A NEW MODIFICATION AND APPLICATION OF THE GRAM STAIN

G. J. HUCKER

New York Agricultural Experiment Station, Geneva, New York

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In making microscopical examinations of the quality of milk received at New York state cheese factories a need arose for a stain which would have a greater differential value than methylene blue, and which would be applicable for quantitative as well as qualitative results. The thought of the Gram stain at once suggested itself. While organisms can be classified only into general groups in methylene blue preparations, and no differentiation can be made between desirable and undesirable types for cheese making, the gas forming groups can be readily distinguished from the desirable lactic acid organisms in slides stained by the Gram method.

In developing a modification of the Gram stain which could be used in staining milk smears, the difficulty has been to secure a decolorizing solution which would allow the Gram positive organisms to retain the stain and still remove the color from the milk and the Gram negative types. The following method has proved satisfactory in our work, and is presented with the hope that it will help solve similar difficulties for other investigators.

The stain is as follows:

Gentian violet solution

Anilin oil	3.0 cc.
Alcohol (absolute)	7.0 cc.
Water	90.0 cc.
Shake; filter	
Gentian violet (Grübler)	2.0 grams

Iodine solution

Iodine	1.0 gram
Potassium iodide	2.0 grams
Water	300.0 cc.

Decolorizing solution

Anilin oil (2 parts) / Xylol (1 part)	mixture	5 parts
Alcohol (95 per cent)		95 parts

Counter stain

Bismarck brown	4.5 grams
Water (boiling)	50.0 cc.
Filter	
Alcohol (95 per cent)	30.0 cc.

The milk smears were prepared by the usual Breed method (Breed and Brew, 1916); i.e., depositing 0.01 cc. of milk on a clean glass slide and spreading with a needle over an area of 1 sq. cm. The smears were dried and placed in xylol until the fat was dissolved, removed, drained, and immersed in 95 per cent alcohol for two minutes for fixing. The slides before being allowed to dry were placed in the gentian violet for forty-five seconds, blotted or allowed to drain after removing from the stain, and immersed in Gram's iodine solution for one minute, destained in the anilin-xylol-alcohol solution until no more stain could be removed; and then counterstained in Bismarck brown for forty-five seconds.

Several formulas of gentian violet solution were used but the particular concentration given has yielded the most consistent and satisfactory results. Satisfactory preparations could not be obtained with "Method 1" (commonly known as Stirling modification) of the Report of the Committee on the Descriptive Chart of the Society of American Bacteriologists (Conn et al., 1919) as light blue and green areas were deposited on the slide when such concentrated gentian violet was used. This was especially true of smears prepared from milk which had developed any degree of acidity. This reaction was probably due to the conversion of the gentian violet into closely related dyes in the presence of the acid and the alcohol of the destaining solution. No definite data are available at present on this point. The stains used in all cases were Grübler's.

The addition of the anilin oil and xylol to the destaining alcohol resulted in retarding the action of the solution sufficiently

to allow the Gram positive organisms to retain the stain while the color was removed from the Gram negative bacteria and the background of milk. Hastings, Evans and Hart (1912), in their cheese work used a decolorizing solution of anilin oil one part and xylol two parts. This solution although removing the stain from the Gram negative organisms and the milk, was slow in action and caused the organisms to appear distended and less brilliant in the final preparation. Consistent results could not be obtained using alcohol as a decolorizer as the stain was removed from the Gram positive bacteria before the milk was sufficiently destained. The results obtained with acetone, as a decolorizer, were similar to those where alcohol was used.

With exception of Bismarck brown,[1] no counter stain exhibited sufficient range of affinity between the nucleo-proteins of the cells and the casein of the milk to allow for different intensities of color even if destained. A few successful smears were made where an aqueous solution of safranin was used as a counter-stain, provided the slides were well washed before the application of the safranin. A heavy precipitate will be deposited on the smear if the Bismarck brown is not frequently dissolved and filtered.

The above method has been used for the routine examination of milk samples for an entire season at a cheese factory where all grades of milk were being received, and it proved helpful in eliminating milk which would develop gassy curds. The smears were checked with duplicate samples stained with methylene blue and no appreciable difference in the count could be observed.

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[1] If Bismarck brown stains the background of milk too deeply, slide may be immersed for a few seconds in a weak aqueous solution of acid fuchsin, after counter staining.