

Formation Of Volatile And Non-Volatile Compounds In Cheese

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Abstract: Flavour development in cheese is a complex process in which major catabolic pathways involved. Initially, the curds of different cheese varieties have almost the same flavours, however the curd produce flavour compounds which lead to discrimination among cheese varieties in terms of flavour throughout ripening. The major biochemical pathways involved throughout ripening of cheese are the followings; liberation of FFA (free fatty acid), associated catabolic reactions, the degradation of the casein matrix to peptides and FAA (free amino acids), the reactions for catabolism of FAA and the metabolism of lactate and citrate. In this review, the general pathway for formation of volatile and non-volatile flavour compounds are stated and detailed knowledge as to products of amino acid catabolism, proteolysis, lipolysis, lactate and citrate metabolism well discussed.

Index Terms: Catabolism, Cheese, Lipolysis, Ripening, Proteolysis

1. INTRODUCTION

The ripening process of all the cheeses is very complex and includes both microbiological and biochemical changes to the curd resulting favourable flavour and texture. Cheese flavour is determined by the balance between many volatile and non-volatile components formed throughout ripening [69]. Microbiological changes to the curd throughout ripening involve the death and lysis of starter microorganism, the growth of nonstarter lactic acid bacteria. In many varieties of cheese, the secondary microflora is of great importance to the flavour and the texture. Some workers discussed microbiological changes throughout ripening of cheese and further discussion not made in this article [6, 8, 42, 88, 93, 98]. The biochemical changes throughout ripening of cheese include primary events and secondary events. The primary events include the metabolism of lactose and citrate, lipolysis and proteolysis. The secondary biochemical events, which follow primary events and contribute many volatile flavour compounds, include the metabolism of fatty acids and of amino acids [42].

2. EFFECT OF AMINO ACID CATABOLISM

Amino acid catabolism is important in all types of cheeses due to end products of the catabolism. The products produced at end of the catabolism include ammonia, amines, aldehydes, phenols, indole and alcohols. The reason of importance of these products is that they contribute formation of cheese flavour. Amino acid catabolism can be split into 3 stages. In the first stage, decarboxylation, deamination, transamination, desulphuration or hydrolysis of amino-acid side chains happen. Amino acids converted to amines by decarboxylation with removing CO₂; to amino acids by transamination or α -ketoacids by oxidative deamination with removing NH₄ (pyridoxal-5-phosphate-dependent enzymes).

In the second stage, first stage's products (amines and α -ketoacids) and amino acids are converted to aldehydes by effect of deaminases on amines. In the last stage, aldehydes produced in second stage reduced to alcohols or oxidised to acids. However, sulphur-containing amino acids have different path. In this path; amino acids first degraded to phenols, indole or CH₃SH, which then converted to sulphur compounds. At the end; some compounds; such as methanethiol other sulphur derivatives, are formed (42, 80, 83). Decarboxylation is the formation of amine from amino acid with the loss of CO₂. Concentration of each amine depends upon cheese's type (concentration of precursor amino acid), the cheese microflora and other factors including ripening temperature, pH, salt concentration [49]. Decarboxylation occurs at an acid pH (optimum about pH 5.5) and usually need PLP (Pyridoxal phosphate) as cofactor [42]. The main amines in cheeses are tyramine and histamine. These are produced from decarboxylation of Tyr and His [60]. Such a relationship between the concentration of amines and amino acids (FAA) has not been found. It could be due to decarboxylation rate of each FAA showing differences. Amines' concentration however can be increased by adjunct lactobacilli. Ney in 1981 [91] showed that some amines including acetamide, propionamide, butyramide, isobutyramide and isovaleramide exist in Cheddar, Emmental, German blue-mould cheese (Edelpilzkase) and Manchego cheese. Dumont, Adda and Roger [29] first found the presence of amines including methylamine, ethylamine, n-propylamine, isopropylamine, n-butylamine, 1-methylpropylamine, n-amylamine, iso-amylamine, anteiso-amylamine, ethanolamine and dibutylamine in Camembert cheese. Liardon et. al. [73] found the presence of 2,5-dimethyl-pyrazine, 2,6-dimethylpyrazine and ethyl-methyl-pyrazine in Swiss Gruyere Cheese. Meinhart and Schreier [84] found the presence of pyridine, pyrazine, 2,3-dimethylpyrazine and 2,3-diethyl-5-methylpyrazine in Parmesan. Sloom and Hofman [104] found 6 alkyl pyrazines in Swiss Emmental. The effect of Deamination in amino acid catabolism is the formation of ammonia and α -ketoacids with the loss of NH₄ by aminotransferases (pyridoxal-5-phosphate(PLP)-dependent enzyme). Even though the concentration of these could show differences among cheeses, in every type of cheeses, these two products of deamination could be found [42]. Br. linens, P.camemberti and G. candidum are the microorganisms which produce ammonia in cheeses including Camembert, Gruyere and Comte. α -keto-3-

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methylbutanoic and α -keto-3-methylpentanoic acids and lead to cheese-like odour in cheeses such as Cheddar, German blue-mould cheeses, Manchego, Parmesan, Gouda, Provolone, Camembert and Fontina. α -ketoacids produced then degraded further to volatile flavour compounds by four different paths. In first path; α -ketoacids enzymatically degraded to hydroxyacid by 2-hydroxyacid dehydrogenases [122]. This path can't form volatile compounds and lead the concentration of α -ketoacid to decreasing. Or α -ketoacids chemically degraded to volatile flavour compounds. Then, the compounds decarboxylated to aldehydes or oxidatively decarboxylated to carboxylic acids. In second path; α -ketoglutarate added to St Paulin-Type cheese by [120]. They saw that amino acid catabolism in cheese with α -ketoglutarate is faster than in the control. These workers stated afterwards that aminotransferase activity can be accumulated in cheese by the addition of α -ketoglutarate. In third path; Shakeel-Ur-Rehman and Fox [40] added α -ketoglutarate to cheese curd. At the end of ripening, flavour development found better in cheese with α -ketoglutarate than control cheese. In fourth path; Rijnen et. al. [101] followed different way to add α -ketoglutarate to cheese. They didn't use exogenous addition, distinctly; they modified *Lactococcus* strain with *Peptostreptococcus asaccharolyticus* to produce α -ketoglutarate from Glu by glutamate dehydrogenase. They found that α -ketoglutarate can be produced in this path. The effect of transamination is the formation of aldehydes. Free amino acids first transaminated intermediate imide which then form aldehyde by Strecker degradation. Just after ripening starts off, free amino acids decarboxylated to amine due to low pH. But later of the ripening, these amines oxidised to aldehydes by Stracker degradation at higher pH. Aldehydes are very important in formation of cheese flavour and thought to contribute the characteristic flavour of Cheddar and Parmesan. However, at high concentration, aldehydes rapidly transformed to alcohols. The Strecker reaction can form Strecker aldehydes including phenylacetaldehyde, isobutanal, 3-methylbutanal and methional from Phe, Leu&Ile, Val and Met, respectively with the loss of CO₂ [42]. The catabolic products of sulphur amino acids contribute to characteristic cheese flavour. Especially, they are important in smear- and surface-ripened cheeses. Dimos et. al. [25] analysed full-fat and reduced-fat cheddar to find a correlation between cheese flavour and concentration of methanethiol, which is main sulphur compounds found in cheese, and they showed that there is a correlation between methanethiol concentration and characteristic cheese flavour by stating that reduced-fat cheddar has the lack of characteristic cheese flavour. Sulphur compounds found in cheese are methanethiol (CH₃SH), hydrogen sulphide (H₂S), dimethylsulphide (DMS: CH₃SCH₃), dimethyldisulphide (DMDS: CH₃SSCH₃), dimethyltrisulphide (DMTS: CH₃SSSCH₃) and carbonyl sulphide (O=C=S) [82]. Br. linens can enzymatically produce methanethiol in smear- and surface-mould-ripened cheeses such as Camembert. However, some types of cheeses do not have surface microflora [42]. Some cheeses without surface microflora can chemically produce methanethiol by their starter or non-starter due to the lack of coryneforms. H₂S's concentration is on increase throughout ripening of cheese [78]. Barlow et. al. [5] first found a correlation between cheese flavour and H₂S

concentration. There are more volatile compounds in cheese. These include Methional (Camembert, premium-quality Cheddar), ethyl-3-methylthio-1-propanoate (Parmesan), S-Methylthioacetate (Limburger cheese), 3-Methylthio-propanal (Cheddar and Parmesan), Benzothiazole (Parmesan), 2,4-Dithiapentane (Camembert) and carbonyl sulphide [112].

3. EFFECTS OF PROTEOLYSIS

Proteolysis is a complex biochemical event which happens throughout ripening of cheeses. Proteolysis in cheese is described as the formation of large water-insoluble peptides and smaller water-soluble peptides (β - , α - and s-casein peptides and other minor proteins) by the action of catalysts including residual coagulant, the milk (plasmin) and, peptides enzymes from starter (LAB) and non-starter (NSLAB). In many cheeses, α s1 -casein faster hydrolysed than β - casein. In blue-veined cheeses; both α s1 -casein and β - casein completely hydrolysed at the end of ripening. The pattern of proteolysis may demonstrate differences among cheeses varieties. Differences could be caused by moisture content, temperature, pH, duration of ripening, cooking temperature and pH at draining [42]. Proteolysis' final products including keto acids, aldehydes or carboxylic acids, ketones, lactones, esters, alcohols, aldehydes, pyrazines, sulphurous and carbonyl compounds, free amino acids (FAA) and salts can contribute characteristic cheese flavour. Ardo and Petterson [2] stated that the composition of amino acid fraction is important for the development of desired cheese flavour and showed that the level of peptides and free amino acid soluble in cheese can be calculated in 5% phosphotungstic acid (PTA). This calculation is used as an indicator of flavour development at any stage of ripening by finding the net outcome of the liberation of amino acids from casein. Fox and Wallace [38] added that the concentration of FAA , which is responsible of brothy, bitter, nutty and sweet taste with peptides, is not correlated with the flavour of cheese by demonstrating that even though different cheeses have approximately the same concentration of FAA, they have different flavours. It may be due to different proportion of FAA. For example, the main amino acids found in cheddar cheese are Glu, Leu, Arg, Lys, Phe and Ser. Concentrations of which are on rise during ripening with the exception of Arg which was reported to be on decrease during ripening [82]. McSweeney et. al. [81] demonstrated that amino acids have different threshold (mg.100mL⁻¹) and taste. These workers showed that Gly, Ser, Thr, Ala, Lys and Pro have sweet taste; His, Asp and Glu have sour taste; Ser, Asp and Glu have umami taste; Thr, Arg, Met, Lys, Val, Leu, Pro, Phe, Tyr and Trp have bitter taste. No salt taste was detected in amino acids [82, 83]. Proteolysis is important in forming textural changes of the cheese curd, decreasing water activity though water binding by the new carboxylic acid and amino groups produced from hydrolysis which increases pH (especially in surface-mould ripened varieties), contributes flavour and off-flavour of cheese by producing peptides and amino acids, liberates substrates such as amino acids for secondary catabolic changes (including deamination, decarboxylation, transamination, desulphuration, catabolism of aromatic compounds such as phenylalanine, tyrosine, tryptophane and reactions of amino acids with other compounds), changes the cheese matrix enabling

the release of sapid compounds throughout mastication and softens cheese texture during ripening because of hydrolysis of the casein matrix of the curd [42]. Proteolysis in cheese during ripening is catalysed by residual coagulant (chymosin, pepsin and plant or fungal acid proteinases), the milk (plasmin, cathepsin D), the starter LAB, the nonstarter (NSLAB) or the secondary starter (P. camemberti, P. roqueforti, Propionibacterium spp., Br. linens and other coryneforms) and exogenous proteinases and/or peptidases which are used to increase the speed of ripening [82]. Residual coagulant, mainly chymosin, is a major source of proteolytic enzymes in many cheeses. Chymosin is used to clot milk and has 88-94% milk clotting activity. The main role of chymosin (like other coagulant) in cheese making is to hydrolyze the Phe105-Met106 bond of the micelle-stabilizing protein (K-casein) during the coagulation of milk. Most of the coagulant activity added to milk is lost in the whey but, Upadhyay et. al. [110] stated that up to 30% of the coagulant activity added to the milk remains active in the curd depending upon some factors including enzyme type, cooking temperature and pH at whey drainage. For example; chymosin can make little contribution to ripening in high-cooked cheese (such as Emmental) because in this kind of cheeses, chymosin is extensively denatured. In solution, chymosin separates β -casein at 7 sites which are close to the hydrophobic-terminal β -casein, and this is able to produce short hydrophobic peptides whose taste is bitter. The separation of β -casein happens in the sites including Leu192 – Tyr193. The main site of chymosin action on α s1-casein is Phe23 – Phe24 [83]. This cleavage site can produce small peptide which can be easily hydrolyzed by starter proteinases. Chymosin also separates α s1-casein in different sites especially including Leu101 – Leu102. α s2-casein is more stable to hydrolysis by chymosin than α s1-casein. Even though para-K-casein has several separation sites, it cannot be hydrolyzed either in solution or in cheese [45]. The reason of this might be due to secondary structures existing in K-casein. The dominant milk proteinase is plasmin. The plasmin active includes active enzyme (plasmin), its zymogen (plasminogen), plasminogen activators and plasmin's inhibitors. All of them exist in milk and, exempt of plasmin's inhibitors, are associated with casein micelles when plasmin's inhibitors present in the serum phase and because of that, plasmin's inhibitors are lost on whey drainage. Plasmin is active at around pH 7.5 and 37°C and affective at peptide bonds on C-terminal side of lysyl and arginyl residues. The physiological role of plasmin in the blood is to degrade fibrin clots during blood-clotting process and plasmin can be produced from plasminogen and inactive precursor. Plasminogen can be activated by somatic cell proteinases (called as plasminogen activators). Plasmin specific activity is on peptide bonds of Lys-X and Arg-X, furthermore, plasmin degrades β -, α s2- and α s1- caseins respectively. In many cheeses, α s1-casein faster hydrolysed than β -casein but; in Swiss-type cheeses; hydrolysis of β -casein is faster than that of α s1-casein. It may be due to the role of plasmin and/or denaturation of chymosin throughout cooking (high plasmin activity and weak chymosin activity). Plasmin can form γ -caseins and proteose-peptones (N-terminal fragments) by degrading of β -casein and α s2-casein. Plasmin hydrolyses β -casein at three sites. These

are Lys28-Lys29, Lys105-His106 and Lys107-Glu108. Plasmin activity is high in the cheeses in which high temperature was used. For example; Parmigiano-Reggiano cheeses ripened at around 20 °C for 24 months. Because of that; plasmin activity is much higher than chymosin activity in this cheese due to the denaturation of chymosin during ripening [42, 60, 80, 83, 91, 111, 122]. Other proteolytic enzymes like plasmin from somatic cells can also produce proteolysis activity in cheese. These are Cathepsin B,D,G,H ,L and elastase [57]. Kaminogawa et al. [55] first found in 1980 that Milk also has an acid proteinase (Cathepsin D) which is heat-stable (can be inactivated at 700 C x 10 min.) with the pH of 4. McSweeney et. al. [80] then added to Kaminogawa et. al. that Cathepsin D (Aspartyl Proteinase) has very poor milk clotting activity even though its activity on α s1-casein is the same with chymosin. Cathepsin B also exists in milk but the presence of other Cathepsins has yet to be discovered [42, 82]. Elastase degrades α s1-casein and β -casein in the first 6 h of incubation of cheese. Elastase activity can produce peptides which can be detected by urea-polycrylamide gel electrophoresis (PAGE). Because of that, elastase is very important in cheese-making from raw milk [48, 122]. LAB, including Lactococcus, Lactobacillus and Streptococcus, show weak proteolytic activity. The LAB have complex proteolytic system which needs many amino acids to liberate for grow to desired level in milk (10⁹-10¹⁰ cfu.mL⁻¹). LABs don't produce carboxypeptidases. LAB system produces peptidases which are important for the final stage of proteolysis throughout the ripening of cheeses owing to liberation of free amino acids which are used as substrates for catabolic reactions. These are a cell envelope-associated proteinase (PrpP), many intracellular oligoendopeptidases (PepO and PepF), 3 aminopeptidases (PepN, PepC and PepG), a glutamyl aminopeptidase (PepA), a pyrrolidone carboxyl peptidase (PCP), a leucyl aminopeptidase (PepL), a prolyl-dipeptidyl aminopeptidase (PepX), a proline iminopeptidase (PepI), aminopeptidase P (PepP), prolinate (PepR), a prolidase (PepQ), a general peptidase (PepV), a general tripeptidase (PepT), peptide system and amino-acid transport system. PrpP produces small peptidases in cheese from the hydrolysis of larger peptides formed by α s1-casein and β -casein. PepA is specific for Glu and Asp residues; PepL moves pyroglutamic residues away from N-terminal of peptides. PepX liberates X-Pro dipeptides from N-terminus of peptides. PepP catalyses liberation of N-terminal amino acid from peptides whose sequence is X-Pro-Pro-(X)_n or X-Pro-(X)_n. PepR can cleave Pro-X dipeptides and PepQ is able to hydrolyses X-Pro dipeptides [42]. The main proteolytic enzyme is Lactococcus which is lactocepin. Lactocepin is a serine proteinase with 5.5-6.5 pH. Lactocepin degrades the caseins to produce short peptides which makes lactococcal cell to grow in milk [42]. Fox and McSweeney [37, 42] first classified Lactoceptins into 2 groups; P1- and P3- type proteinases. P1- type can degrade rapidly β -casein yet slowly α s1-casein and K-caseins. P3- type hydrolyses β -casein differently when degrading fast α s1-casein and K-caseins. NSLAB (non-starter) exist initially at very low level, it grows very fast within 4 weeks of ripening (NSLAB can reach around 10⁷ cfu.g⁻¹) and remain steady afterwards. Hence, NSLAB dominate the viable microflora of Cheddar, extra-mature

Dutch and other cheeses depending upon the death rate of the starter. The proteolytic activity of NSLAB is deemed to supplement the proteolytic activity of the starter because of the production of peptides with almost similar molecular weights and FAA [82]. A secondary microflora (secondary starter) can be added intentionally or encouraged to grow by changing environmental conditions in many cheese variety. It can show a wide range of functions which depends upon the organisms used. For example; in Dutch-type cheese, Cit+ lactococci and Leuconostoc spp. used as a secondary starter to especially produce diacetyl, acetoin and CO₂. In Swiss-type cheeses, Propionibacterium spp.; which even though shows weak proteolytic activity, shows highly peptidolytic activity; used as secondary starter. However, in cheddar cheeses, a secondary starter is not used. Instead, an adjunct starter (Lactobacillus) used to accelerate ripening and volatile forming. Br. Linens excretes extracellular proteinases and aminopeptidases. These lead to proteolysis producing free amino acids on surface smear-ripened cheeses. Aspartyl proteinases and metalloproteinases secreted as an extracellular proteinase by both P.Roqueforti and P. Camemberti. But differently between them; P.Camemberti has an intracellular acid proteinase when P.Roqueforti carboxypeptidase [82].

4. EFFECT OF LIPOLYSIS

Milk fat is important in the development of characteristic flavour of many cheeses. Mulder [89] in 1952 suggested the Component Balance Theory once no compound or class was identified. The theory suggests that a balance of flavours made up by some compounds, which can exist in certain level, contributes to cheese flavour. However, Aston and Dulley [4] in 1982 demonstrated that there is a single compound or class of compounds which are responsible for characteristic cheese flavour in Cheddar cheese. Woo and Lindsay [118] and Molimard and Spinnler [88] supported the study of Aston and Dulley. These workers found that FFAs contribute to characteristic cheese flavour in hard Italian cheese and methyl ketones contribute to characteristic cheese flavour in mould ripened cheeses, respectively. McCarthy et. al. [18, 42, 82] stated that cheeses can't develop their characteristic flavour if they are made from milks in which its fat has been taken away or replaced. Long-chain FFAs (> 12 carbon atoms) play an effective role in cheese flavour due to their high perception threshold, in contrast, short- and intermediate-chain fatty acids (C4:0-C12:0) have much lower perception threshold. However, characteristic cheese flavour is given by short- and intermediate-chain fatty acids when liberated. The impact of free fatty acids on cheese flavour depends upon specificity of lipase, cheese pH, and specificity of free fatty acids. Butanoic acid contributes rancid and cheesy flavour in cheese when octanoic acid can contribute to several identified flavours including wax, soap, goat, musty, rancid and fruity. Hexanoic acid has identified two flavour contributions (pungent and blue cheese) to cheese. In addition to direct contribution of FFAs to cheese flavour; as discussed later, FFAs are also precursors for the production of volatile flavour compounds. More studies as to taste description and threshold values of each FFAs need to be done [42]. Lipids presence in milk can undergo two different degradations (Oxidative or Hydrolytic Degradation) [50]. Malin and Tunick [75] stated that even though oxidative

degradation of cheese can cause off-flavour which is identified as hydrolytic rancidity, this degradation is limited due to weak oxidation/reduction potential (about 250 mV) and the presence of natural antioxidants (Vitamin E) [82], hence, little attention is given to lipid oxidation of cheese. However, hydrolytic degradation of milk fat, namely triglycerides, to free fatty acid, glycerol, mono- and diglycerides is important in development of cheese flavour during ripening. Lipolysis in cheese occurs due to the presence of lipolytic enzymes which splits ester linkage between a fatty acid and glycerol core of triacylglyceride. Lipolytic enzymes classed as esterases or lipases with regards to three main factors: First, length of the hydrolysed acyl ester chain, second, physico-chemical nature of the substrate and third, enzymatic kinetics. Once lipases can hydrolyse acyl ester chain of 10 or more carbon atoms, esterases can only hydrolyse acyl ester chains of more than 2, less than 8 carbon atoms. Lipases can hydrolyse emulsified substrates when esterases just hydrolyse soluble substrates in aqueous solutions. Esterases have Michaelis-Menten type kinetics when lipases have interfacial one of the kinetics [15]. Roquefort, blue-mould and Parmesan cheeses have higher FFA concentration hence, lipolysis in these cheeses is extensive. However; extensive lipolysis of FFA leads to rancidity in cheeses. Extensive lipolysis accepted undesirable in some type of cheeses, such as Cheddar, Gouda and Swiss-type due to rancidity taste. In these cheeses; low lipolysis is balanced with the products of proteolysis and other reactions [36, 114]. Lipases, which is an enzyme that catalyses the hydrolysis of cheese, originate from 6 sources. The sources include milk, rennet preparation and cheese microflora (starter, adjunct starter, non-starter bacteria and exogenous lipases if used) [42]. Milk contains indigenous lipase (lipoprotein lipase, LPL) with esterases. LPL exists in milk due to leakage through the mammary cell membrane from blood. The specificity of LPL is hydrolyse of medium-chain triacylglycerides (MCT). The hydrolyse of LPL is two times faster on MCT containing C6:0, C8:0, C10:0 and C12:0 than long chain triacylglyceride (LCT) containing C16:0, C18:0, C18:1, C18:2, C18:3 and C20:0 [21]. LPL liberates fatty acids from sn-1 and sn-3 positions of mono-, di-, triglycerides and sn-1 position of phospholipids. Under optimum conditions, the concentration of LPL in milk is good enough for rancidity perception within 10s [82, 83]. But Fox et. al. in 2004 [41] added that it can't happen under normal circumstances, because, fat content in milk is protected from LPL action by milk fat globule membrane (MFGM). If anything including mechanical damage (agitation, foaming, homogenization and many more) damages MFGM, lipolysis can occur with the result of off-flavour [39, 41]. LPL is not heat-stable enzyme and lost most of its activity in pasteurized milk. To be completely inactivated, 780x 10 s is needed [27]. Chavarri et. al. (1998) [14] found that LPL activity was on decrease throughout lactation. This may happen due to the effect of lactation on pH. As lactation progresses, pH is on decrease. Even though rennet extracts, which can be used in cheese making, are free of lipase activity, rennet pastes show activity on lipolysis. Rennet pastes (Pregastric esterase, PGE) have been used in cheeses, such as; Provolone, Romano and traditional Greek feta. The rennet pastes used are produced on the farm by macerating the stomach of young dairy animals, such as; calf and lamb in

to a paste containing chymosin and pregastric esterase (PGE). PGE is active at 32-42°C, pH 4.8-5.5 when 0.5 M NaCl present in the environment [18]. PGE's activity on free fatty acids is seen at sn-3 position of triglycerides and is specific for short chain acids. Chaudhari and Richardson in 1971 [13] described a new lipase, named as gastric lipase, in an extract of cleaned gastric tissue by finding superior quality of Cheddar and Provolone. However, later on, Nelson et. al. (1977) [90] the new lipase is indefinite. Many bacterially ripened cheese; such as Cheddar and Gouda, is lack of high lipolytic activity due to pasteurization. But; lipolysis occurs during ripening due to enzymes from the starter and the non-starter. Glucono- δ - lactone; used to chemically acidify cheeses; can produce low level of free fatty acids (FFA) throughout ripening [46]. It has been found a correlation between level of lipolysis of Cheddar and its starter microflora [18, 19]. A fast-lysing starter leads to higher level lipolysis in cheese than a slow-lysing starter. LAB, even though weaker lipolytic than other cheese related microorganisms like Penicillium, are responsible for the liberation of high level of FFA when available in high cell numbers and/or during extended ripening time [18]. Lipolytic enzymes of LAB are intracellular and active at pH 7-8.5 and around 35°C. Lipolytic enzymes of LAB (especially Lactococcus and Lactobacillus spp.) are most effective on short-chain fatty acids. El-soda et. al. (1986) [31] discovered lactobacilli spp. having esterolytic activity show activity on substrates with up to five carbon atoms. But, none of lactobacilli spp. tested in the analyses could hydrolyse o- and p- nitrophenyl (p-NP) substrates with more than 6 carbon atoms. Cheddar cheeses made with Lc. lactic subsp. AM2 had higher level of odd-numbered C3-C15 methyl ketones than the cheese made with Lc. lactic subsp. cremoris HP. What this finding shows is that properties of starter strain can have an effect on level of lipolytic end products in cheese [18, 42, 62, 82]. Later, Fox et. al. [42] added to these workers' finding that strain AM2 is more autolytic than strain HP. That leads to secondary proteolysis much higher in strain AM2 than strain HP. The more secondary proteolysis happens, the more extensive release of intracellular peptidases can be made in cheeses. Collins, McSweeney and Wilkinson [18] found that Cheddar cheese made with Lc. lactis subsp. cremoris AM2 can develop much higher level of FFA including octanoic acid (C8:0), tetradecanoic acid (C14:0), hexadecanoic acid (C16:0) and octadecanoic acid (C18:0) during ripening than cheeses made with Lc. lactic subsp. cremoris HP. Also, these workers noticed that cell free extract made from both strains showed similar level of activity both on lipase and on esterase substrates, concluded that this is evidence for correlation between autolysis of starter bacteria and lipolysis in cheese. Lipolytic activity of adjunct starters including coryneform bacteria (especially Brevibacterium linens), G. Candidum, Propionibacterium freudenreichii and Penicillium spp. affect level of lipolysis. Bonomo, Salzano and Cafaro in 2015 [6] stated that Brevibacterium linens leads to extensive lipolysis in smear-surface ripened cheeses and, ripening environment and the strain used can affect level of lipolysis in Pecorino di Filiano cheese. Sorhaug and Ordal in 1974 [107] found that five strains of Br. linens have esterolytic and lipolytic activities. G. Candidum, which develops on the surface, can lead to much acidification of cheeses at early stages of ripening

and can develop best flavour profile of cheese in semi-hard goat's milk cheese [102]. Propionic acid bacteria (PAB) are much more lipolytic than LAB. Propionibacterium freudenreichii subsp. shermanii (exist in Swiss-type cheese) possesses an intracellular lipase active at pH 7.2 and 47°C [94]. Dupuis, Corre and Boyaval (1993) [30] used some strains of propionibacteria for esterolytic activity and lipolytic activity against several substrates and demonstrated secretion of extracellular esterase and lipase activity during ripening for the first time. But, the extent to which this activity could be raised as result of cell lysis was not studied. Ojala et. al. (2017) [93] found that Propionibacterium freudenreichii can show activity in cold environment. Lipolytic activity of Propionibacterium freudenreichii is mainly on short-chain fatty acids and PF-279 is the main lipolytic esterase of P. Freudenreichii. Penicillium spp. is one the most lipolytic organisms as to cheese ripening [8]. Penicillium spp. are essential lipolytic agents in the mould-ripened cheeses including Brie, Camembert and Roquefort [82]. P. roqueforti possess two lipases, one of which is active at pH 7.5-8.0 and the another is active at more alkaline pH [92]. The first one shows more activity on trihexanoic acid and the last one shows activity mainly on tributanoic acid [86]. P. camemberti has just one extracellular lipase, which is active at pH 9 and around 35°C, and which shows activity more on tributanoic acid [65]. FFA also act as precursor for some catabolic reactions which result in formation of flavour and aroma compounds including methyl ketones, lactones, esters, alkanes and secondary alcohols [82]. Methyl ketones (especially alkan-2-one and nonan-2-one) play an effective role in formation of characteristic cheese flavour, especially in Blue cheese and Camembert cheese. Flavour thresholds of methyl ketones range from 0.09 μ g 100 g⁻¹ for heptan-2-one in water to 4.09 to 50.0 mg 100 g⁻¹ for propan-2-one in water [42]. Dartley and Kinsella (1971) [20] represented that total concentration of methyl ketones in blue-veined cheese was on increase throughout up to 70 day of ripening, but was on decrease afterwards. Thierry, Maillard and Le Quere in 1999 [109] found that total concentration of methyl ketones was on increase during ripening of Emmental cheese. Formation of methyl ketones in cheese, made from action of mould lipases including Penicillium roqueforti [113], Penicillium camemberti and Geotrichum candidum [68, 88]. In addition to mould lipases, spores and vegetative mycelia also can form methyl ketones [11]. Penicillium spp. can metabolise FFA by four steps. In first step; release of FFA by lipases, in second step, oxidation of the released FFA to α - ketoacids. In third step, decarboxylation of keto acids to alkan-2-ones and in the last step, alkan-2-ones degraded to the corresponding alkan-2-ol. The last step is irreversible under anaerobic conditions [61]. Lawrence in 1966 [68] found that Penicillium roqueforti can form methyl ketone when glucose and amino acids are presence. Chaler and Crouzet in 1993 [10] added to Lawrence that Penicillium roqueforti spores can produce methyl ketones when long chain fatty acids, including hexadecanoic acid and cis,cis-9,12-octadecadienoic acid, are presence. Kinsella and Hwang in 1976 [61] first found a positive correlation between FFA level and the concentration of methyl ketones produced and spores of P. roqueforti can oxidize FFAs with 2-12 carbon atoms. Fan, Hwang and Kinsella in 1976 [34] monitored that high concentration of FFA show toxic effect

to *P. roqueforti* spores, and when FFA exist at low concentration in cheeses, FFA mostly oxidized to CO₂ and very low level of methyl ketones can be formed [83]. Chalier and Crouzet (1998) [10] stated that formation of methyl ketones is strain-dependent by showing different amount of methyl ketone production with different strain in copra oil. The rate of formation of methyl ketones in cheese depends on temperature, pH, physiological state of the mould and the concentration of fatty acids. Also, Kinsella and Hwang in 1976 [61] suggested that FFAs are not the only producer of methyl ketones and found that concentrations of heptan-2-one and nonan-2-one were not proportional to quantities of octanoic and decanoic acids present in milk fat. de Llano et. al. in 1992 [24] discovered that the concentrations of heptan-2-one and nonan-2-one were on increase throughout ripening up to max 60 days. Thereafter, their concentration began to be on decrease. Esters and thioesters are also products of fatty acid catabolism. Esters are highly flavoured and formed from the reaction between FFA and alcohols. Esterification reactions forming esters happen between short- to medium- chain fatty acids and the alcohols usually derived from lactose fermentation or amino acid catabolism [42]. Ethyl esters however formed from esterification of ethanol with acetyl-coenzyme A [82]. Arora, Cormier and Lee (1995) [3] found that all of the fatty acid esters existing in Cheddar are ethyl derivatives. Engels et. al. (1997) [32] studied over various cheese varieties and found high level of ethyl butanoate, which contributes fruity flavour, in cheese. However, the fruity flavour is undesirable in Cheddar cheeses [82]. Moreover, *Geotrichum candidum* and *Pseudomonas fragi* are also able to form esters, producing melon odour and fruity flavour, respectively [52, 88]. Thioesters formed from a reaction between FFA and free sulphhydryl groups. Law (1984) [67] demonstrated that thioesters have cheesy flavour. Lamberet, Auberger and Bergere (1997) [66] studied over ability of different strain of coryneform bacteria, *Micrococcaceae*, *Lactococcus lactis* and *Leuconostoc* spp. to produce S-methyl thioesters. All the strains studied produced S-methyl thioesters, which contribute high level of characteristic flavour to various smear-ripened soft cheeses; such as, Tilsit, Limberger, and Havarti. Meinhart and Schreier (1986) [84] identified 38 esters in Parmigiano-Reggiano cheese. Later, Imhof et. al. in 1994 [51] identified 14 different esters in Emmental cheese. Differently, Thierry et. al. in 1999 [109] found that when aqueous phase of Emmental cheese studied by dynamic head space analysis, ester level was on increase throughout warm room stage of ripening and even some esters showed incredible increase (4-20 fold) between 3 days to 62 days. Secondary alcohols; for example, 2-pentanol, 2-heptanol, 2-nonanol; formed in cheese via enzymatic reduction of methyl ketones by *Penicillium* spp.. The study of Urbach in 1993 [111] found in Cheddar cheese that 2-propanol and 2-butanol can be formed by acetone and butanone, respectively. de Llano et. al. (1992) [24] identified that the main alcohols of Blue cheese are 2-heptanol and 2-nonanol. Thierry also found [109] that the level of secondary alcohols was on rise when Emmental was in aqueous phase throughout ripening. Lactones formed by intermolecular esterification of hydroxy fatty acids with the loss of water and at the end of esterification, ring structure formed. α - and β - lactones are highly reactive in cheeses, they have three- and four-sided rings,

respectively. In contrast, δ - and γ -lactones are stable in cheese; they have five- and six-sided rings, respectively. Lactones have a strong aroma but although don't contribute to characteristic cheese flavour, can contribute to overall cheese flavour [82]. Dirinck and De Winne (1999) [26] found that lactones can contribute buttery flavour to cheese. The formation of lactones in cheese has received too little attention. It is highly possible that the concentration of lactones in cheese correlate with the extent of lipolysis. Wong et. al. in 1975 [116] reported that lactones may be formed from keto acids after reduction to hydroxyacids. Eriksen [33] added to the study of Wong et. al. that δ - and γ -lactones spontaneously formed from the corresponding δ - and γ -hydroxyacids. Later, some workers also have suggested their idea as to how lactones formed. Chalier and Crouzet in 1992 [12] stated that C12:0 lactones could be formed from long-chain unsaturated fatty acids (C18:1 and C18:2) by *P. Roqueforti* and vegetative mycelium. Dufosse et. al. in 1994 [28] claimed that lactones may be formed from unsaturated fatty acids by the action of enzymes (lipoxygenases or hydratases). The study of Urbach in 1997 [113] found that sweet flavoured lactones was higher in milk from grain-fed cows than in milk from pasture fed cows. Rehman et. al. in 2000 [100] summarized that potential lactone production is up to feed, season, stage of lactation and breed. Some workers identified lactones presence in some cheeses [1, 43, 47, 54, 85, 87], but, there needs to be studied over the effect of these factors on lactones production and possible mechanism of lactones formation in cheese. Aldehydes formed from amino acids by transamination, which result in production of imide that is later decarboxylated. Keeney and Day in 1957 [56] suggested a different way to formation of aldehydes by Strecker degradation of amino acids. Some workers demonstrated that aldehydes also can be microbially formed [9, 77]. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* spp. *bulgaricus* have an enzyme, named as theonine aldolase, which directly catalyse threonine and glycine to acetaldehyde. Morales et. al. in 2004 [88] studied over the production of flavour compounds in fresh cheese with 10 different strains of enterobacteriaceae. These workers found that all the strains studied can significantly form aldehydes.

5. EFFECT OF LACTATE METABOLISM

Lactose metabolism to lactate is important in cheese making. Lactose is metabolized in two different ways; glycolytic or phosphoketolase. Which pathway is used in lactose metabolism depends upon starter type. The products of the metabolism are L- or D- lactate; or both of them with some other products, for example; ethanol. The importance of lactate in cheese making is it can contribute flavour of acid-curd cheeses and flavour of ripened cheese types especially at first stage of maturation. Acidification of cheeses is important in determining buffering capacity and then the growth of microorganisms and activity of enzymes, which are used in cheese making, throughout ripening [42, 82, 97]. Lactate metabolism is extensive in surface mould-ripened cheeses, for example; Camembert and Brie. Firstly, mesophilic starter bacteria can produce lactic acid; which then metabolized by secondary microflora (*Geotrichum candidum*, *Debaryomyces hansenii*; followed by *Penicillium camemberti* and coryneform bacteria like *Brevibacterium*

linens respectively); in the curd. The yeasts and moulds can swiftly metabolise lactate to CO₂ and H₂O and that leads to pH of cheese surface increases. Deacidification of the cheese surface affects in the growth of coryneform bacteria, plasmin activity (optimum at alkaline pH) and cheese texture. The same deacidification caused by lactate metabolism by yeasts and moulds happens in smear-ripened cheeses. Deacidification is important, because; it affects growth of coryneform bacteria and formation of characteristic cheese flavour during ripening [42, 82, 97]. Lactate produced at the end of lactose metabolism can be further metabolized by various pathways to many compounds which contribute to flavour [108] NSLAB of some cheeses; such as cheddar and Dutch-type, isomerizes the L-lactate to D-lactate. The production of D-lactate is much higher in cheeses produced from raw milk. The difference between L-lactate and D-lactate is the solubility of Ca-D-lactate is less than that of Ca-L-lactate. Lactate is oxidized to acetate and CO₂ by NSLAB in hard cheeses. Oxidization of Lactate depends on the size of cheese, oxygen permeability of rind of cheese and package material. However, the extent of oxidization of cheese is weak due to low redox potential of cheeses (around 250 mV) [42, 48, 82, 97].

6. EFFECT OF CITRATE METABOLISM

Citrate and Lactose metabolisms are subject to the same metabolic regulation. Milk contains around 1750 mg citrate per litre and most of which (around 94%) is in soluble phase. Hence, most of citrate lost on whey drainage throughout cheese making. The amount of citrate available in cheese curd is three times higher than in the whey. Cheddar curd has 0.2-0.5 % citrate (10mmol.kg⁻¹) [82]. Metabolism of citrate is optimum at pH 4.5 [42, 48, 62, 110]. Citrate metabolised by citrate-positive (Cit⁺) strains of lactococci. In Cheddar cheese, the number of lactobacilli is low at initial steps, but is on increase to significant number (from 10-10² cfug⁻¹ to 1x10⁸ cfug⁻¹) within 8-12 weeks of ripening [53]. Cit⁺ microorganisms don't use citrate as an energy source, yet citrate metabolised with lactose and some other sugar. Metabolism of citrate happens faster when lactococci spp. co-metabolise with galactose than with glucose or lactose [58]. Moreover, *Leuconostoc mesenteroides* ssp. *Cremoris* and *Ln. Lactis* can metabolize citrate. However, other LAB used as starter can't metabolize citrate [83]. When citrate metabolized, some enzymes play a role in this metabolism. These are; pyruvate-formate lyase, pyruvate dehydrogenase, α -acetolactate, acetoin reductase and α -acetolactate decarboxylase. The final products of citrate metabolism by Cit⁺ lactococci and *Leuconostoc* spp. show differences [42, 48, 62, 110]. The products of the metabolism by Cit⁺ lactococci are CO₂ and flavour compounds (especially diacetyl). The CO₂ produced at the end of the metabolism is responsible for small eyes formation found in Dutch-type cheeses, however CO₂ produced from citrate metabolism causes undesirable openness in Cheddar and Cottage cheese. Flavour compounds; diacetyl, acetate, acetoin and 2,3-butanediol, can contribute the formation of characteristic cheese flavour. Diacetyl can be used to form acetoin, 2,3- butanediol and 2- butanone [25]. Diacetyl produced from acetate metabolism in small amount when acetoin production is 10 to 50 fold higher than that of

diacetyl [42, 48, 62, 110]. The products of the metabolism by *Leuconostoc* spp. are lactate and acetate. Pure cultures of *Leuconostoc* spp. can't produce acetoin and diacetyl. Pyruvate is an intermediate for citrate metabolism as lactose metabolism and the pyruvate produced from citrate metabolism is diverted to lactate and acetylphosphate (via acetyl-CoA). Acetate produced from acetylphosphate with 1 mol ATP. However, in mixed culture, *Leuconostoc* spp. can produce diacetyl and acetoin most likely due to the ability of *Leuconostoc* spp. to produce lactose, which decreases pH below 5.5 [72]. NSLAB can also metabolize citrate to acetoin, acetate and diacetyl. Thomas in 1987 observed that the level of citrate in Cheddar curd was on decrease to trace level throughout 6 months of ripening. It may be due to NSLAB action on the curd [72].

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