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Evaluation of method bias for determining bacterial populations in bacterial community analyses

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Various methods are used for analyzing a bacterial community. We recently developed a method for quantifying each bacterium constituting the microbiota by combining a next-generation sequencing (NGS) analysis with a quantitative polymerase chain reaction (NGS-qPCR) assay. Our NGS-qPCR method is useful for analyzing a comprehensive bacterial community because it is enables the easy calculation of the amounts of each bacterium constituting the microbiota. However, it has not been confirmed whether the estimated bacterial community obtained using this NGS-qPCR method corresponds to the results obtained using conventional methods. Accordingly, we prepared model bacterial community samples and analyzed them by several methods (NGS-qPCR, species-specific qPCR, flow cytometry, total direct counting by epifluorescent microscopy [TDC], and plate count). The total bacterial cell densities determined by the PCR-based methods were largely consistent with those determined by the TDC method. There was a difference between the amounts of each bacterium analyzed by NGS-qPCR and species-specific qPCR, although the same trend was shown by both species-specific qPCR and NGS-qPCR. Our findings also demonstrated that there is a strong positive correlation between the cell densities of a specific bacterial group in craft beer samples determined by group-specific qPCR and NGS-qPCR, and there were no significant differences among quantification methods (we tested two bacterial groups: lactic acid bacteria and acetic acid bacteria). Thus, the NGS-qPCR method is a practical method for analyzing a comprehensive bacterial community based on a bacterial cell density.

[**Key words:** Next-generation sequencing-quantitative polymerase chain reaction; Bacterial community analysis; Bias; Relative abundance; Bacterial population; Quantification method]

A comprehensive analysis of bacterial diversity by polymerase chain reaction (PCR)-based methods such as PCR-denaturing gradient gel electrophoresis (DGGE) and next-generation sequencing (NGS) has been performed in various fields of study (e.g., food and beverages, the environment, the human gastrointestinal system) (1–4). These methods enable the detection of bacteria that cannot grow on culture medium, and they can be used to analyze detailed bacterial diversity in a relatively short time. However, a PCR-DGGE analysis or a conventional NGS analysis shows only the abundance ratio of each bacterium, not the population of each bacterium. For example, in an investigation of the variation of bacterial cell density during fermentation or for the determination of the correlation between bacterial cell density and the content of a specific component, quantifying the population of each bacterium that is part of a bacterial community is beneficial for the evaluation of that community.

Various quantification methods have been developed and used for quantifying bacterial cell densities; however each quantification method has its merits and demerits. For culture-dependent analyses, several studies have reported that the majority of environmental bacteria were not detected by known culture media, and a bacterial cell density estimated by a culture-dependent method was influenced by a bias based on the culture (5–7). For a culture-independent analysis, flow cytometry combined with *in situ* hybridization or target-specific qPCR have been used for quantifying a target bacterium, but a specific probe for the *in situ* hybridization or the target-specific primer set should be designed for each target bacterium (8–10). Thus, the comprehensive analysis of a bacterial community by the quantification methods described above is not realistic.

We recently developed a new method that is a combination of an NGS analysis and quantitative PCR (qPCR); it enables researchers to calculate the cell density of each bacterium (11). However, it was reported that comprehensive bacterial community analyses by NGS were influenced by the structure of the bacterial community, sequencing technology, and PCR bias (12–18). Accordingly, we attempted herein to confirm whether an estimated abundance of a bacterial community obtained using our NGS-qPCR method corresponds to the results obtained using conventional methods (e.g., the bacterial cell density of a specific bacterium determined by a plate count or a target-specific qPCR, and the total bacterial cell density determined by the total direct count obtained by epifluorescent microscopy [TDC] or flow cytometry).

In this study, we prepared model bacterial community samples and analyzed the samples by several methods: the new NGS-qPCR method, a species-specific qPCR, flow cytometry, TDC, and plate counting. By analyzing a model bacterial community composed of

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known bacteria, we evaluated the bias of these methods including the NGS-qPCR. An overview of the experimental procedure is shown in Fig. 1. In addition, to evaluate whether the NGS-qPCR method is useful for determining the population of a specific bacterial group in complex bacterial flora, we attempted to determine the densities of lactic acid bacteria (LAB) and acetic acid bacteria (AAB) in craft beer samples (Some LAB and AAB, particularly genus *Lactobacillus* [e.g., *Lactobacillus brevis*, *Lactobacillus lindneri*] and genus *Pediococcus* [e.g., *Pediococcus damnosus*], are typical undesirable contaminants of beer). Our goals in conducting the present study were to evaluate the bias of each method and to assess the practical use of the NGS-qPCR method.

MATERIALS AND METHODS

Strains used The nine bacterial strains used in this study are shown in Table 1. All strains other than *Escherichia coli* DH5α were obtained from the NITE Biological Resource Center (NBRC), Japan. *E. coli* DH5α was purchased from Toyobo (Osaka, Japan).

Sample preparation All of the bacterial strains were precultured two times with each appropriate medium and appropriate conditions described as Table 1, and then 100 μL of preculture medium was inoculated into 10 mL of the main culture medium. The main culture was carried out using the same conditions as those for the preculture. Each bacterial suspension was diluted to 10^6 cells mL^{-1} with phosphate-buffered saline (PBS) based on the result of the flow cytometry analysis (see the Flow cytometry section described below).

We prepared six sample suspensions (A–F) by mixing each bacterial suspension (the mixture ratio described in Table 2). Each bacterial mixture suspension was sampled in triplicate, and triplicate samples were analyzed separately by each method as described below. The samples were stored at −30°C until use; however, the plate culture analysis was performed soon after this preparation protocol, and the samples were stored at 4°C until inoculation.

Thirty seven bottles of commercial craft beer were purchased from the market (Supplementary Table S1).

Flow cytometry We filtrated the samples using a 100-μm cell strainer (BD Falcon, Franklin Lakes, NJ, USA). Each sample (1 mL) was centrifuged at 10,000 × *g* at 4°C for 5 min. The pellet was rinsed with 1 mL of 0.85% saline twice, and then the bacterium was suspended in 100 μL of 0.85% saline containing 5 μM SYTO9 dye (Molecular Probes, Thermo Fisher Scientific, Waltham, MA, USA). After the reaction

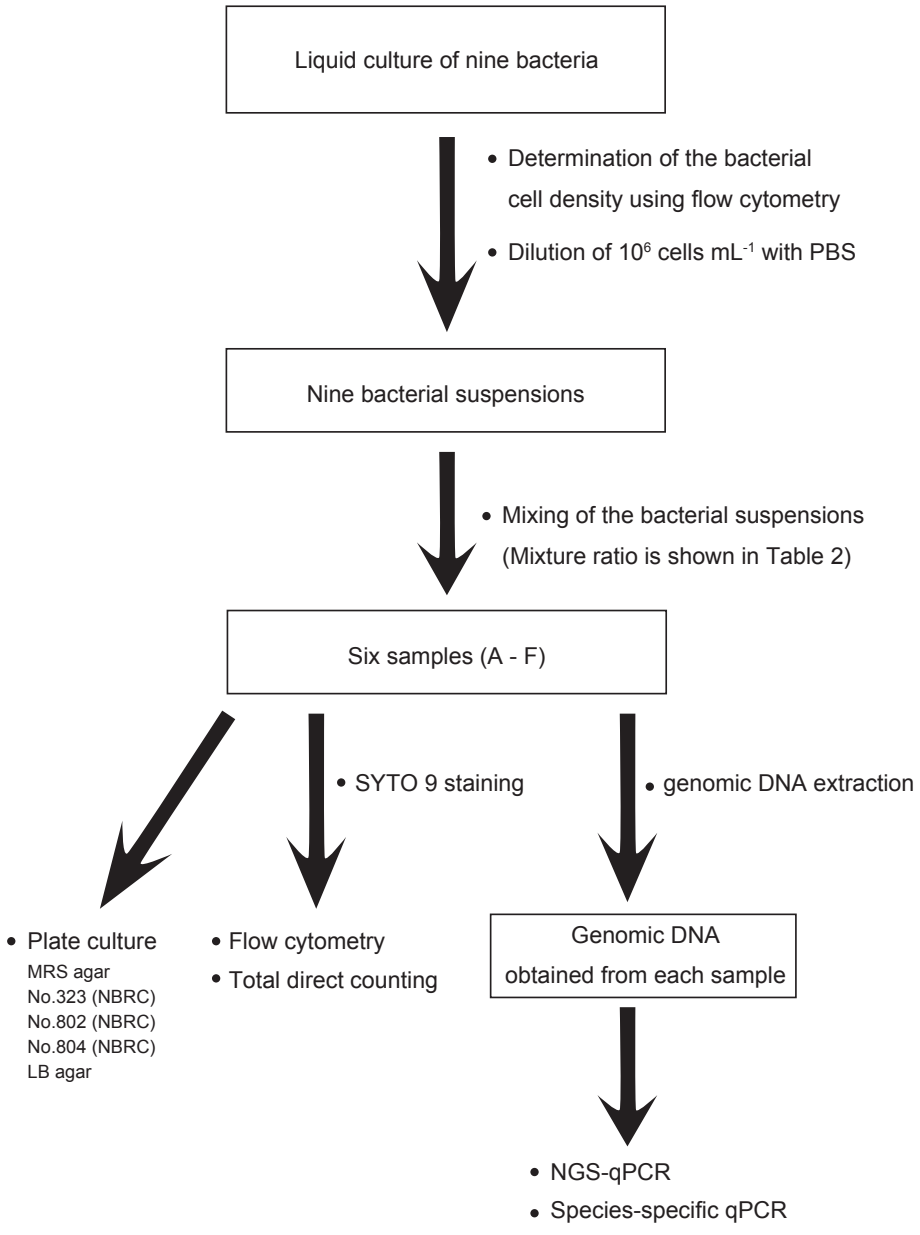

FIG. 1. Overview of experimental procedure.

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