

# A Procedure for the Direct Microscopic Count of Bacteria in Non-fat Dry Milk

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## ABSTRACT

Bacteria in non-fat dried milk (NDM) were enumerated by a method involving preliminary solubilization of the milk proteins in 0.015 N NaOH followed by centrifuging, washing in the NaOH, and microscopically examining stained smears. The method was used to enumerate bacteria in samples of NDM obtained from government surplus stocks or from local retail sources. Bacterial counts from surplus NDM ranged from  $4.64 \times 10^5$  to  $2.83 \times 10^6$ /g (the mean and median were, respectively, 6.23 and  $2.84 \times 10^6$ /g). Counts from retail samples ranged from  $4.48 \times 10^5$  to  $2.42 \times 10^7$ /g (mean and median were 5.57 and  $2.85 \times 10^6$ /g). The predominant bacteria in some samples were paired streptococci; other samples contained rod-shaped bacteria, some with identifiable spores. Comparison of this method with the Levowitz-Weber method indicated that it produced fewer artifacts, was applicable to NDM samples containing a wider range of bacteria, and did not require the use of the potentially carcinogenic tetrachloroethane.

The direct microscopic count (DMC) method for bacterial counts of non-fat dry milk (NDM) (1) involves preliminary reconstitution of the NDM in water and examination of stained smears using the Levowitz-Weber modification of the methylene blue stain (5). The DMC method is potentially useful for determining the bacteriological history of milk before dehydration; high numbers of bacteria may be an indication of time or temperature abuse during storage of milk prior to dehydration, or the presence of mastitis-causing organisms may indicate that the milk was not withheld from market for a long enough period after infection. There are however problems with the Levowitz-Weber method; stained milk smears often show artifacts that interfere with the enumeration of bacteria; the stain colors the background a light blue while bacteria are stained a deeper blue, the lack of contrast makes it difficult to visualize them; the smears may lift off the glass slides if extreme care is not exercised during the staining process; the stain is made with tetrachloroethane which, according to the supplier (Aldrich Chemical Co.) is a strongly suspect carcinogen. The purpose of the work described here was to determine numbers of bacteria in NDM from several sources using a different method for their enumeration. The new method was compared to that out-

lined in the manual "Standard Methods for the Examination of Dairy Products" (1).

## MATERIALS AND METHODS

### *Dry milk samples*

Samples of NDM were obtained from local retail establishments or from surplus stocks of the USDA (Agricultural Marketing Service). The latter represented NDM samples processed in the states of South Dakota, Iowa, Michigan, Minnesota, Oklahoma, Missouri, Massachusetts, Wisconsin, Tennessee, New York, and Louisiana.

### *Milk protein solubilization*

Aliquots of NDM samples were added to pre-weighed polycarbonate centrifuge tubes of 40 ml capacity. To these were added 35 ml of 0.015 N NaOH. Closures were placed on the tubes; they were allowed to stand at room temperature for 1 h with occasional mixing to dissolve the milk proteins.

### *Centrifugation*

The solubilized NDM samples were centrifuged at ambient temperature at 3000 g for 10 min; the supernatant fluids were poured off and 10 ml of the 0.015 N NaOH was added followed by a second centrifugation and supernatant removal. The sediment was made up to a volume of 1.00 ml (1.00 g in the preweighed tubes) and were resuspended by mixing with a vortex mixer.

### *Smears and staining*

A pipettor was used to place portions (usually 2.5 or 10  $\mu$ l) of the resuspended sediments on somatic cell counting slides with 1  $\text{cm}^2$  circles. The smears were dried at 35°C and were stained by either methylene blue (0.3% methylene blue in 95% alcohol mixed with an equal volume of 0.01% KOH) or gram stain. Alternatively methylene blue diluted ten-fold with 0.015 N NaOH was used to resuspend the sediments after centrifuging; using this method the smears could be examined immediately after drying with no further staining.

### *Microscopic examination*

A microscope with an oil immersion objective (numerical aperture 1.25) and wide angle 10X oculars was used to examine the films. The field diameter of the objective was determined with a stage micrometer, from this value the microscope factor was calculated (1). Bacterial counts in the NDM samples were determined by multiplying the microscope factor by the average count

of bacteria from 10 fields and multiplying by the dilution. The fields selected for examination began with that nearest one edge of the smear; from this point every third field in a straight line was examined. The tenth field then was near the center of the smear since the field diameter was determined to be 0.20 mm and the smear radius was 5.7 mm. This method was used to insure that representative counts were obtained even in smears that may have dried unevenly (the smears sometimes dried in concentric rings).

## RESULTS AND DISCUSSION

Preliminary studies were made using one centrifugation step and reconstituting in water; these smears were countable but many artifacts were present; resuspending with 0.015 N NaOH largely eliminated this problem while centrifuging a second time after again resuspending with 0.015 N NaOH produced smears with very few artifacts. The concentration of NaOH was very important; if it was too high the milk proteins formed a gel which prevented effective centrifugation of the bacteria and if it was too low not all of the protein was solubilized. The pH of the solutions with 0.40 to 2.50 g NDM was around 10.2 to 10.8. Centrifuging was at ambient temperature to insure that proteins remained in solution. The sediment could be reconstituted to any desired volume although they were usually made up to 1 ml which was adequate for enumerating bacteria in most samples.

The results of the analyses of the surplus dry milk samples are in Table 1. Two levels of reconstituted sediment were used for these tests, 2.5 and 10  $\mu$ l; both gave nearly the same bacterial counts and had comparable standard deviations. In most cases the replicate smears gave similar results although in 2 samples the replicate differed by 10-fold. The lowest numbers of bacteria were in sample L with approximately 5.2 log N/g, while the highest count was in sample D with about 7.5 log N/g. The results with the retail samples were roughly in the same range (Table 2) ranging from 5.5 to 7.4 log N.

Most of the bacteria in NDM are non-viable thus do not readily stain. Stainability depends on the type of stain employed and on the types of bacteria present; heated gram negative bacteria stain very poorly whereas gram positive bacteria, *Streptococcus faecalis* for example, retain stainability even after heating in the culture medium (6). This may be the reason why most of the bacteria seen in 7/11 samples tested were gram positive cocci (Table 3); one sample of surplus NDM had 98% streptococci, another had 86%, while a sample of retail NDM also had a high streptococcus count of 85%. Other NDM samples showed a variety of bacteria including sporeforming rods, branching rods (possibly lactobacilli or actinomycetes), micrococci, cocci in chains, and occasionally very poor staining gram negative rods.

The ability of bacteria to retain stainability during the solubilization and centrifugation procedure was investigated with the results in Table 4. *S. faecalis* suspended in water had the highest recovery and it was readily distinguishable in the smears after treatment; the numbers were highest however when they were suspended in water. *Escherichia coli* and *S. faecalis* were also recognizable in the stained smears. These

TABLE 1. Direct microscopic count of bacteria in surplus NDM.

Sample	$\mu$ l/cm <sup>2</sup>	Smear #1			Smear #2		
		Field count	mean	SD	Field count	mean	SD
A	10	77	51	7.4	73	51	7.4
A	2.5	12	4.9	7.2	22	13	7.4
B	10	63	55	7.3	26	8.2	6.9
B	2.5	16	11	7.3	4.8	3.2	5.8
C	10	1.6	2.4	5.7	1.8	2.1	5.8
D	10	60	37	7.3	107	162	7.5
D	2.5	19	11	7.4	34	12	7.6
E	10	13	6.7	5.8	16	30	6.7
F	10	2.0	2.2	5.3	2.8	1.3	6.0
G	10	21	10	6.0	46	51	7.2
H	10	13	8.4	5.9	7.4	2.5	6.4
I	10	6.1	3.3	5.5	6.2	3.3	6.3
J	10	4.7	3.3	5.5	1.6	1.3	5.7
K	10	8.1	3.1	5.5	7.1	4.0	6.4
L	10	2.1	0.9	5.0	0.8	0.8	5.4

TABLE 2. Direct microscopic count of bacteria in retail NDM.

Sample	Smear	Field count			log N/g
		Mean	SD	log N/g	
R7	1	16	10	6.7	
	2	22	18	6.9	
R8	1	74	33	7.4	
	2	59	28	7.3	
R9	1	5.7	3.7	6.3	
	2	4.4	2.3	6.1	
R10	1	12	12	6.6	
	2	9.5	15	6.5	
R11	1	17	13	6.7	
	2	10	6.8	6.5	
R12	1	0.8	0.8	5.4	
	2	1.3	1.4	5.6	
R13	1	2.8	2.8	6.0	
	2	8.0	6.2	6.4	

TABLE 3. Streptococci in non-fat dry milk.

Sample	Total bacteria		Streptococci	
	Field count	SD	log N/g	% Total
A	52	23	8.6	98
F	33	4	6.4	53
G	64	27	7.7	86
K	38	15	7.5	75
R1	51	19	7.6	85
R2	14	4.3	7.1	61
R3	1.4	1.3	6.0	45
R4	28	6.6	7.4	68
R5	26	6.2	7.3	45
R6a	2.8	1.8	6.3	50
R6b	12	4.6	7.0	48

TABLE 4. Recovery of bacteria by the SC method.

Culture	Menstruum	Field count		log N/g
		Mean	SD	
<i>S. aureus</i>	Water	110	115	7.5
	NaOH	21	12	6.8
	NDM-NaOH	48	14	7.2
<i>E. coli</i>	Water	41	30	7.1
	NaOH	23	9.0	6.9
	NDM-NaOH	13	12	6.8
<i>S. faecalis</i>	Water	9.1	5.1	6.5
	NaOH	22	21	6.8
	NDM-NaOH	16	5.7	6.7
None	NDM-NaOH	4.4	2.6	6.1

bacteria were not of course subjected to dehydration and their appearance in NDM can only be conjectured.

The greater identifiability of bacteria stained by the gram stain is indicated by the results in Table 5, and the effect of sample size on NDM counts is shown in Table 6. The comparability of counts with sample sizes between 0.4 and 2.5 g shows that this method can be adapted for the determination of bacteria in NDM samples of varying bacterial composition.

A comparison was made of this and the Levowitz-Weber methods. The results (Table 7) were done by counting ten fields by each method; comparable NDM dilutions were used for making the smears. Both methods had large standard deviations indicating that the direct microscope method is inherently subject to large variation. The results in this table suggested that the solubilization-centrifugation (SC) method gave higher counts than the Levowitz-Weber, indicating that bacteria were more readily distinguishable by this technique. The significance of the bacterial types found in NDM is not clear since most of the organisms expected to be present i.e. the gram negative psychrophilic bacteria, apparently do not retain stainability after heating (6). Coulter (2) questioned the public health significance of the DMC of NDM. Our studies were not designed to answer this challenge but it is interesting that some samples had such high numbers of streptococci. In one study (4) the numbers of viable streptococci in 47 samples of raw milk ranged from 39 to 5300/ml. If milk containing these concentrations were dried the numbers in the NDM would be 53000/g at most. Some of the samples we tested had 1000x these numbers; of the seven retail samples for example, the lowest number of paired cocci in any sample was  $1.0 \times 10^6$ /g indicating that the milk from which it was made contained at least  $1.0 \times 10^5$ /ml (assuming that all the bacteria retained stainability after the drying process). Heinemann (3) indicated that the relationship of bacteria in raw milk and that in the derived NDM was not always consistent; the increases in bacterial counts in the NDM using the direct microscopic method ranged from 1.9 to 17 fold with an average of 9.1, which was very close to the value expected when milk is spray dried. Our study indicates that more information is needed to determine the significance of these bacteria in NDM.

TABLE 5. Comparison of methylene blue and gram stains for the SC method for bacteria in NDM.

Sample	Stain	Field count		log N/g
		Mean	SD	
R1	Gram	202	82	7.7
	M.B.	59	24	7.2
R2	Gram	68	9.2	7.2
	M.B.	29	4.7	6.9

TABLE 6. Effect of sample size on bacterial counts by the SC method.

Sample wt (g)	Field count		log N/g
	Mean	SD	
0.4	2.6	1.5	6.3
1.0	14	5.1	6.6
2.5	60	16	6.7

TABLE 7. Comparison of Levowitz-Weber and SC methods for the DMC of bacteria in NDM (No. bacterial/field).

Sample	Levowitz-Weber		SC	
	Mean	SD	Mean	SD
R1	16	3.9	29	5.7
R2	6.1	4.7	14	4.3
R3	0.5	0.7	1.4	1.3
R4	4.6	4.5	28	6.6
R5	7.0	4.2	26	6.2
R6a	0.9	1.1	2.8	1.8
R6b	3.4	3.2	12	4.6

The results of these tests indicate that the SC method for the direct microscopic count of bacteria in NDM has a number of advantages over the recommended method (1). The advantages of the SC method are as follows: it is more sensitive since the final suspension used for counting can be concentrated to any desired volume it is relatively free of artifacts; it is easier to enumerate the microorganisms since they are more deeply stained (if the gram stain is used); there is no tendency for the film to lift when staining and the SC method does not require the use of the potentially carcinogenic tetrachlorane. Many of the samples tested by this method showed leukocytes indicating that it may be a useful method for enumerating them; the method might also be useful for determining the quality of the drying process by enumerating heat denatured insoluble milk particles; it possibly could be used to enumerate other particles such as asbestos.

Our results indicate that some NDM samples obtained from surplus government stocks and some from retail sources showed unusually large numbers of streptococci. The results using a solubilization-centrifugation technique indicated that this method had several distinct advantages over the method currently espoused.

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