RESEARCH ARTICLE

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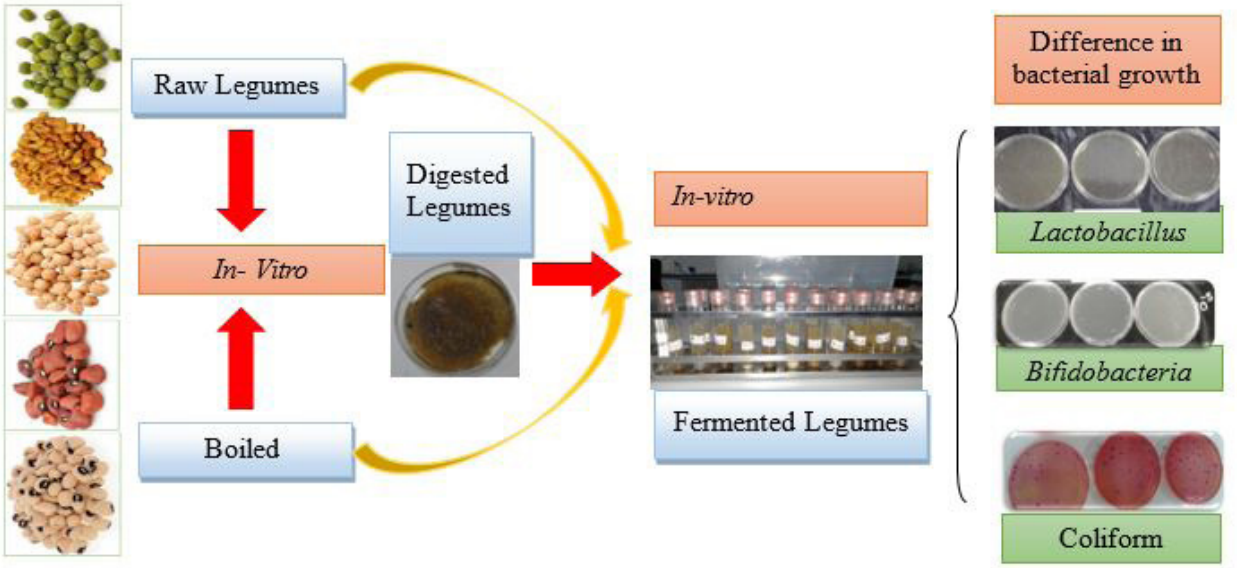

Highlights

- Chickpea showed the highest prebiotic potential.
- Simulated digestion differently modulated prebiotic potential of legumes.
- Boiled legumes showed better prebiotic potential than their raw powders.

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Comparison of fermentation properties in raw and boiled legumes after simulated digestion

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Abstract: This study compared the fermentation properties of selected raw and boiled legumes after simulated digestion. Raw and boiled; mung bean, two cowpea cultivars (*Waruni and Dhawala*), horse gram, and chickpea were subjected to simulated digestion and fermentation using swine gastric and intestinal juices and cecal bacteria. Simulated digestion significantly ($p < 0.05$) reduced prebiotic potential of some legume substrates except boiled; mung bean, *Waruni*, chickpea, *Dhawala*, and raw; *Dhawala* and horse gram. Digested residues of boiled chickpea, horse gram, and *Waruni* showed significantly ($p<0.05$) higher Bifidobacterium proliferation ability when compared to digested residues of boiled mung bean and *Dhawala*. Even after simulated digestion, chickpea showed the highest ($p<0.05$) prebiotic potential compared to other legumes. Findings displayed that simulated digestion differently modulated the fermentation properties of both raw and boiled legumes. In conclusion, boiled legumes are more suitable than their raw powders for preparing prebiotic foods.

Keywords: Legumes; digestion; fermentation; boiled; raw; prebiotic.

INTRODUCTION

The gastrointestinal microbiota plays a vital role in modulating metabolic, immunologic, and protective functions of the human body (Holscher, 2017). Consumption of a variety of dietary fibers and resistant starches is an excellent 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), galactooligosaccharides (GOS), fructooligosaccharides (FOS), xylooligosaccharides (XOS), pectic oligosaccharides (POS), and various oligosaccharides which may act as fermentable carbohydrates for beneficial colon microorganisms (Gómez *et al*., 2016; Scott *et al*., 2013). The metabolic end products synthesized in the lower part of the gastrointestinal tract during fermentation and the bacterial populations present might vary according to the nature and the amount of non-digestible carbohydrate ingested (Rowland *et al*., 2018; Rawi *et al*., 2020). Generally, fermentation of non-digestible carbohydrates, proteins, and other fibers in the large intestine produces beneficial as well as non-beneficial compounds that could affect the bowel health. Short-chain fatty acids (SCFA) such as acetic, propionic, and butyric are some of them which may confer various health benefits to human beings (Aquino *et al*., 2017; Rowland *et al*., 2018). Probiotic bacterial species, mainly *Bifidobacterium* and *Lactobacillus,* are responsible for the production of these short-chain fatty acids, mostly due to the fermentation of dietary

oligosaccharides (Fernando *et al*., 2010). Furthermore, the fermentation process by beneficial microorganisms is vital in reducing the population of pathogenic microorganisms in the gut due to the significant reduction of colonic pH (Aquino *et al*., 2017; Campbell *et al*., 1997).

Legumes are good sources of prebiotic carbohydrates for improving gastrointestinal health (Chen *et al*., 2020; Rengadu *et al*., 2020). Among the Asian countries, legumes such as chickpea and mung bean are widely consumed while cowpea and horse gram are utilized minimally. These legumes are consumed mainly after boiling, and some are used in raw powder form in food product preparations. It is well known that the processing and gastrointestinal digestion modulate fermentation properties of food (Capuano, 2016; Singh, 1988). To our knowledge, comparative information on the prebiotic activity of raw and boiled legumes after *in vitro* enzymatic digestion is scanty, and this information is vital in developing functional food with desired nutritional properties. Hence, the objective of this study is to investigate the fermentation properties of five types of raw and boiled commonly consumed legumes in Sri Lanka (mungbean, two cowpea cultivars (*Waruni, Dhawala*), chickpea and horse gram) after *in-vitro* enzymatic digestion.

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MATERIALS AND METHODS

Materials

Mung bean (MI5) (*Vigna radiata L*), cowpea (*Dhawala & Waruni*) (*Vigna unguiculata L*) grown in Sri Lanka were purchased from the 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*), which is not grown in Sri Lanka, was purchased from Kandy Market, Sri Lanka. Seeds were manually selected to remove impurities before 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 other chemicals used were of analytical grade and purchased from Sigma-Aldrich (Sigma, St. Louis, U.S.A.) unless otherwise stated.

Sample preparation

Legume seeds were soaked overnight in water before boiling. Seeds were boiled until they were sufficiently *softened* to be kneaded with one's *hands.* The amount of water added and the duration required for boiling (Table 1) were decided as previously described (Mtolo *et al*., 2017; Prinyawiwatkul *et al*., 1997). Boiled and raw seeds were ground using a grinder. Then the samples were pre-dried to a constant weight at 60 °C using a drying oven (Mermmert™VO200, Germany) and packed in polythene bags separately and stored in a desiccator for further analysis.

In-vitro digestion of legume samples Collection of gastric and intestinal juices

Gastric juice and intestinal juice were collected from the slaughterhouse 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 the transportation. Juices were stored at -20 °C until the process.

Preparation of gastric enzyme solution

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

Preparation of intestinal enzyme solution

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

Digestion of legumes

According to the methods described by Furuya *et al*. (1979) and Li *et al*. (2004), legume samples were digested with minor modifications. Ground legume sample (2 g) was measured (RADWAG Wagi Elekroniczne, Poland) into 100 mL conical flask, to which 20.0 mL of gastric juice was added, and its pH was adjusted in a range of 4.0-4.6 using a pH meter (HANNA Instrument Inc, Woonsocket). This mixture was incubated in a shaking incubator at 100 rpm (THZ-100 Shaking Incubator, Biocotek, China) for 4 h at 37 °C. At the end of the first incubation period, the contents were neutralized with 1.0 mol dm^{-3} sodium hydroxide solution. In the second stage, 20.0 mL of intestinal fluid prepared as mentioned previously was added, the pH was adjusted (6.9-7.4) and the digestion mixtures were incubated further in a shaking incubator for additional 4 h at 37 °C at 100 rpm. After the second incubation, the contents of the flask were transferred to centrifuge tubes and centrifuged (LEGEND XIR Centrifuge, Germany) immediately at 4500 rpm for 10 min at 5 °C. The supernatant fraction was discarded, and 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 at -20 °C in a refrigerator until use.

In-vitro fermentation of legume samples Preparation of bacterial pellets

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 the last three months were selected and slaughtered after 16 h fast, and ceca were quickly removed and taken into the National

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

Institute of Fundamental Studies by maintaining anaerobic conditions. Twenty five grams of cecal content was measured by an electronic weighing balance (RADWAG Wagi Elekroniczne, Poland) and dissolved in 250.0 mL of phosphate buffer (pH 6.2±0.2 pH). Then the 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 min at a speed of 10000 rpm. Bacterial pellets were resuspended in 250.0 mL of phosphate buffer (pH 6.2±0.2pH).

Fermentation of legume samples

Digested legumes (0.5 g) were accurately measured using an electronic weighing balance (RADWAG Wagi Elekroniczne, Poland) and added to the vacutainer tubes. 8.0 mL from bacterial pellet solution was added into those vacutainers and exposed to a continuous CO_2 flow as described by Calabrò *et al*., (2009). Bacterial suspension (8.0 mL) without the substrate was used as a control. Vacutainers were appropriately sealed and incubated using Mermmert incubator (Mermmert™ IN160, Germany) at 37 °C for 4 h.

Microbial growth analysis

Total anaerobe bacterial counts for each legume fermented with bacterial pellets were taken as previously described by the spread plate method (Garcha *et al*., 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 the 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.

$$\frac{\text{CFU}}{\text{mL}} = \left(\begin{array}{c}\text{Number}\\ \text{of Colonies}\end{array}\right) \times \frac{1}{\left(\begin{array}{c}\text{Dilution}\\ \text{factor}\end{array}\right)} \times \frac{1}{\left(\begin{array}{c}\text{innoculated}\\ \text{volume}\end{array}\right)}$$

Statistical analysis

The tests were performed in triplicate for each sample to be analyzed. All the data were expressed as mean ± standard deviation. Data were analyzed using the S.A.S. software package (S.A.S. institution Inc., 2003, Cary, U.S.A.). Three-Way ANOVA was used for the analysis. Means separations were analyzed along the column and raw using Duncan's Multiple Range Test and Dennet's Test at α = 0.05. Following statistical model was used:

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

RESULTS AND DISCUSSION

Any dietary material that is not digestible by mammalian gastric enzymes and enters the large intestine and degraded by intestinal microflora are considered as prebiotics (Gibson *et al*., 2010; Nakata *et al*., 2017). In this study, before doing *in vitro* fermentation, legumes samples were subjected to simulated gastrointestinal digestion to investigate legumes› actual effectiveness as prebiotic candidates. The findings of this study have shown that *in vitro* digestion modulated the prebiotic potential of both raw and boiled legumes. Simulated digestion significantly reduced ($p<0.05$) prebiotic potential in some legume substrates except boiled, mung bean, *Waruni* cowpea (red cowpea), chickpea, *Dhawala* cowpea and raw; *Dhawala* cowpea and horse gram showing that true prebiotic potential can vary among legumes. Structural and compositional differences in dietary substrates could be the reason behind the differential growth pattern of microorganisms (Holscher, 2017). Observed differences in fermentation properties among different legumes in this study could be due to the differences in physicochemical properties of prebiotic carbohydrates, including sugar composition, branching, chain length, degree of polymerization, and solubility (Holscher, 2017; Siva *et al*., 2019).

Digested residues of boiled chickpea and digested residues of boiled *Waruni* samples showed significantly higher ($p<0.05$) *Bifidobacterium* fermentation ability than digested residues of boiled mung bean and *Dhawala* (Table 2).

Further, digested residues of boiled and undigested raw chickpea showed significantly higher ($p<0.05$) *Lactobacillus* fermentation properties (Table 3) compared to other digested residues of boiled legumes and undigested raw legumes. Significantly higher ($p<0.05$) prebiotic potential shown by digested boiled chickpeas samples in this study may be due to the availability of a higher concentration of readily fermentable components in chickpea, which escape pepsin and pancreatin digestion (Woyengo *et al*., 2017). It has been shown that chickpea contains a significantly higher amount of polyols, a group of low digestible carbohydrates compared to peas, beans, and lentils (Moussou *et al*.,2017), which may be another reason for rapid fermentation shown by chickpea compared to some other legumes.

Prebiotics should be able to selectively metabolize *Lactobacillus* and *Bifidobacterium* at a higher degree compared to pathogenic bacteria. Significantly higher ($p<0.05$) prebiotic potential in chickpea was further supported by a significantly lower ($p<0.05$) *Coliform* population observed for digested boiled residues of chickpea (Table 3) compared to other legume substrates (Akillioglu & Karakaya, 2010). Abundantly available α-galactosides and oligosaccharide; ciceritol in chickpea may have stimulated the growth of probiotic bacteria and inhibited the pathogenic bacteria, as shown previously (Dai *et al*., 2017; Muzquiz *et al*., 2012). Digested residues of raw horse gram showed a significantly higher ($p<0.05$) *Bifidobacterium* fermentation ability compared to digested residues of raw mungbean, *Waruni, and Dhawala* (Table 2).

These observations may be supported higher content of oligosaccharides in cotyledon fractions of horse gram seeds (Prasad & Singh, 2014). Significantly higher ($p<0.05$) *Bifidobacterium* proliferation ability observed for digested residues of boiled *Waruni* and significantly higher ($p<0.05$) *Coliform* suppression ability (Table 4) shown by

Table 2: The effect of *In-vitro* digestion and fermentation on Cecal *Bifidobacterium* growth (log CFU/mL).

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

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	^{a,A} ± 0.58 8.80	^{a,B} ± 0.60 7.89	^{d,C} ± 0.03 6.82	^{a,AB} ± 0.01 8.32
Horse gram	^{b,B} ± 0.02 7.71	^{a,A} ± 0.54 8.27	^{b,B} ± 0.04 7.22	^{d,B} ± 0.01 7.32
Mung bean	^{b,C} ± 0.03 7.61	^{a,A} ± 0.02 7.83	^{b,D} ± 0.04 7.26	^{b,B} ± 0.00 7.71
Waruni	^{b,B} ± 0.01 7.60	^{a,A} ± 0.02 7.83	^{a,C} ± 0.04 7.35	^{c,C} ± 0.01 7.40
Dhawala	$^{b.A}$ ±0.01 7.67	^{a,A} ± 0.01 7.65	^{b,A} ± 0.01 7.25	^{b,A} ± 0.01 7.70
Control	^{c} ±0.03 7.00	^{b} ±0.03 7.00	^{c} ±0.03 7.00	^{e} ± 0.03 7.00

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
Chickpea	^{d,B} ± 0.04 6.94	^{c,C} ± 0.02 6.87	^{b,A} ± 0.02 7.36	^{c,B} ± 0.05 6.70
Horse gram	^{a,A} ± 0.01 7.89	٠,٠4 ± ^{a,C} 7.70	^{a,B} ± 0.01 7.78	^{b,D} ± 0.03 7.18
Mung bean	^{c,A} ± 0.31 7.36	^{b,A} ± 0.17 7.68	^{c,A} ± 0.01 7.26	^{b,A} ± 0.09 7.16
Waruni	^{bc,B} ± 0.01 7.54	^{a,A} ± 0.04 7.68	^{b,C} ± 0.09 7.39	^{a,C} ± 0.02 7.33
Dhawala	^{b,B} ± 0.03 7.63	^{a,A} ± 0.01 7.70	^{d,D} ± 0.02 6.82	^{c,C} ± 0.03 6.97
Control	^{e} ±0.07 6.38	^{d} ± 0.07 6.38	^{e} ± 0.07 6.38	^{d} ± 0.07 6.38

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).

digested residues of both raw and boiled *Dhawala* cowpea were supported by previous findings showing that resistant starch isolated from five cowpea cultivars fulfilled the criteria to be classified as prebiotics (Rengadu *et al*., 2020).

Digested residues of boiled mung bean and *Waruni* cowpea and digested residues of boiled chickpea and mung bean significantly improved the ($p<0.05$) *Bifidobacterium* and *Lactobacilli* growth, respectively and digested residues of boiled chickpea, horse gram and *Dhawala* significantly suppressed ($p<0.05$) the *Coliform* growth compared to their respective raw digested samples suggesting that boiling improved the prebiotic potential of most of the studied legumes. Significantly higher prebiotic potential in digested boiled legumes than digested raw legumes may be due to the increase in soluble fiber composition observed for mung beans upon boiling (Liyanage *et al*., 2018). It has been shown that soluble residues have a much more positive effect on gut microbiota (Chen *et al*., 2020). Further, previous studies have shown that processing not only altered the yield of soluble and insoluble fiber fractions, it also changed the fiber composition and sugar profiles of the two fiber fractions (Chang *et al*., 1989). Moreover, the increase in resistant starch content with the retrogradation of legume flours after boiling may have modulated the growth of probiotic bacteria (Dangsungnoen *et al*., 2012). However, digested residues of raw *Dhawala* and raw horse gram showed significantly higher ($p<0.05$) *Bifidobacterium* proliferation than their digested boiled samples showing that some legumes show higher prebiotic potential in raw forms than when they are boiled. Findings further supported the previous study showing that the prebiotic activity of digested substrates was greatly affected by legume type, boiling, and composition of digesta (Chen *et al.,* 2020).

CONCLUSION

This study displayed that simulated digestion differently modulated the fermentation properties of both raw and boiled legumes. Among all the studied legume samples, boiled chickpea could be considered as the best prebiotic candidate considering both *Bifidobacterium* and *Lactobacillus* proliferation ability. Boiled mung bean, boiled chickpea, and boiled *Waruni* cowpea may be more suitable than their raw forms in preparing prebiotic food.

~~DECLARATION OF CONFLICT OF INTEREST~~

~~The authors declare no conflict of interest.~~

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