PREPARATION, PRODUCTION AND INDUSTRIAL APPLICATION OF CHEESE PROTECTIVE CULTURES

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ABSTRACT

PREPARATION, PRODUCTION AND INDUSTRIAL APPLICATION OF CHEESE PROTECTIVE CULTURES

Cheese industry is always under the risk of microbiological contaminations. These contaminants can be either bacteria or fungal species; both affect the final product quality and acceptance. Globally, 20% of the cheese produced industrially is lost due to microbial contaminations.

Fungal contaminations can be overcome by the usage of chemicals; sorbate, benzoate, natamycine, propionate, etc. or by natural biopreservatives such as protective cultures or some metabolites of antifungal cultures. Chemical preservatives have a legal limit for their usage and these limits are generally not effective to prevent the product from fungal growth. Also consumers demand goes toward the clean-labeled products and force the manufacturers to use bioprotectants if necessary. There are many types of bioprotectants commercially available with defined lactic acid bacteria combinations which are not so effective for some fungal species due to the species that they include.

In this study, several lactic acid bacteria (LAB) strain has been selected to prepare efficient protective culture combinations e.g. *Lactobacillus rhamnosus, Lactobacillus plantarum* and *Lactobacillus paracasei* spp. *paracasei*. The maximum antifungal effect was determined by using mixed culture combination of lactic acid bacteria strains with equal volume. In cheese applications, $3x10^8$ cfu/ml antifungal bacteria mixture including; *Lactobacillus rhamnosus, Lactobacillus plantarum* and *Lactobacillus paracasei* spp. *paracasei*, was inoculated into the curd before fermentation and found more effective than surface applications to the final products. In yoghurt production, same antifungal lactic acid bacteria mixture was found to be effective when applied before fermentation with 10^6 cfu/ml initial cell counts.

ÖZET

PEYNİR KORUYUCU KÜLTÜRLERİNİN HAZIRLANMASI, ÜRETİMİ VE ENDÜSTRİYEL UYGULAMASI

Endüstriyel peynir üretimi prosesin doğası gereği devamlı mikrobiyolojik risk altındadır. Mikrobiyolojik kontaminasyonlar bakteriyel kaynaklı olabildiği gibi küf veya maya kaynaklı da olabilmektedir. Tüm dünyada üretilen peynirlerin yaklaşık % 20'si mikrobiyal kontaminasyonlar sebebi ile tüketilmeden imha edilmektedir ve büyük ekonomik kayıplara sebep olmaktadır.

Bu çalışmada küf ve maya kontaminasyonları üzerinde yoğunlaşmakla beraber, kimyasal koruyucular, biyo-koruyucular ile karşılaştırmalar yapılmış ve yeterince etkili olmadığı bulgulanmıştır. Kimyasal koruyucuların, legal limitlerin üzerine çıkıldığında etkili olduğu tespit edilmiştir. Ancak hem tüketici taleplerinin kimyasal kullanımından ziyade doğal metodlardan yana olması hem de kimyasalların artan konsantrasyonlarının olası sağlık etkileri sebebi ile mikrobiyolojik ajanlar ile koruma metodları önem kazanmıştır.

Çalışmada farklı laktik asit bakterileri içinden, *Lactobacillus rhamnosus, Lactobacillus plantarum* ve *Lactobacillus paracasei* spp. *paracasei*. suşları antifungal kültür kombinasyonu için seçilmiştir. En yüksek antifungal aktivite kültürlerin eşit hacimdeki karışımından elde edilmiştir. Peynirde fermentasyon öncesi uygulamada, $3x10^8$ kob/ml *Lactobacillus rhamnosus, Lactobacillus plantarum* and *Lactobacillus paracasei* spp. *paracasei*, bakterileri karışımı etkin bulunurken, son ürün uygulamalarında etkinin azaldığı gözlemlenmiştir. Yoğurt üretiminde aynı kültür kombinasyonunun 10^6 kob/ml başlangıç hücre sayısında kullanımında yeterli antifungal etki elde edilmiştir.

Dedicated to Arda ŞATANA

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CHAPTER 1

INTRODUCTION

Since prehistoric times; milk and dairy products play an important role related to their nutritive value and health benefits. People have aimed to keep the milk without spoilage and with its own nutritive properties; therefore fermentation process began to gaining more importance.

Cheese, yoghurt, quark, butter, etc. are the main types of dairy products. They are produced from milk by fermentation using starter cultures. Fermented dairy products are produced by using various types of lactic acid starter cultures; mainly *Lactococcus lactis* ssp.*lactis, Lactococcus lactis* ssp. *cremoris, Lactobacillus casei* ssp. *casei, Lactobacillus rhamnosus, Lactococcus lactis* ssp.*lactis* biovar *diacetylactis, Lactobacillus helveticus*, *Leuconostoc mesenteroides* ssp.*cremoris*, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* depending on the product being manufactured.

Microbial flora of the dairy products includes starter and/or adjunct culture and the contaminating microflora. Contamination can occur during processing, handling or storage due to the type of the contaminant flora.

The microbial diversity of a contaminated dairy product may include bacteria or fungal microorganisms. Various types of undesired microorganisms may enter dairy products (cheese, yoghurt, quark are included in this study) during manufacturing and handling. These microorganisms are mainly reported as coliforms, *Eschericia coli, Staphylococcus aureus, Salmonella* ssp., *Listeria monocytogenes, Clostridium perfringens* and many undesired yeast or mold species (Brooks et. al., 2012). In order to prevent the growth of undesired microflora during cheese manufacturing and storage industrially characterized protective cultures, chemical protectants, well designed controlling systems should be used.

There are many types of industrially used protective cultures. Prevention of growth of spore forming bacteria could be achieved by protective cultures, including

Lactobacillus rhamnosus. The commercially available protective cultures against spore forming bacteria generally include *Lactobacillus rhamnosus*. In order to protect cheese from yeast and mold contaminations and prevent the growth of yeast, mold species, protective culture combinations including the species *Propionibacterium freudenreichii* ssp. *shermanii, Propionibacterium jensenii, Lactobacillus rhamnosus, Lactobacillus paracasei* ssp.*paracasei* can be used. Additionally *Lactobacillus plantarum* is generally recommended to protect cheese from gram positive pathogens and fungal contaminations (Varsha and Nampoothiri, 2016).

Even the protective cultures are used as recommended; there could be some quality defects related to the growth of undesired microorganisms in the dairy product. The commercially available antifungal cultures have effects on certain fungal cultures; however the fungal flora of dairy can differ among the product and the globally known flora can be changed. As a result, different culture combination is required for dairy products to keep the product with only desired microflora.

Laref and Guessas had mentioned in their studies about the usage of lactic acid bacteria as protective culture chronologically. They had stated that the studies began to be published firstly at 1958 in which *Lactobacillus acidophilus* was tested against *Candida albicans*. The second study was performed in 1963 to examine the antifungal effects of *Leuconostoc* against yeast cultures. Laref and Guessas have also mentioned about the first study in the literature related to cell free supernatant usage (Laref and Guessas, 2013). On these days there are now many studies according to the increased demand for chemical additive free products.

The main objective of this study is to examine the effects of lactic acid bacteria having antifungal characteristics on undesired fungal species. Within this perspective, aims include to improve their protective power by combining different lactic acid bacteria species. Their influences on some spoiler fungal species were examined by applying these cultures to different type of dairy products such as cheese and yoghurt and by monitoring them during the shelf life.

There are many types of commercially available protective cultures or chemical preservatives with legal usage limitations. The commercial protective cultures are effective on certain fungal species that are generally defined by supplier with antifungal effect spectrum studies. However, dairy contaminants may vary among the type of product, process characteristics, flora of the dairy plant and somehow commercial protective culture usage could be inefficient due to undesirable contaminations.

Additionally using chemical preservatives should not be a choice for producers. The consumers' demands are changed into clean labeled products and promote the producer to use biopreservatives.

This study is focused on the usage of lactic acid bacteria as biopreservative in cheese production. For this purpose, *Lactobacillus paracasei* spp. *paracasei, Lactobacillus rhamnosus, Lactobacillus plantarum* were used against *Debaryomyces hansenii, Pichia membranifaciens, Yarrowia lipolytica, Kluyveromyces marxianus, Geotrichum candidum* and *Penicillium communee* species. The tested fungal species and lactic acid bacteria as antifungal agents have been chosen according to the literature which will be explained in later sections.

The effect was first determined with *in vitro* studies, then *in vivo* studies in product matrix. Finally the usage of designed antifungal culture were tested for the shelf life effect on cheese and the effect on other dairy products; e.g. strained yoghurt, set yoghurt and kashkaval cheese.

CHAPTER 2

CHEESE PROCESSING

White cheese can be classified as soft-cheese related to its water content which is 55-65 g/100g at the beginning. Cheese can be classified in 4 classes, according to its fat in dry matter (FDM); full fat cheese with 45%FDM, semi-skimmed with 30-44 % FDM, low-fat cheese with 20-29% FDM and fatless with <20% FDM (Hayaloglu et al., 2002).

The white brined cheese is a traditional cheese type for Turkey, can be produced from sheep's, cow's or goat's milk. The unavailability for sufficient amounts of sheep or goat milk, generally cow's milk or the mixtures of milks are used (Hayaloglu et al., 2002). According to the TUİK report published in 2018, in the year of 2017, 688061 tons cheese were produced industrially and 96% of this total corresponded to cheeses made from cows' milk (TUİK, 2018).

The cheese production firstly includes the selection of characteristics of the final product. The characteristics of cheese including textural properties, physical parameters, sensory characteristics have to be well designed for further steps. The remaining part of production includes the developing of the process parameters related to the characteristics of cheese. The developed process line has to provide the final products with same quality and sensory characteristics.

Manufacturing the cheese has a very complex nature due to many chemical, physical and microbiological characteristics. Cheese making could be assumed as a concentration process of milk nutritive values. It may be named as a coagulation reaction of milk proteins. The reaction among casein proteins would cause many different properties. Johnson and Lucey have stated that; the interactions among casein micelles might be effected by the pH, solubility of colloidal casein molecules, proteolytic and lipolytic reactions, temperature, humidity and milk contents as milk fat, dry matter, casein, stability of casein, etc. (Johnson and Lucey, 2006). The main parameters to achieve the designed product depends on firstly the milk nutritive composition and secondly the acid development time from cheese milk.

2.1. Stages of Cheese Making

As mentioned before cheese production is generally named as a coagulation process and there are different ways to obtain a good quality gel network in cheese milk during coagulation step. The most frequent three ways are; enzyme usage to obtain destabilized casein micelles and a gel network from its aggregates, decreasing the pH to result in acid coagulated cheese gel network (mainly used in cream cheese and cottage cheese) without any enzymatic reaction by reducing the repulsive charge differences between micelles and combining the acid addition with heat treatments (Law and Tamime, 2010).

Coagulation step is followed by removal of whey from coagulant and the obtained curd is subjected to efficient fermentation step.

2.1.1. Standardization of Milk

The first important step is to start with milk of consistent composition to obtain the final product with desired physical and chemical characteristics. As a result of having non standardized milk, the further steps will not result in the cheese with same standards for all productions.

The milk dry matter or milk fat can be standardized by using milk solids like skimmed milk powder, cream, skimmed milk or by removal of milk cream.

If the product is assumed to contain fat in dry matter value between 50 and 55g per 100g, the process would not require the addition or removal steps. When the desired fat in dry matter is desired to be higher than 50-55 g/100g, some additions like milk powder, skimmed milk powder, condensed skim milk or removal of cream should be performed.

The raw milk accepted by the farm has to fulfill the quality criteria of the farm (antibiotic free, somatic cell and total aerobic mesophilic cell counts, culture tests, etc.) and has to be taken at +4-6 °C to the process which will then be pasteurized at 80-85°C for 2-3 s or at 63°C for 30 min. or at 65°C for 5 min. Some farms use double pasteurization in order to minimize the microbial risks.

2.1.1. Heat Treatment of Milk

Heat treatment is the second important step after standardization to obtain the cheese with desired microbiological quality. It is performed due to the inactivation of pathogenic microorganisms firstly and to increase the cheese yield by denaturing milk proteins (Law and Tamime, 2010).

This process involves heating at 72-75 °C for 15-30 seconds in a continuous flow plate heat exchanger. According to Hickey, heat treatments cause the inactivation of the enzymes naturally occurring milk, coming from lactic acid bacteria. The inactivation of enzymes would lead to prevent undesired sensory profile production in final cheese, and these enzymes are generally lipolytic and proteolytic (Hickey et al, 2007).

2.1.2. Addition of Starter Culture

The preservation method of milk without and UHT process involves generally fermentation step. Fermentation of milk is related to the conversion of lactose into organic acids, mainly lactic acid which can be performed by lactic acid bacteria. They are very common in environment and generally found in foods with high nutritive values (Tunail and Kosker, 1986).

The production of cheese involves complex interactions between milk, rennet and bacteria. However, most cheese is made from pasteurized milk, the only bacteria to be considered is namely starter culture bacteria which is used to reduce pH and give desired quality properties like taste, texture, aroma, etc.

Starter cultures can be produced in the dairy or purchased from a commercial starter culture supplier. Historically, starter cultures were produced in dairy industry using liquid cultures either propagated by the dairy or supplied by local culture producers.

Table 2.1. includes the starter culture types in two classes; thermophilic and mesophilic and the cheese type with lactic acid bacteria used in that cheese. The

difference between starter cultures results in different aroma and texture profiles among cheese varieties.

Table.2.1. Culture Types and Lactic Acid Bacteria Used in Cheese Manufacturing (Source: Tamime et al., 2005)

CULTURE TYPE	SPECIES NAME	CHEESE APPLIED
A. Mesophilic		
O type	*Lactococcus lactis* ssp.*lactis* *Lactococcus lactis* ssp. *cremoris*	Cheddar, Feta, Cottage
LD type	*Lactococcus lactis* ssp.*lactis* *Lactococcus lactis* ssp. *cremoris* *Lactococcus lactis* ssp.*lactis* biovar *diacetylactis* *Leuconostoc mesenteroides* ssp.*cremoris*	Gouda, Tilsitter, mould ripened soft cheeses
B. Thermophilic		
Streptococcus .type	*Streptococcus thermophilus*	Mozarella, Brie, Swiss
Yoghurt type	*Streptococcus thermophilus* *Lactobacillus delbrueckii* ssp.*bulgaricus*	Mozarella, Pizza
Lactobacillus type	*Lactobacillus helveticus* *Lactobacillus delbrueckii* ssp.*lactis*	Swiss, Grana
Mixed Types		
RST Type	*Lactococcus lactis* ssp.*lactis* *Lactococcus lactis* ssp. *cremoris* *Streptococcus thermophilus*	Cheddar
FRC Type	*Lactococcus lactis* ssp.*lactis* *Lactococcus lactis* ssp. *cremoris* *Streptococcus thermophilus* *Lactobacillus delbrueckii* ssp.*bulgaricus*	Feta, White Brined Cheese

The lactic acid bacteria used in cheese industry can be classified in two classes; mesophilic starter cultures and thermophilic starter cultures according to their growth temperatures.

According to Law and Tamime, in the early 1960s, freeze dried liquid cultures and concentrated frozen starters were produced (Law and Tamime, 2010). They were not inoculated into milk. They were firstly inoculated into bulk starter tanks and grown to reach higher cell counts. Differently, technology resulted in directly used culture production. The directly used cultures are; DVS (Direct vat set) and DVI (Direct vat inoculation) cultures. Bulk starter is not produced in starter tanks nowadays (Law and Tamime, 2010).

In a cheese system the starter culture main role is conversion of the lactose into lactic acid basically. Also there have been adjunct cultures (used preferably) with different purposes rather than fermentation. They can be used for aroma formation, texture characteristics, and antimicrobial characteristics or for bacteriocin capabilities.

Mesophilic lactic acid bacteria starter cultures are names as LD and O types. The "LD"cultures contain citrate-fermenting bacteria (L=Leuconostoc species, D= *Lactococcus lactis* ssp.*lactis* biovar.*diacetylactis*) which produce aroma and carbon dioxide from citrate. The O- cultures contain acid producing strains and there is not any gas production. Traditionally O-cultures are used in cheese industry includes *Lactococcus lactis ssp.lactis, Lactococcus lactis* ssp. *cremoris.* LD-cultures are used in most continental semi-hard cheeses such as Gouda, Tilsitter and in some soft cheeses such as Camambert and Port-salud (Tamime et al, 2005).

Thermophilic cultures used in cheese industry are generally; *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactobacillus helveticus*. Using *Streptococcus thermophilus* as a cheese starter, would cause an increase of galactose concentration in cheese milk and affect the final product properties as a result of being unable to metabolize galactose. As in yoghurt fermentation, when *Streptococcus thermophilus* is used with *Lactobacillus bulgaricus*, the lactic acid production rate increases. *Streptococcus thermophilus* is often used with *Lactobacillus helveticus* in Swiss cheese production; since galactose was fermented by *L. helveticus*, eventually specific aroma compounds were formed in combination with *Streptococcus thermophilus* (Law and Tamime, 2010).

2.1.3. Addition of Rennet

As mentioned in the first part of this chapter, cheese can be named as a coagulant obtained from milk caseins. In order to obtain a gel network, milk proteins has to be treated with an effective coagulation type; acid coagulants, enzymes or usage of acid and heat together.

Main types of rennet enzymes or other coagulants, the active enzyme components of them are listed in Table 2.2. depending on their origin.

Table 2.2. Rennet and Coagulants with Their Active Enzyme Compounds, (Source: Tamime and Law 2010)

Group	Source	Rennet and Coagulants	Active Enzyme Compound
Animal	Bovine Stomach	Calf Rennet, Adult Bovine Rennet	Bovine chymosin A, B and C, Pepsin A, Gastriscin
		Rennet Paste	Bovine chymosin A, B and C, Pepsin A, Gastriscin, lipase
	Ovine Stomach	Lamb Rennet, Ovine Rennet	Ovine Chymosin, Pepsin
	Caprine Stomach	Kid-caprine Rennet, Caprine Rennet	Caprine chymosin, Pepsin
Microbial	*Rhizomucor miehei*	Miehei coagulant type L, TL, XL, XGL/XP	*Rhizomucor miehei* aspatic proteinase
	Cryphonectria parasitica	Parasitica coagulant	*Cryphonectria parasitica* aspartic proteinase
Fermented Produced Chymosin	*Aspergillus niger*	CHY-MAX	Bovine chymosin B
		CHY-MAX M	Camelus Chymosin
	Kluyveromyces marxianus var. lactis	Maxiren	Bovine Chymosin B
Vegetable	*Cynara cardunculus*	Cardoon	Cyprosin 1, 2 and 3, Cardosin A,B

In cheese production, coagulants include proteolytic enzymes mainly named as rennet. There are different types of enzymes and the classification depends on the source that the enzymes are extracted. Depend on the sources the enzymes could be obtained from animal origin, from microorganisms that are capable of producing these enzymes or from microorganisms that were genetically treated to produce extracellular pepsin and cymosine enzymes. Law and Tamime has mentioned three different types of coagulating agent in literature including, microbial, fermentative and rennet from animals (Law and Tamime, 2010).

The coagulant is added to the cheese milk after starter culture is added and lowered the pH of cheese milk. A dilution step of the coagulant with chlorine-free good quality tap water is often recommended prior to addition of the cheese milk.

In milk proteins, main part is consisted from casein micelles with a ratio of approximately 80% among other proteins and the remaining part includes serum proteins or whey proteins.

Curd formation is the principle of coagulation which can be observed at pH 4.6 related to the phosphorus content of casein family. At this pH value, serum proteins remain in solution in milk while casein tends to coagulate.

There are types of caseins are α caseins, β casein and κ-casein mainly. The location of the casein micelle includes κ-casein on the surface. Addition of the proteolytic enzyme, firstly κ-casein is hydrolysed and caseinomacropeptide is released. Also casein micelles become destabilized. Aggregation step take part in milk solution.

In cheese process there are several terms relating to the curd formation. The time for clotting, gelation time and cutting time are important parameters for cheese industry. Clotting time can be defined as the time from the additions of enzyme to the first visible flocks are seen. After flocks became visible, without any physical deformation, gel began to be formed. Gelation time is required until all milk in the vat became in gel form. To obtain curd the gel has to be broken at the time of cutting. Cutting time depends on the physical properties of the gel.

Since the coagulant is a proteolytic enzyme, the temperature value affects the coagulant activity. The optimum temperature for curd formation at pH 6.5 is between 34-38 °C for many commercial coagulants. In practice, coagulation occurs at 30-35°C to have adequate control over the curd firmness and cutting. Also the pH has a great effect on coagulation process and on the properties of the curd. The reduction in pH will

increase the rate of the hydrolysis of the κ-casein and aggregation rate (Guinee and Wilkinson, 1992).

2.1.4. Addition of $CaCl_2$

The pH decrease to reduce the clotting and cutting time; $CaCl_2$ is added in cheese milk before coagulant. The concentration of $CaCl_2$ is described both by Law and Tamime and Hayaloglu et al. with 0-20 g per 100 kg milk (Hayaloglu et al., 2002, Law and Tamime, 2010). According to Hayaloglu et al., $CaCl_2$ addition reduces the milk components from loss in whey, this addition would result with reduction in draining time with increased cheese yield (Hayaloglu et al., 2002). Milk also contains calcium itself and when the calcium is low in milk it would cause problems in coagulation step which will then be overcome by addition of $CaCl_2$. It acts by binding the casein micelles and reduces the repulsive forces therefore increase the hydrophobic interactions which affect the gel firmness.

Controlling the cut firmness at cutting is an important parameter in order to control the loss of the dry matter into the whey. Higher firmness at cutting results in the retention of more moisture in the final cheese. After cutting, 5-10 minutes is often used to allow the newly formed cheese grains to form a surface skin, more mechanical stability (Hayaloglu et al., 2002, Fox et al., 2004, Law and Tamime, 2010).

Stirring the cutted aggregates promotes the expulsion of whey which then gives rise to pressure on grains as they collide with each other. Generally after cutting the curd the curd – whey mixture is left without stirring in the vat for nearly 60 minutes, then it is stirred at 5-8 rpm for 10-14 minutes.

2.1.5. Whey Drainage and Remaining Operations

The result of stirring the curd is distribution of curd grains and whey in vat. Curd has to be separated from whey in order to continue the fermentation in cheese molds.

Removal of whey can be performed with many different industrial systems. It only includes the physical separation (Fox et al., 2004).

Generally, perforated screens are used by passing the curd – whey mixture on the screen or in the pipe lines. The whey is drained from perforated area and collected for further operations. Separated curd is molded.

2.1.6. Packaging and Brining

The fermentation of cheese curd requires aerobic conditions with controlled temperature and humidity parameters. Starter bacteria begin to ferment curd into cheese which generally takes 8–12 hours, depending on the activity and concentration of starter bacteria. Fermentation in molds is ended related to the pH of the product, depending on the cheese type. Molds are stored at 4°C to dry the surface of the cheese. Cheese are taken out from molds and placed on the clean wires for moisture equilibrium for 8 hours.

Packaging depends on the cheese type. Brine packaging or vacuum packaging are the most widely used techniques for white cheese. Salting step depends on the packaging type. It can be done before packaging for vacuum packaged products or during brining step for brined white cheeses.

Cheese brine is prepared from the saturated and pre-pasteurized salt solution. The pH of the brine should be similar to the pH of cheese. In order to eliminate the risk of softened cheese surface; calcium chloride might be added in brine solution. Calcium addition depends on the calcium content of cheese. The calcium content of brine should be equal to cheese calcium content. Otherwise calcium in cheese might migrate into brine related to calcium gradient and caseins will further be more soluble on the surface (Fox et al., 2004).

CHAPTER 3

MICROBIOLOGICAL CONTAMINATION OF CHEESE

The unnecessary and undesired microbial growth in food systems are named as microbiological contamination. Microbial contamination is related to the growth of microorganisms; bacteria, yeast, mold, protozoa and even some viruses, are in concern for food industry. In order to prevent the contamination of food system; the main issues has to be taken in concern during production which are classified as unhygienic handling steps, utilization of unsafe water, raw material quality and effective cleaning of the equipment and contact surfaces (Cushen et al., 2012).

The white brined cheese is the most consumed type of cheese among other cheese types. It was underlined in 2006 that the white brined cheese has a production rate of 67% for Turkey with the property of being the most important dairy product which is exported from Turkey to Middle East countries (Temelli et al., 2006).

Choi has stated that, even being a product with high nutritional value and classified as safe; in European Union; the foodborne outcomes were reported from contaminated cheese samples with a ratio of 0,4 % , in 2006 (Choi et al., 2016).

The white cheese production is generally done under unmechanized or artisanal conditions and the process includes many steps performed by handling. As a result of these properties, the process is suitable for microbial contamination during process and handling (Hayaloglu, Guven and Fox, 2002).

In general, white cheese is produced with pasteurized milk industrially. According to efficient pasteurization; starter culture selection becomes important for cheese quality and safety, not for only production time, also for the shelf life of the product. The starter culture types were mentioned in part 2,1,1,3.

There are three different aspects encompassing the microbial surveillance. The first includes the studies of the beneficial microorganisms which contribute the desirable organoleptic properties. The second is to control of microorganisms that lead to quality defects and the last one is to prevent of contamination with agents that can result in foodborne disease.

Different types of cheese carry different types and levels of microbiological risk, pathogens are more likely to survive or grow in soft cheeses rather than hard cheese types. The relation between the cheese maker and milk producer is an important parameter especially if the cheese is made from unpasteurized milk.

The complex nature of stages in cheese manufacture after milk pasteurization exposes the curd to many contamination risks.

The importance of raw milk hygiene is well known to those involved in cheese making; but the microbiological quality of the other ingredients must not be overlooked since these are added after heat treatment of the milk. The main constituents of cheese apart from milk are rennet, starter culture and salt. If these are not produced in dairy, the microbiological safety certificates should be obtained from supplier.

Primarily, the hygiene in a dairy plant has to be controlled due to personal hygiene, air quality, equipment, sanitizing activities, monitoring of water quality and equipment cleanings (Alum et al., 2016).

The maturation of cheese in rooms is a complex step in production of cheese. Many types of microorganisms can contaminate the cheese and grow. Contamination during shelving, from walls, floors, drains can occur. Bacteriological growth can be controlled easily than mold growth. Because controlling the mold contamination and growth requires good environmental hygiene, correct balance of salt, moisture, pH, texture of curd, maturation temperature and humidity and correct air flow (Jervis, 1998, Hayaloglu et al., 2002).

The use of potable water for food manufacturing operations is a legal requirement within the EU. Water does not only carry microbial contamination itself, but can also spread contamination on equipment, floors, and drains in dairy.

The microbiological quality of brine is overlooked by cheese makers. As an example; salt tolerant bacteria such as *Listeria monocytogenes* can grow in brine. In order to overcome this problem, it has to be prepared freshly and controlled regularly (Fratamico and Bayles, 2005).

Airborne mold contamination can arise from external sources such as agricultural air. Also air from rooms in which cheeses are being matured or handled can contain high number of mold spores.

3.1. Defects Related to Microbiology of White Cheese

The process is suitable for enter and growth of coliforms, *Enterobactericaceae*, *Eschericia coli, Staphlococcus aureus, Salmonella* ssp., *Clostridium* ssp., yeast and mold species (Turantas et al., 1989, Bintsis and Papademas, 2017).

As Ross et al. and Bintsis, Papademas have stated in 2002; the secondary microflora has beneficial effect on the flavor and structure development of cheese. Also the same flora can cause some quality defects (Ross et al., 2002, Bintsis and Papademas, 2002).

The important microbiological contamination defects are generally caused by certain groups of microorganisms; Psychrotrophs**,** coliforms, fungi, spore forming bacteria, some undesired lactic acid bacteria and some Enterococcus species (Ledenbach and Marschall, 2009).

Pseudomonas is one of the most important bacteria among Psychrotrops which can reduce diacetyl content. This would cause to breakdown the equilibrium between the diacetyl and acetaldehyde concentrations of the dairy product. As a result, aroma defects would be observed in cheese products. It was stated in 2009 by Ledenbach and Marschall that; "the typical pH is marginally favorable for the growth of Gram-negative psychrotrophic bacteria, with the pH of cottage cheese curd ranging from 4.5 to 4.7 and the pH of creamed curd being within the more favorable pH range of 5.0–5.3". They also mentioned about the salt concentration and insufficient effect of salt on contaminating flora in cottage cheese (Ledenbach and Marschall, 2009).

Depending on the type of cheese, related to the rate of acid production by starter culture, coliform group bacteria can be favored to produce gas holes in cheese when slow acid production by starter occurs.

In white cheese; one of the important defects is to have large gas holes in the cheese structure where the defect is named as "early blowing". The early blowing would cause a spongy structure for the textural characteristic of the cheese. In general the "early blowing" defect is caused by the growth of coliform group bacteria and sometimes by the growth of some yeast species which is determined by Romano et al. (Romano, et al., 1989). The coliform group bacteria cannot survive pasteurization temperatures. However, the bacteria might contaminate the milk after heat treatment by

the contact surfaces of the equipment. It can be underlined that; the cheese having early blowing defect is produced under insufficient cleaned environment even the milk used is pasteurized. In 1987, it was determined that the species *Aerobacter aerogenes* is the most common coliform group bacteria that contaminates the cheese and cause blowing (Abd El, 1987). Also Bintsis and Papademas have stated the salt concentration would be effective on these bacteria to prevent blowing. In addition *Klebsiella aerogenes* growth was stated as the other species that cause early blowing (Bintsis and Papademas, 2017).

The other and important type of blowing is named as "late blowing" which is known to be caused by heterofermentative lactic acid bacteria or Clostridia (D'Incecco, et al., 2018). *Clostridium tyrobutyricum* can tolerate high concentrations of salt and low pH (Bergere and Lenoir, 2000). It can only be prevented with effective heat treatment and sufficient cleaning of the equipments. It will also cause the aroma defects as a result of the butyric acid and carbondioxide which are metabolic products of the bacteria and hydrogen peroxide generation from the lactate fermentation (Bintsis and Papademas, 2002)

Generally fungal growth is known to cause softening and unpleasant yeast or ester-like odour or gas formation (Vivier et al., 1994). If the product is canned, swelling of cans can be caused by lactose fermentation capabilities of yeasts, especially *Kluyveromyces ssp* (Bintsis and Papademas, 2002). Actually, yeast species cause the pH to be increased which decrease the growth of *Staphlycoccus aureus* and some other pathogenic bacteria (Garnier et al., 2017). As stated in the same study of Garnier et al. in 2017; the dairy fungal spoilage is mainly caused by; *Candida, Penicillium, Cladosporium, Debaryomyces, Galactomyces, Yarrowia, Kluyveromyces* for yeasts and molds, respectively (Garnier, et al., 2017).

Since white brined cheeses are stored in packaging materials filled with brine and provided that cheese blocks are completely immersed in brine, the development of molds is rare and cannot be isolated as usually as other species.

Psychrotrops might cause some quality defects; lipolysis lead to formation of free fatty acids and as a result rancid flavor is observed. Kalogridou et al. have studied the effect of storage time of the milk on curd quality. In this study they have examined the psychrotrops in milk during storage and the enzymatic profile that was produced. Lipolytic enzymes produced by psychrotrops are heat labile and shows activity when processing (Kalogridou et al., 1984). The most important group in psychrotrops is *Pseudomonas ssp*. associated with cheese. Champagne et al. have stated the effects of

bacteria during cold strage and determined that; Pseudomonas species produce heat-labile proteolytic and lipolytic enzymes and cause off-flavor and textural defects (Champagne et al., 2007).

In dairy products, especially for white brined cheese; most common yeast species were determined with their percentage as *Debaryomyces hansenii* (32.6%), *Kluyveromyces marxianus* (18.5%) and *Yarrowia lipolytica* (17.4%) (Yalçın, 2007, Garnier et al., 2017). The mold species were isolated from equipment and air samples and identified as mainly belong to *Penicillium* species, especially *Penicillium commune* (Temelli et al., 2006). Since they can produce visible mold colonies on the surface of the cheese mold contamination became an important issue for dairy industry.

According to Chomakov, the formation of ropy substances in brine solution is carried out by the strains of *Lactobacillus plantarum var.viscorum* and they stated the different effects among *Lactobacillus plantarum* strains (Chomakov, 1997). As adjunct cultures, these observations highlight the point to select the true lactobacilli. A detailed study has to be performed for cultures used in white cheese process related to their biochemical activities, aroma profiles, etc.

3.2. Pathogens in White Cheese Manufacturing

Before 1980's dairy products were known as "safe products", but after 1980s studies were performed and reports were generated related to intoxications and infections of human after consumption of contaminated cheese or dairy products. As a result, microbiological quality of the products became in concern and microbiological analysis parameters, hygiene parameters were determined (Temelli, et al., 2006).

In dairy industry; the main raw material; milk is used after pasteurization. As known globally, pasteurization is an important step to eliminate the pathogen flora of the raw milk (IDF, 2004). However; after pasteurization, handling of the cheese or the process steps can generate a sufficient environment for pathogen growth.

The growth and survive of the pathogens in cheese are effected by both intrinsic and extrinsic factors. Intrinsic factors for cheese are; pH, acidity, moisture content, redox potential, water activity, salt content, nutritional contents antimicrobial additives

(sorbate, nitrate, benzoate, hydrogen peroxide, etc.), secondary starter culture activity, main starter culture activity and their metabolites. They all are affective on the growth of microorganisms. Extrinsic factors can be classified as storage conditions (temperature, humidity), package properties (modified atmosphere packaging, vacuum packaging, brine usage, etc.) and production steps.

De Buyser et al. have studied the implications of pathogens and food borne diseases from dairy products and have stated that, the main pathogenic species for cheese industry are; *Salmonella enterica, Listeria monocytogenes, Staphylococcus aureus,* enteropathogenic *Eschericia coli.* These species has pathogenic characteristics and human who consume the food containing these bacteria can be infected (De Buyser et al., 2001).

Microbiological safety of cheese depends not only on milk quality, but also the starter culture or native lactic acid bacterial growth, pH, salt, aging conditions and chemical changes during manufacturing are also affect the microbial flora of the product.

From the reported cheese associated outbreaks of foodborne diseases, it was observed that cheeses made from unpasteurized or improperly pasteurized milks are the most frequent causes.

Some pathogen bacteria can survive at high salt concentrations or at low temperatures. As an example; in a research inactivation kinetics of Listeria was studied and demonstrated that, the survival of *Listeria monocytogenes* was reported as 90 days at 4°C in white brined cheese by Erkmen (Erkmen, 2000).

Even the storage in brine is thought to cause prevent the survival of undesirable contaminants, the brine can also serve as reservoir of certain salt-tolerant pathogens like some strains of *Listeria monocytogenes*.

Ramsaran also reported that *E.coli* O157:H7 has increased nearly 100 fold 10 days after manufacture of feta cheese (Ramsaran et al., 1998). The high numbers of enteropathogenic *Eschericia coli* was found to be due to the lack of proper sanitation and use of unpasteurized milk, but modern processes can prevent this risk by using GMP and HACCP procedures.

Lactic acid bacteria as starter or adjunct culture can prevent growth of undesired microflora with many different ways like; pH decrease or production of many metabolites; lactic acid, diacetyl, phenyl lactic acid and many types of bacteriocins.

Aeromonas hydrophila was found to be inactivated easily during maturation if the initial numbers are smaller than 10^7 cfu/g (Bintsis and Papademas, 2017). Also *Staphylococcus aureus* was found to increase in the first day of manufacture but salting of cheese, decrease in pH cause inhibition of the pathogen. However it was demonstrated that, increased amounts of salt inhibits the lactic starter bacteria and so enhance the survival of *Staphylococcus aureus* (Ledenbach and Marschall, 2009).

There are many yeast species used in food fermentations. However they are used related to their known characteristic, in white cheese production yeast species are classified as undesirable microorganisms since they cause quality defects on cheese surface and flavor. Many of the quality defects are related to yeast or mold growth during cheese manufacturing and shelf life of the product.

However, this study concerns on protecting the cheese from undesired secondary fungal microflora, and yeast/mold are the most difficult microorganisms to eliminate from the process, spoilage fungal species are explained in detail in another section.

3.3. Fungal Species in Cheese Spoilage and Their Properties

Growth of undesired yeast or mold species is more difficult to prevent when compared to growth of contamination caused by bacteria. Njobeh in 2009 stated that; the tested 95 food samples were almost contaminated with *Aspergillus* or *Penicillium*. Also it was stated in the same study; fungal growth clearly indicates the presence of aflatoxin or other fungal toxins in contaminated food samples. Therefore these foods had to be considered as "low quality" food (Njobeh et al., 2009).

In 2003 Boekhout stated that; until their study; approximately 1500 yeast species were defined in literature (Boekhout et al., 2003). Among commercially used microorganisms, yeast is the most important group. Yeasts are commercially used in alcohol production, being the best fermentative organism; the maximum activity is to produce alcohol. Additionally, yeasts are used in bakery, production of oriental foods, pickle, B12 and β-caroten production, polysaccharides as regulators of texture and contents of foods (phosphomannan etc.), production of glycerol, polyhydroxy alcohol,

single cell protein, and lipids (Deak, 1995, Jakobsen-Norvhus, 1996, Hierro et al., 2004).

At the present time, yeast is accepted as the most valuable industrial microorganisms. Chemistry, food, agriculture and drug industries are interested in the usage of yeast. By using molecular biologic techniques related to yeasts, industrial enzymes, interferon and various raw materials of drug industry are produced (Jakobsen-Norvhus, 1996).

Even having these functional properties, yeasts may cause spoilage of food and beverages. Before 30-40 years, it was demonstrated that; yeasts were not important related to public health and also yeasts were reported to being required at high numbers in fermented beverages and foods (Betts et al., 1999, Loureiro and Qerol 1999, Hierro et al., 2004). The effect of yeasts on public health was noticed at 1980s for the first time. After this time, the allergenic effect was supported with literature, even the bowel inflammation was found to be caused by *Saccharomyces cerevisiae* (Jakobsen and Narvhus 1996, Taniwaki et al., 2001).

In recent years, the importance of food spoilage yeast were increased and hold responsible from the large economical lost.

Barnet demonstrated 120 species belong to 30 genus isolated from foods in 1983. Pitt and Hocking demonstrated the important species related to food spoilage as; *Dekkera bruxellensis, Issatchenkia orientalis, Debaryomyces hansenii, Yarrowia lipolytica, Kloeckera apiculata, Pichia membranafaciens, Zygosaccharomyces bailii, Zygosaccharomyces bisporus, Zygosaccharomyces rouxii, Schizosaccharomyces pombe, Saccharomyces cerevisiae, Geotrichum candidum, Candida utilis, Candida tropicalis, Candida zeylanoides, Candida holmii* in 1997.

Although the economical lost is big related to yeast spoilage, there are not so much record related to commercial confidentiality.

The main sources of the yeast caused spoilage are the protectant included foods, olive, cheese, mayonnaise, high fat content foods like milk, vinegar, bread and other fermented foods. Many yeast species are available to cause food spoilage and to conclude an undesired manufacturing process.

Yeast can grow in a broad pH range (pH 2-9), low water activity, low temperatures, high salt or sugar content. They can use pectin and other complex carbohydrates, organic acids, proteins and lipids. The dominant microorganisms decreasing the storage stability in fermented and low pH milk products were detected as

Pichia membranifaciens, Debaryomyces hansenii and *Candida holmii* (Westall and Filtenborg 1998, Prilinger et al., 1999, Welthogen and Viljaen 1999, Taniwaki et al., 2001, Garnier et al., 2017). Cheddar cheese was used to isolate *Debaryomyces hansenii, Cryptococcus albidus, Yarrowia lipolytica, Phodotorula minuta, Rhodotorula glutinis, Torulaspora delbrueckii* and *Kluyveromyces marxianus* (Garnier et al., 2017).

Also as the same with many other authors in 2001 Frank stated the common species isolated from contaminated cheeses were; *Candida* spp., *Kluyveromyces marxianus, Geotrichum candidum, Debaryomyces hansenii,* and *Pichia* spp. as yeast types and *Penicillium spp., Aspergillus spp*. for mold types (Frank, 2001).

The presence of oxygen and the low pH makes the environment selective for many fungal species. In addition; when the product is vacuum packaged; *Penicillium spp. and Cladosporium spp.* have ability to tolerate the low oxygen tension. The chemical preservatives; the sorbate most commonly used in dairy industry can be metabolized some of the fungal species. As an example; the most common mold determined on cheese surface; *Penicillium spp*. can degrade sorbic acid to trans-1,3-pentadiene. The first study concerning this issue was published in 1966; and they also determined the sorbate resistance of Penicillium species. They reported that; Penicillium can tolerate the sorbate concentrations up to 7100 ppm, metabolize the sorbate under this concentration and cause a defect on cheese surface named as "kerosene" flavor (Marth et. al. 1966). Although the sorbate effect on yeast species are still being studied by many researchers; it was found in 1994 that there are some sorbate resistant yeast species (Sensidoni et. al. 1994). In the study of Sensidoni et al., they had stated the undesired flavor on the cheese and margarine products caused by some yeast species (Sensidoni et. al. 1994).

The most dangerous fungal species in food industry can generally from the species which can tolerate osmotic stress and resistant to acidic protectants. Yeast might spoil foods either as a result of inadequate sterility of fermentation conditions or post fermentation contaminations. These spoilage may cause odor defects, turbidity, sediment or pellet formation, eye formation (Taniwaki, 2001).

There are many environmental factor which effects growth of yeast or mold populations like; temperature, Aw, atmospheric conditions, pH, substrates, inhibitors, etc. Because the optimum growth temperature of fungus depends on the type and can vary in between 24°C and 48°C, they are generally recognized as mesophilic microorganisms. Also environmental conditions will affect the growth temperature. An

increase in solute concentration or a decrease in water activity will cause an increase in the growth temperature for 2-6°C (Deak, 2006).

Water activity (Aw) is an important parameter which directly effects the microbial growth. Aw changes according to the solution concentration. There are differences between salt and sugar tolerance or sucrose and glucose tolerance. The vast majority of yeast species show tolerance for higher concentrations of sugar than salt at same water activities. Food spoilage yeast species can grow at minimum water activities between 0.90-0.95. There are a few species belong to osmotolerant genus which can grow at low water activities like *Zygosaccharomyces rouxii* that need water activity 0.62 to grow (Deak, 2006). Also water activity is important to differentiate to mold species. As an example; at water activities 0.80 at 25°C *P. glabrum, P. roqueforti* and *P. communee* can grow but Cladosporium needs increased water activities like 0.86 at same temperature (Deak, 2006 and Hocking 1997).

Although many of the fungal species can be classified as mesophilic; Penicillium species were reported to grown at the storage temperatures in between 2-5 °C (Sørhaug, 2011). Sørhaug stated in their study that the dominant species found in cheese samples were *P. roquefortii, P. camembertii, P. nalgiovense* and *P. communee*. Also in the same study the less isolated other contaminat species were found as; *P. brevicompactum, P. chrysogenum, P. citrinum, P. cyclopium, P. expansum, P. glabrum, P. granulatum, P. palitans, P. solitum, P. verrucosum, P. viridicatum* (Sørhaug, 2011).

The microbial growth also depends on the pH of the growth media for all types of microorganisms. Many of the yeast species are pH tolerant microorganisms and can tolerate the pH values between 3-10 depending on the species, the optimum pH is vary between 4.5 and 6.5 (Stanbury et. al., 2017). It was stated by Hocking and Pitt in 1997 that Penicillium species can tolerate very low pH values, even pH 2.0 can be tolerated by *P. roquefortii* (Hocking and Pitt, 1997). The tolerance of acid is generally related to the type or weakness of acid. Generally organic acids have more inhibitor activity than inorganic acids. Propionic acid has more inhibition activity when comparing with lactic or citric acid. As an example, the minimum pH value for *Pichia membranafaciens* is 3 when acetic acid is the main acidic compound in medium but it can be 2.2 if tartaric or citric acid is used and 1.9 if hydrochloric acid is used.

3.4. Defects Caused by Fungal Species in Cheese

Many different types of foods can be thought as a growth medium for yeast or mold growth according to their content. The food habitat itself can promote the growth of fungal species depending on the type of fungus and the nutritional content of the food.

Yeast species would grow in food system and can cause food quality defects. They cause detectable changes in the textural properties, sensory characteristics and chemical composition of the food depending on their metabolism.

In food industry yeast are important for their two different roles. Their first role is to use them for maturation process regarding to their effect on texture and taste formation. On the other hand yeast can cause quality defects in cheese.

Spoiled cheese can be determined from its physical characteristics; visual microbial growth on cheese surface, odor, texture and taster deformation, biofilm formation, the disfiguration or deformation of the package material or gas formation by fermentative yeast colonies.

Filtenborg and Corbo stated about the limited reports related to the difficulties about to differentiate the yeast colonies whether they are beneficial or not (Westall and Filtenborg, 1998, Corbo et al., 2001). However yeast species can be thought as a contaminant flora for certain dairy products; for kefir the same species are beneficial and has to be alive in the final product. *Kluyveromyces marxianus* is one of the species which can be an example for this situation. If it grows on a cheese surface it will spoil the product and cause deterioration of the cheese.

Even the cheese product has a storage temperature limitation (has to be kept at refrigeration temperature), low pH values, low humidity index and high salt concentration, yeast comprises one of the biggest part of the microflora of many cheese types. Since a yeast contamination affects the quality of the final product; contaminated cheese sample has to be checked to find the source of contamination, beginning from farm to final product storage area.

Extracellular lipolytic or proteolytic enzyme productions, lactose fermentation, usage of lactic and citric acid are main mechanisms of yeast in cheese environments. Especially in maturation processes, much maturation is known as spoilage. Because, permanent hydrolysis of protein and lipids may cause bitter and sour taste while

permanent lactose fermentation may cause increase in acidity, formation of gas and fruity taste (Corbo et al., 2001, Suzzi et al.,2001).

Yeast species have to reach a defined concentration to cause detectable changes. While *Y.lipolytica* cause defects at 10^5-10^6 cfu/g, *G.candidum* cause defects at 10^3-10^4 cfu/g concentrations (Westall and filtenborg, 1998, Prilinger et al., 1999).

Microbial growth in cheese determines the shelf life of the product. Vivier demonstrated the first spoilage in cheese with swelling and texture defects. Also *K. blattae*, *K. thermotolerans* and *C.saphaerica* were responsible for gas formation. (Vivier et al., 1994).

Biofilm formation on cheese surface is generally caused by the *Sporobolomyces roseus* and *Trichosporon sp.* (Deak, 2003). The gas formation and blowing was found to be caused by *Kluyveromyces blattae, Kluyveromyces thermotolerans* and *Candida sphaerica* for the first time in literature by Vivier in 1994 (Vivier et al., 1994).

Generally texture of the cheese is affected by *Yarrowia lipolytica* as a result of milk fat degradation and the high cell counts of *Debaryomyces hansenii* cause the yeast-like flavor.

In the literature there has been never mentioned about outbreaks caused from a yeast contamination, but some species should be considered as opportunistic cause of certain allergic reactions (Garnier et al., 2017). It was mentioned that the illness caused by mycotoxin poisoning has not been documented in 2017 (Garnier et al., 2017).

As a result of visible colonies on the surface of the product from mold contamination; it might cause bigger economical loses when compared to the yeast contamination even at lower concentrations of mold counts. Beside the economic loss; mold contamination itself may even constitute a health risk related to mycotoxins that the mold cells produce (Hymery, 2014).

Basilico et al. had mentioned about the Cheddar cheese contaminant fungal flora in their study and the species all belong to only two different type; *Cladosporium* and *Penicillium* (Basilico et al., 2001).

In 2018 Casquete et al. stated the mold species in a cheese factory as *Penicillium communee, Penicillium solitum, Penicillium roquefortii* and *Penicillium palitans* which were not used as starter and classified as contaminant. The most isolated mycotoxins of the Penicillium spp. mentioned in literature are; roquefortine C, mycophenolic acid, PR toxin, patulin, penicillic acid, citrinin, cyclopiazonic acid (CPA) and ochratoxin (Casquete et al., 2018).

3.5. General Characteristics of Fungal Species Isolated from Cheese

Garnier et al. have stated in their study in 2017 that; there were many documents and studies about food spoilage bacteria, their effects on products and their diversity, whereas there were a limited literature and study about the food spoilage yeast and molds. They cause economic loss for industry and generally they originates from the environment of the plant; air, water, equipment and personal hygiene (Garnier et al., 2017).

Valdes demonstrated that the yeast concentrations were different at cheese produced with rennet addition and cheese produced from coagulum by acid addition. *Debaryomyces hansenii* and *Geotrichum candidum* were isolated from cheese samples produced with rennet addition as coagulant. On the other hand *Kluyveromyces marxianus* and *Pichia membranifaciens* were isolated from the cheese samples produced with acid addition as coagulant (Valder-Stauber et al., 1997).

Şahin and Öztürk examined cheese samples from Tekirdag and Bursa regions and isolate 113 yeast strains. When they analyzed the results of many biochemical and morphological tests, it was observed that samples were belonging to *Debaryomyces, Pichia, Kluyveromyces, Trichosporon* and *Candida* genus (Şahin and Ozturk, 1998). Also they perform total acidity and salt analyses and results showed that acidity between 0.35-1.60 % and salt concentration between 2.8–6.2 % were not enough to inhibit the yeast grow. As a result they demonstrate the importance of pasteurization of milk used in cheese manufacture to avoid contamination from raw milk.

The contamination sources of yeast were examined by Welthagen and Viljoen in a cheese manufacturing environment while cheddar cheese was produced. Results indicate that; dominant species isolated from samples were *Debaryomyces hansenii* and *Cryptococcus albidus*. *Debaryomyces hanseni* was isolated from whey; coagulate before brining and from cheese surface. *Cryptococcus albidus* was isolated from cheese surface only (Welthogen and Viljoen, 1999).

Petersen demonstrated the species related to process steps. At the beginnings of maturation, they isolated *Candida ssp., Trichosporon ssp., Rhodotorula ssp*. at low concentrations while *Debaryomyces hansenii* was at high concentrations from the beginning of maturation. Additionally it was reported the high adaptation ability to environmental conditions of *Debaryomyces hansenii*. Finally Peterson explained it with

lactose fermentation capacity of species and the relation between lactose concentration and yeast concentration (Petersen, 2002).

Also Pereisa-Dias reported the concentrations of *Debaryomyces hansenii* and Candida intermedia 9% at curd and 86% at cheese samples (Pereira-Dias, 2000).

3.5.1. *Debaryomyces* Genus

The foodborne yeast *Debaryomyces hansenii* is the most isolated species from dairy products, especially found in cheese samples. *Debaryomyces hansenii* is generally known as halotolerant, osmotolerant and xerotolerant. It is an ascomycetic yeast species which can be isolated from seawater, salted foods and brine.

Debaryomyces hansenii has its maximum growth rate for the temperature values in between 25–30°C. The minimum temperature value it can grow is 5°C and the maximum value was determined as 32-37 °C (Barnett et al., 2000).

Debaryomyces can produce lytic enzymes and alditols at temperatures between 25-30°C (Barnett et al., 2000). The intracellular and extracellular proteinases were determined and the proteolytic activity of the *Debaryomyces* culture was compared with the proteolytic activity of lactic acid bacteria in skim milk. The results of the study indicated that the proteolytic activity was higher than the proteolytic activity of lactic acid bacteria in skim milk. Also the hydrolyzing effect on casein was not observed at the storage temperatures of cheese samples at 10°C (Van Den Tempel and Jakobsen, 2000).

Since it has been known as a halotolerant species, it is leastly affected yeast at high concentrations of NaCl like brine solutions In addition, like *Pichia guilliermondi, Yarrowia lipolytica* and *Candida parapsilosis*, it is determined that *Debaryomyces hansenii* is a contaminant at low water activities for foods (Prista et al., 2005, Breuer and Harms, 2006).

It was stated that in the research of Sanchez et al., the species of *Debaryomyces hansenii* has low fermentative activity. On the other hand it has high respiratory activity; according to its metabolism for fermentation capability phosphor fructokinase enzyme has the property to limit the fermentation (Sanchez et al., 2018).

Breuer and Harms mentioned the salt tolerance limits of *Debaryomyces hanseni* in their study and stated as; up to 4 M NaCl concentration, it can grow in food systems or in growth media (Breuer and Harms, 2006). *Debaryomyces hansenii* is a heterogenic type. This property may cause many phenotypic differences among strains like. The strains may vary related to the characteristics about metabolism of various carbon sources, lipolytic and proteolytic properties. Additionally the optimum growth conditions may also be differing among strains (Breuer and Harms, 2006). *Debaryomyces* genus members are characterized with limited fermentation capabilities, and inability to assimilate nitrate in order to differentiate the *Debaryomyces* genus, 10% NaCl concentrations can be used in growth media (Breuer and Harms, 2006). Also, for differentiation, lactose fermentation cannot be observed by *Debaryomyces hansenii*.

Cells multiply by multilateral budding. Primitive, also improved pseudomicelle formation can be seen. Conjugation between a cell and its bud is generally before ascus formation. 1 – 4 ascospor is formed in every ascus (Wickerham, 1970, Banwart 1981, Breuer and Harms, 2006). In literature; *Debaryomyces hansenii* was mentioned for its usage a biological control agent for many types of molds in different food systems. It was used with 10^5 cells/ml cell counts and show inhibitory effect on *P. roquefortii* (1×10^5 cells/ml) on solid media (Cordoova et al., 2018).

3.5.2. *Yarrowia* Genus

Yarrowia genus belongs to *Dipodascaceae* family. Yeast species of *Yarrowia* genus can generally been found in food systems rich in lipid and protein as dairy products. Among cheese types it can be found in blue cheese and Camembert at high concentrations. Among *Yarrowia* genus, *Yarrowia lipolytica* is the most frequently observed species isolated from contaminated cheese samples.

Like many other yeast species it has an optimum growth temperature of 25-30°C, but it has ability to grow at low temperatures as 5-10°C. Strains of *Yarrowia lipolytica* can grow at temperatures above 32°C (Boekhout et al., 2003). When compared with other yeast species mentioned in previous part, *Yarrowia lipolytica* is a

sensitive species to high salt concentrations and can be differentiated from *Debaryomyces hansenii* with this property (Barnett et al., 2000).

Yarrowia lipolytica with its high proteolytic activity can disrupt all parts of casein at low temperatures and this is an additional differentiation property with *Debaryomyces hansenii.* Also *Yarrowia* genus cannot tolerate low oxygen concentrations; it is an obligate aerobic microorganism (Tempel and Jakobsen, 2000, Fickers et al., 2005). *Yarrowia* cells multiply with multilateral buddings. Ascospores have ovoid, cap or saturn shapes, having 1-4 ascospors in each ascus. In general, septate hyphae formation can be seen with this genus. Sugar metabolism is weak, since it has rich in protease and lipase activity (Wickerham and Burton 1962, Fickers et al.2005). Besides being undesired yeast species on cheese surface, it has been used industrially as a result of its high lipase activity.

3.5.3. *Kluyveromyces* Genus

Vegetative cells are generally ovoid, ellipsoid, cylindrical or elongated. Budding is performed by asexual reproduction. Ascospores are circular, ovoid or reniform shaped. There are 1-60 ascospores formed in each ascus. Nitrate cannot be assimilated. On the other hand fermentation is observed in this genus. They have aerobic characteristics. As a result alcohol formation can occur by oxygen limitations. In a growth media they do not need external vitamin source. Different from *Debaryomyces* species they cannot tolerate high salt concentrations. It can continue to grow up to 52°C. (Wickerham and Burton 1962, Beneke and Stevenson 1987, Frazier and Westhoff 1988).

Kluyveromyces marxianus is a yeast with both respiratory and fermentative properties. As a result it gains energy by TCA or by fermentation. One of the most important features of this species and *K. lactis* is to assimilate lactose as carbon source different from other contaminating yeast flora of cheese (Lane and Morrissey, 2010).

3.5.4. *Pichia* Genus

Cells multiplying with multilateral budding are globose, ellipsoid or cylindrical. Some of the cells can produce pseudomicelle. Vegetative phase is haploid at the beginning. Conjugations occur between a cell and its bud also rarely between independent cells before ascus formation.

The *Pichia membranifaciens* is mostly isolated species from cheese samples. They can metabolise lactate but cannot metabolise lactose. They have strong fermentation ability. Molecules like starch are not produced. Nitrate can be assimilated. Urease and DBB reactions give negative results. Additionally it has the ability to grow at temperature values up to 44°C. (Beneke and Stevenson 1987, Frazier and Westhoff 1988).

3.5.5. *Geotrichum* Genus

The most frequently isolated species of this genus is *Geotrichum candidum* which is classified as yeast or yeast-like-fungus depending on the colony morphologies. Two main strain dependent characteristics can be observed. The optimum growth temperature of is 25 – 30°C. This type has high proteolytic activity. Differently from other many yeast species, it has a weak proteolytic activity.

3.5.6. *Penicillium* Genus

According to Pitt, *Penicillium* is a large genus consisting approximetely 200 recognized species which can easily been recognized in Genus level on a solid media surface from the characteristic circular gray green org ray blue colonies (Pitt, 2014).

Penicillium species has capability to grow between a wide pH range, from low pH degrees like pH 2 or pH 3 and high pH values like pH 9 or pH 10 (Pitt, 2014).

Penicillium species can tolerate low oxygen concentrations in growth media or in food system that they contaminate. This property has been mentioned in a study in 1992, in which the *Penicillium* species were isolated from vacuum packaged cheese. They demonstrated that growth media with 20% or 40% CO and less than 0.5% Oxygen inhibited growth of *Penicillium* species (Taniwaki et al., 2001).

Cheong et al. studied the antifungal effects of their lactic acid bacteria isolates from different sources. They examined the antifungal activity on certain yeast and mold species, especially *Penicillium commune*. They have stated that the ability of molds to grow mainly on cheese surface depends on their growth characteristics. As they mentioned most of the mold species can grow at refrigeration temperatures and vacuum packaged products with low water activity. These characteristics are generally in agree with the characteristic mentioned in the book chapter of Pitt which is mainly about *Penicillium* spp. (Cheong et al., 2014 and Pitt, 2014).

Penicillium species can produce mycotoxins, and the most frequently known mycotoxins are; roquefortine C, mycophenolic acid, PR toxin, patulin, penicillic acid, citrinin, cyclopiazonic acid and ochratoxin (Casquete et al., 2018) which are vary about their stability in cheese systems. Some of the mycotoxins remain stable during ripening or shelf life of the cheese while others cannot remain stable and do not show biological activity.

Penicillium species show high resistance to high temperature values with heat resistant ascospores. Also the most species belong to the *Penicillium* genus are xerophilic and can grow at water activity values down to 0.82. They can grow at temperature values below 5°C. The main disadvantage of this genus is to be resistant to many antifungal chemical preservatives; especially to sorbate which is most widely used chemical preservative against fungi (Pitt, 2014).

In general, a mold colony on the cheese surface belongs to the *Penicillium* genus. The product with a visible mold colony is thought to be spoiled. Beside this approach, certain *Penicillium* species are used to manufacture some cheese types. As an example *Penicillium camembertii* is used in Camembert cheese and *Penicillium roquefortii* is used for blue cheese manufacturing related to their specific aroma compound production properties.

Among the big *Penicillium* genus; *Penicillium communee* has been chosen for this study since it has been stated in the literature related to being one of the most

frequently isolated mold species from contaminated dairy products (Casquette et al., 2018, Taniwaki et al., 2001, Schnürer and Magnusson, 2005).

CHAPTER 4

ANTIFUNGAL LACTIC ACID STARTER CULTURES

Food manufacturing process, especially dairy processes, maintains suitable conditions for fungal contaminations. However dairy products are rich in nutritive values and promote the growth of contaminating fungal flora.

Fungal contamination itself causes big economical losses for dairy industry. As decrease in shelf life of the product or to cause a quality defected product which cannot be consumed.

To avoid the contamination of yeast or mold species; chemical food preservatives, modified atmosphere or vacuum packaging, chemical disinfectants in plants and antifungal adjunct starter cultures for cultured dairy products are the main solutions. Also the methods which can be combined with Hurdle Technologies are used like; air filtration in plant, effective equipment sanitizing, GMP and HACCP applications in process, water quality, storage temperatures, salt addition (as brine) might be useful for some cases.

In previous chapter, it has been mentioned about the modified atmosphere packaging and vacuum packaging. They cannot fully prevent the fungal growth since the some of the contaminant species can tolerate low oxygen concentrations in growth media. Additionally using salt in food as brine solution is not enough itself. Many yeast species generally isolated from cheese samples have the capability to tolerate salt in growth media.

There are many chemical food preservatives commercially available and legally permitted. The most frequently used chemicals are potassium sorbate, benzoate, propionate, natamycine. In some countries legal permissions or the limits of usage can be different. The regulation about the food additives was constructed and published by European Union. According to this commission regulation, propionate, sorbate and natamycine can be used for hard and semi hard cheese surface applications up to 1000

mg/dm^2 levels (Commission Regulation (EU) No 1129/2011, 2011). In the same regulation a wide range is stated for usage of sorbate for different types of dairy products with different levels of permission in between 300 – 2000 mg/kg. In Turkey, dairy industry is legally permitted to sorbate usage in cheese up to 1000 ppm levels. In 2017, Garnier et al. studied the resistance of fungal species to different chemical preservatives Garnier recommended process lines disinfection and GMP as a first step rather than chemical preservatives. In their study, it can be observed that all chemicals might be effective with very high concentration, but natamycine itself can reduce the number of fungal contaminants cell counts (Garnier et al., 2017).

Agriculture and food industries generally end to use preservatives due to manufacture products with extended storage life and quality.

Many of the yeast and mold strains have the ability to metabolize sorbate; most widely used chemical for dairy. As a result of this mechanism kerosene-like flavor occurs on the cheese surface as mentioned in Chapter 3.

This study also includes the effect of potassium sorbate on selected yeast species and *Penicillium communee* with different concentrations.

4.1. Biopreservatives and Antifungal Lactic Acid Bacteria in Market

Lactic acid bacteria species are known to be safe for human consumption. They can occur in food systems or additionally used in food productions. They are known as GRAS (generally recognized as safe). Some of their metabolic activities are useful tools to prevent fungal contamination makes them useful as adjunct cultures.

Lactic acid bacteria are generally known from dairy products which are responsible from fermentation of the milk product. In addition to their fermentation activity, they produce many different antimicrobial compounds. The most known compounds are lactic acid, acetic acid, hydrogen peroxide, formic acid, propionic acid and diacetyl (Schnürer and Magnusson, 2005).

However the major effect is known to be obtained from organic acids; they also produce proteinaceous compounds and low molecular weight antimicrobial substances (Varsha, 2016).

There are commercially available protective cultures designed with various types of lactic acid bacteria for food industry. Chr.Hansen (Denmark) produced Fresh Q culture for dairy industry which includes *Lactobacillus rhamnosus* and *Lactobacillus paracasei* for cheese and yoghurt protective cultures. Dupont (USA) has a protective culture for dairy industry named as HOLDBAC which includes two types as HOLDBAC YMB with *Lactobacillus rhamnosus, Propionibacterium freudenreichii* spp. *shermanii* and HOLDBAC YMC with *Lactobacillus paracasei* and *Propionibacterium freudenreichii* spp. *shermanii* for antifungal protection. Also there are Sacco (Italy) and CSK (Netherlands) producing antifungal cheese protective cultures named as Lyofest and Dairy Safe. Additionally there are products produced from the metabolites of antifungal lactic acid bacteria. One of them is MicroGARD which has represented at Dupont as preservative addition for fungal contaminations. The other most widely known preservative is DuraFresh produced from Kerry. They are generally produced from the metabolites of propionic acid bacteria, especially from *Propionibacterium shermanii* spp. (Varsha et al., 2016). However, there have been many studies about antifungal lactic acid bacteria, a few commercial cultures are available related to the difficulties to commercialize a microorganism where many differences can occur between *in vitro* and *in vivo* applications or the culture could result in undesirable sensorial characteristics (Hoier et al., 2010, Salas et al., 2018).

4.2. Action Mechanism and Metabolites of Antifungal Cultures

The antimicrobial effect of any culture depends on three mechanisms; production of antagonistic compounds like peptides, diacetyl, benzoic acid, phenyl lactic acid, etc., production of weak organic acids and finally competition for substrate with competing microflora (Bianchini and Bullerman, 2010).

Protective effect of the lactic acid bacteria against fungal species has not fully understood yet. Most important one is about the organic acid production. As a result of increased concentrations of organic acids produced by culture, the food environment begins to be more acidic and it restricts the growth of many types of microorganisms including bacteria, yeast or molds. The antimicrobial effect is generally related with the

decrease in pH and non-dissociated organic acid molecules regarding to their pKa values (Crowley et al., 2013).

The antifungal substances mentioned below act against the growth dynamics of yeast or mold cells. The first study about lactic acid bacteria and fungal interactions was perfomed in 2005 by Strom et al. and the physical interactions between *Aspergillus nidulans* and *Lactobacillus plantarum* were screened. The physical changes were interrupted mycellial growth of *Aspegillus nidulans*, which was determined by microscopic techniques. Also there were changes among the protein structures of *Aspergillus*. The two dimensional structure of proteins was being upregulated which were screened by gel electrophoresis (Strom et al., 2005).

Acetic acic has an inhibitory effect due to its high pKa value (pH 4.76) related to higher dissociation inside cell of fungal species. As mentioned in the study of Crowley et al., the antifungal effect of acetic acid increased with the help of lactic acid. Additionally, propionic acid was mentioned that it has higher antifungal effect than acetic acid with its higher pKa value (4.87) (Crowyley, et al., 2013).

The combination of organic acids included caproic acid, formic acid, acetic acid, lactic acid, propionic acid and butyric acid was found effective on broad spectrum of mold species (Corsetti, et al., 1998). Different carboxylic acids were determined as antifungal in a study of Broberg et al. (Broberg et al., 2007).

Additionally, phenyl lactic acid is the mostly known acid produced by lactic acid bacteria which has high antifungal activity with broad spectrum and was firstly described as a metabolite of *Lactobacillus plantarum* in 2000, stated in the review of Croweley, et al. Phenyl lactic acid was studied in detail in the study of Valero in 2004 and the findings were in agree with the findings of the studies mentioned in the article of Crowley, et al. (Valerio et al., 2004 and Crowley et al., 2013).

Phenyl lactic acid also has antibacterial effect on many Gram negative bacteria and Gram positive bacteria. Crowley et al., have mentioned about many studies from literature which include the broad spectrum of phenyllactic acid and its antifungal effect.

However it was underlined the synergistic effect of other organic acids regarding to the antifungal effect of phenyllactic acid (Valerio et al., 2004, Mu et al., 2009). Since phenyl lactic acid has not got any effect on the sensory characteristics of the food and on human health, it has potential as a preservative for food industry.

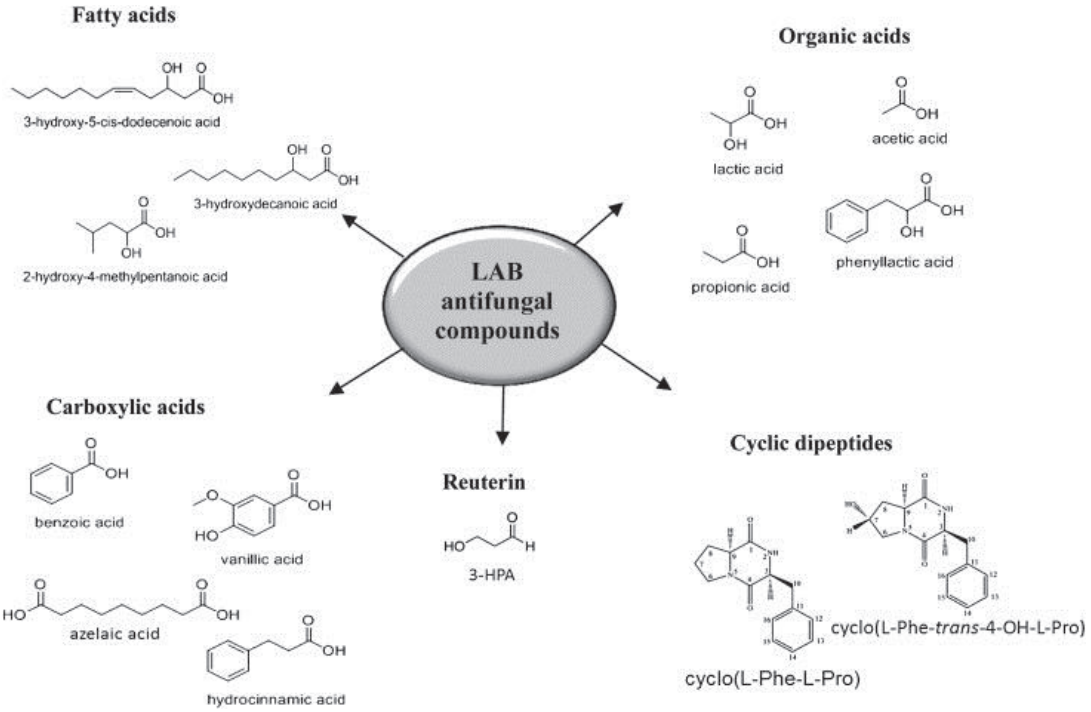

Figure. 4.1. Chemical Structures of Various Antifungal Compounds by Various Lactic Acid Bacteria (Source: Crowley et al., 2013)

Many of the lactic acid bacteria have flavoprotein oxidase to produce hydrogen peroxide. Additionally they can produce hydrogen peroxide from nicotinamide adenine hydroxyl dinucleotide activity.

Lactic acid bacteria cannot produce catalase, and results in the accumulation of the hydrogen peroxide in the growth media. As an antimicrobial agent, hydrogen peroxide in a growth medium causes the inhibition of glyceraldehyde-3-phosphate-dehydrogenase activity, hexokinase activity, and oxidation of sulphydryl groups and affects the glucose transport mechanisms. The studies about hydrogen peroxide concentration and mold growth indicate that the increased concentration of hydrogen peroxide cause decrease in both fungal growth and mycotoxine production (Ponts et al., 2006).

Proteinaceous compounds were identified with antifungal effect after they lost their protective effect with proteolytic enzymes (Crowley et al., 2013). Antifungal peptides of *Lactobacillus plantarum* were investigated by Coda et al. in 2011 and nine different antifungal peptides were identified (Coda, et al., 2011).

In 1999, Niku et al., firstly reported the antifungal lactones of *Lactobacillus plantarum* named as mevanolactone (Niku et al., 1999). After 1999, many other studies have been stated in literature about the lactones of lactic acid bacteria and their effect on yeast and mold species. One of them was about the antifungal effect of dodecalactone on Aspergillus genus and *Penicillium roqueforti* with different minimum inhibition concentrations ranging between 350-6250 microgram per milliliter (Yang et al., 2011).

Reuterin is another metabolite produced by *Lactobacillus reuterin*, known to have effect on gram negative bacteria, yeast and some mold species.

Magnusson et al. determined the increased antifungal activity of *Lactobacillus coryneformis* when the growth medium enriched by glycerol which resulted in the production of 3-hydroxy phenyl lactic acid and it was observed that the increased antifungal effect of reuterin was obtained by the glycerol degradation and 1.3 propanediol and 3-hydroxy phenyl lactic acid was determined in culture supernatant (Magnusson et al., 2003).

Sjogren et al. described some fatty acids with antifungal characteristics. Yeast cells were found to be more sensitive than mold cells against hydroxylated fatty acids with minimum inhibition concentrations ranging between 10-100 μg/ml (Sjogren et al., 2003).

Additionally, it was reported *Lactobacillus hammesi* with antifungal hydroxyl fatty acids from linoleic acids in 2013. It was obtained from the conversion of linoleic acid into mono-hydroxy octadecanoic fatty with 0.7 g/L minimum inhibition concentration on Penicillium species (Crowley et al., 2013).

There are many studies about how antifungal lactic acid bacteria act on fungal growth. By using cell free supernatant, it was determined that the germ tube growth was delayed. Additionally, the hypial branching was reduced and cells were determined with irregular shapes. The studies concerning on microscopic techniques, the fungal cell wall was determined as the first target and the effect were continued with cytolytic activities.

Antifungal metabolites of the lactic acid bacteria act on membrane potential and leakage of the components on membrane. In the same article of Crowley et al., they stated the studies of different authors about lactic acid bacteria and yeast or mold species interactions.

4.3. Factors Affecting the Antifungal Activity of Lactic Acid Bacteria

As a general principle, in microbiological studies; the extrinsic and intrinsic factors are well known to be effective on the growth of microorganisms. But it has to be focused on the factors effective on the production of the metabolites which has antifungal effect. There are limited studies related to the factors affecting antifungal metabolite production of lactic acid bacteria.

The effect of time and temperature parameters for incubation period of the *Lactococcus lactis* ssp. *lactis* on the antifungal metabolites were reported in 1996 by Roy et al. and stated in the review of Bianchini in 2015. According to this study, it was found that, incubating bacteria at 30°C for 48 hours has the maximum effect on the antifungal characteristics of bacteria (Roy, et al., 1996, Bianchini, 2015) However, it was reported that, the maximum yield for antifungal production was achieved after 3-4 days of incubation for *Lactococcus lactis* spp. *diacetylactis* (Reddy and Ranganathan, 1985). Also *Lactobacillus plantarum* was reported to have highest antifungal production capacity at incubation temperature of 30°C (Dalie, et al., 2010 and Bianchini, 2015).

In study of Corsetti et al., it was determined that 48 hours incubation at 30°C, the maximum organic acid production was achieved for *Lactobacillus sanfransisco* with the growth medium pH 6.0 (Corsetti, et al., 1998, Corsetti et al., 2011).

The type of carbon source of the growth medium is effective on the antifungal effect of lactic acid bacteria. It was found that, addition of formic acid, acetic acid or ethanol into the growth medium with defined concentrations have an increasing effect on the antifungal activity of *Lactobacillus coryneformis* in liquid culture. In the same study, glycerol was determined to cause an increase in the antifungal activity against filamentous fungi (Magnusson and Schnürer, 2001).

As mentioned in previous parts, phenyl lactic acid has a great importance among antifungal metabolites. Valerio et al. have studied the effect of enrichment of the growth medium with phenylalanine and tyrosine addition on the antifungal activity. It was reported as increased phenyllactic acid production and therefore increased antifungal activity (Valerio, et al., 2004). To achieve higher phenyl lactic acid production addition of many amino acids and peptides were studied. Since the phenyl lactic acid production occurs in stationary phase and was an ongoing process, it was found that addition of peptides were more effective than addition of amino acids on the phenyl lactic acid

production (Vermeulen, et al., 2006). Additionally, in 2009, as a result of a response surface study related to the effect on different supplements into the growth medium for increased amounts of phenyl lactic acid, phenyl pyruvic acid caused an increase of the phenyl lactic acid production yields (Mu, et al., 2009). Dalie et al. have mentioned about the addition of glucose, yeast extract, NaCl, $CaCl_2$ to modulate the production of antifungal compounds The increased concentrations of NaCl from 0.5% to 3.0% caused higher antifungal activity for *Lactobacillus rhamnosus* and explained by the synergistic effect of NaCl and organic acids. Also calcium was reported to cause an increase in antifungal activity for *Lactobacillus rhamnosus* and *Pediococcus acidilactici* (Dalie, et al., 2010).

The pH of the growth medium was reported as effective on antifungal activity of lactic acid bacteria. All bacteria need a pH range for optimal growth. Beside this property, bacteria need a defined pH range for certain metabolite production. The pH dependence of production of antifungal metabolites was described as to be linked with other factors like temperature, time, substrate, etc. (Dalie, et al., 2010).

4.4. Lactic Acid Bacteria in Cheese Industry

Lactic acid bacteria includes many species belong to different genus; *Lactobacilli, Lactococci, Leuconostoc* and *Streptococci*. This part includes the main properties of this genus.

The most important species in *Lactococcus* genus is the *Lactococcus lactis* ssp.*lactis* and *Lactococcus lactis ssp. cremoris*. The optimum growth temperature of *Lactococcus lactis ssp.lactis and Lactococcus lactis* ssp. *cremoris* is about 26°C, and most strains will not multiply above 38°C. These two bacteria generally consist of 90 % of the mesophilic homofermentative cultures among dairy industry (Cocolin et al., 2018).

Lactococcus lactis strains are used to decrease the pH of the cheese milk at mesophilic temperatures below 5.4. at 6-8 hours. Additionally they are used for aroma formation related to their citrate metabolism.

Lactic acid bacteria do not have the ability to synthesize amino acids or vitamins. Therefore, their nutritional requirements are complex. Besides the presence of a fermentable sugar, they need sources of vitamins, nuclotides and aminoacids. Lactococci require branched-chain aminoacids (leucine, isoleucine or valine) and histidine for growth.

Lactococcus lactis ssp. *lactis* has ovoid cells with a diameter of 0.5-1.0µ. Some strains are found in duplicate while some can be found as long chains. The pH value it decreased is between 4.0-4.5 in glucose broth. It can produce 0.5-0.7 % L-lactic acid in milk. It has proteolytic activity (Cocolin et al., 2018). Also it has ability to produce are organic acid from mannitol, trehalose, sucrose, arabinose, xylose, lactose, maltose and glucose. On the other acid from rafinose, inuline, glycerol and sucrose not produced. Some strains can produce nisin as antmicrobial compound called bacteriocine. It has a broad spectrum to inhibit many Gram (+) pathogens (Favaro et al., 2015)

Lactococcus lactis ssp. cremoris generally has ovoid shaped cells which occur in long chains. The diameter of a typical cell is approximately 0.6-1.0 µ. In lıguid growth media the pH is decreased until 4-4.5. It can produce 0.5-0.7 % L-lactic acid in milk. Also *Lactococcus lactis ssp. cremoris* can produce asetoine, diacetyl and acetaldehyde in low amounts. It can not ferment trehalose, salicine, arabinose, xylose, inuline, glycerol and sorbitol. On the other hand it can ferment maltose, sucrose and raffinose rarely. If fermentable sugars are present in media, some strains can hydrolyse citrate and produce carbondioxide, asetic acid and diacetyl (Favaro et al., 2015 and Cocolin et al., 2018).)

Lactococcus lactis ssp.lactis biovar diacetylactis is important for its ability to form carbondioxide, diacetyl and acetone. It does not show heterofermentative characteristic. It can produce 0.3-0.6 % L-lactic acid in milk. While some strains show proteolytic activity, in general they show little or not proteolytic activity. Many times it produces hydrogen peroxide. Some strains of *Lactococcus lactis ssp. lactis* can also produce malty flavors due to primarily to 3-methyl butanal production from leucine

Leuconostocs are also cocci that occur in pairs and chains and are often ellipsoidal. Morphologicaly similarities make it difficult to distinguish them from Lactococcus. They are catalase negative and generally non-motile. Nicotinic acid, pantotenic acid, thiamin and biotin are necessary compounds for all species of Leuconostocs for growth. They convert glucose to D-lactic acid with heterofermentative

pathway and produce carbondioxide, ethanole by using both hegzose monophosphate and phosphoketolase pathways (Gobbetti et al., 2018).

Leuconostoc mesenteroides ssp. cremoris are ovoid and found in pairs or chains. They produce dextrose and ferment citrate. As a result they produce acetate, carbondioxide and pyruvate which will further be used to produce diacetyl and acetone.

Lactobacilli are a large group of rod-shaped bacteria including many species which can be subdivided in three main groups according to their fermentative metabolism. The lactobacilli in commercial starter cultures are *Lb. delbrueckii bulgaricus, Lb delbrueckii lactis, Lb. helveticus*; all are found in group 1 which indicates obligate homofermenters of hexoses to lactate and do not ferment pentoses.

Lactobacillus delbrueckii ssp. bulgaricus is a thermophilic species which is one of the two important species used in yoghurt manufacturing in combination with *Streptococcus thermophilus*. It converts lactose immediately and produce 1.8% D-lactic acid. *Lactobacillus delbrueckii ssp. lactis* is similar to *Lb.bulgaricus* with most of the properties. It is a thermophilic bacteria, and homofermentative. Also it can produce D-lactic acid 1.8% in growth media. *Lactobacillus helveticus* is a thermophilic species and can be used in combination with *Streptococcus thermophilus* in manufacture of cooked cheese types (Bernardeu et al., 2008).

Other important species of Lactobacilli are; *Lactobacillus paracasei sups. paracasei, Lactobaillus plantarum, Lactobacillus reuteri, Lactobacillus rhamnosus*, etc. which are generally used as adjunct culture in fermentations. Their main role as being adjunct culture is not to ferment lactose, but also they are important for sensory profile and microbial protections.

This study is about to use lactobacilli as adjunct culture to prevent yeast and mold growth related to the antifungal characteristic. For this purpose *Lactobacillus paracasei spp. paracasei, Lactobacillus rhamnosus, Lactobacillus plantarum* have been examined.

Streptococcus genus contains nearly 70 species but only one of them is found as starter culture; *Streptococcus thermophilus* which occurs in pairs and chains. It ferment sugars and produce L-lactic acid. It is a thermophilic bacteria and commonly used in yoghurt production.

Streptococcus thermophilus was found to decrease pH of the milk more rapidly when used in combination with *Lactobacillus bulgaricus* in yoghurt production, *Lactobacillus helveticus* in some types of cheese.

4.5. Nonstarter lactic acid bacteria as protective cultures

There are many types of lactic acid bacteria determined in literature in the genus of Lactococci, Leuconostoc, Lactobacilli and Propionibacteria. Based on the studies, the Genus Lactobacilli seems to have higher effect and potential for dairy industry. However this study is concerned on the effect of Lactobacillius plantarum, Lactobacillus rhamnosus and Lactobacillus paracasei spp. paracasei.

Starter lactic acid bacteria are high in number at the beginning of ripening and decrease regularly during aging, on the contrary NSLAB are present at low concentrations at the beginning but increase about four or five magnitude within a few months (Fox, 2004).

NSLAB have protective role in cheese production and can be considered as microorganisms conferring additional safety factors which result in the microbiological stability of foods, thus minimizing the risk of the growth and survival of food-borne pathogens and food spoilage organisms. Their ability to produce bacteriocins is of basic importance in strategies of biopreservation which results in extension of shelf-life and improvement of the safety of food (Ross et al., 2002).

Bacteriocins are a heterogenous group of ribosomally synthesized peptides or proteins displaying antimicrobial activity divided into 3 classes. Lactobacillus, Pediococcus, Leuconostoc and Enterococcus species can produce a wide range of Class II bacteriocins which are known as heat-stable. Class II bacteriocins are well-known bacteriocins having a strong antimicrobial activity against a broad range of Gram (+) spoilage and food borne pathogens, especially *Listeria monocytogenes* (Cintas et al., 2001, Savadogo et al., 2006).

Class I bacteriocins are the lantibiotics, small and heat-stable peptides containing posttranslationally modified aminoacids (Cintas, 2001). Class I bacteriocins from literature were documented by many researches. De Vuyst and Vandamme examine the *Lactococcus lactis* species to produce bacteriocins and isolated nisinA and nisinZ. Also other reserchers found additional bacteriocins for *Lactococcus lactis* strains as Lactococcin, Lacticin481, Lacticin3147 (de Vuyst and Vandamme, 1994, Piard et al., 1992).

In class II bacteriocins, Lactococcin M is found to be produced by *Lactococcus lactis ssp. cremoris* and Lactococcin A,B,G are found to be produced by *Lactococcus lactis ssp.lactis* (Van Belkum et al.,1991, Nissen-Meyer et al., 1992, Stoddard et al.,1992). From class II bacteriocins *Leuconostoc mesenteroides* ssp. was examined many times and found that three different types of mesentericin are produced (Hechard et al.1992 and 1999, Revol-Junelles et al., 1996).

Lactobacillus plantarum was found to produce Plantaricin S, PlantaricinE and F, Plantaricin J and K, Plantaricin 1.25α, Plantaricin 1.25β (Diep et al.1996). *Lactococcus lactis ssp.lactis biovar diacetylactis* was also found to produce Class II bacteriocin named as Diacetin B (Ali et al.1995*).Lactobacillus plantarum* was documented to produce Pediocin PA.

Additionally, lactic acid bacteria can produce some inhibitor compounds to control microbial growth. In a study with *Lactobacillus plantarum*, it was demonstrated that producing 4-hydroxy-phenyl-lactic acid show a strong inhibition action against mold species (Dalie et al., 2010).

Reuterin is a product of *Lactobacillus reuteri* from the glycerol fermentation which has strong inhibition activity against Aspergillus and Fusarium ssp. The addition of glycerol to some LAB cultures that produce reuterin was reported to increase their antifungal activity.

In a study, Danbo cheese were used to analyze for none starter lactic acid bacteria. They found that *Lactobacillus paracasei* was the most frequently isolated strain and *Lactobacillus plantarum* with *Lactobacillus curvatus* were reported as the main none starter lactic acid bacteria. Their protective effect was also studied and *Lactobacillus paracasei* with *Lactobacillus plantarum* were demonstrated as anti Gram (+) protective cultures (Antosson et al., 2003). These two bacteria were also reported as to have better protective effect when used together related to having a synergistic effect with Lactococci used as starter in cheese (Ortigosa et al., 2006).

Lactobacillus rhamnosus was tested to affect the sensory profile when used as protective culture and found to have and positive affect for both sensory characteristics and protective action (Milesi et al., 2009)

As an anticlostridial protective culture *Lactobacillus paracasei* found to have the highest inhibition action among 12 different Lactobacilli species (Christiansen et al. 2005). Luukkonen compared nitrate as chemical inhibitor and *Lactobacillus rhamnosus* as protective culture to inhibit the growth of *Listeria ssp*. and Enterohemorrhagic *E.coli.*

results indicate that lactobacillus rhamnosus alone is enough to control the contaminating microflora (Luukkonen et al., 2005). Atanassova demonstrate a compound produced by *Lactobacillus casei ssp.casei* named as 2-pyrolidone-5-carboxylic acid which inhibits *Bacillus ssp., Pseudomonas ssp*. and *Enterobacter cloacae* (Atanassova et al., 2003). Also they had mentioned the antifungal properties of same species in their study. The study performed on Boza, showed that *Lactobacillus plantarum, Lactobacillus pentosus, Lactobacillus rhamnosus, Lactobacillus paracasei* have protective effect and inhibit *Eschericia coli, Pseudomonas aeruginosa, Enterococcus faecalis* (Todorov and Dicks, 2006).

Propionicbacterium jensenii is the mostly used bacteria related to its antiyeast-antimold activity. These activities were reported as to depend on a heat stable bacteriocin produced by *Propionibacterium jensenii* (Ginstead and Barefoot, 1992).The inhibitory action of Propionibacterium was believed that it was achieved by propionic acid synthesized in the medium. But Daeschel isolated a heat-stable peptide with 700 Da but not characterized (Daeschel, 1989). After these studies, Ginstead and Barefoot characterized this heat stable polypeptide named as Jenseniin G in 1992.

Cell free supernatants could be used in growth media and they were found to inhibit the germtube formation of Fusarium species. The Fusarium cells were screened in growth medium supplemented with cell free supernatants of Lactobacillus fusarium. The inhibition of germtube formation related to time value was determined with cell free supernatants supplement up to %5 whereas a complete inhibition obtained by supplementing growth medium with 10% cell free supernatants (Mauch et al., 2010).

Also there are many additional studies about the cell free supernatant usage and their properties like heat stability, acid stability, stability against proteolytic enzymes and many of them will be discussed in chapter 6 in detail (Laref et al., 2013, Bazukyan, et al., 2018, Crowley et al., 2013, Guimaraes et al., 2018, etc.).

CHAPTER 5

MATERIALS AND METHODS

5.1. MATERIALS

5.1.1. Lactic Acid Bacteria Cultures

Lactic acid bacteria cultures were obtained from United States Department of Agricultural Research Service (ARS Culture Collection-NRRL Northern Regional Research Laboratory) in lyophilized forms.

Lactobacillus rhamnosus (B1937), *Lactobacillus plantarum* (B4496) and *Lactobacillus paracasei* subs. *paracasei* (B4560) were choosen as non-starter antifungal lactic acid bacteria for this study. They were all chosen according to their origin to be from food sources.

5.1.2. Fungal Cultures

Fungal cultures used in this study, consists of five different yeast species and one mold species. Yeast species were obtained from United States Department of Agricultural Research Service (ARS Culture Collection-NRRL Northern Regional Research Laboratory) in lyophilized forms.

Debaryomyces hansenii (YB 221), *Yarrowia lipolytica* (YB 423), *Pichia membranifaciens* (YB 12990), *Geotrichum candidum* (Y 552) and *Kluyveromyces*

marxianus (Y 1620) propagated in Dichloran rose bengal chloramphenicol agar (DRBCA).

Mold spore suspension was obtained from ATCC (American Type Culture collection). *Penicillium communee* ATCC 10428 was an isolate from cheese surface and propagated in Yeast Mold broths.

5.1.3. Growth Media

Enumeration and propagation of yeast and mold cells were performed with Dichloran Rose Bengal Chloramphenicol Agar (Merck, DRBC, Catalogue number: 100466) and Yeast extract glucose chloramphenicol agar (Merck, YGC, Catalogue number: 116000). Lactic acid bacteria enumerations were performed with de Man, Rogosa and Sharpe Agar (Merck, MRS Agar, Catalogue number: 110660). As liquid culture for lactic acid bacteria, MRS broth was used (Merck, MRS Broth, Catalogue number: 110661) for culture activations and propagation. Cultures were maintained as frozen stock cultures with the addition of 40 % glycerol (Merck, 356352) in to the liquid growth medium. Serial dilutions were carried on with sterile Ringers (Merck, Catalogue number: 115525) solution. Yeast mold growth media were prepared from its ingredients. Whey liquid media with 26 g/L whey powder (results in 20 g/L lactose in growth media) and 11 g/L yeast extract (Merck). Whey Liquid growth media were used for growth and antifungal determination experiments.Whey media was used at pH 5.6. Violet red bile dextrose (VRBD) (Merck) agar was used for Enterobacteriaceae analysis. DRBC agar was used for air sampling analysis for yeast and mold enumaratins. Violet Red Bile (VRB) agar (Merck) was used for total coliform counts, Coagulase positive Staphylococci Petrifilm (3M) was used for Coagulase positive staphylococci counts, Buffered peptone water(Merck), RVS broth (Biomerieux), mKTTn Broth (Biomerieux) and XLT4 (Biomerieux) agar, XLD agar (Becton-Dickinson) were used for *Salmonella* spp. analysis, Fraiser Broth (Merck), PALCAM (Merck) agar, Oxoid Agar (Merck), TSYE Agar (Merck) were used for *Listeria monocytgenes* analysis. The ingredients of growth media are given in Appendices.

5.2. METHODS

5.2.1. Activation of Lactic Acid Bacteria Cultures and Fungal Cultures

Lactic acid bacteria were propagated in MRS broths. Inoculated MRS broths were incubated at 37 °C. *Lactobacillus paracasei* and *Lactobacillus plantarum* were incubated aerobically, *Lactobacillus rhamnosus* was incubated anaerobically by using anaerobic jars (Oxoid Anaerobic Jar, 2,5 L) and anaerojen kit (Oxoid, Anaerojen Kit,for volume of 2,5 L).

After incubation in broth cultures, lactic acid bacteria were streak plated for colony growth. Sterile MRS agar was poured into sterile petri dishes and left for solidification and dry. Active broth cultures were used for streak plate with 0.1 ml of the culture. Activated MRS broth cultures were maintained as frozen stock cultures at broths with 40% glycerol -80 °C.

Yeast cells were propagated in yeast/mold broth and left for incubation for 3-5 days at 25°C. They were streak plated on to DRBC and YGC agar surface by taking 0.1 ml of the activated culture. Cultures were maintained as frozen stock cultures at broths containing 40% glycerol at -80 °C. Mold cells were activated on to agar slants and left for incubation at 25 °C for 5 days. Sterile peptone water (0.2% w/v) was used for vigorous shaking to take the spore suspension.

Spore suspensions were stored at -40° C with 40% glycerol stock solution. OD measurements at 600 nm were performed for further analysis of spore suspensions (Cheong et al., 2014 and Fernandez et al., 2017) Absorbance value with 0.5 at OD 600 nm was defined as $1*10^6$ spore/ml.

Serial dilutions were performed for further analysis from spore suspensions to reach the defined spore concentrations. Since OD measurement was a rapid method to determine the cell concentrations or spore concentrations, it was verified with enumeration on agar for all parts of study.

5.2.2. Growth Curves of Lactic Acid Bacteria and Fungal Cultures

Growth curves were constructed in order to determine the cell counts of the microorganisms at defined time values. Constructed growth curves include yeast mold broth for fungal cultures, whey liquid growth media for lactic acid bacteria cultures in order to mimic cheese environment.

Cultures were inoculated separately. All inoculations were performed in triplicate. Data for average values and standard deviations were analyzed and growth curve constructions were performed according to average value of triplicate analysis.

5.2.2.1. Growth Curves of Fungal Cultures in Whey Solution

Activated yeast cultures were inoculated into sterile growth media which included whey powder and yeast extract, with 2% inoculation ratio (v/v) and left for incubation at 24° C for 3 weeks. Samples were taken in defined time intervals for cell enumeration.

Sampling was performed by taking 1 ml from the broth solution. Samples were serially diluted with sterile peptone water (0.1 %). YGC agar was used for cell enumeration for pour plate technique. Plates were incubated at 25°C aerobically for 5 days.

5.2.2.2. Growth Curves of Lactic Acid Bacteria in Whey Solution

The activated cultures were used for inoculation into the whey growth media. Whey growth media was prepared with 26 g/L whey powder (to obtain final lactose content of 20 g/L) and 11 g/L yeast extract. Whey solution was sterilized by autoclaved before inoculation and pH was adjusted to 5.6.

The 2% inoculated cultures with the beginning cell count of 10^3 cfu/ml approximately were left for incubation at 37°C and cell counts were enumerated by taking samples at 2 hour time intervals. The inoculated cultures' cell counts were maintained with the help of Mac Farland Densitometer (Biosan Den 1B) and serial dilutions were performed. Samples from whey solution were serially diluted with sterile Ringer's solution. 1 ml from serial dilutions were analyzed for cell counts by pour plate method with sterile MRS agar and incubated at 37 °C. By taking logarithms of the cfu/ml counts, growth curves were constructed between logarithms of cell counts and time (hours). All analysis was performed in triplicate, the average data were used for growth curve.

5.2.3. Determination of Fungal Contamination Levels of the Cheese

White brined cheese samples were taken from market with 6 different brands, with different production batches. Totally n=180 samples were analyzed for yeast or mold contamination.

Cheese samples produced with commercially available protective culture; from brands codded as A, B, C, n=120, cheese without protective culture from the brands codded as X, Y, Z; n= 60, were analyzed for yeast and mold contamination levels.

Cheese samples were taken from market or from production plant was serially transferred to the microbiology laboratory. 10 g of cheese sample was taken aseptically and mixed with sterile Ringer's solution.

Ringer and sample solution was mixed with Bagmixer to obtain homogenous sample solution with filtered stomacher bags. Serial dilutions were done and 1 ml of the dilution was taken into petri dish and analyzed with pour plating YGC and DRBC agars, incubated at 24 °C for 5-7 days.

All analyses were performed in duplicate, results were explained as log cfu/g. Samples were named both alphabetic and numeric; UPC indicates cheese samples without protective culture; PC indicates cheese sample with protective culture.

5.2.4. Comparison of the Antifungal Effects of Commercial Protective Culture and Potassium Sorbate

In this study, two commercially available protective cultures were used; their names were codded as PCB and PCC representing two most widely used commercial cultures respectively. These cultures contain different lactic acid bacteria and their activity spectrums were different from each other. Potassium sorbate was also used in this study with two different concentrations; 0 ppm and 1000 ppm.

Potassium sorbate was added in pasteurized brine solution. Brine was prepared with 1000 ppm sorbate and without sorbate. Brine was prepared with NaCl addition into the pasteurized potable water. Effects of PCB, PCC and sorbate usage were screened due to 2-level factorial design, generated by Design Expert software (Stat-Ease).

In the ındustrial plant, those experiments were performed with 4 available cheese vats. For this purpose experiments were done in two separate batches. This caused to design experiments by using block option in software. Two blocks were chosen related to the two different production batches.

Experiments were done for the same quality raw milk. Each production experiment was performed with the steps explained in Chapter 2: Cheese Production Process. Samples were kept at +4 °C for further microbiological analysis. PCB and PCC were added into cheese milk at the same time with cheese starter cultures. Curd was left for fermentation. Selected curd samples were coded according to their contamination situations.

Cheese curd samples were contaminated with *Debaryomyces hansenii* and *Penicillium communee* cultures. Cultures were pre-activated and samples were taken for initial contamination levels.

Table 5.1. Protective Culture and Different Potassium Sorbate Concentrations

Factor Name	Level 1	Level 2
A: PCB	Present	Absent
B: PCC	Present	Absent
Sorbate Concentration	0 ppm	1000 ppm

For all production trials, cheese milk, equipment, personal contact area, water and microbiological quality of the air were analyzed whether mold or yeast contamination occurs or not at the steps of production.

Table 5.2. Factorial Design Table with Run Order

Std	Block	Run	Sorbate	PCB	PCC
7	Block 1	1	1000	present	absent
13	Block 1	2	0	present	present
1	Block 1	3	0	absent	absent
11	Block 1	4	1000	absent	present
9	Block 1	5	0	absent	present
15	Block 1	6	1000	present	present
5	Block 1	7	0	present	absent
3	Block 1	8	1000	absent	absent
8	Block 2	9	1000	present	absent
14	Block 2	10	0	present	present
10	Block 2	11	0	absent	present
4	Block 2	12	1000	absent	absent
6	Block 2	13	0	present	absent
16	Block 2	14	1000	present	present
12	Block 2	15	1000	absent	present
2	Block 2	16	0	absent	absent

The randomized factorial design with 2 blocks was constructed via Design Expert with totally 16 experiments. Responses were the increase in fungal culture cell counts. Analyses of the samples were performed in duplicate and results were given as average of the duplicates.

Samples were taken for yeast and mold analyses during 60^{th} day of the product. The effect of potassium sorbate and protective culture were tested with 23 full- factorial design. Factors were commercial cultures PCB and PCC (2 levels) and potassium sorbate (2 levels). Experiments were performed in replicate due to blocking.

Initial yeast and mold counts were analyzed previously from the samples taken at the after packaging with brine immediately. Results were expressed as average values of logarithms of yeast and mold counts.

At the day 60 of the cheese samples; analyses were performed for yeast and mold counts in order to determine the changes in fungal counts. Responses of the design were determined as decrease in yeast count with unit of log cfu/ml and decrease in mold count with unit of log cfu/ml. The effects of factors were analyzed related to the decrease that they were assumed to cause in fungal growth.

Cheese milk sample was taken from the vats. 100 ml of the standardized, pasteurized milk was taken for yeast, mold, Enterobacteriaceae, analysis.1 ml of the milk sample was taken into separate petri dishes and YGC agar was poured (approximately 20ml) and mixed with milk sample and left for dry. The petri dishes for yeast and mold analysis were incubated at 25 °C for 5-7 days. Yeast and mold colonies were counted separately and results were given in terms of cfu/ml. 1ml of the milk sample was taken into 3 separate petri dishes for Enterobacteriaceae analysis and VRBD agar was poured (approximately 20ml) and mixed with milk sample, left for drying. Petri dishes were poured with second layer of VRBD agar and left for drying. Petri were incubated at 36° C for 18-24 hours. Typical colonies of Enterobacteriaceae were counted and results were given in terms of cfu/ml. Enterobacteriaceae analyses were performed according to the method described in TS EN ISO 21528-2.

Equipment hygiene was tested via sponge swabs with deneutralizing broth (Sponge Sticks, 3M, United States). Sponge sticks were contacted with the surfaces of vats (after disinfection, before milk receipt), surfaces of the mixer of vats, knives, wires, personel hands, molds, walls of the mold storage room and empty packaging material. Sticks were left for effective adsorbtion in their own sterile bags for 30 minutes. 1 ml of the liquid part of the sponge stick sample was taken into 3 separate petri dishes and YGC agar was poured (approximately 20ml) and mixed with sample and left for dry. The petri dishes for yeast and mold analysis were incubated at 25 °C for 5-7 days. Yeast and mold colonies were counted separately and results were given in terms of cfu/ml. 1ml from the liquid part of the sponge stick sample was taken into 3 separate petri dishes for Enterobacteriaceae analysis and VRBD agar was poured (approximately 20ml) and mixed with sample, left for dry. Petri dishes were poured with second layer of VRBD agar and left for dry. Petri were incubated at 36 °C for 18-24 hours. Typical colonies of Enterobacteriaceae were counted and results were given in terms of cfu/ml.

Air hygiene was tested via air sampler machine (Mas 100 Eco, Merck) with the air volume of 400 m^3 for all parts of the plant. Petri dishes including sterile DRBC agar

were put in to the air sampler machine. After the machine works, petri were left for incubation for 5-7 days at 25 °C. Results were given in terms of cfu/400m^3 air.

5.2.5. Agar Spot Test for Screening Antifungal Activity of Single and Mixed Cell Cultures of Lactic Acid Bacteria

It was performed to screen whether the lactic acid bacteria chosen for this study have an antifungal effect or not. For this purpose lactic acid bacteria were grown on MRS agar and fungal cultures with different cell counts were lawn on MRS agar as soft agar and screened for colony formation.

Lactic acid bacteria were activated from stock culture with 5% inoculation ratio by taking in to sterile MRS broths (10 ml) and left for incubation anaerobically for 18-24 hours at 37 °C. Than the second activation was performed by taking bacteria from liquid culture and inoculate them into sterile MRS broth with an inoculation ratio of 2% and left for incubation for 24 hours at 37° C. Samples were taken from cultures to enumerate the cell counts before usage.

Yeast cells were taken from stock and activated in yeast mold broth by incubating them at 24°C for 2 days. Mold cell was activated from stock spore solution by propagating it into yeast mold broth and then plating onto Yeast Mold Agar. Spore suspension was taken into sterile peptone water and filtered by 0.45 mm Millipore sterile filter. The final spore solution was adjusted to a desired spore concentration by measuring absorbance at OD 600 nm. OD 600 nm represented $1*10^6$ spore/ml (Fernandez et al., 2017)

There were many studies performed with agar spot test or overlay method to determine the antifungal effect of lactic acid bacteria. Many of these studies were modified from the method described by Grinstead and Barefoot (Grinstead and Barefoot, 1992). This part of the study was also performed with the method Grinstead and Barefoot with some modifications.

Fungal cultures were tested with different cell concentrations. For this purpose serial dilutions were performed with sterile yeast mold broth. From each dilution, samples were taken to enumerate the cell counts. According to the literature, authors

generally published the studies for screening with this method for a constant fungal concentration. There was not a publication with different yeast and mold counts for overlay method. Additionally this part of study includes mixed culture combination for spot test and the publications generally included single cell spots.

Sterile MRS agar was poured into the petri dishes and left for solidification and dry. From the two times activated culture, cell counts were adjusted at 1.0 MacFarland by Densitometer (BioSan, Densitometer 1B). 0.1 ml of the standardized (1.0 macfarland) cultures were sported onto the center of the MRS agar and left for incubation at 37° C for 2 days (Hassan and Bullerman, 2007). 0.1 ml culture with 1.0 Macfarland results in 10^8 cfu/ml approximately. Tempered soft agar including 0.6% agar was prepared (6 ml) and mixed with 0.1 ml of fungal culture with defined cell counts (serial dilutions were performed to obtain different fungal counts to be tested) and lawn on the MRS agar surface which was spotted previously with lactic acid bacteria. Spot tested petri were left for incubation at 25 °C for 5-7 days. The antifungal effect of lactic acid bacteria were tested as single cell culture, mixture of two bacteria and mixture of three bacteria. Two or three bacterial mixtures were prepared from the same cell counted dilutions by mixing with equal volumes previously.

The inhibition against different cell counts of each fungal culture were recorded as inhibited or not and the antifungal effect related to initial contaminant count was determined.

5.2.6. Screening the Effect of Different Concentrations of Potassium Sorbate on Fungal Cell Cultures

Fungal cultures were activated from stock culture with yeast mold broth and incubated for 72 hours at 25° C and serially diluted.

Whey growth media with 26 g/L whey powder and 11 g/L yeast extract were prepared and autoclaved. Potassium sorbate was solved in sterile deionized water and mixed with whey growth media by filter sterilization. Whey growth media were prepared with 100 ppm, 500 ppm, 1000 ppm and 5000 ppm resulting sorbate concentrations.

Fungal cultures were inoculated from serial dilutions with an initial cell count of approximately between 3- 4 log cfu/ml. Fungal cultures (5 yeast and 1 mold culture) were inoculated into separate flasks and left for incubation at 25 °C for 10 days.
Samples were taken at 12 hours intervals and cell counts were performed by pour plating the serial dilutions on to the YGC agar.

All analysis were performed duplicate and data were calculated as the average value of duplicates, represented as log cfu/ml. Cell counts were plotted against time to observe the changes on the fungal cell counts.

5.2.7. Antifungal Effect of Lactic Acid Bacteria in Whey Growth Media

From the results of the spot test analysis, the most effective culture combination was used for this study. The antifungal effect of the culture was tested in liquid media including whey in order to mimic the cheese environment.

The protective cultures to be tested were prepared with different initial cell counts. The activated lactic acid bacteria were serially diluted with MRS broth to obtain different cell counts. The initial culture tube was standardized with Cell Densitometer (Biosan Den 1B) to 1.0 Macfarland ($3*10^8$ cfu/ml). Serial dilutions were performed to obtain $3*10^4$, $3*10^6$, $3*10^8$, $3*10^9$cfu/ml cell counts.

Activated fungal cultures were inoculated together with protective cultures into whey growth media and left for incubation at 25° C for 35 days. Samples were taken before inoculation to determine the initial fungal count and samples from whey growth media were taken at every defined time intervals (0,1^{st}, 2^{nd}, 3^{rd}, 4^{th},5^{th},6^{th},7^{th},8^{th},9^{th},10^{th}, 15^{th}, 20^{th}, 25^{th}, 30^{th} and 35^{th} day) and cell counts were performed by pour plating the serial dilutions on to the YGC agar.

All analysis were performed triplicate and data were calculated as the average value of triplicates, represented as log cfu/ml. Cell counts were plotted against time to observe the changes on the fungal cell counts. The initial inoculum concentrations of fungal cultures were high compared to a normal industrial contamination levels. For this purpose the activity of fungal cultures were examined on decreased fungal counts in

whey growth media. Fungal counts were maintained at 10^2 cfu/ml levels and the lactic acid bacteria concentration was prepared according to the data obtained from first part. Samples were stored at refrigerator in order to mimic the shelf life of the product

5.2.8. Determination of the Antifungal Activity of Cell Free Supernatants of Lactic Acid Bacteria with Spot Test

Lactic acid bacteria are activated for 18 hours at 37 °C in sterile MRS broths. Activation step was performed twice to obtain more active cells and antifungal metabolites with high concentrations. Second activation step was performed with inoculating culture into MRS broth. Incubation at MRS broth was performed for 72 hours at 37° C.

Broths were mixed via vortex and serially centrifuged at 8000g for 10 minutes. Finally cell free supernatant (CFS) were obtained for each culture by filter sterilization with 0.45 µm sterile filter (Millipore) (Zavaleta et al., 2014).

CFS was mixed with 20 ml YGC agar to a final concentration of 5% and poured into sterile petri dishes. Resulting media was centrally inoculated with 5 µl previously activated yeast/mold culture and incubated at 25°C (10^3 ,10^4 ,10^5 cell counts were used for spots). Control plates were prepared by mixing YGC agar with sterile MRS broth. Analyses were performed in duplicate.

5.2.9. Antifungal Lactic Acid Bacteria Applications on Cheese Samples

The antifungal cultures were tested for white brined cheese and kashar cheese samples. White brined cheese was tested before and after starter culture fermentation. The analyses were performed before and after starter culture fermentation on white cheese curd samples, and before packaging with brine solution, respectively

5.2.9.1. Antifungal Effect of Protective Culture on White Brined Cheese Curd

Cheese samples were taken from an industrial cheese plant. White brined cheese samples were taken at curd step before cheese starter completed the fermentation. After curd was poured into cheese moulds, then samples were taken from the moulds.

Lactobacillus rhamnosus, Lactobacillus plantarum and *Lactobacilus paracasei* were activated separately in MRS broths for two times. From the last activated broths the cultures were separately diluted via MacFarland standards to obtain 1.00 Macfarland standards ($3*10^8$ cfu/ml). Fungal cultures were activated in Yeast Mold broth and diluted to obtain 10^3, 10^4, 10^5 cfu/ml cell counts.

Cheese curd samples were weighted aseptically to 50g and transferred to zipped sterile plastic bags. 50 ml from the active lactic acid bacteria culture (the culture includes the bacteria mixture depending on the results of the spot test) was mixed with cheese curd which was previously placed in the sterile plastic bag (Interscience). The resulting cell concentration of the lactic acid bacteria were approximately $1.5*10^8$ cfu/g. Cheese sample with protective culture were left for effective adsorption at 36°C for 8 hours (equal to reference cheese sample's fermentation time). Samples were contaminated with fungal cultures. The samples were then inoculated with 3 different cell concentrations of 6 separate fungal cultures. For this purpose 10 ml of the fungal culture were added into bags to reach a resulting fungal cell count of 10^2, 10^3, 10^4 cfu/ml (Meile and Schewenninger et al., 2004, Cheong et al., 2014).

Totally 74 different samples were prepared and analyzed including triplicates with both fungal and antifungal inoculations (54 sample for triplicates), non-fungal culture added samples (for each fungal inoculation, totally 18 samples) and 2 for neither protective culture, nor fungal culture added sample as reference.

Samples were left at room temperature for 4 hours; closed bags were carefully mixed for effective adsorption. Cheese bags were taken at 4°C for 4 weeks. Samples were taken at time intervals for 0, 3^{rd}, 5^{th} , 7^{th} , 14^{th} , 21^{st} and 28^{th} days for yeast and mold enumeration. Results were calculated as logarithms of cfu/ml and average value of triplicates were plotted against time. Graphs are used to determine the changes in fungal cell counts.

Yeast and mold counts, *Enterobacteriaceae*, Total coliform, Coagulase positive *Staphylococci*, *Salmonella* spp. and *Listeria monocytogenes* analysis were performed from the samples of curd before fungal culture and protective culture inoculations.

5.2.9.2. Antifungal Effect of Protective Culture on White Cheese

Cheese samples were taken from the packaging step. Samples were not mixed with brine solution were used in order to avoid the effect of brine salt. Cheese samples were weighted 50g and placed in sterile plastic bags.

Lactobacillus rhamnosus, *Lactobacillus plantarum* and *Lactobacilus paracasei* were activated separately in MRS broths twice. From the last activated broths the cultures were separately diluted via MacFarland standards to obtain 1.00 MacFarland standards ($3*10^8$ cfu/ml). Approximately 50 ml of the protective culture were added in plastic sterile bags containing cheese sample in order to make sure that the cheese could be in contact with all surfaces. The resulting cell concentration of the lactic acid bacteria were approximately $1.5*10^8$.

Fungal cultures were activated in Yeast Mold broth and diluted to obtain 10^3, 10^4, 10^5 cfu/ml cell counts.

As a result of being at the end of the fermentation step of the main starter culture, the cheese was soaked in lactic acid bacteria culture solution and left for effective adsorption for only 1 hour at 36° C. Cheese were than taken out into a plastic sterile container aseptically for further parts of the examination.

Samples were contaminated with fungal cultures. They were inoculated with 3 different cell concentrations of 6 separate fungal cultures. For this purpose 10 ml of the fungal culture were added into containers to reach a resulting fungal cell count of 10^2, 10^3, 10^4 cfu/ml.

Totally 74 different samples were prepared and analyzed including triplicates with both fungal and antifungal inoculations (54 sample for triplicates), non-fungal culture added samples (for each fungal inoculation, totally 18 samples) and 2 for neither protective culture, nor fungal culture added sample as reference.

Samples were left at room temperature for 4 hours in closed containers for effective adsorption. Cheese samples were taken at 4°C for 4 weeks. Samples were taken at days 0, 3rd, 5th , 7th , 14th , 21st and 28th for yeast and mold enumeration. Results were calculated as logarithms of cfu/ml and plotted against time. Graphs are used to determine the changes in fungal cell counts.

Yeast and mold counts, *Enterobacteriaceae*, Total coliform, Coagulase positive *Staphylococci*, *Salmonella* spp. and *Listeria monocytogenes* analyses were performed from the samples of curd before fungal culture and protective culture inoculations.

5.2.9.3. Antifungal Effect of Protective Cultures During Shelf Life of White Cheese

The effects of antifungal lactic acid bacteria during shelf life to prevent the growth of yeast or mold species were tested. The cheese samples were prepared by inoculating 10^8 cfu/ml protective lactic acid bacteria into the white cheese curd as mentioned in part 5.2.9.1. The cheese samples after fermentation were left in their original packaging material in brine solution for 3 months in a refrigerator. Different from part 5.2.9.1., samples were not contaminated at the beginning of their shelf life.

Four of the samples were contaminated at the second week of shelf life; couple of them were contaminated with *Debaryomyces hansenii* with 10^3 cfu/ml resulting concentrations and remaining both were contaminated with *Penicillium commune* with 10^3 spore/ml resulting concentration levels.

Second sample set containing four identical cheeses were contaminated at the 4th week of their shelf life; another couple of them were contaminated with *Debaryomyces hansenii* with 10^3 cfu/ml resulting concentrations and remaining both were contaminated with *Penicillium commune* with 10^3 spore/ml resulting concentration.

Third sample set of samples were contaminated at the second month similar with previous contaminations and finally four cheeses were contaminated at the 3rd month of their shelf life. During 3 months of shelf life, control samples were taken into yeast and mold, *Enterobacteriaceae* analyses for their undesired contamination probabilities. The antifungal effect of lactic acid bacteria during shelf life was determined after

contaminations at different time intervals of the shelf life and the contaminated samples were analyzed periodically for three weeks to count the decrease in yeast and mold counts. Experiments were performed in parallels, the value were given as mean of duplicate samples.

5.2.9.4. Antifungal Effect of Protective Culture on Kashkaval Cheese

Samples were taken from an industrial dairy plant at the packaging step. Kashkaval cheese products, which were portioned in 20g were used as final product. Normally, they were vacuum packaged for commercial productions with 20g per package approximately.

In this part of the study, the 20g kashkaval cheese was taken into laboratory and 10 ml of the previously activated and mixed antifungal lactic acid bacteria suspension was used. The culture was streaked on each side of the kashkaval cheese with the help of a sterile spatula.

Lactobacillus rhamnosus, *Lactobacillus plantarum* and *Lactobacilus paracasei* spp.*paracasei* were activated separately in MRS broths for two times. From the last activated broths the cultures were separately diluted via MacFarland standards to obtain 1.00 Macfarland standards ($3*10^8$ cfu/ml). 10 ml of the culture was taken to streak on Kashkaval cheese. The final concentration of lactic acid bacteria on cheese was $1*10^8$ cfu/ml approximately.

The kashkaval cheese samples were left at 36°C for effective adsorption on the surface for 8 hours. Also, this time was necessary for the activation of lactic acid bacteria for both to achieve higher cell counts and starting to synthesize synthesize the antifungal metabolites. The cheese samples were taken into sterile plastic containers with screw caps. The yeast species was *Yarrowia lipolytica* and the mold was *Penicillium communee* for the study according to their growth characteristics. However other yeast species could be isolated from a contaminated kashkaval type cheese sample, *Yarrowia lipolytica* was thought be common related to its ability to grow in high fat products. Cheese samples were further inoculated with resulting cell counts of 10^3 cell/ml *Yarrowia lipolytica* and 10^3 spores/ml *Penicillium communee* cultures.

Cheese samples were left for effective adsorption of fungal cultures on surface and stored at 6 °C for 4 weeks. Yeast and mold cells were determined according to pour plating technique. Changes in fungal counts were determined by enumeration of each sample at days of 1^{st}, 7^{th}, 14^{th}, 21^{st} and 28^{th}. All analysis was performed in triplicate and data were analyzed from mean of triplicate samples.

5.2.10. Applications of Antifungal Lactic Acid Bacteria to Strained Yoghurt and Set Type Yoghurt

Strained and set-type yoghurt products are the other available dairy products for fungal contamination. For this purpose the strained yoghurt and set-type yoghurt were chosen for the industrial application studies.

Strained yoghurt samples were taken after fermentation step because of the closed process lines. Set-type yoghurt samples were taken before fermentation steps and the cultures were inoculated together with yoghurt starter cultures; *Streptococcus salivarius* spp. *thermophilus* and *Lactobacillus delbrueckii* spp. *bulgaricus*.

5.2.10.1. Applications of Antifungal Lactic Acid Bacteria to Strained Yoghurt

Strained yoghurt samples were taken from packaged products of an industrial dairy plant. 90 g strained yoghurt from the package was taken in to sterile plastic containers for analysis.

Lactobacillus rhamnosus, Lactobacillus plantarum and *Lactobacilus paracasei spp.paracasei* were activated separately in MRS broths for two times. From the last activated broths the cultures were separately diluted via MacFarland standards to obtain 1.00 Macfarland standards ($3*10^8$ cfu/ml). Serial dilutions were performed to obtain 10^7

cfu/ml cell counts. 10 ml of the culture was added into yoghurt sample and obtain a final volume of 90 ml with final protective lactic acid bacteria cell count of 10^6 cfu/ml.

Fungal cultures were activated in Yeast Mold broth and diluted to obtain 10^3, 10^4 cfu/ml cell counts approximately. Activated fungal cultures were than used for contamination of strained yoghurt samples. 10 ml from the fungal culture were added by dropping on the surface of the yoghurt and spread by a sterile spatula.

Samples were left at room temperature for effective adsorption for 1 hour, stored for further analysis at +4 °C. Yoghurt samples were analyzed until the end of their shelf life (25 days). Samples were taken at days 0, 1st, 5th, 10th, 20th, 25th for yeast and mold enumerations. Results were calculated as logarithms of cfu/ml and plotted against time. Graphs are used to determine the changes in fungal cell counts. Experiments were performed in triplicate and data were analyzed with the mean value of triplicates.

5.2.10.2. Applications of Antifungal Lactic Acid Bacteria to Set Yoghurt

Yoghurt process includes the inoculation of yoghurt starter bacteria which are *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in to standardized and pasteurized milk. In process line, yoghurt milk and culture is mixed with 2% ratio and filled in packaging material. Packages are sealed and left for incubation at 42°C until pH drops down to 4.4. approximately.

Samples were taken as packaged yoghurt milk with starter bacteria. The seals were opened aseptically and protective culture mixture was added 20 ml to 1000g yoghurt package. Yoghurt was also contaminated with fungal strains and samples were left for incubation at 42° C until pH drops 4.4 approximately.

Lactobacillus rhamnosus, Lactobacillus plantarum and *Lactobacilus paracasei* spp.*paracasei* were activated separately in MRS broths for two times. From the last activated broths the cultures were separately diluted via MacFarland standards to obtain 1.00 Macfarland standards ($3*10^8$ cfu/ml). Initial protective culture concentration resulted in $6*10^6$ cfu/ml approximately.

Fungal cultures were activated in Yeast Mold broth and diluted to obtain 10^3, 10^4 cfu/ml cell counts approximately. Activated fungal cultures were than used for contamination of yoghurt samples. 10 ml from the fungal culture were added by dropping on the surface of the yoghurt and spread by a sterile spatula.

Samples were left at room temperature for effective adsorption for 1 hour, stored for further analysis at +4 °C. Yoghurt samples were analyzed until the end of its shelf life (25 days). Samples were taken at days 0, 1^{st}, 5^{th}, 10^{th}, 20^{th}, 25^{th} for yeast and mold enumerations. Results were calculated as logarithms of cfu/ml and plotted against time. Graphs are used to determine the changes in fungal cell counts. Experiments were performed in triplicate and data were analyzed with the mean value of triplicates.

5.2.11. Sensory Evaluation

White Brined cheese and yoghurt samples were prepared for sensory tests. Triangle test was evaluated in order to differentiate the product that was prepared with antifungal lactic acid bacteria. The test was expressed in detail in the book of Stone and Sidel (Stone and Sidel, 2004)

White brined cheese was prepared by inoculating the antifungal culture at curd that was placed in molds. One of the random production vats was chosen for cheese experiments. Both reference samples and test sample were the cheese produced from same milk and the only difference was the antifungal culture inoculation at curd step. All remaining cheese production procedures were the same. Cheese samples for reference cheese and test cheese were taken to the laboratory and the outer surface was separated to avoid the effect of salt in brine which can cause false results with salt concentration.

Yoghurt samples for reference and test yoghurt were produced in the same production batch with same chemical properties which were produced from same milk. Yoghurt milk and starter culture were filled in the packaging material. The antifungal culture was inoculated at the same time and all yoghurts were proceeding with same process steps.

36 Panelists were selected from the specialist food engineers of the quality assurance department, research and development department and production department of a dairy plant. Training was not performed since all panelists are well trained about the sensory characteristics of cheese types and yoghurt (They were testing the cheese and yoghurt samples in their routine work life).

Actually the aim of the study was to produce the product without detectable effect on the sensory characteristics of the product. For this purpose, the task was to determine which sample was different from two other or which two samples are similar in three coded samples.

Statistical analyses were performed according to results and data were analyzed with 95 % confidence interval.

CHAPTER 6

RESULTS AND DISCUSSIONS

This section of thesis includes the results on the antifungal effects of lactic acid bacteria cultures on defined fungal cultures. Results of studies are also discussed in same sections with literature

6.1. Growth Curves of Fungal Cultures

Activated fungal cultures were separately inoculated in whey liquid media and results were obtained from the average value of triplicate analysis. Figure 1 represents the growth curves of yeast and mold cultures in whey media.

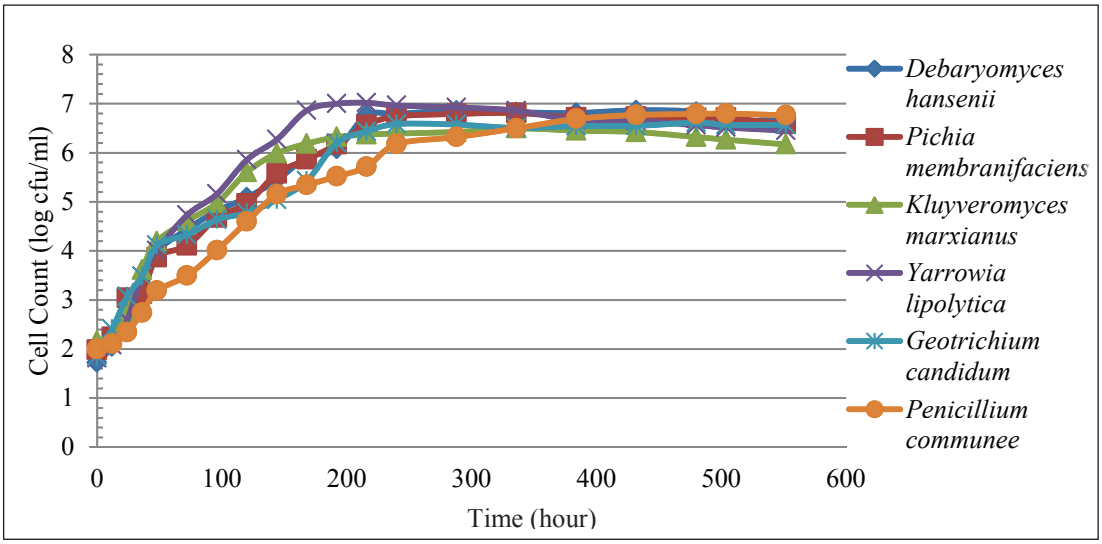

Figure 6.1. Growth Curve of Fungal Cultures in Whey Growth Media

As seen From Figure 6.1., From the growth curves of cultures; it can be observed that stationary phase was attained after 9 days after inoculation. This can be as a result of decreasing substrate concentration in batch culture. The constant substrate concentration was not enough to continue in logarithmic growth phase and they reached into the stationary phase of their growth. Maximum cell counts in logarithmic phase were observed with *Yarrowia lipolytica* and minimum were *Penicillium commune* and *Kluyveromyces marxianus*. Similar observations were done by other researchers (Büchl and Seiler, 2011). Also Table 6.1. includes the cell counts of the cultures with standard deviations.

Table 6.1. Cell Counts of Fungal Cultures with Time in Whey Liquid Media (Cell counts given in table are logarithms of cfu/ml)

Time (Hour)	*D. hansenii* (log cfu/ml)	*P.membranifaciens* (log cfu/ml)	*K. marxianus* (log cfu/ml)	*Y. lipolytica* (log cfu/ml)	*G.candidum* (log cfu/ml)	*P. commune* (log cfu/ml)
0	1.72 ±0.010	1.98 ±0.071	2.17 ±0.112	1.81 ±0.051	1.85 ±0.025	2.00 ±0.044
12	2.05 ±0.096	2.24 ±0.089	2.25 ±0.053	2.08 ±0.108	2.40 ±0.034	2.11 ±0.085
24	2.51 ±0.080	3.04 ±0.034	2.74 ±0.049	2.59 ±0.049	3.07 ±0.055	2.34 ±0.030
36	3.26 ±0.066	3.13 ±0.051	3.62 ±0.035	3.48 ±0.026	3.48 ±0.067	2.74 ±0.055
48	3.98 ±0.061	3.87 ±0.113	4.19 ±0.047	4.01 ±0.034	4.11 ±0.049	3.19 ±0.039
72	4.44 ±0.056	4.11 ±0.088	4.61 ±0.037	4.73 ±0.070	4.33 ±0.041	3.50 ±0.035
96	4.81 ±0.035	4.67 ±0.047	4.98 ±0.037	5.16 ±0.042	4.64 ±0.106	4.01 ±0.026
120	5.09 ±0.074	4.97 ±0.046	5.60 ±0.097	5.86 ±0.059	4.77 ±0.041	4.60 ±0.106
144	5.44 ±0.031	5.58 ±0.069	5.99 ±0.105	6.27 ±0.039	5.04 ±0.072	5.15 ±0.060
168	5.91 ±0.010	5.86 ±0.062	6.19 ±0.034	6.86 ±0.046	5.43 ±0.029	5.34 ±0.055
192	6.07 ±0.085	6.17 ±0.050	6.32 ±0.098	7.00 ±0.040	6.20 ±0.067	5.52 ±0.099
216	6.76 ±0.054	6.56 ±0.070	6.37 ±0.067	7.02 ±0.028	6.44 ±0.033	5.72 ±0.070
240	6.79 ±0.017	6.74 ±0.031	6.39 ±0.042	6.96 ±0.037	6.58 ±0.026	6.18 ±0.053
288	6.86 ±0.019	6.79 ±0.017	6.43 ±0.041	6.92 ±0.063	6.57 ±0.066	6.32 ±0.055
336	6.83 ±0.066	6.81 ±0.027	6.50 ±0.055	6.86 ±0.063	6.51 ±0.073	6.50 ±0.041
384	6.81 ±0.071	6.72 ±0.053	6.45 ±0.087	6.66 ±0.052	6.55 ±0.069	6.69 ±0.018
432	6.87 ±0.024	6.73 ±0.060	6.42 ±0.045	6.65 ±0.054	6.55 ±0.030	6.77 ±0.033
480	6.83 ±0.023	6.70 ±0.033	6.32 ±0.097	6.56 ±0.047	6.61 ±0.024	6.79 ±0.012
504	6.65 ±0.050	6.71 ±0.023	6.27 ±0.070	6.51 ±0.053	6.57 ±0.042	6.80 ±0.026
552	6.67 ±0.056	6.58 ±0.065	6.17 ±0.081	6.45 ±0.064	6.57 ±0.046	6.76 ±0.030

As seen from Table 6.1., standard deviations for *Debaryomyces hansenii* were in between 0.01 and 0.10 with an average value of 0.056, standard deviation for *Pichia membranifaciens* were in between 0.017 and 0.113 with an average value of 0.55, standard deviation for *Kluyveromyces marxianus* were in between 0.034 and 0.112 with an average value of 0.064, Standard deviations for *Yarrowia lipolytica* were in between 0.026 and 0.108 with an average value of 0.051, standard deviations for *Geotrichum candidum* were in between 0.024 and 0.106 with an average value of 0.050, standard deviations of *Penicillium commune* were in between 0.012 and 0.106 with an average of 0.049 respectively.

6.2. Growth Curve of Lactic Acid Bacteria in Whey Growth Medium

Growth curves of *Lactobacillus plantarum*, *Lactobacillus rhamnosus* and *Lactobacillus paracasei* were constructed by taking logarithms of cell counts of the average value of triplicate experiments as seen in Figure 6.2. They reached into a logarithmic phase approximately in 4 hours after inoculation and stationary phase was obtained in between 16 and 20 hours.

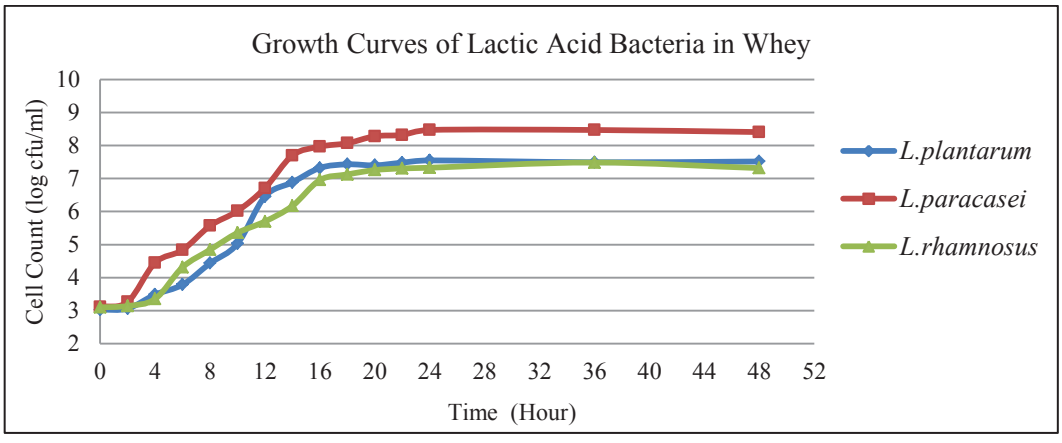

Figure 6.2. Growth Curves of Protective Lactic Acid Bacteria in Whey Growth Media.

Sampling was performed in triplicate and the standard deviations were calculated for each culture among triplicate data for all separate sampling intervals.

Cultures were in lag phase for 3 hours in order to adapt to growth media. After the lag phase, growth entered into the log phase with specific growth rates of 0.30 h^{-1}, 0.39 h^{-1}, 0.37 h^{-1} for *Lactobacillus rhamnosus*, *Lactobacillus plantarum* and *Lactobacillus paracasei* spp. *paracasei* , respectively in the whey medium.

Table 6.2. shows the cell counts of *Lactobacillus rhamnosus*, *Lactobacillus plantarum* and *Lactobacillus paracasei* spp. *paracasei* with standard deviations of triplicate analysis.

Table 6.2 Cell Counts of Lactic Acid Bacteria in Whey Medium

Time (Hour)	*Lactobacillus plantarum* (log cfu/ml)	*Lactobacillus paracasei* (log cfu/ml)	*Lactobacillus rhamnosus* (log cfu/ml)
0	3.025 ±0.023	3.113 ±0.047	3.097 ±0.025
2	3.060 ±0.027	3.263 ±0.084	3.138 ±0.011
4	3.484 ±0.010	4.449 ±0.029	3.352 ±0.014
6	3.784 ±0.018	4.835 ±0.015	4.313 ±0.013
8	4.434 ±0.051	5.570 ±0.015	4.842 ±0.013
10	5.026 ±0.075	6.017 ±0.034	5.355 ±0.023
12	6.458 ±0.016	6.711 ±0.007	5.701 ±0.015
14	6.886 ±0.063	7.701 ±0.009	6.172 ±0.131
16	7.324 ±0.029	7.970 ±0.008	6.956 ±0.015
18	7.432 ±0.044	8.073 ±0.041	7.126 ±0.036
20	7.402 ±0.018	8.283 ±0.008	7.258 ±0.024
22	7.483 ±0.013	8.321 ±0.013	7.309 ±0.004
24	7.551 ±0.009	8.474 ±0.047	7.329 ±0.010
36	7.495 ±0.015	8.473 ±0.015	7.488 ±0.009
48	7.519 ±0.029	8.410 ±0.017	7.318 ±0.088

Time values needed to attain stationary phase for the cultures were compared with literature. Other research related to growth curves were found similar with the findings of this thesis study (Delavenne et al., 2013 and Aunsbjerg et al., 2015).

Mean value for standard deviations of lactic acid bacteria were 0.040, 0.026, 0.029 for *Lactobacillus plantarum*, *Lactobacillus paracasei* spp. *paracasei* and *Lactobacillus rhamnosus*, respectively.

6.3. Determination of Fungal Contamination Levels of Cheese

Cheese samples taken from market or from an industrial plant were analyzed for yeast and mold contaminations. Cheeses of different brands were selected for examinations when commercially available protective cultures were applied. This part of study was performed in order to determine the contamination levels of white brand cheese which were present in markets. Additionally study includes determination the effect of protective culture usage on the fungal contamination levels. By the help of communications with some brands; the protective culture or sorbate usage data were collected and brands were selected according to the information thereafter (cheese brands information related to the protective culture name and brand name cannot be shared according to the privacy policy of the brands). Table 6.3. includes the contaminated sample percentage of commercial cheese products.

Table 6.3. Fungal Contamination Levels of Cheese with/without Protective Culture

Brand	Protective Culture	Yeast Contaminated Sample Number	Mold Contaminated Sample Number	Total Sample Number
A	with protective culture	20	12	40
B	with protective culture	19	16	40
C	with protective culture	17	17	40
X	without protective culture	14	15	20
Y	without protective culture(sorbate used)	13	17	20
Z	without protective culture	17	14	20

The samples including protective culture from 3 brands (A, B and C symbolizes the brand names) with 40 separate production batches were analyzed. Totally 120

samples were contaminated with yeast cells with a ratio of 46.6 % approximately. Contamination levels were in between 10 cfu/ml and 2100 cfu/ml for yeast enumerations. Samples were encountered for mold contamination with the ratio of 37.5% for 120 samples with cell counts between 10 cfu/ml and 150 cfu/ml.

The cheese samples produced without protective culture were from 3 different brands (X, Y and Z symbolizes the brand names) with 20 separate production batches with a resulting number of 60 samples totally. 73.33% of the samples were determined as contaminated with yeast cells with the cell counts between 10 cfu/ml and 2450 cfu/ml. 76.66% of the samples were determined as contaminated with mold cells with the cell counts between 10 cfu/ml and 190 cfu/ml approximately. It was underlined in the package of the brand Y, that sorbate was used at the stage of production. If the results of the brand Y was compared with the results of brands X and Z, the maximum number of cheese samples contaminated with mold were obtained from Y brand with a ratio of 85%. However, it was compatible with the literature since it was determined first in 1966 that mold species belong to *Penicillium* were able to metabolize sorbate (Marth et. al. 1966) and also it was known that, cheese contaminations related to mold growth includes *Penicillium* growth most frequently (Temelli et al., 2006). According to these findings, it was thought that, brand Y samples were generally contaminated with *Penicillium* as a result of sorbate presence in cheese formula.

Also, protective culture usage was determined as effective, when the results of cheese produced with protective culture compared to the results of cheese produced without protective culture.

6.4. Comparison of the Antifungal Effects of Commercial Protective Cultures and Potassium Sorbate

Cheese production is a complex system and involves many factors contributing the microflora of the cheese. As mentioned in previous chapters, cheese includes lactic acid bacteria as starter culture. Beside starter culture usage, cheese producers may prefer to use secondary starter culture for some sensory characteristics of different cheese types and for microbial protection related to antimicrobial characteristics of the

metabolites of lactic acid bacteria. Additional protection can be necessary frequently, protection might be performed by chemical preservatives. Among, potassium sorbate is the most widely used chemical preservative for dairy industry against fungal contamination (Pitt et al., 2014, Fernandez et al., 2017).

In some cases, protective culture and/or sorbate usage may not be able to protect the dairy products against fungal growth even they are used together with the maximum bpermitted levels. Protection highly depends on the type of contaminant flora, because effect of protective cultures limited with the spectrum of bacteria they include. Although being a good antifungal agent, sorbate is found to enhance the growth of some mold species; *Penicillium* was found to be metabolizing sorbate in cheese samples by degrading it via decarboxylation into trans-1,3-pentadiene (Marth et al. 1966, Sensidoni et al. 1994, Pitt et al. 2014, Fernandez et al., 2017).

The commercial cultures used in the study were codded as PCB and PCC. Protective culture PCB includes; *Lactobacillus rhamnosus* and *Propionibacterium freudenreichi* spp. *shermanii,* PCC includes; *Lactobacillus paracasei* and *Propionibacterium freudenreichi* spp. *shermanii,* respectively.

They were used in white brined cheese manufacturing in an industrial dairy plant. They were used according to the constructions of the suppliers. All of the three protective cultures were added together with the starter culture into the cheese milk.

Sorbate was prepared related to the concentrations obtained by the experimental design. The maximum concentration was the legally permitted level of sorbate usage concentration; 1000 ppm. The effect of protective culture and sorbate were performed together and the experimental runs were designed according to the design table obtained by Design Expert, as given in Table 6.4, randomized full factorial design. 16 separate runs were constructed with 2 blocks.

Initial yeast and mold counts were determined by taking 10g of sample from the contaminated cheese. Sample was mixed with sterile Ringer's solution and homogenized with Bagmixer. 1 ml from homogenized sample was taken into petri dish and YGC agar was poured in the same petri, mixed carefully and left for dry. Agars were incubated at 25 °C for 5-7 days. Samples were analyzed in parallels and results were explained as mean value of parallels in terms of log cfu/ml. Initial counts related to experimental runs are listed in Table 6.4 and decrease in cell counts of fungal cultures are listed in Table 6.5. with the mean value of duplicate analysis.

Table 6.4. Initial Fungal Counts of Contaminated Cheese Samples

Std	Block	Run	Sorbate (ppm)	PCB	PCC	Yeast count (log cfu/ml)	Mold count (log cfu/ml)
7	production day 1	1	1000	present	absent	3.806	3.483
13	production day 1	2	0	present	present	3.796	3.447
1	production day 1	3	0	absent	absent	3.806	3.491
11	production day 1	4	1000	absent	present	3.813	3.477
9	production day 1	5	0	absent	present	3.801	3.491
15	production day 1	6	1000	present	present	3.810	3.491
5	production day 1	7	0	present	absent	3.793	3.505
3	production day 1	8	1000	absent	absent	3.820	3.462
8	production day 2	9	1000	present	absent	3.814	3.491
14	production day 2	10	0	present	present	3.799	3.477
10	production day 2	11	0	absent	present	3.816	3.512
4	production day 2	12	1000	absent	absent	3.820	3.505
6	production day 2	13	0	present	absent	3.792	3.508
16	production day 2	14	1000	present	present	3.777	3.519
12	production day 2	15	1000	absent	present	3.799	3.477
2	production day 2	16	0	absent	absent	3.839	3.512

The cheeses were left at +4 °C for further analysis. 60 days later cheese samples were taken for the same analysis. Yeast and mold counts were determined from the average value of duplicates and expressed as log cfu/ml. Since the responses of the experimental design were decreased in yeast counts and decreased in mold counts (presented in Table 6.5), the difference between initial counts and the counts obtained at day 60^{th} were calculated.

The results of the software have determined the effective factors on the responses separately. From the half normal plot of the effects, firstly all of the variables selected as effective, the model F-value was 1.51. This value implies the model is not significant relative to the noise. The half normal probability plot was reanalyzed

according to p-values of the first model. The model was constructed with the factors given in table 6.6 which shows the model as significant due to the F-value.

Table 6.5. Decrease in Fungal Cell Counts (log cfu/ml)

Std	Block	Run	Sorbate (ppm)	PCB	PCC	Decrease in Yeast Count	Decrease in Mold Count
7	production day 1	1	1000	present	absent	0.444	-0.250
13	production day 1	2	0	present	present	0.289	0.430
1	production day 1	3	0	absent	absent	-0.427	-0.569
11	production day 1	4	1000	absent	present	0.493	-0.380
9	production day 1	5	0	absent	present	0.192	0.014
15	production day 1	6	1000	present	present	0.555	-0.414
5	production day 1	7	0	present	absent	0.150	0.014
3	production day 1	8	1000	absent	absent	0.228	-0.652
8	production day 2	9	1000	present	absent	0.432	-0.199
14	production day 2	10	0	present	present	0.255	0.398
10	production day 2	11	0	absent	present	-0.399	-0.585
4	production day 2	12	1000	absent	absent	0.465	-0.403
6	production day 2	13	0	present	absent	0.159	0.045
16	production day 2	14	1000	present	present	0.487	-0.351
12	production day 2	15	1000	absent	present	0.120	0.062
2	production day 2	16	0	absent	absent	0.236	-0.466

Table 6.6 respresents the ANOVA table for the response; decrease in yeast counts. The model terms; sorbate concentration was effective on yeast counts to cause decrease. P-values less than 0.0500 indicate model terms are significant values greater than 0.1000 indicate the model terms are not significant.

The model terms did not include all factors or their combinations depending on the p-value of terms. According to the f-values and p-values of the terms, the model was generated by using the factor A, B and AB. However, the factos C did not used in the

model. The PCB protective culture usage is effective on yeast counts, but not effective as sorbate usage.

Table 6.6. ANOVA Table for Selected Variables for the Response Indicating Decrease in Yeast Count

Source	Sum of Squares	Degree of Freedom	Mean Square	F-value	p-value
Block	0.0018	1	0.0018		
Model	0.7216	3	0.2405	5.14	0.0184
A-Sorbate Concentration	0.4793	1	0.4793	10.23	0.0085
B-PCB Usage	0.1955	1	0.1955	4.17	0.0657
AB	0.0255	1	0.0255	0.5449	0.4759
Residual	0.5152	11	0.0468		
Cor Total	1.24	15			

The ANOVA analysis for the second response corresponding decrease in mold counts is different from the first response. Table 6.7 was prepared according to ANOVA results of the model.

The model did not require selecting the terms. The model included all 3 factors and their combinations. From the first model, the F-Value indicates that the model is significant with all factors. But within the factors and their combinations, insignificants were present.

F-value of the model was 4.36, and the most effective factor on the response was the protective culture PCB usage alone. Sorbate could be thought as affective with p value of 0.06. PCC was not as effective as PCB.

Table 6.7. ANOVA Table for Selected Variables for the Response Indicating Decrease in Mold Count

Source	Sum of Squares	Degree of Freedom	Mean Square	F-value	p-value
Block	0.006	1	0.006		
Model	1.36	7	0.1944	4.36	0.0354
A-Sorbate Concentration	0.218	1	0.218	4.89	0.0626
B-PCB Usage	0.4398	1	0.4398	9.87	0.0163
C-PCC USage	0.1708	1	0.1708	3.83	0.0911
AB	0.3404	1	0.3404	7.64	0.0279
AC	0.0413	1	0.0413	0.9275	0.3676
BC	0.0351	1	0.0351	0.7872	0.4044
ABC	0.1153	1	0.1153	2.59	0.1518
Residual	0.3119	7	0.0446		
Cor Total	1.68	15			

However PCB and PCC were widely used as commercially to prevent fungal contaminations in dairy products. But the species belong to *Penicillium* species might show different sensitivities to different antifungal lactic acid bacteria. In the literature it was reported that, *Lactobacillus plantarum* was the most effective antifungal lactic acid bacteria against *Penicillium commune* in cottage cheese (Cheong et al., 2014). Additionaly, *Lactobacillus plantarum* was stated as the most effective antifungal strain among other lactobacilli but the commercial cultures do not have this species (Crowley et al. 2013, Fernandez et al. 2017). The commercially available protective cultures used in this study have not included *Lactobacillus plantarum*. The minimal effect on mold strain could be explained with the species included in the culture.

As a result of preliminary screening for the industrial preventive actions, this part was only used to determine whether sorbate was highly effective or not. Results were found similar with the studies about the sorbate metabolizing *Penicillium* species (Taniwaki et al., 2001). As seen from the results in Table 6.7, sorbate usage was not as effective as PCB usage on mold growth. However it was found effective on yeast growth.

The results related to the effect of sorbate were similar to the results of Fernandez et al. determined in 2017. They studied the effects of sorbate, propionate and found that propionic acid was more effective than sorbate on the growth of mold species. Also in 2001, Basilico et al. examined the antifungal effects of sorbate, propionate, parabens and natamycin antifungal effects on defined mold strains. They stated that; sorbate had little or no effect on the growth of molds (Basilico et al., 2001).

In 2004, Schwenninger and Meile studied the effects of propionic acid bacteria and lactic acid bacteria on the fungal growth. The propionic acid bacteria and lactic acid bacteria mixtures were the same as the contents of PCB and PCC. They studied the effects of the cultures on yoghurt samples and found that, the mixture of *Propionibacterium shermanii* and *Lactobacillus paracasei* caused a decrease with 2-3 logarithmic units while the mixture of *Propionibacterium shermanii* and *Lactobacillus plantarum* did not cause any antifungal effect (Schwenninger and Meile, 2014). But they were determined that the mixture of *Propionibacterium shermanii* and *Lactobacillus plantarum* had an effect on yeast species as fungistatic agent.

6.5. Spot Test for Screening Antifungal Activity of Single and Mixed Cultures of Lactic Acid Bacteria

Spot tests were performed according to the method described by Grinstead and Barefoot which was applied with the *Propionibacateria* for antifungal screenings and in this study the method was applied with some modifications (Grinstead and Barefoot, 1992). Also the same method was studied with many researchers with modifications (Schwenninger and Meile, 2004, Hassan and Bullerman, 2008, Voulgari et al., 2010, Delavenne et al., 2013, Lynch et al., 2014, Cheong et al., 2014, Delavenne et al., 2015, Fernandez et al., 2017, Buehler et al., 2018,). They generally used single strain of lactic acid bacteria. Also they did not test the fungal strains at different cell counts. However in this part the serial dilutions from stock fungal solution were performed and 3 different concentrations for each fungal strain were tested against indicator lactic acid bacteria culture for their antifungal effect. It was a useful data to compare the antifungal

effect of the culture against different initial fungal counts in order to determine whether the initial count affects or not. The plates were examined whether fungal colony had occurred on the spot or not after incubation at 25°C.The lactic acid bacteria were prepared according to Mac Farland 1.0 standard by using Densitometer (Biosan Den 1B). If necessary, serial dilutions were performed. The Mac Farland 1.0 standard was given as equal to $3x10^8$ cfu/ml cell counts. The table 6.8 was prepared according to the cell counts of lactic acid bacteria which were spotted with 0.1 ml volume onto the agar surface.

Table 6.8. Lactic Acid Bacteria Cell Counts for Spot Tests

Protective Culture Bacteria	Cell Count (cfu/ml)	Log (Cell Count cfu/ml)
B1937 *Lactobacillus Rhamnosus*	2.8×10^7	8.45
B4496 *Lactobacillus plantarum*	3.1×10^7	8.49
B4560 *Lactobacillus paracasei*	2.9×10^8	8.46
B4560 *Lactobacillus paracasei* B4496 *Lactobacillus plantarum*	2.6×10^8	8.42
B4560 *Lactobacillus paracasei* B1937 *Lactobacillus rhamnosus*	3.1×10^8	8.49
B4496 *Lactobacillus plantarum* B1937 *Lactobacillus rhamnosus*	3.3×10^8	8.52
B4496 *Lactobacillus plantarum* B1937 *Lactobacillus rhamnosus* B4560 *Lactobacillus paracasei*	2.9×10^8	8.46

Table 6.9 represents the antifungal effect for *Debaryomyces hansenii,* other tables are Table 6.10 for *Pichia membranifaciens*, Table 6.11 for *Kluyveromyces marxianus*, Table 6.12 for *Yarrowia lipolytica*, Table 6.13 for *Geotrichum candidum* and Table 6.14 for *Penicillium commune*, respectively.

Table 6.9. Antifungal Effect of Lactic Acid Bacteria on *Debaryomyces hansenii*

Protective Culture Bacteria	Cell Count of Protective Bacteria (cfu/ml)	*Debaryomyces hansenii* (cfu/ml)	Inhibition Status
B1937 *Lactobacillus Rhamnosus*	2.8×10^8	4.62×10^5	-
		6.54×10^4	±
		8.1×10^3	±
		7.3×10^2	+
		6×10^1	++
B4496 *Lactobacillus plantarum*	3.1×10^8	4.62×10^5	-
		6.54×10^4	-
		8.1×10^3	±
		7.3×10^2	+
		6×10^1	++
B4560 *Lactobacillus paracasei*	2.9×10^8	4.62×10^5	-
		6.54×10^4	±
		8.1×10^3	+
		7.3×10^2	+
		6×10^1	++
B4560 *Lactobacillus paracasei* + B4496 *Lactobacillus plantarum*	2.65×10^8	4.62×10^5	-
		6.54×10^4	-
		8.1×10^3	++
		7.3×10^2	++
		6×10^1	++
B4560 *Lactobacillus paracasei* + B 1937 *Lactobacillus rhamnosus*	3.1×10^8	4.62×10^5	-
		6.54×10^4	±
		8.1×10^3	±
		7.3×10^2	++
		6×10^1	++
B4496 *Lactobacillus plantarum* + B 1937 *Lactobacillus rhamnosus*	3.3×10^8	4.62×10^5	-
		6.54×10^4	-
		8.1×10^3	±
		7.3×10^2	++
		6×10^1	++
B4496 *Lactobacillus plantarum* + B 1937 *Lactobacillus rhamnosus* +B4560 *Lactobacillus paracasei*	2.9×10^8	4.62×10^5	+
		6.54×10^4	++
		8.1×10^3	++
		7.3×10^2	++
		6×10^1	++

Table 6.10. Antifungal Effect of Lactic Acid Bacteria on *Pichia membranifaciens*

Protective Culture Bacteria	Cell Count of Protective Bacteria (cfu/ml)	*Pichia membranifaciens* (cfu/ml)	Inhibition Status
B1937 *Lactobacillus Rhamnosus*	2.8×10^8	3.95×10^5	-
		4.56×10^4	±
		4.8×10^3	±
		6.1×10^2	+
		5×10^1	++
B4496 *Lactobacillus plantarum*	3.1×10^8	3.95×10^5	-
		4.56×10^4	-
		4.8×10^3	±
		6.1×10^2	+
		5×10^1	++
B4560 *Lactobacillus paracasei*	2.9×10^8	3.95×10^5	-
		4.56×10^4	±
		4.8×10^3	+
		6.1×10^2	+
		5×10^1	++
B4560 *Lactobacillus paracasei* + B4496 *Lactobacillus plantarum*	2.65×10^8	3.95×10^5	-
		4.56×10^4	±
		4.8×10^3	++
		6.1×10^2	++
		5×10^1	++
B4560 *Lactobacillus paracasei* + B 1937 *Lactobacillus rhamnosus*	3.1×10^8	3.95×10^5	-
		4.56×10^4	-
		4.8×10^3	±
		6.1×10^2	++
		5×10^1	++
B4496 *Lactobacillus plantarum* + B 1937 *Lactobacillus rhamnosus*	3.3×10^8	3.95×10^5	-
		4.56×10^4	-
		4.8×10^3	±
		6.1×10^2	++
		5×10^1	++
B4496 *Lactobacillus plantarum* + B 1937 *Lactobacillus rhamnosus* +B4560 *Lactobacillus paracasei*	2.9×10^8	3.95×10^5	+
		4.56×10^4	++
		4.8×10^3	++
		6.1×10^2	++
		5×10^1	++

Table 6.11. Antifungal Effect of Lactic Acid Bacteria on *Kluyveromyces marxianus*

Protective Culture Bacteria	Cell Count of Protective Bacteria (cfu/ml)	*Kluyveromyces marxianus* (cfu/ml)	Inhibition Status
B1937 *Lactobacillus Rhamnosus*	2.8×10^8	2.6×10^5	-
		2.0×10^4	±
		2.28×10^3	±
		2.4×10^2	+
		5×10^1	++
B4496 *Lactobacillus plantarum*	3.1×10^8	2.6×10^5	-
		2.0×10^4	-
		2.28×10^3	±
		2.4×10^2	++
		5×10^1	++
B4560 *Lactobacillus paracasei*	2.9×10^8	2.6×10^5	-
		2.0×10^4	±
		2.28×10^3	+
		2.4×10^2	++
		5×10^1	++
B4560 *Lactobacillus paracasei* + B4496 *Lactobacillus plantarum*	2.65×10^8	2.6×10^5	-
		2.0×10^4	±
		2.28×10^3	++
		2.4×10^2	++
		5×10^1	++
B4560 *Lactobacillus paracasei* + B 1937 *Lactobacillus rhamnosus*	3.1×10^8	2.6×10^5	-
		2.0×10^4	+
		2.28×10^3	++
		2.4×10^2	++
		5×10^1	++
B4496 *Lactobacillus plantarum* + B 1937 *Lactobacillus rhamnosus*	3.3×10^8	2.6×10^5	-
		2.0×10^4	-
		2.28×10^3	±
		2.4×10^2	++
		5×10^1	++
B4496 *Lactobacillus plantarum* + B 1937 *Lactobacillus rhamnosus* +B4560 *Lactobacillus paracasei*	2.9×10^8	2.6×10^5	+
		2.0×10^4	+
		2.28×10^3	++
		2.4×10^2	++
		5×10^1	++

Table 6.12. Antifungal Effect of Lactic Acid Bacteria on *Yarrowia lipolytica*

Protective Culture Bacteria	Cell Count of Protective Bacteria (cfu/ml)	*Yarrowia lipolytica* (cfu/ml)	Inhibition Status
B1937 *Lactobacillus Rhamnosus*	2.8×10^8	6.5×10^5	-
		7.4×10^4	-
		8.5×10^3	+
		6.1×10^2	+
		7×10^1	++
B4496 *Lactobacillus plantarum*	3.1×10^8	6.5×10^5	-
		7.4×10^4	-
		8.5×10^3	+
		6.1×10^2	+
		7×10^1	++
B4560 *Lactobacillus paracasei*	2.9×10^8	6.5×10^5	-
		7.4×10^4	±
		8.5×10^3	+
		6.1×10^2	++
		7×10^1	++
B4560 *Lactobacillus paracasei* + B4496 *Lactobacillus plantarum*	2.65×10^8	6.5×10^5	-
		7.4×10^4	-
		8.5×10^3	+
		6.1×10^2	++
		7×10^1	++
B4560 *Lactobacillus paracasei* + B 1937 *Lactobacillus rhamnosus*	3.1×10^8	6.5×10^5	-
		7.4×10^4	±+
		8.5×10^3	+
		6.1×10^2	++
		7×10^1	++
B4496 *Lactobacillus plantarum* + B 1937 *Lactobacillus rhamnosus*	3.3×10^8	6.5×10^5	-
		7.4×10^4	+
		8.5×10^3	++
		6.1×10^2	++
		7×10^1	++
B4496 *Lactobacillus plantarum* + B 1937 *Lactobacillus rhamnosus* +B4560 *Lactobacillus paracasei*	2.9×10^8	6.5×10^5	+
		7.4×10^4	+
		8.5×10^3	++
		6.1×10^2	++
		7×10^1	++

Table 6.13. Antifungal Effect of Lactic Acid Bacteria on *Geotrichum candidum*

Protective Culture Bacteria	Cell Count of Protective Bacteria (cfu/ml)	*Geotrichum candidum* (cfu/ml)	Inhibition Status
B1937 *Lactobacillus Rhamnosus*	2.8×10^8	3.8×10^5	-
		4.1×10^4	±
		4.6×10^3	±
		3.9×10^2	+
		6×10^1	++
B4496 *Lactobacillus plantarum*	3.1×10^8	3.8×10^5	-
		4.1×10^4	±
		4.6×10^3	±+
		3.9×10^2	++
		6×10^1	++
B4560 *Lactobacillus paracasei*	2.9×10^8	3.8×10^5	-
		4.1×10^4	±
		4.6×10^3	±+
		3.9×10^2	+
		6×10^1	++
B4560 *Lactobacillus paracasei* + B4496 *Lactobacillus plantarum*	2.65×10^8	3.8×10^5	-
		4.1×10^4	±
		4.6×10^3	+
		3.9×10^2	++
		6×10^1	++
B4560 *Lactobacillus paracasei* + B 1937 *Lactobacillus rhamnosus*	3.1×10^8	3.8×10^5	-
		4.1×10^4	±
		4.6×10^3	++
		3.9×10^2	++
		6×10^1	++
B4496 *Lactobacillus plantarum* + B 1937 *Lactobacillus rhamnosus*	3.3×10^8	3.8×10^5	±
		4.1×10^4	+
		4.6×10^3	++
		3.9×10^2	++
		6×10^1	++
B4496 *Lactobacillus plantarum* + B 1937 *Lactobacillus rhamnosus* +B4560 *Lactobacillus paracasei*	2.9×10^8	3.8×10^5	+
		4.1×10^4	+
		4.6×10^3	++
		3.9×10^2	++
		6×10^1	++

Table 6.14. Antifungal Effect of Lactic Acid Bacteria on *Penicillium commune*

Protective Culture Bacteria	Cell Count of Protective Bacteria (cfu/ml)	*Penicillium commune* (cfu/ml)	Inhibition Status
B1937 *Lactobacillus Rhamnosus*	2.8×10^8	1.8×10^5	-
		2.6×10^4	-
		3.1×10^3	±
		2.9×10^2	+
		3×10^1	++
B4496 *Lactobacillus plantarum*	3.1×10^8	1.8×10^5	-
		2.6×10^4	±
		3.1×10^3	±+
		2.9×10^2	++
		3×10^1	++
B4560 *Lactobacillus paracasei*	2.9×10^8	1.8×10^5	-
		2.6×10^4	-
		3.1×10^3	±
		2.9×10^2	+
		3×10^1	++
B4560 *Lactobacillus paracasei* + B4496 *Lactobacillus plantarum*	2.65×10^8	1.8×10^5	-
		2.6×10^4	+
		3.1×10^3	+
		2.9×10^2	++
		3×10^1	++
B4560 *Lactobacillus paracasei* + B 1937 *Lactobacillus rhamnosus*	3.1×10^8	1.8×10^5	-
		2.6×10^4	±
		3.1×10^3	++
		2.9×10^2	++
		3×10^1	++
B4496 *Lactobacillus plantarum* + B 1937 *Lactobacillus rhamnosus*	3.3×10^8	1.8×10^5	±
		2.6×10^4	+
		3.1×10^3	++
		2.9×10^2	++
		3×10^1	++
B4496 *Lactobacillus plantarum* + B 1937 *Lactobacillus rhamnosus* +B4560 *Lactobacillus paracasei*	2.9×10^8	1.8×10^5	±
		2.6×10^4	+
		3.1×10^3	+
		2.9×10^2	++
		3×10^1	++

As mentioned above, there were many studies in literature related to spot test assay, overlay agar test. They generally tested fungal microorganisms with constant cell concentrations; 10^4 cfu/ml approximately. As seen from the tables serial dilutions were tested on agar surface since the initial concentration was important for antifungal activity.

The spot test screening was used to determine the effect of single or mixed culture usage as antifungal culture. As seen from the results, for all fungal cultures tested, mix culture combination of each three lactic acid bacteria resulted in higher antifungal activities.

Delavenne et al. determined the antifungal effects of *Lactobacillus paracasei* and *Lactobacillus rhamnosus* as single cell cultures (Delavenne et al., 2013). The spot tests were performed against *Debaromyces hanseni, Yarrowia lipolytica* and *Kluyveromyces marxianus*. Their results were in agree with the results of this study. *Debaryomyces hanseni* was inhibited with both bacteria, but the inhibition effect of *Lactobacillus paracasei* was smaller than *Lactobacillus rhamnosus*. In this study, among the single cell cultures of three bacteria, maximum antifungal effect was observed from *Lactobacillus rhamnosus* with inhibition zones around colony for the concentration of 10^4 cfu/ml fungal counts. The synergistic effect was observed among *Lactobacillus plantarum*, *Lactobacillus paracasei* and *Lactobacillus plantarum*, *Lactobacillus rhamnosus* for 10^3 cfu/ml yeast counts when comparing the results obtained from single culture of *Lactobacillus plantarum*. The mixture of three lactic acid bacteria resulted in clear inhibition zones around colony at concentrations of 10^5 cfu/ml.

Kluyveromyces marxianus were inhibited with *Lactobacillus paracasei* and *Lactobacillus rhamnosus* single cell cultures with the fungal concentration of 10^4-10^5 cfu/ml whereas the single culture of *Lactobacillus plantarum* could only inhibit 10^2 cfu/ml. The synergistic effects of the cultures were observed by increased antifungal activities.

Pichia membranifaciens were inhibited with *Lactobacillus paracasei* and *Lactobacillus rhamnosus* single cell cultures with the fungal concentration of 10^4 – 10^5 cfu/ml whereas the single culture of *Lactobacillus plantarum* could only inhibit 10^3 cfu/ml. But the two culture combination of *Lactobacillus plantarum* with other two bacteria resulted in higher antifungal activities. Additionally as same with other fungal

cultures tested, the mixture of three bacteria had the maximum effect on yeast cell inhibitions.

Yarrowia lipolytica was the species that were tested many times by many researchers related to its resistance to high concentrations of organic acids and frequency of the isolated from contaminated yoghurt samples (Delavenne et al., 2013, Aunsjberg et al., 2015, Delavenne et al., 2015).

Yarrowia lipolytica culture with different concentrations was inhibited mostly by single cell of *Lactobacillus paracasei* when comparing with other single cell cultures. Inhibition was successed on cell counts of 10^3 – 10^4 cfu/ml yeast cells. The two culture combinations resulted in inhibition at lower cell counts. For the yeast counts at 10^2 – 10^3 cfu/ml the antifungal activity of *Lactobacillus paracasei* was increased by mixing it with *Lactobacillus plantarum.*

Geotrichum candidum (yeast like mold) was inhibited easily by the single cell antifungal cultures when comparing with other fungal species. *Geotrichum candidum* sample with 10^4 cfu/ml cell count was inhibited with all types of single cell culture treatments. From the two bacteria culture of lactic acid bacteria, same results were obtained for the cell counts of 10^4 cfu/ml, but the antifungal effect was increased for lower fungal counts with greater inhibition zones.

Penicillium commune was studied before by Cheong et al. in 2014 and Buehler et al. in 2018. From this study, the inhibition of 10^4 spore/ml *Penicillium commune* ATCC 10428 was inhibited with the single cell cultures of especially *Lactobacillus plantarum.* The antifungal activity of *Lactobacillus plantarum* was observed higher than other single cultures. Also the activity increases synergistically with the combination of other two lactic acid bacteria (Cheong et al. 2014, Buehler et al., 2018).

Results obtained from this part were useful in order to show the increased antifungal effect of mixed lactic acid bacteria cultures on defined fungal species. Additionally this part might be the first study for literature to determine the antifungal effect of culture mixtures and also being first study related to the usage of different concentrations of fungal cells.

6.6. Screening the Effect of Different Concentrations of Potassium Sorbate on Fungal Cell Cultures

Sorbate is a chemical preservative widely used in food industry to prevent fungal growth and *Penicillium* has the ability to metabolize sorbate as mentioned in part 6.4. In Turkey, according to legal limitations sorbate could be used up to 1000 ppm levels for dairy products.

This part of study were constructed to determine the effects of potassium sorbate in liquid growth medium which was inoculated with only 6 fungal cultures separately without antifungal bacteria. For this reason 4 different concentrations of sorbate were prepared with sterile deionized water and added into the previously autoclaved whey growth media by filter sterilization. Resulting sorbate concentrations in 200 ml whey growth media were 100 ppm, 500 ppm, 1000 ppm and 5000 ppm. Table 6.15 represents the cell count versus time (hour) of fungal cultures.

Table 6.15. Cell Counts of Fungal Cultures in Liquid Media with 100 ppm Sorbate Addition (cell counts are given as mean value of triplicate analysis in terms of log cell or spore count/ml with standard deviations)

Time (Hour)	*D.hansenii* (log cfu/ml)	*P. membranifaciens* (log cfu/ml)	*K. marxianus* (log cfu/ml)	*Y.lipolytica* (log cfu/ml)	*G. candidum* (log cfu/ml)	*P. commune* (log spore counts/ml)
0	2.69 ± 0.12	2.53±0.08	2.54±0.09	2.84±0.08	2.34±0.05	2.38±0.01
12	2.88 ± 0.05	2.90±0.08	2.85±0.15	2.83±0.03	2.51±0.03	2.63±0.02
24	3.10 ±0.13	3.28±0.04	3.35±0.04	3.73±0.05	3.53±0.11	2.85±0.06
36	3.44 ±0.06	3.46±0.07	3.73±0.05	3.90±0.01	4.23±0.09	3.11±0.08
48	3.67 ±0.01	3.79±0.11	3.96±0.07	4.23±0.08	4.44±0.03	3.37±0.07
60	3.81 ±.0.01	3.92±0.02	4.12±0.02	4.26±0.08	4.60±0.03	3.72±0.02
72	3.93 ±0.02	4.05±0.04	4.31±0.02	4.34±0.07	4.60±0.06	4.00±0.04
84	4.10 ±0.05	4.28±0.04	4.48±0.01	4.46±0.01	4.59±0.01	4.15±0.10
96	4.34 ±0.03	4.40±0.04	4.51±0.04	4.62±0.02	4.56±0.07	4.40±0.02
108	4.59 ±0.03	4.57±0.09	4.53±0.07	4.75±0.03	4.59±0.02	4.47±0.01
120	4.86 ±0.03	4.57±0.04	4.66±0.03	4.95±0.04	4.57±0.03	4.54±0.01

At the beginning of the incubation cell counts were in between 2.34 and 2.84 $\log_{10}$ cfu/ml. They increased for all types of microorganisms tested. As seen from the table, 100 ppm sorbate was found to have little antifungal effect on the yeast and mold cells. When the data was compared with the growth curve data obtained from the growth curve construction studies mentioned in Section 6.1., nearly the same initial cell concentrations were used.

120 hours after inoculation; the cell counts increased to 2.17 $\log_{10}$ cfu/ml for *Debaryomyces hansenii*, 2,04 $\log_{10}$ cfu/ml for *Pichia membranifaciens*, 2.11 $\log_{10}$ cfu/ml for *Kluyveromyces marxianus* and *Yarrowia lipolytica*, 2.23 $\log_{10}$ cfu/ml for *Geotrichum candidum* and 2.16 $\log_{10}$ cfu/ml for *Penicillium commune* approximately. Figure 6.3. represents the growth curves of culture with 100 ppm sorbate in growth media.

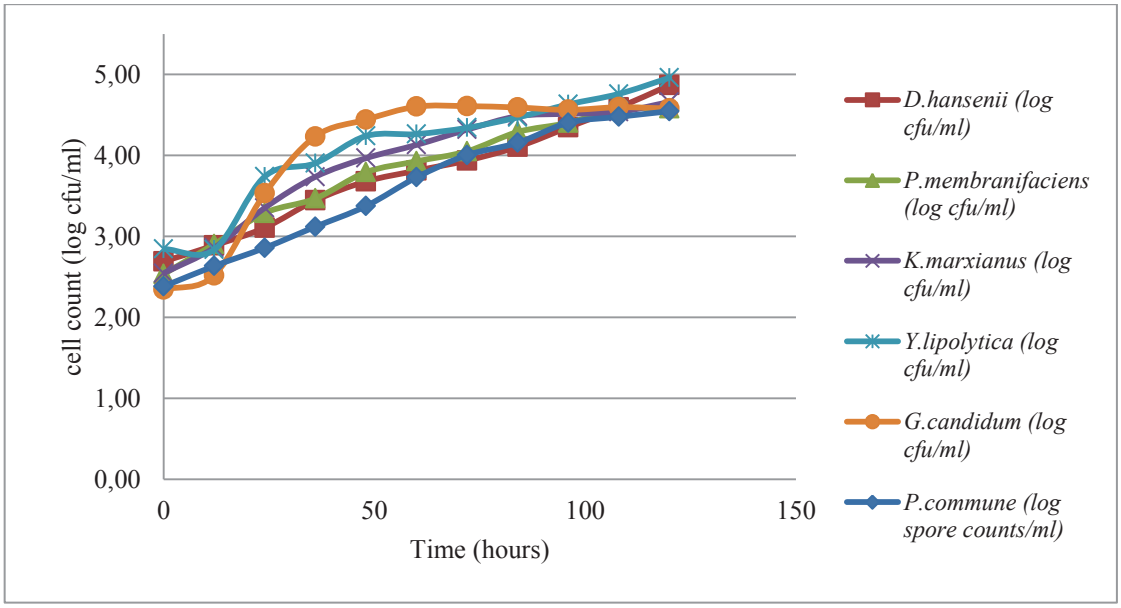

Figure 6.3. Growth of Fungal Cultures in Whey Liquid Media with 100 ppm Sorbate

The increase in cell or spore counts after 120 hours of inoculation was compared with the data obtained in Section 6.1. The growth curve was constructed in that part in liquid growth media without biological or chemical preservatives. According to the growth curve without any preservatives, the cell counts increased 3.37 $\log_{10}$ cfu/ml for

Debaryomyces hansenii, 2.98 $\log_{10}$ cfu/ml for *Pichia membranifaciens*, 3.44 $\log_{10}$ cfu/ml for *Kluyveromyces marxianus*, 4.05 $\log_{10}$ cfu/ml for *Yarrowia lipolytica*, 2.93 $\log_{10}$ cfu/ml for Geotrichum candidum and 2.60 $\log_{10}$ cfu/ml for *Penicillium commune* respectively. Sorbate usage caused to slow down with the increasing rate of cell counts between 0.68 and 1.67 $\log_{10}$ cfu/ml for fungal cultures when comparing with the growth values of the Section 6.1.; a decrease in the rate of growth was detected.

The maximum antifungal effect by the means of decreased growth rate was observed for *Yarrowia lipolytica* culture and the minimum was observed for *Penicillium commune* culture.

From the Figure 6.3, it can be observed that, they had continued to grow up to 10^5 cfu/ml levels. The initial level for inoculum was chosen as 10^2 cfu/ml levels to mimic the real contamination levels. Because in a dairy plant the yeast or mold levels of a contaminated cheese generally counted in the range of 10 – 1000 cfu/ml. Table 6.16. includes the growth data for fungal cultures which were inoculated in growth media including 500 ppm sorbate.

Table 6.16. Cell Counts of Fungal Cultures in Liquid Media with 500 ppm Sorbate Addition (cell counts are given as mean value of triplicate analysis in terms of log cell or spore count/ml)

Time (Hour)	*D.hansenii* (log cfu/ml)	*P.membranifaciens* (log cfu/ml)	*K.marxianus* (log cfu/ml)	*Y.lipolytica* (log cfu/ml)	*G.candidum* (log cfu/ml)	*P.commune* (log spore counts/ml)
0	2.33±0.08	2.41±0.09	2.34±0.12	2.53 ±0.06	2.46 ±0.05	2.19±0.15
12	2.35±0.04	2.51±0.14	2.57±0.03	3.24 ±0.07	2.58 ±0.09	2.68±0.02
24	2.30±0.01	2.63±0.20	2.82±0.06	3.38 ±0.04	2.96 ±0.05	3.03±0.11
36	2.64±0.04	2.82±0.18	2.99±0.02	3.62 ±0.05	3.50 ±0.10	3.05±0.03
48	3.31±0.08	3.31±0.09	3.49±0.04	4.01 ±0.16	3.55 ±0.06	3.19±0.01
60	3.63±0.09	3.80±0.03	3.81±0.04	4.09 ±0.04	3.72±0.16	3.44±0.06
72	3.85±0.08	3.84±0.05	4.00±0.03	4.14 ±0.08	3.88±0.12	3.54±0.00
84	3.87±0.04	3.85±0.08	3.82±0.06	4.14 ±0.10	3.91±0.05	3.78±0.06
96	3.94±0.06	3.77±0.10	3.83±0.02	3.99 ±0.05	3.96±0.07	3.97±0.07
108	4.00±0.03	3.66±0.15	3.71±0.06	3.96 ±0.03	3.98±0.05	4.09±0.04
120	4.05±0.03	3.42±0.05	3.63±0.02	4.01 ±0.09	3.96±0.01	4.22±0.05

500 ppm sorbate concentration is the half of legal usage limit for cheese industry. In general, cheese manufacturers use sorbate with concentration value between 500 and 1000 ppm for white cheese productions.The initial cell concentration was in between 2.19 and 2.53 $\log_{10}$ cfu/ml approximately. Sorbate with 500 ppm concentration did not cause a full antifungal effect, but it caused a higher effect related to the increase in cell counts when compared with 100 ppm sorbate addition. The cell counts increased 1.71 $\log_{10}$ cfu/ml for *Debaryomyces hansenii,* 1.01 $\log_{10}$ cfu/ml for *Pichia membranifaciens,* 1.29 $\log_{10}$ cfu/ml for *Kluyveromyces marxianus*, 1.47 $\log_{10}$ cfu/ml for *Yarrowia lipolytica*, 1.49 $\log_{10}$ cfu/ml for *Geotrichum candidum* and 2.03 $\log_{10}$ cfu/ml for *Penicillium commune* approximately. When the data were compared with the growth data obtained without any preservatives; the increase rates in the cell numbers were slow down with 500 ppm sorbate usage. Although the cultures continued to increase in cell numbers, the sorbate usage caused a decrease in the growth rates of fungal cultures. Also there could be seen from the Figure 6.4. fungal cultures lag phase period became longer with increased sorbate usage.

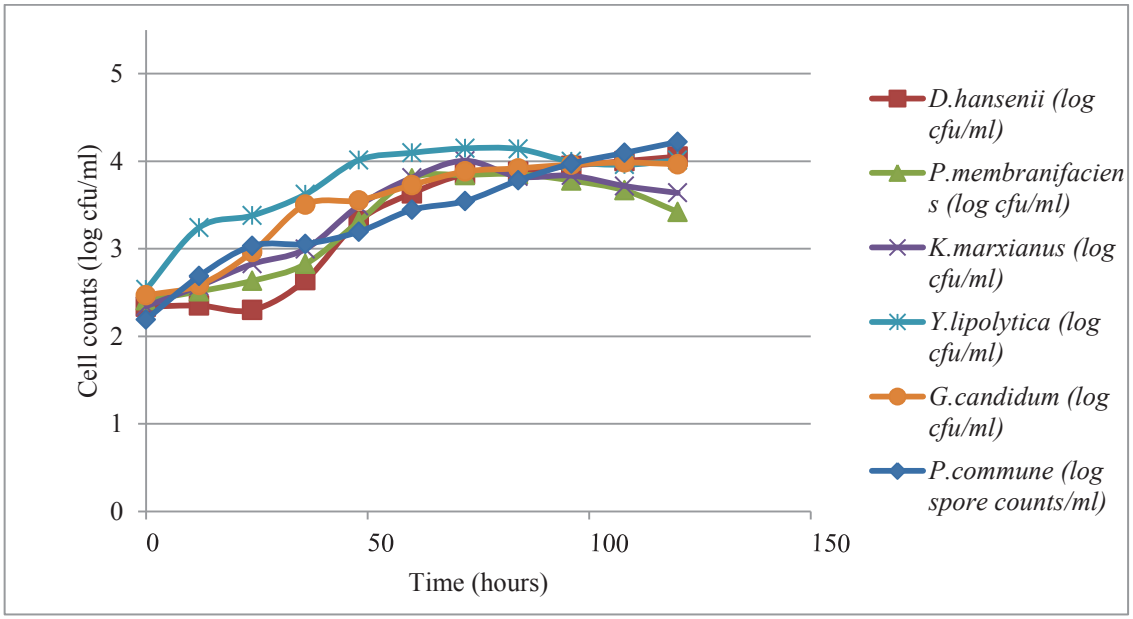

Figure 6.4. Growth of Fungal Cultures in Whey Liquid Media with 500 ppm Sorbate

From the Figure 6.4., it could be observed that 500 ppm would not be the true inhibitory concentration for these cultures. They caused the cultures to grow up to $1x10^3$ – $10x\ 10^3$cfu/ml levels. In a commercial dairy product this would cause quality problem for shelf life of the product. In order to observe the effect of sorbate with its maximum legal limits, cultures were grown in media including 1000 ppm sorbate and Table 6.17 includes the cell counts of fungal cultures with 1000 ppm sobate.

Table 6.17. Cell Counts of Fungal Cultures in Liquid Media with 1000 ppm Sorbate Addition (cell counts are given as mean value of triplicate analysis in terms of log cell or spore count/ml)

Time (Hour)	*D.hansenii* (log cfu/ml)	*P. membranifaciens* (log cfu/ml)	*K. marxianus* (log cfu/ml)	*Y.lipolytica* (log cfu/ml)	*G. candidum* (log cfu/ml)	*P.commune* (log spore counts/ml)
0	2.42±0.06	2.41±0.02	2.41±0.05	2.25±0.06	2.27±0.03	2.42±0.35
12	2.61±0.01	2.62±0.01	2.41±0.09	2.20±0.03	2.19±0.01	2.59±0.05
24	2.63±0.02	2.65±0.05	2.48±0.17	2.42±0.05	2.46±0.01	3.17±0.08
36	2.71±0.03	2.73±0.05	2.55±0.06	2.38±0.12	2.53±0.06	3.76±0.03
48	2.97±0.14	2.75±0.21	2.78±0.01	2.59±0.13	2.49±0.01	3.94±0.06
60	3.15±0.07	3.01±0.08	2.84±0.07	2.54±0.08	2.80±0.04	4.06±0.02
72	3.26±0.05	3.23±0.02	3.00±0.02	2.63±0.03	2.96±0.08	4.19±0.02
84	3.18±0.06	3.19±0.02	2.95±0.06	3.04±0.02	3.07±0.05	4.28±0.04
96	3.13±0.01	3.27±0.03	3.16±0.02	3.11±0.04	2.98±0.07	4.31±0.01
108	3.07±0.06	3.13±0.07	3.03±0.01	3.27±0.03	3.01±0.04	4.36±0.01
120	3.05±0.03	3.11±0.04	3.21±0.05	3.22±0.01	2.88±0.02	4.41±0.01

1000 ppm sorbate is the maximum level for dairy products. But as seen from the table 6.17 which represents the cell numbers of fungal cultures, yeast and mold cells continue to grow with a slower growth rate when compared the data obtained from 100 and 500 ppm sorbate addition.Figure 6.5. represents the growth curves of fungal cultures with 1000 ppm sorbate in growth media.

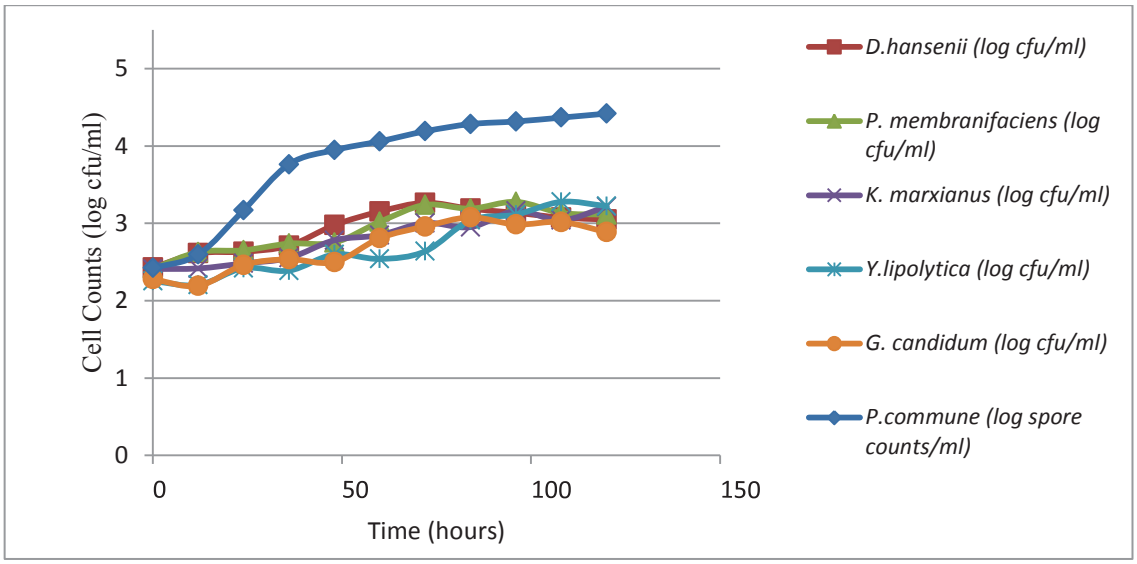

Figure 6.5. Growth of Fungal Cultures in Whey Liquid Media with 1000 ppm Sorbate

Sorbate was also added with a very high concentration; 5000 ppm, in order to observe whether cells would die or not. Table 6.18 includes the cell counts of fungal cultures with the 5000 ppm sorbate in growth media.

Table 6.18. Cell Counts of Fungal Cultures in Liquid Media with 5000 ppm Sorbate Addition (cell counts are given as mean value of triplicate analysis in terms of log cell or spore count/ml)

Time (Hour)	*D.hansenii (log cfu/ml)*	*P. membranifaciens (log cfu/ml)*	*K. marxianus (log cfu/ml)*	*Y. lipolytica (log cfu/ml)*	*G. candidum (log cfu/ml)*	*P.commune (log spore counts/ml)*
0	2.35±0.17	2.31±0.01	2.21±0.01	2.37±0.02	2.18±0.05	2.37±0.01
12	2.52±0.02	2.34±0.05	2.31±0.01	2.28±0.01	2.21±0.05	2.34±0.09
24	2.48±0.01	2.25±0.03	2.11±0.04	2.25±0.03	2.12±0.06	3.08±0.10
36	1.97±0.03	1.90±0.18	2.18±0.05	2.05±0.08	1.88±0.03	3.80±0.04
48	0.65±0.09	0.73±0.04	1.38±0.05	1.79±0.04	2.02±0.02	3.88±0.03
60	-	-	-	0.88±0.02	1.45±0.02	3.82±0.01
72	-	-	-	-	0.50±0.07	3.82±0.05
84	-	-	-	-	-	3.76±0.06
96	-	-	-	-	-	3.45±0.02
108	-	-	-	-	-	3.36±0.09
120	-	-	-	-	-	3.51±0.03

As a result of applying high concentration of sorbate in growth media, all yeast cells were dead after 60-72 hours of inoculation into the growth media except *Penicillium commune.* It continued to grow with increased cell counts up to 10000 cfu/ml levels. Figure 6.6 respresents the growth curves of fungal cultures with 5000 ppm sorbate in growth media.

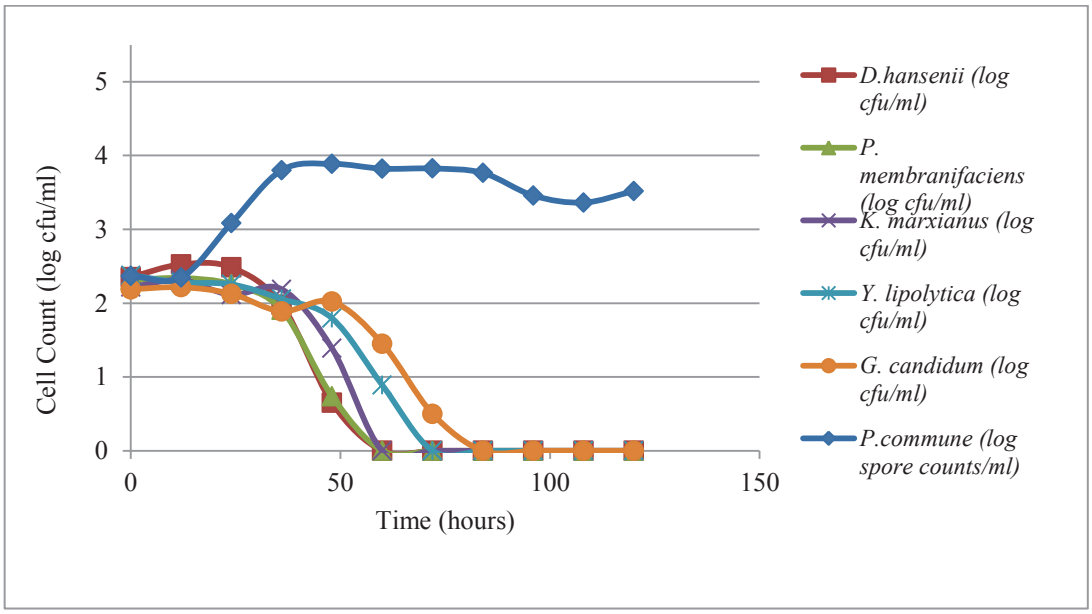

Figure 6.6. Growth of Fungal Cultures in Whey Liquid Media with 5000 ppm Sorbate

As seen from the Figure 6.6, *Penicillium commune* continued to grow. From the Figure 6.7, growth curves of *Penicillium commune* with different concentrations of sorbate were seen.

The control curve was used from the data of Section 6.1 which includes the same growth media without sorbate.

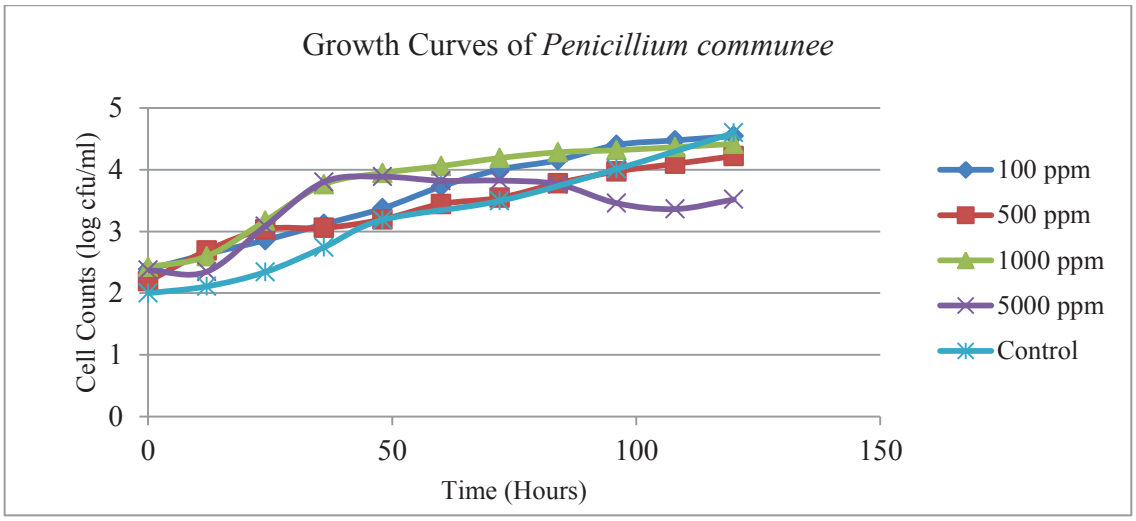

Figure 6.7. Growth Curve of *Penicillium commune* with different Sorbate Concentrations.

Also Figure 6.8, 6.9, 6.10, 6.11, 6.12 respresent the growth curves of yeast or mold cell cultures with different sorbate concentraions and the control curve of the culture. The growth curves of other fungal cultures were placed below includes *Debaryomyces hanseii, Yarrowia lipolytica, Pichia membranifaciens, Kluyveromyces marxianus* and *Geotrichum candidum.*

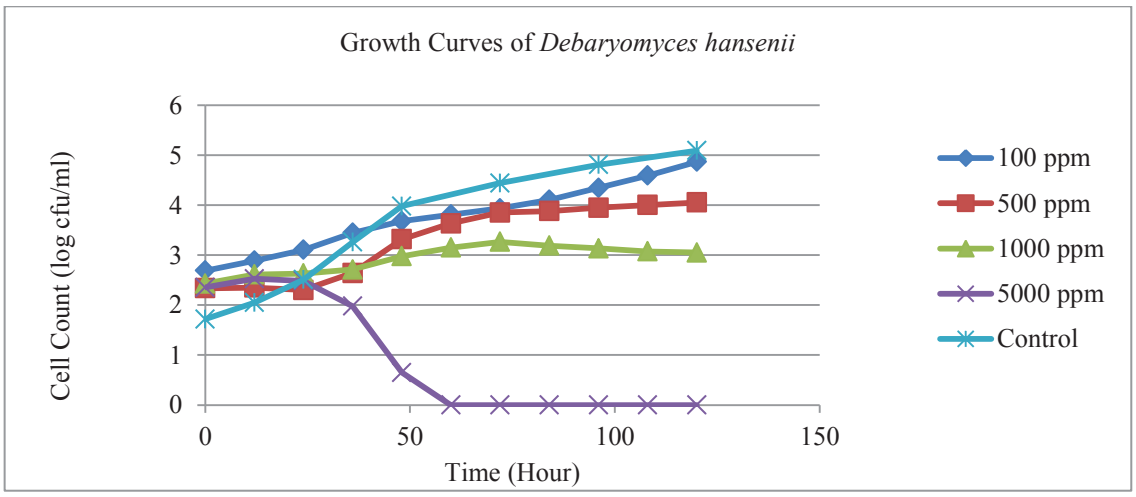

Figure 6.8. Growth Curves of *Debaryomyces hansenii* with Different Sorbate Concentrations

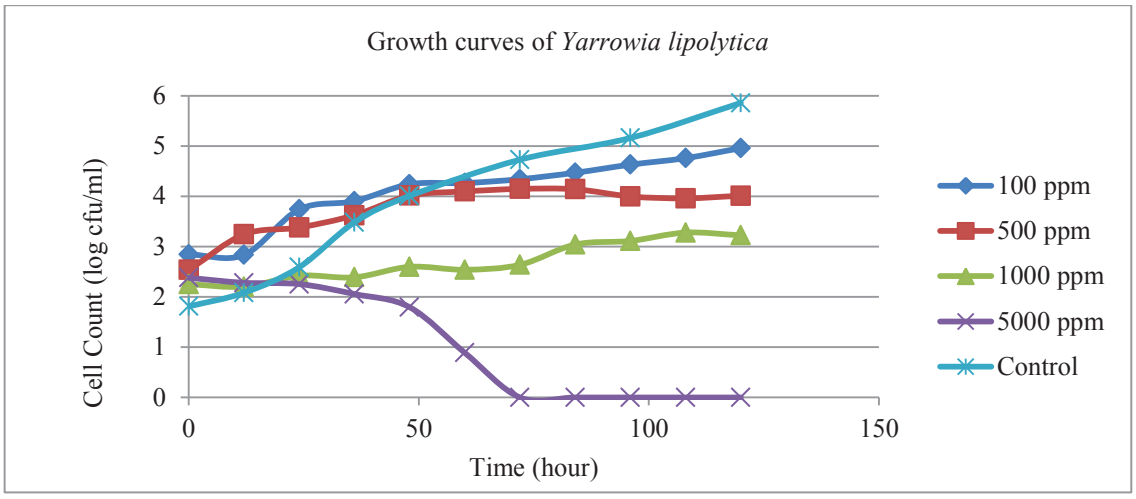

Figure 6.9. Growth Curves of *Yarrowia lipolytica* with Different Sorbate Concentrations

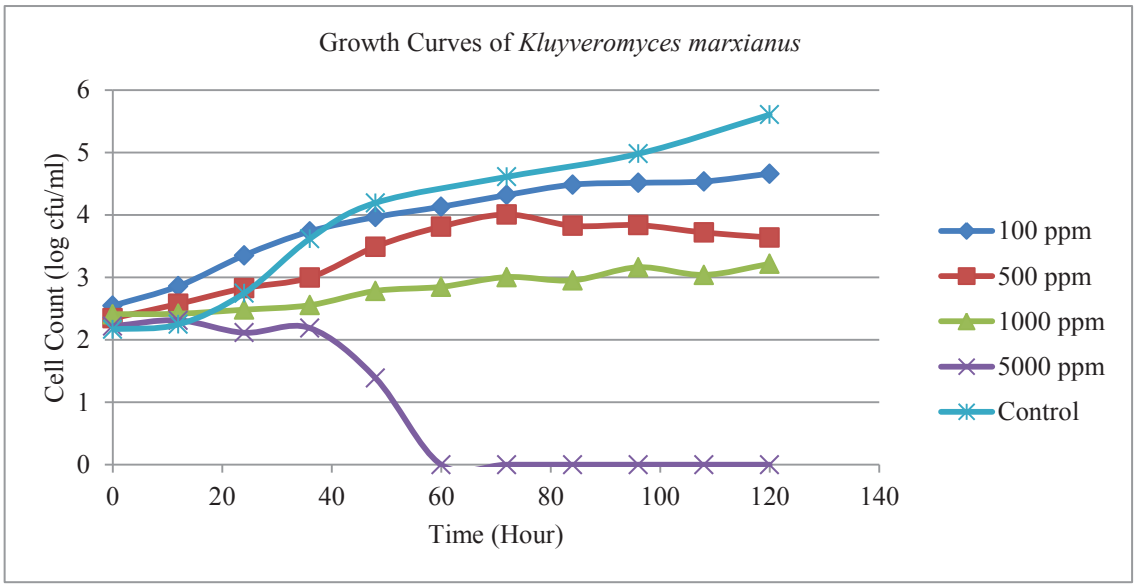

Figure 6.10. Growth Curves of *Kluyveromyces marxianus* with Different Sorbate Concentrations

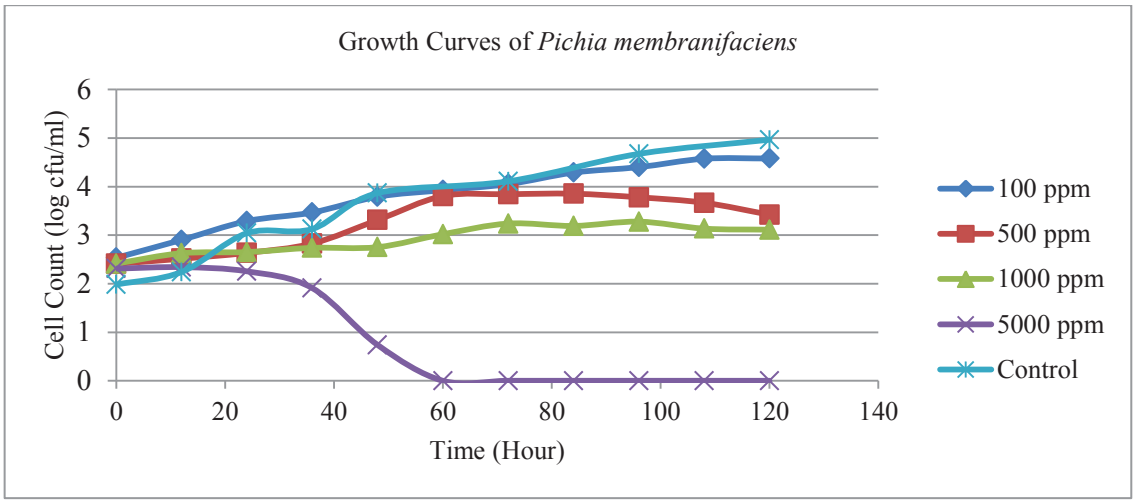

Figure 6.11. Growth Curves of *Pichia membranifaciens* with Different Sorbate Concentrations

From the growth curves of yeast cultures, they continued to growth except 5000 ppm sorbate usage. The concentrations lower than 5000 ppm caused an increased lag phase for the cultures. The growth rates were decreased. Figure 6.12 repsresents the growth curves of *Geotrichum candidum* with different sorbate concentrations.

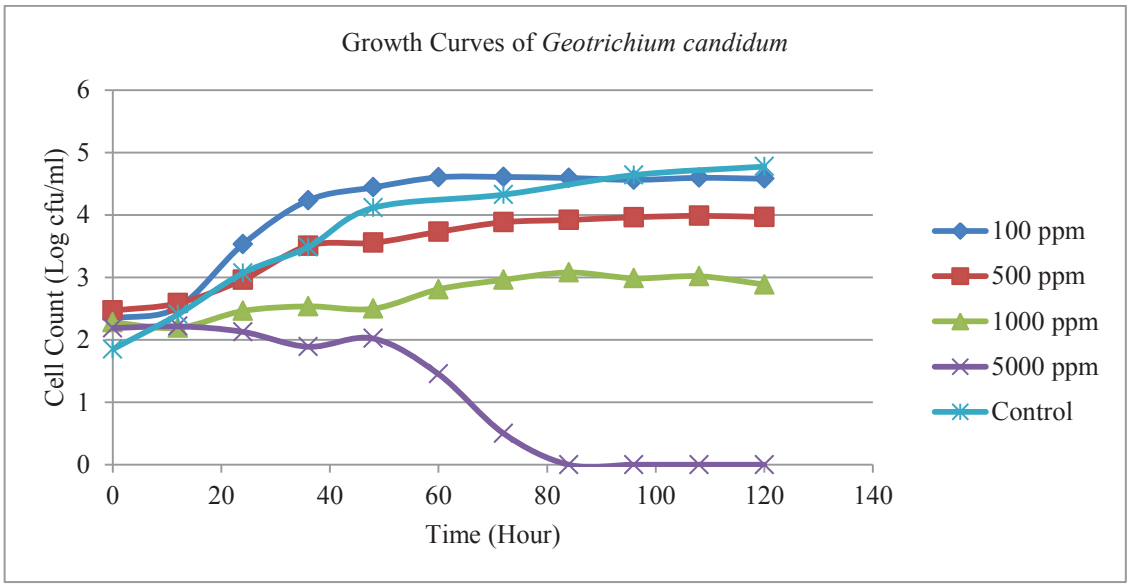

Figure 6.12. Growth Curves of *Geotrichum candidum* with Different Sorbate Concentrations

In food industry, potassium sorbate is widely used as antimicrobial agent; it is a potassium salt of sorbic with the IUPAC-ID name of potassium (2,4-hexa-2,4-dienoate) with the specified name E−202. Potassium sorbate is a white, crystalline substance with molecular mass 150.22 g/mol and melting point of 270 °C (Dehghan et al., 2018).

Antifungal effect of sorbate depends on the dissociation of sorbic acid. Antimicrobial activity mostly occurs at pH below 7.4 related to its pKa degree. The solutions could be prepared with water but the solubility depends on the temperature of the water (Dehghan et al., 2018).

By the increased clean labelled food issue for food industry, it promotes the food producers to use bio preservatives in process. Even sorbate is used in dairy; the legally permitted level would not be enough to inhibit yeast or mold.

6.7. Antifungal Effect of Protective Culture in Whey Growth Media

According to the spot test of the cultures with single strain lactic acid bacteria or with the equal mixture of the two lactic acid bacteria and three bacterial mixtures of lactic acid bacteria, the results were determined as the highest antifungal effect was obtained from the mixed culture of *Lactobacillus rhamnosus*, *Lactobacillus plantarum* and *Lactobacillus paracasei* spp. *paracasei.*

Therefore, the mixture of three bacteria was prepared with 4 different cell counts in order to determine the effective protective culture initial cell count. For this purpose, protective culture with 10^4, 10^6, 10^8 and 10^9 cfu/ml total cell counts were inoculated into whey liquid media.

Protective culture cell counts were maintained by serial dilutions from the culture with 10^9 cfu/ml determined by MacFarland densitometer. At the same time the fungal cultures were added with defined cell counts. The whey solution with inoculations was left for incubation at 25 °C for 35 days.

In literature there were many studies about the antifungal effect of lactic acid bacteria with cell counts of nearly 10^8 cfu/ml. Also there were many study about the synergistic effect of lactic acid bacteria by mixing cultures causing the increased antifungal effect, mentioned in part 6.5.

It was aimed to perform whether by increased cell counts would cause an increased inhibition or not. For this reason 10^9 cfu/ml protective cultures were added and analyzed for its inhibition effect which was determined according to the growth rate data.

Additionally growth curves of fungal cultures with sorbate and without any chemical or biological preservatives, that were determined in previous parts, were used to compare the growth characteristics. All experiments were performed triplicate; data were the mean value of triplicate analysis.

Lactic acid bacteria mixture were prepared with the resulting cell count in 200 ml whey liquid growth media were $\log_{10}$ 4, $\log_{10}$ 6, $\log_{10}$ 8 and $\log_{10}$ 9 cfu/ml approximately. Figures 6.13, 6.14, 6.15, 6.16, 6.17 and 6.18 represents the growth curves of yeast/mol cultures when they were inoculated together with antifungal lactic acid bacteria.All experiments were performed triplicate; data were the mean value of triplicate analysis Growth data are given in Appendix C2 part in detail.

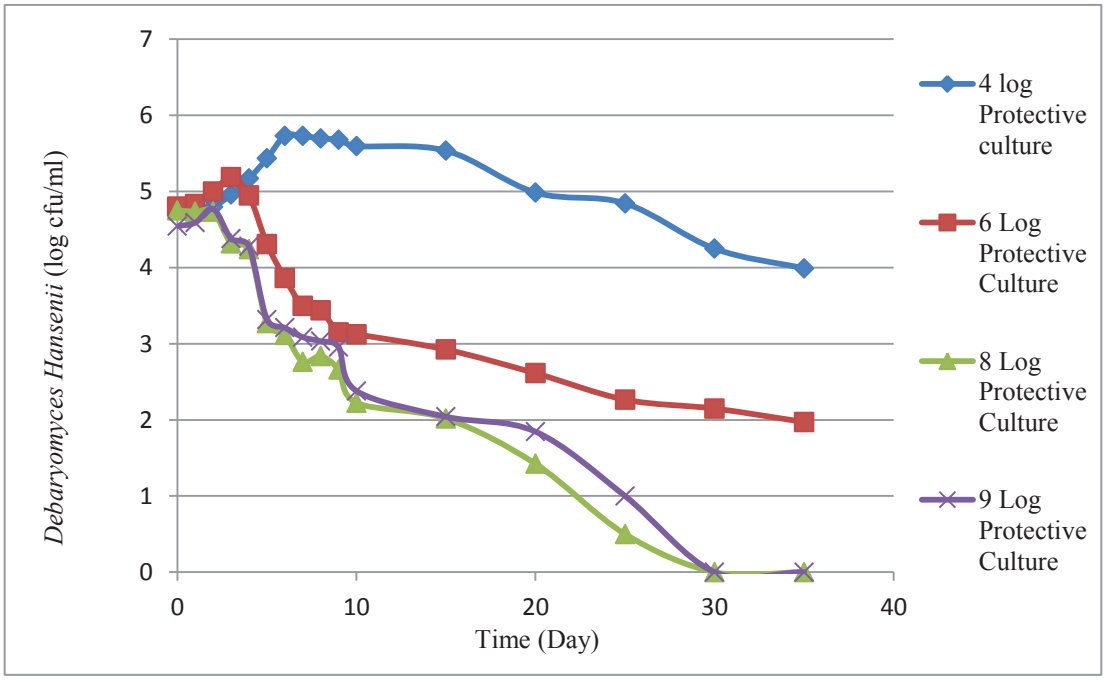

Figure 6.13. Growth Curves of *Debaryomyces hansenii* with Different Protective Culture Additions (Cell counts were given as mean values of triplicates)

Debaryomyces hansenii with an initial cell count of 5.95 x 10^4 cfu/ml (in average) were inoculated together with protective cultures. *Debaryomyces hansenii* was not totally inhibited for the first 35 days of incubation with 10^4 and 10^6cfu/ml protective culture bacteria.

However using 10^8 cfu/ml or higher concentrations caused a sharp decrease in cell counts, even caused total inhibition after 30 days for 10^8 and 10^9 cfu/ml protective cultures.

There were not a significant difference between the usage of 10^9 and 10^8 cfu/ml protective culture related to their antifungal effect on *Debaryomyces hansenii*..

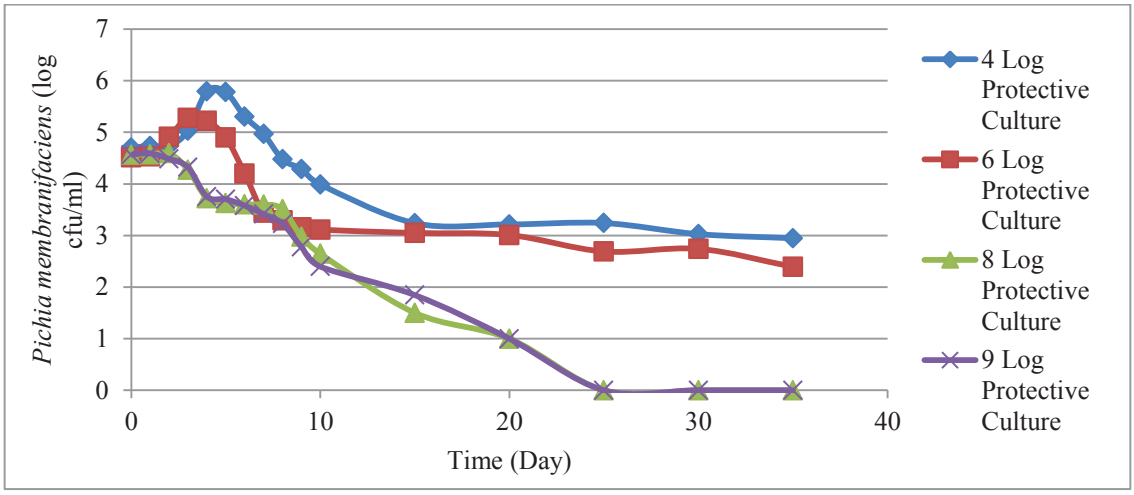

Figure 6.14. Growth Curves of *Pichia membranifaciens* with Different Protective Culture Additions (Cell counts were given as mean value of triplicates)

As seen from the Figure 6.14, *Pichia membranifaciens* culture with an initial average cell count of 50500cfu/ml were totally inhibited with 10^8 and 10^9 cfu/ml protective culture concentrations. Using protective culture with 4 $\log_{10}$ and 6 $\log_{10}$ cfu/ml caused an increase for the first 7 days, and then they began to be inhibited.

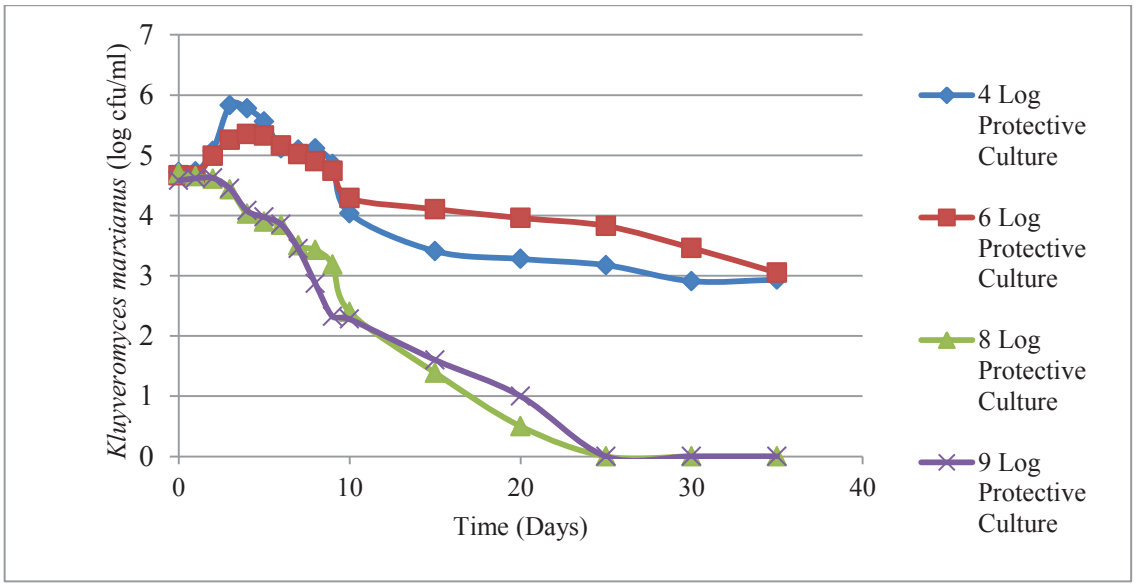

Figure 6.15. Growth Curves of *Kluyveromyces marxianus* with Different Protective Culture Additions (Cell counts were given as mean value of triplicates)

Kluyveromyces marxianus cells were resistant than *Pichia membranifaciens,* and sensitive than *Debaryomyces marxianus* related to the inhibition by antifungal protective cultures.

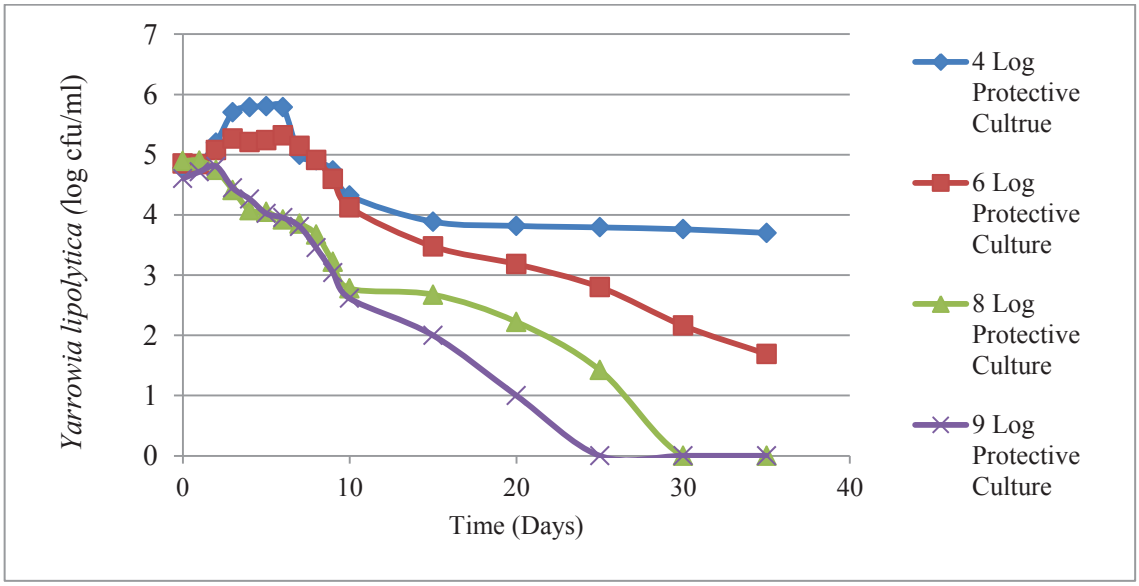

Figure 6.16. Growth Curves of *Yarrowia lipolytica* with Different Protective Culture Additions (Cell counts were given as mean value of triplicates)

There were no significant difference between the usage of 10^9 and 10^8 cfu/ml protective cultures related to their antifungal effect where yeast culture was totally inhibited at 25th day.

Yarrowia lipolytica cells showed similar resistance with *Debaryomyces hanseni* against the usage of 4 $\log_{10}$ cfu/ml protective culture. For the first 35 days, they did not totally inhibited, excepting negligible decrease in cell counts. Increased cell counts of antifungal culture caused a decrease in fungal counts and higher inhibition rates. Using 10^6 cfu/ml protective cultures caused a higher antifungal effect when compared with other yeast strains as examined from their growth curves. *Yarrowia* culture was inhibited at the day 25 with 10^9 cfu/ml protective culture and at the day 30 with 10^8 cfu/ml protective culture usage. Inhibition of *Yarrowia lipolytica* was observed when protective culture usage was increased 1 log. 10^9 cfu/ml protective culture usages caused a total inhibition 5 days before 10^8 culture usage and the inhibition rate after day 9 was higher than 108 cfu/ml culture usage. Figure 6.17 represents the growth curves of *Geotrichum candidum* with different protective culture initial cell couns

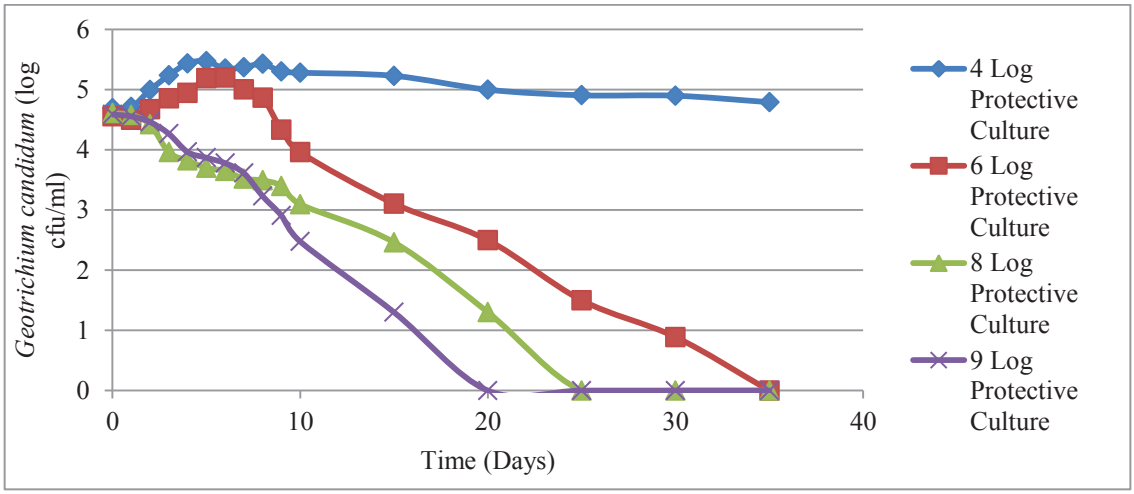

Figure 6.17. Growth Curves of *Geotrichum candidum* with Different Protective Culture Additions (Cell counts were given as mean value of triplicates)

Geotrichum candidum cell counts remained constant after 10^{th} day of incubation with 10^4 cfu/ml protective culture addition as seen from the Figure 6.17. However

Geotrichum culture was totally inhibited with 10^6 cfu/ml antifungal culture usage after 35 days of incubation, 25 days for 10^8 cfu/ml antifungal addition and 20 days for 10^9 cfu/ml antifungal culture applications. This fungal culture seemed to be more sensitive among other fungal species tested.Figure 6.18 represents the inhibition curves of *Penicillium commune* with antifungal lactic acid bacteria in the same growth media.

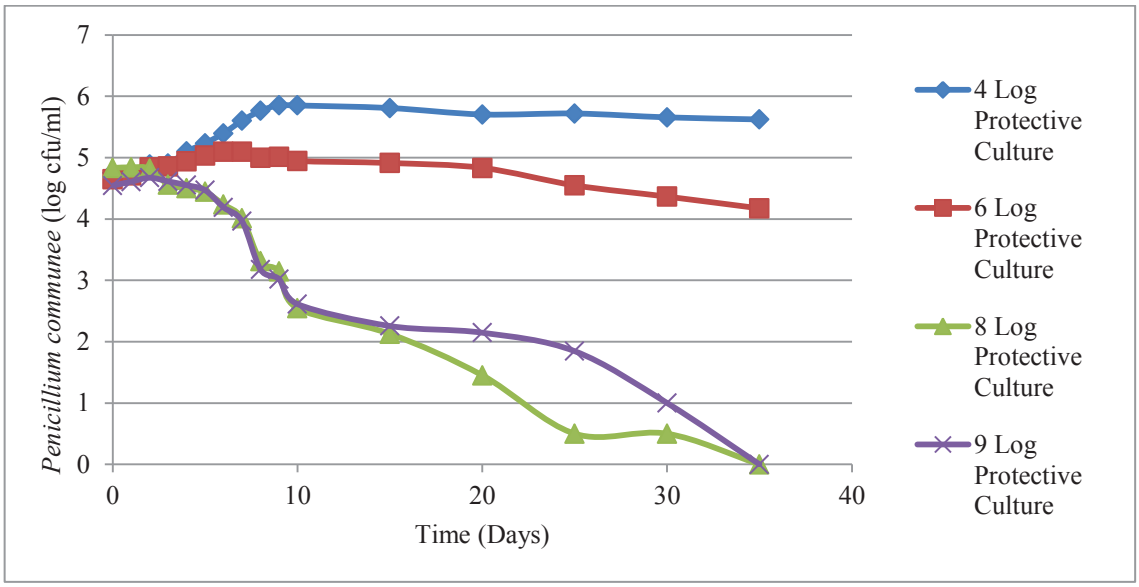

Figure 6.18. Growth Curves of *Penicillium communee* with Different Protective Culture

The most difficult fungal species to inhibit was the *Penicillium communee*. It showed an increase in spore counts when protective culture was used at 10^4 cfu/ml cell concentrations. The decrease in cell count was observed for 10^6 cfu/ml protective culture usage. However, when it was compared with other fungal strains, the inhibition rate was very small for 10^6 antifungal culture dosage. Although *Penicillium commune* was found to be the most sensitive to chemical preservatives, since it is most frequently used mold, the inhibition trend by protective culture was examined and total inhibition was obtained as seen in Figure 6.18.In all experiments performed in whey liquid media, fungal cultures were inhibited with concentrations of initial cell counts. According to the shelf life of the product, the packaged product will be kept at refrigeration temperatures. Thefore activity of protective culture is subjected to decrease according to storage temperatures Figure 6.19 represents the groth curves of fungal cultures with 10^8 cfu/ml antifungal culture usage.

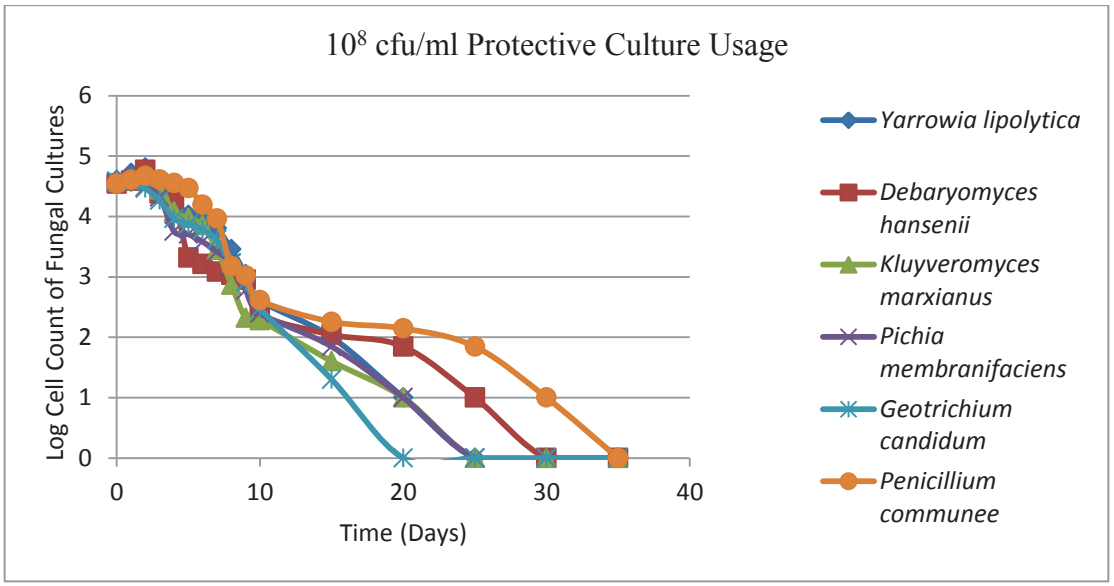

Figure 6.19. Growth Curves of Fungal Cultures with 10^8 cfu/ml Protective Culture Additions (Cell counts were given as mean value of triplicates)

Some examinations were also tested to lower contamination levels of yeast and mold cells in order to determine the inhibition rates at 6°C. The temperature was selected according to the storage parameters of the dairy products.

Mainly fermented dairy products have to be stored at 4 – 8 °C. The antifungal effects against fungal counts were tested at 25°C even for high contamination of yeast and mold cells mentioned in previous paragraphs.

The yeast and mold cells were inoculated with low initial cell counts; 120 cfu/ml for *Debaryomyces hansenii*, 160 cfu/ml for *Pichia membranifaciens*, 140 cfu/ml for *Kluyveromyces marxianus*, 110 cfu/ml for *Yarrowia lipolytica*, 180 cfu/ml for *Geotrichum candidum* and 100 spore/ml for *Penicillium communee*.

Figure 6.20 represents the growth curves of fungal cultures with 10^8 cfu/ml antifungal culture. In this section, the initial contamination levels were chosen lower from previous section.

From the results of high contamination levels incubated at 25°C and low contamination levels incubated at 6°C, culture combination with 10^8 cfu/ml cell count was found effective against yeast and mold species which were tested.

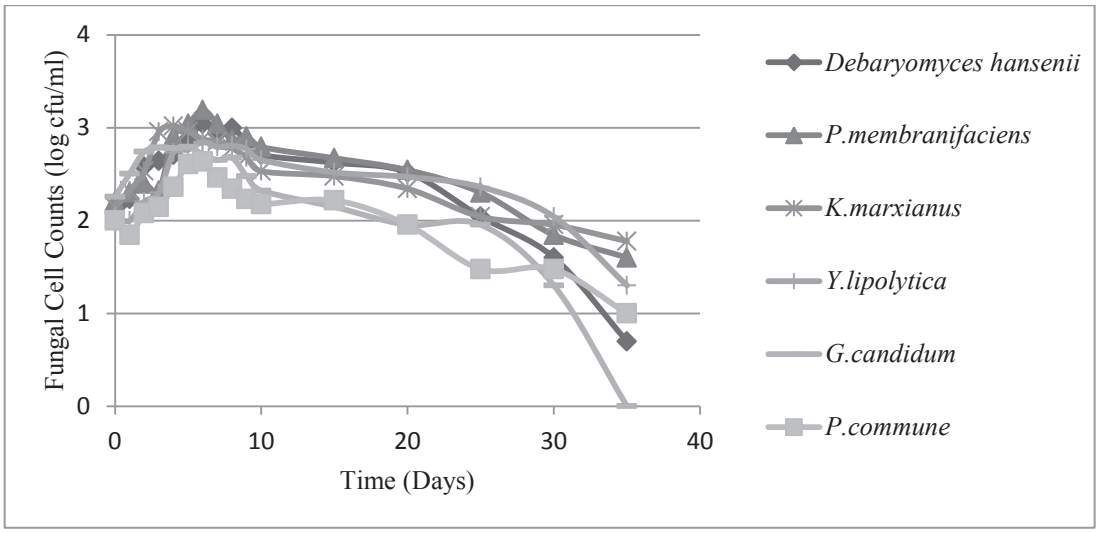

Figure 6.20. Growth Curves of 10^2 cfu/ml Inoculated Fungal Cultures with 10^8 cfu/ml Protective Culture Additions at 6 °C. (Cell counts were given as mean value of triplicates)

There were many studies concerning the antifungal effect of lactic acid bacteria against fungal species performed by agar spot test (Schwenninger and Meile., 2004, Fernandez et al., 2017, Hassan and Bullerman, 2008, Voulgari et al., 2010, Delavenne et al., 2013, Cheong et al., 2014, Lynch et al., 2014, Delavenne et al., 2015, Buehler et al., 2018) or by *in vivo* applications with cheese or yoghurt samples (Taniwaki et al., 2001, Schwenninger and Meile, 2004, Hassan and Bullerman, 2008, Voulgari et al., 2010, Delavenne et al., 2013, Cheong et al., 2014, Lynch et al., 2014, Aunsbjerg, et al., 2015, Delavenne et al., 2015, Fernandez et al., 2017, Gariner et al., 2017, Buehler et al., 2018). The results of spot test according to the same species were generally similar to the results obtained in this part of the study.

The growth of fungal species in combination with protective cultures in a liquid media could not be found to discuss the growth characteristics. But the studies of some authors include *in vivo* applications to compare the effect of antifungal cultures with different inoculum size. Schwenninger and Meile have studied the effect of *Propionibacterium jensenii* and *Lactobacillus paracasei* in yoghurt and cheese samples (Schwenninger and Meile, 2004). They inoculated food samples with 10^2 cfu/ml cell counts of yeast and mold cells and with 10^6 cfu/ml, 10^8 cfu/ml protective cultures. The effect of 10^8 cfu/ml was found to be higher against yeast or mold growths. But the cell

counts of the cells did not reach <10 cfu/ml in yoghurt. This may be due to the lactic acid bacteria type and the storage temperature of the samples. Different from this part of this study, they used different antifungal lactic acid bacteria combinations.

In 2013, Delavenne studied antifungal effect of *Lactobacillus harbinensis, Lactobacillus paracasei* and *Lactobacillus rhamnosus* culture mixture in yoghurt production (Delavenne et al., 2013) They used 10^6 cfu/ml initial concentration in yoghurt production. They found that, *Debaryomyces hansenii, Kluyveromyces marxianus* and *Yarrowia lipolytica* cultures were affected from lactic acid bacteria growth. The cell numbers did not increase and *Lactobacillus rhamnosus* as single culture was found effective on yeast cells than other two lactic acid bacteria. The usage of 10^6 cfu/ml culture effects were similarly observed in this study. It did not cause a total inhibition but the cell counts decrease for the time that samples were taken. Additionally; in the study of Delavenne in 2013, it was mentioned that there were a synergistic effect between yoghurt starter culture and protective cultures tested. The effect of antifungal cultures *in vivo* for cheese and yoghurt were studied and explained in remaining parts.

6.8. Determination of the Antifungal Activity of Cell Free Supernatants of Protective Culture with Spot Test

Cell free supernatants were prepared as mentioned in section 5.2.8. They were not pH adjusted as a result of the study performed by Zavaleta et al. in 2014. In the study of Zavaleta et al., they performed pH adjustment and found a decrease in the antifungal activities of the supernatants due to their acidic nature. .They also autoclaved the supernatants but there were no visible change in their antifungal activities (Zavaleta et al., 2014). For this reason after filtrating the supernatant by 0.45 µm filter, supernatant were directly applied to the agar mixture with 5% resulting ratio.

The supernatants of single cultures from *Lactobacillus plantarum, Lactobacillus rhamnosus* and *Lactobacillus paracasei* spp.*paracasei* and the supernatant from the mixed culture were applied through agar spot test to 3 different cell counts of yeast and mold cells. CFS was mixed with agar and poured into petri dishes. Yeast or mold cells

were spot inoculated with 5 µl volume. Table 6.19 includes the spot test results of CFS on yeast and mold species in double layer agar.

Table 6.19. Effects of Cell Free Supernatants (CFS) on Yeast and Mold Growth with Spot Test

Spotted organism	Spotted cell count or spore count/ml	CFS Used in bottom agar			
		L.Rhamnosus CFS	*L.Plantarum* CFS	*L.paracasei* CFS	Mixed CFS
Debaryomyces hansenii	5.5×10^3	+	-	+	+
	7.1×10^4	+	-	-	-
	6.1×10^5	-	-	-	-
Kluyveromyces marxianus	1.3×10^3	+	+	+	+
	2.4×10^4	+	+	-	+
	1.8×10^5	-	-	-	-
Pichia membranifaciens	2.8×10^3	+	+	-	+
	1.9×10^4	+	+	-	+
	2.6×10^5	+	-	-	-
Yarrowia lipolytica	7×10^3	+	+	+	+
	5.4×10^4	-	-	-	+
	7.7×10^5	-	-	-	-
Geotrichum candidum	1.4×10^3	-	-	-	+
	1.1×10^4	-	+	+	+
	1.4×10^5	-	-	-	-
Penicillium communee	10^3	-	-	+	+
	10^4	-	-	-	-
	10^5	-	-	-	-

As seen from Table 6.19, the cell free supernatant has shown antifungal activity less than the cell culture of lactic acid bacteria. From the results it can be determined the higher effect of mixed culture similar to the spot test of cell cultures of protective bacteria. In 2014, Zavaleta et al. have mentioned the acidic nature of the cell free supernatants by examining the antifungal activity with pH adjustments. Also they used autoclave to sterilize the cell free supernatants (Zavaleta et al., 2014). The results of them indicated that cell free supernatant active compound was not a protein. However if

antifungal compounds were in protein nature, they would be denatured due to heating process and loss their activity.

It was declined that all mold species that they tested were sensitive to the cell free supernatant with a range in agar between 2.65 % and 66.8% (Zavaleta et al., 2014). The big difference between the sensitivities would be explained due to the species specific characters of molds.

In the study of Aunsjberg et al., they mentioned about the water volatile compounds which are lost due to centrifugation and filtration steps of cell free supernatants. The decreased antifungal activities of cell free supernatants compared with cell culture can be explained with this study (Gerez et al., 2013, Aunsjberg et al., 2015).

Cell free supernatant of *Lactobacillus plantarum* was compared with sodium benzoate, potassium sorbate and natamycin against fungal growth in the study of Yang and Chang in 2015. Their studies included to examine by different methods such as paper disc assay, cell free supernatant, 500ppm and 1000 ppm sodium benzoate, 500 ppm and 1000 ppm potassium sorbate, and 20 ppm natamycin to find better performances for antifungal effect tests. Similarly with this study 500 and 1000 ppm sorbate has little or no effect depending on the type of fungal organism tested. Results were the same for benzoate.

Natamycin has shown antifungal effect with 20 ppm concentrations on paper disc. Cell free supernatant had more antifungal activity when compared with sorbate and benzoate (Yang and Chang, 2015).

Guimaraes et al. examined the antifungal effect of cell free supernatants against *Penicillium nordicum* in 2018. Samples were taken from the spore suspension of 10^6 spore/ml with 10 µl volume and spotted on agar surface which was prepared with %10 cell free supernatant addition. Autoclaved samples were found to be more antifungal due to the loss of protein nature and increased organic acids effect (Guimaraes et al., 2018).

Due to the decreased antifungal effect and the preliminary *in vivo* examination on cheese due to the sensorial effect (data not shown), caused not to continue studies with cell free supernatants.

6.9. Antifungal Culture Applications on Cheese Samples

There were many studies related to *in vivo* examination of antifungal lactic acid bacteria. They generally tested by soaking the culture at each side of end product, e.g. cheese was submerged into the starter culture broth. Then samples were contaminated with fungal cultures and examined the lactic acid bacteria's antifungal activity.

Schwenninger and Meile used 100g cheese sample and soaked it into culture solution and examined the antifungal activity through 3 weeks (Schwenninger and Meile, 2004). In 2014, Cheong et al. examined the lactic acid bacteria assumed to be antifungal on cheese samples (Cheong et al., 2014). They used 12g cheese sample and spread 0.1 ml lactic acid bacteria culture on the surface of cheese and contaminate the 2 days incubated cheese samples with mold culture. Cheeses were left to examine the time value to obtain visible mold colony on the surface of the cheese.

In 2017, Fernandez et al. examined the antifungal activity of lactic acid bacteria with 5g of cheese sample. 50 µl of the lactic acid bacteria culture solution was added on the surface of cheese (Fernandez et al., 2017).

This part of study was constructed with two different cheese product; white brined cheese and kashkaval cheese.

Previously activated lactic acid bacteria were applied to white brined cheese before fermentation by inoculating culture into curd in moulds and to brine solution at packaging step. The Kaskaval applications were performed by soaking cheese with each side into culture solution.

6.9.1. Antifungal Effect of Protective Culture on White Brined Cheese Curd

50g cheese curd was taken from cheese moulds before they left for fermentation of the cheese starter cultures. Samples taken before fermentation were mixed with 50 ml protective culture. Protective culture were consisted of *Lactobacillus rhamnosus, Lactobacillus plantarum* and *Lactobacillus paracasei* spp. *paracasei* with cell counts of $3*10^8$ cfu/ml for each bacteria and mixed with equal volumes in previously sterilized

flasks. Before mixing the cultures they were two times activated and gave cell counts of $9*10^8$ cfu/ml according to MacFarland standards (equal to 3 MacFarland standard and measured with Biosan Densitometer) and verified with pour plating the sample with MRS agar.

Cultures were transferred into 200 ml whey liquid growth media and left for incubation. By this step cultures were permitted to synthesize the antifungal compounds efficiently since the usage of whey was to mimic cheese media. Grown cultures were taken for cell count analysis by pour plate technique and the time value regarding to growth curve were similar to the growth curves obtained in section 6.2. The cultures were 10^8 cfu/ml after 24 hours of separate incubations. Equal volumes from cultures were taken into a sterile plastic bag for further steps of analysis. Approximately 50 ml of the culture mixture with cell count of 10^8 cfu/ml was obtained and cell number was verified with enumerating via pour plate technique with MRS agar.

Fungal cultures were propagated in yeast mold broth. Broths were left for incubation 4 days before the experiments. Cell counts were assumed related to the growth curves even the growth curves were constructed with whey liquid media. Cell counts were verified with pour plating the sample's serial dilutions with YGC agar. Fungal cultures were diluted to obtain 3 different initial contamination levels.

Cheese sample with 50g in weight was mixed with 50 ml culture and left for both effective adsorption and fermentation. Fermentation was carried out for 8 hours (time value was determined according to reference cheese fermentation time). After fermentation was ended, fungal contamination was performed due to the values determined before.

Curd samples were analyzed for other microbiological criteria. Analyses for coagulase positive staphylococci, *Enterobacteriaceae,* Total coliform, yeast and mold count before contamination, *Salmonella spp*. and *Listeria monocytogenes* analysis were performed for five of the randomly selected curd samples.

The results were all <10 cfu/g for coagulase positive staphylococci, *Enterobacteriaceae,* Total coliform, yeast and mold counts. Also *Salmonella spp*. and *Listeria monocytogenes* were negative for 25 gram sample for 5 separate curd analysis.

Contaminated samples were left at refrigeration temperatures approximately 6° C and analyzed for 4 weeks. All experiments were prepared in triplicate and the data were the average value of triplicates. Growth data are given in detail in Appendix C3. Figure 6.21 represents the inhibition curves of *Debaryomyces hansenii* with three

different initial cell counts. *Debaryomyces hansenii* initial counts were; 53100 cfu/g, 4200 cfu/g and 460 cfu/g approximately.

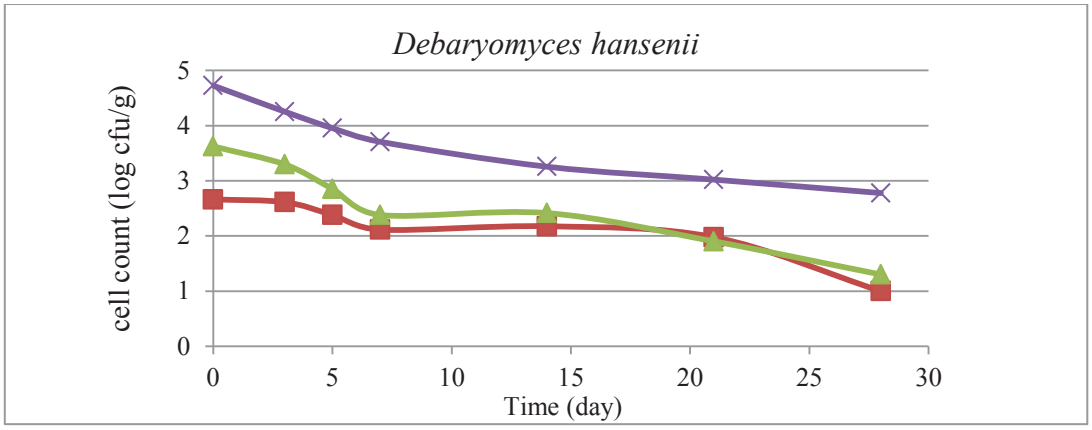

Figure 6.21. *Debaryomyces hansenii* Counts of Cheese Samples with Three Different Initial Contamination Levels

As seen from Figure 6.21, *Debaryomyces hansenii* counts decreased more rapidly during first week and continued to be inhibited. Antifungal culture was determined as effective due to the data and graph above. Figure 6.22 respresents the inhibition curves of Yarrowia lipolytica with three different initial cell counts.

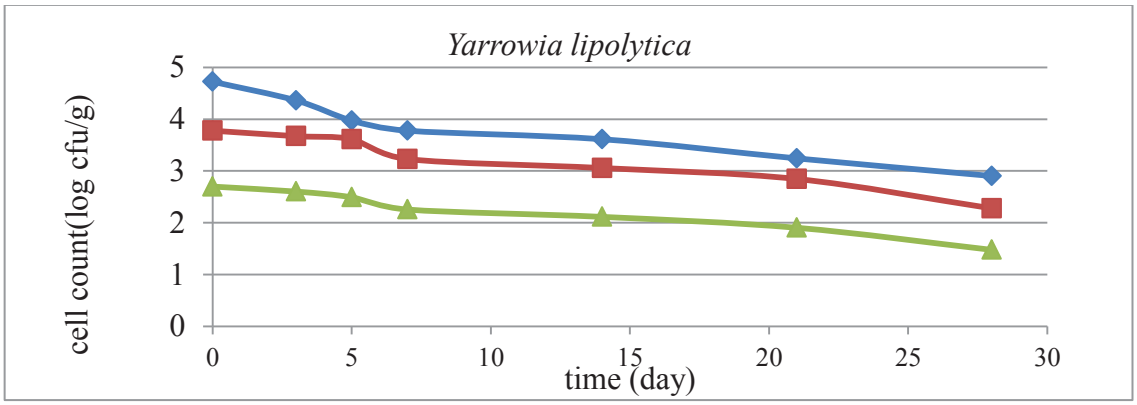

Figure 6.22. *Yarrowia lipolytica* Counts of Cheese Samples with Three Different Initial Contamination Levels

From the Figure 6.22, for *Yarrowia lipolytica*, the similar decrease with *Debaryomyces hansenii* was observed for the first week of the samples. *Yarrowia lipolytica* initial counts were 53000 cfu/ml, 6000 cfu/ml and 500 cfu/ml approximately. *Yarrowia* cells were rapidly inhibited for high initial counts for the first week of the samples. The remaining time for the samples, the inhibition was not as rapid as liquid cultures. This can be explained with the starter culture effect, viscosity of the growth medium and chemical composition of the medium. 500 cfu/ml initial counts were decreased to 30 cfu/ml and the 6000 cfu/ml initial cell count decreased to 190 cfu/ml at the end of the experiment. Figure 6.23 represents the inhibition curves of Kluyveromyces marxianus with three different initial inoculation levels.

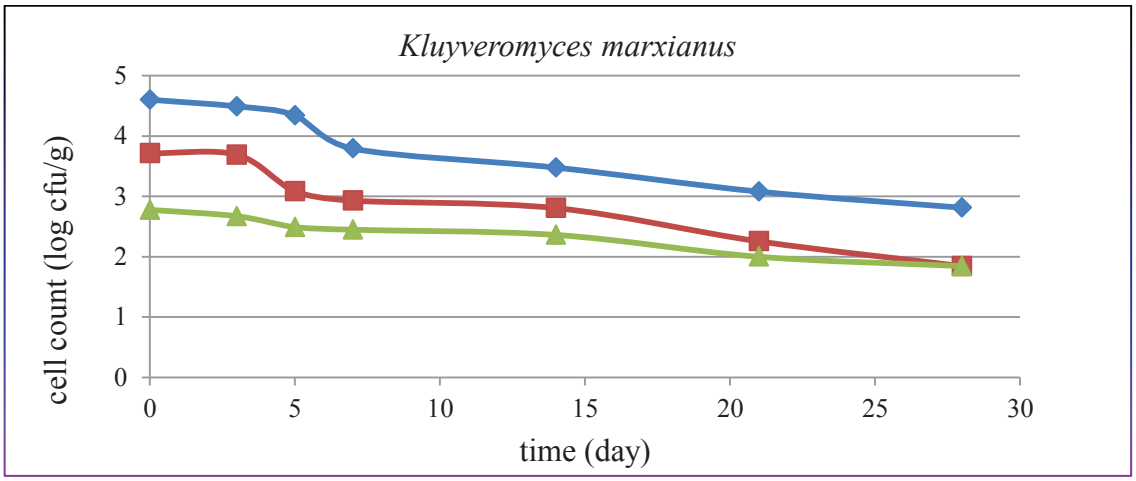

Figure 6.23. *Kluyveromyces marxianus* Counts of Cheese Samples with Three Different Initial Contamination Levels

Kluyveromyces marxianus were added to cheese samples with 40000 cfu/ml, 5200 cfu/ml and 600 cfu/ml initial cell counts. The cell counts decreased to 650 cfu/ml, 70 cfu/ml, 70 cfu/ml respectively. The decrease amount when comparing the differences between initial and final cell count was smallest with the 600 cfu/ml initial contamination rate. Figure 6.24 respresents the inhibition curves of Geotrichium candidum with three different initial inoculation levels.

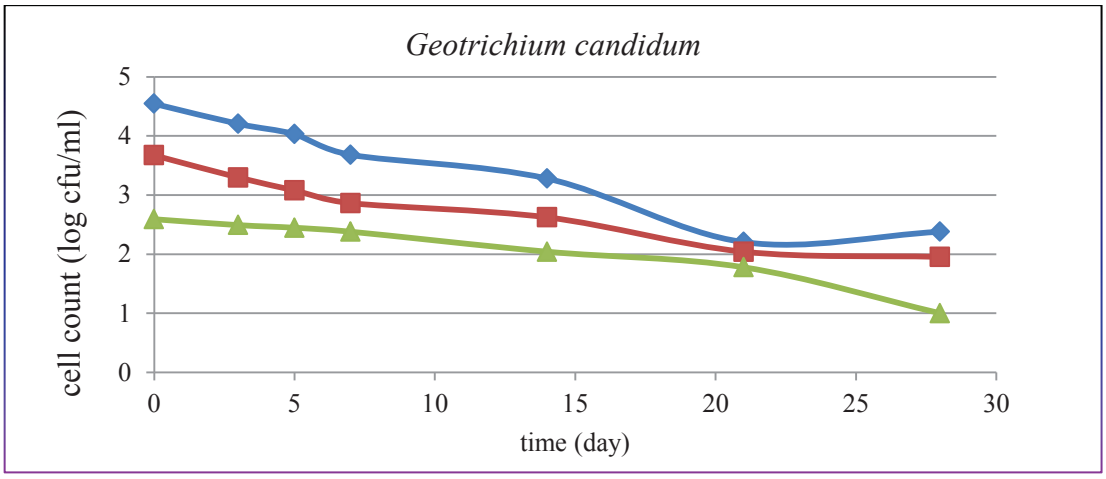

Figure 6.24. *Geotrichum candidum* Counts of Cheese Samples with Three Different Initial Contamination Levels

The inhibition rate of *Geotrichum candidum* is higher when comparing with previously examined yeast species. The initial cell counts were 35000 cfu/ml, 4700 cfu/ml and 390 cfu/ml and the final cell counts of these inocula were 240 cfu/ml, 10 cfu/ml and 10 cfu/ml respectively. Figure 6.25 respresents the inhibition curves of *Pichia membranifaciens.*

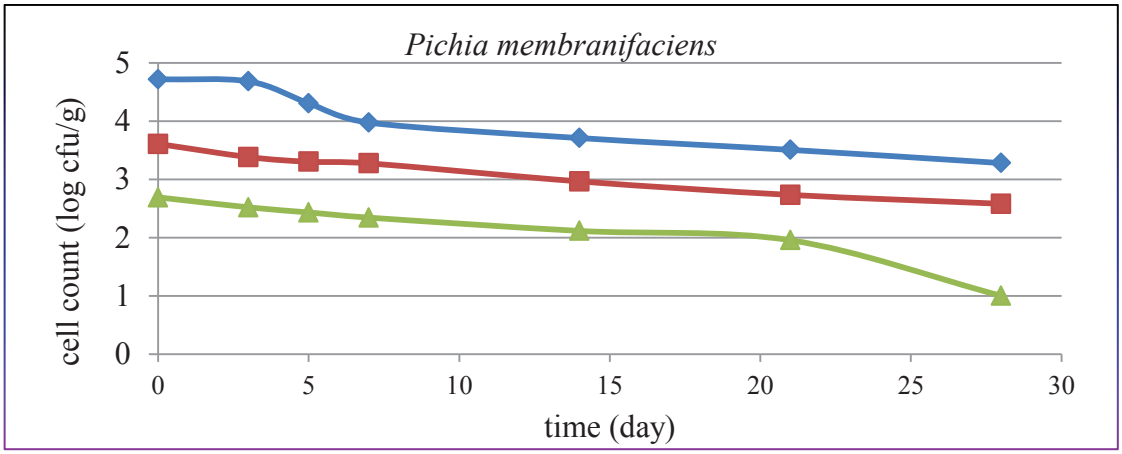

Figure 6.25. *Pichia membranifaciens* Counts of Cheese Samples with Three Different Initial Contamination Levels

Pichia membranifaceins yeast cultures were more sensitive to antifungal cultures according to the growth curves. The initial cell counts were 52000 cfu/ml, 4050cfu/ml and 490 cfu/ml with the final cell counts of 1900 cfu/ml, 380 cfu/ml and 10 cfu/ml respectively. Figure 6.26 respresents the inhibition curves of *Penicilium commune* with three different initial inoculation levels.

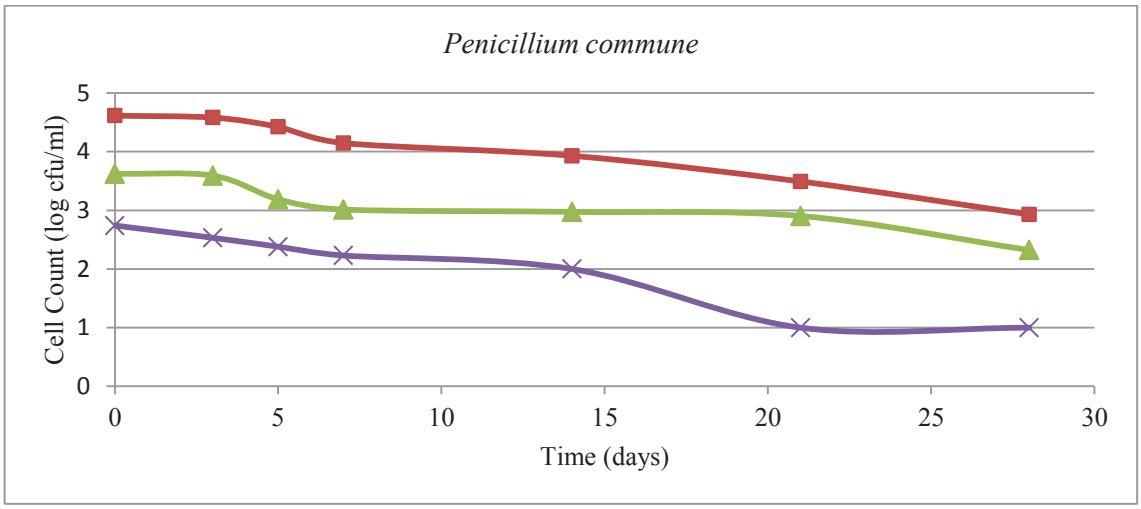

Figure 6.26. *Penicillium communee* Counts of Cheese Samples with Three Different Initial Contamination Levels

Penicillium commune was inoculated with the spore counts of 41000 cfu/ml, 4200 cfu/ml, 550 cfu/ml approximately. The inhibition caused the final spore counts; 850 cfu/ml, 210 cfu/ml and 10 cfu/ml respectively. For low contamination level, cell counts remained constant for the last week of samples.

Schweninger and Meile examined the antifungal effect of *Propionicbacteria* and *Lactobacilli* on cheese surface. They used antifungal culture including $1{,}1*10^6$ cfu/ml *Propionibacteria* and $6{,}9*10^5$ cfu/ml lactobacilli and contaminate the cheese surface with 10^2 cfu/ml yeast cells (Schweninger and Meile , 2014). After 21 days of storage, yeast cells including Candida species were totally inhibited. In this study, yeast cells were inoculated on to cheese samples with three different concentrations. The minimum concentrations of yeast cultures were; 460 cfu/ml, 500 cfu/ml, 600 cfu/ml, 490 cfu/ml, 390 cfu/ml for *Debaryomyces hansenii, Yarrowia lipolytica, Kluyveromyces marxianus, Pichia membranifaciens* and *Geotrichum candidum* with the cell counts after 28 days

10 cfu/ml, 30 cfu/ml, 70 cfu/ml, 10 cfu/ml, 10 cfu/ml respectively. Results were similar to the findings of Schewenninger and Meile. They stated as yeast cells were totally inhibited but the limit of quantification value for their analysis mentioned were 100 cfu/ml. They stated the inhibited samples yeast counts as <100 cfu/ml (<2 log cfu/ml in the paper). The results of this study could be said as similar for this reason. Also they had examined the effects of culture against Candida species different from this study (Schwenninger and Meile, 2004).

Antifungal effect of lactic acid bacteria on cheese surface was studied by Fernandez et al. in 2017. They used *Penicillium chrysogenum* as model mold strain on cottage cheese experiments with lactobacilli strains and Bifidobacterium species. The effect of single culture and mixed culture was stated in this study. They determined the increased effect of mixed culture. Additionally *Lactobacillus rhamnosus* was found to be more effective on yeast cells than they affect the mold cells. They observed the inhibition when the mixed culture was prepared with *Lactobacillus rhamnosus* at 10^6cfu/ml concentration and *Propionibacterium freudenreichii subsp. Shermanii* at 10^9cfu/ml concentration. The increased effect with increased lactic acid bacteria was observed similarly with this study (Fernandez et al., 2017).

Lactobacillus amylovorus was also tested according to its antifungal effect to extend the shelf life of cheddar cheese. Cheese samples were contaminated with 100 ml spore suspension containing 10^4 fungal spores of *Penicillium expansum* which shows the similar characteristics with *Penicillium commune*. Samples were also inoculated with *Lactobacillus amylovorus* with 10^5 cfu/ml initial cell counts. The usage of *Lactobacillus amylovorus* was found to be effective depended on the increase at time interval to observe visible mold colonies for the first 12 days. The difference with this study was the lactic acid bacteria used and the method to determine the antifungal effect. But similarly they stated the presence of antifungal effect even with low initial lactic acid bacteria cell counts (Lynch et al., 2014).

The same mold species with this study, *Penicillium commune*, was examined in cheese whether it will be inhibited or not by the *Lactobacillus plantarum* strains. The mold contaminated was performed with the final count of 10^4 spore/ml and the antifungal effect was detected at the point that the first visible mold colony occurred on the cheese surface. One of the *Lactobacillus plantarum* strain caused antifungal effect up to 18 days, while another strain caused an antifungal effect up to 28 days. The results were similar to this study regarding to the inhibition of *Penicillium commune* was

inihibited by lactic acid bacteria mixture containing *Lactobacillus plantarum* and the results showed an inhibition up to 1.5 $\log_{10}$ cfu/ml cell count decrease. Similarly there were not visible mold colonies at the end of the storage. The different antifungal effect data could depend on the specific effects of strains.

The cheese samples were also prepared without antifungal lactic acid bacteria inoculations as reference. The fungal counts of yeast cells were increased by the means of 2.4 log in average and the *Penicillium commune* cell counts increased 1.8 log in average. Also the visible mold colonies were observed after 8, 11 and 11 days from the first day of inoculation for three different contamination of *Penicillium commune*.

6.9.2. Antifungal Effect of Protective Culture on White Cheese

Cheese samples from packaging part of an industrial plant were taken and 50g of samples were weighted in sterile plastic bags. Fungal cultures and antifungal cultures were prepared as mentioned in part 6.9.1. with same concentrations. Figure 6.27 includes the inhibition curves of yeast and mold cultures at 10^4 cfu/ml levels inoculated in the cheese product with antifungal lactic acid bacteria.

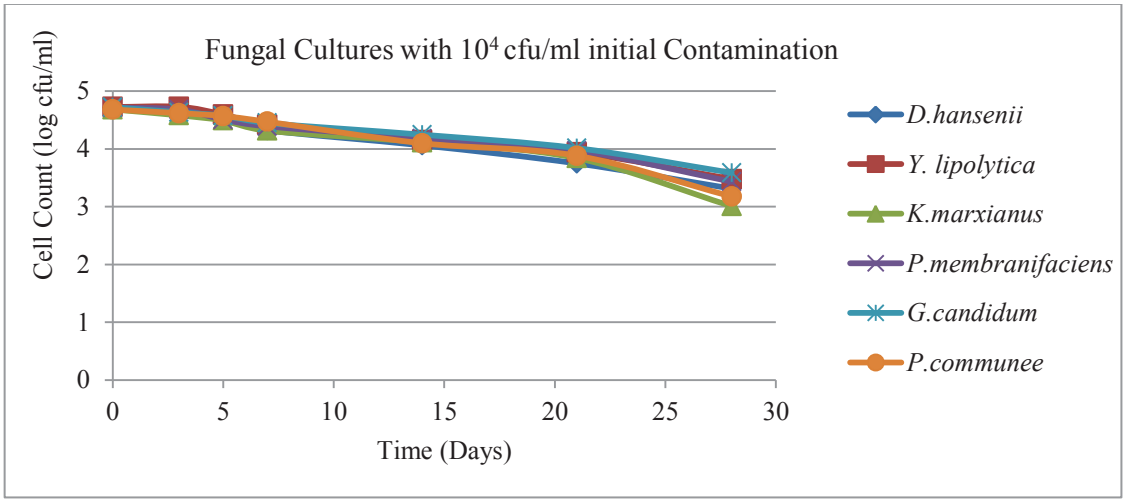

Figure 6.27. Growth Curves of Fungal Cultures with Initial Inoculum Size of 10^4cfu/ml Into the Cheese

Samples from white brined cheese as negative reference without both antifungal and fungal species were analyzed for yeast and mold counts, *Enterobacteriaceae*, Total coliform, Coagulase positive staphylococci, *Salmonella spp*. and *Listeria monocytogenes* to determine the microbiological characteristics of the cheese as reference.

Fungal cultures were applied with different initial inoculation levels on to the cheese sample.

Fungal cultures with 10^4 cfu/ml, 10^3 cfu/ml and 10^2 cfu/ml inoculations were graphed. There was not a significant difference between the inhibition patterns of fungal cultures. Figure 6.28 respresents the inhibition curves of fungal cultures with 10^3cfu/ml.

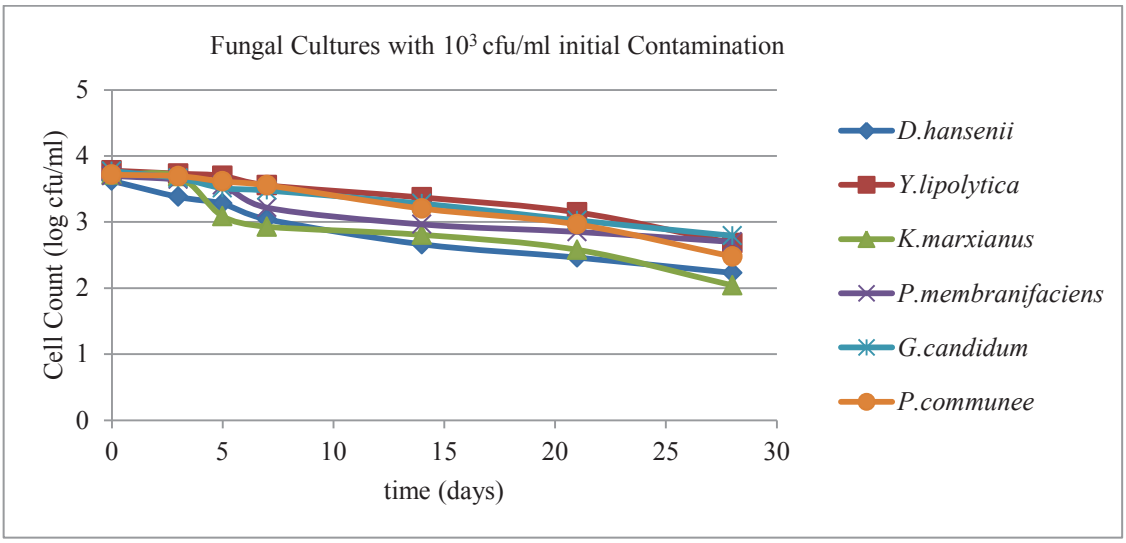

Figure 6.28.Growth Curves of Fungal Cultures with Initial Inoculum Size of 10^3cfu/ml Into the Cheese

Kluyveromyces marxianus culture has been inhibited more rapidly than other fungal cultures for the first week of contamination. Figure 6.29 represents the inhibition curves of fungal cultures with 10^2 cfu/ml initial inoculation levels.

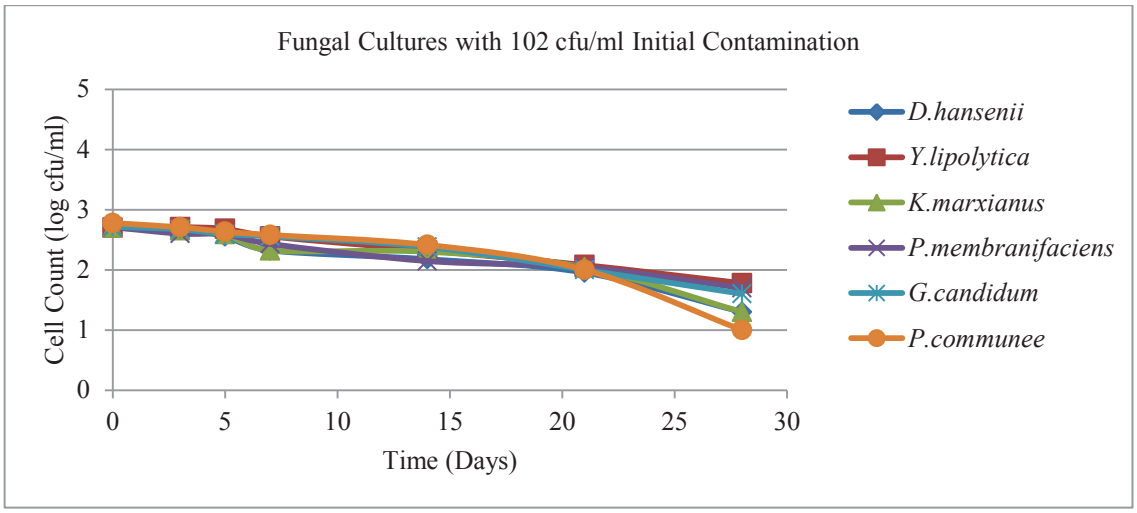

Figure 6.29.Growth Curves of Fungal Cultures with Initial Inoculum Size of 10^2cfu/ml Into the Cheese

The contaminated cheese samples with the most frequently isolated mold species was prevented from contamination by decreasing cell counts from 600 cfu/ml to 10 cfu/ml for 28 days.

It was assumed that the culture metabolites and culture itself would continue to prevent mold or yeast growth on the surface.

The application of antifungal cultures to cheese should be performed at the beginning of the fermentation step. Because the metabolic activities or metabolites of starter cultures were determined to enhance the antifungal properties of antifungal cultures in the study of Delavanne et al., in which the antifungal activity of lactic acid bacteria was found to increase with the fermentation of thermophilic lactic acid bacteria (Delavanne et al., 2015).

The decreases caused by antifungal cultures were compared by the difference in the cell counts given in the table 6.20. The table shows cell count decrease levels of different fungal strains with different initial inoculum levels.

The data were obtained from results obtained in sections 6.9.1. and 6.9.2. They were calculated in relation to the difference between logarithms of initial cell counts and final cell counts for each inoculation of each of yeasts and mold.

Table 6.20. Comparison of the Decrease in Fungal Counts with Different Cheese Applications of Antifungal Starters

Fungal Strain	Initial Inoculum	Curd Application	Cheese Application
Penicillium Commune	4 $\log_{10}$ cfu/ml	1.74	1.51
	3 $\log_{10}$ cfu/ml	1.30	1.23
	2 $\log_{10}$ cfu/ml	1.68	1.77
Geotrichum candidum	4 $\log_{10}$ cfu/ml	2.16	1.13
	3 $\log_{10}$ cfu/ml	2.67	0.97
	2 $\log_{10}$ cfu/ml	1.59	1.11
Pichia membranifaciens	4 $\log_{10}$ cfu/ml	1.43	1.28
	3 $\log_{10}$ cfu/ml	1.02	1.00
	2 $\log_{10}$ cfu/ml	1.69	1.00
Kluyveromyces marxianus	4 $\log_{10}$ cfu/ml	1.78	1.67
	3 $\log_{10}$ cfu/ml	1.87	1.67
	2 $\log_{10}$ cfu/ml	0.93	1.39
Yarrowia lipolytica	4 $\log_{10}$ cfu/ml	1.82	1.24
	3 $\log_{10}$ cfu/ml	1.49	1.09
	2 $\log_{10}$ cfu/ml	1.22	0.92
Debaryomyces hansenii	4 $\log_{10}$ cfu/ml	1.94	1.39
	3 $\log_{10}$ cfu/ml	2.32	1.39
	2 $\log_{10}$ cfu/ml	1.63	1.39

It can be determined from the results shown in Table 6.20, cell count decrease in each of yeasts and mold were higher in curd application than end product application. It could be due to the two different reasons. First reason was the effect of starter culture activity which enhances the antifungal effect and growth of antifungal lactic acid bacteria. The second reason was thought to be the effective distribution of the antifungal culture in curd samples. Therefore, in terms of process efficiency, application of protective cultures to cheese milk in vats would lead to higher antifungal activities.

The real contamination levels in a dairy plant with effective GMP (Good Manufacturing Practices) applications are generally with a maximum level of 10^2 - 10^3 cfu/ml for yeast and 10^1 – 10^2 cfu/ml for mold contamination. Because with an effective air disinfection with pulverized fungicides (should be applied when the plant does not include product and cleaned equipment in contact with air), effective cleaning of

equipment, personal hygiene and the effective pasteurization of raw milk. However the nature of a cheese plant requires higher relative humidity and temperatures than other dairy processes for an effective fermentation (Law and Tamime, 2010).

As mentioned before there are many studies containing the antifungal lactic acid bacteria application to cheese contaminated with different fungal species. Generally the researchers used the method to determine antifungal activity by measuring the time (day) that the visible mold colony had occurred on cheese surface. Also there are some studies in which the cell counts of contaminated cheese samples are encountered.

Aljewicz and Cichosz determined the antifungal effects of single cultures of *Lactobacillus acidophilus* with 0.16 $\log_{10}$ decrease in yeast count, *Lactobacillus paracasei* with 0.21 $\log_{10}$ decrease in yeast count and *Lactobacillus rhamnosus* which caused an increase in yeast counts of the previously contaminated cheese samples. From this research it was determined that the single cultures were less effective than mixed cultures of lactic acid bacteria as was determined in this study. Also Sedaghat et al determined the antifungal effect of *Lactobacillus plantarum* on cheese surface against *Aspergillus flavus* and *Aspergillus paraciticus* by calculating the area on cheese surface with mycelial growth of Aspegillus at defined time periods to observe the changes according to antifungal activity (Sedaghat et al., 2016).

Cheese samples containing 0.1 ml of spore suspension of *Penicillium commune* with $1*10^6$ spore/ml were analyzed for the antifungal effect of *Lactobacillus plantarum* and was found to be effective in order to the none of the samples had visible mold colonies up to 29 days of experiment in the study of Cheong et al. They found *Lactobacillus plantarum* as to be an antifungal strain against *Penicillium commune* (Cheong et al., 2014). Similarly, Lynch et al. determined the antifungal activity with the same method on cheddar cheese samples. They observed the visible mold colony 12 days after the control sample and decline the extension of shelf life by the lactic acid bacteria. The researches, which were found from the literatures, have not been included the cheese applications of mixed culture to the curd cheese samples. Additionally there was not an article published so far including the comparisons of different applications of the antifungal cultures against the defined yeast and mold species

6.9.3. Antifungal Effect of Protective Cultures During Shelf Life of White Cheese

White cheese samples were prepared according to the experimental plan given in part 5.2.9.3. Separate but identical cheese samples of the same production batch, which were inoculated with protective antifungal lactic acid bacteria culture at curd step, were contaminated firstly two weeks after the fermentation and contaminated at the 30th day, 60th day and 90th day separately.

Table 6.21 and 6.22 was prepared with the mean value of duplicates and the data were represented in units of log10 cell or spore per milliliter. Two different fungal species were tested; *Debaryomyces hansenii* which was mentioned in part 3.5.1. as being halotolerant and *Penicillium commune* for mold contamination.

Table.6.21. *Debaryomyces hansenii* Counts in Contaminated Cheese Samples with Different Contamination Time Values

Cell Counts (log cells/ml)	*Debaryomyces hansenii* contamination time			
Time (Days)	14	30	60	90
0	3.02	3.01	3.02	3.01
3	3.01	2.94	3.03	2.97
5	2.98	2.84	2.95	2.95
10	2.76	2.64	2.90	2.89
15	2.23	2.37	2.78	2.81
20	1.80	2.21	2.47	2.42
Decrease from begining	1.22	0.80	0.54	0.58

Cheese samples were stored in their original packaging material and filled with brine solution. This part of study was conducted in order to observe the effects of antifungal lactic acid bacteria in shelf life.

It was assumed to remain the antifungal activity related to their antifungal metabolites which were synthesized during fermentation step. Additionally the effect of brine solution wanted to be tested via this part of the study.

The samples inoculated with yeast cells two weeks after shelf life showed a decrease with 1.22 $\log_{10}$ cell/ml after counting the samples for yeast cells for 20 days. The effect of protective culture was determined to be decreased during shelf life.

At the 90th day of cheese, the contamination with *Debaryomyces hansenii* was performed and the cell counts showed a decrease with 0.58 $\log_{10}$ cell/ml 20 days after storing the samples.

This could be a result of *Debaryomyces hansenii* being a halotolerant species. However, antifungal culture continued to effect the yeast growth.

The result of the study with mold contamination at different days of shelf life demonstrated that, the antifungal of the culture remained with a decreased effect.

The spore counts difference for the 14th day contaminations were 0.71 $\log_{10}$ spore/ml after counting the samples for mold cells for 20 days. But the decrease in the spore counts were 0.38 $\log_{10}$ spore/ml after counting the samples for mold cells for 20 days. The effect was seemed to decrease.

The decrease was thought to be due to the effect of shelf life and brine solution to the starter cultures which was assumed to have a synergistic effect with the antifungal culture.

Table 6.22. *Penicillium commune* Counts in Contaminated Cheese Samples with Different Contamination Time Values

Cell Counts (log cells/ml)	*Penicillium commune* contamination time to the cheese			
Time (Days)	14	30	60	90
0	3.01	3.03	3.01	2.97
3	3.00	3.01	2.97	2.98
5	2.96	2.99	2.96	2.93
10	2.88	2.93	2.91	2.91
15	2.69	2.75	2.82	2.80
20	2.31	2.37	2.51	2.59
Decrease from begining	0.70	0.65	0.50	0.38

6.9.4. Antifungal Effect of Protective Culture on Kashkaval Cheese

The antifungal effects of protective cultures were examined on another type of cheese. The samples were collected from industrial cheese plant's packaging part. Samples with 20g in weight was inoculated with antifungal cultures and further contaminated with fungal cultures. As fungal indicators; *Yarrowia lipolytica* and *Penicillium commune* were chosen. Figure 6.30 represents the inhibition curves of yeast and mold cells.

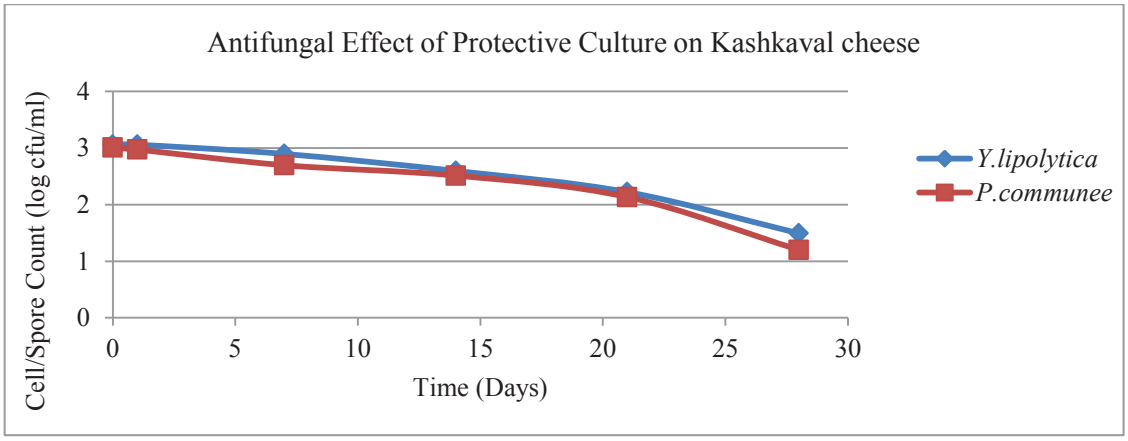

Figure 6.30. Growth Curves of Fungal Cultures on Kashkaval Cheese

The cell counts were decreased for both cultures with similar inhibition pattern as shown in graph 6.30. The cell counts were performed for triplicate analysis and the mean value were used for graph. As seen from the figure, yeast and mold were both determined with the counts between 10 cfu-spore/ml and 50 cfu-spore/ml from an initial contamination rate of 1000-1200 cell-spore/ml. As a result of having thermal process in production steps of kashkaval cheese, starter could not be applied at curd step. For kashkaval cheese, antifungal effect can be maximized by applications after thermal process steps. It could be performed by spraying or soaking to the cheese in to culture solution.

6.10. Applications of Antifungal Lactic Acid Bacteria to Strained Yoghurt and Set Type Yoghurt

Antifungal properties of the selected strains of lactic acid bacteria were determined by spot test, antifungal-fungal interactions in whey liquid media and cheese applications, mentioned in previous parts of this chapter.

This part includes the application of antifungal cultures to other dairy products rather than cheese. For this purpose yoghurt was chosen as dairy product with two different type; strained and set type yoghurt. Growth data for yoghurt applications are given in Appendix C in detail.

6.10.1. Applications of Antifungal Lactic Acid Bacteria to Strained Yoghurt

Strained yoghurt was produced in an industrial plant related to the following steps; pasteurization of the raw milk, standardization the milk to 1,6 (%) fat, inoculation with yoghurt starter culture, fermentation, stirring the fermented product, separation via quark separators, filling into packaging material and finally cold storage of the products.

The samples were taken from the plant before packaging step from the lines into sterile sample containers in order to obtain more fluid sample for efficient distribution of the cultures.

Samples were taken as 90g per container with screw caps. The samples were inoculated with mixed antifungal starter culture with 10 ml volume. The resulting cell counts of antifungal cultures were 10^6 cfu/ml, taken from the culture with 10^7 cfu/ml cell counts (Delavenne et al., 2015). Strained yoghurts were left for effective distribution for 8 hours to mimic the fermentation at room temperature and contaminated with yeast and mold species with 10^2 and 10^3 cells or spores per milliliter. Samples were stored at 6°C for 25 days (shelf life of the product) and analyses were performed at days 0, 5, 10, 15, 20 and 25 respectively.

Yarrowia lipolytica was chosen as indicator yeast strain similarly with the studies of Delavenne et. al. as mentioned with its high resistance to organic acids (Delavenne et al., 2013 and Delavenne et al., 2015). Also *Penicillium commune* was the mold species studied in this part of the study. Figure 6.31 respresents the inhibition curves of yeast and mold cells in strained yoghurt sample.

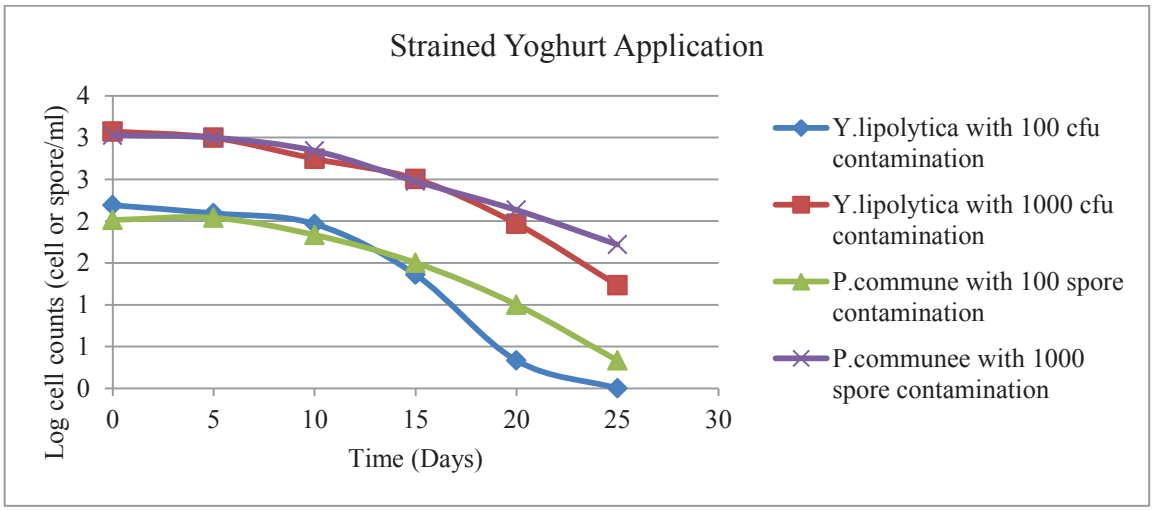

Figure 6.31. Growth Curves of Fungal Cultures with two Different Initial Inoculation Levels in Strained Yoghurt

Fungal contaminations of strained yoghurt generally assumed to be contaminated after fermentation step. There was not enough research found about the application of antifungal lactic acid bacteria to the filtered yoghurt.

This part was performed to examine the antifungal effect with different dairy products. Future studied should be performed for detailed determinations. With this data it can be declined that cultures have had an antifungal effect in filtered yoghurt sample by decreasing cell counts of fungal cultures as shown in Figure 6.31.

6.10.2. Applications of Antifungal Lactic Acid Bacteria to Set Type Yoghurt

Standardized and pasteurized milk was mixed with yoghurt starter culture in line, in the process of an industrial dairy plant. The milk that was mixed with yoghurt starter culture was called as yoghurt milk. The yoghurt milk was filled in yoghurt containers. The containers were also inoculated with antifungal starter culture to a resulting cell count of 10^6 cfu/ml at the beginning of the fermentation. At the end of the fermentation yoghurts were contaminated with *Yarrowia lipolytica* and *Penicillium commune* with resulting cell counts of 100 and 1000 cells or spores per ml respectively. Figure 6.32 represents the inhibition curves of yeast and mold cells with two different initial inoculation levels of each in set yoghurt samples.

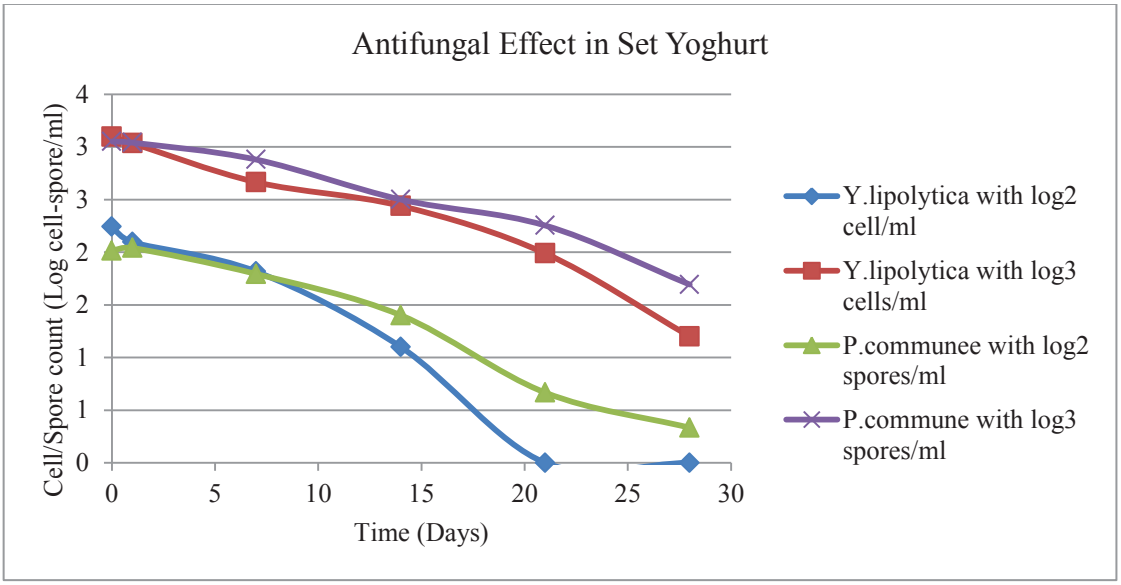

Figure 6.32. Growth Curves of Fungal Cultures with two Different Initial Inoculation Levels in Set-Yoghurt

This study was used to determine the effect of antifungals in combination with yoghurt starter cultures; *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. The antifungal effect was increased when compared with strained yoghurt as a result of both

effective distribution in liquid sample of yoghurt milk in set yoghurt experiments and the increased antifungal effect with thermophilic starter cultures.

Different from strained yoghurt application with nearly same initial contamination levels, *Yarrowia lipolytica* was completely inhibited with 2 $\log_{10}$ inoculation. Both *Penicillium commune* and *Yarrowia lipolytica* were inhibited with 10^6 cfu/ml antifungal starter addition. Similar results were obtained from the study of Delavanne in 2015. They stated the increased antifungal effect of Lactobacillus cultures with yoghurt starter cultures. Also they mentioned the minimum inhibitory concentration for *Yarrowia lipolytica* with 100 cells in yoghurt as 10^6 cfu/ml for single culture of *Lactobacillus harbinensis*. Additionally, they determined the shelf life effect of antifungal starters and stated that the activity was increased one week after from inoculation by the means of the decrease in cell counts of fungal cultures (Delavenne et al., 2015).

Yoghurt was also studied by Aunsjberg et al. in 2015 with *Lactobacillus paracasei* due to its antifungal effect on the product. It was determined that the 10^7 cfu/ml *Lactobacillus paracasei* to be effective on 10^3 spore/ml of mold spore suspensions. Delavenne et. al. had studied the effect of single and mixed cultures of Lactobacilli and Bifidobacteria in yoghurt samples against yeast species *Debaryomyces hanseii, Kluyveromves marxianus, Yarrowia lipolytica* and *Penicillium brevicompactom*. Eight of the eleven isolates could not be able to totally inhibit the growth of cells. But *Lactobacillus harbinensis* and *Lactobacillus rhamnosus* were found to be effective by preventing fungal growth (Aunsjberg et al., 2015, Delavanne et al., 2015).

If the product with “natural yoghurt” label wanted to be produced the lactic acid bacteria should only include *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. For this reason the Turkish set type yoghurts cannot be produced with this protective culture. This part of study was performed to observe the effect of culture in combination with thermophilic lactic acid bacteria fermentations in industrial productions. The protective culture was found to be effective against the contaminating flora of yoghurt samples and the effect was higher when compared with other products experiments. From the scope of this data, culture can be applied in fruit yoghurts, protein fortified yoghurts, sweetened yoghurts. In this study these products’ process lines were not available to examine the effects of the antifungal cultures, since the fermentation is not

performed in packaging materials like yoghurt. The fermentation was performed with its specific starter culture in aseptic tanks.

6.11. Sensory Evaluation

Previously, the samples containing 10^8 and 10^6 cfu/ml protective culture were screened with the same time of the studies mentioned in part 6.9 and 6.10. There were no significant difference between control samples and the samples treated with protective culture. As a result of this prescreening the cultures were applied in the study of cheese and yoghurt applications. But the screenings were performed with 2 or 3 participants. For this reason this data was not shown and used.

The sensory evaluation was performed with 36 panelists who are half trained people with 26-45 ages of range and all were food engineers in an industrial dairy plant. Triangle test was performed in order to differentiate the sample treated with protective culture. The aim of the study was to produce cheese or yoghurt without detectable changes in sensory profile.

Among 36 panelists, 8 people could be able to differentiate the cheese test sample, which were inoculated with antifungal lactic acid bacteria. From the t-test tables, the cheese samples produced with antifungal lactic acid bacteria were not found different from the cheese control samples with 99% confidence interval.

Although the results of the yoghurt triangle test were different from the cheese sensory test results. The antifungal lactic acid bacteria culture usage in set type yoghurt was found to cause more creamy texture in mouth than control samples. The test yoghurt samples, which were inoculated with antifungal lactic acid bacteria, were differentiated from 18 of 25 panelists with a high preference depending on the texture. Since the aim of this part of study was to produce the dairy products without any difference from the original products, yoghurt results indicated that future studies should be performed for antifungal culture applications, whereas the cheese applications were found successful depending on the sensory evaluation results.

CHAPTER 7

CONCLUSIONS

In general this study focuses on the antifungal activity of lactic acid bacteria, *Lactobacillus rhamnosus, Lactobacillus plantarum* and *Lactobacillus paracasei* spp. *paracasei* and their applications in the cheese industry.

Cheese production process involves many microbiological contamination sources. The sources could be bacteria or fungi which were studied in this study. The yeast cultures of *Debaryomyces hansenii, Yarrowia lipolytica, Kluyveromyces marxianus, Pichia membranifaciens, Geotrichum candidum* and the mold *Penicillium commune* were examined for their resistances to selective lactic acid bacteria with potential antifungal property.

The first part of the study was about determination of general contamination levels of industrially produced white cheeses, determining optimum concentration of the most frequently used chemical preservative, e.g. sorbate, and investigating the effects of sorbate with its permitted levels and commercial protective cultures. The cheese samples were determined for their contamination levels. The samples were analyzed in two separate groups; including the cheese produced with commercial protective culture and the cheese produced without commercial protective culture. All of the samples were free from sorbate, benzoate and natamycin and the label statement of being additive free was verified via HPLC analysis (data not shown in the study as it was only performed to be sure about the labels). The results of the cheese without protective cultures are; 73 % of samples tested were contaminated with yeast cells with cell counts between 10 cfu/ml and 2450 cfu/, 76% of the samples were contaminated with mold cells with cell counts of between 10 cfu/ml and 190 cfu/ml. However, the usage of commercial protective culture did not cause a total inhibition and the 46% of the samples were determined as contaminated with yeast cells, 37% of the samples were determined as contaminated with mold cells. The counts of the yeast and molds of the contaminated cheese samples were in between 10-2100 cfu/ml for yeast and 10-150 cfu/ml for mold cells. Results

demonstrated that commercial cultures have an effect to decrease the amount of contamination in white cheese production. The detectable yeast or mold growth was observed even protective culture was used; this was thought to be the result of the bacteria species included in commercial cultures. Because the suppliers always share the effect spectrum of the cultures and do not declare that a commercial culture can inhibit all yeast or mold species.

As mentioned before the first part was about to screen the general contamination levels and the effect of the chemical or biological preservatives. Since potassium sorbate is one of the most widely used chemical protectant and known to inhibit yeast or mold growth (even there were many studies related to the Penicillium which can metabolize sorbate), the different levels of potassium sorbate were examined to the 6 fungal cultures. The sorbate levels tested were100 and 500 ppm which are lower than the legally permitted levels, 1000 ppm, the maximum legally permitted level and 5000 ppm which could not be used in white cheese production according to legislations.100 and 500 ppm levels were found to be ineffective against fungal species and they continued to grow in cell counts in liquid media. Whereas the maximum limit did not inhibit the fungal growth but caused a decrease in growth rates of fungal cultures when compared with the control cultures growths. Only the concentration of 5000 ppm caused a total inhibition except *Penicillium commune* culture which continued to survive in stationary-like growth. The effects of two different commercial culture and sorbate with its maximum level were screened for their effect to decrease counts of *Debaryomyces hanseni* and *Penicillium commune* with factorial design. The results indicated that; one of the commercial cultures was not effective on fungal growth as the other culture and none of the combinations caused a total inhibition of yeast or mold cells which were previously inoculated on the cheese surfaces. The difference between two commercial cultures was the bacteria that they include and the difference between their effects could only be explained by this way. This part of study can be considered as the first study to screen the effect of different sorbate concentrations regarding to their effects on liquid media which were inoculated with single species of yeast or mold cells.

The second part of this study was to screen the effects of selected lactic acid bacteria for their antifungal effect on five yeast and one mold species which were chosen according to the literature and was determined as the most frequently isolated fungal species. Antifungal screenings were performed firstly in order to differentiate the

effect of pure culture and culture combinations of three bacteria; *Lactobacillus rhamnosus, Lactobacillus plantarum* and *Lactobacillus paracasei* spp. *paracasei*. The agar spot tests were performed for different inoculum levels of yeast or mold species. The agar spot screening studies could be the first study to examine the effect of single and mixed cultures against different levels of cell counts of spot for separate yeast or mold cells. According to spot tests, all of the species tested were inhibited with mixture of three lactic acid bacteria with an increased amount when compared with single culture. This was caused by the synergistic effect of lactic acid bacteria and different antifungal compounds synthesized by lactic acid bacteria. The remaining parts of the study were performed with the mixture of three bacteria related to these results.

Antifungal effect of lactic acid bacteria was determined in liquid growth medium prepared with whey and yeast extract as carbon and nitrogen sources. The lactic acid bacteria mixture were prepared and inoculated in whey media with 10^4, 10^6, 10^8 and 10^9 cfu/ml resulting cell counts in order to determine the efficient concentration. The increased concentration of lactic acid bacteria up to 10^8 cfu/ml caused an increased antifungal effect. The 10^9 cfu/ml cell counts of lactic acid bacteria have had the similar antifungal effect against the yeast and mold cultures. The cell count of 10^8 cfu/ml lactic acid bacteria were examined for their antifungal effect at refrigeration temperature and found to be effective for all the cultures tested. The studies stated in the related literature generally focused on the antifungal effect with the method to screen the time value for visible fungal colony on the surface of the product. The growth patterns of the fungal cultures with different antifungal lactic acid bacteria in liquid media could not be found in literature and thought to be a valuable data for dairy industry.

The third part of the study was planned to apply the effective culture in white cheese production. The applications were performed both after fermentation and before fermentation. The maximum effect was obtained in the application in curd, before fermentation. As a result of distributing effectively in curd and synergistic effect of starter lactic acid bacteria, antifungal activity of *Lactobacillus rhamnosus, Lactobacillus plantarum* and *Lactobacillus paracasei* spp. *paracasei* mixture increased. There could not be a study found to compare different applications on white cheese with different initial fungal counts. However three different initial contamination levels of the yeast cultures of *Debaryomyces hansenii, Yarrowia lipolytica, Kluyveromyces marxianus, Pichia membranifaciens, Geotrichum candidum* and the mold *Penicillium commune* was examined for their cell counts at defined time intervals from cheese samples treated

with antifungal cultures. Actually for the general expected levels of fungal contaminations, *Lactobacillus rhamnosus, Lactobacillus plantarum* and *Lactobacillus paracasei* spp. *paracasei* mixture with 10^8 cfu/ml cell counts was found to be effective for 4 weeks. After 4 weeks as a result of total inhibition the cultures were thought to be efficient for the remaining shelf life. Additionally the effect of antifungal lactic acid bacteria mixture with 10^8 cfu/ml initial concentrations was added to cheese at curd step and cheese samples were further inoculated with yeast and mold cells at different time values of the shelf life. The antifungal culture still was active against yeast and mold growth in packaged brined white cheese product. This data can be valuable for dairy industry related to the consumer complaints. In the kitchen air or the refrigerator air mold spores or fungal contamination at very different levels may occur. The consumer who takes the product from market shelf without fungal contamination, hold the product after first opening the package into the refrigerator and depending on the hygiene of equipment or air, the product could be contaminated after first opening and the fungal growth could be observed. Actually this is not a quality defect of the product, if there are no visible fungal growth at the first time of the opening. But with the help of antifungal lactic acid bacteria at different time values of shelf life, the antifungal effect prevents the fungal colonies to rich higher cell counts.

The last part of the study was about the applications of antifungal lactic acid bacteria mixture to dairy products different from white cheese. For this purpose kashkaval cheese, strained yoghurt and set type yoghurt were chosen. The culture was found to be effective against the tested fungal species; *Yarrowia lipolytica* and *Penicillium commune* at 10^8 cfu/ml for kashkaval and 10^6 cfu/ml for yoghurt products.

The culture combination of *Lactobacillus rhamnosus, Lactobacillus plantarum* and *Lactobacillus paracasei* spp. *paracasei* was thought to be more effective when applied at the cheese vats that was assumed from the results of curd applications.

Further studies should be concerned about the characterization of the metabolites with antifungal characteristics and to enhance the antifungal metabolite production of the cultures before commercialization.

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APPENDIX A

CHEMICALS USED AND THEIR CODES

Table A.1. Chemicals Used in Experiments

No	Chemicals	Code
1	MRS Broth	Merck 1.10661
2	MRS Agar	Merck 1.10660
3	Glyserol	Merck 3.56352
4	Agar	Applichem A0949
5	Yeast Mold Broth	Difco 271120
6	Yeast Mold Agar	Difco 271210
7	DRBC Agar	Merck 100466
8	VRBD Agar	Merck 110275
9	VRB Agar	Merck 101406
10	YGC Agar	Merck 1.16000
11	Yeast Extract	Merck 103753
12	Ringer's Tablet	Merck 115525
13	Buffered Peptone Water	Merck 107228
14	RVS Broth	Biomerieux 42210
15	mKTTN Broth	Merck 105878
16	XLT4 Agar	Merck 113919
17	XLD Agar	BD 2554055
18	Fraiser Broth	Oxoid CM0895
19	PALCAM Agar	Oxoid CM0877
20	Oxoid Agar	Oxoid CM1084
21	TSYE Agar	Sigma 93395
22	Coagulase positive staphylococci petrifilm	3M 6491
23	Mac Farland Standarts	Biomerieux 95060
24	Sponge Stick-With Deneutralized Buffer	3M-70200750951

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Table A.1. (cont.)

25	Anaerojar	Oxoid AN0025A
26	Anaerogen kit	Oxoid AN0025A

APPENDIX B

RECIPES FOR CULTURE MEDIA

B.1. MRS Broth

Ingredients	g/L
Pepton	10.0
Lab-Lemco meat extract	10.0
Yeast extract	5.0
D (-) Glucose	20.0
Tween 80	1 ml
K_2HPO_4	2.0
Sodium acetate	5.0
Triammonium citrate	2.0
$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.05
Deionized water	1000 ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.3. Medium was dispensed into test tubes and sterilized by autoclaving at 121° C for 15 minutes.

B.2. MRS Agar

Ingredients	g/L
Pepton	10.0
Lab-Lemco meat extract	10.0
Yeast extract	5.0
D (-) Glucose	20.0

Tween 80	1 ml
K_2HPO_4	2.0
Sodium acetate	5.0
Triammonium citrate	2,0
$MgSO_4.7H_2O$	0,2
$MnSO_4.4H_2O$	0,05
Agar	15.0
Deionized water	1000 ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.3. Medium was sterilized by autoclaving at 121° C for 15 minutes.

B.3. YGC Agar

Ingredients	**g/L**
Yeast extract	5.0
D (-) Glucose	20.0
Chloramphenicol	0.1
Agar	14.9
Deionized water	1000 ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.6. Medium was sterilized by autoclaving at 121° C for 15 minutes.

B.4. DRBC Agar

Ingredients	g/L
Glucose	10.0 g
Bacteriological peptone	5.0 g
Potassium phosphate, monobasic	1.0 g
Magnesium sulfate heptahydrate	0.5 g
RoseBengal (5% soln., w/v)	0.5 ml
Dichloran (2,6-dichloro-4-nitroaniline) solution (0.2%(w/v) in ethanol)	1.0 ml
Chloramphenicol	0.1 g
Distilled water	1 liter
Agar	15.0 g

All ingredients are dissolved in 1000 ml distilled water, and pH was adjusted to 5,6. Medium was sterilized by autoclaving at 121° C for 15 minutes.

B.4. Yeast Mold Agar

Ingredients	g/L
Yeast Extract	3.0 g
Malt Extract	3.0 g
Peptone	5.0 g
Dextrose	10.0 g
Agar	20 g
Distilled water	1000 ml

All ingredients are dissolved in 1000 ml distilled water, and pH was adjusted to 6,2. Medium was sterilized by autoclaving at 121° C for 15 minutes.

B.5. Yeast Mold Broth

Ingredients	g/L
Yeast Extract	3.0 g
Malt Extract	3.0 g
Peptone	5.0 g
Dextrose	10.0 g
Distilled Water	1000 ml

All ingredients are dissolved in 1000 ml distilled water, and pH was adjusted to 6,2. Medium was sterilized by autoclaving at 121° C for 15 minutes.

B.5. VRB Agar

Ingredients	g/L
Pepton	7.0
Yeast extract	3.0
Lactose	10.0
NaCl	5.0
Ox Bile (Bile Salt Mixture)	1.5
Neutral Red	0.03
Cyrstal Violet	0.002
Agar	13
Distilled water	1000

All ingredients are dissolved in 1000 ml distilled water, and pH was adjusted to 7,4. Medium was sterilized by boiling the solution, boiling should continue 2 minutes.

B.6. VRBD Agar

Ingredients	g/L
Pepton	7.0
Yeast extract	3.0
Lactose	10.0
NaCl	5.0
Ox Bile (Bile Salt Mixture)	1.5
Neutral Red	0.03
Cyrstal Violet	0.002
Agar	13
Distilled water	1000

All ingredients are dissolved in 1000 ml distilled water, and pH was adjusted to 7,4. Medium was sterilized by boiling the solution, boiling should continue 2 minutes.

B.7. Yeast Mold Soft Agar

Ingredients	g/L
Yeast Extract	3.0 g
Malt Extract	3.0 g
Peptone	5.0 g
Dextrose	10.0 g
Agar	6 g
Distilled water	1000 ml

All ingredients are dissolved in 1000 ml distilled water, and pH was adjusted to 6,2. Medium was sterilized by autoclaving at 121° C for 15 minutes. Before usage the soft agar media should be melted in water bath.

APPENDIX C

YEAST AND MOLD COUNTS

Table C.1. Cheese samples of different brands without commercial protective culture

Sample ID *	Yeast Count (cfu/ml) **	Mold Count(cfu/ml) **
XUPC1	190	10
XUPC2	1400	20
XUPC3	600	nd
XUPC4	10	nd
XUPC5	nd	10
XUPC6	nd	90
XUPC7	540	140
XUPC8	2060	nd
XUPC9	400	20
XUPC10	710	10
XUPC11	nd	10
XUPC12	nd	30
XUPC13	nd	10
XUPC14	nd	nd
XUPC15	820	nd
XUPC16	230	10
XUPC17	110	30
XUPC18	180	30
XUPC19	1300	70
XUPC20	950	110
YUPC1	nd	80
YUPC2	240	10
YUPC3	1300	nd
YUPC4	1070	20
YUPC5	nd	10
YUPC6	1920	60
YUPC7	nd	40
YUPC8	100	160
YUPC9	180	10
YUPC10	nd	10
YUPC11	210	90
YUPC12	370	nd
YUPC13	nd	40
YUPC14	180	100

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Table C.1. (cont.)

Sample ID	Yeast Count (cfu/ml)	Mold Count(cfu/ml)
YUPC15	nd	nd
YUPC16	350	140
YUPC17	2450	50
YUPC18	460	10
YUPC19	nd	20
YUPC20	690	20
ZUPC1	10	10
ZUPC2	90	190
ZUPC3	nd	10
ZUPC4	330	nd
ZUPC5	10	nd
ZUPC6	940	80
ZUPC7	430	nd
ZUPC8	nd	nd
ZUPC9	750	70
ZUPC10	210	90
ZUPC11	60	nd
ZUPC12	nd	110
ZUPC13	700	30
ZUPC14	1090	10
ZUPC15	470	20
ZUPC16	1840	nd
ZUPC17	1150	90
ZUPC18	300	10
ZUPC19	290	10
ZUPC20	70	60

*Coded names are the brand codes X,Y and Z, UPC indicates without protective culture. ** nd= not detected (<10 cfu/ml)

Table C.2. Cheese produced with commercial protective culture (A, Brand)

Sample ID*	Yeast Count (cfu/ml)**	Mold Count(cfu/ml)**
APC1	nd	nd
APC2	nd	nd
APC3	nd	nd
APC4	nd	nd
APC5	nd	nd
APC6	200	10
APC7	140	nd

(cont. on next page)

Table C.2. (cont.)

Sample ID	Yeast Count (cfu/ml)	Mold Count(cfu/ml)
APC8	20	nd
APC9	90	nd
APC10	170	nd
APC11	490	20
APC12	100	10
APC13	nd	nd
APC14	nd	nd
APC15	nd	nd
APC16	nd	nd
APC17	110	10
APC18	640	60
APC19	nd	nd
APC20	nd	nd
APC21	nd	nd
APC22	60	10
APC23	10	40
APC24	nd	110
APC25	nd	nd
APC26	nd	nd
APC27	290	nd
APC28	200	nd
APC29	60	nd
APC30	nd	nd
APC31	80	nd
APC32	320	80
APC33	nd	10
APC34	nd	nd
APC35	100	nd
APC36	930	nd
APC37	440	30
APC38	40	10
APC39	nd	nd
APC40	nd	nd

*= Coded names are the brand codes A, PC indicates protective culture. ** nd= not detected (<10 cfu/ml)

Table C.3. Cheese produced with commercial protective culture (B, Brand)

Sample ID*	Yeast Count (cfu/ml)**	Mold Count(cfu/ml)**
BPC1	2100	10
BPC2	nd	nd
BPC3	nd	nd
BPC4	nd	nd
BPC5	nd	nd
BPC6	nd	50
BPC7	nd	10
BPC8	nd	30
BPC9	nd	nd
BPC10	nd	nd
BPC11	nd	nd
BPC12	nd	nd
BPC13	nd	nd
BPC14	240	90
BPC15	nd	20
BPC16	nd	nd
BPC17	700	nd
BPC18	10	nd
BPC19	90	10
BPC20	1800	110
BPC21	nd	nd
BPC22	nd	nd
BPC23	nd	nd
BPC24	50	40
BPC25	1050	30
BPC26	280	10
BPC27	700	20
BPC28	nd	nd
BPC29	nd	nd
BPC30	nd	nd
BPC31	610	nd
BPC32	260	10
BPC33	nd	nd
BPC34	450	nd
BPC35	1750	nd
BPC36	290	nd
BPC37	1200	20
BPC38	940	10
BPC39	640	150
BPC40	300	nd

*B indicates the brad B, PC=protective culture usage, **nd=not detected (<10 cfu/ml)

Table C.4. Cheese produced with commercial protective culture (C Brand)

Sample ID*	Yeast Count (cfu/ml)**	Mold Count(cfu/ml)**
CPC1	nd	10
CPC2	nd	nd
CPC3	nd	nd
CPC4	nd	60
CPC5	nd	40
CPC6	nd	10
CPC7	nd	nd
CPC8	nd	nd
CPC9	nd	nd
CPC10	910	20
CPC11	1120	90
CPC12	60	nd
CPC13	nd	nd
CPC14	540	nd
CPC15	nd	nd
CPC16	nd	nd
CPC17	220	110
CPC18	10	10
CPC19	nd	nd
CPC20	880	20
CPC21	1400	nd
CPC22	200	nd
CPC23	nd	nd
CPC24	nd	nd
CPC25	210	70
CPC26	20	10
CPC27	nd	90
CPC28	nd	10
CPC29	90	10
CPC30	140	nd
CPC31	nd	nd
CPC32	300	nd
CPC33	410	nd
CPC34	120	nd
CPC35	380	80
CPC36	nd	nd
CPC37	nd	40
CPC38	nd	nd
CPC39	nd	nd
CPC40	nd	10

*C indicates the brand C, PC=protective culture usage, **nd=not detected (<10 cfu/ml)

Table C.5. Yeast and mold counts in whey media inoculated with 10^4 cfu/ml protective culture (data are represented in mean value of triplicates)

Time (Hour)	*D. hansenii (log cfu/ml)*	*P. membranifaciens (log cfu/ml)*	*K. marxianus (log cfu/ml)*	*Y. lipolytica (log cfu/ml)*	*G. candidum (log cfu/ml)*	*P. commune (log cfu/ml)*
0	4.77	4.70	4.72	4.77	4.68	4.75
1	4.73	4.73	4.72	4.85	4.71	4.75
2	4.79	4.73	5.07	5.20	4.99	4.88
3	4.96	5.03	5.83	5.70	5.23	4.90
4	5.17	5.79	5.77	5.78	5.43	5.09
5	5.43	5.77	5.55	5.80	5.47	5.23
6	5.72	5.30	5.11	5.78	5.34	5.39
7	5.72	4.97	5.09	4.99	5.37	5.60
8	5.69	4.47	5.11	4.90	5.42	5.76
9	5.67	4.28	4.85	4.73	5.29	5.85
10	5.59	3.98	4.03	4.32	5.28	5.85
15	5.53	3.23	3.40	3.88	5.22	5.80
20	4.98	3.21	3.27	3.81	4.99	5.70
25	4.84	3.24	3.17	3.79	4.90	5.72
30	4.24	3.02	2.91	3.76	4.89	5.65
35	3.99	2.94	2.93	3.70	4.79	5.62

Table C.6. Yeast and mold counts in whey media inoculated with 10^6 cfu/ml protective culture (data are represented in mean value of triplicates)

Time (Hour)	*D. hansenii (log cfu/ml)*	*P. membranifaciens (log cfu/ml)*	*K. marxianus (log cfu/ml)*	*Y. lipolytica (log cfu/ml)*	*G. candidum (log cfu/ml)*	*P. commune (log cfu/ml)*
0	4.80	4.51	4.66	4.85	4.55	4.64
1	4.83	4.53	4.66	4.84	4.49	4.71
2	5.00	4.91	4.99	5.07	4.67	4.83
3	5.18	5.28	5.25	5.26	4.85	4.85
4	4.94	5.22	5.35	5.20	4.94	4.93
5	4.31	4.90	5.32	5.24	5.19	5.03
6	3.86	4.20	5.16	5.32	5.19	5.09
7	3.49	3.43	5.01	5.14	5.00	5.09
8	3.43	3.29	4.89	4.91	4.86	4.99
9	3.14	3.15	4.74	4.59	4.33	5.01
10	3.12	3.11	4.28	4.12	3.95	4.94
15	2.92	3.04	4.10	3.47	3.10	4.91
20	2.61	3.00	3.95	3.18	2.49	4.83
25	2.26	2.69	3.82	2.80	1.50	4.54
30	2.14	2.74	3.46	2.16	0.88	4.36
35	1.97	2.39	3.05	1.69	0.00	4.17

Table C.7. Yeast and mold counts in whey media inoculated with 10^8 cfu/ml protective culture (data are represented in mean value of triplicates)

Time (Hour)	*D. hansenii (log cfu/ml)*	*P. membranifaciens (log cfu/ml)*	*K. marxianus (log cfu/ml)*	*Y. lipolytica (log cfu/ml)*	*G. candidum (log cfu/ml)*	*P. commune (log cfu/ml)*
0	4.75	4.56	4.70	4.89	4.61	4.83
1	4.73	4.57	4.65	4.90	4.58	4.84
2	4.73	4.59	4.60	4.74	4.42	4.82
3	4.31	4.27	4.43	4.41	3.96	4.55
4	4.24	3.72	4.02	4.07	3.82	4.50
5	3.26	3.62	3.89	4.04	3.70	4.44
6	3.11	3.60	3.84	3.92	3.64	4.23
7	2.76	3.59	3.50	3.85	3.52	4.01
8	2.83	3.51	3.42	3.67	3.49	3.31
9	2.65	2.97	3.18	3.22	3.39	3.14
10	2.22	2.64	2.40	2.77	3.09	2.54
15	2.01	1.50	1.38	2.67	2.46	2.12
20	1.42	1.00	0.50	2.22	1.30	1.45
25	0.50	0.00	0.00	1.42	0.00	0.50
30	0.00	0.00	0.00	0.00	0.00	0.50
35	0.00	0.00	0.00	0.00	0.00	0.00

Table C.8. Yeast and mold counts in whey media inoculated with 10^9 cfu/ml protective culture (data are represented in mean value of triplicates)

Time (Hour)	*D. hansenii (log cfu/ml)*	*P. membranifaciens (log cfu/ml)*	*K. marxianus (log cfu/ml)*	*Y. lipolytica (log cfu/ml)*	*G. candidum (log cfu/ml)*	*P. commune (log cfu/ml)*
0	4.54	4.56	4.57	4.60	4,58	4,54
1	4.59	4.59	4.61	4.71	4,55	4,60
2	4.77	4.48	4.62	4.80	4,46	4,67
3	4.38	4.32	4.44	4.44	4,26	4,61
4	4.27	3.75	4.07	4.26	3,96	4,5
5	3.31	3.70	3.97	4.02	3,86	4,49
6	3.20	3.57	3.85	3.95	3,77	4,10
7	3.08	3.41	3.44	3.80	3,61	3,93
8	3.03	3.23	2.86	3.45	3,23	3,16
9	2.95	2.77	2.32	3.04	2,90	3,01
10	2.38	2.39	2.27	2.61	2,47	2,62
15	2.04	1.84	1.60	2.00	1,30	2,25
20	1.84	1.00	1.00	1.00	0.00	2,16
25	1.00	0.00	0.00	0.00	0.00	1,85
30	0.00	0.00	0.00	0.00	0.00	1.00
35	0.00	0.00	0.00	0.00	0.00	0.00

Table C.9. Yeast and mold counts with low contamination levels in whey media inoculated with 10^8 cfu/ml protective culture (data are represented in mean value of triplicates)

Time (Hour)	*D. hansenii* (log cfu/ml)	*P. membranifaciens* (log cfu/ml)	*K. marxianus* (log cfu/ml)	*Y. lipolytica* (log cfu/ml)	*G. candidum* (log cfu/ml)	*P. commune* (log cfu/ml)
0	2.07	2.20	2.14	2.04	2.25	2.00
1	2.23	2.30	2.32	2.00	2.50	1.84
2	2.58	2.39	2.53	2.20	2.74	2.07
3	2.64	2.32	2.95	2.30	2.78	2.14
4	2.70	2.90	3.01	2.73	2.77	2.36
5	2.87	3.04	2.95	2.76	2.79	2.60
6	3.04	3.19	2.87	2.84	2.71	2.63
7	2.94	3.04	2.85	2.79	2.65	2.46
8	2.99	2.86	2.77	2.80	2.67	2.34
9	2.84	2.90	2.68	2.77	2.47	2.23
10	2.70	2.79	2.53	2.65	2.32	2.17
15	2.62	2.67	2.47	2.51	2.14	2.21
20	2.51	2.54	2.34	2.47	1.95	1.95
25	2.04	2.30	2.04	2.36	1.95	1.47
30	1.60	1.84	1.95	2.04	1.30	1.47
35	0.69	1.60	1.77	1.30	0.00	1.00

Table C.10. Yeast and mold counts of contaminated cheese curd samples inoculated with 10^8 cfu/ml protective cultures

Fungal Culture	Contamination Levels	0 hour	3rd day	5th day	7th day	14th day	21st day	28th day
D. hansenii	High Level	4.72	4.25	3.95	3.70	3.25	3.02	2.77
	Medium Level	3.62	3.29	2.85	2.38	2.41	1.90	1.30
	Low Level	2.66	2.61	2.38	2.11	2.17	1.97	1.00
Y. lipolytica	High Level	4.72	4.36	3.97	3.77	3.61	3.24	2.90
	Medium Level	3.77	3.67	3.61	3.23	3.05	2.84	2.27
	Low Level	2.69	2.60	2.49	2.25	2.11	1.90	1.47
K. marxianus	High Level	4.60	4.49	4.34	3.79	3.47	3.07	2.81
	Medium Level	3.71	3.69	3.08	2.92	2.80	2.25	1.84
	Low Level	2.77	2.67	2.49	2.44	2.36	2.00	1.84
P. membranif aciens	High Level	4.71	4.68	4.30	3.97	3.70	3.50	3.27
	Medium Level	3.60	3.38	3.30	3.27	2.96	2.73	2.58
	Low Level	2.69	2.51	2.43	2.34	2.11	1.95	1.00
G. candidum	High Level	4.54	4.20	4.02	3.68	3.27	2.20	2.38
	Medium Level	3.67	3.29	3.07	2.86	2.62	2.04	1.00
	Low Level	2.59	2.49	2.44	2.38	2.04	1.77	1.00
P. Commune	High Level	4.61	4.58	4.42	4.14	3.92	3.49	2.92
	Medium Level	3.62	3.59	3.18	3.01	2.97	2.90	2.32
	Low Level	2.74	2.53	2.38	2.23	2.00	1.00	1.00

All results are mean value of triplicates. 3 different level of fungal contamination performed and the growth of fungal cultures observed at defend time (days) value.

Table C.11. Yeast and mold counts of contaminated cheese product samples inoculated with 10^8 cfu/ml protective cultures

Fungal Culture	Contamination Levels	0 hour	3rd day	5th day	7th day	14th day	21st day	28th day
D. hansenii	High Level	4.70	4.69	4.53	4.32	4.06	3.74	3.31
	Medium Level	3.62	3.38	3.27	3.04	2.66	2.46	2.23
	Low Level	2.69	2.68	2.55	2.32	2.17	1.95	1.30
Y. lipolytica	High Level	4.72	4.72	4.59	4.41	4.14	3.94	3.47
	Medium Level	3.77	3.73	3.69	3.55	3.37	3.14	2.68
	Low Level	2.69	2.70	2.68	2.55	2.32	2.07	1.77
K. marxianus	High Level	4.68	4.58	4.49	4.31	4.11	3.83	3.00
	Medium Level	3.71	3.69	3.08	2.92	2.80	2.58	2.04
	Low Level	2.69	2.65	2.59	2.32	2.30	2.04	1.30
P. membranifaciens	High Level	4.71	4.68	4.50	4.38	4.17	3.96	3.43
	Medium Level	3.69	3.64	3.55	3.21	2.96	2.85	2.69
	Low Level	2.70	2.60	2.59	2.43	2.14	2.04	1.69
G. candidum	High Level	4.72	4.63	4.57	4.44	4.24	4.01	3.58
	Medium Level	3.77	3.66	3.51	3.47	3.27	3.02	2.79
	Low Level	2.71	2.67	2.59	2.55	2.38	2.00	1.60
P. Commune	High Level	4.68	4.61	4.56	4.47	4.09	3.88	3.17
	Medium Level	3.71	3.69	3.61	3.55	3.20	2.96	2.47
	Low Level	2.77	2.70	2.63	2.58	2.41	2.00	1.00

Table C.12. Yeast and mold counts of contaminated kashkaval cheese inoculated with 10^8 cfu/ml protective cultures (data were calculated as mean value of triplicates)

Time (Days)	*Yarrowia lipolytica* (log cfu/ml)	*Penicillium commune* (log cfu/ml)
0	3.06	3.01
1	3.05	2.97
7	2.89	2.69
14	2.59	2.51
21	2.22	2.13
28	1.49	1.20

Table C.13. Yeast and mold counts of contaminated stained yoghurt inoculated with 10^6 cfu/ml protective cultures (data were calculated as mean value of triplicates)

Time (days)	Cell counts of *Y.lipolytica* (Log cfu/ml)		Cell counts of *P.communee* (Log cfu/ml)	
0	2.19	3.07	2.01	3.02
5	2.09	2.99	2.04	2.99
10	1.96	2.74	1.83	2.84
15	1.36	2.50	1.50	2.47
20	0.33	1.96	1.00	2.13
25	0.00	1.23	0.33	1.71

Table C.14. Yeast and mold counts of contaminated set yoghurt inoculated with 10^6 cfu/ml protective cultures (data were calculated as mean value of triplicates)

Time (days)	Cell counts of *Y.lipolytica* (Log cfu/ml)		Cell counts of *P.communee* (Log cfu/ml)	
0	2.24	3.09	2.01	3.05
5	2.09	3.03	2.04	3.04
10	1.81	2.66	1.79	2.87
15	1.10	2.44	1.40	2.50
20	0.00	1.98	0.66	2.25
25	0.00	1.20	0.33	1.69

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