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Original article

The influence of lactic acid bacteria on the viability of the reference strain of *Listeria monocytogenes* 123 serotype I in plant foods

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Abstract

Listeria (*L.)* monocytogenes is the causative agent of human listeriosis, the frequent source of which is food of animal origin. The aim of this study was to determine the influence of lactic acid bacteria (LAB) on the viability of Listeria in carrot juice and compound feed inoculated with *L. monocytogenes*. The effect of homogenous cultures of *Streptococcus (Str.) lactis distaticus*, *Str. thermophilus* and *Lactobacillus (Lac.) lactis subsp*. *Cremoris* and the combination of *Str. thermophilus* with *Lac. bulgaricus* in the carrot juice and compound feed samples on viability of inoculated *L. monocytogenes* were examined. There were no statistically significant differences in the results between the experimental groups. Regardless of used LAB, the results showed that the mean pH values in the carrot juice samples decreased from an initial pH of 6.7 to a mean value of 3.7 on 15 experimental day. The *Listeria* concentration in carrot juice samples decreased from average of 4.94 on day 5 to 3.24 log CFU/mL on day 10, and on day 15 achieved <0.01 log CFU/mL. In the compound feed trials, the pH decreased average from initial 6.5 to 3.7 on day 15. The concentration of Listeria decreased, similarly to the carrot juice samples, from average 5.0 on day 5 to 4.68 on day 10, and on day 15 achieved <0.01 log CFU/mL. In control samples, the number of *Listeria* increased throughout the study period and amounted to 9.2-9.84 log CFU/mL/g in all the samples. The activity of LAB has been shown to be antagonistic to *L. monocytogenes.* The results of the study did not show any clear differences between the used LAB strains in limiting the *L. monocytogenes* concentration. Based on the obtained results it can be conducted that the addition of LAB to animal food increases its microbiological safety.

Keywords: *Listeria monocytogenes*, lactic acid bacteria, carrot juice, compound feed

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Introduction

Listeriosis is a disease caused by microorganisms of the genus *Listeria*, which affects both humans and farm and wild animals. It has been established that the causative agent of listeriosis is quite widespread among rodents, fleas, fish, saigas, muskrats, and birds.

In the regions of Kazakhstan where listeriosis of rodents was recorded, it could also be observed among farm animals. Infection was found in 4.8% tested small ruminants, in 4.7% pigs, 3.5% cattle and isolated cases were noted among horses (Musabekova et al. 2011). Due to its widespread, *Listeria* (*L*) *monocytogenes* is also registered in food products, which caused several outbreaks of food poisoning associated with death. This microorganism can be found in several processed foods, dairy, milk, meat and fish, seafood, eggs, fruits, and vegetables (Dhamaa et al. 2015). Potatoes (25.8%) and radishes (30.3%) were most frequently contaminated with the pathogen (Velani and Roberts 1991).

Zilelidou and Skandamis (2018) conducted an analysis of the growth, detection, and virulence of *L. monocytogenes* in the presence of other microorganisms, and their microbial interaction. The authors report that the growth and virulence of the bacteria may depend on the interaction with concomitant microorganisms that have antagonistic potential against *L. monocytogenes*. They indicate the importance in terms of food safety, because this information allows to predict possible risks.

The potential of *Pediococcus (P.) pentosaceus* DT016, as a protective crop, that suppresses the growth of *L. monocytogenes* in vegetables during storage, was evaluated during the experiments on the biopreservation of fresh greens (Ramos et al 2020). The results showed that the pathogen abundance in vegetables inoculated with *P. pentosaceus* DT016 was significantly lower during the entire storage period and on the last day of storage a minimum difference of 1.4 log CFU/g was comparable to vegetables with no protective culture. In addition, when using two levels of *L. monocytogenes* (about 6 and 4 log CFU/g), it was noted that the antagonistic effect of *P. pentosaceus* was higher at lower numbers of the pathogen.

Another study (Prezzi et al. 2020) evaluated the effect of *Lactobacillus (Lb.) rhamnosus* GG on the growth of *Staphylococcus aureus* and *L. monocytogenes*, which were inoculated alone or in combination on the surface of cheeses, when stored for 21 days at 7°C. The percentage survival of each individual bacterial species was also determined after their exposure to simulated *in vitro* gastrointestinal conditions (SGC). The addition of *Lb. rhamnosus* did not affect the pH, the moisture, the fat, the protein, and the texture profile of the cheeses. *Lb. rhamnosus* was able to survive at suitable numbers (>6 log CFU/g) in cheeses from day 7 of storage with a high survival rate (>74.6-86.4%) after SGC. The inhibitory effect of *Lb. rhamnosus* on *L. monocytogenes* was observed in cheese (1.1-1.6 log CFU/g reduction) and after SGC (20% reduction in survival). The authors of the study report that adding of *Lb. rhamnosus* to cheese could potentially inhibit *L. monocytogenes*.

A study of tomatoes and tomato-based products examined the physicochemical properties of fermented tomato juices and assessed the competitiveness of lactic acid bacteria (LAB) against *L. monocytogenes*, *L. innocua* and *Salmonella* spp. in an artificially contaminated tomato juice (Bah et al. 2019). The results have shown an increase in the content of lactic and acetic acids during the fermentation and storage of juices inoculated with *Lb. plantarum* and *Leuconostoc* (*Leu.*) *mesenteroides* at 25°C.

In another experiment the efficiency of two bacteriocin-producing strains – *Lactococcus* (*Lc.*) *lactis* subsp. *lactis* LMG 21206 and *Lb. curvatus* LBPE was studied (Bankerroum et al. 2005). The results have shown that *L. monocytogenes* didn't grow in any of the contaminated lots, but no significant reduction was observed in either the positive control (no starter addition) or the samples fermented with Bac- starter during the fermentation period and up to 15 days of drying. When the Bac+ sourdough contained *Lb. curvatus* LBPE, *L. monocytogenes* cells decreased to below the detectable limit (<10 cfu g) after 4 hours of fermentation, and no survivors could be recovered by enrichment after day 8 of drying. When using Bac+ starter containing *Lc. lactis* LMG21206, after 15 days of drying, a reduction in the number of *Listeria* to a level below the detectable limit was achieved. From the other side Nilsson et al. (2005) conducted a study that elucidates the mechanisms by which the non-bacteriocinogenic *Carnobacterium* (*C.*) *piscicola* inhibits the growth of *L. monocytogenes*. The results have shown that non-bacteriocinogenic *C. piscicola* reduced partially the growth of *L. monocytogenes* by depleting glucose level. As the authors noted, understanding the mechanism of interaction between microorganisms improves the prediction of growth in mixed communities, as well as the use of bioprotection principles for food preservation. Śliżewska et al. (2021) in their study evaluated the safety and probiotic properties of selected strains of lactobacilli, which are intended to feed the animals. *Lactobacillus* spp. proved to be safe as they did not break down mucus and did not exhibit hemolysis. The analyzed strains showed moderate to strong antagonistic activity against *Salmonella* spp., *L. monocyto-*

genes, *Campylobacter jejuni* and *Campylobacter coli*, which was tested using the agar plate method. Coaggregation of lactic acid bacteria strains with pathogens varied from 12.12 to 85.45%. The authors concluded that the analyzed strains have probiotic properties. Since the overuse of antibiotic growth promoters in animal production leads to the spread of antibiotic-resistant pathogens and the accumulation of chemotherapeutic drug residues in animal-derived foods, it is vital to introduce alternative feed additives.

In the study of Riaz et al. (2021) performed on mouse model the antibacterial activity of *Lb. brevis* MF179529, a probiotic bacterial strain was evaluated. As shown by the results of scrambling the probiotic, this led to a significant reduction in the dispersal and multiplication of *L. monocytogenes* in the liver, spleen and intestines. The authors note that the results indicate an ameliorating role of *Lb. brevis* in *L. monocytogenes* infection and suggest that *Lb. brevis* can be used prophylactically.

In the growth of *Listeria* spp. in various types of food and feed products, their pH plays an important role. LAB induce the fermentation process, the product of which is lactic acid, which leads to acidification of the environment. The growth of *Listeria* is inhibited not only by the action of lactic acid, but also by other metabolites of lactic acid microorganisms.

An analysis of the literature data showed that *Listeria* spp. have broad adaptive properties. In many ways, the reproduction of the bacteria in certain environments is influenced by multiple factors such as temperature, physico-chemical conditions and other. There is not much data concerning the influence of other microorganisms on *Listeria* in plant substrates and feeds. Feed is known to be the main link in the spread of listeriosis among animals, therefore we set ourselves the goal of investigating the effect of lactic acid bacteria on the viability of *L. monocytogenes* in the carrot juice and compound feed.

Materials and Methods

Four probiotic preparations were used in the study to determine their effectiveness in the inactivation of *L. monocytogenes* in the carrot juice and in compound feed. The strain of LAB *Streptococcus* (*Str.*) *lactis diastaticus* AMC 23 from the Republican Collection of Microorganisms, starter cultures Lactina LTD-LAT BY (Bulgaria) contained *Str. thermophilus* and *Lb. bulgaricus* and granules of thermophilic dairy culture ST-BODY-3 (Denmark), contained *Str. thermophilus* were tested in the carrot juice. In compound feed *Str. lactis diastaticus* AMC 23, starter cultures Lactina LTD-LAT BY (Bulgaria) contained *Str. thermophilus* and CHN-19 (Denmark) contained *Lb. lactis* subsp. *Cremoris* were tested.

Carrot juice

Local fresh carrots for the carrot juice were selected from agricultural enterprises of the Kostanay region. The carrots were peeled, washed under running water, then ground and the mass was homogenized using a laboratory homogenizer (VELP Scientifica, China). The juice was squeezed out with a mechanical squeezer in the amount of 300 ml. Carrot juice was poured into test tubes in amount of 8 ml (n=36) in each.

Compound feed

The compound feed for cattle (K-60-6) used in the research, containing oats, wheat, barley, wheat bran, table salt, mineral supplements, soybean meal, sunflower meal, was purchased from local producers. After additional grinding in homogenizer, 7 g of feed were placed in test tubes (n=36), and distilled water was added in a ratio of 1:2.

Next, all samples of the juice and feed were sterilized in an autoclave at 120°C , 4 atm., for 15 minutes.

Lactobacilli

The *Str. lactis diastatikus* AMC 23 and starter cultures of lactic acid bacteria were cultured on agar M 17 (Merck, Germany) and MRS agar (Merck, Germany). Next, to obtain sufficient biomass, LAB were multiplied in MRS broth (SRC AMB, Obolensk. Russia).

L. monocytogenes inoculum

The strain *L. monocytogenes* 123 serotype I from the strain collection of the Laboratory of Microbiology of the Department of Veterinary Sanitation in Kostanay was used as a test culture. The *L. monocytogenes* strain was multiplied for 24 h at 37°C in Tryptic Soy Broth (TSB, Merck. Germany) with 0.6 % yeast extract (Fluka, Germany), then washed twice by centrifugation with distilled water in 5000 rpm (4°C, 15 min).

Lactic acid bacteria were introduced into samples of the carrot juice and feed compounds.

The carrot juice test tubes were divided into 4 groups of 6 tubes in each. The lactic acid bacterial cultures of all three preparations used in the study were adjusted to a concentration of 10^8 CFU (colony forming units)/mL. *Str. lactis diastatikus* AMC 23 was introduced to the first group of tubes (n=6), to the second group the starter cultures Lactina LTD-LAT.BY were added (n=6) and to the third group the starter cultures

of ST-BODY-3 were added (n=6). The fourth group of tubes was a control group to which no lactic acid cultures were added (n=6).

The similar model of procedures was used for test tubes containing compound feed. *Str. lactis diastatikus* AMC 23 was introduced to the first group of tubes (n=6), to the second group the starter cultures Lactina LTD-LAT.BY were added (n=6) and to the third group the starter cultures CHN-19. The fourth group of tubes was a control group to which no lactic acid cultures were added (n=6).

In the next step, the strain of *L. monocytogenes* 123 serotype I were introduced into samples of the carrot juice and compound feed. A suspension with a concentration of 10^6 CFU/mL was used for inoculation. Control samples were inoculated only with *L. monocytogenes* at 10^6 CFU/mL. Samples of the carrot juice were stored at 30°C for 15 days and samples of compound feed were stored at room temperature 18-25°C for 15 days. The studied and control samples were evaluated for the growth and viability of the *Listeria* strain on days 5, 10 and 15 of storage.

L. monocytogenes growth was determined every 5 days. One ml of the carrot juice sample and 1 g of feed sample were suspended in 9 ml of PBS. After dilution, the samples were tested on agar medium Palcam (HiMedia, India) for *Listeria* presence by incubation in 30°C for 48 h (carrot juice) and in 18-25°C for 48 h (feed).

Listeria cells were counted using a Scan 100 counter (Interscience, France).

The pH level was determined every 5 days using a pH meter pH-150 MI (Izmtech, Kazakhstan).

Bacteriological examinations were conducted in accordance with ISO 16140-2011 Microbiology of food and animal feeding stuffs standards-Protocol for the validation of alternative methods and ISO 11133:2014(en) Microbiology of food, animal feed and water – Preparation, production, storage, and performance testing of culture media.

Statistical analysis

The results were subjected to determination of the Student's test criterion of significance in three probability thresholds: the first ($p<0.05$), the second ($p<0.01$), the third ($p<0.001$).

Results

In samples of the carrot juice with a strain of *Str. lactis*, the mean initial pH of 6.8 decreased on the 5th day to 5.2 and in next examinations significantly ($p\leq0.01$) decreased to 4.6 and 3.66, respectively. Similar tendency was observed in the changes of pH obtained in the samples with Lactina LTD and ST. Body. In the control group the initial pH of the samples was 6.8 and remained stable on the same level during the entire experiment (Table 1). Comparing the pH of the samples between the groups, the same values were observed on the 0 day, no significant differences between the strains of bacteria were observed in subsequent collections, while from the 5th day the pH of the control samples was significantly higher ($p\leq0.01$) than the others (Table 1).

Mean concentration of *L. monocytogenes* on the day 0 was on the similar level in all the groups (from 2.43 to 2.51 log CFU/ml) and in the second examination significantly increased – the biggest value was found in the control group (5.64 log CFU/ml). In the next examinations it started to decrease in the samples obtained in the strains of bacteria and in the 15th day of the experiment its concentration was under 0.01 log CFU/ml. In the control group concentration of *L. monocytogenes* significantly increased during the experiment and in the last examination achieved 9.21 log CFU/ml.

In samples obtained in concentrated feed with a strain of *Str. lactis*, the mean initial pH of 6.46 decreased on the 5th day to 5.84 and in next examination decreased to 5.72. In the last examination significantly decreased to 3.66 (Table 2). In the case of strain Lactina, the decrease in pH was more pronounced, because after 5 days its value was 4.94 and consequently statistically significantly decreased until the end of the experiment where it was 3.91 (Table 2). The results of pH changes in the case of the strain CHN-19 were similar with the difference, that the decrease in this parameter after five days was smaller, and statistically significant differences were observed only on the 10th and 15th day of the experiment (Table 2).

In the control group the initial pH of the samples was 6.8 and remained stable on the same level during the entire experiment (Table 2). Comparing the pH of the samples between the groups, similar values were observed on the 0 day, no significant differences between the strains of bacteria were observed in subsequent collections, while from the 5th day the pH of the control samples was significantly higher ($p\leq0.01$) than the others (Table 2).

Mean concentration of *L. monocytogenes* on the day 0 was on the similar level in all the groups (from 2.47 to 2.56 log CFU/ml) and in the second examination significantly increased – the biggest value was found in the control group (9.78 log CFU/ml) (Table 2). In the next examinations it started to decrease in the samples obtained in the strains of bacteria and

Table 1. Influence of lactic acid bacteria on pH of the carrot juice and viability of *Listeria* (Mean + SD).

Lactic acid bacteria	*Listeria* concentration, log CFU/ml							
	Day 0		Day 5		Day 10		Day 15	
	pH	concentration	pH	concentration	pH	concentration	pH	concentration
Streptococus lactis diastatikus AMC 23	6.8±0.1	2.49±0.19^{B}	5.2±0.1	4.67±0.19	4.63±0.2^{B}	3.37±0.33	3.66±0.15^{B}	< 0.01^{B}
Lactina LTD LAT. BY	6.8±0.1	2.51±0.17^{B}	5.5±0.2	4.72±0.1	4.7±0,15^{B}	3.45±0.42	3.9±0.1^{B}	< 0.01^{B}
ST. BODY-3	6.7±0.1	2.43±0.18^{B}	5.9±0.15	4.75±0.18	4.5±0.3^{B}	3.24±0.31	3.73±0.4^{B}	< 0.01^{B}
Control	6.8±0.1	2.46±0.17^{B}	^{A}6.8±0,1	5.64±0.06	6.8±0.1^{A}	5.87±0.04	6.8±0.1^{A}	9.21±0.1^{AB}

a, b, c – means for the same item within the same row bearing different superscripts are different at p<0.01
A, B, C – means for the same item within the same column bearing different superscripts are different at p<0.01

Table 2. Influence of lactic acid bacteria on pH of concentrated feed and viability of *Listeria* (Mean + SD).

Lactic acid bacteria	*Listeria* concentration, log CFU/g							
	Day 0		Day 5		Day 10		Day 15	
	pH	concentration	pH	concentration	pH	concentration	pH	concentration
Streptococus lactis diastatikus AMC 23	6.46±0.35	2.47±0.08^{B}	5.84±0.1	5.02±0.06	5.01±0.1	4.72±0.09	3.66±0.15^{B}	< 0.01 ^{B}
Lactina-LAT. BY	6.51±0.23	2.56±0.1^{B}	4.94±0.14^{B}	5.01±0.09	4.51±0.11^{B}	4.81±0.06	3.91±0.1^{B}	< 0.01 ^{B}
CNH-19	6.49±0.31	2.49±0.09^{B}	5.25±0.13	4.94±0.04	4.72±0.1^{B}	4.52±0.19	3.73±0.4^{B}	< 0.01 ^{B}
Control	6.52±0.33	2.51±0.11^{B}	6.46±0.35^{A}	.78±0.04^{AB}	6.46±0.35^{A}	9.79±0.09^{AB}	6.46±0.35^{A}	9.84±0.06^{AB}

a, b, c – means for the same item within the same row bearing different superscripts are different at p<0.01
A, B, C – means for the same item within the same column bearing different superscripts are different at p<0.01

in the 15th day of experiment its concentration was under 0.01 log CFU/ml. In the control group concentration of *L. monocytogenes* significantly increased on the 5th day and after remained on the similar level until the end of the experiment (Table 2).

Discussion

Lactobacillales, producing lactic acid as the major metabolic product, are a natural probiotic. This group includes strains of four genus: *Lactobacillus, Leucanostoc, Pediococcus* and *Streptococcus*. Probiotics are widely used in human medicine as a prophylactic or supportive measure and increasingly in veterinary medicine. Their action on various pathogens found in the feed has been proven (Wang et al. 2021, Zapaśnik et al. 2022). Our research focused on the influence of LAB on the viability of *L. monocytogenes* in the carrot juice and compound feeds.

The main task of animal husbandry is to produce food that is safe for the consumer. Infection of animals or contamination of food products of animal origin by *L. monocytogenes* is one of the threats. The results of our research confirm that the alternative may be LAB. The antagonistic effect of selected LAB to *L. monocytogenes* has been demonstrated which suggests the possibility of using these bacteria in the form of a feed additive to improve their microbiological safety.

Our research was conducted *in vitro*. However, in the studies conducted by other researchers it was shown that *in vivo* the antagonistic properties of probiotic strains were related, inter alia, to the ability to autoaggregate and coaggregate, as well as the ability to adhesion to the intestinal mucosa and inhibition of adhesion of pathogenic microorganisms (Li et al. 2015, Pessoa et al. 2017, Śliżewska et al. 2021). It has also been shown that LAB can form a biofilm constituting a barrier protecting against pathogens. In this way, LAB can compete with gastrointestinal tract pathogens facilitating their excretion (Ben Tahur et al. 2016, Śleżewska et al. 2021).

In the experiment of Muñoz et al. (2019) the antag-

onistic activity and antimicrobial effect of the probiotic supplement *Lb. rhamnosus* GG were studied. Such activity was spotted mainly because of organic derivatives of acids production. The results showed that co-incubation with *Lb. rhamnosus* had a bactericidal effect on *L. monocytogenes* at 15°C and had a bacteriostatic effect on *L. monocytogenes* at 10°C.

The optimal conditions for the growth of *L. monocytogenes* are temperatures in the range of 30-37°C, and a neutral or slightly alkaline pH (Zilelidou et al. 2018). However, the bacterium tolerates a wide range of temperatures, between 1-45°C. Our studies were conducted at temperatures 30°C for the carrot juice samples and 18-25°C for the compound feed. After 15 days of the experiment, the concentration of *L. monocytogenes* was below 0.01 log CFU/g. This indicates that the effect was similar to that obtained by Muñoz et al. (2019), although processing time would be expected to be different at higher temperatures. The growth of *L. monocytogenes* was inhibited under the influence of LAB after 15 days of the experiment, regardless of temperature. A similar result was obtained also by Saucedo-Reyes et al. (2012), where the growth of *L. monocytogenes* was inhibited after 15 days of culture with ascorbic acid at 10 and 4°C and pH 4.0. Therefore, it can be assumed that in case of inhibiting the growth of *L. monocytogenes* in food, the acidification process is more important than temperature. It is difficult to determine the exact critical level of acidity based on literature data. According to Farber et al. (1992, 2021) the minimum pH for the growth of *L. monocytogenes* was reported to be 4.3 using HCL as the acidulent. Saucedo-Reyes et al. (2012) showed that pH 4.5 hindered, but did not inhibit the growth of the bacterium, while growth inhibition was achieved at 4.0 at 10°C. In the study of Boziaris and Nychas (2007), the minimum pH at which growth of *L. monocytogenes* was observed was 4.81 at the temperature range of 25-35°C. Such pH values, and even lower, were achieved in our study already on the 10th day of the experiment, but only on the 15th day of the study a significant decrease in the concentration of *L. monocytogenes* was observed.

Our research investigated the effects of homogeneous cultures of *Str. lactis distaticus*, *Str. thermophilus* and *Leu. lactis* subsp. *Cremoris*, and a combination of *Str. thermophilus* with *Lb. bulgaricus* on the survival of *L. monocytogenes.* The dynamics of the concentration of *L. monocytogenes* decreased in all experimental samples, both the carrot juice and the compound feed, on the 10th day of the study. On the 15th day, the pH in the samples dropped to 3.3-4.0, under these conditions, according to the results of the studies, the number of *L. monocytogenes* reached extremely low level, below 0.01. The results of the study did not show any clear differences in the effect between the used LAB strains in limiting the *L. monocytogenes* concentration, however, the dynamics of the decrease in *L. monocytogenes* concentration was greater in the samples with the carrot juice. Thus, the LAB in the carrot juice and compound feed lowered the pH of the medium to such level that *Listeria* could not multiply in it.

Other results were obtained by Lim et al. (2020), who also studied the effects of LAB mixtures on the survival of *L. monocytogenes*. The Lim et al. (2020) study investigated the effect of LAB such as of *Leu. mesenteroides* and *Lb. curvatus* on the survival of *L. monocytogenes* in cheese. These studies showed that *Leu. mesenteroides* suppressed the growth of *L. monocytogenes* better than *Lb. curvatus*. In contrast, the composition of both LAB in the ratio 7:3 (*Leu. mesenteroides* to *Lb. curvatus*) showed the highest growth control of *L. monocytogenes*.

Other researchers have also studied the effects of different LAB combinations. In the studies by Serna-Cock et al. (2019), antimicrobial activity of homogenous LAB cultures and mixtures of metabolites produced by *Lb. plantarum* and *Weissella* (*W.*) *cibaria* and a combination containing *Lb. brevis, Lb. plantarum and W. cibaria* against *L.* monocytogenes were compared. These studies showed that a three-component mixture containing the metabolites *Lb. brevis, Lb. plantarum and W. cibaria* after 12 hours of fermentation had antimicrobial activity higher than monoculture activity.

In conclusion, based on the results obtained it can be concluded, that the addition of LAB to animal food increases its microbiological safety. LAB, due to the ability of lactic fermentation, lower the pH creating an environment difficult for the majority of contaminating microbes to survive. In contrast to antibiotics, they do not destroy the saprophytic intestinal flora and given to animals do not require the introduction of a withdrawal period for animal origin products.

The present results make it possible to expand the understanding of the causative agent of listeriosis, mainly the influence of certain factors on the survivability of the pathogen. Root crops and concentrated feeds are quite often used in feeding various animals both in industrial farms and in a private farmstead, thereby being one of the factors in the transmission of listeriosis infection.

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