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GROWTH DYNAMICS, ANTAGONISTIC ACTIVITY AND ACID PRODUCTION OF *LACTIPLANTIBACILLUS* (=*LACTOBACILLUS*) *PLANTARUM* KT-L18/1 AND *BACILLUS SUBTILIS BPT-B1* INOCULANTS IN ALFALFA SILAGE

N. O. Kravchenko

Institute of Agricultural Microbiology and Agroindustrial Manufacture,
the National Academy of Agrarian Sciences of Ukraine
97, Shevchenka Str., Chernihiv, Ukraine, 14027

E-mail: nat.probiotik@gmail.com, ismavnaas@ gmail.com

ORCID: https://orcid.org/0000-0001-5090-4276

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Aim. To study growth dynamics, antagonistic activity and acid formation of two inoculant strains, *Lactiplantibacillus* (=*Lactobacillus*) *plantarum* KT-L18/1 and *Bacillus subtilis* BPT-B1, that were introduced into alfalfa silage, up to 30 days of fermentation. **Methods.** A streptomycin-resistant strain of both above-mentioned organisms, obtained in earlier research, was used to achieve the research aims. Alfalfa was artificially wilted and dried to a dry matter content of 39–40 %. Fermentation was conducted under anaerobic conditions in polyethylene bags. Inoculant was added at a concentration of 10^8 CFU/kg alfalfa. Ensiling was achieved in three treatments: 1) control without inoculants, 2) application of *L. plantarum* KT-L18/1^{str} as inoculant, and 3) *B. subtilis* BPT-$B1^{str}$ as inoculant. There were four bags of 300 g alfalfa for each treatment and each treatment was performed in triplicate. The resulting 12 × 3 = 36 bags were kept at room temperature in a dark place for 30 days. At each examination date one sample per treatment was used and examined after cultivation of the two inoculants on elective De Man-Rogosa-Sharpe (MRS) agar and meat infusion agar (MIA), containing streptomycin. The (lg) CFU/g of inoculants and natural background microbiota were determined by dilution plating on these agars at the start of fermentation (day 0) and on days 3, 15 and 30. The antibacterial (ABA) and antifungal activities (AFA) were studied by the agar diffusion method for potentially pathogenic bacterial strains (PPB), namely *Staphylococcus aureus* strain No. 906, *Pseudomonas aeruginosa* ATCC-27853, *Salmonella typhimurium* No. 89 and fungi, a strain each, belonging to the *Penicillium chrysogenum* (strain ch37), *Fusarium oxysporum* (strain ch07), *Rhizopus arrhizus var. arrhizus* (syn. *R. megaspores*) (strain ch37). The pH of the silage was routinely determined in aqueous extract over 30 days. For analysis of background microbiota, 50 g of mixed silage was randomly taken, and 450 ml of sterile water added to prepare suspensions. The (lg) CFU of fungi were determined on Saboraud glucose agar after 3–4 d cultivation at 28 °C. The (lg) CFU/g of clostridia bacteria were determined after 24 h cultivation at 37 °C on Reinforced Clostridial Broth (Himedia, India). The (lg)CFU/g of lactic acid bacteria (LAB) and aerobic spore-forming bacilli (ASB) were determined as described above for *L. plantarum* KT-L18/1^{str} and *B. subtilis* BPT-$B1^{str}$, but without addition of streptomycin to the medium. **Results.** After 30 days of ensiling the alfalfa samples, *L. plantarum* KT-L18/1^{str} and *B. subtilis* BPT-$B1^{str}$ were still present at levels of 9.2 lg CFU/g and 6.9 lg CFU/g respectively. In the natural population of LAB, *L. plantarum* KT-L18/1^{str} had a dominant position. In the variant treated with *B. subtilis* BPT-$B1^{str}$, the number of LAB was 9.2 lg CFU/g, practically equal to the number of LAB in the variant with *L. plantarum* KT-L18/1^{str}. After 30 days of the alfalfa fermentation *L. plantarum* KT-L18/1^{str} and *B. subtilis* BPT-$B1^{str}$ maintained ABA and AFA to the PPB strains (*S. aureus*, *P. aeruginosa*, *S. typhimurium*) and the fungal strains used, one strain each of *Penicillium chrysogenum*, *Fusarium oxysporum* and *Rhizopus arrhizus var. arrhizus.* However, the antagonistic activities of the two inoculant strains decreased during the fermentation, for *L. plantarum* KT-L18/1^{str} from 7–21 %, and for *B. subtilis* BPT-$B1^{str}$ from 16–21 %. Compared to the uninoculated control treatment, the inoculated silage treatments had a lower pH level (5.2–5.3), an increased number of lactic acid bacteria (LAB) (by 32 %), and clostridia were no longer detected. The inoculation impacted fungi down to a level of 10^2–10^3 CFU/g that can ensure the aerobic stability of the feed. **Conclusions.** The inoculant strains and *L. plantarum* KT-L 18/1^{str} and *Bacillus subtilis* BPT-$B1^{str}$ showed a high competitiveness with background microbiota in an ensiling

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experiment with alfalfa. The antagonistic activity of the two strains to PPB after fermentation was preserved at a high level, albeit with a decrease of 7–21 %. *Bacillus subtilis* BPT-B1str kept its AFA against all three fungal strains tested during the 30 days of fermentation. For *L. plantarum* KT-L18/1str this was true for a strain of the *Penicillium chrysogenum* only. The inoculation of and *L. plantarum* KT – L18/1str and *Bacillus subtilis* BPT-B1str caused a decrease in pH of 8–9 % at the end of the fermentation period, an accumulation of LAB with 32 %, and the inhibition of the growth of clostridia up to undetectability in the fermented alfalfa. For both inoculants, compared to uninoculated control variant, fungi decreased down to 10^2–10^3 CFU/g of the feed, which can ensure the aerobic stability of the feed.

Key words: lactic acid bacteria, aerobic spore-forming bacteria, number of microorganisms, antibacterial activity, antifungal activity.

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INTRODUCTION

Alfalfa is an economically valuable forage crop for dairy cattle that can increase the protein content of milk and the general dairy performance (Kent et al, 1988; Zielińska et al, 2015; Ferreira and Teets, 2020). It is highly productive and the least energy-consuming forage crop; one feed unit of the leaf and stem mass of alfalfa contains over 200 g of digestible protein. According to the State Statistics Service of Ukraine, the area under alfalfa is c. 1.8 million hectares, which is 48 % of area cultivated with perennial grasses. In terms of natural moisture supply, the best region for growing alfalfa is the Forest-Steppe region of Ukraine (Hetman et al, 2021). However, the preservation of freshly cut alfalfa for animal feeding by ensiling to produce silage can be complicated due to its high buffering capacity, low amount of water-soluble carbohydrates, high seeping losses, high protein concentration and proneness to secondary fermentation by clostridia bacteria. Therefore, it is generally advised to produce silage with a relatively high dry matter content (≤ 35 to 40 % dm), or sometimes of an even higher dry matter (~40–60 % dm). The high dry matter in the latter silage (also sometimes called haylage), comes with a price of easier heat damage with higher indigestibility of the protein and the need for high density packing of the product (Thomas et al, 1969; Coblentz et al, 2012; Santos and Kung, 2016). The microbial populations present in alfalfa silage are more diverse than those of other crops, such as cereals. Still, most epiphytic microbiota of alfalfa are undesirable for silage, and species of lactic acid bacteria important for fermentation make up only a small percentage (McAllister et al, 2018; Guo et al, 2018; Hu et al, 2020; Yang et al, 2019; Yang et al, 2020).With a sharp change in the redox potential, temperature, humidity, and pH, the taxonomic diversity of the microbiome can reach extreme values, which affects both uncontrolled multiplication and a decrease in the number of individual representatives of the populations (Guo et al, 2023). Therefore, strains of quality-improving microorganisms promising for inclusion in bio preservatives, such as alfalfa silage, should have a number of specific properties such as: dominant growth rate, synthesis of organic acids, homolactic fermentation, osmotolerance and antagonistic activity (Weinberg et al, 2010; Heinritz et al, 2012; Vlková et al, 2012; Muck et al, 2018). Lactic acid bacteria, in the artificially created and unpredictable microbiological ecosystem of silage or haylage, due to their ability to produce biologically active substances (organic acids, vitamins, enzymes, bacteriocins, etc.), enter multifaceted relationships with other microorganisms (Pang et al, 2011; Fabiszewska et al, 2019). Silage quality and the effectiveness of microbial preservatives depend on the competition between lactic acid bacteria (LAB) and bacteria that are undesirable for quality silage, as well as on the result of competition and cooperation between LAB (Ni et al, 2018; Wang et al, 2019; Xu et al, 2018; Fu et al, 2023). In the process of ensilaging forage, under certain conditions, an increased development of fungi (yeast and moulds) is often observed, which leads to a decrease in aerobic stability of the feed and necessitates the search for LAB strains with antifungal activity (O'Brien et al, 2007; Li et al, 2022; Guo et al, 2023). Fortunately bacterial strains of the genus *Bacillus* have been isolated that are capable of rapidly cleaving easily accessible carbohydrates, have a wide range of antagonistic and antifungal activities, and synthesize a number of useful enzymes, amino acids and vitamins (Kaynar et al, 2019; Sumi et al, 2015). These bacteria have long been used as probiotic supplements for animals, which can inhibit the growth of pathogenic bacteria and enhance the intestinal barrier function (Zhang et al, 2016). There are also examples of their successful use as part of feed biopreservatives which are also authorized as technological feed additive in the European Union (EFSA, 2023). It has re-

peatedly been shown that the use of selected strains of for example *Bacillus subtilis, Lentilactobacillus* spp. and *Lactiplantibacillus plantarum* for preservation helps establish the optimal level of feed acidity and the ratio of organic acids, and suppress the development of undesirable microorganisms that cause alfalfa and other feed spoilage (Lara et al, 2015; Perederii et al, 2019a; 2019b; Đorđević et al, 2019; Maksimova, 2019; Kravchenko et al, 2020; Bai et al, 2020; 2021; 2022; Drouin et al, 2022; Kravchenko and Dmytruk, 2022; 2023; Li et al, 2023; Yin et al, 2023). *Lactobacillus plantarum* (Orla-Jensen 1919) Bergey et al 1923 has recently been reclassified and renamed as *Lactiplantibacillus plantarum* (Orla-Jensen 1919) Zheng et al 2020 (Zheng et al, 2022), and will be used here. There are also numerous reports on the study of the impact of silage inoculants on the constellation of the microbiome (mainly bacteria and fungi) of preserved alfalfa, using molecular methods such as 16s rRNA sequence analysis, next generation sequencing (NGS) and single molecule, real-time sequencing (SMRT). (Eikmeyer et al, 2013; Bao et al, 2016; Ni et al, 2017; McAllister et al, 2018; Bai et al, 2020; You et al, 2022). It is known that bacterial metabolism is regulated and maintained physiologically, as it is genetically determined, and therefore, the biochemical activity of bacteria that make up preservatives may also change under fluctuating conditions during the ensilaging process (Bai et al, 2021).However, there are few reports on the growth dynamics and stability of the antagonistic activity of particular strains used as inoculants after introduction into alfalfa. Therefore, the aim of the study was to evaluate the growth characteristics, stability of the antagonistic activity and acid production of streptomycin-resistant mutants of two inoculant strains, viz. *Lactiplantibacillus plantarum* KT-L18/1^{str} and *Bacillus subtilis* BPT-B1^{str} and after the introduction into freshly harvested alfalfa mass and during a 30 day fermentation in a laboratory experiment.

MATERIALS AND METHODS

Bacterial inoculant strains. Lactiplantibacillus plantarum KT-L18/1 and Bacillus *subtilis* BPT- B1, from the Collection of microorganisms of the Laboratory of Probiotics at Chernihiv, isolated from the gastrointestinal tract of young cattle (Perederiy et al, 2019; Kravchenko et al, 2020). In our present study we used streptomycin-resistant mutants of these two strains, which were earlier obtained and described by us, indicated here as *B. subtilis* BPT-B1^{str} and *L. plantarum KT*-L18/1^{str} (Kravchenko et al, 2022).

Ensiling conditions and experimental (inoculation) treatments. Alfalfa (*Medicago sativa*) cv Banat, grown at–1.5 cm at the start of bud formation in August 2023 and left to dry in the sun. The green mass was than further wilted to a dry matter content of 39–40 % in a cabinet drier (2V-151, Ukraine) at 105 °C.

For inoculation *L. plantarum* KT-L18/1^{str} was grown for 7 days at 37 °C in liquid De Man-Rogosa-Sharpe (MRS) medium (De Man et al, 1960) and *B. subtilis* BPT-B1^{str}strain for 24 hours at 37 °C in meat-infusion broth (MIB) (Farmaktyv LLC, Ukraine). Both inoculants were added at 10^8 CFU/kg alfalfa. Ensiling was achieved in three treatments: 1) Control without inoculants, 2) application of *L. plantarum* KT-L18/1^{str} as inoculant, and 3) *B. subtilis* BPT-B1^{str} as inoculant. There were four plastic bags of 300.0 g for each treatment and each treatment was performed in triplicate. All $3 \times 12 = 36$ sample bags were vacuum sealed using a vacuum sealer (Freshpack Pro, China) to create anaerobic conditions and kept for fermentation at room temperature in a dark place for 30 days. At each examination date one sample per treatment was used and examined after cultivation of the two inoculants on elective meat infusion agar (MIA) or MRS agar containing streptomycin at 50 and 5 mg/ml, respectively.

Colony-forming units (CFU) per gram of silage were determined using the formula $A = B/0.1 \times 10^n$, where A is the number of CFU in 1 g of the feed, B is the number of colonies on solid culture medium, and n is the degree of suspension dilution.

Antagonistic bacterial activity (ABA) was studied by the agar diffusion method against potentially pathogenic bacteria (PPB) *Staphylococcus aureus* strain No. 906, *Pseudomonas aeruginosa* ATCC-27853 and *Salmonella typhimurium* No. 89. The test cultures were obtained from the collection of microorganisms of the Chernihiv Regional Sanitary Epidemiological Station, Ukraine and stored before use on nutrient agar under sterile mineral oil at +8 °C.

Handling of PPB was conform national biosafety rules DSP 9.9.5.-80-2002 (https://dnaop.com/html/3108/doc-%D0%94%D0%A1%D0%9F_9.9.5.-080-02). One ml of the potential antagonistic inoculant strain suspended in MIB or MRS was introduced and mixed with 15–20 ml of melted meat infusion agar (MIA) or MRS agar at 45 °C and incubated for 1 and 3 days respectively at 37 °C, respectively. After incubation a small agar slab was taken with a sterile cork borer (8 mm diam.) and placed onto a MIA plate to which a test strain culture of PPB just had been added, as was

done for the inoculant strains. The dishes were kept for 12 h at +8 ^{o}C in a fridge to let antagonistic compounds diffuse into the medium and then incubated at 37 ^{o}C for 24 h. Diameters of inhibition zones were measured. MIA plates with 100 U/ml streptomycin served as positive control. The following scale of inhibition zone/ activity was used: 0–5 mm negative, from 5 to 10 mm low activity, 11–20 mm – medium activity, and over 20 mm – high activity (Strus, 1998; Yrkytova et al, 2012). The experiment was performed in three repeats.

Anti-fungal activity (AFA) was studied using a strain each of the following fungi: *Penicillium chrysogenum* (strain ch37), *Fusarium oxysporum* (strain ch07) and *Rhizopus arrhizus var. arrhizus* (*syn. R. megaspores*) (strain ch37). The fungi were isolated from preserved feed in the Laboratory of Probiotics of the IAMAM of the NAAS, Chernihiv and identification was provided by our colleague Dr. Ye.P. Kopylov. The inoculant bacteria were prepared as described above for ABA. Just before placement of the agar slab a fresh MIA plate was pierced with a sterilized needle holding some mycelium of the fungal test strain grown for 5 days on MIA. Plates were kept, in a fridge at +8 ^{o}C for 12 h to let antagonistic compounds diffuse into the medium and then incubated at 28 ^{o}C for 10 days. Measuring and interpretation of the inhibition zone was as for ABA determination. MIA plates with 100 U/ml nystatin served as positive control. The experiment was performed in three repeats.

Determination of active acidity (pH). The silage pH value was determined from silage aqueous extract using a pH meter (pH-150 MI, Russia).

Effects of inoculants on microbiota of alfalfa silage. For microbiological analysis, bags with silage were opened, their content thoroughly mixed and 50.0 g of silage was randomly taken, and 450 ml of sterile tap water added. Fungi were grown for 3 up to 8 days at 28 ^{o}C on Saboraud glucose agar (Odds, 1991) after which colonies were counted. The numbers of clostridia were determined on Reinforced Clostridial Broth (Himedia, India) after incubation for 24h at 37 ^{o}C (Mossel et al, 1959). Colonies, also penetrating the medium, were counted. The number of LAB and ASB was determined by the method used for re-isolation of *L. plantarum* KT-L18/1^{str} and *B. subtilis* BPT-B1^{str} to determine their growth dynamics in alfalfa silage.

Statistics. Numbers of microbial strains or isolates added or isolated in this study were transferred into base 10 log (lg) per gram wet weight of alfalfa silage. For all repeats (n = 3) of treatments/platings the arithmetic mean and the standard deviation at $p < 0.05$ were calculated.

RESULTS

In our experiment we were able to evaluate growth dynamics and metabolic activity of the two inoculant strains of *L. plantarum* and *B. subtilis* bacteria in the process of alfalfa fermentation via streptomycin resistant mutants of these strains, which were obtained before (Kravchenko et al, 2022). *B. subtilis* BPT-B1^{str} resistant at 50 mg/ml, and *L. plantarum* KT-L18/1^{str} resistant to 5.0 mg/ml. On day 30 of alfalfa fermentation *L. plantarum* KT- L18/1^{str} dominated among the natural population of lactic acid bacteria (LAB), see **Table 1**. During the observation period, the LAB population in the unprocessed alfalfa silage increased from 5.2 lg CFU/g up to 8.2 lg CFU/g of day 15, thereafter decreased to 6.9 lg CFU/g on day 30. In case of inoculation of *L. plantarum* KT-L18/1^{str}, on day 3 the LAB population was already higher than the uninoculated control (6.8 lg CFU/g), and on day 15 of the fermentation, their level increased by two orders as compared to the control (10.6 lg CFU/g). At the same time, at day 3, *L. plantarum* KT-L18/1^{str} exceeded the initial inoculated number by one order and on day 15 it started dominating the natural population (10.3 lg CFU/g).

The number of aerobic spore-forming bacilli (ASB) in the uninoculated control increased a little on day 3: from 5.0 lg CFU/g to 5.8 lg CFU/g. On day 15, their number dropped, and on day 30 their number was only 1.7 lg CFU/g. ASB increase following inoculation was slow, from 5.8 lg CFU/g to 6.9 lg CFU/g after 30 days. However, as of the end of the observation period in the natural population of ASB, Bacillus *subtilis* BPT-B1^{str} had the dominating position (lg 9 out of lg 9.3) in the inoculant treatment.

Anti-bacterial activity (ABA) and anti-fungal activity (AFA) of *L. plantarum* KT-L18/1^{str} and Bacillus *subtilis* BPT-B1^{str} were determined prior to and after the introduction to the alfalfa silage. After the introduction into the green alfalfa mass, these strains preserved ABA and AFA to the strains of the PPB *S. aureus, P. aeruginosa* and *S. typhimurium* and the representative strains of fungi belonging to the genera *Penicillium, Fusarium, Rhizopus* after 30 days of fermentation (**Table 2**).

However, after 30 days of fermentation there was a decrease in ABA to PPB in *L. plantarum* KT-L18/1^{str} : for *S. aureus* 906 this was 21.1 %, for *P. aeruginosa* ATCC 27853 up to 7 % and for *S. typhimurium* 89 it was 8.3 %. The impact of the two inoculant strains

Table 1. The (lg) numbers of *Lactiplantibacillus plantarum* KT-L18/1^{str} and *Bacillus subtilis* BPT-B1^{str} after their introduction into the alfalfa silage and those of background lactic acid bacteria (LAB) and aerobic sporeforming bacilli (ASB) in (un)inoculated alfalfa silage over a period of 30 days

Treatment (n=3)	Number	Day of alfalfa fermentation							
		0		3		15		30	
		CFU/g	lg CFU/g	CFU/g	lg CFU/g	CFU/g	lg CFU/g	CFU/g	lg CFU/g
Treatment I – Control (no inoculation)	Natural population of LAB	(1.6 ± 0.1*) 10^3	3.2 ± 0.06	(8.2 ×105) 10^5	5.91 ± 0.03	(1.4 ± 0.03) 10^8	8.17 ± 0.01	(0.9 ± 0.03 10^7	6.96 ± 0.01
Treatment II *L. plantarum* KT-L 18/1	Natural population of LAB with *L. plantarum* KT-L 18/1^{str}	(2.8 ± 0.1) 10^5	5.5 ± 0.03	(6.3 ± 1.8) 10^6	6.8 ± 0.1	(4.7 ± 1.6) 10^{10}	10.6 ± 0.1	(2.1 ± 0.2) 10^9	9.3 ± 0.03
	L. plantarum KT-L 18/1^{str}	(1.1 ± 0.1) 10^5	5.0 ± 0.03	(1.4 ± 0.2) 10^6	6.1 ± 0.06	(1.8 ± 0.01) 10^{10}	10.3 ± 0.01	(1.5 ± 0.03) 10^9	9.19 ± 0.01
Treatment I – Control (no inoculations)	Natural population of ASB	(1.2 ± 0.1) 10^5	5.0 ± 0.03	(6.6 ± 0.3) 10^5	5.8 ± 0.01	(5.3 ± 0.2) 10^3	3.7 ± 0.03	(5.0 ± 1.15) 10^1	1.7 ± 0.08
Treatment III *Bacillus subtilis* BPT-B1str	Natural population of ASB with *B. subtilis* BPT-B1^{str}	(1.4 ± 0.4) 10^5	5.8 ± 0.4	(6.9 ± 0.5) 10^6	6.8 ± 0.03	(1.3 ± 0.1) 10^7	7.1 ± 0.05	(9.3 ± 0.6) 10^6	6.9 ± 0.1
	B. subtilis BPT-B1^{str}	(1.7 ± 0.2) 10^5	6.2 ± 0.05	(1.6 ± 0.1) 10^6	6.2 ± 0.03	(1.0 ± 0.2) 10^7	6.9 ± 0.03	(9.0 ± 0.2) 10^6	6.9 ± 0.01

Note. * Standard deviation.

Table 2. The antibacterial activity (ABA) and antifungal activity (AFA) of *Lactiplantibacillus plantarum* KT-L18/1^{str} and *Bacillus subtilis* BPT-B1^{str} towards strains of potential pathogenic bacteria (PPB) and some fungal strains before inoculation and as reisolate, after 30 days of fermentation of alfalfa silage under laboratory conditions

Treatment(n = 3)	*Staphylococcus aureus* 906	*Pseudomonas aeruginosa* ATCC 27853	*Salmonella typhimurium* 89	*Penicillium chrysogenum* (strain ch37)	*Fusarium oxysporum* (strain ch07)	*Rhizopus arrhizus var. arrhizus* strain ch37)
L. plantarum KT-L 18/1^{str} prior to inoculation	26.6 * ± 0.3 **	17.1 ± 0.6	26.6 ± 0.3	17.4 ± 0.3	2.3 ± 0.3	1.3 ± 0.3
L. plantarum KT-L 18/1^{str} after 30 days fermentation	22.0 ± 0.5	16.0 ± 0.5	24.4 ± 0.3	16.0 ± 0.6	0.6 ± 0.6	0.6 ± 0.3
B. subtilis BPT-B1str prior to inoculation	33.0 ± 0.2	7.9 ± 0.2	32.7 ± 0.3	22.6 ± 0.7	16.3 ± 0.5	13.6 ± 0.4
B. subtilis BPT-B1str after 30 days fermentation	26.3 ± 0.9	6.4 ± 0.6	27.7 ± 0.3	20.8 ± 0.2	12.9 ± 0.2	12.6 ± 0.3
PC: nystatin 100 U/ml	–	–	–	30.7 ± 0.3	15.3 ± 0.6	15.7 ± 0.3
PC: streptomycin 100U/ml	12.6 ± 1.2	7.5 ± 0.1	10.9–0.1	–	–	–

Note. * Width of the inhibition zone in mm; ** = standard deviation; PC = positive control.

on the fungal microflora was weak. *L. plantarum* KT-L18/1^{str} reduced growth of *Penicillium chrysogenum* (strain ch37) with 8 % after 30 days, but there was no antagonistic effect for the *Fusarium oxysporum* (strain ch07) and *Rhizopus arrhizus var. arrhizus* (strain ch37) strain used in our experiment.

After the introduction into the alfalfa silage for 30 days, *B. subtilis* BPT-B1^{str} also showed a decrease in ABA for *S. aureus* 906, namely 21.1 %, for *P. aeruginosa* ASTCC 27853 it was 19 % and for *S. typhimurium* 89 it was 16 %. The most significant decrease in AFA of *B. subtilis* BPT-B1^{str} was for *Fu-*

Table 3. The pH and the (lg = log10) number of background lactic acid bacteria (LAB), aerobic spore-forming bacteria (ASB), clostridia and fungi after 30 days fermentation of alfalfa with and without inoculation of *Lactiplantibacillus plantarum* KT – L18/1^{str} and *Bacillus subtilis* BPT-B1^{str}

Treatment (n = 3)	pH	(lg) CFU							
		LAB		ASB		Clostridia		Fungi	
		CFU/g	lg CFU/g	CFU/g	lg CFU/g	CFU/g	lg CFU/g	CFU/g	lg CFU/g
Control (no inoculation)	5.7 ± 0.01	(0.9 ± 0.03) * 10^7	7.0 ± 0.03	(6.6 ± 0.9) 10^1	1.8 ± 0.05	(2.7 ± 0.1) 10^7	7.4 ± 0.1	(1.3 ± 0.0) 10^6	6.1 ± 0.03
Inoculation with *L. plantarum* KT-L 18/1^{str}	5.2 ± 0.02	(2.1 ± 0.2) × × 10^9	9.3 ± 0.03	(2.8 ± 0.03) 10^1	1.4 ± 0.03	0	0	(2.2 ± 0.1) × × 10^3	3.3 ± 0.03
Inoculation with *B. subtilis* BPT-B1^{str}	5.3 ± 0.04	(1.7 ± 0.5) × × 10^9	9.2 ± 0.03	(9.3 ± 0.6) 10^6	7.0 ± 0.01	0	0	(4.9 ± 0.6) × × 10^2	2.7 ± 0.00

Note. * = Standard deviation.

sarium oxysporum (strain ch07), namely 38 %, however, its remaining activity was still high, 62 %. Decrease of AFAS for the other two fungal strains was insignificant.

The impact of the two inoculant strains on the pH of alfalfa silage after 30 days of fermentation is presented in **Table 3.** Silage treated with *L. plantarum* KT-L18/1^{str} and Bacillus *subtilis* BPT-B1^{str}, had a stable slightly lower pH level compared to the untreated silage (5.2 and 5.3 respectively, against 5.7 in the control).

In the two inoculant treatments there was a notable increase in the number of LAB with 2.2–2.3 lg CFU/g compared to the uninoculated control, 30 days after fermentation. For Bacillus *subtilis* BPT-B1^{str} there was an increase of 5.2 lg CFU/g in the number of ASB as compared to the control and lg 5.6 as compared to *L. plantarum* KT-L18/1^{str}. The two inoculants reduced the clostridia to nil as compared to the 7.4 lg CFU/g present in the control. Furthermore they decreased the number of fungi with 2.8–3.4 lg CFU/g.

DISCUSSION

Silage inoculants are intended to ensure fast and intense fermentation and can have a considerable impact on microorganisms, undesirable for ensilaging, depending on the stability of the expression of their competitive properties (Zielińska et al, 2015; Muck et al, 2018; Guo et al, 2022). The two inoculant strains *Lactiplantibacillus plantarum* KT-L18/1 and Bacillus *subtilis* BPT-B1used in this study proved to be in this and earlier studies to be probiotics with stable fermentative and antagonistic properties towards pathogenic and potentially pathogenic microorganisms (Perederii et al, 2019a; 2019b; Kravchenko et al, 2022) and they were patented (Patent of Ukraine No. 115938; Patent of Ukraine No. 116292). Streptomycin-resistant mutant strains were used to be able to determine the fate and activity of the inoculants. To our knowledge this has only been done for *L. plantarum* by Jansson (2005) in Sweden for the same reason. For the occurrence of natural streptomycin-resistant strains of L. plantarum, see Zhang et al (2018). The inoculant strains of the two bacteria we used appeared to be stably established and kept a high level of antagonistic activity and lowered the pH of the feed. This was also found earlier for alfalfa and other fodder crops for the same genera and species as we used as for other inoculants, such as *Lactiplantibacillus* (=*Lactobacillus*) *plantarum*, *Lentilactobacillus* (=*Lactobacillus*) *buchneri*, *Pediococcus* and *Enterococcus* species. (Kent et al., 1988; Whiter and Kung, 2001; Zhang et al, 2009; Jatkauskas and Vrotniakiene, 2011; Bao et al, 2016; Bai et al, 2020, 2021 and 2022; Li et al, 2022 and 2023; Liu et al, 2023; Guo et al, 2023; Wang et al, 2023; Günaydin et al, 2023; Peng et al, 2024). In the most recent researches mentioned here, the establishment and influence in/on the microbiome was thoroughly investigated using advanced molecular methods such as 16s rRNA sequencing and next generation sequencing (NGS). However, these recent molecular biological studies have a drawback, because it cannot with certainty be determined if the detected sequences belong to living or dead organisms (Ávila and Carvalho, 2020). On the other hand, modern biotechnology, using metabolomics, proteomics, (meta)genomics, transcriptomics, and genetic manipulation, facilitates identification and improvement of further promising, highly efficient LAB strains in the (near) future. This may lead to increased animal feed quality and sustainability (Okoye et al, 2023). The fact that *B. subtilis* is a facultative anaero-bic bacterium may have contributed to this stable increase in numbers during the ensiling as established by us and others (Bai et al, 2022; Guo et al, 2022).

Antibacterial activity (ABA) and anti-fungal activity (AFA) and AFA of *L. plantarum KT*-L18/1^{str} and *B. subtilis* BPT-B1^{str} was maintained during the 30 days of fermentation, but declined depending on the inoculant and the environmental strain tested, between 7 and 21 %, possibly due to interactions with the local microbiome. This fact has been noted only occasionally before (e.g. Gollop et al, 2005), however, and therefore it has to be investigated in more detail. The antagonistic activity of our *L. plantarum* strain, could possibly be important, too for use in biological plant disease control, as was found for some strains of *L. plantarum*, controlling the plant pathogenic bacteria *X. fragariae*, *X. arboricola* pv. *pruni* and *Pseudomonas actinidiae* in semi-field studies (Daranas et al, 2019). The pH lowering activity of inoculants of both *L. plantarum* and *Bacillus subtilis*, as reported here, has been observed before many times for *L. plantarum* (e.g. Yang et al, 2019; Zhao et al, 2020), but much less for *B. subtilis* (Bai et al, 2020)

An increase in the total number of lactic acid bacteria following inoculation with *L. plantarum* and also for *B. subtilis,* as determined in our experiment (both c. 30 % increase) was also noted by Yang et al (2020) and Guo et al (2023) for *L. plantarum*. For *B. subtilis* it was reported by Bai et al (2022).

When there is access of air to fermented feed it stimulates a rapid development of fungi, and when these

are present at more than 10^4–10^5 CFU/g they impair the aerobic stability of the product and may cause fast spoiling (Auerbach, 1996; O'Brien, 2007; Guo et al, 2023). In our experiment both inoculants, but especially *B. subtilis* BPT-B1 showed a persistent and high AFA during the whole fermentation period. *B. subtilis* is known to produce several metabolites: cyclic lipopeptides, polypeptides, proteins and antibiotics (e.g. subtilosin, sublacin, bacitracin, bacillisin, bacillomycin and subtilisin,), with an inhibiting effect towards pathogenic bacteria and fungi (Hamdache et al, 2011; Kaynar and Beyatli, 2019; Irkitova et al, 2018). LAB strains also synthesize antimicrobial metabolites, including lactic acid, hydrogen peroxide, and bacteriocins. (Pang et al, 2011; Guo et al, 2023). However, the mechanisms of AFA of LAB require additional studies, since the main metabolite of *L. plantarum,* lactic acid, does not have any antifungal effect. Furthermore, the possibility of adding cellulase and/or a cellulase-producing inoculant strain, such as *Bacillus pumilus* (Li et al, 2018; Si et al, 2023) or *B. coagulans* to fasten anaerobic conditions (Wang et al, 2023) in combination with our inoculants, to further improve the quality and digestibility of alfalfa feed, could be investigated.

CONCLUSIONS

Two promising inoculant strains, *Lactiplantibacillus plantarum KT-L18/1* and *Bacillus subtilis* BPT-B1 which were mutated to streptomycin resistance, were followed in their growth dynamics, antagonistic activity and acid production in a 30-day period of fermentation of alfalfa. This towards a background of natural lactic acid bacteria (LAB) and other microbiota.

These two strains proved to be highly competitive and stably present and antagonistic, also to a strain of the potential pathogenic bacterial species *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Salmonella typhimurium* during a 30-day fermentation. The antagonistic activity only slightly decreased over this period. The inoculant strains outnumbered and largely replaced natural LAB at the end of the 30-day fermentation.

B. subtilis BPT-B1^{str} kept its antifungal activity toward the investigated test cultures of fungi after the 30-day alfalfa fermentation. *L. plantarum* KT-L18/1^{str} maintained antifungal activity to only to a strain of *Penicillium* just like prior to inoculation.

Both inoculant strains caused a decrease in pH, an increase in total number of LAB and inhibition of the growth of clostridia in the fermented alfalfa. Furthermore they decreased the number of fungi down to 10^2–10^3 CFU/g, which promoted the aerobic stability of the feed.

Adherence to ethical principles. This article does not contain any studies with human participants and animals performed by any of the authors.

Conflict of interest. The authors declare no conflict of interest.

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Динаміка росту, антагоністична активність та кислотоутворення інокулянтів *Lactiplantibacillus* (=*Lactobacillus plantarum*) КТ-L18/1 і *Bacillus subtilis* ВРТ-В1 у силосі з люцерни

Н. О. Кравченко

Інститут сільскогосподарської мікробіології
та агропромислового виробництва
Національної академії аграрних наук України
м. Чернігів, вул. Шевченко, 97, Україна, 14027

E-mail: nat.probiotik@gmail.com, ismavnaas@gmail.com

orcid: https://orcid.org/0000-0001-5090-4276

Мета. Вивчити динаміку росту, антагоністичну активність та кислотоутворення двох штамів інокулянтів *Lactiplantibacillus (=Lactobacillus) plantarum* КТ-L18/1 та *Bacillus subtilis* ВРТ-В1, які вносили в силос люцерни на 30 діб ферментації. **Методи.** Для досягнення поставлених цілей використовували стрептоміцин-резистентні штами обох вищевказаних мікроорганізмів, отриманий у попередніх дослідженнях. Люцерну штучно пров'ялювали і сушили до вмісту сухої речовини 39–40 %. Ферментацію проводили в анаеробних умовах у поліетиленових мішках. Інокулянт додавали в концентрації 10^8 КУО/кг люцерни. Силосування було досягнуто за трьох обробок: 1) контроль без інокулянтів, 2) застосування *L. plantarum* КТ-L18/1^{str} як інокулянту і 3) *B. subtilis* ВРТ-В1^{str} як інокулянту. Було чотири пакети з 300 г люцерни для кожної обробки, і кожну обробку проводили в трьох повторностях. Отримані 12 × 3 = 36 пакетів витримували за кімнатної температури в темному місці 30 діб. На кожне дослідження використовували один зразок з обробки та вивчали (lg) КУО/г двох інокулянтів після їх культивування на елективному агарі де Мана-Рогози-Шарпа (MRS) та м'ясопептонному агарі (MIA), що містять стрептоміцин. (lg) КУО/г інокулянтів і природної фонової мікробіоти визначали шляхом розведення на цих агарах на початку ферментації (0 доба) і на 3, 15 і 30 доби.

Антибактеріальну (АБА) й антифунгальну активності (АФА) досліджували дифузним методом агарових блоків до потенційно патогенних штамів бактерій (ППБ), а саме *Staphylococcus aureus* штам № 906, *Pseudomonas aeruginosa* ATCC-27853, *Salmonella typhimurium* № 89 та до грибів *Penicillium chrysogenum* штам ch37, *Fusarium oxysporum* штам ch07, *Rhizopus arrhizus var. arrhizus* (syn. *R. megaspores*) штам ch37. pH силосу рутинно визначали у водному екстракті після 30 діб ферментації. Для аналізу фонової мікробіоти випадковим чином відбирали 50 г змішаного силосу та додавали 450 мл стерильної води для приготування суспензій. (lg) КУО грибів визначали на глюкозному агарі Саборо після 3–4-денного культивування за 28 °С. (lg) КУО/г бактерій клостридій визначали після 24 год культивування при 37 °С на середавиші Reinforced Clostridial Broth (Himedia, Індія). (lg)КУО/г молочнокислих бактерій (МКБ) та аеробних спороутворюючих паличок (АСБ) визначали, як описано вище для *L.plantarum* КТ-L18/1^{str} та *B. subtilis* ВРТ-В1^{str}, але без додавання в середовище стрептоміцину. **Результати.** Після 30 днів силосування зразків люцерни, *L. plantarum* КТ-L18/1^{str} та *B. subtilis* ВРТ-В1^{str} все ще були присутні на рівнях 9,2 lg КУО/г та 6,9 lg КУО/г відповідно. У природній популяції МКБ домінуюче місце займав *L. plantarum* КТ-L18/1^{str}. У варіанті, обробленому *B. subtilis* ВРТ-В1^{str} кількість МКБ становила 9,2 lg КУО/г, що практично дорівнювало кількості МКБ у варіанті з *L. plantarum* КТ-L18/1^{str}. Після 30 днів ферментації люцерни штами *L. plantarum* КТ-L18/1^{str} і *B. subtilis* ВРТ-В1^{str} підтримували АБА та АФА до штамів ППБ (*S. aureus, P. aeruginosa, S. typhimurium*) і використовуваних штамів грибів, що належать до *Penicillium chrysogenum, Fusarium oxysporum, Rhizopus arrhizus var. arrhizus*. Проте антагоністична активність двох інокульованих штамів під час ферментації знизилася, для *L. plantarum* КТ-L18/1^{str} від 7–21 %, а для *B. subtilis* ВРТ-В1^{str} від 16–21 %. Порівняно з неінокульованим контролем, інокульований силос мав нижчий рівень pH (5,2–5,3), підвищену кількість молочнокислих бактерій (МКБ) (на 32 %), а клостридії більше не виявлялися. Інокуляція вплинула на зменшення росту грибів до рівня (10^2–10^3 КУО/г), що може забезпечити аеробну стабільність корму. **Висновки.** Інокульовані штами *L. plantarum* КТ-L 18/1^{str} і *Bacillus subtilis* ВРТ-В1^{str} показали високу конкурентоспроможність із фоновою мікробіотою в досліді силосування люцерни. Антагоністична активність двох штамів до ППБ після ферментації збереглася на високому рівні, хоча і зі зниженням на 7–21 %. *Bacillus subtilis* ВРТ-В1^{str} зберігав свою АФК проти всіх трьох протестованих штамів грибів упродовж 30 діб ферментації. Для *L. plantarum* КТ-L18/1^{str} це було вірно лише для штаму *Penicillium chrysogenum.* Інокуляція *L. plantarum* КТ – L18/1^{str} та *Bacillus subtilis* ВРТ-В1^{str} викликала зниження pH на 8–9 % наприкінці періоду ферментації, накопичення МКБ на 32 % та пригнічення росту клостридій до неможливості виявлення у ферментованій люцерні. Для обох інокулянтів порівняно з неінокульованим контрольним варіантом вміст грибів знизився до 10^2–10^3 КУО/г корму, що може забезпечити аеробну стабільність корму.

Ключові слова: молочнокислі бактерії, аеробні спороутворюючі бактерії, чисельність мікроорганізмів, антибактеріальна активність, антифунгальна активність.

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