BEHAVIOUR AND CONTROL OF LISTERIA INNOCUA DURING MANUFACTURE AND STORAGE OF TURKISH WHITE CHEESE

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ABSTRACT

BEHAVIOUR AND CONTROL OF LISTERIA INNOCUA DURING MANUFACTURE AND STORAGE OF TURKISH WHITE CHEESE

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Growth and survival of *L. innocua* and TAC in artificially inoculated Turkish White Cheese during manufacturing and storage periods, with respect to different level of contamination of *L. innocua* were investigated.

Cheese products were manufactured by the short-set procedure in pilot-plant-sized vats, as in AOÇ dairy factory. Pasteurized cow's milk was inoculated with *L. innocua* for obtaining the initial loads of 3.84 and 7.12 log CFU/ml.

Bacterial load of inoculated milk, whey, post-ripened curd and post-salted cheese was determined during processing at 20±5ºC.

Cheeses were stored in 16% saline solution at 4 ± 2ºC for up to 45 days. Samples were taken from each treatment and analysed on 5, 10, 15, 20, 30 and 45 days. Total decrease of *L. innocua* in Turkish White Cheese with each inoculum dose was approximately 2 logs during the storage period. *L. innocua* values were also compared with TAC values.

The results had shown that, if pasteurization is not as sufficient as to kill this bacteria in contaminated raw milk, or if there is post-process contamination, *Listeria* can survive during the manufacture and storage, although they decrease in number. Storage (ripening) period for consumption of cheeses should be at least 90 and 178 days, in low and high inoculum dose, respectively.

Physico-chemical properties of cheese as pH, acidity, salt, fat, moisture contents during storage period were determined. Salt concentration, pH value and storage temperature had a cumulative bactericidal effect on microorganisms.

In this respect, effect of implementing HACCP method on reducing the Listerial contamination of Turkish White Cheese was determined for checking the quality problems in a cheese plant and for directing the companies as a guide.

Key words: Turkish White Cheese, *Listeria innocua*, HACCP

ÖZ

TÜRKİYEDE ÜRETİLEN BEYAZ PEYNİRLERDE
LISTERIA INNOCUA'NIN ÜRETİM VE DEPOLAMA SÜRESİNE BAĞLI
OLARAK CANLILIĞI VE KONTROLÜ

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Bu çalışmada *L. innocua*'nın canlı kalma ve üreme durumunun belirlenmesi amacıyla pilot tip peynir teknesinde, AOÇ Süt Fabrikasındaki üretim esas alınarak beyaz peynir üretimi yapıldı. Pastörize inek sütü 3.84 ve 7.12 log CFU/ ml düzeylerini elde edecek şekilde *L. innocua* ile inokule edildi.

Inoküle süt, peynir altı suyu, olgunlaşmış çökelek ve salamura peynir aşamalarında numuneler alınarak, üretim ve soğuk depolama sırasında Toplam Aerobik Bakteri ve *L. innocua* sayıları tesbit edildi. pH, asitlik, tuz, nem ve yağ miktarları gibi kimyasal analizler yapıldı. Ulaşılan pH değeri, salamura konsantrasyonu ve depolama sıcaklığının *L. innocua* üzerinde toplam bir inhibisyon etkiye sahip olduğunu gösterdi.

Elde edilen verilere göre, 3.84 ve 7.12 log CFU/ml düzeyinde *L. innocua* ile kontamine olan veya bu düzeylerde *L. innocua* içeren pastörize edilmeyen sütten üretilen peynirlerin, güvenli bir tüketim için sırasıyla en az 90 ve 178 gün soğuk koşullarda olgunlaştırma (depolama) periyoduna gerek duyulmaktadır.

Beyaz peynir numuneleri %16 salamura içerisinde 4ºC'deki depo ortamında 45 gün boyunca bekletildi. 5, 10, 15, 20, 30 ve 45. günlerde düşük ve yüksek inokulasyon seviyesinde peynirlerden de numuneler alınarak ekimler yapıldı. Tuzlanan çökelek peynir ile 45 gün depolanan tuzlu peynir arasında toplam *L. innocua* azalışı, yaklaşık 2 log düzeyinde tesbit edildi.

Bu kapsamda *Listerial* kontaminasyonu azaltıp azaltmayacağı hususunda HACCP uygulamasının etkisi, peynir üretim fabrikalarındaki sorunlar kontrol edilerek belirlendi.

Anahtar Kelimeler: Beyaz peynir, *Listeria innocua,* HACCP

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LIST OF ABBREVIATONS

AOÇ	Atatürk Orman Çiftliği
HACCP	Hazard Analyses and Critical Control Points
CCP	Critical Control Point
TSB	Triptic Soy Broth
TSA	Triptic Soy Agar
BHIB	Brain Heart Infusion Broth
BHIA	Brain Heart Infusion Agar
L. innocua	*Listeria innocua*
L. monocytogenes	*Listeria monocytogenes*
TAC	Total Aerobic Count
LAB	Lactic Acid Bacteria
GMP	Good Manufacturing Practises
GHP	Good Hygiene Practises
TSE	Turkish Standart Enstitute
SD	Standart Deviation

CHAPTER 1

INTRODUCTION

1.1 Turkish White Cheese

Turkish White Cheese is a major traditional dairy product in the Turkish market. It is a very similar kind of cheese known widely as Feta Cheese product of Greece. Its consumption is still increasing and over 130.000 tons of Turkish White Cheese per year are produced in Turkey [1]. It can be consumed while fresh, but it is mostly eaten after being ripened in a saline solution. Even today most cheese is made by traditional methods, frequently from raw milk, and is much handled by the cheese makers. Therefore, if contaminated, it could be a major cause of food borne disease [1].

Turkish White Cheese is produced from cow's milk. The major characteristics are the snow white color, the pleasant slightly acid taste and rich flavour. The texture is firm and smooth. The contents for moisture and fat-in-dry-matter contents are 51.5-57.1 % and 46.2-53.3 %, respectively [2].

There are both similarities and differences between Turkish White Cheese that carries Turkish characteristics, and Feta Cheese. Feta is the general name given to white salined cheeses, produced in Greece which is produced from sheep's or mixed sheep's and goat's milk in a ratio up to 7:3, respectively (Greek Codex of Food and Drinks, 1998). The major characteristics of Feta cheese are snow white colour, the pleasant slightly acid taste and the rich flavour. The texture is firm, smooth and creamy and some irregular small mechanical openings are desirable. Feta differs from Turkish White Cheese

majorly in being dry-salted. The production flow chart of the Feta Cheese is the same with Turkish White Cheese, till the drainage of the whey from curd. After the coagulum is transferred into perforated moulds, it is waited until it is firm enough to remove the moulds. The cheese blocks are dry salted on the surface. After 12 h, the blocks are reversed and salted again. This is repeated until the salt content of cheeses reaches 4% on dry basis. The average annual consumption per capita of Feta in Greece is approximately 12 kg. Traditionally, Feta Cheese was manufactured from non-pasteurized milk in small family premises with elementary equipments, as some Turkish White Cheeses in Turkey [3].

1.2 Pathogens in Cheese

Mainly cheese pathogens come from raw milk. The animals may often suffer from clinical and subclinical mastitis. Severe mastitis leads to production of milk with high numbers of pathogens. Some of them are *Staphylococcus aureus, Mycobacterium, Brucella melitensis* or *abortus, Salmonella typhimirium, Listeria monocytogenes, Campylobacter jejuni, Bacillus cereus, Escherichia coli* and *Yersinia enterocolitica* and can be transferred from milk to the cheese thus causing food poisoning. Teat washing and/or disinfection before milking is the suggested as a control measure for the subclinical cases [4].

1.3 Properties of *Listeria* spp

The genus *Listeria* consists of small, non-spore forming, gram-negative rods. *L. monocytogenes* is the primary pathogenic species for man, which causes a foodborne disease called listeriosis in susceptible individuals, including pregnant woman and immunocompromised individuals. *Listeria* spp. are facultative anaerobic, that are psychotropic, motile at 25°C but non-motile at 37°C, growing from 1°C to 44°C [5,6]. The cells are relatively resistant to freezing, and are harmed by pH≥5.0 [6].

Listeria is ubiquitous in the environment and is distributed worldwide. *Listeria* spp. have been isolated from fresh water, wastewater, slaughterhouse waste, milk of normal and mastitic cows [7], the feces of healthy humans, mud, and soil, especially when decaying vegetable material is present [8]. A wide range of animals including mammals, birds, fish and invertebrates have been reported to carry *Listeria* spp [8]. *Listeria* spp. is often present in nature and has been found in milk, in dairy factories, and in soft and semi-soft cheeses [9]. The wide distribution of *Listeria* in the environment is probably related to its ability to grow and survive in extreme conditions [10].

The genus *Listeria* consists of 6 species [11]:

- *L. monocytogenes*
- *L. greyi*
- *L. innocua*
- *L. ivanovii*
- *L. seeligeri*
- *L. welshimeri*

The serotype *L. monocytogenes* is the most important pathogen for a variety of animals including man, however, not all strains of *L. monocytogenes* are pathogenic. *L. ivanovii* has occasionally been found to be associated with infections in humans but is more relevant in relation to infections in domestic animals and sheep. *L. seeligeri* and *L. innocua* have on rare occasions been found in humans and animals respectively. *L. welshimeri* and *L. greyi* are not known to be pathogenic in animals or man. *L. monocytogenes* grows at temperatures of between $<0^0$C and 45^0C and is able to grow slowly at temperatures as low as 5-6^0C. Since *L. monocytogenes* is psychotrophic and can grow at low temperatures, growth of *L. innocua* on cheese can occur.

L. innocua is resistant to certain environmental stresses and can survive in media diverse like wood shavings, fodder, dry straw, animal faeces and soil for several weeks and, in some cases, years. The organism has the ability to withstand light and repeated freezing and thawing.

The bacterium has been found in a variety of raw foods, such as uncooked meats, raw fish, raw shellfish, poultry, and vegetables, as well as in processed foods that become contaminated after processing, such as cook-chill meals, salads, soft cheeses, cold cuts and pates. Unpasteurized (raw) milk, or foods made from unpasteurized milk may contain the bacterium. The consumption of contaminated food is considered to be the principal route of infection and a wide range of food products have been implicated in outbreaks, including soft cheeses and meat-based pates [11].

It is questionable whether *L. monocytogenes* can survive the pasteurization process as defined by the Grade A pasteurized milk ordinance [12]. However, evidence on this issue is conflicting, partially because of 1) the methods used to determine heat resistance of *L. monocytogenes* and 2) the physiological state of the bacterium during heating.

Strains of *L. monocytogenes* differ in their heat resistance. Strains most commonly used for testing include those isolated from the Scott A (serotype 4b), V_7 (serotype 1a) and V37 (serotype 4b) and those most commonly isolated from raw milk-ATCC 19115 (serotype 4b) and F5069 (serotype 4b). Of these, strain Scott A is the most heat resistant [12].

Numerous studies have been carried out to determine the impact of food processing, preservation procedures and disinfectation of surfaces on the survival of *L. monocytogenes*. This microorganism can proliferate at refrigeration temperatures and can also be relatively resistant to normal pasteurization conditions (72°C, 15 sec) [12]. The Decimal Reduction value of *Listeria* in milk

was given as 2.4-4.1 s at 71.7°C ($D_{71.7°C}$: 2.4-4.1 s) but this value changes according to the food material [7]. It was reported that, *L. monocytogenes* inoculated at a level of $1.8x10^4$ CFU/ml of raw milk did not survive upon heating at 67°C for 20 s or more, in a plate pasteurizer, in a small impermeable plastic bag [12]. It was concluded that normal pasteurization of milk would prevent the contamination of cheeses with *Listeria* spp., provided that post-production contamination is prevented. The results suggested that *Listeria* in dairy products is a minor problem if pasteurization of the raw milk is employed [12]. However, early investigations indicated that *L. monocytogenes* might be relatively heat resistant. Doyle [13], reported survival of *L. monocytogenes* in milk after pasteurization at 72.2°C for 16.4 s. It was suggested that the organism may be more heat tolerant when it is enclosed by phagocytes and survival of such *Listeria* in milk after pasteurization at 72.2°C for 16.4 s was reported. The possible difference in heat resistance between *Listeria* freely suspended and those enclosed by phagocytes may have great public health significance [13]. Also, Beckers et al., found survival of *Listeria* spp. after pasteurization at 78°C for 15 s [12].

1.4 Listeria monocytogenes and *Listeria innocua*

L. monocytogenes is an opportunistic haemolytic pathogen of humans and animals recently involved in several outbreaks and sporadic cases of listeriosis associated with the consumption of contaminated foods [9]. It has emerged 14-15 years ago as an important food-borne pathogen. It is appreciably more heat resistant than other non sporing microorganisms such as *Salmonella* and *Campylobacter,* and grow at refrigeration temperatures [14]. It is more acid tolerant than most foodborne pathogens and is able to grow at relatively high NaCl concentrations [15], which facilitates its survival in foods containing NaCl and organic acids as preservatives.

It can cause abortions in pregnant women and meningitis or meningio-encephalitis in immunocompromised men and women [6,13]. In dairy cattle, *L. monocytogenes* can cause mastitis and abortion, leading to excretion of the organism in milk from the infected animal [13].

L. innocua, a species closely related to *L. monocytogenes*, is non-haemolytic and non-pathogenic. Both species share the same natural environments and both has the same properties as gram positive, rod, heat and freeze tolerant [8,14,16], can be frequently isolated from pasteurized milk, soft cheeses, dairy products and other foods [17]. In many articles *L. innocua* was used as an indicator of *L. monocytogenes* [8,13,16,18].

The main route of infection in the human population is the consumption of contaminated food. *L. monocytogenes* is killed by pasteurization and heating procedures used to cook ready-to eat processed foods. However, there is particular concern where the organism is present in refrigerated foods intended for consumption without further cooking. Listeriosis is the name of the illness caused by *Listeria* serotypes including *L. monocytogenes* [10].

1.5. Occurrence of *Listeria* spp. in cheese

L. monocytogenes behaves differently in different kinds of cheese. It has been reported that *L. monocytogenes* survived for more than 140 days in Colby cheese [1], for more than a year in Cheddar Cheese [19] and for more than 90 days in Feta cheese [1]. The microorganism has even grown in Camembert cheese [1]. If Turkish White Cheeses are prepared from raw milk, it is very likely that many of them could be contaminated with *Listeria* spp. The behaviour of the pathogen depends mainly on the strain of *L. monocytogenes* [20], and on different conditions in the cheeses during the manufacture and storage (ripening) period.

Raw milk has been reported as a vehicle for *L. monocytogenes,* some ewes cheese varieties are frequently produced from raw milk [18]. *L. monocytogenes* and *L. innocua* have been detected in samples of ewes milk from 287 farms in Central spain at a rate of 2.19% and 2.00%, respectively [21]. In a survey in Netherlands by Beckers et al.[12], *L. monocytogenes* was detected in 7 of 69 samples of imported soft cheese made from raw milk.

Ryser et al.[13], observed that *L. monocytogenes* survived but did not grow during the manufacture of cottage cheese from contaminated milk, while Piccinin [23], reported that *Listeria* is growing in sterilized cottage cheese during storage at refrigerator temperatures whwn there is post- process contamination. Ryser [19], found when small unsalted cheese was prepared from naturally infected milk containing 5.10^5 *L. monocytogenes*/ ml, the organism survived through 7 d of storage 3 to 5 °C.

It has become a major concern to the food industry because of several reports of listeriosis outbreaks associated with contaminated dairy and food products. Some examples have been associated with consumption of pasteurized milk and dairy products since 1983 [23]. One of these outbreaks was linked to a contaminated Mexican style cheese manufactured in California, which resulted in 142 cases of listeriosis, including 40 deaths [23]. An other evidence for the role of milk in transmission of *L. monocytogenes* from infected dairy cattle to man has appeared in European literature. A massive reported outbreak of human listeriosis occured in Halle, Germany and was followed by epidemics in Jena, Germany and Prague, Czechoslovakia [13]. Consumption of *Listeria* contaminated raw milk was believed to be one of the major cause of human illness. Since then, it has become relatively common to isolate this pathogen from cheese samples [20]. In the US at least 150 cases of listeriosis, including 54 deaths, resulted from consumption of pasteurized milk (72.2°C, 16s) and Mexican style cheeses contaminated with the pathogen [24]. Presence of *Listeria* in cheese may result from contamination and survival after processing or from

post-processing contamination. Refrigeration (2 to 8ºC) slows growth of psychrotroph but doesn't prevent it, and since most cheeses are maintained and ripened in this temperature range, contaminated cheese can present a listeriosis risk. Moreover under optimum conditions, *L. monocytogenes* can multiply at pH values below that of most cheeses [22,25].

According to another article, when cottage cheese was manufactured according to the short-set procedure from pasteurized skim-milk containing 10^4 and 10^5 CFU/ml *L. monocytogenes*, cooking the curd decreased the population to < 10 to 100/ml. The pathogen was recovered by cold enrichment from 52.7% of the cottage cheese samples [26]. The inability of the organism to grow in finished cheese was attributed to the lactic acid present and associated lowering of the pH [26].

1.6 Effect of Lactic Starter Cultures on *Listeria* spp.

Some food borne pathogens are inhibited by growth of a lactic starter culture. According to Pearson and Marth [11] this also is true for *L. monocytogenes* and the other serotypes of *Listeria.* Mesophilic lactic starter cultures (*Lactococcus cremoris* and *Lactococcus lactis*) at 1 to 5% of milk inhibited the pathogen to different degrees at 21 and 30°C. *Lactococcus lactis* inhibited the pathogen slightly more than did *Lactococcus cremoris* otherwise results were similar for both lactic acid bacteria.

Thermophilic lactic starter cultures (*Lactococcus therm*ophilus and *Lactobacillus bulgaricus*) also inhibited the *Listeria* spp. in skim milk. Pearson and Marth [11] found that, *L. bulgaricus* was more inhibitory than *L. thermophilus*. The degree of inhibition was affected by inoculum level of the lactic organisms and temperature of the incubation. As with mesophilic starter cultures, the higher incubation temperature (42°C) resulted in more inhibition

than the lower temperature (37°C). This inhibition may be resulted from the decrease in pH of the medium caused by organic acid production, or from production of antimicrobial substances.

1.7 Implementation of HACCP in Turkish White Cheese

The concept of Hazard Analyses and Critical Control Points (HACCP) is a preventive, structured, systematic and documented approach to ensure food safety [27]. It is generally recognized that the production of cheeses with desirable organoleptic charecteristics and safe for consumption can be assured only when the following factors are continuously controlled and tested:

- The microbiological quality of the raw milk
- Pasteurisation of the raw milk prior to cheese production
- Prevention and recontamination after pasteurisation of the milk and predominance of the desirable microbial flora during storage.

HACCP is a scientifically based system which assures the control of these factors. It is a system aiming at the production of zero defective products which separates the acceptable from non-acceptable or the essential from the non-essential. The conventional way of the ensuring product safety in food processing by end product testing has several drawbacks. In contrast to the classical approach, HACCP establishes control systems that focus mainly on preventative measures rather than relying on end product testing [28]. It targets the identification of specific hazards (microbiological, physical and chemical) [27] and suggests the adoption of preventive measures for their control. The points in the process flow diagram, where the hazards may occur, are critical to consumer safety, and are known as Critical Control Points (CCPs). A flow diagram in a cheese making plant, should include recording of the flow diagram

steps starting from the incoming raw milk till the end packaged cheese. The flow diagrams of cream and whey should be included to the complete diagram.

HACCP was first used in the early 1970's to design regulations on low-acid and acidified canned foods, in order to protect the public health from botulism (Baird-Parker, 1992). Over the last ten years the HACCP concept has rapidly been developed and has found applications in various products such as; chilled and refrigerated foods, seafood and meat and poultry [27]. Milk and milk products such as cheese are historically among the safest foods. However the recent (80's) high number of seperate outbreaks involving *L. monocytogenes, Salmonella* spp*., Escherichia coli* and *Streptococcus* spp. [27] made HACCP also essential for the dairy industry. During the latest years several applications of HACCP in milk and milk products, including cheese, have been reported [3,28].

1.7.1 Critical Control Points in the production line

It is generally recognized that the production of cheeses with desirable organoleptic characteristics and which are safe for consumption can only be assured when certain factors are continuously controlled and tested: the microbiological quality of the raw milk, pasteurization of the raw milk prior to cheese production, prevention of recontamination after pasteurization of the milk and predominance of desirable microbial flora during storage [28].

1.7.1.1 Raw milk

The milk should be obtained from healthy animals under hygienic conditions. The animals may often suffer by mastitis and in 95% of the cases the pathogens held responsible were; *Staphylococcus aureus*, *Staphylococcus epidermis* and some *Micrococcus* strains [3, 28]. These microorganisms

contaminate the nipple of udder because of their presence in environment and milk equipment. The preventive measures are cleaning the udder before and after milking with appropriate antiseptics, controlling the microbial load of milking equipment and the equipment at the industry by through cleaning using a CIP-system (Clean In Place).

An increase in somatic cells indicates an unhealthy animal. Then antibiotics should be given to the animal and its milk is considered inappropriate for collection for at least 72h. The potential existence of antibiotic residues in raw milk prevents the efficiency of starter culture. The animal feeding must be also controlled regarding its content in various metals or other elements (Pb, As, Se, Hg, F, Mb, and Cu), chemical organic substances (aflatoxins, chloride products) and presence of toxic plants [29]. It is suggested that the animal should not be always fed with the same food.

Another hazard at this point is the long exposure of milk to relatively high temperature and temperature variation during transportation. This may favor the growth of pathogens and the production of heat resistant metabolites (toxins, enzymes). Other hazards include chemical substances (aflatoxins antibiotics, pesticide residues) and extraneous material. The filters must be frequently changed, because they can be covered with sediments which can act as milk contaminant [3].

1.7.1.2 Pasteurization & Cooling

The pasteurization process is carried as a continuous operation with the milk heated in a heat exchanger and then held in a prescribed time [5].The heat treatment aims at limiting public health hazards arising from pathogenic microorganisms associated with milk. An adequate pasteurization will destroy all the vegetative forms of bacteria, the psychotropic microorganisms, the yeasts

and the moulds [3]. The surviving microorganisms are *Micrococcus*, *Streptococcus, Lactobacillus, Bacillus* and *Mycobacterium* which constitute indicators of hygienic condition of container or equipment [3]. Therefore, pasteurization constitutes a CCP, because some pathogens and bacteria such as *Mycobacterium* can survive under the ripening conditions (pH, % NaCl) and be risk for public health. The procedure of pasteurization, however, can neither destroy nor eliminate the presence of toxins, bacterial agglomerations and residues of chemical and physical substances, such as antibiotics and metals. Therefore the existence of at least one critical control point before pasteurization is essential (e.g.the reception of raw milk). It was ensured that milk has been correctly pasteurized and afterwards not cross-contaminated by raw milk [3].

1.7.1.3 Addition of Starter Culture

Lactococcus lactis subs. *lactis, Lactococcus lactis* subs. *cremoris, Streptococcus salivaris* subs. *thermophilus* lactic starter cultures were used. The percentage of added culture is approximately 1%. After the starter addition, the mixture remains for half an hour at 32°C to promote the starter growth (curd ripening). Formation of lactic acid bacteria by the starter culture is very important for the appropriate ripening and the preservation of the cheeses. During acidification, records of milk temperature and titratable acidity should be carefully checked since they constitute one of the most critical control points. The preventive measures of this stage consist of monitoring the temperature of milk and controlling the development of acidity (pH reaches 5.0-5.2 within 6-8 hours). The continuous activity of starter should be ensured. Any change in activity may indicate either contamination with bacteriophages or a decreased activity of the starter due to excessive presence of antibiotics and/or disinfectants and considerable variations in the composition of milk [3,28]. Bacteriophage leads to slow acid production from the lactic acid bacteria [3].

For Feta Cheese production usually thermophilic starter cultures are used for acidification. These cultures contain *Str. salivarius* subs. *bulgaricus* and various strains of *Lactobacilli*, such as *Lb. delbruecki* subs. *bulgaricus* and *Lb. delbruecki* subs. *lactis* [3].

Different starter cultures can be used for cheese making. Erkmen [8] was only used *S. cremoris* as starter culture. Zerfiridis [2] were used *Lactobacillus bulgaricus - Streptococcus thermophillus* (1:1 ration), Abd-El Salam, 1993 was used *Lactococcus lactis-Lactobacillus bulgaricus* (1:3 ration) or *Lactococcus lactis- Lactobacillus casei* (1:3 ration). Most cheeses are made by traditional methods in some villages. In these cheeses, yoghurt is used as starter culture.

1.7.1.4 Curd Ripening

The curd remains in the vat, dipped in whey, at 32°C. The starter culture continues to reduce the pH of coagulum. Ripening of the curd is completed within 50-60 min, when the pH reaches 5.1- 5.2. The end of curd ripening should be checked by expierenced personnel. Potential cross contamination of the curd from personnel and environment may favour the growth of pathogens in the final product [3].

1.7.1.5 Storage

During storage the product temperature must be maintained at 5ºC or less in order to ensure the microbiological safety of this product. During storage, psychotrophic bacteria contribute to the continuing ripening thus improving the organoleptic characteristics and killing the pathogens like *Salmonella, Brucella, Staphyloccus aureus* and *coliforms.* These bacteria might contaminate the product after milk has been pasteurized. The pathogen *Mycobacterium* endures

extreme pH conditions and high values of salt concentration. For that reason, pasteurization must ensure the killing of this bacterium [3]. The control measures include the pH of cheeses, storage (ripening of cheese), temperature and R.H % of storage room [28].

1.8 Risk Assessment on CCP's

1.8.1 Risk Analysis

Risk can be defined as "a function of the probability of an adverse effect and the magnitude of that effect consequential to a hazard(s) in food"(FAO/ WHO, 1995)[29].

Risk analysis is the term that has evolved over the past decade to indicate the methodology to approach food-related risks in an objective manner rather than on the basis of feelings and beliefs [30].

The aim of risk analysis is to provide a global standard for the interpretation of the acceptability of risks associated to foods to which consumers might be exposed.

It consists of three components: Risk communication, Risk management (regulation and control) and Risk assessment (scientific advice and information analysis) [31].

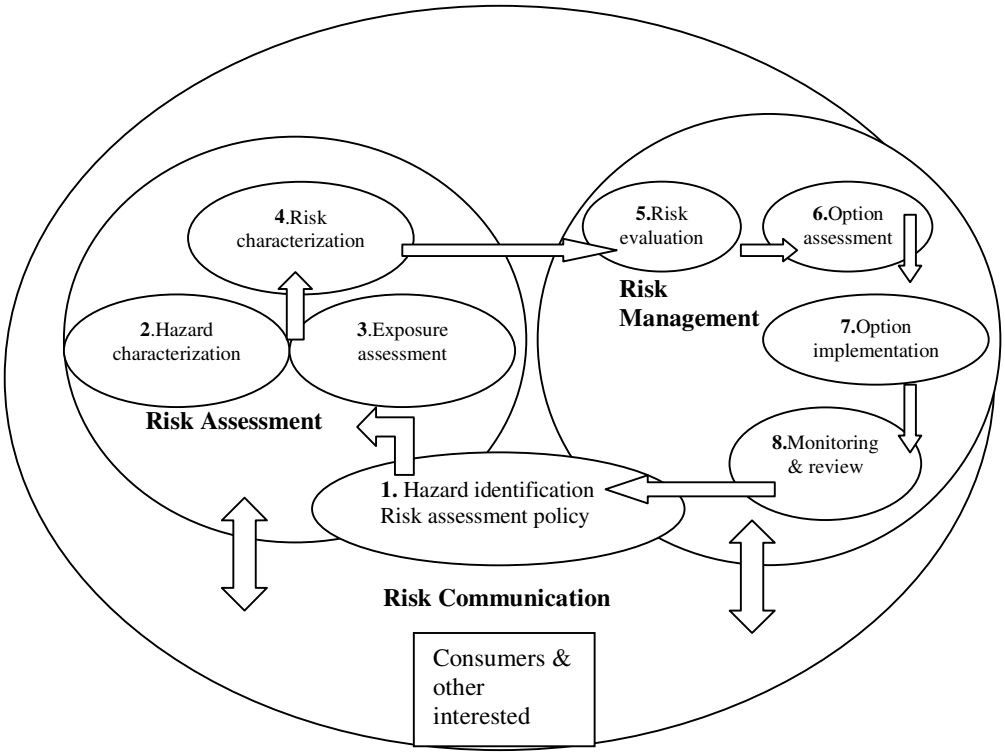


Figure 1.1 Structure of Risk analysis adapted from FAO/WHO report (1997)[31].

1.8.1.1. Risk communication

The interactive exchange of information and opinions throughout the risk analysis process concerning risk, risk related factors and risk perceptions, among risk assessors, risk managers, consumers, industry,the academic community and other interested parties, including the explanation of risk assessment findings and the basis of risk management decisions [31].

1.8.1.2 Risk management

The process, distinct from risk assessment, of weighing policy alternatives, in consultation with all interested parties, considering risk assessment and other factors relevant for the health protection of consumers and for the promotion of fair trade practices, and, if needed, selecting appropriate prevention and control options [31].

According to the outcome of the FAO/WHO Expert Consultation on the Application of Risk Management to Food Safety Matters, Risk management should be governed by the following principles (FAO/WHO, 1997);

- Risk management should follow a structured approach
- The protection of human health should be the primary consideration
- Decisions and practices should be transparent
- Risk assessment policy determination should be a specific component
- The functional separation of Risk Management and Risk assessment should be maintained in order to ensure the scientific integrity of the Risk assessment process
- Decisions should take into account the uncertainty in the output of the Risk assessment
- Risk management should include clear, interactive communication with consumers and other interested parties in all aspects of the process
- Risk management should be a continuing process taking into account all newly generated data in the evaluation and review of management decisions

Risk management comprises four steps (FAO/WHO, 1997);

* Risk evaluation,
* Risk management option assessment,

* Implementation of management decisions, and
* Monitoring and review.

1.8.1.2.1 The use of risk estimates and reduction in risk levels

The initial part of the risk management process sets the stage for a risk assessment, which should result in a risk estimate. Risk estimates should be related to time, i.e. the estimates will change over time as a consequence of changes in level of the hazard in the food, consumer habits etc.

The risk estimate represents the actual risk level, which can be higher or lower than the acceptable risk level. If the actual level is higher than the acceptable risk level, Risk management decisions are necessary to define initiatives to reach the target risk level. The use of the word target risk level reflects the dynamic nature of food-borne microbial disease risk.

The determination of acceptable levels of risk depends not only upon the hazard and risk situation, but also upon a number of socio-economic and technological factors. According to these factors the best management option could be:

* control at the source,
* action plans in the production level,
* introduction of general hygiene measures,
* introduction of specific production control measures (HACCP),
* mandatory criteria in the final product,
* consumer education or a combination of these.

1.8.1.2.2 Risk management and HACCP

Trends in national food safety initiatives show a paradigm shift, moving away from "vertical" detailed legislation, placing more emphasis on Risk analysis and "horizontal" general rulings. The importance of co-operation between different public health and food safety authorities is now emphasised in many countries, and the concept of a total overview of the problems "from farm to table" is taking over. The legalised introduction of HACCP in many countries should be seen in this light.

In general, Risk management decisions can influence all other hygiene initiatives. Information generated as part of the risk management process can be used in the design of HACCP systems, notably some of the Risk assessment information can be used as part of the input into the hazard analysis step of HACCP, as can the establishment of food safety objectives.

The concept of general hygiene rules combined with specific control of critical points along the total chain of food production from farm to table will constitute important parts of most Risk management initiatives in the future [29].

1.8.1.3 Risk assessment

One part of the overall risk analysis procedure, Risk assessment, is the scientific process in which the hazards and the risk factors are identified, and the risk estimate or risk profile is determined [32]. Additionally, Microbiological Risk Assessment is an essential element of Risk analysis because it specifies risks related to pathogenic microorganisms in the food chain on the basis of sound science, combining qualitative and quantitative data in the areas of epidemiology and pathogenicity of microorganisms with food production and handling.

Risk Assessment is a long process, typically occupying several months or years. It is a structured, science-based process to estimate the likelihood and severity of risk which attended uncertainty. This is a global process since the conclusions of a single risk assessment can be applied at any food plant in the world. It is a quantitative process in which numerical degree of risk or potential adverse health effects resulting from exposure to hazardous agents can be calculated [33].

There are two general approaches to risk assessment, described as qualitative and quantitative (FAO/WHO, 1995; CAC, 1999). Qualitative risk assessments are descriptive or categorical treatments of information, whereas quantitative risk assessments are mathematical analyses of numerical data. A quantitative risk assessment is the preferred choise if the necessary quantitative information and resources are available. When data, time and/or other resources are limited, the only option available may be to conduct a qualitative risk assessment. Or, a qualitative risk assessment may be undertaken as a first evaluation of a food safety issue to determine if the risk is significant enough to warrant a more detailed analysis. Qualitative risk assessments should be more than simply a literature review or summary of the available informationabout an issue. A qualitative assessment should follow the same systematic approach as quantitative risk assessment, including sections dealing with hazard identification, exposure assessment, hazard characterization, risk characterization. Ideally a qualitative approach would include a framework for translating qualitative information from different aspects of a risk issue into an objective evaluation of the overall risk [34].

In risk assessment the following simplified formula is often used [35].

Risk: Probability X Effect

Risk is defined as an estimate of the likely occurrence of the hazard. The effect, the damage caused, is often expressed in terms of personal injury, number of victims etc. This has lead to the accepted use of health risk as a measure for expressing the risk of unsafe food [35].

Risk Assessment is a science-based investigation consisting of four steps[36]:

1.8.1.3.1 Hazard Identification: Hazard Identification is the first step in a formal risk assessment. This activity is largely a qualitative evaluation of the risk issue and a preliminary examination of information that is analyzed in more detail in the subsequent steps of the process. In traditional fields of risk assessment, e.g., toxicology and environmental health, the major focus of the hazard identification step is to determine if there is sufficient evidence to consider a substance (e.g. a chemical) as the cause of an adverse health effect .

1.8.1.3.2 Exposure Assessment: Exposure assessment is the estimation of how likely it is that an individual or a population will be exposed to a microbial hazard and what numbers of the microorganisms are likely to be ingested. The exposure assessment phase of microbial risk assessment is faced with a much more dynamic hazard compared to traditional chemical risk assessments because of the potential for microorganisms to multiply and/or die in foods. Factors that must be considered for exposure assessment include the frequency of contamination of foods by the pathogenic agent and its level in those foods over time. Another factor that must be considered in the assessments is patterns of consumption. Other relevant factors include pH, moisture content or water activity (a_w), nutrient content, the presence of antimicrobial substances, and competing microflora.

1.8.1.3.3 Hazard Characterisation: This step provides a qualitative or quantitative description of the severity and duration adverse effects that might result from the ingestion of a microorganism or its toxin in food. A-dose–response assessment should be performed if the data are obtainable.

1.8.1.3.4 Risk Characterisation : Risk Characterization represents the integration of the Hazard Identification, Hazard Characterization, and Exposure

Assessment determinations to obtain a Risk Estimate; providing qualitative or quantitative estimate of the likelihood and severity of the adverse effects which could occur in a given population, including a description of the uncertainties associated with the estimates. The estimates can be assessed by comparison with independent epidemiological data that relate hazards to disease prevalence.

Risk characterization bring together all of the qualitative or quantitative information of the previous steps to provide a soundly based estimate of risk for a given population, Risk Characterization depends on available data and experts judgements. The weight of evidence integrating quantitative and qualitative data may permit only a qualitative estimate of risk.

These steps represents a systematic process for identifying adverse consequences and their associated probabilities arising from consumption of foods that may be contaminated with microbial pathogens and/or microbial toxins [36].

One of the stages of Risk Assessment is the risk occurs as a result of the post-process contamination. If there is any post process contamination of cheese with *L. innocua*, growth curve of *L.innocua* should be observed (Appendix A).

In factory post-processing contamination may be the primary source for introduction of a foodborne hazard. This has often be the case when milk contain *Listeria monocytogenes*, a pervasive environmental contaminant. In this situation, the assessment could focus on characterizing only the events that occur after processing [36].

Risk assessments require major human and monetary resources and result in analyses that may be several hundred pages long. They are usually conducted by a major consortium that includes regulatory, public health, academic and

industry participitation [37]. This consortium contains the scientific literature as a supportive information.

To date only a few comprehensive quantitative risk assessments have been published. Marks and Cassin [36] studied on *Escherichia coli 0157:H7* in home cooked ground beef hamburgers. Another study of Baker [36] is about *Salmonella enteritidis* in shell eggs. Moreover, Buchanon [36] studied on a similar study about liquid pasteurized eggs and lastly a study of Bemrah [36] is so similar with our study about *L. monocytogenes* in soft cheeses made from raw milk. In another paper [37], the focus of the work is to characterize and quantify the factors that contribute to exposure without quantifying the associated human health risk. These include the contamination of milk by *Listeria monocytogenes.*

Risk Assessment and HACCP are related, but fundamentally different processes. Four major elements of risk assessment are described. Some similarities exist between inputs for the first elements of risk assessment (hazard identification) and HACCP (hazard analysis). However, HACCP involves the identification of critical control points of a process for the purpose of producing a 'safe' product, and thus is essentially a risk management procedure that does not estimate risk with attendant uncertainty in the formal structured procedure described for risk assessment [38].

The results of the risk assessment can be used for defining acceptable product characteristics or processing goals for a HACCP program. In the HACCP plan, hazard analysis is the collection and evaluation of information, characteristics and data of contaminants and conditions leading to food safety risks. The results of the hazard analysis is the identification of control measures which are essential to (i) prevent contamination, (ii) prevent unacceptable increase of contaminants and (iii) reduce contaminants to acceptable levels. Next the process steps at which control can be applied and is essential to reduce a food safety risk are defined as critical control points (CCPs) in a HACCP-plan [39].

The identification of risk factors is a crucial step in hazard analysis. Risk factors contribute to the probability of occurrence of hazards in product. At a risk factor the hazard is introduced or there is a probability of increase or decrease. To know the impact of a risk factor the effect can be determined in a qualitative or in a quantitative way. In most HACCP systems a qualitative approach is used.

1.9 Comparison of Turkish White Cheese with Cottage Cheese made from raw milk

Cottage Cheese is also known as pot cheese or farmer's cheese. This type of cheese derives its name from the cottages it is produce in. Cottage Cheese is mostly made by traditional methods in rural areas. Following obtaining milk from cow (approximately at 37°C), starter culture (yoghurt: 1 spoon for 1 kg. of milk) is inoculated immediately. After 30 min, ready enzyme (Trakya Peynir Mayası, Turkey) is added and waited in a hot place approximately 12 hours for ripening. In this type of cheese manufacturing, there is no heat application (pasteurization) to milk, like some Turkish White Cheeses prepared by traditional methods.

1.10 Objectives of the Study

If raw milk is used for cheese process, or if there is post- process contamination, the final populations of bacteria would be hazardous to consumers depending upon the amount of contamination.

Because of the difficulty in preventing contamination of foods that do not receive any treatment to destroy listerial organisms just before consumption, there is a need to assess the risk of their survival and growth. Several studies have focused on the behavior of *L. monocytogenes* in a variety of cheeses

including soft ripened, cheddar and cold pack cheese foods [4, 8, 12, 13, 14, 40, 41], but there is no data on the behaviour of *L. innocua* in Turkish White Cheese. In these studies, cheeses are produced from heat treated milk, but in all types of cheese production there is risk of using traditional methods with untreated milk.

Some Turkish White Cheeses are prepared with raw milk (without any pasteurization) especially at rural areas or at small companies by using the traditional methods as Cottage Cheese, so it is very likely that many of them could be contaminated with *L. innocua* or *L. monocytogenes*.

Especially, if contamination of milk or cheese is in question, storage period leads to the disappearence of the *Listeria* spp. and other unwanted microorganisms. Therefore, storage period emphasizes the ripening period of Turkish White Cheese in this study. According to AOÇ Dairy factory, there should be at least 30-45 day of storage (ripening) period after the production of cheese at refrigeration temperature. In addition, in several studies [3,28] storage is discussed as CCP because of the consumer risk that it carries. Therefore, the effect of storage period on the survival of *L. innocua* was also studied for Turkish White Cheese.

In this study, the growth and survival of *L. innocua and* TAC were investigated with respect to starter activity, salt concentration and different contamination levels during processing and storage (ripening) periods of Turkish White Cheese. The effect of different contamination levels on ripening periods of cheeses were compared. Storage time for consumption of cheeses are determined with each contamination level of *L. innocua,* according to the regression lines. The study was also aimed at establishing methods to prevent the quality problems. The effect of HACCP and risk assessment on reducing the *Listerial* contamination of Turkish White Cheese was also investigated for preventing the quality problems in a cheese plant and for directing the companies as a guide.

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial Strain

Activated culture of *L. innocua* was used throughout the study. This bacterial strain was kindly provided by Hacettepe University, Department of Food Engineering. *L. innocua* culture was activated twice in Brain Heart Infusion Broth (BHIB, Merck) at 37°C for 24h. Activated culture was stored in Micro-bank and in glycerol at -80°C.

100µl activated *L. innocua* culture was inoculated into 50 ml of BHIB in a falcon tube. Overnight growth culture was centrifuged (1500x g/ 30 min). Supernatant was discarded. Pellet was washed twice with 10 ml of 0.1% peptone water. Stock cultures were stored at -80°C and used as inoculum for the rest of the study. Following the addition of 10 ml 0.1 % peptone water, stock culture with cell density of $8.6x10^{10}$ CFU/ml was obtained.

Cow's milk was inoculated with the *L. innocua* culture for obtaining the levels of 3.84 and 7.12 log CFU/ml *L. innocua* into pasteurized milk.

2.2 Milk

Raw milk was supplied from AOÇ Dairy factory, transported to METU Food Engineering Department within ice bags and pasteurized in a water bath at 72°C for 5 min.

2.3 Starter Culture

A commercial lactic starter culture of *Lactococcus lactis* subs. *lactis, Lactococcus lactis* subs. *cremoris* and *Streptococcus salivaris* subs. *thermophilus* was used for the acidification. It was provided from Rhodia (France) in powder form and diluted in pure water by the ratio of 10 / 1 (starter culture/ pure water).

2.4 Rennet enzyme

Rennet enzyme was used for coagulation of the milk. It was provided from Mayasan Lim. Şti. (İstanbul), in liquid form and mixed with pure water by the ratio of 1 /10 (enzyme / pure water). The power of the enzyme is 1/16000.

2.5 Sampling Procedure

Four batches of cheeses were produced. For two batches, 4 L of pasteurized cow milk was divided into two and 2 L was inoculated with 1 ml of *L. innocua* to a final microbial load of 3.84 log CFU/ml. The remaining milk was used (2L) to produce cheese to be used as control which was not inoculated. For the other two batches, again 4 L of pasteurized cow milk was used and 2 L was inoculated with 1 ml of *L. innocua* to a final microbial load of 7.12 log CFU/ml. The remaining milk (2L) was again left to produce cheese to be used as control.

Samples of 10 ml milk or 10 g cheese was taken from pasteurized milk, inoculated milk, whey, post-ripened curd, post-salted cheese and homogenised in 90 ml of Peptone Water (1: 9 ration). Serial 8 fold dilutions were prepared for bacterial analyses. Three replicates were carried out for each analysis and each analysis was repeated 2 times.

2.6 *Listeria* Enumeration

Milk, curd and cheese samples were diluted in sterile 0.1% peptone water. Dilutions were plated on Oxford Agar by spread plating method. Plates were incubated at 37ºC for 24h. Typical colonies of *L. innocua*, which exhibited a black color were counted.

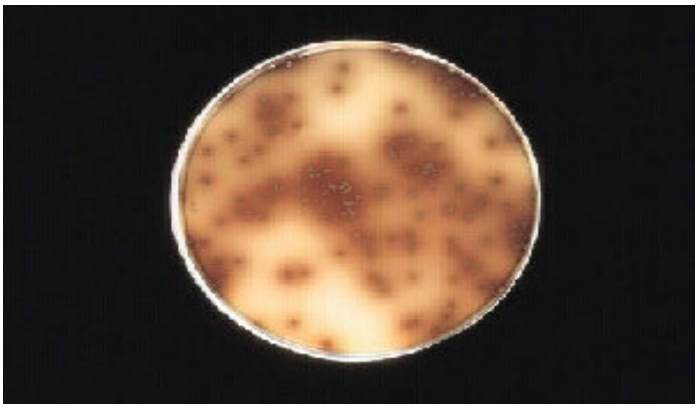


Figure 2.1 Appearence of *L. innocua* in petri dish

2.7 Total Aerobic Count Enumeration

Ready kits of 3M was used for TAC enumeration. The milk, curd, and cheese samples were diluted in sterile 0.1 % peptone water. Inoculation was done by pour plate method. Kits were incubated at 37ºC for 24h and the results were enumerated on the area with the radius of 50 mm.

2.8 Lactic Acid Bacteria Enumeration

MRS agar was used for the enumeration of LAB.

2.9 Composition of cheese

2.9.1 pH: Results were measured with a pH-meter.

2.9.2 Acidity: For the determination of titratable acidity of milk samples, 9 g of milk and 18 g of distilled water were weighed and 0.5 ml of phenolphtalein was added and titrated with 0.1 N NaOH to the first permanent colour change to pink.

For the cheese samples, 10 g of cheese was weighed in a beaker and mixed with 100 ml of distilled water using an electric mixer. Twenty five gram of mixed sample was transferred into an erlenmayer flask and 5 ml of phenolphtalein was added into the erlenmayer and titrated with 0.1 N NaOH for the first permanent colour change to pink. All the titratable acidity values were expressed as percentage of lactic acid.

2.9.3 Moisture content: The moisture contents of the cheeses were determined using the oven drying method. The difference in weight before and after drying for 1 h, at 100°C gives the results of moisture content was recorded.

2.9.4 Fat content: 2.5–3 g cheese sample was weighed into a butyrometer vessel and filled with H_2SO_4 (d: 1.55 g/ cm^3). 1 ml isoamyl alcohol was added. Butyrometer vessel was completed to the level of 35% with H_2SO_4 solution and centrifuged (1100-1200 x g / 5 min / 40-45° C). The oil level was read from butyrometer vessel.

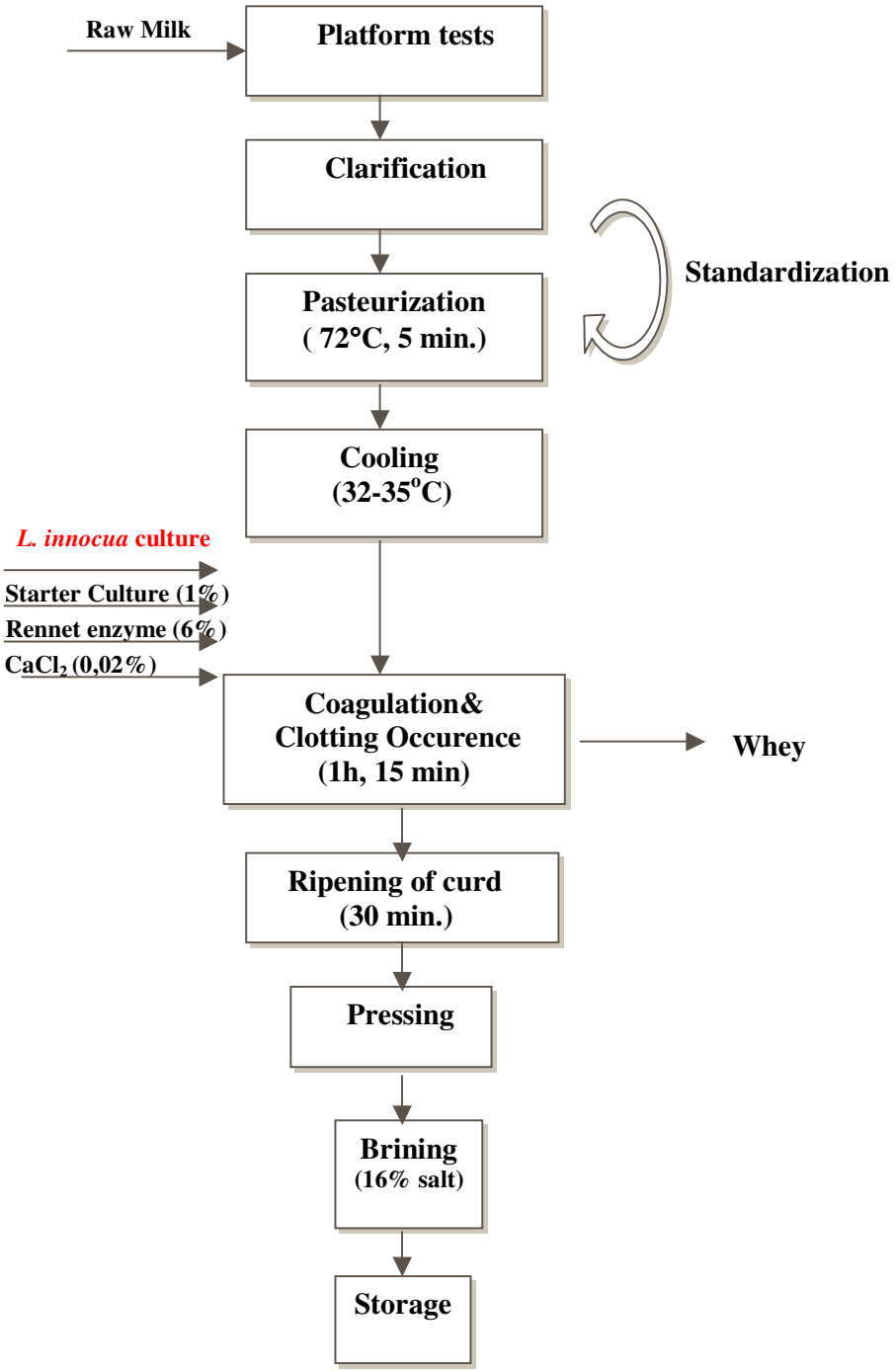
2.9.5 Salt content: 10 g cheese sample was mixed with 15 ml of warm water (50-55°C) and stirred with magnetic stirrer. Twenty five millilitre of distilled water was added and mixed until the sample was dispersed. Dispersed sample was transferred into a 100 ml volumetric flask and the volume was

completed to 100 ml with distilled water. This was then filtered with a filter paper, the collected filtrate was approximately 50 ml. Twenty-five mililiter of filtrate was transferred into a clean flask and added 1 ml of potassium chromate indicator and then titrated with 0.1 N silver nitrate ($AgNO_3$) to the first visible pale red-brown colour lasting 30 s.

2.10 Manufacture of Turkish White Cheese and Sampling Procedure

After milking, the raw milk was chilled to below 4°C and kept at this temperature during its transportation to the dairy factory. Following reception, milk was filtered. The elimination of some straw, grass and other extraneous material can be removed with filtration. It was stored in large silo tanks and sampled for analyses and standardized (casein/fat: 0.7- 0.8). Raw milk was provided from AOÇ Dairy Factory after this stage for production in METU, Department of Food Engineering. It was pasteurized (72 °C for 5 min), and cooled down to 32°C. At this temperature, starter culture (v/v) was added. Formation of lactic acid by starter culture is very important for the appropriate ripening and preservation of the cheese. $CaCl_2$ (v/v) was also added for a firm structure. For the coagulation of the milk and the elimination of the whey, 30 min after the starter culture addition, rennet enzyme (v/v) was added and the milk was coagulated in 50-60 min. The coagulum was cut and pressed (under a 10 kg weight), overnight cheese was salted in 16% (w/v) saline solutions. Cheeses were ripened in this solution in plastic containers at 4°C for 45 days.

Table 2.1 Flow Chart of Turkish White Cheese Production (According to AOÇ Dairy Factory) and the inoculation of *L. innocua*



2.11 Growth of *Listeria innocua* in BHIB

The stock culture was activated in BHIB at overnight. 1 ml of the activated culture was inoculated into an erlenmayer flask containing 100 ml of BHIB medium and then mixed by using a magnetic stirrer. The culture was incubated at 37°C. At specific time intervals, the optical density of the medium was measured at Spectrophotometer at 540 nm by using sterile BHIB medium as blank.

2.12 Pasteurization of the Raw Milk

Pasteurization assay was performed at 72°C for 5 min in a water bath. After allowing time for the content to reach the required temperature and waiting for 5 min, the container was removed and cooled down to about 30-32°C under tap water. 0.1 ml samples were spread plated on three replicate plates of BHIA and Oxford Agar. They were then incubated at 37°C for 24 h. The temperatures were measured by a thermometer (Sınar).

2.13 Inoculation of *Listeria innocua* into Pasteurized milk

A loopful of *L. innocua* cells, obtained by additon of 10 ml 0.1 % peptone water into the stored pellet with the cell density of 8.6x 10^{10} CFU/ml (10.93 log CFU/ml), were added into 10 ml of BHIB and incubated at 37°C for 24 h to give an initial number of 10^{9} – 10^{10} CFU/ml *listeria* cells (OD measured at 540 nm was approximately 1.2).

The numbers of *L. innocua* in pasteurized milk was determined using the appropriate decimal dilutions in 9 ml of 0.1% peptone water. 2 L of pasteurized

milk was inoculated with the third dilution of overnight growth culture of *L. innocua* for obtaining the value of 3.84 log CFU/ml ($4.6x10^3$ CFU/ml =low inoculum dose) and the other batch of 2 L was inoculated with the initial bacterial load after the second activation for obtaining the 7.12 log CFU/ml ($1.4x10^7$ CFU/ml =high inoculum dose) in pasteurized milk. It was mixed for 5 min in Cheese Vat. 0.1 ml samples were spread plated on three replicate plates of BHIA and Oxford Agar. They were then incubated at 37°C for 24 h.

2.14 Analyses of Results

Three replicate samples of two independent trials from control and inoculated cheese samples were tested at each interval, for each inoculation dose during entire experiments. Counts from replicate plates were averaged and converted to log counts of colony-forming-units (CFU/g or log CFU/ml) for graphics and regression analyses.

The log counts of colony-forming-units from three replicate samples of two independent trials were averaged and the mean values plotted. Curves were fitted by linear regression done by Excel. The inactivation time of *L. innocua* over weeks of storage in each inoculum dose was assessed by calculating D_{10} values defined as the negative reciprocal of the slope. Regression equations, coefficients and slopes were defined for all regression lines.

Physico-chemical analyses were done according to the analyses of AOÇ Dairy factory and TSE.

Critical Control Points were determined according to the CCP Decision Tree (Appendix J).

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Microbiological Assay

3.1.1 Behaviour of *L. innocua* and TAC During Processing

Growth of *L. innocua* and TAC of Turkish White Cheese during the processing period with different inoculation levels were shown in Figure 3.1 and 3.2. The data indicated an increase in *L.innocua* and TAC cells during the preparation of cheeses and a drop after salting process.

Listeria counts remained relatively constant in each cheese following the inoculation of *L. innocua* into milk (3.26 log CFU/ml) (Figure 3.1). After the stage of curd pressure overnight at 20±5°C, number of *Listeria* in Turkish White Cheese with low dose inoculation of *L. innocua*, increased to 3.44 log CFU/g. As expected, with the effect of salt treatment with 16% saline solution, a decrease to 2.75 log CFU/g was recorded at the end of the salting process.

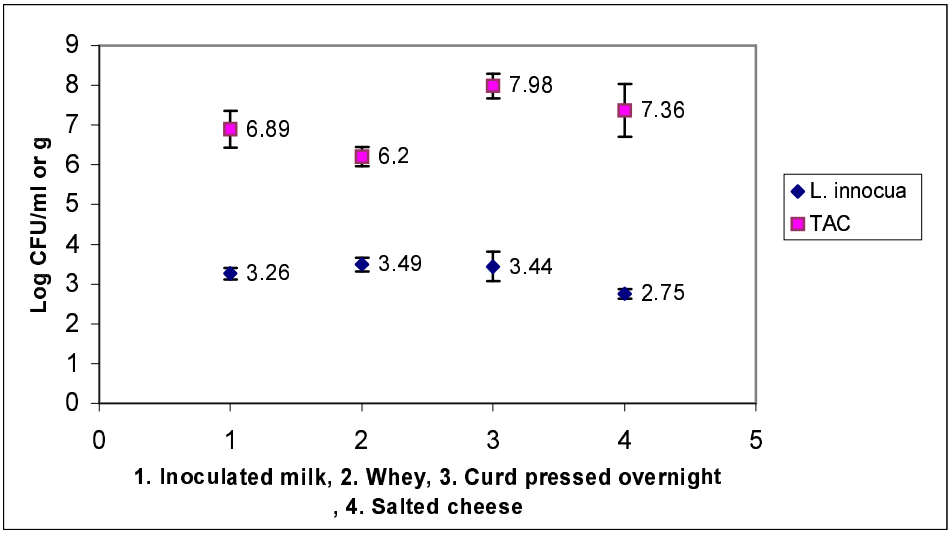


Figure 3.1 Average of *L. innocua* and TAC in Turkish White Cheese after low-dose inoculation of *L. innocua* for obtaining the initial load of 3.84 log CFU/ml

Behaviour of TAC was mainly affected by starter culture. After pasteurization, the bacterial load of milk was approximately 3 logs. However there was an increase in TAC number following the addition of starter culture. After this stage, number of TAC was recorded as 6.89 log CFU/ml in Turkish White Cheese with low-dose inoculation of *L. innocua* (Fig.3.1). The value of 7.98 log CFU/g was obtained in curd pressed overnight. Following the saline treatment of 16%, TAC was drop to 7.36 log CFU/g. Since whey is not used in cheese manufacturing the decrease in TAC of whey is not considered.

In Turkish White Cheese with high dose inoculation of *L. innocua*, *Listeria* number of inoculated milk was recorded as 6.98 log CFU/ml (Fig. 3.2). According to *L. innocua* cell enumeration following overnight pressure at 20±5°C, there was an increase to 7.97 log CFU/g however a decrease to 6.0 log CFU/g was seen, following one day storage of salted cheeses. The same batch of Turkish White Cheese had an initial TAC count of 9.4 log CFU/ml in inoculated milk. Number of TAC in curd was increased to 10.3 log CFU/g, overnight .

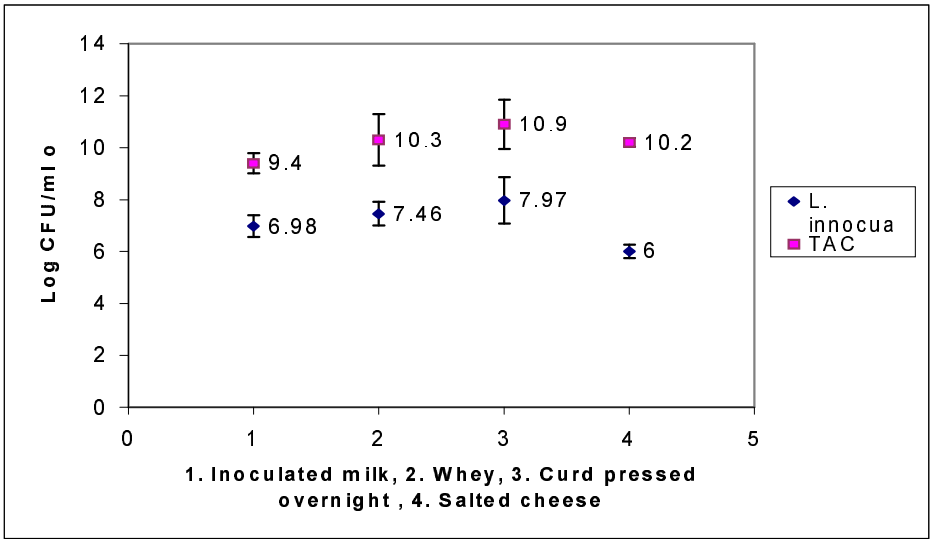


Figure 3.2 Average of *L. innocua* and TAC in Turkish White Cheese after high-dose inoculation of *L. innocua* for obtaining the initial load of 7.12 log CFU/ml

The process of cheese making was carried at a temperature of 20±5°C, because of this there was an increase in the count of *Listeria* cells during the stages of occurrence and elimination of whey and pressing the curd overnight. Neverthless, the total inactivation of *L. innocua* from its inital value in milk to the end of salting stage in 1-day- old cheeses are 15.7 % and 14.1 %, respectively (Appendix E).

The results obtained in this study showed the same trend that has been reported in other studies of cheeses. Erkmen [1] studied the survival of *Salmonella typhimurium* in Turkish White Cheese. In this study, number of *S. tyhimurium* increased during the manufacture of Turkish White Cheese until the salting of cheeses and decreased after salting during the storage conditions.

In a similar study of Ryser and Marth [15], it was reported that *L. monocytogenes* population usually increases due to processing conditions of Brick Cheese.

Some other studies have shown that, the bacterial load of contaminated cheese decreases only in three types of cheeses; Parmesan, Mozzarella and Swiss cheeses. However, during the ripening stage, the results are comparable with those obtained in many other studies including our study, i.e the *Listeria* population gradually decreases [15].

3.1.2 Behaviour of *L. innocua* during storage period

Samples were taken from Turkish White Cheeses with low and high dose inoculation of *L. innocua* and analysed on 5, 10, 15, 20, 30 and 45 days. There was a proportional decrease in *L. innocua* number of Turkish White Cheese (Fig. 3.3). With each inoculation dose, total decrease of 1.8 log CFU/g *L. innocua* was recorded after the storage on day 45. *Listerial* count of Turkish White cheese caused a decrease during 45 days of storage period. However this decrease didn't lead to the complete disappearance of the pathogen and it was able to survive in cheese.

According to the results obtained from the regression lines in Fig. 3.3, storage (ripening) periods of Turkish White Cheeses in refrigeration temperature should be at least least 90 and 178 days, repectively in the cheeses with low and high dose inoculation of *L. innocua,* for a safe consumption.

Lopez and Sanchez [14] reported that *L. monocytogenes* is able to survive even relatively adverse conditions such as high acidity (> 0.5 % as lactic acid) and cold temperatures (10-12°C), for this reason, these bacteria can survive at the manufacturing, ripening processes and can slowly grow at refrigeration temperatures.

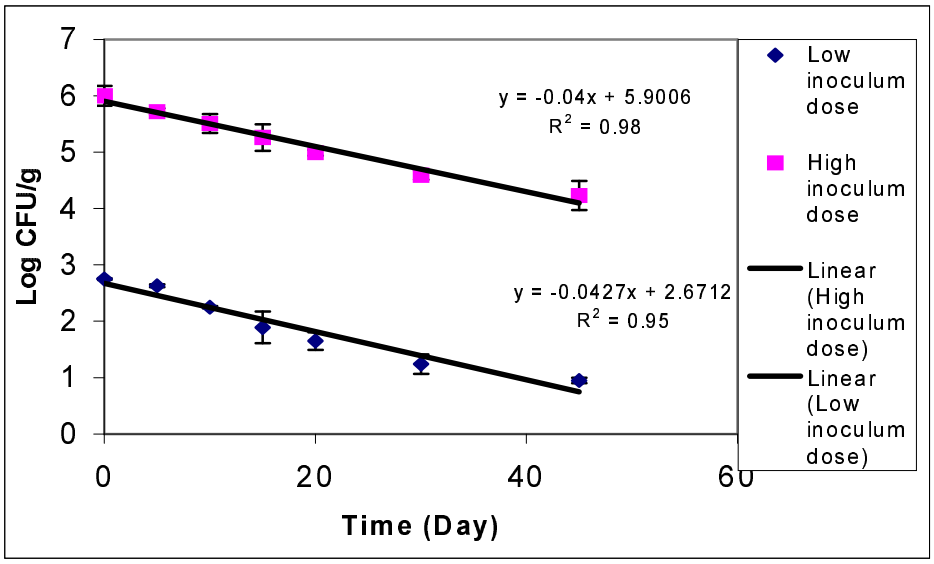


Figure 3.3 Variation of *L. innocua* count in Turkish White Cheese with low (3.84 log CFU/ml) and high (7.12 log CFU/ml) dose inoculations of *L. innocua* during storage of 45 days

If we assess the storage periods of Turkish White Cheeses in a relationship with the previous works, *L. monocytogenes* survived for more than 90 d in Feta Cheese in pH 4.3 [1], more than 28 d in White Cheese made from cow's milk after the salt treatment [4], and 15-60 d in white pickled cheese made from ewes' milk, depending on rate of lactic acid fermentation and temperature of storage [4].

Similarly, Erkmen [1] reported 1 to 2 log cycle increase of *S. aureus* during manufacture of Turkish Feta Cheese from pasteurized cow's milk inoculated with 1.5 % *S. aureus*.

In Manchego cheese from raw ewe's milk, 2 to 3 log cycle increase of *S. aureus* was reported by Nunez et al.[18], who noted the highest counts of *S. aureus* in Manchego cheese were inhibited the growth of *S. aureus* in Manchego cheese from day 1 to day 60 [18].

But in contrast to our study, it was reported that preperation of semi-hard cheese (Manchego type) with milk containing 5.3 and 3.6 log CFU/ml of *Listeria monocytogenes* 4b resulted in the populations of 5.3 and 3.5 log CFU/ml, respectively after 60d of storage. However, only during the salting of cheese a drop in these populations has been observed [18].

Similar results were obtained in the study of Marth and Pearson [26], in which the behaviour of *L. monocytogenes* in Feta Cheese prepared with whole milk containing $5x10^3$ CFU/ml of the pathogen/ml was investigated. When the pH dropped to 4.6 (after 2 d of ripening), growth of the pathogen stopped. However, at the end of the 60 d of ripening *L. monocytogenes* in Feta Cheese was still detectable.

In another report, Back, Langford and Kroll [43] observed the growth of *L. monocytogenes* on Camembert and other soft cheeses at refrigeration temperatures. Camembert cheese prepared from milk containing 500 *L. monocytogenes* strain Scott A, V7, or CA/ml supported increase of populations from $1x10^6$ to $5x\ 10^7$ cfu of the pathogen/g after 65d of ripening with starter cultures. When the pH of some samples of cheese was adjusted, growth of the pathogen was greatest at pH 6.1 rather than pH 4.6 or 7.4.

3.1.3 Regression Analyses of *L. innocua*

Counts of *L. innoca*shown in Fig.3.3 during the storage period of Turkish White Cheese were used to develop regression lines. Decimal reduction times (D Value) and Regression coefficients (R^2) of *L. innocua* with low and high dose of inoculations in Turkish White Cheese were obtained from those regression lines.

Reduction of *L. innocua* in Turkish White Cheese showed a first-order-rate of destruction during storage at 4°C. First order kinetics expressed as;

$\ln (N/ N_0)$: -kt

where N is the number of surviving microorganisms after a storage of cheese for time t (day), N_0 the initial number of microorganisms and k is the inactivation rate constant (day^{-1}). The treatment time in any cheese type given pressure that will result in destruction of 90 % or one $\log_{10}$ of the existing microbial populations, i.e., resulting in one decimal reduction in the surviving population, is referred to as the decimal reduction time or D value. This is generally obtained as the negative reciprocal slope of the $\log_{10} (N/ N_0)$ versus time curve and is therefore reciprocally related to k; D: -1 / k. D values for microbial reduction were obtained from their respective k values using the above relationship.

Storage (ripening) periods for the inactivation of *L. innocua* in Turkish White Cheese with low and high dose inoculation levels were obtained as 90 and 178 days, respectively from the negative reciprocals of the slopes and the values were given below;

D Value of *L. innocua* (day)		R^2	
Low dose	High dose	Low dose	High dose
23.4	25	0.95	0.98

The destruction curves of the Turkish White Cheese fitted the log linear first order model well (R^2>0.91) [6]. The results demonstrated that larger initial populations in Turkish White Cheese resulted in larger final populations. The final populations would be hazardous to consumers depending upon the amount of inoculation or contamination.

For a comparison between the 45 days of storage period in our study and the total inactivation time of *L. innocua* survival (%) and death (%) rates were calculated. The *L. innocua* population was reduced by 65.5 % and 29.5 % during the 45 days of storage period of cheeses with low and high dose inoculations of *L. innocua,* respectively. Survival (%) and death (%) rates are shown in Appendix E.

3.1.4 Variation of TAC during storage period

After the addition of starter culture into the pasteurized milk, TAC were obtained as 6.89 and 9.4 log CFU/ml in Turkish White Cheeses with low and high dose inoculations of *L. innocua,* respectively. Values were increased by around 1 log unit in the 1-day-old cheese pressed at the temperature of 20±5°C (to 7.98 log CFU/ml and 10.9 log CFU/g).

This increase in microbial counts is a normal phenomenon during the manufacture of cheeses, due to physical retention of the microorganisms in the curds (coagulum) and to microbial multiplication during coagulation and whey drainage.

There was a proportional decrease in TAC of Turkish White Cheese during the storage of 45 days (Fig. 3.4), as in *L. innocua* number. The mean value of initial TAC was 6.89 log CFU/g after low dose inoculation of *L. innocua* (Fig.3.1). Following the overnight pressure, an increase to 7.98 log CFU/ml was recorded. And this value drop to 4.89 log cfu/g at the end of the storage period.

Turkish White Cheese with high dose inoculation of *L. innocua* showed a result like the first one (Fig. 3.4). During processing as shown in Fig.3.2, initial TAC was 9.4 log CFU/ml and an increase was recorded to 10.9 log CFU/ml in

overnight pressed curd. In refrigeration conditions, with the effect of salt's antibacterial effect, there was a decrease in the numbers of TAC. Value of 7.41 log CFU/g was recorded at the day of 45.

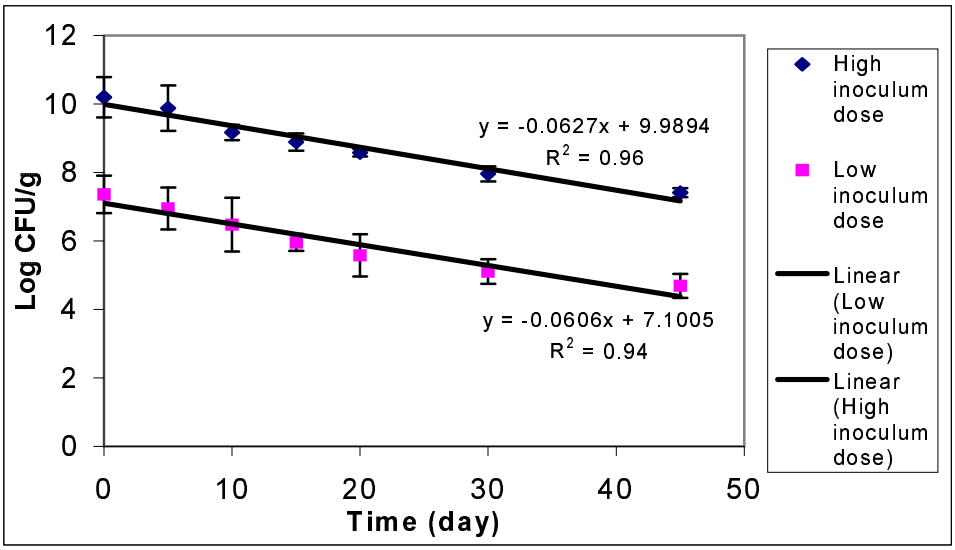


Figure 3.4 Variation of TAC in Turkish White Cheese with low (3.84 log CFU/ml) and high (7.12 log CFU/ml) dose inoculations of *L. innocua* during storage of 45 days

3.1.5 Reasons of regression in *L. innocua* and TAC

During the storage (ripening) of 45 days, numbers of *L. innocua* and TAC decreased substantially in each cheese. This behaviour is contrast to what is seen in other varieties of cheese made using the same sort of technique, such as Manchego, Roncal or Idiazabal [14]. In these cheese types, count of *L. monocytogenes* in 1-day-old cheeses were around 8.5 log CFU/g during the first 2 months of the ripening period.

Because similar procedures are used in the production of Cheddar cheese, other than cooking the curd, the results obtained in this study were compared

with those reported by Ryser et al. [42]. They found that the survival of *L. monocytogenes* in Cheddar Cheese with the initial inoculation level of 2.7 log CFU/ml was about 79% after cooking during ripening conditions on day 1.

Yousef and Marth [41], reported that the population of *L. monocytogenes* remains higher when the microorganism concentration in the milk used for cheese making is higher. This study confirms that result.

In a similar study of Lopez and Sanchez [14], during the ripening period of Manchego Cheese, after commercial pasteurized and homogenized whole milk was inoculated with *L. monocytogenes* (strain ATCC 19114) to a level between $2x10^6$ and $9x10^6$ CFU/ml, D value of 98 days was obtained for inactivation of *L. monocytogenes*. Inactivation rates were influenced by several factors: types of microorganisms, starter culture, initial microbial numbers and salting [6].

These results were also declared for Feta Cheese made in Greece [37], and Brick Cheese [15]. The consumer risk would therefore depend on a) the acceptable level of *L. innocua* in the cheese when it is consumed (Turkish White Cheese should be free from *L. innocua* and *L. monocytogenes* according to TSE) and b) the initial concentration *L. innocua* in the milk and/or the extend to which the cheese would be contaminated by *L. innocua* during processing.

3.1.6 Effect of LAB on *L. innocua* and TAC

Number of LAB showed an increase of 1.5 log units from 2.9 to 4.01 in Turkish White Cheese with low-dose inoculation of *L.innocua* and to 3.84 in cheese with high dose inoculation of *L. innocua,* during coagulation and whey drainage. Lactococci are a very active microbial group as starter culture, breaking down lactose during the first stages of maturation of cheeses, and increasing in number rapidly [43]. They are responsible for the biochemical changes arising in the early stages of storage. Their greater sensitivity to acidity relative to other lactic acid bacteria, such as lactobacilli, makes them less competitive at later stages in storage, during which lactobacilli become the dominant flora [43]. During storage conditions, LAB count was decreased as TAC.

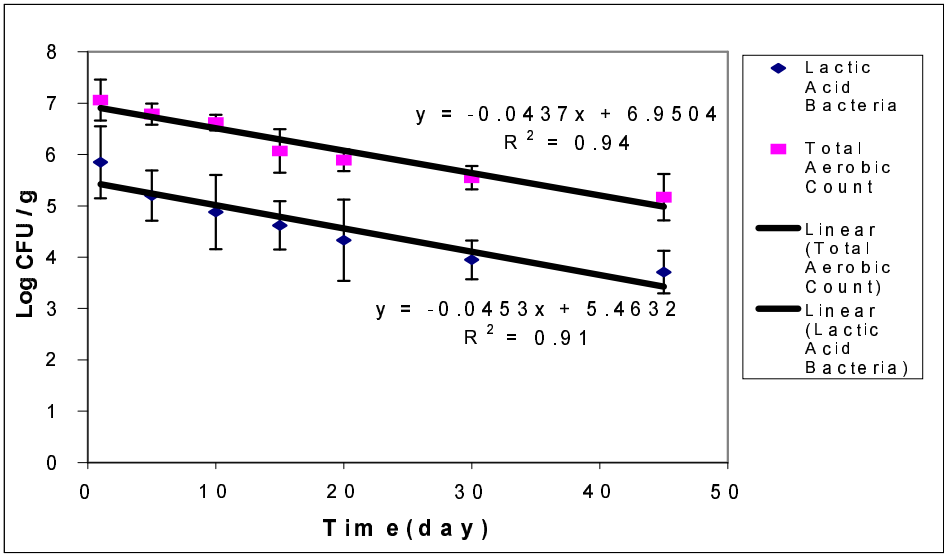


Figure 3.5 Relationship btw LAB and TAC numbers in cheese without the inoculation of *L. innocua*

LAB, were the most abundant microbial groups in milk, being 1-2 log units higher than the other groups (V. Zarate). Without the inoculation of *L. innocua*, major part of the TAC was composed of LAB after the addition of starter culture (Fig.3.5). In Turkish White Cheese with low and high dose inoculations of *L. innocua* was shown in Fig. 3.6 and 3.7.

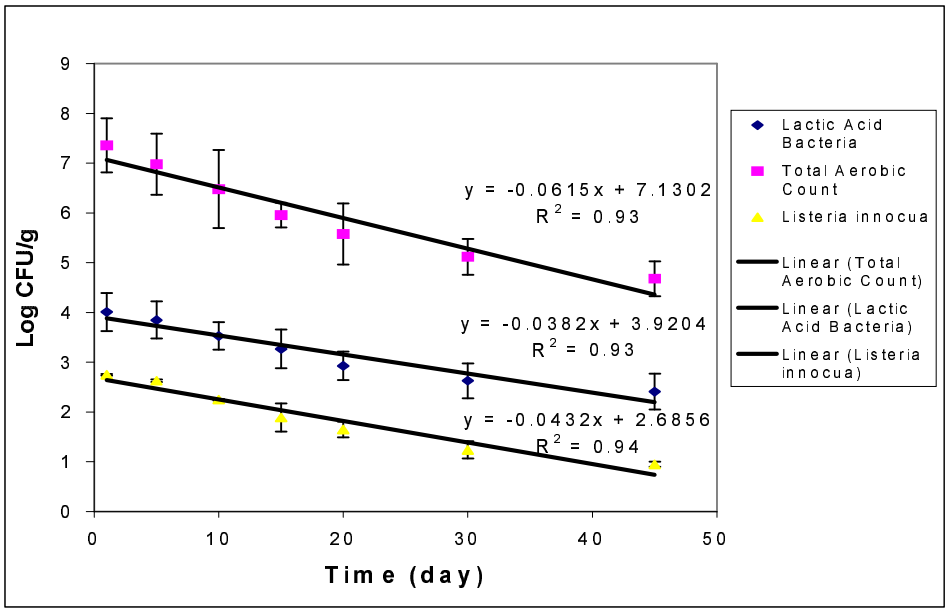


Figure 3.6 Relationship between LAB and TAC numbers in Turkish White Cheese with low-dose (3.84 log CFU/ml) inoculation of *L. innocua* during storage of 45 days

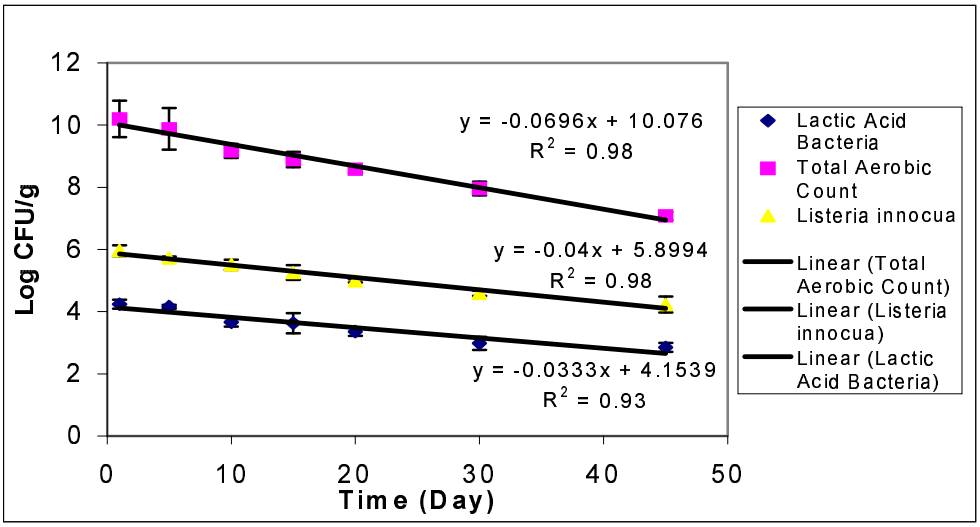


Figure 3.7 Relationship between LAB and TAC numbers in cheese with high-dose (7.12 log CFU/ml) inoculation of *L. innocua* during storage of 45 days

3.2 Physico-Chemical Analyses

Following the salt treatment and during the storage period at refrigeration temperature, physico-chemical characteristics of Turkish White Cheeses were changed as microbiological characteristics. The curd was acidic but an increase in pH was observed during ripening of curd and manufacture. Following the overnight ripening pH of curds with low and high dose inoculations were recorded as 4.84 and 4.90, respectively. pH of the salted cheeses were increased to 4.92 and 5.04 during 5 days in saline solution. These values increase to 6.0 and 6.10 after a storage period of 45 days. In fact, it is known; the decrease in *L.innocua* counts depends on the combined inhibitory effect of salt and the activity of lactic cultures during storage period of time.

A decrease was seen in the moisture content of the cheeses with the effect of salt as the result of dehydration [1]. After brining, salt content of Turkish White Cheese with low dose inoculation was recorded as 5.96 %. The average salt content was 6.8 % at the end of the storage period. In this cheese, there was a reduction in moisture content from 60.23 % in curd to 54.87 % in salted cheese at the end of storage.

In Turkish White Cheese with high dose inoculation, the salt content of curd after brining was 6.2 %. Following the storage of cheese in saline solution during 45 days, the final salt content was recorded as 6.98 %. In a relationship with this result, the moisture content was decreased from 61.49 % in curd to 57 % in stored cheese.

Results of determinations of salt, moisture and fat contents of cheeses with low and high dose inoculations are presented in Table 3.1 and 3.2.

Table 3.1 Changes in physico-chemical composition of Turkish White Cheese with low dose (3.84 log CFU/ml) inoculation of *L. innocua* during storage of 45 days

Day	pH	Acidity	Salt Content (w/v%)	Moisture content (%)	Fat Content(%)
Salted curd	4.92	1.4	5.96	60.23	24.1
5	5.54	1.84	6.03	59.80	30.0
10	5.67	1.82	6.10	59.12	34.2
20	4.68	1.81	6.35	57.86	36.2
30	5.89	1.64	6.42	55.90	36.8
45	6.00	1.59	6.80	54.87	35.4

Table 3.2 Changes in physico-chemical composition of Turkish White Cheese with high dose (7.12 log CFU/ml) inoculation of *L. innocua* during storage of 45 days

Day	pH	Acidity	Salt Content (w/v%)	Moisture content (%)	Fat Content(%)
Salted curd	5.14	1.32	6.20	61.49	24.0
5	5.50	1.76	6.33	60.50	31.0
10	5.64	1.77	6.39	59.89	35.4
20	5.74	1.56	6.45	56.80	37.2
30	5.98	1.42	6.62	57.75	38.8
45	6.10	1.40	6.98	57.0	38.9

3.2.1 pH Variation of Turkish White Cheese during the storage period

During storage, significant increases in pH occurred. On day 45, at the end of the storage period pH value was 6.00 unit in cheese with low dose inoculation and 6.10 unit in cheese with high dose inoculation. This increase in pH may be associated with a reduction in the lactate-to-protein ratio [44] and a loss of buffering capacity of the curd [45] due to removal of lactic acid, soluble Ca, and phosphate in the strech water. Calcium phosphate is a major determinant of buffering capacity of cheese. Hence, reduction in lactate-to-protein ratio and concentration of calcium phosphate are conducive to an increase in pH. Moreover, on subsequent cooling of curd, micellar calcium phosphate dissolves and may result in an increase in pH due to inactivation of H^+ by the phosphate anion [44].

As Turkish White Cheese ripened there was a tendency for the pH to increase slightly, depending on the amount of acid formed initially. Some of this increase may be due to the metabolism of lactic acid into weaker acids or other compounds that will pick up hydrogen ions. Proteolysis or breakdown or protein, forms ammonia and this can also increase pH, although it takes time[46].

pH is one of the hurdles affecting the growth and survival of *L. innocua*. The present results showed that pH value in the range 4.92 to 6.10 inhibited the growth of *L. innocua* in Turkish White Cheese from day 1 to day 45. This is in good agreement with the data of Erkmen [1], who observed maximum pH of 5.7 to 6.3 during the 75 day of storage period of Turkish White Cheese inoculated with *S. aureus*. Additionally, Nunez et al. [18] stated that the pH values 4.9 to 5.2 would be sufficient to decrease the number of *S. aureus* in Manchego cheese. However pH was one of the major factor responsible for a reduction in the viable number of microorganisms in Turkish White Cheese during the processing and storage periods.

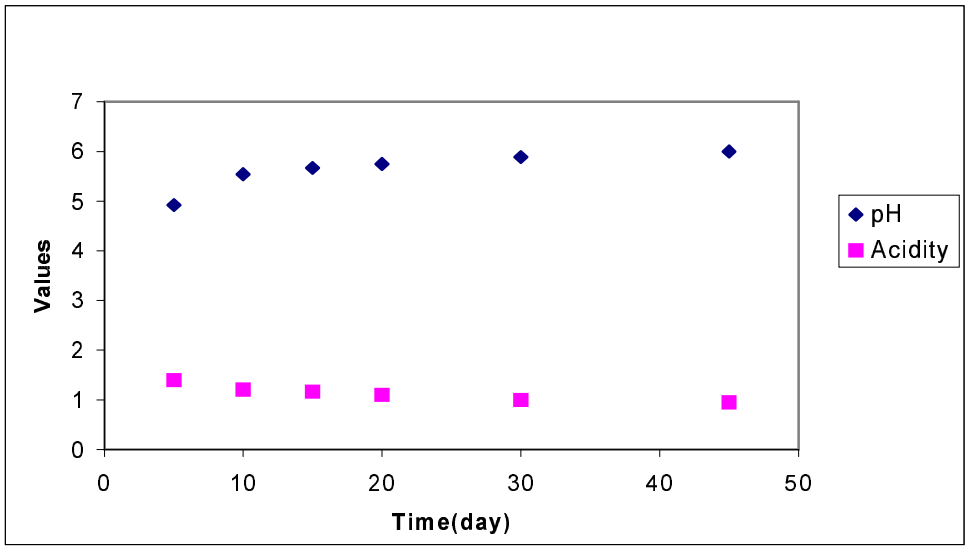


Figure 3. 8 pH-Acidity Variation of Turkish White Cheese with low dose (3.84 log CFU/ml) inoculation of *L. innocua* during storage of 45 days

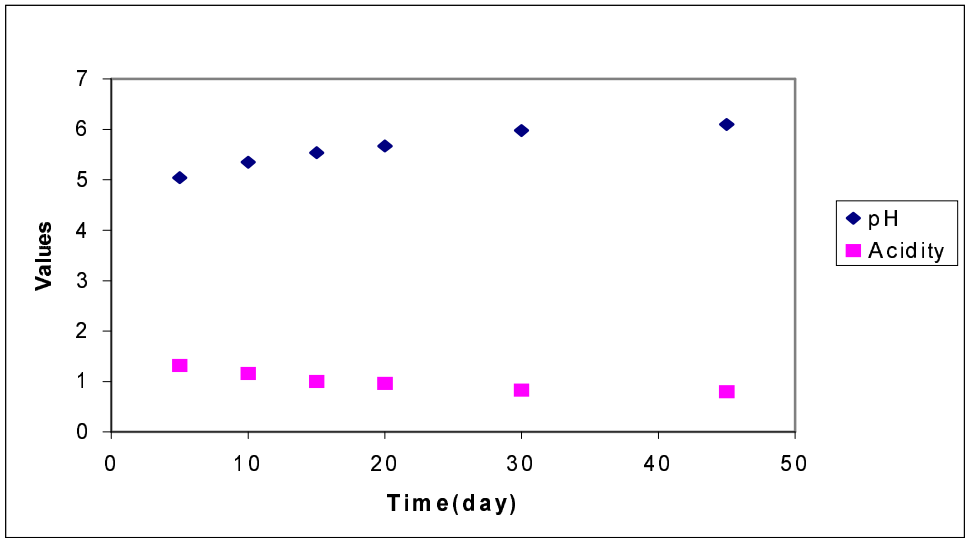


Figure 3.9 pH-Acidity Variation of Turkish White Cheese with high dose (7.12 log CFU/ml) inoculation of *L. innocua* during storage of 45 days

3.3 Risk Assessment and HACCP Plan Development

Under HACCP, eatablishments must analyze their production sytems, identify where hazards microbial contamination (e.g. *L. monocytogenes*) can occur and establish controls to prevent or reduce those hazards.

The first CCP in process flow chart of Turkish White Cheese is the transportation of raw milk. Raw milk is critical because if it is exposed to relatively high temperature and temperature variation during transportation, this may favor the growth of pathogens and the production of heat resistant metabolites (toxins, enzymes). For this reason, transportation should be done at the temperature of 5±2°C to prevent the growth of microorganisms in raw milk. Moreover, there must be some preventive actions on this point. Controls of time and temperature in this stage should be established and systematically monitored to prevent development of a hazard.

As it is known, milking practises should be done by hygienic applications, also following milking contamination of milk should be prevented. After the reception of milk to the factory, the most important application is the changing of the filters frequently, because they can be covered with sediments which can act as milk contaminant. Control measure for raw milk include milk acidity (pH: 6.2-7.5) and TAC count ($<10^6$ CFU/ml in TSA: 30°C for 24 h). In this study, acidity of raw milk was 6.71 and TAC count was 6.45 log CFU/ml ($2.8x10^6$ CFU/ml). Additionally, there were not any *Listeria* species, as obligated by TSE.

Pasteurization constitutes one of the main CCPs, because some pathogens and bacteria such as *Mycobacterium* can survive under the storage (ripening) conditions (pH, % NaCl) in the case of no pasteurization or inadequate pasteurization and can be risk of public health. In this study after the pasteurization of raw milk there were approximately 10^3 CFU/ml (3 log CFU/ml)

TAC number was enumerated, which is appropriate on TSE. These are probably, *Lactobacillus* spp. thermoduric bacteria and other bacteria with spores. Especially, *Clostridium* and *Bacillus* spp. are so resistant to heat.

During pasteurization, corrective actions should be implemented according to the records of pasteurization temperatures and deviations. In our study the holding time of pasteurizator of 5 min is very sensitively applied to milk. Generally, the pasteurization time of milk at 72°C is 15-s. But in AOÇ Dairy Factory, in case raw milk contains *Listeria* spp. including phagocytes, which is so heat resistant, pasteurization time is higher. The flow of milk into the pasteurizer cannot exceed the rate at which the 15-s hold is measured and the holding tube should be uniform. Preventive measures include; automatic safety system to prevent too low or too high temperatures. The pressure difference between pasteurized and untreated milk should be tested and calibrated at 0.5 bar, to avoid the cross contamination of pasteurized milk [3].

Another CCP is the addition and the amount of starter culture. During starter culture addition and acidification, monitoring the temperature of milk and controlling the development of acidity (pH reaches 5.0-5.2 within 6-8 hours) is so important.

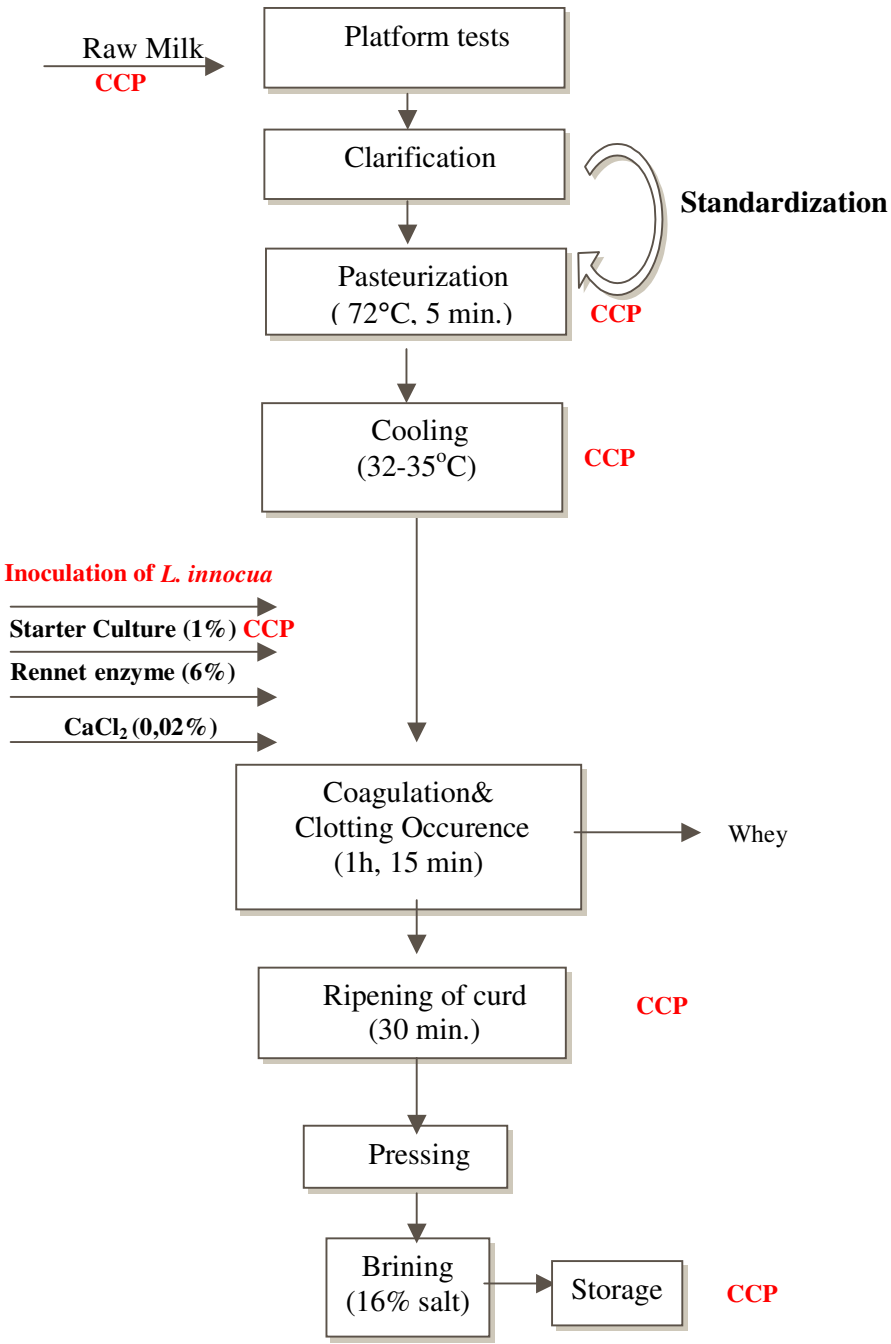
It would be advisable to include starter culture strains of lactococci which are able to produce nisin, as a bacteriosin active against *Listeria*. Under normal conditions of pasteurization, although *Listeria* is inactivated, problems may arise from post-pasteurization contamination. Bacteria can enter cheese at many stages during its processing. The environmental diversity of dairy processing plants provides the microorganism with various sites for colonization. Any pathogen existing in raw milk can potentially make its way into environment of plants processing cheese.

Ripening period of curd in cheese vat is an other CCP. Because during this period, the curd remained in the vat, dipped in whey, at 32 °C. This temperature was so appropriate for multiplication of the TAC and other hazordous bacteria that couldn't be killed by pasteurization. The starter culture continued to reduce the pH of coagulum. Ripening of curd completed overnight, when the pH reaches 5.1- 5.2. Contamination from environment was prevented by covering the vat in this study. In Turkish White Cheese manufacturing factories the end of curd ripening should be checked by expierenced personnel. In addition, potential cross contamination of the curd from personnel should be prevented by trainings in a production factory.

Storage is the last CCP during the process flow chart of Turkish White Cheese[3,28]. During storage the product temperature must be maintained at 5°C or less in order to ensure the microbiological safety of this product. Moreover, if milk and/or cheese is contaminated during the process, the product should be stored at refrigeration temperature according to the contamination level of product. The storage of cheese was considered a CCP for the reason that the reduced temperature inhibited the growth and survival of *L. innocua* and *L. monocytogenes* for a certain time.

This study, with result of behaviour of low and high dose of *L. monocytogenes* during storage as CCP, can even guide the future risk assessment studies on *Listeria* in Turkish White Cheese.

Table 3.3 Flow Chart of Turkish White Cheese Production (According to AOÇ Dairy Factory) with the determined CCPs



CHAPTER 4

CONCLUSIONS

In this study, the physico-chemical changes occuring in Turkish White Cheese and the behaviour of *Listeria innocua*, TAC, LAB during 45 days of storage (ripening) period were reported. Although there are many studies on the survival of *Listeria* in different kinds of cheeses, the aim of studies on the behaviour of *Listeria* in Turkish White Cheese were limited.

During the processing of Turkish White Cheese there was an increase in the number of *L. innocua* and TAC, however in the course of the storage period of 45 days, cells decrease in number although they survive. Total decrease of *L. innocua* after salt treatment of Turkish White Cheese with each inoculation level till the end of the storage period of cheeses were approximately 2 logs.

Inadequate pasteurization of raw milk or post-process contamination of pasteurized milk with *L. innocua* are the main health concern with respect to the listeriosis caused by cheese consumption. However, it was shown that the *Listeria* cells decrease in number during the storage period of cheese. In addition listerial load in cheese product had crucial effect on the time period for the destruction of all *Listeria* cells. Indeed, *L. innocua* population has been reduced by 65.5 % and 29.5 % with low and high dose inoculations of *L. innocua,* respectively, during the 45 days of storage period. Results indicated that, with the contamination level of 3.84 and 7.12 log CFU/ml, suggested storage (ripening) periods at refrigeration temperature would be at least 90 and 178 days, respectively, for a safe consumption of this type of cheese. Reduction of *L. innocua* and TAC in Turkish White Cheese showed a first-order-rate of destruction during storage at 4±2°C.

The salt concentration, pH, starter activity and storage time are the main factors for the decrease in the numbers of *L.innocua* and TAC present in Turkish White Cheese. Moreover, inactivation rates were mainly influenced by initial microbial numbers.

It is confirmed again that, there is a need to use starter culture for the elimination of some milk-borne pathogens. TAC was composed of mostly LAB, and LAB had an inhibitory effect on *L. innocua* and the other microorganisms with the lactic acid production. Milk pH was 6.71 and this value drop to 4.92 in curd after the addition of starter culture. In fact, this drop in the pH of cheese due to lactic acid production was considered as one of the main inhibitory effects on cells of *L. innocua*.

The salt treatment was an other inhibitory effect on *L.innocua* and TAC. After the brining process, the moisture content of cheeses were decreased as a result of dehydration.

In this study, the survival of *L.innocua* as indicator of *L. monocytogenes* in Turkish White Cheese was studied. It was concluded that pH drop due to starter activity caused slightly decrease in number of *Listeria* cells after the addition of starter culture. However, following the salt treatment cells decrease more effectively.

Moreover, storage period has lastly been shown as a critical stage (CCP) for the whole destruction of *Listeria* which somehow contaminated the cheese product. During 45 days of storage, pH was increased to 6.00 and 6.10, respectively in cheeses with low and high dose inoculation levels. *Listeria* cells can't grow at pH values higher than 5.00. Therefore, pH value of cheese is another main factor for the inhibition of *L. innocua,* in this study. This increase in pH may be associated with a reduction in the lactate-to-protein ratio and a loss of buffering capacity of the curd due to removal of lactic acid, soluble Ca, and

phosphate in the strech water. As a consequence, the number of *Listeria* can be reduced drastically by both salt concentration and starter activity with the effect of ripening (storage) at refrigeration temperature.

The presence of *Listeria* species in the production line indicates that post-processing contamination can occur. It is evident that development and use of the GMP and hygienic rules as well as HACCP during handling and cheese processing are needed for all processing plants showing the degree and levels of contamination observed in this study.

CHAPTER 5

RECOMMENDATIONS

Further research may be done to understand the effect of nisin producing starter culture, *Lc. lactis subsp. lactis* ESI 515, with the property of bacteriosin active against *Listeria*, in a comparison with other starter cultures. Also further studies could be done to investigate the effects of starter culture in Turkish White Cheese in a comparison with cheese produced without the addition of starter culture.

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APPENDIX A

CHEMICALS AND SUPPLIERS

Chemicals	**Supplier**
Oxford Agar	Oxoid
Oxford Agar Supplement	Oxoid
Brain Heart Infusion Broth	Merck
Brain Heart Infusion Agar	Merck
Tryptic Soy Broth	Difco
Tryptic Soy Agar	Difco
MRS Agar	Merck
Rennet Enzyme	Mayasan Lim.Şti. (İstanbul)
Starter Culture	Rhodia (France)
Calcium Chloride	MARMARA Industrial Chemicals (İstanbul)

APPENDIX B

COMPOSITION OF CULTURE MEDIA

1. Brain Heart Infusion Broth

Formula (grams per liter)

Brain heart infusion solids	3.5
Casein digest	10.0
Peptone mixture	12.0
Yeast extract	2.0
Dextrose	2.0
Sodium chloride	5.0

Preperation

Suspend 34.5 g of powder in 1 L of deionised water
Mix well to dissolve powder then dispense into container
Autoclave at 121°C at 15 min

2. Brain Heart Infusion Agar

Formula (grams per liter)

Brain Heart Infusion Solids	8.0
Enzymatic digest of animal tissue	5.0
Enzymatic digest of casein	16.0
Dextrose	2.0
Sodium chloride	5.0
Disodium phosphate	2.5
Agar	13.5

Final pH 7.4 ± 0.2 at 25°C

Preparation

Suspend 52 g of medium into 1 L of purified water
Heat with frequent agitation and boil for one minute to completely dissolve the medium
Autoclave at 121°C for 15 min

3. Tryptic Soy Broth:

Formula (grams per liter)

Tryptone	17.0
Phytone	3.0
Sodium Chloride	5.0
Dipotassium phospate	2.5
Dextrose	2.5

Final pH : 7.3 ± 0.2 at 25°C

Suspend 30 g. of the powder and 0.6 g of yeast extract in a liter of distilled water.

Mix thoroghly and then warm gently until solution is complete. Dispense and autoclave for 15 min at 121°C.

4. Tryptic Soy Agar

Formula (grams per liter)

Peptone from casein	17.0
Peptone from soymeal	3.0
D+ Glucose	2.5
Sodium chloride	5.0
Di-potassium hydrogen phosphate	2.5

Final pH: 7.3 ± 0.2 at 25°C

5. MRS Agar

Formula (grams per liter)

Peptone from casein	10.0
Meat extract	8.0
Yeast extract	4.0
D+ Glucose	20.0
Di-potassium hydrogen phosphate	2.0
Tween 80	1.0
Di-ammonium hydrogen citrate	2.0
Sodium acetate	5.0
Magnesium sulfate	0.2
Manganese sulfate	0.04
Agar-agar	14.0

Final pH: 5.7 ±0.2 at 25°C

6. Oxford Agar:

Mode-of-Action

The Oxford Agar formulation is based on Columbia Agar with the addition of lithium chloride, acriflavin, colistin sulfate, cefotetan, cycloheximide and fosfomycin. These ingredients suppress the growth of the common bacteria (e.g. Gram-negative bacteria and a greater part of Gram-positiv-bacteria).

Lithium chloride is one of the ingredients of Oxford Agar base, whereas the other substances derive from the Oxford Listeria Selective Supplement. Listeria serotypes hydrolyses esculin to esculetin and forms a black complex with iron (III) ions. Therefore *L. innocua* produces brown-green coloured colonies with a black halo.

Typical Composition (g/litre)

Peptone	23.0;
Starch	1.0;
Sodium chloride	5.0;
Agar-agar (= Columbia agar)	13.0;
Esculin	1.0;
Ammonium iron (III) citrate	0.5;
Lithium chloride	15.0.

Preparation

29.25 g of Oxford agar was suspended in 500 ml of demin. water, autoclaved (15 min at 121 °C). The lyophilisate of 1 vial Oxford Listeria Selective Supplement was dissolved by adding 5 ml of a 1:1 mixture of ethanol and sterile distilled water. Gently mixed and the contents were added to the culture medium cooled

to 50 °C. The medium were poured into plates and leave to solidify.
pH: 7.0± 0.2 at 25 °C.
The prepared agar was clear and bluish-brown.

Experimental Procedure and Evaluation

The samples were inoculated by spreading on the surface of the medium and incubated at 35 °C up to 48 h aerobically. *Listeria innocua* grows as brown-green coloured colonies with a black halo (esculin splitting).

APPENDIX C

PREPERATION OF SOLUTIONS

1. Peptone Water

Peptone Water solution was prepared to give 0.1% concentration, sterilized in the autoclave for 15 min and at 121°C. pH was adjusted to 7.9 ml of solution was filled into sterile tubes in order to use as a dilution medium. The tubes were then stored in refrigerator.

2. Calcium Chloride Solution

Dissolve 40 g of $CaCl_2$ in 50 ml of distilled warm water then complete to 100 ml of water with distilled water.

3. Saline for Storage (Ripening)

Dissolve 16 g of table salt in 100 ml of distilled warm water and mix thoroughly.

APPENDIX D

EXPERIMENTAL RESULTS

1. Growth of *Listeria innocua* in BHIB

The growth of *Listeria innocua* in BHIB medium at 37°C was demonstrated in Figure 1. The count of *L.innocua* versus time shows that the strain is a rapidly growing microorganism under optimal conditions as in Fig. 2. The culture incubated at 37°C for 24h was used throughout this study. The Optical Densities of *L.innocua* at different log CFU/ml was shown a curve as in Fig.1.

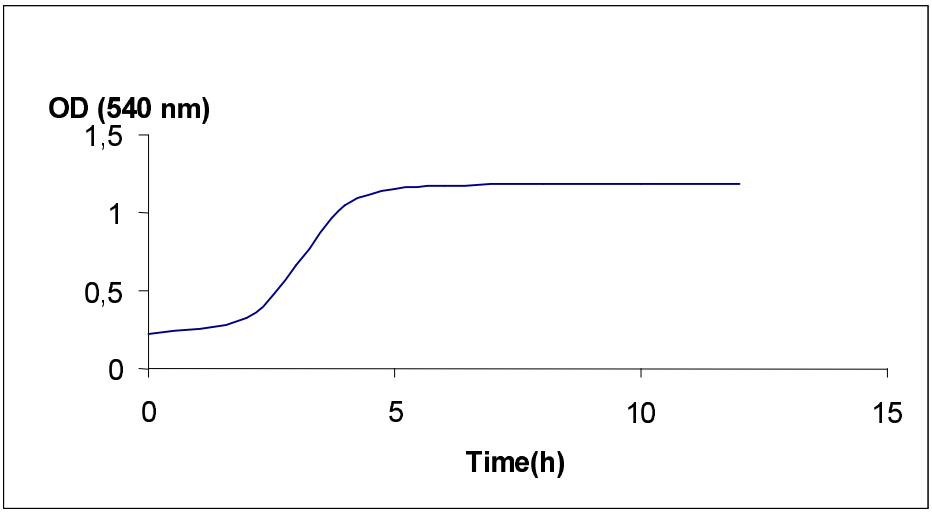

Figure D.1 Growth Curve of *L.innocua*

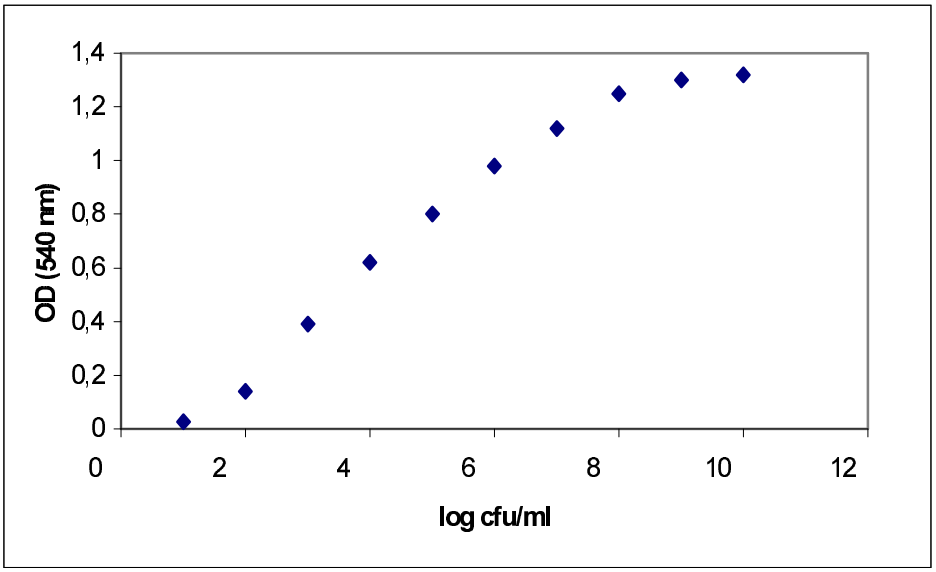

Figure D.2. Count of *L. innocua* versus Optical Density

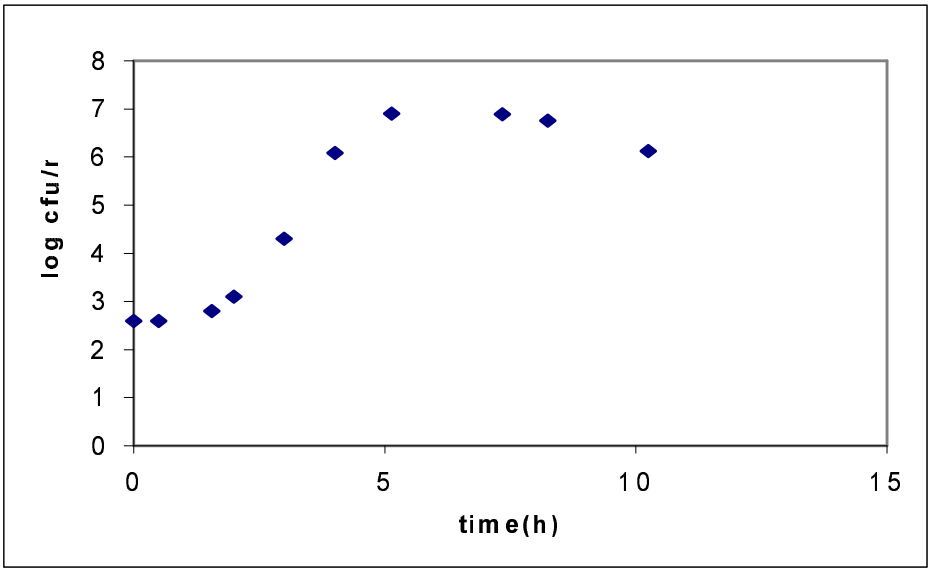

Figure D.3. Count of *L. innocua* versus time

APPENDIX E

VALUES OF ANALYSES

Table E.1 Variation in physico-chemical properties of Turkish White Cheese with low dose inoculation of *L. innocua* during the storage of 45 days

	pH	Acidity	Salt Content (w/v%)	Moisture content (%)	Fat Content (%)
Curd	4.92	1.4	5.96	60.23	24.1
Day 5	5.54	1.84	6.03	59.80	30.0
Day 10	5.67	1.82	6.10	59.12	34.2
Day 20	4.68	1.81	6.35	57.86	36.2
Day 30	5.89	1.64	6.42	55.90	36.8
Day 45	6.00	1.59	6.80	54.87	35.4

Table E.2 Variation in physico-chemical properties of Turkish White Cheese with high dose inoculation of *L innocua* during the storage of 45 days

	pH	Acidity	Salt Content (w/v%)	Moisture content (%)	Fat Content(%)
Curd	5.14	1.32	6.20	61.49	24.0
Day 5	5.50	1.76	6.33	60.50	31.0
Day 10	5.64	1.77	6.39	59.89	35.4
Day 20	5.74	1.56	6.45	56.80	37.2
Day 30	5.98	1.42	6.62	57.75	38.8
Day 45	6.10	1.40	6.98	57.0	38.9

Table E.3 Results of the replicates of *L. innocua* and TAC in Turkish White Cheese after low-dose inoculation of *L. innocua* for obtaining the initial load of 3.84 log CFU/ml

***L. innocua* (log CFU/ml)**

Inoculated milk	Whey	Curd pressed overnight	Salted cheese
3.4- 3.12- 3.25	3.52-3.65- 3.30	3.89- 3.25- 3.24	2.36-3.21-2.68
SD:0,140119	SD:0,176918	SD: 0,372424	SD: 0124862

Total reduction of *L. innocua* during preperation of cheese and after salt treatment (log CFU/ml)

Final count	Initial count	Survival(%)	Death(%)
2.75	3.26	84.3	15.7

TAC (log CFU/ml)

Inoculated milk	Whey	Curd pressed overnight	Salted cheese
7.41- 6.56- 6.7	6.12- 6.46- 6.02	8.21- 7.62- 8.11	6.69- 8.02- 7.37
SD: 0,455741	SD: 0,238607	SD: 0,315753	SD:0,665056

Table E.4 Results of the replicates of *L. innocua* and TAC in Turkish White Cheese after high-dose inoculation of *L. innocua* for obtaining the initial load of 7.12 log CFU/ml

***L. innocua* (log CFU/ml)**

Inoculated milk	Whey	Curd pressed overnight	Salted cheese
7.24- 7.21- 6.50	7.98- 7.23- 7.17	8.64- 6.96- 8.31	6.35- 6.23- 5.42
SD: 0,418848	SD: 0,451331	SD: 0,890112	SD: 0,505866

Total reduction of *L. innocua* during preperation of cheese and after salt treatment (log CFU/ml)

Final count	Initial count	Survival(%)	Death(%)
6.00	6.98	85.9	14.1

TAC (log CFU/ml)

Inoculated milk	Whey	Curd pressed overnight	Salted cheese
9.8- 9.03- 9.37	11.38- 10.08- 9.44	11.9- 10.03- 10.77	10.35- 10.19- 10.06
SD: 0,385876	SD: 0,988534	SD: 0,941754	SD: 0,159478

Table E.5 Values of the replicates of *L. innocua* (CFU/ml) in Turkish White Cheese with low dose inoculation of *L. innocua* during the storage of 45 days

Day	1st trial	2 nd trial	3rd trial	avg. log CFU/ml	SD
1	2.76	2.73	2.74	2.75	0.015275
5	2.67	2.62	2.63	2.63	0.026458
10	2.26	2.23	2.26	2.25	0.017321
15	1.5	1.95	2.21	1.89	0.359212
20	1.48	1.78	1.7	1.65	0.155349
30	1.3	1.4	1.03	1.24	0.191398
45	0.97	0.89	0.98	0.95	0.049329

Total reduction of *L. innocua* during the storage of 45 days (log CFU/ml)

Final count	Initial count	Survival(%)	Death(%)
0.95	2.75	34.5	65.5

Table E.6 Values of the replicates of *L. innocua* (CFU/ml) in Turkish White Cheese with high dose inoculation of *L. innocua* during the storage of 45 days

Day	1st trial	2 nd trial	3rd trial	avg. log CFU/ml	SD
1	6.2	6	5.8	6	0.2
5	5.66	5.77	5.72	5.72	0.055076
10	5.39	5.63	5.5	5.51	0.120139
15	5.48	4.99	5.28	5.26	0.234592
20	4.97	4.9	5.0	4.99	0.051316
30	4.58	4.49	4.68	4.59	0.095044
45	4.26	3.97	4.45	4.23	0.24173

Total reduction of *L. innocua* during preperation of cheese and after salt treatment (log CFU/ml)

Final count	Initial count	Survival(%)	Death(%)
4.23	6	70.5	29.5

Table E.7 Values of the replicates of TAC (CFU/ml) in Turkish White Cheese with low dose inoculation of *L. innocua* during the storage of 45 days

Day	1st trial	2 nd trial	3rd trial	avg. log CFU/ml	SD
1	7.36	7.4	7.34	7.36	0.030551
5	6.98	6.9	6.95	6.95	0.040415
10	6.6	5.84	6.99	6.48	0.584836
15	6.02	5.47	6.4	5.96	0.467582
20	5.58	5.69	5.46	5.58	0.115036
30	4.86	5.48	4.98	5.11	0.328836
45	4.17	5.39	5.09	4.89	0.635715

Table E.8 Values of the replicates of TAC (CFU/ml) in Turkish White Cheese with high dose inoculation of *L. innocua* during the storage of 45 days

Day	1st trial	2 nd trial	3rd trial	avg. log CFU/ml	SD
1	10.48	9.4	10.56	10.2	0.647868
5	10.26	10.02	9.38	9.88	0.454899
10	9.15	9.4	8.98	9.17	0.211266
15	9.13	8.9	8.63	8.89	0.250267
20	8.67	8.47	8.58	8.58	0.100167
30	8.14	7.71	8.02	7.96	0.221886
45	7.36	7.62	7.24	7.41	0.194251

Table E.9 Relationship between LAB and TAC without the inoculation of *L. innocua* (log CFU/ml)

DAY	LAB	TAC
1	5,85	7,06
5	5,2	6,79
10	4,88	6,62
15	4,62	6,07
20	4,33	5,89
30	3,95	5,55
45	3,71	5,17

Table E.10 Values of the replicates of LAB (log CFU/ml) variation in Turkish White Cheese without inoculation of *L.innocua*

Day	**1st trial**	**2 nd trial**	**3rd trial**	**avg. log CFU/ml**	**SD**
1	5.91	5.52	6.12	5.85	0.304467
5	4.67	5.64	5.29	5.2	0.491223
10	4.06	5.43	5.15	4.88	0.723809
15	4.37	5.16	4.33	4.62	0.468081
20	3.81	5.24	3.94	4.33	0.790759
30	3.68	4.38	3.79	3.95	0.376431
45	3.26	4.08	3.79	3.71	0.415812

Table E.11 Values of the replicates of TAC (log CFU/ml) variation in Turkish White Cheese without inoculation of *L. innocua*

Day	1st trial	2 nd trial	3rd trial	avg. log CFU/ml	SD
1	6.95	7.18	7.09	7.06	0.115902
5	6.42	7.13	6.83	6.79	0.351190
10	6.31	6.9	6.64	6.62	0.295690
15	5.56	6.63	6.02	6.07	0.536751
20	5.23	5.64	6.7	5.89	0.758571
30	5.30	6.14	5.20	5.55	0.516272
45	5.12	4.97	5.41	5.17	0.223683

Table E.12 Relationship between LAB and TAC with the low-dose inoculation of *L. innocua* (log CFU/ml)

DAY	LAB	TAC	*L. innocua*
1	4,24	10,2	6
5	4,16	9,88	5,72
10	3,66	9,17	5,51
15	3,63	8,89	5,26
20	3,36	8,58	4,99
30	2,98	7,96	4,59
45	2,85	7,08	4,23

Table E.13 Values of the replicates of LAB (log CFU/ml) in Turkish White Cheese with the inoculation of low dose *L.innocua* during storage of 45 days

Day	1st trial	2 nd trial	3rd trial	avg. log CFU/ml	SD
1	4.68	4.03	4.01	4.24	0.381182
5	4.59	3.91	3.98	4.16	0.374032
10	3.82	3.34	3.82	3.66	0.277128
15	3.65	3.23	4.01	3.63	0.390384
20	3.28	3.12	3.68	3.36	0.288444
30	3.08	3.27	2.59	2.98	0.350856
45	2.59	3.26	2.7	2.85	0.359305

Table E.14 Relationship between LAB and TAC with the high-dose inoculation of *L. innocua* (log CFU/ml)

DAY	LAB	TAC	*L. innocua*
1	3,84	10,2	5,96
5	3,71	9,88	5,72
10	3,46	9,17	5,51
15	3,43	8,89	5,26
20	3,16	8,58	4,99
30	2,78	7,96	4,59
45	2,65	7,08	4,23

Table E.15 Values of the replicates of LAB (log CFU/ml) in Turkish White Cheese with the inoculation of high dose *L.innocua* during storage of 45 days

Day	**1st trial**	**2 nd trial**	**3rd trial**	**avg.log CFU/ml**	**SD**
1	3.86	3.67	3.98	3.84	0.147309
5	3.77	3.65	3.71	3.71	0.06
10	3.32	3.6	3.45	3.46	0.140119
15	3.4	3.08	3.82	3.43	0.371124
20	3.19	3.01	3.28	3.16	0.137477
30	2.98	2.56	2.79	2.78	0.210317
45	2.78	2.52	2.64	2.65	0.14

Table E.16 Results obtained from the regression lines in Figure 3.5 and 3.6.

D Value of *L. innocua* (day)		R^2	
Low dose	High dose	Low dose	High dose
23.4	25	0.95	0.98

APPENDIX F

Figure F.1 CHEESE VAT (ARMFIELD)

Figure F.2 SPECTROPHOTOMETER (PHARMACIA NAVASPEC II)

Figure F.3 COLONY COUNTER (STUART SCIENTIFIC)

Figure F.4 CENTRIFUGE (MSE MISTRAL 1000)

APPENDIX G

Table G.1 Quality Standart of Turkish White Cheese (TSE)

Parameter	Units	Standard
Titration acidity (Lactic acid)	w/w	max.%3
pH		min.4.5
Salt Concentration	w/w	max. % 10
Moisture Concentration	w/w	max. % 60
Coliform Bacteria	CFU/ml	10^3
E. coli	CFU/ml	0
Salmonella	CFU/ml	0
Staph. Aureus	CFU/ml	0
Listeria Monocytogenes	CFU/ml	0

APPENDIX J

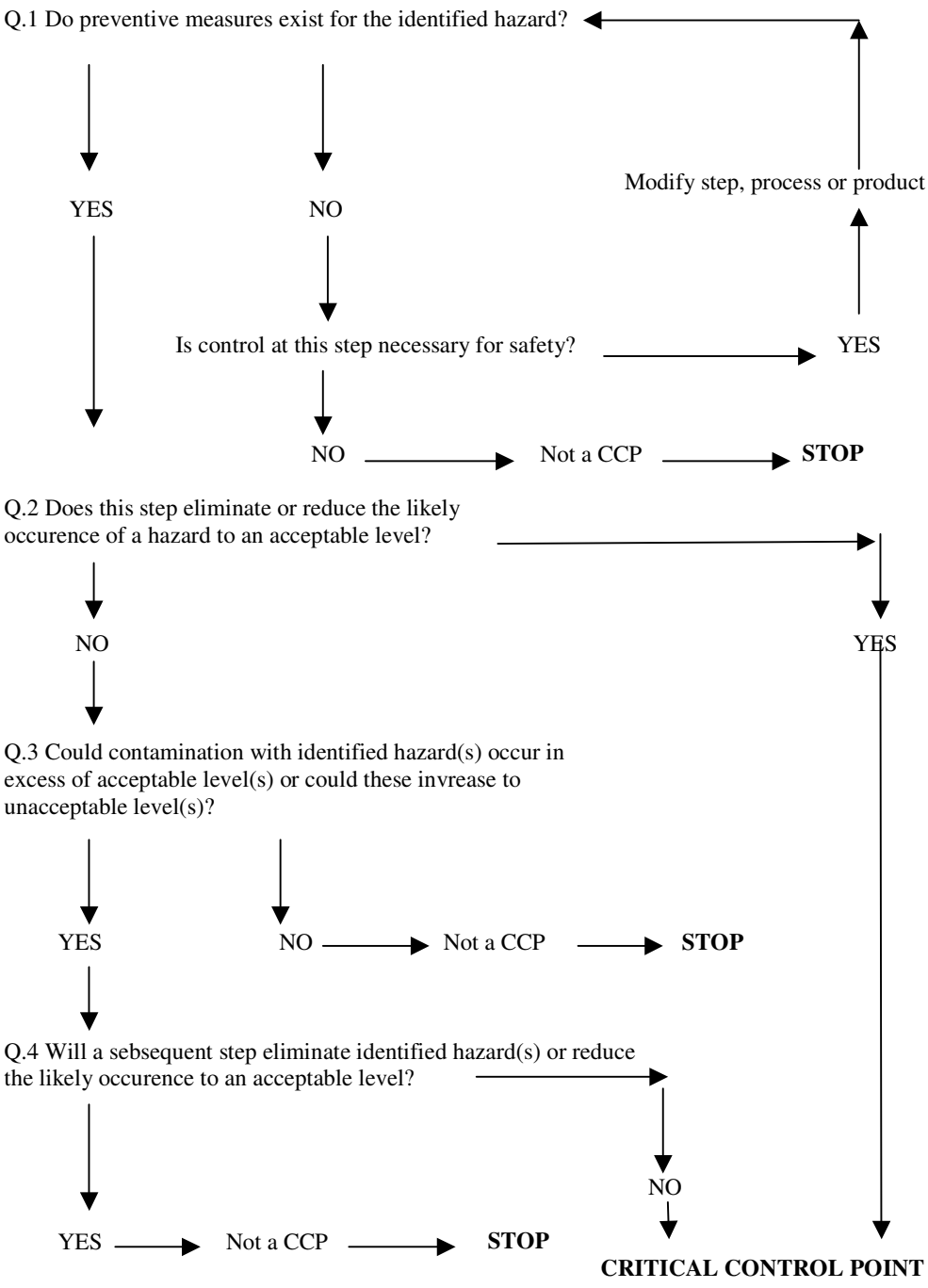

Figure J.1 CCP Decision Tree (NACMCF HACCP System, 1992)