

Enumeration of
Cheese-spoiling Clostridia
in milk
– manual SBS Tube protocol

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1. INTRODUCTION

The quantitative detection of the spore count of *cheese spoiling clostridia* in raw milk is considered a quality indicator for hard cheese production and is widely used as a milk payment criterium. Since the conventional methods are very complex and time-consuming to carry out and there is no ISO standard for this microbiological parameter, alternative methods which permit a rapid, automated and reliable determination and simplify the documentation are preferably applied.

2. INTENDED USE & SCOPE

The AMP-6000[®] analysis system is a platform for enumeration of *cheese spoiling clostridia* by means of a miniaturized and automated MPN (**M**ost **P**robable **N**umber) method. The scope of the method covers milk and milk products. The method results in quantitative results within 48 ± 2 hours with minimal sample preparation.

The method described in the following section is a very simplified version of the AMP-6000[®] clostridia method with rapid visual evaluation but using the very selective AmpMedia 666 formulation and giving results at least as precise as conventional but more complicate MPN-methods.

3. TEST PRINCIPLE

The method described here allows the determination of the *most probable number of microbes* in the range of 86 to > 3700 spores per liter by examining just 16 replicates of pasteurised milk samples in SBS tube strips

Fig. 1: SBS Tube strip rack with 2 strips a 8 tubes (one assay)



The existing level of contamination results in a specific distribution of positive and negative wells. Using statistical calculation a probable bacterial spore count can be established for any number of possible positive wells. During the evaluation of the individual wells the colour changes (red to yellow) is detected by visual inspection and the *most probable spore count* values can be read from the table below.

4. REQUIRED MATERIALS AND EQUIPMENT

- Sterile serologic pipettes (10 ml and/or 25 ml)
- Tubes for sample pasteurisation (20-50 ml)
- AmpMedia 666 -250 ml bottles (*ready-to-use, 4x concentrate*)
- AmpMedia 666 Additive
- Tube racks with sterile SBS tubes
- Sterile reservoir for 4 samples (25 ml volume)
- Multichannel pipette and pipette with sterile 1 ml tips (not provided by SY-LAB)
- Petrisphere gas system and anaerobic gas mixture or anaerobic atmosphere generation kit
- Anaerobic jar
- Rack for anaerobic jar
- Water bath (turbulent)
- Vortex shaker
- Incubator

AmpMedia 666 and Additive has to be stored at 2–8 °C.

5. PROCEDURE

5.1 Sample preparation (day 1)

- a. Dissolve the content of one AmpMedia 666 Additive vial in 6 ml sterile distilled water. The resuspended additive can be stored at 2-8°C for a maximum of 7 days. For prolonged storage aliquots can be stored at -20° to -80°C.
- b. Add the resuspended additive (6 ml) to 250 ml AmpMedia 666 (= 1 bottle for 62 tests). Medium with additive can be stored refrigerated up to 7 days.
- c. Transfer 12 ml of a homogenized (25x inverted) milk sample to a tube (min 20 ml volume) for pasteurisation and add 4 ml AmpMedia 666 including the additive, mix carefully.
- d. Pasteurise the mixture in a turbulent water bath at 80 ± 1 °C for 20 minutes.
- e. Cool the samples for approx. 10 minutes in a bath with fresh tap water.
- f. After pasteurisation and cooling the homogenized sample / medium mixture is poured directly into an empty cavity of the 30 ml 4-fold sample reservoir.
- g. Using a 8-channel pipette with corresponding sterile 1 ml tips transfer 1 ml sample / medium mixture to each tube of two sets of sterile SBS Tube strips (16 vials in total). *Alternatively the sample can be applied directly from the pasteurisation vial to 2 strips (16 vials) of sterile SBS tubes using a single channel micropipette with 1 ml sterile tips* Close processed strips with a breathable foil seal. The seal is applied best for every multiple of sample (1-6) when glued on the tube strips standing in a tube rack. Using a text marker sample ID could be directly written on the breathable seal. Samples can be separated by cutting the seal with a sharp knife after every second tube rack.
- h. A nox-18 anaerobic jar is loaded with the sample racks and connected to the Petrisphere gas system. By pressing the *Start* button, the evacuation process is started, the pot is evacuated to 400 mbar and filled with a synthetic gas mixture (80 % N₂, 10 % CO₂ and 10 % H₂) (six cycles). After the gassing process has ended, the pot is separated from the gas system and incubated in an incubation cabinet at 37 °C for 48 ± 2 h. The use of a palladium catalyst sachet is strongly recommended. In the nox-18 incubator up to 20 SBS racks or a total of 120 samples can be simultaneously incubated.

Chemical anaerobic gas generation systems can be used instead of using the synthetic gas mixture but it must be ensured that they are sufficient for 18 liters anaerobic atmosphere when using the nox-18 anaerobic jar. The gas volumes need to be considered when using other anaerobic jar systems.

5.2 Sample evaluation (day 3)

- i. After the 48 hrs. incubation, the SBS racks are removed from the anaerobic jar and for every sample set of 2 strips the number of vials with a colour change from red to yellow is counted.

IMPORTANT NOTE:

Any colour change from red to yellow even if just visible on the bottom of a vial has to be registered as a positive signal. In case of any doubt examine the vials from the bottom.

- j. Using the total number of positive (yellow vials) per sample you can easily retrieve the clostridia spore count per liter of milk from the following table.

Table 1: MPN result by positive (yellow) vial(s)

Yellow vials	Spores/Liter	lower 95% CV	upper 95% CV
0	< 86		
1	86	12	610
2	180	44	710
3	280	89	860
4	380	140	1000
5	500	210	1200
6	630	280	1400
7	770	360	1600
8	920	460	1900
9	1100	560	2200
10	1300	690	2500
11	1600	830	2900
12	1800	1000	3400
13	2200	1200	4100
14	2800	1500	5200
15	3700	1900	7300
16	> 3700		

Statistical precision parameters are given as the lower and upper 95% confidence intervals using standard MPN statistics

NOTE:

By increasing the number of SBS strips to be used per sample the lower limit of detection can be lowered as well as the statistical precision of the results increased (see Table 2). You can use any MPN calculator (available as

freeware from the internet) to calculate results when using more replicates or request the MPN-tables from SY-LAB.

Table 2: Possible reduction of detection limits in relation to amount of strips/ tubes used

Quantity used		Det. Limit Spores/ Liter
SBS strips	tubes	
2	16	86
4	32	42
5	48	28
8	64	21
10	80	17
12	96	14

6. EXAMPLES FOR EVALUATION

The number of positive (yellow) SBS tubes is counted after incubation and the corresponding spore count is retrieved from Table 1. Any change to yellow (even just on the bottom of the vial) has to be counted as a positive reaction (Fig.3).

Fig. 2: Examples for evaluation



0 yellow = < 86 spores/liter

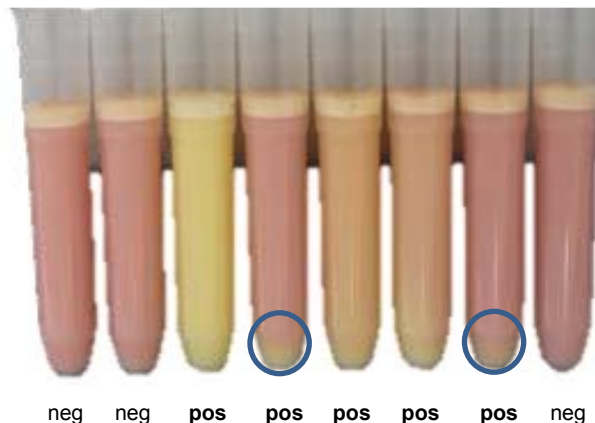


1 yellow = 86 spores/liter



16 yellow = >3700 spores/lite

Fig. 3: Examples for result interpretation



neg neg pos pos pos pos pos neg

7. QUALITY CONTROL

Each batch of medium is checked for functionality and quality using standardised methods and protocols. The release is documented and confirmed with a certificate (quality control certificates can be downloaded at any time at SY-LAB Geräte GmbH website www.sylab.com).

8. SAFETY INFORMATION

Material Safety Data Sheets (MSDS) are available as download on the SY-LAB Geräte GmbH website www.sylab.com for the medium used. The generally accepted safety regulations in the laboratory and the handling of chemicals must also be taken into account and adhered to. Never store medium and/or components of the medium together with food. Always wear gloves, safety goggles and appropriate safety gear when working. Please also take appropriate safety precautions (destruction of the contaminated material by disinfecting and/or autoclaving) to prevent contamination.

9. LIMITED WARRANTY

SY-LAB Geräte GmbH guarantees the functionality and suitability of the product for the intended purpose and in the case of the nutrient medium until the specified expiry date. SY-LAB Geräte GmbH assumes no responsibility whatsoever for consequences (results) or damages of any kind resulting from use. We also reserve the right to modify the product at any time in order to optimise it. Should technical problems occur, you can always contact our technical service (email: supportbio@sylab.com / Tel .: +43 2231 62252-0).