Topics

Simple and rapid selective counting of lactobacillus and coccus in yogurt

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As part of the standardization activities in synthetic biomaterials of VAMAS TWA40 in Japan, we have developed a protocol for simple and rapid isolation and collection of lactic acid bacteria from commercial yogurt, and are attempting to standardize the measurement of lactic acid bacteria counts using this protocol. In this topic, the authors introduce their research on a simple and rapid method to selectively count bacillus and coccus among lactic acid bacteria contained in commercial yogurt by combining image analysis and k-means clustering.

1. Introduction

Probiotics are live microorganisms that provide health benefits to the host when ingested, generally by improving or restoring the intestinal microflora. Lactic acid bacteria, the most common probiotic microorganism, is widely found in probiotic products such as yogurt and pickles and has beneficial effects on maintaining intestinal health. To ensure these beneficial health effects, some countries require a certain number of lactic acid bacteria in yogurt.(In Japan, more than 10^7/g,Ministerial Ordinance Concerning Compositional Standards, etc for Milk and Milk Products.)

Common yogurt contains both the bacillus Bulgaricus (Lactobacillus bulgaricus) and the coccus Thermophilus (Streptococcus thermophilus). Probiotics are mainly classified into the genera Lactobacillus and Bifidobacterium, with the bacillus Bulgaricus being the most commonly used lactic acid bacteria.[1)] The minimum amount recommended to be effective as a probiotic is generally reported to be 10^6～10^7 /g. [2)] To ensure microbiological and nutritional quality and product compliance, manufacturers must be able to identify and selectively quantify each strain.

The above selective quantification is generally achieved by colony counting using lactobacillus media with varying NaCl concentration, carbon source, pH, antibiotic content, and incubation temperature.[3)] On the other hand, it has recently been reported that many of the benefits obtained from live lactic acid bacteria as probiotics can also be obtained from dead populations.[4)] Therefore, it is necessary to evaluate the intestinal regulation and health effects of pasteurized yogurt, pasteurized sour milk, and nutritional supplements using the number of dead bacteria as an indicator. However, dead bacteria cannot be detected or counted by the colony count method, so a technique to quickly and easily detect both live and dead bacteria in probiotic products is needed.

This topic describes a study by the authors[5)] on a simple and rapid method for selectively counting coccus and rods among lactic acid bacteria in commercial yogurt, regardless of whether they are alive or dead.

2. Selective counting of lactobacillus and coccus

Using a previously reported method[6)] for collecting only lactic acid bacteria from commercial yogurt without any foreign substances, we found that the shape and brightness of lactic acid bacteria photographed under an optical microscope were factors that could distinguish between coccus and rods (Figure 1).

The obtained microscopic images of lactobacillus are binarized and each lactobacillus is segmented from the background to obtain the average brightness and shape (aspect ratio) of each lactobacillus by image analysis.(ImageJ, https://imagej.nih.gov/ij/)A two-dimensional plot of the mean luminance and aspect ratio of each lactobacillus in the observation area of 147.43 × 110.57 μm^2 shows that bacillus (numbers 8, 34, 36, 44, 45, 49, 54) localized in the upper left corner of the plot, and coccus in the lower right corner, indicating that the lactic acid bacillus and coccus are well classified(Figure 2). However,when the number of lactobacillus in a photograph or the number ofhs taken

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increases, it is very time-consuming to manually count lactob photograp acilli and coccus.

The k-means clustering method is a form of unsupervised machine learning that aims, simply, to separate groups with

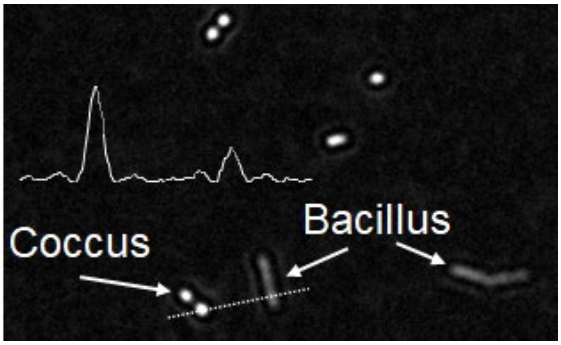

Figure 1. Microscopic images of lactobacillus and coccus in yogurt

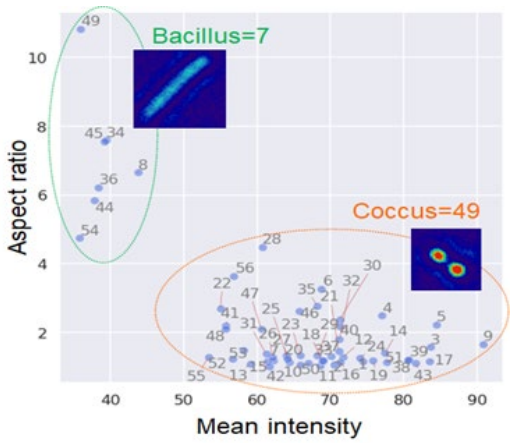

Figure 2. 2-D plot of average luminance vs. aspect ratio for each lactobacillus

similar characteristics and assign them to clusters. To automate the selective counting of bacillus and coccus among the observed lactic acid bacteria, k-means clustering was performed on the plot data set in Figure 2. As a result, the number of bacillus in Figure 2 was 7 and the number of coccus was 49.

The aspect ratio of well-divided coccus is 1, but there are some with an aspect ratio greater than 2 in Figure 2. This is due to the fact that two or more adjacent coccus were recognized as a single coccus during image analysis. The aspect ratios of the coccus recognized as coccus were then converted to integers (rounded down to the nearest whole number) and summed to correct for the total number of coccus (e.g., a coccus with an aspect ratio of 3.5 contains 3 coccus). This resulted in a total bacterial count of 66 in Figure 2. The number of bacillus and coccus counted directly by visual inspection from the original images in Figure 2 was 7 and 60, respectively, and the results after correction for coccus counts showed good agreement. When applied to a larger area ($312.98 \times 239.26\ \mu m^2$), the corrected coccus counts were in good agreement with the visual observations, with an error of approximately 10%.The ratio of bacillus to coccus counted visually and by this method was approximately 1:10, which was consistent with the manufacturer's description.(Bacillus : 1billion/100mg,Coccus:10billion/100,https://www.meiji.com/global/about-us/quality/food-and-nutrition/quality-initiatives/meiji-bulgaria-yogurt/)

This method, including the process of collecting lactic acid bacteria from yogurt, microscopic observation, and image analysis, can be reduced to about 1/30 of the conventional colony counting method. It is also possible to directly observe coccus and bacillus in yogurt, including dead ones, under a microscope. The current problem is that the segmentation of lactobacilli is done manually, and if the segmentation is incomplete, a discrepancy from the actual number of lactobacilli will occur. However, in the future, an optimal algorithm for image segmentation could more accurately count lactobacillus and coccus and be fully automated.

4. Summary

The authors introduced a simple and rapid method for counting lactobacillus and coccus in commercial yogurt using image analysis and k-means clustering. Currently, we are working with members of VAMAS TWA40 in Japan to optimize the protocol for applying this development method to a JIS B 7271-approved portable microbiological observation device.

References

1) M.-R. Van Calsteren, F. Gagnon, J. Nishimura, and S. Makino: Carbohydr. Res., **413**, 115e122 (2015).
2) R. Ashraf and, N. P. Shah: Int. J. Food Microbiol., **149**, 194e208 (2011).
3) A. Talwalkar and K. Kailasapathy: Int. Dairy J., **14**, 143e149 (2004).
4) C. A. Adams: Nutr. Res. Rev., **23**, 37 (2010).
5) H. Nakao, Y. Magariyama: https://doi.org/10.2116/analsci.21P273.
6) H. Nakao, J. -D. Kim: Anal. Sci., **35**, 1065 (2019).