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Identification of Synbiotics Conducive to Probiotics Adherence to Intestinal Mucosa Using an In Vitro Caco-2 and HT29-MTX Cell Model

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Abstract: The ability of bacteria to adhere to the intestinal mucosa is a critical property necessary for the long-term colonization of the intestinal tract. This ability can be highly sensitive to the presence of prebiotics. However, limited data are available in this respect for beneficial bacteria such as probiotics or resident gut microbiota. We previously demonstrated that the presence of prebiotics may decrease adherence in several pre- and probiotic combinations. Thus, characterizing the interactions between numerous combinations involving different classes of pre- and probiotics can be crucial in identifying new synbiotics. Accordingly, here, we extend our prior analyses to evaluate the adhesion of five lactobacilli, six bifidobacteria, and one probiotic *Escherichia coli* strains, as commercial probiotics or promising probiotic candidates, together with the cariogenic *Bifidobacterium dentium* strain. As an in vitro intestinal mucosa model, Caco-2 and mucin-secreting HT29-MTX cells were co-cultured at 9:1 in the presence or absence of prebiotics. Commercial inulin-type fructooligosaccharide prebiotics Orafiti[®] GR, Orafiti[®] P95, and galactooligosaccharide-based prebiotic formula Vivinal[®], including purified human milk oligosaccharides (HMOs) were added into the cultivation media as the sole sugar source (2.5% each). Adherence was tested using microtiter plates and was evaluated as the percentage of fluorescently labeled bacteria present in the wells after three washes. Consistent prebiotics-mediated enhanced adherence was observed only for the commercial probiotic strain *E. coli* O83. For the remaining strains, the presence of HMO or prebiotics Orafiti[®] P95 or Orafiti[®] GR decreased adherence, reaching statistical significance ($p < 0.05$) for three of out of eight (HMO) or five of out of 11 strains tested, respectively. Conversely, Vivinal[®] enhanced adherence in six out of the 12 strains tested, and notably, it significantly attenuated the adherence of the cariogenic *Bifidobacterium dentium* Culture Collection of Dairy Microorganisms (CCDM) 318. To our knowledge, this represents the first report on the influence of commercial prebiotics and HMOs on the adhesion of the cariogenic *Bifidobacterium* sp. Vivinal[®] seems to be a promising prebiotic to be used in the formulation of synbiotics, supporting the adhesion of a wide range of probiotics, especially the strains *B. bifidum* BBV and BBM and the probiotic *Escherichia coli* O83.

Keywords: adherence; prebiotics; probiotics; human milk oligosaccharides; Caco-2; HT29-MTX



Citation: Krausova, G.; Hynstova, I.; Svejstl, R.; Mrvikova, I.; Kadlec, R. Identification of Synbiotics Conducive to Probiotics Adherence to Intestinal Mucosa Using an In Vitro Caco-2 and HT29-MTX Cell Model. *Processes* **2021**, *9*, 569. <https://doi.org/10.3390/pr9040569>

Academic Editor: Massimo Iorizzo

Received: 15 February 2021

Accepted: 20 March 2021

Published: 24 March 2021

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1. Introduction

Probiotics are conventionally defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit to the host” [1]. Nevertheless, the definition and associated terminology are continuously being updated. For example, Zendeboodi et al. [2] recently defined a probiotic as “a viable or inviable microbial cell (vegetative or spore; intact or ruptured) that is potentially healthful to the host”. Moreover, new proposed and introduced categories of probiotics include “paraprobiotics” and “postbiotics” [3], along with “true probiotics”, and “ghost probiotics” [2]. In turn, a prebiotic

is defined as “a substrate that is selectively utilized by host microorganisms conferring a health benefit”, according to The International Scientific Association for Probiotics and Prebiotics (ISAPP) [4]. Generally, prebiotics are categorized as (i) oligosaccharides, (ii) polyols (sugar alcohols), and (iii) soluble fiber [5]. Oligosaccharides constitute the major source of fermentable sugars for microbiota present in the gut. They serve as prebiotics for intestinal microbiota and should selectively stimulate the growth and activity of the beneficial organisms. Established prebiotic carbohydrates include inulin-type fructans, fructooligosaccharides (FOS), galactooligosaccharides (GOS), and lactulose, with several emerging or candidate prebiotics also being considered, such as polydextrose, cellobiose, melibiose, and isomaltulose [6].

Probiotic bacteria are often formulated with prebiotics into “synbiotics”. Both pre- and probiotics function optimally in combination, with the synergistic benefits of pre- and probiotics potentially able to enhance the therapeutic and nutritional value of foods containing these components. In particular, prebiotics should not be broadly metabolized by the gut microbiota, but should instead selectively stimulate the growth of health-promoting microorganisms [4]. Therefore, prebiotics should be comprehensively characterized to assess not only their fermentability, but also their effect on probiotic characteristics, including adherence, as enhanced adhesion ability can prolong bacterial residence in the gastrointestinal tract (GIT) [6]. Thus, the ability to adhere to the intestinal epithelium constitutes an important criterion for probiotics.

Various prebiotics are known to interfere with adhesion by enteric pathogens [7–9], although their effects on the adhesion of beneficial bacteria, such as probiotics, are poorly understood. Previously, we reported that prebiotics generally interfere with, but may also enhance, bacterial adhesion [10,11], with strong species- and strain-specific effects also being observed [8,12]. As GIT microbiota residence may, therefore, be differentially influenced by prebiotics-mediated modification of bacterial adhesion, it is crucial to study the interaction between individual combinations of probiotic strains and prebiotics.

For example, differences in gut microbiota composition between breastfed and formula-fed infants are largely attributable to the presence of human milk oligosaccharides (HMOs) in breast milk [13]. HMOs are complex sugars with unique structural diversity and are known to positively influence infant health [14,15]. Recent progress in HMO manufacturing and chemoenzymatic synthesis has increased the availability of these oligosaccharides, thereby facilitating more in-depth studies of their function and the effects of their supplementation in infant formulae. Notably, a few studies in infants have successfully confirmed the safety and efficacy of HMO-fortified infant formulae [14,16], supporting the regulatory approval of two major HMOs: 2'-fucosyllactose and lacto-N-neotetraose [15]. However, data regarding the effects of HMOs on bacterial adherence are scarce. In healthy breastfed infants, bifidobacteria constitute the predominant bacterial species [17]. Some recent studies reported that HMOs could enhance the adhesion ability by regulating the expression of bacterial adhesins [18]. In particular, Chichlowski et al. [19] reported that the cultivation of a *Bifidobacterium longum* subsp. *infantis* (*B. infantis*) strain on HMOs increased the adherence of this strain to co-cultured Caco-2 and HT29 (human colorectal adenocarcinoma) cells, in an in vitro intestinal model. Subsequently, the fractions of 3'-sialyllactose and 6'-sialyllactose were demonstrated to be responsible for the observed increase in the adherence to the HT29 cell model [20].

Numerous studies have evaluated the microbial utilization of different oligosaccharides, especially focusing on their use as a carbon source for analyzing their growth-promoting effect and survival under GIT conditions. Bacterial adherence is linked to surface properties, which are, in turn, dependent on the structure and composition of the cell wall [21]. Accordingly, the anti-adhesive activity of most oligosaccharides is attributed to their ability to mimic cell receptors that bind pathogens, thereby displacing or flushing pathogens from the GIT [8]. Certain oligosaccharides such as chitooligosaccharides and HMOs contain N-acetyl glucosamine, which is also a component of intestinal mucin and serves as a receptor for bacteria [12]. Indeed, intestinal mucin glycans are used by bacteria

both as a carbon source and attachment sites [22]. Consequently, the presence of intestinal mucus may significantly affect adherence, as observed in studies with cell lines that secrete or do not secrete mucus. Accordingly, adherence is typically tested using different in vitro models, including human Caco-2 and mucus-secreting HT29-MTX cells as the most frequently used model [23]. N-acetyl-glucosamine, together with N-acetyl-muramic acid and other sugars and amino-acids, is also a constituent of peptidoglycan—a major structural polymer of the bacterial cell wall [24]. Peptidoglycan is a thick structure in Gram-positive bacteria (e.g., *Lactobacillus* sp., *Bifidobacterium* sp.), whereas it is thin in Gram-negative bacteria (e.g., *E. coli*) and it also could play a role in overall bacterial adherence [25].

In this study, we aimed to identify potentially effective synbiotics by evaluating the adherence of bacterial strains to a Caco-2 and HT29-MTX co-culture cell line model mimicking the intestinal epithelium. For this purpose, commercial prebiotics (FOS and GOS) and purified HMOs were subjected to testing and their influence on the adhesion ability of probiotic or potentially probiotic strains, together with the cariogenic *Bifidobacterium dentium*, was evaluated. These data will considerably extend the findings of our previous studies and provide a more thorough understanding regarding how commercially available prebiotics and isolated HMOs affect the adhesion of bacteria with real-world relevance.

2. Materials and Methods

2.1. Bacterial Strains and Prebiotics

A total of 12 strains (Table 1) of *Lactobacillus* and *Bifidobacterium* of different origins were tested along with one strain of probiotic *Escherichia coli*. Strains with CCDM identification and the lactobacilli strains DM1TA6-P and PE1TB-P were obtained from the Culture Collection of Dairy Microorganisms Laktoflora® (Tábor, Czech Republic). *Bifidobacterium bifidum* strains BBV and BBM were purchased from the Collection of the Czech University of Life Sciences in Prague, Czech Republic (both isolates from the feces of healthy breast-fed infants). The commercial probiotics *E. coli* O83 and *Bifidobacterium animalis* subsp. *lactis* Bb12 were purchased from the Czech Academy of Sciences (Prague, Czech Republic) and from Chr. Hansen (Starovice, Czech Republic), respectively. Lactobacilli were stored and cultured in De Man Rogosa Sharpe (MRS) broth, pH 5.7 (Merck, Darmstadt, Germany), whereas *bifidobacteria* were stored and cultured in MRS broth, pH 6.2, supplemented with 0.5% L-cysteine hydrochloride (Merck). *E. coli* O83 was cultured in brain heart infusion broth (Merck, Darmstadt, Germany). Strains were grown for 24 h at 37 °C under anaerobic (lactobacilli and bifidobacteria) or aerobic (*E. coli* O83) conditions. All strains were grown overnight before the beginning of the experiment and harvested at an optical density (OD₆₀₀) of 0.5 (corresponding to ~10⁷ CFU/mL).

We utilized three commercially available and widely used prebiotics for the comparison of adherence ability: fructan-based prebiotic Orafti® GR (Beneo, Belgium), a white powder containing mainly chicory inulin with polymeric degrees from 2 to 60 (average of ≥10; composition: >90% inulin, <4% glucose + fructose and <8% saccharose); the prebiotic formula Orafti® P95 (Beneo, Belgium), a white hygroscopic powder containing mainly oligofructose as a product of partial enzyme hydrolysis of chicory inulin (composition: ≥93.2% oligofructose and <6.8% glucose + fructose + saccharose); a GOS-based prebiotic Vivinal® (FrieslandCampina DOMO, Amersfoort, The Netherlands), a non-colored syrup (composition: ≥57% GOS, <23% lactose, <22% glucose, and <0.8% galactose). Glucose and lactose of analytical-grade (Sigma-Aldrich, Czech Republic) were also subjected to testing. Carbohydrate fermentation profiles of the strains were tested using API® 50 CH test (bioMérieux, Inc., Marcy l'Etoile, France). Prior to testing, strains were precultured on Columbia agar with 5% sheep blood (Sigma-Aldrich, Prague, Czech Republic) and the test was performed according to manufacturers' instructions.

Table 1. Bacterial strains used in this study.

Strain	Source
<i>Escherichia coli</i> O83	original culture
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> CCDM 66	yoghurt
<i>Lactobacillus acidophilus</i> CCDM 382	raw goat milk
<i>Lactobacillus fermentum</i> RL-25	human feces
<i>Lactobacillus casei</i> subsp. <i>paracasei</i> DM1TA6-P	colon biopsy
<i>Lactobacillus casei</i> subsp. <i>paracasei</i> PE1TB-P	colon biopsy
<i>B. bifidum</i> CCDM 559	human feces
<i>B. dentium</i> CCDM 318	dental caries
<i>B. breve</i> CCDM 562	gastrointestinal tract of a child
<i>B. bifidum</i> BBM	infant feces
<i>B. bifidum</i> BBV	infant feces
<i>B. animalis</i> subsp. <i>lactis</i> Bb12	original culture

B., *Bifidobacterium*; *L.*, *Lactobacillus*.

2.2. Isolation and Purification of HMOs

Breast milk samples from early lactation were kindly provided by two donors to the Czech University of Life Sciences (informed written consent for analysis of milk samples was obtained from both donors). Milk oligosaccharides were isolated and purified according to Gnoth et al. [26], with some modifications. In brief, 200 mL samples were centrifuged for 30 min at 4 °C and 1800 × g to partially remove lipids, proteins, and cells. Residual proteins were then precipitated for 24 h at 4 °C through ethanol addition (2:1, v/v). Subsequently, samples were centrifuged for 30 min at 4 °C and 1800 × g, dried by rotatory evaporation, and dissolved in deionized water. This process was repeated twice, and the resulting sample was fractionated by gel filtration chromatography in 1% acetic acid at 0.1 mL/min on a Toyopearl HW40F column (Tosoh, Tokyo, Japan). To test for oligosaccharides, individual fractions were resolved by thin-layer chromatography in isopropanol:water:25% ammonia (5:1:2 by volume) and visualized by spraying with 10% sulfuric acid in ethanol and heating. Fractions containing oligosaccharides were collected into vials, cooled at 4 °C for 30 min, frozen at −70 °C for 90 min, and lyophilized using a Cryodos freeze dryer (Telstar, Terrassa, Spain). The yield was 0.8 g purified oligosaccharides from 200 mL breast milk.

2.3. Adhesion to Caco-2 and HT29-MTX Cells

The intestinal mucosa was modeled as a co-culture of HT29 and Caco-2 cells obtained from the American Type Culture Collection (catalog no. HTB-38 and HTB-39, respectively). Caco-2 cells were cultivated in Eagle's minimal essential medium (Lonza, Basel, Switzerland) with 20% fetal bovine serum (Sigma Aldrich), 1% penicillin/streptomycin (Lonza), and 1% L-glutamine (Lonza), whereas HT29 cells were cultivated in Dulbecco's modified Eagle medium (Lonza) with 15% fetal bovine serum (Sigma Aldrich), 1% penicillin/streptomycin (Lonza), and 1% sodium pyruvate (Lonza). The HT29 culture was differentiated using methotrexate to induce constant production of mucin, as previously described [27]. Exponentially growing HT29 cells were incubated with 10 μM methotrexate for 14 days without passage. A phase of higher cell mortality after methotrexate addition followed. Finally, cell division was recovered and as a result, culture of MTX-adapted, mucin-producing cells were obtained. The resulting cells were hereafter referred to as HT29-MTX and were cultivated in the same medium as the parental cells. All cells were incubated at 37 °C and 5% CO₂. To assay bacterial adherence, methods previously described by Kadlec and Jakubec [10] and Krausova et al. [11] were used. For the assays, the mammalian cells of Caco-2 and HT29-MTX were mixed at 9:1 and seeded at 2 × 10⁴ cells per

well in a 96-well microtiter plate between passages 15–21. Cells were used once confluent (usually 24 h) in Caco-2 medium. In turn, bacterial strains were grown overnight in the appropriate culture broth characterized above (2.1) before the beginning of the experiment. Bacterial cells were centrifuged at $10,000 \times g$ for 10 min at room temperature, subsequently, washed twice with physiological saline (pH = 7) and finally resuspended in physiological saline to an optical density of 0.5 at 600 nm. Following this, bacterial suspensions were fluorescently labeled for 1 h with 50 μM Syto 9 (Life Technologies, Carlsbad, CA, USA) at 37 °C in the dark. Subsequently, the cells were washed three times with physiological saline (pH = 7). Labeled samples (100 μL) were then added to 10 wells of co-cultured host cells, along with 100 μL of physiological saline or 5% (*wt/vol*) prebiotic or saccharide (final concentration 2.5%). As a control, bacteria resuspended in tissue culture cultivation media alone, without addition of any carbohydrate source was used. Samples were cultivated in the dark for 1.5 h at 37 °C and 5% CO_2 . In bifidobacteria, anaerobic conditions were created by putting microtiter plates into 2.5 L anaerobic jars using AnaeroGen™ system (Oxoid™, Basingstoke, UK). Subsequently, five wells per sample were selected as controls representing 100% fluorescence. The remaining five wells per sample were washed twice with 200 μL physiological saline and irrigated with physiological saline or 2.5% prebiotic or saccharide. Prior to fluorescence, microtiter plates were washed with physiological saline to remove antibiotics and FBS residues. Fluorescence was then measured using a Synergy 2 microplate reader (Tecan, Männedorf, Switzerland) at an excitation wavelength of 478 nm and emission wavelength of 510 nm. Experiments were repeated in triplicate. The percentage (%) of adherent bacteria was calculated as:

$$X (\%) = ((X_{\text{RFU}} - \text{NC}) / (\text{PC} - \text{NC})) \cdot 100 \quad (1)$$

where X (%) is the percentage of residual fluorescence in the test well, X_{RFU} is well fluorescence in relative fluorescence units, NC is the negative control (nonspecific well fluorescence), and PC is the positive control (bacterial fluorescence without washing).

2.4. Statistical Analyses

Groups were compared using analysis of variance (one-way ANOVA) to test the null hypothesis that prebiotics do not influence bacterial adherence. Significantly different adherence rates (Tables 2 and 3) were subsequently identified by post hoc multiple comparisons using Tukey's Honest Significant Difference method at $p = 0.05$. Data were analyzed using MATLAB (MathWorks, Natick, MA, USA).

Table 2. Adherence (%) of *Escherichia coli* O83 and lactobacilli in the presence of prebiotics.

Prebiotic	O83	CCDM 66	CCDM 382	PE 1TB-P	RL 25	DM1TA6-P
Control	29.1 ± 13.6	35.2 ± 17.1	27.9 ± 6.1	32.8 ± 8.4	18.2 ± 8.2	30.1 ± 7.1
Lactose	45.5 ± 24.0 ↑	13.5 ± 9.2 ↓	9.8 ± 2.4 ↓*	10.8 ± 3.4 ↓*	16.6 ± 5.4 ↓	10.1 ± 3.6 ↓*
Glucose	42 ± 14.1 ↑	10.8 ± 7.1 ↓	7.6 ± 1.6 ↓*	7.9 ± 2.1 ↓*	17.6 ± 8.1 ↓	13.9 ± 2.8 ↓
Orafti® P95	34.1 ± 11.9 ↑	26.8 ± 14.7 ↓	7.4 ± 1.6 ↓*	10.6 ± 4.1 ↓*	13.6 ± 4.9 ↓	17.2 ± 4.0 ↓
Orafti® GR	38.2 ± 10.5 ↑	17.8 ± 8.7 ↓	6.8 ± 2.4 ↓*	7.7 ± 2.4 ↓*	9.5 ± 3.1 ↓	20.2 ± 3.8 ↓
Vivinal®	40.3 ± 22.0 ↑	47.4 ± 19.9 ↑	15.5 ± 3.5 ↓*	23.2 ± 5.1 ↓	30.3 ± 13.2 ↑	30.5 ± 11.9 ↑
HMO	N	16.8 ± 5.1 ↓	18.8 ± 5.0 ↓	N	20.1 ± 6.7 ↑	N

HMO, human milk oligosaccharides. Data represent the means ± SD. ↓, decreased adherence; ↑, increased adherence; * statistically significant loss of adherence; enhanced adherence, however, not statistically significant; N not tested.

Table 3. Adherence (%) of bifidobacteria in the presence of prebiotics.

Prebiotic	CCDM 559	CCDM 318	CCDM 562	BBM	BBV	Bb12
Control	11.1 ± 7.4	24.0 ± 8.0	28.6 ± 7.2	57.8 ± 15.5	48.8 ± 6.8	32.8 ± 13.4
Lactose	5.8 ± 1.2 ↓	6.9 ± 2.5 ↓*	7.3 ± 2.0 ↓*	51 ± 14.0 ↓	40.7 ± 5.9 ↓	8.5 ± 2.7 ↓*
Glucose	4 ± 1.1 ↓	5.0 ± 1.8 ↓*	4.8 ± 0.9 ↓*	45.9 ± 10.1 ↓	31.5 ± 9.7 ↓	6.4 ± 2.2 ↓*
Orafti® P95	6.7 ± 3.2 ↓	5.4 ± 2.0 ↓*	5 ± 1.0 ↓*	46.3 ± 6.9 ↓	38.6 ± 6.8 ↓	6.2 ± 2.0 ↓*
Orafti® GR	4.5 ± 2.7 ↓	4.7 ± 1.5 ↓*	6.5 ± 1.6 ↓*	36.9 ± 11.5 ↓	37.7 ± 4.2 ↓	5.8 ± 1.8 ↓*
Vivinal®	7.2 ± 2.8 ↓	15.9 ± 4.0 ↓	13 ± 7.3 ↓*	72.0 ± 16.4 ↑	50.3 ± 8.2 ↑	17.1 ± 3.2 ↓
HMO	N	8.2 ± 5.1 ↓*	7.4 ± 4.0 ↓*	56.3 ± 16.1 ↓	40.5 ± 7.3 ↓	11.5 ± 3.9 ↓*

HMO, human milk oligosaccharides. Data represent the means ± SD. ↓, decreased adherence; ↑, increased adherence; * statistically significant loss of adherence; enhanced adherence, however, not statistically significant; N not tested.

3. Results

As listed in Tables 2 and 3, all strains tested adhered well to the co-cultures of Caco-2 and HT29-MTX cells. In the absence of prebiotics (control), adherence ranged from 11.1–57.8%, with *B. bifidum* BBM and BBV adhering most efficiently at 57.8% and 48.8%, respectively. In comparison, *B. bifidum* CCDM 559 was the least efficient at 11.1%. In most strains, loss of adherence was observed in the presence of HMOs and the prebiotics Orafti® P95 and Orafti® GR. In particular, the loss of adherence was statistically significant ($p < 0.05$) in strains CCDM 382, PE1TB-P, CCDM 318, CCDM 562, and Bb12. For a clearer presentation of the results, Figures 1 and 2 demonstrate the effect of each prebiotic on all strains separately. In contrast, the GOS-based prebiotic Vivinal® enhanced adherence in six strains and interfered the least with adherence in the remaining six strains, in comparison to other prebiotics (Tables 2 and 3). Overall, *B. bifidum* strains BBM and BBV were the least sensitive to prebiotics, with the enhanced adherence mediated by Vivinal® failing to reach statistical significance. These strains were also the most adherent, even in the presence of fructan-based Orafti® prebiotics. HMOs also interfered with adherence in all eight strains tested, with the decrease being statistically significant ($p < 0.05$) in the three bifidobacteria.

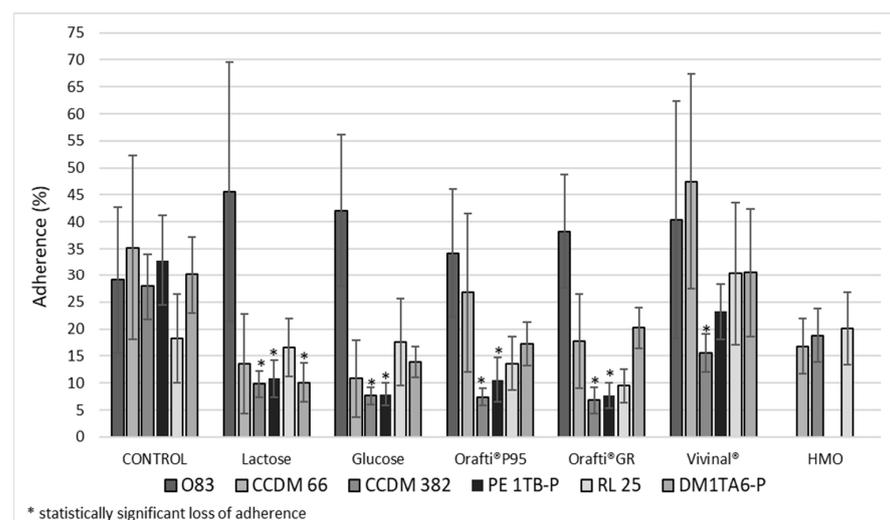


Figure 1. Adherence of *Escherichia coli* O83 and lactobacilli (CCDM 66, CCDM 382, PE1TB-P, RL25 and DM1TA6-P) in the presence of prebiotics and HMOs.

Notably, prebiotics also enhanced adherence in *E. coli* O83, although this increase was not statistically significant. Adherence in this strain was not tested in the presence of HMOs. In contrast, significant loss of adherence was observed in *B. dentium* CCDM 318, which was isolated from dental caries, in the presence of all prebiotics, sugars, and HMOs. Similar

trends were observed in *Bifidobacterium breve* CCDM 562, *Lactobacillus animalis* CCDM 382, *B. animalis* subsp. *lactis* Bb12, and *Lactobacillus casei* subsp. *paracasei* PE1TB-P.

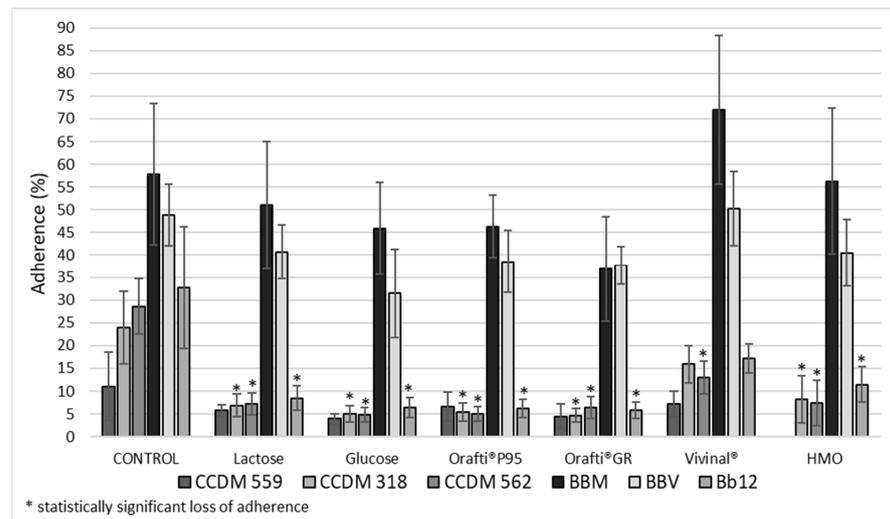


Figure 2. Adherence of bifidobacteria (CCDM 559, CCDM 318, CCDM 562, BBM, BBV and Bb12) in the presence of prebiotics.

The fermentation profiles of bifidobacteria (Table 4) and lactobacilli and the *E. coli* strain (Table 5) demonstrated genetic and phenotypic diversity. In particular, the *E. coli* strain showed positivity to a wide range of carbohydrates. Some differences in carbohydrate fermentation profiles were also observed in the strains within the same species. The relationship and possible effect of individual carbohydrates on bacterial adherence is discussed in more detail in Section 4.

Table 4. Carbohydrate fermentation ability of bifidobacteria using API® 50 CH test.

Bifidobacteria	API® 50 CH Test																									
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	
CCDM 318				+	+	+				+	+	+	+						+			+		+	+	+
CCDM 559										+	+	+														+
CCDM 562			+		+					+	+	+	+						+	+	+		+	+	+	+
Bb12				+	+	+				+	+											+		+		+
BBM										+	+	+														+
BBV										+	+	+														+
	API® 50 CH Test																									
	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	
CCDM 318	+	+	+	+	+	+				+	+	+			+	+										
CCDM 559				+	+																					
CCDM 562	+	+	+	+	+	+				+	+				+					+						
Bb12	+	+	+	+	+	+				+	+			+												
BBM				+	+																					
BBV				+	+																					

Evaluation: + positive, (+) slightly positive. Acid from: 1—glycerol, 2—erythritol, 3—D-arabinose, 4—L-arabinose, 5—D-ribose, 6—D-xylose, 7—L-xylose, 8—D-adonitol, 9—methyl-beta-D-xylopyranoside, 10—D-galactose, 11—D-glucose, 12—D-fructose, 13—D-mannose, 14—L-sorbose, 15—L-rhamnose, 16—dulcitol, 17—inositol, 18—D-mannitol, 19—D-sorbitol, 20—methyl alpha-D-mannopyranoside, 21—methyl alpha-D-glucopyranoside, 22—N-acetyl-glucosamine, 23—amygdalin, 24—arbutin, 25—esculin, 26—salicin, 27—cellobiose, 28—maltose, 29—lactose, 30—melibiose, 31—sucrose, 32—trehalose, 33—inulin, 34—melezitose, 35—raffinose, 36—starch, 37—glycogen, 38—xylitol, 39—gentiobiose, 40—turanose, 41—D-lyxose, 42—D-tagatose, 43—D-fucose, 44—L-fucose, 45—D-arabitol, 46—L-arabitol, 47—gluconate, 48—2-ketogluconate, 49—5-ketogluconate.

Table 5. Carbohydrate fermentation ability of lactobacilli and *E. coli* using API[®] 50 CH test.

Lactobacilli and <i>E. coli</i>	API [®] 50 CH Test																									
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	
DM1TA6-P					+			+		+	+	+	+	+				+	+		+	+	+	+	+	
CCDM 382					(+)					+	+	+	+									+	+	(+)	+	
RL25					+	+				+	+	+	+													
CCDM 66											+	+	+													
PE1TB-P					+			+		+	+	+	+	+				(+)	+	+		(+)	+	+	+	+
<i>E. coli</i> O83				+	+	+					+	+	+	+		+			+	+			+			
	API [®] 50 CH test																									
	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	
DM1TA6-P	+	+	+	+		+	+							+	+		+					(+)				
CCDM 382	+	+	+	+	+	+					+				+											
RL25				+	+	+	+				+												(+)			
CCDM 66				+																						
PE1TB-P	+	+	+	+		+	+							+	+	+	+						+			
<i>E. coli</i> O83				+	+	+		+												+			+			

Evaluation: + positive, (+) slightly positive. Acid from: 1—glycerol, 2—erythritol, 3—D-arabinose, 4—L-arabinose, 5—D-ribose, 6—D-xylose, 7—L-xylose, 8—D-adonitol, 9—methyl-beta-D-xylopyranoside, 10—D-galactose, 11—D-glucose, 12—D-fructose, 13—D-mannose, 14—L-sorbose, 15—L-rhamnose, 16—dulcitol, 17—inositol, 18—D-mannitol, 19—D-sorbitol, 20—methyl alpha-D-mannopyranoside, 21—methyl alpha-D-glucopyranoside, 22—N-acetyl-glucosamine, 23—amygdalin, 24—arbutin, 25—esculin, 26—salicin, 27—cellobiose, 28—maltose, 29—lactose, 30—melibiose, 31—sucrose, 32—trehalose, 33—inulin, 34—melezitose, 35—raffinose, 36—starch, 37—glycogen, 38—xylitol, 39—gentiobiose, 40—turanose, 41—D-lyxose, 42—D-tagatose, 43—D-fucose, 44—L-fucose, 45—D-arabitol, 46—L-arabitol, 47—gluconate, 48—2-ketogluconate, 49—5-ketogluconate.

4. Discussion

Adherence to epithelial cells, and thereby colonization of the GIT, is a desirable property of probiotic bacteria, as transient presence may not confer health benefits to the host. Moreover, probiotics that adhere to the intestinal epithelium and interact appropriately with prebiotics may effectively compete and proliferate in the multispecies intestinal environment, whereas daily intake and long-term use may be required to gain sufficient health effects from non-adhering, transient probiotics. In synbiotics, the interaction between prebiotics and probiotics is also critical, as the former may directly affect the properties of the latter. Another important consideration when formulating synbiotics, is the carbon source, which is crucial for bacterial growth as well as for their adhesion to intestinal epithelial cells, as emphasized in our previous studies [10,11].

The adherence of probiotics has been tested in vitro in several studies; however, it is difficult to directly compare the results due to the use of various tissue models [28]. Currently, Caco-2 cells with added mucin are considered to represent the only reliable and suitable in vitro model to evaluate bacterial adhesion, as they most closely model in vivo physiology [29]. Using this model, incorporating HT29-MTX cells for mucin production, we observed good adherence in the absence of prebiotics (11.1–57.8%), especially by *B. bifidum* BBM (57.8%) and BBV (48.8%) strains, which are of human GIT origin. We note, however, that Laparra and Sanz [29] observed very low adherence by lactobacilli and bifidobacteria (0.72–3.15%) using a similar model. In addition, 14.71% adhesion to Caco-2 cells was observed for *Lactobacillus plantarum* cultured with mannan-oligosaccharides in another study [30], followed by FOS and GOS. Variations in adhesion dependent on carbon source have also been reported in another study [6], with FOS demonstrating the most effective ability to stimulate adhesion of the probiotic strain *Lactobacillus acidophilus* NCFM to intestinal HT29 cells (notably, non-mucin producing ones).

The anti-adhesive effect of individual prebiotics against harmful bacteria is well described in the literature [7–9], especially for oligosaccharides such as D-mannose. This

sugar monomer of aldohexose inhibits bacterial adherence to uroepithelial cells and is effective as a treatment and prophylaxis against urinary tract infections [7] and is thus used as an alternative to antibiotics. Moreover, the decreased adherence of anaerobic pathogens to the HT29 cell line in the presence of FOS, inulin, and lactulose was reported by Sharma and Kanvar [8], which was among the first studies to highlight the anti-adhesive activity against *Bacteroides* and *Peptostreptococcus* species. However, much less is known regarding the adhesive effects of prebiotics on normal gut microbiota and beneficial bacteria, such as probiotics. In one of the first studies addressing this question, Altamimi et al. [12] found that oligosaccharides interfere with adhesion by resident gut microorganisms, such as *Bacteroides fragilis* and *Clostridium difficile*. In mixed cultures, clostridia were the most sensitive to prebiotics, followed by bifidobacteria and *Bacteroides*. In contrast, lactobacilli were the least sensitive.

Our previous data confirmed that the effects of prebiotics on adherence are highly strain-specific [10,11], although anti-adhesive effects were predominantly observed. In the present study, we found that the commercial prebiotics Orafti® P95 and Orafti® GR had no effect or acted anti-adhesively against most strains, in line with the literature [10,11,20,31]. In particular, the loss of adhesion in the presence of prebiotics reached statistical significance ($p < 0.05$) in strains CCDM 382, PE1TB-P, CCDM 318, CCDM 562, and Bb12. Orafti® GR and P95 constitute fructan-based prebiotics derived from chicory roots. Fructan prebiotics differ in the degree of polymerization, with Orafti® GR containing mainly inulin with polymerization degree of 2–60 (average >10), whereas Orafti® P95 primarily contains oligofructose, a product of partial enzymatic hydrolysis of chicory inulin (average degree of polymerization <10). We hypothesize that chain length may contribute to the anti-adhesive properties. Structure is also a factor that is considered critical for fermentability, e.g., molecular branching, glycosidic linkage, type of monosaccharide moieties, or position and conformation of the links between the monosaccharide units [32,33]. The overall structural complexity of sugars is also of relevance. Generally, adhesion is a complex process involving non-specific and specific ligand–receptor interactions. Different structures and specific cell wall components of bacteria, such as fimbriae or pili, adhesins, mucus-binding proteins, fibronectin-binding proteins, or surface layer proteins, provide an advantage for epithelial colonization [34]. Additionally, overall adhesion may be influenced by various environmental factors, for example, pH, temperature, oxygen availability, and bacterial surface components such as lipoteichoic acid, surface proteins, peptidoglycans, and polysaccharides [30,35]. Furthermore, auto-aggregation and hydrophobicity of probiotic bacteria are also important phenotypes for adhesion [36,37].

Milk oligosaccharides constitute the first prebiotics to which infants are exposed, containing complex oligosaccharides that guide the development of neonatal gut microbiota [38] and selectively serving as energy sources for beneficial bacteria [39]. Nevertheless, the effect of HMOs on the adhesion of beneficial bacteria is poorly characterized. Musilova et al. [40] found that the adherence of one of the three bifidobacteria strains tested was strongly sensitive to HMOs, as were both tested strains of clostridia. Consistent with these observations, we found that HMOs interfered—to some extent—with adherence in all eight strains tested, and to a significant extent in three. As with other prebiotic oligosaccharides, HMOs may also exert anti-adhesive properties against both pathogens and beneficial bacteria. The anti-adhesive effects of HMOs may be related to lectins, which are glycan-binding proteins that bind to epithelial oligosaccharides [39]. Adhesion-related virulence factors are often attributed to lectins that bind to oligosaccharides on the epithelial surface and allow bacteria to attach to the surface. Thanks to these glycan-binding determinants, HMOs could serve as soluble analogues of oligosaccharidic binding sites and, thus, block adhesion of pathogens [39]. HMOs may also have glycome-modifying effects, modulating the expression of intestinal epithelial glycans, such as glycocalyx, which serve as attachment sites for bacteria [41]. Individual monosaccharides that constitute HMOs, for example glucose, galactose, sialic acid, N-acetylglucosamine, and fucose, do not affect adhesion individually but are effective in combination against diarrheal pathogens such as *E. coli*,

Vibrio cholerae, and *Salmonella fyris* [42]. This effect is dependent on the oligosaccharide fraction, with the acidic fraction being the most effective. Of sugars that form part of HMOs, D-glucose and D-galactose were fermented by all strains under study. Differences were found in the fermentation ability of L-fucose and N-acetylglucosamine. Of bifidobacteria, notably, only one strain, the *B. breve* CCDM 562 was able to utilize both these sugars. In lactobacilli, only N-acetylglucosamine, not fucose was fermented. The probiotic *E. coli* strain O83 was able to ferment a wide range of carbohydrates, including the mentioned fucose and N-acetylglucosamine. The latter is of importance also for its presence in bacterial cell wall and GIT mucus, as mentioned previously. N-acetyl-glucosamine is known to be involved in biofilm formation by acting as regulatory signal affecting colonization and adherence to intestinal cells [43]. Nevertheless, this effect is strain-dependent, based on genetic diversity and differences in metabolic patterns in *E. coli* strains. In the adherent and invasive *E. coli* LF82 strain, for example, N-acetylglucosamine reduced adherence and biofilm formation [43]. In the probiotic *E. coli* O83 strain tested herein, on the contrary, an increase in adherence in the presence of all tested sugars and prebiotics was noted. Importantly, N-acetylglucosamine was not used as a sole carbohydrate source and, thus, the effect of other sugars cannot be ruled out.

The relationship of fucose and sialic acid to anti-adhesive effects against pathogens was also noted by Bode and Jantscher-Krenn [44]. In addition, 3'- and 6'-sialyllactose, the two predominant oligosaccharides in human milk, enhance the adherence of *B. infantis* ATCC 15,697 [20]. The effects of different types of milk and milk protein fractions, such as albumin and casein, were also found to be substrate- and strain-specific against *Lactobacillus gasseri* and *L. casei* [21], with whole breast milk enhancing adherence in both strains. Notably, this differs from the consistent anti-adhesive effects observed in the present study, which utilized purified HMOs rather than whole milk. Thus, despite the recent progress in the study of the individual HMO components and their influence on bacterial growth (e.g., [45]), additional research is required to clarify their collective influence on functional probiotic characteristics such as bacterial adhesion ability.

Of the strains tested in the present study, only *E. coli* O83 was less adherent in the absence of prebiotics than in the presence of lactose, glucose, and all commercial prebiotics (Orafti® P95, Orafti® GR, and Vivinal®). Consistent with the premise that good adherence, especially in the presence of prebiotics, is characteristic of good probiotics, *E. coli* O83 has been successfully used to control colonization in infants as a preventive strategy against nosocomial infections [46]. Conversely, FOS, sucrose, and inulin significantly suppressed the ability of the probiotic *E. coli* Nissle 1917 strain to adhere to the same Caco-2 and HT29-MTX model used herein [23]. Moreover, we found that prebiotics, lactose, glucose, and HMOs interfered with the adhesion of *B. animalis* subsp. *lactis* Bb12, a well-described and commercially marketed probiotic strain.

Although the genus *Bifidobacterium* is generally recognized as safe, exceptions such as *B. dentium* exist. This species is regarded as an opportunistic cariogenic pathogen that can survive in acidic oral cavities to cause tooth demineralization [47]. Carbohydrate fermentation is an important survival advantage in the oral cavity. However, *B. dentium* strain was not able to efficiently grow on HMOs when used as a carbohydrate source [48]. Based on the metabolic activity testing (API® 50 CH), the *B. dentium* CCDM 318 was proved to ferment neither L-fucose nor N-acetyl-glucosamine. This could explain the poor growth of *E. coli* on HMOs, as the mentioned carbohydrates are forming part of HMOs. However, the repertoire of fermented sugars in *B. dentium* is wide. Additionally, *E. coli* was shown to be able to utilize carbohydrates such as D-glucose, D-galactose, D-fructose and D-mannose, also L-arabinose, D-xylose, starch, amygdalin, arbutin, esculin, turanose and others. Within the family *Bifidobacteriaceae* three species, *Bifidobacterium dentium*, *Parascardovia denticolens*, and *Scardovia inopinata*, are present as an abundant population in the oral cavity [49]. In a recent study [50], *B. dentium* was reported to successfully colonize the germ-free mice ileum and colon. However, the evaluation of the influence of prebiotics on its adherence was not the objective of the study. We found that commercial prebiotics, lactose, glucose, and HMOs

significantly attenuated the adherence of *B. dentium* CCDM 318 to Caco-2 and HT29-MTX cells. To our knowledge this is the first report dealing with the influence of prebiotics and HMOs on the adhesion ability of this cariogenic *Bifidobacterium* species. Nevertheless, to make a stronger conclusion, more bacteria should be subjected to testing. To mention other limitations, the in vitro techniques used in this study are not fully sufficient to justify drawing definite conclusions. Undoubtedly, the use of molecular biology tools could put more light into the mechanisms involved in adhesion of individual strains, as well as the identification of adhesive molecules and their genes. It is also not entirely possible to extrapolate these in vitro results to the multispecies environment of the human GIT where other factor such as peristaltic movements or competition with resident microbiota might play a significant role. On the other hand, although more predictive, in vivo trials tend to use more invasive methods (e.g., colon biopsy samples).

Vivinal[®] enhanced adherence in six of the twelve strains tested. In addition, Vivinal[®] interfered with adherence of all other strains to a lesser extent than other prebiotics. According to the manufacturer, Vivinal[®] syrup contains approximately 60% GOS of three to six sugar monomers, a small amount of galactose, and significant amounts of residual lactose and glucose from which it is synthesized (approximately 20% each). Notably, lactose and glucose individually did not appear to affect adherence, suggesting that these may not contribute to the pro-adhesive properties of Vivinal[®], in agreement with previous findings [10,51]. Furthermore, Vivinal[®] was previously shown to induce the growth of lactobacilli and bifidobacteria both in vitro and in vivo [52–54], in addition to excretion of IgA in the feces after coadministration with the Bb12 strain [55]. Therefore, this prebiotic appears to serve as a suitable synbiotic partner for a wide range of probiotics. In contrast, the anti-adhesive properties of GOS-based prebiotics (Oligomate 55NP[®] and Vivinal[®]) against enteric pathogens have also been documented [51,56], demonstrating the ability to reduce adherence in vitro by as much as 70% in enteropathogenic *E. coli* [9]. In particular, GOS are reported to possess considerable capacity to increase the levels of bifidobacteria and lactobacilli in the gastrointestinal tract (GIT) [57]. Pre- and probiotic combinations are often characterized in terms of the ability of the latter to metabolize the former. Notably, prebiotics should not be broadly metabolized by the gut microbiota but should instead selectively stimulate the growth of health-promoting microorganisms [4]. Otherwise, liberal supplementation with prebiotics may cause imbalances in the gut microbiota. There is some evidence that some prebiotic oligosaccharides can support the growth not exclusively of beneficial bacteria, but as an example, fecal clostridia can also be enhanced [54]. For this reason, especially in cases when the intestinal microbiota is deprived of health-promoting bacteria, the administration of prebiotics should be considered carefully.

The strains *B. bifidum* BBM and BBV, both isolated from infant feces, likely constitute the most suitable probiotics for synbiotic incorporation, as they were the most adherent strains tested, even in the presence of prebiotics, and therefore, appear to be less sensitive to other variables and culture conditions.

Based on the results reported herein, in strains with a smaller repertoire of fermentable sugars a smaller effect of prebiotics on the resulting adherence was noticed. However, this observation applies to bifidobacteria only and more supporting data would be of importance. A hypothesis suggested previously [20] states that in the presence of prebiotics/carbohydrates, the bacterial energy is preferentially shifted to the production of enzymes required for cleavage of these carbohydrates, and this may reduce the formation of adhesins. Nevertheless, it is only speculative as to whether this is the case and further studies should bring more light into such theory.

5. Conclusions

In summary, we conducted an in vitro testing on direct adhesion of twelve bacterial strains to the intestinal cell model Caco-2-HT29-MTX in the presence of various prebiotics, using a fluorescence-based assay. The data show that prebiotics clearly affect bacteria–epithelia interactions, although all strains tested adhered to cultured tissues in vitro. Based

on adherence, the most suitable probiotic candidates are *B. bifidum* BBM and BBV showing the highest adherence to the mammalian cells. Among commercial prebiotics tested, Vivinal® promoted the adherence of the strains tested in vitro and, thus, appears to be a suitable synbiotic partner for a wide range of probiotics. Nevertheless, the effects of individual prebiotics on adherence were found to be highly strain-specific; thus, some combinations of probiotics and prebiotics may be inappropriate, especially if the latter interferes with adherence by the former. Such combinations may also alter the gut microbiota in unfavorable ways, highlighting the need to individually test each combination of probiotics and prebiotics during the development of synbiotics. Additional experiments are needed to simulate more precisely the gut conditions.

Author Contributions: Conceptualization, G.K., I.H., R.S. and R.K.; methodology, I.H., R.S. and R.K.; formal analysis, I.H. and R.K.; investigation, I.M. and I.H.; resources, G.K.; data curation, I.M. and I.H.; writing—original draft preparation, G.K.; writing—review and editing, R.K. and R.S.; supervision, G.K.; project administration, I.H. and I.M.; funding acquisition, G.K. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the Ministry of Agriculture of the Czech Republic [Institutional support no. MZE-RO1421].

Data Availability Statement: The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding author.

Acknowledgments: The authors would like to thank the breast milk donors for their participation.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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