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Evaluation of probiotic and prebiotic-like effects of *Bacillus subtilis* BN on growth of lactobacilli

Masanori Horie,[1,*] Taisuke Koike,[2] Sakiko Sugino,[1] Aya Umeno,[1] and Yasukazu Yoshida[1]

[1] *Health Research Institute (HRI), National Institute of Advanced Industrial Science and Technology (AIST),*
2217-14 Hayashi-cho, Takamatsu, Kagawa 761-0395, Japan
[2] *Mitsubishi-Chemical Foods Corporation, 2-13-10 Nihonbashi, Chuo-ku, Tokyo 103-0027, Japan*

The aim of this study was to determine the probiotic and the prebiotic-like properties of *Bacillus subtilis* BN, a spore-forming bacterium, also known as "natto-kin", which is used for making the Japanese fermented food, natto. We used the spores and vegetative cells of this strain and compared their effects on the growth of lactobacilli. Culture supernatant from *B. subtilis* BN was added to a glucose-free MRS medium used to culture lactobacilli. When lactobacilli were cultured in the supernatant-containing medium, growth was improved. This effect resulted from the digestion of starch by amylase, which was secreted by *B. subtilis*. Moreover, improved amylase-independent growth was also observed. Co-culture with *B. subtilis* improved the growth of lactobacilli, and this effect was only observed with vegetative cells; spores did not improve the growth of lactobacilli. This effect on growth was lost upon heat treatment of the vegetative cells. These results suggest that the surface protein of *B. subtilis* BN vegetative cells participates in the improved growth effect of lactobacilli. It is possible that *B. subtilis* BN could improve the intestinal flora. In addition, *B. subtilis* BN inhibited the growth of *Salmonella enterica*. Thus, it was shown that *B. subtilis* BN has both a probiotic and prebiotic potential. This is the first study demonstrating the selective growth improvement of indigenous intestinal lactobacilli using *B. subtilis* BN.

Key Words: *Bacillus subtilis*; *Lactobacillus*; prebiotics; probiotics; spore

Introduction

With advances in technology for microorganism identification, analyses of intestinal flora have been made, and relationships between the intestinal flora and lifestyle-related diseases, including immunological diseases such as atopic dermatitis, and other diseases, have been reported (Bull and Plummer, 2014; Patterson et al., 2016; Yeom et al., 2015). Obesity and type 2 diabetes are linked to various genetic and environmental factors, and the gastrointestinal flora is one of these important factors. For example, the composition of the microbial gastrointestinal flora between type 2 diabetic volunteers and those with prediabetes was shown to be different (Lambeth et al., 2015). The intake of antibiotics targeting gram-negative bacteria was shown to improve the body weight, plasma glucose, and insulin levels in diet-induced obese mice (Rajpal et al., 2015). This result suggests that gram-positive bacteria, such as lactobacilli, are associated with the inhibition of obesity. Various bacteria, such as lactobacilli and bifidobacteria, have been studied and are used commercially to augment human health (Kato-Kataoka et al., 2016; Szajewska and Kołodziej, 2015; Yanagihara et al., 2014). Although probiotics consist of beneficial bacteria, they do not always have beneficial effects, because their viability and ability to colonize the gastrointestinal tract are sometimes low (Botta et al., 2014; Duary et al., 2011; Gomathi et al., 2014). For example, the survival rate of plant-derived lactic acid bacteria in the stomach is lower than that of animal-derived lactic acid bacteria (Ozawa et al., 2012). The adhesion ability of lactobacilli depends on the environment (Horie et al., 2005; Volstatova et al., 2016). To improve the gastrointestinal flora, promoting

*Corresponding author: Masanori Horie, Health Research Institute (HRI), National Institute of Advanced Industrial Science and Technology (AIST), 2217-14 Hayashi-cho, Takamatsu, Kagawa 761-0395, Japan.
E-mail: masa-horie@aist.go.jp

the growth of beneficial bacteria in the intestine, such as lactobacilli, is effective, in addition to the intake of exogenous probiotics. Prebiotics are effective for promoting the growth of native bacteria in the intestine. The term "prebiotics" was suggested by Gibson and Roberfroid (1995) and is defined as: "microbial food supplements that beneficially affect the host by improving its intestinal microbial balance by changing the composition of colonic microbiota." These prebiotics are not digested in the upper gastrointestinal tract, and they confer benefits to the host through improving the balance of the intestinal flora. Oligosaccharides and dietary fiber are known as the major prebiotics. For example, the intake of galacto-oligosaccharides was shown to reduce serum phenols that were produced from aromatic amino acids by gut bacteria (Miyazaki et al., 2014). The decrease in serum phenols can improve the condition of the skin. Although the associated mechanism is unknown, the intake of galacto-oligosaccharides and long chain fructo-oligosaccharides has been shown to improve atopic dermatitis (Moro et al., 2006).

"Natto" is a traditional Japanese food made from soybeans that potentially contains both probiotics and prebiotics. Natto is produced through the fermentation of soybeans by a type of *Bacillus subtilis*, which is a spore-forming bacterium (Hotta and Sasaki, 2011; Mitsuboshi and Kiuchi, 2007). In the traditional production of natto, straw, containing spores of *B. subtilis*, is boiled to kill other bacteria. Boiled soybeans are wrapped with the straw and maintained at approximately 30°C. The spores then germinate and undergo transition to the vegetative cell form. The vegetative cells of *B. subtilis* ferment the soybeans, producing the natto. Spores of *B. subtilis* exhibit thermal and acid tolerance. Since *B. subtilis* in the natto is in a vegetative cell form, many of the bacteria will be killed by the acidic conditions of the stomach. However, the spores can survive in the stomach, permitting live bacteria to reach the intestine. There have been some reports describing the probiotic effect of *B. subtilis* from natto. For example, the intake of *B. subtilis* C-3102 culture decreased the concentration of p-cresol in feces (Suzuki et al., 2004). In addition, co-culture of *B. subtilis* MA139 and *Lactobacillus reuteri* inhibited the growth of pathogenic *Escherichia coli* K88. The inhibitory effect was more effective when *Lactobacillus* was co-cultured with *B. subtilis* MA 139 than when *Lactobacillus* was cultured alone. This effect might have been caused by an improvement in the growth of *Lactobacillus*, caused by *B. subtilis* (Yang et al., 2015). The intake of natto was shown to improve the composition of *Bifidobacterium* in fecal flora (Terada et al., 1999). However, it has also been reported that the intake of natto did not affect the composition of intestinal *Bifidobacterium*, and could not improve constipation (Takemura et al., 2009). If *B. subtilis* improved the growth of lactic acid bacteria, there are two conceivable mechanisms. One is a growth-improving effect by metabolic products of *B. subtilis*. Another is a growth-improving effect by bacterial materials, such as a surface protein. In the present study, we have examined these two possibilities. *B. subtilis* secretes extracellular α-amylase, and, hence, can convert starch to maltose or glucose (Emori

et al., 1990; Hayashida et al., 1988). In general, lactobacilli, which are a class of extremely beneficial intestinal bacteria, cannot use starch as a carbon sauce. Therefore, a prebiotic-like effect is expected with *B. subtilis*. Specifically, in the intestine, *B. subtilis* can digest starch to sugars, which can be utilized by lactobacilli. Thus, *B. subtilis* would support the growth of intestinal lactobacilli. Although the intake of natto is effective for improving gastrointestinal flora, determining the optimal conditions for improving gastrointestinal flora is difficult. Since natto is a food, it includes vegetative cells, spores, and metabolic substances such as polyglutamic acid. For the probiotic effect of *B. subtilis*, the intake of spores of *B. subtilis* is expected to be the most effective because the spores are chemically stable. In the present study, we studied the prebiotic-like effect of *B. subtilis in vitro*. The effects of the vegetative cells and spores of *B. subtilis* on the growth of lactobacilli were compared. Additionally, the potential probiotic effect, and the growth inhibitory effect, of pathogenic bacteria were also examined.

Materials and Methods

Bacterial strains. Freeze dried *Bacillus subtilis* BN including spores, named "Nattokin powder", was obtained from the Eisai Food & Chemical Co., Ltd. (Tokyo, Japan). The "Nattokin powder" was manufactured by the Meguro Institute Co., Ltd., Kasai Factory (Kasai, Hyogo, Japan) (Lot No. 113501). According to the manufacturer's data sheet, the viable bacteria count in the Nattokin powder was 1.4×10^{11}/g. Vegetative cells of *B. subtilis* BN were obtained by culturing the Nattokin powder in Luria-Bertani (LB) broth (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl in 1 L of distilled water) overnight at 37°C with shaking. Pre-cultured vegetative cells of *B. subtilis* BN were collected by centrifugation at 7000 rpm for 10 min. After the vegetative cells were washed with PBS (phosphate-buffered saline) once, the vegetative cell suspension was prepared at a concentration of 1×10^8 cells/mL with LB broth. The number of bacteria was counted using a bacteria counter (Sunlead Glass Corp., Koshigaya, Japan). *B. subtilis* BN was cultured in LB broth or on LB agar plates. *Lactobacillus crispatus* JCM 8778, *Lactobacillus casei* JCM 1134[T], *Lactobacillus plantarum* JCM 1149[T], and *Salmonella enterica* subsp. *enterica* JCM 1652, were purchased from the Japan Collection of Microorganisms, RIKEN BioResource Center (Tsukuba, Japan). Lactic acid bacteria were cultured in de Man-Rogosa-Sharpe (MRS) broth (Sigma-Aldrich) or on MRS agar plates. Lactic acid bacteria were cultured anaerobically using screw-top test tubes and anaerobic jars with an AnaeroPack® Kenki (Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan). *S. enterica* JCM 1652 was cultured in nutrient broth (5.0 g of bacto peptone, 3.0 g of beef extract, and 5.0 g of NaCl in 1 L of distilled water). For growth inhibition tests, *B. subtilis* BN and *S. enterica* JCM 1652 were cultured on LB agar plates and MLCB agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), respectively, at 37°C overnight.

Spore staining. To confirm the condition of the Nattokin powder, prior to additional examination, spore staining was

performed by the Schaeffer-Fulton modified Wirtz method using a Wirtz spore staining kit (Muto Pure Chemicals Co., Ltd., Tokyo, Japan). The Nattokin powder and cultured *B. subtilis* BN were suspended in PBS. The suspension was spread on a slide glass and fixed by flame. The bacteria were stained by a 5% malachite green solution for 2 min with heating. After water washing, the bacteria were stained by 0.5% of safranine solution for 30 s. The slide was washed and dried.

Survival test in acidic culture medium (Experiment 1). LB broth was adjusted to pH 6.8, 3.0, and 2.0 using HCl. Pepsin from porcine stomach (Wako Pure Chemical Industries, Ltd., Osaka, Japan) (4%) was added to the LB broth, pH 3.0 and 2.0, at a concentration of 110 μL/10 mL. LB broth, pH 6.8, did not include pepsin. The Nattokin powder was suspended in LB broth (pH 6.8) at a concentration of 1×10^8 spores/mL, and 1 mL of the suspension was added to 10 mL of LB medium (pH 6.8, 3.0, and 2.0). *B. subtilis* BN in vegetative cell form was suspended in LB broth (pH 6.8) at a concentration of 1×10^8/mL, and 1 mL of the suspension was added to 10 mL of LB medium (pH 6.8, 3.0, and 2.0). The final concentration of the spores and vegetative cells of *B. subtilis* BN was 1×10^7/mL. The spore and vegetative cell suspensions were statically cultured for 2 or 6 h at 37°C. The culture fluid was then diluted with PBS and spread on LB agar plates. After culturing overnight at 37°C, the number of colonies were counted.

Survival test in simulated gastric juice (Experiment 2). Pre-cultured vegetative cells of *B. subtilis* BN were collected by centrifugation and washed once with PBS. The Nattokin powder was suspended in PBS. For a specific experiment, the Nattokin powder was heated to obtain only spores. The Nattokin powder was suspended in PBS and heated to 65°C for 30 min in a block heater. After washing with PBS three times, the bacteria were further heated at 65°C for 30 min. This suspension was used as a spore suspension. The spores and vegetative cells of *B. subtilis* BN were prepared at a concentration of 1×10^7/mL in 1st Fluid for a dissolution test at pH 1.2 (2.0 g of NaCl and 7.0 mL of HCl in 1 L of distilled water) (Wako Pure Chemical Industries, Ltd.) or 2nd Fluid for a dissolution test at a relatively neutral pH (phosphate buffer solution, pH 6.8). These suspensions were maintained at 37°C for 6 h. The suspension was then serially diluted with PBS and spread on LB agar plates at 100 μL/plate. After culturing overnight at 37°C, the number of colonies were counted.

Growth improving effect on lactic acid bacteria: Growth-improving effect of B. subtilis BN metabolism (Experiment 3). *B. subtilis* BN was pre-cultured in LB broth at 37°C overnight with shaking. Pre-cultured *B. subtilis* BN was inoculated into an amylase production medium (1.0% corn starch, 1.0% peptone, 0.8% $(NH_4)SO_2$, 0.2% $MgSO_4 \cdot 7H_2O$, 0.05% $CaCl_2 \cdot 2H_2O$, 1.4% K_2HPO_4, and 0.6% KH_2PO_4) (Dash et al., 2015) and cultured for 2 days at 37°C with shaking. The culture supernatant was then collected by centrifugation at 13000 rpm for 10 min. After filtration through a 0.22-μm membrane filter (Advantec Toyo Kaisha, Ltd., Tokyo, Japan), 1 mL of the supernatant was added to 9 mL of MRS medium without glucose. As a control, the supernatant of the amylase production medium without bacteria and PBS was also added to glucose-free MRS medium. Subsequently, 10 μL of pre-cultured *L. casei* JCM 1134[T], or *L. crispatus* JCM 8778, was added and cultured overnight at 37°C. The culture fluid was then diluted with PBS and spread on MRS agar and LB agar plates. After culturing overnight at 37°C, the number of colonies were counted.

Growth improving effect on lactic acid bacteria: Growth-improving effect of B. subtilis BN metabolism (Experiment 4). One milliliter of pre-cultured *B. subtilis* BN in LB broth or LB broth without bacteria was added to 9 mL of MRS broth including corn starch (Wako Pure Chemical Industries, Ltd.) instead of glucose and cultured overnight at 37°C with shaking. Culture fluid was transferred to a screw-top test tube and 10 μL of pre-cultured *L. casei* JCM 1134[T], or *L. crispatus* JCM 8778, were added and cultured overnight at 37°C. The culture fluid was then diluted with PBS and spread on MRS agar and LB agar plates. After culturing overnight at 37°C, the number of colonies were counted.

Growth-improving effect of B. subtilis BN (Experiment 5). *B. subtilis* BN was pre-cultured in LB broth at 37°C overnight with shaking. *Lactobacillus* was pre-cultured with stationary growth in MRS broth at 37°C or 30°C overnight. *B. subtilis* BN was collected by centrifugation at 7000 rpm for 10 min. After washing once with PBS, the bacteria (vegetative cell form) were re-suspended in PBS. Nattokin powder (spore) was also suspended in PBS. The number of bacteria in the PBS suspensions were counted using a bacteria counter. In a partial experiment, the bacterial suspensions were treated by heating at 95°C for 10 min with a block heater, or through sonication for 10 min using a Bioruptor UCD-250 (Cosmo Bio Co., Ltd., Tokyo, Japan). Vegetative cells and spores were added to 10 mL of MRS broth at a concentration of 1×10^7/mL. Subsequently, 10 μL of pre-cultured *Lactobacillus* was added to MRS broth and cultured for 6 h at 37°C. The culture fluid was then diluted with PBS and spread on MRS agar and LB agar plates. After culturing overnight at 37°C, the number of colonies were counted.

Growth-inhibitory effect of B. subtilis BN on Salmonella. *B. subtilis* BN was pre-cultured in LB broth at 37°C overnight with shaking. *S. enterica* JCM 1652 was pre-cultured in nutrient broth at 37°C overnight with shaking. After washing once with PBS, the bacteria were re-suspended in PBS. Nattokin powder (spores) was also suspended in PBS. The number of bacteria in the PBS suspensions were counted using a bacteria counter. Each bacterial or spore suspension was added to 10 mL of nutrient broth at a concentration of 1×10^7/mL and cultured for 6 h at 37°C with shaking. The culture fluid was then serially diluted in PBS and spread on MLCB and LB agar plates. The culture fluid before cultivation was also diluted in PBS and spread on MLCB and LB agar plates (0 h control). After culturing overnight at 37°C, the number of colonies were counted. Black colonies on the MLCB agar were considered to be *Salmonella enterica*.
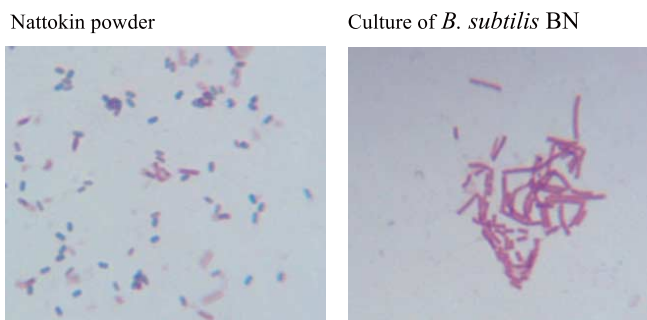
Nattokin powder   Culture of *B. subtilis* BN

Fig. 1. Spore staining of Nattokin powder and culture of *B. subtilis* BN.

The Nattokin powder and cultured *B. subtilis* BN for overnight at 37°C in LB broth were subject to the spore staining method, which was performed using a Wirtz Spore Staining Kit.

Results

Survival of B. subtilis BN in acidic conditions

Results of spore staining using the Nattokin powder and cultured bacteria are shown in Fig. 1. Cultured bacteria included only the vegetative cell form. Vegetative cells and spores of *B. subtilis* BN were treated with media adjusted to pH 6.8, 3.0, and 2.0 for 2 and 6 h (Experiment 1, Fig. 2A). Almost all vegetative cells were killed by treatment with pH 3.0 and 2.0 for 2 h. In contrast, 60% and 50% of spores survived at pH 3.0 and 2.0, respectively, regardless of the treatment time (2 or 6 h). Although the survival rate of spores also decreased with acid treatment, it was time-independent. Moreover, survival of *B. subtilis* BN in acidic conditions was examined using simulated gastric juice of pH 1.2 (Experiment 2, Fig. 2B). Vegetative cells were killed by treatment with simulated gastric juice for 2 h. When compared to treatment with a phosphate buffer solution (pH 6.8), 67% of spores survived treatment with simulated gastric juice (pH 1.2) for 2 h. The decrease in spore survival rate was possibly caused by their coexistence with vegetative cells in the Nattokin powder. Spore staining showed that vegetative cells, as well as spores, were present in the Nattokin powder (Fig. 1). Therefore, in order to remove the influence of the vegetative cells present in the Nattokin powder, they were killed by treatment at 65°C for 30 min. Subsequently, the spore suspension was treated with simulated gastric juice (pH 1.2) for 6 h. The survival rate of the spore suspension was 100% after a 6 h treatment (Fig. 3). As a reference, the same experiment was performed using *Lactobacillus crispatus* JCM 8778 which was isolated from human feces. Here, the survival rate of the suspension was 0 % after a 6 h treatment (Fig. 3).

Growth-improving effect on lactic acid bacteria: Growth-improving effect of B. subtilis BN metabolism

B. subtilis produces amylase (Najafi et al., 2005), and, hence, there is the possibility that *B. subtilis* BN encourages the growth of lactic acid bacteria through the degradation of starch to sugars, such as maltose and glucose, that can be utilized by lactic acid bacteria. Therefore, we have examined this possibility (Experiment 3). *L. crispatus* JCM 8778 and *L. casei* JCM 1134[T], which were isolated
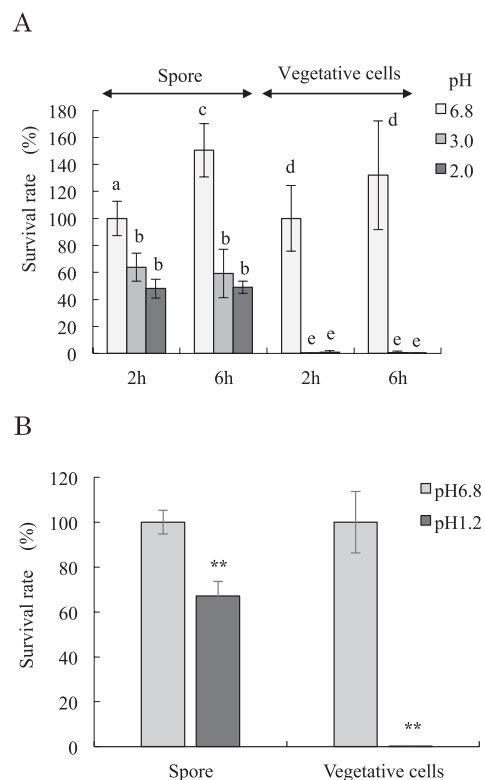

Fig. 2. Survival of *B. subtilis* BN in acidic conditions.

(A) Experiment 1; LB broth was adjusted to pH 6.8, 3.0, and 2.0 using HCl and 0.4% pepsin. Spores and the vegetative cells were suspended in 10 mL of LB medium. The suspension was statically cultured for 2 or 6 h at 37°C. Subsequently, the culture fluid was diluted in PBS and spread on LB agar plates. After culturing overnight at 37°C, the number of colonies were counted. Means indicated by different letters vary in $p < 0.01$ (Tukey's test) significantly. (B) Experiment 2; "Nattokin powder" (spores) and cultured *B. subtilis* BN (vegetative cells) were suspended in 1st Fluid for the dissolution test at pH 1.2 or 2nd Fluid for the dissolution test at pH 6.8, and maintained at 37°C for 6 h. The suspension was diluted in PBS and spread on LB agar plates. After culturing overnight at 37°C, the number of colonies was counted. The survival rate was normalized to the cell growth at pH 6.8 (100%); $**p < 0.01$ vs. pH 6.8. Statistical significance was determined using a Student's *t*-test.
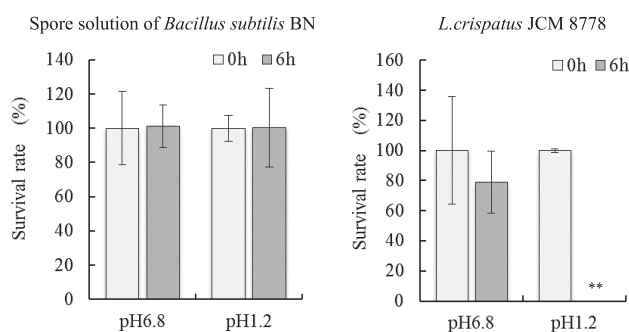

Fig. 3. Survival of *B. subtilis* BN spores and *Lactobacillus* under acidic conditions.

Experiment 2; The Nattokin powder was heated at 65°C for 30 min. This suspension was used as the "spore suspension". The "spore suspension" and *L. crispatus* JCM 8778 were suspended in 1st Fluid for the dissolution test at pH 1.2 or 2nd Fluid for the dissolution test at pH 6.8, and maintained at 37°C for 6 h. The suspension was spread on LB or MRS agar plates. After culturing overnight, the number of colonies was counted. The survival rate was normalized to cell growth at pH 6.8 (100%); $**p < 0.01$ vs. pH 6.8. Statistical significance was determined using a Student's *t*-test.
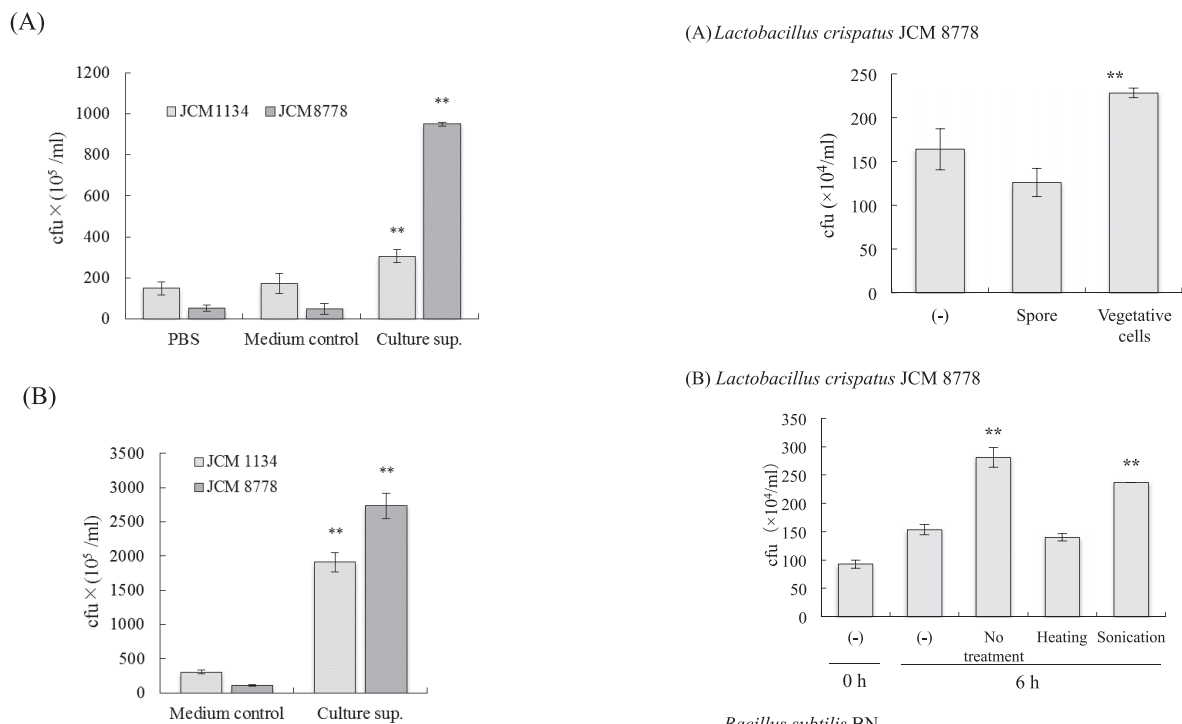
(A)

(B)

Fig. 4. Growth-improving effect on lactobacilli by *B. subtilis* BN metabolism.

(A) Experiment 3; the supernatant of *B. subtilis* BN in an amylase production medium was added to MRS medium without glucose. As a control, the amylase production medium without bacteria and PBS was also added to a glucose-free MRS medium. Subsequently, *L. casei* JCM 1134^T or *L. crispatus* JCM 8778 were added and cultured overnight at 37°C. The culture fluid was then spread on MRS agar and LB agar plates, and the number of colonies was counted. (B) Experiment 4; *B. subtilis* BN in LB broth or LB broth without bacteria was added to MRS broth, containing starch instead of glucose, and cultured overnight at 37°C with shaking. Culture fluid was transferred to screw-top test tubes and *L. casei* JCM 1134^T or *L. crispatus* JCM 8778 were added and cultured overnight at 37°C. The culture fluid was then spread on MRS agar and LB agar plates, and the number of colonies were counted.

(A) *Lactobacillus crispatus* JCM 8778

(B) *Lactobacillus crispatus* JCM 8778

Bacillus subtilis BN

Fig. 5. Growth-improving effect on *L. crispatus* JCM 8778 by *B. subtilis* BN.

Experiment 5; (A) pre-cultured *B. subtilis* BN (vegetative cell form) or Nattokin powder (spore from) was added to MRS broth at a concentration of 1×10^7/mL. Subsequently, 10 μL of pre-cultured *L. crispatus* JCM 8778 was added to MRS broth and cultured for 6 h at 37°C. The culture fluid was then spread on MRS agar plates, and the number of colonies was counted. (B) Pre-cultured *B. subtilis* BN was treated by heating at 95°C for 10 min using a block heater or by sonication for 10 min. The vegetative cells were then cultured with *L. crispatus* JCM 8778 using the same methods as described above. The culture fluid was then spread on MRS agar plates for *L. crispatus* or LB agar plates for *B. subtilis* BN, and the number of colonies were counted.

from human feces and cheese, respectively, were employed as the lactic acid bacteria. The culture supernatant from *B. subtilis* BN growth in an amylase production medium was mixed with a glucose-free MRS medium, and the lactobacilli were inoculated and cultured. When lactobacilli were cultured in a medium including this culture supernatant, growth was improved (Fig. 4A). In contrast, growth improvement was not observed with the addition of only culture medium without *B. subtilis* BN. Starch can be degraded to sugars by amylase that is secreted by *B. subtilis* BN, and these sugars could then be utilized by lactobacilli. Thus, the growth of lactobacilli might be improved by these sugars. Moreover, *B. subtilis* BN was cultured in the MRS broth that included starch instead of glucose. Lactobacilli were then added to the culture fluid and cultured. The culture fluid of *B. subtilis* BN improved the growth of the lactobacilli (Fig. 4B). This effect was less dramatic for *L. casei* JCM 1134 compared to *L. crispatus* JCM 8778. *L. casei* JCM 1134 and *L. crispatus* JCM 8778 were isolated from cheese and human feces, respectively. Lactobacilli of plant origin tend to have the ability to use many kinds of sugar, which is typically not the case 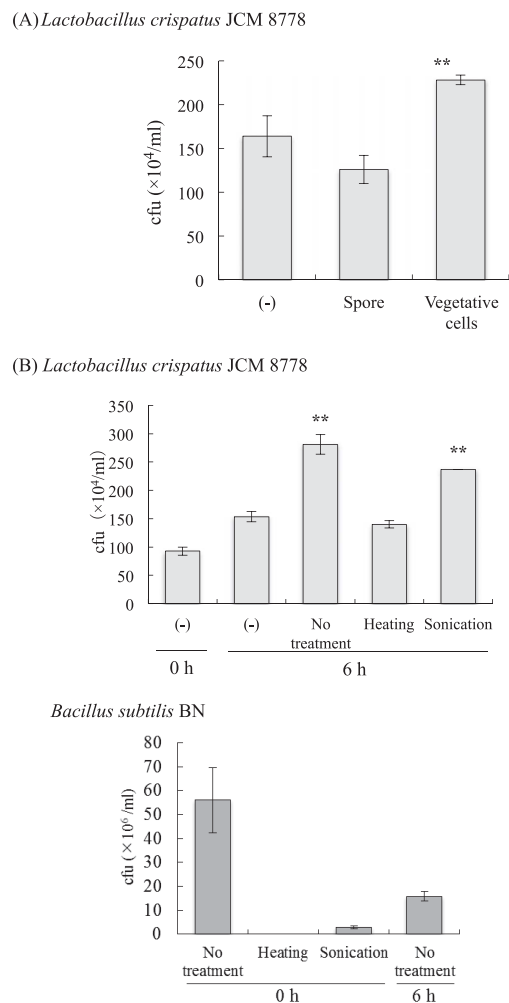for lactobacilli of animal origin (Benno, 1991). These observations suggest that the degradation of starch by *B. subtilis* is effective for improving the growth of intestinal lactobacilli.

Growth-improving effect on lactic acid bacteria: Growth-improving effect of spores

The effect of *B. subtilis* BN spores on the growth of lactobacilli was also examined. Nattokin powder and *L. crispatus* JCM 8778 were mixed and cultured for 6 h at 37°C. The growth-improving effect on lactobacilli was not observed with spores (Fig. 5A). This result suggests the possibility that the bacterial proteins, including the surface protein of vegetative cells, mediate the growth-improving effect.
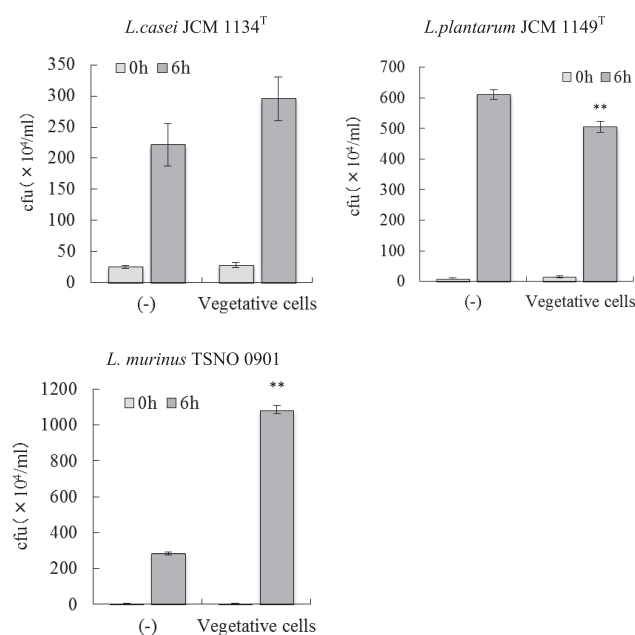
Fig. 6. Growth-improving effect on lactobacilli by *B. subtilis* BN. Experiment 5; Pre-cultured *B. subtilis* BN (vegetative cell form) was added to MRS broth at a concentration of 1×10^7/mL. Subsequently, 10 μL of pre-cultured *L. casei* JCM 1134^T, *L. plantarum* JCM 1149^T, or *L. murinus* TSNO0901, was added to the MRS broth and cultured for 6 h at 37°C. The culture fluid was then spread on MRS agar plates, and the number of colonies were counted.

Growth-improving effect on lactic acid bacteria: Growth-improving effect by *B. subtilis* BN

Next, the growth-improving effect of *B. subtilis* BN was examined. Equal parts of *B. subtilis* BN and *L. crispatus* JCM 8778 were mixed in 10 mL of MRS broth and cultured for 6 h at 37°C. (Fig. 5B). Using these culture conditions, the effect of *B. subtilis* BN metabolism on lactobacilli growth was marginal because *B. subtilis* BN did not grow well in MRS broth. Additionally, the same experiments were performed using *B. subtilis* BN treated by heating at 95°C for 10 min or by sonication for 10 min. Results are shown in Fig. 5B. The growth of *L. crispatus* JCM 8778 was improved by co-culture with untreated and sonicated *B. subtilis* BN. Although the sonication did not affect the growth-improving effect, heated *B. subtilis* BN had no effect on lactobacilli growth. These observations suggested the possibility of the involvement of bacterial components, such as surface proteins, other than metabolites in the growth-improving effect. Additionally, living bacteria were not always necessary to mediate this effect. Thus, the surface protein(s) of the bacteria might be involved in improving the growth of lactobacilli.

This effect was lost through denaturation of the surface protein, which would have been caused by heat-treated *B. subtilis* BN. Moreover, the growth-improving effect of *B. subtilis* BN was also examined using other *Lactobacillus* strains. Three types of lactobacilli, which were isolated from different sources, were used. Specifically, *Lactobacillus casei* JCM 1134 was isolated from cheese, *Lactobacillus plantarum* JCM 1149 was isolated from pickled cabbage, and *Lactobacillus murinus* TSNO 0901 was isolated from mouse feces. Interestingly, the growth-improv-

ing effect was not observed on lactobacilli of a food origin (Fig. 6). In contrast, the growth of intestinal (feces origin) lactobacilli was improved.

Growth inhibitory effect of *B. subtilis* BN on *Salmonella enterica*

The effect of *B. subtilis* BN on *S. enterica*, which is a major food-borne pathogen, was examined. Equal numbers of *B. subtilis* BN and *S. enterica* JCM 1652 were inoculated in nutrient broth and cultured at 37°C for 6 h with shaking. The culture fluid was then spread on MLCB agar plates, and black colonies that formed were counted as *S. enterica*. The growth of *S. enterica* was inhibited through co-culture with *B. subtilis* BN (Fig. 7). The growth inhibitory effect was observed for both vegetative cells and spores of *B. subtilis* BN.

Discussion

In the present study, it has been shown that *B. subtilis* BN has both probiotic and prebiotic potential. Recently, many probiotic strains have been reported. To maintain good conditions for the intestinal flora, increasing the colonization of beneficial bacteria, such as lactic acid bacteria, is important. In recent years, many beneficial bacteria have been reported. For some probiotic effects, such as immunostimulatory actions, living bacteria are not always necessary, as this effect has been observed using dead bacteria (Goto et al., 2013). On the other hand, in many cases, living bacteria are necessary for other probiotic effects, such as intestinal regulation and the prevention of food poisoning. Access of living bacteria to the intestine, and subsequent adhesion and colonization, are important for bacteria to exert beneficial effects. It is also important that these beneficial strains become the predominant species in the intestinal flora. However, this is sometimes virtually impossible, because beneficial probiotic strains do not always have the ability to effectively adhere to intestine and colonize there. For maintaining the health of the host, promoting the colonization of native lactic acid bacteria in the intestinal flora and maintaining a good floral balance is preferable, in addition to the introduction of specific foreign probiotic strains. In the present study, *B. subtilis* BN promoted the growth of lactobacilli. These observations suggest the possibility that *B. subtilis* BN preferentially supports the growth of native lactobacilli in the host intestine. In contrast, *B. subtilis* BN exhibited adverse effects on *S. enterica*. The growth-improving effect on lactobacilli was observed only with the vegetative cell form of *B. subtilis* BN. In contrast, the growth inhibitory effect on *S. enterica* was observed using both the vegetative cell and spore forms. Therefore, the mechanisms of growth improvement and growth inhibition might be different. Since the growth-improving effect on lactobacilli was lost through heating, it is possible that the surface protein of *B. subtilis* BN is involved in mediating this effect. However, we have not determined the mechanism of growth improvement and growth inhibition by *B. subtilis* BN at this time. In order to exert these effects, viable *B. subtilis* BN must have access to the intestine. If *B. subtilis* BN were ingested in vegetative cell form, most

(A) *S.enterica* JCM 1652
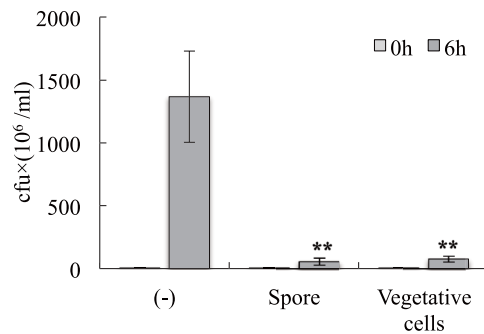
Bacillus subtilis BN
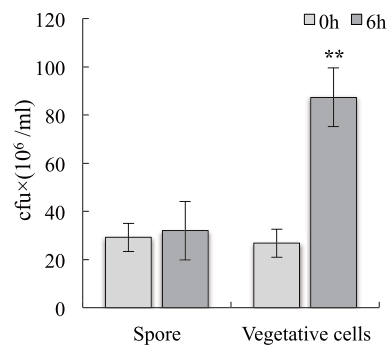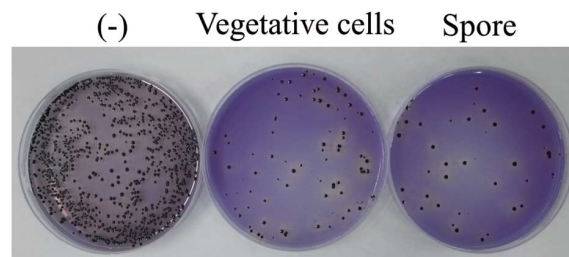

(B) Grown colonies on the MLCB agar plate

(-)   Vegetative cells   Spore

Fig. 7. Growth-inhibitory effect on *S. enterica* by *B. subtilis* BN.

(A) Pre-cultured *B. subtilis* BN (vegetative cells) or Nattokin powder (spores) was added to nutrient broth at a concentration of 1×10^7/mL. Moreover, *S. enterica* JCM 1652 was added at a concentration of 1×10^7/mL and cultured for 6 h at 37°C. The culture fluid was then spread on MLCB agar plates for *S. enterica* JCM 1562 and LB agar plates for *B. subtilis* BN, and the number of colonies were counted. (B) Colonies on an MLCB agar plate. The black colonies on MLCB agar plates were considered to be *Salmonella enterica*.

bacteria would be killed by the acidic conditions in the stomach. However, if *B. subtilis* BN was ingested as a spore, bacteria could travel through the stomach in living form. When *B. subtilis* BN is consumed as spores, sufficient numbers of bacteria could reach the intestine and become vegetative cells. The vegetative cell form enhances the growth of intestinal lactobacilli and inhibits the growth of pathogenic bacteria such as *S. enterica*. Therefore, it is possible that intestinal flora could be improved, and healthy conditions could be maintained by *B. subtilis* BN. From a probiotic perspective, *B. subtilis* BN, ingested as spores, would be more effective than when taken in as fermented soybean "natto". Although *B. subtilis* BN did show probiotic and prebiotic-like effects, whether these effects of *B. subtilis* BN also occur *in vivo* remains unknown, and whether these effects are strain-specific or generic features of *B. subtilis* is unclear. Although some

questions remain, *B. subtilis* BN has the potential to promote human intestinal health.

Conflict of Interest

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