

## Fermentation properties and potential prebiotic activity of HCl-breadfruit resistant starch type III by *Lactobacillus plantarum* ATCC 13649 and *L. brevis* ATCC 8287

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### Abstract

HCl-breadfruit resistant starch type III (HCl-BFRS3) is a type of resistant starch (RS) produced from breadfruit (*Artocarpus altilis*). Generally, RS is the non-digestible starch fraction that resists digestion in the gastrointestinal tract, and is completely or partially fermented in the colon which gives it beneficial physiological effects as a potential prebiotic. The present work assessed the fermentation properties of HCl-BFRS3 produced by local underutilised food crops. HCl-BFRS3 with 57.86% of RS content was analysed for its fermentation properties. *In vitro* fermentability of HCl-BFRS3 with pure cultures of lactic acid bacteria, LAB (*Lactobacillus plantarum* ATCC 13649 and *L. brevis* ATCC 8287), was studied. Their growth patterns, pH changes, and prebiotic activity score (PAS) along with four other different carbohydrate sources (glucose, inulin, fibersol-2, and breadfruit starch) and a control sample against *Escherichia coli* ATCC 11775 was evaluated after 72 h of fermentation. It was found that HCl-BFRS3 selectively supported the growth of both lactobacilli and *E. coli* ATCC 11775, in the range of 6.21 to 9.20 log<sub>10</sub> CFU/mL. HCl-BFRS3 also decreased the pH from the fermentation by *L. plantarum* ATCC 13649 and *L. brevis* ATCC 8287 after 24 h. The highest PAS was obtained by *L. plantarum* ATCC 13649 grown on HCl-BFRS3 (+1.69) as compared to inulin and fibersol-2. In conclusion, HCl-BFRS3 could be exploited as a prebiotic that benefits human health. Nevertheless, further assessment on the suitability of HCl-BFRS3 as a prebiotic material needs to be carried out.

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### Keywords

*Artocarpus altilis*,  
prebiotic,  
lactic acid bacteria,  
prebiotic activity score,  
resistant starch type III

### Introduction

The term prebiotic has been defined by Zhang *et al.* (2014) as a selectively fermented food ingredient, mainly consisting of carbohydrates, that allows specific changes both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon the host health. In other words, prebiotics can be considered as a 'food' for probiotics (Yang *et al.*, 2017). Many carbohydrate-rich foods have been claimed to have prebiotic activity, but not all dietary carbohydrates serve as prebiotics (Lockyer and Nugent, 2017). Therefore, there is a need to establish clear criteria for classifying a food ingredient as a prebiotic. A prebiotic ingredient should (1) resist digestion in the upper gastrointestinal tract, (2) be selectively fermented by intestinal microflora, and (3) selectively stimulate the growth and/or activity of intestinal microflora associated with health and well-being (Wichienchot *et al.*,

2011). Intestinal microflora generally consist of various lactobacilli and bifidobacteria (Zi-Ni *et al.*, 2015a).

The present work assessed two lactobacilli strains (*Lactobacillus plantarum* ATCC 13649 and *L. brevis* ATCC 8287) as representatives of intestinal microflora. These lactobacilli constitute a major part of the normal intestinal microflora in animals and humans besides bifidobacteria (Bratus, 2013). These lactobacilli were compared against *Escherichia coli* ATCC 11775. Various LAB species (*Lactobacillus*, *Streptococcus*, *Enterococcus*, and *Pediococcus*) have been reported as active candidates for probiotic use in humans and animals (Vantsawa *et al.*, 2017). Probiotics can be defined as "live microbial food supplements that benefit the consumers' health by sustaining or improving their intestinal microbial balance" (Arshad *et al.*, 2018). The significance of using *E. coli* in the present work is because it is a predominant aerobic bacterium inhabiting the human

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gut, and among the first bacterial species that colonise the intestine and remain a permanent resident throughout the host's life (Delmas *et al.*, 2015). This species comprises non-pathogenic commensal bacteria and virulent strains. In the present work, *E. coli* ATCC 11775 was the non-pathogenic commensal bacterium.

Nowadays, researchers have been studying various dietary fibres to investigate their prebiotic nature and potentials. To date, however, only inulin, fructo-oligosaccharide, and galacto-oligosaccharide are accepted as prebiotics (Zaman and Sarbini, 2015; Mohanty *et al.*, 2018). Other miscellaneous carbohydrates, such as resistant starch (RS), is still under investigation, and scarce evidence is available to claim it as a potential prebiotic (Villada *et al.*, 2017). Resistant starch (RS) refers to the non-digestible starch fraction that resists absorption and digestion along the gastrointestinal tract, and may be completely or partially fermented in the colon (Jyothsna and Hymavathi, 2017). The primary beneficial effects as well as secondary beneficial effects of RS as a potential prebiotic have been reviewed (Ahuja *et al.*, 2013; Eroglu and Buyuktuncer, 2017). RS could be produced from highly accessible starch sources such as potato, tapioca, yam, banana, and others, but the yields of RS produced have been different. There are several RS and dietary fibres that exist in the market such as Hi-Maize®, Novelose®, and Fibersym, which are extracted from natural breeding, and retrograded high amylose of maize starch that have been chemically modified based on wheat starch. Nevertheless, none of these RS have been extracted from breadfruit [*Artocarpus altilis* (Parkinson) Fosberg], and therefore no study has been reported regarding its potentiality as prebiotic material.

The main objective of the present work was to find the alternative RS sources with prebiotic properties from breadfruit, an underutilised local food crop. The specific objectives were to assess the *in vitro* fermentability of HCl-breadfruit resistant starch type III (HCl-BFRS3) by *L. plantarum* ATCC 13649 and *L. brevis* ATCC 8287, and compare it

against *E. coli* ATCC 11775 by analysing the bacterial growth, the pH changes in the fermentation medium, and the prebiotic activity score. It was hypothesised that HCl-BFRS3 could exhibit potential prebiotic properties, and provide clearer information on the potential use of breadfruit in the healthy food industry.

## Materials and methods

### Bacterial strains

Pure cultures of *L. plantarum* ATCC 13649 and *L. brevis* ATCC 8287 were used as representative probiotic cultures, and *E. coli* ATCC 11775 was used for comparison. The reason *E. coli* ATCC 11775 was chosen in the present work was due to its opportunistic characteristic, whereby it tends to cause harm to the host when there is an ecological disturbance or microbial imbalance (dysbiosis) in the colon (Kho and Lal, 2018).

*Lactobacillus plantarum* ATCC 13649 and *E. coli* ATCC 11775 were purchased from RIKEN BioResource Centre (Ibaraki, Japan) in the form of sterile lyophilised powder, while *L. brevis* ATCC 8287 was purchased from Remel Europe Ltd. (Kansas, USA) in the form of sterile Culti-Loops®. These bacterial strains were revived and cultured at 37°C in the incubator (model Heratherm IGS60, Fisher Scientific (M) Sdn. Bhd., Selangor, Malaysia) on their respective growth media and conditions as shown in Table 1. All the growth media were purchased from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). The bacterial stock cultures were preserved using microorganism preservation beads in a 2.0 mL cryovial containing sterile liquid medium purchased from Apical Scientific Sdn Bhd. (Selangor, Malaysia), sealed with Parafilm, and stored at -20°C prior to use.

### Enzymes and chemicals

A technical grade debranching enzyme, microbial pullulanase (Promozyme® D2), was purchased from Sigma Aldrich (Germany) with an enzyme activity of  $\geq 1,000$  NPUN/g (New

Table 1. Bacterial strains with culture media and conditions.

Bacterial strain	Culture medium (broth/agar)	Culture condition
<i>Lactobacillus plantarum</i> ATCC 13649	de Man, Rogosa and Sharpe (MRS)	Aerobic, 37°C
<i>Lactobacillus brevis</i> ATCC 8287	de Man, Rogosa and Sharpe (MRS)	Aerobic, 37°C
<i>Escherichia coli</i> ATCC 11775	Nutrient	Aerobic, 37°C

Pullulanase Unit Novo), density of 1.10 - 1.20 g/mL, and used upon receipt. All other chemicals used in the present work were of analytical grade, and purchased from Merck Millipore (Germany), Bendosen Laboratory Chemicals (Norway), Nacalai Tesque, Inc. (Kyoto, Japan), Tokyo Chemical Industry (Tokyo, Japan), Sigma Aldrich (Steinheim, Germany), and HiMedia Laboratories Pvt. Ltd. (Mumbai, India).

### Carbohydrates

The five types of carbohydrates used in the present work were glucose (Merck Millipore, Germany), breadfruit starch (BFS), HCl-breadfruit resistant starch type III (HCl-BFRS3), inulin (Orafti® GR, BENEIO-Orafti, Tienen, Belgium), and fibersol-2 (Matsutani Chemical Industry Co., Ltd., Hyogo, Japan). BFS was extracted from breadfruit flour following the methods prescribed by Madzlan *et al.* (2012). HCl-BFRS3 was in a purified form of breadfruit resistant starch type III (BFRS3) that underwent acid hydrolysis. BFRS3 was extracted from breadfruit starch using optimised conditions as performed by Zi-Ni *et al.* (2015a). The RS content of HCl-BFRS3 was 57.86%. Inulin was supplied by DPO Malaysia Sdn. Bhd. (Kuala Lumpur, Malaysia), while fibersol-2 was supplied by Castle Chemical Sdn. Bhd. (Selangor, Malaysia). Inulin (Orafti® GR) was a mixture of oligosaccharides and polysaccharides, which composed of fructose units linked by  $\beta$ -2, 1 linkage, and a glucose unit at the terminal of almost every molecule. The powder was in granulated form with approximately 92% of inulin and 8% of glucose/fructose/sucrose. Fibersol-2 was a soluble resistant maltodextrin (starch-derived dietary fibre) which contained 90% total dietary fibre (TDF). Both inulin and fibersol-2 served as the positive control for comparison, and were commercial prebiotics and dietary fibres, respectively.

### Preparation of HCl-BFRS3

Breadfruit resistant starch type III (BFRS3) was used to produce HCl-BFRS3 through acid hydrolysis. BFRS3 was produced from BFS using several processing conditions involving disruption of starch granules, enzymatic debranching of starch polymers, starch retrogradation, and drying. Briefly, BFS was suspended in distilled water (20%, w/v), gelatinised by boiling at 90°C for 30 min, followed by starch debranching with 20 Pullulanase Unit Novo (PUN) enzyme per gram starch at 60°C for 24 h. The suspension then was autoclaved at 121°C for 1 h before cold storage at 4°C for 24 h. The BFRS3 was oven-dried at 50°C until its moisture content

was less than 13% (Appiah *et al.*, 2011), and finally ground to fine powder with particles size of 125  $\mu$ m (120 mesh size). Finally, BFRS3 underwent acid hydrolysis with 0.5 M HCl acid at 60°C for 24 h, and ground to fine particles size of less than 125  $\mu$ m (120 mesh size); this produced HCl-BFRS3.

### *In vitro* fermentability of HCl-breadfruit resistant starch type III (HCl-BFRS3) by pure cultures

The ability of HCl-BFRS3 to support the growth of *L. plantarum* ATCC 13649 and *L. brevis* ATCC 8287 was assessed following the methods of Zi-Ni *et al.* (2015b).

### Inoculum preparation

The bacterial strains (*L. plantarum* ATCC 13649, *L. brevis* ATCC 8287, and *E. coli* ATCC 11775) were reactivated separately using their respective broth and conditions shown in Table 1 at 37°C, and incubated for 24 h. Then, the incubated bacteria were streaked onto their respective agar and conditions for 24 h (*E. coli* ATCC 11775) and 48 h (*L. plantarum* ATCC 13649 and *L. brevis* ATCC 8287). Bacterial inoculums were prepared separately by transferring a single colony from each culture agar again into respective broth, and incubated at different incubation times.

### Fermentation medium and process

The fermentation medium was similar to MRS broth, except dextrose was replaced with other carbohydrate sources; glucose, BFS, HCl-BFRS3, inulin, and fibersol-2 at 10 g/L (or 1% w/v). Medium without carbohydrate source served as a control. Each of carbohydrate sources were sterilised separately with 254 nm ultraviolet light for 15 min. Fermentations were carried out separately in Universal bottles with a working volume of 20 mL of fermentation media. An inoculum concentration of *L. plantarum* ATCC 13649, *L. brevis* ATCC 8287, and *E. coli* ATCC 11775 was first optimised from  $10^5$  to  $10^7$  CFU/mL, and then added separately at 5% (v/v) in fermentation medium, and fermentations were performed at 37°C for respective times (0, 6, 12, 24, 48, and 72 h) in aerobic condition. The same growth condition for all bacteria was set in aerobic condition because all of them were facultative anaerobes that can survive in both the presence and absence of oxygen to utilise the carbohydrate sources, but prefer oxygen since it provides higher ATP.

### Enumeration of bacterial growth

The viable counts of bacterial cultures were

enumerated by spread-plating method on their respective agar in duplicates. One mL of each culture was ten-fold serially diluted to  $10^{-8}$  dilution, and an aliquot was spread-plated onto their respective agar. Inoculated agar plates were incubated at  $37^{\circ}\text{C}$  for 24 and 48 h for *E. coli* ATCC 11775 and lactobacilli, respectively. Their growth was calculated as  $\log_{10}$  colony forming unit per mL suspension ( $\log_{10}$  CFU/mL). The formulation of bacterial growth was calculated using Eq. 1:

$$N \text{ (CFU/ mL)} = \frac{C}{vd (n1 + 0.1n2)} \quad (\text{Eq. 1})$$

where, C = the sum of colonies on all plates counted; v = the aliquot volume plated onto each plate (0.1 mL); n1 = the number of plates counted as the first dilution; n2 = the number of plates counted at the second dilution; and d = the dilution from which the first count was obtained.

#### Measurement of pH changes

Three mL of samples were directly withdrawn and determined for pH using a pH meter (Mettler Toledo, China) in triplicates. Changes in pH value were calculated as differences between the pH at the initial (0 h) and final of each fermentation period (6, 12, 24, 48, or 72 h).

#### Prebiotic activity score (PAS)

The prebiotic activity score (PAS) of different cultures of *L. plantarum* ATCC 13649 and *L. brevis* ATCC 8287 were calculated against *E. coli* ATCC 11775, as described by Huebner *et al.* (2007). The scores were calculated based on the growth changes, where the growth changes of bacteria were calculated as differences of viable count between specific time (24, 48, and 72 h) and initial time (0 h) of fermentation (in  $\log_{10}$  CFU/mL), using Eq. 2:

$$\text{PAS} = \left[ \frac{\text{Growth changes of lactobacilli on prebiotic}}{\text{Growth changes of lactobacilli on glucose}} \right] - \left[ \frac{\text{Growth changes of E.coli on prebiotic}}{\text{Growth changes of E.coli on glucose}} \right] \quad (\text{Eq. 2})$$

#### Statistical analysis

All the results were expressed as mean  $\pm$  SD (standard deviation). The triplicate data were subjected to two-way analysis of variance (ANOVA) to test the interaction between factors involved (carbohydrates/bacteria strains and fermentation times) and one-way ANOVA to analyse the mean differences among each factor. Duncan *post-hoc* test was performed, and the level of significance was set at  $p < 0.05$  (95% confidence level). The Statistical

Package for Social Science, Version 24.0 (IBM SPSS Inc., USA) was used for the analysis.

## Results and discussions

In the present work, two lactobacilli (*L. plantarum* ATCC 13649 and *L. brevis* ATCC 8287) and one opportunist bacterium (*E. coli* ATCC 11775) were used in order to assess the fermentation properties of HCl-BFRS3 and other carbohydrate substrates (glucose, inulin, fibersol-2, and BFS).

*Growth patterns of L. plantarum* ATCC 13649, *L. brevis* ATCC 8287, and *E. coli* ATCC 11775 at 24, 48, and 72 h

The growth patterns of *L. plantarum* ATCC 13649, *L. brevis* ATCC 8287, and *E. coli* ATCC 11775 are shown in Figures 1(A), 1(B), and 1(C) at 24, 48, and 72 h in the media containing different carbohydrate sources (glucose, inulin, fibersol-2, BFS, and HCl-BFRS3) and the control (without any carbohydrate sources). The substrates fermentability by bacteria was determined by observing the differences in growth patterns of different bacteria in the media containing different carbohydrate sources. Lactobacilli are well-known beneficial saccharolytic bacteria that produce only beneficial end-metabolites, such as lactic acid. Moreover, lactobacilli are the predominant members of the human indigenous microflora.

The ability of lactobacilli to ferment non-digestible carbohydrates could be substrate-strain-dependent, which means different strains possess different metabolic pathways to metabolise different types of carbohydrates. After 24 h of fermentation, *L. plantarum* ATCC 13649, *L. brevis* ATCC 8287, and *E. coli* ATCC 11775 expelled HCl-BFRS3, glucose, and fibersol-2, separately, which were recorded at 7.43, 10.23, and 9.39  $\log_{10}$  CFU/mL, respectively (Figure 1A). Meanwhile, after 48 h of fermentation (Figure 1B), *L. plantarum* ATCC 13649 showed a difference in substrate consumption, where it changed to fibersol-2 (6.93  $\log_{10}$  CFU/mL), which is also a polymeric substrate similar to that of HCl-BFRS3. Other bacteria showed similar growth patterns in the same substrates at 24 h fermentation. However, as the fermentation time progressed to 72 h (Figure 1C), it was demonstrated that *L. plantarum* ATCC 13649 had chosen both polymeric substrates, HCl-BFRS3 and fibersol-2, as the growth rates had not shown any significant difference for both substrates, which were recorded at 6.82 and 6.76  $\log_{10}$  CFU/mL, respectively.

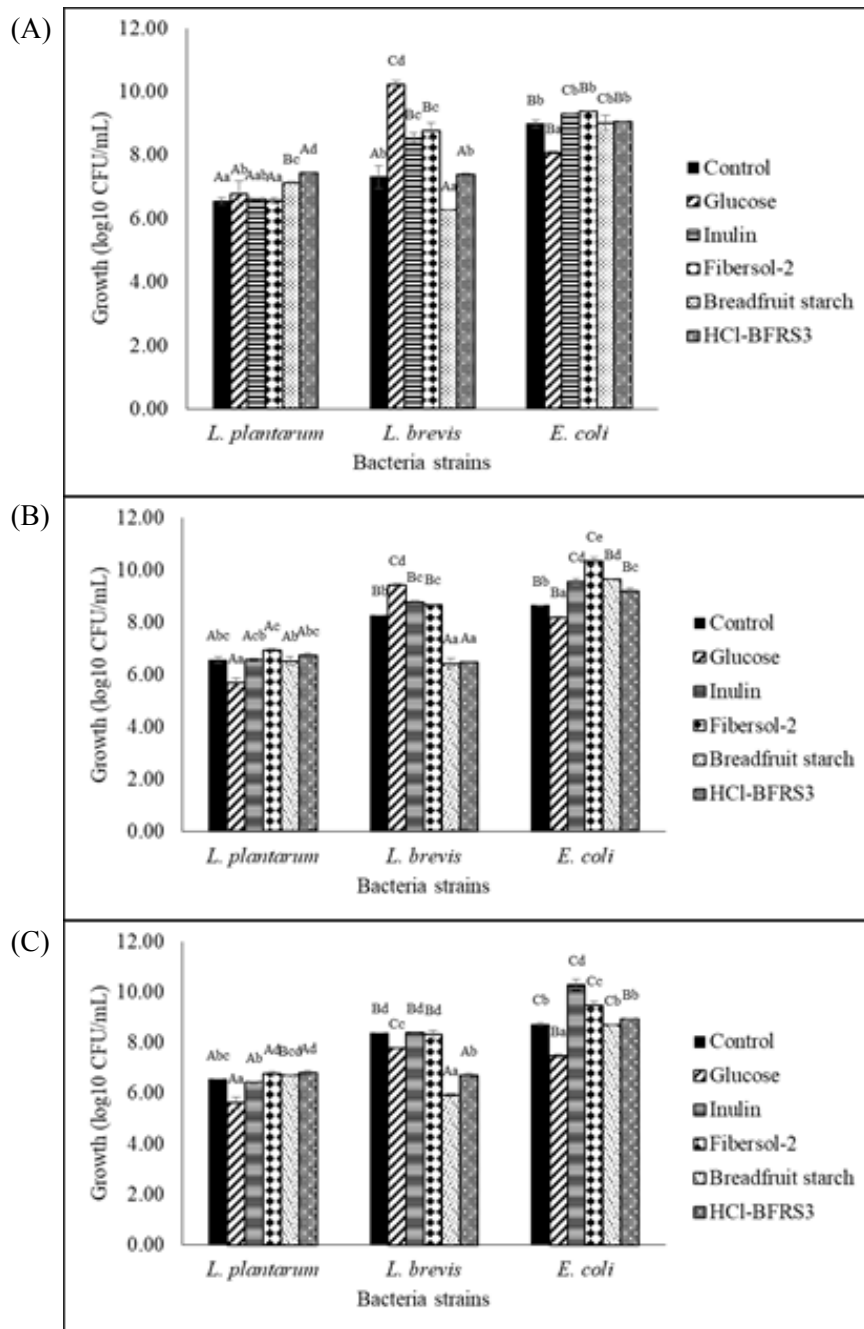


Figure 1. Growth patterns of viable *L. plantarum* ATCC 13649, *L. brevis* ATCC 8287, and *E. coli* ATCC 11775 in different carbohydrate sources (glucose, inulin, fibersol-2, BFS, and HCl-BFRS3) at 24 (A), 48 (B), and 72 (C) h of fermentation. Means with different uppercase and lowercase letters indicate significant difference at  $p < 0.05$  between bacterial strains and carbohydrate sources, respectively.

Bernalier-Donadille (2010) claimed that the bacteria belonging to phylum Firmicutes, specifically LAB, possess hydrolytic enzymes which are vital in aiding the hydrolysis of complex carbohydrates like resistant starch, inulin, and fibersol-2, which then release the simple sugar, glucose. Human hosts do not have this enzyme to breakdown any complex carbohydrates that escape from digestion in the upper gut; thus, LAB are needed (Ciani *et al.*, 2013). Based on the growth

patterns of *L. plantarum* ATCC 13649 at 24, 48, and 72 h, HCl-BFRS3 and fibersol-2 were utilised significantly ( $p < 0.05$ ) by this bacterium as compared to inulin that served as a positive control.

However, this contradicted *L. brevis* ATCC 8287 which favoured simple sugar consumption like glucose at 24 and 48 h fermentation rather than polysaccharides. Although both lactobacilli (*L. plantarum* ATCC 13649 and *L. brevis* ATCC 8287) belong to the same genus, their substrate preferences

are highly varied (Macfarlane *et al.*, 1998). This phenomenon is related to the term substrate-strain-dependence, whereby *L. brevis* ATCC 8287 has selected glucose for their growth due to the simple structure of glucose that can be directly taken for fermentation instead of polymer carbohydrates that must be degraded prior to being fermented. Based on the growth pattern of *L. brevis* ATCC 8287, it can be said that this bacterium has the ability to shift their carbohydrate preferences as the fermentation time progressed to 72 h. This was corroborated by a previous study by Miremedi and Shah (2012) who expressed that the microbial population colonising the small intestine had shifted their selection for fermentation substrates from starch and non-starch polysaccharide to inulin.

A study by Zi-Ni (2016) stated that there were bacteria under the genus *Bacteroides* and phylum Clostridia that could also perform carbohydrate fermentation. The results obtained in the present work revealed that *E. coli* ATCC 11775, an opportunist bacterium, also favoured complex carbohydrates such as fibersol-2 and inulin as well as lactobacilli. In a healthy human, *E. coli* can be found prevalently in the gastrointestinal tract, reaching up to  $10^9$  CFU/g in the faecal matter (Delmas *et al.*, 2015). Most types of *E. coli* are harmless, and even help keep our digestive tract healthy, but some strains that are found in contaminated food or polluted drinking water can cause diarrhoea, which explains why they are known as detrimental bacteria. Their survivability in the colon also depends on the fermentation of carbon sources, leading to the production of organic acids and short chain fatty acids (SCFAs) (Christofi *et al.*, 2019). Inulin and fibersol-2 are two polymeric substrates that could sustain the growth of detrimental bacteria as well as beneficial bacteria for a longer time due to its complex structures.

#### *pH changes*

The pH changes of media from the fermentation of different carbohydrate sources by *L. plantarum* ATCC 13649, *L. brevis* ATCC 8287, and *E. coli* ATCC 11775 are illustrated in Figures 2, 3, and 4, respectively. The reduction in the pH of media containing carbohydrate substrates is another way to assess the fermentability of substrates by bacteria. Based on the results, both lactobacilli and *E. coli* exhibited different trends of pH reduction on different substrates.

#### *pH reduction of different carbohydrate sources by Lactobacillus plantarum ATCC 13649 during*

#### *fermentation*

Theoretically, a reduction in pH values is an indicator of the production of organic acids from carbohydrate fermentation (Napisah, 2018). The utilisation of carbohydrates by LAB during carbohydrate fermentation produces organic acids as their main products, mainly lactic acid (Zi-Ni *et al.*, 2015b). Other organic acids produced included SCFAs, which principally come from carbohydrate fermentation, and branched chain amino acids (BCFAs) produced by protein metabolism (Hijova and Chmelarova, 2007). *In vitro* fermentation of all substrates, including the control (without carbohydrate sources) by *L. plantarum* ATCC 13649 showed a reduction in the pH values (Figure 2). The reduction in pH was observed starting from the first hour until 72 h of *in vitro* fermentation for all the bacteria tested. At first, it was assumed that the longer the fermentation time, the more acids would be produced by these bacterial strains during their proliferation on different carbohydrate sources. However, the results in obtained in the present work showed a contradiction in that assumption, whereby fermentation period did not affect the changes in pH and acids produced. The carbohydrates consumed by the bacteria that determine their end metabolites. A research by Zarinah *et al.* (2019) reported that gut microorganisms use a variety of fermentative pathways to harvest energy, and the pathways depend on many factors, including pH and available fermentation substrates. In the present work, glucose fermentation by all bacteria yielded the greatest reduction in the pH of the media, whereas fermentation of fibersol-2 and BFS yielded the least reduction during the first 12 h of fermentation of *L. plantarum* ATCC 13649. This might be due to the characteristic of glucose as it is a simple sugar that can be used directly for bacterial growth, and this was demonstrated by all bacteria tested in the present work on glucose medium which showed greatest pH reduction.

Additionally, *L. plantarum* is a facultative heterofermenter that converts 1 mol of glucose to 1 mol each of lactic acid, ethanol, acetate, CO<sub>2</sub>, and other by-products only under certain conditions and from specific substrates (Napisah, 2018). Even though the pH of fermentation media by *L. plantarum* ATCC 13649 containing glucose was low, it was previously proven that *L. plantarum* had special adaptation to survive in such low pH conditions. *L. plantarum* had adapted themselves by lowering their intracellular pH values until an equilibrium had been reached between the intracellular and extracellular concentrations of

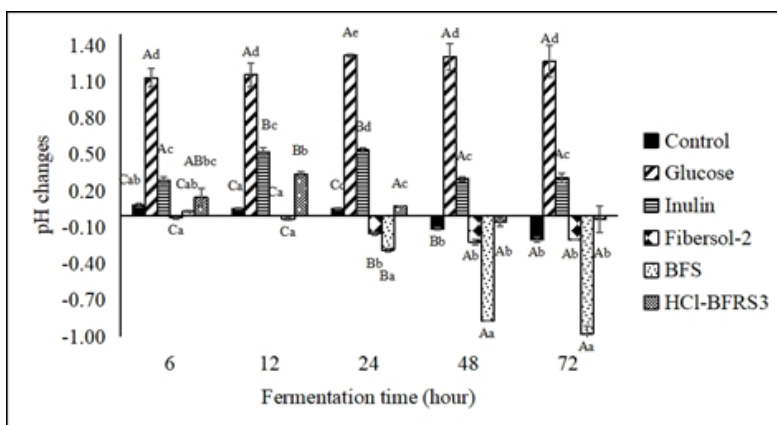


Figure 2. pH reduction of media with different carbohydrate sources (glucose, inulin, fibersol-2, BFS, and HCl-BFRS3) by *L. plantarum* ATCC 13649 during 72 h of fermentation. Means with different uppercase and lowercase letters indicate significant difference at  $p < 0.05$  between fermentation times and carbohydrate sources, respectively.

lactic acid molecules. This made them resistant towards organic acids in the medium (Stevens *et al.*, 2008).

Based on Figure 2, starting at 24 h and further fermentation by *L. plantarum* ATCC 13649, the least pH reduction in media supplemented with fibersol-2 and BFS was observed. This was likely due to protein decomposition in the medium that led to the release of ammonium, hence the pH of the media increased from that at early fermentation time, 0 h. The pH increment was attributed to the ammonium-alkaline compound produced during fermentation, which was the result of the consumption of other nutrient compounds in the media such as biogenic amines comprised of histamine, tyramine, putrescine, and cadaverine (Napisah, 2018; Ruiz Rodríguez *et al.*, 2019). These compounds might be presented as end metabolites of LAB metabolism which increase the pH of media containing fibersol-2 and BFS, thus resulting in

negative pH changes when compared to the initial pH at 0 h. In addition, similar trends of pH reduction were also observed in media containing inulin and fibersol-2 by *L. brevis* ATCC 8287 (Figure 3) and *E. coli* ATCC 11775 (Figure 4).

The reduction in pH in the fermentation media by LAB has been described by Lockyer and Nugent (2017) whereby a greater pH reduction could be an indicator of greater fermentability, as a higher concentration of organic acid is produced. In the present work, this was corroborated by the significantly higher viability of *L. plantarum* ATCC 13649 in media containing HCl-BFRS3 than inulin and fibersol-2 in Figure 1(A) at 24 h, thus resulting in greater pH reduction (Figure 2) at 24 h of fermentation time. Therefore, it was hypothesised that HCl-BFRS3 might have the potential as a prebiotic material.

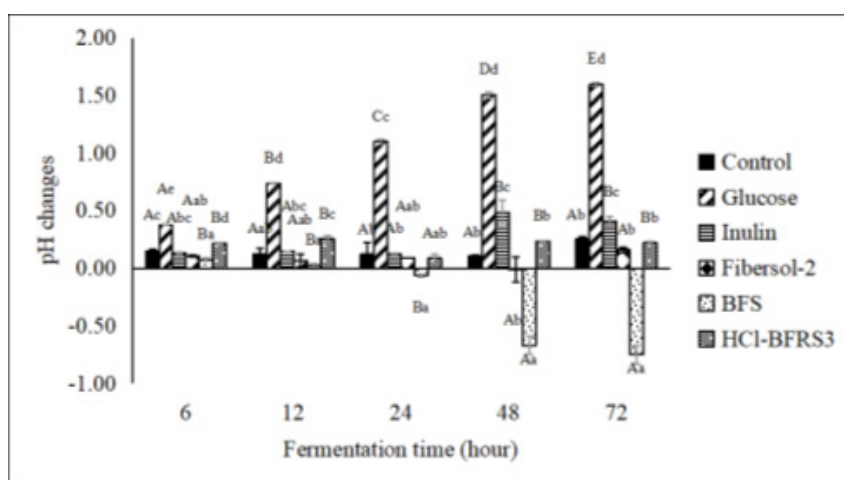


Figure 3. pH reduction of media with different carbohydrate sources (glucose, inulin, fibersol-2, BFS, and HCl-BFRS3) by *L. brevis* ATCC 8287 during 72 h of fermentation. Means with different uppercase and lowercase letters indicate significant difference at  $p < 0.05$  between fermentation times and carbohydrate sources, respectively.

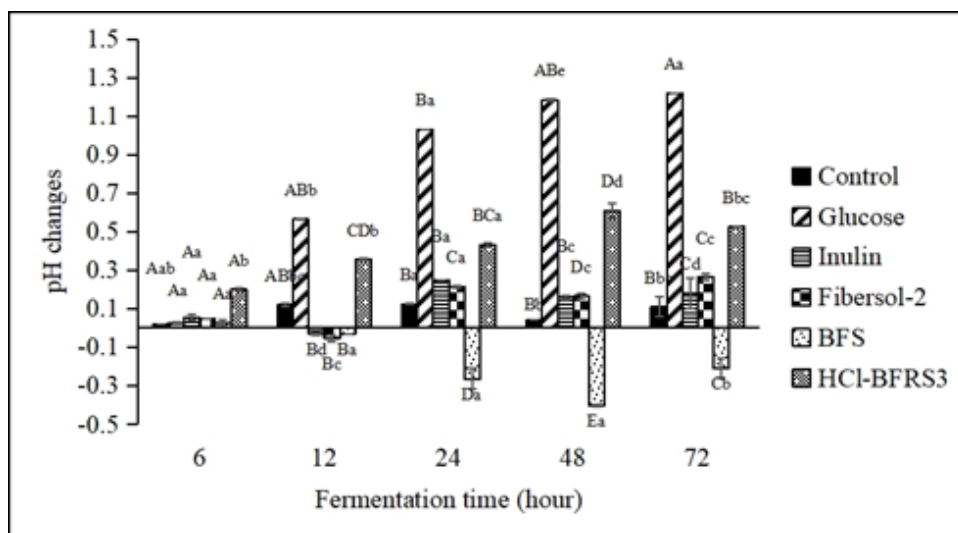


Figure 4. pH reduction of media with different carbohydrate sources (glucose, inulin, fibersol-2, BFS, and HCl-BFRS3) by *E. coli* ATCC 11775 during 72 h of fermentation. Means with different uppercase and lowercase letters indicate significant difference at  $p < 0.05$  between fermentation times and carbohydrate sources, respectively.

#### *pH reduction of different carbohydrate sources by Lactobacillus brevis ATCC 8287 during fermentation*

*Lactobacillus brevis* has been previously listed as a strain used in probiotic products (Quigley, 2018). It is present in various foods, cow manure, humans' and rats' mouths, and intestinal tracts (Quigley, 2018). pH is one of the important factors for the distribution and shifting of microbiota compositions in the human gut (Ilhan *et al.*, 2017).

Bacterial fermentation is a significant process whereby they acquire energy from fermentation of non-digestible carbohydrate sources. A study by Zi-Ni *et al.* (2015b) has pointed out that the fermentability of the substrates by bacteria was varied and strain-dependent. This can be seen by the growth of *L. brevis* ATCC 8287 where it proliferated rapidly in medium containing glucose as the fermentation time progressed, achieving a maximum pH reduction of 1.60 at 72 h fermentation. Glucose is a mono sugar that can be rapidly utilised by bacteria for their growth. In the present work, due to it being a simple sugar, glucose was consumed straightaway by *L. brevis* ATCC 8287. This bacterium is an obligate heterofermentative Gram-positive bacterium that is able to convert 1 mol of glucose through the phosphoketolase pathway to yield 1 mol of lactic acid, ethanol/acetic acid, and carbon dioxide (Fukao *et al.*, 2013; Manini *et al.*, 2015).

In contrast with glucose, fermentation of BFS exhibited the least pH reduction, after 12 h and up until the end of fermentation time, recorded at -0.06 and -0.75 consecutively. This happened due to lesser production of lactic acids from fermentation of BFS by *L. brevis* ATCC 8287. BFS is a native starch

that has intact granules which prohibit it from being digested and fermented by bacteria to produce the acidic end metabolites, lactic acid.

The fermentation of commercial prebiotic (inulin) by *L. brevis* ATCC 8287 was better than breadfruit resistant starch, HCl-BFRS3, at all fermentation times which yielded the highest pH value of 0.49 at 48 h fermentation, except at 12 h. The pH reduction in media containing HCl-BFRS3 (0.26) was higher than inulin (0.16) at 12 h. This indicated that HCl-BFRS3 had higher fermentability than fibersol-2 at 12 h. This was due to different botanical sources of resistant starch obtained. Fibersol-2 was obtained from treated corn starch, while HCl-BFRS3 was produced from breadfruit starch. Previous research added that different starch botanical sources will yield different percentages and quality of the resistant starch even though the same processing methods are employed (Demirkesen-Bicak *et al.*, 2018).

In summary, Ilhan *et al.* (2017) postulated that acidic pH in fermentation media was an important key where it suppressed the growth of other enteropathogens in the gut such as *Veillonella* and *Streptococcus*, whereas some *Bacteroides* and lactobacilli were able to grow and cope well over a wide range of pH values. The lactic acid and SCFA reduced the intraluminal pH which favoured the growth of bifidogenic and LAB (Napisah and Rosma, 2020). This mechanism allowed prebiotics to manipulate the composition of colonic microbiota in the human gut, thus improving the host health in return (Azmi *et al.*, 2012). SCFA were able to stimulate water and electrolyte absorption in the intestine, thus reducing the potential risk of



diarrhoea and dehydration, and increasing colonocyte proliferation and metabolic energy production (Van den Abbeele *et al.*, 2013).

#### *pH reduction of different carbohydrate sources by Escherichia coli ATCC 11775 during fermentation*

Similar to *Lactobacillus* and other gut bacteria, *E. coli* also metabolise different carbohydrate sources to survive, and this depends on many factors, including pH and availability of fermentation substrates (Ilhan *et al.*, 2017). In the present work, the opportunist bacterium, *E. coli* ATCC 11775, demonstrated variations in pH changes towards different substrates at 72 h of fermentation. Overall, *E. coli* ATCC 11775 illustrated their preference towards all carbohydrate sources, glucose, fibersol-2, inulin, and HCl-BFRS3 as they showed great pH reduction, except for BFS (Figure 4). *Escherichia coli* consumed both simple and complex carbohydrates in the present work.

Previous literature had reported that *E. coli* possessed a special adaptation known as carbon catabolite repression (CCR), where it prevented the consumption of other carbon sources, like protein. In other words, this CCR prevented *E. coli* from consuming other carbon sources when it has started consuming glucose. The precise mode of action of CCR in *E. coli* is by regulating the sugar transporters and carbon degradation pathways which enhance their need to consume glucose more efficiently than other carbon sources (Hollinshead *et al.*, 2016).

Besides, *E. coli* ATCC 11775 also showed greater pH reduction when fermented with complex carbohydrate sources (HCl-BFRS3 > fibersol-2 > inulin). Breadfruit resistant starch type III (HCl-BFRS3) was dominated by fibersol-2 and inulin with greater pH reduction of 0.61 at 48 h fermentation. The differences in pH changes among HCl-BFRS3, fibersol-2 and inulin were likely due to the substrate-strain-dependence and chain length of these carbohydrates. It was also noted that *E. coli* ATCC 11775 favoured the degradation of longer chains of polysaccharides similar to LAB rather than shorter ones like inulin which only had a degree of polymerisation (DP) of 10 - 12. DP refers to the numbers of monomeric units such as glucose in a macromolecule of carbohydrate (Jayus *et al.*, 2016). To conclude, the longer chain carbohydrate could sustain the growth of *E. coli* ATCC 11775 for a longer time due to high availability of the simple sugar after the degradation process of complex carbohydrates.

#### *Prebiotic activity score (PAS) of L. plantarum*

ATCC 13649 and *L. brevis* ATCC 8287 against *E. coli* ATCC 11775

The prebiotic activity score (PAS) was employed with the aim to determine to which extent HCl-BFRS3 supported the growth of LAB over *E. coli* ATCC 11775. The PAS of *L. plantarum* ATCC 13649 and *L. brevis* ATCC 8287 against *E. coli* ATCC 11775 in different carbohydrate substrates (glucose, inulin, fibersol-2, BFS, and HCl-BFRS3) and control are presented in Figure 5 (A, B, and C) respectively at 24, 48, and 72 h. LAB strains have been widely used in dairy foods, and deemed to have excellent probiotic properties as can be determined through the PAS (Anprung and Sangthawan, 2012).

PAS can be defined as the ability of given substrates (inulin, fibersol-2, BFS, and HCl-BFRS3) to support the growth of lactobacilli over other organisms and relative to the growth on a non-prebiotic substrate, such as glucose (Huebner *et al.*, 2007). The PAS for *L. plantarum* ATCC 13649 and *L. brevis* ATCC 8287 against *E. coli* ATCC 11775 were significantly different at 24 and 48 h, except for 72 h. The highest PAS of *L. plantarum* ATCC 13649 was observed at 24 h fermentation with the supplementation of HCl-BFRS3 (1.69) and BFS (0.78), whereas negative PAS were detected with supplementation of inulin (-0.12) and fibersol-2 (-0.25). Both HCl-BFRS3 and BFS are derived from breadfruit, and classified as long-chain carbohydrates comprised of amylose and amylopectin linked by glycosidic linkages. Therefore, *L. plantarum* ATCC 13649 required a longer time to breakdown the long-chain carbohydrates and utilise it for their growth, thus leading to the higher PAS for HCl-BFRS3 and BFS as compared to other carbohydrate sources at 24 h.

*L. brevis* ATCC 8287 had negative PAS on all substrates. According to Huebner *et al.* (2007), positive PAS indicated that the potential substrates had supported better growth of beneficial bacteria than detrimental bacteria or pathogens. It can be assumed that those substrates (HCl-BFRS3 and BFS) could support the growth of *L. plantarum* ATCC 13649 specifically at 24 h. Besides these two substrates, other sugar compounds particularly oligosaccharides from *Dioscorea hispida* also demonstrated better growth for *L. plantarum* (Napisah and Rosma, 2020). HCl-BFRS3 dominated over other substrates on *L. plantarum* ATCC 13649, and it was better than *L. brevis* ATCC 8287. The findings were corroborated by a study of Fuentes-Zaragoza *et al.* (2011) and Zaman and Sarbini (2015), where resistant starch had a potential prebiotic function towards human health.

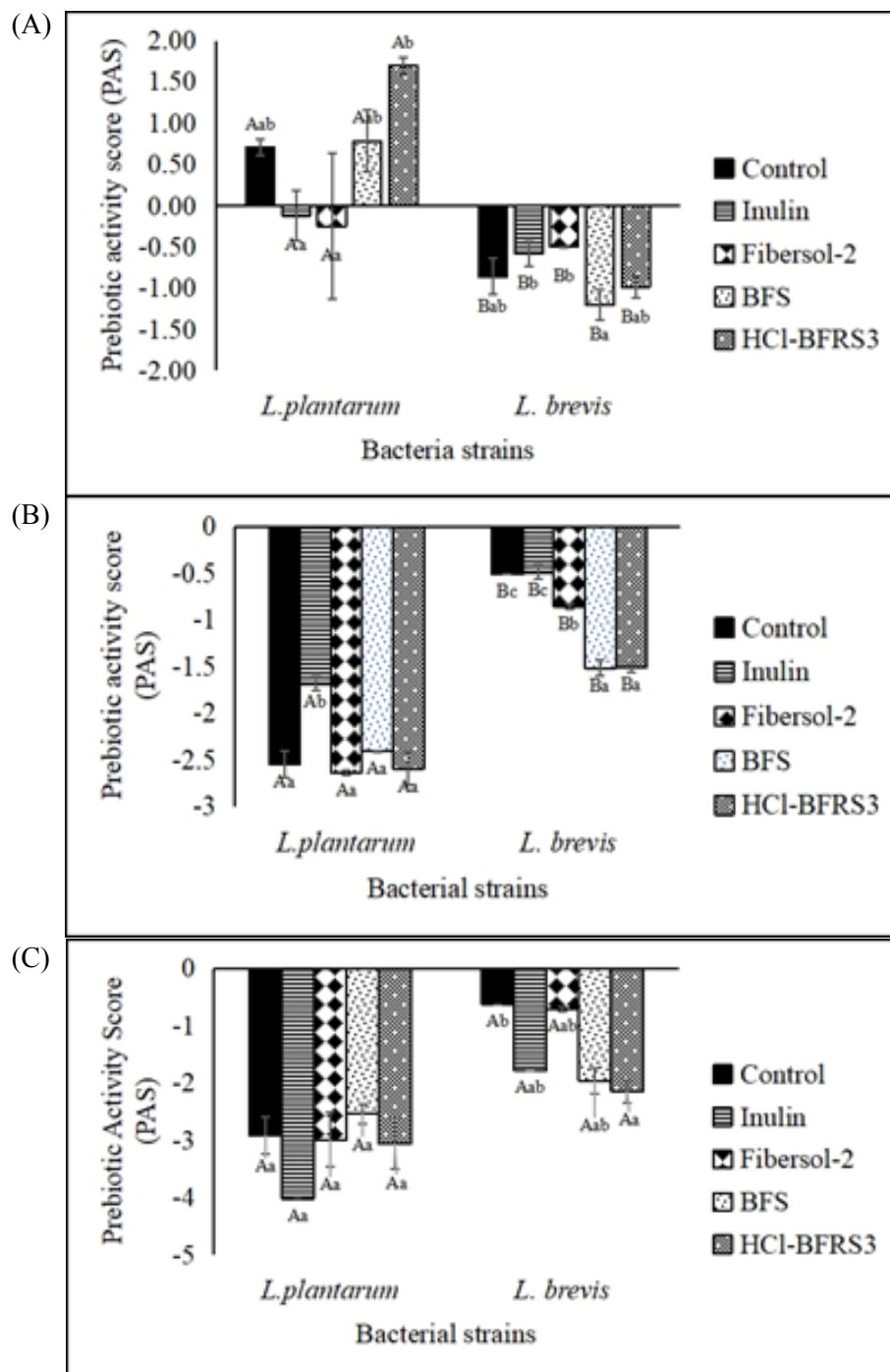


Figure 5. Prebiotic activity scores for different carbohydrate sources (control, inulin, fibersol-2, breadfruit starch, and HCl-BFRS3) by *L. plantarum* ATCC 13649 and *L. brevis* ATCC 8287 against *E. coli* ATCC 11775 at 24 (A), 48 (B), and 72 (C) h of fermentation. Means with different uppercase and lowercase letters indicate significance difference at  $p < 0.05$  between bacterial strains and carbohydrate sources, respectively.

In addition, the susceptibility of a particular carbohydrate to bacterial digestion is dependent on the strains within the same species or so-called as substrate-strain-specific (Zi-Ni *et al.*, 2015a). This was illustrated by the growth changes of *L. plantarum* ATCC 13649 and *L. brevis* ATCC 8287 on the same substrates against *E. coli* ATCC 11775 at 24 h fermentation. *Lactobacillus plantarum* ATCC

13649 had significantly obtained higher positive PAS for all substrates except for inulin and fibersol-2, as compared to *L. brevis* ATCC 8287 which displayed negative PAS at 24 h fermentation. Therefore, it can be concluded that different bacterial strains had different PAS. Anprung and Sangthawan (2012) had postulated that differences in PAS of bacteria were likely due to differences in

the metabolic capacity of bacterial strains, and their prebiotic utilisation that requires the presence of specific hydrolysis and transport systems for a specific prebiotic. This result agrees with a study by Huebner *et al.* (2007) where different PAS by strains within the same species were also observed in lactobacilli cultures for all the prebiotic carbohydrates tested in that study.

Scores lower than 1 or negative indicate that the growth of tested strain is lower on the specific prebiotics as compared to that on glucose and/or its growth is lower than the reference bacteria (*E. coli*) on the prebiotic carbohydrate (Huebner *et al.*, 2007). In the present work, all the lactobacilli tested against *E. coli* ATCC 11775 showed negative PAS when incubated with all substrates at 48 and 72 h of fermentation. This paralleled the growth pattern of *L. plantarum* ATCC 13649 at 48 and 72 h (Figure 1) where its growth was lowest in glucose at these two fermentation times as compared to *L. brevis* ATCC 8287 and *E. coli* ATCC 11775. Therefore, it might be assumed that the incapability of *L. plantarum* ATCC 13649 to grow in glucose had caused the reductions of its PAS. Based on Figure 4, although the growth changes of *L. brevis* ATCC 8287 at 48 and 72 h in the media containing all substrates were higher, the PAS for all substrates at these two fermentation times were negative. These results implied that the growth changes were not necessarily determined by the prebiotic potential of any studied substrates. These results were supported by Zi-Ni (2016) who recorded the same trend, although *Bifidobacterium* sp. FTDC8943 had lower cell density changes than *L. casei* FTCC0442 in tested carbohydrate, the prebiotic scores of *Bifidobacterium* sp. FTDC8943 were higher than *L. casei* FTCC0442.

## Conclusion

In conclusion, different carbohydrate sources influenced the growth of all bacteria tested. HCl-breadfruit resistant starch type III (HCl-BFRS3) had the potential to be further studied as one of prebiotic materials as it exhibited the ability to be fermented by *L. plantarum* ATCC 13649 and *L. brevis* ATCC 8287. This criterion partially fulfilled the prebiotic properties. HCl-BFRS3 could support the selective growth of beneficial bacteria, *L. plantarum* ATCC 13649 and *L. brevis* ATCC 8287, by reducing the pH changes and yielding higher values of the prebiotic activity scores at 24 h of fermentation. However, an increase in bacterial cell density on a particular carbohydrate alone could not

be used to determine the prebiotic potential of the carbohydrate because the tested carbohydrate also supported the growth of pathogenic bacterium (*E. coli* ATCC 11775). Thus, quantitative prebiotic activity scores were applied to determine the prebiotic potential of HCl-BFRS3 against *E. coli* ATCC 11775. Among all substrates, the highest prebiotic activity score was given by *L. plantarum* ATCC 13649 grown on HCl-BFRS3 rather than on inulin and fibersol-2 at 24 h fermentation.

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