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In Vitro Fermentation of *Acacia Senegal* by Fecal Microbiota from lean Donors to Stimulate the Growth of Probiotic

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Abstract:

Gum Arabic is believed to have many health benefits including prevention of gastrointestinal diseases. Prebiotic resist digestion in the upper gastrointestinal tract and allowed for stimulation of bacterial growth in the distal intestine and colon. The prebiotic properties of *Acacia Senegal* was studied using mixed cultures of human fecal bacteria from four lean individuals. The results obtained were compared with inulin as positive prebiotic control. Fermentation studies were carried out using anaerobic, pH-controlled faecal batch cultures, and the changes in the faecal microbial population were monitored at 0, 6, 12, 24 and 36h by fluorescent in situ hybridization (FISH). Quantitative FISH results revealed that *Bifidobacterium*spp. *Bacteroidacea* spp. and *Lactobacillus* spp. were selectively increased ($P<0.05$) after the fermentation of *Acacia Senegal* by the fecal microbiota.*Clostridium* spp.,even have showed slight increase in fermentation of *Acacia Senegal*and inulin but was not significant. The stimulation of growth of probiotic bacteria was accompanied by a high production of acetate acid. The fermentation of *Acacia Senegal* may help to improve health through stimulation of bacteria growth which led to production of acetate. This study provides proof for the prebiotic effectiveness of *Acacia Senegal*, and the result showed that it might have a prolongedbifidogenic effect, thus could prevent certain types of diseases.

Keywords: prebiotic; *Acacia Senegal*; Colonic fermentation; probiotic; SCFA

Introduction:

Gum Arabic, known as acacia gum is a natural gum made of exudation taken from two species of the acacia tree; *Senegalia senegal* and *Vachellia seyal*. The gum is harvested commercially from wild trees throughout Sudan. It is a complex mixture and heteropolysaccharide of high molecular weight of glycoproteins and polysaccharides. It was historically the source of arabinose sugars, which was first discovered and isolated from it (Calame, *et al*.,2008).*Acacia gum*, is traditionally utilized by African populations (Cherbut*et al.* 2003) to prevent and treat intestinal disorders. There has been illustrated that gum feeding can improve intestinal transit and provide digestive comfort (Cherbut*et al.* 2003). Such traditional belief in promoting characteristics of prebiotic have been used and accepted. A prebiotic is a "nondigestible food ingredient that affects the host by selectively targeting the growth and/or activity of one or a limited number of bacteria in the colon, and thus has the potential to improve host health".(Hughes *et al.* 2007). Prebiotic has been shown to improve health and prevent diseases, with the more soluble, fermentable prebiotic sources being proposed to reduce the glycaemic index, insulin sensitivity (Hallfrisch &

Behall 2000) and cholesterol absorption (Kahlon*et al*. 1994) by the simulation of gut microbiota.

The gastrointestinal tract is a complex ecosystem containing up to 10^{11} CFU of bacteria per gram of intestinal content. This large population of bacteria plays a key role in normal gut function, human health and well-being (Van, *et al* 2004). In particular, *Bifidobacteria* and *lactobacilli* are believed to play major important role in maintaining intestinal health and are generally regarded as probiotics due to their effects on maturation and balancing of the immune system (Peran, L. *et al*. 2007; Sartor, R. B. 2008;Zeuthen, L. H.*et al.* 2010) and to their ability in inhibition of pathogens (Collado. M.C.*et al.*2007., Fukuda, S. *et al.* 2011.). However, a sufficient prebiotic intake is required for the desired effect (European Food Safety Authority. 2010). The selective stimulation of specific colonic bacteria is explained by the capability of these bacteria to break down the glycosidic linkages in the prebiotic carbohydrates. These bacteria are able to grow on particular carbon sources, which are less easily fermented by other members of the intestinal community. This provides those bacteria with a selective advantage when competing with other bacterial species in a mixed bacterial community such as the human colon (Sanz, M. L., *et al*. 2005). Hence, there is a great interest in the manipulation of the intestinal microbiota, targeting to increase of the number of *bifidobacteria* and *lactobacilli* and to stimulate the production of short chain fatty acids (SCFA) and lactate in the colon (Gibson G.R & Roberfroid M.B. 1995; Pool-Zobel B.L. 2005). Therefore, this study may provide information regarding prebiotic properties within the *Acacia Senegal*. A small-scale faecal batch culture representing the human gut was used in the experiment. The fermentability and bifidogenic activity of different *Acacia Senegal* was monitored by bacteria enumeration using FISH method. Short-chain fatty acid (SCFA) production was also determined by high performance liquid chromatography (HPLC).

Material and Methods:

Samples*:* The Gum Arabic (*Acacia Senegal*) sample used in this study was purchased from a Sudanese market.Inulin from chicory root (Warcoing, Belgium), being an established prebiotic ingredient, was used as a positive control.

Faecal inoculation : Fecal samples were provided by four healthy adult donors (male, aged 20–30 years old) and (BMI: 18.5-24.9 kg/m2). The donors, had not received antibiotic treatment and had not consumed pre or probiotic supplements for at least six months before the study. Freshly stool samples were collected in sterile plastic pots at The University of Putra Malaysia, Bintulu campus on the day of inoculation of the batch vessels. Samples were diluted (1:10 w/w) with sterile phosphate buffered saline (PBS) (0.1 M, pH 7.4) and homogenized in a stomacher (Stomacher 400, Seward,West Sussex, UK) at normal speed for 2 min.

In vitro fermentations: Anaerobic, pH controlled batch culture fermentations with customised glassvessels (Soham Scientific, Fordham, UK) were used to assess the effect of *Acacia Senegal* on composition of gut microbiota and fermentation characteristics. Fermenter vessels (100 ml) with only 50 ml working volume were aseptically filled with 45 ml of presterilized basal nutrient medium (Sarbini*et al*. 2011). The medium contained the following ingredients: (2 g/l peptone water (Oxoid), 2 g/l yeast extract (Oxoid), 0·1 g/l NaCl, 0·04 g/l K2HPO4, 0·04 g/l KH2PO4, 0·01 g/l MgSO4.7H2O, 0·01 CaCl2.6H2O, 2 g/l NaHCO3, 2 ml Tween 80 (BDH), 0·05 g/l haemin, 10µl vitamin K1, 0·5 g/l cysteine.HCl, and 0·5 g/l bile salts, pH 7·0) and continuously sparged with O_2 free nitrogen (15 ml/min) and each vessel was magnetically stirred. The test sample (*Acacia Senegal*) (1% w/w) were added at 0.5g in 50ml working volume, just before addition of 10% fresh fecal slurry. Control fermentations supplemented with inulin as positive prebiotic control at a concentration of 1% w/w were also included. The temperature of each batch vessel was maintained at 37 C by means of a circulating water bath. The pH was maintained at 6.8 using an automated pH

controller (Fermac 260, Electrolab, Gloucestershire, UK). Batch cultures were run for a period of 36 h and 5 ml samples were obtained from each vessel at 0, 6, 12, 24 and 24 h for FISH and SCFA analysis. The batch experiments were performed in four replicates with four different fecal donors for each substrate.

Bacterial enumeration by Fluorescent in situ hybridization (FISH): Bacterial composition in the gut models was analyzed using Fluorescence *in situ* hybridization (FISH). Synthetic oligonucleotide probes bind with specific regions of the 16 S ribosomal ribonucleic acid molecule and labelled with the fluorescent dye Cy3 were utilised for the enumeration of bacterial groups (Table 1). A volume of 375 µl of samples from each vessel at each sampling time were fixed for 4h at 4 C° with 1125 µl of 4% (w/v) filtered paraformaldehyde (pH 7.2) in a ratio of 1:3 (v/v). Samples were washed twice with phosphate-buffered saline (0.1 M, pH 7.0) and centrifuged at 13 000 g for 5 min and washed twice in 1 ml filter-sterilized PBS. The Pellets were resuspended in 300 µL of filtered PBS/ethanol (99 %) in a ratio of 1:1 (v/v) and stored at -20 C° until further processing. Dilution steps were performed using 10 µL of the fixed samples added to suitable volume of PBS in order to obtain a countable number of fluorescent cells in each field of view. The bacteria were enumerated by adding 20µL to each well of a six-well polytetrafluoroethylene/ poly-L-lysine-coated slide (Tekdon Inc., Myakka City, FL). Slides were dried at 46C° for 15 min. After 15 min of drying, a dehydrated steps were applied, using ethanol with different concentration (50, 80 and 96%) each step of dehydrated take 3min. Slides were returned in the drying oven for 2min to evaporate excess ethanol before addition of hybridisation mixture. Hybridisations were performed by adding 50µLhybridisation mixture consisting of 45µLhybridisation buffer (5 M NaCl, 1 M Tris/HCl pH 8, 30% formamide, ddH2O, 10% SDS) and 5µL probe (Chis150, Lab158, Bac303and Bif164) to each well and left to hybridise for 4h in a microarray hybridisation incubator (Grant-Boekel, Cambridge, UK). Washing buffer was used direct after the hybridization of the slides for 15 min and then followed with dipping in cooled water for few seconds. Slides were dried using compressed air. Subsequently, 5 microliters of polyvinyl alcohol mounting medium with 1,4-diazabicyclo (2,2,2) octane were added onto each well and a cover slip was placed on each slide (20 mm; thickness no. 1; VWR, Lutterworth, UK). Numbers of total bacteria were determined by taking into account viewing each well for fifteen different fields using epifluorescence microscope (CX31; Olympus, Tokyo, Japan) using CX-RFL-2 reflected fluorescence attachment.(Sarbini*et al*. 2011).

Table 1. 16S rRNA oligonucleotide probes used in FISH analysis of bacterial populations.

Probe Code	Target group	Sequence (5'-3')	Reference
Chis150	Most of the Clostridium histolyticum group (Clostridium clusters I and II)	TTATGCGGTATTAATCTYCCTTT	Franks et al. (1998)
Lab158	Lactobacillus–Enterococcus	GGTATTAGCAYCTGTTTCCA	Harmsen et al. (1999)
Bac303	Most Bacteroidaceae and Prevotellaceae, some Porphyromonadaceae	CCAATGTGGGGGACCTT	Manz et al. (1996)
Bif164	Bifidobacterium spp.	CATCCGGCATTACCACCC	Langendijk et al. (1995)

Short chain fatty acids analysis: The short chain fatty acids (SCFA) lactate, acetate, propionate and butyrate, were analysed by ion-exclusion HPLC system (SHIMADZU SPD-20A)equipped with UV-VIS detector. The column used was an ionexclusionRezex ROA-Organic Acid H^+ (8%), 300 X 7·80mm (Phenomenex). Sulphuric acid in HPLC-grade water (0.0025 mmol L^{-1}) was used as the eluent. Samples taken from the batch culture vessels were centrifuged at 13 000 g for 10 min to remove all particulate material. Supernatants were then filtered using 0.2 mm polycarbonate syringe filters (Millipore) and 15 µL was injected into an HPLC system. HPLC was operated at a flow rate of 0·5 ml/min with a heated column at 40^0C. The run time for sample was 40 min. Quantification of the samples was obtained by comparing with calibration curves of lactate, acetate, propionate, and butyrate in concentrations ranging between 6.25 and 100 mM.

Statistical Analysis : Statistical analysis of the data was performed by one-way ANOVA using (SPSS 23 software). Significant differences ($p<0.05$) among gum Arabic samples were analyzed by Duncan triplicates range test Bryman and Crame (2012).

Result:

Bacterial enumeration : Bacterial counts with the duration of 0h, 6h, 12h, 24h and 36h of incubation are shown in Table 2. After fermentation of *Acacia Senegal* and inulin in fecal slurries obtained from healthy donors, FISH method was applied to measure the concentration of selected bacterial. Fermentation of *Acacia Senegal* resulted in a significant increase in the content of *Bifidobacterium*spp.,*Bacteroidetes*spp., and *Lactobacillus* spp. *Acacia Senegal* showed no significant increase in the growth of *Bifidobacterium* from 0 until 12 h and also *Bacteroidetes*at 0h and 6 h, whereas, inulin showed significant growth of *Bifidobacterium* and *Bacteroidetes*from 0 to 24h. The time and speed of fermentation mostly effected by the structure of prebiotic which effect on the growth of probiotic.

Table 2. Mean value of bacterial population (log10 cells/ml batch culture fluid) in the colon model at 0,6, 12, 24 and 36 h inoculated with faecalmicrobiota (n = 4).

	Time	Bif164	Bac303	Lab158	Chis150
Senegal	0h	8.097±0.13	7.846±0.09	8.057±0.02	7.467±0.07
	6h	8.361^{b}±0.05	8.042^{b}±0.04	8.337*±0.06	7.571±0.18
	12h	8.376^{b}±0.1	8.146^{b}*±0.04	8.391*±0.05	7.585±0.07
	24h	8.656^{b}*±0.07	8.351^{b}*±0.05	8.454*±0.04	7.765±0.09
	36h	8.808^{a}*±0.09	8.677^{a}*±0.12	8.463*±0.08	7.612±0.06
Inulin	0h	8.097±0.13	7.846±0.09	8.057±0.02	7.467±0.07
	6h	8.577^{a}*±0.03	8.307^{a}*±0.05	8.308±0.08	7.696±0.09
	12h	8.856^{a}*±0.11	8.532^{a}*±0.07	8.545±0.11	7.687±0.19
	24h	8.938^{a}*±0.04	8.620^{a}*±0.07	8.561±0.12	7.588±0.23
	36h	8.830^{a}*±0.06	8.496^{a}*±0.08	8.489±0.22	7.693±0.26

Bif164: Bifidobacterium spp.; Bac303: Most Bacteroidaceae and Prevotellaceae, some Porphyromonadaceae; Lab158: Lactobacillus/Enterecoccus; Chis150: Clostridium histolyticum. Mean value with unlike superscript letters (a,b) was significantly higher/lower in comparison between substrates in between the same sampling hour ($p < 0.05$).

*Mean value was significantly different from that of 0 h ($p < 0.05$).

However, since *Acacia Senegal* is complex heteropolysaccharide with high molecular-weight so the fermentation might be slow and led to low growth of probiotics. The lower molecular-weight oligosaccharides such as (inulin) are rapidly fermented. This may be because the enzymes produced by Bifidobacterium spp. prefer to utilize non-reducing ends due to the low molecular mass. (Gibson. *et al*. 2004; Sarbini. S.R & Rastall R.A.2011).

Surprisingly, the growth of *Bifidobacterium*, *Bacteroidetes* and *Lactobacillus* start to drop for inulin at 36h (8.83, 8.49, 8.48 Log_{10}) respectively, while the growth of bacteria fermenting*Acacia Senegal* still dramatically increasing. However, the growth of *Bifidobacterium* and *Bacteroidetes* population at 36 h for *Acacia Senegal* showed no significant difference compared to fermentation of inulin. Our findings are in agreement with Wyatt *et al.* 1986, who noted an increase in the numbers of Bacteroides and Bifidobacterium. No significant difference in the growth of *Lactobacillus* population for inulin when compared to 0h.Both *Acacia Senegal* and inulin show no significant difference in the growth of *Clostridium* spppopulation when compared with 0h.However these results showed that, *Acacia Senegal* was selected by the probiotic bacteria.

Short-chain fatty acid (SCFA)analysis: Amounts of SCFA (lactate, acetate, propionate, butyrate) produced by fermentation of *Acacia Senegal* and Inulin is shown in Figure 1. Supplementation of *Acacia Senegal* to gut models inoculated with faecal samples from lean donors led to a significant increase of acetate at 24h and 36h as the major production of the fermentation(95.8 mM and 114.6 mM) respectively, whereas 6 and 12h show no significant growth when compared to 0h(53.06 mM and 46.05mM) respectively. Fermentation of inulin showed significant increase of acetate concentration at all times, but at 36 the production of acetate start to slowdown. Acetate produced may improves intestinal defence mediated by epithelial cells, which could protect the host against enteropathogenic infection (Fukuda. *et al.* 2011).

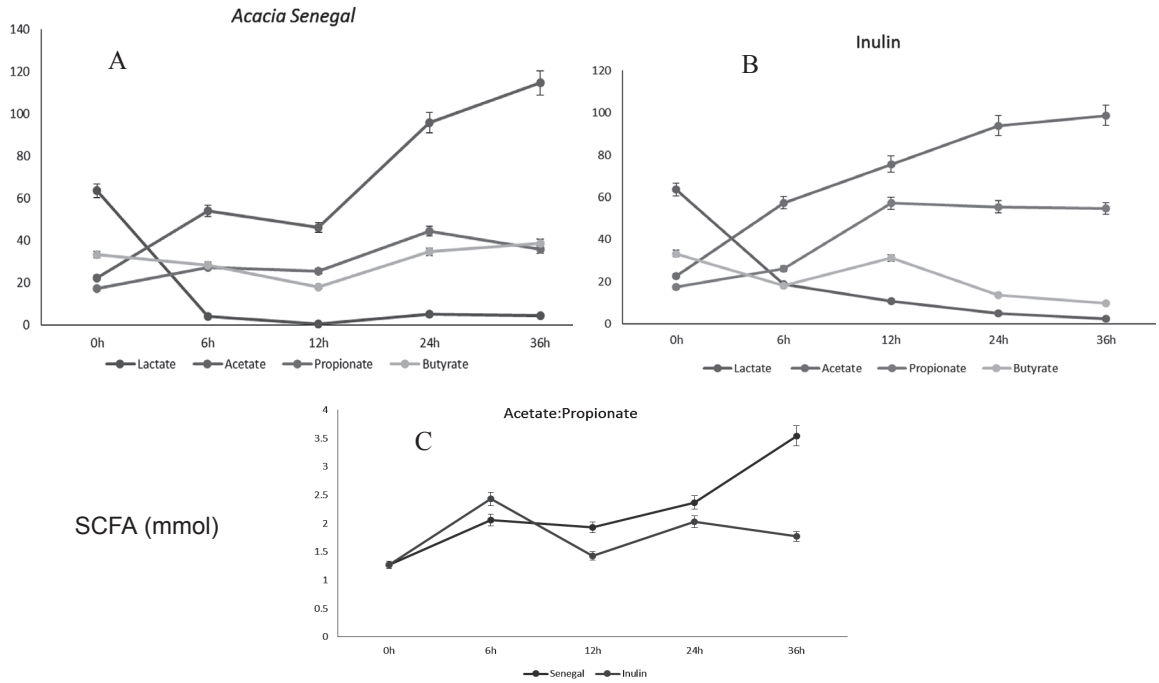

Fig 1: Cumulation production of SCFA (mmol)in pH-controlled batch cultures at 0,6,12 and 24 h (n = 4).

In Vitro Fermentation of *Acacia Senegal* by Fecal

No significant difference was observed in the amount of propionate and butyrate after fermentation of *Acacia Senegal.* Butyrate significantly decreased at *Acacia Senegal* fermentation at 6, 12 h but later elevate at 24, 36 h. No significant increase in butyrate was found in inulin. The butyrate produced may be due to the conversion from other SCFAs, i.e. acetate or lactate by cross-feeding.(Belenguer*et al.* 2006). The acetate to propionate ratio was dramatically increased ($P < 0.05$) by *Acacia Senegal*. Whereas, inulin slightly increased on the acetate to propionate ratio at 6h but later diminished at 12h. After that the ratio elevated with no significant different, this because of the changes on propionate concentration during the fermentation of inulin. The low acetate:propionate ratio may be of interest for regulating serum cholesterol concentrations, because acetate may act as a precursor for cholesterol synthesis, while propionate might inhibit this process (Delzenne. N.M &Kok N 2001; Wolever. *et al*.1995). Lactate production of *Acacia Senegal* and inulin were significantly decreased at all fermentation times from 0h to36h. This is because lactate may be further metabolised to acetate, propionate and butyrate by a number of cross-feeding bacteria.(Vernia. P *et al*. 1988; Sarbini*et al*. 2011).

Conclusion:

The results obtained from this study indicate that *Acacia Senegal* induced great modulation of *in vitro* faecal microbiota and comparable with inulin. *Bifidobacterium* spp and *Lactobacillus* ssp. growth was stimulated by *Acacia Senegal* throughout the fermentation resulting in high production of SCFA.

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