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Full Paper

Adhesion mechanisms of *Bifidobacterium animalis* subsp. *lactis* JCM 10602 to dietary fiber

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Adherence of probiotics to dietary fibers present in the intestinal tract may affect adhesion to intestinal epithelial cells. The properties of the adhesion of bifidobacteria to mucin or epithelial cells have been well studied; however, adhesion of bifidobacteria to dietary fiber has not been investigated. The adhesion ratio of six *Bifidobacterium* strains to cellulose and chitin was examined; among the strains, *Bifidobacterium animalis* subsp. *lactis* JCM 10602 showed high adherence to both cellulose and chitin, and two strains showed high adherence to only chitin. The ratios of adhesion of *B. animalis* to cellulose and chitin were positively and negatively correlated with ionic strength, respectively. These data suggest that hydrophobic and electrostatic interactions are involved in the adhesion to cellulose and chitin, respectively. The adhesion ratios of the cells in the late logarithmic phase to cellulose and chitin decreased by approximately 40% and 70% of the cells in the early logarithmic phase, respectively. Furthermore, the adhesion ratio to cellulose decreased with increasing bile concentration regardless of the culture phase of the cells. On the other hand, the adhesion ratio to chitin of cells in the early logarithmic phase decreased with increasing bile concentration; however, that of cells in the late logarithmic phase increased slightly, suggesting that adhesins differ depending on the culture phase. Our results indicated the importance of considering adhesion to both dietary fibers and the intestinal mucosa when using bifidobacteria as probiotics.

Key words: Bifidobacterium, cellulose, chitin, adhesion, bile, growth phase

INTRODUCTION

A large number of diverse microorganisms form the intestinal microbiota and exert multiple physiological effects on the host. In particular, bifidobacteria and lactic acid bacteria have beneficial effects on the host, such as regulation of intestinal function [1, 2] and the immune system [3, 4]. These bacteria are defined as probiotics, "living microorganisms, which when administered in adequate amounts confer a health benefit on the host" [5]. These health benefits occur due to interactions between the probiotics and the host; thus, it is important to consider the phenomenon of adhesion of probiotics to the intestinal tract.

Intestinal bacteria adhere to the intestinal tract by recognizing carbohydrate moieties of mucin, which is the main component of the mucus layer covering the intestinal epithelial cells. Many studies have shown that specific cell-surface proteins act as adhesion factors, known as adhesins [6]. Several bacterial adhesins have been identified in bifidobacteria, including type IVb tight adherence pili of *Bifidobacterium breve* UCC2003 [7], transaldolase of *Bifidobacterium bifidum* A8 [8], DnaK of *Bifidobacterium animalis* subsp. *lactis* Bi-07 [9], BopA (lipoprotein) of *B. bifidum* MIMBb75 [10], and exopolysaccharides (EPSs) of *B. animalis* IPLA-R1 [11]. Lactic acid bacteria express specific adhesins, including of *Lactobacillus reuteri* 1063 [12], Spa pili of *Lactobacillus rhamnosus* GG [13], and GroEL of *Lactobacillus johnsonii* NCC53 [14]. In particular, proteins that were primarily identified as metabolic enzymes and chaperones (*e.g.*, transaldolase, GroEL and DnaK) are called moonlighting proteins because they act as adhesins on the cell surface.

In the human intestine, there are also dietary fibers from the diet. Dietary fibers are classified into two groups: soluble dietary fibers such as galactooligosaccharides and insoluble dietary fibers such as cellulose. The majority of soluble dietary fibers are metabolized by bacteria in the large intestine to short-chain fatty acids such as butyric acid and propionic acid [15], which can be used as energy sources for colonic epithelial cells and also inhibit

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the growth of pathogens [16]. Insoluble dietary fibers are excreted out of the body by intestinal peristalsis, which is activated to promote fecal excretion; additionally, bile acid that has flowed into the large intestine without being reabsorbed is adsorbed by insoluble dietary fiber and excreted outside the body [17, 18].

Considering that both dietary fiber and mucin have sugar chains as basic structures, the adhesion of probiotics to dietary fiber may affect whether or not it settles in the intestinal tract. Fernando *et al.* mentioned the possibility that dietary fiber can be a vehicle that carries lactic acid bacteria and bifidobacteria, which adhere to dietary fiber, to the intestinal tract [19]. We previously reported that cytoplasmic proteins such as DnaK and glyceraldehyde-3-phosphate dehydrogenase are present on the cell surface of *Lactococcus lactis* IL 1403 and that they also have an affinity for mannan [20]. Subsequent studies have elucidated that several lactic acid bacteria strains adhere to cellulose, a typical insoluble dietary fiber (unpublished data).

The aim of this study was to understand the properties of the adhesion of bifidobacteria abundant in the intestinal tract to the typical dietary fibers cellulose and chitin. We examined whether several bifidobacteria strains derived from the human intestinal tract, feces, and milk products adhered to dietary fiber and then analyzed the effects of pH and bile on the adhesion.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Six *Bifidobacterium* strains were purchased from the Japan Collection of Microorganisms (JCM) of RIKEN BioResource Research Center (Table 1). All strains were cultured anaerobically at 37°C in a de Man, Rogosa, Sharpe (MRS) broth (10.0 g Bacto peptone, Becton, Dickinson and Company [BD], Tokyo, Japan; 10.0 g Bacto beef extract, BD; 5.0 g Bacto yeast extract, BD; 20.0 g glucose; 1.0 g polyoxyethylene (20) sorbitan monooleate; 2.0 g triammonium citrate; 5.0 g sodium acetate; 0.2 g magnesium sulfate heptahydrate; 0.08 g manganese(II) sulfate pentahydrate; and 2.0 g dipotassium hydrogenphosphate per liter) containing 0.05% cysteine, using an AnaeroPack system (Mitsubishi Gas Chemical Company, Tokyo, Japan).

Adhesion assay

The adhesion ratio of bifidobacteria cells to dietary fiber powder was calculated using the difference in sedimentation rate between the cells and the powder. Since dietary fiber powder settles faster than cells, the percentage of cells that adhered and co-settled with dietary fiber powder was calculated.

Cellulose (Nacalai Tesque, Kyoto, Japan) and chitin (Fujifilm Wako Pure Chemical, Osaka, Japan) powders were used as insoluble dietary fibers. Powders with a diameter of 38–100 μm were selected by passing them through sieves and suspended at 30 mg/mL in PC buffer (5.2 mM citrate, 5.8 mM sodium phosphate, 150 mM NaCl) adjusted to pH 5.0 or 7.0 with NaOH. Then, decantation was performed three times to remove fine particles which did not settle naturally. When changing the ionic strength, PC buffer was diluted 1, 3, 9, and 30 times, resulting in ionic strengths of 193, 64, 21, and 6.4 mmol/L, respectively. When investigating the effects of bile on the adhesion of bacteria to fiber, bile (Fujifilm Wako Pure Chemical, Osaka, Japan) was adjusted to 2 g/L with PC buffer and diluted 10 to 1,000 times. The cells were washed twice with PC buffer (9,500 × *g*, 5 min, 4°C) and then suspended at an optical density at 660 nm (OD_{660}) of 2.

Table 1. *Bifidobacterium* strains used in this study

Strains	Origin
Bifidobacterium animalis subsp. *lactis* JCM 10602	Milk product
Bifidobacterium longum subsp. *infantis* JCM 1222	Intestine of infant
Bifidobacterium longum subsp. *longum* JCM 1217	Intestine of adult
Bifidobacterium breve JCM 1192	Intestine of infant
Bifidobacterium pseudocatenulatum JCM 1200	Feces of infant
Bifidobacterium angulatum JCM 7096	Feces of adult

Equal volumes (0.5 mL each) of the cell suspension and the cellulose or chitin powder suspension were mixed in a microtube and incubated at 37°C for 10 min with rotation at 6 rpm. After centrifugation (500 × *g*, 10 sec), the mixture was left for 5 min. A 0.5 mL supernatant sample was collected from the top, and the OD_{660} was measured by using a Ratio Beam Spectrophotometer U-5100 (Hitachi, Tokyo, Japan). To calculate the adhesion ratio, which was the percentage of cells that adhered to the dietary fiber powder, the OD_{660} was compared with the OD_{660} of two controls containing (i) bacteria but no dietary fiber powder and (ii) dietary fiber powder but no bacteria. The adhesion ratio of bifidobacteria to dietary fiber was defined as

$$\text{adhesion ratio}\ (\%) = \left(1 - \frac{A-B}{C}\right) \times 100,$$

where A represents the OD_{660} of the supernatant from the microtube containing a dietary fiber powder plus cells, B represents the OD_{660} of the supernatant from a control tube containing a dietary fiber powder but no cells, and C represents the OD_{660} from a control tube containing cells but no dietary fiber powder.

Statistical analysis

All data are expressed as the mean ± standard error (SE). The level of statistical significance was set at $p<0.05$ based on Student's t-test or Tukey's test.

RESULTS

Adhesion of Bifidobacterium *strains to dietary fiber*

First, the adhesion ratios of six human intestine *Bifidobacterium* strains to cellulose and chitin were examined at pH 7.0 (Fig. 1). *B. animalis* subsp. *lactis* JCM 10602 showed high adhesion ratios to both dietary fibers under conditions in which the adhesion sites of these dietary fiber powders were not saturated (Supplementary Fig. 1). *Bifidobacterium pseudocatenulatum* JCM 1200 and *Bifidobacterium longum* subsp. *longum* JCM 1217 also exhibited high adhesion ratios to chitin; however, all of the tested strains, except *B. animalis*, showed low adhesion to cellulose ratio of less than 10%. Each *Bifidobacterium* exhibited different properties of adhesion to dietary fiber, and our further studies of the phenomenon of adhesion of bifidobacteria to dietary fiber were performed using *B. animalis* subsp. *lactis* JCM 10602, which exhibited a high adhesion ratio to both cellulose and chitin.

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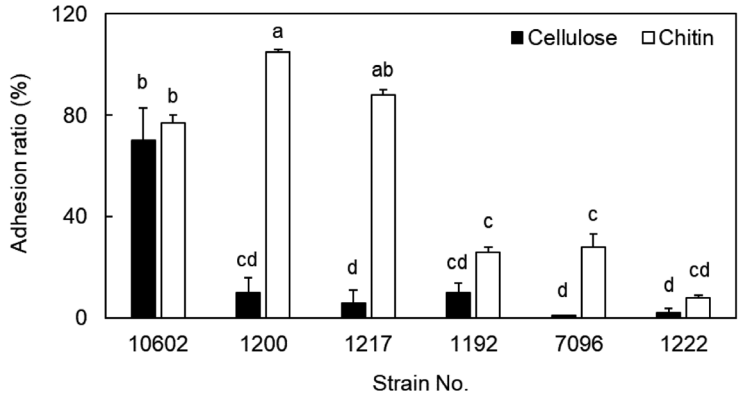

Fig. 1. Adhesion ratios of *Bifidobacterium* strains to dietary fiber. Filled columns, cellulose; open columns, chitin. Adhesion ratios (%) are presented as means ± standard error of triplicate samples. Bars with the same letters are not significantly different from each other ($p<0.05$) by Tukey's test.

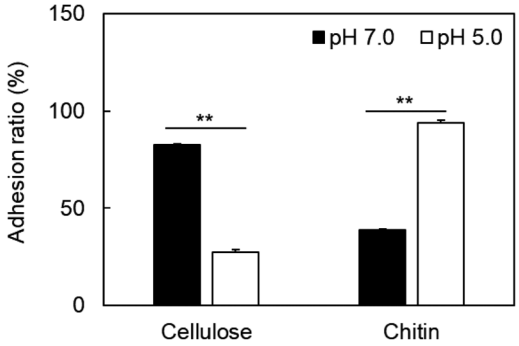

Fig. 2. Effect of pH on adhesion of *B. animalis* to dietary fiber. **p<0.01 by Student's t-test. Filled columns, pH 7.0; open columns, pH 5.0.

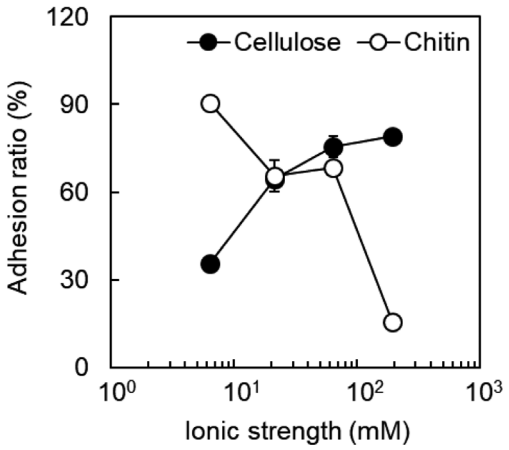

Fig. 3. Effect of ionic strength on adhesion of *B. animalis* to dietary fiber (pH 7.0). Closed circles, cellulose; open circles, chitin.

Effect of pH on the adhesion of B. animalis *to dietary fiber*

When bifidobacteria produce organic acids such as acetic acid in the intestine, the pH is considered to be locally decreased. Therefore, the adhesion ratio of *B. animalis* to dietary fiber was examined not only at the average human intestinal pH of 7.0 but also at pH 5.0. The adhesion ratio to cellulose at pH 7.0 was 82%, whereas at pH 5.0, the ratio was 27%. On the other hand, the adhesion ratio to chitin was 39% at pH 7.0 but increased to 94% at pH 5.0 (Fig. 2). Therefore, the data suggested that the mechanisms of adhesion of *B. animalis* to cellulose or chitin were different.

Effect of ionic strength on the adhesion of B. animalis *to dietary fiber*

In order to estimate what interactions are involved in the adhesion of *B. animalis* to dietary fiber, the effects of ionic strength on the adhesion ratio were investigated. The adhesion ratio to cellulose decreased with decreasing ionic strength (Fig. 3). It was confirmed that glucose, sucrose, and lactose (10 g/L) do not inhibit the adhesion of *B. animalis* to cellulose (data not shown). Considering that crystalline cellulose has a hydrophobic region [21] and that the hydrophobic interaction becomes stronger with increasing ionic strength, the results suggested that a hydrophobic interaction occurs between the bacterial cell and cellulose. On the other hand, the adhesion ratio to chitin increased with decreasing ionic strength. It has been reported that part of the acetamide group of chitin is converted to an amino group and is positively charged [22]. The surface of Gram-positive bacteria is negatively charged due to the presence of phosphate groups contained in lipoteichoic acid (LTA), which possess glycolipids anchored in the cell membrane and a repeating structure of glycerol phosphate that crosses the cell wall [23]. Therefore, it was suggested that the Coulomb force acts between *B. animalis* and chitin and that the difference in the adhesion ratio to chitin among other bifidobacteria depends on the structure or degree of surface exposure of LTA.

Effects of growth phase on the adhesion of B. animalis *to dietary fiber*

The effects of the cell culture phase on the adhesion of *B. animalis* to dietary fiber were evaluated. Various components, such as sortase-dependent pili and surface-associated EPSs, are

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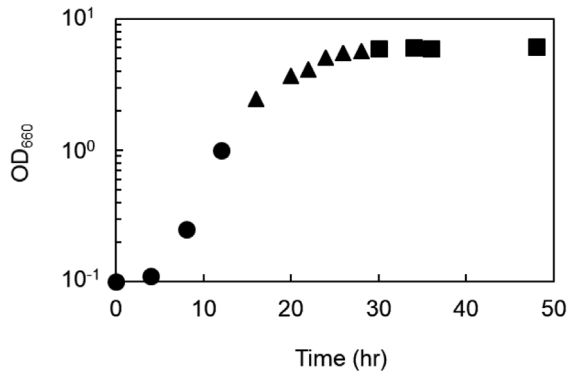

Fig. 4. Time course of the growth of *B. animalis*. Circles, triangles, and squares indicate the values of OD_{660} of the culture broth in different runs.

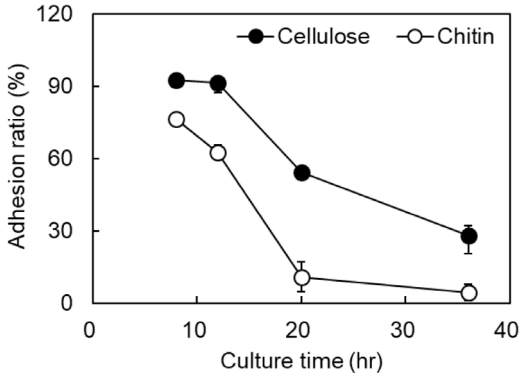

Fig. 5. Effect of culture time on adhesion of *B. animalis* to dietary fiber (pH 7.0). Closed circles, cellulose; open circles, chitin.

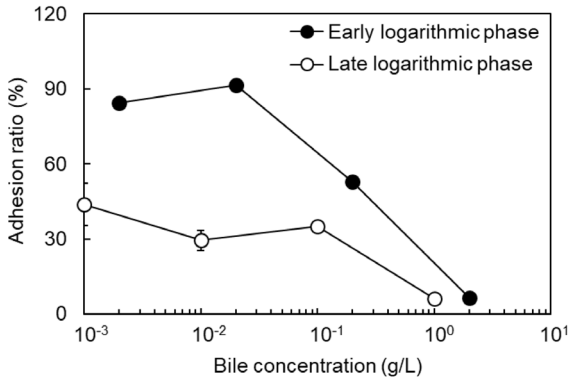

Fig. 6. Effect of growth phase and bile on adhesion of *B. animalis* to cellulose (pH 7.0). Closed circles, early logarithmic phase cells; open circles, late logarithmic phase cells.

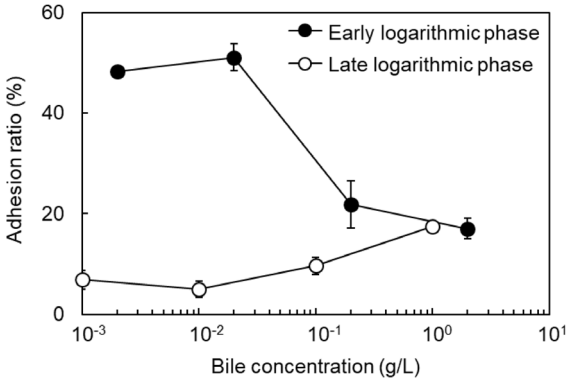

Fig. 7. Effect of growth phase and bile on adhesion of *B. animalis* to chitin (pH 7.0). Closed circles, early logarithmic phase cells; open circles, late logarithmic phase cells.

exposed on the cell wall of bifidobacteria [24]. It is also known that sialidase, which utilizes sialylated mucin and acts as an adhesion factor for mucin, is embedded in peptidoglycan as the culture time progresses [25]. Therefore, based on the growth curve of *B. animalis*, the effects of culture phase on the adhesion ratio to dietary fibers were investigated using cells in the early logarithm (8 hr), middle (12 hr), late (20 hr), and stationary phases (36 hr) (Fig. 4). The adhesion ratio to dietary fiber decreased as the culture progressed (Fig. 5). This result suggested that the state of the cell surface involved in the adhesion to dietary fiber of *B. animalis* also changed as the culture progressed.

Effects of bile on the adhesion of B. animalis *to dietary fiber*

Based on the possibility that hydrophobic interactions were involved in the adhesion of *B. animalis* to dietary fibers (Fig. 3), the effects of bile on the adhesion ratio of the cells in the early and late logarithmic phase were investigated. Bile is mainly composed of bile acids such as cholic acid and chenodeoxycholic acid and forms micelles with diet-derived fat to promote absorption of lipophilic components. If the adhesion of cells to dietary fibers was due to hydrophobic interaction, it would be assumed that bile inhibits the adhesion. The adhesion ratio to cellulose decreased with increasing bile concentration, regardless of the culture phase (Fig. 6). Therefore, it was confirmed that hydrophobic interaction is dominant in the adhesion of *B. animalis* to cellulose. On the other hand, the adhesion ratio to chitin was reduced with increasing bile concentration in early logarithmic phase cells and was slightly increased in late logarithmic phase cells (Fig. 7). Therefore, it was considered that hydrophobic interaction and electrostatic interaction were dominant in the adhesion of the cells to chitin in the early logarithm and late logarithm phases, respectively.

DISCUSSION

There have been several studies on the adhesion of bifidobacteria to intestinal epithelial cells and mucin [26–29]. Since bacteria recognize sugar chains of mucin and adhere to the intestinal tract, the effect of soluble dietary fiber with sugar chain structures such as oligosaccharides on adhesion has been investigated [30, 31]. When bacteria adhere to insoluble dietary fiber, they may not be able to adhere to the intestinal tract; however, there are no reports referring to the adhesion of probiotics to dietary fibers from that perspective. Therefore, in this study, we examined the properties of the adhesion of bifidobacteria to insoluble dietary fiber, as well as the factors that affect them.

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The six *Bifidobacterium* strains showed different properties of adhesion to cellulose or chitin. In particular, *B. animalis* subsp. *lactis* JCM 10602 exhibited a high adhesion ratio to both cellulose and chitin. As various adhesins involved in intestinal epithelial cells and mucins have been reported in several bifidobacteria [7–11], it was suggested that several adhesins were involved in the adhesion to dietary fiber and that the adhesins involved depend on each *Bifidobacterium*. We also showed that the adhesion of *B. animalis* to cellulose involved a hydrophobic interaction between the hydrophobic region of cellulose and the bacterial cells. It has been reported that the adhesion of bifidobacteria to intestinal epithelial cells is correlated with the hydrophobicity of the bacterial cell surface [32–34], suggesting that these adhesins may contribute to the adhesion to dietary fiber.

Furthermore, the adhesion ratio of *B. animalis* to dietary fiber decreased as the culture time progressed. It has been reported that the adhesion ratio of *B. bifidum* S17 to Caco-2 cells is more than 2-fold lower in the stationary phase cells compared with the log phase cells and that the RNA expression levels of proteins such as pili decreased [35]. It has also been reported that LTA of *Lactobacillus gasseri* JCM 1131 is embedded in the cell wall during the logarithmic growth phase; however, it is exposed outside the cells because of cell wall degradation during the stationary and death phases [36]. Therefore, even if the same bacteria are used, the cell surface structure may change during the growth process; it is necessary to consider these contributions when considering adhesion of bifidobacteria to dietary fibers. Incidentally, it is thought that the attachment of bifidobacteria to these insoluble dietary fibers is not a beneficial phenomenon for bifidobacteria. This is because bifidobacteria cannot be assimilated even when attached to insoluble dietary fiber; they are excreted from the intestinal tract, which is rich in substrates. Therefore, it is possible that the bifidobacteria unintentionally adhere to dietary fibers as a result of increased adherence to the intestinal tract.

Bile is made in the liver, secreted into the duodenum, reabsorbed from the small intestine, and shunted back to the liver, while unabsorbed bile flows to the large intestine [37]. Therefore, bile as a surfactant is expected to prevent hydrophobic adhesion of bifidobacteria to cellulose. Our results revealed that the adhesion ratio of *B. animalis* to cellulose decreased to less than 10% with the addition of 1–2 g/L bile. Although the total bile concentration in the intestine is 1–10 g/L, the free bile concentration varies depending on the region of the intestinal tract and the amount of ingested lipid. It is thought that when the amount of bile secretion increases and the free bile concentration increases, bile inhibits the adhesion of bifidobacteria to insoluble dietary fibers. Conversely, the free bile concentration is low in the large intestine after the bile is reabsorbed, so it is hypothesized that bifidobacteria easily adhere to insoluble dietary fiber. Therefore, bile is one of the important factors affecting the adhesion of bifidobacteria to dietary fibers. It is also necessary to understand the effect of bile on the adhesion of bifidobacteria to intestinal epithelial cells.

In addition, since bile acids also show bactericidal effects, stress responses and resistance mechanisms against them are induced in the cells and on the cell surfaces of live intestinal bacteria [38–40]. Therefore, many studies have been conducted on the correlation between the bile acid resistance of bifidobacteria and adhesion to the intestinal tract. For example, bifidobacteria were exposed to bile acids to confer bile acid resistance, and this resulted in a 1.4- to 4-fold increase in the adhesion ratio to mucin [41]. In addition, the acquisition of bile acid resistance by bifidobacteria changed the expression levels of substances that can be adhesins [42]. On the other hand, for bifidobacteria, the bile resistance phenotype was not related to improvement of *in vitro* adhesion capability after gastrointestinal tract transit [43]. In the future, it will be necessary to consider the effects of resistance to bile acids on the adhesion of bifidobacteria to dietary fiber.

In conclusion, this study elucidated that the adhesion of *B. animalis* subsp. *lactis* JCM 10602 to dietary fiber involves hydrophobic interaction and the Coulomb force and that it depends on the growth phase and bile concentration. By clarifying the adhesion of *B. animalis* to dietary fiber, we showed the importance of considering adhesion to dietary fiber as well as adhesion to the intestinal mucosa when using *Bifidobacterium* as a probiotic.

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