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"Green preservatives" – combating fungi in the food industry by applying antifungal lactic acid bacteria

Thesis presented by

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For the degree of

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Under the supervision of

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Declaration

I hereby declare that this thesis is my own work and effort and that it has not been submitted anywhere for any award. Where other sources of information have been used, they have been acknowledged.

Signature:

Date: 05/07/2013

Abstract

Fungal spoilage is the most common type of microbial spoilage in food leading to significant economical and health problems throughout the world. Fermentation by lactic acid bacteria (LAB) is one of the oldest and most economical methods of producing and preserving food. Thus, LAB can be seen as an interesting tool in the development of novel bio-preservatives for food industry. The overall objective of this study was to demonstrate, that LAB can be used as a natural way to improve the shelf-life and safety of a wide range of food products. In the first part of the thesis, 116 LAB isolates were screened for their antifungal activity against four *Aspergillus* and *Penicillium* spp. commonly found in food. Approximately 83% of them showed antifungal activity, but only 1% showed a broad range antifungal activity against all tested fungi. The second approach was to apply LAB antifungal strains in production of food products with extended shelf-life.

L. reuteri R29 strain was identified as having strong antifungal activity *in vitro*, as well as in sourdough bread against *Aspergillus niger*, *Fusarium culmorum* and *Penicillium expansum*. The ability of the strain to produce bread of good quality was also determined using standard baking tests. Another strain, *L. amylovorus* DSM19280, was also identified as having strong antifungal activity *in vitro* and *in vivo*. The strain was used as an adjunct culture in a Cheddar cheese model system and demonstrated the inhibition of *P. expansum*. Significantly, its presence had no detectable negative impact on cheese quality as determined by analysis of moisture, salt, pH, and primary and secondary proteolysis. *L. brevis* PS1 a further strain identified during the screening as very antifungal, showed activity *in vitro* against common *Fusarium* spp. and was used in the production of a novel functional wort-based alcohol-free beverage. Challenge tests performed with *F. culmorum* confirmed the effectiveness of the antifungal strain *in vivo*. The shelf-life of the beverage was extended significantly when compared to not inoculated wort sample. A range of antifungal compounds were identified for the 4 LAB strains, namely *L. reuteri* ee1p, *L. reuteri* R29, *L. brevis* PS1 and *L. amylovorous* DSM20531. The identification of the compounds was based on liquid chromatography interfaced to the mass spectrometer and PDA detector.

Chapter 1

Introduction

1.1 Introduction

Fungal contaminations are a problem in a wide range of food products, leading to spoilage and causing economically important blights, root rots or wilts (Ma, 2010). The production of mycotoxins by fungi in food causes serious public health hazards, including cancerogenic (Bünger *et al.*, 2004), immunotoxic (Sharma, 1993), teratogenic (Pitt, 2000), neurotoxic (Leese-Haley, 2003), nephrotoxic (Fuchs and Peraica, 2005) and hepatotoxic (Lee *et al.*, 2010) effects as well as Kashin-Beck disease (Stone, 2009). In fact, mycotoxins have been recognized as one of the most hazardous contaminants of food and feed (CAST, 1989) and the most important chronic dietary risk factor, being ranked higher than synthetic contaminants, plant toxins, food additives or pesticide residues (Kuiper-Goodman, 1998). Thus, the challenge launched by the world-wide authorities (WHO, FAO, UPEC, etc.) and European Commission through the Directorate of Food Safety, is to track food materials from production through processing, storage and retail in order to respond adequately to the market changes, ensure high-quality food nutrition and high standard safety, and accomplish national and international policies related to food security (Gebbers and Adamchuk, 2010). Due to the recent legislations, the research of antifungal components from lactic acid bacteria (LAB) has generated great interests in the industry. The legislations force not only to reduce significantly the levels of mycotoxins in food products (European Comission, 1995, 1997, 1999, 2002, 2006, 2007), but also to reduce drastically the quantities of synthetic antifungal agents so much that these additives lose their effectiveness as preservatives (Lavermicocca *et al.*, 2000). Among the natural preservation agents, LAB has long been known to improve shelf-life of different fermented food products. Several compounds with antifungal activity have been isolated from LAB.

So far, the majority of identified antifungal metabolites are low-molecular weight compounds consisting of organic acids, diacetyl, ethanol, hydroxylated fatty acids, hydrogen peroxide, cyclic dipeptides, phenolic compounds, reuterin and fungicins (Lavermicocca, 2000, Magnusson and Schnürer, 2001, Ström *et al*., 2002, Lavermicocca *et al.*, 2003, Magnusson *et al.*, 2003, Dal Bello, *et al.*, 2007, Valerio *et al.*, 2009, Ryan *et al.*, 2011). In spite of the high-potential demonstrated by LAB, pilot-scale studies are still missing. Only a limited number of studies report a systematic characterization of LAB antifungal compounds under "*in vitro*" and "*in vivo*" conditions (Stierle *et al.*, 1988, Lee *et al.*, 2006, Broberg *et al.*, 2007, Dal Bello *et al.*, 2007, Ryan *et al.*, 2009a,b, Ryan *et al.*, 2011). Finally, neither the suitability of the antifungal strains as starters for fermentations nor the quality of the final products has always been evaluated to date.

This study aimed at: 1) characterizing the antifungal activity of over 100 strains of LAB, 2) selecting the most promising LAB and 3) implementing new methodologies and techniques in pilot-scale facilities for the production of natural preservatives expected to overcome some of the major modern challenges of the food industry. The main objective was to investigate if LAB can be used as a natural way to improve the shelf-life and safety of different food products. Methodologies and techniques developed in this project can help the food industries to reduce the mycotoxins content and extend the products shelf-life, leading to significant economical savings and increase of the competitiveness of the industry. Since LAB provide a natural way to increase the shelf-life, no chemical additives are needed. Therefore, this study contributes to assure the rights of the consumers to obtain safe food of high nutritional value. The compounds as well as the LAB characterized

during the study have the potential to be used also for other food products where fungal growth is a problem.

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Chapter 2

"Green Preservatives": Combating fungi in the food and feed industry by applying antifungal lactic acid bacteria – a review

2.1 Abstract

Fungal food spoilage plays a pivotal role in the deterioration of food and feed systems and some of fungi are also able to produce toxic compounds for humans and animals. The mycotoxins produced by fungi can cause serious health hazards, including cancerogenic, immunotoxic, teratogenic, neurotoxic, nephrotoxic and hepatotoxic effects and Kashin-Beck disease. In addition to this, fungal spoilage/pathogens are causing losses of marketable quality and hygiene of foodstuffs, resulting in major economic problem throughout the world. Nowadays, food spoilage can be prevented using physical and chemical methods, but no efficient strategy has been proposed so far to reduce the microbial growth, ensuring public health. Therefore, lactic acid bacteria (LAB) can play an important role as natural preservatives. The protection of food products using LAB is mainly due to the production of antifungal compounds such as carboxylic acids, fatty acids, ethanol, carbon dioxide, hydrogen peroxide and bacteriocins. In addition to this, LAB can also positively contribute to the flavour, texture and nutritional value of food products. This review mainly focuses on the use of LAB for food preservation given their extensive industrial application in a wide range of foods and feeds. The attention points out the several industrial patents concerning the use of antifungal LAB as bio-control agents against spoilage organisms in different fermented foods and feeds.

2.2 Introduction

During the past decade there has been an increasing interest in enhancing food quality and safety by replacing the traditional preservation systems with natural alternatives. Biopreservation is defined as the extension of shelf-life achieved by the

use of a natural or controlled microbiota and/or their antimicrobial compounds (Stiles, 1996). This type of preservation is environmentally friendly and represents an ecological approach to food preservation. One of the most popular methods of food biopreservation is fermentation, a process based on the growth of microorganisms in foods, whether natural or added (Ananou *et al.*, 2007). The use of fermentation processes has increased over centuries and now includes many different kinds of food and feed products (Ross *et al.*, 2002). Research performed by Stiles (1996) revealed that 25% of the European diet and 60% of the diet in many developing countries is composed of fermented foods. A major role in these fermentation processes is played by lactic acid bacteria (LAB). They can be found in products such as cheese, yoghurt, fermented meats and vegetables, acidophilus milk, kefir, buttermilk and bread (Caplice and Fitzgerald, 1999, Carr *et al.*, 2002, Hammes *et al.*, 1990).

This trend for natural label friendly preservatives has drastically increased over the last number of years. LAB can play very important role as such preservatives since they have a long history in food production and are generally recognised as safe (GRAS status). Additionally, due to certain specific metabolic properties, LAB are of special interest in food industry, since they can improve flavour, texture and nutritional value of the final food products (Arendt *et al.*, 2007, Guldfeldt *et al.*, 2001, McKay and Baldwin, 1990). In addition to this, they prevent food spoilage and extend the shelf-life of fermented foods (Dal Bello *et al.*, 2007, Sathe *et al.*, 2007, Muhialdin *et al.*, 2011, Ryan *et al.*, 2011), due to production of antimicrobial compounds (Messens and De Vuyst, 2002, Magnusson *et al.*, 2003). One of the main problem associated with food spoilage are fungi. Recent reports revealed that LAB are capable of producing antifungal compounds. Examples of

such compounds are organic acids – lactic acid, acetic acid, formic acid, phenyllactic acid (Corsetti *et al.*, 1998, Gerez *et al.*, 2009, Lavermicocca *et al.*, 2000, Ryan *et al.*, 2009a, Ström *et al.*, 2002), phenolic compounds (Mandal *et al.*, 2007), hydroxy-fatty acids (Sjörgen *et al.*, 2003), esters (Wang *et al.*, 2012), hydrogen peroxide (Venturini *et al.*, 2002), reuterin (Chung *et al.*, 1989, Magnusson, 2003), carbon dioxide (Amanatidou *et al.*, 1999) and proteinaceous compounds (Falguni *et al.*, 2010, Magnusson and Schnürer, 2001, Ryan *et al.*, 2009b, Ryan *et al.*, 2011, Ström *et al.*, 2002, Ström *et al.*, 2005).

Mould spoilage is of enormous economical importance, since it results in huge losses of crop and food stuff worldwide. Fungi are naturally present and can contaminate food crop under favourable conditions of temperature, relative humidity, pH, nutrient availability and oxygen. Besides the loss of crop and foodstuffs, the production of mycotoxins represents a remarkable problem, since it has been associated with a wide range of negative effects for the humans health. In particular, species belonging to the genera of *Aspergillus*, *Fusarium* and *Penicillium* have been associated with the production of aflatoxins, fumonisins, ochratoxins, patulin, trichothecenes and zearalenone (Bhat *et al.*, 2010).

Aflatoxins are naturally occurring, highly toxic compounds that are produced as secondary metabolites by *Aspergillus flavus* and *A. parasiticus* (Bhat *et al.*, 2010). Under favourable temperature and humidity conditions these fungi grow on certain foodstuffs, most commonly groundnuts, dried fruit, tree nuts (almonds, pecans, walnuts, pistachios, Brazil nuts), spices (chillies, blach pepper, coriander, ginger) and a range of cereals (especially maize). Production of aflatoxins is optimal at relatively high temperatures, so contamination is most acute and widespread in warm, humid climates. Although contamination is generally considered to be a

problem in tropical and subtropical regions of Africa, Asia and Latin America (Galvano *et al.*, 1996, Abdularazzaq *et al.*, 2003, Keskin *et al.*, 2009), aflatoxins have also been found in temperate countries of Europe (Markaki and Melissari, 1997, Martin and Martin, 2000). The name aflatoxins was created around 1960 after the discovery that the source of turkey "X" disease in the UK, which resulted in the death of 100000 turkeys and other farm animals, were **A**spergillus **fla**vus **toxins** (Wannop, 1961).

There are at least 18 different types of aflatoxin are produced in nature, however the major ones include aflatoxin B_1, B_2, G_1, G_2 M_1 and M_2 (Bhat *et al.*, 2010). Aflatoxin B_1 is considered the most toxic and is produced by both *A. flavus* and *A. parasiticus*. Aflatoxins G_1 and G_2 are produced exclusively by *A. parasiticus*. Aflatoxins M_1, M_2 were originally discovered in the milk of cows that fed on moldy grain. These compounds are products of a conversion process in the animal's liver.

Fumonisins are a group of mycotoxins produced primarily by *Fusarium verticillioides* and *F. proliferatum*, although a few other *Fusarium* species also may produce them. There are at least 28 different forms of fumonisins, most designated as A-series, B-series, C-series, and P-series. Fumonisin B_1 is the most common and economically important form, followed by B_2 and B_3 (WHO, 2002). Maize is the most commonly contaminated crop, and fumonisins are the most common mycotoxins in maize, although these toxins can occur in a few other crops as well: figs, herbal tea, medicinal plants (Omurtag and Yazicioglu, 2004, Karbancioglu-Guler and Heperkan, 2009, Pietri *et al.*, 2009, Seo *et al.*, 2009). *F. verticillioides* and fumonisins are distributed worldwide (Gelderblom *et al.*, 1992, Castelo *et al.*, 1998).

Ochratoxins are mycotoxins produced by several species of *Aspergillus* and *Penicillium*, especially *P. verrucosum* and *P. carbonarius*. Ochratoxin A is the most

economically important form of ochratoxin, while ochratoxins B and C are less toxic and less common. Ochratoxin A is known to occur in commodities such as cereals, coffee, dried fruit, and red wine. *P. verrucosum* is the leading cause of ochratoxin contamination of cereal grains in temperate climates. Grapes, raisins, and even wines may become contaminated with ochratoxins produced by *A. carbonarius*, the principal causal agent of grape black mold (Zimmerli and Dick, 1996, Battilani *et al.*, 2006). A number of *Aspergillus* spp., including *A. ochraceus*, *A. carbonarius* and *A. niger* may cause ochratoxin contamination in green and processed coffee (Battista *et al.*, 2009). Tree nuts and figs may be infested with *A. ochraceus* and *A. melleus*, the leading producers of ochratoxins in these commodities (Molyneux *et al.*, 2007).

Patulin is a mycotoxin produced by a variety of molds, in particular, *Aspergillus*, *Penicillium* and *Byssochlamys* (Dutton *et al.*, 1984, Fuchs *et al.*, 2008, Moss, 2008). Most commonly it is found in fruits, particularly apples, pears and their products (Frisvad and Thrane, 1996, Kabak *et al.*, 2006, Murillo-Arbizu *et al.*, 2009).

The trichothecenes are the largest group of mycotoxins known to date, consisting of more than 160 chemically-related toxic compounds and are classified into 4 groups depending on their chemical structure. The major ones are T-2 and HT-2 toxins (group A) and nivalenol (NIV) (group B) (Bhat *et al.*, 2010). These mycotoxins are produced by several species of *Fusarium*, *Myrothecium*, *Stachybotrys*, *Trichoderma* and *Trichothecium* (IPCS, 1990). Trichothecene contamination is economically important in cereals (wheat, barley, oats, and maize) and their derivatives (Foround and Eudes, 2009).

Zearalenone (ZEN) is a mycotoxin that mimics the reproductive hormone estrogen. This mycotoxin is produced by *Fusarium* species, primarily by the fungus

F. graminearum. Zearalenone is found worldwide in a number of cereal crops, such as maize, barley, oats, wheat, rice, and sorghum and also in bread (Kuiper-Goodman *et al.*, 1987, Tanaka *et al.*, 1988).Table 2.1 gives an overview of the main mycotoxins found in food and feed, their source, commodities affected and pathologies caused by them.

Table 2.1 *Mycotoxins commonly found in food and feed*[a]

Mycotoxin	Mycotoxinogenic fungus	Food/feed contaminated	Effect on health
Aflatoxins	*Aspergillus flavus* *Aspergillus parasiticus*	cereals, maize, nuts milk and milk products eggs	carcinogenic mutagenic teratogenic immunosuppressive kwashiorkor
Fumonisins	*Fusarium* spp.	cereals, maize figs herbal tea bovine milk silage	apoptosis cancerogenic hepatotoxic nephrotoxic immunosuppressive Kashin-Beck disease[b] leukoencephalomacia[c] pulmonary edema[d] hydrothorax[d] neural tube defects
Ochratoxin A	*Aspergillus* spp. *Penicillium verrucosum*	cereals, maize, coffee, cocoa grapes, wine herbs pork	cancerogenic nephrotoxic immunosuppressive teratogenic
Patulin	*Aspergillus* spp. *Byssochlamys* spp. *Paecilomyces* spp. *Penicillium expansum*	fruits silage	apoptosis cancerogenic genotoxic immunosuppressive oxidative stress response
Trichothecenes	*Fusarium* spp. *Myrothecium* spp. *Stachybotrys* spp. *Trichoderma* spp. *Trichotecium* spp.	cereals	immunosuppressive
Zearalenone	*Fusarium* spp.	cereals	hyperestrogenic

[a]Based on Bhat *et al.*, 2010, except where indicated otherwise.

[b]Stone, R. A., 2009. Medical Mystery in Middle China. *Science* 324, 1378-1381.

[c]Bucci, T., Hansen, D. K., La Borde, J. B., 1996. Leucoencephalomacia and hemmorhage in the brain of rabbits gavaged with mycotoxin fumonisin B_1. *Natural Toxins* 4, 51-52.

[d]Harrison, L. R., Colvin, B. M., Greene, J. T., Newman, L. E., Cole, J. R. Jr., 1990. Pulmonary edema and hydrothorax in swine produced by fumonisin B_1 a toxic metabolite of *Fusarium moniliforme*. *Journal of Veterinary Diagnostic Investigation* 2, 217-221.

As seen from the Table 2.1, mycotoxigenic moulds can cause a wide range of negative effects on human health, which include carcinogenic, immunotoxic, teratogenic, neurotoxic, nephrotoxic, hepatotoxic, and uterotropic. The presence of mycotoxins in foods is therefore potentially dangerous for humans and animals and thus constitutes a serious problem (Dalié *at al.*, 2010).

The use of LAB to control moulds is an interesting alternative approach to physical and chemical preservation methods normally applied in food. Careful selection of specific strains of lactic acid bacteria with antifungal properties can allow the reduction of moulds and yeast genera and can, therefore, improve the shelf-life of many fermented products and reduce the presence of mycotoxins.

In recent years, a range of different reviews on the antifungal activity of LAB have been published (Batish *et al.* 1997, Dalíe *et al.*, 2010, Gourama and Bullerman, 1995, Lowe and Arendt, 2004, Schnürer and Magnusson, 2005). The focus of these reviews has been primarily on the classification and chemical properties of antifungal compounds, their nature, and the factors affecting their production. This review will focus on the application of antifungal LAB as potential food biopreservatives and give an overview on their economical potential, by providing a summary of patents published in this area.

2.3 Food quality and safety

Contamination of food and agricultural commodities by various types of toxigenic moulds is a serious problem which is difficult to eliminate (Bhat *et al.*, 2010). Mycotoxigenic fungi are very common and can grow on a wide range of substrates under a wide range of conditions. For agricultural commodities, the crop contaminations vary from year to year based on weather and other environmental conditions (Bennet and Klich, 2003). Fungal toxins, being natural contaminants, are often unavoidable. They can enter the food chain throughout the entire food production system – in the field, during shipping, handling, storing and they occur in different food products all over the world. Moreover, mycotoxins have been recognized as one of the most hazardous contaminants of food and feed (CAST, 1989). According to Kuiper-Goodman (1998), they are the most important chronic dietary risk factors, higher than synthetic contaminants, plant toxins, food additives or pesticide residues. Indeed, cancer, kidney toxicity, immune suppression are well recognised to be connected to chronic mycotoxicoses (Bünger *et al.*, 2004, Fuchs and Peraica, 2005, Leese-Haley, 2003, Pitt, 2000, Sharma, 1993).

Food quality and safety are topics which are widely and intensively studied by the scientific community. The ability to track food materials from production through processing and storage and to respond to changing market conditions, ensure the success of the food safety monitoring system. The development of national and international policies related to food security is the challenge launched by several world-wide organizations (Council for Agricultural Science and Technology, Food and Agriculture Organization, World Health Organization, European Food Safety Authority, etc.). The problem of food contaminants has been recognized by these

organizations, as well as by the European Union, which implemented new legislations regarding maximum levels of mycotoxins in crops and foodstuffs (EU Commission Regulation, 2003, EU Commission Regulation, 2006, EU Commission Regulation, 2007, EU Commission Regulation 2008, EU Commission Regulation, 2010a, EU Commission Regulation, 2010b). Guidelines for the limits of mycotoxin content in food are based on Joint FAO/WHO Expert Committee on Food Additives data on tolerable daily intake of mycotoxins (JECFA, 2000, JECFA 2001).

FAO estimated that one quarter of the world's food crops, overall, is affected to some extent by mycotoxins (FAO, 1995). Considering that these food crops include cereals, nuts, fruits and vegetables, which comprise a significant part of the European consumer's diet, there is potential for significant exposure to mycotoxins. Exposure of consumers to mycotoxins is mainly via plant foods. However, an additional potential exposure may be throuh the foods of animal origin such as milk, cheese and meat. Animals consuming mycotoxin-contaminated feeds can produce meat, milk and eggs that contain toxic residues and biotransformation products (Bennet and Klich, 2003). This demonstrates the need to control levels of mycotoxins in animal feed as well as food. In fact, the established regulations set the limits of mycotoxins concentration in animal feed at very low levels (Miller *et al.*, 1996) in order to directly avoid the negative impact on animal health and their productivity, and indirectly to ensure that the health of the consumers is not affected by consuming contaminated products.

The economic implications of mycotoxin contamination are profound with estimates extending to billions of dollars (Trail *et al.*, 1995). However, the exact figures revealing the world economic consequences may never be available. Apart from the obvious losses of food and feed, there are losses caused by lower

productivity, losses of valuable foreign exchange earnings, costs incurred by inspections, sampling and analyses before and after shipments, losses attributable to compensation paid in case of claims, farmer subsidies to cover production losses, research, training and extension program costs, cost of detoxification, etc. When combined, these costs are certainly extremely high.

For all the above reasons, any decrease in fungal growth is of great interest. Several preventive measures to minimize mycotoxin contamination in agricultural commodities have been attempted. These can be divided into three broad categories: plant breeding, good agronomic practices and detoxification. Although some of them are effective (cultivating varieties resistant to diseases), others, i.e., appropriate drying techniques, maintaining proper storage facilities, taking care not to exposure the grains to moisture during transport and marketing, can be hard to implement. Furthermore, detoxification can be considered suitable only for animal feed purposes and not for human consumption.

Fungal spoilage in food could be solved by different physical methods and by adding chemical preservatives. The physical treatments, i.e., drying, freezing, freeze-drying, packaging under modified atmosphere, ultraviolet, microwave and infrared radiation usually require expensive multi-faceted approaches that limit their applicability.

The most common classical, chemical preservative agents are the weak organic acids. Use of fungal inhibitors, such as, propionic, acetic, lactic, benzoic and sorbic acids, alter the pH equilibrium of microorganisms causing the inhibition of the microbial growth (Wagner and Moberg, 1989). Propionic acid and its salts are commonly used to extend shelf-life of bakery products. However, according to

Lavermicocca *et al.* (2000), the use of calcium proprionate at the maximum level allowed by European Commission (EU Parliament and Council Directive, 1995) is not very effective. In the case of ethanol addition, its use at levels between 0.5 and 3.5% of loaf weight means an important extension of the shelf-life of bread, but addition higher than 1%, as some sensory tests have indicated, might be unacceptable to the consumers (Legan, 1993). Moreover, it has been proven that many food and feed spoilage moulds are becoming resistant to preservatives like sorbic and benzoic acids, as well as chemical cleaning compounds (Schnürer and Magnusson, 2005).

Historically, chemical preservatives have been used as additional barriers to limit the number of microorganisms capable of growing within a food. However, consumer acceptance of the classical preservation methods is diminishing and this attitude has led researchers to examine possibility of using natural inhibitors from plant and microbial sources. The reasons for this are also to be found partly in the undesirable allergenic potential of many additives. Furthermore, it can not be excluded that the preservatives are metabolized in the foodstuff or *in vivo* to produce toxic substances (Classen *et al.*, 1987).

2.4 LAB as biopreservatives

Lactic acid bacteria is the term used for a range of genera, namely *Lactobacillus*, *Pediococcus*, *Lactococcus*, *Streptococcus*, *Enterococcus*, *Leuconostoc*, *Oenococcus*, *Aerococcus*, *Carnobacterium*, *Tetragenococcus*, *Vagococcus* and *Weisella*. The group is characterized as Gram positive, catalase negative, non-motile, non-respiring and non-spore forming cocci and rods, which produce lactic acid as the major end-product of the fermentation of carbohydrate.

Members that carry out a heterofermentation also produce carbon dioxide, acetic acid or ethanol (Adams, 1999). Their growth results in a reduction of pH, competition for nutrients and possible production of other antagonistic compounds, such as phenolic compounds, hydroxy-fatty acids, hydrogen peroxide, peptide-antibiotics (bacteriocins, e.g., nisin), antibiotics (e.g., reutericyclin) and small antibiotic-like molecules (e.g., reuterin). These all contribute to the preservative effect of LAB (Magnusson *et al.*, 2003). However, the application of these compounds may be limited in some instances to their narrow inhibitory spectrum and their instability under certain conditions. It is noteworthy that the mechanism of antimicrobial action sometimes cannot be satisfactorily explained due to the complex interactive and/or synergistic activity of different compounds (Corsetti *et al.*, 1998, Niku-Paavola *et al.*, 1999).

In general, LAB are considered as safe (Bernardeau *et al.*, 2008), having QPS (Qualified Presumption of Safety - EU) and GRAS (Generally Recognized as Safe - US) status and play an essential role in food preservation. Given that, a broad range of LAB species and strains are employed as starter cultures in the manufacture of dairy, meat and plant-derived products (Leroy and De Vuyst, 2004). This is recognized as improving their quality, bringing nutritional benefits and in some instances therapeutic advantages (El-Nezami *et al.*, 2002, Masood *et al.*, 2011).

Although the prevention of mycotoxin contamination in the field has been the main goal of agriculture and food industries, once the crop becomes infected under field conditions, methods during food processing need to be developed to reduce fungal growth. Therefore, considering present legislations and customers' attitude, the ideal decontamination procedure should be easy to use, inexpensive and should not lead to formation of compounds that may be toxic, nor alter the nutritional and/or

palatability of the product. The need for biopreservation in this context has renewed the interest in the search for food-compatible antimicrobials produced by different microorganisms. As a result, the interest in antifungal LAB has increased in recent years as potential replacements for synthetic antifungal agents. However, while many studies have assessed antibacterial effects of LAB (Castellano *et al.*, 2008, De Vuyst and Leroy, 2007, Dodd and Gasson, 1994, Settanni and Corsetti, 2008), there are relatively few reports explaining specific antifungal activity of LAB and their application.

The following section discusses the application of antifungal LAB in different food and feed systems. It also includes a review of commercially available patented *Lactobacillus* strains, their source, activity spectrum, mode of action and compounds responsible for antimycotic activity, which is summarized in Table 2.2.

Table 2.2 *Lactic acid bacteria with the ability to inhibit mould growth*

LAB isolate	Collection no.[a]	Source of isolation	Activity spectrum	Antifungal compound(s)	Mode of action	Patent number
			Genus *Lactobacillus*			
L. amylovorus FST 2.11	DSM 19280	sourdough	*Aspergillus niger* *Fusarium culmorum* *Penicillium expansum* *Penicillium roqueforti*	cytydine deoxycitidine cyclo(Pro-Pro) cyclo(Leu-Pro) cyclo(Tyr-Pro) cyclo(Met-Pro) cyclo(His-Pro)	bread	EP 2009/056229[b] US 2011/0200569[c]
L. casei ssp. *paracasei*	CNCM I-1518	unknown	*Mucor circinelloides* *Mucor plumbeus* *Penicillium brevicompactum* *Penicillium expansum* *Penicillium roqueforti*	unknown	milk	US 2011/0045134 A1[d]

Table 2.2 *continued*

LAB isolate		Collection no.[a]	Source of isolation	Activity spectrum	Antifungal compound(s)	Mode of action	Patent number
L. casei ssp. *rhamnosus* LC-705[e]		DSM 7061	unknown	*Fusarium* spp.	unknown	bread silage	EP 0576780 A2[f] US 5378458[g]
L. casei ssp. *rhamnosus*		NRRL-B-15792	unknown	*Penicillium oxalicum*	unknown	corn silage cottage cheese	EP 0221499 A2[h] US 4956177[i] US 5965414[j] US 6277374 B1[k]
L. delbrueckii		FERM BP-10663 (FERM P-19705)	unknown	*Penicillium olsonii*	unknown	silage	EP 1726 638 A1[l] US 2008/0286406 A1[m] US 8029779 B2[n]
L. plantarum		ATCC 53187	corn silage	unspecified	unknown	silage	US 4842871[o]
L. paracasei ssp. *paracasei*[p]	SM20	DSM 14514	raw milk	*Candida magnoliae* *Candida parapsilosis* *Candida pulcherrima* *Zygosaccharomyces bailii*	unknown	cheese yoghurt	EP 1308506 A1[q] US 2005/0095318 A1[r] US 7927639 B2[s]
	SM29	DSM 14515	salami				
	SM63	DSM 14516	raw milk				

Table 2.2 *continued*

LAB isolate	Collection no.[a]	Source of isolation	Activity spectrum	Antifungal compound(s)	Mode of action	Patent number
L. sanfranciscencis	FERM P-18244 --- FERM P-18245 --- FERM P-18246	unknown	*Aspergillus aniger* *Aspergillus oryzae* *Mucor hiemalis* *Penicillium citrium* *Penicillium funiculosum* *Rhizopus oryzae*	proteinaceous	bread	US 2003/0035860 A1[t] US 6827952 B2[u]
			Genus *Pediococcus*			
P. acidilactici	ATTC 25742	unknown	*Penicillium oxalicum*	proteinaceous	cheese cottage cheese margarine pepperoni salami spaghetti sauce yogurt	US 4877615[v] US 4906611[w] US 4942032[x]

[a]Collection number given by the depositary institution:

ATTC – American Type Culture Collection, Rockville, USA

FERM – International Patent Organism Depository, Ibaraki, Japan

DSM – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany

NCIMB – National Collection of Industrial and Marine Bacteria Ltd, Aberdeen, UK

NRRL – Northern Regional Research Laboratory, Peoria, USA

[b]Arendt *et al.*, 2009.

[c]Arendt *et al.*, 2011.

[d]Perrier, L., Loysance-Paroux, C., Tirilly, Y., Fuhrmann, B. (2011). Use of *Lactobacillus casei* ssp. *paracasei* as antifungal agent. United States Patent Application US 2011/0045134 A1.

[e]in combination with *Propionibacterium shermanii* JS (DSM 7067)

[f]Mäyrä-Mäkinen *et al*., 1994.

[g]Mäyrä-Mäkinen and Soumalainen, 1995.

[h]King, S. W., Fowler, G. G., Vandenbergh, P. A. (1987). Method for inhibiting fungi. European Patent Application EP 0221499 A2.

[i]King, S. W., Fowler, G. G., Vandenbergh, P. A. (1990). Method for inhibiting fungi. United States Patent Application US 4956177.

[j]Vandenbergh, P. A., King, S. W. (1999). Process for producing yeast and mold inhibiting products by *Lactobacillus*. Unites States Patent Application US 5695414.

[k]Vandenbergh, P. A., King, S. W. (2001). Process for producing by culturing *Lactobacillus casei* var. *rhamnosus* yeast and mold inhibiting products. Unites States Patent Application US 6277374 B1.

[l]Miyamoto and Naito, 2006.

[m]Miyamoto and Naito, 2008.

[n]Miyamoto and Naito, 2011.

[o]Hill, J. E. (1989). Method and inoculant for preserving agricultural products for animal feed. United States Patent Application US 4842871.

[p]in combination with *Propionibacterium jensenii* SM11 (DSM 14513)

[q]Miescher Schwenninger and Meile, 2003.

[r]Miescher Schwenninger and Meile, 2005.

[s]Miescher Schwenninger and Meile, 2011.

[t]Ando, M., Yoshiaki, S., Natsuko, N. (2003). Lactic acid bacteria, fermented seasoning liquid containing the same, and a method for producing bread. United States Patent Application US 2003/0035860 A1.

[u]Ando, M., Yoshiaki, S., Natsuko, N. (2004). Lactic acid bacteria, fermented seasoning liquid containing the same, and a method for producing bread. United States Patent Application US 6827952 B2.

[v]Vandenbergh and Kunka, 1989.

[w]Vandenbergh and Kunka, 1990a.

[x]Vandenbergh and Kunka, 1990b

2.4.1 Application of antifungal LAB in bread

Bread is the most important staple food in the western world and it is generally viewed as a perishable commodity, which is best consumed when 'fresh' (Dal Bello *et al.,* 2007). The loss of freshness is caused by a number of factors (Pateras, 1998) and ,among these, fungal spoilage is the most common and economically important. It has been estimated that fungal spoilage costs the bakery industry more than 200 million euro per year in Western Europe (Legan, 1993). A wide range of moulds have been found in bakery products, examples are *Aspergillus*, *Cladosporium*, *Endomyces*, *Fusarium*, *Monila*, *Mucor*, *Penicillium* and *Rhizopus* (Coda *et al.*, 2008).

The addition of LAB to bread in the form of sourdough has a long history (Hammes and Gänzle, 1998) and is known to have a positive effect on a final product quality, bread volume, crumb structure, shelf-life (Arendt *et al.*, 2007, Corsetti *et al.*, 2000, Clarke *et al.*, 2002, Crowley *et al.*, 2002), flavour (Thiele *et al*., 2002), nutritional value (Liljeberg and Björck, 1994, Liljeberg *et al.*, 1995). With regard to shelf-life specifically, Dal Bello *et al.*, (2007) showed the ability of the strain *Lactobacillus plantarum* FST 1.7 to inhibit bread spoilers such as *F. culmorum* and *F. graminearum.* Analysis of the antifungal components identified lactic acid, phenyllactic acid and the two cyclic dipeptides cyclo (L-Leu-L-Pro) and cyclo (L-Phe-L-Pro) as the main active compounds produced by the strain. Lavermicocca *et al.* (2000) reported the antifungal activity of *L. plantarum* 21B strain against *A. niger* in sourdough and in the resulting breads. In this case, phenyllactic acid was shown to be one of the major compounds occurring in the culture along with lactic acid and acetic acid. This study also revealed that, in some

cases, LAB show even higher preservation efficacy than some chemical preservatives. The use of calcium proprionate at the maximum permitted level of 0.3% of flour weight (EU Parliament and Council Directive, 1995) did not assure preservation. This was borne out by the fact that fungal growth still occurred for several moulds on a conidial germination assay with mould-containing bakery products. A similar effect was observed by Ryan *et al.* (2008), who studied the use of sourdough fermented by the antifungal *L. plantarum* FST 1.7 and *L. plantarum* FST 1.9 strains to reduce amount of calcium propionate in bread against *A. niger*, *F. culmorum*, *P. expansum* and *P. roqueforti*. In this study, a strong synergistic effect was observed when calcium proprionate and antifungal sourdoughs were combined in the bread formulation. The use of reduced calcium proprionate (at 0.1%) only showed significant fungal inhibition when sourdough generated with the antifungal LAB strains was used. Remarkably, the increase in shelf-life achieved by addition of sourdough at 20% was higher than that obtained when only 0.3% calcium proprionate. In addition, Ryan *et al.* (2011) and Arendt *et al.* (2009, 2011) evaluated the antifungal strain *L. amylovorous* DSM 19280 for its ability to delay the growth of *A. niger*, *F. culmorum*, *P. expansum* and *P. roqueforti*, and fungal flora from bakery environment. It was found, that this strain delayed the outgrowth of both indicator and environmental fungi. The breads containing the sourdough fermented with the antifungal *L. amylovorous* DSM 19280 led to a shelf-life extension of 14 days, which a significantly higher than that observed in bread without sourdough and in bread containing 0.3% calcium propionate. The substances responsible for the antifungal activity were identified as cytydine, deoxycytidine, phenyllactic acid, hydroxyphenyllactic acid and five cyclic dipeptides. Moreover, the rheofermentometer and baking tests revealed that addition of sourdough fermented

by *L. amylovorous* DSM 19280 significantly improved bread quality, indicating, therefore, that the strain is a suitable starter culture for wheat sourdough and bread production. Recently, Muhialdin *et al.* (2011) investigated antifungal activity of *Lactobacillus fermentum* Te007, *L. pentosus* G004, *L. paracasei* D5 and *Pediococcus pentosaceus* Te010 against *A. niger* and *A. oryzae* on selected foods and at different incubation temperatures. The studies performed on bread at 30 °C revealed the ability of the strains to inhibit fungal conidia germination of *A. niger* for 21 days − *L. fermentum* Te007, 16 days − *P. pentosaceus* and 12 days *L. pentosus* G004. Fungal growth of *A. niger* and *A. oryzae* was inhibited longer than 27 days at 4 °C. The control (commercial bread with permissible chemical preservatives) only could prevent fungal growth for 4 days at 30 °C and 11 days at 4 °C. The characterization of the antifungal components showed that isolates *L. fermentum* and *P. pentosaceus* possibly produce protein-like compounds, as indicated by the loss of antifungal activity after proteinase K, DNase and RNase treatment.

These studies clearly show that the addition of antifungal LAB, using sourdough technology, can be successfully used by to reduce and/or exclude chemical preservation in the bakery industry, while still ensuring microbiological safety and high quality in the bread.

2.4.2 Application of antifungal LAB in dairy products

Although certain moulds play an important role in cheese production, dairy products are an excellent substrate for the growth of fungi. The most frequent isolated fungi from cheese include *Alternaria*, *Aspergillus*, *Cladosporium*, *Eurotium*, *Fusarium*, *Mucor*, *Penicillium* and *Phoma* (Basílico *et al.*, 2001, Taniwaki *et al.*, 2001). LAB are commonly used as starter cultures in the production

of fermented dairy products. They offer organoleptic, technological, nutritional and health advantages to the food and also contribute to their safety (Leroy and De Vuyst, 2007). Vandenbergh and Kanka (1989), Vandenbergh and Kanka (1990a), Vandenbergh and Kanka (1990b) patented the use of an antifungal peptide produced by a *Pediococcus acidilactici* strain (Table 2.2). The molecule had a molecular weight of between 400 and 500 Da, was heat stable, and was sensitive to treatment with protease. It showed activity against *Penicillium oxallicum* in cheese, cottage cheese, margarine and yogurt. According to Mäyra-Mäkinen *et al.*, (1994), Mäyra-Mäkinen and Suomalainen (1995), Suomalainen and Mäyra-Mäkinen (1999), *L. casei* ssp. *rhamnosus* DSM 7061 showed inhibitory effect against *Fusarium* spp. in fermented milk products. These studies were further supported by investigations of Miescher Schwenninger and Meile (2003, 2004, 2005, 2011) concentrated on the antifungal activity of *L. paracasei* subs. *paracasei* DSM 14514, DSM 14515, DSM 14516 against fungi such as *Candida pulcherrima*, *C. magnoliae*, *C. parapsilosi*, *Zygosaccharomyces bailii* in yoghurt and cheese. Both patents were used by Danisco for the development of HOLDBACK™ Protective Culture. The antifungal bacteria used in this product have a specific inhibitory effect on yeasts and moulds and are designed for use in fresh fermented dairy products such as yoghurt, sour cream, sour milk and cheese. Lately, Muhialdin *et al.* (2011) studied *A. niger* and *A. oryzae* growth inhibition by *Lactobacillus fermentum* Te007, *L. pentosus* G004 and *L. paracasei* D5 and *Pediococcus pentosaceus* Te010 on commercial processed cheese slices. They observed good growth of fungi and conidia production on control cheese without antifungal LAB addition (shelf-life of 4 to 6 days at 20 and 30 °C). However, fungal growth was delayed 6 to 9 days at 20 and 30 °C, compared to 19 to 29 days at 4 °C for all the LAB evaluated.

2.4.3 Application of antifungal LAB in fresh fruits and vegetables

Fruits and vegetables are also affected by mould spoilage (Sathe *et al.*, 2007). This leads to enormous loss of these products. The most commonly isolated yeast and fungi from the product belong to the following genus: *Alternaria*, *Aspergillus*, *Botrytis*, *Candida*, *Cladosporium*, *Colletotrichum*, *Fusarium*, *Penicillium*, *Phoma*, *Phomosis*, *Phytophtora*, *Pythium*, *Rhizopus*, *Rhizoctonia*, *Rhodotorula*, *Sclerotinia* and *Zygosaccharomyces* (Tournas, 2005, Tournas *et al.*, 2006).

Rouse *et al.* (2007) studied the effect of antifungal culture *Pediococcus pentosaceus* against the moulds responsible for apple rot. Model studies performed with apples revealed the effectiveness of these strain against *Penicillium expansum*. Partial characterization of the antifungal compounds revealed that their activity is likely due to antifungal peptides. A similar study performed by Sathe *et al.* (2007) showed the antifungal activity of *L. plantarum* CUK-501 against *A. flavus*, *F. graminearum*, *R. stolonifer* and *B. cinerea*. In this study cucumbers were used as a model system. Both, the cell-free supernatant as well as living cells of this *Lactobacillus* strain were effective and resulted in a significant delay in vegetable spoilage against all tested fungi. Subsequently, before mentioned, Muhialdin *et al.* (2011) reported the antifungal activity of *Lactobacillus fermentum* Te007, *L. pentosus* G004 and *L. paracasei* D5 and *Pediococcus pentosaceus* Te010 in tomato puree. Fungal growth of *A. niger* and *A. oryzae* in commercial tomato puree was observed within 2 days of incubation 20 and 30 °C, and 13 to 18 days at 4 °C. Addition of the LAB supernatants to the sample increased the shelf-life to 5 to 7 days at 20 and 30 °C, and 23 to 40 days at 4 °C. Therefore, all the tested strains can

be seen as possible biopreservation agents for fresh or processed fruits and vegetables.

2.4.4 Application of antifungal LAB in cereal- based beverages

There are several types of cereal-based fermented drinks produced around the world and they can be classified according to the raw materials used or the type of fermentation involved in the manufacturing process. Alcoholic fermented beverages can be classified into wines and beers, while the great majority of non-alcoholic fermentations are souring, mainly lactic acid fermentations (Blandino *et al.*, 2003).

Many non-alcoholic, LAB fermented cereal- and pseudocereal-based beverages represent an evolving generation of functional drinks and commercially available examples include: Grainfiels Wholegrain Liquid® (Australia), Proviva® (Sweden, contains fermented cereal ingredients), Yosa® Smoothies (Bioferme Oy, Finland – contains fermented oats and fruit), and Velle® (Russia – contains fermented oats and fruits). A suitable LAB starter culture is one which dominates in cereal environments, should be capable of biopreservation (Ryan *et al.*, 2008) as well as preventing fungal growth, and thus potentially eliminates the potential for mycotoxin production (Dalié *et al.*, 2010).

LAB have also been extensively used in the malting and brewing industry. An example of applying LAB in malting is the development of LAB starter cultures for use as inoculants during the malting process in order to improve the quality of the malt. Beneficial effects due to LAB addition included increased malt yields, lower viscosity and β-glucan content of wort and improvement of mash filtration and wort filterability were observed by Haikara and Laitila (1995), Laitila *et al.* (2006), Lowe

et al. (2005), Mauch *et al.* (2011). Moreover, the use of antifungal starter cultures in the early stage of malting can inhibit the growth of undesirable moulds and the production of mycotoxins. Studies of Boivin and Malanda (1997) demonstrated how the use of starter cultures could improve the safety of the malt when applied to the steeping water during malting. The investigations of Lowe *et al.* (2004) showed that *Fusarium* spores in the presence of LAB did not negatively affect the quality of malt and wort. In a different study, Haikira *et al.* (1993) employed *Lactobacillus plantarum* and *Pediococcus pentosaceus* as natural preservatives during malting to inhibit the growth of *Fusarium* moulds to prevet gushing. In fact, gushing defined as the quick, uncontrolled, spontaneous over-foaming when opening the bottle or can and is one of the most negative consequences of mould contamination in regard to the quality of malt and beer (Amaha and Kitabatake, 1981). *Fusarium culmorum*, *F. graminearum*, *F. poae* have been proven to be the most active gushing inducers (Haikara and Home, 1991, Munar and Sebree, 1997, Niessen *et al.*, 1992, Schwarz *et al.*, 1996). In addition to this, production of *Fusarium* toxins during malting and their passage into beer have been demonstrated (Schwarz *et al.*, 1995).

2.4.5 Application of antifungal LAB in feed

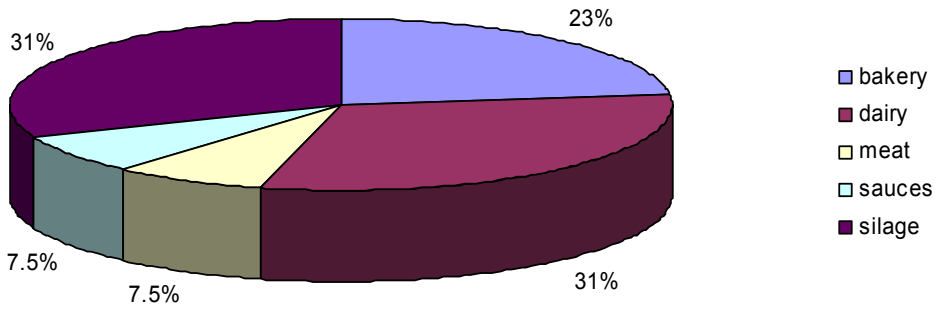
As mentioned previously, fungal spoilage of animal feed is certainly detrimental to its quality but can also give rise to the presence mycotoxins, when feed containing mycotoxins is consumed by animals, and can affect both animal and human health. A wide range of studies have shown that LAB can successfully be applied to reduce fungal growth, and therefore the presence of mycotoxins in these products. Studies conducted by Gourama and Bullerman (1995) and Gourama and Bullerman (1997) revealed that, when silage was inoculated with a combination of

different *Lactobacillus* species (*L. plantarum*, *L. bulgaris*, *L. acidophilus*) an inhibition of *A. flavus* could be observed. Analyses of the antifungal compounds responsible for this effect showed, that a small peptide of less than 1 kDa was the antifungal agent. In similar trials conducted by Miyamoto and Naito (2006, 2008, 2011) *Lactobacillus delbrueckii* FERM BP-10663 was tested in animal feed consisting of 60 wt % of cereal (corn, rye), 26 wt % of oil-seed cake and meal, 2 wt % of chaff and bran, and 12 wt % other materials (sweet stuff waste, molasses and like). This strain had strong antifungal activity against *P. chrysogenum*, *P. roqueforti* and *P. olsonii*, fungi which are frequently associated with animal feeds. Moreover, the authors in the patent claimed that *L. delbrueckii* FERM BP-10663 can also be used in a livestock feed and drink for domestic fowls, domestic animals or pets. In this case, the strain acts as a probiotic, which promotes animal breeding by improvement of deodorization of excretory substances, survival rate of animals, egg-laying rate of hens for egg collection and improvement of cow's milk quality.

In total, there were 18 patents reviewed. These patents show that LAB have considerable potential to be applied as an alternative for commonly used chemical, antifungal agents. Indeed, the number of patents on biopreservatives has increased significantly in recent years.

An overview of the distribution of the patents based on food groups is given in Figure 2.1.

Figure 2.1 *Distribution of the patents within the food and feed fields*[a]

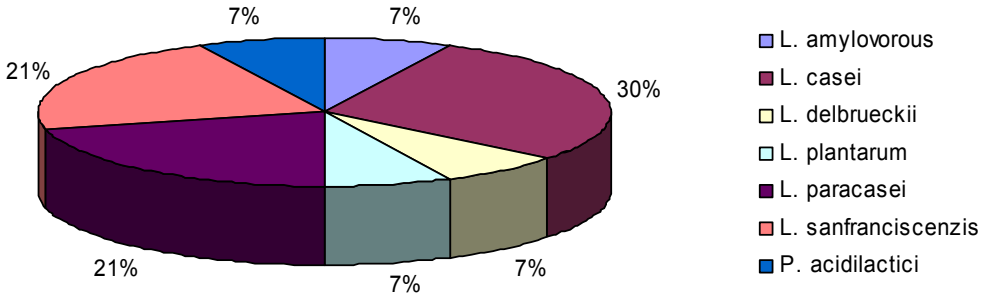


[a]The diagram reports the partition of deposited patents, considering the extensions of a previous patent as one single patent (i.e. the European patent EP 2009/056229 and its extension to US through the patent US 2011/0200569 are considered as one patent only).

As seen in Fig. 2.1, patents on antifungal activity of LAB in foods and feeds were equally distributed among meat products and sauces, and silage and dairy products. Over 20% of patents were filed for bakery goods. In the case of patent distribution

according to LAB species (Fig. 2.2), the largest amount of patents concentrates on *L. casei* strains, followed by *L. paracasei* and *L. sanfraciscensis*, those remaining ones are based on the species *L. amylovorous*, *L. delbrueckii*, *L. plantarum*, *P. acidilactici*.

Figure 2.2 *Distribution of the patents within LAB species*[a]



[a]The diagram reports the partition of deposited patents, considering the extensions of a previous patent as one single patent (i.e. the European patent EP 2009/056229 and its extension to US through the patent US 2011/0200569 are considered as one patent only).

2.5 Conclusions

The use of LAB is one of the oldest methods of food processing and preservation in the whole world going back as far as 7000 years in Babylon (Battcock and Azam-Ali, 1998) when fermentation was used to produce beer, bread, wine, vinegar, yoghurt, cheese and butter. Bacterial antagonism has been recognized

for over a century but in recent decades it has received more scientific attention. The potential of LAB as effective agents in combating food-borne yeasts and moulds, meets the consumers requirements for products, which are high in nutritional value, tasteful, mildly processed, cheap, but with reduced artificial preservatives and extended shelf-life. As well as being safer than existing synthetic antimicrobial agents, LAB and their by-products have been shown to have beneficial effects on human health (Masood *et al.*, 2011).

Moreover, LAB have many traits which promote their industrial importance. They are used for food and beverage fermentation, flavor formation (Urbach, 1995), production of add-in ingredients (Hugenholtz *et al*., 2002), bacteriocins (De Vuyst and Leroy, 2007) and exopolysaccharides (Welman and Madox, 2003). LAB can also be used to produce bulk and fine chemicals, including lactic acid (Kwon *et al.*, 2001), polyols (Wisselink *et al.*, 2002) and vitamins of the B group (Burgess *et al.*, 2004).

Thanks to their QPS status, they can be easily introduced into large-scale food and feed bio-preservation systems and since are not genetically modified they do not require special safety assessments and risk analyses. Thus, the application of the antifungal LAB as food and feed biopreservatives can bring significant commercial benefit. However, to have functional LAB strains with predictable and measurable beneficial effect, strict attention to strain selection and characterisation is needed.

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Chapter 3

Potential of lactic acid bacteria to inhibit common toxigenic food spoilage fungi

3.1 Abstract

Increased concern over chemical food additives has prompted searches for more natural antifungal agents. One potential source of these agents are lactic acid bacteria (LAB). In this study, 116 isolates of LAB isolated from different environments were screened for the antifungal activity. Approximately 83% of the isolates showed inhibitory activity against at least 1 of tested indicator moulds *Aspegillus fumigatus*, *A. niger*, *Penicillium expansum* and *P. roqueforti*. To better understand the factors involved in the antifungal activity of these bacteria, this research studied the profile of antifungal metabolites of *Lactobacillus reuteri* ee1p. The cell-free supernatant of MRS broth fermented by the strain was analysed and 16 known antifungal compounds were identified and quantified.

3.2 Introduction

Contamination of food by various types of toxigenic moulds is a serious and difficult to eliminate problem (Bhat *et al.*, 2010). Mycotoxigenic fungi are very common and can grow on a wide range of substrates under a wide range of conditions. The mould growth may result in several kinds of food spoilage such as off-flavors, toxins, discoloration, rotting and formation of pathogenic or allergenic propagules (Filtenborg *et al.*, 1996). The mycotoxins produced by fungi can cause serious health hazards, including cancerogenic, immunotoxic, teratogenic, neurotoxic, nephrotoxic and hepatotoxic effects and Kashin-Beck disease (Pawlowska *et al.*, 2012). In addition to this, fungal pathogens are causing losses of marketable quality of foodstuffs, resulting in major economic problem throughout the world.

Fungal spoilage in food can be solved by different physical methods (drying, freezing, freeze-drying, packaging under modified atmosphere, ultraviolet, microwave and infrared radiation) and by adding chemical preservatives. However, many food-borne fungi are becoming resistant to antibiotics and traditional chemical preservatives like sorbic and benzoic acids as well as chemical cleaning compounds (Schnürer and Magnusson, 2005). Moreover, consumers' acceptance of classical preservation methods is diminishing and thus, interest in the application of biopreservation in the food industry has increased.

Lactic acid bacteria (LAB) occur naturally in foods or are added as pure cultures to various food products. It has been estimated that 25% of the European diet and 60% of the diet in many developing countries consists of fermented foods (Stiles, 1996). LAB are well known as starter cultures in the manufacture of bread, meat, sauerkraut and dairy products such as acidophilus milk, yoghurt, buttermilk, cottage cheeses, hard and soft cheeses (Carr *et al.*, 2002). It is also shown that LAB contribute towards the safety, flavour, texture and nutritional value of the food product, prevent food spoilage and extend the shelf-life of foodstuffs (Arendt *et al.*, 2007, Dal Bello *et al.*, 2007, Urbach, 1995). Most of their antifungal capacity is due to production of active antimicrobials, like organic acids, carbon dioxide, fatty acids, hydrogen peroxide, phenolic and proteinaceous compounds (Amanatidou *et al.*, 1999, De Vuyst and Leroy, 2007, Sjörgen *et al.*, 2003, Ström *et al.*, 2002, Venturini *et al.*, 2002). In this context, LAB are suggested as promising alternatives to chemical preservatives and antibiotics (Schnürer and Magnusson, 2005).

The aim of the present research was to screen lactic acid bacteria for antifungal activity. The fungal inhibitory spectra of 116 isolates of LAB were

determined. The isolates were screened against 4 mould species, *Aspergillus fumigatus*, *A. niger*, *Penicillium expansum* and *P. roqueforti*. These fungi were selected to represent spoilage organisms of economic significance in the handling of food and feed (Pitt and Hocking, 1999). Finally, one of the best performing strains *L. reuteri* ee1p was further investigated in order to identify the nature of the antifungal activity.

3.3 Materials and Methods

3.3.1 Bacterial cultures

One hundred and sixteen randomly selected strains of lactic acid bacteria were kindly provided from Cork Institute and Technology (Cork, Ireland) and were isolated from cheese, cereal, sourdough and human, porcine, mice and bovine intestines. The strains were grown on MRS5 agar plates (Oxoid, Hampshire, UK) supplemented with 0.05 g/L bromocresol green (Sigma Aldrich Chemie Gmbh, Steinheim, Germany) at 37 °C for 2 days in anaerobic conditions. Working cultures were kept on MRS agar plates at 5 °C, while long-term storage was done at -80 °C in a glycerol/water (1:1) solution. MRS broth (Oxoid, Hampshire, UK) was used as liquid growth medium.

3.3.2 Fungal cultures

Aspergillus fumigatus J9, *Aspergillus niger* FST 4.21, *Penicillium expansum* LTH S46 and *Penicillium roqueforti* FST 4.11 (School of Food and Nutritional Sciences, University College Cork, Ireland) were chosen as representative spoilage fungi. The moulds were cultivated on malt extract agar (MEA) (Oxoid, Hampshire, UK) at 25 °C for 2 to 5 days and stored at 5 °C. The spores were collected after

brushing the plate surface with physiological solution and counted using a Thoma chamber haemacytometer.

3.3.3 *In vitro* antimicrobial activity

The inhibition spectrum of LAB strains was determined by using the overlay method as described by Magnusson and Schnürer (2001) with some modifications. For the screening MRS medium without sodium acetate (NaAc) were used, as NaAc showed antifungal activity in a prescreening. The antifungal activity was achieved by nebulising of 100 µL (10^4 spores per plate) of fungal spore solution on MRS5 agar plates. LAB were inoculated in two 2-cm lines on the MRS agar plates and incubated, first, at 37 °C for 48 h under anaerobic conditions, then under aerobic conditions at room temperature to allow the fungal growth. Afterwards, the plates were subsequently examined for the presence of halos.

3.3.4 LCMS analyses of antifungal compounds

The quality of antifungal compounds produced by *L. reuteri* ee1p in MRS broth was investigated according to Brosnan *et al.* (2012) with some modifications. Fifty mL of cell-free supernatant resulting from the growth of *L. reuteri* ee1p in MRS broth was extracted 3 times with 25 mL of ethyl acetate (EtAc). Three grams of sodium chloride were added to broth in order to ensure the formation of two separate layers. Organic layers were pooled and dried under nitrogen. The samples were reconstituted in H_2O/ACN 9/1 (first 10% ACN was added first to ensure that fatty acids were dissolved and then 90% water was then added). The reconstituted sample was filtered (0.2 µm) and vialed for injection onto LCMS.

L. reuteri M13·10^{-5} strain was used as a negative control and evaluated using the same procedure. Method was developed on a Thermo Accela LC system (Hemel Hampstead, UK) using a Gemini C18 (150 x 2 mm, 5 mm; Phenomenex) column equipped with a Security Guard cartridge (C18, 4 x 2 mm; Phenomenex). A flow rate of 0.3 mL/min was used and the column was maintained at a temperature of 30 °C. A long stepped gradient was employed to ensure adequate separation of the twenty-five known compounds. (Compound A – cytidine, compound B – 2 – deoxycytidine, compound C – glucuronic acid, compound D – 1,2-dihydroxybenzene, compound E – $_{DL}$-*ρ*-hydrophenyllactic acid, compound F – 4-hydroxybenzoic, compound G – 3,4-dihydroxyhydrocinnamic acid, compound H – vanillic acid, compound I – (S)-(—)-2-hydroxyisocapric acid, compound J – caffeic acid, compound K – 3-(4-hydroxyphenyl)-propionic acid, compound L – phenyllactic acid, compound M – 3-(4-hydroxy-3-methoxyphenyl)propionic acid, compound N – *p*-coumaric acid, compound O – benzoic acid, compound P – ferulic acid, compound Q – salicylic acid, compound R – hydrocinnamic acid, compound S – azelaic acid, compound T – methylcinnamic acid, compound U – 3-hydroxydecanoic acid, compound V – decanoic acid, compound W – 2 –hydroxydodecanoic acid, compound X – $_{DL}$-*β*-hydroxymyristic acid, compound Y – $_{DL}$-*β*-hydroxylauric acid). The stepped gradient was as follows mobile phase consisted of A) water with 0.2% acetic acid and B) methanol, 0 minutes – 10% B, 10 minutes – 10% B, 15 minutes – 15% B, 20 minutes – 15% B, 35 minutes – 30% B, 40 minutes - 35% B, 45 minutes -80% B, 58 minutes - 95% B, 60 minutes – 95% B.

LCMS analysis involved the coupling of the Accela LC to the Thermo LTQ Orbitrap XL hybrid mass spectrometer (Hemel Hampstead, UK). The instrument was operated in negative ionisation mode with a resolution of 100,000. The used tune file

employed a capillary temperature of 300 °C, capillary voltage of -50 V, tube lens of -110 V, sheath gas of 45 arb and auxillary gas of 15 arb. The instrument was calibrated on a weekly base as per manufactures instructions for highly accurate mass data.

3.3.5 HPLC analysis of weak organic acids

Organic acids present in 48 h fermented MRS broth were determined by an Agilent 1200 HPLC system with a refractive index detector. Analyses were performed using a Rezex ROA – Organic H^+ (8%) 300 x 7.8 mm column (Phenomenex). The eluent was 0.01 N H_2SO_4 at a flow rate of 0.6 mL/min. The volume of the injection was 10 µL. The column was maintained at 65 °C. Lactate and acetate (Sigma-Aldrich) were used as external standards of calibration. Standard calibration curves were prepared in a concentration range 5 – 100 mM with four different concentration levels (5, 10, 50 and 100 mM). Duplicate injection was made for each concentration level and a weight linear regression was generated. The calibration curves with the external standards were obtained using concentration (mM) with respect to the area obtained from the integration of the PDA peaks. The relation between variables was investigated using linear correlation. The linear regression of the external standards R^2 was 0.9987 for lactate, 1 for acetate and 0.9972 for ethanol, respectively. The amount of the compounds was finally expressed in μg per mL^{-1}.

3.3.6 Determination of Minimum Inhibitory Concentrations (MIC)

MICs were determined for the available commercial compounds, D-glucuronic acid, cytydine, deoxycitidine, sodium decanonate, phenyllactic acid (both

D and L forms), hydroxyphenyllactic acid, *p*-coumaric acid, (Sigma-Aldrich Chemie, Steinheim, Germany), 3-phenylpropionic acid, α-methylcinnamic acid and salicylic acid (Fluka Chemie AG, Buchs, Switzerland) as described under section 2.2 of Apendix 2.

3.4 Results and Discussion

3.4.1 Antifungal activity of lactic acid bacteria

Moulds are common spoilage organisms in different food and feed systems (Magnusson *et al.*, 2003). *Aspergillus* species, included in this study, are frequent contaminants of starchy foods such as bread and potatoes. *Penicillium* species are important spoilage organism in bread and different dairy products (Basílico *et al.*, 2001, Legan, 1993, Taniwaki *et al.*, 2001). Thus, any decrease in fungal growth is of great interest and efficient methods to prevent the mould spoilage are needed. LAB can play very important role as such preservatives since they have a long history in food production and are generally recognised as safe (GRAS status). There have been several reports on antifungal properties of lactobacilli, e.g. *Lactobacillus acidophilus* (Batish *et al.*, 1989, 1990a), *L. amylovorous* (Ryan *et al.*, 2012), *L. brevis* (Mauch *et al.*, 2010), *L. casei* (Suzuki *et al.*, 1991, Gourama, 1997), *L. coryniformis* subsp. *coryniformis* (Magnusson and Schnürer, 2001), *L. lactis* subsp. *lactis* (Roy *et al.*, 1996, Roy *et al.*, 2001), *L. lactis* subsp. *lactis* var. *diacetylactis* (Batish *et al.*, 1989, 1990b, *L. plantarum* (Niku-Paavola *et al.*, 1999, Laitila *et al.*, 2002, Ström *et al.*, 2002, Lavermicocca *et al.*, 2000, 2003, Broberg *et al.*, 2007, Dal Bello *et al.*, 2007), *L. rhamnosus* (Suzuki *et al.*, 1991, Stiles *et al.*, 2002), *L. salivarius* (Stiles *et al.*, 1999), *L. sanfrancisco* (Gobetti and Corsetti, 1997, Corsetti *et al.*, 1998). Some of LAB have also been successfully applied in food and

feed systems. Dal Bello *et al.* (2007), Lavermicocca *et al.* (2000), Ryan *et al.*, (2011) implemented antifungal LAB in the production of bread with extended shelf-life. Miescher Schwenninger and Meile (2004) used LAB to prevent the fungal growth in dairy products. Broberg *et al.* (2007) applied antifungal LAB in feeds.

In the present study, 116 randomly selected isolates were identified and screened for antifungal activity. The antifungal activity of LAB isolated from different sources were investigated by streaking out the bacteria onto MRS plates inoculated with *A. fumigatus*, *A. niger*, *P. expansum* and *P. roqueforti* fungal spores. The tested species and genera were: *Lactobacillus arizonensis*, *L. brevis*, *L. casei*, *L. coryniformis*, *L. fermentum*, *L. gasseri*, *L. paracasei*, *L. plantarum*, *L. reuteri*, *L. rhamnosus*, *Leuconostoc mesenteroides*, *Pediococcus pentosaceus* and *Weisella kimchii* (Figure 3.1). *L. reuteri* was the dominant species. There were 58 *L. reuteri* strains tested and 93% of them were active against at least 1 of tested moulds (Figure 3.2). Other prevalent species were *L. arizonensis* (24 strains) and *L casei* (14 strains). Seventyfive and 93%, respectively of these isolates showed inhibitory action. In total, out of 116 LAB isolates, approximately 83% showed antifungal activity (Figure 3.3). Among this group 47 strains exhibited antifungal action against 1 out of 4 tested fungi, 37 isolates inhibited the growth of 2 out of 4 tested fungi, 11 strains were active against 3 out of 4 fungi and 1 strain inhibited the growth of all tested moulds.

Figure 3.1 *LAB species screened for antifungal activity*

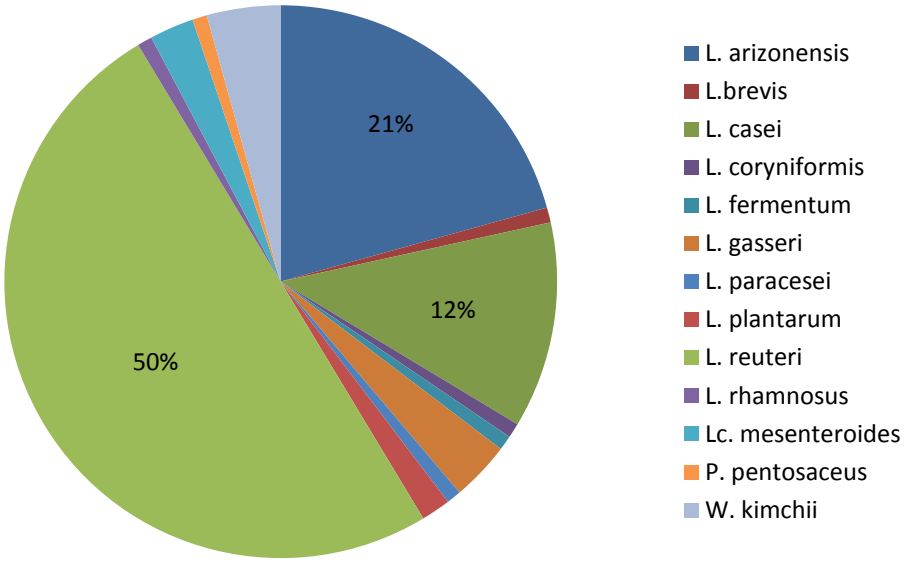


Figure 3.2 *Percentage of each LAB species showing antifungal activity*

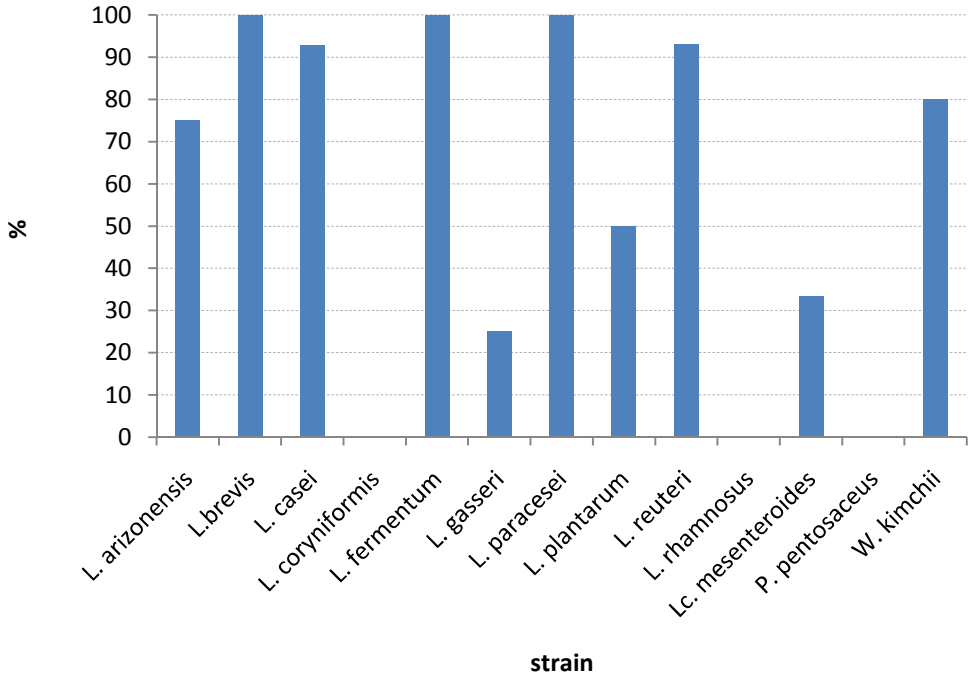


Figure 3.3 *Percentage of LAB showing antifungal activity against common fungi species*

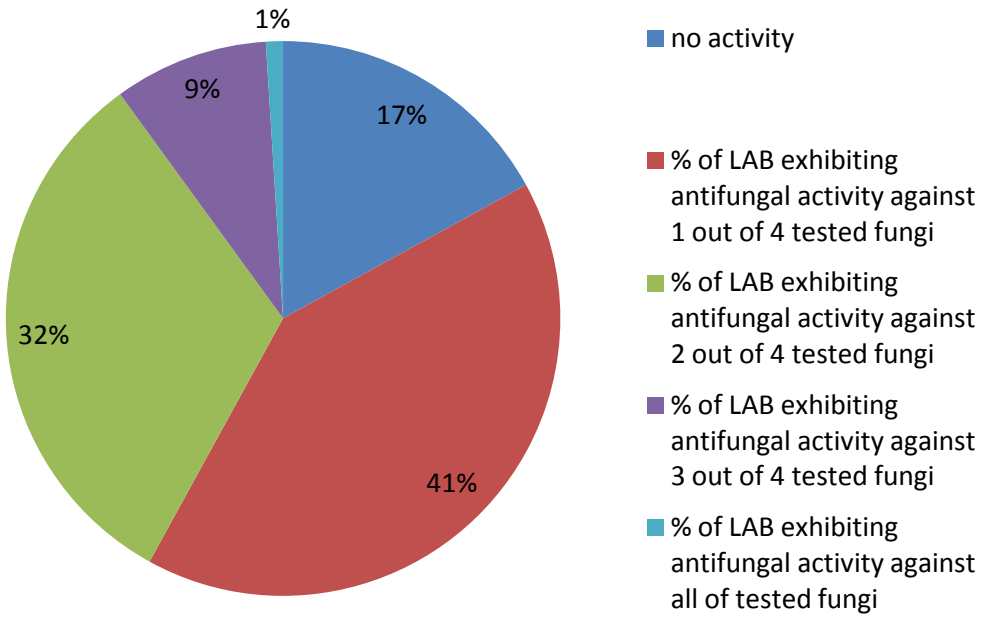


Table 3.1 *Antifungal activity of selected LAB against spoilage moulds. Halo measured in mm.*

Strain	*A.fumigatus*		*A. niger*		*P. expansum*		*P.roqueforti*		Strain origin
	37°C	30°C	37°C	30°C	37°C	30°C	37°C	30°C	
Lactobacillus arizonensis (F1)	1.0	-	-	-	-	-	-	-	sourdough
Lactobacillus arizonensis (F10)	3.0	-	-	-	-	-	-	-	sourdough
Lactobacillus arizonensis (F12)	2.0	-	-	-	-	-	-	-	sourdough
Lactobacillus arizonensis (F13)	-	-	-	-	-	-	3.0	2.0	sourdough
Lactobacillus arizonensis (F17)	0.5	3.0	-	-	-	-	-	-	sourdough
Lactobacillus arizonensis (F2)	-	-	-	-	-	-	-	-	sourdough
Lactobacillus arizonensis (F21)	1.0	-	-	-	-	-	-	-	sourdough
Lactobacillus arizonensis (F22)	-	-	-	-	5.0	4.0	5.0	4.0	sourdough
Lactobacillus arizonensis (F3)	2.0	-	-	-	-	-	-	-	sourdough
Lactobacillus arizonensis (F34)	-	-	-	-	-	-	-	-	sourdough
Lactobacillus arizonensis (F36)	-	-	-	-	1.0	1.0	-	-	sourdough
Lactobacillus arizonensis (F38)	-	-	-	-	3.0	5.0	3.0	3.0	sourdough
Lactobacillus arizonensis (F4)	-	-	-	-	-	-	-	-	sourdough
Lactobacillus arizonensis (F6)	6.0	-	-	-	-	-	-	-	sourdough

Lactobacillus arizonensis (F7)	-	-	-	-	-	-	-	-	sourdough
Lactobacillus arizonensis (F8)	3.0	-	-	-	-	-	-	-	sourdough
Lactobacillus arizonensis (F9)	-	-	-	-	-	-	-	-	sourdough
Lactobacillus arizonensis (R11)	-	-	7.0	2.0	3.0	-	-	-	cheese
Lactobacillus arizonensis (R13)	3.0	-	-	-	-	3.0	-	4.0	cheese
Lactobacillus arizonensis (R14)	-	-	-	-	-	-	-	-	human
Lactobacillus arizonensis (R18)	-	-	5.0	-	-	-	-	-	human
Lactobacillus arizonensis (R25)	-	-	2.0	-	-	-	-	-	human
Lactobacillus arizonensis (R7)	-	-	2.0	-	-	-	-	-	human
Lactobacillus arizonensis (R8)	-	-	7.0	5.0	-	-	-	-	human
Lactobacillus brevis (JJ2p)	3.0	5.0	-	-	-	-	-	6.0	porcine
Lactobacillus casei (bb3)	-	0.5	0.4	1.0	-	-	-	-	porcine
Lactobacillus casei (R1)	-	-	8.0	2.0	-	-	-	-	bovine
Lactobacillus casei (R10)	-	-	5.0	-	-	-	-	1.0	cheese
Lactobacillus casei (R15)	5.0	2.0	1.0	-	-	2.0	-	-	human
Lactobacillus casei (R16)	5.0	-	7.0	-	-	-	-	-	human
Lactobacillus. casei (R17)	-	-	1.0	-	-	-	-	-	human
Lactobacillus casei (R19)	-	-	3.0	1.0	-	-	-	-	human

Lactobacillus casei (R20)	3.0	-	6.0	9.0	-	-	-	-	bovine
Lactobacillus casei (R21)	-	-	-	-	-	-	-	-	human
Lactobacillus casei (R22)	-	-	-	2.0	-	-	-	-	human
Lactobacillus casei (R24)	-	-	2.0	-	-	1.0	-	-	human
Lactobacillus casei (R28)	-	-	4.0	-	-	-	-	-	bovine
Lactobacillus casei (R4)	3.0	-	10.0	-	-	-	-	6.5	human
Lactobacillus casei (R5)	-	-	1.0	1.0	-	-	-	-	human
Lactobacillus coryniformis (E12)	-	-	-	-	-	-	-	-	cereal
Lactobacillus fermentum (F23)	17.0	-	-	-	-	-	-	-	sourdough
Lactobacillus gasseri (M11 10^{-5} 1b)	-	-	-	-	-	-	-	-	murine
Lactobacillus gasseri (M11 10^{-5}1a)	-	-	2.0	-	-	-	-	-	murine
Lactobacillus gasseri (M15 10^{-6} 4)	-	-	-	-	-	-	-	-	murine
Lactobacillus gasseri (M16 10^{-6} 2)	-	-	-	-	-	-	-	-	murine
Lactobacillus paracasei (R3)	-	-	-	-	-	1.0	-	-	bovine
Lactobacillus plantarum (F5)	5.0	3.0	-	-	-	-	-	-	sourdough
Lactobacillus plantarum (R27)	-	-	-	1.0	-	-	-	-	human
Lactobacillus reuteri (R12)	1.0	-	-	-	-	-	-	-	cheese
Lactobacillus reuteri (aa2p)	0.5	-	1.3	0.2	-	-	-	-	porcine

Lactobacillus reuteri (aaIP)	-	-	1.7	-	-	-	-	-	porcine
Lactobacillus reuteri (bb4)	-	-	-	1.0	-	-	-	-	porcine
Lactobacillus reuteri (CC2)	-	-	0.3	1.0	0.2	-	-	-	porcine
Lactobacillus reuteri (d3p)	1.0	-	-	-	-	-	-	-	porcine
Lactobacillus reuteri (dd2p)	5.0	-	15.0	-	-	-	-	-	porcine
Lactobacillus reuteri (ee1p)	8.0	-	-	5.0	-	-	-	2.0	porcine
Lactobacillus reuteri (ee2x)	8.0	-	11.0	-	-	-	-	-	porcine
Lactobacillus reuteri (ee3)	-	-	-	-	-	-	-	-	porcine
Lactobacillus reuteri (ee4p)	-	-	-	-	-	-	-	-	porcine
Lactobacillus reuteri (ff2)	10.0	-	3.0	-	-	5.0	-	-	porcine
Lactobacillus reuteri (ff3)	5.0	-	5.0	1.0	-	5.0	-	-	porcine
Lactobacillus reuteri (ffdp)	-	-	-	-	-	-	-	-	porcine
Lactobacillus reuteri (gg1p)	10.0	-	5.0	5.0	-	-	-	0.1	porcine
Lactobacillus reuteri (gg3p)	6.0	-	-	-	-	-	-	-	porcine
Lactobacillus reuteri (hhIp)	2.0	5.0	-	5.0	-	1.0	-	3.5	porcine
Lactobacillus reuteri (J2p)	7.0	3.0	-	-	-	-	-	-	porcine
Lactobacillus reuteri (K2)	-	3.0	-	5.0	-	-	-	-	porcine
Lactobacillus reuteri (KK1P)	5.0	5.0	7.0	7.0	-	-	-	-	porcine

Lactobacillus reuteri (KK2P)	7.0	-	11.0	2.0	-	-	-	-	porcine
Lactobacillus reuteri (M12 10^{-6} 2)	-	-	10.0	-	-	-	-	-	murine
Lactobacillus reuteri (M13 10^{-5} 3)	-	-	-	-	-	-	-	-	murine
Lactobacillus reuteri (M13 10^{-6} 1)	-	-	-	2.0	-	-	-	-	murine
Lactobacillus reuteri (M14 10^{-5} 2)	5.0	-	5.0	7.0	-	-	-	-	murine
Lactobacillus reuteri (M14 10^{-6} 2)	10.0	10.0	-	6.0	-	-	-	-	murine
Lactobacillus reuteri (M15 10^{-6} 1)	-	-	5.0	-	-	-	-	-	murine
Lactobacillus reuteri (M19 10^{-5} 2)	-	-	1.0	2.0	-	-	2.0	-	murine
Lactobacillus reuteri (M21 10^{-6} 3)	8.0	-	-	1.0	-	-	-	-	murine
Lactobacillus reuteri (M4 10^{-5} 1)	-	-	5.0	-	-	-	-	-	murine
Lactobacillus reuteri (M4 10^{-5} 1)	6.0	-	3.0	-	-	-	-	-	murine
Lactobacillus reuteri (M5 10^{-5} 1)	-	-	5.0	-	-	-	-	-	murine
Lactobacillus reuteri (M5 10^{-6} 2)	-	-	-	1.0	-	-	-	-	murine
Lactobacillus reuteri (M5 10^{-6}1)	20.0	-	1.0	4.0	-	-	-	2.0	murine
Lactobacillus reuteri (M9 10^{-5} 2)	-	-	10.0	-	-	-	-	-	murine
Lactobacillus reuteri (MIP 6)	3.0	-	-	1.0	-	-	-	-	porcine
Lactobacillus reuteri (MIP 9)	-	-	8.0	8.0	-	-	-	-	porcine
Lactobacillus reuteri (N3P)	-	-	3.0	-	-	-	-	-	porcine

Lactobacillus reuteri (OIP)	5.0	-	12.0	-	-	-	-	-	porcine
Lactobacillus reuteri (Q)	15.0	3.0	12.0	1.0	-	-	-	-	porcine
Lactobacillus reuteri (R2)	5.0	-	5.0	6.0	-	10.0	-	-	porcine
Lactobacillus reuteri (R26)	-	-	-	5.0	-	-	-	-	human
Lactobacillus reuteri (R29)	18.0	1.0	-	-	10.0	-	10.0	-	human
Lactobacillus reuteri (R30)	10.0	6.0	10.0	5.0	-	-	-	-	human
Lactobacillus reuteri (R6)	7.0	1.0	5.0	10.0	-	-	-	-	bovine
Lactobacillus reuteri (R9)	-	5.0	13.0	-	-	-	-	-	human
Lactobacillus reuteri (T2P)	-	-	7.0	3.0	-	-	-	-	porcine
Lactobacillus reuteri (UP3)	-	-	15.0	-	-	-	2.0	-	porcine
Lactobacillus reuteri (V1P)	10.0	5.0	6.0	4.0	-	-	-	-	porcine
Lactobacillus reuteri (V2p)	-	-	6.0	-	-	-	-	-	porcine
Lactobacillus reuteri (W2p)	8.0	5.0	-	-	-	-	-	-	porcine
Lactobacillus reuteri (WIP)	-	4.0	-	3.0	-	-	-	-	porcine
Lactobacillus reuteri (x2p)	13.0	-	12.0	7.0	-	-	-	-	porcine
Lactobacillus reuteri (Y2)	10.0	-	-	-	-	-	-	-	porcine
Lactobacillus reuteri (Y3P)	-	-	9.0	-	-	-	-	-	porcine
Lactobacillus reuteri (YIP)	-	-	6.0	7.0	-	-	2.0	-	porcine

Lactobacillus reuteri (Z2p)	10.0	-	-	5.0	-	-	-	-	porcine
Lactobacillus reuteri (ZIP)	5.0	-	10.0	-	-	-	-	-	porcine
Lactobacillus rhamnosus (E9)	-	-	-	-	-	-	-	-	cereal
Leuconostoc mesenteroides (E11)	-	-	-	-	-	-	-	-	cereal
Leuconostoc mesenteroides (E2)	-	-	-	-	-	-	-	-	cereal
Leuconostoc mesenteroides (E4)	3.0	-	-	-	-	-	-	-	cereal
Pediococcus pentosaceus (E6)	-	-	-	-	-	-	-	-	cereal
Weissella kimchii (E7)	-	-	-	-	-	-	-	-	cereal
Weissella kimchii (F27)	-	-	3.0	2.0	1.0	2.0	2.0	4.0	sourdough
Weissella kimchii (F28)	-	-	-	-	1.0	4.0	1.0	1.0	sourdough
Weissella kimchii (F29)	-	-	-	-	-	1.0	1.0	1.0	sourdough
Weissella kimchii (F33)	-	-	-	-	3.0	2.0	-	1.0	sourdough

3.4.2 Antifungal potential of *L. reuteri* ee1p

The inhibition zone obtained with *L. reuteri* ee1p strain showed that this strain is capable of inhibiting the growth of 3 out of 4 tested microorganisms. The results presented in Table 3.1 also revealed that the different mould species showed varying inhibition levels to the antifungal agents produced by *L. returei* ee1p. Especially *A. fumigatus* was very sensitive to the active metabolites of this strain. The halo surrounding the bacterial line was 18.0 mm. However, moderate activity against *P. expansum* and *P. roqueforti* was also observed. Strain *L. reuteri* M13·10^{-5} 3 showed no activity against all tested fungi and thus, was chosen as a negative control.

3.4.3 Production of antifungal substances by *L. reuteri* ee1p

The antifungal components of *L. reuteri* strains were identified by means of LCMS. The ethyl acetate fractions of the cell-free supernatants resulting from fermentation of MRS broth by *L. reuteri* ee1p and M13·10^{-5} 3 strains were screened for presence of 25 known antifungal compounds. Components were detected and identified (Figure 3.4) by comparison of their retention times and exact masses with data recorded for the standards. Chromatographic and spectrometric data of the antifungal components found in MRS broth are summarized in Table 3.2. They were 13 components identified in the supernatant resulting from fermentation of MRS broth by *L. reuteri* ee1p. The observed compounds were as follows: DL-*ρ*-hydrophenyllactic acid, 3,4-dihydroxyhydrocinnamic acid, vanilic acid, (S)-(—)-2-hydroxyisocapric acid, phenyllactic acid, benzoic acid, salicylic acid, hydrocinnamic acid, azelaic acid, 3-hydroxydecanoic acid, decanoic acid, 2-hydroxydodecanoic acid and DL-*β*-hydroxymyristic acid.Vanillic and decanoic acids were not detected in the

MRS fermented by *L. reuteri* M13·10^{-5} 3 (Figure 3.4, Table 3.2). The most abundant compound produced by *L. reuteri* ee1p strain was phenyllactic acid (107.25 mg L^{-1}). *L. reuteri* M13·10^{-5} 3 strain produced only 1.12 mg L^{-1} of that compound. The antifungal potential of phenyllactic acid was confirmed by the results of MIC tests. In more detail, the results of the screening indicated that phenyllactic acid was active against *A. fumigatus* J9 with MIC of 2.5 mg L^{-1} (Table 2, Apendix 2). Although the difference in the quantities of the compound is significant, it not determines the overall antifungal activity of *L. reuteri* ee1p strain. The synergy studies of the LAB compounds have shown that the collective activity of the known compounds is much higher than their individual activity. The antagonistic and synergistic effects of different mixtures of identified compounds was demonstrated by Corsetti *et al.* (1998), Niku-Paavola *et al.* (1999), Ndagano *et al.* (2011). Moreover, the presence of additional inhibitory agents can not be omitted. Other authors, beside from the effect of organic acids, refer to the participation of proteinaceous compounds in the antifungal activity of LAB. Ström *et al.* (2002) reported weak synergistic effects between some cyclic peptides and 3-phenyllactic acid produced by a *L. plantarum* strain. Dal Bello *et al.* (2007) in the cell free supernatant of other *L. plantarum* strain identified lactic acid, phenyllactic acid and two cyclic dipeptides. Similarly, Ryan *et al.* (2011) isolated carboxylic acids, nucleosides, sodium decanoate and cyclic dipeptides from *L. amylovorous*.

The HPLC/PDA analyses of cell-free supernatant of *L. reuteri* fermented MRS broth revealed that both strains produced also lactic and acetic acids and ethanol. Their quantities were comparable for both strains (Table 3.2). This result confirms also the affiliation of *L. reuteri* strains to the obligatory heterofermentative group of LAB (Corsetti and Settani, 2007).

Figure 3.4 *LCMS chromatogram of antifungal compounds found in MRS fermented by (A) L. reuteri ee1p and (B) L. reuteri M13·10^{-5} 3*

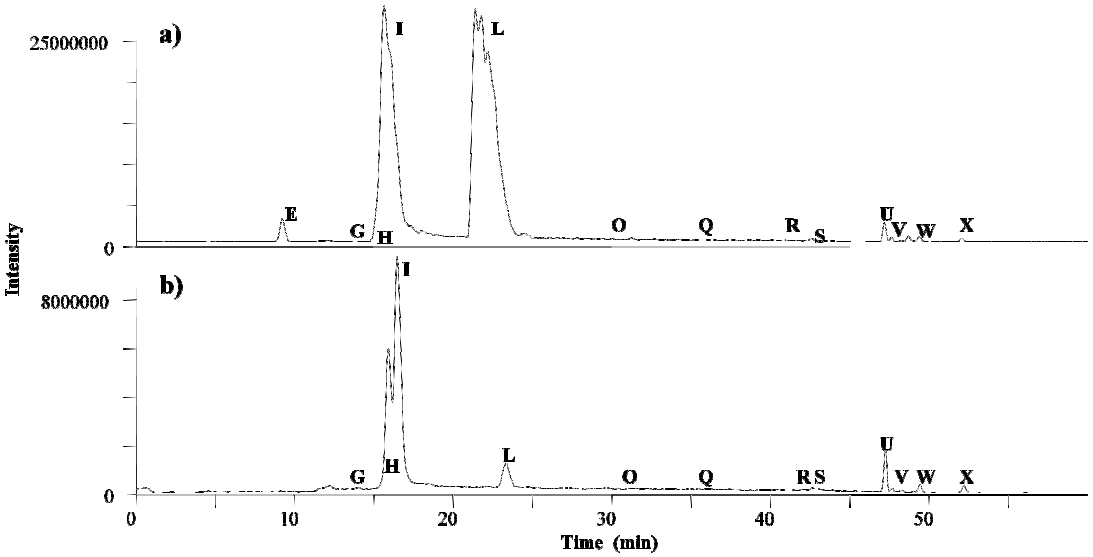


Table 3.2 *Chromatographic, spectrometric and quantitative data (μg L^{-1}) of antifungal components produced by L. reuteri ee1p and M13·10^{-5} 3 strains. Values are means (n = 2): the relative standard deviations for all compounds were <6%*

Peak	Compound	t_R (min)	[M–H]$^-$ (m/z)	Strain	
				L. reuteri ee1p	*L. reuteri* M13·10^{-5} 3
E	DL-ρ-hydrophenyllactic acid	9.23	181.05024	34.76	0.72
G	3,4-dihydroxyhydrocinnamic acid	15.08	181.05027	4.82	-
H	Vanilic acid	16.10	167.03471	4.18	0.27
I	(S)-(—)-2-hydroxyisocapric acid	16.61	131.07114	824.10	40.25
L	Phenyllactic acid	23.39	165.05537	1072.47	11.25
O	Benzoic acid	29.37	121.02927	0.80	0.25
Q	Salicylic acid	36.72	137.02423	0.13	0.07
R	Hydrocinnamic acid	40.98	149.06061	0.24	0.09
S	Azelaic acid	42.53	187.0972	0.56	0.96
U	3-hydroxydecanoic acid	47.20	187.1335	12.87	10.25
V	Decanoic acid	48.97	171.13876	3.78	-
W	2-hydroxydodecanoic acid	49.38	215.1647	1.01	0.12
X	DL-β-hydroxymyristic acid	52.05	243.19594	5.11	2.96
	Acetic acid	-	-	4404.44	3303.09
	Lactic acid	-	-	11834.36	11868.02
	Ethanol	-	-	5191.59	5943.37

3.5 Conclusions

The aim of this study was to evaluate the antifungal capacity of lactic acid bacteria. The study showed that LAB from different environments and from different genera and species can exhibit antifungal activity against a number of common spoilage moulds. The inhibitory activity is caused by several different compounds. Further investigations of the nature of the inhibiting compounds and their mechanism of action, together with development of suitable applications, could have a great potential for the control of spoilage fungi.

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Chapter 4

Characterisation of *Lactobacillus reuteri* R29 as a new antifungal strain improving the quality and shelf life of wheat bread

4.1 Abstract

The antifungal activity of *Lactobacillus reuteri* R29 and *L. reuteri* hhIp strains was investigated *in vitro* against common fungal species found in bakery environment. *L. reuteri* R29 was chosen as a potential fungal growth inhibitor and *L. reuteri* hhIp as a negative control. The LCMS and HPLC/PDA screening of the MRS broth fermented by the antifungal strain revealed the presence of sixteen antifungal components. To characterise the technological properties of dough and bread, fundamental rheology, as well as parameters like pH, TTA and specific volume, were determined. Finally, the ability of the strains to inhibit the fungal outgrowth was verified under pilot-plant conditions. The use of *L. reuteri* R29 as a starter resulted in bread of good quality, with strong ability to inhibit the fungal growth and extend the shelf-life of wheat bread.

4.2 Introduction

Bakery products are important staple foods in most countries and cultures. Baked goods and cereals are a valuable source of nutrients in our diet, which provide us with most of our food calories and approximately half of our protein requirements (Saranraj and Geetha, 2012). However, bakery products, like many other processed foods, are subjected to physical, chemical and microbiological spoilage. Fungal growth is the most frequent cause of spoilage in baked goods and constitutes a serious and costly problem to both manufacturers and consumers. In addition to this, it may cause health problems due to production of toxic and carcinogenic mycotoxins or allergenic conidia, ascospores and mycelia fragments (Filtenborg*et al.*, 1996).

Mould contamination of baked products is influenced by several factors like the type of formulation, leavening source, size and architecture of the bakery, conditioning and packaging of the products. Since fungal spores are generally killed during baking, airborne moulds contaminate baked goods in post bake handling, i.e. during cooling, slicing, wrapping, and storage operations (Knight and Menlove, 2006). Baked goods can be protected from fungal spoilage by destroying spores thatcontaminate the baked products by using physical, chemical and biological control such as. infrared and microwave radiation, using fungal inhibitors such as ethanol, propionic or acetic acid, and using sourdough with antifungal activity. In this regard, Dal Bello *et al.* (2007) showed the ability of the strain *L. plantarum* 1.7 to inhibit bread spoilers *F. culmorum* and *F. graminearum*. Lavermicocca *et al.* (2000) reported the *in situ* antifungal activity of *L. plantarum* 21B strain against *A. niger*. The study also revealed that, in some cases, lactic acid bacteria (LAB) show even higher efficacy with respect to some chemical preservatives. A similar effect was observed by Ryan *et al.* (2008). They studied the interaction of the sourdough addition fermented by antifungal LAB *L. plantarum* FST 1.7 and reduced amount of calcium propionate against *A. niger*, *F. culmorum*, *P. expansum* and *P. roqueforti* and observed a strong synergistic effect when calcium propionate and antifungal sourdoughs were combined into the bread formulation. In addition, in a pilot-scale bakery plant Ryan *et al.* (2011) evaluated the antifungal strain *L. amylovorous* DSM 19280 for its ability to delay the outgrowth of the naturally present fungal flora. The strain delayed the outgrowth of the naturally present fungi, including *A. niger*, *F. culmorum*, *P. expansum* and *P. roqueforti*, even up to 14 days, which is a significantly greater level than bread without sourdough addition and bread containing 0.3% calcium propionate.

The addition of LAB to bread in the form of sourdough has a long history of use in breadmaking (Hammes and Gänzle, 1998) and is known to improve not only shelf-life of bread and bakery products, but to give also the desirable characteristics, such as bread volume, crumb structure, flavor and nutritional value (Crowley *et al.*, 2002, Thiele *et al.*, 2002, Liljeberg*et al.*, 1995). Thus,considering the growth interest from the industry to replace commonly used synthetic antifungal agents by the natural ones to increase the shelf-life of food products preventing the loss of their sensory and nutritional quality, the utilization of LAB with multiple beneficial effects regarding bread quality and bread shelf-life are nowadays particularly desired.The successful application of sourdough in baking primarily relies on the choice of the LAB (and yeasts) to be used as starters (Moroni*et al.*, 2011). The starters used in industrial praxis are composed by a pool of different LAB, which are selected to dominate the fermentation process, to counteract microbial spoilage and staling and to produce desired compounds (aroma constituents, exopolysaccharides) during the fermentation (De Vuyst*et al.*, 2009). Therefore, the aim of this work was to analyse the performance of *L. reuteri* R29 as a starter culture for sourdough bread production and characterize its antifungal activity and bread making performance. For this purpose, the technological quality of sourdough and resulting bread were analysed by means of rheological measurements and baking tests. The inhibitory activity of the strain was tested *in vitro* and confirmed *in situ* through challenges tests during long-term shelf-life performed under pilot conditions.

4.3 Materials and Methods

4.3.1 Bacterial cultures

The antifungal strain *Lactobacillus reuteri* R29 and the negative control strain *L. reuteri*hhIp were kindly provided from Cork Institute and Technology (Cork, Ireland) and were isolated from human and porcine intestines, respectively. The strains were grown on MRS5 agar plates (Oxoid, Hampshire, UK) supplemented with 0.05 g/L bromocresol green (Sigma Aldrich ChemieGmbh, Steinheim, Germany) at 37°C for 2 days in anaerobic conditions. Working cultures were kept on MRS agar plates at 5 °C, while long-term storage was done at -80 °C in a glycerol/water (1:1) solution. MRS broth (Oxoid, Hampshire, UK) was used as a liquid growth medium.

4.3.2 Fungal cultures

Aspergillusniger FST 4.21, *Fusariumculmorum* FST 4.05, *Penicilliumexpansum* LTH S46, *Penicilliumroqueforti* FST 4.11, were provided from School of Food and Nutritional Sciences, University College Cork, Ireland and chosen as representative spoilage fungi. The moulds were cultivated on malt extract agar (MEA) (Oxoid, Hampshire, UK) at 25 °C for 2 to 5 days and stored at 5 °C. The spores were collected after brushing the plate surface with physiological solution and counted using a Thoma chamber haemacytometer.

4.3.3 *In vitro* antimicrobial activity

The inhibition spectrum of LAB strains was determined by using the overlay method as described by Magnusson and Schnürer (2001) with some modifications.

For the LAB antifungal screening MRS medium without sodium acetate (NaAc) were used, as NaAc showed antifungal activity in a prescreening. The antifungal activity of *L. reuteri* strains was achieved by nebulising of 100 µL (10^4 spores per plate) of fungal spore solution on MRS5 agar plates. LAB were inoculated in two 2-cm lines on the MRS agar plates and incubated, first, at 37 °C for 48 h under anaerobic conditions, then under aerobic conditions at room temperature to allow the fungal growth. Afterwards, the plates were subsequently examined for the presence of halos around the LAB spot.

4.3.4 LCMS analyses of antifungal compounds

The quality of antifungal compounds produced by *L. reuteri* R29 in MRS broth was investigated according to Brosnan *et al.* (2012) with some modifications, as described in Chapter 3 under the section 3.3.4.

4.3.5 HPLC analysis of weak organic acids

Organic acids present in 48 h fermented MRS broth were determined as described in Chapter 3 under the section 3.3.5.

4.3.6 Sourdough fermentation

Cells of LAB were cultivated in MRS broth at 37 °C until the late exponential phase of growth was reached (ca. 12 h), then the culture was used to inoculate 100 mL MRS broth at 1% level and incubated for further 24 h. Cells were harvested by centrifugation at 5000 rpm for 10 min and washed twice with sterile tap water. Five hundred grams of wheat flour and 500 g of tap water, containing

bacteria, were used to produce 1000 g of dough (yield of 200). Sourdoughs were incubated for 48 h at 37 °C while being stirred (ca. 200 rpm).

4.3.7 Fundamental rheology

Rheological measurements were performed with a controlled stress and strain rheometer (AntoonPaar MCR 301, Ostifildern, Germany). Samples were measured using a parallel plate geometry, which consisted of a 50-mm diameter corrugated probe and plate. The tests were performed with a 1 mm gap between the plates. After loading, excess of sample was trimmed and a thin layer of paraffin oil was applied to the edges of the sample. Samples were allowed to rest for 5 min prior to being analysed. Tests were performed at the constant temperature of 37°C using a Peltier plate system attached to a water circulation unit. Doughs were prepared by mixing flour and water with 20% addition of sourdough fermented 48 h. Amplitude sweeps were performed in the range from 0.001 to 100% on all the samples to determine the linear viscoelastic region. Frequency sweeps were performed in the range of 1-50 (1/s) angular frequency (ω), with 0.01% strain on all the samples.

4.3.8 Bread making

Sourdough wheat bread was produced according to Ryan *et al.* (2008). In detail, doughs were prepared by replacing 20% of flour with an equivalent quantity of flour in the form of sourdough fermented by selected strain. A non-acidified dough (yeast dough) was prepared as well. According to the formulations (Table 4.1), doughs were mixed in a Kenwood mixer at level 1 for 1 min and level 2 for further 7 min. The doughs were rested in a proofer (Koma BV Roermond, Holland) at 30°C and 85% RH for 15 min, scaled into 450 g portions, moulded in a small scale

moulder, placed in tins (180 mm × 120 mm × 60 mm, Sasa UK Middx, UK) and proofed for 70 min. Baking was carried out at 225°C for 35 min in a deck oven (MIE, Arnstein, Germany). The oven was presteamed before loading with 300 mL of water and once again on loading with 700 mL of water. Loaves were depanned and cooled for 120 min at room temperature.The volume of loaves was measured using the rapeseed displacement method and the specific volume was calculated. Loaves were packed in plastic bags, heat-sealed and stored at room temperature. Analyses, including instrumental textural evaluation of the crumb, pH and TTA measurements, were performed over a 5-day storage period at 3 intervals: 0, 48 and 120 h, as described by Dal Bello *et al.* (2007).

Table 4.1 *Wheat dough formulations*

Ingredient	Non-acidified dough	Biologically acidified dough
Flour (g)	1000	800
Water (g)	600	400
Salt (g)	14.7	14.7
Yeast (g)	20	20
Sourdough (g)	-	200
Acid mix (mL)	-	-
Total liquid (mL)	600	600

4.3.9 Antifungal activity tests

Wheat bread was challenged against four spoilage fungi *A. niger*, *F. culmorum*, *P. expnsum* and *P. roqueforti* as previously described by Dal Bello *et al.*(2007), but with some modifications. Ten slices of each bread were cut and inoculated with fungi suspension (10^2 conidia mL^{-1}). Another 10 slices were left without inoculum. Tests were performed in triplicate. Slices were packed in plastic bags, incubated at room temperature and monitored for mould growth over a

14-day storage period. A bread slice was deemed positive if more than 1% of total surface area was covered by fungi.

4.4 Results

4.4.1 Antifungal activity *in vitro*

Anmodified overlay method (Magnusson and Schnürer, 2001) was adopted to investigate the antifungal activity of *L. reuteri* strains. *L. reuteri* R29 was highly active against all tested fungi, whereas the strain *L. reuteri*hhIp, used as control, showed much lower or no activity, thus was chosen as a negative control. The results of the screening are summarized in Table 4.2. *F. culmorum* and *P. expansum* were the most sensitive to the antifungal compounds produced by *L. reuteri* R29, however strong inhibition with detectable zones around the colonies was also observed against *A. niger* and *P. roqueforti*.

Table 4.2 *Inhibitory activity of L. reuteri R29 and L. reuteri hhIp strains against a range of reference fungi as determined by a modified overlay method*

Mould	**Antifungal performance**[a]	
	***L. reuteri* R29**	***L. reuteri*hhIp**
*A. niger*FST 4.21	+++	-
F. culmorum FST 4.05	++++	+
*P. expanusm*LTH S46	++++	-
P. roqueforti FST 4.11	+++	-

[a]Inhibition was scored as follows; −, no inhibition; +, very weak inhibition around the colonies; ++, low inhibition with little clear zones around the colonies; +++, strong inhibition with detectable zones around the colonies; and ++++, very strong inhibition with large clear zones and nearly no growth around the colonies.

4.4.2 Identification and quantification of antifungal components

The antifungal components of *L. reuteri* strains were identified by means of LCMS. The ethyl acetate fraction of the cell-free supernatant resulting from fermentation of MRS broth by *L. reuteri* R29 was screened for the presence of 25 known antifungal compounds. Components were detected and identified (Figure 4.1) by comparison of their retention times and exact masses with data recorded for the standards. Chromatographic and spectrometric data of the antifungal components found in MRS broth are summarized in Table 4.3. The strain *L. reuteri* hhIp was treated using the same method described above. Caffeic acid and *p*-coumaric acid were absent in broth fermented by the negative strain, but additionally, two other compounds, namely 3-hydroxydecanoic acid and 2-hydroxydodecanoic acid were detected. Their quantities were equal to 12.61 and 4.52 mg mL^{-1}, respectively. The HPLC/PDA analyses of cell-free supernatant of *L. reuteri* fermented MRS broth revealed that both strains produced also lactic and acetic acids. All of the quantitative data are summarized in Table 4.3. The most abundant compounds produced by *L. reuteri* R29 were lactic acid (13.19 g L^{-1}) followed by acetic acid (4.16 g L^{-1}), (S)-(—)-2-hydroxyisocapric acid (3.20 g L^{-1}) and phenyllactic acid (1.87 g L^{-1}). *L. reuteri* hhIp produced significant amounts only of lactic and acetic acids, 9.12 and 3.23 mg L^{-1}, respectively.

Figure 4.1 *LCMS chromatogram of antifungal compounds found in MRS fermented by (A) L. reuteri R29 and (B) L. reuteri hhIp*

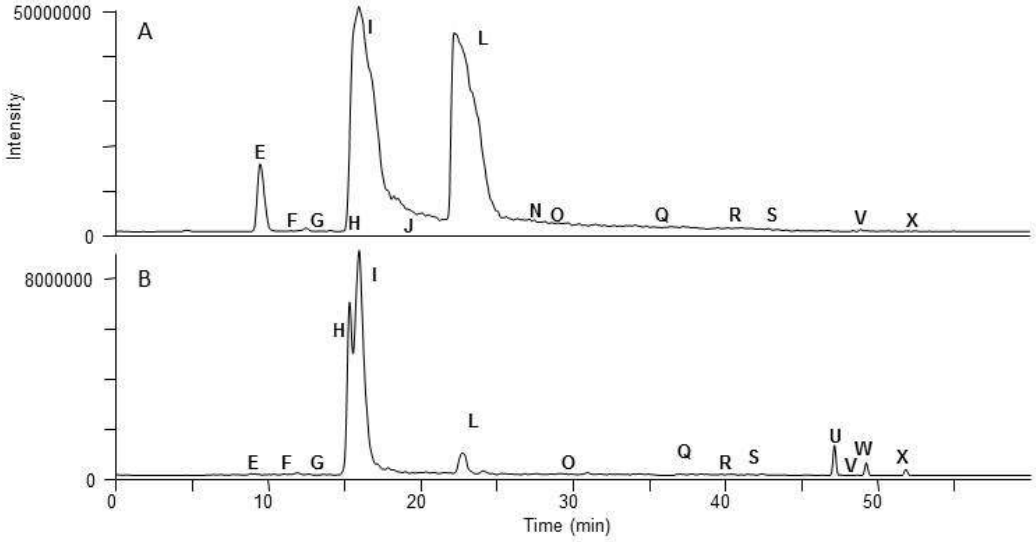


Table 4.3 *Quantitave amounts (mg L^{-1}) of antifungal components produced by L. reuteri strains*

Peak	Compound	t_R (min)	$[M–H]^-$ (m/z)	Antifungal compounds (mg/L)	
				***L. reuteri* R29**	***L. reuteri* hhIp**
E	DL-ρ-hydrophenyllactic acid	9.41	181.05011	398.76	29.57
F	4-hydroxybenzoic acid	11.95	137.02427	1.1	0.46
G	3,4-dihydroxyhydrocinnamic acid	15.27	181.05023	3.26	0.64
H	Vanilic acid	16.77	167.03468	1.23	10.21
I	(S)-(—)-2-hydroxyisocapric acid	18.47	131.07107	3197.18	49.03
J	Caffeic acid	19.31	179.03463	0.15	-
L	Phenyllactic acid	23.67	165.05531	1871.27	44.18
N	*p*-coumaroc acid	28.84	163.03981	0.06	-
O	Benzoic acid	29.81	121.02926	1.23	2.66
Q	Salicylic acid	37.31	137.02427	0.5	0.44
R	Hydrocinnamic acid	41.37	149.06061	0.65	0.46
S	Azelaic acid	42.68	187.09717	0.29	0.21
U	3-hydroxydecanoic acid	47.36	187.13347	-	12.61
V	Decanoic acid	49.15	171.13872	2.22	1.59
W	2-hydroxydodecanoic acid	49.62	215.1647	-	4.52
X	DL-β-hydroxymyristic acid	52.47	243.19591	2.51	12.57
	Acetic acid	-	-	4162.97	3229.13
	Lactic acid	-	-	13191.59	9121.14

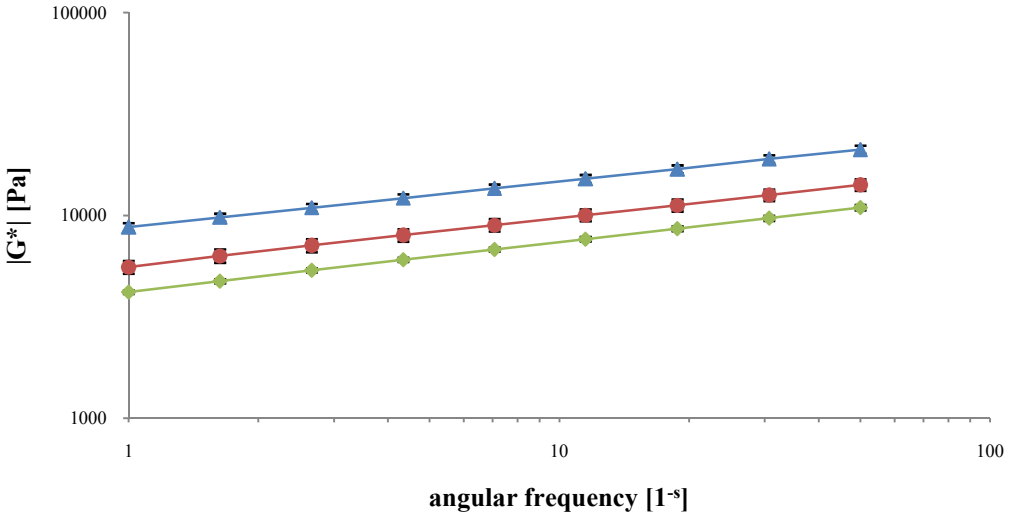
Values are means (n = 2): the relative standard deviations for all compounds were <6%.

4.4.3 Fundamental rheology

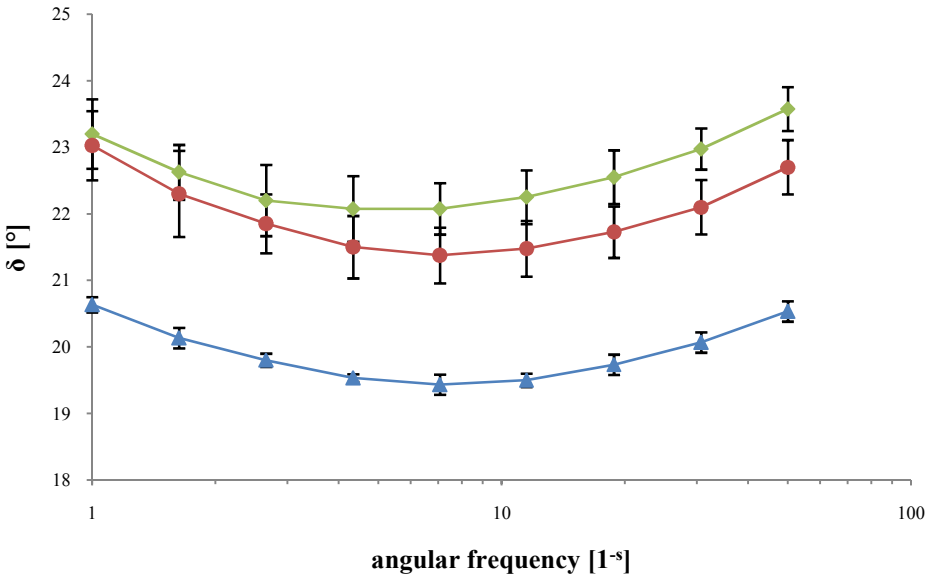
Fundamental rheological studies were performed to evaluate the impact of sourdough addition on the quality and technological properties of dough. In the measurements, doughs with 20% addition of sourdough fermented by *L. reuteri* strains as well as the control without sourdough addition were used. In all tested samples, the elastic modulus (G') was higher than the viscous modulus (G'') indicating than the batters had a solid elastic-like behaviour (data not shown). To determine the resistance to deformation as well as the viscoelastic properties of sourdoughs, complex modulus (|G*|) (Figure 4.2A) and phase angle δ (Figure 4.2B) were calculated. The absolute value of the complex modulus |G*| increased as the

frequency increased and the shape for all curves for all the doughs tested were similar. The complex modulus |G*| of the control wheat batter was higher than the ones containing sourdough in the whole angular frequency range indicating that the control dough was firmer. Among the batters with sourdough addition, batter containing *L. reuteri* R29 sourdough exhibited the lowest |G*|. All doughs showed the same trend for phase angle δover the range of frequencies measured. When the frequency increased, the phase angle values first decreased towards the minimum value before increasing once again. The addition of sourdough did not change this behavior over increasing frequencies.

Figure 4.2 *Effect of sourdough on the complex modulus (A) and phase angle (B) of wheat batters. (♦) sourdough fermented with L. reuteri R29, (●) sourdough fermented with L. reuterihhIp, (▲) control, without sourdough addition*



A



B

4.4.4 Bread quality evaluation

The effects of sourdough addition on loaf volume and crumb hardness of the bread were evaluated. The results summarized in Table 4.4 clearly indicate that the addition of sourdough had a positive effect on bread characteristics when compared with the non-acidified control. The increase of the values of loaf specific volume for the sourdough breads were reflected in a lower crumb hardness as measured using the texture profile analyses. This trend persisted over 5 days of storage. Incorporation of sourdough into bread resulted in a decrease of pH and, at the same time, increase of TTA values compared to the non-acidified control.

Table 4.4 *Characteristics of bread fermented with L. reuteri strains and control bread*

	specific volume [mL/g]	**hardness [N]**			**pH**	**TTA**
		0 h	**48 h**	**120 h**		
R29	3.29±0.08	5.75±0.91	18.65±2.15	25.84±1.8	4.49±0.02	7.5±0.11
hhIp	3.27±0.12	7.08±0.2	20.81±5.84	26.48±4.02	4.58±0.04	6.7±0.17
control	3.27±0.32	7.08±1.63	22.36±0.32	29.21±1.02	5.83±0.01	3.4±0.09

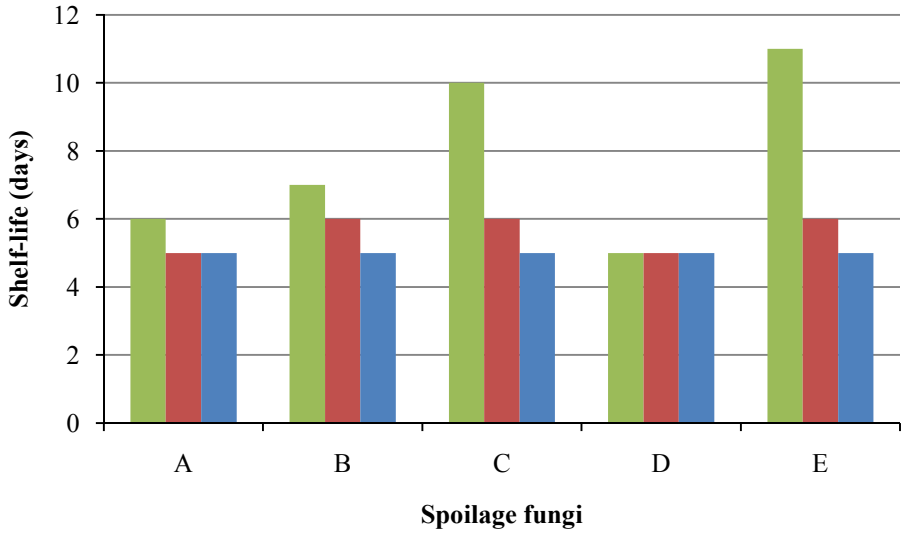
Values are means (n = 3) ± standard errors for three samples.
TTA is reported as ml NaOH (0.1 N)/10 g bread.

4.4.5 Antifungal activity tests

The ability of sourdough bread to delay the rate of fungal growth was investigated by means of challenge tests. The results of the trials are presented in Figure 4.3. Comparison with the spoilage rate in the control breads revealed that *L. reuteri* R29 sourdough bread retarded, with different extent, the outgrowth of both indicator and environmental fungi. Among the indicator moulds the most sensitive fungus to the antifungal compounds produced by *L. reuteri* R29 was *P. expansum* followed by *F. culmorum* and *A. niger*. Although *in vitro* agar plate assay showed

antifungal activity of the strain against *P. roqueforti*, no inhibition on bread slices was observed. The bread with *L. reuteri* R29 sourdough addition, similarly to both controls, spoiled after 5 days of storage. The test against mould flora in a pilot-scale bakery revealed that the bread made with *L. reuteri* hhIp affected the fungal growth by 1 day when compared to the non-acidified control. However, it could not compete with the antifungal abilities of *L. reuteri* R29. The addition of sourdough fermented with this strain resulted in bread which, in the same experimental conditions had increased shelf-life by up to 11 days i.e., 6 days longer than the non-acidified control.

Figure 4.3 *Shelf life of wheat bread challenged against F. culmorum FST 4.05 (A), A. niger FST 4.21 (B), P. expansum FST 4.22 (C), P. roqueforti FST 4.11 (D), and environmental moulds from a pilot scale industrial bakery (University College Cork) (E)*



Bread was prepared using: 20% sourdough started with *L. reuteri* R29 (green bars), 20% sourdough started with *L. reuteri*hhIp (red bars), and control dough (blue bars).

4.5 Discussion

Over the last decade, biopreservation, the control of one organism by another, has gained increasing attention as a means of natural way to improve the shelf-life and safety of baked goods. Antagonistic activity of *Lactobacillus* cultures against bacteria and fungi is a widely observed and frequently reported phenomenon. In this study, the ability of *L. reuteri* R29 to inhibit the outgrowth of *Aspergillus*, *Fusarium* and *Penicillium* spores has been proven both *in vitro* and *in situ* system (Table 4.2, Figure 4.3). Identification of antifungal substances from cell-free supernatants revealed the presence of sixteen organic acids (Table 4.3) as responsible agents for this activity. The antifungal activity of these compounds against a wide range of bacteria, yeast and/or moulds have previously been demonstrated (Corsetti *et al.*, 1998, Lavermicocca *et al.*, 2003, Röcken, 1996, Röcken and Voysey, 1995). Additionally, the presence of other inhibitory components can not be excluded. Beside from the effect of organic acids, other authors refer to the participation of proteinaceous compounds in the antifungal activity of LAB. Ström *et al.* (2002) reported weak synergistic effects between some cyclic peptides and 3-phenyllactic acid. Dal Bello *et al.* (2007) in the cell free supernatant of *L. plantarum* identified lactic acid, phenyllactic acid and two cyclic dipeptides. Similarly, Ryan *et al.* (2011) isolated carboxylic acids, nucleosides, sodium decanoate and cyclic dipeptides from a LAB strain. Thus, the profile of antifungal metabolites produced by *L. reuteri* R29 should be further investigated.

In this study, screening for *in vitro* antifungal activity revealed that *L. reuteri* R29 is active against all tested moulds (Table 4.2). In the wheat bread system the strain showed consistent ability to retard the growth of *A. niger*, *F. culmorum* and

P. expansum but it was not inhibitory against *P. roqueforti* (Figure 4.3). However, it is known already, that transition from *in vitro* to *in situ* system may significantly influence the biotechnological performance of LAB. LAB strains with demonstrated inhibitory activity in agar spot or microtitaer plate assays did not have the same effect in the context of wheat dough (Rosenquist and Hansen, 1998). In fact, many authors described the isolation and characterization of antifungal metabolites under laboratory conditions, whereas their application in food systems is rather limited (Dal Bello *et al.*, 2007, Gerez*et al.*, 2009, Lavermicocca*et al.*, 2000, Ryan *et al.*, 2011).

Following the successful performance of *L. reuteri* R29 as a fungal inhibitor, the research work proceeded with the aim to define the influence of the strain on dough and bread quality. Upon the addition of sourdough prepared from *L. reuteri* R29 strain, there was an increase in loaf specific volume and softer crumb over a 5-day storage period in relation to the control (Table 4.4). Our results are in agreement with those of Hammes and Gänzle (1998), Corsetti *et al.* (2000), Arendt *et al.* (2007), Ryan *et al.* (2011), where the addition of sourdough increased loaf volume. This trend is attributed to the findings obtained from the fundamental rheological tests, where the addition of sourdough yielded a less elastic and a less firm dough. As suggested by Clarke *et al.* (2002), the physicochemical changes in the protein network from the addition of sourdough may have facilitated greater expansion upon proving, due to the altogether softer, more extensible nature of the dough. In summary, the fundamental rheological study and baking test demonstrated that *L. reuteri* R29 was a suitable starter culture for wheat sourdough and bread production.

4.6 Conclusions

In conclusion, *L. reuteri* R29 strain is an efficient starter culture to produce bread of good quality with prolonged shelf-life. It inhibits the fungal growth of *Aspergillus*, *Fusarium* and *Penicillia* species, both under laboratory conditions and in pilot-scale plant. Taking into account the increasing interest in replacing synthetic preservatives with natural ones, *L. reuteri* R29 strain may be a promising bio-preservative, which could be considered for future exploitations not only in the wheat bread context, but also for other cereals or vegetables where fungal growth is a part of the storage problems.

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Chapter 5

Application of *Lactobacillus amylovorus* as an antifungal adjunct to extend the shelf-life of Cheddar cheese

5.1 Abstract

Lactobacillus amylovorus DSM 19280 is an antifungal strain which is inhibitory to a range of fungi including *Penicillium expansum*, *P. roqueforti*, *Aspergillus niger*, *A. fumigatus* and *Fusarium culmorum*. In this study, the strain was used as an adjunct culture in a Cheddar cheese model system. During the ripening period, *P. expansum* spores were applied to the cheese surface to mimic fungal contamination. The presence of the antifungal *L. amylovorus* adjunct resulted in a four-day delay in appearance of *Penicillium* growth on the cheese in comparison to the adjunct-free control. When cheeses were exposed to natural airborne fungi, the presence of the adjunct resulted in a six-day delay in the appearance of mycelia on the cheese surface. Significantly, its presence had no detectable negative impact on cheese quality as determined by analysis of moisture, salt, pH, primary proteolysis (urea-polyacrylamide gel electrophoresis) and secondary proteolysis (free amino acid analysis, HPLC). The results indicate that the strain could have an application for extending the shelf-life of cheeses which are prone to fungal spoilage.

5.2 Introduction

The microflora of Cheddar cheese, the composition of which changes during ripening, plays a central role in cheese-making. At the beginning of the cheese-making process the dominant microbial group is represented by the starter lactococci, that grow rapidly on the day of manufacture reaching high numbers (~10^9 CFU g^{-1}) (Fitzsimons *et al.*, 2001). Their numbers decline as the cheese ripens, primarily due to the adverse environmental conditions (pH, moisture, salt, temperature), present in the cheese and autolysis (McSweeney *et al.*, 1994). Most cheeses contain secondary microflora, which is dominated by adventitious

mesophilic lactic acid bacteria (LAB) referred to as non-starter LAB (NSLAB), that are capable of growing under the selective conditions of ripening cheese. Their presence is probably due to the presence of thermoduric strains that survive pasteurization or from post-pasteurization contamination, ineffective sterilization of cheese making equipment or ingredients (Fox *et al.*, 1998).

Mesophilic lactobacilli are generally the predominant NSLAB species found in Cheddar cheese, although pediococci may also be present (Fitzsimons *et al.*, 2001). Species of *L. paracasei* and *L. plantarum* are commonly found. Other species appearing as minor components of the population, include *L. curvatus*, *L. casei*, *L. brevis* and *L. rhamnosus* (Banks and Williams, 2004). These microorganisms have been shown to contribute to flavour development in some varieties of cheese and may thus be desirable, even if some authors have demonstrated that NSLAB can also develop off flavours and defects in cheese (Lane *et al.*, 1997). In addition, the intentional use of other microorganisms, added as secondary microflora (e.g. *Penicillium roqueforti*, *P. camemberti*, *Brevibacterium linens* or *Propionibacterium freudenreichii*) is sometimes desirable, with the aim to impart particular flavour and aroma properties which give certain cheese varieties their unique characteristics.

As the starter culture decreases there is an increase of the NSLAB counts that vary from a low number (~10 to 10^4 CFU g^{-1}) in 1-day-old Cheddar cheese manufactured from pasteurized milk, to 10^7 - 10^8 CFU g^{-1} in a cheese after three months of ripening (El Soda *et al.*, 2000).

Incorporation of mesophilic lactobacilli, generally specifically selected NSLAB strains, as adjunct cultures in cheese production has industrial and commercial value and remains an important focus of research interest

(Georgieva *et al.*, 2009). The significance of adjunct cultures is primarily to improve cheese quality in some way (Milesi *et al.*, 2008b), perhaps being used to suppress the growth of undesirable, adventitious microorganisms (Banks and Williams, 2004), accelerate ripening, increase aroma and flavour intensity (Fox *et al.*, 1998, El Soda *et al.*, 2000, Azarnia, 2006), or provide probiotic status (Bernardeau *et al.*, 2008).

Dairy products are an excellent substrate for the growth of spoilage fungi. The most frequently isolated fungi from cheese include *Alternaria*, *Aspergillus*, *Cladosporium*, *Eurotium*, *Fusarium*, *Mucor*, *Penicillium* and *Phoma* (Basílico *et al.*, 2001). Research has shown that some LAB have pronounced antifungal activity under laboratory conditions; however, little work has been performed on the application of antifungal LAB in cheese. Miescher-Schwenninger and Meile (2004) reported the antifungal activity of mixed cultures of *Propionibacterium jensenii* and *Lactiobacillus paracasei* subsp. *paracasei* against fungi such as *Candida pulcherrima*, *C. magnoliae*, *C. parapsilosi*, *Zygosaccharomyces bailii* in yogurt and cheese. Recently, Muhialdin *et al*. (2011) studied *A. niger* and *A. oryzae* growth inhibition by *Lactobacillus fermentum* Te007, *L. pentosus* G004 and *L. paracasei* D5 and *Pediococcus pentosaceus* Te010 on commercial processed cheese slices. However, no studies have been performed on the use of antifungal LAB as adjunct cultures in cheese.

This study deals with the use *L. amylovorus* strains of non-dairy origin as adjunct cultures in cheese production, the objectives being to assess the technological properties of the antifungal strain *L. amylovorus* DSM 19280 and *L. amylovous* DSM 20531, to evaluate its effect on the compositional and biochemical properties and proteolysis of cheese and, in particular, to investigate the inhibitory

activity of the strain against *Penicillium*, the predominant genus involved in spoilage of Cheddar-type cheeses.

5.3 Materials and Methods

5.3.1 Cultures and growth conditions

The antifungal strain *L. amylovorus* FST 2.11 patented as DSM 19280 (Arendt *et al.*, 2009; Arendt *et al.*, 2011) was originally isolated from a cereal environment. The strain *L. amylovorus* DSM 20531 was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) and chosen as an antifungal-negative control. Both *Lactobacillus* strains were grown on MRS agar plates (Oxoid, Basingstoke, Hampshire, UK) supplemented with 0.05 g/L bromocresol green (Sigma Aldrich Chemie Gmbh, Steinheim, Germany) at 30°C for 2 days under anaerobic conditions. Working cultures were kept on MRS agar plates at 5°C, while long-term storage was at -80°C in a glycerol/MRS (1:1) solution. MRS broth (Oxoid, Basingstoke, Hampshire, UK) was used as liquid growth medium. The starter culture used was *Lactococcus lactis* R604 DVS (Chr. Hansen, Cork, Ireland).

Penicillium expansum FST 4.22 (Ryan *et al.*, 2011) was chosen as a representative cheese spoilage fungus. The mould was cultivated on malt extract agar (MEA) (Oxoid, Hampshire, UK) at 25°C for 2 to 5 days and stored at 5°C. The spores were collected by brushing the plate surface with physiological solution and counted using a Thoma chamber haemocytometer.

5.3.2 *In vitro* antifungal activity assay

The inhibitory activity of the strains against *P. expansum* was investigated on milk agar plates prepared as follows: 100 g of skimmed milk was dissolved in 700 mL of distilled water. Fifteen grams of agar agar (Merck, Darmstadt, Germany) and 0.05 g of bromocresol green were suspended in 300 mL of water in a separate flask. Milk and agar were autoclaved at 110 °C for 10 min, mixed together and poured into Petri dishes. Overnight cultures of the *Lactobacillus* strains (10^9 CFU mL^{-1}) were placed as 5 µL cell spots on the milk agar and incubated at 30°C for 5 days under anaerobic condition. To test antifungal activity against spoilage mould, plates were overlaid with 10 mL of malt extract soft agar (0.7% agar) containing 10^4 fungal spores per mL. The plates were incubated aerobically at room temperature for 48 h and the antifungal activity was measured as clear zones of inhibition around the bacterial spots.

5.3.3 LC-FTMS analyses of antifungal compounds

The antifungal compounds produced by *L. amylovorus* DSM 19280 grown in MRS broth were measured by the method described by Brosnan, *et al.* (2012). Briefly, a 48 h *L. amylovorus* DSM 19280 culture samples (50 mL) were centrifuged (7,412 g, 10 min, Supra 22K centrifuge, Hanil Science Industrial, Korea), filtered (autoclave filter 0.45 µm, Teknokroma, Barcelona, Spain) and extracted (10 mL aliquot) three times with ethyl acetate (10 mL x 1, 5 mL x 2). Combined ethyl acetate fractions were pooled and dried under nitrogen to dryness (Turbo Vap LV, Zymark, USA). The samples were reconstituted with H2O/ ACN 9/1 (10% ACN was added first and mixed by vortex to ensure fatty acids were dissolved and 90% water was then added). The reconstituted sample (1 mL) was then filtered (0.2 µm filter,

Chromafil PET, 20/15MS, Macherey-Nagel, Düren, Germany) and placed into an amber vial (1.5 mL, Chromacol, Lab Unlimited, Dublin, Ireland) for injection (10 μL) onto the LC-FTMS (hybrid LTQ Orbitrap XL FTMS, Thermo Scientific, UK) instrument.

The chromatographic method was developed on a Thermo Accela LC system (Hemel Hampstead, UK) using a Gemini C18 (150 x 2 mm, 5 mm; Phenomenex) column equipped with a Security Guard cartridge (C18, 4 x 2 mm; Phenomenex). A flow rate of 0.3 mL min^{-1} was used and the column was maintained at 30 °C. A short stepped gradient was employed to ensure adequate separation of isobaric compound from the suite of twenty-five target analytes. The twenty-five target standards included their $[M-H]^-$ theoretical masses are listed as follows: A, cytidine (*m/z* 242.07770 $[M-H]^-$); B, 2–deoxycytidine (*m/z* 226.08278 $[M-H]^-$); C, glucuronic acid (*m/z* 193.03483 $[M-H]^-$); D, DL-*ρ*-hydrophenyllactic acid (*m/z* 181.05008 $[M-H]^-$); E, 1,2–dihydroxybenzene (*m/z* 109.02895 $[M-H]^-$); F, 3,4-dihydroxyhydrocinnamic acid (*m/z* 181.05008 $[M-H]^-$); G, 4–hydroxybenzoic acid (*m/z* 137.02387 $[M-H]^-$); H, caffeic acid (*m/z* 179.03443 $[M-H]^-$); I, vanillic acid (*m/z* 167.03443 $[M-H]^-$); J, (S)-(—)-2-hydroxyisocapric acid (*m/z* 131.07082 $[M-H]^-$); K, 3-(4-hydroxyphenyl)-propionic acid (*m/z* 165.05517 $[M-H]^-$); L, 3-(4-hydroxy-3-methoxyphenyl) propionic acid (*m/z* 195.06573 $[M-H]^-$); M, *ρ*-coumaric acid (*m/z* 163.03952 $[M-H]^-$); N, ferulic acid (*m/z* 193.05008 $[M-H]^-$); O, azelaic acid (*m/z* 187.09703 $[M-H]^-$); P, phenyllactic acid (*m/z* 165.05517 $[M-H]^-$); Q, benzoic acid (*m/z* 121.02895 $[M-H]^-$); R, hydrocinnamic acid (*m/z* 149.06025 $[M-H]^-$); S, methylcinnamic acid (*m/z* 161.06125 $[M-H]^-$); T, 3-hydroxydecanoic acid (*m/z* 187.13342 $[M-H]^-$); U, DL-β-hydroxylauric acid (*m/z* 215.16472 $[M-H]^-$); V, decanoic acid(*m/z* 171.13850 $[M-H]^-$); W, DL-β-hydroxymyristic acid (*m/z*

243.19602 [M-H]$^-$); X, 2–hydroxydodecanoic acid (*m/z* 215.16472 [M-H]$^-$); and Y, salicylic acid (*m/z* 137.02387 [M-H]$^-$). The stepped gradient mobile phase consisted of A) water (HPLC grade) with 0.1% acetic acid (ACN) and B) acetonitrile (HPLC grade) with 0.1% ACN, the gradient conditions were as follows: 0 minutes – 10% B, 3 minutes – 10% B, 10 minutes – 95% B, 13 minutes – 95% B.

LC-FTMS analysis involved coupling the Accela LC to the Thermo LTQ Orbitrap XL hybrid mass spectrometer (Hemel Hampstead, UK). The instrument was operated in negative ionisation mode at 30,000 resolution. Method tune conditions were as follows; capillary temperature of 300 °C, capillary voltage -50 V, tube lens -110 V, sheath gas 45 bar and auxillary gas 15 bar. The instrument was calibrated weekly as per manufactures instructions to ensure accurate high mass data (< 2 ppm) were obtained for all targeted analytes. A lock mass for acetic acid (*m/z* 59.013840) present consistently in the mobile phase was applied to the tune method to ensure reproducible instrument high mass accuracy during each gradient chromatographic run and during sample long sequences.

5.3.4 HPLC analysis of weak organic acids

Organic acids present in 48 h fermented MRS broth were determined as described in Chapter 3 under the section 3.3.5.

5.3.5 Miniature Cheddar cheese manufacture

Miniature Cheddar-type cheeses were manufactured under aseptic conditions from 200 mL batch-pasteurized milk using the procedure of Milesi *et al.* (2008a). Raw milk was obtained from a dairy farm (Cork, Ireland) and pasteurised at 63 °C for 30 min. Cheeses were prepared by incorporating one of three different treatments

as follows; cheeses containing starter culture alone (control cheeses), cheeses containing starter and *L. amylovorus* DSM 19280 as adjunct culture, and cheeses containing starter and *L. amylovorus* DSM 20531 as adjunct. Eighteen cheeses were produced in each treatment, with cheese-making carried out over a number of different days. Fresh adjunct cultures were grown for 18 hours, washed once in phosphate buffer saline (PBS) and inoculated to achieve a final concentration of 10^5 CFU mL^{-1} in the cheese-milk. Starter culture was added to cheese-milk at a level of 0.03% in direct vat set (DVS) form (concentration of 10^7 CFU mL^{-1}). Manufacture was performed under a laminar air-flow hood using sterile utensils on each cheese-making day. Cheeses were salted by immersion in sterile brine (20% NaCl, 0.05% $CaCl_2 \cdot H_2O$, 30 min at room temperature) and then wiped dry, vacuum packed and ripened at 8 ^oC for 90 days.

5.3.6 Microbiological analysis of cheeses

Cheese samples (2 g) were homogenised in 18 mL of 2% (w/v) tri-sodium citrate solution for microbiological analysis and serial dilutions were prepared in tubes containing sterile quarter-strength Ringer's solution. Microbial cell counts were performed in duplicate on days 7, 14, 30, 60 and 90 of ripening. Starter population was determined on lactose M17 (LM17) agar (Merck, Darmstadt, Germany) after incubation at 30 °C for 3 days. The adjunct cultures were enumerated on MRS agar after incubation at 30 °C for 2 days under anaerobic conditions, while NSLAB were enumerated under anaerobic conditions on Rogosa agar (Merck, Darmstadt, Germany) after incubation at 30 °C for 5 days. To obtain preliminary evidence that colonies counted as presumptive adjunct actually belonged to the *L. amylovorus* species, randomly selected colonies from a countable MRS

plate were subsequently grown on selective MRS agar supplemented with 0.05 g/L bromocresol green and their colony colour, morphology and size were examined. In addition, 5 colonies were randomly selected from a countable plate, grown in MRS broth, and following 48 h incubation at 30°C, cell-free supernatants were then analysed by HPLC for organic acids content. Comparison was made with the organic acids pattern obtained from a pure culture of the *L. amylovorus* strains.

5.3.7 Gross composition of cheeses

The pH values of the cheeses were measured in duplicate at 7, 14, 30, 60 and 90 days by inserting a combination electrode connected to a pH meter (Orion, model 720, Orion Research Inc, Boston, MA, USA) into 1:10 cheese slurry. Samples of 30-day-old cheeses were analysed in triplicate for moisture (oven drying at 103 ± 1°C, IDF, 1982) and salt (Fox, 1963).

5.3.8 Assessment of proteolysis

Peptide profiles of pH 4.6-soluble fractions (Kuchroo and Fox, 1982) from 90-day-old cheeses were obtained by reverse-phase HPLC using a system which consisted of a Waters Acquity UPLC H-Class Core System with an Acquity UPLC TUV Detector (dual wavelength) and Acquity Column Heater 30-A. The system was interfaced with Empower 3 software (WatersCorp, Milford, MA, USA). The core system included an Acquity UPLC H-Class quaternary solvent manager, a H-Class Sample Manager-FTN and a CH-A column heater. The column used was an Acquity UPLC BEH C18 1.7µm, 2.1 x 50 mm column. Elution was monitored at 214 nm using a mobile phase of two solvents, A, 0.1% (v/v) trifluoroacetic acid (TFA, sequential grade, Sigma, St Louis, MO, USA) in deionized HPLC grade water

(Milli-Q system, WatersCorp.) and B, 0.1% (v/v) TFA in acetonitrile (HPLC grade, Lab-scan Ltd, Dublin, Ireland). Samples of freeze-dried soluble fractions of the pH 4.6-soluble extracts from cheese were dissolved in solvent A (10 mg mL^{-1}). The samples were centrifuged at 15,000 *g* for 10 min. The samples were then filtered through a 0.45 μm cellulose acetate filter (Sartorius GmbH, Gottingen, Germany) and an aliquot (6.9 μL) of the filtrate was injected into the column at an eluent flow rate of 0.46 mL min^{-1}. Separation was achieved using the following gradient: 100% A for 0.37 min, 50% B (v/v) from 6.6 to 7.28 min. The column was washed with 95% B (v/v) followed by equilibration with 100% A before the next injection. Samples were run three times to measure the reproducibility of the patterns in the above conditions.

Casein degradation was assessed by electrophoresis in polyacrylamide gels (urea-PAGE) (12.5% T, 4% C, pH 8.9) on 90-day-old cheeses using a Protean II vertical slab-gel unit (Bio-Rad Laboratories Ltd, Watford, Herts, UK) according to the method of Andrews (1983) with modifications. Gels were stained directly with Coomassie Brilliant Blue G250 and destained in distilled water until the background was clear, as described by the method of Blaksely and Boezi (1977). Free amino acids (FAA) were determined by trinitrobenzene-sulphonic acid method (TNBS) (Adler-Nissen, 1979). The calibration curve was prepared using leucine (Sigma, St Louis, MO, USA) as standard (range 0.0-1.0 mM L^{-1} of Leu), and results were expressed as mg Leu per cheese.

5.3.9 Antifungal activity of *L. amylovorus* in cheese

L. amylovorus DSM 19280 in cheese was challenged against *P. expansum* FST 4.22, which was chosen as indicative mould commonly isolated from cheese

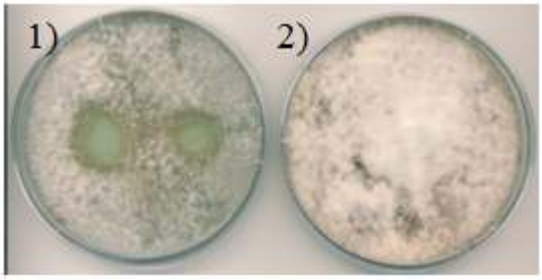
(Basílico *et al.*, 2001, Taniwaki *et al.*, 2001). One hundred µL solution containing 10^4 fungal spores were spread on both sides of 1-week-old cheese. Each cheese was then vacuum packed and heat sealed. A small slot was opened and a filtertip was inserted to ensure comparable aerobic conditions in each bag. The fungal contamination was observed throughout 30 days (cheeses were stored at 5 °C). Three miniature Cheddar-type cheeses were inoculated. A cheese was considered moldy if more than 1% of the total surface area was covered with fungus. A second challenge test was performed in order to evaluate the ability of *L. amylovorus* DSM 19280 to inhibit the outgrowth of airborne mould. Both side of three cheeses were exposed to ambient air for 5 min. Again, each cheese was packaged in a plastic bag and monitored for mould growth as described above. Cheeses with adjunct *L. amylovorus* DSM 20531 and cheeses with no LAB adjunct, were similarly challenged and used as controls. The antifungal trials were performed in triplicate.

5.4 Results and Discussion

5.4.1 *In vitro* antifungal activity of *L. amylovorus* strains

It has been previously reported that *L. amylovorus* DSM 19280 shows antifungal activity against *A. fumigatus*, *A. niger*, *F. culmorum*, *P. expansum* and *P. roqueforti* spoilage moulds following *in vitro* screening (Ryan *et al.*, 2011). In the current study, *in vitro* antifungal activity of *L. amylovorus* DSM 19280 and DSM 20531 strains against *P. expansum* was screened on milk agar plates. As shown in Figure 5.1, strain DSM 19280 was found to be active against the target fungus as shows by the fungal-free clear zone surrounding the bacterial spots, which was 2 mm in diameter. The negative control, *L. amylovorus* DSM 20531 showed no inhibitory activity against spoilage mould.

Figure 5.1 *Antifungal activity of L. amylovorus DSM 19280 (left) and antifungal-negative control L. amylovorus DSM 20531 (right) against P. expansum on milk agar, as differentiated by measuring the zone of clearance surrounding the LAB spot*



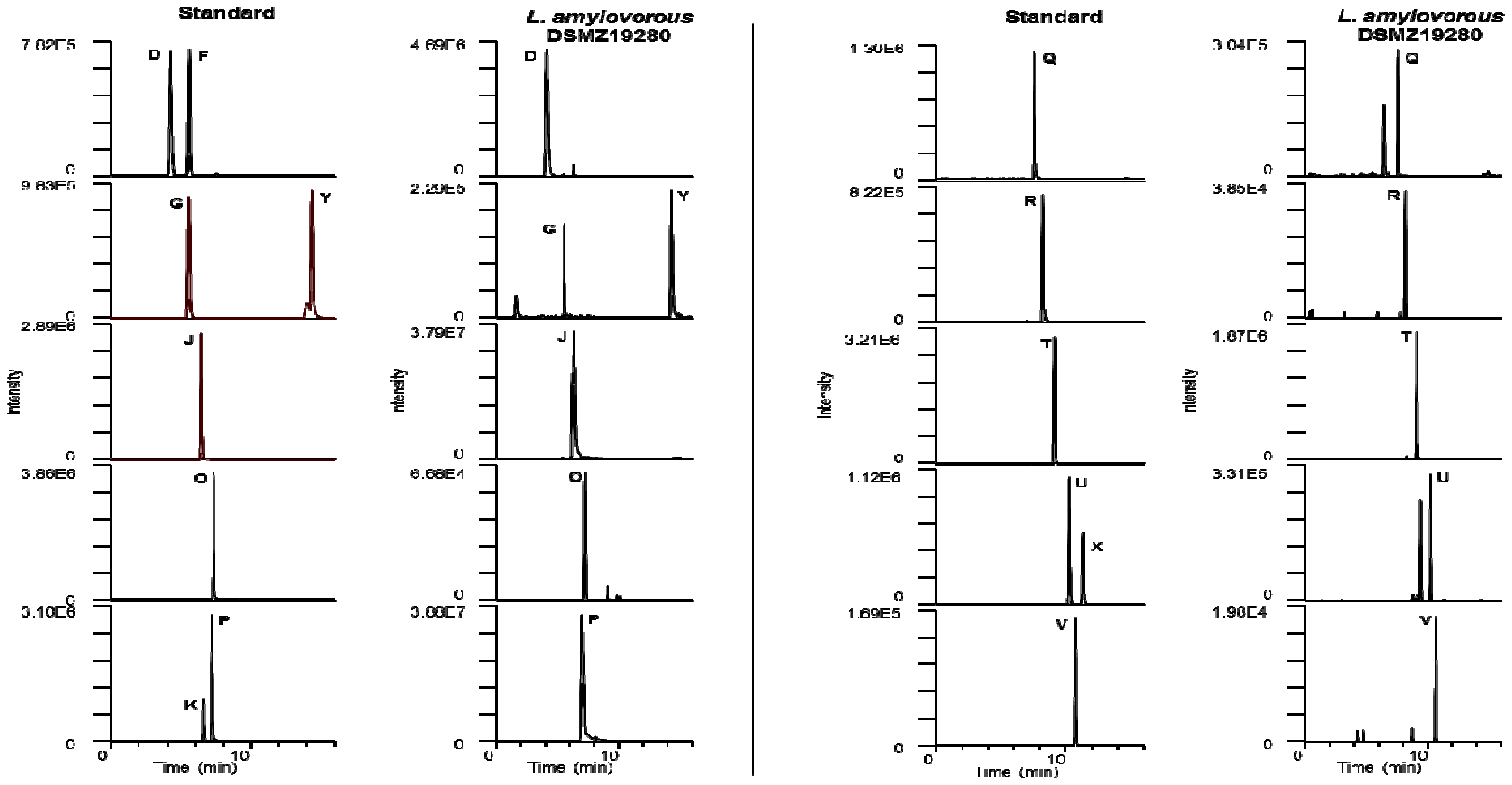
5.4.2 Identification and quantification of antifungal components

A number of antifungal compounds produced by *L. amylovorus* strain DSM 19280 have previously been profiled by Ryan *et al.* (2011), including: 3-phenylpropanoic acid, *p*-coumaric, (*E*)-2-methylcinnamic acid, 3-phenyllactic acid, 3-(4-hydroxyphenyl) lactic acid, lactic acid, acetic acid, D-glucuronic acid, salicylic acid, cytidine, deoxycytidine, sodium decanoate and cyclic dipeptides (cyclo(L-Pro-L-Pro), cyclo(L-Leu-L-Pro), cyclo(L-Tyr-L-Pro), cyclo(L-Met-L-Pro), and cyclo(L-His-L-Pro).

In this study, a different extraction protocol and LC-FTMS method (negative ionisation mode) were employed to identify further potential antifungal components produced by the strain *L. amylovorus* DSM 19280 (Brosnan *et al.*, 2012). An ethyl acetate extract of the *L. amylovorus* DSM 19280 strain was screened for the presence of 25 known antifungal compounds. The standards for these compound were sourced following consultation with peer-reviewed publications that associated these compounds with LAB strains and their antifungal activity (Ström *et al.*, 2002, Sjögren *et al.*, 2003, Broberg *et al.*, 2007, Ryan *et al.*, 2011). The compounds

detected in *L. amylovorus* strain DSM 19280 were identified by comparison of their retention times and exact masses the standards with the commercially available standards (Figure 5.2, Table 5.1). Quantification was obtained by simple calibration against corresponding standards. Compounds observed were as follows: D, DL-*ρ*-hydrophenyllactic acid; G, 4–hydroxybenzoic acid; J, (S)-(-)-2-hydroxyisocapric acid; O, azelaic acid; P, phenyllactic acid; Q, benzoic acid; R, hydrocinnamic acid; 3-hydroxydecanoic acid; U, DL-β-hydroxylauric acid; V, decanoic acid and Y, salicylic acid. Additionally compound I, vanillic acid and compound M, *ρ*-coumaric acid were detected but levels were too low (< 0.01 mg L^{-1}) to quantify. Chromatographic and spectrometric data as well as quantities of the antifungal compounds found in MRS broth are summarized in Table 5.1.

Figure 5.2 *LC-FTMS chromatograms of standard antifungal compounds and those identified in MRS fermented by L. amylovorus DSM 19280*



Peaks represent: D, DL-ρ-hydrophenyllactic acid; G, 4 – hydroxybenzoic; J, (S)-(—)-2-hydroxyisocapric acid; O, azelaic acid; P, phenyllactic acid; Q, benzoic acid; R, hydrocinnamic acid; T, 3-hydroxydecanoic acid; U, DL-β-hydroxylauric acid; V, decanoic acid, and Y, salicylic acid.

Table 5.1 *Chromatographic, spectrometric and quantitative data of antifungal components produced by L. amylovorus DSM 19280 and L. amylovorous DSM 20531*

Peak	Antifungal compound	t_R (min)	$[M–H]^-$ (*m/z*)	Concentration (mg L^{-1})	
				***L. amylovorous* DSM 19280**	***L. amylovorous* DSM 20531**
D	DL-ρ-hydrophenyllactic acid	4.10	181.0502	9.12	5.17
G	4-hydroxybenzoic acid	5.56	137.02428	0.06	0.07
J	(S)-(—)-2-hydroxyisocapric acid	6.35	131.07114	42.28	1.68
O	Azelaic acid	7.30	187.09703	0.04	-
P	Phenyllactic acid	6.98	165.05537	53.32	3.23
Q	Benzoic acid	7.56	121.02926	0.04	0.48
R	Hydrocinnamic acid	8.22	149.06061	1.8	2.41
T	3-hydroxydecanoic acid	9.12	187.13349	6.82	0.17
U	DL-β-hydroxylauric acid	10.28	215.16472	17.35	-
V	Decanoic acid	10.72	171.13873	0.128	0.09
Q	Salicylic acid	14.37	137.02428	0.396	0.37
*	Acetic acid	-	-	3400.57	2955.06
*	Lactic acid	-	-	10493.42	21331.84

20531

Values are means (n = 2): the relative standard deviations for all compounds were <6%. * Quantities detected by HPLC analysis of weak acids rather than by LCMS method.

The improved method of Brosnan *et al.* (2012) allowed for the identification of eight further compounds present in the antifungal, *L. amylovorus* DSM 19280 fermented broth, previously unidentified by Ryan *et al.* (2011). These additional compounds were: D, 4–hydroxybenzoic; I, vanillic acid; J, (S)-(-)-2-hydroxyisocapric acid; O, azelaic acid; Q, benzoic acid; T, 3-hydroxydecanoic acid; U, DL-β-hydroxylauric acid and V, decanoic acid.

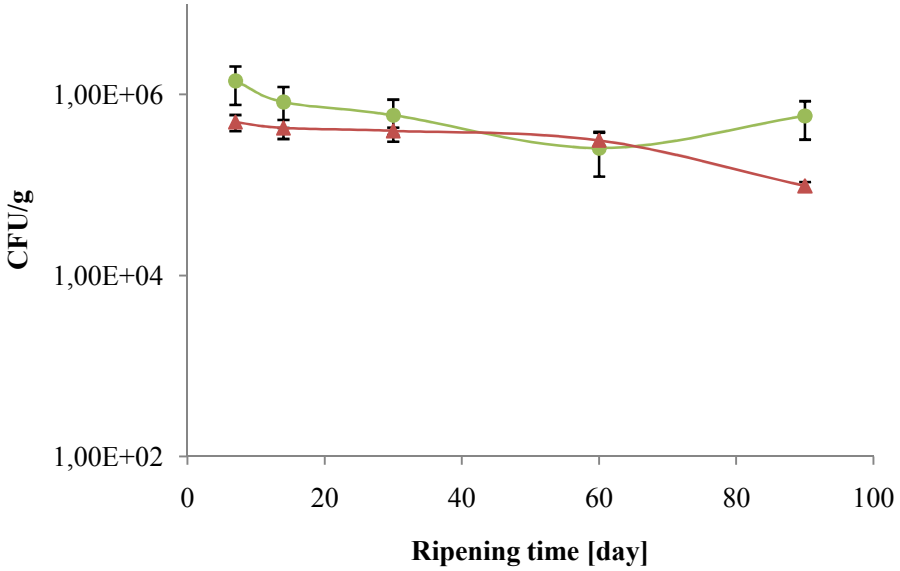
An hypothesis exists that the overall anti-fungal activity of lactic acid bacteria exists due to the combined synergy of multiple compounds. The antagonistic

and synergistic effects of different mixtures of identified compounds was demonstrated in LAB strains (*Lactobacillus sanfrancisco, Lactobacillus plantarum, Weissella cibaria*) by Corsetti *et al.* (1998), Niku-Paavola *et al.* (1999) and Ndagano *et al.* (2011) respectively. These studies revealed that the collective activity of the known compounds is much higher than the sum of their individual activities. Increasing the ability to detect and quantify both known and unknown compounds in antifungal (e. g., *L. amylovorus* DSM 19280) fermented broth will help to provide more insight into possible reasons for the observed antifungal activity of these strains.

5.4.3 Microbiological analyses and cheese composition

Strains of *L. amylovorus* DSM 19280 and DSM 20531 were tested as adjunct cultures in a miniature Cheddar-type cheese model. Cheese without added *Lactobacillus* adjunct was used as a control. In all cheeses, the growth of the starter lactococcal population exhibited a similar trend and showed a typical decrease during ripening (data not shown). In cheeses made with or without adjunct cultures, this typical starter growth was observed, suggesting that the addition of *L. amylovorus* strains did not influence its growth and survival. The growth of the non-starter adjunct lactobacilli during ripening is shown in Figure 5.3. *L. amylovorus* DSM 19280 and DSM 20531 were added to the respective cheese-milks at 10^5 CFU mL^{-1} and were at similar levels at the end of the ripening period. The control cheeses without added adjuncts were free from lactobacilli. This shows the efficacy of the aseptic work conditions used to avoid contamination.

Figure 5.3 *Microbial counts of non-starter adjunct lactobacilli in MRS agar in miniature Cheddar-type cheeses after 7, 14, 30, 60, and 90 d of ripening. Cheeses made using antifungal adjunct L. amylovorus DSM 19280 (●) and L. amylovorus DSM 20531 (▲).*



To obtain preliminary evidence that the colonies which were counted as presumptive adjunct belonged to the species *L. amylovorus*, they were grown on MRS agar supplemented with bromocresol green and identified according to their colony colour, morphology and size. In addition, five colonies were randomly selected from a countable plate and grown in MRS broth. After 48 h incubation at 30°C, cell-free supernatants were analysed by HPLC for their organic acid content and profile. Comparison was made with the organic acid profile obtained from a pure culture of the *L. amylovorus* strains (data not shown) and was consistent.

Table 5.2 presents the compositional analysis of all cheeses. Both cheeses with added LAB adjunct cultures had a similar pH and composition to the control cheeses. However, cheeses made with either *L. amylovorus* strain had higher levels of moisture than the control cheeses. This phenomenon was also observed with Cheddar cheese produced with *L. plantarum* strains (Ciocia, 2010). Nevertheless, Lane *et al.*, 1997 demonstrated that within the ranges typically found in Cheddar cheese, the water and salt content, and pH have little effect on the growth of NSLAB. Increased moisture has been shown to have a positive effect on cheese texture (Mozzi *et al.*, 2006).

Table 5.2 *Biochemical analysis of 90-day-old cheeses*

Cheese adjunct	pH	Salt (%)	Moisture (%)	FAA* (mg Le/cheese)
L. amylovorus DSM 19280	5.46±0.2	1.87±0.67	39.2±1.44	7.90±3.75
L. amylovorus DSM 20531	5.4±0.16	1.79±0.25	37.86±0.57	8.25±2.14
Adjunct-free control	5.38±0.37	1.66±0.77	28.62±7.66	6.37±2.79

Values are means (n = 3)
*FAA; Free amino acids

5.4.4 Antifungal activity of *L. amylovorus* DSM 19280 in cheese

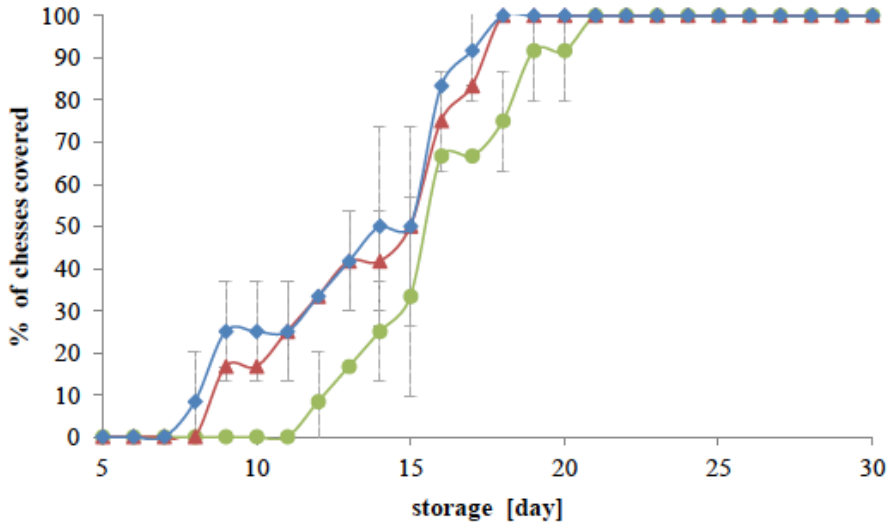
The impact of the antifungal strain *L. amylovorus* DSM 19280 on the shelf-life of miniature Cheddar cheese was evaluated. Comparison with the spoilage rate of the control cheeses revealed that the addition of *L. amylovorus* DSM 19280 to the cheese retarded, to varying degrees, the outgrowth of both the indicator and environmental fungi (Table 5.3). When this strain was included as an adjunct culture in cheeses, the growth of the target mould, *P. expansum* was inhibited for 12 days, which was 4 days longer than the control cheeses containing no adjunct. Additionally, the shelf-life of cheese which contained the DSM 19280, strain when

challenged against airborne, environmental mould from a pilot-scale plant, was up to 18 days while control cheeses spoiled after 12 days. It is worthy of mention that the control antifungal-negative strain *L. amylovorus* DSM 20531, did show a low level of antifungal activity by increasingthe shelf-life of the cheese by 1 day against *P. expansum* and by 4 days against environmental mould flora (Figure 5.4). This is not unexpected as all LAB do exhibit some level of inhibitory activity towards fungi, likely due to acid production, albeit weaker than the more pronounced antifungal strains like *L. amylovorus* DSM 19280. Overall, the use of the antifungal strain resulted in a significant extension of shelf-life.

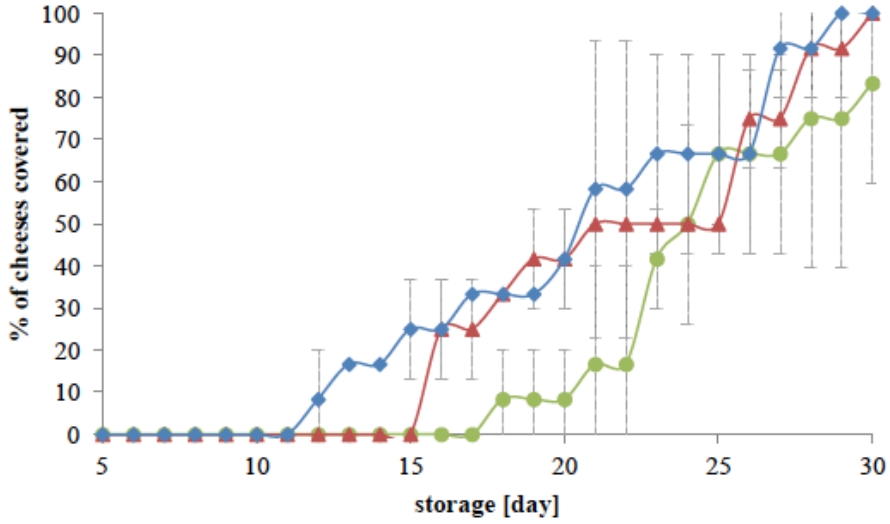
Table 5.3 *Shelf-life of cheeses made with antifungal adjunct L. amylovorus DSM 19280 and L. amylovorus DSM 20531 compared with adjunct-free control cheeses*

Cheese adjunct	**Day of the first mould growth**	
	Airborne contamination test	**Indicator contamination test with *P. expansum***
L. amylovorus DSM 19280	18	12
L. amylovorus DSM 20531	16	9
Adjunct-free control	12	8

Figure 5.4 *Growth of P. expansum (A) and airborne mould (B) on the surface of miniature Cheddar cheeses. Cheese produced using antifungal adjunct L. amylovorus DSM 19280 (●), L. amylovorus DSM 20531 (▲), control adjunct-free cheese (◆).*



A



B

Values are means (n = 18)

Several authors have previously investigated the isolation and characterisation of antifungal components from LAB (Lavermicocca *et al.*, 2000, Magnusson and Schnürer, 2001, Mandal *et al.*, 2007, Sjörgen *et al.*, 2003, Ström *et al.*, 2002). However, the number of studies including food applications of antifungal LAB strains is rather limited, particularly with regard to dairy foods, and methodologies vary, which makes comparison to our findings difficult. In cheese, investigations of Miescher-Schwenninger and Meile (2004) concentrated on the antifungal activity of mixed cultures of *L. paracasei* ssp. *paracasei* and *Propionibacterium jensenii* against fungi such as *Candida pulcherrima*, *C. magnoliae*, *C. parapsilosi*, *Zygosaccharomyces bailii*. Muhialdin *et al.* (2011) studied the inhibition of the growth of *A. niger* and *A. oryzae* by *Lactobacillus fermentum*, *L. pentosus, L. paracasei* and *Pediococcus pentosaceus* on commercial processed cheese slices. In addition, no previous research has used antifungal LAB strains as adjuncts in cheese-making for their bio-preservative effects.

5.4.5 Proteolysis during cheese ripening

Proteolysis was measured as pH 4.6 soluble N, RP-HPLC of pH 4.6 soluble N, TNBS and by urea-PAGE.

The concentration of total free amino acids (FAA) in the pH 4.6-soluble extracts of control and experimental cheeses was determined by the trinitrobenzenesulfonic acid (TNBS) method and expressed as mg Leu per cheese (Table 5.2). Experimental cheeses made with strains *L. amylovorus* DSM 19280 and *L. amylovorus* DSM 20531 demonstrated slightly higher FAA content than the control cheeses. This trend has been previously observed and this may be due either

to increased peptidase activity in cheeses made with adjuncts (Broome *et al.*, 1990, Lynch *et al.*, 1996) or reduced utilization of FAA by the adjunct lactobacilli (Sasaky *et al.*, 1995, Lynch *et al.*, 1996, Lynch *et al.*, 1998, Hynes *et al.*, 2001). FAA have the potential to contribute compounds that may affect the flavour and aroma of cheese (Smit *et al.*, 2005), however, this would be better investigated in large-scale cheese trials where cheese flavour can be accurately assessed.

No differences in the peptide profiles of the cheeses made with or without adjunct lactobacilli were observed after 90 days of ripening, and HPLC chromatograms of the pH 4.6-soluble fraction were similar for all cheeses (data not shown). The *L. amylovorus* adjuncts, from a qualitative point of view, did not affect the level of secondary proteolysis in the cheeses.

Assessment of primary proteolysis by urea-PAGE of the pH 4.6-insoluble extracts demonstrated no differences between the control and the experimental 90-day-old cheeses (data not shown). The results obtained present a similar pattern to those reported by McSweeney *et al.*, 1994, Lynch *et al.*, 1998, Di Cagno *et al.*, 2006, and Ciocia (2010) and suggest that *Lactobacillus* adjuncts are not involved in primary proteolysis in cheese and that the rennet is primarily responsible for the level of proteolysis detectable by urea-PAGE.

Taken together, these results suggest that the adjunct *L. amylovorus* cultures are unlikely to negatively impact on cheese quality. This is critical as protective cultures used for preservation should not affect the technological properties of an established product (Muhialdin *et al.*, 2011).

5.5 Conclusions

The antifungal activity of *L. amylovorus* DSM 19280 has previously been demonstrated in laboratory assays and used in cereal products (Arendt *et al.*, 2009, Arendt *et al.*, 2011, Ryan *et al.*, 2011, Belz *et al.*, 2012). In this study, it has been demonstrated that this antifungal strain can be used as an adjunct culture in Cheddar cheese production. It enhances the cheese quality in terms of shelf-life without changing its composition, biochemical properties or affecting proteolysis. These findings reinforce the potential of *L. amylovorus* DSM 19280 as a natural preservative, which could be easily added by the food fermentation industry. The 6-day shelf-life extension observed may be highly advantageous for dairy products, particularly in the context of environmental fungi.

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Chapter 6

Lactic acid bacteria with antifungal activity and their application in a novel wort-based beverage

6.1 Abstract

Two strains of *L. brevis* were screened for their antifungal activity. *L. brevis* PS1 showed to be active against *F. culmorum in vitro* by the overlay method. *L. brevis* NS strain did not inhibit the fungal growth and was chosen as a negative control. The cell-free supernatant of MRS broth fermented by the *L. brevis* strain PS1 was analysed by means of LCMS and twelve known antifungal compounds were identified. An unhopped barley wort was produced in a pilot scale brewing facility. The wort was inoculated with both lactic acid bacteria (*L. brevis* PS1 and *L. brevis* NS) and the fermentation was monitored. Special emphasis was placed on the shelf-life of the beverage. Challenge tests with *Fusarium culmorum* were performed to determine the effectiveness of the antifungal strain. The shelf-life was monitored over a 14 day period and no fungal growth was detected in the sample containing the antifungal lactic acid bacteria, whereas the sample containing the control strain (*L. brevis* NS) or wort alone showed fungal growth after 2 and 1 day, respectively. LCMS analysis was also used to quantify the antifungal compounds produced by *L. brevis* PS1 in comparison to the control (*L. brevis* NS).

6.2 Introduction

Lactic acid bacteria (LAB) are among the most important groups of microorganisms used in food fermentations. They contribute to the taste and texture of fermented products and inhibit food spoilage bacteria by producing growth-inhibiting substances. As agents of fermentation, LAB are involved in making yogurt, cheese, cultured butter, sour cream, sausage, cucumber pickles, olives and sauerkraut. LAB are also omnipresent in the indigenous microbiota of cereals like barley, wheat, rice, maize, oat, and thus, are involved in fermentation of cereal-based

foods and beverages as well. Cereal fermentations generally differ from most other fermentations because the final product is often not consumed but further processed to beer, bread, crackers etc. (de Valdez *et. al.*, 2010). Contrastingly, several cereals have been consumed for generations in traditional mixed fermentations producing staple food beverages worldwide from a variety of raw materials. Drinks like *cicha*, *boza*, *busa*, *busaa*, *tobwa*, *kaffir beer*, *mahewu*, *sake*, *soybean milk*, *uji*, etc. are widely consumed in Asia and Africa (Blandino *et al.*, 2003), where they are of particular importance since they are shelf stable, have a pleasant taste and lead to improved nutritional properties of the cereal based beverages.

In the Western world, cereal- and pseudocereal-based beverages fermented by LAB are becoming more popular. Commercially available are for example the B. E. Grainfields Wholegrain Liquid® and Proviva®. In addition to the commercially available cereal based beverages, researchers have published on emmer (Coda *et al.*, 2011) and oat based beverages (Angelov *et al.*, 2006, Gupta *et al.*, 2010). LAB have also been extensively used in the malting and brewing industry. An example of applying LAB in malting is the development of LAB starter cultures for use as inoculants during the malting process in order to improve the quality of the malt. Beneficial effects due to LAB addition included increased malt yields, lower viscosity and β-glucan content of wort and improvement of mash filtration and wort filterability were observed by Haikara and Laitila (1995), Laitila *et al.* (2006), Lowe *et al.* (2005), Mauch *et al.* (2011). Moreover, the use of antifungal starter cultures in the early stage of malting can inhibit the growth of undesirable moulds and the production of mycotoxins. Studies of Boivin and Malanda (1997) demonstrated how the use of starter cultures could improve the safety of the malt when applied to the steeping water during malting. The investigations of Lowe *et al.* (2004) showed that

Fusarium spores in the presence of LAB did not negatively affect the quality of malt and wort. In a different study, Haikira *et al.* (1993) employed *Lactobacillus plantarum* and *Pediococcus pentosaceus* as naural preservatives during malting to inhibit the growth of *Fusarium* moulds to prevet gushing. In fact, gushing, defined as the quick, uncontrolled, spontaneous over-foaming when opening the bottle or can, is one of the most negative consequences of mould contamination in regard to the quality of malt and beer (Amaha and Kitabatake, 1981). *Fusarium culmorum*, *F. graminearum*, *F. poae* have been proven to be the most active gushing inducers (Haikara and Home, 1991, Munar and Sebree, 1997, Niessen *et al.*, 1992, Schwarz *et al.*, 1996). In addition to this, production of *Fusarium* toxins during malting and their passage into beer have been demonstrated (Schwarz *et al.*, 1995). Consumption of products contaminated with mycotoxins has been associated with a wide range of negative effects for humans (Pawlowska *et al.*, 2012).

In the brewery, LAB starter cultures are mainly used for the production of acidified mash and wort. Reduction of pH to 5.4 of mash and pH 5.2 for wort by biological acidification improves enzymes activity, thus resulting in better processing and better quality of final beer. The advantages of biological acidification were demonstrated by many authors. Pittner and Back (1995) observed better aroma profile and lower risk of protein haze due to extensive protein breakdown. Lewis and Young (1995) reported improved flavour of beer, a shorter mashing period and more efficient enzymatic processes when uniformly modified malt was acidified. Lowe *et al.*, 2004, noted that the addition of LAB to bioacidify mash containing 50% raw barley and 50% malt resulted in faster filtration times, higher free amino nitrogen levels, lighter wort colour and increased extract levels.

As mentioned above, LAB can act as natural antifungal agents. Their preserving effect is due to production of active antimicrobials, like organic acids, carbon dioxide, fatty acids, hydrogen peroxide, phenolic and proteinaceous compound (Amanatidou *et al.*, 1999, Corsetti *et al.*, 1998, Mandal *et al.*, 2007, De Vuyst and Leroy, 2007, Sjörgen *et al.*, 2003, Ström *et al.*, 2002, Venturini *et al.*, 2002). Thus, considering altogether, LAB antimicrobial potential and consumers' attitude for healthy and nutritious foods, LAB could be an alternative to chemical preservatives commonly used by food producers.

L. brevis species has been already reported in the literature as having antifungal activity (De Muynck *et al.*, 2004, Coda *et al.*, 2008, Gerez *et al.*, 2009, Falguni *et al.*, 2010). The strain *L. brevis* PS1 has been recently described by Mauch *et al.*, 2010 as antifungal agent against *Fusarium* species. Authors studied the antifungal activity of the strain against *F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae* and *F. tricinctum* by the overlay method, investigated the impact of bacterial supernatant on growth of *F. culmorum* as the target organism and characterized the nature of antifungal components, suggesting that the strains produces organic acids and proteinaceous compounds. However, no single compounds were indicated as responsible for inhibitory activity. Thus, the aim of present study was to characterize the antifungal metabolites produced by *L. brevis* PS1 strain and exploit the strain in production of a novel functional barley malt wort-based, alcohol-free beverage with extended shelf-life.

6.3 Material and Methods

6.3.1 Cultures and growth conditions

The antifungal strain *L. brevis* PS1 and the negative control strain *L. brevis* NS (School of Food and Nutritional Sciences, University College Cork, Cork, Ireland) were isolated from bovine intestinal samples and cheese, respectively (Mauch *et al.*, 2010). Both *Lactobacillus* strains were grown on MRS agar plates (Oxoid, Hampshire, UK) at 30°C in anaerobic conditions. Working cultures were kept on MRS agar plates at 5°C, while long-term storage was done at -80°C in a glycerol/water (1:1) solution. MRS broth (Oxoid, Hampshire, UK) was used as liquid growth medium.

Fusarium culmorum FST 4.05 (School of Food and Nutritional Sciences, University College Cork, Ireland) was chosen as representative spoilage fungi. The mould was cultivated on malt extract agar (MEA) (Oxoid, Hampshire, UK) at 25°C for 2 to 5 days and stored at 5°C. The spores were collected after brushing the plate surface with physiological solution. Spores were transferred from this stock solution into a synthetic nutrient-poor medium (Nirenberg, 1976). Vigorous stirring (200 rpm) for 8 days at room temperature provided a fungal cell and conidial suspension with a concentration of 5×10^5 spores mL^{-1}. Mould spores were counted using a Thoma chamber haemacytometer.

6.3.2 *In vitro* antifungal activity

The inhibitory activity of *L. brevis* PS1 against *F. culmorum* was investigated using the overlay method (Magnusson and Schnürer, 2001) with some modifications. *L. brevis* NS was used as a negative control. *Lactobacillus* strains overnight cultures

were placed as 5 μL cell spots on the MRS agar modified as follows (mMRS): pH adjusted to 6.0, sodium acetate as well as potassium dihydrogenphosphate omitted. The plates were incubated at 30°C for 48 h in anaerobic jars. To test antifungal activity against spoilage mould, plates were overlaid with 10 mL of malt extract soft agar (0.7% agar) containing 10^4 spores mL^{-1}. The plates were incubated aerobically at room temperature for 48h and then examined for clear zone around the bacterial spots.

6.3.3 LCMS analyses of antifungal compounds

Lactobacillus brevis PS1 with strong antifungal activity was evaluated to determine specific antifungal compounds produced. Antifungal activity of the cell-free supernatant resulting from growth of *L. brevis* PS1 in MRS broth was investigated as described by Brosnan *et al.*, 2012 in Chapter 3 under the section 3.3.4.

6.3.4 HPLC analysis of weak organic acids and ethanol

Organic acids present in 48 h fermented MRS broth were determined as described in Chapter 3 under the section 3.3.5. Lactate, acetate and ethanol present in fermented for 48 h wort were analysed using the same procedure.

6.3.5 Brewing materials

A mixture of four different malted barley varieties, harvested in 2010 were used for the production of the unhopped wort as follows: 40% Pilsen malt (moisture content: max 5.0%; extract: min. 81.0%; viscosity: 1.525±0.525 mPa·s; friability: min 78.0%; glassiness max 4.0%; protein: 10.5±1% d.m.; soluble nitrogen:

0.695±0.085 g/100 g (4.35±0.55%); Kolbach index: 40.5±4.5%; diastatic power: 300±50 WK), 40% Munich malt (moisture content: max 5.0%; extract: min. 80.0%; viscosity: 1.525±0.525 mPa·s; friability: min 76.0%; glassiness max 4.0%; protein: 10.75±1.25% d.m.; soluble nitrogen: 0.725±0.075 g/100 g (4.5±0.5% d.m.); Kolbach index: 43.5±3.5%), 15% Melanoidin malt (moisture content: max 4.25±1.25%; extract (fine grist): 77.0±2.5% d.m.; protein: 11.0±1% d.m.), 5% Caramel malt dark (moisture content: max 4.25±1.25%; extract (fine grist): min. 75.0%; protein: 11.0±1% d.m.). All malt samples came from Bestmalz AG (Heidelberg, Germany).

6.3.6 Milling

The mixed malted barley was milled with a two-roller mill from Engl Maschinen-Großhandels GmbH (Schwebheim, Germany) set at a 0.7 mm-roller distance. Milling of brewing materials was carried out directly before mashing-in.

6.3.7 Wort preparation

The production of the unhopped wort with malted barley was carried out in a 1,000 litres brewing plant. For mashing, a commonly used infusion process has been chosen as follows: 30 min at 50 °C, 30 min at 62 °C, 40 min at 70 °C and 5 min at 76 °C (mashing-off). In all brewing trials, mashing-in was performed by mixing 120 kg of grist into 450 L of brewing water at 40 °C to achieve a constant liquor-to-grist ratio of 3.8: 1. After reaching 70 °C, saccharification has been checked every 5 min until discoloration of iodine disappeared. Wort separation has been performed in a lauter tun. After a lauter rest of 15 min and turbid wort pumping for approximately 30 min, approx. 250 L of first wort with an extract of 15.35% have been collected. Then three sparging steps (150 L each) were carried out to reach a preboil wort

volume of 700 L (volume measurement at 95 °C) and an extract of 9.01%. After reaching the boiling temperature (>100 °C) the wort samples were hot taken with a containers. All brewing trials were performed in triplicate.

6.3.8 Standard wort analysis

The wort was analysed according to standard methods specified by Mitteleuropäische Brautechnische Analysenkommission (MEBAK) or European Brewery Convention (EBC) (Pfenninger, 1993, Analytica-EBC, 2005). pH of wort has been determined according to MEBAK II methods 2.13.2 and 2.14. Wort extract, apparent extract, apparent degree of fermentation and alcohol have been determined using a Alcolyzer Beer ME in combination with a DMA 4500 M both from Anton Paar GmbH (Graz, Austria).

6.3.9 Beverage preparation

Single colonies grown on MRS agar were subcultured twice in MRS broth at 30 °C. Overnight culture was inoculated in 100 mL to reach an initial concentration of 10^8 CFU/mL. The bacteria were grown for 48 h at 30 °C. Cells were separated from the supernatant by centrifuging at 3000 g for 10 min. Subsequently, to ensure biological purity of the beverage, 50 mL of supernatant was filtered through a sterile 0.45 µm MINISART®-plus filter (Sartorius Stedim Biotech GmbH, Goettingen Germany), 50 mL of supernatant was pasteurized for 1 min at 70 °C according to Kunze (2004).

For samples taken at sixteen intervals (0, 2, 4, 6, 8, 10, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48 h) pH and total titrable acids (TTA) were measured. The LAB growth curve was prepared by optical density (OD_{620}) measurements over time. At the end of

fermentation, microbial cell counts were evaluated. All results are reported as average of values measured at least in duplicate.

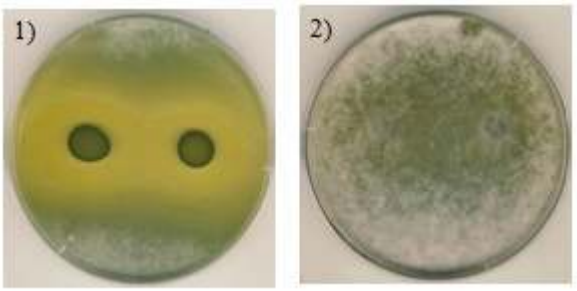
6.3.10 Antifungal activity assay of fermented wort

The antifungal activity of wort fermented by *L. brevis* strains was performed as described previously by Magnusson and Schnürer (2001) with some modifications. In detail, for the microtiter plate well assay, a 100 µL sample of wort fermented by *L. brevis* PS1 containing 10^4 *F. culmorum* spores per mL were added to each well of a sterile 96-well microplate (Sarstedt AG & Co, Nuembrecht, Germany). The wort fermented by *L. brevis* NS showing no antifungal activity as well as uninoculated wort sample were used as negative controls. The plate was sealed with optically clear seal for QPCR and incubated at 25 °C for 14 days inside a Multiscan FC Microplate Photometer (Thermo Fisher Scientic Inc., Waltham, USA). The optical density at 620 nm (OD_{620}) was automatically recorded for each well every hour. The changes in OD over time were used to generate *F. culmorum* growth curves. The degree of inhibition was measured either using the naked eye. The experiment was performed in triplicate.

6.4 Results and Discussion

An overlay method (Magnusson and Schnürer, 2001) was adopted to investigate the antifungal activity of *L. brevis* strains against *F. culmorum* moulds. As shown in Figure 6.1, strain PS1 was found to be active against the target fungus. The clear zone surrounding bacterial spots was 20 mm. In contrary to this, *L. brevis* NS showed no inhibitory activity against tested fungi.

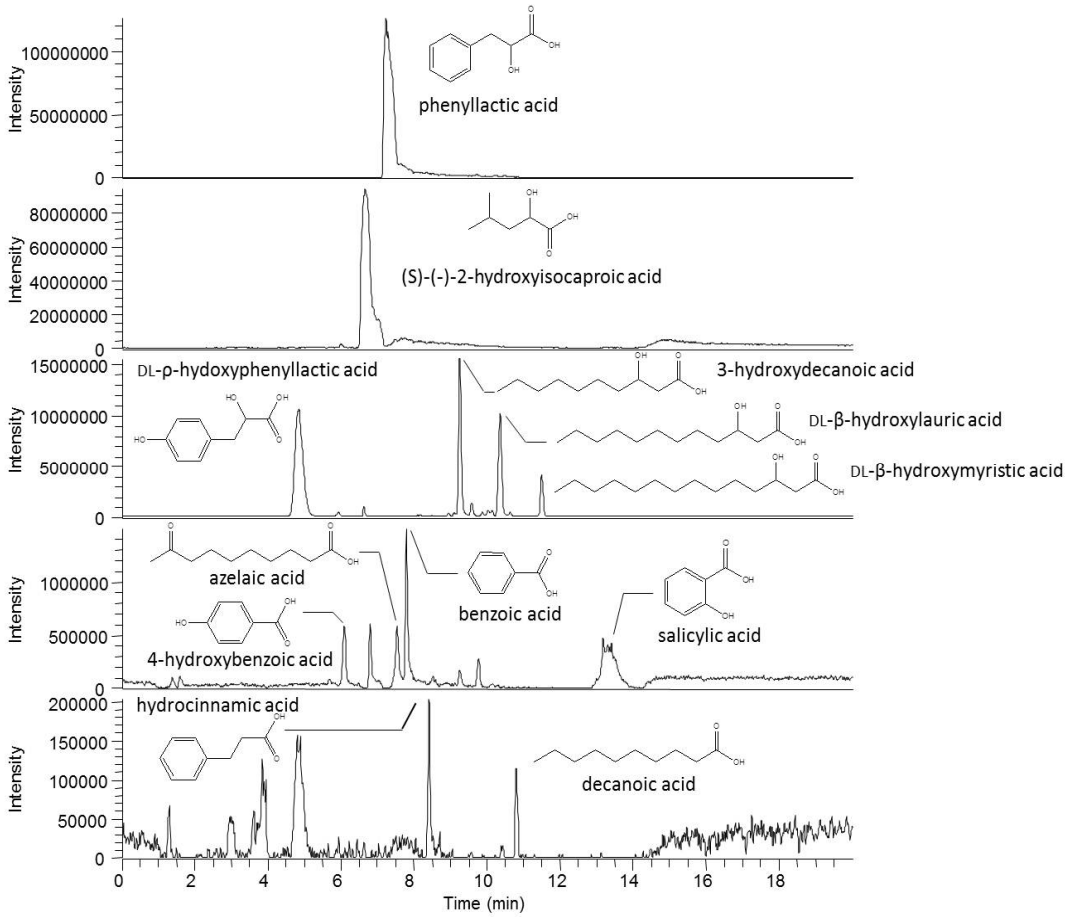
Figue 6.1 *Antifungal activity against F. culmorum of L. brevis PS1 (1) and non-inhibiting control L. brevis NS (2) by overlay method on mMRS5 agar plate*



Antifungal activity of *L. brevis* PS1 was previously investigated by Mauch *et al.* (2010). They demonstrated that the strain could also inhibit *in vitro* the outgrowth of *F. avenaceum*, *F. graminearum*, *F. poae* and *F. tricinctum*. Their further studies evaluated the influence of the freeze-dried cell-free supernatant (cfsP) of *L. brevis* PS1 on the germination of macroconidia and mycelia growth of *F. culmorum*. The addition of cfsP into the growth medium at concentrations ≥2% altered the growth morphology of the target strain, whereas at concentrations >5% the outgrowth of germ tubes from macroconidia was delayed and distorted. The presence of 10% cfsP completely inhibited the outgrowth of *F. culmorum* macroconidia. The activity of the compounds produced by *L. brevis* PS1 was higher at low pH values, i.e. pH<5, indicating that organic acids are active against *Fusarium* spp. This study confirm this hypothesis. Components of *L. brevis* PS1 were identified according to the method of Brosnan *et al.*, 2012. The ethyl acetate fraction of the cell-free supernatant resulting from fermentation of MRS broth by *L. brevis* PS1 strain was screened for presence of 25 known antifungal compounds, namely cytydine, 2-deoxycytydine, $_{D}$-glucuronic acid, $_{DL}$-*ρ*-hydroxphenyllactic acid, 1,2-dixydroxybenzene, 3,4-dixydroxyhydrocinnamic acid, 4-hydroxybenzoic acid, caffeic acid, vanillic acid,

(S)-(—)-2-hydroxyisocaproic acid, 3-(4-hydroxyphenyl)propionic acid, 3-(4-hydroxy-3-methoxyphenyl)propanoic acid, *p*-coumaric acid, ferulic acid, azelaic acid, phenyllactic acid, benzoic acid, hydrocinnamic acid, 3-hydroxydecanoic acid, DL-*β*-hydroxylauric acid, decanoic acid, DL-*β*-hydroxymyristic acid, 2-hydroxydodecanoic acid, and salicylic acid. Twelve of these compounds were detected and identified (see Figure 6.2) by comparison of their retention times and exact masses with data recorded for the standards.

Figure 6.2 *LCMS chromatogram of antifungal compounds found in MRS fermented by L. brevis PS1*



The chromatographic and spectrometric data of compounds identified by LCMS as well as quantities of the compounds are summarized in Table 6.1.

Table 6.1 *Chromatographic, spectrometric and quantitative data (mg L^{-1}) of antifungal components produced by L. brevis PS1 and NS strains*

Compound	t_R (min)	$[M–H]^-$ (m/z)	Strain	
			***L. brevis* PS1**	***L. brevis* NS**
DL-ρ-hydrophenyllactic acid	4.81	181.04994	14.02	-
4-hydroxybenzoic acid	6.07	137.02417	-	-
(S)-(—)-2-hydroxyisocapric acid	6.65	131.07085	363.22	51.44
Phenyllactic acid	7.20	165.05496	419.08	73.09
Azelaic acid	7.53	187.09702	-	-
Benzoic acid	7.78	121.02932	-	
Hydrocinnamic acid	8.39	149.06050	-	-
3-hydroxydecanoic acid	9.24	187.13327	-	-
DL-*β*-hydroxylauric acid	10.35	215.16449	-	-
Decanoic acid	10.78	171.13860	-	-
DL-*β*-hydroxymyristic acid	11.48	243.19562	-	-
Salicylic acid	13.31	137.02414	-	-
Acetic acid	-	-	3012.71	3621.98
Lactic acid	-	-	11769.31	9470.92
Ethanol	-	-	5298.37	2069.42

Values are means (n = 2): the relative standard deviations for all compounds were <6%.

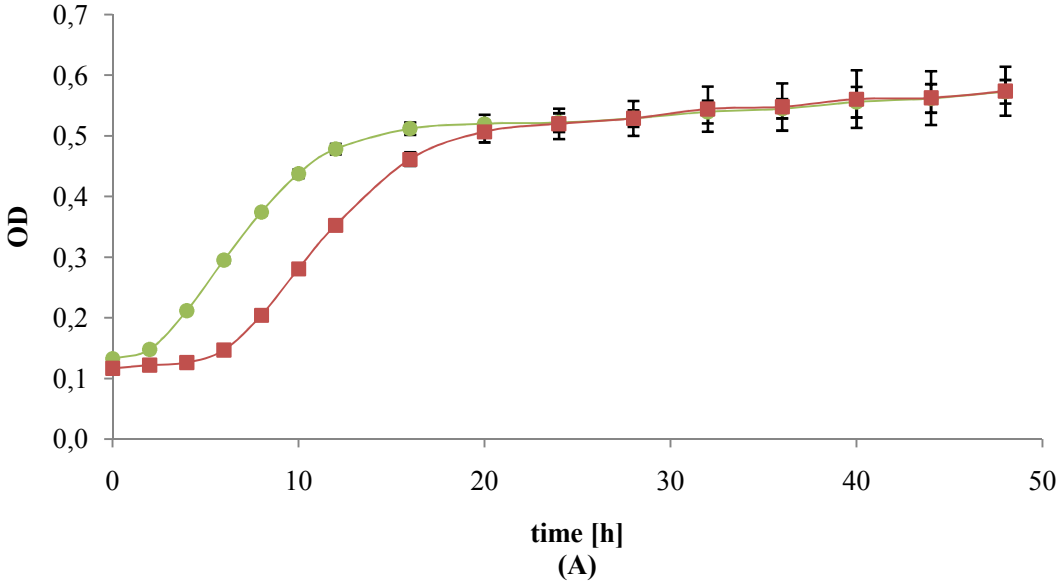
Additionally, the HPLC/PDA analyses of cell-free supernatant of *L. brevis* PS1 fermented MRS broth revealed also the presence of ethanol and lactic and acetic acids (data not shown). They were the major compounds, followed by phenyllactic, (S)-(—)-2-hydroxyisocapric and DL-ρ-hydrophenyllactic acids. Although, other components were detectable, their quantities were out of the calibration range, thus their concentration was >1 ppm mL^{-1}. MRS broth fermented by the non-inhibiting *L. brevis* NS strain was evaluated using the same procedure (data not shown). Interestingly, the quantity of acetic acid produced by the negative NS strain was

slightly higher than the one of the antifungal PS1. However, investigations of Mauch *et al.* (2010) strongly suggest that proteinaceous compounds are also involved in the antifungal activity of *L. brevis* PS1. Both, heating and proteolytic treatments with pronase E and proteinase K reduced the inhibitory activity of *L. brevis* PS1 cfsP. In addition, it is well documented that the antifungal activity of LAB is a complex phenomenon, where numerous and different compounds act synergistically against target organisms. Corsetti *et al.* (1998) reported that the mixture of acetic, caproic, formic, propionic, butyric and *n*-valeric acids, acting in a synergistic way, was responsible for the antimould activity of *L. sanfransisco*. Similar effect was observed by Niku-Paavola *et al.* (1999). Their study demonstrated that benzoic acid, 5-methyl-2,4-imidazolidinedione, tetrahydro-4-hydroxy-4-methyl-2H-pyran-2-one and 3-(2-methylpropyl)-2,5-piperazinedione inhibited growth of *Pantoea agglomerans* (*Enterobacter agglomerans*) by 10–15% when acting separately, but 100% when all were applied together. Newer studies of Ndagano *et al.* (2012) suggested as well that the inhibitory activity of *Weisella* genus is caused by a synergy of phenyllactic acid, 2-hydroxy-4-methylpentanoic acid and other organic acids, since their concentrations produced in the cell-free supernatant was too low in comparison with their minimum inhibitory concentrations (MIC).

Barley malt is a highly nutritious substance. Aqueous extracts of barley malt, wort, now made primarily to be fermented into beer, contain all the essential vitamins in the B family (niacin, riboflavin, pantothenic acid, thiamine, nicotinic acid, vitamin B6 (pyridoxine), biotin and folic acid), amino acids, proteins, minerals such as calcium, magnesium, potassium and zinc, and soluble fiber (glucans). Thus, beverages made using barley malt are attractive from a nutrition standpoint (Owades *et al.*, 1995). Wort used in this study was prepared in a pilot plant of University

College Cork and characterized according to appropriate trials (Table 6.2). Wort was fermented using *L. brevis* PS1 and *L. brevis* NS strains originating from porcine intestine and cheese, respectively. The strains were selected due to their capability to inhibit the outgrowth of *F. culmorum in vitro*. The antifungal strain *L. brevis* PS1 was investigated for its ability to ferment unhopped wort and compared to performing no inhibitory activity isolate *L. brevis* NS. During 48 h of incubation at 30 °C the PS1 strain exhibited slightly faster growth than the negative strain NS. However, after the initial exponential phase (between 0 and 16 h), the plateau was reached on the same level (Figure 6.3A) and LAB cell counts in the final product were around 10^{10} CFU mL^{-1} for both beverages (Table 6.1). The decline of the pH-values was similar for both strains, although the intermediate pH-value of 4.6 was obtained earlier with *L. brevis* PS1, while both strains yielded for similar pH-values after 48 h (Figure 6.3B). Additionally, no significant difference was observed in term of TTA since both *L. brevis* PS1 and *L. brevis* NS strains were able to yield final TTA value of 5.9±0.28 and 4.25±0.07, respectively (Figure 6.3B). HPLC analyses of organic acids indeed demonstrated similar quantitative content of lactate as well as acetate in wort fermented by *L. brevis* strains (Table 6.2). As expected, the uninoculated wort had a significantly higher pH and lower TTA values than fermented drinks (Table 6.2).

Figure 6.3 *Growth of L. brevis PS1 (●) and L. brevis NS (■) in wort (A). Changes in pH (L. brevis PS1 (●), L. brevis NS (■)) and TTA (L. brevis PS1 (○), L. brevis NS (□)) over 48h wort fermentation period by L. brevis strains (B)*



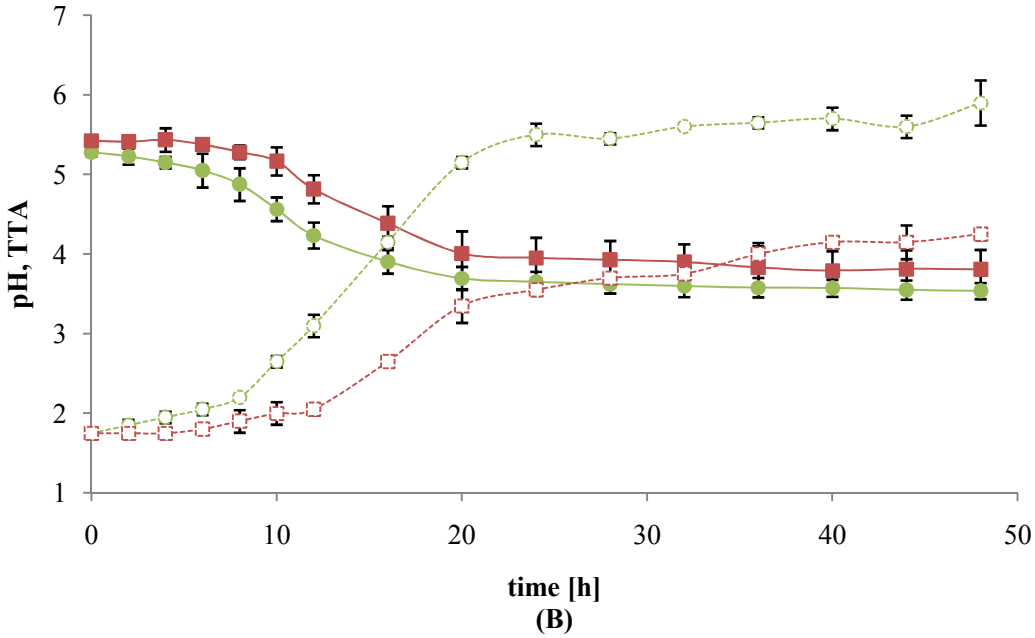
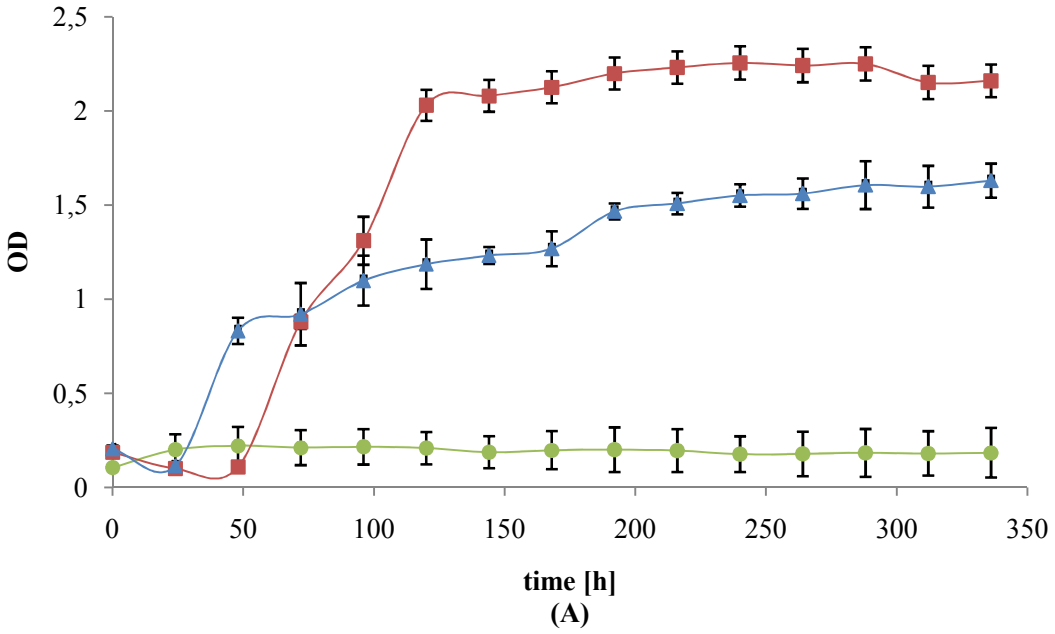


Table 6.2 *Concentrations of organic acids, ethanol, pH and TTA values and viable cell counts in the supernatant of wort inoculated with 10^8 bacteria mL^{-1} and incubated at 30 °C for 48 h*

supernatant	content in mg L^{-1} of:			pH	TTA	CFU×10^{10} mL^{-1}
	lactic acid	acetic acid	ethanol			
uninoculated	728.74±22.50	417.95±4.23	414.63±10.60	5.35±0.0	1.7±0.14	-
L. brevis PS1	3378.90±2.70	918.16±6.01	887.77±4.61	3.54±0.1	5.9±0.28	2.76±0.33
L. brevis NS	2097.96±6.31	633.53±1.20	729.29±5.07	3.81±0.25	4.25±0.07	1.65±0.03

Following the successful demonstration that *L. brevis* PS1 can perform wort fermentation, the next step was to investigate if the strain PS1 can increase the shelf-life of the beverage. The impact of the antifungal strain *L. brevis* PS1 on the shelf-life of the fermented wort was performed using microplate assay method (Magnusson and Schnürer, 2001). The uninoculated control spoiled after 1 day of contamination with *F. cumlomrum* spores. Beverage fermented by *L. brevis* PS1 had an inhibitory effect against the indicator fungus. It delayed the outgrowth of the fungus for more than 14 days, whereas the drink produced by fermentation with *L. brevis* NS retarded the *F. culmorum* growth only for 1 day compared to the uninoculated control. However, from day 3, the rate of mould growth in *L. brevis* NS fermented wort was comparable to the one of the uninoculated sample (Figure 6.4). Two different methods of decontamination, namely sterile filtration and pasteurization, were used during the sample preparation. Both methods did not affect the antifungal activity of the beverage (Figure 6.4). Figure 6.5 presents the microtiter plate after 14-days incubation period.

Figure 6.4 *Growth of F. culmorum in wort fermented by the antifungal strain L. brevis PS1 (●), control strain showing no inhibitory activity L. brevis NS (■), uninoculated wort (▲). (A) sterile filtered sample, (B) pasteurized sample*



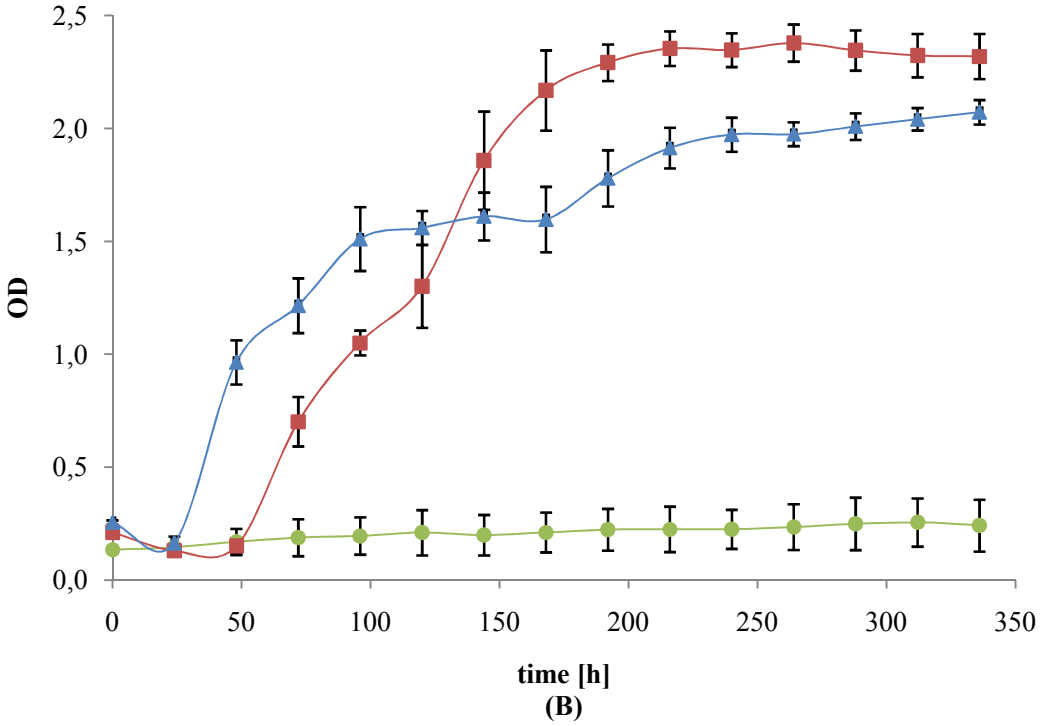
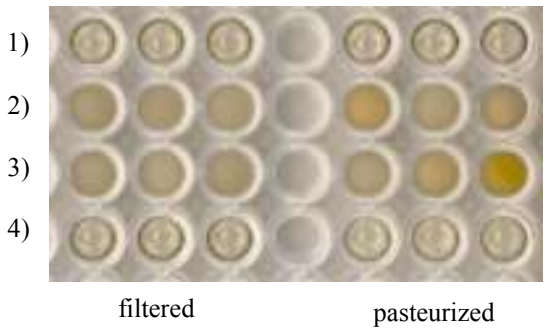


Figure 6.5 *Inhibitory effect against F. culmorum of wort fermented by the antifungal strain L. brevis PS1 (1) and showing no antifungal activity strain L. brevis NS (2). (3) is the sample of uninoculated wort contaminated by F. culmorum, (4) is uninoculated wort with no spores added. Each sample is performed in triplicate.*



The antifungal activity of LAB have already been proven. Numerous studies have described the isolation and characterisation of antifungal components from LAB (Lavermicocca *et al.*, 2000, 2003, Messens and De Vuyst, 2002, Ryan *et al.*, 2009a, 2009b, Ström *et al.*, 2002). The transition from identification of antifungal strains to their application may be difficult, however some authors proposed a technological exploitation of these strains in different foods and feeds. Antimould LAB have been successfully applied in bread (Dal Bello *et al.*, 2007, Lavermicocca *et al.*, 2000, Ryan *et al.*, 2008, Ryan *et al.*, 2011), dairy products (Miescher Schwenninger and Meile, 2004), sauces (Muhialdin *et al.*, 2011) and silage (Broberg *et al.*, 2007). Although, LAB have already been employed in the production of functional wort-based beverages (Back, 1986, Tenge 2002, Krahl, 2010, Mauch 2011), to the best of our knowledge this is the first report describing the antifungal activity of LAB when applied in wort. The investigated strain *L. brevis* PS1 was shown to be another example that the antifungal attributes of LAB exist and have the

potential for being effective food-grade biopreservatives for combating the problem of moulds.

6.5 Conclusions

Interest in food biopreservation has increased in recent years. LAB with antifungal activity, preventing growth of mycotoxigenic moulds are a promising alternative to chemical preservation methods. In this study, the ability of *L. brevis* PS1 to inhibit the outgrowth of *F. culmorum* has been proven both *in vitro* and *in vivo*. Identification of antifungal substances in cell-free supernatant revealed the presence of fifteen known compounds as responsible agents for this activity. However, as suggested by Mauch *et al.* (2010) the presence of proteinaceous components, can not be excluded. In this study, beverage fermented by *L. brevis* PS1 extended the shelf-life 12 days longer than sample containing the control strain (*L. brevis* NS) and 13 days longer than not inoculated wort.

The production of cereal-based beverages is usually based on spontaneous fermentation by indigenous existing microbiota with yeast and LAB being the dominant microorganism. Since spontaneous fermentations are very difficult to control and may influence the quality and stability of the final products, there is an increasing interest in the production of cereal-based beverages with defined starter cultures. The use of antifungal strain *L. brevis* PS1 for the wort fermentation is a promising approach in the development of functional beverages with improved shelf-life. Taking also in consideration that the preparation of the beverage is not expensive nor time consuming, does not require any special equipment and raw materials, it could be an alternative for a healthy, nutritious soft drink.

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Chapter 7

Overall discussion

7.1 Overall discussion

Lactic acid bacteria (LAB) are used in the production of food products for millennia. Traditionally, a wide range of foods were fermented solely for bio-preservation purposes and initially this was thought to be entirely due to the lower pH and organic acid production during LAB fermentation. However, more recently it has become obvious that there are many antimicrobial (anti-fungal/-bacterial) compounds sharing the responsibility for this protective property (Makanjuola *et al.*, 1992, Niku-Paavola *et al.*, 1999, Lavermicocca *et al.*, 2000, Magnusson and Schnürer, 2001, Ström *et al.*, 2002, Magnusson *et al.*, 2003, Sjörgen *et al.*, 2003, Dal Bello *et al.*, 2007, Ryan *et al.*, 2011). Apart from the preservation effect, LAB fermentation provides a natural way to concentrate and enhance nutrients such as vitamins and essential amino acid synthesis (Holzapfel, 1997), destroying undesirable components (mycotoxin, antinutritional factors) and ameliorating sensory qualities. i.e. taste, aroma, texture, consistency and appearance of the food (Thiele *et al.*, 2002, Arendt *et al.*, 2007). Additionally, these factors contribute to an easier preparation (reduced cooking times and lower energy consumption) and enhance product safety (microbial contaminants) (Leroy and De Vuyst, 2004, Simango, 1997). Moreover, during the last decade, consumer lifestyle choices, an increase in adverse reaction to food additives (food intolerance, malabsorption and allergies), have led to an increasing demand for natural preservatives with high acceptance and functionality (Zannini *et al.*, 2012).

Therefore, the aim of this study was to characterize the antifungal activity of over 100 strains of LAB, select the most promising LAB strains and implement these strains in production of high quality food products with extended shelf-life.

In the first part of the research program 116 strains isolated from different environments were screened for antifungal activity against *Aspergillus fumigatus*, *A. niger*, *Penicillium expansum* and *P. roqueforti*. *Aspergillus* and *Penicilia* species belong to the group of storage fungi (Noots *et al.*, 1999) and are frequent contaminants of bakery and dairy products (Basílico *et al.*, 2001, Legan, 1993, Taniwaki *et al.*, 2001). It was shown that, out of 116 LAB isolates, approximately 83% showed antifungal activity. Among this group 9% of the strains exhibited a broad spectrum antifungal action against 3 out of 4 fungi and 1% of the strains inhibited the growth of all tested moulds. These results confirmed that LAB are generally active against fungi (Mauch *et al.*, 2010). However, even the in-depth knowledge about *in vitro* antifungal properties of individual LAB does not determine their suitability as antifungal starter cultures for food fermentations. Many studies have assessed antifungal effects of LAB under the laboratory conditions, but there are relatively few reports explaining specific antifungal activity of LAB and their application (Dal Bello *et al.*, 2007, Gerez *et al.*, 2009, Lavermicocca *et al.*, 2000, Schwenninger and Meile, 2004, Ryan *et al.*, 2011). The suitability of LAB for the application is strongly dependent on the environmental criteria and numerous parameters, including temperature, time of incubation, growth medium, pH and nutritional factors have to be considered (Batish *et al.*, 1997). Therefore, the second part of the research programme dealt with implementation of antifungal LAB strains in different food products.

Chapter 4 described the application of the antifungal strain *L. reuteri* R29 in the sourdough based bread system. It is well documented how sourdough technology can improve the quality of wheat bread volume, shelf-life, crumb structure, flavour and nutritional value (Crowley *et al.*, 2002, Thiele *et al.*, 2002, Liljeberg *et al.*,

1995). The present study confirmed that the addition of sourdough fermented by *L. reuteri* R29 significantly increases the breadmaking performance of wheat dough. Analysis of bread volume confirmed that sourdough addition leads to an increase of loaf volume. The results are in agreement with those of Hammes and Gänzle (1998), Corsetti *et al.* (2000), Arendt *et al.* (2007), Ryan *et al.* (2011), where the addition of sourdough increased loaf volume. The positive impact of the increase in specific loaf volume was reflected in the sourdough breads also having lower crumb hardness values. A similar effect was reported by Ryat *et al.* (2011), who noted that the sourdough breads showed a softer crumb compared with non-acidified bread sample. This trend is attributed to the results obtained from the fundamental rheological tests, where the addition of sourdough yielded a less elastic and less firm dough (Clarke *et al.*, 2002). In terms of shelf-life, *L. reuteri* R29 sourdough bread retarded, the outgrowth of both, environmental and indicator fungi in the challenge test. The addition of sourdough fermented with this strain resulted in bread, which in the same experimental conditions increased the shelf-life up to 11 days, i.e. 6 days longer than the non-acidified control. Numerous studies have described the isolation and characterisation of antifungal metabolites under laboratory conditions, however their application in the bread is rather limited (Dal Bello *et al.*, 2007, Gerez *et al.*, 2009, Lavermicocca *et al.*, 2000, Ryan *et al.*, 2011). In the present study, bread containing 20% sourdough fermented with *L. reuteri* R29 showed remarkable ability to delay the growth of *F. culmorum*, *A. niger* and *P. expansum*.

In summary, the fundamental rheological study, baking tests and antifungal trials demonstrated that *L. reuteri* R29 was a suitable starter culture for production of wheat sourdough and good quality bread with extended shelf-life. Considering that fungal spoilage is the most common and economically important form of microbial

spoilage in bread, with approximately 200 million euro loses to the bakery industry annually (Legan 1993), the prevention of mould growth in baked goods is of great importance.

In chapter 5 strain *Lactobacillus amylovorus* DSM 19280 was used as an adjunct culture in a Cheddar cheese model system. The antifungal activity of *L. amylovorus* DSM 19280 was previously demonstrated in laboratory assays and used in cereal products (Arendt *et al.*, 2009, Arendt *et al.*, 2011, Ryan *et al.*, 2011, Belz *et al.*, 2012). In cheese, the presence of the antifungal *L. amylovorus* DSM 19280 adjunct resulted in a four-day delay in appearance of *P. expansum* growth on the cheese in comparison to the adjunct-free control. When cheeses were exposed to natural airborne fungi, the presence of the adjunct resulted in a six-day delay in the appearance of mycelia on the cheese surface. Up to date, only little research have been carried out on the use of antifungal LAB as adjunct cultures in cheese for their bio-preservative effects. The studies carried out by Miescher-Schwenninger and Meile (2004) reported the antifungal activity of mixed cultures of *Propionibacterium jensenii* and *Lactiobacillus paracasei* subsp. *paracasei* against fungi such as *Candida pulcherrima*, *C. magnoliae*, *C. parapsilosi*, *Zygosaccharomyces bailii* in yogurt and cheese. Recently, Muhialdin *et al.* (2011) reported *A. niger* and *A. oryzae* growth inhibition by *Lactobacillus fermentum* Te007, *L. pentosus* G004 and *L. paracasei* D5 and *Pediococcus pentosaceus* Te010 on commercial processed cheese slices.

Significantly, the presence of *L. amylovorous* DSM 19280 as an adjunct culture had no detectable negative impact on cheese quality as determined by analysis of moisture, salt, pH, primary proteolysis (urea-polyacrylamide gel electrophoresis) and secondary proteolysis (free amino acid analysis, HPLC). This is

critical as protective cultures used for preservation, should not affect the technological or quality properties of an established product (Muhialdin *et al.*, 2011). Moreover, cheeses made with either *L. amylovorus* DSM 19280 and DSM 20531 strains had higher levels of moisture than the control cheeses. This phenomenon was also observed with Cheddar cheese produced with *L. plantarum* strains (Ciocia, 2010) and it has been shown to have a positive effect on cheese texture (Mozzi *et al.*, 2006).

This study confirms the antifungal properties of *L. amylovorous* DSM 19280 strain previously demonstrated in context of cereal products (Arendt *et al.*, 2009, Arendt *et al.*, 2011, Ryan *et al.*, 2011, Belz *et al.*, 2012) and reinforces its potential as a natural preservative, which could be easily implemented by the food fermentation industry.

Chapter 6 deals with the exploitation of *L. brevis* PS1 in production of a novel functional barley malt wort-based, alcohol-free beverage with extended shelf-life. The strain *L. brevis* PS1 has been previously studied by Mauch *et al.*, 2011 and showed to be active against *Fusarium* spp. (*F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae*, *F. tricinctum*) *in vitro*. The investigations on the impact of the bacterial supernatant on the growth of *F. culmorum* suggested that the strains produces organic acids and proteinaceous compounds.

In this study, the antifungal strain was investigated for its ability to ferment unhopped wort and to inhibit the outgrowth of *F. culmorum in vivo*. The shelf-life of the challenge beverage was extended to at least 14 days when compared to notinoculated wort sample. Although, LAB have already been employed in production of functional wort-based beverages (Back, 1986, Tenge 2002, Krahl,

2010), this is the first report describing the antifungal activity of LAB when applied in wort.

In addition, the use of modern instrumental methods allowed the insight into the antifungal components produced by LAB. The antifungal metabolites identified in this thesis were wide range of organic acids: DL-*ρ*-hydrophenyllactic acid, 3,4-dihydroxyhydrocinnamic acid, vanilic acid, (S)-(—)-2-hydroxyisocapric acid, phenyllactic acid, benzoic acid, salicylic acid, hydrocinnamic acid, azelaic acid, 3-hydroxydecanoic acid, decanoic acid, 2-hydroxydodecanoic acid and DL-*β*-hydroxymyristic acid. Synergistic effects in terms of antifungal activity of these compounds in combination with the main metabolites lactic and acetic acids are widely reported in literature. Corsetti *et al*. (1998) found a mixture of acetic, caproic, formic, propionic, butyric and *n*-valeric acids being responsible for the antimould activity of *L. sanfransisco*, Lavermicocca *et al*. (2003) could observe synergistic action of lactic and acetic acids together with phenyllactic acid whereas Ndagano *et al.* (2012) suggested that the inhibitory activity of *Weisella* genus is caused by a synergy of phenyllactic acid, 2-hydroxy-4-methylpentanoic acid and other organic acids. Moreover, the presence of other inhibitory components can not be excluded. Beside from the effect of organic acids, other authors refer to the participation of proteinaceous compounds in the antifungal activity of LAB. Ström *et al.* (2002) reported weak synergistic effects between some cyclic peptides and 3-phenyllactic acid. Dal Bello *et al.* (2007)identified lactic acid, phenyllactic acid and two cyclic dipeptides in the cell free supernatant of *L. plantarum*. Similarly, Ryan *et al.* (2011) isolated carboxylic acids, nucleosides, sodium decanoate and cyclic dipeptides from a LAB strain.

The developed LC-PDA-ESI-MS methods were straightforward, requiring no expensive and time-consuming sample preparation procedure, and convenient in identification of compounds in complex mixtures and small amounts. Increasing the ability to detect and quantify both known and unknown compounds in antifungal mixtures can help to provide more insight into possible reasons for the observed antifungal activity of the strains.

Concluding, this study aimed at: 1) characterizing the antifungal activity of over 100 strains of LAB, 2) selecting the most promising LAB and 3) implementing new methodologies and techniques in pilot-scale facilities for the production of natural preservatives. The main objective, to investigate, if LAB can be used as a natural way to improve the shelf-life and safety of different food products, was achieved. Methodologies and techniques developed in this project can help the food industries to reduce the mycotoxins content and extend the products shelf-life.

Taken together, this thesis emphasizes the great potential and versatility of lactic acid bacteria for their applications in different foods, including the improvement of food safety, increasing economic efficiency and creating products with benefits for human health.

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Appendix

List of Publications

Ryan L. A. M., Dal Bello F., Zannini E., Pawlowska A. M.*, Koehler P., Arendt E. K., 2011. *Lactobacillus amylovorus* DSM 19280 as novel food-grade antifungal agent for bakery products. *International Journal of Food Microbiology*, 146, 276-283.

*Took part in planning and performed part of the microbiological laboratory work. Minor part in writing of the manuscript.

Pawlowska, A. M.*, Zannini, E., Coffey, A., Arendt, E. K., 2012. "Green Preservatives": Combating fungi in the food and feed industry by applying antifungal lactic acid bacteria. *Advances in Food and Nutrition Research*, 66, 217-238.

*Major part in writing the manuscript. Planning of work was done in collaboration with Emanuele Zannini.

Pawlowska, A. M., Lynch, K. .M, Brosnan, B., Coffey, A. Zannini, E., Furey, A., McSweeney, P. L. H., Waters, D., Arendt, E. K., 2013. Application of *Lactobacillus amylovorus* as an antifungal adjunct to extend the shelf-life of Cheddar cheese. *International Dairy Journal*, accepted.

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Pawlowska, A. M.*, Galle, S., Brosnan, B., Furey, A., Coffey, A., Arendt, E. K. Potential of lactic acid bacteria to inhibit common toxigenic food spoilage fungi. *In preparation*.

*Did major part of the laboratory work. Major part in writing the manuscript.

Pawlowska, A. M.*, Brosnan, B., Furey, A., Coffey, A., Arendt, E. K. Characterisation of *Lactobacillus reuteri* R29 as a new antifungal strain improving the quality and shelf life of wheat bread. *In preparation*.

* Did major part of the laboratory work. Major part in writing the manuscript.

Waters, D. M., Pawlowska, A. M.*, Brosnan, B., Oliveira, P. M., Furey, A., Coffey, A., Arendt, E. K., Zannini, E. Multi-compound evaluation of Lactobacillus brevis R2Δ antifungal metabolites in a novelapplication: Extended shelf-life wort beverage base. Journal of Food Microbiology, *submitted*.

* Did major part of the laboratory work.

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Lactobacillus amylovorus DSM 19280 as a novel food-grade antifungal agent for bakery products

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Food biopreservatives

ABSTRACT

Mould spoilage is the main cause of substantial economic loss in bakery industry and might also cause public health problems due to the production of mycotoxins. The reduction of mould growth in bakery products is thus of crucial importance and there is great interest to develop safe and efficient strategies for this purpose. In this study *Lactobacillus amylovorus* DSM19280 has been shown to produce a wide spectrum of antifungal compounds active against common bread spoilage fungi. Among the indicator moulds, *Aspergillus fumigatus* and *Fusarium culmorum* were the most sensitive organisms. Several antifungal compounds were found to be present in synthetic medium inoculated with *L. amylovorus* DSM19280 strain, some of them being reported here for the first time. Wheat doughs fermented with *L. amylovorus* DSM19280 had good rheological properties and the breads thereof were of high quality as shown by rheofermentometer and texture analyser measurements. The results were compared with those obtained with a control non-antifungal *L. amylovorus* DSM20531^T strain, a non-acidified and a chemically acidified dough. The quality of sourdough and bread fermented with *L. amylovorus* DSM 19280 was comparable to that obtained by using *L. amylovorus* DSM20531 ^{T}. Additionally, breads were evaluated for the ability to retard the growth of *Fusarium culmorum* FST 4.05, *Aspergillus niger* FST4.21, *Penicillium expansum* FST 4.22, *Penicillium roqueforti* FST 4.11 and fungal flora from the bakery environment. The biological preservation of bread with *L. amylovorus* DSM 19280 was also compared to the most commonly used antifungal agent Calcium propionate. Breads containing sourdough fermented with *L. amylovorus* DSM 19280 were more effective in extending the shelf life of bread than the calcium propionate.

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1. Introduction

Bread is a staple food world-wide and it is generally considered a highly perishable product. With an average consumption of about 65 kg of bread per capita per year in Europe, it remains an important constituent of a balanced healthy diet (Sievert et al., 2000). However, physicochemical changes (e.g. staling and firming) and microbiological spoilage (e.g. ropiness, mould growth) markedly reduce the shelf-life of bread causing huge economic losses to both the baking industry and the consumer (Gray and Bemiller, 2003). The application of lactic acid bacteria (LAB) in the form of sourdough has been reported to have positive effects on wheat bread quality and shelf-life (Corsetti et al., 2000; Crowley et al., 2002). Concerning the microbiological deterioration, mould spoilage is a serious and expensive problem. For example, in Western Europe, the economic losses related to the presence of moulds in bread are estimated to be more than 200 million Euros per year (Legan, 1993). Beside the repelling sight of visible mould growth, moulds are also responsible for off-flavours formation and the production of mycotoxins and allergenic compounds, that can be formed even before growth is visible. The major fungi involved in bread spoilage belong to the genera *Aspergillus*, *Fusarium*, *Penicillium* and *Mucor* (Legan, 1993).

Bread mould spoilage can be reduced using physical (i.e. UV, infrared or microwave irradiation, and modified atmospheres) and chemical (i.e. active packaging material, propionic, sorbic and acetic acids and their salts) methods; but none of these represent an efficient strategy to control the mould growth. Additionally, some moulds have acquired the ability to grow in presence of potassium sorbate (Davidson, 2001) or even to degrade sorbate (Nielsen and de Boer, 2000).

On the other hand, since the consumers are more concerned about safety and additive content (Prendergast, 1997; Robijn et al., 1996), the bread industry has been working to reduce the number of preservatives, such as calcium propionate (CaP) in an effort to make bread as natural and fresh as possible (Casdagli, 2000). During the last decade biopreservation, the control of one organism by another, has gained increasing attention as a means of naturally controlling the

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shelf life and safety of baked goods. Among natural biological antagonists, lactic acid bacteria (LAB), and in particular lactobacilli, are of particular interest since they have a long history of use in food and are "generally regarded as safe". Several compounds with antifungal activity have been isolated from LAB. So far, the majority of identified antifungal metabolites are low-molecular weight compounds consisting of organic acids, diacetyl, ethanol, hydroxylated fatty acids, hydrogen peroxide, cyclic dipeptides, phenolic compounds, reuterin and fungicins (Dal Bello et al., 2007; Magnusson et al., 2003; Mandal and Mandal, 2007; Schnürer and Magnusson, 2005; Stierle et al., 1988).

Each antifungal compound can act in synergy with others in order to provide an additional hurdle for spoilage moulds. However, the antifungal activity of LAB is not fully elucidated to date. Indeed, most publications on this topic have merely illustrated the LAB inhibitory effects, but have seldom purified and characterized the active compounds. Only a limited number of studies report a systematic characterization of LAB antifungal compounds under "*in vitro*" and "*in vivo*" systems (Broberg et al., 2007; Dal Bello et al., 2007; Lee et al., 2006; Ryan et al., 2009a,b; Stierle et al., 1988). Finally, neither the suitability of the antifungal strains as starters for fermentations nor the quality of the final products has always been evaluated to date.

The present study was undertaken to test the suitability of *Lactobacillus amylovorus* DSM 19280 strain as a starter culture for sourdough bread-making with respect to bread making performance and the effect as a bio-preservative. For this purpose the antifungal compounds produced by the strain *L. amylovorus* DSM 19280 in a synthetic growth medium were isolated and identified.

2. Materials and methods

2.1. Cultures and growth conditions

The strain *L. amylovorus* LA 19280, patented as strain DSM 19280 (Arendt et al., 2009) was originally isolated from a cereal environment and showed inhibitory activity against a wide range of bacteria, yeasts and moulds (data not shown). *L. amylovorus* DSM 20531^T was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) and chosen as a negative control. Both *Lactobacillus* strains were routinely grown on MRS5 medium (Meroth et al., 2003) at 30 °C for 48 h under anaerobic conditions. The cultures were stored anaerobically on MRS5 agar plates at 5 °C or for long term storage at −80 °C in a glycerol/water (1:1 v/v) solution.

The moulds *Aspergillus fumigatus* J9, *Aspergillus niger* FST 4.21, *Fusarium culmorum* FST 4.05, *Penicillium expansum* FST 4.22 and *Penicillium roqueforti* FST 4.11 (Table 1) were kept in the culture collection of the School of Food and Nutritional Sciences, University College Cork, Ireland. The target fungi were chosen as representative bread spoilage fungi. Moulds were cultivated on malt extract agar (MEA) (Oxoid, Hampshire, UK) at 25 °C for 2 to 5 days and stored at 4 °C. The spores were collected after brushing the plate surface with physiological solution. Spores were transferred from this stock solution into a synthetic nutrient-poor medium (Nirenberg, 1976). Vigorous stirring (200 rpm) for 8 days at room temperature provided a fungal cell and conidial suspension with a concentration of 5×10^5 spores mL^{-1}.

Table 1
Inhibitory activity of *L. amylovorus* DSM 19280 and *L. amylovorus* DSM 20531^T strains against a range of reference fungi as determined by the overlay method.

Moulds	Antifungal performance[a]	
	L. amylovorus DSM 20531^T	*L. amylovorus* DSM 19280
A. niger FST 4.21	−	++
A. fumigatus J9	−	++++
F. culmorum TMW 4.0754	−	++++
P. expansum FST 4.22	−	+
P. roqueforti FST 4.11	−	+

[a] Inhibition was scored as follows; −, no inhibition; +, very weak inhibition around the colonies; ++, low inhibition with little clear zones around the colonies; +++, strong inhibition with detectable zones around the colonies; and ++++, very strong inhibition with large clear zones and nearly no growth around the colonies.

2.2. Antifungal activity assays

Two different assays, the overlay method and the microtiter plate well assay were employed to detect antifungal activity. The first one was used to assess the antifungal activity of LAB strains while the second one was performed to determine the MICs (Minimal inhibitory concentration) of the LAB bioactive compounds.

The overlay method was performed as previously described by Magnusson and Schnürer (2001) with some modifications. To avoid any pH effect, the screening was carried out on buffered MRS5 agar plates. The medium was buffered to pH 6.5 by 75 mmol/L KH_2PO_4 solution. LAB strains were added as discrete spots on the plates and incubated at 30 °C for 48 h in anaerobic jars. To investigate antifungal activity, 100 µL of the fungal spore solution, containing ca. 10^4 spores, were sprayed on the surface of plates by nebulisation. These latter were then incubated aerobically at 30 °C for 3 days. The plates were examined for clear zones of inhibition around the bacteria spots.

For the microtiter plate well assay, 100 µL of malt extract broth (MEB), containing testing compounds and 10^3 fungal spores, were added to each well. The plate was incubated at 30 °C for 12 days. The fungal growth was detected by measuring optical density (OD) at 490 nm with Multiskan FC microplate-reader (Thermo Scientific, Waltham, USA). The compounds causing the partial or total delay of fungal growth with respect to the control (MEB containing only fungal spores) were considered as active compounds. The microtiter plate well assay was used to follow the antifungal activity during isolation of antifungal compounds in the present work. The assays of the antifungal activity were performed in duplicate, unless otherwise stated.

2.3. Preparation of LAB cell free supernatant

L. amylovorus DSM 19280 was inoculated to an initial concentration of 10^5 cfu/mL in two litres of MRS5 broth and was grown as still culture at 30 °C for 48 h. Afterwards, broth culture was centrifuged at $2000\times g$ for 15 min and sterile filtrated (0.45 µm pore size filter; Millipore).

2.4. Solid phase extraction (SPE) and High Performance Liquid Chromatography (HPLC)

The isolation of antifungal compounds from cell free supernatant of *L. amylovorus* DSM 19280 was achieved through several purification steps.

Cell free supernatant was applied on a solid phase extraction (SPE) column ($C_{18,}$ 15 g, 5 µm particle size; Merck, Darmstadt, Germany), and eluted first with 5% acetonitrile (ACN) (Sigma) and subsequently eluted with 95% ACN to separate hydrophilic and hydrophobic compounds, respectively. The flow rate was set at 3 mL/min. The hydrophilic phase (5% ACN fraction) was further separated on a HPLC system (Kontron D450) using a semi-preparative reversed-phase C_{18} column (250×10 mm, 30 nm pore size, 5 µm particle size; Phenomenex). Operating conditions were as follows: injection volume of 1 mL, flow rate of 1.5 mL/min and UV detector operating at $\lambda = 210$ nm. The run was performed at 25 °C and the eluent was a mixture of HPLC-grade ACN (Solvent A) and 0.1% aqueous solution of trifluoroacetic acid (Fluka) (Solvent B). Elution was conducted using linear gradient from 0% to 5% of Solvent A in 50 min. The 95% ACN fraction was separated using the same HPLC system reported above, except for the elution that was conducted using linear gradient from 1% to 95% of Solvent A in 40 min. Three fractions were collected; fr1

(retention time from 0 to 17 min), fr2 (retention time from 18 to 26 min) and fr3 (retention time from 27 to 40). Each fraction was concentrated (5 mL) under a gentle stream of nitrogen, and further run on the (RP) C_{18} column used before in order to purify the fractions into individual compounds. After concentration by freeze-drying all compounds from the purification process were resuspended at concentration of 50 mg/mL using malt extract broth (MEB) containing 10% methanol and tested against the indicator mould *A. fumigatus* J9 using the microtiter plate well assay described above. Fractions showing activity in the bioassay were freeze-dried over 48 h. Non-inoculated MRS5 broth was used as a negative control, fractionated, and evaluated in the biotests using the same procedure as for the cell-free supernatant.

2.5. Identification of antifungal compounds

The identification of the antifungal compounds were performed using nuclear magnetic resonance(MNR), mass spectrometer (MS), and gas chromatography (GC) as previously described by Ström et al. (2002). In order to confirm the structure of the isolated compounds, commercially available cyclic dipeptides (Bachem, Weil am Rhein, Germany) and other standard compounds (Sigma, Steinheim, Germany) were purchased and compared to the isolated compounds, with respect to molecular weight and fragmentation patterns, using a quadrupole time of flight mass spectrometer QTOF LC–MS/MS. The isomeric configuration of the isolated 3-phenyllactic acid was determined using a modified version of a published method (Stierle et al., 1988). Briefly, HRGC/MS analyses were performed using a gas chromatograph type HP 5890 series II (Agilent, Waldbronn, Germany) in tandem with a MAT-95S mass spectrometer (Finnigan, Bremen, Germany) using the fused silica capillary DB-FFAP (30 m × 0.32 mm, 0.25 μm film thickness) (J&W Scientific, Folsom, CA, USA). Samples were applied at 40 °C. The temperature was raised at a rate of 3 °C/min to 180 °C and held for 5 min. The GC injector and GC interface were held at 200 °C. Samples (1 to 5 μL) were injected in split mode (1:50) three times, and helium was used as the carrier gas (1 mL min^{-1}). Commercial 3-phenyl-L-lactic acid and 3-phenyl-D-lactic acid were used as references.

The isomeric configuration of the cyclic dipeptides was determined using the same method described above with the exception that GC samples were applied at 120 °C and the temperature was raised at a rate of 2 °C/min to 160 °C and held for 5 min.

2.6. Determination of Minimum Inhibitory Concentrations (MIC)

MICs were determined for the available commercial compounds, D-glucuronic acid, cytydine, deoxycitidine, sodium decanonate, phenyllactic acid (both D and L forms), hydroxyphenyllactic acid, *p*-coumaric acid, (Sigma-Aldrich Chemie, Steinheim, Germany), 3-phenylpropionic acid, α-methylcinnamic acid, salicylic acid, (Fluka Chemie AG, Buchs, Switzerland), cyclo(Pro-Pro), cyclo(Leu-Pro), cyclo(His-Pro), cyclo(Met-Pro), and cyclo(Pro-Tyr) (Bachem AG, Bubendorf, Switzerland).

All the compounds were dissolved in Malt Extract Broth (Merck, Darmstadt, Germany) containing 10% of methanol, and the pH was adjusted to 3.8 with 4 M HCl. The compounds were dissolved at the highest possible concentration in the interval from 2 to 400 mg/mL and diluted in 11 steps in pH adjusted MEB (pH 3.8). A pH-adjusted 5% methanol-MEB and pH-adjusted MEB solutions were used as negative controls. MIC determinations were performed in duplicates using the microtiter plate well method (Lončar et al., 2000) with *A. fumigatus* J9 as a target organism. Spores were suspended in MEB solution to final concentration of 10^4 cells per mL. Fifty microlitres of spore suspension was added to 50 μL of respective substance solution. The inhibition was measured at 30 °C over 48 h in the interval of 30 min by optical density at 620 nm (Multiscan FC, Thermo Fisher scientific, Vantaa, Finland). Measurements using the naked eye were also used for estimation fungal growth in wells. The MIC was determined as the lowest concentration where total inhibition of fungal growth was observed.

2.7. Sourdough fermentation and analysis

The sourdoughs fermented by *L. amylovorus* DSM 19280 and *L. amylovorus* DSM 20531^T were produced as previously described by Ryan et al. (2008) with a dough yield of 200 and an initial inoculum of 10^5 cfu/g. Samples were taken at eight intervals (0, 4, 8, 12, 24, 30, 36 and 48 h) and the LAB cell counts were determined on MRS5 agar plates. At each time point, pH and total titratable acidity (TTA) values were also measured according to a standard method (Arbeitsgemeinschaft Getreideforschung, 1994). At the end of fermentation, three colonies per each type of colony were picked up from MRS5 agar plates containing 30–300 colonies, purified and subjected to the partial 16S rRNA sequencing according to a previously described method (Meroth et al., 2003).

2.8. Baking with L. amylovorus DSM 19280 and L. amylovorus DSM 20531^T strains

The sourdoughs fermented by *L. amylovorus* DSM 19280 and *L. amylovorus* DSM 20531^T were used for the production of wheat sourdough bread following the wheat dough formulation and baking procedure reported by Dal Bello et al. (2007). Sourdough was added at 20% to the dough for bread production. Additionally, non-acidified bread as well as chemically acidified bread was prepared. This latter contained a mixture of lactic and acetic acids to yield the same dough pH as the doughs containing sourdough (Ryan et al., 2008).

2.9. Rheofermentometer test

The gaseous release and the development characteristics of the doughs were examined using a rheofermentometer (Chopin S.A., Villeneuve-La-Garenne, France). Doughs were prepared in the same manner as for baking. Briefly, 300 g of dough was placed in the fermentation chamber and a 1500 g cylindrical weight was placed on top. The dough was evaluated over 3 h at 30 °C. Several characteristics were determined from the dough development and the gaseous release curves obtained, the details of which have been previously described (Gobbetti et al., 1995).

2.10. Bread evaluation tests

Three loaves were utilised for each analysis. Two hours after baking, the loaf volume was measured using the rapeseed displacement method, and after 6 h, the loaf weight was also recorded. Specific loaf volume (mL/g) was calculated. Moisture of the crumb was determined using a one stage drying process (130 °C) while crumb hardness was determined by Texture Profile Analysis (TPA), using the universal testing machine TA-XT2I (Stable Microsystems, Surrey, UK) and following the procedure reported by Dal Bello et al. (2007). Colour of crust (CIE L*a*b* colour system) was determined with a Chroma Meter (Minolta CR-300, Osaka, Japan).

2.11. Antifungal activity in sourdough bread

The antifungal activity of both *L. amylovorus* strains in bread was determined using bread slices as previously described (Ryan et al., 2008). Briefly, wheat bread was challenged against *A. niger* FST 4.21, *F. culmorum* FST 4.05, *P. expansum* FST 4.22 and *P. roqueforti* FST 4.11 moulds. Given that *A. fumigates* J9 could be potentially dangerous for the human health, it was replaced with the above mentioned moulds.

One hundred μL solution containing 10^4 fungal spores were sprayed on both sides of each bread slice. Each slice was then packed

in a plastic bag and heat sealed during such a procedure a small slot was opened and a tip was inserted to ensure comparable aerobic condition in each bag. The fungal contamination of the bread slices was observed throughout 14 days (bread slices were left at room temperature). A series of ten slices was inoculated. Bread spoilage was evaluated based on the percentage of the total surface area of each slice where fungal outgrowth occurred. A bread slice was considered mouldy if more than 1% of the total surface area was covered with fungi.

A second challenge test was performed in order to evaluate the ability of both *L. amylovorus* strains to inhibit the outgrowth of the environmental mould from a pilot scale industrial bakery. Ten slices of wheat bread were left 5 min on both sides at ambient air in the bakery. Afterwards, each slice was packaged in plastic bags and monitored for the mould growth as described above. In both studies, non acidified bread and chemically acidified bread, were also challenged and used as controls (Ryan et al., 2008).

Additionally, bread containing 0.3% of calcium propionate (CaP), a chemical additive usually employed in bakery for bread conservation (Anonymous, 1995), was also prepared in order to compare the inhibitory activity of *L. amylovorus* strains to that of chemical additives. Bread slices were challenged and investigated as described above.

2.12. Statistical analysis

Statistical analyses for rheofermentometer and texture analysis were performed using SPSS 12.0 for Windows computerized statistical analysis package (SPSS Inc., Chicago, IL). Data were examined using one-way analysis of variance (ANOVA). Tukey's honesty significant difference (HSD) test was used for multiple comparisons. Each result is the average of 3 separate experiments with 3 independent samples from each batch. The level of significance was determined at $p<0.05$.

3. Results

3.1. Spectrum of antifungal activity

L. amylovorus DSM 19280 was highly active against fungi; whereas the control strain *L. amylovorus* DSM 20531^T showed no activity. The results depicted in Table 1 also reveal that the different mould species showed varying inhibition levels to the antifungal agents produced by *L. amylovorus* DSM 19280. The *Penicillium* species was the least sensitive to the antifungal compounds produced by *L. amylovorus* DSM 19280.

3.2. Isolation of antifungal compounds

The antifungal activity of cell-free supernatant resulting from growth of *L. amylovorus* DSM 19280 in MRS5 broth was recovered in both 5% and 95% ACN fractions after SPE on a C_{18} column, indicating both hydrophobic and hydrophilic natures of the antifungal compounds. The further separation of the hydrophilic phase by HPLC using a C_{18} column and the following activity assay of the recovered fractions in microtiter wells against *A. fumigates* J9 highlighted the antifungal activity to 3 of a total 23 collected compounds.

The hydrophobic fraction from the SPE analysis was further sub-fractionated and separated by HPLC on a C_{18} column. The activity assay in microtiter wells against *A. fumigatus* J9 highlighted the activity to 14 of a total 141 isolated compounds.

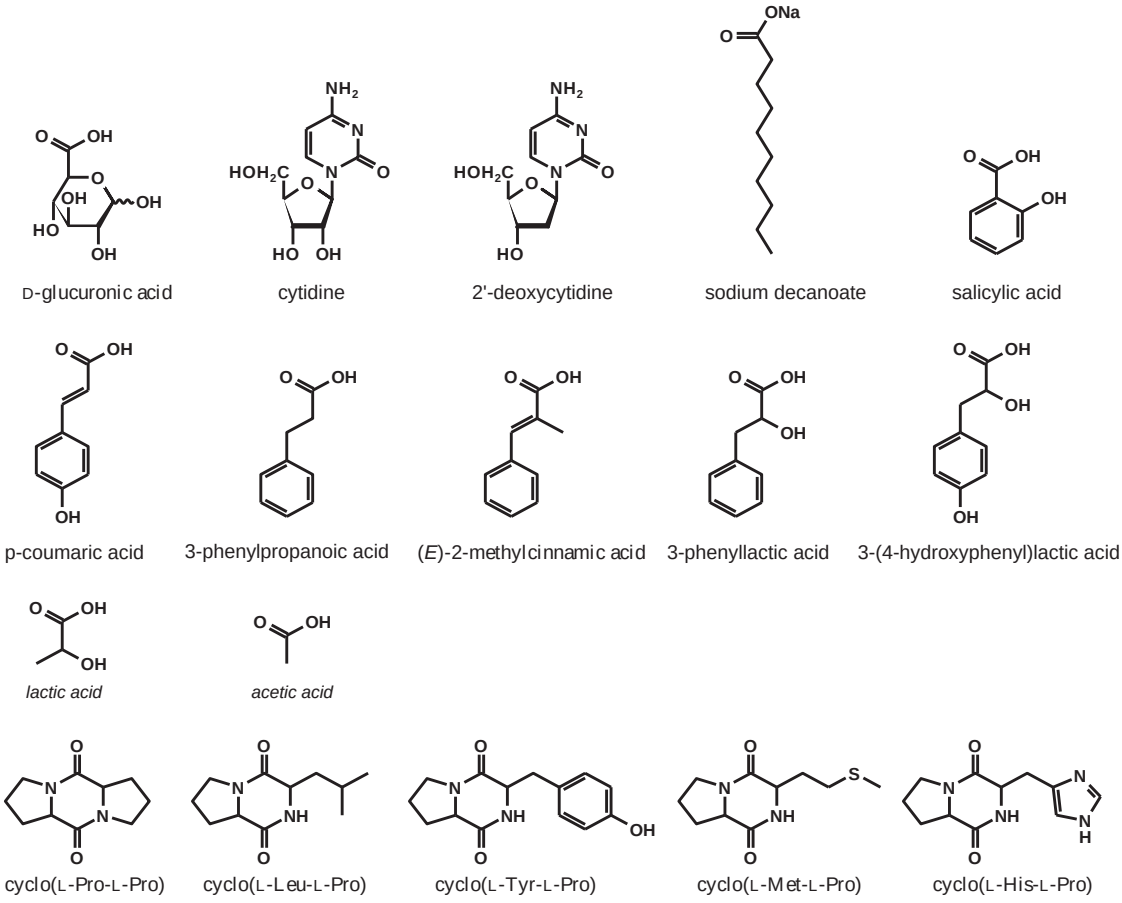


Fig. 1. Antifungal compounds isolated from supernatant of *L. amylovorus* DSM 19280 grown in MRS5 broth.

3.3. Identification of the antifungal compounds

The compounds inhibiting growth of *A. fumigatus* J9 in the bioassay were further characterised and the structures of the compounds were identified. The identification of pure compounds was performed using NMR, QTOF-MS, HRGC–MS and HPLC methods. Apart from lactic and acetic acids, 15 active compounds were identified in the cell free supernatants of *L. amylovorus* DSM 19280 (Fig. 1). The pool of antifungal compounds was composed of: (i) nine carboxylic acids and more in detail, three cinnamic acid derivatives (3-phenylpropanoic acid, *p*-coumaric and (*E*)-2-methylcinnamic acid), 3-phenyllactic acid and its hydroxy derivative (3-(4-hydroxyphenyl)lactic acid), lactic acid, acetic acid, D-glucuronic acid and salicylic acid; (ii) two nucleosides (cytidine and 2′-deoxycytidine); (iii) sodium decanoate and (iv) five cyclic dipeptides (cyclo(L-Pro-L-Pro) cyclo(L-Leu-L-Pro) cyclo(L-Tyr-L-Pro) cyclo(L-Met-L-Pro) and cyclo(L-His-L-Pro)).

The isolated 3-phenyllactic acid was determined to be in a 10/1 ratio of the L vs. the D isomer. All cyclic dipeptides were found to contain only amino acids in the L-configuration. The molecular weights and fragmentation behaviour of all isolated compounds are shown in Table 2.

3.4. Minimal inhibitory concentration (MIC) of antifungal compounds produced by L. amylovorus DSM 19280

Commercial reference samples of the antifungal compounds isolated from *L. amylovorus* DSM 19280 broth were used for determination of fungal inhibitory concentration with *A. fumigatus* J9 as indicator fungi. MICs of antifungal compounds were comprised between 0.05 and >200 mg/mL (Table 2).

In more detail, the results of the screening test indicate that sodium decanoate was the most active compound against *A. fumigatus* J9 with MICs of 0.05 mg/mL, while cytidine and deoxycytidine, were the least active compounds with MICs higher than 200 mg/mL (Table 2).

3.5. L. amylovorus DSM 19280 sourdough analysis

The antifungal strain *L. amylovorus* DSM 19280 was evaluated for the ability to ferment and compete in a sourdough system in comparison to the strain *L. amylovorus* DSM 20531^T. During the 48 h of the fermentation experiments, *L. amylovorus* DSM 19280 exhibited comparable growth to that of *L. amylovorus* DSM 20531^T (data not shown). Based on the 16S rRNA sequencing and colony form comparison, both *Lactobacillus* cultures were present as dominant bacteria at the end of the 48 h of fermentation (data not shown). Growth was characterised by an initial exponential phase between 0 and 24 h, followed by a stationary phase until 30 h and subsequent decline. LAB cells count was around 10^9 cfu/g for both sourdoughs after 48 h.

The decline of the pH-values was similar for both strains, although an intermediate pH-value of 4.6 was obtained earlier with *L. amylovorus* DSM 20531^T, while both strains accounted for similar pH-values (3.7) after 48 h. Additionally, no significant difference was observed in term of TTA since both *L. amylovorus* DSM 19280 and *L. amylovorus* DSM 20531^T strains were able to yield sourdoughs with final TTA value of 14.1 ± 2.3 and 13.5 ± 0.3 respectively (data not shown).

3.6. Rheofermentometer tests

The impact of the *Lactobacillus* strains on dough and bread quality parameters were evaluated and compared to the two control doughs. The results are presented in Table 3. All the parameters obtained from the gas release curve revealed significant differences between doughs containing sourdough and those non acidified or chemically acidified. The total volume of carbon dioxide production, V_t was increased by the addition of sourdough regardless the *Lactobacillus* strain used. The same occurred to the total volume of carbon dioxide retained by dough at the end of the test, V_r. However, the volume of carbon dioxide lost by dough during its fermentation, V_l was significantly higher for both doughs containing sourdough than for the non-acidified or the chemically acidified doughs. Thus, both sourdoughs, caused a reduction in the retention coefficient, RC, which represents the amount of gas retained by dough at the end of fermentation (Table 3).

3.7. Bread evaluation

The effects of sourdough addition on loaf volume, moisture, crumb hardness and crust colour of the bread were evaluated. The results showed in Table 3, clearly highlight that the addition of sourdoughs

Table 2
Molecular weight (MW), main fragment ions, and minimal inhibitory concentration (MIC) of identified compounds isolated form *L .amylovorous* DSM 19280 broth against *A. fumigatus* J9.

Antifungal compound	Chemical formula	MW	Main fragments (*m/z*)	MIC (mg mL^{-1})
Carboxylic acids				
D-Glucuronic acid	$C_6H_{10}O_7$	194.14	105.1, 91.1	25
3-Phenylpropanoic acid	$C_9H_{10}O_2$	150.17	105.1	0.5
p-Coumaric acid	$C_9H_8O_3$	164.16	119.2	>0.1
3-Phenyllactic acid	$C_9H_{10}O_3$	166.17	149.0, 119.8	2.5
3-(4-Hydroxyphenyl)lactic acid	$C_9H_{10}O_4$	182.17	163.1, 135.2	5.0
(*E*)-2-Methylcinnamic acid	$C_{10}H_{10}O_2$	162.19	147.1, 117.1	0.67
Salicylic acid	$C_7H_6O_3$	136.12	93.2	0.25
Fatty acid				
Sodium decanoate	$C_{10}H_{19}O_2Na$	194.25	153.3, 103.0	0.05
Nucleosides				
Cytidine	$C_9H_{13}N_3O_5$	243.22	109.1, 91.1	>200
2′-Deoxycytidine	$C_9H_{13}N_3O_4$	227.22	183.0, 93.1	>200
Cyclic-dipetides				
Cyclo(L-His-L-Pro)	$C_{11}H_{14}N_4O_2$	234.26	207.1, 166.0	50
Cyclo (L-Pro-L-Pro)	$C_{10}H_{14}N_2O_2$	194.23	151.2, 70	50
Cyclo (L-Met-L-Pro)	$C_{10}H_{16}N_2O_2S$	228.3	181.2,	>25
Cyclo (L-Leu-L-Pro)	$C_{11}H_{18}N_2O_2$	210.13	183.2, 155.0	>25
Cyclo (L-Tyr-L-Pro)	$C_{14}H_{16}N_2O_3$	260.3	233.0, 136.1	>25

Table 3
Gaseous release, quality parameters and textural properties of wheat doughs[1].

Property	Non-acidified	Chemically acidified	*L. amylovorus* DSM 20531^T	*L. amylovorus* DSM19280
Gas release curve				
V_t[2] (mL)	1360 ± 82^a	1327 ± 63^a	1630 ± 67^b	1647 ± 96^b
V_r[3] (mL)	1214 ± 56^a	1200 ± 35^a	1334 ± 19^b	1327 ± 8.5^b
V_l[4] (mL)	146 ± 20^a	128 ± 28^a	297 ± 47^b	320 ± 88^b
RC[5] (%)	89 ± 1.5^a	91 ± 2.4^a	82 ± 4.5^b	81 ± 4.2^b
Bread quality				
Loaf specific volume (mL/g)	3,1 ± 0.03^a	3,0 ± 0.04^a	3,4 ± 0.08^b	3,5 ± 0.14^b
Moisture (%)	46 ± 0.30^a	45 ± 0.40^a	44 ± 1.4^a	45 ± 0.60^a
Crust colour	57 ± 4.1^a	57 ± 1.0^a	57 ± 0.30^a	56 ± 3.4^a
Textural evaluation				
Hardness (N)				
Day 0	8.1 ± 1.5^a	9.0 ± 1.5^a	6.6 ± 1.2^a	7.0 ± 1.1^a
Day 2	21 ± 4.1^a	23 ± 3.2^a	15 ± 1.3^b	18 ± 1.5^{ab}
Day 5	28 ± 1.5^a	29 ± 2.9^a	22 ± 3.1^b	25 ± 0.78^{ab}

[1] Mean values ± standard deviation of three replicates is presented. Mean values followed by a common letter within the same row are not significantly different ($P<0.05$).
[2] Total volume of carbon dioxide produced by dough.
[3] Total volume of carbon dioxide retained by dough.
[4] Total volume of carbon dioxide lost by dough during the fermentation process.
[5] Retention coefficient of dough (%) calculated as follow: RC = (Vr/Vt) × 100.

yielded breads with higher specific loaf volume when compared to either non-acidified or chemically acidified doughs. Regarding to crumb hardness values, no significant differences ($p \leq 0.05$) were observed in the hardness of breads measured 2 h post baking (Table 3). However, both sourdough breads, and in particular the one fermented by *L. amylovorus* DSM 20531^T, showed a softer crumb at 48 or 120 h post-baking, compared to non-acidified and chemically acidified breads. Finally, there were no significant differences among the breads in terms of moisture content and crust colour.

3.8. Antifungal activity of sourdough bread

The impact of the antifungal strain *L. amylovorus* DSM 19280 on the shelf life of the sourdough bread was evaluated using appropriate trials (Fig. 2).

Comparison with the spoilage rate in the control breads revealed that *L. amylovorus* DSM 19280 sourdough bread retarded, with different extent, the outgrowth of both indicator and environmental fungi. Among the indicator moulds the most and the least sensitive fungi to the active compounds produced by *L. amylovorus* DSM 19280 were *F. culmorum* FST 4.05 and *P. roqueforti* FST 4.11, respectively. All control breads showed no inhibitory effect against the selected fungi and the bread slices were completely spoiled after 3 days (Fig. 2). The addition of 0.3% CaP to wheat bread affected fungal outgrowth. Compared to the control breads, the addition of CaP increased the shelf-life of the bread by 2 days against *F. culmorum* and *A. niger*, one day against *P. expansum*, and seven days against mould flora of a pilot scale bakery (Fig. 2). However, CaP could not compete with the antifungal abilities of *L. amylovorus* DSM 19280 treated bread which showed the highest antifungal performance (Fig. 2). The maximum shelf life (14 days) was obtained for *L. amylovorus* DSM 19280 sourdough bread when tested against mould flora of a pilot scale bakery (Fig. 2) whereas CaP, in the same experimental conditions, extend the shelf life of bread until 12 days (Fig. 2).

4. Discussion

Interest in food biopreservation has increased in recent years. LAB with antifungal activity, preventing growth of bread spoilage fungi are a promising alternative to chemical preservation. In this study, the ability of *L. amylovorus* DSM19280 to inhibit the outgrowth of *Aspergillus*, *Fusarium* and *Penicillium* spores has been proved both *in vitro* and in wheat bread system (Table 1, Fig. 2). Isolation of antifungal substances from cell-free supernatants identified seventeen bioactive compounds (Fig. 1) as responsible agents for this activity. Among them, 3-Phenylpropanoic acid, (*E*)-2-Methylcinnamic acid, cytidine, deoxycytidine, sodium decanoate, cyclo(L-Met-L-Pro), cyclo(L-Pro-L-Pro), cyclo(L-Tyr-L-Pro) and cyclo(L-His-L-Pro) have never been identified, as antifungal compounds produced by LAB metabolism before. Cyclic dipeptides, also called diketopiperazines, were reported in wheat sourdough by Ryan et al. (2009b). As shown in this paper these compounds are mainly produced as a side effect of the acidification process rather than by the metabolic activity of LAB (Ryan et al., 2009b). Beside their antifungal activity, diketopiperazines, are also known to be involved in the quorum sensing mechanisms, which allow Gram-negative bacteria to monitor their own population density (Holden et al., 1999). To the best of our knowledge there are no publications of Gram-positive organism using diketopiperazines in cell-to-cell communication. However, as suggested by Broberg et al. (2007), the potential interference of diketopiperazines produced by Gram-positive organisms with the quorum sensing system in eukaryotic organisms like fungi, cannot be excluded.

The remaining compounds (*p*-coumaric acid, 3-phenyllactic acid and its hydroxy derivative (3-(4-hydroxyphenyl)lactic acid), D-glucuronic acid, salicylic acid, and cyclo(L-Leu-L-Pro)) produced by strain DSM 19280 have been previously isolated from different LAB and they have been shown to be active against a wide range of bacteria, yeast and/or moulds (Báidez et al., 2006; Broberg et al., 2007; Cheng et al., 2008; Cobo Molinos et al., 2009; Dal Bello et al., 2007; Huberman et al., 2007; Lavermicocca et al., 2003; Messens et al., 2002; Rodríguez-Carvajal et al., 2008; Seong, 2006).

Numerous studies have described the isolation and characterization of antifungal metabolites under laboratory conditions, whereas the application in food system is limited (Dal Bello et al., 2007; Gerez et al., 2009; Lavermicocca et al., 2000). In this study, bread containing 20% sourdough fermented with *L. amylovorus* DSM 19280 showed remarkable ability to delay the growth of *F. culmorum*, *A. niger*, *P. expansum* and *P. roqueforti* (Fig. 2).

CaP is currently the most commonly used antifungal agent used in cereal industry. In this study CaP was used as control to show the antifungal affectivity of the biological fermentation with *L. amylovorus* DSM 19280. It could be seen that CaP was less effective than the selected antifungal strain (Fig. 2).

Interestingly, the longest shelf life was obtained in non inoculated slices of wheat bread containing sourdough fermented with *L. amylovorus* DSM 19280. However, this substantial increase of shelf life when compared to the challenge test is probably due to the lower initial contamination in our pilot scale bakery than in the challenge tests. Indeed, the fungal environmental contamination level in industrial bakeries is one or two orders of magnitude lower than that applied in the challenged tests (Spicher, 1980).

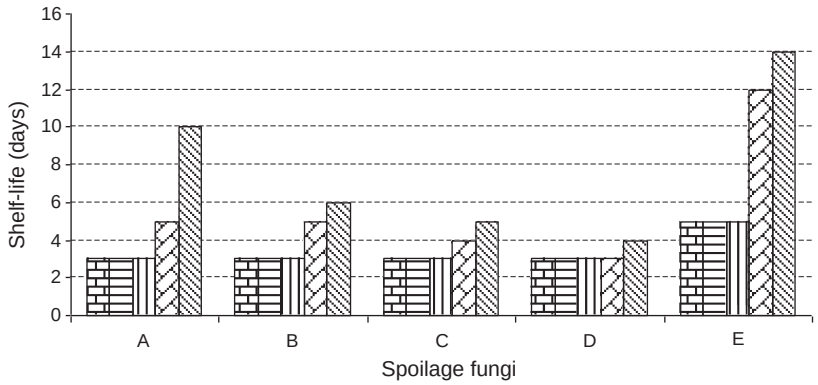


Fig. 2. Shelf life of wheat bread challenged against indicator moulds and environmental moulds from a pilot scale industrial bakery. Shelf life of wheat bread challenged against *F. culmorum* FST 4.05 (A), *A. niger* FST 4.21 (B), *P. expansum* FST 4.22 (C), *P. roqueforti* FST 4.11 (D), and environmental moulds from a pilot scale industrial bakery (E). Bread was prepared using: 20% sourdough started with *L. amylovorus* DSM19280 (▧); 0.3% CaP dough (▨), 20% sourdough started with *L.amylovorus* DSM20531^T (▥); chemically acidified dough (▤), and control dough (▦). For each bread, a series of 10 slices was inoculated by nebulisation with a 10^3 spores of each indicator mould and the other 10 slices were without inoculum. Each bread was analysed in triplicate.

The growth of *L. amylovorus* DSM 19280 in wheat bread may promote the production of antifungal substances other than the compounds isolated from liquid cultures. Their isolation and identification in wheat bread system is particularly difficult due to the presence of interfering compounds both contained in dough and produced during the baking process (Ryan et al., 2009a,b). Despite the authors developed successful protocols for the *in situ* isolation and identification of LAB antifungal compounds (Ryan et al., 2009a,b), the complete profile and importance of the antifungal metabolites produced by *L. amylovorus* DSM 19280 in wheat bread system remains to be investigated.

Following the successful demonstration that *L. amylovorus* DSM 19280 can increase the shelf life of wheat bread the next step was to investigate if the strain DSM 19280 can successfully perform sourdough fermentation and produce a bread of good quality. It is well documented how sourdough technology can improve the quality of wheat bread in terms of bread volume, crumb structure (Clarke et al., 2002; Corsetti et al., 2000; Crowley et al., 2002; Zannini et al., 2009) flavour (Thiele et al., 2002), and nutritional values (Liljeberg et al., 1995).

The present study confirmed that addition of sourdough, either fermented by *L. amylovorus* DSM 19280 or *L. amylovorus* DSM 20531^T significantly increased the breadmaking performance of wheat dough. Rheofermentometer results showed that sourdough improved the gas production and gas retention (Table 3). The increase of gas production in sourdoughs could be related to the high acidification activity of the two LAB strains, (data not shown) which may enhance the capacity of the gluten to retain CO_2 (Gobbetti et al., 1995). Indeed, in the initial phase of the fermentation *L. amylovorus* amylases as well as endogenous flour amylases might contribute to the breakdown of the starch yielding maltose serving as an energy source for microbial growth and thus enhancing the acidification process (Messens et al., 2002). Analysis of loaf volume confirmed that sourdough addition caused an increase of volume not achieved by chemical acidification. The positive impact of an increase in specific loaf volume was reflected in the sourdough breads by lower crumb hardness values (Table 3). Our findings are not in agreement with those of Armero and Collar (1998) who noted that the sourdough process led to harder breads than the straight process. Probably, as shown by Corsetti et al. (1998), the use of a strain of LAB with particular characteristic appears to be a fundamental prerequisite to retard staling. In this study the use of *L. amylovorus*, a LAB species known for its amylase activity, could exert a positive effect in decreasing the staling rate during the storage as reported by Corsetti et al. (2000). In summary, the rheofermentometer and baking tests clearly indicated that *L. amylovorus* DSM 19280 was a suitable starter culture for wheat sourdough and bread production.

The consumers demand for food free from chemical additives increases the needs to look for new food-grade bio-strategies. The results of this study clearly indicate that the sole addition of sourdough fermented with *L. amylovorus* DSM 19280 has the potential to replace the use of calcium propionate as a bread preservative. This is of particular importance since CaP was been associated with potential tumorigenic and neurobiological effects (MacFabe et al., 2007). In conclusion, *L. amylovorus* DSM 19280 can be used as starter culture to perform sourdough fermentations and to produce bread of good quality with extended shelf life.

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