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Research article

ISOLATION AND CHARACTERIZATION OF LACTOBACILLI ISOLATES FROM CANINE *(Canis familiaris)* MILK

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The first food in neonatal puppies is milk. Along other functions as nutritive and purgative, colostrum serves as the induction route for the enteral microbiome. As one of the two most abundant bacteria groups in the colostrum, the lactic acid bacteria are a significant factor due to their attributed functional properties in the digestive system of neonates. Dog lactational microbiome has not been studied in detail and therefore the aim of this work was to isolate, characterize lactobacilli bacteria from canine milk and estimate their antibacterial properties and ability for survival at different temperatures. Four lactic acid bacteria isolates were identified by MALDI TOF and confirmed by 16s sequencing. All four successfully unambiguously identified isolates showed survival at both storage regimes with the best growth at 37 °C in bile salt supplemented medium. The growth of *S. aureus* was impaired by three of four isolates and *E. coli* by two. The obtained results provide a promising base for further probiotic analysis of isolates in both puppies and lactating female dogs.

Keywords: bacteriology, dog, lactation, microbiology, neonates

INTRODUCTION

The lactation period is crucial in canine breeding with early weaning considered to be substantial for puppy mortality and morbidity. Milk offers a rich source of nutrients and energy for the puppies, at the same time providing protection against infectious diseases in the neonatal period. The protective effect is believed to be the result of a plethora of protective factors originating from milk and colostrum such as: lysozyme, lactoferrin, fatty acids with antimicrobial activity, immunoglobulins, immunocompetent cells, fucosylated oligosaccharides, polyamines and commensal bacteria [1]. Canine milk is a natural and continuous source of lactobacilli, originating from the environment, moreover milk has the purpose to fulfil the nutritional requirements and immune protection against infectious diseases for rapidly-growing puppies [2]. The bacteria of

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Lactobacillus genus are considered to be a desirable bacteria for supplementation due to their impact on metabolism and health of the dogs [3-6]. Lactobacilli are Gramm positive, catalase negative, non-spore forming, mostly non-motile and generally rod-shaped bacteria, with lactic acid as the main fermentation end product from sugars, moreover this fermentation property itself has been the basis of the earlier taxonomy of the genus [7]. Following the intensive application of molecular methods many bacterial species from *Lactobacillus* genus were reclassified to several a new genus level under the *Lactobacillaceae* family known as the lactic acid bacteria group (LAB) [7]. The desirable status as probiotic and postbiotic bacteria LAB apparently owe to recognized antibacterial properties of metabolism byproducts consisting of antibacterial peptides subset known as bacteriocins [8] used as biotherapeutic products which output is expanding [9]. Canine milk microbiological studies are scarce and usually aimed at potential pathogenic bacteria, identification of clinical perinatal infections such as septicemia in neonatal puppies [10] or lactational mastitis [2]. The aim of this research was isolation, characterization and focused study of LABs isolated from lactating dogs.

MATERIAL AND METHODS

Ethical approval

Study protocols and involvement of female dogs in the experiment were approved by The Veterinary Directorate, Ministry of Agriculture, Forestry and Water Management (No. 323-07-/2023-05/2) and the Ethical commission for the protection of experimental animal welfare of the Faculty of Veterinary Medicine, University of Belgrade (No. 01-1/12).

Collecting samples

Milk samples were collected from 20 bitches of different breeds (German Shepherd, Dobermnn, Labrador, Šarplaninac, Istrian Shorthaired Hound, Pug, Boston Terrier and Chow Chow). The health status of the bitches was monitored daily, several times a day by observation (appetite, drinking water, willingness to move – walking, playing, caring for the litter – consent for breastfeeding) and carried out by the individual owner or the person responsible for the daily care of the dogs. Bitches with a litter had to be handled gently in order to avoid stress that might affect lactation. At the first sign of anxiety in the bitch, milking was stopped. All animals had met the following criteria: 1) they were observed by a veterinarian and considered to be generally healthy, 2) the pregnancy passed without complications, 3) there was no administration of medication/antibiotics and/or probiotics in the 2 months period before the study, 4) there were no perinatal problems in the litter and 5) the age of the bitches was more than 1.5 years. Milk samples were taken after breastfeeding the puppies in the amount up to 2 mL for bitches larger than 10 kg BW, and up to 1 mL for smaller

ones without reducing the amount of milk which is necessary for breastfeeding. In addition, milk sampling by milking stimulated milk synthesis between breastfeeding of puppies. The bitches were milked by the owner or a soldier in charge for the care and nursing of the bitches in the maternity ward of the military kennel. Preparation of the mammary gland went through the following steps: after a gentle massage and washing of the udder with water the tits were wiped with a disposable paper towel and disinfected with cotton soaked in alcohol (ethanol 68%). In the case bitch witheld the milk, subcutaneous addministration of 2-10 i.u. oxytocin (*Oxytocin®,* Biovet, Bulgaria) was necessary to stimulate the secretion and release of milk into the mammary gland. After whelping, milking into sterile plastic cups took place on the 20th day, the 42nd and the 50th day. Samples were stored in a refrigerator (temperature up to 4 °C), with immediate transport to the laboratory.

METHODS

Isolation and enumeration of lactobacilli in canine milk

For enumeration and isolation of lactobacilli the ISO 15214:1998 method was used, but besides recommended MRS agar, Columbia blood agar (both Himedia, India) was used in parallel, supplemented with 5% v/v sheep blood (CBA), incubated under anaerobic conditions at 37 °C.

Identification of lactobacilli isolates from canine milk

After incubation of MRS and CBA, characteristic colonies were further examined for cell morphology, Gram stain reaction and tested for catalase and oxidase activity. Gram-positive rods with a negative reaction on both catalase and oxidase tests were inoculated into MRS broth for 48h incubation at 37 °C in order to multiply bacteria for further identification and determination of biochemical characteristics. Following MRS broth, isolates were further seeded on MRS and CBA anaerobically for 48 h at 37 °C.

Pure cultures were further identified by the MALDI-TOF method (Bruker MALDI Biotyper, Instrument ID:269944.01833, Server Version 4.1.100 (PYTH) 174 2019-06-158_01-16-09. at the Veterinary University Vienna (Austria).

Sequencing of 16S gene

All lactobacilli isolates were further 16S RNA gene sequenced. The 25µl PCR reaction consisted of the primer set 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'), PCR mastermix (DreamTaq Green PCR Master Mix 2X, Thermo Scientific), 5 µL of bacterial DNA and PCR grade water. The temperature regime of the PCR reaction included: an initial step of 5 minutes at 96 °C,

then 30 cycles of the following regimes: 30 s at 94 °C, 30 s at 57 °C and 60 s at 72 °C, and at the end with a final elongation of 10 minutes at 72 °C. Obtained products were sent commercially for Sanger sequencing commercially (M/s Macrogen Inc., South Korea).

Biochemical properties of lactic acid bacteria isolates from canine milk

Biochemical properties of lactobacilli isolates were determined following earlier described methods [7,12]. The biochemical substrates, reactions and results are presented in Table 1.

Growth and survival in different conditions

The growth of LAB isolates was tested in MRS broth at incubation temperatures of 20, 25, 32, 37 °C and 42 °C (pH 6.2) in the presence of bile salts (MRS broth supplemented with 5% bile salts) (ICE S.p.A, Italy). Growth was assessed by spectrophotometry at 650 nm in 100 µl volume (Sunrise microplate reader, Tecan, Switzerland). Survival of lactobacilli isolates was analyzed after storage at 20 °C for 15 days, at – 71 °C for 30 days (MRS broth supplemented with 15% glycerol) and put into MRS agar after defrosting.

Determination of antibacterial activity

The antibacterial activity was tested toward test strains *Staphylococcus aureus* ATCC 25923, *Salmonella* Typhimurium ATCC 14028, *Escherichia coli* ATCC 25922, *Proteus mirabilis* ATCC 29906 and *Pseudomonas aeruginosa* ATCC 27853. All tests were conducted in triplicate and the results presented as median inhibition diameter.

Direct test

An overlay method was used to determine the ability of the LAB isolates to inhibit the growth of test microorganisms [13]. In brief: MRS agar plates with lactobacilli inoculated in lines and incubated at 37 °C for 48 h in anaerobic jars (Oxoid, Ireland). The plates were overlaid with the test microorganism suspension (10^4 CFU /ml) in 10 mL of soft (0.7%) Cation adjusted Mueller Hinton agar (MH, BBL, USA). Finally, the plates were incubated at 37 °C for 48 h, and the antibacterial activity was assessed based on the zone of test microorganism inhibition.

Bacteriocin test

The bacteriocin production of isolated lactobacilli was tested by Oxford method [5]. The pathogenic strains suspension (10^5 CFU / mL) was laid on MH plates. After allowing absorption of the inoculum for 10 minutes at room temperature, the 7 mm diameter wells were aseptically punctured and 100µl of lactobacilli supernatant product poured in.

The supernatant bacteriocin products were prepared as described earlier [2] in brief: form the broth culture of lactobacillus isolates after MRS broth incubation at 37 °C for 24 h, the samples were centrifuged at 12. 000 rpm for 10 minutes at 4 °C, neutralized with 1 M NaOH to achieve pH 6.2, heated at 100 °C for 5 minutes and sterilized by passing through a 0.22 μm filter.

Statistical analysis

For statistical analysis GraphPad Prism ver.8 was used. Data were checked for normal distribution by Kolmogorov-Smirnov test and nonconforming data were compared by Kruskal-Wallis and Mann-Whitney *post hoc* test. Statistical difference level was set to $p<0.05$.

RESULTS

Isolation and identification

Out of 20 canine milk samples four lactic acid primoisolates were identified, namely *Lacticaseibacillus rhamnosus, Limosilactobacillus reuteri, Lacticaseibacillus paracasei* and *Ligilactobacillus murinus*. All isolates were successfully isolated by MALDI TOF (Anex 1) and 16S gene sequencing (Anex 2).

Enumeration

The average number of lactobacilli in canine milk was 3.59 log CFU/mL with SD 1.10 log CFU/mL and variation coefficient of 0.30, the number ranged from minimal l.11 log CFU/mL up to maximal 7.23 log CFU/mL.

Biochemical reaction

Results of biochemical reactions including sugar acid fermentation, hydrolysis, aminopeptidase and other reactions are presented in Table 1.

Growth and Survival

The data from the growth analysis revealed the largest increase of all four LAB isolates abundance at 37 °C, reaching the OD (650) value of over 1.0 in all isolates (Figure 1). The statistical difference in all four LAB isolates growth was found: for *L. rhamnosus* between 20 °C and 37 °C ($p=0.0102$); *L. reuteri* between 20 °C and 37 °C ($p=0.0162$); *L. paracasei* between 37 °C and 42 °C ($p=0.0102$) and *L. murinus* between 20 °C and 37 °C ($p=0.0100$). Further data are shown in Figure 1. All isolates have survived storage at 20 °C for 15 days, and – 71 °C for 30 days.

Table 1. The biochemical rections of isolated lactobacilli from the canine milk

SUBSTRATE	REACTION	*Lacticaseibacillus rhamnosus*	*Limosilactobacillus reuteri*	*Lacticaseibacillus paracasei*	*Ligilactobacillus murinus*
4-Methylumbelliferyl β-D-glucopyranoside	β- glucosidase activity	-	-	-	-
L-valine- Acetyl-7-Amido-4-Methylcoumarin	aminopeptidase	-	±	±	-
L-Phenylalanine-7-Amido-4-Methylcoumarin	aminopeptidase	+	+	-	+
4-Methylumbelliferyl-α-D-glucopyranoside	α-glucosidase	-	±	-	-
L-pyroglutamic acid 7-amido-4-methylcoumarin	hydrolysis	-	-	-	-
L-tryptophan 7-amido-4-methylcoumarin	glycosidases, phosphatases, or esterases	+	+	+	+
L-Arginine 7-amido-4-methylcoumarin	aminopeptidase B cathepsin H	-	+	-	-
4-Methylumbelliferyl-N-acetyl-β-D-glucosaminide	N-acetyl-β-D-glucosaminidase	-	-	-	-
4-Methylumbelliferyl-phosphate	alkaline or acid phosphatases	-	+	-	-
4-Methylumbelliferyl -β-D-glucuronide	glucuronidase	-	+	-	-
L-isoleucine-7-Amido-4-Methylcoumarin	hydrolysis	±	-	+	-
Trehalose	Acid fermentation	+	+	+	±
Lactose	Acid fermentation	+	-	+	+
Methyl-α & β-glucoside	hydrolysis	+	-	±	+
Sucrose	Acid fermentation	+	+	+	+
Mannitol	Acid fermentation	+	-	+	+
Maltotriose	Acid fermentation	+	-	+	+
Arabinose	Acid fermentation	+	+	±	-
Glycerol	Acid fermentation	+	-	+	+
Fructose	Acid fermentation	+	-	+	+
p-nitrophenyl-β-D-glucoside	hydrolysis	-	-	-	-
p-nitrophenyl-β-D-cellobioside	hydrolysis	-	+	-	-
Proline & Leucine-p-nitroanilide	aminopeptidase	±	-	-	-
p-nitrophenyl-phosphate	phosphatase	-	-	-	-
p-nitrophenyl-α-D-maltoside	α-maltosidase	-	+	-	-
ONPG & p-n-p-α-D-galactoside	β-galactosidase	-	-	-	+
Urea	urease	-	-	-	-
Esculin	Esculin hydrolysis	+	-	+	+
Arginine	hydrolysis	-	+	-	-

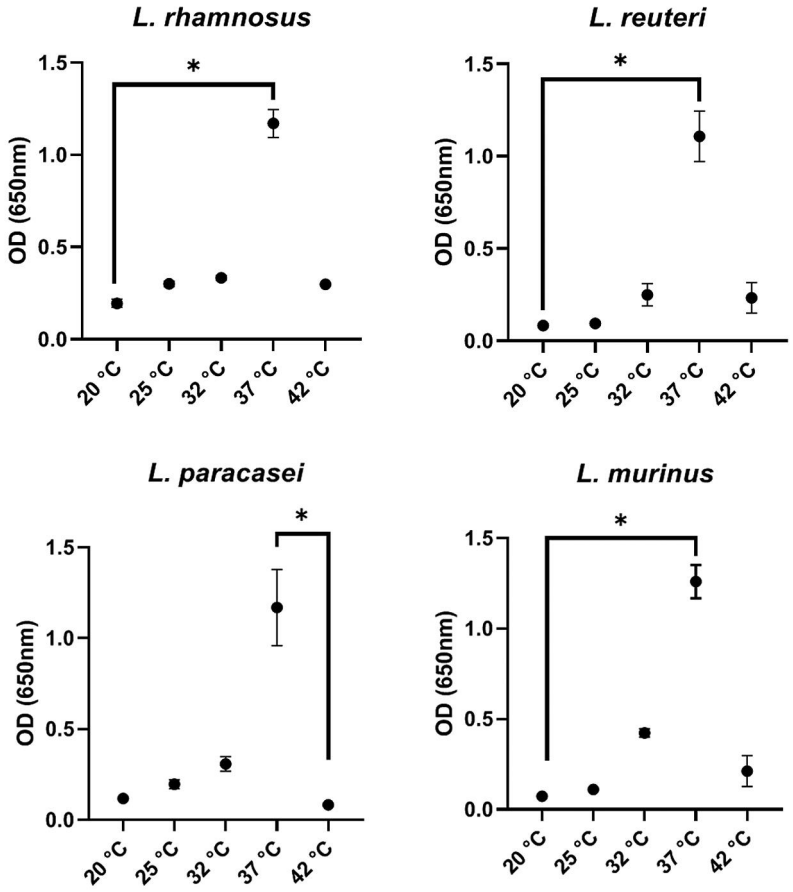

Figure 1. The growth of LAB isolates from canine milk at 5 different temperature regimes X ± SD. Asterisk (*) marks a statistically significant difference ($p<0.05$).

Direct layover test

Most test strains were inhibited by *L. rhamosus* (against *S.aureus*, *S*. Typhimurium and *E.coli*) while *L. murinus* did not produce inhibitory effects for test strains. The most susceptible test strain was *S. aureus* which showed susceptibility to three LAB isolates (*L. rhamnosus, L. reuteri* and *L. paracasei*), while *E. coli* had inhibition under action of two LABs and *S*. Typhimurium to only one. Other test strains showed no inhibition zones. The inhibition zones in the direct test are given in Table 2.

Table 2. Zones of growth inhibition (mm) of test strains by LAB isolates from canine milk-bacteriocin test

Test strains	*Lacticaseibacillus rhamnosus*	*Limosilactobacillus reuteri*	*Lacticaseibacillus paracasei*	*Ligilactobacillus murinus*
S. aureus	5	4	4	n.d.
P. mirabilis	n.d.	n.d.	n.d.	n.d.
S. Typhimurium	3	n.d.	n.d.	n.d.
E. coli	4	n.d.	3	n.d.
P. aeruginosa	n.d.	n.d.	n.d.	n.d.

Median values of direct growth inhibition zones
n.d. – not determined, growth of test strains without inhibition by LAB

Bacteriocin test

In the bacteriocin test the most potent isolate was *L. rhamnosus* which demonstrated inhibition zones against all test strains but *P. aeruginosa*. *L. paracasei* demonstrated zones against 3, *L. reuteri* against 2 and *L. murinus* against 1 test strain. The largest inhibition zone showed *L. rhamnosus* against *E. coli* (19 mm). The inhibition zones recorded in the bacteriocin test are given in Table 3. The photographs of the layover and bacteriocin test are shown in Figure 2.

Table 3. Zones of growth inhibition (mm) of test strains by LAB isolates from canine milk-bacteriocin test

Test strain	*Lacticaseibacillus rhamnosus*	*Limosilactobacillus reuteri*	*Lacticaseibacillus paracasei*	*Ligilactobacillus murinus*
S. aureus	18	18	17	n.d.
P. mirabilis	18	17	14	13
S. Typhimurium	16	n.d.	n.d.	n.d.
E. coli	19	n.d.	17	n.d.
P. aeruginosa	n.d.	n.d.	n.d.	n.d.

Median values of direct growth inhibition zones
n.d. – not determined, growth of test strains without bacteriocin inhibition

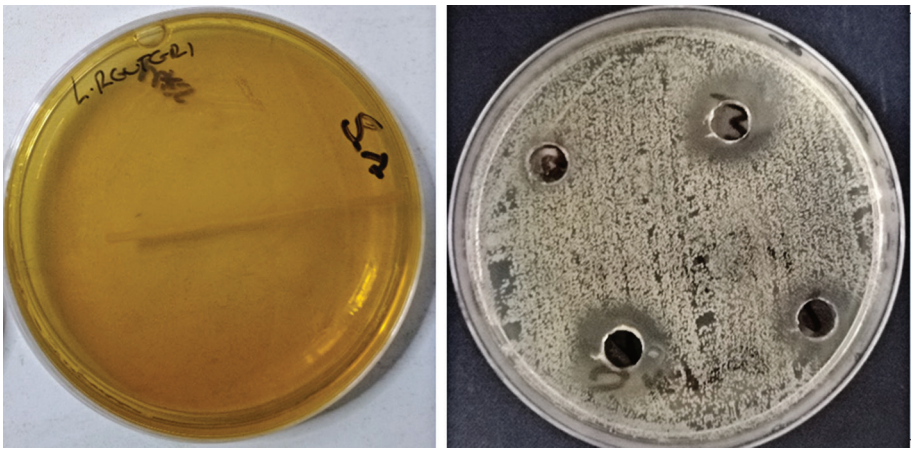

Figure 2. The antibacterial properties test. Left the direct, layover method *L.reuteri* versus *S.aureus*. Right the bacteriocin effect of *L. rhamnosus L. reuteri L. paracasei* and *L. murinus* versus *S. aureus*.

DISCUSSION

The obtained results showed that LAB isolates from canine milk belong to the *Lactobacillaceae* family genera: *Lacticaseibacillus, Limosilactobacillus* and *Ligilactobacillus*. Although now reclassified in three distinctive genera, all four species historically used to belong to the same genus *Lactobacillus* earlier on [12]. Current taxonomic classification is further subject to changes due to the fact that former Lactobacilli members offer a great variety in expressing their phenotypic features under natural biological diversity and that current methods of classification by using of single 'representative' strain per species is constraining that diversity [7]. A more pragmatic approach is the 1919 originated Orla-Jensen classification based on fermentation product properties dividing lactobacilli to obligately homofermentative, facultatively heterofermentative and obligate heterofermenters [12]. Out of 4 LAB isolates from canine milk, *L. reuteri* belongs to the obligately heterofermentative group while others are facultatively heterofermentative which is probably related to their physiological function in dogs gastrointestinal system [2]. Recently the extent of lactobacilli research has been expanded due to the fact that the use of probiotics can normalize the intestinal microbiota in the case of its disfunction [5,14].

The canine milk microbiota may contain LABs which colonize the native digestive tract of puppies during suckling [2]. The obtained results showed that the average count of LABs was 3.59 log CFU/mL which is a higher value than the previously reported range 2.1 – 2.8 log CFU/mL [2]. Our results confirmed the LAB presence in canine milk supporting findings that this biological fluid provides a natural and continuous source of LAB for the suckling puppies [1].

The biochemical characterization revealed that all isolates except *L. reuteri* hydrolyze lactose as the primary milk sugar and only *L. murinus* expressed β-galactosidase activity.

Regarding other sugar to acid fermentation properties the most diverse substrate preferences were expressed by *L. rhamnosus* and *L. paracasei* in the ability to ferment all tested sugars, while *L. reuteri* was the most fastidious, with acid fermentation of only trehalose, sucrose and arabinose revealing its role in food digestion processes if used as a probiotic culture. Although, nowadays considered imprecise for final identification, the biochemical properties of all isolated LABs are in concordance with type strains reported earlier [7,12].

Growth for all tested lactobacilli was most abundant at 37 °C, which presents the probable closest temperature to the real conditions during puppies suckling. Growth was present at all other tested temperature regimes but to a lesser extent. The statistical difference in growth is found in all four isolates at 37 °C and one of the other temperatures, but this issue raises due to triplicates in experiment. If the experiment was conducted with more replicates, most likely that statistical difference would occur between 37 °C and all other temperature regimes.

The obtained growth inhibition zones by direct test were not enough to assess the inhibitory effect. In some inoculated Petri dishes with CBA and MHA a haze around the line of contact with LAB isolates was formed making it difficult to assess the inhibition zone. The next problem was the unclear inhibition zone along the LAB strip. Probably, it is due to the heat stress of the LAB caused by warm molten inoculated media CBA and MHA, so the results were worse than we expected. Similar difficulties with the direct test were reported earlier [2]. The overlay method of the antibacterial effect in *L. rhamnosus* may be influenced by the pH of the medium [1], which was later proven for more LAB species [5] resulting in the issue that the direct test by overlay method is recommended for screening purposes only for future isolates.

On the other hand, the bacteriocin test proved to be easier to perform and read. Bacteriocins are antimicrobial peptides produced either for competitive advantage in microbiome or self-preservation by all major lineages of bacteria [8]. Regarding their function, the most potent *L. rhamnosus* produced antibacterial effects on 4 out of 5 test strains. Toward *P. aerugonosa* no LAB isolate bacteriocin showed effect. The *E. coli* may offer strains that cause neonatal septicemia in puppies [10,15], but it's also worldwide known for enteral diseases of various animal species including humans [16] yet, only *L. rhamnosus* and *L. paracasei* isolates managed to suppress its growth. *S. aureus* is representative of coagulase positive staphylococci which are known to cause serious problems in neonatal period of dogs with significant mortality [10,15,17] and *L. rhamnosus, L. reuteri, L. paracasei* products inhibited its growth. The most abundant colostral bacteria in vaginal delivery of puppies are specifically staphylococci and lactobacilli [18] implicating the significance of gut colonization and interbacteria relations in the neonatal period.

The canine LABs are under researched [2,4], and this gap has been a point of interest in science for the last decade or two. On the other side, the increase in market products based on pet probiotics by 7% is obvious [9]. It has its veterinary medical confirmation

because LABs reduce diarrhea [1], modulate the immune response in canine colostrum [3] as if given to the bitches they strengthen the gastrointestinal performance in younglings [6] and show general immunomodulatory effect in dogs [19]. Our research revealed the good candidates for further lactation immunomodulatory effects in dogs, moreover *L. rhamnosus* showed good survival under simulated canine GI conditions [1].

CONCLUSION

Canine milk represents a growing and interesting field of research, especially due to the One Health concept. All four LAB isolates had a preferable growth temperature of 37° C even in addition of 5% bile salts in medium, and all survived storage conditions of both room and deep freeze temperatures. (-72 °C). Furthermore, the antibacterial effect is confirmed since three of four isolated LABs affected growth of *S. aureus* and 2 isolates affected *E. coli*. These results gave a promising base for probiotic property analysis of canine LABs in both puppies and lactating bitches.

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Authors' contributions

PS carried out the sampling of canine milk, isolation and determination the number of lactobacilli, determination of biochemical characteristics and confirmation of identification by MALDI TOF. NZ carried out the isolation, determined the antibacterial activity and molecular technique methods (PCR), statistical data analyses and draft writing. BA performed microbiological analyses by enumarting the number of lactobacilli in canine milk samples. DŠ assisted in the design of the experiment and data analyses. VD assisted in conselling for the kinology issues. RSR designed the experiment, supervised the sampling, carried out the isolation of lactobacilli isolates, enumeration, and determined the antibacterial activity and ability of survival under different conditions. Also, supervised, reviewed and edited the final version of the manuscript. All authors read and approved the final manuscript.

Declaration of conflicting intrests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Statment of Informed Consent

The owners understood procedure and agreed that the results of analyses of canine milk samples originating from companion and working dogs will be published in the scientific journal Acta Veterinaria-Beograd.

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IZOLACIJA I KARAKTERIZACIJA IZOLATA LAKTOBACILA IZ MLEKA KUJA *(Canis familiaris)*

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Prva hrana za štenad je mleko. Pored ostalih funkcija kao što su hranljiva i purgativna, kolostrum služi za indukciju enteralnog mikrobioma. Kao jedna od dve najzastupljenije grupe bakterija u kolostrumu, bakterije mlečne kiseline predstavljaju značajan faktor zbog funkcionalnih osobina u digestivnom sistemu novorođenih. Laktacioni mikrobiom pasa predstavlja nedovoljno istraženu temu i stoga je cilj ovog rada bio da se izoluju, karakterišu bakterije laktobacila iz mleka kuja i proceni njihova antibakterijska osobine i sposobnost preživljavanja pri različitim temperaturama. Izolovana su četiri izolata bakterija mlečne kiseline, identifikovana pomoću MALDI TOF i potvrđena 16s sekvenciranjem. Sva četiri identifikovana izolata su preživljavala pri temperaturama inkubacije sa najboljim rastom pri 37 °C u podlozi sa dodatkom žučnih soli. Na rast *S. aureus* su uticala tri od četiri izolata, a na *E. coli* dva. Dobijeni rezultati daju obećavajuću osnovu za dalja ispitivanja probiotičkih osobina izolata kod štenadi i kod ženki pasa u laktaciji.