Influence of enzyme-resistant fraction of sorghum (*Sorghum bicolor* L.) flour on gut microflora composition, short chain fatty acid production and toxic substance metabolism

SAMANTHI WATHSALA PELPOLAGE – KYUHO HAN – HIROSHI KOAZE – TETSUO HAMAMOTO – MICHIYO HOSHIZAWA – MICHIHIRO FUKUSHIMA

Summary

Colonic fermentation of carbohydrates such as resistant starch by microbiota has been found to improve host health. Sorghum contains a very high level of resistant starch, due to which it can be a prebiotic substrate, but this property has not been scrutinized yet. Thus, we aimed to investigate the colonic fermentation potential of sorghum dietary enzyme-resistant fraction (ERF) in a laboratory-scale fermenter simulating human colon. ERFs obtained by in vitro digestion by amyloglucosidase and pancreatin were subjected to in vitro fermentation for 48 h with cellulose as a negative control. The secured samples were analysed for the contents of bacteria, short chain fatty acids, pH and ammonia nitrogen. Whole and refined sorghum ERF (ERF-Wh and ERF-Rf) acquired significantly higher contents of anaerobes, *Lactobacillus*, *Bifidobacterium*, lactic acid bacteria, total short chain fatty acids, propionate and butyrate compared to the control at 48 h ($p < 0.05$). Both ERFs demonstrated similar prebiotic behaviour apart from ERF-Rf yielding higher ammonia nitrogen content and higher pH at 48 h. Despite of higher resistant starch contents in ERFs, higher ammonia nitrogen content reflected a prominent protein fermentation suggesting less accessibility to resistant starch, which urges to investigate further on how to improve accessibility of sorghum resistant starch for colonic fermentation.

Keywords

sorghum; in vitro; colonic fermentation; gut microbiota; short chain fatty acids

Sorghum (*Sorghum bicolor* L.) is an indigenous cereal crop in Africa with excellent drought tolerant and low input management capabilities, serving as a staple food for the world's destitute populations in arid and semi-arid areas of Africa, Asia and Latin America [1, 2]. Besides its good agronomical traits, it is long recognized for its unique nutritional properties, despite of being underutilized as a human food source. For instance, it is gluten-free, the flour is characterized by a relatively very low digestibility and is rich in polyphenols such as condensed tannins which possess antioxidant properties, and therefore sorghum has earned interest of nutritionists as a therapeutic or functional food [1, 3].

The low in vitro flour digestibility of sorghum, which is considered as a potential trait for overweight and obesity management, was found to be associated with low digestibility of both starch and protein fractions [1]. Some studies reported that the endosperm proteins (kafirins) are, to a greater extent, responsible for the lower in vitro starch digestibility of sorghum flour [2]. Besides that, endosperm texture and structure, interactions between starch and non-starch components such as tannins and alcohol-soluble proteins, were found to affect in vitro digestibility of sorghum starch [2, 4]. With the low digestibility of starch in sorghum, it becomes an attractive source of resistant starch (RS) with an average content of

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12.0–21.5 %. This RS fraction physically inaccessible to the amylolytic enzymes and water due to the protein matrix in the endosperm [1, 5].

RS-enriched food sources, such as underutilized minor cereals, have recently earned attention of many studies, aiming to mitigate the increase in incidence of chronic metabolic diseases directly associated with diet and lifestyle [6]. For instance, co-morbidities of metabolic syndrome, inflammatory bowel disease and colorectal cancers, are found to be associated with dysbiosis or altered gut microbiota [6]. These diseases are predicted to be prevented or moderated by dietary changes, because diet can significantly affect the etiology of such diseases, as diet composition may stimulate the growth of beneficial bacteria or inhibit unfavourable bacteria [7–9].

Human colon maintains a dense and diverse bacterial community, mainly comprised of anaerobes, with the ability to utilize complex carbohydrates that cannot be hydrolysed by human amylolytic enzymes [10]. *Bacteroidetes* and *Firmicutes* make up the predominant gut bacterial phyla, while *Prevotellaceae*, *Lachnospiraceae* and *Rikenellaceae* account for the most abundant families of colon microbiota [11]. The colonic microbial community is largely shaped up by the availability of microbiota accessible carbohydrates (MACs), such as RS, non-starch polysaccharides and oligosaccharides, which constitute the dietary fibre group [10, 11]. Thus, the dietary intake of fibre becomes a major determinant of the enterotype, as the amount and type of the dietary fibre influence the major taxonomic units of gut microbiome, initially in complement with the available biological niche and, subsequently, by indirect factors such as pH reduction and metabolic cross-feeding ability [7, 12]. Studies involving both humans and rodents revealed changes to the function and abundance of major groups of gut bacteria over diet interventions with RS [13]. Apart from the effect on the microbiome composition, dietary intake of large amounts of fibre or RS increased the fecal bulk, fecal pH, butyrate content, epithelial apoptosis, while reducing cell proliferation markers and colon carcinogenesis [7].

Breakdown of dietary fibre is a fermentation process carried out by the gut microbiota to harvest carbon and energy requirement for their growth, development and proliferation, ascribed to their versatile amylolytic enzyme repertoire that exceeds the capacity of human digestive enzymes [7]. Diet-induced host-beneficial biochemical, biological and physiological effects of the gut microbiota are attributed to secondary metabolites, such as short chain fatty acids (SCFAs) produced during this fermentation process [14]. Reduction of ileal and cecal digesta pH, reduction of cholesterol, fatty acid intake, synthesis and oxidation, improvement of glycemic status, blood glucose homeostasis and insulin sensitivity, improvement of cardiovascular health, attenuation of oxidative stress and inflammation, induction of chemoprotective enzyme activity and hindering growth of pathogenic microorganisms are some of the beneficial effects of RS fermentation [14, 15]. According to the nature of the fermentative substrate such as the type of RS, the above mentioned effects can vary, as these effects are substrate-specific because individual bacterial species inherit differential substrate-binding ability and metabolic pathways [7, 16]. For instance, both RS II and RS IV could increase the abundance of phyla *Actinobacteria* and *Bacteroidetes*, while decreasing the abundance of phylum *Firmicutes*, but each RS type had different effects at species level where RS II increased the abundance of *Ruminococcus bromii* and *Eubacterium rectale* and RS IV influenced *Bifidobacterium adolescentis* and *Parabacteroides distonis* [16, 17]. Subsequently, the beneficial effects also vary. For example, RS II was reported to cause a significant decrease in malondialdehyde, glycosylated hemoglobin, insulin and endotoxin contents, while RS IV influenced reduction of cholesterol, fasting glucose, proinflammatory markers and percentage of body fat [15].

With the previously reported high content of RS, which is in accord with the lower enzymatic digestibility of starch, sorghum is anticipated to be a promising prebiotic. However, its potential in this role has not been explored yet. Thus, the aim of this study was to determine the nature of the influence of sorghum flour enzyme-resistant fraction on selected indicators of gut microbiome and functions, such as microbiota composition, pH, toxic metabolite production and SCFAs fermentation using a mixed culture of swine fecal bacteria in a laboratory-scale fermenter simulating in vitro the human colon. Pig has been identified as a comparatively more suitable model for physiological studies in several areas, including intestinal microbiota, due to similar anatomical structure of gastrointestinal tract, being an omnivorous mammal and having a similar gut microbial composition [18]. It was hypothesized that the use of swine fecal inoculum would represent the human fecal microbiota, by which the digestive enzyme-resistant fraction of sorghum would be utilized as a substrate for their metabolism and could bring about beneficial effects on the above mentioned indicators of microbial function and gut health.

MATERIALS AND METHODS

Materials

Two types of sorghum flour, namely, whole sorghum (S-Wh) flour and refined sorghum (S-Rf) flour were provided by Nakano Industry (Takamatsu, Japan) and cellulose (microcrystalline powder; 20 μm) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

Preparation of sorghum flour enzyme-resistant fractions

Sorghum flour was subjected to enzymatic hydrolysis by a mixture of amyloglucosidase (4-α-D-glucan glucosidase, E.C.3.2.1.3. from *Aspergillus niger*, ≥ 300 U·ml^{-1}, one unit (1 U) corresponding to the amount of enzyme which catalyses the conversion of 1 μmol·l^{-1} of substrate per minute) and pancreatin (E.C.232-468-9 from porcine pancreas, 8× USP specifications, one unit according to United States Pharmacopeia (1 USP) corresponds to the amount of a substance that decomposes a given substrate at a specific rate under standard USP assay conditions) purchased from Sigma-Aldrich.

Four millilitres of amyloglucosidase solution and 3.0 g of pancreatin powder were dissolved in 400 ml of 0.1 mol·l^{-1} maleate buffer (pH 6.0) and centrifuged at 1 600 ×*g* in H-80R (Kokusan, Tokyo, Japan) at 4 °C for 10 min to collect the enzyme-containing supernatant. Twenty grams of sorghum flour were homogenized with 100 ml of enzyme-containing supernatant in a sealed capped 300 ml conical flask and incubated at 37 °C for 16 h with continuous agitation. After incubation, 100 ml of ethanol (99.5%, v/v) was mixed with the flour sample in a beaker, allowed to stand at room temperature for 3 h and centrifuged (1 600 ×*g*, 4 °C, 10 min) in 50 ml centrifuge tubes to remove the supernatant. The pellet was re-suspended in 15 ml of 99.5% ethanol, centrifuged under the same conditions and the supernatant was discarded. Finally, the pellet was suspended in 15 ml of acetone and centrifuged to remove the supernatant. The pellet was dried on aluminium foil at room temperature overnight and then ground, weighed and stored in clean airtight plastic containers at 4 °C until further analysis.

The moisture content (using a method according to AOAC 930.15), protein (AOAC 979.09) with a conversion factor of 6.25, lipid (AOAC 920.85) and ash (AOAC 923.03) were analysed in enzyme-resistant fractions (ERFs) of the two sorghum flours [19]. Resistant starch contents in the sorghum ERFs were determined by Megazyme resistant starch assay procedure (K-RSTAR 08/11, Wicklow, Ireland) according to AOAC 2002.02 method [19]. All the chemicals used were of analytical grade.

Feces and in vitro fermentation

Fresh feces from three 4–5 months old weaned pigs (breed Berkshire) were collected from the herd that belonged to Hokkaido Obihiro Agricultural High School (Obihiro, Hokkaido, Japan). Feces collection, transportation, storage and preparation of fecal slurry were done according to the procedure described by Han et al. [20]. In vitro fermentation was conducted in three laboratory-scale fermenters (220 ml working volume, Able and Biot, Tokyo, Japan) with the ability to control temperature, pH and agitation speed, as well as to maintain anaerobic conditions. Initially, 6.6 g of cellulose (Control), whole sorghum flour ERF (ERF-Wh) and refined sorghum flour ERF (ERF-Rf) were added into the three sterilized fermenter jars containing 170 ml sterilized water. A final carbohydrate concentration of 3.0 g·ml^{-1} and a final protein concentration similar to that of the ERF-Rf sample were adjusted by adding required amount of cellulose and nutrient broth (Difco, Sparks, Maryland, USA), respectively. Finally, 50 ml fecal inoculum (2.0 %, v/v) was added into each jar and it was fixed to the fermenter apparatus. Fermentation was conducted in quintuplets under anaerobic conditions maintained by a continuous supply of CO_2 (0.4 Pa), at a minimum pH level of 5.5 (maintained by 2 mol·l^{-1} NaOH), temperature of 37 °C with an agitation speed of 1.67 Hz for 48 h. Sampling (10 ml each) was done at 0, 6, 12, 24 and 48 h. The samples were later analysed for fecal microbiota, SCFAs and ammonia nitrogen (AN). At every sampling, pH and temperature were recorded.

Microbiological analysis

Respective diluted samples were cultured to enumerate specific bacterial species counts by viable plate count method with selective media. Coliform and lactic acid bacteria (LAB) were enumerated after 24 h of culturing on eosin methylene blue (EMB) agar (Eiken Chemical, Tokyo, Japan) and de Man, Rogosa and Sharpe (MRS) agar (Oxoid, Basingstoke, United Kingdom), respectively. Anaerobes and *Lactobacillus* were enumerated after 48 h of incubation on glucose blood liver (BL) agar (Eiken Chemical) and Rogosa agar (Oxoid), respectively, while *Bifidobacterium* was enumerated on transgalactosylated oligosaccharide (TOS)-propionate agar base (Yakult Pharmaceutical Industry, Tokyo, Japan) after 72 h of incubation. LAB were cultured using pour plate

method, while the other four groups were cultured using spread plate method, all five groups were incubated at 37 °C (TVN680DA Advantec incubator, Toyo Seisakusho Kaisha, Tokyo, Japan). BL, Rogosa and TOS culture plates were stacked in 7 l anaerobic jars along with 3 Anaeropack-Anaero sachets (Mitsubishi Gas Chemical, Tokyo, Japan). Subsequently after the specific incubation period, colonies were visually counted and were expressed as decadic logarithm of colony forming units per millilitre of the working volume.

Short chain fatty acid analysis

Samples were centrifuged (8 000 ×*g*, 4 °C, 15 min), a volume of 450 μl was pipetted out from each supernatant into a 2-ml microtube (Eppendorf, Hamburg, Germany) and 1 ml of 0.5 mol·l^{-1} $HClO_4$ (60%, v/v) was added. After leaving at room temperature for 5 min, the tubes were centrifuged under same conditions for 10 min. Following centrifugation, 300 μl of the supernatant was filtered into a new 1.5 ml microtube using a 1 ml syringe and a cellulose acetate membrane microfilter (pore size 0.45 μm; DISMIC-03CP, Advantec, Toyo Roshi Kaisha, Tokyo, Japan). The prepared samples were analysed by high performance liquid chromatography (HPLC) using Shimadzu LC-10AD chromatograph (Shimadzu, Kyoto, Japan) equipped with a bromothymol blue (BTB) post-column. Analytical specifications were as follows: column, RSpak KC-811 (8.0 mm × 300 mm; Shodex, Tokyo, Japan); eluent and flow rate, 0.003 mol·l^{-1} $HClO_4$ at 1 ml·min^{-1}; column temperature, 47 °C; reaction reagent and flow rate, ST3-R (10-fold dilution, Shodex) at 0.5 ml·min^{-1}; spectrophotometric detector wavelength, 450 nm.

Ammonia nitrogen analysis

Ammonia nitrogen (AN) content in the samples was analysed using a commercially available kit (Wako Pure Chemical Industry, Tokyo, Japan) according to the manufacturer's instructions.

Statistical analysis

All data were analysed for their significance ($p < 0.05$) by analysis of variance (ANOVA) using SPSS statistical software version 17.0 (SPSS, Chicago, Illinois, USA). When significant differences among the test groups were revealed, mean scores were compared by Tukey's test ($p < 0.05$).

RESULTS AND DISCUSSION

In vitro enzymatic digestibility and proximate composition of enzyme-resistant fractions

Proximate composition of ERFs of sorghum is presented in Tab. 1. The two sorghum samples were distinctively different in terms of the protein and resistant carbohydrate contents, where ERF-Rf possessed significantly higher protein content and ERF-Wh had significantly higher resistant carbohydrate and RS contents. Thus, the two substrates were significantly different in terms of the availability of MACs and it was hypothesized that the two ERF fractions would differently affect the in vitro intestinal fermentation. In vitro enzymatic digestibility of starch in sorghum flour was found to be affected by the physico-chemical properties such as starch and protein (sorghum prolamins) interactions, presence of inhibitors (condensed tannis) as well as structure and organization of starch granules [4, 21, 22]. Starch enzymatic hydrolysis is a two-phase reaction, where the degree of starch digestibility will depend on the enzyme's ability to diffuse towards the solid substrate in order to cleave the respective glycosidic linkages [23]. In sorghum flour, the accessibility will depend on the penetration ability through the protein matrix that encapsulates the starch granules [24, 25]. Albeit, most of the dietary proteins are considered to be highly hydrolysable, certain subgroups of sorghum prolamins (β- and γ- kafirin) are found to be resistant to enzymatic hydrolysis and known to form an enzyme-resistant layer of disulfide bonds, which restricts protease diffusion and, subsequently, maintain the integrity of the protein matrix covering the starch granules. This might be the reason behind the very high protein content observed in ERF-Rf as presented in Tab. 1 [26]. Moreover, the channels in the starch granules were found to be lined by prolamins, which are the main routes for enzyme penetration into the central cavity area from where starch di-

Tab. 1. Proximate composition of enzyme-resistant fractions of sorghum flour.

	ERF-Wh	ERF-Rf
Moisture [%]	12.6 ± 0.5^{a}	12.4 ± 0.4^{a}
Carbohydrate [%]	39.3 ± 0.5^{a}	20.2 ± 0.5^{b}
Protein [%]	36.4 ± 0.6^{b}	57.8 ± 1.1^{a}
Fat [%]	1.1 ± 0.2^{a}	0.8 ± 0.2^{a}
Ash [%]	10.5 ± 0.1^{a}	8.9 ± 0.5^{b}
Resistant starch [%]	15.9 ± 0.1^{a}	14.4 ± 0.1^{b}

Values are expressed as percentage on dry weight basis and represent mean ± standard deviation (n = 3). Different letters in superscript in each column represent significant differences ($p < 0.05$) as determined by ANOVA.
ERF-Wh – enzyme-resistant fraction of whole sorghum flour, ERF-Rf – enzyme-resistant fraction of refined sorghum flour.

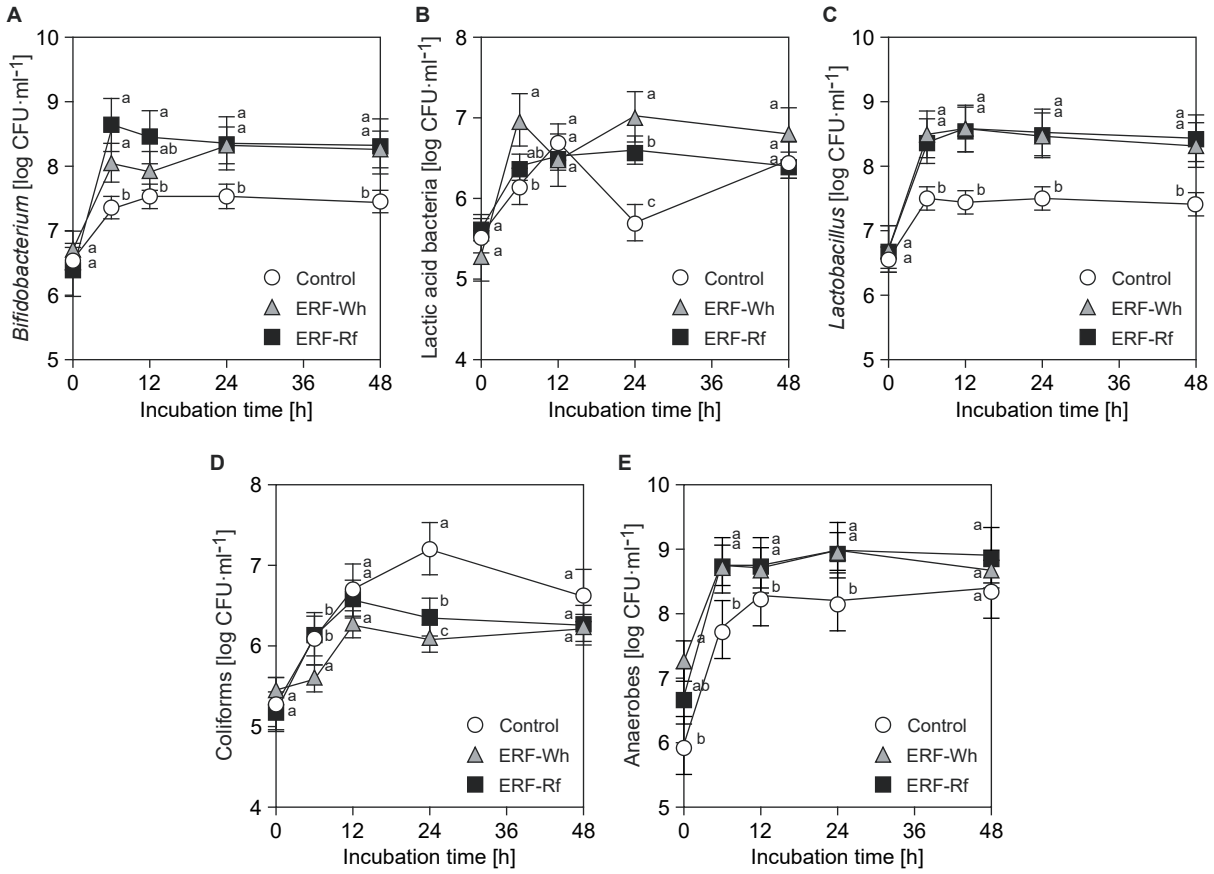

Fig. 1. Microbial populations during in vitro fermentation of sorghum enzyme-resistant fractions.

A – *Bifidobacterium* spp., B – lactic acid bacteria, C – *Lactobacillus* spp., D – coliforms, E – anaerobes.
Values presented are mean ± standard deviation (n = 5). Different letters at each sampling point represent significant differences ($p < 0.05$) as determined by ANOVA coupled to Tukey's test.
ERF-Wh – enzyme-resistant fraction of whole sorghum flour, ERF-Rf – enzyme-resistant fraction of refined sorghum flour.

gestion initiates [27]. And it is suggested that these proteins in the lining would interfere with the inward migration of amylolytic enzymes and, subsequently, the degree of starch digestion [27].

Microbiota composition and pH

Fig. 1 presents data on levels of anaerobes, coliforms, LAB, *Lactobacillus* and *Bifidobacterium* in the fermenters during the entire incubation period.

Anaerobe counts were significantly higher ($p < 0.05$) in ERF-Rf and ERF-Wh in the period of 0–24 h compared to the control, while the anaerobic counts were similar between the two ERFs in the period of 6–48 h. Coliform level of ERF-Wh was significantly lower ($p < 0.05$) than those of the control and ERF-Rf in the period of 6–24 h, while ERF-Rf had similar coliform counts as the control throughout the incubation period except at 24 h, when the counts were significantly lower ($p < 0.05$) than the control, yet higher than ERF-Wh. *Lactobacillus* and *Bifidobacterium* counts were significantly higher ($p < 0.05$) in both ERFs compared to the control in the period of 6–48 h. Results obtained for anaerobic counts, coliforms, *Lactobacillus* and *Bifidobacterium* were comparable with the findings reported by Han et al. [28]. A clear trend could not be observed in LAB counts among the three substrates, yet in ERF-Rf, they increased until 6 h and remained constant since then. LAB counts in ERF-Wh and the control fluctuated in the period of 6–48 h, and all three substrates levelled at 48 h with similar LAB counts. The two ERFs had similar counts for anaerobes, *Lactobacillus* and *Bifidobacterium*, indicating a similar influence on the microbiota specialized in carbohydrate fermentation by the two substrates. This observation is supported by the fact that especially *Bifidobacteria* favour the presence of readily fermentable carbohydrates such as RS [29]. The aforementioned observation suggests that either availabil-

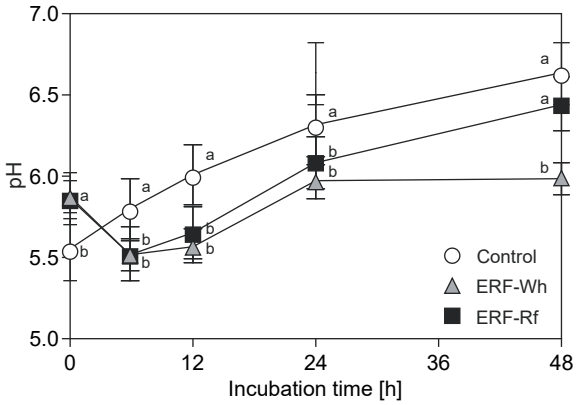

Fig. 2. Variation in pH in the fermentation media during in vitro fermentation.

Values presented are mean ± standard deviation (n = 5). Different letters at each sampling point represent significant differences (p < 0.05) as determined by ANOVA coupled to Tukey's test.
ERF-Wh – enzyme-resistant fraction of whole sorghum flour, ERF-Rf – enzyme-resistant fraction of refined sorghum flour.

ity or accessibility to MACs in the two substrates was similar irrespective of the drastic differences in their proximate composition (Tab. 1). Moreover, anaerobes, *Lactobacillus* and *Bifidobacterium* counts reached the stationary phase at 6 h, where there was no net growth further or only a minimal growth. According to MAIER [30], the prominent protein fermentation, which might have initiated due to either complete exhaustion or the restricted accessibility to MACs as discussed later, and toxicity developed by higher AN production and accumulation, might have limited the growth of beneficial bacteria such as *Lactobacillus* or *Bifidobacterium*.

pH in the control sample continuously increased throughout the incubation period as shown in Fig. 2. ERF-Wh and ERF-Rf showed similar variations in pH in the period of 0–24 h. In the period of 0–6 h, there was a reduction in pH in sorghum ERFs media but, in the period of 6–24 h, pH gradually increased, yet was significantly lower compared to the control. In ERF-Wh, pH stabilized at 24 h, but pH in ERF-Rf continued to increase reaching a value significantly higher than ERF-Wh and similar to that of the control at 48 h. Generally a lower pH in the gut, which is accompanied by a higher organic acid production, which is considered to be healthy and beneficial as it suppresses growth and proliferation of pathogenic microorganisms [20, 31]. In this study, albeit pH of the sorghum ERFs was significantly lower compared to that of the control in the period of 6–24 h, pH continuously increased as described previously. This could be a result of lower availability of MACs and higher protein content in sorghum ERFs, causing lower SCFA fermentation, which subsequently promoted ammonia-producing bacteria resulting in a higher AN production [28]. Similar to these results, previous studies also reported a negative correlation between pH of cecal digesta and colonic ammonia concentration [32].

Short chain fatty acid profile

Resistant carbohydrates that escape the digestion in the small intestine provide energy for the colonic microbiota, bowel epithelium and peripheral tissues via SCFAs, the major end products of colonic fermentation of carbohydrates [31]. These SCFAs exert important biological effects on host such as maintenance of a health-promoting microbiota, suppression of cancer precursors and improvement of nutrient metabolism [7]. Fig. 3 shows the fermentation profiles of major SCFAs during the incubation period. Acetate, propionate and butyrate are the major individual SCFAs, which are generally found in proportions of 3 : 1 : 1 [10]. The concentrations of the three major SCFAs were not significantly different in sorghum ERFs compared to the control sample in the period of 0–12 h. All three SCFAs showed a distinct increase in the rate of production in the period of 12–48 h compared to the period of 0–12 h, which might have been due either to an increase in population of microbiota or to structural changes in RS that improved its accessibility to microbial degradation [33].

Acetic acid is one of the initial organic acids produced along with pyruvate and lactate as a result of the fermentation of readily available fermentable carbohydrates by probiotic bacteria [14]. It was the major fermentation product with the highest proportion among all the three substrates at 48 h, but only in the control sample, it reached 70 % of total SCFAs production. In ERF-Wh and ERF-Rf, it was only 58 % and 53 %, respectively, which was in contrast to the findings reported by GIUBERTI et al. [33]. Acetic acid production was not significantly different among the substrates throughout the whole incubation period at all sampling points, which might have been due to its utilization in the butyric acid biosynthetic pathway [14]. BIRT et al. [7] previously reported a significant correlation between butyrate production and acetate disappearance. Another possible reason for the comparable levels of acetate in the three substrates could be the low availability or accessibility to MACs in the two sorghum ERFs due to a dense protein matrix [10, 24]. Acetic acid pro-

duction is mainly attributed to genera *Bifidobacterium* and *Lactobacillus*, which belong to the group of LAB [14]. Albeit the sorghum ERFs possessed significantly higher counts of *Bifidobacterium* and *Lactobacillus* in the period of 6–48 h, acetic acid production was not significantly different among the three substrates during the mentioned incubation period. According to previous reports, increased counts of *Bifidobacteria* were related to the greater production of acetic acid, which was in contrast to the observations in this study [29]. Higher variability observed for LAB counts during the incubation period might have conferred an effect on the acetic acid production. Additionally, as previously mentioned, bacterial growth reached its stationary phase at 6 h, thus minimal metabolic activity of the microbiota in the medium might be another possible reason for the similar concentrations of acetic acid among the three substrates in the period of 6–48 h.

Propionate and butyrate had a lag phase of 0–6 h and 0–12 h, respectively. This could be due to the time required for acclimatization of the bacterial cells to the culture conditions and the lower availability of acetic acid (precursor of butyrate) in the media [14, 30]. Propionic acid production exhibited a sharp increase in production at 24 h for the two sorghum ERFs and the concentration was significantly higher ($p < 0.05$) at 24 h and 48 h compared to the control. However, there was no significant difference in the concentration between ERF-Wh and ERF-Rf.

Butyric acid production also varied similar to that of propionic acid, where a sharp increase in production was observed at 24 h. At 24 h, butyric acid concentration was significantly higher ($p < 0.05$) in both sorghum ERFs compared to the control but similar between ERF-Wh and ERF-Rf. At 48 h, butyrate concentration exhibited a dramatic increase in ERF-Rf, which was significantly higher ($p < 0.05$) compared to the control and ERF-Wh. Thus, ERF-Rf appeared to

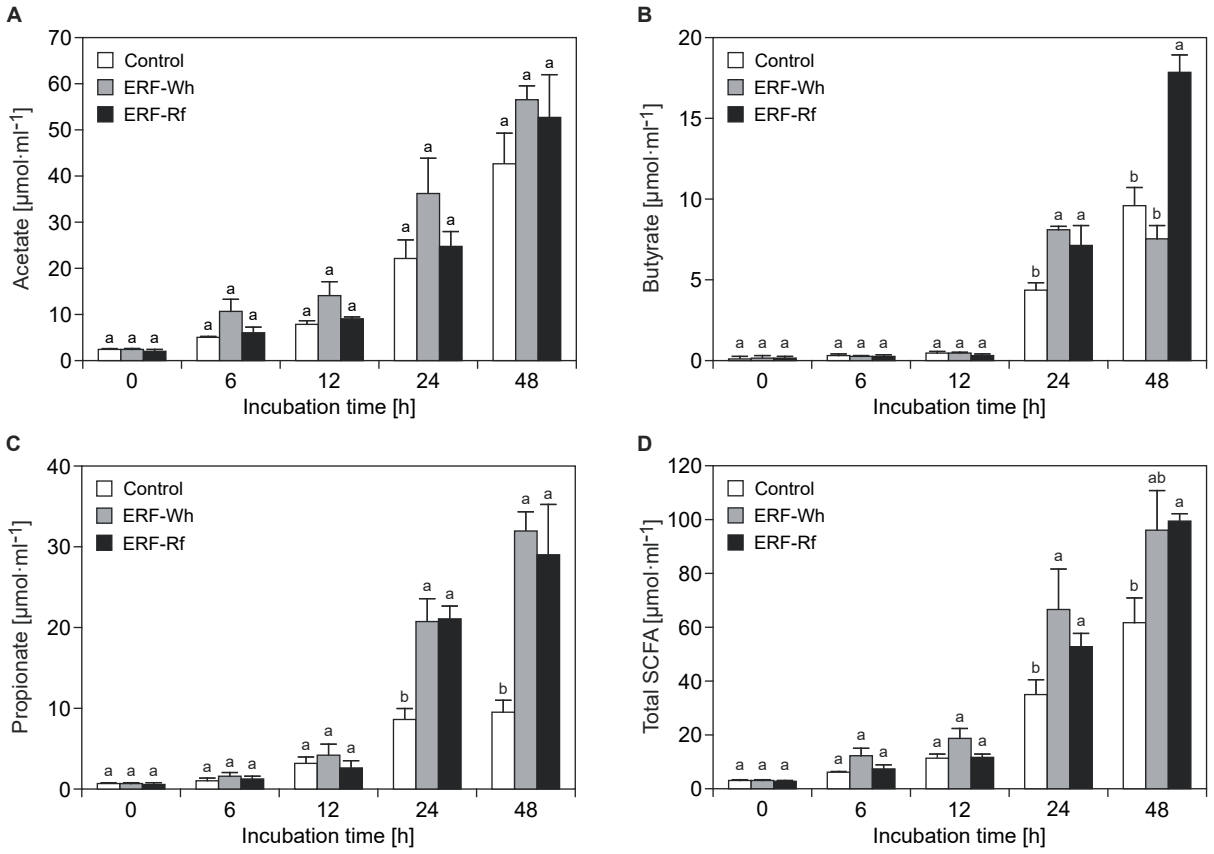

Fig. 3. Short chain fatty acids concentrations during in vitro fermentation.

A – acetate, B – butyrate, C – propionate, D – total short chain fatty acids.
Values presented are mean ± standard deviation ($n = 5$). Different letters at each sampling point represent significant differences ($p < 0.05$) as determined by ANOVA coupled to Tukey's test.
SCFA – total short chain fatty acids, ERF-Wh – enzyme-resistant fraction of whole sorghum flour, ERF-Rf – enzyme-resistant fraction of refined sorghum flour.

be a butyrogenic source as its fermentation led to an almost two-folds higher butyrate concentration than the control, which is an insoluble dietary fibre source [33]. Butyric concentration of ERF-Wh remained almost similar at 24 h and 48 h. The exact underlying reason of this is unclear, yet it could be due to substrate preference, substrate availability to microbiota and competitive abilities that exist in the fermenter environment, might have affected proliferation of butyrate-producing bacteria [16, 33].

Albeit the SCFAs production is reflective of the anaerobic counts, total SCFAs production remained similar among the fermentation substrates in the period of 0–24 h, in spite of the anaerobe counts being significantly higher in sorghum ERFs than the control [10]. This reflected the lower availability of MACs, further supported by the acquisition of stationary phase from 6 h onwards by the anaerobes, which might have been potentially driven by the fermentative substrate deficiency [30]. Later at 48 h sampling point, total SCFAs content was significantly higher ($p < 0.05$) in ERF-Wh and ERF-Rf, where ERF-Rf contained the significantly highest amount of total SCFAs, which might have resulted from accumulation over time [28]. Individual SCFAs and total SCFAs concentrations in the control sample were observed to be similar to those of the sorghum ERFs in the period of 0–12 h, which might be due to the ability of pig gut microbiota to ferment cellulose-like dietary fibre that was highly unlikely to be fermented by the human gut microbiota [20].

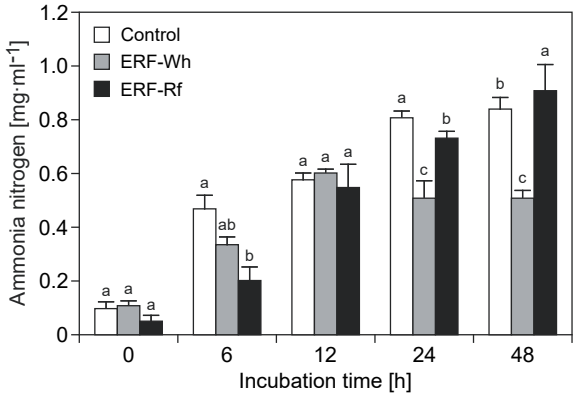

Fig. 4. Ammonia nitrogen concentrations in the fermentation media during in vitro fermentation.

Values presented are mean ± standard deviation ($n = 5$). Different letters at each sampling point represent significant differences ($p < 0.05$) as determined by ANOVA coupled to Tukey's test.
ERF-Wh – enzyme-resistant fraction of whole sorghum flour, ERF-Rf – enzyme-resistant fraction of refined sorghum flour.

Either the individual molar ratios of SCFAs or fermentation rates were not significantly different among the three substrates in the period of 0–12 h (data not shown). Propionate fermentation rates of sorghum ERFs were similar yet significantly higher compared to the control sample in the period of 24–48 h (control: 0.85 μmol·ml^{-1}d^{-1}; ERF-Wh: 7.99 μmol·ml^{-1}d^{-1}; ERF-Rf: 7.91 μmol·ml^{-1}d^{-1}; $p < 0.05$). Butyrate fermentation rate was the significantly highest in ERF-Rf and lowest in ERF-Wh in the said incubation period (ERF-Wh: 3.26 μmol·ml^{-1}d^{-1}; ERF-Rf: 10.70 μmol·ml^{-1}d^{-1}; $p < 0.05$).

At 48 h, molar ratio of acetic acid in the control sample was significantly higher compared to the sorghum ERFs (control: 0.69; ERF-Wh: 0.58; ERF-Rf: 0.52; $p < 0.05$), where the two ERFs had a similar ratio. Propionate molar ratio was similar between the two sorghum ERFs but significantly higher than the control (control: 0.15; ERF-Wh: 0.33; ERF-Rf: 0.29; $p < 0.05$), while butyrate molar ratio was significantly higher in ERF-Rf compared to ERF-Wh (ERF-Wh: 0.09; ERF-Rf: 0.18; $p < 0.05$). Lower molar ratio of acetic acid and higher molar ratios of butyric acid observed in sorghum ERFs reflected the ability of sorghum ERFs to influence the growth and proliferation of gut bacteria, which were able to cross-feed on acetic acid and produce butyrate [10]. Albeit the RS content and carbohydrate contents in sorghum ERFs were higher as presented in Tab. 1, the amount of RS available for fermentation depends on chemical composition, physical structure, degree of polymerization and bond type which affect the SCFAs fermentation pattern [33].

Ammonia nitrogen concentration

AN concentrations in all three fermented substrates gradually increased up to 12 h, as shown in Fig. 4. At 6 h, the control sample had significantly higher ($p < 0.05$) AN concentration compared to sorghum ERFs. Up until 12 h and 24 h, AN concentration in ERF-Rf was either significantly ($p < 0.05$) or comparatively lower than that in ERF-Wh and the control, respectively. After 12 h, AN concentration in ERF-Wh was stabilized, while that in the control and ERF-Rf continued to rise. At 12 h, AN concentration in ERF-Rf exhibited a sharp increase compared to 6 h sampling point. At 24 h, AN concentration in ERF-Rf was significantly lower ($p < 0.05$) compared to the control, but at 48 h, the significant difference between ERF-Rf and control was conversed. At 48 h, ERF-Rf had the highest total SCFA concentration and, at the same time, the highest AN concentration, which might have been due to accu-

mulation of both SCFAs and AN in the fermenters [28]. Generally, a lower pH in the fermentation medium is attributed to higher SCFAs production, yet a rise in pH could be observed in ERFs even with higher SCFAs concentration in the period of 24–48 h. This might have been due to the alkalizing effect of ammonia released to the medium, which is in contrast to the previously reported results [23, 32].

The higher production of ammonia suggested an increase in protein fermentation by bacterial deamination of amino acids, which might have been due to insufficient energy availability for the microorganisms [32, 33]. When energy supply for the growth and development of the microbiota from carbohydrate fermentation becomes deficient, deamination of amino acids is initiated to meet the energy demand [32, 33]. Thus, the higher AN amounts produced by ERF-Rf could be attributed to the lack of MACs for microbial metabolism in the medium as carbon and energy source, thus the microorganisms might have shifted to utilize proteins [29, 32]. Prior to microbial fermentation, the complex carbohydrates are hydrolysed by bacterial glycoside hydrolases or polysaccharide lyases into simple molecules, while the rate of complex carbohydrate depolymerization directly affects the rate of availability of MACs [32, 33]. The lower availability of MACs due to lower depolymerization and, consequently, deprivation of available energy might be the driving factor for higher ammonia production as a repercussion of deamination of amino acids for energy harvesting. Albeit, sorghum is boasted of having the highest RS content available among the known biological sources, and predicted to be an assuring source of MACs, protein fermentation seemed more prominent in the period of 12–48 h, as sorghum RS are physically inaccessible to water or hydrolytic enzymes due to the protein matrix [1, 24].

Additionally, the amount of protein escaping into the colon and the ratio between MACs and proteins, as well as the protein type, affect the extent of microbial fermentation of protein in the colon [32]. Further, according to Taciak et al. [32], protein fermentation progresses when the said ratio decreases, which suggests low availability of MACs at the time. As presented in Tab. 1, the resistant carbohydrate and the resistant protein contents in the two sorghum ERFs were significantly different and the ratio of the carbohydrates (MACs = RS) : protein (without nutrient broth protein) in ERF-Rf was lower compared to ERF-Wh (ERF-Rf: 0.25; ERF-Wh: 0.43). This fact highlights the potentially lower availability of energy from RS fermentation in ERF-Rf, which might be the reason for continuously increasing ammonia production in ERF-Rf since its sharp increase at 12 h. In sorghum, kafirins or prolamins constitute 70 % to 80 % of total endosperm proteins, where the subgroup of γ-kafirin found in the periphery of the protein bodies enclosing the highly digestible α-kafirin sub-group, is found to be highly resistant to hydrolysis by proteases [34–36]. This leads to a reduction in overall protein digestibility of proteins in sorghum, as observed in Tab. 1.

CONCLUSIONS

Both sorghum ERFs demonstrated their prebiotic nature as they positively influenced the growth and proliferation of beneficial gut microbial genera such as *Lactobacillus* and *Bifidobacterium*. The two ERF fractions facilitated a slow initial production of SCFAs, which drastically increased after 12 h of incubation. Albeit the acetate concentration in fermented ERFs were similar to that of the control sample, both ERFs yielded significantly higher levels of propionate, butyrate and total SCFAs in the period of 24–48 h than the control. Butyrogenic nature of ERF-Rf was manifested by its significantly higher butyrate molar percentage and production rate in the period of 24–48 h. Thus as hypothesized, sorghum ERFs behaved as prebiotic substrates as they induced the growth and proliferation of beneficial bacteria with subsequent production of important individual SCFAs via fermentation of MACs in sorghum ERFs. Both ERF fractions caused a progressive fermentation of protein as reflected by the continuously increased AN concentration in the media. Albeit pH values in the sorghum ERF media were significantly lower than that of the control, pH increased successively in the periods of 6–24 h and 6–48 h in ERF-Wh and ERF-Rf, respectively. Though sorghum ERFs had beneficial influence on gut microbiota and SCFAs production, they can infer negative effects on host health because of the higher AN concentration and high pH, in particular regarding ERF-Rf, despite of its butyrogenic nature. Higher protein fermentation in ERFs indicated low availability of MACs regardless of the higher RS contents, which might be due to the structure and organization of starch granules according to previous studies. Thus, we propose to conduct further studies to identify methods to improve MACs in sorghum digestive enzyme-resistant fraction in order to improve its utilization as a human food with therapeutic claims on gut health.

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