

The Use and Effects of Lactic Acid Bacteria in Malting and Brewing with Their Relationships to Antifungal Activity, Mycotoxins and Gushing: A Review

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ABSTRACT

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Several metabolic properties of lactic acid bacteria (LAB) serve special functions, which directly or indirectly have impact on processes such as improved quality and safety and flavour development in the malting and brewing industry. LAB are widely distributed in nature and in spontaneous fermentations, often they are found to be the dominating microflora resulting in both the inhibition of spoilage bacteria and organisms. This review describes the applications of LAB in malting and brewing. Mycotoxins are naturally occurring toxic secondary metabolites of fungi that may be present in cereals. Several of these mycotoxins have been associated with human and animal diseases and are known to survive the brewing process. LAB have been shown to restrict the growth of the most important toxigenic fungi thereby reducing the formation of these harmful toxins. The occurrence of mycotoxins in cereals is discussed and their effect in beer is reviewed. The main features of this review are: (I) LAB starter cultures in malting and brewing (II) production of acid malt; (III) biological acidification of mash and wort in brewing; (IV) bacteriocins produced by LAB in brewing; (V) LAB and antifungal activity; (VI) mycotoxins in cereals.

Key words: Lactic acid bacteria, malting, brewing, biological acidification, *Fusarium*, mycotoxins.

1.0 Introduction

Microbial proliferation has well been established as an indigenous component of the malting and brewing environment¹⁵³, with the resultant microflora having both beneficial and detrimental effects on malt quality¹³⁹. LAB omnipresent on the surface of malt barley may positively influence the quality and safety of the malt and derived products; this property has been exploited for the biological improvement of the malting and beer process^{152,205}.

Contamination of barley by mycotoxigenic and active gushing inducing *Fusaria* are of particular concern to both maltsters and brewers. The ability of LAB to restrict the growth of *Fusarium* moulds in order to prevent gushing is discussed. Gushing of beer is regarded as one of the most negative consequences of moulds with respect to the quality of malt and beer³. LAB are also of particular interest as biopreservation organisms. Their preserving effect relates to the formation of antimicrobial compounds such as lactic acid, acetic acid, hydrogen peroxide and the production of bacteriocins^{112,194}. There have been few reports on the production of specific antifungal compounds from LAB, despite the fact that moulds and yeasts are important spoilage organisms of various food and feed stuffs.

This paper reviews the use of LAB in both the malting and brewing processes. The occurrence of mycotoxins in cereals is discussed along with the implications of these mycotoxins in the malting and brewing industries. Antimicrobial and antifungal activity of compounds produced by LAB are also discussed, identifying where possible, the compounds responsible for the antifungal activity.

2.0 Applications of lactic acid bacteria in malting and brewing

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^{16,74}. This starter culture may be defined as a preparation or material containing large numbers of variable microorganisms, which may be added to accelerate a fermentation process. Being adapted to the substrate, a typical starter facilitates improved control of a fermentation process and predictability of its products⁸¹. The “starter” culture in malting is a relatively new process that controls indigenous microbial growth, and is both technically and economically feasible. Reported beneficial effects on the malting process include a lower viscosity and β -glucan content of wort, increased malt yield, and a pronounced improvement of mash filtration and wort filterability⁷⁴. Boivin and Malanda¹⁷ demonstrated how the application of starter cultures could inhibit the growth of undesirable moulds and the production of mycotoxins when applied to the steeping water during malting.

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The conditions of malting are favourable for the rapid growth and development of bacteria, yeast and fungi, present naturally in barley as well as in the malting house; therefore strict control of incoming barley is vitally important. Steeping is probably the most critical stage at which microbial proliferation begins. Bacteria and yeasts rapidly grow; mould mycelium develops while dormant spores are activated resulting in new growth that can extend from kernel to kernel⁶³. Steep aeration adds to this process⁹⁴ leading to a coat of bacteria, yeasts and fungal spores on steeped grains but particularly damaged kernels. Various systems have been operated to reduce the adverse effects of microbes during steeping such as eliminating aeration during the initial steeping, minimising nutrient supplies to microbes by changing the liquor with air rests²², however the addition of LAB starter cultures is a more effective and natural option. The utilization of LAB as starter cultures in malting reduces the fungal contamination, lowers the aerobic bacterial flora and leads to a higher quality malt regardless of the natural variation of the microflora of barley^{16,17,76}. The addition of starter cultures in the early stage of malting is important due to the intensive growth of *Fusaria* during the very first hours of steeping⁷⁶. Certain *Lactobacillus plantarum* and *Pediococcus pentosaceus* strains have been reported as especially efficient when added to the steeping waters of barley at the level of about 10^7 cells g^{-1} ⁷⁶. It has also been reported that the addition of a pure culture of a bacterial strain described as *Pseudomonas herbicola* during the steeping process resulted in a better quality malt and the reduction of the germination period by one day¹⁵⁶. In addition, characterisation of the molecular mass distribution of β -glucans by gel permeation chromatography revealed a lower molecular size for the wort β -glucans when starter cultures were applied to the first and second steeping waters of barley resulting in faster filtration times and lower wort viscosity¹⁹⁷. The extent of the effectiveness of these starter cultures depends not only on the composition of the barley but also on the contamination level of the barley.

Major metabolic changes in the barley kernel take place during the germination step. Viable counts of bacteria and yeasts reach a maximum as enzymes convert residual carbohydrates to fermentable sugars¹⁵⁸. Although the need for control of the microbial load during germination has been emphasized, varying environmental conditions is not an option without affecting plant physiology²⁰³. LAB starter cultures have positively influenced the malting process by actively contributing to barley germination and/or malt modification. Noots et al.¹³⁹ confirmed that selected starter cultures deliver enzymes in the starchy endosperm that contribute to the modification of the cell wall material. Angelino and Bol⁴ found an unspecified β -glucanase producing starter culture that increased the green malt β -glucanase activity, which ultimately resulted in improved malt quality parameters such as faster filtration time, improved extract difference and reduced wort viscosity. Fluorescence microscopy studies using Calcofluor and Congo Red fluorochromes for staining of malt and mash structural components showed intensive degradation of proteins and cell wall β -glucans when lactic acid bacteria were applied. Xylanase activities of starter malts

are also higher than those of control malts; possibly indicating enhanced modification of the barley kernels¹¹³.

Microbial starter cultures have also been applied in the field by spraying lactic acid bacteria starter cultures during the seed development period of malting barley¹⁷⁰. This resulted in several beneficial effects on the quality of barley and malt made from it such as a decreased proportion of kernels contaminated with *Fusarium*, water sensitivity of barley decreased, falling number increased, extract, FAN and α -amylase activity increased, better malt modification and improved wort filterability were reported while the gushing tendency decreased.

The effect of lactic acid starter cultures in the malting and brewing processes is due to their broad spectrum of microbicidal activity against toxigenic and gushing-active *Fusarium* fungi and also on their other characteristics such as enzyme activities. By addition of lactic starter cultures during the steeping of barley, it has been possible to retard *Fusarium* contamination during malting while reducing the formation of deoxynivalenol and zearalenone mycotoxins¹⁰⁴. Previous to this Haikara and Laitila⁷⁴ also observed a reduction in *Fusaria* contaminations during malting by the addition of LAB while Karunaratne et al.⁹³ and Gourama and Bullerman⁷⁰ both reported that aflatoxigenic moulds produce lower amounts of aflatoxin in the presence of LAB. This is of interest to maltsters and brewers as mould growth and mycotoxin production have been shown to continue to develop during the germination stage of the malting process. The main mycotoxins in cereals and their effects in beer will be discussed in more detail at a later stage.

The brewing industry, although often described as a typical example of traditional or old biotechnology, is applying a whole spectrum of new technical, biochemical, and microbiological inventions. LAB starter cultures are applied in the brewing industry for their ability to improve mash and wort characteristics while ultimately resulting in a better beer. Certain LAB produce antimicrobial substances, which restrict the growth of harmful Gram-negative and positive bacteria^{141,138}. These Gram-negative and positive bacteria compete with grain tissue for dissolved oxygen and may retard mash filtration^{54,73}. The use of lactic acid starter cultures in malting has also led to significant effects in the mashing and brewing processes. β -Glucans are known to cause a number of problems during the brewing process. β -Glucans are complex carbohydrates composed of mixed linkage (1 \rightarrow 3),(1 \rightarrow 4) β -D-glucose polymers. These polysaccharides have been linked with slow lautering, poor beer filtration, and formation of haze during storage of packaged beer⁸³. A significant advantage of the application of lactic starters is improved mash filterability (lautering performance). This is crucial as lautering is often the bottleneck of brewhouse operations. Other advantages observed include lower wort viscosity, and better clarity of wort. Studies show starter technology offers a new tool to avoid lautering problems, especially in years when the presence of split kernels is anticipated due to unfavourable weather conditions¹⁰³. High quality of beer must be ensured when starter cultures are applied in the brewing process. Numerous pilot brewing trials have shown that the aroma profiles of the beers compared well organoleptically with control beers, and their flavour stability was better⁷⁴.

3.0 Acidification of mash and wort

LAB may also be used to biologically acidify mash and wort. Mash and wort acidification has been defined as “the establishment of definite pH levels in the mash, wort and resulting beer that are without the use of some additional acid, beyond the reach of the brewer”¹¹⁰. Typically the average malt will create a mash with a pH of approximately 5.7–5.75. This is higher than the optimum pH for the majority of essential mash enzymes namely β -glucanase (4.5–4.8); α -amylase (5.3–5.7); β -amylase (5.1–5.3) and carboxypeptidases (4.5–4.6). Adjusting the pH to 5.4 enhances the mash enzyme activities and as a result gives rise to a number of beneficial effects including a higher attenuation limit, more extensive protein breakdown and faster lautering times^{100,110}. The pH can be reduced a number of different ways:

3.1 Addition of acids or salts

Phosphoric acids are usually employed but other mineral acids such as sulphuric acid may also be used. Salts such as gypsum (calcium sulphate), Epsom (magnesium sulphate) or calcium sulphate may also be used; these function by acidifying the water. This treatment may lead to flavour defects in the final beer as brewery water is normally treated to achieve calcium levels of 100 ppm. Addition of calcium prevents yeast degeneration and poor break formation, therefore the addition of acids or salts to reduce pH should not interfere with the existing calcium level¹⁰⁰.

3.2 Inclusion of acid malt to the grist

Acid malts are malts enriched with lactic acid to reduce the pH of the mash so as to optimise amylolysis and proteolysis. Such malts may be added at rates from 3–5% of the grist up to a possible 10% where the direct addition of mineral acid or lactic acid either is considered undesirable or forbidden²¹. The use of acid malts not only improve enzymolysis but are reported to increase extract recovery as much as 0.9%; improve break formation; and give a cleaner flavour to pale beers²¹.

There are various methods to prepare acid malt¹¹. An example of such is to hold the germinating barley under anaerobic conditions for at least 24 h, until it becomes acidic due to the action of omnipresent LAB. Another method is to spray green malt with a suspension of the bacterium *Lactobacillus delbrueckii* followed by a holding period of 24–36 h at 50°C prior to kilning²¹. Kunze¹⁰⁰ describes a process which entails steeping the kilned pale malt in water at 45–50°C until the LAB in the malt have formed about 1% lactic acid. The malt is then dried, thus concentrating the lactic acid to between 2 and 4%. The principal of all methods is the same whereby sugars are leached from the malt-allowing LAB to convert these sugars to acid.

Mash acidification using acid malt is reported to reduce pH by 0.15–0.25 units, resulting in improved β -amylase, protease, endo β -glucanase and phosphatase action. Increased buffer capacity, and higher levels of soluble nitrogen were recorded, while less tannin was dissolved. However using acid malts also leads to a higher beer pH.

Therefore it is recommended that mash acidification be followed by wort acidification to lower the final pH of the wort to 5.1¹⁵¹.

3.3 Addition of biologically acidified wort

Under German “Reinheitsgebot” regulations the use of biologically acidified wort is the only technological possibility to correct the pH of mash and wort; in addition, this will improve the taste of the finished beer. LAB that have been isolated from the malt are used to acidify unhopped wort that is then subsequently reintroduced to the brewing process, either at the start of mashing (mash acidification) or during wort boiling (wort acidification) or both¹⁰⁰. According to Nummer¹⁴² the most commonly used strains for biological acidification include *Lactobacillus delbrueckii*, *Lactobacillus amylovorus* and *Pediococcus acidilacti*. These lactic acid bacteria have the ability to grow at 48°C, which gives them a competitive advantage as the growth of most spoilage bacteria is prevented. Acidification is carried out in unhopped wort as hop compounds inhibit LAB. This is principally due to the bacteriostatic properties of hop compounds present in beer²⁰⁷. LAB are anaerobic therefore it is advised to perform culturing in a CO₂ atmosphere¹⁰⁰. Levels of lactic acid produced are variable but normally range between 0.6–0.8%, although levels of up to 1% have been reported for a strain of *Lactobacillus delbrueckii*. A pH drop of 0.2 units is observed for every 2.5% lactic acid added to the main mash. Acidification is normally complete within 24 h¹⁴⁹. Kunze¹⁰⁰ recommends a goal of wort acidification of pH 5.1–5.2. Research has shown that this will result in lighter coloured wort and beer along with a faster fermentation.

3.4 Beneficial effects of biological acidification

The beneficial effects of biological acidification are outlined in Table I¹⁶². Lewis and Young¹¹¹ noted improved organoleptic qualities of the beer, a shorter mashing program, and more efficient enzymatic processes when uniformly modified malt was acidified. Similarly, positive effects were observed by Oliver-Daumen et al.¹⁵¹, such as improvement of beer bitterness, better diacetyl reduction during fermentation, paler beer colour, increased tannin content as well as a noticeable improvement of taste stability. Less protein haze was observed in worts that were biologically acidified due to extensive protein breakdown, while foam stability was enhanced due to the high zinc levels. Zinc is essential for the protein biosynthesis and utilisation in brewer’s yeast. At levels of 0.1–0.15 mg L⁻¹ zinc in cast wort, a quicker fermentation and lower levels of diacetyl result⁷¹. Only 3% of the zinc coming from malt, hops, or water is to be found in the cast wort. This is due to adsorption on spent grains or hot trub and the development of zinc-protein complexes (chelates). Donhauser and Wagner⁵³ explain the high levels of zinc in acidified wort. During biological acidification, more positive charged ions are brought into the wort. These ions react with the chelators rather than the zinc, so that more zinc remains in the wort. Narziss¹³⁴ observed an increase of zinc levels in wort from 0.10 mg L⁻¹ to 0.18 mg L⁻¹ by using biological acidification.

Table I. Positive effects of biological acidification (adapted from Pittner and Back¹⁶²)

Technological benefits	
Enzyme supply	Activation of important mash enzymes
Nutrient supply	Improvement of zinc supply
Elimination of proteins	Better break formation
Redox potential	Lower sensitivity to O ₂
Fermentation	Faster pH decrease, better hot trub precipitation, higher degree of fermentation
Sensory benefits	
Taste	Fuller and smoother flavour profile
Hop bitterness	Pleasant bitterness
Liveliness	Fresh character
Foam	Fine bubbles, stable
Colour	Lighter
Chemical and physical stability	Lower risk of protein haze
Microbiological benefits	
Lower pH	Growth of most potential spoilage organisms (e.g. <i>Megasphaera</i> spp.) will be hindered below pH 4.5. For the obligatory beer spoilage organisms (growth is more retarded, the lower the pH).
Better selection pressure of yeast	Better growth conditions for yeast suppress the growth of competitors.
Higher attenuation of fermentation	Less easily fermentable sugars for spoilage organisms.

3.5 Negative effects of biological acidification

There are disadvantages of using biological acidification, bitter substances are lost, along with higher levels of coagulable nitrogen. Mash acidification increases the buffering capacity of cast out wort and therefore makes it also necessary to acidify the cast out wort. A low pH during wort boiling also leads to lower hop isomerisation and a lower splitting range of DMS precursor^{110,162}. However it is possible to minimise these effects by late addition of acidified wort during wort boiling. Wort acidification seems to be the preferred method of biological acidification; it improves the wort and beer characteristics, but will not reduce the pH of the final beer, whereas mash acidification seems to affect the mash and wort character alone. Oliver-Daumen et al.¹⁵¹ recommend that mash and wort acidification be performed together as the benefits to the brewer are vast.

Biological mash and wort acidification may be carried out in a batch, semi-continuous or continuous process.

3.6 Batch acidification

This is the most widespread and most expensive method. Ten percent of the total stock vessel volume is charged with active acidified wort, and subsequently refilled with wort of varying extract concentration. Temperature is maintained between 46–48°C to permit the lactic strain to propagate, while the contents are gently agitated to ensure faster nutrient introduction. Oliver-Daumen and Schwedhelm¹⁵⁰ found batch acidification provided faster and higher levels of lactic acid if minimal levels of oxygen were incorporated during the fermentation. Batch acidification takes approximately 12–48 h to complete.

3.7 Semi-continuous acidification

In a semi-continuous process acid wort is discontinuously withdrawn for mash or wort acidification. A maximum volume of between 5–10% of the acidified matter is drawn off to acidify the mash or wort, which leaves a permanent volume of 90–95% in the reactor. Vessel temperature is strictly controlled at $48 \pm 1^\circ\text{C}$. Frequency of withdrawal depends on the brew cycles e.g. at 2 h intervals or to fill up the vessels with the first wort spargings¹⁵⁰. Aerobic or anaerobic operation is possible as it is a closed system.

3.8 Continuous acidification

Acidified wort is continually removed from the fermenter and replaced with a nutrient substrate (unacidified wort); therefore the volume of acidified matter remains constant. To prevent end product inhibition of LAB, the lactic acid is precipitated with calcium carbonate. This ensures that the actual lactic acid concentration never exceeds the critical range of greater than 1% acid, and thus ensures optimal acid production¹⁵⁰.

Pittner and Back¹⁶² found this system gave high performance when there was limited brewery space; there was also reduced risk of microbial contamination as the system is closed resulting in superior product quality. It is not however an acceptable form of acidification under the German Purity Law.

3.9 Wort acidification using immobilised LAB

Immobilisation of LAB for use in wort acidification involves restricting the free movement of cells or enzymes by fastening them to a solid carrier, which is then supplied with first wort. When a cell or enzyme is bound to a solid carrier the fluid surrounding the particle can be moved quickly without the fear that the cell or enzyme is washed away. This results in the production of lactic acid. The final desired pH (5.1–5.2) is achieved through a post acidification process. Pittner et al.¹⁶³ applied this idea to produce acidified wort using a *Lactobacillus amylovorus* strain on a DEAE-cellulose carrier. Circulating a bacterial suspension through the carrier bed in the bioreactor carried out immobilisation. The cells are adsorbed on the carrier surface via electrostatic charge. DEAE-cellulose showed excellent performance as a support matrix for the immobilisation. The initial pH reduction was found to be dependent on the carrier material i.e. the amount used. Acid production was dependent on the acid concentration on entrance to, and residence time within, the holding tank. The advantages of this system according to Pittner et al.¹⁶³ are outlined below:

Carrier related advantages

1. The loaded carrier has a high cell density, 10^{10} – 10^{11} cells per gram of dry carrier
2. Immobilisation is carried out by simply recirculating a bacterial suspension through the reactor
3. The carrier is very suitable for immobilisation of LAB, it is regenerable, non compressible and easy to handle.

System related advantages

1. A high productivity in a small volume and factory space is attainable.

2. The closed passage of wort and the high dominance of LAB lead to high microbiological security and therefore to invariably faultless flavour quality
3. The system is well adapted to the brewing environment due to the high flexibility in selecting the flow rate through the reactor.
4. The plant is suitable for automation.

3.10 Acidification requirements

To ensure successful bio acidification wort should be rich in vitamins and amino acids to meet the growth requirements of the lactic acid strain. Strains should have a high viability and be capable of growth at 48°C, to avoid the presence of butyric acid bacteria and yeast. The most appropriate strains have high amylolytic activity, are homofermentative, and capable of producing high levels of L-lactic acid. Lactic acid levels should be monitored during lactic acid fermentation, as levels greater than 1–1.2% lactic acid lead to problems with propagation of the strain. If possible a pH of 5.4–5.6 should be reached for the mash while pH 5.1–5.2 is more beneficial for wort. At these values, enzyme activity is optimised while beer flavour is improved¹⁴⁹.

4.0 Bacteriocins in brewing

Among the variety of antimicrobial substances produced by LAB, bacteriocins are one of the most promising natural food preservatives. According to a classical definition, bacteriocins are proteinaceous compounds that are bactericidal to strains closely related to the producer strain⁹⁵. Bacteriocins are generally only active against Gram-positive cells due to the protective barrier provided by the lipopolysaccharides of the outer membrane of Gram-negative bacteria²⁰⁶. It has become evident that many bacteriocins from LAB and other Gram-positive bacteria have a somewhat broader spectrum of activity, affecting also more distantly related species. Numerous strains of LAB are capable of producing bacteriocins including *Lactobacillus fermenti*, *Lactobacillus helveticus*, *Lactobacillus acidophilus* and *Lactobacillus plantarum*. Several of these bacteriocins are active against *Bacillus cereus*, *Staphylococcus aureus*, *Clostridium botulinum*, *Clostridium perfringens*, *Clostridium difficile* and *Listeria monocytogenes* even under in situ conditions^{82,96}. Bacteriocins of LAB are able to inhibit a wide spectrum of beer spoilage organisms, in particular strains of *Lactobacilli*, *Pediococci*, and *Micrococci*. Certain bacteriocins are also capable of inhibiting Gram-negative beer spoiling organisms such as *Acetobacter pasteurianus* and *Gluconobacter oxydans*¹²⁴. Bacteriocins function by targeting the cytoplasmic membrane; they alter the permeability, thus inhibiting energy production and biosynthesis resulting in cell death. Examples of bacteriocins include lactocin 3147, lactococcin, sakacin P, curvacin A, and nisin^{45,174}.

Idler and Annemuller⁸⁴ screened strains of LAB that were already used in biological acidification for their ability to produce bacteriocins. Findings showed the ability of one strain (B1/2) to inhibit eight beer spoilers while the other strain (B 2/5) inhibited just five of the indicator strains, neither strain were active against Gram-negative bacteria. The study also revealed how the pH of the wort

environment did not affect bacteriogenicity of either strain, while the temperature had a major influence. One strain (B 2/5) showed decreased activity at 48°C, while another (B1/2) could not be used in the brewing trials, as at temperatures greater than 40°C its activity was minimal. It was also observed that when grown in brewer's wort the bacteriocin activity of both strains dropped by 50%, compared to levels when grown in MRS-broth. Brewing trials resulted in a reduction of microbial contamination levels along with partially improved foam stability. Sensory analysis showed no significant difference. Further tests where bacteriocin concentrate was added showed no change in bacteriocin activity during fermentation, followed however by zero activity during beer conditioning.

More recently Idler and Annemuller⁸⁵ isolated a further strain (BB 7/3), which also had bacteriocin and thermophilic properties. Bacteriocin activity was notably improved in comparison to the aforementioned strains, as was activity at 48°C. Activity was again reduced when the bacteria was cultured in brewer's wort, but to a lesser extent than the B 2/5 strain. This newly isolated strain was also active over the pH range that would be encountered in brewing. Tests also showed by adding the culture at various stages during mashing, stability of bacteriocin activity could be as high as 40% if it was added towards the end of the mash cycle. They concluded however, that the acidification properties of the strain alone would not be adequate as a sole acidification culture, and proposed that this strain be used in a mixed culture system along with an acceptable acidification culture.

Bacteriocins have three limitations that reduce their effectiveness as food biopreservatives:

1. They have so far been shown to be ineffective against spoilage and pathogenic Gram-negative bacteria, yeasts and fungi.
2. Their host range can be rather narrow, even within Gram-positive species.
3. Insensitive variants of a sensitive bacterium appear rather frequently in the presence of bacteriocins

Thus from the point of view of safety, it is not possible to completely rely on the antimicrobial effects of bacteriocins.

4.1 Nisin

Nisin is a heat stable bacteriocin produced by *Lactococcus lactis* ssp. *lactis*⁴⁴. Nisin is the only bacteriocin with GRAS (Generally Regarded As Safe) status for use in foods. This was awarded as a result of a history of 25 years of safe use in European countries and was further supported by the accumulated data indicating its non-toxic, non-allergenic nature⁵⁸. All other bacteriocins will require legislative approval in order to acquire GRAS status for use in food products.

Nisin was originally discovered in the late '20s and described in the early '30s^{171,172}. It has been assigned the food additive number E234⁴⁴, and is approved by the FDA⁵. Nisin has a broad inhibitory spectrum and is an effective inhibitor of many Gram-positive bacteria that are pathogenic or involved in food spoilage. The C-terminal region of nisin binds to the cytoplasmic membrane of vegetative cells and penetrates into the lipid phase of the

membrane²⁰, forming pores, which allow the efflux of potassium ions, ATP and amino acids^{39,77,87} resulting in dissipation of the proton motive force and eventually cell death²³. The effectiveness of nisin depends on growth and exposure conditions, such as temperature²⁰⁰, and pH⁸⁸. In general, nisin is more active at lower pH values, whereas the influence of temperature on its effectiveness is controversial.

Ogden et al.¹⁴⁶ found that nisin is effective against a wide range of brewery spoilage organisms and suggest its application as a preservative agent in worts, which would not be boiled immediately after lautering. Tests showed that nisin killed over 99.9% of sensitive lactobacilli and over 95% of sensitive *Pediococci* while also inhibiting the growth of a nisin resistant strain of *Lactobacillus* during fermentation. Subsequently Ogden et al.¹⁴⁶ also reported commercial usage levels of nisin had no adverse effects on the characteristics of brewing yeasts, along with no harmful effects on the taste of beer. Various other studies are concurrent with these results. Previous to this Ogden and Tubb¹⁴⁵ observed the inhibitory effects of nisin against beer spoilage organisms without any detrimental effects on brewing yeasts. More recently the sensitivity of *P. frisingensis* to nisin has been reported³⁰ in contrast to almost all Gram-negative bacteria which are resistant to nisin unless the outer membrane of the cell is damaged by EDTA treatment¹⁹². Synergistic approaches are useful to overcome certain limitations of bacteriocins. In an attempt to broaden the antimicrobial spectrum of bacteriocins, other compounds are applied (e.g. chelating agents such as EDTA) in conjunction with nisin to increase the inhibitory spectrum of the bacteriocin. The increased inhibition is due to the increased permeability of the cell envelope of Gram-negative cells^{43,202}. EDTA is approved as a food additive that is designed primarily as a preservative or to inhibit discolouration. From a regulatory perspective, it should be noted that EDTA is not considered to be a natural additive. Furthermore, EDTA has the drawback that it sequesters a wide range of bi/trivalent metal ions including Fe²⁺, Al³⁺, Cu²⁺, and the transition elements Zn²⁺, Co²⁺, and Sr²⁺.

Nisin may be added at various stages of the brewing process to prevent bacterial contamination or bacterial growth. For example nisin could be used as an alternative to acid washing of yeasts. The yeast cultures are the main reservoir of bacterial infection in the brewing process, and may harbour several different types of beer-spoilage microorganisms¹⁴⁴. In a study by Ogden et al.¹⁴⁶ to determine the effects of post-fermentation treatments on nisin activity such as fining and filtration, it was shown that the use of a variety of filter aids and finings resulted in 8% to 10% loss in nisin activity (nisin was added post-fermentation and the activity was determined after the filtration and fining of the beer respectively).

Nisin may also have a possible application in the ethanol fermentation industry for the control of LAB contamination^{79,125,126}. Periago and Moezelaar¹⁵⁷ studied the combined effect of nisin and carvacrol at different pH and temperature levels on the viability of different strains of *Bacillus cereus*. The five strains tested showed significant differences in sensitivity towards nisin, at pH 7.0 and 30°C. Carvacrol concentrations of 0.3 mmol l⁻¹ had no

effect on viability of *B. cereus* cells. When the same carvacrol concentration was combined with nisin, however, it resulted in a greater loss of viability of cells, than when nisin was applied alone. The concentration of carvacrol played an important role on the bactericidal effect of nisin and, therefore, on the synergistic action of both compounds combined. Other potential applications include the use of nisin as a preservative in sake products to inhibit *Lactobacillus sakei* hiochi-type bacteria, the major spoilage organisms associated with sake⁹².

Like beer, wine can also suffer from spoilage as a result of undesirable LAB growth and subsequent production of detrimental compounds⁴⁰. Sulphur dioxide is often used to suppress LAB in winemaking although its use is strictly regulated. Nisin, although of non-oenological origin, has potential application(s) in winemaking, since it has been shown to restrain the growth of undesirable LAB in wine⁴⁴. Resulting experiments have indicated that nisin has potential in combating spoilage in wines as well as in fruit brandies and fruit mashes, as most strains of *Leuconostoc* and *Pediococcus* are sensitive to it, with *Oenococcus oenus*, the most important strain for malolactic fermentation exhibiting particular sensitivity to nisin^{79,168}. Sensory characteristics of wine remained intact when treated with nisin¹⁶⁹.

5.0 LAB producing antifungal compounds

LAB are known to produce a number of antimicrobial compounds and are important in the biopreservation of food and feed¹³⁰. Their preserving effect due to reduction in pH is mainly related to the production of organic acids, i.e. lactic acid and acetic acid¹⁹⁴, competition for nutrients, and the production of bacteriocins, but other compounds such as hydrogen peroxide, formic acid, propionic acid, acetoin, and diacetyl are also produced¹¹². However, the application of these antimicrobial compounds may be limited due to their narrow inhibitory spectrum, and their instability under certain conditions. The exact mechanism of antimicrobial action can often not be explained due to the complex interactive nature between the different compounds, however synergistic effects are often observed between compounds involved in antimicrobial action^{38,138}.

While many studies have assessed their antibacterial effects⁵², there are few reports on specific antifungal compounds from LAB. This has been complicated by the fact that some fungi are sensitive to the normal by-products of LAB metabolism in particular lactic and acetic acids^{112,160}. In order to evaluate the antifungal potential of lactic acid bacteria, Marth and Hussong¹²² tested four different cultures of *Leuconostoc citrovorum* for their ability to inhibit growth of several organisms at pH 4.5. They found that these cultures inhibited spoilage bacteria but not yeasts. However in a related study Magak'yan and Chuprina¹¹⁵ found *Lactobacillus plantarum* to be the most inhibitory of the different species of lactic acid bacteria tested, as it exhibited antibiotic action against *Aspergillus glaucus*, *Penicillium glaucum* and *Cladosporium herbarum*. In a different study, Collins and Hardt³⁵ were able to demonstrate that filtrates of *L. acidophilus* at pH 7.7 in casitone broth

could retard the growth of *Candida albicans*, although the mould culture was able to grow in nutrient broth containing glucose at pH 4.6. No attempt however was made to explain the cause of discrepancy in the production of antifungal substance under different nutritional conditions. In another study by Batish et al.⁶, 19 standard cultures of LAB were analysed for antifungal activity against a large number of moulds. Out of these, five were able to exhibit a clear antifungal activity against *A. parasiticus*, *A. fumigatus*, *Rhizopus stolonifer* 41, and a wild *Rhizopus* spp.

The reports regarding the role of organic acids from microbial sources in suppressing fungal growth have remained quite insufficient. The inhibitory effects of organic acids are still a complex and unresolved issue, as little purification or characterisation on these compounds has been carried out. Hertung and Drury⁸⁰ studied the relative antifungal activity of volatile fatty acids (VFAs) on grain and found propionic acid to be an effective antifungal substance at a level of 0.8% on grains having 20% moisture. In a different but related study, Paster¹⁵⁵ found propionic acid to be fungicidal against high fungal populations in feeds where *Aspergillus* was the dominant genus. Propionic acid and its salts have also been found to negatively affect fungal growth particularly at low pH²¹³. Similar to lactic and acetic acid, propionic acid can interact with cell membranes to neutralise the electrochemical proton gradient, but it is thought that the effect of propionic and acetic acid are only as a result of the decrease in pH due to lactic acid⁶⁵. Synergistic antifungal effects were reported when lactic acid produced by LAB was combined with sodium acetate of de Mann, Rogosa, Sharpe (MRS), a growth medium for LAB^{25,193}. This may be true too for other antifungal compounds produced¹⁹³. The antimicrobial effect of hydrogen peroxide has been well documented, its concentration in the LAB environment is high since LAB does not produce catalase, although the concentration itself is not inhibitory, it has inhibitory potential in the presence of lactoperoxidase and thiocyanate in natural environments such as milk and saliva³⁶. Hydrogen peroxide has a strong oxidising effect on the bacterial cell, and this is attributed to the destruction of the basic molecular structures of cellular proteins⁴¹. MRS should not be used as a substrate when screening for the antimicrobial substance hydrogen peroxide, as the catalase activity of the yeast extract rapidly degrades hydrogen peroxide¹⁷³.

Diacetyl another end product of fermentation, the molecule responsible for off flavours in beer, is produced by strains of all genera of lactic acid bacteria during citrate fermentation⁵⁶. Diacetyl has antimicrobial activity at levels of 200 mM, especially at pH levels less than 7.0⁸⁹. However, this level of diacetyl will alter the taste and aroma of the product¹⁵⁹.

There are few reports of antifungal peptides produced by lactic acid bacteria. Ström et al.¹⁹⁵ isolated a *Lactobacillus plantarum* strain (MiLAB 393) from grass silage that produced a broad spectrum of antifungal compounds, active against food- and feed-borne filamentous fungi and yeasts. *Fusarium sporotrichioides* and *Aspergillus fumigatus* were found to be the most sensitive of moulds tested, while *Kluyveromyces marxianus* was the most sensitive of yeasts. No inhibitory activity was detected against the mould *Penicillium roqueforti* (the most widespread con-

taminant of bakery products) or the yeast *Zygosaccharomyces bailii*. Three substances responsible for the antifungal activity were identified as 3-phenyllactic acid, and two cyclic dipeptides. Magnusson and Schnürer¹¹⁷ described production of a proteinaceous antifungal compound by a *Lactobacillus coryniformis* strain active against several moulds and yeasts including *Debaromyces hanseii* and *Kluyveromyces marxianus*. The peptide was small (approx 3 kDa), heat stable, active in the pH range 3–6 and totally inactivated by proteinase K or trypsin. Magnusson et al.¹¹⁸ recently screened more than 1200 isolates of LAB from different environments for antifungal activity. Several of the strains showed strong inhibitory activity against the moulds *Aspergillus fumigatus*, *Aspergillus nidulans*, *Penicillium anomala* and *Kluyveromyces marxianus*, and also against the yeast *Rhodotorula mucilaginosa*. However numerous isolates showed reduced antifungal activity after storage and handling. The authors found the degree of fungal inhibition was not only related to production of acetic or lactic acids. In addition, antifungal cyclic dipeptides were identified after HPLC separation and several other active fractions were observed suggesting a highly complex nature of the antifungal compounds. Okkers et al.¹⁴⁷ purified and characterised a medium length peptide TV35b from *Lactobacillus pentosus* with fungistatic effect against *Candida albicans*. They discovered that the peptide caused a reduction in growth of *Candida albicans* and induction of pseudo hyphae, however this peptide was not tested for activity against moulds. Vandenberg and Kanka²⁰⁴ patented the use of an antifungal product produced by a *Pediococcus acidilacti* strain. The inhibitory component was a small peptide (400–500 Da), which was heat stable and could be extracted by butanol. The purified compound contained lactic acid, and valine, and the activity was sensitive to treatment with protease, but not to lipase, lysozyme, DNAase or RNAse. It showed activity against a wide range of filamentous fungi, but the effect on yeast species was variable – inhibiting *Saccharomyces cerevisiae* but not *Candida albicans*. Roy et al.¹⁷⁵ isolated an antifungal compound from *Lactococcus lactis*, after enzymatic treatment with chymotrypsin, trypsin, and pronase E, the antifungal activity disappeared indicating the proteinaceous nature of the antifungal substance. The substance was not however characterised further. Similarly, Gourama⁶⁸ found the inhibitory effect of a *Lactobacillus* strain against two *Penicillium* species was reduced when treated with trypsin, and pepsin, however no further characterisation was carried out. Gourama and Bullerman^{69,70} showed that a combination of *Lactobacillus* species (*L. plantarum*, *L. bulgaricus* and *L. acidophilus*) with a silage inoculant exhibited antifungal activity along with anti-aflatoxin activity against *A. flavus*. At first, it was thought the inhibitory effect was due to a low molecular weight compound, subsequent analysis however revealed that a *Lactobacillus casei* subsp. *pseudoplantarum* was responsible for the antifungal activity. The activity was sensitive to the proteolytic enzymes trypsin and α -chymotrypsin, and it was concluded that inhibition was due to a small peptide of less than 1 kDa. The nature of the antifungal peptide is complex although research suggests that it may be hydrophobic in nature and rapidly adsorbs to the producer cells or forms spontaneous aggregates. Magnusson

and Schnürer¹¹⁷ discovered when a *Lactobacillus coryniformis* strain Si3 was supplemented with either ethanol, formic or acetic acid the total amount of the antifungal peptide increased. Similarly, Callewart et al.²⁶ observed the same response when *Lactobacillus amylovorus* producing amylovorin L471 was treated with ethanol or organic acids.

Purification of the substance is not always possible due to its instability, losing activity after two or three purification steps or after storage. It is also likely that the activity is mediated by a combination of peptides, and is unable to act independently. Purification problems may also be due to the hydrophobic nature of the peptide, this could lead to problems such as binding to glass and plastic material during growth and purification steps¹¹⁷.

More recently, the use of sourdough LAB to inhibit mould growth was studied, which led to the identification of a strain of *Lactobacillus plantarum* 2IB whose culture filtrate showed an important antifungal activity. Phenyl-lactic acid (PLA) was shown to be one of the major compounds occurring in the culture along with lactic acid and acetic acid¹⁰⁸. Subsequently, Lavermicococca et al.¹⁰⁸ tested

the antifungal activity of PLA against a range of fungal species isolated from bakery products and flours. Levels of growth inhibition of 50 to 92.4% were observed for all 13 fungal species, indicating the application of PLA to reduce fungal mass in food systems has a clear advantage compared with the preservatives now commonly used in bakery products such as propionic acid and its salts, which act by a fungistatic mechanism that causes only temporary inhibition of microbial growth¹⁰¹. Dieuleveux et al.⁴⁷ previously isolated PLA from a culture filtrate of *Geotrichum candidum* and characterised it as the main component responsible for the anti-*Listeria* activity shown in the fungal culture. PLA has been reported to be one of the most abundant aromatic acids to which antibacterial properties have been attributed²⁰⁹. Another study on the antifungal activity of *Lactobacillus sanfrancisco* CB1, isolated from sour dough, found that this strain inhibited bread spoilage moulds from the genera *Fusarium*, *Penicillium*, *Aspergillus* and *Monilia*. This antifungal activity was attributed to the formation of several short-chain fatty acids, among which caproic acid was shown to be the most potent inhibitory compound³⁸.

Table II. Publications reporting antifungal activity of lactic acid bacteria (adapted from the 2003 PhD dissertation of Magnusson¹¹⁶).

LAB isolate*	Activity spectrum	Compounds	Reference
<i>Streptococcus lactis</i> C10	<i>Aspergillus parasiticus</i>	ND	Wiseman, D.W. and Marth, E.H., <i>Mycopathol.</i> , 1981, 73 , 49.
<i>Lactobacillus casei</i> ATCC 393	<i>Aspergillus parasiticus</i>	ND	El-Gendy, S.M. and Marth, E.H., <i>J. Food Prot.</i> , 1981, 44 , 211.
<i>L. casei</i> var. <i>rhamnos</i>	Broad spectrum	ND	Coallier-Ascah, J. and Idziak, E.S., <i>Appl. Environ. Microbiol.</i> , 1985, 49 , 163.
<i>L. casei</i> var. <i>rhamnos</i>	Broad spectrum	<1 kDa	Vandenbergh, P.A. and King, S.W., 1988, EP 0,302,300,B1.
<i>Lactobacillus reuteri</i>	Broad spectrum	3-HPA (reuterin)	Talarico, T.L., Casas, I.A., Chung, T.C. and Dobrogosz, W.J., <i>Anti. Agents Chemo.</i> , 1988, 32 , 1854. Chung, T.C., Axelsson, L., Lindgren, S.E. and Dobrogosz, W.J., <i>Microbial Ecol. Health Dis.</i> , 1989, 2 , 137.
<i>Lactobacillus plantarum</i>	Unspecified spoilage mould	ND	Hill, J.E., 1989, US Patent Application 4,842,871.
<i>S. lactis</i> subsp. <i>diacetilactis</i> DRC1	<i>Aspergillus fumigatus</i> <i>Aspergillus parasiticus</i> <i>Rhizopus stolonifer</i>	Possibly proteinaceous	Batish, V.K., Grover, S. and Lal, R., <i>Cul. Dairy Prod. J.</i> , 1989, 24 , 21.
<i>Lactobacillus acidophilus</i> R	<i>Aspergillus fumigatus</i>	ND	Batish, V.K., Lal, R. and Grover, S., <i>Food Microbiol.</i> , 1990, 7 , 199.
<i>Lactococcus lactis</i>	<i>Aspergillus parasiticus</i>	ND	Luchese, R.H. and Harrigan, W.F., <i>J. Appl. Bacteriol.</i> , 1990, 69 , 512..
<i>L. casei</i> subsp. <i>rhamnosus</i> <i>L. plantarum</i> <i>Leuconostoc mesenteroides</i>	<i>Penicillium</i> spp. <i>Aspergillus</i> spp.	ND	Suzuki, I., Nomura, M. and Morichi, T., <i>Milchwissenschaft</i> , 1991, 46 , 635.
<i>L. plantarum</i>	<i>Saccharomyces cerevisiae</i>	ND	Makanjoula, D.B., Tymon, A. and Springham, D.G., <i>Enzyme Microb. Technol.</i> , 1992, 14 , 351.
<i>L. casei</i> subsp. <i>rhamnosus</i> LC-705	<i>Candida lusitanae</i> <i>Aspergillus niger</i> <i>Fusarium</i> spp. <i>Penicillium</i> spp.	ND	Mäyrä-Mäkinen, A.K., Kristianinkatu, A. and Suomalainen, T.V., European Patent Application 0 576 780 A2.
<i>L. lactis</i> subsp. <i>lactis</i> CHD 28.3	<i>Aspergillus parasiticus</i> <i>Aspergillus flavus</i> <i>Fusarium</i> spp.	Possibly proteinaceous	Roy, U., Batish, V.K., Grover, S. and Neelakantan, S., <i>Inter. J. of Food Microbiol.</i> , 1996, 32 , 27.
<i>L. casei</i>	<i>Penicillium</i> spp.	Possibly proteinaceous	Gourama, H., <i>Lebensmittel Wissenschaft und Technologie</i> , 1997, 30 , 279.

ND = Not determined

*Some species have through taxonomic revisions received new species identities, which are not taken into account here.

There are several early reports of LAB producing low molecular weight compounds capable of inhibiting filamentous fungi, and although to a lesser extent yeasts. Coallier-Ascah and Idziak³⁴ isolated a strain of *Lactococcus lactis*, which was seen to inhibit the growth of *Aspergillus flavus* when the two were grown in co-culture. This occurred even when *L. lactis* was grown within a dialysis sac, suggesting that the active agent was a low molecular weight compound, which was secreted into the medium. This compound was partially purified but was seen to lose its activity on storage. In another experiment, an extracellular compound from *L. lactis* was found to inhibit the production of aflatoxin by *A. flavus*. The compound has been reported to be a low-molecular weight (less than 500 D) phosphoglycolipid containing an aromatic ring structure⁸⁶. Haikara and Niku-Pavoola⁷⁵ reported the isolation of a strain of *Lactobacillus plantarum* with antifungal activities against *Fusarium* spp. This antifungal activity was purified by a combination of vacuum evaporation, and ion exchange chromatography. It was concluded that low molecular weight cyclic organic compounds, which were most active at pH values less than 4.0, were responsible

for this action. Subsequent studies by Niku-Paavola et al.¹³⁸ have described the production of low molecular weight antimicrobial compounds from *Lactobacillus plantarum* other than organic acids. The active fraction contained benzoic acid, methylhydantoin, mevalonolactone, and cyclo-(glycl-L-leucyl) and was seen to act synergistically with lactic acid. This fraction inhibited both *Fusarium avenaceum* and the Gram-negative bacterium *Pantoea agglomerans*. Dicks⁴⁶ reported a *Lactobacillus* sp., which released an antifungal compound into the cell free supernatant (CFS). This compound was shown to be inhibitory against *Monilia*, which is a loosely defined term referring to a variety of yeast species (including *Hansenula anomala* and *Candida krusei*). The inhibitory agent exhibited a low molecular weight (<10 kDa), and probably acted by permeating the cell membrane. It resisted degradation by proteolytic enzymes (which suggests that the antifungal compound is non proteinaceous in nature) and was effective within a pH range 3–7.

Perhaps the best-characterised antifungal agent produced by an LAB species is reuterin. Reuterin is produced from glycerol by starving cells under anaerobic condi-

Table II. (continued)

LAB isolate*	Activity spectrum	Compounds	Reference
<i>L. casei</i> subsp. <i>pseudoplantarum</i>	<i>Aspergillus flavus</i>	Possibly proteinaceous <1 kDa	Gourama, H. and Bullerman, L.B., <i>Inter. J. of Food Microbiol.</i> , 1997, 34 , 131. Gourama, H. and Bullerman, L.B., <i>J. of Food Prot.</i> , 1995, 58 , 1249.
<i>L. sanfrancisco</i> CB1	<i>Fusarium</i> spp. <i>Penicillium</i> spp. <i>Aspergillus</i> spp. <i>Monilia</i> spp.	Caproic acid Propionic acid Butyric acid Valeric acid	Corsetti, A., Gobebetti, M., Rossi, J. and Damiani, P., <i>Appl. Microbiol. Biotechnol.</i> , 1998, 50 , 253–256.
<i>L. plantarum</i> VTT E78076	<i>Fusarium avenaceum</i>	Benzoic acid, methylhydantoin, mevalonolactone, cyclo (Gly-L-Leu)	Niku-Paavola, M.-L., Laitila, L., Mattila-Sandholm, T. and Haikara, A., <i>J. Appl. Microbiol.</i> , 1999, 86 , 29.
<i>L. pentosus</i>	<i>Candida albicans</i>	Pentocin TV35b	Okkers, D.J., Dicks, L.M.T., Silvester, M., Joubert, J.J and Odendaal, H.J., <i>J. Appl. Microbiol.</i> , 1999, 87 , 726.
<i>L. casei</i> <i>L. delbreuckii</i> subsp. <i>bulgaricus</i>	<i>Penicillium expansum</i>	ND	Florianowicz, T., <i>Eur. Food. Res. Technol.</i> , 2001, 212 , 282.
<i>L. plantarum</i>	Broad spectrum	Phenyllactic acid 4-hydroxyphenyllactic acid	Lavermicocca, P., Valerio, F., Evidente, A., Lazzaroni, S., Corsetti, A. and Gobetti, M., <i>Appl. Environ. Microbiol.</i> , 2000, 66 , 4084.
<i>L. rhamnosus</i>	<i>Fusarium</i> spp. <i>Penicillium</i> spp. <i>Aspergillus</i> spp. <i>Alternaria</i> spp.	Sodium acetate ¹	Stiles, J., Penkar, S., Plockova, N., Chumchalova, J. and Bullerman, L.B., <i>J. Food Prot.</i> , 2002, 65 , 1188.
<i>L. plantarum</i> MiLab 393	Broad spectrum	3-Phenyllactic acid cyclo(Phe-Pro) cyclo(Phe-OH-Pro)	Ström, K., Sjögren, J., Broberg, A. and Schnurer, J., <i>Appl. Environ. Microbiol.</i> , 2002, 68 , 4322.
<i>L. coryniformis</i> Si3	Broad spectrum	Peptide Phenyllactic acid cyclo(Phe-Pro) cyclo(Phe-OH-Pro)	Magnusson, J. and Schnürer, J., <i>Appl. Environ. Microbiol.</i> , 2001, 67 , 1. Magnusson, J., Ström, K., Roos, S., Sjögren, J. and Schnürer, J., <i>FEMS Microbiology Letters</i> , 2003, 219 , 129.
<i>L. plantarum</i> MiLab 14	Broad spectrum	Hydroxy fatty acids Phenyllactic acid cyclo (Phe-Pro) cyclo (Phe-OH-Pro)	Sjogren, J., Magnusson, J., Broberg, A., Schnurer, J. and Kenne, L., <i>Appl. Environ. Microbiol.</i> , 2003, 69 , 7554. Magnusson, J. and Schnürer, J., <i>Appl. Environ. Microbiol.</i> , 2001, 67 , 1. Magnusson, J., Ström, K., Roos, S., Sjögren, J. and Schnürer, J., <i>FEMS Microbiology Letters</i> , 2003, 219 , 129.

¹Sodium acetate from the MRS substrate was involved in the inhibitory action of lactic acid bacteria towards several moulds: the additional effect of other compounds was not determined.

tions, and the active compound reuterin is an equilibrium mixture of monomeric, hydrated monomeric, and cyclic dimeric forms of 3-hydroxypropionaldehyde, produced by *Lactobacillus reuteri*¹⁹⁸. Its activity is thought to be due to inhibition of ribonucleotide reductase, the enzyme which universally catalyses the first step in DNA synthesis^{51,199}. As well as exhibiting growth inhibiting properties towards a wide range of Gram-negative and Gram-positive bacteria, reuterin has been claimed to be equally effective against lower eukaryotic genera of yeasts and fungi such as *Candida*, *Torulopsis*, *Saccharomyces*, *Saccharomyces*, *Aspergillus* and *Fusarium*³¹, although no data to support this claim was provided. Magnusson and Schnürer¹¹⁷ discovered that accidental addition of glycerol to an overlay assay resulted in a dramatic increase of the inhibitory effect of *Lactobacillus coryniformis* strain Si3 against several filamentous fungi and yeast. During isolation of glycerol metabolites from *Lactobacillus coryniformis*, equal amounts of 3-hydroxypropionic acid and 1,3-propanediol, and only trace amounts of 3-HPA were detected¹¹⁸. The production of reuterin (3-HPA) has earlier been reported from *L. brevis* and *L. buchneri*¹⁸⁰, *L. collinoides*³² and *L. coryniformis*¹³³.

Whilst research has clearly established LAB strains as potential producers of metabolites, other than acetic acid, and lactic acid, capable of inhibiting filamentous fungi, the ability of these LAB to inhibit yeast is less well documented. A patent application by Mäyrä-Mäkinen and Suomalainen¹²⁷ reports on the use of a strain of *Lactobacillus casei* subsp. *rhamnosus* (LC-705) as a broad-spectrum antifungal. The effect was only visualised however when *Lactobacillus casei* (LC-705) was grown in direct competition with the target organism(s). However in these experiments the levels of lactic acid and accompanying drop in pH were not adjusted, making it difficult to assess whether a specific antifungal compound is the reason for this inhibition. Subsequent research claimed that when *Lactobacillus rhamnosus* (LC-705) was used as a starter culture with *Propionibacterium freundenreichii* subsp. *shermanii* JS (as the commercial formulation Bioprofit-preparate) for the production of quark of yoghurt, the growth of inoculated yeast is inhibited¹⁹⁶. However, from the results presented it is impossible to draw any conclusions regarding the nature of the inhibitory compound.

Other work however provides more definitive evidence of the capacity of certain LAB strains to inhibit yeast growth. Makanjola et al.¹²⁰ examined several strains of *Lactobacillus* spp. and *Leuconostoc* spp. for their ability to inhibit growth and ethanol production of distillers yeast. Most strains exhibited some reduced ethanol production however two strains (*Lactobacillus brevis* and *Lactobacillus plantarum*) were able to significantly reduce ethanol production. The *Lactobacillus plantarum* strain caused a marked flocculation of the mixed culture, and this characteristic may have contributed largely to its inhibitory activity. The *Lactobacillus brevis* strain caused no such flocculation; the mechanism of the observed inhibitory effect was not investigated any further.

From the current literature, it can be concluded that antifungal attributes of lactic acid bacteria do exist and have the potential for being effective food-grade biopreservatives for combating the problem of yeasts and moulds.

However before exploring the commercial application of antifungal potentials of lactic cultures, more extensive work is required on these lines to know the exact nature and properties of antifungal substances produced. Most publications on antifungal activity of lactic acid bacteria merely illustrate the activity, but rarely identify active compounds or other reasons for the inhibitory activity. Table II summarises the publications reporting antifungal activity of lactic acid bacteria (as cited by Magnusson¹¹⁶).

6.0 Mycotoxins in cereals

Malted barley is the most common grain used in the beer making process. In recent years, small grains, such as barley, have been greatly affected by the plant disease Fusarium head blight (FHB) also known as scab¹²⁸. *Fusarium graminearum* is the primary pathogen causing FHB on barley. This pathogen causes yield loss primarily by reducing kernel size¹⁶⁷. *F. graminearum* and *F. culmorum* cause root rot, crown rot, foot rot, stem rot and head blight in wheat and barley. Geographic location also determines to a great extent the variety, and quantity of Fusarium species present on barley. Fusarium species have been reported to be the most toxigenic fungi in northern temperate regions^{1,29,105,106,131,154}.

When cereal grains and animal feed are colonised by moulds there's a significant risk of contamination with the secondary metabolites of these fungi. A number of these fungal compounds are endowed with toxic effects towards animals and human beings and are accorded the collective term mycotoxins. Trichothecene mycotoxins including, deoxynivalenol (DON or vomitoxin), nivalenol (NIV) and T-2 toxin, have been detected in FHB infected barley from the upper Midwestern states in the United States¹⁸². In a study by Salas et al.¹⁷⁷, epidemics of FHB in Minnesota, North Dakota and South Dakota were mainly due to the pathogen *Fusarium graminearum* and its mycotoxin DON, however *Fusarium poae*, *Fusarium sporotrichioides* and *Fusarium avenaceum* were also found to be causing infection to a limited extent. Besides trichothecenes, the Fusarium estrogenic toxin zearalenone (ZEN) is also found on barley. The production of these mainly depends on the Fusarium species and the climatic conditions during growth in the field¹³⁵.

Traditionally, mycotoxigenic fungi have been considered to fall into two groups: "field" (or plant pathogenic) and "storage" (or saprophytic) fungi. However in the case of *Aspergillus flavus*, for example, this distinction is merely academic as this fungus is associated with *Aspergillus* ear rot and kernel rot of maize²⁷ and also colonises stored grain when factors such as temperature and water activity are optimised¹⁹¹. In addition, mycotoxins occur in the spores of many fungi, including *Stachybotrys* and *Alternaria* and inhalation therefore represents another route of entry¹⁶⁴. Fusarium species synthesise a wide range of mycotoxins of diverse structure and chemistry. Mycotoxins are also a concern in other by-products of the malting and brewing processes such as various food ingredients and animal feeds⁶¹. The most important, from the point of view of animal health and productivity, are the trichothecenes, zearalenone, moniliformin and the fumonisins⁵⁰. The trichothecenes have been found to be toxic and are

divided into two basic groups, type A and B. Type B trichothecenes include deoxynivalenol (DON) and its 3-acetyl and 15-acetyl derivatives (3-ADON and 15-ADON, respectively), nivalenol and fusarenone-X. Type A trichothecenes include T-2 Toxin (T-2), HT-2 Toxin (HT-2), acetyl T-2 Toxin (AcT-2), deacetoxyscirpenol (DAS), 15-acetoxyscirpenol (MAS) and neosolaniol (NEO). Tests have shown that type A trichothecenes particularly DAS and T-2 toxin are more toxic than type B trichothecenes such as DON and NIV¹⁰⁹. The most toxic trichothecene is T-2 Toxin, which has been reported to cause dermatitis of the nose and buccal commissures in pigs⁴⁹. T-2 Toxin, HT-2 and DAS have also been reported to have extreme toxic effects on skin and mucous surfaces and can induce lesions on the mucosa of the mouth and oesophageal regions in poultry and pigs. Production of type A trichothecenes predominates in *F. sporotrichioides* and possibly also *F. poae*, whereas synthesis of type B trichothecenes occurs principally in *F. culmorum* and *F. graminearum*.

Of all mycotoxins, DON is the most frequently detected and the mycotoxin produced in the greatest quantity in FHB infected grain. Clear et al.³³ also found DON to be the most prevalent mycotoxin in FHB infected barley in Manitoba during recent epidemics, while in Southwest Germany, the predominant mycotoxin reported in barley crops from 1982–1992 was vomitoxin¹³¹. Scott¹⁸⁵ in 1996 reviewed surveys of mycotoxins in commercially available beers and noted that DON, NIV, T-2, HT-2, DAS, ZEN, aflatoxins, ochratoxin A, and fumonisins have in the past been detected in beers at trace (ppb) levels. Fumonisins are secondary metabolites of the fungus *Fusarium moniliforme*, a common mycotoxin found in corn. Fumonisins are known to cause cancer in a number of experimental animals and have been linked to human oesophageal cancer in China and South Africa^{48,129}. Feed refusal, vomiting, and hyperestrogenism are some of the symptoms exhibited by farm animals, particularly swine, when fed with FHB-infected grains^{37,91}.

In addition, *Fusarium* infestation in barley and malt may be associated with “gushing” in the resultant beer¹⁸¹. Gushing may be defined as the quick uncontrolled spontaneous over-foaming immediately when opening a bottle or can³. *Fusarium graminearum*, *Fusarium culmorum* and *Fusarium poe* have all been named as active gushing inducers^{73,132,136,181}. The production of mycotoxins may possibly parallel production of the components responsible for gushing.

The production of mycotoxins during malting was studied for DON. It was revealed that the final concentration of DON in malt was approximately the same or lower than the concentration detected in the barley⁹. DON levels during steeping are reduced possibly due to the washing out or removal of dirt or mould particles¹⁸³. Trenholm et al.²⁰¹ demonstrated that washing contaminated barley with distilled water (3 times for 30 minutes) could reduce DON concentration by 69%. But the levels of DON increase again during subsequent germination and early kilning^{183,184}. Addition of *Lactobacillus plantarum* and *Lactobacillus acidophilus* to steep water resulted in a decreased formation of DON and ZEN¹⁰⁴. Boivin and Malanda¹⁵ however found that a *Lactobacillus plantarum* strain isolated from a malthouse had no significant inhibitory effect

on the production of mycotoxins. In another study, a sharp increase in DON level was only found during the first step of kilning¹³². Schwarz et al.¹⁸² also revealed that DON is uniformly distributed throughout the kernel fractions. Wolf-Hall and Bullerman²¹² revealed a greater production of DON and 15-acetyl DON by certain isolates of *Fusarium graminearum* at 35°C versus 25°C. The rise in temperature in the early stages of kilning may actually stimulate increased production of mycotoxins by certain strains of infecting mould. DON is known to be stable up to 170°C at neutral to acidic pH²¹². In a separate study by Schapira et al.¹⁷⁸ barley was artificially contaminated with DON and DAS. An inhibition of rootlet and coleoptile growth was demonstrated. These toxins also inhibited synthesis of proteolytic enzymes that led to lower levels of α -amino nitrogen in wort and malt. The phytotoxicity of DON was found to be lower than that of DAS and T2-toxin. There are two species shown to be responsible for the production of DON in barley and malt, *Fusarium graminearum*, which is predominant in warmer regions and *F. culmorum*, which is mainly prevalent in more temperate climate zones^{9,104}. However many factors could be affecting the amount of toxin produced at various stages of malting, including strain of mould present, the viability of the infecting mould (injury, dormancy, or death), the amount of infecting mould present, the location of mycelia or spores within the seed structure, and competing and/or antagonistic organisms.

The possibility of transmission of mycotoxins into beer from contaminated grains used in brewing has been of interest since 1972–1973, when the first malting and brewing experiments was surveyed for ochratoxin A (OA) and citrinin⁶⁶. The nephrotoxic OA is a non-*Fusarium* toxin that frequently contaminates barley. Production of this toxin was first demonstrated in *Aspergillus ochraceus*. However, this species occurs mainly in tropical regions, and the responsible organism in contaminated barley was later demonstrated to be *Penicillium verrucosum*¹⁶¹. It has also been established that OA is produced by other fungal species, such as *A. niger*, which has been found as a contaminant of bread⁷⁸. All of these toxic compounds are heat stable and thought not to be affected during wort boiling¹³⁵. However they may be partially removed with the spent grains. This holds in particular for toxins with low water solubility such as ZEN¹⁸⁴. Ochratoxin A along with citrinin are mycotoxins that have been found as causal determinants in a naturally occurring kidney disease, porcine nephropathy⁹⁸. Studies indicate that the natural occurrence of OA in malt has been reported at levels up to 28 ng g⁻¹⁸⁸. The highest levels of OA (1.53 ng mL⁻¹) were found in a strong German beer⁵⁷. This study was part of a larger study reported by Majerus et al.¹¹⁹, showing that the incidence of OA in strong barley beers was 35% compared with 8% in pale barley beers, and at least 5 of the strong beers and one strong wheat beer contained >1 ng mL⁻¹. Kuiper-Goodman and Scott⁹⁹ also found ochratoxin contamination of beer. In a separate study by Visconti et al.²⁰⁸ OA levels were found to range from <0.01 to 0.135 ng mL⁻¹ in an analysis of 10 domestic Italian beers and 51 imported beers. An incidence of 50% contamination was revealed with no substantial difference between strong and pale beers. Baxter et al.⁷ prepared malt contaminated

with OA on a pilot scale. This malt was then used for pilot brewing trials in order to determine the extent at which OA can survive the brewing process. It was concluded that up to 40% OA was lost in the mashing process, while another 16% was eliminated with the spent grains. Some losses of OA were recorded during the fermentation process. Overall, between 13 and 32% of the OA present in the original grist survived into the beer. Several studies have demonstrated that mycotoxins such as OA are nephrotoxic, hepatotoxic, teratogenic, immunosuppressive and carcinogenic in different species, including farm livestock^{121,165}.

The predominant feature of ZEN distribution in cereal grains and animal feed is its co-occurrence with other *Fusarium* mycotoxins, including trichothecenes. This observation is consistent with the confirmed production of ZEN by virtually all toxigenic and plant pathogenic species of *Fusarium*⁵⁰. The highest values for ZEN (11 and 15 mg kg⁻¹) relate to two barley samples from the Fukuoka region of Japan²¹⁴. Zearalenone is metabolised largely to β -zearalenol by brewing strains of *Saccharomyces cerevisiae* and according to Schoental¹⁷⁹ should be looked for in beer. A brewing strain of *S. cerevisiae* transformed 100 μ g mL⁻¹ of zearalenone in koji juice to β -zearalenol (72%) and α -zearalenol (3%) after 8 days with 20% of the zearalenone remaining¹²³. Another brewing strain of *S. cerevisiae*, however, transformed both α - and β -zearalenols equally in a liquid culture medium after 6 days¹⁸. Zearalenone was found to occur naturally in 45% of home brewed African beers¹⁴³. Zearalenone and alpha- or beta-zearalenol have not been found in Canadian, European, or Korean beers with the exception of one French beer, which contained 100 μ g L⁻¹^{148,185,188}. Although fumonisins have only been reported in corn, they may be introduced into beer if contaminated corn or corn products are used as a brewing adjunct²⁴. Corn grists and flaked corn are the most likely sources. To date no study of the brewing process with regard to carryover of fumonisins into beer has been carried out. However, yeast fermentation of fumonisin B₁-contaminated corn used as a substrate for ethanol production showed little degradation of the fumonisin¹⁹. Of the initial fumonisin B₁, 31–35% was recovered in the distillers' dried grains, which actually had increased concentrations of fumonisin B₁, whereas 51–54% was extracted into the whole stillage. The stability of fumonisin B₁ on boiling culture material of *F. moniliforme*² and the losses of only 3–28% fumonisin B₁ and 9–17% fumonisin B₂ during fermentation in wort by three strains of *S. cerevisiae*¹⁸⁶ further indicate that fumonisins could be found in commercial beer. Scott and Lawrence¹⁸⁷ reported the presence of fumonisins in 7 of 30 Canadian beer samples, only four samples tested at >2 ng fumonisin B₁ mL⁻¹. The highest level found was 49 ng fumonisin B₁ mL⁻¹; this sample also contained 12 ng fumonisin B₂ mL⁻¹.

Yeast fermentation is also affected by the presence of mycotoxins. Lafont et al.¹⁰² noted a 50–80% decrease in the rate of fermentation by *Saccharomyces cerevisiae* in fermentation broths containing 10–50 μ g g⁻¹ T-2 toxin. This decrease was a temporary lag and the yeast appeared to recover. Koshinsky et al.⁹⁷ suggest T-2 toxin inhibits mitochondrial function, causing slower oxygen utilization by the yeast resulting in a slower growth rate during the

exponential phase. Flannigan et al.⁶² observed similar results when ZEN was present at 5–50 μ g g⁻¹, DAS at 5–10 μ g g⁻¹²¹⁰, and DON at 50 μ g g⁻¹¹⁷⁶. Whitehead and Flannigan²¹⁰ reported no effect with DON at levels of 20 μ g g⁻¹, while Boeira et al.¹² found no effect of NIV at concentrations less than 50 μ g g⁻¹ on strains of lager and ale yeasts. In a more recent study, the combined interactions of DON, ZEN and fumonisin B₁ on yeast were observed¹². A synergistic interaction between DON and ZEN was noted, but only at very high concentrations. It is unlikely however that any of these mycotoxins would be found at concentrations this high in naturally *Fusarium* infected grain.

Numerous studies have revealed how DON levels increase throughout yeast fermentation. In a baking study using naturally DON contaminated wheat, Young et al.²¹⁵ saw an increase in DON levels in yeast fermented doughnut and attributed this to possible DON precursors in the flour, which were further converted to DON by yeast. Böhm-Schraml et al.¹⁴ demonstrated increasing DON levels within the first 20 h of fermentation, which then subsequently decreased up to 100 h. It was proposed that the decrease in DON was due to absorption by yeast cells or extracellular metabolism.

The effects of DAS on fermentation were minimised by increasing the inoculum density or pitching rate²¹⁰. The authors suggested that certain yeasts might be capable of detoxifying mycotoxins such as T-2 toxin. The development of these mycotoxin resistant yeasts would be of great benefit to the fuel ethanol industry, as heavily mycotoxin contaminated grain could be better utilized since it would not be acceptable for food/feed uses.

6.1 Gushing

Gushing, or sudden over production of foam upon opening a container, is another defect that is closely associated with the use of FHB infected grain in beer making. Gushing is a rare but old phenomenon dating as far back as 1913 when a Danish brewery experienced severe problems. It occurs epidemically from time to time in more wet and cold climates. Gushing can be a serious quality defect resulting in the permanent loss of market share. Primary gushing appears to be caused by the formation and stabilization of large amounts of microbubbles in beer^{28,55,59}. The nucleation centres for these microbubbles seem to be a product of mould growth in the grain, and not only *Fusarium* mould may cause it – others such as *Aspergillus* and *Penicillium* may also contribute to gushing. According to Haikara⁷² however, *Fusarium* species are the most problematic, particularly *Fusarium culmorum*, although *Fusarium avenaceum* may also play a major role. Secondary gushing is due to faulty production processes or to improper treatment of the bottled beer. Schwarz et al.¹⁸³ induced gushing in beer by adding cell-free extracts of *Fusarium graminearum* grown in liquid culture broth and proposed that the gushing factor is a water soluble component produced by the mould. The effects of fungi on the quality of malt and beer have previously been studied only by adding fungal cultures to the barley steeping^{67,190}. This however allows only a very short time for the fungus to grow on the barley grain compared to field conditions, in which the moulds have a much longer time

to develop and excrete metabolically active compounds. In a study by Haikara⁷², barley was artificially contaminated with *Fusarium avenaceum* and *F. culmorum* in the field for two successive years. The most marked changes in the quality of barley, malt and beer were: decrease in germination, extract difference, malt yield, α -amylase activity and diastatic power. The colour of the wort was also intensified. Increases were observed in α -amino nitrogen, zearalenone production and gushing. It was also concluded the most active *F. culmorum* strain with respect to gushing, also caused the most pronounced changes in malt analysis.

Significant correlations exist between the intensity of gushing and levels of DON in barley and malt, ergosterol in malt, and ZEN in malt^{72,181}. As gushing factors are formed before DON during germination of contaminated barley, Munar and Sebree¹³² suggest that they are formed independently. It is only in cases when grain is contaminated solely and heavily with *F. graminearum*, that correlation can be found between DON, FHB symptoms and gushing propensity of malt. In Europe no correlation exists. Although the Fusarium-related gushing mechanism is not yet known, a relationship between high wort nitrogen of malt treated with some *Fusaria* and the gas instability of the beers produced from these malts, coupled with the involvement of a protein factor concentrated on the husk of infected barley or malt are suggested^{132,166}. Munar and Sebree¹³² also suggest the gushing inducing factors are formed through interaction between the viable Fusarium mycelium and the germinating barley kernels. Flannigan⁶⁰ proposes zearalenone sulphate, a derivative of ZEN demonstrated in barley as another possible cause of gushing. No connection was found between gushing and the amount of split kernels¹³⁶.

Mycotoxins originating from barley and/or grain adjuncts survive the malting and brewing processes to different extents. It is important to use naturally contaminated or inoculated barley as substrates in malting experiments, because fungal growth can occur during germination with an accompanying increase in mycotoxin levels (e.g. DON or zearalenone). The mycotoxin that best survives the brewing process is DON, which may even increase in concentration levels during mashing. Zearalenone is largely converted to β -zearalenol during fermentation, while citrinin is mostly destroyed. A small percentage of OA and aflatoxins are recovered in beers.

Various means might be utilized to control FHB, mould growth, and/or mycotoxin production during malting. These include treatment with fungicides in the field, use of resistant barley cultivars, chemical treatment of harvested barley during storage, or biological control during malting⁸. Fungicide application is often expensive for barley, which is a low-value crop. Furthermore, current approved compounds propiconazole, triademefon, and mancozeb have a specific application window that often is outside optimal periods for control of FHB. In addition it is unlikely that all brewers would be receptive to chemical control methods because of concerns over additives, residues, and effects on malt and beer quality. Biological control methods, involving inoculation with *Lactobacillus* starter cultures, have shown the most promise for the control of Fusarium species during malting⁷⁶.

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