October 2022

Enumeration of butyric acid forming (cheese spoiling) clostridia – methodical considerations

Scientific excellence
Industry applicability
Strategic networking
Global influence

Prepared by the experts of the Standing Committee on Harmonisation of Microbiological Methods (SCHMM) – Action Team H26 on Considerations on methods for spore counting of butyric acid forming (cheese spoiling) clostridia.

Concept: Backgrounder

Audience: Expert

Late-blowing in cheese is a significant problem, especially in the manufacture of hard or semi-hard cheeses. Spoilage contributes to wastage in the food chain, decreases efficiency and generates severe economic losses in hard cheese manufacturing. Costs of damage associated with cheese spoiling clostridia have been confirmed to be significant by several IDF National Committees during a combined survey for a new work item proposal to publish an IDF Bulletin dealing with available methods for the enumeration of butyric acid forming clostridia.

Background:

The most severe spoilage defect of hard and semi-hard cheese, so-called late blowing, is caused by undesired microbial activity of butyric acid producing clostridia during cheese ripening. These anaerobic endospore-forming bacteria, above all the species *Clostridium tyrobutyricum*, produce excessive amounts of gasses and organic acids that cause swelling, undesired holes, slits and cracks as well as pronounced off-flavors in cheese (1).

The occurrence of late blowing is to some extent affected by technological parameters, such as salt concentration, pH and ripening conditions. However, the most important influence on hard and semi-hard cheese quality is the raw milk quality. Clostridial endospores are mainly feed born entering raw milk from the barn environment during milking and are not affected by heat treatments like pasteurization due to their thermoduric nature. Thus, for hard and semi-hard cheese producers it is of utmost importance to know the contamination levels of clostridial endospores in the milk that is used for cheese production. Therefore, several countries have integrated the detection and enumeration of clostridial endospores in milk into the routine quality assessment of raw milk. However, due to the lack of internationally standardized detection and enumeration methods, a plethora of different procedures is currently in use. This fact sheet illustrates characteristics of the most important procedures.

The most probable number principle for the detection and enumeration of clostridial endospores in milk.

Only a few or even single clostridial spores per liter raw milk may cause severe spoilage of hard cheese. To reach such low detection and quantification limits, a most probable number (MPN) procedure is suitable (ISO 7218:2014) (1, 2). In general, MPN configurations can be adapted to regulatory requirements and practical considerations. One of the most important parameters in this regard is the number of test tubes or portions as the measurement uncertainty decreases with an increased number of test tubes.

For clostridial enumeration, various MPN procedures including different media formulations are currently in use. A comparison of the most common procedures is provided in Table 1.

Table 1: Characteristics for butyric acid producing clostridia enumeration methods

Method name	**Bryant and Burkey (CNERNA), (3)**	**Dutch Standard (NEN 6877), (4)**	**RCM lactate (VDLUFA M7.18.3.1), (5)**	**AMP-6000 Method (6)**
Abbreviation	**BB**	**NEN**	**RL**	**AMP**
Medium	Bryant and Burkey Broth (with resazurine)	Milk-glucose-lactate medium	Modified Reinforced Clostridial agar (pH 5.4)	Chromogenic AmpMedia 666
Reaction vessel	Glass tubes	Glass tubes	Glass tubes	Microtiter plates, Microtubes
Pasteurisation	75 °C, 10 min	80 °C, 10 min followed by 44-47°C 15 min	75 °C, 10 min	80 °C, 20 min
Anaerobiosis	Paraffin plug	Paraffin plug	Paraffin or agar plug	Anaerobic jar
Incubation	37°C ± 1°C, 7 days	37°C ± 1°C, 96 h ± 4 h	37°C ± 1°C, 3 to 5 days	37°C ± 1°C, 48 h ± 4 h
Evaluation	Gas production leads to lifted paraffin plug	Gas production leads to lifted paraffin plug	Gas production leads to lifted paraffin plug or ruptured agar	Clostridial growth induces colour change of the broth from red to yellow
Automatisation possible	Sample preparation and inoculation	Sample preparation and inoculation	Sample preparation and inoculation	Sample preparation, inoculation and result evaluation
Countries with accredited labs for the respective method	France, Switzerland	Netherlands, Germany	Germany	Austria, Italy, Switzerland
Remarks	• granulated or powdered medium available • no pH adjustment required • incubation with paraffin overlay • low number of replicates tested with routine protocol • (large confidence intervals despite large sample and media volumes) • specificity for *Clostridium spp.* 37% (7)	• inexpensive medium • pH needs to be adjusted • incubation with paraffin overlay • variable number of replicates and volumes tested with routine protocol • short medium shelf life (24 h of milk-glucose-lactate solution) • specificity for *Clostridium spp.* 58% (7)	• granulated or powdered medium available • pH needs to be adjusted • incubation with paraffin overlay • low number of replicates tested with routine protocol • quick inoculation necessary to avoid medium solidification • specificity for *Clostridium spp.* 39% (7)	• ready-to-use medium available • no pH adjustment required • strictly anaerobic incubation in anaerobic jars • quantification of clostridial spores over a wide concentration range • high number of replicates tested with routine protocol • single-use material • highly specific for *Clostridium spp.* > 95% (6)

Other enumeration techniques in use and molecular methods

A colony count based enumeration technique is membrane filtration, mainly used in Switzerland (7). Spores of *Clostridium tyrobutyricum* are quantified after pressure filtration of the pasteurized and prepared raw milk sample on a filter membrane and anaerobic incubation for 3 days at 37 °C in Reinforced Clostridial medium with cycloserine and acid fuchsin (8-11). This filtration method however, cannot be automatised and it is neither applicable for buffalo, sheep or goat milk nor for the analysis of samples, which had been frozen (8).

Molecular methods, such as quantitative PCR or semiquantitative loop-mediated isothermal amplification methods allow the specific detection of *Clostridium tyrobutyricum* (12, 13). These methods reach a lower limit of detection of about 2000 spores/litre milk. Due to the need to detect spore levels down to 100 spores per litre or even less, however, molecular methods currently are not fit to adequately monitor raw milk quality.

Quality payments

MPN Methods described in Table 1 are used in various IDF member conutries to assess an additional parameter for quality payment of milk. As the results are significantly influenced by the method used (1, 7, 8), it is important that spore results as well as the limits applied for quality payment are considered in relation to the method applied for testing.

References

1. Brändle, J., Domig, K. J., & Kneifel, W. (2016). Relevance and analysis of butyric acid producing clostridia in milk and cheese. Food Control, 67, 96-113, https://doi.org/10.1016/j.foodcont.2016.02.038

2. ISO 7218:2014, Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations (ISO 7218:2007 + Amd 1:2013)

3. CNERNA, 1986. Recommandations pour l'estimation de la contamination du lait en spores de Clostridia par la méthode de culture en milieu liquide. Revue Laitière Française, 451, 39–45

4. NEN 6877 (2020) Milk and milk products - Detection of spores of butyric acid bacteria and enumeration of spores of butyric acid bacteria by MPN technique, https://www.nen.nl/en/nen-6877-2020-nl-276199

5. VDLUFA (Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten e.V.), M7.18.3.1 (1996) Bestimmung von Käsereischädlichen Clostridien. Verfahren mit pH-modifiziertem RCM Agar, info@vdlufa.eu

6. Brändle, J., Heinzle, L., Fraberger, V., Berta, J., Zitz, U., Schinkinger, M., Stocker, W., Kneifel, W., & Domig, K. J. (2018). Novel approach to enumerate clostridial endospores in milk. Food Control, 85, 318-326, https://doi.org/10.1016/j.foodcont.2017.10.017

7. Brändle, J., Fraberger, V., Schuller, K., Zitz, U., Kneifel, W. & Domig K.J. (2017). A critical assessment of four most probable number procedures for routine enumeration of cheese-damaging clostridia in milk. International Dairy Journal 73, 109-115, http://dx.doi.org/10.1016/j.idairyj.2017.05.011

8. Jakob, E. & Glauser, D. (2019). Comparaison de méthodes de quantification des bactéries butyriques dans le lait. Recherche Agronomique Suisse 10 (10): 388–395, https://www.agrarforschungschweiz.ch/wp-content/uploads/pdf_archive/2019_10_f_2503.pdf

9. Bourgeois, C.M., Le Parc, O., Abgrall, B., Cleret, J.-J. (1984) Membrane Filtration of milk for counting spores of Clostridium tyrobutyricum. Journal of Dairy Science, 67, 2493-2499

10. Jakob, E. (2011) Analytik rund um die Buttersäuregärung. ALP Forum Nr. 85

11. ALP-Methodenvorgabe (2011) „Quantitative Bestimmung käseschädlicher anaerober Sporen in Milch und Wasser – Membranfiltertechnik mit Selektivmedium" vom 14.12.2011

12. Arnaboldi, S., Benevenia, R., Bertasi, B, Mangeri, L., Tilola, M., Bassi, D.; Cocconcelli,P.S., Stroppa A.& Varisco,G. (2021). Validation of a real-time PCR method on pta gene for Clostridium tyrobutyricum quantification in milk. Food Control, 130 (2021) 108250, https://doi.org/10.1016/j.foodcont.2021.108250

13. Cecere, P., Gatto, F., Cortimiglia, C., Bassi, D., Lucchini, F.,Cocconcelli, P.S., & Pompa, P.P. (2021). Colorimetric Point-of-Care Detection of Clostridium tyrobutyricum Spores in Milk Samples. Biosensors 2021, 11, 293, https://doi.org/10.3390/bios11090293