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RESEARCH PAPER

Effect of solid state fermentation with *Rhizopus oryzae* on biochemical and structural characteristics of sorghum (*Sorghum bicolor* (L.) Moench)

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Abstract

Sorghum (*Sorghum bicolor L. Moench*) is a major cereal consumed widely in Asian and African countries as a human food, while in other countries it is consumed as a feed. Despite having an adequate nutrient content, its use in food is limited, mainly due to the content of antinutritional factors such as condensed tannins. Fermentation technology has been shown to improve the nutritional characteristics of products and help to decrease the content of these anti-nutritional compounds. The study was aimed to evaluate the effect of the solid-state fermentation (SSF) process with *Rhizopus oryzae* (MUCL 28168) on the chemical composition, physical-structural characteristics and nutritional value of sorghum grains. The physical structure of the sorghum starch granules was modified during the fermentation process due to the action of the enzymes, mainly amylases, produced by the microorganism. The results showed that the total carbohydrate content decreased by 1.74%, while the crude protein content increased by 23.87%. Moreover, there was an increase of 19.62% in protein digestibility. For the amino acids, some increased their concentration while others decreased it. An important result was that the lysine and tryptophan content were higher at the end of the fermentation. The fermentation process improved the nutritional characteristics of sorghum, therefore the use of sorghum can be considered for human and animal consumption.

Keywords: Sorghum, condensed tannin, solid-state fermentation, filamentous fungi

Sorghum (*Sorghum bicolor L. Moench*), because of its production and use, is an important cereal in the world, competing for a position in the market against other products such as rice, wheat, barley, and maize. According to USDA (2016), global sorghum production in 2016/2017 was estimated at 62.50 million tons, an increase of 2.9 million tons (4.87%) over the previous year. In Colombia, sorghum is a crop that has gained importance in recent years, improving its production and productivity (FENALCE 2016). Colombia is the number 25 producer, contributing 0.4% of world production (Espinal *et al*. 2005). Due to its agro-climatological and nutritional characteristics, sorghum is a cereal widely used in human food in countries such as Africa and Asia, while in other countries it is basically used in animal feed, which has been a dynamic element in the field of the global consumption of sorghum. Their demand has been the main driving force to raise world production and international trade of this cereal (López Ortiz *et al.* 2011).

Despite the nutritional value, the use of sorghum is

limited due to its content of antinutritional factors, which interfere with the digestibility of proteins and carbohydrates and with the bioavailability of minerals, preventing the absorption of the nutrients present. Therefore, the reduction of these compounds is essential for the improvement of the quality and the promotion of its use as food (Schons *et al.* 2011). Different techniques have been evaluated to reduce the content of antinutritional factors, such as soaking in water, chemical treatments with solvents, mechanical rupture, heat treatments, the use of exogenous enzymes and the processes of germination and fermentation (Savón and Scull, 2006).

Solid state fermentation (SSF) is a biological process carried out by microorganisms which catalyze nutrients, synthesize nutrients, secondary metabolites and other compounds under aerobic or anaerobic conditions. It is fundamentally characterized by being a process carried out in the nearly absence of free water in the substrate. Due to their ability to adapt to the conditions of the process, the most commonly used microorganisms are filamentous fungi (Bhargav *et al.* 2008; Chen, 2013). SSF has been shown to help a significant decrease in the antinutritional factors. The SSF also increase the content of essential amino acids and soluble proteins, reducing the content of mycotoxins, helping to prevent oxidative rancidity, among others (Nout and Aidoo 2010; Savón and Scull 2006). Due to the enzymatic activity of microorganisms, during SSF the texture, flavor and nutritional composition are modified, represented by the change in the content of proteins, lipids and carbohydrates. (Munguia Pérez *et al.* 2008; Perez Quilantan 1996; Mitchell *et al.* 2003; Thomas *et al.* 2013).

Filamentous fungi are the most important group of microorganisms used in SSF processes due to their physiological, enzymatic and biochemical properties. Among the microorganisms most used are those belonging to the genus *Rhizopus*, which have been used for the production of products such as Tempeh, due to the organoleptic and nutritional characteristics that it provides to the product (Nout and Aidoo 2010). Another feature that makes them widely used is that they are categorized as GRAS (Generally Recognized As Safe) according to the USDA since there are no reports of being mycotoxin producers; this classification is highly desirable when it is to be produced for animal and human food (Papini *et al.* 2010; Munguia Pérez *et al.* 2008). Also, the stability of the fermented products is superior, thanks to the release of organic acids during the metabolism of the fungi, which act like antimicrobial agents, due to the decrease of the pH of the product. Thus, this study was aimed to evaluate the effects of the SSF process with *Rhizopus oryzae* (MUCL 28168) on the chemical composition, physical-structural characteristics and nutritional value of sorghum grains.

MATERIALS AND METHODS

Row Materials

Sorghum was obtained from a local market in the city of Cali (Colombia), then it was transported to the laboratory of Microbiology and Applied Biotechnology of the Universidad del Valle, where it was separated and packed in 500 g polyethylene bags, which were vacuum sealed and stored at 5°C.

Composition Analysis

Moisture (AOAC 930.15), fat (AOAC 920.39), ash (AOAC 942.05), were analyzed using the Association of Official Analytical Chemists procedures (A.O.A.C, 1980). The crude protein content (N × 6.25) was estimated using the Kjeldahl method (ISO 1871); the fat was determined by extraction using Soxhlet apparatus; the fiber was measured using the standard method (ISO 6541), and the ash content was analyzed by weight before and after incineration at 550 °C for 24 hours. All analyses were performed in triplicate.

Inoculum preparation

Spores of *Rhizopus oryzae* (MUCL 28168), belonging to Applied Microbiology and Biotechnology laboratory collection growing on potato dextrose agar (PDA) in 250 mL Erlenmeyer flasks (8 days old) were harvested by homogenization with distilled water. Then, the methodology described by Londoño (2015)

was applied to obtain dry spores, which were used during all the experiments. The spores were counted in a Neubauer cell.

Preparation of samples

The sorghum was milled and sieved using a set of ASTM series sieves. The grains retained in the meshes 10, 16 and 30 were selected, corresponding to size ranges from (a) 1.2 to 2.0 mm (b) 0.6 to 1.2 mm and (c) 0.3 to 0.6 mm. Subsequently, the moisture of the sorghum grains was adjusted to 35% and 1 g of dry spores/100 g was added to the substrate and homogenized for 5 min on a sterile aluminum tray.

Solid-state fermentation (SSF) process

The parameters evaluated during the solid-state fermentation process were: temperature (28 °C, 34 °C), flow air (60 mm^3 / min, 100 mm^3 / min), particle size (0.3 mm, 1.2 mm) and wheat bran concentration (0%, 5%). The experiments were carried out in a column bioreactor, consisting of a system of columns 4 cm in diameter and 20 cm in height, which was packed with 100g of previously inoculated moist sorghum. These columns were placed in a thermos regulated water-bath at 28 or 34 °C and were connected to a hose system to allow aeration.

60 g of dry solid was taken, which was moistened with water to the indicated value. The samples were inoculated and incubated under controlled temperature conditions for 24 hours. After fermentation condensed tannins content was measured using the method of vainillina-HCl, total soluble sugars were measured using the Dubois *et al.*, (1956) method; the reducing sugars were measured using DNS method; the soluble proteins were measured using Lowry (Lowry *et al.* 1951) method and pH was measured using AOAC procedures (AOAC 943.02/90).

According to reported by Londoño *et al.* (2016) the parameters selected were: 32.97 °C, air velocity 84.11 mm^3 min^{-1}, 1.16% wheat bran and particle size 0.82 mm. At these process parameters, fermentation kinetics were carried out measuring: CO_2 production, pH, condensed tannins, soluble proteins, total and reducing sugars every 3 h for 24 h. The CO_2 production values were used to calculate the specific growth rate and other kinetic parameters, as described by Raimbault *et al.* (1997).

Characterization of sorghum after solid state processes

The physical characterization of sorghum was carried out using an Electron Scanning Microscope (SEM), G3 ProX reference from Phenom-World (www.phenom-world.com), with a capacity of 100,000X and a resolution of 17 nm per pixel. Amino acid analyses were performed by HPLC using an AGILENT-ZORBAX Eclipse® AAA Analytical 4.6×150 mm-5 μm column.

RESULTS AND DISCUSSION

Sorghum composition

The sorghum had a reddish coloration, which is an indication of the content of condensed tannins. Sorghum presented a spherical shape with mean variable diameters of 4.20 mm ± 0.6 mm. The characterization of the material is presented in Table 1. The macronutrient highest concentration in sorghum was carbohydrates, representing 89.62% of all components. The carbohydrates of this cereal are mainly present in the form of starch, which corresponds to a reserve polysaccharide of the plants. Of the total carbohydrates, crude fiber represented 2.41%. The crude protein of this cereal was found in a range of 7.33%, being the second macronutrient with higher grain content, these proteins are represented mainly by prolamins. Prolamins (kaffirins) are the main storage proteins. These proteins are located along with the starch in the endosperm and constitute approximately 70% of the total protein (Kuo *et al.* 2013). Lipids were 1.86% of the total nutrients in the cereal, with most lipids being polyunsaturated fatty acids (Queiroz *et al.* 2011). The values found here were lower than those reported by López Ortiz *et al.* (2011) but were higher than those found in some maize varieties Betancourt Botero (2014) which shows the potential of this cereal to be used in the industry.

Table 1: Characterization of raw and fermented sorghum

	Result	
Parameter	**Raw sorghum**	**Fermented sorghum**
Moisture (%)	9.98 ± 0.34	30.43 ± 0.34
Total carbohydrates*	89.62 ± 0.50	88.06 ± 0.50
Reducing sugar (g glucose/g substrate)	2.95 ± 1.43	40.17 ± 0.18
Total sugar (g glucose/g substrate)	15.12 ± 2.95	54.50 ± 1.72
Crude protein*	7.33 ± 0.50	9.08 ± 0.50
Soluble protein*	57.34 ± 0.50	68.59 ± 0.50
Crude fiber*	2.41 ± 0.50	2.58 ± 0.50
Ash*	1.19 ± 0.50	1.50 ± 0.50
Fat*	1.86 ± 0.50	1.36 ± 0.50
pH	6.30 ± 0.50	4.50 ± 0.50

** Values are % and each value is an average of three replicates expressed on dry weight basis.*

Solid state fermentation

For each treatment, different values of pH, soluble protein, condensed tannins, total sugars and reducing sugars were obtained (Londoño *et al.* 2016). In general, a pH reduction was observed when there was greater microbial growth, reaching a value of 4.36. Under aerobic conditions, some of the main metabolites of *R. oryzae* are fumaric acid, and lactic acid, which cause the pH of the medium in which it is growing to descend (Ghosh and Rani Ray 2011; Ruiz Badillo 2013). Studies carried out by Correia *et al.* (2005)Lactobacillus bulgaricus, Lactobacillus lactis, Pediococcus pentosaceus and Pediococcus cerevisiae on sorghum flours with other microorganisms have also found a decrease in pH, attributing this behavior to the synthesis of acids such as fumaric, citric and lactic acids.

The total sugars content increased in all treatments, reaching a maximum of 75.09 g / g, due to microbial growth in the substrate. The fungus, following the consumption of simple sugars, initiates a hydrolysis of the starch chains by the action of enzymes such as α-amylase, to perform their metabolic functions. This hydrolysis causes the large chains of starch to split into several units, which is reflected in an increase in sugars (Soccol *et al.* 1998). The hydrolysis of starch during the growth of the fungus in the substrate causes the release of glucose, the main reducing sugar, which results in an increase in this type of sugar for all treatments performed.

Regardless of the treatment, higher values of soluble protein (23.18 g/g) were obtained compared to untreated sorghum. Almost all prolamins of sorghum are linked to phenolic compounds such as tannins, forming complexes tannin-proteins (Montiel *et al.* 2012).

The high affinity of tannins to proteins is due to the numerous phenolic groups present in the tannin molecules that provide many sites for the formation of bonds with the carbonyl groups of peptides. The reactivity and affinity of these linkages are determined by the type, concentration, structure and molecular weight of the tannin, as well as by the degree of polymerization, conformation and molecular weight of the protein. Therefore, proteins with a high proportion of the amino acid proline, and an open and flexible three-dimensional structure show a greater affinity for the tannins (Nogueira 2011). *In vitro* studies have shown that in addition to tannin-protein complexes, phytate-protein complexes, also present in sorghum, are formed by electrostatic interactions involving linkages with the N-amino terminal groups, the s-amino groups of lysine, the imidazole group of histidine and the guanidyl groups of arginine (Taylor and Taylor 2002). Most of these complexes are insoluble, making sorghum digestibility and soluble protein concentration quite low in sorghum without any treatment (Kuo *et al.* 2013; Duodu *et al.* 2003).

During the fermentation process, by the action of tannase enzymes and phytases produced by *R. oryzae* (Londoño-Hernández *et al.* 2017; Chávez-González *et al.* 2012; Ghosh and Rani Ray 2011), the tannin-protein and phytate-protein complexes are hydrolyzed, promoting the release of the bound protein and thus increasing the soluble proteins content. Taylor and Taylor (2002) proposed that during the fermentation

the proteins (prolamin and glutelin), undergo structural changes, that make this type of proteins were more susceptible to the enzymatic attack of the pepsin, increasing, therefore, the digestibility of the sorghum. Similar results to the present study are found by Abdelseed *et al.* (2011), Kuo *et al.* (2013), Elkhalifa *et al.* (2006) in trials with sorghum, and Nnam and Obiakor (2003) with other types of grains. In these studies, the fermentations were carried with different microorganisms.

Likewise, in all fermentations, low values of condensed tannins were found compared to the initial value (2.24%), the lowest is 0.02%. Osman, 2004 and Kuo *et al.* (2013), performing traditional fermentations with sorghum flour, found considerable reductions in tannin and other phenolic compounds after the process, attributing this behavior to the action of enzymes such as tannases, phytases, others.

During fermentation kinetics, sugars and proteins during the first 12 hours showed a decrease in their concentration, increasing gradually after this time. This behavior is attributed to the rapid initial growth of microorganisms, taking soluble proteins and sugars simpler for their energy, the development of their metabolic functions and the production of biomass. Studies carried out by Taylor and Taylor (2002) indicate that soluble proteins are the first to be hydrolyzed during fermentation and that peptides released during this stage are taken by the microorganism for their growth. These results are corroborated by other authors (Nabila and Abdullahih 2003).

Hydrolysis during fermentation of the soluble proteins in the outermost layer of the large structures in which the starch granules are embedded leads to the release of these, leaving the protein matrix unaffected by proteolytic events during the fermentation (Elkhalifa *et al.* 2006), favoring the increase of the same, in the period between 6 and 21 hours of growth. At the end of this time, the decrease in the amount of protein is due to the action of proteases and may be linked to the onset of mycelial autolysis (Soccol *et al.* 1998).

In the case of condensed tannins, there was a total decrease of 86.29%. During the first 12 hours of the process, the concentration of condensed tannins varied from 2.5% to 0.02%, which is attributed to the metabolic activity of the fungus at this time; however, between 12 and 18 hours an increase occurred, that be due to the action of the tannase enzymes on the tannin-protein complexes that act by hydrolyzing these bonds, releasing these compounds, which are subsequently hydrolyzed by the action of these enzymatic complexes. This behavior is similar with represented by the proteins. Schons *et al.* (2011) used the microorganism *Paecilomyces variotii* for the fermentation process, finding a 58% reduction in tannin content. Abdelseed *et al.* (2011), found that after a natural fermentation process applied to different varieties of sorghum, the tannin content decreased by an average of 42.54%. Mugula *et al.* (2003) carried out a controlled fermentation with *Rhizopus oligosporus* in mixtures of sorghum with other cereals and determined that there was a reduction of up to 94% in tannin content. Hassan and El Tinay (1995) concluded that a natural fermentation process in sorghum varieties with the high and low content of condensed tannins presented reduction of 63% and 61.4%, respectively. El Khalifa and El Tinay (1994) achieved through natural fermentation a 92% decrease in the tannin content of sorghum flour.

The behavior of the pH is opposite to that of the other variables, decreasing steadily from 6.0 to 5.5 during the first hours, and drastically between 12 and 15 hours of processing until reaching 4.5, and after 21 hours presents an increase. This decrease in pH is explained by the growth of the microorganism, which used sugars to synthesize some compounds such as fumaric acid, lactic acid, among others, which cause the pH to fall. In addition, the hydrolysis of phenolic compounds also causes the release of acidic molecules. After 15 hours, pH behavior is attributed to the increased activity of proteolytic enzymes, which hydrolyze sorghum proteins releasing amino acids, peptides, and ammonia. The release of these compounds in the medium allows this pH to be maintained, and as the fermentation process continues and by the concentration of these, the pH

is increased. Similar behaviors during fermentation processes have been found by Nnam and Obiakor (2003). CO_2 is a fundamental variable because it is highly related to growth; the concentration of this compound increased with time showing between 12 and 15 hours the highest production.

Fig. 1 shows changes in the development of the fungus during the process. It is possible to observe that the mycelium increases with the passage of time corroborating the results obtained.

During the fermentation time, the kinetic parameters were calculated, which allowed to observe the efficiency of the process (Table 2). Protein/sugar yield was higher compared to other studies, possibly due to the nature of the substrate where the microorganism was developed and the conditions established, which allowed a greater release of proteins compared to the sugar consumed. It was also established that the rate of reduction of tannins, compared to the generation of CO_2 was 2.29. Likewise, it was determined that the appropriate processing time is 21 hours.

Characterization of fermented sorghum

To observe the effects caused by the degradation of the fungus, during the fermentation, on the structural organization of the sorghum, a fermented sample and an unfermented sample were observed by scanning electron microscopy. The image is presented in Fig. 2.

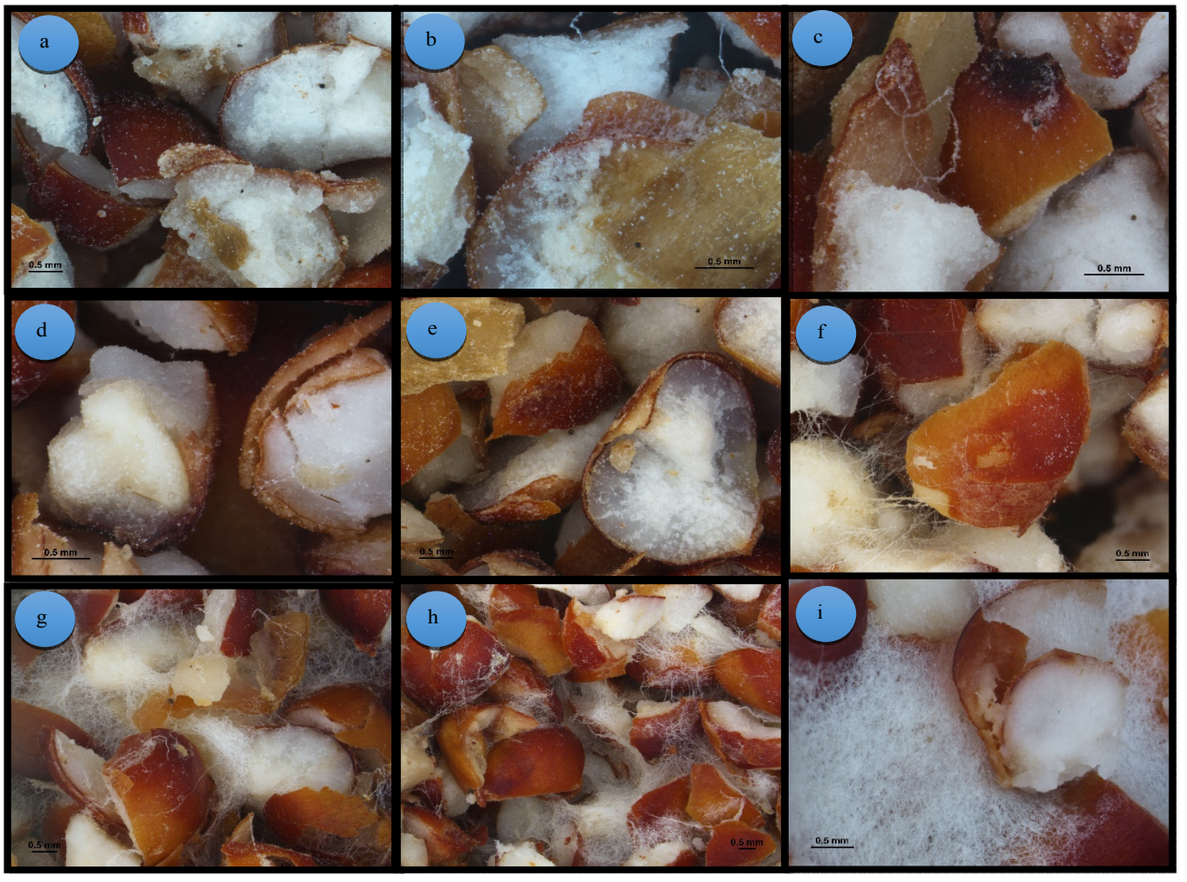
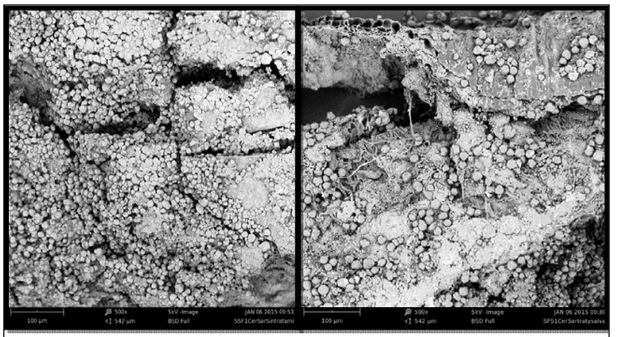


Fig. 1: Macroscopic view of the mycelium developed during the solid fermentation process with *Rhizopus oryzae* (MUCL 28168) on sorghum. (a) Initial (b) 3h; (c) 6h; (d) 9h; (e) 12h; (f) 15h; (g) 18 h; (h) 21 h; (i) 24h

Table 2: Kinetic data for the Rhizopus Oryzae (MUCL 28168) on sorghum

	Initial	Final
Total dry mass	49.56	38.81
Duration of the germination (h)	3	—
Time for maximum rate (h)	18 - 24	—
V Maximum CO_2 evolved rate (mg/h/gs)	1.18	—
Total CO_2 (mg/gs)	0.37	—
Duration of the exponential (h)	15	—
Specific growth rate (μ)	0.22	—
Protein (%wb)	19.96	28.18
Total sugars (%wb)	18.80	58.03
Loss in dry matter (g)	14.07	—
Yield Protein/Sugar	1.18	—
Reduction condensed tannins rate	2.29	—
Yield	0.25	—



(a) (b)

Fig. 2: View of the morphology of sorghum (a) before treatment (b) after the fermentation process, at 500x

It is possible to observe in Fig. 2- (a) how the starch granules in sorghum are completely enclosed in a very compact protein matrix, whereas in Fig. 2 - (b) the proteolytic and amylolytic events occurring during the fermentation impacted this structure deteriorating it and releasing the starch granules that were previously inside the coated structure. Similar observations have been made by Elkhalifa *et al.* (2006) in fermented cooked sorghum flours.

Some authors (Oliveira *et al.* 2010) have observed that the growth of the microorganism in the substrate can change its chemical composition due to the generation of extracellular enzymes to obtain nutrients and additionally produce other metabolites. These metabolites can enrich the substrates depending on the availability of nutrients. Microbial action uses the components available for their chemical and enzymatic processes (Blandino *et al.* 2003; Olanipekun *et al.* 2009; Othman *et al.* 2009).

Table 1 shows the effects on the proximal composition, showing a significant increase in the crude protein content of 23.87%, crude fiber of 7.05% and ash of 26.05%. There was also a decrease in the carbohydrate content of 1,746%, and fats of 26.88%, because of the microbial metabolism. According to the results already described, the reduction of starch is explained by the fact that the fungus uses carbohydrates for its development, being its main source of energy. The increase in fiber content may be related to the production of some polysaccharides by the fungus for the formation of their cellular structures, such as cellulose and chitin, as well as the reduction in fat content, since they must use some lipids for the synthesis of these compounds (Oliveira *et al.* 2010).

Protein digestibility is a nutritional characteristic of grain since high digestibility proteins are more susceptible to hydrolysis by proteolytic enzymes than low digestibility enzymes, thereby providing more amino acids and other nutrients from food (Duodu *et al.* 2003). Sorghum protein digestibility, expressed in pepsin, showed an increase of 19.62%, improving the nutritional quality of the grain, which is attributed to the release of hydrolytic enzymes such as pepsins, amylases and phytases from the microorganism during fermentation; these enzymes are capable of separating the crosslinking of the kaffirin bonds to reduce them to smaller molecules becoming more affordable by the proteolytic enzymes (Kuo *et al.* 2013). Abdelseed *et al.* (2011) found that after a traditional fermentation process in different lines of sorghum, the digestibility increased by 61.50%. Other authors have also found an increase in the digestibility of different varieties of sorghum with high tannin content, after natural fermentation processes (Elkhier and Abd-alraheem 2011; Hassan and El Tinay 1995).

The results of the amino acid profile of a fermented sample compared to an unfermented sample are presented in Table 3.

Table 3: Amino acid profile of raw sorghum and fermented sorghum

Amino acid(mg/100g)	Result	
	Raw sorghum	**Fermented Sorghum**
Aspartic acid	463.0	496.5
Glutamic acid	1352.0	1499.2
Serina	316.1	319.6
Histidine	Not detectable	Not detectable
Glycine	228.1	251.1
Threonine	200.4	232.1
Arginine	237.9	405.0
To the girl	600.8	594.4
Tyrosine	247.2	233.3
Valine	282.3	265.3
Phenylalanine	340.3	347.7
Isoleucine	249.1	235.8
Leucine	855.1	749.6
Lysine	141.5	176.8
Proline	536.0	519.5
Tryptophan	89.7	91.9

It can be observed that the amino acids aspartic acid, glutamic acid, serine, glycine, threonine, arginine, phenylalanine, lysine, and tryptophan increased during the fermentation process, while amino acids tyrosine, valine, isoleucine, and proline decreased. The increase of amino acids during fermentation is due to different reasons: on the one hand it is closely related to the hydrolysis of the tannin-protein, phytate-protein and phenol-protein complexes, since these compounds are bound to the terminal groups of some amino acids such as lysine, histidine and arginine, and when they are released, the content of these amino acids increases; another reason is that during the process proteins, by the action of proteases, are divided into smaller components, amino acids. Other authors (Zhang *et al.* 2007) have also pointed out that during their growth cycle, microorganisms can synthesize some amino acids. Also, the reduction in the content of amino acids is explained by the microbial growth, which degrade the proteins in peptides and amino acids to take advantage of these easily in their development. Mugula and Lyimo (2000) reported that the fermentation of sorghum and some mixtures of cereals produce a significant increase of the amino acids lysine, leucine, isoleucine and methionine.

CONCLUSION

With the process applied under the established conditions of temperature, air velocity, wheat bran (adjuvant) and particle size, a fermented product with improved physicochemical and nutritional characteristics was obtained. There was a notable decrease in the content of condensed tannins (86.26%), one of the main compounds that limits the use of this cereal, as well as the value of the crude protein and its digestibility, which shows that the solid fermentation with *Rhizopus oryzae* (MUCL 28168) has potential application to produce high nutritional quality raw material that can be used in both human and animal feeding.

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DECLARATION OF INTEREST STATEMENT

The authors report no declarations of interest.

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