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Original paper

Effect of milling and sourdough fermentation on bioactive compounds of selected multigrain blends

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Abstract Blends of wheat, rye and barley were used to obtain multigrain milling fractions. The multigrain flours were further subjected to fermentation, and the metabolites profile and the nutritional potential of the obtain sourdoughs were explored. Increasing the level of rye and barley in the multigrain blends resulted in increased DPPH RSA, total phenolic and folic acid contents in flours and brans. The level of panthotenic acid decreased in flours and brans, with the increase of rye and barley levels. Both spontaneous and lactic acid bacteria assisted sourdough fermentation resulted in lower levels of folic acid compared to the corresponding flours, but the concentration increased with the level of rye and barley within blends. The metabolites biosynthesized by lactic acid bacteria varied with the levels of rye and barley in the blends. Multigrain milling and sourdough fermentation are valuable tools for modulating the nutritional profile of the cereal based food products.

Keywords multigrain flours, total phenolic content, antioxidant capacity, vitamins, sourdough

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Introduction

In order to improve the nutritional profile of cereal products one can approach different processing strategies, mainly during roller milling and breadmaking process.

The nutritional profile of the cereals influences to high extent the nutritional composition of the mill streams and flours. The milling extraction rate [1] and selecting specific mill streams to be blend in order to obtain different types of flours [2-4] are considered two of the most important ways to control the levels of bioactive compounds during milling. The high extraction rates allow retaining high levels of vitamins, minerals and fiber in flours, while at low extraction rates most of the bioactive compounds are concentrated into the brans. During roller mill processing the cereals are milled into a number of mill streams that depends by the flow diagram. Accumulation of bioactive compounds in the mill streams is related to the degree of participation of different anatomical components to their formation, the aleurone layer being particularly rich in nutritional components.

One way to obtain flours with high functionality is to take advantage of the bioactive compounds prevailing in different types of cereal by considering the possibility to process blends of cereals. In this respect one can choose the multigrain milling of different blends of cereals [5], or the separate milling of different cereals, followed by blending the flours or different mill streams in different percentages [6]. Anyway, limited information is available in the literature, regarding the use of multigrain milling for increasing the milling efficiency of different cereals, like rye and barley and better usage of their nutrition potential.

Fermentation is probably one the most important processing stage in breadmaking, and allows improving the profile and the content of bioactive compounds. It was reported that increasing the yeast assisted fermentation time favors the gradual increase of the B vitamins levels [7]. In addition, sourdough fermentation is efficient for increasing the minerals and phytochemicals bioavailability, as well as the levels and stability of some vitamins, for producing new bioactive compounds, such as prebiotic oligosaccharides or other metabolites, and also for improving the antioxidant properties [8, 9]. To the best of our knowledge, there are no previous studies on the use of sourdough fermentation for modulating the nutritional profile of the cereal based products prepared with multigrain flours.

The aim of this study was to investigate how the multigrain milling of the blends consisting of different levels of wheat, rye and hulled barley, impacts the total phenolic content, antioxidant capacity, folic acid and pantothenic acid levels of the multigrain flours. In addition, the possibility of using the sourdough fermentation for improving the profile of the bioactive compounds of the multigrain flours was also considered.

Materials and Methods

Chemicals

Folin-Ciocalteu reagent, sodium bicarbonate, ferulic acid, 2,2′-diphenyl-1-picrylhydrazyl (DPPH), methanol, were purchased from Sigma-Aldrich Chemical Co. (Steinheim, Germany). VitaFast® Pantothenic Acid, Ridascreen®Fast Folic Acid, D- and L-lactic acid, acetic acid, ethanol, and glycerol assay kits were purchased from R-Biopharm Rhone Ltd (Boehringer Mannheim, Germany). All chemicals used in the study are of analytical grade.

Multigrain flours preparation

Wheat (Boema variety), rye (Suceveana variety) grown in South East Romanian Plain, harvested in 2016, and hulled barley purchased from a specialized market (BioLogistic, Timişoara, Romania) were used in the experiments. Wheat (W), rye (R) and hulled barley (B) were mixed in the ratios (w:w:w) of 80:10:10 (80W+10R+10B), 70:15:15 (70W+15R+15B) and 60:20:20 (60W+20R+20B), and then milled with Buhler laboratory mill MLU (Buhler, Uzwil, Switzerland), according to Aprodu and Banu [10]. For each blend of cereals considered in the study, the following milling products were obtained: one multigrain flour (F) by blending six flour streams, one bran stream (B), and one short stream (S). The extraction rates were 71.5% (F10), 64.6% (F15) and 60.6% (F20) for multigrain straight flours resulted through multigrain milling the 80W+10R+10B, 70W+15R+15B and 60W+20R+20B blends, respectively.

Sourdough preparation

In order to prepare sourdoughs out of the multigrain flours, the commercial DI-PROX MTTX1 (EDR Ingredients, Romania) starter culture, consisting of three lactic acid bacteria (LAB) *Lactobacillus rhamnosus*, *Lactobacillus brevis* and *Lactobacillus plantarum*, was used. The inoculum was prepared according to producer recommendations such as to attain 10^8 cfu/100 g dough.

Sourdoughs were prepared by first mixing the multigrain flours with tap water and inoculum to get the dough yield (DY, mass of dough/mass of four ×100) of 300, followed by fermentation for 20 hours at 30°C. Parallel sourdough control samples were prepared without inoculum, by spontaneous fermentation for 20 hours at 30°C. The pH of the sourdough was monitored using a Hanna digital pH-meter, according to Romanian standard methods SR 90/2007 [11].

Biochemical analyses

Determination of total phenolic content

The phenolic compounds were extracted using the procedure described by Singleton and Rossi [12] and modified by Gao *et al*. [13]. The extraction was carried out with acidified methanol (HCl/methanol/water, 1:80:10, v/v/v) at room temperature for 2 h on a magnetic stirrer, followed by centrifugation for 15 min at 3000 rpm (refrigerated centrifuged TGL-16M, Xiangyi Laboratory Instrument Co., Ltd., Hunan, China) for 10 min.

The total phenolic contents (TPC) were determined using the Folin-Ciocalteu method described by Singleton and Rossi [12] and modified by Gao *et al*. [13]. After incubating for 90 min the mixture consisting of extract (0.2 mL), 1.5 mL of freshly 10-fold diluted Folin Ciocalteu reagent and 1.5 mL of sodium carbonate solution (60 g/L), the absorbance of was measured at 725 nm. Ferulic acid was used as standard, and the results were expressed as mg ferulic acid equivalent (FAE) per g d.w.

Determination of DPPH-radical scavenging activity

The compounds with antioxidant activity were extracted in 80% (v/v) aqueous methanol solution. After 2 h of constant steering at 37 C, samples were subjected to centrifugation at 10,000 rpm for 15 min [14, 15]. The supernatant was further used for assaying the DPPH-radical scavenging activity (DPPH RSA) using the method proposed by Brand-Williams *et al*. [14] and modified by Beta *et al*. [15]. In short, the extract (0.1 ml) was mixed with 3.9 ml DPPH solution ($6 \cdot 10^{-5}$ mol/l), and the absorbance at 515 nm was recorded after 30 min of resting in the dark at room temperature (A_{30}). The antioxidant activity was calculated as follows:

$$\%DPPH\text{-}RSA=(A_{30}-A_0)/A_0 \cdot 100 \quad (1)$$

where A_0 is the absorbance of the control prepared with 3.9 ml DPPH and 0.1 ml methanol, measured at t = 0.

Determination of the main metabolic end products of sourdough fermentation

The main metabolic end products of sourdough fermentation were quantified using specific R-Biopharm kits (Boehringer Mannheim, Germany), following the procedures indicated by the manufacturer. The enzymatic UV-tests used to quantify the D- and L-lactic acid (limit of detection 5 mg/l), acetic acid (limit of detection 0.15 mg/l), ethanol (limit of detection 1.8 mg/l) and glycerol (limit of detection 1.0 mg/l) are based on the formation of NADH which was measured by the increase in light absorbance at 340 nm.

Determination of the pantothenic acid and folic acid contents

The contents of pantothenic acid and folic acid were determined using the VitaFast® Pantothenic Acid and Ridascreen®Fast Folic Acid kits (Boehringer Mannheim, Germany). The VitaFast® Pantothenic Acid (detection limit of 0.0035 mg/100g) is a microbiological microtiter plate test which quantifies the content of pantothenic acid based on the growth of *Lactobacillus plantarum* used to coat the microtiter plate wells, while Ridascreen®Fast Folic Acid (detection limit of 100 µg/kg) is based on the competitive enzyme immunoassay. The two vitamins were extracted from flour and sourdough matrices and further quantified according to the procedures indicated by the manufacturer. The optical density values of the Ridascreen®Fast Folic Acid and VitaFast® Pantothenic Acid microtiter plates were measured at 450 nm and 610 nm using a plate reader (Stat Fax® 4700, Awarness Technology, Inc.). The RIDA® Soft Win (R-Biopharm AG, Germany) software was finally used to calculate concentration of pantothenic acid and folic acid in the flour and sourdough samples.

Statistical analysis

The experiments were carried out in triplicate, and the results are reported as average values together with standard deviation. Analysis of variance, performed with Microsoft Excel Soft, was used to identify significant differences. Statistical relationships were established by calculating Pearson's correlation coefficients. The differences were quantified using one-way ANOVA with a 95% confidence interval, after assessing the normality and variance equality conditions.

Results and discussion

Characterization of multigrain milling streams

Total phenolic content and antioxidant capacity

The TPC and DPPH RSA of the flours, shorts and brans resulted through milling the multigrain samples are depicted in Figure 1. Increasing the level of wheat substitution by rye and hulled barley within the multigrain blends determined the increase of TPC and DPPH in flours and brans, on the account of shorts. The TPC values of the short streams were lower than the highest amount of TPC found in brans (TPC of 1163.68 mg FAE/g, corresponding to the B20 sample) streams (Figure 1a). On the other hand, the lowest DPPH RSA value of 26.42% (S20) registered among the shorts was beyond the DPPH RSA value corresponding to the B20 sample with the highest antioxidant capacity among all bran streams (Figure 1b).

The trend registered in the levels of TPC found in flour, bran and short streams, varying with the levels of wheat, rye and hulled barley within blends, is obviously related to the TPC of cereals and can be explained by the distribution of the phenolic compounds in the grain. According to Dordevic *et al*. [16] and Zielinski and Kozlowska [17], the TPC increases in cereals in following order: rye < wheat < barley. Our results are in agreement with Liyana-Pathirana and Shahidi [18], who reported that TPC values decreased in the end milling products in the following order: bran > shorts > flour. Moreover, the distribution of phenolic compounds in the mill streams was reported to depend on the techniques used for the separation of the outer layers during milling [19].

The highest amounts of phenolic compounds are located in outer layer, specifically in pericarp and aleurone layer [20, 21]. The phenolic acids, which are the major phenolic compounds found in cereals, are available under three forms: free, soluble conjugated and bound, and the composition and their distribution vary with the anatomical parts of different grains [20, 22, 23]. The bound phenolic acids fraction prevails over the conjugated one, and the free phenolic acids contribute to the lowest extent to the total phenolic acid content of the cereals. Thus, the percentages of conjugated and free forms are about 22, 25 and 35% and 1, 3 and 4%, respectively, in wheat, barley and rye, respectively [24-26]. The same authors reported that the predominant phenolic acids are ferulic, vanillic and p-cumaric acids in wheat, ferulic, vanillic, syringic, and *p*-coumaric acids in barley, and ferulic, sinapic, and 2,4-dihydroxybenzoic acids in rye. The composition and form of the phenolic compounds influence the overall antioxidant activity. Zielinski and Kozlowska [17] reported that, for the same TPC level of different cereals, the antioxidant activities increased in the following order: rye < wheat < barley. In our study a significant positive correlation was established between TPC and DPPH RSA (0.87, $p<0.05$).

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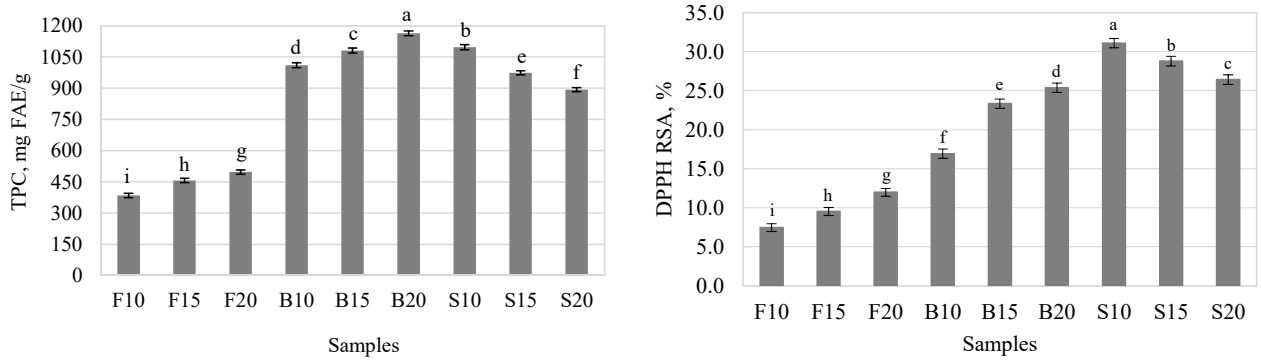
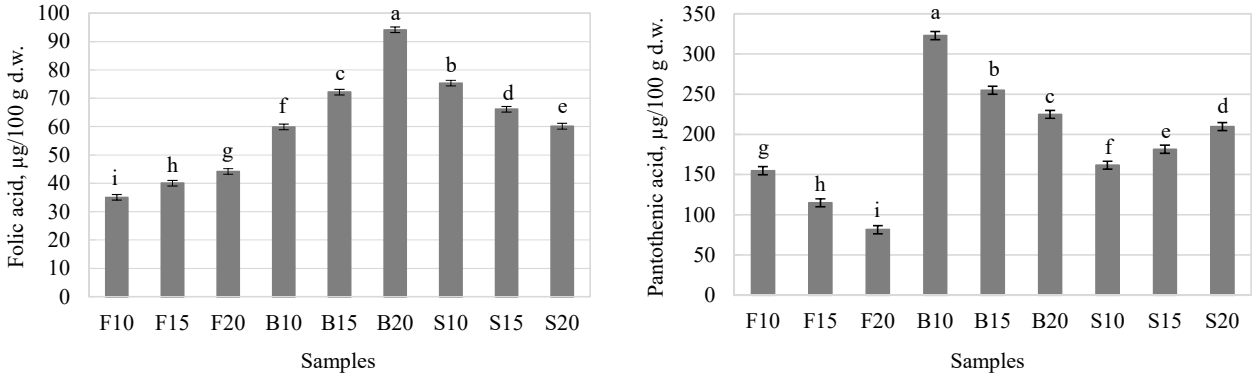


(a) (b)

Figure 1. The total phenolic contents (a) and antioxidant activity (b) of the multigrain flours (F), brans (B) and shorts (S) resulted from milling the multigrain blends. Means that do not share a letter are significantly different at $p<0.05$ (b).

Folic acid and pantothenic acid contents

Cereals are recognised as important sources of B group vitamins. The vitamin levels vary from one cereal to another, and are unevenly distributed within different anatomical parts of the grains. Indeed, our results indicated that milling of the multigrain blends consisting of wheat, rye and hulled barley resulted in streams with varying amounts of folic and pantothenic acids. Substitution of increasing amounts of wheat by rye and hulled barley allowed the increase of the folic acid levels in flours and brans, and the consequent decrease in the shorts (Figure 2a). When compared to the corresponding flours, the contents of folic acid in brans and shorts were about 1.7-2.13 fold and 2.14-1.36 fold higher, respectively. A different trend was registered in the pantothenic acid distribution in the milling streams. The results presented in Figure 2b suggest that the increase of rye and hulled barley levels in the blends subjected to multigrain milling resulted in flours and brans with decreasing contents of pantothenic acid, while the shorts retained increasing amounts of B5 vitamin. Our results are in agreement with Edelmann *et* al. [27] and Mihhalevski et al [28], who showed that folate and panthotenic acid are located in the outer layers and germs. In particular, Hemery *et al*. [29] reported a higher concentration of folate in the aleurone layer than in the other outer layers of wheat. In case of barley, the level of vitamins depends by the pearling and polishing grades [27]. According to Shewry et al. [30], the content of folic acid is similar in barley and rye, and is higher compared to wheat.



(a) (b)

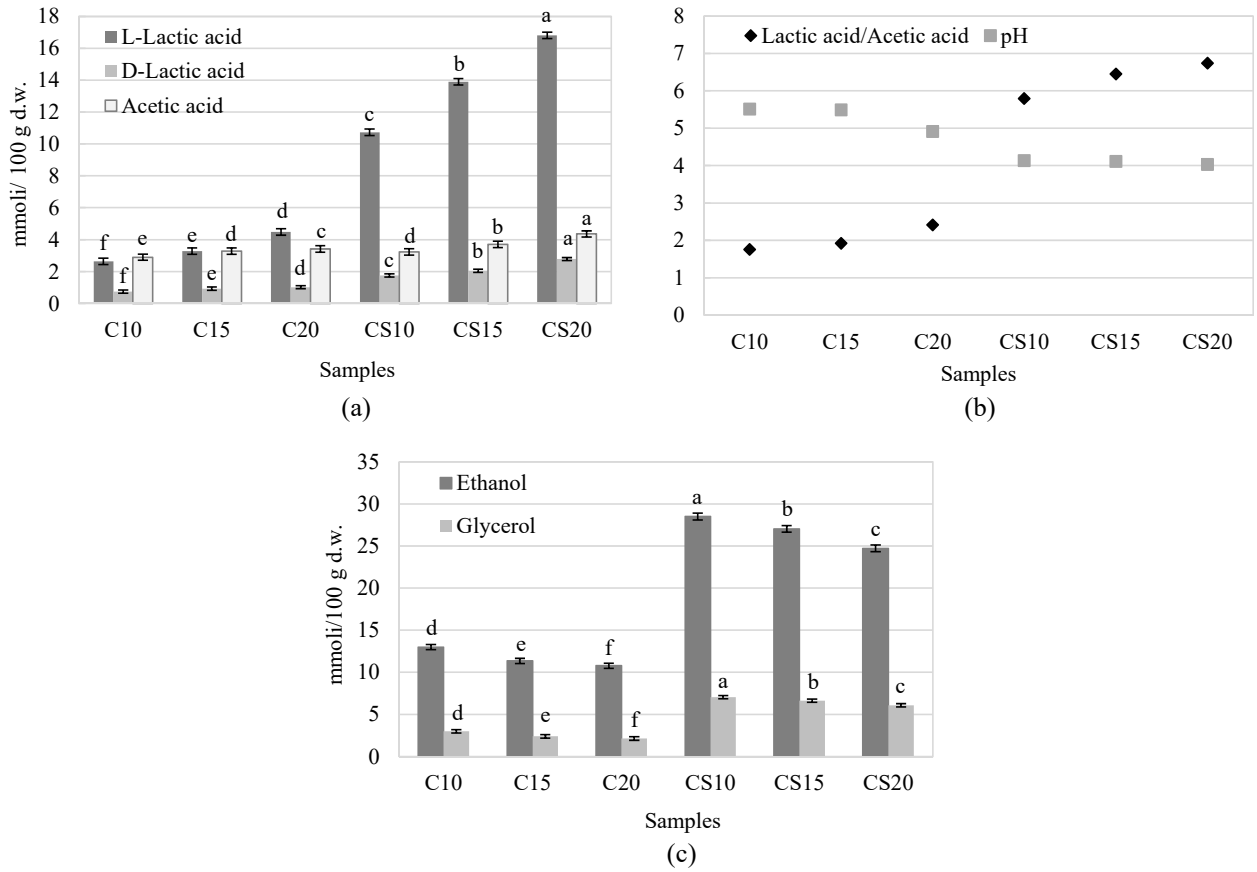
Figure 2. Contents of folic acid (a) and pantothenic acid (b) of the multigrain flours (F), brans (B) and shorts (S) resulted from milling the multigrain blends. Means that do not share a letter are significantly different at $p<0.05$.

Characterization of the sourdoughs

Sourdough fermentation

Accumulation of different metabolic end products during sourdough fermentation play important roles on defining the overall sensorial, nutritional and physical-chemical quality of baked products. In this respect, the composition of the flour subjected to fermentation and the LAB strains selected for starting the fermentation are crucially important. LAB strains have different metabolic pathways for the carbohydrates catabolism [31]. In the present study we used commercial strains consisting of facultative heterofermentative LAB, able to ferment both hexoses, leading to the accumulation of lactic acid, acetic acid, ethanol and CO_2, and pentoses when mostly lactic and acetic acids are formed.

The metabolites biosynthesized by the heterofermentative LAB varied with the level of rye and hulled barley in the wheat flour for both spontaneous and inoculum based initiated fermentations (Figure 3).



(a) (b)

(c)

Figure 3. The production of L,D-lactic acid and acetic acid (a), lactic acid/acetic acid and pH (b) and ethanol and glycerol (c) during sourdough fermented with (CS) and without (C) starter culture. For each metabolite, means that do not share a letter are significantly different at $p<0.05$

The accumulation of lactic and acetic acids caused the decrease of the pH values. Higher decrease was registered in case of samples fermented with LAB strains (pH of 4.13-4.03) compared to the spontaneously fermented sourdoughs (pH of 5.51-4.91) (Figure 3b). The gradual acidification of the medium allows the endogenous enzymes involved in starch and non-starch carbohydrates hydrolysis to become active. These enzymes are responsible for releasing fermentable sugars in the sourdough, which are further metabolise by LAB mainly into L,D - lactic acid and acetic acid. Rye and barley have high contents of amylase that increase the possibility to accumulate high amounts of hexoses in the sourdough, therefore explaining the higher amounts of acids in the samples obtained through the fermentation of multigrain flours, with increasing levels of rye and barley (Figure 3a). Otherwise, Flander *et al.* [32] noted that the activity of the endogenous enzyme from rye flours is higher than of the enzyme from wheat flour. Moreover, the lactic acid/acetic acid molar ratios increased with the increase of the rye and hulled barley levels in the blends (Figure 3b). On the other hand, the concentration of ethanol and glycerol in the sourdoughs decreased with increasing the levels of rye and hulled barley in the blends (Figure 3c). Regardless of the multigrain flour used for preparing the sourdough, significantly higher amounts of both ethanol and glycerol were found in samples fermented with mixture of *Lb. plantarum*, *Lb. brevis* and *Lb. rhamnosus*, compared to the control sourdoughs.

Effect of sourdough fermentation on the total phenolic content and antioxidant capacity of multigrain flours

The extractability of phenolic compounds and the antioxidant activity estimated as radical-scavenging capacity can be modulated by sourdough fermentation [23, 33]. The results presented in Figure 4 highlight that the fermentation processes, carried out with and without starter culture, allowed obtaining sourdoughs with increased TPC and DPPH RSA values in respect to the corresponding flours. The type of fermentation influenced the extent of pH decrease, which highly influence the extractability of different bioactive compounds, because the enzymes involved have different optimum pH [16]. Kariluoto *et al*. [34] noted that the microorganism species, from both epiphytic microflora and starter culture, influence the level of TPC and DPPH RSA values. The pH of control samples (C10, C15 and C20), fermented without starter culture, varied from 4.91 to 5.51, and significant lower pH values ranging between 4.03 and 4.13 were obtained for CS10, CS15 and CS20 samples, fermented with the use of starter culture. When compared to the multigrain flours, the TPC values of the sourdough samples fermented with (CS) and without (C) starter culture were about 2.8-3.3 and 1.4-1.6 fold higher, respectively, while the DPPH RSA values were about 1.7-1.84 and 1.1 fold higher, respectively. These results can be explained by the increase of the amount of easily extractable phenolic compounds, and other bioactive compounds that additionally reduce the DPPH radical. The breakdown of the cell walls fractions occurring during fermentation results in liberation of various bioactive compounds [*35*]. Moreover, the modification of flour composition with the release of bound phenolics as well as synthesis of new compounds assisted by the enzymes derived from cereals and/or LAB should be also considered. The TPC and DPPH RSA values of the sourdoughs increased with the level of rye and hulled barley used to substitute the wheat within blends at multigrain milling (Figure 4).

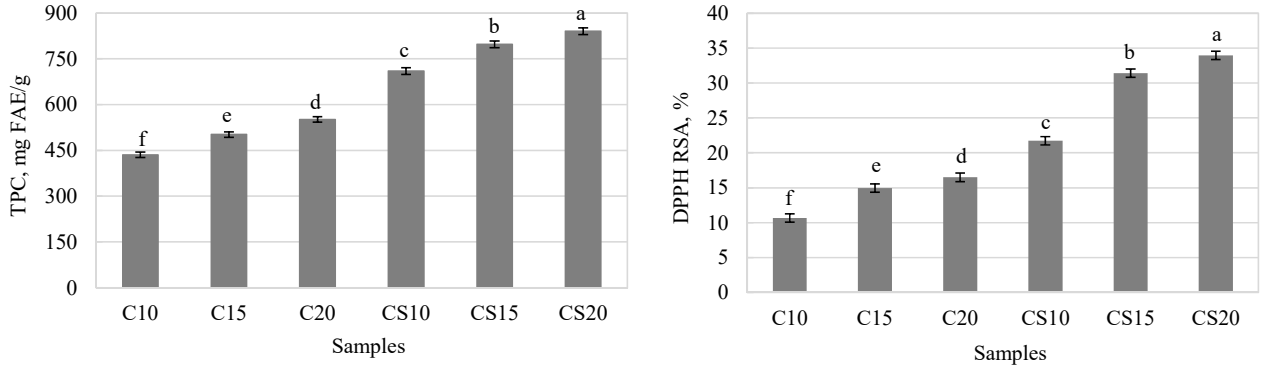


Figure 4. The total phenolic contents (a) and antioxidant activity (b) of the spontaneously fermented sourdoughs (C) and sourdoughs fermented with starter culture (CS). Means that do not share a letter are significantly different at $p<0.05$.

Effect of sourdough fermentation on the folic acid and pantothenic acid contents of multigrain flours

Sourdough fermentation might be a suitable strategy for increasing the vitamin content of the bakery products. According to Capozzi *et al*. [8], some LAB species are able to synthesise B group vitamins.

In our study the fermentation of the multigrain flour based on the endogenous microflora or after inoculation with LAB commercial strains caused the slight decrease of the folic acid contents. The increasing trend of the folic acid level in the flours with the ratio of rye and hulled barley within blends, was also notices in the sourdough samples, regardless of the starter culture presence (Figures 2a and 5a). Anyway, higher contents of folic acid were found in the spontaneously fermented sourdough compared to the samples

fermented after inoculation with LAB, suggesting that the commercial strains prevail over the endogenous microflora and consume higher amounts of acid folic during fermentation. Especially the yeasts have been reported to increase the folate contents in the, whereas the LAB, depending on the species, may synthesize or deplete the vitamin level of sourdoughs [34]. Kariluoto *et al.* [34] indicated the 20% reduction of the acid folic concentration in sourdoughs fermented with *Lb. plantarum* or *Lb. brevis* compared to the sterile rye flour used for preparing it. On the other hand, fermentation of the non-sterile rye flour with *Lb. plantarum* or *Lb. brevis* resulted in 4.7 times lower folate contents compared to the spontaneous fermentation. Anyway, when compared to the rye flour, a slight increase of the folate content was found in the sourdoughs prepared with non-sterile rye flour and fermented with *Lb. plantarum* or *Lb. brevis.* Two possible mechanisms were proposed by Kariluoto *et al*. [34] for explaining these results: the folates produced by the endogenous microflora of the rye flour might be consumed by *Lb. plantarum* or *Lb. brevis*, or the growth of the folate producing endogenous microflora might be retarded by the lactobacilli from the inoculum, as a result of pH decrease or of competition for available nutrients.

The control samples fermented by the spontaneous microflora had slight higher contents of pantothenic acid than corresponding multigrain samples fermented with LAB commercial strains (Figure 5b). Mihhalevski *et al*. [28] reported the decrease of pantothenic acid content in case of fermenting the rye flours with *Lb. panis* compared with a sample fermented without *Lb. panis*.

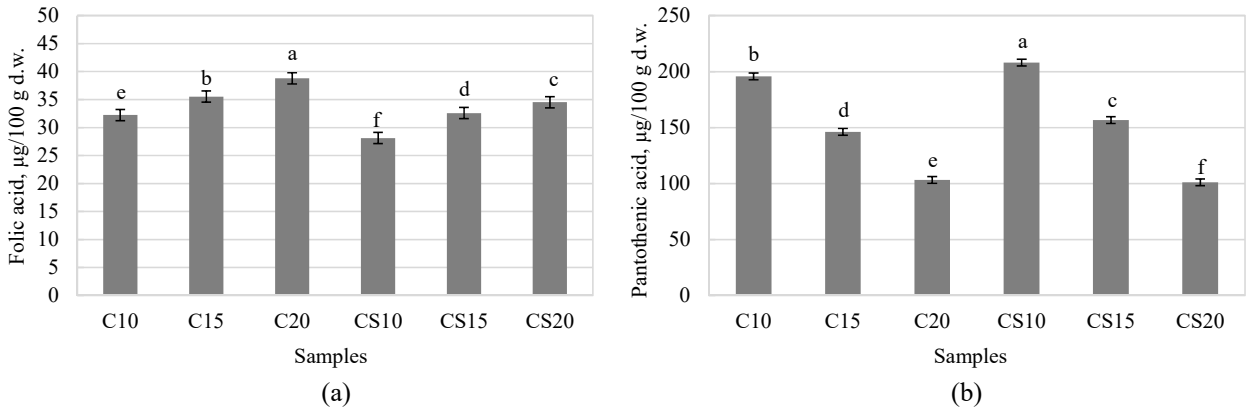


Figure 5. Contents of folic acid (a) and pantothenic acid (b) of the spontaneously fermented sourdoughs (C) and sourdoughs fermented with starter culture (CS). Means that do not share a letter are significantly different at $p<0.05$.

Conclusion

This study showed that increasing the levels of rye and hulled barley in the mixture with wheat at multigrain milling allowed obtaining flours with improved nutritional profile. The distribution of phenolic compounds, antioxidant capacity, folic and pantothenic acids in flours, bran and shorts varied with the wheat substitution by rye and hulled barley. Thus, TPC, DPPH RSA and folic acid increased in flours and brans, and decreased in shorts with the increase of the levels of rye and hulled barley in the multigrain blends subjected to milling. On the other hand, the panthotenic acid appeared to be mostly directed toward shorts when milling blends with increasing levels of rye and hulled barley, a decreasing trend being registered in case of flours and brans. Further fermentation tests indicated that the biosynthesis processes and the profile of metabolites accumulated in the sourdoughs varied significantly with the presence of the starter culture. The mixed starter culture with *Lactobacillus rhamnosus*, *Lactobacillus brevis* and *Lactobacillus plantarum* allowed accumulating higher amounts of L,D-lactic acid, acetic acid, ethanol and glycerol compared to the spontaneously fermented sourdoughs. Regardless of the use of starter culture for inoculating the multigrain flour based slurries, the fermentation process appeared a suitable tool for increasing the antioxidant capacity and the content of phenolic compounds. Moreover, the level of rye and hulled barley in the wheat flour influenced the profile of the metabolites in the sourdoughs. The amount of folic acid increased with the level of rye and hulled barley in the blends, but for all fermented samples the levels of acid folic were slightly below those of the corresponding flours. These findings are useful for developing new cereal based products with improved nutritional profile. Further studies will be conducted for optimising the sourdough bread production using different types of multigrain flours.

Acknowledgments

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