Selection of probiotic bifidobacteria for lambs

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ABSTRACT: Twenty-six bifidobacteria were isolated from faecal samples of lambs. The isolates were identified, functional properties (survival ability at low pH and bile conditions) and antimicrobial activities against potential pathogens were determined. From the isolates with suitable properties (13 strains) rifampicin-resistant mutants were prepared by gradient plate techniques. This property enabled us to differentiate the administered organism from wild strains because resistance to rifampicin is rare among bifidobacteria. Rifampicin-resistant bifidobacteria (RRBifs) were administered to 3-days-old lambs in two trials. In the first trial the strain *B. ruminantium* L29 was applied to 3 lambs and was detected in faecal samples at high counts (6 log CFU/g on average) for one week. In the second trial 3 lambs received a "cocktail" of 12 strains and RRBifs survived in the intestinal tract at counts of about 6 log CFU/g for 25 days. The control group without probiotic treatment consisted of 6 animals. In both treated groups RRBifs dominated among bifidobacteria after their administration. Total bifidobacterial counts (5.64–7.32 log CFU/g) were significantly higher ($P < 0.05$) in treated groups compared to 2.31–2.85 log CFU/g detected in the control group during the first month of lamb life. Lactobacilli counts were also significantly higher ($P < 0.05$) in treated groups compared to the control. The administered bifidobacteria did not affect any other monitored bacterial groups. On the basis of *in vitro* test results, suitable probiotic bifidobacterial strains for lambs were chosen. Some of them survived for 30 days in the gastrointestinal tract of treated lambs, but no tested strain was able to colonise the lamb's tract permanently. The administration of bifidobacterial "cocktail" and consequent identification of the best survived strain seems to be an effective method for selection of potential probiotics.

Keywords: bifidobacteria; lambs; probiotics; faecal flora

The importance of the natural gut microflora for reducing diseases in humans and animals has long been recognised and it is now apparent that the composition of the microflora plays a crucial role both in digestion and in resistance to diseases (Moore, 2004). Different environmental factors may affect the gut microbial ecology; they include diet, medication, stress, age and general living conditions. Many dietary strategies were developed to improve the normal gastrointestinal microbiology; a popular concept is the use of probiotics (Gibson and Fuller, 2000). Probiotics are live microorganisms, generally bacteria but also yeasts that, when ingested alive in a sufficient amount, have a positive effect on the gut microflora resulting in the improved health status (Anadón et al., 2006). A variety of microbial species have been used as probiotics, including the species of *Bacillus*, *Bifidobacterium*, *Enterococcus*, *E. coli*, *Lactobacillus*, *Lactococcus*, *Streptococcus*, a variety of yeast species, and undefined mixed cultures (Simon et al., 2001). Especially bifidobacteria are often incorporated in

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fermented milk products and food supplements for humans and seem to be very effective probiotics (Bergonzelli et al., 2005; Leahy et al., 2005) whereas the species of *Lactobacillus*, *Bacillus*, *Enterococcus* and *Saccharomyces* yeast have been used most extensively in livestock (Wallace and Newbold, 1992; Simon et al., 2001). However, there has been a recent increase in research on feeding bifidobacteria to livestock (Abe et al., 1995; Patterson and Burkholder, 2003; Moore, 2004; Shim et al., 2005). Rada et al. (2006) reported that bifidobacteria are a more numerous bacterial group in the gastrointestinal tract of young ruminants than lactobacilli and there is a reasonable presumption that bifidobacteria could be an effective probiotic for ruminants during the milk-feeding period.

Bifidobacteria are Gram-positive, non-spore-forming, non-motile, anaerobic, saccharolytic, irregular rods. The typical habitat of this genus is the human and warm-blood animal intestinal tract. Bifidobacteria dominate in breast-fed infants being supported by "bifidogenic factors" present in human milk. Their occurrence in other mammal kids during the milk-feeding period is also high (Abe et al., 1995; Vlková et al., 2006). The presence of bifidobacteria in the gastrointestinal tract has been associated with a number of health benefits. Inhibition of pathogens is one of the beneficial effects of bifidobacteria. Proposed mechanisms of pathogen inhibition include competition for nutrients, production of toxic conditions and compounds (volatile fatty acids, low pH, and bacteriocins), competition for binding sites on the intestinal epithelium, and stimulation of the immune system (Servin, 2004; Moroni et al., 2006). Other positive effects that are ascribed to bifidobacteria are vitamin production, degradation and fermentation of food ingredients, stimulation of feeding tolerance, and reduction of allergic symptoms. Bifidobacteria contribute to a reduction in the level of unfavourable metabolites (ammonium and procarcinogenic enzymes) in colon (Gibson and Fuller, 2000; Leahy et al., 2005).

The use of bifidobacterial strains as a probiotic for livestock increased, especially after the application of antibiotics as growth promoters was banned in the European Union. In animal husbandry probiotics are recommended to increase weight gains, to improve feed conversion ratios and to control diarrhoea therapeutically (Moore, 2004). In order to exert a beneficial effect, probiotic bacteria should be viable and present at high numbers in the product at the time of consumption (McBrearty et al., 2001). Moreover, the majority of the bifidobacterial species are host specific and it is recommended that the bifidobacterial strains used as probiotics should originally be isolated from the same species as the intended use ought to have an enhanced chance of survival. Many selection criteria were proposed for assessment of new probiotic strains. Strains should be generally recognized as safe with minimal possibilities for the antibiotic resistance transfer. Probiotic strains should be stable during the gastrointestinal passage (resistance to low pH and bile acids) and also in the product (resistance to oxygen and technological process), and they should maintain good viability and functionality during storage. It is required that probiotics would survive in the gastrointestinal microbial ecosystem; adherence to the gut epithelium would enhance their survival ability. Other probiotic characteristics are inhibition of pathogens, modulation of metabolic activities and immunomodulation. Probably no probiotic strain has all these characteristics, but it is desirable that as many as possible should be present (Vaughan and Mollet, 1999; Gibson and Fuller, 2000; Mättö et al., 2006).

The objectives of this study were to isolate, identify and characterise bifidobacteria from lamb faeces. The main aim of the work was the selection of potentially new probiotic strains for lambs mainly according to their ability to survive in *in vivo* conditions.

MATERIAL AND METHODS

Bacteria isolation and identification

Bifidobacteria were isolated from faecal samples of lambs (Charolais) during the milk-feeding period. Isolations were performed using modified TPY agar (Sharlau, Barcelona, Spain) supplemented with mupirocin (100 g/l) and glacial acetic acid (1 ml/l) according Rada and Petr (2000). Bifidobacterium isolates were identified by the following criteria: (i) they are Gram-positive pleomorphic rods, (ii) they show fructose-6-phosphate phosphoketolase activity (F6PPK) as determined by the method described by Orban and Patterson (2002), (iii) they have a positive reaction with genus-specific primers (Kok et al., 1996), (iv) they have a positive reaction with fluorescence-labelled probe (a kit for *Bifidobacterium* sp., RiboTechnologies, Groningen,

The Netherlands). Bifidobacteria were tentatively identified by their carbohydrate fermentation profiles according to Biavati and Mattarelli (1991). Biochemical profiles were determined by API 50 CHL and API ID 32 A Rapid tests (BioMérieux, France). Five strains originated from the gastrointestinal tract of calves obtained from the German Resource Centre for Biological Material: *B. ruminantium* DSMZ 6489, *B. merycicum* DSMZ 6492, *B. pseudolongum* subsp. *globosum* DSMZ 20092, *B. thermophilum* DSMZ 20210, and *B. boum* DSMZ 20432 were used as control microorganisms. Bifidobacterial cultures were maintained at –70°C in TPY broth (Sharlau, Barcelona, Spain) containing glycerol (20% v/v) and subcultured anaerobically at 37°C for 24 h in the same medium.

Clostridium difficile KK4 was isolated from an infant faecal sample using Reinforced Clostridial Agar (Oxoid) supplemented with novobiocin (8 mg/l) and colistin (8 mg/l; Colado and Sanz, 2007). The strain was characterised by API 20A (BioMérieux, France) and identified to the species level using a fluorescence *in situ* hybridization (FISH) kit for *Clostridium difficile* (RiboTechnologies, Groningen, The Netherlands). Strain *E. coli* O55 was kindly provided by Dr. Igor Šplíchal from the Institute of Microbiology, Academy of Sciences of the Czech Republic, Nový Hrádek.

In vitro testing and characterization of bifidobacteria

Properties predicting the survival of bifidobacteria during passage through the gastrointestinal tract of the animal (acid and bile tolerance) were tested using the method described by Saarela et al. (2003). Briefly: for the acid and bile tolerance test a cell suspension of overnight bifidobacterial cultures was mixed with 2 ml of pH 3 buffer (PBS, pH adjusted with HCl) for acid tolerance test, with 2 ml of PBS buffer (pH 7.2) containing 1.5% bile extract for bile tolerance test, or with 2 ml of PBS buffer (pH 7.2) for the control. The bacterial suspensions were incubated at 37°C for 1 and 2 h (acid tolerance), 2 and 3 h (bile tolerance), or 0 and 3 h (control). The residual viable counts were determined by the standard plate count methods using TPY agar.

The antimicrobial activity of bifidobacterial cell-free supernatants against *E. coli* O55 and *Clostridium difficile* KK4 was tested by the agar-well diffusion method. Cell-free culture supernatants were obtained by microcentrifugation (18 000 × g, 4°C, 20 min) of overnight bifidobacterial cultures, pH of the supernatants was measured with a pH-meter (Schott, Germany). Twenty ml of nutrient agar (Wilkins-Chalgren for *Cl. difficile* or Miller-Hinton for *E. coli* O55, both Oxoid) were added to 1 ml of overnight culture of either *Cl. difficile* or *E. coli*. Plates were dispersed and agar was let solidify. Six wells were created using a 6 mm cork borer. Into the wells 100 µl of the cell-free supernatant of bifidobacteria in triplicate were pipetted, plates were placed into anaerobic jars (Anaerobic Plus System, Oxoid) with palladium catalyst (Oxoid BR 42; atmosphere CO_2:H_2 10:90) and incubated for 10 hours at 4°C to let the supernatant diffuse to agar. After it plates were cultured 24 h at 37°C and zones of inhibition were measured. The antimicrobial activity of the cell-free bifidobacterial supernatant neutralised with 1M NaOH was tested by the same procedure.

Bifidobacterial strains were screened for the ability to ferment cow milk and survive in this condition. Milk was prepared from low-fat dried milk (10 g/100 ml of distilled water), dosed into tubes, boiled for 30 min, hermetically closed, and cooled to 37°C. Overnight growth cultures were inoculated to the milk, milk was fermented for 24 h at 37°C and bifidobacterial counts were determined by cultivation using TPY agar (Sharlau, Barcelona, Spain). Fermented milk was stored at 4°C and bifidobacteria were detected in one-week period.

Preparation of rifampicin-resistant bifidobacteria

From 13 bifidobacterial strains with suitable properties rifampicin-resistant mutants (RRBifs) were prepared by gradient plate techniques. Briefly: Liquid nutrient agar was poured into Petri dishes and dishes were placed at an angle. After agar solidification, liquid nutrient agar with rifampicin (100 mg/l) was poured over the first layer. It solidified and formed an antibiotic gradient. Then, the plates were inoculated with bifidobacteria. After incubation rifampicin-resistant mutant bifidobacteria were isolated from high-drug areas. This property enabled us to differentiate the administered organism from wild strains, because resistance to rifampicin is rare among wild strains of bifidobacteria (Rada et al., 1995). RRBifs showed

the same physiological and biochemical characteristics as original isolates.

Administration of bifidobacteria to lambs

Three-day old sucking lambs from Milan Slavík Farm (Mšec-Háje, West Bohemia Region) were fed milk fermented by RRBifs. Marked strains were subcultured in TPY broth. For the first 3 days of life, lambs with their dams were housed separately and lambs were fed mother's milk exclusively. On the 4th day of life lambs with dams were moved to the flock of sheep where calf starter, hay and water were available *ad libitum*. In the first trial, 3 three-days-old lambs were fed milk fermented by a monoculture of *B. ruminantium* L29. In the second trial, single administration of a mixture of 12 strains ("cocktail" administration) to 3 three-days-old lambs was done. The control group consisted of 6 lambs and was without probiotic treatment.

Sampling, bacteria enumeration, and detection of administered bifidobacteria

Faecal samples were taken using sterile gloves from the rectum, transferred to the tube filled with Wilkins-Chalgren broth (Oxoid) and transported to the laboratory within 2 h. The first samples were taken before the administration of bifidobacteria and lambs were re-sampled in approximately one week periods. Samples were serially diluted in the Wilkins-Chalgren broth (Oxoid) under anaerobic conditions. Rifampicin-resistant bifidobacteria were enumerated using modified TPY agar supplemented with mupirocin (100 mg/l), glacial acetic acid (1 ml/l) and rifampicin at the concentration of 100 mg/l. The administration of the "cocktail" containing 12 different bifidobacterial strains enabled us to select effectively probiotic bacteria with higher survival ability in the intestinal tract. Rifampicin-resistant strains from the highest dilution were identified (as described above) and compared with pure cultures used for probiotic treatment by biochemical tests API 50 CHL and API ID 32 A Rapid (BioMérieux, France).

Faecal bacteria were detected using selective agars. Appropriate dilutions were transferred to sterile Petri dishes, which were immediately filled with the media for bifidobacteria (TPY agar, Sharlau, Spain) supplemented with mupirocin (100 mg/l) and glacial acetic acid (1 ml/l) according to Rada and Petr (2000), lactobacilli (Rogosa agar, Oxoid) and anaerobes (Wilkins-Chalgren, Oxoid). Bifidobacteria and anaerobic bacteria were incubated in anaerobic jars (Anaerobic Plus System, Oxoid) at 37°C for 72 h. Lactobacilli were incubated aerobically at 37°C for 48 h. Petri dishes with TBX agar (Oxoid) for *E. coli* and Slanetz-Bartley (Oxoid) for enterococci were inoculated with 0.1 ml of an appropriate dilution and spread using sterile glass rods. Inoculated plates were incubated aerobically at 37°C for 24 h (*E. coli*) or 48 h (enterococci).

Specific fluorescence in situ hybridisation (FISH) kits for *Bifidobacterium* spp., *E. coli*, *Lactobacillus* sp., and total bacteria by the fluid method (Ribo-Technologies, Groningen, The Netherlands) were used for the quantitative detection of bacteria in faecal samples. After hybridisation, samples were analysed with a Nikon E-800 epifluorescence microscope and software Lucia 5.10.

Statistical analyses

Means and standard deviations of bacterial counts were calculated. The one-sample Kolmogorov-Smirnov test of composite normality was used to confirm normal distribution of data. The significance of differences between the control and experimental group and between the methods used for faecal bacteria detection was evaluated by *t*-test.

RESULTS

Bifidobacteria isolation, identification and characterisation

Sixty-five wild strains of bifidobacteria were isolated from 18 calves aged 2–80 days. The isolates were identified as a member of the genus *Bifidobacterium* by morphological characteristics, F6PPK test, and genus-specific PCR and FISH. Twenty-six strains with different morphology were chosen for detailed characterisation and *in vitro* testing (for results see Table 1). Using a biochemical test 9 strains were identified as *B. merycicum*, 5 as *B. thermophilum*, 5 strains as *B. pseudolongum*, 2 strains belonged to the species *B. ruminantium* and 5 were not identified by the method used.

Table 1. Identification and functional properties of bifidobacteria isolated from lamb faecal samples

Strain	Identified as	Delta log CFU/ml decrease after incubation in				Inhibition zones (mm)*	
		pH 3		1.5% bile			
		1 h	2 h	2 h	3 h	*E. coli* O55	*Cl. difficile* KK4
L1	*B. merycicum*	0.21	0.27	0.11	0.12	6.00	8.67
L2	*B. thermophilum*	0.50	1.99	0.00	0.24	12.00	6.00
L3	*B. merycicum*	0.00	2.88	0.00	0.00	6.00	8.33
L4	*B. merycicum*	0.56	0.66	0.00	0.30	10.67	7.00
L5	*B. merycicum*	0.00	0.12	0.58	0.60	10.67	9.00
L6	*Bifidobacterium* sp.	1.68	> 5	0.75	2.15	6.00	9.00
L7	*Bifidobacterium* sp.	0.00	0.00	0.00	0.00	6.00	8.00
L8	*B. merycicum*	0.00	0.00	0.00	0.00	9.33	7.00
L9	*B. merycicum*	0.53	0.91	0.86	0.93	11.33	6.00
L10	*B. merycicum*	0.40	0.44	0.12	> 5	6.00	8.67
L11	*B. pseudolongum*	0.46	0.79	0.00	0.86	6.00	7.33
L12	*Bifidobacterium* sp.	0.00	0.14	0.18	0.20	6.00	7.00
L13	*B. pseudolongum*	1.61	1.78	0.18	0.21	8.67	8.00
L14	*B. ruminantium*	0.53	1.11	1.15	1.92	14.00	9.00
L15	*B. pseudolongum*	0.95	2.51	0.06	1.66	10.00	6.00
L16	*Bifidobacterium* sp.	0.51	1.47	1.13	1.58	6.00	7.67
L17	*B. merycicum*	0.87	1.51	0.00	0.85	6.00	7.33
L18	*Bifidobacterium* sp.	2.77	4.77	0.00	0.11	6.00	6.00
L19	*B. ruminantium*	0.03	0.21	0.11	0.25	10.67	8.67
L20	*B. pseudolongum*	0.00	1.50	1.52	1.63	8.00	10.00
L21	*B. thermophilum*	> 5	> 5	0.08	0.10	6.00	6.00
L22	*B. pseudolongum*	2.34	> 5	2.48	2.64	12.00	9.00
L23	*B. merycicum*	0.96	> 5	0.00	0.26	13.33	10.00
L24	*B. thermophilum*	0.00	0.00	0.00	0.62	9.33	6.00
L25	*B. merycicum*	0.00	0.00	0.16	0.20	6.00	6.00
L26	*B. thermophilum*	0.00	1.10	0.23	1.43	10.00	9.00
Control strains (DSMZ)							
B. boum 20432		2.80	4.28	0.54	1.50	6.00	8.33
B. merycicum 6492		0.62	0.82	0.56	0.71	6.00	7.33
B. pseudolongum ssp. *globosum* 20099		0.00	0.20	0.00	0.00	6.00	6.67
B. ruminantium 6489		4.93	> 5	0.19	0.19	6.00	8.00
B. thermophilum 20210		1.54	2.43	0.00	0.00	7.33	6.67

*susceptibility of *E. coli* O55 and *Cl. difficile* KK4 to bifidobacterial supernatant (diameters are means of three determinations); strains printed in bold letters were chosen for administration to lambs
DSMZ = German Resource Centre for Biological Material

Table 2. Counts of bifidobacteria (bif) and rifampicin-resistant bifidobacteria (RRBifs) in lamb faecal samples before and after the administration of strain *B. ruminantium* J10IV

Lamb No.	Age (days)	Days after RRBif administration	Bacterial counts log CFU/g		(%) RRBifs*
			bif	RRBifs	
1	3	0	ND	ND	
	12	9	8.91	4.60	0.00049
	18	15	7.49	ND	0
2	3	0	ND	ND	
	11	8	7.45	7.43	95.50
	17	14	7.37	4.13	0.0057
	24	21	6.89	ND	0
3	2	0	ND	ND	
	10	9	6.33	5.36	10.72
	16	15	7.09	6.03	8.71
	23	20	6.14	ND	0

*percentage of RRBifs from the total counts of bifidobacteria
ND = not detected

The bifidobacteria suitable for administration to lambs were chosen by their ability to survive at low pH and in 1.5% bile extract and by their antimicrobial activity against potential pathogens (Table 1). In general, the tested strains were more sensitive to low pH than to bile extract. Eighteen out of the 26 wild strains tested and 4 out of the 5 control strains showed excellent bile tolerance (< 1 log CFU/ml decrease in viability) after 3 hours incubation. Twelve wild lamb isolates showed a decrease in viability even lover than 0.25 log CFU per ml. Only in 2 bifidobacteria a decrease in viability higher than 2 log CFU/ml compared to the control was observed. Wild bifidobacteria exhibited higher viability than reference strains after 2 hours incubation in low pH conditions. The survival of bifidobacteria at low pH was weaker than in 1.5% bile extract. Only 12 out of the 26 wild isolates and 2 out of the 5 control strains showed a decrease in viability lower than 1 log CFU/ml. In nine strains tested unsatisfactory survival ability was detected at pH 3 (decrease > 2 log CFU/ml).

Fifteen out of the 31 bifidobacterial cell-free supernatants tested (including control strains) inhibited the growth of *E. coli* O55 (15 wild and 1 control strain), diameters of inhibition zones varied from 8.00 to 14.00 mm, 10.49 mm on average. Ten strains of bifidobacteria with the antibacterial activity against *E. coli* O55 strongly inhibited this strain resulting in clear inhibition zones with diameter >10 mm around the well. No inhibitory activity of 16 bifidobacteria tested was found against *E. coli* O55 (Table 1). Twenty-four cell-free supernatants tested inhibited *Cl. difficile* KK4 (19 wild and 5 control strains), diameters of inhibition zones around wells ranged between 6.67 and 10.00 mm (8.15 m in average). No inhibitory activity against *Cl. difficile* KK4 was observed in the remaining bifidobacteria (7 strains) (Table 1). The pH values of cell-free supernatants varied between 3.6 and 4.0. The neutralized supernatant did not inhibit either *E. coli* O55 or *Cl. difficile* KK4.

Thirteen strains suitable for the administration to lambs were chosen on the basis of the results described above. The major criterion was the ability to survive passage through the gastrointestinal tract, e.g. resistance to low pH and bile extract. Antimicrobial activity was a minor criterion for bifidobacteria selection. The selected isolates (L1, L4, L5, L7, L8, L9, L11, L12, L17, L19, L20, L24, L25; Table 1) were screened for the ability to ferment cow milk and survive in these conditions, because bifidobacteria were administered to lambs in the form of fermented milk. Bifidobacterial counts

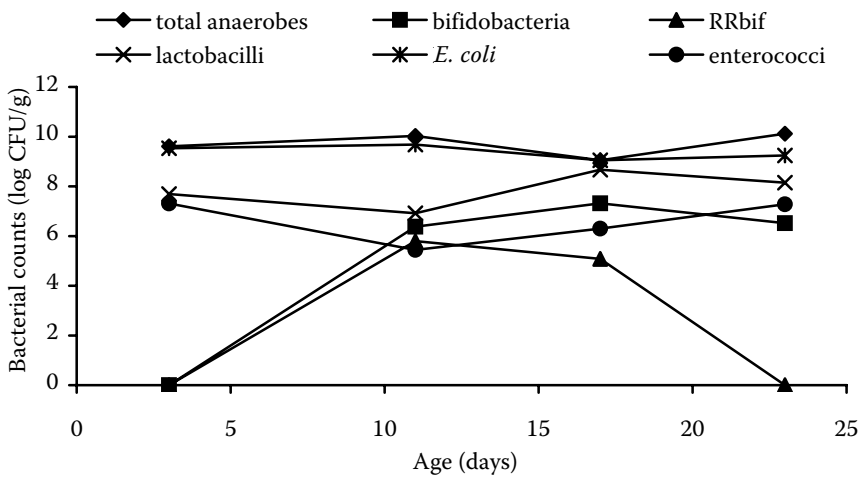

Figure 1. Bacterial counts detected by cultivation in faecal samples of lambs in trial 1 (lambs were fed *B. ruminantium* J10IV at 3 days of age); all data are means of 3 determinations

after 24 hours cultivation in milk ranged from 8.68 to 9.63 log CFU/ml, 9.07 log CFU/ml on average. Twelve out of the 13 strains survived in fermented milk for 1 month at counts > 10^6 CFU/ml, one strain (L17) reached this level only for 10 days. In 6 strains viability higher than 10^6 CFU/ml was observed even for 3 months.

Faecal bacteria enumeration and detection of administered bifidobacteria

In the first trial 3 three-days-old lambs were fed milk fermented by the monoculture of *B. ruminantium* L29. Bifidobacterial counts and viability of RRBifs in faecal samples of treated lambs are shown in Table 2 and Figure 1. In all three lambs no bifidobacteria were detected before the administration of this probiotic (three-days-old lambs). One week after administration, RRBifs accounted for 0.01–100% of total bifidobacteria (Table 2). In the second week after the probiotic treatment RRBifs were not detected in two lambs, in lamb no. 3 RRBifs accounted for 8.77% and were not present in the third week after administration. The average of RRBif counts was the highest in 11-days-old calves (one week after treatment), then they slowly decreased, the strain *B. ruminantium* L29 disappeared approximately 2 weeks after administration. The changes in the numbers of total anaerobic bacteria, bifidobacteria, RRBifs, lactobacilli, *E. coli*, and enterococci in lamb faecal samples in trial one are presented in Figure 1. No bifidobacteria were detected in 3-days-old lambs. Total bifidobacterial counts were 6.38 log CFU/g on the 11th day of life, then they increased to 7.32 log CFU/g on day 17, reaching the counts of 6.52 log CFU/g in the last sampling. Bifidobacterial counts in the treated group were significantly higher ($P < 0.05$) compared to the control group (2.31–2.85 log CFU

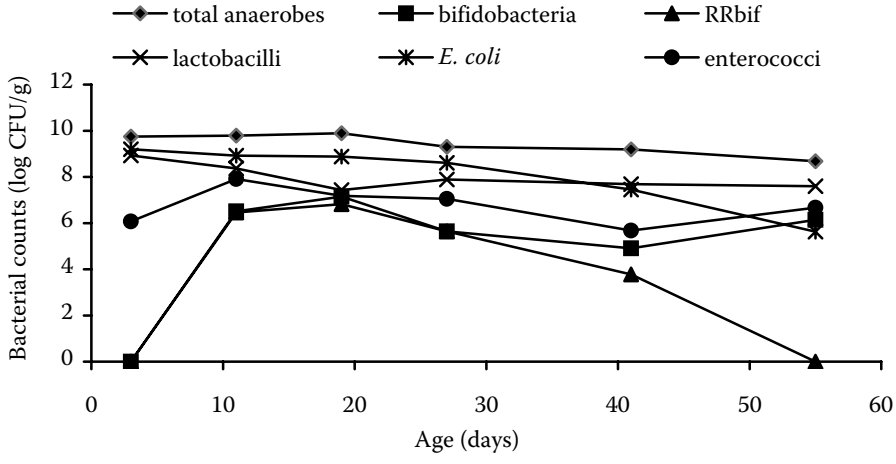

Figure 2. Bacterial counts detected by cultivation in faecal samples of lambs in trial 2 (lambs were fed a bifidobacterial "cocktail" at 3 days of age); all data are means of 3 determinations

Table 3. Counts of bifidobacteria (bif) and rifampicin-resistant bifidobacteria (RRBifs) in lamb faecal samples before and after the administration of bifidobacterial cocktail

Lamb No.	Age (days)	Days after RRBif administration	Bacterial counts log CFU/g		(%) RRBifs*
			bif	RRBifs	
4	3	0	ND	ND	
	11	8	8.14	8.14	100
	20	17	8.64	8.19	35.48
	28	25	5.37	5.37	100
	42	39	ND	ND	
	54	51	7.26	ND	0
5	3	0	ND	ND	
	12	9	6.02	5.87	70.79
	19	16	8.08	7.59	32.35
	28	25	5.90	5.90	100
	40	37	4.91	3.78	7.41
	56	53	5.01	ND	0
6	3	0	ND	ND	
	9	6	5.36	5.37	100
	18	15	4.72	4.67	89.12
	27	24	ND	ND	

*percentage of RRBifs from the total counts of bifidobacteria
ND = not detected

per g) in the first month of lamb life. In the treated group, lactobacilli were the second most numerous group of faecal bacteria, their counts varied from 6.92 to 8.67 log CFU/g. In the control group, lactobacilli were found at significantly lower ($P < 0.05$) counts (5.59 and 4.58 log CFU/g) compared to the treated group at the age of 17 and 23 days. *E. coli* dominated in faecal flora during the whole observation reaching similar counts like total anaerobic bacteria. In 3-days-old lambs enterococci were detected at counts of 7.31 log CFU/g, they decreased to 5.44 log CFU/g on the 11th day of age and their counts increased toward the end of the observation. No significant differences between the treated and control group were detected in entrococci, *E. coli* and total anaerobic bacteria counts.

In the second trial single administration of a mixture of 12 strains ("cocktail" administration) to 3 three-days-old lambs was done, results are shown in Table 3 and Figure 2. In the second experimental group no bifidobacteria were present in 3-days-old lambs either. One week after the administration of RRBifs total bifidobacteria were detected at counts of 6.51 log CFU/g on average, then they increased to 7.15 log CFU/g (maximum value detected in the second trial) and at 4 weeks of lamb age they decreased to 5.64 log CFU/g, these counts were significantly higher ($P < 0.05$) compared to the control group. During the first three observations after the probiotic treatment the cell counts of RRBifs copy the counts of total bifidobacteria, RRBifs accounted for 32.35–100% of total bifidobacteria (Table 3). The average of RRbifs hereafter decreased and the supplied strains were not detected on day 24 after administration in lamb 6, on day 39 in lamb No. 4. The best survival ability of administered bifidobacteria was observed in lamb No. 5, RRBifs disappeared on day 53 after treatment. At the end of the study (55 days of life) total bifidobacterial counts were 6.14 log CFU/g. RRBifs counts in trial 2 were insignificantly higher compared to counts detected in trial 1.

RRBifs were re-isolated from faecal samples of treated lambs and characterised by biochemical tests. The best survival ability in lamb 4 and 5 was observed in strains *Bifidobacterium* sp. L5 and *B. merycicum* L25. From lamb 5 strain *B. merycicum* L1 was also re-isolated in the last sample containing RRBifs. In lamb No. 6 strains *Bifidobacterium* sp. L7 and *B. merycicum* L8 showed the best survival ability.

Development of faecal bacteria of lambs from the second trial is demonstrated in Figure 2. Similarly like in trial one, *E. coli* dominated in the faecal flora of lambs in the first 4 weeks reaching counts of about 10^9 CFU/g. Then their counts decreased to 5.63 log CFU/g on the 55th day of life and were replaced by lactobacilli as well as bifidobacteria and enterococci. Lactobacilli were the second most numerous bacterial group in 3-days-old lambs reaching counts of 8.93 log CFU/g, then they decreased and their level (10^8 CFU/g) was relatively stable during the study, from the fifth week of life lactobacilli dominated in the faecal flora of lambs in trial 2. Their counts were significantly higher ($P < 0.05$) compared to the control group in the first month of life. The level of enterococci varied during the study, in 3-days-old lambs reaching the counts of 10^6 CFU/g, in the second observation it increased to 7.91 log CFU/g, then it decreased to 5.68 log CFU/g, and increased again to counts of 6.67 log CFU/g at the end of the study. There were no significant differences in the counts of *E. coli*, enterococci and total anaerobes between animals in the control and treated group (trial 2).

The development of faecal bacteria in the control group of lambs (without probiotic treatment) is shown in Figure 3. *E. coli* dominated again in the faecal flora of the control group in the first month of life (their counts varied from 9.19 to 8.22 log CFU per g). *E. coli* were replaced by lactobacilli which dominated in faeces, followed by bifidobacteria and enterococci. In 3-days-old lambs lactobacilli and enterococci showed the same counts (8.24 log CFU per g), then both groups decreased to 4.47 log CFU per g (lactobacilli) and 5.91 log CFU/g (enterococci) in 3-weeks-old lambs. Since the 4th sampling a rapid increase of lactobacilli to 10^8 CFU per g was observed at the end of the study. The level of enterococci also increased, but only to counts of 6.70 log CFU/g. Bifidobacteria were found to be a minor group of faecal microflora in lambs aged 3 to 21 days, reaching the counts of 1.01–2.85 log CFU per g. Bifidobacteria rapidly increased in 1-month-old lambs (8.25 log CFU/g) and their counts were 7.11 log CFU/g on the 50th day of observation. There were no diffe-rences in the development of total anaerobes, enterococci and *E. coli* in experimental lambs compared to control animals. In the control group a great reduction in lactobacilli was observed at 3 weeks of age, no such a distinct decline was observed in any of the experimental groups. Higher counts of bifidobacteria in experimental groups in the first 3 weeks were caused by the administration of RRBifs. In lambs included in trial one the high bifidobacteria level ($> 10^6$ CFU/g) was observed already in 3-weeks-old lambs, compared to the control group, where high bifidobacterial counts were found one week later.

Total anaerobic bacteria, bifidobacteria, lactobacilli, and *E. coli* were detected also by FISH (Table 4), but it was not possible to use this pro-

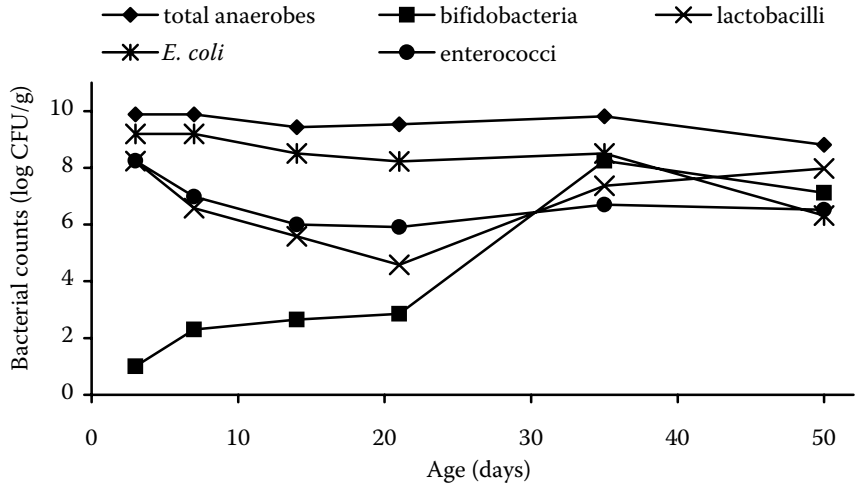

Figure 3. Bacterial counts detected by cultivation in faecal samples of lambs in the control group; all data are means of 6 determinations

Table 4. Comparison of bacterial counts (log CFU/g ± SD) in faecal samples of lambs in the control and experimental group detected by cultivation and FISH

	Control group		Experimental groups	
	cultivation	FISH	cultivation	FISH
Total anaerobes (n = 30)	9.56 ± 0.58	9.89 ± 0.38	9.72 ± 0.56	9.95 ± 0.30
Bifidobacteria (n = 5)	8.15 ± 0.32	8.20 ± 0.68	8.30 ± 1.12	8.46 ± 1.12
Lactobacilli (n = 24)	7.98 ± 0.95	8.36 ± 0.47	8.17 ± 0.68	8.34 ± 0.59
E. coli (n = 27)	8.57 ± 0.64	8.89 ± 0.73	8.68 ± 0.96	8.63 ± 0.64

cedure for bacteria quantification in all samples, because the detection limit of FISH method for ruminant faecal samples is approximately 10^7 CFU/g. In Table 4 the results are presented that were obtained only from samples where bacteria were detected by cultivation as well as by FISH. Bacterial counts determined by FISH are not significantly higher compared to cultivation.

DISCUSSION

To our knowledge, there is a lack of information about the occurrence of bifidobacteria in gastrointestinal tracts of small ruminants. Draksler et al. (2002) studied the development of faecal flora in young Creole goats at 1 to 270 days of age by cultivation methods. A modified selective HHD medium (Merck) was used for bifidobacteria quantification but the modification was not specified. Bifidobacteria were found to be a subdominant group of faecal microflora in 1-day-old goats reaching the counts of 6.41 log CFU/g. Therefore, a significant increase in bifidobacteria was observed between day 1 and 3. On the 3rd day of life the highest bifidobacterial level was found (7.89 log CFU/g), then it decreased to 4.89 log CFU/g at 3 onths. Collado and Sanz (2007) quantified the mucosa-adhered microbiota of lambs (aged 6–9 months) in rumen, duodenum, and colon by culture methods and FISH coupled with flow cytometry (FCM-FISH). Bifidobacteria and lactic acid bacteria were found to be predominant mucosa-adhered bacteria in lambs by both methods. The levels of bifidobacteria were significantly higher in colon and rumen compared to duodenum. Higher counts of analysed bacteria were found using the FCM-FISH method in rumen and colon compared to culture techniques. In our control group of lambs bifidobacteria were found to be a subdominant bacterial group in the first month of animal life by both methods (cultivation and FISH). Bifidobacteria rapidly increased in 1-month-old lambs (8.25 log CFU/g) and their counts were 7.11 log CFU/g on the 50th day of observation.

Bifidobacteria are a desirable bacterial group of intestinal microflora for their positive effects on microbial balance and host's general health. One of the strategies how to improve bifidobacterial counts in the intestine is their administration to animals. For the effective use of probiotic bacteria their ability to survive in *in vitro* conditions for a long period of time is necessary, or even their permanent colonisation of the gastrointestinal tract. A presumption for successful colonisation is the survival ability of probiotics during their passage through the gastrointestinal-tract of the animal. Probiotics must be acid and bile tolerant. Using the *in vitro* test, bifidobacterial strains which fulfil these criterions were found (Table 1) and were administered to lambs. In many studies spontaneously generated rifampicin-resistant mutants were used for differentiation of administered bacteria (Pedersen and Tannock, 1989; Rada et al., 1995; Bredholt et al., 2001). Resistance to antibiotics is stable and effective for differentiation of endogenous bacteria including bifidobacteria because resistance to rifampicin is rare among wild strains of bifidobacteria (Rada et al., 1995). The use of RRBifs seems to be a safe method because resistance to rifampicin is a stable trait and is not coded by plasmids, therefore it is not probably transmissible among bacteria in the natural environment (Pedersen and Tannock, 1989). Pedersen et al. (2003) did not find any differences between the rifampicin-resistant mutants of lactobacilli and their parent strains with respect to colony or cell

morphology, biochemical properties and other phenotypic properties. Neither were any differences in physiological and biochemical characteristics between rifampicin-resistant mutants of bifidobacteria and original strains found in our study.

Numerous studies reported on the inhibitory effect of bifidobacteria against a wide range of pathogenic microorganisms including pathogenic *Escherichia coli* and *Clostridium* sp. These microorganisms were often isolated from young ruminants with diarrhoea (Ishihara et al., 2001; Haschek et al., 2006; Uhde et al., 2008; Herrera-Luna et al., 2009). *Clostridium difficile* may be associated with calf diarrhoea and cattle may be an important source for human *C. difficile* infections (Rodriguez-Palacios et al., 2006). On the other hand, Rodriguez-Palacios et al. (2007) reported that the oral administration of *C. difficile* ribotype 077 to neonatal calves resulted in intestinal colonization but not in detection of toxins or symptoms of enteric disease. Some reports have shown an overlap of the isolates from calves and humans, including two types 027 and 017 of the predominant outbreak. *C. difficile* has also been found in retail meat samples, suggesting that food could be involved in the transmission of *C. difficile* from animals to humans (Rupnik, 2007). Twenty-two isolates of bifidobacteria from lambs showed the antimicrobial activity against *C. difficile* KK4 of human origin in our *in vitro* study. These strains seem to be promising probiotics for young ruminants to reduce the counts of *C. difficile* in their gastrointestinal tract as a source of infections for humans, but these results must be verified in clinical studies.

Several mechanisms have been suggested for the inhibitory action of bifidobacteria towards pathogens including a decrease in local pH by the production of organic acids (De Vuyst et al., 2004). But only few authors reported that the production of acetic and lactic acid is the sole factor responsible for the antagonistic activity of bifidobacteria (Fooks and Gibson, 2003). It has been suggested in numerous reports that other inhibitory substances may contribute to the antagonistic activity as well (Servin, 2004). The results of Makras and De Vuyst, 2005) showed that the inhibitory activity of bifidobacteria towards Gram-negative bacteria is dependent mainly on the lowering of pH, but some *Bifidobacterium* strains produced a bacteriocin in their study. However, the contribution of such inhibitory substances to the antibacterial activity of bifidobacteria was found to be negligible. In our study 15 out of the 31 bifidobacterial cell-free supernatants tested inhibited the growth of *E. coli* O55 and 22 strains were active against *Cl. difficile* KK4. Neutralized supernatants inhibit neither *E. coli* O55 nor clostridia. Our results showed that the antimicrobial activity of tested bifidobacteria is caused especially by a decrease in pH (pH of cell-free supernatants ranged from 3.6 to 4.0) by the production of organic acids, but the production of other antibacterial compounds by bifidobacteria cannot be excluded.

Probiotics are used in animal feeding in order to increase zootechnical performance, such as average daily gain, feed conversion rate, and quality of animal products. There exists a number of reports on desirable effects of probiotics on the health of ruminants. Ripanonti and Stella (2009) suggested the use of spore-forming bacteria such as *Bacillus coagulans* as probiotics for calves. Oral administrations of *Bifidobacterium pseudolongum* or *Lactobacillus acidophilus* to 7-days-old calves improved body weight gain and feed conversion and reduced the frequency of diarrhoea (Abe et al., 1995). The administration of lactic acid bacteria to young animals, including ruminants, has indeed been demonstrated to have a beneficial effect by reducing rates of diarrhoea (Moore, 2004). Chaves et al. (1999) reported the results of *Lactobacillus acidophilus* administration to calves in the first 2 months of life that reduced the diarrhoea occurrence. Experiments of Herich et al. (1998) showed that the application of probiotic lactobacilli to 2–3 months old calves resulted in an increase in nonspecific immune functions. The results of Tkalcic et al. (2003) indicated that the probiotic *E. coli* administered to weaned calves (8 to 10 weeks of age) substantially reduced or eliminated the faecal shedding of *E. coli* O157:H7 and *E. coli* O111:NM. Lema et al. (2001) studied the efficacy of *L. acidophilus*, *L. casei*, *L. fermentum*, *L. plantarum*, and *S. faecium* in reducing the faecal shedding of *E. coli* O157:H7 in experimentally infected lambs. Results indicated that a diet supplemented with probiotics can reduce the total number of *E. coli* O157:H7 in lambs and improve the meat performance of animals as well.

But to our knowledge there are no reports on probiotic bifidobacteria colonisation and consequent microbiological changes in the digestive tract of young ruminants. In our experimental groups,

significantly higher ($P < 0.05$) counts of bifidobacteria (5.64–7.32 log CFU/g) were found during the first 30 days of lamb life compared to the control group (2.31–2.85 log CFU/g). Lactobacilli counts were also significantly higher ($P < 0.05$) in both treated groups (6.93–8.37 log CFU/g) compared to the control group (4.58–6.58 log CFU/g) in the first three weeks after bifidobacteria administration. The administered bifidobacteria did not affect any other monitored bacterial groups. In the first trial the administered strain *B. ruminantium* L29 survived at high counts (6 log CFU/g on average) only for one week (Table 2, Figure 1). In the second trial lambs received a "cocktail" of 12 strains and RRBifs were detected at the count of about 6 log CFU/g for 25 days (Table 3, Figure 2). The administration of the bifidobacterial "cocktail" and consequent identification of the best survived strain seem to be a more effective method for the selection of potential probiotics than the application of only one strain.

On the basis of *in vitro* test results, suitable probiotic bifidobacterial strains for lambs were chosen. Some of them survived in the gastrointestinal tract of treated lambs for 30 days; these strains could be used as probiotics to cover the first month of life, when low bifidobacterial counts were found. No tested strain was able to colonize the lamb's tract permanently, but there is a possibility to dose bifidobacteria continuously during the milk-feeding period.

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