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Effects of Zoovit probiotic on faecal microbiota in cattle

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Abstract: The amount of pathogenic bacteria in feces is an indicator characterizing the health status of animals. This is important to protect the environment and the health of farm workers. The aim of the present study is to determine the effect of probiotic Zoovit on the microbiome of the faecal mass of Holstein cattle. In the experiment, two groups were formed - experimental and control, 30 each. A probiotic was added to the combined feed of the experimental group, but not to the control group. The food ration with the participation of probiotic is placed twice a day. To determine the microbiome in fecal matter in cattle, PCR analyzes were used for the detection of *Lactobacillus delbrueckii subsp. bulgaricus*, a species-specific PCR assay for the presence of total DNA in *L. delbrueckii* and *L. delbrueckii subsp. bulgaricus* and methods for determination of sulfite-reducing colonies. No representatives of *Staphulococccua aureus* and Clostridium sp were isolated in the faecal mass of the animals from both groups. For *Escherichia coli* and Coliforms, a significant decrease in the faecal mass of cows fed with feed containing probiotics was found. The presence of *L. delbrueckii subsp. bulgaricus* in animals receiving a probiotic. Colonies with a colonial characteristic typical of *L. delbrueckii subsp. bulgaricus* were isolated in the animals receiving probiotic Zoovit and in the control group, no colonies typical of *Lactobacillus bulgaricus* were found.

Keywords: probiotics; Holstein; faecal microbiota; PCR analysis; PCR amplification

INTRODUCTION

The continuous application of antibiotics in livestock husbandry both for treatment and non-therapeutic goals results in emergence of antimicrobial resistance and accumulation of antibiotic residues in the animal body. Thus, there is an urgent need to find efficient alternatives to antibiotics in order to avoid additional complications from these effects (Anee et al., 2021; Razzaque, 2021; Zalewska et al., 2021; Elshaghabee and Rokana, 2022).

Xu et al. (2017) have investigated the effects from application of Lactobacillus casei Zhang and Lactobacillus plantarum P-8 on milk yield, milk composition and milk functional component profiles, as well as faecal microbiota of dairy cows. The authors reported significantly increased milk yield and higher content of immunoglobulin G (IgG), lactoferrin (LTF), lysozyme (LYS) and lactoperoxidase (LP) in milk, whereas the somatic cell counts (SCC) were substantially reduced ($P<0.01$); yet no significant effect was found out on milk fat, protein and lactose levels ($P>0.05$).

Research studies have demonstrated the positive effect of beneficial bacteria from the *Lactobacillus* genus and their metabolites on animal health (Zamojska et al., 2021), moreover, the restricted use of antibiotic was reported to have a beneficial effect on the environment and living organisms, including people.

The studies on the influence of probiotics on faecal microbiota are rather few. The reduction of faecal pathogenic microbial load is a parameter of animal health status, and ruminal microbial pathogens counts. This parameter is also impor-

tant for protection of the environment and health of farm workers.

Xu et al. (2017) affirmed that probiotic additives did not alter the composition and diversity of faecal bacteria in supplemented animals.

According to Tufarelli et al. (2017), the addition of probiotics results in reduction of faecal NH3-N concentrations (+15.5%) and butyric acid content, but no effect on levels of acetic and propionic acids was noted. The authors outlined that probiotics increased the proportion of faecal *Lactobacillus* in pigs. Dietary supplementation with probiotic additives improved growth performance and meat quality in pigs, as well as their liveability.

In their study, Kabir et al. (2022) found out that feeding cattle probiotic-fermented rice straw ration improved the growth performance, haematological and serum biochemical parameters and increased the proportion of *Lactobacillus* in the faecal bacterial community. According to the researchers, feeding total mix ration containing probiotic-treated rice straw was a practical approach to promote cattle growth and health as compared to ration with untreated straw.

In a 14-day trial with cattle from a control group and a probiotic-supplemented experimental group, Vadopalas et al. (2021) found no significant between-group differences in faecal pH, but demonstrated considerably greater lactic acid bacteria counts and dry matter content in the experimental group.

After addition of 6 g probiotic containing *Bifidobacterium animalis, Lactobacillus casei, Streptococcus faecalis* and *Bacillus cerevisiae* to the milk replacer of newborn Holstein calves, Guo et al. (2022) found out the that beneficial faecal microflora *(Prevotella)* tended to increase whereas the conditional pathogens *(Dorea)* decreased; the authors affirmed that the predominant species in the faeces of calves belonged to *Bacteroidetes, Firmicutes, Actinomycetes* and *Proteobacteria*.

According to Kawakami et al. (2010) the inclusion of a probiotic based on lactic acid bacteria and yeasts to the diet of Holstein calves increased the daily weight gain, dry matter intake, feed conversion and the faecal scoring.

In feedlot cattle, the faecal microbial community of animals supplemented with a probiotic via the feed was more diverse, with predominance of members of *Firmicutes* (72–98%), *Actinobacteria* (0.8–27%) and significantly lower percentages of *Bacteroidetes* (0.08–4.2%). The authors demonstrated changes in the counts of *Clostridiaceae*, *Lachnospiraceae*, *Ruminococcaceae* and *Bifidobacteriaceae*.

By sequencing analysis of faecal microbiota in Aberdeen Angus cattle, reared on pasture and indoor and only on the pasture, the latter group demonstrated higher abundance of *Firmicutes*, *Cyanobacteria, Elusimicrobia* and *Patescibacteria* (Zhang et al., 2021).

The information about the effect of probiotic bacterial species on faecal microbiota is scarce. More in-depth studies in this field are necessary considering the importance of protection of the environment and agricultural workers' health.

The aim of this study was to evaluate the effect of the Zoovit probiotic on faecal microbiota in Holstein cattle.

MATERIAL AND METHODS

The studies on the effect of the Zoovit probiotic on dairy Holstein cows were conducted in a livestock facility, city of Plovdiv.

The analysis of samples was performed in the Milk and Dairy Products Testing Laboratory, LB LACT", Plovdiv.

In order to evaluate the effect of Zoovit on faecal microbiota, two groups of 30 dairy cattle in each were formed in two barns. The daily ration of both groups consisted of 15 kg concentrate, 20 kg silage and 4 kg alfalfa hay.

The probiotic-supplemented ration was fed twice daily: morning and evening. The ration of group I (experimental) was supplemented with 0.600 kg probiotic (0.020 kg per cow). The second (control) group received no probiotic.

The probiotic preparation Zoovit contains four lactic acid bacterial strains – *Lactobacillus del-*

brueckii subsp. bulgaricus, *Streptococcus salivarius subsp. thermophilus, Lactobacillus acidophilus, Lactobacillus lactis* and one strain *Propinibacterium*.

The determination of bovine faecal microbiota involved PCR assays for detection of *Lactobacillus delbrueckii subsp. bulgaricus,* a species-specific PCR assay for total DNA in *L. delbrueckii* and *L. delbrueckii subsp. bulgaricus,* and methods for enumeration of sulfite-reducing bacterial colonies.

The following microbiological methods for analysis were employed:

- *Escherichia coli*, as per ISO 16649-2:2014;
- *Staphylococcus aureus* as per BSS EN ISO 6888-1:1999/A2:2018;
- Coliforms as per ISO 4832:2006. Microbiology of food and animal feeding stuffs - horizontal method for the enumeration of coliforms;
- *Enterobacteriaceae*, as per ISO 21528-1,2:2017;
- Enumeration of sulfite-reducing colonies, as per ISO 15213:2003.

Six faecal samples from probiotic-supplemented cows were analysed, as well as three randomly selected samples from control (non-supplemented) cows.

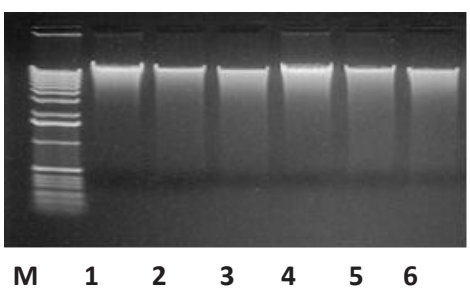

Figure 1. Determination of the purity of the isolated DNA from the six samples of cow faeces

Genomic DNA extraction from colonies of bovine faecal samples was performed with Gene JetTM Genomic DNA Purification Kit (Thermo Fisher Scientific Inc., Waltman, USA).

The extracted DNA purity was determined by 0.7% agarose gel electrophoresis as follows (Figure 1).

M-100 bp molecular marker (Bioneer, Korea);

1-3 DNA isolated from faeces of control cows that did not receive the probiotic;

4-6 DNA isolated from faeces of 3 cows, supplemented with probiotic.

For detection of *L. delbrueckii* and *L. delbrueckii subsp. bulgaricus* in bovine faeces, PCR with species-specific primers was used (Lick et al., 2000; Lick et al., 2001).

The nucleotide sequence of primers is presented in Table 1. The PCR amplification conditions are summarised in Table 2.

Ten /10/ randomly selected colonies from three control cows and three probiotic-supplemented cows were used in the PCR assay for detection of live *L. delbrueckii subsp. bulgaricus* in faecal samples.

The test was performed with DNA isolated from a single lactic acid bacterial colony on MRS agar medium.

RESULTS AND DISCUSSION

The results about the effects of the dietary Zoovit probiotic on changes in *Escherichia coli,* coliforms*, Staphylococcus aureus* and *Clostridium sp.* prevalence in animal faeces are presented

Table 1. Nucleotide sequence of species-specific primers, Fw-forward primer, Rv-reverse primer

Kind	Nucleotide sequence	Size (base units)	Literature
L. delbrueckii	Fw-AAT TCC GTC AAC TCC TCA TC Rv-TGA TCC GCT GCT TCA TTT CA	715	Lick et al., 2000
L. delbrueckii subsp. bulgaricus	Fw-CCT CAT CAA CCG GGG CT Rv-TGA TCC GCT GCT TCA TTT CA	678	Lick et al., 2000; Lick et al., 2001

Table 2. PCR reaction conditions for amplification of species-specific PCR products

Temperature regime	*L. delbrueckii*	*L. delbrueckii subsp. bulgaricus*
Initial denaturation	95°C-10 min	95°C-10 min
	10 cycles	
Denaturation	95°C-20 sec	95°C-20 sec
Hybridization	**55°C-20 sec**	**65°C-20 sec**
Extension	72°C-40 sec	72°C-40 sec
	35 cycles	
Denaturation	95°C-20 sec	95°C-20 sec
Hybridization	**50°C-30 sec**	**60°C-30 sec**
Extension	72°C-1 min	72°C-1 min
Ultimate extension	72°C-10 min	72°C-10 min

Table 3. Effect of probiotics on the content of microorganisms

Microorganism	**Control**	**With probiotic**
Escherichia coli	$1{,}5.10^5$ cfu/g	$5{,}5.10^3$ cfu/g
Coliforms	$3{,}5.10^5$ cfu/g	$2{,}1.10^4$ cfu/g
Staphylococcus aureus	Absence	Absence
Clostridium sp.	Absence	Absence

in Table 3. The presented data demonstrated that neither *Staphylococcus aureus* nor *Clostridium sp.* were present in the faeces of control and probiotic-supplemented cows.

Escherichia coli and coliform counts in the faeces of probiotic-supplemented cows were substantially reduced. In their study with cattle that received a dietary probiotic, Ghazanfar et al. (2018) observed a reduction of faecal coliforms ($P<0.05$). The authors affirmed that the counts of *Lactococcus* species have increased ($P<0.05$). Comparable results were reported by Jatkauska and Vrotniakiene (2010) in a study with calves – the faecal counts of clostridia and enterococci were substantially lower when their ratio was supplemented with a probiotic. Also, the proportion of diarrhoeic probiotic-supplemented calves decreased from 50% to 20%.

A 2 log reduction of active *Escherichia coli* cells was established in probiotic-supplemented animals compared to controls. Similarly, lower faecal *Escherichia coli* counts in the faeces of feedlot calves receiving a probiotic was reported by Mansilla et al. (2023), affirming also the the supplementation with probiotics improved the health and productivity of cattle.

The reduction of coliforms in probiotic-fed cows' vs non-supplemented controls was by one order of magnitude (1 log reduction).

Figures 2÷5 illustrate the results of the present study. In control cows, a dense and profuse growth of *Escherichia coli* and coliforms was observed on the solid nutrient medium, whereas faecal cultures of Zoovit-supplemented animals demonstrated only single colonies consequently to reduction of counts of active microbial cells.

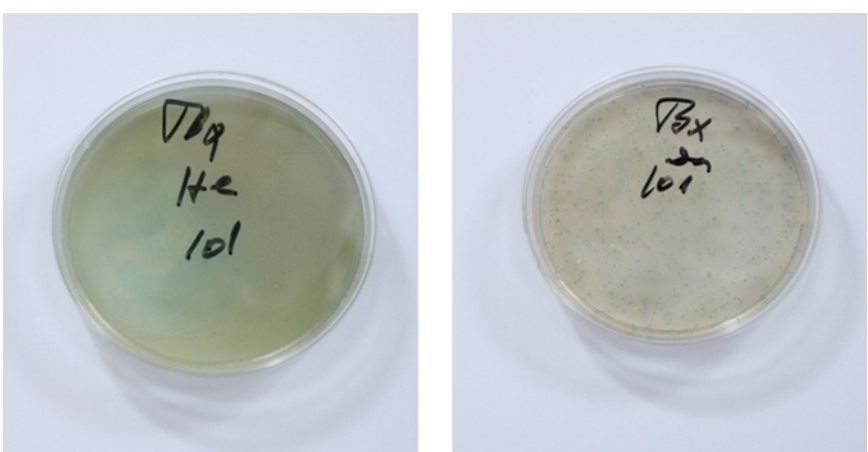

Figure 2. Bactericidal effect of the probiotic product Zoovit on E. Coli

Xu et al. (2017) also found out decreased faecal bacterial counts following dietary supplementation with probiotics based on lactic acid bacteria.

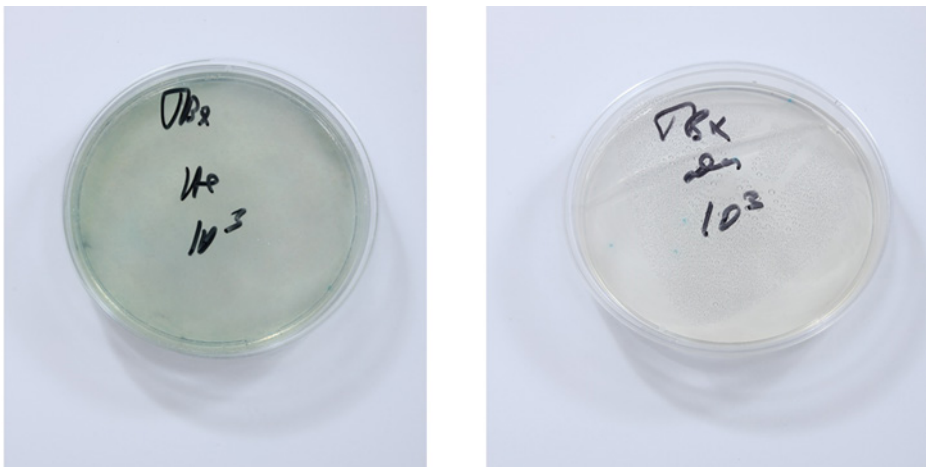

Figure 3. Bactericidal effect of the probiotic product Zoovit on E. Coli

Figure 4. Bactericidal effect of the probiotic product Zoovit on Coliforms

Figure 5. Bactericidal effect of the probiotic product Zoovit on Coliforms

The results from the PCR assay for detection of *Lactobacillus delbrueckii subsp. bulgaricus* showed that lactic acid bacteria in bovine faeces attained 7.17-7.32 log cfu/g in controls as compared to 7.36-7.54 log cfu/g in samples from animals that received the probiotic.

Kawakami et al. (2010) reported that feeding probiotics containing lactic acid bacteria improved faecal microbiota but also decreased the incidence of bovine diarrhoea. Increased *Lactobacillus* counts in heifers supplemented with probiotic were also reported by Ghazanfar et al. (2015).

Using PCR for detection of total DNA from *L. delbrueckii* and *L. delbrueckii subsp. bulgaricus*, fragments with size 715 bp and 678 bp, specific for *L. delbrueckii* and *L. delbrueckii subsp. bulgaricus* were isolated from the faeces of cows supplemented with the Zoovit probiotic (Figure 6 A and B). The DNA isolated from controls exhibited fragments with size of about 150 bp, specific for other lactic acid bacteria. This confirmed that the relative proportion of *L. delbrueckii subsp. bulgaricus* in the gastrointestinal tract of supplemented cows has increased. These findings were entirely in line with the results for detection of *L. delbrueckii subsp. bulgaricus* in the upper digestive tract of Gottingen pigs (Lick, 2001).

In probiotic-fed cows, a colony No. 6 with colonial features typical for *L. delbrueckii subsp. bulgaricus* was found out. The molecular genetic analysis of this colony using species-specific primers for *L. delbrueckii subsp. bulgaricus* revealed a fragment with the desired size of 678 bp (Figure 6 C). In samples from the control group, no colonies with the colonial morphology of *Lactobacillus bulgaricus* were detected.

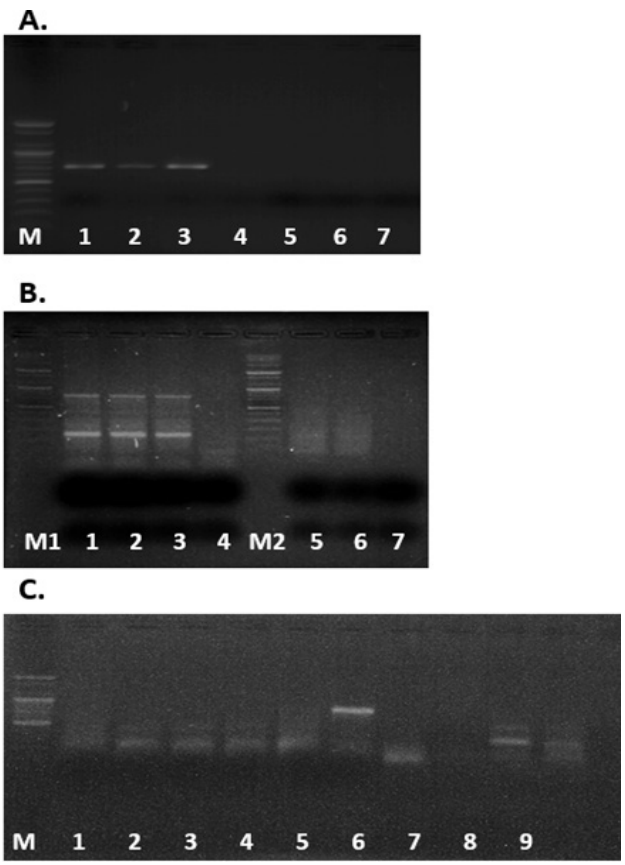

Figure 6. Detection of L. delbrueckii and L. delbrueckii subsp. bulgaricus by PCR using total DNA from cow faecal samples

A. PCR amplification with *L. Delbrueckii*-specific primers.

M-100 bp molecular marker (Bioneer, Korea); **1-3** PCR amplification of DNA isolated from faeces of three probiotic-supplemented cows, **4**-negative control without DNA; **5-7** PCR amplification of DNA isolated from faeces of control cows that did not receive the probiotic.

B. PCR amplification with primers specific for *L. delbrueckii subsp. Bulgaricus.*

M1-100 bp molecular marker (Bioneer, Korea); **1-3** PCR amplification of DNA isolated from faeces of three probiotic-supplemented cows, **4-**negative control without DNA; **M2**-100 bp molecular marker (Bioneer, Korea); **5-7** PCR amplification of DNA isolated from faeces of control cows that did not receive the probiotic.

C. PCR amplification with primers specific for *L. delbrueckii subsp. Bulgaricus* with a single lactic acid bacterial colony grown on selective nutrient medium as template

M-100 bp molecular marker (Bioneer, Korea); **1-10** PCR amplification with a single lactic acid bacterial colony grown on MRS agar as template.

The molecular genetic identification via PCR of total DNA from bovine faeces detected the presence of *L. delbrueckii subsp. bulgaricus*, whereas control samples yielded fragments of

approximate size 150 bp, specific for other lactic acid bacterial species, e.g. confirming the absence of *L. delbrueckii* and *L. delbrueckii subsp. bulgaricus*.

In Zoovit probiotic-fed cows, colonies with colonial features typical for *L. delbrueckii subsp. bulgaricus* were detected. The PCR molecular genetic analysis of this colony with species-specific primers for *L. delbrueckii subsp. bulgaricus* revealed a fragment with the desired size of 678 bp. In the control group, no colonies with the colonial morphology of *Lactobacillus bulgaricus* were present.

The analysis of bovine faecal samples confirmed that neither *Staphylococcus aureus* nor *Clostridium sp.* were present in control and probiotic-supplemented animals.

CONCLUSIONS

The study demonstrated that:

1. The molecular genetic identification via PCR using bovine faecal total DNA detected the presence of *L. delbrueckii subsp. bulgaricus* in cows that received a dietary probiotic.

2. The samples from the animals supplemented with the Zoovit probiotic demonstrated colonies with morphology specific for *L. delbrueckii subsp. bulgaricus*, which was also confirmed by the PCR assay. The typical *Lactobacillus bulgaricus* colonies were not observed in samples from control cows.

3. The analysis of bovine faecal samples confirmed that neither *Staphylococcus aureus* nor *Clostridium sp.* were present in control and probiotic-supplemented animals.

4. The counts of active *Escherichia coli* cells in cows supplemented with probiotic were reduced by 2 log units, whereas the counts of coliforms – by one log unit compared to control group of cows.

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