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Antagonistic properties of bacteria found in sugar beet leaf silage against pathogens naturally occurring in the mouth of cattle

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Summary

The aim of the study was to present antibacterial properties of bacteria found in sugar beet silage against Shigella. The experiment involved bullocks, from which the pathogenic bacteria were isolated, and microorganisms obtained from silage (without additives). It was found that pathogenic bacteria are inhibited by bacteria present in the silage. Experimental subjects included 10 bullocks (crosses of Limousine with Black and White Lowland (BWL) of 700 kg mean body weight. Silage was prepared from sugar beet leaves contaminated with soil. Plant material was ensiled in 6 PCV containers (barrels) of 200 dm^3 in volume closed with a cover allowing the release of gaseous products. The ensiling process lasted 120 days. Samples for chemical and microbiological analyses were collected from three barrel depths (15, 30 and 45 cm) and were subsequently pooled to make a representative sample of 0.9 kg weight. The basic composition of the silage was determined in accordance with AOAC.

The strain antagonistic to Shigella was identified by the molecular method: after isolating bacterial DNA, a PCR reaction was performed. The PCR analysis and the DNA sequence analysis showed that the organism which naturally occurs in sugar beet leaf silage and exhibits antagonistic properties to Shigella bacteria was Bacillus subtilis. Shigella spp., a pathogenic microorganism that is of particular concern to humans, was found in the mouth of cattle.

Keywords: silage, Bacillus subtilis, Shigella, antagonism, inhibition zone

Most intestinal rods are non-pathogenic bacteria which occur in great quantities in the large intestine, although they can also be found on the skin, buccal parts of the throat and in water. Many of them are opportunistic microorganisms which infect only weakened individuals. They comprise non-sporulating or anaerobic bacteria that grow on simple media, ferment glucose, produce acids, are covered with cilia, mobile and encapsulated.

Bacteria classified as *Enterobacteriaceae* have similar structural, genetic and antigen attributes. They frequently exchange genetic information through conjugation, substitution of plasmids or transduction. This contributes to changes in their resistance to many drugs and to the acquisition of genes for the synthesis of toxins and colonising agents.

All *Shigella* species share common invasive properties and cause the cell necrosis of the mucous membrane. The secretion of dysentery toxin (Shiga) is restricted to *S. dysenteriae* type 1, which is responsible for the majority of dangerous infections. *Shigella* enterotoxins (ShET1 and ShET2) are classified as highly contagious – an infectious dose amounts to 100--200 bacterial cells. The high contagiousness of *Shigella* rods is largely attributed to the fact that these bacteria are not destroyed in the acid chyme of the stomach but reach the intestines, where they colonise final sections of the small intestine and the colon. *Shigella* belong to invasive microorganisms capable of intracellular multiplication, which leads to ulceration in the large intestine and the development of inflammatory reaction – hence the presence of blood and leukocytes in the faeces samples of infected patients (10).

Silage manufactured from the leaves of sugar beets – the application of which is discouraged in many handbooks – is still used as cattle feed in agricultural farms

specialising in both plant and animal production (rearing of dairy cows or bullocks) (8). Silage from sugar beet leaves constitutes a significant part of diet in cattle feeding and provides many nutrients, particularly protein and fat as well as microorganisms. Farm animals eat this silage readily. Moreover, it is easily digestible and has lactagogue properties in the case of dairy cows. The presence of microorganisms in silages may exert a positive influence on the rumen microflora as well as on animal health. A disadvantage of this silage is the presence of ketogenic butyric acid. In the case of bullock rearing, its presence in feeds is not as significant as in the case of dairy cattle. Long-term feeding with silages containing high quantities of butyric acid causes diarrhoea, leads to reproduction problems and reduces milk production (5).

The aim of this study was to assess the hygienic status of silage from sugar beet leaves and to verify the research hypothesis about the alleged antagonism between bacteria occurring in the silage (the identification of species exhibiting such properties) and pathogenic bacteria occurring in carrier animals.

Material and methods

Experimental subjects were 10 bullocks (crosses of Limousine with Black and White Lowland (BWL)) of 700 kg mean body weight.

Silages were prepared from sugar beet leaves contaminated with soil. Plant material was ensiled in 6 PCV containers (barrels) of 200 dcm^3 volume closed with a cover allowing the release of gaseous products. The ensiling process lasted 120 days. Samples for chemical and microbiological analyses were collected from three barrel depths (15, 30 and 45 cm) and were subsequently pooled to make a representative sample of 0.9 kg weight. The basic composition of the silage was determined in accordance with AOAC (1).

Statistical analyses regarding the hygienic status of experimental silages and the size of *Shigella* growth inhibition zones were conducted in accordance with glm procedures of the SAS program employing Tuckey and Duncan tests.

The solution for chemical and microbiological analyses was prepared by adding 10 g of silage to 90 cm^3 of the physiological solution of NaCl and homogenising it for a period of 10 minutes. The numbers of mould fungi and yeasts were determined by the plate method on the oxytetracycline-glucose-yeast-extract agar substrate (Oxoid) incubated for 5 days at a temperature of 25°C. Milk fermentation bacteria were determined on the MRS Agar Oxoid for a period of 24-74 hours and at a temperature of 37°C in an environment with 5% CO_2. *Clostridium* was determined on TSC Agar Merck supplemented with D-cycloserine for a period of 18-24 hours and at a temperature of 37°C in anaerobic conditions (Anaerocult® A). *Enterobacteriaceae* were determined on Chromocult Coliform Agar Merck and their incubation time was 24 hours at a temperature of 37°C.

Test bacteria (*Shigella*) were cultured on the Mc Conkey medium. Mouth and anal swabs from bullocks were taken using Cutiplast swabs. After the application of the swabs onto appropriate substrates, plates were incubated for 24 hours at a temperature of 37°C.

DNA was isolated from the developed bacterial colonies with special isolation kits (Genelute Bacterial Genomic DNA Kit®). The purity of the bacterial DNA isolates was confirmed electrophoretically in 1% agarose gel in a buffer with ethidium bromide (1 μg ml^{-1}). Next, a bacterial DNA amplification reaction was carried out in order to obtain a more accurate identification of bacteria which had developed on the selective substrate, using appropriate oligonucleotide primers.

Conditions of the PCR. Volume of the incubation mixture: 25 μl; composition of the incubation mixture: 2.5 mM dNTP, 0.2 U polymerase Taq, 10 × concentrated buffer for PCR, 5 pmol primers (Shigella spp primers were designed on the basis of ipaH gene sequences (GenBank.- 610 bp Shi-1 5' CTT GAC CGC CTT TCC GAT A 3', Shi-2 5' CAG CCA CCC TCT GAG AGT A 3') and 2 μl bacterial DNA supplemented with water up to 25 μl. Amplification conditions: one cycle at 94°C for 15 s, followed by 35 cycles at 94°C for 3 s, 50°C for 10 s, 74°C for 35 s, the last cycle at 74°C for 2 min. PCR products were separated electrophoretically on 2% agarose gel which contained ethidium bromide at a concentration of 1 μg ml^{-1} (12, fig. 1).

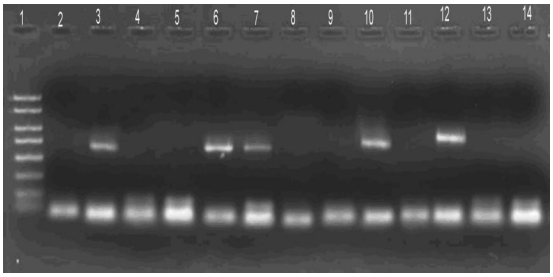


Fig. 1. PCR detection of a part of the *Shigella* gene ipaH (610 bp). Lane 1 – molecular weight marker, lane 3-14 – subsequent specimens

The determination of the antagonism between bacteria from sugar beet leaf silage and pathogenic bacteria. The well method was applied to determine the antagonistic influence of microflora from the sugar beet leaf silage on the bacteria of *Shigella* genera. Deep culture on LB substrate was carried out using *Shigella* bacteria which had earlier been cultured in the form of pellicle. Inoculation was carried out by adding 0.04 cm^3 inoculum of the indicator bacteria from a 24-hour liquid culture on enriched broth to 20 cm^3 of the LB substrate (1.5% agar). The incubation time was 24 hours at a temperature of 37°C.

In order to determine the occurrence of bacteria antagonistic to *Shigella* test strains in the experimental silages, cultures from successive dilutions on the LB substrate were carried out, in the course of which 1 cm^3 of silage solutions diluted in a solution of physiological salt were added to 20 cm^3 of the LB substrate. Incubation was carried out for 24 hours at a temperature of 37°C, and then liquid cultures on the LB substrate were prepared from selected colonies. Next, the developed colonies were tested by the well method in terms of their antagonistic potential against *Shigella* bacteria (11, fig. 2).

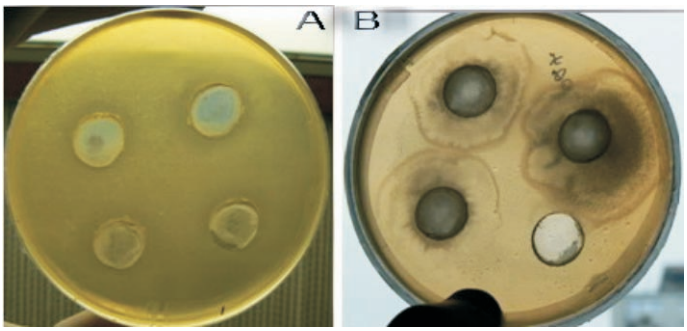


Fig. 2. Well diffusion analysis. Inhibition zones after 24 h (A) and 72 h (B) of incubation

Holes of 11 mm in diameter were cut in the LB agar substrate with *Shigella* cultures. The well bottom was sealed with 50 µl of 1% agar, and then 100 µl of a 24-hour culture of an antagonistic strain was introduced. Subsequently, the well was sealed with 50 µl of 1% agar. After a 24-hour incubation at a temperature of 37°C, inhibition zones of pathogen growth were observed (4).

The identification of the strain antagonistic to *Shigella* bacteria was carried out using the molecular method, whereby after isolating bacterial DNA, the PCR reaction was performed. Universal starters for the bacterial 16S rRNA were used as primers (S-Univ-1492-b-A-21 5' ACG GCT ACC TTG TTA CGA CTT 3', S-D-Bact-0008-a-S-205' AGA GTT TGA TCC TGG CTC AG 3'). Amplification conditions: one cycle at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 53°C for 30 s, 72°C for 90 s, the last cycle at 72°C for 4 min. (fig. 3).

Fragments of bacterial DNA replicated in the PCR were analysed in 1.5% agarose gel with ethidium bromide. The

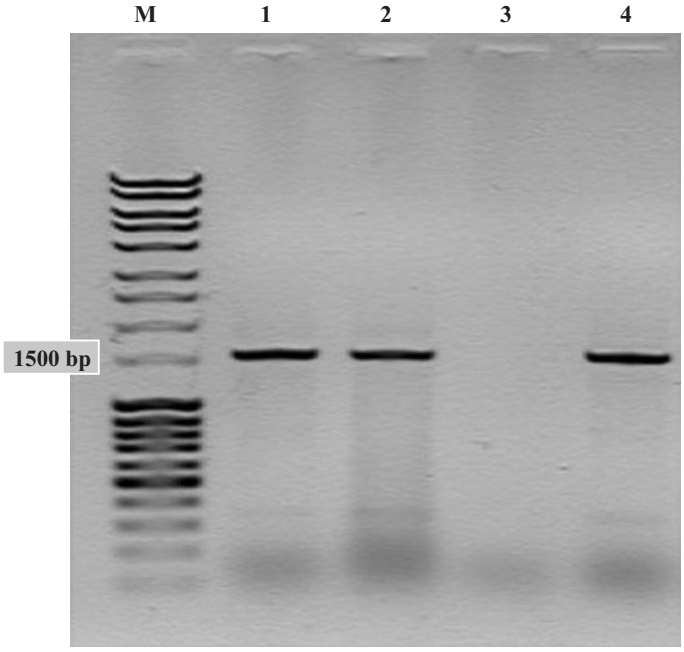
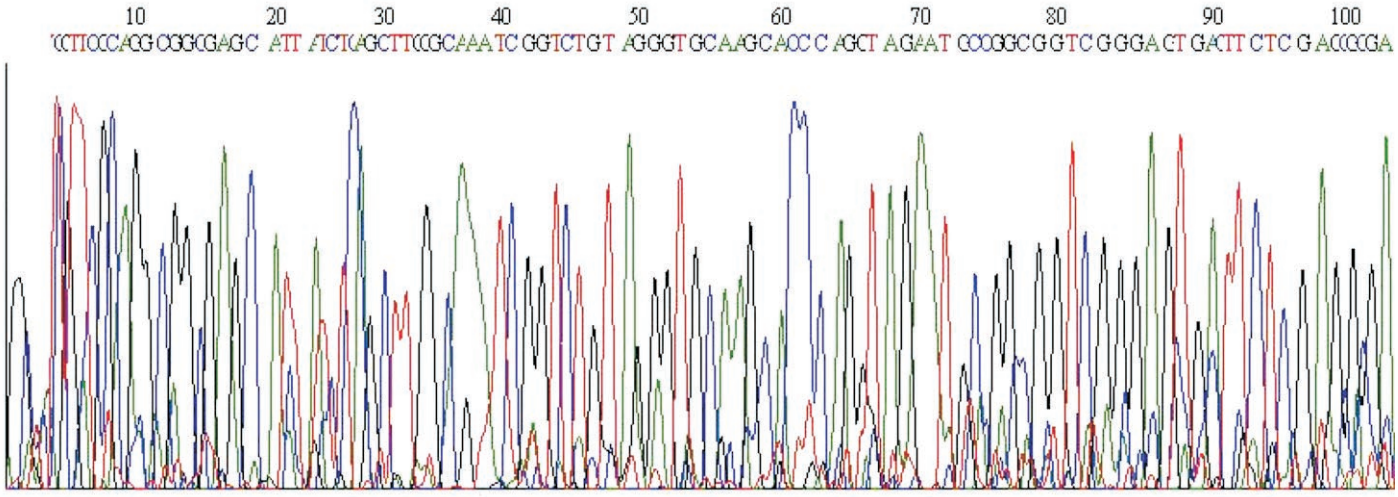


Fig. 3. 1.5% agarose electrophoresis of the silage bacterial DNA amplification product. M – molecular weight marker, line 1-4 – 1500 bp product (16S rRNA) of the PCR obtained from selected colonies

products obtained, 1500 bp in size, were purified on columns and sequenced in a MEGABACE sequencer (Amerscham Bioscience Company). The sequences were recognised with BioEdit computer software, and the microorganism antagonistic to *Shigella* bacteria was identified by feeding the sequences into the NCBI BLAST database (fig. 4).



CCAGTCATCTGTCCACTTCGACNCGGCTGGCTCCTAGAAGGTACTCACCGACTTCGGGTGTTACAAACTCTCGT
GGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGAT
TCCAGCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAACTGAGAACAGATTTGTGGGATTGGCTTAACCTCGC
GGTTTCGCTGCCCTTTGTTCTGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACG
TCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAAGTGCCCAACTGAATGCTGGCAACTAAGATCA
AGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACTGACACGAGCTGACGACAACCATGCACCACCTGT
CACTCTGCCCCCGAAGGGGACGTCCTATCTCTAGGATTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCG
TTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCGAATTCCTTTGAGTTTCAGATCTTGCG
ACCGTACTCCCCAAGGCGGAGTGCTTAATGCGTTAGCTGCAGACATTAAGGGGCCGGAAACCCCTAACATTAA
GACACTCCATCGTTTACGGCGTGGACTACCAGGGGTATCTAATCCTGTTCGTCCCCAGACTTCGATCTCAGCGT

Fig. 4. Amplification products were sequenced and the sequences obtained were identified in the DNA NCBI database

Tab. 1. Chemical composition and total number of microorganisms in the sugar beet silage

Composition and microorganisms	Green forage	Silages						Means 1-6
		1	2	3	4	5	6	
Dry matter g kg^{-1}	171	159^a	155^a	157^a	154^a	159^a	155^a	156.50
Crude protein g kg^{-1}DM	123	144^a	142^a	141^a	150^b	144^a	145^a	144.30
Fat g kg^{-1}DM	17	32^a	31^a	31^a	33^a	32^a	32^a	31.83
Crude fibre g kg^{-1}DM	107	143^a	144^a	143^a	139^b	139^b	145^a	142.17
Sugar g kg^{-1}DM	170	21^a	23^a	25^a	27^b	22^a	28^b	24.33
Lactobacillus log cfu	4.07	5.04^a	5.07^a	5.10^a	5.11^a	4.89^b	5.13^a	5.06
Clostridium log cfu	1.18	nd	nd	nd	nd	nd	nd	–
Enterobacteriaceae log cfu	3.17	2.17^a	1.13^b	1.23^a	1.11^b	1.09^b	1.09^b	1.40
Yeast log cfu	4.00	4.28^a	4.31^a	4.31^a	4.34^a	4.32^a	4.31^a	4.31
Mould log cfu	2.03	1.80^a	1.78^a	1.76^a	1.70^a	1.75^a	1.79^a	1.76

Explanations: a, b – means in rows designated with different letters differ significantly at the level of $p < 0.01$

Results and discussion

Table 1 shows the basic chemical composition and counts of the selected groups of microorganisms in the silages examined. No significant ($p < 0.01$) differences in terms of dry matter and fat concentrations were recorded in the experimental silages. The chemical composition of green mass and feeds was in keeping with DLG standards (2). Counts of microorganisms were similar in all silage samples examined. *Clostridium* bacteria were found (log cfu 1.18) in fresh plant material, but they were absent from the silage after 120 days of ensiling. The ensiling process also reduced counts of *Enterobacteriaceae* and moulds, but it increased populations of lactic bacteria and yeasts. The well method was used to test the antagonism of the bacteria found in the silage to bacteria of the *Shigella genera*. The mean values of the inhibition zones of bacterial growth from the *Shigella* genus affected by the bacteria found in silage samples are presented in table 2. Means obtained in four measurements were analysed statistically, and the differences were examined at a significance level of $p < 0.05$. *Shigella* bacteria were isolated from the mouths, anuses and faeces of all 10 bullocks, which means that the examined animals exhibited carrier traits. Microorganisms which occurred in all 6 silage samples inhibited the growth of the bacterium under examination.

The identified organism, which naturally occurs in sugar beet leaf silage and exhibits antagonistic properties in relation to *Shigella* bacteria, was *Bacillus subtilis.*

Antagonistic properties between different species of microorganisms have been subject of many investigations. Probiotic properties of lactic bacteria, widely attributed to their ability to produce biocins, are commonly recognised. Similar bacteriostatic and fungistatic properties may be exhibited by bacteria from the *Bacillus* genus. Their count in feeds such as silages depends on the extent of contamination of plants with soil, and sugar beet leaves used for ensiling are particularly prone to such soiling. *Bacillus* genus bacteria include Gram-positive microorganisms forming endospores. Their growth takes place primarily in aerobic conditions and differs considerably from that of *Clostridium*. Some facultative anaerobes from the *Bacillus* genus are able to ferment various carbohydrates, producing ethanol, glycerol, 2, 3-butadiol as well as organic acids, including lactic acid (6). These bacteria are capable of producing as much of lactic acid as typical lactic bacteria, and therefore, their role during the ensiling process is not significant. Bacteria from the *Bacillus* genus begin to play an important role during the so-called „feed out" stage, when, together with yeasts, they can cause losses of nutrients in aerobic conditions (3). An important feature of the bacteria from the *Bacillus* genus is their ability to produce antibiotics. In natural environments, production of antibiotics can be a guarantee of survival and proper development in unfavourable conditions. However, numerous microorganisms are able to inactivate and degrade small quantities of antibiotics. Antibiotics produced by *Bacillus* may exhibit a different biological activity. Peptide linear gramicidin and cyclic tyrocidine produced by *Bacillus brevis* disrupt the synthesis of the cytoplasmic membrane in Gram-positive bacteria. In the *Bacillus brevis* itself, acting separately, they inhibit RNA synthesis, whereas their action is reverse when they act together (6). The production of antibiotics by *Bacillus* may be associated with the sporulation process as a genetic coupling of these processes has been observed.

Moreover, antibiotics can also protect spores in natural environments during their germination. So far, a number of species from the *Bacillus* genus have been found to produce polypeptide antibiotics with low masses of several thousand Da. Such antibiotics can be used in medicine to treat bacterial infections; they also show antineoplastic and immunosuppressive properties (4). *Bacillus licheniformis* and *B. subtilis* produce bacitracin, which destroys Gram-positive bacteria but leaves *Pseudomonas aeruginosa* and *Enterobacteriaceae* intact. *Bacillus brevis* produces gramicidin, which inhibits the development of *Mycobacterium* and Gram-positive bacteria. On the other hand, *B. polymyxa* produces polymyxin, harmful to Gram-negative forms. *Bacillus* is also capable of producing lantibiotics (mersacidin – peptide antibiotic) affecting Gram-positive bacteria (9). The above-mentioned compo-

Tab. 2. *Shigella* inhibition zones (mm) under the influence of microorganisms occurring in silage from sugar beet leaves

Silages	Fat stocks									
	1	2	3	4	5	6	7	8	9	10
1	7	6	0	4	7	6	6	4	8	6
	5	3	8	4	8	6	8	6	5	6
	7	5	6	7	7	6	8	7	5	7
	4	0	6	7	8	4	8	7	5	8
Means	5.75^{a}	3.50^{b}	5.00^{a}	5.50^{a}	7.50^{c}	5.50^{a}	7.50^{c}	6.00^{a}	5.75^{a}	6.75^{b}
2	6	6	7	5	4	4	5	3	7	5
	6	0	7	4	3	5	5	6	7	6
	4	5	8	8	8	8	6	7	7	6
	7	6	0	6	9	6	8	7	7	8
Means	5.75^{a}	4.25^{b}	5.50^{a}	5.75^{a}	6.00^{a}	5.75^{a}	6.00^{a}	5.75^{a}	7.00^{c}	6.25^{a}
3	3	6	7	4	5	7	6	8	8	8
	5	6	5	6	5	6	6	4	7	7
	7	7	6	7	6	7	6	6	6	6
	8	5	7	5	7	4	7	7	4	8
Means	5.75^{a}	6.00^{a}	6.25^{a}	5.50^{a}	5.75^{a}	6.00^{a}	6.25^{a}	6.25^{a}	6.25^{a}	7.25^{b}
4	3	6	8	4	7	3	5	4	4	5
	6	7	5	6	7	3	6	7	5	6
	5	6	4	6	3	8	7	7	8	6
	5	5	4	5	5	8	6	8	6	7
Means	4.75^{a}	6.00^{b}	5.25^{a}	5.25^{a}	5.50^{a}	5.50^{a}	6.00^{b}	6.50^{a}	5.75^{b}	6.00^{b}
5	0	4	5	0	5	7	4	6	7	6
	6	6	6	8	5	6	5	6	7	8
	7	7	6	5	5	6	5	6	7	8
	8	6	8	5	7	5	8	7	7	8
Means	5.25^{a}	5.75^{a}	6.25^{a}	4.50^{b}	5.50^{a}	6.00^{a}	6.00^{a}	6.25^{a}	7.00^{c}	7.50^{c}
6	0	6	4	8	8	0	4	7	8	7
	5	6	7	6	7	9	4	5	0	6
	6	7	7	5	6	4	4	6	6	5
	6	5	7	5	4	4	7	3	6	5
Means	4.25^{a}	6.00^{b}	6.25^{b}	6.00^{b}	6.25^{b}	4.25^{a}	4.75^{a}	5.25^{a}	5.00^{a}	5.75^{b}

Explanations: a, b, c – as in tab. 1 at the level of $p < 0.05$

unds arouse considerable interest among scientists and may find application in chemotherapy, food conservation and cosmetic industry (7).

Apart from peptide antibiotics, bacteria from the *Bacillus* genus are also capable of producing „non-ribosomal" antibiotics, e.g. surfactins containing fatty acid chains in their composition and exhibiting antibacterial and antiviral action. These antibiotics are produced only at the stationary phase, and their role has not been fully clarified yet. It is believed that they may have some role in the competition with other microorganisms. Antagonistic properties of the obtained *Bacillus subtilis* strain in relation to the bacteria used as indicator bacteria may have resulted from the inhibiting effect of the metabolism products of these bacteria, such as lactic acid or hydrogen peroxide. Results of our experiments failed to provide an unequivocal answer as to which of these factors plays the key role in inhibiting the growth of bacteria from the *Shigella* genus. It is not known how these bacteria pass into the mouth of cattle – it is possible that infection is caused by bacteria passing from excrement, manure or contaminated feed. Further investigations should aim to discover the dominant factor causing the antagonism.

References

1. AOAC: Association of Official Analytical Chemists: Official Methods of Analysis, Washington, DC 1990.
2. DLG – Futterwerttabellen. Wiederkäuer. DLG-Verl. Frankfurt am Main 1997.
3. *Jonsson A.*: Growth of Clostridium tyrobutiricum during fermentation and aerobic deterioration of grass silage. J. Sci. Food Agric. 1997, 54, 557--568.
4. *Kraszewska J., Wzorek W., Sztando E., Raczyńska-Cabaj A.*: Aktywność antagonistyczna bakterii fermentacji mlekowej z gatunku Lactobacillus plantarum. Acta Sci. Pol. Techno Aliment. 2005, 4, 39-52.
5. *Macgregor Ch. A.*: Directory of feeds and feed ingredients. Hoard's Dairyman Books. USA 2000, 80.
6. *Mannanov R. N., Sattarova R. K.*: Antibiotics produced by Bacillus bacteria. Chemistry of Natural Compounds 2001, 3 7, 117-123.
7. *Markiewicz Z., Kwiatkowski Z. A.*: Bakterie, antybiotyki i lekooporność. Wydawnictwo Naukowe PWN 2006, 250.
8. *McDonald P., Henderson A. R., Heron S. J. E.*: The Biochemistry of Silage. Marlow, Bucks, UK: Chalcombe Publications 1991, 340.
9. *Nagao J.*: Lantibiotics: Insight and Foresight for New Paradigm. J. Biosci. Bioengin. 2006, 102, 139-149.
10. *Stypułkowska-Misiurewicz H., Gonera E.*: Czerwonka bakteryjna w 2002 roku. Przegl. Epidemiol. 2004, 58, 77-84.
11. *Tagg J. R., Mcgiven A. R.*: Assay System for Bacteriocins. Appl. Microbiol. 1971, 21, 943.
12. *Wang R. F., Cao W. W., Cerniglia C. E.*: A universal protocol for PCR detection of 13 species of foodborne pathogens in foods. J. Appl. Microbiol. 1997, 83, 727-736.

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