ISSN 0026-2617, Microbiology, 2013, Vol. 82, No. 4, pp. 475–481. © Pleiades Publishing, Ltd., 2013.
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EXPERIMENTAL ARTICLES

Effect of *Bacillus subtilis* on the Rumen Microbial Community and Its Components Exhibiting High Correlation Coefficients with the Host Nutrition, Growth, and Development

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Received April 10, 2012

Abstract—T-RFLP investigation of the microbial community of the ruminal fluid of calves revealed changes in the microbiocenosis resulting from feeding the animals with biofilm-protected *Bacillus subtilis* cells. In the control animals, which switched from the diary to the vegetable diet, the phylum *Firmicutes* predominated *Firmicutes* (55.11 ± 1.97%), in particular the class *Clostridia* (53.10 ± 2.06%), families *Lachnospiraceae* (25.93 ± 1.41%) and *Clostridiaceae* (9.90 ± 1.35%). Members of the phyla *Bacteroidetes* (11.15 ± 2.88%) and *Actinobacteria* (9.27 ± 1.95%) were also present. Uncultured forms constituted 17.28 ± 2.01%. The share of bacilli (family *Bacillaceae*) was below 2% (1.46 ± 0.41%). Introduction of *B. subtilis* cells into the rumen of experimental animals increased the share of *Bacillaceae* to 2.80 ± 0.30%. The numbers of *Thermoanaerobacteriaceae, Peptostreptococcaceae,* and *Alicyclobacillaceae* increased by an order of magnitude. The numbers of *Pseudomonadaceae, Burkholderiaceae*, and uncultured *Bacteroidetes* increased twofold. Increased numbers of the rumen bacteria and protozoa, elevated fatty acid content, and higher ammonia emission indicated increased efficiency of digestion. Some families, including the domineering ones, included the members with different directions of the correlation with the indices of rumen digestion. The introduced bacilli stimulated the phylotypes with the positive correlation coefficients and suppressed those with the negative correlation. This, the rumen ecosystem was modified in the direction of improved digestion. The functional role of the members of the microbial community, for which the correlations were negative, weakly associated, or unassociated with the indices of rumen digestion are discussed.

Keywords: symbiotic interactions, microbial community, molecular genetic method, T-RFLP analysis, correlation

DOI: 10.1134/S0026261713040127

Development of metagenomic techniques for analysis of the structure of microbial communities [1, 2], of which T-RFLP is the most reliable [3] had a significant effect on the basic research on the symbiotic relationships between a macroorganism and its microbiota. Modern technologies make it possible to investigate the effect of various factors on the host organism and on its microsymbionts and may be used to assess the character of intermicrobial relationships, including those developing in the intestinal ecosystem, e.g., in the case of arrival of a biomass of foreign bacteria as a component of biologically active feed additives. Technologies for the new generation of such additives involving biofilm formation on solid carriers are of great scientific and applied interest. Biofilms in nature play a protective role for biofilm-forming bacteria [4–6]. A biofilm may therefore improve the survival of a microbial population under unfavorable conditions (drying, granulation, etc.). The cells attached to the carrier particles may affect the microbial cenosis of the gastrointestinal tract.

The goal of the present work was to apply T-RFLP analysis modified for investigation of the ruminal bacteria [7] in order to investigate the microbial community of the ruminal fluid of calves switching from the diary diet to the vegetable one and to investigate the effect of *Bacillus subtilis* vegetative cells arriving into the organism of young animals as biofilms of a vegetable carrier on the microbial ecosystem of the rumen chyme and its internal interactions in order to estimate the abundance of the individual components of the microbiota exhibiting high correlation with the indices of host digestion, growth, and development.

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MATERIALS AND METHODS

Bacillus subtilis strain B-8130 was used in the work. Solid-state cultivation of bacteria was carried out on ground sea-buckthorn leaves [8] at 50% water content. After fermentation, the mass was dried at 45°C to the air-dry state. The number of *B. subtilis* cells (CFU/g) was determined by the terminal dilution method. The absence of spores was confirmed by light microscopy of fixed, gentian violet-stained smears under an Axioskop microscope (Zeiss, Germany) using a KS 300 image analysis system.

Electron microscopy was carried out under a Cam-Scan MB2300 scanning electron microscope (Czech Republic). The wet fermentation mass containing bacterial cells was carefully applied on a drop of isotonic (0.5 M) sucrose solution [9] on a coverslip (18 × 18 mm). The preparation was air-dried at room temperature for 24 h. The coverslip was attached to the manipulation table with a double-sided conductive carbon tape. The preparations were sprayed with gold under vacuum according to the standard procedure for the sample preparation for scanning electron microscopy.

Two groups of 10 calves 25–30 days old from the Kelnovo-Chegodaevo experimental farm (Research Institute of Animal Husbandry, Russian Academy of Agricultural Sciences, Dubrovitsy, Russia) were used: group 1 (control) and group 2 (experimental). The animals of group 1 were fed with mixed fodder without additives. The fodder of group 2 was supplemented with 0.1% of dried biomass of *B. subtilis* B-8130 as a biofilm on a vegetable carrier. Experimental feeding was carried out for 105 days. The amounts of the fodder given and of the leftovers were registered daily in order to determine the fodder consumption. The mass of the animals was determined by individual weighting at the beginning and end of the experiment. These data were used to calculate the live weight gain. The indices of digestion were analyzed at the end of the experiment [10]. For characterization of the ruminal digestion, samples of the rumen content were collected with a probe 3 h after feeding. The sample reaction was measured with an Akvilon-410 pH meter. The rumen content was then filtered through four layers of sterile gauze. The filtrate was used to determine total volatile fatty acids (VFA) by steam distillation in a Markham apparatus, ammonium nitrogen the Conway microdiffusion method, and the biomass of protozoa and bacteria by differential centrifugation on a Beckman model J2-21 centrifuge (Germany) [11], and then to get absolutely dry biomass.

The experimental results were statistically treated using the Student *t* criterion.

The species composition of the microbial community from the ruminal fluid of 6 calves (3 from the experimental and the control groups) 4.5 months old was investigated using T-RFLP. DNA was isolated from the rumen content by phenol/chloroform extraction and purified with 2% CTAB. PCR amplification of the 16S rRNA bacterial genes was carried out using the probes 63F (CAGGCCTAACACATGCAAGTC) with a 5'-terminus label (fluorophore D4–WellRed) and 1492R (TACGGHTACCTTGTTACGACTT), which amplify the 16S rRNA gene fragment at *E. coli* positions 63–1492.

For T-RFLP analysis, the fluorochrome-labeled amplicons of the 16S rRNA gene were purified with 3 M guanidine isothiocyanate solution according to the standard procedure [12]. The concentrations of purified gene fragments were determined on a Qubit 2.0 fluorimeter (Invitrogen, Germany) according to the manufacturer's recommendations. Restriction of 16S rRNA amplicons (30–50 ng) was carried out for 2 h at 37°C using the *Hae*III, *Hha*I, and *Msp*I restriction endonucleases (Fermentas, Lithuania) according to the manufacturer's recommendations. The restriction products were precipitated with ethanol, mixed with addition of 0.2 μL molecular mass marker (Size Standart-600, Beckman Coulter, United States) and 10 μL formamide (Sample Loading Solution, Beckman Coulter, United States), and analyzed on a CEQ 8000 (Beckman Coulter, United States) according to the manufacturer's recommendations. The peak sizes and areas were determined using the Fragment Analysis software package (Beckman Coulter, United States). The peaks were grouped into phylotypes with the accepted experimental error pf 1.5 nucleotides, and the percentage of these phylotypes in the microbial community was determined. The phylogenetic position of the bacteria was determined using the Fragment Sorter software package (http://www.oardg. ohiostate.edu/trflpfragsort/index.php).

The casual relationships between the factor and resulting characteristics were established using the Pearson correlation coefficients [13] between the number of rumen microorganisms and the indices of ruminal digestion (pH, emission of ammonia and volatile fatty acids, as well as the number of bacteria and protozoa), which make it possible to determine direct relationships between variables using their absolute values. The following simplified version of the formula was used [14]:

$$\mathbf{r}_{XY} = \frac{\sum (X - \bar{X})(Y - \bar{Y})}{\sqrt{\sum (X - \bar{X})^2 \sum (Y - \bar{Y})^2}},$$

where X, Y, $\bar{X}$, and $\bar{Y}$ stand for the number of bacteria, the variable of LFA amount, ammonia, or bacterial and protozoan biomass, and the averages for X and Y, respectively.

The absolute values of the correlation coefficients $r \geq 0.5$ were considered high. The correlation indices for individual phylotypes were analyzed in the cases when the share of a specific microorganism in the community exceeded 0.05%.

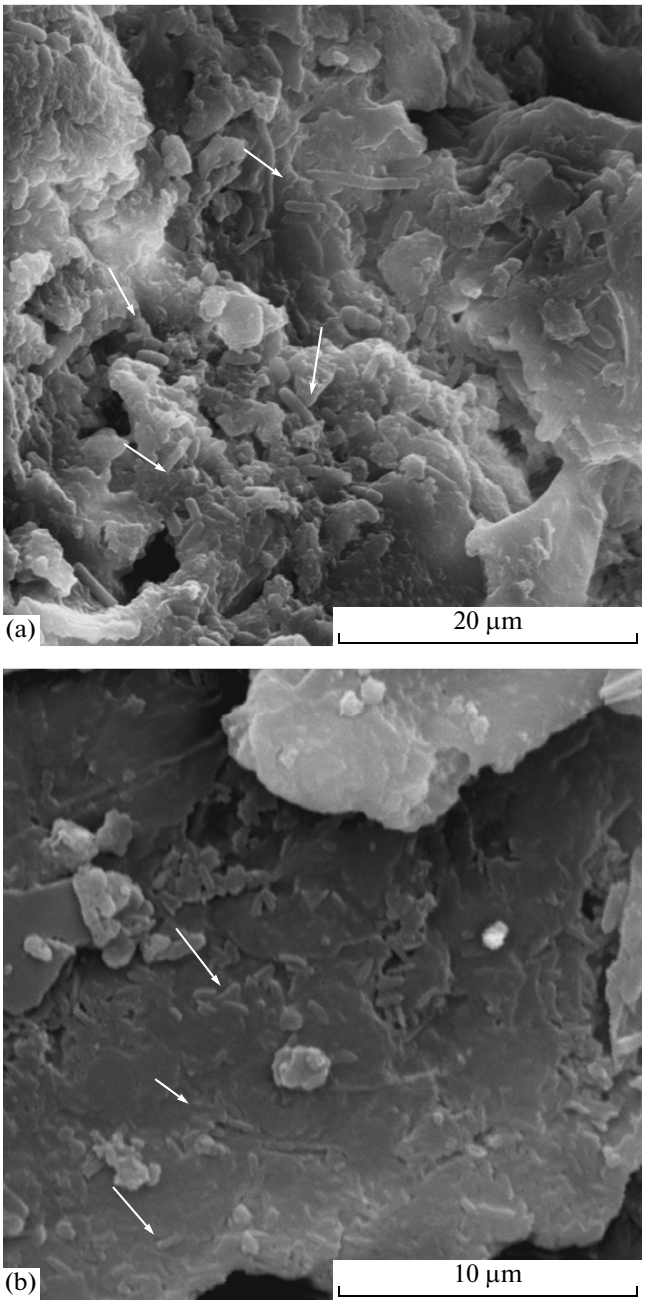
RESULTS AND DISCUSSION

Solid-state cultivation of *B. subtilis* B-8130 resulted in biofilm formation upon the vegetable carrier (figure, a), in agreement with the literature data on *B. subtilis* capacity for biofilm formation [15]. No spores were found in the wet mass or in the dry fermentation product, where the cells on a solid surface were covered with a film providing for protection and additional attachment to the carrier (figure, b). The number of *B. subtilis* cells was $48 \times 10^8 \pm 10\%$ CFU/g dry mass. The mass was added to mixed fodder (0.1%). Bacilli-enriched fodder was used in the ration of the experimental group, while the control calves received mixed fodder without additives. The composition of the microbial community in the ruminal fluid was analyzed after the period of experimental feeding. The rumen microbiota contains various groups, including the bacteria of the ruminal fluid, those adhered to the feed substrates, and those adhered to the mucous membrane of the rumen [16, 17]. In the present work, attention was focused on the bacterial population of the rumen chyme, since a direct correlation exists between the number of bacteria and ciliates in the ruminal content and the productivity of ruminants (the higher microbial number, the higher the animal increment) [18].

Our results revealed the differences in the microbiological characteristics of the ruminal fluid of the experimental and control animals.

In the microbiocenosis of the control calves at 4.5 months, when the animals switched completely from the diary to the vegetable diet [19] and the microbial community was therefore mostly formed, T-RFLP analysis revealed predominance of the phylum *Firmicutes* (55.11 ± 1.97%), mainly of the class *Clostridia* (53.10 ± 2.06%), families *Lachnospiraceae* (25.93 ± 1.41%) and *Clostridiaceae* (9.90 ± 1.35%). Members of the phyla *Bacteroidetes* (11.15 ± 2.88%) and *Actinobacteria* (9.27 ± 1.95%) were present in approximately the same amounts. The share of bacilli (family *Bacillaceae*) did not exceed two per cent (1.46 ± 0.41%). The numbers of bacteria of the families *Bifidobacteriaceae, Enterobacteriaceae, Pseudomonadaceae*, and the phylum *Fusobacteria* were close to those of bacilli. Bacteria of other identified groups, including the family *Lactobacillaceae* (0.32 ± 0.15%) constituted together less than 1%. A significant part of the ruminal microflora belonged to uncultured microorganisms. Unclassified forms together with uncultured bacteroids constituted 17.28 ± 2.01%.

The structure of the microbial community characterized the processes of digestion in this part of the gastrointestinal tract associated with the state of young animals on a vegetable ration. Decomposition of cellulose and starch is associated with mass development of bacteria of the phylum *Bacteroidetes* and families *Lachnospiraceae*, *Ruminococcaceae*, and *Thermoanaerobacteriaceae*, which possess cellulolytic and amylolytic activities. Fermentation of simple and complex carbohydrates also depends on the activity of bacteria of the families *Clostridiaceae, Eubacteriaceae, Veillonellaceae*, and other rumen bacteria [20]. The relatively low abundance of lactobacilli and bifidobacteria may be explained by the age-related characteristics of the calves. Their ontogenesis at the time of sampling was associated with the preferential development



Electron microscopy of the wet product of solid-state fermentation, *B. subtilis* B-8130 cells (arrows) in the biofilm (a) and of the dry preparation, the cells (arrows) mostly covered with a film from above (b).

Table 1. Composition of the bacterial community of calf rumen, T-RFLP analysis, %

Taxonomic rank	Group of animals	
	1 control	2 experimental
Phylum *Bacteroidetes*	11.15 ± 2.88	8.99 ± 1.2
Uncultured *Bacteroidetes*	0.54 ± 0.24	1.22 ± 0.58
Fam. *Bacteroidaceae*	1.48 ± 0.99	0.87 ± 0.17
Fam. *Flavobacteriaceae*	5.74 ± 2.41	4.24 ± 0.54
Fam. *Flexibacteraceae*	2.98 ± 0.73	2.53 ± 0.42
Fam. *Prevotellaceae*	0.42 ± 0.56	0.14 ± 0.1
Phylum *Proteobacteria*	4.33 ± 1.29	6.78 ± 2.12
Fam. *Enterobacteriaceae*	1.93 ± 0.86	2.63 ± 0.14
Fam. *Camphylobacteriaceae*	0.28 ± 0.37	BLRD
Fam. *Pseudomonadaceae*	1.52 ± 0.45	2.99 ± 1.72
Fam. *Burkholderiaceae*	0.63 ± 0.41	1.17 ± 0.05
Phylum *Firmicutes*	55.11 ± 1.97	59.61 ± 2.24
Class *Clostridia*	53.10 ± 2.06	55.72 ± 3.44
Fam. *Thermoanaerobacteriaceae*	1.00 ± 0.06	10.79 ± 3.28***
Fam. *Peptostreptococcaceae*	0.06 ± 0.08	0.77 ± 0.05***
Fam. *Clostridiaceae*	9.99 ± 1.35	7.15 ± 2.02
Fam. *Lachnospiraceae*	25.93 ± 1.41	21.27 ± 5.44
Fam. *Eubacteriaceae*	5.79 ± 1.7	7.83 ± 2.2
Fam. *Ruminococcaceae*	4.83 ± 0.74	2.89 ± 0.26*
Fam. *Veillonellaceae*	5.54 ± 0.62	5.05 ± 0.34
Class *Bacilli*	1.7 ± 0.33	3.48 ± 0.97
Fam. *Bacillaceae*	1.46 ± 0.41	2.8 ± 0.3**
Fam. *Alicyclobacillaceae*	0.05 ± 0.02	0.45 ± 0.52**
Fam. *Paenibacillaceae*	0.2 ± 0.1	0.24 ± 0.17
Order *Lactobacillales*	0.32 ± 0.15	0.42 ± 0.21
Fam. *Lactobacillaceae*	0.32 ± 0.15	0.42 ± 0.21
Phylum *Actinobacteria*	9.27 ± 1.95	8.08 ± 0.72
Fam. *Bifidobacteriaceae*	1.37 ± 0.29	0.4 ± 0.14*
Phylum *Fusobacteria*	1.53 ± 0.24	0.81 ± 0.25*
Uncultured bacteria	16.74 ± 1.77	15.21 ± 0.49

Note: BLRD stands for below the limit of reliable detection.
Reliable at * $p \le 0.05$; ** $p \le 0.1$; *** $p \le 0.001$.

of the microorganisms involved in decomposition of cellulose and starch of the vegetable feed components.

Biofilm-protected cells of *B. subtilis* B-8130 introduced into the host organism with mixed feed caused an increase in the share of the family *Bacillaceae* from 1.46 ± 0.41 to 2.80 ± 0.30%, probably to do growth of the introduced bacilli, and affected the total abundance and ratio of the components of the rumen microbial community. The numbers of *Thermoanaerobacteriaceae*, *Peptostreptococcaceae*, and *Alicyclobacillaceae* increased almost by an order of magnitude. The numbers of uncultured *Bacteroidetes*, as well as of *Pseudomonadaceae* and *Burkholderiaceae*, increased twofold on average. Increased abundance of these forms caused redistribution of the ratios of the components of the microbial community, including a certain decrease in the share of the dominant forms and a reliable decrease in abundance of *Ruminococcaceae* and *Bifidobacteriaceae*, which had, however, no negative effect on the indices of growth and development of the experimental animals an on their digestion (Table 2). Comparison to the indices of ruminal digestion for the control animals revealed 1.3-fold increase in the number of bacteria and protozoa, as well as an increase in VFA synthesis and ammonia emission (the indices

Table 2. Average daily increase in calf body mass ($M \pm m$, $n = 10$) and indices of the processes of ruminal digestion

Animals	Average daily increase, g	pH	Ammonia, mg %	VFA, mmol/100 mL ruminal content	Absolutely dry biomass, g/100 mL ruminal content	
					Protozoa	Bacteria
1 control	753.9 ± 25.25	6.8 ± 0.02	18.12 ± 0.02	11.24 ± 0.03	1.34 ± 0.04	0.40 ± 0.02
2 experimental	852.9 ± 37.65*	6.9 ± 0.04	20.86 ± 0.04	12.44 ± 0.04	1.87 ± 0.05	0.52 ± 0.02

Note: Reliable at $*p \le 0.05$.

characterizing digestion efficiency) in the presence of *B. subtilis* B-8130. These findings required additional investigation for the understanding of the biological background of the phenomenon.

The data obtained by T-RFLP analysis were used to calculate the correlation coefficients between the content of microorganisms and groups in the microbial community and the major indices of ruminal digestion. This approach has been previously successfully applied to investigate the relationship between poultry metabolism and the content of microorganisms in the gastrointestinal tract [21].

Among the *Lachnospiraceae*, which dominated the rumen community, two phylotypes (262 and 279 bp) exhibited high positive correlation, while one phylotype (277 bp) had negative correlation. The correlation coefficients with VFA production, ammonia emission, and total amount of bacteria and protozoa were $r = 0.66$ each for the 262 bp phylotype and $r \ge 0.68$ for the 279 bp phylotype. For the 277 bp phylotype, the coefficients varied from $r = -0.66$ and $r = -0.69$. Importantly, the 264 bp phylotype predominant in this group (*Butyrivibrio fibrisolvens*), which constituted 14.0 and 11.9% of the total microbial number in the control and experimental animals, respectively, exhibited low correlation with the ruminal digestion. Weak positive correlations were found for pH ($r = 0.1$) and number of bacteria in the rumen ($r = 0.03$), while negative correlations were revealed with other digestion indices, with r values not exceeding $r = -0.17$. These results indicated heterogeneity of the family *Lachnospiraceae*, which included the species with opposite functions in the ruminal digestion or unassociated with the investigated characteristics.

In the family *Clostridiaceae*, similar to *Lachnospiraceae*, members with different signs of the correlation dependencies were revealed. For example, the phylotypes 182, 184, 186, and 188 bp exhibited positive correlation with digestion indices (the correlation coefficients varying from $r = 0.59$ to $r = 0.8$), while the phylotype 492 bp showed negative correlation with the correlation coefficients from $r = -0.63$ to $r = -0.68$. The different effects of *Clostridiaceae* members on the ruminal microbial ecosystem may be explained by the broad spectrum of the biochemical activity of the members of this family, which, apart from the species useful for the symbiotic digestion, contains probably those with a negative effect.

Among the *Eubacteriaceae* members, only the phylotypes with high positive correlation with digestion indices were found; for example, the correlation coefficients for the phylotype 199 bp (*Eubacterium ruminantium*) varied from $r = 0.74$ to $r = 0.79$.

The 250 bp phylotype of the family *Ruminococcaceae*, genus *Faecalibacterium* exhibited a negative correlation with all digestive indices ($r = -0.65$). The share of this organism in the ruminal fluid was 2.32 ± 0.82 and 0.70 ± 0.18% for the calves of the control and experimental group, respectively. The factors stimulating host growth and development and intensifying digestion in the microbial ecosystem should result in suppression of the forms inhibiting this process, as was shown in our experiment. The negative correlations of the *Faecalibacterium* sp. phylotype with digestion indicated that this microorganism was probably involved in the synthesis of compounds undesirable for the ruminal digestion. The active preparation with *B. subtilis* shifted the equilibrium of the system, inhibiting this *Ruminococcaceae* species. Since its share in the control animals was 48% of all members of this family, a decrease in the total abundance of *Ruminococcaceae* was observed in the microbial community of the ruminal chyme of the experimental animals (Table 1). Thus, decreased numbers of *Ruminococcaceae* in the presence of bacilli resulted from redistribution of the ratios of the members of this family, with a decrease in abundance of its part playing a negative role in digestion.

Among the *Veillonellaceae* members of the rumen community, the phylotype 259 bp (*Megamonas hypermegale*) exhibited positive correlation with the amounts of ammonia and volatile fatty acids ($r = 0.75$). The share of these bacteria in the ruminal content of experimental animals increased threefold and was 1.82 ± 0.89% compared to 0.60 ± 0.12% in the control. Increased abundance of bacteria of this phylogenetic group in the rumen is known to result in enhanced fermentation of easily degradable carbohydrates due to the stimulation of amylolytic microorganisms [22]. The latter ferment starch and produce high amounts of lactic and succinic acids, while lactate-fermenting bacteria (including *Veillonellaceae* species) convert these acids to VFA with preferential formation of propionic acid. Moreover, a reliable negative correlation was found between the indices of ruminal digestion and the presence of the phylotype

Table 3. Some phylotypes of the microbial community of the ruminal fluid exhibiting high correlation coefficients with the indices of ruminal digestion (T-RFLP analysis)

Fragment length, bp	Microorganism	Character of correlation	Phylotype share, %	
			1 control group	2 experimental group
460	Bacterium of the fam. *Enterobacteriaceae*	+	0.63 ± 0.23	1.93 ± 0.58
182, 184, 186, 188	*Clostridium* sp.	+	0.36 ± 0.05	2.22 ± 0.32
492	*Clostridium* sp.	–	0.77 ± 0.24	0.21 ± 0.07
199	*Eubacterium ruminantium*	+	0.51 ± 0.28	1.91 ± 0.66
262	*Butyrivibrio fibrisolvens*	+	2.61 ± 0.38	3.95 ± 1.27
277	*Pseudobutyrivibrio ruminis*	–	6.29 ± 3.75	2.45 ± 0.36
279	*Pseudobutyrivibrio ruminis*	+	1.01 ± 0.74	2.06 ± 0.63
250	*Faecalibacterium prausnitzii*	–	2.32 ± 0.82	0.7 ± 0.18
259	*Megamonas hypermegale*	+	0.60 ± 0.12	1.82 ± 0.89
265	*Selenomonas* sp.	–	1.17 ± 1.01	0.63 ± 0.15
242	*Amycolatopsis* sp.	–	1.23 ± 0.35	0.06 ± 0.01
243	*Arthrobacter* sp.	–	1.02 ± 0.89	0.28 ± 0.14
246	*Fusobacterium* sp.	–	1.50 ± 0.35	0.60 ± 0.45
430	Uncultured bacterium	–	1.18 ± 0.50	0.08 ± 0.04
431	Uncultured bacterium	–	1.38 ± 0.66	0.53 ± 0.2
462	Uncultured bacterium	+	0.28 ± 0.08	1.12 ± 0.31
Total share of positively associated phylotypes			6.00 ± 1.76	15.01 ± 4.66
Total share of negatively associated phylotypes			16.86 ± 8.57	5.54 ± 1.6

Note: Reliable at $p \le 0.05$.

246 bp (*Fusobacterium* sp. of the phylum *Fusobacteria*), with $r = -0.79$ for VFA and total amount of bacteria and protozoa and $r = -0.80$ for ammonia. The negative correlation between the presence of *Fusobacterium* sp. and digestion may result from competitive relationships between this microorganism and the symbionts involved in ruminal digestion, since it is able to grow at decreased pH utilizing lactate, a product of starch fermentation. Introduction of active *B. subtilis* cells into the ration of the animals resulted in modifications of the microbial composition, increased pH, and enhanced VFA synthesis with preferential production of propionate by *Veillonellaceae*, which suppresses the growth of *Fusobacterium* sp.

Among uncultured species the phylotypes exhibiting negative (e.g., $r = -0.82$ to $r = -0.89$ for the phylotype 430 bp) and positive correlation with digestion indices (e.g., phylotype 431 bp with $r = 0.65$ to $r = 0.74$) were also found. The role of uncultured forms of the microbiocenosis of the ruminal fluid in digestion is therefore not more equivocal than that of other components of the community.

The family *Bacillaceae* was characterized by positive correlation with digestion. Bacilli showed high positive correlation coefficients with ammonia production ($r = 0.83$) and positive correlation with the number of bacteria and protozoa in the rumen ($r = 0.57$). Modification of the rumen microbial ecosystem caused by the vegetative cells of *B. subtilis* B-8130 may be explained by an increase in abundance of the family *Bacillaceae*, which is generally beneficent to the host, resulting in a chain reaction of directional shift in the microbial community, with the preferential development of its members having a positive effect on the digestion of the animals. This suggestion is confirmed by significant stimulation of development of the phylotypes having a positive correlation with digestion (including uncultured ones) and by suppression of those with a negative correlation (Table 3).

The functional role of those components of the rumen community which exhibit a negative correlation with digestion indices in the symbiosis should be different, e.g., associated with pathogen suppression, participation in immunity development [23], or decomposition of toxic low-molecular compounds [24].

The data presented in Table 3 show that 75–80% of the microorganisms of the ruminal fluid exhibited low, if any, correlation with pH, protozoan number, or amounts of ammonia and VFA. Since these microorganisms consume nutrients for their development, they are in relations of commensalism with the host. Bacterial biomass may probably be used to support homoiothermy [24], which is in agreement with the

elevated temperature in the rumen (38–42°C). They may also be consumed as a source of microbial protein. In the latter case, participation of these bacteria in digestion is not required.

The data obtained support the conclusion that the addition of biofilm-protected *B. subtilis* cells into animal feed has an effect on the ruminants by stimulating the ruminal digestion. Not all the microorganisms present in the rumen and even not the domineering organisms are directly involved in digestion, but rather the minor forms, constituting not more than 6% of the microbial community. These were the organisms affected by the probiotic, which caused a directional modification of the microbial ecosystem with a threefold increase in the numbers of bacteria exhibiting a positive effect on digestion, growth, and development of the host organism.

ACKNOWLEDGMENTS

The authors are grateful to N.A. Surovenkova (laboratory of electron microscopy, Severtsov Institute of Ecology and Evolution) for her assistance in their work.

The work was supported by the Russian Federation Ministry of Education and Science, state contract no. 16.M04.11.0012.

REFERENCES

1. Stackebrandt, E. and Goebel, B. M., Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology, *Int. J. Syst. Bacteriol.*, 1994, vol. 44, pp. 846–849.
2. Schütte, U.M., Abdo, Z., Bent, S.J., Shyu, C., Williams, C.J., Pierson, J.D., and Forney, L.J., Advances in the use of terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes to characterize microbial communities, *Appl. Microbiol. Biotechnol.*, 2008, vol. 80, no. 3, pp. 365–380.
3. Hartman, N. and Widmar, F., Reliability for detecting composition and changes of microbial communities by T-RFLP genetic profiling, *FEMS Microbiol. Ecol.*, 2008, vol. 63, no. 2, pp. 249–260.
4. Dunne, W.M., Jr., Bacterial adhesion: seen any good biofilms lately?, *Clin. Microbiol. Rev.*, 2002, vol. 15, no. 2, pp. 155–166.
5. Keller L., Surette, L.G. Communication in bacteria: An ecological and evolutionary perspective, *Nature Rev. Microbiol.*, 2006, vol. 4, pp. 249–258.
6. Rosak, D.B. and Colwell, R.R., Survival strategies of bacteria in the natural environment, *Microbiol. Rev.*, 1987, vol. 51, pp. 365–379.
7. Il'ina, L.A., Balakireva, A.F., Iyldyrym, E.A., and Laptev, G.Yu., Application of T-RFLP analysis for investigation of the bacterial community of the cow rumen, *Meat and Diary Animal Husbandry*, 2011, no. 2, pp. 24–27.
8. Ushakova, N.A., Pavlov, D.S., Chernukha, B.A., Koshelev, Yu.A., Kozlova, A.A., and Nifatov, A.V., RF Patent no. 2346463, *Byull. Izobret.*, 2007, no. 5.
9. Donaldson, D.M., Roberts, R.R., Larsen, H.S., and Tew, J.G., Interrelationship between serum beta-lysin, lysozyme, and the antibody-complement system in killing *Escherichia coli, Infect. Immun.*, 1974, vol. 10, no. 3, pp. 657–666.
10. Kurilov, N.V., Sevast'yanova, N.A., Korshunov, V.N., Firsov, V.I., Mysnik, N.D., Podshibyakin, A.E., Kharitonov, L.V., and Shchegolev, S.Ya., *Izuchenie pishchevareniya u zhvachnykh (Metodicheskie ukazaniya)* (Investigation of Ruminany Digestion (Methodical Recommendations), Borovsk: VNIIFBiP Agr. Anim., 1975.
11. Tarakanov, B.V., Nikolicheva, T.A., and Shavyrina, T.A., Modification of the method of isolation of microbial fractions from the content of the rumen an duodenal chyme, *VNIIFBiP Agr. Anim. Bull.*, 1982, vol. 2, no. 66, pp. 72–75.
12. Maniatis, T., Fritsch, E.F., and Sambrook, J., *Molecular Cloning: A Laboratory Manual*, Clod Spring Harbor Lab. Press, 1982.
13. Lakin, G.F., *Biometriya* (Biometrics), Moscow: Vysshaya shkola, 1990.
14. Gmurman, V.E., *Teoriya veroyatnostei i matematicheskaya statistika: Uchebnoe posobie dlya vuzov* (Probability Theory and Mathematical Statistics: Tutorial for Higher Education), Moscow: Vysshaya shkola, 2004.
15. Kearns, D.B., Chu, F., Rudner, R., and Losick, R., Gene expression in *Bacillus subtilis* surface biofilms with and without sporulation, *Biotechnol. Bioeng.* 2004, vol. 86, no. 3, pp. 344–364.
16. Mc Cowan, R.P., Cheng, K.-J., and Costeron, J.W., Adherent bacterial populations on the bovine rumen wall: distribution patterns of adherent bacteria, *Appl. Environm. Microbiol.*, 1980, vol. 1, pp. 233–241.
17. Dehority, B.A. and Grubb, J., Bacterial population adherent to the epithelium of the roof of the dorsal rumen in sheep, *Environ. Microbiol.*, 1981, vol. 41, no. 6, pp. 1424–1427.
18. Cheng, K.-J. and Costerton, J.W., Adherent rumen bacteria—their role in the digestion of plant material, urea and epithelial cells, in *Digestive Physiology and Metabolism in Ruminants*, Ruckebush, Y. and Thivend, P., Eds., Westport, CT: AVI, 1980, pp. 227–250.
19. Kurilov, N.V. and Kratkova, A.P., *Fiziologiya i biokhimiya pishchevareniya zhvachnykh* (Physiology and Biochemistry of Ruminant Digestion), Moscow: Kolos, 1971.
20. *Biology of the Prokaryotes*, Lengeler, J.W., Drews, G., and Schlegel, H., Eds., Stuttgart: Georg Thieme, 1999.
21. Torok, V.A., Ophel-Keller, K., Loo, M., and Hughes, R.J., Application of methods for identifying broiler chicken gut bacterial species linked with increased energy metabolism, *Appl. Environ. Microbiol.*, 2008, vol. 74, pp. 783–791.
22. Taraqkanov, B.V. and Nikolicheva, T.A., Cellulolytic microflora and metabolic functions in the rumen of young cattle at early introduction of vegetable feed into the ration, *Sel'skokhoz. Biol.*, 1986, no. 4. pp. 89–94.
23. Chung, H. and Kasper, D.L., Microbiota-stimulated immune mechanisms to maintain gut homeostasis, *Curr. Opin. Immunol.*, 2010, vol. 22, no. 4, pp. 455–460.
24. Babin, V.N., Domaradskii, I.V., Dubinin, A.V., and Kondrakova, O.A., Biochemical and molecular aspects of the human–microflora symbiosis, *Ross. Khim. Zh.*, 1994, no. 6, pp. 66–78.

Translated by P. Sigalevich