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Development of Synbiotic Preparations That Restore the Properties of Cattle Feed Affected by Toxin-Forming Micromycetes

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Abstract: A synbiotic preparation based on two strains of *B. licheniformis* was developed to reduce the harmful effects of mycotoxins in postharvest products used for cattle feed. The genomes of these strains were sequenced to analyze their potential for producing bioactive secondary metabolites. An optimal substrate was selected from the variety of plant substrates, with soybeans being identified as the optimal choice. Based on the results of experiments conducted on experimental animals for a month, the introduction of a synbiotic preparation leveled the negative effect of mycotoxins on weight gain in heifer calves, bringing it to the level of the control groups. There was also a decrease in the level of alkaline phosphatase in serum to normal values, which may indicate the probiotic effects of the preparation, not associated with the elimination of the effect of mycotoxins. The activity of these strains against mycotoxins correlates with the presence of lactonase genes in their genomes. Antimicrobial and bioactive metabolites, such as lichenicidin and lichenisin synthesized by strains CL 33 and CL 56, also seem to contribute to the probiotic effect. The results of the study open up prospects for the creation of new synbiotic preparations to fight contamination with mycotoxins.

Keywords: mycotoxins; probiotics; synbiotics; bacillus; cattle; heifer calves; soybean

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1. Introduction

Mycotoxins are toxic secondary metabolites synthesized by mold fungi of the genera *Aspergillus*, *Fusarium*, *Penicillium*, *Claviceps* and *Alternaria*. Mycotoxins have a negative impact on aquaculture and agriculture, entering plant feed as a result of the processing of postharvest products obtained from fields infected with phytopathogenic fungi [1]. In dairy farming, the most negative effects are observed from aflatoxins (AF) and zearalenone (ZEA). Of the four main representatives of aflatoxins, namely (AFB1, AFB2, AFG1, AFG2), aflatoxin B1 (AFB1) is the most toxic and is found in the greatest amounts in feed. At the same time, toxin derivativesformed due to a limited degradation of toxins in the rumen can be just as or even more toxic than the original toxins [2].

The negative effect of mycotoxins, in addition to reducing the productive qualities of animals, lies in their transfer in a biotransformed or unchanged form to animal products, which is dangerous to the health of consumers. The adverse health effects of mycotoxins range from acute poisoning to long-term effects, such as im mune deficiency and cancer [3]. Mycotoxin contamination can lead to reduced carcass yield, decreased average daily gain, poor feed conversion efficiency, increased mortality rate and other negative impacts on meat production [4]. Additionally, mycotoxins can contaminate animal products such as milk and meat (especially pork), leading to human exposure through consumption of these foods [3–5].

There is an approach in which the effects of mycotoxins in feed are reduced by the addition of probiotics; however, most of the work in this area is associated with lactic acid-producing group of probiotics, while the possibility of using probiotic strains of *Bacillus* for these purposes has been insufficiently studied in our opinion.

There is reason to believe that this problem can be solved by using probiotic and synbiotic preparations based on *Bacillus* bacteria. Mold toxins, which have a lactone ring in their composition, can be destroyed by certain enzymes with lactonase activity. These include laccase and AHL lactonase [6,7]. These enzymes are commonly used by bacteria not to fight mycotoxins, but to destroy the signaling molecules of competing species, which they use for intercellular signaling and the development of quorum sensing [8]. Thus, N-acyl homoserine lactone (AHL) is the best known autoinducer of quorum-sensing systems in gram-negative bacteria, and it is known that probiotic Bacilli have a wide range of lactone-degrading enzymes that are potentially useful [9–11].

The most promising approach in terms of modulating the health of animals and humans, as well as their intestinal microbiota, seems to be the combined use of probiotics and prebiotics, both as separate preparations and as synbiotics [12]. Synbiotics are not just a mixture of probiotics and prebiotics; components in their composition are synergistic [13].

The aim of this work is to study the effects of *Bacillus* probiotics on the condition of heifers receiving food contaminated with mycotoxins, and to create a synbiotic preparation based on probiotic *Bacillus* strains and plant substrates that can reduce the harmful effects of mycotoxins in postharvest products used for cattle feed. The main objectives were genetic characterization of selected strains with lactonase activity and study of their metabolites capable of providing an additional probiotic effect, selection of the optimal substrate for obtaining a synbiotic preparation and studying the influence of the synbiotic preparation on the physiological parameters of cattle.

To achieve the above-described goal, a methodology was developed for obtaining synbiotic preparations associated with the use of various plant substrates (peas, chickpeas, food industry waste). A synbiotic preparation based on two strains of *Bacillus licheniformis* was tested on cattle. The results showed that the preparation inhibited the negative effects of mycotoxins and led to an increase in weight gain in heifer calves.

The use of such preparations can have a global positive effect on the health of agricultural consumers, reducing the risks of developing socially significant diseases, such as mycotoxicoses, cancer and others. Reducing the burden associated with the presence of mycotoxins in feed can not only have a direct economic effect by increasing animal productivity, but can also facilitate the processing of postharvest products.

2. Materials and Methods

2.1. Probiotic Strains

The strains of microorganisms *Bacillus licheniformis* CL 33 and CL 56 belonging to Agrokholod LLC were used. The strains from the collection were previously isolated from the feces of healthy cattle and selected by screening for the presence of the *aiiA* gene, as well as for the presence of quorum quenching (QQ) activity. The technique for analyzing QQ activity is protected by a patent, which is currently in the process of registration.

For identification and genetic analysis of the strains, their whole genome sequencing was carried out.

Genomic DNA of strains CL 33 and CL 56 was isolated from fresh culture biomass grown on solid LB medium with 1.5%, using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Sequencing was performed on Genolab-M equipment. Read quality control was performed using HTQC [14]. Low-quality ($Q < 15$), short (<100 bp) reads, and adapter sequences were removed using Trimmomatic version 0.40 [15]. Raw filtered reads were assembled into contigs using SPAdes version 3.15.4 software (Center for Algorithmic Biotechnology, Saint-Petersburg, Russia) [16] with a k-mer size of 77. Contigs shorter than 500 bp were removed. The annotation was performed using the Prokka v. 14.5 (Carlton, Australia) [17].

The search for specific genes was performed using Unipro UGENE. Analysis of the presence of genes for the synthesis of bioactive secondary metabolites was performed using Antismash v 6.0.0 (Leiden, The Netherlands) [18].

2.2. Selection of the Optimal Substrate for Cultivation

MPA medium (agarized meat-peptone broth) was used to cultivate primary Bacillus inoculums.

Liquid sterile media based on soybeans, chickpeas, bell-peppers, zucchini and green peas were used as nutrient substrate options. All media were subjected to centrifugation and filtering through filter paper 2–4 times (followed by autoclaving) to achieve the highest possible optical transparency of the medium.

Suspensions of strains of bacilli with a density of 0.5 according to McFarland were obtained in sterile saline. Next, 10 µL of the suspension were taken and added to each nutrient medium in a ratio of 1 to 100.

Then, the resulting preparations were added to the FLUOstar Omega (BMG Labtech) luminometer plate in a volume of 200 µL per well in accordance with the protocol and incubated for 24 h at a temperature of 37 °C, with optical density measured every 30 min. Additionally, as a negative control, pure nutrient media without the addition of bacilli were introduced.

The optical density was measured at a wavelength of 600 nm (OD 600). All experiments were carried out in 3 independent repetitions. The number of CFU/mL was determined based on the optical density of the culture according to the calibration curve.

2.3. Production of Preparation

The experiment used a preparation containing biochar 700 as a prebiotic (provided by the Southern Federal University), which is often used to reduce the load of toxic substances, including mycotoxins. The preparation contained *B. licheniformis* CL 33 in the amount of 7.4×10^8 CFU/g and *B. licheniformis* CL 56 in the amount of 8.7×10^8 CFU/g.

The preparation of each strain was obtained by solid-phase cultivation on the surface of soybeans according to the method described by the authors earlier [19]. This method allows obtaining biologically active biofilms and a high titer of the drug.

The finished probiotic preparation was obtained by mixing equal weight amounts of preparations of both selected strains and stored in a refrigerator at a temperature of ($4 \pm 2^{\circ}$) and a humidity of not more than 60%.

2.4. Animal Experiments

"Cellobacterin" bioadditive (Biotrof, Saint-Petersburg, Russia) was used as a positive control.

The substrate for experiments was prepared using crushed grain of barley, wheat, corn and wheat bran. The sterile substrate was inoculated with 10% of the volume of the bags with fungal mycelium (micromycetes *Aspergillus parasiticus*, *Aspergillus ochraceus*, Aspergillus flavus, *Fusarium* spp.). Then, they were cultivated at 25 °C for 6–8 days and until the substrate was completely overgrown.

The number of mycotoxins in the feed was determined by competitive enzyme immunoassay according to state standard GOST 31653-2012 [20]. Feed samples in the laboratory after grinding were extracted with an aqueous solution of acetonitrile (1:6) for 24 h. In each sample, the amount and presence of 8 mycotoxins were determined: fusariotoxins (T-2 toxin, fumonisin B1, zearalenone, DON), aspergilotoxins (AB1 aflatoxin, sterigmatocystin, ochratoxin A1) and penicillotoxin (citrinin). Further, their concentration was calculated in µg per 1 kg of feed.

For the study, young heifers aged 1–1.5 years were selected. The choice of animal model was based on the fact that heifers are commonly raised for food production, and therefore the safety and quality of their meat and dairy products are of great concern to consumers. Furthermore, heifers are often used in toxicological research as a model

organism for other livestock species, making the results of this study potentially relevant to a wide spectrum of animal agriculture.

The total number of animals was 70 heads. All animals used were healthy according to standards for cattle breed (state standard GOST R 50848-96) and free of parasites. Since we needed clinically healthy individuals, all animals were evaluated for a number of indicators. Animals were weighed, and the temperature, heart rate and number of respiratory movements were measured.

Blood samples were taken from each animal from the saphenous vein (blood sampling and blood serum were obtained according to generally accepted methods). The blood formula and a number of the most important biochemical blood parameters were monitored: COE (an indicator of inflammation), bilirubin, ALT, AST, α-amylase, alkaline phosphatase (liver condition), urea (excretory system condition), glucose, hemoglobin and total protein. Animals were used only if the values of all the parameters studied were normal.

The young animals were divided into seven groups of ten heads each and placed in separate pens:

1. Control.
2. Control with mycotoxins, 4500 g of bran.
3. Positive control. "Cellobacterin" 600 g, 3900 g bran.
4. Synbiotic, lower dose. 100 g bacteria preparation, 350 g biochar, 4050 g bran.
5. Synbiotic + mycotoxins, lower dose. 100 g bacteria preparation, 350 g biochar, 4050 g bran.
6. Synbiotic, high dose. 450 g of the preparation, 350 g of biochar, 3700 g of bran.
7. Synbiotic, high dose + mycotoxins 450 g of the preparation, 350 g of biochar, 3700 g of bran.

Each animal in the non-mycotoxin groups received: 4 kg of corn silage per day, 1 kg of concentrate (groats, compound feed), hay from herbs and 2 kg straw. Carbamide was introduced into the diet as a mineral supplement according to standard protocol.

In the groups with the introduction of mycotoxins, the same feeding scheme was used, while the food was affected by toxin-forming micromycetes and contaminated with the mycotoxins T-2-toxin and fumonisin B1.

Then, blood was taken from each animal from the saphenous vein (blood sampling and blood serum were obtained according to generally accepted methods).

Clinical examination of animals, as well as control of physiological parameters in each individual (weight, temperature, pulse, number of respiratory movements) was carried out before the start of the experiment and 4 weeks after the start of the experiment.

Body weight was measured using a digital scale to the nearest 0.1 kg. The temperature was measured using a digital thermometer inserted approximately 10 cm into the rectum, and the values were recorded to the nearest 0.1 °C. Pulse rate was measured by palpation of the femoral artery, and the number of beats per minute was recorded. Respiratory rate was measured by observation of the flank movements, and the number of breaths per minute was recorded.

Several of the most important biochemical blood parameters were also monitored: the number of erythrocytes and platelets (as part of the blood formula), bilirubin, ALT, AST, α-amylase, alkaline phosphatase (liver condition), urea (excretory system condition), and glucose, hemoglobin, total protein. Additionally, the immunological status of animals was assessed by two indicators: leukocyte count and ESR.

Morphological and biochemical blood tests were carried out in the laboratory of the North Caucasian Zonal Research Veterinary Institute according to standard methods.

2.5. Statistical Analysis

Statistical analysis of the data was carried out using R version 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria). Statistically significant differences from control groups were assessed using the two-tailed Student's *t*-test, assuming unequal variances. The data were first tested for normal distribution using the Shapiro–Wilk test. For normally distributed data, the *t*-test was applied. To determine whether there was a significant

difference in the means of three or more groups, we used one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. All tests were performed at a significance level of 0.05, and results are reported as means ± standard deviation.

3. Results

3.1. Genetic Characteristics of Strains

In both strains, the absence of genetic determinants of resistance to currently used antibiotics was confirmed.

Based on the results of a search using the Antismash v 6.0.0 algorithm, clusters of genes for the synthesis of secondary (non-ribosomally synthesized) metabolites, as well as a number of ribosomally synthesized metabolites (RSPM, ribosomally synthesized and post-translationally modified peptides) were identified, which can mediate probiotics' activity through modulation of the microbiota composition.

Summarized results for the detected gene clusters of secondary metabolites are presented in Table 1.

Table 1. Genes for the synthesis of secondary metabolites in the genomes of the studied strains.

Strain	Metabolite	Percentage of Similarity with a Known Analogue, %
B. licheniformis CL 33	Lichenicidin	100%
	Lichenisin	100%
	Fengycin-like peptide	53%
	Bacillibactin-like peptide	53%
	Butyrosine-like metabolite	7%
B. licheniformis CL 56	Lichenicidin	100%
	Lichenisin	100%
	Bacillibactin-like peptide	52%
	Fengycin-like peptide	53%

3.2. Selection of the Optimal Substrate

The results of selecting the optimal medium for the *B. licheniformis* CL 33 strain are shown in Figures 1–4 and in Tables 2 and 3.

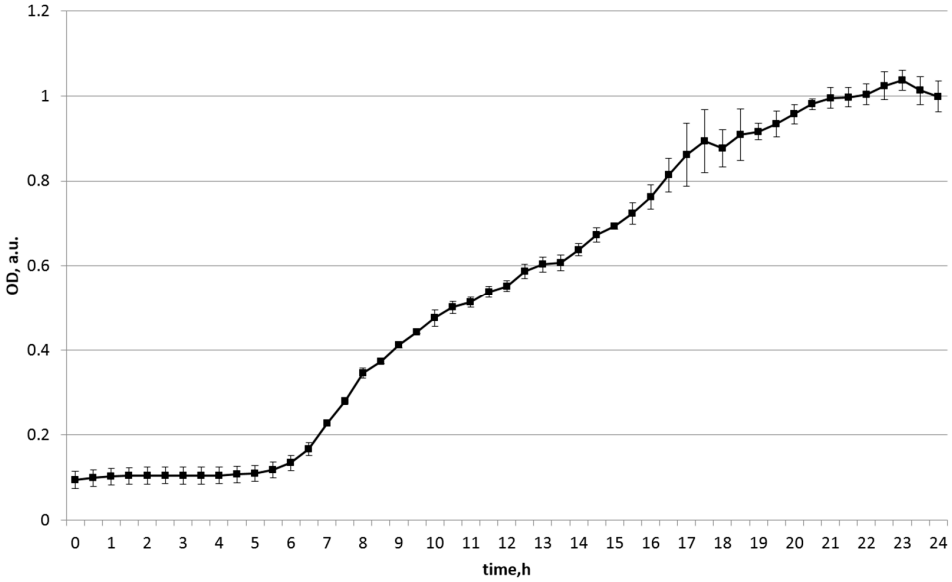

Figure 1. Growth curve of *B. licheniformis* CL 33 on soybean medium.

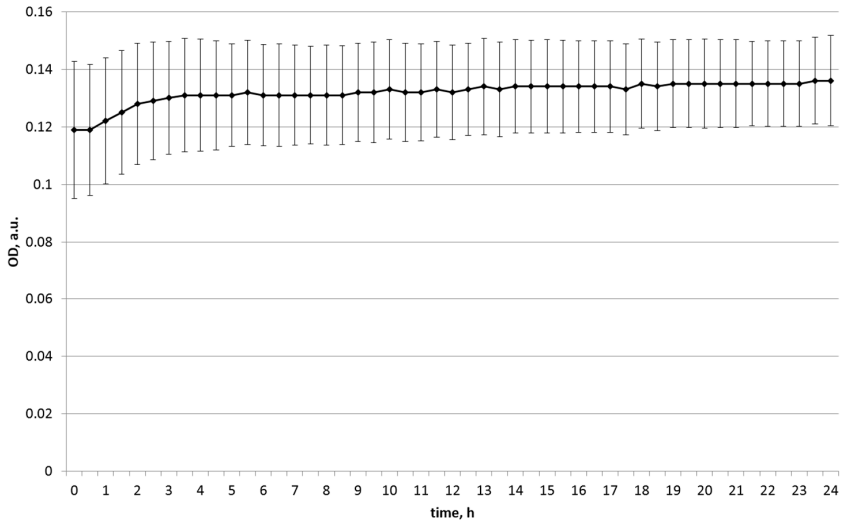

Figure 2. Growth curve of *B. licheniformis* CL 33 on green pea medium.

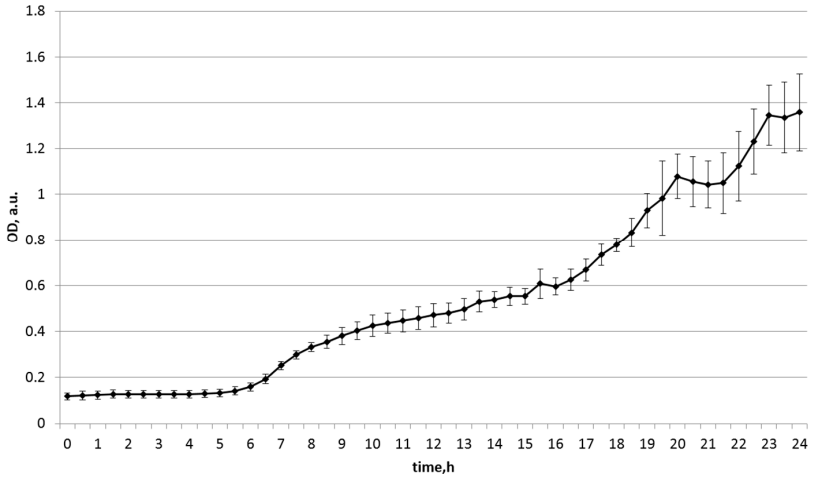

Figure 3. Growth curve of *B. licheniformis* CL 56 on soybean medium.

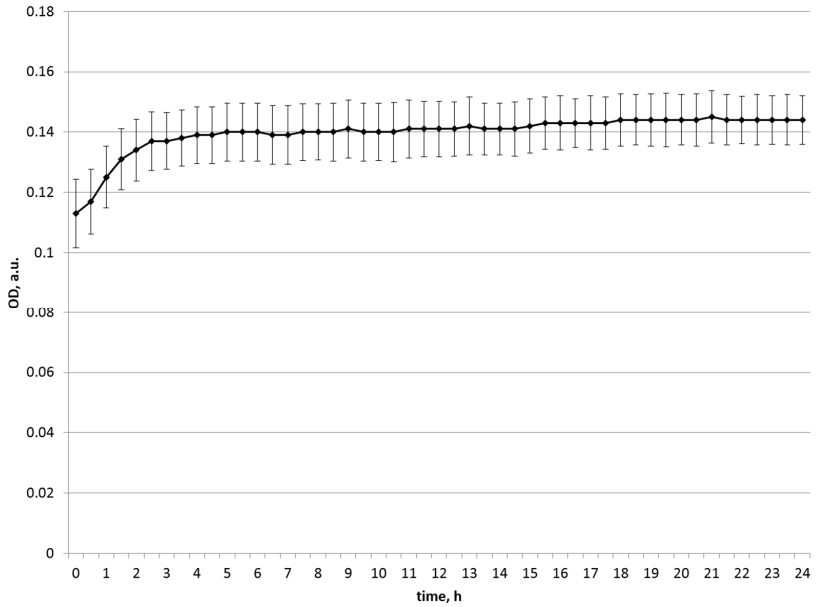

Figure 4. Growth curve of *B. licheniformis* CL 56 on green pea medium.

Table 2. Optical density (OD) of *B. licheniformis* CL 33 cultures on different media.

Substrate Source	Start OD	Maximum OD	Final OD (24 h)
Soy	0.099	1.037	0.999
Chickpea	0.145	0.902	0.531
Bell pepper	0.738	0.856	0.856
Zucchini	0.158	0.254	0.254
Green pea	0.119	0.136	0.136

Table 3. Optical density (OD) of *B. licheniformis* CL 56 cultures on different media.

Substrate Source	Start OD	Maximum OD	Final OD (24 h)
soy	0.118	1.358	1.358
chickpea	0.116	0.733	0.567
bell pepper	0.732	0.846	0.846
zucchini	0.125	0.386	0.386
green pea	0.113	0.144	0.144

As can be seen from the presented data, the most intensive growth of strain CL 33 cells was observed on soy medium. Different initial density was associated with the difference in the initial turbidity of the media due to the substrate; thus, the final density relative to the initial density was estimated, rather than the absolute value of the density. Pea medium was the second-most effective after soy medium, while no significant growth was observed on the other media.

The growth dynamic of *B. licheniformis* 56 was quite similar.

The most intensive growth of strain CL 56 cells was also observed on soy medium. The chickpea medium appeared to be the second-most effective, while no significant increase was observed on the other media. Thus, we can recommend the soy medium as optimal for growing these strains. On the basis of these results, heat-treated hydrated soybeans were used as a substrate for the production of the preparation.

3.3. Influence of the Synbiotic Preparation on the Physiological Parameters of Cattle

The initial morphological and biochemical parameters of the blood of animals in all seven groups were within the reference values. The values of clinical indicators (temperature, pulse, respiration) during the experiment were within the reference values and did not differ statistically when compared between groups. A statistically significant reduction in weight gain was noted in the mycotoxin treated group. The introduction of a synbiotic preparation into the feed leveled this effect (Table 4). At the same time, the average body weight of animals of all experimental and control groups was within the reference values and did not have statistically significant differences with the control group.

Table 4. Body weight and indicators of the clinical status of experimental animals after the end of the experiment.

Indicators, Units	Reference Values	Control	Control + Myco-Toxins	Cello-Bacterin	Synbiotic Low Dose	Synbiotic Low Dose + Mycotoxins	Synbiotic High Dose	Synbiotic High Dose + Mycotoxins
Weight gain, kg	-	12.5 ± 1.2	9.6 ± 2.2	12.5 ± 1.4	13.2 ± 1.3 **	11.9 ± 1.0	13.3 ± 1.3 *	12.4 ± 1.2 *
Weight, kg	280–330	316.7 ± 2.5	317.6 ± 3.8	316.1 ± 2.0	306.4 ± 26.4	309.7 ± 3.6	312.9 ± 3.2	319.6 ± 2.8
Temperature, °C	37.5–39.0	38.3 ± 0.08	38.14 ± 0.09	38.2 ± 0.06	38.1 ± 0.12	38.0 ± 0.09	38.2 ± 0.07	38.1 ± 0.04
Pulse beats/min.	50–80	78.8 ± 2.6	63.8 ± 2.1	75.7 ± 3.66	70.1 ± 2.1	66 ± 1.6	73.7 ± 2.9	76.6 ± 2.8
Breathing in/min	15–30	21.5 ± 0.6	21.9 ± 1.2	20.6 ± 0.5	21.9 ± 1.1	22 ± 0.8	21.1 ± 1.3	23 ± 1.07

Differences from the option with mycotoxins: * $p < 0.05$ ** $p < 0.01$.

At the end of the experiment in all experimental groups, the morphological and biochemical parameters of blood were within acceptable normative values. Blood parameters

that did not have significant differences and deviations from the norm are given in Supplementary Tables. The only significant difference was observed in the level of alkaline phosphatase (Figure 5).

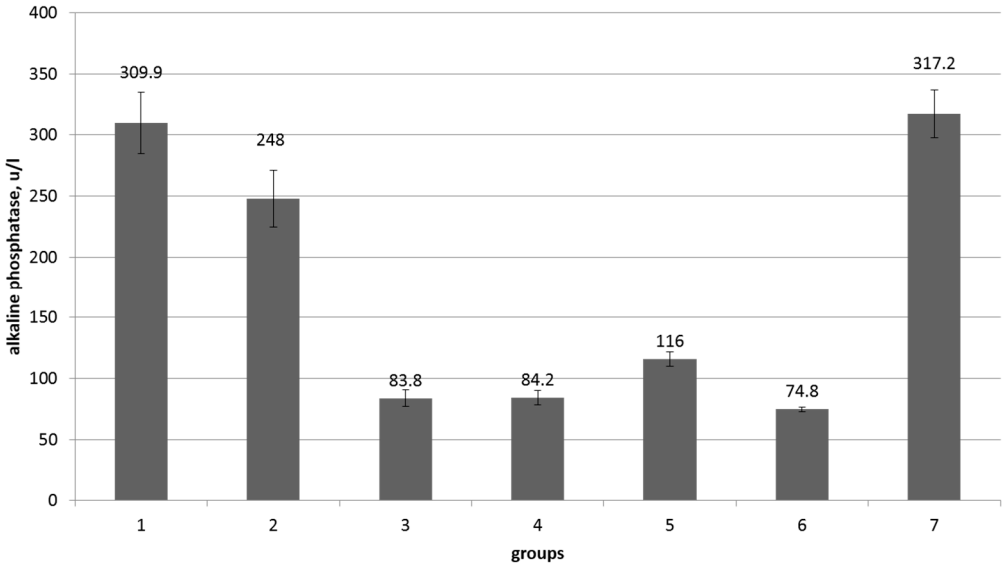

Figure 5. Alkaline phosphatase levels in serum. Groups: 1—control, 2—control with mycotoxins, 3—positive control, 4—synbiotic, lower dose, 5—synbiotic, lower dose + mycotoxins, 6—synbiotic, high dose, 7—synbiotic, high dose + mycotoxins.

4. Discussion

Probiotics are used in cattle breeding to compensate for adverse external influences due to their ability to modulate the immune system and the functioning of the nervous system, reduce the quantities of toxic substances in the blood, participate in the digestion of food at different stages of the digestion process in cows, and also contribute to the normalization of the microbiota [21]. A promising direction is the use of feed with the inclusion of spore-forming probiotic cultures, as well as probiotics based on spore-forming bacteria. In particular, *Bacillus* strains in the spore stage are resistant to high temperatures and survive the processes of extrusion, granulation and expansion. The dormant spore stage allows these probiotics to have longer shelf life without the danger of losing properties. In commercial preparations, two types of strains are mainly used: *Bacillus subtilis* and *Bacillus licheniformis*. They belong to the transient microbiota, which means they should not populate the digestive tract [22].

It appears that the optimal medium for the cultivation of probiotic strains can be prepared based on soybeans. This may be due to the fact that the soy substrate promotes increased production of bioactive lipopeptides in bacilli [23], and thus is a suitable component/substrate for a synbiotic preparation. Soybean meal, soy hydrolysate [24] and soybean flour [25] have also been used as substrates to increase surfactin and other lipopeptides production by *Bacillus*. In general, oily substrates can be applied in combination with hydrophilic substrates to enhance biosurfactant production [26]. Furthermore, the quantitative and qualitative effect of water immiscible and miscible carbon-rich substrates on the production of biosurfactants has been studied [27], showing that these types of substrates can increase lipopeptide production.

A decrease in weight gain was noted in the group treated with mycotoxins, which is natural, since the action of toxins disrupts the physiological processes of animals. It was shown that the introduction of a synbiotic preparation into the feed reduced this effect. At the same time, the average body weight of animals of all experimental and control groups was within the reference values and did not have statistically significant differences with the control group. Thus, it was shown that the introduction of the synbiotic preparation reduces the harm from the presence of mycotoxins in animal feed.

It is known that in the rumen of cattle, food undergoes microbial fermentation, and only after that does it enter the rest of the stomach and intestines. The rumen is inhabited by a diverse microbiota, which not only digests cellulose and forms the necessary protein, but can also subject the substances contained in the feed to various modifications. Rumen microbial composition influences feed efficiency [28], CH_4 emission rate [29], animal health [30] and milk composition [31]. The term "feed efficiency" describes the efficiency with which feed is converted into usable product, and the ratio of feed efficiency is an inherited trait in cattle [32]. Due to the reduced pH, high humidity and temperature, conditions are created in the rumen that are suitable for the development of microorganisms, including those belonging to the genus *Bacillus*. By adding probiotic bacteria to the feed, we contribute to the enrichment of the rumen microbiota with these strains. Mycotoxins that enter the rumen along with the food, in fact, due to the processes taking place in the rumen, are incubated for quite a long time together with mycotoxin-degrading bacilli, which can significantly reduce the toxicity of the feed.

Bacilli can fight mycotoxins in several ways. It is reported that *Bacillus* strains isolated from the aquatic environment of the Brazilian Amazon demonstrated activity against 7 mycotoxigenic fungi [33]. Product of BsDyP gene from *Bacillus subtilis* SCK6 has been shown to enzymatically degrade multiple major mycotoxins [34]. Fengycin produced by *Bacillus amyloliquefaciens* FZB42 has been shown to inhibit *Fusarium graminearum* growth and biosynthesis of its associated mycotoxins [35]. Certain *Bacillus* spp. have also demonstrated decontamination potential on multiple *Fusarium* mycotoxins [36], while other species, such as *B. cereus* 342-2, have been investigated for their biocontrol properties against toxigenic mycobiota and associated toxins found in date palm fruits [37]. Additionally, *B. subtilis* BIOUFLA2 has been evaluated for its ability to reduce fungal colonization and subsequent contamination with rot and/or mycotoxin levels in kernels used for animal feed production [38].

The strains used in the preparation were characterized as having quorum-quenching activity. It is known that some microorganisms have a system of protection against the negative effects of pathogens, based on the destruction of signals from the quorum-sensing system; in particular, homoserine lactones. This enzyme can hydrolyze the lactone ring and the amide bond of AHL into inactive molecules and thereby block quorum-sensing systems and reduce symptoms caused by pathogens [8].

Enzymes capable of inactivating lactones are also able to destroy mycotoxins that include the lactone ring, therefore, selection for this trait makes it possible to find strains that can not only disrupt the sense of quorum in potential pathogens, but also protect the host from mycotoxins from food.

Among bacteria of the genus *Bacillus*, genes encoding AHL lactonase (for example, the *aiiA* gene) are widespread, which makes these microorganisms a promising object for study. Microorganisms belonging to the genus *Bacillus* also contain the laccase gene, which has quorum-inhibiting activity, *cotA* [39].

It can be assumed that the inactivation of mycotoxins was not the only effect of these metabolites, but was also accompanied by a violation of intercellular signaling, which reduces the activity of potential pathogens.

The effect of reducing the content of alkaline phosphatase in the serum of animals deserves a separate discussion. Although when mycotoxins were added to the feed, it decreased slightly compared to the control, it should be noted that its level in the control group was initially high (309.9 units/L), while the norm for cattle is 55–80 units/L [40], and the use of probiotic and synbiotic preparations led to a decrease to exactly this value (except for the group that received a large dose of synbiotic and mycotoxins). This phenomenon was likely due to the systemic effects of probiotics. The increase in control enzyme levels was likely due to sub-optimal diet and should be addressed in further studies. Interestingly, the alkaline phosphatase-lowering effect of probiotics has been observed in human studies [41] and is indicative of improved liver health.

Comparing the effects obtained by us with similar studies, it can be seen that the action of our drug fits into the well-known paradigm of the action of probiotics. It was shown that probiotic bacteria are able to inactivate, adsorb or degrade such substances as AFB1—aflatoxin B1, AFs—aflatoxins, AFB2—aflatoxin B2, AFG1—aflatoxin G1, AFG2—aflatoxin G2 and AFM1—aflatoxin M1 [42]. However, according to this review, most studies in this area have been conducted on strains of lactobacilli, while other bacteria and yeasts are found in a few isolated studies, including as part of complex preparations. Much of this research has been carried out in rat models and not in livestock.

There are in vitro studies on *Bacillus* strains, including a study on *B. licheniformis* showing activity against AFB1 [43], a study characterizing a *B. licheniformis* strain with ZEA-removal ability and a *B. amyloliquefaciens* strain with the same activity [44] and a study on *Bacillus subtilis* and *Bacillus natto* [45]. Authors [46] studied the combination of *B. subtilis*, *Lactobacillus casei* and *Candida utilis* to eliminate AFB1 and ZEA from animal feeding.

As for in vivo studies, there are data on ducks [47] and chickens [48]. No in vivo studies have been performed on heifers in the manner of this research. It is interesting to note, however, a parallel with [49], where the authors noted that bull calves prefer food enriched with the probiotic *B. amyloliquefaciens* H57 over conventional food. The authors attribute this to the ability of the probiotic to prevent the formation of harmful substances including the toxic metabolites of fungi, in the feed that change its organoleptic qualities.

It is worth noting that CL 33 and CL 56 strains contain metabolites that can provide other probiotic properties of *Bacillus* strains. Thus, lichenicidin is a two-peptide lantibiotic produced by *Bacillus licheniformis* [50]. It is composed of the two peptides Blia and Blib, which are linked together by lanthionine bridges [51]. It has antimicrobial activity against Gram-positive bacteria and can inhibit the growth of clinically relevant strains, such as methicillin-resistant *Staphylococcus aureus* (MRSA) [52]. The bactericidal activity of lantibiotics is based on specific binding to the peptidoglycan precursor lipid II, which predetermines its antimicrobial activity [53]. Lichenicidin has been shown to increase the net surface charge of *S. aureus* cells as measured by zeta potential without achieving electrical neutralization. In addition, lichenicidin causes disturbances in the cell surface of pathogen cells, which leads to leakage of internal contents, as observed using atomic force microscopy [54].

Lichenisin is known as a surface active biosurfactant [55]. Lichenisins are anionic cyclic lipoheptapeptide biosurfactants produced by *Bacillus licheniformis* on a carbohydrate-free medium with extreme glucose. They have the ability to reduce the surface tension of water from 72 to 27 mN/m [56].

Fengycin(s) is a type of lipopeptide produced by *Bacillus* species, which has been extensively explored for its biological control properties. It was shown to be able to cause alterations in the bacterial surface topography [57], promote the formation of pores and cause leakage from pathogen cells [58]. Among other properties, fengycins can reduce the mitochondrial membrane potential, induce production of reactive oxygen species (ROS) and downregulate antioxidant enzyme production in fungi cells. Fengycin BS155 can cause chromatin condensation in fungal hyphal cells, which leads to significant changes in protein expression [59]. Fengycins have antimicrobial activity against a wide range of fungi and bacteria [59–61].

Bacillibactin is a catechol-type siderophore. It has been reported to have antibacterial, antifungal and cytotoxic activity [62], as well as broad-spectrum biocontrol potential [63]. Some forms show cytotoxicities against human cancer cell lines HepG2 and MCF7 [64]. Additionally, it may be involved in the production of other antimicrobial compounds, such as fengycin, surfactin, bacillaene and bacillomycin [61].

These compounds can modulate the composition of the microbiota, leading to systemic beneficial effects in animals. There are also suggestions that some of the antimicrobial peptides have a dual effect and can also be used by microorganisms as signaling molecules at naturally achievable subinhibitory concentrations [65].

5. Conclusions

The optimal substrate was selected for obtaining a synbiotic preparation based on two strains of *B. licheniformis*. According to the results of experiments carried out on heifers, the introduction of substrates affected by toxin-forming micromycetes into feed led to a decrease in weight gain, but the introduction of a synbiotic drug, especially at a high dose, leveled this negative effect, bringing it to the level of the control groups. There was also a decrease in the level of alkaline phosphatase in serum to normal values, which may characterize probiotic effects of the drug not associated with the elimination of the effect of mycotoxins.

In the genomes of the studied bacteria, genes for the synthesis of compounds with antimicrobial and other biologically significant activity were found. Their presence may provide the effects of the drug through an indirect effect, that is, through the modulation of the composition of the rumen microbiota.

The results of the study open up prospects for the creation of a new generation of synbiotic preparations for veterinary medicine, which will not only have a general systemic positive effect on the animal organism, but also reduce the negative effects of feed contamination with mycotoxins.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13030523/s1. Table S1: Initial morphological and biochemical blood parameters of experimental animals; Table S2: Morphological and biochemical parameters of the blood of experimental animals at the end of the experiment.

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