**Isolation of Antifungal Lactic Acid Bacteria from Food Sources
and Their Use to Inhibit Mold Growth in Cheese**

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TITLE: Isolation of Antifungal Lactic Acid Bacteria from Food Sources and
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ABSTRACT

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A large amount of cheese is lost every year due to mold contamination. Biopreservation, which is the use of biological entities (microbes) and their metabolites to suppress microbial spoilage instead of chemical preservatives has lately gained increasing interest. Lactic acid bacteria (LAB) have the potential for use in biopreservation, because they are safe to consume and naturally exist in many foods. In this study, fifteen strains of lactobacilli isolated from dairy products, vegetables, and fermented pickles were tested by agar overlay assay for their anti-mold activity. Six strains grown on MRS agar showed strong inhibitory activity against a target mold (*Penicillium* sp. at 10^5 spores/ml) isolated from the surface of Cheddar cheese. The isolates were identified by biochemical tests using API CHL50 strips. Five strains were identified as *Lactobacillus plantarum*, and one strain as *Pediococcus pentasaceus*. Well-diffusion method was used to demonstrate anti-mold activity in *concentrated* cell-free supernatants. Supernatants from all strains showed inhibition of the target mold (indicator). The anti-mold compound(s) produced by all the strains was heat-resistant (100°C for 15 min). Supernatants from 5 strains retained the anti-mold activity when the pH was adjusted to 6.8 ± 0.2 , while one

strain DC2 isolated from cheese lost its anti-mold activity at that pH. Temperature of incubation of cultures affected anti-mold activity. The optimum was 37° C. Very little or no inhibition was noted when cultures were incubated at either 10 or 55 °C.

A preliminary study of applying anti-mold lactobacilli in Cheddar cheese was completed. Anti-mold LAB was added to the cheese milk as *an adjunct* to give 10⁵ cfu/ml. After 1-week and 1-month ripening, mold (10~20 spores) was added on to the surface, and the cheese was wrapped loosely. The appearance of the mold on cheese surface was monitored. Mold was not present on the 1-week old cheese “NB in milk” until the 6th day after the control cheese (made without strain NB) showed signs of mold. The 1-month old cheese “NB in milk ” extended the shelf life 17 days longer than the control cheese.

Keywords: antifungal lactic acid bacteria, cheese, biopreservative, mold

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1.0 INTRODUCTION

Molds can grow on all kinds of food: cheese, milk, meat, cereals, fruit, vegetables, nuts, fats and products of these. The mold 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). It is estimated that between 5 and 10% of the world's food production is lost due to fungal deterioration (Pitt and Hocking, 2009). In Western Europe, mold spoilage of bread alone is estimated to cause annual economical losses of more than £200 million (Legan, 1993).

Cheese is very susceptible to mold growth and is normally kept under refrigeration. Many retail packs are vacuum packaged or gas flushed. However, molds are ubiquitous in cheese plants. Mold mycelia and mold spores are dispersed by air currents in the cheese plants. Molds also grow on damp surfaces such as walls and shelves in cheese curing rooms. Exposed surfaces of cheese are vulnerable to mold contamination from these sources.

Currently mold control in cheese generally involves the use of approved chemical preservatives (generally recognized as safe, GRAS) along with or without “modified atmosphere packaging” (MAPS). These special packaging techniques add to the cost of production, which is passed onto consumers.

Nowadays, consumers are very health conscious. They prefer “clean labels” and don't want those long chemical preservatives and antibiotics words, which they

cannot even pronounce in the list of ingredients declared on the package. The development of a suitable and effective biological control agent for molds that need not be declared specifically on the package would be advantageous to the cheese industry. Biopreservation, the control of one organism by another, has received much attention in the last ten years (Magnusson et al., 2003).

Lactic acid bacteria (LAB) have several potential applications. They are widely used for the production of fermented foods and are also part of intestinal microflora. LAB have a long history of being used in foods and research reports indicate that LAB have beneficial health effects in humans. They produce some antagonistic compounds that are able to control pathogenic bacteria and undesirable spoilage microflora in particular. Using LAB to control mold growth could be an alternative to physical and chemical method that food industry has been using.

2.0 OBJECTIVE

Objective of this study was to:

- (1) Isolate LAB from varies food sources.
- (2) Test the isolated LAB for antifungal activity.
- (3) Define the antifungal LAB on mold inhibition in Cheddar cheese.

3.0 LITERATURE REVIEW

3.1 Microbiology of Molds

The history of the food industry is also a history of fighting with spoilage fungi. The Number 1 problem of food storage is spoilage fungi. Fungi are always able to invade food matrix and bring about acidifying, fermenting, discoloring and disintegrating, rendering nutritious commodities unpalatable or unsafe. Food spoilage is also a main factor that causes huge economical loss every year all over the world. Losses of food to fungal spoilage in Australia, even given a dry climate and advanced technology, is in excess of \$ 10,000,000 per annum; while losses in humid tropical climates and countries with less highly developed technology remain staggering (Pitt and Hocking, 2009). Research into fungal food spoilage and its prevention is clearly an urgent necessity in the food industry.

Molds are very common food spoilage fungi and most of them are strictly aerobic. However some mold can tolerant anaerobic conditions and grow in the presence of low concentrations of CO₂ such as *Pencillium roqueforti* that used in blue cheese. Molds can grow on a variety of substrates and within wide range of pH, water activity (a_w) and temperature. pH is one of the major ambient traits affecting the activity of pathogenicity factors secreted by the pathogen, hence, a pH sensing-response system was developed to enable the pathogen to tailor its arsenal to best fit its host (Prusky and Yakoby, 2003). Ayerst (1969) had reported the delay in germination and subsequent growth rates of thirty isolates from twelve species of

Aspergillus, three species of *Penicillium* and *Stachybotrys atra* throughout the ranges of water activity and temperature that permitted growth. All the isolates were tolerant of low water activity at temperatures close to the optimum and their limit temperature can be from 5 to 10°C or even lower. The minimal water activity for most spoilage mold is 0.80, while xerophilic molds can grow at a_w value as low as 0.65.

Mold growth rate is slower than bacteria reproduction rates. Macroscopic colony formation takes much longer, so early detection of mold contamination is difficult. Besides visible mold growth, mycotoxins, the secondary metabolites produced by certain filamentous fungi, which can be produced in foods are important potential hazards in food industry. Mycotoxins cause a toxic response, termed mycotoxicosis. When digested by humans, mycotoxins can lead to deterioration of liver or kidney function. Some mycotoxins are neurotoxins, while others act by interfering with protein synthesis and produce effects ranging from skin sensitivity or necrosis to extreme immunodeficiency (Sweeney and Dobson, 1998).

However, not all the molds can produce mycotoxins. The profile and amount of mycotoxins, which a mold produces in the food depends completely on the ecological and processing parameters of the particular foodstuff. Some food parameters restrict in the spectrum of species, which are able to grow and spoil the individual food types (Filtenborg et al., 1996). *Aspergillus*, *Fusarium* and *Penicillium* are the main mycotoxigenic fungi found in foods. *Penicillium* and *Asperigillus* are

commonly contaminant foods during storage, while *Fusarium* species are destructive plant pathogens producing mycotoxins before or immediately post harvesting. Table 1 shows different mycotoxins found in foods and feedstuffs.

Table 1 Mycotoxins found in foods and feedstuffs

Commodity	Situation	Potential mycotoxins
Cereals	Pre-harvest fungal infection	Deoxynivalenol, T2 toxin, nivalenol, zearalenone, alternariol, alternariol monomethyl ether, tenuazonic acid fumonisins
Maize and peanuts	Pre-harvest fungal infection	Aflatoxins
Maize and sorghum	Pre-harvest fungal infection	Fumonisin
Stored cereals, nuts, spices	Damp storage conditions (storage abuse)	Aflatoxins and ochratoxin
Fruit juice	Mold growth on fruit	Patulin
Dairy products	Animal consumption of mold contaminated feeds	Aflatoxin M1, cyclopiazonic acid, ochratoxin, compactin, cyclopaldic acid
Meat and eggs	Animal consumption of mold contaminated feeds	Patulin, citrinin, ochratoxin, cyclopiazonic acid, cyclopaldic acid, citromycin, roquefortine fumonisins
Oilseeds	Pre-harvest fungal infection	Tenuazonic acid, alternariol,

Source: Sweeney and Dobson (1998)

Some mycotoxins are only present in the mold, while most are excreted into foods. In liquid food and fruits like peaches, pears and tomatoes, mycotoxins can diffuse very fast and contaminate the whole product. In solid foods like cheese and bread, diffusion is slow, leaving the major of the product uncontaminated.

Mycotoxins are very resistant to physical and chemical treatments. Once the

mycotoxins enter foods, they stay there during processing and storage (Bullerman and Bianchini, 2007). This means all the moldy material used in processing may contribute mycotoxins presence in the final products. Bakirci (2001) reported that pasteurization or UHT treatment of milk did not affect the concentration of aflatoxin M1 in milk because of its heat stability.

Physical and chemical methods have been developed to control the occurrence of molds and their toxins. However, there is no perfect method to reduce the presence of mycotoxins. Moreover, some molds have acquired the ability to resist some preservatives. For example, some *Penicillium* can grow in the presence of potassium sorbate, and other mold can degrade sorbate (Dalie et al., 2009). Thus, controlling food spoilage fungi continues to be a challenge for the food industry.

3.2 Mold contaminations in cheese and during cheese processing

Molds are able to grow on a variety of damp surfaces such as damp walls, shelves, air conditioning duct, air circulation vents, poorly cleaned damp air filters and other moisture-laden dairy plant surfaces. So mold contamination is a big problem in cheese plant, especially for open vat cheese plant.

Cheese is very susceptible to mold growth and is normally kept under refrigeration (4-10°C). Many retail cheeses are vacuum packaged or gas flushed in modified atmosphere packages. Thus, cheese spoilage is generally confined to molds, which are psychrotolerant and can grow under conditions of relatively low oxygen.

Molds are relatively acid resistant. Some molds even use organic acids as carbon sources (Dalie et al., 2009). The low a_w requirements of molds allow them to grow at relatively high salt concentrations. Those properties allow molds to grow well on cheese surface. *P. roqueforti* and *P. commune* are particularly well suited to growth in the cheese environment, having been associated with cheese manufacture for centuries. Shredded cheese is particularly susceptible to spoilage by yeasts and molds due to more exposed cheese surfaces. *P. commune* and *P. roqueforti* have been isolated from moldy shredded cheese in laboratory (Pitt and Hocking, 2009).

Torkar and Vengust (2008) detected yeast and mold contamination in 60 samples of raw milk and 40 samples of curd, soft salted or non-salted cheese and semi-hard cheese manufactured by small artisan food-processing plants. Molds were found in 63.3% of raw milk samples with a mean concentration of $0.6 \log_{10}$ cfu/ml, and isolated mold stains including *Geotrichum* (51.1%), *Aspergillus* (33.8%), *Mucor* (5.9%), *Fusarium* (2.9%). Molds were isolated from 60% of tested cheese samples with average concentrations $2.1 \log_{10}$ cfu/g. The frequencies of isolated genera were *Geotrichum* (91.9%), *Moniliella* (5.4%) and *Aspergillus* (2.7%) were most frequently isolated strains from tested cheese samples. Aflatoxin M1 levels above 50 mg/kg (FDA action level is 0.5 mg/kg) were detected in 10% of cheese samples.

Kure et al. (2004) conducted research in four cheese factories in Norway during 2 years to identify the critical points for mold contamination in the production process, which including air, equipment, plastic film, brine and milk.

Geotrichum (42.6%) and *Penicillium* (33.9%) were the most frequently isolated genera. *Cladosporium cladosporioides*, *C. herbarum*, *P. brevicompactum*, *P. chrysogenum*, *P. commune*, *P. crustosum*, *P. palitans*, *P. spinulosum* and *Phoma* sp. were detected in four factories. The common cheese contaminants such as *P. commune*, *P. palitans*, *P. solitum* and *P. roqueforti* ss. *roqueforti* were found in samples of air and equipment. Their result showed that air was the major source of the important cheese contaminants *P. commune* and *P. palitans* during processing. The cleanliness of cheese plant is the key point to prevent mold contamination.

Kivanc (1990) examined 25 fresh market samples of Van herby cheese (a Turkish cheese) and pickled white cheese for molds and aflatoxins. Over 65% of molds were *Penicillium* species; *Aspergillus* made up 0 to 1.6% and 2.6% to 4.0% of the mold on pickled white cheese and Van herby cheese, respectively. *Mucor*, *Geotrichum* and *Trichoderma* genera were also present. None of the molds produced mycotoxin. Basilico et al. (2001) reported that *P. glomerata* is the main mold-contaminant in vacuum-packed Argentinian-style hard cheese and recommended the sources of contamination need to be controlled by designing a good factory layout.

Kure et al. (2001) reported that the most important contaminating species on Norwegian semi-hard cheeses (Jarlsberg and Norvegia) were *P. commune* and *P. palitans*. The other dominating contaminants were *P. roqueforti* spp. *roqueforti*, *Geotrichum candidum*, *P. solitum* and *P. crustosum*. *P. crustosum* was one of the dominating species on Norwegian cheese.

Lund et al. (1995) studied hard, semi-hard and semi-soft cheeses from Denmark, France, Greece, UK and other countries and found that the main fungal isolates were *Penicillium* species. *P. commune* was the most widespread and frequently occurring species. Most of the isolates (88%) found on cheese were *P. commune*, *P. atramentosum*, *P. chrysogenum* and *P. echinulatum*. Mycotoxin production was also detected. *P. commune* produced cyclopiaonic acid, *P. verrucosum* produced ochratoxin A, *A. versicolor* produced sterigmatocystin and *P. crustosum* produced penitrem A and roquefortine C.

Heat resistant mold can occasionally cause spoilage in heat-processed cheeses such as cream cheese. Ascospores of *Byssoschlamys nivea* and other heat resistant species may be present in raw milk. Ascospores easily survive the pasteurization of milk and cream (Engel and Teuber, 1991).

3.3 Post-manufacture control measures against mold contamination and retarding mold growth in cheese industry

3.3.1 Chemical mold control agents used in cheese

3.3.1.1 Sorbate

The most common preservative agents in cheese are weak organic acids such as acetic, lactic, benzoic and sorbic acid. They have the ability to inhibit the growth of bacterial and fungal cells. Sorbic acid is also reported to inhibit the germination and outgrowth of bacterial spores (Brul and Coote, 1999).

In general, potassium sorbate is the most common chemical agent used in cheese to control mold growth. It is a white, crystalline powder that is highly water-

soluble. Potassium sorbate prevents bacteria, mold and yeast from spoiling cheese. The maximum level of sorbate allowed in cheese for mold control is 0.3% according the code of Federal Regulations. (Sec. 133.118 Colby cheese). Potassium sorbate is “generally recognized as safe” (GRAS) (Morgan, 2011).

Potassium sorbate is known to control *Aspergillus* spp., but many *Penicillium* spp. can tolerant some of potassium sorbate, which limit its effective in the cheese industry because *Penicillium* spp. are the major contaminants in cheese (Pitt and Hocking, 2009). Marth et al. (1966) reported that *P. roqueforti*, *P. notatum*, *P. frequentans* and *P. cyaneo-fulvum* (all recovered from cheese) were able to grow in the presence of up to 5400 ppm potassium sorbate. The degradation of sorbate was identified as 1,3-pentadiene with an unpleasant hydrocarbon-like odor. While Mann and Beuchat’s (2008) study proved that a reduced concentration of potassium sorbate with other antimycotics, particularly natamycin, have potential for controlling the growth of molds thought to be capable of producing 1,3-pentadiene.

Because of the wide use of sorbates a large number of studies followed in the years after 1984. It was reported that sorbic acid and its salts have a potential genotoxin hazard (Abe and Sasaki, 1977, Hasegawa et al., 1984, Ishidate and Odashima, 1977). Mamur’s et al. (2010) results also showed that potassium sorbate is genotoxic to the human peripheral blood lymphocytes *in vitro*.

3.3.1.2 Natamycin

Natamycin, also called pimaricin is an antibiotic produced by *Streptomyces natalensis* and *Streptomyces chattanoogensis*. It is naturally occurring and prevents

growth of yeasts and molds, is considered GRAS for use on cheese. As natamycin is a natural ingredient, it is also used in organic and biological cheeses. Natamycin is very effective in extremely small quantities against most of yeasts and molds that may occur in foods (DSM). It may be applied by dipping or by spraying using an aqueous solution containing 200~300 ppm of the compound (CFR 21-172 Section 155). In shredded cheeses, natamycin is applied to the cheese shreds at the time of application of anti-caking agents. However, natamycin-resistant mold strains *Penicillium discolor*, a species that causes spoilage of hard cheese has been reported by Filtenborg et al (1996) and Cabo et al., (2002).

3.3.2 Modified atmosphere packaging

The use of modified atmospheres to prevent fungal growth reduces chemical preservatives and keep the long shelf life of foods at the same time. The gases used for modified atmosphere packaging (MAP) include carbon dioxide, nitrogen, carbon monoxide and sulphur dioxide. Carbon dioxide (CO₂) with or without other gases is most commonly used. Taniwaki et al. (2001) examined 8 fungi that were grown in atmosphere containing 20% and 40% CO₂ with 1% or 5% O₂, the growth of fungi was reduced by 20~80%, depending on species compared with growth in air. The formation of mycotoxins were greatly decreased but not totally inhibited. Papaioannou et al. (2007) compared the shelf life of a Greek whey cheese under MAP and vacuum package (VP). The results showed that MAP delayed microbial growth compared with VP samples.

3.3.3 Natural antimicrobials

Natural antimicrobials are compounds from herbs and spices, fruits and vegetables, leaves and bark, animal tissues and microorganisms, which have antimicrobial properties (Roller, 1995). Essential oils (EOs) are naturally occurring terpenic mixtures isolated from various parts of plants (Daferera et al., 2000). A technique that has been used since ancient times in cheese to prevent fungal growth, involves physically rubbing the product with certain herbs or spices, or their oils, with well-known antimicrobial properties (Leung, 1978). Vázquez et al. (2001) studied the antifungal effect of eugenol and thymol on the growth and production of citrinin from *P. citrinum*, and proved that they have a strong inhibitory effect; 200 µg/ml of eugenol fully inhibited fungal growth. Gandomi et al. (2009) reported that the EO isolated from *Zataria multiflora* Boiss. (A spice plant growth only in Iran, Pakistan and Afghanistan) had an inhibitory effect against radial fungal growth and aflatoxin production by *A. flavus* in cheese.

3.3.4 Natural biological antagonist

Biopreservation, the control of one organism by another, has received increased attention in recent years. Lactic acid bacteria have a long history of proven safe use in food and beverage fermentations. The existence of LAB in food is not just for nutritional and organoleptic benefits but also act as preservatives. LAB have the ability of extending the shelf life of foods due to the copious antifungal components such as organic acid and bacteriocin produced during fermentation or post fermentation. Halm and Olsen (1996), Shetty and Jespersen (2006) reported

that *S. cerevisiae* together with *Candida krusei* have inhibitory effect on the growth of mycotoxin-producing molds such as *P. citrinum*, *A. flavus* and *A. parasiticus*, mainly due to substrate competition, while inhibition of spore germination might due to the production of high concentration of organic acid (Jespersen, 2003). Shetty and Jespersen (2006) also tested *Saccharomyces cerevisiae* and *Candida krusei* for aflatoxin B1 binding. Some of the isolates bound more than 60% (w/w) of the added toxins (Shetty et al., 2007). Liu and Tsao (2009) reported that some strains of the yeast *Debaryomyces hansenii* have the inhibitory effects on some dairy molds such as *Aspergillus* sp., *Bysschlamys fulva*, *Byssochlamys nivea*, *Cladosporium* sp., *Eurotium chevalieri*, *Penicillium candidum* and *Penicillium roqueforti*.

A lot of research has been done on the antagonistic ability of LAB. Many strains that belong to genera *Lactococcus*, *Lactobacillus*, *Leuconostoc* and *Pediococcus* are known for their antifungal activity (Dalie et al., 2009).

3.4 The antifungal activity of lactic acid bacteria (LAB)

LAB naturally exist in foods (Table 2) and have been used as starter bacteria in fermentations for thousands of years. It has been estimated that 25% of the European diet is contributed by fermented foods; some of them are still produced by natural fermentation. In many developing countries, fermented foods are still produced by traditional means and constitute up to 60% and more of the daily diet (Holzapfel et al., 1995). LAB are also known for their ability of producing antagonism compounds that are able to control pathogenic bacteria and undesirable spoilage mold during fermentation.

Table 2 Foods and their associated lactic acid bacteria

Food types	Lactic acid bacteria
Milk and dairy foods	
Hard cheese without eye formation	<i>Lc. lactis</i> subsp. <i>cremoris</i> and subsp. <i>lactis</i>
Cottage cheese and cheeses with a few or small eyes (Edam)	<i>Lc. lactis</i> subsp. <i>cremoris</i> and subsp. <i>lactis</i> ; and <i>Lc. mesenteroides</i> subsp. <i>cremoris</i>
Cultured butter, buttermilk cheeses with round eyes (Gouda)	<i>Lc. lactis</i> subsp. <i>cremoris</i> and subsp. <i>lactis</i> and var. <i>diacetylactis</i> ; and <i>Le. mesenteroides</i> subsp. <i>cremoris</i>
Swiss-type cheeses	<i>Lb. delbrueckii</i> subsp. <i>bulgaricus</i> ; <i>Lb. helveticus</i>
Dairy products in general	<i>Lb. brevis</i> ; <i>Lb. buchneri</i> ; <i>Lb. casei</i> ; <i>Lb. paracasei</i> ; <i>Lb. fermentum</i> ; <i>Lb. plantarum</i> ; <i>Le. mesenteroides</i> subsp. <i>cremoris</i> ; <i>Le. lactis</i>
Fermented milks	
- yogurt	<i>Streptococcus thermophilus</i> and <i>Lb. delbrueckii</i> subsp. <i>bulgaricus</i> ; <i>Lc. lactis</i> subsp. <i>diacetylactis</i>
- acidophilus milk	<i>Lb. acidophilus</i>
- kefir	<i>Lb. kefir</i> ; <i>Lb. kefirianofaciens</i>
Meats	
Raw	<i>C. divergens</i> ; <i>C. piscicola</i> (<i>maltaromicus</i>) <i>Lb. sake</i> ; <i>Lb. curvatus</i> <i>Le. carnosum</i> and <i>Le. gelidum</i>
Semi-preserved	<i>Lb. viridescens</i> (spoilage) <i>Le. carnosum</i> and <i>Le. gelidum</i> <i>C. divergens</i> ; <i>C. piscicola</i> (<i>maltaromicus</i>)
Fish	
Marinated fish products	<i>Lb. alimentarius</i> <i>C. piscicola</i>
Fermented vegetables	<i>P. acidilactici</i> and <i>P. pentosaceus</i> ; <i>Lb. plantarum</i> ; <i>Lb. sake</i> ; <i>Lb. buchneri</i> ; <i>Lb. fermentum</i> ;
Cucumbers, sauerkraut	<i>Le. mesenteroides</i> (initial fermentation) <i>Lb. bavaricus</i> ; <i>Lb. brevis</i> ; <i>Lb. sake</i> ; <i>Lb. plantarum</i> ;
Olives	<i>Le. mesenteroides</i> , <i>Lb. pentosus</i>
Soy sauce	<i>Tetragenococcus</i> (<i>Pediococcus</i>) <i>halophilus</i>
Baked goods	
Sourdough bread	<i>Lb. sanfrancisco</i> (wheat and rye sourdough) <i>Lb. farciminis</i> ; <i>Lb. fermentum</i> ; <i>Lb. brevis</i> ; <i>Lb. plantarum</i> ; <i>Lb. amylovorus</i> ; <i>Lb. reuteri</i> <i>Lb. pontis</i> (rye sourdough)
Wine (malo-lactic fermented)	<i>Le. oenos</i>
<i>C. carnobacterium</i> ; <i>Lb. Lactobacillus</i> ; <i>Lc. Lctococcus</i> ; <i>Le. Leuconostoc</i> ; <i>P. Pediococcus</i> (Hammes and Tichaczek, 1994)	

3.4.1 Lactic acid bacteria that control mold growth

Antagonism of LAB refers to the inhibition of other microorganism, caused by competition for nutrients, and by the production of antimicrobial metabolites (Holzapfel et al., 1995). These LAB are mainly divided into four genera: *Lactococcus*, *Lactobacillus*, *Leuconostoc* and *Pediococcus*. Extensive research has been done on the antifungal ability and inhibitory components have been detected and identified (Table 3).

Roy et al. (1996) isolated 2100 colonies of LAB from 4 month old Cheddar cheese and raw buffalo milk, six showed antifungal activity against *Aspergillus flavus* IARI and the most promising isolate *Lactococcus lactis* subsp. *lactis* CHD-28.3 had a broad spectrum antifungal activity against *Aspergillus flavus* IARI, *A. flavus* NCIM 555, *A. parasiticus* NCIM 898 and *Fusarium* sp. The neutralized cell-free supernatant of the test culture still had the antifungal activity, but it was sensitive to chymotrypsin, trypsin and pronase E and the antifungal activity disappeared after enzymatic treatment suggesting that the antifungal compound is a peptide. The optimal antifungal activity was observed when the isolate grown was at 30 °C for 48 h in Elliker's broth. *Aspergillus* IARI was the most sensitive indicator mold for this lactic strain.

Magnusson et al. (2003) isolated more than 1200 isolates of LAB from different environments and screened for antifungal activity in a dual-culture agar plate assay. Ten percent of the isolates showed inhibitory activity and 4% showed strong activity against the indicator mold *A. fumigatus*. Seven strains showed strong

and wide antifungal spectrum against *A. fumigatus*, *A. nidulans*, *P. commune* and *Fusarium sporotrichioides*, and also against the yeast *Rhodotorula mucilaginosa*. *P. roqueforti*, while the yeasts *Pichia anomala* and *Kluyveromyces marxianus* were not inhibited. Most of the antifungal isolates were identified by 16S rDNA sequencing as *Lactobacillus coryniformis*. *Lactobacillus plantarum* and *Pediococcus pentosaceus* were also frequently identified among the active isolates. Antifungal cyclic dipeptides were identified and suggesting a highly complex nature of the antifungal activity.

The genus *Lactobacillus* was most frequently isolated and researched. The antifungal strains were isolated from different environments such as sourdough culture (Corsetti et al., 1996), salami (Coloretti et al., 2007), grass silage (Magnusson and Schnurer, 2001), cheeses and milk (Roy et al., 1996) and vegetable products (Sathe et al., 2007). See details in Table 3 below.

Table 3 Lactic acid bacteria isolates with the ability to inhibit toxinogenic mold and yeast growth

LAB isolate	Activity spectrum	Compound(s)	Reference
Genus <i>Lactococcus</i>			
<i>Lactococcus lactis</i> C10	<i>Aspergillus parasiticus</i>	ND	(Wiseman and Marth, 1981)
<i>Lc. lactis</i>	<i>A. flavus</i>	A heat-stable low-molecular-weight compound	(Coallier-Ascah and Idziak, 1985)
<i>Lc. lactis</i> NCFB 496	<i>A. parasiticus</i>	ND	(Luchese and Harrigan, 1990)
<i>Lc. lactis</i> subsp. <i>diacetyllactis</i> DRCI	<i>A. fumigatus</i> , <i>A. parasiticus</i>	ND	(Batish et al., 1989)
<i>Lc. lactis</i> subsp. <i>lactis</i> CHD 28.3	<i>A. flavus</i> , <i>A. parasiticus</i> , <i>Fusarium spp.</i>	Proteinaceous nature substance	(Roy et al., 1996)
<i>Lc. lactis</i> ssp. <i>cremoris</i>	<i>Penicillium expansum</i>	ND	(Florianowicz, 2001)
Genus <i>Lactobacillus</i>			
<i>Lb. sp</i> SK007	Broad spectrum	Phenyllactic acid	(Mu et al., 2009)
<i>Lb. sanfrancisco</i> C57	<i>Fusarium spp.</i> , <i>Penicillium spp.</i> , <i>Aspergillus spp.</i>	ND	(Corsetti et al., 1996)
<i>Lb. plantarum</i> VLT01	<i>A. candidus</i> , <i>P. nalgiovense</i>	Phenyl-lactate, hydroxy-phenyl-lactate	(Coloretti et al., 2007)
<i>Lb. sanfrancisco</i> CB1	<i>Fusarium</i> , <i>Penicillium</i> , <i>Aspergillus</i> and <i>Monilia</i>	A mixture of organic acid	(Corsetti et al., 1998)
<i>Lb. casei</i> ATCC 393	<i>A. parasiticus</i>	ND	(El-Gendy and Marth, 1981)
<i>Lb. casei</i> var. <i>rhamnosus</i>	Broad spectrum	ND	(King et al., 1986)
<i>Lb. reuteri</i>	Broad spectrum	3-HPA (reuterin)	(Chung et al., 1989)
<i>Lb. plantarum</i>	Unspecified spoilage mould	ND	(Hill, 1989)
<i>Lb. acidophilus</i> R	<i>A. fumigatus</i> ,	ND	(Batish et al., 1990)
<i>Lb. casei</i> subsp. <i>rhamnosus</i> , <i>Lb. plantarum</i> , <i>Lb. plantarum</i>	<i>Penicillium spp.</i> , <i>Aspergillus spp.</i>	ND	(Suzuki et al., 1991)
<i>Lb. casei</i> subsp. <i>Rhamnosus</i> LC-705	<i>Saccharomyces cerevisiae</i>	ND	(Makanjuola et al., 1992)
	<i>Candida lusitaniae</i> , <i>A. niger</i> , <i>Fusarium spp.</i> , <i>Penicillium spp.</i> , <i>Cladosporium spp.</i>	ND	(Mäyrä-Mäkinen et al., 2001)

Table 3 (continued). Lactic acid bacteria isolates with the ability to inhibit toxinogenic mold and yeast growth

<i>Lb. casei</i>	<i>Penicillium spp.</i>	Possibly proteinaceous	(Gourama, 1997)
<i>Lb. casei</i> subsp. <i>pseudoplatantarum</i>	<i>A. flavus</i>	Possibly proteinaceous, <1 kDa	(Gourama and Bullerman, 1997)
<i>Lb. plantarum</i> VTT E78076	<i>F. avenaceum</i>	Benzoic acid, methylhydantoin, mevalonolactone, cyclo (Gly-L-Leu)	(Niku-Paavola et al., 1999)
<i>Lb. pentosus</i>	<i>Candida albicans</i>	Pentocin tv35B	(Okkers et al., 1999)
<i>Lb. casei</i> , <i>Lb. delbrueckii</i> subsp. <i>bulgaricus</i>	<i>P. expansum</i>	ND	(Florianowicz, 2001)
<i>Lb. plantarum</i>	<i>Broad spectrum</i>	Phenyllactic acid, 4-hydroxyphenyllactic acid	(Lavermicocca et al., 2000)
<i>Lb. rhamnosus</i>	<i>Penicillium spp.</i> , <i>Aspergillus spp.</i> , <i>Fusarium spp.</i> , <i>Alternaria spp.</i>	Sodium acetate	(Stiles et al., 2002)
<i>Lb. plantarum</i> MiLAB 393	<i>Broad spectrum</i>	3-Phenyllactic acid, cyclo(Phe-Pro), cyclo(Phe-OH-Pro)	(Strom et al., 2002)
<i>Lb. coryniformis</i> Si3	<i>Broad spectrum</i>	Peptide, phenyllactic acid, cyclo(Phe-Pro), cyclo(Phe-OH-Pro), reuterin	(Magnusson, 2003), (Magnusson and Schnurer, 2001)
<i>Lb. plantarum</i> MiLAB 14	<i>Broad spectrum</i>	Hydroxy fatty acids, phenyllactic acid, cyclo(Phe-Pro), cyclo(Phe-OH-Pro)	(Magnusson et al., 2003)
Genus <i>Leuconostoc</i> <i>Ln. mesenteroides</i>	<i>Penicillium spp.</i> , <i>Aspergillus spp.</i>	ND	(Suzuki et al., 1991)
Genus <i>Pediococcus</i> <i>Pc. acidilactici</i> LAB 5	<i>A. fumigatus</i> , <i>A. parasiticus</i> , <i>F. oxyporum</i> , <i>Pencillium spp.</i>	ND	(Mandal et al., 2007)

ND: not determined

3.4.2 Antifungal metabolites produced by lactic acid bacteria

Antifungal Lactic acid bacteria have the ability to producing copious amounts of acidic end products and other compounds with strong antifungal activities. The majority of identified antifungal substances are low molecular weight compounds including organic acids, hydrogen peroxide, proteinaceous compounds, hydroxyl fatty acids and phenolic compounds (Table 4).

Table 4 Metabolic products of lactic acid bacteria with antimicrobial properties

Product	Main target organisms
Organic acid	
- lactic acid	Putrefactive and Gram-negative bacteria, some fungi
- acetic acid	Putrefactive bacteria, clostridia, some yeasts and fungi
Hydrogen peroxide	Pathogens and spoilage organisms, especially in protein-rich foods
Enzymes	
- lactoperoxidase system with H ₂ O ₂	Pathogens and spoilage bacteria (milk and dairy products)
- lysozyme (by recomb, DNA-technology)	Undesired Gram-positive bacteria
Low-molecular metabolites	
- reuterin (3-OH-propionaldehyde)	Wide spectrum of bacteria, molds and yeasts
-diacetyl	Gram-negative bacteria
-fatty acids	Different bacteria
Bacteriocins	
-nisin	Some LAB and Gram-positive bacteria, notably endospore-formers
-other	Gram-positive bacteria, inhibitory spectrum according to producer strain and bacteriocin type

Source: Holzapfel et al. (1995)

3.4.2.1 Organic acids

Lactic acid is the main LAB metabolite that reduces pH of the system. When citrate was present in the medium together with glucose during the initial fermentation, the lactic acid produced was degraded. Citrate was concomitantly degraded, resulting in accumulation of formic, acetic and succinic acids along with CO₂ (Lindgren et al., 1990). The acid production and the accompanying pH decrease extend the lag phase of sensitive organisms. Lipophilic acids such as acetic and lactic acid in their undissociated form can penetrate the microbial cell and interfere with essential metabolic functions such as substrate translocations and oxidative phosphorylation, and reduce the intracellular pH (Lindgren and Dobrogosz, 1990). However, acetic acid is reported to be more inhibitory than lactic acid, especially against yeasts and molds (Eklund, 1989). This can be explained by the extent of dissociation since acetic acid has between two and four times more acid in the undissociated state at pH values between 4.0 and 4.6 compared to lactic acid (Lindgren and Dobrogosz, 1990). Heterofermentative LAB also produce propionic acid in trace amounts. Propionic acid has been described as having membrane-directed activity as its main mode of action against bacteria and fungi. It interacts with cell membranes to neutralize the electrochemical proton gradient, causing bacteriostasis and fungistasis. But the effect of acetic and propionic acid is often dependent on the decrease in pH caused by lactic acid (Eklund, 1989). Moon (1983) reported that the mixtures of lactic, acetic and propionic acid caused a drastic reduction in growth rate of yeast, while yeast still could grow well in relatively high

(100 mmol/l) concentrates of acetate or lactate acid. Propionate was much more inhibitory than acetate or lactate.

Other organic acids that show antifungal activity such as phenyllactic had been found in cultures of *Lactobacillus plantarum* with a wide antifungal spectrum (Lavermicocca et al., 2000, Lavermicocca et al., 2003, Strom et al., 2002). It was hypothesized that phenyllactic acid acted in a synergy pathway with unidentified antifungal substances produced by *Lb. plantarum* MiLAB 393 strain. Lavermicocca et al. (2000) reported that phenyllactic acid was contained at the highest concentration in the bacterial culture filtrate and had the highest activity. It inhibited all the fungi tested at a concentration of 50 mg/ml except for *P. rogueforti* IBT 18687 and *P. corylophilum* IBT 6978 (166 mg/ml).

Benzoic acid is another antifungal organic acid that is one of the oldest chemical preservatives used in the cosmetic, drug and food industries. It was isolated and identified in the culture filtrate of *Lactobacillus plantarum* VTT E-78076 by Niku-Paavola et al. (1999). Benzoic acid was active against *Fusarium avenaceum* VTT D-80147 with 10-15% of inhibition by separate compound in concentration of 10 ppm. It has been assumed that inhibition of growth is due to the elimination of the electrochemical gradient across the cell membrane by undissociated benzoate passing through the membrane and dissociating in the interior (Eklund, 1989).

Antifungal LAB can produce a mixture of organic acids during fermentation, including caproic, formic, butyric and *n*-valeric acid. Corsetti et al. (1998) studied the mixture of organic acids produced by *Lactobacillus sanfrancisco* CB1, and found

that the organic acids act in a synergistic way and that caproic acid played a key role in increasing the antifungal activity of the mixture.

3.4.2.2 Hydroxy fatty acids

Some LAB can produce hydroxy fatty acids that have strong antifungal activity. Sjogren et al. (2003) identified and characterized four antifungal substances, ie., 3-(R)-hydroxydecanoic acid, 3-hydroxy-5-cis-dodecenoic acid, 3-(R)-hydroxydodecanoic acid and 3-(R)-hydroxytetradecanoic acid isolated from *Lactobacillus plantarum* MiLAB 14. Racemic mixtures of the saturated 3-hydroxy fatty acids showed antifungal activity against different molds and yeasts with MICs between 10 and 100 µg/ml. The mechanism behind the antifungal effect hydroxy fatty acids has been suggested that free fatty acids alter membrane fluidity either by partitioning into the lipid bilayer of cells or by inclusion into fatty acyl chains of membrane phospholipids (Avis and Belanger, 2001). This increases membrane permeability and the release of intracellular electrolytes and proteins leading to cytoplasmic disintegration of fungal cells (Sjogren et al., 2003).

3.4.2.3 Hydrogen peroxide

LAB have a small number of flavoprotein oxidase enzymes that enable them to produce hydrogen peroxide (H₂O₂) in the presence of oxygen. Hydrogen peroxide accumulates in the environment since LAB do not produce catalase (Condon, 1987). After accumulation, it oxidizes the lipid membrane and cellular proteins of the target organisms (Lindgren and Dobrogosz, 1990). The antimicrobial effect of hydrogen peroxide at non-inhibitory concentrations is potentiated by lactoperoxide

and thiocyanate present in milk. The lactoperoxidase-thiocyanate-peroxide system involves the reaction of hydrogen peroxide and thiocyanate through catalysis by lactoperoxidase. Hypothiocyanate and other intermediary products then inhibit other microorganisms (Condon, 1987, Schnurer and Magnusson, 2005). Venturini et al. (2002) studied the in vitro antimicrobial activity and the MIC of hydrogen peroxide using different end point methods such as agar diffusion assay, volatility method and agar and broth dilution MIC assays. The effectiveness of 5 and 10% hydrogen peroxide in growth inhibition of *Pc. expansum* in the agar diffusion assay was total, and its MIC as determined by the agar and broth dilution assays was less than 0.025%, suggesting that the application of small quantities of hydrogen peroxide to the apple skin might be an alternative to fungicides to *Pc. expansum*.

3.4.2.4 Reuterin

Reuterin is a broad-spectrum antimicrobial substance originally has been found produced by *Lb. reuteri*. Reuterin is produced from glycerol by starving cells under anaerobic conditions and the active reuterin is an equilibrium mixture of monomeric, hydrated monomeric and cyclic dimeric forms of 3-hydroxypropionaldehyde (3-HPA) (Talarico et al., 1988). It has been postulated that reuterin could play a role in the probiotic effects of *Lb. reuteri*. It is active toward entero pathogens, yeasts, fungi, protozoa and viruses (Axelsson et al., 1989). Reuterin was able to suppress ribonuclease activity, the main enzyme involved in the biosynthesis of DNA, in order to inhibit the growth of target organisms

(Dobrogosz et al., 1989). Chung, Axelsson et al. (1989) reported that reuterin has the ability to inhibit the growth of *Aspergillus* and *Fusarium*.

3.4.2.5 Proteinaceous compounds

There is no clear evidence to explain the role of proteinaceous compounds in the inhibition of mold growth. However, several authors reported that the antifungal metabolites were sensitive to different proteinases. Corsetti et al. (1996) isolated a bacteriocin-like inhibitory substance from *Lactobacillus sanfrancisco* C57, and found that all the antimicrobial compounds produced from strain contained a protein moiety and were sensitive to different proteinases. Coloretto et al. (2007) studied the antifungal activity of lactobacilli isolated from salami and reported that at the end of cell growth, more peptidic compounds that sensitive to the action of proteinase K were released. Coda et al. (2008) identified a mixture of eight peptides from the water-soluble extract of sourdough *L. brevis* AM7, and 5 of these exhibited inhibitory activity. The range of MICs was 3.5 to 8.2 mg/ml. Bread was made by sourdough fermentation with *L. brevis* AM7 plus the addition of the water-soluble extract (27%, v/w; 5 mg of protein/ml) of *P. vulgaris* cv. *pinto*. The bread did not show contamination by fungi until 21 days of storage at room temperature, which is comparable to the same level of afforded by 0.3% (w/w) calcium propionate. Other types of antifungal peptides were identified to be cyclic dipeptides such as cyclo (L-Phe-L-Pro) and cyclo (L-Phe-trans-4-OH-L-Pro). They were produced by *Lb. plantarum* MiLAB 393 (Strom et al., 2002).

Figure 1. Summary of current knowledge of the main antifungal compounds produced by different LAB (Schnurer and Magnusson, 2005).

3.4.3 Factors that influence the antifungal activity of lactic acid bacteria

The antifungal activity of LAB due to the production of metallic compounds that have been greatly influenced by multiple parameters such as incubation temperature and period, nutritional factors and pH of media.

3.4.3.1 Temperature and incubation period

Temperature and incubation period are essential factors that affect the amount of antifungal compounds produced. Magnusson and Schnurer (2001) reported that when *Lactobacillus coryniformis* subsp. *coryniformis* strain Si3 grew in a liquid culture, the maximum mold inhibition occurred in the stationary growth phase, but there was rapid decline in antifungal activity after 48 h. This decrease was due to metabolism of the active compounds or to their enzymatic degradation. However, Coloretti et al. (2007) studied sixty-five strains of Lactobacilli for their antifungal capacity during both fermentative phase (48 h) and late phase (30-day). Only nine strains showed marked inhibitory capacity during fermentative phase, but all the strains tested had activity in the late phase due to cells autolysis. Sathe et al.

(2007) demonstrated that antifungal activity of *Lb. plantarum* CUK 501 was maximal at 30 °C when the culture was at the end of its logarithmic phase.

3.4.3.2 Effect of nutritional factors

Schillinger and Villarreal (2010) studied the influence of medium composition on the antifungal activity of LAB. It was observed that LAB strains producing large zones of inhibition on MRS agar plates were unable to produce inhibition zones on MRS without sodium acetate or on MRS agar with reduced glucose content. For MRS broth, with all strains, an antifungal effect was observed only with normal MRS broth in the presence of 61 mmol/l sodium acetate. Effat (2000) reported an increase in the concentration of NaCl from 0.5% to 3% in *Lb. rhamnosus* growth medium led to a higher antifungal efficiency. A supplementation of broth media with CaCl₂ increased the production of antifungal substance by *Lb. rhamnosus* and *P. acidilactici*, a maximum increase was observed when the concentration of CaCl₂ rose from 0.05% to 0.15% (Effat et al., 2001).

3.4.3.3 Effect of pH

The pH conditions greatly modulate the production of antifungal metabolites by LAB. Sathe et al. (2007) detected the growth of *Lb. plantarum* during 72 h incubation, the pH of broth declined from 6.7 to 4.2. They found that the antifungal titer was high when the pH of the broth was between 4.8 to 4.5, but when the pH was 4.2, a decline in antifungal titer was observed. Magnusson and Schnurer (2001) reported that the antifungal compounds produced by *Lactobacillus coryniformis* subsp. *coryniformis* strain Si3 had the maximum activity at pH values of between 4.5

and 6.0 and was lost at higher pH values. However, the activity was fully regained after readjustment of the pH to the initial value (pH 3.6).

3.5 LAB in cheese system

Lactic acid bacteria have been getting an increasing interest all over the world during the past 30 years. Their antimicrobial activities, growth properties and various compounds that are produced during fermentation have been evaluated. However, little work on the use of antifungal LAB in cheese manufacture and very few commercial protective cultures are available in market. Even though extensive research papers confirmed that some LAB have strong antifungal activity under laboratory conditions, there is no information on the use of anti-mold LAB in actual cheese system. Microorganisms are essential components of all natural cheese varieties and play important roles during both cheese manufacture and ripening. The addition of adjunct LAB could potentially produce new flavors (both good and off-flavor), change the texture of cheese, cause color defects or may lose their antimicrobial activity. The application of antifungal LAB in cheese system needs further investigation.

3.5.1 Cheese microbiological during manufacture and ripening

The primary purpose of cheese manufacture originally was to extend shelf life and conserve the nutritious components of milk. In most cheeses, LAB are used as starter for acidification. High densities of LAB are present in cheese throughout ripening and they play a significant role in the maturation process. The microflora of cheese may be divided into two groups: starter lactic acid bacteria (SLAB) and no

starter lactic acid bacteria (NSLAB). *Lactococcus lactis*, *Streptococcus thermophilus*, *Lactobacillus helveticus* and *Lactobacillus delbrueckii* that involved in acid production during manufacture and contribute to the ripening process are called SLAB. NSLAB along with yeasts and/or molds are considered as secondary microorganism in cheese. In this study, we only focus on NSLAB. NSLAB contribute little to acid production during manufacture, but play a significant role during ripening (Beresford et al., 2001).

SLAB could be defined as isolates which produce sufficient acid to reduce the pH of milk to <5.3 in 6 h at 30~37 °C. They grow during manufacture and typically attain cell densities of 10^8 cfu/g within an hour of the beginning of manufacture and reach $\sim 10^9$ cfu/g of curd at pressing, then decline after salting, due to the presense of salt, the low curd pH and the absence of a fermentable lactose (Lynch et al., 1999). Mesophilic cultures such as *L. lactis* ssp. *cremoris* and *L. lactis* ssp. *lactis* are used in the production of Cheddar, Gouda, Edam, Blue and Camembert cheese.

NSLAB are mesophilic lactobacilli and pediococci, which are dominant microflora during ripening. They are not part of the normal starter flora and generally do not grow well in milk (Cogan et al., 1997) and do not contribute much to acid production in the cheese making. However, concomitant with the decrease of SLAB, NSLAB multiply starting from $10^1 \sim 10^4$ cfu/g of curd on the day of manufacture to become the dominant viable microflora and reaching cell densities of $10^6 \sim 10^8$ cfu/g in the mature cheese (Swearingen et al., 2001). Lynch et al., (1999) reported that the counts of NSLAB ranged from 10^4 to 10^5 cfu/g at milling and

increased to $\sim 5 \times 10^7$ cfu/g during 4 weeks post manufacture. NSLAB are considered to play an important role of flavor development during cheese ripening, but cheeses nowadays are widely made from pasteurized milk due to the sanitary reason, and resulting in reduction in NSLAB in cheese milk. That is why cheese made from raw milk generally ripens faster and develops a stronger more intense flavor than cheese made from pasteurized milk (McSweeney et al., 1993). Many species of NSLAB were isolated from cheese most encountered are *Lb. casei*/*Lb. paracasei*, *Lb. plantarum*, *Lb. rhamnosus* and *Lb. curvatus*, *Pediococcus acidilactici* and *Pc. pentosaceus* are also included.

For antifungal LAB, there is no published research paper on their use in cheese manufacture. Since the antifungal LAB are most likely similar species as NSLAB in cheese (see Table 3), the performance of antifungal LAB should have similar to NSLAB in cheese and become the dominant microflora after 1 month of ripening.

3.5.2 Use of LAB in cheese manufacture

Very limit papers reported the use of antifungal LAB in cheese manufacture. Neugebauer and Gilliland (2005) studied the antagonistic action of *Lactobacillus delbrueckii* ssp. *lactis* RM2-5 toward spoilage organisms in cottage cheese. In Cottage cheese inoculated with *Pseudomonas fluorescens*, the number of spoilage organisms in the control increased 5 log cycles in 7 days, whereas the treatment containing 1.0×10^9 cfu/g *Lb. delbrueckii* ssp. *lactis* RM2-5 had not increased over the course of the 21-day. Schwenninger and Meile (2004) reported the antifungal

activity of a mixed culture of *Propionibacterium jensenii* and *Lactiobacillus paracasei* subsp. *paracasei* in yogurt and cheese. These cultures showed inhibitory activities (more than 10^5 cfu/ml) against yeasts in dairy products such as yogurt or cheese surface at refrigerator temperatures (6°C) without influencing yogurt quality. Initial cell numbers of 5×10^5 cfu/g of propionibacteria and 1×10^8 cfu/g of lactobacilli were the optimal for total inhibition of the spoilage yeast: *Candida pulcherima*, *Candida magnoliae*, *Candida parapsilosis* and *Zygosaccharomyces bailii*.

4.0 MATERIALS AND METHODS

4.1 Cultures and Media

4.1.1 Isolation of mold from cheese surface

Indicator mold was isolated from moldy cheddar cheese. Mold was scrapped off the cheese surface using a sterile inoculation loop and inoculated on acidified potato dextrose agar (PDA, pH=3.2, Hardy Diagnostics, Santa Maria, CA) slants and incubated at room temperature until visible mold growth was seen. This mold was purified and the single colony was picked and transferred to a new PDA slant. The agar slants were stored under refrigerated conditions and used as an indicator mold culture. The mold culture was transferred to a new PDA slant monthly.

4.1.2 Fungal inocula preparation

Mold isolated from Cheddar cheese was grown on PDA slant at room temperature for 7 to 10 days or until sporulation. A sterilized loop was used to scrape the spores from slant and dispersed in 4 ml phosphate buffer (cat #D599, Hardy Diagnostic, Santa Maria, CA) until turbidity. The mold suspension was adjusted to an absorbance of 0.35 measured at 600 nm (A_{600}) using a spectrophotometer (Spectra Max Plus, Molecular Devices, Sunnyvale, CA). The mold spore dilution (1:8 phosphate buffer) was then enumerated by plate counting on PDA agar. Dilution gave $\sim 10^5$ spores/ml (Coloretti et al., 2007).

4.1.3 Isolation of LAB from food

Various food products (e.g. fresh and fermented vegetables, foreign cheeses and sourdough starter) were screened for lactobacilli. The food product (2~5 g) was added in 50 ml of Lactobacillus Selective (LBS) broth (cat #211331, BD BBL™, Sparks, MD) and incubated at 32 °C until heavy turbidity was seen. Suitable decimal dilutions (10^{-6} , 10^{-7} , 10^{-8} were used here) of the culture were surface plated on LBS agar to obtain well-dispersed colonies. The plates were incubated at 32 °C in anaerobic chamber (BBL GasPak Plus BD, Sparks, MD) for 3 days for good colony development.

4.1.4 Screening assay for antifungal activity

Agar plates exhibiting well-dispersed colonies were selected. Individual colonies were replicate plated on deMan-Rogosa-Sharpe (MRS) agar (BD Diagnostics, Sparks, MD) using sterile toothpicks. The replicate plating was done to provide sufficient spacing between the picked colonies (26 colonies for each plate). The plates were incubated in an anaerobic chamber at 32 °C for 3 days to obtain good macroscopic colony growth. One of the replicate plates was used for screening anti-mold activity, and the other corresponding plates served as the “master plate”. Plates chosen for screening were overlaid with 5 ml semi-solid Tryptone Soy Agar (TSA), prepared with Tryptone Soy Broth (TSB, BD BBL™, Sparks, MD) and 0.75% agar, seeded with 1ml of indicator culture of mold. After the overlay soft agar solidified, the plates were incubated at room temperature (about 22 °C) for 2 days or until a uniform mat of mold mycelia growth had been obtained. The plates were

examined to detect any colonies showing a clear zone of inhibition of the mold.

Corresponding colonies from the “master plate” were picked up and transferred to new MRS plates, and then rechecked to confirm anti-mold activity by overlay method.

4.1.5 Identification of strains

Confirmed colonies with anti-mold activity were examined by the Gram-stain reaction, cell morphology, catalase reaction and phenotypical characterization of *Lactobacillus* strains. Six strains with antifungal properties were saved in MRS broth with 20% sterilized glycerol (cat #G33-1, Fisher Sci., Tustin, CA.). The suspension was pipetted into sterile 1.0 ml cryogenic vials (cat #5000-0012, Nalge Co., Rochester, NY) and kept at -50 °C.

Gram stain and microscopic examination: Gram stain kit (Gram Stain kit, BD Difco™ BBL™, Sparks, MD) was used; cell morphology was checked by an optical microscopic (100×/1.25 oil, Olympus CX31, Center Vally, CA).

Catalase test: A drop of broth culture was transferred onto a microscopic slide, a few drops of 3% H₂O₂ was added to the broth culture. Alternatively, 1 to 2 drops of 3% H₂O₂ were directly added on cultures grown on an agar plate. The formation of bubbles were observed and recorded as an indication of catalase production. Generally, lactic acid bacteria are catalase-negative.

Phenotypical characterization of isolated strains was carried out using an API 50CH system (BioMérieux Vitek, Hazelwood, MO).

4.2 Antifungal activity assays

The overlay method and agar well-diffusion method were used to detect antifungal activity. All the experiments were performed in duplicate unless stated specifically.

4.2.1 Detection of the antifungal activity of cells by overlay method

The anti-mold LAB strains were propagated in 10 ml MRS broth and incubated at 32 °C for 20 h. Then, the broth cultures were centrifuged at 4,000 rpm for 25 min to pellet the bacterial cells. The supernatant (9.5 ml) was removed, and then resuspended the pellet with the left over MRS broth. This consolidated dense cell suspension was used to inoculate the MRS agar for overlay method, 3 µL per spot. The plates were then placed in anaerobic chamber and incubated at 32 °C. After 5 days incubation, MRS plates were overlaid with 5 ml TSA soft agar seeded with 1 ml 1:8 mold (isolated from Cheddar cheese, *Penicillium* sp.) suspension (10^5 spores/ml) and incubated at room temperature until a clear zone appeared around a uniform mat of mold mycelia growth. This took approximately 2 days. Inhibitory activity was scored as follow: –, no inhibition; +, very weak inhibition; ++, low inhibition with a little clear zone near the rim of the colony; +++, strong inhibition with a large clear zone near the rim of the colony; +++++, very strong inhibition with no growth near the rim of the colony.

4.2.2 Detection of antifungal activity of cell free extract by well-diffusion method

Preparation of concentrated supernatant: Each strain (1% inoculum) was inoculated in 250.0 ml MRS broth at 32°C for 5 days. The cultures were centrifuged

to sediment the cells, the supernatants were carefully decanted and filter-sterilized (45 µm filter, autoclaved at 121°C for 15 min, Fisher Sci., Tustin, CA). Each of the sterile cell free supernatant (~150 ml) was concentrated by vacuum rotary evaporator (bath temperature of 60 °C, rotational speed of 90 rpm/min under vacuum) to obtain a 15-fold concentrated supernatant.

Well-diffusion method: the anti-mold activity of the cell-free supernatant from lactobacillus strains was determined by a well-diffusion assay in Petri dishes. Several number of oxford cups (8 mm in diameter) were placed on the thin agar layer that covered the bottom of Petri dish. Into each well was poured semi-solid TSA seeded with indicator mold (10^5 cfu/ml). Each well was loaded with 200 µL concentrated supernatant after TSA solidified. The plates were incubated at room temperature for 48 h and then the clear zone around colonies was examined. The clear area of inhibition was scored the same way as 4.2.1.

However, according to Coloretti's et al., (2007) research, not all the antifungal components produced by strains were released from cells, some peptidic components attach to the cell wall. There are two methods to release those peptidic compounds: (1) post fermentation to favor autolysis of cell wall and (2) heat treatment in order to break the cell wall. The inhibitory activity of compounds released during the post fermentative phase was determined by well-diffusion method. Isolates were allowed to grow at 32 °C for 30 days to favor autolysis, concentrated supernatant was prepared using the method described above. The drawback of post fermentation method is time consuming (had to wait at least 30

days). In order to shorten the whole process, the method of breaking cell wall by heat treatment was used. The 5-day incubated culture was heated in a 100 °C water bath for 15 min. The heated culture was inoculated (1 ml) onto LBS agar to check if all the cells were killed. Concentrated supernatant (15-fold) was prepared used the method described above.

4.2.3 Spectrum of antifungal activity

The molds, three *Penicillium* strains, *Aspergillus niger* and *Mucor niemalis*; and yeast, *Geotrichum* sp., *Rhodotorula* sp., *Candida albicans* and *Candida krusei* were target fungi. The overlay method described in 4.2.1 was used to determine the antifungal activity of our isolates. Inhibitory activity was scored as follow: –, no inhibition; +, very weak inhibition; ++, low inhibition with a little clear zone near the rim of the colony; +++, strong inhibition with a large clear zone near the rim of the colony; +++++, very strong inhibition with no growth near the rim of the colony.

4.3 Effect of different fermentation conditions

4.3.1 Growth curve of antifungal strains NB, DC2 and SDR

One percent of each strain was inoculated in MRS broth and incubated at 32°C, samples were taken after inoculation (time 0), and every 2 h for 24 h and 48 h for measurement of OD value (600 nm). The time of incubation was graphed as abscissa and OD value as ordinate to determine the growth curve.

4.3.2 Effect of temperature on growth of antifungal LAB

Inoculated anti-mold strain in MRS broth (1% v/v inoculum) were incubated at different temperatures, 4, 20, 32, 37 and 45 °C for 20 h. OD at 600 nm was measured after incubation at each temperature and graphed to determine growth curve. Blank MRS broth was used as reference.

4.3.3 Effect of pH on growth of antifungal LAB

The pH of MRS broth was adjusted to 2.5, 3.5, 4.5, 5.0, 5.5, 6.0, 6.3, 6.6, 8.5 and 9.5 using 1M HCl or 1 M NaOH. All the MRS broth were autoclaved at 121 °C for 15 min, cooled to room temperature and inoculated with 1% (v/v) anti-mold strains in MRS broth and incubated at 32°C for 20 h. OD₆₀₀ was measured after 20 hours. The pH of MRS broth was plotted on abscissa vs. OD₆₀₀ value was used as ordinate to determine growth curve at different pH values.

4.3.4 Effect of salt on growth of antifungal LAB

In the cheese industry, salt-in-moisture (S/M) is an important quality criterion. NaCl was added to MRS broth to give S/M levels of 0, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 (w/v %). The MRS broth was autoclaved at 121°C for 15 min, cooled to room temperature and inoculated with 1% (v/v) antifungal strains and incubated at 32°C for 20 h. The OD₆₀₀ was measured and growth curve determined by plotting S/M vs. OD₆₀₀.

4.4 Factors affecting antifungal activity of LAB

4.4.1 Heat resistance

Cell-free concentrated culture supernatant were heated at 100 °C for 15 min and the remaining anti-mold activity was determined by well-diffusion method (described in 4.2.2).

4.4.2 pH

The anti-mold activity of 15-fold concentrated supernatant of strains after pH was adjusted to 6.8 ± 0.2 (using 1M NaOH) was measured by well-diffusion method. The concentrated supernatant was also treated with catalase (Sigma, 1mg/ml) to eliminate the inhibitory activity by hydrogen peroxide.

4.4.3 Incubation time

Anti-mold strains NB, DC2 and SDR were inoculated in 100 ml MRS broth and incubated at 32°C. Samples were taken 17 h (log phase), 20 h (stationary phase), 24 h, 35 h, 48 h, 96 h, 120 h and 144 h for determination of anti-mold activity by the well-diffusion method. The cell-free extract from each strain was concentrated 15-fold before use for determination of anti-mold. Then a digital camera was used to record the results. The percentage of clear zone around the wells was measured by program ImageJ (ImageJ is a image processing program developed at the National Institutes of Health that used for image analysis). The area of clear zone and the area of Petri dish were measured separately. Percentage of clear zone (%) = (area of clear zone/ area of Petri dish plate) $\times 100\%$. The ratio of well by plate from 0.5 to 0.6% was considered no antifungal activity.

4.4.4 Incubation temperature

Strains were inoculated in 100 ml MRS broth and incubated at 10, 25, 32, 37, 45 and 55 °C for 5 days. Anti-mold activity of 15-fold concentrated supernatant was determined by well-diffusion method. The percentage of clear zone was measured by program ImageJ. The ratio of well by plate from 0.5 to 0.6% was considered no antifungal activity.

4.4.5 Proteolysis

The effect of proteolytic enzymes on antifungal activity of strains was determined with 10 ml aliquots of 15-fold-concentrated culture filtrate treated with one of the following proteolytic enzymes: proteinase K (Sigma, 1mg/ml) or trypsin (Sigma, 1mg/ml). Samples were adjusted with 1 M HCl and 2 M NaOH to the optimal pH value for each enzyme, 7.6, 7.6 for proteinase K and trypsin respectively. After adjustment of the pH, the supernatants were treated with 100 µl of the respective enzyme per ml and incubated at 37 °C for 1 h. Readjusted the pH of supernatant to the original pH and the determined the remaining antifungal activity by well-diffusion method. Both 15-fold concentrated MRS broth treated with enzymes and pH-adjusted 15-fold-concentrated samples served as controls (Magnusson and Schnurer, 2001).

4.5 Expression of anti-mold activity on milk and cheese

4.5.1 Growth of antifungal LAB in skim milk

Skim milk (10% w/v) was prepared and autoclaved at 110 °C for 15 min, cooled to room temperature and inoculated with anti-mold strains NB, DC2 and SDR, and incubated at 32 °C until coagulated. The time of first coagulation was recorded.

4.5.2 Growth of antifungal LAB in milk agar

Preparation of milk agar (milk-BCP agar): Ten grams of nonfat dry milk was dissolved in 70 ml distilled water (DI water) with a drop of antifoam. Agar (1.5 g) was suspended in 30 ml DI water in a separate flask, and 1.0 ml of Bromocresol purple (BCP) solution (0.1 g BCP in 25 ml DI water) was added, and mixed well. The milk and agar with BCP were autoclaved separately at 121 °C for 10 minutes, followed by tempering in a 48 °C water bath, before mixing together and poured into Petri dishes. The milk agar was allowed to stand for more than 1 h to obtain a firm and dry surface.

The milk agar was spotted with 3 µl of inoculum (10^9 cfu/ml) of anti-mold strains. The plates were incubated up side down at 32°C. After 5 days incubation, the plates were overlaid with semi-soft TSA containing 1 ml mold suspension (10^5 cfu/ml), and then incubated at room temperature until mold growth. Clear zone around colonies indicated mold inhibition.

4.5.3 Extract supernatant and determine antifungal activity when strains were grown in whey powder culture media (including heat treatment)

Preparation of whey powder culture media: whey powder (7 g) were dissolved in 100 ml tap water and centrifuged (4000 rpm/min) to remove undissolved portion. To the supernatant was added the following: 0.5 g of yeast extract, 0.5 g of non-fat dry milk, 0.3 g sodium citrate, 0.1 g $\text{Mg SO}_4 \cdot 7 \text{ H}_2\text{O}$, 0.1 g of MnSO_4 , 0.1 g of K_2HPO_4 and 1 ml 5% (w/v) Tween 80 solution, mixed thoroughly and autoclaved at 121 °C for 15 min.

The medium was cooled to 32°C and inoculated (1% inoculum) with anti-mold strains and incubated at 32 °C for 5 days. The supernatant was removed by centrifuge (4000 rpm/min), filter sterilized (45 µm filter) and concentrated 10-fold (by rotary evaporator). The concentrated supernatant was used for anti-mold activity assay by the well-diffusion method.

Effect of heat treatment of supernatant: The concentrated whey culture supernatants were heated to 100 °C for 15 min prior to being used for anti-mold activity assay by the well-diffusion method.

4.6 Anti-mold strains used in cheese making

4.6.1 NB used in cheese making

One loop of NB was transferred from MRS slant, and inoculated in 10 ml sterilized skim milk culture and incubated at 32 °C until the milk coagulated. One percent inoculum from coagulated skim milk culture was transferred into 10 ml MRS broth, incubated at 32°C for 18~20 h then transferred to another 10 ml MRS broth. Three transfers were done to insure purity. Subcultured 1% culture to skim milk and incubated at 32°C for 20 h. Cell density was then determined by plate

counting. The count result was used to determine the amount of anti-mold culture that added in cheese making.

4.6.2 Cheddar cheese manufacture

Cheddar cheese was made in California Polytechnic State University, Dairy Products Technology Center (DPTC) pilot plant from whole milk obtained from the Cal Poly dairy farm (San Luis Obispo, CA). The milk was pasteurized at 72°C for 15 s. Vats were cleaned and sanitized thoroughly to minimize cross contamination during manufacture of the cheese. The cultures used for cheese manufacture were obtained from Chr. Hansen, INC (Milwaukee, WI). DVS 850 (80×10^9 cfu/g) contained mesophilic strains of *Lactococcus lactis* ssp. *cremoris* and *Lactococcus lactis* ssp. *lactis* were used. Inoculation target of anti-mold strain NB was 10^5 cfu/ml in cheese milk and 10^6 cfu/g in cheese curd (after hand milling and before salting) in order to check the antifungal stains should be added at which step. Cheese curds without any antifungal strains were divided into 4 portions. Anti-mold strains NB, DC2 and SDR (10^6 cfu/g) were added in each portion separately. The last portion was used as control. There were only 2 levels of DC2 and SDR: without anti-mold culture and with anti-mold culture (DC2 or SDR) in curd, in order to check if DC2 and SDR have the ability of growing in cheese and inhibiting the mold growth.

Cheese was made according to standard Cheddar cheese making procedure. The milk was set at 88 °F (31 °C), annatto was added at 46ml/454 kg of milk. A 35% calcium chloride (Chr. Hansen's Lab) solution was added at 87ml/454 kg of milk. The rate at which starter culture was added is 1.092 grams/gallon. Chymosin

(Chymax, Chr. Hansen's Inc., Milwaukee, WI) was added 30 min after inoculation at the rate of 45ml/454 kg of milk. Chymosin was diluted 1:40 in cold water before addition to milk. The temperature of vat was kept at 88°F (31°C) for 30 min. The coagulation was cut using 5/8" wire knives and held for 5 min without stirring to allow the curd to heal. The curds were then cooked in the whey and the temperature was raised slowly from 88°F (31°C) to 102°F (39°C) within 30 min under constant stirring. Whey was drained when pH dropped to 6.1, and then cheddared the curd until pH of curd dropped to 5.5. Followed by hand milling, the curds were salted using NaCl (2.75% of curd) and hooped into 20-lb blocks. The cheese blocks were pressed under 42 psi over night at room temperature, and then were placed in plastic bags, vacuum-sealed, and ripened at 10°C. After one week, the 20-lb block was cut into 1 lb portions, vacuum packaged and stored in ripening room for future use.

Figure 2 shows a flow diagram of the general manufacturing protocol for Cheddar cheese. Due to limit time and material, only strain NB was added both in milk and curd, in order to check which step of adding antifungal strain would be better. Experimental cheese 1 (NB in milk) was made in a 50-gallon open vat, anti-mold strain NB was added in cheese milk 20 min after the addition of starter culture. Experimental cheese 2 (NB in curd) and control cheeses were made together in a 200-gallon size open vat. Cheese curds were split into 4 portions after hand milling and treated as follows. One of them was added NB strain (NB in curd), the other one was control, and no antifungal strains were added. DC2 and SDR were added in each

portion 5 minutes before salting. The 5 groups of cheese samples were labeled as follow:

- 1) Control: No anti-mold strain added;
- 2) Strain NB in milk: Strain NB was added in cheese milk;
- 3) Strain NB in curd: NB was added in curd 5 min before salting;
- 4) Strain DC2 in curd: DC2 added in curd 5 min before salting;
- 5) Strain SDR in curd: SDR was added in curd 5 min before salting

Figure 2. General protocol for Cheddar cheese manufacture

4.6.3 Microbiology analysis of cheese

Samples for bacteriological analysis were taken at 1 d, 1 week and 1 month post manufacture. A sample was aseptically taken from 1 lb cheddar block excluding trim by using a sanitized cheese trier and was placed into a sterile bag. Sampled blocks were then discarded. Number of lactobacillus (NB, DC2 and SDR culture) was estimated with selective media LBS agar after incubation at 32°C for 3 days.

4.6.4 Application of mold suspension on cheese surface

Mold suspensions (10^5 spores/ml) were prepared as previously described and diluted it to 10~20 spores per milliliter. The mold suspension was applied on the surface of 1-week-old cheese blocks of both the control and experimental cheeses. After the mold was added, all the samples were loosely packaged. Samples were prepared in triplicate. Similarly, three separate blocks of cheese, with no added mold spores were loosely sealed in vacuum bags. These cheese blocks were used as control to check the airborne mold contamination and if the antifungal stains can inhibit airborne mold growth. This also served as a control to check if mold spores would be able to grow on cheese surface. All the cheese blocks were placed back into the cheese ripening room and observed every other day after one week of mold inoculation. The date that each cheese block showed signs of mold growth was noted. One-month-old cheese samples were treated the same way as above.

5.0 RESULTS and DISCUSSION

5.1 Isolates of Lactic acid bacteria from food

A total of 15 lactic acid bacteria (LAB) strains were isolated from food sources and tested for their antifungal capacity. The bacterium was observed during the growth phase using overlay method. Six of the LAB were confirmed to have antifungal abilities and produced distinct halos in the Petri dishes with mold growth. These 6 strains were named NB, DC2, SDR, NS, DB and SDC. The bacteria included five *Lactobacillus plantarum* and one *Pediococcus pentosaceus* (SDC). All the strains were Gram positive and catalase negative (Table 5).

Table 5 Identification of six isolates

Name	Source	Gram-stain	Catalase test	Morphology	API 50CHL
NB	Broccoli	G+	Negative	Bacillus	<i>Lactobacillus plantarum</i>
DC2	Sharp cheddar	G+	Negative	Bacillus	<i>Lactobacillus plantarum</i>
SDR	Sourdough starter	G+	Negative	Long bacillus	<i>Lactobacillus plantarum</i>
NS	Sauerkraut	G+	Negative	Bacillus	<i>Lactobacillus plantarum</i>
DB	Soybean sprout	G+	Negative	Bacillus	<i>Lactobacillus plantarum</i>
SDC	Sourdough starter	G+	Negative	Cocci	<i>Pediococcus pentosaceus</i>

5.2 Antifungal activity assays

5.2.1 Overlay method

The concentrated cell suspensions (about 10^{13} cfu/ml, 3 μ l respectively) were spotted on MRS agar and incubated for 5 days with the overlay method. All 6 strains showed strong anti-mold activity (Fig. 3). The diameter of the colonies was 1.0 cm. Strain DB showed the largest clear zone. Strains NB, NS and SDR had similar anti-mold activity. Strains DC2 and SDC had weaker anti-mold activity.

Figure 3. Plates obtained using the overlay method (strain NB, DC2, SDR, NS, DB and SDC)

5.2.2 Inhibitory spectrum of the antifungal strains

All six strains had a broad antifungal inhibitory spectrum due to the ability to inhibit the growth of several taxonomic groups of mold and yeast (Table 6). Mold seemed to be more sensitive than yeast. Antifungal isolates had a strong inhibition to *Penicillium* sp., *Aspergillus niger*, *Mucor niemalis* and *Candida albicans*, and totally inhibited the growth of *Rhodotorula* sp. Antifungal isolates did not inhibit the growth of *Candida krusei* and *Saccharomyces cerevisiae*. Only DC2 and SDR showed a weak inhibition to *Geotrichum* sp.

Table 6 Inhibition of molds and yeasts by antifungal LAB

Fungi	Isolates NB	NS	DB	DC2	SDR	SDC
Mold						
<i>Penicillium</i> sp. (Cheddar)	+++	+++	++++	++	+++	++
<i>Penicillium</i> sp. (cottage cheese)	++++	+++	+++	++++	++++	+++
<i>Penicillium roqueforti</i>	+	+	+	+	+	+
<i>Aspergillus niger</i>	+++	+++	+++	++	+++	+++
<i>Mucor niemalis</i>	+++	++++	++++	+++	+++	++++
Yeast						
<i>Geotrichum</i> sp.	–	–	–	+	+	–
<i>Rhodotorula</i> sp.	++++	++++	++++	++++	++++	++++
<i>Candida albicans</i>	+++	++++	++++	+++	+++	++++
<i>Candida krusei</i>	–	–	–	–	–	–
<i>Saccharomyces cerevisiae</i>	–	–	–	–	–	–

Symbols: –, no inhibition; +, very weak inhibition; ++, low inhibition with a little clear zone near the rim of the colony; +++, strong inhibition with a large clear zone near the rim of the colony; +++++, very strong inhibition with no growth near the rim of the colony.

5.2.3 Well-diffusion method

Inhibitory activity of the supernatant that was produced during the growth phase (48 h incubation) and post fermentative phase (30 days incubation) was determined. Supernatant obtained 5 days after incubation of antifungal strains and thermally treated (100°C for 15 min, in order to release more antifungal components) was also determined by well-diffusion method.

Concentrated supernatant (15-fold) had a stronger anti-mold activity during the growth phase (48 h incubation). However after post fermentation (30 days), the inhibitory activity slightly increased (Fig. 4).

Figure 4 Plates obtained with supernatants of strains during growth phase (48 h) and post fermentative phase (30 days)

Antifungal cultures were incubated for 5 days and thermally treated (100°C for 15 min) to prepare a 15-fold concentrated supernatant. The inhibitory activity was determined by the well-diffusion method. The results showed that after the heat

treatment, anti-mold activity of the supernatant was stronger for all the dilutions compared to the samples without the heat treatment step (Fig. 5).

Figure 5. Plates obtained with supernatant of strains before and after heat treatment (100°C for 15 min)

5.3 Optimal fermentation conditions

Strain NB, DC2 and SDR were selected for further research due to their strong antifungal activity and also because of they represent the lactobacilli isolated from different sources. The growth curve, optimal growth temperature, optimal growth pH and the effect of salt in moisture percentage were determined for each stain.

5.3.1 Antifungal strain NB

NB was incubated under different temperatures, pH and salt levels of the MRS broth in order to determine the effect of different conditions for cell propagation. The optimal growth temperature for NB was 32°C. NB slightly grew at 4°C but increased in number as the temperature of 25°C was reached (Fig. 6). The cells decreased slightly at an incubation temperature above 32°C and decreased at 45°C. This suggests mesophilic property of strain NB. The optimal incubation pH

range was from 5 to 8.5. At pH 6.6, strain NB had the highest OD₆₀₀ value (Fig. 7). The growth rate slightly decreased when salt-in-moisture ratio was increased. The OD values were all above 2 (approximately 10⁹ cfu/ml), which did not change much from the samples that had no salt added (Fig. 8). The growth curve for strain NB at 32°C is shown in Fig. 9.

Figure 6. Effect of temperature on growth of antifungal strain NB

Figure 7. Effect of pH on growth of antifungal strain NB at 32°C

Figure 8. Effect of salt-in-moisture on growth of antifungal strain NB at 32°C

Figure 9. Growth curve of antifungal strain NB at 32°C

5.3.2 Antifungal strain DC2

Strain DC2 was incubated under different temperature, pH and salt level of the MRS broth in order to determine the effect of different conditions for cell propagation. The optimal growth temperature for strain DC2 was in the range 32 and 37 °C; it barely grew at 4 and 45 °C (Fig. 10). The OD-value increased rapidly when the incubation temperature was 30 °C, then sharply decreased at 45 °C (Fig. 10). The optimal growth pH range was from 5.5 to 8.5; at pH 6.6 had the highest OD value indicates high growth at pH 6.6 (Fig. 11). The growth rate slightly decreased when salt in moisture ratio was increased up to 2 % and then kept the same level from S/M 2.5 to 4.5 (Fig. 12). The growth curve for strain DC2 is shown in Fig. 13.

Figure 10. Effect of temperature on growth of strain DC2

Figure 11. Effect of pH on growth of strain DC2 at 32°C

Figure 12. Effect of salt-in-moisture on growth of antifungal strain DC2 at 32°C

Figure 13. Growth curve of antifungal strain DC2 at 32°C

5.3.3 Antifungal strain SDR

Strain SDR was incubated under different temperature, pH and salt level of the MRS broth in order to determine the effect of different conditions for cell propagation. The optimal growth temperature for SDR was between 32 and 37 °C; it barely grew at 4 and 45 °C (Fig. 14). The OD-value increased with temperature up to 30°C then sharply decreased at 45 °C. The OD-value increased with pH up to pH 8.5 then decreased to pH 9.5 (Fig. 15). The growth rate slightly decreased when salt-in-moisture ratio was increased (Fig. 16). Typical growth curve for strain SDR is given in Fig. 18.

Figure 14. Effect of temperature on growth of antifungal strain SDR

Figure 15. Effect of pH on growth of antifungal strain SDR at 32°C

Figure 16. Effect of salt in moisture on growth of antifungal strain SDR at 32°C

Figure 17. Growth curve of antifungal strain SDR at 32°C

5.4 Analysis of concentrated supernatant on antifungal activity

5.4.1 Heat treatment

The antifungal components in supernatant of NB, DC2 and SDR were found to be heat stable after heating at 100 °C for 15 min. The concentrated supernatant retained full inhibitory activity toward the target mold.

5.4.2 pH adjustment and catalase treatment

The activities of strains NB and SDR were partially lost (the inhibitory halo was not very clear) at a pH value of 6.8 ± 0.2 . However, the readjustment of the pH to the starting value (4.0) for the supernatant regained its ability to inhibit mold growth, indicated that the antifungal activity is acidic. The catalase treatment did not change the activity of the supernatants of strain NB, DC2 and SDR. The antifungal compounds found in the supernatant did not show any reaction to the hydrogen peroxide in the catalase treatment, which indicated that hydrogen peroxide was not the main factor to inhibit the growth of mold.

5.4.3 The influence of incubation time

Incubated samples were collected at eight specified times from the anti-mold strain broth. The samples were heat treated, filter sterilized (45 µm filter) and concentrated (15-fold) by rotary evaporator. The collected samples were from the late log phase (17 h), stationary phase (20 & 24 h), death phase (35 & 48 h) and late death phase (96, 120 and 144 h). Strain NB's antifungal activity of the supernatant slowly increased with the incubation time. The maximum antifungal activity was

obtained at the 144 h (6-d) in the incubator. For strain DC2, the antifungal activity increased rapidly from the 17 h to the 24 h and immediately decreased between the 35 and 96 h (4-d). The antifungal activity increased until the 120 h (5-d) was reached and again decreased. At the 24 h, strain DC2's antifungal activity decreased the most. The antifungal activity of strain SDR was similar to that of strain NB; both had stronger antifungal activity with longer incubation times. The only difference was that the SDR strain did not increase after the 120 h (5-d) incubation and was at the same antifungal level when the 144 incubation hours was reached (Fig. 18).

Figure 18. Influence of incubation time on antifungal activity of cell free extract

5.4.4 The influence of incubation temperature

NB, DC2 and SDR were incubated at different temperatures for 5 days and the antifungal activity of supernatant was determined by the well-diffusion method. The maximum antifungal activity for strains NB, DC2 and SDR was observed at

growth temperatures of 45, 25 and 37 °C, respectively. There was no antifungal activity at the growth temperature 10 or 55°C. Strain DC2 did not show any antifungal activity at 45°C (Fig. 19).

Figure 19. Influence of temperature on activity of supernatant
Percentage of clear zone at 0.5~0.6% was considered as no inhibition.

5.4.5 Effects of proteolytic enzymes on antifungal activity

The antifungal activity of concentrated supernatant of stain NB was sensitive to proteinase K and trypsin, suggesting that the antifungal compounds produced by strains NB are peptidic. However, DC2 was not sensitive to proteinase K or trypsin. SDR lost all the inhibitory activity after being treated with proteinase K but the strain did not lose any inhibitory activity with the treatment of trypsin.

5.5 Expression of antifungal activity on milk

5.5.1 The ability of coagulating skim milk

Strains NB, DC2 and SDR can grow in milk and produce acid, but not as fast as starter culture for cheese making. The strains coagulated skim milk at 32-37 °C in 2 days, at 55 °C within 24 h, and 6-7 days at a temperature of 21°C.

Table 7 The ability of strain NB, DC2 and SDR to coagulate skim milk

Strain	Time to coagulate milk at various temperature		
	21 °C	32~37 °C	55 °C
NB	6~7 days	48 h	18 h
DC2	6~7 days	48 h	18 h
SDR	5 days	48 h	18 h

5.5.2 Milk agar test

The six strains all showed certain inhibitory activity to the target mold (Fig. 20). Strains DC2 and SDR showed the strongest antifungal activity.

Figure 20. Antifungal activity of strains NB, DB, SDC, DC2, SDR and NS on milk agar

5.5.3 Antifungal activity of concentrated supernatant produced in whey medium

Concentrated whey (cell free extract) with NB, NS, DB, DC2 and SDR were determined to have antifungal activity by the well-diffusion method. Strain DC2 showed the strongest antifungal activity while strains SDR and DB's did not produce strong inhibitory activity compared to DC2. Cell free extracts (whey medium) of NB and NS had no antifungal activity. After being heat treated (100 °C for 15 min), all of the anti-mold components in the supernatants were deactivated (Fig. 21).

Figure 21. Antifungal activity of concentrated supernatant produced in whey medium culture. (a) before heat treatment; (b) after heat treatment (100 °C for 15 min)

5.6 Cheese making

5.6.1 Microbiology analysis of experimental cheeses

Lactobacilli selective culture medium was used to determine the number of of NB, DC2 and SDR strains in the cheese during ripening. For the control cheese, no lactobacilli were detected on the first day of cheese making or after a week of ripening. However, after the cheese ripened for a month at 10 °C, there was growth on 10^{-2} and 10^{-3} dilution plates, which suggest the growth of non-starter lactic acid

bacteria (NSLAB). It was difficult to compare NSLAB cell densities in control and experimental cheese by plate counts alone.

The expected cell count of strain NB in the cheese curd was 10^5 cfu/g. With the expectation that some of the added NB strain would be lost in the whey, the amount of NB added in the milk was calculated to be about 10^6 cfu/ml. After adding NB in the cheese milk, the milk sample had a plate count of 3.0×10^4 cfu/ml. When the same cheese milk was turned into curd, the curd sample resulted in the same level (3.0×10^4 cfu/ml) as the cheese milk sample. That means almost all the NB strain cells were attached to the casein gel and did not drain with the whey. By staying in the cheese system, the antifungal culture NB was able to propagate during cheese ripening. The cell number of strain NB in cheese reached to 10^7 cfu/g after a week. Strain NB grew fast in the first week of ripening, which was from 10^3 to 10^7 cfu/g. The growth rate steadily decreased after the first week.

The number of strain NB added in curd was calculated to be about 10^5 cfu/g. In fact, the cheese curd sample had cell density of 4.9×10^4 cfu/g, which was higher than the sample NB in milk. The plate count result was lower than expected which may be due to the strain NB lost during salting and pressing. The antifungal culture NB was not totally absorbed by the cheese curd. After salting, more whey was drained and some of the NB cells were drained with the whey at the same time, which explained that there was less strain NB in the “NB in curd” sample than in the “NB in milk” sample. The plate count result was lower than the “NB in milk” sample

after 1 week ripening. The growth of strain NB in the “NB in curd” sample was from 10^3 to 10^6 cfu/g in a week and another 1 log cycle more after 1 month ripening.

The plate count of DC2 and SDR in cheese curd was also determined. The plate count of DC2 started from 10^4 cfu/ml and reached 10^5 cfu/ml after 1 week of ripening, and 10^6 cfu/ml after 1 month. Comparing to DC2, SDR grew faster in the cheese system. The number of bacterium increased 2 log cycles after a week of ripening and another 2 log cycle after 1 month (Table 8).

Table 8 Plate counting results of antifungal cultures in cheese after 1 day, 1 week and 1 month in cheese curing room

Sample	1 day (cfu/g)	1 week (cfu/g)	1 month (cfu/g)
Control	0	0	$10^{-2,-3}$ big and small colonies
NB in milk	3.0×10^4	1.23×10^7	6.4×10^7
NB milk curd	3.1×10^4		
NB in curd	4.9×10^4	3.1×10^6	3.0×10^7
DC2 in curd	1.1×10^4	1.47×10^5	5.5×10^6
SDR in curd	7×10^3	3.20×10^5	2.2×10^7

5.6.2 Ability of inhibiting mold growth on cheese

5.6.2.1 Adding mold on 1-week-old cheese samples

a) Target mold spores were added on cheese surface.

A mold suspension (containing 10 to 20 spores) was applied evenly on the surface of 1-week of old cheese samples and loose. Mold was observed on both control and “NB in curd” samples after 14 days. The “NB in milk” sample did not show mold growth after mold addition indicating shelf life extension of 6 days longer than the control and “NB in curd” sample (Fig. 22). After 35 days of adding mold, samples of control, “NB in curd” were all covered by mold, while “NB in milk”

samples had only 3 mold spots on the surface of the cheese blocks (Fig. 23). “NB in milk” sample showed much better anti-mold activity than “NB in curd” sample.

“DC2 in curd” and “SDR in curd” samples got mold on the same day as control, suggesting they did not have anti-mold activity; the cheeses were all covered by mold after 35 days (figure not shown).

Table 9 Number of days for first mold on cheese inoculated with and without spores after 1 week of ripening

Sample	Mold inoculated (days)				No mold inoculated (days)			
	day 14	day 16	day 18	day 20	day 14	day 16	day 18	day 20
Control	+	+	+	+	+	+	+	+
NB in milk	–	–	–	+	–	–	–	+
NB in curd	+	+	+	+	–	+	+	+
DC2 in curd	+	+	+	+	–	+	+	+
SDR in curd	+	+	+	+	–	–	+	+

Symbol: “–” no mold growth was seen, “+” visible mold growth was seen.

The dimension of cheese block is 5×9.5×8.5 cm

Figure 22. Cheese samples showing visible mold growth after 20 d ripening. The picture was taken at the first day sample of NB in milk got mold, which is 6 days later versus control.

Figure 23. Cheese samples (control and NB in milk) 35 days after adding mold spores.

b) Target mold spores were not added, but was loose packaged

Cheese samples without the added mold were used as a control to check the mold spores from the plant environment (air, equipment and other possibly contamination sources). Also the group control was used as a control of the cheese samples without anti-mold strains but mold was applied on the surface in order to check if target mold spores could grow on the cheese surface. The “NB in curd” cheese sample had the ability of extending the shelf life 2 days longer than the control. “NB in milk sample” showed mold growth 6 days later than the control.

“DC2 in curd” cheese sample extended shelf life 2 days longer than control. “SDR in curd” sample showed mold growth 4 days later than the control. But according to the plate counts in the last section, “SDR in curd” cheese had a lower plate count than “NB and DC2 in curd” cheese. It indicates that SDR may have a better anti-mold activity than NB and DC2 when the bacterium was mixed in cheese curd (Fig. 24).

Figure 24. Cheese samples with no added mold spores in loose packages after 20 days. The picture was taken at the first day sample of NB in milk got mold.

5.6.2.2 Adding mold on 1-month-old cheese samples

a) Target mold spores were added on cheese surface.

The mold suspension (containing 10 to 20 spores) was applied evenly on the surface of 1-month old cheese samples and loosely. The control showed the growth of mold after 4 days. Compared to the control, all the experimental cheese samples for “NB in milk and curd” showed anti-mold activity. “NB in milk” cheese showed mold growth on the 17th day after adding mold, which showed strong inhibitory activity. “NB in curd” cheese showed mold growth after 7 days of adding the mold.

Even though “NB in milk” started growing mold after 17 days, the mold spots did not spread over the cheese. The control and “NB in curd” cheese were completely covered or at least half of the cheese was covered with mold after 23 days of ripening.

“DC2 in curd” cheese sample had a 4 day longer shelf life than the control. “SDR in curd” cheese got mold after 12 days after the control cheese got mold, which showed better anti-mold activity than “NB and DC2 in curd” (Fig. 25).

Table 10 Number of days for first mold on cheese inoculated with spores after 1 month of ripening

	day 4	day 7	day 12	day 15	day 17	day 23
Control	1~2 spot	17 spots	20 spots	39 spots	Mold spots getting bigger	50 spots and keep growing bigger
NB milk	-	-	-	-	2 small spots	18 small spots
NB curd	-	15 spots	20 spots and white mold spread out at least 1 sides of cheese block	25 spots and white mold spread out	Getting bigger	At least 3 sides covered by green mold
DC2 curd	-	20 spots	>30 spots	>30 spots, mold spread out 2 sides	Bigger	All covered by mold
SDR curd	-	-	2 spots	46 spots	Bigger	Bigger

Symbols: “-” no mold observed on surface of cheese. The dimension of cheese block is 5×9.5×8.5 cm

Figure 25. Cheese samples with mold spores after 23 days

b) Target mold spores were not added, but cheese blocks were loose packaged

In loosely packaged cheese without mold addition, the mold growth was observed on the control after 5 days. Addition of strain NB in curd resulted in

delayed mold appearance by 2 days. The mold spread quickly over the NB in curd block of cheese. The cheese in which strain NB was added to milk showed strong anti-mold activity because mold did not appear on the surface of the cheese until after 1 month ripening.

“DC2 and SDR in curd” showed mold growth after 15 days after mold was inoculated, which extended shelf life of 10 more days than the control (Fig. 26), and also have stronger anti-mold activity than “NB in curd” sample.

Table 11 Number of days for first mold on cheese loosely packaged after 1 month of ripening

	d 5	d 7	d 12	d 15	d 20	d 23
Control	1 spot	3 spots	6 spots	4 big spots and white mold spread out 2 sides	10 spots and mold covered one side	11 big spots, green & white mold spread out
NB in milk	–	–	–	–	–	–
NB in curd	–	10 spots	13 spots	9 spots and 2 corner covered by mold	>30 spots and 4 sides covered by white mold	Covered area getting bigger
DC2 curd	–	–	–	1 tiny spot	2 spots	4 spots (small)
SDR curd	–	–	–	2 tiny spots	5 spots and one black mold spot	6 spots and white mold spread out on half side of the block

Symbols: “-” no mold observed on surface of cheese. The dimension of cheese block is 5×9.5×8.5 cm

Figure 26. Cheese samples (without mold spores) loosely packaged after 23 days.

The results above show that mold growth was slower in 1-month-old inoculated with mold spores than 1-week-old cheese, suggesting that more anti-mold compounds were produced and accumulated in a 1-month-old cheese than 1-week-old cheese

6.0 DISCUSSIONS AND CONCLUSION

The aim of this study was to develop a new protective culture based on newly isolated lactobacilli with a focus on the suppression of molds and yeasts in Cheddar cheese. Extensive research has been done on antagonistic lactic acid bacteria and the antifungal compounds that are produced in fermentation media. However, there is little or no information on actual application of lactic acid bacteria in cheese systems for inhibiting mold growth. In this study, the LAB strains were isolated from food sources and screened for antifungal activity. The anti-mold activity and physicochemical characteristics of supernatant produced by these isolates were determined and the most important aspect was the application of anti-mold strains to the cheese making process.

6.1 Antifungal isolates and spectrum of antifungal activity

Lactic acid bacterium naturally exists in vegetables, fermented food and dairy products, but not all of the bacteria have antifungal activity. After screening several food sources for lactic acid bacteria, six strains with antifungal activities were isolated, identified and their growth properties studied. The target mold used was *P. commune* isolated from Cheddar cheese.

Both molds and yeasts are important spoilage organisms in different food systems. The molds evaluated in this study, *P. roqueforti* and *P. commune*, commonly spoil hard cheese, while the yeasts *Candida albicans* and *Rhodotorula* sp. were usually found in soft cheese such as cottage cheese (Pitt and Hocking, 2009). LAB

are known to produce antimicrobial substances, which are mainly in the form of organic acids and bacteriocins. Bacteriocins inhibit the growth of similar or closely related bacterial strains. They are typically considered to be narrow spectrum antibiotics and do not have big effects on the growth of molds and yeasts. This study showed that the six antifungal strains all have a wide antifungal spectrum. Antifungal strains strongly inhibit all the 5 molds in this study, such as *Penicillium* sp., *Aspergillus niger* and *Mucor niemalis*, which are common contaminants in the food industry. Two out of five yeasts, *Rhodotorula* sp. and *Candida albicans* were strongly inhibited by the antifungal isolates. Only strains DC2 and SDR slightly inhibit the growth of *Geotrichum* sp., which is a mold like yeast.

Generally, antifungal strains inhibit a broad range of filamentous fungi (mold) and, to a lesser extent, spoilage yeasts. However, for the specific yeast *Rhodotorula* sp., its growth was totally inhibited by the antifungal strains. This result indicated a potential use of antifungal strains in cottage cheese, which is easier to be contaminated by *Rhodotorula* sp.

6.2 Anti-mold activity of supernatant and the physicochemical characteristics of compounds produce during fermentation

Strains NB, DC2 and SDR were selected for further research, due to their marked inhibitory activity.

The concentrated supernatant of stain NB, DC2 and SDR had anti-mold activity. However, the supernatant concentrates obtained after fermentation for 30 days and thermally treated supernatant showed better inhibitory activity than

supernatant obtained after fermentation for 48 h. This indicated that more anti-mold compounds were released during post fermentation and thermal treatment. Thirty days incubation allowed the cells to totally autolysis so the compounds were released and inhibitory activity increased. This was also observed in previous studies (Coloretti et al., 2007). But using 30 days of incubation would be too long to favor cell autolysis. Therefore, the thermal treated method was used to release more anti-mold compounds. Before using this method, the cell free supernatant of the antifungal strains were tested to make sure the antifungal compounds in the supernatant were heat stable (see 5.4.1). By heating culture to 100 °C for 15 min, the cell wall of bacteria was broken. Consequently, the antifungal compounds were released into the broth. The thermally treated method was used in all the other well-diffusion method.

The production of antifungal substances from 3 strains will be discussed separately below.

Strain NB: the factors that affected the antifungal activity compounds were determined by the incubation time, incubation temperature and effective pH. The production of the antifungal substance from NB started during the exponential growth phase (17 h) and slowly accumulated until the substance reached a maximum activity in the sixth day of incubation (death phase). This result does not agree with Corsetti, et al.'s (1996) who found that the antibacterial compounds produced by *Lb. sanfrancisco* C57 increased progressively during 8~36 h (stationary growth phase) and decreased after 72 h at 28 °C. When strain NB was incubated at

different temperatures, the maximum antifungal activity was observed at 45°C. However, 45°C was not the optimal temperature for strain NB cell propagation (optimal growth temperature was 32°C). This result indicates that even though strain NB reached maximum cell growth at 32°C, the production of antifungal compounds did not reach the maximum levels until 45 °C. A higher incubation temperature may stimulate the production of secondary metabolism, which most likely were antifungal compounds. The active antifungal substances were found to be heat stable, sensitive to proteinase K and trypsin. This result indicates that the main antifungal substance is a peptide. In Corsetti, et al. (1996) also found that *Lb. plantarum* isolated from sourdough culture was sensitive to proteinase K and trypsin. The antifungal substances were only active at a low pH. When pH was adjusted to 6.8±0.2, very weak antifungal activity was observed but when adjusted the pH to 4, the antifungal activity was fully retained. A mixture of organic acid was reported as the main inhibitory substances produced by *Lb. sanfrancisco* CB1. The acids include acetic, caproic, formic, propionic, butyric and n-valeric acids, that acted synergistically in inhibiting mold growth (Corsetti et al., 1998). Similar results were also found by Magnusson and Schnurer (2001). The main antifungal substances produced by strain NB were possibly be organic acids and peptides that were both heat stable and pH dependent.

For the supernatant produced by antifungal strain DC2, the production of antifungal compounds reached the maximum level at 25 °C. The growth curve for strain DC2 was different from NB. Strain DC2 had the ability of producing acid in 24 h. The first peak of antifungal activity was observed after 24 h of incubation but

progressively decreased in activity was observed from 24 to 96 h incubation (stationary and death phase). This result agreed with the research of Corsetti, Gobbetti et al. (1996). The maximum level of bacteriocin produced may occur during different phases of the growth cycle: streptocin STH1 production had the highest level during the exponential growth phase (Schlegel and Slade, 1973), while the production of staphylococcin C55 started in the late logarithmic phase and reached the maximum bacteriocin level between 24 and 48 hours. Similar results were found for the bacteriocins amylovorin L471 from *Lactobacillus amylovorus* (De Vuyst et al., 1996) and Lactocin S from *Lactobacillus sake* (Mortvedt-Abildgaard et al., 1995). However, the antifungal activity of strain DC2 increased again after 5 days of incubation. The proteolytic enzymes test showed that the supernatant of DC2 was insensitive to proteinase K or trypsin, excluding the possibility that the compounds have a peptidic nature. The compounds were, however, sensitive to changes in pH. When the pH of concentrated supernatant was adjusted to 6.8 ± 0.2 , most of the antifungal activity was lost, but still showed weak inhibitory activity. So the antifungal compounds produced by DC2 may be heat stable organic acids, a mixture of fatty acid and bacteriocin. Recent studies (Lavermicocca et al., 2000) confirmed that the inhibitory activity of *L. plantarum* could be attributed to the organic acids phenyl-lactate and 4-hydroxy-phenyl-lactate. This result was also confirmed by Coloretto et al. (2007). The reason that antifungal activity of supernatant declined after 24 h of incubation but increased again after 5 days of incubation may be due to the digestion of organic acids and accumulation of secondary metabolism. Alternatively, the antifungal substances might be highly

hydrophobic that rapidly adsorbs to the producer cells or forms spontaneous aggregates, which has been suggested for the bacteriocin amylovorin L471 and Lactocin S (Magnusson and Schnurer, 2001). Therefore, when the cell wall of DC2 was destroyed by heat treatment, those antifungal compounds were released into the broth, thereby increasing antifungal activity.

Antifungal strain SDR produced most of the inhibitory compounds at 37°C. The concentrated supernatant of SDR had anti-mold activity after 17 h of incubation, but much lower activity compared to strains NB and DC2. However, when the incubation time increased, the antifungal activity of supernatant increased and reached the maximum level after 5 days incubation. The antifungal compounds produced by strain SDR were pH related and sensitive to proteinase K. After the pH of the supernatant was adjusted to a neutral pH, antifungal activity was partially lost but not as much as strain DC2. However, the antifungal activity was totally lost after the supernatant was treated with proteinase K. The above findings indicate that the inhibitory compounds produced by SDR were peptides; and cell lysis results in the release of more antifungal compounds, as suggested by Coloretti, et al. (2007). Organic acids played an important role in inhibiting fungal growth as well.

6.3 Cheese making experiment

In the past 50 years, a lot of research has been done on isolating the antimicrobial LAB and identifying the inhibitory compounds. However, very few papers have addressed their application in the food industry. Furthermore there is little or no research report on the use of antifungal LAB in cheese making. In this

project, a preliminary application study was conducted on Cheddar cheese making. The anti-mold activity of strain NB inoculated cheese was positive.

Before using the antifungal strains NB, DC2 and SDR in cheese making, they were inoculated in milk agar and whey medium to check if there was any growth in a dairy base. Results showed that antifungal strains NB, DC2 and SDR metabolize lactose and can produce antifungal compounds in this matrix.

In Cheddar cheese making, strain NB was added to cheese milk (“NB in milk”) or to curd before salting (“NB in curd”). Control vat of cheese was made without adding strain NB. Both control and experimental cheese samples were made with the same procedures. Selective medium agar was used to determine the plate count of the cheese samples and only lactobacilli could be detected. Strain NB was able to grow in the cheese system, due to the plate count of the experimental samples (both “NB in milk” and “NB in curd”) increasing from 10^4 to 10^7 cfu/g in a month during ripening at 10 °C. Similar results were found by Lynch, et al. (1999) who reported that *Lactobacilli plantarum* grew in Cheddar cheese at a low temperature and low pH while the growth of the starter bacteria was delayed or stopped due to low pH. This suggests that lactobacilli are present in cheese during the late phase of fermentation. For “NB in milk” sample, when the cheese milk was turned into curd, the plate count of strain NB in the curd was at the same level as added to the cheese milk, indicating that NB was well retained in the cheese matrix with minimal losses in the whey. The entrapment of strain NB in the curd allowed it to keep growing to produce antifungal compounds during ripening. Comparing “NB in curd” sample to

“NB in milk”, the cell densities of the former was higher than “NB in milk”. However, growth of strain NB when added to the curd (“NB in curd”) was slow. It might due to salting procedure 5 min after adding NB in the curd. The salt may have inhibited the growth of NB or during salting. Also some of the bacteria may have been lost in the whey during processing.

After one week, the plate count of “NB in milk” was 10^7 cfu/g and “NB in curd” was 10^6 cfu/g. Indicator mold spore suspension (10~20 spores) was applied on the surface of a cheese block. Control and “NB in curd” showed mold growth at the same time, while “NB in milk” sample showed mold growth 6 days later than control. The “NB in milk” sample showed better anti-mold activity than NB in curd. Two reasons that may explain this situation. The first reason is that the level of bacterial cells, 10^7 cfu/ml, should be the minimum plate count for inhibiting mold growth; cell densities lower than 10^7 cfu/ml have overall weak anti-mold activity. The second reason might be adding NB to curd caused cell injury and inhibition during salting, so that the NB cells lost the ability of producing antifungal compounds. When mold spore suspension was added after being ripened for one month, the anti-mold activity increased. For “NB in milk” sample, the shelf life extended was 13 days more than the control. For NB in curd sample the shelf life was extended by 3 days more than control cheese. The plate counts of NB in curd reached 10^7 cfu/ml and at the same time attained the ability of inhibiting mold growth in cheese, which confirmed the assumption above that 10^7 cfu/ml was the minimum level needed to inhibit mold. Extending ripening time enabled the experimental cheese to have a stronger anti-mold activity.

The same control and experimental cheeses were opened and loosely packed after one week and one month ripening (the same day with mold added samples). The only difference was no mold was added in those cheese samples in order to check if the mold spores were able to grow on the cheese (as a control of mold growth) and also could tell if there is a possibility of airborne mold spores contaminants during cheese manufacture. The control cheeses with and without mold additions were observed to have mold growth on the same day. The mold that grew on those samples was determined by the plate observation as the same strain as the target mold. This shows that cheese is easily contaminated by mold spores during manufacture, especially in open cheese vat. When there were spores in cheese, even at low levels, contaminating mold was able to grow within a short period with availability of O₂ in package. But due to lower mold spores contamination, in 1-week old “NB in curd” cheese, the shelf life was extended 2 days longer compared to the control. For “NB in milk” sample, the shelf life was extended 6 days longer than the control. The “NB in milk” samples opened and loosely packaged after one month showed an excellent anti-mold activity. No mold growth was observed after 30 days (the end of observation), while control and NB in curd samples were already completely covered or half covered by mold.

It can be concluded that adding NB to milk gave higher cells densities in cheese and stronger anti-mold activity than adding strain NB to curd. When the cheese curd was salted, the NB cells were well protected in the curd and would not be dispersed and drained with whey.

Besides NB, DC2 and SDR were also added in cheese curd in order to check their anti-mold activity in cheese (preliminary study). The plate counts were low for DC2 (10^5 cfu/ml) and SDR (10^5 cfu/ml) after 1 week ripening. In consequence, no anti-mold activity was shown. The experimented cheeses got mold at the same time as the control. However, after one month ripening, both strains DC2 and SDR in curd samples had the ability of inhibiting mold growth. The plate counts of SDR in curd were 10^7 cfu/ml after one month, and the shelf life was extended 8 days compared to the control. The DC2 in curd (10^6 cfu/ml) had a 3-day shelf life extension compared to the control.

By using antifungal strains in cheese making, the longer ripening time, the higher counts of cells, and the better anti-mold ability was shown.

8.0 Conclusions

In the cheese industry, fungal contamination during cheese manufacture is a major problem that causes huge economic loss each year worldwide. In order to prevent fungi and other food-borne contamination while providing a clean label, more and more research has focused on natural ways of preventing or inhibiting mold growth. LAB antimicrobial compounds have been identified but very little work has been done on their application in cheese.

In this thesis, six antifungal strains were isolated, five were *Lb. plantarum* and one was *Pediococcus pentosaceus*. Three of *Lb. plantarum* strains NB, DC2 and SDR (*Lactobacillus plantarum*) were selected to do further research. The spectrum of antifungal activity was determined, 5 molds and 2 yeasts were inhibited by antifungal strains. The optimal temperature (32 °C), pH (neutral) and the effective salt in moisture (tolerant at 4.5% S/M) of growth of NB, DC2 and SDR were determined.

After screening for antifungal activity of specific strains, concentrated supernatants (15-fold) of the strains NB, DC2 and SDR were used to study the properties of the antifungal compounds by the well-diffusion method. The antifungal compounds produced by strains NB, DC2 and SDR were pH sensitive (active at low pH), heat-stable (100°C for 15 min), insensitive to a catalase and attached to the cell wall. The main factor may be related to the organic acid and bacteriocin. For strains NB and SDR, peptides may play an inhibitory role as well.

The antifungal stains NB, DC2 and SDR were applied in cheese making. Adding strain NB in cheese milk showed that there was a higher count of bacterium and stronger anti-mold activity than adding NB in curd (before salting). Besides, the longer ripening time, a stronger inhibitory activity was observed. For the challenge test (10~20 spores were added on cheese surface), “NB in milk” sample (1-week-old) was able to extend the shelf life by 6 days compared to the control and an extension of 13 days if the mold was added after one month of ripening. For the loosely packaged cheese samples (no mold was added), NB in milk sample did not get mold throughout the experiment (30 days after adding mold). DC2 and SDR in curd also showed different inhibitory activity against mold growth on cheese.

8.0 RECOMMENDATIONS FOR FUTURE WORK

- (1) Isolate molds and yeast samples from the air, equipment and processors of a cheese factory. Do a spectrum of antifungal activity by using those fungi. This will target the main contaminants in cheese industry.
- (2) Identify antifungal strains by sequence analysis of 16 S ribosomal DNA (rDNA).
- (3) Use HPLC, GC-MS, and gel filtration methods to identify the antifungal compounds produced by antifungal strains.
- (4) Design an experiment on cheese making, determine which strain will be suitable to be used in cheese making; how many cells and by which step should be added in; is that possible for antifungal strains inhibit other fungi growth on cheese.
- (5) Do a sensory analysis on cheese with antifungal strains.
- (6) Try to use antifungal strains in other dairy products such as soft cheeses and yogurt.

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