

In vitro evaluation of probiotic bacteria and yeast growth, pH changes and metabolites produced in a pure culture system using protein base products with various added carbon sources

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Abstract

This study was designed to evaluate the changes number of cells of the two selected probiotic bacteria, *B. longum* TISTR2195 and *L. plantarum* TISTR1465, and yeast *S. cerevisiae* TISTR8656. The microorganisms were cultured in two types of media, namely selective media and protein base model. Selective media were divided into 4 sub-groups: with 2% glucose added as a positive control (i), inulin (ii), fructo-oligosaccharide (FOS) (iii), and without added carbon source as negative control (iv). Protein base media had chicken egg and coconut juice, baker's yeast and potassium chloride (i), with 9% FOS (ii), or inulin (iii), or 7.34% whey protein (iv) to verify growth. All the probiotics were able to utilize inulin and FOS to growth. The numbers of cells increased with incubation time. However, *L. plantarum* produced an acidic in the selective culture medium significantly. Protein based with FOS and inulin added supported all probiotics growth. In addition, acetic acid content was the highest among short-chain fatty acids (SCFAs) produced. *B. longum* produced acetic acid very fast within 24 h. *L. plantarum* cultured in inulin added provided the peak level of acetic acid at 72 h. However, *S. cerevisiae* tended to decrease the SCFAs with incubation time.

Keywords: fructo-oligosaccharide (FOS); inulin, prebiotic; probiotic; short-chain fatty acid.

Practical Application: The use of 9% prebiotic added in high protein food processed by retorting can induce the growth of probiotics and their SCFAs produced. The selected product will be marketed for elderly people.

1 Introduction

Probiotics, derived from the Greek words meaning “for life”, are defined as “[...] live microorganisms or their substances produced when administered in adequate amounts confer a promote health benefit on the host [...]” (Hill et al., 2014, p. 506). In addition, Zendeboodi et al. (2020) stated that the definition has been broadened not only live cells of microorganisms (viable/active) but also dead/inactive cells of probiotic and the biochemical metabolites of probiotic. In a recent year, there is added probiotic in several commercial food such as non-dairy foods matrices (Rasika et al., 2021) and dairy (Hadjimbei et al., 2020; Pradeep Prasanna & Charalampopoulos, 2019). Moreover, clinical studies revealed that the addition of probiotic in food product enhance the several mechanisms of action in host health such as altered gut microbiota and restore barrier function (Eor et al., 2020), reduction of postprandial glycemia in healthy (Grom et al., 2020) and prevention of postmenopausal osteoporosis in mouse (Lee et al., 2020).

Probiotics are a group of intestinal microflora that plays an important role for host health by fermenting substrates to end products that directly or indirectly promote mucosal cell function in colon. One type of end products is short-chain fatty acids (SCFAs) with the volatile acid group including acetic acid (C2), propionic acid (C3) and butyric acid (C4). Mostly the effects of SCFA on host health involve changing gene receptor activities (Marques et al., 2019). Among the microbials used as

probiotics are different types of bacteria such as the lactic acid bacteria (LAB), *Lactobacillus* sp. and *Bifidobacterium* sp., while some yeast types, such as *Saccharomyces* sp., are also included (Heyman & Ménard, 2002).

The concept of prebiotics was first defined in 1995 as a “[...] non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria already resident in the colon” (Gibson et al., 2017, p. 492). However, 20 years later in 2016 The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics included “[...] any substrate that is selectively utilized by host microorganisms and giving a health benefit [...]” (Gibson et al., 2017, p. 491).

Prebiotics stimulate the growth or activate specific microbial genera and species in the gut microbiota in order to confer health benefits to the host. However, there are some criteria that must be met in order to allow the classification of a food ingredient as a prebiotic. For instance, it must be neither hydrolyzed, nor absorbed in the upper part of the gastro-intestinal tract, but should promote selective fermentation by potentially beneficial bacteria in the colon and the alteration of the composition of the colonic microbiota towards a healthier composition, preferably inducing effects that are beneficial to the host's health

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(Mumcu & Temiz, 2014). The most well-known prebiotics are non-digestible carbohydrates, particularly oligosaccharides.

Among the oligosaccharide compounds, inulin or β [2,1]-fructans can be hydrolyzed into fermentable fructose and glucose inulinase by probiotics (D. Wang et al., 2016). Although inulin is a fructan monomer, it typically has a more heterogeneous degree of polymerization (DP) of between 3 and 60 ($DP_{\text{average}} = 10$ and 25 in HP grade). In addition, fructo-oligosaccharide (FOS) can be produced from degradation of inulin and oligofructose by endoglycosidase enzymes, yielding a product with a DP of 2–10 ($DP_{\text{average}} = 4$) (Roberfroid, 2007). A FOS structure can be present in which G is glucose and F is fructose and n is the number of fructosyl units, represented by GF_n or F_n (Saulnier et al., 2007).

In a prior study (Kaplan & Hutkins, 2000), it was noted that lactic acid bacteria and bifidobacterium can utilize fructo-oligosaccharide in selected media, as observed by optical density (OD) values, and especially *Lactobacillus plantarum* ATCC4008 gave the highest OD. In addition (Mei et al., 2011) reported that the OD of bifidobacteria grown in selected media with added FOS was higher than that of a control sample grown with glucose. Moreover (Mumcu & Temiz, 2014) stated that prebiotic inulin and FOS can enhance the growth performance of probiotic bacteria.

Based on a review of relevant literature, probiotic growth has been mainly measured by OD, not as a number count. There is less scientific data for growth determined by cell count, OD and changes in pH, as affected by probiotics. Growth conditions without carbohydrates but with sugar have never been reported on for probiotics. Recently, it was proved that using only OD may give inappropriate conclusions, as this measure does not directly reflect the growth.

Generally, the probiotic bacteria such as lactobacilli and bifidobacteria are well-known to have good growth in the selective De Man, Rogosa and Sharpe (MRS) broth, which contains carbon and nitrogen sources in peptone or yeast or meat extract (Horn et al., 2005). This study was aimed to determine the relationship between the cell count of probiotics, their OD, and the pH changes in selected broth media, as affected by the type of prebiotic. In addition, the hypothesis that the final food product made from high protein source, such as chicken egg, and added with prebiotics can stimulate the growth of probiotic bacteria and yeast, needs to be assessed, and the metabolites in an in-vitro system should be determined.

2 Materials and methods

2.1 Microbial strains

The test organisms were lyophilized forms of the bacterial strains, *Bifidobacterium longum* TISTR2195 and *Lactobacillus plantarum* TISTR 1465, and the yeast strain, *Saccharomyces cerevisiae* TISTR8656, which were obtained from the Thailand Institute of Scientific and Technological Research, Thailand. The lyophilized *B. longum* and *L. plantarum* were cultured in deMan, Rogosa and Sharp (MRS) lactobacillus medium and *S. cerevisiae* was cultured in a yeast extract peptone dextrose (YPD) medium.

2.2 Inoculum preparation and probiotic growth in selective media

After the test organisms were sub-cultured to get a healthy condition, a single colony from a pure culture of each bacterial strain was inoculated into 9 mL of MRS broth medium containing proteose peptone (10%), beef extract (1%), yeast extract (0.5%), glucose (2%), Tween 80 (0.1%), $(NH_4)_2$ citrate (0.2%), sodium acetate (0.5%), $MgSO_4$ (0.01%), $MnSO_4$ (0.005%), K_2HPO_4 (0.02%), L-cysteine (0.05%) with adjusted pH of 6.5, and then incubated at 37 ± 2 °C for 48 h under anaerobic conditions. A colony of *S. cerevisiae* yeast colony was inoculated into 9 ml of YPD broth medium containing proteose peptone (2%), yeast extract (1%) and glucose (2%) with adjusted pH of 6.2, before being incubated at room temperature (30 ± 2 °C) for 48 h under constant shaking at 140 rpm. Each organism was sub-cultured twice before being centrifuged at 8,000 xg (Thermo Scientific, Sorvall Primo R, Germany) at 4 °C for 7 min to obtain the cells (Yang et al., 2018). The cell pellets were washed twice using phosphate buffer solution (PBS) and centrifuged again as above. The cells were re-suspended by adding PBS to obtain a final OD of 1.000 ± 0.050 as measured at 660 nm using a UV-vis spectrophotometer (Biochrom Libra S22, Biochrom Ltd. England) (Nakajo et al., 2010).

Selective media without any carbon source, with 2% glucose, inulin and FOS were prepared before a 200 μ L (≈ 5 CFU/mL for bacteria and ≈ 4 CFU/mL for yeast) inoculum of each probiotic was cultured, in preparation of further study.

2.3 Protein base product preparation

The protein base products were prepared by taken chicken egg and coconut juice 45-49%, and baker's yeast and potassium chloride at 0.45-5.00%, as the control or baseline product sample (BPS). The protein base products were then added with 9.00% fructo-oligosaccharide or inulin or 7.34% whey. Approximately 5% of the tested microorganisms were cultured in the mentioned product. 200 μ l of each inoculum of probiotic was added in the mentioned product sample at the start of incubation time.

2.4 Microbial enumeration, optical density and pH measurement

Culturing and incubation were conducted at 37 ± 2 °C for the bacterial treatments and at 30 ± 2 °C under shaking for yeast, with durations of 0, 12, 24, 48 or 72 h. Three replicates were run for each period. 1 ml of each culture condition was diluted in 9 mL of sterile normal saline to make the treatment suspension. The number of cells was measured by the pour-plate technique on MRS agar for bacteria and on YPD agar for yeast, incubated in anaerobic conditions at 37 ± 2 °C, for 48 h for the bacterial strains and at room temperature (30 ± 2 °C) for 48 h for the yeast strain. The enumeration of the colony forming units (CFU) on plates with 30 to 300 colonies was recorded and these are expressed as log CFU/mL. Further, the OD of the culture media was monitored simultaneously by using a spectrophotometer at 660 nm, while pH of the media were also determined during bacterial and yeast growth using a digital pH-meter (Docu-pH+ meter Satorious, Germany) (Gustaw et al., 2011).

2.5 Short chain fatty acid measurement in protein based media

The measurement was modified from the method of (Fernando et al., 2010). An Agilent 7890A gas chromatograph (Agilent Technologies, Santa Clara, California, USA) with flame ionization (FID) was used to analyze SCFAs in the samples. Separation was achieved using a HP-INNOWAX column, 30 m × 0.320 mm × 0.25 μm (Agilent Technologies Inc.) from an initial temperature of 60 °C to the final 230 °C temperature. Flow rates of nitrogen, hydrogen and air were 25, 30 and 300 mL/min, respectively. Mixes of acetic, propionic and butyric acid were prepared to 0, 5, 10, 20, 40, 60, 80 and 100 mmol/L, to make the standard curve. The samples were centrifuged at 10,000 × g for 15 min at 4 °C. The supernatant was mixed with acetonitrile in 1:1 ratio before filtering through a 0.22 μm nylon filter and analysis with the GC.

2.6 Statistical analyses

A completely randomized design (CRD) was used for the experiments. Data were analyzed by one-way analysis of variance with the Tukey test to establish significant differences among the mean values. Statistical analysis was performed using the SPSS program. Differences were considered significant at $p < 0.05$.

3 Result and discussion

3.1 Result

Microbial growth, OD and pH changes in selective media

The number of *B. longum* cells at 0 h was approximately 4.80 ± 0.02 log CFU/mL before it increased to 7.30 ± 0.00 , 7.54 ± 0.004 , 7.56 ± 0.05 and 7.50 ± 0.02 log CFU/mL at 12 h in the control, glucose, inulin and FOS treatments, respectively, and then remained constant up to 24 h (Figure 1A). However, the number of cells in all culture conditions during incubation was at its highest when incubated for 48 h with and without carbon sources added. Later, the number of cells of *B. longum* cultured on MRS with added glucose (positive control) declined significantly from 9.09 ± 0.02 log CFU/mL at 48 h to 7.95 ± 0.01 log CFU/mL at 72 h, while the numbers of cells for the other treatments (negative control, and inulin and FOS added) slightly decreased but not significantly. Surprisingly, the OD of the MRS medium with glucose added significantly increased and reached a level of 2.783 ± 0.004 after being cultured for 72 h, which was higher than for the other conditions (Figure 1B). At 48 h of incubation time, the highest OD was found when the culture media containing glucose. A significant reduction in the pH of the *B. longum*

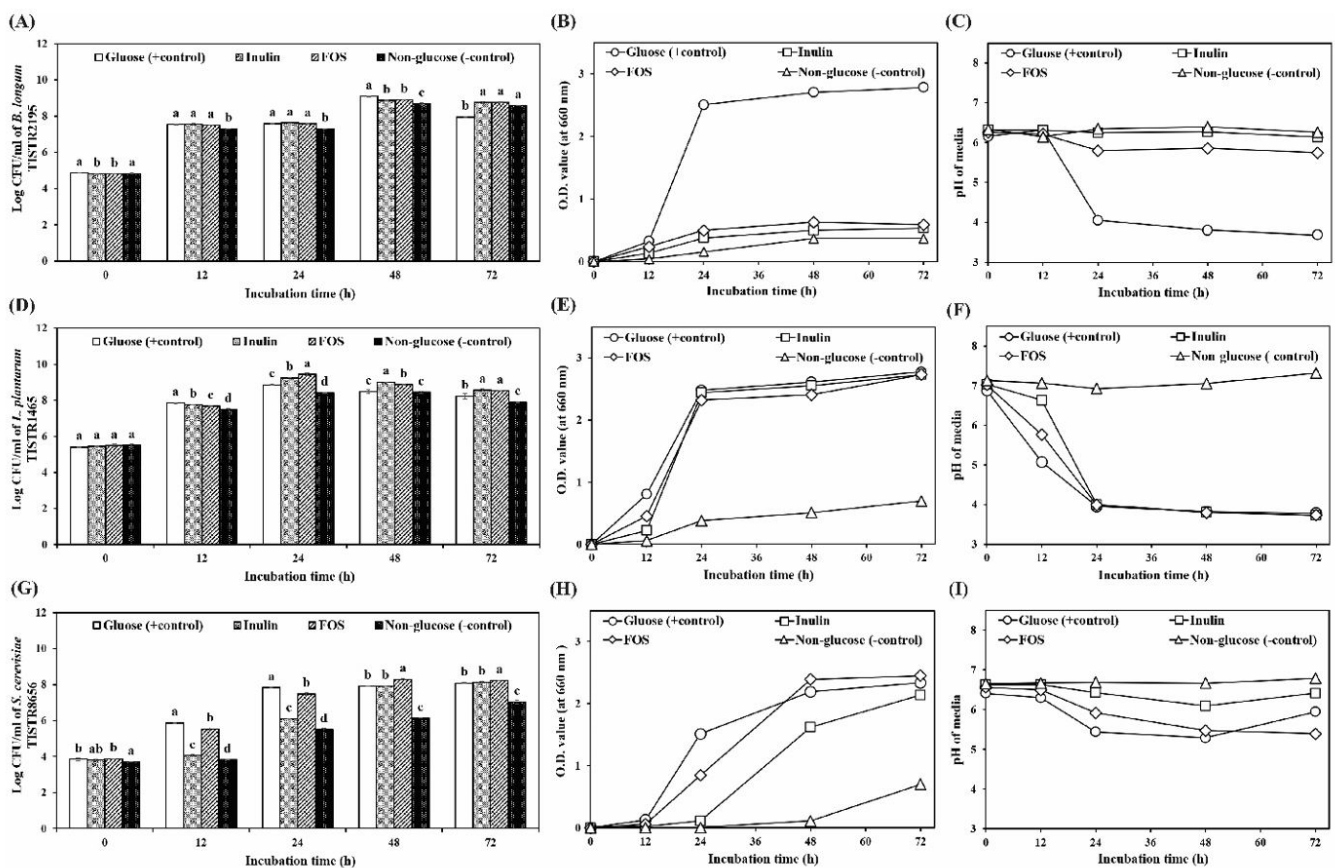


Figure 1. Effects of various added carbon sources on numbers of microbial cells, OD and pH when culturing *B. longum* (A-C), *L. plantarun* (D-F) and *S. cerevisiae* (G-I), respectively. The data are expressed as means, and cases with different letters (a-c) are significantly different ($p < 0.05$).

glucose treatment was noted between 12 and 24 h of culturing, and then it remained constant up to 72 h (Figure 1C).

The *L. plantarum* initial concentration was approximately 5.47 ± 0.05 log CFU/mL at 0 h in all culture treatments, and it thereafter increased and reached the highest numbers of cells of 8.42 ± 0.02 , 8.87 ± 0.02 , 9.25 ± 0.00 and 9.44 ± 0.07 log CFU/mL (at 24 h) for the negative control, glucose (positive control), inulin and FOS treatments, respectively. The growth of *L. plantarum* in MRS medium without any additional carbon source (negative control) was significantly less than in the other treatments after 24 h and thereafter declined at 72 h (Figure 1D). The turbidity of *L. plantarum* increased in all the carbon source-modified treatments with the highest OD observed at 72 h (Figure 1E). Although the turbidity of the control treatment without the addition of a carbon source (negative control) reached its peak at 72 h, it was much lower than that of the three carbon-supplemented treatments. During incubation of *L. plantarum*, the pH of the medium in the three carbon-supplemented treatments decreased within 24 h (Figure 1F). The final pH values of the media with added glucose, FOS and inulin were not significantly different. Without any added carbon source (negative control), the pH of the medium did not decrease, instead it seemed to slightly increase after 72 h of incubation (Figure 1F).

The cell count of *S. cerevisiae* at 0 h was approximately 3.81 ± 0.04 log CFU/mL (Figure 1G), and it rapidly increased when cultured in the glucose and FOS treatments after incubation for 24 h when compared to the negative control and inulin treatments. In addition, at 72 h the growth of *S. cerevisiae* reached the peaks of 8.23 ± 0.01 , 8.13 ± 0.03 , 8.09 ± 0.04 and 7.03 ± 0.06 log CFU/mL for the FOS, inulin, glucose and negative control treatments, respectively. Clearly increased turbidity in the *S. cerevisiae* cultured in glucose (positive control) and FOS treatments was noted after 24 h and the OD of those treatments continued to increase and reached the peak at 48 h in the FOS treatment (Figure 1H). In treatments without glucose (negative control) and inulin, the OD started to increase after 24 h and reached the peak at 72 h. Unsurprisingly, the OD of the control treatment without any added carbon source (negative control) was lower than with the carbon-supplemented treatments and reached a peak of only 0.7017 ± 0.002 , while the other cases did not change during the first 12 h but then started to slightly decrease. However, it was found that pH of glucose added sample (positive control) tended to increase again after 48 h, while pH of FOS sample still kept further increasing. The pH of negative control (without glucose added) was quite stable with a tendency to slightly decrease. The pH of the FOS treatment decreased further during culturing.

Microbial growth and pH changes in protein based product

The cell count of *B. longum* in product sampled at 0 h was approximately 5.50 ± 0.05 CFU/mL (Figure 2A). The highest 9.56 ± 0.02 CFU/mL of cells was found at 24 h in MRS media (positive control) and it then declined until the end of incubation. The protein based product with FOS and inulin added provided the highest cell number at 48 h. Not surprisingly, cell count of *B. longum* in MRS was higher than in the other treatments when incubated for 48 h. However, after 48 h of incubation, the cell

count of *B. longum* in MRS media significantly decreased and was lower than in the other treatments. Obvious pH changes of media during *B. longum* growth were noted at 12 to 24 h (Figure 2B). pH in all treatments significantly dropped from 6.26-5.94 to 3.84-4.15 with incubation for 24 h. However, whey protein added in the sample provided higher pH of medium than the others.

L. plantarum exhibited the significantly highest cell count of 9.22 ± 0.02 CFU/mL at 24 h in MRS medium, before decrease throughout further incubation (Figure 2C). The product added with FOS and inulin provided approximately 1.5 log excess in cell counts during 12 to 24 h. Surprisingly, whey protein added in the sample gave the highest cell count at the end of incubation. pH of cultured *L. plantarum* dropped from about 6 to the lowest pH 3.56 ± 0.01 in MRS at 24 h (Figure 2B). The sample with whey added showed the highest pH at 48 h of incubation.

The highest cell counts of *S. cerevisiae* were found in selective medium with 8.06 ± 0.02 CFU/mL at 48 h and were maintained until end of incubation (Figure 2E). The cell count of *S. cerevisiae* rapidly increased in inulin added sample by 24 h, then declined. Within 24h of incubation, pH of medium from approximately 6.20 ± 0.01 tended to slightly decrease to the lowest pH 5.15 ± 0.02 in FOS added product. A significantly increased pH was found in YPD media at 24 h, with the highest value at the end of incubation (Figure 2F).

Short chain fatty acid content in protein base product

Acetic acid was the main compound among short chain fatty acids produced by the tested three probiotics (Figure 3). Markedly, short chain fatty acids in this study were noticed since the beginning of incubation time, which is not reported in prior literature.

The most acetic acid was produced by *B. longum* at 24 h in selective medium, namely 105.74 ± 5.62 mmol/l (Figure 3A-C). Among protein based product, it was found that FOS significantly stimulated acetic acid production. However, whey protein added into the product seemed to not be utilized and converted to acetic acid. Both propionic and butyric acid productions were also higher in the selective medium than in the others.

All SCFAs produced by *L. plantarum* were significantly higher in the selective medium when compared with the other protein based products (Figure 3D-F). However, the product added with FOS yielded the most acetic acid, 74.21 ± 2.76 mmol/L at 24 h. Interestingly, the base product with inulin added provided the most acetic acid at the end of incubation. In addition, propionic and butyric acid were the highest in selective medium during the incubation.

Surprisingly, *S. cerevisiae* could produce acetic acid more when cultured in the product with FOS added, although the acetic acid content produced by the yeast *S. cerevisiae* was lower than by the other bacterial probiotics (Figure 3A, D and G). Butyric acid content produced by *S. cerevisiae* was similar to *L. plantarum* and was slightly higher than that of *B. longum*.

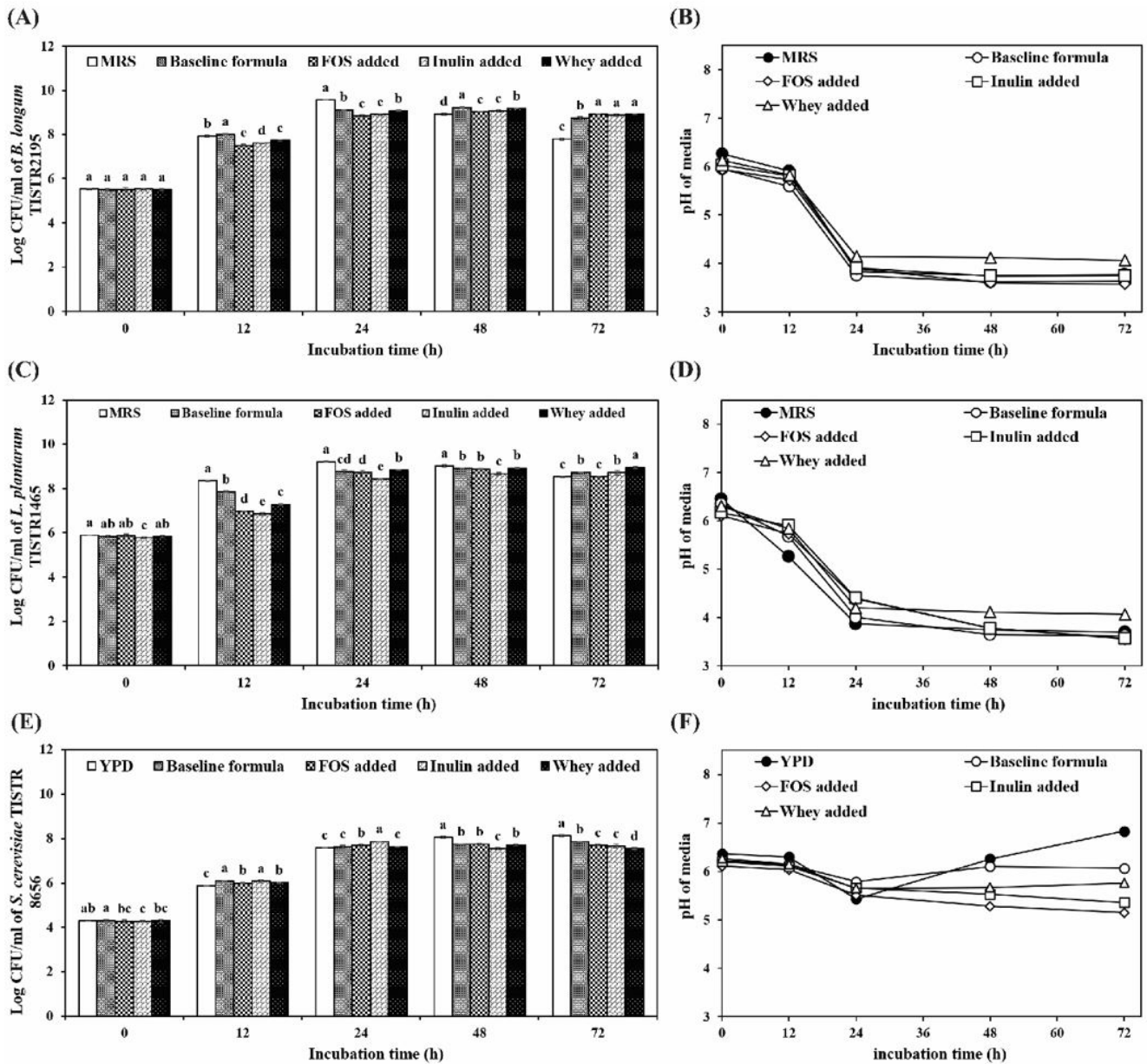


Figure 2. Effects of the various tested media with different added carbon sources on microbial cell growth and the media pH levels when culturing *B. longum* (A-B), *L. plantarum* (C-D) and *S. cerevisiae* (E-F). Means with different letters (a-d) are significantly different ($p < 0.05$).

3.2. Discussion

Microbial growth, OD and pH changes in selective media

It has been previously reported that *B. longum* can grow well even in a low carbon source medium (Abbasiliasi et al., 2017), if carbon/nitrogen for their growth still is sufficient to support the adaptation for survival. Therefore, the growth without any addition of carbon source (negative control sample) as found in this experiment may be due to other ingredients, for instance beef extract, yeast extract, and peptone that contain some carbohydrate, which can be used as carbon source later and for bacterial growth. Additionally, Romano & Nickerson (1958) stated that amino acids could serve as carbon sources for

microbial growth. This may explain why probiotics can grow in the negative control sample. However, the highest cell counts were found in the inulin and FOS treatments after culturing for 72 h, which was slower than that of positive control using glucose as carbon source. This suggests that glucose acted as a fast release source of energy, while FOS and inulin were slower energy sources. Therefore, any source was used easily and then became depleted, leading to lack of food and reduced microorganism growth. The results follow the growth kinetics with faster growth then death rate (Najafpour, 2007). The prebiotics extended the life of probiotic microorganisms and provided enough time to adhere to the villi cells in the gut leading to human health benefits. Goderska et al. (2008), reported that

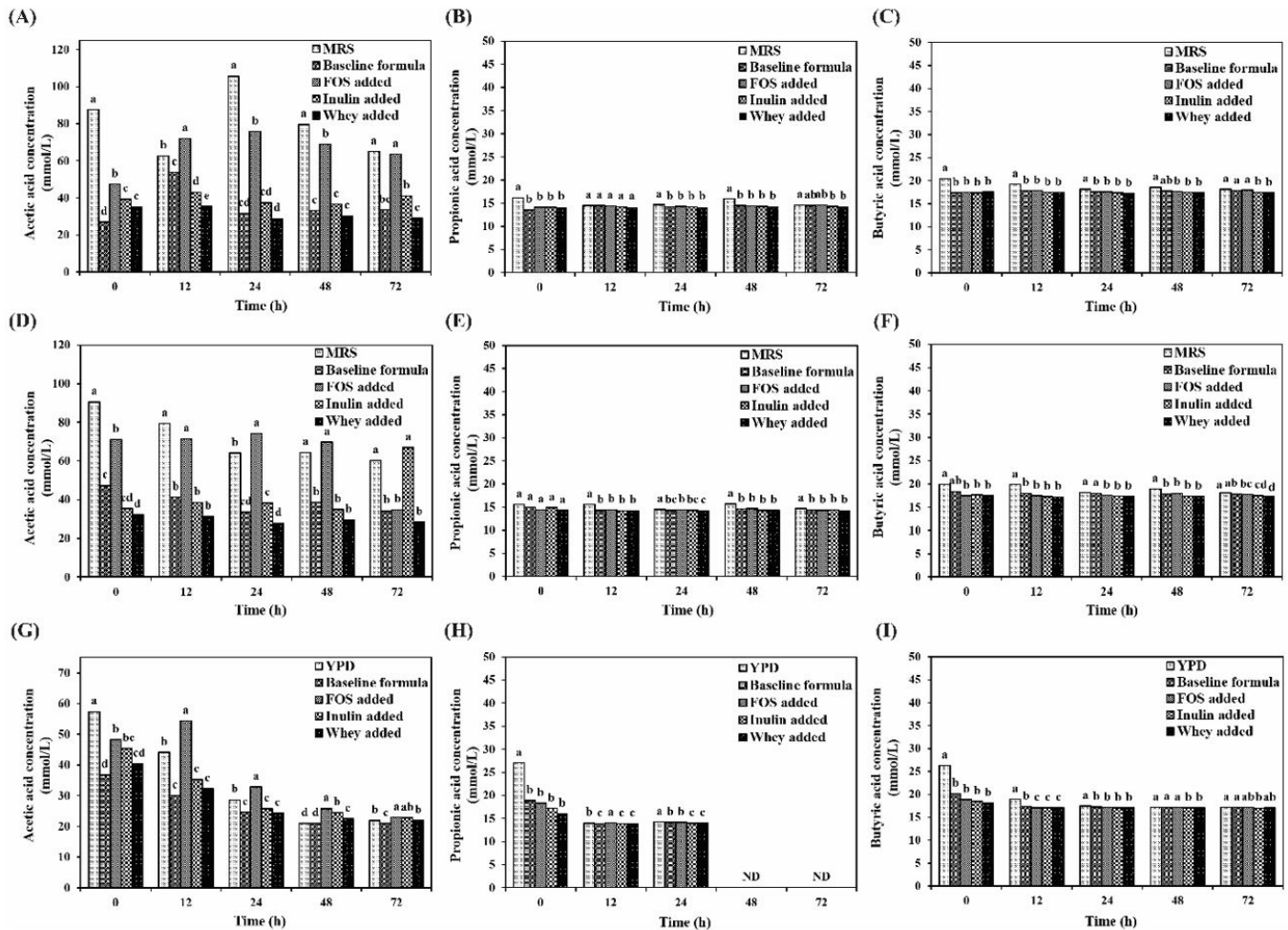


Figure 3. Effects of the various carbon sources on types of SCFA; acetic acid, propionic acid and butyric acid, produced by *B. longum* TISTR2195 (A-C), *L. plantarum* TISTR1465 (D-F) and *S. cerevisiae* TISTR8656 (G-I). Different letters indicate significant differences at a given time between the probiotics ($p < 0.05$).

bifidobacterium strains utilized monosaccharide much faster and more efficiently than other sugars and saccharides including prebiotics. Similarly, other bacteria particularly the pathogenic ones, also prefer monosaccharides and use them faster leading to toxic products and harmful compounds to the host.

Prebiotic compounds probiotic growth due to their digest ability enzyme including inulinase that lack in pathogenic ones. In addition, The difference between probiotics and pathogens are metabolites conferring health benefits via short chain fatty acid, lower pH, bacteriocin compounds etc. (Sun & O’Riordan, 2013).

From observations during incubation, the precipitate or white colored sediment was apparent in the MRS broth added with glucose, but it was not present in the other treatments. It is known that the OD is a reflection of both dead and viable cells as well as other particles, including exo-polysaccharides (Meyers et al., 2018). A significant increase in the OD for glucose sample should parallel with the microorganism growth, but it was found that there was only around 1 log increase compared to the other treatments (Figure. 1B). This indicates that high OD may come from metabolites and dead cells, not so much from viable cells. Therefore, the OD may not be a good approach for

determining the number of cells from bacterial growth in some situations, while plate counts could be more indicative of the cell numbers. Therefore, it is necessary to analyze the supernatant metabolites, particularly SCFAs, in order to evaluate the actual effect of prebiotics.

As previously noted, Bifidobacterium actually utilizes monosaccharides, particularly glucose, for growth better than other bacteria (Rossi et al., 2005). And the end products of the glycolysis pathway as a result of glucose fermentation include lactic acid, which supports the reduction in pH observed in the glucose treatment, whereas the treatments in which prebiotics were added to the media did not have significant changes (Pokusaeva et al., 2011). Yun et al. (2017), noted that after bifidobacterium growth, both the number of cells and the amount of acid, particularly of lactic acid, increased. However, Sánchez et al. (2007), reported that bifidobacteria also have the ability to produce NH_4^+ from intracellular substrates, such as amino acids, which may keep the pH in the medium constant (Nakajo et al., 2010). An increase in acidic compounds leads to a pH drop reflecting the accumulation of organic acids from monosaccharide fermentation. However, Van der Meulen et al. (2006) noted that

B. longum utilized monosaccharides faster than oligo-fructose within 36 h. The fermentation of inulin and FOS by *B. longum* in this experiment were higher than mono saccharide which was not similar to other researchers. As know that, pH is a function of the total hydrogen ions (H^+) in then system, which come from acid production, while alkaline products also can react with H^+ to neutralize acidity. Therefore, SCFAs content really should be measured to assess the bacterial growth under prebiotic conditions (Musara et al., 2003).

In fact, bifidobacteria degrade monosaccharides via the fructose-6-phosphate phosphoketolase pathway, which is termed the *bifid shunt*, and the specific rate of sugar consumption plays a crucial role in the final metabolite products, which consist mainly of lactic acid. In addition, these organisms can also produce acetic acid, ethanol, bacteriocins, exo-polysaccharides and several enzymes (De Vuyst et al., 2014), which may explain the higher OD with added than without added carbon source.

In summary, a significant decrease in the pH of the medium with glucose added (positive control) was matched by increases in both the cell count and the OD, while the inulin, FOS and without added carbon source (negative control) treatments did not show a significant reduction in pH.

The growth of *L. plantarum* was found to rank order the media with FOS, inulin and glucose added (positive control). The cell count in the FOS treatment was the highest within 24 h. This indicates that *L. plantarum* can gradually digest and utilize glucose and fructose in the oligo-fructose ingredients from inulin and FOS, providing acidic compounds that decrease the pH. Saulnier et al. (2007) reported that *L. plantarum* can hydrolyze FOS by using the β -fructofuranosidase enzyme. Further, [11] suggested that β - $(2-1)$ fructans with $DP > 4$, such as FOS, greatly support the growth of probiotics when compared to long chain β - $(2-1)$ fructans ($DP > 8$) such as inulin.

From the observations during incubation, it was noted that the media added with carbon source, such as glucose, FOS or inulin, provided white particles, while without added carbon source (negative control) there was no precipitate. This indicates that the OD of the negative control treatment may directly reflect the number of bacterial cells, while the OD of the carbon-supplemented treatments may reflect the presence of metabolite products, not only the cell count (Lukáčová et al., 2006; Vandenbergh, 1993). Saulnier et al. (2007) noted that sugar fermentation products of *L. plantarum* were mainly lactic acid, which can be hydrolyzed further to acetic acid. The decreased pH during *L. plantarum* growth, therefore indicates the fermentation and acid accumulation. This result also indicates that *L. plantarum* can utilize FOS and inulin in a similar manner to glucose. pH of the negative control sample (without C-source) tended to increase with incubation time. Generally, an increased pH shows alkalinity, which indicates more amine production as a result of the utilization of N-compounds and cell lysis due to cell deaths (Middelboe & Jørgensen, 2006).

A rapid increase in *S. cerevisiae* cell count when cultured in the medium with glucose (positive control) or with FOS within 24 h indicates that yeast growth benefited from smaller simple molecules, easier to utilize or digest. Actually, *S. cerevisiae* is

a unicellular organism that obtains energy primarily through alcohol fermentation, and breaks glucose down as a substrate into ethanol (Walker & Stewart, 2016). The results of this study indicate that the yeast cells in the culture can easier utilize glucose and fructose from fructan hydrolysis of FOS, faster than inulin. Nilsson et al. (1987) reported that the invertase produced by *S. cerevisiae* during incubation was able to degrade oligosaccharides with $DP > 4$. In addition, the study Struyf et al. (2017) indicated that invertase was able to fully degrade FOS with a DP of 2 to 8. It is known that inulin consists of fructo-oligosaccharides with a variety of DPs between 3 and 60, and its molecular weight is normally greater than that of FOS. It is clear that fructo-oligosaccharides with DP below 20 were digested, and given fructose and glucose substrates, the degree of hydrolysis depended on exo-inulinase activity. The study Wang & Li (2013) indicated that *S. cerevisiae* strains were able to utilize mainly glucose and some types of fructo-oligosaccharides with DP up to 6, and provided ethanol as an end product. This study therefore showed that when glucose was used as a carbon source, the growth of *S. cerevisiae* occurred rapidly, and it reduced the pH of the medium faster than in the other conditions, within 24 h (Figure 1G-I).

Taken together, the OD and cell growth (Figure 1G and H) indicate that the higher the cell count, the higher the OD during incubation. However, it was found that the number of cells in the control treatment without added carbon (negative control sample) was similar to those in the other treatments, but its OD was much lower. This may imply that without an added carbon source (negative control), the yeast cells may not be able to provide metabolites as much as in the carbon-supplemented treatments. This means that only main nutrients were used for cell survival, which was similar to starved live cell conditions. This in turn suggests that the cells may not be as big or completely full morphology or in word of in stunted and sickly cell because of limited nutrition (Zakhartsev & Reuss, 2018).

The decreased pH is a function of weak acid production, such as lactic acid accumulation (Valli et al., 2006) and/or amine production (Niu et al., 2019). A reduction of pH at 24 h in the media may indicate that the conversion of carbohydrates to carbon dioxide (CO_2) and/or lactic acid (Sauer et al., 2010) may occur due to autolytic enzymes in the live cells. There was almost no change in the pH of the control treatment without an added carbon source (negative control medium), which suggests that with less carbon source the yeast may not be able to provide enough CO_2 acetic acid and/or lactic acid or may even produce amine products equally to acidic products. In addition, pH of medium during culturing *S. cerevisiae* was higher than with the probiotic bacteria, due to a lesser capacity to produce acids, including lactic acid.

Microbial growth, pH changes and SCFAs as affected by protein based media

It has been well established that the growth of probiotics, including bifidobacteria or lactobacilli, can be stimulated by various substrates. The strongest growth in selective medium could be explained by findings in (Cruz et al., 2002) which

reported that peptone composition in selective media as nitrogen source (in the form of amino acids and peptide) was utilized by yeast in ethanol production.

This current study found that the protein based medium (baseline medium), which is made from chicken egg and commercial coconut juice in approximately 50:50 ratio, could stimulate growth of the probiotics tested. Chicken egg is a versatile natural high protein source with complete nutrients, particularly with a protein content of 12% (Miguel et al., 2005). Romano & Nickerson (1958) stated that microbes utilize amino acids in a medium as carbon sources for cell growth. At 48 h the bacteria and at 72 h the yeast cell counts in positive media (MRS) tended to decrease, while the product sample maintained or increased the counts in prebiotic added media. In addition, McComas & Gilliland (2003) studied whey protein hydrolysate and found that this available nitrogen source can stimulate the growth of *B. longum*. This was similarly noted for *L. plantarum* growth in the current study, as shown in Figure 2C.

The results indicate that glucose was the first substrate that rapidly decreased acid production, but prebiotics could also be hydrolyzed and yielded the lowest pH at the end of incubation. In addition, *S. cerevisiae* produced less acidity than the two tested bacteria based on the measured pH.

SCFAs are saturated aliphatic organic acids from fermentation by some organism, consisting of one to six carbons, with acetate (C2), propionate (C3), and butyrate (C4) usually the most abundant (den Besten et al., 2013). Because inoculum cells in this study were in log phase and cultured in selective medium, the SCFAs can be produced or metabolized within their cells during growth. Therefore, SCFAs were detected since 0 h start time. This is possibly the first report in which SCFAs in the medium were detected at the start of incubation, normally they have only been reported afterwards.

In addition, in the selective medium (MRS) the highest SCFA content, particularly acetic acid content, was noted compared with the other protein based media, due to the simple pathway of glucose fermentation. However, it was found that in protein based media the added FOS supported acetic acid production similarly to control or MRS media, after inoculation with either of the tested probiotic bacteria. Surprisingly, *S. cerevisiae* cultured in the protein base media added with FOS or inulin seemed to be higher in SCFAs than the control. In combination with the observed cell counts (Figure 2 A, C and E) this indicates that the microbial cell metabolites depended on substrate type, especially in protein base product added with FOS, which obviously stimulated microbial SCFA production. Moreover, the ability to use inulin can prolong the utilization so that production of acetic acid by *L. plantarum* was observed still at 72 h.

In addition, *B. longum*, *L. plantarum* and *S. cerevisiae* can produce short chain fatty acids via protein based product. Similar to the findings in Sanchez et al. (2008) that reported that protein fermentation can contribute to the SCFA pool, even though it mostly gives rise to branched-chain fatty acids such as isobutyrate, 2-methylbutyrate, and others.

4 Conclusion

Naturally, microorganisms need sources of carbon and nitrogen as nutrients for growth. The present work demonstrated that the probiotic bacterial strains, *B. longum* and *L. plantarum*, and the yeast, *S. cerevisiae*, were able to utilize prebiotics and increase cell counts, particularly after 24 h. However, *B. Longum*, *L. plantarum*, and the yeast *S. cerevisiae*, were also able to grow in MRS and YPD media without any addition of glucose or other carbon source. Nevertheless, the results showed that FOS seemed to enhance the growth of *L. plantarum* more than glucose in some conditions. Based on these results, FOS was the best substrate, which yielded the highest cell counts of *S. cerevisiae*. Moreover, *S. cerevisiae* was able to utilize FOS better than inulin within a short period. However, at the end of incubation the numbers of viable cells of *B. longum* and *L. plantarum* cultured in the medium added with inulin or FOS were not significantly different ($p < 0.05$). While OD has a good correlation with the number of cells, this is no longer the case with large amounts of exo-metabolites, including exopolysaccharides, weak acids and other substances that are produced during the growth phase. In single culture systems, both inulin and FOS could be utilized and would produce weak acids leading to a lower pH. The protein based media also stimulated probiotic growth, and the prebiotic added in product sample could prolong the probiotic cell growth with acid production. SCFAs produced by those probiotics in this study were the highest in selective medium. In addition, FOS added in protein base product could stimulate fermentation activities of *B. longum*, *L. plantarum* and *S. cerevisiae* better than inulin or whey. In conclusion, FOS seemed to be the preferred prebiotic material supporting probiotics, including the tested bacteria and yeast. However, further investigations should include preclinical animal trials, and later clinical trials, to lead to consumer health products.

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