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ARTICLE

Phenolic Acids and Flavonoids in Nonfermented and Fermented Red Sorghum (*Sorghum bicolor* (L.) Moench)

Louise Svensson, Bonno Sekwati-Monang, Daise Lopes Lutz, Andreas Schieber, and Michael G. Gänzle*

Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta, Canada

This study aimed to identify phenolic acids and flavonoids in the red sorghum variety PAN 3860 and to determine changes in their concentrations during fermentation with lactobacilli. Sorghum sourdoughs fermented with two binary strain combinations, *Lactobacillus plantarum* and *Lactobacillus casei* or *Lactobacillus fermentum* and *Lactobacillus reuteri*, were compared to chemically acidified controls. Four glycerol esters were tentatively identified, caffeoylglycerol, dicaffeoylglycerol, coumaroyl-caffeoylglycerol, and coumaroyl-feruloylglycerol, that have previously not been detected in sorghum. Chemical acidification resulted in hydrolysis of phenolic acid esters and flavonoid glucosides. During lactic fermentation, phenolic acids, phenolic acid esters, and flavonoid glucosides were metabolized. Analysis of ferulic acid, caffeic acid, and naringenin-glucoside contents in single-strain cultures of lactobacilli demonstrated that glucosidase, phenolic acid reductase, and phenolic acid decarboxylase activities contributed to polyphenol metabolism. This study demonstrates that microbial fermentation of sorghum affects the content of polyphenols and can influence the nutritional value and antimicrobial activity of sorghum.

KEYWORDS: Polyphenols; phenolic acids; flavonoids; sorghum; sourdough; *Lactobacillus reuteri*; *Lactobacillus plantarum*

INTRODUCTION

Sorghum (*Sorghum bicolor* (L.) Moench) is the fifth most important crop in the world. It is a staple food in Africa and a good supply of energy, protein, vitamins, and minerals. The grain is traditionally used for food production and opaque beer brewing. Preparation of traditional dishes typically entails cooking of sorghum grains mixed with legumes or boiling of sorghum flour into various types of porridges. For food use, the grains are decorticated to reduce the polyphenol content and milled into coarse flour, which is then cooked directly or after fermentation with lactic acid bacteria. Fermented porridges are the most popular (*1*).

Sorghum contains comparable levels of starch and other major nutrients as other cereals (*2*). However, the availability of these nutrients is limited due to the presence of polyphenolic compounds, particulary tannins, which are located primarily in the testa layer of the grain. Sorghum has a higher content of polyphenols than wheat, barley, millet, or rye (*3*), with phenolic acids, flavonoids, condensed tannins, and deoxyanthocyanidins being the predominant compounds (*4*). The total polyphenol content in sorghum is affected by genetic and environmental factors, such as plant color, thickness of the pericarp, and growth conditions. The grain color varies from white to red. Sorghum with red/purple color has higher total polyphenol contents than tan sorghum grains; the color is mainly attributable to deoxyanthocyanidins (*4*,*5*). The high polyphenol content in red sorghum contributes to a higher resistance to biotic and abiotic stress. Moreover, a correlation between phenolic content and antioxidant activity (*6*) as well as antimicrobial activity (*7*) of sorghum grains has been reported. However, tannins also cause the bitter and astringent taste of some sorghum varieties (*8*), and white sorghum varieties are therefore more commonly used as food.

The nutritive quality of sorghum fermented with lactic acid bacteria is higher in comparison to nonfermented sorghum products. Lactic fermentation may lead to qualitative modification of proteins, often resulting in an increase in water-soluble proteins and amino acids. Moreover, the content of polyphenols and particularly tannins in sorghum decreases through fermentation with lactic acid bacteria (*2*, *6*), and fermented sorghum is less bitter because of the decreased tannin levels. Strains of *Lactobacillus plantarum* metabolize phenolic acids and esters of phenolic acids by tannase (*9*), phenolic acid decarboxylase, and phenolic acid reductase activities (*10*, *11*); however, the conversion of polyphenols by lactobacilli in food fermentations has been characterized in model malt whiskey fermentation only (*12*, *13*)

It was the objective of the present study to identify and quantify phenolic acids and flavonoids in sorghum and to characterize the conversion of phenolic acids and flavonoids in model sorghum fermentations with defined strains of lactobacilli. Fermentation organisms comprised homofermentative and heterofermentative lactobacilli previously isolated from ting, a common dish in

*Address correspondence to this author at the Department of Agricultural, Food and Nutritional Science, 4-10 Ag/For Center, University of Alberta, Edmonton, AB T6G 2P5, Canada (phone (780) 492-0774; fax (780) 492-4265; e-mail mgaenzle@ualberta.ca).

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Botswana that is prepared from sorghum flour by fermentation with lactobacilli.

MATERIALS AND METHODS

External Standards. The following standards were used for analysis: (+)-taxifolin, (+)-catechin, eriodictyol, eriodictyol-7-*O*-glucoside, naringenin, naringenin-7-*O*-glucoside, luteolin, apigenin, 4-coumaric acid, 4-hydroxybenzoic acid, caffeic acid, ferulic acid, gentisic acid, vanillic acid, and (−)-epicatechin, all obtained from Extrasynthése (Genay, France); protocatechuic acid, syringic acid, *trans*-cinnamic acid, salicylic acid, and 3,4-dihydroxybenzaldehyde, all obtained from Sigma (St. Louis, MO).

Bacterial Growth and Media. *Lactobacillus fermentum* FUA3165, *Lactobacillus reuteri* FUA3168, *Lactobacillus plantarum* FUA3171, and *Lactobacillus casei* FUA3166 were previously isolated from ting, a fermented sorghum porridge from Botswana. The strains were separately grown on modified MRS (mMRS) agar for 24 h at 34 °C in modified atmosphere (1% O_2, 5% CO_2, 10% H_2, balance N_2) and subcultured in mMRS broth for 24 h at 34 °C. The mMRS had the following composition per liter: 5.0 g of fructose, 5.0 g of glucose, 10.0 g of maltose, 10.0 g of tryptone, 5.0 g of beef extract, 5.0 g of yeast extract, 2.6 g of H_2KPO_4, 4.0 g of HK_2PO_4, 3.0 g of NH_4Cl, 0.5 g of L-cysteine−HCl, 0.2 g of $MgSO_4$, 0.05 g of $MnSO_4$, 1.0 g of Tween 80, and 1.0 mL of vitamin mix (B12, B1, B2, B6, folic acid, and pantotenic acid). Solid media additionally contained 15 g L^{-1} bacteriological agar.

Bacillus subtlilis FAD-110 and *Listeria monocytogenes* FS-15 were used as indicator strains for the determination of the antimicrobial activity of polyphenol extracts from three different sorghum varieties. The cultures of *B. subtilis* and *L. monocytogenes* were grown overnight at 37 °C in brain−heart infusion (BHI) broth (Oxoid, Unipath Ltd., Basingstoke, Hampshire, U.K.) and tryptic soy broth (TSB) (Difco, Sparks, MD), respectively.

Fermentation of Sorghum Sourdough. *S. bicolor* (L.) Moench PAN 3860, a red sorghum variety, was obtained from the Food Technology Research Centre, Kanye, Botswana. The grains were ground in an Ultra Centrifugal Mill ZM 200 (Retsch, Burlington, Canada) to a size of 0.5 mm. Sterile tap water (50 mL) was added to 50 g of ground sorghum. Overnight cell cultures were washed with peptone saline solution (8.5 g L^{-1} sodium chloride and 1 g L^{-1} peptone), resuspended in 10 mL of sterile tap water, and added to the sorghum doughs. Sorghum sourdoughs were fermented for 24 h at 34 °C. *L. casei* and *L. plantarum* or *L. fermentum* and *L. reuteri* were used in binary strain combinations. These binary strain combinations were chosen because traditional sourdoughs typically contain two to five different strains (*14*). To account for modifications of phenolic acids and flavonoids in the absence of microbial activity, sorghum slurries were prepared with 50 g of ground sorghum and 70 mL of sterile tap water, acidified with 1 mL of lactic and acetic acids in a 4:1 ratio (v/v), and fermented for 24 h at 34 °C. The chemically acidified control had a pH of 4.0, which is similar to the pH for fermented sorghum dough (*15*). Fermented and chemically acidified sorghum doughs were characterized before and after fermentation by measurement of the pH and by determination of viable cell counts. To confirm the identity of the fermentation microbiota with the inoculum, the morphologies of colonies before and after fermentation were compared. Fermentations were carried out in triplicate independent experiments.

Extraction of Phenolic Acids and Flavonoids. Ground sorghum (50 g) or the fermented sorghum dough samples was mixed with 200 mL of 70% (v/v) aqueous methanol. The samples were shaken for 1 h and centrifuged at 4225*g* for 10 min. The supernatant was removed, and the residue was extracted again as described. Methanol was evaporated under vacuum at 30 °C using a Büchi Rotavapor RE21 (Büchi, Flawil, Switzerland); solids were dissolved in Milli-Q water (50 mL) and acidified to pH 1.5 with hydrochloric acid. Ethyl acetate (200 mL, Fisher Scientific, Ottawa, ON, Canada) was added, and the samples were shaken every 10 min for 30 min. The liquid−liquid extraction was repeated, ethyl acetate was evaporated under vacuum at 30 °C, and solids were redissolved in 10 mL of methanol.

For the identification of bound phenolic acids and flavonoids, residues remaining after methanol extraction of 5 g of sorghum flour, or 5 g of sorghum flour after fermentation of chemically acidified doughs, were treated with 20 mL of 2 M HCl at 100 °C for 1 h (*23*). Ethyl acetate was added to the hydrolysate and, after partitioning, the ethyl acetate fraction was separated in a separation funnel and evaporated to dryness. Solids were redissolved in 2 mL of methanol and filtered through a 0.45 μm syringe filter (Fisher Scientific) prior to analysis.

Antimicrobial Activity of Methanolic Sorghum Extracts. The crude methanolic extracts from red sorghum (variety PAN 3860), white sorghum (variety Segaolane), and decorticated commercial North American white sorghum were investigated for their antimicrobial activity. Extracts from unfermented flours were compared to extracts from sorghum sourdoughs fermented with binary strain combinations (*L. reuteri* and *L. fermentum* or *L. casei* and *L. plantarum*). The antimicrobial activity of crude extracts was tested in an agar spot assay using *B. subtilis* and *L. monocytogenes* as indicator strains. Crude methanolic extracts were dried under nitrogen and reconstituted with one-fifth of methanol. TSA and BHI agar plates were inoculated with 0.1 mL of overnight cultures of *B. subtilis* or *L. monocytogenes* by spread-plating. Agar plates were allowed to dry, and 5 μL of the extracts was spotted on the plates. The spots were allowed to dry at room temperature for at least 3 h prior to incubation at 37 °C for 18−24 h. After incubation, the zone of inhibition was measured. Means of two independent experiments are shown.

Identification of Phenolic Acids and Flavonoids. A 1200 series HPLC unit comprising a degasser, binary pump, autosampler, thermostated column compartment, and diode array detector (DAD) (Agilent Technologies, Palo Alto, CA) was connected to a 4000 Q TRAP LC-MS/MS System (MDS SCIEX, Applied Biosystems, Streetsville, ON, Canada). A Luna C18 RP-HPLC column (5 μm, 250 × 4.6 mm, Phenomenex, Torrance, CA) was used together with an AQ 4 × 20 mm C18 precolumn (Phenomenex) for separation of the polyphenols. DAD detection was performed between 190 and 400 nm.

Eluent A consisted of 2% (v/v) acetic acid in Milli-Q water, and eluent B consisted of 0.5% (v/v) acetic acid in Milli-Q water and acetonitrile (50:50, v/v) of HPLC grade (Fisher Scientific). Samples were eluted with the following gradient: 0% B (5 min), 0−40% B (10 min), 40−60% B (40 min), 60−80% B (10 min), 80−100% B (10 min), 100% B (30 min), and 100−0% B (2 min).

Mass spectra were recorded in the negative mode; the flow rate was maintained at 0.5 mL min^{-1} with the pneumatically assisted electrospray probe using high-purity nitrogen gas (99.995%) as the nebulizing (GS1) and heating gas (GS2). The values for optimum spray voltage, source temperature, GS1, GS2, and curtain gases were −4 kV, 600 °C, and 50, 30, and 25 psi, respectively. An information-dependent acquisition (IDA) method, EMS → 4EPI, was used to identify phenolic compounds. Both Q1 and Q3 were operated at low and unit mass resolution. The spectra were obtained over a range from *m*/*z* 50 to 1300 in 2 s. LIT fill time was set at 20 ms. The IDA threshold was set at 100 cps, above which enhanced product ion (EPI) spectra were collected from the eight most intense peaks. The EPI scan rate was 4000 amu s^{-1}. Collision-induced dissociation (CID) spectra were acquired using nitrogen as the collision gas under two different collision energies. The collision energy (CE) and collision energy spread (CES) were −35 and −15 eV for flavonoids and −20 and 0 eV for phenolic acids, respectively. The other MS parameters used were as follows: declustering potential (DP), −70 V; entrance potential (EP), −10 V; and collision exit potential (CXP), −7 V. Data acquisition was interfaced to a computer workstation running Analyst 1.5 (Applied Biosystems).

Quantification of Phenolic Acids and Flavonoids. Quantification was performed by LC-DAD using external standards dissolved in methanol (1 mg mL^{-1}) under the HPLC conditions described above. Phenolic acids and flavonoids were detected at 280 and 320 nm, respectively. Data acquisition, peak integration, and calibrations were performed with the Agilent Chemstation software. The calibration curves were linear over the range of 0.00250−0.600 mg mL^{-1} with a correlation coefficient of ≥0.99. Data are reported as means ± standard deviations of duplicate independent experiments analyzed in triplicate.

Metabolism of Polyphenols by Single Strains in mMRS. The four strains were grown separately in mMRS media containing additionally ferulic acid (0.5 mM), caffeic acid (0.5 mM), or naringenin-7-*O*-glucoside (0.18 mM). Cultures were inoculated with 5% (v/v) overnight cell cultures and fermented for 24 h at 34 °C. Sterile media containing each polyphenol separately were used as controls. The cell-free supernatants were acidified to pH 1.5 with hydrochloric acid, and ethyl acetate (3 mL) was used for

liquid−liquid extraction from the supernatant. Aliquots (20 μL) of the ethyl acetate extract were analyzed by LC-DAD-MS using the conditions described previously. Data are reported as means ± standard deviations of duplicate independent experiments.

RESULTS

Antimicrobial Activity of Sorghum Flours. Crude extracts from three flours were tested for antimicrobial activities against *B. subtilis* and *L. monocytogenes* (**Table 1**). The red sorghum PAN3860 showed higher antimicrobial activity than the white variety Segaolane. Commercial decorticated North American white sorghum flour did not show any activity against the indicator organisms used (data not shown), and fermentation of white or red sorghum flours did not alter the antimicrobial activity. The red sorghum variety PAN 3860 was chosen for further analyses because this grain showed the highest antimicrobial activity.

Identification of Free Polyphenols. Separation of phenolic compounds was achieved by LC-DAD-MS (**Table 2**). An information dependent acquisition (IDA) method was developed to identify phenolic acids and flavonoids in sorghum extracts. A high-sensitivity qualitative scan was obtained by using the 4000 QTRAP system. External standards were analyzed under the same conditions and used for identification by comparison of elution volume, mass spectrum, and UV absorbance. Literature data for mass spectra and UV absorbance were used when external standards were not available (**Table 2**). Mass spectra, elution volumes, and UV absorbance of the identified components are presented in **Table 2**.

The mass spectra, elution volumes, and UV absorbance of protocatechuic acid, catechin, protocatechuic aldehyde, *p*-hydroxybenzoic acid, caffeic acid, *p*-coumaric acid, ferulic acid, taxifolin, eriodictyol, luteolin, naringenin, and apigenin (peaks 1, 4, 7, 8, 11−13, 16, 17, 19, and 20) matched those of the external standards. Peaks 5, 10, and 14 were identified as protocatechuic aldehyde, eriodictyol-7-*O*-glucoside, and naringenin-glucoside, respectively, by comparison of the elution volumes and mass spectra with external standards and literature data (*16*−*18*).

The mass spectra and UV spectra of peaks 6, 9, 15, 18, and 21 matched literature data for glycerol esters (*19*) (see Figure S1C−G of the Supporting Information) and were tentatively identified as caffeoylglycerol, coumaroylglycerol, dicaffeoylglycerol, coumaroyl-caffeoylglycerol, and coumaroyl-feruloylglycerol, respectively. The fragmentation patterns of disubstituted glycerol esters are indicative of 1,3 acylation (**Table 2**; Figure S1E−G of the Supporting Information). UV and mass spectral data for peak 2 matched the mass spectrum and UV absorbance of procyanidin B (*20*) (see Figure S1A of the Supporting Information). The procyanidin dimer B1 has been identified previously in sorghum (*18*). The mass spectrum for peak 3 matched the mass spectrum of taxifolin-7-*O*-*β*-glucoside (*20*) (see Figure S1B of the Supporting Information), and the component was tentatively identified as taxifolin-hexoside.

Quantification of Phenolic Acids and Flavonoids in Sorghum and Sorghum Sourdoughs. The contents of phenolic acids and flavonoids were determined in samples before and after fermentation of sorghum PAN 3860 to investigate the effect of fermentation. Two different binary strain combinations were used, *L. fermentum* with *L. reuteri* and *L. plantarum* with *L. casei*. The pH decreased from 6.3 to 4.8 for sorghum dough fermented by *L. fermentum* and *L. reuteri* and from 5.9 to 4.1 for sorghum dough fermented by *L. plantarum* and *L. casei*. The cell count increased from $(1 \pm 0.6) \times 10^8$ to $(6 \pm 2.7) \times 10^8$ cfu mL^{-1} after 24 h of fermentation with both binary strain combinations. The colony morphology of the strains in fermented sorghum dough matched the morphology of the inoculated strains, confirming that strains used as inoculum dominated throughout the fermentation.

Table 1. Antimicrobial Activity of Methanolic Extracts of Sorghum Flours and Fermented Sorghum Flours

	diameter of zone of inhibition[a] (mm)	
sample	*B. subtilis*	*L. monocytogenes*
unfermented Segaolane	6.6 ± 0.6	6.3 ± 0.6
L. reuteri + *L. fermentum*	6.7 ± 0.6	6.7 ± 1.1
L. casei + *L. plantarum*	6.7 ± 0.6	6.7 ± 0.6
unfermented PAN 3860	9.6 ± 0.6	9.6 ± 1.1
L. reuteri + *L. fermentum*	11.6 ± 1.1	12.6 ± 0.6
L. casei + *L. plantarum*	10.7 ± 1.1	10.3 ± 0.6

[a] Data are means ± standard deviations of two independent fermentations.

Table 2. Identification of Free (Nonbound) Polyphenols in Extracts from Nonfermented Sorghum Flour by LC-DAD-MS

peak	v_R (mL)[a]	identity	*m*/*z* $[M - H]^-$ (intensity, %)	*m*/*z* MS^n (intensity, %)	HPLC-DAD (nm)
1	10.2	protocatechuic acid[b]	153 (100)	109 (79)	230, 260, 294
2	10.4	procyanidin B (*20*)[c]	577 (100)	425 (39), 289 (12)	230, 280
3	10.7	taxifolin hexoside (*20*)[c]	465 (50)	303 (82), 285 (100), 177 (22)	230, 286
4	11.6	catechin[b]	289 (100)	245 (34), 179 (6)	232, 280
5	12.1	protocatechuic aldehyde[b]	137 (100)	109 (2)	228, 280, 310
6	12.4	caffeoylglycerol (*19*)[c]	253 (100)	179 (12), 161 (21), 135 (36)	230, 300sh, 326sh
7	12.7	*p*-hydroxybenzoic acid[b]	137 (32)	93 (100)	228, 256
8	13.3	caffeic acid[b]	179 (14)	135 (100)	228, 294, 322
9	14.9	coumaroyl-glycerol (*19*)[c]	237 (100)	163 (14), 145 (38), 119 (23)	
10	16.1	eriodictyol-7-*O*-glucoside[b]	449 (25)	287 (100), 151 (19)	228, 282
11	17.2	*p*-coumaric acid[b]	163 (12)	119 (100)	226, 308
12	18.3	ferulic acid[b]	193 (100)	134 (38)	226, 294, 322
13	19.0	taxifolin[b]	303 (32)	285 (100), 177 (28), 125 (12)	232, 290
14	20.5	naringenin-7-*O*-glucoside[b]	433 (100)	271 (45), 151 (3)	228, 286
15	29.4	1,3-dicaffeoyl-glycerol (*19*)[c]	415 (100)	253 (19), 179 (2), 161 (7), 135 (6)	226, 300sh, 326sh
16	31.3	eriodictyol[b]	287 (72)	151 (100)	226, 280
17	32.5	luteolin[b]	285 (100)	175 (14), 151 (11)	224, 268, 286, 346
18	34.7	1,3-coumaroyl-caffeoyl-glycerol (*19*)[c]	399 (100)	253 (12), 235 (5), 163 (4), 145 (1)	226, 314
19	36.9	naringenin[b]	271 (100)	151 (18)	224, 288
20	37.1	apigenin[b]	269 (100)	117 (32)	222, 268, 286, 336
21	38.2	1,3-coumaroyl-feruloyl-glycerol (*19*)[c]	413 (100)	235 (5), 193 (2), 163 (2)	222, 316

[a] Elution volume. [b] Identification of compounds with external standards. [c] Tentative identification of compounds on the basis of mass spectra and UV spectra reported in the indicated references.

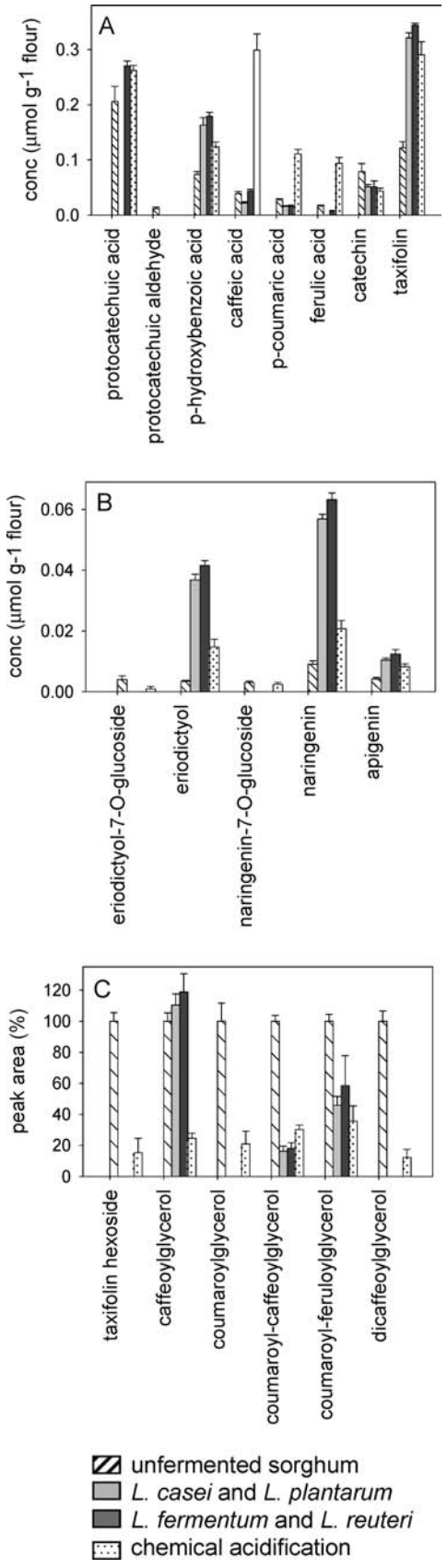

Figure 1. Quantification of free polyphenols in extracts of nonfermented and fermented sorghum flour. Results are expressed as micromoles per gram of sorghum flour (**A** and **B**) and as peak area percent (**C**). Data are shown as means ± standard deviations of three independent experiments analyzed in duplicate.

The concentration of phenolic acids and flavonoids was expressed in micromoles per gram of flour when external standards were available or as percentage relative to unfermented sorghum flour when external standards could not be obtained (**Figure 1**). Luteolin and procyanidin B were not quantified because they were present in small quantities and coeluted with other compounds.

The concentration of all phenolic acids increased during fermentation of chemically acidified doughs, and a corresponding decrease in glycerol esters of phenolic acids to 10−40% of the initial concentrations was observed (**Figure 1A**,**C**). Likewise, the concentrations of all flavonoid glucosides decreased in chemically acidified doughs, and the levels of the corresponding aglycones, taxifolin, eriodictyol, and naringenin, increased. Fermentation with binary strain combinations strongly reduced concentrations of glycerol esters of phenolic acids with the exception of caffeoylglycerol, coumaroyl-caffeoyl-glycerol, and coumaroyl-feruoylglycerol; concentrations of cumaroyl-glycerol and 1,3-dicaffeoylglycerol were below the detection limit after 24 of fermentation (**Figure 1C**). Moreover, most phenolic acids were metabolized, and levels were substantially lower than in the chemically acidified control or below the detection limit after 24 h of fermentation. The only exception was *p*-hydrobenzoic acid, which increased in both fermented sorghum doughs (**Figure 1A**). The increase in the concentration of flavonoid algycons was more pronounced in fermented sorghum doughs compared to the chemically acidified control (**Figure 1B**). Protocatechuic acid was the only compound that showed a different response between the two starter cultures; it was metabolized by *L. plantarum* and *L. casei* but not by *L. fermentum* and *L. reuteri* (**Figure 1A**).

Identification of Bound Polyphenols. Bound polyphenols occurring in the sorghum variety PAN 3860 were quantified before and after fermentation of chemically acidified doughs to investigate whether their release during fermentation contributed to the changes in free polyphenols (**Figure 1**). **Figure 2** shows typical LC-DAD chromatograms obtained for bound polyphenols fraction extracted from sorghum and chemically acidified sorghum dough. Ferulic acid was the major compound in both extracts; protocatechuic acid, protocatechuic aldehyde, caffeic acid, *p*-coumaric acid, ferulic acid, eriodictyol, and naringenin (peaks 1, 5, 8, 11, 12, 16, and 19) were additionally identified (see **Table 2** for peak assignment). Among the bound polyphenols, additional, unidentified peaks that were not present in free polyphenols were observed. The peak at 13.8 mL (peak A in **Figure 2**) showed a $[M - H]^-$ ion at m/z 325. MS and MS^3 analysis of the parent ion demonstrated the presence of fragments at m/z 265.1, 193.1, and 178.0 (**Figure 2** of the Supporting Information); the latter two fragments may represent a ferulic acid moiety. The UV spectrum of the compound matched the UV spectrum of ferulic acid external standard, indicating that the compound is a feruloylpentoside.

Metabolism of Polyphenols. To determine the metabolic activity of the starter cultures, culture media containing ferulic acid, caffeic acid, or naringenin-7-*O*-glucoside were fermented with each of the four strains individually. Different from the extraction of polyphenolic compounds from sorghum doughs, the extraction protocol allowed the detection of volatile metabolites of phenolic acids. An overview on the metabolites formed by the four different strains is provided in **Table 3**. Ferulic acid was metabolized by *L. plantarum* and *L. fermentum* to a metabolite with an elution volume of 15.8 mL. The mass spectrum showed a $[M - H]^-$ ion at m/z 195 and a fragment at m/z 136, corresponding to dihydroferulic acid. Neither *L. casei* nor *L. reuteri* degraded ferulic acid. Caffeic acid was metabolized by *L. plantarum* to two metabolites with elution volumes of 23.6 and 25.9 mL. The mass

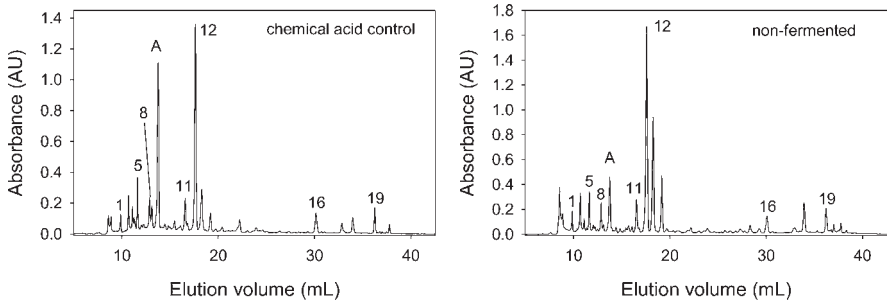

Figure 2. Separation of bound polyphenols in acid-hydrolyzed nonfermented sorghum and chemically acidified sorghum by LC-DAD-MS (280 nm). See **Table 2** for peak assignments. Data are representative of two independent experiments.

Table 3. Metabolites of Single Strains in Laboratory Culture from Ferulic Acid, Caffeic Acid, and Naringenin-7-*O*-glucoside[a]

	ferulic acid	caffeic acid	naringenin-7-*O*-glucoside
L. plantarum	dihydroferulic acid	vinylcatechol, ethylcatechol	naringenin
L. casei	–[b]	–	naringenin
L. fermentum	dihydroferulic acid	vinylcatechol, dihydrocaffeic acid	–
L. reuteri	–	–	–

[a] See text for detection and identification of metabolites. [b] –, no metabolites corresponding to the phenolic substrate were detected.

spectrum of the first peak gave a $[M - H]^-$ ion at m/z 135, matching the mass spectrum of vinylcatechol, and the second peak a $[M - H]^-$ ion at m/z 137, matching the mass spectrum of ethylcatechol. Both compounds were previously identified as metabolites of caffeic acid by *L. plantarum* (*11*). Caffeic acid was metabolized by *L. fermentum* to vinyl catechol and a metabolite with an elution volume of 11 mL. The mass spectrum for the peak at 11 mL showed a $[M - H]^-$ ion at m/z 181 and fragments at m/z 137, corresponding to dihydrocaffeic acid. Neither *L. reuteri* nor *L. casei* metabolized caffeic acid. LC-DAD-MS analyses showed that naringenin-glucoside was partially metabolized by *L. casei* and *L. plantarum* to the aglycon. *L. fermentum* and *L. reuteri* did not metabolize naringenin-7-*O*-glucoside.

DISCUSSION

Phenolic acids and flavonoids in the red sorghum variety PAN 3860 were identified, and their contents in nonfermented doughs and doughs fermented with *L. fermentum* FUA3165 and *L. reuteri* FUA3168 or *L. plantarum* FUA3171 and *L. casei* FUA3166 were compared. Fermentation of chemically acidified doughs as well as lactic fermentation profoundly altered the content and the profile of sorghum polyphenols. The fermentation of sorghum doughs and in vitro metabolism of polyphenols indicated the presence of esterase (tannase), phenolic acid decarboxylase, phenolic acid reductase, and glucosidase activities in the starter cultures. The four strains used different pathways for phenolic acid and flavonoid metabolism.

Phenolic acids and flavonoids present in sorghum PAN 3860 have been identified previously in sorghum (*4*). However, to our knowledge, coumaroyl-glycerol is the only phenolic acid ester of glycerol that was previously found in sorghum (*21*). This study additionally demonstrated that caffeoyl-glycerol, dicaffeoyl-glycerol, coumaroyl-caffeoyl-glycerol, and coumaroyl-feruloyl-glycerol are present in the red sorghum variety PAN 3860. In addition to *p*-hydroxybenzoic aldehyde, which is known to occur in sorghum (*22*), a second phenolic acid aldehyde, protocatechuic aldehyde, was identified. Protocatechuic acid, *p*-hydroxybenzoic

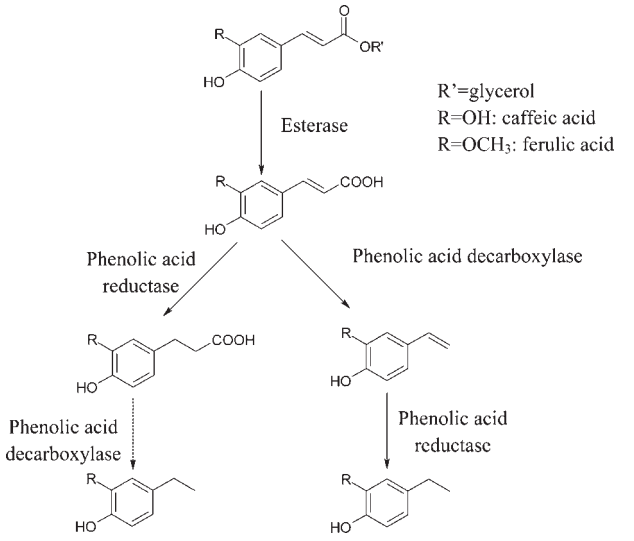

Figure 3. Metabolic pathways of phenolic acid esters and caffeic and ferulic acids. Esterase activity was previously reported for *L. acidophilus* and *L. plantarum* (*9*, *25*). Decarboxylase activity on caffeic and ferulic acids as well as reduction of ferulic acid or vinylphenol to dihydroferulic acid and ethylphenol, respectively, was demonstrated for several *Lactobacillus* spp. (*10*–*12*). For review see ref *13*.

acid, and caffeic acid were the most abundant free phenolic acids, and catechin and taxifolin were the most abundant free flavonoids. The content of free phenolic acids in sorghum PAN 3860 varied between 0.02 and 0.2 μmol g^{-1} of flour and was in same range as previously reported (*23*). In keeping with previous studies, ferulic acid was the most abundant bound phenolic acid (*23*), and a feruloyl-pentoside was present in acid-hydrolyzed sorghum. Because the flour used in this study was obtained from the whole grain rather than decorticated grains, polyphenol levels likely were higher than in those flours generally used for food preparation. However, in countries that use sorghum as a staple in the diet, a large diversity exists relative to the use of raw materials and the use of fermentation or heat-processing steps involved in food preparation (*1*, *2*). The chemical, biochemical, or microbial modifications of phenolic compounds thus are representative of processes used in food preparation.

The total polyphenol content in sorghum decreased significantly after fermentation with traditional starter cultures (*6*, *24*). This study demonstrates that fermentation of sorghum dough profoundly altered the concentration of phenolic acids and flavonoids in sorghum PAN 3860. The partial conversion of phenolic acid esters to phenolic acids in chemically acidified doughs indicates acid hydrolysis or the presence of cereal esterases. Most of the phenolic acid esters were quantitatively metabolized during lactic fermentation, indicating that microbial esterases contribute

to hydrolysis the phenolic acid esters. Tannase and feruloyl esterase activities were identified in *L. plantarum* and *L. acidophilus*, respectively (*9*, *25*). Esterase and glucosidase activities of lactobacilli may furthermore contribute to the release of phenolic acids bound to insoluble cell wall material, particularly protocatechuic and *p*-hydroxybenzoic acids.

The degradation of phenolic acids in lactic fermented sorghum doughs is attributable to phenolic acid decarboxylases and phenolic acid reductases (**Figure 3**). Caffeic acid, coumaric acid, and ferulic acid were metabolized by both binary strain combinations; protocatechuic acid was metabolized by *L. plantarum* and *L. casei* but not by *L. fermentum* and *L. reuteri*. Fermentations with single strains in mMRS demonstrated that ferulic acid was reduced but not decarboxylated by *L. plantarum* and *L. fermentum*. However, *L. fermentum* and *L. plantarum* employed different metabolic pathways for caffeic acid. *L. plantarum* decarboxylated caffeic acid to vinylcatechol, which was partially converted to ethylcatechol (**Figure 3**). In contrast, caffeic acid was decarboxylated to vinylcatechol or reduced to dihydrocaffeic acid by *L. fermentum*, and accumulation of ethyl catechol was not observed. The accumulation of dihydrocaffeic acid by *L. fermentum* indicates that decarboxylase and reductase enzymes of *L. fermentum* and *L. plantarum* have different substrate specificities.

Flavonoid hexosides were quantitatively metabolized during lactic fermentations but only partially hydrolyzed in chemically acidified doughs. The concentrations of eriodictyol and naringenin in lactic fermented doughs were substantially higher than those of the corresponding flavonoid hexosides in flour, indicating release of flavonoids bound to insoluble cell wall material. This study revealed that both naringenin and eriodictyol are present in bound form. *L. plantarum* and *L. casei* exhibited glucosidase activity on naringenin glucoside. Although lactobacilli are known to harbor glycosidase activities, releasing flavonoid and isoflavone aglycones from the corresponding glycosides (*26*, *27*), hydrolysis of naringenin and eriodictyol glycosides by lactobacilli has to date not been described. *L. fermentum* or *L. reuteri* did not hydrolyze naringenin-7-*O*-glucoside during growth in mMRS, but naringenin-7-*O*-glucoside was quantitatively hydrolyzed during growth of these organisms as binary strain combination in sorghum dough. It is possible that glucosidase activity of *L. fermentum* or *L. reuteri* is expressed in sorghum doughs but not in mMRS.

Phenolic acids and flavonoids have antimicrobial activity (*7*, *28*). In keeping with the higher contents of polyphenols, methanolic extracts of the red sorghum variety PAN3860 exhibited higher antimicrobial activity than extracts from the white sorghum variety Segaloane. The content of most phenolic acids decreased after fermentation of sorghum PAN3860 dough but the antimicrobial activity was not changed by fermentation. A previous study revealed that the antimicrobial activity of flavonoids was higher than the activity of the corresponding flavonoid glycosides (*28*). The release of flavonoid aglycones during fermentation of sorghum doughs could thus compensate the decrease in phenolic acids. Sorghum tannins also display antimicrobial activity (*29*) but were not investigated in the present study.

Lactic fermentation is a common technique to prepare and preserve sorghum dishes in Africa, and fermentation increases the palatability and the nutritional value of sorghum-based food products (*1*, *30*). This study demonstrated that *Lactobacillus* species contribute substantially to the conversion of phenolic acids and flavonoid glycosides during fermentation of sorghum doughs. Condensed tannins and deoxyanthocyanidins, which also occur in sorghum, were not taken into account as different extraction and separation methods are required for their quantification from doughs.

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Supporting Information Available: Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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