tissue adherence, intact *Bifidobacteria* cells were observed in the tissue box.

1.6. Antagonistic effects on enteropathogens

Interaction of *Bifidobacterium* strains and various human enteropathogenic bacteria listed in the section on material and methods was studied. The steps of the procedure are as follows:

1. Put 0.1 ml of the pathogen bacteria suspension into Petri dishes and pour in 20 ml of molten Anaerobic basic medium. Mix them up. Wait until they solidify.
2. Put 1 drop of each strain on the surface of the agar.
3. Incubate at 37 °C for 48 h.

There is no growth of pathogen bacteria around the strains with antagonistic activity.

1.7. Metabolism of sugars and oligosaccharides

The method used in fermentation tests seemed adequate for the assessment of the different commercially available oligosaccharides as a prebiotic. The following oligosaccharides were tested:

– Fructooligosaccharides: Raftiline powder, Ratilose powder (ORAFTI).
– Lactosucrose: LS-40L syrup, LS-55L syrup, LS-55 powder (Ensuiko Sugar Refining Co.).
– Isomaltooligosaccharide: OLIGOTIME syrup, OLIGOMT-500 syrup, Isomalto-500 syrup (Showa Sangyo Co.).
– Xylooligosaccharide: Xylo-oligo 70 syrup, Xylo-oligo-95 powder (SUNTORY).

The investigation was carried out both with *Bifidobacteria* and some enteropathogens.

1.8. In vivo feeding experiments with healthy and antibiotic treated mice

Twenty-five male mice were bred separately and divided into five groups. The first group was fed dry nutrient (control 1), the second dry nutrient and food matrix (control 2), the third dry nutrient and food matrix supplemented with Oligotime Syrup 7% (m/m) (isomalto-oligosaccharide) (prebiotic food), the fourth dry nutrient and food matrix supplemented with *B. longum* suspension 10^7 cell g^{-1} (probiotic food), and the fifth group was fed dry nutrient and food matrix supplemented with *B. longum* suspension 10^7 cell g^{-1} and Oligotime Syrup 7% (m/m) (synbiotic food).

Fresh faeces were collected from every mouse on every second day. The faeces were suspended in dilution solution. The suspensions were spread on the surface of Anaerob blood agar, EM agar (LÁNYI, 1980) and RCM agar using the decreasing colony number method. The plates were evaluated after 48 h of anaerobic incubation.

Evaluation: Growth of the bacteria on the Anaerobic agar indicates the total number (CFU) of the colonic microbiota (*Enterobacteriaceae*, Lactic acid bacteria and other bacteria e.g. *Bacteriodes*). Growth of the bacteria on EM agar indicates the presence of the *Enterobacteriaceae* family (*E. coli*). Growth of the bacteria on RCM agar mainly indicates the presence of anaerobic lactic acid bacteria and *Bifidobacteria*. (These microbes were observed under the microscope: if they are Gram positive, non sporing, rods with spatulated, slightly bifurcated or club shaped ends, then they were regarded as *Bifidobacetria.*)

In vivo experiments with 12 antibiotic treated mice, whose colonic microbiota were killed by antibiotic treatment, were grouped and fed as mentioned above. The prebiotic group was left out from the investigation.

2. Results and discussion

2.1. Isolation and identification of Bifidobacteria *from various origin*

Twenty-six strains were isolated from human sources (19 from infants, 7 from adults). The isolated strains were identified at species level. The identification of *Bifidobacteria* species is problematic because of phenotypic and genetic heterogeneity. For the classification of *Bifidobacterium* strains into species biochemical and molecular analysis were used. The results of different identification methods are summarised in Table 1.

Comparing the results gained by fermentation test, degradation of specific complex carbohydrates (CROCIANI et al., 1994) and the 16S rDNA analysis, it can be concluded that they led to identical outcome. It has to be mentioned that some species show very strong similarity on genetic molecular level, so they could not be distinguished at species level. The most controversial problem arises between the species *B. longum* and *B. infantis.* The similarity in their 16S rDNA sequence is 99% (MEILE et al., 1997).

Table 1. Identification of *Bifidobacterium* strains isolated from human sources

Name	Source	Fermentation test	Complex carbohydrate	16S rDNA analysis
B1.1	Infant faeces	*B. angulatum*	*B. pseudocatenulatum*	*B. pseudocatenulatum* (98%~1000b)
B1.2	Infant faeces	*B. bifidum*	*B. bifidum*	*B. bifidum* (97%~1000b)
B2.1	Infant faeces	*B. dentium*	*B. dentium*	*B. dentium* (96%~600b)
B2.2	Infant faeces	*B. longum*	*B. longum*	*B. longum/infantis* (97%~800b)
B2.3	Infant faeces	*B. dentium*	*B. dentium*	*B. dentium* (98%~900b)
B3.1	Infant faeces	*B. bifidum*	*B. bifidum*	*B. bifidum* (97%~1000b)
B3.2	Infant faeces	*B. bifidum*	*B. bifidum*	*B. bifidum* (97%~900b)
B4.1	Infant faeces	*B. angulatum*	*B. catenulatum*	*B. pseudocatenulatum* (97%-900b)
B5.1	Infant faeces	*B. bifidum*	*B. bifidum*	*B. bifidum* (97%~1000b)
B6.1	Infant faeces	*B. longum*	*B. longum*	*B. longum/infantis* (97%~800b)
B7.1	Infant faeces	*B. bifidum*	*B. bifidum*	*B. bifidum* (97%~1100b)
B7.5	Infant faeces	*B. bifidum*	*B. bifidum*	*B. bifidum* (97%~400b)
B8.2	Infant faeces	*B. bifidum*	*B. bifidum*	*B. bifidum* (85%~625b)
B9.1	Infant faeces	*B. breve*	*B. breve*	*B. breve* (97%~1000b)
B9.11	Infant faeces	*B. breve*	*B. breve*	*B. breve* (97%~1000b)
B9.14	Infant faeces	*B. breve*	*B. breve*	*B. breve* (97%~1100b)
B9.15	Infant faeces	*B. breve*	*B. breve*	*B. breve* (97%~1100b)
B10.1	Infant faeces	*B. longum*	*B. longum*	*B. longum/infantis* (97%~900b)
B11.1	Infant faeces	*B. longum*	*B. longum*	*B. longum/infantis* (74%~300b)
A1.2	Adult faeces	*B. longum*	*B. longum*	*B. longum/infantis* (96%~800b)
A3.2	Adult faeces	*B. dentium*	*B. dentium*	*B. dentium* (97%~900b)
A4.3	Adult faeces	*B. longum*	*B. longum*	*B. longum/infantis* (98%~1000b)
A4.4	Adult faeces	*B. longum*	*B. longum*	*B. longum/infantis* (96%~600b)
A4.6	Adult faeces	*B. longum*	*B. longum*	*B. longum/infantis* (97%~800b)
A4.8	Adult faeces	*B. longum*	*B. longum*	*B. longum/infantis* (96%~800b)
A4.9	Adult faeces	*B. longum*	*B. longum*	*B. longum/infantis* (97%~900b)

The isolates derived from infants can be classified as *B. bifidum, B. breve, B. longum/infantis, B. dentium* and *B. pseudocatenulatum.* All of the adults' isolates were *B. longum* except one, which was *B. dentium*.

2.2 Adherence of Bifidobacteria *to human tissue cultures*

The results of investigation of the adherence of *Bifidobacterium* strains to HeLa, HT29 and Hep2 tissue cultures are summarised in Table 2.

No adhesion was shown by the strains of *B. dentium (*A3.2), *B. bifidum* (B1.2), *B. dentium* (B2.1), *B. dentium* (B2.3), *B. pseudocatenulatum* (B4.1) and *B. longum* (B6.1). The strains of *B. bifidum* (B3.2) showed strong adhesion to HeLa tissue. Medium adhesion was observed in case of *B. bifidum* strain (B3.1). Slight adhesion to HeLa tissues of the strains of *B. longum* (A1.2), *B. pseudocatenulatum* (B1.1), *B. longum* (B2.2), *B. longum*^{T}, *B. bifidum*^{T} and *B. infantis*^{T} were observed.

Table 2. Adhesion of *Bifidobacterium* strains to human tissue cultures

Strains	HeLa tissue culture	HT29 tissue culture	Hep2 tissue culture
B. bifidum (B3.2)	+++	+++	+++
B. bifidum (B3.1)	++	–	–
B. bifidum (B5.1)	–	+	+
B. dentium (B2.1)	–	–	–
B. dentium (B2.3)	–	–	–
B. dentium (A3.2)	–	–	–
B. longum (B2.2)	+	–	–
B. longum (B6.1)	–	–	–
B. longum (A1.2)	+	+	++
B. pseudocatenulatum (B 1.1)	+	+	+
B. pseudocatenulatum (B 4.1)	–	–	–
B. adolescentis^T	–	–	+
B. bifidum^T	+	–	–
B. breve^T	–	+	–
B. infantis^T	+	+	+
B. longum^T	+	–	–

+ slight adhesion, 2–3 bacteria/cell
++ medium adhesion, 4–6 bacteria/cell
+++ strong adhesion, more than 6 bacteria/cell
– no adhesion

Investigating the adherence to HT29, the results can be summarised as follows: strong adherence of *B. bifidum* (B3.2), slight adherence of *B. longum* (A1.2), *B. pseudocatenulatum* (B1.1), *B. bifidum* (B5.1), *B. breve*^{T} and *B. infantis*^{T} were observed.

With respect to adherence to Hep2 tissue, the results can be summarised as follows: strong adhesion of *B. bifidum* (B3.2), medium adhesion of *B. longum* (A1.2), and slight adhesion of *B. pseudocatenulatum* (B1.1), *B. bifidum* (B5.1), *B. adolescentis*^{T}and *B. infantis*^{T} were observed.

2.3. Antagonistic effects of Bifidobacterium *strains on human enteropathogenic bacteria*

Out of the 224 treatments in 20 cases, growth inhibition of the enteropathogenic bacteria was detected. Three out of 12 tested isolates and one authentic strain, *B. longum*^{T}, repressed the growth of enteropathogenic bacteria (Table 3).

None of the tested *Bifidobacterium* strains inhibited the growth of *Shigella dysenteriae* and the *Klebsiella pneumonia*. The strain of *B. bifidum* (B1.2) repressed the growth of *Salmonella derby* 10032, *Salmonella typhi* 15001 & 15003, *Shigella flexneri* 20015, *Escherichia coli* 35033 & 35034, *Proteus mirabilis* 60007 and *Yersinia enterocolitica* 98001. The strain of *B. pseudocatenulatum* (B4.1) repressed the growth

of *Salmonella cholerae-suis* 10051, *Salmonella typhi* 15001 & 15003, *Shigella flexneri* 20015, *Proteus mirabilis* 61369 and *Yersinia enterocolitica* 98001. The strain of *B. bifidum* (B5.1) inhibited the growth of *E. coli* 35033 & 35034 and *Proteus mirabilis* 61369 strains. *B. longum*^{T} repressed the growth of *Proteus mirabilis* 61369, *Salmonella cholerae-suis* 10051 and *Yersinia enterocolitica* 98001. The strains of *Proteus mirabilis* and *Yersinia enterocolitica* were the most sensitive to growth of the tested *Bifidobacterium* strains.

Table 3. Interaction of *Bifidobacterium* strains with the HNCMB enteropathogenic strains

Strains	Number of HNCMB of enteropathogenic bacteria													
	Salmonella derby 10032	*S. cholerae-suis* 10051	*S. typhi* 15001	*S typhi* 15003	*Shigella dysenteriae* 20001	*Shigella dysenteriae* 20002	*Shigella flexneri* 20015	*E. coli 055H6* 30213	*E. coli* 35033	*E. coli* 35034	*Klebsiella* sp. 52047	*Proteus mirabilis* 60007	*Proteus mirabilis* 61369	*Yersinia enterocolitica* 98001
B. bifidum (B1.2)	+		+	+			+		++	+		+		+
B. bifidum (B5.1)									++	++			++	
B. pseudocatenulatum (B4.1)		+	+	+			+						++	+
B. longum^{T}		+											++	+

\+ slight inhibition
++ strong inhibition

2.4. Utilisation of oligosaccharides by Bifidobacteria *and enteropathogens*

The applicability of the tested oligosaccharides as prebiotics was defined according to the utilisation by *Bifidobacteria*. In these investigations commercially available oligo-saccharides, regarded as prebiotics, were tested. The results are summarised in Table 4.

Raftiline, Raftilose and the samples of Lactosucrose were utilised by all *Bifidobacterium* strains applied. Some differences were observed between the *Bifidobacterium* strains used in the utilisation of isomalto-oligosaccharides. *Bifidobacterium adolescentis*^{T}, *Bifidobacterium infantis*^{T} and the isolate of *B. longum* (B6.1) did not grow on Isomalto500 as a sole carbon source. Oligotime was not utilised by the isolate of *B. bifidum* (B1.2). None of xylo-oligosaccharides was utilised by the strains of *B. breve*^{T}, *B. bifidum* (B1.2) and *B. breve* (B9.11). It can be concluded that the commercially available prebiotics support the growth of *Bifidobacteria.* These findings prove that the tested materials can be regarded as prebiotics.

Table 4. Utilisation of commercially available prebiotics by *Bifidobacterium* strains

Oligosaccharide	*B. adolescentis*^{T}	*B. breve*^{T}	*B. infantis*^{T}	*B. longum*^{T}	*B. bifidum* (B1.2)	*B. breve* (B9.11)	*B. longum* (A1.2)	*B. longum* (B2.2)	*B. longum* (B6.1)	*B. longum* (A4.3)
Isomalto 500	–	+	–	+	+	+	+	+	–	+
OLIGOTIME	+	+	+	+	-	+	+	+	+	+
OLIGOMT 500	+	+	+	+	+	+	+	+	+	+
LS-44L	+	+	+	+	+	+	+	+	+	+
LS-55P	+	+	+	+	+	+	+	+	+	+
Raftilose	+	+	+	+	+	+	+	+	+	+
Raftiline	+	+	+	+	+	+	+	+	+	+
Xylo-oligo70	+	–	+	+	–	–	+	+	+	+
Xylo-oligo95	+	–	+	+	–	–	+	+	+	+

+ fermentation
– no fermentation

Table 5. Utilisation of commercially available prebiotics by enteropathogenic bacteria

Oligosaccharide	*Salmonella derby* 10032	*Salmonella cholerae-suis* 10051	*Salmonella typhi* 15001	*Shigella dysenteriae* 20001	*Shigella flexneri* 20015	*E coli* 055H6 30213	*E coli* 35033	*Klebsiella* sp. 52047	*Proteus mirabilis* 61369	*Yersinia enterocolitica* 98001
Isomalto 500	+	+	+	+	+	+	+	+	+	+
OLIGOTIME	–	–	+	–	–	–	–	+	–	–
OLIGOMT 500	+	+	+	+	+	–	+	+	–	–
LS-44L	+	+	+	+	+	+	+	+	+	+
LS-55P	–	–	–	–	–	+	+	+	–	+
Raftilose	+	+	+	+	+	+	+	+	+	+
Raftiline	+	+	+	+	+	+	+	+	+	+

+ fermentation
– no fermentation

In course of the design of prebiotic food for the selection of oligosaccharides a secondary screening was carried out applying enteropathogenic bacteria, based on the results gained in the cultivation experiment (Table 5).

Oligotime was selected to be used as a prebiotic, because this oligosaccharide did not support the growth of numerous enteropathogenic bacteria such as *Salmonella derby, Salmonella cholerae-suis, Shigella dysenteriae, Shigella flexneri, E. coli* 055H6, *E. coli, Proteus mirabilis* and *Yersinia enterocolitica.* Maybe it preferably enhances the growth of *Bifidobacteria*. Utilisation of Xylooligosaccharides by enteropathogens has not been tested yet.

2.5. In vivo feeding experiments with healthy and antibiotic treated mice

The effect of functional food was evaluated in *in vivo* feeding experiments where mice were used as test animals. During the experiment the composition of the microbiota in their faeces were investigated.

The feeding experiments with healthy mice lasted for four weeks. During this period the composition of the microbiota of the mice' faeces did not change characteristically. The antagonism of *Bifidobacteria* to the *Proteus* sp. carried by certain test animals could not be proved. The disappearance of heamolyzing bacteria can be regarded as a positive effect in case of mice that were fed by functional food.

In the second investigation the microbiota of the mice was killed with antibiotics. After that yeasts became dominant in their faeces. After the four-week feeding period the microbiota of the group fed with synbiotic was restored and the healthy balance was reinstated.

3. Conclusion

The colon is a highly complex organ, which plays a major role in food assimilation and in the physiological effects of diet, however its role is often underestimated. The composition of colonic microbiota contributes to a great extent to health and well-being of the host organism. The improvement of the intestinal microbiological balance can be reached by implantation of beneficial bacteria or by providing nutrients, which specifically support the growth of resident bacteria with health enhancing effects.

For the creation of functional food *Bifidobacteria* were used as probiotics. The explanation for the selection of *Bifidobacteria* is that *Bifidobacteria* is the most predominant bacteria in the intestinal microbiota. To enhance the probability of the adherence of *Bifidobacteria* derived from external sources, *Bifidobacteria* from human origin were isolated. The isolated strains were identified at genus and species level. The identification of *Bifidobacteria* species is problematic because of phenetic and genetic heterogeneities. From human faeces we managed to isolate various friendly *Bifidobacterium* species: *B. bifidum, B. breve, B. longum, B. pseudocatenulatum*, moreover *B. dentium* which is the only pyogenic species of human *Bifidobacteria*.

This species can be identified easily, and there is no risk to apply as probiotic strain. Some isolates from human origin showed strong adherence to the human tissue cultures, while the authentic *Bifidobacterium* strains showed weak, if any. It is important to note that some isolates had antagonistic effect on various enteropathogenic bacteria.

An attempt was made to create pro-, pre- and synbiotic food. Designed functional foods aimed to improve the balance of colonic microbiota and to favour useful bacteria in it. To fulfil this aim for the preparation of probiotic food, *Bifidobacterium longum*^{T} strain, which is resident in the colon over human life, was used. In the prebiotic food, Oligotime® oligosaccharide, which survives the direct metabolism of the host and reaches the colon, where it serves as a preferential nutrient for *Bifidobacteria*, was applied. Synbiotic food contained both *Bifidobacterium* strain as probiotic and Oligotime® as prebiotic.

The composition of the microbiota isolated from faeces of mice fed with functional food did not change drastically, though after the four-week feeding the haemolyzing enteropathogenic bacteria were not detectable in faeces of mice, which were fed functional food. The antagonistic effect of *Bifidobacteria* on resident *Proteus* species was not observed. It can be regarded as a positive result that *Bifidobacteria* were isolated from the faeces of mice fed pro- or synbiotic food.

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