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 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 (Wassil 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; Nise et al., 2001), cheese (Ahola et al., 2002), and fermented drinks (Hsio et al., 2019). Oral probiotic administration twice daily for 4 weeks yielded significant results in maintaining dental and periodontal health (Zahiradnik 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 multisistant resistant strains (Zommitti 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 used as follows: Streptococcus intermedius DSM-20573, Streptococcus mutans DSM-20525, Porphyromonas gingivalis DSM-28984, and Aggregatibacter actinomycetemcomitans DSM-11122. Streptococci were cultivated in M17 broth (DSM), Streptococcus mutans in M17 containing 0.05% CaCl2, Porphyromonas gingivalis in Tryptic Soy Bile 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, Goshen, 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) 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):

\[ \text{Autoaggregation} \% = \left(1 - \frac{A \text{abs}_{24 h}}{A \text{abs}_{0 h}}\right) \times 100 \]  

(1)

where \( A_{abs} \) represents absorbance at 0 h and \( A_{abs} \) 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 (\( c \)) 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):

\[ \% \text{Inhibition} = \left(1 - \frac{A_{abs \text{sup}}}{A_{abs \text{control}}}\right) \times 100 \]  

(2)

where \( A_{abs} \) represents absorbance at 24 h and \( A_{abs} \) 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](image_url)

**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>
<thead>
<tr>
<th>Tested LAB strain</th>
<th>OD450 value*</th>
<th>Classificationa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus plantarum M1</td>
<td>0.921 ± 0.03</td>
<td>strong</td>
</tr>
<tr>
<td>Lactobacillus plantarum M5</td>
<td>0.878 ± 0.02</td>
<td>strong</td>
</tr>
<tr>
<td>Lactobacillus plantarum KO2</td>
<td>0.762 ± 0.02</td>
<td>strong</td>
</tr>
<tr>
<td>Lactobacillus plantarum KO4</td>
<td>0.955 ± 0.03</td>
<td>strong</td>
</tr>
<tr>
<td>ODC</td>
<td>0.091 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>4 × ODC</td>
<td>0.182 ± 0.02</td>
<td></td>
</tr>
</tbody>
</table>

*Optical density values of LAB strains presented as quadruplicates ± SD. aClassification 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 3 Autoaggregation and coaggregation ability of tested dental pathogens and investigated lactic acid bacteria isolates after 24 h (co)incubation

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Autoaggregation</th>
<th>Coaggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L. plantarum M1</td>
<td>L. plantarum KO4</td>
</tr>
<tr>
<td>S. intermedius</td>
<td>52.16 ± 2.24</td>
<td>71.13 ± 2.11*</td>
</tr>
<tr>
<td>S. mutans</td>
<td>44.28 ± 1.87</td>
<td>66.95 ± 2.43*</td>
</tr>
<tr>
<td>A. actinomycetemcomitans</td>
<td>63.67 ± 1.99</td>
<td>72.17 ± 3.31*</td>
</tr>
<tr>
<td>P. gingivalis</td>
<td>43.11 ± 2.21</td>
<td>52.82 ± 1.91*</td>
</tr>
</tbody>
</table>

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%.

![Graphs A and B showing antimicrobial activity and biofilm formation quantification results.](image-url)
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 out 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 exposed to simulated saliva. However, since many probiotic beneficial effects are strain dependent (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 CPU reduction. Based on the aforementioned, two mare milk (K02 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 (Terrad 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 acidification 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-Knuutila 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 selected probiotic 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. actinomyctecomitan* 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 acidificant supernatant, however, tested streptococci seem to be more susceptible than *P. gingivalis* and *A. actinomycctecomitan* where inhibition was around 50% 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. reuteri* 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. actinomyctecomitan* 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 pathogenic 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 and pathogenic strains 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 possible other inhibitory molecules. Recent study showed high biofilm inhibition 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. actinomyctecomitan* 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 not 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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