SUPPLEMENTARY FILE

RESEARCH COMMUNICATION

Preliminary study on the effect of a cow's teat predip and a teat cup disinfectant on the presence of mesophilic and (proteolytic) psychrotrophic bacteria prior milking

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MATERIAL AND METHODS

The experimental set-up is shown in Figure 1.

Disinfection and sampling procedure for teat orifices

Samples were taken daily during the morning milking session. From day 1 to day 3 (control period), all teats were cleaned with a fresh disposable paper tissue. After cleaning, each teat orifice was sampled using a moistered swab (swab tubes (Copan, Brescia, Italy) were filled before sampling with 5 mL sterile Ringer's solution (Oxoid, Basingstoke, UK) (four swabs per udder) by swabbing an area (+/- 0.5 cm²) around the teat orifice as described previously (De Vliegher et al., 2003). On days 4 to 9 (experimental treatment period), pre-dipping of the teats was applied as an additional treatment. After the standard treatment and sampling as described above, the pre-dip foam (Prefoam +, Hypred SA, Dinard, France) was applied to the teats (during morning and evening milking). The active components of this ready-to-use foam pre-dip were 2% L-(+)-lactic acid and 0.099% salicylic acid. After a contact time of 1 min the teats were dried using a fresh paper tissue to remove excess disinfectant. Then, a swab containing 5 mL Dey-Engley Neutralizing Broth (**DENB**; D3435; Sigma-Aldrich, St-Louis, US) was used

to sample the teat orifice again as described above (four swabs per udder). Swabs were then transferred to the laboratory within 30 minutes and pooled samples were made for each cow:

- one pool of the four teat swabs after standard treatment
- one pool of the four teat swabs after the foam pre-dip treatment.

Each pooled sample consisted of 1 mL from each of the four swabs mixed in one tube.

Disinfection and sampling procedure for the milking machine cluster

Pre and post disinfection of the milking cluster, the four liners of each cluster were sampled once to evaluate the effect of the liner disinfectant. Using a swab prefilled with DENB, the inner part of the liner was sampled by rotating the swab in a spiral trajectory starting from the lower part to the edge, swabbing an estimated surface of 5 cm^2. The disinfectant spray (Perfo Grif, Hypred SA, Dinard, France) contained 5% peracetic acid and 14.5% H_2O_2; it is used in a concentration of 0.8% (diluted in water) as indicated by the manufacturer. The presence of hydrogen peroxide ensures vaporization of the disinfectant after 1 min post application. After spraying the inner surface of the liner with a hand held sprayer, the cups were inverted for 1 min after which the liner was swabbed again as described above. Each liner was processed separately upon arrival in the laboratory.

Bacterial counts

The mesophilic and (proteolytic) psychrotrophic counts were determined on all samples (n=206). For the mesophilic count, 100 µl was plated onto plate count agar (**PCA**; CM0325, Oxoid, Basingstoke, UK) using a spiral plater (Eddy Jet, IUL instruments, Barcelona, ES). After 3 days of incubation at 30°C, a zone with minimal 20 countable colonies was chosen and the number of colony forming units (**CFU**) per mL was calculated according to the manufacturer's instructions.

The enumeration of the (proteolytic) psychrotrophic bacteria was performed according to the IDF Standard 101 (IDF, 2005) on PCA supplemented with 2% commercially available UHT milk. Those proteolytic active psychrotrophs which showed a halo on this medium were then plated onto tryptone soy agar (**TSA**; CM0131; Oxoid, Basingstoke, UK) (maximum three proteolytic colonies per sample) for further identification.

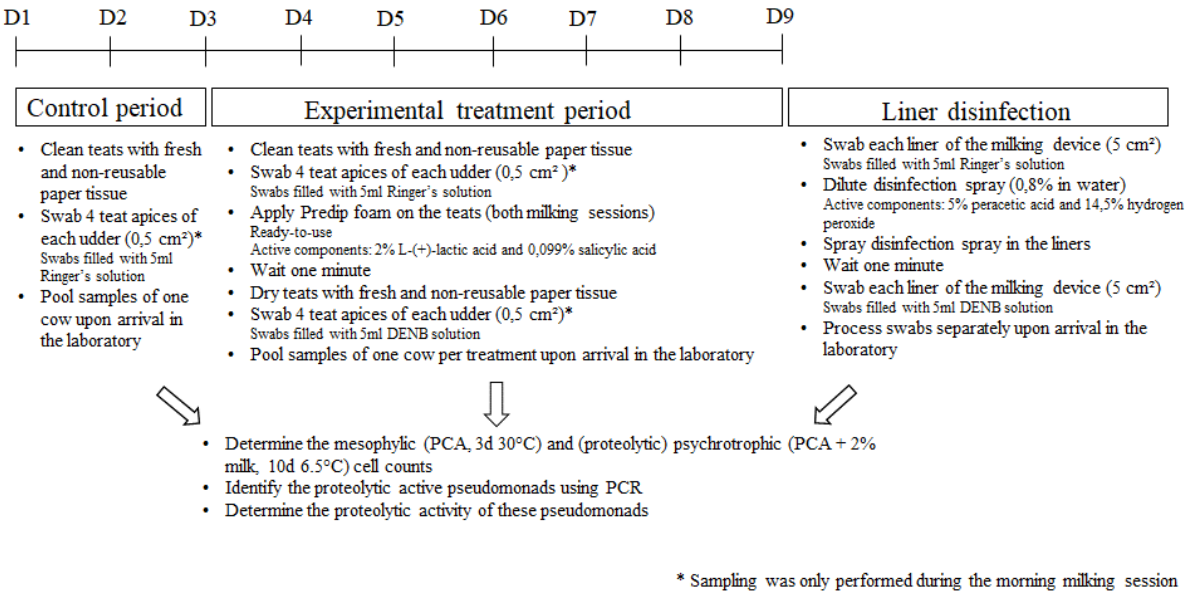
To identify *Pseudomonas* a 16S PCR methodology was used (Locatelli et al, 2002). On *Pseudomonas* positive samples, a *car*A gene PCR (to determine *P. fragi*, *P. lundensis* or *P. putida*) and a *P. fluorescens* 16S PCR were applied (Scarpellini et al., 2004; Marchand et al., 2009).

The proteolytic activity of pseudomonads was determined by the trinitrobenzene sulfonic acid (TNBS) method (Marchand et al., 2009).

Statistical analysis

To determine the effect of the foam pre-dip (pre and post dip), a multilevel regression model with either mesophilic cell count or psychrotrophic cell count was used, with sample as repeated factor within cow, to correct for repeated measures within a cow (SAS 9.4, SAS Institute Inc., Cary, NC, US). Start concentration was determined during the first 3 days. Average concentrations were used as covariate in the model. As well as start concentration, day of measurement (3 to 9), parity number (1 to 3) of each cow and pre and post-dip were tested as fixed factors in a backwards stepwise regression where appropriate Tukey posthoc tests were performed. A two-sided t-test was performed when comparing the bacterial counts pre and post treatment of the liners with the bacterial counts as variable and the two conditions (pre and post treatment) as the grouping variables. A statistical significance was considered at $P<0.05$.

Figure 1: Overview of the experimental design. The experiment consisted of a control period (day 1 to day 3) and an experimental treatment period (day 4 to day 9). All treatments were performed during the morning and evening milking session with sampling during the morning milking session. The liner disinfection occurred after the experimental period (D= day).



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