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Nutritional enhancement of wheat milling by-products: chemical changes and evolution of microbiota during sourdoughlike fermentation of bran

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RIASSUNTO

Numerosi studi epidemiologici dimostrano l'effetto protettivo di una dieta ricca in cereali integrali nei confronti di malattie croniche, quali sindrome metabolica, malattie cardiovascolari e cancro al tratto gastro intestinale. Tali meccanismi di protezione risultano correlati all'elevato contenuto in fibra ed alle proprietà antiossidanti ed anti-cancerogene dei numerosi composti bioattivi, presenti principalmente nella crusca e nel germe delle cariossidi dei cereali.

La rimozione di tali frazioni durante i processi di macinazione, per migliorare la conservabilità delle farine, comporta una significativa riduzione dei contenuti di fibra e composti bioattivi.

Si stima, infatti, che la raffinazione del grano possa condurre ad una riduzione approssimativa del 58% di fibra, 83% di Mg, 61% di folati e del 79% di vitamina E. In particolare, lo strato aleuronico, ossia lo strato più esterno dell'endosperma amilaceo, è ricco di composti bioattivi, ma viene parzialmente rimosso durante il processo di molitura confluendo, insieme ai tegumenti della cariosside, nella crusca, destinata per lo più all'alimentazione animale. Le crescenti pressioni esercitate per garantire lo sfruttamento dei sottoprodotti agro-industriali, hanno suscitato grande interesse per il recupero e la valorizzazione di tali scarti dell'industria molitoria, portando sia all'abbattimento del loro impatto ambientale, sia ad un ritorno economico per l'industria del frumento grazie alla produzione di alimenti/farine arricchite con frazioni di crusca. La consistenza, il sapore ed una palatabilità poco gradevoli conferiti dalla crusca rappresentano i principali limiti alla base del suo scarso utilizzo in panificazione.

Tuttavia, processi di fermentazione della crusca, grazie all'azione di lieviti, batteri lattici e/o di specifici enzimi, risultano trattamenti efficaci al fine di migliorare le proprietà tecnologiche, sensoriali e nutrizionali dei prodotti arricchiti con crusca, così come nel degradare composti antinutrizionali, quali l'acido fitico, incrementando la biodisponibilità dei minerali presenti.

Lo scopo del presente studio è quello di incrementare il contenuto di composti bioattivi presenti nella crusca di frumento, quali fibra solubile, arabinoxilani solubili, acido ferulico libero, attraverso un processo di fermentazione, al fine di utilizzare la crusca fermentata come potenziale ingrediente funzionale. La crusca di frumento è stata sottoposta ad un processo di fermentazione spontaneo caratterizzato da una serie di rinfreschi successivi tipici della produzione di lievito madre (o madre acida, in inglese sourdough). La fermentazione è stata propagata fino al raggiungimento di un microbiota stabile, raggiungendo un elevato numero di batteri lattici e lieviti. Ad ogni rinfresco, batteri lattici e lieviti con differente macromorfologia sono stati isolati, raggruppati in cluster ed analizzati a livello molecolare tramite RAPD-PCR e identificati a livello di specie attraverso il sequenziamento del gene 16S rRNA. *Leuconostoc*

mesenteroides, Lactobacillus brevis, Lactobacillus curvatus, Lactobacillus sakei, Lactobacillus plantarum, Pediococcus pentosaceus e *Pichia fermentans* dominano l'ecosistema del sourdough. Dopo il processo di fermentazione si riscontra un incremento dei contenuti di fibra solubile (+ 30%); inoltre, le quantità di arabinoxilani solubili e di acido ferulico libero risultano rispettivamente quattro e dieci volte maggiori rispetto a quelle presenti nella crusca nativa, mentre la acido fitico appare completamente degradato, probabilmente a causa della attività enzimatiche endogene e microbiche che intervengono durante il processo di fermentazione. Sulla base degli interessanti risultati ottenuti dal punto di vista nutrizionale, alcuni batteri lattici isolati dalla crusca fermentata sono stati caratterizzati al fine di utilizzarli come potenziali culture starter. In particolare, sono state valutate alcune caratteristiche dei ceppi selezionati, tra cui la capacità fermentativa, l'attività antifungina, il metabolismo dei carboidrati, la produzione di esopolisaccaridi e anche l'eventuale resistenza agli antibiotici. Inoltre, sono state testate alcune potenziali attività enzimatiche dei ceppi di batteri lattici e del lievito isolati, quali l'attività degradante gli xilani e l'attività fitasica. Infine, sono state determinate alcune proprietà per valutare la potenzialità probiotica dei batteri lattici, quali la resistenza all'acidità ed ai sali biliari, l'inibizione della crescita di alcuni ceppi di *Listeria* e la capacità di adesione in vitro alle cellule epiteliali intestinali Caco-2.

I risultati evidenziano come i ceppi afferenti alle specie *L. plantarum* e *P. pentosaceus* possano avere interessanti applicazioni tecnologiche, in quanto presentano attività antifungina e produzione di espolissacaridi. Inoltre, alcuni di questi ceppi sono in grado di degradare fitati di calcio e/o sodio, e potrebbero, quindi, essere impiegati come starter al fine di migliorare la disponibilità dei minerali in prodotti fermentati. Inoltre, i ceppi *L. curvatus* (CE 83), *L. brevis* (CE 85) e *P. pentosaceus* (CE 65) sembrano essere possibili candidati per essere utilizzati come probiotici.

In conclusione, il presente studio conferma che il processo di fermentazione può essere considerato un trattamento efficace per incrementare la disponibilità di composti bioattivi presenti nella crusca di frumento. La caratterizzazione di alcuni ceppi di batteri lattici, coinvolti nel processo di fermentazione spontaneo della crusca, rappresenta il primo step nella selezione di culture starter, sulla base delle loro attività metaboliche ed enzimatiche, al fine di condurre processi di fermentazione della crusca mirati ed incrementare le proprietà tecnologiche e nutrizionali dei prodotti con essa arricchiti. I risultati evidenziano, inoltre, come le proprietà testate nei batteri lattici siano altamente ceppo-specifiche. In tal senso, lo studio della diversità in ambito microbiologico rappresenta un'opportunità nello sviluppo biotecnologico e l'utilizzo di un insieme di culture microbiche starter con specifiche proprietà potrebbe essere uno strumento interessante per ottenere prodotti fermentati di maggiore qualità.

ABSTRACT

Several epidemiological studies indicate that high whole grains diets work as protective factors against chronic diseases, such as metabolic syndrome, cardiovascular diseases (CVD), and gastrointestinal cancer. These effects are likely related, at least in part, to their high content of fiber and bioactive compounds, with antioxidants and anti-carcinogenic properties, mainly present in bran and germ of cereal grains. Removal of these fractions during milling to improve shelf-life of the flour results in severe depletion of fiber and bioactive compounds. The loss of about 58% of fiber, 83% of Mg, 61% of folate and 79% of vitamin E has been shown in comparing the content of important nutrients in wholemeal flour and white flour. The aleurone layer (the outermost layer of the endosperm) has been shown to contain many of these functional compounds, but it is partially eliminated in wheat flour milling as a by-product mostly used for the animal feed.

The increasing demand for functional foods and the possibility to take advantage of agro-industrial by-products have attracted great interest in using bran-enriched products. This should lead to a greater value for wheat industries, reducing their environmental impact and getting an economic return. The main reason behind the low utilization rate of wheat bran in baking industry is the gritty texture, bitter and pungent flavour and coarse mouthfeel of bread caused by the bran. However, the fermentation of cereal bran, with yeasts and lactic acid bacteria or with specific enzymes, has been shown to be an interesting pre-treatment to improve technological, sensorial and nutritional properties of bran-enriched products, as well as to degrade anti-nutritive factors, such as phytic acid, in order to increase mineral bioavailability.

This study was aimed to increase the amount of bran’s bioactive compounds, such as soluble fiber, water-extractable arabinoxylans, free ferulic acid, through a fermentation process, in order to use the fermented bran as a potential functional ingredient.

Wheat bran sourdoughlike fermentation processes were conducted through continuous propagation by back-slopping of fermented bran until a stable microbiota was established, reaching high counts of lactic acid bacteria and yeasts. At each refreshment step, bacterial strains were isolated, clustered, molecularly analysed by Randomly Amplified Polymorphic DNA PCR and identified at the species level by 16S rRNA gene sequencing. *Leuconostoc*

mesenteroides, *Lactobacillus brevis*, *Lactobacillus curvatus*, *Lactobacillus sakei*, *Lactobacillus plantarum*, *Pediococcus pentosaceus* and *Pichia fermentans* were dominating the stable sourdough ecosystem.

After fermentation, levels of soluble fiber increased (+ 30%), water-extractable arabinoxylans and free ferulic acid were respectively fourfold and tenfold higher than in raw bran, results probably related to endogenous and microbial enzymatic activities, while phytic acid was completely degraded.

On the basis of the interesting nutritional results, some isolated stains were also characterized in order to select potential starter cultures. The lactic acid bacteria (LAB) were characterized by their bran fermentation capacity, antifungal activity, carbohydrate metabolism, exopolisaccharides production, as well as their antibiotic resistance profiles. The LAB and the yeast were also tested for their potential xylan- and phytate-degrading activities. Moreover, common probiotic properties of the bacterial strains, such as acid and bile tolerance, anti-listeria activity and adhesion to the human intestinal epithelial cells Caco-2 cells were examined. Results suggest that strains belonged to *L. plantarum* and *P. pentosaceus* species could have interesting technological applications, due to their antifungal activity and EPS production. Some of these strains also exhibited phytate degrading activity on calcium and/or on sodium phytate salt and thus they could be exploited to improve mineral bioavailability of fermented products. Moreover, *L. curvatus* (CE 83), *L. brevis* (CE 85), and *P. pentosaceus* (CE 65), seemed to be suitable candidates to be used as probiotics.

In conclusion, the current study supports the hypothesis that fermentation process is an efficient means to increase the amount of bioactive compounds of wheat bran. The characterization of the bacteria involved in bran sourdoughlike fermentation was the first step toward selecting starter cultures, according to their metabolic and enzymatic activities, in order to conduct “tailored” bran fermentation process and improve technological and nutritional properties of bran-enriched products. The present study has shown that investigated properties of the lactic acid bacteria tested are highly strain-specific. In this sense, the study of microbial diversity represents an opportunity for advances in biotechnology and the possibility of mixing strains with different properties and activities could be an interesting approach to obtain fermented products with improved qualities.

1. PREFACE

1. Bioactive compounds in wheat bran

The association between greater intake of whole grains and reduced risk of diseases, including CVD, diabetes and some types of cancer, is one of the most consistent findings in nutritional epidemiology (Ye et al., 2012). Several studies have shown that the regular intake of wholegrain cereals can contribute to reduction of risk factors related to non-communicable chronic diseases (Gil et al., 2011). Giacco et al. (2010) have evaluated in healthy subjects the metabolic effects of a diet rich in whole grain wheat foods versus one based on the same products in refined form; after the whole grain wheat diet both total (- 4.3%; $p<0.03$) and LDL (-4.9%; $p<0.04$) cholesterol levels were lower than after the refined wheat diet. A recent study of Forsberg et al. (2014) have shown that whole grain rye crisp bread for breakfast caused lower self-reported hunger, higher fullness and less desire to eat compared to refined wheat bread. Moreover, an investigation of Connolly et al. (2012) suggests that wholegrain oat-based breakfast cereals may be prebiotics and have low glycaemic index.

In wheat the most significant bioactive compounds, with recognised health benefits, such as minerals, polyphenols (especially phenolic acids), sulfur amino acids, betaine, total choline, alkylresorcinols, vitamins B and E (Fardet, 2010), as well as fibres and others bioactive microcomponents are mainly present in the outer layers of wheat caryopsis (Fig. 1), which are recovered during the milling process in a “technological” fraction called bran. In particular, aleurone is a single cell layer located between the starchy endosperm and the outer layers, which is particularly rich in fiber and bioactive compounds (Brouns et al., 2012), but is removed with bran during the milling process.

Price et al. (2010) show that the incorporation of aleurone-enriched bread into habitual diets lowered plasma levels of major risk factors for cardiovascular diseases such as homocysteine and LDL-cholesterol, probably due to an increased of betaine and phenolic compounds bioavailability.

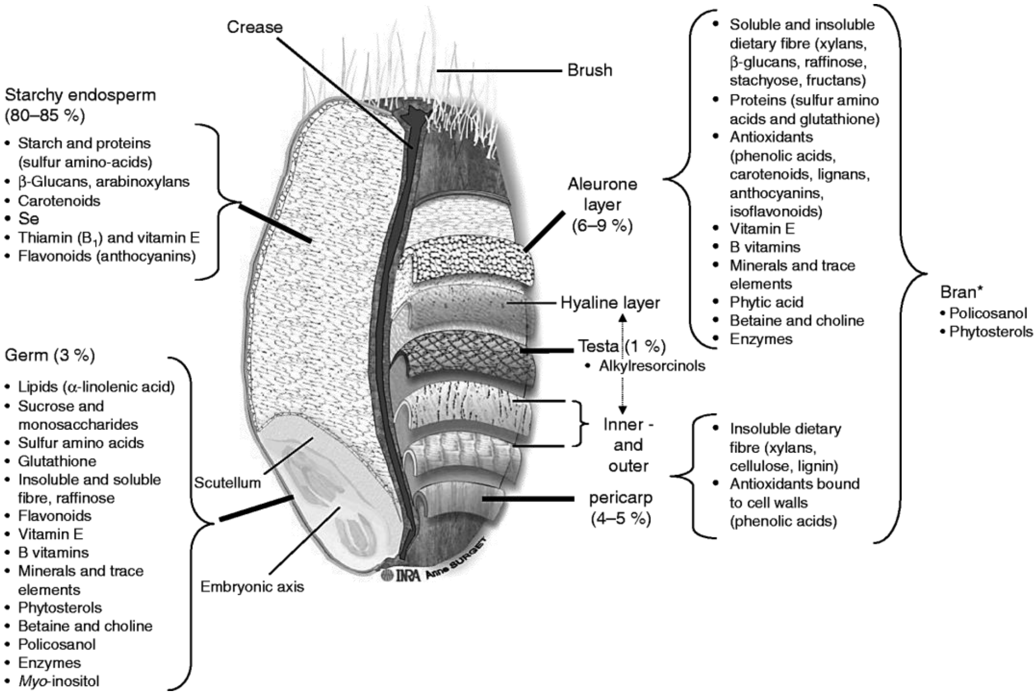

Figure 1. Wheat wholegrain (Fardet 2010).

1.1 Fiber and Arabinoxylans

Trowell (1972) defined dietary fibres as the remnants of edible plant cell polysaccharides, lignin and associated substances, which escape hydrolytic enzymatic digestion in the upper gastrointestinal tract. However, no universally accepted definition exists to date although a Codex definition of dietary fibres was agreed upon in 2009 which defines dietary fibres as ‘‘*carbohydrate polymers with 10 or more monomeric units which are not hydrolysed by the endogenous enzymes in the small intestine of humans*’’ (Codex Alimentarius Commision, 2009).

EFSA (2010) has defined dietary fibre as non-digestible carbohydrates plus lignin, including non-starch polysaccharides (NSP) – cellulose, hemicelluloses, pectins, hydrocolloids (i.e., gums, mucilages, ß-glucans), resistant oligosaccharides – fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), other resistant oligosaccharides, resistant starch – consisting of physically enclosed starch, some types of raw starch granules, retrograded amylose, chemically and/or physically modified starches, and lignin associated with the dietary fibre polysaccharides.

This, along with many other dietary fiber definitions, is linked to analytical criteria, which are good for labelling purposes (particularly solubility in water) but not as informative as for example viscosity and fermentability, as these are physicochemical properties which may affect gastrointestinal function (Kristensen and Jensen, 2011).

The term dietary fibre refers to a vast range of biophysically and biochemically divergent compounds, with varying effects on physiological parameters. In particular, four key factors can be attributed to the range of physiological effects that dietary fibres bring about:

1. The rheological/biophysical properties of dietary fibre within the gut/simulated gastrointestinal conditions, often related to the fiber solubility degree (Jenkins et. al, 2000; Dikeman & Fahey, 2006).
2. The function of fibre within foods as a matrix (Englyst & Englyst, 2005).
3. The biochemical characteristics of various dietary fibres, and their specific effects.
4. The effect of dietary fibres on the large bowel microbiota diversity and the associated by-products of fermentation.

Dietary fibres are recognised for their potential to lower the risk of type II diabetes, colorectal cancer, cardiovascular and diverticular diseases (Collins et al., 2010).

Wholegrain wheat may contain from 9 to 17 g total fibre per 100 g edible portion, which is more than in most vegetables (generally, 6 g/100 g edible portion). Wheat is relatively poor in soluble fibre. It has been found that the soluble:insoluble fibre ratio is about 1:5 for wholegrain wheat, 1:10 for wheat bran and 1:3 for wheat germ (Fardet, 2010).

In particular, the dietary fiber (DF) content of aleurone has been estimated to be 44–50 g/100 g DM (Amrein et al., 2003), depending on the wheat variety. The major polysaccharides present in the fiber fraction are arabinoxylan (65%) and β-glucans (29%), while cellulose plays a minor role (Saulnier et al., 2007).

From a nutritional point of view, arabinoxylans (AX) and compounds resulting from their hydrolysis, such as arabinoxylan-oligosaccharides (AXOS) and xylo-oligosaccharides (XOS), deserve particular attention because of their positive health effects. Arabinoxylan belong to a group of non-starch polysaccharides (NSP) and are an important component of the dietary fiber in cereals (Saeed et al., 2001). These commpounds are present mainly in the bran portion, for example, wheat bran (6.7%). However, the thin aleurone layer surrounding wheat endosperm predominantly contains 60-70% arabinoxylan (Fincher and Stone, 1986). Overall, they constitute 60-69% of NSP in wheat bran and 88% in wheat endosperm (Revanappa et al., 2010).

1.1.1 AX structure

AX consist of a linear backbone of β-D-xylopyranoside units that are either unsubstituted, monosubstituted with a single α-L-arabinofuranoside at either C-(*O*)-2 or C-(*O*)-3, or disubstituted with single *α*-L-arabinofuranoside units at C-(*O*)-2 and C-(*O*)-3 (Gruppen et al. 1993; Izydorczyk & Biliaderis, 1995; Andersson & Aman, 2001).

Less abundant substituents attached to the C-(*O*)-2 position of the xylose residues can be glucuronic acid, 4-*O*-methylglucuronic acid, or short oligomers consisting of L-arabinose, D-xylose, D-galactose, D-glucose, and/or uronic acids, while acetyl groups can be linked to the C-(*O*)-2 and/or C-(*O*)-3 position of the xylose residues (Fig. 2). Hydroxycinnamic acids, mainly ferulic acid, and to a lesser extent dehydrodiferulic acid, *p*coumaric acid, and sinapic acid, are present as substituents as well, and they are generally linked to the C-(*O*)-5 position of terminal arabinose units (Izydorczyk & Biliaderis, 1995; Andersson & Aman, 2001).

The frequency and nature of substituents differs greatly amongst AX from different origin. Clear differences in arabinose to xylose ratio, an indicator of the average degree of arabinose substitution (avDAS), can be found between AX in wheat endosperm (avDAS about 0.5–0.7) and that in the bran tissues, whereby aleurone and seed coat contain lowly substituted AX (avDAS about 0.1–0.4), and outer pericarp contain highly substituted AX (avDAS about 1.1–1.3) (Fig. 3) (Andersson & Aman, 2001; Izydorczyk & Biliaderis, 2005; Barron et al., 2007).

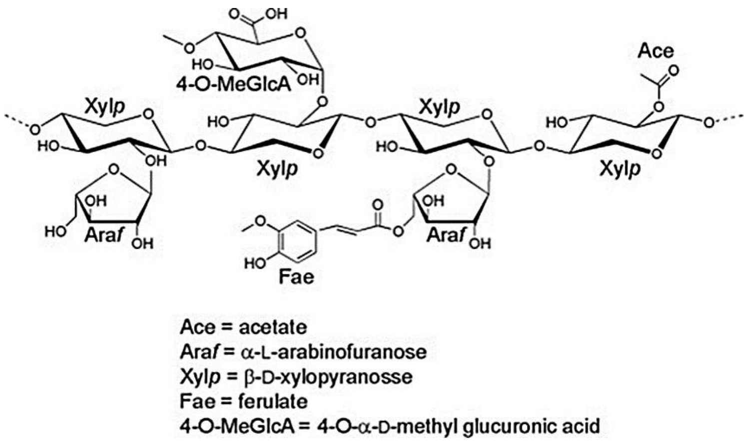

Figure 2. Arabinoxylan molecular structure (Correia et al., 2011)

Part of the AX in cereals are water-extractable, yet the major fraction are water-unextractable (Delcour et al., 1999; Courtin & Delcour, 2001; Maes & Delcour, 2002), the latter probably due to a combination of non-covalent interactions (e.g. hydrogen linkages) and covalent bonds (e.g. dehydrodiferulic acid bridges) with neighboring AX molecules and other cell wall components such as proteins, cellulose, and lignin (Iiyama et al., 1994). According to Saulnier et al. (2007), the total arabinoxylan and water-extractable arabinoxylan content in the common wheat variety are 6.7% and 0.7%, respectively.

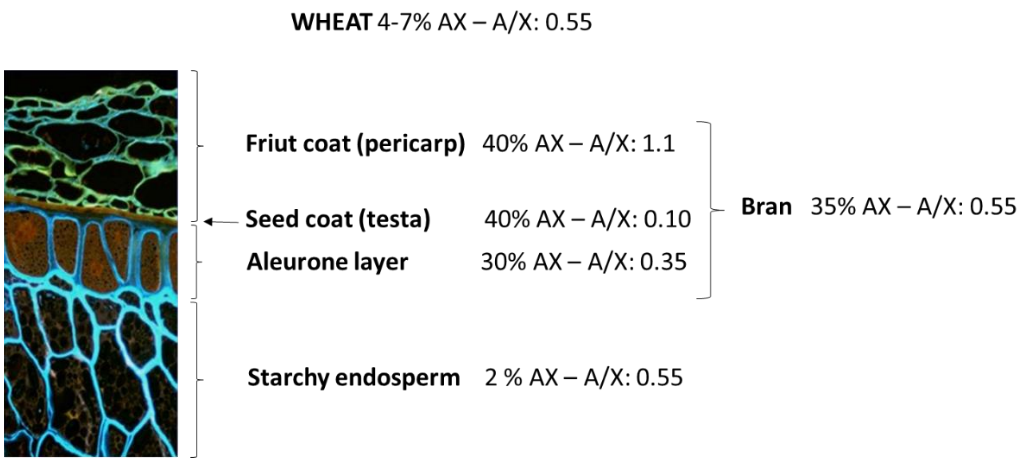

Figure 3. AX in different bran fractions (Autio K., VTT Finland)

1.1.2 AX Hydrolysis

The arabinoxylan-oligosaccharides (AXOS) and the non-substituted xylooligosaccharides (XOS), resulting from an intense hydrolysis of the bran's water-unextractable AX, can be generated in the colon of animals by microbial degradation of AX, or can be present as such in processed food products, or can be prepared and purified from AX-rich sources and used as a food ingredient (Broekaert et al., 2011).

The enzymes involved in AX solubilization are mainly endo-β-1,4-xylanases that cleave β-1,4-glycosyl linkages within the poly-β-1,4-xylose backbone; xylosidases, which release terminal xylose residues from the non-reducing end of the xylan backbone, and alpha-L-arabinofuranosidases, feruloyl esterases, acetyl esterases, and alpha-glucuronidases, which remove arabinose, ferulic acid, acetic acid, and (4-O-methyl) glucuronic acid side chains from the xylan backbone, respectively (Grootaert et al., 2007) (Fig. 4).

The endoxylanases can originate from a combination of different sources including I) endogenous endoxylanases present in cereals (Dornez et al., 2006), II) microorganisms present as natural contaminants on the surface of cereals (Dornez et al., 2006), III) microorganisms added purposely to improve taste, conservation, or leavening properties (e.g. sourdough cultures), IV) purified microbial enzyme preparations added purposely to the food matrix, for instance to increase loaf volume of bread or to increase the filterability of beers (Courtin & Delcour, 2002). In particular, wheat kernel associated endoxylanases are mainly microbial and to a lesser extent wheat endogenous (Dornez et al., 2006).

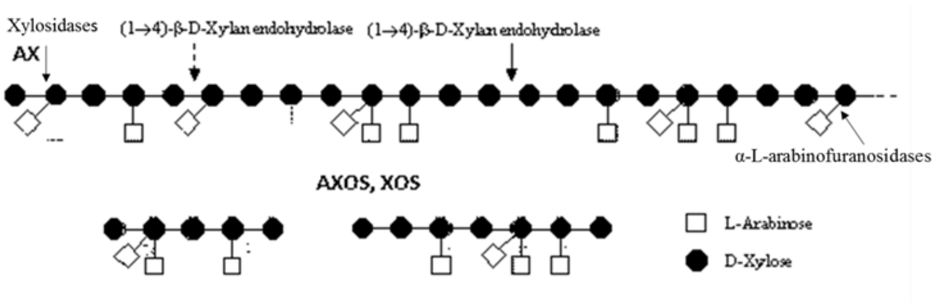

Figure 4. Enzymes involved in AX hydrolysis

1.1.3 Nutritional properties of AX and AXOS

From a nutritional point of view, arabinoxylans (AX) and compounds resulting from their hydrolysis, such as arabinoxylan-oligosaccharides (AXOS) and xylo-oligosaccharides (XOS), deserve particular attention.

AX present different physico-chemical and functional properties depending on their solubility and structural characteristics. Due to their ability to induce viscosity, water extractable arabinoxylan (WE-AX) are able to delay the rate of digestion and absorption of carbohydrates leading to positive effects on the post-prandial glycemic and insulinemic responses (Lu et al., 2000, 2004; Möhlig et al., 2005; Gemen et al., 2011). EFSA panel reports a cause and effect relationship between the consumption of arabinoxylan produced from wheat endosperm and reduction of post-prandial glycemic responses (EFSA 2011).

Several studies indicate that AX, as soluble fiber, can improve glycemic control increasing the viscosity of the stomach content, delaying gastric emptying and nutritient absorption which might result in lower postprandial glycaemic response (Lu et al, 2000, 2004; Garcia et al., 2007). Lu et al., (2000) reported that the addition of 6 g and 12 g of AXrich wheat fibre (ratio of soluble to insoluble = 1.6 and A/X = 0.66) to a breakfast meal allowed a significantly lowered postprandial glucose and insulin response in normal subjects, being the effect dose-dependent.

Another study of Lu et al. (2004) was designed to asses the effect of a 5-week intervention with a diet supplemented with 15 g/day AX (in the form of AX enriched bread and muffins, obtained by a mix of 50% whole wheat, 36% white flour, 14% AX fibre with ratio soluble: total as 0.62) in type II diabetic subjects. In this intervention study with a crossover design, overweight subjects with impaired glucose tolerance received the test diet or a control diet; fasting and 2 h glucose and insulin levels were significantly decreased with the test diet.

In overweight subjects with impaired glucose tolerance, the supplement of 15 g AX (molecular weight = 20–40 kDa; intrinsic viscosity = 80 mL/g; A/X = 0.8) for 6 weeks improved their postprandial serum glucose, insulin and triglyceride response (Garcia et al., 2007).

Utilization of AX-rich fiber also influences plasma lipid concentrations to some extent. Hunninghake et al. (2005) carried out meta-analysis of 67 controlled trials and observed that reduction of 0.045 mmol/L total cholesterol/gram soluble fiber, when fiber supplementation was in the practical range of 2–10 g/day. However, such effects are more pronounced in hypercholesterolemic subjects as compared to the non-significant effects in normolipidaemic subjects.

Moreover, the arabinoxylan-oligosaccharides (AXOS) and the non-substituted xylooligosaccharides (XOS), resulting from an intense hydrolysis of the bran’s water-unextractable AX (WU-AX), have prebiotic function (Van Craeyveld et al., 2008; Vardakou et al., 2008; Cloetens et al., 2010; Broekaert et al., 2011), anti-carcinogenic (Hsu et al., 2004; Femia et al., 2010), antioxidants (Ou et al., 2007) and hypocholesterolemic properties (Broekaert et al., 2011). The available evidence from an extensive range of in vitro, animal, and human studies demonstrates that these fermentable oligosaccharides derived from cereals possess all the hallmarks of prebiotics, including resistance to gastrointestinal hydrolysis and absorption, fermentation by intestinal microbiota, and selective stimulation of the beneficial intestinal microbiota (Grootaert et al., 2009; Broekaert et al., 2011).

The physical and physico-chemical characteristics of AX, such as solubility, hydratation properties, viscosity, molecular weight and branching are involved, also, in their effects on colonic function (Guillon & Champ, 2000; Damen et al., 2011).

Comparing the intestinal fermentation of WU-AX with WE-AX from wheat, WU-AX was only partially fermented in the caeco-colon of rats (around 30 – 40%), whereas 80-90% of WE-AX are fermented (Damen et al., 2011). The degradation of AX seems dependent on the complexity of their structure, which can therefore also affect the production of the beneficial short chain fatty acids (SCFA) and the prebiotic effects during their fermentation. It has been clearly demonstrated that the solubilisation of AX and decrease of its molecular size improved their prebiotic properties. The in vitro fermentation of AX purified from wheat with different molecular sizes (354, 278, and 66 kDa) were associated with a proliferation of the *bifidobacteria*, *lactobacilli*, and *eubacteria* groups, but the 66 kDa AX was particularly selective for *lactobacilli* and presented the higher prebiotic index value (Hughes et al., 2007). Similarly, the treatment of AX (from wheat) with xylanase presented higher prebiotic index than untreated AX during their in vitro fermentation, probably due to the faster metabolization of smaller oligomer fragments by the bacteria (Vardakou et al., 2008). Indeed, many studies have demonstrated the prebiotic effects of arabinoxylan-oligosaccharides (AXOS), which can be prepared and purified by enzymatic hydrolysis of AX. Wheat AXOS presented prebiotic effects in chickens (Courtin et al., 2008) and rats (Van Craeyveld et al., 2008; Damen et al., 2011). In humans, a prebiotic effect of AXOS was observed after the daily consumption of orange juice enriched with AXOS (10 g per day) during 3 weeks (Cloetens et al., 2010). Also when AXOS are consumed in a structured food, as part of a ready-to-eat breakfast cereal (at 0, 2.2 or 4.8 g/day for 3 weeks), they presented prebiotic properties by a selective increased fecal bifidobacteria in a dose dependent manner in healthy men and women, but the amount of lactobacilli remained constant (Maki et al., 2012). Concerning the production of the beneficial SCFA, the consumption of AX (7.6% AX in bread; A/X = 0.87) during three weeks promoted a higher production of total SCFA and in particular of butyrate in the feces of the seven healthy volunteers compared to the control (white bread) or inulin-enriched bread (Grasten et al., 2003). In the study of Anson et al. (2011), the greater solubilization of wheat bran fibres by enzymes and fermentation treatments resulted in a higher production of butyrate in an in vitro model of human colon, as well as observed by Damen et al. (2011), which showed that purified WE-AX (from wheat bran) also provided a higher production of total SCFA in the caecum of rats. Therefore, it has been observed that the depolimerisation of AX can facilitate its degradation and also improve their prebiotic properties. However, the relation between the production of SCFA and the structure of AX is still not fully elucidated and it seems dependent also on the cereal matrix where AX are inserted. Most of the studies found in the literature have been performed with purified fibres, which did not take into account the effects of the matrix structure of cereal. The food matrix can influence the physical accessibility of the fibres to the microbiota, which is the first limiting parameter occurring during the fermentation (Guillon & Champ, 2000).

1.1.4 Technological properties

Arabinoxylans play an important role in end-use quality of flour, mainly through their interaction with water and aptitude to cross-link other arabinoxylan molecules and proteins (Finnie et al., 2006; Du et al., 2009). The functional properties of arabinoxylan are strongly associated with their molecular weights and degrees of branching (Autio, 2006; Revanappa et al., 2010).

The main physical property of arabinoxylan lies in its ability to form viscous aqueous solutions (Fincher & Stone, 1986) that significantly influence the behavior of processed cereal grain, especially bread making (Gamlatha et al., 2008).

The positive impacts of AX include increase the water-holding capacity, the viscosity and the dough development time that further lead to enhance the gas-holding/gas-retention network in the dough (Neukom & Markwalder, 1978). These properties depend on the quantity and molecular size of arabinoxylan, the semi-flexible conformation and ferulic acid contents (Sasaki et al., 2004; Izydorczyk & Biliaderis, 2007).

The hydration ability of arabinoxylan can be further improved with oxidative gelation (Izydorczyk et al., 1990), and cross linkage (Primo-Martın & Martınez-Anaya, 2003).

However, a high degree of crosslinking resulted in a decreased water holding capacities owing to the swelling process (Izydorczyk et al., 1990; Dervilly-Pinel et al., 2004).

The addition of WE-AX, thanks to their viscosity and interfacial activity, results in better retention of gases in the dough owing to enhanced elasticity, and strength of the protein films (Hoseney, 1984). Rao et al. (2007) observed that the addition of WE-AX strengthened the wheat flour dough. Higher molecular weights might have allowed better interaction of AX with the starch-gluten complex. In contrast, addition of WU-AX decreased dough extensibility (Freitas et al., 2003). The mechanism behind their action involves competition for water and cross-linking (Wang et al., 2002).

Solubilization of WU-AX to WE-AX, due to the activity of endogenous or microbial xylan-degrading enzymes, have been reported to improve bread volume and texture in wheat baking (Courtin & Delcour, 2002).

Katina et al. (2012) reported that bread containing yeast-fermented bran, with an increased level of soluble AX (+60%), had improved volume (+10–15%) and crumb softness (25–35% softer) in comparison to unfermented counterparts.

The positive effects of AX on crumb texture can be correlated with increased moisture content. Water acts as plasticizer in gluten-starch composite matrix lowering rigidity in final products (Biliaderis et al., 1995). Simultaneously, decreased gel firming rate and chain ordering of amylopectin was enhanced with the addition of AX in the dough matrix. With the addition of WE-AX in a concentration of 5 g/kg, volume of bread was enhanced significantly and the texture of bread was also improved considerably (Saeed et al., 2011). Moreover, AX has been shown to interfere with the intermolecular re-association of amylose and amylopectin, decreasing retrogradation and increasing the shelf-life of bread (Kim & D'Appolonia, 1977).

However, some studies reported that the addition of non-starch polysaccharides, such as AX, lowers the gluten quality owing to their interaction with other molecules and competition for water (Wang et al., 2002). Labat et al. (2002) suggested an indirect effect of non-starch polysaccharides that would be caused by their ability to form a network limiting the movement of glutenin proteins and the formation of larger aggregates. These undesirable effects can be corrected adding xylanase before mixing (Wang et al., 2002). According to Courtin et al. (2001), the addition of xylanase action results in water re-distribution in dough by breaking the AX molecules.

1.2 Ferulic acid

Whole-grain cereals can protect the body against the increased oxidative stress that is involved and/or associated with all the major chronic diseases: metabolic syndrome (Ford et al., 2003), obesity (Keaney et al., 2002; Higdon & Frei, 2003), diabetes (Maiese K et al., 2007), cancers (Bartsch & Nair, 2006) and CVD (Castelao & Gago-Dominguez, 2008).

Whole-grain cereals are good sources of antioxidants, as shown by measurements made in vitro of the antioxidant capacity of whole-grain, bran and germ fractions (Serpen et al., 2008).

The content of total phenolic acids in bran varies in the range of 761–1384 (mg/100 g), of which around 46–63 (mg/100 g) are extractable (free and conjugated). Ferulic Acid (FA) represents over 95% of the phenolic acids and can be present in wheat bran in monomeric (4.9 – 7.1 mg/g), dimeric (0.7 – 1.0 mg/g) and trimeric (0.1 – 0.2 mg/g) forms (Antoine et al., 2004; Harris et al., 2005; Rosa et al., 2013).

Indeed, wheat grain phenolic acids can be present in three different forms: ester-linked with polysaccharides, conjugated with mono- or oligosaccharides and free.

In the outer layers of wheat caryopsis, phenolic compounds, especially FA, are largely located as structural components of the cell walls of aleurone and pericarp. Most of the FA is covalently bound to complex polysaccharides in the cell walls, mainly AX and lignin (Barron et al., 2007; Anson et al., 2009a) and it is partly responsible for the insolubility of cell wall structures of cereal kernels.

The total phenolic content of wheat fractions is positively correlated with their antioxidant capacity (Liyana-Pathirana & Shahidi, 2006) and FA has been suggested to be the major contributor of the antioxidant capacity (Mateo Anson et al., 2008).

Indeed, FA, due to its aromatic ring, has a well documented antioxidant property in vitro as it has the ability to scavenge free radicals avoiding the oxidation of biologically relevant molecules (Zhao & Moghadasian, 2008).

Moreover, in literature is reported the role of FA as an anti-microbial, antiapoptotic, anti-ageing, anti-inflammatory (Murakami et al., 2002), neuroprotective, hypotensive, pulmonary-protective and cholesterol lowering agent in metabolic diseases such as thrombosis, atherosclerosis (Rakotondramanana et al., 2007), cancer (Kawabata et al., 2000), and diabetes (Jung et al., 2007).

The feruloyl oligosaccharides given intragastrically in diabetic rats at a dose of 50 μmol of bound FA/kg of body weight result in a lower serum lipid peroxidation as well as a greater antioxidant capacity in liver and tested tissues and a higher activity of antioxidant enzymes (glutathione peroxidase and superoxide dismutase) (Ou et al., 2007). The diet supplementation with feruloyl oligosaccharides from wheat bran (prepared by xylanase treatment) at 1% (corresponding to 160 mg of feruloyl oligosaccharides/kg body weight) during 4 weeks also decreased the levels of oxidative stress biomarkers and increased the activities of antioxidant enzymes in rat plasma (Wang et al., 2009).

Although the outermost part of the grain, the bran, is rich in FA, its bioaccessibility or intestinal release from that matrix is very low, thereby reducing its antioxidant action. The low bioaccessibility is explained by the structural position of most of the FA, which is covalently bound to the indigestible polysaccharides of the cell walls constituting the fiber (Anson et al., 2009a), such as AX. In rats, diet enriched with wheat fractions resulted in 90-95% lower FA urine excretion and 5-fold lower plasma FA concentration comparing to rats fed with free FA at similar concentration (Adam et al., 2002).

Innovative processing techniques, such as reduction of particle size (increasing the specific surface area) via ultra-fine grinding or solubilization of cell wall polysaccharides via enzymatic processing have been shown to increase the bioaccessibility of phenolic compounds from wheat bran (Anson et al., 2009b). The FA bioavailability could be improved by enzymatic and fermentation treatments of wheat bran, which released its phenolic acids from polysaccharides (Mateo Anson et al., 2009b; Pekkinen et al., 2014).

If the AX fraction is of small molecular size as for feruloyl oligosaccharides, FA can be released in the rat foregut by the action of the mucosal esterases (Andreasen et al., 2001). Some feruloyl oligosaccharides from larger size and all feruloyl polysaccharides can not be hydrolysed in the foregut and would be fermented by the colonic microbiota (Zhao et al., 2003). In the colon, the fiber fermentation, combined with the action of some microbial feruloyl esterases, allow the release of FA from polysaccharides. Consequently, the fraction arriving in the colon is still interesting, as it could protect the colonic mucosa from inflammatory and oxidative stress, thus potentially protecting this mucosa from cancer (Srinivasan et al., 2007).

1.3 Phytic acid

The outer layers of wheat caryopsis represent a good source of minerals, such as Fe, Mg, Zn, Ca, which are involved in activation of intracellular and extracellular enzymes, in regulation of pH levels in body fluids, and in osmotic balance between cells and their environment. However, mineral availability is limited by the simultaneous presence of phytic acid (PA) (avarage content in wheat bran 4.2%) (Fardet, 2010).

This molecule is highly charged with six phosphate groups extending from the central myo-inositol ring (Fig. 5) (IP6). For this property, PA is considered to be an antinutritional factor for humans and animals as it acts as an excellent chelator of cations such as Ca^{2+}, Mg^{2+}, Fe^{2+} and Zn^{2+} and as it complexes the basic amino acid group of proteins, thus decreasing the dietary bioavailability of these nutrients (Wodzinski & Ullah, 1996; Dvorakova, 1998).

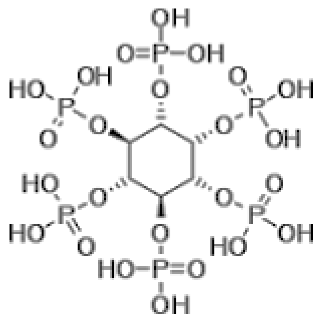

Figure 5. Phytic acid molecular structure

The formation of insoluble mineral-phytate complexes nonabsorbable by human gastrointestinal tract is considered as the main reason for poor mineral availability (Lopez et al., 2002; Konietzny & Greiner 2003).

Thus, enzymatic hydrolysis of PA is desirable; this is possible through the action of some enzymes called phytases. Phytases are meso-inositol hexaphospho- hydrolases that catalyze the stepwise phosphate splitting of phytic acid or phytate to lower inositol phosphate esters and inorganic phosphate (Lei & Porres, 2003). Just like other enzymes, phytase activity or function is affected by the inherent properties of the enzyme (temperature and pH optima, thermostability, proteolysis resistance, molecular mass, isoelectric point, and substrate specificity) and the action conditions.

Therefore, these enzymes have an important role in human diet and are considered useful in increasing the nutritional quality of phytate-rich foods and they could be exploited in producing functional foods (Anastasio et al., 2010).

Phytases can be derived from a number of sources including plants, animals and microorganisms.

Recent research has shown that microbial sources are more promising for the production of phytases on a commercial level and on cereal based foods (De Angeli et al., 2003).

Microbial phytase activity was most frequently detected in fungi, and in particular in some species belonged to genus *Aspergiullus*. Moreover, bacteria belonged to the genera *Bacillus* (Choi et al., 2001) and *Enterobacter* (Yoon et al.,1996) showed extracellular phytase activity.

Nomenclature Committee of the International Union of Biochemistry distinguishes 2 types of phytases: 3- and 6-phytases. This classification is based on the dephosphorylation of IP6 at position D-3 or L-6 of the inositol ring; 3-phytase is typical for microorganisms while 6-phytase has been considered to be characteristic of seeds or higher plants (Cosgrove 1980).

Endogenous phytase activity may be present in the wheat and rye flours but its level greatly varies with the variety and crop year, and, generally, is considered to be insufficient to significantly decrease the amount of phytic acid (Cossa et al., 2000).

Bread making by sourdough fermentation may result in a more suitable pH condition for the degradation of phytic acid by endogenous phytases and sourdough may be a source of microbial phytases (Reale et al., 2007).

1.4 References

Adam A et al., 2002. The bioavailability of ferulic acid is governed primarily by the food matrix rather than its metabolism in intestine and liver in rats. J Nutr 132:1962-1968.

Anastasio M et al., 2010, Selection and Use of Phytate-Degrading LAB to Improve Cereal-Based Products by Mineral Solubilization During Dough Fermentation. J Food Sci 75:M28-M35.

Andersson R, Aman P, 2001, Cereal arabinoxylan: Occurrence, structure and properties. In: Advanced Dietary Fiber Technology. McCleary, B. V. and Prosky, L., Eds. Blackwell Science Ltd., Oxford. pp. 301–314.

Andreasen MF et al., 2001, Esterase activity able to hydrolyze dietary antioxidant hydroxycinnamates is distributed along the intestine of mammals. J Agric Food Chem 49:5679-5684.

Anson NM et al., 2008. Ferulic acid from aleurone determines the antioxidant potency of wheat grain (Triticum aestivum L.). J Agric Food Chem 56:5589-5594.

Anson NM et al., 2009 a, Bioavailability of ferulic acid is determined by its bioaccessibility. J Cereal Sci 49:296-300.

Anson NM et al., 2009 b, Bioprocessing of wheat bran improves in vitro bioaccessibility and colonic metabolism of phenolic compounds. J Agric Food Chem 57:6148-6155.

Anson NM et al., 2011, Effect of bioprocessing of wheat bran in wholemeal wheat breads on the colonic SCFA production in vitro and postprandial plasma concentrations in men. Food Chem 128:404-409.

Antoine, C et al., 2004, Wheat bran tissue fractionation using biochemical markers. J Cereal Sci 39:387-393.

Autio K. 2006, Effects of cell wall components on the functionality of wheat gluten. Biotechnol Adv 24:633-635.

Barron C et al., 2007, Relative amounts of tissues in mature wheat (Triticum aestivum L.) grain and their carbohydrate and phenolic acid composition. J Cereal Sci 45:88-96.

Bartsch H, Nair J, 2006, Chronic inflammation and oxidative stress in the genesis and perpetuation of cancer: role of lipid peroxidation, DNA damage, and repair. Langenbecks Arch Surg 391:499-510.

Biliaderis CG et al., 1995, Effect of arabinoxylans on bread-making quality of wheat flours. Food Chem 5:165-171.

Broekaert WF et al., 2011, Prebiotic and other health-related effects of cereal-derived arabinoxylans, arabinoxylan-oligosaccharides, and xylooligosaccharides. Crit Rev Food Sci Nutr 51:178-194.

Brouns F et al., 2012, Wheat aleurone: separation, composition, health aspects, and potential food use. Crit Rev Food Sci Nutr 52:553-568.

Castelao JE, Gago-Dominguez M, 2008, Risk factors for cardiovascular disease in women: relationship to lipid peroxidation and oxidative stress. Med Hypotheses 71:39-44.

Choi YM et al., 2001, Purification and properties of extracellular phytase from Bacillus sp. KHU-10. J Protein Chem 20:287-92.

Cloetens L et al., 2010, Tolerance of arabinoxylan-oligosaccharides and their prebiotic activity in healthy subjects: a randomised, placebo-controlled cross-over study. Br J Nutr 103:703-13.

Codex Alimentarius Commision, 2009. Report of the 30th Session of the Codex Committee on Nutrition and Foods for Special Dietary Uses. ALINORM 09/32/26 November 2008: para 27–54 and Appendix II.

Collins HM et al., 2010, Variability in Fine Structures of Non cellulosic Cell Wall Polysaccharides from Cereal Grains:Potential Importance in Human Health and Nutrition. Cereal Chem 87:272-282.

Connolly ML et al., 2012, Wholegrain oat-based cereals have prebiotic potential and low glycaemic index. Br J Nutr 108:2198-2206.

Correia, MAS et al., 2011, Structure and Function of an Arabinoxylan-specific Xylanase. J Biol Chem 286: 22510-22520.

Cosgrove DJ, Inositol phosphates: their chemistry, biochemistry and physiology. New York: Elsevier, 1980.

Cossa J et al., 2000, Variabilities of total and phytate phosphorus contents as well as phytase activity in wheat. Tropenlandwirt 101:119-126.

Courtin CM, Delcour JA, 2001, Relative activity of endoxylanases towards water-extractable and water-unextractable arabinoxylan. J Cereal Sci 33:301-312.

Courtin CM, Delcour JA, 2002, Arabinoxylans and endoxylanases in wheat flour bread-making. J Cereal Sci 35:225-243.

Courtin CM et al., 2008, Effects of dietary inclusion of xylooligosaccharides, arabinoxylooligoscaccharides and soluble arabinoxylan on the microbial composition of caecal contents of chickens. J Sci Food Agric 88:2517-2522.

Damen B et al., 2011, Prebiotic effects and intestinal fermentation of cereal arabinoxylans and arabinoxylan oligosaccharides in rats depend strongly on their structural properties and joint presence. Mol Nutr Food Res 55:1862-1874.

De Angelis M et al., 2003, Phytase activity in sourdough lactic acid bacteria: purification and characterization of a phytase from Lactobacillus sanfranciscensis CB1. Int J Food Microbiol 87:259-270.

Dervilly-Pinel G et al., 2004, Investigation of the distribution of arabinose residues on the xylan backbone of watersoluble arabinoxylans from wheat flour. Carbohydr Polym 55:171-177.

Delcour JA et al., 1999, Distribution and structural variation of arabinoxylans in common wheat mill streams. J Agric Food Chem 47:271-275.

Dikeman CL, Fahey GC Jr, 2006, Viscosity as related to dietary fiber: a review. Crit Rev Food Sci Nutr 46:649-663.

Dornez E et al., 2006, Wheat-kernel-associated endoxylanases consist of a majority of microbial and a minority of wheat endogenous endoxylanases. J Agric Food Chem 54:4028-4034.

Du C et al., 2009, Evaluating the feasibility of commercial arabinoxylan production in the context of a wheat biorefinery principally producing ethanol. Part 1. Experimental studies of arabinoxylan extraction from wheat bran. Chem Eng Res Des 87:1232-1238.

Dvorakova J, 1998, Phytase, sources, preparation and exploitation. Folia Microbiol 43:323-338.

EFSA Panel on Dietetic Products, Nutrition, and Allergies (NDA) 2010. Scientific Opinion on Dietary Reference Values for carbohydrates and dietary fibre. EFSA Journal; 8:1462 doi:10.2903/j.efsa.2010.1462.

EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) 2011. Scientific Opinion on the substantiation of health claims related to arabinoxylan produced from wheat endosperm and reduction of post-prandial glycaemic responses (ID 830) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. EFSA Journal,9:2205.

Englyst KN, Englyst, HN, 2005, Carbohydrate bioavailability. Br J Nutr, 94:1-11.

Fardet A. 2010, New hypotheses for the health-protective mechanisms of whole-grain cereals: what is beyond fibre?. Nutr Res Rev 23:65-134.

Femia AP et al., 2010, Arabinoxylan-oligosaccharides (AXOS) reduce preneoplastic lesions in the colon of rats treated with 1,2-dimethylhydrazine (DMH). Eur J Nutr 49:127-32.

Fincher GB, Stone BA 1986, Cell walls and their components in cereal grain technology. In Advances in Cereal Science and Technology, Pomeranz, Y., Ed., American Association of Cereal Chemists, St Paul, MN pp. 207–295.

Finnie SM et al., 2006, Influence of cultivar and environment on water-soluble and water-insoluble Arabinoxylans in soft wheat. Cereal Chem 83:617-623.

Ford ES et al., 2003, The metabolic syndrome and antioxidant concentrations: findings from the Third National Health and Nutrition Examination Survey. Diabetes 52:2346-2352.

Forsberg T et al., 2014, Effects of whole grain rye crisp bread for breakfast on appetite and energy intake in a subsequent meal: two randomised controlled trails with different amounts of test foods and breakfast energy content. J Nutr 13:26.

Freitas RA et al., 2003, A rheological description of mixtures of galactoxyloglucan with high amylose and waxy corn starches. Carbohydr Polym 51:25-32.

Garcia AL et al., 2007, Arabinoxylan consumption decreases postprandial serum glucose, serum insulin and plasma total ghrelin response in subjects with impaired glucose tolerance. Eur J Clin Nutr 61: 334-341.

Gamlatha J et al., 2008, Barley (1-3; 1-4)-bglucan and arabinoxylan content are related to kernel hardness and water uptake. J Cereal Sci 47: 365-371.

Gemen R et al., 2011, Relationship between molecular structure of cereal dietary fiber and health effects: focus on glucose/insulin response and gut health. Nutr Rev 69:22-33.

Giacco R et al., 2010, Effects of the regular consumption of wholemeal wheat foods on cardiovascular risk factors in healthy people. Nutr Metab Cardiovasc Dis 20:186-194.

Gil A et al., 2011, Wholegrain cereals and bread: a duet of the Mediterranean diet for the prevention of chronic diseases. Public Health Nutr 14:2316-2322.

Grasten S et al., 2003, Effects of wheat pentosan and inulin on the metabolic activity of fecal microbiota and on bowel function in healthy humans. Nutr Res 23:1503-1514.

Grootaert C et al., 2007, Microbial metabolism and prebiotic potency of arabinoxylan oligosaccharides in the human intestine. Trends Food Sci Tech 18:64-71.

Grootaert C et al., 2009, Comparison of prebiotic effects of arabinoxylan oligosaccharides and inulin in a simulator of the human intestinal microbial ecosystem. FEMS Microbiol Ecol 69:231-42.

Gruppen H et al., 1993, Waterunextractable cell wall material from wheat flour .3. A structural model for arabinoxylans. J Cereal Sci 18:111–128.

Guillon F, Champ M, 2000, Structural and physical properties of dietary fibres, and consequences of processing on human physiology. Food Res Int 33, 233-245.

Harris PJ et al., 2005, Production and characterisation of two wheat-bran fractions: an aleurone-rich and a pericarp-rich fraction. Mol Nutr Food Res 49:536-545.

Higdon JV, Frei B, 2003, Obesity and oxidative stress –a direct link to CVD? Arterioscler Thromb Vasc Biol 23:365-367.

Hoseney RC 1984, Functional properties of pentosans in baked foods. Food Technol 38:114-117.

Hsu CK et al., 2004, Xylooligosaccharides and fructooligosaccharides affect the intestinal microbiota and precancerous colonic lesion development in rats. J Nutr 134:1523-1528.

Hughes SA et al., 2007, In vitro fermentation by human fecal microflora of wheat arabinoxylans. J Agric Food Chem 55, 4589-4595.

Hunninghake DB et al., 2005, Hypercholesterolemic effect of dietary fiber supplement. Am J Clin Nutr 59:1050-1054.

Iiyama K et al., 1994, Covalent cross-Links in the cell wall. Plant Physiol. 104:315-320.

Izydorczyk MS et al., 1990, Oxidative gelation studies of water-soluble pentosans from wheat. J Cereal Sci 11: 153-169.

Izydorczyk MS, Biliaderis CG, 1995, Cereal arabinoxylans: Advances in structure and physicochemical properties. Carbohydr Polym 28:33-48.

Izydorczyk MS, Biliaderis CG, Arabinoxylans: Technologically and nutritionally functional plant polysaccharides. In Functional Food Carbohydrates, Eds., CRC Press, Boca Raton, FL. 2007, pp. 249–290

Jenkins DJA et al., 2000, Viscous and nonviscous fibres, nonabsorbable and low glycaemic index carbohydrates, blood lipids and coronary heart disease. Curr Opin Lipidol 11:49-56.

Jung EH et al., 2007, Hypoglycemic effects of a phenolic acid fraction of rice bran and ferulic acid in C57BL/KsJ-db/db mice. J Agric Food Chem 55:9800-9804.

Kawabata K et al., 2000, Modifying effects of ferulic acid on azoxymethane-induced colon carcinogenesis in F344 rats. Cancer Letters 157:15-21.

Katina K et al., 2012, Fermented Wheat Bran as a Functional Ingredient in Baking. Cereal Chem 89:126-134.

Keaney JF et al., 2002, Obesity as a source of systemic oxidative stress: clinical correlates of oxidative stress in the Framingham Study. Circulation 106, 467.

Kim SK, D'Appolonia BL, 1977, Effect of pentosans on the retrogradation 558 of wheat starch gels. Cereal Chem 54:150-160.

Konietzny U, Greiner R, Phytic acid: nutritional impact. In Encyclopedia of food science and nutrition. Caballero B, Trugo L, Finglas P, Eds., London, U.K.: Elsevier. 2003, pp 4555-63.

Kristensen M., Jensen MG, 2011, Dietary fibres in the regulation of appetite and food intake. Importance of viscosity. Appetite 56:65-70.

Labat E et al., 2002, Effect of flour water-extractable pentosans on molecular associations in gluten during mixing. LWT. Food Sci Technol 35:185-189.

Lei XG, Porres JM, 2003, Phytase enzymology, applications, and biotechnology. Biotechnol Lett 25:1787-94.

Liyana-Pathirana CM, Shahidi F, 2006, Importance of insoluble-bound phenolics to antioxidant properties of wheat. J Agric Food Chem 54:1256-1264.

Lopez HW et al., 2002, Minerals and phytic acid interactions: is it a real problem for human nutrition? Int J Food Sci Technol 37:727-39.

Lu ZX et al., 2000, Arabinoxylan fiber, a byproduct of wheat flour processing, reduces the postprandial glucose response in normoglycemic subjects. Am J Clin Nutr 71:1123-1128.

Lu ZX et al., 2004, Arabinoxylan fibre improves metabolic control in people with Type II diabetes. Eur J Clin Nutr 58:621-628.

Maes C, Delcour JA, 2002, Structural characterisation of waterextractable and water-unextractable arabinoxylans in wheat bran. J Cereal Sci 35:315-326.

Maiese K et al., 2007, Oxidative stress biology and cell injury during type 1 and type 2 diabetes mellitus. Curr Neurovasc Res 4:63-71.

Maki KC et al., 2012, Digestive and physiologic effects of a wheat bran extract, arabino-xylan-oligosaccharide, in breakfast cereal. Nutrition 28, 1115-21.

Möhlig M et al., 2005, Arabinoxylan-enriched meal increases serum ghrelin levels in healthy humans. Horm Metabc Res 37:303-308.

Murakami A et al., 2002. FA15, a hydrophobic derivative of ferulic acid, suppresses inflammatory responses and skin tumor promotion: comparison with ferulic acid. Cancer Letters 180:121-129.

Neukom H, Markwalder HU, 1978, Oxidative gelation of wheat flour pentosans: A new way of cross linking polymers. Cereal Foods World 23:374–376.

Ou SY et al., 2007, Protection against oxidative stress in diabetic rats by wheat bran feruloyl oligosaccharides. J Agric Food Chem 55:3191-3195.

Pekkinen J et al., 2014, Disintegration of wheat aleurone structure has an impact on the bioavailability of phenolic compounds and other phytochemicals as evidenced by altered urinary metabolite profile of diet-induced obese mice. Nutr Metab 11: 1.

Price R K et al., 2010, Consumption of wheat aleurone-rich foods increases fasting plasma betaine and modestly decreases fasting homocysteine and LDL-cholesterol in adults. J Nutr 140:2153-2157.

Primo-Martın C, Martınez-Anaya MA, 2003, Influence of pentosanase and oxidases on water-extractable pentosans during straight breadmaking process. Food Chem Toxicol 68:31-41.

Rakotondramanana DLA et al., 2007, Synthesis of ferulic ester dimers, functionalisation and biological evaluation as potential antiatherogenic and antiplasmodial agents. Bioorg Med Chem Lett 15:6018-6026.

Rao R et al., 2007, Structural characteristics of water-soluble feruloyl arabinoxylans from rice (Oryza sativa) and ragi (finger millet, Eleusine coracana): Variations upon malting. Food Chem 104:1160-1170.

Reale A et al., 2007, The importance of lactic acid bacteria for phytate degradation during cereal dough fermentation. J Agric Food Chem 55:2993-2997.

Revanappa SB et al., 2010, Structural characterisation of pentosans from hemicellulose B of wheat varieties with varying chapati-making quality. Food Chem 119:27-33.

Rosa NN et al. 2013, Ultra-fine grinding increases the antioxidant capacity of wheat bran. J Cereal Sci 57:84-90.

Saeed F et al., 2011. Arabinoxylans and arabinogalactans: acomprehensive treatise. Crit Rev Food Sci Nutr 51:467-476.

Sasaki T et al., 2004, Effect of water-soluble and insoluble non-starch polysaccharides isolated from wheat flour on the rheological properties of wheat starch gel. Carbohydr Polym 57: 451-458.

Saulnier L et al., 2007, Wheat arabinoxylans: Exploiting variation in amount and composition to develop enhanced varieties. J Cereal Sci 46:261-281.

Serpen A et al., 2008, Direct measurement of the total antioxidant capacity of cereal products. J Cereal Sci 48:816-820.

Srinivasan M et al., 2007, Ferulic acid: Therapeutic potential through its antioxidant property. J Clin Biochem Nutr 44: 275-295.

Trowell H. 1972, Ischemic heart disease and dietary fiber. Am J Clin Nutr 25: 926-932.

Van Craeyveld V et al., 2008, Structurally different wheat-derived arabinoxylooligosaccharides have different prebiotic and fermentation properties in rats. J Nutr 138:2348-55.

Vardakou M et al., 2008, Evaluation of the prebiotic properties of wheat arabinoxylan fractions and induction of hydrolase activity in gut microflora. Int J Food Microbiol 123:166-70.

Wang M et al., 2002, Interaction of water extractable pentosans with gluten protein: effect on dough properties and gluten quality. J Cereal Sci 36:25-37.

Wang J et al., 2009. Wheat bran feruloyl oligosaccharides enhance the antioxidant activity of rat plasma. Food Chem 123: 472-476.

Wodzinski RJ, Ullah AHJ, 1996, Phytase. Adv Appl Microbiol 42:263-302.

Yoon SJ et al., 1996, Isolation and identification of phytase-producing bacterium, Enterobacter sp. 4, and enzymatic properties of phytase enzyme. Enzyme Microb Tech 18:449-454.

Zhao Z et al., 2003, Digestion and absorption of ferulic acid sugar esters in rat gastrointestinal tract. J Agric Food Chem 51:5534-5539.

Zhao Z, Moghadasian MH, 2008, Chemistry, natural sources, dietary intake and pharmacokinetic properties of ferulic acid: A review. Food Chem 109:691-702.

2. STATE OF THE ART

Several epidemiological studies indicate that high whole grains diets work as protective factors against chronic diseases, such as obesity (Koh-Banerjee & Rimm, 2003; van de Vijver et al., 2009) metabolic syndrome (Sahyoun et al., 2006; Aleixandre & Miguel, 2008), type 2 diabetes (de Munter et al., 2007), CVD (Mellen et al., 2008) and gastrointestinal cancer (Chan JM et al., 2007; Schatzkin et al., 2008). These effects are likely related, at least in part, to their high content of fiber and bioactive compounds, with antioxidants and anti-carcinogenic properties, mainly present in bran and germ of cereal grains (Fardet et al., 2010; Okarter et al., 2010). Removal of these fractions during milling, to improve shelf-life of the flour, results in severe depletion of fiber and bioactive compounds.

The loss of about 58% of fiber, 83% of Mg, 61% of folate and 79% of vitamin E has been shown in comparing the content of important nutrients in wholemeal flour and white flour (Truswell, 2002). In particular, the aleurone layer (the most outer layer of the endosperm) has been shown to contain many of these functional compounds (Bronus et al., 2012), such as dietary fibre, phenolic compounds, phytochemicals, vitamins and minerals, but it is partially eliminated in wheat flour milling as a by-product, mostly used for the animal feed (Vitaglione et al., 2008). Current flour mills operate at 70–80% grain to flour conversion yields where the remaining 20–30% constitutes various waste or by-product streams that contain predominantly bran as well as some germ and endosperm (Koutinas et al., 2008). In 2011/2012 the italian annual production of wheat bran was estimated around 987.000 t (Italmopa, 2012).

The continuous search for functional ingredients, providing healthy effects, and the possibility to take advantage of agro-industrial by-products have attracted great interest in using bran-enriched products. This should lead to a greater value for wheat industries, reducing their environmental impact and getting an economic return.

The main reason behind the low utilization rate of wheat bran in baking industry is the gritty texture, bitter and pungent flavour and coarse mouthfeel of bread caused by the bran (Coda et al., 2014).

However, the fermentation of cereal bran, such as wheat and rye, has shown to be an interesting pre-treatment in order to improve sensorial, technological, and nutritional properties of bran-enriched products (Katina et al., 2007; Poutanen et al., 2009; Katina et al., 2012; Coda et al., 2014). The HEALTHGRAIN european project has recently emphasized the possibility to increase the proportion of bioactive compounds in cereals and especially in their by-products such as bran, through biotechnological processes (Shewry, 2009).

Fermentation is considered to be an important biotechnological option to modify the techno-functionality and exploit the potential of wheat, rye, wholegrain flours as well as that of fibre-rich cereal ingredients, such as bran (Katina et al., 2007; Coda et al., 2014). In particular, sourdough fermentation process, traditionally used as a form of leavening, is one of the oldest biotechnological processes in food production and can influence the nutritional quality by decreasing or increasing levels of compounds, and enhancing or retarding the bioavailability of nutrients (Gobbetti et al., 2014).

Pre-fermentation of bran with yeasts, lactic acid bacteria and with specific enzymes has shown to improve loaf volume, crumb structure and shelf life of bread supplemented with fermented bran (Salmenkallio-Marttila et al., 2001; Katina et al., 2006).

Moreover, fermentation, by activating enzymes, can release bound bioactive compounds, synthetize new compounds, degrade antinutrients and increase protein and starch digestibility. Katina et al. (2012) showed that fermentation of wheat bran improves nutritional properties, increasing the level of folates (+40%), free phenolic acids (+500%), and WE-AX (+60%), as well as degrading anti-nutritive factors, such as PA in order to increase mineral bioavailability (Lopez et al., 2001). Nordlund et al. (2013) demonstrated that bioprocessing of rye bran with enzymes and yeast resulted in increased soluble fibre content, caused mainly by AX solubilisation. *In vitro* fermentation studies with human faecal inoculum evidenced that bioprocessed bran promotes also faster SCFA formation and PA release, compared to native bran. Moreover, a recent study of Coda et al. (2014) showed that bran bioprocessed by using sourdough derived microorganisms with enzymes, leads to good textural and sensory properties of high fibre wheat bread containing bran.

Most of the observed changes during fermentation (e.g. AX solubilization, PA reduction) can be explained by the contribution of endogenous and microbial enzymes, such as amylases, proteases, xylanases (Katina et al., 2007; Poutanen et al., 2009) and phytases (Rizzello et al., 2010). From a nutritional point of view, the AX solubilization deserve particular attention because of the WE-AX influence on the post-prandial glycemic and insulinemic responses (Lu et al., 2000, 2004; Möhlig et al., 2005), and the prebiotic potential of the resulting soluble oligosaccharides (AXOS, XOS), that selectively stimulate the beneficial intestinal microbiota (Grootaert et al., 2007; Broekaert et al., 2011; Francois et al., 2012).

A multitude of different microorganisms have evolved enzyme systems which are capable of degrading plant cell wall polysaccharides. In particular, *Trichoderma* and *Aspergillus* species are reported to be efficient in the degradation of xylan by secreting xylanases (Anusha et al., 2013). Moreover, different strains of Bifidobacteria, Lactobacilli and Pediococci are able to degrade arabino-xylooligosaccharides (Madhukumar & Muralikrishna, 2012).

In the bran, phenolic compounds, especially ferulic acid (FA), are largely located as structural components of the cell walls of aleurone and pericarp. Most of the FA is covalently bound to complex polysaccharides in the cell walls, mainly arabinoxylans and lignin (Faulds & Williamson 1999; Anson et al., 2009), and it is partly responsible for the insolubility of cell wall structures of cereal kernels. Therefore, during fermentation process, the AX solubilization could lead to an increased bioaccessibility of easily extractable phenolic compounds, and in particular of ferulic acid (Anson et al 2009, 2010; Katina et al., 2012).

Anson et al. (2009) reported that bioprocessing of wheat bran by the combined action of hydrolytic enzymes and fermentation promote the release of phenolic acids and increased 5-fold the FA bioaccessibility in processed bran. Moreover, in a successive study Anson et al. (2011) reported an higher bioavailabilities of ferulic, vanillic, sinapic, and 3,4-dimethoxybenzoic acids from a whole wheat bread with bioprocessed bran than from a control bread.

These are promising results in order to improve nutritional properties of bran, in fact, some studies highlighted the potential role of ferulic acid as an antioxidant, anti-microbial, antiapoptotic, anti-ageing, anti-inflammatory, neuroprotective, hypotensive, pulmonary-protective and cholesterol lowering agent in metabolic diseases such as thrombosis, atherosclerosis, cancer and diabetes (Ou & Kwok, 2004; Srinivasan et al., 2007; Barone et al., 2009).

Even if aleurone layer is also an important source of minerals such as K, P, Mg, Fe, or Zn, mineral utilization is limited by the simultaneous presence of phytic acid (PA). PA is highly charged with six phosphate groups, and it forms insoluble complexes with dietary cations, thus hindering their intestinal absorption (Lopez et al., 2002). Mineral bioavailability can be improved by the action of phytase, an enzyme capable of hydrolyzing PA to free inorganic phosphate and low myo-inositol phosphate esters. Phytate-degrading enzymes could be endogenous in cereals (Leenhardt et al., 2005), or microbial, produced by yeast (Greiner et al., 2001), *bifidobacterium* (Haros et al., 2005; Palacios et al., 2008,), and lactic acid bacteria (De Angelis et al., 2003). Microbial phytase activity was most frequently detected in fungi, such as in some species belonged to genus *Aspergiullus* (Pandey et al., 2001).

Regarding lactic acid bacteria, the phytase activity could be considered strain specific and largely variable depending on environmental and assay conditions. The screening of a large number of sourdough lactic acid bacteria revealed no intense extracllular phytase activity (De Angelis et al., 2003; Reale et al., 2007). However, bacteria belonged to the genera *Bacillus*

(Choi et al., 2001) and *Enterobacter* (Yoon et al.,1996) showed extracellular phytase activity. Moreover, some *P. pentosaceus* strains have been reported that are able to degrade both sodium and calcium phytate (Bae et al., 1999; De Angelis et al., 2003). Several studies reported a decrease in phytate content after fermentation process (Coda et al., 2010, 2011; Rizzello et al., 2010; Moroni et al., 2012), thus leading to an increased mineral bioavailability. Lopez et al. (2001) demonstrated that a reduction of phytic acid in bread-making can be obtained via sourdough fermentation or prolonged fermentation time, thus leading to improved Mg and P solubility. Furthermore, the sourdough fermentation of wheat germ increased ca. 3.6-fold the phytase activity and enhanced the bioavailability of especially Ca++, Fe++, K+, Mn++, Na+ and Zn++ (Rizzello et al., 2010).

Phytate degrading ability is strictly pH-dependent and the optimum pH for wheat phytases is approximately 5 (Greiner & Konietzny, 2006). The observed reduction in phytate content in the fermented bran might be, therefore, due both to microbial phytases and to an activation of endogenous phytases as a consequence of a fall in pH during fermentation.

Moreover, fermentation of cereal substrates offers an economical way of improving folate content (Liukkonen et al., 2003; Jägerstad et al., 2005). Endogenous or added microbes are known to produce beneficial bioactive compounds, such as folate and vitamin B12 (Kariluoto et al., 2006; Santos et al., 2008). Folate biosynthesis has been studied mainly in lactic acid bacteria, and seems to depend strongly on species, strain, growth time, and cultivation conditions (Lin & Young, 2000; Sybesma et al., 2003).

Herranen et al. (2010) have shown that certain bacteria isolated from oat bran or rye flakes or found in fermenting rye sourdough (Kariluoto et al., 2006) are able to synthesize significant amounts of folate. Moreover, Korhola et al. (2014) concluded that fermentative yeasts together with LAB could be exploited in developing novel high folate content healthy foods from oat bran. For example, some *S. thermophilus* and *S. cerevisiae* strains harbour the genes for pathways in folate biosynthesis and they are able to produce folate and to excrete it into the medium (Rossi et al., 2011; Capozzi et al., 2012).

Moreover, fermentation process, through microbial and endogenous proteolysis activity, could lead to a release of bioactive peptides (Korhonen & Pihlanto, 2007; Coda et al., 2012). These compounds are defined as specific protein fragments that have a positive impact on the body function or condition, and may, ultimately, influence the human health (Kitts & Weiler, 2003). Several studies demonstrated the capacity of sourdough lactic acid bacteria to release peptides with antioxidant activity through the proteolysis of native cereal proteins (Nakamura et al., 2007; Coda et al., 2012; Rizzello et al., 2012). Moreover, Rizzello et al. (2012) showed that fermentation of cereal wholemeal flours by sourdough lactic acid bacteria, such as *L. curvatus* and *L. brevis* strains, increased the concentration of lunasin, a biologically active peptide involved in carcinogenesis suppression.

Therefore, the choice of the starter cultures has a critical impact on the final quality of cereal-based product; in fact, fermentation with well-characterized starter cultures, yeast or lactic acid bacteria (LAB), could be a potential tool to improve the palatability, processability and nutritional attributes of bran and whole-meal flours (Salmenkallio-Marttila et al., 2001). The main criteria used to select microbial starters regard technological, sensory and nutritional aspects. Technological factors of interest for fermentation are growth and acidification rate (Sterr et al., 2009; Coda et al., 2010, 2011), antifungal activity (Coda et al., 2013) and synthesis of exopolysaccharides (e.g. glucan and fructan). Among nutritional properties, synthesis of biogenic compounds (e.g. bioactive peptides), degradation of anti-nutritional factors (e.g. phytic acid) and increase of bioactive compounds (e.g. fiber, WE-AX, total phenols,..) are desirable (Rizzello et al., 2010; Coda et al., 2012).

Interesting strains, to be used as starters, are usually selected from the food matrix they are going to be employed for. Selection of proper strains within the lactic acid bacteria microbiota of cereals is indispensable to choose the more adaptable starter strains to guarantee optimal performance during fermentation and to get desirable properties in cereal-fermented products (Leroy & De Vuyst, 2004, Minervini et al., 2010). In this sense, the screening and the characterization of bacteria involved in spontaneous cereals fermentations are useful in order to select microbial cultures, according to their metabolic and enzymatic activities, to conduct “tailored” fermentation processes and improve bran or whole-meal flours from both nutritional and technological points of view. The study of microbial diversity represents an opportunity for advances in biotechnology.

2.1 References

Aleixandre A, Miguel M, 2008, Dietary fiber in the prevention and treatment of metabolic syndrome: a review. Crit Rev Food Sci Nutr 48:905-12.

Anson NM et al., 2009, Bioavailability of ferulic acid is determined by its bioaccessibility. J Cereal Sci 49:296-300.

Anson NM et al., 2010, Antioxidant and antiinflammatory capacity of bioaccessible compounds from wheat fractions after gastrointestinal digestion. J Cereal Sci 51:110-114.

Anson NM et al., 2011, Effect of bioprocessing of wheat bran in wholemeal wheat breads on the colonic SCFA production in vitro and postprandial plasma concentrations in men. Food Chem 128:404-409.

Anusha M et al., 2013, Isolation, identification and Screening of Xylanase producing fungi from Apple (Pyrus malus L.). Ann Plant Protect Sci 2:119-124.

Bae HD et al., 1999. A novel staining method for detecting phytase activity. J Microbiol Methods 39:17-22.

Barone E et al., 2009, Ferulic acid and its therapeutic potential as a hormetin for age-related diseases. Biogerontology, 10:97-108.

Broekaert WF et al., 2012, Prebiotic and other health-related effects of cereal-derived arabinoxylans, arabinoxylan-oligosaccharides, and xylooligosaccharides. Crit Rev Food Sci Nutr 51:178-194.

Brouns F et al., 2012, Wheat aleurone: separation, composition, health aspects, and potential food use. Crit Rev Food Sci Nutr 52:553-568.

Capozzi V et al., 2012, Lactic acid bacteria producing B-group vitamins: a great potential for functional cereals products. Appl Microbiol Biotech 96:1383-1394.

Chan JM et al., 2007, Whole grains and risk of pancreatic cancer in a large population-based case-control study in the San Francisco Bay Area, California. Am J Epidemiol 166:1174-1185.

Choi YM et al., 2001, Purification and properties of extracellular phytase from Bacillus sp. KHU-10. J Protein Chem 20:287-92.

Coda R et al., 2010, Spelt and emmer flours: characterization of the lactic acid bacteria microbiota and selection of mixed autochthonous starters for bread making. J Appl Microbiol 108:925-935.

Coda R et al., 2011, Utilization of African grains for sourdough bread making. J Food Sci 76:M329-M335.

Coda R et al., 2012, Selected lactic acid bacteria synthesize antioxidant peptides during sourdough fermentation of cereal flours. Appl Environ Microbiol 78:1087-1096.

Coda R et al., 2013, Antifungal activity of *Meyerozyma guilliermondii*: Identification of active compounds synthesized during dough fermentation and their effect on long-term storage of wheat bread. Food Microbiol 33:243-251.

Coda R. et al., 2014, Influence of particle size on bioprocess induced changes on technological functionality of wheat bran. Food Microbiol 37:69-77.

de Munter JS et al., 2007, Whole grain, bran, and germ intake and risk of type 2 diabetes: a prospective cohort study and systematic review. PLoS Med 4:261.

De Angelis M et al., 2003, Phytase activity in sourdough lactic acid bacteria: purification and characterization of a phytase from *Lactobacillus sanfranciscensis* CB1. Int J Food Microbiol 87:259-270.

Dornez E et al., 2008. Contribution of wheat endogenous and wheat kernel associated microbial endoxylanases to changes in the arabinoxylan population during breadmaking. J Agric Food Chem 56:2246-2253.

Fardet A. 2010, New hypotheses for the health-protective mechanisms of whole-grain cereals: what is beyond fibre?. Nutr Res Rev 23:65-134.

Faulds CB, Williamson G, 1999, The role of hydroxycinnamates in the plant cell wall. J Sci Food Agric 79:393-395.

Francois IE et al., 2012, Effects of a wheat bran extract containing arabinoxylan oligosaccharides on gastrointestinal health parameters in healthy adult human volunteers: a double-blind, randomised, placebo-controlled, cross-over trial. Br J Nutr 108:2229-2242.

Gobbetti M et al., 2014, How the sourdough may affect the functional features of leavened baked goods. Food Microbiol 37:30-40.

Greiner R et al., 2001, Stereospecificity of myo-inositol hexakisphosphate dephosphorylation by a phytate-degrading enzyme of baker's yeast. J Agric Food Chem 49:2228-2233.

Greiner R, Konietzny U, 2006, Phytase for food application. Food Technol Biotech 44:123-140.

Grootaert C et al., 2007, Microbial metabolism and prebiotic potency of arabinoxylan oligosaccharides in the human intestine. Trends Food Sci Tech 18:64-71.

Haros M et al., 2005, Phytase activity as a novel feature in Bifidobacterium. FEMS Microbiol Lett, 247:231-239.

Herranen M et al., 2010, Isolation and characterization of folate-producing bacteria from oat bran and rye flakes. Int J Food Microbiol 142:277-285.

Italmopa, Molini d’Italia n° 5/6, maggio-giugno 2012, ANNO LXIII, pp. 16-17.

Jägerstad M et al., 2005, Increasing natural food folates through bioprocessing and biotechnology. Trends Food Sci Tech 16:298-306.

Kariluoto S et al., 2006, Effects of yeasts and bacteria on the levels of folates in rye sourdoughs. Int J Food Microbiol 106:137-143.

Katina K et al., 2006, Effects of sourdough and enzymes on staling of high-fibre wheat bread. LWT-Food Sci Technol 39:479-491.

Katina K et al., 2007, Bran fermentation as a means to enhance technological properties and bioactivity of rye. Food Microbiol 24:175-186.

Katina K et al., 2012, Fermented Wheat Bran as a Functional Ingredient in Baking. Cereal Chem 89:126-134.

Kitts DD, Weiler K, 2003, Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. Curr Pharm Design, 9:1309-1323.

Koh-Banerjee P, Rimm EB, 2003, Whole grain consumption and weight gain: a review of the epidemiological evidence, potential mechanisms and opportunities for future research. Proc Nutr Soc 62:25-9.

Korhola M et al., 2014, Production of folate in oat bran fermentation by yeasts isolated from barley and diverse foods. . J Appl Microbiol 117:679-689.

Korhonen H, Pihlanto A, 2007, Technological options for the production of health-promoting proteins and peptides derived from milk and colostrum. Curr Pharm Design, 13:829-843.

Koutinas AA et al., 2008, A Whole Crop Biorefinery System: A Closed System for the Manufacture of Non-food Products from Cereals. Biorefineries-Industrial Processes and Products: Status Quo and Future Directions, Eds. Birgit Kamm, Patrick R. Gruber, Michael Kamm pp.165-191.

Leenhardt F et al., 2005, Moderate decrease of pH by sourdough fermentation is sufficient to reduce phytate content of whole wheat flour through endogenous phytase activity. J Agric Food Chem 53:98-102.

Leroy F, De Vuyst L, 2004, Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trends Food Sci Tech 15:67-78.

Lin MY, Young CM, 2000, Folate levels in cultures of lactic acid bacteria. Int Dairy J 10:409-413.

Liukkonen KH et al., 2003, Process-induced changes on bioactive compounds in whole grain rye. Proc Nutr Soc 62:117-122.

Lopez HW et al., 2001, Prolonged fermentation of whole wheat sourdough reduces phytate level and increases soluble magnesium. J Agric Food Chem 49:2657-2662.

Lopez HW et al., 2002, Minerals and phytic acid interactions: is it a real problem for human nutrition?. Int J Food Sci Tech 37:727-739.

Lu ZX et al., 2000, Arabinoxylan fiber, a byproduct of wheat flour processing, reduces the postprandial glucose response in normoglycemic subjects. Am J Clin Nutr 71:1123-1128.

Lu ZX et al., 2004, Arabinoxylan fibre improves metabolic control in people with Type II diabetes. Eur J Clin Nutr 58:621-628.

Madhukumar MS, Muralikrishna G, 2012, Fermentation of xylo-oligosaccharides obtained from wheat bran and Bengal gram husk by lactic acid bacteria and bifidobacteria. J Food Sci Technol 49: 745-752.

Mellen PB et al., 2008, Whole grain intake and cardiovascular disease: a meta-analysis. Nutr Metab Cardiovasc Dis 18: 283-290.

Minervini F et al., 2010, Robustness of *Lactobacillus plantarum* starters during daily propagation of wheat flour sourdough type I. Food Microbiol 27: 897-908.

Möhlig M et al., 2005, Arabinoxylan-enriched meal increases serum ghrelin levels in healthy humans. Horm Metabc Res 37:303-308.

Moroni AV et al., Exploitation of buckwheat sourdough for the production of wheat bread. Eur Food Res Technol 235:659-668.

Nakamura T et al., 2007, Isolation and characterization of a low molecular weight peptide contained in sourdough. J Agric Food Chem 55:4871-4876.

Nordlund E et al., 2013, Changes in bran structure by bioprocessing with enzymes and yeast modifies the *in vitro* digestibility and fermentability of bran protein and dietary fibre complex. J Cereal Sci 58:200-208.

Okarter N et al., 2010, Phytochemical content and antioxidant activity of six diverse varieties of whole wheat. Food Chem 119: 249-257.

Ou S, Kwok KC, 2004, Ferulic acid: pharmaceutical functions, preparation and applications in foods. J Sci Food Agric, 84:1261-1269.

Palacios MC et al., 2008, Selection of phytate-degrading human bifidobacteria and application in whole wheat dough fermentation. Food microbiol 25:169-176.

Pandey A et al., 2001, Production, purification and properties of microbial phytases. Bioresource Technol 77:203- 214.

Poutanen K et al., 2009, Sourdough and cereal fermentation in a nutritional perspective. Food Microbiol 26:693-699.

Reale A et al., 2007, The importance of lactic acid bacteria for phytate degradation during cereal dough fermentation. J Agric Food Chem 55:2993-2997.

Rizzello CG et al., 2010, Effect of sourdough fermentation on stabilisation, and chemical and nutritional characteristics of wheat germ. Food Chem 119:1079-1089.

Rizzello CG et al., 2012, Synthesis of the cancer preventive peptide lunasin by lactic acid bacteria during sourdough fermentation. Nutr Cancer 64:111-120.

Rossi M et al., 2011, Folate production by probiotic bacteria. Nutrients 3:118-134.

Sahyoun NR et al., 2006, Whole-grain intake is inversely associated with the metabolic syndrome and mortality in older adults. Am J Clin Nutr 83:124-31.

Salmenkallio-Marttila M et al., 2001, Effects of Bran Fermentation on Quality and Microstructure of High-Fiber Wheat Bread. Cereal Chem 78:429-435.

Santos F et al., 2008, High-level folate production in fermented foods by the B12 producer *Lactobacillus reuteri* JCM1112. Appl Environ Microbiol 74:3291-3294.

Schatzkin A et al., 2008, Prospective study of dietary fiber, whole grain foods, and small intestinal cancer. Gastroenterology 135:1163-1167.

Shewry PR, 2009, The HEALTHGRAIN programme opens new opportunities for improving wheat for nutrition and health. Nutr Bull 34:225-231.

Srinivasan M et al., 2007, Ferulic acid: Therapeutic potential through its antioxidant property. J Clin Biochem Nutr 44: 275-295.

Sterr Y et al., 2009, Evaluation of lactic acid bacteria for sourdough fermentation of amaranth. Int J Food Microbiol 136:75-82.

Sybesma W et al., 2003, Increased production of folate by metabolic engineering of *Lactococcus lactis*. Appl Environ Microbiol 69:3069-3076.

Truswell AS, 2002, Cereal grains and coronary heart disease. Eur J Clin Nutr 56:1-14.

van de Vijver LP et al., 2009, Whole-grain consumption, dietary fibre intake and body mass index in the Netherlands cohort study. Eur J Clin Nutr 63:31-8.

Vitaglione P et al., 2008, Cereal dietary fibre: a natural functional ingredient to deliver phenolic compounds into the gut. Trends Food Sci Tech 19:451-463.

Ye EQ et al., 2012, Greater whole-grain intake is associated with lower risk of type 2 diabetes, cardiovascular disease, and weight gain. J Nutr 142:1304-1313.

Yoon SJ et al., 1996, Isolation and identification of phytase-producing bacterium, Enterobacter sp. 4, and enzymatic properties of phytase enzyme. Enzyme Microb Tech 18:449-454.

3. AIMS OF THE STUDY

The aim of the current study is to improve the amount and the availability of bioactive compounds of wheat bran, through sourdoughlike fermentation process, in order to use the fermented bran as a potential functional ingredient. In particular, this study is designed to evaluate the effects of the fermentation process on fiber solubilization, mainly on AX fraction, and on the availability of other bioactive compounds, such as free ferulic acid and phytic acid.

Moreover, the second goal of the present study is the identification and the characterization of the bacteria involved in sourdoughlike fermentation pocess, in order to select starter cultures, according to their metabolic and enzymatic activities, to conduct “tailored” bran fermentation process aimed at improving technological and nutritional properties of the bran.

4. RESULTS AND DISCUSSION

4.1 Topic 1. Study of the chemical changes and evolution of microbiota during sourdoughlike fermentation of wheat bran.

(Manini F. et al 2014. Study of the Chemical Changes and Evolution of Microbiota during Sourdoughlike Fermentation of Wheat Bran. Cereal Chem, 91:342-349)

Several studies have emphasized the possibility to enhance nutritional properties of cereal by-products through biotechnological processes. Bran fermentation positively affects the bioavailability of several functional compounds. Moreover, bran fermentation could increase water-extractable arabinoxylans (WEAX), compounds with positive effects on glucose metabolism and prebiotic properties. This study was aimed to increase the amount of bran's bioactive compounds through sourdough like fermentation process. Wheat bran fermentations were conducted through continuous propagation by back-slopping of fermented bran (10% inoculum) until a stable microbiota was established, reaching high counts of lactic acid bacteria and yeasts (10^9 and 10^7 CFU g^{-1} respectively). At each refreshment step, bacterial strains were isolated, clustered, molecularly analysed by Randomly Amplified Polymorphic DNA and identified at the species level by 16S rRNA gene sequencing. *Leuconostoc mesenteroides*, *Lactobacillus brevis*, *Lactobacillus curvatus*, *Lactobacillus sakei*, *Lactobacillus plantarum*, *Pediococcus pentosaceus* and *Pichia fermentans* were dominating the stable sourdough ecosystem. After fermentation, levels of soluble fiber increased (+ 30%), WEAX and free ferulic acid were respectively fourfold and tenfold higher than in raw bran, results probably related to microbial xylan-degrading activity, while phytic acid was completely degraded. These preliminary data suggest that fermented bran could be considered as an interesting functional ingredient for nutritional enhancement.

4.2 Materials and methods

4.2.1 Fermentation process and sampling

Spontaneous fermentations (without microbial starters) were developed from commercial native wheat bran (raw, untreated) (mean particle size 475-633 µm -Molino Quaglia, Vighizzolo D'Este, PD, Italy) by mixing 28% of bran and 72% of water in a large beaker (2000 mL), covered with aluminum foil. Fermentation processes were performed as traditional type I sourdough, characterized by low incubation temperatures and daily refreshments to keep the microorganisms in an active state (Meroth et al., 2003; De Vuyst & Neysens, 2005). Fermentation batches were produced in triplicate, at 18 °C, through continuous propagation by back slopping of the fermented bran until a stable microbiota was established (13 days). At every refreshment step (once a day), the fermented bran was used as 10% inoculum for the subsequent fermentation cycle. Fresh samples were taken from unfermented and fermented bran for microbiological analyses. In addition, samples were frozen for later measurements of pH, total titratable acidity (TTA), lactic acid and for the quantification of bioactive compounds (dietary fiber, WE-AX, free ferulic acid and phytic acid).

4.2.2 Microbial quantification and isolation

Lactic acid bacteria (LAB), yeasts and contaminant bacteria were quantified. A sample of 10 g was homogenized for 10 min with 90 mL of sterile saline-tryptone diluent (containing, per liter, 8.5 g of NaCl and 1.0 g of tryptone [pH 6.0]) in a BagMixer 400 stomacher (Interscience, France), serially diluted 1:10 with quarter-strength Ringer's solution and plated on different media. LAB were determined on MRS5 agar (all ingredients were provided by Oxoid Basingstoke, UK) containing 0.001% cycloheximide (Oxoid, Basingstoke, UK) to prevent fungal overgrowth. Plates were incubated anaerobically at 30 °C for 72 h. Yeasts and moulds were determined on Rose Bengal Chloramphenicol (RBC) agar (Biolife, Milan, Italy). Plates were incubated aerobically at 25 °C for 5 days. Contaminant bacteria were determined on CASO agar (Merck KGaA, Darmstadt, Germany) and plates were incubated aerobically at 30 °C for 2 days. At each refreshment step of one batch of sourdough like fermentation, between 10 and 15 colonies of all morphologies were picked from MRS5 and RBC plates and streaked out several times on their respective agar plates to ensure their purity. After microscopic and morphological examination, among a total of 165 isolates, 98 LAB and 13 yeasts were obtained and further characterized.

4.2.3 Molecular characterization of LAB strains by random amplification of polymorphic DNA-polymerase chain reaction (RAPD-PCR) analysis

Randomly Amplified Polymorphic DNA-polymerase chain reaction (RAPD-PCR) profiles were used to perform a first strain differentiation and to explore the genetic diversity of LAB isolated from the fermented bran. Total genomic DNA from the strains was extracted using Microlysis kit (Labogen, Rho, Italy) following the manufacturer's instructions. RAPD-PCR reactions were performed with primer M13 (5'-GAGGGTGGCGGTTCT-3'; Huey & Hall, 1989). Amplification conditions, as well as electrophoresis and analysis of the amplification products, were conducted as previously described by Andrighetto et al (2002) and Morandi et al. (2006). Grouping of the RAPD-PCR profiles was obtained with the BioNumeric 5.0 software package (Applied Maths, Kortrijk, Belgium) using the unweighted pair-group method using arithmetic averages cluster analysis. The value for the repeatability of the RAPD-PCR assay, DNA extraction and running conditions, evaluated by analysis of repeated DNA extracts of the type strains, was 95%.

4.2.4 Molecular identification of the lactic acid bacteria and yeasts strains

For the LAB, a fragment of approx. 800 bp of the 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the primers p8FPL (AGTTTGATCCTGGCTCAG)/p806R (GGACTACCAGGGTATCTAAT) (Hosseini et al., 2009). For the isolated yeasts, a fragment of approx. 500-1300 bp of the D1/D2 domain of the 26S rDNAgene was amplified by PCR using the primers NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG)/NL-4 (5′-GGTCCGTGTTTCAAGACGG) (Kurtzman & Robnett, 1997). PCR reaction was performed in a 25-µL total volume containing 2 unit of Taq DNA polymerase (Finnzymes, Espoo, Finland), 0.5µM of each primer, 200 µM of each dNTP, 1,5 mM $MgCl_2$ and 50–100 ng of genomic DNA. PCR amplifications were performed using a Mastercycler (Eppendorf, Hamburg, Germany). The PCR parameters were as follow: initial denaturation at 94ºC for 5 min; 30 cycles of 94 ºC for 1 min, 56ºC for 1 min and 72ºC for 1 min; final extension at 72ºC for 7 min. The amplified PCR products were visualized by 1% agarose gel electrophoresis stained with SYBR Safe. The gels were photographed under ultraviolet light using a UV transilluminator. Amplicons were sent for sequencing to Macrogene Europe (Amsterdam, Netherlands).

Sequence alignment was carried out using ClustalW software. The BLAST algorithm was used to determine the most related sequence relatives in the National Center for Biotechnology Information nucleotide sequence database (http://www.ncbi.nlm.nih.gov/BLAST).

4.2.5 pH, TTA and lactic acid

pH and total titratable acidity (TTA) were determined on 10 g of fermented bran suspended in 100 mL of distilled water. For the determination of TTA, this suspension was titrated with 0.1M NaOH to a final pH of 8.5, detected by a pHmeter (PHM 250, Radiometer, Copenhagen); TTA was expressed as mL of 0.1 M NaOH needed to achieve the final pH of 8.5. All samples were analyzed in duplicate. Lactic acid was determined by HPLC with RI detection as reported by Lefebvre et al. (2002).

4.2.6 Chemical Analysis

Analysis of moisture, ashes, lipids and proteins was carried out by AACC standard methods (AACC 2001). Sugars were assessed by High-Performance Anion Exchange Liquid Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) (Rocklin & Pohl, 1983). Briefly, 1g of the sample was extracted with 200 mL of distilled water at 60°C for 60 min (Zygmunt et al., 1982); the extract solution was analysed using HPAEC-PAD equipped with a CarboPac PA1 (4x250 mm) column plus guard column CarboPac PA1 Guard (4x50 mm) (Dionex, Sunnyvale, CA, USA) and a pulsed amperometric detector ED50 (Dionex, Sunnyvale, CA, USA). Starch content was calculated by difference (100 – amount of all the other chemical components). Soluble and insoluble dietary fiber were evaluated by the enzymatic–gravimetric procedure (AOAC Method 991.43). Total arabinoxylans (TOTAX) and WE-AX in native and fermented bran were determined by HPAEC-PAD as described by Saulnier & Quemener (2009) with some modification, after hydrolysis with trifluoroacetic acid (TFA) (Courtin et al., 2000; Gebruers et al., 2008). For TOTAX levels, native bran or fermented bran samples (150 mg) were hydrolyzed in 5 mL TFA 2 M for 60 min at 110 °C. For WE-AX evaluation, extracts were prepared by suspending the samples (2 g) in 10 mL deionized water, shaking for 60 min at 7 °C and centrifugation (10000 x g, 10 min, 4 °C). The aqueous extracts were added with 2.5 mL TFA 4 M, and the solution was heated for 60 min at 110 °C. The hydrolysed samples were analyzed by a HPAEC system equipped with CarboPac PA1 (4x250 mm) column plus guard column CarboPac PA1 Guard (4x50 mm) (Dionex, Sunnyvale, CA, USA), a ternary pump (SP8800-Spectra Physics Santa Clara, CA, USA) and a pulsed amperometric detector ED50 (Dionex, Sunnyvale, CA, USA). A gradient elution, with a flow rate of 1 mL/min, was used: 0 min (96% H_2O - 4% NaOH 250 mM), 4 min (100% H_2O - 0% NaOH 250 mM), 22 min (20% H_2O - 80% NaOH 250 mM), 32 min (96% H_2O - 4% NaOH 250 mM), hold up to 41 min. Moreover, 300 mM NaOH postcolumn, with a flow rate of 0.6 mL/min, was added. Pulsed amperometric detection was carried out with the following pulse potentials and durations: E_{OX} =

+0.1 V (t_{OX} = 0.3s), E_{DET} = +0.6 V (t_{DET} = 0.1 s), and E_{RED} = -0.8 V (t_{RED} = 0.3 s). Arabinoxylan content was then defined as 0.88 times the sum of the monosaccharide xylose and arabinose concentrations, determined on external standard basis (Fig.6).

The content of free ferulic acid (FFA) was determined as described by Bartolome & Gomez-Cordoves (1999), with some modifications. After addition of internal standard (d3-hydroxycinnamic acid), samples were extracted for 10 min with 80% ethanol (v/v) in an ultrasonic bath. After centrifugation (20000 x g; 15 min), the supernatant was collected, evaporated to dryness, acidified with HCl 1M and extracted two times with ethyl acetate. The organic solutions were combined and dried under N_2. Samples derivatization was conducted for 1h at 70 °C with N,O-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (1% BSFTA-TMCS, Supelco, Bellafonte, USA). The analytical quantification of FFA was performed by isotope-dilution Gas Chromatography–Mass Spectrometry (GC-MS), by means of a gas chromatograph (GC-17A; Shimadzu, Tokyo, Japan) interfaced with a single-quadrupole mass spectrometer (MS-QP5050; Shimadzu). Gas chromatography separation was performed on a DB-5-MS capillary column (30 m; 0.25 mm i.d., 0.25 µm film thickness, J&W Scientific, Folsom, CA, USA). The analysis of phytic acid was performed by HPLC with spectrophotometric detection as described by Oberleas & Harland (2007). All the analysis were performed in triplicate.

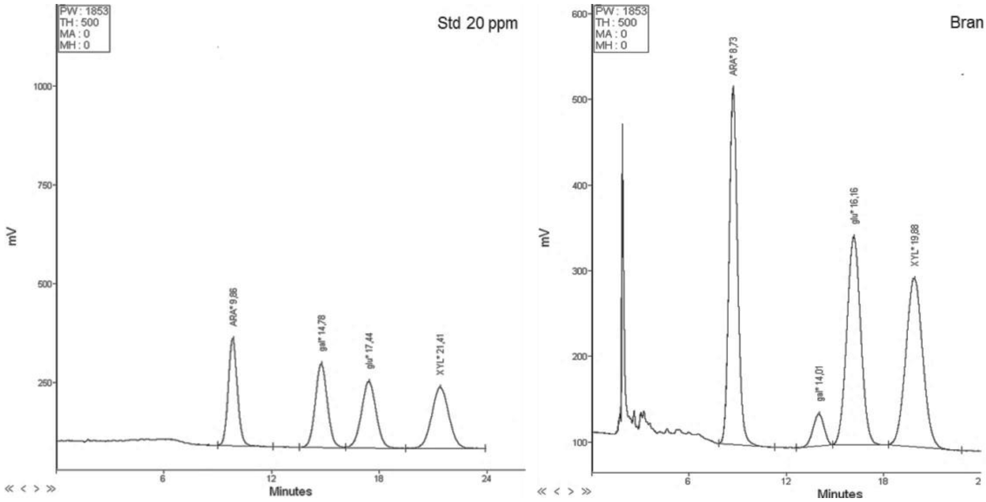

Figure 6. Chromatograms of a standard and a bran sample, obtained by HPAEC-PAD.

4.2.7 Statistical Analysis

Results are expressed on dry weight basis as mean ± standard deviation. One-way ANOVA was used to test the statistical differences in WE-AX content between the different refreshment steps. When the differences among the samples, evaluated by ANOVA, were statistically significant, pairwise comparisons of these samples were assessed with Tukey's test. Paired Student's t-test was used to compare values of FFA and PA levels before and after sourdough like fermentation. The data were processed by GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

4.3 Results and discussion

4.3.1 Microbial counts

At the start of the sourdough like fermentation process, low counts (< 10^6 CFU g^{-1}) were found for both LAB and yeast populations. However, LAB rapidly increased after the first day of bran fermentation, reaching levels of 10^9 CFU g^{-1}. Yeasts population developed more slowly than LAB and fluctuated during the first four days of fermentation. Their counts stabilized at the level of 10^7 CFU g^{-1} after 8 refreshments. The contaminants disappeared after 5 days of fermentation (Table I).

TABLE I
Microbial Counts, pH, Total Titratable Acidity (TTA), Lactic Acid and WE-AX in the different refreshment steps of sourdough like fermentation of wheat bran

refreshment step (d)	contaminants	yeast	LAB	pH	TTA	Lactic Acid	WE-AX
0	5.8^{a}	4.8^{abc}	4.8^{a}	6.6 ± 0.1^{a}	2.9 ± 0.1^{a}	0.02 ± 0.02^{a}	0.5 ± 0.0^{a}
1	7.4^{a}	5.4^{ac}	8.2^{b}	6.5 ± 0.1^{a}	4.6 ± 0.2^{b}	ND	1.7 ± 0.2^{b}
2	7.2^{a}	4.0^{ab}	9.5^{c}	4.3 ± 0.1^{b}	14.9 ± 1.1^{c}	ND	1.8 ± 0.1^{b}
3	6.5^{a}	2.4^{b}	9.7^{c}	4.2 ± 0.1^{bc}	17.5 ± 0.3^{df}	ND	2.7 ± 0.1^{ce}
4	3.5^{b}	3.4^{ab}	9.7^{c}	4.1 ± 0.1^{bc}	18.0 ± 0.9^{def}	ND	3.0 ± 0.2^{cd}
5	$< 2.0^{c}$	3.6^{ab}	9.6^{c}	4.2 ± 0.1^{bc}	17.3 ± 0.2^{d}	ND	3.0 ± 0.3^{cd}
6	$< 2.0^{c}$	4.6^{abc}	9.7^{c}	4.1 ± 0.1^{bc}	18.3 ± 0.4^{def}	ND	2.9 ± 0.1^{cde}
7	$< 2.0^{c}$	5.2^{ac}	9.7^{c}	4.1 ± 0.1^{c}	18.5 ± 0.7^{def}	ND	2.9 ± 0.1^{cde}
8	$< 2.0^{c}$	5.7^{ac}	9.6^{c}	4.1 ± 0.1^{c}	18.8 ± 0.8^{ef}	ND	2.8 ± 0.0^{cde}
9	$< 2.0^{c}$	6.9^{c}	9.6^{c}	4.1 ± 0.1^{c}	18.8 ± 0.8^{ef}	ND	3.0 ± 0.1^{cde}
10	$< 2.0^{c}$	7.1^{c}	9.5^{c}	4.1 ± 0.1^{c}	18.0 ± 0.7^{def}	ND	2.6 ± 0.2^{ce}
11	$< 2.0^{c}$	7.0^{c}	9.6^{c}	4.1 ± 0.1^{c}	18.6 ± 0.5^{def}	ND	3.2 ± 0.4^{d}
12	$< 2.0^{c}$	7.1^{c}	9.6^{c}	4.1 ± 0.1^{c}	18.8 ± 0.4^{ef}	ND	3.0 ± 0.3^{cde}
13	$< 2.0^{c}$	7.1^{c}	9.6^{c}	4.1 ± 0.1^{c}	18.9 ± 0.7^{e}	5.8 ± 0.4^{b}	2.6 ± 0.4^{e}

LAB = lactic acid bacteria; WE-AX = water-extractable arabinoxylan. Microbial counts are measured in log CFU.g^{-1}. TTA is measured in mL of 0.1M NaOH per 10 g. Lactic Acid and WEAX are reported as % db. ND = not determined. Data not shearing the some superscript letters are significantly different for $p < 0.05$.

4.3.2 Molecular characterization of microbial strains

For strains identification, we used a polyphasic approach. First RAPD-PCR was performed on all strains to explore the genetic diversity and the resulting fingerprints were compared to a user-generated BioNumerics database for a preliminary identification. This identification was then confirmed by DNA sequence analysis.

Fig. 7 shows the different banding patterns of the 73 cocci. The strains fell into two main clusters corresponding to *Pediococcus* and *Leuconostoc* genera. Among the Leuconostoc cluster, there were *Ln. mesenteroides* and *Ln. citreum* strains. Intra-specific comparison accomplished by RAPD-PCR profiles revealed a high biodiversity among the strains. Fig. 8 shows the RAPD banding patterns of 25 rod-shaped strains. Almost all the strains were grouped according to species except for two *L. plantarum* strains that did not fall in the cluster of *L. plantarum*. A quite high degree of DNA polymorphism was detected in *L. brevis* where the similarity levels reached only 50% for some of the strains.

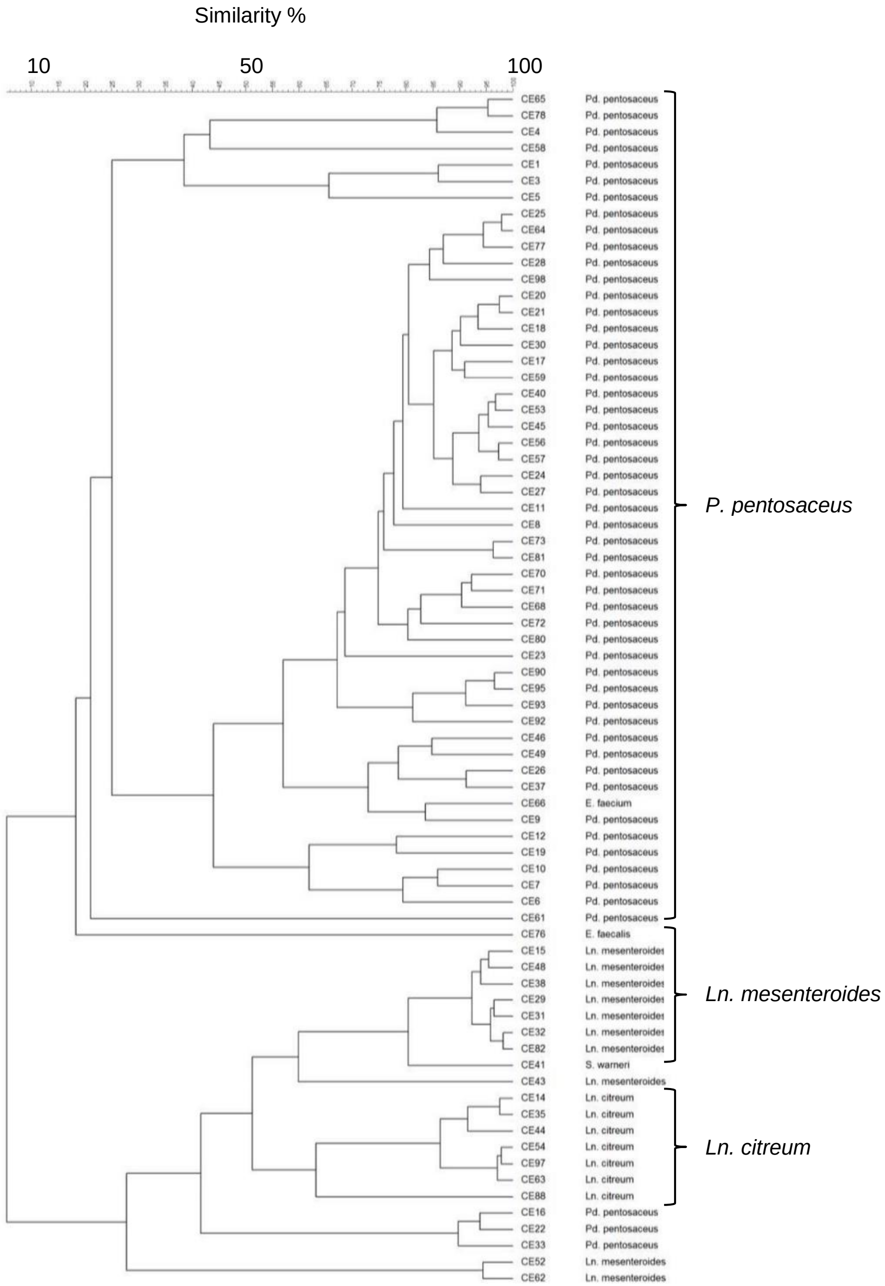

Figure 7. Unweighted pair group arithmetic averages (UPGMA)-based dendrogram derived from the combined RAPD-PCR profiles generated with primer M13 of cocci strains isolated from fermented bran at each refreshment step.

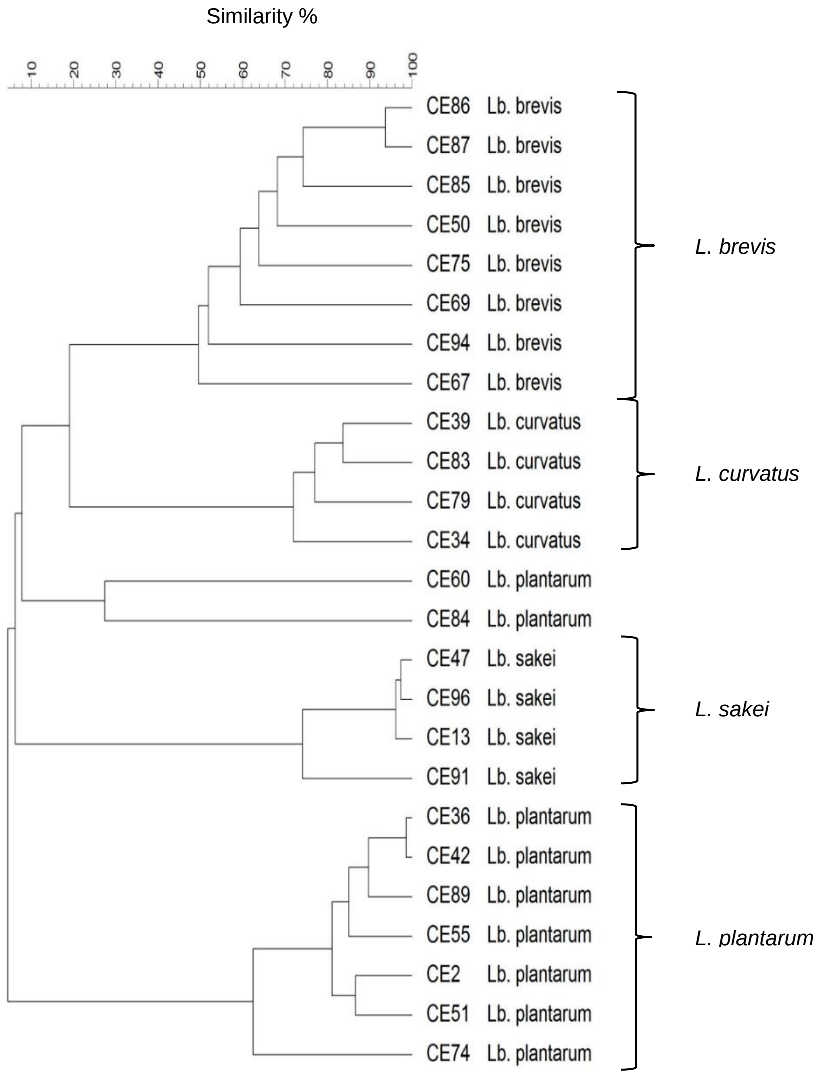

Figure 8. Unweighted pair group arithmetic averages (UPGMA)-based dendrogram derived from the combined RAPD-PCR profiles generated with primer M13 of *Lactobacillus* strains isolated from fermented bran at each refreshment step.

Growth rate and yield of microorganisms are governed by a multitude of ecological factors such as temperature, ionic strength, dough yield, and microbial products (lactate, acetate, CO_2, and ethanol), as well as factors resulting from substrates present in the cereal fraction and from enzymatic reactions (Ganzle et al., 1998; Meroth et al., 2003). Table II shows the endogenous LAB and yeasts development during several refreshments of the sourdough like fermentation of wheat bran. *Ln. mesenteroides*, *L. curvatus* and *P. Pentosaceus* were found from the beginning of the process as bran endogenous bacteria and dominated until the end of the fermentation. *Ln citreum* could be detected as endogenous species until the tenth refreshment but it disappeared at the end of fermentation. On the other hand, *L. plantarum* dominated at the end of fermentation. *L. sakei* sub. *sakei* and *L. brevis* were detectable respectively after four days and seven days of fermentation. Regarding the yeast, *Pichia fermentans* was the only species detected and it was detectable from the beginning up to the end of fermentation.

The yeasts *Pichia* ssp. are frequently associated with positive contribution to the aroma thanks to the production of volatile compounds, mainly ethyl acetate, and glycosidases and xylosidases enzymes (Manzanares et al., 1999).

TABLE II
Bacteria development during sourdough like fermentation (13 refreshments) of wheat bran

Bacteria	0	1	2	3	4	5	6	7	8	9	10	11	12	13
Ln. citreum	x		x		x	x	x	x	x	x	x			
Ln. mesenteroides	x	x	x	x	x	x	x	x	x	x	x	x	x	x
L. sakei* subsp. *sakei					x	x	x	x	x	x	x	x	x	x
L. curvatus	x	x	x	x	x	x	x	x	x	x	x	x	x	x
L. plantarum	x								x			x	x	x
L. brevis								x	x	x	x	x	x	x
P. pentosaceus		x	x	x	x	x	x	x	x	x	x	x	x	x
Pichia fermentans	x	x	x	x	x	x	x	x	x	x	x	x	x	x

Detected bacteria are marked with an x

4.3.3 Characterization of native and fermented bran

4.3.3.1 pH and TTA

Spontaneous bran fermentation of native bran resulted in an intensive acidification, likely related to the growth of LAB. As reported in Table I, pH values decreased from 6.5 to 4.1 during bran fermentation. In particular, pH did not change during the first 24 h of fermentation but after the first back-slopping the pH started to drop slowly reaching pH 4.1, value maintained during the following days (days 3-13). As expected, an inverse relation between pH and TTA values was observed: TTA increased from 2.9 to 18.9 mL NaOH/10 g, values likely related to the parallel accumulation of lactic acid.

4.3.3.2 Chemical composition

At the end of the process (refreshment 13 d), fermented bran contained slightly higher amounts of protein and lipid, and lower amounts of carbohydrates compared with native bran (Table III). In particular, the extimated amount of starch was reduced by about 5%. As expected, the fermentation resulted in a decrease of the total content of sugars, and in particular of sucrose and raffinose, most likely due to the microbial metabolism. These results are in accordance with a previous work on wheat germ fermentation (Rizzello et al., 2010). On the other hand, the fermentation process seemed to promote an increase in total dietary fiber, effect probably related to microbial exo-polysaccharides production (Hassan et al., 2008; Gobbetti et al., 2014; Ganzle, 2014). Moreover, soluble/insoluble fiber ratio increased approx. 20% (0.084 and 0.103 in native and fermented bran respectively) after fermentation. These aspects could be of great interest, from the nutritional point of view, due to the positive effect of the soluble fiber on the health and wellbeing (Anderson et al., 2009). Soluble dietary fiber could increase the viscosity of digesta and slow down the digestive/absorptive processes of nutrients in the small intestine. This may explain the possible effects on carbohydrate metabolism, which could lead to a positive influence on the post-prandial glycemic and insulinemic responses. Moreover, in the stomach, viscosity contributes to the delay of gastric emptying and thus, promoting satiety (Dikeman et al., 2006).

In figures 9 and 10 are reported the micrographs, obtained by means of a light microscope, showing the fermentation effects on bran microstructure. Comparing the bran structure before (Fig. 9) and after fermentation (Fig. 10) the effects of enzymatic and microbial degrading activity on cell material were evident.

TABLE III
Chemical composition (mean ± SD) of native bran and fermented bran (refreshment 13 d)

		Native bran	Fermented bran
Ash		5.3 ± 0.1	5.6 ± 0.1
Proteins		19.2 ± 0.1	20.7 ± 0.1
Lipid		5.6 ± 0.7	7.0 ± 0.4
Carbohydrates		26.0	18.8
of which			
	Starch	21.1	15.4
	Glucose	1.1 ± 0.2	2.5 ± 0.0
	Fructose	0.8 ± 0.1	0.8 ± 0.1
	Raffinose	1.0 ± 0.0	nd
	Sucrose	1.8 ± 0.1	nd
	Maltose	0.1 ± 0.1	nd
Total fiber		43.9 ± 0.3	47.5 ± 0.3
of which	Soluble fiber	3.4 ± 0.2	4.4 ± 0.1

Chemical composition is reported as % db; nd = not detectable.

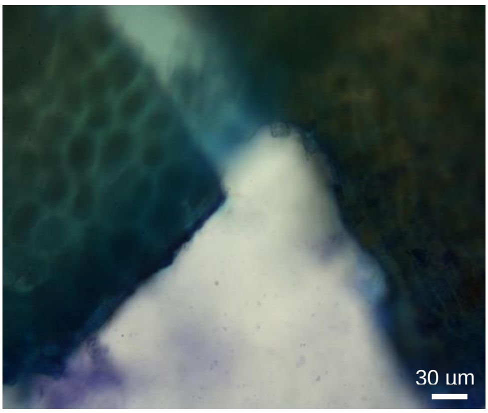

Figure 9. Native bran microstructure

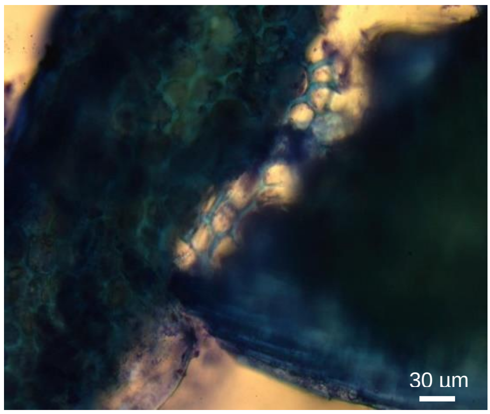

Figure 10. Fermented bran microstructure

4.3.4 Effect of sourdoughlike fermentation on AX and bioactive compounds

Regarding AX, the results obtained clearly illustrate that bran fermentation contribute to fiber solubilization (Table III) and especially to the conversion of WU-AX to WE-AX, in accordance to data reported by Katina et al (2012). As shown in Table I, the amount of WE-AX have already significantly ($p<0.001$) increased from 0.5 to 1.7 g/100g after the first refreshment step, reaching levels of 2.6 g/100g at the end of fermentation.

The percentage of WE-AX/TOTAX in native bran was 3.5% and it reached the level of 14.3% in fermented bran. Fiber solubilization could be explained by the activity of endogenous or microbial enzymes, such as xylanases (Katina et al., 2006). Madrigal et al. (2013) showed xylanase activity in a *P. fermentans* strain. In our work, this same species of yeast was detected in all refreshment steps; thus, the increase evaluated in WE-AX levels could be likely related, at least in part, to the enzymatic activity of this yeast. Endoxylanases cleave β-1,4-glycosyl linkages within the poly-β-1,4-xylose backbone of WE-AX as well as WU-AX, therefore leading to partial solubilization of the latter and to fragmentation of AX into readily soluble AXOS fragments (Dornez et al., 2008). Several studies demonstrate that the extensive fermentation of AX results in WE-AX and/or AXOS with potential prebiotic effect (Broekart et al., 2011; Damen, 2011). However, the physiological impact of AX consumption strongly depends on their structures and properties, since different types of AX have a different impact on the intestinal microbial population (Damen, 2011). Further studies are needed to verify if/how AX modifications, occurred during fermentation, affect bran prebiotic properties.

Clear differences in arabinose to xylose ratio in WE-AX, an indicator of the average degree of arabinose substitution (avDAS), were found. The avDAS in fermented bran was 0.65, significantly ($p< 0.01$) lower than that evaluated in native bran (0.88). The avDAS of the WE-AX was significantly reduced after bran fermentation, indicating that WE-AX in fermented bran were less substituted with arabinose than those from the native bran. This is an interesting result because AX fractions with a low avDAS seem to be more easily degradable by intestinal microbiota (Karppinen et al., 2001; Grootaert et al., 2009; Damen et al., 2011; Brouns et al., 2012). The cell wall structures of wheat kernels are insoluble partially due to phenolic compounds, especially phenolic acids, which form cross-links between polysaccharides and lignin (Faulds & Williamson, 1999). In particular Ferulic Acid (FA), which is the most abundant phenolic compound in grain, and dimers of this acid (diferulates) have an important role in the structural properties of aleurone fiber. These compounds are responsible for the cross-links between cell-wall polysaccharides and, in particular, FA is esterified to cell-wall AX at the C5 position of arabinose residues (Klepacka & Fornal, 2006). The bran cell wall structure is degraded by endogenous and microbial endoxylanases that are activated and/or produced during fermentation. Moreover, other degrading enzymes such as arabinofuranosidases, feruloyl esterases, acetyl esterases, and alpha-glucuronidases, remove arabinose, ferulic acid, acetic acid, and (4-O-methyl) glucuronic acid side chains from the xylan backbone, respectively (Grootaert et al., 2007). Therefore, the fermentation process, through fiber degradation and solubilization, increased the availability of Free Ferulic Acid (FFA), which has well-known antioxidant properties (Katapodis et al., 2003; Fang et al., 2012). In Fig. 11 are reported the levels of FFA detected in bran before and after fermentation. The concentration of FFA in native and fermented bran was respectively 1.99 and 11.38 (mg/100g), with an increase of 82%. Ferulic acid accumulation and its bioconversion to other phenolic derivatives can occur during the growth of LAB, due to ferulic acid esterase and ferulic acid decarboxylase activities. This phenomenon was earlier detected in some LAB species involved in sourdough like fermentation, such as *L. brevis*, *L. plantarum*, and *Pediococcus sp*. (Kaur et al 2013). Moreover, according to Lioger et al. (2007), the fermentation process degraded antinutritive factors, such as phytic acid that was undetectable in fermented bran (Fig. 12), likely through the activation of microbial and endougenous phytases, and this could lead to an increased mineral bioavailability (Lopez et al., 2000; Lioger et al., 2007). Despite most phytate-degrading LAB act on calcium phytate, the most abundant phytate present in cereal and legume-based foods, some *P. pentosaceus* strains have been reported to be able to degrade both sodium and calcium phytate (Raghavendra & Halami, 2009). In contrast, *L. plantarum* is able to produce non-specific acid phosphatase and it showed less specificity towards sodium phytate (Zamudio et al., 2001). Phytate degrading ability is strictly pH-dependent and the optimum pH for plant phytases is approximately 5 (Greiner & Konietzny, 2006). The observed reduction in phytate content in the fermented bran might be, therefore, due to an activation of endogenous bran phytases as a consequence of a fall in pH during fermentation.

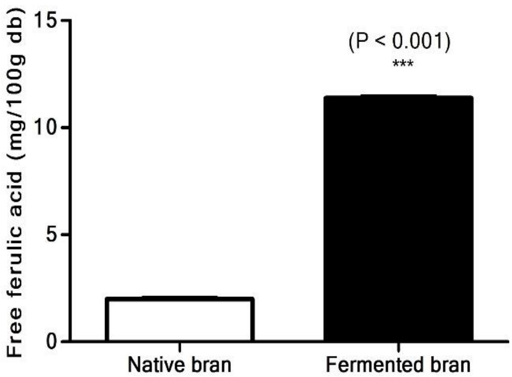

Figure 11. Free ferulic acid content (mg/100g db) in native and fermented bran.

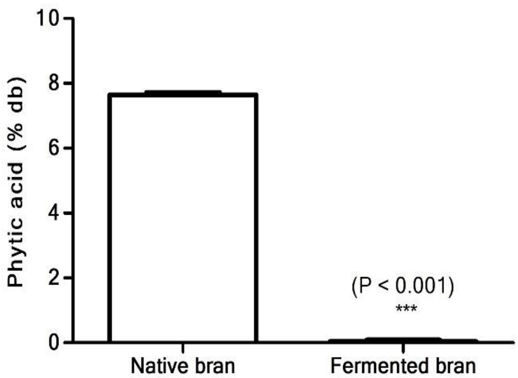

Figure 12. Phytic acid content (% db) in native and fermented bran.

4.4 Conclusions

The first part of the current study supports the hypothesis that sourdoughlike fermentation process is an efficient approach to increase the amount of bioactive compounds of wheat bran. This ancient process, traditionally used as a form of dough leavening, has been exploited in an innovative way to ferment the outer layers of wheat caryopsis. Results suggest that fermentation, through the activation and production of endogenous and microbial enzymes, increase the amount of soluble fiber, WE-AX and FFA, and decrease the content of phytic acid in wheat bran. The identification of the bacteria involved in sourdoughlike fermentation is the first step toward selecting starter cultures, according to their functional properties, in order to conduct "tailored" bran fermentation process. This study provide additional information for the future purpose to add fermented bran as a functional ingredient for bran-enriched products.

4.5 References

American Association of Cereal Chemists. Approved methods of the AACC. 11th ed., St Paul, MN: AACC, 2001.

Anderson JW et al., 2009, Health benefits of dietary fiber. Nutr Rev 67:188-205.

Andrighetto C et al, 2002, Genetic diversity of Streptococcus thermophilus strains isolated from Italian traditional cheese. Int Dairy J 12:141-144.

AOAC International. Approved methods of Analysis 16th ed. Method 991.43. Total, Insoluble and Soluble Dietary Fiber in Food-Enzymatic-Gravimetric Method, MES-TRIS Buffer. Approved 1995 Gaithersburg, MD.

Bartolomé B, Gómez-Cordovés C, 1999, Barley spent grain: release of hydroxycinnamic acids (ferulic and p-coumaric acids) by commercial enzyme preparations. J Sci Food Agric 79:435-439.

Courtin CM et al., 2000, Determination of reducing end sugar residues in oligo- and polysaccharides by gas-liquid chromatography. J Chromatogr A 866:97-104.

Damen B, 2011, Prebiotic effects and intestinal fermentation of cereal arabinoxylans and arabinoxylan oligosaccharides in rats depend strongly on their structural properties and joint presence. Mol Nutr Food Res 55:1862.

De Vuyst L, Neysens P., 2005, The sourdough microflora: biodiversity and metabolic interactions. Trends Food Sci Tech 16:43-56.

Dikeman CL, Fahey GC, 2006, Viscosity as related to dietary fiber: a review. Crit Rev Food Sci Nutr 46: 649-663.

Dornez E et al., 2008, Contribution of wheat endogenous and wheat kernel associated microbial endoxylanases to changes in the arabinoxylan population during breadmaking. J Agric Food Chem 56:2246-2253.

Erkkila AT et al., 2005, Cereal fiber and whole-grain intake are associated with reduced progression of coronary-artery atherosclerosis in postmenopausal women with coronary artery disease. Am Heart J 150:94-101.

Fang HY et al., 2012. Immunomodulatory effects of feruloylated oligosaccharides from rice bran. Food Chem 134:836-840.

Faulds M, Williamson G,1999, The role of hydroxycinnamates in the plant cell wall. J Sci Food Agric 79:393-395.

Ga¨nzle MG et al., 1998, Modeling of growth of *Lactobacillus sanfranciscensis* and *Candida milleri* in response to process parameters of the sourdough fermentation. Appl Environ Microbiol 64:2616-2623.

Ga¨nzle MG, 2014, Enzymatic and bacterial conversions during sourdough fermentation. Food Microbiol 37:2-10.

Gebruers K et al., 2008, Variation in the content of dietary fiber and components thereof in wheats in the HEALTHGRAIN diversity screen. J Agric Food Chem 56:9740-9749.

Gobbetti M et al., 2014, How the sourdough may affect the functional features of leavened baked goods. Food Microbiol 37:30-40.

Greiner R, Konietzny U, 2006, Phytase for food applications. Food Technol Biotech 44:125-140.

Grootaert C et al., 2007, Microbial metabolism and prebiotic potency of arabinoxylan oligosaccharides in the human intestine. Trends Food Sci Tech 18:64-71.

Grootaert C et al., 2009, Comparison of prebiotic effects of arabinoxylan oligosaccharides and inulin in a simulator of the human intestinal microbial ecosystem; FEMS Microbiol Ecol 69:231-242.

Hassan EG et al., 2008, Effect of Fermentation and Particle Size of Wheat Bran on the Antinutritional Factors and Bread Quality. Pak J Nutr 7:521-526.

Hosseini SV et al., 2009, Molecular and probiotic characterization of bacteriocin producing Enterococcus faecium strains isolated from non fermented animal foods. J Appl Microbiol 107:1392-1403.

Huey B, Hall J, 1989, Hypervariable DNA fingerprinting in Escherichia coli. Minisatellite probe from bacteriophage M13. J Bacteriol 171:2528-2532.

Karppinen S et al., 2001, Extraction and in vitro fermentation of rye bran fractions. J Cereal Sci 34:269-278.

Katapodis P et al., 2003, Enzymic production of a feruloylated oligosaccharide with antioxidant activity from wheat flour arabinoxylan. Eur J Nutr 42:55-60.

Katina K et al., 2006, Effects of sourdough and enzymes on staling of high-fibre wheat bread. Lebensm Wiss Technol 39:479-491.

Katina K et al., 2012, Fermented Wheat Bran as a Functional Ingredient in Baking. Cereal Chem 89:126-134.

Kaur B et al., 2013. Biotransformation of rice bran to ferulic acid by pediococcal isolates. Appl Biochem Biotechnol 170:854-867.

Klepacka J, Fornal L, 2006, Ferulic acid and its position among the phenolic compounds of wheat. Crit Rev Food Sci Nutr 46:639-647.

Kurtzman CP, Robnett CJ, 1997, Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5′ end of the large-subunit (26S) ribosomal DNA gene. J Clin Microbiol 35:1216-1223.

Lefebvre D et al., 2002, Simultaneous HPLC determination of Sugars, Organic Acids and Ethanol in Sourdough Process. LebensmWiss Technol 35:407-414.

Lioger D et al., 2007, Sourdough fermentation of wheat fractions rich in fibre before their use in processed food. J Sci Food Agric 87:1368-1373.

Lopez HW et al., 2000, Strains of lactic acid bacteria isolated from sour doughs degrade phytic acid and improve calcium and magnesium solubility from whole wheat flour. J Agric Food Chem 48:2281-2285.

Madrigal T et al., 2013, Glucose and ethanol tolerant enzymes produced by Pichia (Wickerhamomyces) isolates from enological ecosystems. Am J Enol Vitic 64:126-133.

Manzanares P et al., 1999, Screening of non- Saccharomyces wine yeasts for the production of β-d-xylosidase activity. Int J Food Microbiol 46:105-112.

Meroth CB et al., 2003, Monitoring the bacterial population dynamics in sourdough fermentation processes by using PCR-denaturing gradient gel electrophoresis. Appl Environ Microbiol 69:475-82.

Morandi S et al., 2006, Technological and molecular characterisation of enterococci isolated from north‑west Italian dairy products. Int Dairy J 16:867‑875.

Oberleas D, Harland B, 2007, Validation of a column liquid chromatographic method for phytate. J AOAC Int 90:1635-1638.

Raghavendra P, Halami PM, 2009, Screening, selection and characterization of phytic acid degrading lactic acid bacteria from chicken intestine. Int J Food Microbiol 133:129-134.

Rizzello CG et al., 2010, Effect of sourdough fermentation on stabilisation, and chemical and nutritional characteristics of wheat germ. Food Chem 119:1079-1089.

Rocklin RD, Pohl CA, 1983, Determination of carbohydrates by anion exchange chromatography with pulsed amperometric detection. J Liq Chromatogr Relat Technol 6:1577-1590.

Salmenkallio-Marttila M et al., 2001. Effect of bran fermentation on quality and microstructure of high-fibre wheat bread. Cereal Chem 78:429–435.

Saulnier L, Quemener B, Enzymatic mapping of arabinoxylan structure. In Healthgrain Methods: analysis of Bioactive Components in Small Grain Cereals. P. R. Shewry and J. L. Ward, Eds., AACC International: St. Paul, MN 2009, pp. 191-201.

Zamudio M et., 2001, *Lactobacillus plantarum* phytase activity is due to non-specific acid phosphatase. Lett Appl Microbiol 32:181-184.

Zygmunt LC, 1982, High pressure liquid chromatographic determination of mono and disaccharides in presweetened cereals: collaborative study. J Assoc Off Anal Chem 65:256-264.

4.6 Topic 2. Preliminary *in vitro* evaluation of the impact of the fermented bran on fecal microbiota composition and short chain fatty acid production

Dietary fiber and other fermentable carbohydrates are important in maintaining normal large bowel function and the metabolism of intestinal microbiota. Fermentable carbohydrates, which enter the large intestine, may modulate the composition and/or the enzymatic activities of the colonic microbiota, thus having an effect on the host health probably through the end products of bacterial fermentation (Gråsten et al., 2003).

In particular, Non Digestible Oligosaccharides (NDOs) resist digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine (Grootaert et al., 2009). Some of them are considered to be prebiotics. These carbohydrates help to maintain regularity of colonic functions and could possibly contribute to human health by reducing the risk of chronic diseases. Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of beneficial bacteria in the colon, thereby improving the host health (Gibson et al., 1995).

Prebiotic effects in the gut can be evaluated on the basis of the growth of health promoting bacteria, such as lactobacilli and bifidobacteria, the decrease in intestinal pathogens and the increase or decrease in production of health related bacterial metabolites, such as Short Chain Fatty Acids (SCFA) (Yang et al., 2013).

Indeed, the increase in number and/or in metabolic activity of beneficial bacteria can positively influence the host's physiology, by reducing intestinal infections occurrence, improving minerals absorption and inhibiting cancer cells development (Macfarlane et al., 2006; Wong et al., 2006). SCFA produced during colonic fermentation, play an important role in these protective mechanisms; butyric acid, in particular, seem to protect against colon cancer development (Gao et al., 2009; Layden et al., 2013) and possess anti-inflammatory properties (Hamer et al., 2008).

Moreover, butyrate is the preferred energy source for the colonocytes (Donohoe et al., 2011) and it has been reported to be an important factor in maintaining their normal functions (Manning & Gibson, 2004).

AX are selectively degraded in the colon by intestinal bacteria possessing AX degrading enzymes such as xylanases and arabinofuranosidases and represent a new class of potential prebiotics (Broekaert et al., 2011). Existing in different forms, ranging from soluble to insoluble fibers and high molecular weight to enzymatically modified short-chain fractions, the physiological effects of AX are largely unknown (Cloetens et al., 2010). However, several studies indicate that they behave like fermentable fibers in the colon, with different fermentation profiles depending on the physicochemical properties, and, in particular, on their molecular weight and degree of polymerization (Damen et al., 2011; Pollet et al., 2012).

Neyrinck et al. (2011) demonstrated that specific concentrate of WE-AX from wheat can modulate the gut microbiota in high-fat diet-induced obese mice, increasing caecal bifidobacteria content. This effect was accompanied by improvement of gut barrier function and by a lower circulating inflammatory marker.

Damen et al. (2011) showed that WU-AX, WE-AX and AXOS together combined promoted a selective bifidogenic effect in the colon with elevated butyrate levels, reduced pH and suppressed proteolytic metabolites.

Evaluation of structurally different AXOS shows that the AXOS structure has a strong influence on the prebiotic potential and the release of fermentation products. In general, smaller AXOS result in higher increases in intestinal butyrate concentrations and a significant bifidogenic effect (Van Craeyveld et al., 2008).

Huges et al. (2007) investigated the relationship between the molecular weight of AX and its prebiotic effect, through AX fractions in vitro fermentation by human fecal microbiota; it was concluded that the prebiotic effect, that was the selectivity of AX for bifidobacteria and lactobacilli groups, increased as the molecular mass of the AX decreased.

Moreover, Vardakou et al. (2008), using a mixed culture fermentation system, demonstrated that a pretreatment of the WU-AX with endo-β-1,4-xylanase resulted in significantly higher prebiotic index value, indicating that pretreatment provided oligomers that were better utilised by the gut bacteria. Moreover, in a study based on an *in vitro* three-stage continuous fermentation, set up mimicking the human colon, Vardakou et al. (2007) found that wheat AX did not increase Bifidobacterium spp levels when fed to the system, whereas AXOS produced by endoxylanase treatment of the same AX preparation significantly raised *Bifidobacterium* spp levels, while reducing *Clostridium* and *Bacteroides* levels. Hence, these studies collectively suggest that AX is not or only poorly bifidogenic, while its hydrolysis products XOS and AXOS stimulate bifidobacterial growth.

In a recent study, François et al. (2014) showed that the intake of 5 g/day of wheat bran extract, containing AXOS, exerts beneficial effects on gut parameters in healthy preadolescent children: in particular, the Authors assessed an increase in fecal bifidobacteria levels relative to total fecal microbiota, accompanied by a reduction of colonic protein fermentation. Moreover, in an *in* *vitro* model of human colon, Anson et al. (2011) reported a higher production of butyrate induced by wholemeal wheat bread with bioprocessed bran than by native bran.

In this contest, a preliminary *in vitro* fermentation test with human fecal inoculum was conducted in order to test if/how the AX fractions present in the fermented bran could modulate the growth and/or the activity of some intestinal bacteria and, in particular, the SCFA production. The current study did not show a significant influence of the fermented bran on the intestinal microbiota; However the fermented bran seems to promote the in vitro butyrate production, probably thanks to modifications promoted by the bioprocessing of bran.

4.7 Materials and methods

4.7.1 Materials

The commercial native wheat bran (raw, untreated) (mean particle size 475-633 µm) was provided by Molino Quaglia (Vighizzolo D'Este, PD, Italy); oligofructose (Raftilose P95, DP range 2-8) was provided by Orafti (Tienen, Belgium). Fermented bran was obtained as previously described in topic 1. Native and fermented bran were previously sterilized to avoid the influences of their microbiota on the fermentation process operated by fecal microorganisms.

4.7.2 Fecal samples and in vitro fermentation

Faecal samples were obtained from 5 healthy volunteers (2 men and 3 females 40–50 years old) selected to meet the following inclusion criteria: no drug therapy, no laxative of any class, no use of antibiotics within the previous 6 months. Samples of fresh faeces were taken from the first stool passed in the morning and immediately processed into an anaerobic cabinet (Forma Scientific, Marietta, OH, USA) under a $N_2/H_2/CO_2$ (85:10:5, v/v/v) atmosphere.

The different substrated tested (native bran, fermented bran and Raftilose as a control) were added in autoclaved medium to give a final concentration of 1% (w/v), with a control sample prepared without any substrate addition. This medium contained (per liter): 2 g of peptone water (Oxoid Ltd., Basingstoke, United Kingdom), 2 g of yeast extract (Oxoid), 0.1 g of NaCl, 0.04 g of K_2HPO_4, 0.01 g of $MgSO_4.7H_2O$, 0.01 g of $CaCl_2.6H_2O$, 2 g of $NaHCO_3$, 0.005 g of hemein (Sigma), 0.5 g of l-cysteine HCl (Sigma), 0.5 g of bile salts (Oxoid), 2 mL of Tween 80, 10 µL of vitamin K (Sigma), and 4 mL of 0.025% (w/v) resazurin solution (Huges et al., 2007).

Samples were inoculated with 1 % of fecal slurry, which was prepared by homogenizing fresh human feces (10%, w/v) in phosphate-buffered saline (PBS: 8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na_2HPO_4, and 0.2 g/L KH_2HPO_4), pH 7.3 (Oxoid).

The batch cultures were incubated at 37°C in anaerobic conditions: at T_0 and after 24 h of incubation, SCFA and microbial counts were assessed. In vitro fermentation trials were repeated in duplicate five times, one for each healthy faecal donor.

4.7.3 Bacterial Counting by Fluorescent in Situ Hybridization (FISH).

Aliquots of faecal batch cultures were separately fixed for microscopy analysis by DAPI (4,6-diamidine-2-phenylindole) stain and FISH method.

Briefly, 10 mL of batch cultures were vortexed with a dozen glass beads (3 mm diameter) for 3 min to detach microbial cells from particles. The cell suspensions were centrifuged at 200 x g for 5 min to remove large particles and debris. One mL of surnatant was washed once in PBS, diluted 1:3 with 4% paraformaldehyde in PBS and fixed for 4 h at 4°C. Fixed samples were washed twice in filtered PBS, and the pellets, obtained by centrifugation (13.000xg for 10 min at 4°C), were suspended in 200 µl of 50% (v/v) ethanol-PBS and stored at -20°C until use.

DAPI stain was performed according to Kepner & Pratt (1994). Briefly, defrosted samples were tenfold diluted in pre-filtered (0.2 µm) physiological solution in order to obtain the appropriated dilution; samples were then mixed with the stain at a final concentration of 2 µg mL^{-1} and stored in the dark for 15 min at room temperature. Samples were then filtered with 0.2 µm GTBP black polycarbonate membranes (Millipore); the air-dried filters were mounted on a microscope slide with antifading (Citifluor Ltd.), and examined under an epifluorescence microscope (Zeiss Axioskope equipped with the Zeiss 01 set filter).

FISH was performed, as described by Mueller et al. (2006) with minor modifications, with 16S rRNA-targeted oligonucleotide probes (MWG Biotech, Germany) labelled with fluorochrome Cy3 at 5'end. The probes applied, their sequence and their target bacterial groups are listed in Table IV. Unlabeled competitor oligonucleotides were added as required to improve in situ accessibility and specificity (Saumier et al., 2005). The PBS-ethanol stocks were washed twice and diluted 1:2 or 1:5 (according to the expected cell concentration) in filtered PBS.

Cells were pelleted and suspended in 35°C hybridization buffer (900 mM NaCl, 20 mM Tris–HCl pH 8.0, 0,01% SDS, 30% formamide); 50 µl of this suspension were added to 4 µl of oligonucletide probes (50 ng μl^{-1}) and hybridized overnight (16 h) at 35°C. The hybridized cell suspension was washed at 37 °C per 20 min in 1 mL of washing solution (65 mM NaCl, 20 mM Tris–HCl pH 8.0, 5 mM EDTA pH 8.0, 0.01% SDS), then centrifuged and resuspended in PBS before being filtered onto 0.2 µm GTTP polycarbonate filters. The air-dried filters were mounted on microscope slides with antifading. The counts were determined with an Epifluorescence microscope (Zeiss Axioskope) equipped with an HBO-50 W mercury lamp and a Zeiss 15 filter set. The number of bacteria was determined by counting the cells in 20–30 microscopic fields using an eyepiece with a calibrated reticule. The counts are expressed as $\log_{10}$ cell numbers (mL faecal culture)$^{-1}$.

TABLE IV
Probes used for FISH analysis, their sequence, and their target bacterial groups

Probes	Target organisms	Sequence (5'–3')	Reference
Eub338	Bacteria	GCTGCCTCCCGTAGGAGT	Amann et al., 1990
Lab158	*Lactobacillus-Enterococcus* group	GGTATTAGCAYCTGTTTCCA	Harmsen et al., 1999
Bac303	*Bacteroides-Prevotella* group	CCAATGTGGGGGACCTT	Manz et al., 2006
Bif164	*Bifidobacterium* spp.	CATCCGGCATTACCACCC	Langendijk et al., 1995

4.7.4 SCFA determination

SCFA concentrations were assessed in accordance with the method proposed by Weaver et al. (1989), modified as follows. Aliquots (500 µl) of batch faecal cultures were added with 200 µl 85% orthophosphoric acid, 200 µl 2% (v/v) sulphuric acid and 200 µl 2-methylbutyric acid 50 mM as internal standard. SCFA were gently extracted for 1 min with 2 mL ethyl-ether/heptan (1:1 v/v) and centrifuged for 10 min at 800 g to break up the emulsion. The aqueous phase was frozen and the organic layer was removed for analysis by a Varian 3400 CX gas liquid chromatograph equipped with a Varian 8200 CX autosampler and a HP-FFAP fused-silica capillary column (30 m, 0.53 mm i.d. with a 1-mm film). Injector and detector temperatures were 90 and 260°C, respectively. The initial oven temperature was 50°C and was increased by 10°C min-1 to 110°C and then by 5°C min^{-1} and held at 170 for 1 min. Quantification of the SCFA was obtained through calibration curves of acetic, propionic and butyric acids in concentrations between 5 mM and 50 mM (50 mM 2-methylbutyricacid as internal standard).

4.8 Results and discussion

Results did not show any significant change neither in the counts of Bifidobacteria and lactic acid bacteria, microorganisms with potential health effects, nor in those of Bacteroidaceae, family whose members are particularly active in the AX metabolism (Hopkins et al., 2003) (Table V). This lack in statistically significant differences could be related, at least in part, to the high variability among subjects (5 different human faecal inocula) in colonic microbiota, evidenced also by the wide standard deviations range assessed in the different bacterial counts.

In accordance to our results, the study of Grootaert et al. (2009) have not shown any increase in bifidobacteria after AXOS fermentation in a simulator of the human intestinal microbial ecosystem. In contrast, Vardakou et al. (2007) observed a significant increase in bifidobacteria counts after fermentation of xylanase pretreated AX. These conflicting results may be related to the different content and structure between AXOS available in the different studies. It should be noticed that also in the Raftilose fermentation broth, representing the positive control of our protocol, we did not find any significant changes in the microbiota composition.

Moreover, Huges et al. (2007) reported considerable bifidogenic impacts of AX fractions differing in molecular mass on the human fecal microbiota, after 12h of incubation in small-scale fecal batch cultures. In the present study the fermentations were conducted for longer times (24h) and this probably has influenced the availablility of substrates, that likely were readily utilized and become limiting after 24 h.

Table V
Bacterial population (log10 cells/mL) in batch fermentation cultures at t0 and t24 using fermented, Raftilose and native bran as substrate.

	Total cells[1]	**Active cells**[2]	***Bifidobacterium* spp.**[2]	***Bacteroides / Prevetolla***[2]	***Lactobacillus/ Enterococcus***
t_0	9.40 ± 0.3	8.81 ± 0.3	6.95 ± 0.6	7.09 ± 0.3	6.54 ± 0.7
t_{24} Fermented bran	9.41 ± 0.2	8.79 ± 0.1	6.00 ± 0.4	7.87 ± 0.8	6.21 ± 0.8
t_{24} Raftilose	9.38 ± 0.1	8.92 ± 0.2	6.61 ± 0.5	8.09 ± 0.4	6.31 ± 0.8
t_{24} Native bran	9.40 ± 0.2	8.93 ± 0.3	6.32 ± 0.5	7.43 ± 0.7	6.19 ± 0.7
t_{24} Control	9.48 ± 0.4	8.92 ± 0.5	6.24 ± 0.6	7.00 ± 0.6	6.16 ± 0.7

[1] determined by DAPI technique.
[2] determined by FISH technique.

However, we observed a higher production of butyrate (metabolite with protective effects against colon cancer) in the fermented bran batch, result that suggests an increased bacterial metabolic activity induced by this substrate (Fig. 13). An intervention study on healty subjects (Gråsten et al., 2003) demonstrated that wheat bread enriched with AX increases the fecal butyrate concentration. An increase in the butyrate production, after wheat bran consumption, has been shown in previous studies by Molist et al. (2009), in the colonic digesta of piglets, by

Zoran et al. (1997) in faeces of rats, and by Hallert et al. (2003) in the faeces of humans with ulcerative colitis. Some studies have attributed the increment in butyrate concentration to the AX fermentation (Salvador et al., 1993; Hughes et al., 2007). However, it is difficult to attribute a fermenatation product only to a substrate or a specific bacterial group in a mixed culture. It is important to specify that the present study represent only a preliminary *in vitro* test to evaluate the potential effects of the fermented bran on gut bacterial composition and metabolic activity. Thus, for the sake of simplicity, the native and fermented brans were used as substrates for fermentation without a previous *in vitro* treatment simulating gastrointestinal digestion.

Consequently, both the substrates employed contained residual starch (21.1% db and 15.4% db in native and fermented bran, respectively) and traces of sugars, compounds that certainly could influence the growth of the microbiota and the SCFA production. Among carbohydrates, starch is considered the most butyrogenic (Zhou et al., 2013); however it is due to note that we observed the higher increase in butyrate production in the fermented bran batch, even though this substrate contained lower levels of starch than native bran. Therefore, the observed increase in butyrate production could be a result of structural modifications of the fibre, in particular of the AX fraction, that have been probably induced by the bioprocessing of bran. It is likely that bran fermentation might have increased the fibre fermentability by the partial degradation of complex carbohydrates into smaller molecules, with higher solubility and accessibility to the bacterial breakdown, thus leading to an increase butyrate production. Moreover, some evidences have shown that resistant starch could stimulate colonic fermentation and promote, in particular, the production of butyrate (McOrist et al., 2011; Zhou et al., 2013).

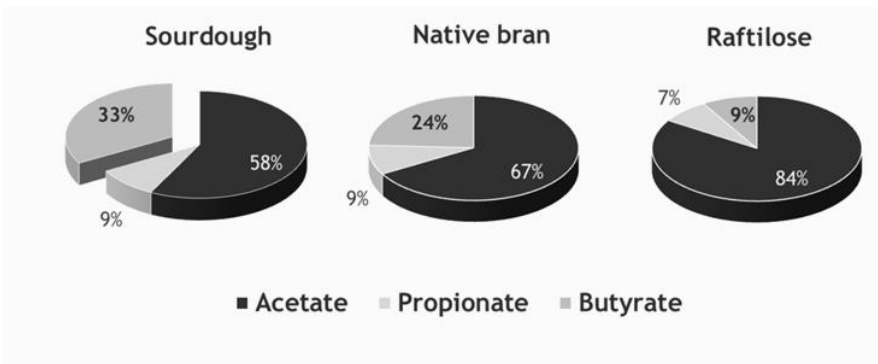

Figure 13. SCFA production (%) in batch fermentation with human faecal inoculum with different substrates (Fermented bran, Native bran, Raftilose).

Using an anaerobic in vitro system, Zhou et al.(2013), investigated the effect of starch structure on the production of metabolites and gut microbiota profile. In this study, normal maize starch was compared with a high amylose maize starch (HAS), either in native or thermally treated forms. Results evidenced that, during the fermentation process, the structure of the normal starch, either in native or thermally treated form, was less organized compared to HAS and was utilized faster, generating more acetate and lactate during fermentation; conversely HAS, with a highly organized structure, was utilized gradually and produced significantly more butyrate, by promoting a significant increase ($P < 0.001$) in the populations of butyrate-producing strains (*Faecalibacterium prausnitzii and Eubacterium hallii*) in the cultures. Therefore, in the current study, the increase in butyrate could also be ascribed to a different starch structure presented in the fermented bran. It might be likely that the microbiota involved in the previous fermentation treatment of bran have used the fraction of ready available starch present in native bran, leaving in this substrate only the fraction of starch with a more organized structure, which induce the production of butyrate during the fermentation trial with faecal inoculum.

4.9 Conclusions

In recent years, an increasing number of studies have focused on the human gut microbiota because of the role played by gut bacteria both in disease and in the maintenance of gut health (Purwani et al., 2012; Scott et al., 2013). Therefore, the modulation of both the intestinal microbial composition and/or SCFA production to improve the host's health is a point of interest.

In particular, there is growing interest in the use of prebiotic oligosaccharides as functional food ingredients. In the present study, even though it represents only a preliminary approach, the fermented bran has not shown any significant influence on the microbiota. However, the prebiotic concept is evolving, and nowadays it is not only related to an increase of the bifidobacteria and lactobacilli counts (Gibson et al, 2004; Rastall et al, 2015). Indeed, also the influence on microbial metabolic activities, such as on SCFA production, seems to be very important to promote the health and the normal functioning of the colon. Butyrate, in particular, has been shown to be an important factor in maintaining normal functions in colonocytes and to be a protective agent against colon cancer (Fung et al,2012).

The current study suggests that the fermented bran could increase the *in vitro* butyrate production, probably thanks to modifications of the fibre (AX fractions in particular), and/or the starch structure promoted by the bioprocessing of bran.

However, further investigations should be conducted to confirm these preliminary results and to better understand the mechanisms involved.

4.10 References

Amann RI et al., 1990, Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic and environmental studies in microbiology. J Bacteriol 172:762-770.

Anson NM et al., 2011, Effect of bioprocessing of wheat bran in wholemeal wheat breads on the colonic SCFA production *in vitro* and postprandial plasma concentrations in men. Food Chem 128:404-409.

Broekaert WF et al., 2011, Prebiotic and other health-related effects of cereal-derived arabinoxylans, arabinoxylan-oligosaccharides, and xylooligosaccharides. Crit Rev Food Sci Nutr 51:178-194.

Cloetens L et al., 2010, Tolerance of arabinoxylan-oligosaccharides and their prebiotic activity in healthy subjects: a randomised, placebo-controlled cross-over study. Br J Nutr 103:703-713.

Damen B et al., 2011, Prebiotic effects and intestinal fermentation of cereal arabinoxylans and arabinoxylan oligosaccharides in rats depend strongly on their structural properties and joint presence. Mol Nutr Food Res 55:1862-1874.

Donohoe DR et al., 2011, The microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon. Cell Metab 13:517-526.

François I EJA et al., 2014, Effects of Wheat Bran Extract Containing Arabinoxylan Oligosaccharides on Gastrointestinal Parameters in Healthy Preadolescent Children. J Pediatr Gastr Nutr 58:647-653.

Fung KIC et al., 2012, A review of the potential mechanisms for the lowering of colorectal oncogenesis by butyrate. Br J Nutr 108, 820–831.

Gao Z, et al., 2009, Butyrate improves insulin sensitivity and increases energy expenditure in mice. Diabetes 58:1509-1517.

Gibson GR et al., 1995, Selective stimulation of bifidobacteria in the human colon by oligofructose and inulin. Gastroenterology 108:975-982.

Gibson GR et al.,2004, Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutr Res Rev, 17, 259–275.

Gråsten S et al., 2003, Effects of wheat pentosan and inulin on the metabolic activity of fecal microbiota and on bowel function in healthy humans. Nutr Res 23:1503-1514.

Grootaert C et al., 2009, Comparison of prebiotic effects of arabinoxylan oligosaccharides and inulin in a simulator of the human intestinal microbial ecosystem. FEMS Microbiol Ecol 69:231-242.

Hallert C et al., 2003, Increasing fecal butyrate in ulcerative colitis patients by diet: Controlled pilot study. Inflamm Bowel Dis 9:116-121.

Hamer HM et al., 2008, Review article: the role of butyrate on colonic function. Aliment Pharm Ther 27:104-119.

Harmsen HJM et al., 1999, A 16S rRNA-targeted probe for detection of lactobacilli and enterococci in faecal samples by fluorescent in situhybridization. Microb Ecol Health Dis 11:3-12.

Hopkins MJ et al., 2003, Degradation of cross-linked and non-cross-linked arabinoxylans by the intestinal microbiota in children. Appl Environ Microbiol 69:6354-6360.

Hughes SA et al., 2007, In vitro fermentation by human fecal microflora of wheat arabinoxylans. J Agr Food Chem 55: 4589-4595.

Kepner RLJR, Pratt JR, 1994, Use of fluorochromes for direct enumeration of total bacteria in environmental samples: past and present. Microbiol Mol Biol Rev 58:603-615.

Langendijk PS et al., 1995, Quantitative fluorescence in situ hybridization of *Bifidobacterium* spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. Appl Environ Microbiol 61:3069-3075.

Layden BT et al., 2013, Short chain fatty acids and their receptors: new metabolic targets. Transl Res 161:131-140.

Manning TS, Gibson GR, 2004, Microbial-gut interactions in health and disease. Prebiotics Best Pract Res Clin Gastroenterol 18:287-298.

Manz W et al., 1996, Application of a suite of 16R rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum Cytophaga-Flavobacterium-Bacteroides in the natural environment. Microbiology 142:1079-1106.

Macfarlane S et al., 2006, Review article: prebiotics in the gastrointestinal tract. Aliment Pharmacol Ther 24:701-714.

McOrist AL et al., 2011, Fecal butyrate levels vary widely among individuals but are usually increased by a diet high in resistant starch. J Nutr 141:883-889.

Molist F et al., 2009, Effects of the insoluble and soluble dietary fibre on the physicochemical properties of digesta and the microbial activity in early weaned piglets. Anim Feed Sci Tech 149:346-353.

Mueller S et al., 2006, Differences in fecal microbiota in different European study populations in relation to age, gender, and country: a cross-sectional study. Appl Environ Microbiol 72:1027-1033.

Neyrinck AM et al., 2011, Prebiotic effects of wheat arabinoxylan related to the increase in bifidobacteria, Roseburia and Bacteroides/Prevotella in diet-induced obese mice. PLoS One 6:e20944.

Pollet A et al., 2012, In vitro fermentation of arabinoxylan oligosaccharides and low molecular mass arabinoxylans with different structural properties from wheat (Triticum aestivum L.) bran and psyllium (Plantago ovata Forsk) seed husk. J Agr Food Chem 60:946-954.

Purwani EY et al., 2012, Fermentation RS3 derived from sago and rice starch with *Clostridium butyricum* BCC B2571 or *Eubacterium rectale* DSM 17629. Anaerobe 18:55-61.

Rastall et al, 2015, Recent developments in prebiotics to selectively impact beneficial microbes and promote intestinal health. Curr Opin Biotechnology 32:42–46

Salvador V et al., 1993, Sugar composition of dietary fibre and short-chain fatty acid production during in vitro fermentation by human bacteria. Br J Nutr 70:189-197.

Saumier K et al., 2005, Enumeration of bacteria from the Clostridium leptum sub-group in human faecal microbiota using Clep1156 16SrRNA probe in combination with helper and competitor oligonucleotides. Syst Appl Microbiol 28:454-464.

Scott KP et al., 2013, The influence of diet on the gut microbiota. Pharmacol Res 69:52-60.

Van Craeyveld V et al., 2008, Structurally different wheat-derived arabinoxylooligosaccharides have different prebiotic and fermentation properties in rats. J Nutr 138:2348-2355.

Vardakou M et al., 2007, In vitro three-stage continuous fermentation of wheat arabinoxylan fractions and induction of hydrolase activity by the gut microflora. Int J Biol Macromols 41:584-589.

Vardakou M et al., 2008, Evaluation of the prebiotic properties of wheat arabinoxylan fractions and induction of hydrolase activity in gut microflora. Int J Food Microbiol 123:166-170.

Weaver GA et al., 1989, Constancy of glucose and starch fermentations by two different human faecal microbial communities. Gut 30:19-25.

Wong JM et al., 2006, Colonic health: fermentation and short chain fatty acids. J Clin Gastroenterol 40:235-243.

Yang et al., 2013, In vitro characterization of the impact of selected dietary fibers on fecal microbiota composition and short chain fatty acid production. Anaerobe 23:74-81.

Zhou Z et al., 2013, Starch structure modulates metabolic activity and gut microbiota profile. Anaerobe 24:71-78.

Zoran DL et al., 1997, Wheat bran diet reduces tumor incidence in a rat model of colon cancer independent of effects on distal luminal butyrate concentrations. J Nutr 127:2217-2225.

4.11 Topic 3. Characterization of lactic acid bacteria isolated from wheat bran sourdough

(Manini F. et al 2015. Characterization of lactic acid bacteria isolated from sourdoughlike fermentation of wheat bran. Submitted to Food Microbiology)

Sourdough fermentation is considered to be an interesting biotechnology approach to modify the techno-functionality and improve the nutritional potential of wheat, rye, wholegrain flours as well as that of of fibre-rich cereal ingredients, such as bran (Katina et al., 2007; Katina & Poutanen, 2013; Coda et al., 2014; Gobbetti et al., 2014).

The demand for faster, more efficient, controllable and large-scale fermentation processes has resulted in proper selection of the starter microorganisms to guarantee the reproducibility of fermentation on an industrial scale and to obtain a product with specific properties (De Vuyst & Neysens, 2005; Carnevali et al., 2007). The choice of the starter cultures has a critical impact on the final quality of cereal-based product; in fact, fermentation with well-characterized starter cultures, yeasts or lactic acid bacteria, could be a potential tool to improve the palatability, processability and nutritional attributes of brans and whole-meal flours (Salmenkallio-Marttila et al., 2001). The main criteria used to select microbial starters regard technological, sensory and nutritional aspects. Technological factors of interest for fermentation are growth and acidification rate (Sterr et al., 2009; Coda et al., 2010, 2011a), synthesis of antimicrobial compounds (Messens & De Vuyst, 2002; Coda et al., 2011b) and antifungal activity (Coda et al., 2013). Among nutritional properties, synthesis of biogenic compounds (e.g. bioactive peptides), degradation of anti-nutritional factors (e.g. phytic acid), increase of bioactive compounds (e.g. fiber, soluble arabynoxylans, total phenols,..), synthesis of exopolysaccharides (e.g. glucan and fructan) are desirable (Rizzello et al., 2010; Coda et al., 2012). Moreover, starter cultures could be also selected because of their probiotic properties in order to contribute to the health and well-being of the hosts by maintaining or improving their intestinal microbial balance (Asahara et al., 2004). In this case the probiotic cultures could be added in the final product and/or their survival and viability must be guaranteed throughout the process steps involved in the manufacture and during the storage conditions.

In accordance to the FAO/WHO Working Group (2002) report, to exert probiotic potential the strains must possess the ability to overcome the extremely low pH and the detergent effect of bile salts and arrive at the site of action in a viable physiological state (Sabir et al., 2010). Furthermore, they should be capable of adhering to the intestinal mucosa (Aslim et al., 2007), and to inhibit pathogenic bacteria (Hudault et al., 1997; Coconnier et al., 1998).

Moreover, the absence of undesirable properties such as virulence factors (Gasser, 1994) and transmissible antibiotic resistances must be considered in the choice of starter cultures (Adimpong et al., 2012). Strains with acquired antibiotic resistances must be avoided because of the potential transferability of resistance traits to other bacteria, including pathogenic microbes (Mathur et al., 2005).

Technologically interesting strains, to use as starters, are usually selected from the food matrix they are going to be employed for. Selection of proper strains within the lactic acid bacteria microbiota of cereals is indispensable to choose the more adaptable starter strains to guarantee optimal performance during fermentation and to get desirable properties in cereal-fermented products (Leroy and De Vuyst, 2004; Minervini et al., 2010). Some recent studies have shown that the use of selected autochthonous lactic acid bacteria to ferment sourdough is a suitable biotechnology to exploit the potential of cereals and pseudo-cereals in bread making (Coda et al., 2010; Sterr et al., 2009; Moroni et al., 2010). Sourdough fermentation is commonly performed as traditional "type I sourdough" characterized by daily propagation through back slopping to keep the microorganisms in an active state (Meroth et al., 2003; De Vuyst & Neysens, 2005). This protocol results in the dominance of the best adapted strain and represents a method for selecting the more adaptable starter strains in order to shorten the fermentation process and to reduce the risk of fermentation failure (Leroy & De Vuyst, 2004). In this sense, the screening and characterization of bacteria involved in spontaneous bran fermentation is useful in order to select some starter cultures, according to their metabolic and enzymatic activities, to conduct "tailored" fermentation process and improve bran or whole-meal flours from both nutritional and technological points of view. The aims of this study was to characterize the strains (13 LAB, 1 yeast) previously isolated from a spontaneous wheat bran sourdoughlike fermentation. LAB strains, belonged to *Leuconostoc mesenteroides*, *Leuconostoc citrum*, *Lactobacillus brevis*, *Lactobacillus curvatus*, *Lactobacillus sakei*, *Lactobacillus plantarum*, and *Pediococcus pentosaceus* species, were phenotypically characterized by their bran fermentation capacity, antifungal activity, carbohydrate metabolism, exopolisaccharides production, as well as their antibiotic resistance profiles. The LAB and the yeast (*Pichia fermenans*) strains were also tested for their potential xylan- and phytate-degrading activities. Moreover, common probiotic properties of the LAB strains, such as acid tolerance, bile tolerance, anti-listeria activity and adhesion to the human intestinal epithelial cells Caco-2 cells were examined. This part of the study was conducted at the Institute of Public Health and Clinical Nutrition, University of Eastern Finland, Kuopio.

Results suggest that *L. plantarum* and *P. pentosaceus* species could have interesting technological applications, due to their antifungal activity and EPS production. Some of these strains also exhibited phytate degrading activity on calcium and/or on sodium phytate salt and thus they could be exploited to improve mineral bioavailability of fermented products. Moreover, *L. curvatus* (CE 83), *L. brevis* (CE 85), and *P. pentosaceus* (CE 65), seemed to be suitable candidates to be used as probiotics. The present study has shown that the investigated properties are highly strain-specific. The characterization of the bacteria involved in bran sourdoughlike fermentation was the first step toward selecting starter cultures, according to their functional aspects, in order to conduct "tailored" bran fermentation process and improve technological and nutritional properties of bran-enriched products.

4.12 Materials and methods

4.12.1 Microorganisms

Lactic Acid Bacteria and yeast strains were isolated from a spontaneous wheat bran sourdoughlike fermentation and identified by phenotypic and molecular techniques (Topic 1- Manini et al., 2014). LAB belonged to the following species: *Leuconostoc citreum* (n = 2), *Lactobacillus plantarum* (n = 3), *Lactobacillus curvatus* (n = 1), *Lactobacillus sakei* (n = 1), *Leuconostoc mesenteroides* (n = 2), *Lactobacillus brevis* (n = 2), and *Pediococcus pentosaceus* (n = 2). The yaest belonged to *Pichia fermentans* species (Table VI). Two strains of *Lactobacillus plantarum* and one of *Lactobacillus casei,* isolated from quinoa seeds, were used as positive controls.

Table VI
Bacterial strains isolated from a wheat bran sourdoughlike fermentation process.

Microorganism	Strain	Isolation source
Ln. citreum	CE88	Wheat bran sourdough
Ln. citerum	CE54	Wheat bran sourdough
L. plantarum	CE42	Wheat bran sourdough
L. plantarum	CE60	Wheat bran sourdough
L. plantarum	CE84	Wheat bran sourdough
L. curvatus	CE83	Wheat bran sourdough
L. sakei	CE47	Wheat bran sourdough
Ln. mesenteroides	CE52	Wheat bran sourdough
Ln. mesenteroides	CE48	Wheat bran sourdough
L. brevis	CE94	Wheat bran sourdough
L. brevis	CE85	Wheat bran sourdough
P. pentosaceus	CE65	Wheat bran sourdough
P. pentosaceus	CE23	Wheat bran sourdough
***Pichia fermentans* (yeast)**	D	Wheat bran sourdough
***L. plantarum*(control)**	Q823	Quinoa seeds
***L. plantarum* (control)**	LGG	Quinoa seeds
***L. casei* (control)**	Q11	Quinoa Inca Pirce seeds

4.12.2 Growth and acidification rate

The fermentation capacity of the strains was evaluated measuring microbial counts, pH and Total Titratable Acidity (TTA) using wheat bran (raw, untreated) (mean particle size 475-633 µm -Molino Quaglia, Vighizzolo D'Este, PD, Italy) as a substrate.

An overnight culture (1% v/v) of each test strain was inoculated into the fermentation substrate (15% w/v of bran and 85% of water) and incubated for 8h at 30°C; the microbial count was evaluated before and after fermentation. LAB were determined on MRS agar (LAB M, Lancashire, UK) and the yeasts were evaluated on Plate Count Agar (PCA) (LAB M, Lancashire, UK). Plates were incubated aerobically at 30 °C for 72 h. pH and TTA were measured, as previously described, during fermentation.

4.12.3 Carbohydrate metabolism and growth at 30 and 37 °C

Carbohydrate fermentation profile of the strains was determined by means of an API 50 CH system (BioMèrieux, Marcy-l'Etoile, France). Test was performed according to the Manufacturer's instructions. The growth performances at 30° C and 37 °C in MRS broth were also monitored using a Thermo Bioscreen C automatic turbidometer (Labsystems Oy, Helsinki, Finland). The cell density was based on values of optical density at 420-580 nm (OD420-580). The growth was measured using 100-well Honeycomb microplates (TermoLabsystems, Helsinki, Finland). Each strain was tested in MRS broth in five replicates in a total volume of 300 µL per well. The plates were then incubated at 30 °C or 37 °C and the absorbance at 420-580 nm of each well was measured every 15 minutes for 24 h. The plates were shaken for 10 s before every measurement to achieve a homologous suspension.

4.12.4 Phytase activity plate assay

The LAB strains were preliminary inoculated in MRS broth (LAB M, Lancashire, UK) and incubated at 30 °C for 24 h, while the yeast were inoculated in Yeast and Mould Broth (LAB M, Lancashire, UK) and incubated at 25 °C for 48 h. Then, the strains were grown at 30 °C for 24 to 48 h in modified Chalmers broth without neutral red and with 1% of sodium phytate (Sigma-Aldrich, Milan, Italy). The microbial suspension was streaked on modified Chalmers agar plates without $CaCO_3$ and with 1% of phytic acid calcium or sodium salt (Sigma-Aldrich). The plates were incubated at 30 °C and examined, after 2 d of incubation, for clearing zones around the streaks. 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 room temperature, the cobalt chloride solution was removed and phytase activity was evaluated by measuring the size of clear haloes (mm) (Bae et al., 1999; Anastasio et al., 2010).

4.12.5 Xylanase activity plate assay

LAB tested were preliminary inoculated in MRS broth (LAB M, Lancashire, UK) and incubated at 30 °C for 24 h, while the yeast were inoculated in Yeast and Mould Broth (LAB M, Lancashire, UK) and incubated at 25 °C for 48 h. The cell cultures were inoculated in holes made in triplicate in the agar plates.

For the screening of xylanase producing microorganisms, the agar medium was prepared by adding 0.1% (w/v) of the dyed (Remazolbrilliant Blue R) substrate Azo-Xylan (birchwood), (Megazyme International Ireland Ltd, Co. Wicklow, Ireland), as the only carbon source, to a sodium phosphate buffer, 100 mM, pH 6 and/or a sodium acetate buffer, 100 mM, pH 4.5 (to test the activity at different pH). The plates were incubated at 30 °C and examined, after 48h of incubation, for clearing zones around the holes.

4.12.6 Antifungal activity

The antifungal activity of the strains was determined using the overlay method described by Magnusson & Schnurer (2001), slighlty modified.

The moulds *Aspergillus oryzae* ATCC 66222 and *Aspergillus niger* 25541 came from the Institute of Public Health and Clinical Nutrition culture collection (University of Eastern Finland, Kuopio). Inocula containing spores or conidia were prepared by growing the moulds on PCA at 30°C for 3-4 days and then collecting spores or conidia after vigorously shaking the slants with sterile peptone water.

The overlay method was performed using MRS agar plates on which LAB were inoculated as two 2-cm-long lines and incubated at 30°C for 48 h. The plates were then overlaid with 10 mL of malt extract soft agar (3% malt extract, 1.5% bacto peptone, 0.75% agar) inoculated with 0.1% (v/v) of peptone water with fungal spores suspension. After solidification, the plates were incubated aerobically at 30°C for 48 h. The plates were examined for clear zones of inhibition around the bacterial streaks, and the area of the zones was scored as follows: - = no inhibition of fungal growth; + = no fungal growth on 0.01-0.3 cm of plate area around bacterial streak; ++ = no fungal growth on 0.3-0.6 cm of plate area around bacterial streak; +++ = no fungal growth on > 0.6 cm of plate area around bacterial streak.

4.12.7 Exopolisaccharides

LAB strains were plated on different MRS agar plate with glucose, sucrose, raffinose, maltose, lactose and starch as the only carbon source. Plates were incubated for 2 days at 30°C. Duplicate plates containing 25 to 250 colonies were scored for mucoid properties (scale of ++ = excess EPS to – = no visible mucoid) (Fig. 14). Colonies were scored as ropy if strings of 5 mm or more were detected when the colony was touched once with a wire-inoculating loop (Dierksen et al., 1997; Ruas-Madiedo & De Los Reyes-Gavilán, 2005).

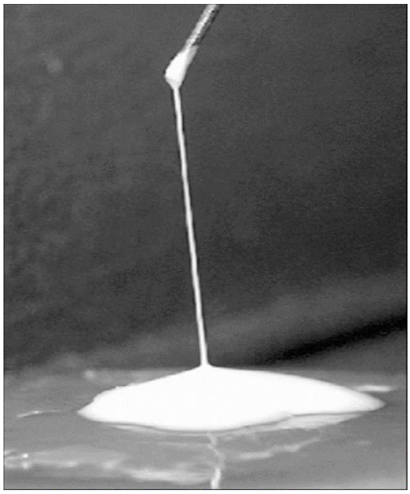

Figure 14. Ruas-Madiedo & De Los Reyes-Gavilán, 2005.

4.12.8 Potential probiotic properties

4.12.8.1 Acid tolerance

The ability of the strains to grow at low pH was evaluated as described by Lee et al. (2011), in acidified MRS broth (final pH 2.5). The pH-adjusted MRS broth was inoculated with an overnight culture of the different LAB strains (0.1% v/v) to a final cell concentration of approximately 1.0 x 10^7 CFU/mL. pH tolerance was evaluated by measuring survival after 2h of incubation at 37 °C to simulate intestinal conditions. Samples (100 µl) were taken at 0h and 2h and plated into duplicate MRS agar plates (LAB M, Lancashire, UK). Finally, colonies were counted after 48 h of incubation at 30 °C. The survival rate was determined as $\log_{10}$ values of colony-forming units per milliliter (CFU/mL).

4.12.8.2 Bile tolerance

Bile tolerance was measured by means of the method described by Sabir et al. (2010). The tolerance of the strains to bile (oxgall) was determined in MRS broth containing 0.3% oxgall (Sigma–Aldrich, Steinheim, Germany). The different LAB strains were inoculated (0.1% v/v) in the modified MRS broth and incubated at 37 °C. Samples were taken at 24h and plated into duplicate MRS agar plates. Colonies were counted after 48 h of incubation at 30 °C. The survival rate was determined comparing the $\log_{10}$ values of the initial colony-former units per milliliter (CFU/mL) and after incubation with bile acids.

4.12.8.3 Adhesion of *L. curvatus* CE83, *L. plantarum* CE84, *L. brevis* CE85, *P. pentosaceus* CE65 to human colon carcinoma cell-line Caco-2

The human colon carcinoma cell-line Caco-2 (ATTC HTB-37) was grown in 75 cm^3 cell culture bottles (Sarstedt, Inc., Newton, NC, USA) using DMEM supplemented with 10% (v/v) heat inactivated fetal bovine serum, 2mM L-glutamine, 1% (v/v) non-essential amino acids, 100 IU penicillin/mL and 100 µg streptomycin/mL (EuroClone, Siziano, Italy). The culture medium was replaced every 2-3 days. Caco-2 cells were subsequently seeded to 24-well culture plates at a concentration of $2.5x10^5$ cells per well. Cells were differentiated for 2 weeks, changing medium every 2-3 days. Cells were always incubated at 37ºC in a 5% CO_2 atmosphere.

Bacterial strains were grown overnight at 37ºC in MRS broth. After incubation, bacterial cells were collected by centrifugation, washed twice with PBS and suspended in PBS to an appropriate dilution (Abs 625 nm of 0.2, approx. $2x10^8$ CFU/mL).

Bacterial strains ($1x10^8$ CFU/mL) were added to each well and incubated for 2 hours. After incubation, the cells were washed four times and lysed with 0.1% Triton X-100 (Sigma-Aldrich). Cell lysates were serially diluted and plated in duplicate on MRS agar plates. Plates were then incubated at 37ºC for 2 days and the bacteria counted. The adhesion capacity of the strains is calculated as percentage of the bacteria counted from the cell lysates divided by the total bacteria added to the well. Three biological replicates made in different days and four replicates for each biological replicates were used for this test.

4.12.8.4 Anti-Listeria activity

The capacity of the strains to inhibit Listeria, a food-borne pathogen, was determined using the agar spot test described by Jacobsen et al. (1999), with some modifications. The assayed strains included *Listeria innocua*, *Listeria monocytogenes* and *Listeria welshimeri*, isolated respectively from three diverse sources (food, animals and humans).

A 100-µl volume of an overnight culture of the pathogen strains was plated on Plate Count Agar (PCA), dried and then the test cultures were spotted (10 µl) in triplicate on the surface of agar plate and incubated at 37°C to develop the spots. After 48 h the inhibition zones were evaluated. A clear zone of more than 1 mm around a spot was scored as positive. Each test was performed twice.

4.12.9 Antibiotic resistance

The minimum inhibitory concentrations (MICs) of fiftteen antibiotics (gentamicin, kanamycin, streptomycin, tetracycline, erythromycin, clindamycin, chloramphenicol, ampicillin, neomycin, vancomycin, quinupristin/dalfopristin, linezolid, trimethoprim, ciprofloxacin and rifampicin) were determined by microdilution as reported by the ISO 10932:2010 standard method (ISO 10932/IDF 223, 2010). Thus, LSM instead of MRS agar was used for the cultivation of the tested strains. Briefly, the LSM agar (LAB susceptibility medium, Klare et al., 2005) consisted of 90% (v ⁄v) of IsoSensitest broth (IST; Oxoid, Basingstoke, UK), 10% (v ⁄v) of MRS broth (LAB M) and 1.5% (w ⁄v) of bacteriological agar n° 1 (Oxoid), adjusted to pH 6.7.

Inocula of the strains were prepared by suspending single colonies (picked up from fresh cultures on LSM agar plates incubated for 48 h at 37°C) in a tube with 3 mL of 0.85% saline suspension and the density was adjusted spectrophotometrically to an OD_{625} of 0.16–0.20 and subsequently diluiting them 1:500 in the medium. This suspension density corresponds approximately to McFarland standard 1 (McF 1), 3 x 10^8 CFU/ mL.

Inoculation of manually premade MIC microtiter test plates (containing the different antibiotic test concentrations in each 50 µl volume of LSM broth per well), with the standardized strain suspensions, was performed by use of a 96-needle multipoint inoculator (50 µl of inoculum per needle was transferred in each well resulting in a final LAB inoculum of 10^2 bacteria mL^{-1}). The inoculated plates were subsequently incubated anerobically at 28°C for 24 h, except for *P. pentosaceus* plates that were incubated at 32°C; the MICs were evaluated as the lowest concentration of a given antibiotic at which no growth of the test organism was observed.

Epidemiological cut-off values were defined according to the committee on Antimicrobial Susceptibility Testing (EUCAST, http://www.eucast.org) and the the FEEDAP Panel (EFSA-FEEDAP, 2012).

4.13 Results and discussion

Functional selection of strains, considering mainly properties such as acidification and growth rate, carbohydrate metabolism and specific enzymatic activities, is the first step to get efficient starter cultures for a bran fermentation process in order to obtain a functional ingredient.

4.13.1 Growth and acidification rate

The fermentation capacity of the strains was expressed by the microbial counts, pH and TTA measured during fermentation using wheat bran as a substrate.

As shown in table VII, the count of all the tested LAB increase after 8 h of fermentation (average increase approximately 2.7 log CFU.g $^{-1}$). In particular, the strains *L. sakei* (CE 47) and *Ln. mesenteroides* (CE48) showed the highest growth (3.6 and 3.7 log CFU.g^{-1} respectively), while for *Ln. citreum* (CE 54) we assessed the lowest growth (1.6 log CFU.g^{-1}).

L. plantarum (CE 84, CE 42, CE 60), *L. curvatus* (CE 83), *L. sakei* (CE 47), and *P. pentosaceus* (CE 65, CE 23) showed the best acidification rate. The lowest acidification rate was obtained with *L. brevis* (CE 94, CE 85) fermentations, in which pH reached values 5.5 and 5.3 respectively. As expected, an inverse relation between pH and TTA values was observed and TTA increased during fermentation.

4.13.2 Carbohydrate metabolism and growth capacity at 30 °C and 37 °C

The carbohydrate metabolism of the LAB strains, which have shown the best growth, tested by API 50 CH system, is reported in table VIII. All the tested strains are able to use D-galactose, D-glucose, D-fructose and D-maltose.

Mixtures of strains with different carbohydrate metabolism are frequently used because they may guarantee optimal acidification and sensory properties (Gobbetti, 1998).

Heterofermentative LAB represent the major LAB in spontaneous fermentations. From previous studies, it is known that obligate heterofermenters, such as *L. fermentum* and *L. brevis*, are able to co-metabolize both arabinose and xylose (Gobbetti et al., 1999; Katina et al., 2012). Among the tested strains, *Ln. citreum* (CE 88), *Ln. mesenteroides* (CE 48), *L. curvatus* (CE 83), *L. brevis* (CE 94, CE 85) and *P. pentosaceus* (CE 65) are able to use both arabinose and xylose. Among *L. plantarum* strains only CE60 is able to use L-arabinose and to a lesser extent also D-arabinose.

Despite none of the *L. plantarum* tested metabolize xylose, these strains showed the widest carbohydrate consumption. This species is commonly found in sourdoughs ecosystems (De

Vuyst & Neysens, 2005), and its prevalence in cereal fermentations has been mainly attributed to the versatile metabolism of carbohydrates (Kleerebezem et al., 2003; Minervini et al., 2010). The tested strains have grown both at 30 °C and 37 °C, except for *P. pentosaceus* (CE 23) that did not grow at 30°C (Table VII).

TABLE VII
pH, Total Titratable Acidity (TTA) and Microbial Counts measured every 2h of wheat bran fermentation.

Bacterial culture		0h	2h	4h	6h	8h
***L. plantarum* CE84**	pH	6.0	5.8	5.5	4.6	4.0
	TTA	2.1	2.7	4.0	6.9	8.6
	log $CFU.g^{-1}$	8.3	n.d.	n.d.	n.d.	11.1
***L. plantarum* CE42**	pH	6.1	6.0	5.8	5.1	4.1
	TTA	1.8	2.5	3.5	5.0	7.8
	log $CFU.g^{-1}$	8.3	n.d.	n.d.	n.d.	11.0
***L. plantarum* CE60**	pH	5.8	5.7	5.4	4.6	3.9
	TTA	2.2	3.1	4.5	6.8	10.2
	log $CFU.g^{-1}$	8.6	n.d.	n.d.	n.d.	11.3
***L. curvatus* CE83**	pH	5.9	5.9	5.6	4.7	4.1
	TTA	1.7	2.4	3.3	5.9	7.5
	log $CFU.g^{-1}$	8.3	n.d.	n.d.	n.d.	11.1
***L. sakei* CE47**	pH	5.9	5.8	5.4	4.5	4.0
	TTA	2.0	2.9	4.5	6.4	9.0
	log $CFU.g^{-1}$	8.0	n.d.	n.d.	n.d.	11.6
***P. pentosaceus* CE65**	pH	5.9	5.9	5.6	4.6	4.1
	TTA	2.1	2.9	4.4	6.8	9.4
	log $CFU.g^{-1}$	8.4	n.d.	n.d.	n.d.	11.0
***P. pentosaceus* CE23**	pH	6.0	5.9	5.5	4.4	3.9
	TTA	1.8	2.4	3.7	7.3	9.5
	log $CFU.g^{-1}$	7.8	n.d.	n.d.	n.d.	10.8
***L. brevis* CE94**	pH	6.1	6.1	6.0	5.8	5.5
	TTA	1.8	2.2	2.7	3.6	4.4
	log $CFU.g^{-1}$	7.8	n.d.	n.d.	n.d.	10.5
***L. brevis* CE85**	pH	6.0	6.0	5.9	5.7	5.3
	TTA	1.9	2.4	3.1	3.6	4.4
	log $CFU.g^{-1}$	7.9	n.d.	n.d.	n.d.	10.7
***Ln. citreum* CE88**	pH	6.0	5.9	5.6	4.9	4.4
	TTA	1.8	2.7	3.8	6.4	8.2
	log $CFU.g^{-1}$	8.3	n.d.	n.d.	n.d.	11.0
***Ln. citreum* CE54**	pH	6.1	6.0	5.7	4.8	4.3
	TTA	2.0	2.7	4.2	8.4	8.6
	log $CFU.g^{-1}$	8.5	n.d.	n.d.	n.d.	10.1
***Ln. mesenteroides* CE52**	pH	6.0	5.9	5.7	5.2	4.8
	TTA	1.9	2.5	3.6	5.0	6.5
	log $CFU.g^{-1}$	8.0	n.d.	n.d.	n.d.	10.6
***Ln. mesenteroides* CE48**	pH	6.0	6.0	5.8	5.1	4.4
	TTA	1.9	2.5	3.1	5.2	7.0
	log $CFU.g^{-1}$	7.5	n.d.	n.d.	n.d.	11.2

Microbial counts are expressed in log $CFU.g^{-1}$. TTA is measured in mL of 0.1 M NaOH per 10 g. n.d.= not determined.

TABLE VIII

Carbohydrate metabolism of LAB tested by API 50 CH system and growth capacity at 30°- 37°C.

	CE88[a]	CE42[b]	CE60[b]	CE84[b]	CE83[c]	CE48[d]	CE94[e]	CE85[e]	CE65[f]	CE23[f]
CONTROL	-	-	-	-	-	-	-	-	-	-
GLICEROL	-	+	+	+	-	-	-	-	+	+
ERYTHRITOL	-	-	+	-	-	-	-	-	-	-
D-ARABINOSE	-	-	+	-	-	-	-	-	-	-
L-ARABINOSE	+++	-	+++	-	+++	+++	+++	+++	+++	+++
D-RIBOSE	-	+++	+++	+++	+++	+++	+++	+++	+++	+++
D-XYLOSE	++	-	-	-	+++	+++	+++	+++	+++	-
L-XYLOSE	-	-	-	-	-	-	-	-	-	-
D-ADONITOL	-	-	-	-	-	-	-	-	-	-
METHYL-ßd-xYlopiranoside	-	-	-	-	-	-	-	-	-	-
D-GALACTOSE	++	+++	+++	+++	++	++	+++	++	+++	+++
D-GLUCOSE	+++	+++	+++	+++	+++	+++	+++	++	+++	+++
D-FRUCTOSE	+++	+++	+++	+++	++	++	++	++	+++	+++
D-MANNOSE	+++	+++	+++	+++	-	-	-	-	+++	+++
L-SORBOSE	-	-	-	-	-	-	-	-	-	-
L-RHAMNOSE	-	-	-	+	-	-	-	-	-	++
DULCITOL	-	-	-	-	-	-	-	-	-	-
INOSITOL	-	-	-	-	-	-	-	-	-	-
D-MANNITOL	++	+++	+++	+++	+	+	+	-	-	-
D-SORBITOL	-	+++	+++	+++	-	-	-	-	-	-
METHYL-αD-mannopyranoside	-	+++	+++	-	-	-	-	-	-	-
METHYL-αD-glucopyranoside	++	-	-	-	++	++	++	++	-	-
N-ACETYLGLUCOSAMINE	+++	+++	+++	+++	++	++	+	++	+++	+++
AMIGDALIN	+	+++	+++	+++	-	-	-	+++	+++	+++
ARBUTIN	++	+++	+++	+++	-	-	-	-	+++	+++
ESCULIN	+	+	+	+	+	+	+	+	+	+
SALICIN	+++	+++	+++	+++	-	-	-	-	+++	+++
D-CELLOBIOSE	-	+++	+++	+++	+	+	+	+++	+++	+++
D-MALTOSE	+++	+++	+++	+++	++	+++	+++	+++	+++	+++
D-LACTOSE	-	+++	+++	+++	-	-	-	-	+++	++
D-MELIBIOSE	+++	+++	+++	+++	-	-	-	++	-	+++
D-SACCHAROSE	+++	+++	+	+++	-	-	-	-	-	+++
D-TREHALOSE	+++	+++	+++	+++	-	-	-	-	+++	+++
INULIN	-	-	-	-	-	-	-	-	+	-
D-MELEZITOSE	-	-	+++	+++	-	-	-	+++	-	-
D-RAFFINOSE	+++	+++	+++	+++	-	-	-	+++	-	+++
AMIDON	-	-	-	-	-	-	-	-	-	-
GLYCOGEN	-	-	-	-	-	-	-	-	-	-
XYLITOL	-	-	-	-	-	-	-	-	-	-
GENTIOBIOSE	-	+++	+++	+++	-	-	-	-	+++	+++
D-TURANOSE	+++	-	+++	+++	-	-	-	+++	-	-
D-LYXOSE	-	-	-	-	-	-	-	-	-	-
D-TAGATOSE	-	-	-	-	-	-	-	-	+++	+++
D-FUCOSE	-	-	-	-	-	-	-	-	-	-
L-FUCOSE	-	-	-	-	-	-	-	-	-	-
D-ARABITOL	-	++	++	+	-	-	-	++	-	-
L-ARABITOL	-	-	-	-	-	-	-	-	-	-
POTASSIUM GLUCONATE	++	++	-	++	++	+	++	++	++	++
POTASSIUM 2-KETOGLUCONATE	++	-	-	-	-	-	-	-	-	-
POTASSIUM 5-KETOGLUCONATE	++	-	-	-	+	+	++	++	-	-
Growth at 30 ° C	+	+++	++	+++	++	+	+	+	++	-
Growth at 37 ° C	+	+++	+++	+++	++	+	+	+	+++	++

[a] *Ln. citreum,* [b] *L. plantarum,* [c] *L. curvatus,* [d] *Ln. mesenteroides,* [e] *L. brevis,* [f] *P. pentosaceus.* Interpretation of LAB growth in API 50 CH system +++ = high growth (yellow); ++ = quite growth (green); + = little growth (dark green); - = not growth (blue). LAB growth at 30° C and 37°C mesured as Abs 420-580 nm at the beginning of stationary phase: +++ = > 2.0; ++ = 1.9-1.7; + = < 1.7; - = < 0.1.

4.13.3 Enzymatic activities

The indigenous microbiota of sourdough is a source of considerable genetic diversity representing different enzymatic activities useful in biotechnological applications (Pepe et al., 2004). Enzymes, such as xylanase and phytase are examples of the technological potential of the microbial biomass of sourdough. Xylanolytic enzymes are a group of enzymes that are involved in the hydrolysis of xylans and arabinoxylan polymers, and consequently in their solubilization (Gruppen et al., 1993; Narbutaite et al., 2009). Moreover, in bread and bakery industry, xylanases are used to increase the dough viscosity, bread volume and shelf life (Haros et al., 2001; Romanowska et al., 2003; Poutanen et al., 2009).

The strains were screened for their endo-xylanase activity and their phytate degrading ability using modified Chalmers agar supplemented with phytate salt (calcium or sodium) (Table IX).

Although all the strains were able to grow in a minimal broth, with xylan as the only carbon source, none of them showed endo-xylanase activity in the plate assay (Figure 15).

Madhukumar & Muralikrishna (2012) reported xylanase activity in *L. plantarum*, *P. pentosaceus* and *L. brevis* strains, quantifying the activity using wheat bran xylo-oligosaccharides as carbon source.

The differences in the xylanase activity results obtained in the present study, compared to those reported in the cited study (Madhukumar & Muralikrishna 2012), are likely related to the difference in the degree of polymerization of the carbon source used. In fact, in the present study the cell wall polysaccharide xylan was used as substrate; instead, the Authors of the other work used xylo-oligosaccharides. Concerning the yeast, in the present study *P. fermentans* did not show xylanase activity. Madrigal et al. (2013) observed xylanase activity in only one of the two *P. fermentans* strains tested, confirming the strain-specifity of this activity.

Considering the nutritional importance of this enzymatic activity, related to the bran fiber fraction "solubilization", further investigation at a genetic level have been planned to evaluate the presence and the expression of genes codifying for this activity.

Regarding phytate degrading activity, the ability to degrade sodium phytate was prevalent among the LAB tested, infact all the strains showed this enzymatic activity except of *L. plantarum* (CE60). This is consistent with an investigation on 12 species of sourdough lactic acid bacteria, in which although with some differences, the degrading activity on sodium phytate was largely distributed in all the species (De Angelis et al., 2003).

Moreover, 8 out of 13 LAB strains tested were able to hydrolyze both hexacalcium and sodium phytate (phy+), the most abundant forms in which phytates are present in cereal and legume-based foods (Raghavendra & Halami, 2009), forming a translucent zone around the colonies

(Figure 16; Figure 17). In particular, *Ln. citerum* (CE 54) exhibited particularly potent activities both on calcium and sodium phytates. Although some *P. pentosaceus* strain have been reported to be able to degrade both sodium and calcium phytate (Bae et al., 1999; De Angelis et al., 2003), in the present study the two *P. pentosaceus* tested strains have shown phytate degrading activity only on sodium phytate and no activity on calcium phytate, reflecting a intraspecific variability among strains belonged to the same species (Olstorpe et al., 2009). The strains that have shown phytate degrading ability could be exploited as starter cultures in fermented foods to improve the mineral bioavailability (Anastasio et al., 2010), thus upgrading the nutritional quality of phytate-rich foods.

TABLE IX
Phytase and endo-xylanase activities of LAB and yeast

Bacterial culture		**Phytase activity**		**Endo-xylanase activity**
		phytic acid calcium salt	**phytic acid sodium salt**	
CE88	***Ln. citreum***	++	+	-
CE54	***Ln. citerum***	+++	+++	-
CE42	***L. plantarum***	+	+++	-
CE60	***L. plantarum***	++	-	-
CE84	***L. plantarum***	+	++	-
CE83	***L. curvatus***	-	++	-
CE47	***L. sakei***	+	+	-
CE52	***Ln.mesenteroides***	++	++	-
CE48	***Ln. mesenteroides***	-	++	-
CE94	***L. brevis***	+	+++	-
CE85	***L. brevis***	+	+++	-
CE65	***P. pentosaceus***	-	+	-
CE23	***P. pentosaceus***	-	+	-
D	***P. fermentans***	-	-	-
Q11 (control)	***L. casei***	+++	++	-
2 (control)	***Clostridium***	-		+++

Interpretation of zone diameter of inhibition: - = no inibition; + = 0.01-0.1 cm; ++ = 0.1-0.3 cm; +++ = > 0.3 cm.

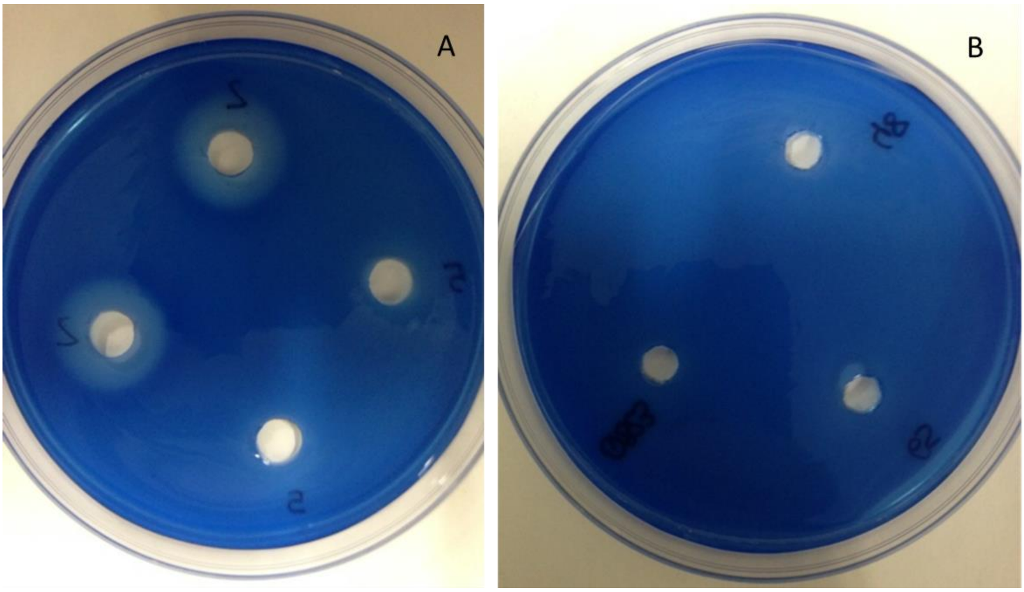

Figure 15. Screening for xylanolytic activity of the control strain *Clostridium* (A) and of tested LAB strains (B) on Azo-xylan agar medium.

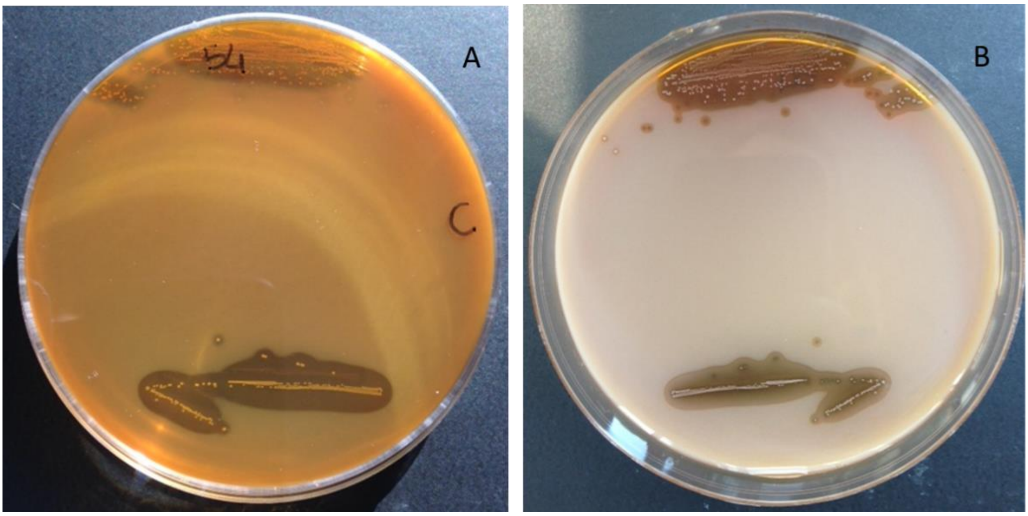

Figure 16. (A) Modified Chalmers agar with hexacalcium phytate showing the zones of clearing produced by phytase activity of *Ln. citerum* (CE 54).
(B) Elimination of false positive results by cobalt chloride solution treatment.

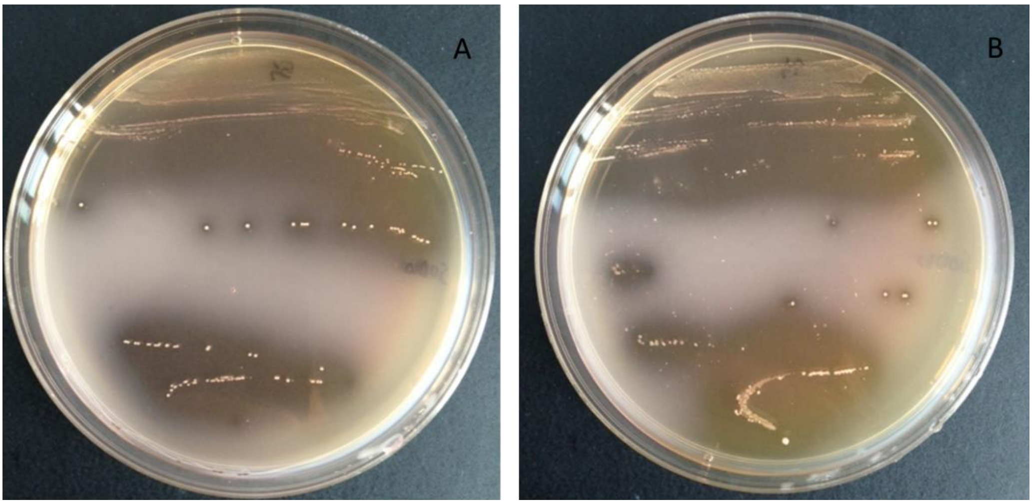

Figure 17. Modified Chalmers agar with sodium phytate showing the zones of clearing produced by phytase activity of *Ln. citerum* CE 54 (A) and *L. plantarum* CE 42 (B), after cobalt chloride solution treatment.

4.13.4 Antifungal activity

Another valuable characteristic of the starter strains is their antifungal activity; during the last few years, in fact, there has been a growing interest in the use of microorganisms and/or their metabolites to prevent spoilage and to extend the shelf-life of bakery products (Magnusson et al., 2001; Garofalo et al., 2012). The preserving effect of LAB is mainly related to the formation of lactic acid, acetic acid, and hydrogen peroxide, to the competition for nutrients and the production of bacteriocins (Gupta & Srivastava, 2014).

The culture overlay assay carried out on the LAB strains showed different level of inhibition against the two fungal target strains *Aspergillus oryzae* and *Aspergillus niger* (Table X), which are capable of rapid growth on the surface of bakery products (Smith et al., 2004).

L. plantarum strains (CE 42, CE 60, CE 84), *L. curvatus* (CE 83) and *P. pentosaceus* (CE 65, CE 23) showed the highest activity against *Aspergillus oryzae* and a moderate activity against *Aspergillus niger*. The latter was particularly inhibited by *Ln. mesenteroides* (CE 52) and *L. brevis* (CE94). In previous studies, some *L. plantarum* strains have already shown to possess antifungal activity (Coda et al., 2011b; Gupta & Srivastava, 2014).

TABLE X
Antifungal activity of lactic acid bacteria

Bacterial culture		***Aspergillus oryzae* ATCC 66222**	***Aspergillus niger* 25541**
CE88	***Ln. citreum***	+	-
CE54	***Ln. citerum***	++	+
CE42	***L. plantarum***	+++	++
CE60	***L. plantarum***	+++	++
CE84	***L. plantarum***	+++	++
CE83	***L. curvatus***	+++	++
CE47	***L. sakei***	+	-
CE52	***Ln.mesenteroides***	++	+++
CE48	***Ln. mesenteroides***	++	+
CE94	***L. brevis***	++	+++
CE85	***L. brevis***	++	+
CE65	***P. pentosaceus***	+++	++
CE23	***P. pentosaceus***	+++	++
Q823	***L. plantarum*(control)**	++	+
Q11	***L. casei* (control)**	++	-
LGG	***L. plantarum* (control)**	+++	+

Interpretation of zone diameter of inhibition - = no inibition; + = 0.01-0.3 cm; ++ = 0.3-0.6 cm; +++ = > 0.6 cm.

4.13.5 Exopolisaccharides production

An interesting property of sourdough LAB is their ability to synthesize a large structural variety of exo-polysaccharides (EPS), such as glucan and/or fructans. In fact, the large structural varieties of EPS isolated from sourdough include mainly homopolysaccharides (HoPS), which consists of one monosaccharide (mostly fructose or glucose) with the resulting EPS designated glucans or fructans, respectively. The biosynthesis of HoPS is cellwall bound or extracellular through the activity of glycansucrases and requires the specific substrate sucrose. In contrast to HoPS, heteropolysaccharides (HePS) are composed of irregular repeating units that are synthesized from sugar nucleotides by the activity of intracellular glycosyltransferases (Galle & Arendt, 2014).

Most LAB-producing EPS belong to the genera *Streptococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Pediococcus* (Ruas-Madiedo & De Los Reyes-Gavilán, 2005; van Hijum et al., 2006), some of which were investigated in the present study.

Suitability of EPS produced by sourdough LAB to replace or reduce plant hydrocolloids used in the bread-making process has been suggested in order to improve dough rheological parameters and bread quality (Tieking et al., 2003; Tieking & G¨anzle, 2005; Di Cagno et al., 2006; Lacaze et al., 2007; Schwab et al., 2008; Katina et al., 2009). Additionally, oligosaccharides and other metabolites generated during EPS formation from sucrose have also shown to effect physiological (health promoting) and technological properties in baked goods (Korakli et al., 2002; Kaditzky et al., 2008). Indeed, EPS exhibit a positive effect on the texture, mouthfeel, taste perception, and stability of fermented food and for certain EPS prebiotic effects have also been described (Dal Bello et al., 2001; Korakli et al., 2002; Tieking & G¨anzle, 2005; Schwab et al., 2008).

The EPS production was tested using MRS medium with different carbon sources. In accordance to Tieking & G¨anzle (2005), which postulated that the probability of any sourdough flora containing at least one EPS producing strain is high, in this study out of the 13 LAB only 3 strains didn’t produce EPS (CE 47; CE 52; CE 85). In the present study we found that the major EPS producer are *L.plantarum* and *P. pentosaceus* strains. As shown in Table XI the two *P. pentosaceus* strains (CE 65; CE 23) were able to produce EPS in particular using glucose and raffinose, and maltose respectively. *L. plantarum* (CE 84) was able to produce EPS in presence of different carbon sources, such as sucrose, raffinose, maltose and in particular lactose, while *L. plantarum* (CE 42, CE 60) were able to produce EPS in presence of starch, usually the most abundant carbon source in cereals products. The *L. plantarum* strains tested produced EPS with different charbohydrates, indicating that in each case the most suitable carbohydrate is largely dependent on the strain tested (Ruas-Madiedo & De Los Reyes-Gavilán, 2005). Di Cagno et al. (2006) demonstrated that a sourdough started with EPS forming *W.cibaria* and *L. plantarum* increased the viscosity of the sourdough and, when added at 20%, the resulting bread had higher specific volume and lower firmness. *L. curvatus* strain (CE 83) showed to produce EPS using maltose. To date, only one study reported the production of HePS from a sourdough isolated which was a *L. curvatus* strain. The produced HePS was composed of galactosamine, galactose, and glucose in a ratio of 2:3:1, respectively (Van der Meulen et al., 2007).

The strain *Ln mesenteroides* (CE 48) produced EPS using glucose and maltose as carbon source and probably it synthesized dextran as reported in other studies (Lacaze et al., 2007); in Panettone, a traditional Italian sweet bread, dextran from *Ln. mesenteroides* is responsible for the long storage stability (Decock & Cappelle, 2005). However, the other *Ln mesenteroides* strain tested (CE 52) did not show any EPS production. Strains identified as same genus did not exhibit exopolysaccharide production with the same substrates.

Differences in EPS production related to the carbon source of the medium have been attributed to the presence of different sugar transport systems in the LAB strains (Chervaux et al., 2000)

In the present work, only a preliminary qualitative screening for EPS production was conducted; the characterization of the EPS produced by our strains, in terms of structure and amounts, need further investigations in order to use the isolated bacteria as starters cultures in cereal fermentations, thus promoting the availablility of these polymers for food applications, through the in situ synthesis during processing (Tieking & Gaenzle, 2005; Bounaix et al., 2009).

TABLE XI

Exopolisaccharides production of lactic acid bacteria in MRS medium with different carbon source

Bacterial culture		Glucose	Sucrose	Raffinose	Maltose	Lactose	Starch
CE88	***Ln. citreum***	-	-	+	-	-	-
CE54	***Ln. citerum***	+	-	-	-	-	-
CE42	***L. plantarum***	-	-	-	-	-	+
CE60	***L. plantarum***	++	-	-	-	-	+
CE84	***L. plantarum***	-	+	+	+	++	-
CE83	***L. curvatus***	-	-	-	++	-	-
CE47	***L. sakei***	-	-	-	-	-	-
CE52	***Ln.mesenteroides***	-	-	-	-	-	-
CE48	***Ln. mesenteroides***	+	-	-	+	-	-
CE94	***L. brevis***	-	-	-	+	-	-
CE85	***L. brevis***	-	-	-	-	-	-
CE65	***P. pentosaceus***	++	+	-	+	-	-
CE23	***P. pentosaceus***	-	+	++	++	-	-

- = no sticky; + = sticky; ++ = very sticky.

4.13.6 Screening for probiotic properties

Spontaneously fermented foods, such as sourdough, may constitute a reservoir for new LAB spp. strains with potential probiotic characteristics (Sabir et al., 2010; Ramos et al., 2013).

The LAB isolated from sourdough are inherently able to survive the harsh fermentation conditions, and are, therefore, likely to be able also to survive the passage through the gastro intestinal tract (GIT). The LAB strains were screened for their ability to survive to acid and bile salt, to adhere to epithelial surfaces (Table XII) and for their antagonistic activity towards intestinal pathogens.

4.13.6.1 pH and bile resistance

According to Fuller (1992), bile, even at low concentrations, can inhibit the growth of microorganisms. Gilliland et al. (1984) reported that 0.3% is considered to be a critical concentration for screening for resistant strains. In the present study, all of the strains were able to survive in 0.3% (w/v) bile and *L. plantarum* (CE 60, CE 84), *L. curvatus* (CE 83), *Ln. mesenteroides* (CE 48) and *L. brevis* (CE 94, CE 85), were even able to replicate/grow in presence of bile salt for 24h (Table XII). These results are in agreement with observations of Lee et al. (2011), which reported strong survival under bile conditions for several LAB strains, such as *L. plantarum* and *L. brevis* strains, isolated from a traditional Korean fermented vegetable. Results about the effects of a low pH on the LAB strains (number of viable cells after 2h of incubation at pH 2.5) are reported in table XII.

In accordance with Delgado et al. (2007), the acidic condition (pH 2.5) seemed to be more damaging to the bacteria, with only 6 out of 13 strains surviving 2h of exposure and none of them growing.

Conclusively, *L. curvatus* (CE 83), *Ln. mesenteroides* (CE 48), both *L. brevis* strains (CE 94; CE 85) and *P. pentosaceus* (CE 65; CE 23) were able to survive when exposed to the conditions of GIT, in terms of the low pH and the presence of bile salts.

According to previous reports, our study showed that the acidic tolerance is not necessarily related to the species of LAB, but may also be strain-specific (Maldonado et al., 2012). In fact, differences were observed among strains belonged to the same species, such as *L. plantarum*, *Ln mesenteroides* and *P. pentosaceus*, in terms of acid and bile tolerance.

Moreover, despite strains of *L. plantarum* have previously been proven to be able to survive gastric transit (Georgieva et al., 2008; Mathara et al., 2008), our results revealed that *L. plantarum* tested strains present a strong bile tolerance but lower ability to survive at low pH.

These tests are, however, rather qualitative, and the resistances of probiotic cultures to low pH and bile in food matrices during passage through the GIT might be greater than those seen in the physiological solutions used in the present study (Dunne et al., 2001).

4.13.6.2 Adhesion to Caco-2 cells

The capacity of probiotics to adhere to the intestinal mucosa is a key factor in a strain's ability to survive and function as desired in the intestine (Dunne et al., 2001).

The adhesion ability to Caco-2 cells, which express the morphological and physiological characteristics of human enterocytes (Blum et al., 1999), was evaluated for 4 LAB strains, belonged to different species, selected according to their ability to survive to the conditions of the GIT: *L. curvatus* (CE 83), one of the *L. brevis* strains (CE 85), one *P. pentosaceus* (CE 65), and also one *L. plantarum* (CE 84) was tested although its scarce resistance to low pH.

L. plantarum (LGG; Q823) and *L. plantarum* (CE 42) were used as positive and negative control, respectively. All the tested strains, strongly adhered to the Caco-2 cells, with adhesive properties even higher than those assessed in the positive control. *L. curvatus* (CE 83), *L. brevis* (CE 85) and *P. pentosaceus* (CE 65), thanks to their ability to survive to the conditions of the GIT and thier capacity to adhere to the intestinal mucosa could be considered as suitable candidates to be used as probiotics.

TABLE XII
Tolerance to low pH conditions (pH 2.5 for 2h of incubation) and to bile salt (0.3 % oxgall for 24 h of incubation) and Adhesion to to human colon carcinoma cell line Caco-2

Bacterial culture		pH 2.5		Oxgall 0.3%		Adhesion to Caco-2 cell-line[b]
		0h log CFU.mL^{-1}	2h log CFU.mL^{-1}	0h log CFU.mL^{-1}	24h log CFU.mL^{-1}	log CFU.mL^{-1}/ %
CE88	***Ln. citreum***	7.8 ± 0.4	0.0 ± 0.0	6.7 ± 0.5	4.5 ± 0.7	ND
CE54	***Ln. citerum***	7.9 ± 0.6	0.0 ± 0.0	5.9 ± 0.5	3.4 ± 0.2	ND
CE42	***L. plantarum***	5.8 ± 0.8	0.0 ± 0.0	7.0 ± 0.6	5.3 ± 1.5	0.0 ± 0.0
CE60	***L. plantarum***	7.8 ± 0.3	0.0 ± 0.0	7.0 ± 0.6	8.2 ± 0.3	ND
CE84	***L. plantarum***	8.1 ± 0.2	0.0 ± 0.0	6.5 ± 0.7	8.2 ± 0.3	6.5 ± 0.0 / 81.4%
CE83	***L. curvatus***	7.5 ± 0.2	3.7 ± 0.2	7.1 ± 0.0	8.1 ± 0.0	6.3 ± 0.1/ 79,4%
CE47	***L. sakei***	6.7 ± 0.2	0.0 ± 0.0	3.2 ± 1.0	1.0 ± 1.4	ND
CE52	***Ln. mesenteroides***	7.2 ± 0.8	0.0 ± 0.0	7.2 ± 0.3	0.5 ± 0.8	ND
CE48	***Ln. mesenteroides***	7.8 ± 0.2	4.6 ± 1.0	7.3 ± 0.2	8.4 ± 0.0	ND
CE94	***L. brevis***	7.3 ± 0.1	6.0 ± 0.7	5.8 ± 0.5	7.3 ± 1.2	ND
CE85	***L. brevis***	7.3 ± 0.1	3.4 ± 1.1	5.6 ± 0.5	6.6 ± 1.1	6.1 ± 0.0 / 76.4%
CE65	***P. pentosaceus***	7.4 ± 0.1	3.4 ± 0.9	7.1 ± 0.1	3.9 ± 0.7	6.5 ± 0.1 / 81.6%
CE23	***P. pentosaceus***	7.7 ± 0.1	1.3 ± 0.9	6.0 ± 0.0	2.7 ± 0.4	ND
Q823	***L. plantarum***	7.5 ± 0.1	7.2 ± 0.1	6.2 ± 0.4	5.2 ± 0.1	5.9 ± 0.4 / 73.7%
Q11	***L. casei***	7.2 ± 0.3	1.5 ± 0.3	7.1 ± 0.2	5.3 ± 0.4	7.4 ± 0.3 / 92.5%
LGG	***L. plantarum***	ND	ND	ND	ND	5.5 ± 0.2 / 69.3%

[b] Average log no. of adhering lactobacilli in Caco-2 cell after 2 h incubation.
Initial inoculums at approximately 1×10^{8} CFU/mL (log 8.0). ND, not determined.

4.13.6.3 Anti-listeria activity

L. plantarum (CE 42, CE 60, CE 84), *Ln. mesenteroides* (CE52) and *P. pentosaceus* (CE 65) isolated strains showed intense inhibition activity against all the pathogenic bacteria tested (*L. innocua, L. monocytogened, L. welshimeri*) (Table XIII).

The inhibition of undesirable and pathogenic bacteria, causing diarrhea or other diseases in the human intestine (Temmerman et al., 2003), is a desirable property for probiotics (Bernet-Camard et al., 1997; Delgado et al., 2007), in order to balance the intestinal environment, and thereby improve host health.

This inhibition could be due to the production of inhibitory substances, such as organic acids, bacteriocins or H_2O_2 (Juven et al., 1992). At present, the nature of the inhibitory substances involved in the antagonistic activities of the tested strains is unknown and it will be investigated. Moreover, the probiotic candidate strains do require further in vitro and in vivo investigations, in order to confirm their probiotic characteristics and evaluate the health-promoting effects in the human intestinal tract.

TABLE XIII
Anti-listeria activity of lactic acid bacteria.

Bacterial culture		***L. innocua***	***L. monocytogenes***	***L. welshimeri***
CE88	***Ln. citreum***	-	+	-
CE54	***Ln. citerum***	-	+	-
CE42	***L. plantarum***	++	++	++
CE60	***L. plantarum***	++	++	++
CE84	***L. plantarum***	++	++	++
CE83	***L. curvatus***	-	-	-
CE47	***L. sakei***	-	+	+
CE52	***Ln.mesenteroides***	+	+	+
CE48	***Ln. mesenteroides***	-	-	-
CE94	***L. brevis***	-	-	-
CE85	***L. brevis***	-	-	-
CE65	***P. pentosaceus***	++	++	++
CE23	***P. pentosaceus***	+	-	++

Interpretation of zone diameter of inhibition - = no inibition; + = 0.1- 0.2 cm; ++ = 0.2- 0.3 cm.

4.13.7 Antibiotic resistance

Because of their long-time use in various food and feed preparations, LAB have been classified as GRAS 'generally recognized as safe' (Adams & Marteau, 1995; Boriello et al., 2003). However, it has been shown that genes coding for antibiotics resistance can be transferred among bacteria of different genera and thus to human commensal flora and to pathogenic bacteria, temporarily residing in the hosts, which consequently cannot be treated with previously successful antibiotics (Mathur & Singh, 2005; Adimpong et al., 2012).

According to Kastner et al. (2006), out of 200 starter cultures and probiotic bacteria isolated from 90 different food sources, 27 isolates exhibited resistance patterns that could not be ascribed as an intrinsic feature of the respective genera.

Therefore, it is very important to verify that probiotic and nutritional LAB strains used as starter cultures lack acquired antimicrobial resistance properties prior to considering them safe for human consumption. The results of antibiotic susceptibility testing are shown in Table XIV. The bacteria were considered resistant to a particular antibiotic when the MIC (mg/L) values obtained were higher than the recommended breakpoint value defined at species level by the FEEDAP Panel (EFSA-FEEDAP, 2012) and the committee on Antimicrobial Susceptibility Testing (EUCAST, http://www.eucast.org).

In the present study *L. plantarum* (CE 84), *L. curvatus* (CE 83) and both *L. brevis* strains (CE94; CE 85) were resistant to Clindamycin. These strains may require further molecular investigation to ascertain the cause of these resistance patterns before their utilization. Our results showed that the investigated strains were resistant to high concentration of vancomycin (MIC values 128 μg mL^{-1}). In a previous study, Danielsen & Wind (2003) shown that *L. plantarum/pentosus* strains were resistant to higher concentrations of vancomycin (MIC ≥ 256 μg/mL). Furthermore, *L. plantarum* and *L. brevis* strains resistant to high concentrations of vancomycin (MICs ≥256 μg/mL) was also reported by Delgado et al. (2005). According to Ammor et al. (2007) the resistance of *Lactobacillus*, *Pediococcus* and *Leuconostoc* species to vancomycin (MIC values 128 μg mL^{-1}) is due to the absence of D-Ala-D-lactate in their cell wall which is the target of vancomycin. Thus the resistance mechanisms observed among these strains is inherent or intrinsic to their species and could therefore not be attributed to acquisition of resistance genes. In fact, intrinsic resistance is not horizontally transferable as it is chromosomally encoded and related to the general physiology or anatomy of an organism.

The the MIC values obtained for trimethoprim and ciprofloxacin for some of the strains were higher than the recommended FEEDAP Panel's breakpoint values. However, Lactobacilli are generally intrinsically resistant for quinolones, trimethoprim and ciprofloxacin (Danielsen & Wind, 2003; Nawaz et al., 2011). Moreover, the data available (Korhonen et al., 2007) indicate that within species of lactobacilli the range of apparent trimethoprim resistances can be wide with no clear breakpoint values. Therefore, the MIC testing of trimethoprim for lactic acid bacteria was not considered relevant. Furthermore, testing for linezolid and neomycin are no longer considered necessary (EFSA, 2008).

Table XIV
Minimal inhibitory concentration (MICs) of tested antibiotics in LAB

	Ln. citreum		*Ln.mesenteroides*			*L. plantarum*				*L. curvatus*	*L. sakei*		*L. brevis*			*Pd. pentosaceus*		
	CE88	**CE54**	**CE52**	**CE48**	Breakpoint (µg ml^{-1})	**CE42**	**CE60**	**CE84**	Breakpoint (µg ml-1)	**CE83**	**CE47**	Breakpoint (µg ml^{-1})	**CE94**	**CE85**	Breakpoint (µg ml-1)	**CE65**	**CE23**	Breakpoint (µg ml^{-1})
	MIC (µg ml^{-1})		MIC (µg ml^{-1})			MIC (µg ml^{-1})				MIC (µg ml^{-1})			MIC (µg ml^{-1})			MIC (µg ml^{-1})		
Gentamicin[a]	4	4	4	4	16	4	4	4	16	4	4	16	4	4	16	4	4	16
Kanamycin[a]	16	16	16	16	16	16	16	64	64	16	16	64	16	16	32	16	64	64
Streptomycin[a]	4	4	4	4	64	4	16	32	n.r.	4	4	64	4	4	64	4	16	64
Tetracycline[a]	1	1	1	1	8	8	8	8	32	8	1	8	8	8	8	4	4	8
Erythromycin[a]	0.5	0.5	< 0.25	< 0.25	1	< 0.25	0.5	< 0.25	1	< 0.25	< 0.25	1	< 0.25	< 0.25	1	< 0.25	0.5	1
Clindamycin[a]	0.25	0.25	0.25	0.25	1	0.25	0.25	8	2	8	0.25	1	8	8	1	0.25	0.25	1
Chloramphenicol[a]	1	1	4	1	4	4	4	4	8	4	4	4	1	1	4	4	1	4
Ampicillin[a]	0.25	0.25	0.25	0.25	2	0.25	0.25	0.25	2	0.25	1	4	0.25	0.25	2	1	1	4
Neomycin[a]	4	4	4	4	n.r.	4	4	4	n.r.	4	4	n.r.	4	4	n.r.	4	4	n.r.
Vancomycin[a]	128	128	128	128	n.r.	128	128	128	n.r	128	64	n.r.	128	128	n.r.	128	128	n.r.
Quinupri/Dalfopri[b]	2	2	1	1	4	1	2	4	4	4	4	4	4	2	4	2	4	4
Linezolid[b]	1	1	2	1	4	1	2	1	4	1	1	4	2	1	4	2	2	4
Trimethoprim[b]	16	32	8	4	4	1	1	1	4	1	4	4	1	1	4	8	16	4
Ciprofloxacin[b]	2	2	2	8	4	8	16	16	4	2	2	4	2	2	4	32	64	4
Rifampicin[b]	0.25	0.25	0.25	0.25	0.5	0.25	0.25	0.25	0.5	0.25	0.25	0.5	0.25	0.25	0.5	0.25	0.25	0.5

a FEEDAP Breakpoint (EFSA Journal 2012)
b EUCAST Clinical Breakpoint
n.r.= not required

4.14 Conclusions

The aim of producing a wide variety of high-quality standardized fermented ingredients/food products has generated a demand for specialized starters. In this sense, the study of microbial diversity represents an opportunity for advances in biotechnology. Moreover, the possibility of mixing strains with different properties and activities could be an interesting procedure to obtain fermented goods or fermented bran-enriched products with improved technological and nutritional qualities.

Our results suggest that *L. plantarum* and *P. pentosaceus* species could have interesting technological applications, due to their antifungal activity and EPS production. Some of these strains also exhibited phytate degrading activity on calcium and/or on sodium phytate salt and they could be exploited to improve mineral bioavailability of fermented products. Moreover, *L. curvatus* CE 83, *L. brevis* CE 85, and *P. pentosaceus* CE 65 seemed to be suitable candidates to be used as probiotics. Further studies should be conducted in order to test the effectiveness of these strains in improving fermented bran and sourdoughs qualities and to better determine their potential applications. Moreover, the screening and selection of other lactic acid bacteria strains, isolated from cereals sources, belonged to the same well adapted species analyzed in the present study, and showing interesting metabolic and enzymatic activities, represent a future perspective in order to improve the final properties of the fermented bran.

4.15 References

Adams MR, Marteau P, 1995, On the safety of lactic acid bacteria from food. Int J Food Microbiol 27:263-264.

Adimpong DB et al., 2012, Genotypic characterization and safety assessment of lactic acid bacteria from indigenous African fermented food products. BMC microbiol 12:75.

Ammor MS et al., 2007, Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria. Food Microbiol 24:559-570.

Anastasio M et al., 2010, Selection and Use of Phytate-Degrading LAB to Improve Cereal-Based Products by Mineral Solubilization During Dough Fermentation. J Food Sci 75:M28-M35.

Asahara T et al., 2004, Probiotic bifidobacteria protect mice from lethal infection with Shiga toxin-producing *Escherichia coli* O157: H7. Infect Immun 72:2240-2247.

Aslim B et al., 2007, Factors influencing autoaggregation and aggregation of *Lactobacillus delbrueckii* subsp. *bulgaricus* isolated from handmade yogurt. J Food Protect 70:223-27.

Bae HD et al, 1999, A novel staining method for detecting phytase activity. J Microbiol Methods 39:17-22.

Bernet-Camard MF et al., 1997, The human *Lactobacillus acidophilus* strain LA1 secretes a non-bacteriocin antibacterial substance(s) active in vitro and in vivo. Appl Environ Microbiol 63:2747-2753.

Blum S et al., 1999, Adhesion studies for probiotics: need for validation and refinement. Trends Food Sci Technol 10:405-410.

Borriello SP et al., 2003, Safety of probiotics that contain lactobacilli or bifidobacteria. Clin Infect Dis 36:775-780.

Bounaix MS et al., 2009, Biodiversity of exopolysaccharides produced from sucrose by sourdough lactic acid bacteria. J Agric Food Chem 57:10889-10897.

Carnevali P et al., 2007, Liquid sourdough fermentation: industrial application perspectives. Food Microbiol 24:150-154.

Chervaux C et al., 2000, Physiological study of *Lactobacillus delbrueckii* subsp. *bulgaricus* strains in a novel chemically defined medium. Appl Environ Microbiol 66:5306-5311.

Coconnier MH et al., 1998, Antagonistic activity against Helicobacter infection in vitro and in vivo by the human *Lactobacillus acidophilus* strain LB. Appl Environ Microbiol 64:4573-4580.

Coda R et al., 2010, Spelt and emmer flours: characterization of the lactic acid bacteria microbiota and selection of mixed autochthonous starters for bread making. J Appl Microbiol 108:925-935.

Coda R et al., 2011a, Utilization of African grains for sourdough bread making. J Food Sci 76:M329-M335.

Coda R et al., 2011b, Antifungal activity of *Wickerhamomyces anomalus* and *Lactobacillus plantarum* during sourdough fermentation: identification of novel compounds and longterm effect during storage of wheat bread. Appl Environ Microbiol 77:3484-3492.

Coda R et al., 2012, Selected lactic acid bacteria synthesize antioxidant peptides during sourdough fermentation of cereal flours. Appl Environ Microbiol 78:1087-1096.

Coda R et al., 2013, Antifungal activity of *Meyerozyma guilliermondii*: Identification of active compounds synthesized during dough fermentation and their effect on long-term storage of wheat bread. Food Microbiol 33:243-251.

Coda R et al., 2014, Sourdough lactic acid bacteria: Exploration of non-wheat cereal-based fermentation. Food Microbiol 37:51-58.

Dal Bello F et al., 2001, In vitro study of prebiotic properties of levan-type exopolysaccharides from lactobacilli and non-digestible carbohydrates using denaturing gradient gel electrophoresis. Syst Appl Microbiol 24:232-237.

Danielsen M, Wind A, 2003, Susceptibility of *Lactobacillus* spp. to antimicrobial agents. Int J Food Microbiol 82:1-11.

De Angelis M et al., 2003, Phytase activity in sourdough lactic acid bacteria: purification and characterization of a phytase from *Lactobacillus sanfranciscensis* CB1. Int J Food Microbiol 87:259-270.

De Vuyst L, Neysens P, 2005, The sourdough microflora: biodiversity and metabolic interactions. Trends Food Sci Technol 16:43-56.

Decock P, Cappelle S, 2005, Bread technology and sourdough technology. Trends Food Sci Technol 16:113-120.

Delgado S et al., 2005, Antibiotic susceptibility of *Lactobacillus* and *Bifidobacterium* species from the human gastrointestinal tract. Curr Microbiol 50:202-207.

Delgado S et al., 2007, Subtractive screening for probiotic properties of *Lactobacillus* species from the human gastrointestinal tract in the search for new probiotics. J Food Sci 72:M310-M315.

Di Cagno R et al, 2006, Glucan and fructan production by sourdough *Weissella cibaria* and *Lactobacillus plantarum*. J Agric Food Chem 54:9873-9881.

Dierksen KP et al., 1997, Expression of ropy and mucoid phenotypes in *Lactococcus lactis*. J Dairy Sci 80:1528-1536.

Dunne C et al., 2001, In vitro selection criteria for probiotic bacteria of human origin: correlation with in vivo findings. Am J Clin Nutr 73:386S-392S.

EFSA-FEEDAP, 2012, Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA J 10:2740-2749.

EFSA, 2008, Technical guidance. Update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance. EFSA J 732:1-15.

FAO/WHO Working Group. Guidelines for the evaluation of probiotics in food. London Ontario, Canada, April 30 and May 1, 2002. Rome: FAO; 2002.

Fuller R, 1992, Probiotics: the Scientific Basis. Chapman and Hall, London, pp. 1-9.

Galle S, Arendt EK, 2014, Exopolysaccharides from Sourdough Lactic Acid Bacteria. Crit Rev Food Sci Nutr 54:891-901.

Garofalo C et al., 2012, Selection of sourdough lactobacilli with antifungal activity for use as biopreservatives in bakery products. J Agric Food Chem 60:7719-7728.

Gasser F, 1994, Safety of lactic acid bacteria and their occurrence in human clinical infections. Bull Inst Pasteur 92:45-67.

Georgieva R et al., 2008, Identification and vitro characterization of *Lactobacillus plantarum* strains from artisanal Bulgarian white brined cheeses. J Basic Microbiol 48:234-244.

Gilliland SE et al., 1984, Importance in bile tolerance of *Lactobacillus acidophilus* used as a dietary adjunct. J Dairy Sci 67:3045-3051.

Gobbetti M, 1998, Interactions between lactic acid bacteria and yeasts in soudoughs. Trends Food Sci Technol 9:267-274.

Gobbetti M et al., 1999, Added pentosans in breadmaking: Fermentations of derived pentoses by sourdough lactic acid bacteria. Food Microbiol 16:409-418.

Gobbetti M et al., 2014, How the sourdough may affect the functional features of leavened baked goods. Food Microbiol 37:30-40.

Gruppen H et al., 1993, Enzymic degradation of waterunextractable cell wall material and arabinoxylans from wheat flour. J Cereal Sci 18:129-143.

Gupta R, Srivastava S, 2014, Antifungal effect of antimicrobial peptides (AMPs LR14) derived from *Lactobacillus plantarum strain* LR/14 and their applications in prevention of grain spoilage. Food Microbiol 42:1-7.

Haros M et al., 2001, Use of fungal phytase to improve breadmaking performance of whole wheat bread. J Agric Food Chem 49:5450-5454.

Hudault S et al., 1997, Antagonistic activity exerted in vitro and in vivo by *Lactobacillus casei* (strain GG) against *Salmonella typhimurium* C5 infection. Appl Environ Microbiol 63:513-518.

International Standard ISO 10932/IDF 223 (2010): Milk and milk products – Determination of the minimal inhibitory concentration (MIC) of antibiotics applicable to bifidobacteria and non-enterococcal lactic acid bacteria (LAB).

Jacobsen CN et al., 1999, Screening of probiotic activities of forty-seven strains of *Lactobacillus* spp. by in vitro techniques and evaluation of the colonization ability of five selected strains in humans. Appl Environ Microbiol 65: 4949-4956.

Juven BJ et al., 1992, Antagonistic compounds produced by a chicken intestinal strain of *Lactobacillus acidophilus*. J Food Prot 55:157-161.

Kaditzky S et al., 2008, Performance of *Lactobacillus sanfranciscensis* TMW 1.392 and its levansucrase deletion mutant in wheat dough and comparison of their impact on bread quality. Eur Food Res Technol 227:433-442.

Kastner S et al., 2006, Antibiotic susceptibility patterns and resistance genes of starter cultures and probiotic bacteria used in food. Syst Appl Microbiol 29:145-155.

Katina K et al., 2009, In situ production and analysis of *Weissella confusa* dextran in wheat sourdough. Food Microbiol 26:734-743.

Katina K et al., 2012, Fermented Wheat Bran as a Functional Ingredient in Baking. Cereal Chem 89:126-134.

Katina K, Poutanen K, Nutritional aspects of cereal fermentation with lactic acid bacteria and yeast. In Handbook on Sourdough Biotechnology, M. Gobbetti, M. Gaenzle eds., Springer 2013, New York.

Klare I et al., 2005, Evaluation of new broth media for microdilution antibiotic susceptibility testing of lactobacilli, pediococci, lactococci, and bifidobacteria. Appl Environ Microbiol 71:8982-8986.

Kleerebezem M et al., 2003, Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc Natl Acad Sci 100: 1990-1995.

Korakli M et al., 2002, Metabolism by bifidobacteria and lactic acid bacteria of polysaccharides from wheat and rye, and exopolysaccharides produced by *Lactobacillus sanfranciscensis*. J Appl Microbiol 92:958-965.

Korhonen JM et al., 2007, Characterization of dominant cultivable lactobacilli and their antibiotic resistance profiles from faecal samples of weaning piglets. J Appl Microbiol. 103, 2496-2503.

Lacaze G et al., 2007, Emerging fermentation technologies: Development of novel sourdoughs. Food Microbiol 24:155:160.

Lee J et al., 2011, Evaluation of probiotic characteristics of newly isolated *Lactobacillus* spp.: Immune modulation and longevity. Int J Food Microbiol 148:80-86.

Leroy F, De Vuyst L, 2004, Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trends Food Sci Technol 15:67-78.

Madhukumar MS, Muralikrishna G, 2012, Fermentation of xylo-oligosaccharides obtained from wheat bran and Bengal gram husk by lactic acid bacteria and bifidobacteria. J Food Sci Technol 49:745-752.

Madrigal T et al., 2013, Glucose and ethanol tolerant enzymes produced by Pichia (Wickerhamomyces) isolates from enological ecosystems. Am J Enol Vitic 64:126-133.

Magnusson J, Schnürer J, 2001, *Lactobacillus coryniformis* subsp. *coryniformis* strain Si3 produces a broad-spectrum proteinaceous antifungal compound. Appl Environ Microbiol 67:1-5.

Maldonado NC et al., 2012, Lactic acid bacteria isolated from young calves e characterization and potential as probiotic. Res Vet Sci 92:342-349.

Manini F et al., 2014, Study of the chemical changes and evolution of microbiota during sourdough like fermentation of wheat bran. Cereal Chem 91:342-349.

Mathara JM et al., 2008, Functional properties of *Lactobacillus plantarum* strains isolated from Maasai traditional fermented milk products in Kenya. Curr Microbiol 56:315-321.

Mathur S, Singh R, 2005, Antibiotic resistance in food lactic acid bacteria: a review. Int J Food Microbiol 105:281-295.

Meroth CB et al., 2003, Monitoring the bacterial population dynamics in sourdough fermentation processes by using PCR-denaturing gradient gel electrophoresis. Appl Environ Microbiol 69:475-82.

Messens W, De Vuyst L, 2002, Inhibitory substances produced by Lactobacilli isolated from sourdoughs e a review. Int J Food Microbiol 72:31-43.

Minervini F et al., 2010, Robustness of *Lactobacillus plantarum* starters during daily propagation of wheat flour sourdough type I. Food Microbiol 27:897-908.

Moroni AV et al., 2010, Solubility of proteins from non-gluten cereals: a comparative study on combinations of solubilising agents. Food Chem 121:1225-1230.

Narbutaite V et al., 2009, The xylanolytic profile of lactic acid bacteria with antimicrobial properties in novel fermentation media enriched with dietary fibre. In Proceedings of the 5th International Congress Flour-Bread'09. 7th Croatian Congress of Cereal Technologists, Opatija, Croatia, 21-23 October, 2009. Faculty of Food Technology, Osijek, University of Josip Juraj Strossmayer, pp. 394-401.

Nawaz M et al., 2011, Characterization and transfer of antibiotic resistance in lactic acid bacteria from fermented food products. Curr Microbiol 62:1081-1089.

Pepe O et al., 2004, Technological and Molecular Diversity of *Lactobacillus plantarum* Strains Isolated from Naturally Fermented Sourdoughs. Syst Appl Microbiol 27:443-453.

Poutanen K et al., 2009, Sourdough and cereal fermentation in a nutritional perspective. Food Microbiol 26:693-699.

Raghavendra P, Halami PM, 2009, Screening selection and characterization of phytic acid degrading lactic acid bacteria from chicken intestine. Int J Food Microbiol 133:129-134.

Ramos CL et al., 2013, Strain-specific probiotics properties of *Lactobacillus fermentum*, *Lactobacillus plantarum* and *Lactobacillus brevis* isolates from Brazilian food products. Food Microbiol 36:22-29.

Rizzello CG et al., 2010, Effect of sourdough fermentation on stabilisation, and chemical and nutritional characteristics of wheat germ. Food Chem 119:1079-1089.

Romanowska I et al., 2003, The application of fungal endoxylanase in bread-making. Commun Agric Appl Biol Sci 68: 317-320.

Ruas-Madiedo P, De Los Reyes-Gavilán CG, 2005, Invited Review: Methods for the Screening, Isolation, and Characterization of Exopolysaccharides Produced by Lactic Acid Bacteria. J Dairy Sci 88:843-856.

Sabir F et al., 2010, Assessment of potential probiotic properties of *Lactobacillus* spp., *Lactococcus* spp., and *Pediococcus* spp. strains isolated from kefir. J Food Sci 75:M568-M573.

Salmenkallio-Marttila M et al., 2001, Effect of bran fermentation on quality and microstructure of high-fibre wheat bread. Cereal Chem 78:429-435.

Schwab C et al., 2008, Formation of oligosaccharides and polysaccharides by *Lactobacillus reuteri* LTH5448 and *Weissella cibaria* 10M in sorghum sourdoughs. Cereal Chem 85:679-684.

Smith JP et al., 2004, Shelf life and safety concerns of bakery products—a review. Crit Rev Food Sci Nutr 44:19-55.

Sterr Y et al., 2009, Evaluation of lactic acid bacteria for sourdough fermentation of amaranth. Int J Food Microbiol 136:75-82.

Tamang JP et al., 2009, Functional properties of lactic acid bacteria from ethnic fermented vegetales of the Himalayas. Int J Food Microbiol, 135:28-33.

Temmerman R et al., 2003, Identification and antibiotic susceptibility of bacterial isolates from probiotic products. Int J Food Microbiol 81:1-10.

Tieking M et al., 2003, In situ production of exopolysaccharides during sourdough fermentation by cereal and intestinal isolates of lactic acid bacteria. Appl Environ Microbiol 69:945-952.

Tieking M, G¨anzle MG, 2005, Exopolysaccharides from cereal-associated lactobacilli. Trends Food Sci Technol 16:79-84.

Van der Meulen R et al., 2007, Screening of lactic acid bacteria isolates from dairy and cereal products for exopolysaccharide production and genes involved. Int J Food Microbiol 118:250-258.

van Hijum S et al., 2006, Structure-function relationships of glucansucrase and fructansucrase enzymes from lactic acid bacteria. Microbiol Mol Biol Rev 70:157.

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APPENDIX I

The V International Symposium on Sourdough - Cereal Fermentation for Future Foods, 10-12th October 2012, Helsinki, Finland - (oral presentation)

Wheat Bran Sourdough as a Functional Ingredient

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Several studies indicate that high-content whole grains diets work as a protective factor to chronic diseases. This decreased risk is related to the high content of fiber and bioactive compounds found mainly in the bran, which is usually removed during milling because of its fast rancidity. The increasing demand for functional foods and the pressure to ensure the exploitation of agro-industrial by-products have attracted great interest in bran-enriched foods/flours. The Healthgrain European Project recently emphasized the possibility to increase the amount of bioactive compounds in cereals by-products through biotechnological processes. Bran fermentation has been shown an efficient pre-treatment in order to enhance technological and nutritional properties of high fiber products (Katina *et al.* 2007). From a nutritional point of view, the fermentation effect on water-extractable arabinoxylans (WEAX) deserve particular attention, because of the positive effects on glycaemic and insulinaemic responses (Lu *et al.* 2000). Moreover, microbial xylan-degrading activity positively affects the bioavailability of functional compounds commonly found in the bran. This study aims to develop an innovative biotechnological process of wheat bran stabilization by microbial acidification. Briefly, bran sourdoughs were produced at 18 °C through continuous propagation by back-slopping of ripe dough (10% inoculum) until a stable microbiota was established. At each refreshment step (every 24h), analysis of the bacterial content and the acidity of the dough, measured as pH and total titratable acidity (TTA), were performed on the ripe sourdough. Furthermore, the amounts of fiber and bioactive compounds, such as WEAX, ferulic and phytic acids, were determined before and after bran fermentation. Lactic acid bacteria (LAB) rapidly increased after the first day of bran fermentation and reached high amounts (10^9 CFU g^{-1}). Yeasts population fluctuated during propagation, but after 8 refreshments it stabilized at the level of 10^7 CFU g^{-1}. The TTA and pH developments followed the LAB growth with the pH rapidly decreasing from 6.5 to 4.1. Results suggest that wheat bran sourdough is a "stable" functional ingredient for bakery products that can be used to improve their nutritional and technological properties.

References

1. Lu Z X, Walker K Z, Muir J G, O'Dea K (2000): Arabinoxylan fiber, a byproduct of wheat flour processing, reduces the postprandial glucose response in normoglycemic subjects. Am. J. Clin. Nutr., 71:5, p. 1123.

2. Katina K, Laitila A, Juvonen R, Liukkonen K H, Kariluoto S, Piironen V, Landberg R, Aman P, Poutanen K (2007): Bran fermentation as a means to enhance technological properties and bioactivity of rye. Food Microbiol., 24:2, p. 175.

Cereals & Europe Spring Meeting 2013, Leuven, Belgium 29-31th May, 2013
(poster presentation)

Nutritional enhancement of grain milling byproducts

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The increasing demand for functional foods and the opportunity to take advantage of agro-industrial by-products have attracted great interest in bran-enriched products. The Healthgrain European Project has recently emphasized the possibility to enhance nutritional properties of cereal by-products through biotechnological processes. Bran fermentation has been shown to contribute to dietary fiber solubilisation and positively affect the bioavailability of functional compounds. The fermentation effects on arabinoxylans (AX) deserve special attention because of the water-extractable arabinoxylans (WEAX) positive effects on glycemic and insulinemic responses and the arabyoxylan-oligosaccharides (AXOS) potential prebiotic properties. This study aims to improve bran nutritional properties through an innovative biotechnological process of fermentation involving sourdough in order to use the fermented bran as a functional ingredient. Wheat bran sourdoughs were produced through continuous propagation by back-slopping of ripe dough until a stable microbiota was established, reaching high counts of Lactic Acid Bacteria (LAB) and yeasts (10^9 and 10^7 CFU g^{-1} respectively). At each refreshment step, bacterial strains were isolated and identified by sequence analysis of partial 16S rRNA gene for LAB and D1/D2 domain of 26S rDNA for yeasts. Furthermore, the amounts of fiber, WEAX, free ferulic acid and phytic acid were measured. The amount of soluble fiber and WEAX significantly increased after sourdough, as did the level of free ferulic acid, probably due to microbial xylan-degrading activity, while the concentration of phytic acid decreased. Results suggest that fermented bran could be considered as an interesting functional ingredient for nutritional enhancement.

The 13th European Young Cereal Scientists and Technologists Workshop, Freising, Germany 13-16th May 2014 – (poster presentation)

Study of the nutritional changes and evolution of microbiota during sourdough like fermentation of wheat bran

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Several studies have emphasized the possibility to enhance nutritional properties of cereal by-products through biotechnological processes. Bran fermentation positively affects the bioavailability of several functional compounds and it could increase water-extractable arabinoxylans (WEAX), compounds with positive effects on glucose metabolism and prebiotic properties. This study was aimed to increase the amount of bran's bioactive compounds through sourdough like fermentation process. Wheat bran fermentations were conducted through continuous propagation by back-slopping of fermented bran (10% inoculum) until a stable microbiota was established, reaching high counts of lactic acid bacteria and yeasts (10^9 and 10^7 CFU g^{-1} respectively). After fermentation, levels of soluble fiber increased (+ 30%), WEAX and free ferulic acid were respectively fourfold and tenfold higher than in raw bran, results probably related to microbial xylan-degrading activity, while phytic acid was completely degraded. At each refreshment step, bacterial strains were isolated, clustered, molecularly analysed by Randomly Amplified Polymorphic DNA and identified at the species level by 16S rRNA gene sequencing. *Leuconostoc mesenteroides*, *Lactobacillus brevis*, *Lactobacillus curvatus*, *Lactobacillus sakei*, *Lactobacillus plantarum*, *Pediococcus pentosaceus* and *Pichia fermentans* were dominating the stable sourdough ecosystem. These strains were characterized by their bran fermentation capacity, antifungal activity, carbohydrate metabolism, exopolisaccharides production, as well as their antibiotic resistance profiles. These isolated were also tested for their potential xylan- and phytate-degrading activities. Moreover, common probiotic properties of the bacterial strains, such as acid tolerance, bile tolerance, anti-listeria activity and adhesion to the human intestinal epithelial cells Caco-2 cells were examined. These preliminary data suggest that fermented bran could be considered as an interesting functional ingredient for nutritional enhancement. Moreover, the characterization of the bacteria involved in sourdough like fermentation is the first step toward selecting starter cultures, according to

their functional aspects, in order to conduct “tailored” bran fermentation process and improve its nutritional properties.

XVII Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, University of Bologna, Cesena, 19-21th September, 2012 - (poster presentation).

XVIII Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Universities of Padova and Udine, Conegliano 25-27th September, 2013- (poster presentation).

XIX Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, University of Bari, Bari 23-26th September, 2014 - (oral presentation).

Publications

Study of the chemical changes and evolution of microbiota during sourdoughlike fermentation of wheat bran

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