COMPARISON OF FERMENTATIVE PROPERTIES IN RAW AND BOILED LEGUMES AFTER SIMULATED DIGESTION

Running title: Simulated digestion and prebiotic potential

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This study compared fermentative properties of selected raw and boiled legumes before and after simulated digestion. Mung bean, two cowpea cultivars (Waruni and Dhawala), horse gram and chickpea were subjected to *in-vitro* digestion using swine gastric and intestinal juices and then subjected to fermentation using swine cecal bacteria. Simulated digestion reduced ($P<0.05$) prebiotic potential in all legume substrates except boiled; mungbean, Waruni, chickpea, Dhawala and raw; Dhawala and horsegram. Digested boiled chickpea showed higher ($P<0.05$) prebiotic potential compared to all digested boiled legumes except horsegram and Waruni. Digested boiled chickpea suppressed ($P<0.05$) Coliform growth compared to digested boiled mungbean, Dhawala and horsegram. Digested boiled mungbean and Waruni showed higher ($P<0.05$) prebiotic potential compared to their digested raw samples. Digested boiled chickpea, horsegram and Dhawala suppressed ($P<0.05$) the coliform growth than their raw digested samples. Findings displayed that simulated digestion differently modulated the fermentative properties of both raw and boiled legumes.

Keywords; Legumes, *In-vitro* Digestion, *In-vitro* Fermentation, boiled, raw, prebiotic potential

Practical Application

This study compares the fermentative properties of selected raw and boiled legumes subjected to simulated digestion. Findings would be useful in making dietary guidelines and developing functional food products for a healthy life. Even after simulated digestion chickpea showed highest prebiotic potential and could be considered as the best prebiotic legume for developing prebiotic food. Boiled legumes may be more suitable in preparing prebiotic foods than their raw powders.

INTRODUCTION

The gastrointestinal microbiota plays an important role in modulating metabolic, immunologic and protective function of human body (Holscher, 2017). Consumption of variety of dietary fibers and resistant starches is a good dietary strategy for modulating compositional variability as well as the metabolic activity of the intestinal microorganisms (Holscher, 2017). Prebiotics found in natural food sources are non-digestible carbohydrates such as resistant starch (RS), galacto-oligosaccharides (GOS), fructo oligosaccharides (FOS), xylo oligosaccharides (XOS), pectic oligosaccharides (POS), and various oligosaccharides that provide carbohydrates fermentable by the beneficial colon micro-organisms (Gómez, Gullón, Yáñez, Schols, & Alonso, 2016, Scott, Martin, Duncan, & Flint, 2014). The metabolic end products which are synthesized in the lower part of gastrointestinal tract during fermentation and the bacterial populations present vary with respect to the nature and the amount of non-digestible carbohydrate sources ingested(Rowland et al., 2018). Generally, fermentation of non-digestible carbohydrates, proteins and fibers in large intestine will produce beneficial as well as non-beneficial compounds which could affect the bowel health. Short chain fatty acids (SCFA) such as acetic, propionic, and butyric are among them which confer various health benefits to the human beings (Aquino et al., 2017,Rowland et al., 2018).Probiotic bacterial species, mainly *Bifidobacterium* and *Lactobacillus* are right behind the production of these short chain fatty acids mostly due to fermentation of dietary oligosaccharides which are identified as prebiotics (Fernando et al., 2010). Furthermore, fermentation process by beneficial micro-organisms are important in reducing the population of pathogenic microorganisms in the gut due to the significant reduction of colonic pH (Campbell, Fahey and Wolf, 1997,Jailane et al, 2017).

Legumes have been identified as a good source of prebiotic carbohydrates for improving gastrointestinal health. Among legumes; chickpea and mungbean are widely consumed in Asian countries while cowpea and horsegram are utilized minimally. These legumes are consumed mainly after boiling and some are used as raw powder in food product preparations. It is well known that the processing and the, gastrointestinal digestion modulate fermentative properties of food (Singh, 1988,Capuano, 2017). To our knowledge, comparative information on prebiotic activity of raw and boiled legumes after *in vitro* enzymatic digestion is scarce and this information is vital in developing functional food with desired nutritional properties. Hence, the main objective of this study is to focus on the fermentative properties of selected five types of raw and boiled legumes in Sri Lanka (mungbean, two cowpea cultivars (Waruni, Dawala), chickpea and horsegram) after *in-vitro* enzymatic digestion.

3.0 MATERIAL AND METHODS

3.1 Materials

Fresh and non-fumigated legume seeds (Mung bean (*Vigna radiate L*), Dhawala &Waruni cowpea (*Vigna unguiculata L*) were purchased from Government Seed Centre, Dambulla, Sri Lanka. Horse gram (*Macrotyloma uniflorum)* was purchased from Grain Legumes and Oil Seed Crops Research and Development Centre, Agunakolapelessa (GLOSCRD), Sri Lanka. Chickpea (*Cicer arietinum L*) was purchased from Kandy, Sri Lanka. Seeds were manually selected to remove foreign material and damaged once prior to the experiment. The selected legume seeds were stored at -4 °C until use. Bifidobacterium AGAR (HiMedia Laboratories PVT, Ltd., India), M.R.S. AGAR (OXOID LTD, England) and MacConkey AGAR (OXOID LTD, England) were used for culturing of Bifidobacterium, Lactobacillus and Coliform bacterial species, respectively. Peptone water solution (M028-100G HIMEDIA peptone water, HiMedia Laboratories Pvt, Ltd., India) was used to prepare serial dilutions from fermented solution. All the other chemicals used were of analytical grade and purchased from Sigma-Aldrich (Sigma, St. Louis, USA) unless otherwise stated.

3.2 Methods

3.2.1 Sample preparation

Legume seeds were soaked overnight prior to boil and the amount of water and the time duration required for boiling depend on the type of legume species as shown in the Table 1. Boiled and raw seeds were ground using a grinder. Then the samples were pre dried at 60°C using a drying oven (Mermmert™ VO200, Germany) and packed in polythene bags separately and stored in a desiccator for further analysis.

3.2.2 *In-vitro* digestion of legume samples

3.2.2.1 Collection of gastric and intestinal juices

Gastric juice and intestinal juice were collected from slaughter house of Mawelawattha livestock field station, Faculty of Agriculture, University of Peradeniya, Sri Lanka. Stomach and intestinal content of slaughtered pigs were squeezed out to collect gastric and intestinal juice and filtered through a clean muslin cloth. A cool box was used to store collected samples during transportation. Juices were stored at -20°C until process.

3.2.2.2 Preparation of gastric enzyme solution

Collected gastric juice was centrifuged (LEGEND XIR Centrifuge, Germany) at 1250 rpm for 10 minutes at 5°C (Furuya et al., 1979). Then the supernatant was collected and stored at -20°C refrigerator until use.

3.2.2.3 Preparation of intestinal enzyme solution

Collected intestinal juice was centrifuged using (LEGEND XIR Centrifuge, Germany) at 4500rpm for 10 minutes at 5°C (Furuya et al., 1979).Then the supernatant was collected and stored at -20°C refrigerator until use.

3.2.2.4 Digestion of legumes

Legume samples were digested according to the methods described by (Li, Feng, Xu, & Yang, 2004) and (Furuya et al., 1979) with minor modifications. 2g of each ground legume sample was measured (RADWAG WagiElekroniczne, Poland) into 100 ml conical flask, to which 20 ml of gastric juice was added and its pH was adjusted into a range of 4.0-4.6 using a pH meter (HANNA Instrument Inc, Woonsocket) and incubated with shaking at 100rpm in a shaking incubator (THZ-100 Shaking Incubator, Biocotek, China) for 4 hrs at 37°C. At the end of the first incubation period, the contents were neutralized with 1M sodium hydroxide. In the second stage, 20 ml of intestinal fluid prepared as mentioned previously was added, pH was adjusted (6.9-7.4) and the digestion mixtures were incubated in shaking incubator for additional 4 hrs at 37°C at 100rpm. At the completion of the second incubation the contents of the flask were transferred to centrifuge-tubes and centrifuged (LEGEND XIR Centrifuge, Germany) immediately at 1250 g for 10 minutes at 5°C. The supernatant fraction was discarded. The precipitate was mixed with a little water and filtered through a filter paper (Toyo-Roshi No. 5A, Toyo-Roshi, Tokyo, Japan) and oven dried(Mermmert™VO200 , Germany) at 60°C and stored in -20°C refrigerator until use.

3.2.3 *In-vitro* fermentation of legume samples

3.2.3.1 Preparation of bacterial pellets for fermentation study

Ceca were collected from two healthy swine of slaughter unit of Mawelawattha Livestock field station, Department of Animal Science, Faculty of Agriculture, University of Peradeniya, Sri Lanka. Two healthy swine which had not received any antibiotic treatment within last 3 months were selected and slaughtered after 16 hours fast, ceca were quickly removed and taken into the National Institute of Fundamental Studies by maintaining anaerobic conditions.25g of cecal content was measured by an electronic weighing balance (RADWAG WagiElekroniczne, Poland) and dissolved in 250ml of phosphate buffer (pH 6.2±0.2 pH). Then cecal solution was filtered twice using a pre sterilized muslin cloth. Bacterial pellets were obtained by centrifuging using (LEGEND XIR Centrifuge, Germany) the filtrate at 5°C for 15 minutes at a speed of 10000rpm. Bacterial pellets were re suspended in 250ml of phosphate buffer (pH 6.2±0.2pH).

3.2.3.2 Fermentation of legume samples

0.5g of digested legumes were accurately measured using an electronic weighing balance (RADWAG WagiElekroniczne, Poland) and added to the vacutainer tubes.8ml from bacterial pellet solution was added into those vacutainers and exposed to a continuous CO_2 flow as described by (Calabrò et al., 2009). Vacutainers were sealed properly and incubated using Mermmert incubator (Mermmert™ IN160, Germany) at 37°C for 4 hours.

3.2.3.3 Microbial growth analysis

Total anaerobe bacterial counts for each legume fermented with bacterial pellets were taken as previously described by spread plate method (Garcha, Katyal, & Sharma, 2016). Fermented legume samples were serially diluted with peptone water and inoculated on plates prepared from Bifidobacterium AGAR, M.R.S. agar and MacConkey agar. All the plates were placed in Mermmert incubator (Mermmert™ IN160, Germany) under anaerobic conditions at 37°C. Plates having bacterial colonies within a range of 25-250 were selected for calculations.

$$\frac{\text{CFU}}{\text{ml}} = \text{Number of Colonies} \times \frac{1}{(\text{Dilution factor})} \times \frac{1}{(\text{innoculated volume})}$$

3.3 Statistical analysis

Data were analyzed using SAS software package (SAS institution Inc., 2003, Cary, USA). Three-Way ANOVA was used for the analysis. Means separations were analyzed by Duncan's Multiple Range Test and Dennet's Test at $\alpha = 0.05$. Following statistical model was used.

$$Yijkl = \mu + \alpha i + \varepsilon j + \gamma k + \alpha\varepsilon ij + \alpha\gamma ik + \varepsilon\gamma jk + \alpha\varepsilon\gamma i$$

4.0 RESULTS

4.1 The effect of *in-vitro* digestion and boiling on *Bifidobacterium* fermentation in legumes

4.1.1 *In vitro* digestion and *Bifidobacterium* fermentation

In vitro digestion has significantly reduced (P<0.05) the *Bifidobacterium* fermentation in boiled chickpea, boiled horsegram ,boiled Dhawala ,raw chickpea, raw mung bean and raw Waruni samples (Table 2)compared to their respective undigested samples. Digested raw horsegram showed a significantly high (P<0.05) *Bifidobacterium* fermentation ability compared to digested raw mungbean, Waruni and Dhawala. Digested boiled chickpea and digested boiled Waruni samples showed significantly higher (P<0.05) *Bifidobacterium* fermentation compared to digested boiled Mungbean and Dhawala. There was no significant effect (P<0.05) of *in vitro* digestion on *Bifidobacterium* fermentation in boiled mungbean and boiled Waruni.

4.1.2 Boiling and *Bifidobacterium* fermentation

Both digested and undigested boiled chickpea and horsegram showed significantly low (P<0.05) *Bifidobacterium* growth compared to their respective raw samples (Table 2).Whereas, digested boiled mungbean and digested boiled Waruni cowpea samples showed significantly high (P<0.05) *Bifidobacterium* growth compared to their digested raw samples. Boiling has reduced the *Bifidobacterium* fermentation in chickpea and horse gram whereas boiling has improved the *Bifidobacterium* fermentation in mung bean and Waruni cowpea samples.

4.2 The effect of *in-vitro* digestion and boiling on *Lactobacillus* fermentation in legumes

4.2.1 *In vitro* digestion and *Lactobacillus* fermentation

Digestion significantly reduced (P<0.05) the *Lactobacillus* fermentation in both raw and boiled Mungbean and Waruni and boiled horsegram and raw chickpea (Table 3) compared to their undigested samples. Digested boiled and undigested raw chickpea showed significantly higher (P<0.05) *Lactobacillus* fermentation properties compared to other digested boiled legumes and undigested raw legumes. However, digested raw chickpea showed significantly lower *Lactobacillus* fermentation properties compared to other raw digested legumes.

4.2.2 Boiling and *Lactobacillus* fermentation

Lactobacillus fermentation (Table 3) was significantly ($P<0.05$) high in undigested boiled horsegram, mungbean and Waruni samples and in digested boiled chickpea and mungbean compared to their respective raw samples. Digested boiled chickpea showed the highest ($P<0.05$) *Lactobacillus* fermentation ability and digested boiled horsegram showed the lowest ($P<0.05$) fermentation ability compared to other digested boiled legumes.

4.3 The effect of *in-vitro* digestion and boiling on Coliform fermentation in legumes

4.3.1 *In vitro* digestion and coliform fermentation

Digestion has significantly increased ($P<0.05$) the Coliform fermentation (Table 4) in both raw and boiled chickpea while it has significantly reduced ($P<0.05$) the coliform fermentation in raw and boiled horse gram, Waruni and Dhawala. Both raw digested and raw undigested horse gram showed the significantly highest ($P<0.05$) coliform fermentation compared to other respective legume samples.

4.3.2 Boiling and Coliform fermentation

Coliform fermentation was significantly ($P<0.05$) low (Table 4) in both undigested and digested boiled chickpea and horsegram. Whereas, Coliform fermentation was significantly increased ($P<0.05$) in undigested boiled Waruni samples and digested and undigested boiled Dhawala samples. Digested boiled chickpea and Dhawala samples showed lowest ($P<0.05$) Coliform fermentation compared to other digested boiled legume samples. Thus, digested boiled chickpea and Dhawala cowpea showed better prebiotic properties in terms of coliform fermentation.

4.2 The effect of *in vitro* digestion and fermentation on pH

Digestion has significantly increased ($P<0.05$) the pH of both raw and boiled fermented legume samples (Table 5). Lowest pH ($P<0.05$) was observed in undigested boiled chickpea sample. All the legume samples had a lower ($P<0.05$) pH value compared to control. Fermentation has decreased the initial pH of all the legume samples.

5.0 Discussion

Any dietary material that is not digestible by gastric mammalian enzymes and enters the large intestine are prebiotics (Gibson et al., 2010). In this study before doing *in vitro* fermentation, legumes samples were subjected to simulated gastrointestinal digestion to investigate the true effectiveness as prebiotic candidates. Findings of this study have shown that *in vitro* digestion modulated the prebiotic potential of both raw and boiled legumes. Structural and compositional differences in dietary substrates could be the reason behind the differential growth pattern of microorganisms. In fact, observed differences in fermentative properties among different legumes in this study could be due to the differences in prebiotic carbohydrate concentrations among legumes (Siva, Thavarajah, Kumar, & Thavarajah, 2019). Simulated digestion reduced ($P<0.05$) prebiotic potential in all the legume substrates except boiled; Mungbean, Waruni cowpea, chickpea, Dhawala cowpea and raw; Dhawala and horsegram showing that true prebiotic potential is lower in most of the studied legume samples after gastrointestinal digestion.

Bifidobacterium fermentation was significantly high in digested boiled chickpea and digested boiled Waruni cowpea compared to digested boiled mung bean and digested boiled Dhawala cowpea showing that boiled chickpea and boiled Waruni cowpea are better prebiotic candidates than boiled mung bean and boiled Dhawala cowpea. Digested boiled chickpea showed higher ($P<0.05$) prebiotic potential compared to all digested boiled legume substrates except horsegram and Waruni cowpea and this may be due to the presence of readily fermentable components in chickpea which escape pepsin and pancreatin digestion (Woyengo, Jha, Beltranena, & Zijlstra, 2017). It has been shown that chickpea contains significantly higher amount of polyols,: a group of low digestible carbohydrate compared to peas, beans and lentils(Moussou et al., 2017) which may be another reason for rapid fermentation shown by chickpea compared to other legumes. Further, abundantly available α-galactosides and oligosaccharide; ciceritol in chickpea, may have simulated the growth of probiotic bacteria and inhibited the pathogenic bacteria as shown previously (Muzquiz et al., 2012)(Dai et al., 2017). Further, the higher content of oligosaccharides present in cotyledon fractions of horsegram seeds could be the reason for observed higher prebiotic capacity in horsegram (Prasad & Singh, 2015). Digested boiled mungbean and Waruni cowpea showed higher ($P<0.05$) prebiotic potential compared to their digested raw samples showing that boiled mung bean powder and boiled Waruni cowpea powder are more suitable in preparing

prebiotic foods than their raw powder. Higher prebiotic potential in digested boiled mung beans compared to digested raw mung beans may be due to the increase in soluble fiber composition upon boiling (Liyanage et al., 2018). Moreover, the increase in resistant starch content with the retro gradation of legume flours after boiling may modulate the growth of prebiotic bacteria (Dangsungnoen, Moongngarm, & Deeseenthum, 2012).

Higher prebiotic potential in chickpea was further supported by lower ($p<0.05$) coliform population showing that chickpea was the best prebiotic candidate compared to other legume substrates (Akillioglu & Karakaya, 2010). Digested boiled chickpea, Waruni cowpea, mungbean and horsegram showed lower ($P<0.05$) coliform growth compared to their respective digested raw samples showing that boiling has suppressed ($P<0.05$) the coliform growth and improved the prebiotic potential. However, digested raw Dhawala and raw horsegram showed better prebiotic potential than their digested boiled samples showing that some raw legumes show better prebiotic properties than when they are boiled. This study displayed that simulated digestion differently modulated the fermentative properties of both raw and boiled legumes.

6.0 CONCLUSION AND RECOMMENDATIONS

Among all the studied legume samples boiled chickpea could be considered as the best prebiotic candidate in terms of both *Bifidobacterium* and *Lactobacillus* growth. Boiled mungbean, and boiled Waruni cowpea are more suitable than their raw forms in preparing prebiotic food.

7.0 ACKNOWLEDGEMENT

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Table 1. Requirements for boiling of different legume seeds

Legume seed	W/V ratio	Time period(minutes)
Mungbean	1: 3	30
Waruni	1: 6	30
Dhawala	1: 6	30
Horsegram	1 :7	47
Chickpea	1: 5	40

Table 2.The effect of *in vitro* Digestion and fermentation on Cecal *Bifidobacterium* growth (log CFU/ml)

	Bifidobacterium			
Substrate	Undigested		Digested	
	Raw	Boiled	Raw	boiled
Chickpea	8.55±0.01^{a}	8.43±0.01^{a}	8.15±0.04^{ab}	8.04±0.05^{a}
Horse gram	8.30±0.01^{b}	8.09±0.03^{b}	8.30±0.02^{a}	7.90±0.04^{ab}
Mung bean	7.73±0.09^{c}	7.89±0.04^{c}	7.56±0.15^{c}	7.78±0.03^{b}
Waruni	8.30±0.02^{b}	8.04±0.02^{b}	7.53±0.02^{c}	7.98±0.06^{a}
Dhawala	7.83±0.02^{c}	7.83±0.04^{c}	8.01± 0.05^{b}	7.18± 0.09^{c}
Control	6.40±0.17^{d}	6.40±0.17^{d}	6.40±0.17^{d}	6.40±0.17^{d}

Values are expressed as means±SD.
Mean values within a column with lowercase superscript letters are significantly different at P<0.05.
Mean values within a raw with different uppercase superscript letters are significantly different at P<0.05.

Table 3. The effect of *in vitro* Digestion and fermentation on Cecal *Lactobacillus* growth (log CFU/ml)

Substrate	**Undigested**		**Digested**	
	Raw	Boiled	Raw	Boiled
Chickpea	8.80±0.58^{a}	7.89±0.60^{a}	6.82±0.03^{d,}	8.32±0.01^{a,ab}
Mung	7.61±0.03^{b}	7.83±0.02^{a}	7.26±0.04^{b}	7.71±0.00^{b}
Horse gram	7.71±0.02^{b}	8.27±0.54^{a}	7.22±0.04^{b}	7.32±0.01^{d}
Dhawala	7.67±0.01^{b}	7.65±0.01^{a}	7.25±0.01^{b}	7.70±0.01^{b}
Waruni	7.60±0.01^{b}	7.83±0.02^{a}	7.35±0.04^{a}	7.40±0.01^{c}
Control	7.00±0.03^{c}	7.00±0.03^{b}	7.00±0.03^{c}	7.00±0.03^{e}

Values are expressed as means±SD.
Mean values within a column with lowercase superscript letters are significantly different at P<0.05.

Mean values within a raw with different uppercase superscript letters are significantly different at P<0.05.

Table 4. The effect of *in vitro* Digestion and fermentation on Cecal Coliform growth (log CFU/ml)

Substrate	Undigested		Digested	
	Raw	Boiled	raw	boiled
Horse gram	7.89±0.01^{a}	7.70±0.004^{a}	7.78±0.01^{a}	7.18±0.03^{b}
Dhawala	7.63±0.03^{b}	7.70±0.01^{a}	6.82±0.02^{d}	6.97±0.03^{c}
Mung bean	7.36±0.31^{c}	7.68±0.17^{b}	7.26±0.01^{c}	7.16±0.09^{b}
Waruni	7.54±0.01^{bc}	7.68±0.04^{a}	7.39±0.09^{b}	7.33±0.02^{a}
Chickpea	6.94±0.04^{d}	6.87±0.02^{c}	7.36±0.02^{b}	6.70±0.05^{c}
Control	6.38±0.07^{e}	6.38±0.07^{d}	6.38±0.07^{e}	6.38±0.07^{d}

Values are expressed as means±SD.
Mean values within a column with lowercase superscript letters are significantly different at P<0.05.
Mean values within a raw with different uppercase superscript letters are significantly different at P<0.05.

Table 5. pH of Fermented Legume Samples

Substrate	Undigested		Digested		Control
	raw	boiled	Raw	boiled	
Chickpea	4.94±0.05^{d}	3.84±0.01^{e}	5.80±0.01^{c}	5.93±0.01^{b}	6.15±0.01^{a}
Mung	5.45±0.04^{d}	4.79±0.01^{e}	5.85±0.01^{b}	5.85±0.09^{c}	6.15±0.01^{a}
Dhawala	5.40±0.01^{d}	4.65±0.07^{e}	5.77±0.01^{c}	5.79±0.02^{b}	6.15±0.01^{a}
Waruni	5.11±0.01^{d}	5.44±0.02^{c}	5.83±0.01^{b}	5.79±0.01^{b}	6.15±0.01^{a}
Horse Gram	5.66±0.03^{d}	4.74±0.01^{e}	5.76±0.01^{c}	5.87±0.02^{b}	6.15±0.01^{a}

Values are expressed as means±SD. Mean values within a raw with different superscript letters are significantly different at P<0.05.