doi: 10.17221/8727-CJAS

# Persistence of bifidobacteria in the intestines of calves after administration in freeze-dried form or in fermented milk

M. Geigerová, E. Vlková, V. Bunešová, V. Rada

Department of Microbiology, Nutrition and Dietetics, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Prague, Czech Republic

**ABSTRACT**: In order to improve the gut microbiome of calves, probiotic bacteria can be fed as active living-cells (fermented milk), or as live but inactive (freeze-dried) cultures. Ten bifidobacterial strains with suitable probiotic properties (as determined in our previous study) were tested for survival during the freeze-drying process, and screened for their ability to ferment cow's milk. The viability of both freeze-dried and live-cell cultures during storage was also tested. All of the strains tested were able to ferment cow's milk, with average counts of 8.26 ± 0.62 log CFU/ml. Eight out of the ten strains were able to survive in milk for 2 months in counts higher than $10^6$ CFU/ml. Bifidobacteria showed high viability following the freeze-drying process, with average numbers of 9.03 ± 0.22 log CFU/vial and did not decrease after 12 months of storage. The mixture of rifampicin-resistant variants of bifidobacteria (RRBs) was fed to 2-day-old dairy Charolais calves in the form of living-cells, or as freeze-dried bacteria. The control group was given no probiotics. Survival of the RRBs administered and the numbers of other bacterial groups in faecal samples was monitored by culturing. Bifidobacteria that were administered passed successfully through the upper parts of the gastrointestinal tract, and were found in numbers higher than $10^9$ CFU/g for two weeks. RRBs colonized the intestines of calves for at least 63 days in both treatment groups. Significantly higher total counts of bifidobacteria were found in the treated groups, compared to the control group. Reduction in *Escherichia coli* and total coliforms numbers, and an increase in lactobacilli counts were observed in both experimental groups following the application of the probiotic mixtures. Our results show that both forms of administering probiotic bifidobacteria to calves are effective, but that the freeze-dried form is more suitable from a practical viewpoint.

**Keywords**: probiotic bacteria; technological properties; storage conditions; gut; young ruminants

## INTRODUCTION

Antibiotics have been used to prevent and control intestinal infections in young ruminants for many years. However, the widespread usage of antibiotics in livestock has led to antibiotic residues found in animal products, and increased the emergence of drug-resistant bacteria in human beings (Abu-Tarboush et al. 1996). In addition to the need to reduce infectious disease in cattle, other important aspects are considered, including animal welfare, quality control of animal products, public health issues, and odours from animal farms, which are related to the gastrointestinal microbiota, and need to be addressed (Awati 2014). Therefore, there is a need to replace antibiotics in animal feeds with other additives that would positively influence the composition of intestinal microbiota and improve livestock health. Many additives have been proposed for these purposes (Roodposhti and Dabiri 2012; Del Razo-Rodriguez et al. 2013; Hu et al. 2014). One strategy becoming more common

Supported by the Czech Science Foundation (GA ČR) (Grant No. 14-31984P) and by the Grant Agency of the Czech University of Life Sciences Prague (CIGA) (Project No. 20142013).

doi: 10.17221/8727-CJAS

with cattle is the administration of probiotics. Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO 2002). Many studies have focused on the effect of probiotic applications on growth performance and animal health (Timmerman et al. 2005; Frizzo et al. 2010). A meta-analysis conducted by Frizzo et al. (2011) showed that probiotics increase body weight gain and improve feed efficiency. Recent research has demonstrated that the effects of probiotic bacteria can reach far beyond the gastrointestinal tract. Documented benefits include decreased mortality, improved immune function, and increased milk production (Maamouri et al. 2014). Although the positive impact of probiotic preparations has been clearly demonstrated, studies focused specifically on the ability of applied probiotic bacteria to colonize the intestinal tract are limited.

Different microorganisms are used as probiotics in ruminants. More than 60 bacterial, fungal, and yeast species are available for commercial use. Frequently used probiotic microorganisms include strains of lactic acid bacteria, *Propionibacterium* spp., *E. coli*, *Saccharomyces* yeast, and undefined mixed culture (Simon et al. 2001). Selection of suitable probiotic species is dependent on the age of the host. The rumen of dairy calves is not developed yet; therefore, probiotics are selected to target the intestines. Bifidobacteria and lactobacilli are suitable probiotic bacteria for calves, as these genera are an important part of their intestinal microbiota (Uyeno et al. 2010). Thus, it is an appropriate strategy to attempt to increase the counts of these potentially beneficial bacteria of young ruminants. Some strains of bifidobacteria are host-specific (Bunesova et al. 2014). Therefore, it is important for the donor and recipient animals to be of the same species. Probiotic strains can be administered as active living-cells or in an inactive form, including spray-dried, frozen, or freeze-dried microorganisms. All listed variants have been verified as suitable for preservation and distribution of probiotics (Carvalho et al. 2004).

In a previous study from our group, *in vitro* tests were used to identify specific strains of bifidobacteria originating in calves as having suitable functional properties to act as probiotics. These strains temporarily colonized the gastrointestinal tract of calves after their administration in fermented milk (Vlkova et al. 2010). However, for the large-scale application of bifidobacteria to calves, a freeze-dried variant of the bacteria is preferred. The question is whether the form, in which the probiotics are administered, would affect their ability to colonize the digestive tract of calves. Therefore, the aim of this study was to compare the survival ability of bifidobacteria in the gastrointestinal tract, applied to 2-day-old dairy calves in the form of live-cells or as freeze-dried bacteria.

## MATERIAL AND METHODS

***Bifidobacterial strains used and determination of stability in fermented milk or during storage as freeze-dried cultures.*** Bifidobacterial strains administered to calves in this study were collected in a previous experiment (Vlkova et al. 2010). Briefly, bifidobacteria were isolated from faecal samples of calves during the milk-feeding period and characterized by *in vitro* tests. Ten strains with suitable physiological properties were identified by sequencing the *16S rRNA* gene. Six strains were identified as *B. animalis* subsp. *animalis* (strains code: 023 II, 805 P4, 012 II1, 017 III, 805 III, 813 P2), two as *B. thermophilum* (strains code: 017 III2, 025 II), one strain was identified as *B. longum* subsp. *suis* (strain code: 022 II), and one as *B. choerinum* (strain code: 023 I2). We then generated rifampicin-resistant mutants (RRBs) from these strains, using a gradient plate technique. No differences in physiological and biochemical characteristics were found between RRBs and the original strains (Vlkova et al. 2010). Rifampicin resistance is rare among bifidobacteria and enabled us to differentiate microorganisms administered for the study from the endogenous wild-type strains (Rada et al. 1995).

Bifidobacterial strains were screened for their ability to ferment cow's milk and survive under these culture conditions. Milk was prepared from low-fat dried milk (10 g/100 ml of distilled water), 10 ml aliquots were distributed into tubes, boiled for 30 min, hermetically closed, and cooled to 37°C. Overnight growth cultures were inoculated at about $1 \times 10^7$ CFU to the milk, the milk was anaerobically fermented for 24 h at 37°C, and bifidobacterial counts were determined by anaerobic cultivation on modified Wilkins-Chalgren agar

doi: 10.17221/8727-CJAS

(Oxoid, Basingstroke, UK) supplemented with soya peptone (5 g/l; Oxoid). Fermented milk was stored at 4°C and survival of bifidobacteria was determined at approximately one-week intervals for 6 months by cultivation as described above.

The ability of bifidobacteria to survive the freeze-drying process was tested as follows. Ten ml of overnight cultures at density of $10^8$ CFU/ml were collected by centrifugation and resuspended in 10 ml of 10% skim-milk, which served as a lyoprotectant. Glass vials with 10 ml of bacterial suspensions were frozen to –75°C for 30 min, freeze-dried (HetoPowerDry LL3000, Thermo Fisher Scientific, Waltham, USA) under vacuum for 24 h, and hermetically closed with rubber stoppers before opening the drying chamber. Freeze-dried bifidobacteria were kept at room temperature, and their survival during storage was monitored in three-month intervals for 12 months by cultivation on modified Wilkins-Chalgren agar (Oxoid) supplemented with soya peptone (5 g/l; Oxoid). For each analysis, a new vial with freeze-dried bacteria was used.

***Animals, bacteria administration, and sampling.*** Bifidobacteria were administered to 2-day-old Charolais calves from a local farm ("Chov Charolais", Slabce, Czech Republic). There were two experimental groups. In the first group (fermented milk; FM), eight animals were fed from bottle with a single dose of a mixture of ten 10% skim-milk cultures (10 ml) fermented by the 10 different RRBs strains listed above. The total amount of fermented milk fed was 100 ml, and contained approximately $10^{10}$ bifidobacterial cells. The second experimental group (lyophilized bacteria; LB) also comprised eight animals, and they were fed from bottle with a single dose of the mixture of freeze-dried bacteria, resuspended in 100 ml of 10% skim-milk immediately before feeding. Identically to the FM group, $10^9$ of each of the 10 strains of RRBs were administered, for a total dose of $10^{10}$ cells. Eight calves from the same farm with no probiotic treatment were used as a control (C). All groups were housed separately, and none of the animals (both calves and their dams) in this study was treated with antibiotics, coccidiostats or other inhibitory substances. Calves were housed with their dams, and suckled with no additional feed. Water was available *ad libitum*.

Survival of administered RRBs and other bacterial groups was monitored in faeces of all calves included in experiment by cultivation. Faecal samples were collected from the rectum using sterile gloves, transferred to a tube with Wilkins-Chalgren broth (Oxoid), and transported within 2 h to the laboratory. Samples were collected from 2-day-old calves and animals were re-sampled at 5, 10, 14, 21, 35, 49, and 63 days of age.

***Microbiological assays.*** Samples were serially diluted in the Wilkins-Chalgren broth (Oxoid) under anaerobic conditions. RRBs were enumerated using Wilkins-Chalgren agar (Oxoid) supplemented with soya peptone (5 g/l; Oxoid), L-cystein (0.5 g/l; Sigma-Aldrich, St. Louis, USA), Tween 80 (1 ml/l; Sigma), mupirocin (100 mg/l; Merck, Kenilworth, USA), rifampicin (80 mg/l; Sigma), and glacial acetic acid (1 ml/l). Total bifidobacterial counts were determined by the same medium without rifampicin (Rada and Petr 2000), and total anaerobes were cultivated on Wilkins-Chalgren (Oxoid). Anaerobic bacteria were incubated in anaerobic jar (Anaerobic Plus System, Oxoid) at 37°C for 72 h. To enumerate lactobacilli, cells were cultured on Rogosa agar (Oxoid) adjusted to pH 5.4 ± 0.2 with acetic acid, and plates were incubated under micro-aerophilic conditions at 37°C for 72 h. To create micro-aerophilic conditions, the first agar layer was covered with a second layer of Rogosa agar, before incubation. For enumeration of *E. coli* and total coliforms, 0.1 ml of a diluted sample was inoculated to Petri dishes with TBX agar (Oxoid), and spread using sterile glass rods. Plates were incubated aerobically at 37°C for 24 h.

***Statistical analyses.*** Bacterial counts were expressed as the mean with standard deviation. Analysis of variance (one way ANOVA) was applied to determine the statistical significance between tested groups of calves with a 95% confidence interval. Scheffe's method (post-hoc test) was used to determine differences between tested groups. The Shapiro-Wilk test was used to test normality of the population. The results were processed using STATISTICA software (Version 12.0, 2013).

## RESULTS

***Bifidobacteria survival in fermented milk or when stored as freeze-dried cultures.*** The tested bifidobacteria showed good ability of growth in cow's milk and after 24 h of cultivation in milk they were present in counts ranging from 7.02 to 9.41 log CFU/ml, with an average of 8.26 ± 0.62 log CFU/ml.

doi: 10.17221/8727-CJAS

Eight out of 10 strains survived in fermented milk for 2 months with counts > $10^6$ CFU/ml; one strain (025 II) attained this level for only 12 days, and one (strain 805 P4) survived for 26 days with counts >$10^6$ CFU/ml. Viability higher than $10^6$ CFU/ml for 4 months was observed for five of the strains.

Resistance to the freeze-drying process and the stability of lyophilized cultures during storage was similarly assayed. Immediately following freeze-drying (approximately $10^9$ CFU of each strain was freeze-dried), bacterial numbers varied between 8.84 and 9.35 log CFU/vial, with an average of 9.03 ± 0.22 log CFU/vial. Bacterial viability during storage at room temperature was stable for the duration of the study. The means of bacterial counts were 8.80 ± 0.13, 8.78 ± 0.38, 8.80 ± 0.47, and 8.93 ± 0.50 log CFU/vial, after 3, 6, 9, and 12 months, respectively.

***Enumeration of faecal bacteria from calves and detection of administered bifidobacteria.*** The counts of RRBs and additional groups of bacteria determined from faecal samples collected from all experimental calves are shown in Table 1. Approximately $10^7$ CFU/g of bifidobacteria were detected in the faeces of 2-day-old calves in both experimental groups, which was significantly ($P < 0.05$) lower than numbers of bifidobacteria detected in control calves. Three days following administration of the probiotic mixtures, the numbers of bifidobacteria increased to 9.95 log CFU/g, as detected in the FM group, and to 9.55 log CFU/g in the LB group (Table 1). These counts were significantly ($P < 0.05$)

Table 1. Bacterial counts (log CFU/g ± SEM, $n = 8$) in faeces of calves fed a mixture of lyophilized bacteria (LB) or fermented milk (FM), and of calves in the untreated control (C) group at various times of experiment

| Age (days) | Group | Total anaerobes | Bifidobacteria | RRBs | Lactobacilli | *E. coli* | Coliforms |
|---|---|---|---|---|---|---|---|
| | FM | 10.01 ± 0.39$^{A}$ | 7.03 ± 0,02$^{A}$ | < 2.00 | 6.11 ± 1.84$^{A}$ | 8.99 ± 0.10$^{A}$ | 9.02 ± 0.14$^{A}$ |
| 2 | LB | 9.49 ± 0.21$^{A}$ | 7.03 ± 0.01$^{A}$ | < 2.00 | 6.48 ±0.18$^{A}$ | 8.58 ± 0.07$^{AB}$ | 8.68 ± 0.11$^{A}$ |
| | C | 9.59 ± 0.01$^{A}$ | 7.83 ± 0.31$^{B}$ | < 2.00 | 7.41 ± 0.60$^{A}$ | 8.05 ± 0.22$^{B}$ | 8.52 ± 0.07$^{A}$ |
| | FM | 10.68 ± 0.21$^{A}$ | 9.95 ± 0.03$^{A}$ | 9.79 ± 0.32$^{A}$ | 9.08 ± 0.23$^{A}$ | 8.50 ± 0.34$^{A}$ | 9.14 ± 0.07$^{A}$ |
| 5 | LB | 10.10 ± 0.13$^{AB}$ | 9.55 ± 0.46$^{A}$ | 9.60 ± 0.35$^{A}$ | 8.30 ± 0.47$^{A}$ | 8.81 ± 0.10$^{A}$ | 8.98 ± 0.01$^{A}$ |
| | C | 9.31 ± 0.36$^{B}$ | 7.83 ± 0.36$^{B}$ | < 2.00 | 7.95 ± 0.64$^{A}$ | 8.58 ± 0.31$^{A}$ | 8.92 ± 0.10$^{A}$ |
| | FM | 10.42 ± 0.15$^{A}$ | 9.46 ± 0.40$^{A}$ | 9.41 ± 0.14$^{A}$ | 8.90 ± 0.31$^{A}$ | 7.92 ± 0.45$^{A}$ | 8.53 ± 0.33$^{A}$ |
| 10 | LB | 10.10 ± 0.28$^{A}$ | 9.36 ± 0.13$^{A}$ | 9.29 ± 0.15$^{A}$ | 9.09 ± 0.00$^{A}$ | 8.03 ± 0.07$^{A}$ | 8.91 ± 0.07$^{A}$ |
| | C | 10.03 ± 0.26$^{A}$ | 9.12 ± 0.66$^{A}$ | < 2.00 | 8.68 ± 0.31$^{A}$ | 9.03 ± 0.26$^{A}$ | 9.10 ± 0.18$^{A}$ |
| | FM | 10.34 ± 0.22$^{A}$ | 9.40 ± 0.31$^{A}$ | 9.15 ± 0.00$^{A}$ | 8.83 ± 0.14$^{A}$ | 8.08 ± 0.13$^{A}$ | 8.31 ± 0.16$^{A}$ |
| 14 | LB | 10.20 ± 0.07$^{A}$ | 9.22 ± 0.01$^{AB}$ | 9.15 ± 0.23$^{A}$ | 9.12 ± 0.05$^{A}$ | 8.05 ± 0.35$^{A}$ | 8.69 ± 0.63$^{A}$ |
| | C | 10.10 ± 0.02$^{A}$ | 8.51 ± 0.19$^{B}$ | < 2.00 | 8.95 ± 0.20$^{A}$ | 8.33 ± 0.11$^{A}$ | 8.97 ± 0.08$^{A}$ |
| | FM | 10.41 ± 0.16$^{A}$ | 9.16 ± 0.49$^{A}$ | 8.86 ± 0.52$^{A}$ | 8.14 ± 0.24$^{A}$ | 8.31 ± 0.13$^{A}$ | 8.41 ± 0.15$^{AB}$ |
| 21 | LB | 10.25 ± 0.44$^{A}$ | 9.20 ± 0.16$^{A}$ | 9.13 ± 0.14$^{A}$ | 8.75 ± 0.29$^{A}$ | 7.41 ± 0.07$^{B}$ | 7.49 ± 0.07$^{B}$ |
| | C | 10.13 ± 0.02$^{A}$ | 8.53 ± 1.01$^{A}$ | < 2.00 | 8.50 ± 0.51$^{A}$ | 8.57 ± 0.05$^{A}$ | 9.16 ± 0.41$^{A}$ |
| | FM | 9.95 ± 0.03$^{A}$ | 7.60 ± 0.00$^{A}$ | 7.33 ± 0.19$^{A}$ | 8.13 ± 0.36$^{A}$ | 8.21 ± 0.01$^{A}$ | 8.23 ± 0.02$^{A}$ |
| 35 | LB | 9.76 ± 0.04$^{A}$ | 8.21 ± 0.13$^{B}$ | 8.04 ± 0.16$^{B}$ | 8.92 ± 0.16$^{A}$ | 5.90 ± 0.01$^{B}$ | 6.19 ± 0.18$^{B}$ |
| | C | 9.90 ± 0.02$^{A}$ | 6.16 ± 0.03$^{C}$ | < 2.00 | 8.11 ± 0.54$^{A}$ | 8.60 ± 0.23$^{A}$ | 8.62 ± 0.21$^{A}$ |
| | FM | 9.40 ± 0.09$^{A}$ | 7.30 ± 0.30$^{AB}$ | 5.78 ± 0.03$^{A}$ | 8.44 ± 0.10$^{A}$ | 7.39 ± 0.60$^{A}$ | 7.66 ± 0.07$^{A}$ |
| 49 | LB | 9.30 ± 0.11$^{A}$ | 8.04 ± 0.46$^{A}$ | 6.00 ± 0.12$^{A}$ | 8.32 ± 0.06$^{A}$ | 6.08 ± 0.21$^{A}$ | 6.22 ± 0.12$^{A}$ |
| | C | 9.10 ± 0.26$^{A}$ | 6.12 ± 0.14$^{B}$ | < 2.00 | 7.73 ± 0.55$^{A}$ | 7.61 ± 0.07$^{A}$ | 7.67 ± 0.27$^{A}$ |
| | FM | 9.05 ± 0.04$^{A}$ | 7.07 ± 0.12$^{A}$ | 5.29 ± 0.58$^{A}$ | 7.63 ± 0.09$^{A}$ | 7.15 ± 0.05$^{A}$ | 7.19 ± 0.05$^{A}$ |
| 63 | LB | 9.11 ± 0.01$^{A}$ | 7.56 ± 0.09$^{A}$ | 5.55 ± 0.34$^{A}$ | 7.96 ± 0.23$^{A}$ | 6.19 ± 0.01$^{A}$ | 6.42 ± 0.00$^{A}$ |
| | C | 9.21 ± 0.58$^{A}$ | 5.58 ± 0.24$^{B}$ | < 2.00 | 8.13 ± 0.13$^{A}$ | 7.68 ± 0.30$^{A}$ | 7.82 ± 0.31$^{A}$ |

RRBs = rifampicin-resistant variants of bifidobacteria

$^{A–C}$values in columns at the same age with no common superscripts significantly differ ($P < 0.05$)

doi: 10.17221/8727-CJAS

higher compared to the control group (7.83 log CFU/g) (Table 1). Numbers of total bifidobacteria were higher in both experimental groups than in the control for the duration of the study; statistical significance for the differences was observed for days 5, 14, 35, 49, and 63. Non-significantly higher counts of bifidobacteria were seen in the FM group, in comparison with LB group, for the first 3 sampling dates after treatment. Thereafter, bifidobacteria were consistently more numerous in the LB group through to the end of the study, with a significant difference on day 35. No RRBs were detected in faecal samples from all calves before their administration, or in the control group for the duration of the study. The RRBs administered demonstrated robust survival in the gastrointestinal tracts of calves in both experimental groups; three days after application, RRBs reached counts of 9.79 and 9.60 log CFU/g in the FM and LB groups, respectively (Table 1). Their levels gradually decreased as the trials continued, but 2 months following treatment, RRBs were still found in numbers higher than $10^5$ CFU/g. RRBs were more numerous in the FM group on days 5 and 10; thereafter, the numbers were identical until day 21 of the experiment, where RRBs showed higher number in LB group, and remained higher until the end of the study. The difference was significant only on day 35.

Total anaerobic bacteria were found in similar numbers in all calves, reaching a maximum on day 5 in the FM group, and on day 21 in the LB and control groups. The highest counts were found in calves fed fermented milk during the whole experiment, and the numbers were significantly ($P < 0.05$) higher compared to the control group on day 5. Lactobacilli were present in the lowest numbers in 2-day-old calves, and their counts increased rapidly, particularly in both experimental groups 3 days following bifidobacteria application. Numbers were relatively stable during the whole experiment reaching counts between 7.63 and 9.12 log CFU/g (Table 1). The numbers of lactobacilli exceeded bifidobacteria in the control group on days 5 and 14, and in all groups from 35-day-old calves. Coliform bacteria, including *E. coli*, varied in counts between $10^8$ and $10^9$ CFU/g in the first 2 weeks of the calves' life. Decreasing numbers were measured, starting from the 3rd week of life in calves treated with lyophilized bacteria (LB group); a similar reduction of coliforms was delayed in the FM and control groups on day 49. On days 21 and 35, significantly ($P < 0.05$) lower counts of *E. coli* and total coliforms in the LB group compared to both the FM and control groups were detected.

## DISCUSSION

Probiotic bacteria may be applied to a host as active living-cells, or as inactive, usually freeze-dried, cells (Fasoli et al. 2003). Because milk is an appropriate nutritive source for microbial growth (Quigley et al. 2013), one way to administer probiotics to calves is by feeding them milk fermented by probiotic cultures. After the fermentation process and during the storage period, the number of probiotic microorganisms in the product should remain at least at $10^6$ CFU/ml to achieve the desired functions in the gut (Vinderola et al. 2000). All bifidobacteria tested in this study were able to ferment cow's milk, reaching counts higher than $10^7$ CFU/ml, and most of the strains tested remained viable at the required levels for at least 2 months. The advantage of using fermented over non-fermented (sweet) milk is that the milk is preserved for several weeks by bacterial acidification (Bayram et al. 2007). A disadvantage of feeding probiotics in the fermented milk form is the large volume required, compared to freeze-dried probiotics. Expanding interest in the usage of probiotics as a regular contributor to livestock nutrition has placed greater emphasis on promoting high cell viability during storage, and maintaining this high activity at the site of action. A suitable approach for probiotics preservation is freeze-drying (Carvalho et al. 2004), but this process sometimes causes the loss of bacterial viability due to ice crystal formation and rupture of cell membranes (Poddar et al. 2014). The viability of dried bacteria depends also on the method used for their rehydration (Champagne et al. 2010). Our results indicate that the bifidobacteria tested were resistant to the freeze-drying process, and their viability was stable for at least one year. Moreover, bacteria fed to calves passed the upper parts of the gastrointestinal tract successfully, and colonized the gut for more than 2 months.

Numerous studies have reported beneficial effects of probiotics on the health status and performance of calves (Mudgal and Baghel 2010; Bayatkouhsar et al. 2013; Qadis et al. 2014; Soto et al. 2014). Most of the experiments showed increased weight

doi: 10.17221/8727-CJAS

gain and improved feed conversion ratios, but few studies also monitored a long-term survival of the probiotics administered. Moreover, a detailed description of inoculum used in studies was often missing. Therefore, our study was focused on monitoring the persistence of bifidobacteria fed to calves in faecal samples. Bifidobacteria administered in both forms (as active live-cells in fermented milk or as freeze-dried inactivated cells) showed high survivability in the gastrointestinal tract, being found in faecal samples in numbers higher than $10^7$ CFU/g five weeks after the treatment. It has been suggested that some components of milk, especially milk proteins, enhance the survival of bacterial strains under different conditions (Livney 2010; Saxelin et al. 2010). Therefore, the administration of probiotics in milk, or as bacteria freeze-dried using milk as a cryoprotectant, may help improve their survival in the digestive tract. Rochet et al. (2008) assessed the survival of *B. animalis* in adults after ingestion in fermented milk or as a freeze-dried product. The gastrointestinal survival of the strain tested was equally good for both applications. Our results showed that there were significant differences in the survival of bacteria if administered live or freeze-dried. Bifidobacteria were found in faecal samples in higher counts after their application in fermented milk, 14 days after treatment. From day 21 there was a higher number of bifidobacteria in the freeze-dried group than in the fermented milk group. This difference was probably due to the form of administration, as freeze-dried bacteria need time for re-activation in the intestine. The osmotic conditions and pH of the intestine, and the availability of an appropriate nutritional energy source may affect the rate of recovery to a viable state (Costa et al. 2000).

The total numbers of bifidobacteria in the calf faeces samples rapidly increased after the administration of RRBs strains in both treatment groups. An increase was also measured in the control group, but at later times in the study. The counts of bifidobacteria in both experimental groups were significantly higher than counts in the control group during the whole study, except for day 2. We additionally observed the effect of administration of bifidobacteria on other bacterial groups in the digestive tract of calves. One group of intestinal bacteria we examined were lactobacilli, as they are also probiotic bacteria, and a common constituent of the intestinal microbiota of calves (Maldonado et al. 2012). Three days after the application of probiotics, the number of lactobacilli was insignificantly higher in both experimental groups compared to the control, but the differences were not significant (Table 1). This finding is consistent with the results obtained by Vlkova et al. (2009), who observed a slight increase in lactobacilli numbers after feeding bifidobacteria to lambs.

Coliform bacteria, particularly *E. coli*, are causal agents of diarrhoea in calves and it is therefore desirable to reduce their numbers (Moore 2004). In this study, faecal samples from calves fed freeze-dried bifidobacteria had significantly lower numbers of coliform bacteria and *E.coli* on days 21 and 35, compared to untreated calves and calves treated with probiotic fermented milk (Table 1). The group receiving fermented milk also had non-significantly lower counts of *E. coli* and coliforms in their faecal samples, compared to the control group, after the 5$^{th}$ day of life. Roodposhti and Dabiri (2012) reported similar results after administration of a multi-strain probiotic mixture to calves, finding significantly reduced *E. coli* numbers in faeces compared with untreated controls. Two mechanisms by which probiotic microorganisms can reduce *E.coli* and other bacteria present in the environment have been proposed. One is the production of inhibitory substances by probiotic bacteria. Bifidobacteria are able to synthesize organic acids, and some strains also produce bacteriocins (Martinez et al. 2013). The second proposed mechanism of probiotic action against pathogenic or potentially pathogenic bacteria is competitive inhibition of adherence to the intestinal mucus and epithelial cells (Roodposhti and Dabiri 2012).

## CONCLUSION

Experimental data from *in vivo* testing of bifidobacteria administration as a freeze-dried product or in fermented milk demonstrated that both forms of probiotics are suitable for feeding to calves. At least some strains from the bifidobacterial mixture applied in this study were stable during gastrointestinal passage, and were able to colonize the digestive tract of calves for at least 63 days. Nevertheless, due to a longer shelf life, and the possibility to feed probiotics in smaller volumes, the freeze-dried form of bacteria is preferable.

doi: 10.17221/8727-CJAS

## REFERENCES

Abu-Tarboush H.M., Al-Saiady M.Y., Keir El-Din A.H. (1996): Evaluation of diet containing lactobacilli on performance, fecal coliform, and lactobacilli of young dairy calves. Animal Feed Science and Technology, 57, 39–49.

Awati A.A. (2014): Think outside the gut! Effects of probiotics on animal performance and the environment. International Scientific Conference on Probiotics and Prebiotics – IPC2014, Budapest, Hungary, 14.

Bayatkouhsar J., Tahmasebi A.M., Naserian A.A., Mokarram R.R., Valizadeh R. (2013): Effect of supplementation of lactic acid bacteria on growth performance, blood metabolites and fecal coliform and lactobacilli of young dairy calves. Animal Feed Science and Technology, 186, 1–11.

Bayram B., Yanar M., Guler C., Metin J. (2007): Growth performance, health and behavioural characteristics of Brown Swiss calves fed a limited amount of acidified whole milk. Italian Journal of Animal Science, 6, 273–279.

Bunesova V., Vlkova E., Rada V., Killer J., Musilova S. (2014): Bifidobacteria from the gastrointestinal tract of animals: differences and similarities. Beneficial Microbes, 5, 377–388.

Carvalho A.S., Silva J., Ho P., Teixeira P., Malcata F.X., Gibbs P. (2004): Relevant factors for the preparation of freeze-dried lactic acid bacteria. International Dairy Journal, 14, 835–847.

Champagne C.P., Raymond Y., Tompkins T.A. (2010): The determination of viable counts in probiotic cultures microencapsulated by spray-coating. Food Microbiology, 27, 1104–1111.

Costa E., Usall J., Teixido N., Garcia N., Vinas I. (2000): Effect of protective agents, rehydratation media and initial cell concentration on viability of Pantoea agglomerans strain CPA-2 subjected to freeze-drying. Journal of Applied Microbiology, 89, 793–800.

Del Razo-Rodriguez O.E., Ramirez-Bribiesca J.E., Lopez-Arellano R., Revilla-Vazquez A.L., Gonzalez-Munoz S.S., Cobos-Peralta M.A., Hernandez-Calva L.M., McDowell L.R. (2013): Effect of dietary level of selenium and grain on digestive metabolism in lambs. Czech Journal of Animal Science, 58, 253–261.

FAO/WHO (2002): Guidelines for the Evaluation of Probiotics in Food. Report of a Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food. Food and Agriculture Organization of the United Nations/World Health Organization, London, UK/Ontario, Canada.

Fasoli S., Marzotto M., Rizzotti L., Rossi F., Dellaglio F., Torriani S. (2003): Bacterial composition of commercial probiotic products as evaluated by PCR-DGGE analysis. International Journal of Food Microbiology, 82, 59–70.

Frizzo L.S., Soto L.P., Zbrun M.V., Bertozzi E., Sequeira G., Armesto R.R., Rosmini M.R. (2010): Lactic acid bacteria to improve growth performance in young calves fed milk replacer and spray-dried whey powder. Animal Feed Science and Technology, 157, 159–167.

Frizzo L.S., Zbrun M.V., Soto L.P., Signorini M.L. (2011): Effect of probiotics on growth performance in young calves: a meta-analysis of randomized controlled trials. Animal Feed Science and Technology, 169, 147–156.

Hu L., Che L., Su G., Xuan Y., Luo G., Han F., Wu Y., Tian G., Wu C., Fang Z., Lin Y., Xu S., Wu D. (2014): Inclusion of yeast-derived protein in weanling diet improves growth performance, intestinal health, and anti-oxidative capability of piglets. Czech Journal of Animal Science, 59, 327–336.

Livney Y.D. (2010): Milk proteins as vehicles for bioactives. Current Opinion in Colloid and Interface Science, 15, 73–83.

Maamouri O., Selmi H., M'hamdi N. (2014): Effects of yeast (Saccharomyces cerevisiae) feed supplement on milk production and its composition in Tunisian Holstein Friesian cows. Scientia Agriculturae Bohemica, 45, 170–174.

Maldonado N.C., de Ruiz C.S., Otero M.C., Sesma F., Nader-Macias M.E. (2012): Lactic acid bacteria isolated from young calves – characterization and potential as probiotics. Research in Veterinary Science, 92, 342–349.

Martinez F.A.C., Balciunas E.M., Converti A., Cotter P.D., Oliveira R.P.D. (2013): Bacteriocin production by Bifidobacterium spp. A review. Biotechnology Advances, 31, 482–488.

Moore J. (2004): The use of probiotics in the calf: an overview. Cattle Practice, 12, 125–128.

Mudgal V., Baghel R.P.S. (2010): Effect of probiotic supplementation on growth performance of pre-ruminant buffalo (Bubalus bubalis) calves. Buffalo Bulletin, 29, 225–228.

Poddar D., Das S., Jones G., Palmer J., Jameson G.B., Haverkamp R.G., Singh H. (2014): Stability of probiotic Lactobacillus paracasei during storage as affected by the drying method. International Dairy Journal, 39, 1–7.

Qadis A.Q., Goya S., Ikuta K., Yatsu M., Kimura A., Nakanishi S., Sato S. (2014): Effect of a bacteria-based probiotic on ruminal pH, volatile fatty acids and bacterial flora of Holstein calves. Journal of Veterinary Medical Science, 76, 877–885.

Quigley L., O'Sullivan O., Stanton C., Beresford T.P., Ross R.P., Fitzgerald G.F., Cotter P.D. (2013): The complex microbiota of raw milk. FEMS Microbiology Reviews, 37, 664–698.

Rada V., Petr J. (2000): A new selective medium for the isolation of glucose nonfermenting bifidobacteria from hen caeca. Journal of Microbiological Methods, 43, 127–132.

doi: 10.17221/8727-CJAS

Rada V., Marounek M., Rychly I., Santruckova D., Vorisek K. (1995): Effect of Lactobacillus salivarius administration on microflora in the crop and caeca of broiler chickens. Journal of Animal and Feed Sciences, 4, 161–170.

Rochet V., Rigottier-Gois L., Ledaire A., Andrieux C., Sutren M., Rabot S., Mogenet A., Bresson J.L., Cools S., Picard C., Goupil-Feuillerat N., Dore J. (2008): Survival of Bifidobacterium animalis DN-173 010 in the faecal microbiota after administration in lyophilised form or in fermented product – a randomised study in healthy adults. Journal of Molecular Microbiology and Biotechnology, 14, 128–136.

Roodposhti P.M., Dabiri N. (2012): Effect of probiotic and prebiotic on average daily gain, fecal shedding of Escherischia coli, and immune system status in newborn female calves. Asian-Australasian Journal of Animal Sciences, 25, 1255–1261.

Saxelin M., Lassig A., Karjalainen H., Tynkkynen S., Surakka A., Vapaatalo H., Jarvenpaa S., Korpela R., Mutanen M., Hatakka K. (2010): Persistence of probiotic strains in the gastrointestinal tract when administered as capsules, yoghurt, or cheese. International Journal of Food Microbiology, 144, 293–300.

Simon O., Jadamus A., Vahjen W. (2001): Probiotic feed additives – effectiveness and expected mode of action. Journal of Animal and Feed Sciences, 10, 51–67.

Soto L.P., Zbrun M.V., Frizzo L.S., Signorini M.L., Sequeira G.J., Rosmini M.R. (2014): Effect of bacterial inoculants in milk on the performance of intensively reared calves. Animal Feed Science and Technology, 189, 117–122.

Timmerman H.M., Mulder L., Everts H., van Espen D.C., van der Wal E., Klaassen G., Rouwers S.M.G., Hartemink R., Rombouts F.M., Beynen A.C. (2005): Health and growth of veal calves fed milk replacers with or without probiotics. Journal of Dairy Science, 88, 2154–2165.

Uyeno Y., Sekiguchi Y., Tajima K., Takenaka A., Kurihara M., Kamagata Y. (2010): An rRNA-based analysis for evaluating the effect of heat stress on the rumen microbial composition of Holstein heifers. Anaerobe, 16, 27–33.

Vinderola C.G., Bailo N., Reinhemier J.A. (2000): Survival of probiotic microflora in Argentinian yoghurts during refrigerated storage. Food Research International, 33, 97–102.

Vlkova E., Grmanova M., Rada V., Homutova I., Dubna S. (2009): Selection of probiotic bifidobacteria for lambs. Czech Journal of Animal Science, 54, 552–565.

Vlkova E., Grmanova M., Killer J., Mrazek J., Kopecny J., Bunesova V., Rada V. (2010): Survival of bifidobacteria administered to calves. Folia Microbiologica, 55, 390–392.

Received: 2015–01–06
Accepted after corrections: 2015–09–18

*Corresponding Author*

prof. Ing. Eva Vlková, Ph.D., Czech University of Life Sciences Prague, Faculty of Agrobiology, Food and Natural Resources, Department of Microbiology, Nutrition and Dietetics, Kamýcká 129, 165 21 Prague 6-Suchdol, Czech Republic
Phone: +420 224 382 755, e-mail: vlkova@af.czu.cz