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RESEARCH ARTICLE

Probiotic viability in the gastrointestinal tract in a randomised placebo controlled trial: combining molecular biology and novel cultivation techniques

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Abstract

Understanding the viability of ingested probiotics within the gastrointestinal tract is essential for evaluating their efficacy and deciphering their mechanisms of action. Detecting *Bifidobacterium longum* subspecies *longum* BB536 is particularly challenging owing to its indistinguishability from the naturally abundant *B. longum* species in the human gut. We aimed to address this challenge by developing a selective culture medium for *B. longum* BB536 and employing a propidium monoazide-quantitative polymerase chain reaction (PMA-qPCR) method to verify the survival of the probiotic after consumption. To achieve this, we designed a novel lactose-mupirocin-trimethoprim (LMT) medium that facilitates the cultivation of *B. longum* BB536 under anaerobic conditions at 42 °C. We screened 52 healthy adults and enrolled 39 who met the eligibility criteria. The participants were randomised into two groups, with 34 completing the protocol: 17 received commercial yogurt containing *B. longum* BB536 (9.30 $\log_{10}$ cfu/day) and 17 received a placebo. Prior to the intervention, *B. longum* BB536 was undetectable in all participants. However, following supplementation, LMT culturing identified viable *B. longum* BB536, with average counts of 6.33 ± 0.69 $\log_{10}$ cfu/g on day 3 and 6.16 ± 0.74 $\log_{10}$ cfu/g on day 17. PMA–qPCR further validated these results, showing viable cell counts of 6.09 ± 0.68 $\log_{10}$ cells/g wet faeces on day 3 and 6.44 ± 0.64 $\log_{10}$ cells/g wet faeces on day 17. While each method detected *B. longum* BB536 in some participants where the other did not, no participant tested negative by both methods at any time point. This complementarity between LMT culturing and PMA–qPCR ensures a comprehensive detection strategy, confirming the presence and resilience of *B. longum* BB536 in the gastrointestinal tract and underscoring its potential as a beneficial probiotic strain (UMIN000052110).
Japan Conference of Clinical Research: registration number: BYG2B-01; University Hospital Medical Information Network: study protocol registration UMIN000052110.

Keywords

Bifidobacterium longum BB536 – selective medium – viability – probiotics

1 Introduction

Probiotics are live microorganisms consumed for their health benefits (Sanders *et al.*, 2019). They function by producing metabolites that strengthen the intestinal epithelial barrier and modulate the immune system (Abdulqadir *et al.*, 2023). However, ingested probiotics face numerous environmental stresses, such as digestive enzymes and competition for nutrients from other gut bacteria. Consequently, their survival in an active state until they reach the gut is not guaranteed.

Bifidobacterium longum subsp. *longum* BB536 is a globally researched probiotic strain known for its health benefits, including alleviating constipation, exhibiting anti-allergy effects, and modulating immune functions (Wong *et al.*, 2019). This strain produces metabolites such as acetic acid and lactic acid, which help maintain intestinal integrity and suppress inflammation (Abdulqadir *et al.*, 2023). *B. longum* BB536 also produces indole lactic acids, which exert anti-inflammatory effects on intestinal cells via the aryl hydrocarbon receptor (Sakurai *et al.*, 2019). Additionally, *B. longum* BB536 supports the production of butyric acid through interactions with other gut microbiota (Sugahara *et al.*, 2015). Clinical trials have detected *B. longum* BB536 in faeces using quantitative polymerase chain reaction (qPCR); however, this method does not distinguish between live and dead bacteria (Akatsu *et al.*, 2013; Kondo *et al.*, 2013; Rougé *et al.*, 2010; Tremblay *et al.*, 2021).

To accurately quantify live ingested probiotics, the propidium monoazide (PMA)–qPCR method is employed, which combines bacteria-specific primers with a PMA reagent that binds to the DNA of bacteria with damaged cell membranes, inhibiting PCR amplification (Nocker and Camper, 2006). This method has been widely used to evaluate the viability of probiotics (Fujimoto *et al.*, 2011, 2013; Palaria *et al.*, 2012). However, the PMA-qPCR method determines viability based on cell membrane integrity, neglecting proliferation capacity. Another approach for detecting live probiotics involves culturing on agar plates supplemented with species-specific nutrients and antibiotics, enhancing efficiency. This method, which includes identification by PCR using species- or strain-specific primers, has also been utilised to evaluate the viability of various probiotic strains. Moreover, this method has been used to evaluate the viability of multiple probiotic strains (Christensen *et al.*, 2006; Dommels *et al.*, 2009; Yuki *et al.*, 1999). However, differentiating *B. longum* BB536 from other strains of the same species remains challenging owing to its prevalence in the gut across all age groups (Kato *et al.*, 2017).

Therefore, we aimed to develop a selective culture method for *B. longum* BB536 and evaluate its viability in healthy adults who consumed fermented milk containing the strain. We used the newly developed selective medium and PMA-qPCR methods to provide a comprehensive evaluation of the probiotic's viability.

2 Materials and methods

Selective medium development for bacteria culture

The *B. longum* strains used in this study were obtained from the Japan Collection of Microorganisms (JCM), American Type Culture Collection (ATCC), Morinaga Culture Collection (MCC). These strains were cultured under anaerobic conditions using AnaeroPack Kenki (Mitsubishi Gas Chemical Co., Tokyo, Japan) at 37 °C for 16 h in de Man-Rogosa-Sharpe (MRS) medium (Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with 0.05% (w/v) L-cysteine hydrochloride (Kanto Chemical Co., Tokyo, Japan). Following incubation, the cultures were diluted 10^7 times with saline, and 100 µl of each dilution was plated on lactose–mupirocin–trimethoprim (LMT) agar (Supplementary Table S1) and TOS propionate agar (Yakult Pharmaceutical, Tokyo, Japan) supplemented with equivalent amounts of trimethoprim and mupirocin as in LMT agar. Plates were incubated anaerobically at temperatures ranging from 37 to 42 °C for 3 days.

Detection of live Bifidobacterium longum BB536 using LMT agar and PCR identification

Faecal samples were diluted 20-fold (w/v) with 0.85% saline and cultured on LMT agar at 42 °C for 3 days under anaerobic conditions using the AnaeroPack Kenki. The cultured plates were stored at 4 °C until analysis. For quantification, colonies LMT agar were selected and suspended in 20 µl sterile water. Each suspended colony was treated with 40 µl of InstaGene Matrix (Bio-Rad, Hercules, CA, USA), heated at 99 ° C for 30 min, and centrifuged at 1000×*g* for 5 min at room temperature. PCR analysis was performed using TB Green® Premix Ex Taq™ (Tli RnaseH Plus, Takara, Kyoto, Japan) with specific primers for *B. longum* BB536 (Forward; GAACAGGGTGTGCTGAGTGA, Reverse; CAAGCGAGAAGATCATCGAA, Gianotti *et al.*, 2006) and analysed using an Applied Biosystems 7500/7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Milford, MA, USA). Only the colonies identified as *B. longum* BB536

by strain-specific primers were counted in the quantification. The detection limit was 4.30 $\log_{10}$ cfu/g of wet faeces.

Detection of live Bifidobacterium longum BB536 using PMA–qPCR

A 1-ml sample of the faecal suspension was mixed with 5 µl of 20 mM PMAxx reagent (Biotium, Fremont, CA, USA) and incubated in the dark on ice for 10 min. Subsequently, 800 µl of the PMA-treated suspension was exposed to blue LED light for 15 min using the PhAST Blue photoactivation system (GenIUL, Barcelona, Spain). Following photoactivation, 400 µl of the suspension was centrifuged at 10,000×*g* for 5 min at 4 °C. The pellet was stored at –80 °C until DNA extraction, which was performed using the bead-beating method, as previously described (Murakami *et al.*, 2021). Quantitative PCR was conducted in two technical replicates as described previously, with a detection limit of 4.80 $\log_{10}$ cells/g of wet faeces.

Comparative validation of LMT culture and PMA-qPCR techniques for assessing viable Bifidobacterium longum BB536 spiked into faecal suspension

The *B. longum* BB536 culture medium, as described above, was centrifuged at 4,000×*g* for 5 min at room temperature. The resulting bacterial pellet was resuspended and diluted saline. The bacterial solution was then incorporated into the faecal suspension, leading to a 20-fold (w/v) dilution of the faecal matter in saline. The count and viability of *B. longum* BB536 introduced into the faecal suspension were then determined by using both LMT agar medium and the PMA-qPCR technique.

Clinical trial design

A clinical trial was conducted to investigate the effect of *B. longum* BB536 on the safety and intestinal environment of healthy adults, with the viability of *B. longum* BB536 being evaluated as the efficacy outcome. This randomised, placebo-controlled, double-blind trial was conducted at the Matsumoto health laboratory in Nagano, Japan, from September 2023 to December 2023. The approval of this clinical trial was obtained from the Ethics Committee of Japan Conference of Clinical Research (registration number: BYG2B-01, date of board: August 17, 2023). The study protocol was registered with the University Hospital Medical Information Network (UMIN000052110). The study was conducted in accordance with the Declaration of Helsinki (Fortaleza, revised in 2013) and the Ethical Guidelines for Life Science and Medical Research Involving Human Subjects (Ministry of Education, Culture, Sports, Science and Technology, Ministry of Health, Labour and Welfare, and Ministry of Economy, Trade and Industry Notification No. 1, 2021). Prior to study commencement, all participants provided written informed consent after a thorough explanation of the procedures.

The trial consisted of a 1-week pre-observation period followed by a 17-day intervention period. Participants were randomly assigned to one of two groups: a placebo or *B. longum* BB536 group. The sample size were calculated by taking into consideration the maximum number of cases that could be performed at the institution.

They were randomly divided 1:1 into two groups using stratified randomisation by an independent investigator. The allocation sequence was sealed to both participants and investigators until the end of the study, and the database was locked. Throughout the intervention period, participants consumed their assigned products once daily after meals. Faecal samples were collected on days 0, 3, and 17 following the protocol outlined in the sample collection instructions.

Participants

Healthy adults, aged 18–64 years old, were recruited. The exclusion criteria included: (1) regular use of medications affecting gut microbiota; (2) inability to discontinue consumption of foods or supplements containing lactic acid bacteria, bifidobacteria, or oligosaccharides; (3) presence of hepatic, renal, cardiac, pulmonary, gastrointestinal, hematologic, endocrine, or metabolic diseases, or a significant history thereof; (4) severe drug or food allergies; (5) lactose intolerance or milk allergy; (6) pregnancy, lactation, or likelihood of pregnancy; (7) participation in other clinical trials; and (8) any conditions deemed by the investigator as inappropriate for participation. Prior to study commencement, participants completed dietary records and healthcare questionnaires to assist in screening. Only those meeting all criteria were enrolled. Throughout the pre-observation and intervention periods, participants maintained diaries to document subjective symptoms, bowel movements, and dietary intake.

Supplementation

The intervention involved a commercial fermented milk containing 20 million cfu/g of *B. longum* BB536. The placebo was identical in composition except for the absence of *B. longum* BB536. These test foods were provided by Morinaga Milk Industry (Tokyo, Japan). The

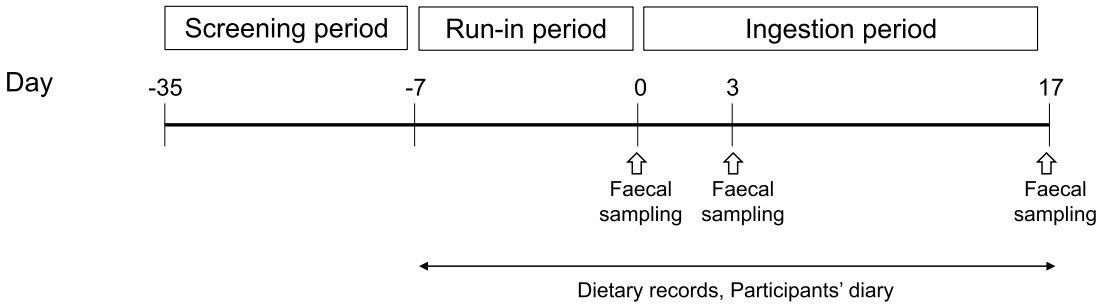

FIGURE 1 Clinical trial schedule.

taste, smell, and appearance of the test foods were confirmed to be indistinguishable by an independent investigator. Participants consumed 100 g daily from 400-g packages, which were refrigerated and replaced every 2-3 days. Compliance was monitored by checking the remaining quantity of fermented milk and diary entries.

Sample collection

Faecal sampling was performed as previously described with slight modification (Tamura *et al.*, 2017). Briefly, faecal samples were collected on days 0, 3, and 17 (Figure 1) using Raku-Ryu cups to prevent contamination of urine and other toilet bowl contents. Samples were immediately transferred to a faecal collection tube type-C (Asiakizai, Tokyo, Japan) and enclosed in the AneroPouch Kenki (Mitsubishi Gas Chemical Co.). They were stored in a cold container (4-8 °C) and transported to the laboratory within 24 h.

Statistical analysis

The safety analysis set (SAF), which was defined as all randomly assigned participants who consumed the test food, was used to compare baseline characteristics and evaluate the safety of the test food. Per protocol set (PPS) analysis, which was defined as all randomly assigned participants who consumed the test food for more than 80% of the test period, was also performed to evaluate the viability of ingested *B. longum* BB536. Baseline characteristics are shown as the mean ± standard errors of the mean. Baseline characteristics at screening were analysed using Student's t-tests for age and BMI between groups, and a chi-squared test for sex. Regarding safety assessment, the incidence rate (frequency; number of participants with confirmed adverse events/total number of participants) were calculated for each group. Differences in incidence rates between the groups were tested by Fisher's exact test. Tolerance of the time to submit samples was set to ± 1 days. When data were missing, they were treated as missing values. The statistical analyses of Student's t-test and chi-squared test were conducted using IBM SPSS Statistics (ver. 24.0; Armonk, NY, USA), and that of Fisher's exact test was conducted using R (ver. 4.3.2). *P*-value of <0.05 was considered statistically significant.

3 Results

Development of BB536 selective medium

Initially, our objective was to assess the viability of *B. longum* BB536 in the intestinal tract post-ingestion as probiotics. However, using the TOS propionate medium, a common selective medium for bifidobacteria, incubation at any temperature between 37 to 42 °C did not result in the selective cultivation of *B. longum* BB536 (Table 1). To address this, we developed a specific culture medium for *B. longum* BB536 by modifying the existing TOS propionate medium. We termed this modified medium with two added antibiotics as modified TOS (mTOS). Our modification involved the addition of mupirocin, an antibiotic effective against a wide range of gram-positive bacteria, which does not affect bifidobacteria. Thus, it was added to suppress the growth of lactic acid bacteria (Bunesova *et al.*, 2015; Serafini *et al.*, 2011). Additionally, our previous research indicated that *B. longum* BB536 has relatively high resistance to trimethoprim compared with other *Bifidobacterium* strains (Xiao *et al.*, 2010). Based on these findings, we evaluated the efficacy of these antibiotics in enhancing the selective growth of *B. longum* BB536 in the mTOS medium. However, not only *B. longum* BB536, but also other *B. longum* strains could thrive in the mTOS medium (Figure 1). Therefore, we replaced galactooligosaccaride in the mTOS medium with lactose, which resulted in exclusive growth of *B. longum* BB536 only at 42 °C (Table 1, Figure 2). Furthermore, the growth of *B. longum* BB536 was not impaired in the LMT medium compared to the unmodified TOS propionate medium. Subsequently, the use of LMT medium

TABLE 1 Colony forming ability of *Bifidobacterium longum* subsp. *longum* BB536 and JCM1217^T in TOS and LMT medium.[1]

Medium	Strains	37 °C	40 °C	41 °C	42 °C
TOS	JCM1217^T	8.97 ± 0.17	9.19 ± 0.09	9.08 ± 0.07	9.05 ± 0.005
	BB536	9.28 ± 0.07	9.31 ± 0.07	9.30 ± 0.04	9.26 ± 0.07
LMT	JCM1217^T	7.95 (2/3)	7.30 (1/3)	7.45 (2/3)	ND
	BB536	9.30 ± 0.03	9.29 ± 0.04	9.17 ± 0.03	9.25 ± 0.05

1 Average ± standard deviation of Log_{10} cfu/ml values (n = 3) are shown. ND = not detected in all triplicated samples. If there were not detected samples, the average value with the number of detected samples described in at 42 °C () were shown.

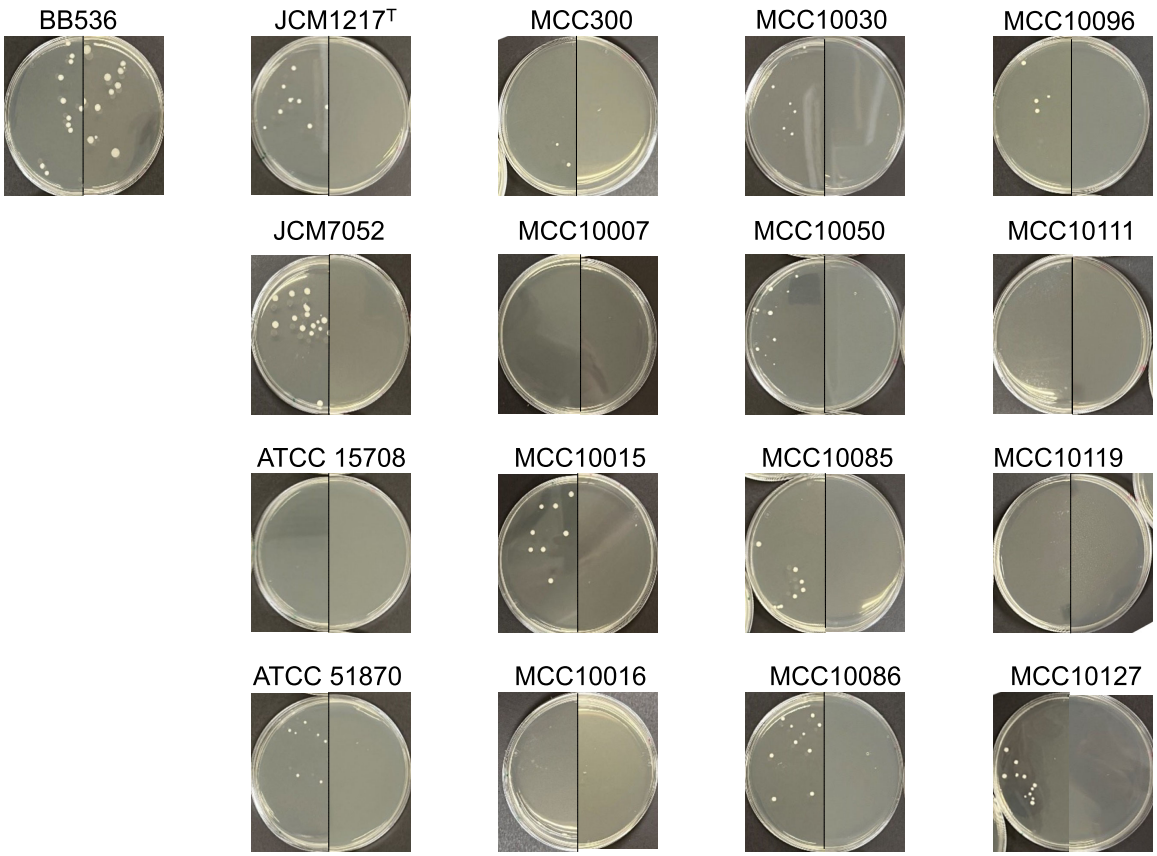

FIGURE 2 Colony forming ability of *Bifidobacterium longum* in mTOS and LMT medium. Bacterial colonies of 17 strains of *B. longum* grown on the mTOS agar plates (left panel) and LMT agar medium (right panel). Each bacteria were cultured anaerobically at 42 °C for 3 days.

facilitated the efficient detection of viable *B. longum* BB536 spiked into faecal matter (Supplementary Table S2). We also confirmed the result were comparable to those obtained using the PMA-qPCR method. These outcomes demonstrate that the LMT medium is an effective tool for detecting viable *B. longum* BB536.

Clinical trial to measure Bifidobacterium longum BB536 viability in the intestinal tract

Following the development of the selective LMT medium, specifically designed to facilitate the growth of *B. longum* BB536, we conducted a clinical trial to measure the viability of this strain in the intestinal tract post-ingestion. During the recruitment periods (September 2023), of the 52 individuals screened, 39 met the inclusion criteria and were enrolled in the study (Figure 3). After randomisation and allocation, one participant dropped out owing to illness prior to intervention. Therefore, we included 38 participants in the SAF. No participants withdrew after the initiation of the intervention. In PPS analysis to evaluate viable *B. longum* BB536 in the faecal samples, we excluded four participants owing to low compliance, evidenced by <80% intake of the test food during the trial period.

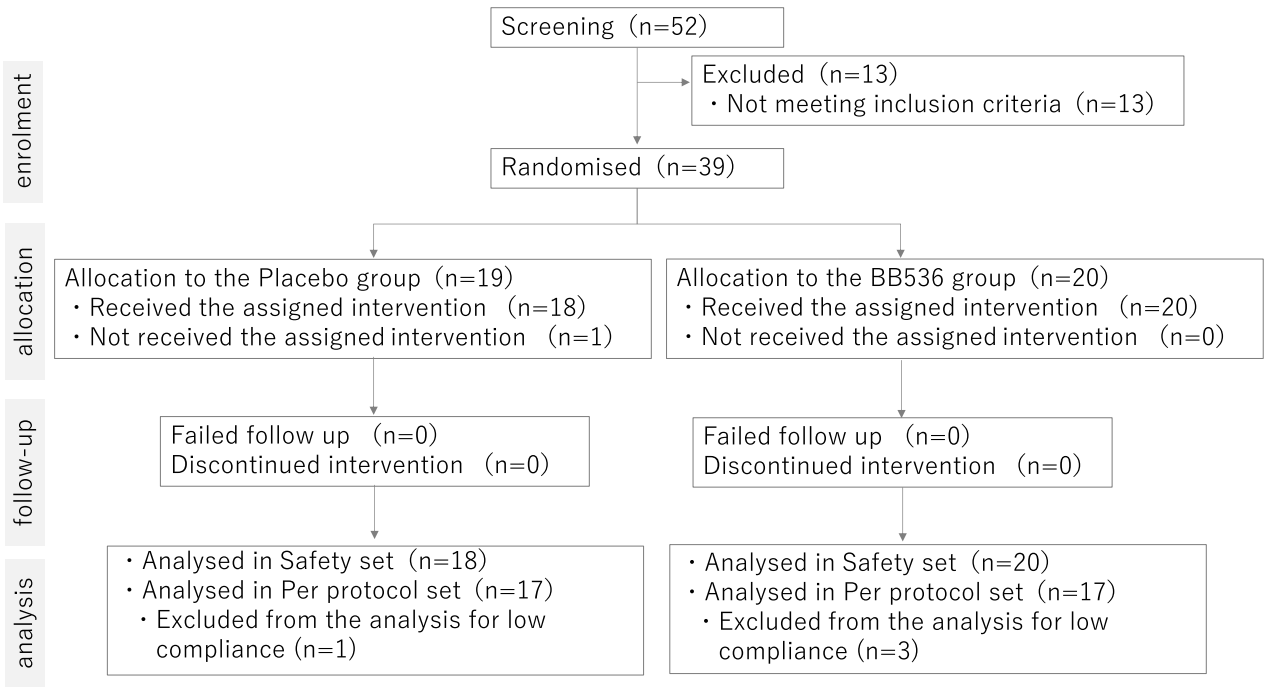

FIGURE 3 Flow diagram of the clinical trial.

TABLE 2 Baseline characteristics of participants of the safety set.[1]

	Placebo group (n = 18)	BB536 group (n = 20)	*P*-value
Age (year)	19.6 ± 0.45	19.1 ± 0.16	ns
Male/female (male %)	8/10 (44.4%)	10/10 (50.0%)	ns
Body mass index (kg/m^2)	21.8 ± 3.3	20.5 ± 2.1	ns

1 ns = not significant. Results are shown as mean ± standard error. Age and body mass index were performed using the t-tests, sex was performed using chi-squared test.

Consequently, 34 participants were analysed in the PPS for viability of the ingested probiotics. The baseline characteristics of the participants are detailed in Table 2. There were no significant differences in baseline characteristics between the two groups.

Quantification of viable Bifidobacterium longum BB536

To determine the efficacy of the intervention, we quantified viable *B. longum* BB536 in participant samples. Prior to the start of supplementation, no participants exhibited detectable levels of *B. longum* BB536. In the placebo group, no participants showed detectable levels of *B. longum* BB536 on days 3 or 17. In the BB536 group, the viable cell counts of *B. longum* BB536 were 6.33 ± 0.69 $\log_{10}$ cfu/g wet faeces (14/15 analysed participants) on day 3 and 6.16 ± 0.74 $\log_{10}$ cfu/g wet faeces (13/16 analysed participants) on day 17 (Figure 4). Additionally, using the PMA-qPCR method, we observed comparable levels of viable *B. longum* BB536 cells: 6.09 ± 0.68 $\log_{10}$ cells/g wet faeces (12/15 analysed participants) on day 3 and 6.44 ± 0.64 $\log_{10}$ cells/g wet faeces (15/16 analysed participants) on day 17, suggesting that PMA-qPCR could provide accurate measurements of live bacteria. In contrast, when we used the qPCR method without PMA treatment, which quantifies both dead and live cells, we observed a significant difference in bacteria counts: 7.38 ± 0.86 $\log_{10}$ cells/g wet faeces (12/15 analysed participants) on day 3 and 7.56 ± 0.45 $\log_{10}$ cells/g wet faeces (14/16 analysed participants) on day 17.

Remarkably, by combining the results of both the culturing and PMA-qPCR methods, we were able to detect the presence of the probiotic in every participant on both day 3 and day 17 (Figure 5). This 100% detection rate across all participants and time points underscores the robust survivability of the BB536 strain throughout the gastrointestinal environment and the thoroughness of our detection protocol.

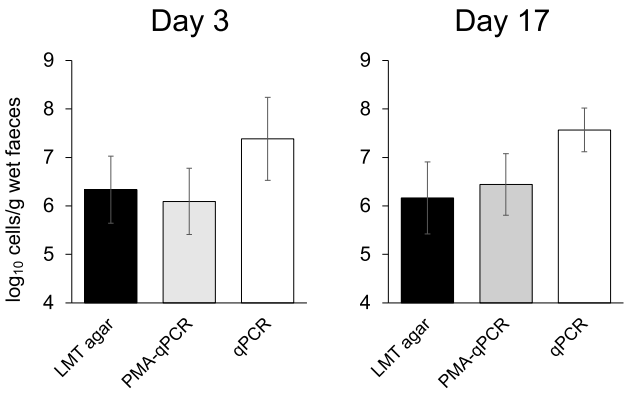

FIGURE 4 Quantification of *B. longum* BB536 in faecal samples. Viable cell counts of *Bifidobacterium longum* BB536 at day 3 and day 17, as determined by culture-based, PMA-qPCR, and q-PCR methods. The data are presented as the average ± standard deviation.

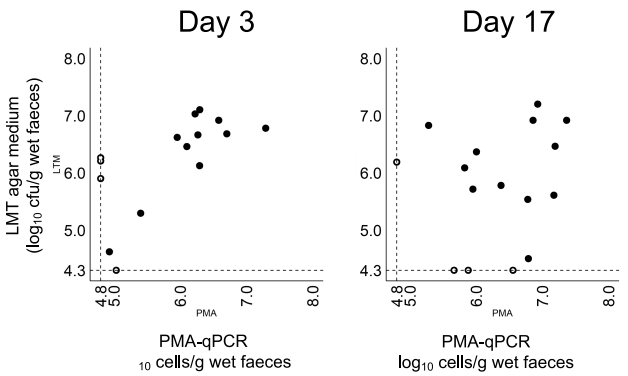

FIGURE 5 Comparison of viable cell counts detected in LMT agar medium or PMA-qPCR. Scatter plot of viable cell counts of *Bifidobacterium longum* BB536 detected by LMT agar medium versus those detected by PMA-qPCR on day 3 and day 17. Dotted lines indicate the detection limits of PMA-qPCR and the LMT agar medium. White circles indicate samples that were below the detection limit for at least one of the methods.

Safety assessment

We recorded 19 adverse events by participants during the study period, none of which were serious. There was no difference in the incidence of adverse events (Placebo: 0.28, BB536: 0.25, *P*-value = 1.0) between the two group, and no adverse events were attributed to consumption of the test foods.

4 Discussion and conclusions

The efficacy of *B. longum* BB536 has been well-documented across numerous human clinical trials, possibly owing to its production of beneficial metabolites. Our study confirms that *B. longum* BB536 can survive transit to the intestine when consumed in fermented milk, offering valuable insights for further exploration of its mechanisms of action. Notably, we were able to detect *B. longum* BB536 in faecal samples in a viable state, indicating its potential to interact with the host throughout the entire gastrointestinal tract. This finding underscores the importance of investigating its specific functions across different digestive regions in future studies.

In our efforts to quantify live *B. longum* BB536 from faecal samples, we faced significant challenges owing to the presence of endogenous *B. longum* strains in the gut (Derrien *et al.*, 2022). Developing a strain-specific selective medium was crucial for accurately quantifying ingested *B. longum* BB536. We successfully enhanced the selectivity of the conventional TOS propionate medium, used for bifidobacteria, by modulating the carbohydrate source, adding antibiotics, and adjusting the culture temperature. Supplementation of trimethoprim effectively suppressed susceptible *Bifidobacterium* species, such as *Bifidobacterium breve* (Kim *et al.*, 2018; Xiao *et al.*, 2010). Additionally, altering the culture temperature significantly improved selectivity; while the optimal temperature for human-derived *Bifidobacterium* is 36-38 °C (Ruiz *et al.*, 2011), several strains of *B. longum* could not grow at 42 °C. These modifications helped achieve greater selectivity for *B. longum* BB536, although the exact mechanisms driving this increased selectivity remain to be elucidated. One hypothesis is that stress response genes in *B. longum* BB536 may be influenced by the composition of the culture medium, contributing to its robust stress tolerance, particularly against gastric acids (Liong *et al.*, 2005). However, further research, including transcript analysis, is required to uncover the detailed mechanisms involved.

Our newly developed selective medium was generally as effective as the PMA–qPCR method in detecting *B. longum* BB536 in participants. However, discrepancies arose with *B. longum* BB536 being detected in some participants by only one of the methods. The issue was that the growth of non-selective bacteria, which could potentially interfere with *B. longum* BB536, posed a limitation when using the selective medium. To overcome these obstacles, it is crucial to further refine the selective medium to improve its accuracy. Nevertheless, the high selectivity observed in most cases supports the effectiveness of the LMT agar medium.

Furthermore, it is recognised that some bifidobacteria can enter a viable but non-culturable (VBNC) state under certain conditions, where they maintain cell membrane integrity but are not culturable (Sibanda

et al., 2024). This VBNC state, along with the presence of dead cells, could contribute to the bacteria cell number detected by PMA treatment. Conversely, *B. longum* BB536 detected only by the LMT agar method suggests that these cells, while having damaged cell membranes, remain viable under optimal growth conditions. Damage to cell membranes, potentially caused by host-derived factors or other gut bacteria, or due to delays in sample processing, may allow PMA staining but still permit culture in favourable conditions. The detection of bacteria in this intermediate state of viability may explain the discrepancies between PMA-qPCR and culture-based methods. However, the fact that no participant samples had undetectable *B. longum* BB536 by either method indicates a high survival rate for *B. longum* BB536.

In these clinical samples, the two analysis methods showed a good correlation on day 3, but a slight divergence on day 17 (Figure 5). This discrepancy could be attributed to variations in the viability conditions of *B. longum* BB536 in the test foods. Previous studies have reported that certain probiotic bifidobacteria can enter a viable but VBNC state in fermented foods during storage periods (Lahtinen *et al.*, 2008), indicating that different batches of test foods may impact the state of *B. longum* BB536, potentially leading to variations in the results obtained from the culturing and PMA–qPCR methods. Furthermore, it has been observed that stress environments can also induce the VBNC state in bacteria (Wendel, 2022). This implies that changes in the gut environment during the test period may also influence the state of ingested *B. longum* BB536, further contributing to the observed divergence in results on day 17. Given these considerations, the combined use of culturing and PMA-qPCR methods could be beneficial for the comprehensive detection of viable probiotic strains, as it may help account for potential variation in the bacteria's state due to factors such as differences in test food batches or changes in the gut environment.

In conclusion, our findings demonstrate the effectiveness of the LMT agar medium for quantifying *B. longum* BB536 in faecal samples and affirm the probiotic's viability and functional activity in the gastrointestinal tract following daily consumption of fermented milk. Moreover, the complementary roles of LMT culturing and PMA-qPCR ensures a comprehensive detection strategy. These findings underscore the potential of *B. longum* BB536 as a beneficial probiotic strain, capable of surviving transit through the digestive system and potentially exerting positive effects on gut health. The robustness of this strain observed in our study highlights its promise for further exploration and utilisation in promoting human well-being.

Supplementary material

Supplementary material is available online at: https://doi.org/10.6084/m9.figshare.27755214

Table S1. The composition of LTM medium.

Table S2. Comparison of culture method using LMT agar medium and PMA-qPCR to detect spiked *B. longum* BB536.

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Authors' contribution

Conceptualization: AS, TO, MT. Data curation: AS, MK, RE, TO. Formal analysis: AS, MK, SA, TO. Investigation: AS, MK, RE, AS, EM, HK, RM, MNakan, AH. Methodology: AS, MK, RE, SA, NM, AH, KK, MNakan, TO. Project administration: NI, MNakan, TO. Resource: YK, HM. Supervision: MNakam, MT. Validation: AS, TO. Visualization: AS, TO. Writing – original draft: AS, MK. Writing – review & editing: SA, KK, NI, MNakan, TO, MT. All authors approved the submitted version of manuscript.

Conflict of interest

This study was sponsored by Morinaga Milk Industry Co., Ltd. (Tokyo, Japan). AS, MK, RE, SA, EM, HK, RM, NM, AH, KK, YK, HM, NI, MNakan, TO, MT are employed at Morinaga Milk Industry Co., Ltd.

References

Abdulqadir, R., Engers, J. and Al-Sadi, R., 2023. Role of *Bifidobacterium* in modulating the intestinal epithelial tight junction barrier: current knowledge and perspectives. Current Developments in Nutrition 7: 102026. https://doi.org/10.1016%2Fj.cdnut.2023.102026

Akatsu, H., Iwabuchi, N., Xiao, J.-Z., Matsuyama, Z., Kurihara, R., Okuda, K., Yamamoto, T. and Maruyama, M., 2013. Clinical effects of probiotic *Bifidobacterium longum* bb536 on immune function and intestinal microbiota in elderly patients receiving enteral tube feeding. Journal of Parenteral and Enteral Nutrition 37: 631-640. https://doi.org/10.1177/0148607112467819

Bunesova, V., Musilova, S., Geigerova, M., Pechar, R. and Rada, V., 2015. Comparison of mupirocin-based media for selective enumeration of bifidobacteria in probiotic supplements. Journal of Microbiological Methods 109: 106-109. https://doi.org/10.1016/j.mimet.2014.12.016

Christensen, H.R., Larsen, C.N., Kæstel, P., Rosholm, L.B., Sternberg, C., Michaelsen, K.F. and Frøkiær, H., 2006. Immunomodulating potential of supplementation with probiotics: a dose–response study in healthy young adults. FEMS Immunology and Medical Microbiology 47: 380-390. https://doi.org/10.1111/j.1574-695X.2006.00109.x

Derrien, M., Turroni, F., Ventura, M. and Van Sinderen, D., 2022. Insights into endogenous *Bifidobacterium* species in the human gut microbiota during adulthood. Trends in Microbiology 30: 940-947. https://doi.org/10.1016/j.tim.2022.04.004

Dommels, Y.E.M., Kemperman, R.A., Zebregs, Y.E.M.P., Draaisma, R.B., Jol, A., Wolvers, D.A.W., Vaughan, E.E. and Alberset, R., 2009. Survival of *Lactobacillus reuteri* DSM 17938 and *Lactobacillus rhamnosus* GG in the human gastrointestinal tract with daily consumption of a low-fat probiotic spread. Applied and Environmental Microbiology 75: 6198-6204. https://doi.org/10.1128/AEM.01054-09

Fujimoto, J. and Watanabe, K., 2013. Quantitative detection of viable *Bifidobacterium bifidum* BF-1 cells in human feces by using propidium monoazide and strain-specific primers. Applied and Environmental Microbiology 79: 2182-2188. https://doi.org/10.1128/AEM.00354-14

Fujimoto, J., Tanigawa, K., Kudo, Y., Makino, H. and Watanabe, K., 2011. Identification and quantification of viable *Bifidobacterium breve* strain *Yakult* in human faeces by using strain-specific primers and propidium monoazide. Journal of Applied Microbiology 110: 209-217. https://doi.org/10.1111/j.1365-2672.2010.04873.x

Gianotti, L., Morelli, L., Galbiati, F., Rocchetti, S., Coppola, S., Beneduce, A., Gilardini, C., Zonenschain, D., Nespoli, A. and Brag, M., 2010. A randomized double-blind trial on perioperative administration of probiotics in colorectal cancer patients. World Journal of Gastroenterology 16: 167-175. https://doi.org/10.3748/wjg.v16.i2.167

Kato, K., Odamaki, T., Mitsuyama, E., Sugahara, H., Xiao, J.-Z. and Osawaet, R., 2017. Age-related changes in the composition of gut *Bifidobacterium* species. Current Microbiology 74: 987-995. https://doi.org/10.1007/s00284-017-1272-4

Kim, M.J., Ku, S., Kim, S.Y., Lee, H.H., Jin, H., Kang, S., Li, R., Johnston, T.V., Park, M.S. and Ji, G.E., 2018. Safety evaluations of *Bifidobacterium bifidum* BGN4 and *Bifidobacterium longum* BORI. International Journal of Molecular Sciences 19: 1422. https://doi.org/10.3390/ijms19051422

Kondo, J., Xiao, J.-Z., Shirahata, A., Baba, M., Abe, A., Ogawa, K. and Shimoda, T., 2013. Modulatory effects of *Bifidobacterium longum* BB536 on defecation in elderly patients receiving enteral feeding. World Journal of Gastroenterology 19: 2162. https://doi.org/10.3748/wjg.v19.i14.2162

Lahtinen, S.J., Ahokoski, H., Reinikainen, J.P., Gueimonde, M., Nurmi, J., Ouwehand, A.C. and Salminen, S.J., 2008. Degradation of 16S rRNA and attributes of viability of viable but nonculturable probiotic bacteria. Letters in Applied Microbiology 46: 693-698. https://doi.org/10.1111/j.1472-765X.2008.02374.x

Liong, M.T. and Shah, N.P., 2005. Acid and bile tolerance and the cholesterol removal ability of bifidobacteria strains. Bioscience and Microflora 24: 1-10. https://doi.org/10.12938/bifidus.24.1

Murakami, R., Hashikura, N., Yoshida, K., Xiao, J. and Odamaki, T., 2021. Growth-promoting effect of alginate on *Faecalibacterium prausnitzii* through cross-feeding with *Bacteroides*. Food Research International 144: 110326. https://doi.org/10.1016/j.foodres.2021.110326

Nocker, A. and Camper, A.K., 2006. Selective removal of DNA from dead cells of mixed bacterial communities by use of ethidium monoazide. Applied and Environmental Microbiology 72: 1997-2004. https://doi.org/10.1128%2FAEM.72.3.1997-2004.2006

Palaria, A., Johnson-Kanda, I. and O'Sullivan, D.J., 2012. Effect of a synbiotic yogurt on levels of fecal bifidobacteria, clostridia, and enterobacteria. Applied and Environmental Microbiology 78: 933-940. https://doi.org/10.1128/aem.05848-11

Rougé, C., Goldenberg, O., Ferraris, L., Berger, B., Rochat, F., Legrand, A., Göbel, U.B., Vodovar, M., Voyer, M., Rozé, J.-C., Darmaun, D., Piloquet, H., Butel, M.-J. and De La Cochetièreet, M.F., 2010. Investigation of the intestinal microbiota in preterm infants using different methods. Anaerobe 16: 362-370. https://doi.org/10.1016/j.anaerobe.2010.06.002

Ruiz, L., Ruas-Madiedo, P., Gueimonde, M., De los Reyes-Gavilán, C.G., Margolles, A. and Sánchez, B., 2011. How do bifidobacteria counteract environmental challenges? Mechanisms involved and physiological consequences. Genes and Nutrition 6: 307-318. https://doi.org/10.1007%2Fs12263-010-0207-5

Sakurai, T., Odamaki, T. and Xiao, J., 2019. Production of indole-3-lactic acid by *Bifidobacterium* strains isolated

from human infants. Microorganisms 7: 340. https://doi.org/10.3390/microorganisms7090340

Sanders, M.E., Merenstein, D.J., Reid, G., Gibson, G.R. and Rastall, R.A., 2019. Probiotics and prebiotics in intestinal health and disease: from biology to the clinic. Nature Reviews Gastroenterology and Hepatology 16: 605-616. https://doi.org/10.1038/s41575-019-0173-3

Serafini, F., Bottacini, F., Viappiani, A., Baruffini, E., Turroni, F., Foroni, E., Lodi, T., Van Sinderen, D. and Ventura, M., 2011. Insights into physiological and genetic mupirocin susceptibility in bifidobacteria. Applied and Environmental Microbiology 77: 3141-3146. https://doi.org/10.1128%2FAEM.02540-10

Sibanda, T., Marole, T.A., Thomashoff, U.L., Thantsha, M.S. and Buys, E.M., 2024. *Bifidobacterium* species viability in dairy-based probiotic foods: challenges and innovative approaches for accurate viability determination and monitoring of probiotic functionality. Frontiers in Microbiology 15: 1327010. https://doi.org/10.3389/fmicb.2024.1327010

Sugahara, H., Odamaki, T., Fukuda, S., Kato, T., Xiao, J.-Z., Abe, F., Kikuchi, J. and Ohno, H., 2015. Probiotic *Bifidobacterium longum* alters gut luminal metabolism through modification of the gut microbial community. Scientific Reports 5: 13548. https://doi.org/10.1038/srep13548

Tamura, M., Hoshi, C., Kobori, M., Takahashi, S., Tomita, J., Nishimura, M. and Nishihira, J., 2017. Quercetin metabolism by fecal microbiota from healthy elderly human subjects. PLoS ONE 12: e0188271. https://doi.org/10.1371/journal.pone.0188271

Tremblay, A., Fatani, A., Ford, A.L., Piano, A., Nagulesapillai, V., Auger, J., MacPherson, C.W., Christman, M.C., Tompkins, T.A. and Dahl, W.J., 2021. Safety and effect of a low- and high-dose multi-strain probiotic supplement on microbiota in a general adult population: a randomized, double-blind, placebo-controlled study. Journal of Dietary Supplements 18: 227-247. https://doi.org/10.1080/19390211.2020.1749751

Wendel, U., 2022. Assessing viability and stress tolerance of probiotics – a review. Frontiers in Microbiology 12: 818468. https://doi.org/10.3389/fmicb.2021.818468

Wong, C.B., Odamaki, T. and Xiao, J., 2019. Beneficial effects of *Bifidobacterium longum* subsp. *longum* BB536 on human health: modulation of gut microbiome as the principal action. Journal of Functional Foods 54: 506-519. https://doi.org/10.1016/j.jff.2019.02.002

Xiao, J., Takahashi, S., Odamaki, T., Yaeshima, T. and Iwatsuki, K., 2010. Antibiotic susceptibility of bifidobacterial strains distributed in the Japanese market. Bioscience, Biotechnology, and Biochemistry 74: 336-342. https://doi.org/10.1271/bbb.90659

Yuki, N., Watanabe, K., Mike, A., Tagami, Y., Tanaka, R., Ohwaki, M. and Morotomi, M., 1999. Survival of a probiotic, *Lactobacillus casei* strain Shirota, in the gastrointestinal tract: selective isolation from faeces and identification using monoclonal antibodies. International Journal of Food Microbiology 48: 51-57. https://doi.org/10.1016/s0168-1605(99)00029-x