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Novel approach to enumerate clostridial endospores in milk

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ABSTRACT

Clostridial contamination of raw milk causes late-blowing, a severe quality defect in cheese. Consequently, milk containing high numbers of cheese-damaging clostridial spores should not be used for the production of certain types of hard and semi-hard cheese. Currently, there is no officially standardised method available to monitor clostridial spore levels in milk, and major drawbacks like long analysis time, labour intensity, uncertainty of results and insufficient selectivity for clostridia exist for usually used conventional MPN (most probable number) techniques. Therefore, an optimised medium in combination with a semi-automated application for the enumeration of clostridia in milk was developed. The aim of this study was to evaluate this new methodology in comparison with a conventional method (using Bryant and Burkey broth) based on the analysis of 84 milk samples. Method inclusivity was further tested using pure clostridial cultures, and selectivity was assessed by molecular identification of isolates obtained from the new assay. The novel approach proved to be suitable for the detection of clostridia in both suppliers' and processed milk, also indicating that it is superior in selectivity, sensitivity and analysis time compared to conventional techniques.

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1. Introduction

Butyric acid-producing clostridia are undesired contaminants in cheese production due to their ability to cause late-blowing, a severe quality defect in hard and semi-hard cheeses. During the cheese ripening process, anaerobic spore formers of the genus *Clostridium* can convert lactic acid into butyric acid, acetic acid and gases (CO_2 and H_2). As a consequence, contaminated cheeses exhibit not only pronounced deficiencies in structural quality, such as irregular eye formation, slits and cracks but also develop unpleasant rancid off-flavours, which makes them practically unmarketable (Brändle, Domig, & Kneifel, 2016).

Clostridial contaminations in cheese production usually originate from raw milk. Due to the ubiquitous presence in nature and soil in particular, clostridia are found in the stable environment and easily enter the milk during the milking process (Doyle et al., 2015). The implementation of good farming management practices and the avoidance of silage feed can minimise the risk of late-blowing, because clostridial spore levels in animal feed (especially silage) strongly correlate with those found in raw milk (Dasgupta & Hull, 1989; Vissers, Driehuis, Te Giffel, De Jong, & Lankveld, 2007; Zucali et al., 2015). In some areas, silage feed is even prohibited, when the use of raw milk is intended for certain types of cheese (usually registered PDO (protected origin of designation) cheeses) (Ivy & Wiedmann, 2014; Murphy, Martin, Barbano, & Wiedmann, 2016). Furthermore, in some regions or countries such as the Netherlands, for instance, a penalty system has been established to control suppliers' milk, whereas milk is required to contain less than 1000 spores per litre (Heyndrickx, 2011).

To date there is no international standard method to reliably control compliance with these regulations or to determine spore levels of cheese-damaging clostridia in milk. In fact, the enumeration of clostridial endospores in milk poses several challenges limiting the analytical strategies. In particular, a low detection limit of approximately 10^2 clostridial spores per litre milk is required in order to successfully prevent late-blowing (Brändle et al., 2016). Currently, microbiological MPN (most probable number)-based procedures, which indicate clostridial growth and gas production by a raised paraffin or agar plug in parallel tubes, are mainly used for routine monitoring purposes. They provide a method sensitivity

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of about 200–300 spores per litre milk, albeit there is an ongoing debate about whether this limit of detection is low enough. Besides, these conventional methods are extremely time-consuming, labour-intensive, and results can be highly uncertain due to large confidence intervals (Brändle et al., 2017; Driehuis, 2013). Furthermore, facultatively anaerobic spore-formers interfere with the detection of clostridia due to the insufficient medium selectivity of conventional MPN methods (Brändle et al., 2017).

Alternatively, spores can be detected in milk by membrane filtration and subsequent incubation of the membranes on a chromogenic solid medium (Bourgeois, Le Parc, Abgrall, & Cleret, 1984; Reindl, Dzieciol, Hein, Wagner, & Zangerl, 2014). However, this method has not become internationally established, probably due to complex sample pre-treatments and limited filterability of raw milk despite previous enzymatic pre-digestion. The specificity of this method for *Clostridium tyrobutyricum* and the low detection limit of 25 spores L^{-1} are the main advantages of the filtration method (Jakob, 2011). Practical experience, however, showed that the filtration method yields more false negative results than MPN methods using liquid media (Jakob, 2011). This indicates that the inclusion of other butyric acid producing clostridial species than *C. tyrobutyricum* is crucial and that clostridial spore levels may be underestimated when solid media are used.

For the rough estimation of clostridial spore levels in raw milk, combined colour change and gas production in a liquid chromogenic modified RCM medium with a paraffin overlayer has been used in some countries (Christiansson, Ogura, Ekelund, Persson, & von Below, 1995). However, due to the high inoculation volume of 10 mL per tube and the low number of replicates, an accurate quantitative result cannot be obtained, and the determination of higher spore levels is not possible.

In brief, the dairy industry lacks a fast and sensitive method for the selective and reliable quantification of clostridial spores in milk across a broader concentration range. Therefore, the aim of this study was to develop and evaluate a new analytical strategy that overcomes the described obstacles inherent to conventional methods. For this purpose, the following targets were considered: improving the inclusivity and selectivity of the growth medium, reducing the analysis time, obtaining higher sample throughputs, improving the certainty of results and enhancing method sensitivity.

By using a prototype assembly, we evaluated the suitability of the developed method for monitoring of suppliers' and processed milk. Due to the lack of an official reference method, one of the most commonly applied conventional MPN procedures using Bryant and Burkey broth containing resazurin and lactate was selected for comparison. In addition, molecular identity check of collected isolates and a practically oriented application trial were included in the study to investigate the quality of the new method for routine purposes.

2. Materials and methods

2.1. Development of selective medium

2.1.1. Production of clostridial spore cultures

Pure cultures of clostridia were obtained by cultivation in RCM broth (Reinforced Clostridial Medium, Merck; Darmstadt, Germany) at 37 °C under anaerobic conditions. One millilitre of bacterial culture was inoculated into tubes containing 9 mL sterile sporulation broth (modified Brain Heart Infusion with 0.002 $g.L^{-1}$ resazurin sodium salt and 0.05 $g.L^{-1}$ manganese sulphate or RCM broth). After laboratory pasteurisation in a water bath at 80 °C for 15 min, the tubes were incubated in anaerobic jars (using a gas mixture containing 80% N_2, 10% CO_2, and 10% H_2; Don Whitley Scientific; West Yorkshire, UK) for 10 days at 30 °C. After incubation, bacterial cultures were centrifuged at 4 °C and 1300xg for 10 min, washed with 5 mL of sterile deionised water, and centrifuged again under the same conditions. The centrifugation and washing procedure was repeated twice. The resulting spore pellet was then resuspended in sterile water and pasteurised in a water bath at 80 °C for 15 min. Obtained spore suspensions were stored at −20 °C until use.

2.1.2. Optimisation of clostridial growth medium

In preliminary tests, germination and outgrowth of clostridial spores (aqueous suspensions) in various broth recipes were monitored by measuring the change in optical density of cultures over time in a Bioscreen C device (Oy Growth Curves Ab Ltd.; Helsinki, Finland). Due to potential matrix influences of milk, additional media performance tests were conducted using an impedimetric method in a BacTrac 4300 device (SY-LAB; Neupurkersdorf, Austria). In this method, UHT milk (3.5% fat) was spiked with clostridial spore suspensions at different levels and inoculated into different cultivation broths as described by Fontana et al. (2002). Based on the optical density and impedance levels, a chromogenic RCM-based cultivation broth of optimised selectivity for clostridia was developed (AmpMedia666, SY-LAB). A European patent application describing the medium and application for clostridial analysis has been deposited by SY-LAB on October 5th, 2016 (EP 16192488.1).

2.1.3. Inclusivity testing of the new medium

Inclusivity of the developed growth medium was tested by observing germination and outgrowth of 63 clostridial strains. For this purpose, clostridial spores were suspended in UHT milk (dilution 1:100) and inoculated into microwell plates loaded with fourfold-concentrated AmpMedia666 (SY-LAB), reaching a ratio of 3:1 (milk-spore-suspension:medium). Each strain test was performed in triplicate. The microwell plates were incubated anaerobically (85% N_2, 10% CO_2, and 5% H_2) for 72 h at 37 °C and recorded photographically. Growth was indicated by a change in the colour of the cultivation broth from red to yellow.

2.2. Development of the novel method

Twenty-one millilitres of homogenised milk sample (inverted 25 times according to ISO, 2010) were transferred into a sterile falcon tube (50 mL) with a customised filter element (SY-LAB) and pasteurised in a water bath at 80 °C for 20 min. After cooling to approximately 50 °C, 7 mL of fourfold-concentrated AmpMedia666 were added to the milk sample, and the milk-medium mixture was homogenised using a vortex mixer. The following volumes of the mixture were then transferred into a sterile 96-well plate using an AMP-6000®-APS pipetting device (SY-LAB): 32 replicates of 0.32 mL, 32 replicates of 0.16 mL and 32 replicates of 0.08 mL. When a lower detection limit was required, 37.5 mL of homogenised milk sample and 12.3 mL of fourfold-concentrated AmpMedia666 were treated according to the instructions described above. The following volumes of the mixture were then pipetted into a sterile 96-deepwell plate: 32 replicates of 0.8 mL, 32 replicates of 0.4 mL and 32 replicates of 0.2 mL.

The inoculated 96-well plates were closed with a lid or sealed with a gas-permeable foil and incubated in an anaerobic jar (jar gassing system using a gas mixture containing 80% N_2, 10% CO_2, and 10% H_2; Don Whitley Scientific). After incubation at 37 °C for 48 h, the plates were vortexed at 1000 rpm for 1 min using a microplate shaker (Neuation Technologies Pvt. Ltd, Gandhinagar, India). Subsequently, the plates were evaluated using a customised reader (AMP-6000® LabImager TR, SY-LAB). The reader was equipped with

a software, which calculated clostridial spore counts immediately based on the number of positive wells per dilution according to the formula reported by Hurley and Roscoe (1983). A change in the colour of the chromogenic broth from red to yellow indicated a positive result due to clostridial growth, whereas wells without clostridial growth kept the initial red colour.

2.3. Evaluation of the novel method

To verify the performance of the new procedure (N), it was compared with a conventional MPN method using Bryant and Burkey broth containing resazurin and lactate (CNERNA, 1986). This method served for reference purposes in our study and is hereinafter referred to as the BB method. As a practical prerequisite, the operator who performed the analyses for this study, had participated in a proficiency test on clostridial spores in raw milk using the BB method (organised by ACTALIA Cecalait; France) in April and November 2016. The calculated standard deviation of the obtained results from the reference value was below 0.3 log, proving the correct application of the method. The following procedure was used: test tubes were filled with paraffin pellets (solidification point 54–56 °C) and autoclaved at 121 °C for 15 min (resulting in a paraffin layer of approximately 1.5–2 cm in height in each tube). Subsequently, these tubes were filled with 10 mL of sterile Bryant and Burkey broth containing resazurin and lactate (Merck; Darmstadt, Germany). In total, 10 tubes were inoculated as follows: 5 tubes were prepared with 1 mL milk each and 5 tubes with 0.1 mL of milk each according to the recommendations of Jakob (2011). Inoculated tubes were pasteurised for 20 min at 80 °C to kill vegetative cells and to activate the sporulation of endospore-forming bacteria. After adjusting to 37 °C in a water bath, test tubes were incubated at 37 °C for 7 days. A positive reaction was registered by a lifted paraffin plug. MPN counts were calculated according to Hurley and Roscoe (1983) and are expressed as spores L^{-1}.

Fig. 1 illustrates the workflow of the novel method using a chromogenic cultivation broth in the developed semi-automated application compared with the conventional method based on the detection of gas production in Bryant and Burkey broth containing resazurin and lactate.

2.3.1. Milk samples

The method evaluation was performed by simultaneously analysing milk samples using both methods. Seventy-nine raw milk samples from milk tankers and 5 processed milk samples (after bactofugation) were collected from two Austrian dairy companies. Sample aliquots of approximately 500 mL were collected and transported to the laboratory under cooled conditions (below 8 °C). All 84 samples were stored at 4 °C and analysed within 3 days of sampling applying the new method (N) and the conventional method (BB) in parallel. Analyses were performed in duplicate. Additionally, and also in order to collect practical experience with the new method (N), 93 milk samples were collected during different stages of the cheese production chain from a dairy producing Austrian PDO mountain cheese (silage feeding is prohibited when milk is used for the production of this cheese type) and at different time periods. These samples included suppliers' milk ($n = 55$), skim milk ($n = 2$), milk from bulk tanks or milk tankers ($n = 23$), vat milk before rennet coagulation ($n = 9$) and milk collected during milking ($n = 4$). These samples were frozen, transported to the laboratory and stored at −30 °C until analysis. Spore levels in these samples were determined only using the new method (N).

2.3.2. Assessment of selectivity of the new method

From the cultured wells of the semi-automated method, a loop of selected bacterial suspensions from red and yellow cavities was spread onto non-selective RCA (Reinforced Clostridial Agar) and incubated anaerobically (80% N_2, 10% CO_2, and 10% H_2) at 37 °C. Alternatively, 0.1 mL of incubated suspension was pipetted onto RCA and streaked using a drigalski spatula. Single colonies were inoculated into RC broth and incubated anaerobically (for anaerobic conditions see above) at 37 °C for 24–48 h. Subsequently, 1.5 mL of the bacterial culture were centrifuged at 8000x*g* for 6 min at 4 °C. DNA was extracted from the cell pellet using the peqGOLD bacterial DNA Kit (PEQLAB GmbH; Erlangen, Germany). A 1508 bp-sized fragment of the 16S rDNA was amplified using the universal primer pair bak4 and bak11w according to the protocols previously described (Brändle et al., 2017). Subsequently, PCR products were examined on a 2% agarose gel and sequenced by Eurofins Genomics. The obtained sequences were subjected to a BLAST analysis for identification (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3.3. Statistical analysis

In total, data from the comparison tests of 84 milk samples using the two methods (N and BB method; with both methods each milk sample was analysed in duplicate) were considered for statistical analysis. Instead of MPN results >16.000, <180, or <75 spores L^{-1} milk, the values of 16.000, 180 and 75 were used for statistical calculations. After having checked all raw spore count data for plausibility and validity, statistical evaluation was carried out using Bias for Windows, Version 11 (Epsilon Verlag; Hochheim Darmstadt, Germany). Pairwise comparisons of logarithmic values from duplicate determinations were performed to elucidate any significant differences between the methods tested. For this purpose, Bland-Altman's method comparison, a parametric test method, and the Passing Bablok Regression, a non-parametric test method, were used to assess the level of agreement between the new method (N) and the comparison method (BB method). Data correlation was evaluated using the Pearson's correlation coefficient. Differences were considered significant if associated with a p value < 0.05.

3. Results and discussion

3.1. Inclusivity testing of the growth medium

The results of the inclusivity testing are provided in Table 1. Clostridial test strains were able to grow in AmpMedia666 and induced a visible change in colour within 48 h. The results confirmed the detectability of the most relevant butyric acid-producing clostridia associated with late-blowing within less time than conventional methods (3–10 days).

3.2. Agreement of the novel procedure with a conventional method

Statistical analysis showed that the results obtained using the new method (N) are strongly correlated with those obtained using the Bryant and Burkey method (BB) (Pearson's correlation coefficient $r = 0.92$ with $p \ll 0.05$). However, when analysing identical raw milk samples, significantly lower spore levels ($p \ll 0.05$) were obtained using the new method (N). This result is illustrated by the Bland-Altman plot (Fig. 2) indicating a constant systematic effect expressed as mean value of difference of -0.39 log L^{-1} (with a 95% confidence interval between -0.43 and -0.34). The limits of agreement ranged from -1.01 to $+0.24$ log L^{-1}. A slightly positive trend of the regression line was observed at higher mean spore levels, indicating a proportional systematic effect depending on spore levels. Due to the presence of outliers (Grubbs' test for outliers $p < 0.05$, Z-test $p < 0.01$) and because spore count data did not

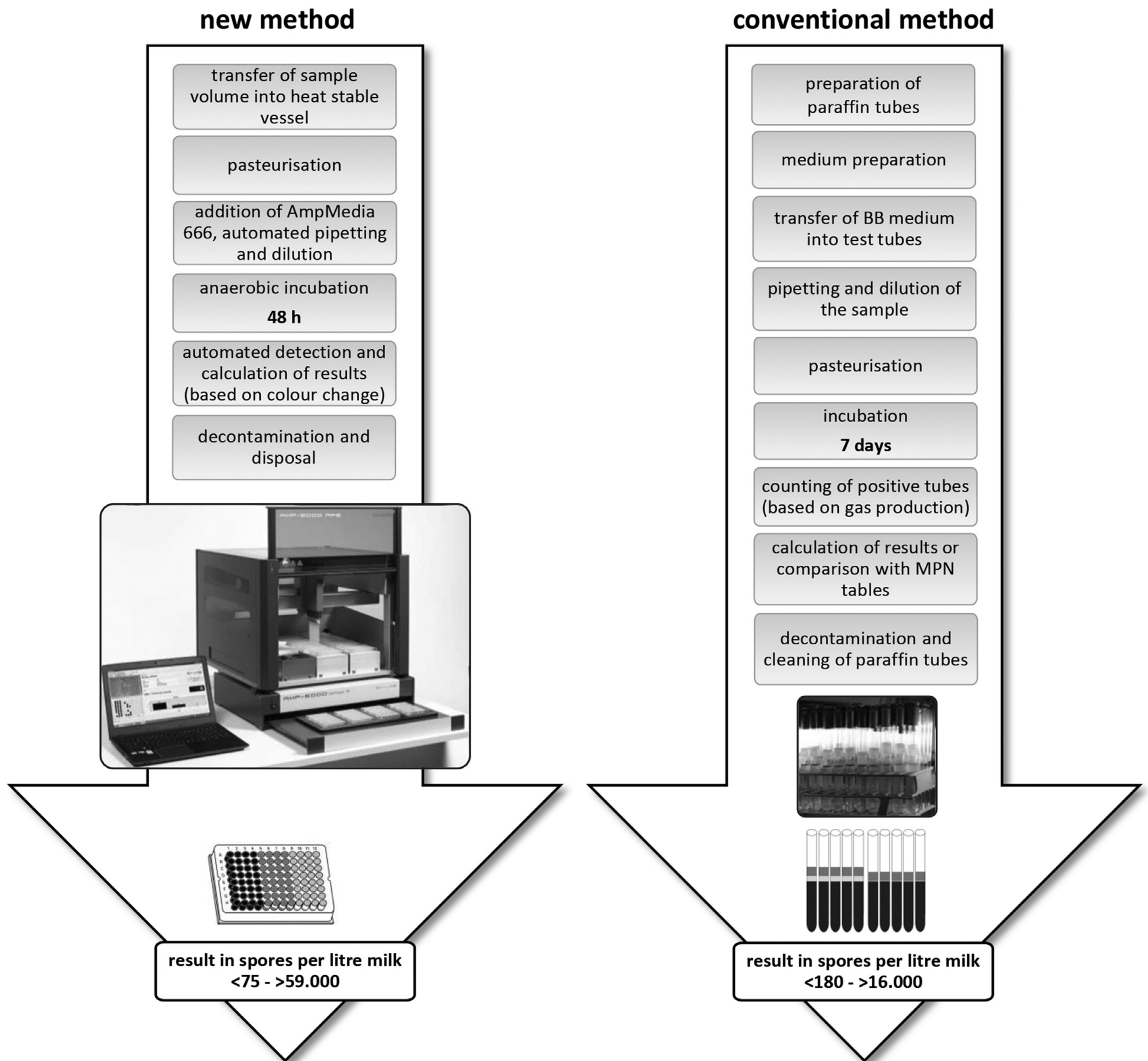


Fig. 1. Comparison between workflows of a novel MPN (most probable number) application (N) and a conventional MPN method (using Bryant and Burkey broth containing resazurin and lactate). The detectable ranges correspond to the following pipetting schemes: new method (N): 32 × 0.24 mL, 32 × 0.12 mL and 32 × 0.06 mL of milk sample, BB method: 5 × 1 mL and 5 × 0.1 mL of milk sample.

always obey a normal distribution, a Passing Bablok regression analysis following a robust, non-parametric model was performed (Fig. 3). The regression equation obtained was $N = -0.54$ (-0.68 to -0.42) + 1.06 (1.02–1.11) x BB (with N and BB representing the spore levels (log L^{-1}) obtained with the new method and the BB method, respectively). This model also yielded a significant constant (-0.5 log L^{-1}) but also a proportional systematic effect between the two methods ($P = 95\%$).

Constant and proportional systematic effects were observed using both parametric and non-parametric tests. The systematic deviation between the new (N) and the conventional method (BB) can be partly explained by different detection principles and methodological differences (details are listed in Table 2 and described below), even though both methods detect the same target (*i.e.* the presence of clostridial spores).

Based on the higher overall sample volume of the new method (N) and the preparation of three dual dilution steps (32 replicates each) instead of two decimal dilution steps (5 replicates each), a more detailed grading of spore levels is achieved. Improved result accuracy is mirrored by smaller confidence intervals of the new method (N) compared to the conventional method (BB).

The lower limit of detection of the new method (N) (LOD: 30 spores L^{-1} (deepwell plates) and 75 spores L^{-1} (standard microwell plates)) enables the observation of variations of spore levels in milk below the LOD of conventional MPN methods (<180–300 spores L^{-1}).

Surprisingly, standard deviations of repeatability (s_r) of both methods were in good agreement (0.20 log L^{-1} for N and BB; s_r was

Table 1
Sensitivity of the new broth AmpMedia666 in the cultivation and detection of clostridial strains (results are based on 3 replicates); + colour change within 48 h; - no colour change within 48 h, ^{T} type strain.

Sample code	Clostridial species	Strain number	Origin	Colour change in AmpMedia666
Cl 37	*C. acetobutylicum*^{T}	LMG 5710; DSM 792		−/+/+
Cl 13	*C. beijerinckii*	IMB 4132 202, NIZO strain 103		+/+/+
Cl 22	*C. beijerinckii*	IMB 4132 2008, Liebefeld-Bern R 1357		+/+/+
Cl 24	*C. beijerinckii*	IMB 4132 2007, Liebefeld-Bern P 1320		+/+/+
Cl 26	*C. beijerinckii*	IMB 4132 2023, Liebefeld-Bern 1453		+/+/+
Cl 34	*C. beijerinckii*	DSM 1820	soil	+/+/+
Cl 36	*C. beijerinckii*^{T}	DSM 791	soil	+/+/+
Cl 40	*C. beijerinckii*	LMG 1219; ATCC 6015		+/+/+
Cl 201	*C. beijerinckii*	IMB 4132 201	draff silage isolate	+/+/+
Cl 2002	*C. beijerinckii*	IMB 4132 2002	grass silage isolate	+/+/+
Cl 2005	*C. beijerinckii*	IMB 4132 2005	grass silage isolate	+/+/+
Cl 2006	*C. beijerinckii*	IMB 4132 2006	grass silage isolate	+/+/+
Cl 2016	*C. beijerinckii*	IMB 4132 2016, Liebefeld-Bern 1548		+/+/+
Cl 31	*C. beijerinckii*	IMB 4132 2014, Liebefeld-Bern 1544		+/−/+
Cl 17	*C. butyricum*	DSM 2477 4P1	wetwood	+/+/+
Cl 18	*C. butyricum*	DSM 2478 MMP3	lake sediment	+/+/+
Cl 19	*C. butyricum*^{T}	DSM 10702; ATCC 19398	pig intestine	+/+/+
Cl 9	*C. sporogenes*^{T}	LMG 8421; ATCC 3584; DSM 795		+/+/+
Cl 12	*C. sporogenes*	ATCC 19404	gas gangrene	+/+/+
Cl 1904	*C. sporogenes*	NCDO 1791, NCIMB 701791	cheese	+/+/+
Cl 2	*C. sporogenes*	8260 University of Graz		+/+/+
M88.2.	*C. sporogenes*		raw milk isolate	+/+/+
Cl 2071	*C. tertium*	NCDO 1802, NCIMB 701802	processed cheese	+/+/+
Cl 2072	*C. tertium*	NCDO 1804, NCIMB 701804	milk	+/+/+
M55.16	*C. amygdalinum*		raw milk isolate	+/+/+
M85.2.	*C. bifermentans*		raw milk isolate	+/+/+
Cl 3	*C. tyrobutyricum*	NCDO 1759, NCIMB 701759	cheddar cheese	+/+/+
Cl 14	*C. tyrobutyricum*	DSM 663	Emmental cheese	+/+/+
Cl 15	*C. tyrobutyricum*	DSM 664	raw milk	+/+/+
Cl 16	*C. tyrobutyricum*	IMB 4132 2020, NIZO strain BZ 15		+/+/+
Cl 20	*C. tyrobutyricum*^{T}	DSM 2637		+/+/+
Cl 25	*C. tyrobutyricum*	IMB 4132 2022		+/+/+
Cl 29	*C. tyrobutyricum*	IMB 4132 2010, Liebefeld-Bern 1520/S2		+/+/+
Cl 33	*C. tyrobutyricum*	IMB 4132 2018, Liebefeld-Bern 1559		+/+/+
Cl 2009	*C. tyrobutyricum*	IMB 4132 2009, Liebefeld-Bern 1520/S1		+/+/+
Cl 2021	*C. tyrobutyricum*	NCDO 1755, NCIMB 701755	cheddar cheese	+/+/+
I 18	*C. tyrobutyricum*		cheese isolate	+/+/+
I 19	*C. tyrobutyricum*		cheese isolate	+/+/+
I 20	*C. tyrobutyricum*		cheese isolate	+/+/+
I 22	*C. tyrobutyricum*		cheese isolate	+/+/+
I 23	*C. tyrobutyricum*		cheese isolate	+/+/+
I 52	*C. tyrobutyricum*		cheese isolate	+/+/+
I 53	*C. tyrobutyricum*		cheese isolate	+/+/+
I 54	*C. tyrobutyricum*		cheese isolate	+/+/+
I 56	*C. tyrobutyricum*		cheese isolate	+/+/+
I 57	*C. tyrobutyricum*		cheese isolate	+/+/+
I 62	*C. tyrobutyricum*		cheese isolate	+/+/+
I 63	*C. tyrobutyricum*		cheese isolate	+/+/+
K 6.5	*C. tyrobutyricum*		cheese isolate	+/+/+
K 8.1	*C. tyrobutyricum*		cheese isolate	+/+/+
K 8.3	*C. tyrobutyricum*		cheese isolate	+/+/+
K 10.3	*C. tyrobutyricum*		cheese isolate	+/+/+
K 11.2	*C. tyrobutyricum*		cheese isolate	+/+/+
K 13.2	*C. tyrobutyricum*		cheese isolate	+/+/+
K 13.5	*C. tyrobutyricum*		cheese isolate	+/+/+
K 16.3	*C. tyrobutyricum*		cheese isolate	+/+/+
K 18.1	*C. tyrobutyricum*		cheese isolate	+/+/+
K 18.2	*C. tyrobutyricum*		cheese isolate	+/+/+
K 25.1	*C. tyrobutyricum*		cheese isolate	+/+/+
K 26.1	*C. tyrobutyricum*		cheese isolate	+/+/+
K 27.1	*C. tyrobutyricum*		cheese isolate	+/+/+
K 27.9	*C. tyrobutyricum*		cheese isolate	+/+/+
K 28.5	*C. tyrobutyricum*		cheese isolate	+/+/+

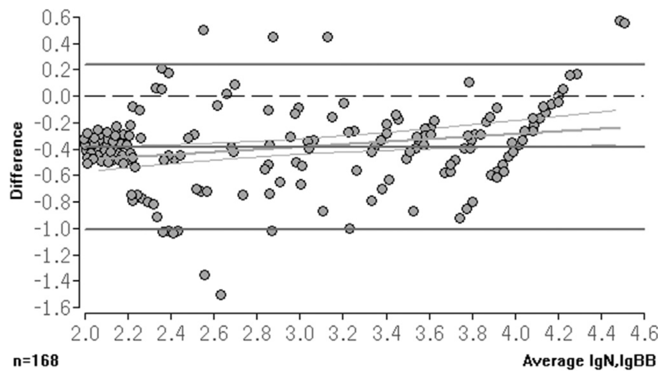


Fig. 2. Bland-Altman plot of the difference in spore levels obtained by the novel most probable number (MPN) method (N) and the conventional MPN method (BB) for the test set (n = 84 samples, 2 replicates per sample). The outer black solid lines represent the 95% confidence limits of agreement. The dashed line represents the identity line (in case of identical results from both methods, the regression line would coincide with the identity line) and the inner solid black line represents the mean difference between the N (new) and BB (Bryant and Burkey) methods. The grey line represents the regression line.

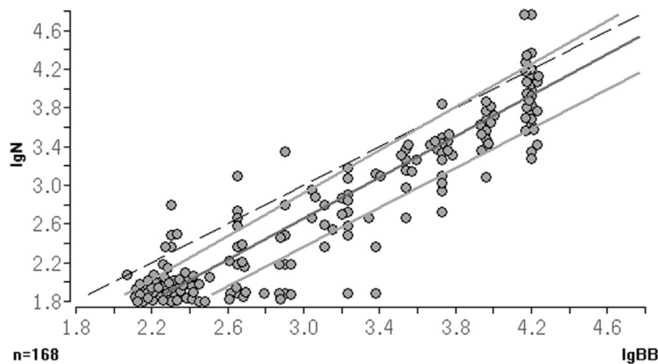


Fig. 3. Passing-Bablok plot of the difference in spore levels obtained by the new most probable number (MPN) method (N) and the conventional MPN method (BB) for the test set (n = 84 samples, 2 replicates per sample). The dashed line represents the identity line; the inner dark grey line represents the regression line.

calculated based on duplicate determinations of 59 samples for N and 62 samples for BB; values at/below the lower detection limit and at/above the upper detection limit had been excluded). Taking into account the methodological characteristics described in more detail above, a higher s_r would be expected for the BB method than for the new method (N). However, the reduced number of possible outcomes due to fewer dilutions and replicates of the BB method probably resulted in many value pairs with a standard deviation of 0 (13/62 pairs for BB and 0/59 pairs for N) leading to an underestimation of s_r. It should be emphasised that the experimentally determined s_r concerns only the data obtained within this study and additional studies using larger datasets would be encouraged to specifically compare standard deviations of repeatability of both methods.

We hypothesise that the generally lower spore counts obtained with the new method (N) result from better selectivity towards clostridia in the new broth, due to the use of selective agents. The proportional effect shows that the impact of improved selectivity of the new broth is even stronger at higher spore levels, probably due to the false inclusion of several other spore-formers in the BB method.

In general, the endospore-forming microbiota of milk not only consists of clostridia but also of facultatively anaerobic genera such as *Bacillus* and *Paenibacillus*. In particular, species of the genus *Bacillus* may interfere with clostridial detection, because germination and growth of bacilli usually proceeds faster than clostridial germination and outgrowth (Xiao, Francke, Abee, & Wells-Bennik, 2011). According to previous findings, more bacilli than clostridia are found in positive MPN tubes containing BB broth (Brändle et al., 2017). Likewise, Driehuis, Hoolwerf, and Rademaker (2016), identified paenibacilli from positive MPN tubes also containing BB broth.

Jonsson (1990) and Christiansson et al. (1995) were able to reduce the interference of bacilli by adding neutral red and D-cycloserine to clostridial culture media. However, the media required an incubation time of 7 and 4 days respectively and to the best of our knowledge, the selectivity of these media has not been verified by means of molecular methods.

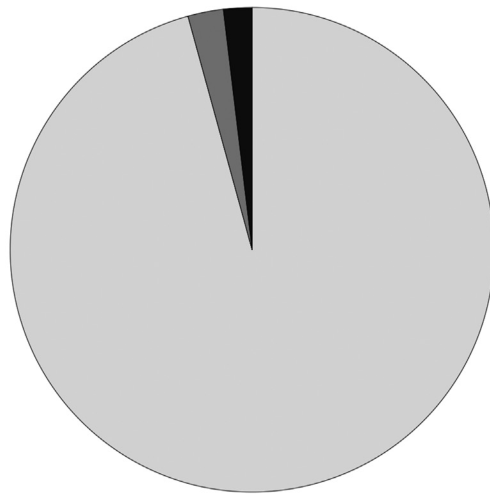
One of the key questions of the present study was whether the improvement of selectivity of the faster, new method (N) was adequate for the sufficiently specific detection of clostridia in raw milk.

3.3. Selectivity of the novel method

To assess the selectivity of the new method (N), 421 isolates derived from 153 milk samples were identified by 16S rDNA sequencing. Most of the isolates were obtained from bacterial suspensions originating from wells with positive reactions, whereas another series of isolates were grown from suspensions from negative reactions. Identified species were grouped into three categories: (1) species belonging to the genus *Clostridium*; (2) species belonging to other spore-forming genera, in particular *Bacillus* and *Paenibacillus*; and (3) non-spore-forming species. Corresponding results are presented in Fig. 4. From all positive reactions, 95.7% were identified as clostridia, 2.4% were assigned to other spore-forming genera, and 1.9% were non-spore-forming species. On the contrary, we were not able to isolate clostridia from wells

Table 2
Major differences between the new (N) and the conventional method (BB) using Bryant and Burkey broth for the enumeration of butyric acid producing clostridia in milk.

	new method	conventional method
medium composition	contains selective agents	contains no selective agents
detection principle	colour change of indicator dye	gas detection using paraffin plugs
sample volume	13.4 mL per microtiter plate; 33.6 mL per deepwell plate	5.5 mL
dilution	3 dual dilution steps	2 decimal dilution steps
replicates per dilution	32	5
confidence intervals (95%)	small microtiter plate 1-0-0; 75 (11–530) deepwell plate 1-0-0; 30 (4.2–210)	large 1-0; 200 (28–1400)
incubation time	48 h	3-10 days



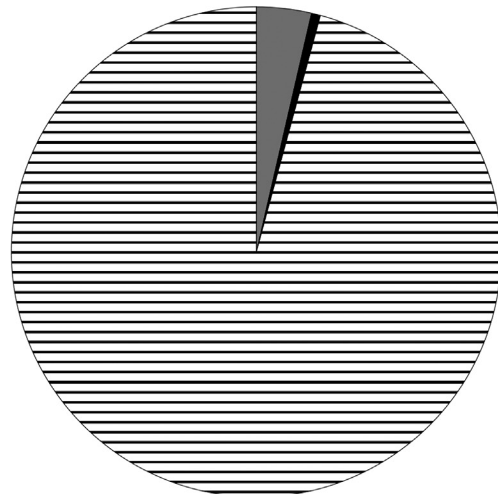


Fig. 4. Relative abundance of clostridial, other endospore-forming and non-spore-forming species, which have been isolated from a) positive reactions (n = 421) and b) negative reactions (n = 166). Classification based on the results of 16S rDNA sequencing. No growth: no colonies were obtained from solid growth medium.

yielding negative reactions. In fact, 95.8% of the microbial suspensions originating from negative wells and thereafter streaked onto solid RCA did not produce any bacterial colonies.

The most abundant species derived from raw milk samples was *C. tyrobutyricum*, representing 77.9% of the isolates of the genus *Clostridium*, followed by *C. sporogenes* (10.9%), *C. bifermentans* (4.0%), *C. subterminale* (2.5%) and *C. perfringens* (1.2%). Additionally, six clostridial species with a relative abundance below 1% were isolated (see Fig. 5). Interestingly, *C. tyrobutyricum* accounted for 93.1% of the isolates obtained from raw milk samples from cows fed with silage, whereas this species only appeared to 19.3% within isolates originating from raw milk produced without silage feeding (Fig. 5).

Advantageously, clostridia could almost exclusively be isolated from positive wells. The high prevalence of *C. tyrobutyricum* in milk from silage fed cows is in agreement with studies of Driehuis et al. (2016) and Bermúdez et al. (2016) referring to cow milk and of Reindl et al. (2014) to goat milk. In another study dealing with ovine milk, however, *C. sporogenes* and *C. perfringens* were predominantly isolated (Arias et al., 2013; Garde, Arias, Gaya, & Nuñez, 2011; Turchi et al., 2016). Within the present study, apart from *C. tyrobutyricum* and *C. sporogenes*, several other clostridial species were detected, in particular in milk from not silage-fed cows. The contribution of those clostridial species to late-blowing defects, however, remains under investigation. Some authors concluded that *C. tyrobutyricum* is the sole causative agent of late-blowing (Jakob, 2011; Klijn, Nieuwenhof, Hoolwerf, Van der Waals, & Weerkamp, 1995), whereas others suggested that several clostridial species play some role in the evolution of such defects (Garde et al., 2011; Gómez-Torres, Garde, Peirotén, & Ávila, 2015; Ingham, Hassler, Tsai, & Ingham, 1998; Le Bourhis et al., 2007). We identified eleven clostridial species, of which the following species already had been isolated from cheese milk or were associated with late-blowing: *C. tyrobutyricum*, *C. sporogenes*, *C. bifermentans*, *C. perfringens*, *C. cadaveris*, *C. sordellii*, and *C. butyricum* (Bassi, Puglisi, & Cocconcelli, 2015; Reindl et al., 2014). To our knowledge, *C. subterminale*, *C. amygdalinum*, *C. argentinense* and *C. aestuarii* have not been isolated from late-blown cheeses yet, but they might play some role in spoilage due to their potential ability to produce butyrate and hydrogen gas (Kim, Jeong, & Chun, 2007; Liou, Balkwill, Drake, & Tanner, 2005; Parshina et al., 2003; Rainey, Hollen, & Small, 2009; Suen, Hatheway, Steigerwalt, & Brenner, 1988). The low relative abundance in the analysed milk samples, however, indicates a limited significance of these species in dairy products. Considering not only species-specific but also strain-dependent variations within costridial metabolism, further research would be required to elucidate the influence of individual clostridial strains on cheese quality.

Based on our findings, we conclude that the new method facilitates the quantification of a broad range of butyric acid clostridia, but primarily detects *C. tyrobutyricum*, thus representing a useful tool for monitoring cheese-damaging spore levels in raw milk.

3.4. Clostridial monitoring in suppliers' milk

To assess the novel method under practical conditions, a series of suppliers' milk samples delivered to a small-scale producer of Austrian PDO mountain cheese were investigated. The production of this cheese type requires the use of raw milk obtained without

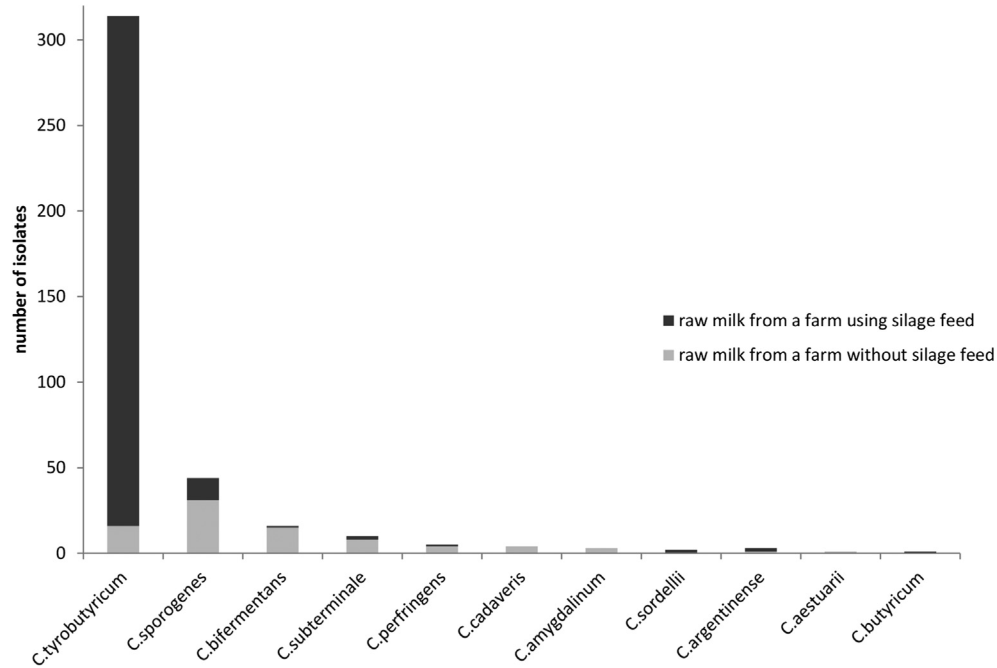


Fig. 5. Number of isolates of respective clostridial species derived from raw milk samples produced with silage feeding (n = 74) or without silage feeding (n = 75). Species identification was conducted by 16S rDNA sequencing.

using silage feed and thus containing low clostridial spore levels. An illustration of the results of this case study is given in Fig. 6. Due to marked spoilage of several lots of hard cheese by late-blowing observed during spring 2016, raw milk samples from six suppliers (A-F; milk produced without using silage feed) were investigated for clostridial spores in July 2016. Low spore counts (<75 L^{-1}) were observed in the milk samples from suppliers A-E, but elevated spore counts were detected in the sample received from supplier F. However, as the detected spore level was still below the threshold value defined based on conventional MPN procedures (the identical sample was analysed in parallel using the BB method, yielding spore counts of <180 L^{-1}), no further action was planned. As a consequence to these observations, a second monitoring series was performed in November and December

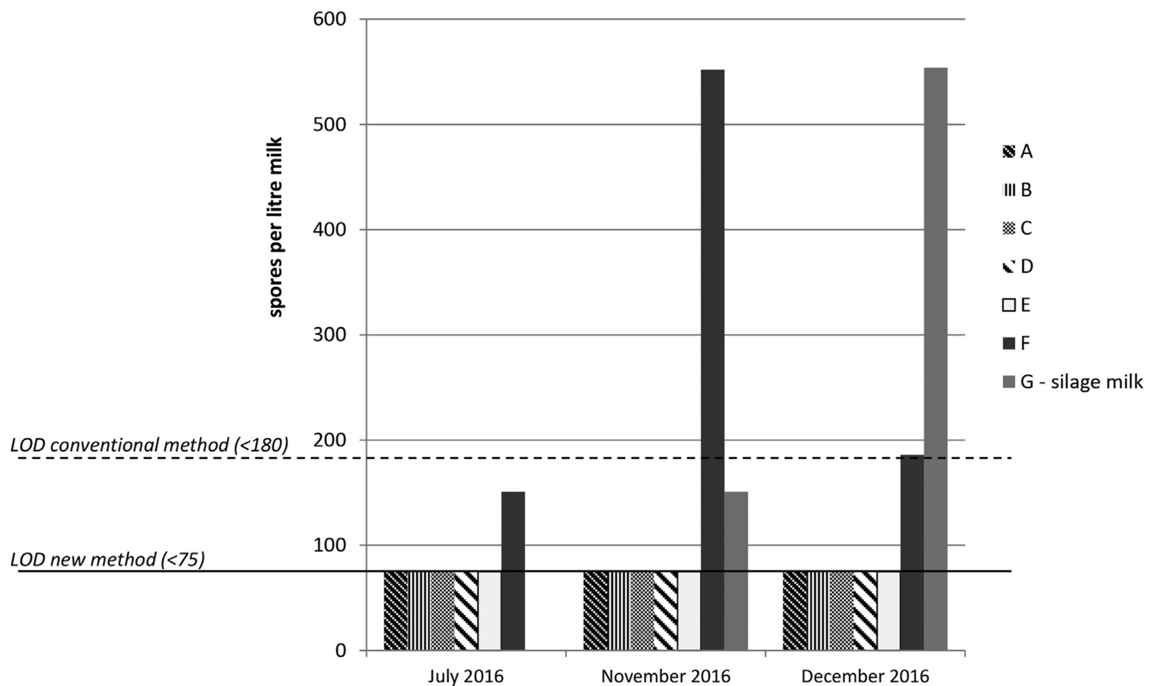


Fig. 6. Spore counts of raw milk samples from suppliers A-F and supplier G (milk sample obtained from silage-fed cows) at different periods and examined using the new method. Horizontal solid line: limit of detection (LOD) of the new method; horizontal dashed line: LOD of the conventional method using Bryant and Burkey broth with resazurin and lactate. For further explanation see text section 3.4.

2016, whereas raw milk samples from silage-fed cows were also included for comparison purposes. With the novel methodology, higher clostridial spore levels in raw milk delivered by supplier F than in the samples from suppliers A-E were observed again. Consequently, advice was given to exclude milk from supplier F from cheese production. Expectedly, also the milk samples from the silage-fed cows showed elevated clostridial spore numbers. Hence, the results obtained prove that the new method (N) is capable of identifying milk samples with elevated spore counts and for monitoring milk for its appropriateness for cheese-making.

4. Conclusion

Based on the elaborated findings, the method presented possesses several advantages. Firstly, it provides for a reliable quantification of clostridial spores in raw milk over a wide concentration range from 75 to 59.000 L^{-1} (30–23.000 spores L^{-1} in a deepwell plate assay). Secondly, the use of higher sample volumes and a higher number of dual dilution steps and replicates as well as enhanced selectivity reduce the uncertainty of single test results. Significantly shorter incubation times and semi-automated pipetting can be further mentioned as important features. Thus, the integration of this technique into microbiological routine assessment of cheese milk constitutes a powerful alternative to using conventional, time-consuming and less sensitive methods to enumerate clostridial spores in milk.

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