Low-temperature centrifugation of milk for manufacture of raw milk cheeses: impact on milk debacterization and cheese yield

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ABSTRACT

Centrifugation is occasionally applied to clean cheese milk, particularly to remove Clostridia's spores that may cause the "late blowing" of cheese. The sludge separated by centrifugation also contains fat and protein, thus is sterilized and added back to cheese milk. In manufacture of raw milk cheeses, centrifugation shall be performed at temperature below 40 °C and no sterilized sludge can be added to vat milk. Both these limitations negatively affect cheese yield. To evaluate process sustainability, three different centrifugation configurations were tested at a factory producing a traditional raw-milk extra-hard cheese. Either a single or double centrifugation, the latter with two different volumes of discharged sludge, were tested over 3-week periods each. Efficiency of spore removal, decrease of total bacterial count, loss of milk solids and cheese yield were evaluated daily with respect to not-centrifuged milk from the same batch. Double centrifugation with low-volume sludge gave highest efficiency of spore removal, i.e. 98.2 %, while the single process minimized the loss of cheese yield. Impedometric analysis indicated that centrifugation caused a preferential removal of rod-shaped lactic acid bacteria, regardless of configuration. This finding was confirmed by microscopy and suggested that a different bacteria population would operate during cheese ripening.

Key words: centrifugation, spore, raw milk cheese, impedance microbiology, cheese yield

1. INTRODUCTION

Cheese milk centrifugation at high centrifugal force (8,000-10,000 *g*) allows removal of bacterial spores (Guinee & O'Callaghan, 2010) and decreases bacterial and somatic cells counts (Sant'Ana, 2014). This effect exploits differences in density and size between milk and non-milk solids. The density of bulk milk (4 % fat and 8.95 % solids-non-fat) at 20 °C is 1.028-1.033 kg/dm^3 which is lower than that of bacterial cells (1.07-1.13 kg/dm^3) and spores (1.2-1.3 kg/dm^3) (Te Giffel & van der Horst, 2004; Sant'Ana, 2014; Fox, Uniacke-Lowe, McSweeney, & O'Mahony, 2015). Modern disc centrifuges intended for milk pre-treatment are third generation machines with optimum operating temperature in the range 50-60 °C (McCarthy, 2011). During milk centrifugation, spores and bacterial cells are concentrated and periodically ejected (every 15-20 min) as sludge. Removal efficiency varies widely depending on the type and operating conditions (speed, flow rate, temperature, etc.) but also on the characteristics of cells and spores, primarily density but also size and shape (Te Giffel & van der Horst, 2004).

Pre-treatment of cheese milk using a dedicated centrifuge, also named Bactofuge (Tetra Pak, Sweden) or bacteria-removing centrifuge (Westfalia-GEA, Germany), was developed for the removal of *Clostridium* spores causing the "late blowing" defect in hard and semi-hard cheeses (Bisig, Fröhlich-Wyder, Jakob, & Wechsler, 2010; Guinee and O'Callaghan, 2010; Lamichhane, Kelly, & Sheehan, 2018; Waes & Van Heddeghem, 1990). More recently, this process was also adopted in drinking milk manufacture to extend the shelf-life of the product without increasing pasteurization or sterilization temperature (Te Giffel, Van Asselt, & De Jong, 2006; Gesan-Guiziou, 2010). The sludge normally represents 2.5-3.5 % of the processed milk volume and contains up to 12-13 % protein, namely casein (GEA, 2018; Gesan-Guiziou, 2010; Waes & Van Heddeghem, 1990). Thus, to avoid loss of these solids, that would result in a decreased cheese yield, the sludge is sterilized and re-added to the cheese milk (Walstra, Geurts, Noomen, Jellema, & van Boekel, 1999).

The described centrifugation process shall not be adopted in the manufacture of raw milk cheeses, such as traditional or Protected Designation of Origin (PDO) cheeses, whose product specification

requires the use of milk that has not been heated above 40 °C (Bisig et al., 2010; D'Amico 2014; Egger, Nicolas, & Pellegrino, 2016). In these cases, both configuration and operating conditions of the centrifuge have to be optimized in order to obtain the required spore reduction and minimize the protein loss while respecting the raw milk requisite.

The aim of this study was to assess the impact of adopting a milk centrifugation process conducted at 39 °C and with sludge elimination in the technology of a raw-milk hard cheese. Three different centrifugation configurations were tested at a cheese factory with the aim of identifying the one representing the best compromise between the bacteria removal efficiency and cheese yield. During three experimental periods, three-week each, 20 cheese vats were dedicated daily to experimental stream and 20 vats to control stream (no milk centrifugation applied).

2. MATERIALS AND METHODS

2.1 Experimental design and sampling

The experimentation outlined in Fig. 1 was carried out at a cheese factory adopting the traditional process of a raw milk extra-hard cheese described by D'Incecco, Pellegrino, Hogenboom, Cocconcelli, & Bassi, 2018b. Raw bulk milk was received daily from three local farms, pooled in a tank and sampled for analyses. Fat was separated by batch gravity creaming at 8-12 °C for around 10 hours. Partly skimmed (fat 2.2 g/100 mL) milk was divided into two streams: one stream entirely followed the traditional cheese-making (control, C); the other stream (experimental, E) was submitted to centrifugation adopting three different configurations: one centrifuge (configuration 1); two centrifuges in series (configuration 2); two centrifuges in series and reduced sludge volume (configuration 3). This last only implied a changing in the setting of the sludge volume produced by both centrifuges which is related to the amount of discharged solids. The different configurations were tested for three-week periods each. The centrifuges were one-phase CSI-230-01-772 Westfalia

(Germany) operating at 39 °C and flow rate 21,000 L/h. After centrifugation, milk was held in a degassing tank at ~13 °C for 4 hours and then conveyed to the vat. A total of 40,000 L milk was worked daily in 20 vats for the experimental stream and 20 vats for the traditional one. For each stream, on nine different days of the testing period, milk was sampled from two vats (replicates) just before the addition of the natural whey starter and calf rennet and, after extraction of the obtained curd, the whey was sampled as well. Samples were immediately frozen with liquid Nitrogen and brought to laboratories for analyses. All cheeses obtained during each testing period (two cheeses per vat × 40 vats per day × 9 days) were weighted after the 16-day brine salting and 3-day drying at room temperature to have an early estimate of cheese yield.

2.2 Gross composition of milk and cheese whey

Samples of vat milk and cheese whey were analysed for fat, total protein, casein and lactose contents using a Milkoscan 134 (Foss, Denmark) (IDF, 1996). Analyses were run in triplicate.

2.3 Cheese yield calculation

Cheese yield was calculated for single vat as the obtained amount of cheese (weight of two loaves) with respect to the amount of poured milk. This last was measured using a flow meter and the corresponding weight was calculated using the milk density value.

2.4 Determination of total bacterial count, coliforms and spores

Spores of anaerobic clostridia were determined by the most probable number (MPN) technique. Aliquots (1 mL) from serial dilutions of samples prepared using sterile Ringer solution (Oxoid, Ltd., UK) were inoculated into 5 tubes containing 5 mL of reconstituted (10% wt/vol) skim milk (Oxoid) that was supplemented with yeast extract (1% wt/vol), sodium lactate (3.36% wt/vol), sodium acetate

(1% wt/vol), and cysteine (0.2% wt/vol) and sterilized. Each tube was overlaid with 1.5 mL of sterile melted paraffin-vaseline mixture (1:1, wt/wt) and heated at 80 °C for 10 min to kill vegetative cells. Tubes were incubated at 37 °C for 7 d and daily inspected for gas production. The MPN counts were expressed as spores per litre. Percent spore reduction was calculated by referring the spore count in each vat milk to that in the respective raw whole milk. Total bacterial count and coliforms (cfu/mL) were determined using Milk Plate Count Agar (Oxoid), with incubation at 30 °C for 72 hours (IDF, 1991), and Violet Red Bile Agar (Oxoid), with incubation at 37 °C for 24 hours (ISO, 2006), respectively. Analyses were carried out in triplicate.

2.5 Determination of lactic acid bacteria

The LAB count of milk samples was determined by impedance analysis. An accurate milk thawing protocol was used. The 50-mL Falcon® tubes containing the frozen samples were kept in a water bath at 37 °C for 13 min to obtain a complete and homogeneous melting of the milk. Thawed milk (6 ml) was immediately aliquoted into pre-sterilized measuring vial and analyzed at 25 °C for the quantification of mesophilic LAB using a BacTrac 4300® Microbiological Analyzer system (Sylab, Austria) as described by Bancalari et al. (2016). The impedometric curves describing LAB growth were visualized as the relative change of impedance in the culture medium (M%). The parameter M% was recorded every 10 min for 48 hours. Data obtained from all the measurements were elaborated using the Gompertz equation, as previously reported by Bancalari et al. (2016), to derive three kinetic parameters (Suppl. File 1): (i) Lag, representing the time (hours) cells require to adapt to the medium and start growing; (ii) Rate, corresponding to the exponential phase, is useful to evaluate LAB acidification rate in milk; (iii) yEnd, corresponding to the maximum variation of impedance recorded, describes the capability of LAB cells to modify medium impedance value due to lactate ion accumulation during growth. Analyses were carried out in triplicate and the final pH of milk samples was measured by means of pH-meter Beckman Instrument mod Φ350 (Furlenton, CA, USA).

2.6 Microscopy of vat milk

Confocal laser scanning microscopy (CLSM) was used to investigate the microstructure of both E and C vat milk. Samples were stained with Fast green FCF (0.1 mg/mL) (Sigma-Aldrich, St Louis, USA) to visualize proteins and with Nile red (0.1 mg/mL) (Sigma-Aldrich) to detect the triacylglycerol core of fat globules as described by D'Incecco, Rosi, Cabassi, Hogenboom, & Pellegrino, (2018c). Briefly, stock solutions of Fast green (1mg/mL) and Nile red (1mg/mL) were prepared in water and in 80% (v/v) DMSO (Sigma-Aldrich), respectively. Solutions were kept protected from light until use. Samples were observed using an inverted CLSM instrument (Nikon A1+, Minato, Japan) after 10-min staining in the dark at room temperature. Nile red was excited at 488 nm and the emission filter was set at 520-590 nm while Fast Green was excited at 633 nm and the emission filter was set at 660-740 nm. Images are presented as maximum projection of 25 layers of 1024 × 1024 pixel images stacked together. Image analysis was performed using Vision4D software (Arivis, AG, Germany) on maximum projection of CLSM z-stack images. Image analysis data are means of three independent evaluations.

2.7 Microscopy of bacterial population

Shape of bacteria cells in both E and C vat milk was evaluated by means of CLSM. Cells were stained by Hoechst 34580 (Sigma-Aldrich, St Louis, USA) at the final concentration of 3 µg/mL. Hoechst 34580 was excited at 405 nm and the emission filter was set at 410-485 nm. The same equipment as for milk microscopy was used.

2.8 Statistical analysis

Statistical difference (t-Test; two-tailed distribution) between E and C samples was evaluated using the SPSS Win 12.0 program (SPSS Inc. IBM Corp., Chicago, IL). Differences at $p < 0.05$ (*); $p < 0.01$ (**) and $p < 0.001$ (***) were considered significant.

3. RESULTS

3.1 Effect of milk centrifugation on vat milk composition and cheese yield

Gross composition was determined for vat milks derived from the same batch of incoming milk and obtained using either the traditional natural creaming (C) or with an additional centrifugation (E). Centrifugation was performed using three different configurations (Fig. 1): 1 centrifuge, 2 centrifuges or 2 centrifuges with a reduced sludge volume (improved). Single centrifugation did not cause any changes in fat, protein, lactose and casein contents ($p > 0.05$) (Table 1). Differently, all components significantly decreased ($p < 0.01$ for fat, lactose and casein; $p < 0.001$ for protein) in E vat milk obtained adopting 2 centrifuges whereas, adopting the improved configuration, only protein and casein were significantly lower ($p < 0.01$). Cheese weight showed the opposite trend than milk casein content while the lowest weight difference (1.05 kg) between C and E loaves was obtained with the single centrifugation (Table 1).

The amount of fat lost in cheese whey was always significantly higher in the E samples compared to the respective C samples, irrespective of the configuration tested (Table 2). However, statistical strength progressively decreased from double to single centrifugation. No significant differences ($p > 0.05$) were observed for protein.

3.2 Effect of centrifugation on microbiological quality of vat milk

Spore content of vat milk was the most important parameter to monitor, due to its relation with the possible insurgence of late blowing in the derived cheese. The positive effect of natural creaming was

195 evident in this regard confirming that, although this process is principally adopted for lowering the
196 fat content of milk in making numerous traditional cheeses, it also allows around 90% spore removal
197 (D'Incecco, P., Faoro F., Silvetti T., Schrader K., & Pellegrino, 2015). The additional centrifugation
198 of milk improved the efficiency of spore removal compared to the natural creaming itself (Table 3).
199 When referred to the spore content of raw bulk milk, efficiency was significantly ($p < 0.05$) higher
200 for both processes involving double centrifugation. Conversely, the single centrifugation did not
201 provide any improvements ($p > 0.05$). As expected, percent spore reduction obtained by
202 centrifugation was much less variable than that of natural creaming. Overall, coefficients of variation
203 (CV) for centrifugation and natural creaming were 1.7% and 7.5% respectively.
204 Generally, both TBC and coliforms counts had a great daily variability in bulk milk, due to the
205 different contamination of incoming raw milk, and some values further increased during natural
206 creaming due to microbial growth (Table 3). Both TBC and coliform average counts in milk were
207 variable among treatments, being the variability highest with the single centrifugation and lowest
208 with the improved double centrifugation (Table 3). Considering single day samples, the reduction
209 reached values up to 79.8% for TCB and 99% for coliforms (data not shown). Overall, despite the
210 large variability, both milk hygiene parameters were significantly improved by centrifugation at low
211 temperature only when the double process was adopted (Table 3). Furthermore, data obtained by
212 mean of agar plate count showed that the effect of milk centrifugation at the tested temperature was
213 not directly related to that of spore reduction, irrespective of type of centrifugation treatment. Thus,
214 to study the effect of centrifugation on lactic acid bacteria, a different approach was used.
215
216 3.3 Effect of centrifugation on autochthonous LAB

217 The presence of mesophilic NSLAB was characterized in E and C vat milk samples by the respective
218 kinetic parameters Lag, Rate and yEnd derived from the conductimetric curves obtained using a
219 BacTrac 4300® (Bancalari, D'Incecco, Savo Sardaro, Neviani, Pellegrino, & Gatti, 2019) (Table 4).

220 The Lag value is inversely proportional to LAB cell number. Mean values of Lag were significantly
221 higher ($p < 0.05$) for E milk samples than for the C samples, principally when two centrifugations
222 were carried out. This indicated that LAB cell numbers were lower in the E samples, thus cells needed
223 more time to reach the minimum concentration required to record a variation in the electrical signal.
224 Differently, the value of acidification rate (Rate) does not depend on LAB cell number, but mostly
225 on the presence of different types of LAB. For all configurations, mean value of Rate was lower in E
226 milk samples than in C samples, although with a different level of significance, indicating a faster
227 acidification rate of LAB present in the former. This means that milk centrifugation was able to
228 modify LAB composition. On the other end, mean values of yEnd obtained for E vat milk and the
229 corresponding C vat milk were not significantly different, indicating that LAB in the two types of
230 milk were not different in their acidification capability.
231 Considering the differences between E and C samples in terms of Lag and Rate parameters, the
232 microbial populations in the two types of milk was further investigated using CLSM. Interestingly, a
233 difference in the shape of bacteria was found between E and C samples, regardless the type of
234 centrifugation. Since such a different picture was confirmed for all samples, only the sample
235 centrifuged once and the respective control are shown in Fig. 2. Only cocci bacteria were observed
236 in E sample while also rod-shaped bacteria were present in C.
237
238 3.4 Effect of centrifugation on microstructure of milk

239 Continuous centrifugation of milk is usually carried out between 52 °C and 60 °C to operate at a low
240 milk viscosity thus decreasing risk of fat globule damaging (GEA, 2018). Within this
241 experimentation, centrifugation was carried out at 39 °C in order to manufacture raw milk cheese.
242 Vat milk microstructure was evaluated by comparing CLSM images of E samples with the respective
243 C samples for the tested configurations. The typical microstructure of partly-skimmed raw milk was
244 observed (D'Incecco, Ong, Pellegrino, Faoro, Barbiroli, & Gras, 2018a) in C samples, characterized

by casein micelles occupying the whole milk volume and individual fat globules dispersed in (Fig. 3). Diameter, circularity and area of fat globules were evaluated through image analysis (Table 4). Single centrifugation did not cause a significant ($p > 0.05$) difference in fat globule characteristics. Differently, both globule diameter and area were remarkably ($p < 0.001$) lower in the E samples obtained with double centrifugation, either with or without reducing sludge volume. Circularity of fat globules in E milk was significantly ($p < 0.001$) lower than in the corresponding C milk only with the double centrifugation. Small quantity of free fat was present in all samples, either C or E, and regardless the centrifugation configuration involved (not shown).

4. DISCUSSION

In order to manufacture raw milk cheeses, low-temperature centrifugation conditions should be optimized in view of removing spores while keeping the reduction of cheese yield as low as possible. Independently of the configuration tested in this study, milk centrifugation brought to a remarkable loss of protein in the sludge. Protein loss is directly related to the volume of the sludge itself (GEA, 2018) and, in principle, would decrease as the centrifugation temperature decreases (McCarthy, 2011). The protein content was around 8% (wt/wt) for the sludge ejected when the single-centrifuge or two centrifuges-improved configurations were used while it increased up to 10% with the two-centrifuges (not shown). These values are within the range 2.5-12.8% reported in the literature (Gesan-Guiziou, 2010; Lamichhane et al., 2018) for milk centrifugation at 50 °C, although different types of equipment were used. Due to the difficulty of precisely measuring the amount of sludge ejected during operation, a more accurate evaluation of protein loss was achieved by comparing the casein content of vat milk samples derived from the two streams, E and C respectively. We recorded an increased casein content of vat milk by 0.13 g/100 mL ($p < 0.05$) when the sludge volume was decreased in the double centrifugation and a further casein increase, up to 0.17 g/100 mL of vat milk, was achieved by adopting the single milk centrifugation. The fat content displayed the same behaviour. Consequently, cheese yield progressively increased from double-stage centrifugation to

double-stage improved up to single centrifugation. In contrast, however, low-temperature centrifugation resulted in a lower spore removal efficiency due to a higher milk viscosity (Te Giffel & van der Horst, 2004; McCarthy, 2011). In fact, the single centrifugation at 39 °C gave an average spore reduction of 95.9%, i.e. remarkably lower than values reported in literature, such as 97.4-98.7% at 48°C (Te Giffel & van der Horst, 2004) or 97.8% at 62 °C (Gesan-Guiziou, 2010). To the authors knowledge, no recent data on removal efficiency of two centrifuges in series are available in literature. However, the efficiencies we obtained with the double centrifugation at 39 °C were fully comparable to those obtained with a single centrifugation at much higher temperatures. Interestingly, the reduction of sludge volume had a positive effect on both spore removal efficiency (from 97.9 to 98.2%) and reduction of protein loss.

In raw milk cheeses, no matter whether traditional or PDO, the autochthonous LAB population is believed to play a unique role which is essential for the development of their quality and typicality. In fact, natural cultures are usually added to milk whereas both selected starters and adjunctive cultures are excluded (Franciosi, Settanni, Cavazza, & Poznanski, 2009; Gobbetti, De Angelis, Di Cagno, Mancini L., & Fox, 2015). Both TBC and coliform count were reduced by milk centrifugation at 39 °C, although data were extremely variable. Based on their recognized role in raw milk cheeses, a more revealing comparison of NSLAB in vat milk derived from E and C streams was considered to be advisable. The adopted impedometric method measures the change in the electrical conductivity of milk where NSLAB and also non-LAB genera are naturally present. During bacteria growth, metabolic processes release small charged compounds (mainly lactic acid) that increase the milk electrical conductivity (Bartinou, Katsogiannos, Koustoumpardis, & Spiliotis, 2005; Bancalari et al., 2016). Therefore, the variation in conductivity signal mainly depends on the number of bacterial strains and on their ability and rapidity to metabolize lactose. LAB can reasonably be considered as the main responsible of the impedance variation. On the other hand, non-LAB also generate acetate or ethanol in different ratios (Quigley et al., 2013).

296 The longer Lag times recorded for E vat milk samples, compared to the respective C samples, allowed
297 us to evidence that LAB were partly removed by centrifugation. Moreover, the differences in Rate
298 values between the two types of milk lead us to hypothesize that the treatment also removed LAB
299 cells selectively. In fact, CLSM observations of milk samples at the end of incubation for the
300 impedometric analysis confirmed that milk centrifugation selectively retained bacterial cells having
301 a spherical shape much more than the rod-shaped ones, regardless the configuration adopted. In
302 contrast, a mix of the two types of cells was observed in the C samples, as it is expected for natural
303 raw milk. Similar selective effect of milk centrifugation towards lactic acid bacilli was observed by
304 Faccia, Mastromatteo, Conte, & Del Nobile, (2013). These authors found lactic bacilli to grow less
305 than coccus-shaped LAB in mozzarella cheese made from milk that underwent to centrifugation.
306 Selection of bacteria cells by centrifugation, according to their shape, size or density was speculated
307 by Te Giffel and van der Horst (2004). All in all, we could conclude that milk centrifugation mainly
308 removes rod-shaped LAB, which have a lower Rate parameter and thus a faster acidification. Both
309 aspects are mostly relevant in the production of long-ripened raw-milk cheeses, where rod-shaped
310 NSLAB, mainly belonging to *Lactobacillus casei* group (Bancalari et al., 2017; Bottari, Levante,
311 Neviani, & Gatti, 2018), become dominant during the cheese ripening (Gatti, Bottari, Lazzi, Neviani,
312 & Mucchetti, 2014; D'Incecco, Gatti, Hogenboom, Bottari, Rosi, Neviani, & Pellegrino, 2016). Thus,
313 a lower number of these essential LAB during ripening could result in a cheese with different sensory
314 properties. In addition, differences in fat globule integrity caused by double centrifugation could
315 impair the typical cheese structure that deeply characterizes raw-milk hard cheeses at the end of their
316 long ripening.
317
318 **5. CONCLUSION**
319 The results of this work suggested that centrifugation of cheese milk can be successfully applied at
320 temperature compatible with the manufacture of raw milk cheeses. However, a compromise has to be
321 reached between spore removal efficiency and protein loss because these two parameters have shown

an opposite behaviour while changing centrifugation configuration and sludge volume. The scenario derived from this study suggests that using two centrifuges in series can be a superior solution as long as the sludge volume is kept low. Major concern of adopting this process deals with the prevailing removal of rod-shaped bacteria that causes the unbalancing of NSLAB species in cheese milk. Thus, the possible impacts on proteolysis and lipolysis behaviour as well as on flavour will be evaluated in the hard cheeses of this study at the end of the ripening.

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Tables

Table 1. Effect of milk centrifugation configurations on vat milk composition (g/100 mL) and cheese yield (loaf weight)

Vat milk sample	Configuration	Fat	Protein	Lactose	Casein	Loaf weight (kg)	Δ (kg)
C	1	2.8 ± 0.05	3.55 ± 0.02	4.93 ± 0.04	2.76 ± 0.02	42.26 ± 0.91	-1.05
E		2.78 ± 0.04^{ns}	3.52 ± 0.02^{ns}	4.91 ± 0.04^{ns}	2.74 ± 0.01^{ns}	41.45 ± 0.95^{ns}	
C	2	2.63 ± 0.03	3.36 ± 0.02	4.99 ± 0.03	2.62 ± 0.04	41.57 ± 0.43	-1.43
E		2.59 ± 0.03**	3.32 ± 0.02***	4.94 ± 0.03**	2.57 ± 0.04**	40.14 ± 0.41***	
C	3	2.78 ± 0.06	3.51 ± 0.04	4.88 ± 0.05	2.73 ± 0.03	41.98 ± 0.54	-1.37
E		2.74 ± 0.07^{ns}	3.47 ± 0.06**	4.85 ± 0.06^{ns}	2.7 ± 0.04**	40.61 ± 0.64***	

Data are presented as mean value ± standard deviation. Statistical analysis was performed comparing the experimental sample (E) with the respective control (C) within each configuration. The asterisks indicate the significance levels (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; t-Test). ns: not significant difference.

473 **Table 2.** Fat and protein content (g/100 mL) in cheese whey

Cheese whey samples	Configuration	Fat	Protein
C	1	0.38 ± 0.03	0.95 ± 0.01
E		0.42 ± 0.02**	0.96 ± 0.02^{ns}
C	2	0.39 ± 0.03	0.88 ± 0.01
E		0.44 ± 0.02***	0.87 ± 0.03^{ns}
C	3	0.39 ± 0.03	0.94 ± 0.03
E		0.43 ± 0.02***	0.94 ± 0.03^{ns}

474

475 Data are presented as mean value ± standard deviation. Statistical analysis was performed comparing
476 the experimental sample (E) with the respective control (C) within each configuration. The asterisks
477 indicate the significance levels (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; t-Test). ns: not significant
478 difference.

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Table 3. Effect of milk natural creaming (C) or centrifugation using different configurations (E) on microbiological quality of vat milk and spore removal efficiency with respect to bulk milk

Sample	Configuration	TBC (cfu/mL)	Coliforms (cfu/mL)	Spores (MPN/L)	Spore removal efficiency (%)
Bulk milk		15,270 ± 6,263	749 ± 640	451 ± 248	
Vat milk C	1	18,250 ± 18,007	860 ± 965	47 ± 27	88.8 ± 7.6
Vat milk E		4,925 ± 1,350^{ns}	63 ± 71^{ns}	17 ± 9*	95.9 ± 3^{ns}
Bulk milk		85,550 ± 63,598	1,299 ± 1,396	807 ± 605	
Vat milk C	2	72,750 ± 46,949	845 ± 531	85 ± 53	92.3 ± 4.5
Vat milk E		14,913 ± 18,084**	34 ± 36**	16 ± 13**	97.9 ± 0.9*
Bulk milk		28,109 ± 10,102	1,278 ± 2,102	924 ± 623	
Vat milk C	3	48,400 ± 43,887	338 ± 342	80 ± 60	89.5 ± 8.1
Vat milk E		6,133 ± 4,711**	39 ± 50**	13 ± 7***	98.2 ± 1.3**

Statistical analysis was performed by comparing each experimental sample (E) with the respective control (C) within each configuration. Spore removal efficiency is the mean of daily efficiencies. The asterisks indicate the significance levels (*p < 0.05; **p < 0.01; ***p <0.001; t-Test). ns: not significant difference.

Table 4. Effect of centrifugation configurations on kinetic parameters of autochthonous LAB determined by impedance analysis

Vat milk sample	Configuration	Lag	Rate	yEnd
C	1	15.01 ± 3.87	2.54 ±0.63	29.78 ± 1.35
E		17.71 ± 1.61 *	2.01 ± 0.27 *	29.72 ± 2.39 ns
C	2	16.25 ± 1.48	3.65 ± 0.59	27.99 ± 0.53
E		18.79 ± 1.15 ***	3.22 ± 0.61 ns	28.28 ± 0.70 ns
C	3	16.60 ± 1.46	3.62 ± 0.44	28.93 ± 1.04
E		20.35 ± 2.68***	2.56 ± 0.42***	29.22 ± 1.43ns

Statistical analysis was performed comparing the experimental sample (E) with the respective control (C) within each configuration. The asterisks indicate the significance levels (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; t-Test). ns: not significant difference.

512 **Table 5.** Effect of milk centrifugation with different configurations on fat globule characteristics

Vat milk samples	Configuration	Area	Circularity	Diameter
C	1	6.1 ± 7.02	0.94 ± 0.19	3.0 ± 1.51
E		6.55 ± 6.83^{ns}	0.92 ± 0.11^{ns}	2.98 ± 1.57^{ns}
C	2	6.85 ± 5.26	0.92 ± 0.10	3.12 ± 1.36
E		5.38 ± 6.61***	0.89 ± 0.15***	2.62 ± 1.75***
C	3	6.47 ± 6.68	0.96 ± 0.09	3.01 ± 1.56
E		5.75 ± 6.09***	0.95 ± 0.10^{ns}	2.85 ± 1.55***

513

514 Statistical analysis was performed comparing the experimental sample (E) with the respective control
515 (C) within each configuration. The asterisks indicate the significance levels (*$p < 0.05$; ** $p < 0.01$;
516 ***$p < 0.001$; t-Test). ns: not significant difference.

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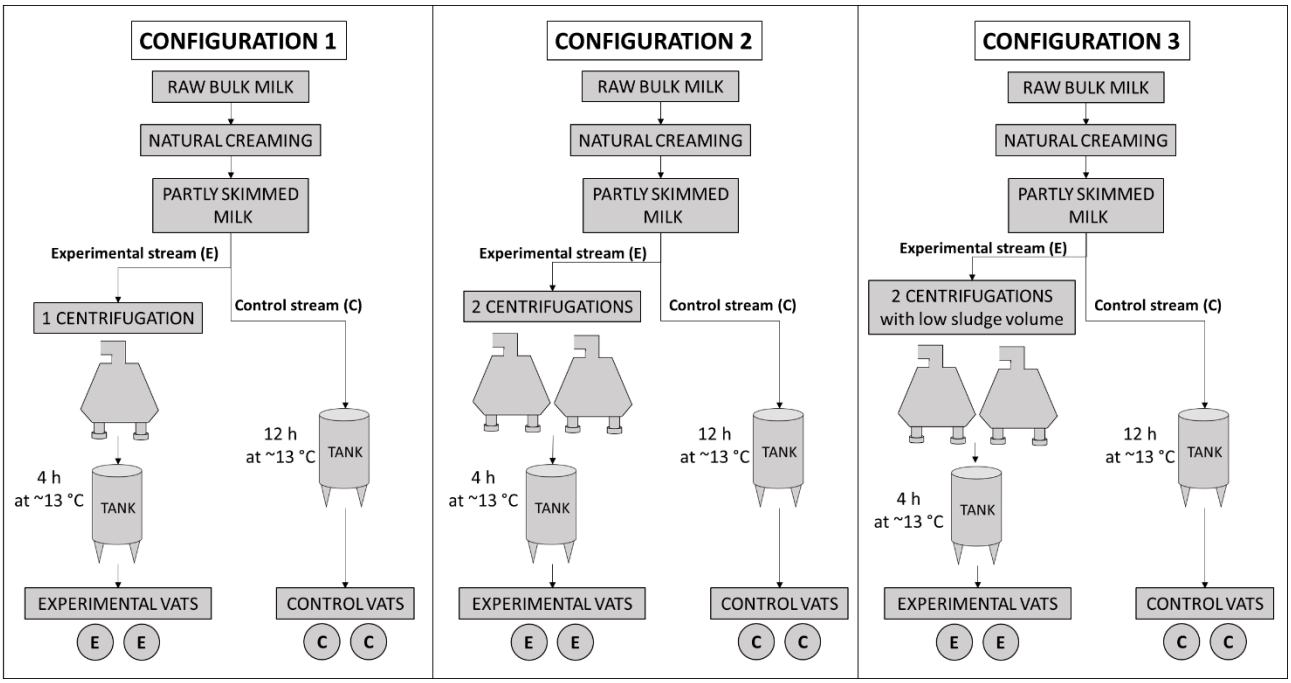
518

519 **Figures**



520

521 **Fig. 1.** Flow diagram showing process steps milk went through from raw milk collection to vat
522 cheese-making for both streams: experimental (E) and control (C). Three centrifugation
523 configurations were tested within the experimental stream: single centrifuge (configuration 1), two
524 centrifuges in series (configuration 2) or two centrifuges in series with reduced volume of the sludge
525 (configuration 3). Control samples were produced within each configuration by using the same raw
526 bulk milk used for the experimental stream.

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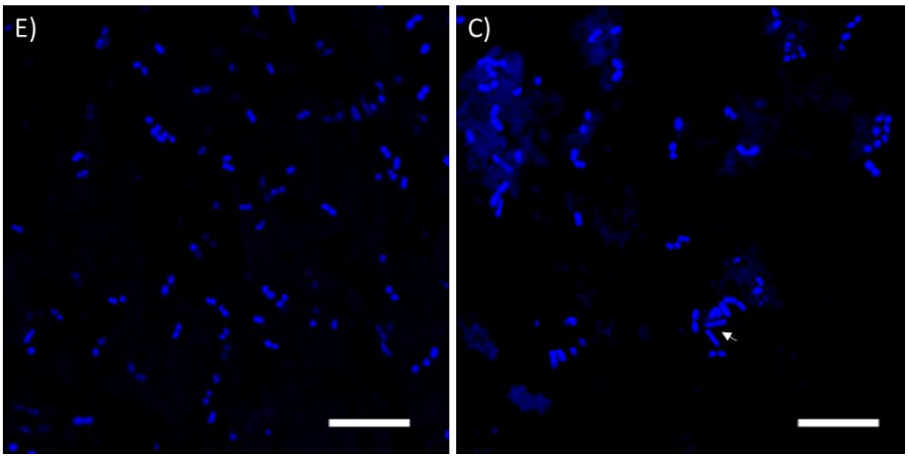


Fig. 2. Confocal laser scanning microscopy of bacteria cells in single centrifuged (E) or control (C) vat milk. Rod-shaped cells were present in the C sample (arrow). Bars are 10 μm in length.

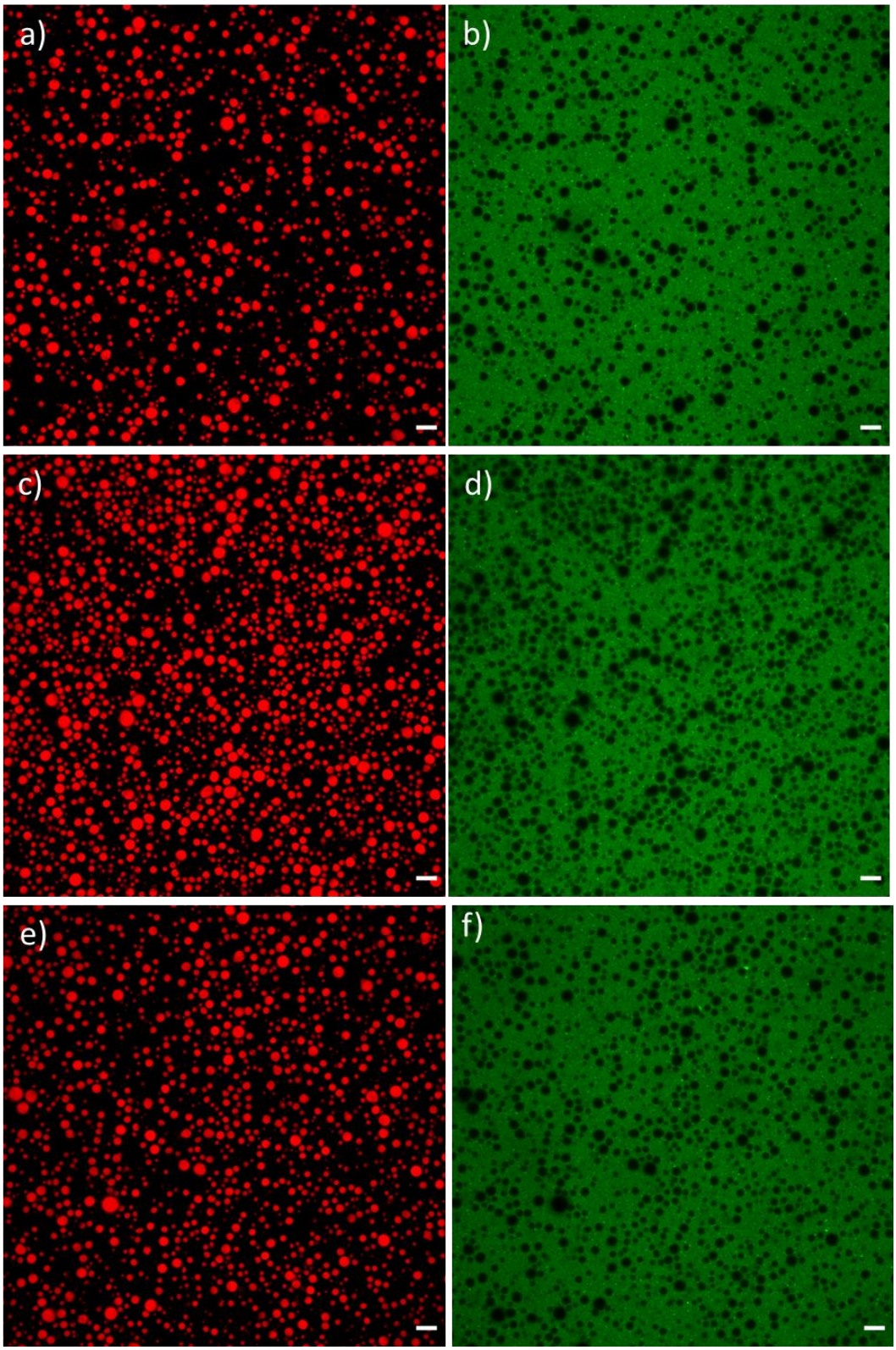
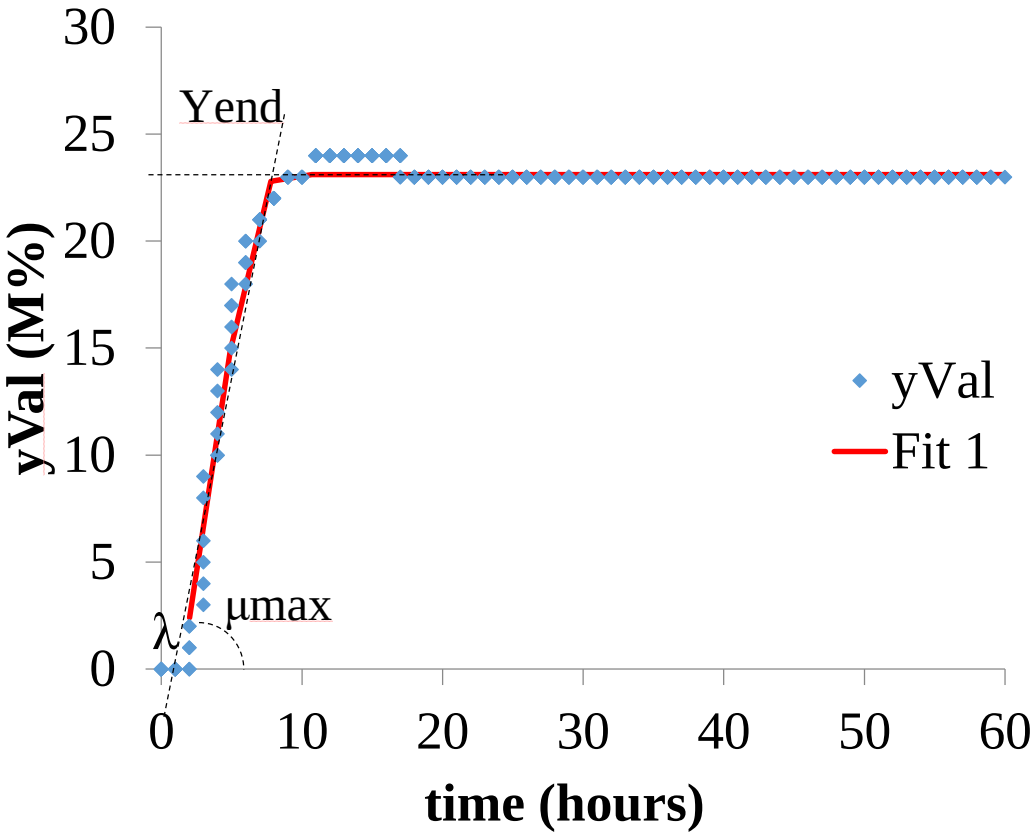


Fig. 3. CLSM images of fat globules (red) and protein (green) of vat milk after double (a, b) or single (c, d) centrifugation; control sample (e, f). Bar is 10 µm in length.



Suppl. File 1. Example of impedance curve fitted to the Modified Gompertz equation (Gibson et al., 1988) using DMfit version 2.1 Excel add-in (http://www.combase.cc/index.php/en/tools). Blue diamond symbols are the y Values that DMfit uses to represent the M% data recorded by the BacTrac4300® during the whole analysis. Red solid line (Fit 1) is the fitted curve described by Modified Gompertz equation. The fitted curve is represented by a sigmoidal curve that well describes the original one. For this reason, three parameters, are easily calculated by the ComBase tool and they are useful to describe the curve: i) lag time (λ), ii) maximum specific M% rate (μmax), and iii) maximum value of M% (Yend). The possibility to fit the original data to the Modified Gompertz equation is tied to the necessity that the two curves overlap.

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