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In Vitro Functional and Immunomodulatory Properties of the *Lactobacillus helveticus* MIMLh5-*Streptococcus salivarius* ST3 Association That Are Relevant to the Development of a Pharyngeal Probiotic Product

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The use of proper bacterial strains as probiotics for the pharyngeal mucosa is a potential prophylactic strategy for upper respiratory tract infections. In this context, we characterized *in vitro* the functional and immunomodulatory properties of the strains *Lactobacillus helveticus* MIMLh5 and *Streptococcus salivarius* ST3 that were selected during previous investigations as promising pharyngeal probiotics. In this study, we demonstrated *in vitro* that strains MIMLh5 and ST3, alone and in combination, can efficiently adhere to pharyngeal epithelial cells, antagonize *Streptococcus pyogenes*, and modulate host innate immunity by inducing potentially protective effects. In particular, we found that the strains MIMLh5 and ST3 activate U937 human macrophages by significantly inducing the expression of the proinflammatory cytokine tumor necrosis factor alpha (TNF-α). Nonetheless, the induction of the anti-inflammatory interleukin-10 (IL-10) by MIMLh5 or ST3 was never lower than that of TNF-α, suggesting that these bacteria can potentially exert a regulatory rather than a proinflammatory effect. We also found that the strains MIMLh5 and ST3 induce cyclooxygenase 2 (COX-2) expression and demonstrated that toll-like receptor 2 (TLR-2) participates in the recognition of the strains MIMLh5 and ST3 by U937 cells. Finally, we observed that these microorganisms grow efficiently when cocultured in milk, suggesting that the preparation of a milk-based fermented product containing both MIMLh5 and ST3 can be a practical solution for the administration of these bacteria. In conclusion, we propose the combined use of *L. helveticus* MIMLh5 and *S. salivarius* ST3 for the preparation of novel products that display probiotic properties for the pharyngeal mucosa.

The administration of living microbial cells to humans or animals to promote or maintain health is commonly known as a "probiotic approach." Traditionally, probiotic products are explicitly proposed to be effective in the gastrointestinal tract (GIT). However, several probiotics have been designed for the vaginal mucosa (35), the urinary tract (5), the skin (20), and the oral cavity (38). The use of probiotics for the oropharyngeal tract (OPT) is particularly promising. OPT dysfunctions are often related to the presence of microbial pathogens (for instance, *Streptococcus mutans*, group A streptococci, or *Porphyromonas gingivalis*) or to microbial dysbiosis. Furthermore, compared with the distal GIT, the OPT is a more accessible site for microorganisms of exogenous origin. For these reasons, the OPT is a potential target for new, specifically designed probiotic products.

An excellent example demonstrating the potential of the probiotic approach for the OPT is represented by the research activity of J. R. Tagg and coworkers, who isolated the *Streptococcus salivarius* strain K12 (45). Strain K12 has a marked ability to inhibit pathogenic bacteria, mainly due to the production of three different bacteriocins, salivaricins A2, B, and 9 (44). Strain K12 was also demonstrated to be able to colonize the upper respiratory tract (17) and to downregulate the innate immune responses of human epithelial cells (8). The scientific results from the Tagg lab studies supported the creation of a set of probiotic pharmaceutical products (lozenges, powders, and chewing gum), commercialized under the name of BLIS, that were specifically designed for the prevention or treatment of dysfunctions such as cavities, periodontitis, halitosis, and pharyngitis. In July 2011, the oral probiotic products BLIS K12 were granted generally recognized as safe (GRAS) status by the United States Food and Drug Administration (FDA), enabling this probiotic to be included as an ingredient in food products within the United States.

Recently, we published two studies in which a selection of probiotic and dairy bacteria and new oral isolates were screened *in vitro* for their ability to adhere to human pharyngeal cell layer and keratinocytes, antagonize *Streptococcus pyogenes*, and modulate host immune system responses (13, 14). As a result of these studies, we selected the oral isolate *Streptococcus salivarius* ST3 and the dairy strain *Lactobacillus helveticus* MIMLh5 as new probiotic candidates for the pharyngeal mucosa. In this report, we show the

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results of further investigations of these bacteria. We analyzed *in vitro* the potential probiotic properties of these bacteria when employed in combination. Specifically, we examined their ability to adhere to FaDu human pharyngeal cells, to antagonize *Streptococcus pyogenes*, and to modulate the immune response of macrophages. Particularly, since the immunological study of the interaction between probiotic bacteria and the oropharyngeal mucosa has been based exclusively on epithelial cells, in this study we assessed the responses induced by bacteria in host cells properly belonging to the immune system. To explore the potential of combining strains ST3 and MIMLh5 in a fermented food product, we also studied the growth of *S. salivarius* ST3 in the presence of different sugars and in milk in coculture with strain MIMLh5. Our results support the possibility of producing a novel milk-based fermented food product to be used as probiotic for the oropharyngeal mucosa.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and growth experiments. *Lactobacillus helveticus* MIMLh5 was grown at 42°C in MRS broth (Difco Laboratories Inc., Detroit, MI). *Streptococcus salivarius* ST3 was cultivated at 37°C in M17 broth (Difco Laboratories) supplemented with 2% glucose. The growth curves of strain ST3 were determined with 384-well microtiter plates using a BioTek Synergy HT multimode microplate reader (AHSI S.p.A., Bernareggio, Italy) to measure growth in M17 broth supplemented with 2%, 1%, 5‰, 2.5‰, 1.25‰, or 0.625‰ of one of the following sugars: fructose, galactose, glucose, inulin, lactose, lactulose, sucrose, or fructooligosaccharides (FOS). All sugars were purchased from Sigma-Aldrich (Steinheim, Germany), with the exception of FOS, which were from Actilight (Giulio Gross S.p.A., Trezzano sul Naviglio, Italy). The preparation of 384-well microtiter plates was conducted with an ep*Motion* automated pipetting system (Eppendorf, Milan, Italy). Acidification curves were determined by inoculating skim milk (Difco) (1% inoculum) with an overnight culture of MIMLh5 and ST3 alone or in coculture and recording the pH every hour for 24 h with a pH meter recorder (XS instruments pH 2100; Opto-Lab, Concordia, Italy). Recombinant *S. pyogenes* strain C11^{LucFF} (*emm* type 77) was grown at 37°C in BHI medium (Difco) supplemented with 0.3% yeast extract and 5 μg ml^{-1} of chloramphenicol.

FaDu cell layer preparation. *In vitro* cultivation. FaDu cells (human pharynx carcinoma cell line; ATCC HTB-43) were routinely grown in Eagle's minimum essential medium supplemented with 10% (vol/vol) heat-inactivated (30 min at 56°C) fetal calf serum, 100 U ml^{-1} penicillin, 100 mg ml^{-1} streptomycin, 0.1 mM nonessential amino acids, and 2 mM L-glutamine (EMEM) and were incubated at 37°C in a water-jacketed incubator in an atmosphere of 95% air and 5% carbon dioxide.

Bacterial adhesion on the FaDu cell layer. FaDu cell monolayers were grown in 3-cm petri plates on microscope cover glasses until they reached confluence. Next, the cell layers were carefully washed twice with phosphate-buffered saline (PBS), pH 7.3. The bacterial cell concentrations of overnight cultures were determined microscopically with a Neubauer Improved counting chamber (Marienfeld GmbH, Lauda-Königshofen, Germany). Bacterial cells were recovered by centrifugation and resuspended in PBS to obtain a concentration of 10^8 cells per ml. Two milliliters of the bacterial suspension were incubated with a FaDu cell monolayer for 1 h at 37°C. Afterward, the monolayers were washed 3 times with PBS, fixed for 8 min with 2 ml of methanol, and stained with 2 ml of Giemsa's stain (Carlo Erba Reagenti S.p.A., Rodano, Italy). The cover glasses were then washed 5 times with 1 ml of PBS, dried, and examined microscopically (100× magnification, with oil immersion). The adherent bacteria from 20 randomly selected microscopic fields were counted, and average counts were determined.

Bacterial antagonism assay on the FaDu cell layer. Antagonism against *Streptococcus pyogenes* was studied using the bacterial exclusion method on FaDu cell layers as previously described (13). In brief, 2 × 10^8 cells ml^{-1} of a tester strain suspension in PBS were preincubated with the FaDu cell layer for 1 h. The FaDu cells were then washed with PBS and incubated with 2 × 10^8 cells ml^{-1} of the indicator strain (*S. pyogenes* bioluminescent recombinant strain C11^{LucFF}) for 1 h. After the second incubation, FaDu cell layers were quickly washed twice with 1 ml of PBS (pH 7.3), and D-luciferin (Sigma-Aldrich) was added at a final concentration of 12.5 μM in citrate buffer, pH 5. Immediately, bioluminescence was measured with a Victor 3 luminometer (PerkinElmer, Monza, Italy). Each tester strain was analyzed in triplicate in at least two independent experiments. Unpaired Student's *t* test was used to detect statistically significant differences.

Study of NF-κB activation in FaDu cells. Stably transfected FaDu cell lines were generated by transfecting cells with the plasmid pNiFty2-Luc (Invivogen, Labogen, Rho, Italy) as described by Guglielmetti et al. (13). This plasmid contains a promoter combining five nuclear factor-κB (NF-κB)-binding sites and the *luc* firefly luciferase reporter gene. Stimuli that activate NF-κB promote its binding to the vector promoter, resulting in the expression of the luciferase gene. After growth in the presence of 50 μg ml^{-1} zeocin, the FaDu cells were detached by trypsinization and then resuspended in EMEM at a concentration of 250,000 cells ml^{-1} in the presence of 100 mM HEPES (pH 7.4). Subsequently, 50 μl of a tester bacterial suspension containing 2.5 × 10^9 or 2.5 × 10^8 cells ml^{-1} was added to 450 μl of the FaDu cell suspension, resulting in a multiplicity of infections (MOI) of approximately 1,000 or 100, respectively. After incubation at 37°C for 4 h, the samples were kept in ice and sonicated at maximum power for 5 s using a Bandelin Sonoplus ultrasonic homogenizer (Bandelin Electronic GmbH & Co., Berlin, Germany). Insoluble particles were removed by centrifugation, and 100 μl of the supernatants were dispensed in duplicate into the wells of a 96-well white microtiter plate (PerkinElmer, Monza, Italy) using an ep*Motion* automated pipetting system (Eppendorf). Next, 12.5 μl of a 10 mM ATP solution (1 mM final concentration) and 12.5 μl of 0.1 mM D-luciferin were added to the wells, and bioluminescence was immediately measured with a Victor^3 1420 multilabel counter (PerkinElmer). The maximum of the light production curve was considered for comparison of results. All conditions were analyzed in triplicate in at least two independent experiments. Unpaired Student's *t* test was used to detect statistically significant differences.

Cell culture, growth conditions, and stimulation of the U937 human macrophage cell line. The cell line U937 (ATCC CRL-1593.2) was derived from a human histiocytic lymphoma (37). These cells are maintained as replicative, nonadherent cells and have many of the biochemical and morphological characteristics of blood monocytes (15). When treated with phorbol myristate acetate (PMA), U937 cells differentiate to become adherent, nonreplicative cells with characteristics similar to those of tissue macrophages, including similar isoenzyme patterns (33) and other phenotypic markers (15). The normal growth medium for the U937 cells consisted of RPMI 1640 medium (Lonza, Basel, Swiss) supplemented with 10% (vol/vol) fetal bovine serum (FBS) (Gibco-BRL, Life Technologies, Milan, Italy), 2 mM L-glutamine, 100 units ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin (Sigma-Aldrich). U937 cells were seeded at a density of 5 × 10^5 cells well^{-1} in 12-well plates and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Differentiation was induced by the addition of PMA (Sigma-Aldrich) into the cellular medium at a final concentration of 100 nM and was allowed to proceed for 48 h. Afterward, cells were washed once with sterile PBS buffer to remove all nonadherent cells. One hour before the bacteria were added to the cells, the culture media was replaced with RPMI 1640 medium supplemented with 1% (vol/vol) FBS to allow the cells to adapt. Bacteria were used at MOIs of 100 and 1,000, and lipopolysaccharide (LPS; final concentration of 1 μg ml^{-1}) from *Escherichia coli* 0127:B8 (Sigma-Aldrich) was used as the positive control for proinflammatory stimulus in U937 cells. An untreated sample, i.e., only RPMI 1640 medium with 1% (vol/vol) FBS, was used as the control.

Inhibition assay with Toll-like receptor neutralizing antibodies. Human anti-Toll-like receptor 2 (anti-TLR-2) antibody (Invivogen) was

added to U937 cells 1 h before the stimulation with bacterial cells. A human immunoglobulin A2 (IgA2) isotype control (Invivogen) was used as the control to exclude nonspecific binding and blocking activity of the antibody. Both the anti-TLR2 and IgA2 isotype control were used at 5 μg ml^{-1}. This concentration was determined by examining the neutralizing efficacy of anti-TLR2 with zymosan from *Saccharomyces cerevisiae* (Invivogen) as the ligand.

Preparation of RNA and reverse transcription. After incubating U937 cells at 37°C for 4 h, the supernatant was carefully removed from each well and the total cellular RNA was isolated from the adhered U937 cells with an RNeasy Minikit (Qiagen, Inc., Valencia, CA). Afterward, RNA concentration and purity were determined with a NanoDrop spectrophotometer (ND-1000, Thermo Fischer Scientific), and reverse transcription to cDNA was performed with an iScript Select cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) using the following thermal cycle: 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C.

Real-time quantitative PCR. The mRNA expression levels of cytokines were analyzed with SYBR green technology in real-time quantitative PCR (qPCR) using SsoFast EvaGreen Supermix (Bio-Rad) on a Bio-Rad CFX96 system according to the manufacturer's instructions. The primers used are as follow (5′→3′): 18srRNA forward, ATCCCT GAAAAGTTCCAGCA; 18srRNA reverse, CCCTCTTGGTGAGGTCA ATG; interleukin-10 (IL-10) forward, AGCAGAGTGAAGACTTTCT TTC; IL-10 reverse, CATCTCAGACAAGGCTTGG; tumor necrosis factor alpha (TNF-α) forward, TCAGCTCCACGCCATT; TNF-α reverse, CCCAGGCAGTCAGATCAT; cyclooxygenase 2 (COX-2) forward, CCC TTGGGTGTCAAAGGTAA; COX-2 reverse, TGAAAAGGCGCAGTTT ACG. All primers were selected using Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), and the specificity of the primers was tested with melting curves during amplification and by 1% agarose gels. Quantitative PCR was carried out according to the following cycle: initial hold at 96°C for 30 s and then 40 cycles at 96°C for 2 s and 60°C for 5 s. Gene expression was normalized to that of the 18S ribosomal DNA (rDNA) gene. The amount of template cDNA used for each sample was 12.5 ng. All results regarding cytokine mRNA expression levels are reported as the fold of induction (FOI) respective to the control (namely, unstimulated U937), to which we attributed an FOI of 1.

RESULTS AND DISCUSSION

In previous studies, we selected *Streptococcus salivarius* ST3 and *Lactobacillus helveticus* MIMLh5 from several oral bacterial isolates and probiotic/dairy bacteria as potential probiotics for the pharyngeal mucosa (13, 14). In this study, we evaluated the possibility of using strains ST3 and MIMLh5 in combination by assessing their *in vitro* probiotic and immunologic properties.

ST3/MIMLh5 cosuspension did not affect adhesion and antagonistic properties of the individual strains on the FaDu cell layer. In previous studies, strains ST3 and MIMLh5 showed similar efficient adhesive and antagonistic features, but they displayed different behaviors both in the activation of NF-κB and in the profile of induced cytokines (13, 14). Therefore, we evaluated *in vitro* the potential probiotic properties of strains ST3 and MIMLh5 used in combination. We first studied the interaction with FaDu human pharyngeal cell line in terms of adhesion and ability to antagonize *Streptococcus pyogenes* (Fig. 1). The adhesion of each strain was not affected when the other was also present, indicating that there is no competition for adhesion sites between MIMLh5 and ST3 in our test conditions (MOI of 1000 for each bacterium) (Fig. 1A). The adhesion index (the number of bacteria adhered per 100 FaDu cells) for the two strains, in fact, was not significantly different when a strain was alone or in coculture (*P* value of 0.835 or 0.507, respectively); both conditions resulted in an adhesion index of approximately 2,000 (data not shown).

In the following series of experiments, we measured the bioluminescence produced by *S. pyogenes* $C11^{LucFF}$ as an indication of the exclusion antagonism exerted by strains ST3, MIMLh5 and their mixed suspension. *L. helveticus* MIMLh5 showed better antagonism against *S. pyogenes* (72% reduction of luminescence), but *S. salivarius* strain ST3 could also effectively antagonize the pathogen (53% luminescence reduction). When the tester bacteria were used in combination (MOI of 1,000 each), the antagonistic activity was maintained at the highest level (72% reduction), indicating that the two strains can coexist without reducing their *in vitro* exclusion ability (Fig. 1B).

The effect of strain MIMLh5 on NF-κB activation in FaDu epithelial cells is predominant over that of strain ST3. An increasing number of *in vitro* and *in vivo* experiments support the idea that probiotic bacteria confer their health benefits to the host by interacting with the immune system (7, 23). The ability to modulate host immune responses *in vitro* has also been shown for bacteria that have been proposed as probiotics for the OPT, such as *Streptococcus salivarius* K12 (8) and the two strains included in this study (13, 14). Particularly, *in vitro* immunological tests with FaDu cells revealed different responses to *S. salivarius* ST3 and *L. helveticus* MIMLh5 (13, 14). While strain MIMLh5 reduced IL-8 and IL-6 secretion (14), ST3 had only a limited effect on these cytokines but drastically inhibited IL-1β and stimulated macrophage inflammatory protein 1β (MIP-1β) (13). It has been proposed that the reduction in IL-8 and IL-6 secretion resulting from exposure to strain MIMLh5 can be attributed to the inhibition of NF-κB activation (14). In contrast, *S. salivarius* ST3 promoted the activation of NF-κB (13). In this study, we explored the effect of strains ST3 and MIMLh5 in combination on NF-κB activation in FaDu cells. As observed in previous investigations (13, 14), *L. helveticus* MIMLh5 decreased the NF-κB-dependent production of bioluminescence, while ST3 showed a stimulatory effect (Fig. 1C). When the two bacterial strains were used in combination at the same multiplicity of infection (MOI of 1,000 each), activation of NF-κB was reduced to an extent similar to that determined by MIMLh5 alone (Fig. 1C), indicating that the inhibitory effect of this bacterium on NF-κB-mediated responses is predominant over the outcome induced by strain ST3.

Effect of the strains MIMLh5 and ST3 on activation of U937 human macrophages. Studies on the immunomodulatory effects of probiotics for the oropharyngeal mucosa have thus far concentrated mainly on immune responses at the epithelial cell level (8, 13). An evaluation of the responses by cells involved in the immune system is thus required. In this study, we quantified via reverse transcription (RT)-qPCR the gene expression of TNF-α (a cytokine involved in inflammatory responses [4]) and IL-10 (a potent anti-inflammatory interleukin that inhibits the production of proinflammatory cytokines in several cell types [9, 11]) in PMA-differentiated U937 human macrophages. We tested the strains MIMLh5 and ST3 alone and in combination (coincubation) at MOIs of 100 and 1,000. In all tested conditions, strains MIMLh5 and ST3 induced a higher IL-10/TNF-α ratio than lipopolysaccharide (LPS) from *Escherichia coli*, a potent proinflammatory stimulus. Unlike LPS, the induction of IL-10 by MIMLh5 or ST3 alone was never lower than that of TNF-α (Fig. 2A). Strain ST3, in particular, triggered profound IL-10 expression. In coincubation experiments, we used a total MOI (i.e., the sum of the bacterial cell numbers of both strains) corresponding to the MOI of the bacterial strains employed individually to see possible syn-

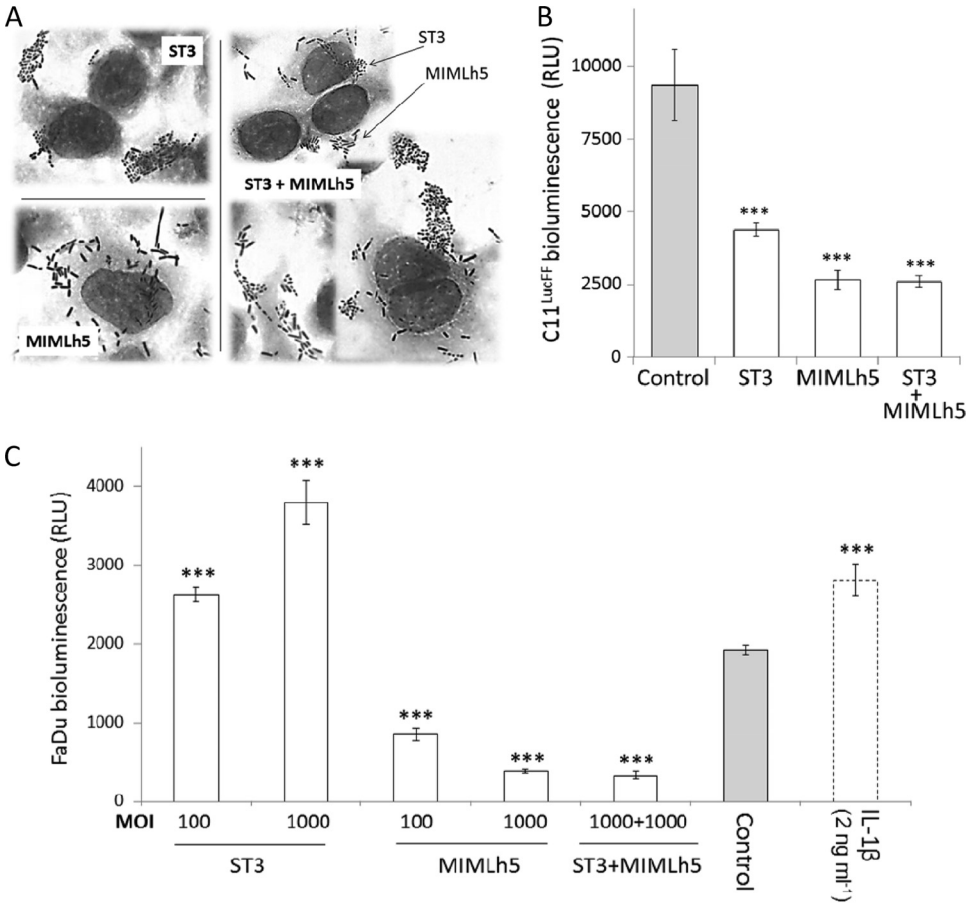

FIG 1 Probiotic properties of *Lactobacillus helveticus* MIMLh5 and *Streptococcus salivarius* ST3 on a FaDu human pharyngeal cell layer. (A) Bacterial adhesion as observed with Giemsa staining under a light microscope; FaDu cell nuclei appear in red. (B) Antagonistic exclusion activity against bioluminescent *Streptococcus pyogenes* $C11^{lucFF}$; the control is a cell layer treated only with PBS before incubation with *S. pyogenes*. (C) Effect of bacteria on FaDu cells stably transfected with an NF-κB/luciferase reporter vector. The control is a FaDu cell layer incubated without bacterial cells. MOI, multiplicity of infection (bacterial cells per FaDu cell). IL-1β was used as the positive control for NF-κB activation. Data in histograms are the means (± standard deviations) from at least three independent experiments conducted in triplicate. Bacterial and FaDu cell luciferase activities are expressed as relative luminescence units (RLU). Asterisks indicate statistically significant differences compared to the control ($P < 0.001$).

ergistic effects. At the lower MOI tested, the mixed suspension of MIMLh5 and ST3 resulted in an intermediate cytokine induction compared to that of the single strains (Fig. 2A). In contrast, synergism was observed at the MOI of 1,000; the combination of MIMLh5 and ST3 caused a significant increase in the expression of both TNF-α and IL-10 (Fig. 2A). These data show that the bacterial cell concentration affects the immune response of U937 cells; a dose-dependent approach should be therefore considered when defining a specific immunological effect of a bacterial strain, as demonstrated in previous works (10).

In this study, the strains MIMLh5 and ST3 significantly induced the expression of the proinflammatory cytokine TNF-α. Previous studies have shown that different *Lactobacillus* species can trigger pronounced proinflammatory activity in DCs (6, 43) and in macrophages (26). Macrophages are professional phagocytes that reside in the secondary lymphoid organs as well as in almost all tissues, including the tonsils (47), serving as sentinels to detect microbial invaders (1). Upon the recognition of pathogen-associated molecular patterns (PAMPs [18]), macrophages produce inflammatory mediators, such as cytokines and chemokines, that alert the immune system to the infection of injury. A proinflammatory behavior that results in the activation of cell-mediated (Th1-type) immunity, like that observed for MIMLh5 and ST3, could thus be crucial in combating intracellular pathogen attacks and viral infections and alerting the host's immune system. Other studies have reported that most lactic acid bacterial strains that have been tested could induce a Th1 response, even though this capability is strictly dependent on the specific strain and dose used (29, 31). Notably, in the same studies, some strains of *S. thermophilus*, a species phylogenetically close to *S. salivarius*, induced a high level of IL-10 (29). In our experiments, *S. salivarius* ST3 induced a higher level of IL-10 than TNF-α. Furthermore, the increased production of the anti-inflammatory cytokine was more pronounced in ST3 than in MIMLh5 at the highest MOI tested (Fig. 2A). Therefore, we speculate that the presence of both bacteria, even at high doses (MOI of 1,000), might not result in detrimental effects. The regulatory effect of the significant IL-10 expression could protect against secondary outcomes that can occur after streptococcal infections, namely, reactive inflammatory conditions such as rheumatic disease (32).

The strains MIMLh5 and ST3 induce cyclooxygenase 2 (COX-2) expression in U937 cells and in BMDCs. Cyclooxygen-

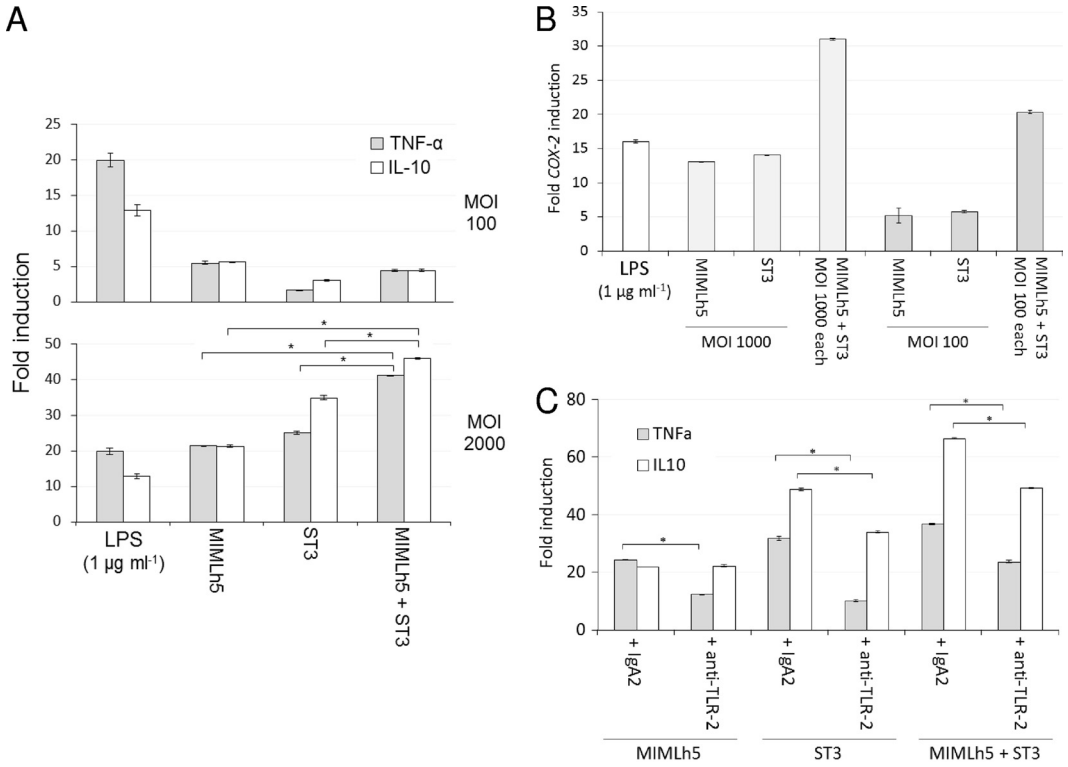

FIG 2 Transcription analysis of cytokine genes in U937 cells stimulated with *Lactobacillus helveticus* MIMLh5 and *Streptococcus salivarius* ST3 after 4 h of incubation with bacterial strains used alone or in association at two different MOIs. Lipopolysaccharide (LPS) was used as the positive control at a concentration of 1 μg ml^{-1}. The values are the means (± standard deviations) for a result representative of three independent experiments, expressed as the fold change in induction relative to the result for the control (U937 unstimulated cells), which was set at a value of 1. Asterisks indicate statistically significant differences compared to results for the corresponding control (*, $P < 0.05$). (A) Expression levels of IL-10 and TNF-α. (B) Expression levels of *COX-2*. (C) Expression levels of IL-10 and TNF-α in the presence of a neutralizing antibody against TLR-2 (anti-TLR2). Anti-TLR2 was added to U937 cells 1 h before stimulation with bacteria. Immunoglobulin-A2 isotype (IgA2) was used as the control for nonspecific blocking activity.

ase (COX), or prostaglandin synthase H (PGH), is a homodimer enzyme involved in the synthesis of prostaglandins (PGs) from arachidonic acid (12). PGs are involved in several physiological processes and contribute to the protection of the gastrointestinal mucosa (46, 27). Two isoforms of the COX enzyme have been identified and described (21). COX-1 is constitutively expressed in a wide range of tissues, whereas COX-2 is constitutively expressed in very few tissues but is induced by several stimuli, including bacterial components (16). We investigated the effect of the strains MIMLh5 and ST3 on *COX-2* gene activation in the U937 cell model. *L. helveticus* MIMLh5 and *S. salivarius* ST3 induced *COX-2* gene expression to similar levels. The expression levels for MIMLh5 and ST3 were 5.20 and 5.75, respectively, at an MOI of 100, and 13.08 and 14.12, respectively, at an MOI of 1,000 (Fig. 2B). The association of the two bacteria resulted in an enhanced transcription of the gene (Fig. 2B). In similar experiments, we also observed a strong increase in *COX-2* gene expression in murine bone marrow-derived dendritic cells (BMDCs) (see Fig. S1 in the supplemental material).

Although other studies have suggested that lactic acid bacteria could affect COX-2 secretion, there is no clear consensus in the literature. For instance, several probiotic preparations were found to reduce the amount of prostaglandins and levels of *COX-2* gene expression at the intestinal level in murine models (3, 41) and *in vitro* (24). In contrast, other papers reported a significant upregulation of *COX-2* expression levels by probiotic bacteria *in vitro* (19, 30) and *in vivo* (19, 22). *Lactobacillus acidophilus*, a species phylogenetically related to *L. helveticus*, was reported to significantly increase *COX-2* expression and PGE_2 secretion in the human colon cancer cell line Colo320 (30). Differences in tested strains and model systems likely explain the lack of consensus found in the literature.

The ability of the strains MIMLh5 and ST3 to induce *COX-2* expression suggests that these bacteria could directly affect inflammatory processes not only by modulating cytokine secretion but also by inducing prostaglandin production. COX-2 is involved in the induction of oral tolerance through the action of PGs in guiding T cells toward an immunosuppressive phenotype (28) and in resolving inflammation (42). The observed effect of MIMLh5 and ST3 on the activity of the *COX-2* gene could thus be of immediate benefit for the host's mucosa; furthermore, rapid *COX-2* upregulation in response to injury or inflammation has been reported to restore mucosal integrity (40).

TLR-2 participates in the recognition of the strains MIMLh5 and ST3 by U937 cells. To gain information on possible signaling pathways involved in the immune responses induced by *L. helveticus* MIMLh5 and *S. salivarius* ST3, we investigated which Toll-like receptor (TLR) recognizes these bacteria. TLRs are a class of transmembrane proteins involved in innate immunity (2); in mammals, they work as pattern recognition receptors (PRRs) and participate in detecting various conserved microbial molecules, such as lipopolysaccharide (LPS), lipoteichoic acid (LTA), pepti-

doglycan (PGN), bacterial lipoproteins, lipoarabinomannan, and zymosan (25). More specifically, TLR-2 forms heterodimers with TLR-1 or TLR-6 and primarily interacts with Gram-positive bacteria by recognizing PGN, LTA, and lipoproteins (39).

In this study, as preliminary information needed to set up the experiment, we observed that 5 μg ml^{-1} of zymosan induced in U937 cells a 5-fold reduction in the expression level of TNF-α, a cytokine known to be induced in the downstream pathway activated by TLR-2. We then performed experiments with a neutralizing antibody against TLR-2 (anti-TLR2-Ab) to partially block its ability to bind ligands. An IgA isotype control was also used to exclude nonspecific binding to U937 cell receptors. After a 1-h preincubation with an anti-TLR2 neutralizing Ab (5 μg ml^{-1}), we stimulated the U937 cells for 4 h with bacteria. We then evaluated the expression of the genes that encode TNF-α and IL-10 by using RT-qPCR. When TLR-2 was blocked with anti-TLR2, we observed significantly less TNF-α mRNA, both when strains MIMLh5 and ST3 were used individually and when they were used in combination (Fig. 2C). These results indicate that TLR-2 is involved in mediating the immunostimulatory activity of both strains. These data are consistent with previous studies that showed that the immunomodulatory activity elicited by lactobacilli involves TLR-2 in macrophages (36) and in dendritic cells (43, 49). However, preincubation with anti-TLR2 resulted in a reduction of IL-10 expression only with strain ST3, whereas MIMLh5-mediated expression of IL-10 was not affected (Fig. 2C), indicating that receptors independent from TLR-2 may be involved in the induction of this cytokine. The interference of the anti-TLR2-Ab with IL-10 induction that we observed in our study with strain ST3 is in agreement with the literature. Molecules able to induce IL-10 in a TLR2-dependent manner have been already described (34, 48). It is plausible that a similar response mechanism is triggered by *S. salivarius* ST3 in U937 cells.

When the strains were combined, the reduction of the proinflammatory cytokine TNF-α was similar to that of ST3 alone (1.43- and 1.34-fold reduction, respectively). Even though anti-TLR2 generally reduced TNF-α induction, the relative increase of TNF-α gene expression caused by the bacteria in combination compared to bacteria employed individually was markedly higher when TLR-2 was blocked with anti-TLR2 than that in control conditions (i.e., in the presence of IgA2) (Fig. 2C). In other words, the synergistic effect of using both strains was intensified by the presence of the anti-TLR2 antibody. As a consequence, when TLR-2 was blocked, the IL-10/TNF-α ratio increased only slightly (approximately 18%) with bacteria in combination, whereas the ratio was nearly doubled with the use of the individual strains. We can therefore speculate that PRRs other than TLR-2 are involved in the recognition of these bacteria. It might also be hypothesized that blocking TLR-2 can impact the formation of heterodimers with other TLRs and/or the cooperation of TLR-2 with other types of receptors (such as scavenger receptors), therefore causing different cytokine responses.

Strains ST3 and MIMLh5 can be used in combination to ferment bovine milk. In light of the *in vitro* functional properties of *L. helveticus* MIMLh5 and *S. salivarius* ST3 in combination, we examined the possibility of cocultivating these bacteria in bovine milk and potentially employing MIMLh5 and ST3 in dairy products. *L. helveticus* MIMLh5 is a natural whey starter culture that is well adapted to grow in bovine milk. In contrast, *S. salivarius* is a human oral commensal isolate that cannot proliferate when cultivated in milk due to its limited ability to use lactose as the sole carbon and energy source. We determined the growth curve of ST3 with eight different carbohydrates at six concentrations in M17 medium (see Fig. S2 in the supplemental material). Strain ST3 displayed optimal growth in the presence of glucose, sucrose, fructose and fructooligosaccharides (FOS); its growth was significantly reduced with inulin and lactose but was not detectable with galactose and lactitol (Fig. 3A). Interestingly, strain ST3 metabolized Actilight (Fig. 3B), a commercial product consisting of a mixture of short-chain FOS (1-kestose, nystose, and fructosylnystose) that is frequently used as a prebiotic supplement. When we added one of the four sugars that are efficiently metabolized by ST3 to skim milk, ST3 growth became similar to that in M17 supplemented with the same sugar. Subsequently, we performed acidification curves by monitoring the pH in skim milk supplemented with 2% glucose after a 1% inoculum of the overnight cultures of strains ST3 and MIMLh5 alone and in coculture. The association of the two strains resulted in a faster acidification of

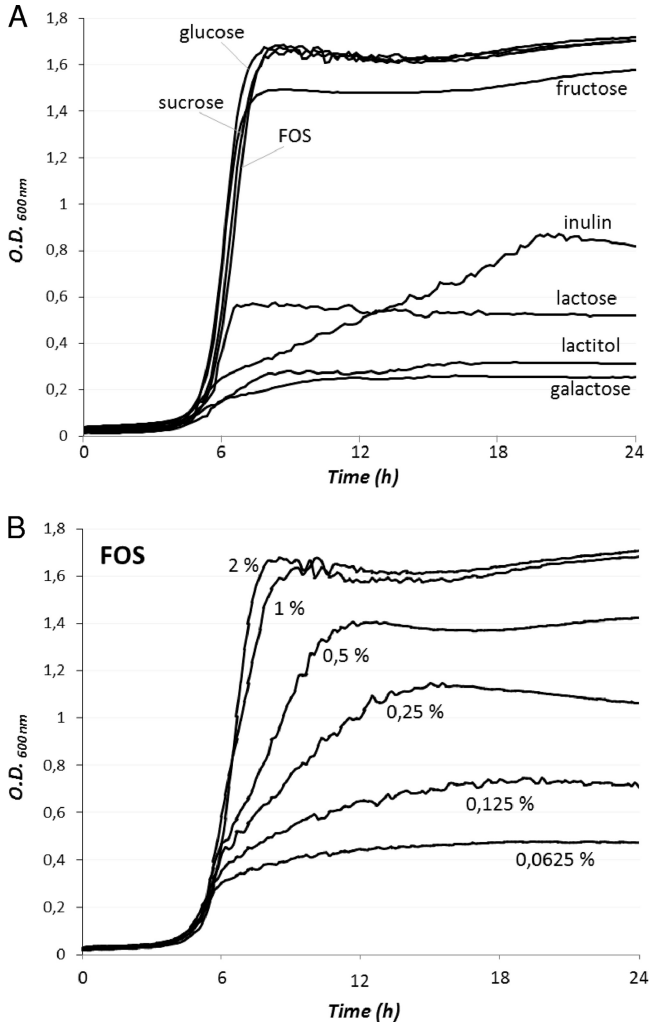

FIG 3 Growth curves of *Streptococcus salivarius* ST3. (A) Growth in M17 medium supplemented with 2% of 8 different carbon sources. (B) Growth in M17 medium supplemented with 6 different concentrations of Actilight fructooligosaccharides (FOS). The curves are representatives of two experiments carried out in sextuplicate.

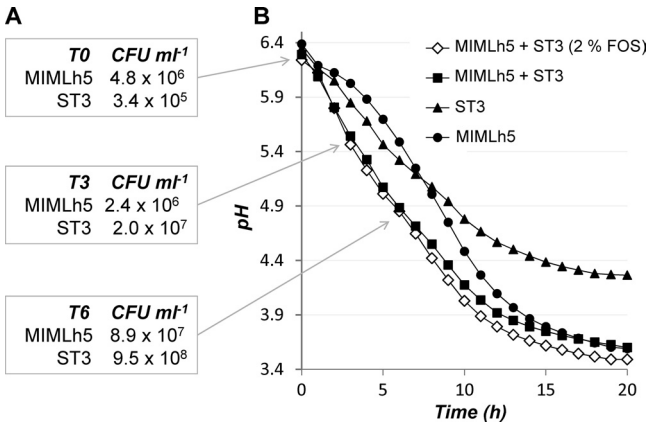

FIG 4 Growth of *Lactobacillus helveticus* MIMLh5 and *Streprococcus thermophilus* ST3 (single culture and coculture) at 42°C in skim milk supplemented with 2% glucose or FOS. (A) Bacterial plate counts of coculture; *T0*, immediately after 1% inoculum; *T3* and *T6*, after 3 and 6 h, respectively. (B) Acidification curves.

the medium (Fig. 4). In fact, after 6 h of incubation at 42°C, ST3 and MIMLh5 monocultures reached pH 5.2 and 5.5, respectively, whereas their coculture reduced the pH to 4.9 in skim milk supplemented with 2% glucose or FOS (Fig. 4). After 6 h of coincubation in skim milk supplemented with glucose at 42°C, ST3 and MIMLh5 reached a number of viable cells of 9.5×10^8 and 8.9×10^7 CFU ml^{-1}, respectively (Fig. 4), indicating that both strains contributed to the milk acidification. These data support the potential of using a combination of strains ST3 and MIMLh5 to ferment milk for the production of a novel food product.

Conclusion. There is an increasing interest in prophylactic strategies effective for upper respiratory tract infections, which represent a major cause of medical prescription for antibiotics, especially in children. The identification of bacterial strains that could be used as probiotics for the pharyngeal mucosa is one potential strategy.

This study is part of an ongoing research project designed to identify new potential probiotic bacteria for the pharyngeal mucosa and to develop novel food products for an effective administration of these probiotics. We presented a further characterization of the strains MIMLh5 and ST3 that were selected during previous investigations as the most promising pharyngeal probiotics (13, 14). We demonstrated *in vitro* that strains MIMLh5 and ST3, alone and in combination, can efficiently adhere to pharyngeal epithelial cells, antagonize *S. pyogenes*, and modulate host innate immunity by inducing potentially protective effects. We gave particular attention to bacterial immunomodulatory properties because modulation of the host's immunity is one of the most commonly purported benefits of the consumption of probiotics and is supported by an increasing number of *in vitro* and *in vivo* studies (7). We found that the combination of MIMLh5 and ST3 resulted in a synergistic effect, according to cytokine induction, that might help the host immune system react to potential pathogens while maintaining a balance between pro- and anti-inflammatory cytokines, thus preventing possible exaggerated responses. Finally, we observed that these microorganisms grow efficiently when cocultured in milk, suggesting that the preparation of a milk-based fermented product containing both MIMLh5 and ST3 can be a practicable solution for the administration of these bacteria.

In conclusion, we propose the combined use of *Lactobacillus helveticus* MIMLh5 and *Streptococcus salivarius* ST3 for the preparation of novel products that display probiotic properties for the pharyngeal mucosa. A clinical study will soon be carried out to confirm that the *in vitro* properties exerted by strains MIMLh5 and ST3 can result in an actual benefit to the human host in terms of preventing oropharyngeal infections.

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