Improving the functional and sensorial profile of cereal-based fermented foods by selecting Lactobacillus plantarum strains via a metabolomics approach

Improving the functional and sensorial profile of cereal-based fermented foods by selecting *Lactobacillus plantarum* strains via a metabolomics approach

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A B S T R A C T

Metabolomics was recently applied in food science for monitoring the quality, processing, safety, and microbiol-ogy of both raw materials and final products to improve the consumer's health and confidence.
The present work aimed at using a metabolomics approach to detect the flavour and antioxidant profiles charac-teristic of different *Lactobacillus plantarum* strains in sourdoughs of durum wheat and KAMUT® khorasan wheat. The study was developed to improve sensorial and functional properties of cereal-fermented foods through the selection of optimal flour-microbial strain combinations. A large set of metabolites, including volatile compounds, polyphenols and flavonoids, was taken into consideration and the antioxidant activity was determined. The met-abolic profiles were combined by using heat maps to visualise the result of a hierarchical clustering of profile data. In KAMUT® khorasan wheat-fermented dough, a high correlation was found between a group of volatiles (5 alcohols, 6 carbonils, dodecanoic acid and 1,3-hexadiene) and the polyphenolic compounds gallic acid (GA), epi-gallocatechin-gallate (EGCG), epigallocatechin (EGC), flavonoids (Flav), protocathecuic acid (Prot ac) and total polyphenols (Polyp). In durum wheat dough a different pattern of volatile molecules (10 alcohols, 5 carbonils, 3 acids and 3 hydrocarbons) resulted highly correlated with EGC, EGCG, Flav and Polyp. The results also evi-denced a simultaneous increase of sensorial and health promoting compounds when using *L. plantarum* strains 98A and 6BHI in fermented durum wheat dough, while 94A and 206 strains had their highest performance with KAMUT® khorasan wheat dough. Our data show that metabolomics may represent an important tool for the rapid selection of strain/substrate combinations to simultaneously increase sensorial and health beneficial characteristics.

Keywords:
Cereals
Functional foods *Lactobacillus plantarum* Sourdough
Metabolomics
Strain selection
KAMUT® khorasan wheat

1. Introduction

Cereal-based foods are an essential component of the daily human diet, and in particular wheat, together with rice and maize, is one of the most used grains. Nutritionally, they are an important source of carbohydrates, proteins, dietary fibres, vitamins and many non-nutrients (such as, phenolics, flavonoids, carotenoids and fructans). Although the increased consumption of whole grains and their derived food products has been associated with protection against several Western diseases, such as cardiovascular disorders, type 2 diabetes and some cancers, in vivo studies on the antioxidant potential of fermented versus unfermented cereals are still inadequate (Masisi, Beta, & Moghadasian, 2016).

Fermentative processes play a key role in the production of cereal-derived products due to their influence on the organoleptic characteristics (such as, taste, aroma and texture) and on the improvement of nutritional properties with a final positive effect on human health. In fact, depending on the process type and microorganism species, microbial fermentative activity in contact with dough components can increase the level of several bioactive molecules with nutraceutical functions (Dey, Chakraborty, Jain, Sharma, & Kuhad, 2016; Gianotti et al., 2011; Heiniö et al., 2016). Overall, lactic acid bacteria (LAB) are a small part of the autochthonous microbiota of raw vegetables and they have to be adapted to the intrinsic characteristics of the raw materials to obtain desirable properties of fermented vegetable food products.

Abbreviations: AA, ascorbic acid; ABTS, 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; CAT, catechin; EC, epicatechin; ECG, epicatechin-3-gallate; EGC, epigallocatechin; EGCG, epigallocatechin-gallate; FW, fresh weight; GA, gallic acid; Polyp, polyphenols; Flav, flavonoids; Fer ac, ferulic acid; Van ac, vanillic acid; Prot ac, protocathecuic acid; LAB, lactic acid bacteria.

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The crucial point is that the health promoting effects of phenolic compounds depend on the consumed amount and on their bioavailability. On the other hand, while consumers are increasingly demanding products with functional properties, the consumption patterns of new products are strongly determined by their sensorial characteristics (Breslin, 2001). Therefore, bacterial strain selection for vegetable food fermentations faces both health promoting and sensorial requirements.

Metabolomics, i.e. the systematic determination of low-molecular weight metabolites within a biological sample, has been applied in food science for monitoring quality, processing, safety, and microbiology of both raw materials and final products to improve the consumer's health and confidence (Cevallos-Cevallos, Reyes-De-Corcuera, Etxeberria, Danyluk, & Rodrick, 2009). Metabolic profiling of fermented foods has also been used to record metabolite modifications during fermentation and the possibility to predict, among others, the sensory and nutritional quality of the final product (Mozzi, Ortiz, Bleckwedel, De Vuyst, & Pescuma, 2013). Specifically, the kinetics of physicochemical parameters, microbiota population and their metabolites were investigated in cheese and yoghurt using NMR (Piras et al., 2013; Settachaimongkon et al., 2015). More recently, the metabolomics approach has permitted to study volatile metabolites in fermented bakery products in order to understand the role of flour and the fermentation process in increasing rheological and volatile metabolites (Balestra et al., 2015). Furthermore, Chen, Ye, Chen, and Yan (2016) applied metabolic profiling to investigate the quality shift in crab paste fermentation, and Pogačić et al. (2015) adopted this approach to screen various cheese-related LAB strains for their ability to produce aroma compounds.

Fruits, vegetables and beverages are the main sources of phenolic compounds in the human diet. Among these, bread and wine represent the main fermented foods of the Mediterranean diet with phenolic compounds being responsible for their sensorial and nutritional characteristics. In a previous study, Kamut® khorasan bread-fed rats showed a better response to oxidative stress than those fed with durum wheat bread, especially when sourdough bread was supplied (Gianotti et al., 2011; Carnevali et al., 2014). The present work aimed at using a metabolomics approach to detect the effect of these two wheat flours and different *L. plantarum* strains, isolated from bread and wine, on the flavouring and antioxidant characteristics of fermented sourdough. Volatile, polyphenols, flavonoids, several individual phenolic compounds and antioxidant activity were taken into consideration as parameters to improve sensorial and functional properties of cereal-fermented foods.

2. Material and methods

Durum wheat and KAMUT® khorasan wheat (a registered trademark of Kamut International, Ltd. and Kamut Enterprises of Europe, bvba) are closely related. The main differences are represented by their protein, polyphenol and selenium content (Supplementary Table S1) which are more concentrated in Kamut® khorasan wheat, bread and pasta products (Carnevali et al., 2014; Gianotti et al., 2011).

All the *Lactobacillus plantarum* strains used in this study belong to the collection of the Department of Agri-Food Science and Technologies (University of Bologna, Bologna, Italy). Ten strains of *L. plantarum* were selected among 44 different strains isolated from sourdough and wine based on the high content of naturally occurring polyphenols in the original food source. These strains were previously characterised by RADP-PCR on the basis of their molecular profiles obtained by amplification of DNA with M13 primer according to Serrazanetti, Ndagijimana, Miserocchi, Perillo, and Guerzoni (2013). Another selection parameter consisted of both high and low similarity values between the strains in order to test target strains representing the different clusters obtained (Supplementary Fig. S1). Strains 94A, 133, 124, 189A, 98A, 98B, 206 and 801 were isolated from sourdough, while strains 5BHI and 6BHI were isolated from red wine.

2.1. Inoculum and dough preparation

Strains were cultivated at 37 °C for 24 h in De Man-Rogosa-Sharp (MRS) agar (Oxoid, Basingstoke, UK) and then centrifuged at 4000 × *g* for 10 min. The pellet was washed and resuspended in 10 mL of physiological solution (0,9% v/v NaCl) for the subsequent dough preparation.

Doughs were prepared in a sterile environment. Twelve grams of each flour (durum wheat or KAMUT® khorasan wheat) were mixed with 6.5 mL of saline solution and inoculated with bacterial cells (10^7 CFU/mL initial dough cell load) (Vernocchi et al., 2008). The doughs were incubated for 16 h at 35 °C. The controls were represented by uninoculated dough before (Control 0 h) and after (Control 16 h) incubation. At the end of incubation, inoculated samples accounted for a cell load of around 10^9 CFU/mL. All the doughs were placed in sterile vials (3 g/vial) and stored at −80 °C until analysis. At least two biological replicates were prepared for each sample.

Samples were subsequently analysed for polyphenolic compounds with potential health beneficial effects (total polyphenols, flavonoids, several individual phenolic compounds), specific molecules having sensorial effects (flavouring compounds), and antioxidant activity.

2.2. Extraction of free phenolics

Dough samples were powdered in liquid nitrogen and stored at −20 °C. Aliquots of 0.5 g of powder were incubated overnight in the dark with shaking at 4 °C with 5 mL of 98% methanol +2% (v/v) 12 N HCl (Ferri, Gianotti, & Tassoni, 2013). Samples were then centrifuged to separate the solid material from the supernatants which were successively used for polyphenol and flavonoid spectrophotometric quantifications.

Other aliquots of 0.5 g of powder of each sample were incubated overnight in the dark with shaking at room temperature with 5 mL of 95% (v/v) methanol (Ferri et al., 2013). Samples were centrifuged to remove the solid material and the obtained supernatants were used for antioxidant activity analyses and HPLC separation.

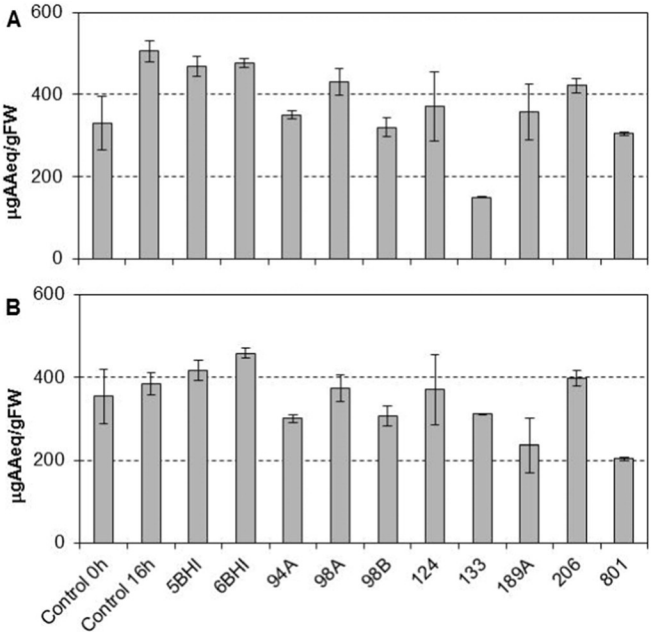

Fig. 1. Total antioxidant activity in sourdoughs of (a) durum wheat and (b) KAMUT® khorasan wheat flours fermented with ten different strains of *L. plantarum*. Data are expressed as µg of ascorbic acid equivalent per gram of fresh weight (µgAAeq/gFW) ± SD (n = 4).

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2.3. Quantification of total polyphenols and total flavonoids and determination of antioxidant activity

The spectrophotometric methods for total phenolic (by using Folin–Ciocalteu method; Singleton, Orthofer, & Lamuela-Raventos, 1999) and total flavonoid (Zhishen, Mengcheng, & Janming, 1999) quantifications were previously optimised for cereal food components (Ferri et al., 2013). The results were expressed as gallic acid (GA) or catechin (CAT) equivalents, respectively, per gram of dough fresh weight (μgGAeq/gFW and mgCATeq/gFW).

Antioxidant activity was measured using the 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) method (Re et al., 1999) with minor modifications, following the protocol previously optimised for cereal samples (Ferri et al., 2013). The results were expressed as ascorbic acid (AA) equivalents per gram of dough fresh weight (μgAAeq/gFW).

2.4. Quantification of specific polyphenols by HPLC-DAD

Polyphenols were extracted from 0.5 g of dough powder with the neutral extraction described above and analysed by reverse-phase HPLC-DAD separation (column Luna C18(2), 5 μm particles 250 mm × 4.6 mm, pre-column Security-Guard Ea, Phenomenex, Torrence, CA, USA) equipped with an online diode array detector (DAD) (MD-2010 Plus, Jasco Instruments, Großumstad, Germany). The adopted HPLC-DAD separation procedure (Ferri et al., 2009) allowed the simultaneous analysis of the following compounds: (+)-catechin (CAT), (−)-epicatechin (EC), (−)-epigallocatechin-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG), quercetin, rutin, (±)-naringenin, and gallic, *trans*-cinnamic, *p*-coumaric, caffeic, ferulic, sinapic, chlorogenic, vanillic, protocatechuic, syringic acids. HPLC standards were purchased from Sigma–Aldrich (Milano, Italy).

2.5. SPME–GC analysis and mass spectrometry

Solid phase micro extraction-gas chromatography–mass spectrometry (SPME-GS–MS) analysis was performed by a method previously developed (Serrazanetti et al., 2011). All the GC–MS raw files were converted to netCDF format via Chemstation (Agilent Technologies) and subsequently processed with the XCMS toolbox (http://metlin.scripps.edu/download/). XCMS software allows automatic and simultaneous retention time alignment, matched filtration, peak detection and peak matching. The resulting table containing information such as peak index (retention time-*m*/*z* pair) and normalised peak area was exported

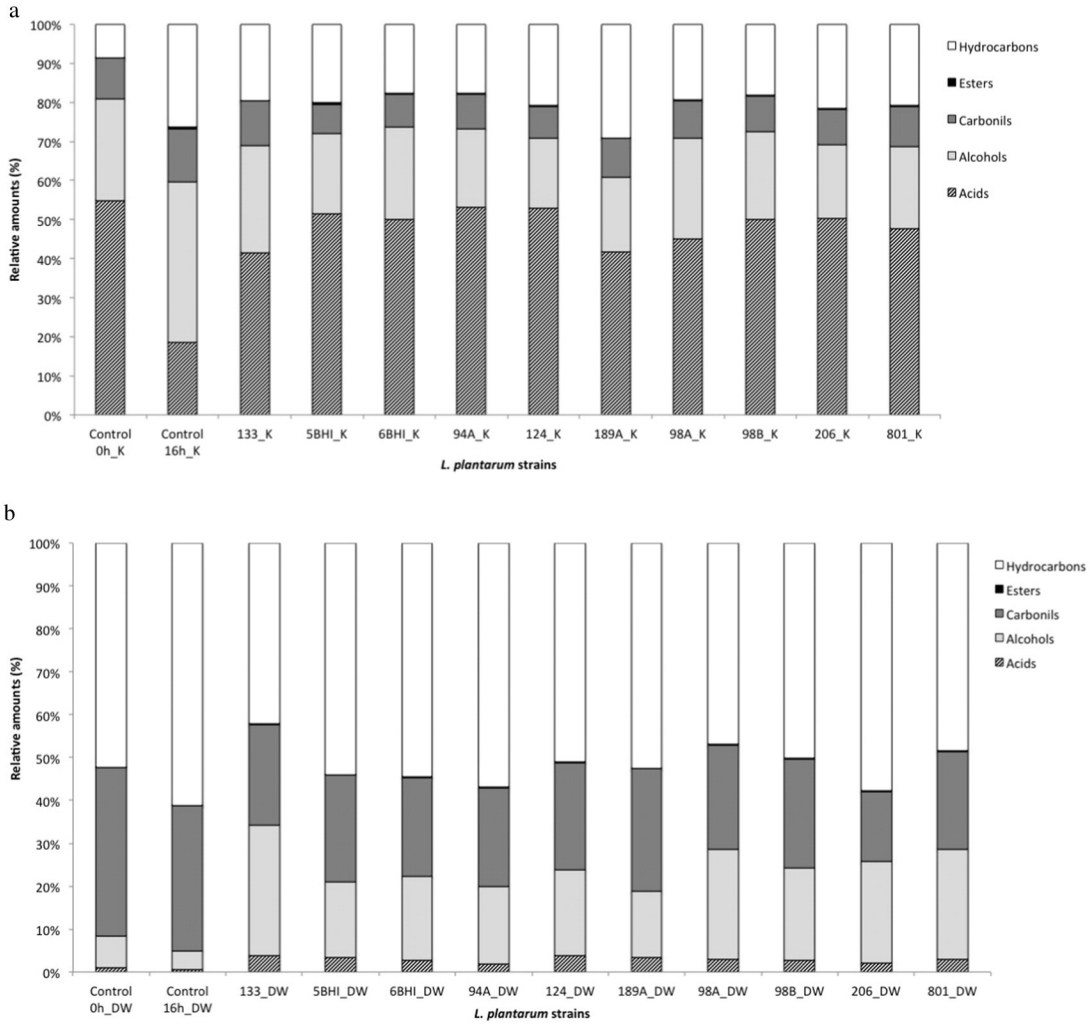

Fig. 2. a) Relative amounts of volatile compounds grouped for molecular class (hydrocarbons, esters, carbonils, alcohols and acids) in dough obtained with durum wheat flour, uninoculated (Control 0 h and Control 16 h) and inoculated with different strains of *L. plantarum*; b) relative amounts of volatile compounds grouped for molecular class (hydrocarbons, esters, carbonils, alcohols and acids) in dough obtained with KAMUT® khorasan wheat flour, uninoculated (Control 0 h and Control 16 h) and inoculated with different strains of *L. plantarum*.

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into R (www.r-project.org) for subsequent statistical or multivariate analyses (Serrazanetti et al., 2011). Volatile compounds were expressed as % peak area.

2.6. Statistical analysis

Each dough was prepared twice at the same conditions and following the same procedure. Each sample was analysed in at least two biological replicates. Experiments were repeated twice with similar results. The data reported are the means of all the replicates ± SD.

To elaborate the heat map, the quantitative data obtained from metabolite determinations were previously used to build a single matrix, which was submitted to a two-way hierarchical clustering analysis (Serrazanetti et al., 2011). A heat map visualization of the data set was also performed after a logarithmic function transformation of each GC peak area (Alves et al., 2015) using R (www.r-project.org). The heat maps, visualizing metabolite concentration or antioxidant data which significantly characterised each strain, were then obtained. The values are represented by cells coloured according to the *Z*-scores, where Z = (observed value - mean)/standard deviation. Moreover, the relationship between volatile and antioxidant metabolites was also visualised as correlation heat maps using R (www.r-project.org).

3. Results

3.1. Effect of LAB fermentation on phenolic compound content

The total polyphenol (Polyp) content was similar in the two controls (0 h and 16 h incubation of dough without LAB), while all LAB-treated samples (both durum and KAMUT® khorasan wheat) displayed significantly increased levels (Supplementary Tables S2 and S3). The best result was obtained with *L. plantarum* 98A strain, which, in both types of dough, led to a 50% higher Polyp content with respect to the controls (Supplementary Tables S2 and S3).

The total flavonoid (Flav) content in durum wheat doughs was not significantly different from the two controls (Supplementary Table S2), except for those inoculated with *L. plantarum* 98B and 124 strains which showed a 30% increase in Flav content (Supplementary Table

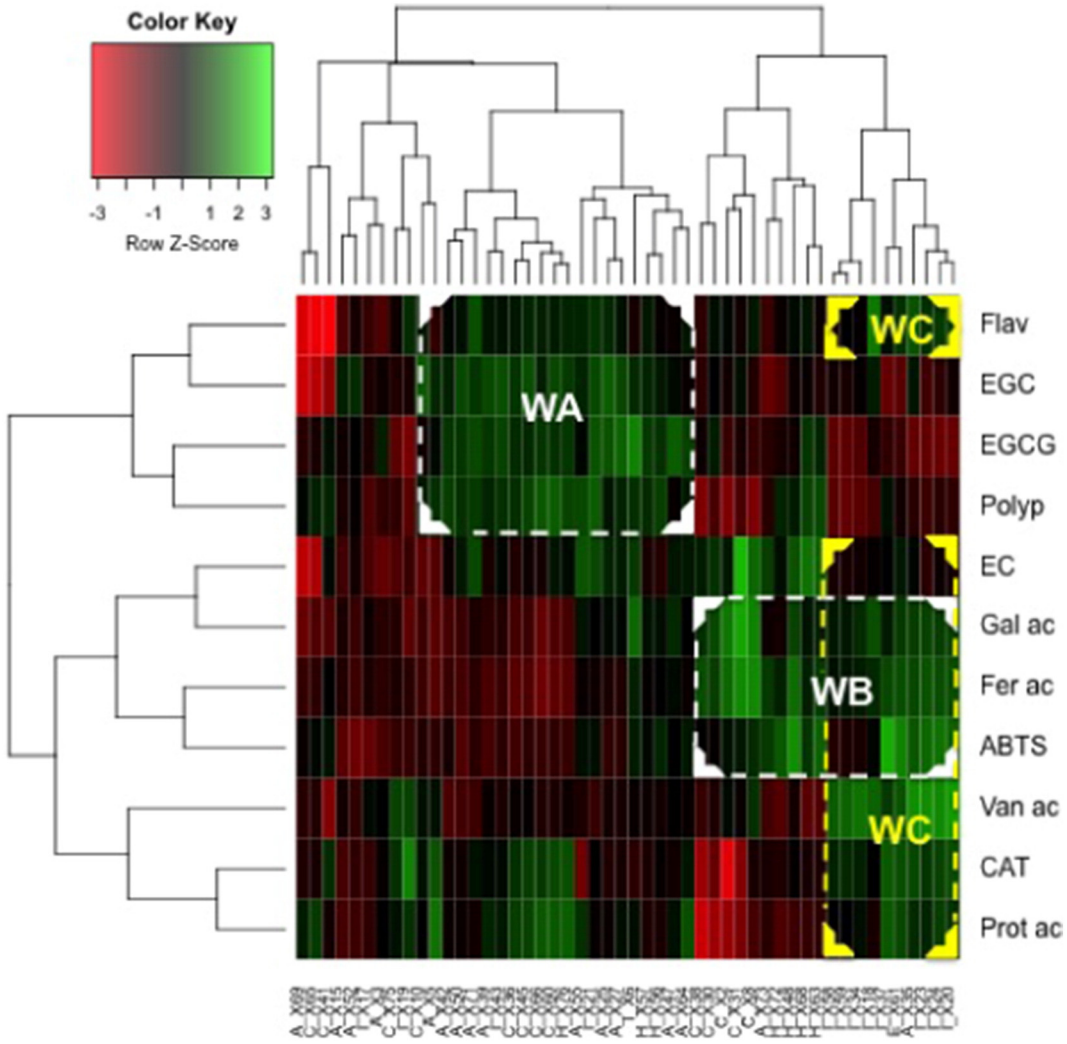

Fig. 3. Heat map representation of the correlation obtained between volatile molecules and antioxidant compounds released by *L. plantarum* strain-inoculated durum wheat doughs. Areas were normalised by applying a logarithm function. *A = Alcohols:* A_X15 4-methyl-3-heptanol; A_X25 isoamyl alcohol; A_X27 1-pentanol; A_X3 X3 alcohol; A_X35 2-methyl-2-buten-1-ol; A_X39 1-hexanol; A_X42 3,5-octadien-2-ol; A_X47 1-octen-3-ol; A_X5 ethyl alcohol; A_X50 1-octanol; A_X52 1,2-dodecanediol; A_X55 3,7-dimethyl-1-octanol; A_X64 1-decanol; A_X69 7-methyl-4-octanol; A_X71 phenethyl alcohol; A_X73 1-dodecanol; *C = Carbonils:* C_X10 hexanal; C_X2 2-methyl-pentanal; C_X30 2-octanone; C_X31 3-hydroxy-2-butanone (acetoin); C_X36 2-heptenal; C_X38 6-methyl-5-hepten-2-one; C_X41 2-nonanone; C_X45 2-octenal; C_X60 2,4-nonadienal; C_X65 2-dodecenal; C_X66 2,4,decadienal; C_X75 1,1,3-trimethyl-3-cyclohexene-5-one; C_X8 pentanal; *E = Ester:* E_X61: unidentified ester; *H = Acids:* H_X48 acetic acid; H_X56 3-methylbutanoic acid; H_X57 2-methylbutanoic acid; H_X63 pentanoic acid; H_X68 hexanoic acid; H_X72 heptanoic acid; H_X76 4 penten-1-yl-ester, dodecanoic acid; *I = Hydrocarbons:* I_X17 1,2 dimethyl-benzene; I_X18 2-methyldecane; I_X19 10-methylnonadecane; I_X20 5-methyl dodecane; I_X21 2-penthyl-furan; I_X23 4-methyl-dodecane; I_X24 3-methyl-dodecane; I_X34 2(5H)-furanone; I_X37 10-methyl-eicosane; I_X43 1,3 hexadiene, 3-ethyl-2-methyl; I_X58 2(3H) furanone (A); I_X59 2(3H) furanone (B); I_X6 2-ethyl-furan.

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S2). The KAMUT® khorasan wheat control displayed a 20% increase in Flav content after 16 h of incubation with respect to the Control 0 h (Supplementary Table S3). The effect of specific LAB strains was similar in KAMUT® khorasan and durum wheat doughs, with strains 98B and 124 leading to an increase of 30% in Flav content compared to Control 0 h (Supplementary Table S3).

The free-fraction of some polyphenols was also analysed by HPLC-DAD (Supplementary Tables S2 and S3). Ferulic acid (Fer ac) content in uninoculated durum wheat samples showed a 14% increase after 16 h of incubation (final concentration of 88.6 nmol/gFW), while in LAB inoculated samples it was generally lower than in the Control 0 h (Supplementary Table S2), probably due to metabolic transformation. Vanillic acid (Van ac) was detected in all fermented samples and in the Control 16 h (Supplementary Table S2). Protocatechuic (Prot ac) and gallic acid (GA) levels were differently affected by each *L. plantarum* strain. In particular, a large amount of Prot ac was quantified in 98A-inoculated samples, i.e. a 25-fold increase compared to Control 16 h (Supplementary Table S2). In uninoculated KAMUT® khorasan wheat, the increase in Fer ac content after 16 h (380%) was larger than in durum wheat, but the relative amount was 10-times lower (final concentration of 8.9 nmol/gFW) (Supplementary Table S3). Similarly to durum wheat results, Van ac was detected in all fermented KAMUT® khorasan wheat samples, while Prot ac and GA and catechins were differently affected by specific bacterial strains (Supplementary Table S3).

The final amount and composition of different types of catechins (CAT) were also analysed (Supplementary Tables S2 and S3). In the durum wheat Control 0 h, only epigallocatechin (EGC) was present although it decreased 2.2-fold in Control 16 h in which CAT was also detected. After 16 h of fermentation with the different *L. plantarum* strains, CAT, epicatechin (EC), EGC and, in lower amount, epigallocatechin-gallate (EGCG) were detected. CAT was not found in Control 0 h, while it represented the most abundant compound in almost all the treated samples (except 133) reaching a maximum amount of 41.3 nmol/gFW in 98A-treated sample (Supplementary Table S2). In

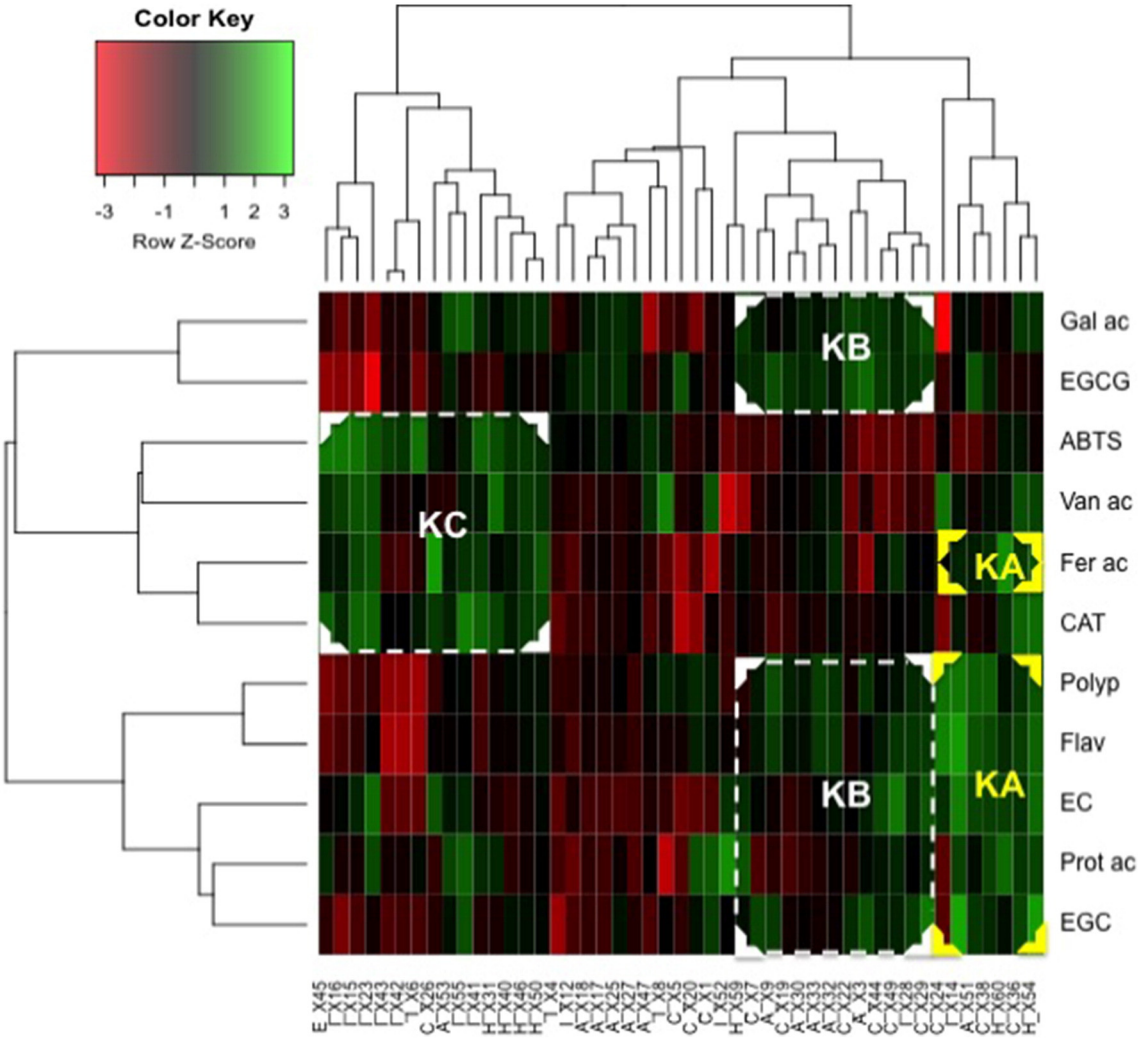

Fig. 4. Heat map representation of the correlation obtained between volatile molecules and antioxidant compounds released by *L. plantarum* strain-inoculated KAMUT® khorasan wheat doughs. Areas were normalised by applying a logarithm function. *A = Alcohols:* A_X17 isoamyl alcohol; A_X18 1-pentanol; A_X25 1-hexanol; A_X27 3,5-octadien-2-ol; A_X3 ethyl alcohol; A_X30 1-octen-3-ol; A_X32 1-octanol; A_X33 1,2-dodecanediol; A_X47 1-decanol; A_X51 7-methyl-4-octanol; A_X53 phenethyl alcohol; A_X9 4-methyl-3-heptanol; *C = Carbonils:* C_X1 2-methyl-pentanal; C_X19 2-octanone; C_X20 3 hydroxy-2-butanone (acetoin); C_X22 2-heptenal; C_X24 6-methyl-5-hepten-2-one; C_X26 2 nonanone; C_X29 2-octenal; C_X36 2-decenal; C_X38 benzeneacetaldehyde; C_X44 2,4-nonadienal; C_X49 2,4-decadienal; C_X5 pentanal; C_X7 hexanal; *E = Ester:* E_X45 unidentified ester; *H = Acids:* H_X40 2 methylbutanoic acid; H_X46 pentanoic acid; H_X50 hexanoic acid; H_X54 heptanoic acid; H_X59 dodecanoic acid; H_X60 octanoic acid; H_X31 acetic acid; *I = Hydrocarbons:* I_X12 10-methylnonadecane; I_X14 2-penthyl-furan; I_X15 4-methyl-dodecane; I_X16 3-methyl-dodecane; I_X23 10-methyl-eicosane; I_X28 1,3-hexadiene, 3-ethyl-2-methyl; I_X4 2-ethyl-furan; I_X41 nonadecane; I_X42 2(3H) furanone (A); I_X43 2(3H) furanone (B); I_X52 3-nonen-5-yne, 4-ethyl; I_X55 2(3H) furanone, 5-butyldihydro-; I_X6 toluene; I_X8 1-chloro octane. Polyp: total polyphenols; Flav: total flavonoids; Fer acid: ferulic acid; GA: gallic acid; Prot acid: protocathecuic acid; Van acid: vanillic acid; CAT: catechin; EC: epicatechin; EGC: epigallocatechin; EGCG: epigallocatechin gallate; ABTS: antioxidant activity.

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KAMUT® khorasan wheat controls (0 and 16 h), only CAT and EGC were detected, with a slight increase at the end of the 16 h incubation. As observed in wheat samples, CAT, EC, EGC and, in lower amount, EGCG were differently detected after treatment with specific LAB strains, in general with lower amounts with respect to durum wheat samples. CAT was measured in all KAMUT® khorasan wheat-fermented doughs and represented the most abundant catechin, with best results found in the 133-treated sample (17.4 nmol/gFW) (Supplementary Table S3).

Other phenolic compounds (epicatechin-3-gallate (ECG), quercetin, rutin, naringenin, *trans*-cinnamic, *p*-coumaric, caffeic, sinapic, chlorogenic and syringic acids) were absent or present in trace amounts both in durum wheat and KAMUT® khorasan wheat samples.

3.2. Effect of LAB fermentation on antioxidant activity

The total antioxidant activity of controls and inoculated samples was measured by the ABTS assay (Fig. 1a, b). The antioxidant capacity increased by 52.5% after 16 h in uninoculated durum wheat controls (Fig. 1a) and, similarly, in the 5BHI and 6BHI samples, while all the other inoculated samples displayed a lower antioxidant activity (Fig.

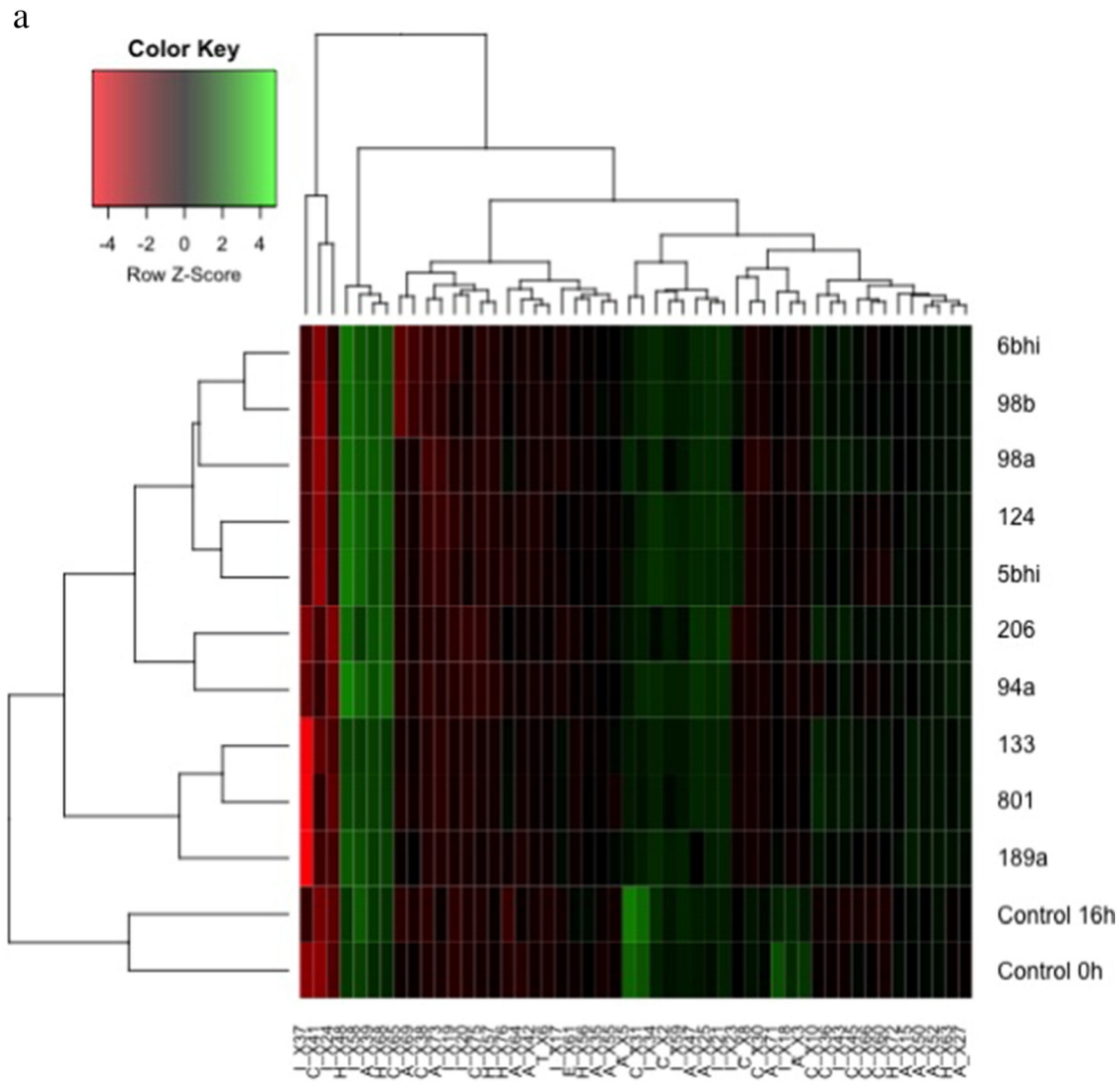

Fig. 5. a Heat map representation of GC peak areas of metabolites from *L. plantarum* strain-inoculated durum wheat flour doughs. The metabolites were organised by chemical families, with the number of compounds per family indicated. Areas were normalised by applying a logarithm function. *A = Alcohols:* A_X15 4-methyl-3-heptanol; A_X25 isoamyl alcohol; A_X27 1-pentanol; A_X3 alcohol; A_X35 2-methyl-2-buten-1-ol; A_X39 1-hexanol; A_X42 3,5-octadien-2-ol; A_X47 1-octen-3-ol; A_X5 ethyl alcohol; A_X50 1-octanol; A_X52 1,2-dodecanediol; A_X55 3,7-dimethyl-1-octanol; A_X64 1-decanol; A_X69 7-methyl-4-octanol; A_X71 phenethyl alcohol; A_X73 1-dodecanol; *C = Carbonils:* C_X10 hexanal; C_X2 2-methyl-pentanal; C_X30 2-octanone; C_X31 3-hydroxy-2-butanone (acetoin); C_X36 2-heptenal; C_X38 6-methyl-5-hepten-2-one; C_X41 2-nonanone; C_X45 2-octenal; C_X60 2,4-nonadienal; C_X65 2-dodecenal; C_X66 2,4,decadienal; C_X75 1,1,3-Trimethyl-3-cyclohexene-5-one; C_X8 pentanal; *E = Ester:* E_X61: unidentified ester; *H = Acids:* H_X48 acetic acid; H_X56 3-methylbutanoic acid; H_X57 2-methylbutanoic acid; H_X63 pentanoic acid; H_X68 hexanoic acid; H_X72 heptanoic acid; H_X76 4 penten-1-yl-ester, dodecanoic acid; *I = Hydrocarbons:* I_X17 1,2 dimethyl-benzene; I_X18 2-methyldecane; I_X19 10-methylnonadecane; I_X20 5-methyl dodecane; I_X21 2-penthyl-furan; I_X23 4-methyl-dodecane; I_X24 3-methyl-dodecane; I_X34 2(5H)-furanone; I_X37 10-methyl-eicosane; I_X43 1,3 hexadiene, 3-ethyl-2-methyl; I_X58 2(3H) furanone (A); I_X59 2(3H) furanone (B); I_X6 2-ethyl-furan. b Heat map representation of GC peak areas of metabolites from *L. plantarum* strain-inoculated KAMUT® khorasan wheat doughs. The metabolites were organised by chemical families, with the the number of compounds per family indicated. Areas were normalised by applying a logarithm function. *A = Alcohols:* A_X17 isoamyl alcohol; A_X18 1-pentanol; A_X25 1-hexanol; A_X27 3,5-octadien-2-ol; A_X3 ethyl alcohol; A_X30 1-octen-3-ol; A_X32 1-octanol; A_X33 1,2-dodecanediol; A_X47 1-decanol; A_X51 7-methyl-4-octanol; A_X53 phenethyl alcohol; A_X9 4-methyl-3-heptanol; *C = Carbonils:* C_X1 2-methyl-pentanal; C_X19 2-octanone; C_X20 3-hydroxy-2-butanone (acetoin); C_X22 2-heptenal; C_X24 6-methyl-5-hepten-2-one; C_X26 2-nonanone; C_X29 2-octenal; C_X36 2-decenal; C_X38 benzeneacetaldehyde; C_X44 2,4-nonadienal; C_X49 2,4-decadienal; C_X5 pentanal; C_X7 hexanal; *E = Ester:* E_X45 unidentified ester; *H = Acids:* H_X40 2 methylbutanoic acid; H_X46 pentanoic acid; H_X50 hexanoic acid; H_X54 heptanoic acid; H_X59 dodecanoic acid; H_X60 octanoic acid; H_X31 acetic acid; *I = Hydrocarbons:* I_X12 10-methylnonadecane; I_X14 2-penthyl-furan; I_X15 4-methyl-dodecane; I_X16 3-methyl-dodecane; I_X23 10-methyl-eicosane; I_X28 1,3-hexadiene, 3-ethyl-2-methyl; I_X4 2-ethyl-furan; I_X41 nonadecane; I_X42 2(3H) furanone (A); I_X43 2(3H) furanone (B); I_X52 3-nonen-5-yne, 4-ethyl-; I_X55 2(3H) furanone, 5-butyldihydro-; I_X6 toluene; I_X8 1-chloro octane.

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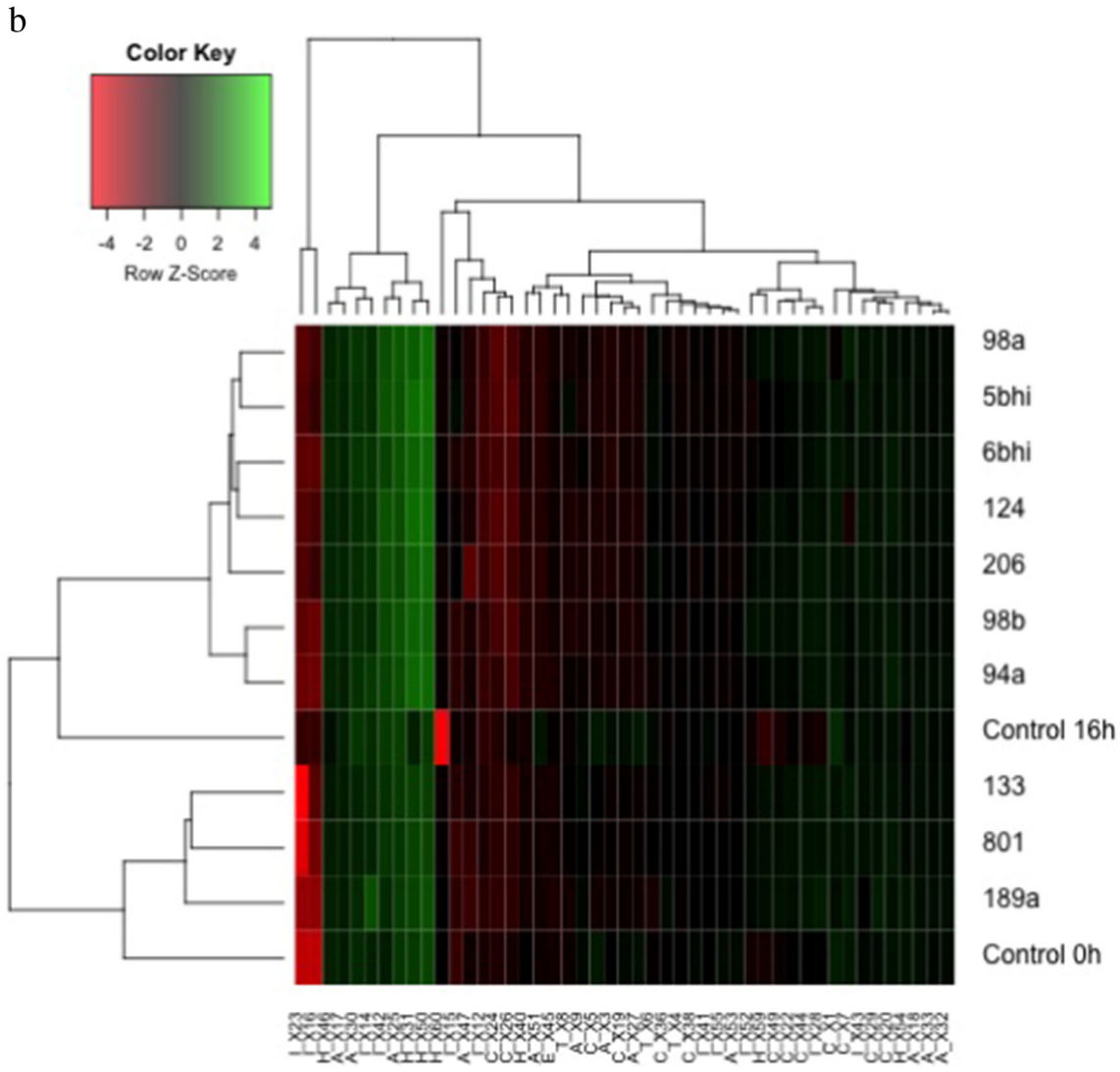

Fig. 5 (*continued*).

1a). KAMUT® khorasan wheat controls at 0 and 16 h of incubation did not display significantly different results, while antioxidant activity was increased by fermentation with 5BHI and 6BHI LAB strains (+13% and +24% respectively) (Fig. 1b), confirming these strains as the most effective.

3.3. Effect of LAB fermentation on volatile compounds

The total release of metabolites was evaluated taking into consideration their comparison as a group and, specifically, by summarizing the chromatographic areas (expressed as percentage) of each molecule belonging to alcohols, hydrocarbons, carbonils, acids and esters (Fig. 2a,b). Different molecular patterns were evidenced in durum wheat and KAMUT® khorasan wheat flour-fermented dough depending on the matrix and also on the strain inoculated.

The presence of *L. plantarum* strains in durum wheat dough was clearly evidenced by the release of alcohols and acids, while esters, carbonils and hydrocarbons did not characterise the fermented samples. In particular, 1-pentanol, 2-methyl-2-buten-2-ol, 1-decanol and ethanol were mainly released in the fermented dough (Supplementary Table S4). On the other hand, KAMUT® khorasan wheat flour-fermented by *L. plantarum* strains evidenced a generally higher relative amount of acid compounds such as acetic and hexanoic acids (Supplementary Table S4).

3.4. LAB strain selection: combination of metabolic profiles of antioxidant and volatile compounds

A metabolomics approach was adopted to evaluate the best combination of LAB strain and cereal substrate (durum wheat flour and KAMUT® khorasan wheat flour) to increase the functional and sensorial potential of *L. plantarum* strains. In particular, it was assessed whether the substrate/bacteria interaction may provide flavouring and health beneficial effects to final bakery foods. Specifically, for each strain, the metabolic profiles of polyphenolic and volatile compounds were combined by using heat maps to visualise the result of a hierarchical clustering of profile data. Two heat maps, one for each matrix tested (durum wheat or KAMUT® khorasan wheat) were constructed (Figs. 3 and 4, respectively) in order to identify any positive or negative quantitative relationships (green and red, respectively) between volatile and polyphenolic molecules.

In Fig. 3, the three most important green areas (WA, WB and WC) evidenced a high correlation between volatiles and antioxidant compounds. In particular, the group of volatile molecules WA, which includes 10 alcohols, 5 carbonils, 3 acids and 3 hydrocarbons, is highly

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correlated with EGC, EGCG, Flav and Polyp. Another group of volatile compounds (WB), represented by 2 alcohols, 4 carbonils, 5 acids and 8 hydrocarbons, is highly correlated with GA, Fer ac and antioxidant activity (ABTS). A third group (WC) characterised by only few volatiles, i.e. 2-methyl-2-buten-ol, 8 hydrocarbons and one unidentified ester, was correlated with Flav, GA, Fer ac, Van ac, CAT, Prot ac and antioxidant activity (ABTS). In KAMUT® khorasan wheat-fermented dough, three main areas in the heat map (KA, KB and KC) evidenced a high correlation between volatile and polyphenolic compounds. The KA area is the result of the correlation between Polyp, Flav, EC, Prot ac, Fer ac and EGC with a group of volatile compounds including 7-methyl-4-octanol, 3 carbonils, octanoic and heptanoic acids and 2 hydrocarbons. A second area (KB) evidenced a high correlation between a group of volatiles, including 5 alcohols, 6 carbonils, dodecanoic acid and 1,3-hexadiene, and polyphenolic compounds, such as GA, EGCG, EGC, Flav, Prot ac and Polyp. This correlation group (KB) seemed to efficiently respond to both sensorial and health beneficial requirements. Finally, a third area (KC) comprising phenethyl alcohol, 2-nonanone, 4 acids, 8 hydrocarbons and 1 unidentified ester resulted highly correlated with CAT, Fer ac, Van ac and ABTS.

In general, all the considered groups deriving from *L. plantarum* fermentation seemed to delineate a combination of sensorial and health beneficial properties, in particular the KAMUT® khorasan wheat dough group KB. Moreover, it was possible to note that alcohols and carbonils, which represent the most volatile compounds among those identified, were associated mainly to groups WA and KB both correlated with the presence of polyphenols. On the other hand, the highest number of polyphenolic compounds was associated to groups WC and KA, but the combination between *L. plantarum* and KAMUT® khorasan wheat flour seemed to provide a large spectrum of volatile molecules, whereas in durum wheat doughs hydrocarbons represent the majority of volatile compounds.

The volatile molecules and polyphenolic compounds released by each strain of *L. plantarum* in the different tested flours were represented in two heat maps including the uninoculated dough at the beginning (Control 0 h) and at the end of fermentation (Control 16 h) (Figs. 5 and 6, respectively). In durum wheat, as evidenced by the stronger brightness of green correlations, the main differences were related to the control samples which appeared in a cluster clearly separated from the samples inoculated with *L. plantarum* (Fig. 5a). In particular, acetic acid (H_X48), phenethyl alcohol (A_X71), ethanol (A_X5) and acetoin (C_X31) affected this clusterization. This behaviour was probably due to the metabolism of endogenous yeasts

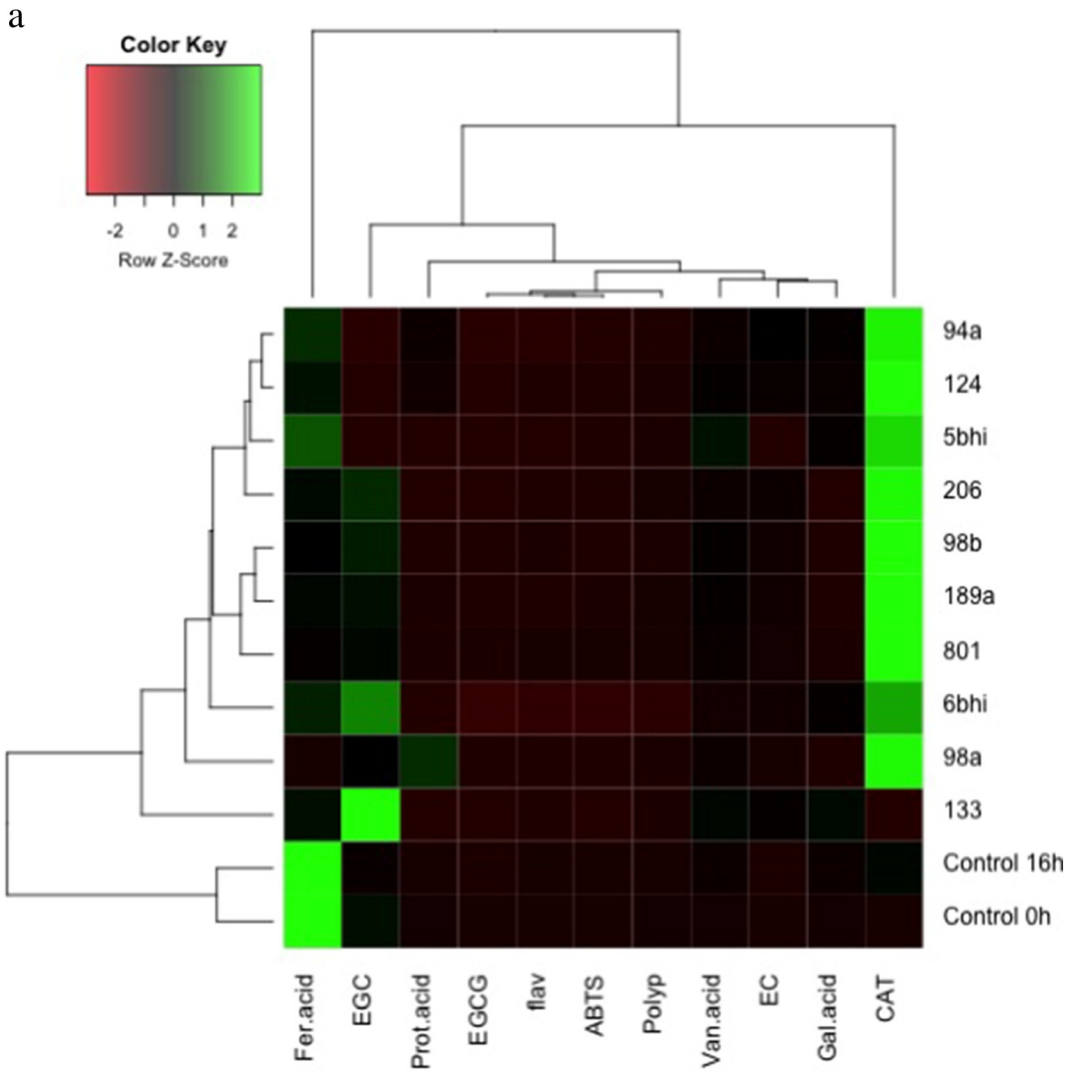

Fig. 6. a Heat map representation of antioxidant compounds released by *L. plantarum* strain-inoculated durum wheat flour doughs. Polyp: total polyphenols; Flav: total flavonoids; Fer acid: ferulic acid; GA: gallic acid; Prot acid: protocathecuic acid; Van acid: vanillic acid; CAT: catechin; EC: epicatechin; EGC: epigallocatechin; EGCG: epigallocatechin gallate; ABTS: antioxidant activity. b Heat map representation of antioxidant compounds released by *L. plantarum* strain-inoculated KAMUT® khorasan wheat doughs. Polyp: total polyphenols; Flav: total flavonoids; Fer acid: ferulic acid; GA: gallic acid; Prot acid: protocathecuic acid; Van acid: vanillic acid; CAT: catechin; EC: epicatechin; EGC: epigallocatechin; EGCG: epigallocatechin gallate; ABTS: antioxidant activity.

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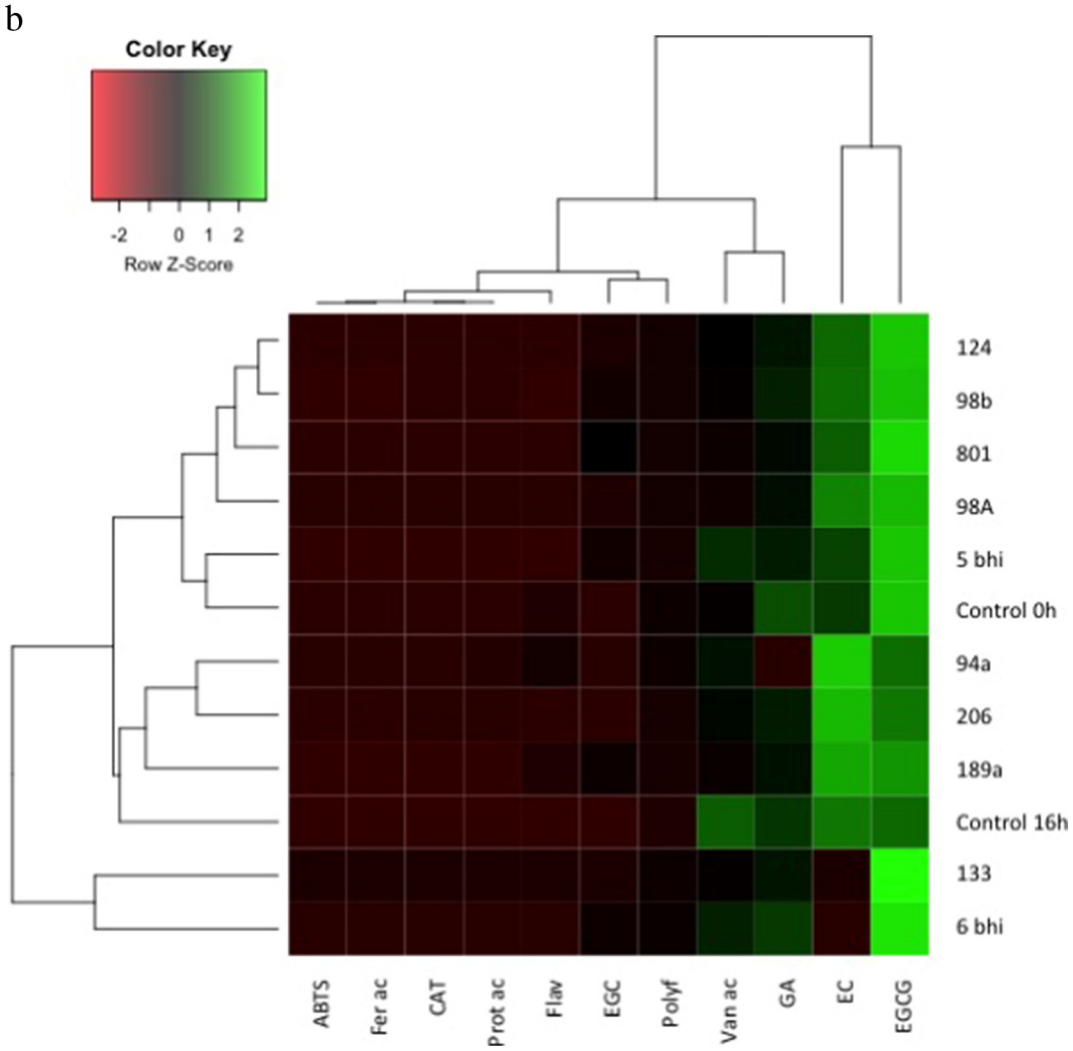

Fig. 6 (*continued*).

(10^6 CFU/g) (data not shown). In fact, their metabolic role was clearly more significant in the absence of *L. plantarum*. Besides acetic acid, many minor alcohols also seem directly correlated with dough inoculated with the strains 6BHI, 98B, 98A, 124, 5BHI, 206 and 94A. Within the cluster of inoculated doughs, it is possible to evidence a main grouping of volatile compounds including hexanal, 2-heptenal, hexadiene, 4-methyl-3-heptanol, 1-octanol, 1,2-docanediol, pentanoic acid and 1-pentanol. In fact, in the control samples their concentration was significantly lower.

Fig. 5b evidenced the influence of metabolites on the grouping of sourdoughs obtained with *L. plantarum* strains and KAMUT® khorasan wheat flour. The unfermented control (Control 0 h) and some inoculated doughs (189A, 801 and 133) clustered together. This group of doughs was characterised by a negative correlation with two different hydrocarbons: 10-methyl-eicosane and 3-methyl-dodecane. On the other hand, the inoculated samples grouped together mainly due to the influence of acetic acid (H_X31), haxanoic acid (H_X50), octanoic acid (H_X60), 1-hexanol (A_X25), 2-pentyl furan (I_X14) and 2(3H)-furanone (I_X42). The naturally fermented sample (Control 16 h) presented a unique volatile compound profile mainly affected by the negative correlation with octanoic acid (H_X60). Moreover, in this figure a cluster containing *L. plantarum* strains 98 A, 5BHI, 6BHI and 124 outlined a weak correlation to strain origin and volatile compounds released in the system considered. In fact, *L. plantarum* strains 5BHI and 6BHI were isolated from red wine, while all the others *L. plantarum* strains were isolated from sourdough.

The same approach was adopted to represent the polyphenolic compounds released in fermented samples of durum wheat and KAMUT® khorasan wheat flours in comparison to controls (Fig. 6a,b respectively). In Fig. 6a a high correlation between the control samples (Control 0 h and Control 16 h) accounted for by a higher content of Fer ac compared to the inoculated durum wheat doughs. The latter samples were characterised by a high correlation between CAT and EGC contents and strains 133 and 6BHI.

The antioxidant data of doughs obtained by the fermentation of KAMUT® khorasan wheat flour with the selected strains of *L. plantarum* (Fig. 6b) showed the highest correlation between all inoculated and control samples (Control 0 h and Control 16 h) and EGCG. Interestingly, strains 133 and 6BHI were separated from the other samples due to the negative correlation with EC.

The comparison between data obtained with the two different wheat varieties evidenced that the effect of *L. plantarum* strains was stronger in durum wheat than in KAMUT® khorasan wheat fermentation and the metabolic pattern was directly correlated to the strain inoculated. In fact, the cluster separation of control and inoculated samples was quite evident. On the contrary, with KAMUT® khorasan wheat flour fermentation *L. plantarum* strains were less able to modify the substrate while a strong effect of the substrate compounds was observed.

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4. Discussion

The present work represents a first approach to an in situ study of *L. plantarum* strains as a cell factory to improve health beneficial and sensorial requirements of cereal-based fermented foods. According to our findings, antioxidant activity and polyphenol content may represent comprehensive indicators of those requirements. In fact, besides acids which resulted equally correlated to polyphenol content and antioxidant activity, volatile molecules such as alcohols and carbonils were more correlated with polyphenols, whereas antioxidant activity appeared more correlated with hydrocarbons and esters. However, for the two considered flours, correlated molecules were not the same for each chemical family evidencing specific strain/substrate interactions.

In general, fermentation by *L. plantarum* promotes an increase in polyphenol content (such as, hydroxycinnamic acids, catechins and other flavonoids), due to the depolymerisation of present compounds, consequently improving the antioxidant potential (Hassani, Procopio, & Becker, 2016). The ability of fermentation to improve antioxidant capacity is primarily due to an increase in the amount of phenolic compounds and flavonoids. These antioxidant compounds can act as free radical terminators, metal chelators, singlet oxygen quenchers or hydrogen donors to radicals (Hur, Lee, Kim, Choi, & Kim, 2014). In fact, bioprocessing can remarkably increase the bioavailability of phenolic acids and their circulating metabolites, which have immunomodulatory effects ex vivo (Nelson et al., 2016; Valli et al., 2015). Salmeron, Fucinos, Charalampopoulos, and Pandiella (2009) studied the production of volatile compounds by the probiotic strain *L. plantarum* NCIMB 8826 in cereal- based media (oat, wheat, barley and malt). They observed that inoculation with probiotic lactic acid bacteria caused a significant change in the aroma profile of the four cereal broths. In particular, oat and barley broths were most affected by the fermentation process, while volatile production depended more on the substrate than on the microorganism. Analogously, in our results the two substrates did not produce the same metabolic profile in fermented doughs. In particular, durum wheat fermentation provided a higher metabolic diversity with the tested strains, whereas KAMUT® khorasan wheat itself seemed to play an important role in determining the metabolic traits of fermented dough probably due to the higher quali-quantitative profile of flour compounds. Regardless, *L. plantarum* fermentation was significantly effective in the production of sensorial and health beneficial compounds in both the considered flours. In particular, in durum wheat flour a compromise between good sensorial and excellent health beneficial characteristics may be represented by *L. plantarum* strains 98A, and 6BHI. On the other hand, strain 94A and 206 seemed to provide the best sensorial and health beneficial performances in KAMUT® khorasan wheat dough.

5. Conclusion

In conclusion, an improvement of sensorial and health beneficial characteristics of cereal-fermented products requires a specific combination of *L. plantarum* strains and cereal wheat flour. According to our data, the effect of *L. plantarum* fermentation on sensorial and health beneficial compounds was significant in both durum and KAMUT® khorasan wheat flours. However, KAMUT® khorasan wheat itself represents a highly specific source of volatile and phenolic compounds. Any standardised and simple indicator that provides a predictive tool to screen strain performances represents a plus value in both scientific and applied research. In the present work, we applied for the first time a metabolomics approach to correlate specific groups of volatile molecules with antioxidant activity and polyphenols content in fermented cereals. Metabolomics may represent an important information tool suitable for a rapid selection of strain/substrate combinations able to simultaneously increase sensorial and health promoting characteristics of cereal-fermented foods.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.foodres.2016.08.044.

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