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DEVELOPMENT OF NEW SYSTEMS OF BIO-PRESERVATION FOR THE SAFETY AND SHELF-LIFE OF FOOD

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State of art

To assure food quality is necessary to have a quality production of the food, and this could be seen as the sum of all the choices that are needed to take to have a genuine and well-conservable product output (appreciated by the consumer and with good nutritional profile).

The quality concept is crossed to the healthy one, taking part of the food safety. About the quality food approach, nowadays, several sectors of the food industry especially the fermentative one, can rely on microbial starter cultures whom application in different production processes allows to maintain, through a controlled fermentation, both quality product standards and risk control. However, the availability of a few starter cultures, which are adapted to different production processes, has brought to products with poor sensorial fingerprinting.

Furthermore, the microbial associations available today do not include Non-Starter Microorganisms, also known as adjunct cultures that in several fermentation processes play the major role in producing peculiar sensory features.

The implementation of existing processes and the development of new products and new process technologies need new "high performance" strains, in order to satisfy the request of the process and quality, respecting the sustainability (less working waste, by product recycling). Moreover, the microorganisms that play a central role in obtaining fermented foods can be excellent vehicles to confer beneficial effects to the consumer's health, through their use as efficient "cell factories" to bring functional biomolecules.

Fungal threat to Food safety

Food safety is one of major challenge for food science. Many diseases find the etiology in the biological contaminated food (Painter *et al.* 2013). For these reason, the public attention has been focused in improving food safety, and more generally the whole quality of food. Cereal derived products are one of the most consumed staple food worldwide (USDA 2018), and it has been like this for millennia. However, abiotic and biotic stresses are very common in cereal production from cultivation to final food. Contamination of filamentous toxigenic and/or spoilage fungi is a contingent issue especially in this field, where humid and warm environmental conditions are extremely fitting for the mold development. Conidio-spores of fungi are ubiquitous in the biosphere and are dispersed by air unless contamination is controlled by clean room technology (Denyer *et al.* 2004).

Bakery products are easily colonized by fungal spores from diverse genera including *Aspergillus*, *Cladosporium*, *Endomyces*, *Fusarium*, *Monilia*, *Mucor*, *Penicillium*, and *Rhizopus* (Dal Bello *et al.* 2007). If on one hand the economic losses are dramatically consistent, (Axel, Zannini and Arendt 2017), on the other hand public health is fully involved. Fungal contaminants of crops are responsible of the synthesis and accumulation of toxic secondary metabolites, known as mycotoxins. There are several classes of mycotoxins, among which aflatoxin B1 and fumosin B are the most dangerous ones. Aflatoxin B1 is produced by *Aspergillus flavus* and *A. parasiticus*, the genus *Fusarium* is known for the synthesis of different mycotoxins among which deoxynivalenol, produced by *F. graminearum* and fumonisin B1 produced by *F. verticillioides*, well adapted in maize crop (Deepa and Sreenivasa 2017). This constitutes a relevant health problem in countries where maize is the staple food (Janse van Rensburg *et al.* 2015). A lack of attention in the application of good agricultural practices is the main reason for accumulation of mycotoxins in raw materials during harvest and storage (Neme and Mohammed 2017). These toxins, through the production chain, can easily come to the final consumer, with great health risks.

Lactic acid bacteria as bio-control agents

With the aim of improving the healthiness and quality of food, in the last decade, a great attention has been paid to the possibility of adopting bio-control strategies, through a targeted use of food grade microorganisms capable of enhancing the nutritional quality and controlling the development of pathogenic microorganisms along the whole chain, without interfering with the process itself (Dalié, Deschamps and Richard-Forget 2010). This approach should reduce not only the growth of undesirable microorganisms and the amount of their toxic metabolites, but also decrease substantially the use of chemicals and preservatives (towards which, among other substances, several microorganisms have acquired resistance mechanisms). Microbial fermentation is one of the oldest and ecologically friendly methods of preserving foods. LAB are known for their ability to produce not only discrete amount of different organic acids, but also other active compounds with demonstrated antifungal activity (Quattrini et al., 2018).

Recently, through extensive programs of screening, new LAB biotypes, isolated from different ecological niches and food matrices, have been studied. Some of these strains, showing high performance and competitiveness, are used as probiotics for the development of new food formulations with functional characteristics (Batista *et al.* 2017) and for the control of the growth of undesirable microorganisms. It refers, for example, to the antifungal properties of strains of *Lactococcus* spp. and *Lactobacillu*s spp. (Crowley and Mahony 2013; Stoyanova, Ustyugova and Netrusov 2012), and to another promising bio-control strategy, linked to the ability of selected strains to interact with preformed mycotoxins, reducing the bioavailability of the toxic compounds (Haskard *et al.* 2001; Peltonen *et al.* 2001; Zhu *et al.* 2016). Investigations about the ability of LAB to remove mycotoxins suggested that binding to cell wall components is more likely to be the mechanism of removal, rather than metabolic processes. (Dalié, Deschamps and Richard-Forget 2010;Shetty 2006). Recent studies, in dairy sector mainly, have shown that selected probiotic strains are able to remove aflatoxin from contaminated milk and during yogurt production and storage (Elsanhoty *et al.* 2014; Sarlak *et al.* 2017;Serrano-niño *et al.* 2013). Studies regarding the use of probiotic strains as detoxification agents in other food sectors are still limited. Among *Lactobacillus* species, different strains of *L. plantarum, L. reuteri, L. sanfranciscensis, L. coryniformis* have been characterized for antifungal properties and different antimold metabolites have been identified and applied in food products, e.g. cyclic dipeptides, organic acids, phenyllactic acid, propionic acid, ferulic acid (Dalié, Deschamps and Richard-Forget 2010).

Bran valorisation

Cereal bran valorisation has, for many reasons, a noticeable interest. By-products generated by cereal agro-industrial sector are rich in dietary fibre, minerals and bioactive compounds and could represent beneficial ingredients for human nutrition. The main reasons behind the low utilization of native brans as ingredient for cereal-based products are due to their poor safety (heavy metal and mycotoxins), technological (increased dough stickiness, reduced volume) and sensory qualities (dark colour, taste, chewy/hard texture) (Rose *et al.* 2010; Heinio *et al.* 2016). The spontaneous sourdough-like fermentation process, characterized by a consortium of yeasts and lactic acid bacteria (LAB),

has shown to be an interesting pre-treatment in order to ameliorate sensory and textural quality, as well as to improve the microbial safety of bran (Dalié, Deschamps and Richard-Forget 2010; (Manini *et al.* 2011; Messia *et al.* 2016). Through fermentation, an increased bioavailability of bioactive compounds and a decreased level of some anti-nutritional compounds can be obtained (Katina *et al.* 2012); Manini *et al.* 2011). To date, the majority of published literature in this field regards fermented wheat bran. Little is known about the effects of this "bio-approach" on properties of others cereal, as maize. This cereal and its by-products could represent attractive alternative raw materials for fibre enrichment of gluten-free products. According to A.I.R.E.S (Associazione Italiana Essiccatori Raccoglitori Stoccatori di Cereali e Semi oleosi, http://www.micotossine.it/), about 82% of maize production is used for animal feed and only 18% is devoted to human consumption. At the milling stage, dry maize mechanical processing creates whole or fractionated products, accounting about for up to 25%, generally used as animal feed or biofuel. In this context, the valorisation of maize by-products as valuable food ingredients to be exploited in the production of new food formulation could represent an opportunity. Ferulic acid is diffused in cereals and maize bran appears as one of the richer sources, containing about 26–33 g/kg (Zhao and Moghadasian 2008). Moreover, maize bran contains hemicellulose, cellulose and a small amount of lignin, thus representing a source of dietary fibre. The hemi-cellulosic fraction, generally denominated arabinoxylans (AX), has a particular heterogeneous nature, with extensive cross-linkages between ferulic acid and arabinose/xylose hetero-polysaccharides. This particular feature promoted a complex and rigid structure of maize cell wall (Rose *et al.* 2010). The potential of native microorganisms and their enzymatic activities of increasing the fraction of soluble dietary fibres and the content of free ferulic acid deserves to be tested. Recently the EFSA Panel published a positive Opinion about the request of a Health Claim related to the positive effects on the post-prandial glycemic and insulinemic responses elicited by water-extractable arabinoxylans (WEAX) (EFSA Panel on Dietetic Products 2011). This Opinion was founded on results from different studies reporting that this fraction can delay the rate of carbohydrates digestion/absorption, thus positively affecting glucose metabolism (EFSA Panel on Dietetic Products 2011; Ai and Jane 2016). Moreover, it is almost recognized in literature that arabino-xylanoligosaccharides (AXOS) and xylan-oligosaccharides (XOS) obtained by the hydrolysis of arabinoxylans present in brans can exert prebiotic properties (Broekaert *et al.* 2011). Very little is known about the autochthonous of LAB and yeast characterizing this by-product. The spontaneous fermentation could allow to isolate and identify potential functional strains (of both LAB and yeast) in order to be applied in guided fermentations to improve the bran and allow a possible re-use in food industry.

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Aim of the project

The aim of the project was the selection and application of new biotypes of Lactic Acid Bacteria (LAB), to be used in the fermented food production, in order to improve food nutritional, safety and functional quality. To achieve the research goal, molecular profiling approaches, genomic and post-genomic techniques, mass spectrometry were combined with the development of extended screening programs to a significant number of LAB biotypes. The biotypes were identified and studied for characteristics of bio-control and non-conventional functional properties, to be exploited mainly in the cereal and bakery sector.

Chapter 1. Functional characterization of Lactobacillus plantarum ITEM 17215: a potential biocontrol agent of fungi with plant growth promoting traits, able to enhance the nutritional value of cereal products

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This chapter is a functional characterization of strains belonging to *Lactobacillus plantarum* species, in order to develop strategies of biocontrol in cereal products.

ABSTRACT

In this work, we explored the potential of 25 *Lactobacillus plantarum* strains isolated from cereals and milk-based products, testing characteristics related to antifungal activity and to nutritional quality. The tested strains demonstrated interesting beneficial traits, such as the ability to utilize fructo-oligosaccharides, prebiotic substances that help probiotic microorganisms to grow in the human gut, and to reduce phytate, an antinutrient present in cereal sector. Regarding mould inhibition, we highlighted the ability of the strains to inhibit *Penicillium roqueforti, Mucor circinelloides* and mycotoxinogenic moulds associated with cereal grains as Aspergillus *flavus, A. niger, Fusarium verticillioides*. Moreover, a moderate reduction of the bioavailability of aflatoxin AFB1 was detected. The selected *L. plantarum* strain ITEM 17215, showed a strong inhibitory ability towards fungal growth and was able to produce 1,2-dihydroxybenzene, benzoic acid, p-hydroxyphenyllactic acid and 3-phenyllactic acid. The latter compound, already described as efficient antifungal inhibitor, was the most abundant and its concentration was further increased by adding phenylalanine and phenylpyruvic acid in the growth medium. The metabolites produced by strain ITEM 17215 could also be related to the ability of the strain to induce cereal germination and promote plant growth. This aspect, not yet investigated in *L. plantarum,* could have interesting applications in the agro-food sector.

Keywords: Lactobacillus plantarum, antifungal activity, 3-phenyllactic acid, aflatoxin, plant-growth promoting activity, phytate reduction, cereal sector

1. Introduction

With the aim of improving the healthiness and quality of food, in the last decade, a great attention has been paid to the possibility of adopting bio-control strategies, through a targeted use of food grade microorganisms capable of enhancing the nutritional quality and controlling the development of pathogenic microorganisms along the whole chain, without interfering with the process itself (Dalié, Deschamps, & Richard-Forget, 2010). This approach should reduce not only the growth of undesirable microorganisms and the amount of their toxic metabolites but should also reduce substantially the use of chemicals and preservatives (towards which, among other substances, several microorganisms have acquired resistance properties).

In fermented foods, Lactic Acid Bacteria (LAB) are the main protagonists, both in terms of use and commercial value. Recently, through extensive programs of screening, new LAB biotypes, isolated from different ecological niches and food matrices, have been studied. Some of these strains, showing high performance and competitiveness, are used as probiotics (Hill et al., 2014) for the development of new food formulations with functional characteristics (Batista et al., 2017; Lollo et al., 2015) and for the control of the growth of undesirable microorganisms. It refers, for example, to the antifungal properties of strains of *Lactococcus* spp. and *Lactobacillus* spp. (Crowley, Mahony, & van Sinderen, 2013; Stoyanova, Ustyugova, & Netrusov, 2012), and to another promising bio-control strategy, linked to the ability of selected strains to interact with preformed mycotoxins, reducing the

bioavailability of the toxic compounds (Bovo Campagnollo et al., 2016; El-Nezami, Kankaanpa, Salminen, & Ahokas, 1998; Meca, Ritieni, & Mañes, 2012; Peltonen, El-Nezani, Haskard, Ahokas, & Salminen, 2001; Zhu, Hassan, Watts, & Zhou, 2016). Investigations about the ability of LAB to remove mycotoxins suggested that binding to cell wall components is more likely to be the mechanism of removal, rather than metabolic processes. (Dalié et al., 2010; Shetty & Jerspersen, 2006). Recent studies, in dairy sector mainly, have shown that selected probiotic strains are able to remove aflatoxin from contaminated milk and during yoghurt production and storage (Elsanhoty, Salam, Ramadan, & Badr, 2014; Sarlak et al., 2017; Serrano-Niño et al., 2013). Studies regarding the use of probiotic strains as detoxification agents in other food sectors are still limited.

A major issue in the cereal sector was and still is the deterioration of the raw material by filamentous fungi. The fungal growth causes not only significant economic losses but also has important implications for food safety. Indeed, fungal contamination can cause loss of a whole grain stock, as well as an accumulation of mycotoxins, toxic secondary metabolites of which synthesis is favoured at high temperatures and humid conditions (Ahlberg, Joutsjoki, & Korhonen, 2015; Błajet-Kosicka, Twarużek, Kosicki, Sibiorowska, & Grajewski, 2014; Neme & Mohammed, 2017). Among mycotoxins, aflatoxins (AF) and fumonisins (F) are one of the most dangerous mycotoxin found in cereals. The aflatoxin AFB1, produced by *Aspergillus flavus* and *A. parasiticus*, is the most carcinogenic of the natural occurring aflatoxins (Strosnider et al., 2006). The genus *Fusarium* is known for the ability of synthesizing several mycotoxins, among which deoxynivalenol, produced by *F. graminearum* (Fung & Clark, 2004), and fumonisin B1 produced by *F. verticillioides* frequently found in cereals, particularly in maize (Deepa & Sreenivasa, 2017). This constitutes a relevant health problem in countries where maize is the staple food (van Rensburg, McLaren, Flett, & Schoeman, 2015). A lack of attention in the application of good agricultural practices is the main reason for accumulation of mycotoxins in raw materials during harvest and storage (Neme & Mohammed, 2017). These toxins, through the production chain, can easily come to the final consumer, with great health risks.

Another aspect related to cereal consumption is the presence of anti-nutritional factors. Among these factors, phytate is the most important, because it has strong ability to complex multi-charged metal ions (Coulibaly, Kouakou, & Chen, 2011).

Multiple publications have highlighted the positive properties of LAB strains in cereal sector. Within LAB group, *Lactobacillus plantarum* is considered one of the most suitable protective microorganisms in cereal production chain. *L. plantarum* is a versatile and competitive species, adapting to different environmental conditions. These features can explain its presence, as a member of the complex microbiota, in many sourdoughs (De Vuyst et al., 2014). The growth inhibition of fungi is mainly related to the production of organic compounds during fermentation metabolism. The interest dedicated to *L. plantarum* is related to the ability of producing antimicrobial compounds, especially antifungal metabolites, among which 3-phenyllactic acid (PLA), 4-hydroxyphenyllactic acid (Cortés-Zavaleta, López-Malo, Hernández-Mendoza, & García, 2014; Lavermicocca, Valerio, & Visconti, 2003; Poornachandra Rao et al., 2017; Russo et al., 2016; Zhang, Zhang, Shi, Shen, & Wang, 2014) and cyclic dipeptides (Dal Bello et al., 2007), the production of which in cereal-based products increases their safety and shelf-life (Dalié et al., 2010; Oliveira, Zannini, & Arendt, 2014; Russo, Fares, Longo, Spano, & Capozzi, 2017). Among them, PLA, derived from the phenylalanine catabolism, has raised a noticeable interest (Chaudhari & Gokhale, 2016; Valerio, Lavermicocca, Pascale, & Visconti, 2004). This compound has been also related to growth promotion of rice (*Oriza sativa*), increasing water and nutrient absorption and seed endosperm utilization efficiency (Adachi

et al., 2013). The antimicrobial mechanism of action of PLA is still unclear. However, the possibility to enhance its production in LAB by adding suitable precursors has been reported (Li, Jiang, & Pan, 2007; Valerio, Di Biase, Lattanzio, & Lavermicocca, 2016).

This study aimed to be a further exploration of the potential of the species *L. plantarum*, through the evaluation of properties not yet deepened: (i) the ability of inhibition *in vitro* and *in situ* of moulds not investigated up to now, (ii) the aflatoxin binding, (iii) the potential to induce cereal germination and to promote plant growth, (v) the phytate degradation ability and fructo-oligosaccharides utilization. Moreover, studies have been carried out on antimicrobial compounds produced by the selected *L. plantarum* ITEM 17215, and on the conditions influencing the PLA production.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Twenty-Five *Lactobacillus plantarum* strains were studied. The strains were previously isolated from different sources:CR62, CR63 CR67, CR70, CR71, CR73, CR74, CR75, CR77, CR78 from maize bran, CE42, CE60, CE84 (deposited in the Agro-Food Microbial culture Collection of the Institute of Sciences of Food Production, CNR, Bari, Italy as ITEM 17215) from wheat bran, 93, MD143, MD123, MD147, VS516, VC114, VC194, VC233, SE90, SE140 from raw milk cheeses, AC, LC1 from cocoa beans. *Lactobacillus rhamnosus* strain GG (ATCC 53103) was used as a positive control in aflatoxin binding experiments. Strains were routinely grown in anaerobic conditions (Anaerocult A, Sigma, St Louis, MO, USA) in MRS broth/agar (Difco Lab., Augsburg, Germany), for 24-48 h at 30 °C.

Growth in milk was studied using 9% RSM (Reconstituted Skim Milk- Difco) incubated at 30 °C. For studies of 3-phenyllactic acid (PLA) production, MRS broth and RSM, supplemented with 1.5 g L^{-1} phenylalanine (Phe) (Sigma) and 3.0 g L^{-1} phenylpyruvic acid (PPA) (Sigma) were used. Bacterial concentration was measured as CFU mL^{-1}. Acidifying activity of the strains was tested in MRS broth; the pH was measured and recorded automatically, throughout the 48-h incubation period.

2.2. Fungal strains and culture conditions

Fusarium verticillioides FSq1, *Aspergillus niger* FS11, *Aspergillus flavus* FS13, *Mucor circinelloides* FSq2 and *Penicillium roqueforti* FS 22 (from the Collection of the Department of Health, Animal Science and Food Safety, University of Milan, Italy) were routinely grown on Malt Extract Agar (MEA) (Merck, Darmstadt, Germany) at 25 °C for 5-7 days and stored at 4°C until further use. Fungal spore suspensions were harvested by adding 15 mL of sterile milli-Q water and stored at 4° C until further use. Concentration of the spores was assessed by flow cytometer estimation (BD Accuri C6 Flow Cytometer, BD Biosciences, Franklin Lakes, NJ USA).

2.3. Antifungal activity in vitro

Antifungal activity of the *L. plantarum* strains was investigated with an overlay assay (Axel et al., 2016). Bacteria, grown for 16 h in MRS broth at 30 °C, were inoculated in 2-cm lines on MRS agar plates and allowed to grow anaerobically for 48 h at 30°C. Subsequently, plates were overlaid with 10 mL of cooled soft (0.7%) MEA containing mould spore suspension (10^4 spores mL^{-1}). Plates were

incubated for 4 days at 25°C, and the antifungal activity was evaluated as clear zones of inhibition around the bacterial smears. The degree of inhibition was calculated on the basis of the inhibition zone as no inhibition (-) for inhibition zone smaller than 3 mm, moderate (+/-) for inhibition zone ranging from 3 to 10 mm or strong (+) for inhibition zone larger than 10 mm. The typical aspect of the inhibition halos is shown in Fig. 1.

Antifungal activity was also tested on cell-free supernatant (CFS): *L. plantarum* strains were inoculated in MRS broth to an initial concentration of 10^6 CFU mL^{-1} and incubated at 30°C for 48 h. CFS was obtained by centrifugation (3000 x *g*, 5 min) and sterilized by filtration (0.2 μm pore-size cellulose acetate filter). Plates of MEA containing 10^4 mould spores mL^{-1} were supplemented with different concentrations of CFS (10%-50%-80% v/v), incubated at 25 °C for 5 days and subsequently examined for inhibitory activity against *F. verticillioides*, *M. circinelloides* and *A. flavus.*

2.4. Antifungal activity in cereals and milk-based products

L. plantarum strains that displayed the highest antifungal activity were further tested for their ability to inhibit the moulds during cereal storage in simulated micro-silos and micro-yogurt preparations.

A micro-silo consisted of a test tube (3 x 11 cm) containing 20 g of a compacted mix of cereals (wheat, rice and oat in equal proportion) pre-treated with 20% of water for 48 h at 4°C. In one micro-silo the mixture was co-inoculated with 10^4 spores g^{-1} of *F. verticillioides* or *M. circinelloides*, or *A. flavus* and 10^6 CFU g^{-1} of *L. plantarum.* Another micro-silo, inoculated only with mould spores, was used as a control. The micro-silos were placed at room temperature (comprised between 19 and 22 °C) and mould growth was daily monitored for 10 days.

For micro-yogurt preparation, 20 mL of 9% RSM were inoculated with commercial starter cultures of *Streptococcus thermophilus* and *L. delbrueckii* subsp. *bulgaricus* (15 mg L^{-1}) (Lyofast –Sacco System, Cadorago, Italy) and 10^6 CFU mL^{-1} of *L. plantarum*. Fermentation was performed at 43 °C for 5-6 h, until the pH reached a value of 4.7. Subsequently, the resulting micro-yogurt preparations were transferred into petri plates, inoculated with *P. roqueforti* to a final concentration of 10^4 spores mL^{-1} and incubated at 25 °C. Samples were analysed for mould growth inhibition after 7 days of incubation. Yogurt without adding *L. plantarum* was used as negative control.

2.5. Effect of L. plantarum on growth rate of A. flavus, M. circinelloides and F. verticillioides

Twenty-five mL of malt extract broth in 100 mL Erlenmeyer flask were inoculated with 10^9 CFU mL^{-1} of overnight *L. plantarum* cultures and 10^4 mould spores mL^{-1}. Fungal mycelial mass, harvested after different incubation times at 25°C, was separated by filter paper and weighed after drying at 105°C.

2.6. Identification of targeted antifungal compounds

L. plantarum strain ITEM 17215 was grown in MRS broth at 37 °C for 48 h. CFS was ultrafiltered (1kDa) and subsequently purified using the Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) procedure, as described by Brosnan, Coffey, Arendt, & Furey (2014a) using a dispersive solid phase extraction (dSPE) kit (Agilent Technologies, Santa Clara, CA, USA). According to the procedure reported by Brosnan, Coffey, Arendt, & Furey (2012) and Brosnan,

Coffey, Arendt, & Furey (2014b), purified extracts were subjected to the Ultra Performance Liquid Chromatography - Photo Diode Array - High Resolution - Tandem Mass Spectrometry (UPLC/PDA-ESI-HR-MS/MS) analysis for targeted identification of 16 potential antifungal compounds including 1,2-dihydroxybenzene, allyl phenylacetate and the following acids: DL-p-hydroxyphenyllactic, 4-hydroxybenzoic, 3,4-diydroxyhydrocinnamic, vanillic, caffeic, 3-(4-hydroxyphenyl) propionic, PLA, (E)-p-coumaric, 3-(4-hydroxy-3-methoxyphenyl), propanoic, benzoic, (E)-ferulic, salicylic, hydrocinnamic and α-methylcinnamic. The UPLC-PDA-HR-MS/MS analyses were carried by coupling an Acquity UPLC separation module (Waters, Milford, MA, USA), equipped with a Gemini C18 column (150 x 2.0 mm. 3 µm, 110 Å, Phenomenex, Torrance, CA, USA) to an Acquity PDA eλ Detector (Waters) and (in-line) a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer through a HESI-II probe for electrospray ionisation (Thermo Fisher Scientific, San Jose, CA, USA). Data were processed using the Xcalibur software (version 3.0, Thermo Fisher Scientific).

2.7. Quantification of organic acids

3-Phenyllactic, lactic and acetic acids were quantified through an HPLC system (L 7000, Merck Hitachi) equipped with RI and UV (210 nm) detectors serially connected, using a SH1821 column (300 x 8 mm, Shodex, München, Germany) maintained at 50 °C and eluted with 5 mM H_2SO_4 at 0.5 mL min^{-1}. Analytical grade organic acids were used as standards (Sigma). Four-point external calibration curves ($R^2 > 0.998$) were adopted to quantify PLA, acetate, and lactate in MRS broths. LOQ of 0.010 g L^{-1} and LOD of 0.005 g L^{-1} (S/N = 3) for PLA were obtained, respectively.

2.8. Determination of MIC values

The MIC values of PLA, lactic acid and acetic acid were determined. Dilution of each acid, with concentration range between 0.5 and 7.5, 6.0 and 50.0, 0.1 and 3.0 mg mL^{-1} for PLA (Sigma), lactic acid (Sigma) and acetic acid (Sigma) respectively, were added to MEA medium inoculated with 10 spores mL^{-1} of *A. flavus*, *M. circinelloides* or *F. verticillioides*. MIC was defined as the lowest concentration where no growth could be observed after the incubation period.

2.9. Aflatoxin binding assay

Aflatoxin binding assay was carried out as described by Haskard, El-Nezami, Kankaanpa, Salminen, & Ahokas (2001). Solid AFB1 (Sigma) was suspended in benzene-acetonitrile (97/3; v/v) to obtain an AFB1 concentration of approximately 2 mg mL^{-1}. A solution of 5 µg mL^{-1} AFB1 was prepared in PBS (pH 7.3), and the benzene-acetonitrile was evaporated by heating in a water bath (80 °C, 5 to 10 min). For each bacterial strain, a volume of the culture broth corresponding to 10^{10} cells was centrifuged (3000 x *g*, 15 min, 10 °C) and the bacterial pellets were washed with 5 mL of Milli-Q water, suspended in 1.5 mL of AFB1 solution (5µg mL^{-1}) and incubated at 37°C. After 24 h of incubation, the bacteria were pelleted (3000 x g, 10 to 15 min, 10°C), and the supernatant (200 µL) containing AFB1 was collected and stored at -20 °C. For each strain, a bacterial control (bacteria suspended in PBS) and an AFB1 control (5 µg mL^{-1} of AFB1 in PBS) were tested. For strains showing the highest binding ability, the effect of incubation time was evaluated at 0, 24 and 48-h time points. The analysis was carried out on HPLC system consisting of two 1580 HPLC pump (Jasco, Easton, MD, USA), a degaser unit DG 2080-53 (Jasco), a FP 1520 fluorimeter detector set up at 365 nm

excitation and 415 nm emission, a manual sampler with a loop of 20 µL (Rheodyne). The analyses were performed isocratically with water/acetonitrile/methanol (6/3/1); flow rate was set at 1 mL min^{-1} room temperature. The analyses were run on a C18 column (Supelco, 150 mm x 4.6 mm, 5 µm). The chromatograms were analysed with Autochrom-3000 system (Young Lin Instrument Co., Ltd, Anyang, Korea) version 2.0.15. AFB1 solutions ranging from 2.5 to 30 µg mL^{-1} were used for the calibration curve determination: the detection limit was 0.01 µg mL^{-1}. The percentage of AFB1removed was calculated using the equation 100 × [1 − (peak area of AFB1 in the supernatant)/(peak area of AFB1in the positive control)].

2.10. Plant growth promotion assays

To assess the *L. plantarum* ITEM 17215 ability to promote growth of wheat plants, a preliminary lab-scale test was set up. Bread wheat (*Triticum aestivum*) kernels were soaked for 24 hours in either (i) sterile distilled water, (ii) non-inoculated MRS broth, or (iii) sterile distilled water and 10 mL of cultural broth containing 10^7 cells of *L. plantarum* ITEM 17215, grown in MRS medium for 24 hours at 30 °C. After soaking, the kernels were placed in plastic boxes, with natural lighting and temperature conditions, and kept wet by periodically spraying them with sterile tap water. Each treatment was set up in three distinct plastic boxes for replicates. After 7 days, the height of 100 randomly selected plants per treatment was measured.

2.11. Lactate dehydrogenase gene expression

Lactate dehydrogenase gene expression was evaluated through RT-qPCR experiments. Cells were grown at 30 °C for 48 h in MRS broth, RSM and MRS and RSM supplemented with PPA (3 g L^{-1}) and Phe (1.5 g L^{-1}). RNA extraction was performed using the NucleoSpin RNA II extraction kit (Macherey–Nagel GmbH, Düren, Germany). Residual contaminating DNA was hydrolysed with DNaseI, Rnase free kit (Thermo Fisher Scientific) at 37°C for 30 min. Subsequently, 0.2 µg of RNA was rewritten into cDNA using RevertAid First strand cDNA Synthesis Kit (Thermo Fisher Scientific) in accordance with manufacturer's instructions. Amplification, detection, and real-time analyses were performed using a Linegene 9620 Real-Time PCR (Bioer Technology, Hangzhou, China). SG qPCR Master Mix (EURx, Gdansk, Poland) was used for real-time amplification and detection of the cDNA. The nucleotide sequences of the primers used in this study for *ldh* and reference gene (*recA*) are as reported by Marco, Bongers, de Vos, & Kleerebezem (2007). Specifically:

-5' TGATCCTCGTTCCGTTGATG 3'
-5' CCGATGGTTGCAGTTGAGTAAG 3' for *ldh* gene;
-5' GGCAGAACAGATCAAGGAAGG 3'
-5' TATCCACTTCGGCACGCTTA 3' for *recA.*

One µL of cDNA was used as a PCR template in a 15 µL reaction mixture containing 4.78 µL water, 0.72 µL primer mix (containing 0.3 µM of each primer) and 7.5 µL of the SG qPCR master mix (EurX). The following experimental run protocol was used: initial denaturation step at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 59 °C for 30 s and extension at 72 °C for 20 s. A melting curve analysis was performed to verify the identity of the PCR products. Melting points (T_m) of 76.3 and 77.3 were obtained for *ldh* and *recA* respectively. Standard

curves had good linearity (R^2 =0.999) and efficiency (slopes -2.99 and -3.13 for *ldh* and *recA* respectively). The amplification of the two genes was conducted in triplicate. The relative expression ratios were calculated by the mathematical model of Pfaffl (2001), which included an efficiency correction for real-time PCR efficiency of the individual transcripts.

2.12. Phytate degradation

Strains were preliminary grown at 30 °C for 24-48 h in modified Chalmers broth without neutral red and with 1% of sodium phytate (Sigma). Five µL of the microbial suspension were spotted on modified Chalmers' agar plates without $CaCO_3$ and with 1% of hexacalcium phytate (Sigma) (Anastasio et al., 2010). The plates were incubated at 30 °C and examined after 2 days of incubation for clearing zones around the spots. To eliminate false positive results, caused by microbial acid production, Petri plates were flooded twice with 2% (w/v) aqueous cobalt chloride solution. After 20 min of incubation at 25 °C, the cobalt chloride solution was removed and phytase activity was evaluated.

2.13. Utilization of fructo-oligosaccharides

Fermentation of carbohydrates was tested in MRS basal medium (MRS without carbohydrate); fructo-oligosaccharides (FOS - Actilight, Tereos, Lille, France) were autoclaved separately (112 °C for 30 min) and added to the sterile basal medium to obtain the final concentration of 10 g L^{-1}. Cells from 24-h MRS cultures were washed twice with sterile isotonic saline solution, resuspended in the same isotonic solution, inoculated (1%) in MRS basal medium and in MRS-FOS and incubated at 30°C for 48 h. Fermentation of FOS was evaluated by measuring the increase in absorbance at 600 nm (A_{600}).

2.14. Statistical analysis

Three independent replicates of each experiment were performed, and their results were expressed as mean values ± standard deviation. When necessary, the data were compared through one-way ANOVA, followed by Tukey's exact test ($p < 0.05$), or through a principal component analysis (PCA), performed in SPSS statistical package for Windows, v. 22.0 (IBM Corporation, Armonk, NY, USA). The dataset used for these analyses included the 25 isolates as separate rows, and 8 columns for the different parameters evaluated: the inhibitory effect against the 5 fungal pathogens used (which were used for the PCA analysis), aflatoxin binding, production of organic acids, and phytate degradation.

3. Results and discussion

3.1 Selection of highly performant L. plantarum strains

Selection of highly performant *L. plantarum* strains was carried out testing different properties. As reported in Table 1, we tested characteristics related to antifungal activity and to beneficial traits.

3.1.1. Antifungal activity

A first screening of the 25 *L. plantarum* strains was performed against five fungal target strains, *Fusarium verticillioides*, *Aspergillus niger*, *A. flavus*, *Mucor circinelloides* and *Penicillium roqueforti.* Varying degrees of inhibition were detected against the tested moulds in the overlay method. On the basis of the inhibition zones, *L. plantarum* strains were classified into three groups with no, moderate or strong antifungal activity. *P. roqueforti* FS 22 was the most resistant strain: only four strains out of 25, one from cereals and three from cheeses, were able to inhibit its growth. In contrary, *F. verticillioides* FSq1 was inhibited by all *L. plantarum* strains, with only one exception and *M. circinelloides* FSq2 by 60% of the tested strains, most of which isolated from cereals. The data regarding fungal inhibition, analysed through the PCA, showed that the strains isolated from maize were clearly distinguishable from those isolated from other substrates and very similar to one another (Fig 2). In contrast, the strains obtained from wheat, cheese, and cocoa do not clearly segregate and have a more varied antifungal effect. These data are of interest, because growth inhibition of these contaminants has not been extensively studied and to the best of our knowledge, no reports on this antifungal ability in *L. plantarum* are available in literature.

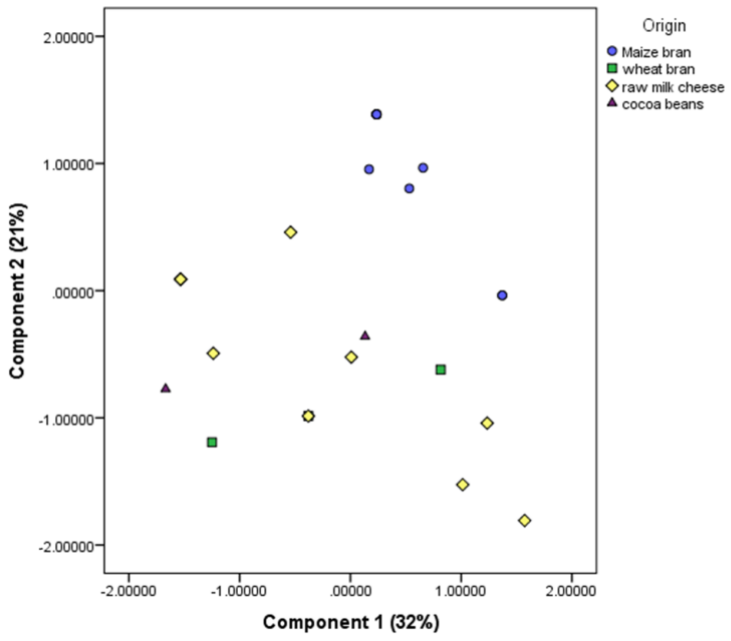

Fig. 2. Graphical representation of the two-major component obtained from principal component analysis carried out on the fungal inhibition results. The principal component 1, on the x axis, explains 32% of variability, while principal component 2, on the y axis, explains 21% of variability.

Fusarium species are associated with cereal grains. A few LAB strains have been shown to inhibit growth and production of mycotoxins by *F. graminearum*, which is abundant in various cereal crops and processed grains. *F. verticillioides* is the most prevalent fungus associated with maize, causing several diseases, and producing a wide range of mycotoxin that includes fusaric acid, fusarins and fumonisins (Deepa & Sreenivasa, 2017) Fumonisin B1 is considered the most toxic one; epidemiological data indicate a possible correlation between the consumption of fumonisin/*F. verticillioides* contaminated maize and the high incidence of esophageal cancer in countries where maize is a dietary staple (Oldenburg, Höppner, Ellner, & Weinert, 2017; van Rensburg et al., 2015). *M. circinelloides* is frequently isolated from food matrices (Lee et al., 2014) and it is considered one of the causal agents of the fungal infection mucormycosis (Pitt & Hocking, 2009). Moreover, most of the *L. plantarum* analysed (68%) exerted a strong inhibition against *A. flavus* FS13, while *A. niger* FS11 was inhibited by approximately 36% of the *L. plantarum* strains. Also, these data are of interest, since the results of previous studies showed the inability of *L. plantarum* to inhibit *Aspergillus* growth (Cortés-Zavaleta et al., 2014; Russo et al., 2016). In particular, as reported below, selected *L. plantarum* ITEM 17215 can completely inhibit mould development when grown in co-culture, and this ability could be effective in limiting the mycotoxin production.

L. plantarum strains were further tested for their ability to bind aflatoxin B1 (AFB1). Although in cereal sector the potential of using microbial strains with mycotoxin binding abilities is considered of great value in reducing the mycotoxin exposure (Dalié et al., 2010; Shetty & Jespersen, 2006), up to now very few *L. plantarum* have been characterized for their toxin binding ability. Overall, in the present study, *L. plantarum* strains, also tested at different incubation times, moderately bound the AFB1. The 80 % of the strains showed a binding

level ranging from 5 to 7.5 μg mL^{-1}, and only a single strain (SE90) bound 29 % (14.5 μg mL^{-1}). However, in the condition of the assay, *L. rhamnosus* GG, chosen as a positive control, showed a similar binding ability, in contrast to other studies in which a percentage of 70-80% was reported (El-Nezami et al., 1998; Haskard et al., 2001). This aspect needs to be further investigated.

Fig.1. Antifungal effect of *L. plantarum* ITEM 17215 against: A) *Aspergillus niger*; B) *A. flavus*; C) *Fusarium verticillioides*; D) *Mucor circinelloides*; E) *Penicillium roqueforti*

3.1.2. Enhancement of food quality

L. plantarum strains have been tested for targeted properties linked to improvement of food quality, as phytate degradation ability and FOS utilization. As shown in Table 1, 19 of the 25 strains were able to hydrolyse calcium phytate, an important characteristic in the cereal sector. Phytate is known to chelate several essential nutrients and can negatively influence the activity of digestive enzymes by chelation of mineral cofactors or by interacting with proteins. Moreover, 23 out of the 25 tested *L. plantarum* strains were able to grow in presence of FOS, prebiotic oligosaccharides used in combination with probiotic microorganisms for the development of functional food ingredients (Rastall & Maitin, 2002). These characteristics could be exploited for a potential use of selected *L.*

plantarum strains as new probiotic cultures. Further studies are underway to find other functional properties in *L. plantarum* strains, to be followed by clinical and *in vivo* studies to confirm the strains as probiotics. Finally, all tested strains showed a high acidification rate, with a pH value, after 16 h of incubation at 30°C, ranging from 3.9 to 4.2.

Table 1. Antifungal activity, aflatoxin binding, acidifying activity, phytate degradation and FOS utilization by *Lactobacillus plantarum* strains.

Strains	Antifungal activity[1]					Aflatoxin B1 binding[2]	Acidifying activity[3]	Phytate degradation	FOS utilization (A_{600})	
	Mucor circinelloides	*Fusarium verticillioides*	*Aspergillus niger*	*Aspergillus flavus*	*Penicillium roqueforti*				Basal MRS medium	MRS +FOS
CR62	+	+	-	+	-	14±4.9	4.00±0.01	+	0.61±0.05	1.91±0.12
CR63	+	+	-	+	-	12±0.4	4.16±0.00	+	0.57±0.07	1.73±0.15
CR67	+	+	-	+	-	10±0.7	4.06±0.07	+	0.58±0.03	1.80±0.05
CR70	+	+/-	-	-	-	13±0.1	3.94±0.01	+	0.95±0.04	1.61±0.06
CR71	+	+	+/-	+	-	13±0.05	3.99±0.02	+	0.94±0.02	1.86±0.10
CR73	+	+/-	-	-	-	11±1.9	3.93±0.01	+	0.94±0.05	1.79±0.02
CR74	+	+	-	+/-	-	13±0.7	4.00±0.00	+	0.66±0.06	1.65±0.01
CR75	+	+	-	+	-	10±0.8	3.97±0.00	+	0.92±0.01	1.86±0.11
CR77	+	+	-	+	-	9±0.7	3.97±0.00	+	0.90±0.03	1.53±0.07
CR78	+	+/-	-	+	-	8±1.0	3.97±0.02	+	0.96±0.03	1.63±0.09
CE42	-	+/-	+	+	-	10±1.3	3.95±0.02	+	0.97±0.02	1.80±0.14
CE60	-	+	+	+/-	-	11±1.4	3.91±0.04	-	0.78±0.07	0.72±0.10
ITEM 17215	+	+	+	+	+	14±0.6	4.21±0.01	+	0.85±0.10	1.82±0.04
93	-	+	-	+	-	12±1.9	3.88±0.01	-	0.87±0.08	1.75±0.06
MD143	+/-	-	-	+/-	+	12±0.5	3.92±0.00	+	0.89±0.00	1.79±0.05
MD123	-	+/-	-	+	-	14±0.2	3.90±0.02	-	1.01±0.05	1.76±0.10
MD147	-	+	-	+	-	7±1.6	3.87±0.02	+	0.85±0.04	1.77±0.11
VS516	-	+	-	+	-	11±1.7	3.92±0.00	-	0.98±0.01	1.84±0.10
VC114	-	+/-	+	+	-	10±0.8	3.93±0.01	+	0.78±0.08	1.68±0.01
VC194	-	+	-	+	-	11±0.2	3.90±0.01	-	0.92±0.07	1.68±0.05
VC233	+	+	+	+/-	+	11±0.6	4.10±0.01	+	0.66±0.03	1.67±0.05
SE90	+/-	+/-	+	+	-	29±0.8	3.88±0.01	+	0.81±0.03	0.82±0.06
SE140	-	+	-	-	+	11±2.1	3.90±0.00	+	0.78±0.01	1.72±0.16
AC	-	+	+	+	-	15±1.1	3.87±0.05	-	0.90±0.09	1.61±0.13
LC1	+/-	+	+	+/-	-	5±0.5	3.96±0.00	+	0.85±0.07	1.76±0.06

[1] Strains were classified as no (-), moderate (+/-), or strong (+) inhibitors by using the overlay method.

[2] Expressed as percentage of binding.

[3] Measured as pH value after 16 h of incubation at 30°C in MRS broth

3.2. Characterization of L. plantarum ITEM 17215

The initial screening data indicate that the tested *L. plantarum* strains demonstrated a good performance, regarding either antifungal activity or beneficial properties. Among them, the strain ITEM 17215 showed the strongest inhibitory activity towards all the tested fungal strains (Fig.3), and the ability to degrade phytate and utilize FOS. Its multi-properties, exploited in a specific process or sector, as cereal conservation and fermentation, can improve, at the same time, safety and quality of the final product. For these reasons, the subsequent experiments were directed towards the study of this strain.

3.2.1. Identification and quantification of antifungal compounds

L. plantarum ITEM 17215 completely inhibited mycelial growth of *A. flavus* and *F. verticillioides* and reduced by 25% the mycelial mass of *M. circinelloides* after 9 days of simultaneously cultivation in malt extract broth. The antifungal activity of the strain was also tested in plates of MEA supplemented with increasing concentrations of untreated CFS*:* CFS was able to inhibit the growth of *F. verticillioides* strain when supplemented at 10% (v/v). The complete inhibition of *A. flavus* and *M. circinelloides* strains was reached when CFS was supplemented at 80%.

With the aim to identify the active antifungal compounds, CFS from the strong inhibitor strain ITEM 17215 was analysed by UPLC/PDA-ESI-HR-MS/MS. *L. plantarum* ITEM 17215 produced 1,2-dihydroxybenzene, benzoic acid, p-hydroxyphenyllactic acid and PLA (Fig.4). The latter compound, already described as efficient antifungal inhibitor, was the most abundant and its concentration, as determined by HPLC, resulted equal to 99.6±4.0 mg L^{-1}. HPLC analysis also revealed the ability of the strain ITEM 17215 to produce a discreet amount of acetic acid (1.1±0.2 g L^{-1}), combined with high lactic acid yield (21.5±1.4 g L^{-1}).

In parallel, we studied the effect of organic acids on germination of the mould spores in solid media. Acetic acid and PLA were the compounds more effective for the inhibition of growth of the studied moulds. Indeed, MIC values of 2.35, 0.58, 0.29 g L^{-1} for acetic acid were obtained for *M. circinelloides*, *A. flavus* and F. *verticillioides*, respectively. MIC values for PLA were 5.0 g L^{-1} for *A. flavus*, 3.0 g L^{-1} for *M. circinelloides* and 1.5 g L^{-1} for *F. verticillioides.* Against lactic acid, the tested moulds required higher amounts of this organic acid, being M. *circinelloides* more resistant (MIC 25.0 g L^{-1}) than A. *flavus* and *F. verticillioides* (MIC 12.5 g L^{-1}). In any case, most of the MIC obtained for organic acids against the tested moulds were greater than those estimated with the producer strain. The data of the obtained MIC are in accordance with the previous observations that more metabolites produced by LAB can contribute to synergistically inhibit mould growth (Cortés-Zavaleta et al., 2014; Russo et al., 2016).

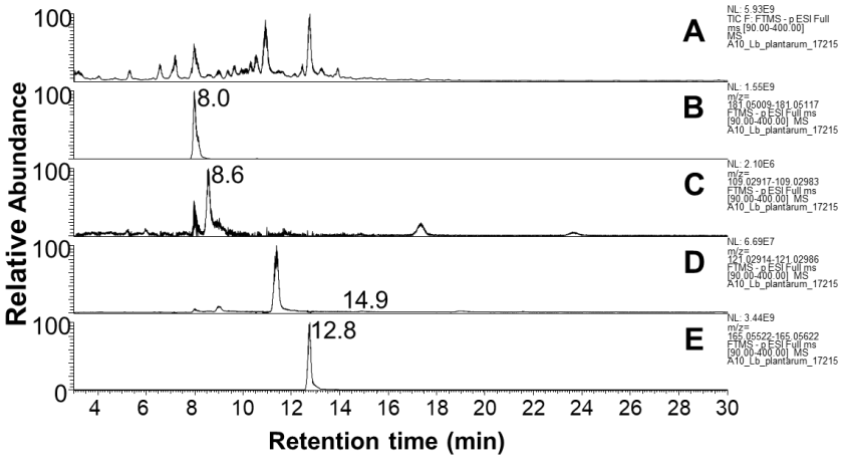

Fig. 4. HR-MS chromatograms of potential antifungal compounds identified in CFS from the strong inhibitor strain L. plantarum ITEM 17215: A, HR-MS total ion current chromatogram; B, extracted ion chromatogram ([M–H+]– exact mass ± 3 ppm) (XIC) of p-hydroxyphenyllactic acid (181.0506); C, XIC of 1,2-dihydroxybenzene (109.0295); D, XIC of benzoic acid (121.0295); E, XIC of PLA (165.0557). Retention times of corresponding compounds are indicated. Identity of compounds was confirmed by HR-MS/MS (spectra not shown).

3.2.2. L. plantarum ITEM 17215 mould inhibition activity in cereals and milk-based products

When inoculated in micro-silos contaminated with *A. flavus*, *F. verticillioides* or *M. circinelloides*, *L. plantarum* strain ITEM 17215 was able to inhibit the growth of the moulds (Fig. 5): after 10 days of incubation the count in MEA decreased from 10^8 CFU g^{-1} in the control micro-silo inoculated with 10^4 spores g^{-1} to <10 CFU g^{-1}.

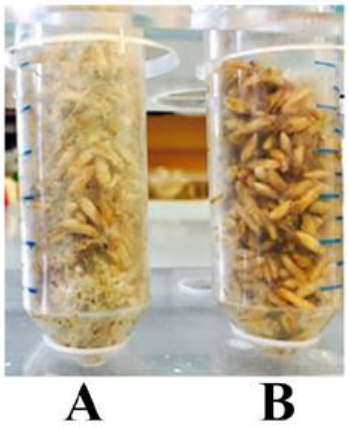

Fig. 5. Growth inhibition of *Fusarium verticillioides* by *L. plantarum* ITEM 17215. A) micro-silo inoculated with 10^4 fungal spores g^{-1}; B) micro-silo inoculated with 10^4 fungal spores g^{-1} and 10^6 CFU g^{-1} of strain ITEM 17215.

Moreover, germination tests indicated that the *L. plantarum* strain ITEM 17215 strongly induced wheat germination and promoted plant growth (Fig. 6). Seven days after the soaking treatment, wheat kernels germinated in the plastic boxes and their heights were significantly (p <0.05) different based on the treatments. In particular, non-treated controls and MRS non-inoculated-treated kernels were approximately of the same height, with an average of 3.8±0.4 cm and 3.5±0.5 cm, respectively, while the kernels treated with the cultural broth were of 6.6±0.3 cm. The statistical analysis confirmed that the cultural broth treatment is significantly different from the other two treatments (p = 0.000).

Plant growth promoting effects of some rhizobacteria have been studied: the bacteria may secrete organic acids, such as succinic and lactic acids, and these acids may increase plant growth under conditions in which the populations of pathogens are reduced (Lugtenberg & Kamilova, 2009). Little data are available on LAB as plant growth promoting bacteria: they were found as endophytic components of durum wheat plant (Minervini et al., 2015) and some studies indicate their potential for biocontrol and rice growth promotion. Adachi et al. (2103) demonstrated that PLA can promote root and shoot growth in rice seedling and hypothesize that this acid could be involved in the regulation of the cell enlargement and division in synergy with other plant growth substances. Considering the reported data, it is possible to relate the superior seedling growth we observed, with the production of organic acids, particularly PLA secreted by the strain ITEM 17215. This fact needs to be further examined before proposing a potential application of the strain as a plant growth-promoting bacterium.

L. plantarum ITEM 17215 did not show evident antagonistic effects against *P. roqueforti* used as a test mould in micro-yogurt preparation. This last result was related to a poor ability of the strain to produce PLA when grown in milk, as verified by HPLC analysis: when the strain was grown in RSM, the PLA production was not quantifiable.

Fig.6. Effect of L. plantarum ITEM 17215 on wheat germination. A) wheat grains soaked in water (control); B) wheat grains soaked in water with 10 mL cultural broth of L. plantarum ITEM 17215.

3.2.3. Improvement of PLA production

As previously reported (Valerio, Di Biase, Lattanzio, & Lavermicocca, 2016), PLA production can be improved by the addition of Phe to the growth medium and/or by the addition of the precursor PPA, with lactate LDH activity responsible for the efficient conversion from PPA to PLA. In this regard, it has been suggested that differences in the LDH amino acid sequences could be responsible for the disparity in the ability of LAB strains to produce PLA (Zhang et al., 2014). When the *L. plantarum* ITEM 17215 was grown in MRS supplemented with Phe and PPA, the production of PLA increased from about 0.1g L^{-1} to 2.2 ± 0.2 g L^{-1}, 22-fold more than in MRS broth without PLA synthesis precursors. The gene expression experiments also showed that the expression of *ldh* gene was boosted with the addition of the precursors. Statistically significant difference ($p< 0.05$) was noticeable between *ldh* expression levels (relative expression ratio of 7.84) in the two different tested conditions (Fig.7).

To verify the inability of the strain to produce PLA when grown in RSM, the *ldh* expression experiments were also carried out to evaluate the different expression level in RSM and RSM supplemented with the precursors. Transcription of *ldh* gene was poorly induced in response to precursors (relative expression rate of 1.3) (Fig.7). Moreover, *ldh* gene was over-expressed only in MRS broth (15.5-fold) in comparison to RSM. The inability of the strain to produce high levels of PLA when grown in RSM deserves to be further investigated, also at the species level.

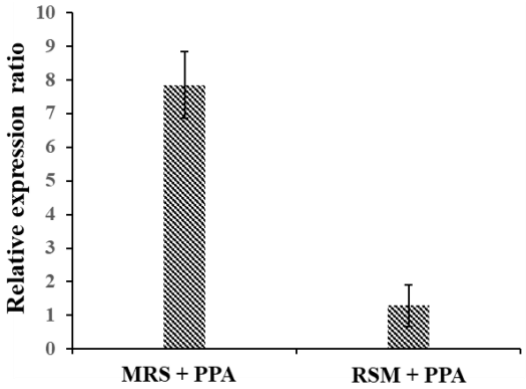

Fig.7. Relative expression ratio of *ldh* gene in *L. plantarum* ITEM 17215, grown in MRS and RSM in presence of 3.0 g L^{-1} phenylpyruvic acid (PPA), calculated relative to its expression in control sample. Data were normalized using *recA* gene. The bars represent the standard deviations.

4. Conclusions

Many reports describe the ability of *L. plantarum* strains to inhibit mould growth, related to the production of PLA and other organic acids. Moreover, the potential use of selected *L. plantarum* strains in sourdough fermentation has been described by many authors. This study aimed to further explore the potential of the species. The tested strains not only showed a good spectrum of inhibition against food spoiling moulds but had interesting beneficial traits such as the ability to utilize FOS, prebiotic substances, that help probiotic microorganisms to grow in the human gut, and to reduce phytate, an antinutrient present in cereal sector. Phytic acid is the major storage form of phosphorous comprising 1–5% by weight in cereals (Schlemmer, Frølich, Prieto, & Grases, 2009), but represents an antinutritive factor for its ability to chelate dietary minerals, reducing their bioaccessibility and bioavailability. Several studies have shown that the fermentation process can significantly reduce phytic acid, not only by an activation of endogenous phytases but also by specific microbial biotypes able to produce extracellular phytases (Lopez et al., 200; Manini et al., 2014). Regarding mould inhibition, we described, for the first time, the ability of the *L. plantarum* strains to inhibit moulds that have not been studied yet: the food borne pathogen *M. circinelloides* and the mycotoxigenic *F. verticillioides*. Moreover, most of the analysed *L. plantarum* strongly inhibited *A. flavus*. The selected *L. plantarum* ITEM 17215 showed the highest inhibitory ability towards all the tested fungal strains. Further analyses demonstrated that the strain produced two known fungal growth inhibitors, PLA and p-hydroxyphenyllactic acid as well as lactic and acetic acids. Regarding PLA, the presence of Phe and PPA in the growth medium, further increased its production, allowing to achieve a very high concentration of PLA. The metabolites produced by the strain ITEM 17215 could also explain its ability to induce wheat germination and to promote plant growth. This aspect, not investigated yet in *L. plantarum*, could have interesting application in the agro-food sector.

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Chapter 2. Evaluation of microbial consortia and chemical changes in spontaneous maize bran fermentation

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In this chapter, we evaluated the microbial population of maize bran to reconsider the by-product in industrial exploitation and as a possible source of interesting antifungal biotypes of lactic acid bacteria and yeasts.

ABSTRACT

Sustainable exploitation of agro-industrial by-products has attracted great interest in cereal bran valorization. In this research, a polyphasic approach has been carried out to characterize maize bran at microbiological and chemical level during a sourdough like fermentation process, in order to enhance its technological and nutritional properties. Autochthonous microbiota was isolated at different refreshment steps and subjected to identification and molecular characterization. Fermentation was characterized by a rapid increase in lactic acid bacteria and yeasts, with a co-dominance, at the initial stage, of *Weissella* spp., *Pediococcus* spp. and *Wickerhamomyces anomalus.* At the end of the fermentation, a natural selection was produced, with the prevalence of *Lactobacillus plantarum, Lactobacillus brevis* and *Kazachstania unispora.* This is the first time that a specific association between LAB and yeasts is reported, during the maize bran fermentation process. Enzymatic activities related to this microbial consortium promoted a "destructuration" of the fiber fraction, an increase in soluble dietary fiber and a reduction of phytic acid content. Our data also evidenced a noticeable increment in ferulic acid. The results obtained indicate that fermentation processes represent an efficient biotechnological approach to increase nutritional and functional potential of maize bran. Moreover, the characterization of microbiota involved in natural fermentation process will allow the selection of specific biotypes, with appropriate metabolic and enzymatic activities, to conduct "tailored" fermentation processes and improve brans or whole-meal flours from both nutritional and technological points of view.

Keywords: by-product; sustainability; spontaneous fermentation; maize bran; lactic acid bacteria; yeast.

1.Introduction

Cereal bran valorization has, for many reasons, a noticeable interest. The dietary recommendations call for an increased intake of functional foods: by-products generated by cereal agro-industrial sector are rich in dietary fiber, minerals and bioactive compounds and could represent beneficial ingredients for human nutrition. The main reasons behind the low utilization of native brans as ingredient for cereal-based products are due to their poor safety (heavy metal and mycotoxins), technological (increased dough stickiness, reduced volume) and sensory qualities (dark color, taste, chewy/hard texture) (Rose et al. 2010; Heinio et al. 2016). The spontaneous sourdough-like fermentation process, characterized by a consortium of yeasts and lactic acid bacteria (LAB), has shown to be an interesting pre-treatment in order to ameliorate sensory and textural quality**,** as well as to improve the microbial safety of bran (Dalié et al. 2010; Manini et al. 2014; Messia et al. 2016). Recently, several studies have shown the ability of many LAB to inhibit mold growth and mycotoxin biosynthesis (Dalié et al.

2010). These data highlight the potential offered by LAB as natural agents for decontamination of food frequently contaminated by toxigenic fungal strains, particularly cereals. Moreover, through fermentation, an increased bioavailability of bioactive compounds and a decreased level of some anti-nutritional compounds can be obtained (Katina et al. 2012; Manini et al. 2014).

To date, the majority of published literature in this field regards fermented wheat bran. Little is known about the effects of this "bio-approach" on properties of others cereal, as maize. This cereal and its by-products could represent attractive alternative raw materials for fiber enrichment of gluten-free products. According to A.I.R.E.S (Associazione Italiana Essiccatori Raccoglitori Stoccatori di Cereali e Semi oleosi, http://www.micotossine.it/), about 82 % of maize production is used for animal feed and only 18% is devoted to human consumption. At the milling stage, dry maize mechanical processing creates whole or fractionated products, accounting about for up to 25%, generally used as animal feed or biofuel. In this context, the valorization of maize by-products as valuable food ingredients to be exploited in the production of new food formulation could represent an opportunity.

Ferulic acid is diffused in cereals and maize bran appears as one of the richer sources, containing about 26-33 g/kg (Zhao and Moghadasian 2008). Moreover, maize bran contains hemicellulose, cellulose and a small amount of lignin, thus representing a source of dietary fiber. The hemicellulosic fraction, generally denominated arabinoxylans (AX), has a particular heterogeneous nature, with extensive cross-linkages between ferulic acid and arabinose/xylose eteropolisaccharides. This particular feature promoted a complex and rigid structure of maize cell wall (Rose et al. 2010). The potential of native microorganisms and their enzymatic activities of increasing the fraction of soluble dietary fibers and the content of free ferulic acid deserves to be tested.

Recently the EFSA Panel published a positive Opinion about the request of an Health Claim related to the positive effects on the post-prandial glycemic and insulinemic responses elicited by water-extractable arabinoxylans (WEAX) (EFSA 2011). This Opinion was founded on results from different studies reporting that this fraction can delay the rate of carbohydrates digestion/absorption, thus positively affecting glucose metabolism (Ai and Jay-lin 2016; EFSA 2011). Moreover, it is quite recognized in literature that arabino-xylanoligosaccharides (AXOS) and xylan-oligosaccharides (XOS) obtained by the hydrolysis of arabinoxylans present in brans can exert prebiotic properties (Broekaert et al. 2011).

Little data are available in literature on microbial population during maize fermentation. They mainly refer to maize-based spontaneously fermented doughs produced in West African countries (Oguntoyinbo et al. 2011; Okeke et al. 2015; Assohoun-Djeni et al. 2016). Very lacking information exists on microbial population during maize bran fermentation and on their contribution in promoting nutritional and functional properties of this cereal by-product. Thus, this study was performed to characterize maize bran, at microbiological and chemical level during a sourdough like fermentation process.

2.Materials and methods

2.1 Fermentation process and chemical analyses

Two commercial native maize brans (C1 and C2 - average particle size 500-600 µm -Molino Perteghella, Solarolo di Goito, MN, Italy; Molino Spoletini, Arcevia, AN, Italy) were subjected to spontaneous fermentations (without microbial starters), according to Manini et al. (2014). Fermentation experiments were carried out in duplicate, at 30 °C, through continuous propagation

until a stable microbiota was established (12 days). Unfermented samples and fermented bran samples, collected at different refreshment steps were stored at -20 °C for further analysis.

Proximate composition, ferulic acid, total (TOTAX) and water-soluble (WEAX) arabinoxylans, were assessed, in duplicate, on native and fermented maize brans as previously reported (Manini et al. 2014).

2.2 Evaluation of microbial population

Using selective media and conditions as previously reported (Manini et al. 2014), we quantified and isolated lactic acid bacteria (LAB), non-lactic acid bacteria (NLAB), yeasts and molds.

For each fermented bran, between 5-20 colonies representing all morphologies were picked from the respective plates at the refreshment steps 1, 7 and 12, purified by successive streaking, and stored in glycerol at -80 °C for further experimentations. A total of 135 presumptive LAB, 93 yeast isolates, 12 NLAB and three molds were selected and subjected to identification and molecular characterization.

2.3 Molecular identification of the isolates

Total bacterial DNA was extracted from 100 µL of an overnight culture, using the Microlysis kit (Labogen, Rho, Italy) following the manufacturer's instructions. For LAB identification, a first clustering step was reached by a PCR amplification of the 16S–23S rDNA spacer region (RSA). Molecular identification of LAB isolates with different RSA patterns, and NLAB was carried out by partial 16S rDNA gene sequencing, species-specific amplification and/or restriction analysis of the 16S–23S rDNA spacer region. For the isolated yeasts and molds, total DNA was extracted in a PRECELLYS®24-DUAL lyser/homogeniser (Bertin-technologies, Saint Quentin en Yvelines, France) (3 cycles of 30 s, 30-s break) with a mixture of glass beads (Ø < 106 µm). Molecular identification was carried out by restriction digestion of the Internal Transcribed Spacer (ITS) and/or partial 26S rDNA gene sequencing. The DNA sequences for the primers used, their corresponding specificities and the thermal cycle parameters employed are reported in Table 1. Amplification was carried out in a Mastercycler (Eppendorf, Hamburg, Germany). PCR was performed in a 25 µL reaction mixture containing 50 to 100 ng DNA template, 2.5 µL 10 x reaction buffer (Thermo Fisher Scientific, Vilnius, Lithuania), 200 µmol·L-1 of each dNTP, 2.5 mmol·L-1 MgCl2, 0.5-mmol·L-1 of each primer, and 0·5-U Taq polymerase (Thermo Fisher Scientific). The amplification products were separated on a 1.5-3.0 % agarose gel stained with ethidium bromide and photographed. Amplicons were purified using NucleoSpin® Extract II (Macherey-Nagel, Düren, Germany) and sequenced at Eurofins Genomics (Ebersberg, Germany). Sequence alignment was carried out with ClustalW software. The BLAST algorithm was used to determine the most relate sequence relatives in the National Centre for Biotechnology Information nucleotide sequence database (www.ncbi.nlm.nih.gov/BLAST). The 16S rRNA gene restriction digestion was carried out employing AluI, CfoI, and DraI (Thermo Fisher Scientific), according to the supplier's instructions. The products of the PCR-amplified Internal Transcribed Spacers ITS region were digested with the restriction endonucleases HinfI and TaqI. Restriction digests were subsequently analyzed by agarose electrophoresis, as above reported.

Table 1 PCR primers and conditions used for isolates identification

Primers	Target	Sequence (5'-3')	Thermal Conditions	Amplicon (bp)	Reference
RSA_F RSA_R	16S–23S rDNA spacer region (RSA).	GAAGTCGTAACAAGG CAAGGCATCCACCGT	94 °C × 45 s 54 °C × 1 min × 35 cycles	Variable	Lane *et al.* (1991)
16S_F 16S_R	*16S* rDNA gene	AGAGTTTGATCCTGGCTCAG CTACGGCTACCTTGTTACGA	94 °C × 45 s 55 °C × 45 s × 35 cycles	1540	Lane *et al*. (1991)
p8FLP p806R	partial *16S* rDNA gene	AGTTTGATCCTGGCTCAG GGACTACCAGGGTATCTAAT	94°C x 1 min 56°C x 1 min x 30 cycles	800	McCabe *et al*. (1995)
*L. plant*_F *L. plant*_R	*Lact. plantarum*	CCGTTTATGCGGAACACC TCGGGATTACCAAACATCAC	94 °C × 2 min 56 °C × 1 min × 35 cycles	318	Torriani *et al* (1991)
Pedio23S_F Pedio23S_R	*Pediococcus* spp.	GAACTCGTGTACGTTGAAAAGTGCTGA GCGTCCCTCCATTGTTCAAACAAG	94 °C × 45 s 64 °C × 1 min × 35 cycles	701	Pfannebecker *et al*. (2008)
P23S_R PPE23S_F	*Ped.pentosaceus*	CTGTCTCGCAGTCAAGCTC CCAGGTTGAAGGTGCAGTAAAAT	94 °C × 1 min 67 °C × 1 min × 35 cycles	1640	Pfannebecker *et al*. (2008)
P23S-R ArgentF	*Ped. argentinicus*	CTGTCTCGCAGTCAAGCTC GATATTCCTGTACTAGTTAGAT	94 °C × 1 min 60 °C × 1 min × 35 cycles	948	This study
Lbrev 1391F	*Lact. brevis*	TAATGATGACCTTGCGGTC TGTACACACCGCCCGTC	94 °C × 45 s 48 °C × 45 s × 35 cycles	330	Coton *et al*. (2008)
Weiss_F Weiss_R	*Weissella* spp.	CGTGGGAAACCTACCTCTTA CCCTCAAACATCTAGCAC	94 °C × 45 s 54 °C × 1 min × 35 cycles	725	Jang *et al.* (2002)
1 RL LaccreR	*Lact. lactis*	TTTGAGAGTTTGATCCTGG GGGATCATCTTTGAGTGAT	94 °C × 2 min 54 °C × 1 min × 35 cycles	238	Pu *et al.* (2002)
LeuclacF LeuclacR	*Leuc. lactis*	AGGCGGCTTACTGGACAAC CTTAGACGGCTCCTTCCAT	94 °C × 2 min 58 °C × 1 min × 35 cycles	742	Lee *et al.* (2000)
ITS1 ITS4	ITS1-5.8S-ITS2internal transcribed spacer (ITS)	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	94 °C × 45 s 60 °C × 1 min × 35 cycles	Variable	Jespersen *et al.* (2005)
NL1 NL4	*26S* rDNA gene	GCATATCAATAAGCGGAGGAAAAG GGTCCGTGTTTCAAGACGG	94 °C × 2 min 52 °C × 1 min × 35 cycles	778	Kurtzman *et al.* (1998)

2.4 Molecular fingerprinting of lactic acid bacteria

LAB strains were typed by random amplification of polymorphic DNA-PCR (RAPD) typing with primers M13 (5′-GAGGGTGGCGGTTCT-3′) and LP1 (5'ACGCGCCCT-3'). An annealing temperature of 38° and 48 °C for M13, and LP1 respectively, and an amplification protocol of 35 cycles were used. The PCR products were analyzed by electrophoresis and photographed as reported earlier. Grouping of the RAPD-PCR profiles was obtained with the BioNumeric 5.0 software package (Applied Maths, Kortrijk, Belgium), following the unweighted pair-group method with arithmetic averages cluster analysis. The value for the reproducibility of the assay, evaluated by the analysis of repeated DNA extracts of representative strains was > 93 %.

2.5 Carbohydrate utilization

The utilization of different carbon sources was carried out using a basal medium (containing Peptone 1,5% wt/v, Yeast extract 0.6 % wt/v, chlorophenol red 0.004 % wt/v, Tween 80 1 ml L^{-1}, pH 6.4) and the desired filter-sterilized carbohydrate at a final concentration of 0.5% (wt/v).

2.6 pH measurement

Measurement of pH was determined on 10g of fermented bran suspended in 100 mL of distilled water by a pH meter (PHM 250, Radiometer, Copenhagen, Denmark).

2.7 Statistical analysis

All data are reported as mean ± standard deviation. One-way ANOVA was performed on chemical data; if significant (p<0.05) effects were found, pairwise comparisons between samples were checked with Tukey's test (Statgraphics XV version 15.1.02, StatPoint Inc., Warrenton, VA, USA).

2.8 Nucleotide sequence accession numbers

The *16S* rDNA sequences determined during the present study have been deposited in the GenBank database under the accession numbers MF348245- MF348248 and MF399041- MF399049. The *26S* rDNA sequences determined during the present study have been deposited in the GenBank database under the accession numbers MF348249- MF348254.

3. Results

3.1 Microbiological analysis

In this study, two commercial native maize brans were used to prepare spontaneous laboratory sourdoughs. The values of microbial counts as well as the pH during fermentation are shown in Table 2. Fermentation was characterized by a rapid increase in LAB number after the first day of bran fermentation, reaching levels of 108-109 CFU/g, for both bran samples; these levels remained constant during all refreshment steps. The trend for the yeasts paralleled those of the LAB population throughout fermentation. From an initial concentration of 102-103 CFU/g an increasing and gradual

growth was observed, reaching levels of 10^7-10^8 CFU/g, after three-five days of fermentation. The count was stable until the end of fermentation. The data obtained indicated that, even if the LAB count stayed higher than that of yeasts, LAB and yeasts had similar growth throughout the fermentation. Contrarily to these fermenting microorganisms, the molds population, with initial load of 10^4 CFU/g, decreased rapidly after two-three days of fermentation.
Referring to NLAB, they increased of about four and five log cycles in C1 and C2 fermentation respectively. This high value remained constant in C2 fermentation, until the end of the refreshments, while in C1 fermentation decreased after nine days, at about 10^3 CFU/g. The observed different trend could be related to a lower pH value measured in C1 fermentation, which decreased from 5.1 at the beginning of the fermentation to 3.7 after two refreshment steps; it could contribute to control the growth of non-acidophilic bacteria.

3.2 Molecular characterization of microbial isolates

With the aim to characterize the natural microbial population involved in maize bran fermentation, 135 colonies of presumptive LAB were selected from different MRS plates, obtained by the analysis of the two different maize bran samples subjected to sourdough like fermentation process, as previously reported. Specifically, 68 isolates (36 cocci and 32 rods) from C1 and 67 isolates (15 cocci and 52 rods) from C2 samples were selected.

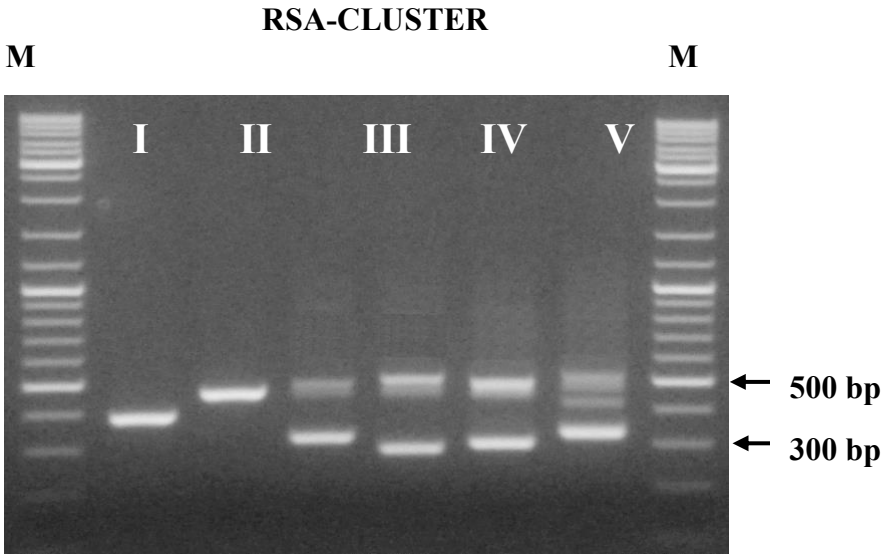

Figure 1 RSA profiles of representative LAB isolates of each cluster obtained. M= DNA ladder mix.

The 135 new isolates were submitted to molecular analysis for their identification. A first clustering step was reached by PCR amplification of the 16S–23S rRNA spacer region (RSA). Within 51 coccal isolates, three different profiles were obtained (Fig. 1). Clusters I and II grouped three isolates each one, whose profiles were referable to Lactococcus lactis and Leuconostoc lactis species, characterized by one band migrating approximately at 380 and 460 bp, respectively; these profiles were confirmed by species-specific amplifications (Lee et al. 2000; Pu et al. 2002). Cluster III grouped the majority of the coccal isolates (88 %); the RSA profile, characterized by three bands of about 310, 480, 500 bp, was associated to Pediococcus genus. A genus-specific PCR showed positive signals for all 45 isolates (Pfannebecker et al. 2008). Through 16S rRNA analysis of representative strains of the

cluster, specific PCR for *P. pentosaceus* (Pfannebecker et al. 2008) and a PCR assays developed in this study for identification of *P. argentinicus* (based on primer set designed within variable regions of *23S* rRNA gene (Table 1), seven strains have been ascribed to *P. argentinicus* species and the remaining 38 isolates to *P. pentosaceus*. *P. argentinicus*, isolated from Argentinean wheat flour is a described species of *Pediococcus*, showing a high phylogenetic relatedness with *P. pentosaceus* (De Bruyne et al. 2008). Fig. 2a shows the RAPD-PCR banding patterns of the 45 *Pediococcus* isolates; a high degree of genetic variability can be observed.

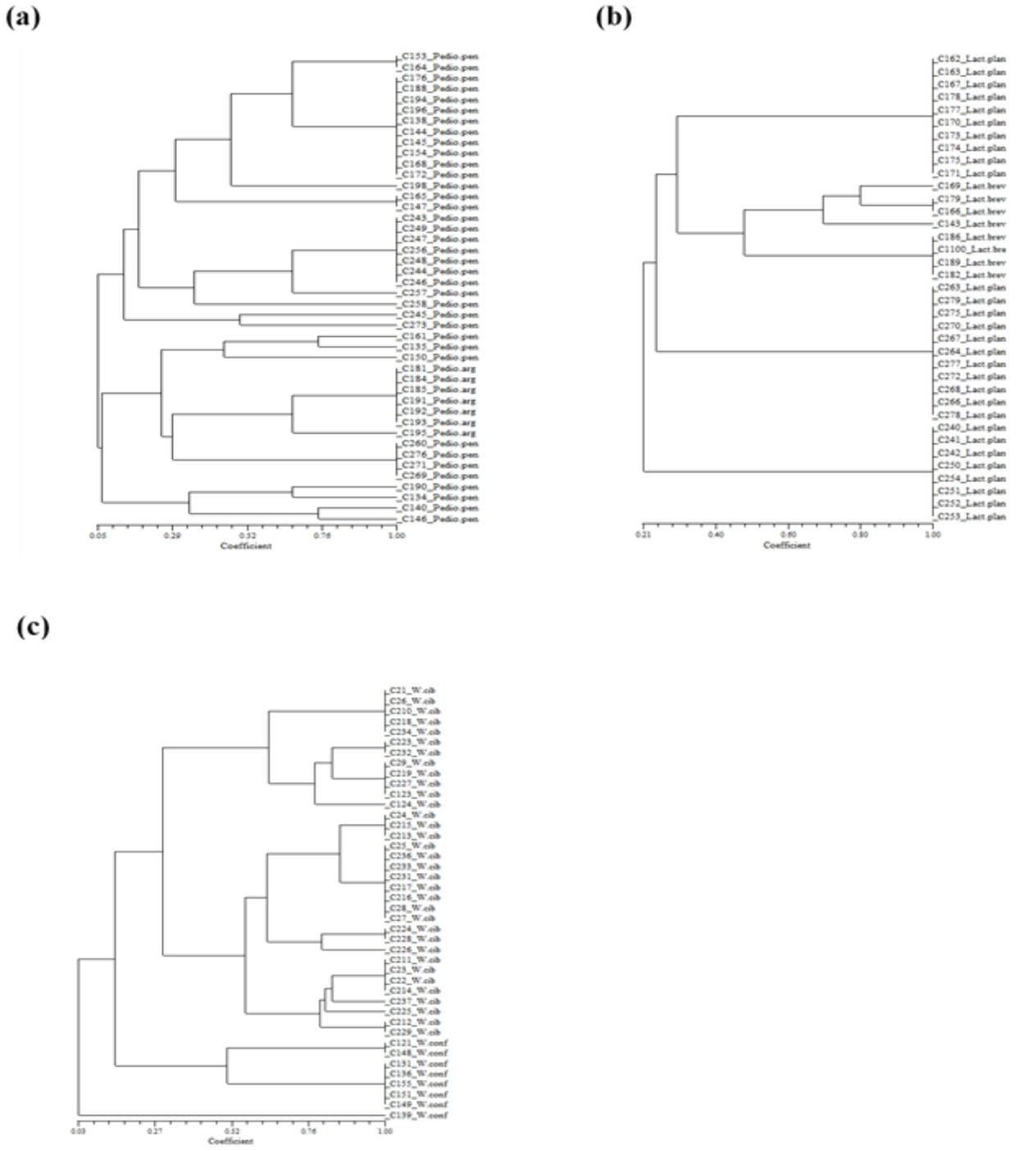

Figure 2 RAPD-PCR profiles of LAB isolated during maize bran sourdoughs fermentation. Primers M13 (a and b) and LP1 (c)

The 84 rod isolates were grouped in three different clusters (IV, V, VI in Fig. 1). Clusters IV and V included 29 and 8 isolates respectively, whose characteristic profiles for the species *Lactobacillus plantarum* and *L. brevis* have been confirmed by species-specific amplifications (Torriani et al. 2001; Coton et al. 2008). Their RAPD profiles are presented in Fig. 2b. Differently from the high polymorphism found in *Pediococcus*, the degree of intra-species variability in *L. plantarum* was very low, despite the isolates came from two different samples of bran and from different refreshments. Cluster VI grouped 47 isolates with a RSA profile constituted by three bands of about 340, 440 and 530 bp: *16S* rRNA sequencing of a representative strain of the cluster indicated the belonging to the species *Weissella confusa.* A subsequent genus-specific PCR (Jang et al. 2002) showed positive signals for all 47 isolates. Since no species-specific probes are available for the identification of *Weissella* species, we carried out L-arabinose utilization test and a restriction analysis of the *16S* rDNA with restriction endonucleases able to underline the polymorphism existing within the gene of related species (*Alu*I, *Dra*I and *Cfo*I) (Table 3). The results obtained permitted to ascribe 12 isolates to *W. confusa* (L-arabinose negative) and 35 isolates to *W. cibaria* (L-arabinose positive), the species most phylogenetically related to *W. confusa.* Their differentiation was also obtained by RAPD-PCR experiments with primer LP1 (Fig. 2c).

A total of 12 NLAB isolates were collected from CASO agar plates. The isolates from the refreshments of dough C1 belonged to the Gram negative *Acetobacter* genus, being *A. cibinongensis* (two strains) and *A. orientalis* (one strain) the isolated species, along with *Enterobacteriaceae*, primarily *Enterobacter asburiae* (two strains), *E. ludwigii* (one strain), *E. cloacae* (one strain) and then *Escherichia coli* (one strain). Differently, NLAB identification from the doughs of maize bran C2 demonstrated a representative presence of Gram positive bacteria belonging to *Staphylococcus* genus, being *S. warneri* (two strains) and *S. pasteuri* (one strain) the isolated species. The next step was the identification of the 93 yeast isolates (51 from C1 and 42 from C2). Also, in this case a first clustering has been obtained through the amplification of the Internal Transcribed Spacers. Four clusters were distinguishable: cluster I grouped five isolates with a PCR product of 440 bp, cluster II 32 isolates with a PCR product of 600 bp, cluster III 12 isolates with a PCR product of 720 bp and cluster IV 44 isolates with a PCR product of 740 bp. *26S* rDNA gene sequencing of representative strains of each cluster allowed to ascribe the isolates of the four clusters to the species *Pichia fermentans, Wickerhamomyces anomalus, Kluyveromyces marxianus* and *Kazachstania unispora,* respectively. A further digestion of the amplified ITS products with *Taq*I and *Hin*fI, permitted the obtainment of a specific restriction profile for all isolates of the same species (Table 3). Finally, three representative fungal isolates were identified as *Mucor circinelloides*, *Mucor irregularis* and *Fusarium verticillioides*. These fungal species, causing several diseases and producing a wide range of mycotoxins, have been associated with various cereal crops and processed grains (Murillo-Williams and Munkvold, 2008; Pitt and Hocking, 2009).

Table 2 Viable cell counts and pH values during spontaneous fermentation of maize bran C1 and C2.

Days	Maize bran C1					Maize bran C2				
	Contaminants	Yeasts	Moulds	LAB	pH	Contaminants	Yeasts	Moulds	LAB	pH
0	4.5 ± 0.2	2.5 ± 0.7	3.7 ± 0.1	3.6 ± 0.6	5.1 ± 0.1	3.3 ± 0.1	2.0 ± 0.5	4.0 ± 0.1	3.1 ± 0.3	5.1 ± 0.1
1	8.8 ± 0.3	4.0 ± 0.4	2.9 ± 1.3	8.8 ± 0.1	4.0 ± 0.1	7.8 ± 0.2	5.5 ± 0.4	4.7 ± 0.1	8.1 ± 0.1	5.2 ± 0.1
2	8.4 ± 1.3	5.0 ± 0.4	3.8 ± 0.3	9.0 ± 0.2	3.7 ± 0.1	7.8 ± 0.3	7.8 ± 0.3	2.0 ± 1.3	9.2 ± 0.3	4.3 ± 0.1
3	8.6 ± 0.6	6.1 ± 2.2	3.0 ± 1.4	9.0 ±0.0	3.7 ± 0.1	8.2 ± 0.3	8.2 ± 1.2	2.0 ± 0.3	9.1 ± 0.2	4.3 ± 0.1
4	8.4 ± 1.5	6.9 ± 0.2	2.5 ± 0.7	9.1 ± 0.2	3.7 ± 0.1	8.0 ± 0.6	8.1 ± 0.4	2.0 ± 1.4	9.0 ± 0.2	4.5 ± 0.1
5	8.4 ± 0.4	7.2 ± 0.4	2.5 ± 0.7	8.8 ± 0.1	4.0 ± 0.1	7.9 ± 1.2	7.8 ± 0.4	2.0 ± 0.7	9.1 ± 0.1	4.3 ± 0.1
6	7.9 ± 0.9	7.4 ± 0.3	2.5 ± 0.7	8.8 ± 0.4	3.7 ± 0.1	7.6 ± 0.3	7.8 ± 0.2	2.0 ± 0.7	8.9 ± 0.1	4.3 ± 0.1
7	7.7 ± 0.6	7.7 ± 0.0	2.5 ± 0.7	9.1 ± 0.3	3.7 ± 0.1	8.0 ± 0.4	8.0 ± 0.3	2.0 ± 0.7	9.3 ± 0.4	4.3 ± 0.1
8	7.6 ± 1.8	7.8 ± 0.1	2.5 ± 0.7	9.1 ± 0.2	4.0 ± 0.1	7.8 ± 0.6	7.7 ± 0.1	2.0 ± 0.7	9.2 ± 0.2	4.3 ± 0.1
9	6.8 ± 1.5	7.9 ± 0.8	2.5 ± 0.7	9.0 ± 0.1	3.7 ± 0.1	7.6 ± 1.6	7.6 ± 0.8	2.0 ± 0.7	9.1 ± 0.1	4.1 ± 0.1
10	3.3 ± 1.8	7.2 ± 0.8	2.7 ± 0.4	8.9 ± 0.3	3.7 ± 0.1	7.8 ± 1.3	7.8 ± 0.5	2.0 ± 0.7	9.2 ± 0.1	4.1 ± 0.1
11	3.5 ± 0.7	7.6 ± 0.0	2.5 ± 0.7	8.7 ± 0.0	3.7 ± 0.1	8.1 ± 0.8	7.6 ± 0.2	2.0 ± 0.4	9.3 ± 0.4	4.1 ± 0.1
12	3.3 ± 0.4	7.6 ± 0.1	2.5 ± 0.7	8.6 ± 0.0	3.7 ± 0.1	7.7 ± 0.7	7.6 ± 0.1	2.0 ± 0.7	9.2 ± 0.1	4.3 ± 0.1

3.3 Dynamics of microbial population during fermentation

Species composition and LAB and yeasts succession during the fermentation of the two samples of maize bran are reported in Fig. 3. As shown, the fermentation of both bran samples was characterized by a microbial succession. The initial stage of the fermentation was characterized by co-dominance of *Weissella* sp. and *Pediococcus* sp. in C1 sample and predominance of *Weissella* sp. in C2 sample. *W. anomalus* was the dominant yeast species in both fermentations. At the refreshment step 7, a natural selection was produced, with the disappearing of *Weissella* in both samples and the presence of *L. plantarum* (53% of the isolates in C1 and 45% in C2 samples), who became the main species in C2 (69%) at refreshment step 12. In C1 samples a concomitant presence of *L. brevis* was observed. *P. pentosaceus*/*argentinicus* occurred at all stage of refreshment, even if with different incidence. Regarding yeast succession, *W. anomalus* disappeared from the community, while *K. unispora* was subsequently detected. In C1 fermentation this was the only yeast found at the end of the fermentation; in C2 sample a significant presence of *K. marxianus* (56% and 19% at refreshment steps 7 and 12 respectively) was also observed.

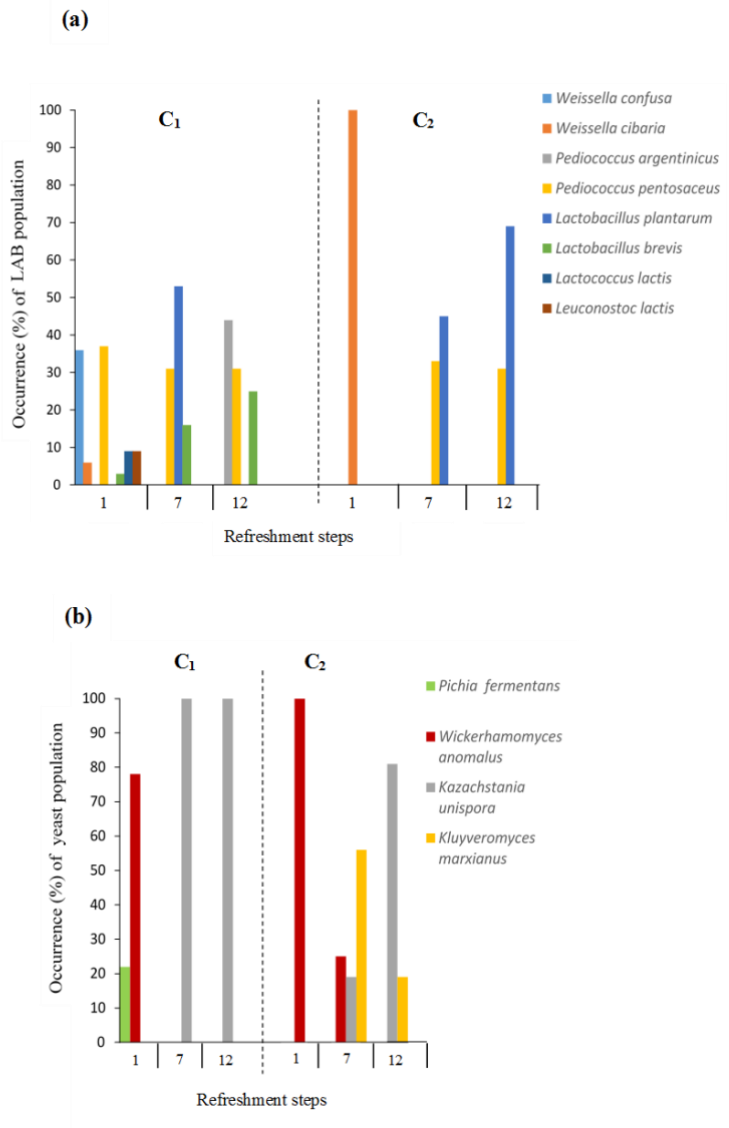

Figure 3 Changes in microbial community structure during sourdough-like fermentation of maize bran

3.4 Characterization of native maize bran and fermented bran

Table 4 reports the proximate composition detected in native and fermented samples. In native maize bran C2, ash, protein and sugars content were significantly (p<0.05) higher than in C1, differences that could probably be related to cultivars and pedo-climatic conditions (Sokrab et al. 2011). At the end of the sourdough-like fermentation process (refreshment 12), both the tested brans contained slightly higher amounts of lipid and significantly lower (p<0.05) protein levels from native sample only for C2. As expected, sourdough-like fermentation resulted in a significant (p<0.05) decrease of the total content of sugars. On the other hand, this process induces a threefold significant (p<0.05) increase in soluble dietary fiber in both C1 and C2 samples, without affecting their insoluble fraction. Regarding TOTAX, the results obtained suggest that bran sourdough-like fermentation contribute to their solubilization, with a significant (p<0.05) reduction of this hemicellulosic fraction, accompanied only by a slight increase of WEAX. The arabinose to xylose ratio in WEAX, an indicator of the average degree of arabinose substitution (avDAS), showed a little reduction, indicating that, in both fermented brans, WEAX were slightly less substituted with arabinose than those present in the native brans (Damen et al. 2011; Broekaert et al. 2011). The levels of FFA detected in C1 and C2 maize bran before and after sourdough-like fermentation are shown in Fig. 4. The concentration of FFA assessed in native brans was not statistically different (0.7 ± 0.2 and 1.6 ± 0.4 mg/100g for C1 and C2 respectively) and showed a significant (p<0.05) increase after the sourdough-like fermentation (twofold and fivefold in C2 and C1 respectively). Phytate is known to chelate several nutritionally essential nutrients and can negatively influence the activity of digestive enzymes by the chelation of mineral cofactors or by interacting with protein. In accordance with our previous study on wheat (Manini et al. 2014) the sourdough-like fermentation process promoted a degradation of phytic acid (Fig. 5) also in maize brans. In particular, this antinutrient compound was significantly reduced of about 50% in both C1 and C2, likely through the activation of microbial and endogenous phytases.

4. Discussion

The continuous search for functional ingredients providing health effects and the possibility to take advantage of agro-industrial by-products have attracted great interest in using bran-enriched products. In this research, a polyphasic approach has been carried out to characterize at microbiological and chemical level maize bran in order to enhance its technological and nutritional properties.

Little data are available in literature on microbial composition of maize and maize bran: to our knowledge up to now researches were carried out on the evolution of fermenting microbiota in wheat or rye brans (Katina et al. 2007; Manini et al. 2014) and in ethnic food products, such as tarhana (Settanni et al. 2011). Very lacking information exists on microbial population during maize bran fermentation. In our study, microbial population colonizing the first steps of natural fermentation of maize bran is characterized by a predominance of species belonging to the *Pediococcus* and *Weissella* genus. These physiological groups have also been selected during maize-based spontaneously fermented doughs produced in West African countries (Oguntoyinbo et al. 2011; Okeke et al. 2015; Assohoun-Djeni et al. 2016). Even if little data are available, this microbial population could be considered maize/maize bran endogenous.

Table 4 Chemical composition (mean ± SD; expressed as % of dry weight) of native and fermented (refreshment 12) brans. Data in the same row not sharing common letters are significantly different ($p<0.05$).

	Native maize bran C1	Fermented maize bran C1	Native maize bran C2	Fermented maize bran C2
Ash	0.9 ± 0.0^{a}	1.1 ± 0.0^{a}	2.2 ± 0.1^{b}	1.8 ± 0.2^{c}
Proteins	6.4 ± 0.3^{a}	7.2 ± 0.2^{a}	9.0 ± 0.3^{b}	7.7 ± 0.5^{a}
Lipids	2.7 ± 0.0	4.2 ± 0.1	3.5 ± 0.8	4.6 ± 0.2
Sugars	1.7 ± 0.1^{a}	0.2 ± 0.0^{b}	4.3 ± 0.4^{c}	0.5 ± 0.0^{b}
Glucose	0.3 ± 0.0	0.2 ± 0.0	2.6 ± 0.2	0.2 ± 0.0
Fructose	0.3 ± 0.0	nd*	1.4 ± 0.2	0.2 ± 0.0
Sucrose	1.2 ± 0.1	nd	nd	nd
Maltose	nd	nd	0.4 ± 0.0	nd
Insoluble Fiber	35.2 ± 3.0	36.0 ± 1.8	41.2 ± 3.7	45.6 ± 1.6
Soluble Fiber	0.6 ± 0.1^{a}	1.7 ± 0.4^{b}	0.7 ± 0.2^{a}	2.3 ± 0.3^{b}
Total arabinoxylans (TOTAX)	20.1 ± 1.5^{a}	13.1 ± 0.1^{b}	21.3 ± 1.1^{a}	10.6 ± 1.2^{b}
Arabinose/Xilose in TOTAX	0.75	0.74	0.70	1.28
Sol. Arabinoxilans (WEAX)	0.1 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
Arabinose/Xilose in WEAX	1.43	1.07	2.98	2.35

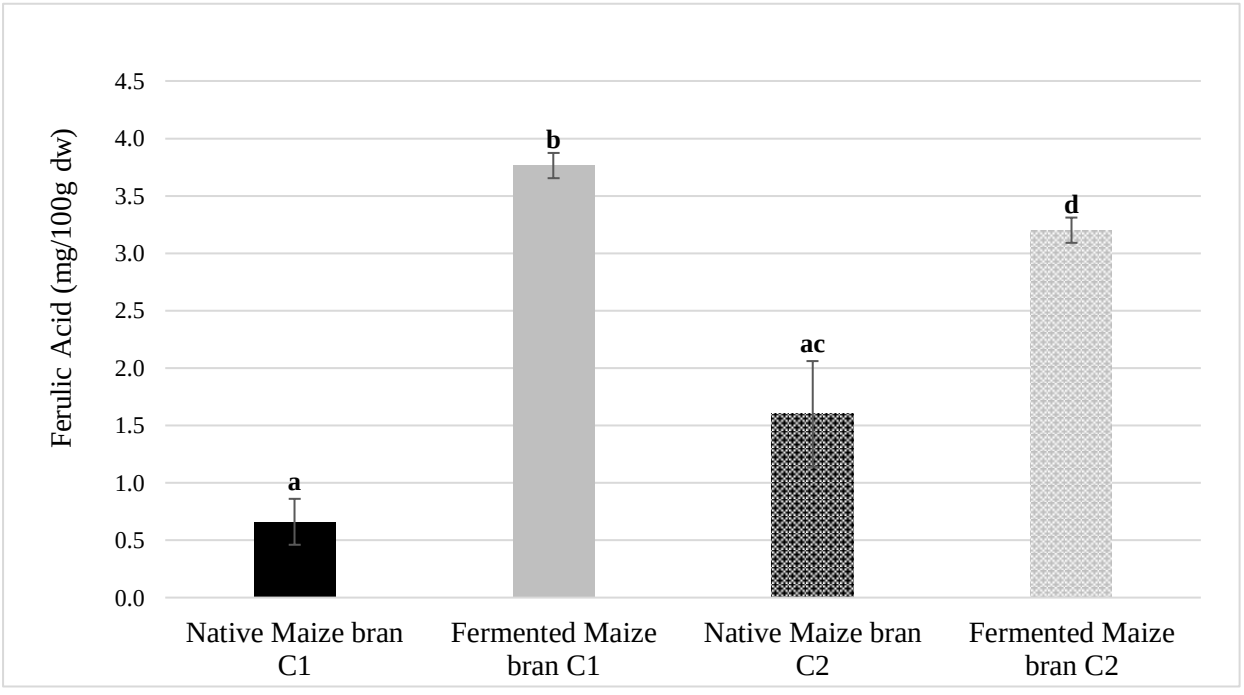

Figure 4. Free Ferulic Acid (mean ± SD; expressed as mg/100 g of dry weight) content in native and fermented (refreshment 12) brans. Bars not sharing common letters are significantly different ($p<0.05$).

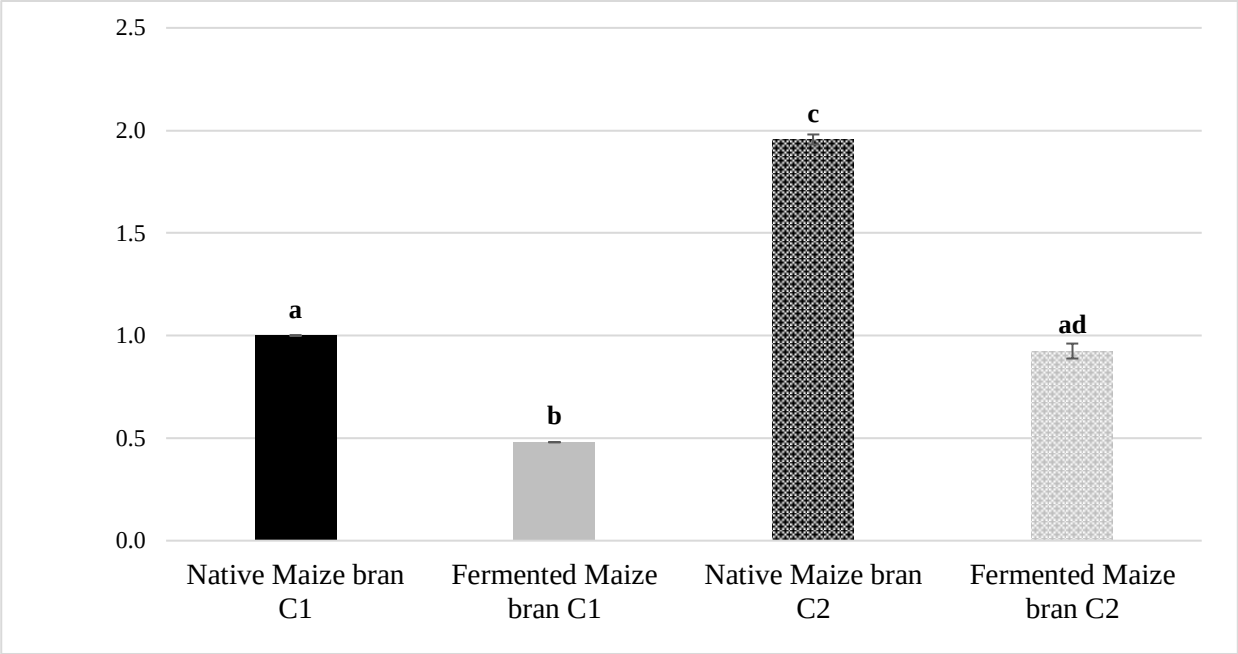

Figure 5. Phytic acid (IP6) (mean ± SD; expressed as g/100 g of dry weight) content in native and fermented (refreshment 12) brans. Bars not sharing common letters are significantly different ($p<0.05$).

During daily propagation of sourdoughs, *L. plantarum* became the main LAB species. *L. plantarum* is a versatile and competitive species, that can adapt to different environmental conditions (Minervini et al. 2010) and these features can explain its presence, as member of the complex microbiota, in many sourdoughs (De Vuyst et al. 2014). The interest dedicated to this species, particularly in the bakery sector, is related to the ability of producing antimicrobial compounds, especially antifungal metabolites (Crowley et al. 2013).

During the maize bran fermentation, we found several species of yeasts, some of who predominate in the first step of fermentation, as *W. anomalus*, other at the end of the refreshments, as *K. unispora*. This is the first report on association between LAB and yeasts in maize bran fermentation process. *W. anomalus* was shown to inhibit the growth of fungi in airtight-stored cereal grains (Coda et al. 2011). Its presence in maize bran sourdoughs, in association with *L. plantarum* could explain the decrease of vital molds we observed during fermentation. *K. unispora* plays a significant role both in cheese ripening and in fermented milk production; it has been documented to exist in few sourdough ecosystems, as artisan wheat and rye sourdough albeit in low abundance (Bessmeltseva et al. 2014). On the contrary, our data suggest that this species can be considered representative of the yeast microbiota selected during maize bran refreshment steps.

Evident differences in dominant NLAB were found between C1 and C2 samples; this could be a consequence of differences in the environmental conditions (i.e., temperature, water, and maize provenance) and in the evolution of dough pH; this parameter has a strong and decisive influence on the control and selection of bacteria. Among the identified NLAB, the family *Enterobacteriaceae* was the predominant in C1 doughs, followed by *Acetobacter* genus, differently from the doughs C2 where *Staphyloccocus* genus was the prevalent. Regarding *Enterobacteriaceae,* strains of *E. asburiae* were previously identified among thermotolerant wheat associated bacteria from a peninsular zone of India (Verma et al. 2016) and one *E. ludwigii* strain isolated from *Lolium perenne* rhizosphere showed plant growth promoting properties (Shoebitz et al. 2009). The genus *Acetobacter* has been previously

reported to dominate, together with the genus *Lactobacillus*, the first step of fermentation of whole crop maize silage (Sträuber et al. 2012). Different species of the genus *Acetobacter* were also found in a preliminary study to explore the bacterial microbiota in Colombian maize fermented dough *Masa Agria* (Chavez-Lopez et al. 2016). Lactic and acetic acid provide sour taste in maize, but, at the same time, these fermentation products promote the activation of the phytases naturally present in grains and in bacteria, by a decrease of the pH value. The action of phytases plays a key role in improving nutritional value by increasing the bioavailability of essential dietary minerals. In addition, as it was observed in this study, *Acetobacter* species can be present in the later stage of cereal fermentation for their ability to utilize molecules other than sugars and to tolerate low pH conditions (Chavez-Lopez et al. 2016). Our findings on *Staphylococcus* isolated from C2 doughs are in agreement with other researchers who stated that *Staphylococcus* sp. bacteria were isolated and identified from fresh yellow grains and roots of maize in Nigeria (Orole and Adejumo 2011). Their persistence during the fermentation steps can be explained by the higher pH values profile of C2 doughs (4.3 - 4.1) that did not lead to selective environmental conditions.

The pH reduction during the process activated the enzymatic activities related to the microbial consortium characterizing spontaneous maize bran fermentation, providing a "destructuration" of the fiber fraction and a reduction of phytic acid content in both the tested brans. Moreover, the fermentation process provided for an increase in the soluble dietary fiber that can be attributed to microbial exo-polysaccharides production, as evidenced in previous studies on wheat and sorghum sourdoughs (Galle et al. 2010; Ganzle 2014). Our results also suggest that bran fermentation promoted AX solubilization since the percentage of WEAX/TOTAX was increased from 0.5% (native bran) to 2% (fermented bran). The data obtained appears interesting from a nutritional point of view, since soluble fiber positively affect post-prandial glucose metabolism and satiety (Anderson et al. 2009; Dikeman and Fahey 2006).

As already reported (Katina et al. 2006), endogenous and microbial xylanases could have a key role in fiber solubilization. Xylanase activity has been found in a *P. fermentans* strain (Madrigal et al. 2013), in *Weissella* spp. strains isolated from Indian fermented foods (Patel et al. 2013) and in *L. plantarum* and *P. pentosaceus* starter cultures (Laitila et al. 2006). These microorganisms were detected in all refreshment steps, suggesting a potential role of their enzymatic pool in increasing WEAX concentration, as assessed in fermented brans. Moreover, even if maize bran has been reported as a substrate resistant to enzymatic digestibility (Agger et al. 2010), our results suggest an AX "destructuration", during maize bran fermentation. These data, interesting from a functional/nutritional point of view, deserve to be deepened by further studies.

Ferulic acid is a bioactive antioxidant compound abundant in maize bran (Zhao and Moghadasian 2008), where it is strictly cross-linked to arabinose residues in AX (Saulnier et al 1999). The suggested destructuration of cell walls material, could also partly explain the increased FFA content that we assessed in fermented brans. In addition to microbial endoxylanases, other degrading enzymes, such as arabinofuranosidases, feruloyl esterases, acetyl esterases, and alpha-glucuronidases, can be involved (Grootaert et al. 2007). Even if these enzymatic activities are poorly studied in *Lactobacillus* genus, a few papers show the presence of feruloyl esterases in *L. plantarum* strains (Esteban-Torres et al. 2015) and of arabinofuranosidases in selected strains of *L. brevis* (Michlmayr et al. 2013).

Phytic acid is the major storage form of phosphorous comprising 1–5% by weight in cereals, located in bran fraction and pericarp in rice and wheat, and found mainly in the germ of maize kernels (Schlemmer et al. 2009). From a nutritional point of view, this compound is considered an

antinutritive factor, for its ability to chelate dietary minerals, thus reducing their bioaccessibility and bioavailability. Our data show that the fermentation process promotes a significant reduction of phytic acid, likely related to an activation of endogenous phytases and to the presence of specific microbial biotypes able to produce significant extracellular phytase activities.

In conclusion, the sourdough-like fermentation of maize bran seems a suitable bio-approach in promoting nutritional and functional properties of this cereal by-product. Further studies are ongoing for the characterization of the isolated microorganisms and for understanding their role in specific activities related to the enhancement of the properties of fermented maize bran as well as their safety. These studies will allow the selection of starter cultures, according to their metabolic and enzymatic activities, in order to conduct "tailored" fermentation process and improve brans or whole-meal flours from nutritional, functional and technological points of view.

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Chapter 3. *Exploiting synergies of sourdough and antifungal organic acids to delay fungal spoilage of bread*

[Results obtained from Visiting Research at Dept. of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, CA; tutor: Professor Michael Gänzle]

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In this chapter we studied a possible application of antifungal LAB in sourdough bread making, firstly defining the antimicrobials MICs and then setting up new formulations. This research project was conducted at Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton (CA), under the tutorship of the kind Professor Michael Gänzle.

ABSTRACT

Fungal spoilage of bread remains an unsolved issue in bread making. This work aimed to identify alternative strategies to conventional preservatives in order to prevent or delay fungal spoilage of bread. The minimum inhibitory concentration (MIC) of bacterial metabolites and chemical preservatives was evaluated *in vitro* and compared to their *in-situ* activity in baking trials. Calcium propionate, sorbic acid, 3-phenyllactic acid, ricinoleic acid, and acetic acid were tested both individually and in combination at their MIC values against *Aspergillus niger* and *Penicillium roqueforti*. The combination of acetic acid with propionate and sorbate displayed additive effects against the two fungi. For these reasons, we introduced sourdough fermentation with specific strains of lactobacilli, using wheat or flaxseed, in order to generate acetate in bread. A combination of *Lactobacillus hammesii* and propionate reduced propionate concentration required for shelf life extension of wheat bread 7-fold. Flaxseed sourdough bread fermented with *L. hammesii*, excluding any preservative, showed a shelf life 2 days longer than the control bread. The organic acid quantification indicated a higher production of acetic acid (33.8 ± 4.4 mM) when compared to other sourdough breads. Addition of 4% of sucrose to sourdough fermentation with *L. brevis* increased the mould free shelf-life of bread challenged with *A. niger* by 6 days. The combination of *L. hammesii* sourdough and the addition of ricinoleic acid (0.15% or 0.08%) prolonged the mould free shelf-life by 7-8 days for breads produced with flaxseed or wheat sourdoughs. In conclusion, the *in vitro* MIC of bacterial metabolites and preservatives matched the *in situ* antifungal effect. Of the different bacterial metabolites evaluated, acetic acid had the most prominent and consistent antifungal activity. The use of sourdough fermentation with selected strains able to produce acetic acid allowed reducing the use of chemical preservatives.

Keywords: Bread, fungal spoilage, propionic acid, acetic acid, lactobacillus; flaxseed; ricinoleic acid.

1. Introduction

Fungal spoilage is a key limiting factor for the shelf life of bread and causes considerable economic losses. Bakery products are easily colonized by fungal conidiospores from diverse genera including *Aspergillus*, *Cladosporium*, *Endomyces*, *Penicillium*, and *Rhizopus* (Dal Bello et al., 2007). Conidiospores of filamentous fungi are ubiquitous in the biosphere and are dispersed by air unless contamination is controlled by clean room technology (Denyer and Baird, 2006). The water activity and pH of bread support growth of mycelial fungi on bread that is stored at ambient temperature (Belz

et al., 2012; Zhang et al., 2010). Refrigeration delays fungal growth but also accelerates starch retrogradation and bread staling (Gray and Bemiller, 2003).

UV light and pulsed light technology reduce spore contamination of bread but find only limited commercial application (Smith et al., 2004). Chemical preservatives are more commonly used to extend the shelf life of bread. Ethanol vapors delay germination of fungal spores (Salminen et al., 1996), calcium propionate and sorbic acid are widely used as preservatives in pre-packed and sliced bread (Smith et al., 2004). However, the use of preservatives conflicts the aim to develop "clean label" products that avoid the use of additional chemicals. (Anonymous, 2018a and 2018b).

Lactic acid bacteria are used in baking applications as leavening agents, to achieve dough acidification, or to improve specific quality attributes of bread (Gobbetti et al., 2014; Hammes and Gänzle, 1998). Lactic acid bacteria produce metabolites with antifungal activity; however, their antifungal metabolites are uncharacterized, unproven in food, or negatively impact bread flavour (Axel et al., 2017; Black et al., 2013; Quattrini et al., 2018). Acetic acid produced in primary carbohydrate metabolism has antifungal activity but also impacts flavour and texture of bread (Drews, 1953; Gerez et al., 2009; Kaditzky et al., 2008). The levels of acetic acid produced in sourdough fermentations is readily adjusted by addition of pentoses, or by addition of sucrose as electron acceptor in heterofermentative metabolism (Gänzle, 2015). fermentation of *L. diolivorans* and *L. buchneri* produced propionic acid in sourdough; however, propionic acid also impacts bread flavour when added at effective concentrations (Zhang et al., 2010). 3-Phenyllactic acid and cyclic dipeptides have antifungal activity *in vitro* but their contribution to the inhibition of fungal growth on bread remains unproven (Axel et al., 2017; Ryan et al., 2009a and 2009b; Vermeulen et al., 2006). Hydroxylated unsaturated fatty acids have antifungal activity in bread but their accumulation to active concentrations in sourdough remains to be demonstrated (Liang et al., 2017; Black et al., 2013). *In situ* preservative effects of lactic acid bacteria have often been attributed to synergistic activities of uncharacterized compounds (Axel et al., 2017; Mandel et al., 2013).

Plant-derived antifungal compounds support the antifungal activity of bacterial metabolites. For example, hop extract was recently demonstrated to be an effective antifungal ingredient in bread making (Nionelli et al.,2018); compounds with antifungal activity isolated from legume flours (*Pisum sativum*, *Phaseolus vulgaris*) were also successfully employed to extend the mould-free shelf life of what bread (Rizzello et al., 2015 and 2017). Flaxseeds have a high oil content with a high proportion of linoleic acid, a substrate for enzymatic or microbial conversion to antifungals fatty acids (Black et al., 2013). The microbial and enzymatic conversion products, 10-hydroxy-12-octadecenoic acid and coriolic acid, respectively, have similar antifungal activity acid (Black et al., 2013; Liang et al., 2017).

The use of multiple antifungal metabolites to exploit synergies improves the antifungal effect of sourdough while minimizing the impact of organic acids on bread flavour (Ryan et al., 2008; Zhang et al., 2010). However, synergistic effects of different antifungal metabolites have not been systematically assessed by comparison of the correlation of *in vitro* MIC and *in situ* preservative effects (Axel et al., 2017). This study therefore aimed to compare the inhibitory concentration of antifungal compounds to their antifungal effect in bread. Antifungal compounds were assessed bread produced with straight dough process, and in sourdough bread. Wheat sourdoughs were compared to flaxseed sourdoughs.

2. Materials and methods

2.1 Strains and growth conditions

Lactobacillus hammesii DSM16381 from French sourdough (Valcheva et al., 2006) and *Lactobacillus plantarum* C264 and *Lactobacillus brevis* C186 from maize bran (Decimo et al., 2017) were cultivated on modified MRS (mMRS) medium (Black et al., 2013) 30 °C. Representative of common fungal spoilage in bread *Aspergillus niger* FUA5001 and *Penicillium roqueforti* FUA5005 were used as target strains for the antifungal assay, *P. roquefortii* is an isolate from mouldy bread with high resistance to antifungal interventions (Zhang et al., 2010). Fungal strains were cultivated on malt extract agar medium at 25 °C for 72 h, and spores were collected by adding physiological solution (0.85% NaCl, 0.01 % Tween80). After filtration with Whatman N.1 filter paper, the suspensions were stored at -20° C until further use. Spore suspensions were diluted to proper spore density (10^2 or 10^4 spores/mL) counted with a hemocytometer (Fein-Optik, Jena, Germany).

2.2 Antifungal activity assay

Minimum inhibitory concentrations (MIC) were determined with serial 2-fold dilutions of ricinoleic acid, 3-phenyllactic acid, acetic acid, calcium propionate and sorbic acid (Merck, Darmstadt, Germany) in 96-well microtiter plates (Magnusson and Schnürer, 2001). In the MIC assays, the pH was controlled at pH 4.5 by adjustment of the pH of the medium and the stock solutions of antifungal compounds. Microtiter plates were inoculated with mMRS broth containing 10^4 spores/ mL of *A. niger* or *P. roqueforti* and incubated at 25 °C for 5 days. The MIC was determined as the lowest concentration of compound inhibiting the mould growth. Ethanol, which was used as solvent for ricinoleic acid, was removed by evaporation under a laminar flow hood before the addition of the fungal spores.

A checkerboard procedure (Gänzle et al., 1999) was carried out to determine the combined inhibitory activity of two compounds. The plates were inoculated and incubated at 25 °C for 5 days. The MIC was determined as the lowest concentration of the two compounds inhibiting the mould growth. Experiments were performed in triplicate.

2.3 Sourdough fermentation and bread preparation

L. hammesii, *L. plantarum* and *L. brevis* were used to prepare sourdough bread. Cells from an overnight culture in mMRS medium were washed twice and suspended in sterile tap water to a concentration of 10^8 CFU/mL. Sourdough was prepared by mixing white wheat flour or flaxseed flour, sterile tap water, and culture in a ratio of 2:1:1 (wt/wt/wt). The dough was fermented at 30 °C for 24 h. Samples were taken at time 0 and after 24 hours for determination of cell counts and pH values, and for quantification of organic acids. Colony morphology and uniformity were used to verify the identity of fermentation microbiota with the inoculum. Cell counts for the three strains reached 10^9-10^{10} CFU/g after 24 h.

Bread formulations shown in Tables 1 and 2. Sourdough bread was prepared with 10% addition of sourdough. Bread with chemical preservatives was prepared with different concentrations

according to MIC results. Bread making procedure was described by Black et al. (2013). After baking, the breads were cooled to 20°C on racks for 120 min, and samples were taken for challenge test, pH determination, and quantification of organic acids.

The same protocol was used in the bread experiments to investigate the antifungal effect of the combination of *L. hammesii* sourdough and ricinoleic acid, with minor modifications. Sourdough was fermented for 2 days and 50 g-flour breads (i.e. all the ingredients were used in the same proportion shown in Table 1 and 2, but half of the amount) were made for these experiments. Bread was hand-kneaded for extra 3 min after mechanical mixing. The second proofing was 85min. Bread experiment groups include control without addition of sourdough and ricinoleic acid ([control]); *L. hammesii* fermented sourdough bread with addition 2% linoleic acid during sourdough fermentation; *L. hammesii* sourdough bread with addition of 0.03%, 0.08% and 0.15% ricinoleic acid added at the bread stage, respectively.

2.4 Bread challenge test against P. roqueforti *and* A. niger

Mould challenge test was conducted as described by Black et al. (2013). Bread samples were sliced in 25-mm thick slices and inoculated with a suspension containing 10^2 spores/mL. The spore suspensions were sprayed on each corner of the slice and in the middle, delivering 90 μL of suspension or about 10 spores on each spot. The inoculated slices were placed into sterile plastic bags with filter tips ensure aerobic conditions. Slices were incubated for 12 d at 20 °C and monitored every 12 h. The last day before visible mycelial growth is reported as mould-free shelf life. The effect of chemical preservatives or sourdough fermentation or the combination of the two was determined in triplicate independent experiments (triplicate sourdough fermentation and baking). Statistical analysis was done with Tukey's test with Graphpad Software or SPSSStatistics Software. Significant differences were reported at a confidence level of *P* values of 0.05.

2.5 Quantification of acetic acid with high performance liquid chromatography (HPLC)

Acetic acid was determined by HPLC with an Aminex HPX-87 column (300 mm × 7.8 mm, Biorad, USA) at a temperature of 80 ˚C and a flow rate of 0.4 mL/min with 5 mM H_2SO_4 as the eluent. The injection volume was 10 μL. Refractive index detector and UV detector (210 nm) were used for detection. For sample preparations, 2 g of bread was diluted with 10 mL of MilliQ water and incubated for 3 h at 80 °C. After centrifugation, 7% perchloric acid were added and the solution incubated at 4 °C overnight. Precipitated protein was removed by centrifugation. The samples were filtered before injection in the column.

3. Results

3.1 MIC of preservatives and combination effects

The individual MIC for each of the five compounds was tested *in vitro* against the two indicator strains *A. niger* and *P. roqueforti* at pH of 4.5. Sorbic acid was the strongest inhibitor (0.4 ± 0.1 and

0.2 ± 0.0 mM for *A. niger* and *P. roquefortii,* respectively), followed by calcium propionate (1.3 ± 0.2 and 12.0 ± 0.0 mM), ricinoleic acid (1.7 ± 0.0 and 3.5 ± 0.0 mM) and acetic acid (8.2 ± 3.4 and 25.0 ± 5.5 mM). 3-Phenyllactic acid was the weakest inhibitor with MIC values of 30 ± 10 and 50 ± 0 mM against *A. niger* and *P. roquefortii*. Synergistic activities of acetic acid with other inhibitors were determined with checkerboard assays. Acetic acid exhibited additive activity with calcium propionate, sorbic acid and ricinoleic acid (Figure 2). MIC values of calcium propionate and acetic acid combination were lower than the individual MICs, respectively, with 0.6 + 6.2 mM against *A. niger* and 3.1 + 6.2 mM against *P. roquefortii* (Fig. 2). The combination of sorbic acid and acetic acid was active at 0.2 + 3.1 mM against *A. niger* and 0.2 + 6.2 mM against *P. roqueforti* (Fig. 2).

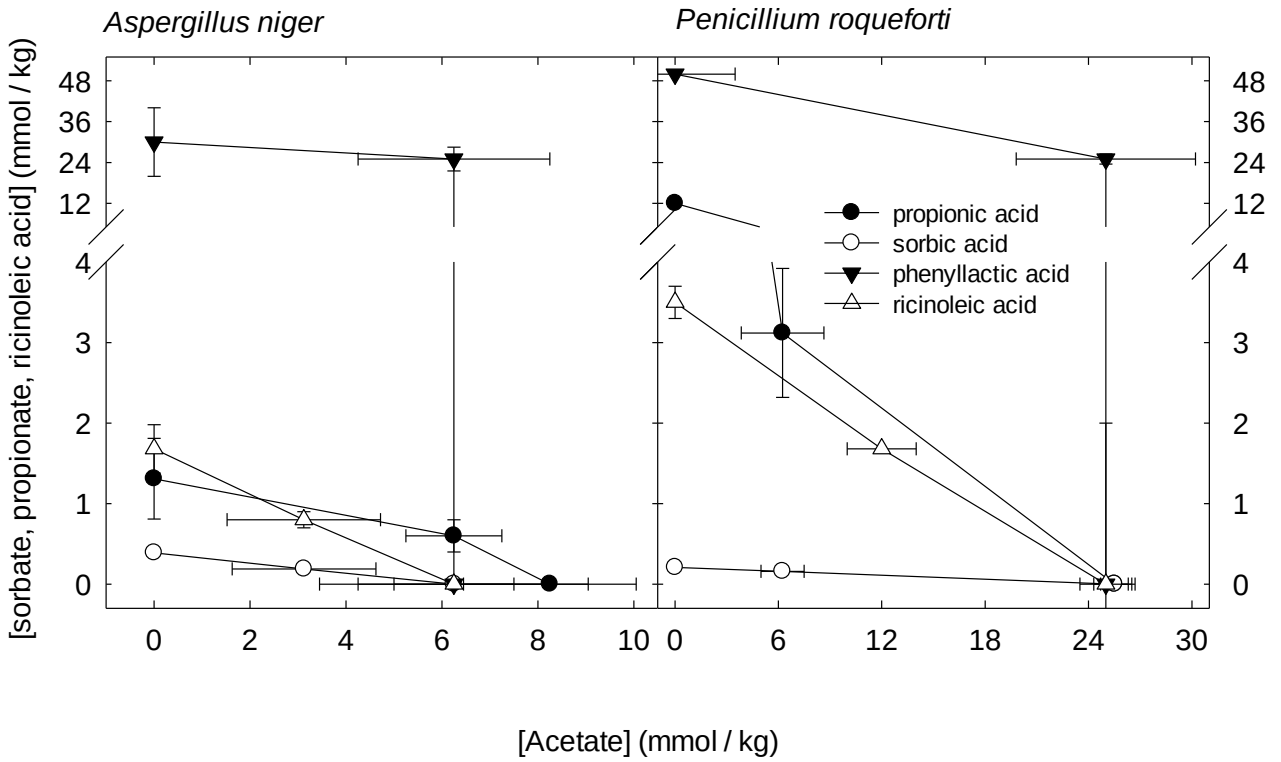


Figure 1. Minimum inhibitory concentration of acetic acid in combination with sorbic acid, propionic acid, phenyllactic acid, or ricinoleic acid. The minimum inhibitory concentrations were evaluated at a pH of 4,50. The results are shown as means ± standard deviations of three independent experiments.

3.2 Antifungal effect of organic acids addition to bread

The organic acids were used in baking trials; compounds or combination of compounds were added approximately at the level of their respective MIC. Bread was challenged by inoculation with *A. niger* or *P. roqueforti* and stored until visible mycelial growth, or for 12 days. The results are shown in Table 3. With the exception of ricinoleic acid, the results obtained *in vitro* are comparable

with the data obtained *in situ*. 3-Phenyllactic acid, the weakest inhibitor *in vitro*, showed no antifungal effect *in situ* when added at a level corresponding to 20 mmol / kg bread (Table 3). Acetate, calcium propionate and sorbic acid significantly extended the mould-free shelf life of bread; sorbic acid and acetic acid extended the shelf life by 5-6 days. Acetic acid extended the shelf life of bread by three days (*p<0.05)* in combination with propionic acid; acetic acid in combination with sorbic acid extended the shelf life only by two days (*P<0.1*) relative to the control (Table 3).
To determine whether the antifungal effects relate to the pH, the pH of breads is also shown Table 3. The pH of control bread was 5.5. Addition of acetic acid and phenyllactic acid reduced the pH to values below 4.5 while other organic acids had no major effect on the pH.

Table 3. Effect of preservatives alone or in combination on the mould-free shelf life of bread. Preservatives were added as indicated in Table 2 to match their MIC *in vitro*. Data are shown as means ± standard deviations of three independent experiments. Values in the same row that do not share a common superscript differ significantly ($p<0.05$).

Additive	Control	3-PLA	Ricinoleic acid	Acetic acid	Prop.	Sorb.	Prop. + acetic	Sorb. + acetic
pH	5.4±0.1^{a}	4.4±0.0^{b}	5.3±0.0^{a}	4.4±0.0^{b}	5.4±0.0^{a}	5.1±0.1^{a}	4.8±0.7^{ab}	4.9±0.5^{ab}
Indicator	Bread mould-free shelf life (d)							
A. niger	3.6±1.1^{b}	5.3±0.5^{b}	4.3±1.1^{b}	9.7±0.5^{a}	8.3±1.1^{a}	10.0±1^{a}	8.5±0.7^{a}	6.0±0.0^{ab}
P. roqueforti	4.3±0.1^{b}	5.0±1.0^{b}	4.7±1.1^{b}	9.3±0.5^{a}	8.0±1.0^{a}	9.0±0.7^{b}	7.5±0.3^{ab}	6.5±0.7^{ab}

PLA = 3 phenyllactic acid; Prop. = Ca propionate; sorb. = sorbic acid

3.3. Antifungal effect of sourdough addition to bread

The effect of sourdough alone or in combination with preservatives on the mould-free shelf life was also assessed in challenge studies with *P. roqueforti* and *A. niger*. A first series of sourdoughs was prepared with wheat flour, fermented with *L. plantarum*, or *L. brevis* or *L. hammesii*. Use of wheat sourdough fermented with these three lactobacilli moderately but significantly extended the shelf life of bread challenged with *A. niger* but was ineffective against *P. roqueforti* (Table 4). The acetic acid concentrations in breads produced with *L. hammesii*, *L. plantarum* and *L. brevis* sourdoughs were 12.6 ± 3.4, 13.2 ± 4.7 and 16.2 ± 2.3 mmol/kg, respectively.
The use of flaxseed sourdough in baking reduced the shelf life of bread except for sourdoughs fermented with *L. hammesii*. The acetate concentrations in bread produced with flaxseed sourdoughs fermented with *L. hammesii*, *L. plantarum* and *L. brevis* were 33.8 ± 4.4, 17.8 ± 6.3 and 23.8 ± 3.8 mmol/kg of bread, respectively, which was substantially higher than acetate concentrations obtained with wheat sourdoughs.
Addition of calcium propionate (3.1 mM) to *L. hammesii* sourdough bread prolonged the shelf life of wheat bread challenged with *P. roqueforti* and *A. niger*; the combination of *L. hammesii* sourdough with addition of sorbic acid (0.2 mM) extended the shelf life of bread challenged with *A. niger* but not with *P. roquefortii*.
To additionally evaluate the effect of acetic acid concentrations, wheat or flaxseed sourdoughs were fermented with addition of 4% sucrose. Remarkably, the addition of sucrose to sourdough did not increase the concentration of acetic acid in bread relative to the bread without sucrose addition (data

not shown). The mould-free shelf life of bread nevertheless increased, particularly for *L. brevis* sourdoughs, which increased the shelf life to 8.5 and 9 days for bread challenged with *P. roquefortii* and *A. niger*, respectively. A similar shelf-life was only obtained with the addition of chemical preservatives.

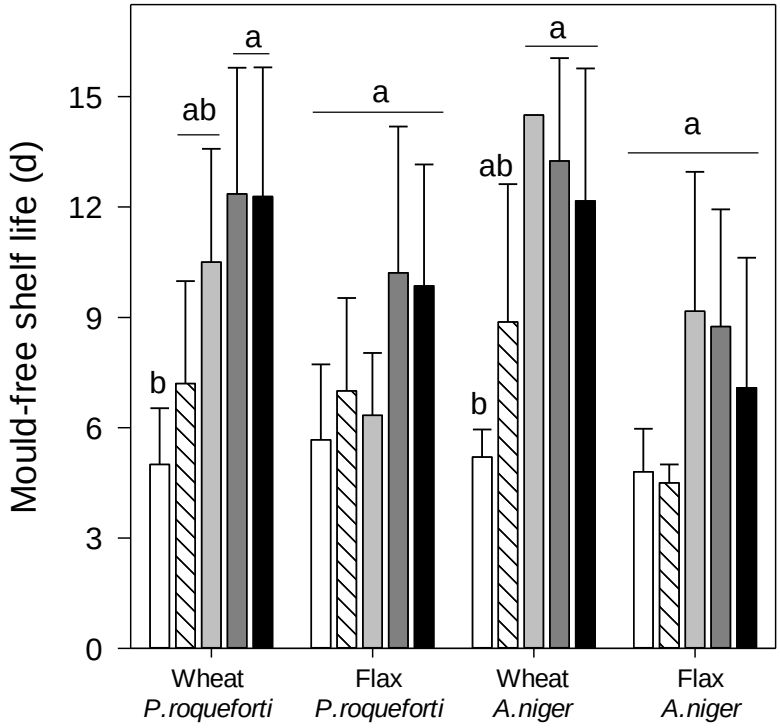
Ricinoleic acid inhibited fungal growth *in vitro* (Figure 1) but did not delay fungal growth when added as sole preservative to bread (Table 3). To determine its activity in combination with *L. hammesii* sourdough, 0.03% to 0.15% ricinoleic acid, corresponding to 1 to 5 mM, were added to bread produced with *L. hammesii* wheat and flaxseed sourdoughs. Sourdough fermented with addition of 2% linoleic acid, the substrate for formation of the antifungal 10-hydroxy-12-octadecenoic acid by *L. hammesii*, was additionally evaluated. Addition of 0.08 or 0.15% ricinoleic acid increased the shelf life of wheat bread challenged with *A. niger* or *P. roquefortii* to more than 12 days (Figure 2); addition of 0.03% ricinoleic acid was effective only against *A. niger*. Addition of linoleic acid to sourdoughs fermented with *L. hammesii* did not delay fungal growth (Fig. 2). An extension of the shelf life by sourdough in combination with ricinoleic acid was not observed in wheat bread with flaxseed sourdough; the increase of the average shelf life was less than experimental error (Figure 2).

Table 4 Effect of sourdough on the pH and the mould-free shelf life of bread. The sourdough was fermented with *L. hammesii, L. plantarum* or *L. brevis*, with or without addition of 4% sucrose; *L. hammesii* sourdough was combined with calcium propionate (3.1 mM) or sorbic acid (0.16 mM). The challenge test was with two indicator strains. Data are shown as means ± standard deviations of three independent experiments. Values obtained for different breads with the same indicator strain differ significantly if they do not share a common superscript ($p<0.05$).

	Not fermented	*L. hammesii*	*L. plantarum*	*L. brevis*	*L. hammesii* + propionate	*L. hammesii* + sorbic acid	*L. hammesii* + sucrose	*L. plantarum* + sucrose	*L. brevis* + sucrose
					A. niger				
Wheat	3.0±0.6^{c}	4.8±0.3^{b}	4.3±0.6^{b}	4.7±0.6^{b}	10.5±0.7^{a}	7.0±1.4^{a}	5.5±0.7^{b}	5.0±0.0^{b}	9.0±0.0^{a}
Flaxseed	3.0±0.0^{c}	5.0±0.6^{b}	3.6±0.6^{c}	3.7±0.6^{c}	n.d.	n.d.	6.5±0.0^{b}	5.0±0.0^{b}	9.0±0.0^{a}
					P. roqueforti				
Wheat	5.3±0.6^{b}	5.3±0.6^{b}	5.0±0.0^{b}	5.0±0.0^{b}	8.3±0.3^{a}	5.5±0.7^{b}	6.5±0.7^{ab}	5.5±0.7^{b}	8.5±0.7^{a}
Flaxseed	3.3±0.6^{c}	5.0±0.0^{b}	3.6±0.6^{c}	4.3±0.6^{c}	n.d.	n.d.	6.5±0.7^{ab}	5.5±0.7^{b}	8.5±0.0^{a}
					pH				
Wheat	5.4±0.6^{a}	4.3±0.1	4.3±0.1	4.3±0.0	4.2±0.2	4.1±0.2	4.5±0.3	4.3±0.1	4.6±0.2
Flaxseed	5.3±0.1^{a}	4.6±0.1	4.5±0.1	4.5±0.6	n.d.	n.d.	4.4±0.0	4.3±0.0	4.3±0.1

n.d., not determined.

Figure 2. Effect of sourdough in combination with ricinoleic acid on the mould-free shelf life of bread. Control bread was produced without addition of sourdough (white bars); *L. hammesii*-fermented sourdough bread was produced with addition 2% linoleic acid during sourdough fermentation (white hatched bars); or with addition of 0.03% (gray bars), 0.08% (dark gray bars) or 0.15% ricinoleic acid (black bars) added at the bread stage. Experiments were done with wheat sourdough or flaxseed sourdough as indicated and *Penicillium roqueforti*



and *Aspergillus niger* were used as challenge organisms. Data are shown as mean ± standard deviations of seven independent experiments. Values produced with the same sourdough and challenged with the same organism differ (p<0.05) if they do not share a common superscript.

4. Discussion

Bread is a perishable product and subject to rapid deterioration after baking**.** Fungal spoilage is one of the main causes of bread spoilage**.** Moreover, formation of mycotoxins production by filamentous fungi represents a health risk (Sirot et al., 2013). *P. roquefortii,* one of the challenge strains used in this study, is resistant to biological or chemical preservation; this organism also often occurs as spoilage agent in bread (Axel et al., 2017). An inoculum of 100-1000 spores per slice of bread, (Nionelli et al., 2018, Ryan et al., 2011, Zhang et al., 2010) is substantially higher than the environmental contamination in industry practice. Environmental mould contamination is difficult to control and to reproduce, however, studies on the mould-free shelf life of bread consistently demonstrate that spoilage by environmental contaminants is substantially slower and more readily controlled by preservatives when compared to bread challenged with *Penicillium* spp. (Axel et al., 2015; Belz et al., 2012; Black et al., 2013). Challenge studies with *P. roqueforti* therefore represent a worst-case scenario but nevertheless allow comparative assessment of different sourdoughs or additives.

In this work, we compared the *in vitro* MIC of antifungal bacterial metabolites and chemical preservatives. Phenyllactic acid has the weakest antifungal activity at pH 4.5. In keeping with prior observations, inhibition of fungal growth was observed only at concentrations exceeding 30 mmol / L, corresponding to 4 g / L (Axel et al., 2016; Ryan et al., 2009a). During growth in sourdough, lactobacilli produce phenyllactate from phenylalanine, however, the concentration of phenyllactate

in sourdough remains below 0.2 mmol / kg or less than 1% of the MIC (Axel et al., 2016; Ryan et al., 2009; Vermeulen et al., 2006). The combination of different organic acids displays additive rather than synergistic activity when adjusting for the pH (this study); therefore, phenyllactate is not likely to make a contribution to inhibition of fungal growth in bread.

Calcium propionate, sorbic acid, ricinoleic acid and acetic acid displayed antifungal activity in the range of 1 – 24 mmol / L and the *in-situ* activity matched the *in vitro* activity when assayed at the same pH. The pH plays a key role for the activity of weak organic acids (Lind et al., 2005). Undissociated acids penetrate the fungal membrane and acidify the cytoplasm, leading to cell death (Stratford and Eklund, 2003). The pKa of ricinoleic acid, acetic acid, sorbic acid, and propionic acid is 4.74, 4.75, 4.76, and 4.90, respectively, indicating that their activity in sourdough bread with pH < 5.0 is much higher than their activity in yeast-leavened bread with a pH of 5.5. Indeed, ricinoleic acid was ineffective in bread with a pH of 5.5 but displayed antifungal activity in sourdough bread. Sourdough fermentation thus has a double role in preservation as it accumulates antifungal organic acids and reduces the pH, thus increasing their antifungal activity.

Lactic acid bacteria produce multiple metabolites with *in vitro* activity against fungal spores, including organic acids, cyclic dipeptides, and long-chain hydroxyl fatty acids (Axel et al., 2017; Black et al., 2013; Gerez et al., 2009). The present study identified acetic acid as the most relevant antifungal compound produced by lactic acid bacteria, as it is readily accumulated to concentrations matching the MIC against fungal spores. Acetate formation by heterofermentative lactic acid bacteria can be adjusted by addition of sucrose, providing fructose to allow regeneration of co-factors and increased acetate formation in heterofermentative metabolism (Stolz et al., 1995; Gänzle, 2015). Addition of acetic acid to bread delays fungal spoilage (Drews, 1953); however, excess levels of acetic acid also result in an unacceptable flavor (Hansen and Schieberle, 2005) and interfere with development of the gluten network in wheat baking, (Kaditzky et al., 2008). Acetic acid in concetrations of 10 – 30 mmol kg^{-1} has a beneficial impact on bread flavor (Hansen and Schieberle, 2005); the current study demonstrates that this range of acetic acid concentration also substantially contributes to the mould-free storage of bread.

The combination of acetate with other antifungal compounds reduces or prevents the adverse impact of individual organic acids on bread flavour. Proof of concept was provided by prior studies using sourdough containing propionic and acetic acids (Zhang et al., 2010), or using sourdough in combination with propionate (Ryan et al., 2008). We extended prior observation by demonstrating additive activity of sourdough or acetic acid with propionic acid, ricinoleic acid and sorbic acid. The antifungal effect of acetic acid in combination with other antifungal organic acids is attributable to the additive antifungal activity of organic acids (Tables 3 and 4). In combination with acetic acid or sourdough, the propionate or sorbate concentration required for shelf life extension of wheat sourdough bread was reduced 7-fold when compared to the amount required for preservation of straight dough bread. Remarkably, ricinoleic acid was effective only in combination with sourdough.

The additive activity of *L. hammesii* sourdough and ricinoleic acid, an unsaturated hydroxy-fatty acid present in castor oil, was further explored by adding different levels of ricinoleic acid to bread produced with *L. hammesii* sourdough. The antifungal activity of ricinoleic acid is comparable to other unsaturated hydroxy fatty acids including coriolic acid and 10-hydroxy-12-octadecenoic acid, which are produced by enzymatic or microbial conversion of linoleic acid in sourdough (Black et al., 2013; Liang et al., 2017). The addition of 0.1% coriolic acid to bread also significantly increased the

mould-free shelf life of bread (Black et al., 2013). Our study demonstrates that a combination of sourdough and ricinoleic acid displayed a similar antifungal performance at a ricinoleic acid concentration of 0.08%.

Of note, sucrose addition to sourdough did not substantially increase the acetate concentration in bread. The availability of sucrose and other substrates for co-factor regeneration in sourdough supports formation of 10 – 20 mM acetate in wheat sourdough; the acetate concentration can be increased by addition of sucrose (Korakli et al., 2001). With a sourdough addition of 10%-30%, the carry-over of the acetic acid from sourdough accounts for only 2 – 6 mmol/g, and most of the acetic acid that is present in bread 10 – 20 mmol/g, was produced after the final mixing in the bread dough where sucrose levels were not different. Heterofermentative lactobacilli produce acetate rather than ethanol as long as electron acceptors are available (Korakli et al., 2001; Stolz et al., 1995). In artisanal and industrial practice, the sourdough addition to bread dough ranges from as little as 3% for high acidity, long time fermented type II sourdoughs to more than 30% for metabolically active type I sourdough with a relatively high pH and low acidity (Brandt, 2007; Ganzle and Zeng, 2018; Lacaze et al., 2007). Independent on the level of addition, however, antifungal compounds present in sourdough are diluted three-fold to more than 10-fold. Sourdoughs that are propagated in bakaries tipycally are fermented to warrant a high metabolic activity of lactobacilli in bread dough (Brandt, 2007; Lacaze et al., 2007). In brief, the impact of sourdough on the mould-free shelf life of bread necessitates metabolic activity of sourdough microbiota during proofing and hence depends strongly on the sourdough technology employed.

Replacement of wheat with other substrates for sourdough fermentation and / or baking significantly impacts the mould-free shelf life of bread (Axel et al., 2015 and 2016). Different substrates support formation of different levels of organic acids (Axel et al., 2015) and are a potential source of plant bioactives with antifungal activity (Gänzle, 2014). We explored the use of flaxseed sourdough; flaxseed is rich in linoleic acid (Dubois et al., 2007) and may support the enzymatic or microbial formation of antifungal hydroxy fatty acids from linoleic acid. In addition, flaxseed offers health benefits in relation to cardiovascular diseases that are derived from its high fibre content and the content of ω-3 fatty acids (Caligiuri et al., 2014; Cunnane et al., 1995; Kajla et al., 2015). Fungal growth on bread produced with flaxseed or flaxseed sourdoughs was equal or faster when compared to the wheat counterparts. Bread produced with flaxseed sourdoughs contained higher levels of acetate than the corresponding wheat breads; however, flaxseed also contains mucilage with high water binding capacity (Kaewmanee et al., 2014). Hydrocolloids may increase the water activity of bread and hence accelerate fungal spoilage. Our data suggest that linoleic acid bound in triglycerides does not support formation of the antifungal 10-hydroxy-12-octadecaenoic acid by *L. hammesii* in flaxseed sourdoughs. Bacterial hydration of free unsaturated fatty acids is a mechanism of detoxification (Volkov et al., 2010) and past studies aiming to convert plant oil to bioactive lipids by lactic acid bacteria employed lipase to achieve hydrolysis of triglycerides (Ogawa et al., 2005).

In conclusion, we demonstrate that the *in vitro* MIC of bacterial metabolites and preservatives is matches the *in situ* antifungal effect. We also demonstrated that the accumulation of antifungal metabolites in sourdough is a difficult proposition – because sourdough is used at a dosage of only 10 –to 20%, antifungal metabolites are relevant only if they are produced in bread dough, or if the concentration of antifungal metabolites in sourdough need to exceed the MIC 5 – 10-fold. Acetic acid is the most significant antifungal metabolite of lactobacilli, mainly because it is rapidly produced

during mixing and proofing of the bread dough and is thus present in bread at concentrations close to the MIC. Irrespective of the presence of antifungal metabolites, however, the use of sourdough greatly enhances the activity of weak organic acids through the reduction of pH, and allows to exploit additive antifungal activities of different organic acids. We demonstrated additive activity of sourdough use with sorbic acid, propionic acids, and ricinoleic acid; in addition, the study provides a conceptual template for exploration of synergistic or additive effects of sourdough with other antifungal additives or ingredients.

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Chapter 4. Safety and functional significance of *Weissella cibaria* and *W. confusa* in food: a polyphasic approach

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In this chapter we investigated the safety and the beneficial traits -biocontrol and probiotic potential- of two controversial species Weissella cibaria and Weissella confusa.

ABSTRACT

Weissella cibaria and *Weissella confusa* are controversial species of lactic acid bacteria (LAB) found in food products. They are naturally present in in many fermentation processes of vegetables and cereals, with a positive implication for the quality of food. On the other hand, *Weissella* species have been associated to possible human infections, and for this reason the strains of the species are not yet used as starter cultures and are not included in Qualified Presumption of Safety status of European Food Safety Authority (EFSA). A deepening of the physiological and genetic characteristics of *Weissella* species could help to select suitable strains for possible practical applications. A comparative genome analysis of 15 sequenced *W. cibaria* and 5 *W. confusa* genomes to date available was carried out, in parallel with a polyphasic study of twelve strains of *W. cibaria* and eight strains of *W. confusa* previously isolated from sourdough-like maize bran fermentation. The comparative genomic analysis resulted in absence of severe pathogenicity factors. Although some virulence genes were found, these, for homology and function, were present in other LAB species/strains, considered safe by EFSA and commonly used as probiotics. The phenotypic tests carried out on our strains corroborated the genomic results. Moreover, interesting functional and pro-technological traits were highlighted in the tested strains, for both the species.

Keywords: Weissella cibaria, *Weissella confusa*, Comparative genomic analysis, Functional characteristics, Antifungal activity, Virulence traits, IS molecular typing

1. Introduction

The different species ascribed to the genus *Weissella* are known for a long time for their presence in various food matrices and in many spontaneous fermentation processes of vegetables and cereals, in particular the species *W. cibaria* and *W. confusa* (Fusco et al. 2015; Decimo et al. 2017; Fessard and Remize 2017). Their constant presence indicates that they are part of the microbial natural population occurring in different fermented products. Nevertheless, *W cibaria* and *W. confusa* are not yet used as selected starter cultures, are not included in the list of taxonomic units proposed by the European Food Safety Authority (EFSA) for Qualified Presumption of Safety (QPS) status, and no specific antibiotic breakpoints for these species were suggested by the Clinical and Laboratory Standards Institute (CLSI) or the EFSA.

The researches published on *Weissella* spp. are not few, but they are mainly concentrated on the production and characterization of exopolysaccharides (EPS) (Di Cagno et al. 2006; Katina et al. 2009; Ahmed et al. 2012; Wolter et al. 2014; Hu and Ganzle 2018). The use of EPS synthesized by starter cultures is a common practice in the dairy industry and, in sourdough fermentation, improves texture and storage life of bread. Moreover, *Weissella cibaria* and *W. confusa* are able of producing

in situ high molecular weight dextrans. These homopolysaccharides improve the softness of the fresh bread, and their use is promising in gluten-free backing (Wolter et al. 2014).

Other studies on *Weissella* spp. are fragmentary. However, from these publications it is clear that, although strain-specific, other properties are of interest, both for the quality and safety of food, as the production of bacteriocins (Srionnual et al. 2007; Masuda et al. 2011), the ability to overcome the gastric barrier (Le and Yang, 2018) and to inhibit micotoxinogenic moulds (Valerio et al. 2009; Ndagano et al. 2011). In this regard, a strain of *W. cibaria* has been used in probiotic yoghurt to reduce aflatoxin poisoning in Kenyan children (Nduti et al., 2016). These characteristics suggest a possible use of specific strains as potential probiotic cultures, also supported by the hypothesis that the genus may represent a common inhabitant of our intestine (Lee et al. 2012).

On the other hand, *Weissella* species have been associated to possible human infections (Kamboj et al. 2015), even if their clinical significance remains unclear, as they have been mainly associated to polymicrobial infection and/or to immune-compromised patients. Little information is available on the mechanism and factors related to their pathogenicity, apart the intrinsic resistance to vancomycin and fosfomycin.

Today highlighting possible virulence factors is easier, for the availability of genomes sequenced and deposited in public databases. However, genomic data on *Weissella* are restricted to a few publications (Abriouel et al., 2015; Figueiredo et al. 2015; Li et al. 2017). These comparative genome analyses highlighted several genes putatively involved in virulence, as genes encoding haemolysins, collagen adhesins and antibiotic resistance-encoding genes. The role of these genes and their transferability in *Weissella* is still unknown. In fact, the presence of some adhesins, considered a virulence factor in pathogenic microorganisms, may be a desirable feature in probiotic bacteria, as a fibronectin-binding protein (FbpA) that in *W.cibaria* inhibits biofilm formation of *Staphylococcus aureus* (Wang et al., 2017), or mucus-binding proteins that may play an important role in the adhesion of the probiotic strain to the host surfaces. Comparative genomic studies on *W. cibaria* (Lynch et al. 2015) focalized the attention on useful metabolic traits, as the bacteriocin gene cluster, dextransucrase genes and genes related to an efficient proteolytic system. No specific virulence factor genes were detected.

It follows that a deepening of the physiological and genetic characteristics of the species of *Weissella* could help to select suitable strains for which to evaluate the status of QPS and possible practical applications.

In a previous work based on the characterization of the native population of natural fermentation of corn bran (Decimo et al. 2017), different strains of *W. cibaria* and *W. confusa* were isolated. They were found mainly in the last refreshment steps, where their presence was significantly abundant. The aim of the present work was a polyphasic study of these isolates, with particular regard to potential probiotic properties. In parallel, a comparative genome analysis of 15 sequenced *W. cibaria* and 5 *W. confusa* genomes to date available was carried out.

2. Materials and methods

2.1 Bacterial strains and culture conditions

Twelve strains of *Weissella cibaria* and eight strains of *Weissella confusa* previously isolated from sourdough-like maize bran fermentation (Decimo et al. 2017) were used in this study. The strains were routinely sub-cultured in MRS broth/agar (Difco Lab., Augsburg, Germany) medium for 24/48 hours at 30 °C. The strains were deposited in the culture Collection of the Department of Food, Environmental and Nutritional Sciences, University of Milan, Italy, at −80 °C in MRS with 15% glycerol. Growth in milk was studied using 9% RSM (Reconstituted Skim Milk- Difco) incubated at 30 °C.

2.2 Growth at different cultural conditions

The growth performance of the strains was evaluated in MRS broth at 10 °C, at 45 °C, at pH 9.6 and with the addition of 4.0 and 6.5% NaCl. Growth was evaluated with OD_{600} measurement.

2.3 Acidifying activity

Each strain was inoculated at 1% in MRS broth and in RSM. The pH was measured and recorded automatically, throughout the 24 h incubation period at 30 °C. Two trials for each strain were carried out.

2.4 Redox potential

The variation in redox potential was determined over 24 h during waterbath incubation at 30°C, according to Brasca et al. (2007). Briefly, a redox meter (pH302 Hanna Instruments, Villafranca Padovana, PD, Italy) was used to monitor the oxidoreduction values in the inoculated MRS, and the data were recorded every 30 min for 24 h. The redox electrodes were standardized using two redox solutions (240 mV and 470 mV; Hanna Instruments). In order to avoid atmospheric oxygen interference with the redox measurement the cultures were carried out under static conditions. The Eh values were calculated according to Jacob (1970). Two replicates for each bacterial strain were used to estimate the reduction activity, evaluated by determining the maximum difference between two measures [Dmax (mV)] over 24 h (Brasca et al. 2007).

2.5 Carbohydrate fermentation assay

Weissella strains were tested for the ability to ferment glucose, xylose, L-arabinose, threalose, sucrose, ribose and galactose. Bacterial cells grown in MRS broth at 30°C for 16 h were harvested by centrifugation (5000 *g*, 15 min, 4 °C), washed twice with sterile saline solution (NaCl 0,85%) and re-suspended in the same volume of diluent. The fermentation assay was performed in Basal Sugar Medium (BSM) broth (containing g/L: polypeptone 15, yeast extract 6, tween80 1 ml, chlorophenol-red 0.04, pH 6.4) in microtiter plates containing 200 µL of BSM (with and without supplement of

0.5% sugar) and 1% washed cellular suspensions. The plates were incubated at 30°C and were visually examined for color changes after 24 and 48 h of incubation. Two replicates were carried out for each *Weissella* strain.

2.6 FOS and fructose utilization

To assess the ability of the strains to ferment prebiotic substances as fructose and fructo-oligosaccharides (FOS), cells grown in MRS broth at 30°C for 16 h were harvested by centrifugation (5000 *g*, 15 min, 4°C), washed twice with saline solution and inoculated in 5 mL MRS broth pH 6.8 (with and without supplement of 1.0 % sugar). After 24h of incubation at 30 °C, the OD_{600} and pH were measured.

2.7 Screening for EPS production

Exopolysaccharide (EPS) biosynthesis was evaluated as described by Bounaix et al. (2009) with slight modifications. The strains were streaked on agar plates containing modified MRS medium with 60 g/L sucrose instead of 20 g/L of glucose (namely MRS-sucrose) and incubated at 37°C for 48 h, Mucoid growth exhibiting slime production was evaluated.

2.8 Hydrophobicity assay

Bacterial adhesion to hydrocarbons was determined according to Kos et al. (2003) with slight modifications. Bacteria grown in MRS broth at 30 °C for 24 h were harvested by centrifugation (5000 *g*, 15 min, 4 °C), washed twice in sterile saline solution and re-suspended in 0.1 M KNO_3 (pH 6.2) to approximately 0.5 OD_{600} (A_0). 1 mL of xylene was added to 3 mL of cell suspension. After 10-min of pre-incubation at room temperature, the two-phase system was mixed by vortexing for 2 min. The aqueous phase was removed after 20 min of incubation at room temperature, and its absorbance at 600 nm (A_1) was measured. The percentage was calculated using the relation of the absorbance at 600 nm measured before and after the contact with the xylene through the following formula: Adhesion (%) = $[1 – (A_1 / A_0)] * 100$, where A1 was the absorbance at 600 nm measured after the contact with the xylene and A_0 was the absorbance at 600 nm before the contact with the xylene.

2.9 Bile tolerance

Bile tolerance was measured in triplicate in MRS broth containing 0.3% or 1% oxgall (Sigma–Aldrich, Steinheim, Germany), inoculated and incubated at 30 °C. The growth was evaluated with OD_{600} measurement.

2.10 Tolerance to simulated gastric juice

The method of Charteris et al. (1998) was used with slight modifications. Overnight cultures (4 mL) were centrifuged (5000 *g*, 15 min, 4 °C), washed twice in 50 mM K_2HPO_4 (pH 6.5) and resuspended in 4 ml of the same buffer. One milliliter of washed cell suspension was harvested by

centrifugation and resuspended in 10 mL simulated gastric juice (pepsin 0.3% w/v, NaCl 0.5% w/v) adjusted to pH 2,5 and 3. Total viable counts were performed, on MRS agar before and after an incubation period of 1 and 3 h (for pH 2.5 and 3, respectively) at 37 °C.

2.11 Antifungal activity

The *Weissella* isolates were tested for their antifungal activity against *Fusarium verticillioides, Mucor circinelloides* and *Aspergillus flavus*. The moulds (from the Collection of the Department of Health, Animal Science and Food Safety, University of Milan, Italy) were grown on Malt Extract Agar (MEA) (Merck, Darmstadt, Germany) at 25°C for 5–7days. Then, spore suspensions were harvested by adding 15mL of sterile milli-Q water and counted by flow cytometer estimation (BD Accuri C6 Flow Cytometer, BD Biosciences, Franklin Lakes, NJ USA). Antifungal activity was evaluated with an overlay assay (Quattrini et al. 2018). Bacteria, grown for 16 h in MRS broth at 30°C, were inoculated in 2-cm lines on MRS agar plates. After incubation for 48 h at 30 °C, plates were overlaid with cooled soft (0.7%) MEA containing mould spore suspension (10^4 spores/mL) and incubated for 4 days at 25°C. The antifungal activity was evaluated as clear zones of inhibition around the bacterial smears.

2.12 Antibiotic resistance

Antibiotic susceptibility tests were performed by the microdilution method in MRS broth incubated at 30 °C for 24 h. The minimum inhibitory concentration (MIC) was calculated after growth in MRS broth at 30 °C, using 10^5 cells/mL as initial inoculum. Interpretative criteria for susceptibility status were the Clinical and Laboratory Standards Institute (CLSI) guidelines and the microbiological breakpoints defined by EFSA (FEEDAP, 2012).

A breakpoint has not been recommended for the genus *Weissella*. We therefore considered the values for *Lactobacillus* and *Leuconostoc* together, because the phylogenetically determined taxonomic position of *Weissella* is between the genera *Leuconostoc* and *Lactobacillus* (Collins et al. 1993).

2.13 Biogenic amine production

The biogenic amine production was correlated to amino acids decarboxylation. The enzymatic decarboxylation was investigated with the method described by Bover-Cid and Holzapfel (1999). Four amino acids were investigated: histidine, lysine, ornithine and tyrosine. *Morganella morganii* DSMZ 30164^T was used as positive control. The strains were streaked on the plates and incubated at 37°C for 24 hours. The decarboxylation activity resulted in a pH increase of the medium, and a consequent change of the indicator pH colour from yellow to purple.

2.14 Insertion Sequences

The search of Insertion Sequences (IS) along the genomes of *Weissella* strains was performed using the NCBI BLAST and ISfinder (http://www-is.biotoul.fr) (Siguier et al. 2006). Positive matches

for transposase were confirmed manually to determine which family they belong by the comparison of the element size, presence of terminal IRs and DRs, number of *orf*s and DDE consensus region with related elements. For new elements, IS names were kindly provided by ISfinder.

Genomic copy number and distribution of ISs were determined by digestion of the total DNA from the 20 strains tested with *Hind* III restriction enzyme. The resulting fragments, separated on a 0.8% w/v agarose gel were transferred to a nylon membrane by Southern blotting. The primers used for the production of IS-specific probes are listed in Table 1. The primers were obtained from PRIMM srl (Milano, Italy). The PCR amplification procedure was performed as described previously (Ricci and Fortina, 2006). The DIG DNA Labelling and Detection Kit (Roche Diagnostic GmbH, Mnnheim, Germany) was used for digoxigenin labelling of the probes. Prehybridization and hybridization were performed in 50% (w/v) formammide at 42 °C. The probes were detected by chemio-luminescent detection using CSPD (Roche) and the signals were visualized by exposure to X-ray film for 2 h.

Table 1. Primers and conditions used for IS PCR amplification.

IS name	Accession number	Primer	Tm	Length
ISWci2	CP012873	5'-TGCATCTCGACAAGAGATTG -3'	58°C	946 bp
		5'- GAGAGCTTCCATTCGCTCAT -3'		
ISWci1	CP012873.1	5'- TCCAGGATTGCCTCTTGTTT -3'	58°C	841 bp
		5'- CACCGTCGTTTCAAGACTGA -3'		
IsWco1	CAGH01000055	5'- TTCTTGATCTTGTCGTGTTC -3'	58°C	502 bp
		5'- GATCGACCATATCAGAAGGT -3'		

2.15 Data source for comparative genome analysis

Information of available *W. cibaria* and *W. confusa* genomes was retrieved from the National Center for Biotechnology Information (NCBI, available at http://www.ncbi.nlm.nih. gov/). Genome analysis was carried out using the Rapid Annotation using Subsystem Technology (RAST) Server (Aziz et al. 2008). The NCBI BLAST software was used for sequence similarity search (Altschul et al. 1997).

2.16 Statistical analysis

Three independent replicates of the experiments were done, and their results were expressed as mean values ± standard deviation. When necessary, the data were compared through one-way ANOVA, followed by Tukey's test ($p < 0.05$).

3. Results

3.1 Comparative genomic analysis

A comparative genomic analysis on 15 sequenced *W. cibaria* and 5 *W. confusa* genomes was carried out. Among the 20 genomes available in GenBank, four were related to strains coming from healthy infant saliva, two from kimchi, three from sourdough, two from insects and other sources. The analysis was carried out by Rapid Annotation Server using Subsystems Technology annotation tool, NCBI-Basic Local Alignment Search Tool, NCBI-Conserved Domain Database looking for functional genes and for possible virulence related factors.

The search of hypothetical virulence factors showed the presence of genes that could be involved in pathogenicity. In particular, the search for annotated genes resulted in three different haemolysins: α-haemolysin (*hly*A), haemolysin C (*hlyC*) and haemolysin III. Haemolysin III is described as predicted membrane channel-forming protein YqFA, previously described in *B. cereus* (NP_835109.1). The related gene was present in all genomes tested and its nucleotide sequence seemed highly conserved among the genomes (99 %). The multiple alignments showed that this protein, largely distributed among Gram positive bacteria, showed similarity with haemolysin III of several genera as *Leuconostoc* (WP_004911898.1) (64%), *Lactobacillus* (WP_107739861.1) (45%), *Enterococcus* (OJG68906.1) (43%), *Bacillus* (RBJ50015.1) (43%), *Listeria* (WP_036096723.1) (41%). The putative α-haemolysin is described as RNA methyltransferase. The α-haemolysin has been already well-characterized in *Staphylococcus aureus* (*hla*, also known as α-toxin). In *S. aureus*, the level of expression of *Hla* is tightly controlled by the accessory gene regulator (*agr*), a quorum-sensing (QS) regulator that control the expression of specific virulence genes. Among *Weissella* species, only the aquaculture pathogenic *W. ceti* contains genes encoding the two-component system regulators, *agrA* (WS105_0510, annotated as LytTr DNA-binding domain protein) and *agrC* (WS105_0511, annotated as sensor protein CitS); these genes are not present in any other *Weissella* species (Figueiredo et al., 2015) and should be considered species-specific genes. Consequently, the only presence of this *hlyA* gene has not to be seen as a virulence factor in *W. cibaria*/*confusa*. The haemolysin C gene is annotated as *hlyC*/*CorC* (CP012873.1)*;* analysing the conserved domain of the protein the main function predicted seems to be related to magnesium and cobalt transporter, rather than haemolytic activity.

Among the candidate genes encoding cell surface adhesins, we only found two annotated genes encoding a fibronectin binding protein (WP_010373731.1) and a mucus binding protein (NZ_CP012873.1). The gene for fibronectin binding protein was detected in all *W. cibaria* and *W. confusa* genomes analysed (94% amino acidic identity). The alignments resulted in high identities in closer genera as *Leuconostoc* (WP_036068220.1) (60%), *Lactobacillus* (WP_017261841.1) (56%), *Pediococcus* (WP_057748137.1) (54%). In the last decade, several studies have revealed that a wide range of bacteria possess adhesin-like proteins, able to bind to fibronectin, that could play a direct role in bacterial colonization and bacteria–host interactions (Henderson et al. 2010). Their presence in pathogenic strains can be considered the first step of infection; on the contrary, in strains with probiotic potential this characteristic should not be an excluding factor for its use, but regarded as a key factor for the attachment of probiotic bacterial cell in the human host. In this context, Wang et

al., 2017 demonstrated that a fibronectin-binding protein of *W. cibaria* isolates was able to inhibit Staphylococcus *aureus* colonization on host tissues.

In *W. cibaria* genomes there is an annotated gene codifying for a "mucus binding protein" (NZ_CP012873.1), which is a huge complex of about 6000 amino-acids, with multiple conserved domains mainly correlated to external viral teguments (Mcnabb and Courtney, 1992). This gene was found also in *W. confusa* (WP_004560401.1) with 87% of identity, in *Leuconostoc* (WP_077282836.1) with 40% and in *Streptococcus pneumoniae* (COI29454.1) (90%). The functionality of this atypical gene could be controversial and further studies are necessary to know its potential role in pathogenicity.

Regarding antibiotic resistance determinants, as already reported, *Weissella* spp., as many lactic acid bacteria, possess an intrinsic resistance to vancomycin. This antibiotic interferes with precursors of peptidoglycan synthesis, binding D-Ala/D-Ala dipeptide, inhibiting the polymerization. In *Weissella*, the terminal D-Ala is substituted with a D-lactate or D-Ser, avoiding the antibiotic to bind in that site, and conferring the resistance phenotype (Gueimonde et al., 2013). A multidrug efflux pomp related to fosfomycin resistance was found (CP012873.1) (TC 2.A.1) in *Weissella cibaria* CH2 genome. The gene was present in all genomes analysed. Further analysis showed that the efflux pomp is widespread in different Gram-positive and Gram negative genera: *Leuconostoc* (WP_036067854.1) (66%), *Lactobacillus* (WP_010622689.1) (58%), *Listeria* (WP_096926801.1) (50%), *Staphylococcus* (WP_000610059.1) (42%), *Salmonella* (WP_050189798.1) (34%) *Escherichia* (WP_001612799.1) (29%). As for vancomycin, also fosfomycin have to be considered an intrinsic resistance. No genes related to tetracycline resistance were found in the genomes of *W. cibaria* and *W. confusa.* In *W. cibaria* genomes one gene present in two copies (CP012873.1) was annotated as a methicillin resistance protein. Two similar genes with an amino acid identity of 81 and 58% were also found in the *W. confusa* genomes. Methicillin is a ß-lactamic antibiotic targeting the enzymes responsible for peptidoglycan synthesis. Indeed, a search on NCBI-CDD indicates that the gene encodes a protein having a catalytic domain related to enzymes involved in cell wall peptidoglycan synthesis, specifically a transpeptidase involved in pentaglycin bridge formation. This protein was present in all-related Gram-positive genera (*Leuconostoc*, *Lactobacillus*, *Pediococcus*) with high identities (>80%) and supposedly with the same function. The nucleotide sequence was aligned with the known *mecA* gene (Katayama and Hiramatsu, 2000), responsible of the methicillin resistance in *S. aureus* (KC243783.1), showing no homology. Moreover, the two copies of the gene in *Weissella*, are located in two different chromosomal loci and they appear not to be into a transferable cassette, as previously described for the methicillin resistance gene cassette *mecA* in *S. aureus*.

No genes encoding decarboxylases, related to biogenic amines production were detected in all genomes analysed.

From the data obtained *W.cibaria* and *W. confusa* seem associated to low virulence profiles and their presence in food could be considered not only a low health risk, but also an adjunct advantage. Indeed several functional traits were detected. The first functional trait investigated was the arabinoxylan catabolism. The arabinoxylan degradation is related to the activity of several enzymes, as endo-1,4-β-xilanases, α-l-arabinofuranosidase, β-xilosidase, α-glucuronidase e feruroil esterase. Their combined action allows the obtainment of oligosaccharides with pre-biotic properties, an increase of soluble fiber and, with the action of feruloil esterase, the increase of free ferulic acid. All *W. cibaria* genomes harbour the gene (WP_010373933.1) encoding a β-xylosidase. This gene is also

present in all *W. confusa* genomes tested (nucleotide similarity 95%). The protein exhibited significant similarity to the known sequences of β-xylosidase of *Leuconostoc* (WP_029509980.1) (84% amino acidic identity), *Lactobacillus oligofermentans* (WP_057890071.1) (78%) and *Serratia* spp. (WP_015673394.1) (75%). The gene encoding the feruloyl esterase was no detected. The other genes related to arabinoxylans degradation were differently distributed. The α-N-arabinofuranosidase gene (CP012873.1) was found only in *W. cibaria* genomes, with predicted double function of both β-xylosidase/α-N-arabinofuranosidase activity, as reported by NCBI-CDD. The alignment resulted in high amino acidic identity with *Weissella bombi* (WP_092461590.1) (80%), *Lactococccus lactis* (WP_058219862.1) (76%), *Lactobacillus brevis* (WP_021741280.1) (75%), *Pediococccus acidilactici* (WP_063504605.1) (75%). The endo-1,4-β-xylanase gene was present only in all *W. confusa* genomes. Lower aminoacidic identities were found, with *Enterococcus timonensis* (WP_071130632.1) (44%), *Bacillus circulans* (WP_095258370.1) (44%), *Bifidobacterium adolescentis* (WP_107646029.1) (40%). Therefore, it is possible to hypothesize that the degradation of this polymer could be obtained by a potential synergistic action of selected strains of the two species, which are isolated most of the times from the same cereal fermented products (Bjorkroth et al.,2002).

Another functional trait, well documented in *Weissella* species, is the dextran production, mainly correlated to dextransucrase activity (Galle et al., 2010). In the genomes analysed, dextransucrase gene was found in all *W. cibaria* (GU237484.3) *and W. confusa* (KP729387.1) strains, with an aminoacidic identity of 75%. In addition, an EPS gene cluster, characterized by different enzymes (tyrosine-protein kinase transmembrane modulator, undecaprenyl-phosphate galactose phosphotransferase, glycosyl transferase, tyrosine-protein kinase EpsD) was found in both species These genes, not deeply investigated, could be related to the production of other EPS, as glucans and fructans (Di Cagno et al. 2006; Malik et al. 2009).

Finally, the published *W. confusa* and *W. cibaria* genomes were searched for the presence of Insertion Sequence (IS) elements. To date, no insertion elements have been described for the two species in the IS element database (http://www-is.biotoul.fr). Studies regarding the occurrence and distribution of these Mobile Genetic Elements can represent a species-specific approach to evaluate the genetic diversity among related species and /or strains. Analysis of the genomes revealed the presence of three IS elements, two in *W.cibaria* genomes and one in *W.confusa* genomes. IS names were kindly provided by ISfinder and designated IS*Wci1* (CP012873.1), IS*Wci2* (CP012873) and IS*Wco1* (CAGH01000055) respectively. These IS elements are menbers of the IS*3* family. IS*Wci1* is 56% aa similar to IS*Bce19* found in *Bacillus cereus* and was present in all the genomes of *W. cibaria* and in one of *Weissella confusa*. IS*Wci2* is 66% aa similar to IS*1520* in *Lactobacillus sakei* and to IS*981* identified in several lactococci. The sequence was found in all the genomes tested. IS*Wco1* is 60% aa similar to IS*Lsa2*, another IS element found in *Lactobacillus sakei*. This IS element was found in all *W. confusa* genomes and in one of *W. cibaria*.

3.2 Characterization of W. cibaria and W. confusa strains isolated from sourdough-like maize bran fermentation

3.2.1 Physiological and technological properties

The strains tested showed a good adaptability towards cultural stresses, as the temperature, the NaCl concentration and the pH value.

All strains were able to growth at 10°C, in presence of 4% NaCl and a pH 9.6. The growth at 45°C was more evident for *W. confusa* strains.

All strains were able to utilize glucose, xylose and sucrose as sole carbon sources. Lactose and trehalose were not fermented. Galactose and ribose were only fermented by strains belonging to *W. confusa*. This ability, which occurred after a longer incubation period (48-72 h), could be due to a selection inside the population and, for galactose, to the activation of an inducible Leloir pathway. The fermentation of L-arabinose also allowed to discriminate between the two species: all *W. cibaria* strains were able to utilize this carbon source, contrarily to *W. confusa* strains.

As reported in Table 2, all tested strains showed a high acidification rate in MRS medium, with a pH value, after 24 h of incubation at 30°C, ranging from 3.5 to 4.9. On the contrary, a limited capacity of growing and acidifying in milk was observed, probably due to the inability to ferment lactose, and for the obligate heterofermentative metabolism.

The 20 *Weissella* strains were also screened to evaluate the changing in the redox potential during grow in MRS liquid medium, a parameter not yet investigated in these species. All strains had high reducing ability (Table 2), in particular the strains CM18, CM10 and CR36 which could reach values close to -400 mV. In sourdough environment, Capuani et al. (2012), described a potential use of this interesting parameter, coupled with the traditional pH measurement, to successfully control the baking process. It allows to control that the fermentation process is going in the prefigured direction, ensuring the desired quality of the product and monitoring possible contaminations. Moreover, the redox potential has been described to influence the aromatic profile (Kieronczyk et al., 2006), as well as protein structure and texture of baked products. In dairy productions, this trait is also of noticeable interest, because the low reduced environment can provide a good barrier against the possible development of contaminant and pathogenic microorganisms.

The ability to synthesize exopolysaccharides (EPS) by *W. confusa* and *W. cibaria* is well documented by many authors. In this work we qualitatively screened the ability of EPS production in solid medium added with high percentage of sucrose (6%), resulting in sticky and viscous colonies. All strains were able to produce polysaccharides, as shown in Figure 1. This technological trait is highly requested for sourdough fermented products, e. g. gluten free doughs, where hydrocolloids are crucial to improve the texture and the specific volume of the bread. EPS are also favourable in yogurt to reach a creamy texture.

Table 2. pH values in MRS and RSM media and maxim redox potential difference in MRS $\Delta E_{max\ (mV)}$ after 24 hours of incubation at 30°C.

	Strains	pH MRS	pH RSM	$\Delta E_{max\ (mV)}$
W. cibaria	CM1	4.37 ± 0.4^{b}	$6,01\pm0.08^{a}$	-334 ± 13^{d}
	CM6	4.92 ± 0.39^{b}	$5,96\pm0.10^{a}$	-378 ± 55^{d}
	CM10	4.59 ± 0.19^{b}	$6,06\pm0.19^{a}$	-355 ± 38^{d}
	CM18	4.7 ± 0.4^{b}	$6,05\pm0.32^{a}$	-385 ± 29^{d}
	CM34	4.61 ± 0.34^{b}	$6,05\pm0.25^{a}$	-87 ± 24^{a}
	CM23	4.21 ± 0.17^{ab}	$6,06\pm0.04^{a}$	-182 ± 25^{b}
	CM32	4.09 ± 0.28^{ab}	$6,11\pm0.28^{a}$	-365 ± 42^{d}
	CM9	3.49 ± 0.28^{a}	$6,07\pm0.05^{a}$	-148 ± 18^{b}
	CM19	3.54 ± 0.12^{a}	$6,02\pm0.15^{a}$	-157 ± 1^{b}
	CM27	4.09 ± 0.26^{ab}	$6,07\pm0.14^{a}$	-114 ± 16^{b}
	CR23	4.40 ± 0.13^{b}	$6,04\pm0.05^{a}$	-182 ± 32^{b}
	CR24	3.93 ± 0.23^{ab}	$6,02\pm0.12^{a}$	-187 ± 16^{b}
W. confusa	CR21	4.47 ± 0.30^{b}	$5,90\pm0.03^{a}$	-224 ± 31^{c}
	CR31	3.70 ± 0.11^{a}	$5,95\pm0.24^{a}$	-260 ± 6^{c}
	CR36	4.06 ± 0.14^{ab}	$5,98\pm0.28^{a}$	-376 ± 33^{d}
	CR39	4.45 ± 0.08^{b}	$5,95\pm0.35^{a}$	-192 ± 35^{b}
	CR48	4.12 ± 0.29^{ab}	$5,96\pm0.37^{a}$	-258 ± 15^{c}
	CR49	3.83 ± 0.32^{ab}	$6,03\pm0.38^{a}$	-242 ± 32^{c}
	CR51	4.03 ± 0.28^{ab}	$5,96\pm0.35^{a}$	-255 ± 32^{c}
	CR55	4.15 ± 0.1^{ab}	$5,98\pm0.05^{a}$	-232 ± 2^{c}

Fig. 1. Sticky and viscous colonies of *W. cibaria*/*confusa* in MRS-sucrose agar after 24 h at 30°C.

3.2.2 Functional traits

The ability of the strains tested to grow in presence of FOS, is shown in Table 2. All *W. cibaria* and *W. confusa* strains were able to ferment fructose, decreasing the pH value. FOS were used as carbohydrate source by all *W. confusa* strains and by 10/12 *W. cibaria*. The utilization of prebiotic

compounds is one of the indicator of probiotic potential of a bacterial strain (Kaplan and Hutkins, 2000). These data highlight that generally these species possessed a high attitude to use prebiotics as growth source, in a future probiotic perspective.

An essential trait of a probiotic microorganism is its ability to reach and survive in the large intestine, overcoming the intestinal barrier. The strains tested showed a moderate tolerance to simulated gastric juice, many strains decreasing their viability of 3 log cycles (Table 4) at pH 3. In simulated gastric juice at pH 2.5 a progressive reduction in viability was observed but recovered viability after 1 h was about 10^2-10^4 CFU/ml for all strains. Moreover, all strains showed a noticeable ability to resist to bile salts, reaching values between 30% and 60% of residual growth also when the highest concentration of bile salts (1%) was added to the medium (Table 5). At the concentration of 0.3% the inhibition was minimal, with the residual growth ranging from 48% to 86%.

Table 3. Prebiotics utilization. Data are shown as mean ± standard deviations of triplicates. Values differ if they do not share a common superscript ($p < 0.05$).

		MRS basal medium (control)		MRS + FOS (1%)		MRS + fructose (1%)	
	Strains	OD_{600nm}	pH	OD_{600nm}	pH	OD_{600nm}	pH
W. cibaria	CM1	0.57±0.12^a	6.74±0.19^a	1.4±0.09^b	5.65±0.05^a	1.70±0.05^b	5.1±0.20^a
	CM6	0.47±0.16^a	6.84±0.29^a	1.44±0.12^b	5.66±0.12^a	1.76±0.02^b	5.09±0.11^a
	CM10	057±0.13^a	6.78±0.23^a	1.44±0.16^b	5.65±0.12^a	1.76±0.04^b	5.08±0.09^a
	CM18	0.58±0.10^a	6.81±0.14^a	1.50±0.05^b	5.6±0.10^a	1.76±0.19^b	5.12±0.22^a
	CM34	0.61±0.09^a	6.68±0.23^a	1.45±0.03^b	5.66±0.01^a	1.76±0.01^b	5.11±0.15^a
	CM23	0.37±0.00^a	6.83±0.27^a	0.49±0.11^a	6.69±0.11^b	1.62±0.18^b	5.22±0.10^a
	CM32	0.40±0.14^a	6.82±0.09^a	0.46±0.00^a	6.76±0.06^b	1.62±0.00^b	5.24±0.16^{ab}
	CM9	0.32±0.18^a	6.79±0.14^a	1.17±0.08^b	5.61±0.10^a	1.57±0.01^b	5.04±0.18^a
	CM19	0.41±0.12^a	6.72±0.29^a	1.22±0.09^b	5.65±0.15^a	1.62±0.09^b	5.01±0.07^a
	CM27	0.37±0.10^a	6.84±0.07^a	1.13±0.19^b	5.55±0.16^a	1.50±0.18^b	5.11±0.19^a
	CR23	0.37±0.20^a	6.83±0.16^a	1.22±0.05^b	5.63±0.03^a	1.68±0.03^b	4.95±0.23^a
	CR24	0.37±0.04^a	6.85±0.22^a	1.19±0.02^b	5.58±0.08^a	1.84±0.07^b	4.82±0.25^a
W. confusa	CR21	0.31±0.00^a	6.87±0.13^a	1.38±0.11^b	5.73±0.16^a	1.88±0.17^b	4.89±0.04^a
	CR31	0.35±0.20^a	6.87±0.16^a	1.14±0.08^b	5.85±0.17^a	0.99±0.12^a	5.94±0.09^c
	CR36	0.34±0.18^a	6.89±0.17^a	1.33±0.14^b	5.82±0.04^a	1.42±0.19^b	5.63±0.14^{bc}
	CR39	0.31±0.03^a	6.84±0.18^a	1.24±0.13^b	5.62±0.11^a	1.07±0.03^a	5.54±0.02^b
	CR48	0.41±0.14^a	6.94±0.07^a	1.38±0.17^b	5.67±0.18^a	1.51±0.05^b	5.26±0.09^b
	CR49	0.48±0.10^a	6.97±0.17^a	1.51±0.18^b	5.75±0.08^a	1.31±0.03^{ab}	5.78±0.10^{bc}
	CR51	0.45±0.00^a	6.94±0.22^a	1.51±0.16^b	5.74±0.01^a	1.36±0.05^b	5.78±0.02^{bc}
	CR55	0.38±0.01^a	6.89±0.05^a	1.29±0.0^b	5.73±0.0^a	1.13±0.17^a	5.78±0.01^{bc}

Hydrophobicity properties of the strains was evaluated as percentage of adhesion to a hydrophobic solvent, the xylene. This bacterial trait could be predictive of adhesiveness of probiotic bacteria, the first step of the colonization of the epithelium. The results are shown in Table 4. Generally, the data obtained indicate a low potentiality of the strains to adhere to the solvent, even if this ability could be

considered strain specific: high values (55.6, 48.0 and 44.3%) were obtained for strains CM9, CM27 (*W. cibaria*) and CR21 (*W. confusa*), respectively.

Table 4. Simulate gastric juice tolerance after 3 h at pH 3 and 1 h at pH 2.5. The results are expressed log cfu/mL. Data are shown as mean ± standard deviations of triplicates. Values differ if they do not share a common superscript ($p < 0.05$).

	Strains	T_0	T3 pH 3	T1 pH 2.5
W. cibaria	CM1	9.00 ± 0.08^{a}	4.00 ± 0.08^{b}	3.49 ± 0.05^{b}
	CM6	8.98 ± 0.16^{a}	5.80 ± 0.29^{c}	4.18 ± 0.17^{c}
	CM10	9.23 ± 0.11^{a}	6.26 ± 0.41^{c}	2.00 ± 0.15^{a}
	CM18	9.11 ± 0.10^{a}	3.78 ± 0.14^{ab}	3.23 ± 0.75^{b}
	CM34	9.20 ± 0.23^{a}	4.15 ± 0.20^{b}	2.00 ± 0.79^{a}
	CM23	9.04 ± 0.31^{a}	6.04 ± 0.46^{c}	3.43 ± 0.08^{b}
	CM32	8.98 ± 0.02^{a}	6.61 ± 0.16^{c}	3.04 ± 0.06^{b}
	CM9	9.08 ± 0.04^{a}	6.04 ± 0.35^{c}	2.70 ± 0.46^{b}
	CM19	8.98 ± 0.18^{a}	6.18 ± 0.18^{c}	2.30 ± 0.25^{a}
	CM27	9.11 ± 0.03^{a}	5.79 ± 0.13^{c}	2.95 ± 0.53^{b}
	CR23	9.11 ± 0.20^{a}	6.34 ± 0.12^{c}	2.00 ± 0.13^{a}
	CR24	9.20 ± 0.04^{a}	6.23 ± 0.02^{c}	1.70 ± 0.41^{a}
W. confusa	CR21	9.18 ± 0.00^{a}	6.40 ± 0.16^{c}	4.18 ± 0.00^{c}
	CR31	9.00 ± 0.00^{a}	3.00 ± 0.42^{a}	2.60 ± 0.07^{ab}
	CR36	8.85 ± 0.10^{a}	4.43 ± 0.09^{b}	2.00 ± 0.09^{a}
	CR39	9.26 ± 0.05^{a}	4.32 ± 0.12^{b}	1.70 ± 0.27^{a}
	CR48	9.08 ± 0.10^{a}	6.26 ± 0.06^{c}	2.48 ± 0.18^{ab}
	CR49	9.04 ± 0.00^{a}	5.09 ± 0.47^{b}	3.53 ± 0.87^{c}
	CR51	8.30 ± 0.40^{a}	4.70 ± 0.28^{bc}	3.76 ± 0.30^{c}
	CR55	9.00 ± 0.02^{a}	4.32 ± 0.24^{b}	3.93 ± 0.09^{c}

Table 5. Bile tolerance and hydrophobicity of the tested strains. Data are shown as mean ± standard deviations of triplicates. Values differ if they do not share a common superscript ($p < 0.05$).

	Strains	MRS control (OD_{600})	Bile 0,3% (OD_{600})	% Growth	Bile 1% (OD_{600})	Growth%	Hydrophobicity (xylene adhesion %)
W. cibaria	CM1	4.37 ± 0.05^{b}	2.23 ± 0.43^{a}	51.03	1.40 ± 0.33^{a}	32.04	7.4%
	CM6	4.92 ± 0.10^{b}	3.25 ± 0.02^{a}	66.06	1.96 ± 0.19^{b}	39.84	14.3%
	CM10	4.59 ± 0.20^{b}	2.65 ± 0.02^{a}	57.73	1.9 ± 0.12^{b}	41.39	6.4%
	CM18	4.7 ± 0.36^{b}	2.27 ± 0.74^{a}	48.30	1.49 ± 0.08^{a}	31.70	5.9%
	CM34	4.61 ± 0.22^{b}	2.37 ± 0.04^{a}	51.41	1.69 ± 0.11^{ab}	36.66	3.7%
	CM23	4.21 ± 0.05^{ab}	2.19 ± 0.72^{a}	52.02	1.74 ± 0.02^{ab}	41.33	30.8%
	CM32	4.09 ± 0.12^{ab}	2.51 ± 0.55^{a}	61.37	1.58 ± 0.39^{ab}	38.63	27.8%
	CM9	3.49 ± 0.35^{a}	3.01 ± 0.17^{a}	86.25	2.08 ± 0.2^{b}	59.60	55.6%
	CM19	3.54 ± 0.18^{a}	2.69 ± 0.55^{a}	75.99	1.64 ± 0.27^{ab}	46.33	41.7%
	CM27	4.09 ± 0.40^{ab}	2.82 ± 0.41^{a}	68.95	2.15 ± 0.28^{b}	52.57	48%
	CR23	4.40 ± 0.01^{b}	3.32 ± 0.42^{a}	75.45	2.00 ± 0.17^{b}	45.45	0.0%
	CR24	3.93 ± 0.60^{ab}	3.35 ± 0.7^{a}	85.24	2.04 ± 0.33^{b}	51.91	0.0%
W. confusa	CR21	4.47 ± 0.25^{b}	2.92 ± 0.45^{a}	65.32	1.8 ± 0.27^{ab}	40.27	44.3%
	CR31	3.70 ± 0.10^{a}	2.51 ± 0.86^{a}	67.84	1.64 ± 0.05^{ab}	44.32	1.9%
	CR36	4.06 ± 0.23^{ab}	3.01 ± 0.87^{a}	74.14	1.73 ± 0.3^{ab}	42.61	15.8%
	CR39	4.45 ± 0.01^{b}	3.21 ± 0.44^{a}	72.13	2.46 ± 0.09^{b}	55.28	2.6%
	CR48	4.12 ± 0.22^{ab}	2.73 ± 0.22^{a}	66.26	2.18 ± 0.14^{b}	52.91	19.1%
	CR49	3.83 ± 017^{ab}	3.02 ± 0.01^{a}	78.85	1.78 ± 0.04^{ab}	46.48	11.2%
	CR51	4.03 ± 0.15^{ab}	2.86 ± 0.13^{a}	70.97	1.95 ± 0.14^{b}	48.39	3.8%
	CR55	4.15 ± 0.05^{ab}	2.81 ± 0.15^{a}	67.71	1.84 ± 0.13^{ab}	44.34	15.1%

3.2.3 Antifungal activity

Antifungal activity is an appreciated additional feature of starter and adjunct cultures, in several food sectors, as cereal sector, where moulds are widely present and can represent an extra risk for the possible production and accumulation of mycotoxins. Several publications have highlighted the positive use of LAB strains as agents of biocontrol, for their ability to secrete compounds as organic acids, phenyllactic acid, cyclic dipeptides, hydroxy-fatty acids, able to inhibit mould development, limiting the mycotoxin production (Quattrini et al., 2018; Lavermicocca et al., 2000). Little information is available regarding antifungal potential of *Weissella* strains. Interestingly, all *Weissella* strains tested were able to inhibit the growth of *Fusarium verticillioides*, the most prevalent fungus associated with maize, producing a wide range of mycotoxins, including fumonisin B1 (Deepa & Sreenivasa, 2017), considered the most toxic one. (Table 6- Figure 2). Moreover, most of the strains analysed (17/20) exerted a strong inhibition against the aflatoxigenic *Aspergillus flavus*. *Mucor irregolaris* and *M. circinelloides* were inhibited by approximately 55 and 45% of the strains respectively. Also, these data are of interest, because *Mucor* species are frequently isolated from food matrices and *M. circinelloides* is considered one of the causal agents of the fungal infection

mucormycosis (Lee et al., 2014). Further analysis will allow to understand the mechanism of action of this fungal inhibition.

Table 6. Antifungal activity of *Weissella* strains against *M. irregularis*, *M. circinelloides*, *F. verticillioides* and *A. flavus*. Strains were classified as no (-) or strong (+) inhibitors by using the overlay method.

		Antifungal activity			
	Strains	*Mucor irregularis*	*Mucor circinelloides*	*Fusarium verticillioides*	*Aspergillus. flavus*
W. cibaria	CM1	+	+	+	+
	CM6	+	+	+	+
	CM10	+	+	+	+
	CM18	+	+	+	+
	CM34	-	-	+	+
	CM23	-	-	+	+
	CM32	-	+	+	+
	CM9	-	-	+	-
	CM19	-	-	+	+
	CM27	-	-	+	+
W. confusa	CR23	-	-	+	-
	CR24	-	-	+	+
	CR21	-	-	+	+
	CR31	-	-	+	+
	CR36	+	-	+	-
	CR39	+	+	+	+
	CR48	+	+	+	+
	CR49	+	+	+	+
	CR51	-	-	+	+
	CR55	+	+	+	+

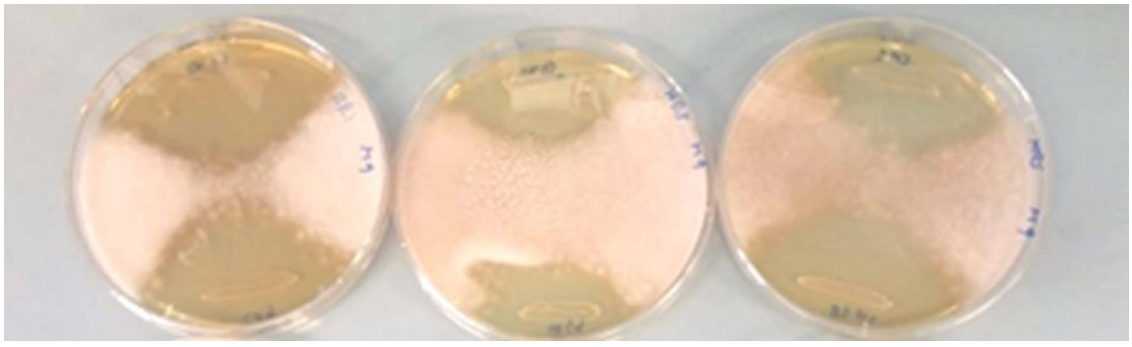

Fig. 2. Antifungal effect of *Weissella* strains against *Fusarium verticillioides.*

3.2.4 Antibiotic resistance and virulence traits

One of the criteria for strains selection for suitability for food industry is about their safety. Antibiotic resistance is regarded with always increasing attention from EFSA and OMS, for the spread of microbial resistances. To test antibiotic resistance profiles of the strains, we considered the breakpoints established by EFSA (EFSA 2012) for *Leuconostoc* and *Lactobacillus*, which are the closest genera to *Weissella*. Indeed, up to now no specific antibiotic breakpoints for these species are suggested by the CLSI or the EFSA. According to the breakpoints listed in Table 7, the strains were identified as either sensitive (S, MIC < breakpoint) or resistant (R, MIC > breakpoint). Since intrinsic resistance to vancomycin and fosfomycin are known, these antibiotics were not tested. Results showed that all tested strains were susceptible to tetracycline, ampicillin and chloramphenicol. Lincomycin had effect on 12/20 strains tested. On the other hand, all the strains were resistant to aminoglycosides (AG; gentamycin, kanamycin and streptomycin). The most common mechanism of AG resistance is chemical modification by aminoglycoside-modifying enzymes (AMEs) (Garneau-Tsodikova and Labby, 2016). However, along the tested genomes available in database no genes encoding AG-acetyltransferases, AG-nucleotidyltransferases and AG-phosphotransferases were found. For these reasons, it is possibile to hypothesize two other potential, acquired mechanisms of resistance: mutations of the ribosome or enzymatic modifications of the ribosome (Garneau-Tsodikova and Labby, 2016). In this case AG resistance should be considered intrinsic and not transferable. This data was in agreement with previous findings (Hummel et al., 2007; Katla et al.,2001), where several lactic acid bacteria starters and not-starters (*Lactobacillus* spp., *Leuconostoc* spp., *Lactococcus* spp., *Pediococcus* spp.) showed 70-80% of resistant phenotypes, and indicating that this can be considered an intrinsic resistance. The sulphonamide resistance can also be due to intrinsic modification of the dihydropteroate synthetase enzyme. As for aminoglycoside, many lactic acid bacteria showed a natural reduced sensibility towards this antimicrobials since most of lactic acid bacteria lack of the complete pathway of folic acid ex-novo biosynthesis (the target of the sulphonamides) (Katla et al., 2001). Regarding methicillin, we evaluated MIC values of oxacillin, according to EUCAST break point related to methicillin resistant bacteria (> 4 µg/mL), in comparison with three *Lactobacillus* strains, commercially used as probiotic cultures, *Lactobacillus rhamnosus* GG, *Lactobacillus paracasei* ATCC 5622, *Lactobacillus plantarum* ATCC 4008. All *Weissella* and *Lactobacillus* strain tested showed MIC values ranging from 4 to 8 µg/mL. These values seem to indicate a methicillin resistance, which, if related to transpeptidase enzyme or to a modification of the protein (as specified above, in 3.1 section), could be considered an intrinsic resistance not easily transferred horizontally. Nevertheless, further studies are needed to fully disclose the mechanism of resistance towards this antimicrobial.

Finally, according to genotypic traits, any *W. cibaria* and *W. confusa* strains tested showed the ability to produce biogenic amines.

Table 7. Antibiotic susceptibility test. The antimicrobials were used at break-point concentrations for related *Leuconostoc*/*Lactobacillus* genera (EFSA, 2012). (S, sensible, MIC < breakpoint; R, resistant, MIC > breakpoint).

Strains	Tetracyclin (8 µg/mL)	Streptomycin (64 µg/mL)	Kanamycin (64 µg/mL)	Gentamycin (16 µg/mL)	Lincomycin (8 µg/mL)	Chloramphenicol (4 µg/mL)	Trim+ Sulfa (8 +160 µg/mL)	Oxacillin (4 µg/mL)	Ampicillin (4 µg/mL)
CM1	S	R	R	R	R	S	R	R	S
CM6	S	R	R	R	R	S	R	R	S
CM10	S	R	R	R	S	S	R	R	S
CM18	S	R	R	R	S	S	R	R	S
CM34	S	R	R	R	R	S	R	R	S
CM23	S	R	R	R	R	S	R	R	S
CM32	S	R	R	R	R	S	R	R	S
CM9	S	R	R	R	S	S	R	R	S
CM19	S	R	R	R	S	S	R	R	S
CM27	S	R	R	R	S	S	R	R	S
CR23	S	R	R	R	S	S	R	R	S
CR24	S	R	R	R	S	S	R	R	S
CR21	S	R	R	R	R	S	R	R	S
CR31	S	R	R	R	S	S	R	R	S
CR36	S	R	R	R	S	S	R	R	S
CR39	S	R	R	R	R	S	R	R	S
CR48	S	R	R	R	R	S	R	R	S
CR49	S	R	R	R	S	S	R	R	S
CR51	S	R	R	R	S	S	R	R	S
CR55	S	R	R	R	S	S	R	R	S

3.2.5 IS: molecular typing

IS*Wci1*, IS*Wci2* and IS*Wco1*, previously found along the available genomes of the two species, were tested for frequency and distribution on the genomic DNA of the strains studied.

The IS*Wci2* seemed the most representative, with several copies distributed in all genomes of *W.cibaria* and *W.confusa* strains (Figure 2). The results indicate a copy number ranging from 2 to at least 8. Of the 12 *W.cibaria* strains tested almost 7 different restriction patterns were identified; almost 4 different profiles were distinguishable for the 8 *W. confusa* strains. IS*WCi1* and IS*Wco1* (Figure 3-4) were present in a minor copy number and did not highly differentiate the strains. Further studies are in progress to understand the contribute of these IS on the genome plasticity and on the adaptive response of their host.

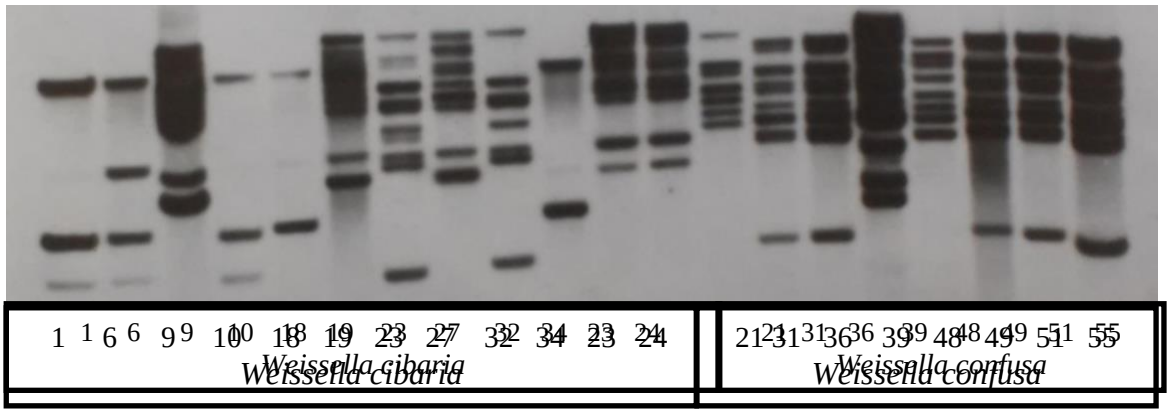

Figure 2. IS*WCi2* fingerprints of *W. cibaria* and *W. confusa* strains

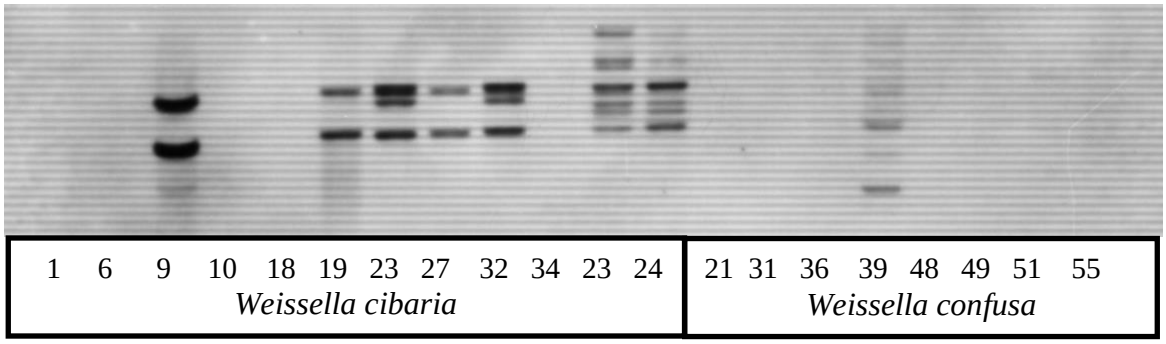

Figure 3. IS*WCi1* fingerprints of *W. cibaria* and *W. co*nfusa strains

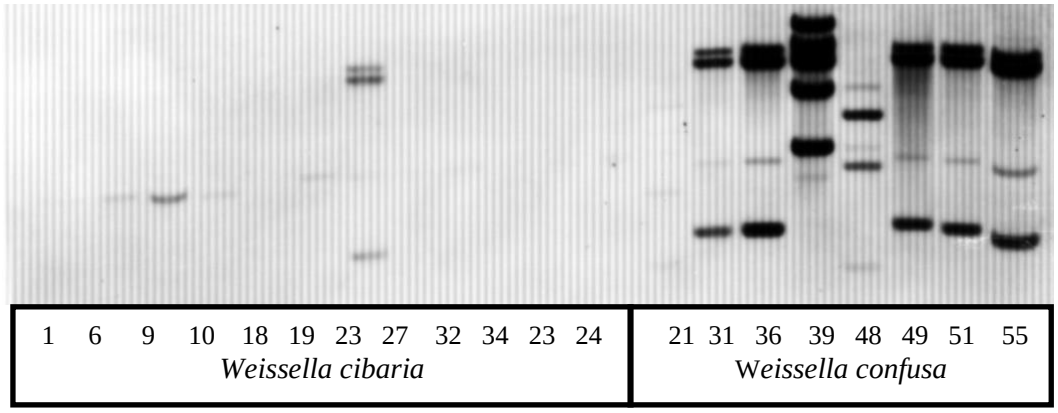

Figure 4. IS*WCo1* fingerprints of *W. cibaria* and *W. confusa* strains

4. Conclusions

In conclusion, our results support the idea that selected strains of *W.*cibaria and *W.* confusa can represent interesting adjunct culture to be exploited in food sector and in probiotic preparations. The comparative genome analysis carried out in parallel with a polyphasic study on 20 strains previously isolated from natural fermentation, seem indicate the absence of severe virulence factors. Moreover,

even if antibiotic resistance studies deserve to be further investigated, it is possible to hypothesize an intrinsic resistance to many antibiotics, trait present in other LAB commonly used as probiotics, and not easily transferable to other bacteria strains. Finally, interesting functional and pro-technological traits were highlighted in the tested strains, for both the species. For these reasons, further studies are in progress on selected strains to obtain the QPS status necessary for food applications.

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Conclusions and perspectives

The most known food fermentation processes are driven by simple or complex community of microorganisms. Their impact on food production is well known. Nowadays more and more population groups understand the importance of microorganisms in food, both in a positive and in a negative way, and their implications in obtaining high-standard quality and healthy products; this increasing demand is due to the new deep consciousness that a good health is also related to the consumption of fresh and genuine food.

Moreover, the request of the so-called "functional" food is constantly improving; this kind of food is believed to add health benefits and positively act on reducing the risk of chronicle diseases risk (cardio-vascular diseases, diabetes).

This explains why food industry is one of the most dynamic sector in new technologies and product's development, in order to ensure the ask of good and healthy nutrition.

The development of new starter cultures represents an area of huge growth potential for the food industry. Microbial fermentation is one of the oldest and ecologically friendly methods of producing and preserving foods, and plays several functions in food processing, such as typicity, through development of a diversity of flavour and aromas in food substrates, preservation and detoxification during food fermentation processing, enrichment of food biologically with compounds that originate from either biotransformation reactions or biosynthesis.

In this context, the present research was focused on the selection of new LAB biotypes that can be used in the fermented food production, to design innovative bio-control strategies and new food formulations.

Our findings highlighted that the fermentation of maize bran could be a source of high LAB biodiversity, where interesting functional strains can be found and applied in new fermentation processes. This bio-approach is suitable for a nutritional and functional improvement of this valuable by-product, which is nowadays mainly allocated to feed industry.

We studied and highlighted some interesting antifungal and nutritional features of *Lactobacillus plantarum* ITEM17215, which can be exploited by food industries, for cereal-fermented products.

We also demonstrated that could be possible to prolong the mould-free shelf life of the bread, using selected hetero-fermentative *Lactobacillus* strains in sourdough preparation with wheat or flaxseed flour and sucrose.

Finally, through a polyphasic approach, we characterized strains belonging to the species *Weissella cibaria* and *Weissella confusa*, isolated from spontaneous fermentation of maize bran. These strains showed a likely probiotic potential, and at the same time a low virulent gene pool. For selected strains, we suggest a QPS status for a future industrial application.

In the light of these findings, the next step would be the identification of a suitable food-making process, in order to apply the selected strains as bio-control agents and/or as nutritional improver. For *L. plantarum* the QPS status is already obtained and so the industrial application would be quicker. For *Weissella* strains, the EFSA approval is still required, but the features highlighted in this Phd project are valuable and promising.

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