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EQUID MILK IS A SOURCE OF PROBIOTIC BACTERIA WITH POTENTIAL IN CARIES REDUCTION AND PRESERVATION OF PERIODONTAL HEALTH

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ABSTRACT

Dental caries and periodontitis are the most common diseases of the oral cavity and can develop into life-threatening health conditions. Both diseases are multifactorial chronic diseases in which pathogenic microorganisms play an important role in disease progression. Several studies suggest that probiotics may play an important role in preventing of such diseases. In this study, lactic acid bacteria isolated from mare and donkey milk were evaluated for their potential as dental probiotics. The ability to survive and form biofilms in the oral cavity, as well as the antimicrobial and coaggregative abilities against cariogenic streptococci and periodontitis-related *P. gingivalis* and *A. actinomycetemcomitans* were investigated. Two *L. plantarum* strains showed high probiotic potential. Together with high aggregation and antimicrobial activity, investigated strains displayed the ability to prevent biofilm formation of the tested pathogens. Our results suggest that there are several ways to exert antimicrobial activity and that the tested probiotic characteristics are strain-dependent. This study characterizes for the first time naturally occurring bacteria from mare and donkey milk for use in the preservation of dental health. Equid milk appears to be a potent new source of probiotic bacteria that can be used to reduce or prevent dental disease.

Keywords: dental, probiotic, caries, periodontitis, equid milk, *Lactobacillus*

INTRODUCTION

The most common diseases in the oral cavity caused by oral flora dysbiosis are periodontitis and caries (**Zhu *et al.*, 2018**). According to global burden of disease study in 2016, periodontal disease was affecting more than 750 million people, and caries affected 2.44 billion worldwide (**Vos *et al.*, 2017**). Apart from genetic, nutritional, and hygiene factors, multiple microorganisms play an important role in the development of such diseases. *Streptococcus mutans*, due to virulence factors and resulting imbalance between microbiota and host, greatly contribute to dental caries (**Wasfi *et al.*, 2018**). Periodontitis is classified as an inflammatory condition in the gingival tissue and can result in tooth loss. Periodontitis is caused by bacterial accumulation on the teeth around the gingival margin (**Alvarenga *et al.*, 2015**). Several microorganisms were found to play a role in periodontitis development. *Aggregatibacter actinomycetemcomitans* has been associated with several forms of periodontal disease (**Izano *et al.*, 2007**). *Porphyromonas gingivalis* causes chronic periodontitis through changes in commensal bacterial community resulting in dysbiosis (**Mei *et al.*, 2020**) and also could have an important role in the development of oral squamous cell carcinoma (**Lafuente Ibáñez de Mendoza *et al.*, 2020**). Additionally, if periodontal *Streptococcus intermedius* infection occurs, it could result in brain and liver abscesses (**Wagner *et al.*, 2006**).

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer health benefits on the host **(FAO/WHO, 2002**). Probiotic consumption has many documented health benefits such as immunomodulatory (**Frece *et al.*, 2009**), antioxidative **(Wu *et al.*, 2019**), anti-inflammatory and anticancerogenic (**Han *et al.*, 2015**) effects. Although most of the research focuses on probiotic effects on intestinal health (**Coqueiro *et al.*, 2018; George Kerry *et al.*, 2018; Li *et al.*, 2020**), several studies have shown the potential of probiotics in caries reduction (**Hedayati-Hajikand *et al.*, 2015; Stensson *et al.*, 2014**) and the maintenance of periodontal health **(Nguyen *et al.*, 2021**). As probiotic market is in constant growth (**Zucko *et al.*, 2020**), development of new probiotic formulas for oral health is strongly encouraged (**Amargianitakis *et al.*, 2021**). Most of the studies investigating probiotic associated caries reduction focused on probiotic enriched milk (**Juneja and Kakade, 2012; Näse *et al.*, 2001**), cheese (**Ahola *et al.*, 2002**), and fermented drinks (**Hu *et al.*, 2019**). Oral probiotic administration twice daily for 4 weeks yielded significant results in maintaining dental and periodontal health **(Zahradnik *et al.*, 2009**). Proposed probiotic activities in the oral cavity include antagonistic and aggregation activities in interaction with pathogens, and direct interaction with oral epithelium (**Mahasneh and Mahasneh, 2017**).Similarly to intestinal application, more studies are necessary to fully understand the mechanisms and impacts of dental probiotics, together with obtaining documented and investigated strains from new sources as probiotic characteristics are strain-specific (**Campana *et al.*, 2017**). Several randomized clinical trials have demonstrated the efficacy of probiotics in tablet form against oral pathogens, and the genus *Lactobacillus* appears to be the most commonly used along with *Streptococcus salivarius* (**Mahasneh & Mahasneh, 2017**). Potential of probiotic application for numerous health issues is unquestionable and World Health Organization (WHO) stated that probiotics will be an important tool in fighting against a range of noninfectious and infectious diseases instead of antibiotics that showed many negative effects in addition to rising resistance and occurring multiresistant strains (**Zommiti *et al.*, 2020**).

Equid milk (mare and donkey) was traditionally widely used and generally considered beneficial to health (**Salimei and Fantuz, 2012**). Recently, we studied *Lactobacillus* strains isolated from mare and donkey milk, and two isolates exhibited anti-inflammatory and probiotic potential (**Kostelac *et al.*, 2021**). In this study, we aimed to investigate the potential of isolates from equid milk for a protective role in caries and periodontitis. The ability of probiotic candidates to survive in saliva and to form biofilms was determined. Furthermore, we investigated the coaggregation and antimicrobial ability along with the potential biofilm obstruction against common caries and periodontitis causing microorganisms. The methods selected in this study served the dual purpose of selecting the necessary criteria for the first time, the potential of naturally present lactic acid bacteria in donkey and mare milk for use as dental probiotics.

MATERIALS AND METHODS

Bacterial strains and growth conditions

In this study, ten lactic acid bacteria (LAB) strains previously isolated from mare (*L. plantarum* KO1, *L. plantarum* KO2, *L. plantarum* KO3, *L. plantarum* KO4, *L. plantarum* KO5) and donkey (*L. plantarum* M1, *L. plantarum* M2, *L. plantarum* M3, *L. plantarum* M4, *L. paracasei* M5) milk (**Kostelac *et al.*, 2021**) were used. Strains were cultivated in de Man, Rogosa, and Sharp (MRS) broth (Biolife, Milan, Italy) at 37 °C for 24 – 48 h prior to the experiments.

Dental pathogens were obtained from the German Collection of Microorganisms and Cell Cultures GmbH as lyophilized cultures and were as follows: *Streptococcus intermedius* DSM-20573, *Streptococcus mutans* DSM-20523, *Porhyromonas gingivalis* DSM-28984, and *Aggregatibacter actinomycetemcomitans* DSM-11122. Streptococci were cultivated in M17 broth (Biolife) for 24 – 48 h. *P. gingivalis* was cultivated in Tryptic soy broth (Biolife)

anaerobically and *A. actinomycetemcomitans* was cultivated in Tryptic soy broth (Biolife) supplemented with 5 % fetal calf serum (Merck, Darmstadt, Germany) for 48 h at 37 °C.

Survival of LAB isolates in simulated saliva

After 24 h cultivation in MRS broth at 37 °C, LAB cells were harvested by centrifugation (Hermle, Gosheim, Germany) at 6000 rpm for 15 min, washed twice with 0.5 sterile saline, and resuspended in simulated saliva. All suspensions were calibrated at optical density (OD_{600}) of 0.25. Simulated saliva was prepared according to **Marques *et al.*, 2011**, designated as simulated saliva 1 (SS1). Viable cell count was determined after 24 h in simulated saliva and control (sterile deionized water) by pour plate method and CFU ml^{-1} was calculated.

Biofilm formation quantification of LAB isolates and dental pathogens

Biofilm formation ability was carried out according to **Gómez *et al.*, 2016** and modified by **Kostelac *et al.*, 2021**. Briefly, biofilm classifications were done after 48 h incubation in an appropriate growth medium in a 12-well polystyrene microtiter plate (Corning, Amsterdam, Netherlands). After washing, adherent cells stained with crystal violet were released and OD was measured at 595 nm using a spectrophotometer (UNICAM HELIOS E, Thermo Electron, Waltham, Massachusetts, USA). OD values were compared with the mean OD value of the negative control (ODC) and classified according to **Borges *et al.*, 2012** as non-biofilm producers (OD ≤ ODC), weak (ODC < OD ≤ 2 × ODC), moderate (2 × ODC < OD ≤ 4 × ODC), strong producers (4 × ODC < OD).
In order to assess the biofilm formation strength of tested pathogens in experimental conditions, biofilm formation quantification was done as described above using appropriate pathogen culture media.

Autoaggregation and coaggregation assay

After 24 – 48 h cultivation in appropriate media, bacterial cells were harvested by centrifugation, washed two times, and re-suspended in simulated saliva as described above. Autoaggregation ability of dental pathogens was determined as described in **Kos *et al.*, 2003** with modifications. Prior to measurement, OD values of bacterial suspensions were adjusted to 0.2 ± 0.05 to standardize the number of bacterial cells. Percentage of autoaggregation was expressed as (1):
Autoaggregation (%) = $1 - (A_t/A_0) \times 100$; (1)
where A_0 represents absorbance at 0 h and A_t represents absorbance at 24 h.
Coaggregation assay was done equivalent to autoaggregation assay with the exception of mixing equal volumes of LAB suspensions and suspensions of investigated dental pathogens. The coaggregation ratio (%) was calculated by comparing the absorbance of the pathogen suspension with the absorbance of the mixed suspensions.

Antimicrobial activity of LAB supernatants

The antimicrobial activity of equid milk LAB isolates was determined by a modified method of **Kostelac *et al.*, 2021**. LAB supernatant was extracted by centrifugation at 6000 rpm, 20 min, and filter sterilized (0.22 µm). After extraction, the supernatant was divided and one half was neutralized with 0.1 M NaOH. The experiment was set in 96-well microtiter plates and samples consisted of 200 µL of pathogen cultivation media and 40 µL tested LAB supernatant. Samples were inoculated with 10 µL of pathogen suspension. Controls were samples without the addition of LAB supernatant and blanks were uninoculated samples. Absorbance was measured at 0 h and 24 h at 620 nm on a microplate reader (Tecan, Sunrise, Männedorf, Switzerland). The experiment was done in quadruplicate. Inhibition was calculated as follows (2):
$\%_{inhibition} = (1 - A_1/A_0) \times 100$ (2)
where A_1 represents absorbance at 24 h and A_0 starting absorbance at 0 h.

Inhibition of biofilm formation measurement

Biofilm formation ability was determined for dental pathogens in the presence of undiluted, 3 times diluted, and 20 times diluted LAB supernatants. LAB supernatants were prepared as previously described. The experiment was set in the 12 well microtiter plates and samples consisted of 2 ml pathogen media, 400 µl of LAB supernatant, and inoculated with 100 µL pathogen suspension. Uninoculated sterile MRS was added in the controls instead of supernatants and uninoculated samples served as blanks. Biofilm quantification was done as described in the previous section. Biofilm formation ability was expressed as a percentage of control and experiments were conducted in quadruplicates in three independent experiments.

Hemolytic activity

The hemolytic activity of LAB isolates was determined according to **Halder *et al.*, 2017**. Investigated LAB strains were streaked on blood agar plates (Biolife) and incubated at 37 °C for 24 - 48 h. After incubation, plates were observed for the presence or absence of hemolytic zones around LAB colonies.

Statistical analysis

Experiments were conducted in triplicate. All values are expressed as means ± standard deviation (SD). Statistical differences between groups were determined using t-test. Statistical significance was set at $p \leq 0.05$ and all comparisons were done using STATISTICA 14.0.0.15 (Tibco, Palo Alto, USA).

RESULTS

Survival of LAB isolates in simulated saliva

Equid milk isolates displayed different survival rates in simulated saliva (Figure 1). The survival percentage span for all isolates was between 65 and 97%. Two strains from donkey (M1, M5) and two from mare milk (KO2, KO4) had a survival rate above 95% thus were selected for the next phase experiments.

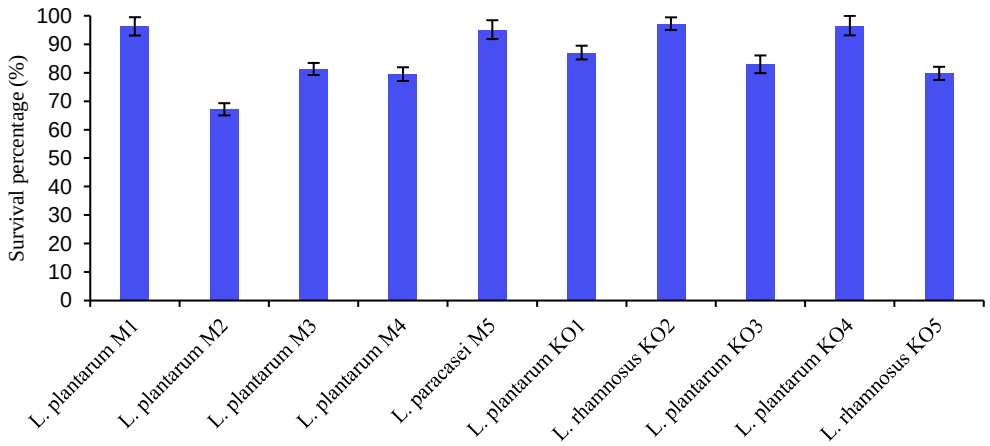


Figure 1 Survival of equid milk LAB isolates after 24 h exposure to simulated saliva conditions. *Results are expressed as a percentage of the control ± SD

Biofilm formation quantification of LAB isolates and dental pathogens

Results for the biofilm formation ability are presented in Table 1. All four tested *Lactobacillus* strains are classified as strong biofilm producers. Tested pathogen strains were also classified as strong biofilm producers (Table 2.)

Table 1 Biofilm ability classification of investigated lactic acid bacteria after 48 h cultivation

Tested LAB strain	OD_{540} value[a]	Classification[b]
Lactobacillus plantarum M1	0.921 ± 0.03	strong
Lactobacillus plantarum M5	0.878 ± 0.02	strong
Lactobacillus plantarum KO2	0.762 ± 0.02	strong
Lactobacillus plantarum KO4	0.955 ± 0.03	strong
ODC	0.091 ± 0.02	
4 × ODC	0.182 ± 0.02	

[a]Optical density values of LAB strains presented as quadruplicates ± SD. [b]Classification after comparison to OD cut off (ODC) and ODC × 4 according to (**Borges *et al.*, 2012**)

Autoaggregation and coaggregation assay

Autoaggregation of dental pathogens and coaggregation with investigated LAB strains are presented in Table 3. The presence of *L. plantarum* M1 resulted in a significant increase in aggregation for all tested pathogens. *L. plantarum* KO4 displayed an equivalent significant increase for *S. intermedius* and *S. mutans* however, in the mixture with *A. actinomycetemcomitans* and *P. gingivalis*, although present, the increase was not statistically significant. The opposite effect was noticed for *L. plantarum* KO2, where aggregation rate was decreased compared to pathogen control and was significant for *S. mutans* and *A. actinomycetemcomitans.* Since *L. plantarum* KO2 did not positively affect pathogen aggregation rate it was excluded from further experiments.

Table 2 Biofilm forming ability classification of selected dental pathogens

Tested pathogen strain	Result[a]	Classification[b]
Streptococcus intermedius DSM 20573	0.404 ± 0.02	strong
Streptococcus mutans DSM 20523	1.631 ± 0.01	strong
ODC	0.087 ± 0.02	
4 × ODC	0.348 ± 0.02	
Porhyromonas gingivalis DSM 28984	0.372 ± 0.06	strong
ODC	0.094 ± 0.03	
4 × ODC	0.376 ± 0.03	
Aggregatibacter actinomycetemcomitans	0.521 ± 0.04	strong
ODC	0.098 ± 0.03	
4 × ODC	0.392 ± 0.03	

[a]Optical density values of LAB strains presented as quadruplicates ± SD. [b]Classication after comparison to OD cutt off (ODC) and ODC × 4 according to Borges *et al.* (**Borges *et al.*, 2012**)

Table 3 Autoaggregation and coaggregation ability of tested dental pathogens and investigated lactic acid bacteria isolates after 24 h (co)incubation[a]

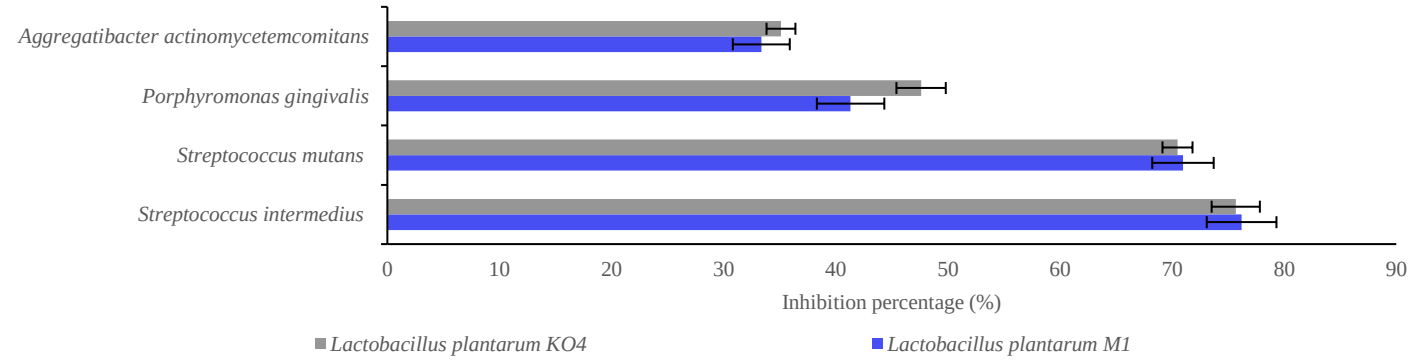
Pathogen	Autoaggregation	Coaggregation		
		L. plantarum M1	*L. plantarum* KO4	*L. plantarum* KO2
S. intermedius	52.16 ± 2.24	71.13 ± 2.11*	60.74 ± 2.03*	49.87 ± 3.11
S. mutans	44.28 ± 1.87	66.95 ± 2.43*	55.61 ± 3.47*	39.64 ± 1.74*
A. actinomycetemcomitans	63.67 ± 1.99	72.17 ± 3.31*	66.89 ± 2.32	46.34 ± 1.52*
P. gingivalis	43.11 ± 2.21	52.82 ± 1.91*	46.23 ± 3.12	41.21 ± 2.76

[a]Values (in %) are the mean percentage of triplicate experiments. *Significantly different from autoaggregation ($p<0.05$)

Antimicrobial activity of LAB supernatants

The antimicrobial activity of untreated LAB supernatants, expressed as an inhibitory percentage, is presented in figure 2A. Investigated LAB supernatants significantly inhibited the growth of dental pathogens. There was no detected difference between the inhibitory activity of the two *Lactobacillus* strains. Both isolates showed higher inhibition of streptococci than *A. actinomycetemcomitans* and *P. gingivalis.* After neutralization, inhibition was significantly reduced for both LAB strains (Figure 2B). Inhibitions under 10 % were observed for *P. gingivalis* in the presence of both LAB supernatants and *A. actinomycetemcomitans* (KO4 supernatant) and *S. intermedius* (M1 supernatant). The highest inhibition after neutralization was observed for *S. mutans* in the presence of *L. plantarum* KO4 supernatant exceeding 20%.

A



B

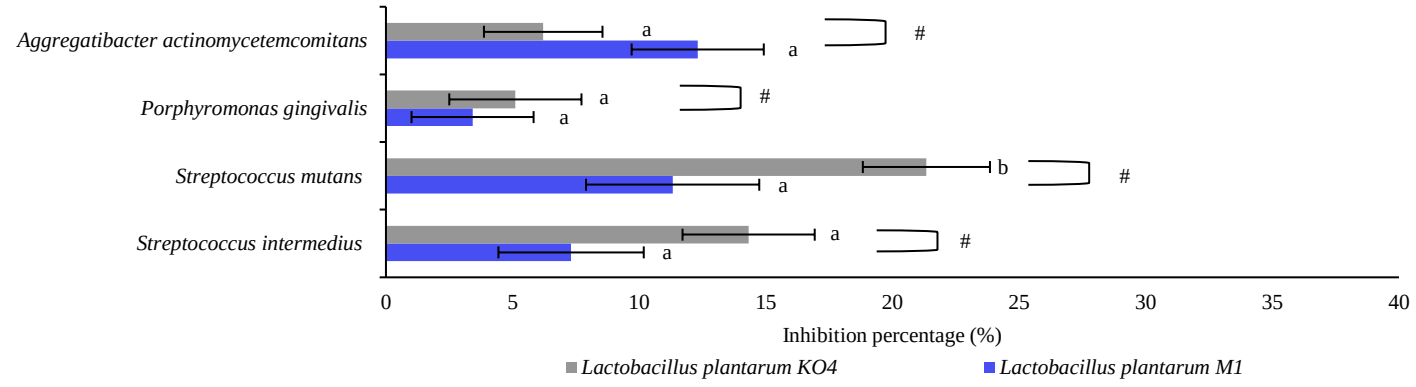


Figure 2 Antagonistic activity of A) acidic and B) neutralized lactic acid bacteria supernatants on dental pathogens after 24 h cultivation expressed as a percentage of the control sample ± SD. Results are expressed as a percentage of the control ± SD. [#]Statistically different from the acidic supernatant ($p \leq 0.05$). [a,b]Different letters indicate statistical difference between strains ($p \leq 0.05$)

Inhibition of biofilm formation measurement

Biofilm formation of three dental streptococci, *P. gingivalis*, and *A. actinomycemcomitans* was measured in the presence of different dilutions of tested LAB supernatants. Biofilm formation percentages are presented in Table 4. Given percentages are biofilm formations compared to controls. Both LAB supernatants inhibited biofilm formation of tested pathogens and since there is a statistical difference in the inhibition ability among dilutions, results point out to the concentration dependent inhibition. Drop of inhibition due to dilution is higher when *L. plantarum* KO4 was used.

Table 4 Biofilm forming ability[a] of selected dental pathogens the presence of different dilutions of untreated LAB supernatans.

	Lactobacillus plantarum M1		
	original	3x diluted	20x diluted
S. intermedius	83.38 ± 3.52	75.41 ± 3.45[*]	50.21 ± 2.78[*#]
S. mutans	64.83 ± 2.11	16.69 ± 1.32[*]	9.17 ± 1.57[*#]
A. actinomycetemcomitans	65.34 ± 4.52	15.78 ± 2.45[*]	4.34 ± 1.31[*#]
P. gingivalis	45.11 ± 3.72	12.17 ± 2.71[*]	2.65 ± 1.11[*#]
	Lactobacillus plantarum KO4		
	original	3x diluted	20x diluted
S. intermedius	80.81 ± 3.42	10.32 ± 1.54[*]	6.41 ± 2.12[*#]
S. mutans	43.61 ± 1.33	25.42 ± 2.42[*]	7.81 ± 1.14[*#]
A. actinomycetemcomitans	13.97 ± 2.74	3.98 ± 1.42[*]	1.65 ± 0.91[*#]
P. gingivalis	17.31 ± 0.91	4.54 ± 0.91[*]	2.35 ± 0.92[*#]

[a]Biofilm formation is expressed as a percentage of the untreated control ± SD; [*]statistically different from the original; [#]statistically different from the 3x diluted sample

Hemolytic activity

L. plantarum M1 and *L. plantarum* KO9 displayed no visible zones on blood agar thus exerting γ-hemolytic (no hemolytic) activity.

DISCUSSION

Most of the probiotic research focuses on the interactions of probiotics with gut flora and the beneficial effects that result from such interactions. The role of probiotics in oral health received much less attention compared to gut health (**Chen *et al.*, 2020**). The purpose of the present study was to investigate a new probiotic source for its potential in dentooral protection. Ten isolates from equid milk were investigated, and their probiotic characterization was conducted to select the strains with the highest potential. Since the probiotics are intended for oral use, they must withstand the conditions oat the desired site of action. Probiotics for oral protection should have a high survival rate in saliva. Our results show that the tested equid milk isolates survive well when exposes to simulated saliva. However, since many probiotic beneficial effects are strain dependant (**Ishikawa *et al.*, 2021**) we excluded all strains that showed a reduction greater than 5% in viable number in order to select the most potent probiotic strains. Tolerance to saliva conditions is not surprising for LAB strains however, differences among strains were observed previously in the study of **Haukioja *et al.* (2006)** where several *Lactobacillus* and *Bifidobacterium* strains survived well during 24 h in saliva and differences among strains were observed through CFU reduction. Based on the aforementioned, two mare milk (KO2 and KO4) and two donkey milk (M1 and M5) isolates were selected for further characterization. Apart from surviving, the ability to adhere to the target site is also important as it allows exertion of beneficial effects and competitive exclusion of pathogens on site. The ability to colonize and to adapt to different stresses is correlated to biofilm formation and it is considered a beneficial property as it promotes colonization (**Terraf *et al.*, 2012**). In this study, tested LAB strains were classified as strong biofilm producers thus satisfying probiotic criteria. Although such results point to colonization potential, it is very important to understand that previously conducted *in vivo* studies found only transient colonization in the oral cavity, stating that probiotic bacteria display lower competitiveness in a weakly acidic environment (**Ravn *et al.*, 2012**). Such transient colonization during probiotic therapy reduces possible negative effects of probiotic acididification and lowers probiotic risks for oral health (**Söderling, 2012**). The most studied probiotic *Lactobacillus rhamnosus* GG (LGG) showed only temporary presence after the probiotic treatment and was gradually reduced **(Yli-Knuuttila *et al.*, 2006**) so permanent colonization seems improbable. Despite that, several studies indicate beneficial effects of LGG in caries reduction (**Nase *et al.*, 2001**) and improvement of periodontal health (**Toiviainen *et al.*, 2015**). The exact role of mentioned transient colonization in the improvement of dental health should be further investigated. During therapy, probiotics are present in the oral cavity and can interact with present pathogens. Probiotics can modulate pathogenic biofilms and exert inhibitory activity through coaggregation **(Chugh *et al.*, 2020**). In our study, donkey milk isolate *L. plantarum* M1 showed the highest coaggregation rates with dental pathogens during 24 h treatment. A significant increase in aggregation compared to control indicates desired interactions. As expected, the ability is strain-dependent and also depends on the investigated pathogen. Coaggregation of commercial probiotics was measured for cariogenic *S. mutans* in the study of **Twetman *et al.* (2009)** where maximum coaggregation after 24 h was observed for *Lactobacillus acidophilus* CCUG 5917 and *S. mutans* GS-5 reaching 70% and also noticing a difference between coaggregation of several probiotic strains and oral streptococci. Coaggregation of lactobacilli against *P. gingivalis* and *A. actinomycetemcomitans* was also measured by **Samot *et al.* (2017)**. Lactobacilli used in their study displayed low coaggregation rates against two mentioned pathogens with a maximum of 4.5% for *P. gingivalis*. Based on our results, we selected two strains (M1 and KO4) for further characterization as they displayed the highest coaggregation rates with all tested pathogens and *L. plantarum* KO2 was excluded from further experiments due to reduced aggregation in suspension with pathogens. Supernatants of selected LAB strains were further tested for antimicrobial activity. All pathogens were inhibited in the presence of acidic supernatant, however, tested streptococci seem to be more susceptible than *P. gingivalis* and *A. actinomycetemcomitans* where inhibition was around 30% lower for both LAB strains. Neutralization of supernatants greatly reduced inhibition rates. Previous studies on probiotic effects on foodborne pathogens are connected to pH reduction due to lactic acid production (**Bungenstock *et al.*, 2020**). However, an investigation of *L. reuterii* effects on dental pathogens concluded that antimicrobial activity may not be due to lactic acid only (**Yang *et al.*, 2021**). Our results confirm that since the inhibitory activity was still present after neutralization with significant over 20% inhibition of cariogenic *S. mutans* in the presence of *L. plantarum* KO4 supernatant and around 12 % for *A. actinomycetemcomitans* in the presence of *L. plantarum* M1. Further research on equid milk isolates and the composition of their active supernatants should be performed. Furthermore, with the assumption of transient colonization discussed above, we wanted to assess the effects of untreated LAB supernatants on biofilm formation of dental pathogens, firstly we measured untreated biofilm formation of pathogen strains. All tested pathogens formed strong biofilms in measured conditions so we could further investigate the effect of LAB supernatants on biofilm formation. Biofilm formation inhibition was present for both probiotic candidates and it was concentration dependant as dilutions of supernatants reduced the inhibitory activity. Based on previously measured antimicrobial activity, inhibition could be the result of such activities against pathogens and could be result of acidification, coaggregation and posibble other inhibitory molecules. Recent study showed high biofilm inhibiton of *S. mutans* in the presence of probiotic bacteria isolated from traditional Sichuan pickles (**Chugh *et al.*, 2020**). Interactions between probiotics and dental pathogens are not fully investigated but it can be assumed that they are strain dependant (probiotic and pathogen). **Ishikawa *et al.* 2020** detected probiotic reduction of *P. gingivalis* in biofilms proposing mode of action through regulation of transcriptional and virulence associated factors. Furthermore, colonization ability of *A. actinomycemcomitans* was shown to be reduced by lactobacilli postbiotics (**Ishikawa *et al.*, 2021**).

CONCLUSION

Based on our results, we can conclude that two isolates from equid milk, *L. plantarum* M1 and *L. plantarum* KO4, meet the tested probiotic criteria for dentooral protection. Further studies on the mode of action and *in vivo* experiments are needed to better understand the interaction of probiotics with the host microbiome. Based on present findings, probiotics could be used for prevention but further research is needed to evaluate them as oral therapeutics. The safety prerequisites are met as none of the LAB strains displayed hemolytic activity. Although the milk of equid has long been known for its health benefits, it appears

to be a great potential source of probiotic bacteria whose health applications have not yet been adequately studied.

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REFERENCES

Ahola, A. J., Yli-Knuuttila, H., Suomalainen, T., Poussa, T., Ahlström, A., Meurman, J. H., & Korpela, R. (2002). Short-term consumption of probiotic-containing cheese and its effect on dental caries risk factors. *Archives of Oral Biology*, *47*(11), 799–804. https://doi.org/10.1016/S0003-9969(02)00112-7

Alvarenga, L. H., Prates, R. A., Yoshimura, T. M., Kato, I. T., Suzuki, L. C., Ribeiro, M. S., Ferreira, L. R., Pereira, S. A. dos S., Martinez, E. F., & Saba-Chujfi, E. (2015). *Aggregatibacter actinomycetemcomitans* biofilm can be inactivated by methylene blue-mediated photodynamic therapy. *Photodiagnosis and Photodynamic Therapy*, *12*(1), 131–135. https://doi.org/10.1016/j.pdpdt.2014.10.002

Amargianitakis, M., Antoniadou, M., Rahiotis, C., & Varzakas, T. (2021). Probiotics, prebiotics, synbiotics and dental caries. New perspectives, suggestions, and patient coaching approach for a cavity-free mouth. *Applied Sciences*, *11*(12), 5472. https://doi.org/10.3390/app11125472

Borges, S., Silva, J., & Teixeira, P. (2012). Survival and biofilm formation by Group B streptococci in simulated vaginal fluid at different pHs. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, *101*(3), 677–682. https://doi.org/10.1007/s10482-011-9666-y

Bungenstock, L., Abdulmawjood, A., & Reich, F. (2020). Evaluation of antibacterial properties of lactic acid bacteria from traditionally and industrially produced fermented sausages from Germany. *PLOS ONE*, *15*(3), e0230345. https://doi.org/10.1371/JOURNAL.PONE.0230345

Campana, R., Van Hemert, S., & Baffone, W. (2017). Strain-specific probiotic properties of lactic acid bacteria and their interference with human intestinal pathogens invasion. *Gut Pathogens*, *9*(1), 1–12. https://doi.org/10.1186/s13099-017-0162-4

Chen, Y. T., Hsieh, P. S., Ho, H. H., Hsieh, S. H., Kuo, Y. W., Yang, S. F., & Lin, C. W. (2020). Antibacterial activity of viable and heat-killed probiotic strains against oral pathogens. *Letters in Applied Microbiology*, *70*(4), 310–317. https://doi.org/10.1111/lam.13275

Chugh, P., Dutt, R., Sharma, A., Bhagat, N., & Dhar, M. S. (2020). A critical appraisal of the effects of probiotics on oral health. *Journal of Functional Foods*, *70*, 103985. https://doi.org/10.1016/j.jff.2020.103985

Coqueiro, A. Y., Bonvini, A., Raizel, R., Tirapegui, J., & Rogero, M. M. (2018). Probiotic supplementation in dental caries: is it possible to replace conventional treatment? *Nutrire*, *43*(1), 1–9. https://doi.org/10.1186/s41110-018-0064-3

FAO/WHO (2002) Guidelines for the Evaluation of Probiotics in Food. Food and Agriculture Organization of the United Nations/World Health Organization, London, Ontario.

Frece, J., Kos, B., Svetec, I. K., Zgaga, Z., Beganović, J., Leboš, A., & Šušković, J. (2009). Synbiotic effect of *Lactobacillus helveticus* M92 and prebiotics on the intestinal microflora and immune system of mice. *Journal of Dairy Research*, *76*(1), 98–104. https://doi.org/10.1017/S0022029908003737

George Kerry, R., Patra, J. K., Gouda, S., Park, Y., Shin, H. S., & Das, G. (2018). Benefaction of probiotics for human health: A review. *Journal of Food and Drug Analysis*, *26*(3), 927–939. https://doi.org/10.1016/j.jfda.2018.01.002

Gómez, N. C., Ramiro, J. M. P., Quecan, B. X. V., & Franco, B. D. G. de M. (2016). Use of Potential Probiotic Lactic Acid Bacteria (LAB) Biofilms for the Control of *Listeria monocytogenes*, *Salmonella Typhimurium*, and *Escherichia coli* O157:H7 Biofilms Formation. *Frontiers in Microbiology*, *7*, 863. https://doi.org/10.3389/FMICB.2016.00863

Halder, D., Mandal, M., Chatterjee, S. S., Pal, N. K., & Mandal, S. (2017). Indigenous probiotic *Lactobacillus* isolates presenting antibiotic like activity against human pathogenic bacteria. *Biomedicines 2017, Vol. 5, Page 31*, *5*(2), 31. https://doi.org/10.3390/BIOMEDICINES5020031

Han, K. J., Lee, N. K., Park, H., & Paik, H. D. (2015). Anticancer and anti-inflammatory activity of probiotic *Lactococcus lactis* NK34. *Journal of Microbiology and Biotechnology*, *25*(10), 1697–1701. https://doi.org/10.4014/jmb.1503.03033

Haukioja, A., Yli-Knuuttila, H., Loimaranta, V., Kari, K., Ouwehand, A. C., Meurman, J. H., & Tenovuo, J. (2006). Oral adhesion and survival of probiotic and other lactobacilli and bifidobacteria *in vitro*. *Oral Microbiology and Immunology*, *21*(5), 326–332. https://doi.org/10.1111/j.1399-302X.2006.00299.x

Hedayati-Hajikand, T., Lundberg, U., Eldh, C., & Twetman, S. (2015). Effect of probiotic chewing tablets on early childhood caries - a randomized controlled trial. *BMC Oral Health*, *15*(1), 1–5. https://doi.org/10.1186/s12903-015-0096-5

Hu, X., Huang, Z., Zhang, Y., Hong, Y., & Zheng, Y. (2019). Effects of a probiotic drink containing *Lactobacillus casei* strain Shirota on dental plaque microbiota. *Journal of International Medical Research*, *47*(7), 3190–3202. https://doi.org/10.1177/0300060519853655

Ishikawa, K. H., Bueno, M. R., Kawamoto, D., Simionato, M. R. L., & Mayer, M. P. A. (2021). Lactobacilli postbiotics reduce biofilm formation and alter transcription of virulence genes of *Aggregatibacter actinomycetemcomitans*. *Molecular Oral Microbiology*, *36*(1), 92–102. https://doi.org/10.1111/omi.12330

Ishikawa, K. H., Mita, D., Kawamoto, D., Nicoli, J. R., Albuquerque-Souza, E., Lorenzetti Simionato, M. R., & Mayer, M. P. A. (2020). Probiotics alter biofilm formation and the transcription of *Porphyromonas gingivalis* virulence-associated genes. *Journal of Oral Microbiology*, *12*(1), 1805553. https://doi.org/10.1080/20002297.2020.1805553

Izano, E. A., Wang, H., Ragunath, C., Ramasubbu, N., & Kaplan, J. B. (2007). Detachment and killing of *Aggregatibacter actinomycetemcomitans* biofilms by dispersin B and SDS. *Journal of Dental Research*, *86*(7), 618–622. https://doi.org/10.1177/154405910708600707

Juneja, A., & Kakade, A. (2012). Evaluating the effect of probiotic containing milk on salivary mutans streptococci levels. *Journal of Clinical Pediatric Dentistry*, *37*(1), 9–14. https://doi.org/10.17796/jcpd.37.1.tq91178m7w876644

Kos, B., Šušković, J., Vuković, S., Šimpraga, M., Frece, J., & Matošić, S. (2003). Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. *Journal of Applied Microbiology*, *94*(6), 981–987. https://doi.org/10.1046/j.1365-2672.2003.01915.x

Kostelac, D., Gerić, M., Gajski, G., Markov, K., Domijan, A. M., Čanak, I., Jakopović, Ž., Svetec, I. K., Žunar, B., & Frece, J. (2021). Lactic acid bacteria isolated from equid milk and their extracellular metabolites show great probiotic properties and anti-inflammatory potential. *International Dairy Journal*, *112*, 104828. https://doi.org/10.1016/j.idairyj.2020.104828

Lafuente Ibáñez de Mendoza, I., Maritxalar Mendia, X., García de la Fuente, A. M., Quindós Andrés, G., & Aguirre Urizar, J. M. (2020). Role of *Porphyromonas gingivalis* in oral squamous cell carcinoma development: A systematic review. *Journal of Periodontal Research*, *55*(1), 13–22. https://doi.org/10.1111/jre.12691

Li, C., Niu, Z., Zou, M., Liu, S., Wang, M., Gu, X., Lu, H., Tian, H., & Jha, R. (2020). Probiotics, prebiotics, and synbiotics regulate the intestinal microbiota differentially and restore the relative abundance of specific gut microorganisms. *Journal of Dairy Science*, *103*(7), 5816–5829. https://doi.org/10.3168/jds.2019-18003

Mahasneh, S. A., & Mahasneh, A. M. (2017). Probiotics: A promising role in dental health. *Dentistry Journal*, *5*(4), 1–10. https://doi.org/10.3390/dj5040026

Marques, M. R. C., Loebenberg, R., & Almukainzi, M. (2011). Simulated Biological Fluids with Possible Application in Dissolution Testing. *Dissolution Technologies*, *18*(3), 15–28. https://doi.org/10.14227/DT180311P15

Mei, F., Xie, M., Huang, X., Long, Y., Lu, X., Wang, X., & Chen, L. (2020). *Porphyromonas gingivalis* and its systemic impact: Current status. *Pathogens*, *9*(11), 1–23. https://doi.org/10.3390/pathogens9110944

Nase, L., Hatakka, K., Savilahti, E., Saxelin, M., Pönkä, A., Poussa, T., Korpela, R., & Meurman, J. H. (2001). Effect of Long-Term Consumption of a Probiotic Bacterium, *Lactobacillus rhamnosus* GG, in Milk on Dental Caries and Caries Risk in Children. *Caries Research*, *35*(6), 412–420. https://doi.org/10.1159/000047484

Näse, L., Hatakka, K., Savilahti, E., Saxelin, M., Pönkä, A., Poussa, T., Korpela, R., & Meurman, J. H. (2001). Effect of Long–Term Consumption of a Probiotic Bacterium, *Lactobacillus rhamnosus* GG, in Milk on Dental Caries and Caries Risk in Children. *Caries Research*, *35*(6), 412–420. https://doi.org/10.1159/000047484

Nguyen, T., Brody, H., Radaic, A., & Kapila, Y. (2021). Probiotics for periodontal health—Current molecular findings. *Periodontology 2000*, *87*(1), 254–267. https://doi.org/10.1111/prd.12382

Ravn, I., Dige, I., Meyer, R. L., & Nyvad, B. (2012). Colonization of the oral cavity by probiotic bacteria. *Caries Research*, *46*(2), 107–112. https://doi.org/10.1159/000336960

Salimei, E., & Fantuz, F. (2012). Equid milk for human consumption. *International Dairy Journal*, *24*(2), 130–142. https://doi.org/10.1016/j.idairyj.2011.11.008

Samot, J., Belkhelfa, H., Haddioui, L., & Badet, C. (2017). Probiotic Properties of Lactobacilli that could be Used Against Periodontitis. *Journal of Probiotics & Health*, *05*(03). https://doi.org/10.4172/2329-8901.1000180

Söderling, E. (2012). Probiotics and dental caries. *Microbial Ecology in Health & Disease*, *23*(0), 55–56. https://doi.org/10.3402/mehd.v23i0.18582

Stensson, M., Koch, G., Coric, S., Abrahamsson, T. R., Jenmalm, M. C., Birkhed, D., & Wendt, L. K. (2014). Oral administration of *Lactobacillus reuteri* during the first year of life reduces caries prevalence in the primary dentition at 9 years of age. *Caries Research*, *48*(2), 111–117. https://doi.org/10.1159/000354412

Terraf, M. C. L., Tomás, M. S. J., Nader-Macías, M. E. F., & Silva, C. (2012). Screening of biofilm formation by beneficial vaginal lactobacilli and influence of culture media components. *Journal of Applied Microbiology*, *113*(6), 1517–1529. https://doi.org/10.1111/J.1365-2672.2012.05429.X

Toiviainen, A., Jalasvuori, H., Lahti, E., Gursoy, U., Salminen, S., Fontana, M., Flannagan, S., Eckert, G., Kokaras, A., Paster, B., & Söderling, E. (2015). Impact of orally administered lozenges with *Lactobacillus rhamnosus* GG and *Bifidobacterium animalis* subsp. *lactis* BB-12 on the number of salivary mutans streptococci, amount of plaque, gingival inflammation and the oral microbiome in healthy adults. *Clinical Oral Investigations*, *19*(1), 77–83. https://doi.org/10.1007/s00784-014-1221-6

Twetman, L., Larsen, U., Fiehn, N. E., Steckésn-Blicks, C., & Twetman, S. (2009). Coaggregation between probiotic bacteria and caries-associated strains: An *in vitro* study. *Acta Odontologica Scandinavica*, *67*(5), 284–288. https://doi.org/10.1080/00016350902984237

Vos, T., Abajobir, A. A., Abate, K. H., Abbafati, C., Abbas, K. M., Abd-Allah, F., ... & Criqui, M. H. (2017). Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. *The Lancet*, 390(10100), 1211-1259. https://doi.org/10.1016/S0140-6736(17)32154-2

Wagner, K. W., Schön, R., Schumacher, M., Schmelzeisen, R., & Schulze, D. (2006). Case report: brain and liver abscesses caused by oral infection with *Streptococcus intermedius*. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology*, *102*(4), 2005–2007. https://doi.org/10.1016/j.tripleo.2006.02.010

Wasfi, R., Abd El-Rahman, O. A., Zafer, M. M., & Ashour, H. M. (2018). Probiotic *Lactobacillus* sp. inhibit growth, biofilm formation and gene expression of caries-inducing *Streptococcus mutans*. *Journal of Cellular and Molecular Medicine*, *22*(3), 1972–1983. https://doi.org/10.1111/jcmm.13496

Wu, Y., Wang, B., Zeng, Z., Liu, R., Tang, L., Gong, L., & Li, W. (2019). Effects of probiotics *Lactobacillus plantarum* 16 and *Paenibacillus polymyxa* 10 on intestinal barrier function, antioxidative capacity, apoptosis, immune response, and biochemical parameters in broilers. *Poultry Science*, *98*(10), 5028–5039. https://doi.org/10.3382/ps/pez226

Yang, K. M., Kim, J.-S., Kim, H.-S., Kim, Y.-Y., Oh, J.-K., Jung, H.-W., Park, D.-S., & Bae, K.-H. (2021). *Lactobacillus reuteri* AN417 cell-free culture supernatant as a novel antibacterial agent targeting oral pathogenic bacteria. *Scientific Reports 2021 11:1*, *11*(1), 1–16. https://doi.org/10.1038/s41598-020-80921-x

Yli-Knuuttila, H., Snäll, J., Kari, K., & Meurman, J. H. (2006). Colonization of *Lactobacillus rhamnosus* GG in the oral cavity. *Oral Microbiology and Immunology*, *21*(2), 129–131. https://doi.org/10.1111/j.1399-302X.2006.00258.x

Zahradnik, R. T., Magnusson, I., Walker, C., McDonell, E., Hillman, C. H., & Hillman, J. D. (2009). Preliminary assessment of safety and effectiveness in humans of ProBiora3™ a probiotic mouthwash. *Journal of Applied Microbiology*, *107*(2), 682–690. https://doi.org/10.1111/j.1365-2672.2009.04243.x

Zhu, B., Macleod, L. C., Kitten, T., & Xu, P. (2018). *Streptococcus sanguinis* biofilm formation & interaction with oral pathogens. *Future Microbiology*, *13*(8), 915–932. https://doi.org/10.2217/fmb-2018-0043

Zommiti, M., Feuilloley, M. G. J., & Connil, N. (2020). Update of probiotics in human world: A nonstop source of benefactions till the end of time. *Microorganisms*, *8*(12), 1–33. https://doi.org/10.3390/microorganisms8121907

Zucko, J., Starcevic, A., Diminic, J., Oros, D., Mortazavian, A. M., & Putnik, P. (2020). Probiotic – friend or foe? *Current Opinion in Food Science*, *32*, 45–49. https://doi.org/10.1016/J.COFS.2020.01.007