International Journal of Food Microbiology 302 (2019) 24–34

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

# Characterization of indigenous *Pediococcus pentosaceus*, *Leuconostoc kimchii*, *Weissella cibaria and Weissella confusa* for faba bean bioprocessing

Carlo G. Rizzello[a,*,1], Rossana Coda[b,c,1], Yaqin Wang[b], Michela Verni[a], Ilkka Kajala[d], Kati Katina[b], Arja Laitila[d]

[a] Department of Soil, Plant, and Food Sciences, University Aldo Moro of Bari, Via Amendola 165/a, 70125 Bari, Italy
[b] Department of Food and Nutrition, University of Helsinki, Agnes Sjoberginkatu 2, 00014 Helsinki, Finland
[c] Helsinki Institute of Sustainability Science, University of Helsinki, P.O. Box 3, 00014 Helsinki, Finland
[d] VTT Technical Research Centre of Finland Ltd., P.O. Box 1000, FI-02044 VTT, Finland

ARTICLE INFO

*Keywords:*
Lactic acid bacteria
Dextrans
Faba bean fermentation

ABSTRACT

The interest towards legumes in food applications has risen over the past decades. However, the presence of antinutritional factors (ANF) and the poor technological performances still restricts their application in food fortification. In this study, four lactic acid bacteria (LAB) isolated from faba bean were applied as starter cultures for faba bean bioprocessing. None of the strains employed produced exopolysaccharides from raffinose, on the contrary, they did with sucrose as substrate. The fermented doughs were characterized and the strains were compared for their adaptation capacity and metabolic performance including the formation of dextrans, the degradation of ANF and the ability to improve antioxidant activity and in vitro protein digestibility (IVPD). A contribution to the proteolysis was given by the presence of endogenous enzymes, responsible for the increase of peptides and amino acids in dough from irradiated flour. However, the LAB strains further enhanced proteolysis. *Weissella cibaria* VTT E-153485 led to the highest peptide release and consequentially to the highest IVPD. In doughs fermented with *Pediococcus pentosaceus* VTT E-153483 and *Leuconostoc kimchi* VTT E-153484, phytic acid was reduced to more than half the initial concentration. Inoculated doughs had significantly lower content of oligosaccharides after 24 h of incubation compared to the controls. The most efficient raffinose consumption was found for *Leuc. kimchi* and *W. cibaria.* Doughs inoculated with weissellas contained > 1% of dextrans. *Weissella confusa* VTT E-143403 induced a significant increment in viscosity (ca. 7 times higher than the controls). This study revealed that well-characterized, indigenous LAB provided beneficial biotechnological features in faba bean dough processing and contributed to its implementation in the food production.

## 1. Introduction

In the last years, the studies on legumes as protein-sources in food applications have increased. The main reasons for this incremental interest are their worldwide cultivation and good nutritional profile including important micronutrients (Multari et al., 2015). Research has demonstrated that high-protein (predominantly animal-based) diets are likely to be harmful to gut health in longer term, because of a reduction in cancer-protective compounds and an increase in hazardous metabolites (Windey et al., 2012). A possible reason is the lack of adequate fiber and associated phytochemicals. Thus, replacement of animal proteins with plant proteins may affect the nutrient adsorption at gut level, allowing changes in amino acids and triacylglycerol plasma concentration and exerting hypoglycemic and hypotriacylglycemic activity (Fernández-Quintela et al., 1998).

Faba bean (*Vicia faba* L.) is the second most cultivated legume and has been widely utilized as feed and food in both raw and processed forms. However, in spite of its potential as protein source for human

*Abbreviations:* ANF, antinutritional factors; E3403, faba bean dough fermented with *Weissella confusa* VTT E-143403; E3483, faba bean dough fermented with *Pediococcus pentosaceus* VTT E-153483; E3484, faba bean dough fermented with *Leuconostoc kimchii* VTT E-153484; E3485, faba bean dough fermented with *Weissella cibaria* VTT E-153485; EPS, exopolysaccharides; FAA, free amino acids; FQ, fermentation quotient; IF, dough from irradiated faba bean flour; IVPD, in vitro protein digestibility; LAB, lactic acid bacteria; L-DOPA, L-3,4-dihydroxyphenylalanine; ME, methanolic extract; NF, dough from native faba bean flour; TTA, total titratable acidity; WSE, water/salt-soluble extracts

* Corresponding author.
*E-mail address:* carlogiuseppe.rizzello@uniba.it (C.G. Rizzello).
[1] These authors contributed equally to this work and are joint first authors.

https://doi.org/10.1016/j.ijfoodmicro.2018.08.014
Received 4 June 2018; Received in revised form 27 July 2018; Accepted 4 August 2018
Available online 24 August 2018
0168-1605/ 

diet, faba bean still remains underutilized, particularly in Western countries (Tijhuis et al., 2012). In addition to proteins, faba bean is a rich source of fiber and functional secondary metabolites (Aune et al., 2011), such as L-3,4-dihydroxyphenylalanine (L-DOPA), the precursor to the neurotransmitter catecholamine (Ramya and Thaakur, 2007).

Besides healthy aspects, the regular intake of plant-based foods such as faba bean in substitution of animal proteins in the diet is encouraged due to sustainability reasons (Multari et al., 2015). Indeed, the intensive cereals food and feed production is a major cause of soil degradation and crops such as faba bean, which have the ability to fix nitrogen, can offer an effective strategy to tackle the environmental damages of monoculture practices (Chapagain and Riseman, 2015).

Faba bean, its fractions, and its processing products (grains, hulls, and flours) contain compounds potentially having negative impact on nutrition, defined antinutritional factors (ANF) such as saponins, condensed tannins, protease inhibitors, α-galactosides, phytic acid and vicine and convicine (Multari et al., 2015).

Different strategies have been proposed to decrease the ANF content in faba beans and other legumes, ranging from crop genetic improvement to processing (for review see Multari et al., 2015). In particular, bioprocessing and fermentation are successful strategies to decrease the content of ANF in faba bean while at the same time enhancing its technological and nutritional properties and thereby opening new application scenarios for the food industry (Adebiyi et al., 2017; Chandra-Hioe et al., 2016; Coda et al., 2015, 2017a; Rizzello et al., 2016a).

In the past decade, studies on innovative food products have explored the metabolic traits of starters isolated from the matrix to be processed, most often displaying the best adaptation and most suitable performance (Corbo et al., 2017; Di Cagno et al., 2013). Typically, these starters have been referred to as indigenous or “autochthonous” intending strains isolated from and used to ferment the same raw-matrix.

Based on the expected growth of the vegetarian product market within the next few years (Multari et al., 2015) and the recognized potential of the lactic acid bacteria (LAB) fermentation to exploit more of faba bean as food ingredient, the selection of proper starters for industrial processing is needed. In a recent characterization of LAB species isolated from spontaneous faba bean fermentation, some relevant features related to its transformation were assessed allowing the identification of technologically relevant biotypes (Verni et al., 2017).

In this study, four LAB strains belonging to *Pediococcus pentosaceus*, *Leuconostoc kimchii*, *Weissella cibaria* and *Weissella confusa* species, originating from faba bean were characterized and compared for their adaptation capacity and metabolic performance including formation of exopolysaccharides (e.g. dextrans), reduction of α-galactosides and phytic acid content, generation of antioxidant activity, and in vitro protein digestibility.

## 2. Materials and methods

### 2.1. Microorganisms and raw materials

The faba bean (*Vicia faba major*) flour used in this study (CerealVeneta, San Martino di Lupari, PD, Italy) had the following composition: moisture, 9.50 ± 0.08%; protein, 24.21 ± 0.09% of dry matter (d.m.); fat, 1.33 ± 0.05% of d.m.; total carbohydrates, 57.45 ± 0.80% of d.m. of which starch, 44.90 ± 0.20% of d.m.; dietary fibers, 9.80 ± 0.40% of d.m. and ash, 3.60 ± 0.04% of d.m.

A batch of faba bean flour was irradiated with a gamma radiation dose of 11.8 kGy (Scandinavian Clinics Estonia OÜ, Tallinn, Estonia) in order to inactivate the indigenous microbiota and was used as control as described below.

Four LAB strains (*Pediococcus pentosaceus* VTT E-153483, *Leuconostoc kimchii* VTT E-153484, *Weissella cibaria* VTT E-153485, and *Weissella confusa* VTT E-143403) isolated from the faba bean flour were deposited to the VTT Culture Collection, Espoo Finland (http://culturecollection.vtt.fi/). LAB were routinely propagated in MRS broth (Oxoid Ltd., Basingstoke, Hampshire, UK) in anaerobic conditions at 30 °C and used as described below.

### 2.2. Faba bean fermentation

After propagation in MRS broth in anaerobic conditions at 30 °C for 24 h, LAB cells were harvested by centrifugation (10,000 × *g*, 10 min), washed once with Ringer's solution (Oxoid) and re-suspended in sterile tap water at the cell density of ca. 8.0 log cfu/mL. Semi-liquid doughs (300 g) were obtained by mixing faba bean flour and sterile tap water (ratio flour:water, 1:2), and were inoculated with the LAB cells suspensions at the initial cell density of ca. 6 log cfu/g.

Doughs were fermented with *P. pentosaceus* VTT E-153483 (E3483), *Leuc. kimchii* VTT E-153484 (E3484), *W. cibaria* VTT E-153485 (E3485), and *W. confusa* VTT E-143403 (E3403) and incubated in static conditions at 25 °C for 48 h. Two spontaneously fermented (not inoculated) doughs were included in this study: a) one obtained with native faba bean flour (NF) and one with irradiated flour (IF). These doughs were incubated in the same conditions as the LAB started doughs and were used as control samples in order to discriminate the potential effect of the autochthonous microbiota and of the endogenous enzymes, respectively. The fermentation experiments were performed as triplicates. Aliquots of each dough were withdrawn after 24 and 48 h of fermentation and used for chemical characterization.

### 2.3. Microbiological analyses

Microbiological analyses of the doughs were performed by plate count at the beginning and end of incubation time. LAB were determined on MRS agar (Oxoid) after incubation in anaerobic conditions at 30 °C for 3 days. MRS was supplemented with 0.01% cycloheximide (Sigma Chemical, St. Louis, MO, USA) and 0.2% 2-phenylethanol to suppress the growth of fungi and Gram-negative bacteria. EPS production ability of LAB was assessed on MRS supplemented with 2% sucrose or 2% raffinose (w/v) (Juvonen et al., 2015). Yeast and moulds were determined on YM agar (Difco Laboratories), after incubation in aerobic conditions at 25 °C for 3–5 days. Chlortetracycline and chloramphenicol (both at 0.01%) were added to YM medium to prevent bacterial growth. In addition, 0.02% of Triton-X 100 (BDH) was used to limit the spreading of fungal colonies on YM-agar. Gram-negative coliforms were determined on Chromocult® coliform agar (Merck Millipore) after incubation in aerobic conditions at 37 °C for 24 h.

### 2.4. Kinetics of acidification

Kinetics of acidification during LAB fermentation of faba bean doughs were determined and modelled in agreement with the Gompertz equation as modified by Zwietering et al. (1990): y ¼ k + A exp.{ − exp [($V_{max}$ e/A)(l − t) + 1]}; where y is the acidification rate expressed as dpH/dt (units of pH/h) at the time t; k is the initial level of the dependent variable to be modelled (pH units); A is the pH (units) variation (between inoculation and the stationary phase); $V_{max}$ is the maximum acidification rate expressed as dpH/h, respectively; λ is the length of the lag phase measured in hours. The experimental data were modelled by the non-linear regression procedure of the Statistica 8.0 software (Statsoft, Tulsa, USA).

### 2.5. Chemical characterization

Total titratable acidity (TTA) was determined after homogenization of 10 g of samples with 90 mL of distilled water, and expressed as the amount (mL) of 0.1 M NaOH required to neutralize the solution, using phenolphthalein as indicator (official AACC method 02-31.01) (AACC, 2003).

Water/salt-soluble extracts (WSE) of freeze dried sourdoughs were prepared according to Weiss et al. (1993) and used to analyze organic

acids, ethanol, peptides, and free amino acids (FAA). Organic acids were determined by High Performance Liquid Chromatography (HPLC), using an ÄKTA Purifier system (GE Healthcare, Buckinghamshire, UK) equipped with an Aminex HPX-87H column (ion exclusion, Biorad, Richmond, CA), and an UV detector operating at 210 nm (Rizzello et al., 2010). The fermentation quotient (FQ) was determined as the molar ratio between lactic and acetic acids.

The concentration of peptides and FAA was determined on the WSE, to evaluate the degree of proteolysis of the native proteins of doughs. For the peptide analysis, WSE were treated with trifluoroacetic acid (0.05% wt/vol) and subject to dialysis (cut-off 500 Da) to remove proteins and FAA, respectively. Then peptides concentration was determined by the *o*-phtaldialdehyde (OPA) method as described by Church et al. (1983). FAA were analyzed by a Biochrom30 series Amino Acid Analyzer (Biochrom Ltd., Cambridge Science Park, UK) with a Na-cation exchange column (20 cm × 0.46 cm internal diameter) as reported in Rizzello et al. (2010).

### 2.6. Sugars and mannitol

One hundred milligrams of freeze-dried samples were mixed with 5.0 mL of Milli-Q water and vortexed for 5 min to allow the complete dissolution of free sugars and mannitol. Then, the suspensions were boiled for 5 min. After cooling, samples (400 μL) were filtered using Amicon Ultra-0.5 centrifugal filter units (Millipore, Billerica, MA) at 12,000 ×*g* for 10 min to remove polymeric molecules (above 10 kDa). Before further analysis, samples were diluted with Milli-Q water. Mono-, di- and oligo-saccharides in faba bean doughs were analyzed by high performance anion exchange chromatography with pulse amperometric detection (HPAEC-PAD) system. A CarboPac PA1 column (250 × 4 mm i.d., Dionex, Sunnyvale, CA) and a Waters 2465 pulsed amperometric detector (Waters, USA) were used (Xu et al., 2017a). Glucose, fructose, sucrose (Merck, Germany), galactose, melibiose, raffinose, stachyose (Sigma-Aldrich) and verbascose (Megazyme, Ireland) were used as standards and 2-deoxy-D-galactose (Sigma-Aldrich) was used as the internal standard for quantification. Mannitol was quantified by the HPAEC-PAD system equipped with a CarboPac MA-1 analytical column and a DECADE detector (Antec Leyden, The Netherlands) as reported by Xu et al. (2017a). Mannitol (Sigma-Aldrich) was used as the standard for quantification. Under the experimental conditions, a detection limit of 0.02% (on dough dry weight) was determined for sugars and mannitol.

### 2.7. Dextran production and viscosity

The amount of dextran in freeze-dried and homogenized sourdough samples was determined by an enzyme-assisted method using an enzyme mixture of dextranase (Sigma-Aldrich, Germany) and α-transglucosidase (Megazyme, Ireland) as described by Katina et al. (2009). Commercial dextran from *Leuconostoc* spp. was purchased from Sigma-Aldrich (Sweden) and was hydrolyzed in the same conditions of the samples to verify the enzyme activity.

Viscosity of doughs was measured at 23 °C. Constant rate measurement of viscosity as a function of shear rate was performed with a rotational rheometer (Rheolab QC, Anton Paar GmbH, Vienna, Austria) using a ST22.02-4V probe in a beaker (100 mL). The measuring profile was set to increase shear rates from 2 $s^{-1}$ to 100 $s^{-1}$ and return to 2 $s^{-1}$ at 23 °C. Two replicate measurements were conducted, and the average value was reported. Only viscosities that were measured at 100 s/1 were presented to reveal viscosity variations in the studied sourdoughs.

### 2.8. In vitro protein digestibility

The in vitro protein digestibility (IVPD) of doughs was determined by the method originally described by of Akeson and Stahmann (1964) with some modification (Coda et al., 2017a). The IVPD was expressed as the percentage of the total protein, which was solubilized after the sequential enzymatic hydrolysis with pepsin and pancreatin.

### 2.9. Phytase activity and phytic acid

Phytase activity of sourdough samples was determined according to the method of De Angelis et al. (2003). The extract was prepared by suspending of 1 g of freeze-dried sourdough samples in 4 mL of Tris-HCl buffer (0.05 M, pH 7.5). After centrifugation, the supernatant was mixed with substrate and kept in 45 °C for 2 h. The colour reagent was added and the released inorganic phosphate was measured by a spectrophotometer. Phytic acid concentration was measured using Megazyme kit K-PHYT 05/07 (Megazyme International Ireland Limited, Bray, Ireland) following the manufacturer's instructions.

### 2.10. Total phenols and antioxidant activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was determined on the methanolic extract (ME) of freeze dried faba bean doughs and the concentration of total phenols was determined as described by Slinkard and Singleton (1997), and expressed as gallic acid equivalent. The free radical scavenging capacity was determined using the stable radical DPPH· (Rizzello et al., 2010). and expressed as follows: DPPH scavenging activity (%) = [(blank absorbance − sample absorbance) / blank absorbance] × 100. The value of absorbance was compared with 75 ppm butylated hydroxytoluene (BHT), which was used as the antioxidant reference.

### 2.11. Statistical analysis

The results were calculated as means of at least three replicates. The data were analyzed by one-way ANOVA; pair-comparison of treatment means was obtained by Tukey's procedure at $P < 0.05$, using the statistical software Statistica 8.0 (StatSoft Inc., Tulsa, USA). Significantly different data were indicated with a different superscript letter. Biochemical and nutritional properties of the doughs after 48 h of fermentation were analyzed through principal component analysis (PCA), using the software Statistica 8.0 (StatSoft Inc., Tulsa, USA).

## 3. Results

### 3.1. Faba bean fermentation

Prior to fermentation, cell density of presumptive LAB was 3.6 ± 0.2 and 2.5 ± 0.1 log cfu/g in native and radiated faba bean flours, respectively. After 24 h of fermentation at 25 °C, LAB in inoculated doughs ranged between 8.4 ± 0.4 and 9.5 ± 0.1 log cfu/g (Fig. 1S). The lowest value was observed for the dough fermented by *P. pentosaceus* VTT E-153483, while no significant differences were found for the other doughs. Cell densities of 7.00 ± 0.26 and 3.20 ± 0.15 were observed for NF and IF doughs (Fig. 1S).

A further increase of ca. 1 log cycle of LAB cell density was retrieved for both IF and NF doughs after 48 h of incubation. However, also after 48 h of fermentation, the cell number of LAB in the inoculated doughs was significantly higher than the spontaneously fermented controls (Fig. 1S). Compared to native flours, the radiation treatment caused a reduction of 1 log cycle of all microbial groups analyzed. Coliforms remained constant below 2 log cfu/g after 24 and 48 h of fermentation in IF dough, while their number increased up to 6.1 ± 0.1 log cfu/g during 24 h spontaneous fermentation of NF (Fig. 1S). Compared to the data obtained after 24 h, decreases in coliforms from 1 to 2 log cycles were observed after 48 h of fermentation. Yeasts and moulds were stably lower than 2.6 log cfu/g in all fermented samples throughout all the incubation time.

Based on the kinetics of acidification (Table 1), both *W. cibaria* and *W. confusa* behaved similarly, showing both the shortest latency phase

**Table 1**
Kinetics of acidification as modelled according to the Gompertz equation as modified by Zwietering et al. (1990) of faba bean dough inoculated with *P. pentosaceus* VTT E-153483, *Leuc. kimchii* VTT E-153484, *W. cibaria* VTT E-153485, and *W. confusa* VTT E-143403 and fermented at 25 °C for 24 h.

| Strain | $V_{max}$ (dpH/h) | λ (h) | ΔpH |
|---|---|---|---|
| *P. pentosaceus* VTT E-153483 | 0.12 ± 0.03$^{b}$ | 7.94 ± 0.15$^{a}$ | 1.38 ± 0.08$^{a}$ |
| *L. kimchii* VTT E-153484 | 0.20 ± 0.03$^{a}$ | 8.04 ± 0.7$^{a}$ | 1.41 ± 0.09$^{a}$ |
| *W. cibaria* VTT E-153485 | 0.10 ± 0.0$^{b}$ | 4.91 ± 0.5$^{b}$ | 0.99 ± 0.05$^{b}$ |
| *W. confusa* VTT E-143403 | 0.08 ± 0.01$^{b}$ | 5.75 ± 0.6$^{b}$ | 0.80 ± 0.04$^{b}$ |

The data are the means of three independent experiments ± standard deviations (n = 3). $^{a–b}$Values in the same column with different superscript letters differ significantly ($P < 0.05$).

(< 6 h), Vmax of approximately 0.1 dpH/h and similar pH variation. *P. pentosaceus* VTTE-153483 and *L. kimchii* VTT E153484 had λ values of ca. 8 h, and showed the highest ΔpH (Table 1).

At the beginning of fermentation, doughs had a pH of ca. 6.20. The pH value was stable in IF dough during the 48 h of incubation, while it decreased in all the other samples from 22 to 30% (Table 2). The lowest pH values at the end of the fermentation were found for doughs fermented with *P. pentosaceus* VTT E-153483 and *Leuc. kimchii* VTT E-153484 (Table 2).

In agreement with pH values, the TTA of E3483, E3484 and E3485 after 24 h were significantly higher than IF and NF, while E3403 showed a TTA similar to NF dough (Table 2). After 48 h, all inoculated doughs had TTA values significantly higher than the controls (Table 2). In both the fermentation times, the doughs fermented with the starters showed similar results with the exception of E3404 which had significantly lower TTA.

After the first 24 h, the highest synthesis of lactic acid was observed in E3483 and E3484 with 104.29 ± 4.91 and 97.78 ± 3.63 mmol/kg, respectively (Table 2). A marked further increase (46%) was observed in E3483 after 48 h of fermentation. The lowest lactic acid concentration was obtained when *W. cibaria* VTT E-153485 was the starter (Table 2).

A slight increase in acetic acid concentration occurred in IF during the incubation, whereas it significantly increased after 48 h in NF dough reaching values higher than E3483 and E3403 (Table 2). *L. kimchii* VTT E-153484 and *W. cibaria* VTT E-153485 led to the highest production of acetic acid (Table 2). FQ values after 24 h ranged from 5.64 to 36.80 with E3483 showing the highest value (Table 2). After 48 h of fermentation, FQ remained constant in E3483, E3484, and E3403, while a slight increase was found in E3485 (Table 2).

Peptides and FAA concentrations were analyzed before and after incubation to estimate the degree of proteolysis (Table 2). During the first 24 h of fermentation, the peptide concentration increased for all the doughs, up to 26 g/kg. Significantly higher concentrations were found in E3485 and E3403 compared to the other doughs. The peptide concentration further increased from 6 to 71% during 48 h for all the inoculated doughs, while a moderate increase was noticed in NF dough (Table 2).

Total FAA (TFFA) concentration after 24 h of fermentation increased from 16 to 97% in all the samples, and particularly for the inoculated doughs (Table 2). TFFA kept increasing in all the samples during further incubation until 48 h. The dough started with *W. confusa* VTT E-143403 showed the highest TFAA concentration of ca. 4 g/kg after 48 h.

Prior incubation, the most abundant amino acids in faba bean doughs were Glu and Arg, in amounts of 400 ± 19 and 450 ± 23 mg/kg, respectively. After 24 h of fermentation with *P. pentosaceus* VTT E-153483, *Leuc. kimchii* VTT E-153484 and *W. cibaria* VTT E-153485 several FAA reached concentrations significantly higher than those found in IF (Thr, Glu, Gly, Ala, Cys, Val, Leu, Phe, GABA, His and Arg) (Fig. 1A). Asp in E3484 was > 10 times higher than in both the controls, reaching 142 ± 6 mg/kg. A marked decrease of Tyr (> 70%) was observed in all the inoculated doughs compared to the controls. In E3403 after 48 h, the amount of all the amino acid was significantly higher than that found in the other fermented doughs with few exceptions (Lys, Tyr and Arg) (Fig. 1B). The FAA profiles of the doughs fermented with *Pediococcus* and *Leuconostoc* strains were almost similar, while E3485 was characterized by relevant lower concentration of almost all the essential FAA (Fig. 1B).

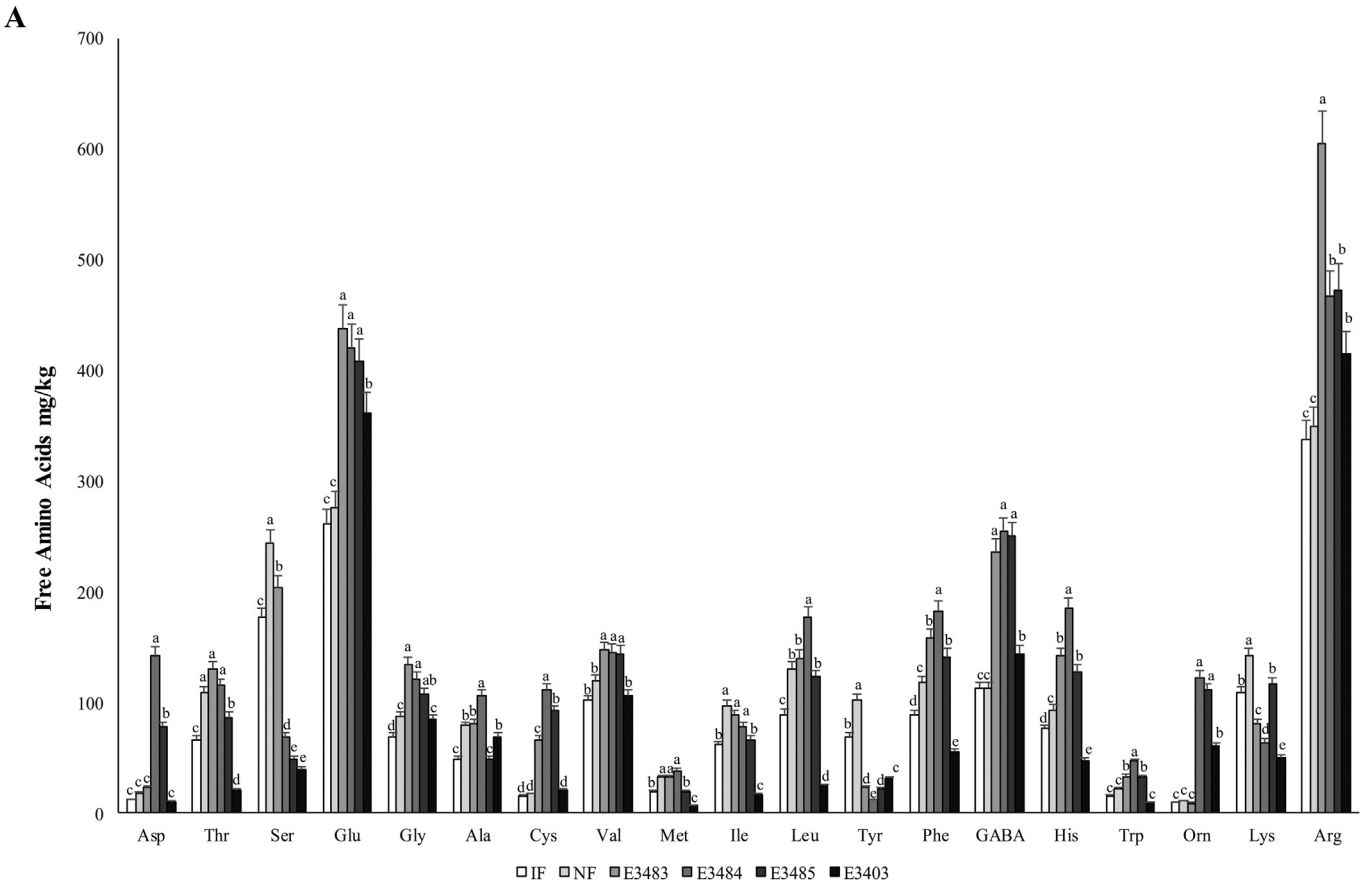
### 3.2. Sugars and mannitol

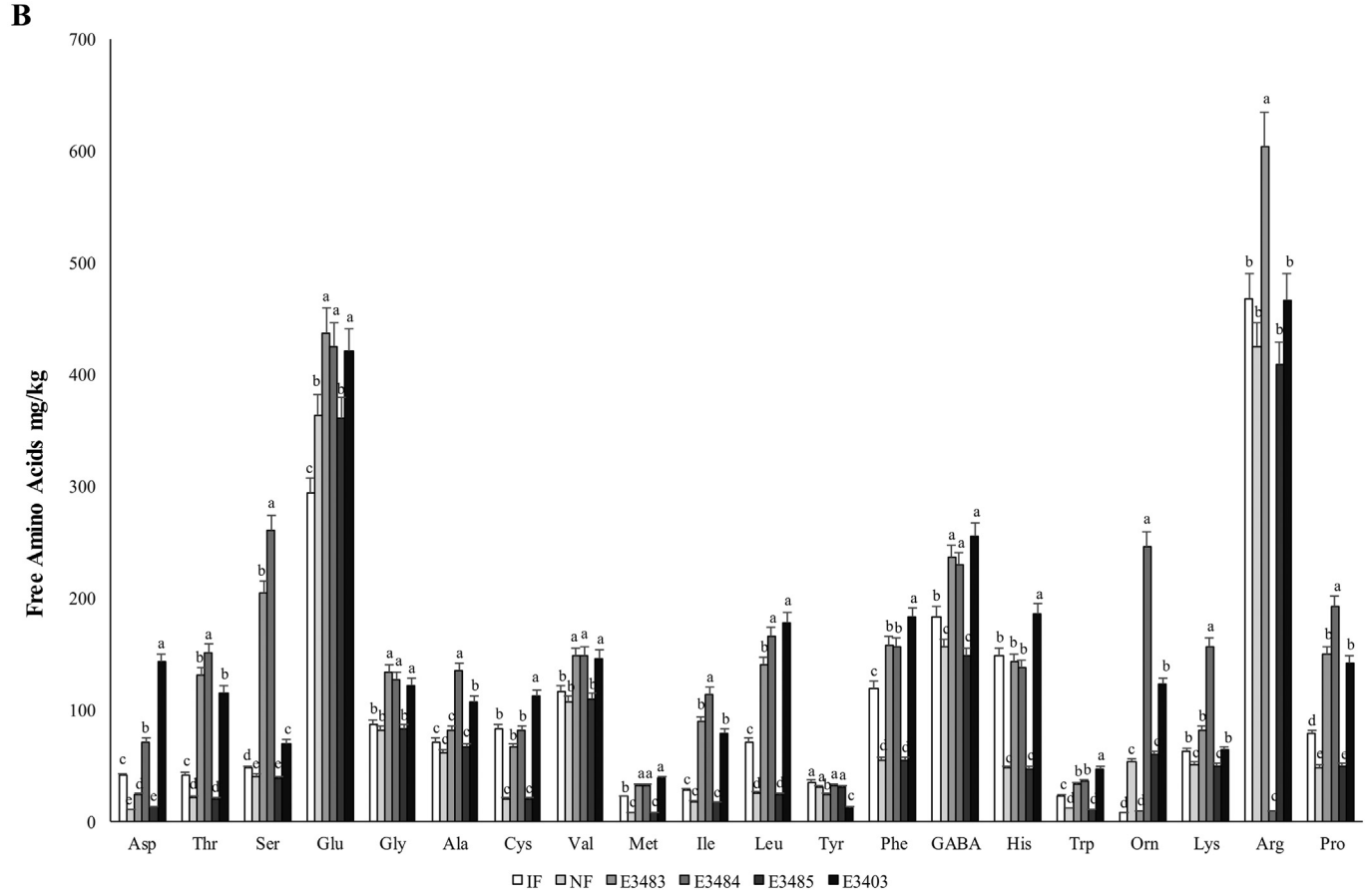
Prior fermentation, verbascose, sucrose and stachyose were the most abundant sugars in not incubated faba bean doughs (Table 3). Raffinose was ca. 0.60%, while glucose, fructose and galactose were not detected. After 24 h fermentation, sucrose was not detected in the doughs E3484, E3485, E3403 (Table 3), while it decreased in E3483 (44%) and NF (26%).

**Table 2**
Biochemical characterization of faba bean doughs inoculated with *P. pentosaceus* VTT E-153483 (E3483), *Leuc. kimchii* VTT E-153484 (E3484), *W. cibaria* VTT E-153485 (E3485), and *W. confusa* VTT E-143403 (E3403) after 24 and 48 h of fermentation at 25 °C. Two not inoculated doughs, one obtained with native faba bean flour (NF) and one with irradiated flour (IF) were produced and incubated in the same conditions and used as controls.

| | | pH | TTA | Lactic acid (mmol/kg) | Acetic acid (mmol/kg) | FQ | Peptides (g/kg) | FAA (g/kg) |
|---|---|---|---|---|---|---|---|---|
| IF | *0* | 6.14 ± 0.02$^{a}$ | 3.1 ± 0.1$^{a}$ | 7.26 ± 0.34$^{a}$ | 0.45 ± 0.02$^{a}$ | 15.97 ± 0.75$^{a}$ | 15.80 ± 0.78$^{a}$ | 1.58 ± 0.08$^{a}$ |
| | *24* | 6.21 ± 0.03$^{a}$ | 3.5 ± 0.2$^{d}$ | 7.73 ± 0.36$^{e}$ | 0.71 ± 0.05$^{e}$ | 10.87 ± 0.51$^{c}$ | 18.31 ± 0.90$^{b}$ | 1.83 ± 0.10$^{d}$ |
| | *48* | 6.08 ± 0.04$^{a}$ | 3.7 ± 0.2$^{d}$ | 8.49 ± 0.40$^{e}$ | 0.78 ± 0.03$^{e}$ | 10.82 ± 0.43$^{c}$ | 18.88 ± 0.92$^{c}$ | 2.41 ± 0.12$^{c}$ |
| NF | *0* | 6.24 ± 0.04$^{a}$ | 2.8 ± 0.1$^{a}$ | 7.24 ± 0.30$^{a}$ | 0.53 ± 0.02$^{a}$ | 13.66 ± 0.74$^{a}$ | 15.94 ± 0.76$^{a}$ | 1.59 ± 0.07$^{a}$ |
| | *24* | 5.47 ± 0.03$^{b}$ | 7.5 ± 0.4$^{c}$ | 43.23 ± 1.73$^{d}$ | 7.66 ± 0.38$^{c}$ | 5.64 ± 0.24$^{e}$ | 18.42 ± 0.91$^{b}$ | 2.30 ± 0.12$^{c}$ |
| | *48* | 4.85 ± 0.03$^{b}$ | 11.7 ± 0.6$^{c}$ | 66.8 ± 2.84$^{d}$ | 9.74 ± 0.45$^{c}$ | 6.86 ± 0.37$^{de}$ | 23.60 ± 1.19$^{b}$ | 2.98 ± 0.16$^{b}$ |
| E3483 | *0* | 6.20 ± 0.02$^{a}$ | 3.0 ± 0.2$^{a}$ | 7.38 ± 0.29$^{a}$ | 0.46 ± 0.02$^{a}$ | 16.04 ± 0.53$^{a}$ | 16.19 ± 0.84$^{a}$ | 1.67 ± 0.07$^{a}$ |
| | *24* | 4.70 ± 0.04$^{c}$ | 12.2 ± 0.3$^{a}$ | 104.29 ± 4.91$^{a}$ | 2.83 ± 0.11$^{d}$ | 36.80 ± 1.71$^{a}$ | 18.64 ± 0.92$^{b}$ | 3.01 ± 0.17$^{a}$ |
| | *48* | 4.39 ± 0.03$^{c}$ | 16.0 ± 0.9$^{a}$ | 152.89 ± 6.28$^{a}$ | 4.36 ± 0.17$^{d}$ | 35.05 ± 1.46$^{a}$ | 31.98 ± 1.65$^{a}$ | 3.19 ± 0.19$^{b}$ |
| E3484 | *0* | 6.19 ± 0.01$^{a}$ | 3.1 ± 0.1$^{a}$ | 7.42 ± 0.29$^{a}$ | 0.49 ± 0.08$^{a}$ | 15.14 ± 0.45$^{a}$ | 16.03 ± 0.94$^{a}$ | 1.66 ± 0.10$^{a}$ |
| | *24* | 4.74 ± 0.03$^{c}$ | 12.7 ± 0.7$^{a}$ | 97.78 ± 3.63$^{a}$ | 16.33 ± 0.77$^{a}$ | 5.98 ± 0.56$^{e}$ | 18.96 ± 0.98$^{b}$ | 3.24 ± 0.18$^{a}$ |
| | *48* | 4.48 ± 0.04$^{c}$ | 15.3 ± 0.9$^{a}$ | 116.41 ± 4.86$^{b}$ | 21.06 ± 0.95$^{a}$ | 5.52 ± 0.76$^{e}$ | 20.18 ± 1.12$^{b}$ | 3.62 ± 0.22$^{a}$ |
| E3485 | *0* | 6.21 ± 0.03$^{a}$ | 3.4 ± 0.2$^{a}$ | 7.40 ± 0.42$^{a}$ | 0.47 ± 0.07$^{a}$ | 15.74 ± 0.65$^{a}$ | 16.35 ± 0.80$^{a}$ | 1.68 ± 0.12$^{a}$ |
| | *24* | 5.13 ± 0.02$^{b}$ | 9.3 ± 0.6$^{b}$ | 62.53 ± 3.24$^{c}$ | 9.16 ± 0.39$^{b}$ | 6.83 ± 0.34$^{d}$ | 26.42 ± 1.37$^{a}$ | 2.84 ± 0.17$^{b}$ |
| | *48* | 4.64 ± 0.05$^{b}$ | 15.3 ± 0.9$^{a}$ | 100.56 ± 4.41$^{c}$ | 11.66 ± 0.51$^{b}$ | 8.62 ± 0.42$^{d}$ | 35.70 ± 1.89$^{a}$ | 3.23 ± 0.20$^{b}$ |
| E3403 | *0* | 6.22 ± 0.04$^{a}$ | 3.7 ± 0.3$^{a}$ | 7.45 ± 0.43$^{a}$ | 0.50 ± 0.09$^{a}$ | 14.90 ± 0.59$^{a}$ | 16.45 ± 0.92$^{a}$ | 1.67 ± 0.10$^{a}$ |
| | *24* | 5.36 ± 0.03$^{b}$ | 7.8 ± 0.5$^{c}$ | 75.70 ± 3.56$^{b}$ | 5.34 ± 0.32$^{dc}$ | 14.17 ± 0.33$^{b}$ | 23.80 ± 1.28$^{a}$ | 3.29 ± 0.19$^{a}$ |
| | *48* | 4.76 ± 0.03$^{b}$ | 13.8 ± 1.0$^{b}$ | 120.10 ± 5.31$^{b}$ | 8.20 ± 0.37$^{c}$ | 14.60 ± 0.27$^{b}$ | 34.07 ± 1.35$^{a}$ | 4.06 ± 0.25$^{a}$ |

The data are the means of three independent experiments ± standard deviations (n = 3). $^{a–e}$Values referring to the same incubation time, in the same column, with different superscript letters, differ significantly ($P < 0.05$). Italics was used for the scientific taxonomic names of the bacteria used as starters for fermentation.




(caption on next page)

**Fig. 1.** Free amino acids concentrations (mg/kg) in faba bean doughs inoculated with *P. pentosaceus* VTT E-153483 (E3483), *Leuc. kimchii* VTT E-153484 (E3484), *W. cibaria* VTT E-153485 (E3485), and *W. confusa* VTT E-143403 (E3403) after 24 (A) and 48 h (B) of fermentation at 25 °C. Two not inoculated doughs, one obtained with native faba bean flour (NF) and one with irradiated flour (IF) were produced and incubated in the same conditions and used as controls. Data are the means of three independent analyses. Error bars indicating the standard deviation are represented. [a–e]Values of the same FAA having different superscript letters differ significantly ($P < 0.05$).

Fructose was present after 24 h in E3485 and E3403 (Table 3), but not anymore detectable after 48 h, while glucose was detected in IF and NF doughs until the end of fermentation (Table 3). Galactose was retrieved in all the doughs after the first 24 h of fermentation (Table 3). Stachyose, a tetrasaccharide consisting of two α-D-galactose units, one α-D-glucose unit, and one β-D-fructose unit, was found at a concentration of ca. 2.80% at the beginning of incubation. Its concentration progressively decreased below 1% in E3483 and E3485. Although a decrease was observed also in IF and NF doughs, final amounts were markedly higher than the inoculated doughs (Table 3). A similar trend was observed for the pentasaccharide verbascose (Table 3), with the lowest concentration in E3483. Raffinose amount significantly decreased during fermentation, and it was not detectable in all the inoculated doughs at 48 h, while in IF and in NF decreases of 43 and 58% were found, respectively (Table 3). At 24 h, the most efficient raffinose consumption was observed in E3484 and E3485 (Table 3).

Mannitol was found in all the doughs after 24 h of fermentation. In particular, after 24 h, the highest concentration was found in E3484 and E3485, while no significant differences were found between E3483 and E3403 (Table 3). With the exception of E3483, mannitol further increased in the following 24 h of fermentation. The highest final concentration was found in E3484. A relevant mannitol concentration was also found in NF fermented dough (Table 3).

### 3.3. Dextran production and viscosity

The preliminary screening of the ability to synthesize EPS showed that all the LAB strains produced slime in different amount on MRS agar supplemented with sucrose, but none of them produced EPS in presence of raffinose, as no sliminess or ropyness was observed. Being raffinose a substrate for levansucrase, but not for glucansucrase, dextran but not fructan synthesis was hypothesized (Semjonovs and Zikmanis, 2008).

After 24 h of fermentation, the amount of dextrans in the doughs ranged from 0.24 to 1.47% of dry weight (dw) (Table 4). The highest

**Table 4**
Dextrans concentration (expressed as % of dry weight) and viscosity (Pa/s) as determined on faba bean doughs inoculated with *P. pentosaceus* VTT E-153483 (E3483), *Leuc. kimchii* VTT E-153484 (E3484), *W. cibaria* VTT E-153485 (E3485), and *W. confusa* VTT E-143403 (E3403) after 24 and 48 h of fermentation at 25 °C. Two not inoculated doughs, one obtained with native faba bean flour (NF) and one with irradiated flour (IF) were produced and incubated in the same conditions and used as controls. Data are the means of three independent analyses. Error bars indicating the standard deviation are represented. [a–e]Values in the with different superscript letters within the same time of incubation differ significantly ($P < 0.05$).

| | | Dextrans (%) | Viscosity (Pa/s) |
|---|---|---|---|
| IF | *0* | nd | 0.17 ± 0.02$^{a}$ |
| | *24* | 0.24 ± 0.03$^{d}$ | 0.89 ± 0.02$^{c}$ |
| | *48* | 0.28 ± 0.04$^{d}$ | 0.77 ± 0.0$^{c}$ |
| NF | *0* | nd | 0.16 ± 0.04$^{a}$ |
| | *24* | 0.63 ± 0.03$^{c}$ | 0.89 ± 0.04$^{c}$ |
| | *48* | 0.66 ± 0.03$^{c}$ | 0.58 ± 0.06$^{d}$ |
| E3483 | *0* | nd | 0.17 ± 0.02$^{a}$ |
| | *24* | 0.60 ± 0.04$^{c}$ | 1.66 ± 0.03$^{b}$ |
| | *48* | 0.81 ± 0.03$^{c}$ | 2.17 ± 0.09$^{b}$ |
| E3484 | *0* | nd | 0.17 ± 0.01$^{a}$ |
| | *24* | 0.25 ± 0.03$^{d}$ | 0.39 ± 0.04$^{d}$ |
| | *48* | 0.28 ± 0.04$^{d}$ | 0.41 ± 0.09$^{d}$ |
| E3485 | *0* | nd | 0.16 ± 0.03$^{a}$ |
| | *24* | 1.01 ± 0.02$^{b}$ | 0.39 ± 0.06$^{d}$ |
| | *48* | 1.02 ± 0.05$^{b}$ | 0.46 ± 0.09$^{a}$ |
| E3403 | *0* | nd | 0.16 ± 0.04$^{a}$ |
| | *24* | 1.37 ± 0.03$^{a}$ | 6.20 ± 0.05$^{a}$ |
| | *48* | 1.70 ± 0.03$^{a}$ | 6.73 ± 0.08$^{a}$ |

The data are the means of three independent experiments ± standard deviations (n = 3). [a–d]Values referring to the same incubation time, in the same column, with different superscript letters, differ significantly ($P < 0.05$). nd: not detected. Italics was used for the scientific taxonomic names of the bacteria used as starters for fermentation.

**Table 3**
Sugars and mannitol concentration in faba bean doughs inoculated with *P. pentosaceus* VTT E-153483 (E3483), *Leuc. kimchii* VTT E-153484 (E3484), *W. cibaria* VTT E-153485 (E3485), and *W. confusa* VTT E-143403 (E3403) after 24 and 48 h of fermentation at 25 °C. Two not inoculated doughs, one obtained with native faba bean flour (NF) and one with irradiated flour (IF) were produced and incubated in the same conditions and used as controls. Data are expressed as % on dry weight.

| | | Glucose | Fructose | Galactose | Sucrose | Stachyose | Verbascose | Raffinose | Mannitol |
|---|---|---|---|---|---|---|---|---|---|
| IF | *0* | nd | nd | nd | 3.08 ± 0.12$^{a}$ | 2.84 ± 0.10$^{a}$ | 4.57 ± 0.18$^{a}$ | 0.61 ± 0.04$^{a}$ | nd |
| | *24* | 0.56 ± 0.02$^{b}$ | nd | 0.66 ± 0.03$^{b}$ | 2.94 ± 0.13$^{a}$ | 1.81 ± 0.07$^{a}$ | 2.61 ± 0.11$^{a}$ | 0.36 ± 0.02$^{a}$ | 0.12 ± 0.01$^{c}$ |
| | *48* | 0.82 ± 0.04$^{a}$ | nd | 0.91 ± 0.05$^{b}$ | 2.91 ± 0.12$^{a}$ | 1.55 ± 0.05$^{a}$ | 2.43 ± 0.12$^{a}$ | 0.34 ± 0.01$^{a}$ | 0.12 ± 0.02$^{d}$ |
| NF | *0* | nd | nd | nd | 3.03 ± 0.11$^{a}$ | 2.78 ± 0.06$^{a}$ | 4.68 ± 0.15$^{a}$ | 0.62 ± 0.04$^{a}$ | nd |
| | *24* | 0.67 ± 0.03$^{a}$ | nd | 0.82 ± 0.04$^{a}$ | 2.24 ± 0.09$^{b}$ | 1.51 ± 0.06$^{b}$ | 2.22 ± 0.09$^{b}$ | 0.36 ± 0.02$^{a}$ | 0.74 ± 0.03$^{ab}$ |
| | *48* | 0.87 ± 0.03$^{a}$ | nd | 0.99 ± 0.06$^{b}$ | 1.95 ± 0.06$^{b}$ | 1.29 ± 0.04$^{b}$ | 1.89 ± 0.07$^{b}$ | 0.26 ± 0.01$^{b}$ | 0.73 ± 0.03$^{b}$ |
| E3483 | *0* | nd | nd | nd | 3.05 ± 0.12$^{a}$ | 2.84 ± 0.09$^{a}$ | 4.61 ± 0.16$^{a}$ | 0.60 ± 0.03$^{a}$ | nd |
| | *24* | nd | nd | 0.66 ± 0.03$^{b}$ | 1.69 ± 0.07$^{c}$ | 1.63 ± 0.05$^{b}$ | 1.88 ± 0.08$^{c}$ | 0.22 ± 0.01$^{b}$ | 0.22 ± 0.02$^{c}$ |
| | *48* | nd | nd | 1.05 ± 0.06$^{b}$ | 1.65 ± 0.05$^{c}$ | 0.76 ± 0.03$^{d}$ | 0.71 ± 0.06$^{d}$ | nd | 0.24 ± 0.03$^{d}$ |
| E3484 | *0* | nd | nd | nd | 3.05 ± 0.12$^{a}$ | 2.81 ± 0.08$^{a}$ | 4.66 ± 0.16$^{a}$ | 0.61 ± 0.03$^{a}$ | nd |
| | *24* | nd | nd | 0.87 ± 0.05$^{a}$ | nd | 1.71 ± 0.06$^{ab}$ | 2.18 ± 0.08$^{b}$ | 0.14 ± 0.01$^{c}$ | 0.86 ± 0.04$^{a}$ |
| | *48* | nd | nd | 1.35 ± 0.07$^{a}$ | nd | 1.02 ± 0.05$^{c}$ | 1.19 ± 0.08$^{c}$ | nd | 1.24 ± 0.10$^{a}$ |
| E3485 | *0* | nd | nd | nd | 3.01 ± 0.11$^{a}$ | 2.83 ± 0.10$^{a}$ | 4.50 ± 0.17$^{a}$ | 0.61 ± 0.04$^{a}$ | nd |
| | *24* | nd | 1.50 ± 0.13$^{a}$ | 0.85 ± 0.04$^{a}$ | nd | 1.53 ± 0.05$^{b}$ | 2.16 ± 0.09$^{b}$ | 0.17 ± 0.01$^{c}$ | 0.63 ± 0.03$^{b}$ |
| | *48* | nd | nd | 1.01 ± 0.05$^{b}$ | nd | 0.90 ± 0.06$^{cd}$ | 1.10 ± 0.09$^{c}$ | nd | 0.99 ± 0.09$^{ab}$ |
| E3403 | *0* | nd | nd | nd | 3.00 ± 0.12$^{a}$ | 2.86 ± 0.10$^{a}$ | 4.60 ± 0.18$^{a}$ | 0.61 ± 0.04$^{a}$ | nd |
| | *24* | nd | 1.28 ± 0.10$^{ab}$ | 0.62 ± 0.03$^{b}$ | nd | 1.73 ± 0.07$^{ab}$ | 2.15 ± 0.08$^{b}$ | 0.29 ± 0.03$^{b}$ | 0.21 ± 0.02$^{c}$ |
| | *48* | nd | nd | 0.99 ± 0.05$^{b}$ | nd | 1.02 ± 0.05$^{c}$ | 1.12 ± 0.10$^{c}$ | nd | 0.52 ± 0.03$^{c}$ |

The data are the means of three independent experiments ± standard deviations (n = 3). [a–d]Values referring to the same incubation time, in the same column, with different superscript letters, differ significantly ($P < 0.05$). nd: not detected. Italics was used for the scientific taxonomic names of the bacteria used as starters for fermentation.

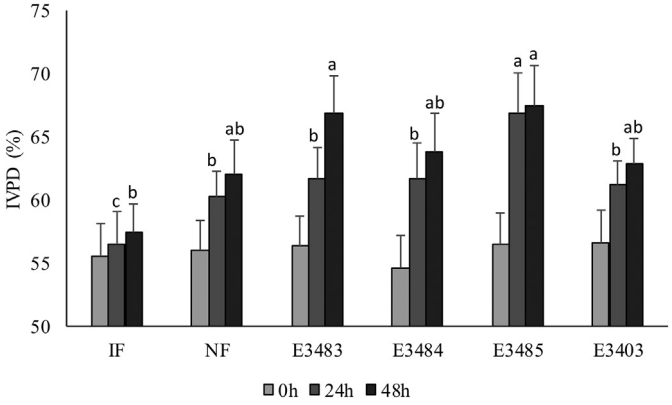



**Fig. 2.** In vitro protein digestibility (IVPD) determined on freeze dried faba bean doughs before and after 24 and 48 h of fermentation. IF, irradiated faba bean flour; NF, native faba bean flour; E3483: *Pediococcus pentosaceus* VTT E-153483 (E3483), *Leuc. kimchii* VTT E-153484 (E3484), *W. cibaria* VTT E-153485 (E3485), and *W. confusa* VTT E-143403 (E3403). Data are the means of three independent analyses. Error bars indicating the standard deviation are represented. $^{a-c}$Values in the with different superscript letters within the same time of incubation differ significantly ($P < 0.05$).

amount was found in the doughs inoculated with *W. cibaria* and *W. confusa*, containing > 1% (of dw). In the following 24 h the amount further increased in E3483 (of ca. 24%), and E3403, while it remained stable in all the other cases.

Viscosity of the doughs increased during incubation and, after 24 h, it was similar for the two control doughs IF and NF. Compared to those, viscosity of E3484 and E3485 was significantly lower, while a viscosity ca. 7 fold higher was found in E3403. E3403 had the highest viscosity also at 48 h, followed by E3483, having a value ca. 3 fold lower (Table 4).

### 3.4. In vitro protein digestibility

The protein digestibility was determined on faba bean doughs through a protocol mimicking the digestion in the gastrointestinal tract (Akeson and Stahmann, 1964). Prior to fermentation IVPD values ranged from 54.54 to 56.57%, with no difference among the controls and the inoculated samples. A slight but not significant increase was observed in IF dough during incubation, while IVPD significantly increased (from 7 to 18%) during the first 24 h of fermentation in all the other samples (Fig. 2). Compared to t0, at the end of 48 h of incubation, increases of 10–19% were found in the inoculated doughs. A significant increase was also observed in NF during incubation, although the final value was lower, but not significantly, than those of the inoculated doughs (Fig. 2).

### 3.5. Total phenols and antioxidant activity

Prior fermentation, doughs contained ca. 1.57 mmol/kg of total phenols. With the only exception of IF dough, the extracted total phenols increased during incubation (Fig. 3A). E3485 was characterized by the highest increase of total phenols in the first 24 h and a final concentration of 3.19 mmol/kg (Fig. 3A). Total phenols further increased in E3484 and E3403 after 48 h showing ca. 3.20 mmol/kg, while no significant differences were found for E3485. As shown in Fig. 3B, there were no significant differences in the antioxidant activity among the doughs before fermentation, although relevant DPPH scavenging activity was detected (81–83%). Proportionally to the phenols concentration, the antioxidant activity increased during fermentation. Increases up to 14% were observed for the inoculated doughs, especially those fermented with *Leuc. kimchii* VTT E-153484, *W. cibaria* VTT E-153485 (Fig. 3B).

### 3.6. Phytase activity and phytic acid concentration

A weak phytase activity (Fig. 4A) was found in faba bean doughs before and after 24 h of fermentation. The activity was > 10 times higher in E3483 and E3484 after 24 h of fermentation. A significant increase was observed in E3485 and E3403, only after 48 h of fermentation. An opposite trend was shown in E3483 and E3484, in which decreases up to 36% were found after 48 h (Fig. 4A). The concentration of phytic acid in doughs was ca. 0.40 g/100 g before fermentation. With the exception of IF, phytic acid content decreased during fermentation. The most efficient degradation was observed in E3483 and E3484, having final values of ca. 0.18 g/100 g of phytic acid. Significantly higher concentrations were found in NF doughs compared to all the inoculated doughs (Fig. 4B).

### 3.7. Principal component analysis

Data collected from the nutritional characterization of faba bean doughs were subjected to principal component analysis (PCA) as showed in Fig. 5. The first and second factors explained, respectively, the 66.61 and 18.88% of the total variance. Factor 1 clearly separated the inoculated from the control doughs. Inoculated doughs shared several features (lower amount of phytic acid and raffinose while higher content in FAA, and lactic acid with an increase in protein digestibility) and were grouped in the left area of the plot. The difference between NF and IF is explained by the activity of endogenous microbiota which contributed, in NF dough, to the higher synthesis of organic acids and proteolysis compared to IF. A partial reduction of phytic acid and oligosaccharides was observed in the controls, slightly higher for NF than IF. Among the inoculated doughs, E3484 and E3485 were very similar, especially for the amount of lactic acid produced and the high antioxidant activity. Whereas, dough fermented with *W. confusa* VTT E-143403, placed on the bottom left area of the plot, stood out for the highest proteolytic activity and a more balanced ratio of organic acids. E3403 dough also showed the highest viscosity. *P. pentosaceus* VTT E-153483 was located in the middle of the left area because of its discrete ability to influence viscosity and highest lactic acid production. This analysis suggested that the use of *W. confusa* VTT E-143403 represents the best compromise between nutritional and technological properties, with a relevant increase of antioxidant activity.

## 4. Discussion

The growing importance of legumes as food ingredient and the confirmed advantages of fermentation, as an affordable method for improving nutritional and nutraceutical properties require the set-up novel biotechnological protocols for legume bioprocessing applied in the industrial scale.

Recent studies revealed that LAB fermentation of different legumes improved the protein digestibility, the mineral and phenols bioavailability, leading to the partial or complete degradation of the anti-nutritional factors α-galactosides, tannins, phytic acid and trypsin inhibitors and pyrimidine glucosides causing favism (vicine, convicine and its derivatives) (Bartkiene et al., 2014; Chandra-Hioe et al., 2016; Coda et al., 2015; Rizzello et al., 2016a). Another area of growing interest concerning legume flours is the synthesis in situ of EPS such as dextrans, which have shown the ability to modify the rheological properties of different fermented legumes, promoting their usability for several food applications (Xu et al., 2017a, 2017b). Additionally, the nutritional advantages of the fortification of cereal-based foods with fermented faba bean as ingredient (e.g. bread and pasta) were also demonstrated (Coda et al., 2017a, 2017b; Rizzello et al., 2017). For all these reasons, the evaluation of the metabolic and pro-technological performances of LAB strains potentially employable as starters for faba bean bioprocessing is crucially important in order to find the best candidates for food and feed applications. A list of the main

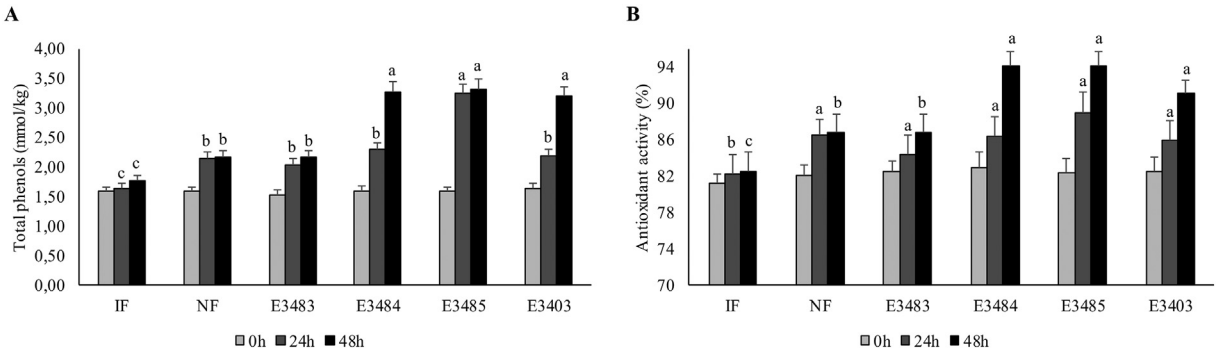



**Fig. 3.** Total phenols concentration (A) expressed as mmol of gallic acid equivalents/kg and antioxidant activity (B) expressed as DPPH radical scavenging activity (%) determined on methanolic extracts from freeze-dried faba bean doughs inoculated with *P. pentosaceus* VTT E-153483 (E3483), *Leuc. kimchii* VTT E-153484 (E3484), *W. cibaria* VTT E-153485 (E3485), and *W. confusa* VTT E-143403 (E3403) after 24 (A) and 48 h (B) of fermentation at 25 °C. Two not inoculated doughs, one obtained with native faba bean flour (NF) and one with irradiated flour (IF) were produced and incubated in the same conditions and used as controls. Data are the means of three independent analyses. Error bars indicating the standard deviation are represented. [a–c]Values in the with different superscript letters within the same time of incubation differ significantly ($P < 0.05$).

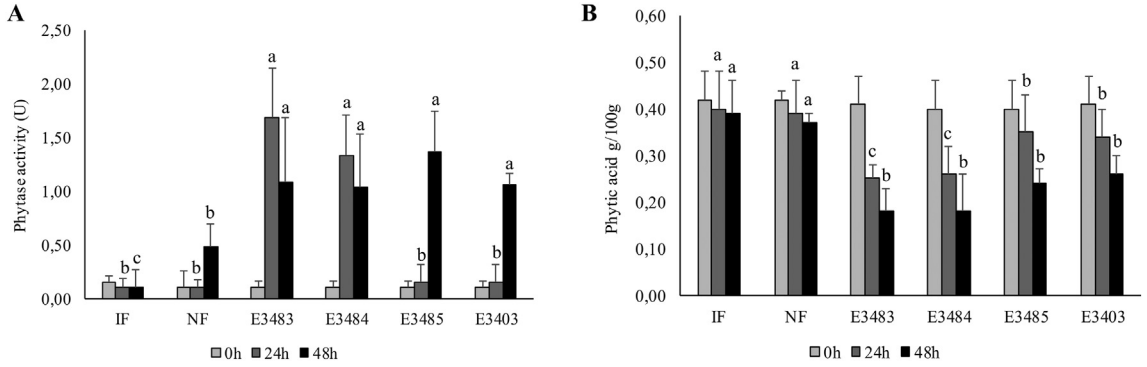



**Fig. 4.** Phytase activity (A) and phytic acid concentration (B) in freeze-dried faba bean doughs inoculated with *P. pentosaceus* VTT E-153483 (E3483), *Leuc. kimchii* VTT E-153484 (E3484), *W. cibaria* VTT E-153485 (E3485), and *W. confusa* VTT E-143403 (E3403) after 24 (A) and 48 h (B) of fermentation at 25 °C. Two not inoculated doughs, one obtained with native faba bean flour (NF) and one with irradiated flour (IF) were produced and incubated in the same conditions and used as controls. One unit (U) of phytase activity was defined as the amount of enzyme required to release 1 μmol of phosphate from Na-phytate per min under the assay conditions. Data are the means of three independent analyses. Error bars indicating the standard deviation are represented. [a–c]Values in the with different superscript letters within the same time of incubation differ significantly ($P < 0.05$).

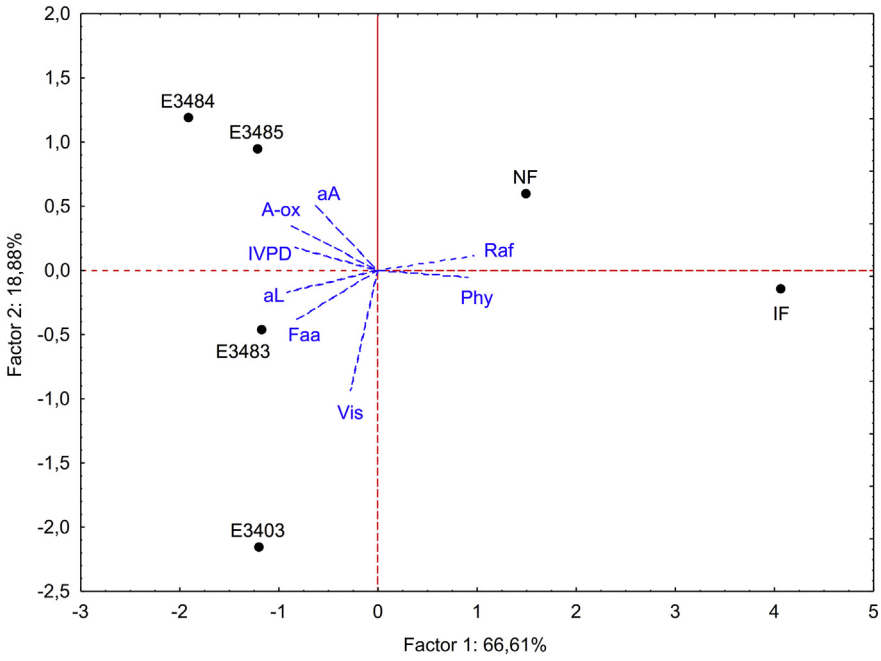



**Fig. 5.** Principal component analysis (PCA) on the basis of the biochemical and nutritional characteristics of faba bean doughs inoculated with *P. pentosaceus* VTT E-153483 (E3483), *Leuc. kimchii* VTT E-153484 (E3484), *W. cibaria* VTT E-153485 (E3485), and *W. confusa* VTT E-143403 (E3403) after 48 h of fermentation at 25 °C. Two not inoculated doughs, one obtained with native faba bean flour (NF) and one with irradiated flour (IF) were produced and incubated in the same conditions and used as controls. aL: lactic acid concentration; aA: acetic acid concentration; Faa: free amino acids; IVPD: in vitro protein digestibility; Aox: antioxidant activity; Vis: viscosity; Raf: raffinose consumption; Phy: phytic acid concentration.

characteristics of the lactic acid bacteria considered in this study is reported in Table 1S.

Fermentation process can rely on the microbiota characterizing raw materials and the environment or can be controlled by inoculation of selected, well-characterized starters. Several research outcomes have shown that the LAB microbiota associated with the food matrix to be processed represents an optimal source for potential starter cultures, thanks to the faster adaptation to the specific matrix, and the marked influence on the nutritional and technological properties (Corbo et al., 2017; Filannino et al., 2018; Sáez et al., 2017).

In a former study the LAB previously found to be dominant in spontaneously fermented faba bean doughs mainly belonged to *Pediococcus* and *Leuconostoc* genera and, to a lesser extent, to *Weissella*, *Enterococcus*, and *Lactobacillus* spp. (Coda et al., 2017b). In the present study, four strains, belonging to *P. pentosaceus*, *Leuc. kimchii*, *W. cibaria* and *W. confusa*, originating from faba bean, were applied as starters for fermentation and compared based on characteristics of great interest for the development of specific faba bioprocessing protocols. The four strains are able to produce EPS of the glucan type, most probably dextran. Dextran synthesis was largely reported for LAB strains belonging to *P. pentosaceus* (Shukla and Goyal, 2013), *Leuc. kimchii* (Schleifer, 2009), *W. cibaria* and *W. confusa* (Galle et al., 2010; Xu et al., 2017a). Based on previous observation on EPS synthesis in situ (Kajala et al., 2016), fermentation was carried out at 25 °C. The sub-optimal temperature for LAB growth was chosen to avoid excessive acidification and, as consequence, potential negative repercussions on the dextransucrase activity.

All the starters used in this study showed a growth higher than 2 log cycles during the 48 h of fermentation exceeding, with the exception of *P. pentosaceus* VTT E-153483, a cell density of 9 log cfu/g. A markedly lower LAB cell density was observed in spontaneously fermented dough obtained with irradiated flour, while native, untreated flour harboured a population of ca. 8 log cfu/g at the end of the incubation. In agreement with the previous findings (Coda et al., 2017b), yeasts were at low cellular density before and after fermentation, in all the samples.

The *Weissella* spp. strains showed the shortest latency phase and the lowest Vmax, while *P. pentosaceus* VTT E-153483 and *Leuc. kimchii* VTT E-153484 showed the highest variation of pH and production of lactic acid. *P. pentosaceus* is a homofermentative species, and its use as starter led to the highest FQ. The marginal concentration of acetic acid found in E3483, lower than that found in the spontaneous fermented control, could be related to the contribution of the indigenous microbiota. Doughs fermented with *Leuc. kimchii* VTT E-153484 had the lowest FQ, due to the relevant amount of acetic acid produced, most likely due to the reduction of fructose to mannitol, as previously reported for *Leuconostoc* spp. (Wisselink et al., 2002). Relevant acetic acid concentration was also observed in E3485, also in this case together with mannitol release. Overall, most *Weissella* spp. are not able to reduce fructose to mannitol (Gänzle, 2015), nevertheless, some *W. cibaria* strains are able to produce mannitol from sucrose (Galle et al., 2010). Indeed, the mannitol production has already been reported as both strain- and substrate-dependent (Galle et al., 2010). The ratio between lactic and acetic acid can positively influence sensorial properties of the final product, therefore a proper FQ is considered an important parameter for starters selection (Gänzle, 2015).

Sucrose was the most abundant disaccharide in faba doughs and enabled the formation of EPS. Theoretically, sucrose can be first utilized for microbial growth during the exponential phase, then for EPS production during the stationary phase (Plante and Shriver, 1998). Residual fructose was found at concentration higher than 1% in the two doughs fermented with *Weissella* spp. This is in agreement with previous studies on faba bean showing that, through the activity of glucansucrase on sucrose, glucan is formed, while fructose is released (Xu et al., 2017b). Faba bean contains relevant concentrations of the raffinose family oligosaccharides (RFO), the α-galactosides raffinose, verbascose, and stachyose which can act as antinutritional factor (Teixeira et al., 2012). RFO are not degraded in the gastrointestinal tract due to the lack of α–galactosidase (α-Gal) activity, and, despite favouring the metabolism of beneficial intestinal microorganisms at low concentration, they can be fermented in the large intestine by the intestinal microbiota, causing gastrointestinal symptoms (Teixeira et al., 2012). Fermentation can efficiently lead to raffinose elimination in different legumes including faba bean (Xu et al., 2017a, 2017b). The possibility to degrade RFO during fermentation is an important selection criteria for faba bean starters (Connes et al., 2004). Under the conditions of this study, all the fermented doughs showed a decrease of RFO content. Previously Xu et al. (2017b) showed the role of endogenous α-Gal in RFO degradation in faba bean flour. Therefore, we can hypothesize a combined activity of the endogenous and microbial enzymatic activity. As the consequence of the RFO hydrolysis, galactose, was detected after the first 24 h of fermentation. Stachyose progressively decreased during fermentation, especially in the inoculated doughs. Verbascose followed a similar trend, nevertheless *P. pentosaceus* VTT E-153483 caused intensive degradation.

The accumulation of raffinose was previously observed and correlated to the partial hydrolysis of verbascose and stachyose during faba bean fermentation (Coda et al., 2015, 2017b; Teixeira et al., 2012). It is hypothesizable that the longer fermentation time applied in this study favoured intensive degradation of raffinose. The capability to use raffinose is not widespread among LAB. However, it has been reported that in *P. pentosaceus* the fermentation of some sugars, including raffinose, is plasmid encoded and the presence of α-Gal is inducible (Gonzalez and Kunka, 1986). Furthermore, *Weissella* spp. strains able to use raffinose were previously characterized (Verni et al., 2017).

Dextran concentration in doughs fermented by *Weissella* strains was notably increased, ranging from 1.02 to 1.70% and relatively close to the theoretical amount expected (1.5%) based on the presence of native sucrose. Theoretically, the total content of residual fructose, liberated from sucrose, and mannitol, should be roughly equal to the content of glucan formed. In our conditions, the content of glucan was lower in E3483 and E3485. Probable reasons for this could be the use of sucrose-liberated glucose for growth and other metabolic activities, and the formation of oligosaccharides by glucansucrase through acceptor reaction, as reported earlier (Xu et al., 2017b). Despite the relative similarity in dextran content, a very different rheological behaviour was found for E3485 and E3403, since the latter showed a markedly higher viscosity. The different thickening ability of EPS might be due to the structure and molecular weight of the dextrans produced by the two strains and, possibly, by the different interactions between the other faba bean components (e.g. starch and proteins) (Xu et al., 2017a, 2018).

Together with acidification, proteolysis is considered a LAB key-feature in food biotechnology, since the degradation of native proteins is of great importance for improving the digestibility of polypeptides and bioavailability of free amino acids, but also for the release of potential bioactive peptides (Rizzello et al., 2016b) and for the development of taste and flavor (Leroy and De Vuys, 2004). A discrete contribution to the proteolysis was given by the presence of endogenous enzymes, responsible for the increase of peptides and amino acids (ca. 16%) occurring during the first 24 h of incubation of the dough obtained with radiated flour, having the lowest microbial cell density among the samples. The use of LAB led to a more intense proteolysis; doughs fermented with *W. cibaria* VTT E-153485 and *W. confusa* VTT E-143403 showed the highest peptide release. The use of *W. confusa* VTT E-143403 also induced the highest release of FAA. In previous studies, *Weissella* spp. strains isolated from faba bean displayed high endopeptidase activity (Verni et al., 2017). The final TFAA in faba bean doughs resulted markedly higher than what commonly found in cereals (Lattanzi et al., 2013; Verni et al., 2017). Moreover, the fermentation with *Leuc. kimchii* VTT E-153484 and *W. confusa* VTT E-143403 increased the concentration of free Met and Cys, the limiting amino acids of faba bean (Multari et al., 2015).

In agreement with the proteolysis driven by the starters, the IVPD values at the end of the fermentation were significantly higher than those of the control doughs. The partial degradation of the proteins makes the sequences more susceptible to further degradation by the digestive enzymes (Rizzello et al., 2016b). *W. cibaria* VTT E-153485, able to cause the highest release of peptides, led to the highest increase of IVPD in the first 24 h of fermentation.

The interest for the presence of antioxidant compounds in foods has increased, based on the recognized role in the prevention mechanisms of the oxidative stresses (Adebiyi et al., 2009) and to the ability to prolong the shelf-life, through the inhibition of the oxidative processes (Shahidi and Ambigaipalan, 2015). The antioxidant activity found in faba doughs was markedly higher than that found for cereal doughs, mainly thanks to the high total phenols content (El-mergawi and Taie, 2014). Overall, significant increases of the antioxidant activity were observed in inoculated doughs, with the only exception of that fermented by *P. pentosaceus* VTT E-153483, having the same activity of the spontaneously fermented NF. It was reported that *P. pentosaceus* is particularly efficient to degrade phenol compounds, for example through decarboxylases, leading to a loss of antioxidant activity (Rodríguez et al., 2009). Nevertheless, the contribution to the antioxidant activity provided by the high concentration of peptides and FAA found in inoculated samples, should be considered (Rizzello et al., 2016b).

Phytic acid is widely present in legume flours where it acts as mineral chelator (Campos-Vega et al., 2010) also reducing the digestibility of protein, starch, and lipids (Frias et al., 2003). Phytase activity, catalyzing the hydrolysis of phytic acid, is described as lower in legumes than in cereals (Frias et al., 2003; Steiner et al., 2007). Overall, acidification activates cereal endogenous phytases due to more suitable values of pH, however, it was reported that the acidification conditions did not improve the activity of the legume endogenous phytases (Coda et al., 2015; Gustafsson and Sandberg, 1995). For this reason, the contribution of microbial phytases to the degradation of phytic acid in faba bean is highly relevant. LAB possess phytase activity to a certain extent, largely investigated in previous studies (De Angelis et al., 2003; Zotta et al., 2007). Among the faba bean LAB characterized, *P. pentosaceus* VTT E-153483 and *Leuc. kimchii* VTT E-153484 caused a significant degradation of phytic acid already during the first 24 h of fermentation.

All strains showed a proper aptitude (growth, acidification and proteolysis performances) to be applied as starters for faba bean bioprocessing. Investigation of the metabolic differences highlighted crucial information for the choice of the suitable starter strains (to be used as a single or mixed cultures) aiming at obtaining specific biotechnological targets to overcome issues regarding the use of faba bean in human nutrition. Overall, *W. confusa* VTT E-143403 showed the best combination among the characteristics tested (optimal fermentation quotient, high amino acids release and dextran synthesis/texture modifying potential, intense phytase and antioxidant activities).

### 4.1. Future prospective

One of the reasons of the rising demand for healthier plant-based food is the increasing awareness of the adverse risks associated with the consumption of animal proteins. Among legumes, faba bean has great potential as functional ingredient. However, despite its positive impact on human health and agroecosystem, faba bean has been underutilized for decades mainly due to the presence of ANF that interfere with nutrient absorption and sometimes cause pathologic conditions. A broad variety of methods were effective in their reduction and some of the strategies proposed resulted in an efficient and affordable way to diminish, and in some cases completely eliminate, ANF. The efficiency of fermentation and its impact on the sensory and technology properties (beside nutritional aspects) strongly depends on the pro-technological and metabolic traits of the starters used, as described by an increasing number of scientific reports on faba bean bioprocessing. The incorporation of faba bean, whether raw or prior treatments, in cereal-based food formulas has been studied over the years. When added as raw flour, it was mostly responsible of increasing the protein content of the composite product, but technological properties were negatively affected and ANF increased. On the contrary, the fortification of bread, pasta, extruded snacks and gluten-free products with fermented faba bean flour allowed an overall improvement of the nutritional, functional and technological qualities. On the basis of the promising results already obtained, a significant increase of the use fermented faba bean in food industry is expected.

## Conflict of interest

The authors declare no conflict of interest.

## Funding

This research has been developed under the European Project “BIOPROT – Novel multifunctional plant protein ingredients with bioprocessing” (FP7-ERA-Net - SUSFOOD).

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2018.08.014.

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