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Adhesion Conditions of *Bifidobacterium pseudocatenulatum* KAKii to Human Enterocyte-like Caco-2 Cell Lines

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Abstract

Attachment ability of bifidobacteria strains to the human intestinal surface is an important criterion as a probiotic candidate. However, attachment activity is influenced by external and internal conditions. This study was conducted to screen cell surface hydrophobicity and adhesion scores of bifidobacteria strains. Attachment conditions (pH and exposure time) of selected strains to human enterocyte-like Caco-2 cell lines were subsequently investigated. Three different solvents (n-hexadecane, Toluene, and Xylene) were used in cell surface hydrophobicity analysis. Based on the results obtained, xylene presented consistent cell hydrophobicity activity in all strains used. *Bifidobacterium pseudocatenulatum* KAKii (wild type strain) gave promising cell hydrophobicity activity with no significant difference ($p > 0.05$) when compared to *Lactobacillus plantarum* NBRC 3070 with xylene as a solvent, and also presented a significantly higher attachment score ($p < 0.05$) compared to all strains used. The influence of pH and time exposure on adhesion of *B. pseudocatenulatum* KAKii to Caco-2 cells revealed that this strain was favored to attach to the intestinal cell line at pH 6 and after 120 min of exposure. Further optimization of attachment conditions will be carried out.

Keywords: *Bifidobacterium* strains, cell-surface hydrophobicity, pH conditions, time of exposure, Caco-2 cell lines

Introduction

Probiotics are defined as beneficial microorganisms that are able to give benefits to human health [1]. The number of live probiotics in the human gastrointestinal tract (GIT) decreases as humans grow older, due to food consumption, lifestyle, environment, and surrounding effects. The presence of probiotics is reported to inhibit the colonization of harmful pathogens and increase nutrient uptake by the human body [2,3]. A probiotic must be able to attach to and colonize the human GIT. The attachment of probiotics to the surface of the human intestine plays an important role in their being able to exert their healthful effects [4]. To date, *Bifidobacterium* strain is one of the most well-known probiotics and has attracted researcher interest. *Bifidobacterium* strain is a common probiotic which has been isolated from healthy infant feces [5] and is believed to colonize our human gastrointestinal tract (GIT) [3,6]. *Bifidobacterium* strain is a strict anaerobic Gram positive bacterium, generally rod-branched in shape. The variety of *Bifidobacterium* strains and subspecies explains the different morphology between these bacteria.

Cell surface hydrophobicity is a preliminary test to observe the interaction between *Bifidobacterium* strains and hydrocarbons. Various compositions of bacterial cell walls, consisting of hydrophobic and hydrophilic substances, mediate the initial contact between the bacteria and the human intestine [7]. One

of the most common methods used to screen the interaction is Microbial Adhesion To Hydrocarbons (MATH), which was first developed by Rosenberg in 1980 [8,9]. The most applicable solvents used in MATH are n-hexadecane, toluene and xylene [9,10].

Several *in vitro* approaches have been developed in order to assess the adhesion ability of probiotics, with the use of cell line cultures such as Caco-2, HT-29, and HT-29 MX [1,11]. Caco-2 cell line is an *in vitro* model that has been widely used by many researchers to study the interaction mechanisms between bacteria and the human gastrointestinal tract (GIT) [12,13]. Caco-2 cell line originates from human colonic adenocarcinoma cells and is normally used in attachment studies of bacteria *in vitro*.

Many commercialized probiotics have been utilized in other industries, such as the dairy and food industries [14]. However, there is a lack of research on the adhesion mechanism between wild-type *Bifidobacterium* strains and GIT cell lines. Therefore, it is imperative to justify the capability of potential wild-type *B. pseudocatenulatum* KAKii to adhere to the human intestinal tract. This understanding will enhance the fundamental knowledge on the wild-type strain to become a substitute probiotic to be introduced and applied in future commercialization. The aim of this study is to investigate the adhesion activity between wild-type *B. pseudocatenulatum* KAKii to the GIT model Caco-2 cells.

Materials and methods

A local isolate *B. pseudocatenulatum* KAKii was used in this study and was grown on de Man, Rogosa and Sharpe (MRS) broth supplemented with 0.25 % (w/v) L-cysteine (Merck, Germany). The identification of the strain was done by using 16S rRNA polymerase chain reaction (PCR) prior to gene sequencing [15]. The strain was prepared for the late exponential phase by growing the cell in MRS media supplemented with L-cycteine for 12 - 16 h at 37 °C under anaerobic conditions. Initial strain concentration was maintained at approximately 10^8 CFU/mL prior to attachment. Probiotic strains used in this study are shown in **Table 1**. *Bifidobacterium pseudocatenulatum* B1279 (ATCC: 27919) and *Lactobacillus plantarum* NBRC 3070 (ATCC: 8014) were selected as comparative strains.

Table 1 Bacterial strains used in this study

Bacterial strains	Sources
L. plantarum NBRC 3070	Commercial
B. pseudocatenulatum B1279	Commercial
B. pseudocatenulatum KAKii	Infant feces

Human colon adenocarcinoma Caco-2 cell line (HTB-37) was purchased from American Type Culture Collection (ATCC). The cells were cultivated and cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM; NECC, Sigma, Basel, Switzerland) supplemented with 10 % (v/v) fetal bovine serum, 100 U mL^{-1} penicillin, and 100 mg mL^{-1} streptomycin (Sigma, Switzerland) at 37 °C in atmosphere at 5 % CO_2 - 95 % air. For adhesion assays Caco-2 monolayers were prepared on glass cover slips and placed in tissue culture plates. The cells were maintained until they reached 80 - 90 % confluency to be used in adhesion assays. The cell culture media was changed every other day and replaced by fresh non-supplemented DMEM at least 3 h before the adhesion assays.

Cell surface hydrophobicity of *B. pseudocatenulatum* KAKii was determined by Microbial Adhesion To Hydrocarbons (MATH) using n-hexadecane, toluene, and xylene as the solvents. Briefly, *B. pseudocatenulatum* KAKii was grown in MRS broth for 16 - 18 h at 37 °C. Cultures were harvested by centrifugation (2000 X g, 15 min, 4 °C), washed twice in PUM buffer (K_2HPO_4: 22.2 g/L; KH_2PO_4: 7.26 g/L; urea: 1.8 g/L; $MgSO_4$: 0.2 g/L; pH 7.1 ± 0.2), and finally suspended in the same buffer. The initial absorbance (A0) at 600 nm of the suspension was adjusted to 0.70 ± 0.02 units. Five mL of cell suspension in PUM buffer was dispensed in clean and dry round bottom test tubes, followed by the

addition of one mL of n-hexadecane, toluene, or xylene. The contents were vortexed for 2 min. The tubes were left undisturbed for 1 h at 37 °C to allow phase separation. The lower aqueous phase was carefully removed with a sterile Pasteur pipette, and absorbance (A1) was recorded at 600 nm. Cell surface hydrophobicity in terms of per cent (H %) was calculated using the following formula;

$$H\ \% = \left(1 - \frac{A1}{A0}\right) \times 100, \quad (1)$$

All bacteria used in this study were grown in 10 mL of MRS broth under 37 ˚C for 24 h anaerobically. Cells with approximately 10^8 CFU/mL were harvested by centrifugation after 24 h of cultivation. The pellets were rinsed with phosphate-buffered saline (PBS) and resuspended in DMEM medium (pH 7) without antibiotics. The bacterial suspensions were added in a ratio of 1:10 bacteria to epithelial cells. The plates were incubated for 2 h at 37 °C in 5 % CO_2 in a CO_2 incubator. After the incubation period, the cell culture plates were washed 5 times with PBS (pH 7.2), fixed with methanol at room temperature, then dried under room conditions, with Gram staining performed. Adhesion of *B. pseudocatenulatum* KAKii to Caco-2 cells was detected using a light microscope by counting twenty randomized fields per cover slip. The results were expressed as an average number of bacteria adhered in twenty randomized fields per cover slip for each bacteria strain. Experiments were repeated at least twice and each sample on each plate was tested in duplicate. Percentage of adherence for each bacterium was calculated by using the following formula;

$$Adherence\ \% = \frac{Final\ number\ of\ bacteria}{Initial\ number\ of\ bacteria} \times 100, \quad (2)$$

During exposure of *B. pseudocatenulatum* KAKii to Caco-2 cells, 2 conditions of attachment were prepared; different pH, and time of exposure. Phosphate-buffered saline (PBS) was prepared and adjusted to different pH (5, 6, 7, and 8) by using 1 molar of hydrochloric acid (HCL) and sodium hydroxide (NaOH) prior to sterilization at 121 °C for 15 min. The bacteria used was exposed to different pH of PBS prior to attachment. Subsequently, bacteria used were exposed to Caco-2 cells at different time exposures (30, 60, 120, and 180 min).

All experiments were carried out at least in duplicate, and the results expressed as (mean ± SD). Standard deviations were also indicated as error bars in the graphs. Statistical analysis was performed using MINITAB software (Minitab Inc.). Statistical comparisons were made by one-way analysis of variance (ANOVA). In all statistical analysis, $p < 0.05$ was considered as statistically significant.

Results and discussion

As shown in **Table 2**, different strains with different solvents gave different percentages in hydrophobicity activity. Based on the results, it was revealed that *B. pseudocatenulatum* KAKii was able to adhere to hydrocarbons, as compared to other commercialized probiotics, with 8.01 % adhesion to n-hexadecane, 21.63 % adhesion to toluene, and 25.57 % adhesion to xylene.

Table 2 Cell surface hydrophobicity of different bacteria towards different solvents

Bacteria	n-hexadecane, H %	Toluene, H %	Xylene, H %
L. plantarum NBRC 3070	*40.31 ± 0.28[a]**	18.32 ± 0.42[a]	26.31 ± 0.81[a]
B. pseudocatenulatum B1279	10.08 ± 0.10[b]	13.93 ± 0.34[b]	34.43 ± 1.87[b]
B. pseudocatenulatum KAKii	8.01 ± 0.30[c]	21.63 ± 0.58[c]	25.57 ± 1.52[a]

Note: [a]Values are mean ± standard deviation of duplicate independent runs.

**Values in the same column with different letters were significantly different ($p < 0.05$).

The adhesion of probiotics to human GIT is one of essential criteria for the bacteria to give benefits to health. Adhesion ability of probiotic is strain dependent and is influenced by their physical and chemical characteristics [16,17]. Adherence activity involves a contact mechanism related to Van der Waals and electrostatic forces between bacteria and cell surfaces. The study of cell surface hydrophobicity is important in order to observe the possible interconnection between physico-chemical characteristics and the ability to adhere to the human intestine [18]. Different adhesion percentages obtained correlate with the hydrophobic and hydrophilic capacities of cell envelopes, and the usage of different solvents might also affect the adhesion percentages [19,20]. This might be due to different solvent structures affecting the hydrocarbon adherence of *B. pseudocatenulatum* KAKii. This finding is supported by researchers who have reported that, in different chemical compounds and compositions between bacteria, hydrophilic strains are poorly adhered, while hydrophobic bacteria are well adhered [9]. The results indicate that *B. pseudocatenulatum* KAKii is able to adhere to hydrocarbon, likewise, other commercialized probiotics, and shows no significance difference ($p > 0.05$) when xylene is used as a solvent (**Table 2**).

The adhesion of bacteria toward human gastrointestinal tract (GIT) cell line was demonstrated *in vitro* by using Caco-2 cell lines. **Table 3** shows the number of bacteria used that adhered to Caco-2 cell lines after 120 h exposure and at pH 5.6 conditions.

Table 3 Number of bacteria that adhered to Caco-2 cell lines

Bacteria	Number of bacteria, cells/ml		Adherence, %
	Initial	Final	
L. plantarum NBRC 3070	*$2.80\times10^{9} \pm 0.04$ [a]**	$3.30\times10^{7} \pm 0.02$ [a]	1.2 ± 0.07 [a]
B. pseudocatenulatum B1279	$2.76\times10^{9} \pm 0.04$ [a]	$3.70\times10^{7} \pm 0.01$ [a]	1.3 ± 0.04[a]
B. pseudocatenulatum KAKii	$1.81\times10^{9} \pm 0.01$ [b]	$2.90\times10^{7} \pm 0.03$ [b]	1.6 ± 0.03[c]

Note: [a]Values are mean ± standard deviation of duplicate independent runs.

** Values in the same column with different letters were significantly different ($p < 0.05$)

Based on the result obtained, *B. pseudocatenulatum* KAKii showed the highest adherence percentage as compared to other commercialized probiotics. This might be due to the source of *B. pseudocatenulatum* KAKii being isolated from healthy breast-fed infant feces, where the bacteria were more inclined to adhere to human GIT models, as compared to sub-cultured commercialized bacteria. The origin of the wild-type bacteria was correlated with the optimum surroundings and environment conditions, hence, affecting the highest reading observed [5,9].

Probiotics must be able to survive at low pH and gastric acidity, as these represent the conditions of the human intestine [19]. In addition, probiotics can adhere well to human intestine significantly in a given time period [1]. *B. pseudocatenulatum* KAKii was selected for further study on adherence condition optimization to Caco-2 cells, as shown in **Figure 1**. Prior to attachment *B. pseudocatenulatum* KAKii to Caco-2 cells, the bacterium was exposed to different pH conditions ranging from pH 5 to pH 8. It was observed that *B. pseudocatenulatum* KAKii exposure to pH 6 was preferred 6 prior to adherence towards Caco-2 cells, with the attachment observed relatively higher than other pH conditions of exposure (**Figure 1**). This might be due to pH 6 demonstrating the environment factors in the human GIT and suiting the conditions of adhesion between *B. pseudocatenulatum* KAKii towards Caco-2 cell lines [1]. This study was done in order to further identify the best time of attachment *B. pseudocatenulatum* KAKii to the gastrointestinal tract.

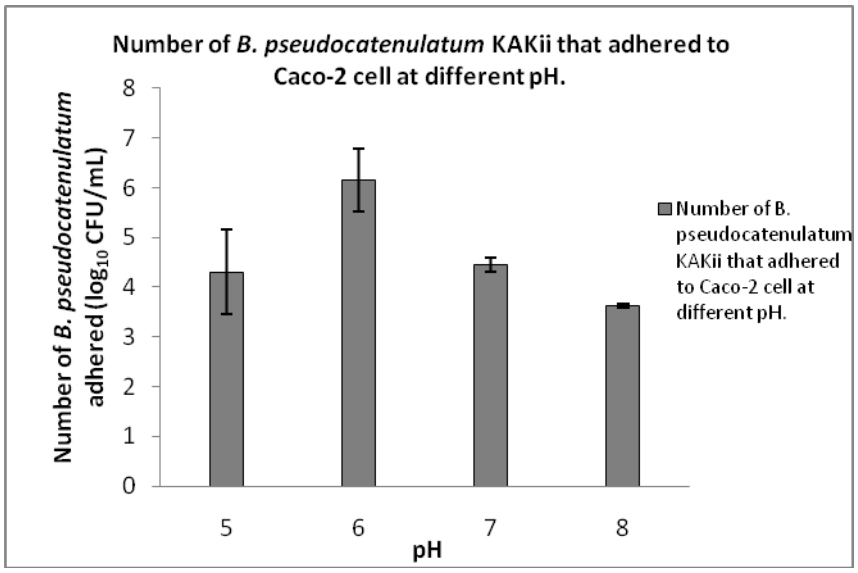

Figure 1 The adhesion ability of *B. pseudocatenulatum* KAKii to Caco-2 cell lines at different pH. The figure shows that *B. pseudocatenulatum* KAKii adhered well to Caco-2 cell lines at pH 6, while pH 8 showed poor adherence activity. Error bars show means ± standard deviation.

B. pseudocatenulatum KAKii was exposed to Caco-2 cells at different times of exposure after being treated at optimum pH (pH 6) prior to attachment to Caco-2 cells, with the results shown in **Figure 2**. Based on the results obtained, it was observed that 120 h of exposure was preferred for better attachment towards Caco-2 cells (**Figure 2**). The optimal time of exposure was needed by the bacteria for the adhesion activity to take place. This data proves that the optimal conditions for *B. pseudocatenulatum* KAKii were pH 6 and 120 min, with no significant differences with other studies [1,21]. This might be due to the mechanisms of signaling, penetration, and colonization by the probiotics, where better understanding could be obtained by further study on bacterial proteins [4-6,14,21]. The origin of *B. pseudocatenulatum* KAKii was from breast-fed infant feces, which are believed to be already acclimatized with GIT conditions, and this would assist the adherence activity of bacterium to the intestinal lining [1,5].

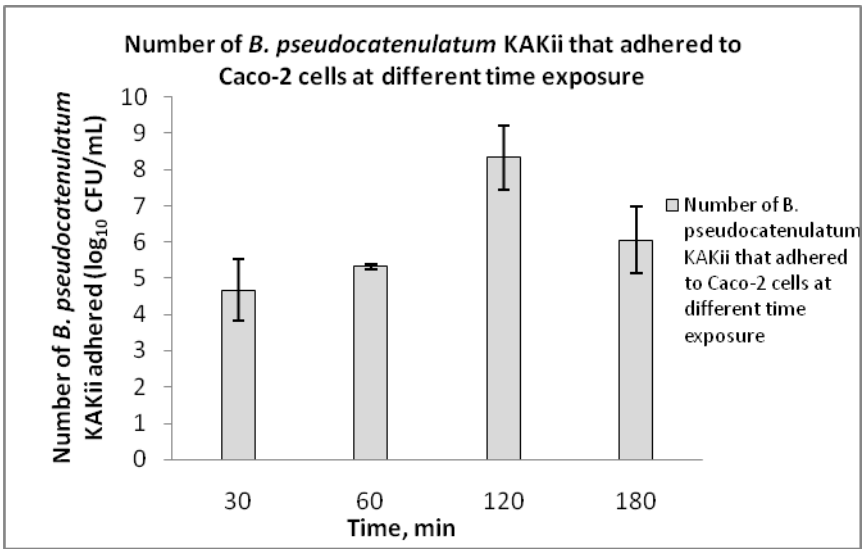

Figure 2 The adhesion ability of *B. pseudocatenulatum* KAKii to Caco-2 cell lines at different times of incubation. The figure shows that *B. pseudocatenulatum* KAKii adhered well to Caco-2 cell lines at 120 min of incubation, while 30 min of incubation showed poor adherence activity. Error bars show means ± standard deviation.

Conclusions

In conclusion, *B. pseudocatenulatum* KAKii was able to adhere to Caco-2 cell culture, as shown by other commercialized probiotics. Nonetheless, it has the potential to be a substitute of commercialized probiotics, based on the findings that the strain has the ability to adhere to hydrocarbons and cell lines, despite other factors such as temperature and types of cell lines used that may affect the attachment. Future research may explore the mechanisms involved in bacterium attachment towards Caco-2 cells, which include the expression of surface proteins and the biochemical pathways incorporated during the attachment.

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