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Research Article

Effects of Fermentation by Yeast and Amylolytic Lactic Acid Bacteria on Grain Sorghum Protein Content and Digestibility

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Despite many advantages to its cultivation, grain sorghum is an underutilized crop because of low nutrient availability, particularly protein digestibility, due to antinutritional compounds in the grain and by moist-heat cooking. Some of these concerns can be mitigated by how the grain is processed. Fermentation is one processing method that can improve digestibility and at the same time concentrate protein in a substrate. In this experiment, grain sorghum was subjected to different treatments and fermented with baker's yeast *(Saccharomyces cerevisiae)* and an amylolytic species, *Lipomyces kononenkoae*, to improve and increase protein content. The effects of pasteurization or sterilization of the substrate, nitrogen supplementation, amyloglucosidase addition, and coculture with *Lactobacillus amylovorus* were examined. After fermentation, baker's yeast samples treated with enzyme increased in crude protein, from 9% in unfermented grain to approximately 27% after treatment. Nitrogen supplementation accelerated protein enrichment and was a significant factor at 24 hours of fermentation. Both types of yeast increased pepsin digestibility of sorghum protein compared to thermally processed control samples. The ratio of phytate to protein was reduced by both yeast species. *L. kononenkoae* reduced phytates in the substrate but did not enrich protein content. The lactic coculture had no significant effect on measured responses.

1. Introduction

Grain sorghum is a staple food for people and their livestock all over the world, with particular importance in arid parts of Africa and Asia. Sorghum is a significant source of dietary protein despite being relatively low in protein and, like many cereals, deficient in lysine as well [1]. Additionally, the presence of antinutritional factors interferes with digestion of the protein the grain does contain [2]. Wet heat processing also reduces protein digestibility even further through various mechanisms including protein crosslinking, which may cause resistance to the digestive enzymes of monogastric organisms [3]. These issues limit the use of sorghum in food and feed applications; though grain sorghum contains roughly the same amount of protein as maize, the digestibility of sorghum protein is generally lower [3].

Sorghum's dietary importance and potential for expanded applications have driven work for improving its protein quality. Cultivars with low or no tannin content generally have better protein digestibility than high-tannin grain, but at the cost of reduced pest resistance, which increases losses in the field. Breeding for cultivars with higher protein is another approach; however, increased protein in the grain does not necessarily translate into more protein metabolized by the consumer [4]. Postharvest processing of the grain can be used to improve protein quality and digestibility. Chemical and physical processing such as alkali treatment and dehulling can decrease tannins and phytates; removal of these compounds correlates with higher protein digestibility [5].

Fermentation is another way of increasing protein digestibility. Many traditional preparation methods for sorghum foods involve a fermentation step, and studies of these methods show nutritional improvements [4]. Lactic fermentation is commonly employed in making porridges, breads, and other sorghum food products; studies of these fermented foods have shown higher digestibility and improved amino acid profile compared to foods prepared from unfermented grain [6, 7]. Fermentation has also been used to increase the

protein content of plant matter, generally for feed applications.

Yeasts are often used in protein production applications; yeast biomass can be used directly as a protein supplement [8, 9] or as part of fermentation byproduct as with distiller's grain [10, 11]. Protein is increased either by the accumulation of microbial biomass or by the concentration of protein already in the substrate as carbohydrates are consumed. The mechanism by which enrichment occurs is dependent on the substrate system and the organism used.

The development of an inexpensive processing method that could both improve the digestibility of protein in sorghum grain and increase the protein content could make it a more desirable ingredient for food or feed applications. The goal of this research was to determine conditions for a fermentation process with yeast and lactic acid bacteria to enrich and improve protein content in grain sorghum. An amylolytic strain of yeast was compared to commercial baker's yeast in the presence of nitrogen supplements, amyloglucosidase, and coculture with an amylolytic *Lactobacillus* species. This paper is based on the Master of Science thesis by Day [12].

2. Materials and Methods

2.1. Culture Preparation. Dry baker's yeast *(Saccharomyces cerevisiae)* (AWG Brands, Kansas City, KS) was purchased from a local grocery store. Cultures of *Lactobacillus amylovorus* NRRL B-4540 and amylolytic yeasts were obtained from the USDA Agricultural Research Service. Five yeast strains documented to have the ability to produce amylases and glucoamylases were available: *Pseudozyma tsukubaensis* NRRL Y-7792, *Piskurozyma capsuligena* NRRL Y-6355, *Saccharomycopsis fibuligera* NRRL Y-1062, *Schwanniomyces occidentalis* var. *occidentalis* NRRL Y-2477, and *Lipomyces kononenkoae* NRRL Y-11553. The cultures arrived as lyophilized pellets, which were aseptically transferred to cryovials containing 0.6 ml sterile YM broth. Each vial was streaked to its own YM agar plate and grown at 26 ± 2°C for approximately 3 days. The plates were stored refrigerated and used to propagate the inoculation cultures. The baker's yeast was similarly cultured, with an aqueous suspension of yeast granules initially streaked to YM agar plates. An isolated colony was streaked to a second plate, which was used to propagate inoculum for the rest of the experiment.

2.2. Amylolytic Yeast Selection. The amylolytic yeasts were screened for fermentation activity of the sorghum meal. New YM agar plates were streaked for each organism. After growth, 20 mg of cells were collected from each plate with a sterile loop. These cells were transferred to Erlenmeyer flasks containing 50 ml sterilized YM broth. The flasks were incubated in a MaxQ 4450 orbital shaker (ThermoScientific, Waltham, MA, USA) at 250 rpm and 27°C for 9 hours. Then 18 ml of fresh YM was added to each flask and the shaking was increased to 275 rpm to ensure aeration. Incubation was continued for another 9 hours, which brought the total incubation time to 18 hours total. Cells were counted with a hemocytometer to choose the inoculum volume, between 15 and 30 ml of broth for approximately 10^8 cells. The inoculum was transferred to centrifuge tubes and centrifuged in an Allegra X-22R (Beckman Coulter, Brea, CA, USA) at 1450 g for 10 minutes. The growth medium was decanted and the cells suspended in 3 ml sterile DI water to transfer them to the sorghum slurry.

Sterile bottles containing a 25% w/v sorghum meal slurry were cooked in an 86°C bath for 90 minutes, cooled, and inoculated with each organism. The inoculated samples were held in a water bath at 35°C for 48 hours. The sample bottles were weighed at 0, 24, and 48 hours of fermentation, and changes in weight were recorded. Decrease in substrate weight as the fermentation progressed was used as an indicator of the ability of the microorganism to metabolize the substrate. The amylolytic organism associated with the most substrate weight lost was selected for the experiment; protein content was tested as well.

2.3. Preparation of Inoculum for Fermentation Process. Approximately 48 hours before a fermentation run, frozen vials containing approximately 5×10^7 colony forming units (CFU) of *Lactobacillus amylovorus* were thawed and incubated in 100 ml of sterile MRS broth at 37°C for 18 hours. Aliquots containing at least 1×10^8 cells were stored in centrifuge tubes at −20°C until fermentation.

Yeast inoculum for the experiment was prepared by harvesting 0.08 grams of isolated yeast colonies from YM agar plates that were previously streaked and incubated at room temperature for approximately 2 days. The yeasts were placed in flasks containing 200 ml of YM broth and incubated at 26 ± 2°C with continuous shaking at 250 to 300 rpm. The baker's yeast was incubated for 18 hours and the amylolytic yeast was incubated for 9 hours. After incubation, the cells were counted using a Bright Line hemocytometer (Hausser Scientific, Horsham, PA, USA). The broth cultures were divided into aliquots of equal volume, containing approximately 1×10^8 baker's yeast cells or 1×10^9 amylolytic yeast cells. A large number of amylolytic yeasts were used because the cells were much smaller than the other yeast species. A suspension of centrifuged cells in sterile water was used to inoculate the sorghum substrate.

2.4. Fermentation Process. A five-factor two-level factorial experimental design with a total of 32 treatments (Table 1) was used to test the effects of yeast type, thermal process, nitrogen supplementation, amyloglucosidase treatment, and influence of a lactic coculture on the final protein content in the fermented sorghum. For the preparation of the substrate, a single lot of food-grade USDA organic white grain sorghum (Pleasant Hill Grain, Hampton, NE, USA) was ground and sieved through #18 mesh screen. The substrate was prepared as follows: forty grams of the sieved sorghum meal was weighed into sterile glass media bottles. For the samples that received nitrogen supplementation, a 3 : 1 blend of ammonium sulfate and yeast extract was added to the dry sorghum meal at a rate of 2% of the grain weight, which was equivalent to 0.8 grams per sample. Next, sterile deionized water was added to each bottle at a 3 : 1 v/w ratio (120 ml) and the

Table 1: Experimental design.

Yeast species	Thermal treatment	Nitrogen supplement	Enzyme addition	Lactic coculture
S. cerevisiae (baker's yeast)	Pasteurization	Yes	Yes	Yes
				No
			No	Yes
				No
		No	Yes	Yes
				No
			No	Yes
				No
	Sterilization	Yes	Yes	Yes
				No
			No	Yes
				No
		No	Yes	Yes
				No
			No	Yes
				No
L. kononenkoae	Pasteurization	Yes	Yes	Yes
				No
			No	Yes
				No
		No	Yes	Yes
				No
			No	Yes
				No
	Sterilization	Yes	Yes	Yes
				No
			No	Yes
				No
		No	Yes	Yes
				No
			No	Yes
				No

thermal treatments were applied immediately. Sterilized treatment bottles were autoclaved at 121°C for 15 minutes. Pasteurized treatment bottles were placed in a water bath maintained at 86°C for 90 minutes. The pasteurized and sterilized substrate samples were cooled to room temperature while the yeast and *Lactobacillus* cultures were prepared for inoculation. Amyloglucosidase was added to the bottles designated for enzyme treatment. Frozen *L. amylovorus* cultures were thawed, centrifuged, and decanted, and the cells were resuspended in 2 ml of phosphate buffered saline (PBS) and added to the coculture treatment samples. The substrates were then inoculated with one species of yeast.

The bottles were capped loosely to allow fermentation gases to vent and the initial weight of each bottle was recorded before placement in a 35°C water bath. After 9 hours of incubation, the bottles were removed from the bath, wiped dry, and weighed again. The pH of each slurry sample was taken, and any pH below 4.5 was adjusted to pH 5 with calcium carbonate. One gram or less of calcium carbonate was generally sufficient to raise the pH. After pH adjustment was complete, all bottle weights were recorded again before they were returned to the bath. Bottle weights were measured before and after 20-gram slurry samples were collected, at 24-hour intervals. Slurry samples were dried in an oven at 60°C, transferred to sterile containers, and held at room temperature until analyses were conducted.

2.5. Chemical and Physical Analyses. Dried samples were analyzed for percent moisture using an Omnimark μ-wave moisture analyzer (Sartorius Corporation, Bohemia, NY, USA) and assayed for total nitrogen by combustion using an Elementar Rapid N III unit (Elementar Analysensysteme GmbH, Langenselbold, Germany). Ammonia nitrogen was determined by placing one gram of sample and 30 ml of a 2 N solution of potassium chloride in a centrifuge tube. The tubes were shaken in the high setting for 10 minutes on an Eberbach shaker (Eberbach Corporation, Ann Arbor, MI) to extract ammonia from the samples. The filtered extract was analyzed

for ammonia nitrogen content with a Skalar Autoanalyzer (Skalar Analytical, Breda, The Netherlands). Crude protein was calculated from the difference between total nitrogen and ammonia nitrogen, using a factor of 6.25 to convert elemental nitrogen to protein.

Protein digestibility was determined by a version of AOAC method 971.09 modified to use three concentrations of pepsin (0.002%, 0.02%, and 0.2%) to make the method more sensitive, instead of the single concentration used in the standard AOAC method. Phytate was quantified by a colorimetric method [13]. Amino acid profiles of select samples were obtained using a hydrolysis method based on AOAC 994.12 without performic acid oxidation, which is used to determine cysteine and methionine. The hydrolyzed samples were analyzed by LC/MS using a Phenomenex EZFaast amino acid analysis kit (Phenomenex Inc., Torrance, CA). The amino acid analysis was performed by the Tyson Foods, Inc., Food Safety and Research Laboratory.

2.6. Statistical Analysis. Statistical analyses were performed using SAS 9.4 software (SAS Institute Inc., Cary, NC, USA). Effects of individual treatments and treatment interactions on crude protein were determined using analysis of variance (ANOVA), and means were compared with Fisher's least significant difference (LSD) test. The same method was used to compare mean phytate content for select samples.

3. Results and Discussion

3.1. Amylolytic Yeast Selection. After 48 hours of incubation, substrates inoculated with all strains of amylolytic yeasts showed weight loss. However, the flask inoculated with *Lipomyces kononenkoae* exhibited the greatest decrease in sample weight (Figure 1). In preliminary experiments performed with baker's yeast (procedure not shown), it was found that weight loss correlated with increasing amounts of protein (Figure 2). Therefore, the amount of weight lost was used as a basis to judge how well the fermentation could accumulate protein. Based on these results, *L. kononenkoae* was selected to be the amylolytic yeast species in subsequent experiments. The fermented substrate was tested and showed a modest improvement in percent protein (12%) compared to a control (9%); substrate fermented by other organisms showed no difference from the control. *L. kononenkoae* is documented to produce both extracellular amylases and glucoamylases [14, 15], which may make it more effective at assimilating carbon from starch.

3.2. Fermentation. Of the five factors tested, only yeast type and enzyme treatment had a significant effect on the final crude protein content ($p > 0.05$) (Table 2). Samples fermented with baker's yeast and treated with amyloglucosidase increased crude protein from 9% to 27%. No significant increase in protein was seen in samples subjected to one of these treatments but not the other, and no samples fermented with *L. kononenkoae* showed any significant increase in protein content (Figure 3). None of the other treatments had

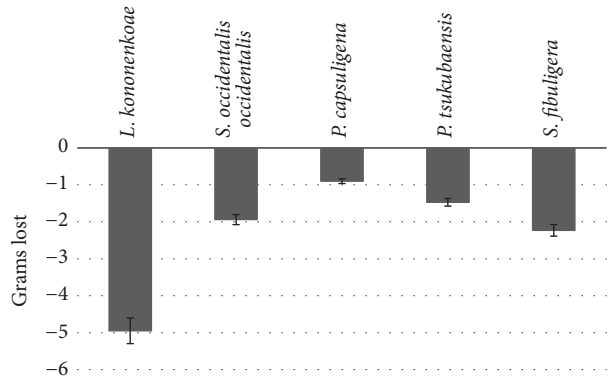

Figure 1: Substrate weight loss (mean ± standard error) taken as an indicator of yeast ability to ferment the sorghum substrate. Original substrate was approximately 160 grams (40 g sorghum and 120 g sterile deionized water).

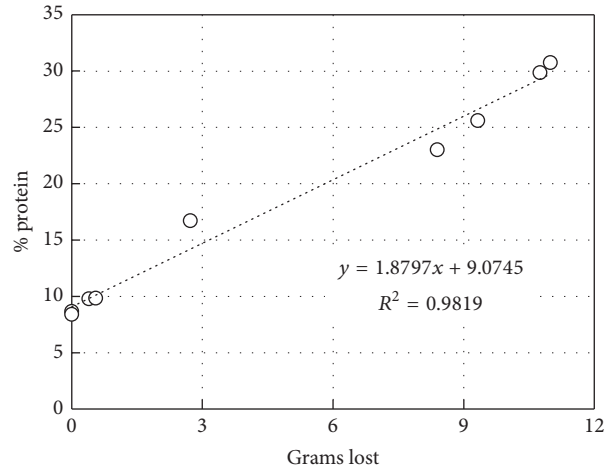

Figure 2: A correlation between substrate mass loss, at expenses of carbon dioxide escaping the flasks, and percent protein increase due to biomass production observed in baker's yeast fermentation. Original substrate was approximately 160 grams (40 g sorghum and 120 g sterile deionized water).

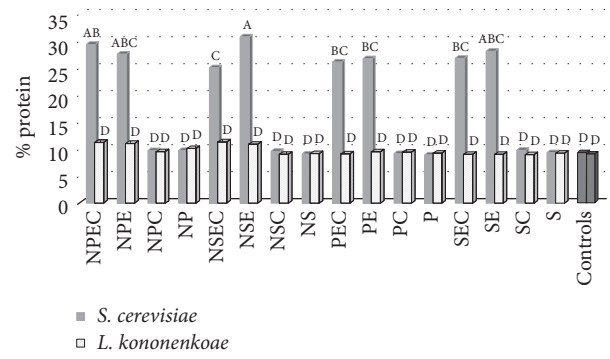

Figure 3: Crude protein by yeast species after 48 hours of fermentation, compared to pasteurized and sterilized controls. N: nitrogen supplement; P: pasteurized; S: sterilized; E: enzyme treatment; C: coculture. Same letters are not significantly different, $p < 0.05$.

Table 2: Significance of treatments on percent crude protein at 48 hours of incubation. Y: yeast species; N: nitrogen supplement; T: thermal treatment; E: enzyme; C: coculture. Significant factors ($p < 0.0001$) are Y, E, and Y ∗ E.

Source	ANOVA SS	F value	Pr > F
Y	1819.362	321.4	<.0001
N	0.138928	0.02	0.876
Y ∗ N	1.471635	0.26	0.6119
T	19.61861	3.47	0.0672
Y ∗ T	0.730108	0.13	0.7207
N ∗ T	2.449287	0.43	0.5130
E	2158.123	381.3	<.0001
Y ∗ E	1801.939	318.3	<.0001
N ∗ E	0.410555	0.07	0.7886
T ∗ E	10.94310	1.93	0.1692
C	2.307640	0.41	0.5254
Y ∗ C	1.353275	0.24	0.6265
N ∗ C	4.532704	0.8	0.3742
T ∗ C	0.237009	0.04	0.8385
E ∗ C	3.365257	0.59	0.4435

a significant effect on percent protein; however, some treatments affected phytate content and protein digestibility.

Overall protein digestibility decreased in thermally processed samples compared to raw grain, an effect observed in other studies [16]. The mechanisms behind this are not completely understood but may include formation of protein-starch complexes, as well as crosslinking of proteins in general and of sulfur-containing amino acids in particular. These structural changes can cause resistance to digestive proteases. Digestibility of fermented samples increased compared to samples that were thermally processed but not fermented. Digestibility at the lowest pepsin concentration of 0.002% was ten to twenty percent higher in both the fermented and the raw grain samples compared to the thermally processed unfermented controls. The percent digestibility results obtained here were similar to literature values for digestibility of sorghum grain [3]. Increased digestibility compared to thermally processed unfermented controls was observed after fermentation by both species of yeast. Similar results have been seen in yeast fermentation of other grains [17]. The digestibility of pasteurized samples fermented with baker's yeast was similar to raw grain, while that of sterilized baker's yeast samples was slightly lower (Figure 4). Natural fermentation was observed in the pasteurized control samples (evidenced by gas production, off-odors, and other signs of microbial growth) and appeared to increase digestibility compared to the initial condition of that sample, a result seen in other studies on fermentation by indigenous microorganisms [18, 19]. Fermentation by deliberate yeast inoculation resulted in slightly higher protein digestibility compared to digestibility after wild fermentation.

3.3. Phytate Levels. In this experiment, the baker's yeast samples with the highest protein enrichment had higher phytate levels than the unfermented control samples (Figure 5). This result was somewhat unexpected, as microorganisms use phytases to degrade phytates in plant materials to obtain phosphorus [20, 21]. For a protein enrichment fermentation, simply evaluating the phytate content could give an incomplete view of the effect of the fermentation process. The carbohydrate reduction that resulted in higher percent protein in these samples would concentrate other components present in the grain substrate as well. The amount of phytate relative to the amount of protein should have more of an effect on protein digestibility than absolute phytate quantity. Therefore, the amount of phytate was compared to the amount of protein in the samples. The phytate/protein ratio was lower in the fermented samples than the control samples, as seen in Figure 5. For baker's yeast, the ratio of phytate to protein is much lower than in the control samples. The *L. kononenkoae* samples that showed growth despite the lack of protein concentration were also tested for phytate. These samples exhibited decreased phytate/protein ratios compared to the control samples, even without protein enrichment; absolute phytate in these samples was lower than phytate in raw grain and control samples as well. This shows that the fermentation did reduce phytate content.

3.4. Amino Acids. The amino acid assay showed the most abundant amino acids to be glutamic and aspartic acids, leucine, proline, and valine. A high amount of these amino acids is typical in many grains [22]. Without the performic acid oxidation step, this method was unable to detect cysteine or methionine. The results seen here are similar to amino acid profiles of nontannin sorghum found in the literature [23, 24]. Some studies show changes in amino acid contents as a result of fermentation. Kazanas and Fields [25] noted increased lysine, leucine, isoleucine, and methionine without any increase in protein. In this work, however, the percentages of amino acids in fermented and unfermented sorghum are very similar (Table 3). This would imply the bulk of the protein is native sorghum protein, rather than yeast or other microbial proteins which would have different amino acid profiles [8]. The fermentation process does not significantly alter the sorghum protein composition, as the fermentation organisms do not metabolize entire proteins in large amounts. There was also little difference between the ratios of amino acids in raw and thermally processed controls, indicating that the type of thermal process had little effect on protein structure. There is some speculation that a contributing factor to reduced digestibility in cooked sorghum is isomerization of amino acids [3]; however, this effect is not implied by the results here.

4. Conclusion

The combined effect of the increase in protein content and digestibility yielded over 20% digestible protein in the pasteurized sorghum fermented with baker's yeast, at even the lowest concentration of pepsin used.

This work has shown the potential for improvement of sorghum grain protein through yeast fermentation. Though the process reduces the total grain mass, the increase in protein could yield a more valuable product than the original

Table 3: Selected amino acids by sample treatment. B: baker's yeast; L: *L. kononenkoae*; N: nitrogen supplement; P: pasteurized; S: sterilized; E: enzyme; C: coculture; Con: control samples at 0 or 48 hours.

	Raw Con	PCon-0	PCon-48	BNPEC	BNPE	BNSEC	BNSE	LNSEC	LNSE	Serna-Saldivar and others 1987 raw	Serna-Saldivar and others 1987 cooked
Aspartic acid	9.29	8.85	9.60	9.08	7.69	7.02	8.88	8.99	9.19	6.83	6.18
Glutamic acid	40.87	33.4	33.76	29.90	23.90	19.66	24.42	36.75	29.70	21.74	21.52
Isoleucine	4.23	5.11	5.24	5.13	5.32	5.32	5.29	4.37	5.26	4.20	3.97
Leucine	5.93	6.63	6.29	6.85	7.62	6.86	7.74	6.00	6.80	14.08	13.80
Lysine	3.25	2.82	2.29	3.17	1.88	5.82	1.79	2.35	1.60	2.21	2.32
Proline	5.83	7.35	7.47	7.09	8.98	7.95	8.61	8.04	8.44	8.82	9.27
Serine	3.35	4.38	4.17	4.67	6.39	6.40	5.87	5.29	5.02	4.20	4.42
Threonine	2.78	3.49	3.59	4.12	5.31	5.41	4.90	4.10	3.96	3.15	3.20
Valine	5.18	6.46	6.75	7.27	7.99	7.89	7.75	6.42	7.33	5.25	5.30

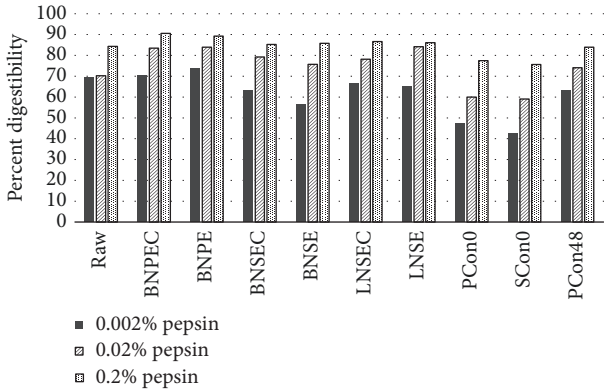

FIGURE 4: Pepsin digestibility of select samples at three pepsin concentrations. B: baker's yeast, L: *L. kononenkoae*, N: nitrogen supplement; P: pasteurized; S: sterilized; E: enzyme; C: coculture; Con: control samples at 0 and 48 hours.

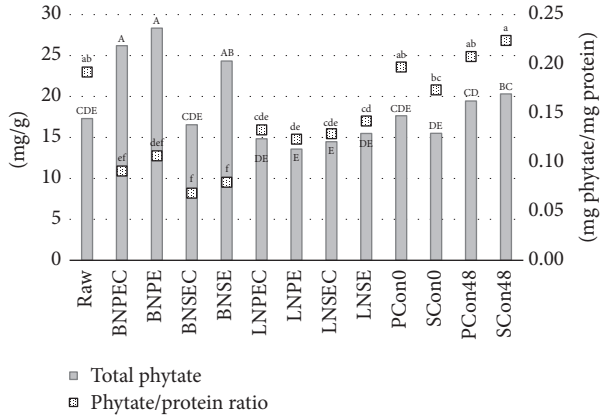

FIGURE 5: Total phytate and phytate/protein ratio for select samples. B: baker's yeast; L: *L. kononenkoae*; N: nitrogen supplement; P: pasteurized; S: sterilized; E: enzyme; C: coculture. Same letters are not significantly different, $p < 0.05$.

grain. Increased demand for sorghum ingredients could allow commercial farmers to shift production to sorghum during drought conditions and produce a profitable harvest even if other more water-intensive crops fail. One potential application of the work here could be to test the effect of yeast fermentation on the types of tannin sorghum commonly grown in other areas of the world. Development of an efficient fermentation process that could increase both protein digestibility and content in these grains could improve the nutritional quality and utility of this staple crop.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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