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Inhibition of *Staphylococcus aureus* Invasion into Bovine Mammary Epithelial Cells by Contact with Live *Lactobacillus casei*

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Staphylococcus aureus **is a major pathogen that is responsible for mastitis in dairy herds. *S. aureus* mastitis is difficult to treat and prone to recurrence despite antibiotic treatment. The ability of *S. aureus* to invade bovine mammary epithelial cells (bMEC) is evoked to explain this chronicity. One sustainable alternative to treat or prevent mastitis is the use of lactic acid bacteria (LAB) as mammary probiotics. In this study, we tested the ability of *Lactobacillus casei* strains to prevent invasion of bMEC by two *S. aureus* bovine strains, RF122 and Newbould305, which reproducibly induce acute and moderate mastitis, respectively. *L. casei* strains affected adhesion and/or internalization of *S. aureus* in a strain-dependent manner. Interestingly, *L. casei* CIRM-BIA 667 reduced *S. aureus* Newbould305 and RF122 internalization by 60 to 80%, and this inhibition was confirmed for two other *L. casei* strains, including one isolated from bovine teat canal. The protective effect occurred without affecting bMEC morphology and viability. Once internalized, the fate of *S. aureus* was not affected by *L. casei*. It should be noted that *L. casei* was internalized at a low rate but survived in bMEC cells with a better efficiency than that of *S. aureus* RF122. Inhibition of *S. aureus* adhesion was maintained with heat-killed *L. casei*, whereas contact between live *L. casei* and *S. aureus* or bMEC was required to prevent *S. aureus* internalization. This first study of the antagonism of LAB toward *S. aureus* in a mammary context opens avenues for the development of novel control strategies against this major pathogen.**

Staphylococcus aureus is an opportunistic pathogen with a broad host range, and it is a leading cause of chronic and acute infections in humans and domesticated animals worldwide (1–4). Among these infections mastitis is a major disease, affecting dairy herds and resulting in huge economic losses all along the milk production chain (5–7). In milk production, *Staphylococcus* species are the main contagious pathogens responsible for clinical and subclinical mastitis in lactating cows (5). *S. aureus* generally causes more acute infections than other staphylococcal species, which can be linked to its ability to colonize the host tissue and thus cause persisting and relapsing infections (8).

To date, intramammary administration of antibiotics is the most common method to treat bovine mastitis (9, 10). However, antibiotic treatments have a low cure rate during lactation for many mastitis pathogens and especially for *S. aureus*, frequently resulting in chronic and recurrent infections. The mechanism of persistence of *S. aureus* in its host is still not fully understood. One confirmed mechanism used to evade host defenses is internalization into host cells. It is now well established that *S. aureus* can adhere to and internalize into mammary gland epithelial cells (8). A variety of surface-exposed (protein A and fibrinogen- and fibronectin-binding proteins) and secreted (enterotoxins, hemolysins, and coagulase) virulence factors allow it to colonize, invade, and multiply in host tissues (8, 11–14).

Many strategies have been proposed to counteract the infectious cycle of *S. aureus* within the mammary gland. Critical steps, like adhesion and invasion of the host cells, can be targeted by innovative strategies that take account of the increasing social demand for a sustainable agriculture with reduced inputs such as antibiotics. In recent years, the concept of biological control has emerged as one interesting sustainable alternative to fight against pathogens. The range of applications of probiotic bacteria thus has broadened, and they are now considered a possibility for alternative treatments against mastitis (15, 16). The inhibitory activities of lactic acid bacteria (LAB) with a GRAS (generally recognized as safe) status against pathogens have been under scrutiny to address the problem of pathogen colonization in different ecosystems. Lactobacilli are known to have a protective effect against some infections. This ability has been related to the adhesion properties of epithelial cells, which inhibit pathogen adhesion by specific competition or by steric hindrance, as well as to growth inhibition of pathogens by the secretion of bacteriocins, H_2O_2, or other antimicrobial compounds, and to competition for nutrients and modulation of the host immune response (17). Such properties are harnessed in the development of vaginal probiotics used to prevent urogenital infections (18). Recently, the use of a bacteriocin-producing *Lactococcus lactis* strain was reported to be as efficient as a conventional antibiotic therapy to treat staphylococcal mastitis (19, 20). Encouraging results were also obtained with a *Lactobacillus perolens* strain which was able to inhibit several mastitis-causing pathogens *in vitro*, to coaggregate with all of them, and to adhere to bovine teat canal epithelial cells without affecting udder aspect or the appearance of milk (21). These alternative insights into intramammary infections provide new leads in the fight against mastitis.

In this work, we evaluated the ability of *Lactobacillus casei* to counteract *S. aureus* adhesion to and internalization into bovine mammary epithelial cells (bMEC). *L. casei* CIRM-BIA 667 was selected for this study on the basis of (i) its probiotic effects in the intestinal ecosystem (22, 23) and (ii) its inhibitory activity against

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staphylococcal biofilm formation (personal observation). The main results were further confirmed with two additional strains, BL23 and CIRM-BIA 1542, a strain isolated from bovine teat canal.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Two bovine strains of *Staphylococcus aureus* were used in this study: *S. aureus* RF122 (renamed ET-3 in Herron-Olson [24]) and *S. aureus* Newbould 305 (here referred to as NB305). These strains are well characterized and reproducibly induce severe or mild mastitis in experimental infections (24, 25). The *Lactobacillus casei* strain, CIRM-BIA 667 (here referred to as 667 and also known as CNRZ 313 and ATCC 393), was used to assess inhibitory capabilities against staphylococcal infection *in vitro*. It is the type strain for *L. casei*. Two additional *L. casei* strains were included: *L. casei* BL23, known for its probiotic properties (26, 27), and *L. casei* CIRM-BIA 1542 (here referred to as 1542), isolated from bovine teat canal (this study).

S. aureus strain RF122 carrying the plasmid pCtuf-*gfp* (28) was constructed in this study to allow constitutive expression of green fluorescent protein (GFP) in this strain and direct visualization of *S. aureus* adhered to or internalized into MAC-T cells by confocal microscopy (see below). Subcultures prior to invasion assays were performed overnight as follows. For *S. aureus* strains, culture was carried out in brain heart infusion medium (BHI; pH 7.4; BD, Le Pont de Claix, France) at 37°C under agitation (180 rpm), and *L. casei* was cultured in Man Rogosa Sharpe medium (MRS; pH 6.8; BD, Le Pont de Claix, France) at 30°C without shaking. Subcultures were washed once with phosphate-buffered saline (PBS) and suspended at different concentrations in Dulbecco's modified Eagle's medium (DMEM; pH 7.4; D. Dutscher, Brumath, France).

Bacterial concentrations in subcultures were estimated by spectrophotometric measurements at 600 nm with a VWR V-1200 spectrophotometer. They were further confirmed by determination of the bacterial population using a micromethod as previously described (29). The *S. aureus* population (in CFU/ml) was determined on mannitol salt agar (MSA; D. Dutscher, Brumath, France) after 24 h of incubation at 37°C. The *L. casei* population was determined on MRS (pH 5.4) and incubated anaerobically for 48 h at 37°C in an anaerobic jar.

Mammary epithelial cells and culture conditions. The established bovine mammary epithelial cell (MAC-T) line (30) (Nexia Biotechnologies, Quebec, Canada) has been widely used for invasion assays (8) and thus was retained for this study. MAC-T cells were cultured in T75 cell culture flasks using MAC-T medium: DMEM containing 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 10 mg/ml streptomycin, and 5 μg/ml insulin (D. Dutscher). Cells were incubated at 37°C in a humidified incubator with 5% CO_2. They were cultured to a confluent monolayer, treated with 0.05% trypsin (Gibco-BRL, Grand Island, NY), and suspended in fresh MAC-T medium at a concentration of 2×10^5 cells/ml. For adhesion and internalization assays, cells were then seeded in 12-well plates (2×10^5 cells/well) and incubated overnight at 37°C in 5% CO_2 to obtain a confluent monolayer.

Adhesion assays. Adhesion assays were adapted from Almeida et al. (8) and modified as follows. Confluent monolayers of MAC-T cells (2.5×10^5 cells/well) were washed twice with PBS and incubated at 37°C in 5% CO_2 with 1 ml of *S. aureus* suspension in DMEM at 2.5×10^6, 1×10^7, or 2.5×10^7 CFU/ml to achieve a multiplicity of infection (MOI; ratio of *S. aureus* organisms to cells) of 10:1, 40:1, or 100:1, respectively. Adhesion assays with *L. casei* were performed by adding 1 ml of *L. casei* at 1×10^8 CFU/ml or 5×10^8 CFU/ml to achieve a ratio of interaction (ROI; ratio of *L. casei* organisms to cells) of 400:1 or 2,000:1. *S. aureus* and *L. casei* adhesion was measured 1 h postinfection.

For adhesion inhibition assays, cells were primarily incubated with *L. casei* at an ROI of 200:1, 400:1, or 2,000:1 for 2 h at 37°C with 5% CO_2 and washed twice with PBS prior to infection with *S. aureus* for 1 h. When specified, the *L. casei* suspension was separated from the cell monolayer using a 0.4-μm cell culture insert filter (Millicell; Millipore Corporation, Switzerland). After incubation steps, MAC-T monolayers were washed four times with PBS and treated with 0.05% trypsin for 10 min at 37°C. Cells were centrifuged for 5 min at $800 \times g$ and lysed using 100 μl of 0.01% Triton in sterile water. The population of *S. aureus* that adhered (CFU/ml) was determined using a micromethod as described above.

The adhesion assay of *S. aureus* alone was used as a reference. Adhesion rates were then defined as the adhered *S. aureus* population in the presence of *L. casei* relative to the adhered *S. aureus* population in the reference experiment.

For some experiments, heat-killed *L. casei* 667 cells were prepared by incubating the *L. casei* suspension in DMEM at 95°C for 15 min prior to addition to bMEC. Supernatant samples were prepared from a 2-h culture on DMEM of *L. casei* 667 inoculated at 5×10^8 CFU/ml, and the pH was adjusted to 7.4.

Internalization assays. Internalization assays were adapted from Almeida et al. (8) and modified as follows. Confluent monolayers of MAC-T cells (2.5×10^5 cells/well) were washed twice with PBS and incubated at 37°C in 5% CO_2 with 1 ml of *S. aureus* and/or *L. casei* suspension in DMEM at an MOI of 10:1, 40:1, or 100:1 for *S. aureus* and an ROI of 200:1, 400:1, or 2,000:1 for *L. casei*. *S. aureus* and *L. casei* internalizations were measured 2 h postinfection. For internalization inhibition assays, *L. casei* and *S. aureus* were simultaneously added to the cells for 2 h. When specified, *L. casei* was separated from the cell monolayer and *S. aureus* using a 0.4-μm cell culture insert filter (Millicell; Millipore Corporation, Switzerland). *S. aureus* internalization was measured 2 h postinfection following an additional 2-h incubation step with DMEM supplemented with gentamicin (100 μg/ml). This step resulted in the killing of extracellular bacteria and allowed the numeration of the internalized bacterial population only. Subsequently, MAC-T monolayers were washed four times with PBS, treated with trypsin, centrifuged for 5 min at $800 \times g$, and lysed in 0.01% Triton. *S. aureus* and *L. casei* populations were determined as described above.

The internalization assay of *S. aureus* alone was used as a reference. Internalization rates were then defined as the internalized *S. aureus* population in the presence of *L. casei* relative to the internalized *S. aureus* population in the reference experiment. Heat-killed *L. casei* 667 and supernatant of *L. casei* 667 cells were prepared as described above.

Intracellular survival assays. Internalization assays were performed as described above with *S. aureus* RF122 at an MOI of 10:1, 40:1, or 100:1 and in the absence or presence of *L. casei* 667 (ROI of 2,000:1). The internalized *S. aureus* population measured after these 2 h of infection was used as the starting point for intracellular survival assay. Cells were further incubated in DMEM containing gentamicin (25 μg/ml) at 37°C in 5% CO_2, and the remaining internalized *S. aureus* population was measured 24, 48, and 72 h postinfection. DMEM-gentamicin medium was changed every 24 h.

Cell counting and cell viability assays. Cell density and viability were determined using a hemocytometer by the trypan blue exclusion method 2, 24, 48, and 72 h postinfection.

MTT cell viability assays. Cell viability was measured during the intracellular survival assay (see above) at 2, 24, 48, and 72 h postinfection using methylthiazolyldiphenyltetrazolium bromide (MTT) as previously described (31). Briefly, following incubation with DMEM containing 25 μg/ml gentamicin, cells were washed four times and incubated in 0.5 mg/ml MTT in PBS for 4 h at 37°C in 5% CO_2. The medium was removed, and isopropanol was added for 30 min with shaking at 350 rpm. Absorbance was read at 570 nm with a background at 690 nm. Uninfected cells were used as a negative control (100% viability), and cells treated with 0.01% Triton served as a positive control of mortality (0% viability). Relative viability was expressed with regard to uninfected cells.

Analysis of cellular morphology during internalization assay by confocal microscopy. MAC-T cells were cultured in 8-well Labtek chamber slides (NalgeNunc International, Naperville, IL). A total of 5×10^4 cells were seeded in each well and incubated overnight at 37°C with 5% CO_2. Internalization assays were performed as described above, including

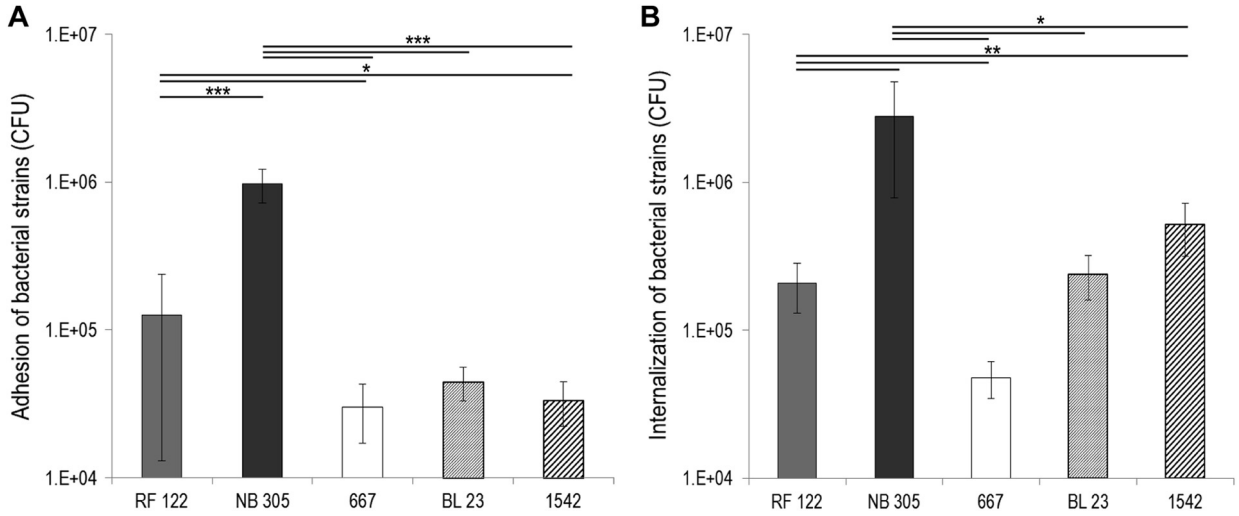

FIG 1 Adhesion to and internalization into bMEC of *S. aureus* strains RF122 and NB305 and *L. casei* strains CIRM-BIA 667, BL23, and CIRM-BIA 1542. *S. aureus* (MOI of 100:1) and *L. casei* (ROI of 2,000:1) populations adhered to (A) and internalized into (B) bMEC were determined after 1 and 2 h of interaction, respectively. Data are presented as mean populations per well (i.e., corresponding to 2.5×10^5 bMEC) ± standard deviations. Each experiment was done in triplicate, and differences between groups were compared using Student's *t* test. *, $P < 0.05$; ***, $P < 0.0005$.

a 2-h step with gentamicin (100 μg/ml) to kill extracellular bacteria, with an MOI of 100:1 for *S. aureus* RF122 and an ROI of 2,000:1 for *L. casei* 667. Following the internalization assay, cells were washed four times with PBS, fixed for 30 min in PBS containing 4% paraformaldehyde, and permeabilized with PBS containing 0.1% saponin for 10 min. Staining was performed in darkness at room temperature. Cells were stained using the fluorescent nucleic acid stain SYTO 9 from the LIVE/DEAD BacLight stain kit (Molecular Probes Inc., Leiden, The Netherlands) for 30 min, which allowed staining of both bacteria and MAC-T cells. Alternatively, the *S. aureus* strain RF122 carrying the plasmid pCtuf-*gfp* was used and combined with the staining of cell actin cytoskeleton with phalloidin (Interchim, Montluçon, France) at 1 U/ml in PBS containing 1% bovine serum albumin for 30 min. This allowed visualization of *S. aureus* (green) and cytoskeleton (red). Images were acquired using the confocal Nikon C1Si microscope (Nikon, Tokyo, Japan) with an excitation wavelength of 488 (for SYTO 9 and GFP) and of 543 nm (for phalloidin) and using a lens with ×100 magnification. Emission of fluorescence was monitored at 515 nm (± 15 nm) for SYTO 9 and GFP and 590 nm for phalloidin. Image analysis was performed with Image J (National Institutes of Health, Bethesda, MD; http://imagej.nih.gov/ij/).

Statistical analysis. Each experiment was done in triplicate (biological repeats). Statistical analysis was performed with R software (R Development Core Team, 2007). The differences among the groups were assessed using Student's *t* test with Bonferroni's correction considering a *P* value lower than 0.05.

RESULTS

Adhesion and internalization capacities of *S. aureus* and *L. casei*. We tested *in vitro* the adhesion and internalization abilities of two *S. aureus* bovine strains, namely, RF122 and NB305, which induce severe and mild bovine mastitis, respectively. Interestingly, we found that the adhesion and internalization capacities of NB305 were higher than those of RF122. Adhered and internalized *S. aureus* populations were 5- and 40-fold larger, respectively, for strain NB305 than for RF122 at an MOI of 100:1 (Fig. 1). As a comparison, adhesion and internalization capacities of *L. casei* were also assessed. The three *L. casei* strains used exhibited poor adhesion capacities compared to *S. aureus*, as illustrated in Fig. 1. Indeed, despite a higher bacterium/cell ratio (2,000:1 and 100:1 for *L. casei* and *S. aureus*, respectively), the adhered and internalized populations were smaller for *L. casei* than for *S. aureus*.

***L. casei* 667 reduced the adhesion of *S. aureus* RF122.** Several conditions of adhesion were tested to evaluate the capacities of *L. casei* to prevent adhesion of *S. aureus* RF122 and NB305 to bMEC: preincubation of bMEC with *L. casei* prior to infection by *S. aureus* or coinfection of both species. Three MOIs were tested for *S. aureus* in combination with three ROIs for *L. casei* 667. Conditions leading to *L. casei*-mediated inhibition were preincubation of MAC-T cells for 2 h with *L. casei* at an ROI of 200:1, 400:1, or 2,000:1, followed by the addition of *S. aureus* at an MOI of 100:1. Under these conditions, a significant reduction of the adhesion rate was observed for *S. aureus* RF122, down to ~60% of the adhesion observed with *S. aureus* RF122 alone (Fig. 2). It should be noted that during this experiment and subsequent assays of adhesion and internalization, the density of the MAC-T cell monolayer was conserved, as confirmed by direct microscopic observation and cell counting. Thus, the lower adhered population of *S. aureus* RF122 to MAC-T cells did not result from a smaller amount of attached MAC-T cells in wells when incubated with *L. casei*. Under the same experimental conditions, *L. casei* 667 did not significantly affect the adhesion rate of *S. aureus* NB305 (Fig. 2). Similarly, no significant inhibition of *S. aureus* RF122 or NB305 was observed with the two additional *L. casei* strains tested, BL23 and 1542 (see Fig. 4A). This result indicates that the inhibitory efficacy of *L. casei* depends on both the *S. aureus* and *L. casei* strains used.

***L. casei* reduced internalization of *S. aureus* RF122 and NB305.** Beyond *L. casei*'s ability to impair *S. aureus* adhesion, we tested its inhibitory potential against *S. aureus* RF122 and NB305 internalization into MAC-T cells. As mentioned for adhesion assays, several conditions were tested. Conditions resulting in *L. casei*-mediated inhibition were the coincubation of *L. casei* at an ROI of 2,000:1 with *S. aureus* at an MOI of 10:1, 40:1, or 100:1. Under these conditions, coinfection of *S. aureus* RF122 or NB305 with *L. casei* led to a significant decrease of their internalization

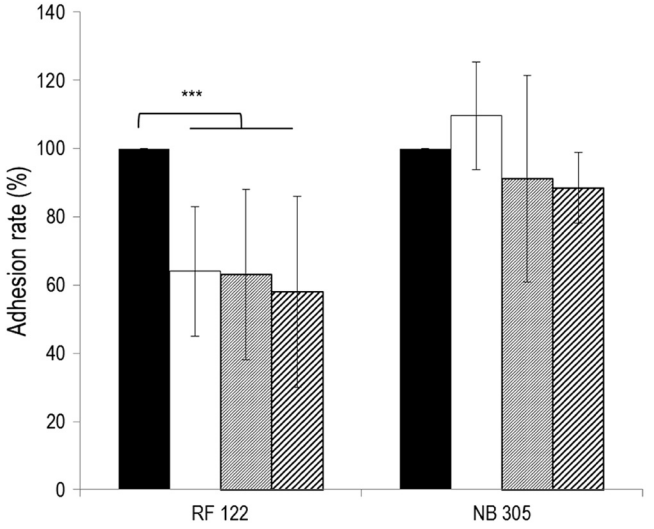

FIG 2 Inhibition of *S. aureus* RF122 and NB305 adhesion to bMEC by *L. casei* CIRM-BIA 667. Shown are adhesion rates of *S. aureus* strains after 1 h of interaction with bMEC and following 2 h of preincubation of cells with *L. casei* at an ROI of 200:1 (white bars), 400:1 (gray bars), and 2,000:1 (hatched bars). *S. aureus* was used at an MOI of 100:1. An adhesion assay of *S. aureus* alone was used as a reference (black bars). Adhesion rates were then defined as the adhered *S. aureus* population in the presence of *L. casei* relative to the adhered *S. aureus* population in the reference experiment. Data are presented as means ± standard deviations. Each experiment was done in triplicate, and differences between groups were compared using Student's *t* test with Bonferroni's correction. ***, $P < 0.0005$.

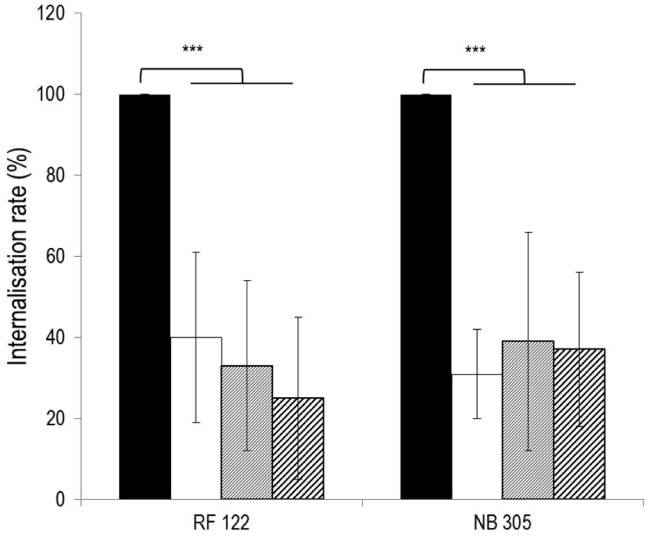

FIG 3 Inhibition of *S. aureus* RF122 and NB305 internalization into bMEC by *L. casei* CIRM-BIA 667. Shown are internalization rates of *S. aureus* strains after 2 h of interaction with bMEC with coincubation with *L. casei* at an ROI of 2,000:1. *S. aureus* strains were used at an MOI of 10:1 (white bars), 40:1 (gray bars), or 100:1 (hatched bars). The internalization assay of *S. aureus* alone was used as a reference (black bars). Internalization rates were then defined as the internalized *S. aureus* population in the presence of *L. casei* relative to the internalized *S. aureus* population in the reference experiment. Data are presented as means ± standard deviations. Each experiment was done in triplicate, and differences between groups were compared using Student's *t* test with Bonferroni's correction. ***, $P < 0.0005$.

rates by 61 to 75% (Fig. 3). This result was further confirmed with the two additional *L. casei* strains (Fig. 4B). Of note, due to the strong acidification of the medium by mixed culture of *L. casei* 1542 and *S. aureus*, the ROI was only 400:1 in mixed cultures for this *L. casei* strain, compared to an ROI of 2,000:1 for strains 667 and BL23. Despite this lower ROI, *L. casei* 1542 efficiently inhibited *S. aureus* internalization. It should be observed here that the total number of viable staphylococci was unaffected by the presence of *L. casei* in adhesion or internalization assays. *S. aureus* was

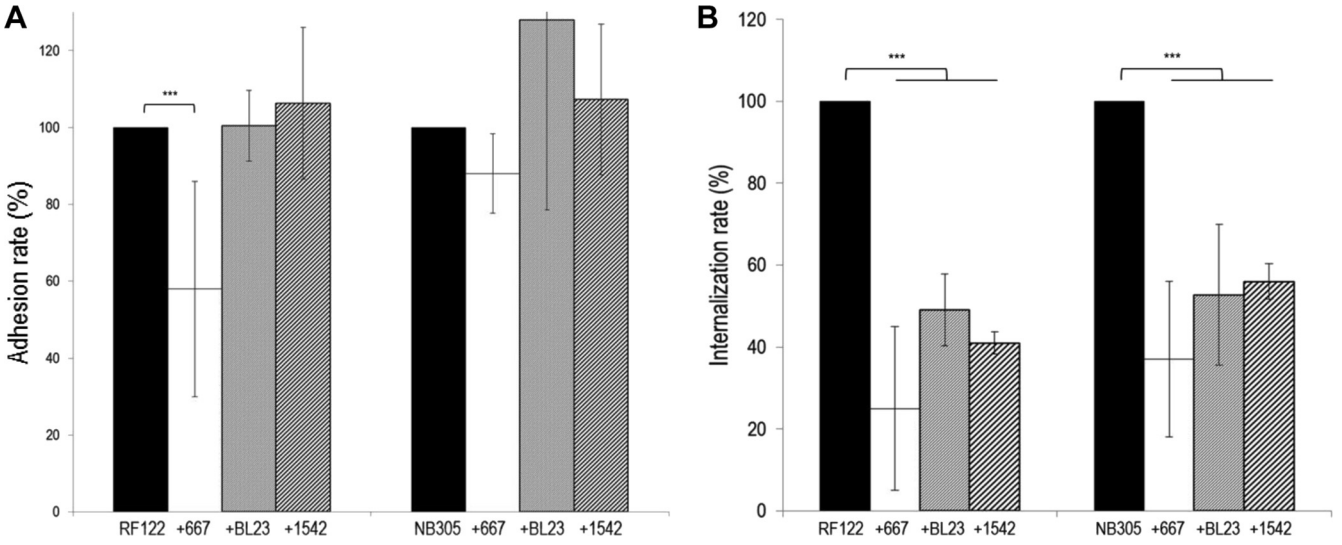

FIG 4 Inhibition of adhesion and internalization of *S. aureus* RF122 and NB305 by *L. casei* strains. (A) Rates of adhesion of *S. aureus* RF122 and NB305 strains to bMEC following preincubation of cells with *L. casei* CIRM-BIA 667 (white bars), BL23 (gray bars), and CIRM-BIA 1542 (hatched bars) at an ROI of 2,000:1. (B) Rates of internalization of *S. aureus* RF122 and NB305 into bMEC in the presence of *L. casei* CIRM-BIA 667 (white bars) and BL23 (gray bars) at an ROI of 2,000:1 and CIRM-BIA 1542 (hatched bars) at an ROI of 400:1. Adhesion and internalization assays were performed with an *S. aureus* MOI of 100:1. Adhesion/internalization assays of *S. aureus* alone were used as a reference (black bars). Adhesion/internalization rates were then defined as the adhered/internalized *S. aureus* population in the presence of *L. casei* relative to the adhered/internalized *S. aureus* population in the reference experiment. Data are presented as means ± standard deviations. Each experiment was done in triplicate, and differences between groups were compared using Student's *t* test with Bonferroni's correction. ***, $P < 0.0005$.

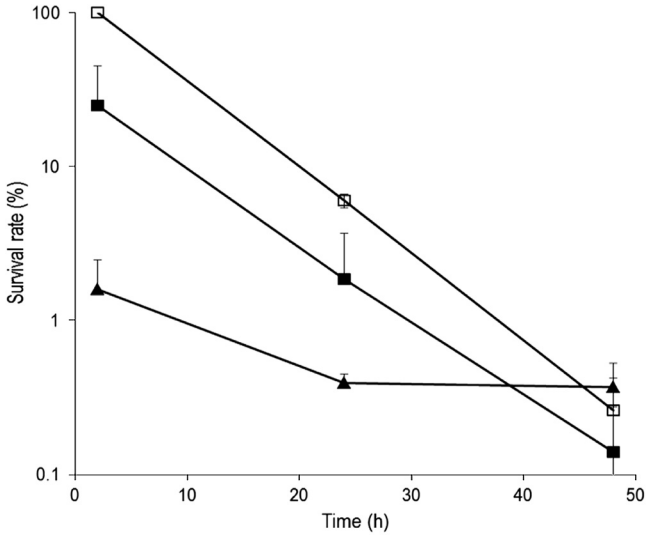

FIG 5 Survival rate of *S. aureus* RF122 and *L. casei* CIRM-BIA 667 within bMEC. bMEC were incubated for 2 h with *S. aureus* RF122 (MOI, 100:1) with or without *L. casei* CIRM-BIA 667 (ROI, 2,000:1) in DMEM. Following a 2-h incubation step with gentamicin (100 μg/ml) to kill extracellular bacteria, cells were further incubated with gentamicin (25 μg/ml) for 24 and 48 h. The initial internalized *S. aureus* population measured after the 2 h of infection by *S. aureus* alone was used as the reference. The remaining internalized population of *S. aureus* alone (□) or in coinfection with *L. casei* (■) and *L. casei* (▲) then were measured and expressed relative to an *S. aureus* reference population. Data are presented as the mean survival rate ± standard deviations. Each experiment was done in triplicate, and differences between half-lives were compared using Student's *t* test.

indeed able to grow in DMEM during incubation with bMEC, and the population reached after 1 or 2 h of infection was similar with or without preincubation or coinfection with *L. casei* (see Fig. S1 in the supplemental material). Interestingly, inhibition of internalization was reciprocal. Hence, the rate of *L. casei* 667 internalization was reduced by 58 and 50% in coinfection experiments with *S. aureus* RF122 and NB305, respectively.

***L. casei* 667 did not alter intracellular survival of *S. aureus* RF122.** To investigate the fate of internalized bacteria, intracellular survival of *S. aureus* RF122 was monitored 24, 48, and 72 h postinfection. A rapid decrease of the *S. aureus* internalized population was observed with only 6 and 0.26% of the initial internalized *S. aureus* population after 24 and 48 h of infection (Fig. 5). The *S. aureus* internalized population was lower in the presence of *L. casei* but a similar decrease of the *S. aureus* internalized population was observed, as illustrated by half-lives of internalized *S. aureus* into MAC-T of 6.1 and 5.1 h with and without *L. casei*, respectively ($P = 0.12$). This result was confirmed with *S. aureus* MOIs of 40:1 and 10:1 (data not shown). Intracellular survival of *L. casei* was greater than that of *S. aureus* RF122, as illustrated by a lower rate of decrease of the *L. casei* population ($P = 0.002$) (Fig. 5).

***L. casei* 667 treatment did not affect MAC-T cell viability.** We showed that *L. casei* was able to inhibit *S. aureus* invasion into MAC-T cells without affecting the cell monolayer density. Additional studies were done to investigate the effect of *L. casei* 667 on cell viability and morphology. Cell viability was estimated by trypan blue exclusion and indicated that viability was above 99% for all of the bacterial concentrations tested (data not shown). In agreement, cell viability assessed by MTT assays revealed the same decrease of viability for infected and uninfected cells, i.e., a drop in cell viability of 20 to 25% from 48 h onwards (see Fig. S2 in the supplemental material). Finally, direct observation of bMEC by confocal microscopy during internalization assays confirmed that the general cell architecture was similar in untreated control cells and cells treated with *L. casei* or *S. aureus* RF122 either alone or in combination (Fig. 6).

Inhibition of internalization required live *L. casei*, whereas adhesion did not. To further characterize the inhibition observed

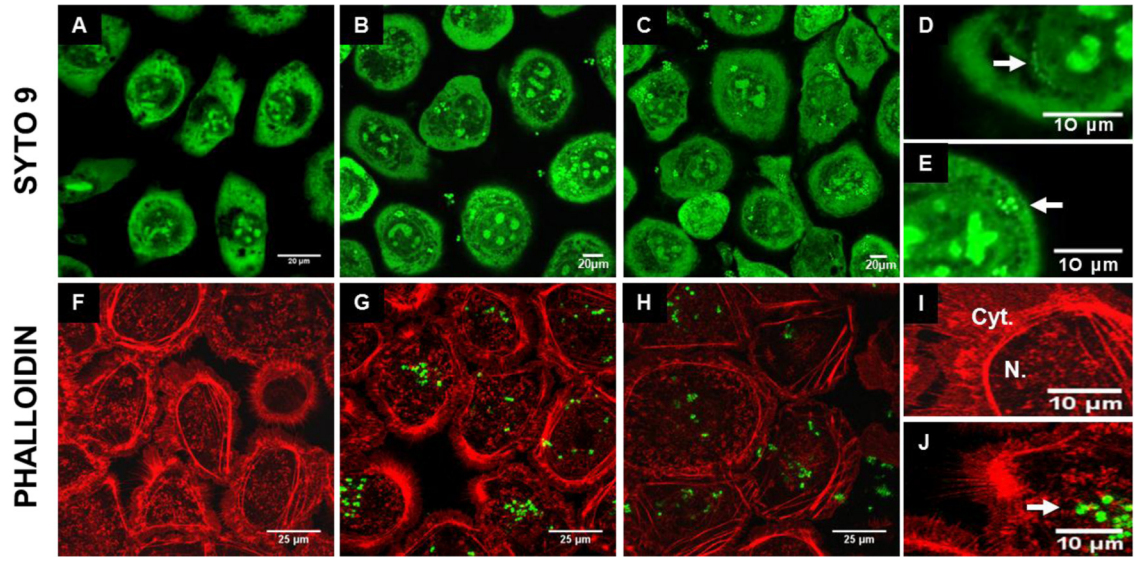

FIG 6 Fluorescent confocal microscopy of mammary epithelial cells during bacterial infections. SYTO 9 (A to E) and phalloidin (F to J) stainings were used to observe bMEC structure following internalization assays with *S. aureus* RF122 (carrying plasmid pCtuf-*gfp* in the case of phalloidin staining) at an MOI of 100:1. MAC-T cells were either untreated (control; A, F, and I) or treated with *S. aureus* alone (B, E, G, and J), *L. casei* CIRM-BIA 667 alone at an ROI of 2,000:1 (D), or *S. aureus* and *L. casei* in cocultures (C and H). A lens with a ×100 magnification was used, and panels D to E and I to J are electronically zoomed. Arrows indicate internalized *L. casei* (D) or internalized *S. aureus* (E and J). Cyt., cytoplasm; N., nucleus.

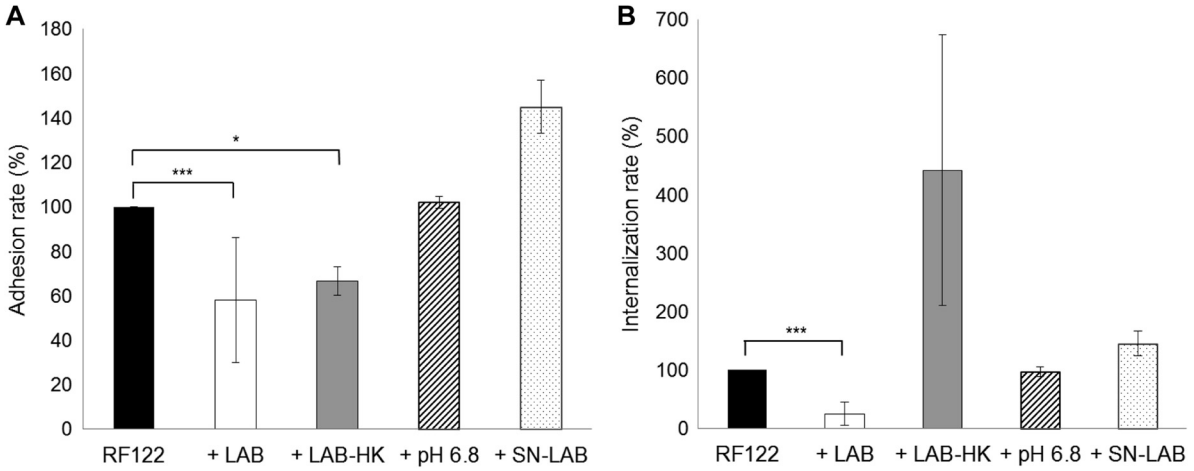

FIG 7 Adhesion and internalization rates of *S. aureus* RF122 with different treatments. (A) Adhesion rates of *S. aureus* RF122 to bMEC at an MOI of 100:1, either alone or with preincubation of cells with *L. casei* CIRM-BIA 667 at an ROI of 2,000:1 (+LAB), heat-killed *L. casei* CIRM-BIA 667 at an ROI of 2,000:1 (+LAB-HK), DMEM acidified to pH 6.8 with lactic acid (+pH 6.8), or *L. casei* CIRM-BIA 667 supernatant (+SN-LAB). (B) Internalization rates of *S. aureus* RF122 into bMEC at an MOI of 100:1, either alone or with coincubation with *L. casei* CIRM-BIA 667 at an ROI of 2,000:1 (+LAB), heat-killed *L. casei* CIRM-BIA 667 at an ROI of 2,000:1 (+LAB-HK), DMEM acidified to pH 6.8 with lactic acid (+pH 6.8), or *L. casei* CIRM-BIA 667 supernatant (+SN-LAB). Adhesion/internalization assays of *S. aureus* alone were used as references. Adhesion/internalization rates were then defined as the adhered/internalized *S. aureus* population in the presence of *L. casei*, lactic acid, or supernatant relative to the adhered/internalized *S. aureus* population in the reference experiment. Data are presented as means ± standard deviations. Each experiment was done in triplicate, and differences between groups were compared using Student's *t* test with Bonferroni's correction. *, $P < 0.05$; ***, $P < 0.0005$.

with *L. casei*, additional adhesion and internalization experiments were carried out by replacing *L. casei* with either (i) DMEM artificially acidified to pH 6.8 with lactic acid (corresponding to the pH reached after 2 h of incubation of MAC-T cells with *L. casei* at an ROI of 2,000:1), (ii) supernatant of *L. casei* grown in DMEM, or (iii) heat-killed *L. casei*. Neither DMEM containing lactic acid nor *L. casei* supernatant affected *S. aureus* RF122 adhesion or internalization rates (Fig. 7). Heat-killed *L. casei* was still able to inhibit *S. aureus* RF122 adhesion, whereas the inhibitory effect of *L. casei* on *S. aureus* internalization occurred only in the presence of live *L. casei* (Fig. 7). Surprisingly, heat-killed *L. casei* even seemed to favor *S. aureus* internalization, although the difference was not statistically significant ($P = 0.13$).

Inhibition of adhesion and internalization required contact with *L. casei*. To further characterize the mechanism of inhibition, additional adhesion and internalization experiments were carried out using cell culture insert filters in order to separate *L. casei* from bMEC and *S. aureus*. *L. casei*-mediated inhibition of adhesion was released when *L. casei* was separated from bMEC during the preincubation step (Fig. 8A). Likewise, *L. casei*-mediated inhibition of internalization was not retained using cell culture insert filters, indicating that the contact of live *L. casei* with bMEC and/or *S. aureus* was required (Fig. 8B).

DISCUSSION

In this work, we established the ability of *L. casei* to reduce adhesion to and/or internalization into MAC-T cells of two bovine *S. aureus* strains. The ability of *L. casei* to affect adhesion was strain dependent. *L. casei* 667 was the only *L. casei* strain able to inhibit adhesion of *S. aureus* RF122 by 40%. This was not confirmed on the highly adherent *S. aureus* strain NB305, indicating that *L. casei* 667 inhibition of *S. aureus* adhesion to bMEC was restricted to the *S. aureus* strain with low adhesion capacity (i.e., RF122). The poor capacity of *L. casei* to inhibit *S. aureus* adhesion is probably related to its low adhesion capacity compared to that of *S. aureus*. Rates of adhesion to MAC-T cells were 4- to 30-fold lower for *L. casei* than for *S. aureus*, although the bacterium/bMEC ratio was 20-fold higher. Such low-adherence properties of *L. casei* 667 to epithelial cells had been previously reported (22). More striking in this work was the ability of *L. casei* to impair *S. aureus* internalization. As of this time, very few studies have investigated the ability of probiotic lactic acid bacteria to modulate internalization of pathogens within host cells (32). One interesting outcome was the inhibition of internalization of the two *S. aureus* strains tested by the three *L. casei* strains tested, whereas inhibition of *S. aureus* adhesion to bMEC was limited to one *S. aureus*/*L. casei* couple. Following internalization, the *S. aureus* bacterial population decreased. This was in agreement with a study by Martinez-Pulgarin et al., who reported that after a short period of intracellular replication (2 h), internalized *S. aureus* concentration in MAC-T cells gradually decreased over time (33). In fact, the fate of internalized *S. aureus* was similar with or without *L. casei*, suggesting that *L. casei* did not affect *S. aureus* physiology once internalized.

During these internalization assays, we also established the capacity of *L. casei* to internalize, and this was strongly strain dependent. Interestingly, *L. casei* 1542, isolated from the teat canal, internalized more efficiently into bMEC cells. This might reflect an adaptation of some *L. casei* strains to the bovine host, as previously shown for *S. aureus* ruminant isolates (34–36). The internalization capacities of the three *L. casei* strains were lower than those of *S. aureus* NB305 and, to a lesser extent, *S. aureus* RF122. Contrary to the adhesion capacity of LAB, which is well documented, only a few studies report the internalization of lactic acid bacteria (37). A fibronectin-binding protein has been identified in the genomes of *Lactobacillus* species, suggesting a capacity to adhere and to be internalized (38–41). Interestingly, the survival rate of *L. casei* in MAC-T cells was better than that of *S. aureus*. This improved

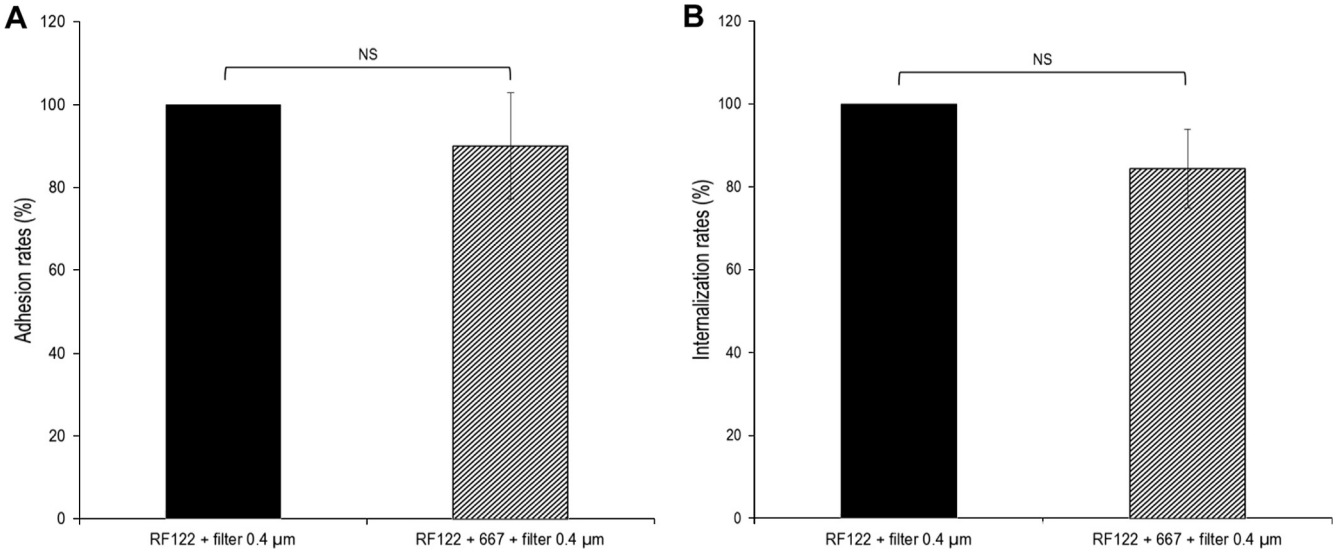

FIG 8 *L. casei* inhibition of *S. aureus* adhesion and internalization requires contact with bMEC and/or *S. aureus.* Adhesion and internalization assays were performed, as previously described (see the legends to Fig. 2 and 3), using *S. aureus* RF122 at an MOI of 100:1 and *L. casei* CIRM-BIA 667 at an ROI of 2,000:1, except that *L. casei* was separated from *S. aureus* and bMEC using a cell culture insert. Adhesion/internalization assays of *S. aureus* alone with the cell culture insert were used as references. Adhesion/internalization rates were then defined as the adhered/internalized *S. aureus* population in the presence of *L. casei* relative to the adhered/internalized *S. aureus* population in the reference experiment. Data are presented as means ± standard deviations. Each experiment was done in triplicate, and differences between groups were compared using Student's *t* test. NS, not significant.

survival may be due to a better resistance to acid and oxidative stresses (42).

L. casei inhibition of *S. aureus* adhesion and internalization required specific conditions. Two features were common to adhesion and internalization inhibition: (i) in all cases, posttreatment of *S. aureus*-adhered or -internalized bMEC with *L. casei* did not alter *S. aureus* adhesion or internalization rates (data not shown), implying that the use of *L. casei* was indicated for prevention rather than for treatment of *S. aureus* mastitis; and (ii) in all cases, contact with *L. casei* cells was required, indicating that inhibition did not rely on diffusible compounds. However, other features differed between adhesion and internalization inhibition. Inhibition of *S. aureus* RF122 adhesion required preincubation with *L. casei*, whereas inhibition of internalization occurred only when *S. aureus* was coincubated with *L. casei*. In addition, live *L. casei* was required to inhibit internalization, whereas heat-killed *L. casei* still was able to affect *S. aureus* adhesion. This suggests that the mechanisms of adhesion and internalization inhibition involve two distinct processes.

The results of adhesion inhibition are in agreement with a competitive exclusion mechanism. Preincubation of MAC-T cells with live or heat-killed *L. casei* allowed saturation of adhesion sites prior to inoculation by *S. aureus*. This is consistent with other studies where a similar decrease in the *S. aureus* adhesion rate (approximately 40%) was observed by competition with viable or heat-killed *Lactobacillus* strains (43–46).

L. casei inhibition of *S. aureus* internalization probably involves one or more means acting alone or in combination: (i) modulation of bMEC physiology or integrity induced by contact; (ii) direct effect on *S. aureus*, including coaggregation, as observed for vaginal lactobacilli (47, 48), although preliminary experiments indicated that *L. casei* 667 exhibited poor aggregative abilities (data not shown); (iii) inhibition of *S. aureus* virulence expression, including major virulence regulators, as previously reported (48–51); and (iv) competition for attachment sites involved in internalization. In agreement with a competition mechanism, the *L. casei* internalization rate was also affected by *S. aureus*. To our knowledge, this is the first time such reciprocal competition for internalization has been demonstrated.

In conclusion, the basic requirements for a strain to be used as a probiotic against epithelial cell infection are that it must be able to adhere to the host epithelium, have no cytotoxic effect on host cells, and show antagonistic activity toward pathogenic bacteria (16, 17, 52). In this study, we showed that *L. casei* meets all of these criteria. *L. casei* was able to adhere to and even internalize into MAC-T cells and to prevent *S. aureus* internalization and, to a lesser extent, adhesion without modifying cell viability and morphology. The use of LAB to prevent *S. aureus* invasion into bMEC leads to interesting perspectives on new topical strategies to improve the efficiency of mastitis treatment and to reduce the chronicity of *S. aureus* infection.

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