Available online at www.sciencedirect.com
SCIENCE DIRECT®
Food Chemistry 90 (2005) 853–859
Food Chemistry
www.elsevier.com/locate/foodchem

Sorghum fermentation followed by spectroscopic techniques

Isabel Correia, Alexandra Nunes, Iola F. Duarte, António Barros, Ivonne Delgadillo *

Departamento De Quimica, Campus Univeritario De Santiago, Universidade De Aveiro, 3810-193 Aveiro, Portugal

Received 12 April 2004; accepted 14 May 2004

Abstract

The effect of lactic bacteria fermentation on sorghum was followed by spectroscopic techniques (^{1}H NMR and FT-IR) and chemical analysis.

Wet-cooked sorghum flour was inoculated with lactic bacteria (*Lactobacillus fermentum*, *Lactobacillus bulgaricus*, *Lactobacillus lactis*, *Pediococcus pentosaceus* and *Pediococcus cerevisiae*) and a mixture of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* from a commercial natural yogurt. Only *L. fermentum* and the commercial yogurt inoculum were able to grow in sorghum.

The induced lactic bacteria fermentations were compared with a spontaneous traditional fermentation carried out with flour used for fermentation purposes.

In all fermentations, a decrease in pH was noticed and consequently an increase in titratable acidity was detected. Also observed were an increase in free amino acids and total protein content. Reducing sugars, soluble protein and starch decreased during the fermentation processes. These chemical results were confirmed by ^{1}H NMR and FT-IR.

This study showed that spectroscopic methods are suitable and less time-consuming than chemical methods for following fermentation processes, giving the same relevant information and allowing large screening experiments.

Keywords: *Sorghum bicolor* (L.) Moench; Spectroscopy; Fermentation and lactic bacteria

1. Introduction

Sorghum [*Sorghum bicolor* (L.) Moench] is one of the major food crops of the semiarid regions of Africa and Asia. In those regions, sorghum grains are an important source of energy, proteins, minerals and vitamins for millions of people. This cereal is considered to be the poor people's food and is generally consumed in rural areas where it may comprise more than 70% of the food intake (Hulse, Laing, & Pearson, 1980). As in those areas the populations are frequently undernourished, the nutritional quality of sorghum is particularly important.

The poor nutritional quality of sorghum meal is due to the deficiency of certain essential amino acids, such as methionine, isoleucine and lysine (Neucere & Sumrell, 1979), low starch availability (Hall, Absher, Toluske, & Tillaah, 1968) and low protein digestibility (Hamaker, Kirleis, Mertz, & Axtell, 1986; MacLean, Lopez de Romana, Placko, & Graham, 1981), and is also related to the presence of certain anti-nutritional factors, such as tannins (Price & Butler, 1977).

Several studies, based on sorghum fermentation with naturally-occurring microflora, have shown that this treatment improves the nutritional quality of sorghum. The sorghum fermentation increases the protein content (Chavan, Chavan, & Kadam, 1988), enhances protein quality by improving digestibility (Hassan & El Tinay, 1995), provides a better essential amino acid composition as result of de novo production of some important

* Corresponding author. Tel.: +351-234370718; fax: +351-234370084.
E-mail address: ivonne@dq.ua.pt (I. Delgadillo).

0308-8146/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2004.05.060

amino acids (Au & Fields, 1981), makes the starch more available (Kazanas & Fields, 1981), decreases the tannin content (Hassan & El Tinay, 1995), and increases the vitamin content (El Tinay, Abdel Gadir, & El Hidai, 1979). However, some of the endogenous sorghum microorganisms are pathogenic or may produce toxic substances, such as mycotoxins (Gassem, 1999).

In this study, some lactic bacteria (*Lactobacillus fermentum*, *Lactobacillus bulgaricus*, *Lactobacillus lactis*, *Pediococcus pentosaceus* and *Pediococcus cerevisiae)* and a natural yogurt were tested as inocula to ferment sorghum flour. The purpose of this work was to develop a controlled fermentation and to evaluate the use of secure lactic bacteria fermentation for improvement of sorghum nutritional quality. Spectroscopic methods were used to study the fermentation process and chemical methods were applied to establish comparison.

2. Materials and methods

2.1. Sorghum flours

For the traditional fermentation, a South Africa commercial flour (with sorghum endogenous microorganisms) was used (King Korn Mabele – King Food Corporation, South Africa).

For the induced fermentation, sorghum grains (KLW-South Africa cultivar) were ground with a coffee mill to pass through a 0.3 mm screen. This variety was chosen because its digestibility is particularly affected by cooking (Nunes, Correia, Barros, & Delgadillo, 2004).

2.2. Bacterial strains and growth conditions

L. fermentum, *L. bulgaricus*, *L. lactis*, *P. pentosaceus* and *P. cerevisiae* were obtained from the Instituto Nacional de Engenharia e Tecnologia Industrial (INETI) in lyophilized form. In order to promote their re-hydration, these bacteria were first cultured in a milk liquid medium composed of 8 g/l of dehydrated nutrient broth (Difco) and 100 ml skim milk (10 g/l, separately sterilized). After re-hydration the bacteria were cultured in a solid medium aseptically prepared by the addition of 15.0 g/l of granulated agar (Fluka) to a tryptic soy medium (Merck) composed of 17.0 g/l of casein peptone, 3.0 g/l of soy meal peptone, 2.5 g/l of glucose, 5.0 g/l of sodium chloride and 2.5 g/l of dipotassium hydrogen phosphate (pH 6.5 ± 0.2), and incubated at 37 °C. Natural yogurt was used directly for inoculation.

2.3. Flour fermentation

For the traditional fermentation, Mabele sorghum flour (10 g) was mixed with 20 ml of cool sterilized water to obtain a thick paste. Then, previously fermented dough (2 g) was added to act as a starter. This mixture was left to ferment at room temperature (ca. 25 °C) with the natural microflora to pH 4, which happened after two days. After this period of time moulds started to grow in the sample.

For lactic fermentation, 6 samples of sorghum flour (10 g each) were mixed with sterilized water (1:10 w/v), in sealed E-flasks, and boiled for 1 min under manual stirring. This procedure eliminates endogenous microorganisms. After being cooled at room temperature (ca. 25 °C), one of the samples was inoculated with 5 ml of a yogurt suspension (20% v/v). Each one of the other mixtures was inoculated, respectively, with 5 ml of *L. fermentum*, *L. bulgaricus*, *L. lactis*, *P. pentosaceus*, and *P. cerevisiae* suspensions. These suspensions presented approximately the same number of cells (30.0×10^7 cells/ml); counted on a Neubauer Chamber. All the samples were incubated at 37 °C for five days.

After fermentations were completed (two days for the traditional and five days with the lactic bacteria), the volumes were adjusted to 150 ml. The samples were centrifuged at 15,000 rpm for 20 min (Sigma 3K30 centrifuge) and the residues were freeze-dried.

2.4. pH and titratable acidity measurement

The pH of the supernatant of the fermented material was measured with a glass electrode. The titratable acidity was determined by titration with 0.1 N NaOH to an end-point of 8.2. The titratable acidity was expressed as the volume of sodium hydroxide solution required to neutralize 1 ml of supernatant.

2.5. Reducing sugars determination

The supernatant reducing sugars were determined by the 3,5- dinitrosalicylic acid (DNS) colorimetric method, with glucose as the standard (Miller, 1959). One ml of DNS reagent was added to 1 ml kept of sample. The mix was kept in a boiling water bath for 5 min. After cooling to room temperature (ca. 25 °C) in a cold water bath, 10 ml of distilled water was added. The absorbance at 540 mm was measured (Shimadzu UV-160A spectrophotometer), interpolating the value obtained with calculated values for glucose solutions of known concentration. The blanks were prepared by substituting sample solution for distilled water. All measurements were made in triplicate.

2.6. Total sugars determination

A modified phenol–sulfuric acid method was used to determine total sugars present in the supernatant of the fermented material (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). One ml of 5% phenol was added to

100 μl of sample. Then, 1 ml of concentrated sulfuric acid was added and the mixture was kept in a boiling water bath for 10 min. After cooling to room temperature (ca. 25 °C), in a cold water bath, the absorbance at 490 nm was measured (Shimadzu UV-160A spectrophotometer). The amount of sugars was then determined by reference to a standard curve prepared with glucose. The blanks were prepared by substituting sample solution for distilled water. All measurements were made in triplicate.

2.7. Soluble protein determination

Soluble proteins of the supernatant were determined with a BCA kit for protein determination (Sigma).

2.8. Total amino acids determination

The quantitative measurement of free amino acids of the supernatant was done using the ninhydrin reaction (Plummer, 1978). Two ml of buffered ninhydrin reagent (0.8 g of ninhydrin and 0.12 g of hydrindantin dissolved in 30 ml of 2-methoxyethanol plus 10 ml of acetate buffer 4 M, pH 5.5) were added to 2 ml of sample and heated in a boiling water bath for 15 min. The mixture was cooled to room temperature (ca. 25 °C), 3 ml of 50% ethanol were added and the absorbance was read at 570 nm after 10 min (Shimadzu UV-160A spectrophotometer). The amount of amino acids was determined by reference to a standard curve previously prepared with arginine. The blanks were prepared by substituting sample solution for distilled water. All measurements were done in triplicate.

2.9. Insoluble protein determination

The residues were submitted to determination of total N by elementary analysis. The percentage of protein was determined by multiplying by 6.25.

2.10. Starch determination

The amount of starch of the residue was determined using a total starch determination kit (Megazyme).

2.11. ^1H NMR

High resolution nuclear magnetic resonance (NMR) spectroscopy was used to evaluate the effects of fermentation on the chemical composition of the supernatants. The samples were prepared to contain 10% D_2O, used as the internal lock, and 0.02% sodium 3-(trimethylsilyl)propionate-d_4(TSP-d_4), used as chemical shift and intensity reference, and transferred to 5 mm o.d. NMR tubes. The ^1H 1D NMR spectra were recorded at 27 °C on a Bruker Avance DRX-500 spectrometer, operating at 500.13 MHz for proton, using the 'noesyprld' pulse sequence: RD–90°–t_1–90°–t_m–90° – acquire FID (Bruker library), where RD is the relaxation delay (5.0 s), t_1 represents the first increment in a NOESY experiment (3 μs), and t_m is the mixing period (100 ms). The water signal was suppressed by presaturation during the relaxation delay and mixing time. Each ^1H 1D spectrum consisted of 128 scans of 32 K data points with a spectral width of 8012.82 Hz and an acquisition time of 2.04 s.

2.12. Fourier transform infrared

Fourier transform infrared (FT-IR) spectroscopy was used to evaluate the effects of fermentation on the residues of the samples. The FT-IR spectra were obtained using a Golden Gate single reflection diamond ATR system in a Bruker IFS-55 spectrometer. The spectra were recorded in absorbance mode from 4000 to 500 cm^{-1}, co-adding 128 scans at 8 cm^{-1} resolution. Ten replicates were collected for each sample. The obtained spectra were transferred into a data analysis package (Barros, 1999). For principal component analysis (PCA), the 1780-800 cm^{-1} region was selected and each spectrum was standard normal variate (SNV) corrected. The PCA allowed the characterization of the sample relationships (scores plans) and the recovery of their subspectral profiles (loadings) (Jolliffe, 1986).

2.13. Statistical analysis

All values are expressed as means and standard deviations for three replicates, with the exception of those obtained by elementary analysis. Mean values of treatments were compared by Student's t test. Differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Traditional fermentation – supernatant

Changes in pH, titratable acidity, reducing sugars, total sugars, soluble protein and free amino acids are presented in Table 1.

The pH of the supernatant of traditionally fermented sorghum decreased from 6.4 to 4.1. Consequently, the titratable acidity showed an increase from 0.05 to 0.19 ml/ml. This is the result of the production of acids by the microorganisms. Indeed, the ^1H NMR spectrum for the spontaneous fermented sample (Fig. 1(b)) shows the drastic increase of lactic, acetic, formic and succinic acids.

The reducing sugars decreased from 3392 to 473 mg/l, and the total sugars from 4013 to 694 mg/l. These results are in agreement with the ^1H NMR

Table 1
Chemical analysis results of the control sample and traditional fermented supernatants

Chemical parameter	Control sample	Fermented sample
pH	6.4	4.1
Titratable acidity (mg/ml)[a,b]	0.05 ± 0.0007	0.19 ± 0.0018
Reducing sugars (mg/l)[a,b]	3392 ± 29	473 ± 8
Total sugars (mg/l)[a,b]	4013 ± 108	694 ± 26
Free amino acids (mg/l)[a,b]	436 ± 32	704 ± 76
Soluble proteins (mg/l)[a,b]	4609 ± 28.3	1045 ± 8

[a] Means of replicates.
[b] Differences were considered significant at $p < 0.05$.

Table 2
Chemical analysis results of the control sample and traditional fermented residues

Chemical parameter	Control sample	Fermented sample
Insoluble proteins (%)	7.0	8.3
Starch (%)[a,b]	69.6 ± 0.4	60.3 ± 2.5

[a] Means of replicates.
[b] Differences were considered significant at $p < 0.05$.

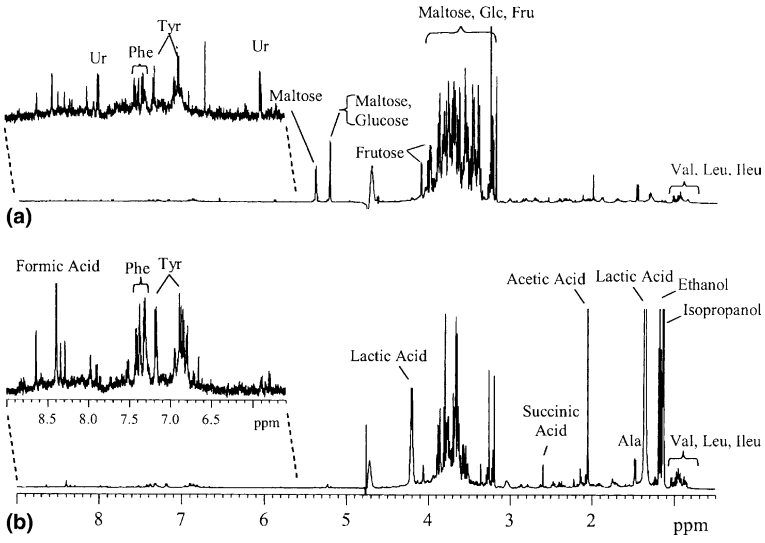

Fig. 1. ^{1}H NMR spectra of (a) control sample, and (b) spontaneously by fermented sample.

spectra (Fig. 1), which show decreases of glucose, fructose and maltose signals with fermentation. However, there are contradictory reports on this topic. According to some studies (Kazanas & Fields, 1981), the level of reducing sugars should increase as a consequence of starch hydrolysis; however, some studies show the opposite, that is a decrease in reducing (Mugula, 1992; Taur, Pawar, & Ingle, 1984) and total sugars (El Tinay et al., 1979).

The increase in free amino acids (436–704 mg/l) and the decrease in soluble proteins (4609–1045 mg/l) are the result of protein hydrolysis by microbial proteases (Table 1). The ^{1}H NMR spectra (Fig. 1) indeed show increases of valine, leucine, isoleucine, alanine, phenylalanine and tyrosine in the fermented sample. The microorganisms are able to hydrolyze proteins into usable amino acids and peptides. Additionally, during their growth cycle, they can synthesize amino acids from metabolic intermediates (Au & Fields, 1981).

Other changes revealed by ^{1}H NMR are life increases of ethanol and isopropanol and decrease of uridine, between the control and the fermented sample.

3.2. Traditional fermentation – residue

With fermentation, the starch content decreases 13.4% due to microbial amylase hydrolysis (Table 2). Similar results were obtained by El Tinay et al. (1979) and Chavan et al. (1988).

The insoluble proteins showed an increase of 18.6%, as a consequence of microorganism activity. The microorganisms concentrate nitrogen, i.e., in fermented meal the amount of protein increases as a consequence of starch consumption (Table 2). These results are similar to those reported by Kazanas and Fields (1981). The starch decrease and protein increase can be observed by the FT-IR/PCA analysis. PCA distinguishes the control sample (unfermented flour) from fermented flour along the PC2 axis (Fig. 2(a)). The loadings profile (Fig. 2(b)) shows that the control sample is characterized by signals from lipids (1743 cm^{-1}) and carbohydrates (1168, 1130, 1110 and 976 cm^{-1}). When starch is enzymaticaly attacked by α-amylases, the maximum of its characteristic peak shifts from 976 to 1018 cm^{-1} (unpublished results). In the fermented flour, the shift occurs from 976 to 1045 and 1072 cm^{-1}, which could

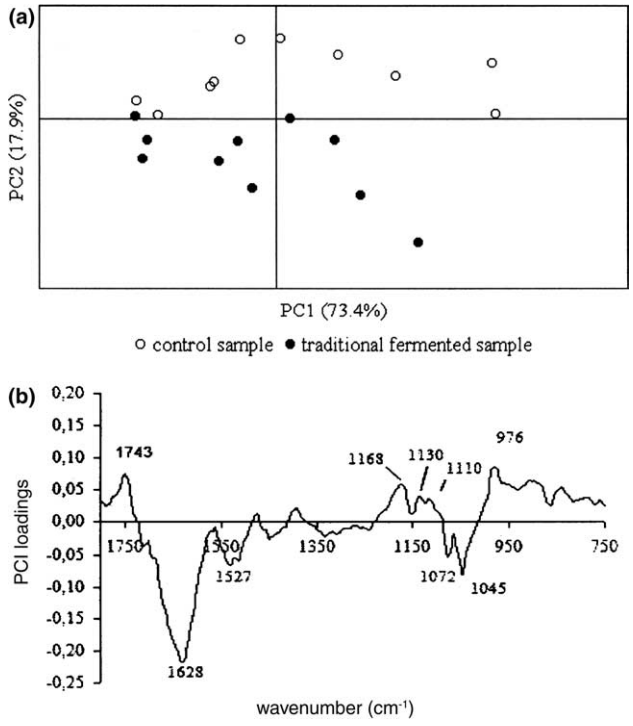

Fig. 2. (a) PCA scores scatter plot (PC1 vs. PC2) and (b) PCA loadings plot profile (PC2) of the FT-IR spectra of control sample and traditional fermentation.

be related to strong starch hydrolysis. Other characteristic signals from this sample are found at 1628 and 1527 cm^{-1}. These peaks correspond to proteins (amide I and amide II, respectively) and suggest an accumulation of protein in the fermented sample. Therefore, PCA of FT-IR shows that the fermentation caused a decrease in lipid content, an increase in protein content and structural changes in starch.

3.3. Lactic fermentation – supernatant

The *Streptococcus thermophilus*/*L. bulgaricus* from the natural yogurt and the specie *L. fermentum* were able to grow in sorghum flour. The species *L. bulgaricus*, *L. lactis*, *P. pentosaceus* and *P. cerevisiae* did not growth in sorghum flour.

Table 3 presents the results of fermentation with lactic bacteria. The pH of the supernatant of the sorghum fermented by *S. thermophilus*/*L. bulgaricus* (commercial yogurt) decreased from 5.7 to 4.3. The titratable acidity showed an increase from 0.04 to 0.10 ml/ml. In the case of *L. fermentum* the pH decreased to 4.7 and, consequently, the titratable acidity increased (0.04–0.90 ml/ml) (Table 3). In both fermented samples, the production of acids can be observed in the ^{1}H NMR spectra, which show the increase of lactic, acetic, pyruvic, succinic and formic acids (Figs. 3(b), (e), (c)).

In reducing sugars, a decrease was observed (1031–318 mg/l for the yogurt inoculum and to 378 mg/l for *L. fermentum*) as a result of its consumption as an energy source. In contrast to traditional fermentation, total soluble sugars suffer a large increase, from 2268 to 33,116 mg/l for the yogurt inoculum and to 17,918 mg/l for *L. fermentum* (Table 3). The ^{1}H NMR spectrum of the control sample (Fig. 3(a)) shows a relatively broad signal at 5.4 ppm, arising from glucose H1 protons involved in $\alpha(1 \rightarrow 4)$ glycosidic linkages. This indicates that part of the starch was solubilized by cooking, causing visible signals in the spectrum. With the fermentation process, these signals increase as a result of starch hydrolysis which allows more dextrins to pass into the liquid phase. The glucose signals also decrease in both lactic fermentations.

The increase in the amino acid level and the decrease in soluble proteins result from the protein hydrolysis by bacterial proteases (Table 3). The ^{1}H NMR spectra reveal an increase in valine, leucine and isoleucine in both fermented samples. For the case of the *L. fermentum* inoculum, an increase in phenylalanine and tyrosine (Fig. 3) is observed. Further information given by the spectra is the increase in ethanol and isopropanol and a decrease in γ-amino-butyric acid (GAB) for both fermented samples.

3.4. Lactic fermentation – residue

As can be seen in Table 4, there was a decrease of 14.3% in the starch content of the sample fermented with yogurt inoculum as a result of hydrolysis by microbial amylases. This hydrolysis was also detected by

Table 3
Chemical analysis results of the control sample and lactic fermented supernatants

Chemical parameter	Control sample	Yogurt inoculum	*L. fermentum*
pH	5.7	4.3	4.7
Titratable acidity (mg/ml)[a,b]	$0.04 \pm 0.02 \times 10^{-2}$	$0.10 \pm 0.07 \times 10^{-2}$	$0.90 \pm 0.07 \times 10^{-2}$
Reducing sugars (mg/l)[a,b]	1031 ± 15	318 ± 24	378 ± 29
Total sugars (mg/l/)[a,b]	2268 ± 18	33,116 ± 40	17,918 ± 70
Free amino acids (mg/l)[a,b]	171 ± 7	227 ± 5	353 ± 28
Soluble proteins (mg/l)[a,b]	977 ± 166	229 ± 48	369 ± 46

[a] Means of replicates.
[b] Differences were considered significant at $p < 0.05$.

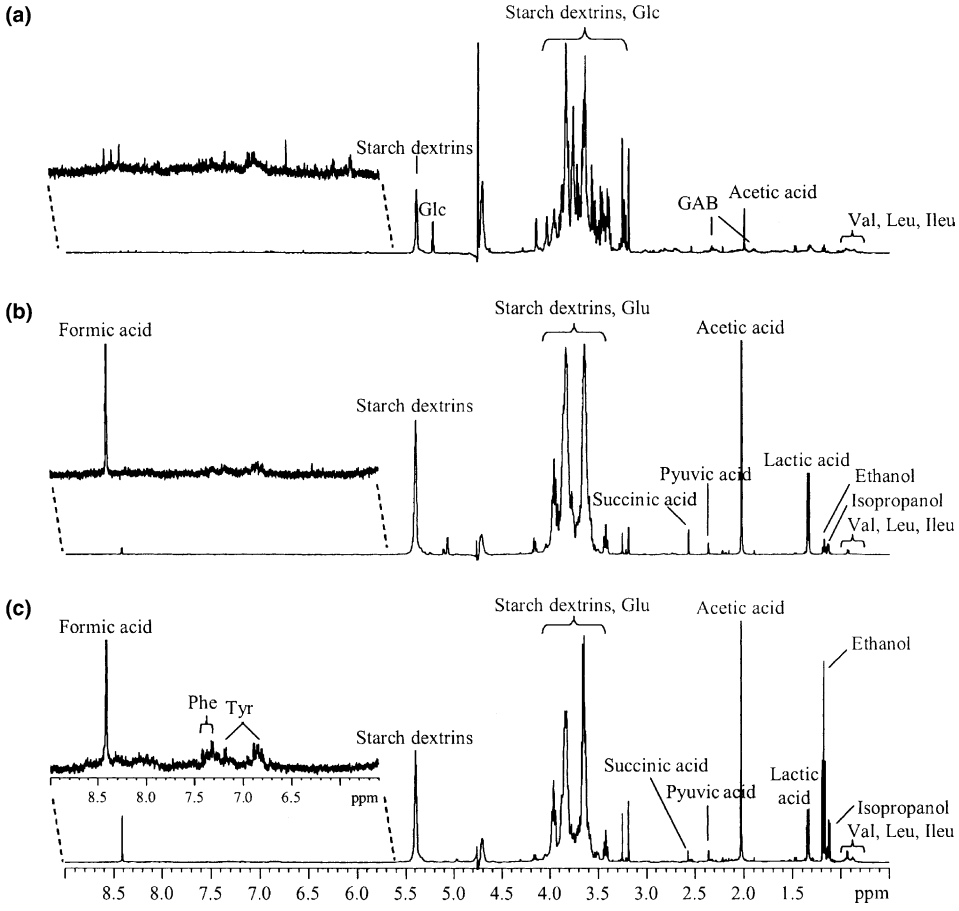

Fig. 3. ^{1}H NMR spectra of (a) control sample, (b) sample fermented with natural yogurt, and (c) sample fermented with *L. fermentum.*

Table 4
Chemical analysis results of the control sample and lactic fermented residues

Chemical parameter	Control sample	Yogurt inoculum	*L. fermentum*
Insoluble proteins (%)	7.1	12.8	7.7
Starch (%)[a]	75.4 ± 0.9	64.6 ± 0.6[b]	75.1 ± 3.2

[a] Means of replicates.
[b] Differences were considered significant at $p < 0.05$.

the increase in the supernatant total sugars (Section 3.3). In the case of *L. fermentum,* the decrease in starch content does not seem to be significant. However, as before, the ^{1}H NMR spectra (Fig. 3) indicate the occurrence of starch hydrolysis in both fermentations. This discrepancy may arise from the consumption of non-starch components by the microorganisms, which may mask the real starch decrease due to hydrolysis.

Insoluble protein increases (7.1–12.8%) in the sample fermented with yogurt inoculum. This is a consequence of both production of bacterial mass and, mainly, of protein concentration by the starch decrease. The protein increase was smaller (7.1–7.7%) in the sample fermented with *L. fermentum* (Table 4).

The FT-IR/PCA analysis shows a distinction, along the PC1 axis, between the three groups of samples, control sample, sample fermented with yogurt inoculum and with *L. fermentum* (Fig. 4). The greatest difference can be observed between control and natural yogurt samples. The loadings profile shows that the sample fermented with yogurt is characterized by signals arising from protein (1635 and 1527 cm^{-1}, amide I and amide II, respectively) and from starch hydrolysis (1149 and 1022 cm^{-1}). The unfermented control sample is characterized by the peak of the undamaged starch at 976 cm^{-1} (Fig. 4). As the *L. fermentum* samples are located around the PC1 origin, this suggests that the protein and starch changes are between the two extreme samples (control and natural yogurt).

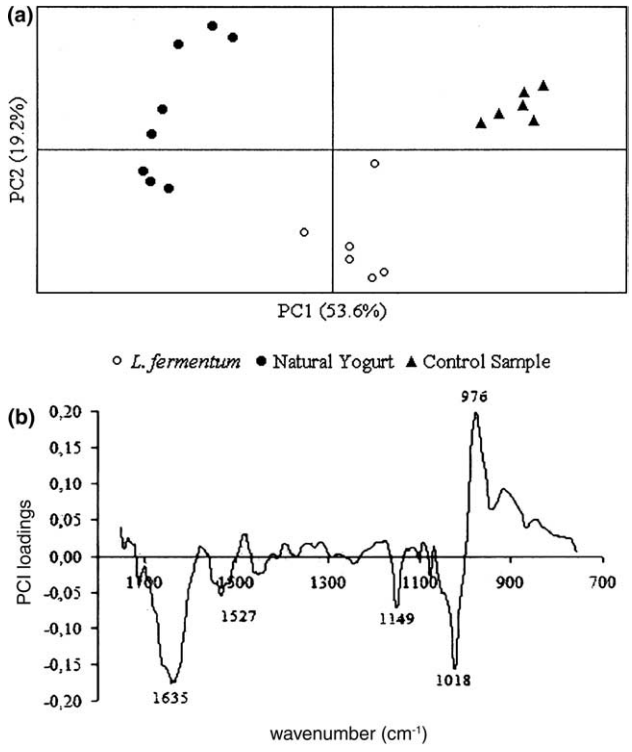

Fig. 4. (a) PCA scores scatter plot (PC1 vs. PC2) and (b) PCA loadings plot profile (PC1) of the FT-IR spectra of control sample and lactic fermentations.

4. Conclusions

The use of natural yogurt and *L. fermentum* as inocula for sorghum flour fermentation suggests that lactic bacteria could be a secure and alternative way to improve some sorghum nutritional characteristics.

The use of ^{1}H NMR, and FT-IR, combined with PCA, proves to be an extremely useful tool for monitoring the effects induced by fermentation processes on the chemical compositions of the samples. These techniques are complementary and give additional information. They are less time-consuming and allow a more complete insight into the overall process. Therefore, they are suitable for following fermentation and to test different experimental conditions for developing new food products.

Acknowledgements

We thank Prof. John Taylor and Janet Taylor, University of Pretoria, South Africa (for providing the sorghum samples and the Mabele flour) and Dr. Isabel Fernandes, INETI, Lisbon, Portugal (for the microorganisms). Alexandra Nunes thanks FCT (Portugal) for the PhD Grant No. SFRH/BD/1071/2000. Isabel Correia acknowledge the Eurospec Grant No. G7/RT/CT/2001/05063.

References

Au, P. M., & Fields, M. L. (1981). Nutritive quality of fermented sorghum. *Journal of Food Science, 46*, 652–654.

Barros, A. (1999). Contribution á la sélection et la comparaison de variables caractéristiques. PhD Thesis. Paris, France: Institut National Agronomique Paris-Grignon.

Chavan, U. D., Chavan, J. K., & Kadam, S. S. (1988). Effect of fermentation on soluble proteins and in vitro digestibility of sorghum, green gram and sorghum green gramblends. *Journal of Food Science, 53*, 1574–1575.

Dubois, M, Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry, 28*(3), 350–356.

El Tinay, A. H., Abdel Gadir, A. M., & El Hidai, M. (1979). Sorghum fermented kisra bread. I. Nutritive value of kisra. *Journal of the Science of Food and Agriculture, 30*, 859–863.

Gassem, M. M. A. (1999). Study of the micro-organisms associated with the fermented bread (khamir) produced from sorghum in Gizan region, Saudi Arabia. *Journal of Applied Microbiology, 86*(2), 221–225.

Hall, G. A. B., Absher, C. W., Toluske, A. R., & Tillaah, A. D. (1968). Net energy of sorghum grain and corn for fattening cattle. *Journal of Animal Science, 27*, 32–37.

Hamaker, B. R., Kirleis, A. W., Mertz, E. T., & Axtell, J. D. (1986). Effect of cooking on protein profiles and in vitro digestibility of sorghum and maize. *Journal of Agricultural and Food Chemistry, 34*, 647–649.

Hassan, I. A. G., & El Tinay, A. H. (1995). Effect of fermentation on tannin content and in vitro protein and starch digestibilities of two sorghum cultivars. *Food Chemistry, 53*(2), 149–151.

Hulse, J. H., Laing, E. M., & Pearson, O. E. (1980). *Sorghum and the millets: Their composition and nutritive value*. London: Academic Press.

Jolliffe, I. T. (1986). *Principal component analysis*. New York: Springer.

Kazanas, N., & Fields, M. L. (1981). Nutritional improvement of sorghum by fermentation. *Journal of Food Science, 46*, 819–821.

MacLean, W. C., Lopez de Romana, G., Placko, R. P., & Graham, G. G. (1981). Protein quality and digestibility of sorghum in preschool children: Balance studies and plasma free amino acids. *Journal of Nutrition, 111*, 1928–1981.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry, 31*, 426–428.

Mugula, J. K. (1992). The nutritive quality of sorghum–commonbean tempe. *Plant Foods for Human Nutrition, 42*(3), 247–256.

Neucere, N. J., & Sumrell, G. (1979). Protein fractions from varieties of grain sorghum: Amino acid composition and solubility properties. *Journal of Agricultural and Food Chemistry, 27*(4), 809–812.

Nunes, A., Correia, I., Barros, A., & Delgadillo, I. (2004). Sequential in vitro pepsin digestion of uncooked and cooked sorghum and maize samples. *Journal of Agricultural and Food Chemistry, 52*(7), 2052–2058.

Plummer, D. T. (1978). *An introduction to practical biochemistry*. London: McGraw-Hill.

Price, M. L., & Butler, L. G. (1977). Rapid visual estimation and spectrophotometric determination of tannin content of sorghum grain. *Journal of Agricultural and Food Chemistry, 25*(6), 1268–1273.

Taur, A. T., Pawar, V. D., & Ingle, U. M. (1984). Effect of fermentation on nutritional improvement of grain sorghum [*Sorghum bicolor* (L.) Moench]. *Indian Journal of Nutrition and Diet, 21*, 129–136.