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Chapter 27
The Search for Sources of Enterobacteria and Clostridia Endotoxins in Russian Dairy Farms: Possible Transfer of Endotoxins Through the Feed-Cow-Milk Chain

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Contents

Abstract Here, samples to identify bacterial endotoxins were collected from two commercial dairy farms in Leningrad Oblast: farm A (samples of feces and milk were taken) and farm (where samples were taken from the feeding table, milk and rumen chyme). The study comprised four groups (A1, A2, B1, B2) where 1 was the control and 2 the test group. A1 were healthy, A2 showed signs of pathologies of the limb joints, B1 received the basic diet (BD) and B2 were fed this, plus the feed

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additive AntiKlos. Using PCR, samples were examined for the presence of genes for Shiga toxins (*stx1A*, *stx2B*), intimin (*eae*) and enterohemolysin (*ehxA*) produced by enterobacteria; for alpha (*cpa1*), beta (*cpb*) and epsilon toxin (*etx*) produced by *Clostridium perfringens*, plus binary toxin (*cdtB*), toxin A (*tcdA*) and toxin B (*tcdB*) produced by *Cl. difficile*. In the test Group A2, one animal out of six studied (16.7%) had the intimin (*eae*) and enterohemolysin (*ehxA*) genes produced by enterobacteria that were not found in the control A1. The epsilon toxin gene (*etx*) was the most common and present in 100% of the examined fecal samples from both farms and 100% of milk samples from farm A.

27.1 Introduction

Increasingly intensive agricultural production systems may increase the risk of the spread of pathogens, primarily those that form toxins, that are potentially dangerous to both animals and humans [1]. The process of agriculture intensification serves to create conditions conducive to an increased pathogenic load. In parallel, the tendency in modern dairy farming to overload of the rumen with available forms of energy (starch and monosaccharides), while simultaneously reducing the proportion of fiber in the diet, can lead to conditions that are contrary to the physiological needs of the animal. Indeed, when highly concentrated feeding is employed, the composition of the microbiome in the rumen is usually disrupted. This often leads to colonization of the gastrointestinal tract by opportunistic and pathogenic microbiota [2] and the subsequent release of endotoxins [3].

Foodborne diseases pose a serious threat to human health and are often associated with faults at various stages of livestock industry. This starts with the feed production process [4] in that it has reported that the use of cattle manure as fertilizer can lead to the contamination of agricultural products with pathogens [5]. The leading causes of most foodborne disorders include pathogens such as *Escherichia coli*, *Salmonella enterica*, *Staphylococcus aureus*, *Campylobacter* spp., *Yersinia enterocolitica*, *Clostridium perfringens* and others all fall into this category [1, 6].

There are indications that the introduction of low-quality silage into the diet of cows is closely associated with the contamination of milk by microorganisms such as *Clostridium* spp. [7]. It was later shown [8] that, after feeding ryegrass haylage and silage, outbreaks of botulism B were observed in cattle. It was established that *C. botulinum* multiplied and produced a neurotoxin in the silage when the pH level of the plant mass was at least 5.3. In general terms, however, little attention has been paid to the issue of the circulation of pathogens and their toxins along the various links of the technological chain in farm conditions. Research devoted to the health, safety and management of herd issues in livestock production is thus of paramount importance (e.g., [9–12]). In particular, research related to the spread of such dangerous pathogens as *Clostridium* spp. and bacteria of the Enterobacteriaceae family that produce various toxins causing diseases in humans and animals should take priority [1].

The first step toward assessing the risk to human and animal health associated with microorganisms circulating in a farm environment is to develop a set of tools for detecting pathogens and their toxins. At the same time, introducing can reduce the risk of pathogens spreading within farms. Pathogen detection, coupled with biosecurity measures in the production chain plus high fiber, controlled starch/monosaccharide diets that are beneficial to the microbiome are thus the key to controlled and sustainable dairy production.

In this respect, the purpose of this investigation was to study the distribution of endotoxins from pathogenic enterobacteria and clostridia of feces, rumen contents, feed (from the feeding table) and milk of cows using bespoke PCR test techniques. A second aim was to assess the effect of a natural feed additive (AntiKlos—BIOTROF LLC, which has a high level of antagonistic activity against clostridia and enterobacteria) on the presence of endotoxins in the body of dairy cows.

27.2 Materials and Methods

Samples for the study of endotoxins were collected on two dairy farms in the Leningrad Oblast in 2023 from four potential sources (two sites, both with a control and a test group) of bacterial endotoxins, i.e., feces, rumen contents, feed from the feed table, and milk of black-and-white Holstein cows of the 2nd and 3rd lactation. In both farms, the animals were kept in the same environment and in tethered conditions. Cow rations were calculated automatically using the AMTS.Cattle.Professional program in accordance with generally accepted requirements [13]. All procedures on animals were performed in compliance with the Animal Ethics Committee recommendations of the L. K. Ernst Federal Research Center for Animal Husbandry.

In a commercial farm of the Pushkin District (farm A), samples of feces and milk were taken from cows during decline of their lactation, with an average productivity of 8500 kg, and with a live weight of 600 kg. The diet included silage (25 kg), haylage (15 kg), hay (1.6 kg), and mixed feed (11.3 kg). The following groups were formed: A1, control (clinically healthy cows), and A2, test group who showed clinical signs of pathologies of the joints of the limbs, including lameness and others (10 animals in each group).

In a commercial farm of the Gatchina District (farm B), samples of feed were taken from the feeding table, while milk and rumen chyme were collected from the cows with an average productivity of 9200 kg and a live weight of 600 kg. Groups were formed as follows: B1, control group that received the basic diet; and B2, test group in which the cows were fed BD supplemented with the feed additive AntiKlos (BIOTROF LLC, St. Petersburg, Russia). The feed additive AntiKlos contains a strain of the microorganism *Bacillus* spp., as well as organic acids with antibacterial and anti-inflammatory effects. The *Bacillus* spp. strain, as part of the additive, has a high level of antagonistic activity against clostridia and enterobacteria, which was confirmed by in vitro experiments using test strains of *C. perfringens* obtained from the ATCC collection (USA) and the *E. coli* strain K-12 F + Str. R (KS-507) from the

All-Russian Collection of Industrial Microorganisms (VKPM). Again, there were 10 animals in each group. BD included silage (20 kg), hay (2.0 kg) and mixed feed (8.06 kg). The cows of Group B2 received AntiKlos at the rate of 50 g per 1 cow per day for 90 days and from 130 days after calving, distributed manually to each animal in the experimental group.

Collection of all samples was carried out following the maximum possible compliance with aseptic conditions for the methods used. Sampling for feed analysis was carried out from the feeding table. Five-point samples were taken from different places (at equal distances) of the feeding table. From the point samples, a combined sample was made, the mass of which was 3 kg. From the combined food sample, an average sample weighing 100 g was isolated using the quartering method.

Milk, feces and rumen contents were collected individually. Milk was aseptically collected from the mammary glands into sterile conical vials. The collection procedure was preceded by the preparation of the mammary glands of cows before taking milk samples, including sanitary treatment. When collecting milk samples for research, the average sample was made up of proportional fractions of all daily milk yields (morning and evening). Three to five streams of milk were taken from each quarter of the udder. To create a pooled sample, approximately 12 ml of milk was decanted from each quarter, i.e., only 48 ml per cow. Fecal samples were collected from the rectum. Sampling of rumen contents (30–50 g) was performed in triplicate from each cow from the upper part of the ventral rumen sac with the maximum possible compliance with aseptic conditions for this method used and manually using a sterile probe. The number of animals studied from each group is indicated in Tables 27.2 and 27.3. Three samples were studied from each animal (i.e., three replicates per cow).

The collected samples of feces, rumen chyme, milk and feed were immediately placed in sterile plastic bottles. All samples were frozen at −20 °C and transported in dry ice to the molecular genetic research laboratory of BIOTROF LLC (St. Petersburg, Russia) for subsequent DNA extraction.

Total DNA from the samples was isolated using the Genomic DNA Purification Kit (Thermo Fisher Scientific, Inc., USA) according to the manufacturer's instructions. Analysis of bacterial toxin genes in samples was carried out using a PCR testing method. Herewith, samples were examined for the presence of genes for Shiga toxins (*stx1A*, *stx2B*), intimin (*eae*), enterohemolysin (*ehxA*) produced by enterobacteria; alpha toxin (*cpa1*), beta toxin (*cpb*) and epsilon toxin (*etx*) produced by *C. perfringens*; and binary toxin (*cdtB*), toxin A (*tcdA*) and B (*tcdB*) produced by *Clostridium* (*Clostridioides*) *difficile*. Primers for detection of toxin genes (Table 27.1) were designed using the NCBI/Primer-BLAST program [14]. The housekeeping gene *Eub339-518* (16S rRNA) was used as a control. Amplification reactions were produced using a DTlight amplifier (DNA-Technology, Russia) and a Tersus Plus PCR kit (JSC Evrogen, Russia) in accordance with the manufacturer's protocol. The amplification mode and conditions were as follows: 3 min at 94 °C (preheating); 40 s at 94 °C, 60 s at 55 °C, and 90 s at 72 °C (34 cycles).

Mathematical and statistical processing of the results was implemented using the method of multifactor Analysis Of Variance (ANOVA) in Microsoft Excel XP/2003

Table 27.1 Sequences of primers used in PCR studies of endotoxin genes

Genes	Primer sequences (5′–3′)	Amplicon length, bp
stx1A	F: CCTTTCCAGGTACAACAGCGGTT R: GGAAACTCATCAGATGCCATTCTGG	478
stx2B	F: AAATATGAAGAAGATATTTGTAGCGGC R: CAGCAAATCCTGAACCTGACG	251
eae	F: GGAACGGCAGAGGTTAATCTGCAG R: GGCGCTCATCA TAGTCTTTC	346
ehxA	F: GCATCATCAAGCGTACGTTCC R: AATGAGCCAAGCTGGTTAAGCT	534
cpa1	F: GCTAATGTTACTGCCGTTGA R: CCTCTGATACATCGTGTAAG	324
cpb	F: TCCTTTCTTGAGGGAGGATAAA R: TGAACCTCCTATTTTGTATCCCA	611
etx	F: TGGGAACTTCGATACAAGCA R: TTAACTCATCTCCCATAACTGCAC	396
cdtB	F: TTGACCCAAAGTTGATGTCTGATTG R: CGGATCTCTTGCTTCAGTCTTTATAG	262
tcdA	F: GCATGATAAGGCAACTTCAGTGGTAa R: AGTTCCTCCTGCTCCATCAAATG	629
tcdB	F: CCAAARTGGAGTGTTACAAACAGGTG R: GCATTTCTCCATTCTCAGCAAAGTA	410

Table 27.2 Presence of endotoxins in samples of feces and milk of cows from farm A, of the Pushkino District ($n = 3$)

Group of cows	Toxin-producing taxa and genes									
	Enterobacteriaceae				*C. perfringens*			*C. difficile*		
	stx2B	*stx1A*	*eae*	*ehxA*	*cpa1*	*cpb*	*etx*	*cdtB*	*tcdA*	*tcdB*
Fecal samples										
A1 (control)	1*/3**	1/3	0/3	0/3	2/3	0/3	3/3	0/3	0/3	0/3
A2 (test)	1/6	0/6	1/6	1/6	3/6	0/6	6/6	0/6	0/6	0/6
Milk samples										
A1 (control)	0/2	1/2	0/2	0/2	0/2	0/2	2/2	0/2	1/2	0/2
A2 (test)	0/3	0/3	0/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3

Note *The numerator indicates the number of animals whose samples contained toxin genes. **The denominator indicates the total number of animals studied

Table 27.3 Presence of endotoxins in samples of feed, rumen and milk of cows from farm B in the Gatchina District ($n = 3$)

Group of cows	Toxin-producing taxa and genes									
	Enterobacteriaceae				*C. perfringens*			*C. difficile*		
	stx2B	*stx1A*	*eae*	*ehxA*	*cpa1*	*cpb*	*etx*	*cdtB*	*tcdA*	*tcdB*
Feed samples										
Average mixed samples from Groups B1 and B2	3*/3**	2/3	0/3	0/3	3/3	0/3	3/3	0/3	2/3	0/3
Rumen chyme samples										
B1 (control)	2/3	0/3	2/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3
B2 (test)	1/3	0/3	2/3	0/3	0/4	0/5	3/3	0/3	0/3	0/3
Milk samples										
B1 (control)	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
B2 (test)	0/3	0/3	0/3	0/3	0/3	0/3	3/3	0/3	0/3	0/3

Note *The numerator indicates the number of animals whose samples contained toxin genes. **The denominator indicates the total number of animals studied

and RStudio (Version 1.1.453; [15]). The significance of differences was determined using Student's t-test; differences were considered statistically significant at $p < 0.05$.

27.3 Results and Discussion

As a result of PCR analysis, the presence of genetic determinants of Shiga toxins (*stx1A*, *stx2B*) of enterobacteria and those of alpha (*cpa1*) and epsilon (*etx*) toxins produced by *C. perfringens* is shown in Table 27.2. *E. coli* O26 strains that produce Shiga toxins that have been previously isolated from livestock [16] and are capable of causing diseases in both animals [17] and humans [18]. Shiga toxin is a major factor in the Enterobacteriaceae group responsible for severe human diseases [19]. Other studies have demonstrated [20] that the cattle and human *E. coli* O26 strains in Scotland were genetically identical, and isolates from cattle in Scotland are closely related to highly pathogenic isolates from humans in Germany and Italy. Our discovery of the presence of endotoxin genes in samples from clinically healthy cows once again confirms the likelihood of asymptomatic carriage of pathogens.

Interestingly, the genes for intimin (*eae*) and enterohemolysin (*ehxA*), produced by enterobacteria, were also found in our experiment in Group A2 (that included cows with joint diseases), in one animal out of six studied (corresponding to 16.7%), while they were not detected in the group of clinically healthy animals (control Group A1). Bacteria commonly associated with septic arthritis in cattle are known to be

E. coli in particular [21]. In recent years, to improve the milk productivity of dairy cows on farms, it has been common to use diets with a high content of mixed feed that causes a number of metabolic diseases, such as lactic acidosis. It has been established that acidosis often leads to inflammatory diseases, such as laminitis, mastitis, endometritis, hepatitis and others [22]. Limb diseases are the most important clinical sign in dairy herds suffering from acidosis [23]. Delarocque et al. [24] established that intestinal bacteria and their metabolites can migrate to extraintestinal distal organs through endogenous pathways. Dysbiosis of the rumen microbiota, in parallel with acidosis, leads to disruption of the rumen epithelial barrier, which causes the penetration of bacteria and/or metabolites into the body through the bloodstream or lymph flow. Once in the blood, bacterial pathogenic factors can induce the production of haptoglobin, serum amyloid A, and the inflammatory cytokines TNF-α and IL-1β [25], leading to associated inflammatory diseases. Thus, the presence of the *eae* and *ehxA* genes in the feces of cows in the experimental Group A2 could be associated with joint diseases.

Note that in some samples of cow milk, we found the *stx1A* and *etx* genes identified in feces. However, the epsilon toxin (*etx*) gene produced by *C. perfringens* was the most common and was present in 100% of the feces and milk samples examined. The most likely explanation for this observation is that this this microorganism is often found in the soil, water, intestinal contents and feces of mammals [26]. It has also been detected in meat such as pork, beef and chicken [27]. Previously, when analyzing the intestinal contents of broiler chickens, the presence of *C. perfringens* was detected in 75–95% of cases [28]. The presence of toxigenic *E. coli* strains in raw milk has been reported [29], as has the role of *E. coli* in bovine mastitis [30]. According to Owusu-Kwarteng et al. [31], endogenous milk contamination can occur as a result of direct transfer of pathogens from the blood of an infected animal into the milk (as a systemic infection) or through infection of the udder. In milk samples from one of our cows, we identified the *tcdA* gene produced by *C. difficile*; this was not present in the feces and thus may be associated with a local udder infection.

Among the samples of feed, rumen and milk of cows from farm B (Gatchina District), the samples of feed from the feeding table had the greatest contamination with endotoxin genes (Table 27.3). Hereby, the genes for type 2 Shiga-like, alpha and epsilon toxins of *C. perfringens* and toxin A of *C. difficile* were identified in 66.7–100% of cases. In the rumen of cows in the control Group B1 and experimental Group B2, the genes for type 1 Shiga-like toxin, intimin of enterobacteria, alpha and epsilon toxins of *C. perfringens* were also identified. *C. difficile* toxin genes were not detected in the rumen of cows of both groups. By analogy with farm A, the *etx* gene was the most represented and was detected in 100% of the studied samples of rumen chyme and feed. In the group B2 with the introduction of a feed additive, the content of Shiga toxin (*stx2B*) genes in the rumen was detected in one animal out of three studied, whereas it was found in two animals of the three studied in the control.

Our data indicate that the infection of animals with toxin-producing strains of enterobacteria and clostridia most likely occurs through feed. It is known that Shiga toxin-producing *E. coli* (STEC) can colonize plants through seeds [32]. Later, during the growing season of crops, infection with these pathogens can occur during the use

of various agricultural practices [33], including the application of organic fertilizers to the soil [34]. Crozier et al. [35] showed that a number of intestinal pathogens have effective defense mechanisms against the plant immune system. For example, genes involved in stress responses in *E. coli* O157:H7 cells are differentially expressed when exposed to spinach and lettuce extracts. It is also known that errors in the process of forage procurement of canned feed (slow decrease in pH, insufficient compaction of plant mass, etc.) can lead to the development of clostridia and enterobacteria [36].

It is worth noting that, in contrast to farm A, the transfer of genetic determinants of the studied endotoxins into milk did not occur under conditions of farm B. This demonstrates the effect of the importance of the "sanitary barrier" and may be associated with the individual immune status of the flock determined by feeding and/or housing conditions.

Here, we have thus demonstrated that the circulation of toxin-producing pathogens occurs between two different large agricultural systems for crop and livestock production. This indicates the complexity and versatility of the existing problem. Our studies highlight the possibility of transfer of some endotoxins of enterobacteria and clostridia through the feed-cow-milk chain, which depends on the specific conditions for the generation of livestock products.

27.4 Conclusion

Prevention of infectious diseases in cattle is important for both economic efficiency of livestock production and public health. The results of our study may be useful for implementing foodborne pathogen control measures on farms. Gaining an understanding of likely sources of infection is critical to preventing disease. Further development of methods for quantifying endotoxin genes will allow more accurate choice of prevention and treatment measures.

Consumption of unpasteurized milk and dairy products continues to pose a significant health risk to consumers due to the persistence of pathogenic microorganisms that pose a serious safety risk. Therefore, it is necessary to purposefully implement the appropriate systems to ensure the safety of milk and dairy products, starting with the cultivation of feed crops.

Further research into the ability of pathogen toxin genes to be transmitted through the milk production chain and associated environments will help develop effective measures to combat unwanted contamination, primarily focused on the sources of contamination.

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