



J. Dairy Sci. 100:3497–3512
<https://doi.org/10.3168/jds.2016-12355>
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Reduction of *Mycobacterium avium* ssp. *paratuberculosis* in colostrum: Development and validation of 2 methods, one based on curdling and one based on centrifugation

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ABSTRACT

The aim of this study was to develop and validate 2 protocols (for use on-farm and at a central location) for the reduction of *Mycobacterium avium* ssp. *paratuberculosis* (MAP) in colostrum while preserving beneficial immunoglobulins (IgG). The on-farm protocol was based on curdling of the colostrum, where the IgG remain in the whey and the MAP bacteria are trapped in the curd. First, the colostrum was diluted with water (2 volumes colostrum to 1 volume water) and 2% rennet was added. After incubation (1 h at 32°C), the curd was cut and incubated again, after which whey and curd were separated using a cheesecloth. The curd was removed and milk powder was added to the whey. Approximately 1 log reduction in MAP counts was achieved. A reduction in total proteins and IgG was observed due to initial dilution of the colostrum. After curd formation, more than 95% of the immunoglobulins remained in the whey fraction. The semi-industrial protocol was based on centrifugation, which causes MAP to precipitate, while the IgG remain in the supernatant. This protocol was first developed in the laboratory. The colostrum was diluted with skimmed colostrum (2 volumes colostrum to 1 volume skimmed colostrum), then skimmed and centrifuged (at 15,600 × *g* for 30 min at room temperature). We observed on average 1.5 log reduction in the MAP counts and a limited reduction in proteins and IgG in the supernatant. To obtain a semi-industrial protocol, dairy pilot appliances were evaluated and the following changes were applied to the protocol: after 2:1 dilution as above, the colostrum was skimmed and subsequently clarified, after which the cream was heat

treated and added to the supernatant. To investigate the effect of the colostrum treatment on the nutritional value and palatability of the colostrum and the IgG transfer, an animal experiment was conducted with 24 calves. Six received the dam's colostrum, 6 were given untreated purchased colostrum (control), and 2 groups of 6 calves received colostrum treated according to both of the above-mentioned methods. No significant differences were found between the test groups and the dam's colostrum group in terms of animal health, IgG uptake in the blood serum, milk, or forage uptake. Two protocols to reduce MAP in colostrum (for use on-farm or at a central location) were developed. Both methods preserve the vital IgG.

Key words: *Mycobacterium avium* ssp. *paratuberculosis*, colostrum decontamination, curdling, centrifugation

INTRODUCTION

Mycobacterium avium ssp. *paratuberculosis* (MAP) is the causal agent of Johne's disease, a contagious, chronic, and often lethal enteritis. Johne's disease results in reduced milk yields and negative economic consequences. Calves younger than 1 yr are the most susceptible to MAP infection. Possible transmission routes for calves are contaminated colostrum and milk; contaminated feed or water; environmental contamination (barn environment, manure, dust, and aerosols) and to a lesser extent *in utero* or through contaminated sperm (Sweeney, 1996; Diéguez et al., 2008; Eisenberg et al., 2010, 2015; Mortier et al., 2013).

The present study focused on the elimination of colostrum transmission because colostrum is the first milk the calf receives after parturition and can be considered as a first possible MAP source. Colostrum is vital to the calf as it contains maternal immunoglobulins (IgG), which neonatal calves lack as they are agammaglobulinemic

Received November 25, 2016.

Accepted January 23, 2017.

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at birth. Calves gain their immunity through ingestion and absorption of colostrum IgG, which is characterized by the presence of at least 10 mg of IgG per mL of blood serum 24 to 48 h after birth. Colostrum also contains essential nutrients such as vitamins, growth factors, and maternal cells (Gauthier et al., 2006; Godden, 2008; Houser et al., 2008). At present, only one research group enumerated the MAP bacteria present in the colostrum of subclinical and clinical cows: results obtained by PCR demonstrated up to 24 ± 12 cfu/mL and 254 ± 63 cfu/mL in colostrum of subclinical and clinical cows, respectively (Stabel et al., 2014). At present, the infectious dose of MAP is unknown. It is expected that low numbers of MAP are sufficient to infect a calf (Begg and Whittington, 2008; Eisenberg et al., 2010). Reducing the number of MAP in colostrum could reduce the intake of MAP and subsequently reduce the risk of infection. Farmers would benefit by either decontaminating the colostrum on-farm or treating their cows' colostrum at a nearby central location.

Several methods have the potential to decontaminate colostrum such as pasteurization. Pasteurization has important drawbacks: Godden et al. (2015) found no long-term benefits when feeding heat-treated colostrum to calves and pasteurization equipment is prohibitively expensive for on-farm use. Curdling is effective and more appropriate for on-farm use as it is easy, inexpensive, and does not require any special equipment. Van Brandt et al. (2010) demonstrated that after curdling the majority (80–90%) of MAP cells can be found in the curd, whereas the IgG remain in the whey. Centrifugation is another valuable option, one often used to concentrate MAP for improved detection. Gao et al. (2005) increased the detection sensitivity of MAP by centrifuging bulk tank milk and analyzing the pellet and cream fraction. Because centrifugation is already frequently applied under (semi-)industrial circumstances for separating or clarifying milk and cream, centrifugation has potential for use within a semi-industrial protocol (SIP) for MAP decontamination.

In the current project, we developed 2 protocols for MAP decontamination: an on-farm protocol (FP) based on curdling and a SIP based on centrifugation with potential application in a central location. First, we aimed to develop and validate both methods to reduce MAP in colostrum under laboratory conditions with special attention to the conservation of IgG and other essential nutrients. Second, we adapted the protocols for on-farm use and at the central location, validated them with naturally contaminated colostrum, and performed an animal experiment to assess the influence of the treated colostrum on the calves' health (several parameters) and IgG uptake in their blood.

MATERIALS AND METHODS

Bacterial Strains and Culture Preparation

In the first part of the project (experiments to optimize MAP removal from colostrum), a *Mycobacterium smegmatis* strain (MB4355; ILVO collection) was used as a replacement for MAP, because this bacterium forms colonies on tryptone soy agar (TSA; CM0131; Oxoid, Basingstoke, UK) after 3 d of incubation at 37°C. This choice facilitated the laboratory experiments (Hussain et al., 2016). One week before an experiment, the *M. smegmatis* strain was inoculated on TSA. After 3 d of incubation at 37°C, one loopful of this culture was added to tryptone soy broth (TSB; CM0129, Oxoid) with glass beads (to prevent clumping of the culture). This suspension was incubated for 3 d on a shaker at 37°C. Subsequently, the culture was drawn into a syringe and expelled 20 times through a 0.8×40 mm needle (BD Microlance; BD Biosciences, Louth, Ireland) to break up any large clumps. Finally, the optical density at 540 nm of this suspension was measured and adjusted with TSB to a value of 1.15, which corresponds to approximately 10^9 cfu/mL. A decimal dilution of the suspension was made to dilution 10^{-8} . Next, the colostrum sample was inoculated with the appropriate dilution. The inoculated colostrum was incubated for 1 h at 40°C to allow the bacteria to bind to the colostrum particles and subsequently incubated overnight at 4°C on a shaker.

The protocol was validated using a MAP strain (MB3840). This strain was grown on Herrold's egg yolk medium for 3 mo at 37°C. For 1 L of Herrold's egg yolk medium, 9 g of bacteriological peptone (LP0037; Oxoid), 4.5 g of NaCl (CL00.1249.5000; ChemLab, Zedelgem, Belgium), 15.3 g of technical agar (LP0011; Oxoid), 2.7 g of beef extract (B4888; Sigma-Aldrich, Steinheim, Germany), 4.1 g of sodium pyruvate (P2256; Sigma-Aldrich), and 27 mL of glycerol (8.18709.1000; Merck, Darmstadt, Germany) were added to 970 mL of distilled water. The pH was adjusted to 7.8 with 10 M NaOH (1.06462.1000; Merck) and the medium was boiled to dissolve the agar. After sterilization (121°C for 15 min) and cooling to 56°C, the following supplements were added: one vial of 2 mg of Mycobactin J (Synbiotics, Lyon, France) resuspended in 4 mL of 95% ethanol (CL00.0505.500, ChemLab); 6 egg yolks and 5 mL of a filter-sterilized (0.22- μ m Millipore filter) 2% Malachite green solution (1.01398.0025; Merck). The day before the experiment, Ringer's solution was applied onto the plate and the culture was scraped off using a loop after which the bacterial culture was drawn into a syringe and treated as described above for *M. smegmatis*.

For the *M. smegmatis* experiments, the number of *M. smegmatis* was determined for the culture, the inoculated colostrum (after overnight incubation), and the obtained whey and supernatant samples (see below). For the culture, 100 μL of the 10^{-5} to 10^{-8} dilutions were plated out in triplicate onto TSA. For the inoculated colostrum, whey and supernatant 10-fold dilutions were made in Ringer's solution and 100 μL of each dilution was plated out in triplicate on TSA. After 3 d of incubation at 37°C, colonies were counted.

For the MAP experiments, the MAP presence was determined as described above on Middlebrook agar (7H10; Becton Dickinson, Le Pont de Claix, France), supplemented with one vial of 2 mg of Mycobactin J and 1 bottle of oleic albumin dextrose catalase supplement (212240; Becton Dickinson and Company, Lovington, UK) with an incubation period of 3 mo at 37°C.

Colostrum Samples

Colostrum was obtained from Melkcontrole Centrum Vlaanderen (Lier, Belgium). This colostrum originated from various farms. It was delivered in 10-L containers, thawed, and poured into 1-L bottles. All colostrum was sterilized by gamma rays (dose: 15 kGy; Synergy Health, Etten-Leur, the Netherlands). Before each experiment, the sterility of the colostrum was confirmed by bacteriological analysis.

For some centrifugation experiments, skimmed colostrum was needed. Colostrum was diluted in a 2:1 ratio of 2 volumes colostrum to 1 volume water (abbreviated below as "diluted 2:1 with water" or "diluted 2:1 with skimmed colostrum" as appropriate) to approach the viscosity of milk. The diluted colostrum was then heated to 43°C to dissolve all fat globules and skimmed using a separator (Van Houte, Gent, Belgium). The skimmed colostrum was divided into 1-L bottles and gamma sterilized as described above.

For validation of the methods, naturally MAP-contaminated colostrum ($n = 16$) was obtained from lactating seropositive cows ($n = 7$), a heifer born from a seropositive cow ($n = 1$), or lactating cows in a paratuberculosis (paraTBC) positive herd ($n = 8$). Diergezondheidszorg Vlaanderen (Lier, Belgium) kindly provided this colostrum from farmers registered in the Belgian paraTBC program.

Viscosity Measurement of Colostrum, Milk, and Diluted Colostrum

Skim milk, whole milk, colostrum ($n = 1$) and 12 dilutions of this colostrum sample [9 volumes colostrum (C):1 volume water (W), 5C:1W, 4C:1W, 3C:1W,

2C:1W, 1C:1W, 1C:2W, 1C:2W, 1C:3W, 1C:4W, 1C:5W, and 1C:9W, respectively] were measured with a Brookfield digital viscometer (DV-II + Pro Brookfield Engineering Labs Inc., Stoughton, MA) using the small sample adapter (Brookfield Engineering Labs Inc.).

Curdling Experiments for Optimizing the On-Farm Protocol

A flowchart with an overview of the curdling experiments is given in Figure 1.

Experiment 1: Best Rennet Type and Optimization of the Rennet Concentration for Curdling of Colostrum. Three rennet types were purchased at Brouwmarkt (Almere, the Netherlands): rennet powder [2,200 international milk-clotting units (IMCU)/g], vegetarian rennet (190 IMCU/g), and calf rennet (100 IMCU/g). In the first experiment, various concentrations of each rennet type were added to 40 mL of colostrum ($n = 1$): 0.03% (vol/vol), 0.06, 0.1, 0.5, 1, and 2%. The curdling treatment was to incubate the colostrum for 1 h at 32°C, cut the curd, incubate for another hour at 32°C, and separate whey and curd using a cheesecloth. After curdling, the presence/absence of whey and (if present), whey volume was noted.

Experiment 2: Effect of Curdling Undiluted and 2:1 Diluted Colostrum on the Presence of *M. smegmatis*. The effect of 2 concentrations (1 and 2%) of the 3 rennet types were compared for undiluted ($n = 3$) and colostrum diluted 2:1 with sterile water ($n = 3$) to which approximately 10^8 or 10^5 cfu *M. smegmatis*/mL were added as described above. Both undiluted and diluted colostrum were divided over 18 sterile 50-mL tubes (containing 1 or 2% of the 3 rennet types, 3 tubes per condition). Curdling was performed as described in experiment 1. The presence of *M. smegmatis* in the inoculated colostrum and all whey samples was determined as described above.

Experiment 3: Effect of Diluting Colostrum with Milk or Water on *M. smegmatis* Presence. Colostrum ($n = 3$) was diluted in a 2:1 ratio with sterile water or purchased UHT milk to determine whether this had an effect on the curdling and the *M. smegmatis* presence in whey. Approximately 10^8 or 10^5 cfu of *M. smegmatis*/mL were added as described above. Each colostrum sample was subdivided into three 50-mL tubes containing 2% calf rennet and curdling was performed as described above. *Mycobacterium smegmatis* counts were determined on the inoculated colostrum and whey fractions as described above.

Experiment 4: Validation of the Curdle Protocol with MAP. Nine colostrum samples were used. For validation, we used a concentration close to naturally

contaminated colostrum (10^2 – 10^3 cfu/mL; Stabel et al., 2014). In addition, 10^7 cfu/mL was added as a control concentration. Colostrum with the lowest MAP dose

was divided into three 50-mL tubes and each colostrum sample with the highest MAP dose into 2 tubes; each tube contained 2% calf rennet. Curdling was performed

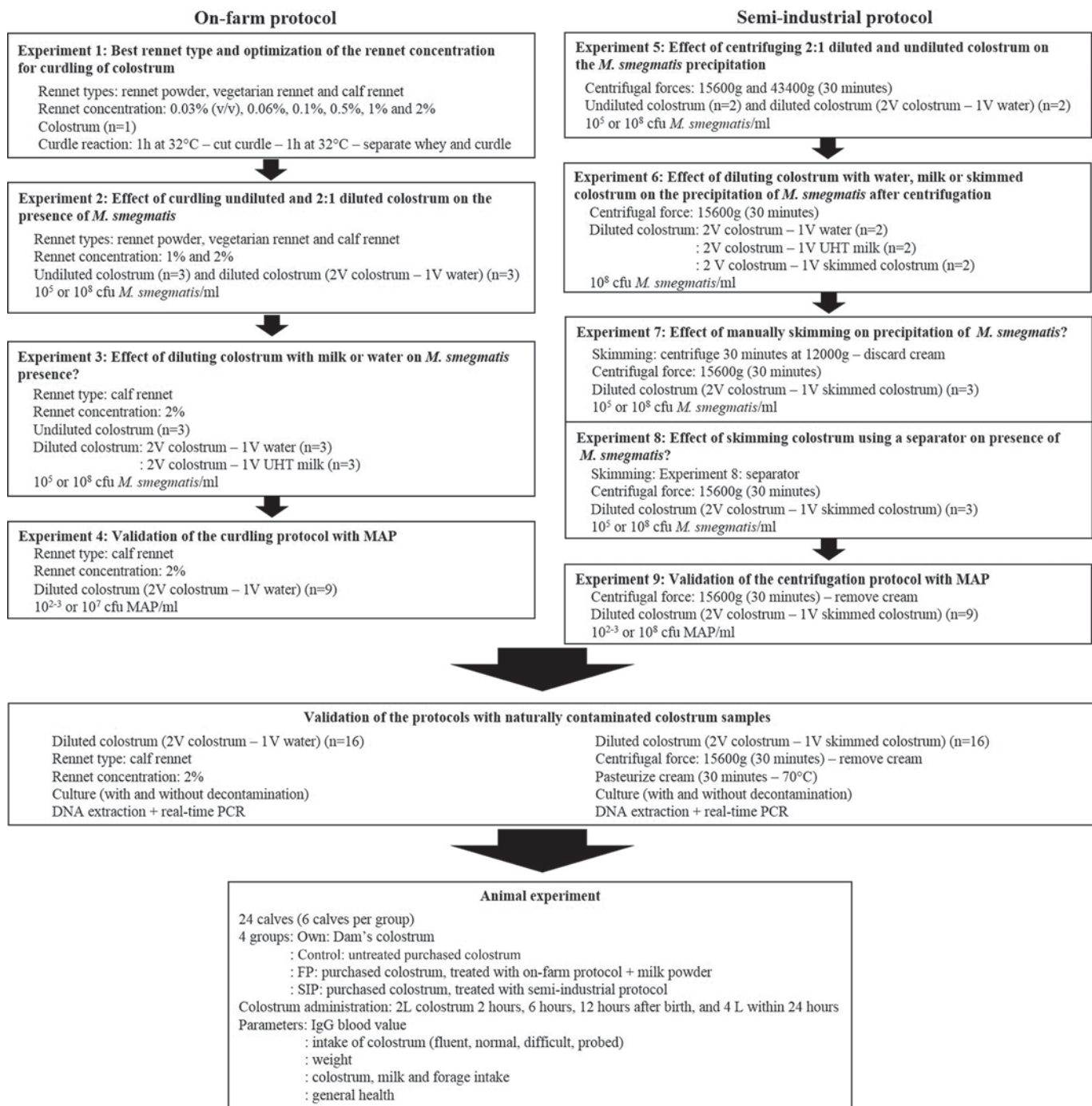


Figure 1. Flowcharts of the experiments performed to obtain the on-farm protocol (FP) and semi-industrial protocol (SIP), validation, and animal experiment. MAP = *Mycobacterium avium* ssp. *paratuberculosis*. *M. Smegmatis* = *Mycobacterium smegmatis*. All centrifugations were performed at room temperature.

as described above. The MAP counts were determined on Middlebrook agar for all inoculated colostrum and whey samples as previously described.

Centrifugation Experiments for Optimizing the Semi-Industrial Protocol

We also optimized a SIP with the aim of using it at a central location. First, we determined the specifications for centrifuges used in the dairy processing: milk separators have a relative centrifugal force around $15,600 \times g$ and semi-industrial centrifuges operate at approximately $43,400 \times g$. An overview of the centrifugation experiments is given in Figure 1.

Experiment 5: Effect of Centrifuging 2:1 Diluted and Undiluted Colostrum on the *M. smegmatis* Precipitation. We added approximately 10^8 or 10^5 cfu *M. smegmatis*/mL to colostrum ($n = 3$) and colostrum diluted 2:1 with sterile water ($n = 3$). Three subsamples of each colostrum type ($V = 40$ mL per subsample) were centrifuged for 30 min at $15,600 \times g$ and $43,400 \times g$ at room temperature (RT) using a Sorvall centrifuge (Rotor: T29; Sorvall Lynx 6000, Thermo Scientific, Waltham, MA). The *M. smegmatis* counts were determined for the inoculated colostrum and all supernatants on TSA as previously described.

Experiment 6: Effect of Diluting Colostrum with Water, Milk, or Skimmed Colostrum on the Precipitation of *M. smegmatis* After Centrifugation. Colostrum ($n = 3$) was diluted 2:1 with water, purchased UHT milk, or skimmed colostrum (see above). Approximately 10^8 cfu of *M. smegmatis*/mL were added to the colostrum. Three subsamples ($V = 40$ mL) of the 6 diluted colostrum were centrifuged for 30 min at $15,600 \times g$ at RT. The *M. smegmatis* counts on the supernatants were done as described above.

Experiment 7: Effect of Manually Skimming Colostrum on Precipitation of *M. smegmatis*. Two colostrum samples were spiked with *M. smegmatis* (10^8 or 10^5 cfu *M. smegmatis*/mL) after which the colostrum was divided into 2 parts. One part was skimmed (i.e., the colostrum was centrifuged for 30 min at $12,000 \times g$ at RT), after which the samples were put on ice. After 15 min, we removed the cream using a spatula and the supernatant was put in a centrifugation tube. Then 2 subsamples of each skimmed and whole colostrum ($V = 40$ mL) were centrifuged for 30 min at $15,600 \times g$ at RT to remove *M. smegmatis*. Presence of *M. smegmatis* in the supernatants was determined as described above.

Experiment 8: Effect of Skimming Colostrum Using a Separator on Presence of *M. smegmatis*. This experiment was a scaled-up version of experiment 7. Due to the large volumes needed for this process (V

$= 10$ L), we only skimmed 3 colostrum samples. All colostrum were diluted in a 2:1 ratio with skimmed colostrum and 10^5 cfu of *M. smegmatis*/mL was added to 2 samples and 10^8 cfu/mL to 1 sample. These colostrum samples were heated to 43°C to dissolve all fat globules and then skimmed in the separator. Three subsamples of the skimmed colostrum was centrifuged for 30 min at $15,600 \times g$ at RT. The *M. smegmatis* counts were performed on the obtained cream, skimmed colostrum, and supernatants as described above.

Experiment 9: Validation of the Centrifugation Protocol with MAP. During this experiment, the final method was validated with MAP to mimic naturally contaminated colostrum. For this experiment, we used 9 colostrum samples. All samples were diluted 2:1 with skimmed colostrum. Half of each sample was inoculated with 10^8 cfu of MAP/mL; the other half was inoculated with 10^2 to 10^3 cfu of MAP/mL. One 50-mL tube of colostrum with the highest MAP dose and two 50-mL tubes of colostrum with the lowest MAP dose were centrifuged for 30 min at $15,600 \times g$ at RT, after which we manually removed the cream and determined the MAP counts in the supernatants on Middlebrook agar.

Validation of the Protocols with Naturally Contaminated Colostrum Samples

We divided the received colostrum samples into 2 parts (Figure 1). One part, diluted 2:1 with sterile water, was subjected to the on-farm method. The other part, diluted 2:1 with skimmed colostrum, was subjected to the SIP. After applying the SIP, we pasteurized the cream at 70°C for 30 min in a hot water bath and determined the MAP presence after inoculating 100 μL in triplicate on Middlebrook agar. We determined the MAP concentration of all untreated colostrum, diluted colostrum, whey, and reconstituted supernatant samples (mixing supernatant with pasteurized cream) as follows: a dilution series was made up to 10^{-2} and 100 μL of each dilution was plated in triplicate on Middlebrook agar. We also chemically decontaminated these samples with *N*-acetyl-L-cysteine as described by Bradner et al. (2013) and Stabel et al. (2014) to improve MAP detection, after which a decimal dilution up to dilution 10^{-2} was plated (100 μL) in triplicate on Middlebrook agar.

On all culture-positive samples (diluted colostrum, whey, or supernatant), we also performed a DNA isolation procedure using the Adiapure Paratb milk kit (BioMerieux/Adiagene, Saint-Brieuc, France). Subsequently, we performed an in-house developed and validated real-time PCR based on the IS900 sequence

for the quantification of MAP. We added 5 μ L of the obtained DNA to 20 μ L of PCR mixture consisting of 12.50 μ L of TaqMan Environmental Mastermix (Applied Biosystems, Warrington, UK), 0.75 μ L of forward (**F**) primer (600 nM, DH2 F: 3'-GCCTTCGACTACAA-CAAGAGC-5'; Eurogentec), 0.75 μ L of reverse (**R**) primer (600 nM, DH2 R: 3'-GCGTCGGGAGTTTG-GTAG-5'; Eurogentec), 0.25 μ L of probe (200 nM, 3'-GCCGCGCTG ATCCTGCTTACT-5'; Eurogentec) and water. The PCR reaction was as follows: 1 cycle of 10 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C followed by on cycle of 15 min at 40°C. A DNA standard curve of MAP strain MB 3835 (ILVO collection, 10^6 to 10^2 genomes/ μ L) was used to convert the obtained C_t -values into genome and subsequent cell concentrations.

Protein and IgG Content of the Samples

From each colostrum, diluted colostrum, whey, and supernatant sample, the total protein and IgG concentration and changes in protein fractions were determined.

The Coomassie Plus (Bradford) Assay kit (23236; Thermo Scientific, Rockford, IL) was used to determine the total protein concentration of the samples and was performed according to the manufacturer's protocol (https://tools.thermofisher.com/content/sfs/manuals/MAN0011203_CoomassiePlus_Bradford_Asy_UG.pdf).

To study the various protein fractions in the samples, a SDS-PAGE was performed as described by Laemmli (1970). Six standards (all from Sigma-Aldrich, St. Louis, MO) were included for identification of the protein fractions: α -casein (C6780), β -casein (C6905), β -LG (L-0130), lactoferrin (L4765), gamma-globulin (G5009), and protein standard (P5369). The low molecular weight calibration kit (17-0446-01; Amersham Pharmacia Biotech, Buckinghamshire, UK) was used as a marker for weight determination.

To determine the IgG concentration in the samples, an in-house-developed gel filtration method was used. In short, the samples were prepared according to the International Dairy Federation standard 178:1996 (IDF, 1996) and were subsequently diluted 20 times with elution buffer (20 mM Tris and 150 mM NaCl). Gel filtration was performed using a Yarra 3u SEC-2000 column (00H-4512-K0; Phenomenex, Torrance, CA) connected with the HPLC PE series 200 equipped with a Detector UV/VIS series 200 set at 280 nm (Perkin Elmer, Waltham, MA). An IgG standard curve (concentration range between 2 and 10 mg/mL; G5009-25G; Sigma-Aldrich) was measured and the response (ratio of peak area) was plotted against the concentration. This curve

was used to determine the IgG concentration in the samples.

Animal Experiment

This animal experiment was approved by ILVO Ethics Committee (application number: 2015/246) and the animals were treated in an ethical manner according to the guidelines for care and use of agricultural animals in research and teaching. After the experiment, the calves remained in the ILVO herd.

We purchased 220 L of colostrum at European Colostrum Industry (Marloie, Belgium). This commercially available colostrum is sterile, has specified characteristics (70 g/L of Ig; 14% proteins; 6.0% fat and infectious bovine rhinotracheitis free) and was delivered in 1-L bottles. All colostrum was thawed and pooled. Sixty liters of the pooled sample was immediately poured into 1-L bottles and frozen at -20°C (control). The FP was performed on 90 L of colostrum: 45 L of tepid water was added and the resulting mixture was heated to 32°C in a cheese tank. Subsequently, we added 2% of calf rennet, and after 1 h of incubation at 32°C , we cut the curd and continued incubation for another hour. The whey and curd were then separated using a cheesecloth. The whey was poured into 1-L bottles and frozen. Before feeding the thawed whey to the calf, 120 g of milk powder (Excellent, Denkavit, the Netherlands) was added. The SIP was performed on 70 L of colostrum. First, 20 L of colostrum was diluted 2:1 with water, heated to 43°C , and skimmed. The skimmed colostrum was added to the remaining 50 L; this mixture was again heated to 43°C and then skimmed. The cream was pasteurized for 30 min at 70°C above boiling water (*au bain marie*). The skimmed colostrum was clarified with the Easyclean I (GEA Westfalia, Oelde, Germany). We added the inactivated cream to the clarified colostrum and homogenized the mixture, after which it was poured into 1-L bottles and frozen. One bottle of each type was used to determine the protein and IgG concentration as described above.

Four groups consisting of 6 Holstein-Friesian heifer calves were included in the experiment (Figure 1): one group receiving colostrum from its respective dam (own group), one group receiving nontreated colostrum from the pool (control group), one group receiving colostrum treated according to the FP, and one group receiving colostrum treated according to the SIP. The calves were born into the ILVO herd between April and December 2015. At birth, the calves were successively allocated to 1 of the 4 groups in order: control, SIP, FP, and own group. Only female calves and calves originating from cows, not heifers (to ensure sufficient colostrum production), were included in the experiment.

At birth, all calves were weighed. All calves were fed at the same time: each received 2 L of colostrum within 2 h after birth, another 2 L around 6 h after birth, another 2 L around 12 h after birth, and the remaining 4 L within the next day. When a calf belonged to one of the experimental groups, the respective colostrum was thawed in a warm water bath at 40°C. The IgG content of the colostrum was estimated using a digital refractometer (Obione calf, Obione, Charentay, France). When the calves did not voluntarily consume the colostrum within 6 h after birth, probe feeding was applied. The consumed volume of each calf was noted and the intake of the colostrum was noted (very easy, normal, difficult, or probed). We lack intake data for last 3 calves, born into the own group. After the colostrum period, the calves were all fed twice daily with a commercial milk formula (Denkavit Excellent milk powder, 1 L/10 kg of weight). They also received an ILVO-made concentrate (F09–53, maximum 3 kg), water ad libitum, and hay ad libitum. Weaning happened around 8 wk of age, when the calf had consumed a total of 5.25 kg of concentrate the week before. The calves were housed in individual pens until the age of 4 mo, after which they were grouped. In the groups, they received silage feed (maize/prewilted grass silage) and an ILVO-made concentrate (F14–33, maximum 1.5 kg per calf).

Blood samples were taken approximately 72 h after birth and sent to DGZ Vlaanderen to determine the amount of IgG in blood serum. The calves were weighed every 2 wk up to the age of 16 wk and then monthly until the end of the experiment (6 mo of age). The daily milk and concentrate intake was individually noted. The general health (presence of diarrhea or illness) and antibiotic intake of each calf were also monitored.

Statistical Analysis

The number of replicates was 3 in all experiments, except in the validation experiments (experiment 4 and 9). This number of colostrum samples was calculated, using the power procedure (SAS, overall *F*-test for 1-way ANOVA). The group means of the preliminary experiments (results from experiments 2 and 5), a 95% interval and a nominal power of 0.8 were used. For validation purposes (experiments 4 and 9), we used 9 colostrum samples, to which a low and high MAP concentration had been added. The colostrum was divided into subsamples (3 with low concentration and 2 with high concentration). A 2-sided *t*-test was performed when comparing the bacterial counts as dependent variable in experiments 2, 5, 7, and 8 with the bacterial counts as variable and the 2 conditions (for example, adding 1 or 2% of rennet) as the grouping variables. For

experiments 3 and 6, we performed an ANOVA to compare the bacterial count results (dependent variable) of various treatments (independent variables) on the colostrum samples. During the animal experiment, a general linear model analysis was done using treatment group and time as fixed effects and cow as random effect to determine the effect on the dependent variables: IgG blood value, weight gain, and colostrum, milk, and forage intake. For all analyses, a *P*-value smaller than 0.05 was considered significant.

RESULTS AND DISCUSSION

Mycobacterium avium ssp. *paratuberculosis* causes a lethal, chronic enteritis known as Johne's disease that reduces milk yields and results in negative economic consequences. Intake of contaminated colostrum is one of the possible contamination routes for young calves. Decontamination of colostrum (with conservation of the IgG) before feeding the calves might reduce this contamination route. Our goal was to develop 2 decontamination protocols, one with the potential to be used on the farm (FP; a cheap, easy-to-use, and practical method based on curdling) and one with the potential to be used at a central location (SIP; based on centrifugation). To the best of our knowledge, this study is the first attempt to decontaminate colostrum by means of curdling and centrifugation.

On-Farm Protocol (Based on Curdling)

Experiment 1 involved adding various concentrations of 3 rennet types (rennet powder, vegetarian rennet, and calf rennet) to colostrum. When less than 0.1% of rennet was added, no curdling reaction was observed. Addition of 0.5% rennet resulted in a curdle reaction, but without clear separation of whey and curd. Upon addition of 1 or 2% of rennet, a clear separation of whey and curd occurred. These results were the similar for all 3 rennet types.

The density of colostrum (4 times denser than milk, see below) may explain the higher rennet percentage needed for curdling. We measured the viscosity of water, milk, colostrum, and different dilutions of colostrum (diluted with water). For milk, we observed a viscosity of 2.28 cP, which is 4 times lower than the viscosity of colostrum. When the colostrum was diluted either 2:1 or 1:1 with water, this resulted in a viscosity similar to milk (data not shown). As our goal was to maintain as many IgG in the decontaminated colostrum as possible, we used the 2:1 dilution ratio in subsequent experiments.

During the second experiment, we assessed the influence of (1) the rennet concentration (1 or 2%), (2) the

rennet type (rennet powder, vegetarian, or calf rennet), and (3) diluting the colostrum on the *M. smegmatis* counts (Figure 2). When comparing addition of 1 and 2% rennet to the diluted inoculated colostrum, we found no statistically significant differences (P -values between 0.19 and 0.95, 2-sided t -test). For the undiluted colostrum, a statistical difference was observed for the rennet powder (for the sample with the high dose, $P = 0.040$, 2-sided t -test) and the calf rennet ($P = 0.027$ and $P = 0.002$ for the low and high dose, respectively, 2-sided t -test). Using 2% rennet guarantees an effective curdle reaction. For the 2% rennet concentration, we observed a statistical difference between the calf rennet and the other rennet types: calf rennet gave better results than the others ($P = 0.021$ calf vs. powder, $P = 0.009$ calf vs. vegetarian, 2-sided t -test). We therefore decided to use calf rennet during subsequent experiments. Compared with the results of Van Brandt et al. (2010), our rennet percentage was remarkably higher (0.03 vs. 2%, respectively). The only difference between both studies is that they curdled skimmed colostrum,

whereas we curdled whole colostrum. After curdling, we observed for the undiluted colostrum with the low *M. smegmatis* dose on average a 1.22 ± 0.12 log reduction and for the high dose on average a 1.00 ± 0.28 log reduction. For the diluted colostrum, we observed on average a 0.79 ± 0.21 log and 0.97 ± 0.21 log reduction for the samples with the low and high dose of *M. smegmatis*, respectively (Figure 2). We found that diluting the colostrum did not have an additional effect on the *M. smegmatis* reduction (P -values between 0.05 and 0.98, 2-sided t -test). However, throughout preliminary experiments, we observed that when using undiluted colostrum not all samples curdled (2 of 10 colostrum samples), whereas all samples of the 2:1 diluted colostrum curdled. Despite little additional reduction in *M. smegmatis*, we chose to include diluting the colostrum in the protocol to be sure that curdling happens for each colostrum.

We assessed the effect of curdling colostrum diluted 2:1 with water versus milk on the *M. smegmatis* counts in experiment 3. Diluting with milk would supplement

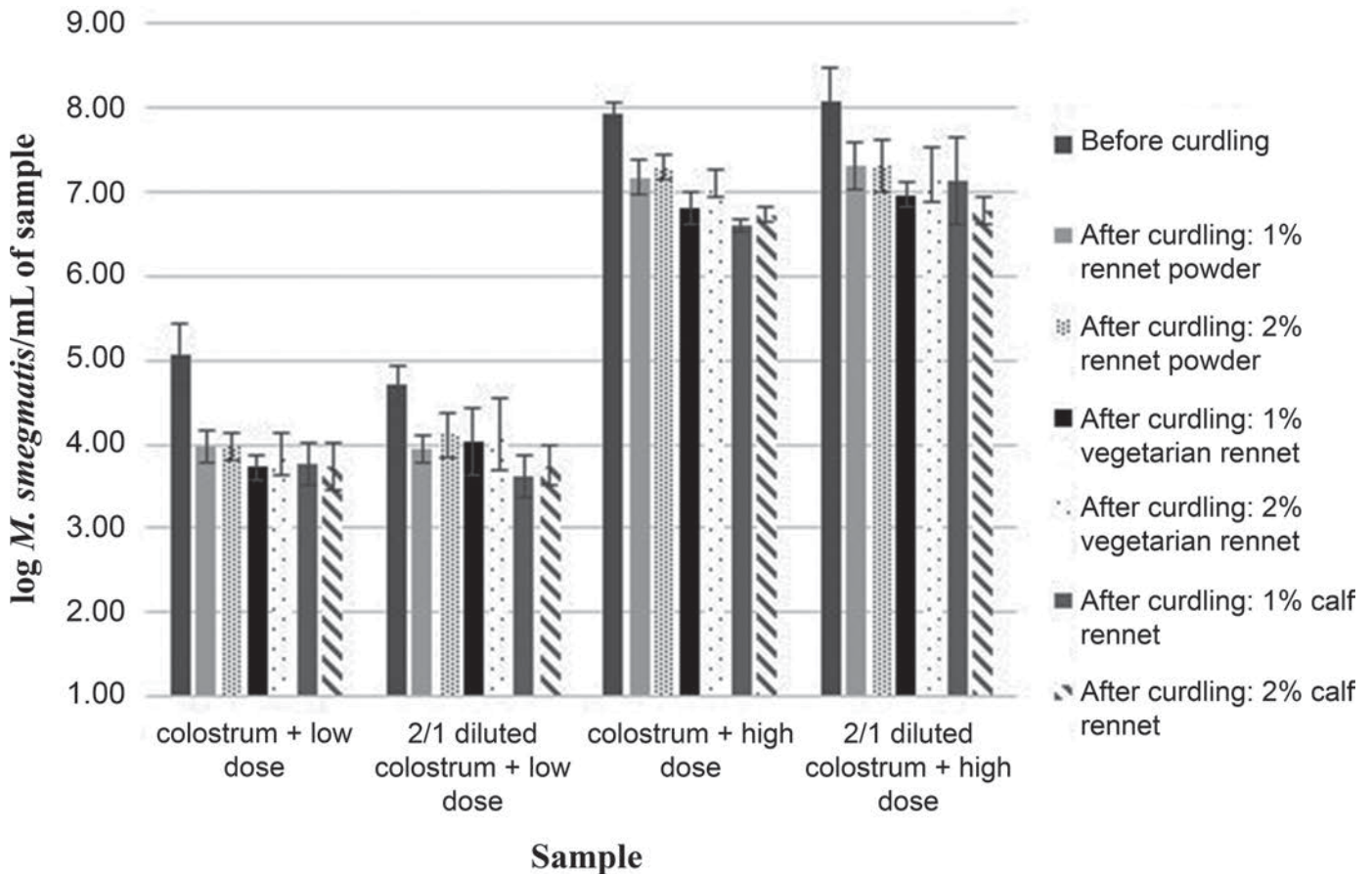


Figure 2. Results (mean \pm SD, $n = 3$) of the *Mycobacterium smegmatis* counts of the (2:1 diluted) inoculated colostrum before curdling and of the whey samples after curdling with either 1 and 2% rennet powder, 1 and 2% of vegetarian rennet, or 1 and 2% of calf rennet.

the colostrum with additional proteins, which could be beneficial for calves. When using the highest *M. smegmatis* dose, we observed a 1.95 and 1.63 log reduction after curdling of colostrum diluted with milk and water, respectively, whereas a 0.98 and 1.84 log reduction, respectively, was obtained when the low dose was used. No significant difference was observed when comparing the log reductions in colostrum inoculated with the high dose ($P = 0.89$, ANOVA), and a significant difference was observed for the low dose ($P < 0.001$, ANOVA). Because milk addition would represent an additional cost to the farmers and the milk would curdle as well, dilution with water was chosen.

Besides observing reduction in *M. smegmatis*, we also investigated the effect of curdling on the proteins and more specifically IgG present in the whey. In general, we observed a first decrease in total protein concentration when adding water to the colostrum, which also affected the IgG concentration. After the diluted colostrum was curdled, a further decline in total protein concentration was observed, mainly due to the formation of the curd by the caseins. During the curdle step, the majority of the IgG remained in the whey (on average throughout the experiments, we found no more than a 5% loss; Figure 3).

From these results, we obtained a protocol that is cheap, easy to use, and practical for farmers. The protocol, validated during experiment 4, is as follows. First, dilute the colostrum 2:1 with water and add 2% of calf rennet. Incubate for 1 h at 32°C, cut the curd, and incubate again for 1 h at 32°C. Afterward, separate the whey and curd using a cheesecloth (or similar). The added concentrations in the validation experiment (experiment 4) mimicked the natural contamination of colostrum. On average, we found 0.85 log MAP reduction (between 0.46 and 1.59 log) in the samples with the low MAP dose and 1.03 log reduction (between 1.02 and 1.27 log reduction) in the samples with the high MAP dose. We observed lower reductions compared with Van Brandt et al. (2010): they described 2.65 to 3.32 log reduction after curdling. Several explanations are possible for the observed difference. Van Brandt et al. (2010) skimmed colostrum, which reduced the number of MAP by about 1 log before curdling. In addition, they separated the whey and curd after a centrifugation step, which differs from our separation step with the cheesecloth. It is likely that centrifugation resulted in a more clear whey, whereas small pieces of curd could pass through the cheese cloth. However, skimming and centrifugation were not included in the optimization of our FP as this protocol should be as practical and as inexpensive as possible for the farmer.

Stabel et al. (2014) determined the amount of MAP in colostrum of subclinical and clinical cows and found 1.3

and 2.3 log cfu/mL, respectively. The present protocol seems to be sufficient to decontaminate colostrum from subclinical cows but not from clinical cows. Because the infectious dose is unknown, it is possible that the present FP is sufficiently efficient to decrease the number of MAP in colostrum of clinical cows to amounts below the infectious dose.

Semi-Industrial Protocol (Based on Centrifugation)

To investigate whether diluting the viscous colostrum has an effect on the presence of *M. smegmatis*, we centrifuged undiluted and 2:1 diluted colostrum with water at 2 centrifugal forces (experiment 5). When centrifuging the undiluted colostrum inoculated with 10^8 cfu/mL for 30 min at $15,600 \times g$ and $43,400 \times g$, we observed a 0.96 and 0.92 log reduction, respectively. In case of the diluted colostrum, we found 2.60 and 2.30 log reduction with the same conditions, respectively. When the lowest *M. smegmatis* concentration was added to both undiluted and diluted colostrum, the results were below the detection limit (<2 log/mL). The diluted colostrum contained 5 log *M. smegmatis* per mL before centrifuging and the supernatant contained less than 2 log, implying that at least 3 log reduction was achieved. The observed differences in log reduction between undiluted and diluted colostrum were statistically significant ($P < 0.001$ for both forces, 2-sided *t*-test). As no statistical significant difference was observed between the 2 gravitational forces on the *M. smegmatis* of the diluted colostrum ($P = 0.71$, 2-sided *t*-test), $15,600 \times g$ was used for the following experiments.

Milk or skimmed colostrum can also be used to dilute the colostrum. Diluting with milk or skimmed colostrum will add proteins, nutrients, and in case of the latter also IgG, as investigated in experiment 6. When centrifuging diluted colostrum, we found 2.40, 1.98, and 2.77 log reduction after centrifuging colostrum diluted with water, milk, and skimmed colostrum, respectively (Figure 4). The log reduction, obtained after centrifuging colostrum diluted with skimmed colostrum was statistically different from the others ($P = 0.0002$ for skimmed vs. water and $P = 0.0001$ for skimmed vs. milk, ANOVA). We decided to use diluting colostrum with skimmed colostrum in the protocol, because of the addition of IgG (see below).

Skimming also reduces the viscosity of colostrum. After manual skimming, a reduction of approximately 0.84 log was found when centrifuging the colostrum with a high *M. smegmatis* dose, a significant difference ($P = 0.0001$, 2-sided *t*-test; data not shown). These results are comparable to Van Brandt et al. (2010) who noted counts of 0.81 and 1.02 log MAP retrieved

from manually derived cream. For technical reasons, no results could be obtained for the colostrum with the low concentration of *M. smegmatis*.

We also assessed mechanical skimming using a separator in experiment 8, where 0.34 to 0.72 log *M. smegmatis* was found in the cream fractions. A statistical difference was found when comparing *M. smegmatis* counts for the colostrum before and after skimming ($P = 0.002$, 2-sided *t*-test; Figure 5). When we centrifuged

the skimmed colostrum, we found 0.95 and 1.62 log reduction with the colostrum inoculated with the low dose and high dose, respectively. During this process, a total reduction of 1.5 to 2 log could be observed.

The effects of centrifugation on the proteins and more specifically IgG present in the supernatant were also examined. As expected, diluting the colostrum with skimmed colostrum had less effect on the total protein and IgG concentration than diluting with water. After

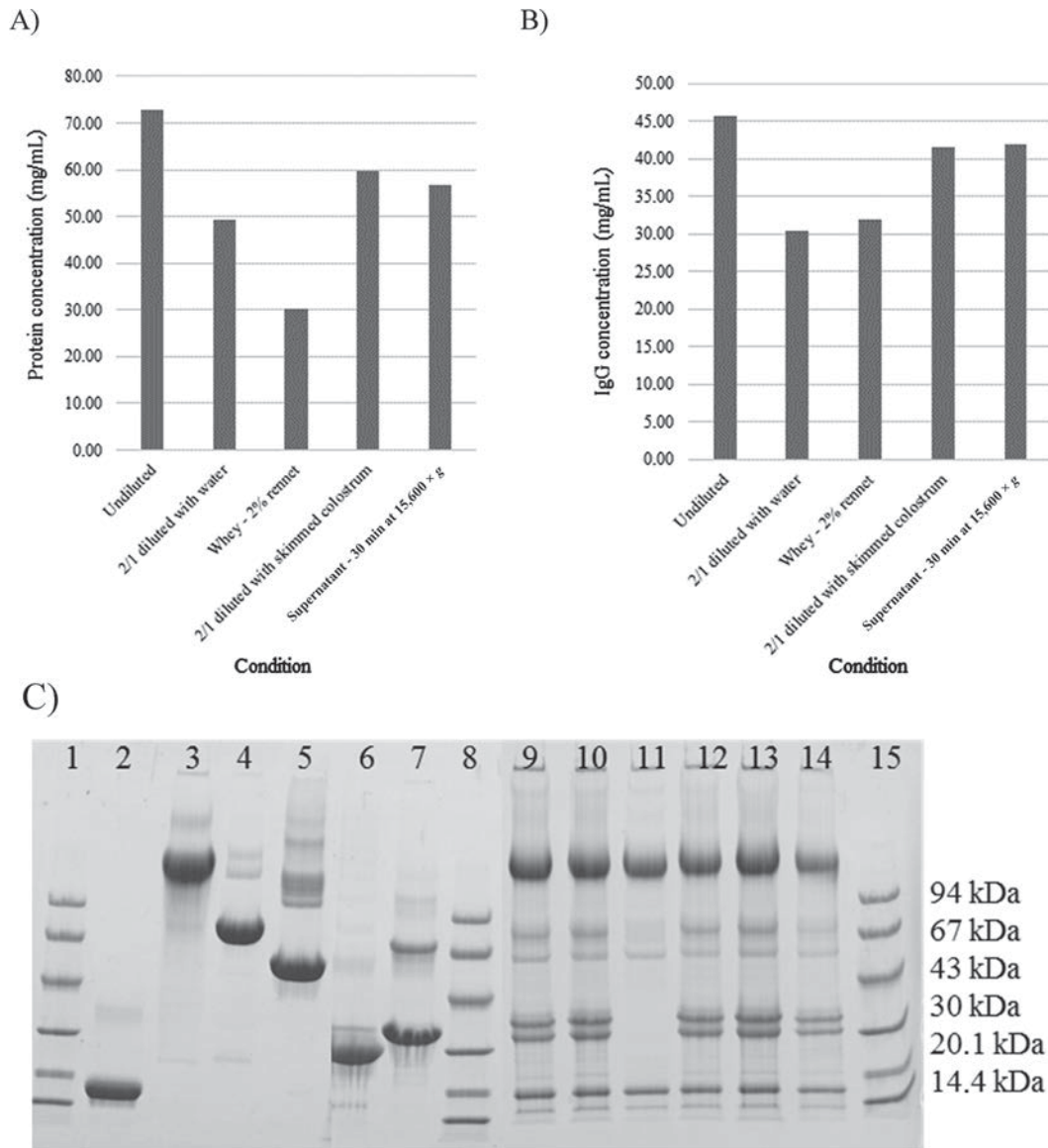


Figure 3. Example of the total protein and IgG determination of colostrum 3 during the validation experiment for the centrifugation and curdle protocol. For this colostrum, the results are shown for (A) the total protein determination using the Coomassie blue protein assay, (B) the IgG determination using gel filtration, and (C) the identification of the protein fractions, using SDS-PAGE (lane 1: reference proteins, lane 2: β-LG, lane 3: gamma-globulin, lane 4: lactoferrin, lane 5: protein standard, lane 6: β-casein, lane 7: α-casein, lane 8: reference proteins, lane 9: skimmed colostrum, lane 10: colostrum 3, lane 11: colostrum 2:1 diluted with water, lane 12: whey after curdling with 2% rennet, lane 13: colostrum 2:1 diluted with skimmed colostrum, lane 14: supernatant obtained after centrifuging for 30 min at 15,600 × g, and lane 15: reference proteins with the molecular weights). All centrifugations were performed at room temperature.

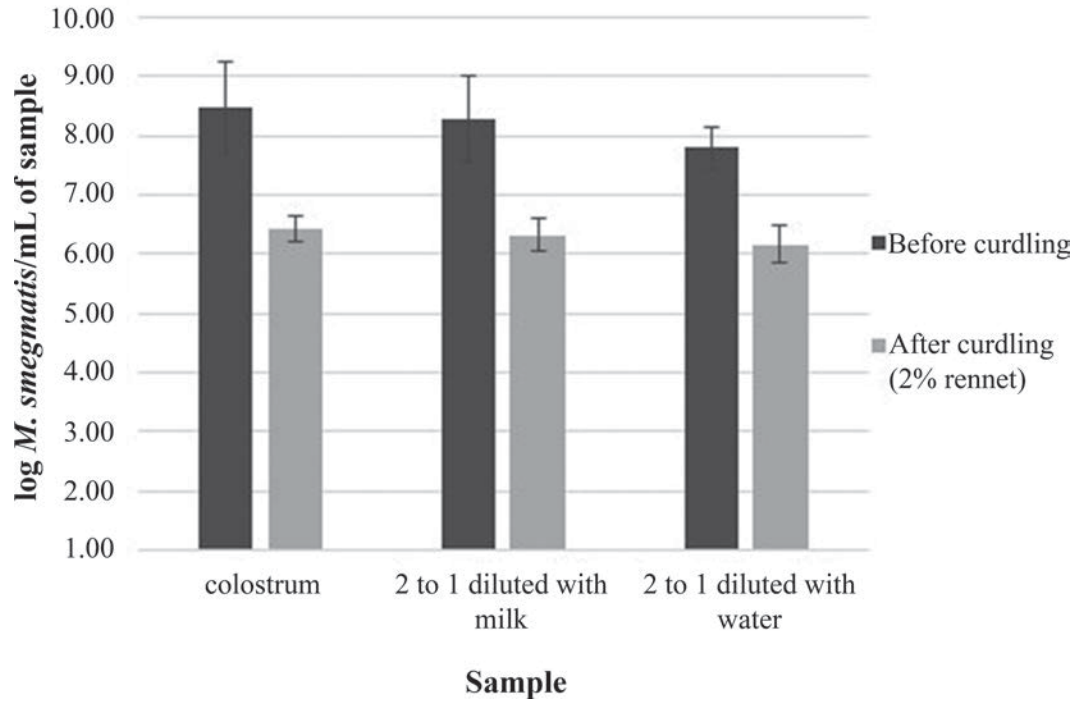


Figure 4. Results (mean ± SD, n = 3) of the *Mycobacterium smegmatis* counts before and after centrifugation of colostrum 2:1 with water or milk and inoculated with the highest *M. smegmatis* concentration.

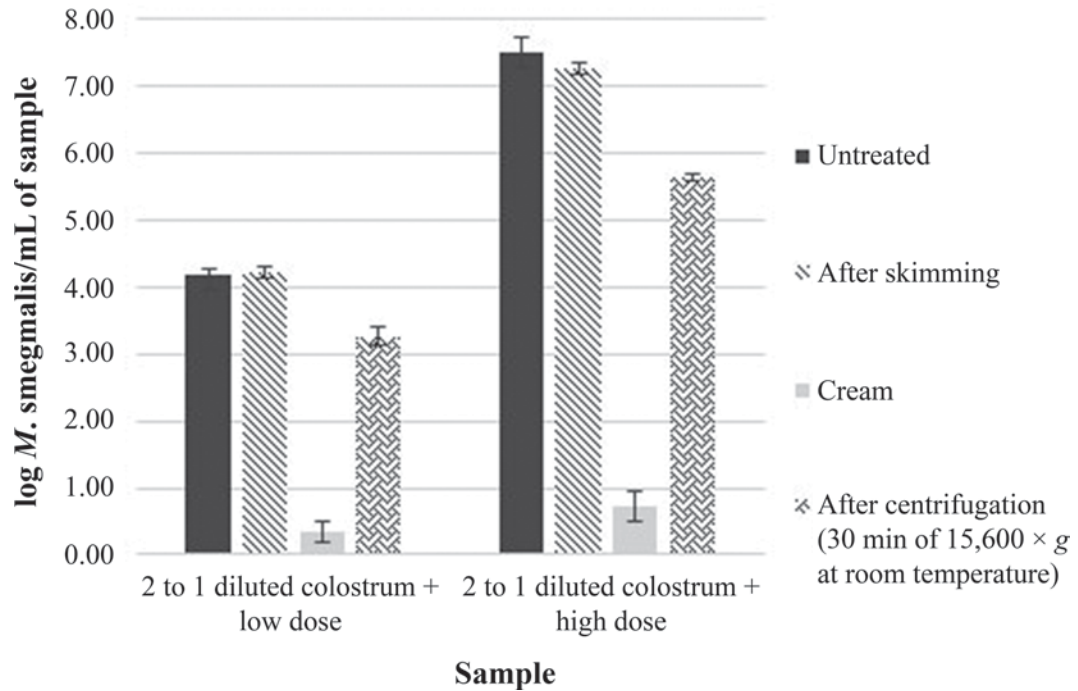


Figure 5. Results (mean ± SD, n = 6) of the *Mycobacterium smegmatis* counts before and after skimming and after centrifuging of the colostrum diluted 2:1 with skimmed colostrum and inoculated with a low or high dose of *M. smegmatis*.

centrifugation, the majority of the IgG remained in the supernatant (in general we found no more than 5% loss throughout the experiments; Figure 3).

Based on the results, the following SIP was proposed and validated with MAP (experiment 9). First, colostrum is diluted 2:1 with skimmed colostrum. Next, the colostrum is centrifuged for 30 min at $15,600 \times g$ to separate cream and supernatant separated. On average, a 1.29 ± 0.27 log reduction (between 0.94 and 1.65 log) in MAP was detected when centrifuging the 2:1 diluted colostrum with the low dose of MAP. For the colostrum with the high dose of MAP, on average 1.27 ± 0.15 log reduction was observed.

For the decontamination of colostrum originating from cows with a subclinical infection (1.3 log cfu/mL), this protocol suffices. For the decontamination of colostrum originating from cows with symptoms (2.3 log cfu/mL), this protocol will not suffice (Stabel et al., 2014). Note that this protocol was optimized under laboratory conditions. In those experiments, for practical reasons skimming occurred manually during the validation. In a separator, however, the circulating system physically separates supernatant and cream throughout the skimming process.

Validation with Naturally Contaminated Samples

We obtained 16 colostrum samples: 7 originating from a seropositive cow (colostrum 1, 2, 3, 5, 7, 9, and 16), 1 from a heifer born from a seropositive cow (colostrum 15), and 8 from cows in a paraTBC-positive herd (colostrum 4, 6, 8, 10, 11, 12, 13, 14, and 15). Six colostrum samples (colostrum 2, 7, 12, 14, 15, and 16) were considered negative (the results of all MAP counts were <10 cfu/mL). We performed MAP counts on the samples with and without a chemical decontamination procedure as described by Bradner et al. (2013): MAP was found in 7 colostrum samples (colostrum 1, 3, 5, 9, 10, 11, and 13) for both treatments, in 2 samples only after decontamination, and in 1 sample without decontamination (Table 1). On average, we observed 2.2 ± 0.16 cfu of MAP/mL in the positive colostrum samples, close to the counts from colostrum of clinical cows as reported by Stabel et al. (2014). Although the farmers in this study were all subjected to the Belgian paraTBC program, the presence of “clinical” cows was not mentioned. It is possible that these “subclinical” cows intermittently shed more MAP as previously reported (Mortier et al., 2014). Another possibility is that our methodology is more sensitive compared with the one used by Stabel et al. (2014) due to the medium used.

No MAP was observed in the whey and supernatants of these samples, indicating that both protocols resulted

in a MAP reduction of at least 1.2 to 2.2 log reduction, which appears sufficient for the decontamination of these samples. After pasteurization of the cream, we retrieved no MAP. When we performed the quantitative (q)PCR on the original samples, we unexpectedly detected no MAP-DNA. The samples contained approximately 2 log of MAP, which is more or less the detection limit of the qPCR. It is possible that the number was too low to detect in the qPCR.

Adaptation of the Protocols for On-Farm Use and Use at a Central Location

After performing the final FP, we obtained whey with immunoglobulins, but without caseins, an im-

Table 1. Overview of *Mycobacterium avium* ssp. *paratuberculosis* (MAP) counts, determined with and without initial chemical decontamination procedure of the colostrum as described by Bradner et al. (2013)¹

Sample	MAP count (average log cfu/mL \pm SD)	
	No decontamination procedure	Decontamination procedure
Colostrum 1	2.28 \pm 0.08	2.59 \pm 0.07
Whey (FP)	<1	<1
Supernatant (SIP)	<1	<1
Colostrum 3	2.07 \pm 0.16	1.98 \pm 0.19
Whey (FP)	<1	<1
Supernatant (SIP)	<1	<1
Colostrum 4	<1	2.10 \pm 0.14
Whey (FP)	<1	<1
Supernatant (SIP)	<1	<1
Colostrum 5	2.65 \pm 0.20	2.62 \pm 0.31
Whey (FP)	<1	<1
Supernatant (SIP)	<1	<1
Colostrum 6	2.33 \pm 0.03	<1
Whey (FP)	<1	<1
Supernatant (SIP)	<1	<1
Colostrum 8	<1	1.85 \pm 0.19
Whey (FP)	<1	<1
Supernatant (SIP)	<1	<1
Colostrum 9	2.38 \pm 0.09	2.16 \pm 0.12
Whey (FP)	<1	<1
Supernatant (SIP)	<1	<1
Colostrum 10	2.21 \pm 0.12	2.05 \pm 0.25
Whey (FP)	<1	<1
Supernatant (SIP)	<1	<1
Colostrum 11	2.36 \pm 0.08	2.27 \pm 0.08
Whey (FP)	<1	<1
Supernatant (SIP)	<1	<1
Colostrum 13	2.03 \pm 0.51	2.01 \pm 0.15
Whey (FP)	<1	<1
Supernatant (SIP)	<1	<1

¹The counts are shown for each colostrum sample (as received from the farmer), for each whey sample obtained after performing the on-farm method (FP), and for each supernatant obtained after performing the semi-industrial protocol (SIP). The MAP counts of colostrum samples 2, 7, 12, 14, 15, and 16 were all negative. Negative results are indicated in the table as <1 log cfu/mL.

portant protein source for the calves. To supplement this whey, we added the milk powder which the calves would receive at an older age to the whey after thawing and before administration to the calf. This step aims to add extra nutrients to the whey to partially correct for the lost proteins. Visually this colostrum was less dense than the regular colostrum and it is difficult to estimate the effect of the treatment on the composition and palatability of this reconstituted whey.

We also adapted the validated SIP based on centrifugation with equipment used in the dairy industry. In a first step, colostrum is diluted 2:1 with skimmed colostrum, heated to 43°C (to dissolve the fat globules), and skimmed with a separator. The obtained cream is pasteurized at 70°C for 30 min over boiling water (*au bain marie*). We choose this heating protocol because pasteurization for 1 h at 60°C or 15 s at 72°C has been reported as insufficient to consistently reduce MAP in milk and colostrum (Grant et al., 2005; Hammer et al., 2014). We also performed a preliminary experiment to determine the temperature effective in eliminating MAP (data not shown). The obtained skimmed colostrum was subsequently clarified using the Easycream I. After clarification, cream and clarified colostrum were mixed and homogenized. We expect to achieve a higher log reduction than observed in the experiments as 2 subsequent centrifugation steps are used, which will be sufficient for the decontamination of colostrum originating from clinical cows. In addition, these appliances have a closed centrifugation system, where the supernatant is centrifuged more thoroughly than in the laboratory.

Animal Experiment

For the animal experiment, colostrum was prepared according to the developed protocols described above. The calves need at least 200 g of IgG to obtain a IgG blood value of at least 10 g of IgG/L of blood serum (lower limit of passive transfer; Godden, 2008; Godden et al., 2012). The pool (control colostrum) contained 61 g of IgG/L, whereas the colostrum prepared with the curdle and centrifugation protocol contained 49 and 54 g of IgG/L, respectively. These values were considered high enough as the calves received 6 L of colostrum within 24 h after birth.

We evaluated different parameters during this animal experiment. Approximately 72 h after birth, we determined the IgG amount in the blood serum (Figure 6). Lower values were observed in the 3 test groups compared with the group that received colostrum from their dam. However, this difference was not statistically significant, given the large variation in the own group. This is probably due to intercow variations

in the colostrum quality or intercalf variations in the blood values as described previously (Alley et al., 2012; Morrill et al., 2012; Yang et al., 2015). For the test groups, we worked with standardized purchased colostrum, which should have a fixed IgG content of 70 g of IgG/L. In spite of this, the blood values in these groups were lower, which indicates that these groups received fewer IgG than expected. One possibility is that the IgG amount decreased during the preparation process. It has been reported that the IgG amount decreases if the colostrum has undergone 3 freeze-thaw cycles, which was the case during the preparation (Morrill et al., 2015). Measurement of the IgG amount revealed only 61 g of IgG/L instead of the standardized 70 g/L. Another possibility is that the treatment of the colostrum changes the structure of the colostrum, resulting in a lower IgG uptake. During the animal trial, we estimated the IgG content of each bottle of colostrum with a refractometer. Because of the changes in structure (e.g., dilution, addition of milk powder), the results of the treated colostrum samples are not useful.

Besides the blood values, we also assessed the ease of intake of the colostrum (5 intakes per calf). All calves consumed 10 L of colostrum within 24 h after birth, except for one calf from the HP group that consumed 9 L and 2 calves from the own group that consumed 9.5 L of colostrum each. The majority of the own group drank very easily or normally (Figure 7) and no calves needed probe feeding. We observed that the majority of intakes (67 and 83%) of the control and SIP group, respectively, happened either easily or normally. One calf of the control group (1 intake) and one calf of the SIP group (2 intakes) required probe feeding. The results of the FP group differed from the other groups: only 34% of the feedings were categorized as very easy or normal, whereas 40% were difficult. Three out of 6 calves required one probe feeding and 2 calves required 4 probe feedings each (27% of all feedings of this group). Normally the calves of the ILVO herd only require very sporadic probe feedings as was observed in the own group (internal communication).

The calves had a birth weight of 38.8 ± 0.97 kg and an average weight of 117.9 ± 8.72 kg after 16 wk. We observed no statistically significant difference in weight gain between the groups, except at 16 wk, where a significant difference was observed between the own group (1.18 kg/wk) and the others (control: 0.91 kg/wk, FP: 0.78 kg/wk, and SIP: 0.86 kg/wk; $P = 0.008$). No significant differences were observed between the milk and forage intakes of the calves in the test groups.

One calf of the SIP group died from colitis and one calf from the control group died from intestinal perforation. In total, 19 antibiotic treatments were given: one in the own group, 5 in the control group, 6 in the

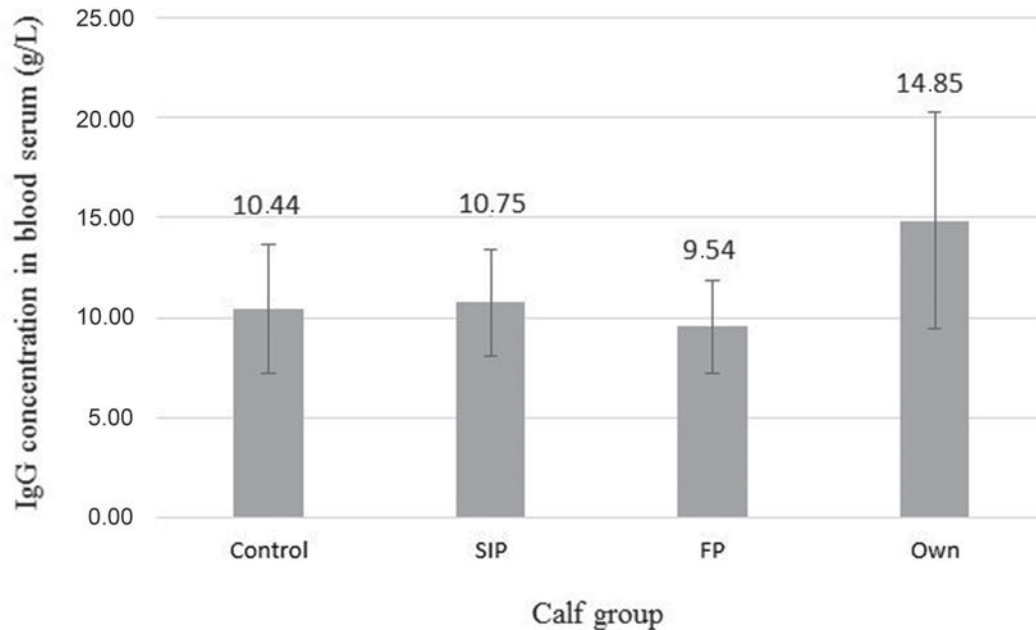


Figure 6. IgG blood serum results (mean \pm SD, $n = 3$) of the calves belonging to the 4 test groups (own, control, SIP, and FP) in the animal experiment. The average of each group is indicated. SIP = semi-industrial protocol; FP = farm protocol.

SIP group (3 treatments were given to the calf that later died), and 7 in the FP group (2 calves received 3 treatments each). When comparing the number of treatment days relative to the total duration of the ex-

periment, the calves of the control, SIP, and FP groups received on average 3.83, 4.33, and 5.17 d of treatment, respectively, as opposed to 0.50 d in the own group. Unfortunately, during the time period when the first 9

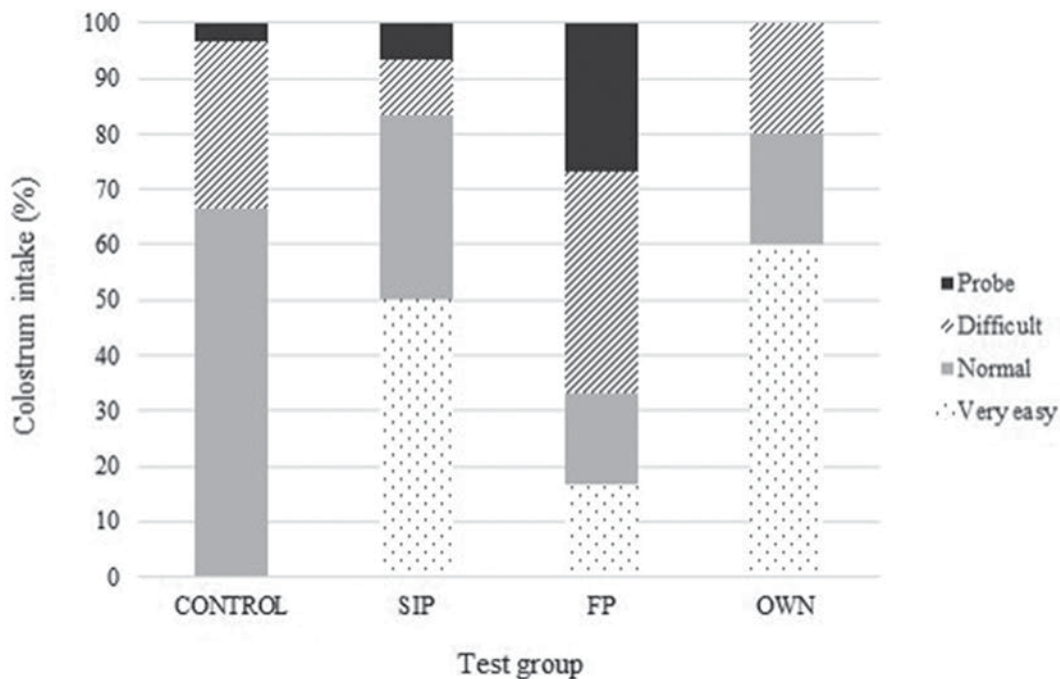


Figure 7. Total colostrum intake of all calves in the animal experiment. The results are shown as a percentage. SIP = semi-industrial protocol; FP = farm protocol.

calves were born and allocated to the 3 test groups, a *Salmonella* infection was diagnosed on the farm where the experiment was conducted. This resulted in diarrhea and antibiotic treatment of some calves. After the birth of the first 9 calves, this infection was absent for the remaining period of the experiment. Therefore, we cannot exclude that the higher occurrence of illness and antibiotics treatment in the test groups is related to a higher infection risk during the first weeks/months of the animal experiment. For the remaining duration of the experiment, the observed diarrhea scores were the same for all groups (data not shown).

We compared different parameters and found that even though the on-farm colostrum was less attractive to the calves and more calves of this group needed to be probed, in general the animals from this group performed well. On some Belgian farms, calves are routinely probed to be certain of the ingested volume of colostrum; in such cases this protocol could be applied. On farms, where probing is uncommon, the on-farm treatment protocol could negatively affect colostrum intake. This study was based on the use of purchased sterile colostrum; treatment of fresh and naturally contaminated colostrum and its effect on colostrum intake must also be studied.

CONCLUSIONS

We have optimized 2 decontamination protocols for MAP under laboratory conditions. The FP, based on curdling, has the potential to be used on the farm and results in at least 1 log reduction in MAP counts with a limited loss of IgG. The SIP was developed to be used at a central location and resulted in at least 1.5 log reduction in MAP counts and a limited loss of IgG. Both protocols were adapted for use based on place of treatment: for on-farm use, we added milk powder to the whey fraction before giving it to the calf. For application in a central location, we used equipment from the dairy industry (resulting in 2 centrifugation steps, which will increase the MAP reduction). Both methods were validated using artificially and naturally contaminated colostrum. We also administered these uninfected treated colostrum to calves, and found no significant differences between the test groups. For future work, it would be interesting to assess whether these treatments have an effect on other pathogens as well.

ACKNOWLEDGMENTS

This research was funded by the Institute for the Promotion of Innovation by Science and Technology in Flanders (VLAIO) project 110784. We thank Melkcontrole Centrum Vlaanderen and DGZ for their kind

contribution of the colostrum used in this study. Special thanks go to the laboratory technicians at ILVO. We also gratefully acknowledge the contribution of the animal caretakers and students at ILVO and Miriam Levenson for English-language editing.

REFERENCES

- Alley, M. L., D. M. Haines, and G. W. Smith. 2012. Short communication: Evaluation of serum immunoglobulin G concentrations using an automated turbidimetric immunoassay in dairy calves. *J. Dairy Sci.* 95:4596–4599.
- Begg, D. J., and R. J. Whittington. 2008. Experimental animal infection models for Johne's disease, an infectious enteropathy caused by *Mycobacterium avium* ssp. *paratuberculosis*. *Vet. J.* 129:129–145.
- Bradner, L., S. Robbe-Austerman, D. C. Beitz, and J. R. Stabel. 2013. Chemical decontamination with N-acetyl-L-cysteine-sodium hydroxide improves recovery of viable *Mycobacterium avium* ssp. *paratuberculosis* organisms from cultured milk. *J. Clin. Microbiol.* 51:2139.
- Diéguez, F. J., I. Arnaiz, M. L. Sanjuán, M. J. Vilar, and E. Yus. 2008. Management practices associated with *Mycobacterium avium* subspecies *paratuberculosis* infection and the effects of the infection on dairy herds. *Vet. Rec.* 162:614–617. <https://doi.org/10.1136/vr.162.19.614>.
- Eisenberg, S. W. F., A. P. Koets, J. Hoeboer, M. Bouman, D. Heederik, and M. Nielen. 2010. Presence of *Mycobacterium avium* ssp. *paratuberculosis* in environmental samples collected on commercial Dutch dairy farms. *Appl. Environ. Microbiol.* 76:6310–6312. <https://doi.org/10.1128/AEM.00998-10>.
- Eisenberg, S. W. F., V. P. M. G. Rutten, and A. P. Koets. 2015. Dam *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection status does not predetermine calves for future shedding when raised in a contaminated environment: A cohort study. *Vet. Res.* 46:70.
- Gao, A., J. Odumera, M. Raymond, and L. Mutharia. 2005. Development of improved method for isolation of *Mycobacterium avium* ssp. *paratuberculosis* from bulk tank milk: Effect of age of milk, centrifugation, and decontamination. *Can. J. Vet. Res.* 69:81–87.
- Gauthier, S., Y. Pouliot, and J. L. Maubois. 2006. Growth factors from bovine milk and colostrum: Composition, extraction and biological activities. *Lait* 86:99–125. <https://doi.org/10.1051/lait:2005048>.
- Godden, S. 2008. Colostrum management for dairy calves. *Vet. Clin. North Am. Food Anim. Pract.* 24:19–39. <https://doi.org/10.1016/j.cvfa.2007.10.005>.
- Godden, S. M., D. J. Smolenski, M. Donahue, J. M. Oakes, R. Bey, S. Wells, S. Sreevatsan, J. Stabel, and J. Fetrow. 2012. Heat-treated colostrum and reduced morbidity in preweaned dairy calves: Results of a randomized trial and examination of mechanisms of effectiveness. *J. Dairy Sci.* 95:4029–4040.
- Godden, S. M., S. Wells, M. Donahue, J. Stabel, J. M. Oakes, S. Sreevatsan, and J. Fetrow. 2015. Effect of feeding heat-treated colostrum on risk for infection with *Mycobacterium avium* ssp. *paratuberculosis*, milk production, and longevity in Holstein dairy cows. *J. Dairy Sci.* 98:5630–5641.
- Grant, I. R., A. G. Williams, M. T. Rowe, and D. D. Muir. 2005. Efficacy of various pasteurization time-temperature conditions in combination with homogenization on inactivation of *Mycobacterium avium* ssp. *paratuberculosis* in milk. *Appl. Environ. Microbiol.* 71:2853–2861.
- Hammer, P., C. Kiesner, and H.-G. C. Walte. 2014. Short communication: Effect of homogenization on heat inactivation of *Mycobacterium avium* subspecies *paratuberculosis* in milk. *J. Dairy Sci.* 97:2045–2048.
- Houser, B. A., S. C. Donaldson, S. I. Kehoe, A. J. Heinrichs, and B. M. Jayarao. 2008. A survey of bacteriological quality and the occurrence of *Salmonella* in raw bovine colostrum. *Foodborne Pathog. Dis.* 5:853–858.

- Hussain, A., S. K. Singh, P. R. P. Verma, N. Singh, and F. J. Ahmad. 2016. Experimental design-based optimization of lipid nanocarrier as delivery system against *Mycobacterium* species: *In vitro* and *in vivo* evaluation. *Pharm. Dev. Technol.* <https://doi.org/10.1080/10837450.2016.1212879>.
- IDF. 1996. Determination of acid soluble β -lactoglobulin content. International Dairy Federation, Brussels, Belgium.
- Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head bacteriophage T4. *Nature* 227:680–685.
- Morrill, K. M., E. Conrad, A. Lago, J. Campbell, J. Quigley, and H. Tyler. 2012. Nationwide evaluation of quality and composition of colostrum on dairy farms in the United States. *J. Dairy Sci.* 95:3997–4005.
- Morrill, K. M., K. E. Robertson, M. M. Spring, A. L. Robinson, and H. D. Tyler. 2015. Validating a refractometer to evaluate immunoglobulin G concentration in Jersey colostrum and the effect of multiple freeze–thaw cycles on evaluating colostrum quality. *J. Dairy Sci.* 98:595–601. <https://doi.org/10.3168/jds.2014-8730>.
- Mortier, R. A., H. W. Barkema, J. M. Bystrom, O. Illanes, K. Orsel, R. Wolf, G. Atkins, and J. De Buck. 2013. Evaluation of age-dependent susceptibility in calves infected with two doses of *Mycobacterium avium* subspecies *paratuberculosis* using pathology and tissue culture. *Vet. Res.* 44:94–103. <https://doi.org/10.1186/1297-9716-44-94>.
- Mortier, R. A., H. W. Barkema, K. Orsel, R. Wolf, and J. De Buck. 2014. Shedding patterns of dairy calves experimentally infected with *Mycobacterium avium* subspecies *paratuberculosis*. *Vet. Res.* 45:71.
- Stabel, J. R., L. Bradner, and S. Robbe-Austerman. 2014. Clinical disease and stage of lactation influences shedding of *Mycobacterium avium* subspecies *paratuberculosis* into milk and colostrum of naturally infected dairy cows. *J. Dairy Sci.* 97:6296–6304. <https://doi.org/10.3168/jds.2014-8204>.
- Sweeney, R. W. 1996. Transmission of paratuberculosis. *Vet. Clin. North Am. Food Anim. Pract.* 12:305–312.
- Van Brandt, L., K. Coudijzer, G. Vlaemynck, M. Hendrickx, C. Michiels, W. Messens, and L. Herman. 2010. Localization of *Mycobacterium avium* subspecies *paratuberculosis* in artificially inoculated milk and colostrum by fractionation. *J. Dairy Sci.* 93:4722–4729. <https://doi.org/10.3168/jds.2009-2895>.
- Yang, M., Y. Zou, Z. H. Wu, S. L. Li, and Z. J. Cao. 2015. Colostrum quality affects immune system establishment and intestinal development of neonatal calves. *J. Dairy Sci.* 98:7153–7163.

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