

Efforts to standardize the measurement of the number of lactic acid bacteria in yogurt

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Fermented dairy products utilize microorganisms such as lactic acid bacteria, and are expected to have beneficial effects such as intestinal regulation. Since the number of lactobacilli contained in dairy products is often used as an indicator in their evaluation, it is necessary to collect lactobacilli from fermented dairy products in high yield. In this report, we introduce our recent work on the standardization of lactobacillus count using a protocol developed for the simple and rapid isolation and collection of lactobacilli from commercial yogurt as part of the standardization activities of VAMAS TWA40 for synthetic biomaterials.

1. Introduction

The health-promoting effects of consuming lactic acid bacteria contained in fermented foods such as yogurt have been known for a long time, and live lactic acid bacteria and bifidobacteria, in particular, are called probiotics and are thought to have a positive effect on the body. According to the strict definition of yogurt by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO), "Yogurt is made from milk and lactic acid bacteria, containing Bulgarian and thermophilus bacteria, and produced by their fermentation."¹⁾ According to the Ministerial Ordinance concerning Compositional Standards etc. for Milk and Milk Products in Japan, "fermented milk" (Article 2, Paragraph 40 of the Ministerial Ordinance on Milk, etc.) is defined as containing more than 10 million lactic acid bacteria or yeast cells per 1 ml. ²⁾ Furthermore, yogurt approved as a food for specified health uses (FOSHU) has undergone a review of the scientific basis for its effectiveness and safety in demonstrating physiological functions and specific health functions, and there is a regulation that "100g of the product must contain at least 1 billion Bulgarian bacteria and at least 10 billion Thermophilus bacteria. ³⁾ Therefore, each food manufacturer must check the number of lactobacilli daily by conducting sampling inspections of the finished products.

The general confirmation method described above is to inoculate a certain amount of the finished yogurt into a special agar medium, incubate it at a predetermined

temperature and time, and then count the number of colonies (lactic acid bacteria) that appear on the medium (colony counting). However, this type of test usually takes two to three days, and a faster method of confirmation is desired.

On the other hand, in recent years, although it has become clear that not only viable bacteria but also dead bacteria produce various physiological effects ⁴⁾, it is impossible to culture dead bacteria by colony counting. Therefore, a technique to quickly and easily quantify lactobacilli regardless of whether they are alive or dead is necessary for experimental protocols.

Optical microscopy is a relatively inexpensive instrument that can be used to observe various types of microorganisms, including lactic acid bacteria as small as 1 μm in size. Unlike conventional colony counting, it can visualize viable as well as dead microorganisms quickly and directly on site. Against this background, the Ministry of Economy, Trade and Industry (METI) enacted the Japanese Industrial Standard (JIS) for portable microbiological observers, JIS B 7271, in 2019. This JIS aims to establish a simple inspection tool for microbial observation using smartphones and is expected to be used in a wide range of fields including food processing facilities, medical facilities, restaurants, and educational facilities. Observational instruments certified by JIS are already on the market. It is expected that the use of such an observation device will enable quick and easy quantification of lactobacilli, regardless of whether they are alive or dead, in the field.

Since microorganisms in food samples such as yogurt

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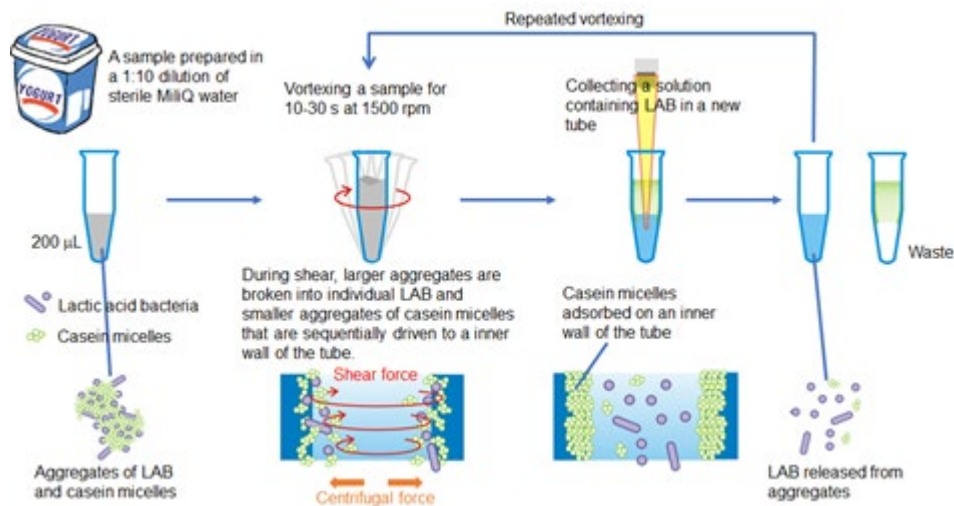


Figure 1. Schematic representation for separating LAB from yogurt samples by vortexing procedures.

coexist with foreign substances, the foreign substances must be removed from the sample during microbiological observation.

In particular, a large amount of casein micelle aggregates coexist with lactobacilli during the fermentation process of yogurt, and this prevents the collection of lactobacilli by centrifugation or dilution.⁵⁾ Casein micelle aggregates are physically pulverized using a homogenizer, etc., and then dissolved by chemical treatment with alkali or surfactant, and lactic acid bacteria can be collected by centrifugation.⁶⁾ However, the lactobacilli collected in such a process will not only be damaged in shape but will also differ greatly from their original characteristics in yogurt due to rapid environmental changes. On the other hand, there is a method to isolate and collect lactic acid bacteria by magnetic microparticles using immunoassay⁷⁾, but the immunoassay kit used is very expensive. A quick, easy, and inexpensive method to collect lactobacilli is desired as a protocol for on-site lactobacilli collection.

In this topic article, we introduce our recent efforts to develop a protocol for easy and rapid isolation and collection of lactobacilli from commercial yogurt and to standardize the measurement of lactobacilli using this protocol.

2. Protocol for the collection of lactic acid bacteria from yogurt

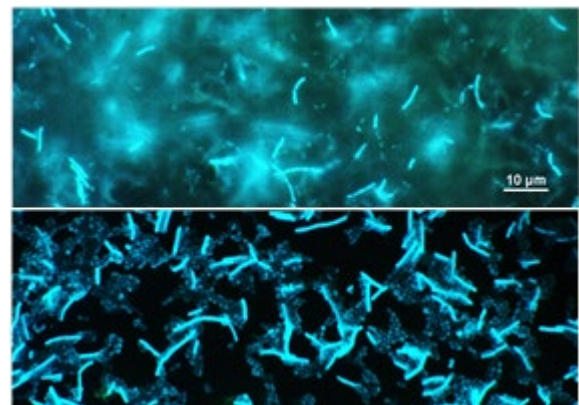


Figure 2. Fluorescence micrographs of yogurt samples before (top) and after (bottom) application of vortex mixer.

The author has recently developed a method for the direct isolation and collection of lactic acid bacteria from commercial yogurt without the use of special mechanical equipment (Fig. 1).⁸⁾

We collected 200 µL of commercial yogurt diluted to 1/10 with sterile water in a 1.5 mL centrifuge tube and agitated the contents with a vortex mixer. The shear force generated by the vortex mixer breaks down the large aggregates into smaller aggregates and isolated lactic acid bacteria, which are continuously driven to the inner tube wall. At this time, the smaller casein micelle aggregates are preferentially

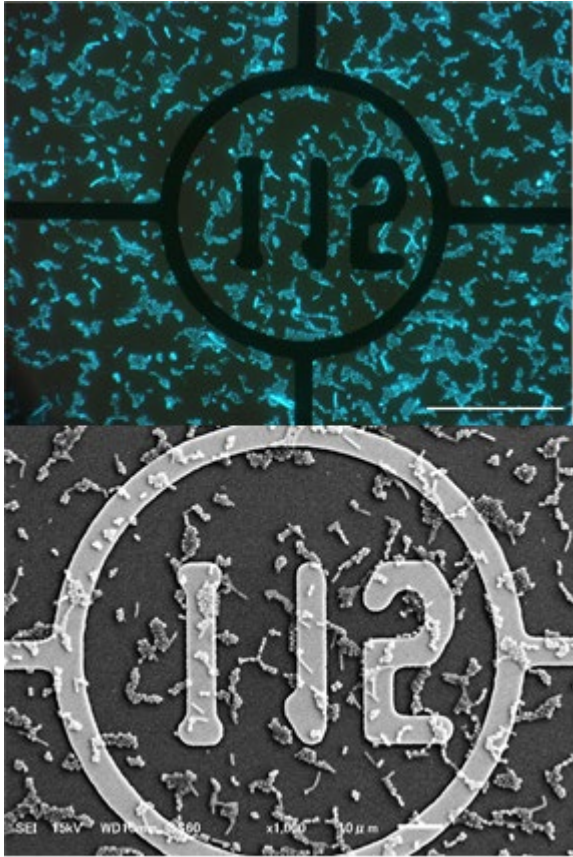


Figure 3. Fluorescence micrograph (top) and electron micrograph (bottom) of the same area of the extracted lactic acid bacteria immobilized on the substrate surface.)

adsorbed onto the inner tube wall, resulting in an internal solution with a reduced amount of contaminants. By repeating this process, an aqueous solution containing only lactobacilli was ultimately obtained, and after three or more processes, an aqueous solution containing only lactobacilli with almost no foreign matter was obtained (Fig. 2). This collection process takes only about 10 minutes and can be completed within 30 minutes to an hour, including the observation procedure using an optical microscope, which is much faster than colony counting that requires 2-3 days.

3. Toward the measurement and standardization of lactobacilli count by optical microscopy

Using the protocol described above, it is possible to directly measure the number of lactic acid bacteria in yogurt.



Figure 4. Bacteria Self-checker "mil-kin"
<https://www.mil-kin.com/product>

1 μ L of the lactobacillus collection solution diluted 1/10 to 1/100 is dropped onto a cover glass substrate and air-dried. By hydrophilizing the surface of the cover glass with a 2mm diameter circle pattern in advance, a sample of lactobacilli dispersed and fixed in a certain area can be obtained (Fig. 3). The center of the sample is photographed at several points to measure the number of lactic acid bacteria. As a result, the number of lactic acid bacteria per mL of yogurt was approximately 650 million. The lactic acid bacteria in the yogurt included rod-shaped Bulgarian bacteria and spherical Thermophilus bacteria, with approximately 63 million and 590 million cells, respectively. The ratio of Bulgarian bacteria to thermophilus bacteria was about 1:10, indicating that the yogurt met the conditions specified in FOSHU.

Considering in-situ measurement in the food industry, the installation and use of conventional biological microscopes for research are somewhat difficult. The above-mentioned portable microbiological observatory approved by JIS B 7271 is small and lightweight at 175 mm \times 113 mm \times 146 mm and weighs 450 g (including the smartphone stand for photography), making it portable (Fig. 4). The power source is a battery, and the installation location is not limited by the power supply. In addition, the image resolution is less than 1 nm, which is sufficient to identify individual lactic acid bacteria. Furthermore, by using a smartphone to take pictures, it is possible to instantly measure the number of lactobacilli in the application and consolidate the measurement data on the cloud. In order to standardize the measurement of the number of lactobacilli in yogurt in the future, we believe that the use of such an observation device is essential.

4. Summary

We have developed a protocol for the simple and rapid isolation and extraction of lactic acid bacteria from commercial yogurt and introduced our recent efforts to standardize the measurement of lactic acid bacteria using this protocol. In the future, we are considering conducting round-robin tests using the developed protocol in collaboration with an observation device manufacturer and a yogurt manufacturer.

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