Research article

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Growth Analysis of *Lactobacillus Acidophilus* Using Different Non-Digestible Carbohydrates

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ABSTRACT

Probiotics are live microorganisms and offer health benefits to the digestive system and used in the production of many fermented foods. Non-digestible carbohydrates are dietary fibers which cannot be digested and absorbed by the small intestine. Strains of *Lactobacillus*, are among the most common and popular group of probiotics and added to many dairy products and dietary supplements. Besides, *Lactobacillus acidophilus* can exhibit many useful benefits such as showing thermostability, maintaining the growth activity at a wide pH range, and offering strong inhibition actions against spoilage of food and pathogenic bacteria. Aims of this study are to analyse the ability of non-digestible carbohydrates to act as a carbon source in enhancing the growth activity of *L. acidophilus in vitro* and to determine which type of non-digestible carbohydrate sources contributed a high biomass production. *L. acidophilus* was grown on de Man, Rogosa and Sharpe (MRS) medium. The optical density and pH of the cell biomass produced were measured and cell dry weight was determined. The highest biomass production recorded was for barley 10.02 g. L^{-1} followed by yam 8.79 g. L^{-1}, 7.17 g. L^{-1} for garlic, 6.81 g. L^{-1} for banana and 4.86 g. L^{-1} for sweet potato, while positive control (glucose) recorded 4.20 g. L^{-1} of cell biomass. The results also showed a decreasing in the pH values which indicated the formation of lactic acids in the medium after 24 h of incubation at 37°C on rotary shaker set at 200 rpm. The overall results, confirmed that *L. acidophilus* helps in the hydrolysis of non-digestible carbohydrates and subsequent conversion of the sugars to cell biomass and decrease the pH compared to the negative control (without carbon source). This shows that in future, production of a synbiotic products using these non-digestible carbohydrates and probiotics strains is promising to offer many benefits to human's health.

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Introduction

Probiotics are live microorganisms that play an important role in the digestive system by keeping the gut healthy and balancing the beneficial microflora in the gut [1]. The use of probiotics in the last two decades has been increased significantly due to their ability in conferring many health benefits to human's digestive system such as protecting the host from different harmful microorganisms, and making the immune system stronger. In

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addition, probiotics are known for their ability in improving feed digestibility and decreasing the metabolic disorders [2]. Non-digestible carbohydrates are dietary fibres (prebiotics) and cannot be digested and absorbed by the small intestine. Prebiotic is defined as non-digestible food which helps in improving and balancing the growth of the beneficial microflora in the digestive system. Plants are considered the common source of these non-digestible carbohydrates and contain a mixture of polysaccharides which are the integral components in the cell wall of the plants [3]. Gut microbiota offer many positive effects to the function, metabolism and integrity of the intestine. Gut microbiota can also fight many pathogens by the induction of immunomodulatory molecules which have antagonistic properties and can be gained from the production of lactic acid bacteria for instance *Bifidobacterium* and *Lactobacillus* genera [3]. Choosing substrate specificities for probiotics is important for the optimization of the density of probiotics in the intestine. The increase of probiotics population is influenced by the composition of the monosaccharide of the non-digestible carbohydrates, the glycosidic linkage between the monosaccharide residues, and the degree of polymerization [4]. Many researchers proved the ability of prebiotics (non-digestible carbohydrates) in inhibiting the attachment of pathogens to epithelial cells and it is depending on the secretion of the peptides which are responsible in enhancing the absorption of minerals, stimulating the appetite, and preventing from diseases [3]. The term “synbiotic” was introduced by Gibson and Roberfroid (1995) which referred to a combination of both probiotics and prebiotics [5]. Consuming synbiotic can stimulate and activate the metabolism of a physiological intestinal microbiota [6]. Thus, using synbiotic can help in overcoming problems related to the survival of probiotics in the gastrointestinal tract. The combination of both probiotics and prebiotics as a single product can lead to great influence compared of using probiotic or prebiotic alone [7]. Thus, current studies are focusing on studying and selecting carbohydrates components which can be utilized by probiotic strains in high levels to produce synbiotic products to enhance the growth of beneficial bacteria in the gastrointestinal tract. Recently, many people prefer a good quality in purchasing food products and they search for specific features such as longer shelf-life products that had been produced using natural fermentation process. The process of natural food fermentation is carried out by using fermenting microbial

communities such as lactic acid bacteria (LAB) as an energy source to enhance the quality of the product [8]. LAB are among the most common and significant groups of probiotic used in food processing industries, particularly in dairy products. These microorganisms can boost the digestion of lactose and stimulate the immune system, and prevent from diarrhea [9]. Functional food ingredients offer many beneficial effects for humans and these benefits are gained from the bioactive components present in the products. These ingredients in functional food are probiotics, prebiotics, soluble fibers, and others. Nowadays, the demand for fermented probiotic foods is increasing significantly and > 500 of the probiotic products have been introduced in the world, since probiotic products offer many benefits to humans, therefore the most common way to consume probiotics is mostly through the intake of food products and some fermented products may not last longer than a month and the cells may die or not be active for a period of time [10]. Thus, it is important to maintain probiotics strains live longer and also indicates the importance of dietary fibers to be used as substrates for sustaining the growth and increasing the shelf-life of probiotic strains along with the processing and storage conditions [11]. Therefore, the aims of this study were, to prepare raw non-digestible carbohydrates and seeding with *L. acidophilus* in the prepared MRS media. The growth of the bacteria was analysed by measuring cell dry weight of *L. acidophilus* over specific time frame with pH.

Material and Methods

Preparation of non-digestible carbohydrates samples

Selected sources of non-digestible carbohydrates samples have been used in this study namely, sweet potato, yam, garlic, barley and banana. Garlic and barley are commercially available. For the preparation of sweet potato, yam and banana samples, 500 g of each was obtained as a raw material from a local market. The samples were washed, peeled and sliced thinly. Then, both sweet potato and yam samples were placed on a dehydrator's tray and dried at 68°C for 24 h. For banana sample the drying process was carried out at 45°C for 72 h. After the drying process, all the samples were ground into a fine powder. On the other hand, 100 g of fine powder of barley and garlic samples were obtained from commercially available product in the supermarket.

Microorganism

The *Lactobacillus acidophilus* (ATCC 4356) strain was provided by the Institute of Bioproduct Development, Universiti Teknologi Malaysia. Originally, it was purchased from a German Culture Collection Center (Leibniz Institute DSMZ, Braunschweig, Germany).

Seed culture of *L. acidophilus* and inoculum preparation

The de Man, Rogosa and Sharpe (MRS) medium was prepared according to the standard procedure [12]. For the preparation of the inoculum, the seed culture was grown into a 250 mL Erlenmeyer shake flask, containing 40 mL of the prepared MRS broth, 1 g of glucose (already dissolved in 10 mL of distilled water) and 1 mL of *L. acidophilus* stock culture. The inoculum preparation was carried out as first and second inoculum. The flask of the first inoculum was incubated for 24 h at 200 rpm and 37°C on a rotary shaker (Innova 4080, New Brunswick, NJ, USA). On the second day, 5 mL of the first inoculum seed cultures of *L. acidophilus* was transferred into the second inoculum using sterile tip and further incubated following the same incubation conditions as first inoculum. After the cultivation of the second inoculum, the production medium was prepared by adding 1 g of the prepared and the commercialized non-digestible carbohydrates samples mixed with 10 mL of distilled water into 250 mL Erlenmeyer shake flasks with a volume of 40 mL of the previous prepared liquid MRS broth. The experiment was carried out in duplicates and two controls been used; one as positive (glucose) and other one as negative (no carbon source). 5 mL of the cultivated second inoculum was pipetted into all the Erlenmeyer shake flasks containing the samples and then all the flasks were agitated for 24 h at 200 rpm at 37°C on a rotary shaker (Innova 4080, New Brunswick, NJ, USA).

Optical density and pH measurement

After 24 h of incubation, cell growth was observed by measuring the optical density (OD) of the cultivated stocks in a single beam spectrophotometer (DR 6000, Hach Co., Loveland, CO, USA) at Absorbance 600 nm. For better accuracy, the stocks were diluted into ratio of 1:100. The OD of the culture was converted to dry cell mass through a previously prepared linear correlation between OD and CDW. One $OD_{600\ nm}$ was almost equal to 0.3 g. L^{-1} for this culture. The pH was measured after 24 h of incubation period using sterilizable pH probe (TOLEDO, Delta 320 pH Meter).

Results and Discussion

Non-digestible carbohydrates and seeding of *L. acidophilus* in the growth media

In this study, growth of *L. acidophilus* in MRS medium was monitored for the production of biomass in every sources of non-digestible carbohydrate and commercially available carbon sources and compared to that of the positive control using glucose as a carbon source and negative control without any carbon source. *L. acidophilus* in MRS medium was grown in an orbital shaker over 24 h period at 37°C. The growth activity was recorded as shown in (Table 1). Based on (Table 1), the data showed that the values were significant ($p<0.05$) and this indicates that *L. acidophilus* was able to grow in the provided carbon source.

Table 1 Mean and standard deviation values of maximum $OD_{(600nm)}$ reading for the growth cultures over 24 h of incubation period

Carbon source	$OD_{600\ nm}$ reading
Negative control	0±0.00
Positive control	0.14±0.016
Yam	0.293±0.014
Sweet potato	0.162±0.039
Banana	0.227±0.014
Commercial Barley	0.334±0.002
Commercial Garlic	0.239±0.022

*Mean ± standard deviation of duplicates analysis
*Note: positive control = (glucose); negative control = (no carbon source)

Measurement of cell dry weight based on an OD reading and pH analysis

Cell dry weight (CDW) and pH were recorded in order to analyse the correlation between the two readings over 24 h of cultivation in shake flask culture. The OD of culture was converted to dry cell mass through a linear correlation standard curve. 1 $OD_{600\ nm}$ was almost equivalent to 0.3 g L^{-1} [13]. pH reading was taken and each pH was measured as in (Table 2). The pH values and CDW values were plotted using bar chart as presented in (Figure 1). The correlation between the two were compared. Based on (Figure 1) pH values are low except for negative control and the decrease in pH indicated that there was

a cell biomass accumulation and the provided carbon sources were fermented by *L. acidophilus* effectively.

Table 2 Mean and standard deviation values of pH for the growth cultures over 24 h of incubation

Carbon source	pH
Negative control	8.5±0.098
Positive control	5.17±0.028
Yam	4.81±0.056
Commercial Garlic	4.86±0.021
Banana	4.97±0.014
Commercial Barley	4.22±0.021
Sweet potato	4.96±0.014

*Mean ± standard deviation of duplicates analysis
*Note: Positive control = (glucose); Negative control = (no carbon source)

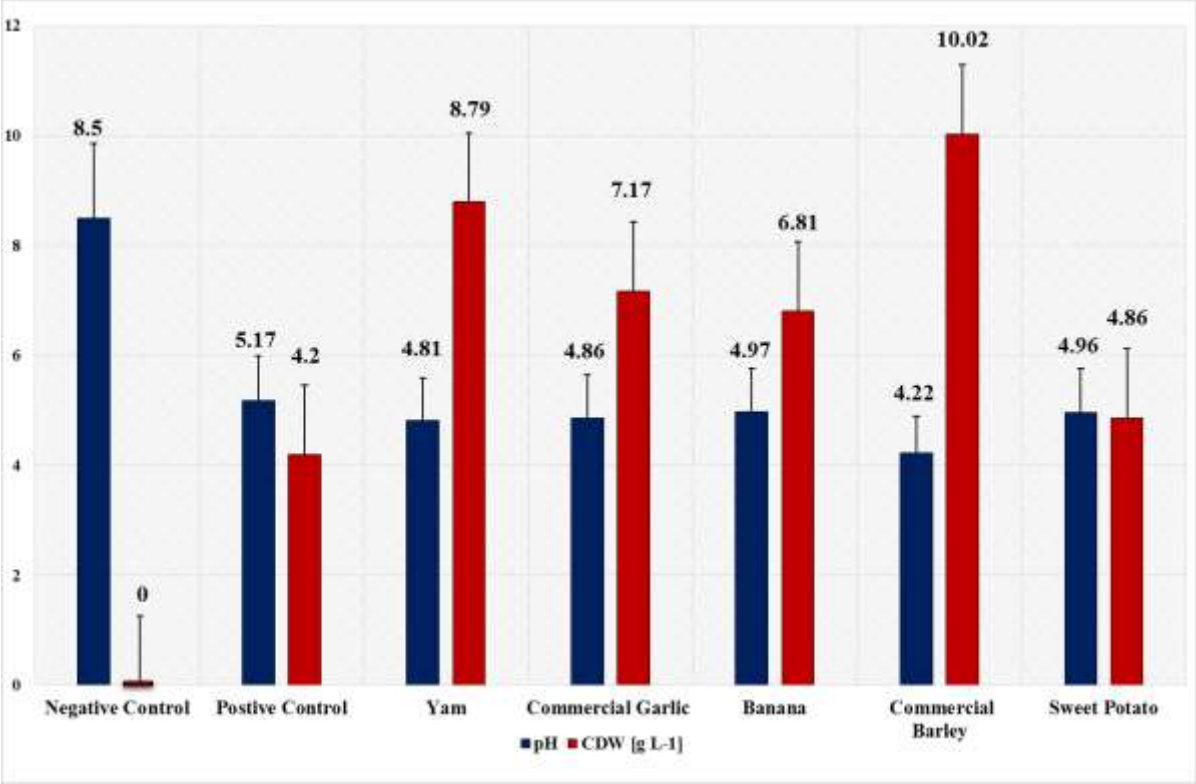

Fig 1 pH and cell dry weight (CDW) of the cultivated samples over 24 h of incubation period

From (Figure 1), we can conclude that using commercial barley allowed the highest production of the cell biomass recorded as 10.02 g L^{-1} and this is due to the structure of

barley since it is consisting of heterogeneous complex molecules and this leads in improving the growth and the activity of *Lactobacillus* strains [14]. Moreover, [15], stated the lactic acid bacteria can grow well in the presence of any cereal source especially barley, and this makes it a good substrate. In addition, the increase of the cell biomass could be due to the decrease in the content of starch during fermentation of barley with *Lactobacilli* as it was previously reported [16]. The approximate amount of starch content in barley is about 70%, this indicated that *L. acidophilus* had fermented the starch efficiently during the 24 h of cultivation. Moreover, the biochemical characteristics of *β*-1,3-1,4-glucanase; which is an endoglucanase enzyme and mainly found in *Lactobacillus* species played a role as well in increasing the production of cell biomass by hydrolyzing barley *β*-glucan compound completely [17]. According to one study [14] , *β*-glucans were reported to be fermented completely by the intestinal microbiota and were able to enhance the growth rate and the production of lactic acid bacteria. Yam, produced cell biomass of 8.79 g L^1. The high cell biomass produced can be due to α-amylase by *L. acidophilus* with yam. The production of α-amylase results in the breaking down of starch into fermentable carbohydrates and yield lactic acid as a final product [18]. Furthermore, yam generally is consist of 60-80% starch content and 20-30% of amylose; which makes it a good source for *L. acidophilus* to utilize it and used it as a carbon source [19]. The high content of starch leads to the accumulation of lactic acid, which had formed due to the use of α-amylase which degraded it effectively. According to the analysis done by [20] it was reported that yam when it was applied *in vivo* to mice it had inhibited the growth of pathogenic bacteria of *Clostridium perfringens*. The growth of *Lactobacillus* has been observed and it was reported that addition of yam as prebiotic source had increased and enhanced *Lactobacillus* in the gut of mice. Commercial garlic recorded 7.17 g L^1 of cell biomass. The increase of the produced cell biomass is could be due to the fructan compound present in garlic which is an important storage carbohydrate and it was recorded that the fructan amount in garlic is more than 75% [21]. Fructooligosaccharides, shown in (Figure 2) can be found in an amount of 10-16% in garlic and it has a degree of polymerization from 3-50 which makes garlic a good source of prebiotic and promotes the survival of *L. acidophilus* [22]. Thus, to influence and enhance the growth of probiotics strains it is mainly depending on the length of degree

polymerization and it is believed that *L. acidophilus* uses the fructan as a source of carbon. The total amount of fibers in garlic is nearly 26% which makes it a good source to be utilized and used as a carbon source during fermentation process. Garlic fructan can work as prebiotic and energy source for *L. casei.* Hence, specific amount of garlic particularly those which are below the antimicrobial activity concentration can promote the growth of beneficial bacteria present in the gut [23].

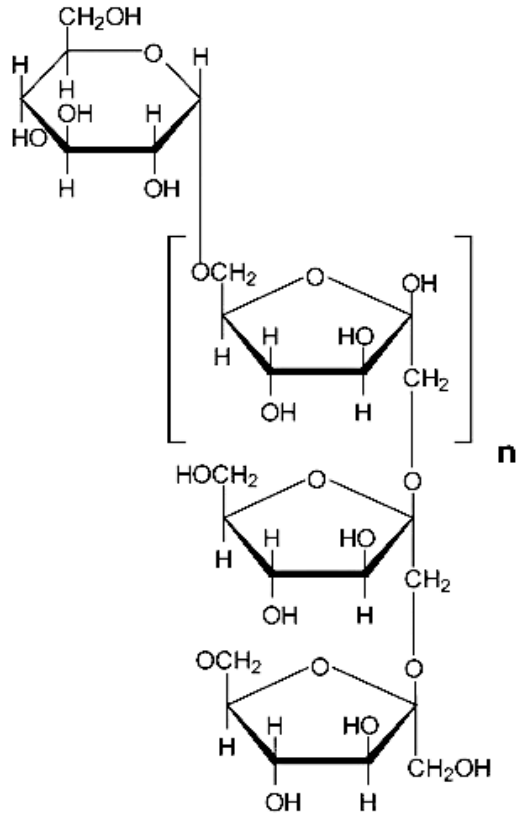

Fig 2 General chemical structures for inulin and inulin-like oligosaccharides [24]

For banana, *L. acidophilus* records cell biomass of 6.81 g L^{-1}. It was concluded that *L. acidophilus* can produce cellulase and hemicellulase enzymes which leads in the utilization of the carbohydrates in the banana and increase the growth activity of the *L. acidophilus* [25]. In addition, the use of banana powder as non-digestible carbohydrate source, contributed in positive effects due to the high amounts of dietary fibers in banana and this makes it suitable for the growth of *L. acidophilus*. Furthermore, sucrose, fructose and glucose in banana also play a role in increasing the cell biomass and producing lactic

acid and pyruvic acids, the pathway used to produce these compounds is Embden-Meyerhoff-Parnas, which used NADH as the co-factor and the enzyme lactate dehydrogenase. The biomass produced for sweet potato sample was 4.86 g L^{-1} which is somewhat low value compared to barley, yam and garlic. The utilization of sweet potato as a carbon source by *L. acidophilus* is due to the capability of the strain in producing exogenous enzymes such as amylase, protease, and lipase in order to degrade the carbohydrates in the sweet potato such as sucrose, maltose, and glucose [26]. The degradation of these carbohydrates resulted in the production of lactic acid and promote the survival of *L. acidophilus* because of the nitrogen and carbon sources offered during the fermentation of the carbohydrates. Moreover, based on the data, it was indicated that the fermentation process was effective by the enzymes produced by *L. acidophilus* which utilized the fibers or starch in sweet potato as an energy source. On the other hand, for the positive control containing glucose, the biomass of cells produced was as low as 4.20 g L^{-1} and pH 5.17. This was due to *L. acidophilus* fermented glucose and the value of cell biomass decreased slightly due to the simple building blocks of glucose compound and the number of the molecules 1-2 in glucose [27]. The overall results indicated that non-digestible carbohydrates samples were utilized significantly and no growth of *L. acidophilus* in medium without sugar (negative control) was recorded. CDW and pH were correlated to each other. Low in pH value during growth was due to *L. acidophilus* since it is a lactic acid bacteria and used the provided carbohydrate and transformed it into the simplest form of glucose and then converted it back as lactic acid during the fermentation. The decrease of pH values indicates the formation of titratable acidity in the cultivated culture. Based on the results, the differences in the values of CDW for the samples is mainly depending on the chemical composition and structure for each carbon source used. According to Healthline American website (https://www.healthline.com/about) barley is consisting of 73.5 g of carbohydrates; mainly polysaccharides and 17.3 g of fibers. The different types of polysaccharides in barley such as cellulose and β-glucans are playing a critical role in enhancing the fermentation process and increasing the biomass production in *L. acidophilus* effectively and this is linked to β and α -glycosidic bonds [28]. Moreover, the amount of carbohydrates and fibers in yam are 37 g and 5 g respectively. Garlic contains 33 g of

carbohydrates and 3.1 g of fibers. While, banana contains 24 g of carbohydrates and 3.1 g of fibers. Lastly, for sweet potato it contains 20 g of carbohydrates and 3 g of fibers. All these different ranges of carbohydrates and fibers content in each sample indicated their ability to be utilized by *L. acidophilus* and increase the biomass production after 24 h of incubation and indicated the differences of the cell dry weight values and pH values. Based on the results, we can conclude that barley is considering one of the important source that can be used in future to produce fermented products and act as a natural supplier or substrate for enhancing the growth of probiotics or can be used in the production of synbiotics products because it helps in sustaining the growth and shelf-life of probiotics strains and the beneficial bacteria in the gut if it is consumed regularly.

In the future, it is recommended to study the chemical composition, structures and characteristics of non-digestible carbohydrates (barley, yam, garlic, banana and sweet potato) to understand how *L. acidophilus* can utilize these non-digestible carbohydrates effectively. This study focuses on using selected non-digestible carbohydrates in food industries to act as a carbon source for probiotic strains to increase shelf-life and enhance the growth activity. In addition, it will potentially help in producing symbiotic products which consist of both probiotics and prebiotics and this will help in providing many health benefits to the consumers and used as a treatment for any disorder in human health.

Conclusion

Thus, combining non-digestible carbohydrates and *L. acidophilus* can gain great benefits for human's health because probiotics can survive the acidic gastric environment in the gut, whereby non-digestible carbohydrates (barley) can enhance the growth activity and produce high cell biomass. In future, production of synbiotic products using non-digestible carbohydrates and *L. acidophilus* can help in sustaining the product's quality and increase the shelf-life.

Abbreviations

LAB: lactic acid bacteria; CDW: cell dry weight; OD: optical density; MRS: de Man, Rogosa and Sharpe; dp: degree of polymerization; NADH: Nicotinamide adenine dinucleotide (NAD) + hydrogen (H); rpm: Rotary per minute; nm: Nanometers; h: Hours.

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