Lactobacilli Interfere with *Streptococcus pyogenes* Hemolytic Activity and Adherence to Host Epithelial Cells

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**Keywords:** *Streptococcus pyogenes*, *Lactobacillus*, streptolysin, microbiota, adherence

**INTRODUCTION**

A range of bacterial communities including commensals and pathogens colonize the nasopharynx. The pharyngeal inhabitant *Streptococcus pyogenes* [group A streptococcus (GAS)], an adapted human pathogen, a common colonizer of the mucosa of the mouth, nose, and pharynx, is among the many pathogens that most often colonize their host asymptomatically and only occasionally cause disease. Local pharyngeal infection with GAS manifests as pharyngitis and, if spread from the local site, can cause the systemic diseases sepsis, streptococcal toxic shock syndrome, and necrotizing fasciitis (Luca-Harari et al., 2009). *S. pyogenes* produces a wide array of virulence factors, enabling it to adhere, invade, and spread within the human host (Cunningham, 2008). One of the characteristic features of *S. pyogenes* is the ability to lyse red blood cells (RBC), referred to as...
β-hemolysis. *S. pyogenes* produces two different hemolysins/streptolysins: streptolysin S (SLS) and streptolysin O. The reason why *S. pyogenes* sometimes causes disease is not entirely understood, but both bacterial virulence factors and host factors are thought to contribute (Cole et al., 2011). The microbiota is one such host factor that needs further investigation. Attachment to epithelial cells is the crucial initial step of colonization because non-adherent GAS is removed by mucus and saliva flow. Additionally, bacterial interaction with the epithelial cells elicits multiple responses in the host cells, including cell signaling events, and modification of the host cell transcriptome (Nakagawa et al., 2004). The host responses regulate the bacterial colonization and play a significant role in the pathogenesis of the infection (Ribet and Cossart, 2015).

The microbiota prevents colonization with pathogenic bacteria and represents an important first line of defense. Mechanisms describing the probiotic effects of *Lactobacillus* strains include upregulation of mucin production in the host cells, interference with host pattern recognition receptors, competition for essential metabolites, production of antibacterial molecules, and co-aggregation between the bacteria of the microbiota and invading pathogenic bacteria, leading to interference with pathogen adherence to host cells (Lebeer et al., 2010; Reid et al., 2011; Lievin-Le Moal and Servin, 2014). *Lactobacillus* species are known to play a significant role in protection against many gastrointestinal and urogenital pathogens (Osset et al., 2001; Ostad et al., 2009). However, less is known about their antagonistic capacity against oral-pharyngeal pathogens. Additionally, the detailed mechanisms behind the anti-adhesive properties of lactobacilli and their effect on the expression of virulence-associated genes are far from fully understood.

Different species of *Lactobacillus* are part of the microbiota of the mucosal membranes in the pharyngeal tract (Badet and Thebaud, 2008). The study, therefore, aimed to investigate whether *Lactobacillus* strains interfere with the expression of the virulence-associated factors of *S. pyogenes* and its ability to adhere to pharyngeal epithelial cells.

**MATERIALS AND METHODS**

**Bacterial Strains and Growth Conditions**

All *Lactobacillus* strains were isolated from healthy human individuals. *Lactobacillus rhamnosus* Kx151A1, isolated from a gastric biopsy, has been described previously (Roos et al., 2005). The *Lactobacillus salivarius* LMG9477, a type strain from the Belgian Coordinated Collections of Micro-organisms (BCCM), and *Lactobacillus reuteri* ATCC: PTA-5289, a kind gift from BioGaia AB, Stockholm were isolated from the oral cavity and have been described previously (de Klerk et al., 2016). Lactobacilli were grown on Rogosa agar plates and cultured in and have been described previously (de Klerk et al., 2016). *Lactobacillus reuteri* Kx151A1, isolated from a gastric biopsy, has been described previously (Roos et al., 2005). *Lactobacillus rhamnosus* is a species known to play a significant role in protection against many gastrointestinal and urogenital pathogens (Osset et al., 2001; Ostad et al., 2009). However, less is known about their antagonistic capacity against oral-pharyngeal pathogens. Additionally, the detailed mechanisms behind the anti-adhesive properties of lactobacilli and their effect on the expression of virulence-associated genes are far from fully understood.

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**Cell Lines and Culture Conditions**

The pharyngeal epithelial cell lines FaDu (ATCC: HTB-43), and Detroit 562 (ATCC: CCL-138) were cultured in DMEM with GlutaMAX and pyruvate (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Sigma–Aldrich, St. Louis, MO, USA). The cells were maintained at 37°C and 5% CO₂ in a humidified environment. In all experiments, epithelial cells were seeded into cell culture plates the day before experiments to reach 90% confluence. The cell medium was replaced with serum-free DMEM half an hour before the experiments.

**Conditioned Media (CM)**

Lactobacilli were grown on Rogosa agar plates. The bacteria were scraped off, washed twice with Todd–Hewitt broth (THB), and resuspended in THB. From this suspension, the growth of lactobacilli was initiated by adjusting to A₆₀₀ ≈ 0.1 in THB. The growth was monitored until the density reached A₆₀₀ ≈ 1.0. The lactobacilli were removed by centrifugation. The supernatant was passed through a 0.2 μm filter, adjusted to pH 7.8 and supplemented with 2% tryptone to obtain the Conditioned Media (CM, Saroj and Rather, 2013).

**Hemolytic Activity**

Group A streptococcus was cultured in THB, co-incubated with lactobacilli, or in CM. For co-incubation, GAS and lactobacilli from agar plates were suspended in THB at A₆₀₀ = 0.1 in the proportion of 1:1. At the desired time point, the bacteria were harvested by centrifugation; the supernatants were passed through a 0.2 μm filter and used for the hemolytic assay. RBCs for hemolytic assays were prepared from horse blood (Håtunalab AB, defibrinated) by washing three times in phosphate buffered saline (PBS) and diluting to 2% in PBS. The supernatants were diluted 1:10, mixed 1:1 with RBCs and incubated at 37°C in 5% CO₂ for 1 h. The RBCs were removed by centrifugation at 2000 g for 3 min, and the supernatant was analyzed at A₄₀₄ for hemolysis. Hemolytic activity was reported as the percentage relative to water alone. The SLS-mediated hemolytic activity was confirmed using trypan blue and cholesterol binding assays as SLS gets inhibited in the presence of trypan blue and SLO by cholesterol (Sierig et al., 2003). For all assays, the hemolytic activity was examined in the presence of trypan blue and cholesterol. The hemolytic activity of GAS in lactobacilli supernatants was measured by harvesting the supernatants at 1 h intervals until the growth reached the stationary phase. For hemolytic activity of GAS supernatant in co-incubation with CM, supernatant from the growth of GAS in THB from A₆₀₀ ≈ 0.1 to A₆₀₀ ≈ 1.0 was obtained. The supernatant was mixed with THB, or with CM from L. salivarius (L.sa), L. rhamnosus (L.rh), or L. reuteri (L.re) in a proportion of 1:1 and incubated for 30 min at 37°C with 5% CO₂ prior to measuring the hemolytic activity.
**Viable Count**

To assess the viability of GAS, viable count was performed by serial dilutions and plating on GC agar. In co-incubation experiments, the bacteria could be distinguished by colony size; GAS formed colonies larger than lactobacilli.

**Quantitative PCR Assays**

Group A streptococcus was grown alone or co-incubated with lactobacilli in THB or in CM to an MOI of 100. The cells were harvested by centrifugation and total RNA was isolated using an RNeasy mini kit (Qiagen) after treatment with mutanolysin. Total RNA 200 ng was reverse transcribed to cDNA using SuperScript VILO master mix (ThermoFisher Scientific). The primers sagA-RT-(F) 5′GCTACTAGTGCTGAAACAACCTCAA-3′ and sagA-RT-(R) 5′AGCAACGTTAAGGCAAAGT3′ were used for the qPCR. The following conditions were used: an initial denaturation step at 95°C for 10 min; 45 cycles at 95°C for 15 s and 60°C for 1 min. The fold change was measured in relation to the housekeeping gene gyrA.

**Luciferase Assay**

The transcriptional fusion for the luciferase assay was prepared using pKSM720. The resulting construct, pKSM720-sagApro-luc, was electroporated into GAS. Spectinomycin at 50 µg/ml was used to maintain the plasmid. The GAS with the construct was grown in THB or CM from lactobacilli from A600 ≈ 0.1 to A600 ≈ 1.0, and the luciferase activity measured according to the manufacturer’s instructions (Promega). The assay was conducted in the absence of spectinomycin. The maintenance of the plasmid construct was confirmed from the viable counts on THY plates with or without spectinomycin.

**Adherence Assays**

Adherence assays were performed as previously described (Maudsdotter et al., 2011). FaDu (ATCC: HTB-43) or Detroit 562 (ATCC: CCL-138) pharyngeal epithelial cells were cultured in a 48-well plate and were inoculated with GAS at an MOI of 100 or together with lactobacilli at an MOI of 100 for 2 h. In competition assays, GAS and lactobacilli were added simultaneously. In exclusion assays, lactobacilli were pre-incubated with epithelial cells for 2 h followed by washing to remove unbound bacteria before the addition of GAS. In displacement assays, GAS was pre-incubated with the epithelial cells for 2 h followed by washing before addition of lactobacilli. When mixtures of lactobacilli were used, an MOI of 100 for each strain was added to the epithelial cells. After incubation, unbound bacteria were removed by washing in the medium.

Adhered bacteria were quantified from viable counts obtained by lysing the epithelial cells for 5 min in 1% saponin and plating on GC plates. The adherence of GAS alone was normalized to 1.

**Statistical Analysis**

All the experiments were performed in triplicate and repeated three times. Analysis of variance (ANOVA) and Tukey’s HSD (honestly significantly different) test (Statistica) was used to analyze differences between the groups for statistical significance. Statistical analysis of ratios or relative values was performed on log ratios. A p-value below 0.05 was considered statistically significant. Error bars represent standard deviation.

**RESULTS**

**Certain Lactobacilli Inhibit the Hemolytic Activity of S. pyogenes**

We investigated the influence of lactobacilli on the hemolytic activity of GAS. GAS was co-incubated with different Lactobacillus strains in THB and assayed for hemolytic activity. L. rhamnosus Kx151A1 (L. rh) and L. reuteri PTA-5289 (L. re) inhibited the hemolytic activity of GAS (Figure 1A). However, L. salivarius LMG9477 (L. sa) had no effect. The hemolytic activity was mediated by SLS because the presence of trypan blue, but not cholesterol, blocked the hemolytic activity (data not shown). To rule out the possibility that lactobacilli may inhibit the growth of GAS or affect the pH, we performed viable counts and measured the pH in the medium. The incubation of GAS with lactobacilli did not affect the survival of GAS (Figure 1B). Additionally, the pH of the growth media was not significantly affected during the co-incubation (Figure 1C). These results show that certain lactobacilli block the SLS-mediated hemolytic activity of GAS.

**Lactobacillus Growth Supernatant Inhibits the Hemolytic Activity of S. pyogenes**

To examine whether the inhibition of hemolytic activity was due to molecules released from lactobacilli, a conditioned medium (CM) was prepared from the growth supernatants of lactobacilli. The hemolytic activity of GAS in the CM was assessed at regular intervals. The SLS-mediated hemolytic activity was inhibited in GAS grown in the CM from L. rhamnosus (L. rh) and L. reuteri (L. re) throughout the growth period (Figure 2A). As previously reported, hemolytic activity was detected during the logarithmic phase and reached a maximum in the stationary phase (Kinkel and McIver, 2008). The growth of GAS was not affected in the CM (Figure 2B). These results indicate that the inhibitory molecule is a component released from lactobacilli.

To examine whether the released effector molecule binds directly to SLS, supernatants from the growth of GAS in THB were mixed with the CM of lactobacilli and assayed for hemolytic activity. There was no observable difference in the hemolytic activity of the GAS supernatant mixed with fresh THB and the GAS supernatant mixed with CM from lactobacilli (Figure 2C).
These data suggest that \textit{Lactobacillus} strains release or secrete a soluble effector molecule that inhibits the hemolytic activity of GAS.

\section*{Lactobacilli Interfere with the Expression of the \textit{sag} Operon}

Streptolysin S is chromosomally encoded by a contiguous nine-gene \textit{sag} operon (Borgia et al., 1997; Molloy et al., 2011). The first gene of the operon is \textit{sagA}, which encodes a 53 amino acid precursor. The expression of \textit{sagA} during the co-incubation of GAS with lactobacilli was monitored by qPCR. There was no significant ($P \geq 0.05$) change in the expression of \textit{sagA} when GAS was co-incubated with \textit{L. salivarius} (L. sa) (Figure 3A). However, when GAS was co-incubated with cultures of \textit{L. rhamnosus} (L. rh) and \textit{L. reuteri} (L. re), a significant ($P < 0.01$) decrease in \textit{sagA} expression was observed. A similar effect was observed in the expression of \textit{sagA} with GAS cultured in the CM of lactobacilli (Figure 3B). CM from \textit{L. rhamnosus} (L. rh) and \textit{L. reuteri} (L. re), but not \textit{L. salivarius} (L. sa), inhibited \textit{sagA} expression. The results indicate that the decrease in the hemolytic activity of GAS by lactobacilli is due to a decrease in the expression of the \textit{sag} operon.

\section*{Lactobacilli Interfere with the Promoter Activity of the \textit{sag} Operon}

To confirm that the effector molecule released from Lactobacillus inhibits the SLS at the transcriptional level, we constructed a transcriptional fusion of the promoter region of the \textit{sag} operon to a promoterless luciferase reporter gene, \textit{sagApro-luc}. GAS harboring \textit{sagApro-luc} exhibited the expected luciferase activity when grown in THB. However, the luciferase activity was inhibited when the GAS with \textit{sagApro-luc} was cultured in the CM from \textit{L. rhamnosus} (L. rh) and \textit{L. reuteri} (L. re) (Figure 4), whereas the CM of \textit{L. salivarius} (L. sa) had no effect. The data demonstrate that a lactobacilli-released effector molecule regulates the GAS SLS-mediated hemolytic activity at the transcriptional level.
Lactobacilli Inhibit the Adherence of GAS to Host Epithelial Cells

In addition to their effect on hemolytic activity, we also investigated if lactobacilli have an effect on the initial adherence of GAS to pharyngeal epithelial cells. All the Lactobacillus isolates, i.e., L. rhamnosus (L. rh), L. reuteri (L. re), and L. salivarius (L. sa), significantly ($P \leq 0.01$) reduced the adherence of GAS by approximately 35% in a competition assay (Figure 5A). Additionally, all three Lactobacillus isolates significantly ($P \leq 0.01$) inhibited streptococcal adherence when pre-incubated with the host cells for 2 h before the addition of GAS in exclusion assays. However, in the exclusion assays, a difference between the Lactobacillus isolates was observed: L. rhamnosus and L. reuteri inhibited streptococcal adherence by approximately 35%, whereas approximately 20% inhibition was observed with L. salivarius. Additionally, the ability of lactobacilli to displace already-adhered GAS was tested. L. salivarius significantly ($P \leq 0.01$) inhibited streptococcal adherence by 35% in displacement assays. In contrast, neither L. rhamnosus nor L. reuteri displaced adhered GAS. In a previous study, we showed that co-incubating GAS with lactobacilli for longer time points (4–16 h) reduced GAS viability and thereby reduced the number of viable GAS adhered to host cells (Maudsdotter et al., 2011). However, co-incubating host cells with GAS and lactobacilli for 2 h did not affect streptococcal viability (Figure 5B). These data show that lactobacilli significantly reduce GAS adherence to epithelial cells.

Effect of Different Combinations of Lactobacillus Strains on the Adherence of GAS

Different combinations of Lactobacillus isolates were co-incubated with GAS to evaluate whether a combination of lactobacilli enhanced the anti-adhesive effect. A combination of L. rhamnosus (L. rh) or L. reuteri (L. re) with L. salivarius (L. sa) conferred a significantly ($P \leq 0.01$) higher level of inhibition compared to co-incubation with any of the Lactobacillus isolates alone (Figure 6A). However, a combination of L. rhamnosus (L. rh) and L. reuteri (L. re) did not enhance the anti-adhesive effect compared to each species alone (Figure 6A). The effect of different combinations of lactobacilli on GAS adherence was tested in an additional cell line, pharyngeal epithelial Detroit 562 cells, and the same effects were observed (Figure 6B). Thus, a combination of Lactobacillus strains can increase the anti-adhesive effect.

DISCUSSION

Group A streptococcus and lactobacilli are both part of the pharyngeal microbiota. This study aimed to investigate...
Lactobacilli are a heterogeneous group of bacteria. Additionally, it has been previously reported that different lactobacillus isolates vary widely in their ability to interfere with the virulence mechanisms of pathogens (Osset et al., 2001; Ekmecki et al., 2009; Ostad et al., 2009). GAS infections have been attributed to the variety of secreted and surface-bound virulence factors. One of the most important virulence factors

how lactobacilli affect the virulence phenotypes of GAS in terms of hemolytic activity and adherence to host epithelial cells.

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**FIGURE 5** | Lactobacilli inhibit GAS adherence to pharyngeal epithelial host cells. (A) Adherence of GAS to FaDu epithelial cells in a competition, exclusion and displacement assay (B) Recovery of GAS after co-infection with lactobacilli for 2 h. Significant differences are marked with an asterisk.

**FIGURE 6** | Group A streptococcus adherence to epithelial cells in competition with different combinations of lactobacilli. GAS adherence to (A) FaDu cells and (B) Detroit 562 cells after infection alone or together with L. salivarius (L.sa), L. rhamnosus (L.rh), and L. reuteri (L.re) for 2 h. Significant differences are marked with an asterisk.
encoded by GAS is SLS (Ginsburg, 1999). In addition to
the ability to lyse RBCs, SLS has been shown to damage
the cell membranes of lymphocytes, neutrophils, platelets,
lysosomes, and mitochondria, indicating an important role
for SLS in S. pyogenes pathogenesis (Ginsburg, 1999; Molloy
et al., 2011). In this study, we found that lactobacilli were
able to inhibit the hemolytic activity of GAS. Moreover,
CM from the growth of lactobacilli was sufficient to inhibit
SLS production. The transcriptional fusion and qPCR data
revealed that this effect takes place at the transcriptional
level. The secreted components from lactobacilli negatively
regulate the promoter activity and down-regulate the sag
operon.

Incoming pathogens must compete with the resident
microbiota for nutrients and space to colonize the mucosal
membranes in the body. Additionally, the microbiota modulate
immune responses, produce inhibitory substances and prevent
the attachment of pathogens and in this way protect the
host from infections (Lebeer et al., 2010). Lactobacilli of
different species belong to the microbiota of the pharynx,
the gastric tract, and the urogenital tract and inhibit the
adherence of many pathogens (Alexandre et al., 2014; Ribet and
Cossart, 2015). In a few studies, the Lactobacillus components
mediating adherence inhibition have been identified; lactobacilli
produce biosurfactants (Servin, 2004; Rizzo et al., 2013),
S-layer proteins (Velraeds et al., 1996, 1998; Spurbeck and
Arvidson, 2010), and surface-located enolase (Ren et al.,
2012), which have been reported to inhibit the adherence
of different pathogens. However, the mechanisms by which
Lactobacillus species inhibit the adherence of pathogens are
still largely unknown. Some studies have reported a similar
level of adherence inhibition when lactobacilli are added
to host cells together with the pathogen as that observed
when lactobacilli pre-colonize the host cells (Lee et al.,
2003; Zhang et al., 2010). Other studies have reported a
stronger inhibition of lactobacilli pre-colonizing the host
cells when compared to lactobacilli added simultaneously
with the pathogen (Vielfort et al., 2008). In this study, we
demonstrate that L. rhamnosus Kx151A1, L. reuteri PTA-
5289, and L. salivarius LMG9477 reduced the adherence of
GAS to the host epithelial cells. In addition, we observed that
L. salivarius conferred stronger protection when added
in competition with GAS compared to when it pre-
colonized the host cells. Further, L. salivarius was the only
Lactobacillus species with the ability to displace already-
adhered GAS. Our results highlight the differences between
Lactobacillus species in the mechanism by which they confer
colonization resistance as well as the effects they exert on host
cells.

Various Lactobacillus species have previously been found
to exhibit bactericidal activities against GAS in co-infection
studies. In a previous study, we showed that co-incubation
with lactobacilli for longer time points caused elevated lactic
acid concentrations that are bactericidal against GAS and
thereby reduce the number of viable GAS adhered to host
cells (Almengor et al., 2007). In this study, we investigated the
influence of lactobacilli on GAS adherence at a shorter time
point at which GAS was not killed and observed inhibition of
the initial adherence that was independent of compromised
viability.

Thus, colonization with Lactobacillus species impairs GAS
pathogenicity at different stages of infection, both by reducing
the adherence at earlier stages and by affecting the viability after
prolonged incubation and attenuating the hemolytic activity.

Probiotics are live microorganisms that confer health benefits
on the host. Lactobacillus is a well-studied genus for probiotic
use. Many criteria have been suggested for the selection
of probiotics, including their ability to prevent the adherence
of pathogens to target cells (Lee et al., 2003; Lebeer et al.,
2010). The effects of probiotics are strain-specific, and the
mechanisms of protection are largely unknown. The current
study reveals that lactobacilli interfere with the virulence
phenotypes of GAS in terms of SLS production and adherence
to the host epithelial cells. Furthermore, a combination of
lactobacilli has a greater effect in reducing the adherence of
GAS to host epithelial cells and thus has an enhanced protective
effect.

The findings may have a potential application in the use
of lactobacilli strains as a probiotic for oral health purposes
to combat GAS infections. Also, the secreted component
from the lactobacilli holds a therapeutic value to fight GAS
infections.

The identification of the Lactobacillus-secreted product
interfering with the transcription of the sag operon is under
study. Greater understanding of the molecular mechanisms by
which probiotics function will help in developing enhanced
future therapeutics.

AUTHOR CONTRIBUTIONS
Conceived and designed the experiments: SS, LM, A-BJ. Performed experiments: SS, LM, RT. Analyzed the data and wrote
manuscript: SS, LM, A-BJ.

FUNDING
This work was supported by the Swedish Research Council, the
Swedish Cancer Society, Knut and Alice Wallenberg’s Stiftelse,
and Torsten and Ragnar Söderbergs Stiftelse.

ACKNOWLEDGMENTS
We thank Prof. Kevin S. McIver, University of Maryland for
providing pKSM720. We thank Nazeer Kanakryyh for his
technical assistance.
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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