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Improved Bioavailability of Dietary Phenolic Acids in Whole Grain Barley and Oat Groat following Fermentation with Probiotic *Lactobacillus acidophilus*, *Lactobacillus johnsonii*, and *Lactobacillus reuteri*

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ABSTRACT: The aim of this study was to improve the bioavailability of the dietary phenolic acids in flours from whole grain barley and oat groat following fermentation with lactic acid bacteria (LAB) exhibiting high feruloyl esterase activity (FAE). The highest increase of free phenolic acids was observed after fermentation with three probiotic strains, *Lactobacillus johnsonii* LA1, *Lactobacillus reuteri* SD2112, and *Lactobacillus acidophilus* LA-5, with maximum increases from 2.55 to 69.91 $\mu g\ g^{-1}$ DM and from 4.13 to 109.42 $\mu g\ g^{-1}$ DM in whole grain barley and oat groat, respectively. Interestingly, higher amounts of bound phenolic acids were detected after both water treatment and LAB fermentation in whole grain barley, indicating higher bioaccessibility, whereas some decrease was detected in oat groat. To conclude, cereal fermentation with specific probiotic strains can lead to significant increase of free phenolic acids, thereby improving their bioavailability.

KEYWORDS: *phenolic acids, barley, oat, lactic acid bacteria, probiotic fermentation, esterase*

INTRODUCTION

Phenolic acids are associated with a low risk for chronic diseases and protection against carcinogenesis and mutagenesis.[1−3] Fruits, berries, and vegetables are rich sources of phenolic acids and have been investigated in several human studies showing a potential relationship between consumption of phenolic acids and health.[4−6] Interestingly, the content of phenolic acid in cereal grains is comparable with or even higher than that found in fruits, berries, and vegetables, with a concentration in the range of 600−3600 mg kg^{-1} in cereals[7−9] compared to 10−1000 mg kg^{-1} in fruits, berries, and vegetables.[10,11] However, little attention has been given to the health benefits of cereal consumption attributed to their high content of phenolic acids. The bioavailability and bioaccessibility of phenolic acids in fruits, wines, and vegetables is good, as the majority of phenolic acids are present in free form or as conjugates with quinic, tartaric, and malic acids and sugar moieties, which can be hydrolyzed in the upper intestinal tract. On the other hand, phenolic acids in cereals are mostly present in bound form through ester bonds to arabinoxylan chains or through ether bonds to lignin. Unlike the hydrolyzable free phenolic acids in fruits and vegetables, these fiber-bound phenolic acids are not hydrolyzed by human digestive enzymes but reach the colon intact, where they can be released by the action of bacterial enzymes such as microbial esterases and xylanases.[12] Indeed, animal and human studies have shown an increase in the concentration of ferulic acid and phenolic acid metabolites produced by colonic microflora in blood samples after the intake of cereal products.[13,14] However, the recovery of phenolic acids in plasma is still lower after cereal intake than after the intake of, for example, fruits, coffees, and wines.[15]

Processing is important for both sensorial and nutritive properties of cereal-based products. The content of phenolic compounds in cereal grains can be markedly affected by milling, extrusion, germination, and sourdough baking.[16,17] For example, the content of easily extractable phenolic compounds has been shown to significantly increase during germination, whereas the level of alkylresorcinols has not been affected.[17] It has been shown that extrusion cooking significantly increases the content of free phenolic acids in cereal grains.[18] Moreover, treatments of wheat bran with feruloyl and *p*-coumaryl esterases purified from various sources have been shown to increase the content of free phenolic acids.[19,20] In addition, fermentation of cereals with lactic acid bacteria (LAB) is experiencing increased interest. During fermentation, the grain constituents are modified by the action of both endogenous and bacterial enzymes, including esterases, xylanases, and phenoloxidases, thereby affecting their structure, bioactivity, and bioavailability. Cereal-based LAB fermentation has been shown to increase the levels of nutrients including folates, soluble dietary fiber, and total content of phenolic compounds in cereals[17,21] and to improve the protein digestibility and SCFA production in vitro.[22,23] Most studies have focused on the fermentation of rye and wheat in the baking industry, although oat and barley have

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Table 1. Content of Phenolic Acids (Micrograms per Gram Dry Matter) in Free and Bound Form in Flours from Whole Grain Barley and Oat Groat Flours

flour	caffeic acid	*p*-coumaric acid	ferulic acid	sinapic acid	5,5-diferulic acid	8-o-4-diferulic acid	8,5-diferulic acid[a]	total free/ bound phenolic acids	total free and bound phenolic acids
whole grain barley									947.7
free	0.21 ± 0.01	0.85 ± 0.19	1.32 ± 0.15	0.17 ± 0.02	BD[b]	BD	BD	2.55	
bound	6.39 ± 3.43	191.68 ± 10.3	674.48 ± 7.51	8.31 ± 0.64	16.19 ± 1.06	28.96 ± 1.34	21.14 ± 3.49	947.15	
oat groat									381.41
free	0.5 ± 0.09	0.65 ± 0.11	1.25 ± 0.21	1.73 ± 0.57	BD	BD	BD	4.13	
bound	6.96 ± 4.12	51.39 ± 5.10	271.32 ± 8.02	16.52 ± 1.47	7.47 ± 0.83	12.41 ± 1.52	11.21 ± 1.51	377.28	

[a]Benzofuran form. [b]BD, below detection limit.

been shown to be appropriate substrates for LAB fermentation. Interestingly, oat-based fermented products have been reported to decrease total cholesterol and increase fecal *Bifidobacterium* spp. in healthy volunteers.[24] However, the effect of LAB fermentation on the content of free and bound phenolic acids and their bioavailability has been poorly studied.

The aim of the present study was to increase the bioavailability of dietary phenolic acids by increasing the content of free phenolic acids in whole grain barley and oat groat flours following fermentation with LAB strains exhibiting esterase activity. Both probiotic and nonprobiotic bacterial strains were used to investigate the possibility of developing synbiotic (i.e., combination of pre- and probiotics) cereal-based products with additional health benefits in terms of improved bioavailability of dietary phenolic acids.

MATERIALS AND METHODS

Chemicals. Ferulic acid and *p*-coumaric acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Caffeic acid and sinapic acid were obtained from Fluka (Buchs, Switzerland). Methanol and acetonitrile of chromatographic grade, ethyl acetate, glycerol 87%, sodium chloride, hydrochloric acid fuming 37%, acetic acid (GR for analysis), and sodium hydroxide (pellets GR for analysis) were purchased from Merck (Darmstadt, Germany). Lactic acid (a variable mixture of D- and L-lactic acids, AnalaR) was obtained from BDH, Poole, U.K., and MRS broth and MRS agar were from Oxoid, U.K.

Cereal Samples. Whole grain barley of variety Olve and whole grain oat of variety Belinda were obtained from Lantmännen Cerealia, Moss, Norway. Oat grains were dehulled before milling using a laboratory dehuller (Streckel & Schrader KG, Germany) to produce oat groats. Oat groats and the whole barley grains were milled using a Retsch Mortar Grinder model RM100 with a 0.5 μm sieve (USA). Prepared samples were used for analysis and fermentation trials immediately after processing.

Bacterial Strains and Growth Conditions. A collection of 56 LAB strains was evaluated for their ferulic acid esterase (FAE) activity (data not shown). The following 8 strains were further used in this study, and their origin is shown in parentheses: *Bifidobacterium animalis* ssp. *lactis* BB-12 (Chr. Hansen, Denmark) (for convenience referred to as LAB), *Lactobacillus acidophilus* LA-5 (Chr. Hansen), *Lactobacillus johnsonii* LA1 (NCC 533), *Lactobacillus reuteri* SD2112 (ATCC 55730, kindly supplied by BioGaia Biologics, Sweden), *Lactobacillus fermentum* NCDO 1750 (ATCC 14931), *Lactobacillus plantarum/pentosus* AD2,[25] *L. plantarum* NC8,[26] and *L. plantarum* WCFS1.[27] BB-12, LA-5, LA1, and SD2112 are probiotic bacteria used in commercialized probiotic products. All strains were routinely grown in de man, Rogosa, Sharpe (MRS) medium at 37 °C except AD2, NC8, and WCFS1, which were incubated at 30 °C.

Ferulic Acid Esterase (FAE) Assay. Feruloyl esterase (or ferulic acid esterase, FAE) activity of the LAB strains was measured by an agar plate screening assay as described by Donaghy.[28] The protocol for Gram-positive bacteria (not *Bacillus*) was followed, including MRS agar omitting glucose, pH 6.5, and with ethyl ferulate (Sigma-Aldrich), for which the amount of released ferulic acid was determined by measurement of clearing zones.

Preparation of Starter Cultures. The bacterial strains were subcultured three times overnight in MRS broth to make the starter culture inocula. The broth cultures were washed twice with 5 mL of 0.9% NaCl aqueous solution and finally resuspended in 0.9% NaCl to obtain a bacterial suspension of approximately 9 log colony-forming units (cfu) mL^{-1}.

Fermentation of Whole Grain Barley and Oat Groat Flours. The fermentations of whole grain barley flour and oat groat flour were prepared by mixing 9 g of flour with 11 g of distilled water and 200 μL of starter culture inoculum using a laboratory shaker (MS2 minishaker, IKA, Germany). Two different controls were included in the fermentation setup: (1) water control without starter culture and (2) acid control, in which distilled water was replaced by 11 g of a 1:1 mixture of lactic acid (0.5 M) and acetic acid (0.5 M). All of the fermentation cultures were incubated at 37 °C for 18 h. Samples were collected before and after fermentation for measurements of pH and enumeration of bacteria, whereas samples for analysis of phenolic acids were frozen at 80 °C and then freeze-dried (Alpha 1-2 LD plus, Christ, Germany). Fermentation of each sample was performed in parallel.

Enumeration of Bacteria. The viability of the starter culture inocula and the numbers of cells in the cultures before and after fermentation were ascertained by traditional plate counts on MRS agar. All agar plates were incubated for 3 days at 37 °C.

pH Measurements. pH was measured in duplicate samples of unfermented and fermented flours (Φ 32 pH-meter, Beckman Coulter, Brea, CA, USA) by mixing 0.5 g of the samples with 4.5 mL of distilled water.

Extraction of Free and Bound Phenolic Acids. Extraction of free and bound phenolic acids from whole grain barley and oat groat was performed as described in our previous study.[7]

Quantitative Analysis of Free and Bound Phenolic Acids in Extracts. Identification and quantification of phenolic acids were carried out using a rapid separation high-performance liquid chromatography (HPLC) system (UltiMate 3000 Rapid Separation LC system, Dionex, Sunnyvale, CA, USA) with a diode array detector (DAD) (UltiMate 3000 RS, Dionex) and an Acquity UPLC BEH C8, 1.7 μm, 2.1 × 150 mm column (Waters, Milford, MA, USA). A gradient elution program was used with 1% acetic acid in Milli-Q water as eluent A and 1% acetic acid in acetonitrile as eluent B.[7] The injection volume was 10 μL, and the flow rate was 0.450 mL min^{-1}, with a column temperature of 50 °C. The identification and quantification of phenolic acids were carried out using external standards (caffeic, *p*-coumaric, ferulic, and sinapic acid) and UV spectrum characteristics (the Chromeleon Chromatography Information Management System (Dionex)). Quantification of diferulic acids (5,5′-diferulic, 8-o-4-diferulic and 8,5-diferulic acids) was estimated using the response factor for *p*-coumaric acid. Each sample was prepared in parallel.

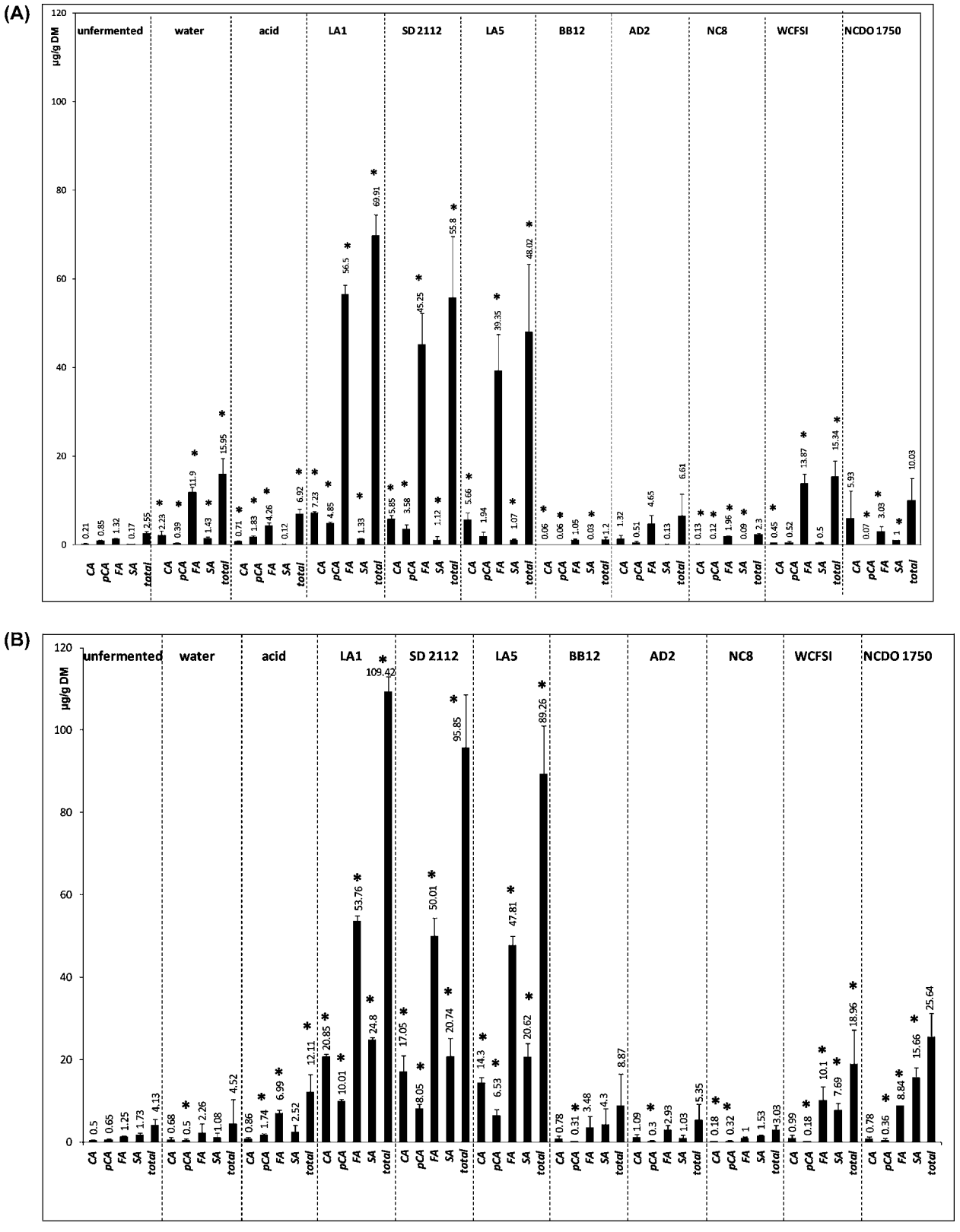

Figure 1. Content of free phenolic acids: caffeic (CA), *p*-coumaric (p-CA), ferulic (FA), and sinapic acids (SA) after LAB fermentation of flours from whole grain barley (A) and oat groat (B), μg g^{-1} DM (dry matter). Unfermented control, water control, and acid control are included. The strain numbers of the LAB are indicated: *L. johnsonii* LA1, *L. reuteri* SD2112, *L. acidophilus* LA-5, *B. animalis* BB-12, *L. plantarum/pentosus* AD2, *L. plantarum* NC8, *L. plantarum* WCFSI, *L. fermentum* NCDO 1750. Each bar represents the mean of at least two experiments performed in triplicates ± SEM. ∗ = $p < 0.05$.

Statistical Analysis. All experiments were performed at least in duplicate, and data are expressed as the mean ± SEM (HPLC analysis was performed in duplicate when variation in data was ≤10% and more than in triplicate when variation in data was >10%). For each

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flour and strain combination (barley, oat, free phenolic acids, bound phenolic acids) ANOVA was performed using Minitab 13.30 (Minitab Inc., State College, PA, USA) and the Statistical Analysis System SAS 9.2 (SAS Institute, Inc., Cary, NC, USA). The main factor in the model was type of strain, with two to four parallels (nested factor) and two replicates for each parallel.

F tests were used to examine effects of fermentation on content of phenolic acids in flour. Dunnett's test was used to identify differences between fermented and unfermented samples ("control"). Pearson correlation coefficients were calculated and tested at the 0.05 level.

RESULTS AND DISCUSSION

Phenolic Acids in Flours from Whole Grain Barley and Oat Groat. The content of free and bound phenolic acids in flours from whole grain barley and oat groat is presented in Table 1. Phenolic acids are mostly found in bound form in cereals, giving the ratio of the content of bound to the content of free phenolic acids of nearly 400:1 in flour from whole grain barley and over 100:1 determined in flour from oat groat (Table 1). Flour from whole grain barley contained higher amounts of phenolic acids (2.5-fold) than flour from oat groat (Table 1). This is most likely a consequence of the dehulling process of oat, which is known to decrease the content of phenolic acids,[29] as phenolic acids are concentrated in the outer layers of the cereal kernel. However, oat groat flour contained more total free phenolic acids than whole grain barley flour, 4.13 μg g^{-1} compared with 2.55 μg g^{-1}, as oat is known to have a higher content of free phenolic acids.[7] Ferulic and *p*-coumaric acids were the predominant phenolic acids in both flours (Table 1). Generally, the content of phenolic acids in whole grain barley and oat groat flours determined in this study is in agreement with other studies.[7]

Effect of Fermentation on the Level of Free Phenolic Acids in Flours from Whole Grain Barley and Oat Groat. Bacterial feruloyl esterases (FAE) are able to release bound phenolic acids from cereal fiber matrices into free forms;[30] thus, 56 LAB strains were screened for FAE activity. The screening (data not shown) resulted in seven promising strains: four with high activities (*L. johnsonii* LA1, *L. reuteri* SD2112, *L. acidophilus* LA-5, and *B. animalis* BB-12) and three with intermediate activities (*L. fermentum* NCDO 1750, *L. plantarum/pentosus* AD2, and *L. plantarum* NC8) that were further evaluated for their capacity to release bound phenolic acids during fermentation. One strain with no measured FAE activity was also included as a negative control, that is, *L. plantarum* WCFS1.

Fermentation of whole grain barley and oat groat flours by the selected strains resulted in an increase of viable cell numbers from approximately 7 to 9 log colony-forming units (cfu) mL^{-1} after 18 h, independent of strain and type of cereal flour. The pH decreased from 6.1 (whole grain barley) and from 6.6−6.7 (oat groat) to pH 3.6 and 3.9, respectively.

To ensure that the fermentation effects on the content of free phenolic acids in flours from whole grain barley and oat groat relate to specific bacterial fermentation, the impact of treatment by only water or acid was studied (Figure 1). Interestingly, control experiments with water and acid treatment resulted in 5−6-fold increases of total free phenolic acids (Figure 1). This increase of free phenolic acids has previously been shown in other studies.[31] The results indicate a significant but small effect on the bioavailability of cereal phenolic acids following hydrolysis and/or activation of cereal esterases during acid/water treatments widely used in processing of cereal foods.

In contrast, fermentation with the probiotic strains *L. johnsonii* LA1, *L. reuteri* SD2112, and *L. acidophilus* LA-5 gave an increase in the content of free phenolic acids of ∼20-fold in both flours (Figure 1), with the highest absolute increase following oat groat fermentation. The maximum increase of free phenolic acids was obtained with *L. johnsonii* LA1, with an increase from 2.55 to 61.91 μg g^{-1} DM and from 4.13 to 109.42 μg g^{-1} DM in whole grain barley and oat groat, respectively. Interestingly, these levels of free phenolic acids are comparable to that found in fruits and vegetables.[32] Ferulic acid was the predominant phenolic acid that increased in both flours, reaching a content in the range of 39−56 μg g^{-1}, although significant increases of free caffeic, *p*-coumaric, and sinapic acid were also detected.

Fermentation with the other LAB strains had much less effect on the level of free phenolic acids (Figure 1). Similar to the effects obtained with water and acid controls, a 4−6-fold significant increase of total free phenolic acids was detected with *L. plantarum* WCFS1, the negative control strain in terms of FAE activity, and with *L. fermentum* NCDO 1750 following fermentation of both flours. The total content of free phenolic acids was not affected in both flours following fermentation with *L. plantarum* NC8, *L. plantarum/pentosus* AD2, and *B. animalis* BB-12.

The reason the content of free phenolic acids in the flours was not affected equally following fermentation with all LAB strains exhibiting FAE activity could be due to degradation of the phenolic acids by an inducible phenolic acid decarboxylase (PAD), as previously shown in strains of *L. plantarum*, *L. fermentum*, and *B. animalis*.[31,33,34] In contrast, strains of *L. acidophilus* and *L. reuteri* have been shown to not degrade either ferulic or caffeic acids.[32] As a consequence, we tested the stability of phenolic acids during incubation of their standard solutions with *L. acidophilus* LA-5 and *L. plantarum* NC8 and observed that the content of phenolic acids significantly decreased after incubation with *L. plantarum* NC8 compared with *L. acidophilus* LA-5 (data not shown). Another possibility could be that these LAB strains or some of them did not possess FAE activity under the cereal fermentation conditions used, as optimal activities of the FAEs is known to have broad ranges of pH (4−8) and temperature (30−65 °C)[30] dependent on the original source. Moreover, FAEs from various sources have been shown to differ in their specificity against hydroxycinnamic acids and to be highly inducible depending on the growth substrate.[30,35] It has, for instance, been shown that FAE activity is carbon source dependent in *Bifidobacterium* sp. and *L. acidophilus*, with a much lower activity of FAE during fermentation of wheat or rye bran compared to synthetic methyl ester.[36,37] As FAEs from the LAB strains used in the present study were not purified and characterized, the optimal conditions for their best activities are still unknown. Another reason could be a consequence of low activity of carbohydrate-degrading enzymes such as xylanase and arabinofuranosidase. Several studies indicate the importance of interaction of these enzymes with FAE to effectively release ferulic acid.[38]

Content of Bound Phenolic Acids in Fermented Flours from Whole Grain Barley and Oat Groat. The effect of fermentation on the level of bound phenolic acids in flours from whole grain barley and oat groat was studied using four selected strains: *L. reuteri* SD2112 and *L. acidophilus* LA-5 (large increase in free phenolic acids), *L. fermentum* NCDO 1750 (slight increase in free phenolic acids), and *L. plantarum* WCFSI (no FAE activity detected) (Figure 2).

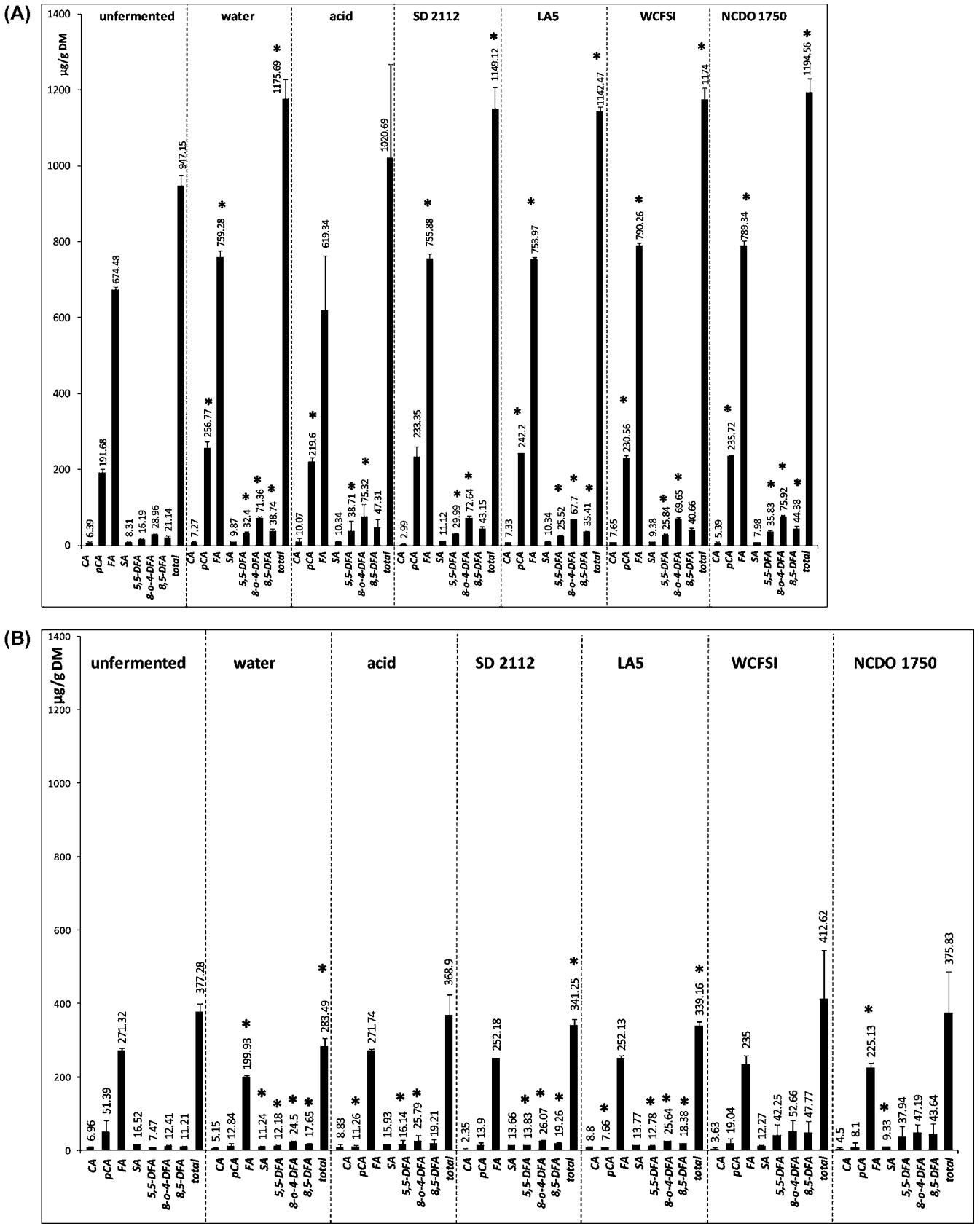

Figure 2. Content of bound phenolic acids (caffeic (CA), *p*-coumaric (p-CA), ferulic (FA), sinapic (SA), and diferulic acids 5,5′-diferulic acid (5,5′-DFA), 8-o-4-diferulic acid (8-o-4-DFA), 8,5′-diferulic acid (8,5′-DFA)) after LAB fermentation of flours from whole grain barley (A) and oat groat (B), $\mu g\ g^{-1}$ DM (dry matter). Unfermented control, water control, and acid control are included. The strain numbers of the LAB are indicated: *L. reuteri* SD2112, *L. acidophilus* LA-5, *L. plantarum* WCFSI, *L. fermentum* NCDO 1750. Each bar represents the mean of at least three experiments performed in triplicates ± SEM. ∗ = $p < 0.05$.

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Interestingly, both water treatment and fermentation with all four LAB strains increased the total content of bound phenolic acids in flours from whole grain barley by 23 ± 3% (Figure 2A). The increase was related to the increased content of bound *p*-coumaric acid, ferulic acid, and dimers of ferulic acid (5,5′-diferulic, 8-o-4-diferulic, and 8,5′-diferulic acids). A similar trend was observed with the acid control, although the changes in the total content of bound phenolic acids were not significant. This increased detection of bound phenolic acids is in agreement with previous studies on processed barley[29] and on fermented wheat and rye.[17,22] On the other hand, improved accessibility of bound phenolic acids to hydrolysis can be attributed to the increased ratio of soluble dietary fiber to insoluble dietary fiber in the cereal product.[39] Increased content of soluble dietary fiber in cereal-based products is desirable from a nutritional point of view. Soluble dietary fiber seems to be a better substrate for feruloyl esterases present in the human intestine compared with insoluble dietary fiber,[40] thereby leading to improved bioaccessibility of bound phenolic acids from soluble dietary fiber. The increase in soluble dietary fiber could be a consequence of the action of various endogenous nonstarch polysaccharide-hydrolyzing enzymes, as previously demonstrated in wheat and barley.[29,41] These enzymes seem to be activated after various mechanical, water, and thermal treatments[29,42,43] and diminish after dehulling, debranning, and peeling of cereal grains.[44,45] Thus, this might explain the decrease of bound phenolic acids obtained in our study for oat groat flour after both water treatment and fermentation with *L. reuteri* SD2112 and *L. acidophilus* LA-5 (Figure 2B), which is in contrast to the increase of the bound phenolic acids in whole grain barley flour (Figure 2A).

In summary, in this study we have shown that fermentation of flours from whole grain barley and oat groat with probiotic LAB strains can significantly increase the content of free phenolic acids, thereby improving their bioavailability. To our knowledge, this is the first study that shows such features of highly commercialized probiotic strains during fermentation of flours from whole grain barley and oat groat. Moreover, we also showed that FAE activity exhibited by LAB strain cannot be a clear indicator for the effective release of phenolic acids in cereal grains following fermentation. The increase of bound phenolic acids in whole grain barley upon fermentation and water treatment is presumably due to increased content of soluble dietary fiber and thus higher bioaccessibility. This is in contrast to some diminished content of bound phenolic acids in oat groat flour, which indicates that the type of preprocessing and type of flour are crucial factors affecting the content of bound phenolic acids after fermentation and water treatment. Further experiments could include other probiotic strains or mixtures of probiotic strains in addition to measurements of important nutritional values such as the content of soluble dietary fiber, β-glucan, starch, and arabinoxylan. Thus, this study clearly shows the potential to develop synbiotic cereal-based products with improved bioavailability of dietary phenolic acids.

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Notes

The authors declare no competing financial interest.

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