	FOOD SAFETY LAB / MILK QUALITY IMPROVEMENT PROGRAM *Standard Operating Procedure*	

Title: Butyric Acid Bacteria Most Probable Number SOP			
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Name of Protocol

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SECTION 1 INTRODUCTION

1.1 Purpose

The purpose of this document is to set forth standard guidelines for the Butyric Acid Bacteria Most Probable Number method used to detect Clostridium sp. and other butyric acid bacteria in raw milk.

1.2 Scope

This SOP applies to the Milk Quality Improvement Program and the Food Safety Lab. The protocol may also be used by laboratory members from other locations.

1.3 Definitions

Butyric Acid Bacteria (BAB): anaerobic sporeforming bacteria that ferment sugar (lactate) and produce acetic and butyric acids along with carbon dioxide and hydrogen gas.

Most Probable Number (MPN): a statistical method used to estimate the number of bacteria in a sample through a series of dilutions.

1.4 Safety

Wear gloves, safety glasses, and other appropriate personal protective equipment for the entire procedure.

SECTION 2 MATERIALS

- **Molten paraffin wax**
- **Bryant and Burkey agar:** this media should be distributed in 9mL aliquots in 16x150 mm tubes with yellow caps; 20 tubes will be needed for each raw milk sample
- **Water bath capable of reaching and maintaining 75ºC**
- **Water bath capable of reaching and maintaining 63ºC**
- **50mL serological pipettes**
- **1mL pipettor (1000uL) and pipette tips**
- **Aluminum foil**
- **Incubator set to 36ºC**
- **Bunsen burner and striker or lighter**
- **Beakers for molten paraffin wax**

SECTION 3 PROCEDURES

3.1. Preparing Paraffin Wax

3.1.1 Paraffin wax should be autoclaved in beakers on a liquid 20 cycle. Cover the beakers with aluminum foil prior to autoclaving.
   a. There are beakers already "ruined" by wax that should be used. These beakers are located on an end cap shelf on bench 15.

3.2.1 After the autoclave cycle is complete, beakers of wax should be placed in a 63°C water bath, so the wax does not solidify.

3.2. Preparing Bryant and Burkey Media

3.2.1 Bryant and Burkey media should be prepared by the media room following their protocol for preparing the media.
   a. Media should be distributed in 9ml aliquots in 16x150mm tubes with yellow caps prior to autoclaving.

3.2.2 If the media has been prepared in advance, it may need to be boiled again to eliminate oxygen.
   a. If Bryant and Burkey agar is dark/purple/brown at the top (more than 1/3 of the tube), oxygen is present, and the tubes should be placed in a boiling water bath (100°C) for 5-10 minutes until agar is golden/yellow.

3.3. Butyric Acid Bacteria Most Probable Number

3.3.1 For each raw milk sample, arrange tubes filled with 9ml of Bryant and Burkey agar into 2 rows of 10 (20 tubes per sample).

3.3.2 Light a Bunsen burner.
   a. Fill the first row (10 tubes) with 5ml of raw milk sample per tube using a 50ml serological pipette.
   b. Fill the second row (10 tubes) with 0.5ml of raw milk sample per tube using a 1ml pipette.

3.3.3 Cover your bench space with aluminum foil to prevent wax from dripping on your bench.

3.3.4 Pour about 2cm of molten wax from the beakers into each tube.
   a. The paraffin wax solidifies quickly, and it is recommended that all caps for an entire sample are removed at once and that you move quickly.
   b. The beaker of wax should be returned to the water bath when not in use as it will solidify quickly at room temperature.

3.3.5 Once all sample tubes are filled with wax, place them in a 75°C water bath for 15 minutes.

3.3.6 After the heat treatment, incubate the samples at 36°C for 6 days. Check the samples every 2 days and mark the tubes that have gas production as positive.

3.3.7 Use BAB MPN tables to calculate the MPN (attached at the end of this document).

SECTION 4 TROUBLESHOOTING

Problems previously encountered with this test include:

(1) Wax in the water baths: be sure to clean water baths frequently after performing this test since wax will melt in the 75°C water bath and will cause the water bath to look dirty. It is easiest to remove wax from the water bath once the wax solidifies (check the day after performing the SOP).
(2) Sometimes small bubbles appear in or below the wax plug during incubation. Do not mark the tube as positive unless the wax plugged has moved above the level of the liquid in the tube.
(3) Different volumes and dilutions of the sample can be used for the most probable number method, but the tables will have to be recalculated for accurate results.

SECTION 5 REFERENCES

SECTION 6 METHOD VERSION & CHANGES

VERSION	DATE	EDITOR	COMMENTS
Version 1	5/9/24	Rachel	Original SOP
Version 2			
Version 3			

5mL/0.5mL 10-replicate MPN Table

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August 10, 2017

The following MPN table applies to the setup of 10 tubes of appropriate media with 5mL sample each and 10 tubes of appropriate media with 0.5mL each. Results in spores/L.

# positive 5mL tubes	# positive 0.5mL tubes	spores/L
0	0	< 18
0	1	18
0	2	37
0	3	55
0	4	74
0	5	93
0	6	110
0	7	130
0	8	150
0	9	170
0	10	190

# positive 5mL tubes	# positive 0.5mL tubes	spores/L
1	0	19
1	1	38
1	2	58
1	3	78
1	4	97
1	5	120
1	6	140
1	7	160
1	8	180
1	9	200
1	10	220

# positive 5mL tubes	# positive 0.5mL tubes	spores/L
2	0	40
2	1	61
2	2	81
2	3	100
2	4	120
2	5	150
2	6	170
2	7	190
2	8	210
2	9	240
2	10	260

# positive 5mL tubes	# positive 0.5mL tubes	spores/L
3	0	64
3	1	86
3	2	110
3	3	130
3	4	150
3	5	180
3	6	200
3	7	230
3	8	250
3	9	280
3	10	300

# positive 5mL tubes	# positive 0.5mL tubes	spores/L
4	0	90
4	1	110
4	2	140
4	3	160
4	4	190
4	5	210
4	6	240
4	7	270
4	8	300
4	9	320
4	10	350

# positive 5mL tubes	# positive 0.5mL tubes	spores/L
5	0	120
5	1	150
5	2	170
5	3	200
5	4	230
5	5	260
5	6	290
5	7	320
5	8	350
5	9	380
5	10	420

# positive 5mL tubes	# positive 0.5mL tubes	spores/L
6	0	160
6	1	190
6	2	220
6	3	250
6	4	280
6	5	310
6	6	350
6	7	390
6	8	420
6	9	460
6	10	510

# positive 5mL tubes	# positive 0.5mL tubes	spores/L
7	0	200
7	1	240
7	2	270
7	3	310
7	4	350
7	5	390
7	6	430
7	7	480
7	8	530
7	9	580
7	10	630

# positive 5mL tubes	# positive 0.5mL tubes	spores/L
8	0	260
8	1	300
8	2	340
8	3	390
8	4	440
8	5	500
8	6	560
8	7	620
8	8	690
8	9	770
8	10	850

# positive 5mL tubes	# positive 0.5mL tubes	spores/L
9	0	340
9	1	400
9	2	460
9	3	530
9	4	600
9	5	690
9	6	800
9	7	1000
9	8	1100
9	9	1200
9	10	1400

# positive 5mL tubes	# positive 0.5mL tubes	spores/L
10	0	480
10	1	570
10	2	700
10	3	860
10	4	1100
10	5	1400
10	6	1800
10	7	2400
10	8	5000
10	9	5000
10	10	> 5000