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INFLUENCE OF NISIN TO THE LACTIC ACID BACTERIA GROWTH AND FLAVOUR COMPOUNDS DURING RIPENING OF RAW MILK-CHEESE

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INFLUENCE OF NISIN TO THE LAB GROWTH AND FLAVOUR COMPOUNDS DURING RIPENING OF RAW MILK-CHEESE

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ABSTRACT

The development of flavour compounds in raw milk-cheese are influenced by the presence of microflora. This study was designed to evaluate the effect of NSLAB and SLAB to the formation of flavour compounds in raw milk-cheese. In addition, the influence of nisin to the growth of NSLAB and SLAB, and subsequent effect to the flavour compounds during ripening was also studied. The resulting data support the conclusion that in early ripening, NSLAB presented at a low initial level and substantially increase during further ripening, while SLAB also showed increase growth, but indicated slow-down growth near the end of ripening. There is another indication that non-LAB microflora grew in a high number in cheese without starter addition due to lack of competition. However, no significant inhibition of nisin found in relation to the growth of both SLAB and NSLAB. In relation with flavour profile, it was found that cheese without starter generally produces higher concentration of volatile compounds compare to cheeses with starter addition. This results indicated not only NSLAB presented and involved in the flavour development, other microflora such as yeast/mold/other bacteria also suspected to be present. Moreover, cheese with nisin-producer did not have significant difference with cheese with no nisin-producer in relations to the flavour profile. This is expected, because as stated before, no inhibition of nisin happened. Difference of flavour profile between cheese with similar starters (D227 and D447) suggested either spoilage by other microflora, or different enzyme activities occurred during ripening. In conclusion, more analysis are worthwhile to prove the assumption made in this study, such as measurement of nisin activity, enzymatic activity analysis, more accurately LAB quantification, and identification of potential spoilage in raw milk-cheese.

Keywords: NSLAB; SLAB; nisin; raw milk-cheese; cheese ripening

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LIST OF ABBREVIATIONS

AA = Amino Acid

Phe = Phenylalanine

Tyr = Tyrosine

Trp = Tryptophan

Met = Methionine

LPL = Lipoprotein Lipase

SIM = Selected Ion Monitoring

FFA = Free Fatty Acid

LAB = Lactic Acid Bacteria

MRSA = de Man, Rogosa and Sharpe Agar

MRSA-V = MRS medium supplemented with Vancomycin

LM17 = Lactose-Medium 17

PDA = Potato Dextrose Agar

PCA = Plate Count Agar

YGC = Yeast Extract Glucose Chloramphenicol

TCBS = Thiosulfate Citrate bile salts Sucrose

PAB = Propionibacterium

CHAPTER 1 INTRODUCTION

1.1 BACKGROUND INFORMATION

More than 500 varieties of cheeses are exist around the world is a prove that cheese is a popular dairy product (IDF, 1982). Research on microbiology, chemistry, and technology of cheese are still advancing as the results of people's interest in cheese making (Sgarbi *et al*., 2013). Cheese undergoes significant changes during the ripening periods. Freshly-made cheese of many varieties have similar, bland flavours. It is during the ripening periods that each cheese varieties will produce their own distinctive flavours (McSweeney and Sousa, 1999). Remeker cheese is another new development in cheese industry. Aside from using raw milk for their cheese production, other unique characteristic from this cheese is the type of milk they are using. As most of cheese are made out of Holstein cows' milk,(Capper and Cady 2012) Remeker cheese is made out of Jersey cows' milk, as Jersey cow's milk produced higher fat and protein content (Capper and Cady, 2012).

The flavour profile of raw milk-cheese and pasteurized milk-cheese are distinctively different. Beuvier *et al.,* (1997) reported greater growth of NSLAB in raw milk-cheese compare to pasteurized milk-cheese. NSLAB will barely present in cheese made from pasteurized milk as they could not resist pasteurization (Turner *et al*., 1986). NSLAB generally do not involved in the curd acidification. They do, however, partake in flavour development during maturation of cheese as secondary flora, as of shown in semi hard-type cheese like Cheddar and Gouda and hard Swiss-type cheese such as Emmental, Gruyère, and Comté (Beresford *et al*., 2001; Kołakowski *et al*., 2012).

Although the role of NSLAB is still relatively unclear, some reports demonstrated that NSLAB possess wide range of peptide hydrolytic enzymes, which causes increase of the amount of short peptides and free amino acids (De Pasquale *et al*., 2014; Williams and Banks, 1997; McSweeney *et al*., 1993). In addition, NSLAB possess higher lipase and esterase activities compare to SLAB, leading to higher production of free fatty acids (FFA) (McSweeney *et al*., 1993). The high contribution of NSLAB during ripening is strengthened by the fact that NSLAB could survive the harsh condition of cheese ripening (low pH, low water activity and high salt content), while the amount of SLAB decrease significantly (Broadbent and Steele, 2005; Gobbetti *et al*., 2015).

SLAB not only participate at flavour profile in cheese, they also have an important role in the cheese preservation. Some of these bacteria produce antagonistic substances, called bacteriocins, which in small amounts are very active against pathogens (Moreno et al, 2000). Nisin is one of the bacteriocins that is produced by certain strains of *Lactococcus lactis* subsp. *lactis,* and is known to exhibits antimicrobial activity against a wide range of microorganisms, mainly against Gram positive bacteria. Although the use of raw milk for cheese manufacturing increases the flavour intensity, it will also concern the safety risk of the cheese itself, as many pathogens might also grow, therefore the presence of bacteriocin is important (Poli *et al*., 2007; Hayes *et al.,* 1986; Lovett *et al.,* 1987).

On the other hand, the use of bacteriocin may cause negative consequences to the growth of NSLAB. Research conducted by Ryan *et al.,* (1996), Ryan *et al*., (2001) and Rossi and Veneri (2016) showed that a high initial inoculum of the bacteriocinogenic culture caused inhibition to the growth of most NSLAB in cheese such as *Lactobacillus paracasei* and L. *plantarum*. It is already known that most of

NSLAB present in cheese are nisin-sensitive species. The inhibition of NSLAB could influence the flavour profile of cheese.

1.2 PROBLEM DEFINITION

Several research agree that cheese made from raw milk are generally more intensely flavoured than cheese made from pasteurized milk (Roy et al., 1997; Shakeel-Ur-Rehman *et al*., 1999). Both SLAB and NSLAB have their own perspective role to the flavour development. However the role of both NSLAB and SLAB for metabolism of volatile compounds that lead to the flavour formation in raw milk-cheese still need to be studied further. In addition, nisin-producing starters are often used in raw milk-cheese for prevent growth of pathogens (De Vuyst and Vandamme, 1994). However, the effects of nisin might inhibit the growth of NSLAB as well, which lead to changes in flavour profile. The effect of nisin to the growth of NSLAB and SLAB are still unclear. Therefore studying the impact of nisin to the growth of both LAB might give better understanding about the changes of flavour compounds during raw milk-cheese ripening.

1.3 OBJECTIVES

The objectives of this study are to investigate if nisin will affect NSLAB and SLAB growth, thus subsequently impacted the flavour compounds of raw milk-cheese during ripening as well.

1.4 RESEARCH QUESTIONS

How does non starter lactic acid bacteria (NSLAB) differ with starter lactic acid bacteria (SLAB) regarding the metabolism of flavour compounds of raw milk-cheese during ripening?

- Is there any effect of nisin to NSLAB and SLAB growth?
- What would be the influence of nisin in relation to the overall flavour profile?
- Will different SLAB cultures will give similar trends and values to the flavour profile?

1.5 HYPOTHESIS

In general, it is expected that SLAB and NSLAB combination would give better overall flavour profile of raw-milk cheese rather than with only NSLAB. The growth of nisin-producing SLAB is expected to be not influenced by the presence of nisin, as it is known that *Lactococcus lactis* subsp. *lactis* is nisin-resistant (Kramer *et al*., 2006). The same hypothesis is also expected between different nisin-producing SLAB. It is expected that different nisin-producing SLAB with similar characteristics will provide similar results of LAB growth, thus give similar flavour profile. Nisin is expected to have inhibiting effect to NSLAB, as many of them are supposedly sensitive to the presence of nisin (Rossi and Veneri, 2016). Therefore, the inhibition of NSLAB will subsequently decrease the concentration or type of important flavour compounds, especially the compounds derived from lipid metabolism during cheese ripening.

1.6 APPROACH

This thesis project will focus more on the impact of nisin to the growth of both NSLAB and SLAB, in correlation with the formation of flavour compounds in raw milk-cheese. Raw milk which is produced by Jersey Cow and obtained from Remeker Farm will be used. Milk samples will be added with two nisin- and one no nisin-producing SLAB, separately. Another milk sample without any SLAB addition will also be included. Milk samples will be then fermented for 4 hours before cheese manufacturing.

Fermentation was done longer than normal cheese making to faster the growth of NSLAB, thus the influence of NSLAB for flavour formation in cheese could be seen faster and more obvious during ripening (Kusuma's research, 2015). Cheese manufacturing will be done in FQD Lab through microcheese-making system. The cheese will be left for ripening for 52 days. Sampling will be done to both milk and cheese at specific sampling points, and analysis will be performed. Volatile compounds will be extracted and identified using SPME and GC-MS analysis. Flavour profile will be created through analytical method using MetAlign, MetAlignID, AMDIS, and Ms. Excel. By the end of this project, it is expected to see if nisin can affect or inhibit the growth of LAB (SLAB and NSLAB) and if the effect will cause difference in the flavour compounds of raw milk-cheese during ripening.

CHAPTER 2 LITERATURE REVIEW

2.1 RAW MILK-CHEESE

Traditionally, cheeses are made from raw milk, but due to safety reasons, nowadays cheeses mostly made from pasteurized milk. However, raw milk cheese slowly develops more intense flavour during cheese ripening (Buchin *et al*., 1998), due to higher concentration of amino acid and fatty acids (Price and Call, 1969; McSweeney *et al*., 1993). In many cheeses, especially Gouda-type cheese, microbial activity is of main important factor for flavour development. Starter culture basically involve to the production of lactic acid in the early stage of raw and pasteurized milk-cheese manufacture, they also largely contribute to the proteolysis, hence the flavour development during ripening (Broom and Limsowtin, 1998; Kieronczyk *et al*., 2003). However, in especially raw milk-cheese, non-starter lactic acid bacteria (NSLAB) play important role during cheese ripening. Initially present at low numbers, NSLAB gradually become the dominant microflora in long ripening cheese (Fox *et al*., 1998). High lipolysis rate, especially production of free fatty acid (FFA) in raw milk-cheese are attributed to the activity of bacterial's esterases and lipases (Kebary *et al*., 1996). On the other hand, most starter cultures used in fermented milk products have weak intracellular lipase and esterase activities (Paulsen *et al*., 1980). Remeker cheese is similar to Gouda-type raw milk-cheese where the presence of LAB are highly involved in flavour development. But other microflora, that is mold, is also responsible for flavour development in this cheese due to the use of ghee, which increases mold growth. However, in this study, ghee is not implemented during cheese making, therefore the discussion will focus more on the LAB involvement to the flavour development of raw milk-cheese.

2.2 LACTIC ACID BACTERIA

Cheese manufacture and ripening involves the action of enzymes and microorganisms. Traditionally, the microflora of cheese consisted of adventitious microorganisms either from milk environment or contaminate the cheese during manufacture and/or ripening. Currently, however, most cheeses were made through addition of highly defined starters. Lactic acid bacteria are considered to be the most important microflora for Gouda-type cheese. Most naturally-present microflora in cheese mainly comprises as non-starter lactic acid bacteria (NSLAB), while the starter commonly referred to as starter lactic acid bacteria (SLAB) (Fox *et al*., 1998).

In the case of Dutch-type cheeses, the starters usually dominated by mesophilic LAB, although some strains of thermophilic LAB were reported to sometimes used as well. The strains of *Lactococcus lactis* subsp*. lactis*, *Lactococcus lactis* subsp*. cremoris, Leuconostoc mesenteroides*, *Streptococcus thermophilus*, *Lactobacillus helveticus,* and *Lactobacillus delbruecki* are commonly used individually or in combination as mesophilic and thermophilic bacterial starters for cheese manufacture (Hoier *et al*., 2010). The SLAB are responsible for acid production and grow fast during the early cheese production. During ripening, however, the number of starters generally decreases rather quickly during cheese ripening. During cheese ripening, the environment becomes too harsh for SLAB to grow: low pH, a relatively high salt content, and lacks of a fermentable carbohydrate (Elek, 2014; Fox *et al*., 1998; Settanni and Moschetti, 2010). It is known that SLAB greatly influence the flavour formation in cheese, especially increasing proteolysis (Lynch *et* al., 1997; Tavaria and Malcata, 2003).

In contrast with SLAB, NSLAB normally will not contribute during the early stage of cheese ripening, mainly due to the low amount present. However, many research showed that cheese made from raw

milk is more intensely flavoured and ripens faster that cheese made from pasteurized milk, mostly caused by lipolysis (McSweeney and Fox, 1999; William and Banks, 1997; Van Hoorde *et al*., 2008; Van Leuven *et al*., 2008). While SLAB growth decrease during ripening, NSLAB grow faster as NSLAB are more resistant to the harsh environment during cheese ripening. The group of NSLAB is particularly heterogeneous with mesophilic lactobacili being mostly represented in semi hard-type cheese: *Lactobacillus farciminis, Lactobacillus pentosus, Lactobacillus paracasei, Lactobacillus plantarum, Lactobacillus casei, Lactobacillus curvatus,* and *Lactobacillus rhamnosus*. Some non-*Lactobacillus* species of NSLAB were commonly found but in a lesser extent during cheese ripening, such as *Pediococcus acidilactici, Pediococcus pentosaceus*, *Enterococcus durans*, and *Enterococcus faecalis*. *Leuconostoc* spp. and *Lactococci* species may also be identified (Fitzsimons et al., 1999; Gobbetti *et al*., 2002; Coeuret *et al.,* 2004). Most NSLAB possess enzymes with high esterase activity, which in turn, increase the production of free fatty acid (FFA) that will contribute on flavour development (William and Banks, 1997; Lynch et al., 1997). Aside from bacterial enzymes, other enzymes like milk lipase and renneting enzyme also contribute to the FFA production.

Different role of SLAB and NSLAB to flavour development in cheese were discovered through many experiments. *Lactococcus* spp. are often used as SLAB due to their ability to metabolize citrate into CO2, required for the eye formation, and important flavour compounds such as diacetyl, acetate and ethanol (Walstra *et al.,* 1993; McSweeney, 2004). Freshly made curds of several type cheese have similar and blend flavours. During the early ripening, the SLAB grow abundantly, produce acid, lower the pH, hence trigger proteolysis. Thus resulting the formation of small peptides or amino acids, lactate, FFA, and other cell's lysis by-products (McSweeney and Sousa, 2000). Nevertheless, in long-ripened cheese, SLAB and NSLAB develop dynamically (Broadbent and Steele, 2005). At some point of ripening, the SLAB undergo autolysis, and NSLAB start to grow significantly. NSLAB development during ripening can be attributed to their ability to use the major compounds which have been produced by SLAB, such as small peptides and amino acid (AA) for further metabolization, thus enhancing SLAB's proteolysis (Gatti *et al.,* 2008, Fox and McSweeney, 2004). Moreover another distinction of NSLAB ability to SLAB, is their high rate of lipolysis. Research conducted by Awad, (2005), El-Soda *et al.,* (1992), Madkor *et al.,* (1999) showed that higher FFA level discovered in raw milk-cheese. NSLAB is believed to possess and able to release intracellular lipases and esterases enzymes that SLAB do not possess, and breakdown lipid into free fatty acids, such as methyl ketones (especially 2-nonanone, 2-heptanone and 2-pentanone). In general, both SLAB and NSLAB have their own influence to the flavour development in cheese. However in raw milk-cheese, the presence of NSLAB is more obvious, thus the combination of both LAB is believed to affect greatly to the falvour development of raw milk-cheese.

2.3 NISIN

Use of raw milk in cheese manufacturing is hygienically hazardous and although good practices of milking and milk storage can reduce the risk of pathogen growth, but cannot nullify the risk completely (Poli *et al*., 2007). The possibility of exploiting the antimicrobial potential of bacteriocin, produced from lactic acid bacteria (LAB), to improve the safety of these products has been studied. Studies of the effect of bacteriocinogenic cultures of SLAB showed their ability to induce bacterial's cell to lysis (Garde *et al*., 2002; Martínez-Cuesta and Requena, 2001).

Nisin is one of bacteriocins, produced by certain strains of *Lactococcus lactis* subsp. *lactis.* Nisin is the only one of two bacteriocins approved by WHO and FDA as generally regarded as safe (GRAS) for use

in pasteurized products and processed cheese (Parada *et al*., 2007). It is is usually used by food manufacturer to inhibit pathogen growth in food products. It has received attention because of its inhibitory effect against both Gram-positive and Gram-negative bacteria (Stevens *et al*., 1991). It is a complex protein, formed by 34 amino-acids, with molecular weight of 3.5 kDa, and isoleucine (NH2) and lysine (COOH) as terminal amino acids (Jozala *et al*., 2015). Nisin solubility, stability, and biological activity are highly dependent on pH. The solubility and stability of nisin increase with increasing acidity (Delves-Broughton, 1990). Under acid conditions, more than 80% of the produced nisin is release from the cells. On the other hand, under neutral or alkaline conditions, nisin is almost insoluble, thus its activity decreased significantly (Penna *et al*., 2006).

Figure 2.1 Primary Structure of Nisin

High attention of nisin attracted many research, especially to synthesize nisin. Thus, nisin was synthesized successfully and already used as a commercial nisin called Nisaplin (Aplin and Barret, Ltd.). Many food industries, especially cheese industries, add nisin to the food products by inducing a nisin producer, or through synthesized nisin. Nisin biosynthesized is considered complex and costly, and utilization of commercial nisin might cause immediate inhibitory effect to the advantageous microflora present in cheese, such as non-starter lactic acid bacteria, which is important for flavour development during raw milk-cheese ripening (Ryan *et al*., 2001). However, although nisin was proved to have inhibitory effect to nisin-sensitive NSLAB, it should be noted that the low initial level of nisin producer will cause small effect to NSLAB inhibition (Rossi and Veneri, 2015).

2.4 FLAVOUR FORMATION IN CHEESE

Volatile flavour compounds in cheese originate from degradation of major milk constituents, that is lactose, citrate, milk lipids, and milk proteins (casein) during ripening. The degradation process of all compounds depending on the ripening time, some compounds might need few weeks, others might need months or years (Singh *et al*., 2003). The major biochemical pathways which lead to the flavour formation of a whole range of precursors of flavour compounds are the following: metabolism of lactose, lactate, and citrate (often referred as glycolysis), proteolysis, and lipolysis (McSweeney, 2004).

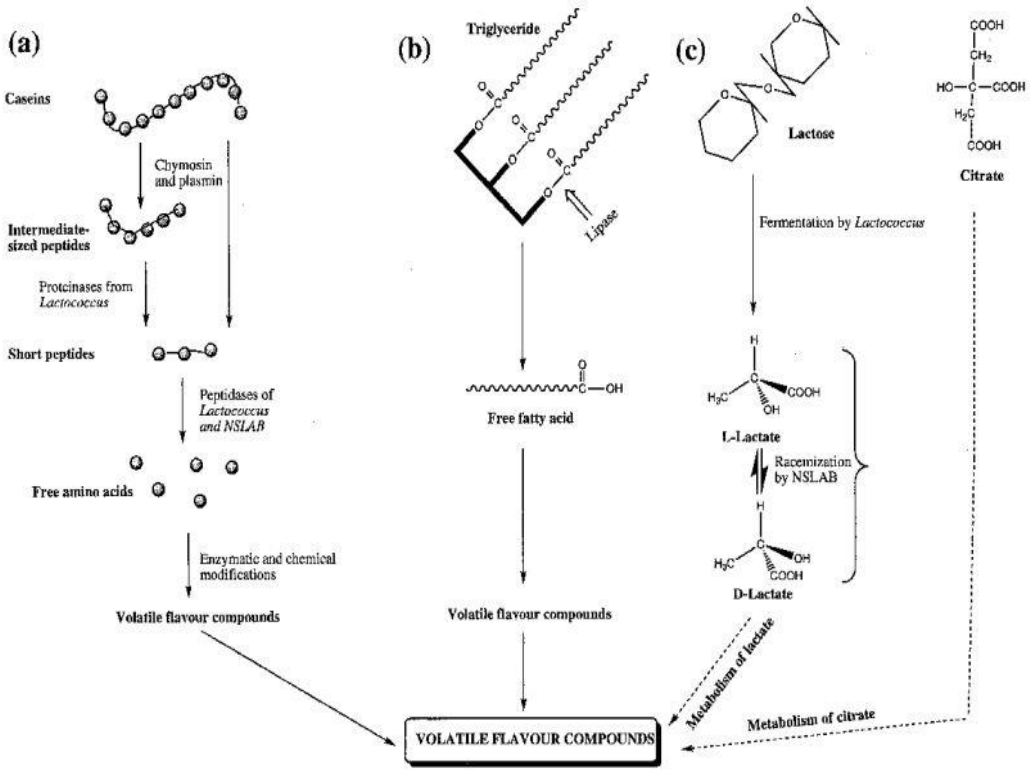


Figure 2.2 General Pathway of Cheese Metabolism (McSweeney and Sousa, 1999)

2.4.1 METABOLISM OF LACTOSE, LACTATE, AND CITRATE (GLYCOLYSIS)

Most of lactose in milk are lost in the whey during cheese manufacture. However low levels of lactose remains in the curd after cheese making (e.g 0.8 – 1% in cheddar cheese manufacture) (Huffman and Kristoffersen, 1984). Glycolysis metabolism starts from degradation of lactose into lactate by most starter culture and NSLAB. *Leuconostoc* spp. do not contribute to the degradation of lactose into lactate, however, they contribute to the degradation of lactose into other compounds, such as ethanol (Vedamuthu, 1994). The degradation of lactose by SLAB especially lactococci strains produce L-lactate, while lactose can be degraded into D-lactate by NSLAB (Thomas and Crow, 1983). The latter could also racemize L-lactate into DL-Lactate, however it has little impact to the flavour profile.The oxidation of lactate by both SLAB and NSLAB also produce several important flavour compounds such as acetate, ethanol, formate and CO_2 (Fox *et al*., 2000).

Citrate is water-soluble, thus most of it will lost during draining of whey process. Nevertheless, low number of citrate are still in great importance, as it will converted into several important flavour compounds during cheese ripening. Metabolism of citrate involve the use of certain mesophillic SLAB (Fox *et al.*, 1990; Cogan and Hill, 1993; Parente and Cogan, 2004; McSweeney and Fox, 2004). Citrate is metabolize by certain SLAB, namely *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*. Some *Leuconostoc* spp also believed to be able to convert citrate. However, other SLAB and NSLAB could not metabolize citrate, although some lactobacili strains from NSLAB group might be able to metabolize citrate. Metabolism of citrate resulting to the production of acetoin, acetate, diacetyl, and 2,3-butanediol which are important flavour compounds (Palles *et al*., 1998).

Metabolism of lactate by SLAB is most important in surface mold-ripened cheese. Lactate produced by SLAB will be further metabolized by yeast and mold, e.g *Geotrichum candidum*, *Debaryomyces hansenii*, followed later by *Penicillium camemberti* to CO2 and O2, thus deacidifying the cheese surface. The deacidifying process have a role in increasing proteolysis rate by yeast and mold (McSweeney and Fox 2004). As Remeker cheese is made with combination of LAB and mold, it is expected that interaction between SLAB and mold will cause high lactose metabolism in general.

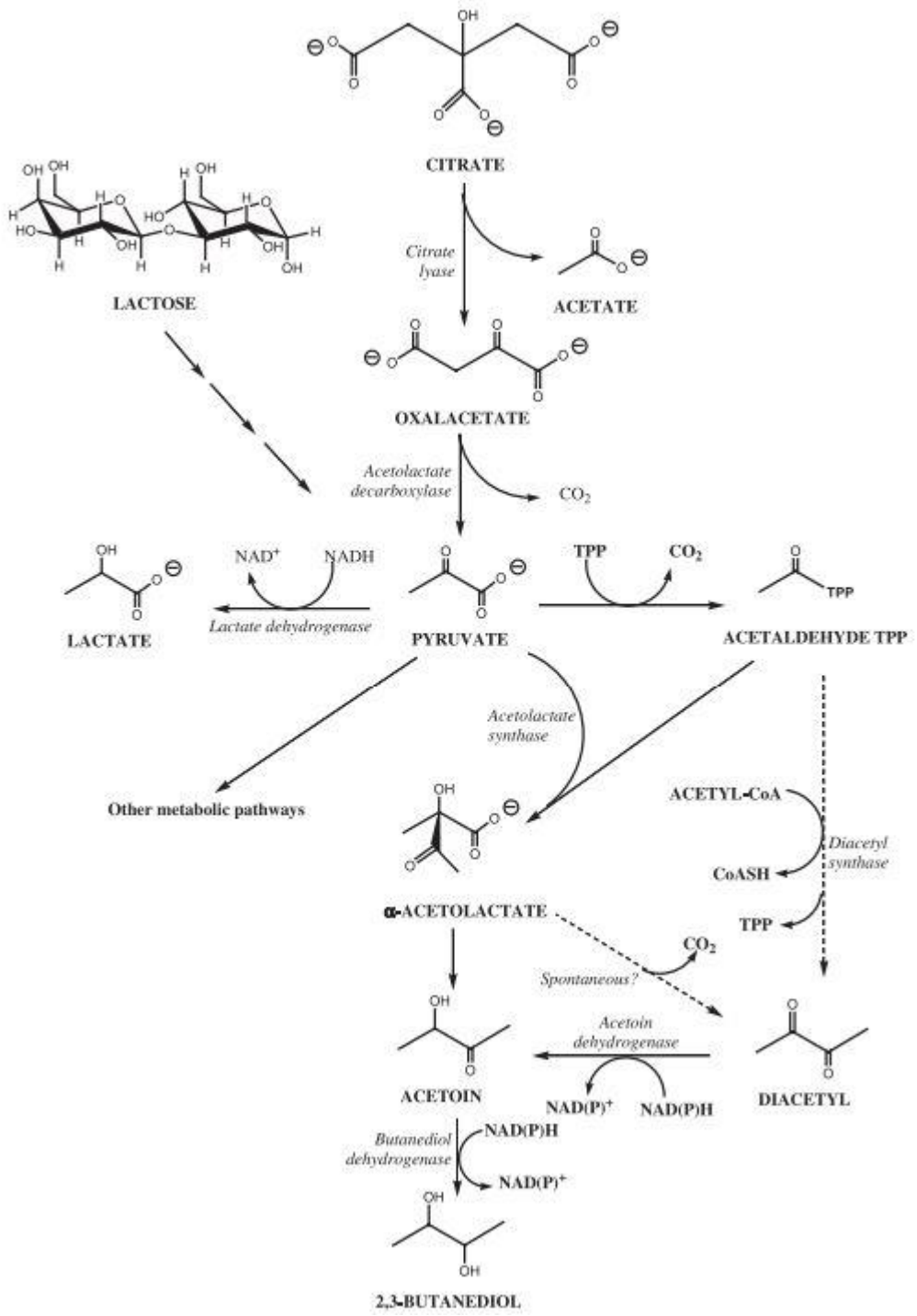


Figure 2.3 Pathway for Metabolism of Citrate (Fox et al., 2004)

2.4.2 METABOLISM OF AMINO ACID (PROTEOLYSIS)

During the manufacture and ripening of cheese, a gradual decomposition of casein occurs due to combined action of various proteolytic enzymes. These include enzymes from coagulant, milk enzymes, and bacterial proteases (Singh *et al*., 2003). In semi hard-type of cheese such as Cheddar, coagulant is responsible for the initial hydrolysis of casein. Coagulant activity mostly done to the α_{s1}-casein, with limited hydrolysis of β-casein. Subsequently, milk and starter proteases appears to be important for production of small peptides and amino acids (Fox *et al*., 1994). SLAB, especially *Lactococcus* spp. possess a very comprehensive proteolytic system. Due to possession of high level of soluble N (peptides and free amino soluble in various aqueous extracts), SLAB are believed to be capable of attacking paracasein in cheese and converting it into soluble products (Visser, 1977). NSLAB also possess a wide range of proteolytic enzymes (Atlan *et al*., 1993) and may contribute to the formation of short peptides and free amino acids during longer ripening.

Metabolism of amino acids by SLAB was most extensively studied. First step of amino acids degradation by SLAB is done with help of enzyme transaminases through transamination pathway. Breakdown of many amino acids, namely phenylalanine (Phe), tyrosine (Tyr), tryptophan (Trp), and methionine (Met) reported to produces many important flavour compounds (Gao *et al*., 1998), for example phenyl pyruvic acid. Phenyl pyruvic acid is then further degraded by lactococcal cells in vitro to phenyl lactate, phenyl acetate, and benzaldehyde which are significantly important flavour compounds in semi hard-type of cheese (Yvon *et al*., 1998).

In contrast with a normal Gouda cheese, in Remeker cheese not only LAB are the major factor of flavour development, but mold also have important role in it. Although less important than Blue-type cheese, proteolysis in surface mold-ripened cheese is quite significant, especially in raw milk-cheese (Spinnler and Gripon, 2004). A study done by Desmazeaud *et al.*, (1976) showed high production of varieties peptides and free amino acids by *P. camemberti* in Camembert cheese. This is due to high production of extracellular endo- and exo-peptidases by the mold. Those enzymes activity shows increase trends after 15 days of ripening. Thus, the presence of mold is expected to increase the proteolytic activity in Remeker cheese compare to Gouda and Camembert cheese by itself.

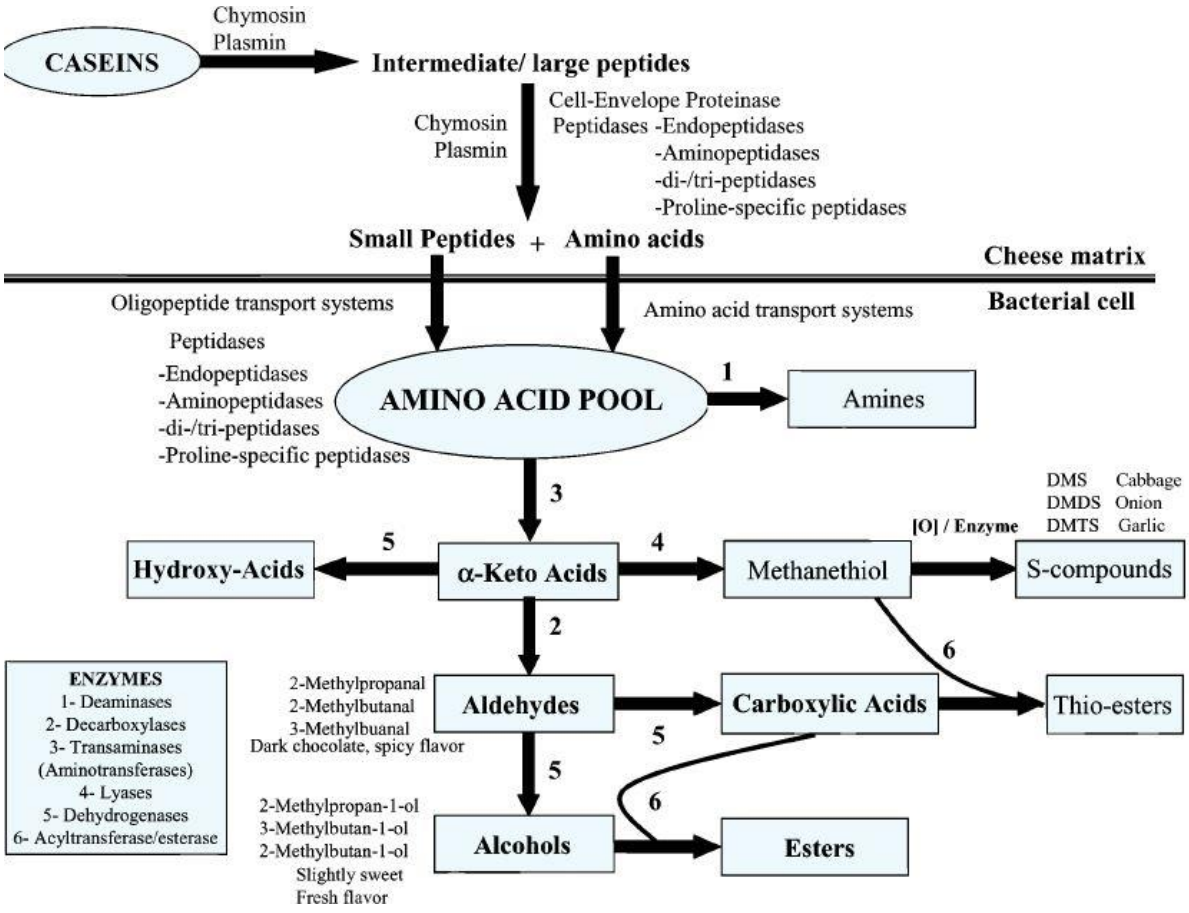


Figure 2.4 General Overview of Flavour Compounds Generation Through Proteolytic Pathway of Milk Protein
DMS = dimethyl sulfide; DMDS= dimethyl disulfide; DMTS= dimethyl trisulfide
(Modification by Singh *et al*., (2003) from Kranenburg *et al*., (2002))

2.4.3 METABOLISM OF LIPID (LIPOLYSIS)

Metabolism of lipid is induced by the present of enzyme lipases, which can be originated from milk lipase e.g lipoprotein lipase (LPL) which is an important enzyme for raw milk-cheese, rennet, or bacterial lipase. Number of esterases also contribute to the flavour development (Olivecrona *et al*., 1992). Lipids may undergo hydrolytic or oxidative degradation, although in cheese, oxidation is seldom happen due to the low oxidation/reduction potential (Fox and Wallace, 1997; McSweeney and Sousa, 2000). Oxidative degradation in cheese resulting in production of methyl ketones, which also contribute to cheese flavour, although further degradation of methyl ketones to secondary alcohol do not contribute in cheese flavour (Gripon *et al*., 1991). In all cheese varieties, triglycerides undergo hydrolysis by the action of the mentioned lipases, resulting in the liberation of most free fatty acids (FFA). It is to be note however, that low lipolysis level was desired for hard-type of cheese e.g Cheddar and Gouda. High level of lipolysis would cause rancidity taste and flavour perception, especially by LPL (McSweeney and Sousa, 2000; Collins *et al*., 2003). Although most production of fatty acid metabolism is FFA, aldehydes, and alcohol are also produced is lower amount but give significant effect to the flavour development.

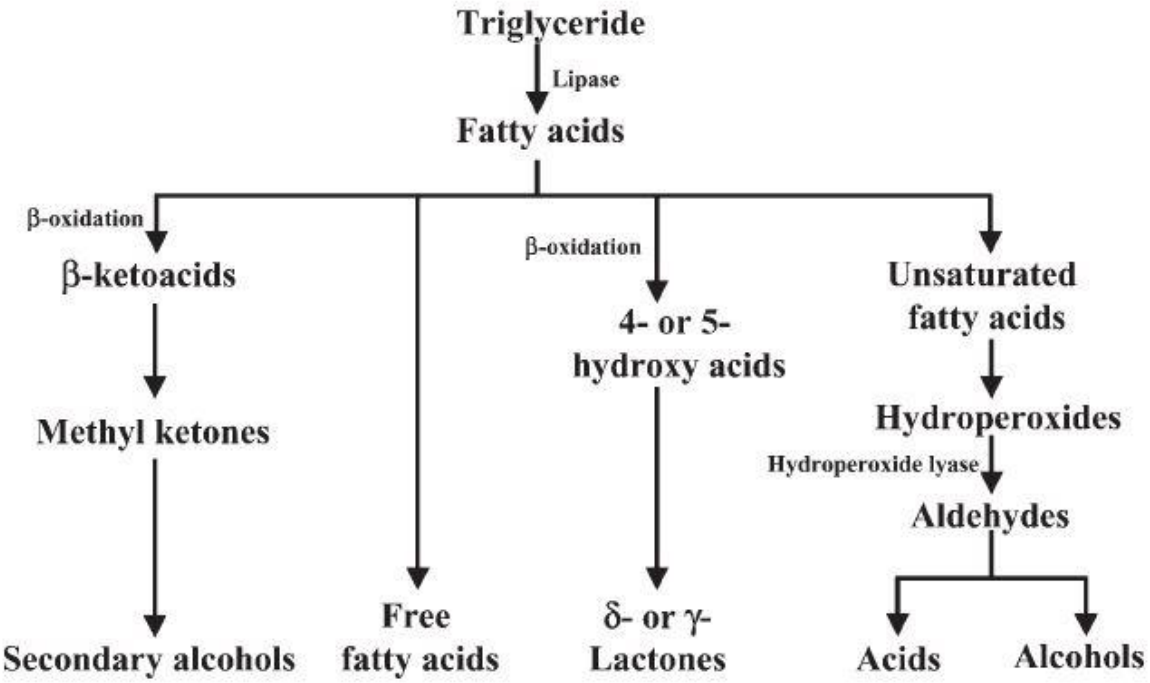


Figure 2.5 General Pathway of Fatty Acids Metabolism (Singh et al., 2003)

Lipolysis of cheese in correlation with microflora was extensively studied. Evidence showed that in bacterial-ripened cheeses such as Gouda and Cheddar made from pasteurized milk, level of lipolysis is low. Aside from the inactivation of the milk lipase, the activity of bacterial lipases are also low (Collins *et al*., 2003). Both SLAB and NSLAB possess intracellular esterolytic/lipolytic enzymes which are responsible for the liberation of significant levels of fatty acids (especially short-chain fatty acids) during the long ripening period (Fernandez *et al.,* 2000, Kamaly *et al.,* 1990; Gobbetti *et al.*, 1996). However it is known that SLAB especially lactococci strains are more weakly lipolytic/esterolytic compare to NSLAB strains (Bhownik and Marth, 1990; Chick *et al*., 1997). In general, both SLAB and NSLAB are more weakly lipolytic compare to other microflora, e.g mold, although mold enzymes still have not been studied extensively (Kman *et al*., 1966; Niki *et al*., 1966).

As mention before, intense degradation of fat is a common characteristics in mold-ripened cheese. Molds are able to secrete a large diversity of lipase. Although currently no specific explanation about their hydrolysis synthesized system, but molds can hydrolyse triglycerides more or less rapidly compare to other microflora. However those actions are highly influenced by the molecular weight of fatty acids and the pH conditions (Spinnler and Gripon, 2004). *G. candidum* preferentially liberates longer chain fatty acids, while *P. camemberti* preferentially shorter and low molecular weight fatty acids. Both molds active at pH 6.0-9.0 during ripening (Bertolini *et al*., 1995). However some FFA not only formed through lipolysis, acetic acid and propionic acid mostly degraded by the same microorganisms through glycolysis and proteolysis. Therefore, it is expected that in general, lipolysis in Remeker cheese will be higher compare to Gouda cheese. Nevertheless, in this study, ghee is not used to coat the microcheese, which will boost mold growth. Therefore less or no mold is expected to grow in microcheese.

CHAPTER 3 MATERIALS AND METHODS

3.1 OVERVIEW OF EXPERIMENTAL PROJECT

The summary of this experimental project is described in Figure 3.1.

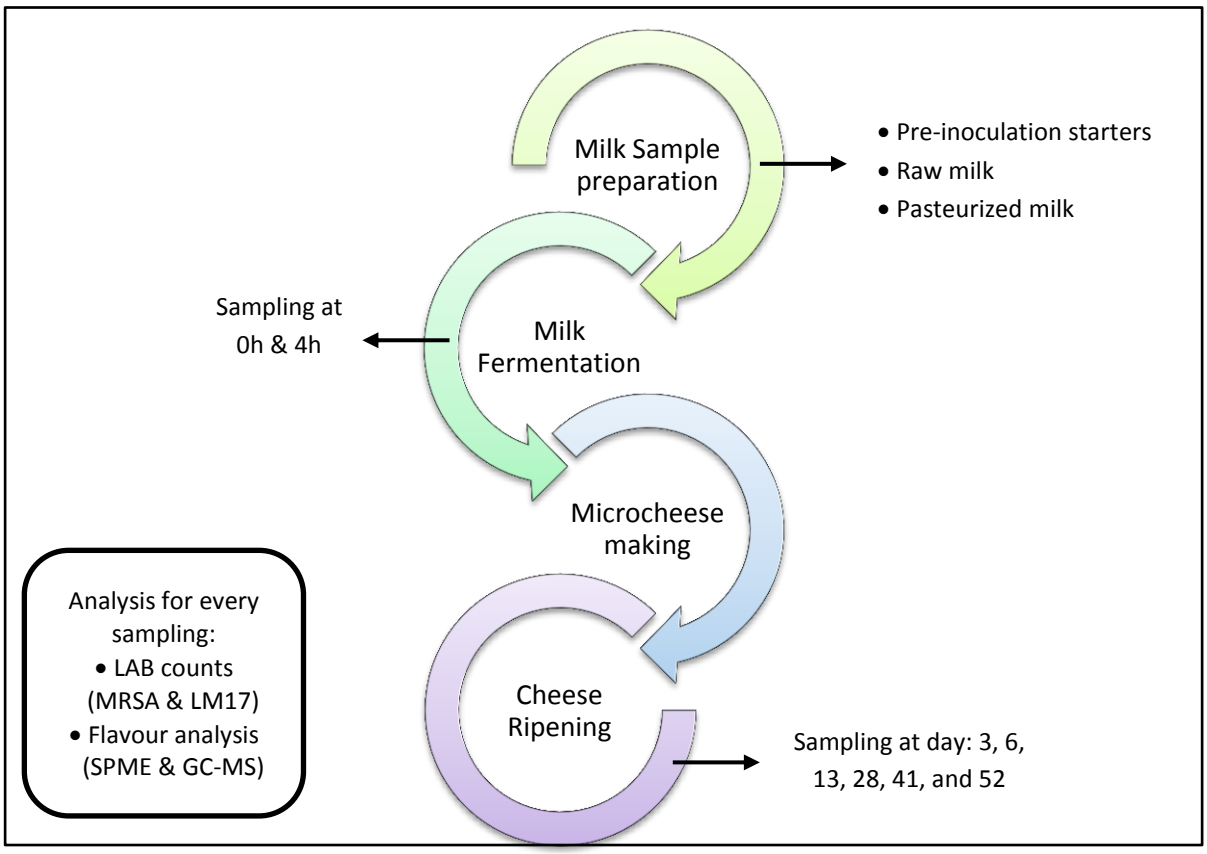


Figure 3.1 Overview of Experimental Design

3.2 SAMPLE PREPARATION

The milk sample were produced by Jersey cows from Remeker Farm. The raw milk was then pasteurized in water bath at 80°C for 30s for the making of pasteurized milk-cheese. The starter LAB used in this thesis project were nisin-producing LAB and no nisin-producing LAB. The nisin-producing starter were mixed-culture D227.7 and D447.7 (CSK, the Netherlands), which were given by Remeker Farm. The starter D447 consists of several lactococci strains, including *Lactococcus lactis* spp *cremoris* and *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*. Starter D227 description was unknown, although it is known both starter D227 and D447 have similar function and characteristic, therefore it is assumed that both starters contain similar strains of LAB. The no nisin-producing starter was starter culture C02 (CSK, the Netherlands). Starter C02 is described as LD-type of starter, although the list of LAB is unknown, it is known that the starter also include *Leuconostoc* species. The milk samples were taken for sampling after the addition of starter culture (0 hour) and after 4 hours of fermentation. Seven types of cheese were created through microcheese making. The cheese were ripened for 52 days, and sampling were done in different days until end of ripening.

Table 3.1 Design of the Sampling

Sample	Sampling Time
Milk:	
Raw milk with starter D227.7	0 and 4 h of fermentation
Raw milk with starter D447.7	0 and 4 h of fermentation
Raw milk with starter C02	0 and 4 h of fermentation
Raw milk without starter	0 and 4 h of fermentation
Pasteurized milk with starter D227.7	0 and 4 h of fermentation
Pasteurized milk with starter D447.7	0 and 4 h of fermentation
Pasteurized milk with starter C02	0 and 4 h of fermentation
Cheese:	
Made of raw milk with starter D227.7	3, 6, 13, 28, 41, 52 days of ripening
Made of raw milk with starter D447.7	3, 6, 13, 28, 41, 52 days of ripening
Made of raw milk with starter C02	3, 6, 13, 28, 41, 52 days of ripening
Made of raw milk without starter	3, 6, 13, 28, 41, 52 days of ripening
Made of pasteurized milk with starter D227.7	3, 6, 13, 28, 41, 52 days of ripening
Made of pasteurized milk with starter D447.7	3, 6, 13, 28, 41, 52 days of ripening
Made of pasteurized milk with starter C02	3, 6, 13, 28, 41, 52 days of ripening

3.3 INOCULATION OF STARTER CULTURES

The starter cultures were obtained as Direct Vat Inoculation (DVI) cultures, with D227.7 and D447.7 in a frozen pellet form and C02 in a frozen liquid form. All starters were stored at -45°C, therefore they were reactivated before use for fermentation. Approximately 10^8 CFU/mL of each starters were inoculated in a reconstituted, low-heat, 10% (wt/vol) NILAC skim milk (NIZO, Ede, the Netherlands) individually, and incubated at 20°C for 20 hours (Wemmenhove *et al.*, 2013). The milk cultures then used for raw and pasteurized milk fermentation during cheese manufacture.

3.4 MICROCHEESE-MAKING PROCEDURE

The microcheese-making protocol was modified from Bachmann *et al.*, (2009) and Smit *et al.*, (2004) for suitable condition in Laboratory. 1% of milk culture was served as the inoculum for both raw and pasteurized milks. In the first step, milk cultures were inoculated individually for each milk samples. For pasteurized milk, the milk was pasteurized first at 80°C for 30s before addition of milk culture. After addition of inoculum, both milks were fermented at 30°C, for 4 hours. Sampling were done right after inoculation and 4 hours after fermentation of milk. Uninoculated raw milk was also incubated with the same conditions. Fermentation was done longer than normal cheese making to faster the growth of NSLAB, thus the influence of NSLAB for flavour formation in cheese could be seen faster and more obvious during ripening (with special thanks for Kusuma's research, 2015).

After 4 hours, rennet (230µL/L of milk) and 33% (wt/vol) $CaCl_2$ were added to the milk samples, and the cheese making procedure followed as the microcheese making procedure (modified from Bachmann *et al.,* 2009; Smit *et al.,* 2004). 1.7 mL of milk samples were pipetted into each well of MASTERBLOCK® 96-Well Deep Well Microplates (Greiner Bio-One GmbH, Austria), and the microplates were incubated for 60 min in incubator at 30°C. After the incubation, the cutting and stirring of the curd began manually, by using a custom-made stirring device, as it can be seen in the Figure 3.2. The cutting and stirring process together took 20 min. During these 20 min, cutting/stirring was done for 20 s and resting for 3 min were alternated. The microplate then closed with a sterile Capmat (Greiner Bio-One GmbH, Austria) and centrifuged (1000x g) at 22°C for 15 min

to slightly compact the curds and facilitate whey removal. Following centrifugation, 800 µL of whey was removed from each well and replaced with 620 µL of MiliQ water at 55°C. This will gave the temperature in the wells around 36°C. Afterwards, the microplates were incubated in a water bath at 35.5°C for 40 min with stirring, while 40 s cutting and 6 min resting were alternated. The microplates were further left in water bath for additional 20 min without stirring. Subsequently, the plates were subjected to second centrifugation (3626x g) at 30°C for 75 min. This caused complete separation between curd and supernatant. The supernatant then was discarded by turning the microplates upside down for 60 min. Finally, the microplates were covered with Capmat, Microseal 'B'Adhesive Film (Bio-Rad, California, United States), and Gas Permeable Adhesive Plate Seals (Thermo Fisher Scientific, Massachusetts, USA) to ensure complete protection of the microcheeses. The microplates were stored in a cooling stove at 16°C. After overnight incubation, approximately 17 µL of a 20% sodium chloride solution (wt/vol) and 10 µL of 1% natamycin solution (wt/vol) were added to each wells. This treatment was done to ensure minimum contamination of unwanted bacteria and mould to the microcheeses. The microplates were once again stored in the cooling stove for ripening. Sampling were done during cheese ripening in every sampling points until 52 days. About 200-300 mg (approximately 1-2 microcheeses) of each microcheese samples were taken in every sampling for further analysis (Figure 3.3).

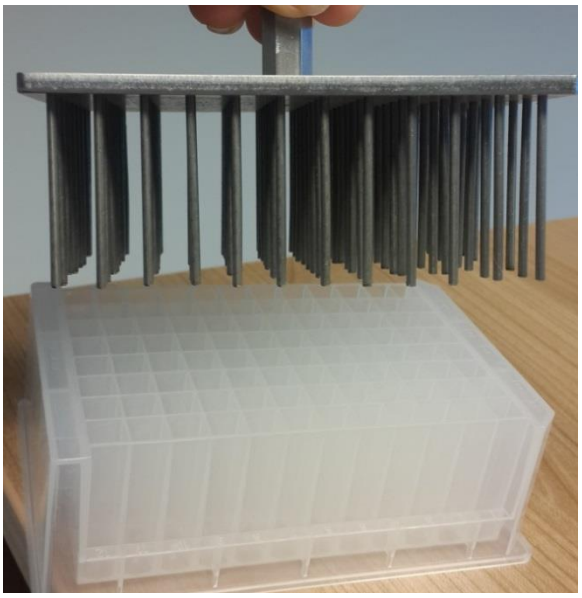


Figure 3.2 Microplate and Stirring Device Used For Microcheese Making

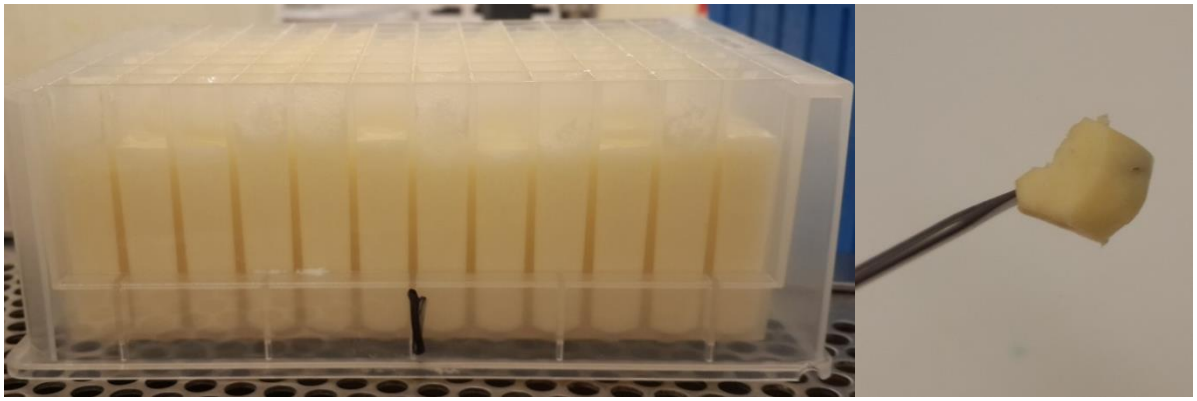


Figure 3.3 Microcheese Making Process From Milk Addition (Left) Until Microcheese Forming (Right)

3.5 LACTIC ACID BACTERIA COUNTS

Lactic acid bacteria counts followed spread plating technique. Before plating, samples and media were prepared beforehand. Two types of media were used, de Man, Rogosa, and Sharpe (MRS) agar (Merck KGaA, Darmstadt, Germany) for the presumptive lactobacilli, and M17 agar (Oxoid Ltd., Hampshire, England) (with 10% w/v lactose) for the presumptive lactococci (LM17). Both agar were poured to petri dish (brand, origin) separately and stored at room temperature until used.

LAB counts were as outlined by Teshome and Tefera (2016). For milk sample, about 1 mL of each milk samples were pipetted and in a sterile 9 mL peptone-physiological salt (PPS) solution (Tritium Microbiologie, Eindhoven, the Netherlands), and serial dilutions were made. For the cheese samples, approximately 100 mg of cheese were weighted, and placed in a sterile stomacher bag. Then 10 mL of a sterile PPS solution was added, and subsequently homogenized for 5 minutes at 230 rpm with Stomacher® 400 Circulator (Seward Ltd., Worthing, United Kingdom). This will create a dilution of 10^{-2}. 1 mL of the homogenate was then taken to create the next desired serial dilutions. Approximately 0.1 mL of each dilution was pipetted and spread evenly to the surface of the petri dish that contain media agar.

The plating were done in duplicate, and all MRSA plates were incubated at 37°C for 72 hours for lactobacili counts, while all LM17 agar plates were incubated at 30°C for 48 hours for lactococci counts (Kirmaci *et al.,* 2015). The total counts of bacteria were enumerated and the results were expressed as colony forming units (CFU) per gram of cheese. Log value was adapted for graph display in order to compare the increase or decrease of the microbiological growth in cheese.

3.6 SPME EXTRACTION

The extraction process was done to obtain volatile compounds in cheese. About 100-200 milligrams of cheese was weighed and placed in the 10 ml (46x22.5 cm) GC vials (Figure 3.4). Blank samples (MiliQ water) were also measured to check the absence of carry-over. The vials were immediately sealed with 20mm PTFE/Silicone/Silver caps. A 75 µm Carboxen/Polydimethylsiloxane (non-polar) black fibre (Bellefonte, Pennsylvania, USA) was inserted in the vials through a septum. The fibre then exposed to the headspace vial for 40 minutes. A temperature of 45°C was maintained during headspace extraction. The fibre was then transferred into the GC injection port for volatile compounds identification.

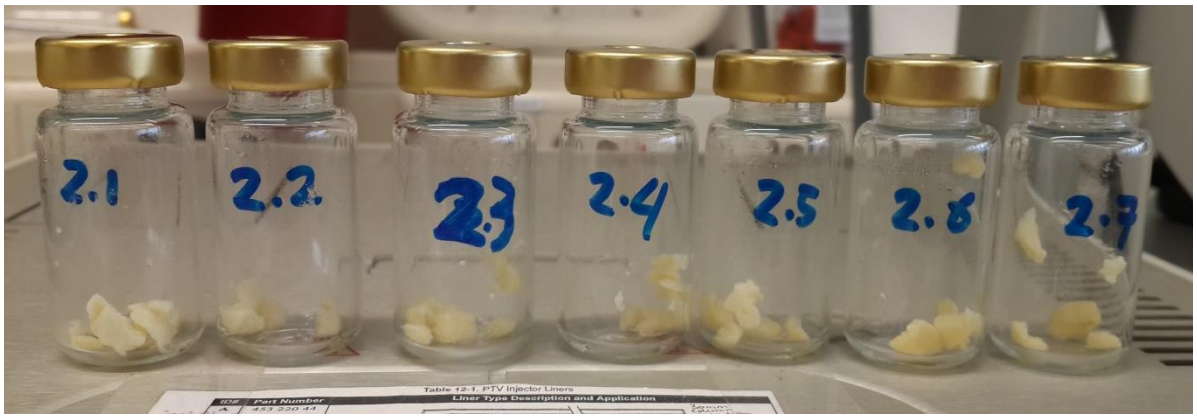


Figure 3.4 Microplate and Stirring Device Used For Microcheese Making

3.7 GC-MS ANALYSIS

GC-MS analysis was carried out to help with identification of volatile compounds in cheese samples. The fibre that transferred into GC injection port was held for 10 minutes for desorbtion proces). The temperature programming of the gas chromatograph oven started at 40°C for 3 minutes, followed by a 15°C/min ramp increase up to 220°C, maintaining this temperature for 1 minute. The injector used was the split type with manual injection, maintained at a temperature of 225°C. Helium was used as the carrier gas at a constant flow rate of 1.5 mL/min. Full scan MS was utilized instead of Selected Ion Monitoring (SIM) as the instrument analysis. The goal for full scan MS mode is to consider all peaks obtained from the GC-MS analysis, while SIM mode only considers selected peaks associated with a specific substances. When deciding the full scan MS mode, the mass fragments can be decided manually. The mass range was set at the range 33-250 m/z. The fragmentation pattern is dependant to the electron impact energy, therefore the electron ionization was applied to the system, used a typical 70 eV (electron volt). The column used was Stabilwax®-DA 30m; 0.25mmID (Restek, USA).

In order to avoid any pause and error during the GC-MS running, it is better to set one of the setting in GC-MS programme at maximum condition. The preparation run timeout (min) in Oven Section of Trace GC Ultra, should be changed to the maximum time (400 min). This setting was done to prevent the problem (analysis abruptly stopped during run) that could occur during the extraction period and Gas Chromatogram analysis.

3.8 ANALYTICAL METHOD

The eluted compounds obtained from GC-MS analysis were identified using Xcalibur software (Thermo Fisher Scientific, Massachusetts, USA) by peak integration. The resulting data sets were afterwards processed using MetAlign software programme (Wageningen UR, Wageningen, the Netherlands). The peaks were aligned and annotated using MetAlignID. The resulting peak list was further processed with AMDIS32 software (NIST, Gaitherburg, MD, USA) in combination with library files (provided by Hettinga, 2008), in order to sum up the important volatile compounds and their retention times. The compounds and their retention times (min) were then created using Microsoft Excel file (as Comma Delimited CSV file). The GC-MS library was then created based on the Excel file to obtain the value for each compounds. In the last step, the value for each compounds was obtained from “Macro For MetAlignID Using Ret File Output” macro excel (.xlsm) file after processing it. From the macro file, the final %TIC and TIC value was obtained. The TIC value was interpreted as volatile compound’s concentration.

Better step-by-step of how to analyse GC-MS data was explained well through “Handbook for GCMS analysis” protocol (provided by Rijswijck, 2015). Some addition important things to note about processing GC-MS data:

- Before run the MetAlign program, it is better to prepare this following folders first to avoid any confusion: Parameters, Raw data, Output 10A, Output for MetAlignID, Output MetAlignID, and Output Macro folders.
- During the compilation of the compounds using AMDIS32 programme, the chosen compounds were based on: Weighted of match should be higher than 70, and R.Match factor should be higher than 700-800.

CHAPTER 4 RESULTS AND DISCUSSION

4.1 CHANGES OF THE EXPERIMENTAL DESIGN

As stated in "Chapter 3 Materials and Methods" seven samples were originally prepared, which consisted of 4 raw milk-cheese and 3 pasteurized milk-cheese samples. However, the pasteurization step was not done properly, as it did not reach the desired temperature of 80°C after 30s of heating. It led all pasteurized milk-cheese analysis (both LAB counts and GC-MS) to have similar results with raw milk-cheese. A test analysis was performed to show that there should be an effect to the LAB growth, had the pasteurization was done properly. The results can be seen in Appendix 1, which shows the expected LAB counts before and after proper batch pasteurization (63°C for 30 min) treatment to the raw milk. The results showed that before the pasteurization, about 3 log of LAB was detected in MRSA, and after pasteurization no LAB was detected. Therefore, only the results of four samples of raw milk-cheese were decided to be displayed and discussed without the other 3 pasteurized milk-cheese samples.

Table 4.1 New Design of the Cheese Experiment

Sample no.	Sample	Sampling Time
Milk:		
1	**Raw milk with starter D227.7**	0 and 4 h of fermentation
2	**Raw milk with starter D447.7**	0 and 4 h of fermentation
3	**Raw milk with starter C02**	0 and 4 h of fermentation
4	**Raw milk without starter**	0 and 4 h of fermentation
Cheese:		
1	**Made of raw milk with starter D227.7**	3, 6, 13, 28, 41, 52 days of ripening
2	**Made of raw milk with starter D447.7**	3, 6, 13, 28, 41, 52 days of ripening
3	**Made of raw milk with starter C02**	3, 6, 13, 28, 41, 52 days of ripening
4	**Made of raw milk without starter**	3, 6, 13, 28, 41, 52 days of ripening

4.2 MICROBIOLOGICAL GROWTH EVALUATION IN RAW-MILK CHEESE

The growth of SLAB and NSLAB were monitored from cheese-milk fermentation until the end of ripening (days 52) through bacterial count with spread plate technique. Two microbiological media were used for the bacterial counts, MRS agar, and M17 agar (10% lactose) (LM17). It was mentioned in "Materials and Methods" section that MRSA had been used for lactobacili counts, which could represent most NSLAB strains, while LM17 had been used selectively for lactococci counts, which represent the SLAB. Although different incubation temperatures was implemented during experiment (37°C for MRSA, 30°C for LM17) to prevent the growth of lactococci strains in MRSA, it does not eliminate a chance for other LAB and microflora, such as yeast, to grow in MRSA. Therefore, it is possible that some MRSA results might give unexpected results. LAB growth in MRSA during cheese ripening can be seen on Figure 4.1.

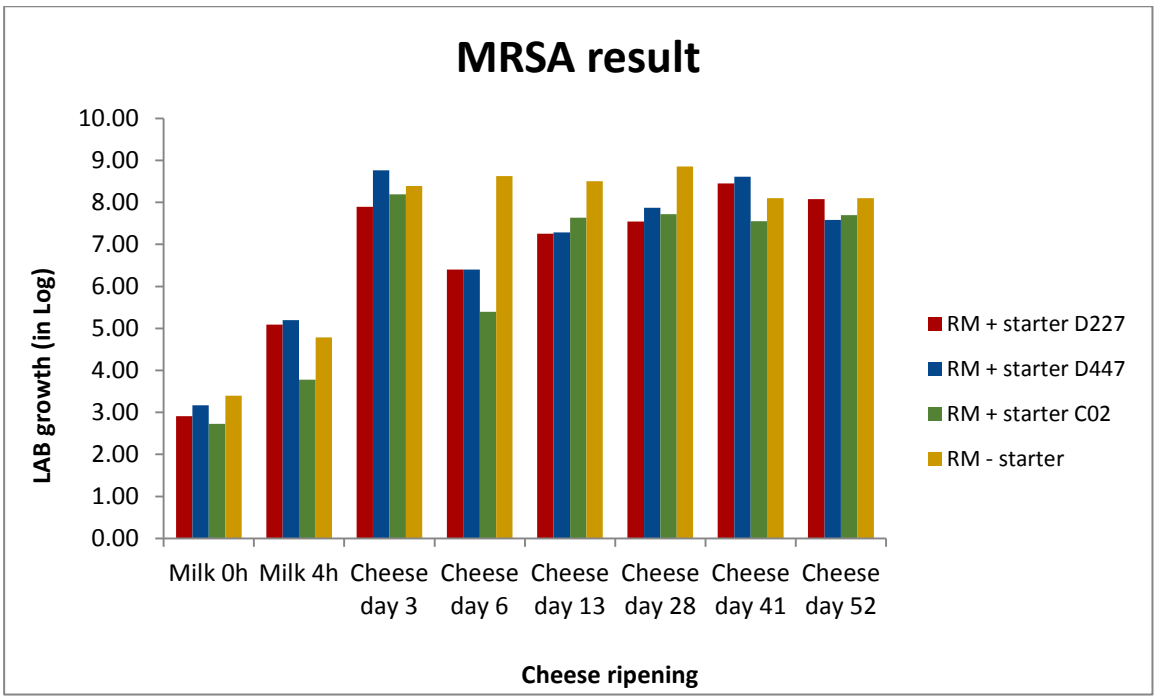


Figure 4.1 LAB Growth in MRSA During Cheese Ripening

Microorganism counts in MRS agar showed that there were a huge increase of LAB even after 4 hours of milk fermentation and during early of cheese making. Before further explanation about the result of LAB growth in MRS agar, there was an unexplainable problem of LAB growth in the 3-day-old cheese. Except the result in day 3, the graph showed a steady increase of LAB growth during 52 days of cheese ripening. It is assumed that the LAB growth should be in line with the concentration of volatile compounds. However there is no indication of unusual trendline of volatile compounds concentration during 3 days of cheese ripening (referred to Figure 4.5). Therefore it is believed that the cause of the sudden growth of LAB in 3-day-old cheese is the error during LAB analysis.

The first two samples (with addition of starter D227 and starter D447, respectively) have similar trends of LAB growth. Both trends showed steady increase during cheese ripening from 3 log in the beginning of milk fermentation, and increased until about 8 log in 52-day-old cheese. Cheese with starter C02 started from the lowest LAB amounts during milk fermentation, but during cheese ripening, the growth increased in fast-paced and achieved similar results of LAB growth with other cheeses at the end of ripening. Cheese without starter achieved the fastest growth of LAB, as it already reached 8 log during 6 days of ripening, continued with slow fluctuation until the end of ripening.

The similar results from the first two cheeses (with starter D227 and D447, respectively) were as expected because both starters were expected to contained similar LAB strains and similar functions. The high growth of LAB in cheese with starter C02 was expected. As previously mentioned that starter C02 consists of LD-type LAB with addition of Leuconostoc strains, it is assumed that more lactobacili strains presented in the starter of this cheese compare to cheese with starter D227 and D447 that has already been known to contained only lactococci strains. Hence, the high number of LAB detected in MRSA for cheese with starter C02 was expected.

The fast growth of LAB in cheese without starter compare to other cheeses could be the indication of competition between microflora presented in the cheeses. Starter culture, especially *Lactococcus lactis*, could grow rapidly during the initial cheese making, utilize lactose and carbon-source as nutrition, and produce acid in cheese (Irlinger and Mounier, 2011). The decrease of pH, lack of food source, and dominant presence of starter put the other acid-intolerant, non-starter microorganisms in unfavorable condition, and as they unable to compete with the starter, the number decrease significantly (Mounier *et al.*, 2008). With cheese without starter addition, the acid production and the competition between microorganism was slow, therefore many non-starter microorganisms grew well. And as stated previously, although MRSA is a media used for LAB growth, other microorganisms could also grow in it, leading to higher overall counts. Figure 4.2 showed number of species grew in the petri dish of cheese sample without starter, although further identification of the microorganisms is needed to validate the presumption.

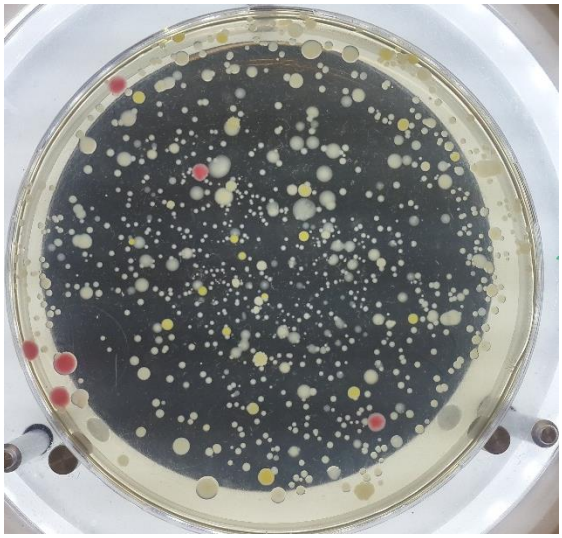


Figure 4.2 Various Microorganisms Detected in MRSA

The bacterial counts in LM17 is presented on Figure 4.3.

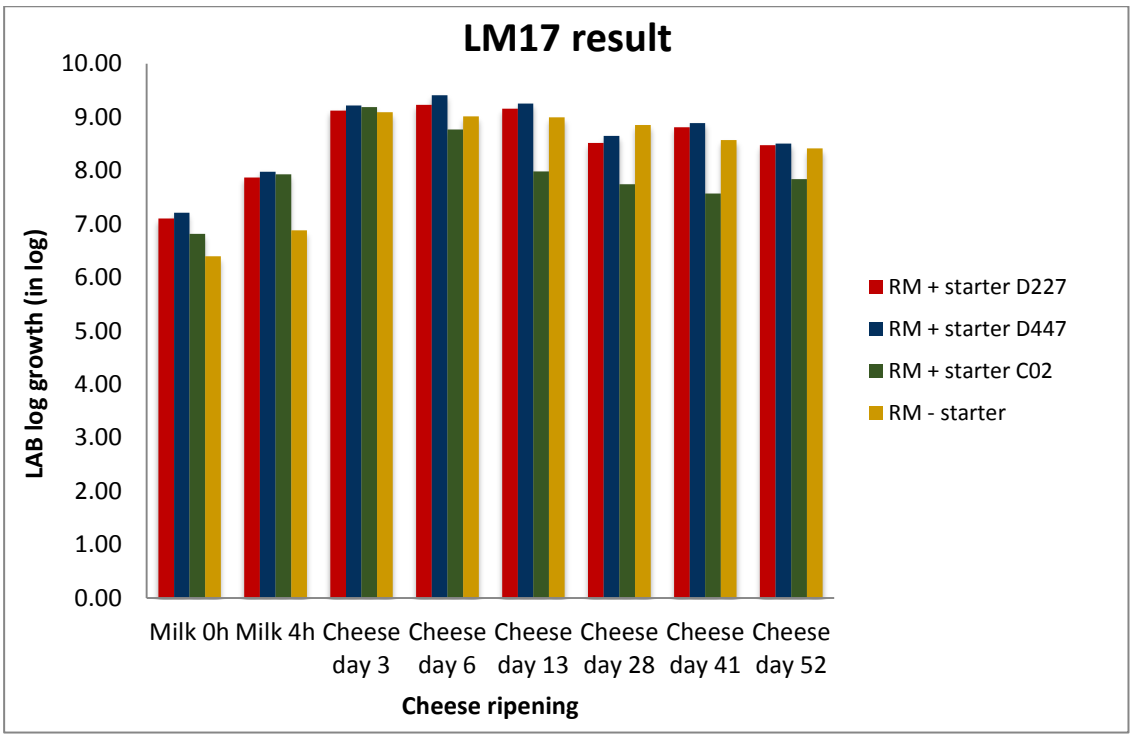


Figure 4.3 LAB Growth in LM17 During Cheese Ripening

Results showed that there was already high number of lactococci strains in early of milk fermentation with addition of starters (7 log) and without starter (6 log). After 3 days of cheese ripening, there was an increase by 2-3 log cycles, but then a slow steady decrease by 1-2 log cycles at 52-day-old cheese. This results were expected as according to research conducted by Zago *et al*. (2007) and Franciosi *et al*., (2008), that population of SLAB are high in number during the early of cheese ripening process, but will gradually decrease during aging.

The highest amount of LAB was identified in cheese with starter D227 and D447. The results were expected, as the starter used were dominated with lactococci strains. The fast decrease of lactococci amount in cheese with starter C02 after 3 days of ripening was assumed to be the effect of competition between microflora. The reason can be explained by comparing lactococci counts in milk with starter C02 and milk without starter, and analyzing the trendline of the LAB counts in MRSA (Figure 4.1). It is noted that in the beginning, lactococci amounts presented in milk with starter C02 (6.8 log) was only slightly higher compare to milk without starter (6.4 log), therefore it is assumed that only small amount of lactococci strains presented in starter culture C02. And by looking at Figure 4.1, it can be concluded that in cheese with starter C02, lactobacili strains dominated the growth, and therefore suppressed the growth of other microflora, including the lactococci strains.

Cheese without starter addition was considered to have a high amount of LAB in LM17 media. Casey *et al*., (2006) stated that other than mesophilic lactobacili and *Leuconostoc*, pediococci and enterococci are also two main NSLAB found in cheese. This could be the idea why LAB amount in LM17 media in cheese without starter was high.

Overall, the growth of NSLAB and SLAB in their perspective medium, MRSA and LM17, were as expected. The number of NSLAB started in low amount, followed by significant increase during 52 days of cheese ripening, while the SLAB amount started in high amount, and increase during 6

days of ripening, before slowly decreased for the rest of the ripening. Cheeses with the first two starters were expected to give similar trendline of LAB growth. It is suspected for cheese with starter C02 to contain more lactobacili strains than lactococci strains, hence high growth in MRSA. And high amount of LAB detected for cheese without starter in both medium were assumed to be the growth of other non-LAB microorganism.

4.2.1 NISIN INFLUENCE TO THE GROWTH OF NSLAB AND SLAB IN CHEESE

Nisin activity analysis was not conducted in this experiment, therefore to see the effect of nisin to the growth of both NSLAB and SLAB in cheese, standard deviation error bar was implemented. Standard deviation error bar was made based on each samples in relations to the average LAB counts during 52 days of ripening. The average LAB was counted started from milk until the end of ripening. Day 3 of ripening result was not included in the graph, as it was previously mentioned that the data was assumed as invalid.

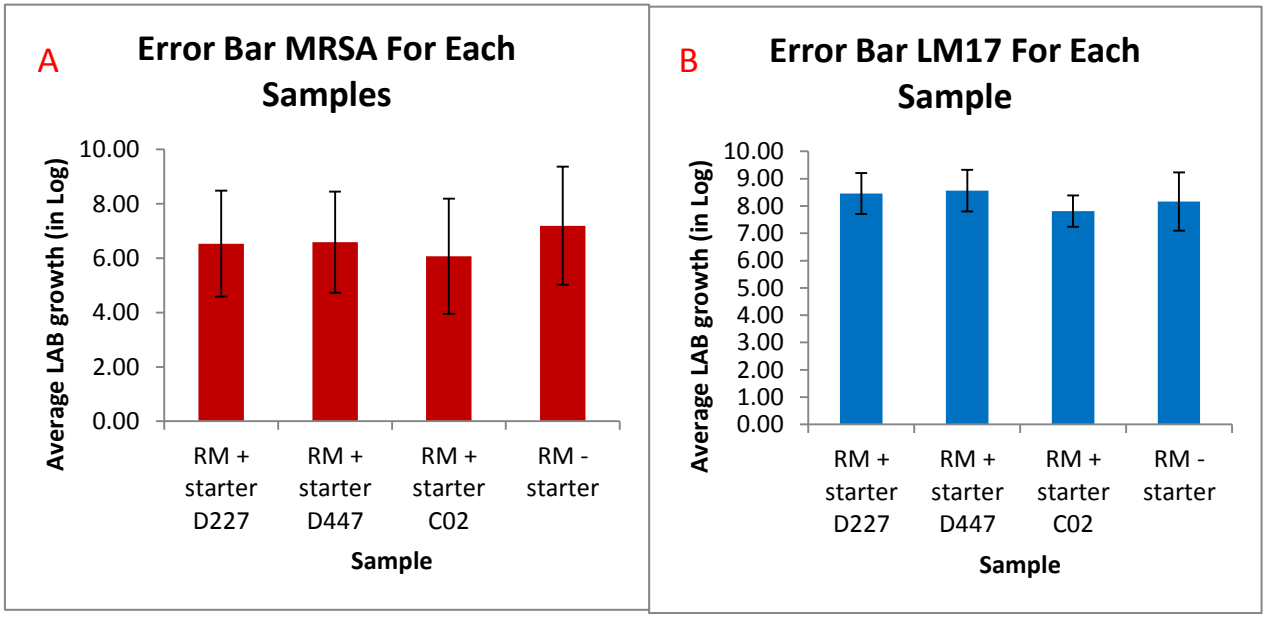


Figure 4.4 Standard Deviation Error Bar of LAB Growth In MRSA and LM17 Media

Graph (A) and (B) show standard deviation of all samples based on the average of LAB counts. By ignoring the result from day 3, the average of LAB counts were obtained from the average of LAB counted from milk 0h until day 52 of cheese ripening.

Standard deviation of MRSA showed no significant different of the average LAB counts in all cheese samples. Standard deviation of LM17 showed no significant difference of total LAB counts between cheese with starter D227, D447, and cheese without starter, while slight difference in cheese with starter C02. Both MRSA and LM17 results showed lowest total viable LAB counts in cheese with starter C02.

Both starters D227 and D447 were expected to have similar trendline and highest amount of LAB in LM17. It was already known that starter D447 consists of *L. lactis* subsp. *lactis*, and *L. lactis* subsp. *cremoris,* which has been known of their high tolerance againts nisin (Benech *et al*., 2002). Therefore no or little effect of nisin to the growth of lactococci strains in cheese with starter D227 and D447 was expected, thus proving the fast growth of SLAB.

Despite the fast growth of SLAB in cheese with starter D227/D447, it was expected for both cheeses to have lower amounts of LAB growth in MRSA, which means lower growth of NSLAB. No significant difference found between cheese with starter D227/D447 and starter C02 in MRSA suggested that either most NSLAB presented in those cheeses are nisin-tolerant, or the nisin activity was too low to inhibit the NSLAB (Rossi and Veneri, 2015; Benech *et al.*, 2003). In conclusion, there was no nisin inhibition could be found to the NSLAB in this cheese experiment.

4.3 FLAVOUR COMPOUNDS IN RAW-MILK CHEESE

The GC-MS and MetAlign's profiling of the raw milk and raw milk-cheese resulted in the total identification of 46 volatile compounds, as shown in the Table 4.2. However, due to some identified compounds did not reach the standard criteria for mass spectral matching, only 23 of all 46 compounds are going to be presented in this report, and the rest will be excluded..

Table 4.2 List of Volatile Composition Identified in Raw Milk-cheese Based on Their Chemical Class and Aroma Note (van Leuven et al., 2008; Molimard and Spinnler, 1996; Zheng et al., 2012)

Volatile Compounds	Aroma Note
Alcohol	
1-Hexanol	fatty, floral
1-Butanol	floral, fragrant, fruity
1-Pentanol	fruity
Ethanol	mild, etheric, alcohol
3-Pentanol	fruity
2-Propyn-1-ol	-
2-Propanol, 1-methoxy- (1-Methoxypropan-2-ol)	-
1-Butanol, 3-methyl- (Isoamyl alcohol)	fruity, alcohol, solvent-like, grainy
Linear Aldehydes	
Hexanal	-
Alkene (Cyclic aromatic compounds)	
1-Butene	-
Hexane, 2,4-dimethyl- (2,4-Dimethylhexane)	-
1,3-Butadiene, 2-methyl- (Isoprene)	sweet, floral, rose-like
Limonene	citrus
Styrene	plastic-like
Branched-chain and aromatic aldehydes	
Pentanal, 2-methyl- (2-Methylpentanal)	-
Benzaldehyde	almond
Benzeneacetaldehyde (Phenylacetaldehyde)	rosy
Acetic acid	vinegar
Acetaldehyde	sweet, pungent
Ketones	
2-Heptanone	fruity, musty, soapy, identical in blue cheese
2-Pentanone	acetone, sweet, fruity
Acetone	-
2-Nonanone	green, earthy, fatty, fruity, musty, identical in blue cheese
2-butanone	etheric
2-Butanone, 3-hydroxy- (acetoin)	buttery

2,3-Butanedione (diacetyl)	buttery
2-Propanone, 1-hydroxy- (Hydroxyacetone)	pungent, sweet, caramellic, etheric
Free Fatty Acids	
Butanoic acid	cheesy
Pentanoic acid	cheesy, identical in swiss cheese
n-Decanoic acid	rancid
Propanoic acid	-
Hexanoic acid	pungent, identical in blue cheese
Octanoic Acid	wax, soapy-like, musty, fruity, rancid
Nonanoic acid	-
Heptanoic acid	-
Other Carboxylic group	
Acetic Acid	vinegar, pungent
Branched-chain volatile acids	
Butanoic acid, 3-methyl- (3-Methylbutanoic acid)	waxy, old, identical in swiss cheese
Butanoic acid, 2-methyl- (2-Methylbutanoic acid)	-
Propanoic acid, 2-methyl- (2-Methylpropanoic acid)	sweat-like
Octane, 3,5-dimethyl- (3,5-Dimethyloctane)	-
Esters	
2-Propenoic acid, butyl ester (Butyl acrylate)	sweet, fruity
Sulfur compounds	
Dimethyl sulfide	sulfurous, cabbage-like
Disulfide, dimethyl	sulfurous, onion-like
Dimethyl sulfone	-
Amine (nitrogen compounds)	
Pyrrolidine	ammoniacal, fishy, shellfish-like and seaweed-like
Azetidine	-

Total TIC value of selected volatile compounds was created to see differences of concentration before and after cheese ripening.

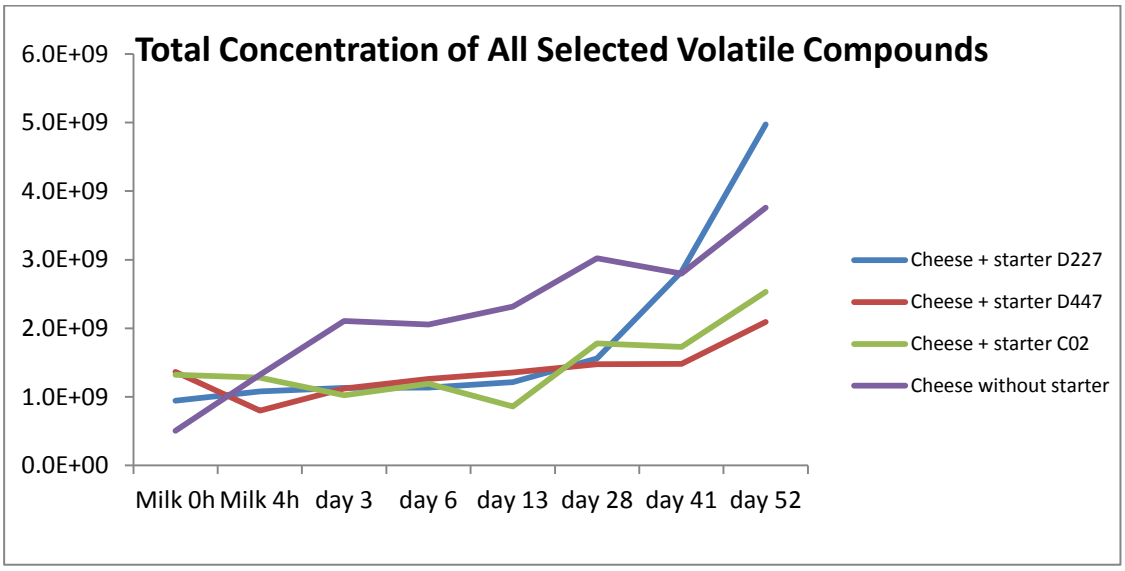


Figure 4.5 Total TIC Value of All Volatile Compounds During Cheese Ripening

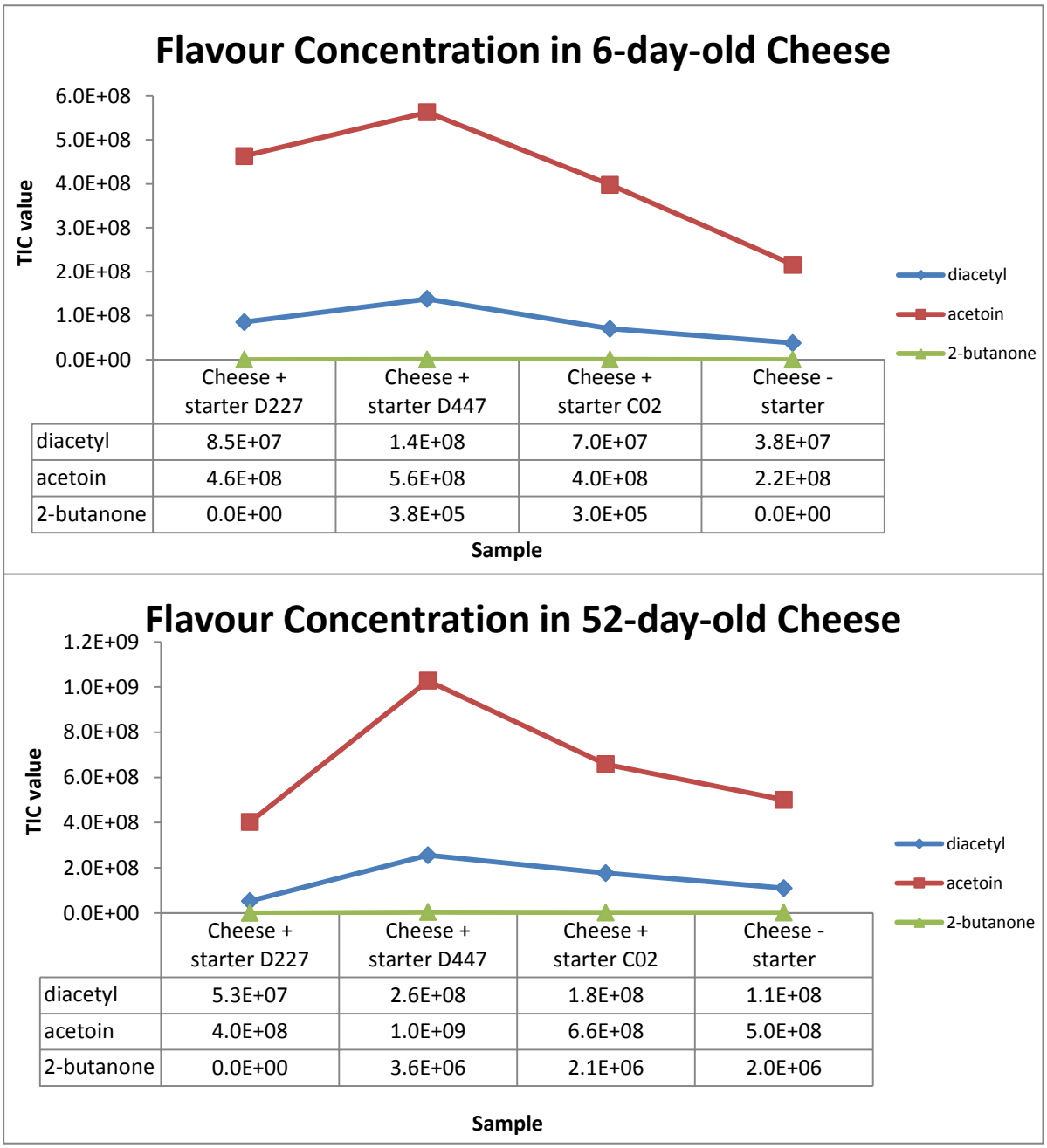
The total TIC from the beginning of all milk fermentation (milk 0h) started in a similar trendline. The similar overall trendline from all cheeses during ripening was also observed, although it can be seen that cheese without starter demonstrated higher concentration of volatile compounds compared to other cheeses. The steady increase of volatile compounds was also mentioned by previous study of Chen (2016), where she mentioned that after 54 days of ripening, there was an average increase of 2-fold in total volatile compound in Remeker cheese. Both the increase and the total concentration of volatile compounds (which is 10^9) in this project have similar value with Chen's result.

Another thing to note is after 28 days of ripening, the concentration of volatile compounds in cheese with starter D227 started to increase faster than cheese with starter D447. After investigated the individual trendline of volatile compounds (Figure 4.7-4.14), an interesting discovery was found between cheese with starter D227 and cheese without starter. The fast increase of cheese with starter D227 after 28 days of ripening, and overall high concentration of volatile compounds in cheese without starter during ripening were caused by same compound group, which is free fatty acid (FFA). This indicated that cheese without starter produced higher overall amount of FFA. The further explanation is described in Figure 4.12. Further explanation about higher concentration of FFA in cheese with starter D227 compare to cheese with starter D447 after 28 days of ripening is better explained in section 4.3.4.

4.3.1 METABOLISM OF LACTATE AND CITRATE

The metabolism of lactose into lactate is one of the most important metabolism pathway to create distinctive flavour of all cheese varieties. Lactate can be derived into producing many important flavour compounds such as acetate, ethanol, and acetaldehyde (McSweeney and Sousa, 1999).

Co-metabolism of lactose with citrate will also produce several important flavour compounds in cheese, resulting in the formation of diacetyl, acetoin, 2,3-butanediol, and 2-butanone (Cogan, 1985). The concentration of diacetyl, acetoin, and 2-butanone from 6-day-old and 52-day-old cheese were compared to see the interaction between those compounds over time of ripening.



	Cheese + starter D227	Cheese + starter D447	Cheese + starter C02	Cheese - starter
diacetyl	8.5E+07	1.4E+08	7.0E+07	3.8E+07
acetoin	4.6E+08	5.6E+08	4.0E+08	2.2E+08
2-butanone	0.0E+00	3.8E+05	3.0E+05	0.0E+00

	Cheese + starter D227	Cheese + starter D447	Cheese + starter C02	Cheese - starter
diacetyl	5.3E+07	2.6E+08	1.8E+08	1.1E+08
acetoin	4.0E+08	1.0E+09	6.6E+08	5.0E+08
2-butanone	0.0E+00	3.6E+06	2.1E+06	2.0E+06

Figure 4.6 Comparison of Diacetyl, Acetoin, and 2-butanone Concentration in 6-day-old Cheese With 52-day-old Cheese

The trendline production of diacetyl, acetoin, and 2-butanone in all cheeses except cheese with starter D227, were expected. The trend line shows increase in all 3 compounds during ripening, although 2-butanone have relatively higher increase (10-fold larger) of concentration compare to acetoin and diacetyl (2-fold larger). Dimos *et al.*, (1996) stated that diacetyl will be further converted to acetoin, 2,3-butanediol and then further into 2-butanone during cheese ripening. Low concentration of 2-butanone suggested further ripening time is needed to confirm the metabolism of diacetyl. What could be the unexpected result was the decrease of acetoin and diacetyl, while no production of 2-butanone during ripening of cheese with starter D227. The reason was unclear, as it was expected for both cheese with starter D227 and D447 to have similar starter cultures, hence similar production of volatile compounds should be obtained.

So, in general, cheese with SLAB addition, especially the ones that contained lactococci strains (D447) produced higher amount of diacetyl, acetoin, and 2-butanone compare to cheese without starter addition, which is expected. However the low amounts of all compounds in cheese with starter D227 was not expected, and the reason remains unknown. Higher amount of acetoin compare to diacetyl also as expected. Lower amount of acetoin was also expected, as it needs further ripening time for it to be produced. Further explanation will be explained in a separate discussion about each compounds.

2,3-BUTANEDIONE / DIACETYL

2,3-butanedione, or usually called Diacetyl, is an important flavour compounds in many cheese varieties, especially Dutch-type cheese, gives a 'buttery note' aroma to the cheese (van Leuven *et al*., 2008). Diacetyl is mainly derived from citrate metabolism by specific LAB strains, *Lactococcus lactis* subsp*. lactis* biovar *diacetylactis* and subsp. *cremoris*, and mixed-culture of *Leuconostoc* spp. (Singh *et al*., 2003; van Leuven *et al*., 2008). Several strains of mesophilic lactobacilli, such as *Lactobacillus viridescens, Lactobacillus fermenti*, and *Lactobacillus plantarum*, could also metabolize citrate to produce diacetyl (Drinan *et al*., 1976). Those lactobacili strains might present in cheese as part of NSLAB or from starter culture. Diacetyl is considered to be produced in small amounts compare to other metabolites from citrate metabolism, but it is considered to be very important.

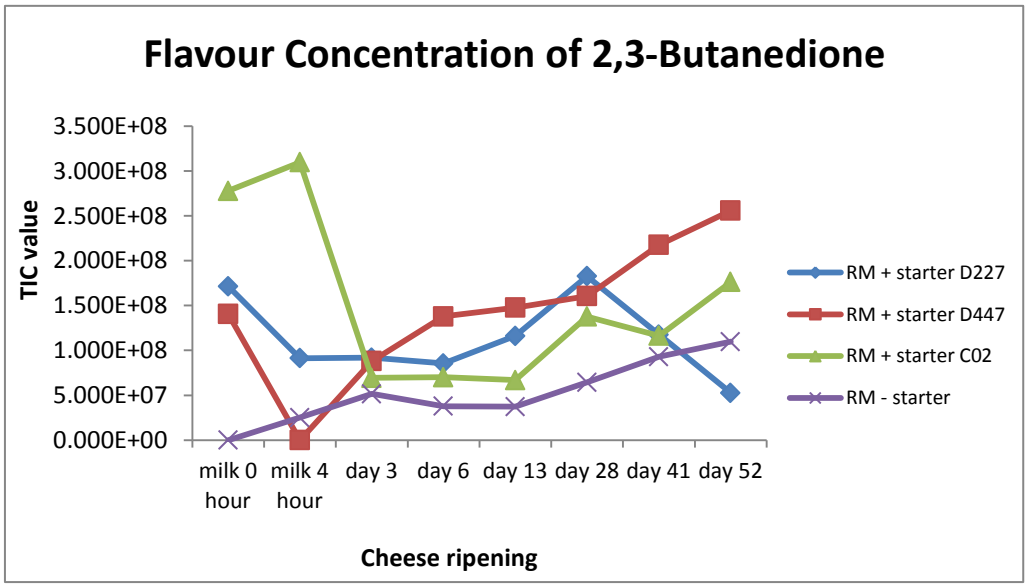


Figure 4.7 2,3-Butanedione Quantification During Cheese Ripening

Rapid decline of diacetyl production was noted during milk fermentation, and gradual increase during cheese ripening. In fresh-made cheese, it was noted that the flavour intensity of diacetyl was 10^7, and except cheese with starter D227, other cheeses were observed to have an increase until 10^8 (Figure 4.7). Neither the results nor the trends were as expected. First of all, lower concentration were expected to be present in milk before fermentation and then an increase during cheese-making. It is also expected that after early of cheese ripening, the amounts of diacetyl will decrease as it should be converted into other flavour compounds. There can be few possibilities to this phenomenon. As it is known, diacetyl is metabolized further into other compounds through citrate metabolism, with the help of LAB strains. This could be the reason of the decrease of diacetyl during fermentation of milk. Physical factors, such as shaking, and

aeration might also be the reasons of the lack amounts of diacetyl production. Aerobic condition is needed to increase the formation of diacetyl. Inconsistencies trends of diacetyl production was probably the result of volatilization. According to van Leuven *et al*., (2008), diacetyl is a compound with high volatilization. The compound might volatized during the manufacture of cheese or during GC-MS analysis.

Comparison between type of cheese with different starters were as expected. The cheese with starter D227 and D447 were expected to have higher amount of diacetyl compare to cheese with starter C02. Cheese without addition of starter also recorded with the lowest amount of diacetyl. As stated by McSweeney and Sousa (1999), diacetyl is formed mainly by culture of lactococci strains. Pure cultures of *Leuconostoc* spp. do not produce diacetyl. The same reason is implemented to the cheese without starter, as only few lactobacili strains could produce diacetyl. Starter D227 and D447 consist of lactococci strains, while starter C02 consist of mixed culture of lactococci and lactobacilli strains, therefore it is assumed that cheese with starter D227 and D447 could produce higher concentration of diacetyl.

In conclusion, higher diacetyl concentration in cheese with starter D227 and D447 in general compare to other cheeses were expected as the work of SLAB, especially lactococcis strains. It was suggested that lactococci strains possess the required enzyme to metabolize citrate into diacetyl. A further degradation of diacetyl into other compounds or volatilization could be the reason why diacetyl production was lower after milk fermentation.

2-BUTANONE-3-HYDROXY / ACETOIN

Another example of citrate metabolism, 2-butanone-3-hydroxy or acetoin, is a compound that belongs to methyl ketone group. It has similar aroma note, with 'buttery' odour (van Leuven *et al*., 2008). Acetoin is a resulted metabolite from diacetyl metabolism by acetoin dehydrogenase. Although acetoin can be form through diacetyl metabolism, the main precursor of acetoin formation is pyruvate. Pyruvate is metabolize into acetolactate and further degraded into acetoin by enzyme acetolactate synthase (Gunsalus, 2012; McSweeney, 2004).

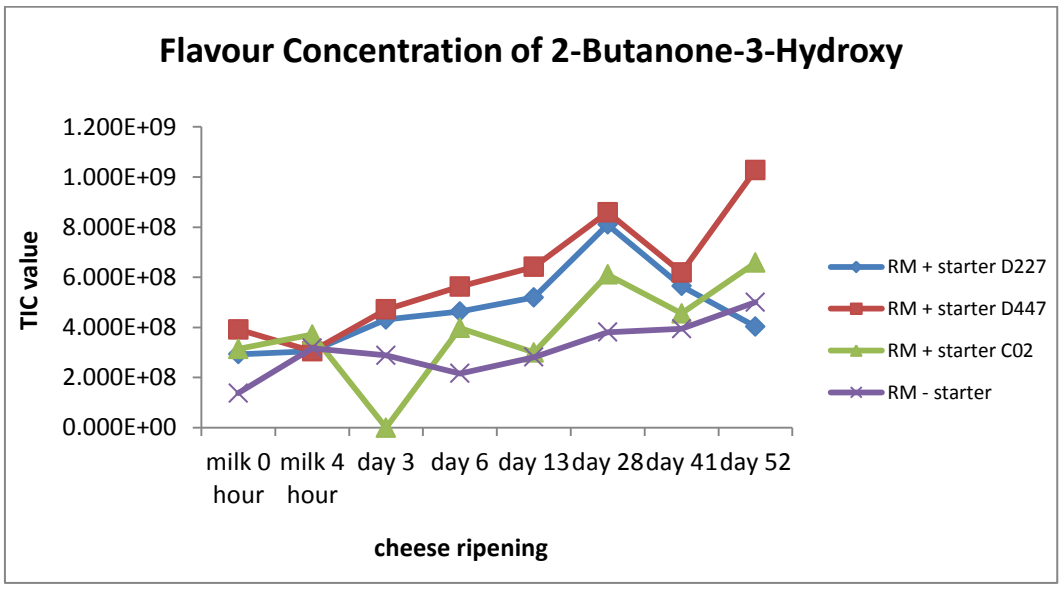


Figure 4.8 2-Butanone-3-Hydroxy Quantification During Cheese Ripening

Figure 4.8 shows the concentration value of acetoin. Acetoin amounts were in par with acetic acid as the highest volatile compounds detected in the raw milk-cheese, make up until 57% (maximum amounts detected) of the total volatile compounds found in the cheeses. Highest amount of acetoin was recorded from cheese with starter D447, accumulating 10^9 at 52 days of ripening. The results were as expected, with cheese with starter D227 and D447 have similar trendline (although different at later ripening time) and higher compared to cheese with starter C02. Cheese without starter have lowest amount of acetoin, accumulating $5x10^8$ at 52 days of ripening. Similar to diacetyl, acetoin production is influenced by starter LAB. Starter culture which include *L. lactis* strains produce high amounts of acetoin, while pure culture of *Leuconostoc* spp. do not produce acetoin, although decent amounts could be produced with combination starter of *Leuconostoc* spp. and *Lc. lactis* strains McSweeney and Sousa (1999). Thus, higher acetoin concentration of acetoin in cheese with starter D227/D447 than C02 was expected.

ETHANOL

Since the beginning of cheese-making, ethanol only comprise in a low amounts, comprising in average of 10^6, with $6x10^6$ as the highest value detected from cheese without addition of starter. Excluding cheese with starter D227, other cheeses showed similar increase of ethanol concentration after 3 days of ripening (with the average of 10^7), followed by drastic decrease for the rest of the ripening (with the average of 10^6) (Figure 4.9).

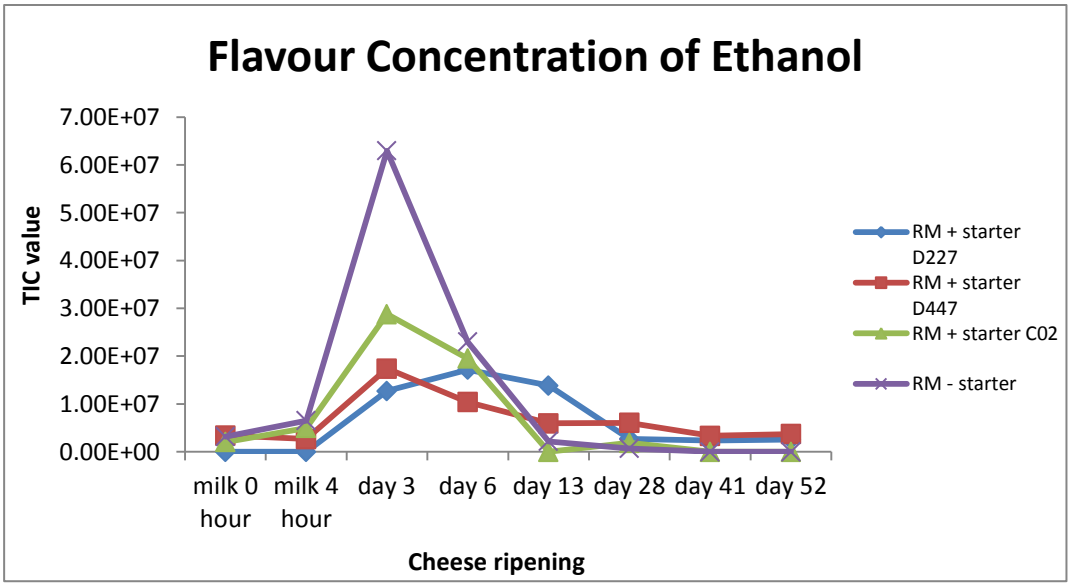


Figure 4.9 Ethanol Quantification During Cheese Ripening

Cheese with addition of starter C02 was expected to have higher amount of ethanol compared to cheese with addition of starter D227 and D447. Ethanol is a compound which came from alcohol group, and derived from acetaldehyde by some strains, especially *Leuconostoc* spp. Although some lactococci and lactobacili strains can also break down acetaldehyde and produce ethanol, *Leuconostoc* spp. strains could produce higher amount of ethanol (Vedamuthu E. R., 1994). It was known that starter D227 and D447 do not consists of *Leuconostoc* spp., while starter C02 does contain *Leuconostoc* spp.

Cheese without addition of starter recorded highest amount of ethanol. The possible explanation could be that as no SLAB added to the raw milk, less competition happened to the non-starter microorganisms, therefore non-starter microorganism other than NSLAB could grow easier during ripening. Yeast is known to resides in the milk source as other natural non-LAB microorganism, moreover it is known that yeast can rapidly metabolize lactate into ethanol and other alcohol molecules (Ghanadzadeh and Ghorbanpour, 2012). High production of ethanol was detected in Cheddar cheese without or low starter activity, which enabled *Candida* species to grow. Aside from ethanol, other compounds such as ethyl esters (ethyl acetate, ethyl butyrate) was also produced in high concentration (Horwood *et al*., 1987).

The decrease in ethanol concentration during cheese ripening correlated to the esters production. Esters are formed via the transesterification of ethanol and FFA. Ethanol is a limiting factor in production of various esters as it is the most common alcohol presented in cheese. Therefore esters concentration will be increased and ethanol concentration will be decreased over time (Holland *et al.,* 2002; Urbach, 1997). The only ester detected in this experiment was 2-propenoic acid, butyl ester, and from Figure 4.16 it can be seen the production of 2-propenoic acid, butyl ester was increased during ripening, which was expected.

ACETATE

Acetate, or acetic acid is a very important flavour compound in many cheeses, mainly because the abundant amount of it in cheese. The results showed that acetic acid comprised about 24.6-53.7% of all selected volatile compounds in the raw milk-cheese. Results showed that acetate have inconsistent change in every ripening time, although since the start of cheese making, a high amount of acetate already detected (10^8), meaning they are produced very fast even in the milk source (Figure 4.10). Previous research done by Bao (2016 and Chen (2016) on Remeker cheese also showed higher concentration of acetate detected during cheese ripening (with average of 10^8) .

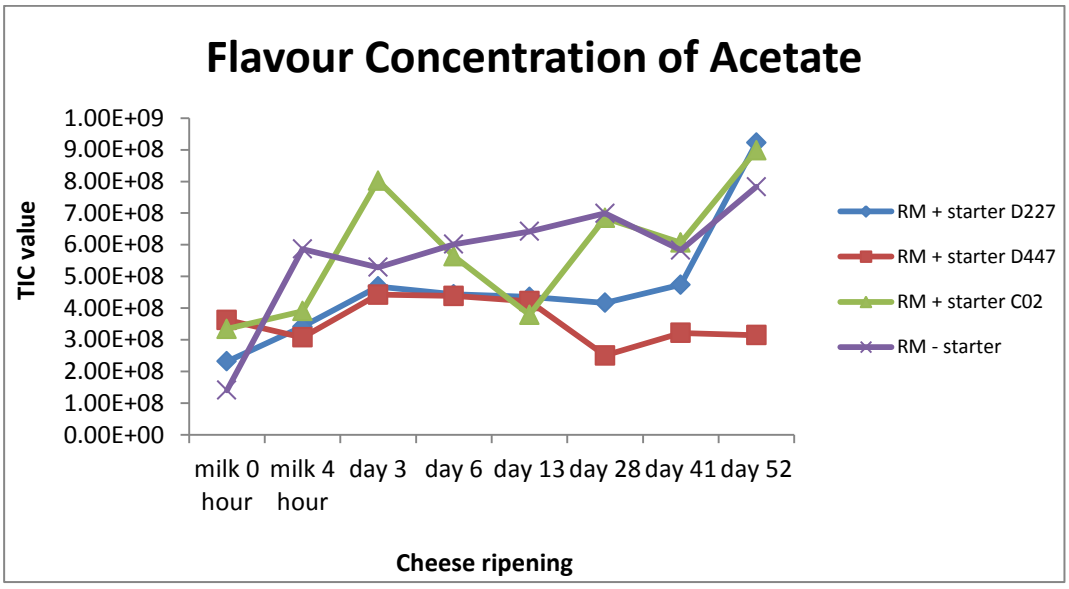


Figure 4.10 Acetate Quantification During Cheese Ripening

Higher concentration detected in cheese with starter C02 than in cheese with starter D227 and D447 was expected, because products of citrate metabolism by lactococci and *Leuconostoc* spp

differ. Starter culture that contained mostly lactococci strains could produce a decent amount of diacetyl, acetoin, and lactate, but the latter culture could produce large amounts of lactate and acetate (McSweeney and Sousa, 1999). High concentration in cheese without starter was also expected, as various microorganisms presented in the cheese might be able to metabolize lactate and citrate into acetate (Cogan, 1995; Singh *et al*., 2003).

An inconsistency amount of acetate could be the result of amino acid metabolism. Acetic acid is one of the major component produced from amino acid phenylalanine (Phe), and Tryptophan (Trp) catabolism. Catabolism of Trp produces indole pyruvate, which can be catabolized further to indole acetic acid. Catabolism of Phe also goes through transamination pathway, where Phe is catabolized with the help of aminotransferase into several compounds, including phenyl acetate (Fox *et al*., 1995). These reactions were found to be reversible (Gummalla and Broadbent, 2001), therefore, the concentration might be fluctuate during ripening.

4.3.2 METABOLISM OF AMINO ACIDS

The important flavour compounds in raw milk-cheese which derived from amino acid metabolism were from aldehydes, alcohol and sulfur groups. Degree of proteolysis is heavily influenced by presence of enzymes and LAB in cheese.

ALDEHYDES

The most notable flavour compounds from aldehydes group which was detected came from benzaldehyde. Although benzaldehyde have arguably smaller amounts compared to other flavour compounds, it has an unique semi-hard cheese's aroma, which is 'bitter-almond' note, and this compound grows rapidly and have major impact in cheese with long ripening time (van Leuven *et al*., 2008).

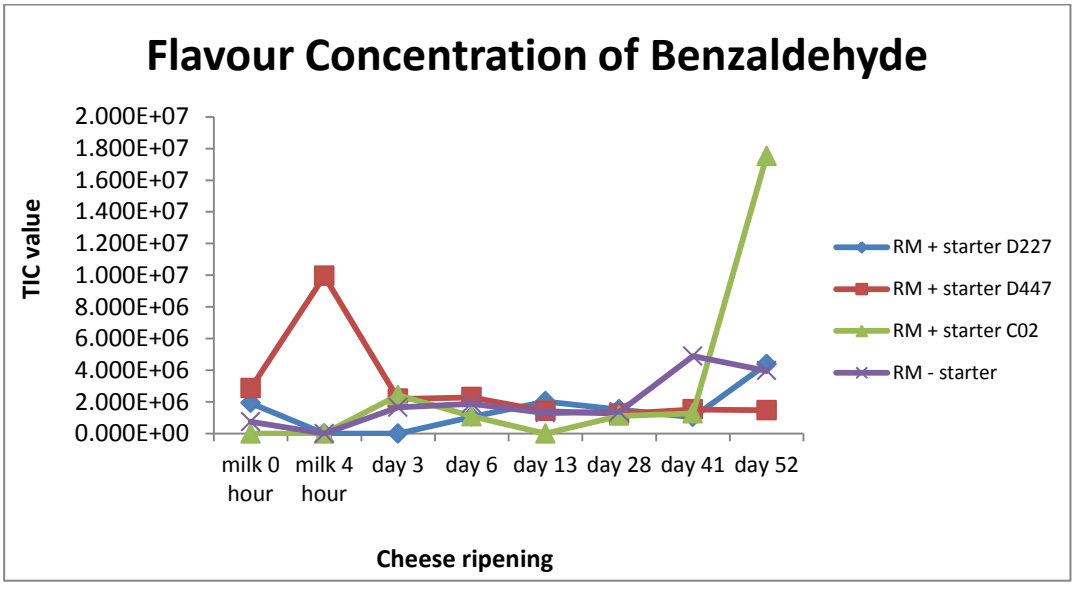


Figure 4.11 Benzaldehyde Quantification During Cheese Ripening

The results showed an inconsistent trend line, which is not as expected. All cheese samples accumulate similar average amounts of benzaldehyde during ripening and showed slight increase amounts after 41 days of ripening, except cheese with starter C02 which has steep increase after 41 days of ripening. The slow increase amounts of benzaldehyde was expected, since the

ripening process includes low oxygen concentration, low ripening temperature, and low pH, which are not a favourable condition for benzaldehyde catabolism (Groot and Bont, 1998).

The slow formation of benzaldehyde in this research was in line with previous research of Thevissen (2015) and Bao (2016), where results showed a significance increase in benzaldehyde after 4 months of ripening. Benzaldehyde could be formed through metabolism of Phe or Trp. Both amino acids undergoes transamination and further dehydrogenation pathways before benzaldehyde formed. Those pathways were heavily influenced by the presence of LAB. Phe and Trp metabolism to benzaldehyde are faster with the presence of lactococci and lactobacili strains (Yvon *et al*., 1997). The difference of benzaldehyde concentration in this research could not be seen, as it needs longer ripening time to produce benzaldehyde.

ALCOHOL

Aside from ethanol, the raw milk-cheese also contain several alcohol groups. Primary alcohol detected from cheese samples were 1-butanol, 3-methyl-, 1-hexanol, and 1-pentanol, while 2-propanol, 1-methoxy- and 2-propyn-1-ol were identified as secondary alcohol. 3-pentanol also detected as tertiary alcohol. 1-butanol, 3-methyl- or isoamyl alcohol was most notable as the most abundant alcohol isomers aside from ethanol. It is the main volatiles in fresh cheese and held responsible for 'fruity, floral' note (Carbonell *et* al., 2002; Sablé *et al*., 1997).

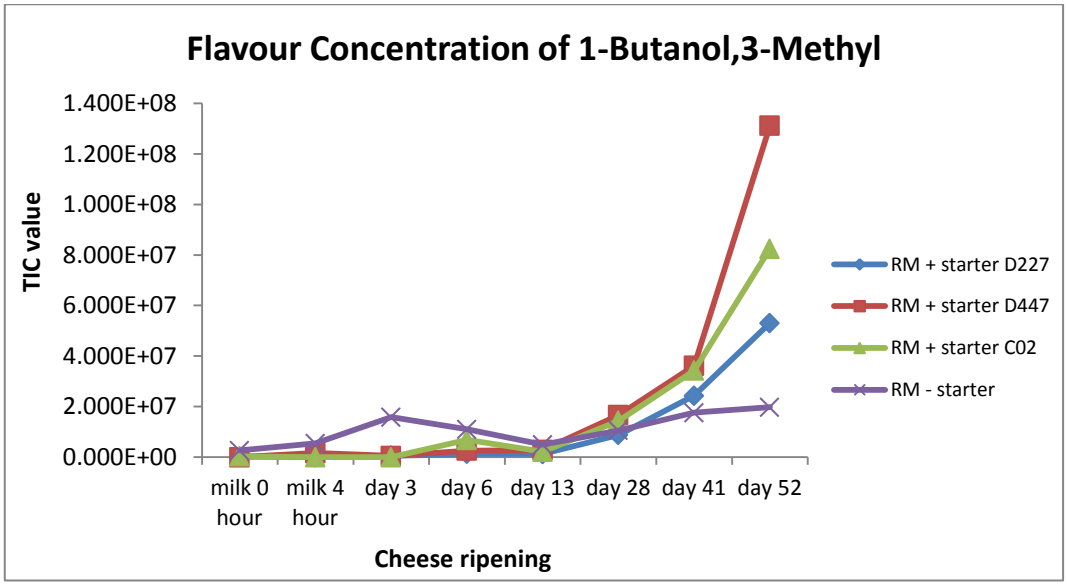


Figure 4.12 1-Butanol,3-Methyl Quantification During Cheese Ripening

The trendline showed increase production of 1-butanol, 3-methyl- during ripening. Several studies reported that 1-butanol, 3-methyl increases during cheese ripening and at some points, the amounts decrease (Mikelson and Ciprovica, 2011; Singh *et al*., 2003; Ocak *et al*., 2015). Previous research of Chen (2016) also showed the increase of 1-butanol, 3-methyl started to happen after 84 days of ripening in Remeker cheese.

Highest amount was recorded in cheese with starter D447 at 52 days of ripening which is 10^8 and lowest amount was recorded in cheese without starter addition, accumulated 10^7, which is as expected. Early investigation showed that activity of enzymes in combination with microorganisms are the main factor for 1-butanol, 3-methyl production. Alanine, valine and leucine was catabolized into 3-methylbutanal, then subsequently converted, by the hydroxyl acid

dehydrogenase produced by several strains of microorganisms, into 3-methyl 1-butanol (Smit *et al*., 2004). 3-Methylbutanal as 1-butanol, 3-methyl's precursor was not detected in this research for unknown reason, therefore no correlation could be made between compounds. Jollivet *et al*., (1994) stated that cheese that was inoculated by starter, especially lactobacili strains-culture, produces higher amount of 3-methyl 1-butanol. Therefore higher concentration of 1-butanol, 3-methyl in cheese with addition of starter compared to cheese without addition of starter was as expected.

Another unexpected results also happen in this compound between cheese with starter D227 and D447. After 41 days of ripening, although both cheeses showed an increase in concentration, cheese with starter D447 recorded 10-fold increase more than cheese with starter D227. As it happened with several compounds in this research, the reason remains unknown.

SULFUR COMPOUNDS

Disulfide, dimethyl was found to be the most notable sulfur compound detected from the raw milk-cheese. Flavour intensity of disulfide, dimethyl can be seen in Figure 4.13.

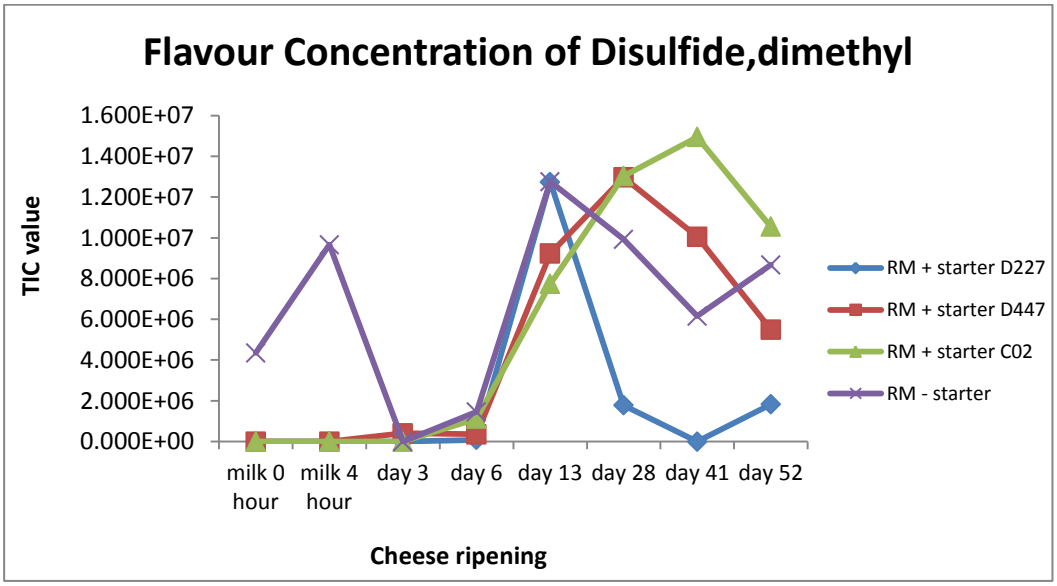


Figure 4.13 Disulfide,dimethyl Quantification During Cheese Ripening

All cheeses showed different trendline and fluctuation of disulfide, dimethyl. One similarity however, could be noted. All cheeses showed production of disulfide, dimethyl after 6 days of ripening. A further steep decreased was also noted in all cheese, although in different ripening time, which was not expected, as stated by van Leuven *et al.,* (2008) that sulfur concentration increases during long ripening of cheese.

It is known that disulfide, dimethyl is a volatile compound derived from methanethiol. The metabolism of disulfide, dimethyl starts from metabolism of amino acid methionine (Met) (Singh *et al*., 2003). The metabolism of Met converts Met by either enzymatic conversion or chemical decomposition to methanethiol. Methanethiol then easily oxidized to disulfide, dimethyl. Kim and Olson (1989) suggested that methanethiol may interact with other volatile compounds such as acetaldehyde, pentanol, diacetyl, ethanol and others to form other compounds. This might be the reason of the fluctuation production of disulfide, dimethyl.

The conversion rate of amino acid Met into disulfide, dimethyl also influenced by starter culture, especially lactococci strains. *Lactococcus lactis* strains play role in producing enzymes needed for Met metabolism, and accelerate disulfide, dimethyl production. Therefore it was expected to acquire higher amounts of disulfide, dimethyl in cheese with addition of starter D227 and D447, which were not seen through this experiment, and the reason remains unknown. Another thing to be noted, is a different trendline between starter D227 and D447 after 13 days of ripening. Again, the mentioned starters should have produced similar trendline of compounds. The possible explanation will be presented by comparing several compounds, as it can be seen in Figure 4.17.

4.3.3 METABOLISM OF FATTY ACID

Metabolism of fatty acid in cheese occurs in the form of hydrolytic and oxidative degradation. The important flavour compounds produced from fatty acid metabolism are Free Fatty Acid (FFA), ketones and esters Singh *et al*., (2003). It is expected for all cheeses with starter additions to show slow increase of FFA, as it is believed in bacterial-ripened cheese most fatty acid metabolism come from NSLAB. Most NSLAB is expected to actively participate in flavour development during later stage of ripening. Nevertheless, for cheese without starter, it is expected to have faster increase as many microflora are expected to grow inside, which will affect the fatty acid metabolism.

FREE FATTY ACID (FFA)

Several FFA have been identified in most cheese samples, including propanoic acid, butanoic acid, pentanoic acid, hexanoic acid, heptanoic acid, octanoid acid and nonanoic acid. Acetic acid, despite recorded as organic acid compound, it is mostly formed by lactate or amino acid metabolism. Butanoic acid (C4:0), hexanoic acid (C6:0), and octanoic acid (C8:0) have been recorded as having most abundant amounts in the microcheese compare to other FFA, and they recorded similar trendline, therefore they are chosen to be presented.

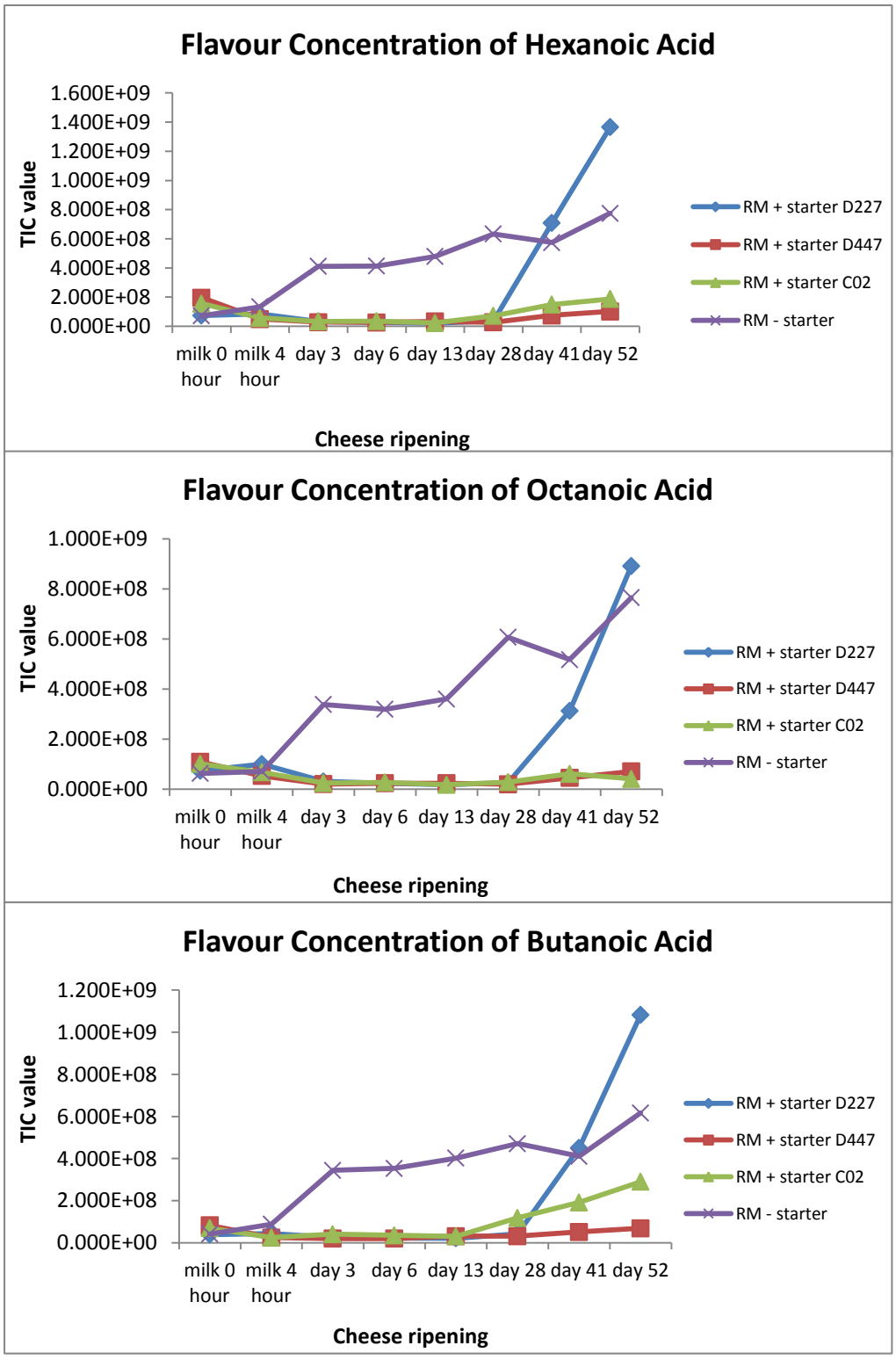


Figure 4.14 Various FFA Quantification During Cheese Ripening

Figure 4.14 showed similar trend line between all FFA results. All FFA showed slow increase of amounts during cheese ripening, except cheese without starter. The trendlines were expected, as according to research of lipolysis in Cheddar cheese by Dimos (1992), the rate of lipolysis was small, even in full-fat cheeses. Attaie and Richter (1996) also stated that FFA formation, that is, the extent of lipolysis is usually increases after 6 weeks of cheese ripening. Previous research of Wen-hui also showed the huge increase of most FFA in Remeker cheese was after 54 days of

ripening. It is believed through her research, especially in acetic acid, that the increase production was due to fermentation by NSLAB.

FFA are one of the precursor of esters formation (the other being alcohol) (McSweeney, 2004), therefore results of both groups could be correlated. However as it can be seen in Figure 4.16, only propionate butyl ester was detected in this experiment, which is unexpected. Therefore the correlation between both groups could not be explained. Nevertheless, it is suggested that the further degradation by lipase was not happened yet, and propionate butyl ester formation was due to the work of other microflora, e.g. yeast/mold/propionibacterium. Further explanation about this spoilage can be seen in section 4.3.4.

Lower amounts of FFA found in cheese with starters than cheese without starter were expected. Major lipolysis are caused mainly by milk lipase and microbial lipolytic enzymes. Lipases and esterases of LAB appear to be the main principal lipolytic agents in Dutch-type of cheese (Fox *et al*., 2000). To hydrolyse milk fat in milk and cheese, LAB possess esterolytic/lipolytic intracellular enzymes such as tributyrin lipase, capable of hydrolyzing a range of fatty acids (Holland and Coolbear, 1995). Despite the presence of these enzymes, most SLAB, especially *Lactococcus* are generally considered to be weakly lipolytic, although some lactobacili species showed high lipolysis activity (Desmazeaud, and Ismail, 1986). Further research of De Wit *et* al., (2005), Fox *et al*., (1993), Baillargeon *et al*., (1989), and Freitas *et al*., (1999) also suggested that other natural microflora such as yeast *Yarrowia lipolytica* was reported to have higher lipolytic activity compare to LAB strains. Those nonstarter microflora could be presented in cheese without starter, therefore higher concentration of FFA found in cheese without starter was expected.

In conclusion, higher concentration of FFA were detected from cheese without starter than other cheeses. The rate of lipolysis is mostly affected by the presence of microorganisms. In general, NSLAB have much higher lipolysis compare to SLAB (McSweeney *et al*., 1993). Moreover, it is believed that yeast were present in higher amount in cheese without starter, thus influence the FFA production. Huge difference in FFA production from cheese with similar starters (D227 and D447) near the end of ripening indicated either a spoilage by other microflora (yeast/mold/propionibacterium) in cheese with starter D227, or different enzym activity between both starters. Further explanation can be seen in section 4.3.4.

KETONES

Main ketones which have been identified belongs to 2,3-butanedione and 2-butanone,3-hydroxy. However, other ketones also play a role in flavour development in cheese, such as acetone and methyl ketones, including 2-heptanone, 2-nonanone and 2-butanone. Of all this ketones, 2-heptanone was detected the most during this research. 2-heptanone along with other methyl ketones, were regarded as important flavour compounds, as they are responsible for various ‘floral-fruity’ aroma (Singh *et al*., 2003).

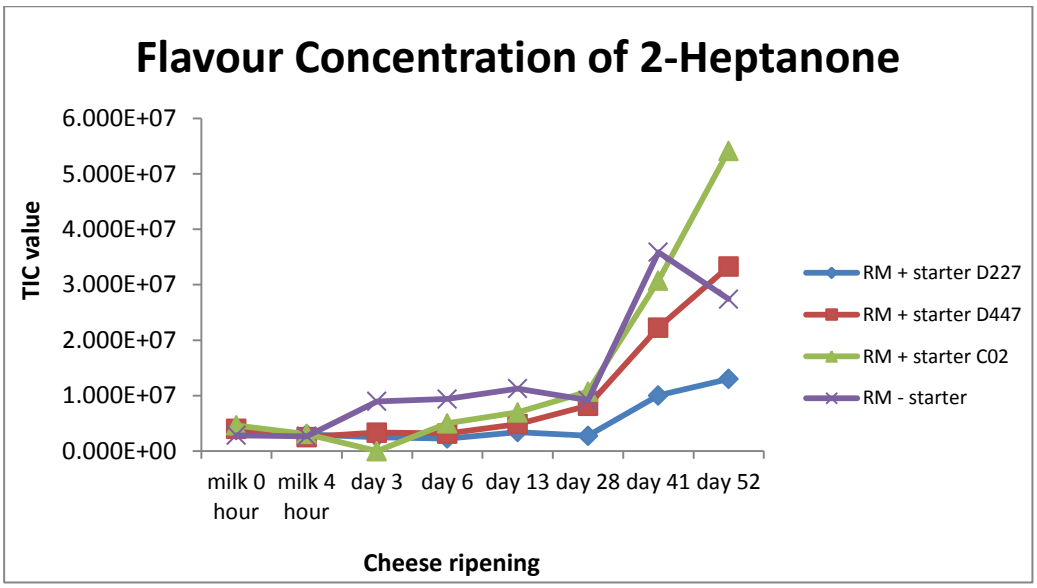


Figure 4.15 2-Heptanone Quantification During Cheese Ripening

The highest concentration of 2-heptanone was obtained from cheese with starter C02 ($5x10^7$). It can be seen that over time, 2-heptanone concentration in all cheeses have similar patterns of slow increased. Only after 28 days of ripening, the increased was decent. Previous research of Remeker cheese by Qiao and Wen-hui showed the slow increase of methyl ketone compounds, followed by steep increased after 84 days of ripening. From this comparison, it can be concluded that mold play a significant role in production of 2-heptanone. The research done by Collins *et al*., (2003), Urbach (1997), and Molimard and Spinnler, (1996) also support the assumption. Those research stated that formation of methyl ketones are heavily influenced by mould presence. Methyl ketones are formed in cheese due to the action of mould lipases, that is, *Penicillium roqueforti Penicillium camemberti* and *Geotrichum candidum*). Spores of several moulds, especially *P. roqueforti* have also been found to oxidize fatty acids into methyl ketones. The lack of methyl ketones in this research compared to previous research probably because lack growth of mould in cheese, as it has been known that the cheese making procedures in this experiment was conducted through microcheese-making procedure. The cheese were ripened in a sealed space, completely covered by combination of several non-gas permeable seals, which minimize gas permeation and aeration, which are unfavourable conditions for mold's growth (Kinsella & Hwang, 1976).

ESTERS

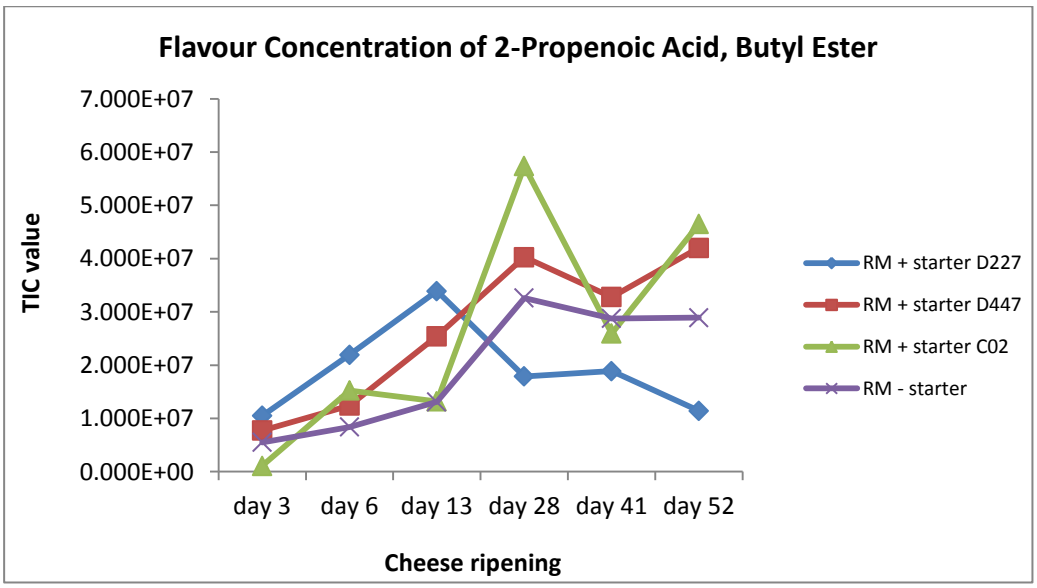


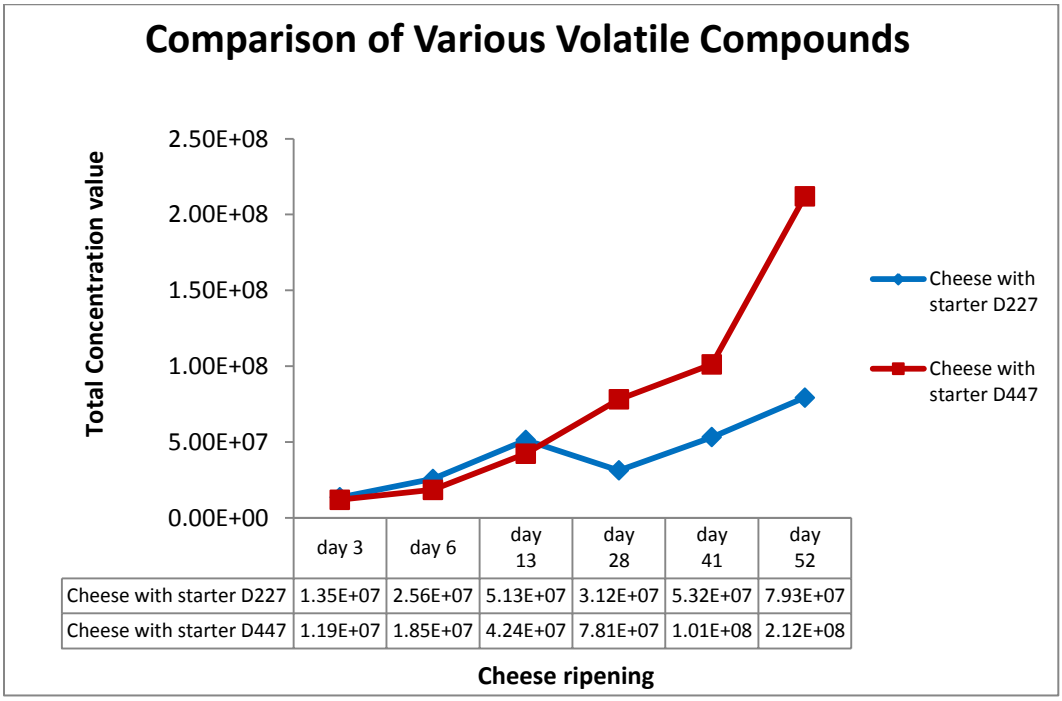
Figure 4.16 2-Propenoic Acid, Butyl Ester Quantification During Cheese Ripening

The only ester compounds that were presented in all cheeses is 2-propenoic acid, butyl ester. This result was unexpected. It was expected that ester compounds which presented the most should be from ethyl ester group, as ethanol was the most abundant alcohol presented in the cheeses (Alewijn *et al.*, 2003). Previous Remeker cheese's experiment of Bao (2016) and Chen (2016) also showed several ethyl esters were detected through their cheese ripening. The unexpected discovery is remains unknown, considering that various FFA's were also detected in this experiment. Could be the production of ethyl ester in this experiment was not yet happen, since the production of FFA was also still low even after 42 days of ripening (Figure 4.14).

It is suggested that despite lack of other ester compounds present in the cheeses, 2-propenoic, butyl ester was detected alone because its precursor, which is propanoic acid, is not only metabolized through lipolysis, but through glycolysis or proteolysis as well (Mallatou *et al*., 2003). However this statement could not be proved because the propanoic acid concentration (referred to Appendix 4) was even lower compared to other FFA such as butanoic, hexanoic, and octanoic acid. Nevertheless, previous research by Thevissen (2015) showed that higher acetic acid production will cause higher propionic acid production, because propionic acid is a major by-product of acetic acid formation.

4.3.4 POSTULATION APPROACH BETWEEN STARTER D227 AND D447

As it can be seen in many figures, there were unexpected difference trendline of several compounds production between cheese with starter D227 and starter D447. The trendline were unexpected because both starters should have contained similar strains (refer to Chapter 3 Materials and Methods). Most of the compounds showed different trendline after 41 days of ripening. Two suggested explanations are given after comparing the total production of disulfide, dimethyl, 2-heptanone, 2-propenoic acid, butyl ester, and 3-methyl-1-butanol compounds at 41-52 days of ripening (Figure 4.17).



	day 3	day 6	day 13	day 28	day 41	day 52
Cheese with starter D227	1.35E+07	2.56E+07	5.13E+07	3.12E+07	5.32E+07	7.93E+07
Cheese with starter D447	1.19E+07	1.85E+07	4.24E+07	7.81E+07	1.01E+08	2.12E+08

Figure 4.17 Comparison of Total Concentration Value of Several Compounds Between Cheese With Starter D227 and D447

4.3.4.1 SPOILAGE BY YEAST/MOLD APPROACH

Figure 4.17 shows the different trendline of several important volatile compounds between cheese with starter D227 and D447. It can be seen that at 41 days of ripening, cheese with starter D447 had 10-fold concentration higher than cheese with starter D227. One possible explanation might be correlated to the growth of some yeast and mold in cheese with starter D447. *Geotrichum candidum, Yarrowia lipolitica,* and *Kluyveromyces lactis* are mold and yeast mostly present in mold surface-ripened cheese. The reason why only disulfide, dimethyl, 2-heptanone, 2-propenoic acid, butyl ester, and 3-methyl-1-butanol compounds that were summarized is because both mold and yeast are responsible for the highest production of those compounds (Martin *et al*., 2001). Furthermore, the growth of mold could be seen clearly during the trial experiments (Figure 4.18), although no visible mold growth were seen during the real experiment. The influence of yeast could be seen by analysing the production of ethanol and ester, especially ethyl ester, which unfortunately could not be quantified in this study. Further quantification of mold/yeast growth should be implemented to verify the theory that yeast/mold were the reason of the difference in concentration of compounds between both cheeses. Yeast/mold quantification/identification should also be done to cheese without starter, as it is believed that those microorganisms grew in there as well.

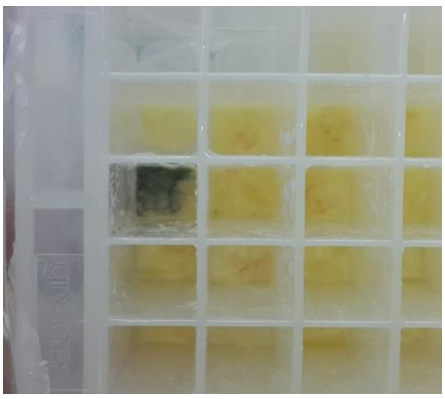


Figure 4.18 Growth of Mold During Trial Experiment of Microcheese Making

4.3.4.2 SPOILAGE BY PROPIONIBACTERIUM APPROACH

However, the oddness of flavour formation, especially in lipid metabolism, between cheese with starter D227 and D447 might be caused by something else other than yeast/mold spoilage. Van Leuven *et al*., (2008) stated that the most ester presents in semi hard-type of cheese like Gouda belongs to ethyl ester group. The same results also happened in previous Remeker's study by Bao (2016) and Chen (2016). However, the high concentration of propenoic ester (Referred to Figure 4.16) instead of ethyl ester in this project indicated a spoilage by Propionibacterium strains (PAB). Presence of PAB is essential in Swiss-type of cheese, however high amount in Gouda-type of cheese is considered to be a spoilage. Spoilage by PAB is usually recognize by the presence of brown or red spots in cheese, which was visible in the microcheeses in this project (Steffen *et al.,* 1987; Turner *et al*., 1983). It is indicated from this study that metabolism of fatty acids by PAB led to production of popanoic acid, hence faster production of propenoic ester compare to other ester compounds. Therefore it can be concluded that in especially cheese with starter D227, spoilage by PAB occurred, hence affected the flavour formation (mostly lipid metabolism).

Nonetheless, the spoilage of PAB did not simply answered all odd lipolysis results. When comparing Figure 4.14 and Figure 4.17 altogether, another strange result shows. In Figure 4.14 it shows high production of FFA in cheese with starter D227, but metabolites from further FFA degradation show high concentration in cheese with starter D447 instead of cheese with starter D227 (Figure 4.17). Further research by Thierry *et al.,* (2005) observed ester synthesis rate with and without the presence of PAB. The result suggests that enzymes from LAB starters are involved in many FFA degradation. PAB are therefore not necessarily directly involved in FFA degradation, especially in ester formation, but may only act as providers of the precursor. In summary, high production of FFA by PAB does not necessarily mean high production of metabolites of FFA degradation, which happened in this study. The role of starter LAB are also important in degradation of FFA.

Following the previous statement, the bacterial lipase of starter LAB might be the reason of different concentration of lipid metabolites between cheese with similar starter LAB (D227 and D447). Although little information about the effect to the lipid metabolism, several research suggested that even in the same *Lactobacillus lactis* species, the metabolic activities and fermentation end products might different. Rattray (2002) stated that different *Lactococcus lactis* species, e.g. *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* produce different amount of volatile compounds, especially lactate metabolites.

Another discovery by research of Ayad *et al*., (2001) showed different flavour compounds's concentration from amino acid metabolism by several *Lactococcus lactis* strains indicated that some *Lactococcus lactis* strains do not possess same enzymatic activity for compounds metabolism. Thus, in conclusion, different concentration of some compounds in cheese with starter D227 and D447 in this project may correlate to the different enzymatic activities of the two starters. Deeper analysis of enzymatic activity between the two starters might be recommended to prove the suggestion, especially in raw milk-cheese, where the fatty acid metabolism will influence greatly to the flavour development during ripening. The activity of microflora lipase and esterase, can be detected by growing the selected microflora, e.g. propionibacterium strains in specific substrates (Dupuis *et al*., 1993). Determination of the lipase activity and esterase assay can be done in every sampling points during ripening

CHAPTER 5 CONCLUSION

This study was designed to evaluate the effect of NSLAB and SLAB to the formation of flavour compounds in raw milk-cheese, as well as to investigate the influence of nisin to the growth of NSLAB and SLAB, thus subsequently affect the flavour development during cheese ripening. Taken all accounts of growth in MRSA and LM17 medium, the resulting data support the conclustion that in early ripening, NSLAB presented at a low initial level and substantially increase during further ripening, while SLAB also showed increase growth, although near the end of ripening there was indication of slow-down growth. There was another indication that non-LAB microflora grew in a high number in cheese without starter addition due to lack of competition supposedly cause by SLAB.

Presence of nisin for NSLAB inhibition has not had success in this study. No inhibition of nisin to SLAB is expected, as *Lactococcus lactis* spp. are expected to be nisin-tolerant. However, most NSLAB are expected to be nisih-sensitive. Similar NSLAB growth in cheese that contained nisin-producing starter (starter D227 and D447) with cheese that contained no nisin-producing starter (starter C02) indicated that the activity of nisin was too low to significantly inhibit NSLAB. Due to the unsuccesfull inhibition of NSLAB by nisin, the flavour development during cheese ripening cannot be correlated to the nisin.

Despite the unsuccesfull attempt to correlate nisih influence to the flavour development, the presence of NSLAB and SLAB indeed play roles to the overall flavour profile in raw milk-cheese. The most prominent different effect of NSLAB and SLAB can be seen from FFA production. The FFA production in cheese without starter was averagely higher compare to other cheese with starter addition during ripening. This results prove that NSLAB in general cause higher rate of lipolysis compare to SLAB, although other microflora such as yeast/mold or propionibacterium also suspected to play important role to the lipolysis rate. On the other hand, higher amounts of products from lactate and citrate metabolism were discovered from cheese with starter addition, especially starter that consists *lactococcus* spp. However in general, different results were obtained, especially in last stage of ripening, in cheese with similar SLAB addition (starter D227 and D447). It is suggested that both starters, although have similar characteristics, are suspected to have different enzymatic activity, thus led to the different flavour formation.

To conclude, NSLAB and SLAB will indeed produce different flavour profile during cheese ripening. However longer ripening period is needed to strengthen the results. Nisin is also believed to cause inhibition to NSLAB growth, and might influence the flavour development in later ripening of cheese, especially fatty acid metabolism, which is important for raw milk-cheese. Higher activity of nisin, thus higher inoculation of SLAB during cheese manufacturing is needed to confirm the statement. It is difficult to find the explanation for the exact cause of different results from flavour profile between cheese with similar starter culture. Further study about the growth of mold/yeast/propionibacterium and enzyme activity of the starter cultures might provide a more definite explanation.

CHAPTER 6 RECOMMENDATION

Several problems occured during this study in relation to the effect of LAB and nisin to the flavour compounds, and also the implementation of the microcheese making. Therefore some recommendations can be proposed to prevent similar problems for future study.

6.1 DIFFERENT METHODS OF LAB IDENTIFICATION

Two methods of LAB counts were implemented to determine the growth of NSLAB and SLAB separately. However, because of wide range of LAB's optimum temperature and media growth, the MRSA media could not be used to determine the exact growth of NSLAB. There are two methods that can be recommended for more precise NSLAB and SLAB counts.

6.1.1 DIFFERENT MEDIA FOR NSLAB GROWTH (Oberg *et al.*, 2011, Kołakowski *et al.*, 2004)

Other media can be used to obtain a more accurate LAB counts exclusively for NSLAB (most lactobacilli strains). Although the use of MRSA medium is too broad for the total of LAB counts, it can be combined with other chemical compound to prevent the growth of SLAB (lactococci strains). The use of MRSA that is supplemented with 200 µg/mL vancomycin (MRSA-V) proved to obtain more accurate results of NSLAB growth rather than the use of MRSA medium only.

6.1.2 LAB ISOLATION AND IDENTIFICATION (Referred to LAB identification protocol provided by Nicolette Klijn)

LAB identification can be performed to characterize the LAB in cheese. In this way, the NSLAB and SLAB can be identified separately and more precise rather than LAB counts method. Serial dilutions of sample are prepared, spread plate method is then implemented. All plates then incubated at 25°C for 24 to 48 hours. Afterwards, colonies that grew in plates are transferred in MRS broth medium, and incubated at 25°C for 24 hours. Several characterization analysis on the strains should be performed to obtain accurate result of LAB identification.

- Microscopy
- Growth at different temperatures (15, 25, 35 and 45°C)
- Salt resistance (4% NACl)
- Casein breakdown / milk fermentation (using milk agar plates or sterile milk)
- CH50 API for identification of LAB strains

6.2 DETERMINATION OF NISIN ACTIVITY (Rossi and Veneri, 2016)

Nisin quantification was not done in this thesis project, as it is difficult to quantify the easily-to-degrade nisin, especially in small form of cheese. However during this experiment, some difficulties occurred, such as the growth of NSLAB was not successfully inhibited by the presence of nisin. This problem suggested that either nisin activity was too low, or NSLAB presented were resistant againts nisin. Therefore it is recommended to quantify the amount of nisin, as prove for the assumption made in this study. Several methods can be used to quantify nisin, however, agar diffusion assay is often performed for approximate nisin activity.

6.3 YEAST AND MOLD QUANTIFICATION OR IDENTIFICATION IN MICROCHEESE IN RELATION TO THE FLAVOUR DEVELOPMENT

In some cheese varieties such as Camembert and blue-veined cheese, the high growth of yeast and mold is expected, and will positively contribute to the development of flavour and texture. However in other cheeses, the high number of those microorganisms is described as spoilage and could cause unwanted sensory characteristics and texture of the cheese (Roostita and Fleet, 1996).

Even though several precaution were implemented during microcheese making to prevent the spoilage of microorganisms other than LAB, it is still suspected that yeast/mold were grown inside the microcheese in decent amounts. Those yeast/mold were believed to influence the flavour development of microcheese, hence caused odd results in flavour profile, that is, to cheese with similar starters (D227 and D447). To prove the presence of the responsible yeast/mold, several methods can be applied to identify the microorganisms.

6.3.1 YEAST IDENTIFICATION (Welthagen and Viljoen, 1999)

Several medium can be used for yeast identification. Potato Dextrose Agar (PDA) is often used for determination of yeast counts, while Plate Count Agar (PCA) can be used for the total count of microorganism. PDA is best used because of its accurativeness for samples with high a_w (>0.95), such as Remeker cheese (according to research by Chen (2016) that Remeker cheese recorded a high a_w of 0.9-0.98), while PCA is often prefferred because its compability with any type of samples. For yeast identification, the selective medium Yeast Extract Glucose Chloramphenicol (YGC) is often implemented. Aside from identification, YGC media could be used for yeast colony morphology and microscopy characterization. Further explanation of yeast identification can be seen in literature (Welthagen and Viljoen, 1999).

6.3.2 MOLD IDENTIFICATION (Referred to Mold identification protocol provided by Nicolette Klijn)

As neither yeast nor mold analysis were done in this study, the potential spoilage in the microcheese could not accurately determine came from yeast spoilage. Mold could also be the potential microflora that spoiled the microcheese. Therefore, mold identification analysis is worthwhile to be implemented as well. Mold growth and identification are often done using PDA media. Isolatee is swab and growth in a separate PDA plates. Similar to LAB identification, mold identification also consists of several characterization analysis.

- Microscopy
- Colony morphology
- Growth on milk agar plates
- Thiosulfate Citrate bile salts sucrose (TCBS) agar medium for interpretation of mold species

6.4 PROPIONIBACTERIUM QUANTIFICATION (El-Kholy *et al.*, 2006)

It is suggested that propionibacterium were presented in microcheeses in this project. Those bacteria has potential to influence the flavour compounds in the microcheese. However it is not known if the influence is significant or not. Therefore propionibacterium counting is recommended for future study. PAB counting is done on APT agar medium. The plates are incubated at 30°C for 3-4 days under anaerobic conditions.

6.5 ENZYMATIC ACTIVITY ANALYSIS FOR BOTH STARTER CULTURES

It is believed that enzymatic activity between the two similar nisin-producing stater cultures (D227 and D447) contribute to the different flavour profile of both cheeses, especially in the metabolism of lipid. Therefore, to prove the theory, enzymatic activity analysis of both starters is recommended. Several method of enzyme activity analysis is better seen in the following.

- Determination of aminotransferase and decarboxylase activity (Ayad *et al*., 2001)
- Lipase and esterase assays (Lopes *et al*., 2011; Dupuis *et al*., 1993)

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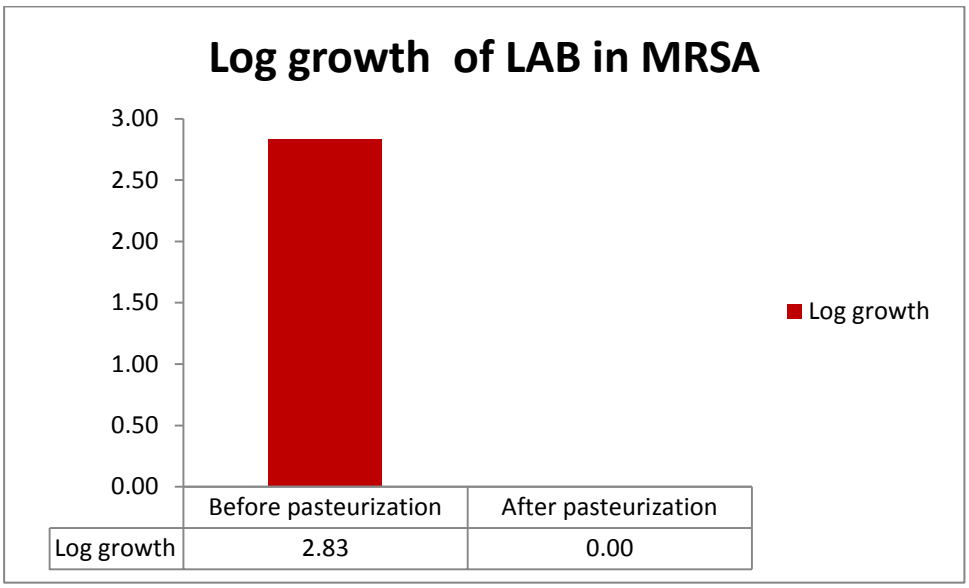
APPENDIX

Appendix 1. Difference LAB Growth Before and After Pasteurization

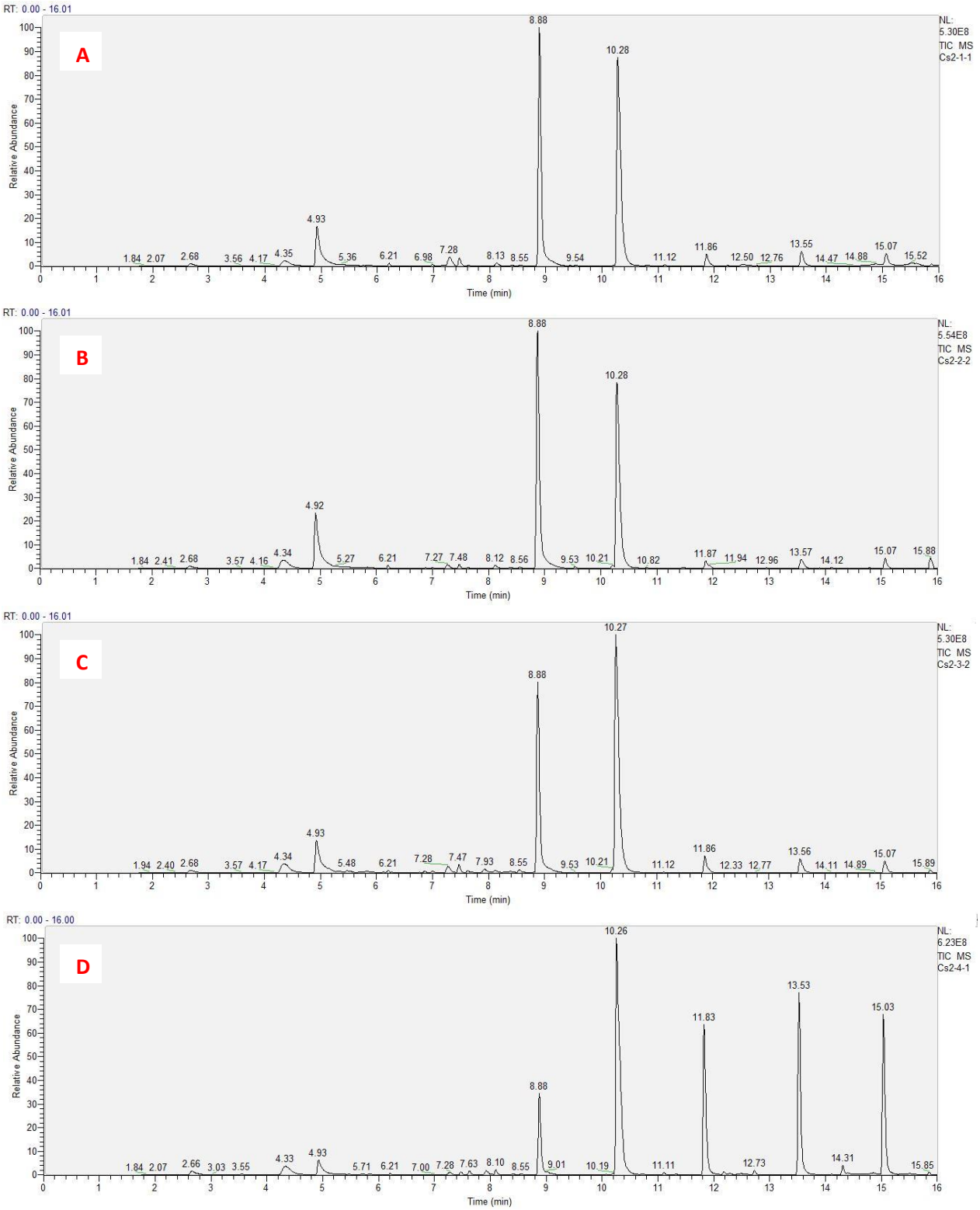


Appendix 2. Total Percentage Selected Volatile Compounds From 6-day-old and 52-day-old Raw Milk-cheese

	6-day-old Cheese				52-day-old Cheese			
	Cheese + starter D227	Cheese + starter D447	Cheese + starter C02	Cheese-starter	Cheese + starter D227	Cheese + starter D447	Cheese + starter C02	Cheese-starter
Hexane, 2,4-dimethyl-	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0
Acetone	0.5	0.0	0.3	0.0	0.0	0.0	0.7	0.0
2-Butanone	0.0	0.0	0.0	0.0	0.0	0.2	0.1	0.1
Ethanol	1.6	0.8	1.6	1.1	0.1	0.2	0.0	0.0
2,3-Butanedione	7.6	10.9	5.9	1.9	1.0	12.2	6.9	2.9
Disulfide, dimethyl	0.0	0.0	0.1	0.1	0.0	0.3	0.4	0.2
2-Propanol, 1-methoxy-	0.4	0.0	0.0	0.4	0.1	0.3	0.6	0.0
2-Propenoic acid, butyl ester	2.0	1.0	1.3	0.4	0.2	2.0	1.8	0.8
2-Heptanone	0.2	0.2	0.4	0.5	0.3	1.6	2.1	0.7
1-Butanol, 3-methyl-	0.1	0.2	0.6	0.5	1.1	6.1	3.3	0.5
2-Butanone, 3-hydroxy-	40.7	44.7	33.3	10.7	7.9	49.3	26.1	13.3
2-Nonanone	0.0	0.0	0.0	0.0	0.1	0.1	0.3	0.3
Acetic acid	39.1	34.8	47.3	29.5	18.6	14.9	35.5	20.9
1-Pentanol	0.1	0.0	0.0	0.0	0.4	0.0	0.0	0.3
Propanoic acid	0.3	0.2	0.2	0.3	0.5	0.1	0.4	0.2
Benzaldehyde	0.1	0.2	0.1	0.1	0.1	0.1	0.7	0.1
Butanoic acid	2.0	1.6	3.0	17.2	21.8	3.3	11.4	16.4
3-Pentanol	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0
Pentanoic acid	0.1	0.1	0.2	0.5	0.9	0.5	0.4	0.5
Hexanoic acid	2.3	2.1	3.0	20.0	27.5	4.9	7.4	20.6
Heptanoic acid	0.1	0.1	0.2	0.9	1.2	0.2	0.1	1.3
Octanoic Acid	2.1	1.9	2.3	15.2	17.9	3.4	1.6	20.4
Nonanoic acid	0.8	1.1	0.4	0.7	0.3	0.0	0.0	0.5

Appendix 3. Overall Chromatogram Results From 6-day-old and 52-day-old Raw Milk-cheese*

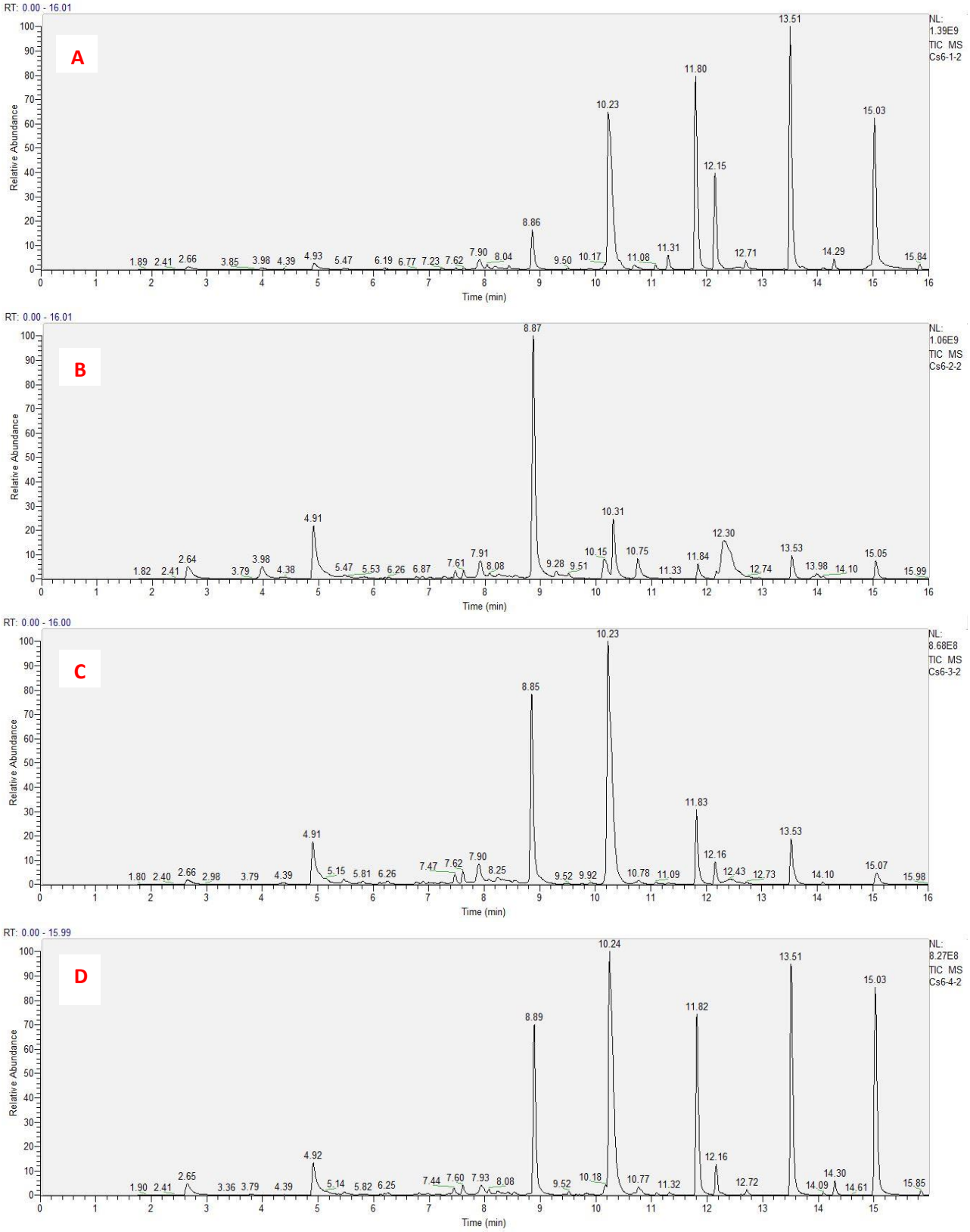
6-day-old Cheese Chromatogram**



*After baseline reduction

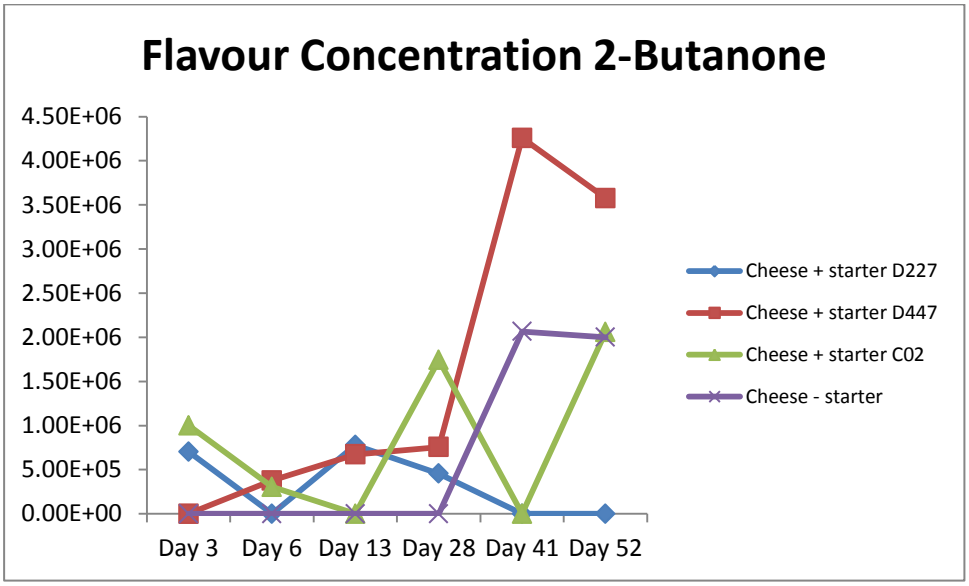
**Denotes to 4 types of cheese: (A) Cheese with starter D227, (B) Cheese with starter D447, (C) Cheese with starter C02, (D) Cheese without starter

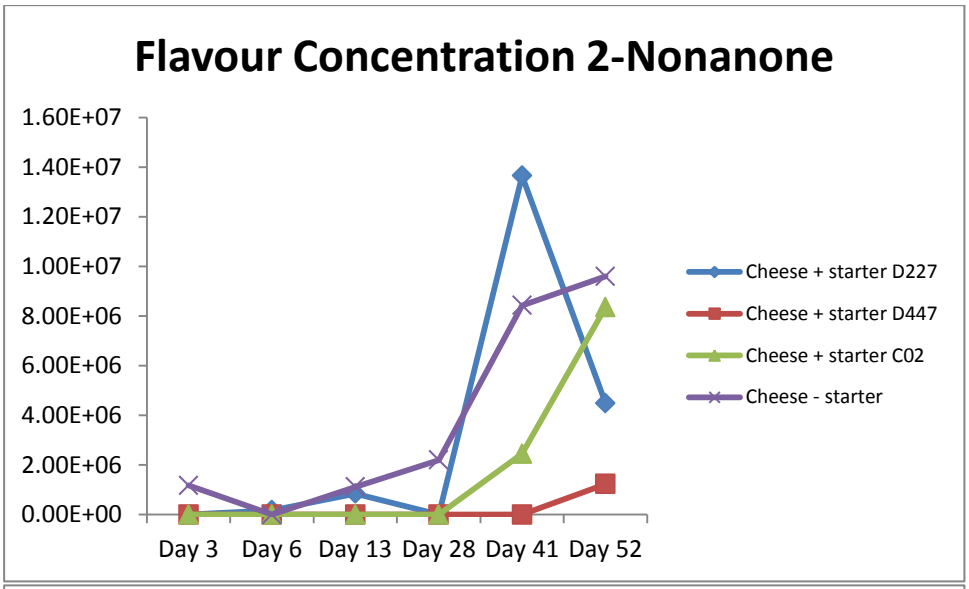
52-day-old Cheese Chromatogram*

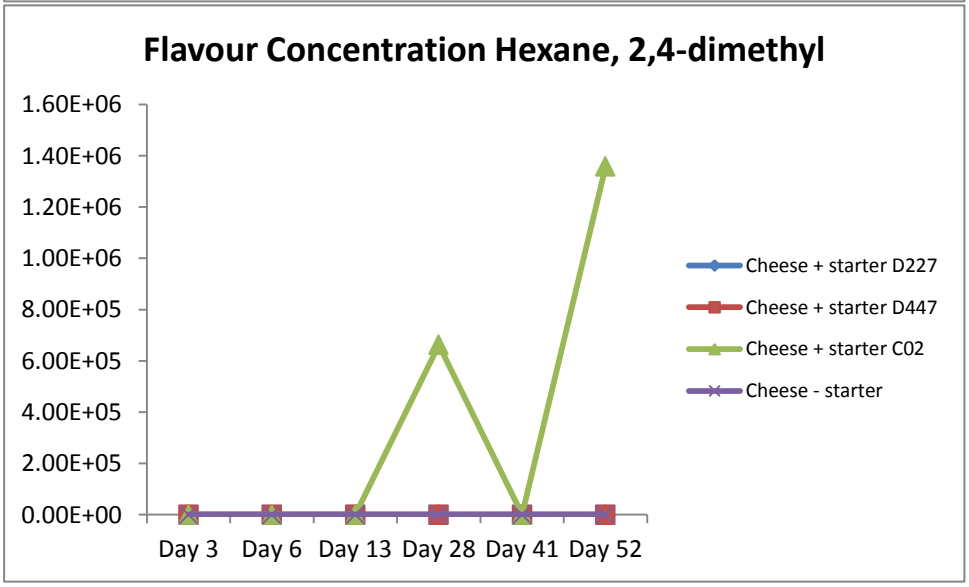


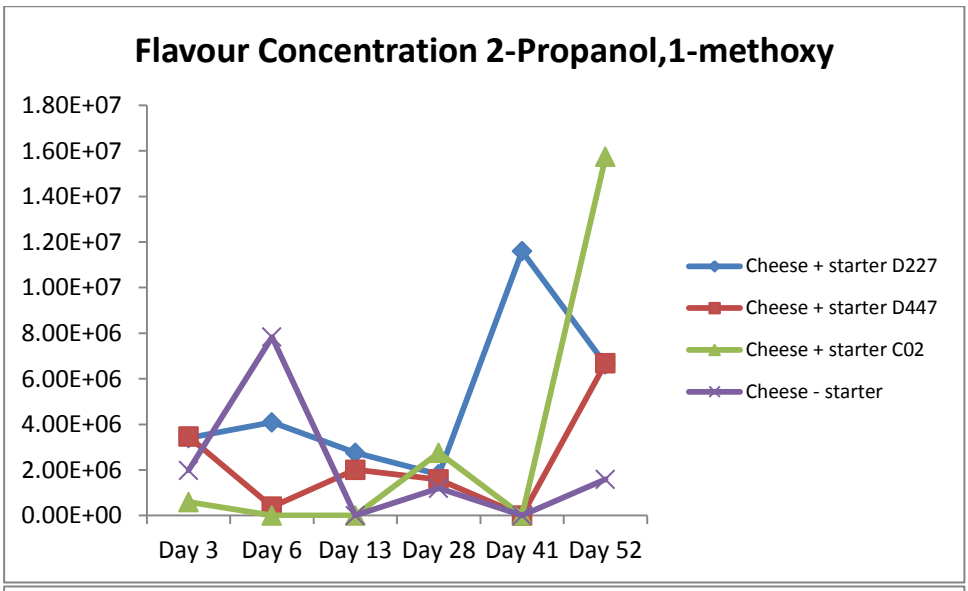
*Denotes to 4 types of cheese: (A) Cheese with starter D227, (B) Cheese with starter D447, (C) Cheese with starter C02, (D) Cheese without starter

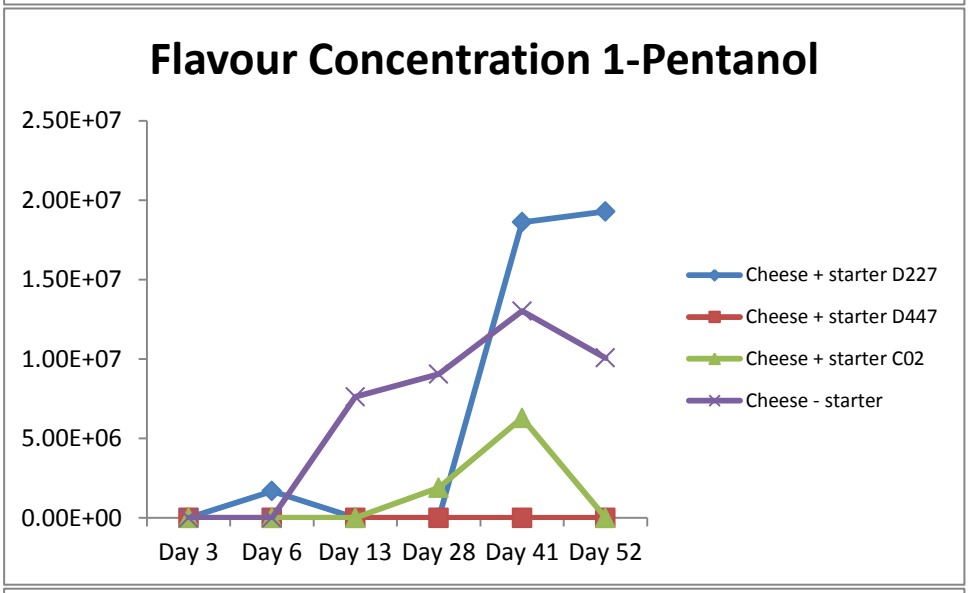
Appendix 4. Flavour Concentration Production During Raw Milk-cheese*

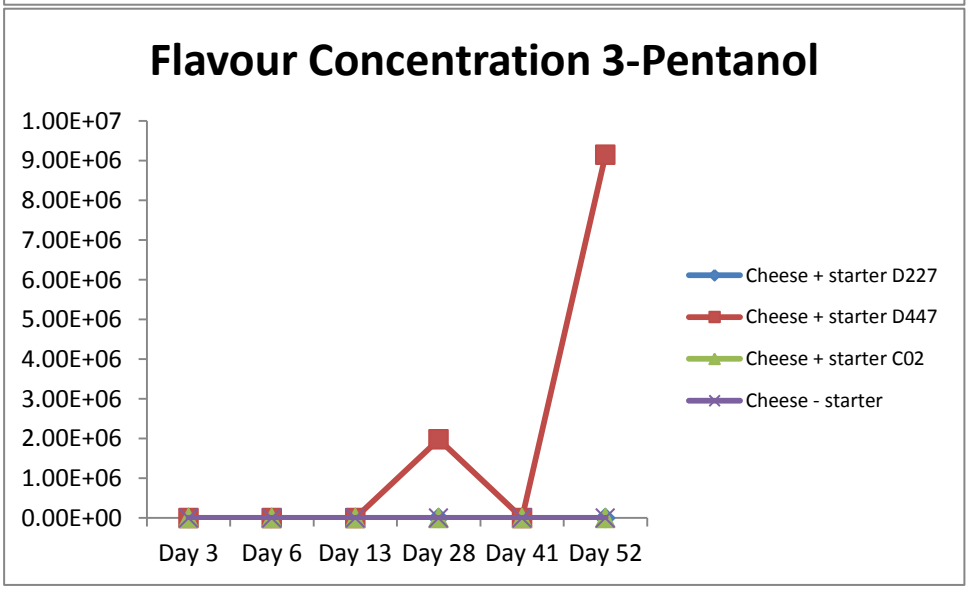


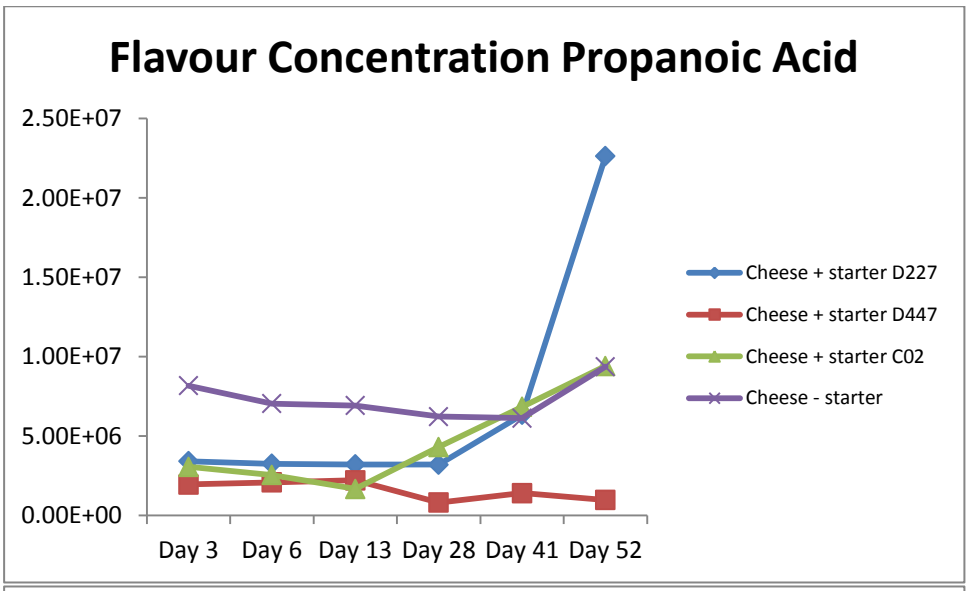


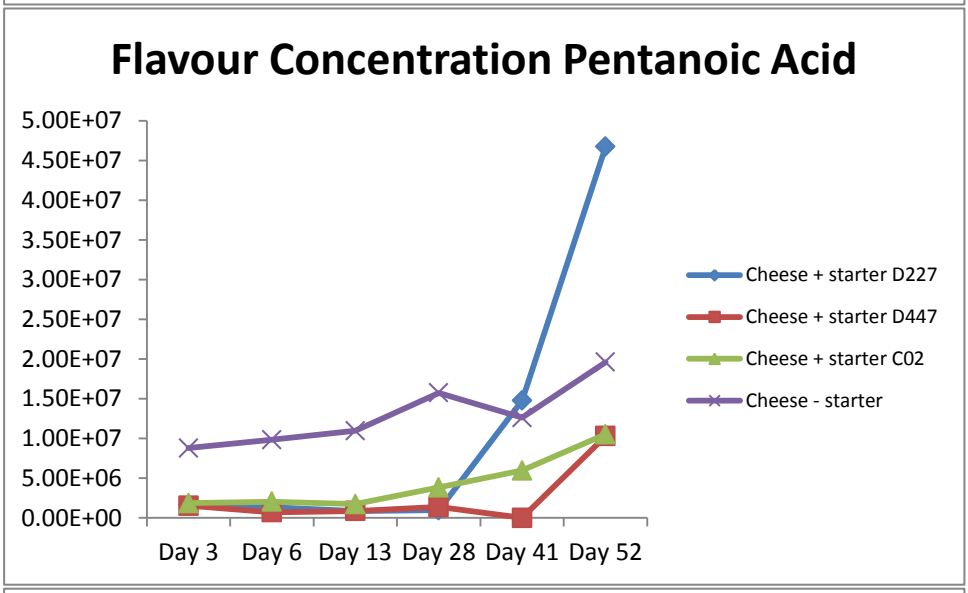


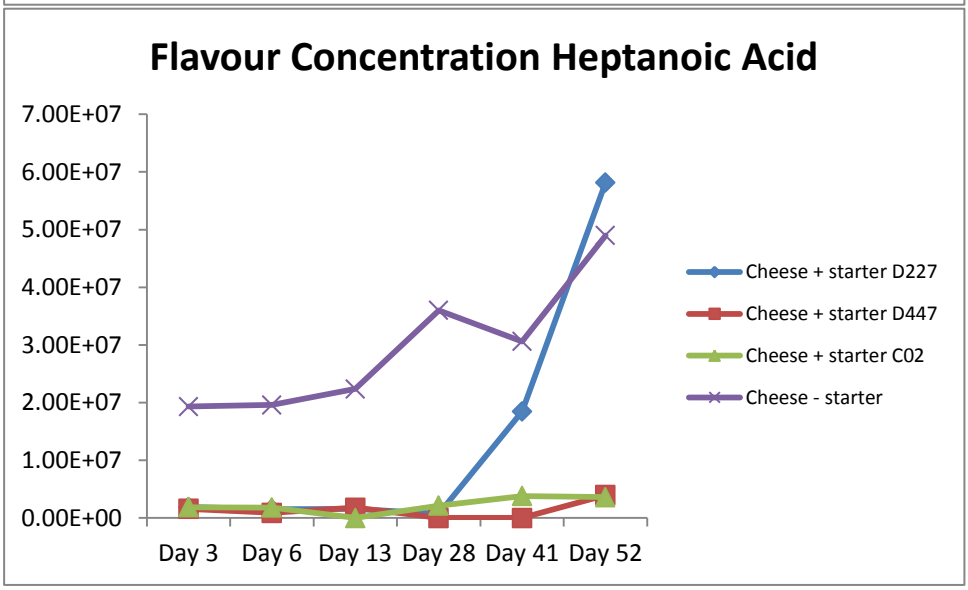

Flavour Concentration 2-Propanol,1-methoxy
1.80E+07
1.60E+07
1.40E+07
1.20E+07
1.00E+07
8.00E+06
6.00E+06
4.00E+06
2.00E+06
0.00E+00
Day 3
Day 6
Day 13
Day 28
Day 41
Day 52
Cheese + starter D227
Cheese + starter D447
Cheese + starter C02
Cheese - starter

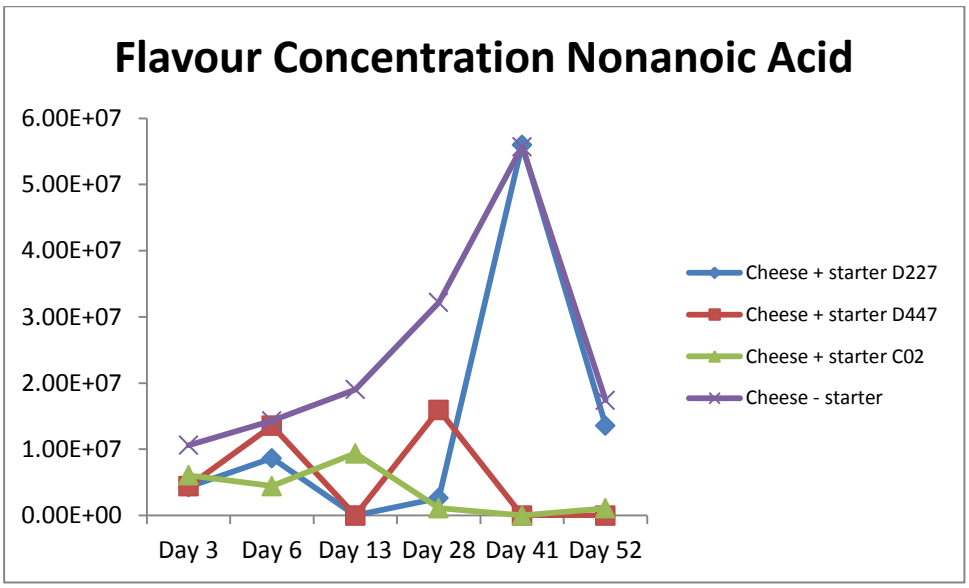

Flavour Concentration 1-Pentanol
2.50E+07
2.00E+07
1.50E+07
1.00E+07
5.00E+06
0.00E+00
Day 3
Day 6
Day 13
Day 28
Day 41
Day 52
Cheese + starter D227
Cheese + starter D447
Cheese + starter C02
Cheese - starter


Flavour Concentration 3-Pentanol
1.00E+07
9.00E+06
8.00E+06
7.00E+06
6.00E+06
5.00E+06
4.00E+06
3.00E+06
2.00E+06
1.00E+06
0.00E+00
Day 3
Day 6
Day 13
Day 28
Day 41
Day 52
Cheese + starter D227
Cheese + starter D447
Cheese + starter C02
Cheese - starter


Flavour Concentration Propanoic Acid
2.50E+07
2.00E+07
1.50E+07
1.00E+07
5.00E+06
0.00E+00
Day 3
Day 6
Day 13
Day 28
Day 41
Day 52
Cheese + starter D227
Cheese + starter D447
Cheese + starter C02
Cheese - starter


Flavour Concentration Pentanoic Acid
5.00E+07
4.50E+07
4.00E+07
3.50E+07
3.00E+07
2.50E+07
2.00E+07
1.50E+07
1.00E+07
5.00E+06
0.00E+00
Day 3
Day 6
Day 13
Day 28
Day 41
Day 52
Cheese + starter D227
Cheese + starter D447
Cheese + starter C02
Cheese - starter


Flavour Concentration Heptanoic Acid
7.00E+07
6.00E+07
5.00E+07
4.00E+07
3.00E+07
2.00E+07
1.00E+07
0.00E+00
Day 3
Day 6
Day 13
Day 28
Day 41
Day 52
Cheese + starter D227
Cheese + starter D447
Cheese + starter C02
Cheese - starter



*x-axis = Cheese ripening time; y-axis = Flavour concentration