

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

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Supplementary methods

Sample collection

Teats were swabbed with sterile swabs (D. Dutscher, Brumath, France) moistened with a sterile 0.9% (w/v) saline solution. The swabs were immediately placed in sterile 15 ml tubes containing 2.5 ml of sterile 0.9% (w/v) saline solution. Foremilk (5 ml) and CM (15 ml) samples were then collected by quarter as previously described, except for D15E (Rault *et al.*, 2020). For FM sampling at D15E, teat skin was cleaned with a paper towel but thorough cleaning with ethanol was omitted to limit the stimulation of the mammary gland and avoid milk leaking, thus allowing the collection of the true FM.

Design of the *Lacticaseibacillus paracasei* CIRM BIA 1542-specific primers

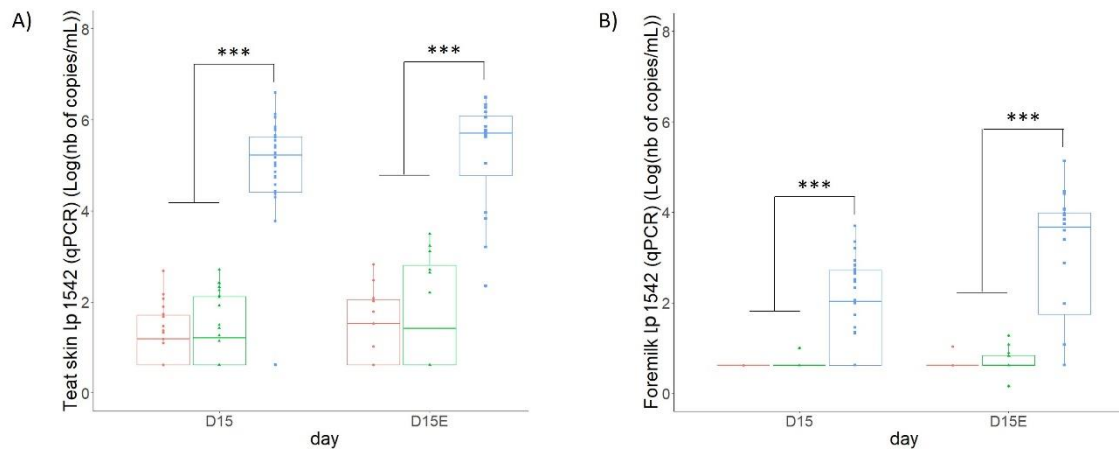
Firstly, the sequence of Lc1542 was compared to the 50 ‘nearest neighbours’ available in the PATRIC database using the similar genome finder tool, followed by the protein family sorter tool in order to identify strain-specific proteins. Nine candidate genes were thus selected. Secondly, a Blast of these 9 genes was performed on the NCBI databases. Three candidate genes were retained, presenting the fewest homologous genes. Finally, a qPCR was performed on 10⁶ copies of gDNA of 10 phylogenetically close or more distant strains (*Lacticaseibacillus casei* CIRM BIA 667 and 769, *L. paracasei* CIRM BIA 2516 and BL23, *Lacticaseibacillus rhamnosus* CIRM BIA 930, *Lactococcus lactis* CIRM BIA 2553, *Streptococcus uberis* CIRM BIA1637, *Lactiplantibacillus plantarum* CIRM BIA 2654, *L. brevis* CIRM BIA 1595 and *Staphylococcus aureus* NB305) to confirm the specificity of the strain-specific primers. A range of 10² to 10⁶ copies of gDNA of Lc1542 was used.

The forward primer (5'-TGTTGGATACCGAGACTCAATGAA-3') and the reverse primer (5'-ATTTCTTTAGCTTTATCCTTCCCGT-3') targeting the Lp1542_504 gene allow to specifically amplify the DNA of Lp1542, whereas no signal was obtained with the 10 tested strains.

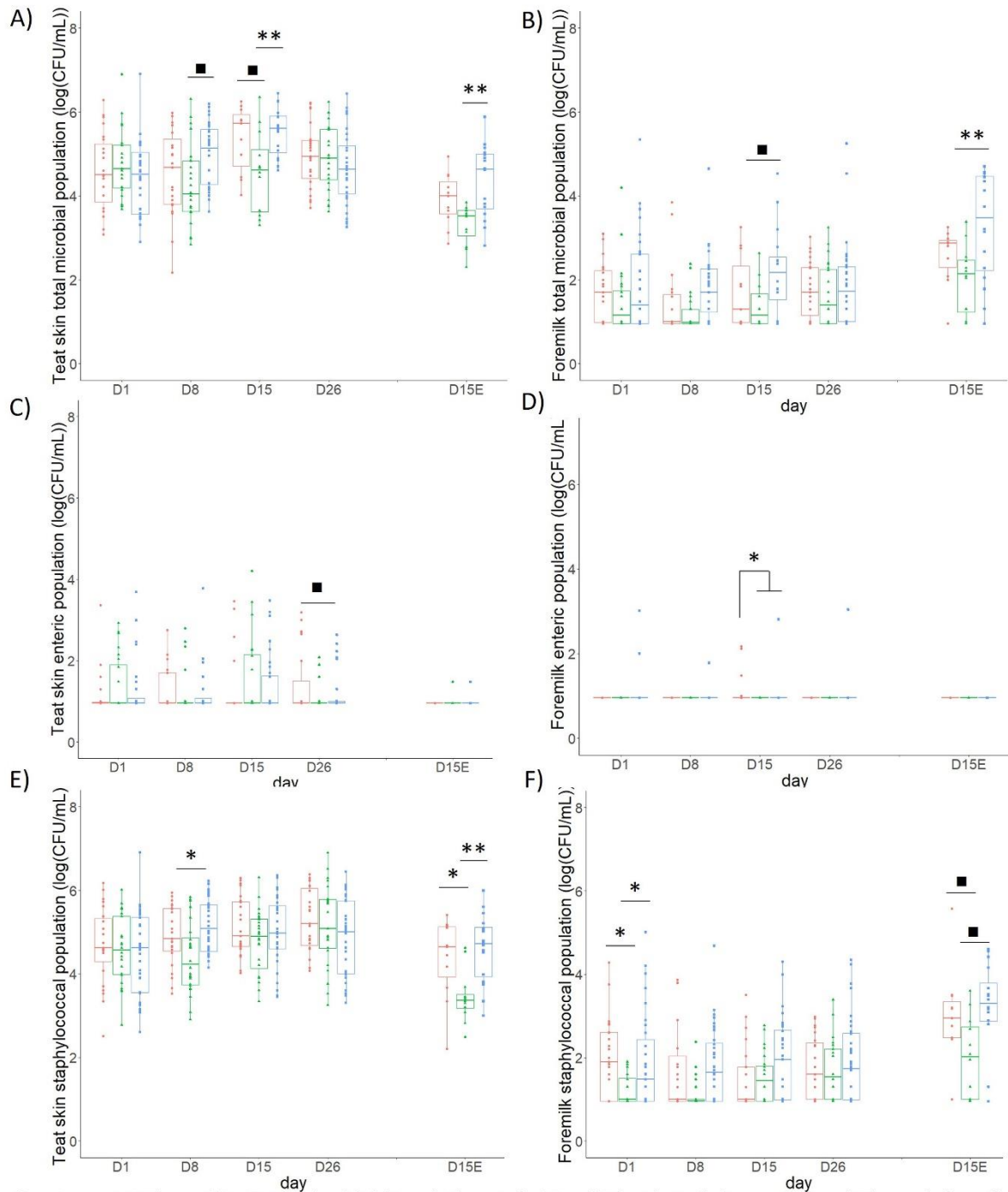
Milk somatic cell typing

The milk samples were filtered using a 70 µm filter and centrifuged at 3,000 g for 5 min at 4°C. The pellet was then resuspended in MACSQuant® Running Buffer (130-092-747, Miltenyi Biotech, Bergisch Gladbach, Germany) to obtain a solution at approximately 1.5 million cells/ml. A volume of 90 µl of the solution was transferred in Fluorescence Minus One (FMO) CD45, FMO CH138 and multi-labelled tubes and 100 µl in the unlabelled tube. FMO and multi-labelled tubes were supplemented with 10 µl of FcR Blocking reagent (130-059-901 Miltenyi Biotech). The tubes were placed in an ice bath and protected from light for all the following procedure. The incubation of the tubes was performed in 2 steps. A first 30-min incubation was done after addition of the non-conjugated primary antibodies (anti-CH138 antibodies; Supplementary Table S1). Isotype control antibodies were also used as gating controls. After the addition of 1 ml of FACS buffer (Running buffer supplemented with 5% (v/v) BSA), the tubes were centrifuged at 300×g, for 5 min at 4 °C and the pellets were then resuspended in 400

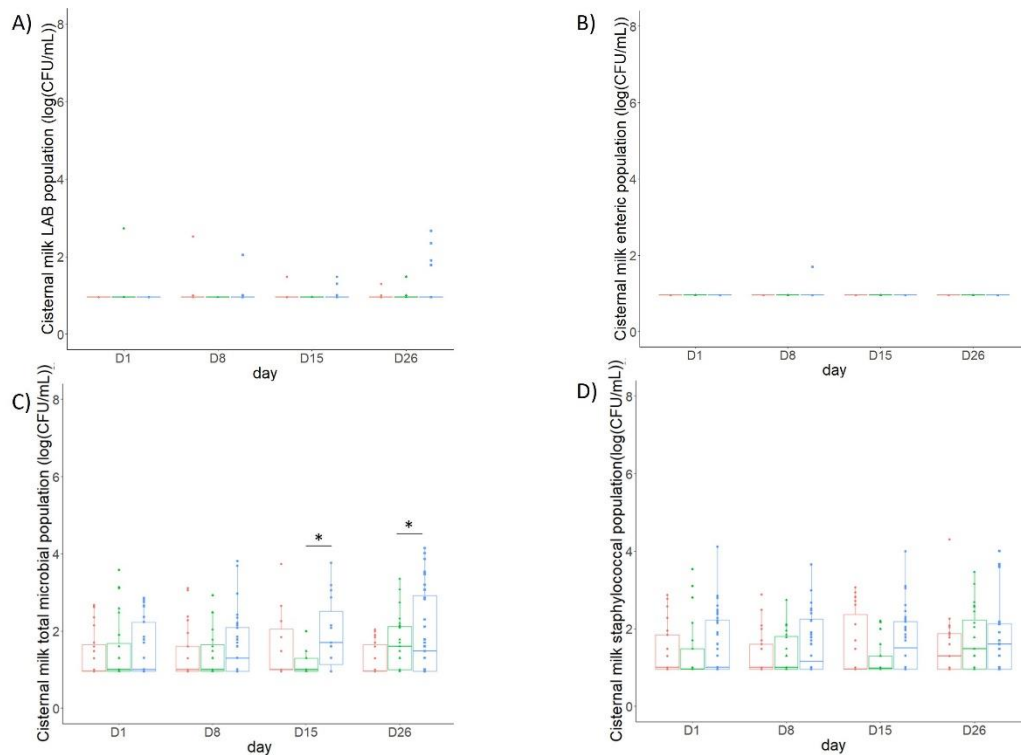
μl (unlabelled tubes) or 100 μl (other tubes) of FACS buffer. A second 20-min incubation was done after addition of the secondary antibodies (except for the CD49f which is already conjugated; Supplementary Table S1). After the addition of 1 ml of FACS buffer, the tubes were centrifuged at $300\times g$, for 5 min at 4 °C and the pellets were then resuspended in 400 μl of FACS buffer. The tubes were then passed through a flow cytometer (MACS Quant® 10 analyzer, Miltenyi Biotec) firstly without Propidium Iodide (PI, 130-093-233, Miltenyi Biotec) and secondly with 1 $\mu\text{g/ml}$ of PI. A flow rate of 500 cells per second) was used to avoid bias related to a rapid flow rate allowing a better visualization of the singlets.



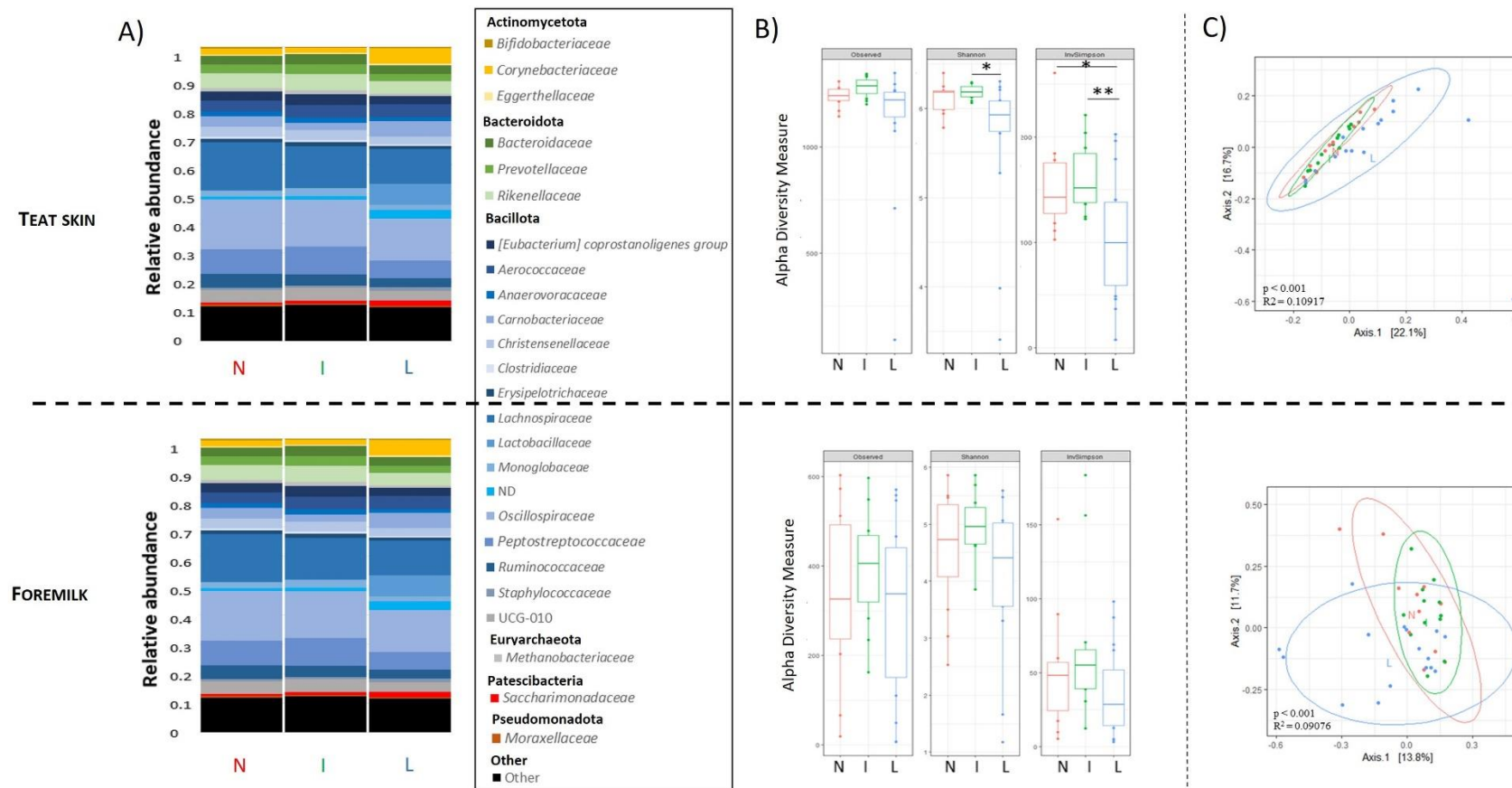
Supplemental Figure S1. *Lactacisibacillus paracasei* CIRM BIA 1542 (Lp1542) population was determined on teat skin (A) and in foremilk (B) at D15 and D15E by qPCR using specific primers and is expressed in log (number of copies/mL). Boxplots were used to represent the data distribution. Boxes extend 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.



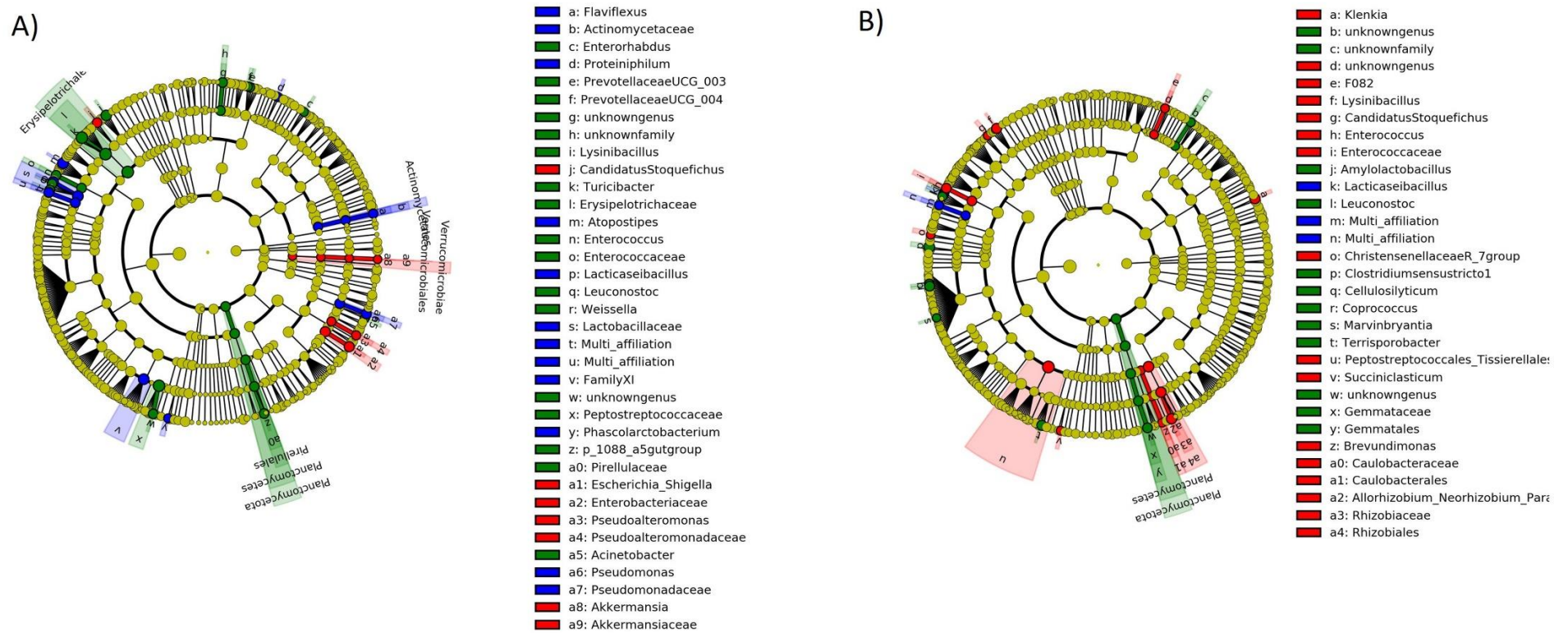
Supplemental Figure S2 . Total microbial (A and B), enteric (C and D) and staphylococcal (E and F) populations (log (CFU/mL)) on teat skin (A, C and E) or in foremilk (B, D and F) before treatment (D1), during treatment (D8, D15, D15E) and following treatment (D26). Cow quarters were either treated with *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; *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ■ $p < 0.1$.



Supplemental 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). 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; * $p < 0.05$.



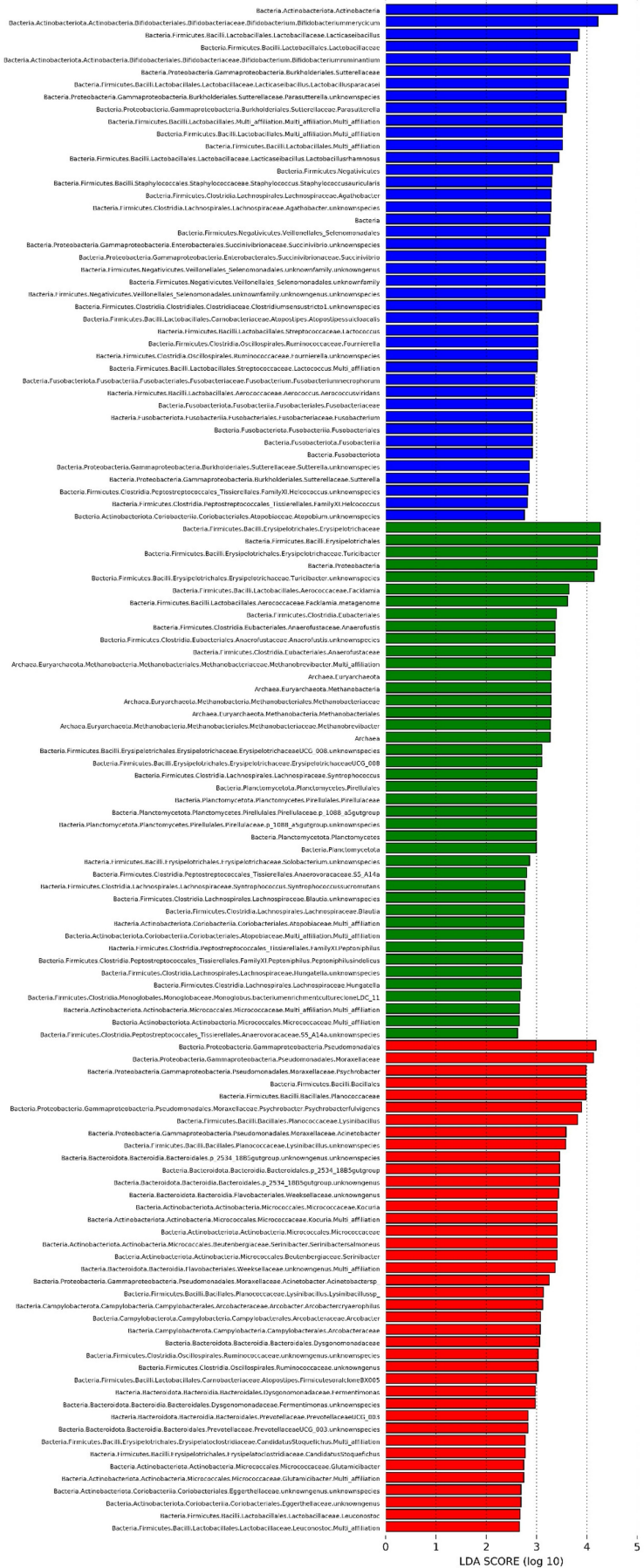
Supplemental Figure S4. Impact of post-milking treatment on alpha- and beta-diversity of the teat skin and foremilk microbiota at D15E. A) Taxonomic profiles of the total microbiota. The barplots represent the 20 dominant families of each condition. B) Observed, Shannon and Inversed Simpson indices were used to represent the alpha-diversity of each condition. Each point represents a cow quarter either treated by *Lactacaseibacillus. 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.



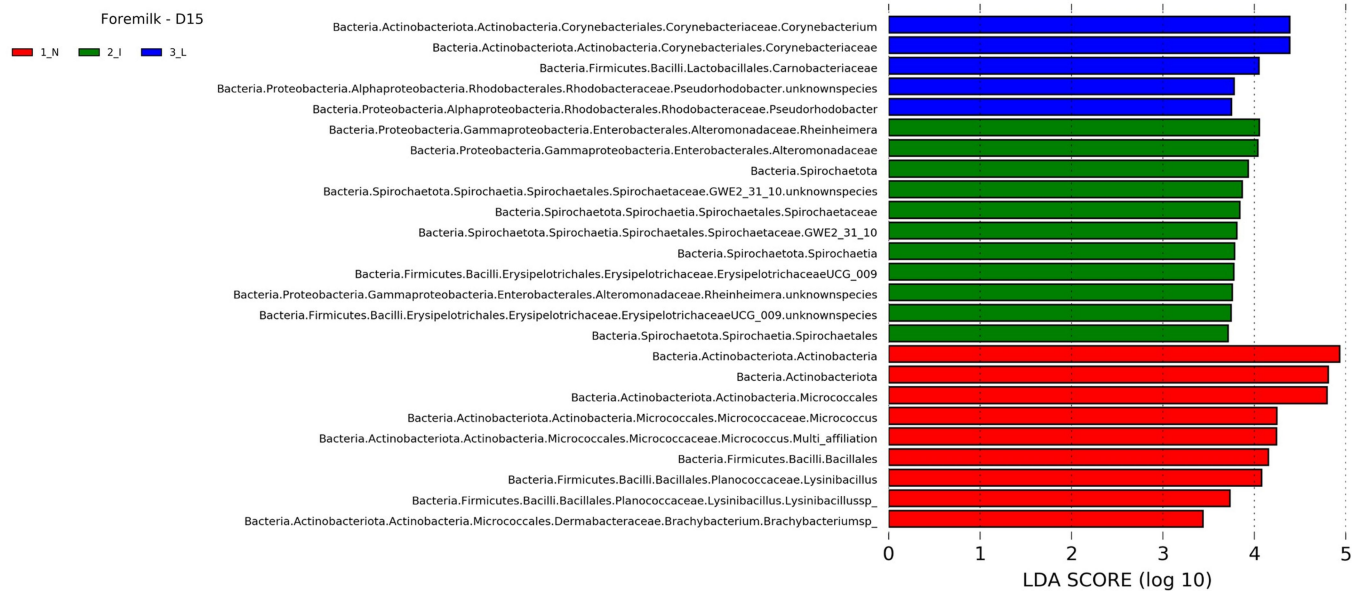
Supplemental Figure S5. Linear discriminant analysis effect size (LEfSe) of the total microbiota on teat skin (A) or in foremilk (B) at D15E. A cladogram was used to represent the difference of family composition between cow quarters treated by *Lactocaseibacillus paracasei* CIRM BIA 1542 (blue), iodine (green) or not treated (red).

Teat skin - D15

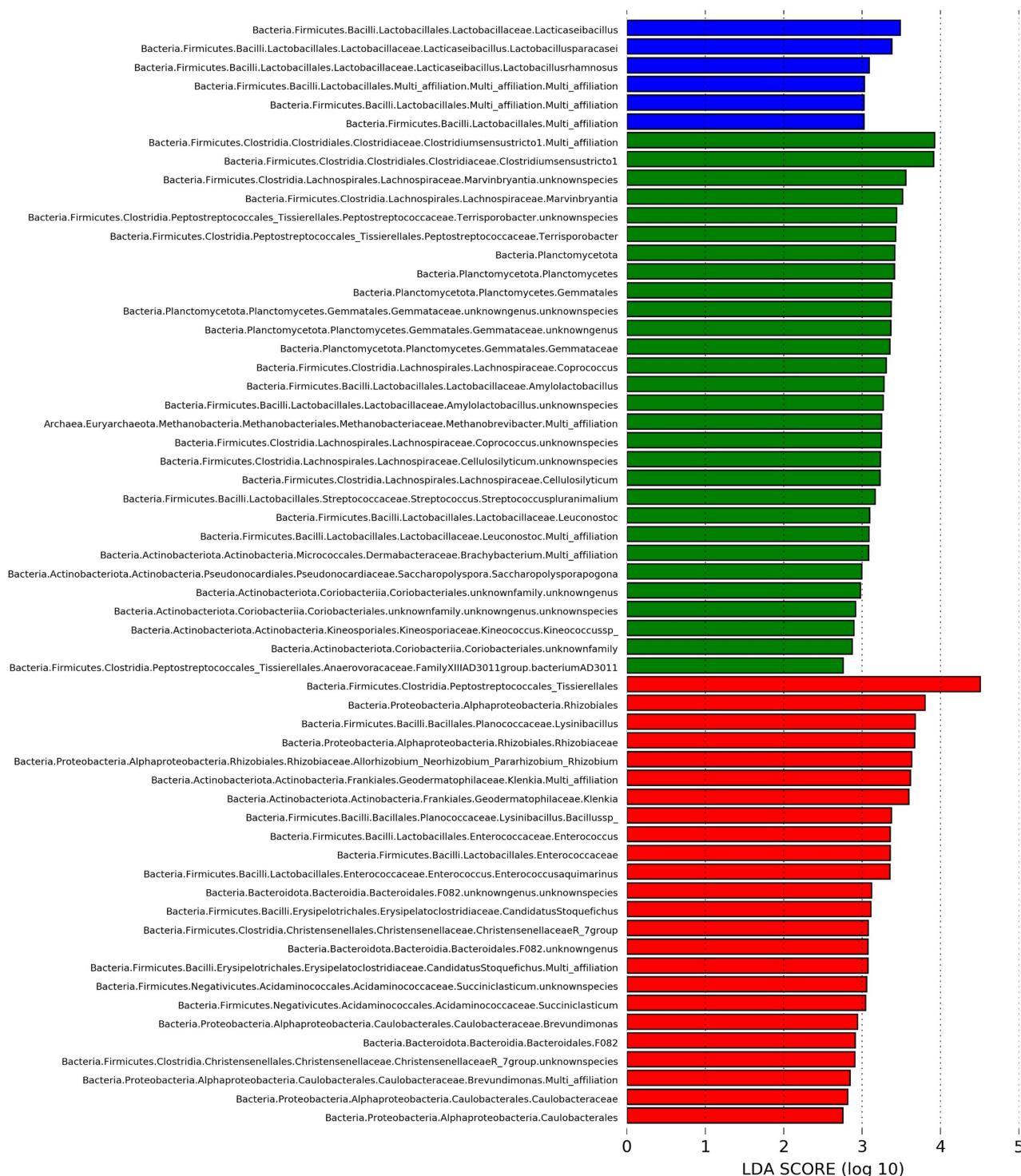
1_N 2_I 3_L



Supplemental Figure S6. Linear discriminant analysis effect size (LEfSe) of the total microbiota on teat skin at D15. LDA score (log 10) was used to quantify the difference of family composition between cow quarters treated by *Lactocaseibacillus paracasei* CIRM BIA 1542 (blue), iodine (green) or not treated (red).



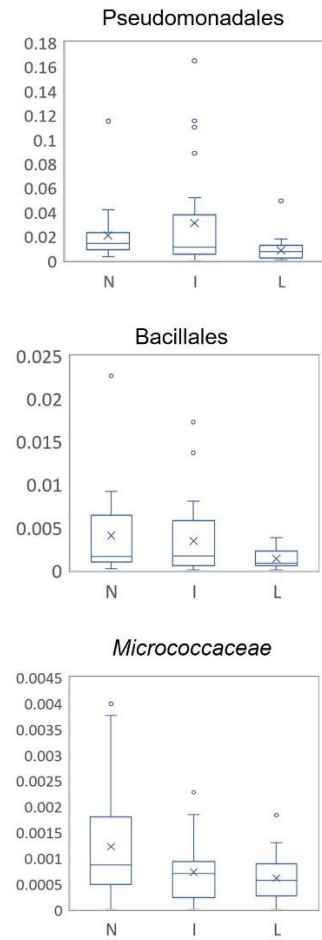
Supplemental Figure S8. Linear discriminant analysis effect size (LEfSe) of the total microbiota in foremilk at D15. LDA score (log 10) was used to quantify the difference of family composition between cow quarters treated by *Lacticaseibacillus paracasei* CIRM BIA 1542 (blue), iodine (green) or not treated (red).



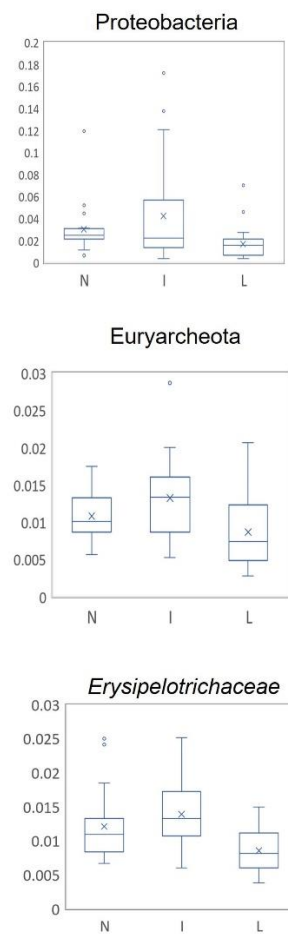
LDA SCORE (log 10)

Supplemental Figure S9. Linear discriminant analysis effect size (LEfSe) of the total microbiota in foremilk at D15E. LDA score (log 10) was used to quantify the difference of family composition between cow quarters treated by *Lactocaseibacillus paracasei* CIRM BIA 1542 (blue), iodine (green) or not treated (red).

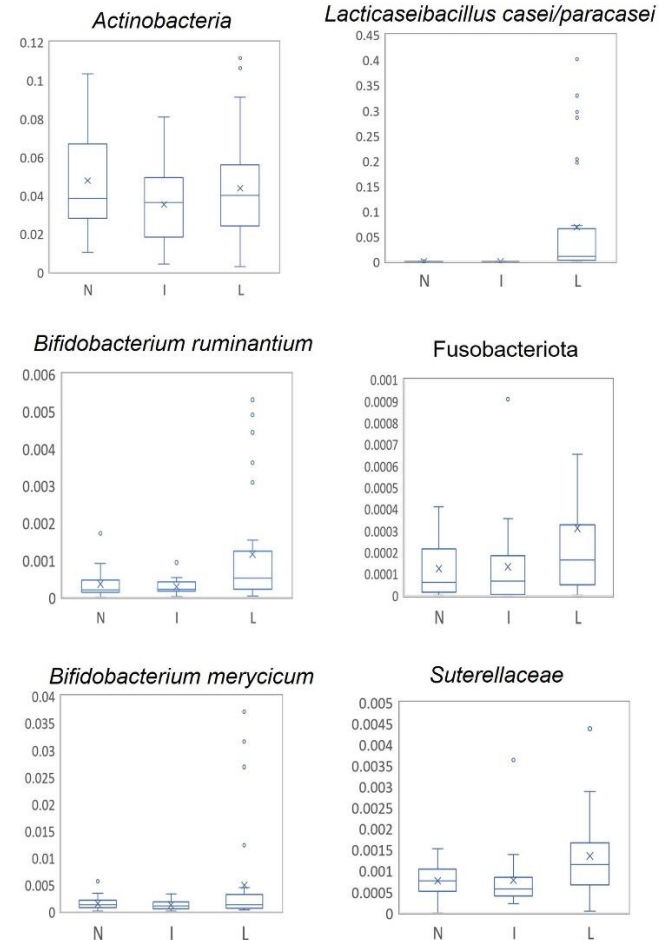
Taxa more abundant in N quarters



Taxa more abundant in I quarters



Taxa more abundant in L quarters



Supplemental Figure S10. Relative abundance of discriminant taxa between the three treatments (*Lactocaseibacillus paracasei* CIRM BIA 1542, iodine or no treatment), as determined by a Linear discriminant analysis effect size (LEfSe) on the TS microbiota at D15. Boxplots were used to represent the relative abundance 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 and the cross represents the mean relative abundance.