Preliminary evaluation of potential prebiotic capacity of selected legumes and seed mucilage on the probiotic strain *Lactobacillus rhamnosus* GG

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Received 2nd November 2020 / Accepted 26th January 2021

Abstract. The characterization of the prebiotic potential of legumes and mucilage are essential and crucial for the development of symbiotic food products. The aim of the present study was to compare and to determine the prebiotic capacity of selected legumes, namely adzuki bean, mung bean, black cowpea, and mucilages from chia seed and flaxseed on the growth of *Lactobacillus rhamnosus* GG. Resistance towards acid, pancreatin hydrolysis, and the prebiotic scores of the tested substances was determined based on growth promotion after 24 h of incubation. Results revealed that flaxseed mucilage was more resistant against hydrolysis by acid (1.47%) and pancreatin (2.64%) compared to the other samples (5.64 – 18.06% for acid and 10.34 – 15.57% for pancreatin). The relative prebiotic scores for flaxseed mucilage and black cowpea were 98% and 94%, respectively, which were higher than commercial prebiotics including inulin, fructooligosaccharides, and isomaltooligosaccharides. The optimum concentrations of flaxseed mucilage and black cowpea during 36 h of fermentation were 0.8% and 0.4% (w/v), respectively. The findings indicated that flaxseed mucilage was partially digested during gastrointestinal transit and it promotes the growth of the *L. rhamnosus* GG. The potential prebiotic capacity of flaxseed mucilage and its symbiotic relationship with *L. rhamnosus* GG suggests that they can be incorporated together for the development of functional foods.

Keywords: Functional foods, *Lactobacillus rhamnosus* GG, legume flours, prebiotics, prebiotic capacity, seed mucilage

INTRODUCTION

The symbiotic relationship between the indigenous microbial community in the digestive tract is crucial in maintaining intestinal homeostasis, thereby keeping the body healthy (Gagnière *et al.*, 2016). However, the imbalance in the composition of gut microbiota (or dysbiosis)

is associated with both intestinal (irritable bowel syndrome and celiac disease) and extra-intestinal disorders such as metabolic syndrome and obesity (Carding *et al.*, 2015; Heeney *et al.*, 2018; Ramakrishna, 2013). Therefore, administration of prebiotics and/or probiotics has been suggested

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as one of the approaches to modulate the equilibrium of the intestinal microbiota (Tungland, 2018).

Probiotics are living non-pathogenic strains of strictly selected microbes that confer therapeutic benefits to the host when administered in an adequate amount (Chew *et al.*, 2019; Hill *et al.*, 2014). Prebiotics has been defined as 'a substrate that is selectively utilized by a host conferring health benefits' (Gibson *et al.*, 2017). The consumption of prebiotics has been associated with the prevention of constipation, infectious and antibiotic-associated diarrhoea, inflammatory bowel disease, metabolic syndrome, obesity, and enhancing the uptake of certain mineral (Slavin, 2013; Taiseer *et al.*, 2014; Zhang *et al.*, 2018). Due to increasing health awareness among consumers, the demand for functional foods, namely probiotics and prebiotics has expanded greatly (Lai *et al.*, 2020a; Singla & Chakkaravarthi, 2017; Zaman & Sarbini, 2016). Common prebiotics such as mannitol, isomaltooligosaccharides, and oligofructose had been reported to protect the viability of probiotics (Chan & Pui, 2020; Ng *et al.*, 2019; Siang *et al.*, 2019; Yee *et al.*, 2019; Yong *et al.*, 2020).

The increase in consumers' awareness on the importance of the human microbiota has led to an upsurge in the demand for probiotics and prebiotics due to their beneficial effects on overall health by maintaining gut homeostasis. Recently, more studies were focusing on the potential prebiotic effects from legumes and seed mucilage (Bustamante *et al.*, 2015; Monk *et al.*, 2017). Legumes are rich in slowly digestible oligosaccharides and resistant starch that may exert potential prebiotics effects on the different probiotic strains (Winham *et al.*, 2017; Wu *et al.*, 2015). Furthermore, various legumes such as mung bean and adzuki bean have also been reported to maintain the viability of *L. rhamnosus* GR-1 in yoghurt throughout cold temperature storage (Soltani *et al.*, 2018). In addition, mice fed with cooked black bean altered the gut microbiota community by stimulating the growth of the beneficial bacteria and enhanced the production of short-chain fatty acids (Monk *et al.*, 2017).

On the other hand, mucilage is the gel-like soluble fibre that envelops a seed and is secreted by mucilage secreting cells upon hydration (Kreitschitz & Gorb, 2018). Previous studies had

also reported that mucilage from basil seed (Wongputtisiri & Khanongnuch, 2015), psyllium husk seed (Esmailzadeh *et al.*, 2016), and bush mint seed (Mueller *et al.*, 2017) showed high prebiotic potential on stimulating the growth of probiotics. Flaxseed is composed of about 11–15% of mucilage and it consists of a mixture of rhamnogalacturonan I and arabinoxylan, where the latter has been found to have prebiotic activity (Lai *et al.*, 2020b; Lai *et al.*, 2020c; Naran *et al.*, 2008; Pastell *et al.*, 2009; Singer *et al.*, 2011). In addition, chia seed contains about 5–6% of mucilage that is made up of β -D-xylopyranosyl, α -D-glucopyranosyl, and 4-O-methyl- α -D-glucopyranosyl-uronic acid unit in the ratio 2:1:1 (Muñoz *et al.*, 2012). Hence, both legumes and mucilage-bearing seed may be regarded as potential sources of prebiotics.

Since there is little published data on the comparison of the potential prebiotic effects of legumes and seed mucilage on a specific bacteria strain, it is worthwhile to study the prebiotic effects of selected legumes and seed mucilage on the growth of probiotics such as *Lactobacillus rhamnosus* GG. Thus, this study aims to determine and compare the prebiotic effects of adzuki bean (*Vigna angularis*), mung bean (*V. radiata*) and black cowpea (*V. unguiculata*) and seed mucilage from chia seed (*Salvia hispanica*) and flaxseed (*Linum usitatissimum*) on the growth of *L. rhamnosus* GG. The optimum concentration of the selected legumes and seed mucilage during fermentation was also determined.

MATERIALS AND METHODS

Materials

LactoGG capsules (LactoGG, USA) containing *L. rhamnosus* GG ATCC 53103 were purchased from a local pharmacy in Malaysia. Adzuki bean (*Vigna angularis* (Willd.) Ohwi & H. Ohashi), mung bean (*Vigna radiata* L.), black cowpea (*Vigna unguiculata* (L.) Walp), chia seed (*Salvia hispanica* L.) and flaxseed (*Linum usitatissimum* L.) were purchased from the local supermarket in Cheras, Kuala Lumpur, Malaysia. The scientific names and type of legumes used in this study were identified and verified by the Institute of Bioscience, Universiti Putra Malaysia.

Preparation of legume powder and seed mucilage

The legume powder of adzuki bean, mung bean, and black cowpea was obtained by milling using a miller (Panasonic MX-800S, Japan) and sieved through a 0.5 mm sieve to obtain the fine powder (Wani *et al.*, 2010). Flaxseed mucilage was produced by using hot water extraction (90°C, pH 5.0, 30 min under 100 rpm agitation) at a ratio of 1:10 for seed: water (Esparza *et al.*, 2011), while the chia seed mucilage was hot water-extracted at 80°C, pH 6.0, 2 h under 100 rpm agitation at a ratio of 1:40 for seed: water (Muñoz *et al.*, 2012). After agitation, the mucilage was separated from the seed by filtration through a piece of cheesecloth. The filtrate containing extracted mucilage was transferred into petri dishes and dried in a conventional oven at 60°C for 16 h. The dried mucilage was peeled from the petri dishes and ground into a fine powder using mortar and pestle.

Determination of resistance towards acid and pancreatic enzymatic hydrolysis

The resistance of the samples towards simulated acid and pancreatic enzymatic hydrolysis was determined according to Azmi *et al.* (2012) and Majeed *et al.* (2018b), respectively, with some

modifications. Artificial human gastric acid juice (g/mL) was prepared to contain 6.2 g NaCl, 2.2 g KCl, 0.22 g CaCl₂, 1.2 g NaHCO₃ and 0.3% (w/v) pepsin (Chemsoln, India) in 1 L of distilled water with pH adjusted to 1 and 5 using 5 M HCl. To prepare artificial pancreatin solution, pancreatin (Chemsoln, India) at 1 mg/mL was dissolved in 50 mM phosphate buffer, pH 7.4. The sample solution at a concentration of 1% (w/v) was prepared by dissolving 0.1 g of the sample powder into 10 mL of distilled water and placed in a sonicator bath (WUC-A06H, Daihan Scientific Co. Ltd., Korea) for 3 min. To perform the test, 5 mL of artificial gastric acid juice or the enzymatic pancreatin solution was added to 5 mL of the sample solution and incubated in a shaking water bath (Cole-Parmer, USA) at 37°C for 240 min at 100 rpm. Aliquots (1 mL) were withdrawn periodically at every hour to determine the reducing sugar content using the dinitrosalicylic acid (DNS) method and total sugar content by phenol-sulphuric acid assay according to Miller (1959) and Dubois *et al.* (1956), respectively. Fructooligosaccharides (FOS) (Sensus, Netherlands) was used as the reference. The percent of hydrolysis was calculated using the formula represented by Equation 1:

$$\text{Percent of hydrolysis (\%)} = \frac{\text{Reducing sugar released}}{\text{Total sugar content} - \text{initial reducing sugar content}} \times 100 \quad (1)$$

where the reducing sugar is the difference between its final and initial content, while the initial reducing sugar was the reducing sugar at 0 h.

Determination of prebiotic activity score

The prebiotic effect of samples was determined according to Thuaytong and Anprung (2011) with slight modifications. The reaction mixture (0.5% w/v) for the selected legumes and seed mucilage was prepared by dissolving the respective powder into 30 mL of distilled water in a sonicator bath for 3 min. After that, 0.3 mL of an overnight *L. rhamnosus* GG culture (1.0 % v/v) was added to each reaction mixture. Glucose (0.5% w/v) was used as the reference for this test, while FOS, inulin (Sensus, Netherlands), galactooligosaccharides (GOS) (Chemsoln, India) and isomaltooligosaccharides (IMO) (CK Ingredient, Malaysia) served as the positive

controls. De Man, Rogosa & Sharpe (MRS) broth devoid of glucose was used as the negative control. Viable cell counts before (0 h) and after incubation for 24 h at 37°C were determined by the drop plate method (Yeung *et al.*, 2016). An aliquot (1 mL) of the broth cultures was withdrawn and serially diluted with 9 mL of phosphate buffer saline (PBS, Lonza, USA). The enumeration was determined after 48 h of incubations at 37°C. The log viable cell count and the prebiotic scores were calculated using Equations 2 and 3 (Khorasani & Shojaosadati, 2016):

$$\text{Log viable cell count (CFU/ml)} = \text{Log} \frac{\text{Mean number of cells in triplicates}}{\text{Volume plated} \times \text{dilution factor}} \quad (2)$$

$$\text{Prebiotic scores} = \frac{[\log (\text{CFU/ml}) \text{ on prebiotic at 24 h} - \log (\text{CFU/ml}) \text{ on prebiotic at 0 h}]}{[\log (\text{CFU/ml}) \text{ on glucose at 24 h} - \log (\text{CFU/ml}) \text{ on glucose at 0 h}]} \times 100 \quad (3)$$

Determination of selected ingredients' concentration with optimum prebiotic capacity

The selected ingredients with the highest prebiotic score were then evaluated at different concentrations (0.2–1.0% w/v) to determine the optimum concentration in growth stimulatory effect after 36 h of incubation in MRS broth (Azmi *et al.*, 2012). The viability of *L. rhamnosus* GG was determined by drop plate method after 48 h of incubation at 37°C with the initial (0 h) cell population fixed at 10⁴ CFU/mL in 100 mL growth media with the selected ingredient (Yeung *et al.*, 2016). Changes in the pH of the broth cultures were measured every 3 h using a calibrated pH meter. Titratable acidity (TA) was determined every 3 h based on AOAC (1998) method by titrating 10 mL of sample broth diluted with 40 mL of distilled water with 0.1 N NaOH to a phenolphthalein endpoint. The volume required was recorded and the TA was expressed as percent lactic acid.

Statistical analysis

All tests were carried in triplicates and the results were reported as mean $\pm$ standard deviation. One-way variance analysis (ANOVA) and Tukey's post hoc test was performed and when $p \leq 0.05$ is considered significantly different using IBM SPSS Statistics version 24.

RESULTS AND DISCUSSION

Figure 1a, 1b and 1c present the percent of hydrolysis of the selected legumes and seed mucilage in artificial acid solution (pH 1 and pH 5) and pancreatic enzyme at pH 7.0, respectively. The percent of hydrolysis for the all selected legumes and seed mucilage was significantly ($p \leq 0.05$) higher at lower pH (pH 1) as compared to pH 5, for the artificial acid solutions. This could be due to the acid hydrolysis on the

polysaccharides in the samples into reducing sugars such as glucose (Miloski *et al.*, 2008). It was also observed that the percent of hydrolysis increases as the incubation time increases, indicated by the production of more reducing sugars. A similar trend was also reported when bamboo shoot crude polysaccharides and arrowroot starch was treated with simulated gastric juice, more reducing sugars were produced as the incubation time increases (Azmi *et al.*, 2012; Faridah *et al.*, 2017).

In the present study, fructooligosaccharides (FOS) exhibited high tolerance toward simulated gastrointestinal conditions with more than 97% remaining intact after 4 h of incubations at both conditions (Figure 1). The percent of hydrolysis of selected legumes ranged from 10.8% for adzuki bean to 18.1% for black cowpea at pH 1 (Figure 1a) while 5.7% for adzuki bean to 13.1% for black cowpea at pH 5 (Figure 1b). This may be attributed to the breakdown of sucrose in the legumes by HCl from the artificial acid solution while soluble fibre of seed mucilage slowed down gastric emptying (Bernacchia *et al.*, 2014; Miloski *et al.*, 2008). The release of reducing sugars from the legumes could be enhanced by high sucrose content (in the legume) and at low pH of gastric juice (Chavan *et al.*, 2015; Miloski *et al.*, 2008). Hence, the high hydrolysis demonstrated by black cowpea in this study could be due to its higher amount of sucrose as compared to both adzuki bean and mung bean (Fan *et al.*, 2015). Nevertheless, the 3 legumes (black cowpea, adzuki bean, mung bean) in this study demonstrated lower hydrolysis as compared to red kidney bean at low pH after 4 hours (~25%) (Saad *et al.*, 2020).

In contrast to legumes, the degree of hydrolysis at the end of incubation at pH 1 ranged from 1.5% for flaxseed mucilage to 5.6% for chia seed mucilage (Figure 1a) and both seed mucilages exhibited more than 98% of resistance towards gastric acid at pH 5 (Figure 1b). The degree of hydrolysis of the selected legumes was higher than

both seed mucilage and FOS, indicating that the seed mucilage has a higher resistance towards acid hydrolysis as compared to the selected legume flour. The lower degree of hydrolysis displayed by flaxseed mucilage could be due to the higher hetero-polysaccharide (70%) such as galactomannan as compared to chia seed mucilage (65%) (Cruz-Rubio *et al.*, 2018). Galactomannan is resistance towards gastric acid and digestive enzymes, hence improving the degree of hydrolysis (Majeed *et al.*, 2018a).

Mucilage is the soluble dietary fibre extracted from the seed that could retain in gastrointestinal for a longer period (Singer *et al.*, 2011; Suri *et al.*, 2016). Similar results were reported by Emaga *et al.* (2012) where flaxseed mucilage subjected to low pH of 0.2 M sulphuric acid for 4 hours showed approximately 1% of hydrolysis. Furthermore, higher hydrolysis (approximately 9%) were also found when the acid concentration was increased to 1 M and 2 M. Moreover, Bustamante *et al.* (2017) also demonstrated chia seed and flaxseed mucilage protective effect towards probiotics throughout gastrointestinal digestion. This support that flaxseed and chia seed mucilage are not easily digested by the gastric acid.

Figure 1c shows that the degree of hydrolysis using pancreatic enzymes ranged from 2.64% (flaxseed mucilage) to 10.34% (chia seed mucilage) and from 12.65–15.57% (for the legumes) after 4 h of incubation. The present findings revealed that only flaxseed mucilage showed the highest tolerance towards enteric digestion, which was similar to the control (FOS). The highest percent of hydrolysis was obtained by black cowpea (15.57%) possibly due to higher carbohydrate composition in black cowpea (61–69%) than the adzuki bean and mung bean (54–59%) (Adamu *et al.*, 2016; Brishti *et al.*, 2017; Hamid *et al.*, 2016). Hence, more carbohydrates could be broken down from acid and enzyme hydrolysis (Amezcuza-Allieri *et al.*, 2017). Thakur *et al.* (2018) reported that the pancreatic hydrolysis rate of bamboo shoots and potato peel (about 7%) which were similar to those of legume flour obtained in the present study.

Approximately 82% of the legumes examined remain undigested in the artificial acid solutions (Figure 1a and 1b) and the pancreatin enzymatic solution (Figure 1c) due to the present of non-digestible oligosaccharides. The reported total

non-digestible oligosaccharides for adzuki bean, mung bean and black cowpea were 49.8 g/kg, 45.5 g/kg and 69.8 g/kg, respectively (Fan *et al.*, 2015). In addition, the results may be due to the presence of α -amylase inhibitors in adzuki bean, mung bean and black cowpea (Piergiovanni & Gatta, 1994; Ueda *et al.*, 2004; Wisessing *et al.*, 2010). These findings suggested that the selected legumes and seed mucilage were partially digested during the gastric and intestinal region and the remaining undigested substrate could possibly serve as the prebiotic source for the commensal bacteria in the gut including *Lactobacillus rhamnosus* GG.

The prebiotic scores are used to estimate the health effects of prebiotic in humans through its ability to stimulate growth of probiotics (Figueroa-González *et al.*, 2019). Figure 2 depicts the comparison of the prebiotic scores on the legumes and seed mucilage examined, commercial prebiotics and glucose. Glucose has the highest prebiotic score (100%) due to its highest growth promotion in *L. rhamnosus* GG after 24 h of incubation. Glucose is the most common carbon source that supports a good growth rate of many lactobacilli (Sánchez *et al.*, 2010; Succi *et al.*, 2017). Results revealed that the prebiotic scores of adzuki bean, mung bean and commercial prebiotics including FOS, isomaltooligosaccharides (IMO) and inulin were in the range of 79–82% which are not significantly different ($p > 0.05$) with each other. However, MRS broth without prebiotic has the lowest prebiotic score (43.51%) due to lack of carbohydrates sources for growth. This highlights the importance of carbohydrates that is required for the growth of *L. rhamnosus* GG (Azmi *et al.*, 2012).

The prebiotic scores of flaxseed mucilage and black cowpea were not significant ($p > 0.05$) different from glucose and were higher compared to commercial prebiotics particularly FOS, inulin, and IMO. The present findings are consistent with the study conducted by Watson *et al.* (2013) who reported that *L. rhamnosus* GG grew moderately when either inulin or FOS was the sole carbon source. The high prebiotic score of flaxseed mucilage (98.08 %) was possibly attributed to the monosaccharide residues (i.e. fucose, arabinose, fructose and galactose) in the flaxseed mucilage which were utilized by *L. rhamnosus* GG for growth (Kankainen *et al.*, 2009;

Petrova *et al.*, 2018). Flaxseed mucilage has been reported to increase the generation of short-chain fatty acids like acetate, propionate and butyrate in batch cultures by pig intestinal digesta which are necessary for growth (Lin *et al.*, 2011). Previous studies also demonstrated that the novel prebiotic

source from fenugreek seed fibre, cranberry seed fibre, and fungi polysaccharides could exhibit a growth stimulatory effect on the tested strains similar to the commercial prebiotic (Majeed *et al.*, 2018a; Majeed *et al.*, 2018b; Nowak *et al.*, 2016).

Figure 1. Percent of hydrolysis (%) of selected legume powder and seed mucilage at artificial acid solution at (a) pH 1, (b) pH 5, and (c) artificial pancreatin enzyme solution at pH 7.0. Error bars indicate the

standard deviation of triplicate experiments ($n = 3$). FOS, fructooligosaccharides. FOS serves as positive control.

Figure 2. Prebiotic scores (%) for *Lactobacillus rhamnosus* GG with different type of prebiotics. ^{abcd} means significantly different at $p \leq 0.05$ via ANOVA and Tukey's test as post hoc test. Error bars indicate the standard deviation of triplicate experiments ($n = 3$). IMO, isomaltooligosaccharides; GOS, galactooligosaccharides; FOS, fructooligosaccharides. Glucose serves as reference; while inulin, FOS, IMO, and GOS serve as positive control. No prebiotic refers to de Man, Rogosa & Sharpe (MRS) broth devoid glucose which serves as negative control.

On the other hand, the prebiotic score of black cowpea powder was 94% (Figure 2), which was approximately 15% greater in prebiotic capacity than the adzuki bean and mung bean. This is probably due to higher total oligosaccharides content in black cowpea (100.3 g/kg) as compared to adzuki bean (62.5 g/kg) and mung bean (65.1 g/kg) (Fan *et al.*, 2015). Besides that, *L. rhamnosus* GG has been reported to ferment in different leguminous porridges (white bean, speckled bean and green lentil) after 10 h at 37°C and storage at 5°C for 21 days (Petruláková & Valík, 2015). Since black cowpea and flaxseed mucilage illustrated the highest prebiotic score (Figure 2), they were included to determine the optimal concentration of the growth promotion of *L. rhamnosus* GG based on its fermentation for 36 h at 37°C.

Figure 3a and 3b show the growth curves of *L. rhamnosus* GG supplemented with different concentrations (0–1.0% w/v) of flaxseed mucilage and black cowpea, respectively during 36 h of fermentation at 37°C with MRS broth at 0% (w/v) of each sample as the negative control. Immediately after inoculation of *L. rhamnosus* GG

into the respective media, *L. rhamnosus* GG cells experienced a lag phase for approximately 3 h (4.63–4.92 log CFU/mL) and the cells started to grow in exponential phase, reaching maximum viability (11.25 log CFU/mL) at 9 h for 0.8% (w/v) flaxseed mucilage (Figure 3a) and (10.50–10.82 log CFU/mL) at 12 h for 0.4–1.0% (w/v) black cowpea (Figure 3b).

The *L. rhamnosus* GG cells in glucose-free MRS broth (0.0% w/v flaxseed mucilage or black cowpea) reached stationary phase after 15 h of incubation ($\sim 10^9$ CFU/mL). As the concentration of flaxseed mucilage (from 0.0% to 0.8% (w/v) and black cowpea (from 0.0% to 0.4% (w/v)) increase, more nutrients are available for cell division, therefore accelerated growth curve was observed by at least 33% as compared to the negative control. These outcomes were compatible with the study from Majeed *et al.* (2018a), where the concentrations of galactomannan from fenugreek seed fibre were proportional to the growth stimulation effect of *Bacillus coagulans* MTCC 5856 after 24 h of fermentation. The rapid growth of *L. rhamnosus* GG in the media supplemented with flaxseed

mucilage and black cowpea during the log and stationary phases could result in the decline in the cell population (27–36 h of incubation) due to the exhaustion in nutrients and accumulation of toxic waste compounds from the cell metabolic activities (Ho *et al.*, 2017).

Flaxseed mucilage at 0.8% (w/v) and black cowpea at 0.4% (w/v) provide the optimal growth stimulatory effect in *L. rhamnosus* GG. The slow growth of the *Lactobacillus* strain at 1.0% (w/v) flaxseed mucilage concentration may probably attribute to the increase in the viscosity (Bustamante *et al.*, 2015). Besides that, a similar result was also reported by Savedboworn *et al.*

(2017) where 2% inulin showed higher probiotic growth than 3% inulin. The study explained that the probiotic with higher prebiotic concentration may produce a higher amount of acid and resulted in lower probiotic growth. The growth curve of high black cowpea concentrations (0.6–1.0%) were not significantly different ($p > 0.05$) from the 0.4% (w/v). These findings were supported by Wang *et al.* (2006) who underlined that no difference in bacteria densities of lactobacilli and bifidobacterial were detected in the media supplied with 1.0%–3.0% (w/v) of alginate oligosaccharides

Figure 3. Growth curve of *Lactobacillus rhamnosus* GG based on viable cell count over time (0–36 h) under different concentrations (0–1.0% w/v) of (a) flaxseed mucilage and (b) black cowpea powder. Error bars indicate the standard deviation of triplicate experiments ($n=3$). FM, flaxseed mucilage; BC, black cowpea.

The fermentation of both flaxseed mucilage and black cowpea was evident by the reduction in pH of the media (Figure 4) and an increase in the titratable acidity (TA) (Figure 5). Figure 4 shows the initial pH of all the growth media were at 5.5–5.6. The pH of the growth media dropped to 3.5–3.6 for flaxseed mucilage (Figure 4a) and 3.5–3.7 for black cowpea (Figure 4b) at the 27th h of incubation as the cells entered the death phase.

This demonstrates that the low pH of the growth media resulted in the decline in *L. rhamnosus* GG's viability as the optimum pH of *L. rhamnosus* GG ranged from 4.5 to 6.4 (Valík *et al.*, 2008).

After 9 h of incubation, pH of the growth media containing 0.8% (w/v) flaxseed mucilage was the lowest which emphasized the high production of the lactic acid (Figure 4a). In contrast, the decrease in pH of the media with

0.0% (w/v) flaxseed mucilage was less pronounced due to the slower cell division in the log phase. Furthermore, the reduction in pH was not significantly different ($p > 0.05$) for black cowpea concentrations above 0.4% (w/v), implying that the synthesis of the short-chain fatty

acids was comparable as shown in Figure 5b. The obtained results were compatible with the pH obtained from the 24 h fermentation of flaxseed mucilage by *L. acidophilus* La-5 at different concentrations (Bustamante *et al.*, 2015).

Figure 4. The pH of growth medium containing *Lactobacillus rhamnosus* GG and 0–1.0% (w/v) of (a) flaxseed mucilage and (b) black cowpea powder at 36 h of fermentation. Error bars indicate the standard deviation of triplicate experiments ($n=3$). FM, flaxseed mucilage; BC, black cowpea.

Lactic acid was synthesized as a major by-product from the metabolism of carbon source by *L. rhamnosus* GG (de Keersmaecker *et al.*, 2006). Both TA curves showed similar pattern such that the percent of TA is directly proportional to the incubation time, especially during the exponential phase. Figure 5 shows that the percent of TA was elevated as the concentration of flaxseed mucilage and black cowpea increases. This is probably due to rapid utilization of the nutrients for growth and more lactic acid was produced, with an exception for 1.0% (w/v) flaxseed mucilage (Figure 5a) while the concentration of black cowpea above 0.4% (w/v) has no significant effect ($p > 0.05$) on the increase in percent TA (Figure 5b).

At the end of the fermentation (36 h), the final TA for 0.8% flaxseed mucilage was 2.32% (Figure 5a) and 2.03–2.07% for 0.4–1.0% black cowpea (Figure 5b), respectively. The results were also consistent with Ho *et al.* (2017) highlighted that higher population of the *L. acidophilus* and *B. bifidum* at 24 h of incubation as a result of higher enzymatic activity to hydrolyse the oligosaccharides of citrus pectin enzyme hydrolysate into monosaccharides and greater ability to utilize monosaccharides. Thus, a higher amount of lactic acid was produced. Flaxseed mucilage at 0.8% (w/v) and black cowpea at 0.4% (w/v) was the optimum concentration in providing most favourable growth stimulatory effect in *L. rhamnosus* GG.

Figure 5. Titratable acidity (TA) of growth medium containing *Lactobacillus rhamnosus* GG and 0–1.0% (w/v) of (a) flaxseed mucilage and (b) black cowpea powder at 36 h of fermentation. Error bars indicate the standard deviation of triplicate experiments (n=3). FM, Flaxseed mucilage; BC, black cowpea.

CONCLUSION

This study provides the comparison of different selected legumes and seed mucilage on the resistance towards digestibility and the prebiotic capacity on the *L. rhamnosus* GG. The flaxseed mucilage exhibited similar resistance towards artificial acid and pancreatic hydrolysis as compared to FOS. However, flaxseed mucilage and black cowpea presented the highest prebiotic score that was not significantly different from the glucose reference. The optimum concentration of flaxseed mucilage and black cowpea were 0.8% and 0.4% (w/w), respectively due to the highest in *L. rhamnosus* GG growth promotion. The current study provides an alternative prebiotic source that is suitable for the production of symbiotic functional food products along with the commercial probiotic strains of *L. rhamnosus* GG.

ACKNOWLEDGEMENTS

This work was supported by Pioneer Scientist Incentive Fund (PSIF) grant (Proj-In-FAS-055) and UCSI Research Excellence and Innovation Grant (REIG) (REIG-FAS-2020-003) from UCSI University.

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