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Changes in carotenoids, phenolic acids and antioxidant capacity in bread wheat doughs fermented with different lactic acid bacteria strains

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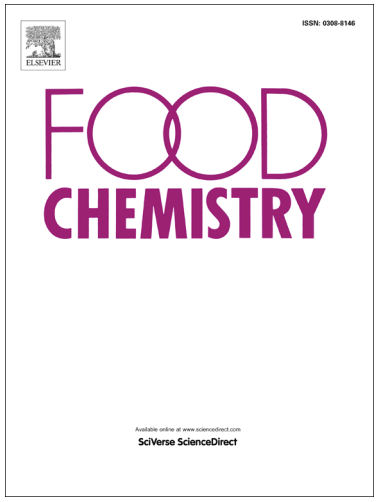

Short communication

Changes in carotenoids, phenolic acids and antioxidant capacity in bread wheat doughs fermented with different lactic acid bacteria strains

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Changes in carotenoids, phenolic acids and antioxidant capacity in bread wheat doughs fermented with different lactic acid bacteria strains

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Abstract

Amongst the processing technologies able to improve the functional features of cereal-based foods, sourdough fermentation using Lactic Acid Bacteria (LAB) has been recently rediscovered for its beneficial effects. Wheat (*Triticum aestivum* L.) bread doughs were prepared using LAB strains belonging to different *Lactobacillus* species and changes in phenolic acid, carotenoid content and antioxidant capacity were evaluated. Two *L. plantarum* strains out of six were able to significantly increase carotenoid content in the dough, suggesting that a higher mobilization/solubilisation of these antioxidant compounds occurs. Within different fractions (free, soluble-conjugated, insoluble-bound), the relative distribution of ferulic acid and antioxidant activity changes depending on the specific strain. Overall, results indicate that some LAB strains cause in situ changes significantly increasing the content of functional compounds in doughs during fermentation. This, in turn, could improve the functional features of bakery foods characterised by a high content in carotenoids and other bioactive compounds.

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Introduction

Plant secondary metabolites act in the human body as micronutrients or phytochemicals, with possible antioxidant, anti-inflammatory, anti-microbial and neuroprotective effects (Morrissey & O'Brien, 1998, Anson, Havenaar, Bast, & Haenen, 2010).

Cereals are particularly interesting: they are among the foods with the highest content of phenolic acids, which are well-known antioxidants, and can also contain carotenoids (Brandolini et al., 2013, Zilic et al., 2012). Cereals are almost never consumed as such; they are often subjected to technological treatments that can affect the original grain composition, thus changing its phytochemical content. Probably, the single most important transformation of cereals, and in particular of soft wheat (*Triticum aestivum* L.), is bread production, or bakery. In bakery, processing can contribute to functionality.

Sourdough fermentation with Lactic Acid Bacteria (LAB) has recently gained a renewed interest, in view of the increased nutritional value and potential health benefits it can bring about to the final product (Poutanen, Flander, & Katina, 2009). During sourdough fermentation, indeed, the grain constituents are subjected to the action of both endogenous and bacterial enzymes of different classes, and this causes the solubilisation of some components and the production of new nutritionally active molecules (Hur et al., 2014). Overall, sourdough fermentation improves nutrient bioaccessibility and produces compounds with anti-oxidant and anti-inflammatory activity (Greco et al., 2011). Moreover, several LAB are able to produce molecules of interest as a result of particular bacterial synthetic pathways (Turpin et al., 2016). Some of them are also endowed of their own antioxidative activity (Kaizu et al., 1993).

Changes in primary and secondary metabolites occurring in the cereal matrix after sourdough fermentation have been investigated through both a targeted (Rizzello et al., 2007), and a non-targeted methodological approach (Mozzi et al., 2013, Ferri et al., 2016). The overall metabolism of small molecules during sourdough fermentation has been recently studied by a non-targeted method in different cereals-based doughs, such as rye and wheat (Koistinen et al., 2018), durum wheat and KAMUT® Khorasan wheat (Ferri et al., 2016), and both studies highlighted the significance of this food processing approach in the final composition of the product.

Phenolic acids are a class of simple phenolic compounds associated with a lower risk for chronic diseases and with protection against various types of cancer (Chang et al., 2006). In cereals, they are mostly present in the bound form, covalently bonded to cell wall components such as arabinoxylans and lignin, through ester or ether bonds, respectively. Under this form, human digestive enzymes do not hydrolyse them, but they reach intact the colon, where they can be

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released by the action of bacterial enzymes (Saura-Calixto, 2011). Indeed, both animal and human studies have reported 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 (Kern et al., 2003).

Besides phenolic compounds, carotenoids have recently gained much attention, due to their recognized antioxidant properties. Hydroxy-containing carotenoids, such as lutein and zeaxanthin, in particular, have a fundamental role in maintaining an optimal eye functionality (Olmedilla et al., 2001); other beneficial actions include the prevention of cardiovascular diseases (Osganian et al., 2003).

In this work, a standard soft wheat flour has been kneaded and subjected to sourdough fermentation with LAB strains belonging to different species in order to evidence possible differences in their ability to change the phytochemical content of the dough. Levels of carotenoids, phenolic acids and their relative distribution in three different forms (free, soluble-conjugated, and insoluble-bound), as well as antioxidant capacity, were evaluated in the doughs, with the aim of selecting the best performing strain to be used for a functional final product.

Keywords: Carotenoids, Cereal-fermented food, *Lactobacillus* strains, Phenolic acids, Sourdough fermentation.

2. Material and Methods

2.1. Material

Phenolic acids (4-hydroxybenzoic acid, caffeic acid, chlorogenic acid, ferulic acid, gallic acid, *p*-coumaric acid, synapic acid, syringic acid, and *trans*-cinnamic acid) pure standards (≥ 99.5% purity) in powder form; HPLC-grade methanol, acetonitrile, acetone, diethyl ether, ethyl acetate and water; hydrochloric acid (37%, w/w), sodium hydroxide beads (≥ 98%), Folin-Ciocalteu's reagent, sodium carbonate (≥ 99%), ferric chloride hexahydrate (≥ 98%), ferrous chloride (≥ 98%), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4"-disulfonic acid sodium salt (FerroZine, ≥ 97%), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, ≥ 97%). Stock and standard solutions of phenolic acids were prepared in methanol. Standard solutions were obtained by diluting stock solutions with the mobile phase (initial composition). Certified stock solutions of carotenoids (neoxanthin, violaxanthin, antheraxanthin, zeaxanthin, lutein, β-cryptoxanthin, β-carotene) in

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acetonitrile were purchased from DHI (Hørsholm, Denmark) and diluted with methanol before injection.

An XS Instruments (Carpi, Italy) pH 50 pHmeter, a Thermo Scientific CL10 centrifuge, an IKA (Staufen, Germany) A11 Basic knife mill and a Christ Alpha 1-4 LD freeze dryer were used.

2.2. Microorganisms and dough preparation

Wheat flours (*Triticum aestivum* L.) was purchaised in a local market.

Lactic Acid Bacteria (LAB) strains, belonging to the Department of Agricultural and Food Science and Technology of the University of Bologna, were selected based on their ecological characteristics and their source of isolation (all of them were isolated from fermented products). The strains used were *Lactobacillus fermentum* (MR13), *L. rhamnosus* (C249, C1272), *L. plantarum* (LB102, LB124, LB126, LB245, 29DAN, 83DAN, 6BHI, 98A) and *L. brevis* (3BHI). LAB strains were grown separately in Man Rogosa Sharpe (MRS) broth (Oxoid, Milan, Italy) at 37°C for 24 h. Microbial cells and dough were prepared according to Taneyo Saa et al. (2018). The inoculated dough was incubated at 30°C for 24 h to obtain a mature sourdough starter which was used (30% of total dough) to ferment the final dough (approximately 10^7 CFU/g). The final fermentation of each specific LAB dough was performed at 30°C for 24 h.

Finally, all dough samples were freeze-dried. Immediately before analysis, the samples were finely ground in a knife mill for 4 × 30 s periods, with 20-s intervals.

2.3. HPLC Determination of Phenolic Acid Content

2.3.1. Extraction

Phenolic acids were extracted according to the protocol described by Moore et al. (2005) with modifications (Antognoni et al., 2017). A 2-g aliquot of powder was transferred into a knife mill with 20 mL of a methanol/acetone/water (7/7/6, v/v/v) mixture; after a 30-s mixing, the mixture was left to rest for 10 min, then centrifuged for 5 min at 1400×*g*. Three fractions (free: F, soluble-conjugated: SC, insoluble-bound: IB) were extracted from the sample supernatant (F, SC), and the pellet (IB). The F fraction was obtained by analysing the supernatant as such, while the SC fraction was obtained by difference after alkaline hydrolysis.

2.3.2. HPLC analysis

Aliquots of 20 µL of the extracts were injected into the HPLC system. This included a Jasco (Tokyo, Japan) PU-4180 quaternary chromatographic pump, MD-4015 photodiode array detector and AS-4050 autosampler. The stationary phase was an Agilent (Santa Clara, CA, USA) Zorbax Eclipse Plus C18 reversed-phase column (100 mm × 3 mm I.D., 3.5 µm).

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The chromatographic method for analysis of phenolic acids was adapted from Mattila, Pihlava, & Hellstrőm, (2005), with modifications (Antognoni et al., 2017). Full spectral data were recorded at all times; for quantitative purposes, the signals at 254 nm (for 4-hydroxybenzoic acid), 280 nm (for gallic acid, *p*-coumaric acid, syringic acid and *trans*-cinnamic acid) and 329 nm (for caffeic acid, chlorogenic acid, ferulic acid and synapic acid) were used. The recovery values of phenolic acids in spiked samples ranged from 78.8 to 92.2% (RSD < 9.81%, n = 6).

2.4. HPLC Determination of Carotenoid Content

2.4.1. Extraction

Carotenoids were extracted as follows. A 5-g aliquot of the sample powder was transferred in a knife mill with 20 mL of an ethanol/water (8/2, v/v) mixture; after 30 s of mixing, the mixture was left to rest for 20 min, then mixed again for 30 s, and centrifuged for 10 min at 1400×*g*. The supernatant was filtered through a syringe filter (nylon, pore size 0.2 µm) and 20 µL of this solution were injected into the HPLC system, which used the apparatus described above.

2.4.2. HPLC Analysis

The method for carotenoid analysis was adapted from Hidalgo et al. (2006), with modifications (Antognoni et al., 2017). Carotenoid concentrations of sample extracts were extrapolated from pure analyte standard curves. Full spectral data were recorded at all times; the signal at 450 nm was used for quantitative purposes. The recovery values of carotenoids in spiked samples ranged from 80.1 to 90.3% (RSD < 11.1%, n = 6).

2.5.1. Total phenolic content

Total phenolic content was assayed in F, SC, and IB fractions using Folin-Ciocalteu's reagent according to the procedures described by Zhou & Laux (2004) and by Yu et al. (2002), with modifications. A mixture containing 200 µL (F) or 50 µL (SC, IB) of dough extract and 250 µL of Folin-Ciocalteu's reagent (diluted 1:10 with water) was completed to 800 µL with water and left to react for 1 min at RT. Then, 800 µL of 20% sodium carbonate were added and the mixture was incubated for 30 min at 40°C. The absorbance at 765 nm was measured on a Jasco V-730 UV-Visible spectrophotometer, and used to calculate the total phenolic content, using ferulic acid and Trolox as standards.

2.5.2. Ferric reducing antioxidant power

This assay was carried out using the FRAP-Ferrozine method according to the procedure described by Berker et al. (2010) with modifications. 100 µL of dough extract (F, SC, and IB fractions) were mixed with 100 µL of 10 mM $FeCl_3$ aqueous solution and 100 µL of 2.5 mM Ferrozine aqueous solution. After 30 min at RT, the absorbance at 562 nm was measured in a Perkin Elmer (Turku,

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Finland) Viktor X3 multilabel plate reader, and used to calculate the FRAP, interpolating on a $FeCl_2$ calibration curve and using ferulic acid and Trolox as standards.

Results and discussion

Strains used belonged to different species: *L. fermentum*, *L. plantarum*, *L. rhamnosus*, *L. brevis* (see Materials and methods). To assess their capacity to ferment wheat flour, pH decrease and microbial cell increase were measured in the inoculated dough (Fig. 1). pH decreased by at least two units and microbial load increased by around two log cycles confirmed the quite good fermentative ability of all LAB strains.

Carotenoid content in fermented doughs is shown in Fig. 2. Lutein was the major carotenoid found in all samples, while zeaxanthin was present at much lower concentrations, the difference being about tenfold. These levels fall within the range reported by other authors in wheat flour (Leenhardt et al., 2006). Changes in lutein and zeaxanthin content in doughs fermented by the different LAB strains were variable: most of them show similar or lower levels of lutein compared to unfermented dough, while fermentation with two *L. plantarum* strains (29DAN and 83DAN) brought about a significant increase (60 % and 100%, respectively) in lutein and zeaxanthin levels in the dough (Fig. 1). During sourdough fermentation, variations in flour components are mediated by both endogenous enzymatic activities and microbial conversion. Thus, the decrease in lutein content in some doughs fermented by LAB belonging to different species and strains (LB124, LB126, LB245, 6BHI, 98A, C1272, 3BHI) could be due to a higher degradation of this compound during fermentation, probably because of a higher lipid oxidation involving the endogenous lipoxygenase (LOX)/linoleate system (Mc Donald, 1979). Indeed, the kneading process results in a huge incorporation of oxygen in the dough, which facilitates the LOX-mediated oxidation of polyunsaturated fatty acids, with co-oxidation of xantophylls (Drapon et al., 1974). Moreover, the metabolism of LAB in sourdough can also favour lipid oxidation during fermentation, through the production of hydrogen peroxide (Vermuelen et al., 2007), bringing to a further reduction in lutein levels. The significant increase of this carotenoid caused by 29DAN and 83DAN, compared to control, is more difficult to explain. Several *L. plantarum* strains can produce the C30 carotenoid 4,4-diaponeurosporene (Garrido-Fernandez et al., 2010), but production of xantophylls has never been reported. On the other hand, the metabolism of some LAB can exert strong antioxidative effects, through a decrease in the oxidation-reduction potential of sourdough, as well as a specific accumulation of glutathione or related thiol compounds (Capuani, Behr, & Vogel, 2012). This provides enough reducing power to convert lipid peroxides to hydroxides (Jänsch et al., 2007), and

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could cause a lower lipid oxidation compared to other strains. Furthermore, increased bioaccessibility of these carotenoids from their localization in the aleurone layer in the grain could also raise their free concentration.

Total polyphenol content (TPC) in the doughs was evaluated in the free (F), soluble-conjugated (SC) and insoluble-bound (IB) forms. The only significant increase in TPC compared to control was found in the SC fraction of doughs fermented with *L. rhamnosus* C1272 strain (data not shown). In a previous work Ferri et al. (2016) analysed TPC in doughs prepared from both durum wheat and KAMUT® Khorasan wheat using different *L. plantarum* strains, and a 50% increase was found in the dough fermented with the 98A strain.

The three fractions were also analysed by HPLC-DAD for their phenolic acid content. *p*-coumaric and cinnamic acids were detected in the doughs fermented with all LAB strains, mainly as insoluble-conjugates, with levels ranging from 2.0 to 60.0 µg/g DW. Caffeic, sinapic, *p*-hydroxybenzoic, gallic acids were detected at much lower levels, in the range 0.4 to 23.0 µg/g DW, and only in some dough samples (data not shown). Ferulic acid was the most abundant compound, reaching total levels in the 110-400 µg/g DW range, and its relative distribution within the three fractions is reported in Fig. 3. In all doughs, the IB ferulic acid was by far the largest contributor to total content, amounting to nearly 80%. Only in a few cases (LB124, LB126, LB245, 29DAN), LAB fermentation led to a significant decrease in total ferulic acid content to levels lower than 200 µg/g, which was basically due to a decrease in the IB form. In doughs fermented with the other LAB strains, IB ferulic acid is either not statistically different (MR13, LB102, 98A, C1272, 3BHI) or significantly higher (83DAN, 6BHI, C249, 3BHI) compared to unfermented dough; in the last three strains, total ferulic acid content was higher than 300 µg/g. This increased detection of bound phenolic acids agrees with previous studies on fermented wheat (Anson et al., 2009) and other cereals (Liukkonen et al., 2003; Hole et al., 2012), and can be explained by an increased content of ferulic acid dimers, as well as by an improved accessibility of bound phenolic acids to hydrolysis, possibly due to an increased ratio of soluble to insoluble dietary fibers in fermented cereal products (Arora, Jood, & Khetarpaul, 2010). Three out of eight *L. plantarum* strains (LB126, 29DAN, 98A) were able to enrich the doughs in F ferulic acid with a higher human bioaccessibility, even though it still remained at levels much lower than in IB and SC fractions (Fig. 3, insert). This different behaviour can be due to a different capacity of bacterial strains to degrade phenolic acid esters or tannins by esterase activity, as well as to a different inducible phenolic acid decarboxylase activity, which has been demonstrated in *L. plantarum* species (Gury et al., 2004; Topakas, Vafiadi, & Christakopoulos, 2007). Overall, this indicates a strict strain-specificity in the ability to

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metabolize/degrade/hydrolyze phenolic acids, as already reported in literature (Hole et al., 2012; Ferri et al., 2016).

The antioxidant activity of the three fractions of dough samples was tested by using the FRAP-Ferrozine assay, which is the only assay that allows a direct measure of total electron-donating antioxidants in a biological sample (Halvorsen et al., 2002). Fermentation with most LAB strains did not have a significant effect on total FRAP (Fig. 4). Only two *L. plantarum* strains (29DAN, 98A) showed a significantly higher FRAP value compared to control, which is specifically attributable to the SC fraction, while LB 245 and both *L. rhamnosus* strains C249 and C1272 had significantly lower FRAP values, in the latter case mostly due to a drastic decrease in F antioxidant levels (Fig. 4). The positive influence of 29DAN and 98A on antioxidant activity can be due either to its capacity to liberate active compounds from the cereal matrix during fermentation, or to its own intrinsic antioxidant capacity (Hur et al., 2014).

The antioxidant activity turned positively correlated with total ferulic acid levels (*P* value 0.0085, Pearson r 0.70), but not correlated with lutein levels (*P* value 0.189, Pearson r 0.389). A negligible contribution of carotenoids to the antioxidant capacity measured by the most common used *in vitro* assays has often been reported (de Ancos et al., 2002; Choi et al., 2007), and this might be related to the lipophilic nature of these compounds and to their different mode of action compared to phenolic compounds (Rodriguez-Amaya, 2010). However, it has to be taken into consideration that other compounds, different from those identified and quantified in this study, can contribute to the total antioxidant activity. For example, tannic acid, which has been reported to possess a strong antioxidant capacity (Zhang et al., 2015), can be a good candidate, since an intense tannin metabolism is known to occur during sourdough fermentation (Gänzle, 2014).

In conclusion, our study shows that fermentation of bread wheat with some LAB strains can enhance the levels of bioactive compounds, thus potentially improving its beneficial health effects. A bioconversion of the non-free forms of phenolic compounds can occur during fermentation, leading to changes in the potential bioaccessibility/bioavailability of functional compounds. Moreover, an increase in carotenoid levels was also observed by some LAB strains, probably due to an increased mobilization of these membrane-associated lipophilic compounds from the cereal matrix. This process is strictly strain-specific, probably linked to the intrinsic metabolic machinery of lactobacilli.

In LAB, the factors responsible for regulating carotenoid production and other damage repair systems remain unknown. However, a first example of transcriptome analysis and sequencing of oxidative stress response genes in *Enterococcus gilvus* described the upregulation of isoprenoid

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biosynthesis genes as a potential explanation of carotenoid-based stress response mechanism in LAB (Ohki et al., 2018). Indeed, the expression of genes responsible for pyruvate dehydrogenase complex (which converts pyruvate into acetyl-coA, the first compound in isoprenoid biosynthesis) was reported to show the same pattern of upregulation than carotenoid biosynthesis genes after aerobic treatment. In addition, the expression of transcriptional regulator *spx* and genes encoding UvrABC system protein was also upregulated. These findings support the present work and suggest further investigation on LAB strain performances in order to set up bioprocesses aiming to produce cereal food or ingredients with an increased concentration of carotenoids and other bioactive compounds.

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Authors' contributions
G.P., F.A., R.M. carried out analysis of carotenoids and phenolic acids, and antioxidant assay; D.L.T.S. and A.G. grew L.A.B. strains and prepared wheat doughs; A.G., F.A., R.M. designed the study and wrote the paper. All authors critically contributed to the writing of manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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Figure legends

Fig. 1. pH decrease and microbial cell increase in wheat dough fermented by LAB strains

Fig. 2. Zeaxanthin and lutein content in bread wheat doughs fermented with the different LAB strains. Data represent the mean ± S.E. of two extractions with triplicate samples. Different letters indicate significant differences at P<0.05.

Fig. 3. Free (insert), SC, IB, and total ferulic acid content in the doughs fermented by the different LAB strains. Data represent the mean ± S.E. of two extractions with triplicate samples. Different letters on the black bar (total ferulic acid) indicate significant differences at $P<0.05$.

Fig. 4 Ferric-reducing antioxidant activity (expressed as µM FRAP values) in doughs fermented with the different LAB strains. Data represent the mean ± S.E. of two extractions with triplicate samples. Different letters indicate significant differences at $P<0.05$.

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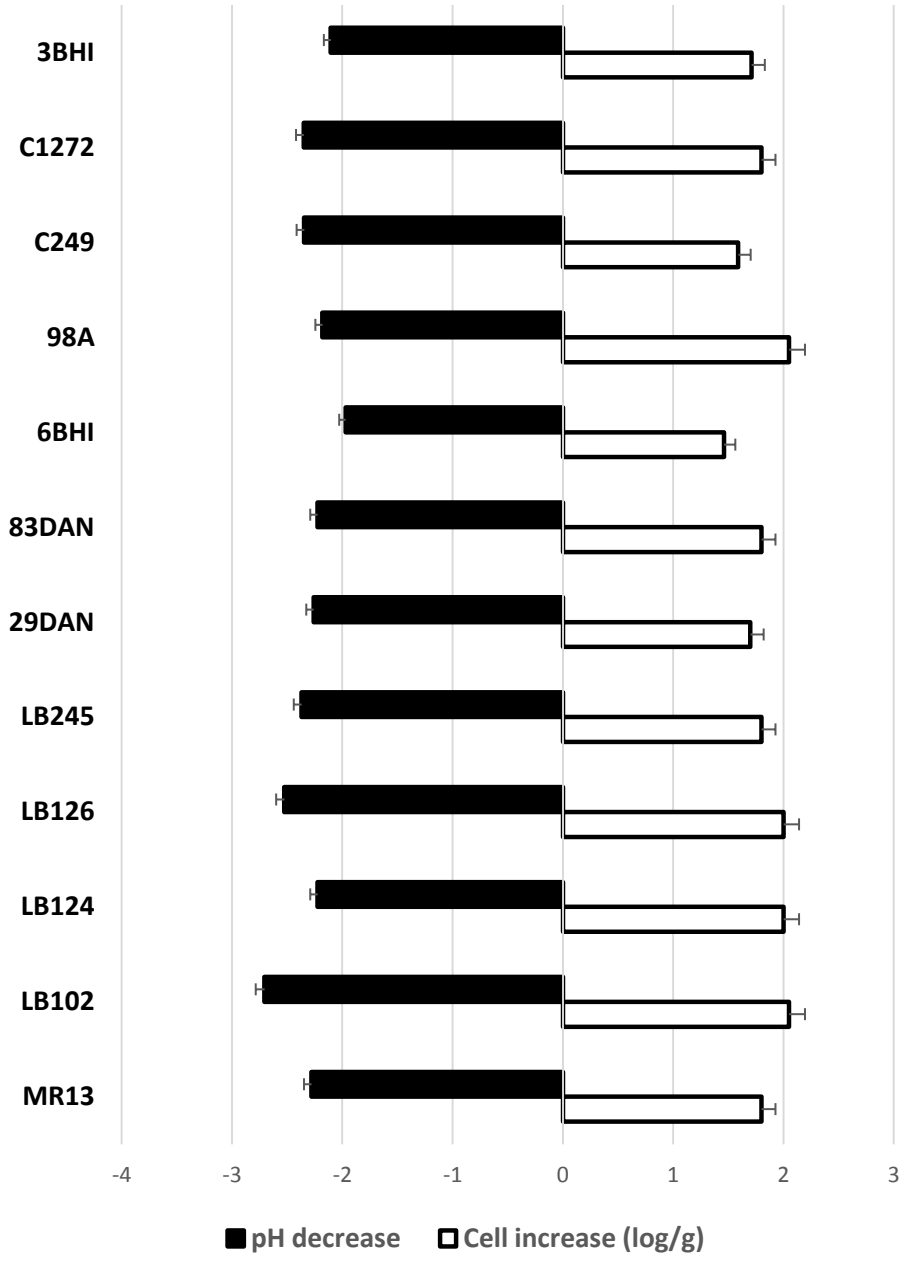

Fig. 1

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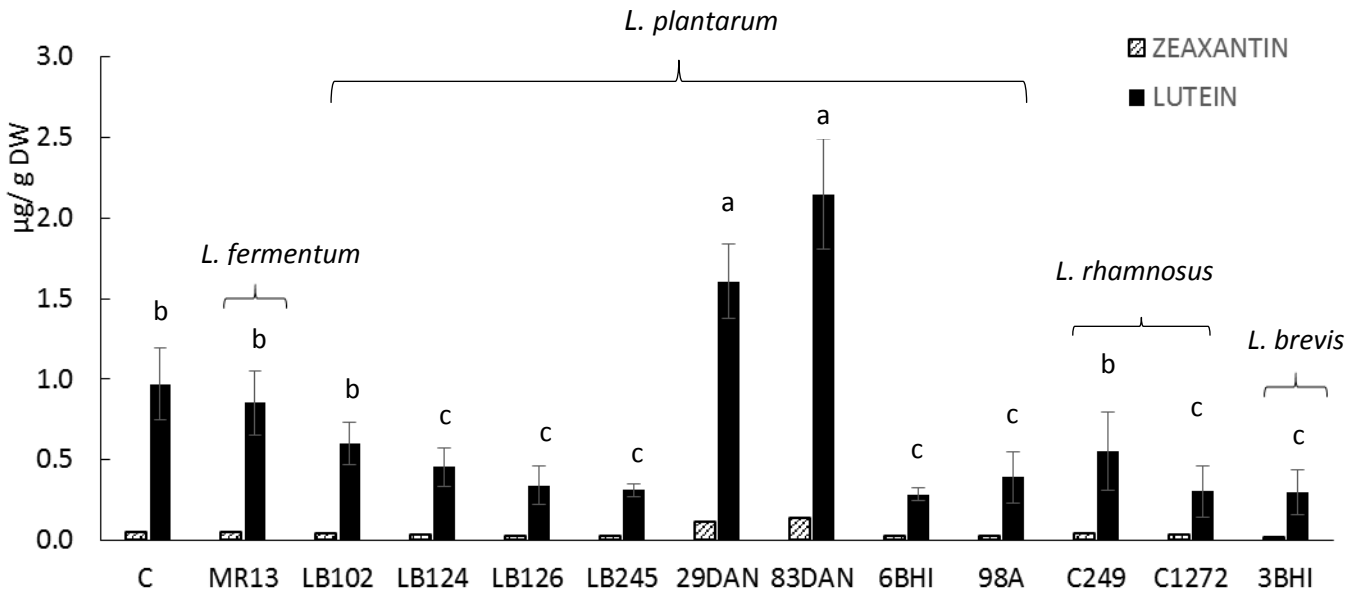

Fig. 2

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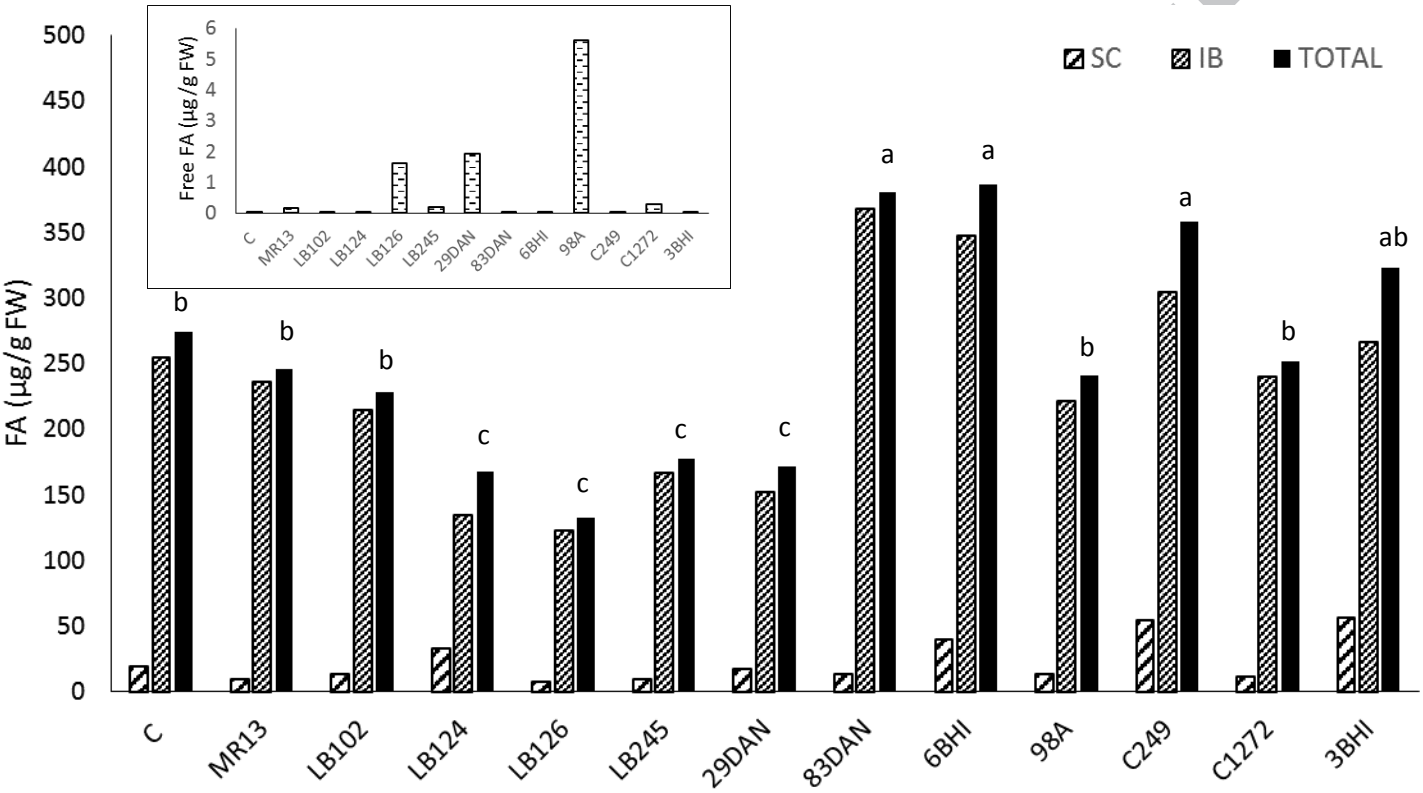

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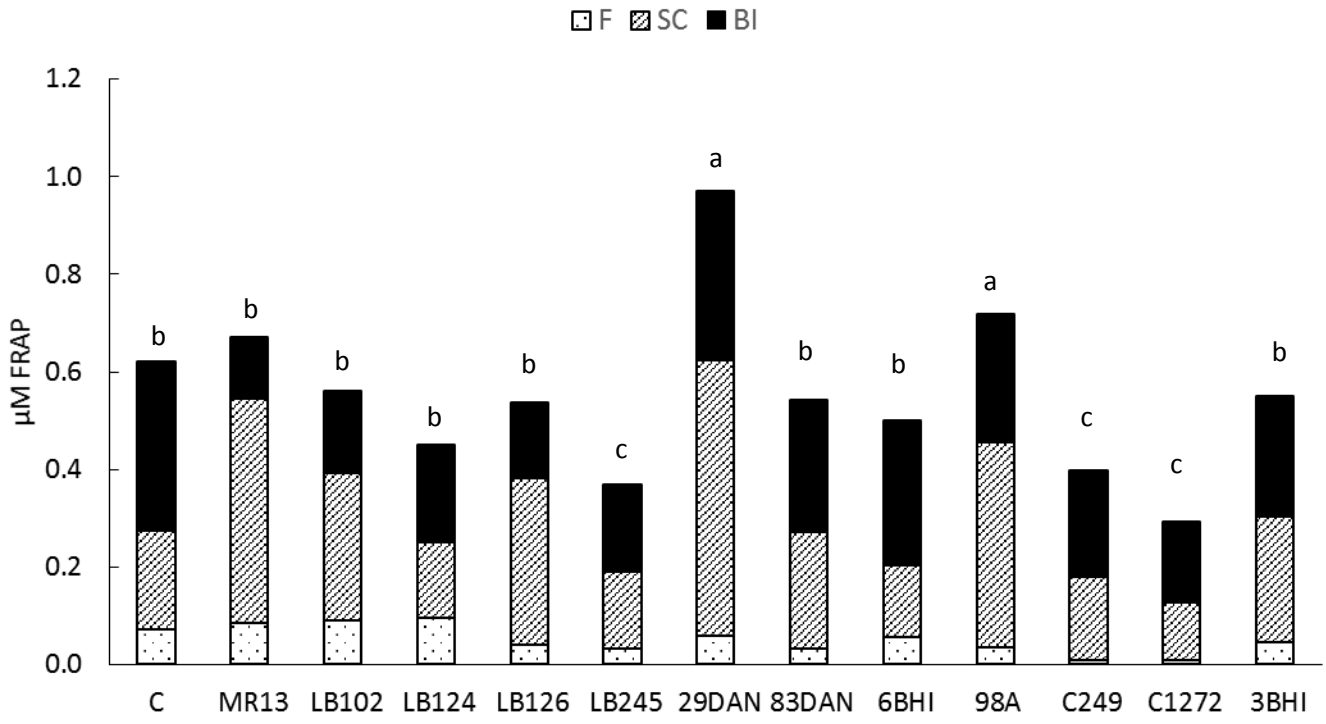

Fig. 4

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Highlights

Carotenoid levels change in wheat dough fermented with some Lactic Acid Bacteria

Two out of twelve Lactobacillus strains increased lutein content in fermented dough

Changes in bioavailable ferulic acid during LAB fermentation are strain-specific

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