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Prebiotic efficiency of custard apple seeds

JK Pallavi*[1], J Arcot[2] and Usha Antony[3]

[1] Post doctoral Fellow (UGC PDFw),
Department of Biotechnology,
Anna University,
Chennai, India

[2] Associate Professor,
ARC Training Centre for Advanced Technologies in Food Manufacture, School of Chemical Engineering,
UNSW, Sydney, Australia

[3] Professor, Center for Food Technology,
Department of Biotechnology,
Anna University,
Chennai, India

***Abstract* - A comprehensive study on the seeds of custard apple was done to identify innate prebiotic potential. Among the different components of seeds, low molecular weight carbohydrates are good substrates for growth of probiotics as they are likely to have prebiotic activity. The high amount of crude fiber (28g/100g) identified from the proximate analysis of the seed was selected for further testing and analyses. The seeds were found to contain 9.6g/100g low molecular weight sugars, 23.26g / 100g soluble dietary fiber and 54.43g / 100g insoluble dietary fiber. These promising components were isolated, purified and tested *in vitro*. Prebiotic activity score was calculated to evaluate the prebiotic efficiency of the selected components. PAS were 1.91 for the soluble fiber fraction, 0.63 for the insoluble fiber fraction and 0.19 for the LMWC.**

Key Words - Custard apple seeds, prebiotic, low molecular weight carbohydrates, dietary fiber, prebiotic activity score

I. INTRODUCTION

According to Gibson and Roberfroid a prebiotic is "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 in the colon" [1]. Prebiotics are characterized by - (a) resistance to gastric acidity, hydrolysis by mammalian enzymes, and gastrointestinal absorption; (b) fermentation by intestinal microflora; and (c) selective stimulation of the growth and/or activity of intestinal bacteria associated with health and well-being. Chemically, they are oligo- and polysaccharides that are indigestible by the human digestive system and reach the intestine to stimulate the growth of beneficial bacteria over harmful ones. *Bifidobacteria* and *Lactobacillus* that share a commensal relationship with human hosts are considered beneficial and referred to as 'probiotics'. Since prebiotics act as energy substrates for probiotics and allow for their selective proliferation, they ensure a competitive advantage over the harmful ones like *Clostridia*.

Commonly studied prebiotics are fructooligosaccharides (FOS), galactooligosaccharides (GOS) and xylooligosaccharides (XOS) that are present in fruits and vegetables as structural / soluble components. Though they have natural sources of origin, they are being produced on a commercial basis. FOS are produced by transfructosylation of sucrose; GOS, by transgalactosylation of lactose; XOS, by chemical-enzymatic treatment of ligno-cellulosic materials of plant cells. Apart from these, there many low molecular weight carbohydrates (LMWC - kestose, nystose, fructosylnystose, etc.) that have promising function as prebiotics. Some of them are synthetically produced by transfructosylation of sucrose as a family of GF_n compounds.

With the onus on identification of natural / indigenous plant sources for prebiotics, research has taken up pace on these lines. Custard apples are considered super fruits, in that they possess many therapeutic and medicinal properties [2] and [3]. The seasonal fruit is underutilized in India, often consumed fresh and not processed. The present study was focused to identify the prebiotic potential in seeds that are generally thrown away as waste.

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II. MATERIALS AND METHODS

Custard apple fruits were collected from the local market, washed and seeds separated from the fruit. The seeds were washed and air dried. The seed coat was removed mechanically by peeling out and the peeled seed made into fine powder in a blender (particle size < 1 mm). DNS, sulfuric acid, hydrochloric acid, MRS broth and MRS agar were purchased from HiMedia (India); sodium hydroxide from Fisher Scientific; pepsin, pancreatin, bile salts and amylase from Sigma (USA); ethanol from Hayman (USA); amyloglucosidase from Megazyme (USA). The bacterial strains ATCC4356 (*L. acidophilus*) and MTCC728 (*E. coli*) were procured from the Institute of Microbial Technology (Chandigarh, India). Standard solutions and dilutions were prepared daily. For gas chromatography, milliQ water was used to prepare all the solutions. All experiments were done in triplicates.

A. Proximate Analysis of the seed:

For proximate analysis of the seed, standard procedures were followed to estimate moisture, protein, fat, crude fiber, total ash and acid insoluble ash [4]. Carbohydrates were estimated as the difference remaining after estimating all the above components.

B. Extraction of prebiotic components

Samples were defatted in petroleum ether (BP 60 °C – 80 °C) for 4 h and the residue was dried and saved until further use.

- LMWC: LMWC were extracted from the seeds according to the methods described in [5]. Ten gm of fine defatted seed powder was weighed (Shimadzu, UX420H) and 50 % ethanol was added just enough to cover the sample thoroughly. The extractions were carried out at 30°C with continuous shaking (60 rpm, Orbitek Shaker). After 72 h, the samples were filtered using a muslin cloth and the filtrate was collected. The solvent in the filtrate was evaporated in a rotary evaporator (Equitron, Evator). The extract that remained with the LMWC, was freeze dried in a lyophilizer (Scanvac) and stored at -20 °C until further use.

  The sugars in the LMWC were tested for their resistance to gastric digestion *in vitro* by the modified method of [6]. Four grams of the seed powder was taken in a conical flask and 40 mL distilled water was added. To this solution, 4 mL pepsin (0.576 g of pepsin powder in 12 mL of 0.1 M HCl) was added and kept in a shaker incubator at 37 °C for 2 h at 60 rpm. The pH was adjusted to 6.8 with I M NaOH and 8 mL of bile-pancreatin solution (0.12 g of pancreatin and 0.5 g of bile salts added in 30 mL of 0.1 M $NaHCO_3$) was added. 4 U of α-amylase (1 mg/mL in Phosphate Buffer Saline) was also added and incubated for 2 h at 37 °C with constant shaking (60 rpm). The solutions were centrifuged (3000 g, 15 min) in a REMI centrifuge and supernatants collected. The sugar concentration in the lyophilized LMWC powder (before digestion) and the supernatants collected above (after digestion) were determined using the modified DNS method [7].

  The specific sugar composition (Fructose oligomers – F_2, F_3, F_4, F_5, F_6, F_7; glucose-fructose oligomers – GF_2, GF_3, GF_4, GF_5, GF_6; individual sugars – glucose, fructose, galactose, lactose, sucrose and maltose) in the LMWC extract were identified by HPAEC-PAD (High Performance Anion Exchange Chromatography - Pulsed Amperometric Detection) at Eurofins Analytical Services India Private Limited, Bengaluru, India.

- Dietary Fiber: A suitable protocol was employed based on methods described by [8] and [9]. Five g of the seed powder was mixed with 20 mL distilled water. Pepsin, 4 mL (pH 1.5, 0.576 g of pepsin powder in 12 mL of 0.1 M HCl) was added and incubated for 1 h at 37 °C in a shaker incubator (60 rpm). The pH was adjusted to 6.8 with 1 M NaOH and 8 mL of α-amylase in phosphate buffer saline (1 mg/mL in PBS) was added to the mixture. The reaction was allowed for 16 h in the shaker incubator (60 rpm) at 37 °C. 80 µL amyloglucosidase (3260 U/mL) was added after adjusting the pH to 4.4 and allowed to react for 1 h at 60 °C. Whatman Filter paper (No.1) was used to separate the residue and filtrate. The residue was washed twice with 95% ethanol, air-dried and kept overnight in a hot air oven (70 °C). This fiber is the insoluble dietary fiber fraction (ISF). To the filtrate, 4 volumes of 95% ethanol were added and allowed to stand for 40 minutes. The precipitate was collected and air-dried. The resulting fine powder was the soluble fraction (SF). For estimating the total dietary fiber, the sample, after amyloglucosidase treatment was subjected to precipitation with 4 volumes ethanol, filtered and the

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precipitate was dried. The weight was corrected for undigested protein and ash.

- Resistant Starch: Resistant starch in the seeds was estimated by the method described in [10].

- Inulin: Concentration of inulin in the seeds was estimated by resorcinol method [11].

- Non-Starch Polysaccharides (NSP's): For the estimation of soluble and insoluble NSP, the protocol of Englyst was followed [12]. The sugars in the fractions were separately isolated, purified, acid hydrolyzed and derivatised. They were identified and quantified by gas chromatography with a Supelco SP-2380 wide bore capillary column (30 m x 0.53 mm i.d., d_f 0.20 µm) and a Flame Ionization Detector (Agilent, 7890A, USA) [13].

C. Estimation of the prebiotic potential

The prebiotic activity scores of the selected components - LMWC, SF and ISF were determined according to methods of Huebner et al., (2007). Prebiotic activity score ={(probiotic log cfu/mL on the prebiotic at 24h - probiotic log cfu/mL on the prebiotic at 0 h) / (prebiotic log cfu/mL on glucose at 24 h - probiotic log cfu/mL on the glucose at 0 h)} - {(enteric log cfu/mL on the prebiotic at 24 h - enteric log cfu/mL on the prebiotic at 0 h) / "("enteric log cfu/mL on glucose 24 h - enteric log cfu/mL on the glucose at 0 h")"}.

Accordingly, for 0 h and 24 h (overnight) growth count, cultures of *L. acidophilus* (ATCC4356) (in MRS broth, 37 °C, anaerobic) and *E. coli* (MTCC728) (in Luria broth, 37 °C, aerobic) supplemented separately with 1% glucose and 1% potential prebiotic were enumerated (using spread plate technique) on MRS agar (for *L. acidophilus*) and Luria agar (for *E. coli*) after appropriate serial dilutions to check for the bacterial growth count. The number of colony forming units (cfu) was enumerated with a Colony counter (Lapiz digital colony counter).

III. RESULTS AND DISCUSSION

Custard apple seeds were chosen to identify an economically viable and sustainable source for prebiotics. Reports are available on the composition of different parts of the fruit [3] and [14]. This is the first time that a detailed study on the individual components of the seeds is being reported with the specific aim of identifying prebiotics. A preliminary proximate analysis was necessary to understand the composition of the seed, the results of which are presented in Table 1. There are reports on the proximate composition of the peel and seed of in terms of ash, foreign matter and moisture content [14].

Though the carbohydrate fraction of the seed was less than 15 %, studies were done to identify the percentage composition of LMWC in this fraction because a range of oligosaccharides and monosaccharides are promising prebiotics. Many LMWC are not digested by the human body. The gut flora have specialized enzymes like β-fructosidase (that catalyzes breakdown of FOS) that facilitate utilization of these sugars.

Since a high amount of crude fiber (~ 28 %) was identified in the seeds, a detailed study on dietary fiber and the indigestible fiber fractions was pursued. Dietary fiber is approximately one-fifth to one-half of the total crude fiber content in plants. The American Association of Cereal Chemists (2001) defined dietary fiber as, "the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. Dietary fiber includes polysaccharides, oligosaccharides, lignin, and associated plant substances. Dietary fibers promote beneficial physiological effects including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation." [15].

TABLE 1. PROXIMATE COMPOSITION OF CUSTARD APPLE SEED

Parameter	**Percentage (w/w)[a]**
Moisture	28.85 ± 0.05
Total ash content	1.82 ± 0.12
Acid insoluble ash	0.14 ± 0.07
Total fat	15.12 ± 0.03
Protein	11.95 ± 0.02
Crude fiber	27.88 ± 0.06
Carbohydrates (by difference)	14.24 ± 0.04

[a]The individual components of the custard apple seeds are presented as their percentage composition with standard deviation for three replications.

Dietary fiber has been classified in many ways over the years [16], [17] and [18]. The most commonly accepted and physiologically useful way of classification is based on their solubility in water – soluble fiber and insoluble fiber. Soluble

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fiber is easily dissolved in water and rapidly utilized by the bacteria. It consists of non-cellulosic polysaccharides like hemicelluloses, pectin and oligosaccharides. Insoluble fiber has its role in fecal bulking and consists of cell wall components like cellulose, hemicelluloses, pectin and lignin.

Inulin type prebiotics are members of a larger group called 'fructans' (polymers in GF_n) and because of their β (2,1) linkages, they are not digested by the human digestive system. Since inulin and oligofructose reach the colon for selective utilization by the gut flora, they can be considered as soluble dietary fiber [19]. Resistant starch includes starches that are incompletely digested / partially hydrolyzed in the small intestine and reach the large intestine to become substrates for probiotics; and are also classified as soluble dietary fibers [20].

There is a concept of "indigestible fraction" consisting of resistant starch, pectin, lignin, cellulose, hemicelluloses and residual proteins and minerals that reach the gut to provide a beneficial effect on the host by positively altering the growth of bacteria [8]. NSPs are a group of non-α- glucan polysaccharides of the plant cell wall. Insoluble NSP include cellulose, galactomannans, xylans, xyloglucans, and soluble NSP consist of pectins, arabinogalactans, arabinoxylans, and β-(1,3)(1,4)- D-glucans (β-glucans) [21].

In the present study, promising prebiotics with better yield - LMWC, SF and ISF were isolated from custard apple seeds using non destructive methods and subsequently analyzed (Table 2). LMWC were extracted from the seeds using 50% ethanol as solvent. Three different concentrations of ethanol (50%, 85% and 95%) using different durations and temperatures were examined to identify the best possible settings for extraction.

In the present study, though the yield of LMWC was low (9.6%), it was the highest achievable when 50 % ethanol was utilized as a solvent at 30 °C. The individual sugars and oligosaccharides in the LMWC were depicted by the HPAEC-PAD analysis and negligible amounts of oligomers and monomers were identified (Table 3).

Prebiotics are fermented by probiotics into lactic acid and other short chain fatty acids (SCFA's like acetic acid, butyric acid and propionic acid) through saccharolytic fermentation. The SCFA's are then easily absorbed by the host as energy sources. So, it is essential that the carbohydrates reach the intestine after escaping gastric digestion. A study was therefore conducted to determine the *in vitro* digestibility of the LMWC prior to estimating its prebiotic potential. The amount of reducing sugar (% w/w) in the LMWC before and after *in vitro* digestion of the extract was 35.5 ± 3.9 and 26.7 ± 2.2 respectively. This meant that more than 70 % of the sugars in LMWC were found to be resistant to *in vitro* acid-bile digestion, suggesting their prebiotic potential. Prebiotic components extracted from various parts of fruits and vegetables (banana, jackfruit, potato, etc.) were studied for their resistance to *in vitro* digestibility [5].

TABLE 2. PERCENTAGE COMPOSITION OF PREBIOTIC COMPONENTS

Parameter	**Percentage (w/w)**
Total LMWC	9.6 ± 1.5
Total dietary Fiber (TDF)	78.5 ± 0.6
Soluble Dietary Fiber (SF)	23.3 ± 0.5
Insoluble Dietary Fiber (ISF)	54.4 ± 0.9
Resistant Starch	2.5 ± 0.1
Inulin	1.8 ± 0.1

TABLE 3. INDIVIDUAL SUGAR COMPOSITION OF THE LMWC FROM HPAEC-PAD (LOQ 0.1 for individual sugars and 0.2 for the oligomers)

Sugar	**Percentage (w/w)**
Fructose oligomers F_2, F_3, F_4, F_5, F_6, F_7	Each < 0.2
Glucose-Fructose oligomers GF_2, GF_3, GF_4, GF_5, GF_6	Each < 0.2
Galactose	< 0.1
Glucose	< 0.1
Lactose	< 0.1
Maltose	< 0.1
Fructose	0.69
Sucrose	0.95

The largest component of the seed was total dietary fiber (Table 2). The amount of the ISF in the seed was much higher (~ 55%) than SF (~ 24%). Since the seed coat was removed while performing these studies, it can be understood that approximately 80% of the peeled seed contained dietary fiber. This reinstates that custard apple seeds are a good natural source of dietary fiber. Resistant starch and inulin were also identified in the seeds, but at much lower levels and since they fall into the category of soluble dietary fiber, individual analysis of their

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prebiotic efficiency was not performed in this study. Much of the reported work is on the edible portion/pulp of fruits and there are very few studies on the fibre components from the seeds of fruits. The dietary fiber content in the custard apple fruit, but not the seed has been reported [22].

The ability of a substrate to be efficiently metabolized by the beneficial bacteria in comparison to the harmful ones is the key factor to recognize the substrate as a prebiotic. The prebiotic activity score enables the quantification of the prebiotic potential of substrates [23]. The present study could identify the individual prebiotic potential of the LMWC, SF and ISF isolated from the seeds of custard apple. The fractions could be collected immediately without any loss, in the purest form to facilitate direct addition as an active carbon source in the bacterial growth medium (without negatively hindering the growth of the bacterial species under study). The structure-function relationship was elucidated in the specific components and how they could affect the prebiotic activity when isolated from the food matrix. Growth of the bacteria on glucose was used as a standard to allow for comparison.

From the bacterial cell growth (Table 4), it can be assumed that soluble fiber, insoluble fiber and LMWC are all being utilized by *Lactobacillus* efficiently. The ability of all the three fractions to promote the growth of *L. acidophilus* was better than that of *E. coli*. This data was used to calculate the prebiotic activity score (PAS). The prebiotic activity score of soluble fiber fraction was 1.91, insoluble fiber fraction 0.63 and LMWC was 0.19. A positive prebiotic activity score indicated that the substrates are being utilized by the probiotic as efficiently as or better than glucose and are therefore potential prebiotics. A negative score would indicate that they are being underutilized by the probiotic or are being better utilized by the negative control (pathogenic strains).

With respect to LMWC, though the rate of growth of *L. acidophilus* was much higher in comparison to *E. coli*, the limited amount of 'available' low molecular weight sugars (added as a carbon source) into the medium could have affected the duration of log phase, and therefore the prebiotic activity score. Therefore, the low yield can probably explain the low PAS for LMWC. Similar studies on the prebiotics like LMWC isolated from germinated rice, garlic, etc were conducted [24]. Though the prebiotic score of insoluble fiber was positive, the data from Table 4 suggests that there is only a marginal difference between the rate of growth of *E. coli* and *L. acidophilus* with ISF as the carbohydrate source. When soluble fiber was the substrate, the rate of growth of *L. acidophilus* was much higher when compared to *E. coli*. So, in comparison, soluble fiber fraction would be a preferred prebiotic over the insoluble fiber fraction.

An exploration of the individual sugars in the NSP fractions from custard apple seeds was done through a GC-FID study [13]. The study was indicative as it allowed identification and quantification of six monomers (xylose, fucose, arabinose, glucose, arabinose and mannose) in the total and insoluble NSP fractions (results not shown). However, a further detailed analysis of all other individual sugars of the fractions is necessary for a complete understanding of the complex fiber profile of custard apple seeds.

Different prebiotics are utilized differently by the probiotics and it is important to establish that a particular prebiotic is being effectively utilized. The present study was able to quantify the prebiotic utilization in terms of bacterial cell growth over a period of 24 h. A future study on fermentation of LMWC and individual dietary fiber fractions by *Lactobacilli* to produce SCFA would definitely corroborate the present findings to establish the substrates from custard apple seeds as prebiotics.

TABLE 4. BACTERIAL GROWTH IN THE CARBOHYDRATE FRACTIONS (log cfu/mL)

Carbohydrate	***E. coli***		***L. acidophilus***	
	0 h	**24 h**	**0 h**	**24 h**
Glucose[a] (standard)	7.68±0.03	8.69±0.02	7.98±0.02	9.31±0.03
Soluble Fiber	7.04±0.02	8.07±0.01	7.27±0.01	9.84±0.02
Insoluble Fiber	7.42±0.13	8.10±0.09	7.16±0.14	8.90±0.11
LMWC	6.91±0.09	7.51±0.07	7.01±0.03	8.06±0.04

[a] Growth on glucose was used as a reference.

IV. CONCLUSION

There is a huge potential for the studies on prebiotics derived from plant sources as they have a natural origin and traditional knowledge associated with them that encourages end user preference over commercial products. A non-destructive approach was successfully used for extraction of dietary fiber fractions from the seeds of custard apple. Further, the study was able to identify the prebiotic efficiency of the fractions and

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establish that custard apple seeds are a sustainable natural source of prebiotics.

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