Post-milking application of a Lacticaseibacillus paracasei strain impacts bovine teat microbiota while preserving the mammary gland physiology and immunity

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RESEARCH ARTICLE

Post-milking application of a *Lacticaseibacillus paracasei* strain impacts bovine teat microbiota while preserving the mammary gland physiology and immunity

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Abstract

Bovine mastitis (BM) is a major disease in dairy industry. The current approaches – mainly antibiotic treatments – are not entirely effective and may contribute to antimicrobial resistance dissemination, rising the need for alternative treatment. The present study aims to evaluate the impact of post-milking application of *Lacticaseibacillus paracasei* CIRM BIA 1542 (Lp1542) on the teat skin (TS) of 20 Holstein cows in mid lactation, in order to reinforce the barrier effect of the microbiota naturally present on the teat. Treatment (Lp1542, iodine or no treatment) was applied post-milking twice a day on the 4 teats of healthy animals for 15 days. Blood and milk samples, and TS swabs were collected at day (D)1, D8, D15 and D26 before morning milking and at D15 before evening milking (D15E) to evaluate Lp1542 impact at the microbial, immune and physiological levels. Lp1542 treatment resulted in a higher lactic acid bacteria and total microbial populations on TS and in foremilk (FM) at D15(E) compared with iodine treatment. Metabarcoding analysis revealed changes in the composition of TS and FM microbiota, beyond a higher *Lacticaseibacillus* abundance. This included a higher abundance of *Actinobacteriota*, including *Bifidobacterium*, and a lower abundance of *Pseudomonadota* on TS of Lp1542 compared with iodine-treated quarters. In addition, Lp1542 treatment did not trigger any major inflammatory response in the mammary gland, except interleukin 8 production and expression which tended to be slightly higher in Lp1542-treated cows compared with the others. Finally, Lp1542 treatment had no impact on the mammary epithelium functionality (milk yield and composition) and integrity (epithelial cell exfoliation into milk and milk Na^+/K^+ ratio). Altogether, these results indicate that a topical treatment with Lp1542 is safe with regard to mammary gland physiology and immune system, while impacting its microbiota, inviting us to further explore its effectiveness for mastitis prevention.

Keywords

bovine mastitis – prevention strategy – lactic acid bacteria – teat skin microbiota – milk microbiota

1 Introduction

Several studies highlighted in human and animals a relationship between the microbiota and the health or functionality of the related organ (Bicalho *et al.*, 2017; Gaeta *et al.*, 2017). Strategies for modifying or restoring the microbiota, based on the administration of one or more strains of the microbiota, have been explored, mostly in human (Doré *et al.*, 2013; Martín *et al.*, 2019). Similarly, in bovine, a relationship was found between

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the richness of teat cistern microbiota and the udder health (Falentin *et al.*, 2016; Rault *et al.*, 2020). Bovine mastitis (BM) is the most common and detrimental disease in dairy industry worldwide affecting approximately 40% of cows in France each year (CNIEL, 2021). BM is caused by an intramammary infection, which triggers an inflammation of the bovine mammary gland (Blowey *et al.*, 1995; Halasa *et al.*, 2007). Prophylactic treatments including post-milking teat disinfection and/or antimicrobial therapy at the end of lactation are currently used to prevent infections (Gruet *et al.*, 2001). The use of chemical products for post-milking dipping such as chlorine or iodine is contested because they can provoke a teat skin (TS) irritation (Sadakane and Ichinose, 2015) and the presence of iodine residues was reported in milk, with safety concerns especially for young consumers (Borucki Castro *et al.*, 2010, 2012). On the other hand, antimicrobials used to treat the cows are unfortunately not entirely effective (Sharun *et al.*, 2021), and increase the risk to spread antimicrobial resistance (Oliver and Murinda, 2012). Therefore, researchers are working on the development of alternatives to current approaches to prevent or treat BM (Bennett *et al.*, 2022; Brouillette *et al.*, 2023). One of them is to preserve the mammary microbiota (Teat skin, foremilk) to act as a barrier against pathogens. Besides, although they are not dominant, lactic acid bacteria (LAB) are very interesting components of the bovine mammary microbiota. Their abundance has been associated with a healthy mammary gland (Oikonomou *et al.*, 2014). Furthermore, the use of LAB as a barrier flora has shown *in vitro* encouraging results to prevent BM by modulating the innate immune response and by preventing the adhesion and/or internalisation of pathogens (Armas *et al.*, 2017; Souza *et al.*, 2018). *Lacticaseibacillus paracasei* CIRM BIA 1542 (Lp1542) is one promising candidate, due to its ability to inhibit *Staphylococcus aureus* internalisation into bovine mammary epithelial cells (bMEC) (Bouchard *et al.*, 2013, 2015). Lp1542 was isolated from teat canal microbiota and belongs to a species with a Qualified Presumption of Safety (QPS) status (EFSA, 2023). In the present study, we postulated that the application of Lp1542 would allow maintaining a diverse microbiota on TS and in foremilk (FM) and strengthening its barrier effect in relation to the *in vitro* beneficial properties of the strain. An *in vivo* assay was thus conducted to evaluate the impact of a post-milking application of Lp1542 on the bovine TS at the microbial, immune and physiological levels, in comparison with iodine and the absence of treatment.

2 Materials and methods

The strain used in this assay was *Lacticaseibacillus paracasei* CIRM BIA 1542 (hereafter referred as to Lp1542), isolated from bovine mammary gland microbiota. Lp1542 was cultured on Man Rogosa Sharpe medium (MRS; pH 5.4; BD, Le Pont de Claix, France) and incubated without agitation for 24 h at 37 °C. After the incubation, the bacterial cells were harvested by centrifugation at 6,000×*g* for 10 min at 4 °C, washed twice with 0.9% (w/v) saline solution and resuspended in UHT skimmed milk at 1×10^9 cfu/ml. The Lp1542 suspension was aliquoted (10 ml) and stored at –20 °C until use.

Animals and experimental design

The trial was performed on 20 Holstein cows (2 batches of ten animals according to calving date) at the INRAE PEGASE experimental farm (IEPL, 35650 Le Rheu, France; https://doi.org/10.15454/yk9q-pf68); See Supplementary Table S1 for the complete list of cows, their batches and their treatments). All procedures involving animals were approved by the local Ethics Committee in Animal Experiment of Rennes and the French Ministry of Higher Education, Research and Innovation (APAFIS #22154-201909261613485 V3). The cows were separated into 3 treatment groups that were balanced by milk production, body weight, lactation rank, days in milk (DIM), milk composition and somatic cell count (SCC), measured during 2 weeks before treatment. Three treatments were tested: (1) absence of post-milking treatment (group N, n = 6 cows), (2) post-milking teat spraying with iodine as conventional treatment (group I, n = 6 cows) and (3) post-milking teat spraying with Lp1542 suspension at ~1×10^9 cfu/ml (group L, n = 8 cows) (Figure 1). Treatment was applied twice a day for 15 days on the 4 teats of the animals, treating each animal with one treatment (N, I or L) to avoid cross contamination between teats with the Lp1542 and to observe the effect of treatment also at the systemic level. The trial was carried out on healthy animals (SCC ≤ 100,000 cells/ml) in mid lactation (110 ± 22 DIM) treated with iodine before the trial (twice a day, for 1 week). The animals were followed for an additional period of 11 days in order to assess the persistence of the effect. During this period, iodine was used twice a day as a conventional treatment.

Sample collection

Teat skin (side and teat end) swabs, foremilk (5 ml) and cisternal milk (CM, 15 ml) were collected manually at the quarter level (4 × 6-8 cows per treatment group) just

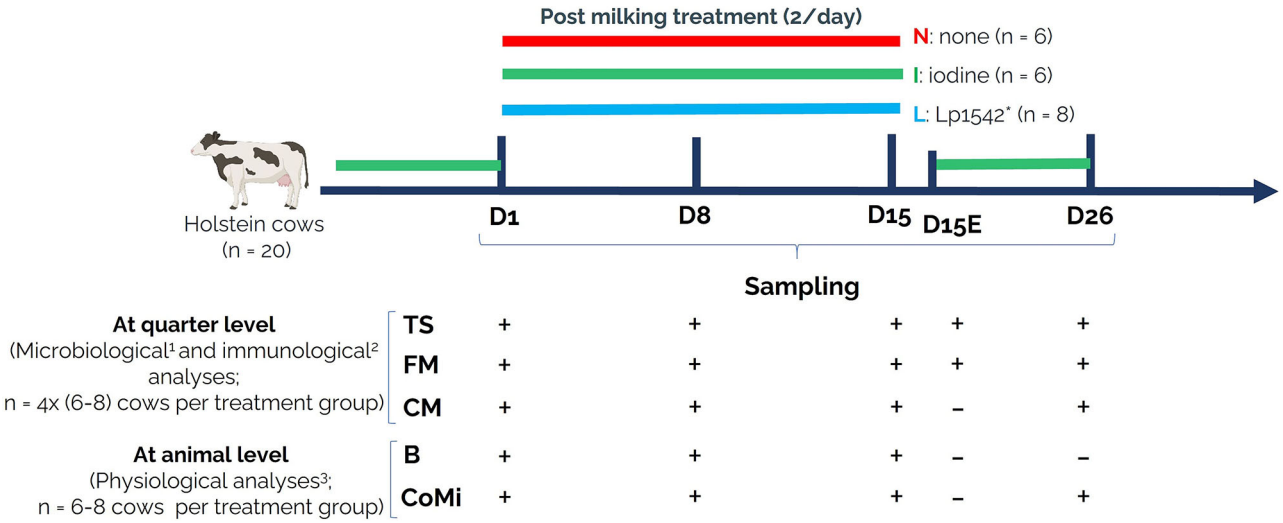

FIGURE 1 Experimental design of the study. The trial was performed on 20 Holstein cows. The cows were separated into 3 treatment groups: (1) absence of post-milking treatment (group N, n = 6 cows), (2) post-milking teat spraying with iodine as conventional treatment (group I, n = 6 cows) and (3) post-milking teat spraying with Lp1542 suspension (group L, n = 8 cows). Treatment was applied twice a day on the 4 teats of the animals for 15 days. The animals were followed for an additional period of 11 days with iodine treatment twice a day, in order to assess the persistence of the effect. Teat skin (TS) swabs, foremilk (FM) and cisternal milk (CM) were collected just before (D1), during (D8, D15) and 11 days after the differential treatment period (D26). Teat skin swabs and FM samples of individual quarters were also collected before the evening milking at D15 (D15E) for the second batch of animals (n = 10). Composite milk (CoMi) and blood samples (B) were collected at D1, D8, D15 and D26 (only for CoMi). [1] Microbial analyses were performed at the quarter level at all timepoints, except microbiota analyses through metabarcoding and determination of Lp1542 by quantitative PCR, which were performed at D15 and D15E only. [2] Immunological analyses were performed at the quarter level at all timepoints (except D15E) only in CM. [3] Milk performances were evaluated at the cow level at all timepoints (except D15E). Other physiological analyses were performed at the cow level at all timepoints (except D15E and D26).

before (D1), during (D8, D15) and 11 days after the differential treatment period (D26). Sampling was performed blindly and randomly before the morning milking, corresponding to 15 hours following the post-milking treatment (see Supplementary Methods for details on sampling). Teat skin swabs and FM samples of individual quarters were also collected before the evening milking at D15 (D15E) for the second group of animals (n = 10). These additional samples were included to limit milk leaking, that occurred frequently before morning sampling and that biased the sampling of FM. Besides, it allows monitoring the impact of treatment on TS and FM microbiota 8 h following the treatment. Composite milk and blood samples were collected at cow level (6-8 samples per treatment group) at D1, D8, D15 and D26 (only for composite milk). Composite milk corresponds to the milk of the 4 quarters that was collected by the milking machine. All milk samples (FM, CM, and composite milk) and TS samples were stored on ice until use. Blood was collected in two lithium-heparin tubes (Sarstedt, Numbrecht, Germany) immediately after milking from the coccygeal vein of each cow. The plasma was recovered by centrifugation at 3,000×*g* for 15 min, aliquoted and stored at –20 °C until use.

Microbial analysis

Microorganisms recovered from TS, FM or CM were quantified by diluting serially and plating on different selective agar plates using the conventional plate-counting technique or its miniaturised method (Baron *et al.*, 2006). MRS agar was used for LAB isolation, Plate Count Agar (PCA, Blokar, Beauvais, France) for total bacteria, Mannitol Salt Agar (MSA; CONDALAB, Madrid, Spain) for *Staphylococcus* spp. and Mac Conkey (CONDALAB) for enteric bacteria. The plates were incubated 48 h at 37 °C under anaerobic conditions, 48-72 h at 30 °C, 24-48 h at 37 °C and 24 h at 37 °C for MRS, PCA, MSA and Mac Conkey, respectively. Bacterial population was expressed in log (cfu/ml). A value of 0.95 was attributed when no colony was counted, corresponding to a threshold detection of 1 colony detected in 100 µl of milk or TS microbial suspension.

Determination of Lacticaseibacillus paracasei *CIRM BIA 1542 by quantitative PCR*

Lp1542 population was determined by qPCR from total DNA extracted from TS and FM at D15 and D15E (used for microbiota analysis, see below). Strain-specific primers were designed using the tools available on the

Pathosystems Resource Integration Center (PATRIC) platform (https://www.bv-brc.org/; see Supplemental methods for details on the design of Lp1542 specific primers). The forward primer (5′-TGTTGGATACCGAGA CTCAATGAA-3′) and the reverse primer (5′-ATTTCTTTA GCTTTATCCTTCCCGT-3′) targeting the Lp1542_504 gene were used to quantify the Lp1542 strain in the samples, using a range of DNA of the strain and a detection threshold corresponding to 0.62 log cfu/ml of Lp1542.

Presence of pathogenic microorganisms in cisternal milk

The presence of pathogenic microorganisms was investigated on CM pooled from the 4 quarters at D1 and D15. The analysis was performed by an accredited laboratory (Labocea, Fougères, France) using the PathoProof™ Mastitis Complete-16 Kit (Thermo Scientific™, Waltham, MA, USA), allowing the detection of 15 of the main contagious mastitis-causing microorganisms.

Microbiota analysis

An analysis of the TS and FM microbiota was performed at D15 (morning and evening) by PCR amplification of the V3-4 region of the gene encoding 16S rRNA using the universal primers S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21. DNA extraction, PCR amplification and amplicon sequencing on the Illumina MiSeq PE250 platform (Illumina Inc., San Diego, CA, USA) of Genome Quebec (Montreal, Canada) were performed as described previously (Mariadassou *et al.*, 2023). Negative controls undergoing all steps from extraction to sequencing but without bacterial suspension were included for each set of extractions, resulting in 13 negative controls that were further used to determine the kitome (i.e. potential contaminant Operational Taxonomic Unit (OTU) originating from extraction, amplification and sequencing steps).

Sequence library analysis was performed using the FROGS pipeline hosted on the INRAE MIGALE bioinformatics platform. Pre-processing, clustering and chimera removal steps were performed with the FROGS pipeline (Galaxy Version 3.2.3+galaxy2) essentially as previously described (Rault *et al.*, 2020). The FROGS clustering step was performed with Swarm with an aggregation distance of 1 (Mahé *et al.*, 2014). Additional filter tool (FROGS OTU filter) was used to apply an abundance filter before the taxonomic affiliation process and to keep OTU with a minimum proportion of 0.00005. Affiliation was then performed with the FROGS affiliation OTU tool based on Blastn+ using the 16S SILVA 138.1 database (Camacho *et al.*, 2009; Quast *et al.*, 2013). Data were filtered to remove the kitome, defined as OTUs present in at least one negative control and whose abundance in the negative control was >1%. This threshold allowed to deplete 97,6% of OTU in the negative controls, while limiting the risk to remove OTUs that were really present in samples. Finally, samples with a number of reads < 1,500 were removed. A rooted phylogenetic tree was created with FastTree and Phangorn R package implemented on FROGS pipeline.

Markers of inflammation

Mammary gland inflammation was estimated by measuring interleukin 8 (IL-8) concentration in milk at the quarter level but also plasma concentrations of cortisol and haptoglobin (i.e. at animal level). IL-8 levels were determined by enzyme-linked immunosorbent assay (ELISA) as described previously (Roussel *et al.*, 2015). The plasma concentration of cortisol was determined using the competitive ELISA method previously developed (Komara and Marnet, 2009). Haptoglobin was measured with the PHASE haptoglobin colorimetric assay kit (Tridelta Development Ltd, Maynooth, Ireland) using a multiparameter analyser (Kone Instrument Corp., Espoo, Finland).

Isolation and count of milk somatic cells

Milk somatic cells were isolated at D1, D8 and D15 and mammary epithelial cells (MEC) were purified from milk as described previously by immunomagnetic separation using Dynabeads (Pan Mouse IgG, Dynal Biotech, Invitrogen) previously coupled to an anti-cytokeratin antibody (Clone 34βE12, Dako, Trappes, France) (Herve *et al.*, 2019). The cells bound with the Dynabeads were resuspended in 1 ml phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) and aliquoted into two microtubes. An aliquot was used to determine the viability and the concentration of MEC in the milk with a Vi-Cell XR analyzer (Vi-CELL XR, Cell Viability Analyzer, Beckam Coulter, Brea, CA, USA) and the other aliquot was fixed with 1 ml of trizol and stored at –80 °C until RNA extraction.

Milk somatic cell typing

Milk somatic cell typing was performed by fluorescence-activated cell sorting (FACS) using a panel of antibodies designed to granulocytes and MEC (Supplementary Table S2 and Supplementary Methods). The cells labelled with CD49f antibody were identified as MEC and those with CH138 antibody as granulocytes.

Evaluation of mammary epithelium integrity

Mammary epithelium integrity was estimated by measuring plasma concentration of lactose but also Na^+:K^+ ratio in milk and MEC exfoliation rate into milk as described previously (Herve *et al.*, 2019). Lactose was quantified using the kit for the detection of lactose/D-galactose (Roche Diagnostics, Basel, Switzerland) and a multiparameter analyser (Kone Instrument Corp.). Milk concentration of Na^+ and K^+ were measured by atomic absorption spectrophotometry (Spectra AA220, Varian, Palo Alto, CA), allowing the calculation of Na^+:K^+ ratio in milk. The MEC exfoliation rate was defined as the number of MEC exfoliated per day and calculated as follows:

$$MEC\ exfoliation\ Rate = MY \times MEC,$$

where MY = daily milk yield (l) and MEC = MEC concentration (cells/l of milk) measured during morning milking (see above).

Milk performances

Milk yield of each animal was recorded during the morning and the evening milking. A volume of 50 ml was taken to determine the SCC and milk composition (fat, protein, and lactose content) using an infrared method (MyLab, Châteaugiron, France). The somatic cell score was then calculated as follows:

$$Somatic\ cell\ score\ (SCS) = \log\ base\ 2(SCC/100{,}000) + 3.$$

Expression of genes involved in the immune response and in the milk production by epithelial cells collected from milk

RNA extraction and RNA quality analysis of the purified MEC were performed as described previously (Boutinaud *et al.*, 2013). Gene expression analysis of IL-8 (*CXCL8*) and 6 (*IL6*), related to the immune response, and of cadherin 1 (*CDHI*), alpha lactalbumin (*LALBA*) and kappa casein (*CSN3*), related to epithelium integrity and functionality in epithelial cells was performed by quantitative RT-PCR as described previously (Herve *et al.*, 2019). The primers used for real-time PCR have been described in previous studies (Ben Chedly *et al.*, 2009; Boutinaud *et al.*, 2008, 2012, 2013; Finot *et al.*, 2018; Souza *et al.*, 2018). NormFinder macro (Andersen *et al.*, 2004) was used to assess the variability of candidate reference genes (Andersen *et al.*, 2004). The mRNA levels of the studied genes were expressed relative to the geometric mean of 3 selected reference genes: *RPLP0*, *RPS5* and *RPL19* and expressed as a semi-absolute number of mRNA molecules, multiplied by 10^8 and log10-transformed, using the method described previously (Boutinaud *et al.*, 2004).

Statistical analysis

A one-way analysis of variance (ANOVA) based on mixed model followed by a post-hoc test was performed using R software (Version 4.2.2, R Core Team, 2022) to compare the impact of treatments at D8 and D15 at the microbial, immune, and physiological levels. Two mixed models were used in our study, as the treatment was not applied during the entire experiment. One model was used to evaluate the effects during the treatment period (D8, D15) and another to evaluate the effects post-treatment. These mixed models take into account days, treatment and the interaction of both, but also parity and batches of animals, as fixed effects, and cow as a random effect. The impact of treatment at D1 was used as the covariate, especially because a treatment effect was observed for certain variables at D1, indicating that our treatment groups were initially not fully homogeneous (Figures 1 and 4, Supplementary Figure S2 and S3 and Supplementary Tables S1, S8, S9 and S10). Additional mixed models were used at D1 (before treatment), D15E (group of ten cows) and D26 (following treatment) and take into account treatment, parity and batches of animals (except at D15E) as fixed effect and cow as a random effect. Differences were considered statistically significant at $P < 0.05$. Of note, a trend-level significance was defined at $P < 0.10$.

Regarding microbiota, statistical analyses were performed using R and specialised packages: phyloseq (v. 1.34), DESeq2 (v 1.30.1) and custom scripts (Love *et al.*, 2014; Mariadassou, 2023; McMurdie and Holmes, 2013; R development Core Team, 2013). Data were rarefied to the same depth before computing alpha and beta diversity indices but not for differential abundance studies. Observed, Shannon and Inversed Simpson indices were used to represent the alpha-diversity of each condition. The impact of treatment on each index was assessed using a one-way analysis of variance (ANOVA) followed by a Tukey's HSD post-hoc test to find significant pairwise differences (adjusted P-value < 0.05). Beta diversity analyses were performed on the Bray-Curtis, Jaccard, UniFrac and wUniFrac distances. A Multi-Dimensional Scaling (MDS) was performed on the Bray-Curtis distance matrix to represent the samples on the principal plane, labelled by treatment. The impact of treatment on beta-diversity was assessed using multivariate analysis of variance (PERMANOVA), as implemented in the adonis2 function from the vegan package. Differences

were considered statistically significant at $P < 0.05$. Finally, a discriminant analysis was performed between the treatments using the linear discriminant analysis (LDA) effect size (LEfSe) pipeline, hosted on the INRAE galaxy GENOTOUL bioinformatics platform as previously described (Rault *et al.*, 2020). This analysis was completed by a differential abundance analysis performed with the DESeq2 R package implemented in the Easy16S R-shiny interface hosted on the Shiny Migale platform (Love *et al.*, 2014).

Data availability

Data files related to metagenomic analysis are available at the Sequence Read Archive of the National Center for Biotechnology Information under the accession number PRJNA1050055.

3 Results

Lacticaseibacillus paracasei *CIRM BIA 1542 is transiently present on teat skin and in foremilk*

The impact of Lp1542 post-milking treatment (L) was first evaluated on LAB populations present on TS and in FM, revealing a LAB population in FM 1.5 and 30-fold higher in L quarters than iodine post-milking treatment (I) quarters at D15 and D15E, respectively ($P < 0.01$; Figures 2A and 2B). Similarly, a 49-fold increase of the LAB population was observed on TS of L compared with I quarters, but only at D15E. The LAB population in CM was close to-and most generally below-the threshold whatever the treatment was, suggesting that no live Lp1542 was present in the mammary gland cistern (Supplementary Figure S3A). Since MRS medium is not species or strain-specific, the presence of Lp1542 specifically on L quarters was confirmed by qPCR using specific primers of Lp1542 (Supplementary Methods, Supplementary Figures S1A and S1B). At D26, the LAB population on TS or in FM was similar for all treatments, indicating no persistence of Lp1542 11 days after the treatment (Figures 2A and 2B).

Lp1542 treatment preserves the total microbial population compared with iodine treatment, without negative impact on the presence of pathogens in milk and teat skin

Interestingly, the total microbial population was also modulated by the treatment (Supplemental Figures S2A and S2B), with a lower total microbial population on TS and in FM in I than in L quarters at D15 ($P < 0.05$) and D15E ($P < 0.1$) but also in CM at D15 and D26 ($P < 0.05$) (Supplementary Figure S3C). No significant differences were found between L and N (absence of post-milking treatment) quarters, while the total microbial population on TS at D15 also tended to be lower in I compared with N quarters. This suggests that iodine treatment decreased the total microbial population on TS compared with the two other conditions (L and N), rather than a major increase of the microbial population induced by Lp1542 treatment. Similarly, the staphylococcal population was lower at D8 and D15E on TS of I compared with L and N quarters ($P < 0.05$; Sup-

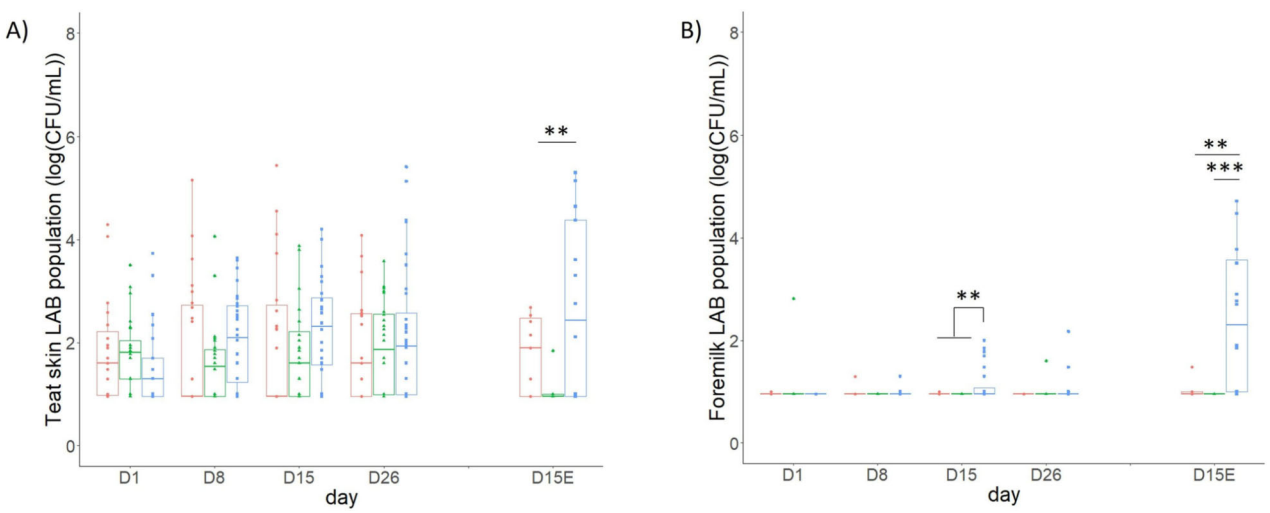

FIGURE 2 Lactic acid bacteria population (log (cfu/ml)) on teat skin (A) and in foremilk (B) before treatment (D1), during treatment (D8, D15, D15E), and following treatment (D26). Cow quarters were either treated with *Lacticaseibacillus paracasei* CIRM BIA 1542 (L; blue square) or iodine (I; green triangle) or not treated (N; red circle). Boxplots were used to represent the data distribution. Boxes extend from the 25th to the 75th percentile of each group's distribution of values. Within each box, horizontal line represents median value. ANOVA analysis based on mixed models followed by a post-hoc test was used to obtain statistical data; *** $P < 0.001$, ** $P < 0.01$.

plementary Figure 2E). However, no significant differences were found in FM during treatment regarding the staphylococcal population (Supplementary Figure S2F). Furthermore, the treatment did not significantly impact the enteric bacteria population with a median at the threshold detection level whatever the treatment was (Supplementary Figures S2C and S2D). No significant difference was observed in CM for enteric bacteria and staphylococcal population (Supplementary Figures S3B and S3D).

Finally, the presence of pathogens causing BM was evaluated in CM using the PathoProof™ Mastitis Complete-16 Kit, indicating no or a very low detection of pathogen DNA as revealed by a very high number of amplification cycles (Ct > 30 in all cases). Low levels of *Staphylococcus* spp. DNA were detected in cows of the 3 treatment groups at D1 and or D15 (30 < Ct < 40), as well as a low level of *Enterococcus* spp. DNA in cow C22 at D1 (Ct = 31) (Supplementary Table S1).

Lacticaseibacillus paracasei *CIRM BIA 1542 impacts the teat skin and foremilk microbiota*

The impact of the Lp1542 treatment on TS and FM microbiota was evaluated using metataxonomic approach at D15 and D15E on 234 samples from 20 cows (Figure 3). The sequencing of 16s rRNA produced 15,687,803 pairs of read. After the data were filtered, 7,878,144 of reads were finally obtained corresponding to 220 samples: group N (n = 39 at D15 and n = 22 at D15E), group I (n = 43 at D15 and n = 24 at D15E) and group L (n = 60 at D15 and n = 32 at D15E). TS and FM microbiota was dominated by *Bacillota*, followed by *Bacteroidota*, *Actinobacteriota*, *Euryarchaeota*, *Pseudomonadota* and *Patescibacteria* regardless the treatment applied (Figure 3A and Supplementary Figure S4A for dominant phyla and families at D15 and D15E, respectively).

Regarding the alpha-diversity, Lp1542 treatment had no significant effect on the FM microbiota (Figure 3B and Supplementary Figure S4B), whereas for TS microbiota, the Shannon and InvSimpson indexes were lower in L compared with I and or N quarters ($P < 0.05$; Figure 3B and Supplementary Figure S4B). Regarding beta-diversity, although separation of samples by treatment was not fully obvious from the MDS representation associated with Bray-Curtis distance (Figure 3C and Supplementary Figure S4C), an effect ($P < 0.05$) of treatment was found on the TS at D15 and D15E with the four distances used (Bray-Curtis, UniFrac, wUniFrac and Jaccard distances), except with the wUniFrac distance (only a trend at D15) (Supplementary Table S3). Similarly, a treatment effect ($P < 0.05$) was observed on the FM microbiota beta-diversity at D15 with Bray-Curtis and Jaccard distances (and a trend with wUniFrac) and at D15E with all distances except UniFrac (Supplementary Table S3).

The impact of post-milking treatment on TS and FM microbiota at D15 and D15E was further explored though a differential abundance analysis and a linear discriminant analysis effect size (LEfSe) (Figure 4, Supplementary Tables S4-S7 and Supplementary Figures S5-S9). Both methods confirmed that *Lactocaseibacillus paracasei* was more abundant in L quarters than in I and N quarters on TS and in FM (Figure 4, Supplementary Tables S4-S7 and Figures S5-S9).

Additional significant differences were observed between treatments, with globally more differences between L and I quarters than between L and N or I and N. The most discriminant taxa in TS microbiota at D15, as revealed by the LEfSe analysis, included a higher abundance of *Actinobacteriota* (previously named *Actinobacteria*) (including two *Bifidobacterium* species), *Negativicutes*, *Fusobacteriota*, and *Suterrellaceae* in L quarters, whereas *Pseudomonadota* (previously named *Proteobacteria*), *Euryarcheota*, *Planctomycetota* and *Erysipelotrichaceae*, were more abundant in I quarters and *Pseudomonadales*, *Bacillales* and *Micrococcaceae* in N quarters (Figure 4A, Supplementary Figure S6 and S10). These differences in TS microbiota composition at D15 were corroborated by the differential abundance analysis, which revealed a higher abundance of two OTUs related to *Bifidobacterium merycicum* and *Bifidobacterium ruminantium* and several OTUs related to *Staphylococcus chromogenes*, *Corynebacterium*, *Fusobacterium*, *Alloiococcus* or *Oscillospiraceae* in L quarters compared with I and or N quarters (Supplementary Table S4). Conversely, the abundance of several OTUs related to *Euryarcheota*, *Acinetobacter* and *Escherichia-Shigella* were more abundant in I than L quarters. Albeit less numerous, differences between treatments were observed on TS at D15E as well (Supplementary Figures S5 and S7 and Table S5), and in FM at D15 and D15E (Figure 4B, Supplementary Figures S5, S8 and S9, Supplementary Tables S6 and S7), with most differential OTU being more abundant in L or N compared with I quarters.

Lacticaseibacillus paracasei *CIRM BIA 1542 application on the teat hardly affected the local or systemic immune level*

The impact of Lp1542 at the immune level was investigated through the SCS and IL-8 concentration in milk

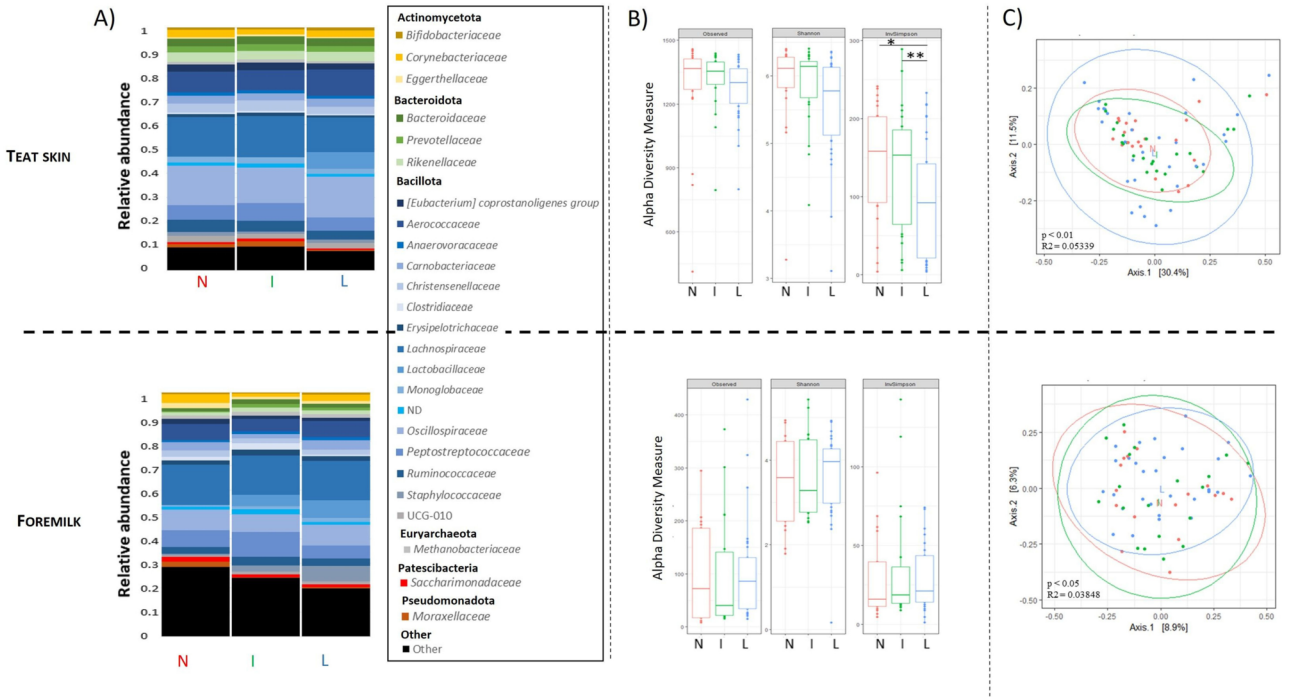

FIGURE 3 Impact of post-milking treatment on alpha- and beta-diversity of the teat skin and foremilk microbiota at D15. (A) Taxonomic profiles of the total microbiota. The barplots represent the 20 dominant families of each condition. (B) Observed, Shannon and Inversed Simpson indices were used to represent the alpha-diversity of each condition. Each point represents a cow quarter either treated by *Lacticaseibacillus paracasei* CIRM BIA 1542 (blue), Iodine (green) or not treated (red). ANOVA analysis followed by a Tukey test was used to obtain statistical data; ** $P < 0.01$, * $P < 0.05$. (C) Multi-dimensional scaling (MDS) performed on the measurement of the Bray-Curtis distance were used to represent the beta diversity. PERMANOVA analysis revealed a treatment effect on both teat skin and foremilk microbiota ($P < 0.05$). The R2 value indicates the contribution of the treatment to the beta-diversity of microbiota.

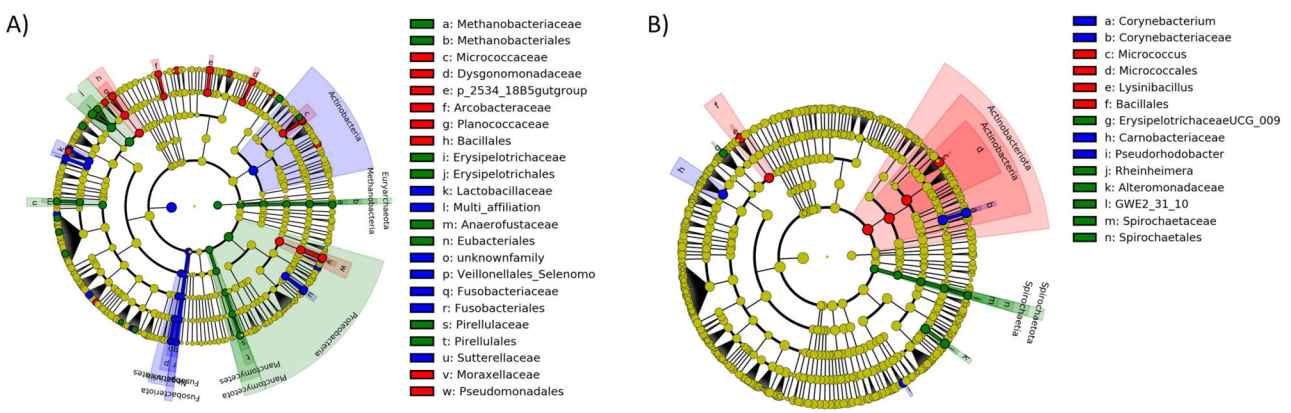

FIGURE 4 Linear discriminant analysis effect size (LEfSe) of the total microbiota on teat skin (A) or in foremilk (B) at D15. A cladogram was used to represent the difference of family composition between cow quarters treated by *Lacticaseibacillus paracasei* CIRM BIA 1542 (blue), iodine (green) or not treated (red).

at the quarter level (Figure 5). No significant difference was further observed for the SCS between the treatments. Albeit low in all cases, the IL-8 concentration tended to be higher at D8 and D15 in L compared with I quarters ($P < 0.1$), while no significant difference was found with N quarters. A similar impact of treatment was observed at D26, after the treatment was stopped. Interestingly, a higher expression of *CXCL8* in exfoliated milk MEC was observed in L cows compared with N cows (9.45 versus 8.12 log, corresponding to a 20-fold higher expression) at D15 (Table 1). In contrast, the abundance of *IL6* was not impacted by the treatments. Finally, the proportion of granulocytes in milk but also the plasma concentration of haptoglobin and cortisol at

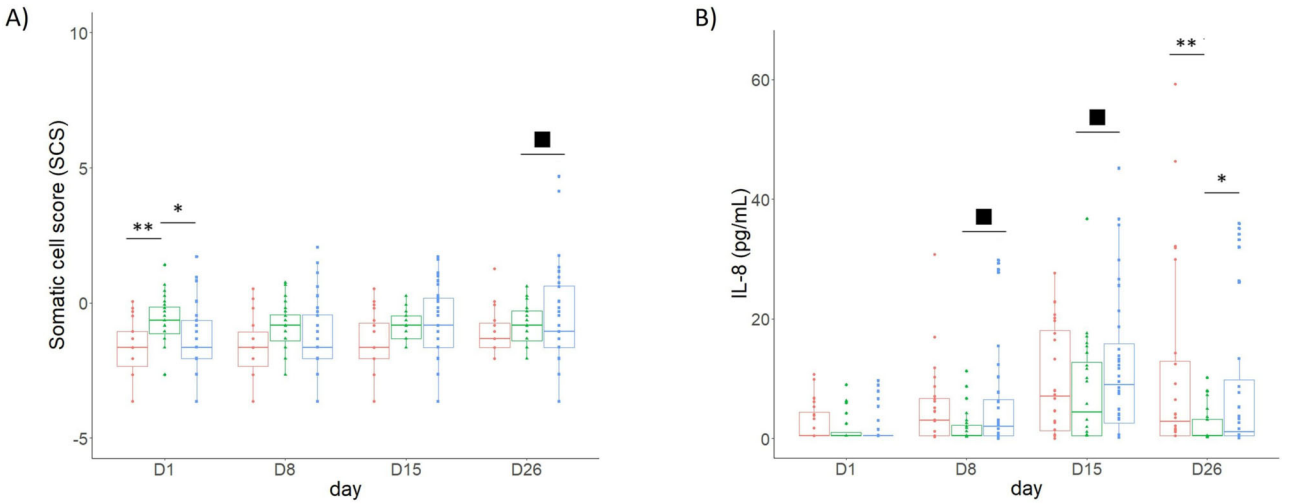

FIGURE 5 (A) Somatic cell score (SCS) and (B) interleukin (IL)-8 concentration (pg/ml; B) before treatment (D1), during treatment (D8, D15, D15E) and following treatment (D26). Cow quarters were either treated with *Lacticaseibacillus paracasei* CIRM BIA 1542 (L; blue square) or iodine (I; green triangle) or not treated (N; red circle). Boxplots were used to represent the data distribution. Boxes extend from the 25th to the 75th percentile of each group's distribution of values. Within each box, horizontal line represents median value. ANOVA analysis based on mixed models followed by a post-hoc test was used to obtain statistical data; ** $P < 0.01$, * $P < 0.05$, black square: $P < 0.1$.

TABLE 1 Impact of post-milking treatment (D8 and D15) on the gene expression of interleukins 8 (*CXCL8*) and 6 (*IL6*), related to the immune response, and cadherin 1 (*CDHI*), alpha lactalbumin (*LALBA*) and kappa casein (*CSN3*), related to epithelium integrity and functionality in epithelial cells recovered from composite milk samples[1]

		D8			D15				Treat-ment[2]	Day	Tt × Day
		N	I	L	N	I	L	SEM			
Immune response[3,4]											
Interleukin 8	*CXCL8*	8.60[a]	9.15[ab]	9.52[b]	8.12 [a]	8.55[a]	9.45[b]	0.292	***	.	NS
Interleukin 6	*IL6*	5.60	5.78	6.07	5.80	5.37	5.64	0.435	NS	NS	NS
Mammary epithelium integrity and synthesis[3,4]											
Kappa casein	*CSN3*	10.1	9.91	10.2	9.43	9.17	9.17	0.240	NS	***	NS
Alpha lactalbumin	*LALBA*	12.2	12.2	12.7	9.77	9.76	9.78	0.330	NS	***	NS
Cadherin 1	*CDH1*	7.01	7.13	7.40	7.69	7.98	7.90	0.175	NS	***	NS

[1] Cows were either not treated (N; n = 6) or treated with iodine (I; n = 6) or *Lacticaseibacillus paracasei* CIRM BIA 1542 (L; n = 8).

[2] ANOVA analysis based on mixed models followed by a post-hoc test was used to obtain statistical data; *** $P < 0.001$, $P < 0.1$. NS = not significant. Values with different superscript letters means significant difference ($P < 0.05$) at a specific day.

[3] Data are expressed in Emmean ± SEM where Emmean is the mean estimated by the statistical model using D1 as covariate and SEM, the maximal standard error.

[4] The mRNA levels of the studied genes were expressed relative to the geometric mean of 3 reference genes: *RPLP0*, *RPS5* and *RPLI9* and expressed as a semi-absolute number of mRNA molecules, multiplied by 10^8 and log-transformed.

the cow level, were not affected by the treatment (Table 2 and Supplementary Table S8).

Lacticaseibacillus paracasei *CIRM BIA 1542 application on the teat did not affect the mammary gland epithelium integrity nor the milking performances*

The impact of Lp1542 treatment the mammary gland integrity was investigated through the MEC exfoliated into milk, the proportion of MEC and the Na^+/K^+ ratio in milk and the blood lactose concentration, revealing no significant impact of the Lp1542 application on those parameters (Table 3).

The impact of the treatment on the mammary gland functionality was investigated through the milk yield and milk fat and protein contents and lactose concentration, revealing no difference at D8 and D15 (Table 3). Of note, the lactose concentration had the tendency to be slightly higher at D26 for the Lp1542-treated cows in comparison with the iodine-treated cows ($P < 0.1$;

TABLE 2 Impact of post-milking treatment on different immune markers at the animal level at D8, D14 and D15. Cows were either not treated (N; n = 6) or treated with iodine (I; n = 6) or *Lacticaseibacillus paracasei* CIRM BIA 1542 (L; n = 8)

	D8			D14[1]			D15			SEM	Treat-ment[2]	Day	Day × Tt
	N	I	L	N	I	L	N	I	L				
Granulocytes (%)[3,4]	52.1	60.5	61.0	NA[5]	NA	NA	55.9	55.3	44.7	5.09	NS	.	.
Cortisol (ng/ml)[4,6]	15.1	10.5	8.15	11.9	9.51	11.4	14.4	10.8	12.2	2.42	NS	NS	NS
Haptoglobin (mg/ml)[4,6]	0.41	0.48	0.42	0.44	0.44	0.41	0.42	0.45	0.38	0.06	NS	NS	NS

[1] An additional blood sampling was realised at D14 to avoid an effect of stress induced by sampling.
[2] ANOVA analysis based on mixed models followed by a post-hoc test was used to obtain statistical data; $\cdot$ $P < 0.1$. NS = not significant.
[3] Milk somatic cells typing was performed by fluorescence-Activated Cell Sorting (FACS) using a panel of antibodies. The cells labelled with the anti-CH138 antibody were identified as granulocyte.
[4] Data are expressed in Emmean ± SEM where Emmean is the mean estimated by the statistical model using D1 as covariate and SEM, the maximal standard error.
[5] NA means that no sample was analysed at this time.
[6] Plasma concentrations.

TABLE 3 Impact of post-milking treatment on integrity and functionality of mammary epithelium at D8 and D15.[1]

	D8			D15					Day	Tt × Day
	N	I	L	N	I	L	SEM	Treat-ment[2]		
Mammary epithelium integrity[3]										
MEC exfoliation in milk rate (×10³ cells/day)	116	76.3	144	143	121	144	51.9	NS		NS
Milk Na^+ concentration (mg/kg)	276	288	285	290	297	299	7.82	NS	***	NS
Milk K^+ concentration (×10³ mg/kg)	1.71	1.67	1.67	1.83	1.79	1.76	0.035	NS	*	NS
Milk Na^+/K^+ ratio	16.3	17.2	16.8	16.0	16.5	16.7	0.319	NS	NS	NS
Lactose in blood (mg/l)	44.5	50.6	39.2	52.5	51.8	48.1	6.29	NS	***	NS
MEC (%)[4]	7.36	11.66	6.34	5.61[a]	11.69[b]	9.05[ab]	1.84	*	NS	NS
M. e. functionality[3]										
Milk yield (kg/day)	30.6	30.0	30.6	30.8	30.2	30.5	0.472	NS	NS	NS
Milk fat content (g/kg)	41.6	37.2	38.7	42.6	40.6	42.5	1.97	NS	.	NS
Milk protein content (g/kg)	36.4	34.1	33.4	37.6	36.2	36.8	1.64	NS	.	NS
Lactose in milk (g/kg)	49.3	49.1	48.8	49.5	49.7	49.0	0.301	NS	*	NS

[1] Cows were either not treated (N; n = 6) or treated with iodine (I; n = 6) or Lacticaseibacillus paracasei CIRM BIA 1542 (L; n = 8).
[2] ANOVA analysis based on mixed models followed by a post-hoc test was used to obtain statistical data; *** $P < 0.001$, * $P < 0.05$, $\cdot$ $P < 0.1$. NS means not significant. Values with different superscript letters means significant difference (P < 0.05) at a specific day.
[3] Data are expressed in Emmean ± SEM where Emmean is the mean estimated by the statistical model using D1 as covariate and SEM, the maximal standard error.
[4] Milk somatic cells typing was performed by fluorescence-Activated Cell Sorting (FACS) using a panel of antibodies. The cells labelled with CD49f were identified as MEC.

Supplementary Table S9). Finally, the absence of any treatment effect was further confirmed on the expression of *CDH1*, *LALBA* and *CSN3* in MEC recovered from milk (Table 1).

4 Discussion

Most *in vivo* assays addressing the LAB potential for bovine mammary gland health explore the ability of intramammary infusions of LAB to treat mastitis (i.e. curative treatment) (Beecher *et al.*, 2009; Crispie *et al.*, 2008; Kitching *et al.*, 2019). Preventive strategies based on topical application of LAB on TS have been poorly explored, despite promising results when a combina-

tion of four LAB strains were applied on TS (Paduch *et al.*, 2020). In the present study, we evaluated the impact of a topical post-milking application of a *Lacticaseibacillus paracasei* strain on the bovine TS during lactation as a preventive strategy and deeply assessed, for the first time, the impact of such treatment at the microbial (through culture-dependent and independent approaches), immune and physiological levels before further exploring its effectiveness to prevent BM.

Lacticaseibacillus paracasei *CIRM BIA 1542 application modulate teat skin and foremilk microbiota: implications for the prevention of mastitis*

Lactic acid bacteria were present on TS, and to a lesser extent in FM, whatever the treatment was. This was not surprising as LAB are commonly present in dairy environment such as silage (Gagnon *et al.*, 2020). However, the LAB population increased on TS and in FM of Lp1542 treated quarters and the presence of Lp1542 on these quarters was specifically confirmed with qPCR. Its presence on TS and in FM was transient, highlighted by a Lp1542 population higher at D15E than at D15, and no more differences in LAB population at D26, once the treatment was stopped. Furthermore, Lp1542 was not present in CM, suggesting that the latter did not reach the cistern or that the immune system prevented this phenomenon. Altogether, these results are reassuring for the dairy producers who could be refractory to use a live culture of bacteria. Beyond the presence of Lp1542 on TS and in FM, the Lp1542 treatment allowed to preserve a microbial load on TS and in FM close to the one that naturally occurs in non-treated quarters. Conversely, the bactericidal effect of the iodine treatment, highlighted on total and staphylococcal populations, was confirmed (Bennett *et al.*, 2022; Gibson *et al.*, 2008).

A major question was to evaluate whether the Lp1542 treatment could impact the presence of pathogens. The staphylococcal population was higher on the TS of Lp1542-treated and non-treated quarters in comparison with iodine-treated quarters, which may be seen as a risk of intramammary infection development (Dufour *et al.*, 2012). Nevertheless, the presence of staphylococci, mainly coagulase negative staphylococci (CNS) on teat skin has also been proposed to prevent colonisation by other major pathogens responsible for mastitis (De Vliegher *et al.*, 2004; Lam *et al.*, 1997). At least, no pathogens causing mastitis were found in cisternal milk as confirmed using the Pathoproof kit, except *Staphylococcus* spp. that were detected in the cisternal milk of few cows belonging to the 3 groups of treatment, and at a very low level (Ct > 30). Additional experiments are required to further explore the potential protective effect of the Lp1542 application on teat skin against subclinical and clinical mastitis.

The metataxonomic analysis enabled to deeply investigate changes in teat microbiota. Only a moderate impact was found on alpha-diversity, with indexes such as InvSimpson and Shannon that were lower in L quarters. These 2 indexes consider both the richness and evenness and their decrease in Lp1542 treated quarters may be due to the relative abundance of *Lacticaseibacillus* which was strongly increased in these quarters. However, the richness itself (number of OTU) was not modified. Several studies highlighted a positive correlation between the alpha-diversity and the mammary gland health (Braem *et al.*, 2012; Falentin *et al.*, 2016; Rault *et al.*, 2020). Thus, although Lp1542 treatment does not increase richness, it maintains it at a level similar to the non-treated control, with potential benefits to health. Of note, although similar richness was also found in iodine-treated quarters, it may be associated to both alive and dead bacteria.

Several changes occurred in TS and FM microbiota composition, beyond the higher abundance of *Lacticaseibacillus casei* on Lp1542 treated quarters. Most changes were observed on TS at D15, and mostly between L and I, with an intermediate situation of non-treated quarters. The differential analyses on TS and in FM samples at D15 revealed that *Actinobacteriota* were more abundant in L but also in N quarters (only in FM) whereas *Pseudomonadota* were more abundant in I quarters. *Pseudomonadota* have previously been associated with a higher rate of mastitis using metataxonomic analysis (Rault *et al.*, 2020). It's worth mentioning here that metataxonomics, like other DNA-based approaches, does not differentiate live and dead bacterial cells. At least we did not observe a higher population of Enterobacteria on iodine treated quarters by culture. Furthermore, the differential abundance analysis also highlighted that *Staphylococcus chromogenes*, *Corynebacterium* spp. but also *Lachnospiraceae*, *Bifidobacterium merycicum* and *Bifidobacterium ruminantium* were more abundant in L than I quarters. This is of interest considering that *Bifidobacterium* and *Lachnospiraceae* were associated to a healthy state (Falentin *et al.*, 2016; Oikonomou *et al.*, 2014; Rault *et al.*, 2020). Furthermore, several studies highlighted the beneficial proprieties of *Bifidobacterium*, especially in human intestinal context, including their anti-inflammatory properties, and their ability to reinforce the epithelial barrier (Al-Sadi *et al.*, 2021; Bergmann *et al.*, 2013). In the

opposite, *S. chromogenes* and *Corynebacterium* spp. are both considered as minor pathogens causing BM (Reyher *et al.*, 2012). However, a meta-analysis highlighted a significant protective effects by those minor pathogens, specifically when major pathogens were introduced into the mammary gland via methods bypassing the teat end (Reyher *et al.*, 2012). Altogether, these results suggest that the addition of an exogenous strain modulates the balance between species within the microbiota and/or the recolonisation of the TS and FM microbiota after each post-treatment.

Safety assessment of Lp1542 treatment at the immune and physiological levels

At the immune level, our results showed that post-milking Lp1542 teat application did not induce any major local nor systemic inflammatory response. Indeed, the haptoglobin concentrations and somatic cell scores were not impacted by the Lp1542 treatment, contrary to other studies which have administrated LAB into the bovine mammary gland (Beecher *et al.*, 2009; Crispie *et al.*, 2008; Pellegrino *et al.*, 2017). Our results are consistent with one study observing no association between the external application of a teat dip containing LAB and the SCC (Paduch *et al.*, 2020). Furthermore, immune cell proportion was unchanged between Lp1542-treated cows and the others cows. Several studies have reported that the quantification of the different types of immune cells in addition to the SCC provide a better representation of the udder health status (Schwarz *et al.*, 2011; Widmer *et al.*, 2022). Finally, blood cortisol concentration was not impacted by the treatment with Lp1542, suggesting that its application does not induce a stress for the animal. This finding is of interest for the dairy producers because an increase blood cortisol concentration may lead to a milk yield loss (Grelet *et al.*, 2022). In contrast, the production of the proinflammatory cytokine, IL-8 but also the expression of the corresponding gene, tended to be slightly higher in Lp1542 than in iodine-treated quarters, indicating a local low-level stimulation of the immune response by Lp1542, without measurable consequences on somatic cell counts. As mentioned above, intramammary infusion of LAB generally lead to a proinflammatory response – including an increase in IL8 secretion in milk – which was proposed to help eliminating the pathogen (Kitching *et al.*, 2019; Mignacca *et al.*, 2017). In our case, no significant differences in IL-8 secretion in milk were observed between the untreated quarters and quarters treated with Lp1542 or iodine, suggesting an intermediate state for the non-treated quarters. The presence of a live microbiota (in non-treated and Lp1542-treated quarters) may help to maintain a basal inflammatory level contrary to iodine treatment. In the literature, researchers have proposed that cows with an intermediate SCC values (21,000-50,000 cells/ml) have a lesser risk to develop intramammary infections, due to the presence of an 'optimal' microbiome (Oikonomou *et al.*, 2014).

At the physiological levels, our results firstly showed that Lp1542 did not impact the integrity of the mammary epithelium. The integrity of the mammary epithelium, allowed by the existence of tight junctions between MEC, ensures the cohesion of the tissue needed to the synthesis of milk and the establishment of a barrier against the invasion by pathogenic agents. Furthermore, the MEC contribute to the innate immune response (Günther *et al.*, 2017). In case of an intramammary infection, the integrity of the epithelium is broken, leading to variations in milk yield and composition, in the plasmatic concentration of lactose and the Na^+:K^+ ratio in milk as well as an increase in the rate of MEC exfoliation and spread of infection (Bruckmaier and Wellnitz, 2017). Our results showed that the application of Lp1542 preserve the integrity of mammary epithelium. Secondly, Lp1542 did not affect the functionality of the mammary epithelium nor the milking performances. Likewise, the quantification of genes involved in the integrity of mammary gland (*CDHI*) but also genes related to the synthesis of milk component (*LALBA* and *CSN3*) supports the safety of the Lp1542 application with regard to the functionality and integrity of the mammary epithelium. To our knowledge, this is the first study to evaluate the impact of LAB application at the physiological level.

In conclusion, this study shows that a topical treatment with *L. paracasei* CIRM BIA 1542 is safe with regard to mammary gland physiology and immune system, while significantly impacting its microbiota. Altogether, these results invite us to further explore on a larger scale and on longer treatment periods, the effectiveness of this new microbial strategy to prevent mastitis, possibly in relation to the changes in microbiota and low-level immune stimulation. Whether a protective effect of the Lp1542 application were to be confirmed, it would contribute to the development of more sustainable control strategies that are more respectful of the environment, the human health and well-being of animals.

Supplementary material

Supplementary material is available online at: https://doi.org/10.6084/m9.figshare.25689357

Supplementary methods. Sample collection, Determination of *Lacticaseibacillus paracasei* CIRM BIA 1542 by quantitative PCR and Milk somatic cells typing.

Figure S1. *Lacticaseibacillus paracasei* CIRM BIA 1542 population was determined on teat skin (A) and in foremilk (B) at D15 and D15E by qPCR using specific primers and is expressed in log (number of copies/ml).

Figure S2. Total microbial (A and B), enteric (C and D) and staphylococcal (E and F) populations (log (cfu/ml)) on teat skin (A, C and E) or in foremilk (B, D and F) before treatment (D1), during treatment (D8, D15, D15E) and following treatment (D26).

Figure S3. Lactic acid bacteria (A), enteric (B), total bacterial (C) and staphylococcal (D) populations (log (cfu/ml)) in cisternal milk before treatment (D1), during treatment (D8, D15) and following treatment (D26).

Figure S4. Impact of post-milking treatment on alpha- and beta-diversity of the teat skin and foremilk microbiota at D15E.

Figure S5. Linear discriminant analysis effect size (LEfSe) of the total microbiota on teat skin (A) or in foremilk (B) at D15E.

Figure S6. Linear discriminant analysis effect size (LEfSe) of the total microbiota on teat skin at D15.

Figure S7. Linear discriminant analysis effect size (LEfSe) of the total microbiota on teat skin at D15E.

Figure S8. Linear discriminant analysis effect size (LEfSe) of the total microbiota in foremilk at D15.

Figure S9. Linear discriminant analysis effect size (LEfSe) of the total microbiota in foremilk at D15E.

Figure S10. Relative abundance of discriminant taxa between the three treatments (*Lacticaseibacillus paracasei* CIRM BIA 1542, iodine or no treatment), as determined by a Linear discriminant analysis effect size (LEfSe) on the TS microbiota at D15.

Table S1. List of cows and associated metadata and impact of post-milking treatment on the presence of pathogens causing bovine mastitis at D1 and D15 using the PathoProof™ Mastitis Complete-16 Kit.

Table S2. Antibody panel used for milk cell typing by fluorescence-activated cell sorting (FACS).

Table S3. Impact of post-milking treatment on beta-diversity of the total microbiota on teat skin or in foremilk at D15 and D15E.

Table S4. Differential abundance analysis of the total microbiota on teat skin at D15. Cows were either not treated (N; n = 6) or treated with iodine (I; n = 6) or *Lacticaseibacillus paracasei* CIRM BIA 1542 (L; n = 8).

Table S5. Differential abundance analysis of the total microbiota on teat skin at D15E.

Table S6. Differential abundanceanalysis of the total microbiota in foremilk at D15.

Table S7. Differential abundance analysis of the total microbiota in foremilk at D15E.

Table S8. Impact of post-milking treatment on different immune markers at the animal level before treatment (D1).

Table S9. Expression of genes of interleukins 8 (*CXCL8*) and 6 (*IL6*), related to the immune response, and cadherin 1 (*CDH1*), alpha lactalbumin (*LALBA*) and kappa casein (*CSN3*), related to epithelium integrity and functionality in epithelial cells recovered from composite milk samples before treatment (D1).

Table S10. Impact of post-milking treatment on integrity and functionality of mammary epithelium before (D1) and following treatment (D26).

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Authors' contribution

Conceptualization, MB and SE; methodology, CG, LR, JC, PP, LF and GB; software, CG, JC, PP, LF, MB and SE; validation, MB and SE; formal analysis, CG, JC, MB and SE; writing-original draft preparation, CG; writing-review and editing, CG, MB and SE; supervision, MB and SE; funding acquisition, SE. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

Al-Sadi, R., Dharmaprakash, V., Nighot, P., Guo, S., Nighot, M., Do, T. and Ma, T.Y., 2021. *Bifidobacterium bifidum* enhances the intestinal epithelial tight junction barrier and protects against intestinal inflammation by targeting the Toll-like receptor-2 pathway in an NF-κB-independent manner. International Journal of Molecular Sciences 22: 8070. https://doi.org/10.3390/ijms22158070

Andersen, C.L., Jensen, J.L. and Ørntoft, T.F., 2004. Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Research 64: 5245-5250. https://doi.org/10.1158/0008-5472.CAN-04-0496

Armas, F., Camperio, C. and Marianelli, C., 2017. *In vitro* assessment of the probiotic potential of *Lactococcus lactis* LMG 7930 against ruminant mastitis-causing pathogens. PLoS ONE 12: e0169543. https://doi.org/10.1371/journal.pone.0169543

Baron, F., Cochet, M.-F., Ablain, W., Grosset, N., Madec, M.-N., Gonnet, F., Jan, S. and Gautier, M., 2006. Rapid and cost-effective method for micro-organism enumeration based on miniaturization of the conventional plate-counting technique. Le Lait 86: 251-257. https://doi.org/10.1051/lait:2006005

Beecher, C., Daly, M., Berry, D.P., Klostermann, K., Flynn, J., Meaney, W., Hill, C., McCarthy, T.V., Ross, R.P. and Giblin, L., 2009. Administration of a live culture of *Lactococcus lactis* DPC 3147 into the bovine mammary gland stimulates the local host immune response, particularly IL-1beta and IL-8 gene expression. Journal of Dairy Research 76: 340-348. https://doi.org/10.1017/S0022029909004154

Ben Chedly, H., Lacasse, P., Marnet, P.-G., Wiart-Letort, S., Finot, L. and Boutinaud, M., 2009. Cell junction disruption after 36 h milk accumulation was associated with changes in mammary secretory tissue activity and dynamics in lactating dairy goats. Journal of Physiology and Pharmacology 60: 105-111.

Bennett, S., Fliss, I., Ben Said, L., Malouin, F. and Lacasse, P., 2022. Efficacy of bacteriocin-based formula for reducing staphylococci, streptococci and total bacterial counts on teat skin of dairy cows. Journal of Dairy Science 105: 4498-4507. https://doi.org/10.3168/jds.2021-21381

Bergmann, K.R., Liu, S.X.L., Tian, R., Kushnir, A., Turner, J.R., Li, H.-L., Chou, P.M., Weber, C.R. and De Plaen, I.G., 2013. Bifidobacteria stabilize claudins at tight junctions and prevent intestinal barrier dysfunction in mouse necrotizing enterocolitis. The American Journal of Pathology 182: 1595-1606. https://doi.org/10.1016/j.ajpath.2013.01.013

Bicalho, M.L.S., Santin, T., Rodrigues, M.X., Marques, C.E., Lima, S.F. and Bicalho, R.C., 2017. Dynamics of the microbiota found in the vaginas of dairy cows during the transition period: Associations with uterine diseases and reproductive outcome. Journal of Dairy Science 100: 3043-3058. https://doi.org/10.3168/jds.2016-11623

Blowey, R.W., Blowey, R.W. and Edmondson, P., 1995. Mastitis control in dairy herds: An illustrated and practical guide. Farming Press Books, Ipswich, UK.

Borucki Castro, S.I., Berthiaume, R., Laffey, P., Fouquet, A., Beraldin, F., Robichaud, A. and Lacasse, P., 2010. Iodine concentration in milk sampled from Canadian farms. Journal of Food Protection 73: 1658-1663. https://doi.org/10.4315/0362-028x-73.9.1658

Borucki Castro, S.I., Berthiaume, R., Robichaud, A. and Lacasse, P., 2012. Effects of iodine intake and teat-dipping practices on milk iodine concentrations in dairy cows. Journal of Dairy Science 95: 213-220. https://doi.org/10.3168/jds.2011-4679

Bouchard, D.S., Rault, L., Berkova, N., Le Loir, Y. and Even, S., 2013. Inhibition of *Staphylococcus aureus* invasion into bovine mammary epithelial cells by contact with live *Lactobacillus casei*. Applied and Environmental Microbiology 79: 877-885. https://doi.org/10.1128/AEM.03323-12

Bouchard, D.S., Seridan, B., Saraoui, T., Rault, L., Germon, P., Gonzalez-Moreno, C., Nader-Macias, F.M.E., Baud, D., François, P., Chuat, V., Chain, F., Langella, P., Nicoli, J., Le Loir, Y. and Even, S., 2015. Lactic acid bacteria isolated from bovine mammary microbiota: Potential allies against bovine mastitis. PLoS ONE 10: e0144831. https://doi.org/10.1371/journal.pone.0144831

Boutinaud, M., Ben Chedly, M.H., Delamaire, E. and Guinard-Flament, J., 2008. Milking and feed restriction regulate transcripts of mammary epithelial cells purified from milk. Journal of Dairy Science 91: 988-998. https://doi.org/10.3168/jds.2007-0587

Boutinaud, M., Galio, L., Lollivier, V., Finot, L., Wiart, S., Esquerré, D. and Devinoy, E., 2013. Unilateral once daily milking locally induces differential gene expression in both mammary tissue and milk epithelial cells revealing mammary remodeling. Physiological Genomics 45: 973-985. https://doi.org/10.1152/physiolgenomics.00059.2013

Boutinaud, M., Lollivier, V., Finot, L., Bruckmaier, R.M. and Lacasse, P., 2012. Mammary cell activity and turnover in dairy cows treated with the prolactin-release inhibitor quinagolide and milked once daily. Journal of Dairy Science 95: 177-187. https://doi.org/10.3168/jds.2011-4461

Boutinaud, M., Shand, J.H., Park, M.A., Phillips, K., Beattie, J., Flint, D.J. and Allan, G.J., 2004. A quantitative RT-PCR study of the mRNA expression profile of the IGF axis during mammary gland development. Journal of Molecular Endocrinology 33: 195-207. https://doi.org/10.1677/jme.0.0330195

Braem, G., De Vliegher, S., Verbist, B., Heyndrickx, M., Leroy, F. and De Vuyst, L., 2012. Culture-independent exploration of the teat apex microbiota of dairy cows reveals a wide bacterial species diversity. Veterinary Microbiology 157: 383-390. https://doi.org/10.1016/j.vetmic.2011.12.031

Brouillette, E., Millette, G., Chamberland, S., Roy, J.-P., Ster, C., Kiros, T., Hickey, S., Hittle, L., Woolston, J. and Malouin, F., 2023. Effective treatment of *Staphylococcus aureus* intramammary infection in a murine model using the bacteriophage cocktail StaphLyse™. Viruses 15: 887. https://doi.org/10.3390/v15040887

Bruckmaier, R.M. and Wellnitz, O., 2017. Triennial lactation symposium/BOLFA: Pathogen-specific immune response and changes in the blood-milk barrier of the bovine mammary gland. Journal of Animal Science 95: 5720-5728. https://doi.org/10.2527/jas2017.1845

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K. and Madden, T.L., 2009. BLAST+: Architecture and applications. BMC Bioinformatics 10: 421. https://doi.org/10.1186/1471-2105-10-421

CNIEL, 2021. Les chiffres clés des mammites en élevage laitier: Quel est l'impact des infections mammaires en élevage ? Les mammites, j'anticipe! Available at: https://les-mammites-j-anticipe.com/informations/chiffres-cles/

Crispie, F., Alonso-Gómez, M., O'Loughlin, C., Klostermann, K., Flynn, J., Arkins, S., Meaney, W., Paul Ross, R. and Hill, C., 2008. Intramammary infusion of a live culture for treatment of bovine mastitis: Effect of live lactococci on the mammary immune response. The Journal of Dairy Research 75: 374-384. https://doi.org/10.1017/S0022029908003385

De Vliegher, S., Opsomer, G., Vanrolleghem, A., Devriese, L.A., Sampimon, O.C., Sol, J., Barkema, H.W., Haesebrouck, F. and de Kruif, A., 2004. *In vitro* growth inhibition of major mastitis pathogens by *Staphylococcus chromogenes* originating from teat apices of dairy heifers. Veterinary Microbiology 101: 215-221. https://doi.org/10.1016/j.vetmic.2004.03.020

Doré, J., Simrén, M., Buttle, L. and Guarner, F., 2013. Hot topics in gut microbiota. United European Gastroenterology Journal 1: 311-318. https://doi.org/10.1177/2050640613502477

Dufour, S., Dohoo, I.R., Barkema, H.W., Descôteaux, L., Devries, T.J., Reyher, K.K., Roy, J.-P. and Scholl, D.T., 2012. Epidemiology of coagulase-negative staphylococci intramammary infection in dairy cattle and the effect of bacteriological culture misclassification. Journal of Dairy Science 95: 3110-3124. https://doi.org/10.3168/jds.2011-5164

EFSA Panel on Biological Hazards (BIOHAZ), 2023. Update of the list of qualified presumption of safety (QPS) recommended microbiological agents intentionally added to food or feed as notified to EFSA 17: Suitability of taxonomic units notified to EFSA until September 2022. EFSA Journal 21: e07746. https://doi.org/10.2903/j.efsa.2023.7746

Falentin, H., Rault, L., Nicolas, A., Bouchard, D.S., Lassalas, J., Lamberton, P., Aubry, J.-M., Marnet, P.-G., Le Loir, Y. and Even, S., 2016. Bovine teat microbiome analysis revealed reduced alpha diversity and significant changes in taxonomic profiles in quarters with a history of mastitis. Frontiers in Microbiology 7: 480. https://doi.org/10.3389/fmicb.2016.00480

Finot, L., Chanat, E. and Dessauge, F., 2018. Molecular signature of the putative stem/progenitor cells committed to the development of the bovine mammary gland at puberty. Scientific Reports 8: 16194. https://doi.org/10.1038/s41598-018-34691-2

Gaeta, N.C., Lima, S.F., Teixeira, A.G., Ganda, E.K., Oikonomou, G., Gregory, L. and Bicalho, R.C., 2017. Deciphering upper respiratory tract microbiota complexity in healthy calves and calves that develop respiratory disease using shotgun metagenomics. Journal of Dairy Science 100: 1445-1458. https://doi.org/10.3168/jds.2016-11522

Gagnon, M., Ouamba, A.J.K., LaPointe, G., Chouinard, P.Y. and Roy, D., 2020. Prevalence and abundance of lactic acid bacteria in raw milk associated with forage types in dairy cow feeding. Journal of Dairy Science 103: 5931-5946. https://doi.org/10.3168/jds.2019-17918

Gibson, H., Sinclair, L.A., Brizuela, C.M., Worton, H.L. and Protheroe, R.G., 2008. Effectiveness of selected premilking teat-cleaning regimes in reducing teat microbial load on commercial dairy farms. Letters in Applied Microbiology 46: 295-300. https://doi.org/10.1111/j.1472-765X.2007.02308.x

Grelet, C., Vanden Dries, V., Leblois, J., Wavreille, J., Mirabito, L., Soyeurt, H., Franceschini, S., Gengler, N., Brostaux, Y., HappyMoo Consortium and Dehareng, F., 2022. Identification of chronic stress biomarkers in dairy cows. Animal 16: 100502. https://doi.org/10.1016/j.animal.2022.100502

Gruet, P., Maincent, P., Berthelot, X. and Kaltsatos, V., 2001. Bovine mastitis and intramammary drug delivery: Review

and perspectives. Advanced Drug Delivery Reviews 50: 245-259. https://doi.org/10.1016/s0169-409x(01)00160-0

Günther, J., Petzl, W., Bauer, I., Ponsuksili, S., Zerbe, H., Schuberth, H.-J., Brunner, R.M. and Seyfert, H.-M., 2017. Differentiating *Staphylococcus aureus* from *Escherichia coli* mastitis: *S. aureus* triggers unbalanced immune-dampening and host cell invasion immediately after udder infection. Scientific Reports 7: 4811. https://doi.org/10.1038/s41598-017-05107-4

Halasa, T., Huijps, K., Østerås, O. and Hogeveen, H., 2007. Economic effects of bovine mastitis and mastitis management: A review. Veterinary Quarterly 29: 18-31. https://doi.org/10.1080/01652176.2007.9695224

Herve, L., Quesnel, H., Veron, M., Portanguen, J., Gross, J.J., Bruckmaier, R.M. and Boutinaud, M., 2019. Milk yield loss in response to feed restriction is associated with mammary epithelial cell exfoliation in dairy cows. Journal of Dairy Science 102: 2670-2685. https://doi.org/10.3168/jds.2018-15398

Kitching, M., Mathur, H., Flynn, J., Byrne, N., Dillon, P., Sayers, R., Rea, M.C., Hill, C. and Ross, R.P., 2019. A live bio-therapeutic for mastitis, containing *Lactococcus lactis* DPC3147 with comparable efficacy to antibiotic treatment. Frontiers in Microbiology 10: 2220. https://doi.org/10.3389/fmicb.2019.02220

Komara, M. and Marnet, P.G., 2009. Endocrine responses and milk emission characteristics in high yielding Alpine dairy goats under once daily milking management. Small Ruminant Research 87: 64-69. https://doi.org/10.1016/j.smallrumres.2009.10.003

Lam, T.J., Schukken, Y.H., van Vliet, J.H., Grommers, F.J., Tielen, M.J. and Brand, A., 1997. Effect of natural infection with minor pathogens on susceptibility to natural infection with major pathogens in the bovine mammary gland. American Journal of Veterinary Research 58: 17-22.

Love, M.I., Huber, W. and Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biology 15: 550. https://doi.org/10.1186/s13059-014-0550-8

Mahé, F., Rognes, T., Quince, C., de Vargas, C. and Dunthorn, M., 2014. Swarm: Robust and fast clustering method for amplicon-based studies. PeerJ 2: e593. https://doi.org/10.7717/peerj.593

Mariadassou, M., 2023. Custom scripts [Computer software]. https://github.com/mahendra-mariadassou/phyloseq-extended/tree/dev

Mariadassou, M., Nouvel, L.X., Constant, F., Morgavi, D.P., Rault, L., Barbey, S., Helloin, E., Rué, O., Schbath, S., Launay, F., Sandra, O., Lefebvre, R., Le Loir, Y., Germon, P., Citti, C. and Even, S., 2023. Microbiota members from body sites of dairy cows are largely shared within individual hosts throughout lactation but sharing is limited in the herd. Animal Microbiome 5: 32. https://doi.org/10.1186/s42523-023-00252-w

Martín, R., Chamignon, C., Mhedbi-Hajri, N., Chain, F., Derrien, M., Escribano-Vázquez, U., Garault, P., Cotillard, A., Pham, H.P., Chervaux, C., Bermúdez-Humarán, L.G., Smokvina, T. and Langella, P., 2019. The potential probiotic *Lactobacillus rhamnosus* CNCM I-3690 strain protects the intestinal barrier by stimulating both mucus production and cytoprotective response. Scientific Reports 9: 5398. https://doi.org/10.1038/s41598-019-41738-5

McMurdie, P.J. and Holmes, S., 2013. phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. PLoS ONE 8: e61217. https://doi.org/10.1371/journal.pone.0061217

Mignacca, S.A., Dore, S., Spuria, L., Zanghì, P., Amato, B., Duprè, I., Armas, F., Biasibetti, E., Camperio, C., Lollai, S.A., Capucchio, M.T., Cannas, E.A., Di Marco Lo Presti, V. and Marianelli, C., 2017. Intramammary infusion of a live culture of *Lactococcus lactis* in ewes to treat staphylococcal mastitis. Journal of Medical Microbiology 66: 1798-1810. https://doi.org/10.1099/jmm.0.000641

Oikonomou, G., Bicalho, M.L., Meira, E., Rossi, R.E., Foditsch, C., Machado, V.S., Teixeira, A.G.V., Santisteban, C., Schukken, Y.H. and Bicalho, R.C., 2014. Microbiota of cow's milk; distinguishing healthy, sub-clinically and clinically diseased quarters. PLoS ONE 9: e85904. https://doi.org/10.1371/journal.pone.0085904

Oliver, S.P. and Murinda, S.E., 2012. Antimicrobial resistance of mastitis pathogens. Veterinary Clinics of North America – Food Animal Practice 28: 165-185. https://doi.org/10.1016/j.cvfa.2012.03.005

Paduch, J.-H., Lücking, J., Mansion-de Vries, E., Zinke, C., Wente, N. and Krömker, V., 2020. Prevention of intramammary infections by prepartum external application of a teat dip containing lactic acid bacteria with antimicrobial properties in dairy heifers. Pathogens 9: 288. https://doi.org/10.3390/pathogens9040288

Pellegrino, M., Berardo, N., Giraudo, J., Nader-Macías, M.E.F. and Bogni, C., 2017. Bovine mastitis prevention: Humoral and cellular response of dairy cows inoculated with lactic acid bacteria at the dry-off period. Beneficial Microbes 8: 589-596. https://doi.org/10.3920/BM2016.0194

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J. and Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. Nucleic Acids Research 41: D590-596. https://doi.org/10.1093/nar/gks1219

R development Core Team, 2013. R: a language and environment for statistical computing [Computer software].

R Foundation for Statistical Computing. Available at: https://www.R-project.org

Rault, L., Lévêque, P.-A., Barbey, S., Launay, F., Larroque, H., Le Loir, Y., Germon, P., Guinard-Flament, J. and Even, S., 2020. Bovine teat cistern microbiota composition and richness are associated with the immune and microbial responses during transition to once-daily milking. Frontiers in Microbiology 11: 602404. https://doi.org/10.3389/fmicb.2020.602404

Reyher, K.K., Haine, D., Dohoo, I.R. and Revie, C.W., 2012. Examining the effect of intramammary infections with minor mastitis pathogens on the acquisition of new intramammary infections with major mastitis pathogens – A systematic review and meta-analysis. Journal of Dairy Science 95: 6483-6502. https://doi.org/10.3168/jds.2012-5594

Roussel, P., Cunha, P., Porcherie, A., Petzl, W., Gilbert, F.B., Riollet, C., Zerbe, H., Rainard, P. and Germon, P., 2015. Investigating the contribution of IL-17A and IL-17F to the host response during *Escherichia coli* mastitis. Veterinary Research 46: 56. https://doi.org/10.1186/s13567-015-0201-4

Sadakane, K. and Ichinose, T., 2015. Effect of the hand antiseptic agents benzalkonium chloride, povidone-iodine, ethanol and chlorhexidine gluconate on atopic dermatitis in NC/Nga mice. International Journal of Medical Sciences 12: 116-125. https://doi.org/10.7150/ijms.10322

Schwarz, D., Diesterbeck, U.S., König, S., Brügemann, K., Schlez, K., Zschöck, M., Wolter, W. and Czerny, C.-P., 2011. Flow cytometric differential cell counts in milk for the evaluation of inflammatory reactions in clinically healthy and subclinically infected bovine mammary glands. Journal of Dairy Science 94: 5033-5044. https://doi.org/10.3168/jds.2011-4348

Sharun, K., Dhama, K., Tiwari, R., Gugjoo, M.B., Iqbal Yatoo, Mohd., Patel, S.K., Pathak, M., Karthik, K., Khurana, S.K., Singh, R., Puvvala, B., Amarpal, Singh, R., Singh, K.P. and Chaicumpa, W., 2021. Advances in therapeutic and managemental approaches of bovine mastitis: A comprehensive review. Veterinary Quarterly 41: 107-136. https://doi.org/10.1080/01652176.2021.1882713

Souza, R.F.S., Rault, L., Seyffert, N., Azevedo, V., Le Loir, Y. and Even, S., 2018. *Lactobacillus casei* BL23 modulates the innate immune response in *Staphylococcus aureus*-stimulated bovine mammary epithelial cells. Beneficial Microbes 9: 985-995. https://doi.org/10.3920/BM2018.0010

Widmer, J., Descloux, L., Brügger, C., Jäger, M.-L., Berger, T. and Egger, L., 2022. Direct labeling of milk cells without centrifugation for counting total and differential somatic cells using flow cytometry. Journal of Dairy Science 105: 8705-8717. https://doi.org/10.3168/jds.2022-22038