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RESEARCH ARTICLE

Abstract

Probiotics could help combat infections and reduce antibiotic use. As use of live bacteria is limited in some cases by safety or regulatory concerns, the potential of inactivated bacteria is worth investigating. We evaluated the potential of live and heat-inactivated *Lactobacillus gasseri* LA806 to counteract *Staphylococcus aureus* and *Escherichia coli* infection cycles in an *in vitro* model of bovine mastitis. We assessed the ability of live and inactivated LA806 to impair pathogen colonisation of bovine mammary epithelial cells (bMECs) and to modulate cytokine expression by pathogen-stimulated bMECs. Live LA806 induced a five-fold decrease in *S. aureus* adhesion and internalisation (while not affecting *E. coli* colonisation) and decreased pro-inflammatory cytokine expression by *S. aureus*-stimulated bMECs (without interfering with the immune response to *E. coli*). The ability of inactivated LA806 ability to diminish *S. aureus* colonisation was two-fold lower than that of the live strain, but its anti-inflammatory properties were barely impacted. Even though LA806 effects were impaired after inactivation, both live and inactivated LA806 have barrier and immunomodulatory properties that could be useful to counteract *S. aureus* colonisation in the bovine mammary gland. As *S. aureus* is involved in various types of infection, LA806 potential would worth exploring in other contexts.

Keywords: probiotics, mammary infection, immunomodulation, lactic acid bacteria

1. Introduction

In the last decades, probiotics have been widely investigated for the treatment and prevention of human and animal diseases. Bacteria belonging to the *Lactobacillus* and *Bifidobacterium* genera are the most commonly used microorganisms due to their long history of safe use and valuable benefits for health (Brodmann *et al.*, 2017; Melo Pereira *et al.*, 2018). Some bacteria display properties useful for fighting pathogens, such as competitive adhesion to the epithelium, production of antimicrobial substances, competition for nutrients, coaggregation with pathogens, modulation of virulence expression in pathogens and/or modulation of the host immune system (Bermudez-Brito *et al.*, 2012; Even *et al.*, 2014; Melo Pereira *et al.*, 2018). Consequently, probiotics are considered as a means of combating infectious diseases and reducing massive antibiotic use in both humans and domesticated animals.

Bovine mastitis is a major infectious disease responsible for considerable economic losses in dairy farms and throughout the milk production chain (Bouchard *et al.*, 2013). Mastitis is an inflammation of the mammary gland generally due to bacterial infection. *Staphylococcus aureus* and *Escherichia coli* are among the main pathogens involved in ruminant mastitis. Prophylactic and curative treatments that are predominantly based on hygienic procedures and antibiotics are not fully effective, prompting the need for alternative or complementary strategies (Francoz *et al.*, 2017; Peton and Le Loir, 2014). Several studies have reported valuable characteristics of selected bacterial strains in the context of bovine mastitis (Armas *et al.*, 2017; Crispie *et al.*, 2008; Klostermann *et al.*, 2008; Malvisi *et al.*, 2016; Nader-Macías *et al.*, 2008; Pellegrino *et al.*, 2017; Souza *et al.*, 2018; Wang *et al.*, 2017). Our group showed that certain *Lactobacillus* strains can impair pathogen adhesion to and internalisation into bovine mammary epithelial cells

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(bMECs) and exhibit anti-inflammatory properties when incubated with pathogen-stimulated bMECs by decreasing the expression of several pro-inflammatory cytokines (Bouchard *et al.*, 2013, 2015; Souza *et al.*, 2018).

Although these results are promising and the bacteria used have a Generally Recognized as Safe (GRAS) or a Qualified Presumption of Safety (QPS) status, the use of live bacteria is a source of concern as such bacteria could have at least a transient negative impact on animal health by causing inflammation and/or infection. This belief is corroborated by the results of certain *in vivo* studies. For example, intra-mammary infusion of a live *Lactococcus lactis* strain in mastitic dairy cows was as effective as antibiotic treatment in a field trial (Crispie *et al.*, 2008; Klostermann *et al.*, 2008), but this benefit was associated with a transient inflammation following stimulation of the mammary immune system (Beecher *et al.*, 2009; Crispie *et al.*, 2008). Likewise, infusion of another *L. lactis* strain into the ewe mammary gland led to a transient clearance of the pathogens in the gland, but also to an increase in the inflammatory status of the gland (Mignacca *et al.*, 2017). A *Lactobacillus perolens* strain similarly provoked a transient pro-inflammatory reaction when infused into cows during lactation or the dry period (Frola *et al.*, 2012, 2013). Whilst stimulation of the immune system by intra-mammary infusion of live bacteria is likely to favour elimination of the pathogen, at least transiently, an exacerbated immune stimulation may provoke clinical symptoms of mastitis (Mignacca *et al.*, 2017). These contrasting results blur the boundary between the beneficial and deleterious effects of live bacteria infusion and challenge the use of live probiotic bacteria in the context of mastitis.

Bacterial viability is generally considered as a prerequisite for the functionality of probiotics (Aguilar-Toalá *et al.*, 2018; Terpou *et al.*, 2019). According to the WHO/FAO definition, probiotics are 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2001). This definition therefore stipulates that probiotics must be 'live' to provide health benefits. However, whereas some studies have reported the need for bacteria to be alive to exert their effects (Aguilar-Toalá *et al.*, 2018; Brodmann *et al.*, 2017; Douillard and Vos, 2019; Terpou *et al.*, 2019), others have demonstrated that viability is not mandatory for all probiotic effects, as not all mechanisms are directly linked to viability, particularly when the effect depends on surface-exposed components (Aguilar-Toalá *et al.*, 2018; Brodmann *et al.*, 2017; Douillard and Vos, 2019; Terpou *et al.*, 2019). In addition, it cannot be denied that non-viable (inactivated) bacteria may have advantages over live bacteria in terms of safety, by reducing the risk of microbial translocation, infection and inflammatory responses (Aguilar-Toalá *et al.*, 2018; Catozzi *et al.*, 2019; Piqué *et al.*, 2019). The use of inactivated bacteria may therefore be safer – particularly in humans or animals with a weak immune system or under inflammatory conditions – and still valuable, provided that the beneficial effects of the bacteria are at least partially preserved.

In this *in vitro* study, we investigated the potential of *Lactobacillus gasseri* LA806 to counteract the infection cycle of *S. aureus* and *E. coli* within bMECs and determined whether the properties of this strain were preserved after heat inactivation. We evaluated the ability of *L. gasseri* LA806, either alive or inactivated, to impair pathogen adhesion to and internalisation into bMECs and to modulate bMEC inflammatory response. *L. gasseri* LA806 was selected for this study on the basis of previous observations *in vitro* showing its strong ability to adhere to intestinal epithelial cells (internal data) and to restore and reinforce the epithelial barrier (Alard *et al.*, 2018).

2. Materials and methods

Bacterial strains and culture conditions

The strain *L. gasseri* LA806 from the Larena collection (PiLeJe Industrie, Paris, France) was used in this study, as well as two well-characterised strains, representative of two major mastitis pathogens: *S. aureus* Newbould 305 (hereafter referred to as NB305) and *E. coli* P4 (Bouchard *et al.*, 2015; Peton and Le Loir, 2014).

L. gasseri LA806 was cultured in Man Rogosa Sharpe medium (MRS; pH 6.8; BD, Le Pont de Claix, France) at 37 °C without shaking. *S. aureus* NB305 and *E. coli* P4 were cultured in brain heart infusion medium (BHI, pH 7.4; BD, Le Pont de Claix, France) at 37 °C under agitation (180 rpm). Subcultures were washed once with phosphate-buffered saline (PBS) and suspended at different concentrations in a cell culture medium (DMEM/F12) for cell infection assays (see below). Bacterial concentrations in subcultures were estimated by spectrophotometric measurements at 650 nm.

For some experiments, *L. gasseri* LA806 was inactivated by heat treatment at 70 °C for 10 min in DMEM, just before its addition to bMECs. Preliminary tests had identified these conditions as the mildest conditions capable of reducing the viable *L. gasseri* LA806 population below 20 cfu/ml (detection limit).

Epithelial cells and culture conditions

Two epithelial cell lines were used in this study. The MAC-T cell line ((Huynh *et al.*, 1991); Nexia Biotechnologies, Quebec, Canada) has been widely used for invasion assays and was therefore retained for this study (Almeida *et al.*, 1996). Cells were grown in T75 cell culture flasks in MAC-T medium corresponding to DMEM/F12 advanced medium (D. Dutscher, Brumath, France) supplemented with

10% heat-inactivated foetal 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. After attaining a confluent monolayer, they were treated with 0.05% trypsin (Gibco-BRL, Grand Island, NY, USA), and suspended in fresh MAC-T medium. For adhesion and internalisation assays, cells were seeded in 12-well plates (2×10^5 cells/well) and incubated for 24 h at 37 °C in 5% CO_2 to obtain a confluent monolayer.

The second epithelial cell line, PS, was used for immunomodulation assays. The PS cell line reportedly presents a better immune response to infection-associated stimuli compared to the MAC-T response (Roussel *et al.*, 2015). PS cells were grown in PS growth medium corresponding to DMEM/F12 advanced medium (D. Dutcher) containing 10 ng/ml of insulin-like growth factor-1 (Peprotech, Rocky Hill, CT, USA), 5 ng/ml of fibroblast growth factor (Peprotech), 5 ng/ml of EGF (Sigma-Aldrich, Saint Louis, MO, USA), 1 µg/ml of hydrocortisone (Sigma-Aldrich), 20 mM of HEPES buffer (D. Dutcher) and 2 mM of glutamine (Gibco, Waltham, MA, USA). Cells were incubated at 37 °C in a humidified incubator with 5% CO_2. After attaining a confluent monolayer, they were treated with 0.05% trypsin (Gibco-BRL), and suspended in fresh PS growth medium. For immunomodulation assays, cells were seeded in a 12-well plate at a concentration of 2×10^5 cells/well and incubated at 37 °C in a humidified incubator with 5% CO_2 for 48 h to obtain a confluent monolayer. They were then washed with Hank's Balanced Salt Solution (HBSS, Sigma-Aldrich) and incubated for 24 h in a stimulation medium: DMEM/F12 advanced medium containing 4 ng/ml hydrocortisone, 20 mM HEPES buffer and 2 mM glutamine.

Adhesion and internalisation assays

Adhesion and internalisation assays were adapted from those described by Bouchard and collaborators (Bouchard *et al.*, 2013). Confluent monolayers of MAC-T cells (2.5×10^5 cells/well) were washed twice in PBS. Cells were incubated at 37 °C in 5% CO_2 in 1 ml DMEM with *L. gasseri* LA806 at a multiplicity of infection (MOI) of 400:1 or 2,000:1 and *S. aureus* NB305 or *E. coli* P4 at a MOI of 100:1. Adhesion and internalisation were measured 1 and 2 h after infection, respectively.

For the adhesion assays, MAC-T monolayers were washed four times with PBS and treated with 0.05% trypsin for 5 min at 37 °C. Cells were centrifuged for 5 min at 800 *g* and lysed by adding 100 µl of 0.01% Triton in sterile water. The population of *S. aureus* NB305 and *L. gasseri* LA806 that adhered (cfu/ml) was determined using a previously described micromethod (Baron *et al.*, 2006; Bouchard *et al.*, 2013). The *L. gasseri* LA806 population was determined on MRS (pH 5.4) and incubated anaerobically for 48 h at 37 °C. The *S. aureus* NB305 and *E. coli* P4 populations (cfu/ml) were determined on mannitol salt agar (MSA; D. Dutscher) and MacConkey agar (Torrejon de Ardoz, Madrid, Spain), respectively, after 24 h of incubation at 37 °C. It should be noted that the adhered and internalised populations of *S. aureus* NB305 1 h post-infection were taken into account in the final count of the adhered population. The internalised population was indeed very small compared to the adhered population 1 h after infection (data not shown) and even 2 h after infection (~10 fold smaller, see Figure 1 legend) and could therefore be neglected. The results of an adhesion assay of *S. aureus* NB305 alone were used as the reference. Adhesion rates were defined as the adhered *S. aureus* NB305 population in the presence of *L. gasseri* LA806 relative to the adhered *S. aureus* NB305 population in the reference experiment.

For the internalisation assays, cells were washed four times with PBS, incubated for an additional 2 h in DMEM containing gentamicin (100 µg/ml; Pan Biotech, Aidenbach, Germany). This step resulted in the killing of extracellular bacteria and allowed numeration of the internalised bacterial population only. The MAC-T monolayers were subsequently washed four times with PBS, treated with trypsin, centrifuged for 5 min at 800×*g* and lysed in 0.01% Triton. Internalised populations of *L. gasseri* LA806, *S. aureus* NB305 and *E. coli* P4 (cfu/ml) were determined as described for the adhesion assays.

Determination of adhered heat-inactivated *Lactobacillus gasseri* LA806 population

The adhered heat-inactivated *L. gasseri* LA806 population was determined by quantitative PCR in adhesion assays as described above. After Triton treatment, samples were washed in 9 volumes of buffered peptone water, centrifuged for 5 min at 8,000×*g* and stored at -20 °C until DNA extraction. The cell pellets were suspended in 50 µl TE buffer (20 mM Tris HCl, 2 mM EDTA) and transferred into tubes containing 200 µl lysis buffer (TE buffer containing 1% Triton) and 200 mg zirconium beads (VWR, Fontenay-Sous-Bois, France). Cells were disrupted using a Precellys Evolution homogeniser (Bertin Instruments, Montigny-le-Bretonneux, France) by performing three cycles of 30 s at 6,800 rpm and 30 s on ice. After centrifugation at 4 °C for 5 min at 16,000×*g*, lysates were treated with proteinase K and DNA was extracted using a DNeasy blood and tissue kit and a Qiacube automated purification system (Qiagen, Hilden, Germany) according to the supplier's recommendations.

The *L. gasseri* LA806 population adhering to bMECs was determined by qPCR on the total DNA extracted using a standard range of pure *L. gasseri* LA806 DNA (expressed as the copy number of chromosome). The reaction mixture for qPCR contained SsoAdvanced Universal SYBR® Green

Supermix (1×, Bio-Rad, Hercules, CA, USA), each primer (0.5 µM) and a cDNA template. The primers LgBLX_gpmA_FOR (5'-TCCACACATCGTTCCTAAGGC-3') and LgBLX_gpmA_REV (5'-GCCGTCAAGTAAATCTGGAGC-3'), targeting phosphoglyceromutase, were used as forward and reverse primers, respectively. Thermal cycling consisted of 30 s at 98 °C, followed by 40 cycles of 10 s at 95 °C and 30 s at 60 °C. Primer specificity was confirmed by lack of amplification on *S. aureus* genomic DNA.

Immunomodulation assays

The immunomodulatory properties of *L. gasseri* LA806, either alive or heat-inactivated, were explored essentially as previously described (Souza *et al.*, 2018). Confluent monolayers of PS cells at 2.5×10^5 cells/well were washed twice with HBSS and incubated with *L. gasseri* LA806 at a MOI of 100:1 or 1000:1 alone and with *S. aureus* NB305 or *E. coli* P4 at a MOI of 30:1 in DMEM. After 2 h, cells were washed twice with HBSS and incubated with stimulation medium containing 100 µg/ml gentamicin for 2 h to kill adhered extracellular bacteria. They were then washed three times with HBSS and incubated with stimulation medium containing 25 µg/ml gentamicin for an additional 4 h to complete a total period of 8 h post-infection. After the incubation period, either 2 or 8 h post-infection, cells were washed once with HBSS and then lysed with the RLT buffer of the RNeasy Mini Kit (Qiagen) for subsequent RNA extraction.

Total RNA was extracted using an RNeasy Mini Kit (Qiagen) according to the manufacturer's recommendations. Contaminating DNA was eliminated using DNA-free™ DNase Removal Treatment and Reagents kit (Life Technologies, Waltham, MA, USA). 1 µg DNAse-treated RNA was used for reverse transcription using a qScript™ cDNA Synthesis Kit (Quanta Biosciences, Beverly, MA, USA), according to the manufacturer's recommendations.

qRT-PCR was performed using a CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, USA). Targeted genes included interleukin (IL)-1α (IL-1α), IL-6, IL-8, tumour necrosis factor-α (TNF-α), lingual antimicrobial peptide (LAP), tracheal antimicrobial peptide (TAP), β-defensin 1 (DEFβ1), nuclear factor κB (NFκB) and nucleotide-binding oligomerisation domain 2 (NOD2). Primer sequences have already been published (Souza *et al.*, 2018). PCR were performed as previously described. The genes coding for tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ), ribosomal protein L19 (RPL19) and peptidylprolyl isomerase A (PPIA) were used for normalisation as previously described (Souza *et al.*, 2018).

Statistical analysis

Statistical analysis was performed using R software (http://www.R-project.org). Effects of *L. gasseri* LA806 on pathogen adhesion and internalisation were assessed by a one-way ANOVA, followed by Tukey's multiple comparison test. Differences between treatments were assessed considering as statistically significant a *P*-value of less than 0.05. Effects of pathogens and *L. gasseri* LA806 on interleukin and defensin expression were assessed by two-way ANOVA, using the presence of pathogens (none/*E.coli/S.aureus*) and *L. gasseri* LA806 (none/*L.gasseri* MOI 100×/ *L. gasseri* MOI 1000×) as factors. Differences between treatments were assessed considering as statistically significant a *P*-value of less than 0.05. Each experiment was conducted in biological triplicate.

3. Results

Live *Lactobacillus gasseri* LA806 inhibit colonisation of epithelial cells by *Staphylococcus aureus* NB305 but not by *Escherichia coli* P4

The assay was performed with *S. aureus* NB305 and *E. coli* P4, two representative strains of major pathogens implicated in bovine mastitis. *L. gasseri* LA806 at a MOI of 400:1 or 2000:1 was co-incubated with the pathogens on epithelial cells (MAC-T cell line) for 1 and 2 h for the assessment of adhesion and internalisation, respectively. A significant five-fold decrease in both the adhesion and internalisation of *S. aureus* NB305 was observed after its co-incubation with *L. gasseri* LA806 at a MOI of 2000:1 (Figure 1A and B), *L. gasseri* LA806 having no effect at a MOI of 400:1. In contrast, regardless of the MOI, *L. gasseri* LA806 had no significant effect on the adhesion or internalisation of *E. coli* P4 (Supplementary Figure S1).

Live *Lactobacillus gasseri* LA806 displayed anti-inflammatory properties

The immunomodulatory properties of *L. gasseri* LA806 were determined by evaluating the immune response of epithelial cells (PS cell line) after infection by *S. aureus* NB305 and *E. coli* P4 in the presence or absence of *L. gasseri* LA806. The expression of pro-inflammatory cytokines (IL-1α, IL-6, IL-8 and TNF-α), antimicrobial peptides (DEFβ1, LAP, TAP) and transcription factors (NOD2 and NFκB) was measured. A low but significant expression of IL-8 was induced by *L. gasseri* LA806 at a MOI of 1000:1 at 2 h, independently of the presence of a pathogen (Figure 2A). A similar trend was observed for IL-6 (Figure 2C). *L. gasseri* LA806 alone had no significant effect on the other cytokines at 2 h (Figure 2E, G) and no effect on any of the genes tested at 8 h (Figure 2B, D, F, H).

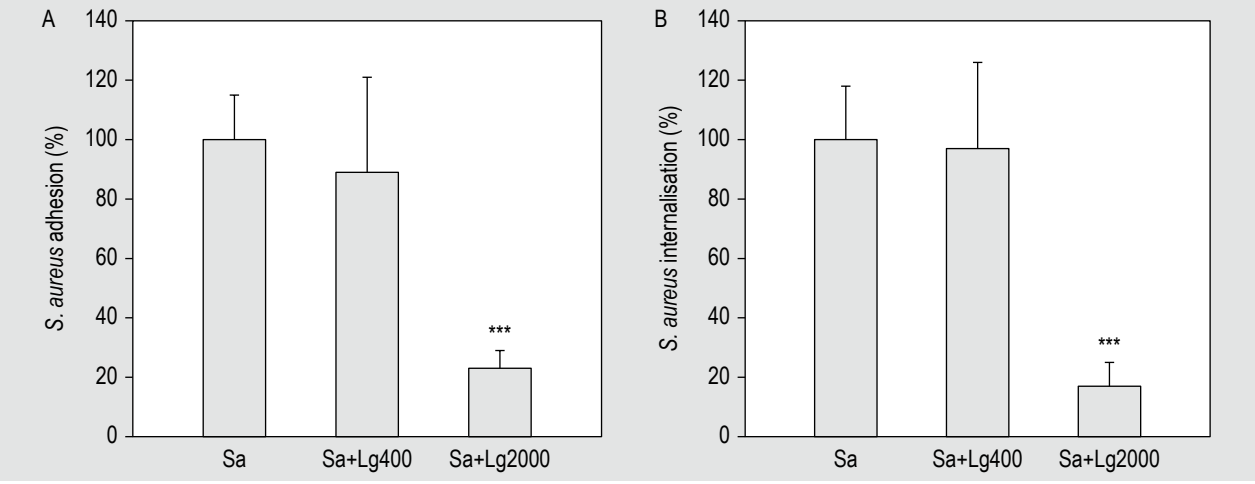

Figure 1. Effect of live *Lactobacillus gasseri* LA806 on *Staphylococcus aureus* N305 adhesion to and internalisation into bovine mammary epithelial cells. Pathogen adhesion (A) and internalisation (B) rates were measured at 1 h and 2 h post-infection, respectively. *S. aureus* NB305 (Sa) was used at a MOI of 100:1 and *L. gasseri* LA806 (Lg) at a MOI of 400:1 or 2,000:1. Adhesion and internalisation assays of *S. aureus* NB305 alone were used as a reference (control) and rates were then defined as the adhered or internalised *S. aureus* NB305 population in the presence of *L. gasseri* LA806 relative to the adhered or internalised *S. aureus* NB305 population of the reference experiment. Adhesion and internalisation rates of *S. aureus* NB305 alone were 3.4×10^6 and 4.8×10^5 cfu/well respectively. Data are presented as means ± standard deviations. Each experiment was performed in triplicate. The effects of *L. gasseri* LA806 on *S. aureus* NB305 adhesion and internalisation were assessed by a one-way ANOVA, followed by Tukey's multiple comparison test. * *P*<0.0005. MOI: multiplicity of infection.**

Whereas no effect was observed at 2 h, *S. aureus* NB305 induced a significant increase in the expression of all pro-inflammatory cytokines at 8 h (Figure 2). This induction was significantly reduced by *L. gasseri* LA806 at a MOI of 1000:1. *E. coli* P4 significantly induced the expression of IL-1α and TNF-α at 2 h and a similar trend was observed for IL-8 (Figure 2A, C, E, G). *E. coli* P4 did not induce the expression of any pro-inflammatory cytokine at 8 h (Figure 2B, D, F, H). *L. gasseri* LA806 did not interfere with the effects of *E. coli* on pro-inflammatory cytokines at either 2 or 8 h. The significant effects are summarised in Table 1.

A significant induction of antimicrobial peptides (DEF β1, LAP and TAP) was observed when cells were infected by *E. coli* at 8 h, with no modulatory effect of *L. gasseri* LA806 (Supplementary Figure S2). This effect was not observed with *S. aureus*. No variation in expression of NOD2 and NF-κB was detected under the conditions tested (data not shown).

Heat inactivation of *Lactobacillus gasseri* LA806 partially preserved its ability to inhibit colonisation by *Staphylococcus aureus*

L. gasseri LA806 had no effect on *E. coli* P4 colonisation. Competition assays between heat-inactivated (HI) *L. gasseri* LA806 and pathogens for bMEC colonisation were therefore performed solely with *S. aureus* N305. A HI *L. gasseri* LA806 MOI of 2000:1 was used, which was effective in adhesion and internalisation competition experiments with the live strain. Heat inactivation reduced the ability of *L. gasseri* LA806 to inhibit both the adhesion and internalisation of *S. aureus* NB305 to bMECs (Figure 3); the ability of the HI strain was two-fold lower than that of the live strain.

To determine whether this lesser effect was due to a reduced ability of HI *L. gasseri* LA806 to adhere to epithelial cells, in line with a competition mechanism, adhesion of HI *L. gasseri* LA806 was assessed. Adhered populations of live and HI *L. gasseri* LA806 were quantified by qPCR after 1 and 2 h of incubation. Compared to the live strain, there was a four to five-fold decrease in the amount of HI *L. gasseri* LA806 cells that adhered to bMECs 1 and 2 h post-incubation (Figure S3). As a control, we evaluated by qPCR the effect of heat inactivation on the *L. gasseri* LA806 population using a pure culture of the strain (without incubation with epithelial cells; data not shown). Heat-inactivation induced a two-fold loss of the *L. gasseri* LA806 population as determined by qPCR. This latter result suggests that the four to five-fold decrease in the amount of HI *L. gasseri* LA806 cells that adhered to epithelial cells was partially, yet not entirely due to this population loss. Inactivation therefore induced a two-fold decrease in the ability of *L. gasseri* LA806 to adhere to epithelial cells.

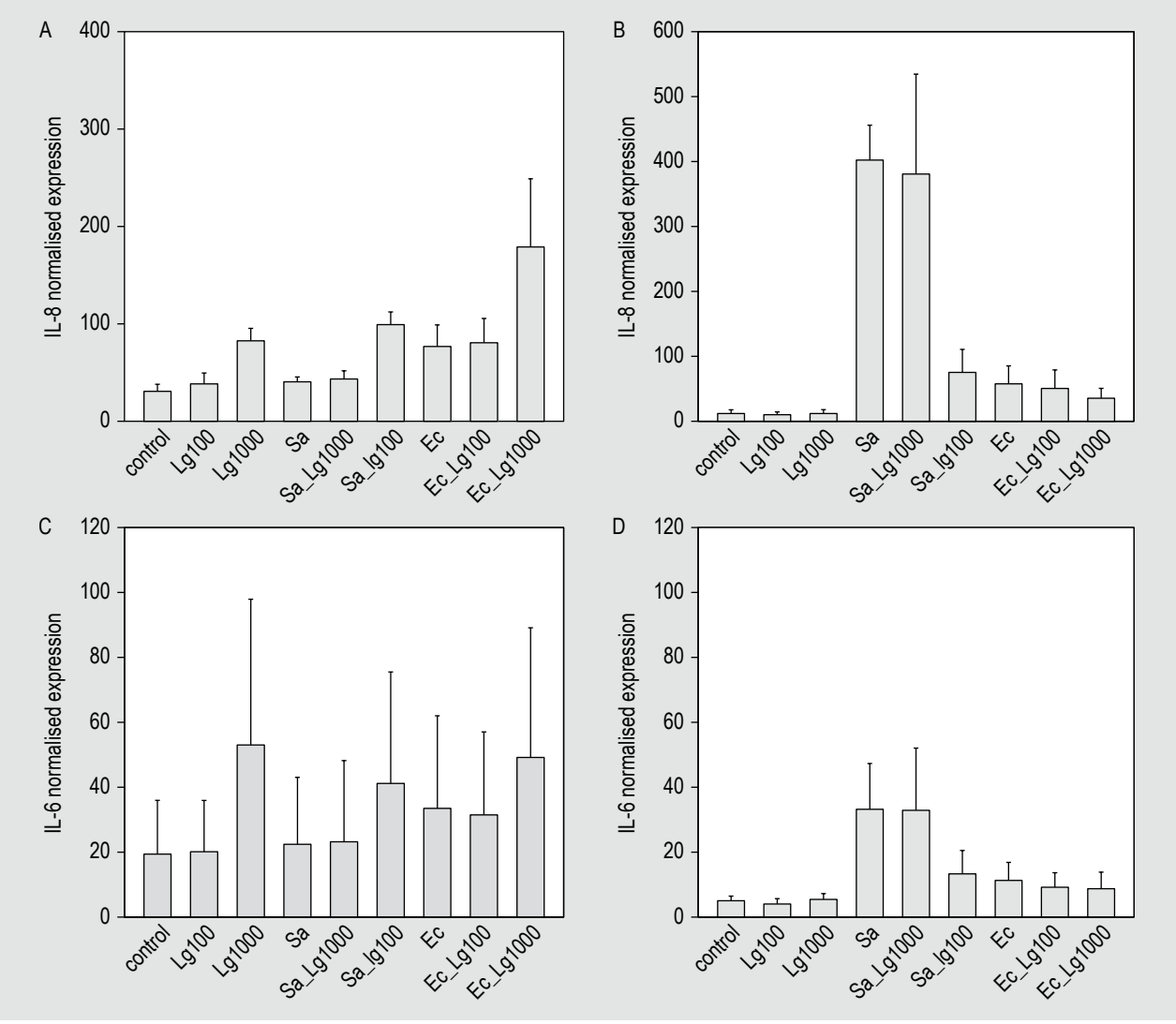

Figure 2. Impact of *Lactobacillus gasseri* LA806 (Lg) on interleukin (IL)-8 (A, B), IL-6 (C, D), tumour necrosis factor (TNF)-α (E, F) and IL-1α (G, H) expression by bovine mammary epithelial cells (bMECs) during infection by *Staphylococcus aureus* NB305 (Sa) or *Escherichia coli* P4 (Ec). bMECs were stimulated with pathogens at a MOI of 30:1 either alone or in the presence of *L. gasseri* LA806 at a MOI of 100:1 and 1.000:1. Expression was measured at 2 h (A, C, E, G) and 8 h (B, D, F, H) post-infection. Expression was determined as the normalised expression with regard to three control genes. Each experiment was performed in triplicate. The effects of each factor (i.e. the presence of a pathogen or *L. gasseri* LA806) and interactions between the two factors were assessed using a two-way analysis of variance. MOI = multiplicity of infection.

Heat inactivation of *Lactobacillus gasseri* LA806 scarcely affected its immunomodulatory properties

Overall review of the immunomodulatory properties of HI *L. gasseri* LA806 compared to the live strain indicates that most of the immunomodulatory effects of live *L. gasseri* LA806 were conserved following heat inactivation of the bacterial cells (Figure 2 and Supplementary Figure S4). Addition of HI *L. gasseri* LA806 at a MOI of 1000:1 induced a moderate increase in IL-6 at 2 h, independently of the presence of a pathogen (Supplementary Figure S4C), in agreement with the trend observed with live *L. gasseri* LA806 (Figure 2C).

HI *L. gasseri* LA806 showed a significant inhibitory effect at 8 h on *S. aureus*-stimulated bMECs (Supplementary Figure S4A,B), which is in line with the experiments with live *L. gasseri* LA806. The expression of all cytokines tested was indeed significantly reduced in the presence of HI *L. gasseri* LA806 at a MOI of 1000:1 and even at a MOI of 100:1 for IL-8 (Supplementary Figure S4B,D,F,H). Interestingly, HI *L. gasseri* LA806 at a MOI of 1000:1 significantly decreased the induction of TNF-α expression in the presence of *E. coli* at 2 h (Supplementary Figure S4E). This inhibition was not observed with live *L. gasseri* LA806 (Figure 2E, F).

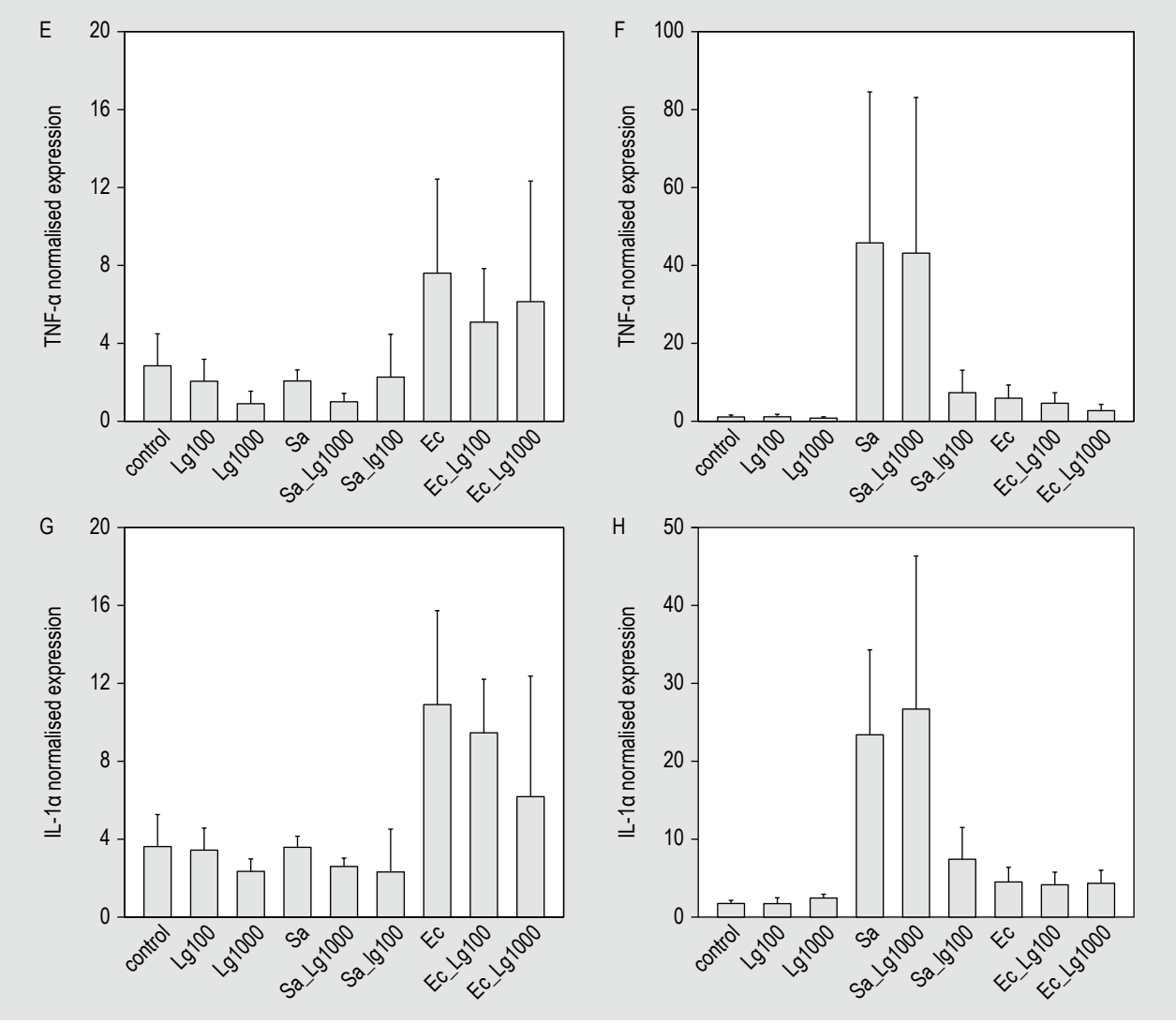

Figure 2. Continued.

Table 1. Summary of the significant effects (*P*-values) of *Lactobacillus gasseri* LA806 (Lg) on cytokine expression by bovine mammary epithelial cells during infection by *Staphylococcus aureus* NB305 or *Escherichia coli* P4.[1]

Factor	Significant effect on gene expression at 2 h	Significant effect on gene expression at 8 h
Lg 1000	IL-8 (**), IL-6 (*P*=0.0547)	
S. aureus		IL-8 (***), IL-6 (***), IL-1α (***), TNF-α (***)
E. coli	IL-8 (*P*=0.0513), IL-1α (***), TNF-α (*)	
S. aureus × Lg 1000		IL-8 (***), IL-6 (*), IL-1α (*), TNF-α (*)

[1] IL = interleukin; TNF = tumour necrosis factor. * *P*<0.05, ** *P*<0.005, *** *P*<0.0005.

As observed in the experiments with live *L. gasseri* LA806, the HI bacteria did not modulate *E. coli* P4 induced antimicrobial peptide expression at 8 h (Supplementary Figure S5), or the expression of NOD2 and NF-κB in bMECs (data not shown).

4. Discussion

The highly problematic rise in antibiotic resistance forces us to consider alternative human and animal health strategies in order to reduce the use of antibiotics (WHO, 2015).

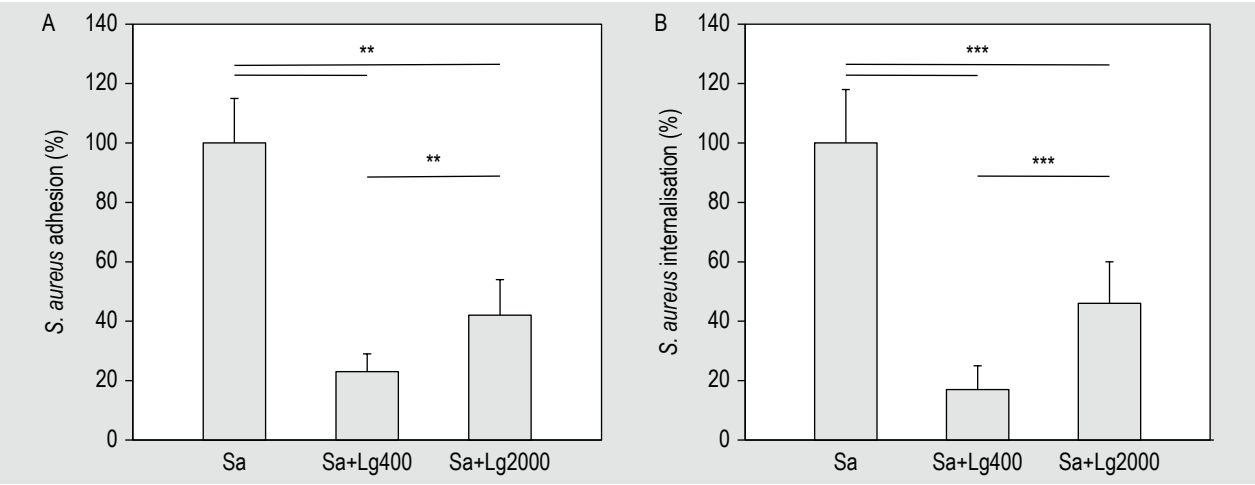

Figure 3. Effect of heat-inactivated (HI) *Lactobacillus gasseri* LA806 on *Staphylococcus aureus* N305 adhesion to and internalisation into bovine mammary epithelial cells. *S. aureus* adhesion (A) and internalisation (B) rates were measured 1 h and 2 h post-infection, respectively. *S. aureus* NB305 (Sa) was used at a MOI of 100:1 and *L. gasseri* LA806, either heat-inactivated (HI-Lg) or alive (Lg), at a MOI of 2000:1. The results of adhesion and internalisation assays with *S. aureus* alone were used as the reference (control). Rates were then defined as the adhered or internalised *S. aureus* NB305 population in the presence of *L. gasseri* LA806 either heat-inactivated or live, relative to the adhered or internalised *S. aureus* NB305 population of the reference experiment. Data are presented as means ± standard deviations. Each experiment was performed in triplicate and differences between groups were assessed using a one-way analysis of variance followed by Tukey's range test. ** $P<0.005$, *** $P<0.0005$. MOI: multiplicity of infection.

In this context, the exploration of strategies involving probiotics has attracted interest in many areas, including bovine mammary gland health. Although probiotics are recognised to confer health benefits on the host, the WHO/FAO also consider that they have four types of putative side effects: systemic infections, deleterious metabolic activities, gene transfer and excessive activation of the immune system, especially in immunocompromised individuals and neonates (Doron and Snydman, 2015; Piqué *et al.*, 2019; Sanders *et al.*, 2016). Bacterial inactivation could alleviate some of these safety concerns, while retaining the value of these bacteria if their beneficial effects are at least partially preserved. The results presented here reveal that the probiotic properties of *L. gasseri* LA806 assessed in an *in vitro* model of bovine mastitis are in part preserved after heat inactivation.

Live *Lactobacillus gasseri* LA806 inhibits *S. aureus* colonisation of bMECs while hardly affecting *Escherichia coli* colonisation

Interaction with *L. gasseri* LA806 resulted in a five-fold reduction of *S. aureus* NB305 adhesion to and internalisation into bMECs, whereas it did not alter those of *E. coli* P4. This ability of lactic acid bacteria (LAB) to prevent *S. aureus* colonisation of bMECs has been previously reported for several *Lactobacillus casei* strains (Bouchard *et al.*, 2013) and for *Lactococcus lactis* V7 (Assis *et al.*, 2015). Interestingly, as observed with *L. gasseri* LA806, *L. lactis* V7 did not modify *E. coli* adhesion rate but in contrast to *L. gasseri* LA806, it was able to reduce *E. coli* P4 internalisation, confirming that the ability to compete with pathogens for bMEC colonisation is species- and even strain-dependent (Bouchard *et al.*, 2013). In accordance with this finding, one study did not report such a property of the *L. lactis* strain tested (Mignacca *et al.*, 2017). Nevertheless, it should be noted that in this study, competition assays were performed with another bovine mammary epithelial cell line and with a lower LAB:cell ratio. Strain dependency, especially with regard to the pathogen, leads us to reject the hypothesis of a competition solely based on steric hindrance, as in such a case similar inhibition of adhesion should have been observed for *E. coli*. It rather supports a competition for specific sites on bMECs. This is in agreement with the existence of different mechanisms of interaction with bMECs for *E. coli* and *S. aureus*: different pathogen-associated molecular patterns (PAMPS) interact with different pattern recognition receptors (PRR) (Gilbert *et al.*, 2013; Schukken *et al.*, 2011; Védrine *et al.*, 2018).

Complex immunomodulatory properties of live *Lactobacillus gasseri* LA806: from pro- to anti-inflammatory profiles

The immunomodulatory properties of *L. gasseri* LA806 were investigated on bMECs in the presence or absence of the pathogens at 2 and 8 h post-infection. This kinetic approach revealed complex interactions of *L. gasseri* LA806 with bMECs. At 8 h, *L. gasseri* LA806 had anti-inflammatory effects, as revealed by a decrease in the expression of several pro-inflammatory cytokines (IL-8, IL-6, IL-1α and TNF-α) by *S. aureus*-stimulated bMECs.

A similar anti-inflammatory profile was reported for *L. casei* BL23 at a lower *S. aureus* MOI (10:1) (Bouchard *et al.*, 2013; Souza *et al.*, 2018). However, a slight but yet significant increase in IL-8 was observed at 2 h in the presence of *L. gasseri* LA806 (either alone or during co-infection with *S. aureus* or *E. coli*) and a similar trend was observed for IL-6, suggesting a transient pro-inflammatory profile of this *L. gasseri* strain. These results clearly emphasise the importance of analysing cytokine expression over time rather than at a single time-point to better understand the immunomodulatory properties of a given strain, as previously reported for pathogens (Fu *et al.*, 2013; Günther *et al.*, 2011) and LAB strains (Souza *et al.*, 2018). In human peripheral blood mononuclear cells, *L. gasseri* LA806 induced a slight increase in IL-12 and interferon-γ expression, while scarcely affecting IL-10 expression, suggesting a moderate pro-inflammatory profile in this model as well (Alard *et al.*, 2018). We propose that in the mammary gland context, *L. gasseri* LA806 did not display anti-inflammatory properties *per se*, but rather provoked an early and moderate stimulation of the innate immune system. By adhering to bMECs and thus inhibiting *S. aureus* adhesion and internalisation, *L. gasseri* LA806 likely induced a lesser stimulation of pro-inflammatory cytokine expression by *S. aureus*.

E. coli-stimulated bMECs displayed cytokine expression kinetics differing from those of *S. aureus*-stimulated cells and notably, an earlier stimulation of pro-inflammatory cytokine expression, as previously reported (Günther *et al.*, 2017; Schukken *et al.*, 2011). Indeed, *E. coli* led to a transient induction of IL-1α, TNF-α and IL-8 at 2 h post-infection. This effect, that was lost by 8 h post-infection, was followed by an induction of LAP, TAP and DEFβ1 defensin expression at 8 h. *L. gasseri* LA806 did not significantly interfere with the bMEC immune response to *E. coli*. This observation is consistent with the lack of effect of *L. gasseri* LA806 on the colonisation ability of the pathogen. At least *L. gasseri* LA806 did not impair the induction of defensins that contribute to combating pathogens. To a certain extent, it somehow complies with the *primum non nocere* principle.

Heat-inactivated *Lactobacillus gasseri* LA806 retains beneficial properties

Bacteria can be inactivated using different methods, such as heating, chemical treatment, gamma or UV radiation and sonication (Piqué *et al.*, 2019). These treatments can affect cell structural components and most of the inactivated bacterial products contain more cellular lysate than cells *per se* (Piqué *et al.*, 2019). Interaction of LAB with epithelial cells was reported to involve surface components of the bacteria (Sengupta *et al.*, 2013). *L. gasseri* LA806 was thus inactivated with moderate heating (10 min at 70 °C) in order to limit denaturation and preserve the ability of the strain to interact with bMECs.

Moderate heating led to partial loss of the ability of *L. gasseri* LA806 to inhibit *S. aureus* adhesion to and internalisation into bMECs (two-fold lower than that of the live bacteria). This is clearly the consequence of an impaired ability of HI *L. gasseri* LA806 to adhere to epithelial cells. The adhesion rate of HI *L. gasseri* LA806 at 1 and 2 h, as determined by qPCR, was approximatively two-fold lower than that of live *L. gasseri* LA806 after correction for the loss of signal due to heat treatment. Although the heat treatment was moderate, it likely denaturised cell wall surface components including those involved in the interaction with bMECs. Various cell surface components, such as proteins, peptidoglycans, teichoic acids and polysaccharides contribute to the interaction of LAB with epithelial cells (Piqué *et al.*, 2019; Taverniti and Guglielmetti, 2011). A *L. casei* BL23 *srtA2* mutant, *srtA2* encoding a sortase, was less effective in inhibiting *S. aureus* adhesion to and internalisation into bMECs (Souza *et al.*, 2017). This characteristic was associated with an altered abundance of LPXTG and moonlighting proteins at the surface of the *srtA2* mutant strain as well as with modifications of the cell wall structure. Cell surface components also contribute to the immunomodulatory and anti- and pro-inflammatory properties of the strains. For example, components associated with peptidoglycans, such as teichoic acids, have been shown to elicit pro-inflammatory responses induced by LAB themselves and to inhibit the excessive inflammatory response induced by pathogens (Piqué *et al.*, 2019; Taverniti and Guglielmetti, 2011).

HI *L. gasseri* LA806 was still able to significantly inhibit *S. aureus* colonisation, although with diminished efficacy. Interestingly, the immunomodulatory properties of HI *L. gasseri* LA806 were scarcely affected. The beneficial properties of *L. gasseri* LA806 were therefore at least partially preserved following heat inactivation. Inactivated strains have already been assessed in mastitis. One study showed a transient pro-inflammatory effect of intra-mammary infusion of a heat-inactivated *Lactobacillus rhamnosus* strain, revealed by an increase in somatic cell count and a slight modification of milk microbiota after six days (Catozzi *et al.*, 2019). However, despite stimulation of the innate immune system, administration of the heat-inactivated *L. rhamnosus* strain did not enhance pathogen clearance in this study. The use of inactivated bacteria has also been tested in gastrointestinal, dermatological and respiratory allergic diseases (Adams, 2010; Piqué *et al.*, 2019), oropharyngeal and vaginal candidiasis (Pericolini *et al.*, 2017; Roselletti *et al.*, 2019), development of caries (Schwendicke *et al.*, 2014), osteoarthritis (Henrotin *et al.*, 2019), as well as for the stimulation of human immune cells or for their anti-inflammatory properties (Jensen *et al.*, 2017). Beneficial properties maintained after inactivation include prevention of infections and competition with pathogens (Canducci *et al.*, 2000). The results of these studies varied greatly, bacterial inactivation resulting

either in conservation (Roselletti *et al.*, 2019) or partial loss (Pericolini *et al.*, 2017) of beneficial properties and even, in some studies, in superior efficacy of the inactivated bacterial strain evaluated *versus* the live strain (Jensen *et al.*, 2017; Schwendicke *et al.*, 2014). This variability probably reflects the mechanisms involved as well as differences in the protocol of inactivation used (Piqué *et al.*, 2019).

In conclusion, this study revealed that *L. gasseri* LA806 has barrier and immunomodulatory properties that could be useful to counteract mammary gland infections caused by *S. aureus* and that the properties of this strain were at least partially preserved following moderate heat treatment. The use of an inactivated probiotic in this context could limit pathogen colonisation, attenuate inflammation and reduce tissue damage caused by *S. aureus* infection, likely with a lower risk of side effects due to the use of an inactivated strain. The potential of live and inactivated *L. gasseri* LA806 to counteract the infection cycle of *S. aureus* warrants confirmation *in vivo*. Furthermore, as *S. aureus* is implicated in numerous types of infection in both animals and humans, the potential of *L. gasseri* LA806 would be worth exploring in other contexts.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/BM2020.0146.

Figure S1. Effect of *Lactobacillus gasseri* LA806 on *Escherichia coli* P4 adhesion to and internalisation into bovine mammary epithelial cells.

Figure S2. Impact of *Lactobacillus gasseri* LA 806 on DEFβ1, LAP and TAP expression by bovine mammary epithelial cells following infection by *Staphylococcus aureus* NB 305 (or *Escherichia coli* P4).

Figure S3. Effect of heat inactivation on *Lactobacillus gasseri* LA806 adhesion to bovine mammary epithelial cells.

Figure S4. Impact of heat inactivated *Lactobacillus gasseri* LA806 on IL-8, IL-6, TNF-α and IL-1α expression by bovine mammary epithelial cells following infection by *Staphylococcus aureus* NB305 (or *Escherichia coli* P4).

Figure S5. Impact of heat inactivated *Lactobacillus gasseri* LA806 on DEFβ1, LAP and TAP expression by bovine mammary epithelial cells following infection by *Staphylococcus aureus* NB305 (or *Escherichia coli* P4).

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Conflict of interest

The authors declare no conflict of interest.

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