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NOVEL STRATEGIES FOR OPTIMIZATION OF THE CHEDDAR CHEESE MANUFACTURING PROCESS

Thesis presented by

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BSc in Biological Engineering (Beijing Technology and Business University)

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For the degree of

Doctor of Philosophy

(PhD in Food Science and Technology)

2018

DECLARATION BY THE CANDIDATE

Novel Strategies for Optimization of the Cheddar Cheese Manufacturing Process

Jia Hou

I hereby declare that work described in this thesis is my own and has not been submitted for another degree, either in University College Cork, or elsewhere.

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Jia Hou

2018

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THESIS ABSTRACT

The effects of Cheddar cheese quality has been studied previously on pH, composition, ionic strength (NaCl) and temperature; however, physicochemical and biochemical parameters of cheese such as milk composition, processing parameters, residual lactose/lactate and galactose (in cheeses made with starter systems containing *Streptococcus thermophilus* as adjunct) and calcium phosphate levels, have to date received little attention. The objective of this project is to investigate the effects of such parameters during cheese production and maturation on Cheddar cheese quality which could develop future strategies for optimization of the Cheddar cheesemaking process, reducing variation in quality and maximizing Cheddar quality. Curd washing during cheese manufacturing had significant effect on controlling cheese residual sugar and lactate contents hence affected Cheddar cheese pH, which significantly influenced the cheese texture, sensory and volatile profiles of Cheddar cheeses. Cheddar cheese made with curd washing step had flavours described as 'fruity', 'buttery' and 'sweet' compared to non-washed cheese. CaP content in Cheddar can be controlled with different pH at whey drainage has a major impact on cheese texture and functionality. Reduced calcium in Cheddar cheese resulted in a more pungent, onion, rancid, buttery and caramel aromas and a more bitter taste than the standard calcium cheeses. Milk protein level is another very important factor to Cheddar cheese quality which can be standardised/concentrated by ultrafiltration (UF) process. Increasing milk protein level by UF significantly increased cheese yields, cheese firmness and fracture stress while decreased the cheese moisture and primary proteolysis at later stage of ripening. Cheddar cheese made with UF milk was more fruity, buttery and caramel than the standard milk cheeses. Due to modern Cheddar cheese practice, *Sc. thermophilus* strains are often used as an adjunct culture. Hence,

during this study, one galactose-positive and one galactose-negative *Sc. thermophilus* strain were selected based on their phenotypes for Cheddar cheese making in combination with standard mesophilic cultures. Higher drain pH was also used in this study to be comparable with recent commercial cheese making practise. Cheddar cheese made using Gal^+ and Gal^- *Sc. thermophilus* strains as adjunct cultures affected the levels of residual sugars, the profile of volatile compounds and sensory properties of Cheddar cheese.

Overall, the experimental work reported in this thesis generated new knowledge and theories about how to control and optimise Cheddar cheese quality during cheese manufacture and ripening.

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Jia Hou

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Chapter 1: LITERATURE REVIEW

Cheddar Cheese Quality: The Influence of Milk Quality, Processing Method and Ripening Process

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1.1.Introduction: Overview of the parameters that affect Cheddar cheese quality

Cheese production and consumption is increasing globally due to the importance in providing excellent source of protein, fat-soluble vitamins, calcium and can play an important role in a well-balanced human diet for all age groups (Robinson & Wilbey, 1998a; O'Brien & O'Connor, 2004). In the European Union (27) alone, cheese consumption increased from 8190 to 9062 thousand tonnes between 2006 and 2016. In Ireland, 195,000 tonnes of cheese is made *per annum*, mostly Cheddar, from a pool of milk drawn from a small geographical area using starters and coagulants obtained from a limited number of commercial sources and often with identical technology. Cheddar cheese production involves the conversion of milk to cheese curd and after the fresh cheese curd been made, they will be stored in ripening rooms in order to allow flavour and texture development; ripening times can vary from 3 month for mild cheese to over 2 years for extra-mature Cheddar cheese (Fox & McSweeney, 1998a). Several studies have shown seasonal variations in composition of milk (Chapman & Burnett, 1972; Auldist et al., 1998; O'Brien, Mehra, Connolly, & Harrington, 1999a,b). The pH and gross composition of cheese milk, especially the concentrations of protein, casein,fat and mineral content has a major influence on several aspects of cheese manufacture, including rennet coagulability, gel strength, curd syneresis, cheese composition, yield and quality (Chapman, 1974; Grandison, Ford, Owen, & Millard, 1984; Fox & Guinee, 2000a; Fox & McSweeney, 2004; Guinee, O'Kennedy, & Kelly, 2006). The milk for cheesemaking should be of a high microbiological and chemical quality and free from antibiotics, which may inhibit the growth of starter cultures and delay the fermentation/acidification process (Robinson & Wilbey, 1998c; Fox, Guinee, Cogan & McSweeney, 2000b).

1.1.1. Bovine milk composition: protein, fat and lactose

Milk composition varies considerably, depending on factors such as the animals' health, nutritional status, feeding regimen, stage of lactation and age (Fox, 2003a). The average gross composition of bovine milk is 3.7 % (w/w) fat, 3.4 % (w/w) protein and 4.8 % (w/w) lactose (Fox, Guinee, Cogan & McSweeney, 2016a).

The principal families of proteins in milk are the caseins and whey proteins which constitute ~80 and 20 %, respectively, of the total protein in bovine milk (Fox et al., 2016a). Caseins in bovine milk have been defined as phosphoproteins which precipitate from raw milk by acidification to their isoelectric precipitation at pH 4.6, leaving behind the whey proteins, which remain soluble in solution at this pH (Eigel, Butler, Ernstrom, Farrell, Harwalkar, Jenness, & Whitney, 1984; Stelwagen, 2003; Fox, 2003b). Caseins become concentrated in curd during cheese manufacturing while the whey proteins are mostly lost during whey drainage, hence, caseins are the principal proteins in cheese (Fox et al., 2016a).

Bovine milk contains about 3 to 5 % (w/w) fat in globular form and emulsified in the aqueous phase (87 %, w/w) of milk (Jensen, 2002). The basic components of milk fat consist of glycerol backbone to, which 1, 2 or 3 fatty acids that are esterified to give mono-, di- and triglycerides, respectively, and over 400 individual fatty acids have been identified, in which only 15 or 16 fatty acids are present in milk at concentration above 1 % (Taylor & MacGibbon, 2003a; Taylor & MacGibbon, 2003b). During cheesemaking and maturation, milk fat is degraded by lipolysis in to different fatty acids which contribute directly to Cheddar cheese flavour (Singh, Drake, & Cadwallader, 2003).

Lactose is the major carbohydrate in the milk of most mammals and it is generally accepted that non-mammalian sources of lactose are very rare hence, it's

commonly referred to as 'milk sugar' (Holsinger, 1997; Mustapha, Hertzler & Savaiano, 1997; Muir, 2003). The lactose content of milk is generally in the range 4.4 to 5.2 %, averaging 4.8 % of anhydrous lactose (Holsinger, 1988). Lactose is a disaccharide composed of D-galactose and D-glucose (Thelwall, 1997), the concentration of which decreases progressively and significantly during lactation (Fox & McSweeney, 1998b). As lactose is water-soluble, most is lost in the whey during cheese manufacture (Schaafsma, 2003). However, low concentrations of lactose in cheese curd have implications for the quality of the resultant cheese, as discussed in Section 1.2.2 below. Moreover, lactose in milk and dairy products can cause abdominal discomfort in humans who have insufficient lactase (β-galactosidase) in their digestive tract to hydrolyse lactose into its constituent monosaccharides (lactose intolerance) (Fox & McSweeney, 1998b).

1.1.2. Technology of cheese manufacture

Cheesemaking involves complex chemical and physical phenomena. It is essentially a concentration process which involves dehydration of milk to give a 6- to 12-fold, beginning with the coagulation of the major milk protein (casein), then proceeding with manufacturing steps designed to control the chemistry of the casein molecules depending on the type of cheese required (Fox & McSweeney, 2004: Lawrence, Gilles, Creamer, Crow, Heap Honoré, Johnston, & Samal, 2004). The physical or rheological characteristics of cheese are governed by interactions between casein molecules (Johnson & Lucey, 2006). While the individual cheese manufacturing procedure is different, the principle steps are common to many cheese types (Robinson & Wilbey, 1998b; Fox, 2003c). In this study, Cheddar cheese quality has been studied and the following discussion will concentrate on the procedures

employed in its manufacture. A general overview of the Cheddar cheese manufacturing process is shown in Figure. 1.

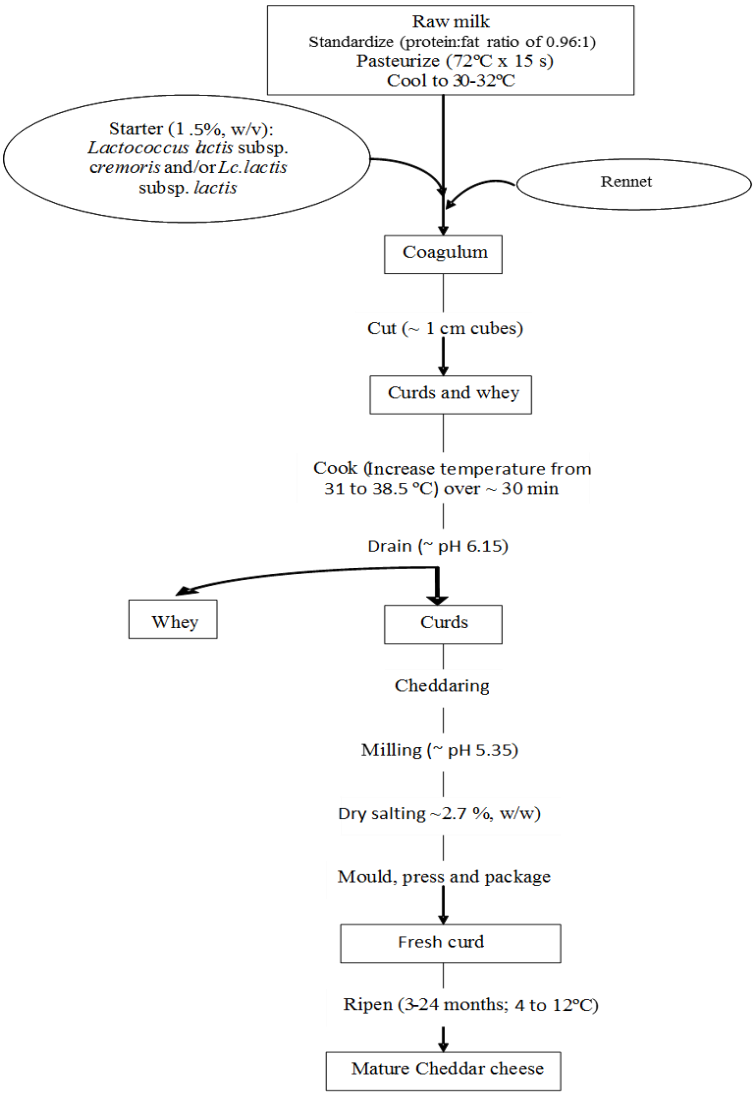

Figure 1.1. Flow diagram of Cheddar cheese process (modified from Fox & McSweeney, 2004).

The key elements in producing the desired cheese are: (a) milk composition (in part of final cheese composition) and (b) the rate and extent of acid development during manufacture (which influences the moisture loss, the extent of dissolution of colloidal calcium phosphate and the lowest pH obtainable in the cheese, all key factors in deciding the texture of the finished cheese) (Johnson, & Law, 2010). To ensure

cheese with a consistent quality, it is necessary to pre-treat or process the cheese milk prior to cheesemaking. The most common milk treatments include standardization and pasteurization process. Moreover, other processes are also available to process cheese milk depending on various objectives such as homogenization (increase rate of gel firming and curd firmness, increase cheese yield) and other thermal treatments (Eck & Gillis, 2000).

Standardization

To produce a cheese of consistent composition, the first thing that needs to be considered is to start with milk of a consistent composition. Bovine milk composition varies considerably depending on factors such as stage of lactation, feed and nutritional status of the cow and climatic conditions (Fox & McSweeney, 1998c; Robinson & Wilbey, 1998c). Hence, to obtain a desired milk composition, the cheese milk must be standardized to an appropriate protein to fat level, for example, in Cheddar cheese production, milk needs to be standardised to a protein to fat ratio of 0.96:1. This can be done by either, removal of cream (fat) or the addition of protein source (e.g., addition of skim milk, condensed skim milk, membrane retentates or milk powders such as skim milk powder) (Robinson & Wilbey, 1998d; Fox et al., 2000b; Maubois, 2000). The effects of milk standardization with ultrafiltration on the properties of the resultant cheese are discussed in Section 1.2.1 below.

Pasteurization

Raw milk naturally contains microorganisms in which some are beneficial for cheesemaking and others such as coliforms, which may cause health issues and food spoilage (Robinson & Wilbey, 1998d; Richard & Desmazeaud, 2000). Pasteurization

of milk for cheesemaking is primarily for public health reasons (*Mycobacterium tuberculosis*, the organism that causes tuberculosis, is killed by pasteurization) but also to provide a milk supply of more consistent bacteriological quality and to increase the shelf-life of the cheese. Batch pasteurization at low temperature, long time (LTLT) involves heat treatment of cheese milk at 63-65 ºC x 30 min which was used initially (still in use in farmhouse) but then replaced by continuous high temperature, short time (HTST) pasteurization (72 ºC x 15 s) (Fox, Guinee, Cogan, & McSweeney, 2016b). Pasteurization also inactivates several enzymes in the milk, including lipase and alkaline phosphatase. Lack of alkaline phosphatase activity in milk indicates that the milk has been properly pasteurized (Fox et al. 2016b). It should be noted that, pasteurization of cheese milk at temperature > 72 ºC will result in partial denaturation of whey proteins, which affects its rennet coagulability and curd-forming properties, and may also cause inactivation of indigenous milkenzymes that may be important in cheese ripening (Fox & McSweeney, 2004).

Following milk pre-treatments, the cheese milk is cooled to 30-32 ºC and ready to be converted into cheese curd by the following steps:

- Starter culture addition and fermentation/acidification:

The starter cultures are acid-producing bacteria used during cheesemaking. They reduce the milk pH by fermenting lactose to lactic acid (Canteri, 2000; Parente & Cogan, 2004) which is the first step to convert milk into cheese. The reduction in pH increases the rate of enzyme activity of the coagulant, the rate of syneresis, slows the growth of bacteria (including pathogens) and causes the dissolution of colloidal calcium phosphate (CCP) from the casein. In addition to the loss of calcium phosphate, the net charge repulsion between casein molecules increases initially but then decreases as the pH nears the isoelectric point of casein (Johnson, & Law, 2010).

Starter strains differ in their sensitivity to salt, temperature and pH hence they need to be carefully selected, as the rate of acid production may have significant effect on cheese composition and quality (Fox et al., 2000b; Powell, Broome & Limsowtin, 2003). It is desirable to have most of the acid develop in the curd during pressing, to aid in better curd fusion, better eye development (Swiss cheese) and retention of CCP to buffer against low pH (Johnson, & Law, 2010). Mesophilic starter cultures (mainly lactococci) are generally more salt-tolerant than thermophilic strains (e.g. *Streptococcus thermophilus*). Mesophiles are also more likely to ferment residual sugar (lactose and galactose) at cheese ripening temperatures (8–12 °C) (Johnson, & Law, 2010). In Cheddar cheese manufacture, defined-strain mesophilic starter cultures, consisting of *Lactococcus lactis* subsp. *lactis* and/or *Lc. lactis* subsp. *cremoris* are normally used (Robinson & Wilbey, 1998e; Lawrence et al., 2004). The amount of starter added to milk for cheesemaking depends on the rate and extent of acid development and conditions of culture propagation (media, pH control and age). Bulk starter cultures are normally inoculated at levels in the range 1.5-3.0 % (w/w) into cheese milk at 31 ºC and allowed to ripen for up to 60 min for them to adjust the milk environment and produce low levels of lactic acid which assists the coagulation process following rennet addition (Kosikowski & Mistry, 1997; Parente & Cogan, 2004). The effect of starter cultures on cheese quality will be discussed more in detail in Section 1.3.1. below.

Secondary starter 'adjunct' cultures are sometimes added either to the primary starter culture or separately to milk in the vat (Powell et al., 2003) not for acid development but for production of specific flavour compounds, texture or gas of the resultant cheese (Rattray, 2003; Chamba & Irlinger, 2004; Johnson, & Law, 2010).

The effect of starter cultures on cheese quality will be discussed in more detail in Section 1.3.1. below.

- Coagulation and cutting:

After starter culture is added and fermentation produced, rennet (activity at 200 IMCU mL^{-1}) is added to the milk with up to 10 times dilution in water (aid its dispersion) and added at a rate of 0.18 ml/ L of milk which contains 3.4 % protein at ~31 ºC (Robinson & Wilbey, 1998f). Coagulation of milk is due to the aggregation of caseins to form a gel that also entraps the milk fat (Fox & McSweeney, 2004; Horne & Banks, 2004). This can also be achieved by addition of other enzymes from both animal and microbial sources (Ramet, 2000a; Andrén, 2003). In Cheddar cheese production, calf rennet is most commonly used which contains the milk-clotting enzyme, chymosin, an acid proteinase with an optimum activity at pH 4.0 (Kosikowski & Mistry, 1997; Robinson & Wilbey, 1998j; Lucey, 2003).

After the required gel firmness is achieved, the gel is then cut into small pieces using knives (Robinson & Wilbey, 1998b, g; Ramet & Scher, 2000). This increases the curd surface area and results in curd particle beginning to shrink rapidly, especially at the surface, swiftly expelling large amounts of whey (syneresis), which is initially trapped in the interior of the curd particle but can be squeezed out (Kosikowski & Mistry, 1997; Johnson, & Law, 2010). After cutting, the curds are allowed to stand in the whey for ~ 10 min during which time they 'heal' and the curd forms a membrane-like/skin (Robinson & Wilbey, 1998b; Robinson & Wilbey, 1998h). At the cut surface, the fat globules that are too large to be trapped or surrounded by the casein network are lost in whey. The skin prevents further fat loss but contains small pores, through which whey from the interior of the curd particle can be squeezed out. The skin also

makes the curd more resilient to stress and less likely to break or tear which prevents further moisture losses from the curd (depending on the type of knifves/ cutting mechanism). If the curd was cut into too small pieces, very small curd particles may not be incorporated into the curd mass when whey and curd are later separated, and thus represent a loss in cheese yield (Johnson, & Law, 2010). The effect of gel firmness at cutting will be further discussed in detail below in Section 1.2.2.

- Stirring and cooking/heating

After the curd is cut, it is stirred and heated at a controlled rate. The starter continues to produce acid, and the combination of stirring, heating and acid development, causes protein matrix contraction and it also has a profound effect on moisture (syneresis) and dissolution of calcium phosphate, which have major implications for the characteristics of the cheese (Kosikowski & Mistry, 1997; Lawrence et al., 2004; Johnson, & Law, 2010). During cooking, the temperature of the curd/whey mixture increases gradually at a rate of ~ 0.1 ºC min^{-1} until the temperature reaches maximum scald, which is generally in the range 37-39 ºC for Cheddar cheese (Robinson & Wilbey, 1998b; Fox & McSweeney, 2004). As the temperature increases, the starter bacteria start to grow rapidly and convert the remaining lactose in the curd particles to lactic acid. The lactic acid then diffuses into the whey and the pH continues to drop (Robinson & Wilbey, 1998b; Lawrence et al., 2004).

The curd-whey mixture is stirred continuously during cooking at an increased speed to keep the curd particles separate from each other in the whey and hence assists in whey removal (syneresis) (Robinson & Wilbey, 1998h; Ramet, 2000b). Syneresis rearranges and tightens the casein matrix and results in moisture being squeezed out

from the casein network. The most important factors influencing syneresis are: (a) cooking temperature, (b) the rate of acid development after cutting (drop in pH) and (c) pressure exerted on the curd (Fox et al., 2000b; Dejmek & Walstra, 2004).

- Whey drainage, cheddaring and milling

The whey and curd separation can play an important role in the texture, colour and flavour of the cheese (Johnson, & Law, 2010). When the pH of the curd-whey mixture reaches the desired drain pH (~ pH 6.15 for Cheddar cheese), whey is removed from cheese vat while the stirrers remain mixing the contents in the vat (Robinson & Wilbey, 1998b, Bennett & Johnston, 2004). The pH of the whey at drainage is one of the most important control point during cheesemaking as it determines the final moisture, pH, mineral content and thus structure of the resultant cheese (Lawrence et al., 2004; Upreti, & Metzger, 2006a). After the whey is drained, the curd is allowed to mat together and then cut into slabs. The slabs are kept warm, turned and piled on top of each other at desired pH which is called 'cheddaring'. The faster the slabs are stacked, the more moisture will be trapped within the curd (Johnson, & Law, 2010). Once the desired pH is reached (e.g., 5.3 – 5.4 for Cheddar), the slabs are milled into curd chips (typically 6-10 g) and whey and fat are released from the newly exposed surfaces.

- Salting, moulding and pressing

After the curd is milled, salt is added at a rate of ~ 2.7 % (w/w) (for Cheddar) and mixed thoroughly to ensure uniform absorption and also to avoid the development of "mottled" cheese (with presence of irregular shaped spots or blotches) (Kosikowski & Mistry, 1997; Robinson & Wilbey, 1998f; Guinee & Fox, 2004). Salting of the curd during Cheddar cheesemaking process can inhibit growth of lactic microorganisms

(stop acid production), promote syneresis of whey during pressing, and it imparts essential flavour to the final product (Robinson & Wilbey, 1998c; Robinson & Wilbey, 1998h; Hardy, 2000; Fox & McSweeney, 2004; Guinee & Fox, 2004). Following salting, the curd is allowed to mellow for 20 min and the salty whey is allowed to drain. During mellowing, salt adsorption continues on the curd surface (Bennett & Johnston, 2004; Lawrence et al., 2004). The effect of salting on cheese quality is discussed in detail below in Section 1.2.2.

After the 20 min mellowing process, the curd chips are then weighed into moulds and placed on a hydraulic press for up to 18 h (Kosikowski & Mistry, 1997; Ramet, 2000b). The aims of pressing are (a), to form the curd into the desired shape, (b), to expel any free whey and (c) to complete the curd knitting process. The time, pressure and efficiency of pressing are mostly related to the curd at the time of pressing and the decrease in pH (loss of CCP) during pressing (Johnson, & Law, 2010). Following pressing, the cheeses are vacuum-packaged and stored at 4-12 ºC throughout ripening (Robinson & Wilbey, 1998b; Bennett & Johnston, 2004).

1.1.3. Cheese microbiology: general aspects

Cheddar cheese contains a diverse variety of microorganisms, which can be broadly divided into 2 groups - starter bacteria and non-starter bacteria (Cogan, 2003; Beresford & Williams, 2004). Starter culture plays a critical role during all phases of cheesemaking and during the maturation process. Lactic acid starter bacteria can ferment the lactose to lactic acid, to ensure the correct pH during coagulation, pressing and in the final cheese curd during cheesemaking (as discussed in Section 1.3.1). During the maturation period, the starter culture influences the development of flavour, aroma, texture and functionality of the cheese, where their enzymes play an

important role in proteolysis, lipolysis and amino acid breakdown, as discussed in Section 1.3.1 below (Lawrence et al., 2004; Parente & Cogan, 2004; Høier, Janzen, Rattray, Sørensen, Børsting, Brockmann, & Johansen, 2010). Those enzymes are called intracellular enzymes which are only released into the cheese matrix after the cell lyses (Beresford & Williams, 2004; Fox & Cogan, 2004a). Starter bacteria can grow rapidly during cheese manufacture and achieve $\geq 10^8$ cfu.g^{-1} within one day post manufacture (Hannon, Wilkinson, Delahunty, Wallace, Morrissey & Beresford, 2003; Shakeel-Ur-Rehman, Waldron, & Fox, 2004; Beresford & Williams, 2004). At the early stage of maturation, the starter bacteria is able to continue to metabolize residual lactose in the cheese, largely dependent on the salt-in-moisture content of the cheese (Cogan, 2003). As the vital substrate (lactose) is utilised and the lactic acid is produced (lowering cheese pH), the starters begin to lose viability (Farkye, 2000; Beresford, Fitzsimons, Brennan & Cogan, 2001; Parente & Cogan, 2004). When the starter cells start to die off, they release a number of intracellular peptidases and some intracellular proteinases which varies from strain to strain and is largely predetermined by factors such as the cook temperature, pH, salting and final cheese composition (Beresford & Williams, 2004; Lawrence et al., 2004; Parente & Cogan, 2004).

Non-starter lactic acid bacteria (NSLAB) can gain access to Cheddar cheese from the raw milk or through cross contamination from the cheesemaking environment (Jordan & Cogan, 1999). They are present in cheese initially at a low number but increases during ripening and can reach 10^6-10^8 cfu.g^{-1} within 2-3 months and predominate especially when the starters have died (Peterson & Marshall, 1990; Banks, 2003; Cogan 2003). The growth rate of NSLAB in Cheddar cheese is independent of lactose content (Fox, McSweeney, & Lynch, 1998). NSLAB can also metabolise energy sources other than lactose in cheese (e.g., sugars released on

autolysis of starter cells, sugars derived from the casein glycomacropeptide and milk fat globule membrane, peptides, and amino acids) to a degree dependent on species and strain type (Diggin, Waldron, McGoldrick, Cogan, & Fox, 1999; Peterson & Marshall, 1990; Williams, Withers, & Banks, 2000; Asteri, Robertson, Kagkli, Andrewes, Nychas, Coolbear, Holland, Crow, & Tsakalidou, 2009), hence, NSLAB growth would not be expected to be totally impeded by reduced lactose levels. The NSLAB population of Cheddar cheese is mainly comprised with mesophilic facultatively heterofermentative lactobacilli, especially *Lb. casei, Lb. paracasei, Lb. plantarum and Lb. curvatus* (Fitzsimons, Cogan, Condon, & Beresford, 1999; Beresford et al., 2001; Fox & Cogan, 2004a). A previous study on NSLAB population of 8 week old Irish Cheddar cheese showed that it was composed of 55 % *Lb. casei*, 28 % *Lb. plantarum* and 14 % *Lb. curvatus* (Jordan & Cogan, 1993). Another study on mature Irish Cheddar cheese showed that NSLAB flora consisted of 96.4 % *Lb. paracasei*, 2.1 % *Lb. plantarum*, 0.3 % *Lb. curvatus* and 0.3 % *Lb. brevis* (Fitzsimons et al, 1999). It has been noted that there are difficulties to control the numbers and species of lactobacilli in cheese and they may have various contribution towards final cheese quality (Fox & McSweeney, 1995b; Fox & Cogan, 2004a).

1.1.4. Biochemical changes during cheese ripening

Cheese maturation is a highly complex process which involves changes to physical and chemical properties of cheese under a temperature-controlled environment (4 to 12 ºC) for up to 2 years depending on the flavour, texture and appearance development (Kosikowski & Mistry, 1997; Law, 2010). During ripening, the primary biochemical reactions which involve the metabolism of residual sugars, lipids (lipolysis) and proteins (proteolysis) and those primary changes are typically

followed by secondary changes which involve catabolism of the primary metabolised products (lactic acid, fatty acids and amino acids) (Fox & McSweeney, 2004). The key changes during ripening of Cheddar cheese are discussed as below.

- **Glycolysis: carbohydrate fermentation**

Lactose is converted to lactic acid (mainly to L(+) -lactic acid) in Cheddar cheese at the early stage of cheese ripening, mainly by the metabolism of the starter bacteria (Fox, Lucey & Cogan, 1990). This leads to a drop in milk pH and precipitation of casein which has a major impact on cheese texture (via loss of minerals from casein micelles) (Creamer, Lawrence, & Gilles, 1985; Lawrence, Creamer, & Gilles, 1987; Creamer, Gilles, & Lawrence, 1988). It also affects cheese proteolysis due to a greater retention of chymosin in a more acidic environment in curd (Creamer et al, 1985) and/or due to the increase in susceptibility of casein to proteolysis, hence deplete minerals from the curd (O'Keeffe, Fox, & Daly, 1975) (as discussed in Section 1.4.1). Under certain conditions (e.g., S/M > 6 %, inhibit activity of starters), non-starter lactic acid bacteria (NSLAB) can also metabolize residual lactose to lactic acid (mainly the D(-)-lactic acid). D(-)-lactate can be also produced by racemization of L(+)-lactate to D(-)-lactate by NSLAB during ripening which involves oxidation of L(+)-lactate by l-lactate dehydrogenase (L-LDH) to pyruvate, which is then reduced to D(-)-lactate by d-lactate dehydrogenase (D-LDH) (Thomas & Crow, 1983; McSweeney & Fox, 2004). Racemization of lactate in cheese has little or no effect on cheese flavour, however, D(-)-lactate can form calcium lactate pentahydrate which crystallizes and appears as white deposits on the cheese surface (Pearce, Creamer, & Gilles, 1973; McSweeney & Fox, 2004). Moreover, D(-)-lactate is metabolised by human slower than L(+)-lactate which can result in nutritional consequences such as loss of weight, dehydration, regurgitation and vomiting(Ballabriga, Conde, & Gallart-Catala, 1970).

After all sugars have been utilised, NSLAB can also further convert the lactates to acetate, ethanol, formate and CO_2, which may have a role to play in cheese flavour (McSweeney & Fox, 2004; Law, 2010).

- **Lipolysis**

Milk fat is essential for flavour development in cheese during ripening. Lipolysis in cheese occurs with the presence of lipolytic enzymes which cleave the ester bond of triacylglycerides into their constituent fatty acids and glycerol, mono- or diacyglycerides (Deeth, & Touch, 2000). Lipolytic enzymes in cheese may originate from the milk, starter bacteria, secondary bacteria (adjunct cultures), NSLAB, rennet, or other exogenous enzymes, if added. Indigenous milk lipase, LPL mostly (~90%) in bovine milk, is associated with the casein micelles and incorporated into the curd during cheese manufacture. The activity of LPL, is significantly reduced by pasteurization (72 ºC x 15 s), however, it still contributes to lipolysis in pasteurised milk since heating at 78 ºC for 10 s is required for complete inactivation (McSweeney, & Sousa, 2000; Olivecrona, Vilaró, & Olivecrona, 2003; Collins, McSweeney, & Wilkinson, 2004). During Cheddar cheese ripening, only moderate levels of lipolysis occur compared to varieties such as Blue mould-ripened cheeses and hard Italian cheeses, e.g., Romano and Parmesan (Fox, Law, McSweeney, & Wallace, 1993; Collins et al, 2004). Starter bacteria has weak lipolytic properties, however, their esterase and lipase activities which liberate fatty acids in cheese, increases depending on the populations and the cheese ripening period (Fox et al., 1993; Collins et al., 2004). Free fatty acids liberated during lipolysis can contribute directly to cheese flavours by imparting flavour notes or indirectly as precursors for metabolic reactions which generate other flavour compounds such as ketones, lactones, esters, alkanes and

secondary alcohols (McSweeney, & Fox, 1993; Engels, Dekker, De Jong, Neeter, & Visser, 1997; Partidario, Barbosa, & Boas, 1998; McSweeney, & Sousa, 2000). However, an excess of free fatty acids or an incorrect balance of free fatty acids may lead to off flavour/rancidity in cheeses, especially in mild-flavoured cheese varieties (McSweeney, & Fox, 1993). The effect of lipolysis on cheese quality will be further discussed below in 1.4.2.

- **Proteolysis and amino acids catabolism**

Proteolysis is the most complex and important of the primary biochemical reactions occuring during cheese ripening which results in the production of peptides and free amino acids from the degradation of casein by proteolytic/peptidolytic enzymes and those changes have important influence on cheese texture, functionality and flavour (Fox et al., 1993; McSweeney, 2004). In cheese, proteolysis is catalysed by the action of a wide range of proteinases and peptidases which originate from coagulant/rennet, cheese milk, starter lactic acid bacteria, NSLAB, secondary starter cultures and exogenous proteinases and/or peptidase if added (McSweeney, 2004; McSweeney, & Sousa, 2000). Proteolysis in cheese has been studied and reviewed extensively over the last few decades (Grappin, Rank & Olson, 1985; Fox et al., 1993; Fox, Singh & McSweeney, 1994; Fox, Singh & McSweeney, 1995b; McSweeney, 2004; Sousa, Ardö, & McSweeney, 2001; Upadhyay, McSweeney, Magboul, & Fox, 2004; Upreti, Metzger, & Hayes, (2006b). Proteolysis contributes cheese flavour directly by the production of peptides and free amino acids which impart flavour and can also act as precursors for secondary catabolic reactions. Proteolysis can also change cheese texture by breakdown of the protein matrix and increase in the pH and water binding capacity of the cheese as a result of the newly formed amino and

carboxyl groups as peptide bonds are cleaved (Lawrence et al, 1987). Proteolysis during cheese ripening occurs in two stages as primary proteolysis, which involves breakdown (hydrolysis) of caseins to a range of large- (water-insoluble) and intermediate- (water soluble) sized peptides, and secondary proteolysis, which involves further breakdown of large and intermediate peptides into smaller peptides and amino acids (McSweeney, 2004; Upadhyay et al., 2004). The primary proteolysis involves hydrolysis of *para*-casein protein due to the action of residual coagulant (chymosin) on α_{s1}-casein (CN), in which the Phe_{23}-Phe_{24} bond is its primary cleavage site (Fox, 2003d; McSweeney, 2004). During cheese ripening, further hydrolysis is generally carried out by the proteolytic system of lactic acid bacteria (LAB) which consist of proteinases (mainly the cell envelope-associated proteinase), and results in the formation of small peptides and amino acids (Fox, 2003d; Upadhyay et al., 2004). Amino acids are the final products of proteolysis in cheese and the main amino acids found in Cheddar cheese are glutamic acid, leucine, arginine, lysine, phenylalanine and serine (McSweeney, 2004). Studies showed that the concentration of free amino acids significantly correlated with flavour intensity of Cheddar cheese and can be used as an index of ripening (Aston, Grieve, Durward, & Dulley, 1983; Puchades, Lemieux, & Simard, 1989; McSweeney, & Fox, 1997). Details of proteolysis is discussed in Section 1.4.3.

1.2. Effect of manufacturing parameters and milk/cheese compositions on the quality and yield of Cheddar cheese

1.2.1. Milk protein standardization: Ultrafiltration

Membrane processing has affected the dairy industry in many ways which led to significant new process and product development. This technology involves

ultrafiltration (UF) and more recently, microfiltration (MF). Using membrane technology has opened up significant advances in cheesemaking, such as developing a continuous process, improving plant efficiency, cheese yield and potentially creating new cheese varieties. As a result, numerous factories all over the world (mainly Europe) now use UF to manufacture a wide range of cheeses (Korolczuk, Maubois, and Fauquant, 1986; Maubois, 2002; Kelly, 2003; Mistry, & Maubois, 2004; Pouliot, 2008).

UF is a membrane process which separates macromolecules having a molecular weight of 1000-200 000 Da from solvent and dissolved solutes. With a relatively low pressure (less than 70 – 170 kPa), UF produces from milk (a), a permeate/ultrafiltrate which contains water, lactose, soluble minerals, non-protein nitrogen and water-soluble vitamins, and (b), a retentate in which the proteins, fat and colloidal salts are increased in concentrations to the amount of permeate removed (Glover, 1985; Mistry, & Maubois, 2004).

UF is widely used in cheesemaking and there are three major methods: low concentration (protein standardization, 1-2x), medium concentration (2-6x), and high concentration (pre-cheese concept, 6-8x) (Mistry, 2003; Pouliot, 2008). UF can alter some physicochemical properties of cheese milk including viscosity, buffering capacity and rennet coagulation properties. Firstly, as protein is concentrated in milk after UF, the milk viscosity increases which is important for cheese factories to meet the pumping requirements when the milk protein in the retentate is at high levels (Mistry, 2003). Also efficient mixing is required for further mixing of other ingredients such as starter cultures and rennet to prevent lumping in vat. Secondly, during UF process, milk protein and colloidal salts are concentrated simultaneously which leads an increase in the buffering capacity (Mistry, 2003). The changes in

buffering capacity directly affects acid production by lactic acid bacteria when the aqueous phase of milk becomes more concentrated with lactose.Hence the pH of cheese falls which offers the potential for growth of spoilage and pathogenic organisms during cheese making and ripening. It also influences the rennet coagulation properties and the changes during cheese ripening (Mistry, 2003; Salaün, Mietton, & Gaucheron, 2005). The low pH of cheese may result in undesired acid taste of cheese and also create an imbalance in calcium equilibrium which will cause poor cheese texture and functionality (O'Mahony, Lucey, & McSweeney, 2005; Johnson & Lucey, 2006; Lee, Johnson, Govindasamy-Lucey, Jaeggi, & Lucey, 2010). This defect can be accomplished by reducing the milk pH to 5.6 to 6 during UF process (Mistry, 2003). Moreover, UF process can reduce the rennet coagulation time (RCT) and increase the firmness of the coagulum due to the higher level of hydrolysate (κ-casein) in UF milk. The change would be useful to improve poor rennet coagulation properties for high heat treated milks (Guinee, Gorry, O'Callaghan, O'Kennedy, O'Brien, & Fenelon, 1997; Waungana, Singh & Bennett, 1998; Mistry, 2003; Sandra, Cooper, Alexander, & Corredig, 2011).

1.2.2. Effect of gel firmness at cutting, curd washing, pH at whey drainage and salting on the quality of Cheddar cheese

After addition of starter culture and rennet to milk, the curd is cut, stirred and cooked (heated). During this period, fermentation of starter bacteria keep bringing down the curd pH and syneresis happens. The gel firmness at cutting is critical as it may significantly affect the cheese yield. If the gel is cut at a low gel firmness, there will be higher losses of fat which is due to the curd shattering, whereas if the gel is cut too firm there will be high losses of casein protein which is due to tearing of the gel

(Fox et al., 2000c). The greater pH drops after cutting the coagulum, the more syneresis will occur and the higher the temperature used to cook the curd and whey mixture after cutting, the lower the moisture will remain in the curd (Johnson, & Law, 2010).

The pH of cheese is controlled by the interactive effects of a number of critical factors including the amount of lactic acid, calcium phosphate and protein, the salt sensitivity of starter culture, and the level and duration of salting (Fox, Lucey, & Cogan, 1990; Guinee, 2004; O'Connor, 1974; Turner & Thomas, 1980). Curd washing/whey dilution is used in the manufacture of brine-salted cheeses such as Gouda and Edam to control the lactose, and hence lactic acid concentration in the curd and pH of the resultant cheese. Curd washing is also used in the manufacture of cheeses such as Colby and Monterey, whereby cold water is added to, and mixed with, the drained curd for a relatively short period (5-15 min) before being drained off (Lee, Johnson, Govindasamy-Lucey, Jaeggi & Lucey, 2011). The main purpose here is to reduce curd temperature, depress syneresis and modify texture (Fox & Guinee, 2013). The degree and mode (batch or continuous) of stirring has been found to significantly influence lactic acid content, cheese pH and ratio to soluble ofcasein-bound calcium phosphate (Lee et al., 2011). Cheddar cheese is not a washed curd variety, hence, seasonal variation in the concentration of lactose in milk, which can range from ~ 4.0 to 4.8 % (w/w) (O'Brien et al, 1999b) is expected to influence the composition and quality of the cheese. Huffman and Kristoffersen, (1984) investigated the effect of altering the lactose content of Cheddar cheese, either by adding lactose to the curd-whey mixture (high lactose, HL) or by curd washing (replacing whey with simulated milk ultrafiltrate, low lactose, LL). The residual lactose contents of the control (CL), HL and LL cheeses at 1 day were 0.27, 0.41 and 0.06 % (w/w), respectively. Shakeel-

Ur-Rehman et al, (2004) also prepared HL and LL cheeses, with lactose levels of 2.3 and 0.25 % (w/w) at day one, by fortifying the milk to 8.4 % (w/w) lactose with lactose powder (HL) or by washing of the curds from control milk (LL; lactose concentration not given), respectively. Although the pH of the HL cheese decreased significantly during maturation from ~ 5.3 at day one to 4.8 at day 180, that of the LL cheese remained relatively constant at ~ 5.3 to 5.4. Despite only minor differences in the levels of primary proteolysis, the HL cheese had higher levels of total free amino acids and on grading, was found to have a harsh coarse flavour and a crumbly body. Moynihan, Govindasamy-Lucey, Molitor, Jaeggi, Johnson, McSweeney and Lucey, (2016) also standardized lactose to 3 different levels in low-moisture, part-skim Mozzarella cheesemilk by mixing milk and retentate/permeate. The three levels used were at a high level, 1.8 (HLC, the normal level in milk), medium level, 1.3 (MLC); and level, 1.0 (LLC) of lactose-to-casein ratios. LLC and MLC cheeses had lower levels of lactose, galactose, lactic acid, and insoluble calcium compared with HLC cheese. Cheese pH was higher with LLC milk compared to other cheeses throughout ripening which led to a firmer, chewier texture and lower meltability compared to other cheese during ripening.

Calcium in cheese contributes to the crosslinking of the *para*-casein network, and thereby, its integrity and rigidity (Lawrence, Heap & Gilles, 1984, Lucey & Fox, 1993). CCP is the main buffering agent in milks and cheeses and will increase the potential to develop an excessively low pH (<5.0) in the cheese by removing it during cheesemaking (Lucey & Fox, 1993; Johnson & Lucey, 2006). Hence, the level of total calcium, or more specifically the level of casein-bound calcium, as influenced by cheese pH, has a major impact on cheese texture and functionality (Lawrence, Creamer & Gilles, 1986; Guinee, Feeney, Auty & Fox, 2002). The calcium content of

cheese is controlled principally by the pH at set, the pH at whey drainage, and in the case of dry-salted cheeses, the pH at salting (Lawrence et al., 1984). Lower pH values at rennet addition leads to greater solubilization of CCP during the stirring and cooking of the curd particles in the whey, and its removal in the drain of whey (Hooydonk, Hagedoorn, & Boerrigter, 1986; Guinee et al., 2002; Upreti & Metzger, 2006a). Upreti and co-workers investigated the interactive effects of variations in levels of calcium phosphate (CaP), residual lactose plus lactic acid, and salt-in-moisture on the composition of Cheddar cheese and changes in proteolysis, levels of water-soluble organic acids, and pH during ripening (Upreti & Metzger, 2006a, 2007). The level of CaP in the cheese was varied by altering the pH of the milk at set (6.6, 6.2) and the curd at whey drainage (6.4, 5.7), the level of lactose and lactic acid in the curd by the addition of lactose powder or by curd washing using a pH-adjusted solution of calcium and phosphate, and the salt-in-moisture content by different salting rates.

1.2.3 Cheese composition

Cheese composition is one of the major determinants of cheese quality (Fox & Cogan, 2004a) and it is affected by several parameters, such as raw milk composition, milk standardization, pasteurization condition and the manufacturing process (Lawrence et al., 2004). According to the Code of Federal Regulations (CFR, 2008), the moisture content of Cheddar cheese must be ≤ 39 % (w/w). A grading chart published by Gilles & Lawrence (1973) is used to give an indication of compositional parameters which relate to good quality of Cheddar cheese (Figure. 1.2).

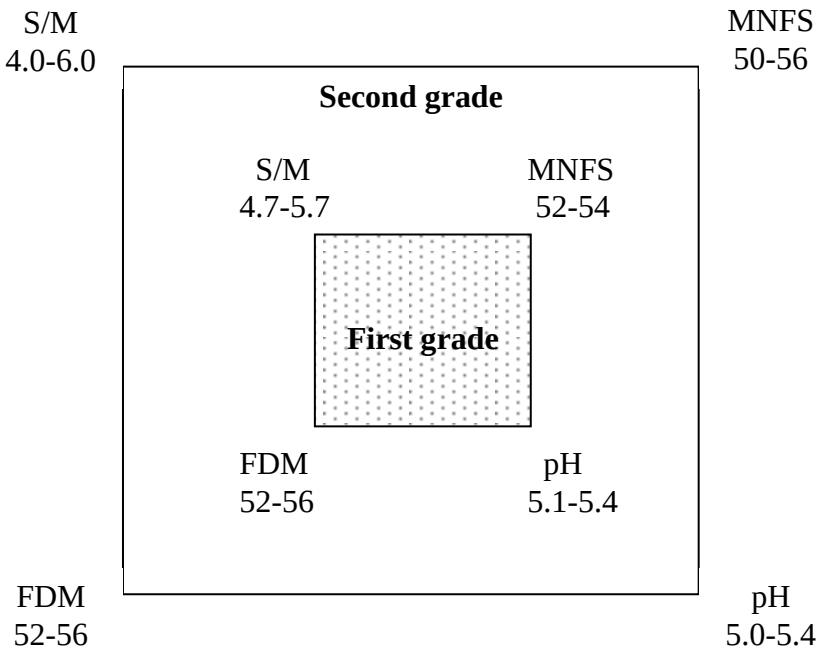

Figure. 1.2. Cheddar cheese quality chart with the range of salt-in-moisture (S/M), moisture in non-fat substance (MNFS), fat-in-dry matter (FDM) and pH for first- (shaded) and second-grade Cheddar cheese analysed at 14 day. (Modified from Gilles & Lawrence, 1973; Lawrence et al., 2004).

The salt-in-moisture (S/M) content of cheese is a major determinant of cheese quality as it affects water activity (a_w), which affects the growth of bacteria and enzyme activity (salt tolerance in starter cultures, see below section 1.3.). When the S/M value is low (< 4.5 %), the starter bacteria is able to grow to high levels which may result in off-flavours in final cheese (Fox & Cogan, 2004a). The fat-in-dry matter (FDM) affects the moisture-in-non-fat-substance (MNFS) which influences indirectly on cheese quality. A desired FDM content in cheese can be achieved by suitable standardization of the casein-to-fat ratio of the cheese milk prior to cheesemaking (Lawrence & Gilles, 1987). Cheese pH is one of the key determinants of cheese quality during ripening which can provide an indication of the acid production during the cheese-making process (Lawrence & Gilles, 1987; Fox & Cogan, 2004a). Typically, Cheddar cheese pH increases during maturation which is due to factors such as the production of ammonia, via proteolysis (details see below, Section 1.4.3.) and/or

oxidative deamination of amines (Fenelon & Guinee, 2000) and to a relatively high protein-to-lactate ratio in the resultant cheese (Guinee et al., 1995).

1.2.4. Cheese yield

Cheese yield and manufacturing efficiency affect the profitability of cheese manufacturing plants (Guinee et al., 2006). Consequently, the factors affecting the recovery of milk fat and protein, and cheese yield had been extensively studied and reviewed over the last few decades (Olson, 1977; Lundstedt, 1979; Gilles & Lawrence, 1985; Emmons, Ernstrom, Lacroix & Verret, 1990; IDF, 1991; Lolkema, 1993; Lucey & Kelly, 1994; Fox, Guinee, Cogan & McSweeney, 2000c; Vandeweghe, 2000; Guinee, 2003a). Cheese yield is often defined as the amount of cheese obtained from a given amount of cheese milk, e.g., kg cheese 100 kg^{-1} milk. Usually, it is preferable to report cheese yield independent of the actual moisture content, such as moisture adjusted yield (Y_{ma}), or as dry matter yield (Johnson, & Law, 2010). Some of the more important factors include the composition and quality of the raw milk, milk handling and storage practices, milk pre-treatments (e.g., standardization to protein-to-fat ratios and protein contents, pasteurization temperature), firmness of the gel at cutting and the speed/duration of cutting programme, the rate of cooking of the curd-whey mixture and the temperature to which it is cooked, the duration of stirring and cooking before the physical separation of whey from the curd, design of cheese vat, and curd handling conditions following whey removal will affect cheese yields.

Casein content contributes directly to cheese yield as it is present as a continuous matrix within which fat and moisture are retained. In addition, the curd moisture phase contains dissolved solids such as whey proteins, κ-casein, glycomacropeptide, lactates and soluble salts, which all contribute indirectly to cheese

yield (Guinee, 2003a). Cheese yield generally increases linearly in the range 24 to 37 g.kg^{-1} with increasing milk casein content (e.g., from 30 to 46 g protein kg^{-1}). Similarly, for a fixed casein level (~25g kg^{-1}), the yield of cheese increases linearly with increasing fat content (from 5 to 330 g kg^{-1}) in the range 4.0 to 33 g kg^{-1} fat (Guinee, 2000).

1.3. Effect of starter and adjunct cultures on Cheddar cheese quality

1.3.1. Starter cultures on Cheddar cheese quality

The starter cultures are the acid-producing bacteria used in cheesemaking, which not only contribute to the pH reduction but also influence the taste, aroma and texture of the cheese. The reduction in pH is due to the metabolism of lactose to lactic acid by the lactic acid bacteria (LAB) which increases the rate of enzyme activity of the coagulant, hence, increases the rate of syneresis, prevents the growth of undesirable bacteria (including pathogens) and causes the dissolution of CCP from the casein. Lawrence et al. (1984) indicated that the sensitivity of the starter culture also plays a key role in fermentation of residual lactose after salting and consequently lowering the cheese pH. Nevertheless, due to the loss of calcium phosphate with decreasing pH, the net charge repulsion between casein molecules which increase initially but then decreases as the pH moves towards the isoelectric point of casein (pH 4.6). Both of above influence the chemistry of the casein network, which affect the physical properties of cheese such as firmness, smoothness of mouth-feel and even the colour of the cheese (Johnson, & Law, 2010). LAB and NSLAB cooperate in aroma formation in Cheddar cheese: the conversion of amino acids to keto- and hydroxyl acids is initiated by lactobacilli, while *Lactococcus* strains further convert these products to carboxylic acids. This cooperation between LAB and NSLAB leads to an

enhanced cheese flavour (Murtaza, Ur-Rehman, Anjum, Huma & Hafiz 2014). The selection of LAB also affects the activity of various starter-derived enzymes in the cheese during ripening and consequently the rate of ripening (proteolysis and lipolysis, see below, Section 1.4) and the quality of the cheese (Fox, Guinee, Cogan, McSweeney, 2016c).

1.3.2. Adjunct cultures on Cheddar cheese quality

- *Streptococcus thermophilus*

The use of adjunct cultures in addition to the normal starters which were traditionally used is to improve and/or accelerate flavour development in full-fat cheeses (Fox et al, 1998; Law, 2001). In Irish Cheddar cheese plants, the routine use of starter culture adjuncts, including *Streptococcus thermophilus* (*Sc. thermophilus*), which is used primarily for its thermo- and phage- resistance properties, but also apparently to affect flavour. *Sc. thermophilus* is also reported to give faster acid production during Cheddar manufacture (Michel & Martley, 2001), which may be likely associated with a more effective protein hydrolysis and peptide uptake (Law & Haandrikman, 1997; Cogan, Beresford, Steele, Broadbent, Shah & Ustunol, 2007) and with the non-utilization of the galactose moiety of lactose, by most *Sc. thermophilus* strains (Tinson, Hillier, & Jago, 1982a; Thomas & Crow, 1984).

Most strains of *Sc. thermophilus* used in the dairy industry are unable to metabolise galactose (Thomas & Crow, 1984; Hutkins, Morris & McKay, 1985; Hutkins, Halambeck, & Morris, 1986; Vaillancourt, Moineau, Frenette, Lessard & Vadeboncoeur, 2002; Robitaille, Moineau, St-Gelais, Vadeboncoeur & Britten, 2007). De Vin, Rådström, Herman and De Vuyst (2005) reported that only ~ 16 % of 49 strains of *Sc. thermophilus* evaluated on M17 medium supplemented with galactose

were galactose positive. Similarly, preliminary studies in the thesis's authors' laboratory found that only ~ 8 % strains of *Sc. thermophilus* from the Moorepark culture collection metabolised galactose. Thomas and Crow (1984) investigated the galactose-metabolizing ability of *Sc. thermophilus* from different sources and found that most were galactose-negative (Gal^-) because of failure to induce galactokinase, resulting in the excretion of galactose when grown in lactose-containing broth. When grown under lactose limitation in J8 broth containing 20 mM galactose, partial galactose utilization occurred and the proportion of galactose used depended on the generation time of cells during incubation. Hence, the use of *Sc. thermophilus* (which primarily metabolizes only the glucose moiety of lactose) as an adjunct culture usually results in the accumulation of galactose during cheese manufacture (Thomas, Turner & Crow, 1980; Tinson et al., 1982a, Michel and Martley, 2001). Bley, Johnson and Olson (1985) reported that the use of a 0.5 % (w/w) non-galactose-fermenting *Sc. thermophilus* as an adjunct resulted in higher level of residual galactose in one month-old stirred curd Cheddar and intensified the degree of browning in processed cheese made therefrom. Similarly, Michel and Martley (2001) found that Cheddar cheese made using *Sc. thermophilus* as an adjunct culture to *Lc. lactis* subsp. *cremoris* or *lactis* strains had a high residual galactose level of ~ 26.6 mmol/ kg (0.48 %, w/w) at 1 d. Moreover, the residual galactose content increased with an increase in the scald temperature from 38 °C to 41 °C (data not reported by authors). Tinson, Ratcliffe, Hillier and Jago (1982b) reported that high levels of residual galactose (33 mmol kg^{-1}, 0.56 %, w/w) in 8 wk-old Cheddar cheese made using *Sc. thermophilus* (0.5 %, w/w) as an adjunct to *Lc. lactis* subsp. *cremoris* coincided with a higher production of CO_2, leading to the development of slits and fractures in the cheese at 8 and 14 wks, this

may be due to the growth of NSLAB which are able to metabolise the galactose hence, causing the defect.

The accumulation of galactose in cheese can lead to problems such as (i) providing a readily fermentable carbohydrate which can influence the development of the non-starter lactic acid bacteria (NSLAB) flora possibly leading to defects, (ii) the presence of a reducing sugar in cheese can cause excessive Maillard browning on heating as in Mozzarella cheese, and (iii) early gas production in Cheddar cheese (Mullan, 2000; Ortakci, Broadbent, Oberg & McMahon, 2015). Nevertheless, the presence of galactose can interfere with the drying and crystallization properties of lactose during whey processing (Harju, Kallioinen, & Tossavainen, 2012).

- *Lactobacillus*

The other commonly used adjunct cultures are *Lactobacillus* (*Lb*.) spp., in particular mesophilic *Lactobacillus casei*., thermophilic *Lactobacillus helveticus.* and *Lactobacillus delbrueckii subsp. bulgaricus* (Drake & Swanson, 1995; Fox et al., 1998; Midje, Bastian, Morris, Martin, Bridgeman & Vickers, 2000; Johnson, & Law, 2010). Selection of lactobacilli into cheese are dependent on their ability to ferment the residual lactose in cheese, after the initial acidification by the starter culture in the vat. Johnson, & Law (2010) reported that when the residual lactose is converted to enzyme-rich biomass *in situ* and to 'secondary' lactose metabolites, such as carbon dioxide, acetic acid and ethanol, those compounds can affect the cheese flavour profile by their mechanisms. For example, ethanol reacts with free fatty acids (FFA) to produce fruity esters, such as ethyl hexanoate in cheese and acetic acid that contributes sharpness to cheese reacts with methane thiol to produce a cheesy thiomethyl ester. Fenelon, Beresford & Guinee (2002) used *Lactococcus lactis* spp. *lactis* and *cremoris*

as starter cultures in combined with adjunct cultures including of (i) *Lb. helveticus*, (ii) *Lb. helveticus/casei* and (iii) *Leuconostoc cremoris/Lc. lactis* var. *diacetylactis/Sc. thermophilus/Lb. helveticus*, in the manufacture of reduced-fat Cheddar cheeses in which they found that the cheeses made using adjunct cultures had higher concentrations of low molecular mass peptides (< 0.5 kDa) and free amino acids (FAA) and also graded (with commercial grader) with higher flavour scores, more acceptable compared to control cheeses made without adjunct cultures. Banks, Hunter & Muir (1993) reported that the use of an adjunct culture at an optimum salt content (1.8 %) achieved desired flavour for reduced-fat Cheddar cheese (16 %, w/w, fat) to be comparable to commercial full-fat Cheddar cheeses.

1.4. Biochemical changes during cheese ripening and their impact on cheese quality

1.4.1. Glycolysis and Cheddar cheese quality

Glycolysis involves the fermentation of lactose to lactates which has been discussed above in section 1.1.4. Seasonal variation in the concentration of lactose in milk, which can range from ~ 4.0 to 4.8 % (w/w) (O'Brien et al, 1999b) is expected to influence largely on the final pH of the cheese, which affects enzyme activity (proteolysis and lipolysis), cheese texture, and also the non-starter microflora. Nevertheless, cheese flavour is likely to be affected due to variations in the production of lactic and acetic acids and the metabolic activity of the cheese microflora (Fox et al, 2016c).

Very few studies have been done on the effect of residual sugars in cheese on cheese quality. Huffman & Kristoffersen (1984) washed the curd or added lactose to the curd and whey mixture after cutting the coagulum, however, due to the small

molecular size and the solubility of lactose, it turned out to be lost in whey by syneresis and hence, the increase in the concentration of lactose in curd was quite small. However, lowering the levels of lactose at day 1 led to lower levels of lactate and higher pH values in the mature 9 month-old cheese; flavour developed more slowly in the washed curd cheeses, which were described as being less sharp. Similarly, Waldron (1997) reduced the lactose content of Cheddar cheese by curd washing which involved replacing 35–45 % of the whey by an equal volume of warm water during cooking process. Residual lactose in cheese varied from 0.03 % (washed) to 1 % (control) and 2.5 % (lactose added) at day 1. The concentration of residual lactose in washed curd cheese was completely metabolized within ~2 weeks while it still persisted in the high-lactose cheeses throughout 180-d ripening. The cheese pH in washed curd cheeses was inversely proportional to the concentration of lactose in the curd. Sensory analysis showed that flavour development was significantly faster in lactose added cheeses compared to the washed-curd cheeses, but harsh due to the low pH while the washed curd cheeses were cleaner and milder. Both studies suggested that the concentration of residual lactose in cheese has a substantial effect on the quality of Cheddar cheese. The residual lactose in cheese can also lead to Maillard (non-enzymatic) browning when cheese is heated especially when cheese is made using thermophilic cultures e.g., *Sc. thermophilus* which are mostly galactose-negative as discussed in Section 1.3.2. However, if a galactose-positive strain of *Lactobacillus* is used as an adjunct culture, the residual galactose can be metabolized to L - or D - lactate. Moreover, the presence of residual sugars during ripening can also lead to undesirable secondary fermentations which may cause off-flavour, gas production and/or reduced quality (Fox et al, 2016c).

1.4.2. Lipolysis and Cheddar cheese quality

The degree of lipolysis in cheese varies widely between varieties, from ~6 meq/100 g free fatty acids (FAAs) in Gouda to 45 meq/100 g in Danish Blue (Gripon 1987, 1993). The source of lipases in cheese are from milk, rennet, starter bacteria, adjunct starter cultures and/or non-starter bacteria (Fox & Cogan, 2004b). Lipolysis plays a major role in the quality of cheese which affects cheese rheology and texture (see Section 1.4.4) and also cheese flavour (see Section 1.5.1).

1.4.3. Proteolysis and Cheddar cheese quality

Proteolysis in Cheddar cheese during ripening is an important indicator for the maturation progress and affects cheese flavour and texture development. The total amount and composition of the amino acids in cheese has been used as an index of cheese ripening (Fox et al., 1995a). The formation of peptides and free amino acids during ripening is accomplished essentially by LAB and is responsible for aroma formation in Cheddar cheese either directly or by acting as a flavour precursor for other compounds that are formed through transamination, deamination, decarboxylation, desulfuration, etc. and degraded into flavourcompounds such as amines, aldehydes, alcohols, and ammonia (Fox et al., 1996; Law, 2001; Sousa et al., 2001; Smit & Engels,2005). In previous studies, researchers attempted to enhance FAA content in Cheddar cheese by adding amino acids directly to cheese (Wallace & Fox, 1997) and modifying of lactococci genetically with increased aminopeptidase N activities (McGarry, Law, Coffey, Daly, Fox & Fitzgerald, 1994; Christensen, Johnson & Steele, 1995). However, those approaches did not affect the Cheddar cheese flavour development, which may indicate that the cheese flavour development in flavour biogenesis is not due to the release of amino acids, but their further conversion to

aroma compounds (Yvon et al., 1998). Cheese starter bacteria, lactococci, are able to degrade amino acids via transamination (Gao, Oh, Broadbent, Johnson, Weimer & Steele, 1997), which results in the formation of α-keto acids (α-KA) and those acids are corresponding to almost every amino acid in Cheddar cheese (Ney, 1981). Aromatic aminotransferase enzymes have been previously found in *Lactococcus lactis* ssp. *cremoris* (Yvon, Thirouin, Rijnen, Fromentier & Gripon, 1997; Rijnen, Bonneau & Yvon, 1999a) and *Lactococcus lactis* ssp. *lactis* (Gao & Steele, 1998). These enzymes which are known as flavour precursors have been found to initiate the degradation of certain amino acids, including Val, Leu, Ile, Phe, Tyr, Trp, and Met. Inactivation of those enzymes by breakdown of amino acids with *Lactococci* strains has shown a reduction in cheese flavour development during ripening (Rijnen, Delacroix-Buchet, Demaizieres, Le Quere, Gripon & Yvon 1999b).

Proteolysis also affects the cheese texture during ripening as the intact casein is degraded into polypeptides and smaller water-soluble peptides. Moreover, carboxyl and amine groups that are liberated during proteolysis cause a decrease in a_w by binding water molecules (Sousa et al., 2001).

1.4.4. Cheese texture and rheology

Cheddar cheese is a hard type cheese which has a texture intermediate between solid and liquid and hence, referred to as being viscoelastic in nature (Guinee, 2003b). Cheese texture can be measured using several different sensory and mechanical methods such as creep, stress, compressiom, melt and relaxation tests, which have been reported previously (Guinee, 2002; Foegeding, Brown, Drake & Daubert, 2003; Guinee & Kilcawley, 2004; O'Callaghan & Guinee, 2004). Cheese texture is an important indicator of overall quality cheese, while rheological properties influence

cheese texture, eating quality and physical behaviour when subjected to processing operations (e.g., grating, slicing, shredding) (Guinee, 2003b; Guinee & Kilcawley, 2004; O'Callaghan & Guinee, 2004). They are affected by various factors such as manufacturing process, cheese composition, cheese microstructure and physico-chemical state of the cheese components (e.g. degree of casein hydration, ratio of solid-to-liquid fat, proportion of intact-to-hydrolyzed casein, level of fat coalescence) (Foegeding et al., 2003; Guinee, 2003a). Proteolysis is one of the main factors affecting cheese texture during ripening (Guinee, 2002; O'Callaghan & Guinee, 2004). Decades ago, researchers stated that cheese texture changes intensively during the first 30 days of ripening due to the residual chymosin activity in cheese cleaving α_{s1}-casein at the Phe23-Phe24 peptide bond producing water-soluble peptides and amino acids which do not contribute to the cheese texture by protein matrix, and thus result in softening of cheese texture (Visser, 1991; Kindstedt, 1995; Guinee, 2003a; Laurence et al, 2004). Now it is thought to be mainly due to solubilisation of calcium phosphate (O'Mahony et al, 2005). Cheese texture further softens throughout ripening as proteolysis proceeds with residual rennet activity, plasmin and also the presence of starter and non-starter proteinases. Another important factor affecting cheese texture is the solubilisation of CCP (Lucey, Johnson & Horne, 2003). In previous studies, researchers reported that softening of Cheddar cheese texture was highly correlated with the level of insoluble Ca during ripening (Lucey, Mishra, Hassan & Johnson 2005; O'Mahony et al, 2005). Solubilisation of CCP results in rearrangement of casein particles, reduces CCP crosslinking and an increase in electrostatic repulsion between casein micelles. This leads to loose casein-casein bonds which would be conducive to softening of cheese texture (Lucey et al., 2003; Hassan, Johnson & Lucey, 2004). Cheese pH also plays an important role in cheese textural changes either by influence

on *para*-casein hydration or by affecting the activity of residual chymosin and plasmin and hence, casein hydrolysis (Guinee, 2003a; Pastorino, Hansen & McMahon, 2003). Lee et al, (2010) also studied the rheological properties of Colby cheese with different insoluble calcium content during ripening. The insoluble calcium content in cheese was reduced by lowering the pH at key manufacturing steps, hence causing a reduction in buffering capacity of curd, and lower cheese pH. Residual lactose was also controlled by reverse osmosis (concentrate) or curd washing (dilution). The higher lactose content in cheese resulted a lower cheese pH which showed at very low pH values (<4.9) meltability reduced during ripening.*1.4.5. Colorimetry*

Colour is important for measuring the quality of food as it is considered by consumers to be related to product freshness, ripeness, desirability and food safety (McCraig, 2002; Jeliński, Du, Sun & Fornal, 2007). Cheese colour can be measured using a colorimeter (HunterLab meter or Minolta Chroma meter) which is developed by transforming or filtering reflected spectra to produce reproducible colour space coordinates which are called: L* (index of whiteness), a* (index of redness) and b* (index of yellowness) (MacDougall, 2001). Colorimetry is widely used in quality control and also for product development to measure the colour of curd and cheese. Cheese colour is related to cow's diet, addition of colouring and cheese variety. Measuring cheese colour can also be used to detect defects, such as browning, during cheese maturation (Carreira, Dillinger, Eliskases-Lechner, Loureiro, Ginzinger, & Rohm, 2002).

1.5. Cheese flavour and sensory properties

The sensory properties of food are important determinants for consumer to choose what food to purchase and consume. Flavour plays a major role in this context.

Flavour may be defined as the combination of taste and odour (Urbach 1997a). Cheese flavour has been studied and reviewed extensively over the past few decades (Ohren & Tuckey, 1969; Aston & Dulley, 1982; Seitz, 1990; Fox & Wallace, 1997; Urbach, 1997a, b; Forde & Fitzgerald, 2000; McSweeney & Sousa, 2000; Singh et al, 2003; Collins et al., 2003; Holland, Liu, Crow, Delabre, Lubbers, Bennet & Norris, 2005; Smit & Engels, 2005). It is considered to be the result of a balance of a complex blend of volatile and non-volatile compounds (amino acids and fatty acids) derived from the breakdown of proteins (mainly casein), lipids and carbohydrates (mainly lactose) during maturation (Fox & Wallace, 1997; Forde & Fitzgerald, 2000; Holland et al., 2005). The compounds which influence cheese flavour the most are alcohols, aldehydes, esters, ketones, dicarbonyls, short- to medium-chain free fatty acids (FFA), methyl ketones, lactones and phenolic compounds, nitrogen- and sulphur-containing compounds (Aston & Dulley, 1982; Fox & Wallace, 1997; Urbach 1997a, b).

Cheese flavour changes with ripening time; cheese has a mild flavour at early stage of ripening to strong mature flavour after ripening for 12-24 months. During ripening, a series of biochemical reactions occurs which generates flavour compounds by milk enzymes, starter bacteria, NSLAB and rennet (Forde & Fitzgerald, 2000; McSweeney & Sousa, 2000; Collins et al., 2003). In agreement with previous studies, proteolysis is considered to be the most important biochemical pathway which contributes most to flavour compounds in Cheddar cheese during ripening (Forde & Fitzgerald, 2000; Sousa et al., 2001). Proteolysis produces numerous peptides and amino acids that contribute to the background savoury flavour of Cheddar. Amino acids also act as flavour precursors for the formation of important flavour compounds such as esters, thiols, aldehydes and ketones (Sousa et al., 2001; Smit et al., 2005). However, bitterness is one of the major defects in Cheddar cheese flavour which is

due to the high level of high molecular weight peptides formed by proteolysis (McSweeney & Sousa, 2000; Bank 2003; Lawrence et al., 2004).

Lipolysis is another major contributor to cheese flavour development during ripening by producing aromatic fatty acids, which contribute directly and positively to cheese flavour (Fox & McSweeney, 1995a). Research shows that long-chain FFAs (>12 carbon atoms) have a minor role in flavour of Cheddar cheese while short- and medium-chain FFAs (4 - 12 carbon atoms) contribute more to cheese flavour with their individual characteristic profiles (Collins et al., 2003; Le Quéré & Molimard, 2003). Those FFAs also act as substrates to aid in the formation of typical Cheddar flavour compounds such as methyl ketones, alcohols, lactones and esters (Fox & McSweeney, 1995b; McSweeney, 2004). However, excessive lipolysis can also lead to rancid flavour in cheese (Fox & McSweeney, 1995b) and the ethyl and hexyl esters derived from short-chain FFAs can result in a fruity defect in Cheddar cheese (Guinee & Law, 2002).

The use of cheese starter, adjunct cultures and their rate of autolysis (enzyme release) affect cheese flavour directly by their effect on proteolysis and lipolysis to produce amino acids and fatty acids (Torres, Bouzas, Kirby, Almonacid Merino, Kantt, Simpson, & Banga, 1995; Laurence et al., 2004; Holland et al., 2005; Peláez & Requena, 2005; Smit et al., 2005). They are also reported to affect cheese flavour indirectly by producing optimial conditions for enzyme activities (e.g., redox potential, pH) (Lawrence et al., 2004). NSLAB is also reported to have a significant effect on cheese flavour and is able to reduce the harshness and bitterness caused by some starter cultures (Banks, 2003; Beresford & Williams, 2004).

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OBJECTIVES

The objectives of this thesis were to:

The main objective of the following experimental chapters was to further develop the understanding of the novel strategies for improving Cheddar cheese quality by optimization of its manufacturing process. These experimental chapters investigated a number of different strategies to explore this topic such as applying curd washing during manufacture to control lactose levels, altering milk treatments and cheese processing parameters and using selected adjunct culture.

- In **Chapter 2 and 3**, curd washing was applied during cheese manufacture process to give four different targets of lactose plus lactic acid in cheese moisture phase levels which in range of typical Irish milk lactose seasonal variations. The effect of curd washing on cheese composition, biochemistry, microbiology, rheology and sensory was studied.
- In **Chapter 4**, combination effect of curd washing and controlling calcium phosphate levels was carried out in Cheddar cheese as a continuation of **Chapter 2 and 3**. This this study would try to develop the known theories available on residual lactose and levels of calcium phosphate and its relate impacts on the quality of Cheddar cheese.
- In **Chapter 5**, a similar approach to **Chapter 4** was used to investigate the effect of curd washing and milk protion fortification by UF process on Cheddar cheese quality.
- In **Chapter 6**, a large number of lactic acid bactira of dairy origin was screened in order to identify their acidification profiles, sugar

metabolism and salt sensitivity to be used for further cheese production as adjunct cultures. The cultures studied in this study were *Sc. thermophilus and Lactobacillus*.

- In **Chapter 7**, selected galactose positive and galactose negative strains of *Sc. thermophilus* were used in Cheddar cheese manufacture as adjunct cultures at different drain pH. The effect of using those adjunct cultures on the impact of Cheddar cheese was studied.

Chapter 2: Effect of curd washing on composition, lactose metabolism, pH, and the growth of non-starter lactic acid bacteria in full fat Cheddar cheese[1]

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Abstract

Cheddar cheese was manufactured in triplicate from mid-lactation milk and analysed over a 270-day ripening period. The curd was washed in the cheese vat to give target levels of lactose plus lactic acid in cheese moisture ($LLAM_C$) in the final cheese of 5.3 (control), 4.5, 4.3 and 3.9% (w/w); these values correspond to the expected $LLAM_C$ levels in non-washed cheeses made from milk with lactose levels of 4.8 (control), 4.6, 4.3 and 3.8 % (w/w), respectively. Increasing curd washing from 0 to 33% of milk volume significantly reduced mean levels of total lactate and $LLAM_C$ over the ripening period. Conversely, it increased the mean cheese pH, by ~ 0.3-0.4 at times ≥ 90 days. The $LLAM_C$ was ~ 1.0 – 1.6 units lower than predicted based on levels of milk lactose and curd washing. Otherwise, alteration of curd washing generally did not affect gross composition or microbiology of the cheese.

2.1. Introduction

The pH of cheese is controlled by the interactive effects of a number of critical factors including the amount of lactic acid, calcium phosphate and protein, the salt sensitivity of starter culture, and the level and duration of salting (Fox, Lucey, & Cogan, 1990; Guinee, 2004; O'Connor, 1974; Turner & Thomas, 1980). Curd washing is used in the manufacture of brine-salted cheeses such as Gouda and Edam to control the lactose, and hence lactic acid concentration in the curd and pH of the resultant cheese. In Gouda and Edam, the whole moulded cheeses, which vary in dimensions and weight (e.g. 0.2 to 100 kg), are submerged in brine (NaCl) and salt penetrates slowly during brining and the subsequent storage period. Consequently, ample time remains for virtual complete fermentation of lactose to lactic acid by the starter culture before the salt concentration in the centre of the cheese becomes inhibitory (although inhibition may occur in the surface layer). Hence, the pH in brine-salted cheese is determined by the degree of curd washing which determines the levels of lactose, and ultimately the levels of lactic acid. By contrast, in dry-salted cheeses such as Cheddar and related types (such as Cheshire and Colby cheese), salt is added directly to the curd (milled chips, typically 6-10 g) at a desired target pH (e.g., 5.3 – 5.4 for Cheddar). Owing to the larger surface area of the dry-salted curd, salt reaches all parts rapidly, becoming inhibitory to starter cultures to a degree dependent on salt sensitivity. This inhibits the fermentation of lactose (to lactic acid) by the starter culture prior to the completion of its fermentation (Broadbent, Houck, Johnson, & Oberg, 2003; Sheehan, Wilkinson, & McSweeney, 2008). Hence, it is important that the fermentation proceeds close to the target pH prior to salting otherwise the pH may not decrease adequately and the cheese may not ripen correctly. Cheddar curd at salting contains 0.5–0.8 % (w/w) lactose, which is fermented during early maturation via continued

activity of the starter, but this depends on the levels of salt-in-moisture (S/M) and the salt tolerance of the added starter culture strains. Despite the conversion of residual lactose to lactic acid in pressed dry-salted Cheddar cheese, the pH generally does not decrease significantly during maturation because of its high buffering capacity, associated *inter alia* with the high retention of calcium phosphate, and the addition of salt prior to fermentation of the residual lactose. Dry salting of curds at the higher-than-ultimate pH (e.g., 5.3 – 5.4 for Cheddar) reduces the risk of the pH of the resultant cheese being too low, excessive proteolysis, and defective quality.

Cheddar cheese is not a washed curd variety, hence, seasonal variation in the concentration of lactose in milk, which can range from ~ 4.0 to 4.8 % (w/w) (O'Brien, Mehra, Connolly, & Harrington, 1999) is expected to influence the composition and quality of the cheese. Nevertheless, relatively few studies have investigated the potential relationship between the lactose content of milk, and the levels of residual lactose and lactate, on Cheddar cheese quality. Huffman and Kristoffersen, (1984) investigated the effect of altering the lactose content of Cheddar cheese, either by adding lactose to the curd-whey mixture (high lactose, HL) or by curd washing (replacing whey with simulated milk ultrafiltrate, LL). The residual lactose contents of the control (CL), HL and LL cheeses at 1 day were 0.27, 0.41 and 0.06% (w/w), respectively. Lower levels of lactose at day one led to lower levels of lactate and higher pH values in the mature 9 month-old cheese; flavour developed more slowly in the LL cheeses, which were described as being less sharp. Shakeel-Ur-Rehman, Waldron and Fox, (2004) also prepared HL and LL cheeses, with lactose levels of 2.3 and 0.25% (w/w) at day one, by fortifying the milk to 8.4% (w/w) lactose with lactose powder (HL) or by washing of the curds from control milk (LL; lactose concentration not given), respectively. Although the pH of the HL cheese decreased significantly during

maturation from ~ 5.3 at day one to 4.8 at day 180, that of the LL cheese remained relatively constant at ~ 5.3 to 5.4. Despite only minor differences in the levels of primary proteolysis, the HL cheese had higher levels of total free amino acids and on grading, was found to have a harsh coarse flavour and a crumbly body. In both of the above studies, it is unclear what the levels of lactose in the cheese on day one would correspond to in terms of the lactose content of milk used in the manufacture of standard (non-washed) Cheddar cheese. Lee et al, (2010) also studied the rheological properties of Colby cheese with different insoluble calcium content during ripening. The insoluble calcium content in cheese was reduced by lowering the pH at key manufacturing steps, hence causing a reduction in buffering capacity of curd, and lower cheese pH. Residual lactose was also controlled by reverse osmosis (concentrate) or curd washing (dilution). The higher lactose content in cheese resulted a lower cheese pH which showed at very low pH values (<4.9) meltability reduced during ripening.

The aim of the current study was to determine the effect of altering the levels of lactose and lactic acid in cheese moisture (LLAMc), by varying the degree of curd washing in the cheese vat, on gross composition, sugar metabolism, pH, and the growth of starter and non-starter lactic acid bacteria (NSLAB) in full fat Cheddar cheese. The levels of curd washing were chosen to coincide with LLAMc levels in standard (non-washed) Cheddar made from milk with levels of lactose ranging from 4.8 to 3.8% (w/w) and which, therefore, represent the natural seasonal variation of lactose in Irish milk (O'Brien, et al., 1999).

2.2. Materials and methods

2.2.1. Preparation of cheese milk

Holstein-Friesian cows' milk (2000 kg) was obtained from a spring-calving, herd (Moorepark, Co. Cork, Ireland). Milk samples were standardized to a protein to fat ratio of 0.95:1, stored overnight at 8 °C, pasteurized at 72 °C for 15 s, cooled to 31°C, and pumped to four cheese vats (500 L).

2.2.2. Starter cultures for cheesemaking

Defined strain starter cultures were used in cheesemaking (*Lactococcus lactis* subsp. *cremoris* strains 223 and 227; Chr. Hansen Ireland Ltd., Rohan Industrial Estate, Little Island, Co. Cork, Ireland). Both cultures were grown overnight at 24 °C in reconstituted 10% (w/v), antibiotic-free skim milk powder solution (Kerry Ingredients Ltd., Charleville, Co. Cork, Ireland) which had been heat treated at 95 °C for 30 min. When the pH of the inoculated milk reached between pH 4.5 to 5.0, the cultures were cooled and stored at 4°C until required for cheesemaking (~1 day).

2.2.3. Cheese manufacture and treatments

Cheesemilk (31 °C) was inoculated at a level of 0.75 % (w/w) with each of the cultures and left to stand for 30 minutes. The pH was measured and adjusted, if necessary, to pH 6.55 using 5 % (v/v) lactic acid solution to have same starting milk pH. Rennet (Chymax Plus, Chr. Hansen Ireland Ltd.) was diluted 1:10 in de-ionized water, and added to the milk at a level of 0.18 mL kg^{-1} based on a protein level of 3.3 % (w/w). Following gelation, the gel was cut at standard Cheddar curdfirmness at 54 Pa (determined using a CSL2 500 Carri-Med rheometer; TA Instruments, Inc., New Castle, DE, USA) and allowed to heal for 10 min. Then the curd-whey mixture was

stirred continuously, cooked at a rate of 0.2 °C min^{-1} to 34 °C. Agitation was then stopped and different quantities of whey were withdrawn from each of the four vats and filtered through a nylon mesh (to remove any curd particles/fines, which were returned to the cheese vat), and replaced by equivalent amounts of pasteurised (80 °C for ~ 5 min) reverse osmosis-treated water at 38°C. Replacement of whey with an equal weight of wash water was considered important to eliminate the effect of the altered curd-to-whey ratio on cheese composition, especially moisture which tends to decrease as the latter is increased (Guinee, O'Kennedy, & Kelly, 2006). The levels of whey removed to achieve the levels of lactose and lactic acid in cheese moisture (LLAMc) were 0 (Control, 5.3LLAMc), 16.1 (4.5LLAMc), 21.6 (4.3LLAMc) and 33 (3.9 LLAMc) L 100 kg^{-1} cheese milk to give target LLAMc levels in the curd/cheese moisture of 5.3, 4.5, 4.3 and 3.9 % (w/w), respectively. Following water addition, the curd-whey mixture was further cooked to 38.5 °C at 0.2 °C min^{-1} and the whey (control) or diluted whey (washed curd) was separated from the curd when the pH of the curd reached 6.15. The curds were cheddared, milled at pH 5.35, salted at a level of 2.7 % (w/w), mellowed for 20 min, placed in rectangular moulds (23 kg), and pre-pressed at 0.13 kPa for 30 min. The moulded cheeses were then placed in a horizontal press and pressed overnight at 2.5 kPa. Cheeses were vacuum packed and stored at 4 °C for 14 days and at 8 °C thereafter. The cheeses were coded as 5.3LLAMc, 4.5LLAMc, 4.3LLAMc and 3.9LLAMc, according to target lactose level in the moisture phase of the cheese. Cheesemaking trials were carried out in triplicate (trials 1-3) over a four-week period from April 15 to May 08, 2009.

2.2.4. Changes in composition of curd and whey during manufacture

Analysis of cheeses from trials 1 and 2, indicated that the concentration of sugars in cheese moisture were lower than those predicted based on the level of lactose in milk and levels of curd washing (Figure 2), hence, it was decided to monitor the partition of moisture, lactose and lactic acid between the curd and whey during manufacture in trial 3. Samples of curd and whey were taken at key stages of manufacture: at maximum scalding temperature (when cooking temperature was reached), immediately before whey drainage, after whey drainage, and before salting. For curd samples from the cheese vat, samples (200 g) of the stirred curd-whey mixture were poured through a 1 mm stainless steel sieve and agitated thoroughly to remove surface whey. The curd sample (~ 20 g) was immediately placed in a sterile stomacher bag (Grade Packaging Ltd, 8 Vulcan Court, Coalville, Leicestershire, England). A sub-sample was immediately withdrawn and analysed for moisture, as described below. The stomacher bag, containing the remaining curd was folded and taped, and stored at -20 °C to prevent further microbial growth and lactose degradation; the curd was then thawed at 4 °C and analysed for sugars within 1 week. Samples of whey or whey-wash water mixtures were taken directly from the cheese vats, sieved (1 mm), placed in a 30 mL Universal sterile container (Ramboldi Ltd, Limassol, Cyprus), stored at -20 °C, and analysed as for curd samples.

2.2.5. Sampling of cheese

Cheeses were sampled at different times over the 270-day ripening period. Samples for compositional analysis were grated by passing through a Krups Rotary 350 food processor, with a universal blade (Robert Krups GmbH & Co, Solingen, Germany), which produced particles of <1 mm. The samples were analysed for lactose,

lactic acid and pH. Samples for microbiological analysis were taken aseptically at different times using a sterile cheese trier and placing the resultant sample (~ 10 g) in a sterile stomacher bag.

2.2.6. Composition analysis of curds, whey and cheese

Curd and whey samples were analysed for moisture using standard International Dairy Federation (IDF) methods (Guinee, Auty, & Fenelon, 2000) and lactose and lactate as described below. Grated cheese samples were analysed at 14 days for protein, fat, NaCl, moisture, ash, Ca and P using standard IDF methods (Guinee, et al., 2000). The pH was measured after each sampling date on cheese slurry prepared from 20 g of grated cheese and 12 g distilled water (Guinee, et al., 2000).

2.2.7. Microbial counts in cheese

Following sampling, a 10 g sample of cheese was diluted 1:10 in sterile 2 % (w/v) trisodium citrate, and macerated using a stomacher (Stomacher, Lab-Blender 400, IUL, S.A., Barcelona, Spain) for 4 min. Serial dilutions of the resultant slurry were prepared depending on the stage of maturation and the bacterial population being measured (i.e., starter culture or NSLAB). Starter lactococci were enumerated on LM17 agar (Terzaghi & Sandine, 1975) after incubation at 30 °C for 3 days. NSLAB, which in Cheddar cheese mainly comprise mesophilic *Lactobacillus* spp. (Jordan & Cogan, 1993; Williams & Banks, 1997), were enumerated on LBS agar (Rogosa, Mitchell, & Wiseman, 1951) incubated anaerobically, with an overlay, at 30 °C for 5 days.

2.2.8. Sugars in cheese

The concentrations of lactose, galactose, and D (-) - and L (+)-lactate in the cheeses were measured using the Boehringer Mannheim UV test kit (Cat No. 0176 303 and Cat No. 11 112 821 035, Boehringer Mannhein/R-Biopharm AG, Darmstadt, Germany; Rynne, Beresford, Kelly, & Guinee, 2007). The total lactate was calculated as the sum of D (-)- and L (+)-lactate contents, and total sugars and derived acids as the sum of lactose, galactose, and total lactate. Each analysis was carried out in duplicate.

The predicted level of LLAMc in cheese was calculated based on lactose levels in milk moisture, and on volumes of whey removed and wash water added, using the formula:

$$LLAMc = \frac{LIMM \ x \ volume\ whey}{volume\ whey + volume\ wash\ water} \qquad (1)$$

where LLAMc is lactose plus lactic acid in the cheese moisture phase, and LIMM is the lactose in the moisture phase of milk.

2.2.9. Statistical analysis

Three replicate cheesemaking trials were undertaken in which the cheeses were manufactured from milk with 4.8 % (w/w) lactose. In each trial, four treatment cheeses were made: control cheese with 5.3 % LLAMc, and the three remaining cheeses with target LLAMc values of 4.5LLAMc, 4.3LLAMc and 3.9LLAMc, which were obtained by diluting the whey in the cheese vat to varying degrees with added wash water. A randomised complete block design incorporating the four treatments

(percentage of lactose in moisture phase) and three blocks (replicate trials) was used for data analysis. Analysis of variance (ANOVA) was carried out using a SAS (SAS® version 9.1.2) procedure (SAS Institute, 2004) where the effects of treatment and replicates were estimated for all response variables. Tukey's multiple-comparison test was used as a guide for pair comparisons of the treatment means; the level of significance in all the treatments was determined at $P < 0.05$.

A split-plot design was used to determine effect of treatments, storage time and their interactions on the specific variables measured at regular intervals during cheese ripening, such as lactose, D (-) - and L (+)-lactate, total lactate content, total sugar content, pH and starter and NSLAB counts. Analysis of variance for the split-plot design was carried out using a general linear model (GLM) procedure of SAS (SAS Institute, 2004). Statistically significant differences ($P < 0.05$) between different treatment levels were determined by Fisher's least significant difference.

Simple linear regression (SLR) was also used to establish possible correlations between different response variables (e.g. total sugars, LLAMc, lactose, lactic acid, pH and curd wash water). The regression equation and correlation coefficient (r) were determined using Excel 2003 (Microsoft Office Excel 2003); the significance of correlation was determined by applying Student's *t* test to r with *n*-2 df, where *n* is the actual number of data points and the df is the degrees of freedom.

2.3. Results and discussion

2.3.1. Compositional analysis of milks

The mean levels % (w/w) of protein, fat, casein and lactose in the pasteurised cheese milk were 3.21 (± 0.04), 3.31 (± 0.06), 2.84 (± 0.02) and 4.89 (± 0.07),

respectively; the levels of protein and lactose are typical of those reported elsewhere for early/mid-lactation milk.

2.3.2. Cheese composition

The composition of cheese at 14 days is shown in Table 2.1. All cheeses had similar levels of moisture, fat, protein, S/M, pH, lactate, ash, and calcium. The results agree with the findings of others (Huffman & Kristoffersen, 1984; Shakeel-Ur-Rehman, et al., 2004) showing that curd washing did not influence the gross composition of the cheese. The concentration of lactose at 14 days decreased significantly with curd washing, from ~ 0.58 % (w/w) in the unwashed curd, control cheese (5.3LLAMc) to ~ 0.15% (w/w) in the 3.9LLAMc cheese, which had the highest level of curd washing.

Table 2.1. Effect of curd washing on the composition and pH of full-fat Cheddar cheese at 14 days[d, e]

Compositional Factors	Target level of LLAMc (%, w/w)[f]			
	5.3	4.5	4.3	3.9
Moisture (%, w/w)	37.8[a]	38.0[a]	37.8[a]	37.9[a]
Fat (%, w/w)	30.7[a]	30.9[a]	31.0[a]	30.9[a]
Protein (%, w/w)	25.5[a]	25.8[a]	25.9[a]	25.8[a]
Ash (%, w/w)	4.23[a]	3.97[a]	4.05[a]	4.03[a]
MNFS (%, w/w)[g]	54.6[a]	55.0[a]	54.7[a]	54.9[a]
FDM (%, w/w)[g]	49.5[a]	49.8[a]	49.8[a]	49.8[a]
S/M (%, w/w)[g]	5.27[a]	4.91[a]	4.98[a]	5.03[a]
Total lactate (%, w/w)	1.07[a]	1.11[a]	0.99[a]	0.85[a]
Lactose (%, w/w)	0.58[a]	0.26[b]	0.25[b]	0.15[b]
Total sugars (%, w/w)	1.68[a]	1.41[ab]	1.27[bc]	1.02[c]
LLAMc (%, w/w)	4.28[a]	3.52[b]	3.32[bc]	2.95[c]
Ca (mg 100 g^{-1})	793[a]	789[a]	785[a]	792[a]
P (mg 100 g^{-1})	522[a]	520[a]	520[a]	458[b]
L/P (%, w/w)[g]	4.23[a]	4.21[a]	3.83[a]	3.28[a]
pH	5.33[a]	5.30[a]	5.36[a]	5.41[a]

[d]Lactose content was adjusted by curd-washing at cooking stage of 34°C; results are presented as the mean values of three replicate trials.
[e]The composition and pH were measured at day 14 ripening time.
[f]Target level of lactose plus lactic acids in cheese moisture (%, w/w), LLAMc. Values within a row not sharing a common differ significant, $P < 0.05$.

[g]Abbreviations: S/M, salt in moisture; LLAMc, lactose plus lactic acids in cheese moisture phase; MNFS, moisture in non-fat substances; FDM, fat in dry matter; L/P, lactate to protein ratio.

2.3.3. Sugar metabolism in cheese

2.3.3.1. Total sugars

The mean levels of total sugars and derived acids (lactic acids) on a total weight basis, comprising residual un-fermented lactose, lactic acid and galactose, did not change significantly during ripening time but decreased significantly with level of wash water (Figure 2.1; Table 2.2). The relationship between total LLAMc and the level of washing of curd from milk with 4.8 % (w/w) lactose is shown in Figure 2.2. Regression analysis of all data points (treatments at different ripening times) indicated that total sugars decreased at a mean rate of 0.0179 % (w/w) per kg of wash water per 100 kg milk (r ~ 0.92). A similar trend was observed for the concentrations of LLAMc, which decreased at a rate of 0.0442 % (w/w). The decrease in content of total sugar and derived acids in the curd with washing is consistent with the solubility of all sugars in the moisture (serum) phase of the curd, and the diffusion of lactic acid and lactose from regions of high concentration (curd moisture) to low concentration (diluted whey).

The concentration of LLAMc in the control, non-washed cheese (4.14 %, w/w) was ~1.0 % (w/w) less than that of the lactose in the moisture phase of milk (LIMm; 5.32 % w/w). Similarly, analysis of data from previous studies (Czulak, Conochie, Sutherland, & van Leeuwen, 1969; Guinee, Kilcawley, & Beresford, 2008; Huffman & Kristoffersen, 1984; Martley & Crow, 1993; Rynne, et al., 2007; Turner & Thomas, 1980) indicated that the reported concentrations of LLAMc (3.4 to 4.6 %, w/w) for Cheddar cheese are lower than those expected (~ 4.3 to 5.2 %, w/w) based on the seasonal variations in the concentration of lactose in milk of ~ 4.0 to 4.8 %, w/w

(O'Brien, et al., 1999). The lower levels of LLAMc compared with that of LIMm may reflect a more rapid diffusion rate of lactic acid from the moisture phase of the curd particle (where lactose is being fermented to lactic acid by the starter culture) than that of lactose from the whey into curd particle because of the lower molecular mass of the former compared to the latter (~ 90 and 342 Da, respectively) and the higher concentration of lactate in the curd particle moisture. Measurement of the lactate and lactose in the curd and whey at different stages (from maximum scald to curd milling) during the manufacture of the 5.3LLAMc and 3.9LLAMc cheeses, show that the lactate-to-lactose ratio in the moisture phase of the curd particles was significantly higher than that in the whey moisture at all times (Figure 2.3). It is assumed that the depletion of lactose in the moisture phase of the curd particle (by the starter culture) results in a counter current diffusion of lactose from the whey into the curd particle.

The above suggestion that the diffusion of lactate from the curd particle to the whey is more rapid than the diffusion of lactose in the reverse direction is supported by the analysis of lactose and lactic acid during the manufacture of the 5.3LLAMc and 3.9LLAMc cheeses. This shows that the concentration of lactose plus lactic acid in the moisture phase of the curd particles was lower than that in the moisture phase of the whey at different stages (maximum scald to curd milling) of cheesemaking (Figure 2.4). Apart from at maximum scald in the 3.9LLAMc cheese, which may be due to insufficient time (~ 23 min between washing and maximum scald) to allow for much diffusion of lactate from the curd particles to the whey. The difference in LLAMc between the cheese moisture and the whey became more pronounced as the moisture content of the curd particles decreased over the course of cheese manufacture, i.e. from the time that the curd-whey mixture was cooked to 38.5 °C (maximum scald, pH ~ 6.5) to removal of whey from curd (whey drainage, pH ~ 6.15) through to the time

when the curds were milled just prior to salting (pH ~ 5.35). As curd particles synerese (Figure 2.4a) and become denser over the course of cheesemaking, it is envisaged that the impedance of the curd matrix (e.g., tortuousity and the sieve effect exerted on the diffusing lactose/lactic acid molecules) to lactose molecules migrating from the whey into the curd particle increases, with the expected effect being greater for lactose than lactic acid (Geurts, Walstra, & Mulder, 1974; Floury, El Mourdi, Silva, Lortal, Thierry, & Jeanson, 2015). A similar trend was noted for the control and washed curds, i.e., that the LLAMc was generally higher in the whey or whey-wash water mixture than in the corresponding curd particles at the different stages of curd manufacture (Figure 2.4). In the above context, it is noteworthy that the transport of solutes between cheese moisture and salting medium (e.g., concentrated brine around dry-salted curd chip) occurs by an impeded diffusion process (Fox, McSweeney, Cogan, & Guinee, 2004).

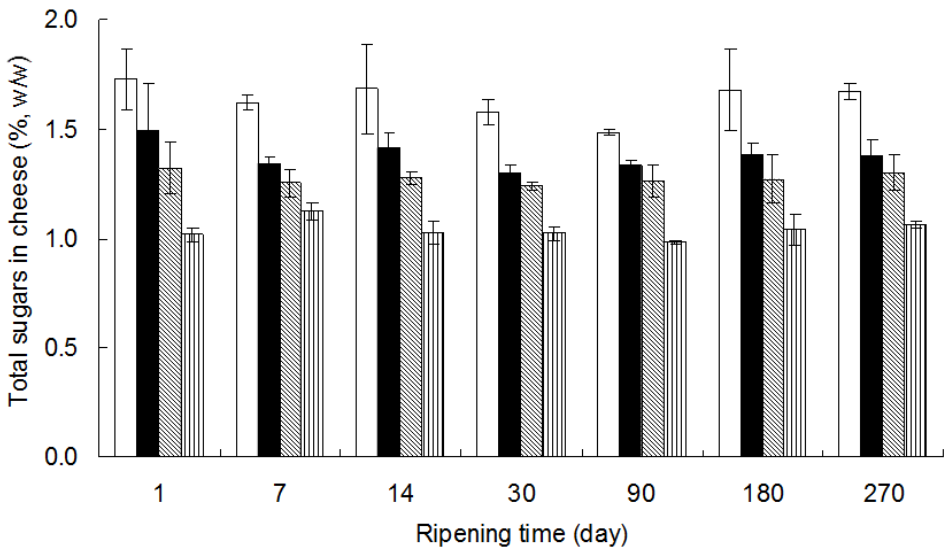

Figure 2.1. Total sugar content, as a function of ripening time, in Cheddar cheeses in which curds were washed to varying degrees during manufacture to give target levels of lactose plus lactic acid in cheese moisture (LLAMc) of: 5.3 (control, non-washed, □), 4.5 (■), 4.3 (▧) and 3.9 (▥), % (w/w). Values represent the means of three replicate trials; error bars show standard deviations of the mean.

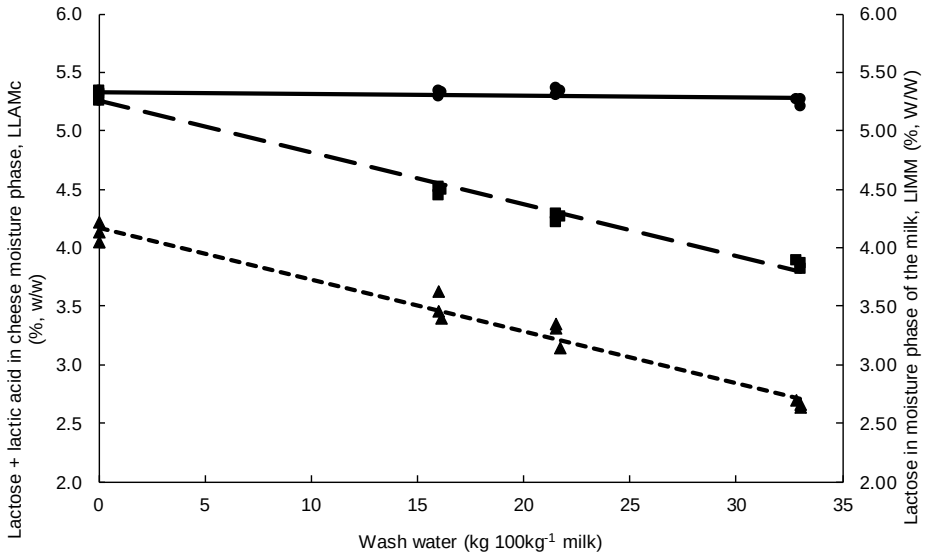

Figure 2.2. Experimental (▲) and predicted (■) levels of lactose plus lactic acid in cheese moisture (LLAMc) as a function of level of curd washing in the cheese vat. The levels of lactose in moisture phase of the milk (LIMM,●) are also shown. For each LLAMc curve, the 12 data points correspond to the four treatment cheeses from the three replicate trials, and each data point represents the mean of the values at different time point in vat. For the LIMM curve, the 12 data points correspond to the 12 vats of milk used in cheese manufacture.

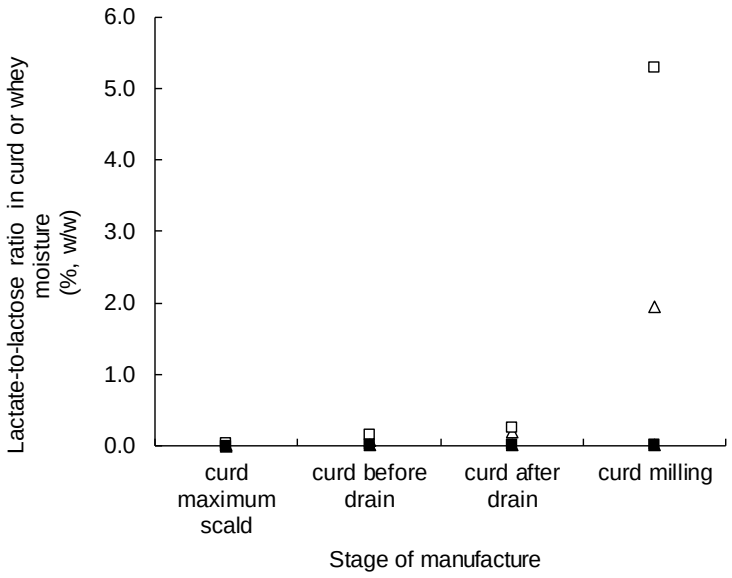

Figure 2.3. Lactate-to-lactose ratio in moisture phase of Cheddar cheese curds and whey at different steps of manufacturing process: at maximum scald (when cooking temperature reached 38.5°C), before whey drainage, after whey drainage, and curd milling. Data are shown for curds with target LLAMc levels of 5.3 (□, control, unwashed curd) and 3.9 (△, washed curd), % (w/w), and whey from the corresponding cheese curds 5.3 (■, control, unwashed whey) and 3.9 (▲, washed curd). n=3.

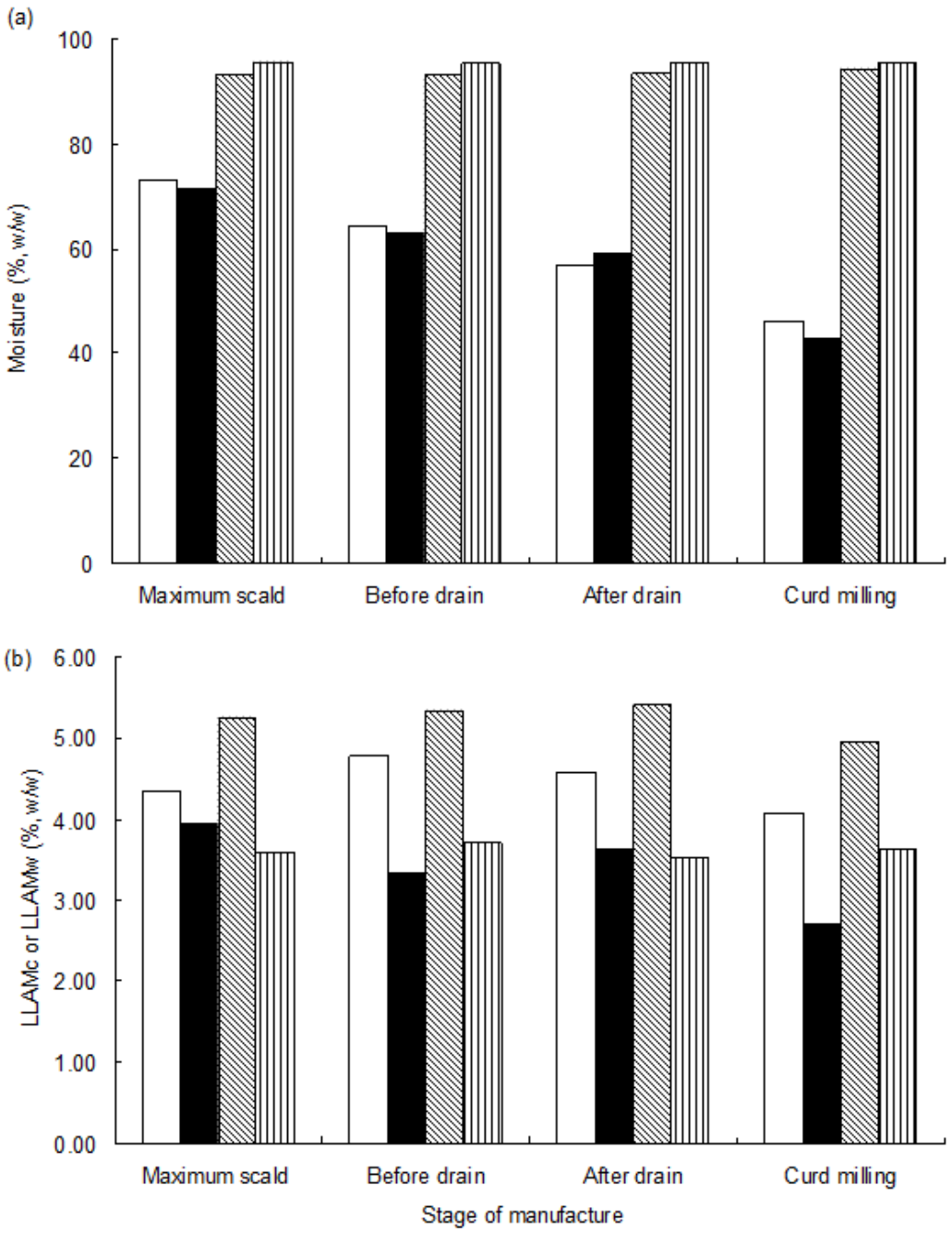

Figure 2.4. Moisture content (a) and lactose plus lactic acid in cheese or whey moisture (LLAMc or LLAMw) (b) of Cheddar cheese curds and whey at different manufacturing times: at maximum scald (when cooking temperature reached 38.5 °C), before whey drainage, after whey drainage, and curd milling. Data are shown for: curds with target LLAMc levels of 5.3 (□, control, unwashed curd) and 3.9 (■), % (w/w), and whey from the corresponding cheese curds with target LLAMc levels of 5.3 (▨), control, unwashed curd and 3.9 (▥), % (w/w).

Table 2.2. Degrees of freedom (df) and statistical significances (*P* - values) for changes in lactose, D(-)-lactate, L(+)-lactate, total lactate, and pH in full fat Cheddar cheeses with different target LLAMc levels[a].

Factor	Lactose		D(-)-Lactate		L(+)-Lactate		Total lactate		Total sugars		pH	
	df	*P*	df	*P*	df	*P*	df	*P*	df	*P*	df	*P*
Main plot												
Curd Washing	3	0.0022	3	0.2549	3	0.1151	3	0.0064	3	<0.0001	3	0.032
Sub-plot												
Ripening time	6	<0.0001	6	<0.0001	6	<0.0001	6	<0.0001	6	0.0230	6	0.0035
Interaction (Curd washing x time)	18	0.0452	18	0.2538	18	0.0973	18	0.2055	18	0.7162	18	0.0001

[a] LLAMc: lactose plus lactic acid in cheese moisture. Lactose content was adjusted by curd-washing at the cooking stage of 34 °C; results are presented as the mean values of three replicate trials.

2.3.3.2. Lactose

The mean level of residual lactose in the different cheeses decreased significantly with level of curd washing and ripening time (Figure 2.5, Table 2.2). Regression analysis indicated a linear relationship ($r \geq 0.7$, df =10) between the extent of curd washing and levels of residual lactose at ripening times up to 180 days, but especially during the first month. Similarly, previous studies (Huffman & Kristoffersen, 1984; Shakeel-Ur-Rehman, et al., 2004; Upreti & Metzger, 2006) have noted that residual lactose levels in cheese at 1 to 30 days after manufacture decreased with curd washing. Such a trend is expected as lactose is a water-soluble solute. The only constraint to lactose reduction within the curd particle on washing is its diffusivity through the curd matrix to the wash water surrounding the curd particles; the extent of diffusion increases with time of contact of the curd particle with the wash water but is also likely to be affected by temperature and curd particle size.

The high levels of residual lactose in all cheeses throughout ripening, especially in the control (which did not include a curd washing step), is somewhat surprising and may be attributed to a high salt sensitivity of the starter culture used. The starter culture used in cheesemaking, *Lactococcus lactis subsp. cremoris* 223 and 227, has been found to have a relatively low salt tolerance compared to other *Lactococcus lactis* strains (Fox, et al., 2004; Rulikowska, Kilcawley, Doolan, Alonso-Gomez, Nongonierma, Hannon, & Wilkinson, 2013). Thomas and Pearce (1981) reported that when using salt-sensitive starter cultures (paired strains of *Lactococcus cremoris* strains 134/582 or 134/584), residual lactose in Cheddar cheese increased from ~ 0.2 to ~ 0.6 % (w/w) as S/M was increased from 4.5 to 5.5 % (w/w).

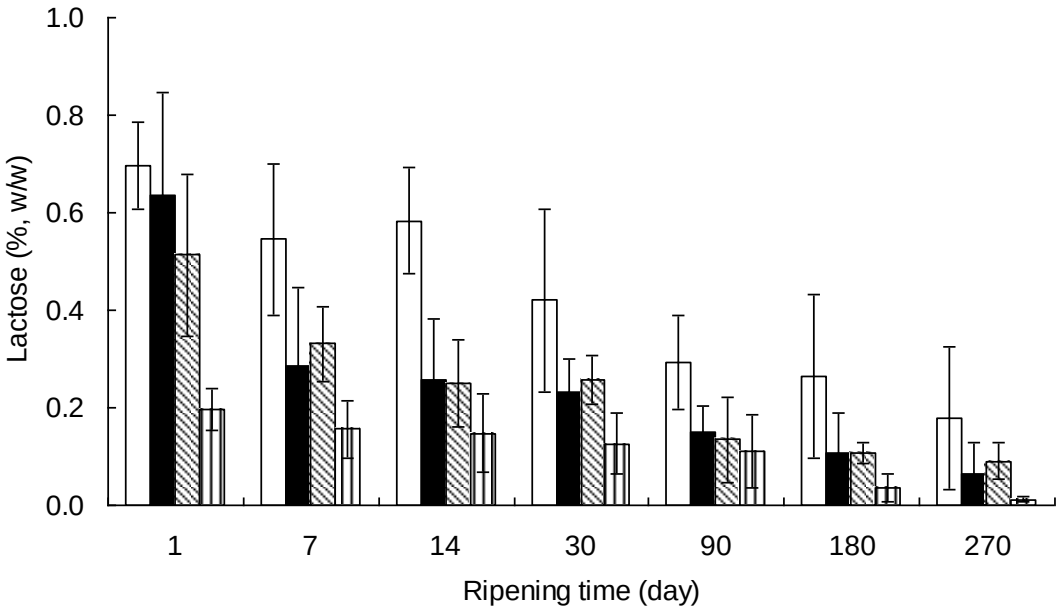

Figure 2.5. Lactose content as a function of ripening time in Cheddar cheeses in which curds were washed during manufacture to give target levels of lactose plus lactic acid in cheese moisture (LLAMc) of 5.3 (control, non-washed, □), 4.5 (■), 4.3 (▧) and 3.9 (▥), % (w/w). Values represent the means of three replicate trials; error bars show standard deviations of the mean.

2.3.3.3. Total lactate

The overall mean levels of total lactate increased significantly with ripening time, decreased with extent of curd washing, and were not significantly affected by the interaction between treatment and ripening time (Figure 2.6, Table 2.2). The increase in lactate content with ripening time is consistent with the decrease in lactose levels, which is metabolised to lactic acid during ripening by starter and NSLAB (Turner & Thomas, 1980). The population of the latter increased from ~ 10^2 to ~ 10^6-10^7 cfu g^{-1} of cheese over the 270 day ripening period, as discussed below. The increase in lactate along with the simultaneous decrease in residual lactose agrees with the trends reported in previous studies on Cheddar cheese in which S/M content is within recommended levels of 4 – 6 % (w/w) (Jordan & Cogan, 1993; Rynne, et al., 2007; Thomas & Pearce, 1981; Turner & Thomas, 1980).

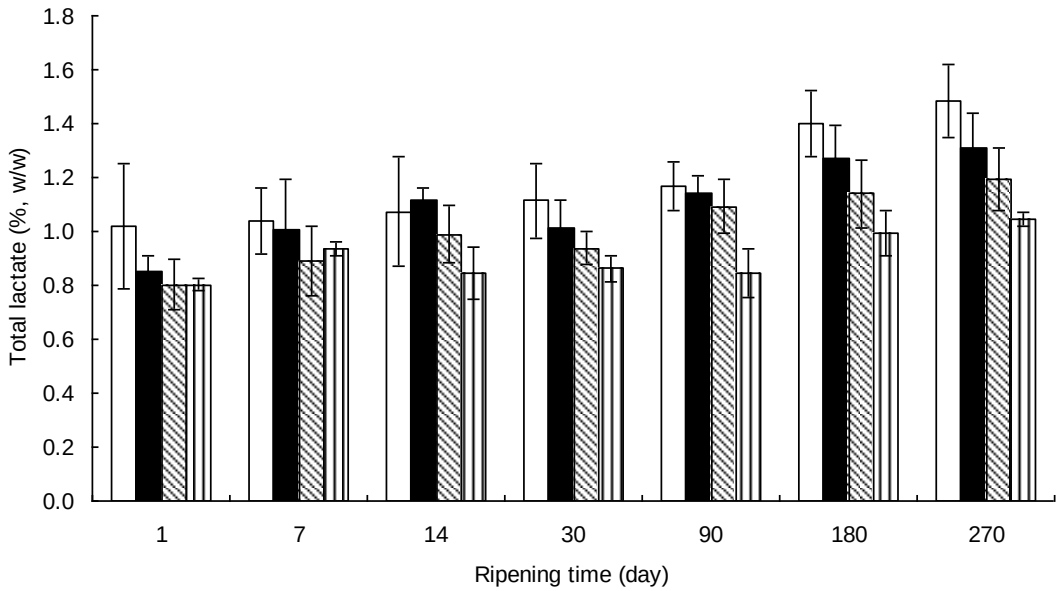

Figure 2.6. Total lactate content as a function of ripening time in Cheddar cheeses in which curds were washed during manufacture to give target levels of lactose plus lactic acid in cheese moisture (LLAMc) of 5.3 (control, non-washed, □), 4.5 (■), 4.3 (▧) and 3.9 (▥), % (w/w). Values represent the means of three replicate trials; error bars show standard deviations of the mean.

2.3.3.4. D (-) - and L (+)-lactate

The mean levels of D (-)- and L (+)-lactate over the 270 days ripening period were significantly affected by ripening time but not by the extent of curd washing. The mean concentration of L (+)-lactate decreased significantly during ripening up to 180-day, while that of D (-)-lactate increased to ~ 35 to 45 % of total lactate at times ≥ 180 days (Figure 2.7, Table 2.2). The progressive racemisation of L (+)-lactate to D (-)-lactate proceeded most rapidly between 30 to 180 days, and thereafter more slowly. In agreement with previous findings for reduced-fat (Rynne, et al., 2007) and full-fat (Jordan & Cogan, 1993; Turner & Thomas, 1980) Cheddar cheeses, the increase in D(-) lactate coincided with an increase in NSLAB to counts of ~ ≥ 10^5cfu g^{-1}. Factors that impede the growth of NSLAB in Cheddar cheese, such as reduction in pressing

temperature, rapidity of cooling post pressing, and higher S/M level, prolong the time at which equilibrium between L(+)- and D (-)- lactate concentrations is attained (Thomas & Pearce, 1981; Turner & Thomas, 1980).

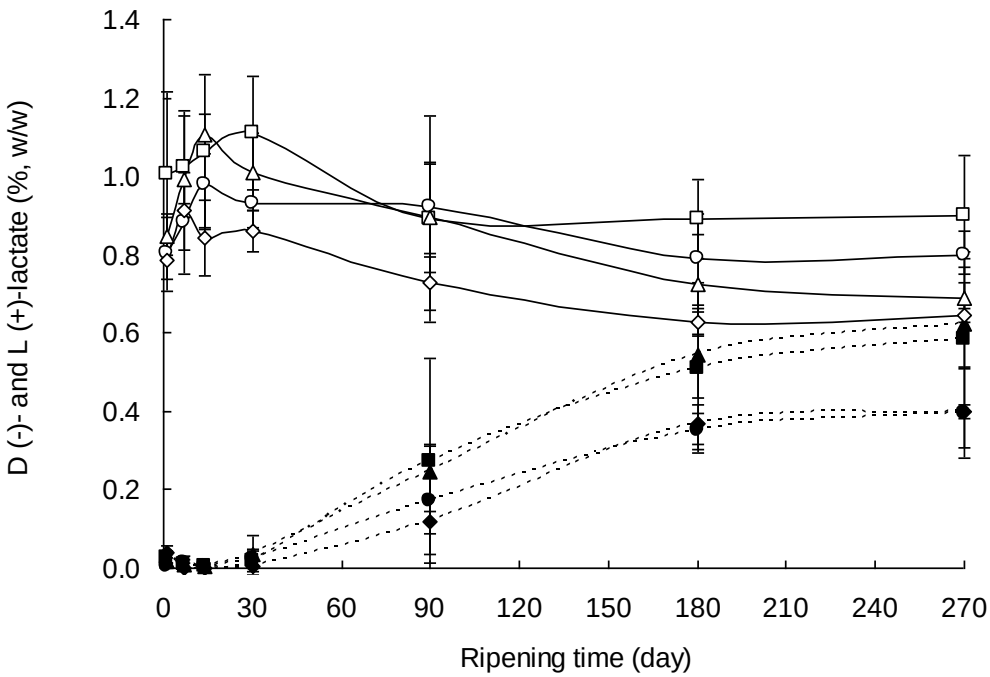

Figure 2.7. D(-)-lactate (broken lines) and L(+)-lactate (solid line) content as a function of ripening time in Cheddar cheeses in which curds were washed during manufacture to give target lactose plus lactic acid in cheese moisture (LLAMc) of: 5.3 (■, □), 4.5 (▲, △), 4.3 (●, ○) and 3.9 (◆, ◇) % (w/w). Values represent the means of three replicate trials; error bars show standard deviations of the mean.

2.3.4. pH Changes during ripening

Cheese pH was significantly affected by curd washing, ripening time, and their interaction (Figure 2.8, Table 2.2). The mean pH over the 270 days ripening period increased significantly on curd washing, with the pH of the 3.9 LLAMc cheese being ~ 0.4 units higher than that of the 5.3 LLAMc (control) cheese at 270 days. Linear regression of the data from the three replicate trials at individual ripening times indicated no relationship between pH and level of curd washing at times ≤ 14 days, but a positive correlation at times ≥ 30 days that became increasingly more significant

with ripening time, i.e., at 180 days, r = 0.87 (df, 10) while at 270 days, r = 0.9 (df, 10). The higher pH in washed curd cheeses may be attributed to the significantly lower levels of total lactate at ripening times ≥ 90 days (Figure 2.6). The lower lactate at these times would be expected to reduce the ratio of lactate to buffering capacity at times ≥ 90 days (Lucey & Fox, 1993; Salaün, Mietton, & Gaucheron, 2005; Upreti & Metzger, 2007). Regression analysis indicated significant relationships between pH and total lactate at all ripening times. The present results concur with those from previous studies, which indicates an inverse relationship between pH of Cheddar cheese and levels of lactic acid (Guinee, et al., 2008; Huffman & Kristoffersen, 1984). Moreover, the inverse relationship between Cheddar cheese pH and total lactate content parallels inverse relationship between pH and cheese moisture, which is the solvent for lactose and lactate (Rynne, et al., 2007).

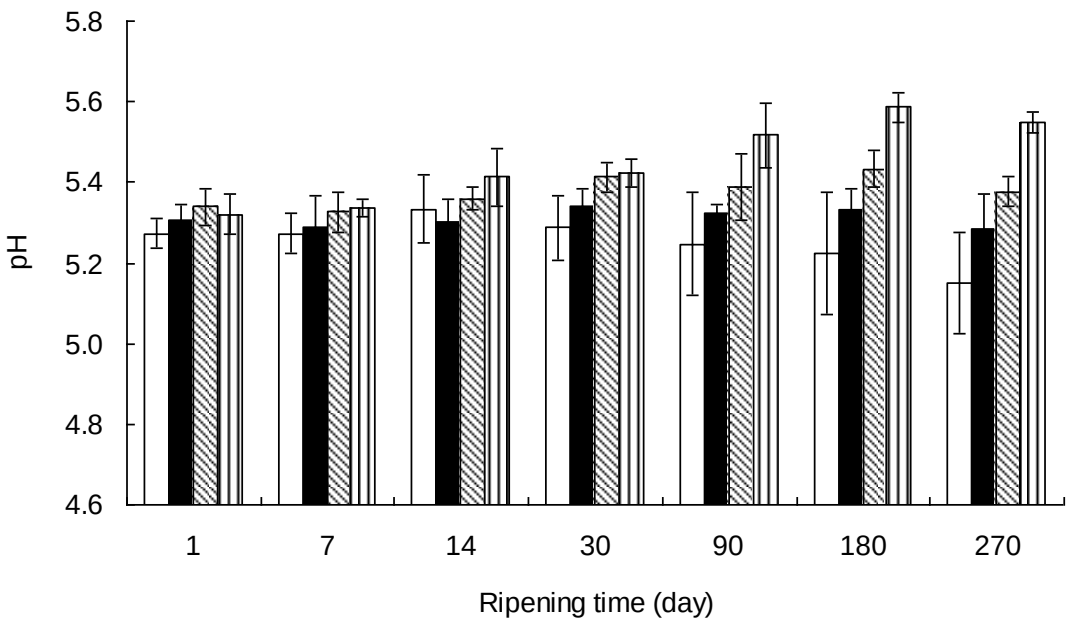

Figure 2.8. Cheese pH as a function of ripening time in Cheddar cheeses in which curds were washed during manufacture to give target levels of lactose plus lactic acid in cheese moisture (LLAMc) of 5.3 (control, non-washed, □), 4.5 (■), 4.3 (▨) and 3.9 (▥), % (w/w). Values represent the means of three replicate trials; error bars show standard deviations of the mean.

2.3.5. Microbial counts of starter and non-starter lactic acid bacteria (NSLAB) in cheese

2.3.5.1. Starter bacteria

In agreement with previous studies on Cheddar (Fenelon, O'Connor, & Guinee, 2000; Hannon, Wilkinson, Delahunty, Wallace, Morrissey, & Beresford, 2003; Shakeel-Ur-Rehman, et al., 2004), starter population decreased significantly with ripening time, but was not affected by curd washing (Table 2.3, Figure 2.9). The decrease in starter cell count may be attributed to permeablisation and autolysis (Sheehan, O'Cuinn, FitzGerald, & Wilkinson, 2009).

It is noteworthy that the starter counts in all cheeses at day 1 ($10^{7.5}$ to 10^{8} cfu g^{-1}) were ~ 1 log unit lower than those reported in previous studies of Cheddar in which starter counts at day one were ~ 10^{9} cfu g^{-1} cheese (Fenelon, O'Connor, & Guinee, 2000; Hannon, et al., 2003; Shakeel-Ur-Rehman, et al., 2004). The relatively low starter cell counts in the current study may be due to the relatively high salt sensitivity of starter culture strains (*Lactococcus lactis* subsp. *cremoris* strain 223 and 227) used. The lack of a significant relationship between starter cell count and level of curd washing is expected on the basis that all cheeses contained residual lactose, even after 270 days ripening (0.05 to 0.25 %, w/w) and that lactose as an energy source was not limiting. A similar trend was noted by Shakeel-Ur-Rehman, et al., (2004), who reported that variation in the content of residual lactose in Cheddar cheese (0.25 to 2.2 %, w/w, at 14 days) did not significantly influence the growth of starter bacteria or NSLAB.

2.3.5.2. Non-starter lactic acid bacteria (NSLAB)

The mean counts of NSLAB over the 270 days increased significantly with ripening time but were not affected by curd washing (Table 2.3, Figure 2.9). NSLAB populations at day one were similar in magnitude to those previously reported for Cheddar cheese (Jordan & Cogan, 1993; Turner & Thomas, 1980).

The lack of significant effect between LLAMc content or residual lactose level at day one and NSLAB counts concurs with results from previous studies (Shakeel-Ur-Rehman, et al., 2004; Turner & Thomas, 1980), which concluded that the rate of NSLAB growth in Cheddar cheese was independent of lactose content (Fox, McSweeney, & Lynch, 1998). NSLAB can also metabolise energy sources other than lactose in cheese depending on the different populations (e.g., sugars released on autolysis of starter cells, sugars derived from the casein glycomacropeptide and milk fat globule membrane, peptides, and amino acids) to a degree dependent on species and strain type (Asteri, Robertson, Kagkli, Andrewes, Nychas, Coolbear, Holland, Crow, & Tsakalidou, 2009; Diggin, Waldron, McGoldrick, Cogan, & Fox, 1999; Peterson & Marshall, 1990; Williams, Withers, & Banks, 2000), hence, NSLAB growth would not be expected to be totally impeded by reduced lactose levels.

Table 2.3. Degrees of freedom (df) and statistical significances (*P* - values) for changes in starter counts and non-starter lactic acid bacteria (NSLAB) counts in full fat Cheddar cheeses with different target LLAMc levels [a].

Factor	Starter counts		NSLAB counts	
	df	*P*	df	*P*
Main plot				
Curd washing	3	0.9229	3	0.9243
Sub-plot				
Time	4	<0.0001	5	<0.0001
Interaction (curd washing x time)	12	0.0865	15	0.0001

[a] LLAMc: lactose plus lactic acid in cheese moisture. Lactose content was adjusted by curd-washing at the cooking stage of 34°C; results are presented as the mean values of three replicate trials.

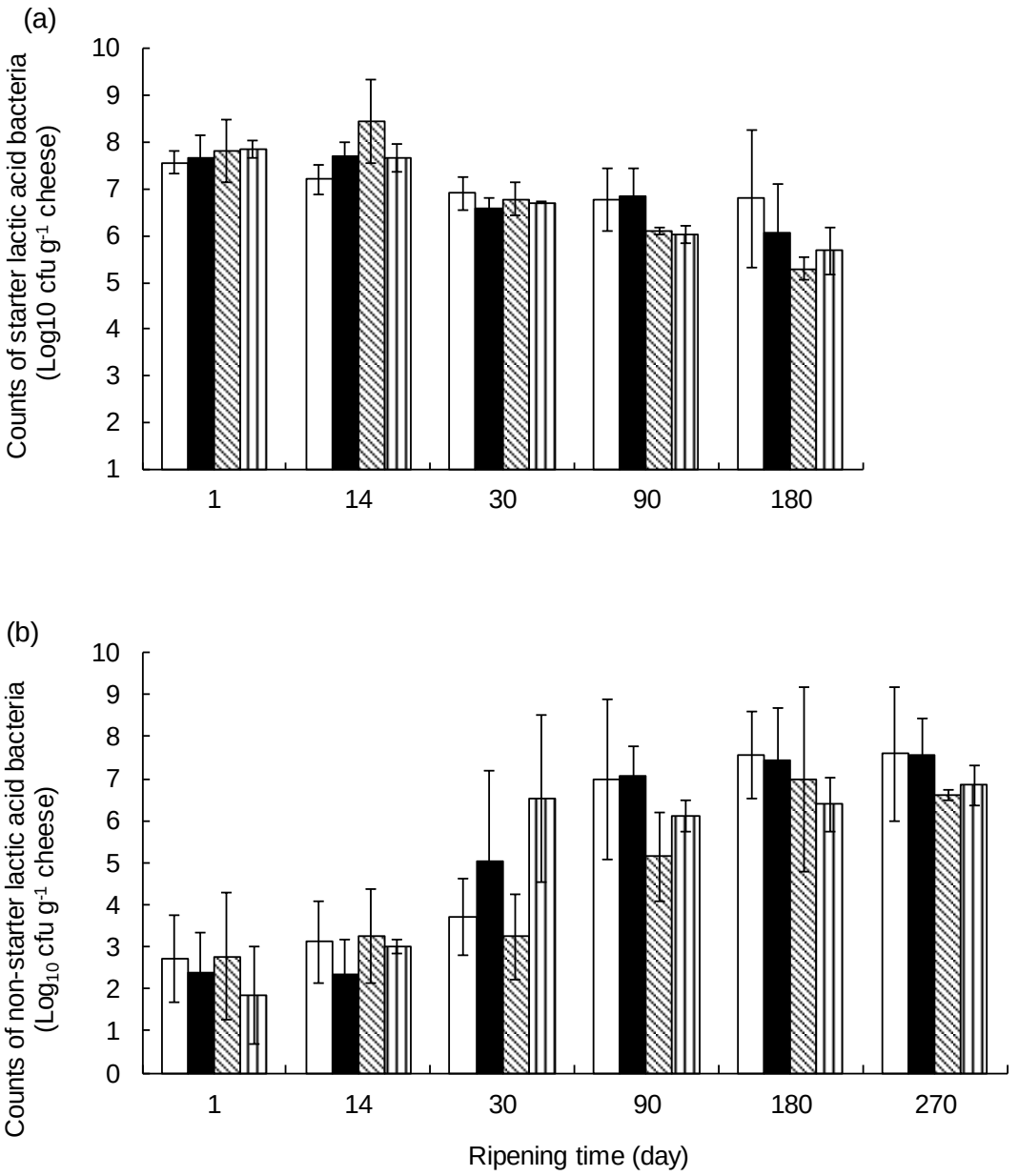

Figure 2.9. Counts of starter lactic acid bacteria (a) and of non-starter lactic acid bacteria (b) as a function of ripening time in Cheddar cheeses in which curds were washed during manufacture to give target levels of lactose plus lactic acid in cheese moisture (LLAMc) of 5.3 (control, non-washed, □), 4.5 (■), 4.3 (▨) and 3.9 (▥), % (w/w). Values represent the means of three replicate trials; error bars show standard deviations of the mean.

2.4. Conclusions

Curd was washed to varying degrees during the manufacture of Cheddar cheese, by replacing whey, at levels ranging from 0 to 33 % of milk weight, with equivalent weights of water in the cheese vat. Increasing the level of curd washing

significantly reduced levels of residual lactose and lactic acid and, hence, the concentration of LLAMc in the cheese, which decreased linearly at a rate of around 0.0179 % (w/w) per kg of wash water added per 100 kg milk. However, the LLAMc level in all cheeses was significantly lower (by 1 to 1.5 %, w/w) than that predicted on the basis of lactose content in the moisture phase of cheese milk and extent of wash water added. During maturation, L (+)-lactate was increasingly converted to D (-)-lactate, which accounted for 35-45 % of total lactate at 180-270 days. As expected, cheese pH was inversely related to concentration of total lactate and increased with degree of curd washing, the latter effect being significant at ripening times ≥ 90 days. Curd washing had no effect on the populations of starter bacteria and NSLAB. Consequently, it may be employed as a method of reducing both the levels of total lactate and unfermented lactose content in Cheddar cheese; reduction in residual lactose is important in minimising the risk of discomfort to consumers suffering from lactose intolerance as high level of residual lactose would present in cheese even at advanced ripening times if salt sensitive starter culture being used. The lower content of total lactate in washed curd Cheddar may also prove advantageous in reducing the incidence of calcium lactate crystals associated with the conversion of L(+)-lactate to D (-)-lactate (Kubantseva, Hartel, & Swearingen, 2004). Owing to its effects on pH, curd washing is also likely to influence casein hydration, proteolysis, texture and hence quality; these effects are currently being studied and will be reported later.

2.5. Acknowledgement

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Chapter 3: Effect of curd washing on cheese proteolysis, texture, volatile compounds, and sensory grading in full fat Cheddar cheese[1]

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Abstract

Curd was washed to varying degrees during Cheddar cheese manufacture, by partial replacement of whey with water at the early stages of cooking, to give target levels of lactose plus lactic acid in cheese moisture ($LLAM_C$) of 5.3 (control), 4.5, 4.3 and 3.9 % (w/w). The cheeses were matured at 8 °C for 270 days. While curd washing had little effect on composition or the mean levels of proteolysis (as measured by pH 4.6 soluble nitrogen and levels of free amino acids), it led to cheeses that were overall firmer and less brittle. Curd washing resulted in cheeses having lower levels of some volatile compounds, and being less acid, more buttery, sweeter, saltier and creamier than non-washed cheeses which had more 'sweaty', pungent and farmyard-like sensory notes. The results suggest that curd washing during Cheddar manufacture may be used as a means of creating variants with distinctive flavour profiles.

3.1. Introduction

The fermentation of residual lactose in cheese to lactic acid has a significant effect on cheese pH. Curd washing is used to control the ratio of lactose to lactic acid in the resultant cheese and hence cheese pH. This is a common practise in the manufacture of Dutch style cheeses such as Gouda and Maasdammer types (van den Berg, Meijer, Düsterhöft, & Smit, 2004) and involves the dilution of cheese whey with water during the cooking/stirring stages of manufacture. In practice, some of the whey is removed from the vat shortly after cutting and replaced with water; the levels of whey removal, water addition, and water temperature vary according to cheese type (variant) and manufacturing technology. Diluting the whey with wash water to varying degrees reduces the levels of residual lactose and lactic acid in Cheddar cheese, and increases the pH (e.g., by up to 0.1- 0.3 units when wash water was added at a rate of 33 g/100 g cheese milk) after ripening times of ≥ 90 day (Huffman & Kristoffersen, 1984; Shakeel-Ur-Rehman, Waldron, & Fox, 2004). The drained curds of Cheddar (Colby and washed-curd Cheddar) and Monterey cheese are also washed by adding cold water, ~ ≤ 18 °C, to the drained curd, at a level of ~ 25% of the milk volume, when the whey has been drained to the top of the curd bed; the mixture of curd and wash water is agitated, but the contact time of the water with the curd before wash water drainage is relatively short, typically 5 -15 min. The primary function here is curd cooling and depression of moisture loss. Owing to the fact that the casein is quite dehydrated at this stage of cheese manufacture, little dilution of lactic acid or lactose, by diffusion from the moisture phase within the curd to the wash water surrounding the curd particles in the short contact time, is unlikely (Fox, McSweeney, Cogan, & Guinee, 2004). Nevertheless, the level of lactic acid in Colby cheese may be

influenced by the mode of curd washing (batch, continuous) and the degree of agitation during stirring (Lee, Johnson, Govindasamy-Lucey, Jaeggi, & Lucey, 2011).

In the manufacture of conventional Cheddar cheese, the curd is generally not washed. However, owing to seasonal variation in the content of lactose in milk, the absence of washing can lead to significant variations in the levels of lactic acid in Cheddar (Guinee, et al., 2008), which in turn is likely to affect overall cheese quality. Little information is available on the effect of curd washing on proteolysis, rheological properties and flavour development in Cheddar cheese. In a previous study, Hou, Hannon, McSweeney, Beresford, and Guinee, (2012) studied the effect of curd washing on the levels of sugars and changes in pH during ripening of Cheddar cheese. Washing involved removal of part of whey during cooking/stirring and its replacement with an equal weight of water. The degree of curd washing was varied to give target levels of lactose plus lactic acid in cheese moisture ($LLAM_C$) in the final cheese of 5.3 (control), 4.5, 4.3 and 3.9 % (w/w), respectively. Increasing the levels of curd washing significantly reduced mean levels of lactose (from 0.7 %, w/w, in the control non-washed cheese to 0.2 %, w/w, in the most highly washed cheeses), total lactate and $LLAM_C$ over the ripening period. Conversely, it increased the mean cheese pH, by ~ 0.3-0.4 at times ≥ 90 days. Otherwise, alteration of curd washing generally did not affect gross composition or microbiology (starter or non-starter lactic acid bacteria population) of the cheese.

The objective of the current study, which was a continuation of the previous study (Hou, et al., 2012), was to evaluate the impact of curd washing on proteolysis, texture, volatile compounds, and sensory characteristics of Cheddar cheese.

3.2. Materials and Methods

3.2.1. Cheese manufacture

Cheddar cheese were manufactured in triplicate as previously described by Hou et al., (2012). Essentially, milks were standardized to a protein to fat ratio of 0.95:1, pasteurized at 72 °C for 15 s, cooled to 31 °C, and pumped to 4 cheese vats (500 L). Cheesemilk was inoculated at a level of 1.5 % (w/w) with cultures (*Lactococcus lactis* subsp. *cremoris* strains 223 and 227; Chr. Hansen Ireland Ltd., Rohan Industrial Estate, Little Island, Co. Cork, Ireland) and left for 30 minutes prior to addition of rennet (Chymax Plus, Chr. Hansen Ireland Ltd) at a level of 0.18 mL kg^{-1}. The gel was cut at a firmness of 54 Pa and the curd particle/whey mixture was allowed to heal for 10 min and then stirred continuously while heating to 34 °C at a rate of 0.2 °C min^{-1}. Stirring was then stopped and varying quantities of whey (0, 16, 23 and 33 kg per 100 kg cheese milk) were removed and replaced by equivalent weights of water at the same temperature. The curd-whey mixture was again stirred and further cooked to 38.5 °C at a rate of 0.2 °C min^{-1} and the whey was drained when pH at 6.15. The curds were cheddared to pH 5.35, milled, salted at 2.7 % (w/w), mellowed for 20 min, placed in rectangular moulds (23 kg), pre-pressed at 0.13 kPa for 30 min, and pressed overnight at 2.5 kPa.

A total of three trials were undertaken, and in each trial 4 different treatment cheeses were manufactured in which the target concentration of lactose + lactic acid in the moisture phase of the cheese (LLAMc) was varied by washing the curd to different levels in the cheese vat (Hou, et al., 2012). The four treatment cheeses with LLAMc concentrations of 5.3, 4.5, 4.3 and 3.9 % were denoted 5.3 LLAMc, 4.5 LLAMc, 4.3 LLAMc and 3.9 LLAMc, respectively.

3.2.4. Proteolysis

Cheese samples were taken periodically (day 14, 30, 90, 180, 270) during ripening and grated; a portion (60 g) was immediately placed in a sterile stomacher bag (Grade Packaging Ltd, 8 Vulcan Court, Coalville, Leicestershire, England) and stored at -20 °C until analyzed.

3.2.4.1. Levels of pH 4.6-soluble N (pH 4.6-SN) and chymosin activity in cheese

The water-soluble extract (WSE) was prepared making a cheese slurry comprised of 60 g cheese and 120 mL distilled water (55 °C) using a stomacher (Stomacher, Lab-Blender 400, IUL, S.A., Barcelona, Spain) for 5 min, as described by Kuchroo & Fox, (1982). Following centrifugation of the homogenate, the filtrate was passed through glass wool to remove fat. A portion of the resultant WSE was adjusted to pH 4.6 using 2.7 N HCl and centrifuged at 3000 g for 20 min at 4 °C; the filtrate was passed through glass wool to yield the pH4.6-soluble extract (pH4.6-SE). The N contents of the pH4.6SE were determined in duplicate by the macro-Kjeldahl method (International Dairy Federation, 1993) to obtain the levels of pH4.6-soluble N. The chymosin activity in cheese was measured as described in previous studies (Hurley, O'Driscoll, Kelly, & McSweeney, 1999). Finely grated cheese samples (50 mg) were weighed into a 1.5 mL microcentrifuge tube to which 1 mL of 0.1 M trisodium citrate was added. The tubes were incubated in a water bath at 37 °C for 30 min and agitated for 15 s at 5 min intervals. The samples were then centrifuged at 1000 *g* in a Micro Centaur centrifuge (Sanyo, Gallenkamp, Leister, UK) for 1 min to separate the fat. A sample (70 μL) of the subnatant aqueous layer was used for analysis of chymosin activity. This involved addition of 1 mg mL^{-1} aqueous solution of a synthetic heptapeptide substrate (Pro-Thr-Glu-Phe-[NO_2-Phe]-Arg-Leu; Bachem

Feichemikalien AG, Switzerland) to the sample to form the substrate solution. A portion of the latter (30 μL) was then added to 200 μL of 100 mM sodium formate buffer, pH 3.2 (BDH, Poole, Dorset, UK). The mixture was incubated at 37 °C for 5 h and terminated by heating at 70 °C for 10 min. Samples were centrifuged at 16,000 g for 10 min and the supernatant was analysed by HPLC for the chymosin-induced hydrolysis product ([NO_2-Phe]-Arg-Leu).

3.2.4.3. Phosphotungstic acid (5 % (w/v)) -soluble cheese nitrogen (PTA-SN) and free amino acids

The degree of secondary proteolysis was quantified by measuring the levels of PTA-SN, which includes low molecular mass peptides (< 15 kDa) and free amino acids (Jarrett, Aston, & Dulley, 1982). PTA-SN was measured using the method of Rank, Grappin, & Olson, (1985), and expressed as percentages of total nitrogen (TN). A portion (30 mL) of the pH4.6-SN extract was mixed with 18 mL of 9.2 N H_2SO_4 , 9 mL of 33.3 % dodeca-tungstophosphoric acid (commonly referred to as phosphotungstic acid; BDH, VWR International Ltd., Poole, BH151TD, England), and 3 mL of distilled water, which gave a total volume of 5 % phosphotungstic acid. Then the mixture was allowed to stand at room temperature for 18 hours and filtered through Whatman No. 42 filter paper (Whatman International Ltd., Maidstone England). The filtrate was analysed for N using the macro-Kjeldahl.

The levels of individual free amino acids in the pH4.6-SN extract were determined by first deproteinizing the extract by blending with an equal volume of 24 % (wt/vol) trichloroacetic acid. The mixture was allowed to stand for 10 min and then centrifuged at 14,400 × g (Microcentaur, MSE UK Limited, London, UK) for 10 min. The supernatant was analysed using a Beckman 6300 High Performance

Analyzer (Beckman Instruments Ltd., High Wycombe, UK), as described by Fenelon, Guinee, Delahunty, Murray, & Crowe, (2000).

3.2.5. Rheology

Six 25 mm cube samples were cut from each cheese, wrapped in tin foil, and kept at 4 °C overnight. Each cheese cube was taken from the refrigerator and immediately compressed to 70 % of original height in a single bite on a TA-HDi Texture Profile analyzer (Stable Micro Systems, Vienna Court, Lammas Road, Godalming, Surrey, UK) at room temperature at a compression a rate of 60 mm min^{-1} using a 100-kg load cell. The firmness was defined as the maximum force value on the resultant force-displacement curve. Fracture stress was defined as the force per unit area required to induce fracture of the cheese cubes, and fracture strain was defined as the fractional displacement at fracture.

3.2.6. Volatile analysis

The cheeses were analysed for volatile compounds and descriptive sensory analysis at 270 d, which based on previous studies was considered the most opportune time to reflect mature Cheddar cheese (Hannon, Kilcawley, Wilkinson, Delahunty, & Beresford, 2007; Kilcawley, Nongonierma, Hannon, Doolan, & Wilkinson, 2012).

The volatile compounds contained in each cheese were analysed by solid phase microextraction coupled to gas chromatography-mass spectrometry (SPME GC-MS). For volatile analysis, a 5 g grated sample of each cheese was added to a 20 mL SPME vial (Apex Scientific Ltd., Maynooth, Co. Kildare, Ireland) and equilibrated to 40 °C for 5 min with pulsed agitation for 4 s at 250 rpm. Sample introduction was accomplished using a CTC Analytics CombiPal Autosampler (CTC Analytics AG,

Riedstrasse 4222 Zwingen Switzerland). A single 1 cm x 50/30 µm StableFlex divinylbenzene/Carboxen/polydimethylsiloxane (DVD/Carboxen/PDMS) fiber was used for all analysis (Supelco, Bellefonte, PA, USA). The SPME fiber was exposed to the headspace above the samples for 20 min. The fiber was retracted and injected into the GC inlet at 250 °C and desorbed for 2 min. Splitless injections were made on a Varian 450 GC (Varian Analytical Instruments, Harbour City, CA, USA) with a Zebron ZB-5msi (60 m x 0.25 mm ID x 0.25 µm) column (Phenomenex, Macclesfield, Cheshire, UK). Volatile compounds were separated using gas liquid chromatography, using conditions defined by Hannon, et al. (2007). The detector used was a Varian 320 triple quad mass spectrometer (Varian Analytical Instruments) operating in the scan mode within a mass range of m/z 30-350 amu at 2.5 scans s^{-1}. Ionisation was performed by electron impact at 70 eV; calibration was performed by auto-tuning. Individual compounds were identified using mass spectral comparisons to the NIST 2005 mass spectral library. Individual compounds were assigned quantification and qualifier ions to ensure that only the individual compounds were identified and quantified. Quantification was performed by integrating the peak areas of the extracted ions using the Varian Star MS workstation, version 6.9.2 (Varian Analytical Instruments). The results presented are the averages of duplicate analysis.

3.2.7. Descriptive sensory analysis

Descriptive sensory analysis was carried out at University College Cork, as described by Hannon, Wilkinson, Delahunty, Wallace, Morrissey, & Beresford, (2003) in cheese matured for 270 days, the typical age when Irish Cheddar is ready for consumption. Duplicate cheese samples (~300 g portion) were taken for sensory analysis and were frozen at − 20 °C until the day of analysis. All cheese samples

(including duplicates) were then analysed for sensory characteristics using a one balanced design experiment. Thirteen trained assessors participated in a series of group discussions during which the panel evaluated the odour, taste and flavour of each of the cheeses and added new descriptors to the previously existing vocabulary where necessary. Descriptive sensory analysis was carried out using a final vocabulary of 15 odour, 5 taste and 19 flavour terms. Cheeses were coded with randomly selected 4-digit numbers and the order of tasting between and within days was balanced. Samples (stored at –20 °C), required for analysis on the following day, were thawed overnight at 4 °C. On the day of assessment, the outer layer (5 mm) of each cheese was discarded and each cheese was cut into 5 g cubes and allowed to warm to room temperature and presented in a glass tumbler covered with a clock glass. Sensory attributes were scored for odour, then flavour and taste on unstructured 100 mm line scales labelled at both ends (at 5 % and 95 %) with extremes of each descriptive term.

The sensory scores received from all of the individual sensory assessors for the different odour, taste and flavour attributes of the treatment cheeses were averaged; the means for the triplicate trials were obtained from these averages. The mean scores for the different flavour and aroma attributes were standardised (1/Standard Deviation of the mean score for each attribute) and analysed using principal component analysis (PCA) by Unscrambler V 6.1 (CAMO AS,N-7041 Trondheim, Norway). The results are presented as a principal component (PC) plot.

3.2.8. Statistical analysis

Four treatment cheeses, varying in the level of lactose + lactic acid in cheese moisture, were made in each trial, and three replicate trials (blocks) were undertaken. Data for changes in individual response variables (e.g., pH4.6 soluble N) at different

times throughout ripening were analysed using a split-plot design to determine the separate effects of treatment and ripening time, and the effect of the interaction among treatments and ripening time. Analysis of variance for the split-plot design was carried out using SAS (SAS Institute, 2004), and the significance of the differences was determined by Fisher's least significant difference test, as described by Hou, et al., (2012). The data for volatile compounds, which were measured at 270 d only, were analysed using a randomised complete block design incorporating the four treatments and three blocks.

Analysis of variance (ANOVA), was carried out using a SAS (SAS® version 9.1.2) procedure (SAS Institute, 2004), to examine the data at specific ripening times (e.g., PTA-SN) for the effect of treatment. Tukey's multiple-comparison test was used as a guide for pair comparisons of the treatment means; the level of significance in all the treatments was determined at $P < 0.05$.

3.3. Results and discussion

3.3.1. Cheese composition

The gross compositions of the cheeses, previously described by Hou, et al., (2012), were not significantly influenced by curd washing; the mean levels of moisture, fat, protein, salt and ash were 37.89 (±0.1), 30.88 (±0.1), 25.74 (±0.16), 1.89 (±0.09), and 4.07 (±0.1) % (w/w), respectively.

3.3.2. Proteolysis

3.3.2.1. Changes in pH4.6-soluble N (pH4.6-SN)

The mean levels of pH4.6-SN in the different cheeses increased significantly over the 270-day ripening period (Table 3.1, Figure 3.1), with the magnitude of the

increase being comparable to that reported previously (Guinee, Auty, & Fenelon, 2000; Lee, et al., 2011; Reville & Fox, 1978; Sheehan, Fenelon, Wilkinson, & McSweeney, 2007). This increase is attributed to the hydrolysis of intact casein by residual chymosin and, to a lesser extent the indigenous milk proteinase, plasmin and the proteolytic activity of the cheese starter culture (Sousa, Ard, & McSweeney, 2001). In agreement with Shakeel-Ur-Rehman, et al., (2004), curd washing did not significantly affect the level of pH4.6-soluble N. Analysis of the cheeses from one of the three replicate trials at different times over the 270 day ripening period, indicated that chymosin activities in all cheeses were similar (~14.4–15 CU kg^{-1} cheese) and were not influenced by curd washing or ripening time; the chymosin activities were comparable to those previously (~14–20 CU kg^{-1} cheese) reported by Hurley, et al., (1999) for Cheddar. This suggests that chymosin activity was the main agent controlling the development of pH4.6-SN in all of the cheeses despite the differences in pH (5.2-5.6) in the mature cheeses (Hou, et al., 2012). The proteolytic activity of chymosin in both dilute casein dispersions (Fox & Mulvihill, 1982; Møller, Rattray, Sørensen, & Ardö, 2012; Mulvihill & Fox, 1978, 1980) and in cheese (Mulvihill & Fox, 1980) increases with reduction in pH.

In contrast to the above, others (Upreti, Metzger, & Hayes, 2006; Upreti & Metzger, 2007) found that a lower pH in control cheeses compared to washed-curd cheeses (5.0-5.1 compared to 5.3–5.4) resulted in higher levels of pH4.6-SN; however, in these studies the former had significantly higher contents of moisture and moisture-non-fat-substances (MNFS) and lower levels of salt-in-moisture. Nevertheless, previous study by Lee, Johnson, Govindasamy-Lucey, Jaeggi, & Lucey, (2010) showed that Cobly cheese made with RO milk (higher lactose) had lower pH and

higher proteolysis. At 3 month during ripening, cheese pH was decreased to below 5 which is lower than current study even though the moisture content was lower.

Table 3.1. Degrees of freedom (df) and statistical significances (*P*-values) for changes in primary/secondary proteolysis and free amino acids in full-fat Cheddar cheeses with different target lactose in moisture levels[a].

Factor	pH4.6-SN[b]		PTA-SN[b]		FAA[b]	
	df	*P*	df	*P*	df	*P*
Main plot						
Curd washing	3	0.0907	3	0.7238	3	0.0771
Sub-plot						
Time	4	<0.0001	4	<0.0001	4	<0.0001
Interaction (Target lactose levels x time)	12	0.0122	12	0.0087	12	0.0421

[a]Lactose content was adjusted by curd-washing at cooking process to 34 °C; results are presented as the mean values of three replicate trials.
[b] Abbreviations: SN: soluble nitrogen; PTA: phosphotungstic acid; FAA: free amino acids.

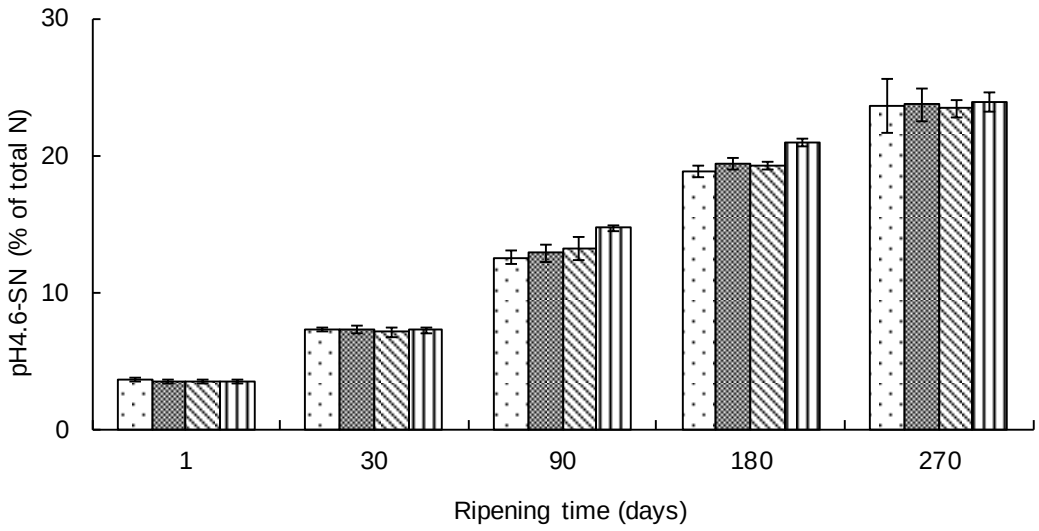


Figure 3.1. Age-related changes in levels of pH4.6-SN in full-fat Cheddar cheeses with different levels of lactose plus lactic acid in cheese moisture (LLAMc), as affected by different curd washing procedures used during manufacture. The LLAMc levels were: 5.3 (control, non-washed,), 4.5 ( ), 4.3 ( ) and 3.9 ( ), % (w/w). Presented values are the means of three replicate trials; error bars represent standard deviations of the mean.

3.3.4.2. Changes in 5 % (w/v) phosphotungstic acid-soluble nitrogen (PTA-SN) and free amino acids (FAA)

The mean levels of PTA-SN in the different cheeses were not significantly affected by curd washing treatment, but were significantly affected by the ripening time, and their interaction (Figure 3.2, Table 3.1). The mean levels of PTA-SN in all cheeses increased from ~0.4 % total N at day 1 to 3.3 % total N at day 270; these levels are similar to those reported previously (Hannon, et al., 2005). Similarly, the mean levels of FAA over the 270-day ripening period were not significantly affected by curd washing, but increased significantly with ripening time (Figure 3.3a, Table 3.1). ANOVA of data from the 270 day-old cheese indicated that the FAA levels in 3.9 LLAMc cheese were significantly lower than those in the other cheeses which had similar concentrations. In contrast, Shakeel-Ur-Rehman, et al., (2004) found that the levels of FAA in Cheddar cheese made from milk fortified with lactose and with a low

pH (~4.8-5.0) were significantly lower than those in the control cheese or in washed curd cheese (pH 5.3-5.4) at ripening times ≥ 120 days. This discrepancy may reflect differences in the peptidase system of the starter cultures used in both studies.

In agreement with previous studies (Guinee et al, 2008; Hannon et al, 2007; Swearingen, O'Sullivan, & Warthesen, 2001), the FAA present at highest levels in all cheeses were glutamic acid, leucine, phenylalanine, arginine and lysine (Figure 3.3b). At 270 days of ripening, the highly washed cheese (3.9LLAMc) had significantly lower levels of aspartic acid, leucine, phenylalanine, proline and lysine, and higher levels of cysteine and arginine compared to non-washed cheeses. We have no explanation for differences in FAA profile between the cheeses, but contributing factors could reflect differences in factors such as peptidase activities as affected by pH, NSLAB species (Gobbetti, et al., 1999a; Gobbettia, et al., 1999b), and degrees of autolysis and permeability of starter and non-starter bacteria (Doolan & Wilkinson, 2009).

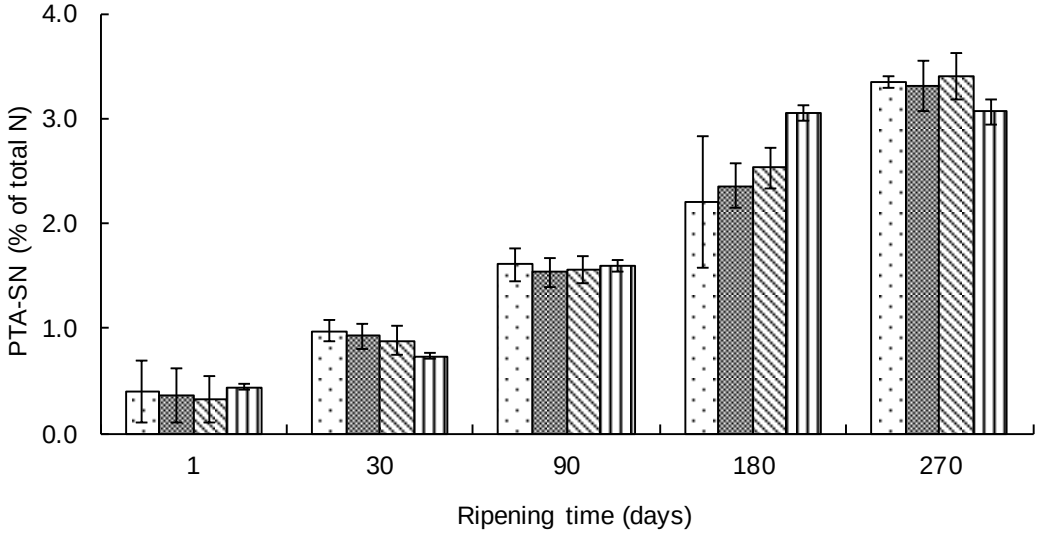
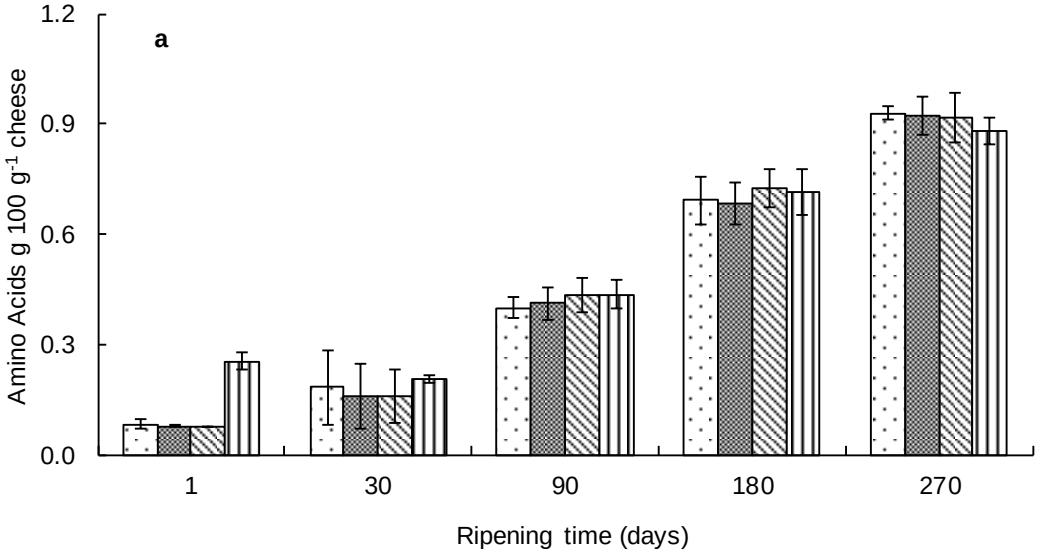


Figure 3.2. Age-related changes in levels of PTA-SN in Cheddar cheeses with different levels of lactose plus lactic acid in cheese moisture (LLAMc) as affected by different curd washing procedures using during manufacture. The LLAMc levels were: 5.3 (control, non-washed, (□), 4.5 (■), 4.3 (▧) and 3.9 (▥), % (w/w). Presented values are the means of three replicate trials; error bars represent standard deviations of the mean.



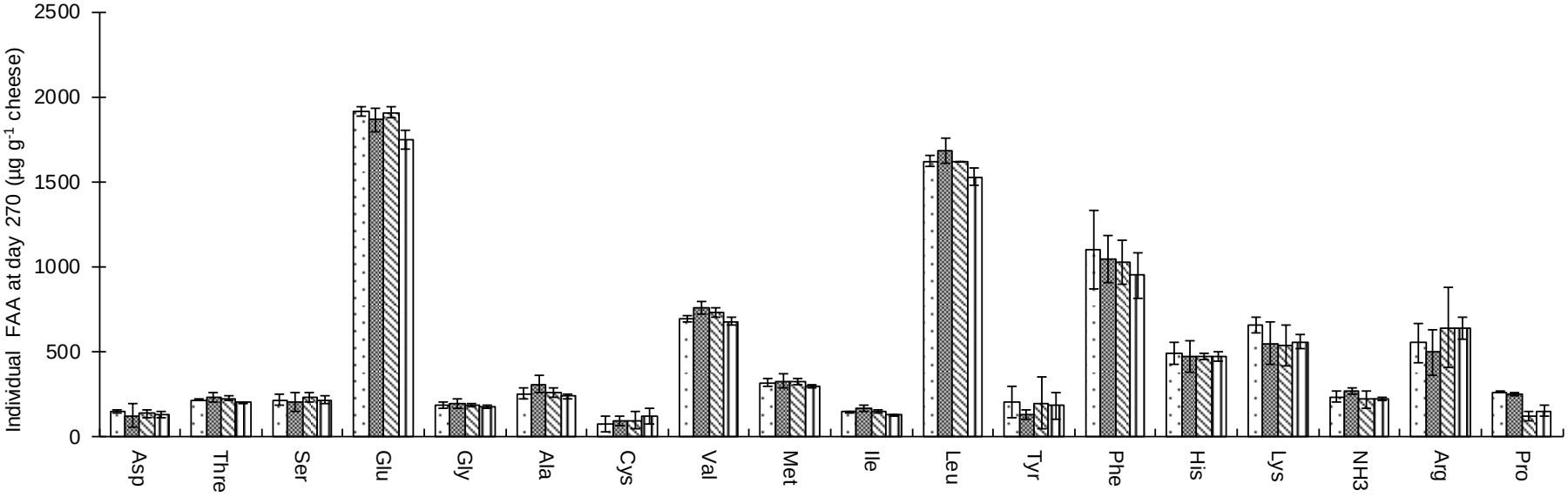


Figure. 3.3. Age-related changes in the concentrations of total free amino acids (FAA) (a) and individual FAA at day 270 (b) in Cheddar cheeses with different LLAMc: 5.3 (control, non-washed, □), 4.5 (■), 4.3 (▨) and 3.9 (▥), % (w/w). LLAMc refers to level of lactose plus lactic acid in cheese moisture, as affected by different curd washing procedures used during manufacture. Presented values are the means of three replicate trials; error bars represent standard deviations of the mean.

3.3.5. Rheology

The mean levels of firmness and fracture stress over the ripening period was affected by curd washing (Figure 3.4-3.6, Table 3.2); moreover, the mean firmness and fracture stress were significantly affected by the interaction between curd washing and ripening time. Similar to previous studies of both full fat and half fat Cheddar cheese (Guinee, et al., 2000), the mean values of firmness, fracture stress and fracture strain over the 270 day ripening period decreased significantly ($P < 0.001$) during ripening (Table 3.2). This effect is consistent with the reduction in the content of intact casein, as reflected by the age-related increase in pH4.6-SN (Costa, et al., 2010; Guinee, et al., 2000). The mean firmness of the 3.9 LLAMc was significantly higher than that of the other cheeses; ANOVA indicated that the differences among the cheeses were most pronounced at times ≤90 days and diminished with time. The differences in firmness cannot be attributed to gross composition, which was the same for all cheeses (Hou, et al., 2012). A tentative explanation may be an increased degree of calcium binding by the *para*-casein with increasing pH (Guinee, et al., 2000) in the 3.9 LLAMc cheese, which would be expected to increase the degree of *para*-casein aggregation and the force required to deform the cheese matrix. Calcium could be bound either as Ca ions electrostatically linked to glutamic and aspartic residues on the casein, and/or as insoluble calcium phosphate bound to serine phosphate groups on the casein. Cortez, Furtado, Gigante, & Kindstedt, (2008) reported that an increase in the pH of Mozzarella cheese, following exposure to ammonia vapour, reduced the level of serum-soluble calcium and significantly increased the firmness. Similarly, Visser, (1991) reported that the firmness of experimental Gouda cheese, where pH was varied and composition was otherwise fixed, increased markedly as the pH was increased from ~5.2 to 5.43-5.58. Moynihan, Govindasamy-Lucey, Molitor, Jaeggi,

Johnson, McSweeney and Lucey, (2016) also adjusted residual lactose to 3 different levels in low-moisture, part-skim Mozzarella cheesemilk by mixing milk with different portions of retentate/permeate. The three levels used were at a high level, 1.8 (HLC, the normal level in milk), medium level, 1.3 (MLC); and level, 1.0 (LLC) of lactose-to-casein ratios. LLC and MLC cheeses had lower levels of lactose, galactose, lactic acid, and insoluble calcium compared with HLC cheese. Cheese pH was higher with LLC milk compared to other cheeses throughout ripening which led to a firmer, chewier texture and lower meltability compared to other cheese during ripening.

The higher fracture stress in the 3.9LLAMc cheese may also be attributed to its high pH (Hou, et al., 2012); hence, a plot of fracture stress as a function of cheese pH at 180 and 270 days (when the pH difference between the cheeses was most pronounced) indicated that fracture stress decreased as the pH was increased from 5.15 to 5.35, and thereafter increased significantly as the pH increased further to 5.45-5.5 (Figure. 3.7). This trend was consistent with that reported by Watkinson, et al., (2001) who reported that the fracture stress of model cheeses (41 to 43%, w/w, moisture) made using chemical acidification (using glucono-delta-lactone) increased linearly in the pH 5.4 to 5.8, despite the higher moisture content of the higher pH cheeses. Similarly, they concur with the results for Gouda cheese, showing that fracture stress increased as cheese pH was increased from 5.2 to 5.6, and reduced from 5.2 to 5.0 (Visser, 1991). The fracture results in the current study suggest that more highly washed cheeses were less brittle and require a higher force to fracture (Zoon, 1991).

Table 3.2. Degrees of freedom (df) and statistical significances (*P*-values) for changes in rheology properties in full fat Cheddar cheeses with different target lactose in moisture levels[a].

Factor	Firmness (N)		Fracture stress (N cm^{-2})		Fracture strain (-)[b]	
	df	*P*	df	*P*	df	*P*
Main plot						
Curd washing	3	0.0092	3	0.0001	3	0.1814
Sub-plot						
Time	4	<0.0001	4	<0.0001	4	<0.0001
Interaction (Target lactose levels x time)	12	0.0566	12	0.0416	12	0.4330

[a]Lactose content was adjusted by curd-washing at cooking process to 34°C; results are presented as the mean values of three replicate trials.
[b]Fracture strain is a dimensionless quantity.

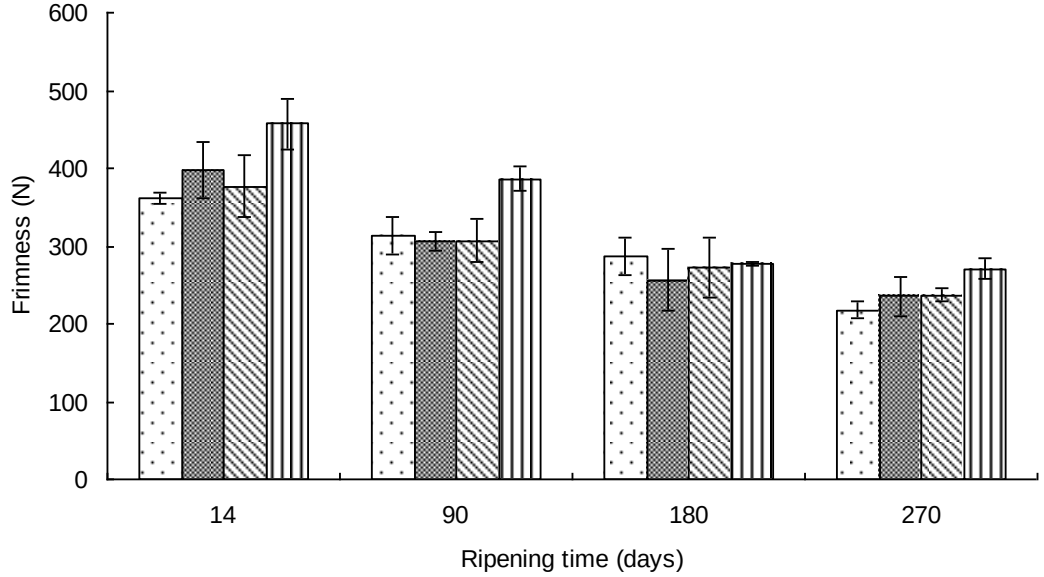


Figure 3.4. Age-related changes in firmness in Cheddar cheeses with different levels of lactose plus lactic acid in cheese moisture (LLAMc), as affected by different curd washing procedures during manufacture: 5.3 (control, non-washed,), 4.5 ( ), 4.3 ( ) and 3.9 ( ), % (w/w). Presented values are the means of three replicate trials; error bars represent standard deviations of the mean.

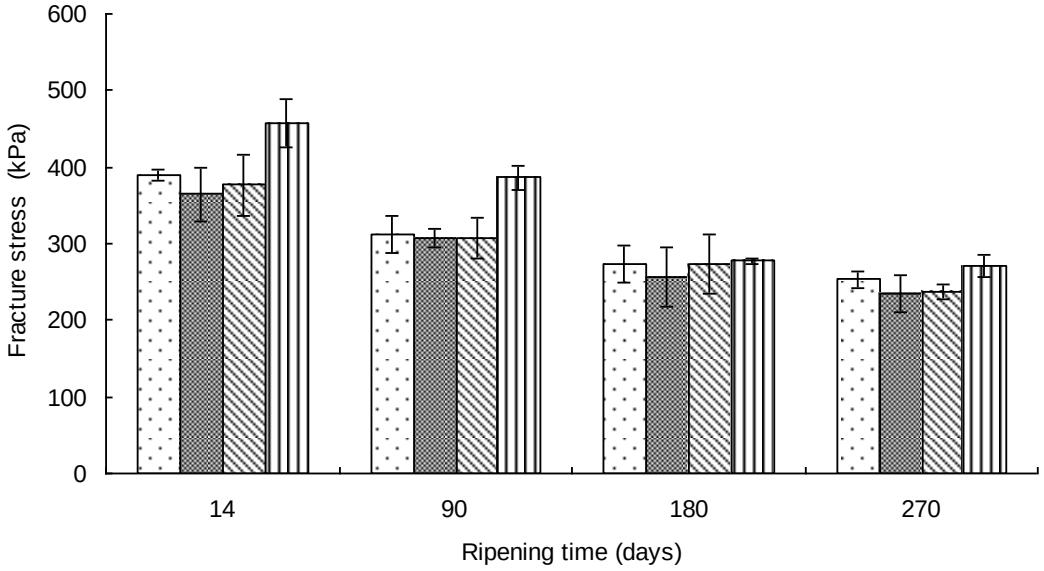


Figure 3.5. Age-related changes in fracture stress in Cheddar cheeses with different levels of lactose plus lactic acid in cheese moisture (LLAMc), as affected by different curd washing procedures during manufacture: 5.3 (control, non-washed,), 4.5 ( ), 4.3 ( ) and 3.9 ( ), % (w/w). Presented values are the means of three replicate trials; error bars represent standard deviations of the mean.

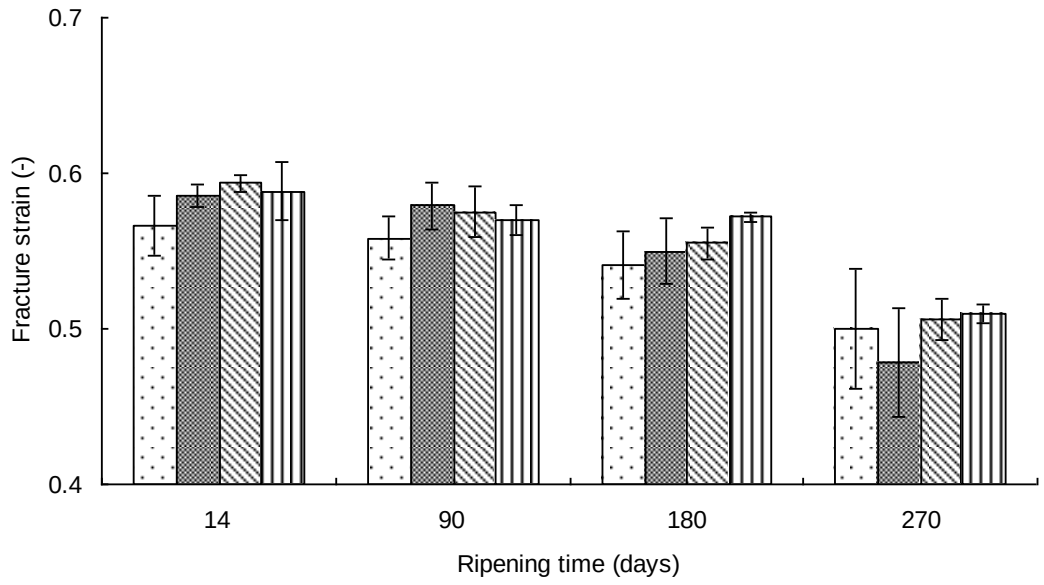


Figure 3.6. Age-related changes in fracture strain in Cheddar cheeses with lactose plus lactic acid in cheese moisture (LLAMc), as affected by different curd washing procedures during manufacture: 5.3 (control, non-washed,), 4.5 ( ), 4.3 ( ) and 3( ), % (w/). Presented values are the means of three replicate trials; error bars represent standard deviations of the mean.

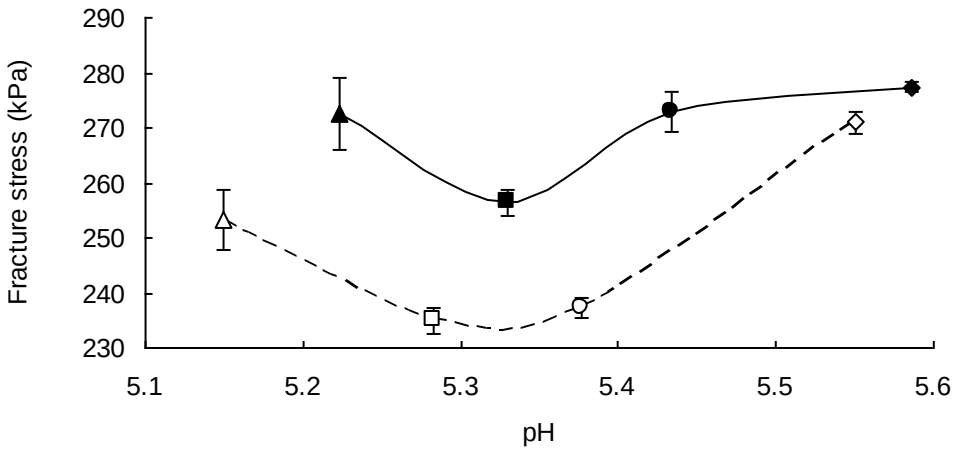


Figure 3.7. Fracture stress as a function of pH for 180 (solid line, full symbols) and 270 (broken line, open symbols) day-old Cheddar cheeses with different levels (%, w/w) of lactose plus lactic acid in cheese moisture (LLAMc): 5.3 (▲,△), 4.5(■,□), 4.3 (●,○) and 3.9 (◆,◇).

3.3.6. Volatile analysis

Over 40 compounds were identified by solid phase micro-extraction of the headspace followed by gas chromatography in tandem with mass spectroscopy of the 270-day old cheese. Twenty one major compounds, consisting of 5 alcohols, 6 ketones, 3 esters, 4 aldehydes, 4 acids and 1 sulphur compound were identified in all of the cheeses (Table 3.3). All of these have been previously identified in Cheddar cheese (Hannon, et al., 2007). Although all the compounds were identified in all the cheeses, the PCA of the volatile compounds was performed to assess how the individual compounds contributed to the profile of each cheese and how they discriminated between each of the cheeses (Figure 3.8). Principal components, PC1 and PC2, which accounted for 57 % of and 23 % of explained variance respectively, separated the cheeses on the basis of the levels of wash water used. The more highly washed cheeses, i.e., 3.9 LLAMc and 4.3 LLAMc, scored negatively on PC1 while 4.5 LLAMc and 5.3 LLAMc scored positively; however, in contrast to the other cheeses, the 3.9 LLAMc cheese scored negatively on PC2. These results indicate that the highly washed cheese (3.9 LLAMc) had the lowest concentrations of all volatile aroma compounds.

The PCA biplot shows that the control (non-washed) 5.3 LLAMc cheese had higher levels of ketones (acetone, 2-butanone and 2- nanonone), ester compounds (ethyl butanoate and ethyl hexanoate), aldehydes (3-methyl-butanal and benzeneacetaldehyde) and acids (butanoic acid and octanoic acid). The highly washed 3.9LLAMc cheese had lower levels of all these compounds but had higher levels of 2 butanol and 2, 3-butanediol. Each compound is associated with different odours, depending on the concentration and it is the balance of each of the compounds present in the cheese at a point in time that gives the overall perception of odour of each of the individual cheeses. For example in the 3.9 LLAMc cheese, the acetone and 2,3-

butanediol are associated with a sour milk, fruity, creamy and buttery odour, while the aldehydes, 2- and 3-methyl butanal, are associated with a fruity and malty odour and are formed by the degradation of leucine and isoleucine. In contrast, in the 5.3 LLAMc cheese, 2-butanone contributes an etheric aroma while 2-nanonone imparts a green, earthy aroma (Singh, Drake, & Cadwallader, 2003); the esters contribute to 'sweet' and 'fruity' odours while carbon disulphide contributes a sulphur, boiled cabbage or garlic odour. The mean levels of some compounds (acetone, ethyl butanoate, ethyl hexanoate, butanoic acid, octanoic acid and carbon disulphide) were significantly lower in the highly washed cheese (3.9 LLAMc) in comparison to the unwashed cheese (5.3 LLAMc). Interestingly, previous studies (Hickey, Kilcawley, Beresford, Sheehan, & Wilkinson, 2006; Hickey, Kilcawley, Beresford, & Wilkinson, 2006) have reported that starter cultures actively produce FFA in the cheese vat, and some of these FFA will be lost in the whey. Hence, it is likely that the concentration of volatile compounds derived from the shorter chain fatty acids (C4, C8) (Fox, et al., 2004; Singh, et al., 2003), which are more water soluble than the longer chain fatty acids (Webb & Johnson, 1972) would decrease as the level of wash water added to the cheese vat increases. This is in agreement with the data shown on the PCA plot where products of lipolysis (aldehydes and ketones) were higher in the unwashed cheese in comparison to the highly washed cheese.

Table 3.3. The effect of lactose content and curd-washing[c] on the concentrations of volatile compounds in full-fat Cheddar cheese[d]

Compounds	Target level of LLAMc[e] (%, w/w)[e]			
	3.9	4.3	4.5	5.3
Alcohols				
Ethanol	2.28E+07[a]	3.14E+07[a]	5.06E+07[a]	6.15E+07[a]
2-butanol	1.15E+07[a]	5.60E+06[a]	7.82E+06[a]	3.36E+06[a]
2-Me-1-Propanol	8.48E+05[a]	1.17E+06[a]	1.31E+06[a]	9.91E+06[a]
2,3-Butanediol	1.69E+07[a]	1.88E+07[a]	2.69E+06[a]	3.60E+06[a]
Ketones				
Acetone	2.02E+06[b]	6.70E+05[b]	2.47E+06[ab]	4.08E+06[a]
2-Butanone	1.11E+07[ab]	9.51E+06[b]	3.34E+07[a]	1.62E+07[ab]
2-Pentanone	4.49E+05[a]	1.33E+06[a]	1.32E+06[a]	2.15E+06[a]
2-Heptanone	7.56E+06[a]	1.15E+07[a]	6.88E+06[a]	8.34E+06[a]
2-Nonanone	1.56E+06[b]	2.89E+06[ab]	3.58E+06[a]	2.73E+06[ab]
Esters				
Ethyl Acetate	1.94E+06[a]	6.78E+06[a]	5.04E+06[a]	6.15E+06[a]
Ethyl Butanoate	1.60E+06[b]	2.62E+06[ab]	2.71E+06[a]	3.04E+06[a]
Ethyl Hexanoate	1.27E+06[b]	1.79E+06[ab]	2.15E+06[a]	2.11E+06[ab]
Aldehydes				
3-Methyl-Butanal	8.55E+05[b]	2.60E+06[a]	1.96E+06[ab]	2.23E+06[ab]
2-Methyl-Butanal	9.26E+04[a]	6.51E+05[a]	3.67E+05[a]	5.99E+05[a]
Octanal	1.76E+06[a]	1.14E+06[a]	1.38E+06[a]	2.18E+06[a]
Benzeneacetaldehyde	1.21E+06[b]	2.65E+06[a]	2.68E+06[a]	2.80E+06[a]
Acids				
Acetic acid	2.64E+07[a]	2.22E+07[a]	4.22E+07[a]	2.46E+07[a]
Butanoic acid	3.90E+06[b]	1.07E+07[ab]	1.97E+07[ab]	3.52E+07[a]
Hexanoic acid	6.26E+06[a]	6.30E+06[a]	1.02E+07[a]	9.72E+06[a]
Octanoic acid	3.77E+06[b]	8.45E+06[a]	1.01E+07[a]	9.81E+06[a]
Sulphur compounds				
Carbon Disulphide	2.20E+06[a]	2.18E+06[a]	1.88E+06[a]	2.60E+06[a]

[c]Lactose content was adjusted by curd-washing at cooking process to 34 °C; results are presented as the mean values of three replicate trials.
[d]Units for concentrations of volatile compounds: averaged peak areas in arbitrary units.
[e]Values within a row not sharing a common superscirpt differ significant, $P<0.05$.
[f]Abbreviations: LLAMc, lactose plus lactic acid in cheese moisture phase.

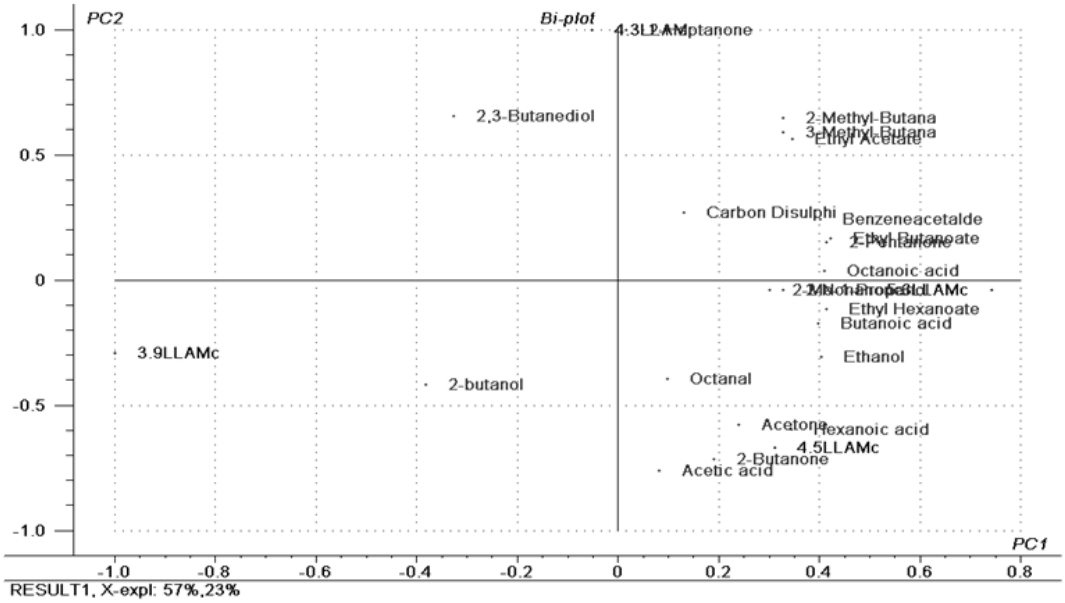


Figure 3.8. Principal Component Analysis (PCA) showing the first two principal components of volatile compounds in 270 day-old Cheddar cheeses made with different levels of lactose plus lactic acid in cheese moisture (LLAMc), as affected by different curd washing procedures during manufacture. Presented values are the means of the three replicate trials.

3.3.7. Descriptive sensory analysis

The PCA plot for the different odour and flavour attributes of the 270 day-old cheeses reveal a wide variety of attributes **(**Figure 3.9**)**. As for the volatile compounds, all of the odour and flavour attributes were detected in each of the cheeses. However, the washing treatments influenced the balance and proportions (intensities) of the different attributes, and thereby, the perception of flavour for the different cheeses. The first two PCs discriminated significantly between the cheeses and accounted for a cumulative explained variance of 80 % and significantly ($P < 0.05$) discriminated between cheeses. PC1 (47 % of explained variance) and PC2 (33 % of explained variance) separated the cheese on the basis of the levels of wash water, with the highly washed cheeses scoring positively and the less washed cheeses scoring negatively across PC1 and PC2. The 3.9 LLAMc and 4.3 LLAMc cheeses were described as having a very similar profile and were associated with a ‘fruity’, ‘buttery’ ‘sweet’,

‘nutty’, ‘chemical’, and ‘fermented’ flavour, a ‘buttery’, ‘savoury’, ‘fruity’, ‘chemical sulphur’ odour and a ‘sweet’, ‘salt’, ‘bitter’, ‘astringent’ taste, while the 4.5 LLAMc cheese had a more ‘nutty’ and ‘vinegar’ odour and the 5.3 LLAMc cheese was characterised as having a ‘rancid’, ‘farmyard’ flavour, a 'sweaty/cheesy' odour and an ‘acid taste'.

The results demonstrated that the highly washed cheeses tended to be creamier, have sweeter taste, were nuttier, more buttery, and were less rancid and pungent, and had less ‘farmyard’ aroma. This result concurs with that of Shakeel-Ur-Rehman, et al., (2004), who found that washed-curd cheeses had a significantly lower intensity of unclean, acidic tastes of the corresponding control and 'high-lactose' cheeses and suggested that this was due to the partial removal of the certain compounds by replacing the whey with water during the curd washing process. It is unclear why the sensory attributes of the current cheeses differed despite the absence of differences in primary proteolysis and total free amino acids between the cheeses. A tentative explanation for the sweeter flavour of the highly washed cheese may reside in its significantly lower concentrations of lactic acid (Hou, et al., 2012), glutamate, leucine, phenylalanine and lysine, which contribute to acid, bitter and umami flavours (Nishimura & Kato, 1988; Solms, 1969). Moreover, it is expected that the intensity of acid taste contributed by lactic and glutamic acids decreases at the higher pH (Neta, Johanningsmeier, Drake, & McFeeters, 2009), because of their higher degree of dissociation into the salt forms (lactate, glutamate). It is noteworthy that the reported dissociation constants of these acids are ~4.5 and 4.9, respectively and hence the acid/salt ratio are expected to decrease significantly as the pH increased from 5.2 (control cheese) to 5.6 in the most highly washed cheese. An additional factor contributing to the sweeter, more butter odour of the washed curd cheeses could be

due to a difference in the species/strains of NSLAB microflora, or a different microbial metabolism owing to the altered environment (e.g., pH, lactate) in the cheese. While, the mean counts of starter bacteria and NSLAB throughout the entire ripening period were not significantly affected by level of curd washing, the mean levels at 90 and 180 days were lower in the cheeses with the 2 highest levels of curd washing (Hou et al., 2012). In this context, a comparative study of the NSLAB microflora and their metabolism in control (unwashed) and washed curd cheeses of similar composition would be of interest.

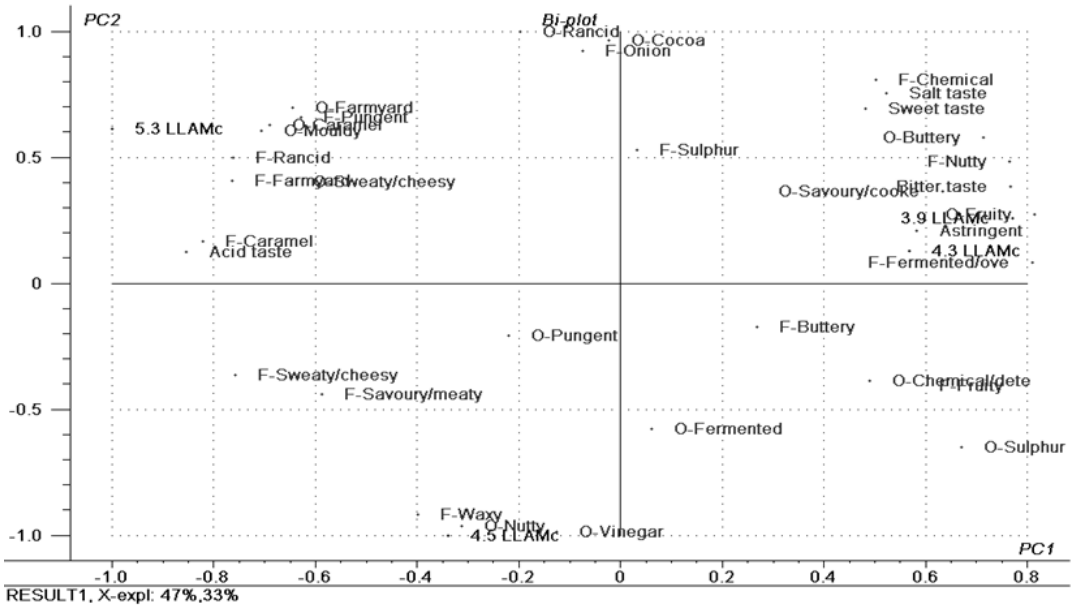


Figure 3.9. Principal Component Analysis (PCA) showing the first two principal components of descriptive sensory odour (O) and flavour (F) attribute scores in 270 day-old Cheddar cheeses made with different levels of lactose plus lactic acid in cheese moisture (LLAMc), as affected by different curd washing procedures during manufacture. Presented values are the means of the three replicate trials. O = odour; F = flavour.

3.4. Conclusion

Curd washing, involving partial removal of whey from the cheese vat and its replacement with water, was used to vary the levels of lactose plus lactic acid in Cheddar cheese. This study indicated that curd washing significantly influenced the texture, sensory and volatile profiles of the cheeses. While the mean levels of proteolysis during ripening, as measured by levels of pH4.6-SN, PTA-SN and total FAA, were not affected by curd washing, the levels of some free amino acids (glutamic acid, leucine, phenylalanine and proline) were lowest in the 3.9 LLAMc cheeses which received the highest level of curd washing. Curd washing also resulted in higher mean levels of firmness and fracture stress throughout ripening, indicating that it led to harder, less crumbly cheese.

High wash levels significantly reduced the concentration of some of the volatile compounds (e.g., acetone, ethyl butyrate, carbon disulphide) in the 270 day-old cheese. Descriptive sensory analysis showed that higher levels of curd washing coincided with a higher intensity of cheese flavours described as ‘fruity’, ‘buttery’ and ‘sweet’, while the absence of a curd washing step (control cheese) led to more ‘rancid’ and ‘farmyard’ aroma. The results suggest that curd washing may be applied during Cheddar cheese manufacture as a means of creating distinct cheese variants with subtle flavour differences suited to niche markets. Further studies on the effect of calcium phosphate levels on the Cheddar cheese quality is discussed in the next Chapter.

3.5. Acknowledgment

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Chapter 4: Effect of curd washing on the properties of reduced-calcium and standard-calcium Cheddar cheese[1]

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Abstract

Washed (W) and non-washed (NW) variants of standard (SCa)- and reduced (RCa)- calcium Cheddar cheeses were made in triplicate, ripened for a 270-day period, and analysed for composition and changes during maturation. Curd washing was applied to cheeses to give a target level of lactose plus lactic acid in cheese moisture (LLAMc) of 3.9 g/100 g in the washed cheese, compared to a value of 5.3 g/100 g LLAMc in the control non-washed cheeses. The four cheese types were denoted standard-calcium non-washed (SCaNW), standard-calcium washed (SCaW), reduced-calcium non-washed (RCaNW), and reduced-calcium washed (RCaW). The mean calcium level was 760 mg/100 g in the SCaNW and SCaW, and 660 mg/100 g in the RCaNW and RCaW cheeses. Otherwise, the gross composition of all cheeses was similar, each with protein, fat and moistures levels of ~ 26, 32 and 36 g/100 g, respectively. Curd washing significantly increased the losses of fat from milk to cheese for both the standard- and reduced-calcium cheeses, and reduced the losses of protein in the standard calcium cheese. Otherwise, the cheese yields (calculated by six different equations) showed either curd washing or reducing the calcium levels had any significant effect (data not shown). Curd washing significantly reduced the mean level of lactic acid in the SCaW cheese, and residual lactose in both SCaW and RCaW cheeses. The mean pH of the standard-calcium cheese over the 270-day ripening period was significantly higher with curd washing and ripening time, in contrast to the reduced-calcium cheese which was not affected by the latter parameters. Otherwise, curd washing had little effect on changes in populations of starter bacteria or non-starter lactic acid bacteria, proteolysis, rheology, or color of the cheese during ripening. Descriptive sensory analysis at 270 days indicated that the SCaW cheese had a nuttier, sweeter, less fruity and less rancid taste than the corresponding SCaNW cheese. In

contrast, curd washing was not as effective in discriminating between the RCaW and RCaNW cheeses. The RCaW cheese had a more buttery, caramel odour and flavour, and a more bitter, less sweet and nutty taste than the SCaW cheese, while the RCaNW had a more pungent and less-fruity flavour, a less fruity odour, a saltier, more-bitter and less-acid taste, and a more astringent mouthfeel than SCaNW. Washing of curd during manufacture provides a means of reducing the contents of lactic acid and residual lactose, increasing pH, and altering the sensory properties of Cheddar cheese, with the level of these effects being significantly less pronounced as the calcium content was reduced.

4.1. Introduction

Curd washing is practiced in the manufacture of some cheese varieties, for example in brine-salted, Dutch-style cheeses such as Edam, Gouda and Maasdammer, to control the level of lactose and lactic acid and properties of the final cheeses. In the manufacture of these varieties, salting is undertaken by immersion of the moulded cheese curd in brine, typically for 1 to 5 days depending on size. As salt diffusion into cheese is slow (Guinee & Sutherland, 2011), the growth of starter bacteria and the fermentation of residual lactose to lactic acid could potentially proceed uninhibited into the inner regions of the cheese before the salt has migrated inwards, and lead to an excessively low pH. This in turn is conducive to a higher degree of calcium solubilization, higher proteolysis by residual coagulant, and impaired texture, taste and quality (Lawrence, Creamer & Gilles, 1986). Such an occurrence is prevented by reducing the level of residual lactose in the cheese curd prior to salting, by diluting the whey during the cooking/stirring phase of manufacture by removal of whey and adding water, a process referred to as curd washing. The proportions of whey removed and water added are varied according to the lactose level in the milk, which varies with stage of lactation (O'Brien, Mehra, Connolly, & Harrington, 1999). Curd washing is also used in the manufacture of cheeses such as Colby and Monterey, whereby cold water is added to, and mixed with, the drained curd for a relatively short period (5-15 min) before being drained off (Lee, Johnson, Govindasamy-Lucey, Jaeggi, Lucey, 2011). The main purpose here is to reduce curd temperature, depress syneresis and modify texture (Fox & Guinee, 2013). The degree and mode (batch or continuous) of stirring of curd-whey mixture has been found to significantly influence lactic acid content, cheese pH and ratio to soluble calcium phosphate (Lee et al., 2011). Moynihan, Govindasamy-Lucey, Molitor, Jaeggi, Johnson, McSweeney and Lucey,

(2016) also found lower the levels of lactose in low-moisture, part-skim Mozzarella cheesemilk resulted cheeses had lower levels of lactose, galactose, lactic acid, and insoluble calcium compared with high lactose cheese.Curd washing is not conventionally applied in the manufacture of Cheddar cheeses, which is typically salted by adding dry crystalline salt to the milled curd at pH 5.2-5.4, prior to moulding and pressing; dry-salting is considered to arrest starter culture growth and greatly slow down the metabolism of residual lactose, and thereby prevent the pH from decreasing excessively. Nevertheless, the lactic acid content and pH of commercial Cheddar cheese vary significantly (Guinee, Kilcawley & Beresford, 2008), probably as a result of the interactive effects of a number of factors such as rate of acidification, pH at set (rennet addition) and whey drainage, pH at salting, level of lactose in the cheese milk, salt sensitivity of the starter culture, moisture content of the curd, and salt content (Czulak, Conochie, Sutherland & van Leeuwen, 1969; O'Brien et al., 1999). Hence, washing the curd during the manufacture of cheddar, by partial removal of whey during cooking and stirring and its replacement by water, has been investigated as a means of achieving more consistent levels of lactose and lactic acid, and flavour of the final cheese (Shakeel-Ur-Rehman, Waldron & Fox, 2004; Upreti & Metzger, 2006a; Hou, Hannon, McSweeney, Beresford & Guinee, 2014). In previous studies on the effect of curd washing on Cheddar cheese (Hou, Hannon, McSweeney, Beresford, & Guinee,.2012; 2014), the current authors found that increasing curd washing from 0 to 33 % of milk volume significantly reduced the mean levels of total lactate and increased the mean cheese pH by 0.3 to0.4 units at ripening times greater than 90 days. Overall, washed-curd cheeses were firmer and less brittle, had lower levels of some volatile compounds, and were less acid, more buttery, sweeter, saltier

and creamier than non-washed cheeses. The authors concluded that curd washing may provide a means of creating Cheddar cheese variants with distinctive properties.

The calcium content of commercial Cheddar cheese varies significantly, as indicated in serval surveys of different retail Cheddar brands: 673- 761 mg/100 g (Fenelon, Guinee, Delahunty, Murray & Crowe, 2000a; Guinee, Harrington, Corcoran, Mulholland & Mullins, 2000a), 638-778 mg/100 g (Guinee et al., 2008), and 631-871 mg/100g (McCarthy, Wilkinson, & Guinee, 2017); using the data from these three studies, involving 88 cheeses, the mean calcium level in commercial Cheddar was 758 ± 54 mg/100 g.

Calcium in cheese contributes to the crosslinking of the *para*-casein network, and thereby, to its integrity and rigidity (Lawrence, Heap & Gilles, 1984; Lucey & Fox, 1993). Hence, the level of total calcium, or more specifically the level of casein-bound calcium, as influenced by cheese pH, has a major impact on cheese texture and functionality (Lawrence et al., 1986; Guinee, Feeney, Auty & Fox, 2002). The calcium content of cheese is controlled principally by the pH at set, whey drainage, and in the case of dry-salted cheeses, the pH at salting (Lawrence et al., 1984; Lee, Johnson. Govindasamy-Lucey, Jaeggi, Lucey, 2011). Lower pH values at rennet addition lead to greater solubilization of colloidal calcium phosphate during the stirring and cooking of the curd particles in the whey, and its removal in the drain whey (Hooydonk, Hagedoorn & Boerrigter, 1986; Guinee et al., 2002; Upreti & Metzger, 2006a). Upreti and co-workers investigated the interactive effects of variations in levels of calcium phosphate (CaP), residual lactose plus lactic acid, and salt-in-moisture on the composition of Cheddar cheese and changes in proteolysis, levels of water-soluble organic acids, and pH during ripening (Upreti & Metzger, 2006a, 2007). The level of CaP in the cheese was varied by altering the pH of the milk at set (6.6, 6.2) and the

curd at whey drainage (6.4, 5.7), the level of lactose and lactic acid in the curd by the addition of lactose powder or by curd washing using a pH-adjusted solution, and the salt-in-moisture content by different salting rates. Curd washing significantly reduced the lactic acid level in the reduced CaP cheese, but not in the high CaP cheese over 50 weeks ripening; the pH of the washed-curd high- and low CaP cheeses was ~0.1 unit higher than the corresponding non-washed cheeses after 50 weeks of ripening. Nevertheless, interpretation of the results is somewhat confounded by compositional differences (moisture, fat, protein, phosphorous) between the washed and non-washed cheeses (Upreti & Metzger, 2006a, 2007).

Cheese yield and manufacturing efficiency affect the profitability of cheese manufacturing plants (Guinee, O'Kennedy & Kelly, 2006). Consequently, the factors affecting the recovery of milk fat and protein, and cheese yield have been extensively studied and reviewed (Lolkema, 1993; Lucey & Kelly, 1994; Fox, Guinee, Cogan & McSweeney, 2000). Some of the more important factors include the composition and quality of the raw milk, milk handling and storage practices, milk pre-treatments (e.g., standardization of protein-to-fat and protein contents, pasteurization temperature), firmness of the gel at cutting and the speed/duration of cutting programme, the rate of cooking of the curd-whey mixture and the temperature to which it is cooked, the duration of stirring and cooking before the physical separation of whey from the curd, design of cheese vats, and curd handling conditions following whey removal. However, the effect of curd washing on Cheddar cheese yield haven't been studied before.

The current study investigates the effect of curd washing on the composition, yield, rheology, volatile compounds and sensory properties of Cheddar cheeses with

different calcium levels, but otherwise similar gross composition; calcium levels (763 and 664 mg/100 g) were chosen to reflect those found in retail cheese.

4.2. Materials and methods

4.2.1. Cheese manufacture and treatments

Four different treatment cheeses were manufactured in each of three replicate trials undertaken over a three-week period from April 28 to May 12, 2011: standard-calcium non-washed (SCaNW), standard-calcium washed (SCaW), reduced- calcium non-washed (RCaNW), reduced-calcium washed (RCaW).

Raw milk samples were separated and standardized to a protein-to-fat ratio of 0.96, pasteurized at 72 °C for 15 s, cooled to 31 °C, and pumped to the cheese vats (500 L). The manufacture of the SCaNW cheeses involved inoculation of cheesemilk with a commercial DVS culture, R-704 (50 U), comprising a mixture of *Lactococcus lactis* subsp. *cremoris* and *lactis* strains (Chr. Hansen Ireland Ltd., Rohan Industrial Estate, Little Island, Co. Cork, Ireland). Thirty minutes later, rennet (Chymax Plus, Chr. Hansen Ireland Ltd., 200 IMCU/ml), diluted 1:10 in de-ionized water, was added at a level of 0.18 mL/kg based on a protein level of 3.3 g/100 g.

Otherwise, the cheese-making procedure described by Hou et al. (2012) was used for the manufacture of all cheeses, apart from differences in set pH, drainage pH and in extent of curd washing between the treatment cheeses. In the case of the washed SCaW cheese, agitation was stopped at 34 °C and a portion of whey (28.8 % of cheese milk) was withdrawn from the cheese vat and replaced by an equal weight of pasteurised de-ionised water at 35 °C. The level of washing (whey removal and replacement with water) was chosen to give a target level of lactose plus lactic acid in cheese moisture phase (LLAMc) of (3.9 g/100) g, compared to 5.3 g/100 g cfor the

non-washed cheese, respectively. Following water addition, the curd-whey mixture was further cooked to 38.5 °C. The whey (SCaNW curd), or diluted whey (SCaW curd), was drained from the curd mass when the curd pH reached 6.3. The manufacturing procedure of the RCaNW and RCaW cheeses were similar to that of the corresponding SCaNW and SCaW cheeses, apart from the following changes. The pasteurised milk was cooled to 20 °C to minimise risk of localised acid-induced coagulation on subsequent pre-acidification to pH 6.30 using 10% (wt/v) lactic acid solution; the pH-adjusted milk was then re-heated to 31 °C. The pH values of the milk at set and curd at whey drainage were 6.3 and 5.85, respectively.

4.2.2. Sampling of cheese

Treatment cheeses (20 kg blocks) were sampled at different times (1, 14, 30, 90, 180, 270 d) over the 270-day ripening period. On each sampling occasion, a vertical slice (0.5 cm thick) was removed from one of the vertical outside faces of the block and discarded, and a slab (~ 8 cm thick, ~ 2 kg), including the freshly-exposed surface, was taken for analysis of composition (day 14), texture, proteolysis, color, volatile compounds (day 270), and descriptive sensory analysis (day 270). Following sampling, cheeses were analysed within 48 h, except in the case of volatiles and descriptive sensory analysis where rectangular samples of ~ 100 g and 300 g, respectively, were individually sealed in foil, placed in plastic bags, vacuum wrapped (Model C10H, Webomatic®, Bochum, Germany), and stored at -20 °C for ~ 30-35 days prior to analysis due to planning with external sensory experts.

4.2.3. Composition analysis of milk, whey and cheese yields

The following samples (~ 200 g) were taken during cheese manufacture: (i) pasteurised cheeses milks (from the vats), (ii) pre-wash whey (PWW) withdrawn from the cheese vats at 34 °C prior to removal of whey and addition of water, in the case of washed-curd cheeses, (iii) bulk whey (BW) which corresponds to the whey (in the case of non-washed cheese) or a mixture of whey and wash water (for washed-curd cheese) expressed during whey drainage and cheddaring, and (iv) white whey (WW) expressed from the curd during milling, salting, pre-pressing and pressing. All wheys were filtered by passing through a 1 mm sieve to remove curd fines (which put back to cheese curd for further processing) and stored at 4 °C prior to analysis within 2 - 4 days after collection. Milks were analysed for total solids (International Dairy Federation (IDF), 1987), contents of lactose, protein and fat using a Milkoscan (Slangerupgade 69, DK-3400 Hillerød, Denmark), casein content (IDF, 1964), non-casein nitrogen levels (IDF, 1964) and non-protein nitrogen by Kjeldahl analysis of a filtrate obtained from a mixture of equal volumes of milk and 24 % trichloroacetic acid. Whey samples were analysed for total solids, fat by the Röse-Gottlieb (IDF, 1996) and protein by Kjeldahl (IDF, 1993). The percentage of fat lost in whey (% FLW) streams (e.g., in the BW streams) was calculated from the weight of fat in whey as a percentage of the fat in milk (Guinee et al., 2006). The % FLW during the manufacture of the non-washed (control) cheese was calculated from the combined losses of fat in the BW and WW, while that in the washed cheese was calculated from the total losses in the PWW, BW and WW. The yield of cheese was calculated as weight (kg) of pressed cheese per 100 kg of cheese milk (Guinee et al., 2006).

4.2.4. Composition analysis of cheese

Cheese and whey samples were analysed for moisture using standard IDF methods (Guinee, Auty, & Fenelon, 2000b) and lactose and lactate as described below. Grated cheese samples were analysed at 14 days for protein, fat, NaCl, moisture, ash, Ca and P using standard IDF methods (Guinee, et al., 2000b). The pH was measured after each sampling date on cheese slurry prepared from 20 g of grated cheese and 12 g distilled water (Guinee, et al., 2000b).

4.2.5. Microbial counts in cheese

Cheeses were analysed for counts of starter *lactococci* and non-starter lactic acid bacteria (NSLAB) on LM17 agar and LBS agar respectively, as described previously (Hou et al., 2012). Coliform were enumerated by pour-plating on Violet Red Bile Agar (VRBA) incubated at 30 °C for 24 h, and enterococci were plated on Kanamycin esculin azide agar at 27 °C for 24 h.

4.2.7. Sugar and lactate in cheese

Portions (5 g) of finely grated cheese samples were stored in a stomacher bag (Grade Packaging Ltd, 8 Vulcan Court, Coalville, Leicestershire, England) at -20 °C until analysed, thawed at 4 °C, and analysed for lactose, glucose and galactose. The sugars were extracted and measured using the HPLC method described by Zeppa, Conterno and Gerbi, (2001), apart from a modification in the equipment used and flow rate. Lactose, glucose and galactose were separated and eluted on a 300 x 7.8 mm Aminex HPX-87C cation exchange carbohydrate column (Bio-Rad Laboratories, Richmond, CA), and detected with a Waters 2414 Refractive Index Detector (Waters, Bray, Ireland). Sample temperature was set to 12 °C and the column oven temperature

of the HPLC unit was at 60 °C. The mobile phase was 0.009 N sulphuric acid at a flow rate of 0.5 mL/min. Standards solutions of lactose, glucose (VWR International Ltd, Northwest Business Park, Ballycoolin, Blanchardstown, Dublin 15, Ireland) and galactose (Sigma-Aldrich Ireland Ltd, Arklow, Ireland) were prepared by dissolving each at a level of 1 mg/mL in Milli-Q water (18.2 MΩ cm) (Millipore Ireland B.V., Tullagreen, Carrigtwohill, County Cork, Ireland). Each solution was then further diluted to give reduced- and high-concentration standards of 10 and 100 mg/L, respectively. These solutions were used to calibrate the concentration against peak height, and the retention times for the individual sugars. The concentrations of sugars in the cheeses were calculated by comparing the peak area of samples to standard curves. Sugar concentrations were calculated as g/100 g cheese.

Similarly, D (-)- and L (+)- lactate were extracted by the above procedure, and separated on a phenomenex chirex 3126 cation exchange silica column (Phenomenex, Hurdsfield Ind. Est., Macclesfield, Cheshire, UK) fitted with a Waters 2487 Dual λ Absorbance Detector (Waters, Bray, Ireland). The mobile phase was 0.001 M copper sulphate at a flow rate of 1.0 mL/min.

The concentration of total lactate was calculated as the sum of D (-)- and L (+)- lactates, and total sugars plus its derivatives as the sum of lactose, galactose, glucose and total lactate. Each analysis was carried out in duplicate.

4.2.8. Proteolysis

The level of pH 4.6-soluble nitrogen (pH4.6-SN) and levels of 5 % phosphotungstic acid-soluble cheese nitrogen (PTA-SN) were measured, as described previously (Hou et al., 2014). The levels of individual free amino acids (FAA) in the pH4.6-SN extract were determined by using a Beckman 6300 High Performance

Analyser (Beckman Instruments Ltd., High Wycombe, UK), as described by Fenelon et al., (2000a).

4.2.9. Rheology

Six cheese cubes (25 mm^3 cubes) were cut from each treatment cheese using a Cheese Blocker (Bos Kaasgreedschap, Bodengraven, Netherlands), wrapped in tin foil, and stored at 8 °C overnight prior to analysis. Each cube was compressed by 70 % on a texture analyser (model TA-HDI, Stable Micro Systems, Godalming, UK) with a 5-mm compression plate and a 100-Kg load cell, as described previously (Hou et al., 2014). The following rheological parameters were calculated from the resultant force/displacement curves: fracture stress (KPa), the stress at fracture, as indicated by the inflection point of the curve; fracture strain, the fractional displacement at fracture; and firmness, the force required to compress the cheese to 30 % of its original height.

4.2.10. Cheese color analysis

The color characteristics of the cheeses were evaluated after 30, 90, 180 and 270 days ripening. On each occasion, a 500 g block was cut from each cheese, and evaluated for color at 6 different locations across the freshly-cut face using a Colorimeter (CR-400 Chroma Meter, Konica Minolta, Osaka, Japan) with a condition of illuminant D65 and a 2° observer. The measurements from the colorimeter test were color space coordinates, namely, L*-value (an index of whiteness), a*-value (an index of redness), and b*-value (an index of yellowness). Prior to measurement, the Colorimeter was calibrated using the white Konica Minolta Calibration Plate for the following color space parameters Y, y and x, as defined by the International Commission on Illumination (CIE).

4.2.11. Volatile compounds

The 270 day-old cheeses were analysed in triplicate on same block at different places for volatile compounds. For each cheese, a 5 g sample was added to a 20 mL solid phase microextraction (SPME) vial (Apex Scientific Ltd., Maynooth, Co. Kildare, Ireland) and analysed by SPMEcoupled to a gas chromatograph (GC)-mass spectrometer (MS). The samples were transferred from the sample holder to the incubation unit of the GC using a CTC Analytics CombiPal Autosampler (CTC Analytics AG, Riedstrasse 4222 Zwingen Switzerland) and equilibrated at 40 °C for 5 min with agitation. The single 1 cm x 50/30 µm StableFlex divinylbenzene/carboxen/polydimethylsiloxane (DVD/Carboxen/PDMS) fibre (Supelco, Bellefonte, PA, USA) was exposed to the headspace above the samples for 20 min, retracted and automatically injected into the GC inlet at 250 °C and desorbed for 2 min. Volatile compounds were separated using gas-liquid chromatography, using conditions defined by (Hannon, Kilcawley, Wilkinson, Delahunty & Beresford, 2007). The detector used was a Varian 320 triple quad mass spectrometer (Varian Analytical Instruments) performed by electron impact at 70 eV.

4.2.12. Descriptive sensory analysis

The sensory properties of the 270 day-old cheeses from each of the three replicate trials were evaluated using descriptive sensory analysis, as described previously (Hou et al., 2014). Duplicate samples (~ 300 g blocks) were taken from each cheese and stored at -20 °C until required for analysis; samples were thawed by holding overnight at 4 °C. A trained panel, comprising 13 external experts who routinely perform sensory analysis on Cheddar cheeses, assessed the cheeses for 7 odour, 9 flavour and 5 taste attributes. The sensory scores awarded to each of the

treatment cheeses from any one trial by the individual assessors were averaged; the means for the triplicate trials were obtained from these averages. The mean scores for the different sensory attributes were standardised (1/Standard Deviation of the mean score for each attribute) and analysed using principal component analysis (PCA) by Unscrambler V 6.1 (CAMO AS, N-7041 Trondheim, Norway). The results are presented as a principal component (PC) plot. Attributes scored for odour included pungent, sweaty/cheesy, rancid, fruity, buttery, caramel and estery; attributes for flavour were pungent, rancid, fruity, buttery, caramel, waxy, sweaty/cheesy, nutty and onion, while those for taste comprised sweet acid, salt bitter and astringent.

4.2.13. Statistical analysis

Three replicate cheesemaking trials (blocks) were undertaken, each with 4 treatment cheeses, namely SCaNW, SCaW, RCaNW and RCaW. Analysis of variance (ANOVA) was used to determine if the treatment cheeses differed with respect to response variables, such as compositional factors, at specific time points (e.g., 14 days). ANOVA was undertaken using SAS® version 9.1.2 (SAS Institute, 2004), where the effects of treatment (curd washing or calcium reduction) and replicates were estimated for all response variables. Tukey's multiple-comparison test was used for paired comparison of treatment means and the level of significance was determined at $P < 0.05$.

The data for changes in individual response variables (such as sugars and proteolysis) in the SCa and RCa cheeses as a function of ripening were analysed using a split-plot design in order to determine the effects of treatment, ripening time, and their interaction. Analysis of variance for the split-plot design was carried out using a

general linear model (GLM) procedure of SAS (SAS Institute, 2004), and significance at $P < 0.05$ determined using Fisher's least significant difference test.

The data for volatile compounds and descriptive sensory analysis, measured at day 270 only, were analysed using PCA and are shown on the PC plot.

4.3. Results and discussion

4.3.1. Gross Composition

The gross composition of all cheeses complied with that for Cheddar cheese as specified by legislation (Table 4.1;CFR, 2008). Washing significantly reduced the levels of lactose, total lactate, total sugars and lactate to protein ratio, and increased the pH and fat in dry mater (FDM) in the SCa cheeses at 14 days. The reduction in the concentrations of lactose and lactate is consistent with the results of previous studies (Shakeel-Ur-Rehman et al., 2004; Hou et al., 2012). In contrast to the SCa cheeses, washing did not significantly affect the concentrations of lactose, total lactate or lactate to protein ratio in the RCa cheeses. There were no different between the cheeses as a result of the altered make procedure on the composition or pH of the 14 day-old cheeses (Table 4.1). Nevertheless, it is noteworthy that the level of salt and salt-in-moisture in reduced-calcium cheeses (RCaNW, RCaW) were non-significantly higher than those in the corresponding standard calcium cheeses (SCaNW, SCaW), despite the curds being salted at the same rate and at the same pH. It is possible that reducing the calcum content of the curd at salting leads to more extensive development of curd fibres (Guinee, 2003), a structural change (less rigid structure) which allowing salt to be absorbed better during mellowing (Gilles, 1976).

The mean calcium content of the SCa cheeses was significantly higher than that of the RCa cheeses. This trend concurs with previous studies showing reduction

of pH at set and at whey drainage as effective means of reducing calcium content of cheese (Guinee et al., 2002). In contrast, the phosphate content of both SCa- and RCa cheeses did not differ significantly. This indicates that the reduction in pH at whey drainage leads to more extensive loss of calcium than phosphorous from curd particles to whey. The higher loss of calcium at the lower whey drainage pH concurs with the results of Czulak et al., (1969), who concluded that when high acidity in the curd is reached quickly, sufficent calcium is lost to alter the texture properties of the cheese, but insufficient phosphorous is lost to significantly alter the buffering capacity. The authors suggested that this was due to a greater mobility of the calcium ion than the phosphate ion from curd to whey. A similar observation was made by Upreti and Metzger, (2006a) who reported that the reduction in calcium content on reducing set pH (6.6 to 6.2) and drain pH (from 6.2 to 5.7) was much higher than the corresponding reduction in phosphate content; the authors attributed this to a lower degree of solubilization of phosphate. Much of the phosphate (~ 40 %) is organic P (serine phosphate) that is covalently linked to the casein and remains with the casein on pH reduction (White & Davies, 1958); in contrast, all the micellar calcium is inorganic calcium phosphate, attached electrostatically to the casein and solubilized as the pH is reduced.

Table 4.1. The composition of 14 day-old, non-washed and washed-curd, Cheddar cheeses with standard- or reduced-calcium levels[1, 2, 3]

Cheese composition[3]	SCaNW	SCaW	RCaNW	RCaW
Moisture (g/100 g)	36.8^{aA}	36.3^{aA}	35.8^{aA}	36.8^{aA}
Fat (g/100 g)	31.8^{aA}	32.4^{aA}	32.6^{aA}	32.1^{aA}
Protein (g/100 g)	25.9^{aA}	26.4^{aA}	26.5^{aA}	26.3^{aA}
Lactose (g/100 g)	0.29^{aA}	0.02^{bA}	0.50^{aA}	0.26^{aA}
Total lactate (g/100 g)	1.13^{aA}	0.91^{bA}	0.88^{aA}	0.84^{aA}
Lactic acid + sugars (g/100 g)[4]	1.63^{aA}	1.08^{bA}	1.59^{aA}	1.27^{bA}
Lactate/ Protein (%)	4.37^{aA}	3.44^{bA}	3.33^{aA}	3.19^{aA}
Salt (g/100 g)	1.75^{aA}	1.79^{aA}	1.85^{aA}	1.93^{aA}
Calcium (mg/100 g)	763^{aA}	763^{aA}	675^{aB}	654^{aB}
P (mg/100g)	475^{aA}	462^{aA}	454^{aA}	449^{aA}
Calcium/ protein (mg/g protein)	29.4^{aA}	29.0^{aA}	25.5^{bB}	24.9^{bB}
S/M (g/100 g)	4.60^{aA}	4.90^{aA}	5.20^{aA}	5.30^{aA}
MNFS (g/100 g)	53.9^{aA}	53.7^{aA}	53.2^{aA}	54.2^{aA}
FDM (g/100 g)	50.3^{bA}	50.8^{aA}	50.9^{aA}	50.8^{aA}
pH at day14	5.24^{bA}	5.35^{aA}	5.23^{aA}	5.34^{aA}

[1]The cheese treatments, described in detail in Materials and Methods, included non-washed (control) curd cheeses with standard (SCaNW) or reduced calcium (RCaNW) content, and washed-curd cheese with standard (SCaW) or reduced-calcium (RCaW) content.
[2]Analysis of variance (ANOVA) was used to determine differences between treatments.
[3]The statistical effect of washing on the standard calcium cheeses (SCaNW, SCaW) and on the reduced-calcium cheeses (RCaNW, RCaW) is indicated by lower case superscripts, while the effect of reducing calcium content of the non-washed (SCaNW, RCaNW) and washed (SCaW, RCaW) cheeses is indicated by the upper case superscripts. Values not sharing a common superscript differ significantly, $P < 0.05$.
[3]Abbreviations: S/M, salt in moisture; MNFS, moisture in non-fat substances; FDM, fat in dry matter; SIM, total sugars in cheese moisture.
[4]Sugars comprise lactose, glucose and galactose; the concentrations of the latter 2 sugars were ~ 0.21 % and 0.15 % for the SCa and RCa cheeses, respectively.

4.3.2. Composition analysis of milk, whey and cheese yields

The composition of the pasteurised cheese milks did not differ significantly between cheese treatments (data not shown), as expected, because the same milk was for used all cheese treatments in each of the replicate cheese making trials. The RCa milks coagulated more rapidly than the SCa milks (Table 4.2), which is expected because of their lower pH, which leads to a reduction in the net charge on the casein micelles, and increases in the concentration of ionic calcium and rennet activity (Fox

et al., 2000). The time from cutting to whey drainage (cut to drain time), which represents the length of time that the curd particles are in contact with the whey, was significantly longer for the RCa cheeses, owing to the greater pH difference between pH at set (rennet addition) and whey drainage. The rate of pH reduction between set and whey drainage was more rapid for the RCa cheeses than for the SCa cheeses (Table 4.2); this may reflect a lower buffering capacity (insoluble calcium) of the RCa curd because of the lower levels of calcium phosphate (Lucey & Fox, 1993; Lee, Johnson, Govindasamy-Lucey, Jaeggi, & Lucey, 2010). Washing significantly reduced the rate of pH reduction between cut and whey drainage in the SCa cheese, but not in the RCa cheese.

Table 4.2. The pH and manufacture time of washed-curd (W) and non-washed (NW) Cheddar cheeses with standard- (SCa) and reduced-(RCa) calcium levels[1, 2]

	SCaNW	SCaW	RCaNW	RCaW
pH at different stages of manufacture				
pH at set	6.56[a]	6.56[a]	6.30[b]	6.30[b]
pH at drainage	6.30[a]	6.30[a]	5.87[b]	5.87[b]
pH at milling	5.35[a]	5.35[a]	5.35[a]	5.35[a]
pH difference between set-to-drain	0.26[b]	0.26[b]	0.43[a]	0.43[a]
Times for cheesemaking stages (min)				
Ripening period (starter addition to set)	30[a]	30[a]	30[a]	30[a]
Set (rennet addition) to cut	37[a]	37[a]	23[b]	22[b]
Cut-to-wash time	-	30[a]	-	30[a]
Cut-to-drain time	130[c]	153[b]	194[a]	202[a]
Starter-to-drain time	207[b]	231[b]	257[a]	264[a]
Rate of acidification (pH reduction with time)	0.0020[b]	0.0017[c]	0.0022[a]	0.0021[ab]

[1]The cheese treatments, described in detail in the Materials and Methods, comprised washed and unwashed variants of Cheddar cheeses with standard (~ 760 mg Ca/100 g cheese) or reduced (~ 660 mg Ca/100 g cheese) contents of calcium. Washing involved the withdrawal of some whey replacement by water during the cooking stage of manufacture to reduce the concentration of lactose in moisture from ~ 5.3 % in the control non-washed cheeses to ~ 3.9 % in the washed cheeses.

[2]Values within a row not sharing a common superscript differ significantly, $P < 0.05$Table 4.3. The composition of cheese wheys of control non-washed, and washed-curd, Cheddar cheeses with standard- or reduced-calcium levels[1, 2]

Composition	SCaNW	SCaW	RCaNW	RCaW
Pre-washed whey (PWW)				
Fat, g/100 g	-	0.58^{A}	-	0.58^{A}
Protein, g/100 g	-	0.55^{A}	-	0.52^{B}
Fat, % of total milk fat	-	4.53^{A}	-	4.54^{A}
Protein, % of total protein	-	4.59^{A}	-	4.28^{B}
Bulk whey (BW)				
Bulk whey, kg/100 kg	$89.22^{a,B}$	$89.49^{a,A}$	$89.99^{a,A}$	$89.98^{a,A}$
Fat, g/100 g	$0.35^{a,A}$	$0.23^{b,A}$	$0.29^{a,B}$	$0.24^{a,A}$
Protein, g/100 g	$0.88^{a,A}$	$0.63^{b,A}$	$0.88^{a,A}$	$0.60^{b,A}$
Fat, % of total milk fat	$8.52^{a,A}$	$5.76^{b,A}$	$6.91^{a,B}$	$5.99^{a,A}$
Protein, % of total milk protein	$23.18^{a,A}$	$16.49^{b,A}$	$23.03^{a,A}$	$15.81^{b,A}$
Pre-washed whey +bulk whey (PWW+BW)				
Fat, % of total milk fat	$8.52^{b,A}$	$10.30^{a,A}$	$6.91^{b,B}$	$10.53^{a,A}$
Protein, % of total milk protein	$23.18^{a,A}$	$21.08^{b,A}$	$23.03^{a,A}$	$20.09^{b,A}$
White whey (WW)				
White whey, kg/100 kg	$0.66^{a,A}$	$0.66^{a,A}$	$0.65^{a,A}$	$0.66^{a,A}$
Fat, g/100 g	$2.92^{a,A}$	$3.10^{a,A}$	$3.25^{a,A}$	$2.93^{a,A}$
Protein, g/100 g	$0.92^{a,A}$	$0.80^{b,A}$	$0.98^{a,A}$	$0.87^{a,A}$
Fat, % of total milk fat	$0.53^{a,A}$	$0.56^{a,A}$	$0.57^{a,A}$	$0.54^{a,A}$
Protein, % of total protein	$0.18^{a,A}$	$0.16^{b,A}$	$0.19^{a,A}$	$0.17^{a,A}$

[1]The cheese treatments, described in detail in the Materials and Methods, included control, non-washed curd cheeses with standard (SCaNW) or reduced calcium (RCaNW) content, and washed-curd cheese with standard (SCaW) or reduced-calcium (RCaW) content.

[2]Analysis of variance (ANOVA) was used to determine differences between treatments. The statistical effect of washing on the standard calcium cheeses (SCaNW, SCaW) and on the reduced-calcium cheeses (RCaNW, RCaW) is indicated by lower case superscripts, while the effect of reducing calcium content of the non-washed (SCaNW, RCaNW) and washed (SCaW, RCaW) cheeses is indicated by the upper case superscripts. Values not sharing a common superscript differ significantly, $P < 0.05$.

While the fat content of the PWWs from the RCaW and SCaW were similar, the protein content of the PWW for the RCaW cheese was slightly, but significantly, lower than that of the SCaW cheese (Table 4.4). The lower protein in the PWW from the RCaW cheese may reflect a lower degree of κ-casein hydrolysis due to the shorter time between rennet addition and washing, in turn due to the lower pH at set which enhances casein aggregation and gelation of rennet-treated milk, hence the less gel network formation took place and caused further curd shattering during cooking (McMahon, Paulson, & Oberg, 2005).

The composition of the BW for non-washed cheeses was similar to that reported previously for Cheddar cheese (Guinee et al., 2006). Curd washing significantly reduced the level of protein in the BW for the standard-calcium and reduced-calcium (SCaW, RCaW) cheeses, and fat in the BW for the standard-calcium cheese (SCaW); the fat content of the BW from the RCaW cheese was numerically, but not significantly, lower than that from the RCaNW cheese (Table 4.4). The lower fat and protein levels in BWs from washed-curd cheeses reflect the dilution of the whey during the curd washing process. Reducing the calcium content of the non-washed cheese resulted in a significantly higher quantity of BW with a lower fat content and percentage of total milk fat, as seen by comparing the SCaNW and RCaNW cheeses (Table 4.4). In contrast, reducing calcium of the washed-curd cheese did not affect the quantity of BW or its fat content which may favour the further whey processing to keep consistent quality and energy comsumption.

The combination of PWW and BW, the total whey expressed prior to salting and pressing, is denoted PWW+BW. Curd washing significantly increased the percentage of total fat lost in the PWW+BW. A tentative explanation for the higher fat loss may be due to an increased stress on, and some physical damage to, the curd

particles owing to the whey removal and water addition stages (within 5-10 mins). During whey removal, the curd particle:whey volume ratio is increased and is only restored when all the water (used to replace the whey removed) was added to the cheese vat. Consequently, there is a temporary increase in curd: whey volume ratio during curd washing when the curd particles are still relatively fragile, which is likely to cause higher collision frequency of curd particles during water addition. Analogously, an increase in the curd-to-whey ratio following low concentration factor ultrafiltration of cheese milk leads to higher fat losses during manufacture, especially at milk protein levels of 4.0%, w/w (Guinee, Pudja, & Mulholland, 1994, Guinee, O'Callaghan, Mulholland, & Harrington, 1996). In contrast to the trend for fat loss, curd washing significantly reduced the quantity of protein lost in the PWW+BW whey (Table 4.4). This lower protein loss reflects a reduction in the diffusion-induced permeation of whey proteins through the calcium-phosphate *para*-casein network of the curd particles to the surrounding whey when the whey is diluted.

Although the mechanisms are not clear enough, in general, it would due to complex steric and electrical effects or electroneutrality principle (Donnan Effect) (Bowers, Fulton, & Thompson, 1984).

The levels of fat and protein in the WW are similar to those reported by Fenelon and Guinee (1999). The fat content or % of total milk fat in the WW was not influenced by either curd washing or altering the Ca levels of the cheese. However, curd washing reduced the protein content of the WW from the SCa cheese significantly and the RCa cheese non-significantly.

Curd washing did not significantly affect the actual yield of the SCa or RCa cheeses, however, the higher fat losses in the washed-curd cheeses coincided with

numerically lower yield (Table 4.4), even all curd fines were collected and returned back to cheeses.

Table 4.4. The losses of milk fat and protein in whey and yield of cheese for control non-washed, and washed-curd, Cheddar cheeses with standard- or reduced-calcium levels[1,2]

Whey losses/recoveries and cheese yield	SCaNW	SCaW	RCaNW	RCaW
FLW (% total milk fat)[3]	$9.05^{b,A}$	$10.86^{a,A}$	$7.47^{b,B}$	$11.06^{a,A}$
PLW (% total milk protein)[3]	$23.36^{a,A}$	$21.24^{b,A}$	$23.22^{a,A}$	$20.26^{b,A}$
Ya (kg/ 100 kg)[3]	$10.07^{a,A}$	$9.96^{a,A}$	$10.01^{a,A}$	$9.94^{a,A}$

[1]The cheese treatments, described in detail in Materials and Methods, included control, non-washed curd cheeses with standard (SCaNW) or reduced calcium (RCaNW) content, and washed-curd cheese with standard (SCaW) or reduced-calcium (RCaW) content.

[2]Analysis of variance (ANOVA) was used to determine differences between treatments. The statistical effect of washing on the standard calcium cheeses (SCaNW, SCaW) and on the reduced-calcium cheeses (RCaNW, RCaW) is indicated by lower case superscripts, while the effect of reducing calcium content of the non-washed (SCaNW, RCaNW) and washed (SCaW, RCaW) cheeses is indicated by the upper case superscripts. Values not sharing a common superscript differ significantly, $P < 0.05$.

[3]Abbreviations: FLW, percentage milk fat lost in whey streams; PLW, percentage milk protein lost in whey streams; Ya, actual cheese yield (kg/ 100 kg of milk).

4.3.3. Changes in sugars and its derivatives during ripening

4.3.3.1. Lactose and total lactate

Curd washing significantly reduced the mean lactose concentration over the 270-day ripening period in the SCaW and RCaW cheeses (Figure 4.1). This trend concurs with the findings of previous studies (Upreti & Metzger, 2006a; Hou et al., 2012).

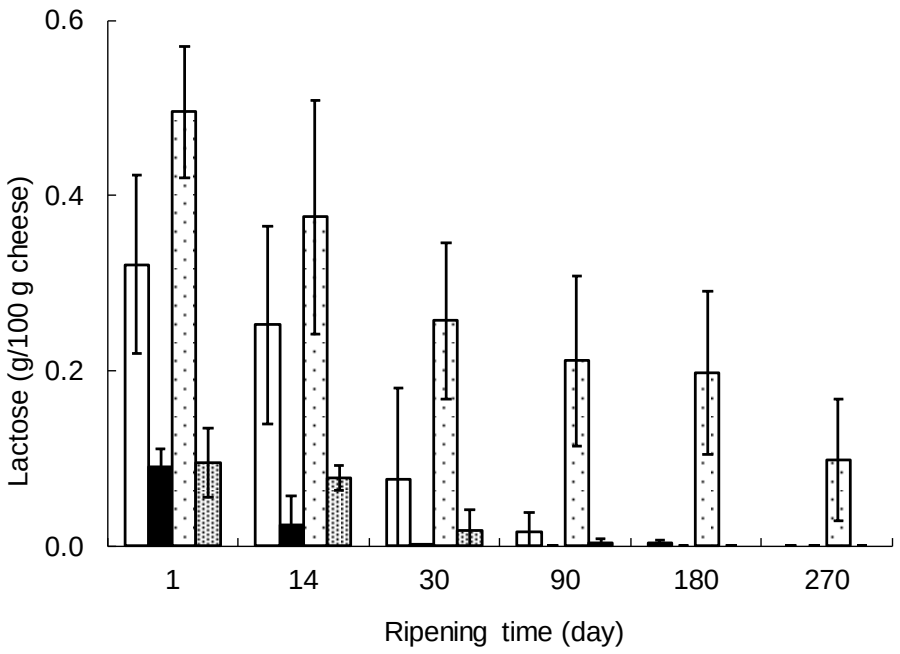

Figure 4.1. Changes in the levels of residual lactose in control, non-washed curd cheeses with standard (SCaNW, □) or reduced calcium (RCaNW, ▒) content, and washed-curd cheese with standard (SCaW, ■) or reduced-calcium (RCaW, ▒) content. Presented values are the means of three replicate trials; error bars represent standard deviations of the mean.

The mean level of residual lactose decreased in all cheeses with maturation time (Figure 4.1, Table 4.5), coinciding with a concomitant increase in the concentration of lactate (Figure 4.2). While virtually concentration of lactose had decreased to zero in the SCaNW, SCaW and RCaW cheeses at 90 d, ~ 0.1 to 0.2 % remained in the RCaNW cheeses after 90, 180 and 270 d ripening. Analysis of variance (ANOVA) of the data indicated no significant difference between the lactose

content of SCaNW and RCaNW, or SCaW and RCaW at these times (data not shown). Nevertheless, the non-significantly higher level of residual lactose in the RCaNW cheese compared to the SCaNW cheese may reflect the non-significantly higher mean level of S/M of the former (Table 4.1). This suggestion is supported by the work of Thomas and Pearce, (1981), which showed that the residual lactose in Cheddar cheese at 14 days after manufacture increased linearly by ~ 0.025% per 0.1% increase in S/M in the range 4 to 6% S/M. A similar trend to that in the current study was reported by Upreti and Metzger, (2006a) who attributed the higher residual lactose in reduced-calcium cheeses (~ 0.25 g/100 g compared to 0 g lactose/100 g in the standard-calcium cheeses after 350 d) to differences in composition between the cheeses, including the higher moisture content (1.5-2.0 g/100 g) of the reduced-calcium cheeses.

Reducing calcium level in the washed curd or non-washed curd cheeses did not significantly influence the gross composition (S/M, MNFS, FDM, pH at day 14, moisture) of corresponding pairs of washed or non-washed SCa and RCa cheeses in the current study.

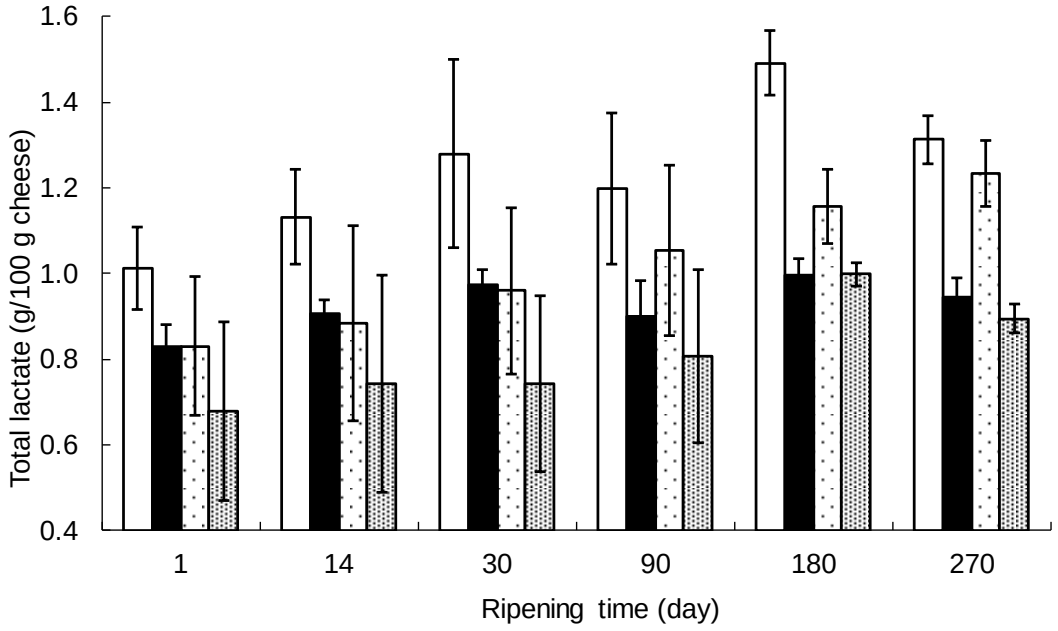

Figure 4.2. Age-related changes in the levels of total lactates in control, non-washed curd cheeses with standard (SCaNW, □) or reduced calcium (RCaNW, ▒) content, and washed-curd cheese with standard (SCaW, ■) or reduced-calcium (RCaW, ▒) content. Presented values are the means of three replicate trials; error bars represent standard deviations of the mean.

Table 4.5. Degrees of freedom (df) and statistical significances (*P*-values) for changes in lactose, D (-)- lactate, L (+)- lactate, total lactate, and pH in control non-washed, and washed-curd, Cheddar cheeses with standard- or reduced-calcium level.

Factor	Lactose		D(-)-Lactate		L(-)-Lactate		Total lactate		pH	
(Standard calcium)	df	*P*	df	*P*	df	*P*	df	*P*	df	*P*
Main plot										
Curd washing	1	0.0113	1	0.0248	1	<.0001	1	0.0155	1	<.0001
Sub-plot										
Ripening time	5	<.0001	5	<.0001	5	<.0001	5	<.0001	5	0.0256
Interaction (curd washing x ripening time)	5	0.0001	5	0.0006	5	0.5518	5	0.0360	5	0.0026

Factor	Lactose		D(-)-Lactate		L(-)-Lactate		Total lactate		pH	
(Reduced calcium)	df	*P*	df	*P*	df	*P*	df	*P*	df	*P*
Main plot										
Curd washing	1	0.0413	1	0.1140	1	0.1424	1	0.0565	1	0.1710
Sub-plot										
Ripening time	5	0.0006	5	<.0001	5	0.0098	5	0.0004	5	0.2697
Interaction (curd washing x ripening time)	5	0.3610	5	0.6353	5	0.6374	5	0.7017	5	0.1288

[1]The cheese treatments, described in detail in Materials and Methods, included control, non-washed curd cheeses with standard (SCaNW) or reduced calcium (RCaNW) content, and washed-curd cheese with standard (SCaW) or reduced-calcium (RCaW) content.
[2]Analysis of variance was carried using a general linear model (GLM) procedure of SAS, where the effects of treatment, ripening time and their interactions.

4.3.3.2. D (-)- and L (+)- lactate

Curd washing significantly reduced the mean concentrations of L (+)- lactate and D (-)- lactate in the SCaW cheeses over the 270-day ripening period, but not in the RCaW cheeses (Table 4.5). The mean levels of L (+)- lactate decreased significantly in all cheeses with ripening time, while those of D (-)- lactate increased (Figure 4.3). The changes in the concentrations of L (+) and D (-) occurred most rapidly between 30 and 180 days, after which the concentrations changed little. The racemisation of L (+)- lactate to D (-)- lactate, which is a feature in Cheddar cheese maturation (Jordan & Cogan, 1993; Hou et al., 2012), coincided with an increase in the numbers of NSLAB from ~ 10^5 cfu/g at day 30 to ~ 10^8 cfu/g at day 270 (data not shown).

The mean concentrations of D (-)- and L (+)- lactate in the SCaNW and SCaW cheeses over the 270 day ripening period were higher than those in the corresponding RCaNW and RCaW cheeses (Figure 4.3), a trend consistent with the higher total lactate and lower residual lactose in the SCaNW and SCaW cheeses.

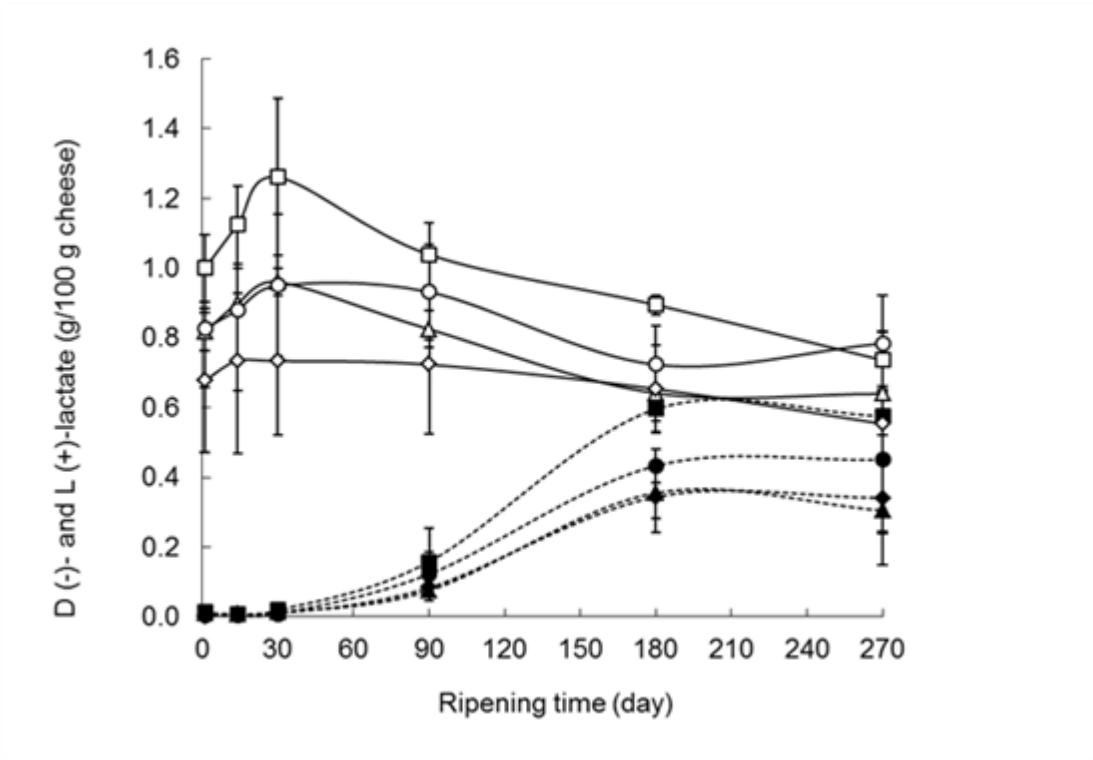

Figure 4.3. Age-related changes in the levels of D (-)- Lactate (broken lines) and L (+)- lactate (solid line) content in control, non-washed curd cheeses with standard (SCaNW, ■, □) or reduced calcium (RCaNW, ●, ○) content, and washed-curd cheese with standard (SCaW, ▲, △) or reduced-calcium (RCaW, ◆, ◇) content. Presented values are the means of three replicate trials; error bars represent standard deviations of the mean.

4.3.3.3. Glucose and galactose

The mean concentration of glucose (0.15 g/100 g) in the SCaNW and RCaNW cheeses over the 270-day ripening period was significantly higher than that in the corresponding SCaW (0.12 g/100 g) or RCaW (0.1 g/100 g) cheeses (data not shown). Glucose decreased significantly in all cheese during ripening, with the decrease (0.01-0.04 g/100 g cheese) depending on treatment.

The mean concentration of galactose over the 270-day ripening period was very low in all cheeses. The levels in SCaNW and RCaNW cheeses were similar at 0.03 g/100 g and significantly higher than those in the SCaW and RCaW cheeses (which were also identical at 0.02 g/100 g). Galactose was absent in the 270 day old

cheese. We have no explanation for the presence of unexpected glucose and galactose at these concentrations in the cheese.

4.3.4. pH changes during ripening.

The mean pH of the SCaW cheeses increased significantly with curd washing and ripening time; moreover, it was significantly affected by the interaction of curd washing and ripening time, as reflected by the increase in the magnitude of the pH difference between the SCaW and SCaNW cheeses as ripening time progressed (Figure 4.4, Table 4.5). The pH of SCaW increased by ~ 0.2 units, from ~ 5.33 at day 1 to 5.52 at day 270, whereas that of SCaNW decreased significantly by ~ 0.07 units, from 5.25 to 5.18 over the same time period. The increase in the pH of the SCaW cheese during ripening is probably associated with proteolysis, and the commensurate hydrolysis of peptide bonds resulting in the generation of $–COO^-$ and NH_3^+ in the cheese environment and also buffering by solublisation of calcium phosphate. At the normal lactate concentrations of Cheddar cheese, as in the SCaNW cheese in the current study, the protonation of amino groups does not lead to an increase in pH because of the excess H^+ ions from the lactate which is expected to be fully dissociated in the cheese environment (pKa ~ 3.86). It is noteworthy that the pH of Gouda, a washed-curd cheese, increases with ripening time and that for a given washing rate (withdrawal of fixed amount of whey and replacement with equal volume of water) the increase with ripening time is most pronounced when the cheese is made from late lactation milk when the lactose level is likely to be at its lowest (Lawrence et al., 1986). Hence, the divergence between the pH of the SCaW and SCaNW cheeses probably reflects the lower ratio of lactic acid-to-buffering substances (protein and phosphate

salts) in the SCaW cheeses (Lucey & Fox, 1993; Salaün, Mietton & Gaucheron, 2005; Kim, Oh, & Imm, 2018).

In contrast to the SCa cheeses, the mean pH of the RCa cheeses over the 270 day ripening period was not significantly affected by curd washing, ripening time or their interaction. This trend is consistent with that showing that curd washing did not significantly affect the level of total lactate in the RCa cheeses. However, ANOVA at 270 days indicated that the pH of the RCaW cheese was significantly higher than that of the RCaNW cheeses, a result consistent with the lower concentration of total lactate in the former.

Similar to the SCaNW cheeses, the pH of the RCaNW cheese decreased by ~ 0.05 units between day 1 and day 270, while that of the RCaW cheese increased by ~ 0.1 units, from 5.25 at day 1 to 5.35 at day 270.

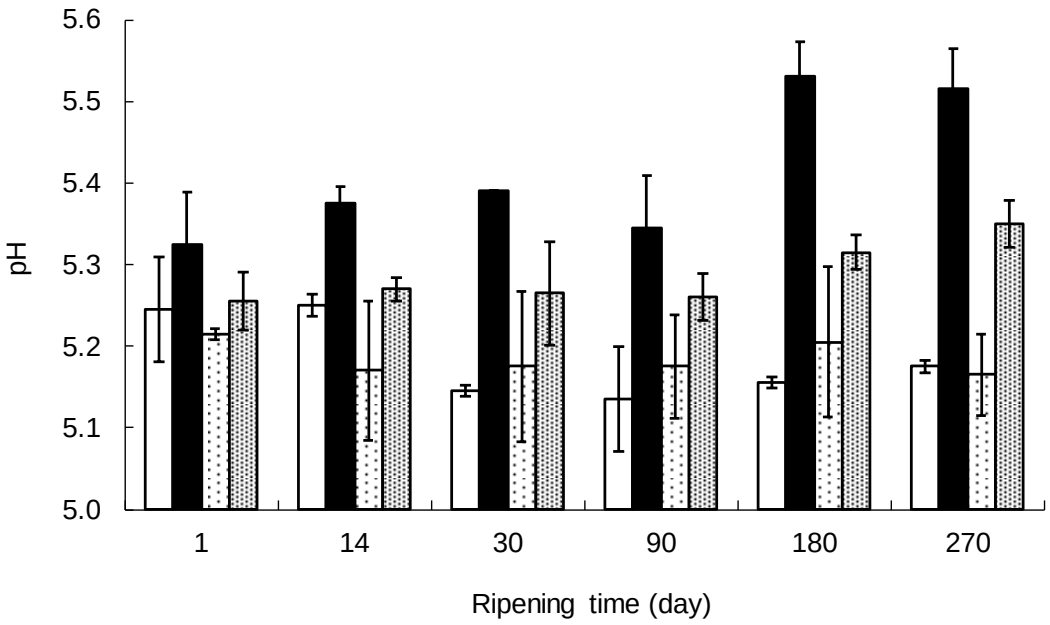

Figure 4.4. Age-related changes in the pH in control, non-washed curd cheeses with standard (SCaNW, □) or reduced calcium (RCaNW, ▒) content, and washed-curd cheese with standard (SCaW, ■) or reduced-calcium (RCaW, ▒) content. Presented values are the means of three replicate trials; error bars represent standard deviations of the mean.

4.3.5. Microbial counts of starter and non-starter lactic acid bacteria (NSLAB) in cheese

4.3.5.1. Starter bacteria

Starter bacteria counts at day one were ~ $10^{9.5}$ cfu/g cheese. The mean starter count of all cheeses decreased significantly with ripening time (Figure 4.5). The magnitude of the decrease in cell count, from ~ $10^{9.5}$ cfu/g at day 1 to ~ $10^{7.5}$ cfu/g at day 270, is typical of that reported elsewhere for Cheddar cheese made using *Lactococcus lactis* subsp. *cremoris* and *lactis* (Turner & Thomas, 1980).

In agreement with previous studies on Cheddar (Shakeel-Ur-Rehman et al., 2004, Hou et al., 2012), the mean starter count in all the cheeses over the 270 day ripening period was not significantly affected by curd washing (Table 4.6).

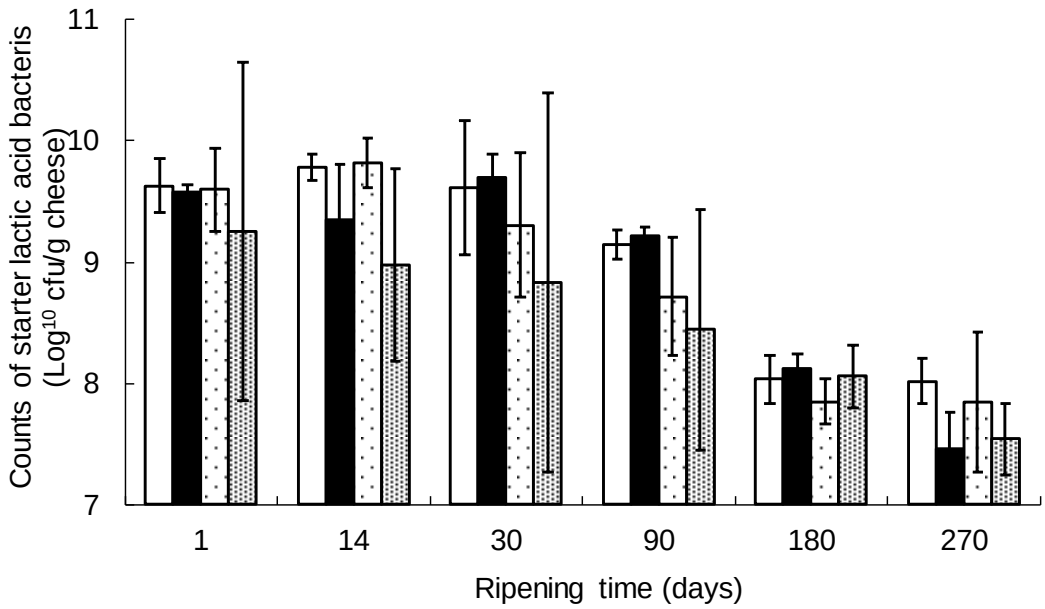

Figure 4.5. Age-related changes in the counts of starter lactic acid bacteria in control, non-washed curd cheeses with standard (SCaNW, □) or reduced calcium (RCaNW, ▒) content, and washed-curd cheese with standard (SCaW, ■) or reduced-calcium (RCaW, ▒) content. Presented values are the means of three replicate trials; error bars represent standard deviations of the mean.

Table 4.6. Degrees of freedom (df) and statistical significances (*P*-values) for changes in starter and NSLAB counts in control non-washed, and washed-curd, Cheddar cheeses with standard- or reduced-calcium levels[1].

Factor	Starter counts		NSLAB counts	
(Standard calcium)	df	*P*	df	*P*
Main plot				
Curd washing	1	0.2648	1	0.4732
Sub-plot				
Time	5	<.0001	5	<.0001
Interaction (Curd washing x time)	5	0.1560	5	0.1111

Factor	Starter counts		NSLAB counts	
(Reduced calcium)	df	*P*	df	*P*
Main plot				
Curd washing	1	0.5333	1	0.0576
Sub-plot				
Time	5	<.0001	5	<.0001
Interaction (Curd washing x time)	5	0.7058	5	0.0806

[1]The cheese treatments, described in detail in Materials and Methods, included control, non-washed curd cheeses with standard (SCaNW) or reduced calcium (RCaNW) content, and washed-curd cheese with standard (SCaW) or reduced-calcium (RCaW) content.

4.3.5.2. Non-starter lactic acid bacteria (NSLAB)

The mean counts of NSLAB increased significantly in all cheeses during ripening, from ~ 10^2-10^3 cfu/g at day 1 to ~ 10^8 cfu/g at day 270 (Figure 4.6), and were not affected by curd washing. This trend corroborates findings from New Zealand, where it was reported that NSLAB growth in Cheddar was independent of residual lactose content (Turner & Thomas, 1980).

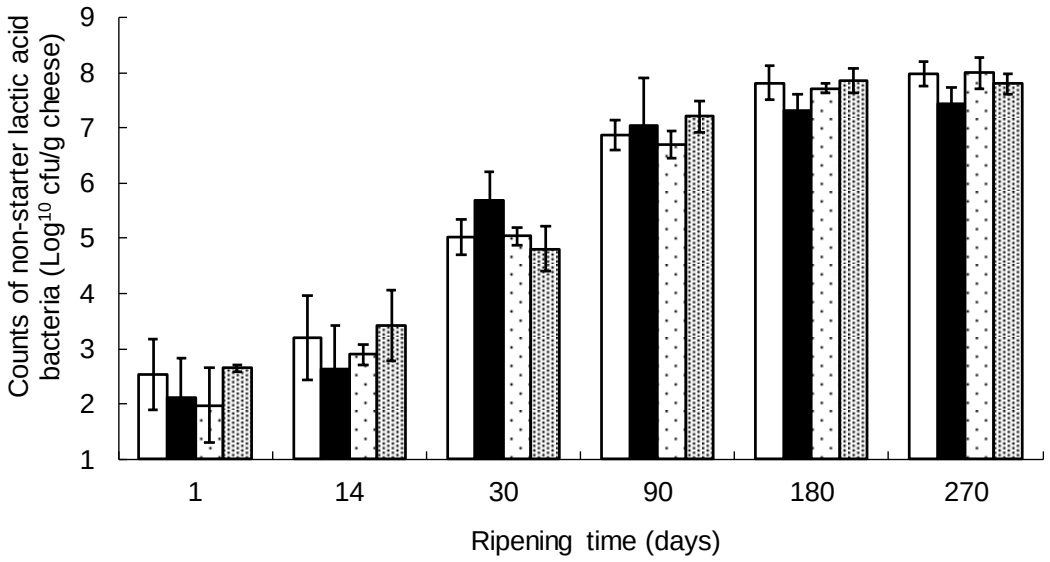

Figure 4.6. Age-related changes in the counts of non-starter lactic acid bacteria (b) in control, non-washed curd cheeses with standard (SCaNW, □) or reduced calcium (RCaNW, ▨) content, and washed-curd cheese with standard (SCaW, ■) or reduced-calcium (RCaW, ▩) content. Presented values are the means of three replicate trials; error bars represent standard deviations of the mean.

4.3.5.3. Coliforms and Enterocci

Coliforms and enterococci were present in all cheeses at 1 day, ranging from 10^3 cfu/g and $10^{3.5}$ cfu/g in the non-washed cheeses to 10^4 and $10^{4.5}$ cfu/g in the washed curd cheeses, respectively. These organisms decreased rapidly to < 10 cfu/g at 180 days. Coliform bacteria and enterocci are killed by pasteurization and, therefore, their presence in cheese indicates post-pasteurization contamination. The relatively high

numbers of these bacteria initially present in the cheeses are a reflection of the considerable amount of manual handling of the milk prior to pilot-scale cheese manufacture. The initial levels in the washed-curd cheeses were generally higher, suggesting that the wash water (RO water) and transport pipes were also sources of contamination. Rea, Franz, Holzapfel & Cogan, (2004) investigated the effect of including different strains of Enterococcus (e.g., 3 strains of *E. faecalis* and 1 strain each of *E. faecium*, *E. durans* and *E. casseliflavus*) to a level of 10^7 cfu/g in 1 day-old cheese and concluded that enterococci had little impact on proteolysis and flavour of Cheddar. Hence, the populations in the current study (< $10^{4.5}$ cfu/g) are unlikely to have had a significant effect on the quality of the cheese. Similarly, it can be concluded from the findings of Kosikowski and Fox, (1969) that coliforms at population of < 10^5 cfu/g have no impact on Cheddar cheese flavour.

4.3.6. Proteolysis

4.3.6.1. Changes in primary proteolysis

The mean levels of pH4.6-SN in all cheeses increased significantly over the 270-day ripening (Figure 4.7a), but were not affected by curd washing. The magnitude of increase during ripening (from ~ 2.0 % of total N at 1 day to ~ 25 % of total N at day 270) is typical of that previously reported for Cheddar cheese matured at 8 °C (Fenelon et al., 2000a) and is indicative of hydrolysis of the insoluble intact calcium phosphate *para*-casein into water soluble peptides by residual chymosin (Fenelon, O'Connor, & Guinee, 2000b).

ANOVA analysis showed that the RCaNW cheeses had significantly higher levels of pH4.6-SN than the corresponding SCaNW cheeses at 30, 90 and 180 days (data not shown); however, such differences were not observed between the SCaW

and RCaW cheeses. A similar trend was reported by (Upreti, Metzger, & Hayes, 2006b) who indicated that cheeses with low Ca and P had a higher ($P < 0.05$) level of proteolysis than their corresponding high Ca and P treatments which was due to the higher residual chymosin activity and moisture content in the low Ca + P cheeses. Moreover, the higher level of primary proteolysis in the RCaNW cheese at 30, 90 and 180 day compared to corresponding SCaNW cheese may be associated with a lower degree of calcium-induced interaction, and aggregation, between the casein molecules, which would increase their susceptibility to chymosin-mediated proteolysis (Fox, Guinee, Cogan, & McSweeney, 2000). Hence, O'Keeffe, Fox, and Daly, (1975) reported that a reduction in the level of calcium phosphate in Cheddar cheese curd resulted in a significantly higher degree of casein degradation in 1-d-old cheese. Similarly, Fox, (1970) reported that the caseins in milk, especially the αs_1-CN, became increasingly susceptible to proteolysis at pH 6.6 as the level of micellar calcium phosphate was reduced; the effect was attributed to the concomitant disruption of the casein micelle structure, increases in the solubility of individual caseins, and their accessibility to rennet. A tentative explanation for the similar levels of proteolysis in the SCaW and RCaW cheeses, in contrast to the SCaNW and RCaNW cheeses, may be due to the higher mean pH of the latter cheeses over ripening; it is feasible that the differences associated with calcium level on casein dissociation may not be as pronounced at the higher pH (pH 5.8-6.4), which would be less conducive to casein hydrolysis (Mulvihill & Fox, 1977).

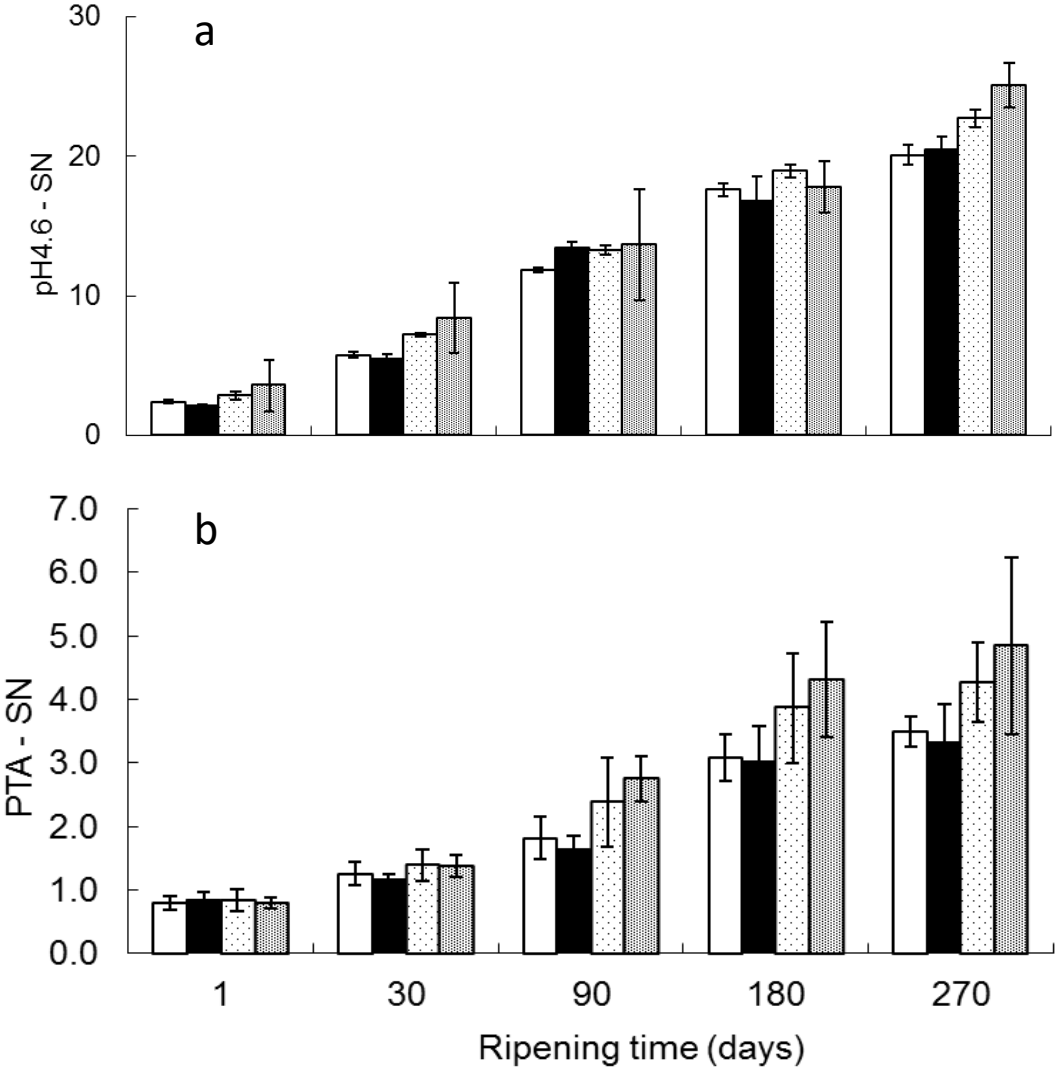

Figure 4.7. Age-related changes in (a) pH4.6-SN and (b) PTA-SN, in control, non-washed curd cheeses with standard (SCaNW, □) or reduced calcium (RCaNW, ▨) content, and washed-curd cheese with standard (SCaW, ■) or reduced-calcium (RCaW, ▩) content. Presented values are the means of three replicate trials; error bars represent standard deviations of the mean.

4.3.6.2. Changes in secondary proteolysis

PTA-SN and FAA were used as measures of the degree of secondary proteolysis, reflecting the degradation of large molecular weight peptides produced by residual coagulant and plasmin into low molecular weight peptides and free amino

acids (Jarrett, Aston & Dulley, 1982; Fenelon et al., 2000b). The mean levels of PTA-SN increased significantly in all cheeses during ripening but were not affected by curd washing (Figure 4.7b). A similar trend was found for FAA, the concentrations of which were comparable to those reported for experimental full-fat Cheddar cheeses of similar ages (Fenelon et al., 2000b) but lower than the mean values (1.0 - 5.6 %) reported for retail Cheddar cheeses depending on brand type (Fenelon et al., 2000a, Guinee et al., 2008) (data not shown). The FAA content of Cheddar varies with brand type, as influenced by composition, types of culture and culture adjuncts, and maturation time and temperature.

Comparison of the data at 90 and 180 days using ANOVA indicated that the levels of PTA-SN/TN in the RCaNW and RCaW were higher than in the corresponding SCaNW and SCaW cheeses (data not shown), despite the similar composition and microbiology of all cheeses. PTA-N (as % of pH4.6-SN) in the RCaNW and RCaW was also significantly higher at 30, 90 and 180 day (data not shown), suggesting more rapid degradation of the relatively large molecular weight peptides in the pH4.6-SN extract by starter culture peptidases released on autolysis (Fenelon & Guinee, 1999). A tentative explanation for the relatively high PTA-SN in the RCaNW and RCaW cheeses could be a higher proteolytic activity resulting from a higher degree of autolysis of the non-viable starter cells due to the higher S/M (Sheehan, Cuinn, FitzGerald & Wilkinson, 2006), or a greater release of starter cell envelope proteinase (CEP) from starter cells into the cheese matrix (Coolbear, Reid & Pritchard, 1992; Exterkate & Alting, 1999).

Free amino acid N (FAAN) as a % of PTA-SN increased from a mean of ~ 40 % in all cheeses at day 1 to ~ 65 % at day 270, and was higher in the SCaNW and SCaW cheeses than the corresponding RCaNW and RCaW cheeses (data not shown).

4.3.7. Rheological properties

The mean fracture stress and firmness of all cheeses decreased significantly during ripening (Table 4.7, Figure 4.8). This trend is consistent with the increase in primary proteolysis of calcium phosphate *para*-casein network (Figure. 4.7a), which is the main structural component of the cheese matrix resisting the stresses applied during compression (Guinee, 2003). Solubilisation of calcium during maturation may be also a contributory factor to ripening-induced softening (O'Mahony, Lucey & McSweeney, 2005).

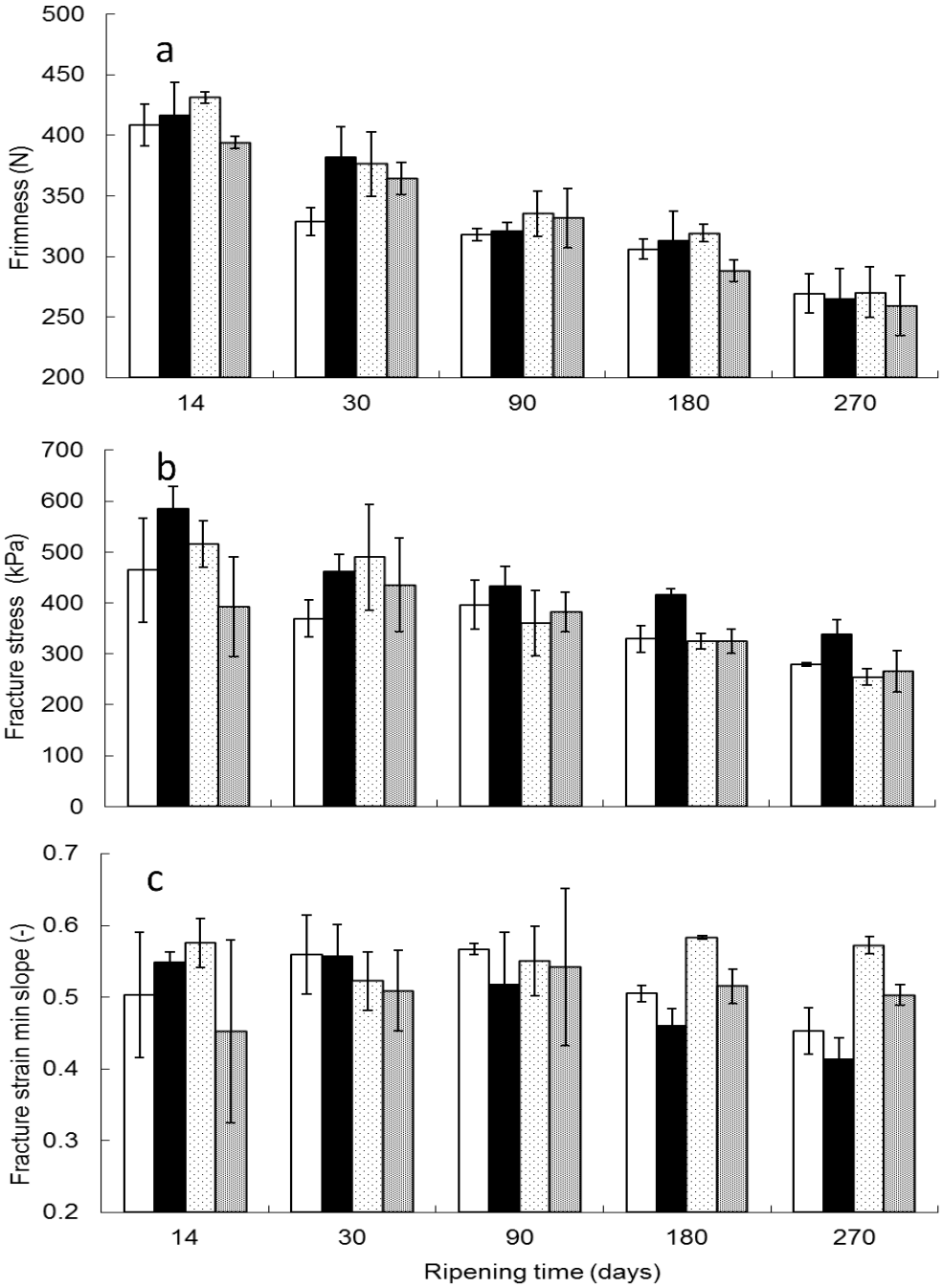

Figure 4.8. Age-related changes in firmness (a), fracture stress (b) and fracture strain (c) in control, non-washed curd cheeses with standard (SCaNW, □) or reduced calcium (RCaNW, ▢) content, and washed-curd cheese with standard (SCaW, ■) or reduced-calcium (RCaW, ▩) content. Presented values are the means of three replicate trials; error bars represent standard deviations of the mean.

The mean values for fracture strain and firmness of all cheeses over the 270-day ripening period were not affected by curd washing, a trend which is consistent with the absence of significant differences in the mean levels of pH4.6-SN. However, the mean fracture stress of the SCaW cheese over 270 days was significantly higher than that of the SCaNW cheese. This may reflect the higher pH of the SCaW cheese, which would favor a higher level of casein bound calcium (Lee et al., 2011) and be expected to give a more cohesive, rigid *para*-casein cheese network that requires higher stress to fracture (Visser, 1991). In contrast, the latter effect was not observed in the reduced-calcium cheeses, which may reflect the absence of a significant difference in pH between RCaNW and RCaW cheeses.

Though the mean fracture strain of all cheeses over the 270-day ripening period was not influenced by curd washing, ANOVA showed that curd washing significantly reduced the mean fracture strain of both the SCaW and RCaW cheeses at 180 and 270 d (data not shown). These findings concur with the observations of Visser, (1991), who reported that the fracture strain of Gouda cheese as a function of pH increased to a maximum at pH values of 5.3-5.4 depending on age, and thereafter decreased on further increasing pH. An increase in pH to values > 5.3 (as in the SCaW and RCaW at times ≥ 180 days) is conducive to a higher ratio of colloidal calcium and phosphate-to-soluble calcium and phosphate, and a higher degree of calcium phosphate-mediated protein aggregation and contraction, which decreases hydrophobic the interactions and is therefore likely to favor a less continuous microstructure (Visser, 1991; Guinee et al, 2000b; Lucey, Johnson, & Horne 2003).

Table 4.7. Degrees of freedom (df) and statistical significances (*P*-values) for changes in rheology properties in control non-washed, and washed-curd, Cheddar cheeses with standard- or reduced-calcium levels[1,2].

Factor	Firmness (N)		Fracture stress (N/cm^2)		Fracture strain (-)	
(Standard calcium)	df	*P*	df	*P*	df	*P*
Main plot						
Curd washing	1	0.0780	1	<.0001	1	0.1235
Sub-plot						
Time	4	<.0001	4	<.0001	4	0.3375
Interaction (Curd washing x time)	4	0.1424	4	0.4605	4	0.0067

Factor	Firmness (N)		Fracture stress (N/cm^2)		Fracture strain (-)	
(Reduced calcium)	df	*P*	df	*P*	df	*P*
Main plot						
Curd washing	1	0.3561	1	0.2115	1	0.8473
Sub-plot						
Time	4	<.0001	4	<.0001	4	0.1522
Interaction (Curd washing x time)	4	0.3600	4	0.2498	4	0.0603

[1]The cheese treatments, described in detail in Materials and Methods, included control, non-washed curd cheeses with standard (SCaNW) or reduced calcium (RCaNW) content, and washed-curd cheese with standard (SCaW) or reduced-calcium (RCaW) content.
[2]Analysis of variance was carried out using a general linear model (GLM) procedure of SAS, where the effects of treatment and replicates were estimated.

4.3.8. Cheese colour

Colour of cheese, as defined by L* (whiteness), a* (redness), b* (yellowness) or the Hue angle, was not affected by curd washing or by calcium content of the cheese (Table 4.8). Similarly, ripening time did not affect the whiteness of any of the cheeses, but significantly increased the redness and reduced the yellowness in all cheeses. These changes coincided with a reduction in moisture content (data not shown) and pH, and an increase in levels of fat and non-protein N (% of total cheese N). Regression analysis of the data (from all ripening times) indicated that levels of both pH4.6-SN and FAA were positively correlated with a* (df = 14; r = 0.63 and 0.58, respectively) and negatively with b* (df = 14; r = 0.56 and 0.56), respectively.

Table 4.8. Degrees of freedom (df) and statistical significances (*P*-values) for changes of colour in control non-washed, and washed-curd, Cheddar cheeses with standard- or reduced-calcium levels[1].

Factor	L*		a*		b*		Hue angle	
(Standard calcium)	df	*P*	df	*P*	df	*P*	df	*P*
Main plot								
Curd washing	1	0.4491	1	0.7190	1	0.0072	1	0.5339
Sub-plot								
Time	3	0.4595	3	<.0001	3	0.0263	3	<.0001
Interaction (Curd washing x time)	3	0.8366	3	0.2904	3	0.0913	3	0.2942

Factor	L*		a*		b*		Hue angle	
(Reduced Ca)	df	*P*	df	*P*	df	*P*	df	*P*
Main plot								
Curd washing	1	0.6977	1	0.1316	1	0.2881	1	0.2215
Sub-plot								
Time	3	0.3948	3	0.0373	3	0.0005	3	0.0447
Interaction (Curd washing x time)	3	0.1958	3	0.8968	3	0.3892	3	0.9221

[1]The cheese treatments, described in detail in Materials and Methods, included control, non-washed curd cheeses with standard (SCaNW) or reduced calcium (RCaNW) content, and washed-curd cheese with standard (SCaW) or reduced-calcium (RCaW) content.

4.3.9. Volatile compounds analysis

Volatile compounds are associated with different odours (Singh, Drake & Cadwallader, 2003), and contribute to cheese flavour to an extent dependent on the concentration and the balance of each of the volatile compounds and non-volatile taste compounds present in the cheese, and the properties of the cheese matrix (Le Quéré, Pierre, Riaublanc & Demaizières, 1998).

In the current study, over 40 compounds, typical of those found in Cheddar cheese (Singh et al., 2003; Carunchia Whetstine, Luck, Drake, Foegeding, Gerard & Barbano, 2007), were identified in the 270-day old cheeses. Thirty four major compounds, consisting of 10 alcohols, 6 ketones, 4 esters, 6 aldehydes, 5 acids and 3 sulphur compounds, were identified in all of the cheeses.

Pinciple Component Analysis (PCA) was undertaken to establish if the different cheeses could be separated by the types and concentrations of volatile compounds; a biplot of the scores of the cheeses and the loadings of the attributes is presented in Figure 4.9. Principal components, PC1 and PC2, accounted for 39 and 36 % of explained variance between the cheeses, respectively. PC1 separated the cheeses on the basis of calcium content, with SCa cheeses scoring negatively and the reduced-calcium cheeses scoring positively. PC2 separated the cheeses on the basis of the wash treatment, with non-washed cheeses scoring higher than the washed-curd cheeses. The SCaNW and SCaW cheeses scored negatively on PC1 and PC2, and had significantly lower concentrations of total aroma compounds than the corresponding RCaNW and RCaW cheeses.

The PCA biplot shows that SCaNW and SCaW cheeses grouped together and associated with volatiles such as dimethyl disulphide, 2-butanone, 2-propanol, 2-methyl-propanal, and ethyl acetate. The four latter compounds are associated with

etheric, pungent, malt, and fruity-sweet odour s in Cheddar cheese (Singh et al., 2003) and are likely to have contributed to the fruity and pungent flavour identified on sensory analysis of the SCaNW and SCaW cheeses (Figure 4.10). The RCaNW cheese were discriminated as a group, and were more associated with alcohols (1-propanol, 2-nonanol, 2-heptanol), ketones (nonanal, heptanal, 2-pentanal), and acids (propa-, octa- and hexanoic acids) (Figure 4.9). These were associated with fruity, pungent, waxy sensory attributes. RCaW cheese, separated as a third grouping, were characterized by volatiles such as 3-methyl-butanal, 2-methyl-1-propanol and 3-methyl-1-butanol, and by flavour attributes including onion, pungent and rancid.

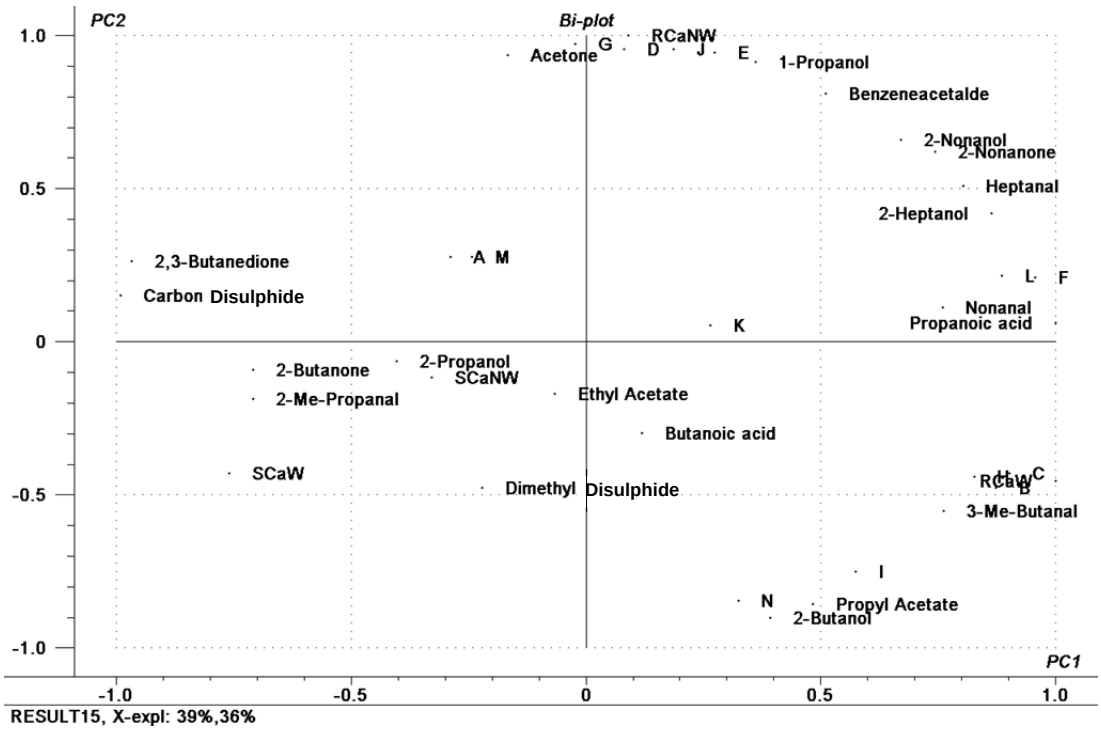

Figure 4.9. PCA showing the first two Principal Components of volatile compounds in 270 day-old control, non-washed curd cheeses with standard (SCaNW) or reduced calcium (RCaNW) content, and washed-curd cheese with standard (SCaW) or reduced-calcium (RCaW) content. Presented values are the means of the three replicate trials. The names of some volatile compounds that were overlapping in the PCA plot are abbreviated as follows to facilitate viewing: A = 1-pentanol; B = Me-1-butanol; C = 2-Me-1-propanol; D = 2,3-butanediol; E =ethanol; F = 2-heptanone; G = 2-pentanone; H = hexanoic acid, ethyl ester; I = octanoic acid, ethyl ester; J = acetic acid; K = hexanoic acid; L = octannic acid; M = methanethiol; N = 2-butanal.

4.3.10. Descriptive sensory analysis

The PCA plot for the different odour and flavour attributes of the 270 day-old cheeses is shown in Figure 4.10. The first two PCs discriminated significantly between the cheeses and accounted for a cumulative explained variance of 80 %. PC1 (46 % of explained variance) and PC2 (34 % of explained variance) separated the cheeses on the basis of the calcium content with the SCaNW and SCaW cheeses scoring negatively and the reduced- calcium cheeses scoring positively on PC2. The cheeses were classified into 3 major groupings by PC1 and PC2: RCaNW and RCaW, SCaNW, and SCaW. The RCaNW and RCaW cheeses had flavours described as pungent, onion, rancid, buttery and caramel, odours as caramel and buttery, and taste as bitter; however, compared to the RCaNW cheese, the flavour and odour of the RCaW cheese was slightly more buttery and caramel flavour. These attributes are consistent with the presence of various volatile compounds (Singh et al., 2003), e.g., pungent (2-butanol, 2-pentanol, 1-propanol) and bitter (benzaldehyde, 2-hexenal). The SCaNW cheeses had flavour and odour described as sweaty, cheesy, fruity, and taste as salty and acid. The SCaW cheeses had a waxy flavour, pungent, 'estery' odour , and a sweet nutty taste. The fruity and pungent odour perceived in both cheeses is consistent with the presence of 2-propanol and ethyl acetate in their volatile fractions (Figure 4.9). Nevertheless, the fact that the SCaW and SCaNW belong to the same PCA grouping for volatile compounds (Figure 4.9), but a different grouping for the corresponding descriptive sensory analyses (Figure 4.10), confirms that sensory perception of cheese at any time is complex, being determined by volatile compounds, taste compounds, texture and their interactions (Szczesniak, 2002).

The results demonstrated that curd washing differentiated the sensory characteristics of the 270-d old SCaNW and SCaW cheeses, with the latter cheeses

having a waxier flavour, a more ‘estery’, less fruity, less sweaty cheesy odour, and nuttier and sweeter taste than the SCaNW cheeses. This finding concurs with previous studies (Shakeel-Ur-Rehman et al., 2004; Hou et al., 2014), who found that washed-curd cheeses had a significantly lower intensity of unclean and acid tastes than the corresponding cheeses from control, or lactose-fortified, milks, probably as a consequence of the lower levels of lactic acid and higher pH of the washed-curd cheeses. In contrast, curd washing was not as effective in discriminating between RCaW and RCaNW cheeses. The discrepancy in the effect of curd washing between the SCaNW and SCaW cheeses on the one hand, and RCaNW and RCaW cheeses on the other may be due to the smaller differences in lactic acid and pH between the RCaNW and RCaW cheeses compared to the SCaNW and SCaW cheeses.

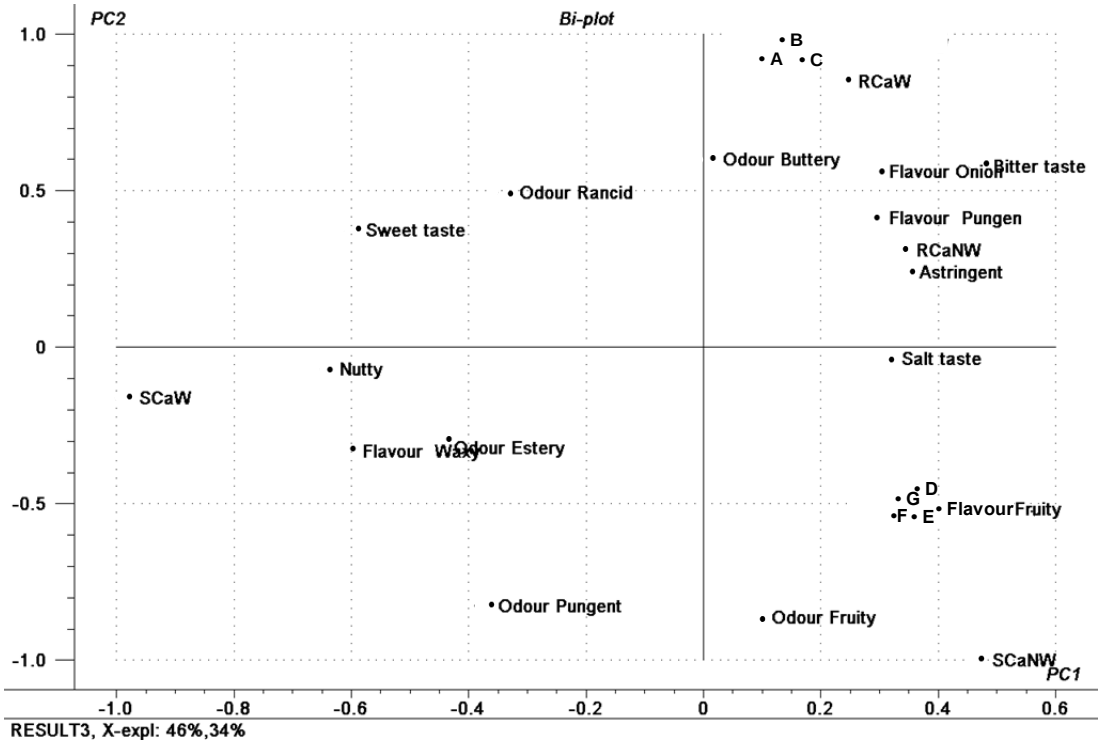

Figure 4.10. PCA showing the first two Principal Components of descriptive sensory odour and flavour attribute in 270 day-old control, non-washed curd cheeses with standard (SCaNW) or reduced calcium (RCaNW) content, and washed-curd cheese with standard (SCaW) or reduced-calcium (RCaW) content. Presented values are the means of the three replicate trials. Abbreviations: A = flavor caramel; B = odor caramel; C = flavor buttery; D = odor sweaty/cheesy; E = acid taste; F = flavour rancid; G = flavor sweaty/cheesy.

Reducing calcium content resulted in discrimination between the washed cheeses (SCaW, RCaW) and to a lesser extent between the non-washed cheeses (SCaNW, RCaNW). The RCaW cheeses had a more buttery, caramel odour and flavour, and a more bitter, less sweet and nutty taste than the SCaW cheeses. This trend is consistent with the lower pH of the RCaW cheese which would favor a lower degree of dissociation of acids such as lactic, glutamic and aspartic to the salt forms, and a corresponding increase in the intensity of acid flavour (Table 4.9) (Neta, Johanningsmeier, Drake & McFeeters, 2009). Moreover, the higher level of primary proteolysis in RCaW, as indicated by the higher level of pH4.6-SN, could also be a contributory factor to its more bitter flavour. ANOVA of PTA-SN and FAA nitrigen,

as percentages of pH 4.6 SN, in the 270-day-old cheeses indicated no difference between the mean values for the RCaW and SCaW cheeses (data not shown), suggesting a possible greater accumulation of medium-to-low molecular weight bitter peptides in the RCaW cheese (Stadhouders & Hup, 1975; Visser, 1977; Exterkate, Alting & Slangen, 1995). The RCaNW cheese had a more pungent and lessfruity flavour, a less fruity odour, a saltier, more bitter and less acid taste, and a more astringent mouthfeel than the SCaNW cheese. This may reflect the non-significantly higher concentration of glutamate, leucine, phenylalanine and lysine, which contribute to acid, bitter and umami flavours in the RCaNW cheeses (Solms, 1969; Nishimura & Kato, 1988).

Table 4.9. Biochemical parameters of 270-day-old, control non-washed, and washed-curd, Cheddar cheeses with standard- or reduced-calcium levels[1,2, 3]

Parameters	SCaNW	SCaW	RCaNW	RCaW
Lactose (g/100 g)	0.00±0.00^{aA}	0.00±0.00^{aA}	0.10±0.07^{aA}	0.00±0.00^{aA}
Lactic acid (g/100 g)	1.31±0.06^{aA}	0.94±0.04^{bA}	1.23±0.08^{aA}	0.89±0.03^{bA}
pH	5.18±0.01^{bA}	5.52±0.05^{aA}	5.17±0.05^{bA}	5.35±0.03^{aA}
pH4.6-SN (g/100 g)	20.09±0.7^{aB}	20.48±0.96^{aB}	22.68±0.61^{aA}	25.08±1.58^{aA}
Total FAA (g/100 g)	0.64±0.00^{aA}	0.73±0.07^{aA}	0.69±0.08^{aA}	0.72±0.08^{aA}
Individual FAA(g/100 g)				
Glutamate	1205±163^{aA}	1470±156^{aA}	1326±115^{aA}	1361±115^{aA}
Leucine	1279±61^{aA}	1221±99^{aB}	1419±115^{aA}	1457±38^{aA}
Phenylalanine	1167±100^{aA}	1469±164^{bA}	1514±249^{aA}	1545±82^{bA}
Lysine	423±45^{aA}	686±115^{aA}	469±122^{aA}	575±85^{aA}
Sum of volatile compounds	3.17E+06^{bA}	4.05E+06^{aA}	4.49E+06^{aA}	3.37E+06^{aA}

[1]The cheese treatments, described in detail in Materials and Methods, included control, non-washed curd cheeses with standard (SCaNW) or reduced calcium (RCaNW) content, and washed-curd cheese with standard (SCaW) or reduced-calcium (RCaW) content.

[2]Analysis of variance (ANOVA) was used to determine differences between treatments.

[3]The statistical effect of washing on the SCa and on the RCa cheeses is indicated by lower case superscripts, while the effect of reducing calcium on the non-washed (SCaNW, RCaNW) and washed (SCaW, RCaW) cheeses is indicated by the upper case superscripts. Values not sharing a common superscript differ significantly, $P < 0.05$.

4.4. Conclusion

Cheddar cheeses were manufacture with standard calcium (SCa, ~ 762 mg/100 g) and reduced calcium (RCa, ~654 or 675 mg/100 g) levels, to reflect those previously found in surveys of retail brands of Cheddar cheese (758 ± 54 mg/100 g) (Guinee, Kilcawley, & Beresford, 2008). Curd washing was used to vary the levels of LLAMc in the SCa and RCa cheeses from ~ 5.3 to 3.9 (g/100 g) moisture, which would correspond to the range of LLAMc in cheese made from a seasonal milk supply from predominantly spring-calved herds, as for example in Ireland, New Zealand and parts of Australia. The levels of lactose plus lactic acid on a weight basis were 1.42 and 0.93, 1.38 and 1.1 (g/100 g) SCa non-washed (SCaNW) and washed (SCaW) cheese, and RCa non-washed (RCaNW) and washed (RCaW) cheeses. While residual lactose was metabolised rapidly to lactic acid in all cheeses during the first 30 days of ripening, significant quantities of unfermented lactose remained in the RCaNW cheese even after ripening for 180 (~ 0.2 g/100 g) or 270 (~ 0.1 g/100 g) days. Curd washing significantly reduced the mean concentration of lactic acid, and increased the mean values of pH and fracture stress of the SCaW cheese over the 270-day ripening period, but did not affect these parameters in the RCaW cheese. Nevertheless, curd washing significantly reduced the lactic acid content and increased the pH of the RCa cheese at 270 days (though not at other ripening times). The 270 day-old RCaW cheese had more buttery and caramel flavour than the corresponding RCaNW cheese, while the SCaW cheese had a waxier flavour, a more ‘estery’, less fruity, less sweaty cheesy odour, and nuttier and sweeter taste than the corresponding SCaNW cheese. Otherwise, curd washing did not significantly affect gross composition, microbiology, proteolysis, or color.

Reducing the calcium level in the cheese from ~ 762 to 665 (mg/100 g) significantly increased the levels of pH4.6-SN at most ripening times in the non-washed cheese (RCaNW), but only at day 270 in the washed-curd cheese (RCaW). Descriptive sensory analysis of the 270 day-old cheeses indicated that RCaNW and RCaW cheeses had more pungent, onion, rancid, buttery and caramel flavours, more caramel and buttery odours, and a more bitter taste than the corresponding SCaNW and SCaW cheeses. Otherwise, calcium reduction had little effect on Cheddar cheese characteristics.

This study demonstrated that Cheddar cheeses with relatively low calcium levels (~ 665 mg/100 g) had significant quantities of residual lactose, even after advanced ripening times of 180-270 days. The higher residual lactose levels in the reduced-calcium cheeses coincided with a non-significantly higher level of S/M, possibly because of a possible structural alteration to the curd at salting which enhanced salt uptake. Residual lactose may be undesirable to those predisposed to lactose intolerance which is a metabolic disorder and affects large numbers of the world population. The exclusion of cheese (a high protein, calcium-rich food) from the diet in such populations could have nutritional and economic consequences. The current study, however, showed that curd washing is a very effective means of reducing and controlling the levels of residual lactose in Cheddar cheeses with different calcium levels. Apart from the latter effect, curd washing is an effective tool to reduce residual lactose in Cheddar cheese, and to differentiate the sensory properties of aged (270 day-old) Cheddar cheese to an extent that varies with calcium content. Moreover, it may be employed as a method of reducing both the calcium and unfermented lactose content in Cheddar cheese within the seasonal variations may be

applied during Cheddar cheese manufacture as a means to control losses during manufacture and achieve more consistent yields.

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Chapter 5: Effect of curd washing on the quality of full-fat Cheddar cheeses made from control or protein-standardized milk

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Abstract

Cheese milk was standardised to 3.3 % (w/w) or 4.0 % (w/w) protein by ultrafiltration and the resulting curds were washed to varying degrees during Cheddar cheese manufacture (by partial replacement of whey with water at the early stages of cooking) to give target levels of lactose plus lactic acid in cheese moisture ($LLAM_C$) of 5.2 (control), 4.2 and 3.9 % (w/w) in both standardized milk(SM) and protein-fortified (PFM) milk cheeses. The six cheese types were denoted as 5.2LLAMcSM, 4.2LLAMcSM, 3.9LLAMcSM and 5.2LLAMcPFM, 4.2LLAMcPFM, 3.9LLAMcPFM. The cheeses were made in triplicate and matured at 8 °C for 270 days and analysed for composition and changes during maturation. Curd washing significantly reduced the mean level of residual lactose, lactic acid, total sugars in cheese and protein and fat in bulk whey and protein loss in bulk whey. Curd washing also increased the fat loss in bulk whey and levels of free amino acids in cheese. The mean cheese pH over the 270-day ripening period increased significantly with extent of curd washing. Otherwise, curd washing did not significantly affect gross composition, microbiology, proteolysis or texture of cheese. As expected, increasing the milk protein levels from 3.3 % (w/w) to 4.0 % (w/w) significantly increased the levels of protein and fat in standardised cheese milk, actual cheese yield, cheese firmness and fracture stress, while decreased the cheese moisture and pH4.6-SN at ≥ 90 days. Descriptive sensory analysis on 270 day-old washed curd cheeses showed to have ‘fruity’, ‘buttery’ and ‘sweet’ aroma while standarized non-washed cheeses (5.2LLAMcSM and 5.2LLAMcPFM) had ‘rancid’ and ‘farmyard’ aroma. When higher levels of milk protein were used in cheesemilk, cheeses were more fruity, buttery and caramel than the corresponding low protein milk cheeses. This study indicated that curd washing significantly reduced the residual sugars, but increased the

cheese pH and FAA during ripening which also contributed to cheese flavours such as ‘fruity’, ‘buttery’ and ‘sweet’. Increasing the milk protein levels significantly increased cheese yields, firmness and fracture stress, while decreased the cheese moisture (unless corrective steps taken) and pH4.6-SN at ≥ 90 days and indicated that high milk protein cheeses had fruity, buttery and caramel flavours and odours.

5.1. Introduction

Consistent cheese quality is important for cheese manufacturers and consumers. Thus cheese quality needs to be kept as uniform as possible. The single most important factor affecting cheese quality is milk composition, especially its lactose and protein content. However, there are several factors which contribute to the milk composition including the stage of lactation (which alters udder physiology and metabolism), plan of nutrition (especially for cows fed on pasture), somatic cell count, and season (Guinee, O'Kennedy & Kelly, 2006). Seasonal changes in milk composition (lactose, protein and fat) and quality, especially in late lactation have a major impact on curd forming properties, cheese yield and composition of the resultant cheeses (O'Keeffe, 1984; Banks and Tamime, 1987; O'Brien, Mehra, Connolly, & Harrington, 1999), and are thus conducive to inconsistencies in cheese quality (Lawrence, Heap & Gilles, 2004). Those effects in milk composition and their impact on cheesemaking can be reduced through several ways such as optimization of dairy husbandry practices, calving patterns, diet management, and cheesemaking procedures (e.g., applying curd washing, the standardization of protein to fat ratio etc.).

Curd washing is used to control the ratio of lactose to lactic acid in the resultant cheese and hence cheese pH. It is practiced in the manufacture of some cheese varieties, for example in brine-salted, Dutch-style cheeses such as Edam, Gouda and Maasdammer, to control the level of lactose and lactic acid and properties of the final cheeses (Hou, McSweeney, Beresford, & Guinee,2014b). This involves reducing the level of residual lactose in the cheese curd prior to salting, by diluting the whey during the cooking/stirring phase of manufacture by removal of whey and adding water. The proportions of whey removed and water added are varied according to the lactose level in the milk which varies with stage of lactation (O'Brien et al., 1999). Curd washing

is not conventionally applied in the manufacture of Cheddar cheeses, in which dry salt is added to the milled curd at pH 5.2-5.4, prior to moulding and pressing which greatly slows down the metabolism of residual lactose, and thereby prevents the pH from decreasing excessively. However, the pH of commercially produced Cheddar cheese varies significantly (Guinee et al., 2008), which may be due to a number of factors such as acidification rate, pH at rennet addition and whey drainage, pH at salting, level of lactose in the cheese milk, salt sensitivity of the starter culture, moisture content of the curd, and salt content (Czulak et al., 1969; O'Brien et al., 1999). Moreover, diluting the whey with wash water to varying degrees, reduces the levels of residual lactose and lactic acid in Cheddar cheese, and increases the pH (e.g., by up to 0.1- 0.3 units when wash water was added at a rate of 33 g per 100 g cheese milk) after ripening times of ≥ 90 day (Huffman & Kristoffersen, 1984; Shakeel-Ur-Rehman, Waldron, & Fox, 2004). Hence, washing the curd during the manufacture of Cheddar has been investigated as a means of achieving more consistent levels of lactose and lactic acid, and flavour of the final cheese (Shakeel-Ur-Rehman et al., 2004; Hou et al., 2014a). In a previous study, Huffman and Kristoffersen, (1984) investigated the effect of altering the lactose content of Cheddar cheese, by adding lactose to the curd-whey mixture (high lactose, HL) and by curd washing (replacing whey with simulated milk ultrafiltrate, LL). Lower levels of lactose at day one led to lower levels of lactate and higher cheese pH in the 9 month-old cheese. In addition, flavour developed more slowly in the LL cheeses and the cheeses were less sharp. Similarly, Shakeel-Ur-Rehman, et al, (2004) also found that the pH of HL cheese decreased significantly during maturation from ~ 5.3 at day one to 4.8 at day 180, where as LL cheese pH remained relatively constant at ~ 5.3 to 5.4. Curd washing had little effect on the levels of primary proteolysis; however, the HL cheese had higher levels of total free amino

acids and was found to have a harsh flavour and a crumbly body. Moreover, Hou, McSweeney, Beresford, & Guinee, (2012; 2014a) also used curd washing by replacing 33 % of whwy with equal quantiy of water for Cheddar cheese and found that it significantly reduced mean levels of total lactate and increased the mean cheese pH by 0.3 to 0.4 units at ripening times greater than 90 days. Overall, washed-curd cheeses were firmer and less brittle, had lower levels of volatile compounds, and were less acid, more buttery, sweeter, saltier and creamier than non-washed cheeses.

The seasonal variation in milk protein levels has a significant influence on curd forming properties and cheesemaking characteristics. For practical reasons, in modern dairy plants, the curd cutting program is set based on time rather than on gel firmness or gel firming rate. Hence, standardization of the milk protein level to a target value across the cheese-making season (with constant casein to whey ratio) would provide a very effective means of minimizing the effects of seasonal related variations in milk composition on cheese composition, quality and manufacturing efficiency. Moreover, standardization of milk protein to higher than normal levels offers the advantage of increasing plant output without capital expenditure on extra cheese vats (Guinee et al., 2006). Hence, the use of membrane processes such as ultrafiltration (UF) to standardise the milk protein level for cheesemaking has been introduced and investigated extensively (IDF, 1994; Guinee, O'Callaghan, Mulholland, & Harrington,1996). Nevertheless, in a previous study, Broome, Tan, Alexander, & Manser, (1998) increased the milk protein level to 4.0 % and 4.5 % (w/w) for cheese manufacture and found that the moisture in non-fat substances (MNFS) was reduced effectively which resulted in a better quality Cheddar cheese (Lawrence, Gilles, Creamer, Crow, Heap, Honoré, Johnston, & Samal, 2004).

Even though altering the milk protein level in cheesemaking and using curd washing to achieve a target residual lactose in cheese have been studied previously, there is little information on the combined effect of using curd washing and UF to concentrate milk protein to standardise cheese milk composition on Cheddar cheese quality over the seasonal variations. Hence, the objective of the current study was to compare the effect of applying different levels of curd washing to cheeses made with standardized milk (3.3 %, w/w) and protein-fortified milk (4 %, w/w) on the composition, sugar metabolism, pH, microbiology, proteolysis, rheology, volatile compounds and sensory properties of Cheddar cheeses.

5.2. Materials and methods

5.2.1. Preparation of cheese milks

Holstein-Friesian cows' milk (3000 kg), with a 3.25 % (w/w) protein level, was obtained from a spring-calving herd (Moorepark, Co. Cork, Ireland) in April 2010. The milk was separated at 55 °C and the resultant cream and skim milk were cooled to 4 °C. Half of the resultant skim milk was passed through ultrafiltration (UF) process with a Pasilac plact and frame unit (Model 36, APV Pasilac AS, DK-85600 Silkeborg, Denmark) fitted with GR61PP polysulphone membranes having a surface area of 9 m^2 and a molecular mass cur-off of 20 kDa. to concentrate the protein to 4.0 % (w/w). Then the retentate was standardised by mixing with cream to a protein to fat ratio of 0.96:1. The other part of untreated skim milk was also standardized with cream to a protein to fat ratio of 0.96:1. Both standardised milks were stored overnight at 8 °C, pasteurized at 72 °C for 15 s, cooled to 31 °C, and pumped to 500 L cheese vats of which three vats contained untreated milk and the other three vats were filled with UF milk.

5.2.2. Cheese manufacture and treatments

Defined strain starter cultures, *Lactococcus lactis* subsp. *cremoris* strain 227 and 303, (Chr. Hansen Ireland Ltd., Rohan Industrial Estate, Little Island, Co. Cork, Ireland) were both grown overnight at 24 °C in reconstituted 10 % (w/v), antibiotic-free skim milk powder solution (Golden Vale Food Products Ltd., Charleville, Co. Cork, Ireland) which had been heat treated at 95 °C for 30 min. When the pH of the inoculated milk reached between pH 4.5 to 5.0, the cultures were cooled and stored at 4 °C until required for cheesemaking (~1 day). Pasteurised milk (31 °C) was inoculated with *Lactococcus lactis* subsp. *lactis* strain 227 and 303 cultures at a level of 0.75 % (w/w) for each culture. After a 30 min ripening period at 31 °C, rennet (Chymax Plus, Chr. Hansen Ireland Ltd.) was diluted 1:10 in de-ionized water, and added to the milk at a level of 0.18 mL kg^{-1} based on a protein level of 3.3 % (w/w). Following rennet addition, the mixing was stopped for gelation and the gel was cut at a firmness of 54 Pa. Then the curd-whey mixture was stirred continuously, and the cooking was started at a rate of 0.2 °C min^{-1} to 34 °C. The stirring was stopped and different quantities of whey were withdrawn from two vats with the untreated standardised milk and two vats containing the UF milk retentate, and filtered through a nylon mesh (to remove any curd particles/fines, which were returned to the cheese vat), and replaced by equivalent amounts of pasteurised (80 °C for ~ 5 min) reverse osmosis-treated water at 34 °C. To give target levels of lactose and lactic acid in cheese moisture (LLAMc) in the curd/cheese moisture of 5.3, 4.3 and 3.9 % (w/w), the levels of whey removed from the untreated standardised milk were 0 (Control, 5.2LLAMcSM), 23.5 (4.3LLAMcSM) and 28.7 (3.9 LLAMcSM) L 100 kg^{-1} cheese milk and the amount of whey removed from UF milk was 0 (Control, 5.2LLAMcPFM),

25.0 (4.3LLAMcPFM) and 30.1 (3.9 LLAMcPFM) L 100 kg^{-1} cheese milk, calculated based on the in-vat milk lactose content. After curd washing, the curd-whey mixture was further cooked to 38.5 °C and the whey (control) or diluted whey (washed curd) was drained from the curd when the pH of the curd reached 6.15.

The curds were cheddared, milled at pH 5.35, salted at 2.7 % (w/w), mellowed for 20 min, placed in rectangular moulds (23 kg), and pre-pressed at 0.13 kPa for 30 min, and then pressed overnight at 2.5 kPa in horizontal press. Cheeses were vacuum packed and stored at 4 °C for 14 days and at 8 °C thereafter. The cheeses were coded as 5.3LLAMcSM, 4.3LLAMcSM and 3.9LLAMcSM for the cheeses made with untreated standardised (control) milk and 3LLAMcPFM, 4.3LLAMcPFM and 3.9LLAMcPFM for the cheeses made with protein-standardised milk. Cheesemaking trials were carried out in triplicate (trials 1-3) over a four-week period from April 28 to May 21, 2010.

5.2.3. Composition of milk and whey during manufacture

Cheese milk samples taken ex-vat at the beginning of cheese manufacture, while whey samples were taken immediately before whey drainage (bulk whey), and after pressing (white whey). Milk samples (100 g) were analysed through a Milkoscan (Foss, Warrington, Cheshire, UK) for their lactose, protein and fat contents. Milk samples were also analysed for their casein number and non-protein nitrogen levels (NPN) using Kjeldahl method.

The whey samples (~ 100 g) were taken directly from the cheese vats, sieved (1 mm), placed in a 30 mL universal sterile container (Ramboldi Ltd, Limassol, Cyprus), stored at 4 °C to prevent further microbial growth and lactose degradation.

Samples of whey or whey-wash water mixtures were then analysed for fat and protein within two days.

5.2.4. Sampling of cheese

Cheeses samples were taken at different ripening times (1, 14, 30, 90, 180 and 270 days) over the 270-day period. A block of sample (~200 g) was taken for compositional analysis which were described in the previous study (Hou et al., 2012). The samples for microbiological analysis (starter bacteria and non-starter lactic acid bacteria, NSLAB) were taken aseptically at the different stages of ripening using a sterile cheese trier and placing the resultant sample (~ 10 g) in a sterile stomacher bag in duplicate. A block of cheese (~ 2000 g) was taken for texture, proteolysis, volatile compounds and descriptive sensory analysis.

5.2.5. Composition analysis of milk, whey and cheese yields

Ex-vat milk and whey samples were analysed for fat and protein as described below. Lactose, glucose and galactose were measured using high performance liquid chromatography (HPLC) method as described by Zeppa, Conterno, & Gerbi, (2001).

Cheese milk, refers to pasteurized milk with added starter culture and, was analyzed for fat (IDF, 1996), total nitrogen (IDF, 1993), and casein (CN) (IDF, 1964). As the weight fraction of pasteurized milk and starter inoculua were the same by using the bulk culture, the protein content in the milk was calculated using the following formula (Fenelon and Guinee, 1999):

$$Pcm = (Ppm \times 0.985) + (Psi \times 0.015)$$

Where Pcm, Ppm, and Psi are correspond to protein contents of the milk, pasteurized milk, and starter inocula, respectively. Similarly, the fat content of the milk was calculated using the formula:

Fcm = (Fpm × 0. 985) + (Fsi × 0.015)

Where Fm, Fpm, and Fsi are correspond to fat contents of the milk, pasteurized milk, and starter inocula, respectively.

The percentage of milk fat lost in whey streams (% FLW) (e.g., bulk whey) and the percentage fat recovered in to cheese (% FRC) were calculated on the basis of the fat content of cheese milk, whey and cheese as described by Guinee et al. (2006). The % FLW during the manufacture of washed-curd cheese was calculated by the following formula:

$$\%\,FLW = \frac{100\ x\left(weight\ of\ \ fat\ in\ whey\ 1 + weight\ of\ \ fat\ in\ whey\ 2\right)}{\left(weight\ of\ \ cheese\ milk\ x\ \%, w/w,\ \ fat\ in\ cheese\ milk\right)}$$

Where whey 1 refers to undiluted whey and whey 2 refers to diluted whey.

The percentages of milk protein lost to whey and recovered in cheese were calculated using similar formulas.

The actual cheese yield, Y_a, was determined from the weights of cheese milk and weight of pressed cheese, and expressed as kg per 100 kg of cheese milk (Guinee et al., 2006). Cheese yields were also expressed in a number of different formats, which have been described previously (Guinee et al., 2006) which refers to Y_{afpam}, yield per 100 kg of milk normalized to reference fat (3.4 %, w/w) and protein (3.3 %, w/w) levels; Y_{ma}, moisture-adjusted yield; Y_{mafpam}, moisture-adjusted (38.5 %, w/w) yield per 100 kg of milk normalized to reference fat and protein levels; Y_{afcam}, yield per 100 kg of milk normalized to reference fat (3.4 %, w/w) and casein (2.53 %, w/w)

levels; and Y_{mafcam}, moisture-adjusted yield per 100 kg of milk normalized to reference fat and casein levels.

5.2.6. Cheese compositions

Finely grated cheese samples were analysed for moisture, protein, fat, NaCl, ash, Ca, P and pH at 14 days using IDF standard methods (Guinee, Auty, & Fenelon, , 2000).

5.2.7. Microbial counts in cheese

Following sampling, the counts of starter lactococci and NSLAB were determined on selective media (LM17 and LBS agar) as described in a previous study (Hou et al., 2012).

5.2.8. Sugars and lactates in cheese

Five grams of finely grated cheese samples was stored at -20 °C for the further sugar analysis. The samples were thawed, 25 mL of 0.013 N sulphuric acid was added, macerated for 10 min and then centrifuged at 7,000 g at 4 °C for 5 min and the supernatant was filtered through a 0.2 µm nylon syringe filters (Lab Unlimited, Whitestown, Dublin 4, Ireland) into a 1.5 mL clear screw neck glass HPLC vial (APEX Scientific, Maynooth Business Campus, Maynooth, Co. Kildare, Ireland). The concentration of residual lactose, glucose and galactose were detected using a HPLC method under the conditions as described by Hou et al. (2014b).

D (-) - and L (+)-lactate were also measured by HPLC method using the same extraction method as described by Hou et al. (2014b).

Total lactate was calculated as the sum of D (-)- and L (+)-lactate contents, and total sugars and derived acids as the sum of lactose, galactose, glucose and total lactate. Each analysis was carried out in duplicate.

The predicted level of LLAMc in cheese was calculated based on lactose levels in milk and on volumes of whey removed and wash water added, using the formula described by Hou et al. (2012).

5.2.9. Proteolysis

Cheese samples (60 g) were taken periodically during ripening for analysis of primary and secondary proteolysis. Primary proteolysis was measured by pH 4.6-soluble nitrogen (pH4.6-SN) and secondary proteolysis was measured by 5 % (w/v) phosphotungstic acid-soluble cheese nitrogen (PTA-SN) and free amino acids (FAA), both methods were as described in our previous study (Hou et al., 2014a). The nitrogen contents of the pH 4.6-SN and PTA-SN were determined in duplicate by the macro-Kjeldahl method (IDF, 1993) to obtain the levels of primary and secondary proteolysis.

5.2.10. Rheology

Texture samples were taken on day 14, 30, 60, 90, 180 and 270. Cheese samples were cut to six 25 x 25 x 25 mm cubes by using cheese cutter (Cheese Blocker; Bos Kaasgreedschap, Bodengraven, Netherlands) and stored at 4 °C overnight before analysis to obtain the equilibrium temperature. The compression test was carried out by using a 5 mm compression plate and a 100 kg load cell, on a texture analyzer (model TA-HDI, Stable Micro Systems, Godalming, UK) as described in Rynne, Beresford, Kelly, & Guinee, (2004). The rheological parameters measured were the Young's Modulus and the true stress, true strain. Texture profile analysis parameters frimness

(N, force at maximum compression on the first bite), fracture stress (kPa, force per unit area at the point of fracture on the first bite) and fracture strain (dimensionless, as the strain corresponding to the minimum slope on the force-displacement curve) were calculated as described previously (Bourne, 1978).

5.2.11. Volatile compounds

The volatile compounds of each cheese were analysed at day 270 of ripening. Five grams grated sample of each cheese was added to a 20 mL SPME vial (Apex Scientific Ltd., Maynooth, Co. Kildare, Ireland) and was analysed by solid phase microextraction coupled to gas chromatography-mass spectrometry (SPME GC-MS) under the condition of which was described in a previous study (Hou et al., 2014a).

5.2.12. Descriptive sensory analysis

Descriptive sensory analysis was carried out on the 270-day cheeses from each trial. Sensory testing was conducted at University College Cork by a trained panel who were experienced in the sensory analysis of Irish Cheddar cheese, as described by Hou et al. (2014a). In this study, the sensory attributes determined included 9 odours, 12 flavours and 5 tastes. The sensory data obtained from each individual sensory assessor for the different treated cheeses were averaged and the means of the triplicate trials were obtained from these averages.

5.2.13. Statistical analysis

Three replicate cheesemaking trials were undertaken in which the cheeses were manufactured from milk with two different protein levels (3.3 and 4.0 %, w/w) and washed to a lactose plus lactic acid in cheese moisture phase at 5.3, 4.3 and 3.9 %

(w/w), respectively. Compositional data and individual volatile compounds were carried out by using a randomised complete block design that incorporated the 3 treatments (curd washing levels), 2 milk protein levels and 3 blocks (replicate trials) was used for the analysis of response variables. Analysis of variance (ANOVA) was carried out on the data using the general linear model procedure of SAS (SAS® version 9.1.2) (SAS Institute, 2004). Tukey's multiple-comparison test was used for pair comparisons the means of treatments and the level of significance was determined at $P < 0.05$.

A split-plot design was used to monitor the effects of treatments (milk protein levels and curd washing levels) and ripening time and their interaction on the response variables measured at regular intervals during ripening (such as sugars, pH, microbial counts and proteolysis). The split-plot design was carried out using SAS (SAS Institute, 2004), and significance of differences ($P < 0.05$) was determined by Fisher's least significant difference test, as described by Hou et al., (2012).

The data for volatile compounds and descriptive sensory analysis were measured at day 270 only, and the mean scores for the different volatile compounds and sensory attributes were standardised (1/standard deviation of the mean score for each attribute) and analysed using principal component analysis (PCA) by Unscrambler V 6.1 (CAMO AS, N-7041 Trondheim, Norway). The results were presented as principal component (PC) plots.

5.3. Results and discussion

5.3.1. Milk composition

The mean composition % (w/w) of raw milk were protein 3.39 (±0.11), fat 3.99 (±0.22), lactose 4.82 (±0.02), respectively, which are typical for the commercial Irish

milks in April and May (Mehra, O'Brien, Connolly, & Harrington, 1999; O'Brien et al., 1999).

The composition of standardized pasteurised milks and of cheese milks calculated to include bulk starter are presented in Table 5.1. For the both experimental series, increasing the milk protein levels from 3.3 % (w/w) to 4.0 % (w/w) significantly increased the milk fat content from 3.5 % (w/w) to 4.25 % (w/w) which is due to the standardization of the protein-to-fat ratio by mixing skim milk and cream and a reduction in the level of non-protein nitrogen (NPN), due to the dilution effect of added protein and also being removed from during UF process (Guinee et al., 2006). The casein numbers and lactose were not affected by the milk protein levels; however, the casein numbers in the protein-fortifiedmilk were generally 0.5 to 0.6 % (w/w) higher than the standarized milk (data not shown). The fat content in cheese milk was numerically lower than in the corresponding pasteurised milk which may due to the addition of bulk starter culture made from reconstituted skim milk powder.

Despite the statically significant difference in fat, protein, casein numbers and lactose that was found in both ex-vat pasteurised milk and cheese milk, the differences was small (<0.1 %, w/w) so was not considered likely to affect cheese parameters.

Table 5.1. Composition of pasteurised milks and cheese milks used to make Cheddar cheeses with different milk protein levels and target levels of lactose plus lactic acids in cheese moisture

	Target level of lactose for Standarized and Protein-fortified cheese milks[1]					
	Standarized milks cheese			Protein-fortified milks cheese		
Milk composition	5.2LLAMcSM	4.2LLAMcSM	3.9LLAMcSM	5.2LLAMcPFM	4.2LLAMcPFM	3.9LLAMcPFM
Pasteurized milk						
Fpm (% w/w)[3]	$3.50^{a,B}$	$3.45^{a,B}$	$3.47^{a,B}$	$4.22^{b,A}$	$4.25^{ab,A}$	$4.27^{a,A}$
Ppm (% w/w)[3]	$3.27^{a,B}$	$3.28^{a,B}$	$3.30^{a,B}$	$3.96^{a,A}$	$3.98^{a,A}$	$4.00^{a,A}$
Cpm (% w/w)[3]	$77.92^{a,A}$	$77.90^{a,A}$	$76.65^{b,A}$	$78.52^{b,A}$	$78.62^{ab,A}$	$78.87^{a,A}$
Lpm (% w/w)[3]	$4.81^{a,A}$	$4.84^{a,A}$	$4.89^{a,A}$	$4.85^{b,A}$	$4.90^{ab,A}$	$4.91^{a,A}$
Cheese milk[2]						
Fcm (% w/w)[3]	$3.45^{a,B}$	$3.40^{a,B}$	$3.42^{a,B}$	$4.16^{b,A}$	$4.19^{ab,A}$	$4.20^{a,A}$
Pcm (% w/w)[3]	$3.27^{a,B}$	$3.28^{a,B}$	$3.30^{a,B}$	$3.95^{a,A}$	$3.97^{a,A}$	$3.99^{a,A}$
Ccm (% w/w)[3]	$77.92^{a,A}$	$77.90^{a,A}$	$76.65^{b,A}$	$78.52^{b,A}$	$78.62^{ab,A}$	$78.87^{a,A}$
Lcm (% w/w)[3]	$4.82^{a,A}$	$4.85^{a,A}$	$4.89^{a,A}$	$4.86^{b,A}$	$4.90^{ab,A}$	$4.91^{a,A}$

[1]Analysis of variance (ANOVA) was used to determine differences between treatment milks. The statistical effect of curd washing on cheeses made with standarized milk (5.2LLAMcSM, 4.2LLAMcSM, 3.9LLAMcSM) or protein-fortified milk (5.2LLAMcPFM, 4.2LLAMcPFM, 3.9LLAMcPFM) is indicated by lower case superscripts, while the effect of milk protein level on cheeses made with no curd washing (5.2LLAMcSM, 5.2LLAMcPFM), with curd washing to 4.2 LLAMc (4.2LLAMcSM, 4.2LLAMcPFM) and 3.9 LLAMc (3.9LLAMcSM, 3.9LLAMcPFM) is indicated by the upper case superscripts. Values not sharing a common superscript differ significantly, $P < 0.05$.

[2]Cheese milk refers to pasteurized milk with bulk starter culture; the levels of fat, protein and casein number in the cheesemilk were estimated pro-rata from the compositions of the pasteurised milk and starter culture. Protein calculation factor used was total nitrogen x 6.38.

[3]Abbreviations: Fpm, fat content in pasteurized milk; Ppm, protein content in pasteurized milk; Cpm, Casein number (casein expressed as percentage of total protein) in pasteurized milk; Lpm, lactose content in pasteurized milk; Fcm, fat content in cheese milk; Pcm, protein content in cheese milk; Ccm, Casein number in cheese milk; Lcm, lactose content in cheese milk.

5.3.2. Cheese composition

The day 14 gross composition of all cheeses complied with that for Cheddar cheese as specified by US legislation (Table 5.2) (CFR, 2008). Curd washing significantly reduced residual lactose, total sugars, total sugar to protein and sugar in moisture (SIM) in all cheeses and also total lactate and fat in dry matter (FDM) in the protein-fortified milk cheeses at day 14. Application of curd washing to cheeses made of both standarized and protein-fortified milk significantly reduced the residual lactose concentration in cheese at day 14 from ~0.37 % (w/w) for the control cheeses (5.3LLAMcSM and 5.3LLAMcPFM) to 0.1 % (w/w) for the most highly washed cheeses (3.9LLAMcSM and 3.9LLAMcPFM). The reduction in the concentrations of lactose and lactate is consistent with the results of previous studies (Shakeel-Ur-Rehman et al., 2004;Hou et al., 2012). Otherwise, curd washing did not significantly affect the levels of moisture, fat, protein, salt-in moisture, ash, and moisture in non-fat substances (MNFS) which is in agreement with the results of previous studies (Huffman and Kristoffersen, 1984; Shakeel-Ur-Rehman et al., 2004; Hou et al., 2012).

In agreement with previous studies (Bush, Caroutte, Amundson, & Olson,1983; Kealey and Kosikowski, 1985; Guinee, Pudja, & Mulholland,1994; Broome et al., 1998; Guinee et al., 2006), increasing milk protein content from 3.3 to 3.6 or 4.0% (w/w) resulted in significant decreases in the levels of moisture by ~ 1.6 % (w/w) and MNFS in control unwashed cheeses. This decrease in cheese moisture may be due to the concomitant increase in the ratio of casein to soluble salts with the increase of milk protein which leads to a rapid aggregation of *para*-casein and also the higher number and volume fraction of curd particles in the curd/whey mixture results in a higher collision between the curd particles, vat knives and walls consequently the moisture is reduced (Guinee et al., 2006). In contrast to moisture, the fat levels in cheese increased

numerically, but not significantly, as the milk protein increased which is consistent with findings from previous studies (Fenelon and Guinee, 1999; Guinee et al., 2006) who indicated that the fat globules may prevent the whey exuding from the *para*-casein matrix. As a result, the decrease in MNFS will reflect the moisture associated with protein matrix and lower in its magnitude compared to the percentage reduction in moisture at higher milk protein levels. Otherwise, altering milk protein levels from 3.3 % (w/w) to 4 % (w/w) did not have effects on other parameters of cheese gross composition.

Table 5.2. Effect of curd washing and different milk protein levels on the composition and pH of full-fat Cheddar cheese[1,2]

	Target level of lactose for Standarized and Protein-fortified milk cheese[3]					
	Standarized milk cheese			Protein-fortified milk cheese		
Cheese composition	5.2LLAMcSM	4.2LLAMcSM	3.9LLAMcSM	5.2LLAMcPFM	4.2LLAMcPFM	3.9LLAMcPFM
Moisture (%, w/w)	$38.03^{a,A}$	$37.95^{a,A}$	$37.78^{a,A}$	$36.13^{a,B}$	$36.26^{a,B}$	$36.47^{a,A}$
Fat (%, w/w)	$30.73^{a,A}$	$30.99^{a,A}$	$31.04^{a,A}$	$31.98^{a,A}$	$32.25^{a,A}$	$32.22^{a,A}$
Protein (%, w/w)	$25.45^{a,A}$	$25.69^{a,A}$	$24.91^{a,A}$	$25.63^{a,A}$	$25.21^{a,A}$	$24.53^{a,A}$
Lactose (%, w/w)	$0.35^{a,A}$	$0.13^{b,A}$	$0.13^{b,A}$	$0.41^{a,A}$	$0.07^{b,A}$	$0.08^{b,A}$
Total lactate (%, w/w)	$0.99^{a,A}$	$0.97^{a,A}$	$0.94^{a,A}$	$1.19^{a,A}$	$1.00^{ab,A}$	$0.93^{b,A}$
Total sugars (%, w/w)	$1.50^{a,A}$	$1.24^{b,A}$	$1.22^{b,A}$	$1.79^{a,A}$	$1.22^{b,A}$	$1.15^{b,A}$
Lactate/Protein (%, w/w)	$3.86^{a,A}$	$3.77^{a,A}$	$3.72^{a,A}$	$4.61^{a,A}$	$3.90^{a,A}$	$3.71^{a,a}$
Tsugars/Protein (%, w/w)[4]	$5.89^{a,A}$	$4.86^{b,A}$	$4.83^{b,A}$	$6.90^{a,A}$	$4.80^{b,A}$	$4.64^{b,a}$
Salt (%, w/w)	$1.63^{a,A}$	$1.72^{a,A}$	$1.80^{a,A}$	$1.72^{a,A}$	$1.71^{a,A}$	$1.69^{a,A}$
Ash (%, w/w)	$4.07^{a,A}$	$3.92^{a,A}$	$4.08^{a,A}$	$4.13^{a,A}$	$4.01^{a,A}$	$3.99^{a,A}$
Ca (mg 100 g^{-1})	$732^{a,A}$	$731^{a,A}$	$727^{a,B}$	$790^{a,A}$	$789^{a,A}$	$775^{a,A}$
P (mg 100g^{-1})	$470.8^{a,A}$	$464.9^{a,A}$	$458.5^{a,A}$	$497.9^{a,A}$	$493.1^{ab,A}$	$475.8^{b,A}$
S/M (%, w/w)[4]	$4.30^{a,A}$	$4.54^{a,A}$	$4.76^{a,A}$	$4.77^{a,A}$	$4.73^{a,A}$	$4.63^{a,A}$
MNFS (%, w/w)[4]	$54.89^{a,A}$	$54.99^{a,A}$	$54.78^{a,A}$	$53.12^{a,B}$	$53.52^{a,A}$	$53.80^{a,A}$
FDM (%, w/w)[4]	$49.58^{a,A}$	$49.94^{a,A}$	$49.88^{a,A}$	$50.07^{b,A}$	$50.59^{ab,A}$	$50.71^{a,A}$
SIM (%, w/w)[4]	$4.94^{a,A}$	$3.28^{b,A}$	$3.22^{b,A}$	$4.95^{a,A}$	$3.38^{b,A}$	$3.17^{b,a}$
pH at day14	$5.26^{a,A}$	$5.30^{a,A}$	$5.36^{a,A}$	$5.36^{a,A}$	$5.36^{a,A}$	$5.39^{a,A}$

[1]Milk protein was concentrated by ultrafiltration; Lactose content was adjusted curd-washing at cooking process to 34 °C; results are presented as the mean values of three replicate trials.

[2]The compositions and pH were measured at day 14.

[3]Analysis of variance (ANOVA) was used to determine differences between treatment cheeses. The statistical effect of curd washing on cheeses made with a standarized milk (5.2LLAMcSM, 4.2LLAMcSM, 3.9LLAMcSM) or Protein-fortified milk (5.2LLAMcPFM, 4.2LLAMcPFM, 3.9LLAMcPFM) is indicated by lower case superscripts, while the effect of milk protein level on cheeses made with no curd washing (5.2LLAMcSM, 5.2LLAMcPFM), with curd washing to 4.2 LLAMc (4.2LLAMcSM, 4.2LLAMcPFM) and 3.9 LLAMc (3.9LLAMcSM, 3.9LLAMcPFM) is indicated by the upper case superscripts. Values not sharing a common superscript differ significantly, $P < 0.05$.

[4]Abbreviations: Tsugars/Protein, total sugars to protein ratio; S/M, salt in moisture; MNFS, moisture in non-fat substances; FDM, fat in dry matter; SIM, total sugars in cheese moisture.

5.3.3. Whey composition

5.3.3.1. Bulk whey

The mean compositions of bulk wheys are shown in Table 5.3 which were similar to those reported previously (Fenelon and Guinee, 1999; Guinee et al., 2006). Applying curd washing to the cheeses made with standarized and protein-fortified milk did not significantly affect the weight of bulk whey, but significantly reduced the fat and protein in bulk whey and also protein as % of total milk protein and fat as % of total milk fat. The lower fat and protein levels in bulk wheys from washed-curd cheeses reflect the dilution of the whey during the curd washing process.

Increasing the milk protein level from 3.3 to 4.0 % (w/w) decreased the weight of bulk whey significantly for the curd-washed cheese while for the control unwashed cheeses, the bulk whey weight was similar but was significantly lower in protein-fortified milk cheeses. The fat level in bulk whey was similar in all cheese which is in agreement with previous results (Guinee et al., 1994) that increasing the milk protein content from 3.3 to 4.0% (w/w) did not significantly influence the fat level in the bulk cheese whey. The consistent level of fat in the bulk whey was expected due the consistancy of the milk standardization protein to fat ratio, which avoids dilution or concentration of the casein matrix relative to fat hence its ability to occlude or to compress/deform embedded fat globules (Guinee et al., 1994). Moreover, all the cheeses were manufactured at similar condition during renneting and cutting which reduced the potential for differences in curd particle shattering at the early stages of stirring. The protein level (%, w/w) of the bulk whey increased significantly as the milk protein content was increased from 3.3 to 4.0 % (w/w). This trend is consistent with the concomitant increases in the levels of whey proteins and glycomacropeptide

(which accounts for 4 to 5% of total casein) during UF treatment, which are both lost in the cheese whey (Guinee et al., 2006).

Table 5.3. The effect of altering lactose and milk protein levels[1] on the composition of cheese wheys[2]

	Target level of lactose for Standarized and Protein-fortified milk cheese [3]					
	Standarized milk cheese			Protein-fortified milk cheese		
Conposition	5.2LLAMcSM	4.2LLAMcSM	3.9LLAMcSM	5.2LLAMcPFM	4.2LLAMcPFM	3.9LLAMcPFM
Bulk whey						
Bulk whey/100 kg of milk, kg	$90.70^{a,B}$	$90.79^{a,A}$	$90.73^{a,A}$	$88.69^{a,A}$	$88.90^{a,B}$	$88.77^{a,B}$
Fat, % w/w	$0.32^{a,A}$	$0.21^{b,A}$	$0.19^{b,A}$	$0.36^{a,A}$	$0.21^{ab,A}$	$0.17^{b,A}$
Protein, % w/w	$0.88^{a,B}$	$0.72^{ab,A}$	$0.61^{b,B}$	$1.06^{a,A}$	$0.81^{b,A}$	$0.70^{c,A}$
Fat, % of total milk fat	$9.06^{a,A}$	$6.08^{b,A}$	$5.33^{b,A}$	$8.52^{a,A}$	$4.90^{b,B}$	$3.99^{b,A}$
Protein, % of total milk protein	$26.93^{a,A}$	$22.06^{ab,A}$	$18.39^{b,A}$	$26.82^{a,A}$	$20.26^{b,A}$	$17.48^{c,A}$
White whey						
White whey/100 kg of milk, kg	$0.95^{a,A}$	$0.79^{a,A}$	$0.90^{a,A}$	$0.87^{a,A}$	$0.83^{a,A}$	$0.88^{a,A}$
Fat, % w/w	$2.83^{a,A}$	$3.06^{a,A}$	$2.77^{a,A}$	$2.78^{a,A}$	$2.50^{a,A}$	$2.59^{a,A}$
Protein, % w/w	$1.02^{a,B}$	$0.98^{a,A}$	$0.95^{a,B}$	$1.15^{a,A}$	$1.01^{a,A}$	$1.03^{a,A}$
Fat, % of total milk fat	$80.95^{a,A}$	$88.53^{a,A}$	$79.40^{a,A}$	$65.92^{a,A}$	$58.54^{a,A}$	$60.71^{a,A}$
Protein, % of total milk protein	$31.29^{a,A}$	$29.78^{a,A}$	$28.75^{a,A}$	$29.03^{a,A}$	$25.24^{a,B}$	$25.66^{a,B}$

[1]Milk protein was concentrated by ultrafiltration; Lactose content was adjusted by curd-washing at cooking process to 34 °C; results are presented as the mean values of three replicate trials.

[2]The whey composition were measured during cheese-making. Bulk whey was collected at whey drainage and white whey was collected after pressing.

[3]Analysis of variance (ANOVA) was used to determine differences between treatment cheeses. The statistical effect of curd washing on cheeses made with a standarized milk (5.2LLAMcSM, 4.2LLAMcSM, 3.9LLAMcSM) or Protein-fortified milk (5.2LLAMcPFM, 4.2LLAMcPFM, 3.9LLAMcPFM) is indicated by lower case superscripts, while the effect of milk protein level on cheeses made with no curd washing (5.2LLAMcSM, 5.2LLAMcPFM), with curd washing to 4.2 LLAMc (4.2LLAMcSM, 4.2LLAMcPFM) and 3.9 LLAMc (3.9LLAMcSM, 3.9LLAMcPFM) is indicated by the upper case superscripts. Values not sharing a common superscript differ significantly, $P < 0.05$.

5.3.3.2. White whey

The weight of white (press) whey, which accounted for only ~1.5 % of total whey weight, was not affected significantly by milk protein level or curd washing (Table 3). The compositions of white whey were similar to those reported previously (Fenelon and Guinee, 1999) for Cheddar cheese made under similar conditions. Curd washing had no effect on fat and protein content in white whey while increasing the milk protein level from 3.3 to 4.0 % (w/w) significantly increased the protein in control non-washed cheeses (5.2LLAMcSM and 5.2LLAMcPFM) and adversely decreased the protein as % of total milk protein in the washed curd cheeses.

5.3.4. Losses and recoveries of milk fat and protein

Applying curd washing to the cheeses made with standarized and protein-fortified milk significantly increased the protein and fat losses in whey streams (Table 5.4). This trend is consistent with the curd washing effect on the actual fat and protein levels in the bulk whey. Increasing curd washing levels also decreased the fat recoveries to cheese which is consistent with the increasing in percentage of total milk fat lost in the bulk whey. The explanation for the increasing in fat loss in washed curd cheeses may be due to an increased stress on, and some physical damage to, the curd particles owing to whey removal and water addition stages. During whey removal, the curd particle-to-whey volume ratio is increased and is only restored when all the water (used to replace the whey removed) was added to the cheese vat. Consequently, there is a temporary increase in curd:whey volume ratio during curd washing when the curd particles are still relatively fragile, which is likely to cause higher collision frequency of curd particles during water addition.

Increasing the level of protein milk from 3.3 to 4.0 % (w/w) had no significant effects on the losses or recoveries to all cheeses which may due to the similar manufacture condition and making procedure for all the cheeses.

Table 5.4. The effect of lactose content and milk protein levels[1] on the losses[2] and recoveries[2] of milk fat and protein and yield on full-fat Cheddar cheese.

	Target level of lactose for Standarized and Protein-fortified milk cheese [3]					
	Standarized milk			Protein-fortified milk		
Whey compositional factors and cheese yields	5.2LLAMcSM	4.2LLAMcSM	3.9LLAMcSM	5.2LLAMcPFM	4.2LLAMcPFM	3.9LLAMcPFM
FLW (% total milk fat)[4]	$8.21^{b,A}$	$9.43^{a,A}$	$9.28^{ab,A}$	$7.92^{b,A}$	$9.05^{a,A}$	$8.84^{ab,A}$
PLW (% total milk protein)[4]	$0.24^{a,A}$	$0.20^{ab,A}$	$0.16^{b,A}$	$0.23^{a,A}$	$0.18^{b,A}$	$0.15^{c,A}$
FRC (% total milk fat)[4]	$91.79^{a,A}$	$90.57^{b,A}$	$90.72^{ab,A}$	$92.08^{a,A}$	$90.95^{b,A}$	$91.16^{ab,A}$
PRC (% total milk protein)[4]	$66.55^{a,A}$	$67.30^{a,A}$	$66.09^{a,A}$	$66.64^{a,A}$	$66.21^{a,A}$	$64.43^{a,A}$
Y_a (kg/100kg)[4]	$9.76^{a,B}$	$9.79^{a,B}$	$9.83^{a,B}$	$11.75^{a,A}$	$11.73^{a,A}$	$11.83^{a,A}$

[1]Milk protein was concentrated by ultrafiltration; Lactose content was adjusted by curd-washing at cooking process to 34 °C; results are presented as the mean values of three replicate trials.

[2]The contents and recoveries were measured during cheese-making at day 1 after manufacture and cheese protein and fat contents were measured at day 14 of ripening.

[3]Analysis of variance (ANOVA) was used to determine differences between treatment cheeses. The statistical effect of curd washing on cheeses made with a standarized milk (5.2LLAMcSM, 4.2LLAMcSM, 3.9LLAMcSM) or Protein-fortified milk (5.2LLAMcPFM, 4.2LLAMcPFM, 3.9LLAMcPFM) is indicated by lower case superscripts, while the effect of milk protein level on cheeses made with no curd washing (5.2LLAMcSM, 5.2LLAMcPFM), with curd washing to 4.2 LLAMc (4.2LLAMcSM, 4.2LLAMcPFM) and 3.9 LLAMc (3.9LLAMcSM, 3.9LLAMcPFM) is indicated by the upper case superscripts. Values not sharing a common superscript differ significantly, $P < 0.05$.

[4]Abbreviations: FLW, percentage milk fat lost in whey streams; PLW, percentage milk protein lost in whey streams; FRC, percentage fat recovery in cheese; PRC, percentage protein recovery in cheese; Y_a, actual cheese yield (kg $100kg^{-1}$ of milk).

5.3.5. Cheese yields

The yields of cheeses are given in Table 5.5. In an attempt to explain potential differences in cheese yield to the direct effect of treatments rather than to inter-treatment differences associated with milk composition (levels of fat, protein, or casein) or cheese composition (moisture), cheese yield was expressed in a number of formats as defined earlier and discussed separately below. Generally, curd washing had no significant effect on cheese yields.

Table 5.5. The effect of lactose content and milk protein levels[1] on the yield of full-fat Cheddar cheese.

	Target level of lactose for Standarized and Protein-fortified milk cheese [3]					
	Standarized milk cheese			Protein-fortified milk cheese		
Cheese Yields[2]	5.2LLAMcSM	4.2LLAMcSM	3.9LLAMcSM	5.2LLAMcPFM	4.2LLAMcPFM	3.9LLAMcPFM
Y_a (kg/100kg)[4]	$9.76^{a,B}$	$9.79^{a,B}$	$9.83^{a,B}$	$11.75^{a,A}$	$11.73^{a,A}$	$11.83^{a,A}$
Y_{afpm}[4]	$9.73^{a,A}$	$9.81^{a,A}$	$9.81^{a,A}$	$9.72^{a,A}$	$9.63^{a,A}$	$9.68^{a,A}$
Y_{ma}[4]	$9.83^{a,B}$	$9.87^{a,B}$	$9.95^{a,B}$	$12.20^{a,A}$	$12.16^{a,A}$	$12.22^{a,A}$
Y_{amfpam}[4]	$9.91^{a,B}$	$9.96^{a,B}$	$10.06^{a,B}$	$12.67^{a,A}$	$12.60^{a,A}$	$12.63^{a,A}$
Y_{afcam}[4]	$8.61^{a,A}$	$8.69^{a,A}$	$8.68^{a,A}$	$8.60^{a,A}$	$8.52^{a,A}$	$8.57^{a,A}$
Y_{mafcam}[4]	$8.68^{a,A}$	$8.76^{a,A}$	$8.78^{a,A}$	$8.93^{a,A}$	$8.83^{a,A}$	$8.85^{a,A}$

[1]Milk protein was concentrated by ultrafiltration; Lactose content was adjusted by curd-washing at cooking process to 34 °C; results are presented as the mean values of three replicate trials.

[2]The contents were measured during cheese-making and day 1 after manufacture and cheese protein and fat contents were measured at day 14 of ripening.

[3]Analysis of variance (ANOVA) was used to determine differences between treatment cheeses. The statistical effect of curd washing on cheeses made with a standarized milk (5.2LLAMcSM, 4.2LLAMcSM, 3.9LLAMcSM) or Protein-fortified milk (5.2LLAMcPFM, 4.2LLAMcPFM, 3.9LLAMcPFM) is indicated by lower case superscripts, while the effect of milk protein level on cheeses made with no curd washing (5.2LLAMcSM, 5.2LLAMcPFM), with curd washing to 4.2 LLAMc (4.2LLAMcSM, 4.2LLAMcPFM) and 3.9 LLAMc (3.9LLAMcSM, 3.9LLAMcPFM) is indicated by the upper case superscripts. Values not sharing a common superscript differ significantly, $P < 0.05$.

[4]Abbreviations: Y_{afpam}, yield per 100 kg of milk normalized to reference fat (3.4 %, w/w) and protein (3.3 %, w/w) levels; Y_{ma}, moisture-adjusted yield; Y_{mafpam}, moisture-adjusted (38.5 %, w/w) yield per 100 kg of milk normalized to reference fat and protein levels; Y_{afcam}, yield per 100 kg of milk normalized to reference fat (3.4 %, w/w) and casein (2.53 %, w/w) levels; Y_{mafcam}, moisture-adjusted yield per 100 kg of milk normalized to reference fat and casein levels.

5.3.5.1. Y_a and Y_{afpam}

Actual yield, Y_a, increased significantly (~ 2 %, w/w) as milk protein increased from 3.3 to 4.0 % (w/w), due to proportional increases in the content of additional protein and milk fat. The increase in Y_a with increase in milk protein agrees with that reported previously for skim milk cheese (0.33 kg per 0.1 %, w/w milk protein; Gilles and Lawrence, (1985) and for full-fat Cheddar cheese from milk standardized to a protein-to-fat ratio of 0.97 (0.33 kg per 0.1 % w/w milk protein; Fox, Guinee, Cogan & McSweeney, 2000).

The normalized yield, Y_{afpam}, per 100 kg of milk with reference levels of fat (3.4 % w/w) and protein (3.3 %, w/w) was not affected by either curd washing or milk protein levels. This trend concurs with the small differences in recoveries of milk fat and protein in cheese.

5.3.5.2. Y_{ma} and Y_{mafpam}

In the current study, the moisture content of the cheeses decreased significantly with increasing standardization of milk protein levels from 3.3 % to 4.0 % (w/w). During cheesemaking, there was no attempt made to intervene in the manufacture process to offset the moisture decrease, as the objective of the study was to consider the effect of two treatment variables: method of protein standardization and curd washing. However, for commercial purposes, the adverse effect on reduction in cheese moisture on cheese yield can be ameliorated by standardising the protein in milks. The moisture content of cheeses from the protein-standarised milk could be increased by a number of alternative processes, such as increasing milk pasteurization temperature (depend on cheese type), gel firmness at cutting, curd particle size, cooking rate of the curd/whey mixture or pH at curd milling, and reducing temperature of maximum scald

(Mayes and Sutherland, 1989; IDF, 1991, 1994; Fox et al., 2000; Dejmek and Walstra, 2004; Guinee et al., 2005).

The moisture-adjusted yield, Y_{ma}, normalizes cheese moisture to a reference level, and thereby facilitates the comparison of the yields of treatment cheeses with different moisture contents. The Y_{ma} increased significantly from ~9.95 % (w/w) to 12.2 % (w/w) on increasing the milk protein from 3.3 to 4.0 % (w/w) with all cheeses and the reasons were similar to that discussed above for Y_a.

Similar to Y_{ma}, the normalized moisture-adjusted (38.5 % w/w) yield, Y_{mafpam}, per 100 kg of fat (f) and protein (p) adjusted milk (am), also increased significantly with milk protein increase from 3.3 to 4.0 % (w/w) which may also due to the higher total solids in the cheese milk.

5.3.5.3. Y_{afcam} and Y_{mafcam}

The normalized yield per 100 kg of milk with reference levels of fat (3.4 % w/w) and casein (2.53 %, w/w), Y_{afcam}, was not significantly affected by increasing milk protein content or by curd washing (Table 5.5). This indicates that milk casein and protein recovery in cheese (Table 5.1 and 5.4) were similar in all cheeses; casein is essentially the protein fraction (apart from denatured whey protein complexed with the casein) recovered in the protein matrix of the Cheddar cheese (Guinee et al., 2006).

Similar to Y_{afcam}, the moisture-adjusted yield per 100 kg of fat and casein-adjusted milk, Y_{mafcam}, was not significantly affected by milk protein content or curd washing, which indicates that at the similar moisture level, yield of cheese from all cheese milks were similar due to the same fat and protein recovery.

5.3.6. Sugars in cheese

5.3.6.1. Lactose

The mean level of residual lactose was not significantly affected by curd washing, but significantly affected by ripening time and their interaction in the cheeses made with standarized milk, where as it decreased significantly with curd washing, ripening time and their interactions for cheeses made with protein-fortified milk (Figure 5.1a and 5.1b, Table 5.6**)**. Similar levels of residual lactose in cheese were found by previous researchers (Huffman and Kristoffersen, 1984; Shakeel-Ur-Rehman et al., 2004; Upreti, McKay, & Metzger, 2006a; Hou et al., 2012; Hou et al., 2014b) where cheese curds were washed during manufacturing. The lack of significant effects of curd washing on the cheeses made with standarized milk is may be due to the lactose be used up fast during the first 30 days of ripening (Figure 5.1a), thereafter, only less than 0.1 % (w/w) residual lactose remained in the cheeses and the levels of residual lactose did not change during the 270 days of ripening period. The cheeses made with protein-fortified milk contained lower levels of residual lactose (~ 0.09 %, w/w at day 1).

The residual lactose reduced rapidly during the first 30 days of ripening especially in cheeses made with curd washing. Similar trend was found by other authors (Huffman & Kristoffersen, 1984; Shakeel-Ur-Rehman, et al., 2004; Upreti & Metzger, 2006a) who noted that residual lactose levels in cheese from 1 to 30 days after manufacture decreased with curd washing. The high levels of residual lactose in control cheeses throughout ripening (which did not include a curd washing step), may be due to the higher retention of lactose in the cheese moisture phase (which was not diluted during curd washing) as lactose is a water-soluble solute.

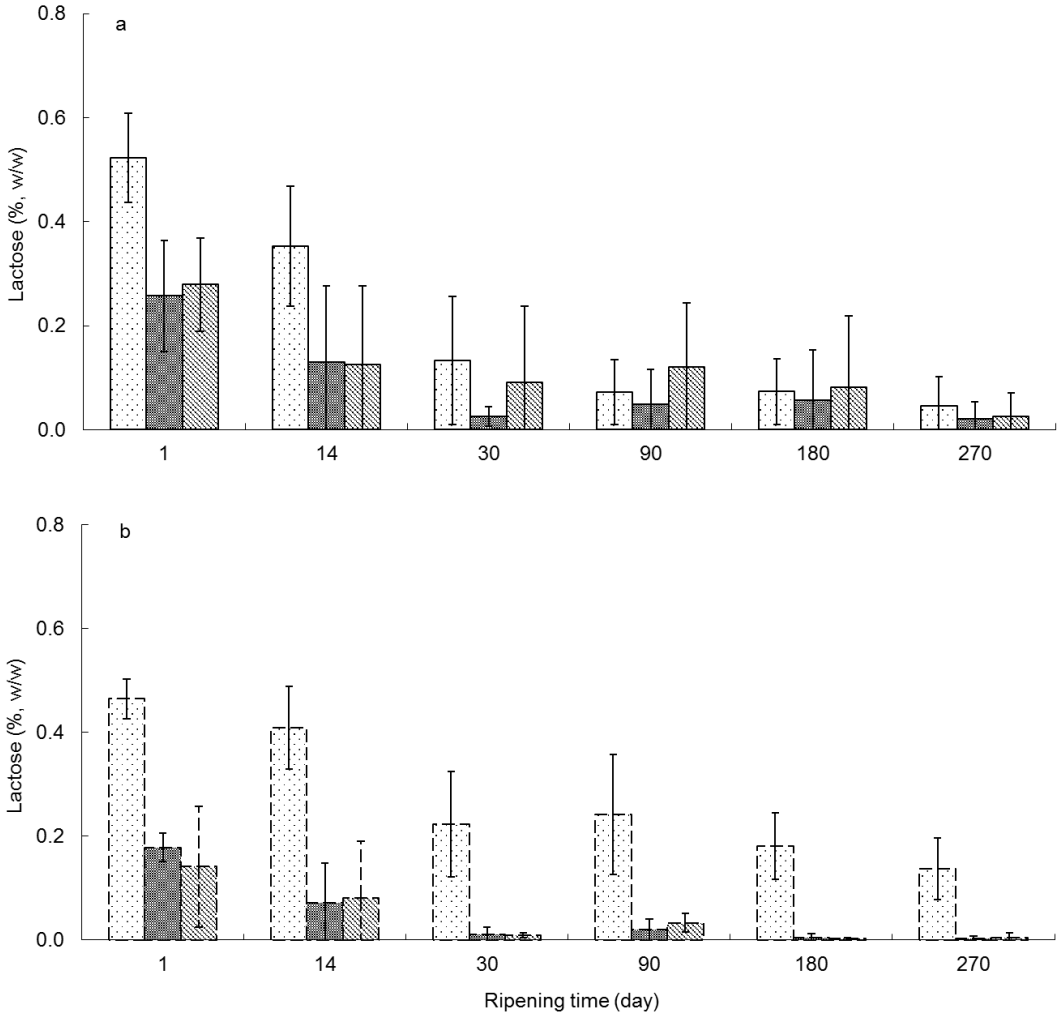

Figure 5.1. Age-related changes in the levels of residual lactose in Cheddar cheese made from standarized milk (3.3 % protein; a, solid bars) or protein-fortified milk (4.0 % protein; b, broken bars) and with curd washing to different levels of lactose + lactic acid in cheese moisture: 5.2 % (Control, non-wash, 5.2LLAMcSM, ; 5.2LLAMcPFM,), 4.2 % (4.2LLAMcSM, ; 4.2LLAMcPFM,) or 3.9 % (3.9LLAMcSM, ; 3.9LLAMcPFM,). Protein-fortified milk was prepared by low-concentration factor membrane filtration. Presented values are the means of three replicate trials; error bars represent standard deviations of the mean.

Table 5.6. Degrees of freedom (df) and statistical significances (*P*-values) for changes in lactose, D(-)-lactate, L(+)-lactate, total lactate, and pH in full fat Cheddar cheeses with different milk protein and lactose content[1,2].

Factor	Lactose		D(-)-Lactate		L(+)-Lactate		Total lactate		Total sugars and derivatives		pH	
(Standarized milk cheese)	df	*P*	df	*P*	df	*P*	df	*P*	df	*P*	df	*P*
Main plot												
Curd washing	2	0.1645	2	0.0062	2	0.1017	2	0.0256	2	<.0001	2	0.0263
Sub-plot												
Time	5	<.0001	5	<.0001	5	0.0146	5	0.0121	5	0.0098	5	0.6435
Interaction (curd washing x time)	10	0.0005	10	0.0003	10	0.9668	10	0.2494	10	0.9484	10	0.0153

Factor	Lactose		D(-)-Lactate		L(+)-Lactate		Total lactate		Total sugars and derivatives		pH	
(Protein-fortified milk cheese)	df	*P*	df	*P*	df	*P*	df	*P*	df	*P*	df	*P*
Main plot												
Curd washing	2	<.0001	2	0.0027	2	0.0011	2	0.0001	2	<.0001	2	0.0260
Sub-plot												
Time	5	<.0001	5	<.0001	5	0.0002	5	0.0750	5	0.0241	5	<.0001
Interaction (curd washing x time)	10	0.1177	10	0.0035	10	0.9336	10	0.8619	10	0.6068	10	0.0509

[1]Milk protein was concentrated by ultrafiltration; Lactose content was adjusted by curd-washing at cooking process to 34 °C; results are presented as the mean values of three replicate trials.
[2]Analysis of variance was carried using a general linear model (GLM) procedure of SAS, where the effects of treatment, ripening time and their interaction were estimated.

5.3.6.2. Total sugars

The mean levels of residual lactose, lactic acids, glucose and galactose summarized as total sugars and derived acids, were significantly affected by the curd washing and ripening time for all cheeses (Figure 5.2; Table 5.6). Similar levels of total sugars in a 270-day ripening period were found by our previous study (Hou et al., 2012) for Cheddar cheeses where curd washing was applied during cheese manufacture. Curd washing significantly decreased the total sugars in cheese from a mean value of ~1.76 % (w/w) for non-washed cheeses to ~ 1.28 % (w/w) for the curd-washed cheeses. The decrease in total sugars was due to the solubility of all the sugars in and the diffusion process of lactose and lactic acids during the curd washing and whey drainage. Other than that, the increase of milk protein levels by UF did not significantly affect the total sugar content in the cheese.

The concentration of LLAMc in the control cheeses (~ 4.15 %, w/w) was ~ 1.0 % (w/w) less than that of the lactose in the moisture phase of milk (LIMm; 5.2 % w/w) (see below). Similarly, data found in the previous study (~1 % less LLAMc than LIMm) which indicated that the lower levels of LLAMc compared to that of LIMm may reflect a more rapid diffusion rate of lactic acid from the moisture phase of the curd particle than that of lactose from the whey into curd particle because of the lower molecular mass of the former compared to the latter (~ 90 and 342 Da, respectively) and the higher concentration of lactate in the moisture of the curd particle (Hou et al., 2012).

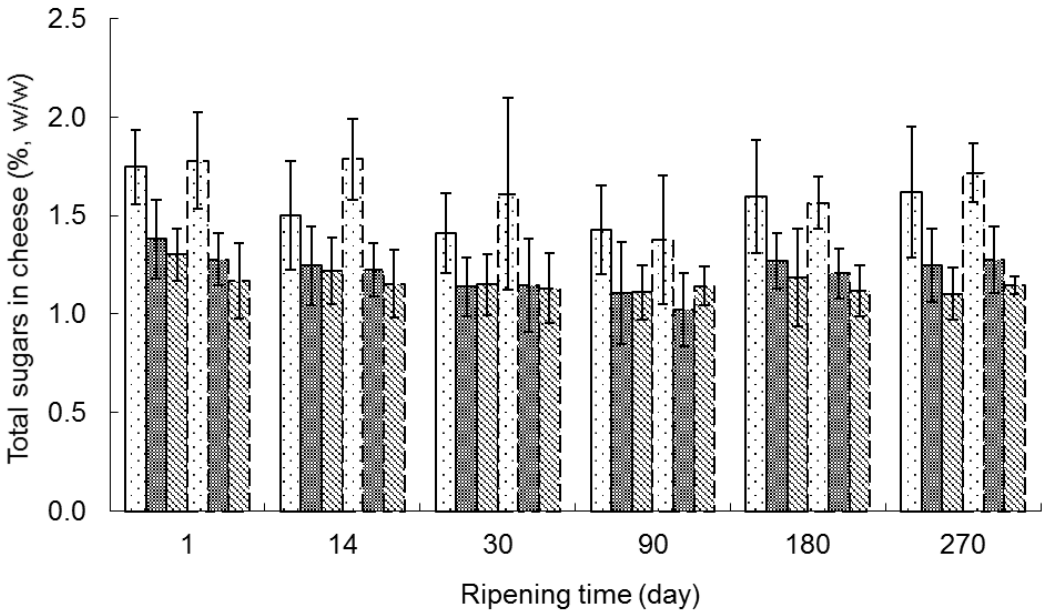

Figure 5.2. Age-related changes in the levels of total sugars in Cheddar cheese made from standarized milk (3.3 % protein; a, solid bars) or protein-fortified milk (4.0 % protein; b, broken bars) and with curd washing to different levels of lactose + lactic acid in cheese moisture: 5.2 % (Control, non-wash, 5.2LLAMcSM, ; 5.2LLAMcPFM,), 4.2 % (4.2LLAMcSM, ; 4.2LLAMcPFM,) or 3.9 % (3.9LLAMcSM, ; 3.9LLAMcPFM,). Protein-fortified milk was prepared by low-concentration factor membrane filtration. Presented values are the means of three replicate trials; error bars represent standard deviations of the mean.

5.3.6.3. D (-) - and L (+)-lactate

The mean levels of D (-) - lactate over the 270 days ripening period was significantly affected by curd washing, ripening time and their interaction for all cheeses. However, the mean levels of L (+) - lactate were only significantly affected by curd washing and ripening time for the cheeses made with protein-standardised milk, but only significantly affected by ripening time for the standarized milk cheeses (Figure 5.3; Table 5.6). Concentrating milk protein from 3.3 % (w/w) to 4 % (w/w) did not significantly affect the levels of D (-) - and L (+)-lactate over 270-day ripening period.

During the 270-day ripening period, the concentration of L (+) - lactate was related inversely to that of D (-)-lactate. These results are consistent with the findings reported previously for full-fat Cheddar cheese (Jordan and Cogan, 1993; Hou et al., 2012). During maturation, the racemisation of L (+)-lactate to D (-)-lactate was due to the growth of NSLAB count from < 10^1 to ~ 10^6 cfu g^{-1} of cheese over the 270 day ripening period (as discussed below), which was slow at the beginning, but proceeded rapidly between 30 to 180 days, and thereafter more slowly. The D (-)-lactate was probably not formed from residual lactose which was essentially all utilised by 30 days, when both the NSLAB counts (10^1 to ~ 10^2 cfu g^{-1} of cheese) and the concentrations of D (-) -lactate were very low (~ 0.005 %, w/w). The concentration of L (+) - lactate was the major end-product, and being ~0.3–0.4 g 100 g^{-1} higher than D (-)-lactate when the concentrations of the isomers had stabilised, at ripening times > 180 day which is in agreement with Turner and Thomas (1980) for full-fat Cheddar cheese with a salt-in-moisture level of ~4 % (w/w).

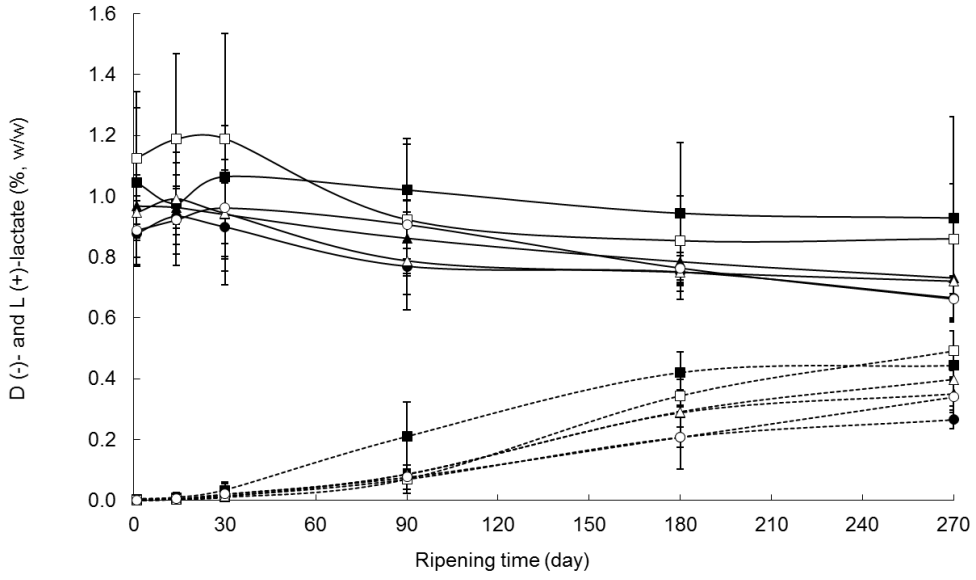

Figure 5.3. Age-related changes in the levels of D (-)-Lactate (broken lines) and L (+)-lactate (solid line) content in standarized milk (3.3 %, w/w) or protein-fortified milk (4.0 %, w/w) Cheddar cheeses made with curd washing to a lactose plus lactic acid levels of 5.2 % (Control, non-wash, 5.2LLAMcSM, ■; 5.2LLAMcPFM, □), 4.2 % (4.2LLAMcSM, ▲; 4.2LLAMcPFM, △) and 3.9 % (3.9LLAMcSM, ◆; 3.9LLAMcPFM, ◇). Protein-fortified milk was prepared by low-concentration factor membrane filtration. Presented values are the means of three replicate trials; error bars represent standard deviations of the mean.

5.3.6.4. Total lactate

The lactate levels in all cheeses increased during the first 30 days of ripening, and thereafter plateaued at values of ~ 1.1 ± 0.1 % (w/w) between 90 – 270 days. The latter values are slightly lower but similar in magnitude (1.4 – 1.5 %, w/w) to those reported previously for Cheddar (Thomas and Pearce, 1981; Hou et al., 2014b; McCarthy, Wilkinson, Kelly & Guinee, 2015).

The overall mean levels of total lactate increased significantly with ripening time and decreased significantly with curd washing in all cheeses (Figure 5.4, Table 5.6). The increase in lactate content with ripening time is consistent with the decrease in lactose levels, which is metabolised to lactic acid during ripening by starter and

NSLAB (Turner and Thomas, 1980; Rynne et al., 2004; Hou et al., 2012) as discussed below.

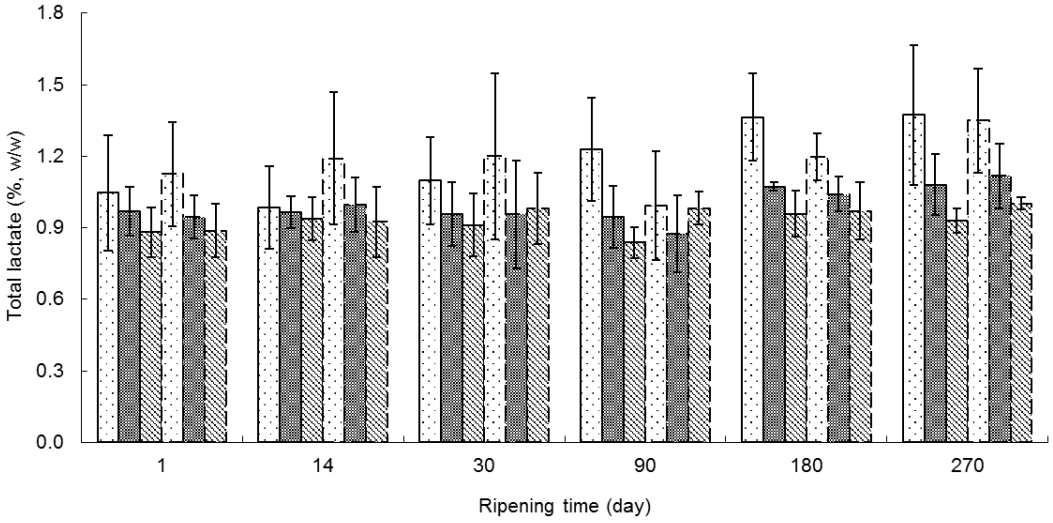

Figure 5.4. Age-related changes in the levels of total lactates in Cheddar cheese made from standarized milk (3.3 % protein; a, solid bars) or protein-fortified milk (4.0 % protein; b, broken bars) and with curd washing to different levels of lactose + lactic acid in cheese moisture: 5.2 % (Control, non-wash, 5.2LLAMcSM, ; 5.2LLAMcPFM,), 4.2 % (4.2LLAMcSM, ; 4.2LLAMcPFM,) or 3.9 % (3.9LLAMcSM, ; 3.9LLAMcPFM,). Protein-fortified milk was prepared by low-concentration factor membrane filtration. Presented values are the means of three replicate trials; error bars represent standard deviations of the mean.

5.3.7. pH changes during ripening period

Cheese pH was significantly affected by curd washing for all cheeses (Figure 5.5, Table 5.6). The mean pH over the 270 days ripening period increased significantly with curd washing, with the pH of the 3.9LLAMcSM cheese being ~ 0.33 units higher than that of the 5.2LLAMcSM cheese and ~ 0.2 units higher in the 3.9LLAMcPFM cheese than that of the 5.2LLAMcPFM cheese at 270 days. A similar trend was found in previous studies on curd-washing in full fat Cheddar cheese (Hou et al., 2012); the higher pH in washed curd cheeses may be attributed to the significantly lower levels

of total lactate at ripening times ≥ 90 days (Figure 5.4). The present results concur with those from previous studies, which indicates an inverse relationship between pH of Cheddar cheese and levels of lactic acid (Huffman & Kristoffersen, 1984; Guinee et al., 2008). Moreover, the inverse relationship between Cheddar cheese pH and total lactate content parallels inverse relationship between pH and cheese moisture, which is the solvent for lactose and lactate (Rynne, Beresford, Kelly, & Guinee,, 2007).

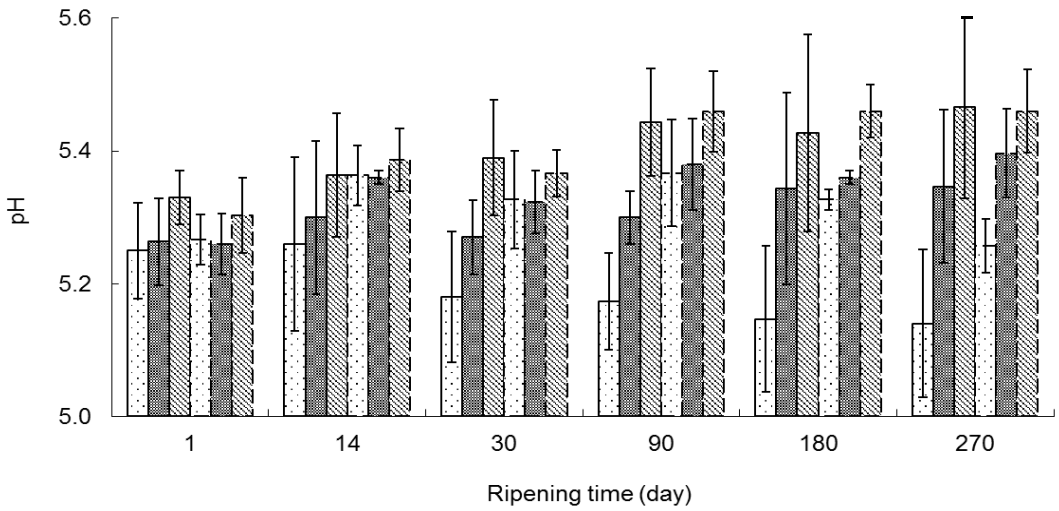

Figure 5.5. Age-related changes in the levels of pH in Cheddar cheese made from standarized milk (3.3 % protein; a, solid bars) or protein-fortified milk (4.0 % protein; b, broken bars) and with curd washing to different levels of lactose + lactic acid in cheese moisture: 5.2 % (Control, non-wash, 5.2LLAMcSM, ; 5.2LLAMcPFM,), 4.2 % (4.2LLAMcSM, ; 4.2LLAMcPFM,) or 3.9 % (3.9LLAMcSM, ; 3.9LLAMcPFM,). Protein-fortified milk was prepared by low-concentration factor membrane filtration. Presented values are the means of three replicate trials; error bars represent standard deviations of the mean.

5.3.8. Microbial counts of starter and NSLAB in cheese

5.3.8.1. Starter bacteria

Starter bacteria counts in all cheeses at day 1 were ~ $10^{9.5}$ cfu/g cheese. The mean starter count of all cheeses decreased significantly with ripening time (Figure

5.6a, Table 5.7). The magnitude of the decrease in cell count, from ~ $10^{9.5}$ cfu/g at day 1 to ~ $10^{6.8-7.5}$ cfu/g at day 270, is typical of that reported elsewhere for Cheddar cheese made using *Lactococcus lactis* subsp. *cremoris* and *Lc. lactis* subsp. *lactis* as starter (Turner and Thomas, 1980; Hou et al., 2012). The decrease in starter cell count may be attributed to permeablisation and autolysis (Sheehan, O'Cuinn, FitzGerald, & Wilkinson, 2009). Curd washing significantly decreased the mean counts of starter bacteria in the cheeses made from standarized milk but had no effect on the protein-standardised milk cheeses (Figure 5.6a, Table 5.7). The significant effect of curd washing on the standarized milk cheeses may be due to the numerically higher S/M content in the washed cheeses, hence limited the growth of starter strains.

5.3.8.2. NSLAB

NSLAB populations at day 1 were similar in magnitude to those previously reported for Cheddar cheese (Jordan & Cogan, 1993; Turner & Thomas, 1980). The mean counts of NSLAB over the 270 days increased significantly with ripening time from ~ $10^{0.3}$ cfu/g at day 1 to ~ 10^{6} cfu/g at day 270, but were not affected by curd washing for all cheeses (Table 5.7, Figure 5.6b). The lack of significant effect of curd washing on NSLAB counts agrees with results from previous studies (Shakeel-Ur-Rehman, et al., 2004; Turner & Thomas, 1980), which concluded that the rate of NSLAB growth in Cheddar cheese was independent of lactose content (Fox, McSweeney, & Lynch, 1998). NSLAB can also metabolise energy sources other than lactose in cheese to a degree dependent on species and strain type (Hou et al., 2012), hence, NSLAB growth would not be expected to be totally impeded by reduced lactose levels. Noticeably, the NSLAB count in the wash curd cheeses was ~10^{1} cfu/g lower than the control, non-washed cheeses especially at day ≥ 30 for standarized milk

cheeses and 30 and 90 day for protein-standardised milk cheeses. Even though the growth of NSLAB is independent of residual lactose in cheese, the increase in curd pH and decrease in total lactate by curd washing, the other available carbohydrate and oxidation-reduction potential will also affect the NSLAB growth (Fuquay, John, Fox, and McSweeney, 2011).

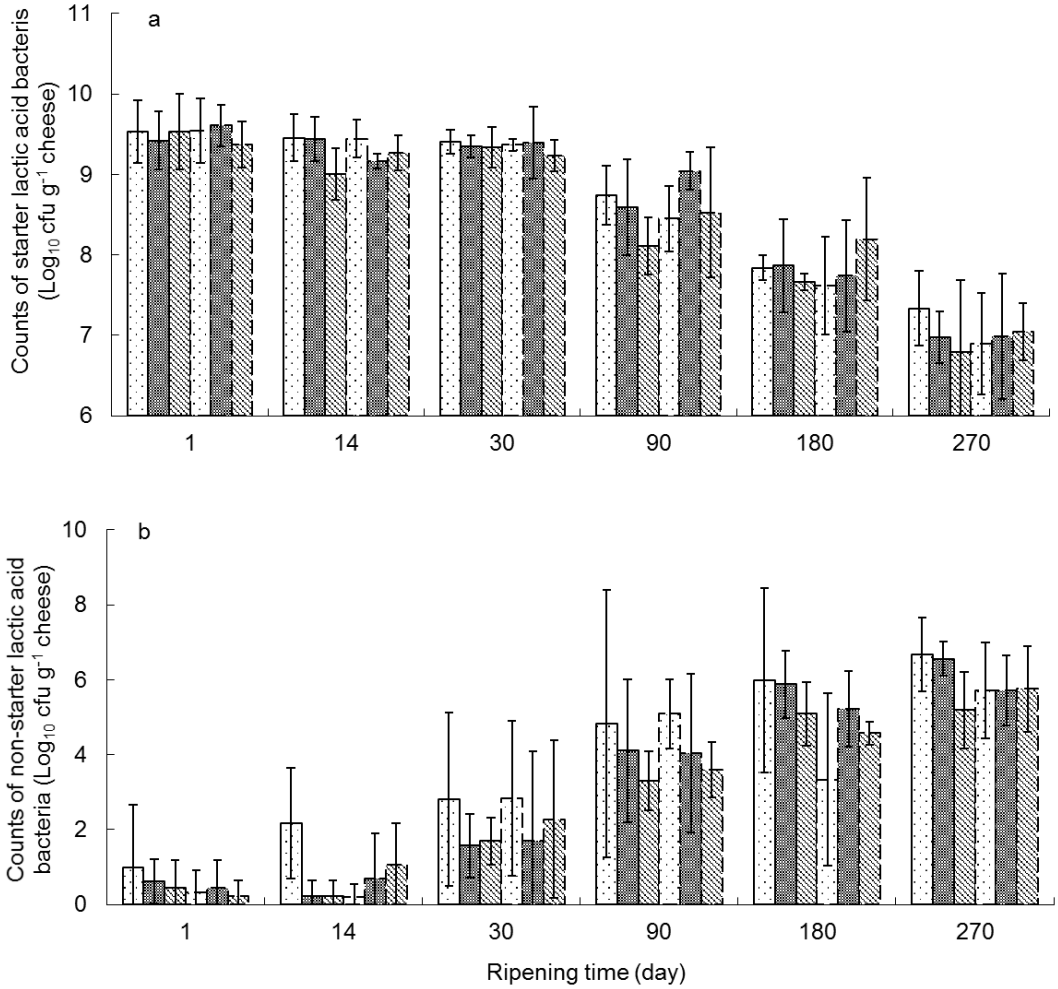

Figure 5.6. Age-related changes in the counts of starter lactic acid bacteria (a) and of non-starter lactic acid bacteria (b) in Cheddar cheese made from standarized milk (3.3 % protein; a, solid bars) or protein-fortified milk (4.0 % protein; b, broken bars) and with curd washing to different levels of lactose + lactic acid in cheese moisture: 5.2 % (Control, non-wash, 5.2LLAMcSM, ; 5.2LLAMcPFM,), 4.2 % (4.2LLAMcSM, ; 4.2LLAMcPFM,) or 3.9 % (3.9LLAMcSM, ; 3.9LLAMcPFM,). Protein-fortified milk was prepared by low-concentration factor membrane filtration. Presented values are the means of three replicate trials; error bars represent standard deviations of the mean.

Table 5.7. Degrees of freedom (df) and statistical significances (*P*-values) for changes in starter counts and NSLAB count in full fat Cheddar cheeses with different milk protein and lactose contents[1,2].

Factor	Starter counts		NSLAB counts	
(Standarized milk cheeses)	df	*P*	df	*P*
Main plot				
Curd washing	2	0.0358	2	0.4196
Sub-plot				
Time	5	<.0001	5	<.0001
Interaction (Curd washing x time)	10	0.8235	10	0.8616

Factor	Starter counts		NSLAB counts	
(Protein-fortified milk cheeses)	df	*P*	df	*P*
Main plot				
Curd washing	2	0.7782	2	0.9913
Sub-plot				
Time	5	<.0001	5	<.0001
Interaction (Curd washing x time)	10	0.6876	10	0.5200

[1]Milk protein was concentrated by ultrafiltration; Lactose content was adjusted by curd-washing at cooking process to 34 °C; results are presented as the mean values of three replicate trials.
[2]Analysis of variance was carried using a general linear model (GLM) procedure of SAS, where the effects of treatment, ripening time and their interaction were estimated.

5.3.9. Assessment of Proteolysis

5.3.9.1. Changes in primary proteolysis, pH 4.6-SN

The mean levels of pH4.6-SN in the different cheeses increased significantly over the 270-day ripening period from ~2.6 % at day 1 to ~21-23 % of total nitrogen at day 270 (Table 5.8, Figure 5.7), with the magnitude of the increase being comparable to that reported previously (Guinee, Auty, & Fenelon, 2000; Lee, Johnson, Govindasamy-Lucey, Jaeggi, Lucey, 2011; Hou et al., 2014a) which is due to the hydrolysis of intact casein by residual chymosin and, to a lesser extent by the indigenous milk proteinase, plasmin and the proteolytic activity of the cheese starter culture (Sousa, Ardo, & McSweeney, 2001). Similar effect was found by Shakeel-Ur-Rehman et al. (2004) who indicated that pH4.6-SN increases during ripening whilecurd washing did not significantly affect the level of pH 4.6-SN.

Increasing the milk protein level from 3.3 % to 4.0 % (w/w) by UF decreased over ripening at the average level of pH4.6-SN in cheese by 1.15 % of total N (Figure 5.7). This trend may be due to the significantly higher moisture in the standarized milk cheeses which favours the residual chymosin activity and also possibly by reducing protein but having same level of enzymes (Fox, 1975). Similar results were reported by Upreti, Metzger, and Hayes (2006b) that the control cheeses had significantly higher moisture content and moisture-non-fat-substances (MNFS) than the washed-curd cheeses, which resulted in higher levels of pH4.6-SN.

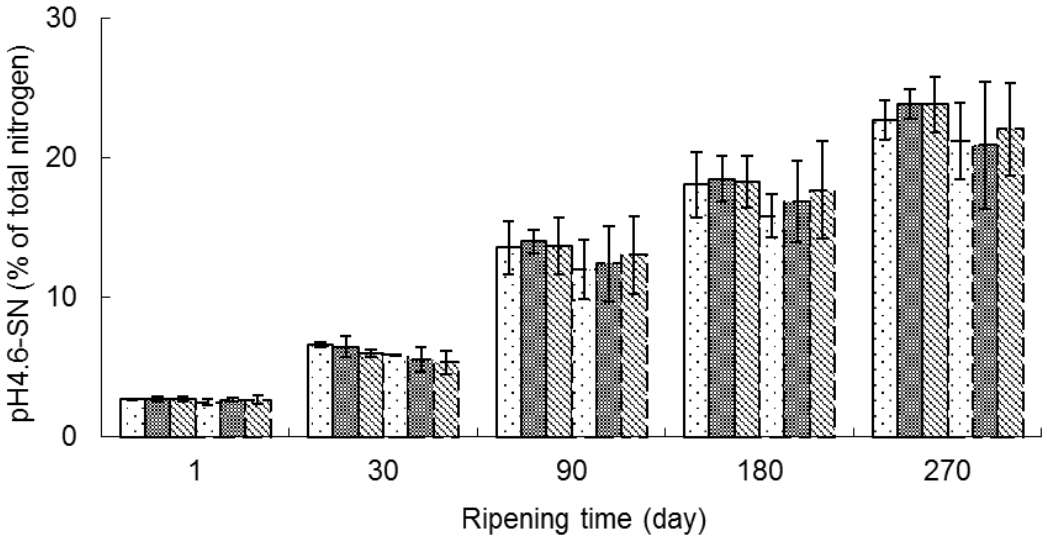

Figure 5.7. Age-related changes in the levels of pH4.6-SN in Cheddar cheese made from standarized milk (3.3 % protein; a, solid bars) or protein-fortified milk (4.0 % protein; b, broken bars) and with curd washing to different levels of lactose + lactic acid in cheese moisture: 5.2 % (Control, non-wash, 5.2LLAMcSM, ; 5.2LLAMcPFM,), 4.2 % (4.2LLAMcSM, ; 4.2LLAMcPFM,) or 3.9 % (3.9LLAMcSM, ; 3.9LLAMcPFM,). Protein-fortified milk was prepared by low-concentration factor membrane filtration. Presented values are the means of three replicate trials; error bars represent standard deviations of the mean.

Table 5.8. Degrees of freedom (df) and statistical significances (*P*-values) for changes in primary and secondary proteolysis in full fat Cheddar cheeses with different milk protein and lactose content[1,2].

Factor	pH4.6-SN		PTA-SN		FAA	
(Standarized milk cheeses)	df	*P*	df	*P*	df	*P*
Main plot						
Curd washing	2	0.6234	2	0.6144	2	0.0030
Sub-plot						
Time	5	<.0001	5	<.0001	5	<.0001
Interaction (curd washing x time)	10	0.9902	10	0.1126	10	0.3814

Factor	pH4.6-SN		PTA		FAA	
(Protein-fortified milk cheeses)	df	*P*	df	*P*	df	*P*
Main plot						
Curd washing	2	0.5760	2	0.3406	2	0.2379
Sub-plot						
Time	5	<.0001	5	<.0001	5	<.0001
Interaction (curd washing x time)	10	0.9849	10	0.8156	10	0.5095

[1]Milk protein was concentrated by ultrafiltration; Lactose content was adjusted by curd-washing at cooking process to 34 °C; results are presented as the mean values of three replicate trials.
[2]Analysis of variance was carried using a general linear model (GLM) procedure of SAS, where the effects of treatment, ripening time and their interaction were estimated.

5.3.9.2. Changes in secondary proteolysis, PTA-SN and FAA.

PTA-SN and FAA were used as measures of the degree of secondary proteolysis, reflecting the degradation of large molecular weight peptides produced by residual coagulant and plasmin into low molecular weight peptides and free amino acids by the action of microbial enzymes (Jarrett, Aston, & Dulley, 1982; Fenelon, Guinee, Delahunty, Murray, & Crowe, 2000). The mean levels of PTA-SN in the different cheeses significantly increased with ripening time but were not affected by curd washing treatment (Figure 5.8, Table 5.8). The mean levels of PTA-SN in all cheeses increased from ~0.4 % total N at day 1 to > 4.10 % total N at day 270; these levels are similar to those reported previously for mature Cheddar cheese (Guinee, Wilkinson, Mulholland & Fox, 1991; Fenelon et al., 2000; Hou et al., 2014b). The used of increased milk protein levels in the cheesemilk during manufacturing had little effect on PTA-SN.

Similarly, the mean levels of FAA increased significantly over the 270-day ripening period for all cheeses. Curd washing significantly increased the FAA content of standardized milk cheese but notin the protein-fortified milk cheeses (Figure 5.9, Table 5.8). This may be due to the higher pH in washed-curd cheese that affected the peptidase activity from the starter cultures. The non-significant effect of curd washing in protein-standardised milk cheeses may be due to several factors such as peptidase activities, NSLAB species (Gobbetti, et al., 1999), and degrees of autolysis and permeability of starter and non-starter bacteria (Doolan & Wilkinson, 2009).

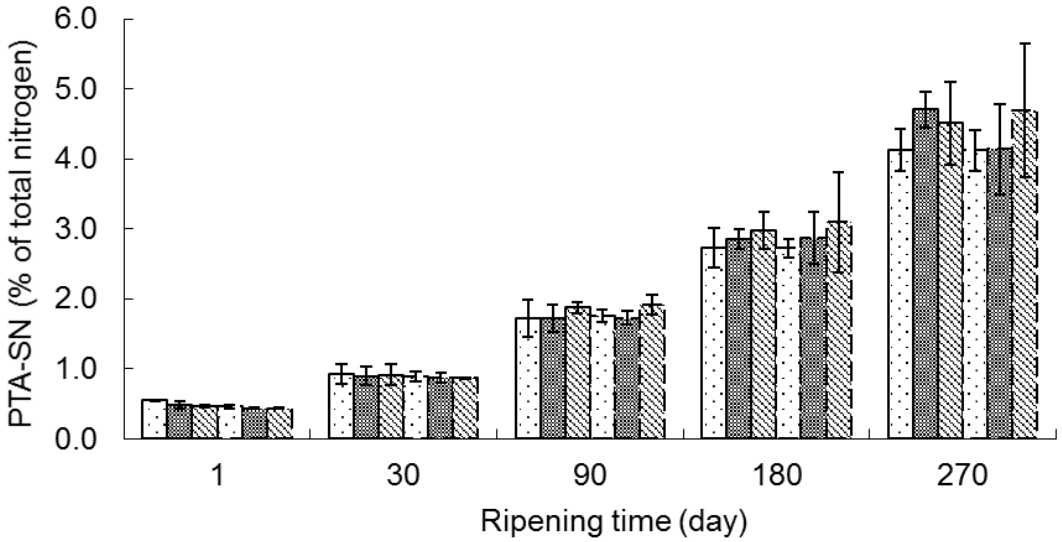

Figure 5.8. Age-related changes in the levels of PTA-SN in Cheddar cheese made from standarized milk (3.3 % protein; a, solid bars) or protein-fortified milk (4.0 % protein; b, broken bars) and with curd washing to different levels of lactose + lactic acid in cheese moisture: 5.2 % (Control, non-wash, 5.2LLAMcSM, ; 5.2LLAMcPFM,), 4.2 % (4.2LLAMcSM, ; 4.2LLAMcPFM,) or 3.9 % (3.9LLAMcSM, ; 3.9LLAMcPFM,). Protein-fortified milk was prepared by low-concentration factor membrane filtration. Presented values are the means of three replicate trials; error bars represent standard deviations of the mean.

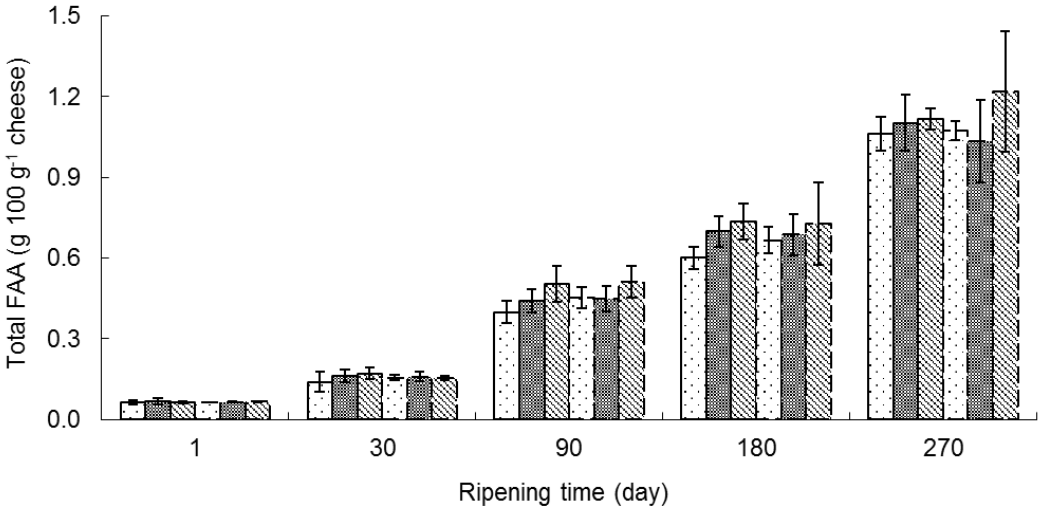

Figure 5.9. Age-related changes in the levels of total free amino acids (FAA) in Cheddar cheese made from standarized milk (3.3 % protein; a, solid bars) or protein-fortified milk (4.0 % protein; b, broken bars) and with curd washing to different levels of lactose + lactic acid in cheese moisture: 5.2 % (Control, non-wash, 5.2LLAMcSM, ; 5.2LLAMcPFM,), 4.2 % (4.2LLAMcSM, ; 4.2LLAMcPFM,) or 3.9 % (3.9LLAMcSM, ; 3.9LLAMcPFM,). Protein-fortified milk was prepared by low-concentration factor membrane filtration. Presented values are the means of three replicate trials; error bars represent standard deviations of the mean.

5.3.10. Rheology properties

The mean values of firmness and fracture stress over the 270 day ripening period decreased significantly ($P < 0.0001$) during ripening (Table 5.9, Figure 5.10-5.11) while fracture strain was not significantly affected by ripening time (Table 5.9, Figure 5.12). This trend was similar to previous studies of both full-fat and half-fat Cheddar cheese and which effect is consistent with the reduction in the content of intact casein, as reflected by the age-related increase in pH4.6-SN (Guinee, et al., 2000).

The mean levels of firmness and fracture stress over the ripening period were not significantly affected by curd washing (Table 5.9). This non-significant effect may be due to the similar gross composition in the curd-washed standardzed and protein-

fortified milk cheeses (Hou et al, 2012). Increasing the milk protein levels from 3.3 % to 4.0 % (w/w), increased the cheese firmness at an average level of 40 - 60 N and fracture stress at 110 – 140 kPa and this effect was more pronounced at early stages of ripening period. This results consist with the lower cheese moisture and higher proteolysis which associated with protein break down causing a softer, pasty body.

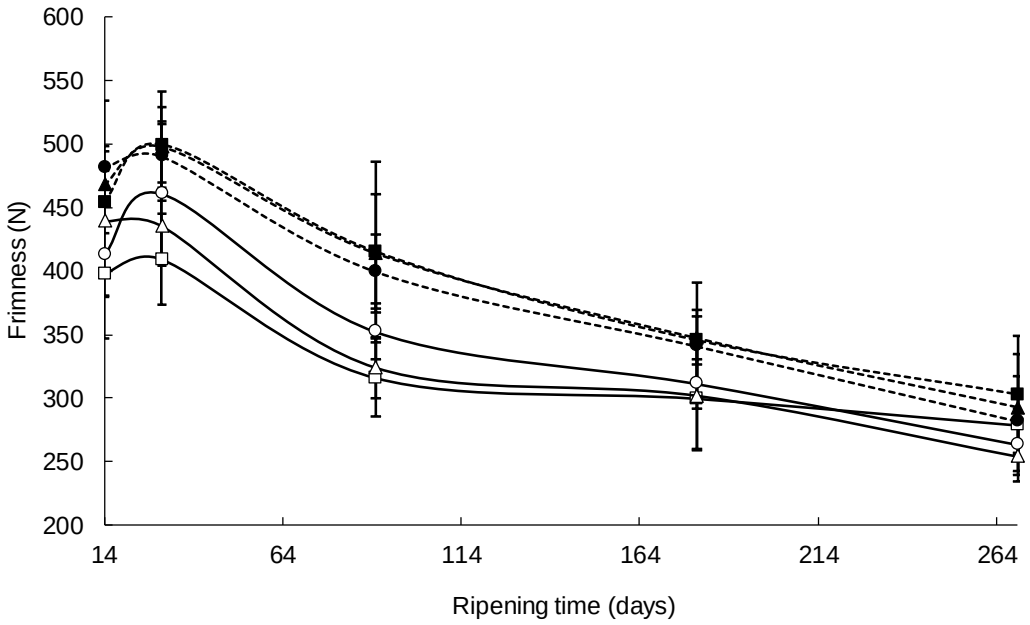

Figure 5.10. Age-related changes in firmness values of Cheddar cheese made from low protein milk (3.3 %, w/w, solid line) and high protein milk (4.0 %, w/w, broken line) Cheddar cheeses made with curd washing to a lactose plus lactic acid levels of 5.2 % (Control, non-wash, 5.2LLAMcLP, ■; 5.2LLAMcHP, □), 4.2 % (4.2LLAMcLP, ▲; 4.2LLAMcHP, △) and 3.9 % (3.9LLAMcLP, ◆; 3.9LLAMcHP, ◇). Presented values are the means of three replicate trials; error bars represent standard deviations of the mean.

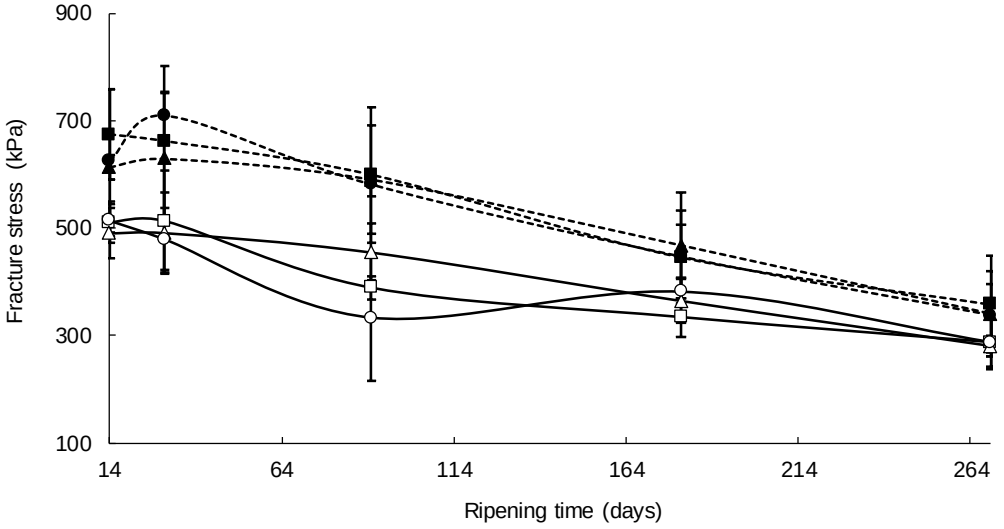

Figure 5.11. Age-related changes in the fracture stress values of Cheddar cheese made from low protein milk (3.3 %, w/w, solid line) and high protein milk (4.0 %, w/w, broken line) Cheddar cheeses made with curd washing to a lactose plus lactic acid levels of 5.2 % (Control, non-wash, 5.2LLAMcLP, ■; 5.2LLAMcHP, □), 4.2 % (4.2LLAMcLP, ▲; 4.2LLAMcHP, △) and 3.9 % (3.9LLAMcLP, ◆; 3.9LLAMcHP, ◇). Presented values are the means of three replicate trials; error bars represent standard deviations of the mean.

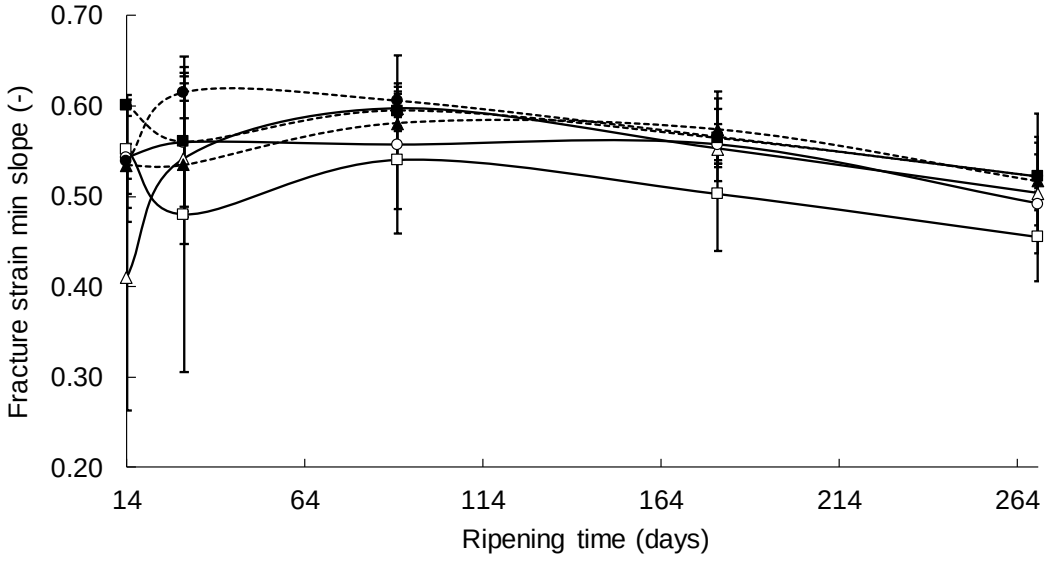

Figure 5.12. Age-related changes in fracture strain valuea of Cheddar cheese made from low protein milk (3.3 %, w/w, solid line) and high protein milk (4.0 %, w/w, broken line) Cheddar cheeses made with curd washing to a lactose plus lactic acid levels of 5.2 % (Control, non-wash, 5.2LLAMcLP, ■; 5.2LLAMcHP, □), 4.2 % (4.2LLAMcLP, ▲; 4.2LLAMcHP, △) and 3.9 % (3.9LLAMcLP, ◆; 3.9LLAMcHP, ◇). Presented values are the means of three replicate trials; error bars represent standard deviations of the mean.

Table 5.9. Degrees of freedom (df) and statistical significances (*P*-values) for changes in rheological properties in full fat Cheddar cheeses with different milk protein and lactose content[1,2].

Factor	Firmness (N)		Fracture stress (N cm^{-2})		Fracture strain (-)[3]	
(Standarized milk cheese)	df	*P*	df	*P*	df	*P*
Main plot						
Curd washing	2	0.6488	2	0.7494	2	0.2438
Sub-plot						
Time	4	<.0001	4	<.0001	4	0.1089
Interaction (Curd washing x time)	8	0.5351	8	0.1659	8	0.2196

Factor	Firmness (N)		Fracture stress (N cm^{-2})		Fracture strain (-)[2]	
(Protein-fortified milk cheese)	df	*P*	df	*P*	df	*P*
Main plot						
Curd washing	2	0.9905	2	0.8005	2	0.3077
Sub-plot						
Time	4	<.0001	4	<.0001	4	0.0087
Interaction (Curd washing x time)	8	0.9721	8	0.9560	8	0.0862

[1]Milk protein was concentrated by ultrafiltration; Lactose content was adjusted by curd-washing at cooking process to 34 °C; results are presented as the mean values of three replicate trials.
[2]Analysis of variance was carried using a general linear model (GLM) procedure of SAS, where the effects of treatment, ripening time and their interaction were estimated.
[3]Fracture strain is a dimensionless quantity.

5.3.11. Volatile compounds analysis

A total of 29 compounds were identified by solid phase micro-extraction of the headspace followed by gas chromatography in tandem with mass spectroscopy of the 270-day old cheese; 7 alcohols, 7 ketones, 2 esters, 2 aldehydes, 4 acids, 2 sulphur, 2 alkanes, 2 alkene and 1 terpene (limonene) compound were identified in all of cheeses (Figure 5.13). All of these compounds have previously been identified in Cheddar cheese (Hannon, Kilcawley, Wilkinson, Delahunty, & Beresford, 2007; Hou et al. 2014a). Although all compounds were identified in all the cheeses, the PCA of the volatile compounds was performed to assess how the individual compounds contributed to the profile of each cheese and how they discriminated between each of the cheeses (Figure 5.13). Each compound is associated with different odours, depending on its concentration and it is the balance of each of the compounds present in the cheese at a point in time that gives the overall perception of odour of each of the individual cheeses (Thomsen, Martin, Mercier, Tournayre, Berdagué, Thomas-Danguin, & Guichard, 2012; Singh, Drake, & Cadwallader, 2003). Principal components, PC1 and PC2, which accounted for 45 % and 27 % of explained variance respectively, separated the cheese on the basis of the levels of milk protein levels and wash water used. PC1 separated the cheeses on the basis of milk protein levels with standarized milk cheeses positively while protein-standardised milk cheeses scoring negatively. PC2 separated the cheeses on the basis of the different curd wash levels, with higher wash levels, lower the scoring on PC2.

The PCA biplot shows that the control (non-washed) 5.2LLAMcPFM cheese had higher levels of ketones (acetone, 2-heptanone and 2-nanonone), ester compounds (ethyl hexanoate and ethyl octanoate), aldehydes (benzeneacetaldehyde) and acids (octanoic acid). Those compounds are associated with almond, fruity, fatty, sweaty,

blue cheese and musty flavour, which was consistent with sensory attributes as fruity, buttery, sweaty/cheesy and rancid (Figure 5.14), as discussed below. Application of curd washing to the cheeses made from protein-standardised milk decreased the concentrations of the compounds which were associated with 5.2LLAMcPFM, with 4.2LLAMcPFM having the lower levels of all these compounds. In the standarized milk cheeses, alkenes (1-octene and *p*-xylene), alcohols (3-methyl-2-buten-1-ol, 3-methyl-3-buten-1-ol and ethanol), ketones (acetoin, 2,3-butanedione and 2-butanone), alakane (heptane), terpene (limonene), dimethyl sulphide and acetic acid were associated with 5.2LLAMcSM. These compounds are associated with to fruity, alcohol, buttery, and citrus flavour attributes (Singh et al, 2003), the esters contribute to 'sweet' and 'fruity' odours while carbon disulphide contributes a sulphur, boiled cabbage or garlic odour. 4.2LLAMcSM and 3.9LLAMcSM cheeses were grouped together which are associated with pentane (pungent, almond), carbon disulphide, and alcohols such as 2-butanol (alcoholic), 2-heptanol (fatty, green, blue cheese-like and fruity), and 1- and 2-pentanol (sweet, fruity, nutty and alcoholic). Interestingly, the effects of curd washing on the cheeses were described in previous studies that starter cultures actively produce free fatty acids (FFA) in the cheese vat, and some of these FFA will be lost in the cheese whey (Hickey, Kilcawley, Beresford, & Wilkinson, 2006), would decrease as the level of wash water added to the cheese vat increases.

Increasing the milk protein level from 3.3 % to 4.0 % (w/w) led to cheeses tending to contain more esters, acids and ketone compounds which may be due to their lower moisture content.

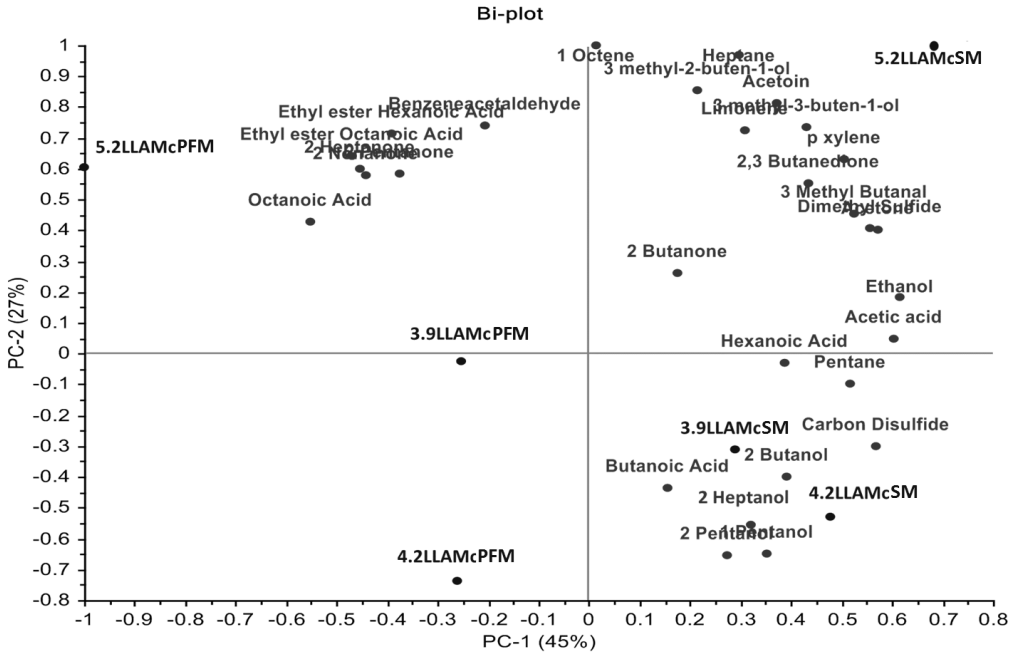

Figure 5.13. Score plot from principal component analysis of volatile data from 270 day-old standarized milk and protein-fortified milk Cheddar cheeses with curd washing to a lactose plus lactic acid levels of 5.2 % (Control, non-wash, 5.2LLAMcSM; 5.2LLAMcPFM), 4.2 % (4.2LLAMcSM; 4.2LLAMcPFM) and 3.9 % (3.9LLAMcSM; 3.9LLAMcPFM). Presented values are the means of three replicate trials.

5.3.13. Descriptive sensory analysis

The PCA plot for the different odour and flavour attributes of the 270 day-old cheeses is shown in Figure 5.14. The first two PCs discriminated between the cheeses and explained a cumulative variance of 67 %. PC1 (47 % variance) and PC2 (20 % variance) separated the cheeses on the basis of the milk protein content with the protein-standardised milk cheese scoring negatively and the standarized milk cheese scoring positively across PC1. The cheeses were classified into 4 major groupings by PC1 and PC2: 3.9LLAMcPFM; 4.2LLAMcPFM and 5.2LLAMcPFM; 3.9LLAMcSM and 5.2LLAMcSM; and 4.2LLAMcSM.

The 3.9LLAMcPFM cheeses had flavours described as caramel, waxy and buttery, odours as savoury/cooked and a sweet taste. The 4.2LLAMcPFM and 5.2LLAMcPFM cheeses had fruity, rancid and sweaty/cheesy flavours, buttery,

sweaty/cheesy, rancid, fruity and caramel odour and salty, bitter and astringent taste. The standardized milk cheeses were grouped closely to each other with 3.9LLAMcSM and 5.2LLAMcSM cheeses scored positively on PC 2 and 4.2LLAMcSM cheese scored negatively on PC 2. 3.9LLAMcSM and 5.2LLAMcSM cheeses had savoury/meaty, onion and farmyard flavour and a sweet taste for 3.9LLAMcSM cheese. The 4.2LLAMcSM cheese had mouldy, pungent and oily flavour, mouldy, pungent and farmyard odour and acid, salt, astringent and bitter taste. These attributes are consistent with the presence of various volatile compounds (Singh et al., 2003), e.g., pungent (pentane, 2-butanol, 2-pentanol, 1-propanol). These differences were associated with a higher level of alcohols (1-pentanol, 1-hexanol, 2-butanol, 2-heptanol) and the terpene, limonene in the low milk protein cheeses, and low levels of octanoic acid and benzeneacetaldehyde and ketones (2-heptanone, 2-noanone and acetone) in the protein-standardised milk cheeses.

The results also demonstrated that the highly washed cheeses tended to be sweeter and more buttery, and were less rancid and pungent, and had less ‘farmyard’ aroma. This result agrees with the findings from previous studies (Shakeel-Ur-Rehman et al., 2004; Hou et al., 2014a) who found that washed-curd Cheddar cheeses had a significantly lower intensity of unclean, acidic tastes compared to the corresponding control and 'high-lactose' cheeses. The sweet note in the washed curd cheeses may be due to their significantly lower concentrations of lactic acid during ripening (Table 5.6) and individual free amino acids such as glutamate, leucine, phenylalanine and lysine (data not shown), which contribute to acid, bitter and umami flavours (Solms, 1969; Nishimura & Kato, 1988). Moreover, the intensity of acid taste contributed by lactic and glutamic acids decreases at the higher pH (Neta, Johanningsmeier, Drake, & McFeeters, 2009), which is found in the washed curd

cheese, because of the greater dissociation of the acids and the formation of salts (lactate, glutamate).

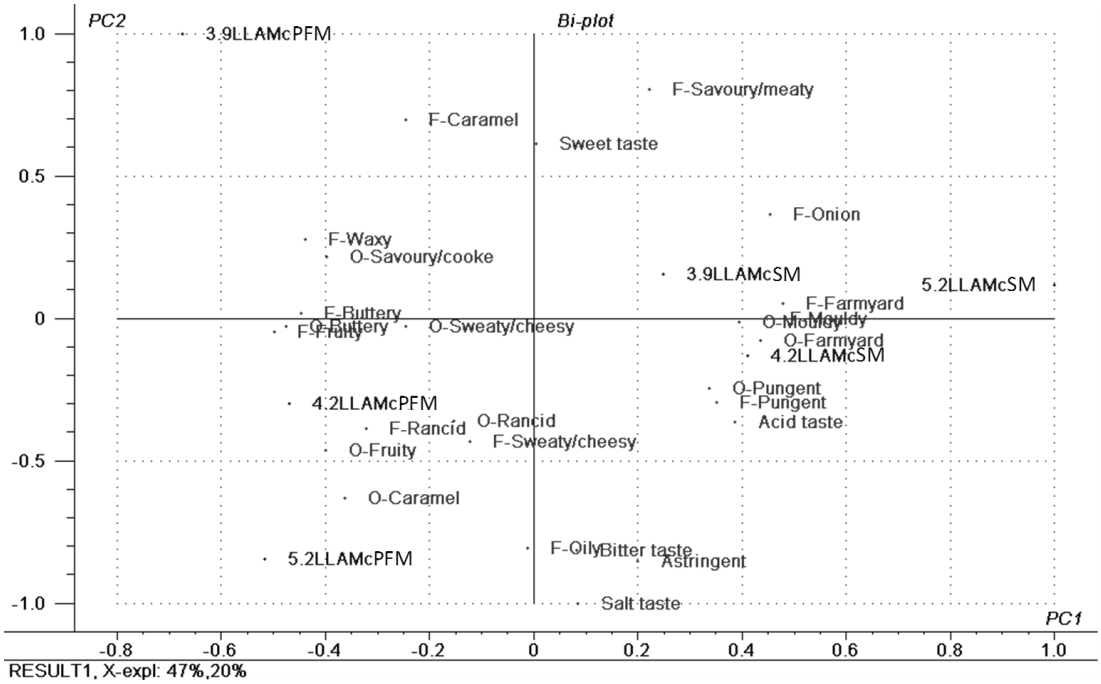

Figure 5.14. Score plot from principal component analysis of descriptive sensory odour (O) and flavour (F) attribute scores in 270 day-old Cheddar cheeses made with standarized milk and protein-fortified milk with curd washing to a lactose plus lactic acid levels of 5.2 % (Control, non-wash, 5.2LLAMcSM; 5.2LLAMcPFM), 4.2 % (4.2LLAMcSM; 4.2LLAMcPFM) and 3.9 % (3.9LLAMcSM; 3.9LLAMcPFM). Presented values are the means of three replicate trials. O = odour; F = flavour.

5.4. Conclusion

Curd washing, involving partial removal of whey from the cheese vat and its replacement with water, was used to vary the levels of lactose plus lactic acid in Cheddar cheese to 5.2, 4.2 and 3.9 % (w/w) which would correspond to the range of LLAMc in cheese made from a seasonal milk supply from predominantly spring-calved herds, as for example in Ireland, New Zealand and parts of Australia (Hou et al., 2014a). This current study indicated that curd washing significantly reduced the residual lactose, total lactate, total sugars, protein and fat in bulk whey and protein loss in bulk whey, but increased the cheese pH over ripening, fat loss in bulk whey and FAA.

High wash levels significantly reduced the concentration of most of the volatile compounds in the 270 day-old cheese. Descriptive sensory analysis on 270 day-old

cheeses showed that higher levels of curd washing coincided with a higher intensity of cheese flavours described as ‘fruity’, ‘buttery’ and ‘sweet’, while the absence of a curd washing step (control cheese) led to more ‘rancid’ and ‘farmyard’ aroma. Otherwise, curd washing did not significantly affect gross composition, microbiology, proteolysis or texture of cheese.

Increasing the milk protein level from 3.3 % (w/w) to 4.0 % (w/w) by UF significantly increased the levels of protein and fat in standardized cheese milk, protein in bulk whey, Y_a, Y_{ma}, Y_{amfpam}, cheese firmness and fracture stress, while decreased the cheese moisture and pH4.6-SN at ≥ 90 days. Descriptive sensory analysis of the 270 day-old cheeses indicated that protein-standardised milk cheeses had more fruity, buttery and caramel flavours and odours than the corresponding standard protein milk cheeses which had more mouldy, farmyard flavours and odour and bitter taste. Otherwise, altering milk protein level from 3.3 to 4.0 % had little effect on Cheddar cheese characteristics.

The results suggest that washing cheese curds made from protein-fortilised milk may be used as a means of creating Cheddar cheese variants with distinctive flavour profiles (from more savoury to sweeter), and can also avoid the accumulation of high levels of residual lactose during ripening, which can lead to undesirable browning during cooking applications or allergy reactions such as lactose intorlerance.

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Chapter 6: Screening and selection of different strains of *Sc. thermophilus* and *Lactobacillus* as adjunct cultures for use in future Cheddar cheese processing

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Abstract

Fifty-one dairy *Sc. thermophilus* and 103 *Lactobacillus* species from Moorepark culture collection were studied for their sugar metabolism ability, salt sensitivity and fermentation rate. Only 8 % of the *Sc. thermophilus* strains were galactose-positive. In a LM-17 medium, most of the *Sc. thermophilus* strains excreted galactose into the medium after lactose depletion. The *Sc. thermophilus* strains grew in the presence of 0-2 % salt in MRS broth but were unable to tolerate up to 3 % salt. They had various acidification rates in 10 % recosititeted skim milk (RSM) under the same condition, while galactose-positive strains were generally faster lactic acid producers than the galactose-negative strains. Meanwhile, *Lactobacillus* strains were mostly galactose-positive and when used in combination with galactose-negative *Sc. thermophilus* strains, they accelerated the fermentation process. Based on sugar metabolism, salt sensitivity and acidification ability, two *Sc. thermophilus* strains (one galactose positive and one galactose negative) and one *Lactobacillus* strain (galactose positive) were selected for further cheese making.

6.1. Introduction

The composition of culture systems used for Cheddar manufacture has changed in recent years, principally due to the common inclusion of *Sc. thermophilus* for its thermo- and phage resistance properties. Since *Sc. thermophilus* primarily metabolizes only the glucose moiety of lactose, galactose accumulates during manufacture (Thomas, Turner, & Crow 1980; Tinson, Hillier, & Jago, 1982a; Michel & Martley, 2001) leading to problems such as browning during cooking of cheeses. However, De Vin, Rådström, Herman & De Vuyst (2005) reported that ~ 16 % of 49 strains of *Sc. thermophilus* evaluated on M17 medium supplemented with galactose were galactose-positive. Thomas & Crow (1984) investigated the galactose-metabolizing ability of *Sc. thermophilus* from different sources and found that most were galactose-negative (Gal^-) because of failure to induce galactokinase. Bley, Johnson & Olson (1985) reported that the use of a 0.5 % (w/w) non-galactose-fermenting *Sc. thermophilus* as an adjunct resulted in higher level of residual galactose in one month-old stirred curd Cheddar (compared to the control cheese) and intensified the degree of browning in processed cheese made therefrom. Similarly, Michel and Martley (2001) found that Cheddar cheese made using *Sc. thermophilus*, as an adjunct culture to *Lactococcus. lactis* subsp. *cremoris* or *lactis* strains, had a high residual galactose level of ~ 26.6 mmol/kg (0.48 %, w/w) at 1 day. Moreover, the residual galactose content increased as the scald temperature was increased from 38 to 41 °C (data not reported). Tinson, Ratcliffe, Hillier & Jago (1982b) reported that high levels of residual galactose (33 mmol/kg, 0.56 %, w/w) in 8 wk-old Cheddar cheese made using *Sc. thermophilus* (0.5 %, w/w) as an adjunct to *Lc. lactis* subsp. *cremoris* coincided with a higher production of CO_2, leading to the development of slits and fractures in the cheese at 8 and 14 wks.

Mesophilic lactobacilli bacteria constitute the major non-starter lactic acid bacteria (NSLAB) populations in Cheddar cheese (Jordan & Cogan, 1993, Fitzsimons, Cogan, Condon, & Beresford, 1999). The addition of lactobacilli as an adjunct culture in Cheddar cheese making has been studied previously by many researchers, who reported that using lactobacilli as an adjunct culture increases the concentration of free amino acids during ripening and enhances flavour development (Lee, Laleye, Simard, Holley, Emmons & Giroux, 1990; Kiernan, Beresford, O'Cuinn, & Jordan, 2000; Fenelon, Beresford, & Guinee, 2002; Hannon, Wilkinson, Delahunty, Wallace, Morrissey & Beresford, 2003). Lee et al. (1990) added homofermentative lactobacilli (*Lactobacillus casei* subsp. *casei*, *Lb. casei* subsp. *pseudoplantarum* and *Lb. plantarum*) in conjunction with the normal starter cultures to Cheddar cheese and observed a reduction in the maturation time of 2 months when ripening for 8 months period. They also studied the influence on sensory properties in Cheddar cheese and found certain strains of *Lb. casei* subsp. *casei*, *Lb. casei* subsp. *pseudoplantarum*, *Lb. plantarum* and *Lb. casei* subsp. *rhamnosus* produced several defects in cheese including high acidity, bitterness, off-flavour and openness. Kiernan et al. (2000) also added seven strains of mesophilic lactobacilli bacteria individually as adjuncts to Cheddar cheese with normal starter cultures to determine the autolysis of those strains during cheese ripening. They also added a thermophilic *Lactobacillus helveticus* strain as an adjunct culture. The results showed that mesophilic lactobacilli bacteria did not lyse during cheese ripening while the thermophilic *Lb. helveticus* strain lysed and released its intracellular enzymes which improved the cheese flavour. Fenelon et al. (2002) compared six different culture systems for their effect on proteolysis and flavour of reduced-fat Cheddar cheese. They reported that using *Lb. helveticus* as an adjunct culture increased the low molecular mass peptides, free amino acids and

flavour scores at day 90 and 180. Hannon et al. (2003) manufactured Cheddar cheese with three related starter system with warying autolytic properties. The level of proteolysis and intracellular enzyme, lactate dehydrogenase was higher in the cheeses made with adjunct culture and they also had a more balanced and stronger flavour.

The objective of the current study was to evaluate the galactose phenotype of 51 *Sc. thermophilus* strains and 103 *Lactobacillus* strains from dairy origin and also their sugar metabolism ability, salt sensitivity and fermentation rate to select suitable adjunct cultures for further Cheddar cheesemaking trials.

6.2. Material and Methods

6.2.1. Strains of Sc. thermophilus and Lactobacillus

Over 200 strains of *Sc. thermophilus* from the Moorepark Food Research Centre culture collection from dairy and intestinal origin were screened on their genotype using Pulsed Field Gel Electrophoresis (PAGE) previously (Hannon et al, unpublished; O'Sullivan, & Fitzgerald, 1998) and a total of 51 strains were selected and screened for their sugar metabolism, salt sensitivity and acid production rate. All strains were maintained at -80 °C in MRS or LM17 (Oxoid Ltd., Basingstoke, United Kingdom) containing 20 % glycerol.

A total of 103 species of *Lactobacillus* from the Moorepark Food Research Centre culture collection from dairy and intestinal origin were screened for their growth rate, galactose metabolism and rate of acidification as an adjunct culture. All strains were maintained at -80 °C in MRS or LM17 (Oxoid Ltd., Basingstoke, United Kingdom) containing 20 % glycerol.

6.2.2. Strain activation

6.2.2.1. Activation of Sc. thermophilus strains

Strains of *Sc. thermophilus* were taken aseptically from culture stock at -80 °C and grown overnight at 42 °C in reconstituted 10 % (w/v), antibiotic-free skim milk powder solution (Kerry Ingredients Ltd., Charleville, Co. Cork, Ireland) which had been heat treated at 95 °C for 30 min. All strains were streaked individually on LM17 plates (Oxoid Ltd., Basingstoke, United Kingdom) using inoculation loops and incubated at 42 °C for 3 days. All strains were propagated twice in LM17 agar plate to ensure the activity. *Enterococcus* contamination was checked on kanamycin esculin azide agar (KAA) plates at 30 °C for 24 hours.

6.2.2.1. Activation of Lactobacillus strains

Strains of *Lactobacillus* were taken aseptically from culture stock in -80 °C freezer and grew twice on de Man, Rogosa, and Sharpe (MRS) plates (Oxoid Ltd., Basingstoke, United Kingdom), which had previously been autoclaved at 121 °C for 15 minutes; then incubate anaerobically using the GasPak™ EZ Gas Generating Pouch Systems (Becton, Dickinson and Company, 1 Becton Drive, Franklin Lakes, New Jersey) at 30 °C for 3 days.

6.2.3. Sugar metabolism ability

6.2.3.1. Sugar metabolism ability of Sc. thermophilus strains

Activated *Sc. thermophilus* strains were grown in sterilised L-M17 broth overnight at 37 °C.

Sterilised M17 broth (~ 4.5 %, w/v, no sugar content) was prepared in glass test tubes (5 ml each tube). The glass test tubes with M17 broth was then added with

1 ml of 10 % (w/v) glucose, lactose or galactose solutions separately and was autoclaved for 15 minutes at 121 ºC.

Activated *Sc. thermophilus* strains were inoculated at the level of 0.1 % to each M17 broth containing individual sugars. The samples were then incubated at 37 °C for 3 days. Control samples (M17 broth + individual sugars, un-inoculated) were also prepared. Optical density (OD) at 600 nm was measured after 24 hours incubation. All experiments were carried out in duplicate.

6.2.3.2. Sugar metabolism ability of Lactobacillus strains

Lactobacillus strains from culture collection were activated twice on MRS agar which was incubated anaerobically using the gas pack system (Anaerocult® A, Merck, Ireland) at 30 °C for 3 days. One colony of each test strain was inoculated in to 10 ml Gal-MRS broth tube and incubated at 30 °C for 2 days. OD_{600nm} reading was measured after 24 and 48 hours of incubation. Control tubes were un-inoculated. If the OD_{600nm} reading was > 1.0, the sample was diluted with Gal-MRS broth to obtaining an OD reading lower than this value and final OD_{600nm} reading was corrected accordingly. All experiments were carried out in duplicate.

Gal-MRS broth was prepared as previously described by Korakli, Pavlovic, Ganzle, & Vogel, (2003) with modifications (modified MRS broth containing 5 % of galactose instead of glucose, without the vitamin supplement). The composition of 1 litre of the MRS was as follows: peptone from casein, 10 g; yeast extract, 4 g; meat extract, 8 g; $K_2HPO_4 \cdot 3H_2O$, 2 g; KH_2PO_4, 5 g; $MgSO_4 \cdot 1H_2O$, 0.2 g; $MnSO_4 \cdot 4H_2O$, 0.05g; L-Cys, 0.5 g; Tween 80, 1 ml. The final pH was adjusted to 6.2 using 1 N HCl. Gal-MRS broth was made by mixing 9 ml of broth with 1 ml of 25 (%, w/v) galactose

solution. MRS broth and the galactose solution (25 %, w/v) were autoclaved separately at 121 °C for 15 min. After sterilization, all media components were mixed aseptically.

6.2.4.. Salt tolerance of Sc. thermophilus strains

Activated *Sc. thermophilus* strains from LM-17 plate were inoculated into 5 ml single-strength MRS broth tube and incubated overnight at 37 °C. After the growth of strains in single-strength MRS broth, 0.1 % (v/v) of the overnight strain was inoculated into 10 ml of double-strength MRS broth containing different levels of NaCl: 0, 1, 2, 3, 4, 5, 6 and 6.5 % (w/v). Those strains were incubated at 37 °C and the pH of MRS broth was measured after 2 and 7 days of incubation. A control tube with each concentration of salt and without inoculation was also included. All experiments were done in duplicate.

6.2.5. Acidification profiles

6.2.5.1. Acidification profiles of Sc. thermophilus strains

The acidification profiles of strains were measured using the CINAC pH monitoring system (Ysebaert, Frépillon, France)using the Cheddar cheese making temperature profile. The system monitored the reduction in pH by recording values every 5 min. Strains of *Sc. thermophilus* were grown overnight at 37 °C in heat-treated 10 % (w/v) reconstituted antibiotic-free skim milk powder solution. After growth, 1 % (v/v) activated culture was inoculated into 100 ml heat-treated 10 % (w/v) reconstituted skim milk powder solution bottle at 30 °C. A previously calibrated and sterilised CINAC pH probe was placed into each of the inoculated milk and covered with parafilm (Bemis Corporate, One Neenah Center, Neenah, WI).

pH changes were measured over 5 hours under same temperature profile used during Cheddar cheese manufacture. Samples were incubated at 30 °C for the first 80 min (in cheese: time from starter addition to cut curd) and the samples were cooked as Cheddar cheese manufacture procedure which was to increase the temperature at 1 °C per 5 minutes until 38.5 °C (42 mins) was reached. Temperature was maintained at 38.5 °C for the rest of 178 mins.

6.2.5.2. Acidification profiles of combination of selected strains

Based on sugar metabolism, salt sensitivity and acidification ability, selected strains of *Sc. thermophilus* and *Lactobacillus* was tested on CINAC pH monitoring system with combination of defined strain starter cultures, which were used in cheesemaking (*Lactococcus lactis* subsp. *lactic* strain 227 and 303; Chr. Hansen Ireland Ltd., Little Island, Co. Cork, Ireland). The strain combinations tested were:

- *Lactococcus lactis* subsp. *lactis* strain 227 and 303
- *Lactococcus lactis* subsp. *lactis* strain 227 and 303 + *Sc. thermophilus* DPC5095 (Gal^-)
- *Lactococcus lactis* subsp. *lactis* strain 227 and 303 + *Sc. thermophilus* DPC1796 (Gal^+)
- *Lactococcus lactis* subsp. *lactis* strain 227 and 303 + *Sc. thermophilus* DPC5095 (Gal^-) + 10 Gal^+ *Lactobacillus* strains
- *Lactococcus lactis* subsp. *lactis* strain 227 and 303 + *Sc. thermophilus* DPC1796 (Gal^+)+ 10 Gal^+ *Lactobacillus* strains

The temperature profile was the same as described above.

6.2.6. Fermentation profiles of Sc. thermophilus strains

Fermentation profiles of *Sc. thermophilus* strains was measured by measurement of OD_{600nm} and levels of residual sugars (lactose, glucose and galactose) remaining, each hour during incubation at 42 °C for 8 hours using the modified method as reported by De Vin et al, (2005). Activated *Sc. thermophilus* strain were inoculated at 1 % into 0.5 % (w/v) lactose LM-17 medium and incubated at 42 °C. Sample of 20 ml incubated LM-17 medium was transferred aseptically into sterilised containers each hour over 8 hours and OD_{600nm} was determined immediately after the sample was taken and the rest of the sample was frozen at -20 ˚C to avoid further sugar degradation.

The sugars were extracted and measured using the HPLC method described by Zeppa, Conterno & Gerbi, (2001), apart from a modification in the equipment used and flow rate as described by Hou, McSweeney Beresford, & Guinee, (2014). Lactose, glucose and galactose were separated and eluted on a 300 x 7.8mm Aminex HPX-87C cation exchange carbohydrate column (Bio-Rad Laboratories, Richmond, CA), and detected with a Waters 2414 Refractive Index Detector (Waters, Bray, Ireland). The mobile phase was 0.009 N sulphuric acid at a flow rate of 0.5 mL/min. The concentrations of sugars in the cheeses were calculated by comparing the peak area of samples to standard curves. Sugar concentrations were calculated as g/100 g cheese.

Three fermentation profile was identified based on the galactose metabolism ability of the strains.

6.3. Results and Discussion

6.3.1. Strain activation and identification

Sixty-two *Sc. thermophilus* strains were activated from culture collection and, before any further test, they were checked for purity by streaking on a KAA plate to check for the any presence of *Enterococcius* contamination (black colonies). Strains found to be impure because of *Enterococcus* contamination on KAA plate or under microscope were not used in further screening (Table 6.1). Species specific polymerase chain reaction (PCR) analysis was undertaken for strain identification (data not shown).

Table 6.1. Summary of all 53 *Sc. thermophilus* strains selected from Moorepark culture collection with activation and identification.

# DPC	Stock at -20°C	*Microscopy*	*KAA*	PCR
1155	x	Pure	Clear	√
1796	x	Pure	Clear	√
1148	-	Pure	Clear	√
1283	-	Pure	Clear	-
1831	x	Pure	Clear	√
1167	-	Pure	Clear	√
1170	-	Pure	Clear	-
1151	x	Pure	Clear	√
2579	x	Pure	Clear	√
1176	x	Pure	Clear	√
1172	x	Pure	Clear	√
2224	x	2 types of coccus	Clear	-
5010	x	Pure	Clear	√
1149	x	Pure	Clear	√
1797	x	Pure	Clear	√
1150	x	Rods	Clear	√
1829	x	Pure	Clear	√
1834	x	Pure	Clear	√
1835	x	Pure	Clear	√
2218	x	Pure	Clear	√
1171	x	Pure	Clear	√
1177	x	Pure	Clear	√
2546	x	Pure	Clear	√
2544	x	Unpure (cocci+rods)	Black	-
1842	x	Pure	Clear	√
1795	-	Pure	Clear	√
2220	x	Pure	Clear	√
5095	x	Pure	Clear	√
1154	x	Pure	Clear	√
1173	x	Pure	Clear	√
1818	x	Pure	Clear	√
5061	x	Pure	Clear	√
1775	x	Pure	Clear	√
1147	x	Pure	Clear	√
1853	-	Pure	Clear	-
1153	x	Pure	Clear	√
1156	x	Pure	Clear	√
1169	x	Pure	Clear	√
1802	-	2 types of coccus	Black	-
1805	-	2 types of coccus	Black	-
1808	-	Unpure	Black	-
1174	x	Pure	Clear	√
1179	x	Pure	Clear	√
1285	x	Unpure	Black	√
1287	x	Pure	Clear	-
1777	x	Pure	Clear	√
1780	-	Pure	Clear	-

1781	x	Unpure	Black	-
3544	-	Unpure	Black	-
1809	x	Pure	Clear	√
1821	x	Pure	Clear	√
1822	x	Pure	Clear	√
1823	x	Unpure	Black	-
1828	x	Pure	Clear	√
5279	x	Pure	Clear	√
2214	x	2 types of coccus	Black	-
2216	x	Pure	Clear	√
2219	x	Pure	Clear	√
1150	x	Pure	Clear	√
1173	x	Pure	Clear	√
1835	x	Pure	Clear	√
5062	x	Pure	Clear	√

6.3.2. Sugar metabolism

6.3.2.1. Sugar metabolism ability of Sc. thermophilus strains

Fifty-one strains were screened for their ability to metabolise lactose, galactose and glucose in M17 media containing single source of sugar (Figure 6.1a, 6.1b and 6.1c). All the strains were able to metabolise lactose with 96 % strains had OD_{600nm} reading > 1.0 after 3 days incubation at 37 °C. However, after the 3 days fermentation, only 7.7% of strain was able to metabolism galactose in a galactose containing media. This is consistent with previous studies which reported most strains of *Sc. thermophilus* used in the dairy industry are unable to metabolise galactose (Thomas & Crow, 1984; Hutkins, Morris & McKay, 1985; Hutkins, Halambeck, & Morris, 1986; Vaillancourt, Moineau, Frenette, Lessard, & Vadeboncoeur, 2002; Robitaille, Moineau, St-Gelais, Vadeboncoeur & Britten, 2007). Moreover, De Vin et al, (2005) reported that only ~ 16 % of 49 strains of *Sc. thermophilus* evaluated on M17 medium supplemented with galactose were galactose-positive.

Based on the OD_{600nm} readings on galactose, all strains were divided into three groups (Table 6.2): OD_{600nm} reading below 0.5 indicated that the strain is not able to

metabolise galactose or only to metabolise the sugar to a very limited extent were grouped with "-" symbol. Between OD_{600nm} reading 0.50 and 1.0, strains can use galactose efficiently were called "+", while those strains demonstrating an OD_{600} >1.0, were called "++".

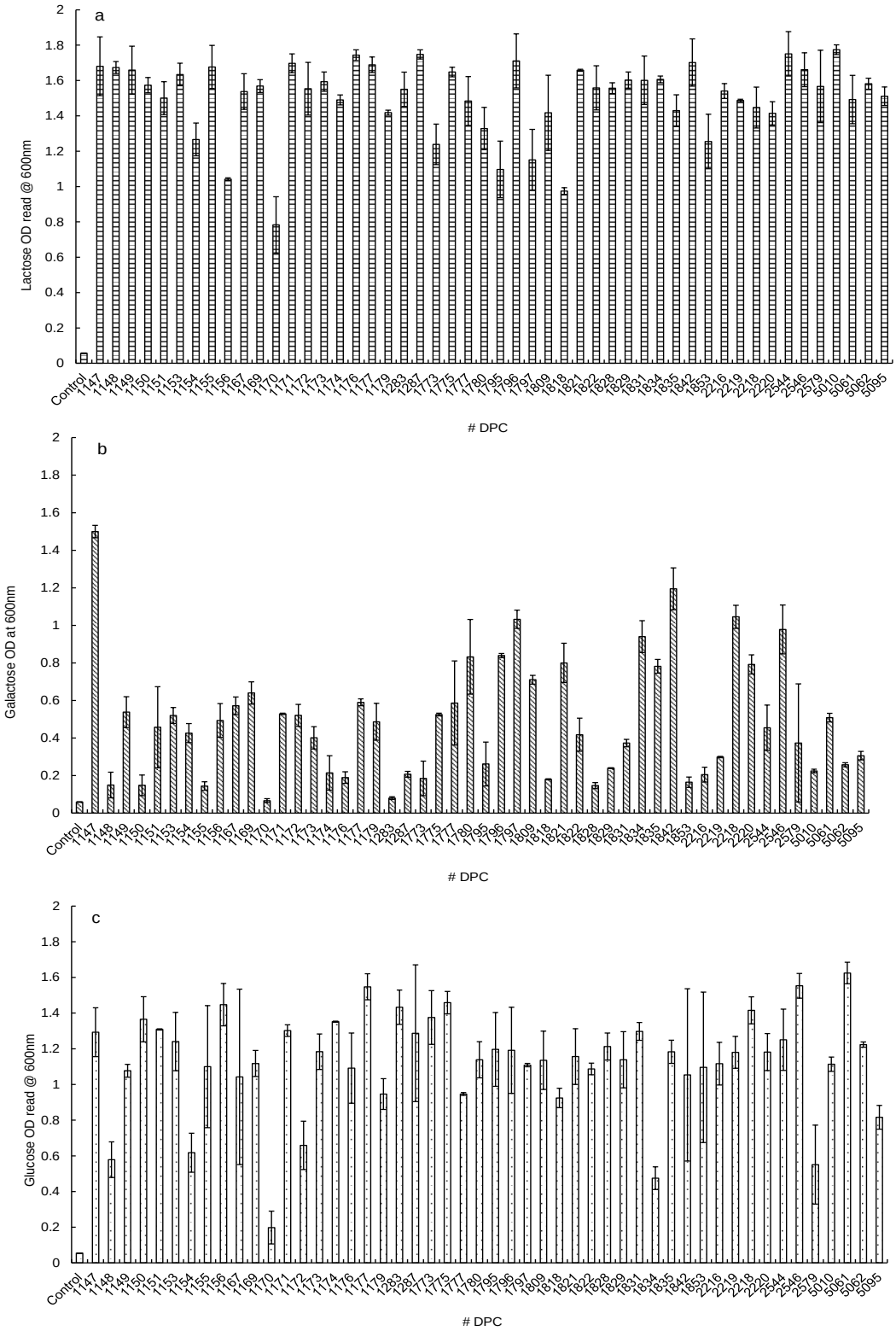


Figure 6.1. Sugar metabolism ability of *Sc. thermophilus* strains in LM17 broth supplemented with (a), lactose, (b), galactose and (c), glucose using OD_{600nm} reading.

Table 6.2. Summary of different characteristics including galactose metabolism, acidification rate and salt sensitivity of *Sc. thermophilus* strains.

# DPC	Galactose	Acidifier	Salt Sensitivity
1147	++	Slow	+
1148	-	Very Slow	+
1149	+	Slow	-
1150	-	Very Slow	++
1151	-	Slow	++
1153	+	Fast	-
1154	-	Slow	+
1155	-	Slow	+
1156	-	Very Slow	+
1167	+	Medium	+
1169	+	Fast	+
1170	-	Slow	++
1171	+	Very Slow	++
1172	+	Medium	+
1173	-	Very Slow	++
1174	-	Medium	-
1176	-	Medium	-
1177	+	Very Slow	++
1179	-	Fast	+
1283	-	Very Slow	+
1287	-	Medium	++
1773	-	Very Slow	++
1775	+	Medium	++
1777	+	Fast	+
1780	+	Very Slow	++
1795	-	Very Slow	+
1796	+	Fast	+
1797	++	Very Slow	-
1809	+	Medium	+
1818	-	Very Slow	+
1821	+	Fast	+
1822	-	Fast	++
1828	-	Very Slow	+
1829	-	Fast	++
1831	-	Fast	++
1834	+	Very Slow	+
1835	+	Fast	++
1842	+	Medium	+
1853	+	Medium	+
2216	-	Fast	++
2219	-	Very Slow	+
2218	++	Fast	++
2220	+	Medium	+
2544	-	Fast	++
2546	+	Medium	+
2579	-	Medium	+
5010	-	Fast	+
5061	-	Very Slow	++
5062	-	Fast	-
5095	-	Fast	++
5279	-	Fast	++

6.3.2.2. Sugar metabolism ability of Lactobacillus strains

103 strains were screened for their galactose metabolism ability in MRS media containing galactose and the OD_{600nm} reading were taken after 1 and 2 day fermentation (Table 6.3). After day 1 fermentation, 14.6 % of *Lactobacillus* strains increased the OD reading to between 5 to 7 and 47.6 % of strains reached 3 to 5, however, 37.9 % of strains stayed between 0 to 3. Continuing fermentation on day 2, over 50 % stains reached high OD reading to above 5, while 8.74 % strains still remained at 0 to 3.

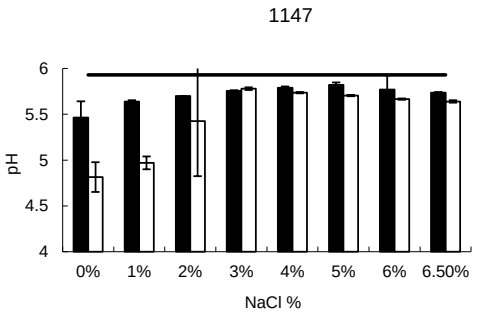
Table 6.3. Galactose metabolism of 103 *Lactobacillus* strains based on OD_{600nm} reading after 1 and 2 day fermentation in galactose containing MRS broth.

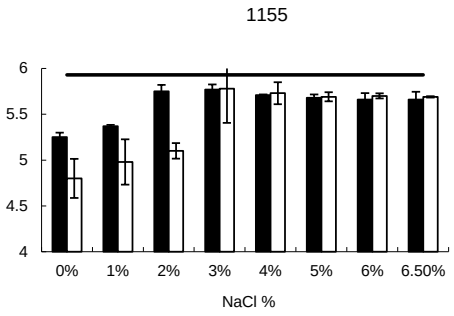
Day 1			Day 2		
# DPC	Strains	OD_{600nm} reading	# DPC	Strains	OD_{600nm} reading
6190	*Lactobacillus*	0.04	6191	*Lactobacillus*	0.05
6191	*Lactobacillus*	0.04	6192	*Lactobacillus*	0.12
4808	*Mesophilic Lb*	0.05	6149	*Lactobacillus*	0.12
6149	*Lactobacillus*	0.05	6126	*Lactobacillus*	0.28
6192	*Lactobacillus*	0.05	4687	*Unidentified*	0.56
4805	*Lb. paracasei*	0.06	5622	*Lb.acidophilus*	0.68
5622	*Lb.acidophilus*	0.07	6190	*Lactobacillus*	1.20
4687	*Unidentified*	0.11	4815	*Lactobacillus*	1.35
6126	*Lactobacillus*	0.19	6135	*Lactobacillus*	2.84
6123	*Lactobacillus*	0.24	4778	*Lb. paracasei*	3.00
4775	*Lb. paracasei*	0.35	4780	*Lb. paracasei*	3.07
4780	*Lb. paracasei*	0.36	5253	*Lactobacillus*	3.13
4814	*Lactobacillus*	0.49	4816	*Lactobacillus*	3.16
6060	*Lb.acidophilus*	0.51	6060	*Lb.acidophilus*	3.26
4815	*Lactobacillus*	0.58	6137	*Lactobacillus*	3.31
4767	*Lb. paracasei*	0.62	4726	*Lb. paracasei*	3.33
4813	*Lactobacillus*	0.64	4740	*Lb. paracasei*	3.35
6135	*Lactobacillus*	0.65	6123	*Lactobacillus*	3.37
4778	*Lb. paracasei*	0.70	4775	*Lb. paracasei*	3.38
5260	*Lactobacillus*	0.80	4748	*Lb. paracasei*	3.42
6057	*Lb. ruteri*	1.34	4736	*Lb. paracasei*	3.43
4710	*Lb. casei*	1.35	4803	*Lb. paracasei*	3.45
5570	*Lactobacillus*	1.47	4671	*Lb. paracasei*	3.59
4821	*Lb. paracasei*	1.67	4805	*Lb. paracasei*	3.60
4693	*Lb. rhamnosus*	1.82	4730	*Lb. paracasei*	3.93
4806	*Lb. paracasei*	1.88	4667	*Lb. paracasei*	4.11
6137	*Lactobacillus*	1.99	4715	*Lb. paracasei*	4.17
4819	*Lb. paracasei*	2.14	4693	*Lb. rhamnosus*	4.17
6084	*Lb. ruteri*	2.21	4721	*Lb. paracasei*	4.22
6059	*Lb. casei*	2.22	4712	*Lb. paracasei*	4.26
4852	*Lb. casei*	2.25	6057	*Lb. ruteri*	4.29
4802	*Lb. paracasei*	2.28	4745	*Lb. paracasei*	4.30
4816	*Lactobacillus*	2.38	4742	*Lb. paracasei*	4.34
4770	*Lb. paracasei*	2.49	4673	*Lb. paracasei*	4.37
5253	*Lactobacillus*	2.58	4767	*Lb. paracasei*	4.40
4810	*Mesophilic Lb*	2.69	4729	*Lb. paracasei*	4.41
4759	*Lb. paracasei*	2.73	4738	*Lb. paracasei*	4.49
4673	*Lb. paracasei*	2.87	4813	*Lactobacillus*	4.63
4853	*Lb. casei*	2.91	4806	*Lb. paracasei*	4.69
6136	*Lactobacillus*	3.05	4727	*Lb. paracasei*	4.70
5270	*Lactobacillus*	3.06	4733	*Lb. paracasei*	4.83
4818	*Lb. paracasei*	3.08	5570	*Lactobacillus*	4.92
5264	*Lactobacillus*	3.09	4710	*Lb. casei*	4.98
4762	*Lb. paracasei*	3.22	4734	*Lb. paracasei*	5.11
4712	*Lb. paracasei*	3.26	4773	*Lb. paracasei*	5.23
4854	*Lb. casei*	3.35	6136	*Lactobacillus*	5.31
4764	*Lb. paracasei*	3.40	4692	*Lb. rhamnosus*	5.56
4674	*Lb. paracasei*	3.44	4808	*Mesophilic Lb*	5.63
6130	*Lactobacillus*	3.44	4749	*Lb. paracasei*	5.64
5269	*Lactobacillus*	3.46	4812	*Lactobacillus*	5.66

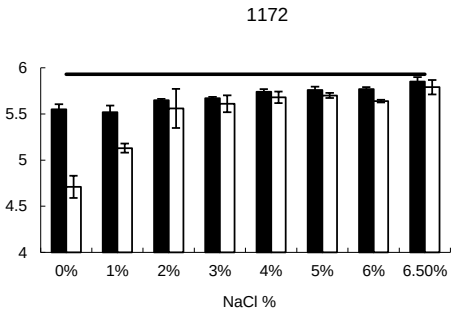
4768	*Lb. paracasei*	3.48	4810	*Mesophilic Lb*	5.67
5276	*Lactobacillus*	3.49	4768	*Lb. paracasei*	5.80
5266	*Lactobacillus*	3.51	4674	*Lb. paracasei*	5.83
4671	*Lb. paracasei*	3.54	5411	*Lactobacillus*	5.94
4748	*Lb. paracasei*	3.56	5269	*Lactobacillus*	5.97
4667	*Lb. paracasei*	3.61	5264	*Lactobacillus*	6.06
4803	*Lb. paracasei*	3.62	5071	*Lb. casei*	6.06
6080	*Lactobacillus*	3.72	5270	*Lactobacillus*	6.08
6120	*Lactobacillus*	3.73	5255	*Lactobacillus*	6.10
5411	*Lactobacillus*	3.74	5266	*Lactobacillus*	6.12
5071	*Lb. casei*	3.76	5276	*Lactobacillus*	6.12
4923	*Lb. casei*	3.87	5375	*Lb. paracasei*	6.14
4730	*Lb. paracasei*	3.87	5260	*Lactobacillus*	6.15
4734	*Lb. paracasei*	3.91	4762	*Lb. paracasei*	6.16
4820	*Lb. paracasei*	4.01	4690	*Lb. rhamnosus*	6.16
6070	*Lactobacillus*	4.07	5258	*Lactobacillus*	6.16
4726	*Lb. paracasei*	4.12	4925	*Lb. pentosus*	6.17
4851	*Lb. plantarum*	4.21	4926	*Lb. paracasei*	6.19
4925	*Lb. pentosus*	4.21	4923	*Lb. casei*	6.19
4749	*Lb. paracasei*	4.24	4922	*Lp. plantarum*	6.22
4688	*Lb. rhamnosus*	4.28	5251	*Lactobacillus*	6.23
4773	*Lb. paracasei*	4.32	6130	*Lactobacillus*	6.25
5255	*Lactobacillus*	4.35	4921	*Lb. casei*	6.26
4715	*Lb. paracasei*	4.40	4814	*Lactobacillus*	6.27
5375	*Lb. paracasei*	4.41	4924	*Lb. paracasei*	6.30
4771	*Lb. paracasei*	4.48	4759	*Lb. paracasei*	6.31
4926	*Lb. paracasei*	4.48	4852	*Lb. casei*	6.34
4721	*Lb. paracasei*	4.48	4853	*Lb. casei*	6.39
5251	*Lactobacillus*	4.57	4818	*Lb. paracasei*	6.39
4755	*Lb. paracasei*	4.59	4770	*Lb. paracasei*	6.40
4812	*Lactobacillus*	4.59	4851	*Lb. plantarum*	6.44
4921	*Lb. casei*	4.59	4755	*Lb. paracasei*	6.45
4850	*Lb. plantarum*	4.61	4854	*Lb. casei*	6.46
4760	*Unidentified*	4.63	4750	*Lb. paracasei*	6.50
4692	*Lb. rhamnosus*	4.68	4850	*Lb. plantarum*	6.52
4817	*Lb. paracasei*	4.73	4819	*Lb. paracasei*	6.52
6058	*Lb. plantarum*	4.82	6059	*Lb. casei*	6.53
4738	*Lb. paracasei*	4.99	4821	*Lb. paracasei*	6.62
4750	*Lb. paracasei*	5.02	4820	*Lb. paracasei*	6.67
4742	*Lb. paracasei*	5.03	6084	*Lb. ruteri*	6.67
5412	*Lactobacillus*	5.10	6070	*Lactobacillus*	6.82
4727	*Lb. paracasei*	5.11	4771	*Lb. paracasei*	6.87
4733	*Lb. paracasei*	5.28	4802	*Lb. paracasei*	7.00
4690	*Lb. rhamnosus*	5.30	4688	*Lb. rhamnosus*	7.16
4736	*Lb. paracasei*	5.45	5413	*Lactobacillus*	7.21
4740	*Lb. paracasei*	5.52	4817	*Lb. paracasei*	7.30
4729	*Lb. paracasei*	5.53	4760	*Unidentified*	7.38
4745	*Lb. paracasei*	5.79	5412	*Lactobacillus*	7.47
4924	*Lb. paracasei*	5.90	6120	*Lactobacillus*	7.58
6124	*Lactobacillus*	6.05	6080	*Lactobacillus*	7.60
5413	*Lactobacillus*	6.23	6058	*Lb. plantarum*	7.70
5258	*Lactobacillus*	6.71	6124	*Lactobacillus*	7.73
4922	*Lp. plantarum*	6.92	4764	*Lb. paracasei*	7.85

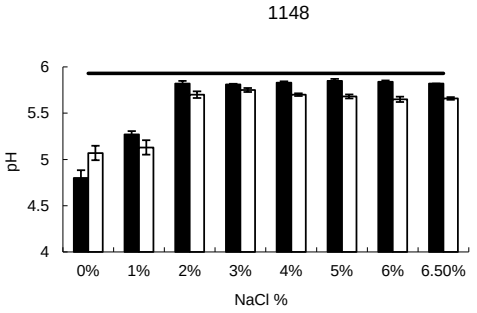
6.3.3. Salt tolerance ability of Sc. thermophilus strains

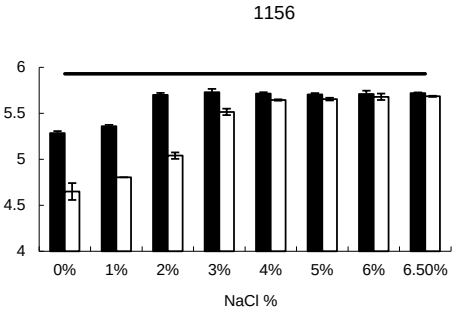
Salt is added in dry form or as brine during cheese manufacture to add flavour and avoid excess growth of lactic acid bacteria which produces lactic acid in cheese during manufacture and ripening. . Hence, salt sensitivity test was done on *Sc. thermophilus* strains which shows the ability of the strain to deal with an osmotic stress. The salt sensitivity of *Sc. thermophilus* strains were tested by measuring pH after 2 and 7 days incubation period in MRS broth containing different levels of NaCl (Figure 6.2). Six strains of *Sc. thermophilus* (DPC1149, 1153, 1174, 1176, 1797 and 5062) were very salt sensitive as they were not able to decrease the pH below 5 within 2 days. Most of the strains were able to grow and decrease the pH at 2 % salt concentration under a 7 day incubation period at 37 °C and very few of them were able to reduce pH at salt concentration > 3 %.

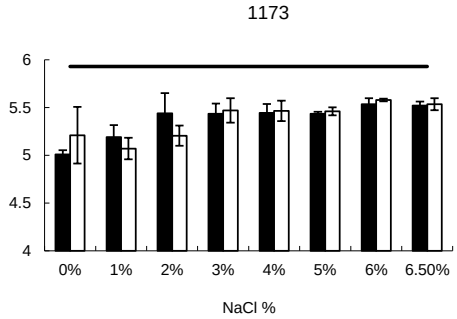

1147
pH
6
5.5
5
4.5
4
0%
1%
2%
3%
4%
5%
6%
6.50%
NaCl %

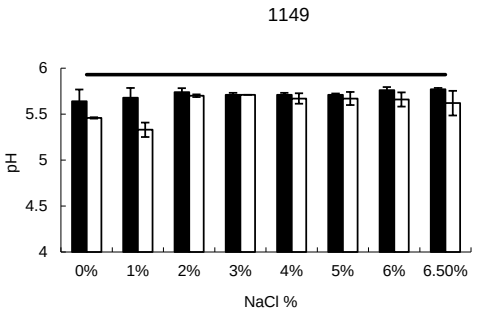

1155
6
5.5
5
4.5
4
0%
1%
2%
3%
4%
5%
6%
6.50%
NaCl %

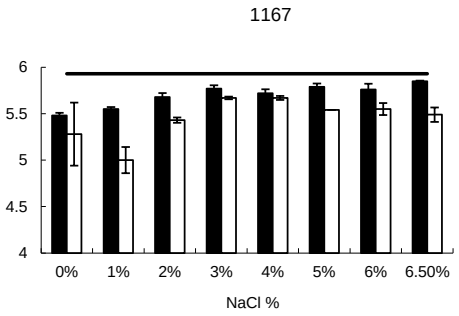

1172
6
5.5
5
4.5
4
0%
1%
2%
3%
4%
5%
6%
6.50%
NaCl %

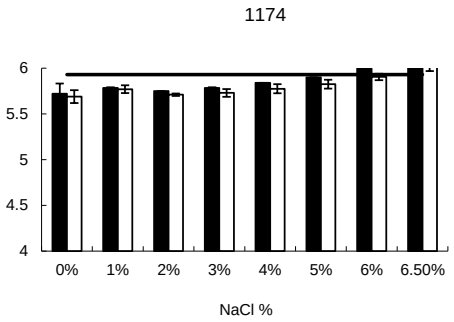

1148
pH
6
5.5
5
4.5
4
0%
1%
2%
3%
4%
5%
6%
6.50%
NaCl %


1156
6
5.5
5
4.5
4
0%
1%
2%
3%
4%
5%
6%
6.50%
NaCl %


1173
6
5.5
5
4.5
4
0%
1%
2%
3%
4%
5%
6%
6.50%
NaCl %


1149
pH
6
5.5
5
4.5
4
0%
1%
2%
3%
4%
5%
6%
6.50%
NaCl %


1167
6
5.5
5
4.5
4
0%
1%
2%
3%
4%
5%
6%
6.50%
NaCl %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4%
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6%
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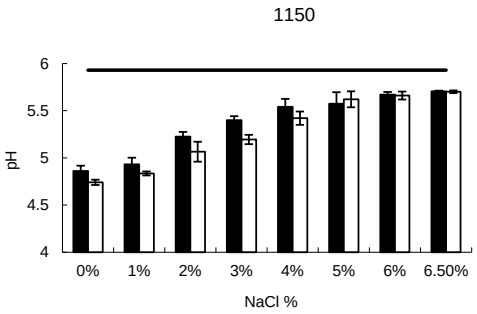
NaCl %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pH
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1%
2%
3%
4%
5%
6%
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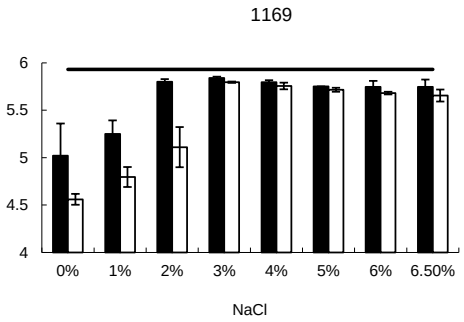
NaCl %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2%
3%
4%
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6%
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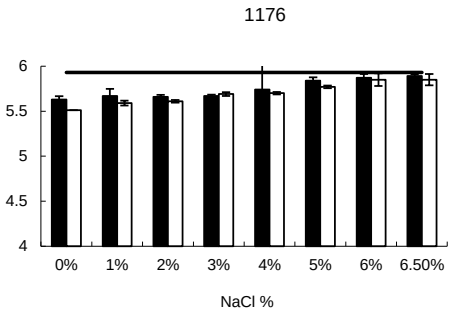
NaCl


1176
6
5.5
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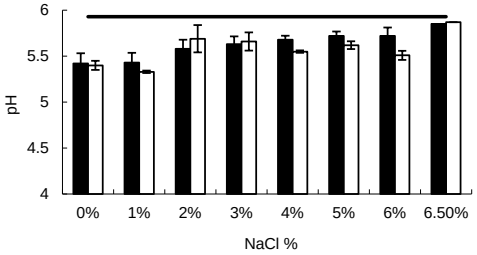
4.5
4
0%
1%
2%
3%
4%
5%
6%
6.50%
NaCl %

1151



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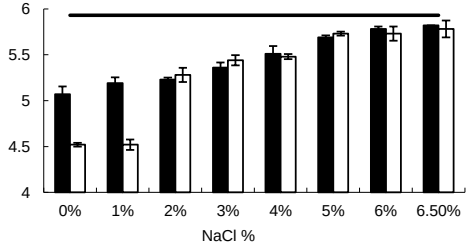
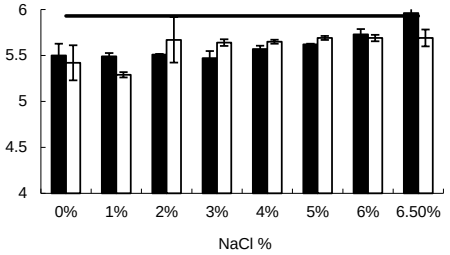


1177



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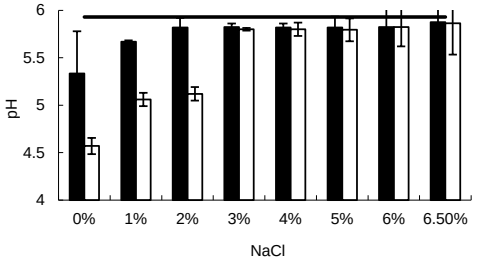

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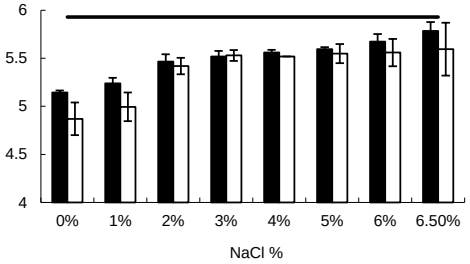

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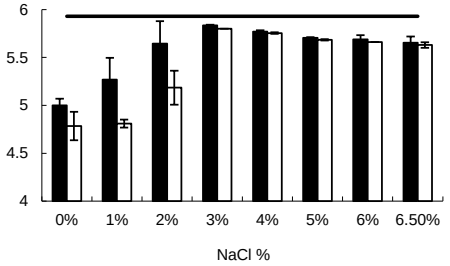
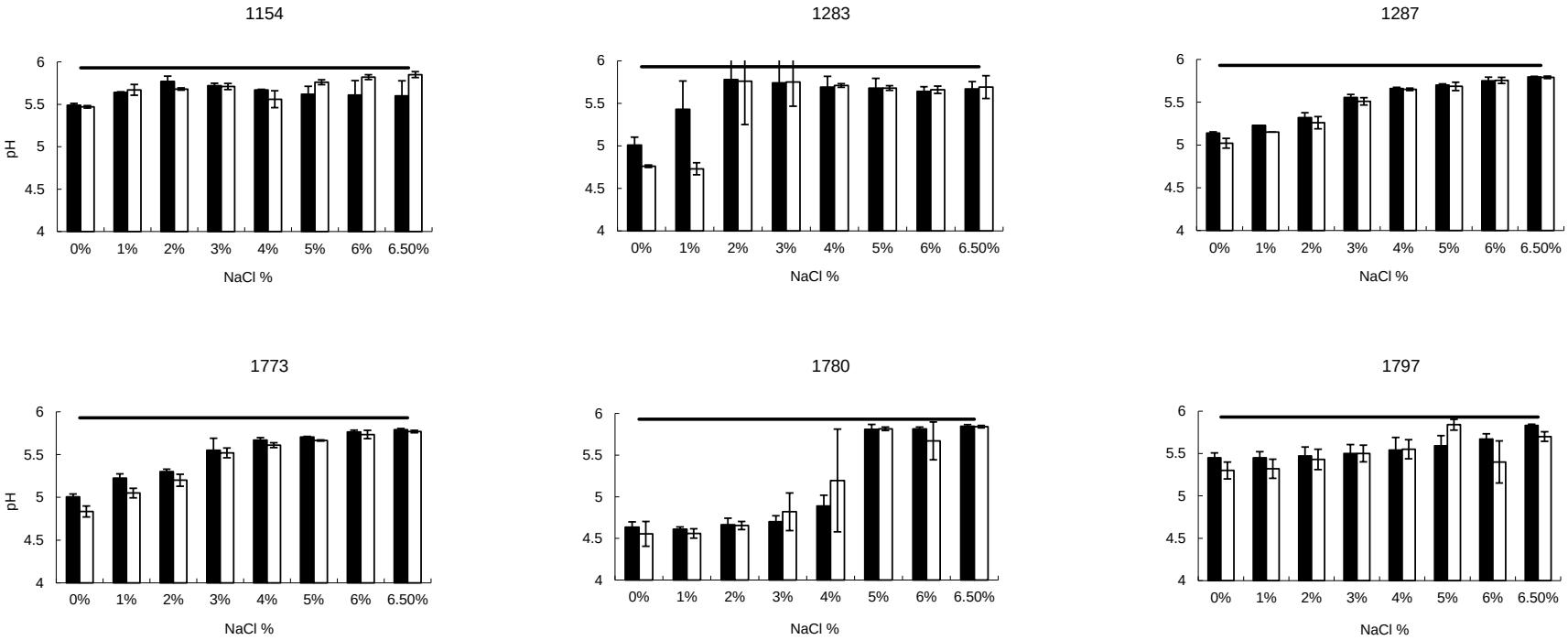
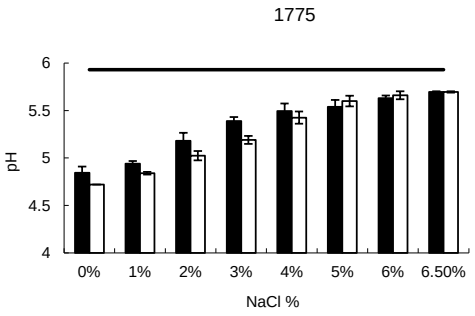



1154
1283
1287
1773
1780
1797
pH
6
5.5
5
4.5
4
0%
1%
2%
3%
4%
5%
6%
6.50%
NaCl %


1775
pH
6
5.5
5
4.5
4
0%
1%
2%
3%
4%
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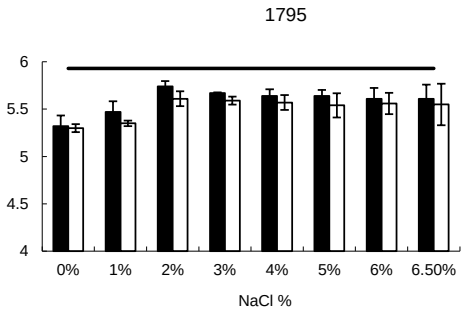
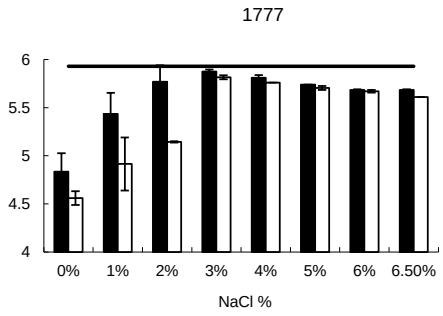
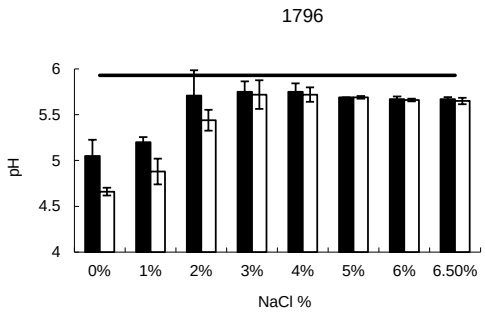
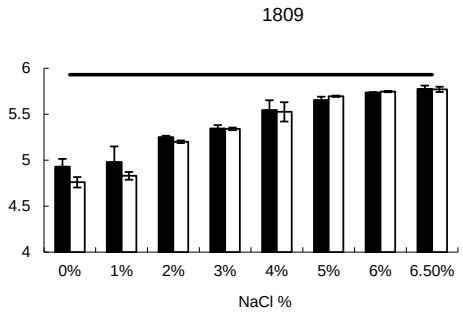
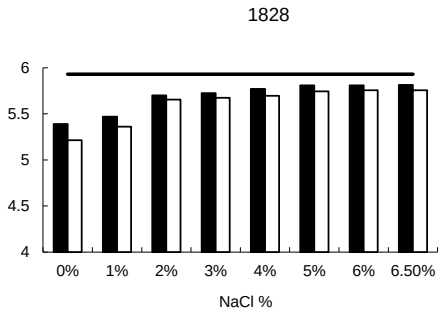
6%
6.50%
NaCl %

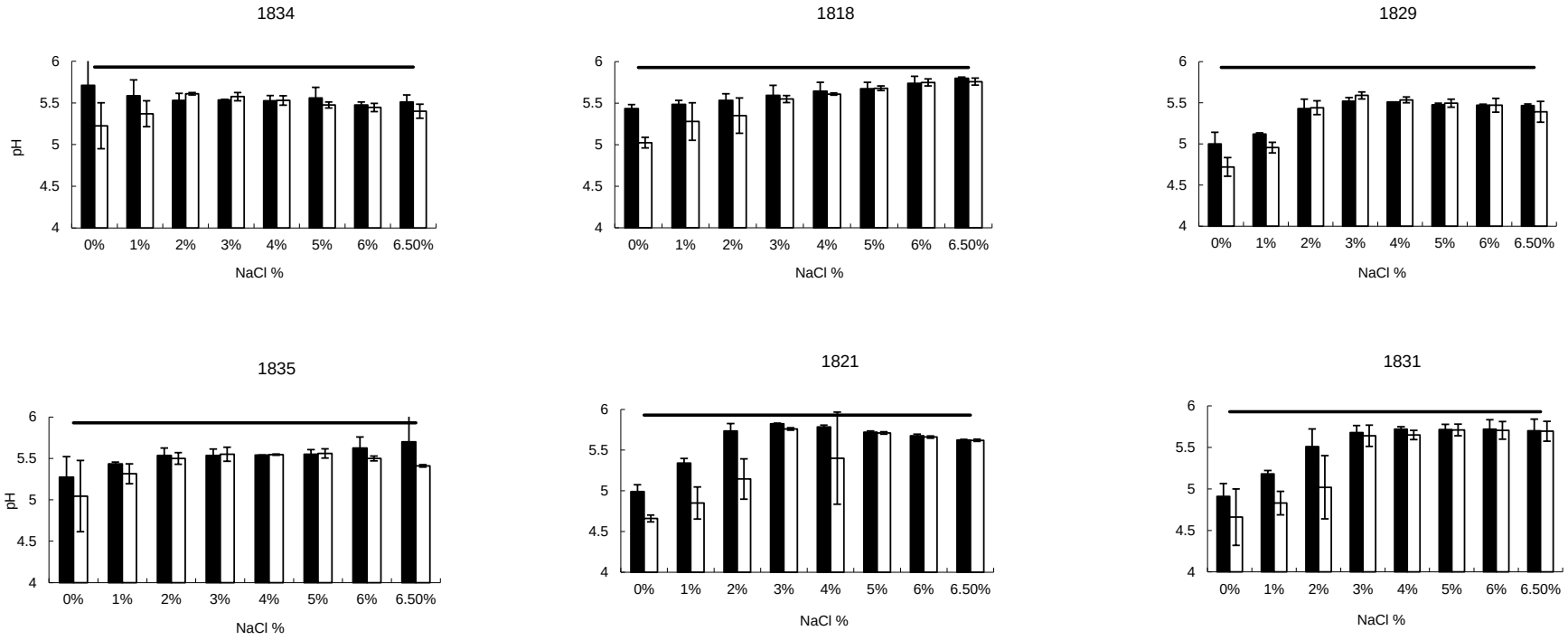

1795
6
5.5
5
4.5
4
0%
1%
2%
3%
4%
5%
6%
6.50%
NaCl %

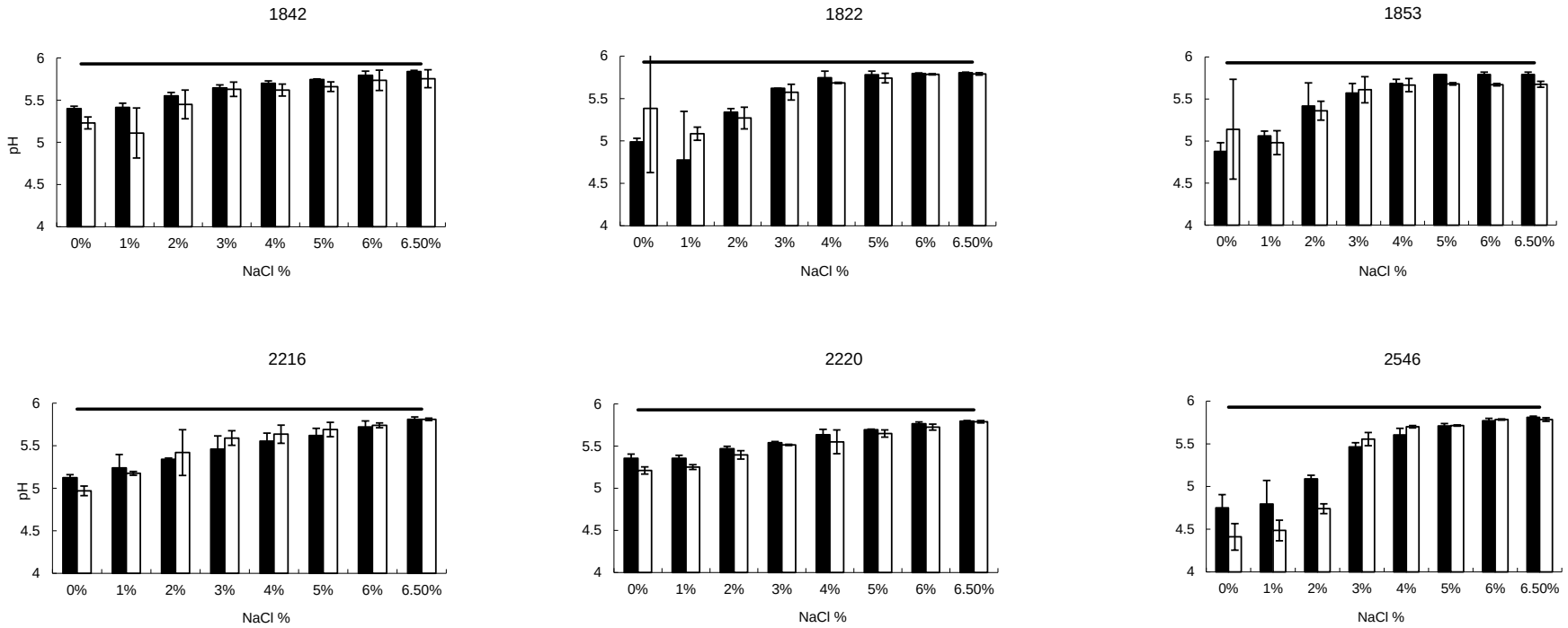

1777
6
5.5
5
4.5
4
0%
1%
2%
3%
4%
5%
6%
6.50%
NaCl %

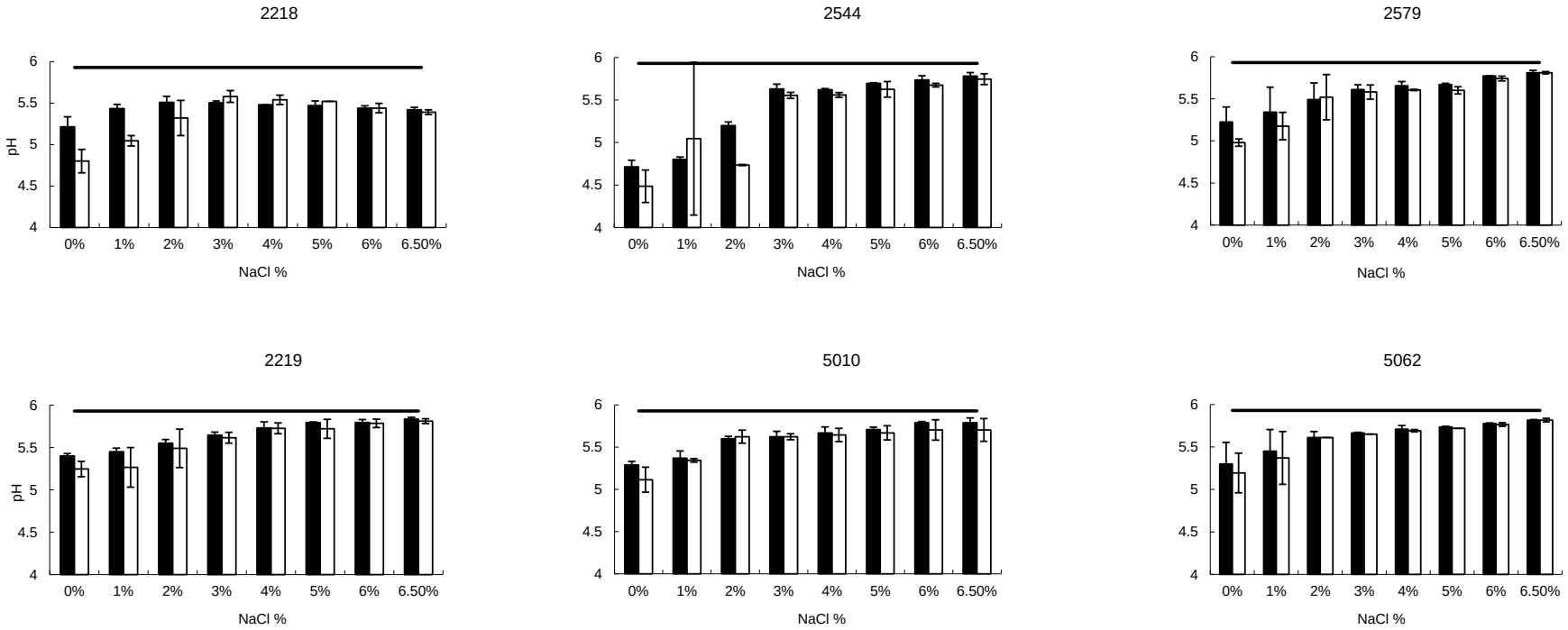

1796
pH
6
5.5
5
4.5
4
0%
1%
2%
3%
4%
5%
6%
6.50%
NaCl %


1809
6
5.5
5
4.5
4
0%
1%
2%
3%
4%
5%
6%
6.50%
NaCl %


1828
6
5.5
5
4.5
4
0%
1%
2%
3%
4%
5%
6%
6.50%
NaCl %


1834
1818
1829
1835
1821
1831
pH
6
5.5
5
4.5
4
0%
1%
2%
3%
4%
5%
6%
6.50%
NaCl %


1842
1822
1853
2216
2220
2546
pH
6
5.5
5
4.5
4
0%
1%
2%
3%
4%
5%
6%
6.50%
NaCl %


2218
2544
2579
2219
5010
5062
pH
6
5.5
5
4.5
4
0%
1%
2%
3%
4%
5%
6%
6.50%
NaCl %

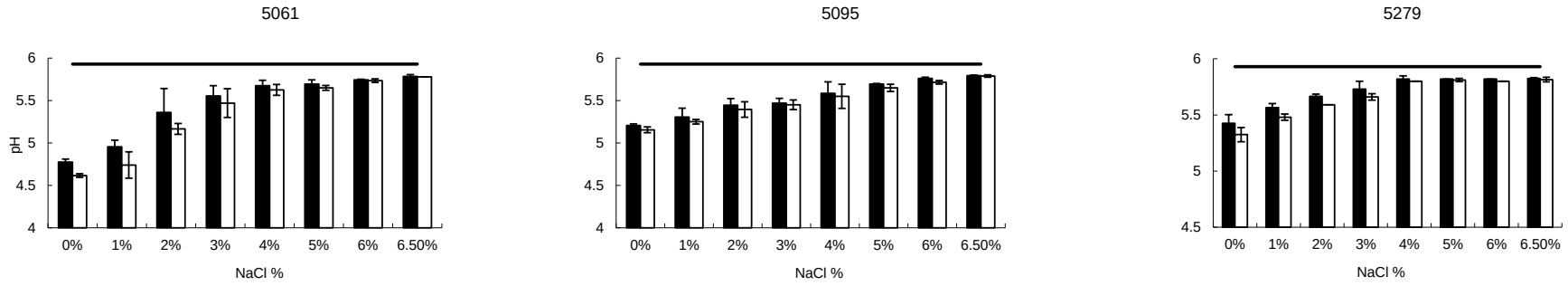


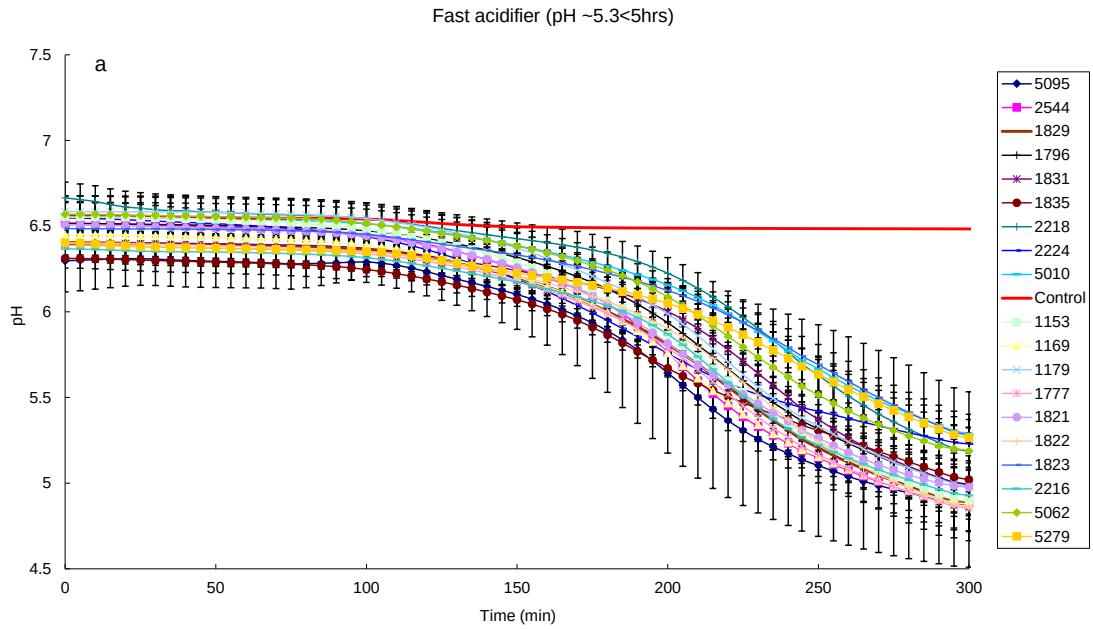
Figure 6.2. The ability of *Sc. thermophilus* strains to grow in reaction osmotic stress conditions with different levels of salt after 2 (black bar) and 7 (white bar) days. Control (not inoculated borth) reading is shown as a solid line in figures.

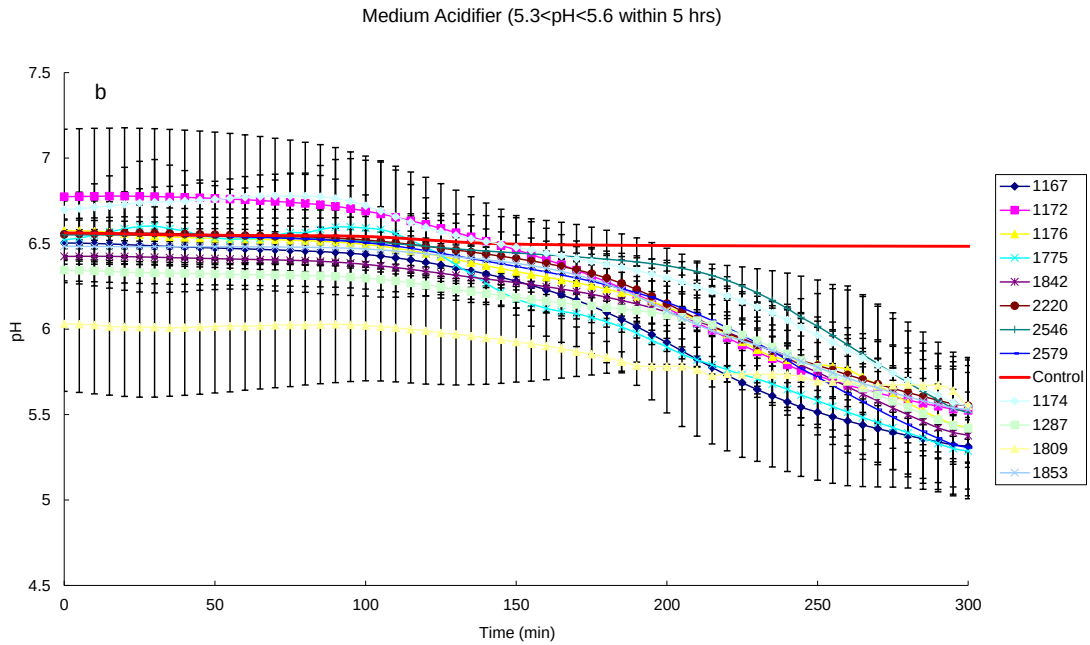
6.3.4. Acidification profiles

6.3.4. 1. Acidification profiles of Sc. thermophilus strains

The pH of *Sc. thermophilus* strains in the milk samples decreased with the 5 hours incubation time at different rates, namely fast acidifiers (pH<5.3), medium acidifiers (5.3<pH<5.6), slow acidifiers (5.6<pH<5.9) and very slow acidifiers (pH>5.9) (Figure 6.3a, 6.3b, 6.3c and 6.3d). The greatest decrease of pH was observed from time 2 to 5 hours.

Based on the results of sugar metabolism, salt sensitivity and acidification rate, one galactose-metabolising strain (DPC 1796) and one galactose non-metabolising (DPC 5095) *Sc. thermophilus* were selected for further analysis.


Fast acidifier (pH ~5.3<5hrs)
a
pH
Time (min)
7.5
7
6.5
6
5.5
5
4.5
0
50
100
150
200
250
300
5095
2544
1829
1796
1831
1835
2218
2224
5010
Control
1153
1169
1179
1777
1821
1822
1823
2216
5062
5279


Medium Acidifier (5.3<pH<5.6 within 5 hrs)
b
pH
Time (min)
7.5
7
6.5
6
5.5
5
4.5
0
50
100
150
200
250
300
1167
1172
1176
1775
1842
2220
2546
2579
Control
1174
1287
1809
1853

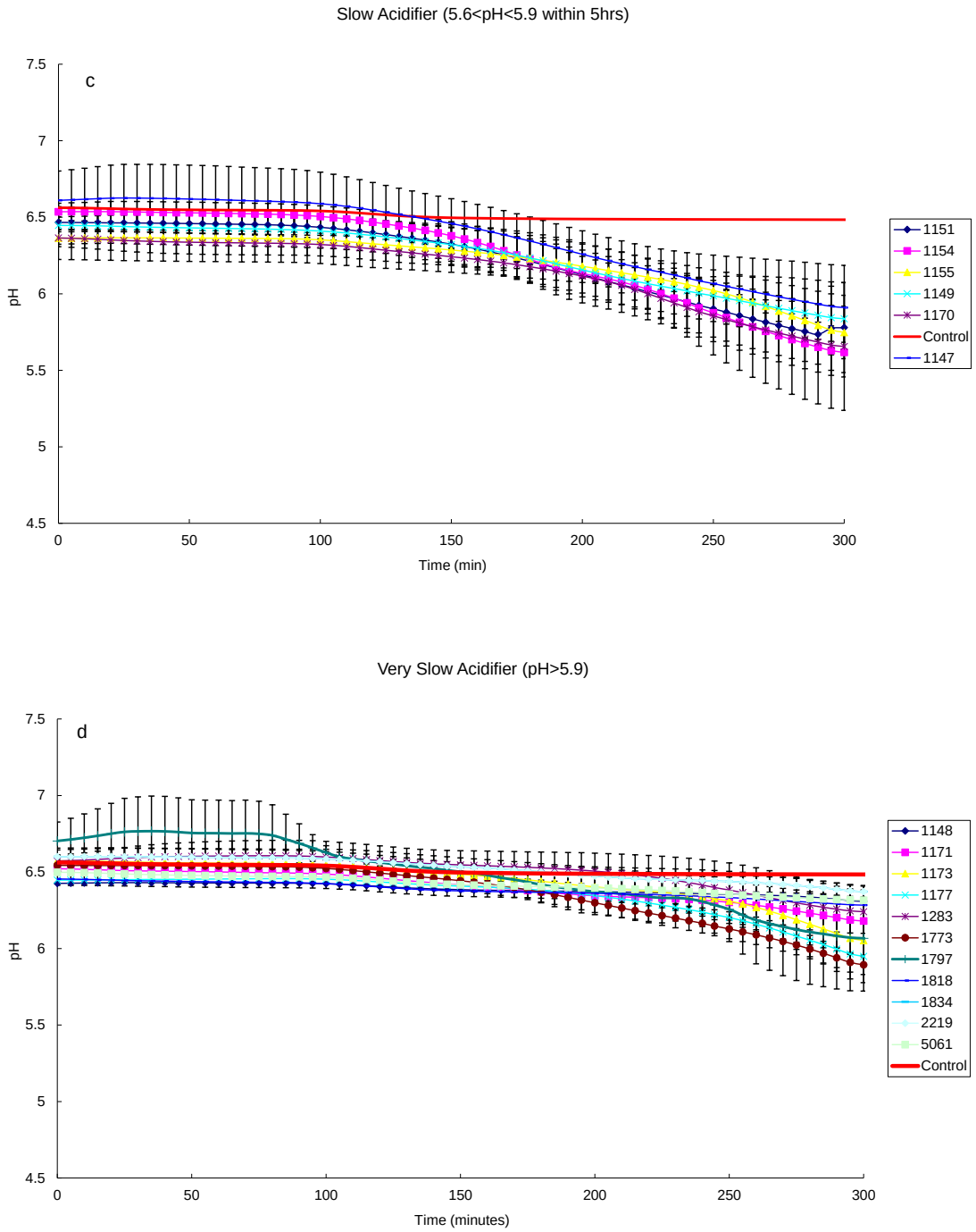
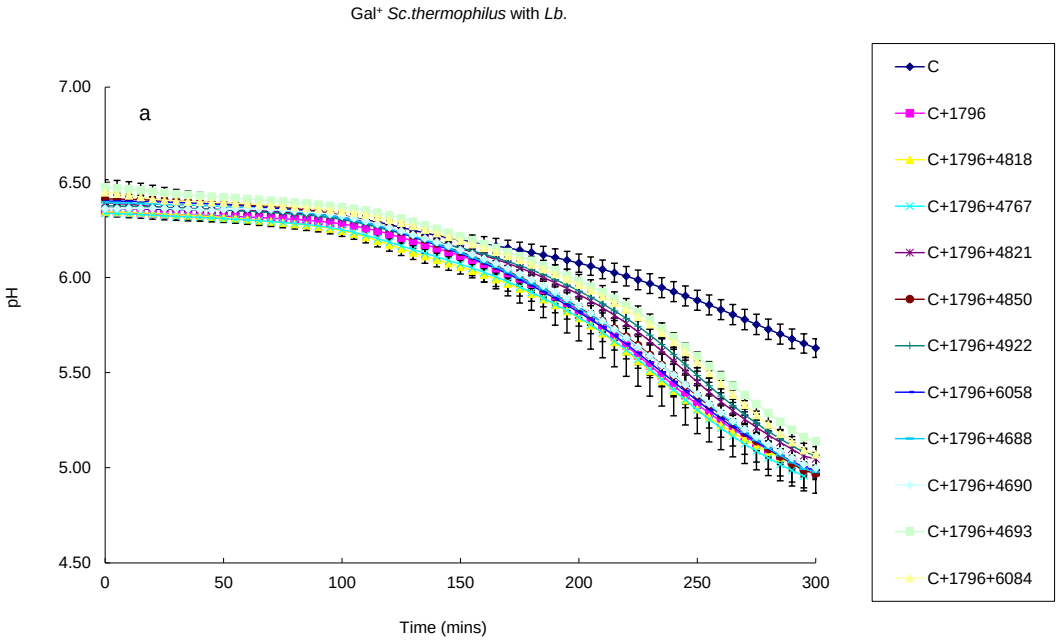


Figure 6.3. The acidification rate (pH changes) of *Sc. thermophilus* strains growth in 10 % RSM solution over 5 hours, (a), fast acidifiers (pH<5.3), (b), medium acidifiers (5.3<pH<5.6), (c), slow acidifiers (5.6<pH<5.9) and (d), very slow acidifiers (pH>5.9).

6.3.4.2. Acidification profiles of combination of selected strains

Two strains of *Sc. thermophilus* (DPC1796 and 5095) and ten *Lactobacillus* (DPC4767, 4818, 4821, 4850, 4922, 6058, 4688, 4690, 4693 and 6084) were selected and tested on CINAC pH equipment in combination with defined strain starter cultures which were used in cheesemaking: *Ls. lactis* subsp. *lactic* strain 227 and 303 as control (C) (Chr. Hansen Ireland Ltd., Rohan Industrial Estate, Little Island, Co. Cork, Ireland) for their acidification ability based on their ability to survive/tolerate Cheddar cheese making temperature profile as mentioned above.

Galactose fermenting *Sc. thermophilus* DPC1796 accelerated the pH drop during incubation especially after cooking the milks to 38.5 °C (its preferred growth temperature). Combination of galactose-fermenting *Lactobacillus* strains with *Sc. thermophilus* DPC1796 did not affect the acidification rate (Figure 6.4a). In contrast, galactose non-fermenting *Sc. thermophilus* DPC5095 had similar acidification rate as C cultures, but addition of *Lactobacillus* strains varied the pH decrease (Figure 6.4b). This indicated that galactose-fermenting *Sc. thermophilus* DPC1796 is a fast acidifier while DPC5095 had no effect on acceleration of pH drop. *Lactobacillus* strains can be used in combination of galactose non-fermenting *Sc. thermophilus* DPC5095 to accelerate acidification rate.



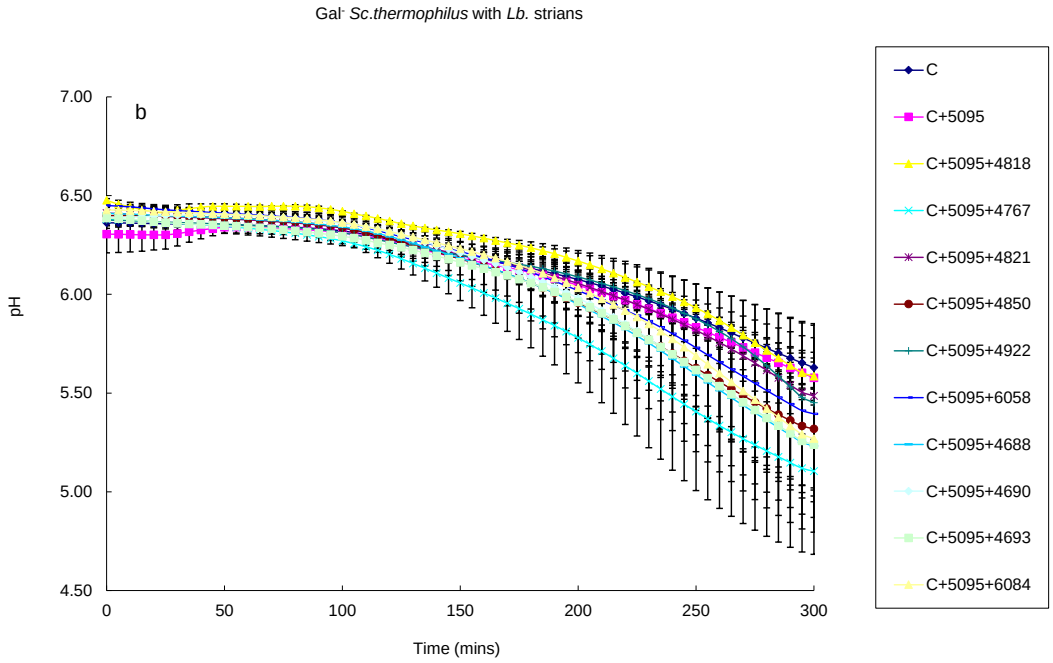


Figure 6.4. Acidification profiles of selected strains of *Sc. thermophilus* and *Lactobacillus* in combination with *Lactococcus lactis* subsp. *lactic* strain 227 and 303, (a), Gal^{+} *Sc. thermophilus* in combination with *Lactobacillus* strains; (b), Gal^{-} *Sc. thermophilus* in combination with *Lactobacillus* strains.

6.3.5. Fermentation profile of Sc. thermophilus strains

Based on the previous study by De Vin et al, (2005) (Figure 6.5), we divided the fermentation profile of 49 dairy *Sc. thermophilus* strains in lactose-limited fermentation with respect to galactose consumption after lactose depletion. The fermentation profiles of Moorepark collection *Sc. thermophilus* strains were also tested their fermentation ability for 8 hours in LM 17 media. The OD_{600nm}, lactose, galactose and glucose concentrations were measured every hour during incubation (Figure 6.6). The 51 *Sc. thermophilus* strains can be divided into the 4 profiles as shown below (Figure 6.6):

- Fermentation profile A (15 strains): DPC1171, 1173, 1174, 1177, 1179, 1775, 1780, 1818, 1831, 2218, 2219, 2220, 2579, 5061 and 5279.
- Fermentation profile B (27 strains): DPC1148, 1150, 1151, 1152, 1153, 1155, 1156, 1167, 1169, 1172, 1176, 1283, 1287, 1777, 1795, 1796, 1797, 1809, 1821, 1822, 1828, 1829, 1842, 1853, 2546, 5062 and 5095.
- Fermentation profile C (8 strains): DPC1147, 1154, 1170, 1773, 1834, 1835, 2216 and 5010.
- Fermentation profile D (1 strain): DPC1149

The strains within profile A and D are galactose-negative while B had galactose metabolism ability, but not as effective as D.

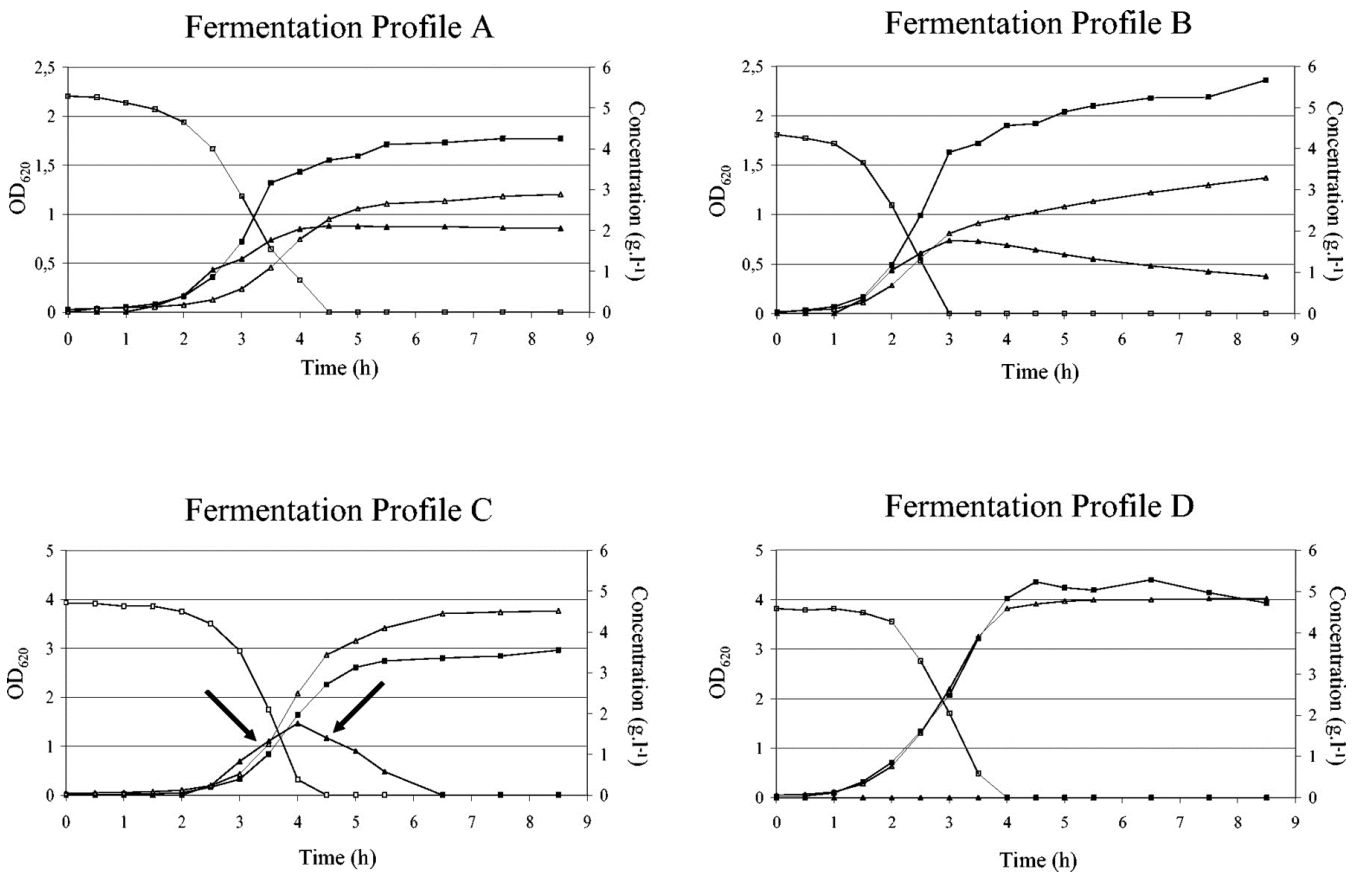
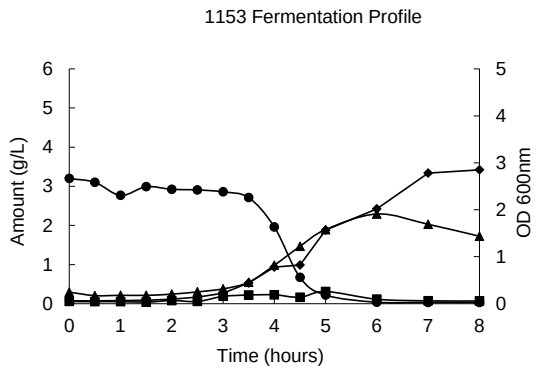
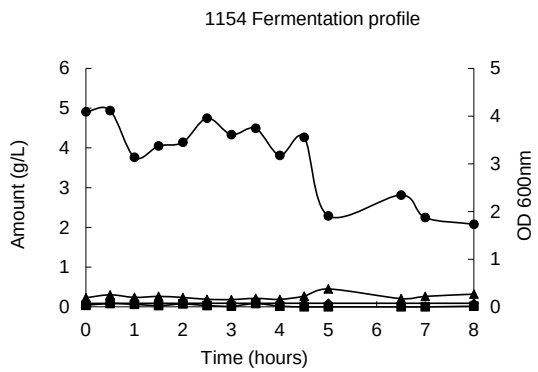
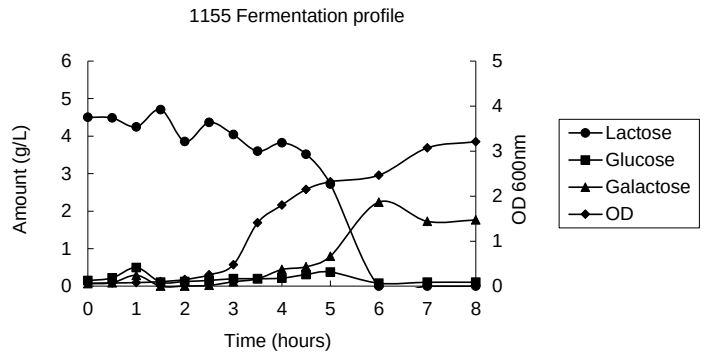
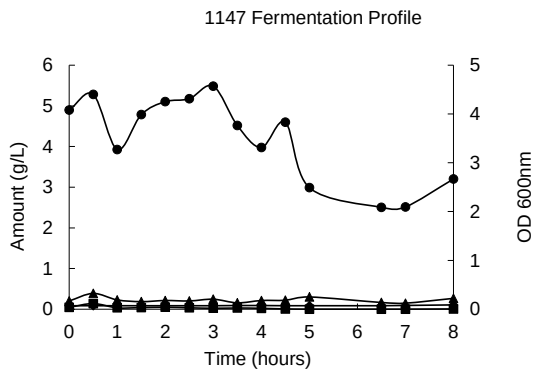


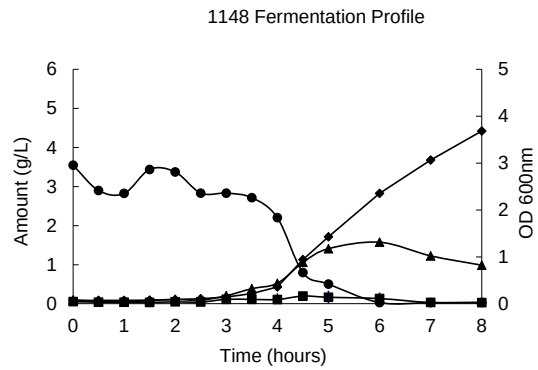
Figure 6.5. Four typical batch fermentation profiles as found among 49 *S. thermophilus* strains when grown in M17 medium with 0.5% (wt/vol) lactose at 42°C. *S. thermophilus* IMDOST04, *S. thermophilus* IMDOST10, *S. thermophilus* IMDOST07, and *S. thermophilus* IMDOST40 are representative strains for fermentation profiles A, B, C and D, respectively. The black arrows in fermentation profile C indicate the different sampling points for enzyme activity measurements. Symbols: ▲, galactose; △, lactic acid; ■, OD_{620}; □, lactose. (De Vin, Rådström, Herman, & De Vuyst, 2005).

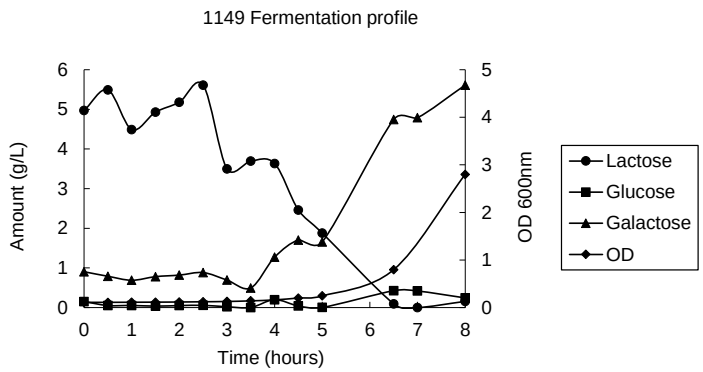

1153 Fermentation Profile
Amount (g/L)
OD 600nm
Time (hours)

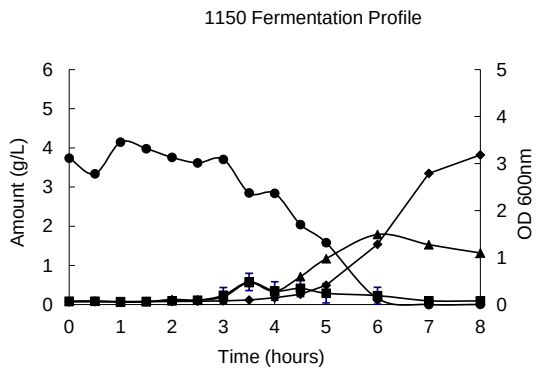

1154 Fermentation profile
Amount (g/L)
OD 600nm
Time (hours)

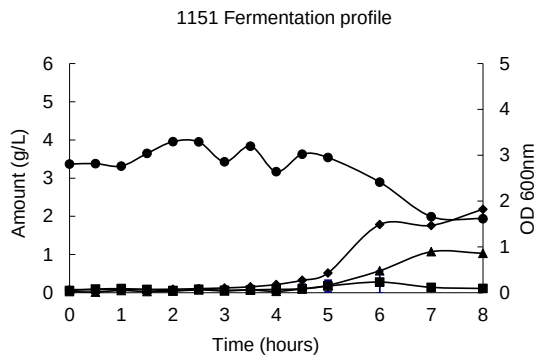

1155 Fermentation profile
Amount (g/L)
OD 600nm
Time (hours)
Lactose
Glucose
Galactose
OD

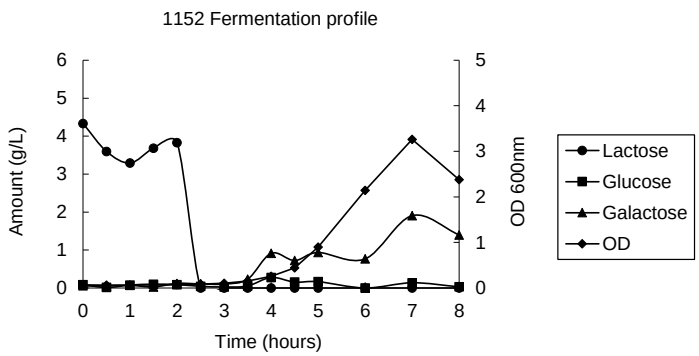

1147 Fermentation Profile
Amount (g/L)
OD 600nm
Time (hours)

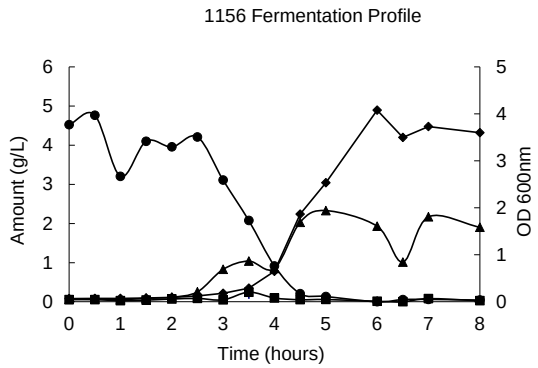

1148 Fermentation Profile
Amount (g/L)
OD 600nm
Time (hours)

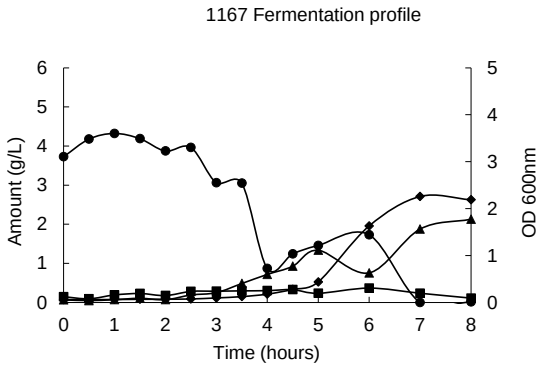

1149 Fermentation profile
Amount (g/L)
OD 600nm
Time (hours)
Lactose
Glucose
Galactose
OD


1150 Fermentation Profile
Amount (g/L)
OD 600nm
Time (hours)


1151 Fermentation profile
Amount (g/L)
OD 600nm
Time (hours)


1152 Fermentation profile
Amount (g/L)
OD 600nm
Time (hours)
Lactose
Glucose
Galactose
OD


1156 Fermentation Profile
Amount (g/L)
OD 600nm
Time (hours)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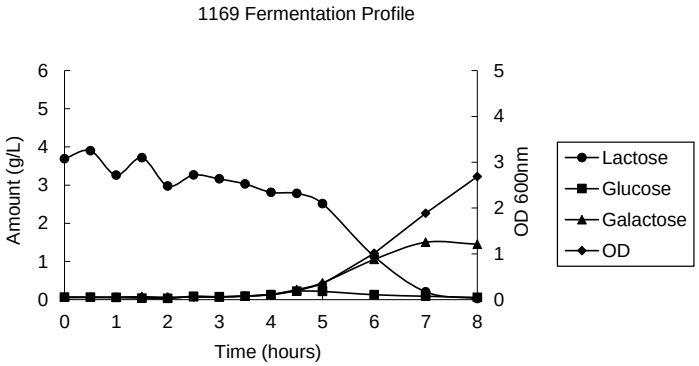
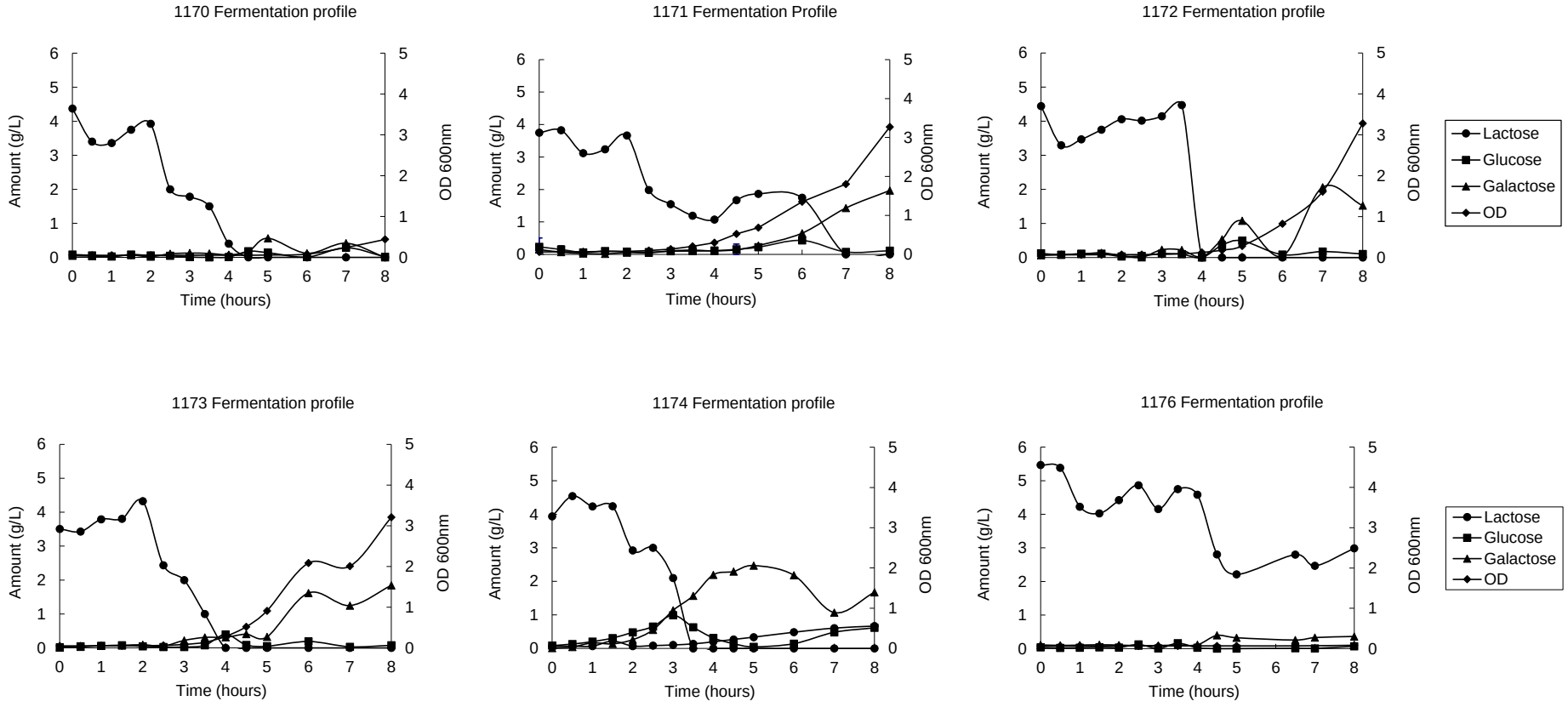
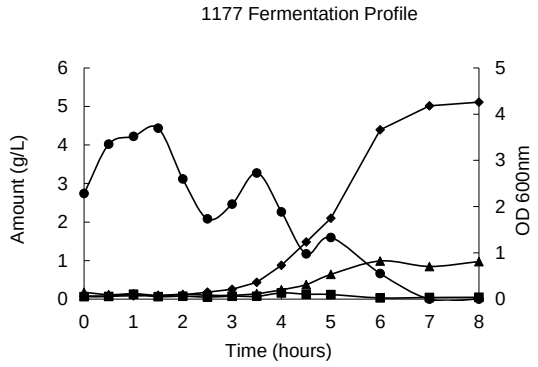
Time (hours)


1169 Fermentation Profile
Amount (g/L)
OD 600nm
Time (hours)
Lactose
Glucose
Galactose
OD


1170 Fermentation profile
1171 Fermentation Profile
1172 Fermentation profile
1173 Fermentation profile
1174 Fermentation profile
1176 Fermentation profile
Amount (g/L)
OD 600nm
Time (hours)
Lactose
Glucose
Galactose
OD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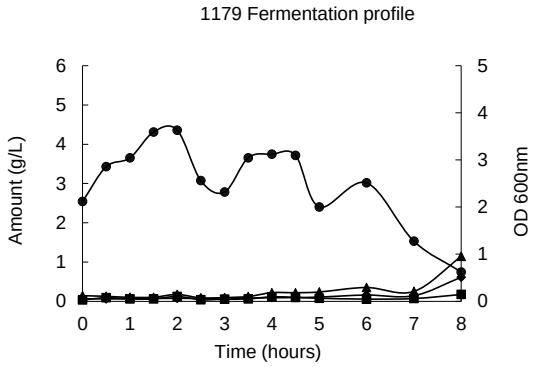
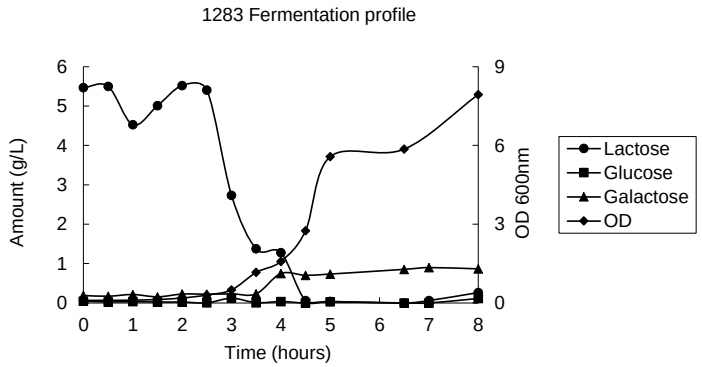
Amount (g/L)
OD 600nm
Time (hours)


1179 Fermentation profile
Amount (g/L)
OD 600nm
Time (hours)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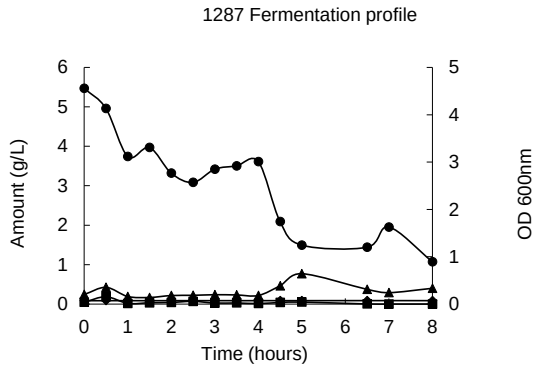
Amount (g/L)
OD 600nm
Time (hours)
Lactose
Glucose
Galactose
OD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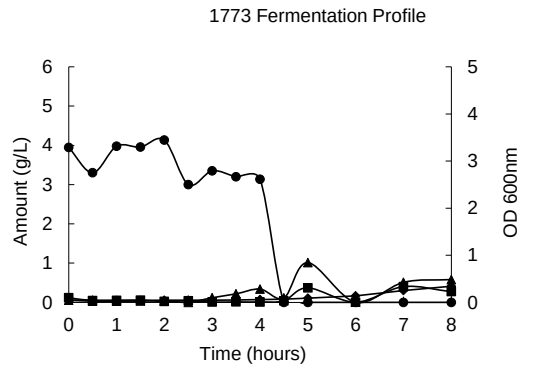
Amount (g/L)
OD 600nm
Time (hours)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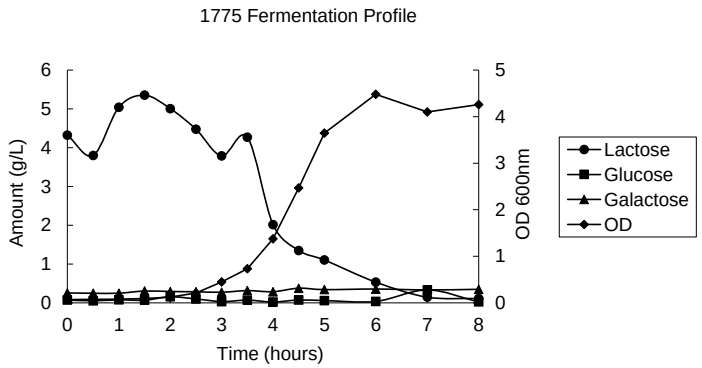
OD 600nm
Time (hours)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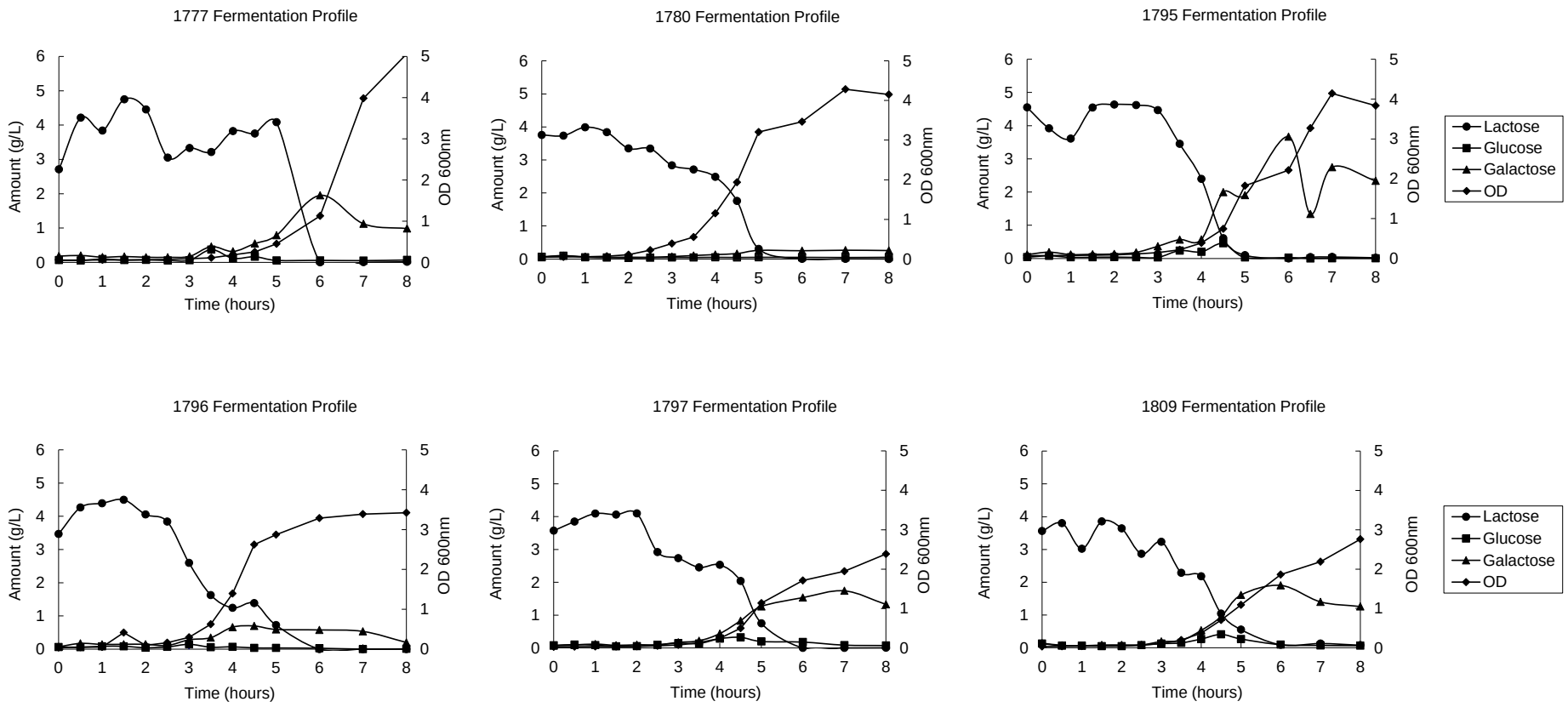
Time (hours)
Lactose
Glucose
Galactose
OD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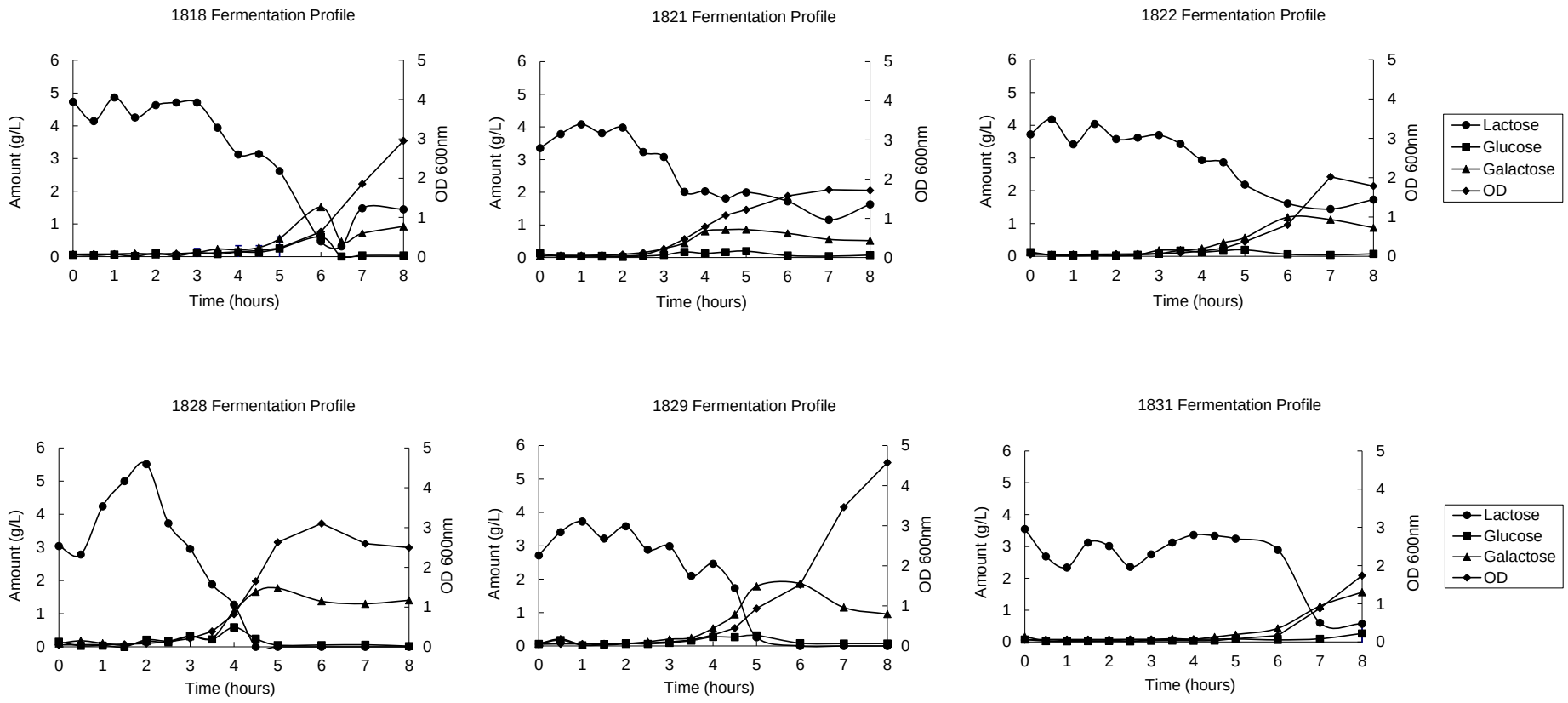
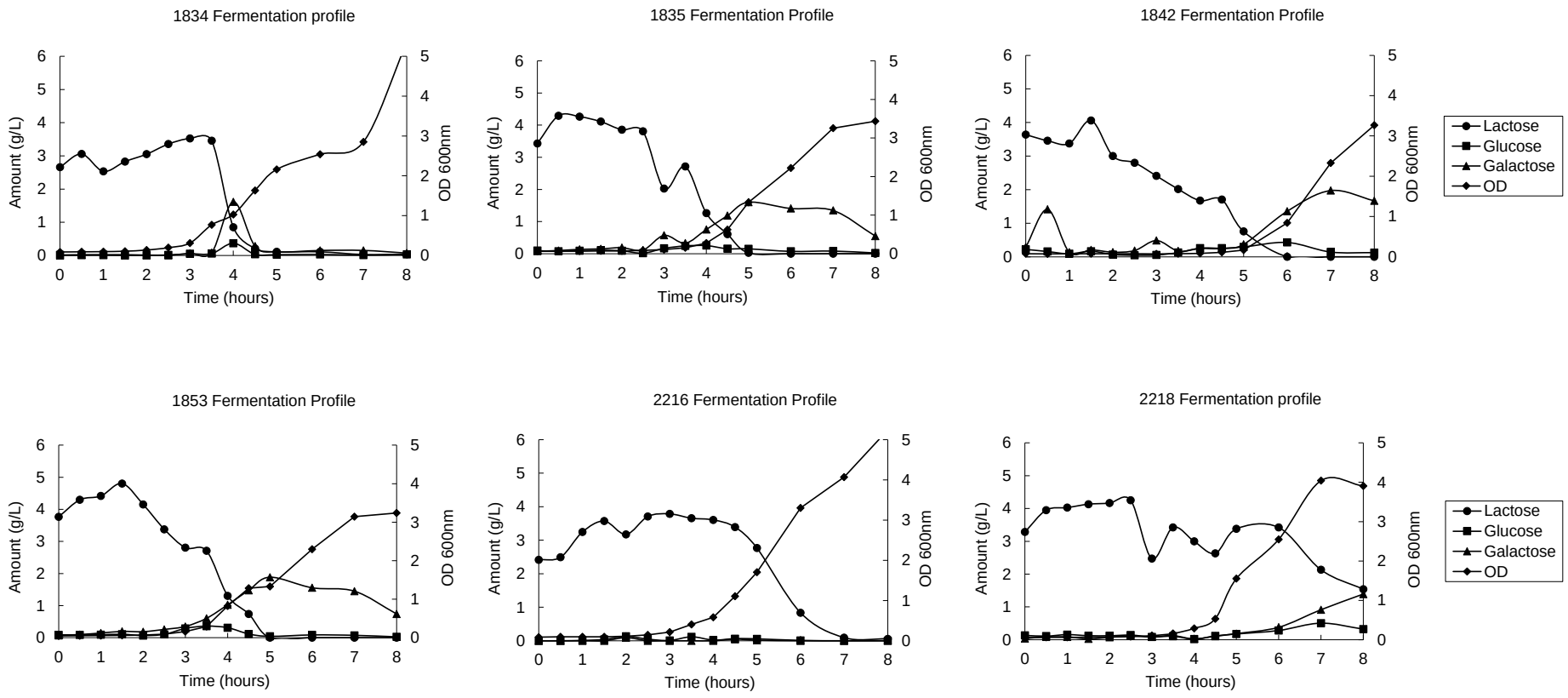
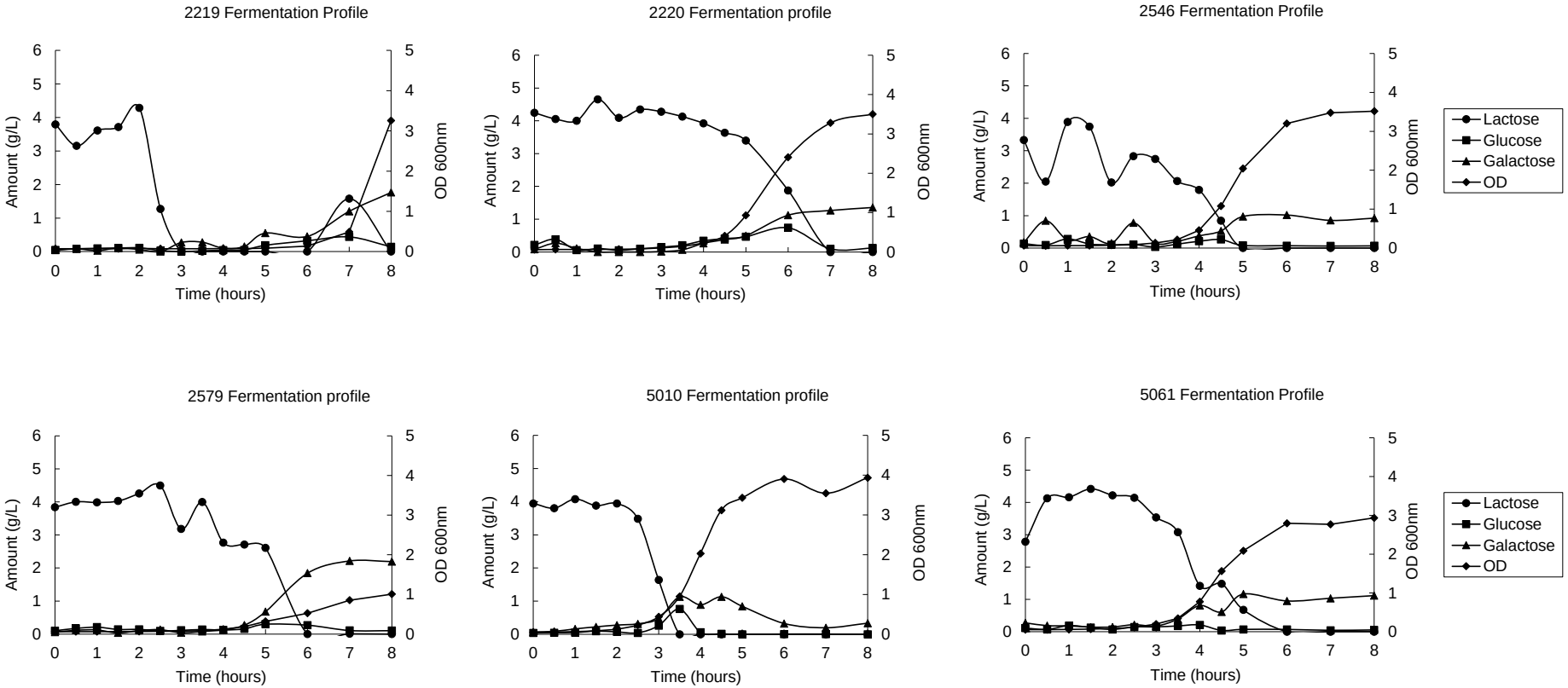
1780 Fermentation Profile
1795 Fermentation Profile
1796 Fermentation Profile
1797 Fermentation Profile
1809 Fermentation Profile
Amount (g/L)
OD 600nm
Time (hours)
Lactose
Glucose
Galactose
OD


1818 Fermentation Profile
1821 Fermentation Profile
1822 Fermentation Profile
1828 Fermentation Profile
1829 Fermentation Profile
1831 Fermentation Profile
Amount (g/L)
OD 600nm
Time (hours)
Lactose
Glucose
Galactose
OD


1834 Fermentation profile
1835 Fermentation Profile
1842 Fermentation Profile
1853 Fermentation Profile
2216 Fermentation Profile
2218 Fermentation profile
Amount (g/L)
OD 600nm
Time (hours)
Lactose
Glucose
Galactose
OD


2219 Fermentation Profile
2220 Fermentation profile
2546 Fermentation Profile
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5010 Fermentation profile
5061 Fermentation Profile
Amount (g/L)
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Time (hours)
Lactose
Glucose
Galactose
OD

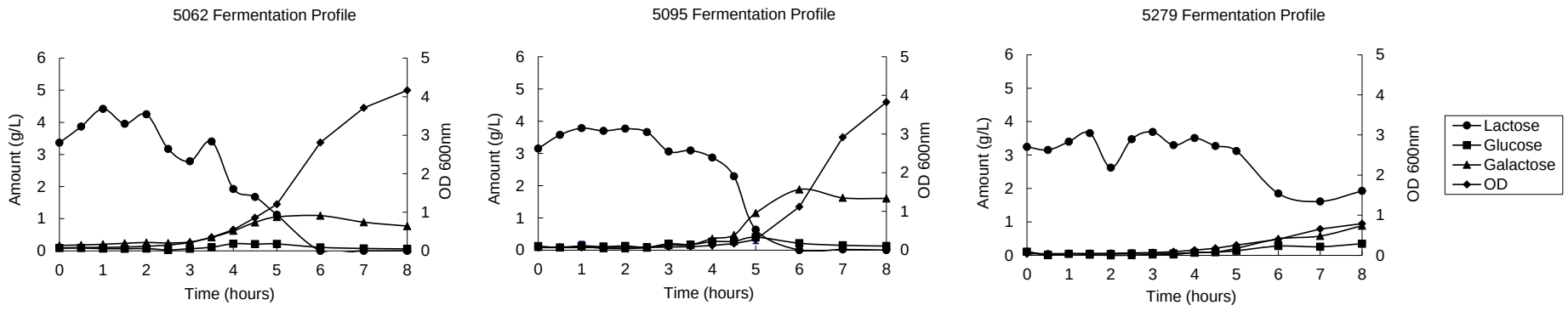

Figure 6.6. Fermentation profiles of strains of *Sc. thermophilus* with lactose (●), glucose (■), galactose (▲) and OD_{600nm} reading (◆).

6.4. Conclusion

The current study provides some relevant intrinsic properties of the *Sc. thermophilus* and *Lactobacillus* strains which can further our understanding of their behaviour in fermented milk products. *Sc. thermophilus* strains were mostly able to metabolise lactose better than galactose while only 8 % of total 51 strains were galactose-positive. They can tolerate up to 3 % salt in MRS broth and the optimum growth showed between 0 - 2 %. Moreover, *Sc. thermophilus* strains had various acidification rates in 10 % RSM under the same condition, while galactose-positive strains were generally faster than the galactose-negative strains. *Lactobacillus* strains were mostly galactose-positive and when used in combination with galactose-negative *Sc. thermophilus* strains, they were able to accelerate the fermentation process. Based on sugar metabolism, salt sensitivity and acidification ability, one galactose-positive *Sc. thermophilus* strain, DPC1796, one galactose-negative *Sc. thermophilus* strain, DPC5095 and one galactose-positive *Lactobacillus* strain, DPC4818 were selected for use with defined strain starter cultures *Lactococcus lactis* subsp. *lactic* strain 227 and 303 for further cheesemaking trials.

6.5. Acknowledgement

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Chapter 7: Effect of galactose metabolising and non-metabolising strains of *Streptococcus thermophilus* as a starter culture adjunct on the properties of Cheddar cheese made with low or high pH at whey drainage[1]

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Abstract

Cheddar cheeses were made using control culture (C, *Lactococcus lactis* subsp. *lactis*), or with control culture plus a galactose-metabolising (Gal^+) or galactose-non-metabolising (Gal^-) *Sc. thermophilus* adjunct; for each culture type, the pH at whey drainage was either low (pH 6.15) or high (pH 6.45). *Sc. thermophilus* affected the levels of residual lactose and galactose, and the volatile compound profile and sensory properties of the mature cheese (270 d) to an extent dependent on the drain pH and phenotype (Gal^+ or Gal^-). For all culture systems, reducing drain pH resulted in lower levels of moisture and lactic acid, a higher concentration of free amino acids, and higher firmness. The results indicate that *Sc. thermophilus* may be used to diversify the sensory properties of Cheddar by descriptive sensory analysis, for example from a fruity buttery odour and creamy flavour to a more acid taste, rancid odour, and a sweaty cheese flavour at high drain pH.

7.1. Introduction

Cheddar cheese manufacture has changed much in recent decades with advances in mechanization and the increase in plant throughput. Specific features of large-scale modern manufacture is the production of different variants (e.g., mild, mature, vintage), the extensive use of direct vat starter cultures (DVS), and a higher pH at whey drainage, for example ~6.4-6.5 compared to ~6.1-6.2 in traditional Cheddar cheese made using bulk starter culture. In such factories, even where milk protein standardization using membrane filtration is not practiced, the various cheesemaking steps, such as starter addition, rennet addition, gel cutting and whey drainage, tend to be performed on the basis of time rather than on some objective parameter such as pH of milk or curd at different stages of manufacture, or gel firmness at cutting. Another feature, at least in Irish Cheddar cheese plants, is the routine use of starter culture adjuncts, including *Sc. thermophilus*, which is used primarily for its thermo- and phage- resistance properties, but also apparently to affect flavour. *Sc. thermophilus* is also reported to give faster acid production during Cheddar manufacture (Michel & Martley, 2001), which may be likely associated with a more effective protein hydrolysis and peptide uptake (Law & Haandrikman, 1997; Cogan, Beresford, Steele, Broadbent, Shah & Ustunol, 2007) and with the non-utilization of the galactose moiety of lactose, by most *Sc. thermophilus* strains (Tinson, Hillier, & Jago, 1982a; Thomas & Crow, 1984).

Most strains of *Sc. thermophilus* used in the dairy industry are unable to metabolise galactose (Thomas & Crow, 1984; Hutkins, Morris & McKay, 1985; Hutkins, Halambeck, & Morris, 1986; Vaillancourt, Moineau, Frenette, Lessard, & Vadeboncoeur, 2002; Robitaille, Moineau, St-Gelais, Vadeboncoeur & Britten, 2007). De Vin, Rådström, Herman and De Vuyst (2005) reported that only ~ 16 % of 49

strains of *Sc. thermophilus* evaluated on M17 medium supplemented with galactose were galactose positive. Similarly, preliminary studies in the authors' laboratory found that only 8 % of 51 strains of *Sc. thermophilus* from the Moorepark culture collection metabolised galactose. Thomas and Crow (1984) investigated the galactose-metabolizing ability of *Sc. thermophilus* from different sources and found that most were galactose negative (Gal^-) because of failure to induce galactokinase, resulting in the excretion of galactose when grown in lactose-containing broth. When grown under lactose limitation in J8 broth containing 20 mM galactose, partial galactose utilization occurred and the proportion of galactose used depended on the generation time of cells during incubation.

Hence, the use of *Sc. thermophilus* (which primarily metabolizes only the glucose moiety of lactose) as an adjunct culture usually results in the accumulation of galactose during cheese manufacture (Thomas, Turner & Crow, 1980; Tinson et al., 1982a, Michel & Martley, 2001). Bley, Johnson and Olson (1985) reported that the use of a 0.5 % (w/w) non-galactose-fermenting *Sc. thermophilus* as an adjunct resulted in higher level of residual galactose in one month-old stirred curd Cheddar (compared to the control cheese) and intensified the degree of browning in processed cheese made therefrom. Similarly, Michel and Martley (2001) found that Cheddar cheese made using *Sc. thermophilus*, as an adjunct culture to *Lc. lactis* subsp. *cremoris* or *lactis* strains, had a high residual galactose level of ~ 26.6 mmol/ kg (0.48 %, w/w) at 1 d. Moreover, the residual galactose content increased as the scald temperature was increased from 38 °C to 41 °C (data not reported). Tinson, Ratcliffe, Hillier and Jago (1982b) reported that high levels of residual galactose (33 mM kg^{-1}, 0.56 %, w/w) in 8 wk-old Cheddar cheese made using *Sc. thermophilus* (0.5 %, w/w) as an adjunct to *Lc. lactis* subsp. *cremoris* coincided with a higher production of CO_2, leading to the

development of slits and fractures in the cheese at 8 and 14 wks. This was most probably caused by the growth of NSLAB that are able to metabolise galactose.

The accumulation of galactose in cheese can lead to problems such as (i) providing a readily fermentable carbohydrate which could influence the development of NSLAB flora and possibly lead to defects, (ii) the presence of a reducing sugar in cheese which can cause excessive Maillard browning on heating, and (iii) early gas production in Cheddar cheese (Mullan, 2000; Ortakci, Broadbent, Oberg & McMahon, 2015). Moreover, the presence of galactose in whey can affect the rate of growth of lactose crystals during whey processing and increase the propensity of the resultant whey powder to browning during storage (Dattatreya, Lee , & Rankin, 2010; Paterson & Smakman, 2011). While many of the foregoing studies (Bley et al., 1985; Hutkins et al., 1986; Michel & Martley, 2001) studied the effects of *Sc. thermophilus* as an adjunct on composition and sugar metabolism, we are unaware of any that investigated their effects on proteolysis, rheology or sensory properties despite its apparent impact on flavour development. Moreover, there appear to be few, if any, studies on the comparative effect of galactose positive (Gal^+) and galactose negative (Gal^-) *Sc. thermophilus* as adjunct culture on the latter aspects of cheese quality.

The objective of the current study was to compare the effect of Gal^+ and Gal^- strains of *Sc. thermophilus* as an adjunct culture on the composition, sugar metabolism, pH, proteolysis, volatile compounds, texture, microbiology and sensory properties of Cheddar cheeses prepared made with a high drain pH (6.45), as in modern manufacture, or a low drain pH (6.15), as in more traditional manufacture.

7.2. Materials and methods

7.2.1. Preparation of cheese milk

Holstein-Friesian cows' milk (3000 kg) was obtained from a spring-calving, herd (Moorepark, Fermoy, Ireland). Milk samples were standardized to a protein to fat ratio of 0.96:1, stored overnight at 8 °C, pasteurized at 72 °C for 15 s, cooled to 31 °C, and pumped to cheese vats (500 L).

7.2.2. Starter cultures for cheesemaking

Defined strain starter cultures were used in cheesemaking (*Lc. lactis* subsp. *lactis* strain 227 and 303; Chr. Hansen Ireland Ltd., Rohan Industrial Estate, Little Island, Ireland). Both cultures were grown overnight at 24 °C in reconstituted 10 % (w/v), antibiotic-free skim milk powder solution (Kerry Ingredients Ltd., Ireland) which had been heat treated at 95 °C for 30 min. When the pH of the inoculated milk reached between pH 4.5 to 5.0, the cultures were cooled and stored at 4 °C until required for cheesemaking (~1 d).

Adjunct starter cultures of *Sc. thermophilus* from the Moorepark culture collection were screened on the basis of sugar metabolism, acidification rate and salt sensitivity as described in previous chapter. One galactose metabolising (DPC 1796) and one galactose non-metabolising (DPC 5095) *Sc. thermophilus* strains were selected for cheesemaking. Both cultures were grown overnight at 37 °C in reconstituted 10 % (w/v), antibiotic-free skim milk powder solution (Golden Vale Food Products Ltd.) as described above.

For convenience, the cultures used in cheesemaking were denoted as follows: control culture C, consisting of *Lc. lactis* subsp. *lactis* strains 227 and 303, each inoculated at a level of 0. 75 % (w/w); Gal^+ culture, consisting of the control culture

and a galactose-metabolising *Sc. thermophilus* DPTC 1796 (inoculated at a level of 0.25 %, w/w)*;* and Gal^- culture, consisting of the control culture C plus galactose non-metabolising *Sc. thermophilus* DPTC 5095 (inoculated at a level of 0.25 %, w/w).

7.2.3. Cheese manufacture and treatments

Six different treatment cheeses were manufactured in each of three replicate trials undertaken over a three-wk period from October 20 to November 11, 2011. The cheeses were denoted as: high-drain pH (6.45) made using culture C (HDpHC); high-drain pH with Gal^+ culture ($HDpHGal^+$); high- drain pH with Gal^- culture ($HDpHGal^-$); low-drain pH (6.15) with culture C (LDpHC); low-drain pH with Gal^+ culture ($LDpHGal^+$); low- drain pH with Gal^- culture ($LDpHGal^-$).

The manufacture of cheese involved inoculation of cheesemilk with *Lc. lactis* subsp. *lactis* strain 227 and 303, each at a level of 0.75 % (w/w). Additionally, *Sc. thermophilus* 1796, was added to milk for the $HDpHGal^+$ and $LDpHGal^+$ cheeses, and *Sc. thermophilus* 5095 to milk for the $LDpHGal^-$ and $HDpHGal^-$ cheeses; *Sc. thermophilus* 1796 and 5095 were each inoculated at a level of 0.25 % (w/w). The mean initial count of the *Lc. lactis* subsp. *lactis* was ~ 1 x 10^7 cfu mL^{-1} in all milks, while that of the *Sc. thermophilus* was ~ 6.2 x 10^6 cfu mL^{-1} in the $LDpHGal^+$, $LDpHGal^-$, $HDpHGal^+$ and $HDpHGal^-$ milks. Thirty minutes later, rennet (Chymax Plus, Chr. Hansen Ireland Ltd., 200 IMCU mL^{-1}), diluted 1:10 in de-ionized water, was added at a level of 1.8 mL kg^{-1} based on a protein level of 3.3 g per 100 g milk and mixed in for 1.5 min to ensure uniform distribution. Immediately, a sample of the rennet-treated cheese milk was taken from the cheese vat, and placed in an insulated glass container. Within 2 min, a 13-g subsample was placed in the cell of a controlled stress rheometer (CSL2 500 Carri-Med TA Instruments, Inc., New Castle, DE) located

in an adjacent laboratory, and subjected to a low oscillating strain of 0.025 at a frequency of 1Hz at 31 °C. The development of elastic shear modulus, G', a measure of gel stiffness, was measured as a function of time; when G' reached 54 Pa, cutting of the gel in the cheese vat was initiated. Following a 1.5 min cutting programme, the resultant curd particle-whey mixture was allowed to stand quiescently (heal) for 10 min, then stirred continuously, cooked at a rate of 0.2 °C min^{-1} from 31 to 38.5 °C, which is typical of the scald temperature used in commercial practice for Cheddar cheese made with, or without, *Sc. thermophilus* as an adjunct culture. Whey was separated from the curd when the pH of the curd reached 6.45 for the high-drain pH cheeses (HDpHC, $HDpHGal^+$, $HDpHGal^-$) and 6.15 for the low-drain pH cheeses (LDpHC, $LDpHGal^+$, $LDpHGal^-$). The curds were cheddared, milled at pH 5.35, salted at a level of 2.7 % (w/w), mellowed for 20 min, placed in rectangular moulds (23 kg), and pre-pressed at 0.13 kPa for 30 min. The moulded cheeses were then placed in a horizontal press and pressed overnight at 2.5 kPa. A total of two cheeses, each weighing ~ 20 kg, was obtained for each treatment on each of the three separate cheesemaking occasions (trials). Cheeses were vacuum-packed and stored at 4 °C for 14 d and at 8 °C thereafter.

7.2.4. Sampling of cheese

Cheeses (from 20 kg blocks) were sampled at different times (1, 14, 30, 90, 180, 270 d) over the 270-d ripening period, as described by Hou, McSweeney, Beresford and Guinee (2014b).

7.2.5. Composition analysis of cheese

Grated cheese samples were analysed at 14 d for moisture, protein, fat, NaCl, moisture, ash, Ca and P using standard IDF methods (Guinee, Harrington, Corcoran, Mulholland & Mullins, 2000). The pH was measured after each sampling date on cheese slurry prepared from 20 g of grated cheese and 12 g distilled water (Guinee et al., 2000).

7.2.6. Microbial counts in cheese

Starter *lactococci* were enumerated on LM17 agar after incubation at 20 °C for 5 d and the *Sc. thermophilus* adjunct cultures were also enumerated on LM17 agar after incubation at 43 °C for 3 d (ISO/IDF, 2010).

Cheeses were analysed for counts of non-starter lactic acid bacteria (NSLAB) on LBS agar, as described previously (Hou, Hannon, McSweeney, Beresford & Guinee, 2012). Coliform were enumerated by pour-plating on Violet Red Bile Agar (VRBA) incubated at 30 °C for 24 hours.

7.2.7. Lactose and lactate in cheese

Extraction and analysed of lactose, glucose and galactose were measured using the high performance liquid chromatography (HPLC) method which eluted on a 300 x 7.8mm Aminex HPX-87C cation exchange carbohydrate column (Bio-Rad Laboratories, Richmond, CA, USA), and detected with a Waters 2414 Refractive Index Detector (Waters, Bray, Ireland) as described previously by Hou et al. (2014b). The concentrations of sugars in the cheeses were calculated by comparing the peak area of samples to standard curves. Sugar concentrations were calculated as g 100 g^{-1} cheese.

Similarly, D (-)- and L (+)- lactate were extracted by the above procedure, and separated on a phenomenex chirex 3126 cation exchange silica column (Phenomenex, Hurdsfield Ind. Est., Macclesfield, UK) fitted with a Waters 2487 Dual λ Absorbance Detector (Waters, Bray, Ireland) as described previously by Hou et al. (2014b). The concentration of total lactate was calculated as the sum of D (-)- and L (+)- lactates. Each analysis was carried out in duplicate.

7.2.8. Proteolysis

The level of pH 4.6-soluble nitrogen (pH4.6-SN) was measured as described by Hou, Hannon, McSweeney, Beresford & Guinee (2014a). The concentration of individual free amino acids (FAA) in the pH4.6-SN extract were determined using cation-exchange chromatograhy on a Beckman 6300 High Performance Analyser (Beckman Instruments Ltd., High Wycombe, UK), as described by Fenelon, Guinee, Delahunty, Murray and Crowe (2000).

7.2.9. Rheology

Six cheeses cubes (25 mm^3 cubes) were cut from each treatment cheese using a Cheese Blocker (Bos Kaasgreedschap, Bodengraven, Netherlands), wrapped in tin foil, and stored at 8 °C overnight prior to analysis. Each cube was compressed by 70 % on a texture analyser (model TA-HDI, Stable Micro Systems, Godalming, UK) with a 5-mm compression plate and a 100-Kg load cell, as described previously (Hou et al., 2014a). The following rheological parameters were calculated from the resultant force/displacement curves: fracture stress (KPa), the stress at fracture, as indicated by the inflection point of the curve; fracture strain, the fractional displacement at fracture; and firmness, the force required to compress the cheese to 30 % of its original height.

7.2.10. Volatile compounds

The 270 d-old cheeses were analysed in triplicate for volatile compounds. For each cheese, a 5 g sample was analysed by solid phase micro-extraction (SPME) coupled to a gas chromatograph (GC)-mass spectrometer (MS). Volatile compounds were separated using gas-liquid chromatography, at the conditions defined by Hannon, Kilcawley, Wilkinson, Delahunty and Beresford (2007).

7.2.11. Descriptive sensory analysis

The sensory properties of the 270 d-old cheeses from each of the three replicate trials were evaluated using descriptive sensory analysis, as described previously (Hou et al., 2014a). The results are presented as a principal component (PC) plot. Attributes scored for odour included pungent, sweaty/cheesy, rancid, fruity, buttery and caramel; attributes for flavour were pungent, farmyard, creamy, rancid, fruity, buttery, caramel and sweaty/cheesy, while those for taste comprised throat burn, sweet, acid, salt, bitter and astringent.

7.2.12. Statistical analysis

Three replicate cheesemaking trials were undertaken, each with 6 treatment cheeses, namely LDpHC, LDpHGal^{+}, LDpHGal^{-}. HDpHC, HDpHGal^{+} and HDpHGal^{-}. Analysis of variance (ANOVA) was used to determine if the treatment cheeses differed with respect to response variables, such as compositional factors, at specific time points (e.g., 14 d). ANOVA was undertaken using SAS® version 9.1.2 (SAS Institute, 2004), where the effects of treatment (different drain pH or starter system) and replicates were estimated for all response variables. Tukey's multiple-

comparison test was used for paired comparison of treatment means and the level of significance was determined at $P < 0.05$.

The data for changes in individual response variables (such as sugars and micro counts) in the high and low drain pH cheeses over the duration of ripening were analysed using a split-plot design in order to determine the effects of treatment, ripening time, and their interaction. Analysis of variance for the split-plot design was carried out using a general linear model (GLM) procedure of SAS (SAS Institute, 2004), and significance at $P < 0.05$ determined using Fisher's least significant difference test.

The data for volatile compounds and descriptive sensory analysis, measured at 270 d only, were analysed using PCA by Unscrambler V 6.1 (CAMO AS, N-7041 Trondheim, Norway). The results are presented as a principal component (PC) plot.

7.3. Results

7.3.1. Cheese manufacturing time

The use of *Sc. thermophilus* adjunct cultures and alteration of pH at whey drainage had varying effects on the times for the different stages of manufacture, namely the drain time (time from starter culture addition to whey drainage), curd residence time in cheese vat (time from gel cutting to whey drainage), cheddaring time (time from whey drainage to curd milling), and total make time (time from starter culture addition to curd milling).

The mean drain time for the HDpH cheeses (118-121 min) was significantly shorter than that for the LDpH cheeses (170-207 min). The curd residence time in the cheese vat for the HDpH cheeses (53-54 min) was significantly lower than that for the LDpH cheeses (105-135 min). However, the cheddaring time for the HDpH cheeses

(125- 150 min) was significantly longer than that of the LDpH cheeses (71 -87 min) (Table 7.1).

Starter culture had a significant effect on the total make time of the HDpH chesses, but not the low drain pH (LDpH, 6.15) cheeses. The make time for the HDpHGal^{+} cheese (253 min) was significantly shorter (about 30 mins) than the corresponding control HDpHC (205 min) or HDpHGal^{-} cheeses (284 min).

Table 7.1. Effect of different starter culture and pH at whey drainage on the composition of 14 d-old Cheddar cheeses and the times required for different stages of manufacture[1, 2, 3]

	Cheese treatment					
	LDpHC	LDpHGal$^+$	LDpHGal$^-$	HDpHC	HDpHGal$^+$	HDpHGal$^-$
Cheese composition						
Moisture (%, w/w)	38.5^{a,A}	38.7^{a,A}	38.7^{a,B}	40.1^{a,A}	40.9^{a,A}	41.0^{a,A}
Fat (%, w/w)	30.7^{a,A}	30.5^{a,A}	30.7^{a,A}	29.5^{a,A}	29.1^{a,A}	29.1^{a,B}
Protein (%, w/w)	25.4^{a,A}	25.1^{a,A}	25.3^{a,A}	24.8^{a,A}	24.4^{a,A}	24.3^{a,B}
Salt (%, w/w)	1.74^{a,A}	1.93^{a,A}	1.84^{a,A}	1.73^{a,A}	1.66^{a,A}	1.64^{a,A}
Ca (mg 100 g^{-1})	755^{a,A}	757^{a,A}	742^{a,A}	771^{a,A}	769^{a,A}	746^{a,A}
Calcium to protein (mg g^{-1})	29.7^{a,A}	30.2^{a,A}	29.3^{a,A}	31.1^{a,A}	31.5^{a,A}	30.7^{a,A}
P (mg 100 g^{-1})	486^{a,A}	480^{a,A}	476^{a,A}	487^{a,A}	471^{a,A}	472^{a,A}
S/M (%, w/w)	4.54^{a,A}	5.01^{a,A}	4.74^{a,A}	4.30^{a,A}	4.08^{a,A}	3.99^{a,A}
MNFS (%, w/w)	55.5^{a,A}	55.6^{a,A}	55.8^{a,B}	56.9^{a,A}	57.6^{a,A}	57.8^{a,A}
FDM (%, w/w)	49.9^{a,A}	49.7^{a,A}	50.0^{a,A}	49.2^{a,A}	49.2^{a,A}	49.3^{a,A}
pH	5.25^{a,A}	5.34^{a,A}	5.29^{a,A}	5.25^{a,A}	5.24^{a,A}	5.19^{a,B}
Time for different stages of cheese manufacture (min)						
Cut-to-drain	133^{a,A}	104^{b,A}	122^{ab,A}	54^{a,B}	54^{a,B}	53^{a,B}
Drain-to-mill	71^{a,B}	75^{a,A}	87^{a,B}	150^{a,A}	124^{b,A}	151^{a,A}
Cut-to-mill	204^{a,A}	179^{a,A}	210^{a,A}	205^{a,A}	178^{b,A}	204^{a,A}
Total manufacture: Starter addition-to-mill	281^{a,A}	252^{a,A}	279^{a,A}	280^{a,A}	253^{b,A}	284^{a,A}

[1] Low drain pH (LDpH) or high drain pH (HDpH) cheeses made with control culture (LDpHC, HDpHC), galactose-metabolising *Sc. thermophilus* culture (LDpHGal$^+$, HDpHGal$^+$), or galactose-metabolising *Sc. thermophilus* culture (LDpHGal$^-$, HDpHGal$^-$).

[2] Values within a row relating to LDpH cheeses (LDpHC, LDpHGal$^+$, LDpHGal$^-$) or HDpH cheeses (HDpHC, HDpHGal$^+$, HDpHGal$^-$) and not sharing a common lower-case superscript differ significantly ($P < 0.05$) for effect of starter culture; values within a row relating to cheeses made with control culture (LDpHC, HDpHC), galactose-metabolising *Sc. thermophilus* culture(LDpHGal$^+$, HDpHGal$^+$) or galactose-non-metabolising *Sc. thermophilus* culture (LDpHGal$^-$, HDpHGal$^-$), and not sharing a common upper-case superscript letter differ significantly ($P < 0.05$) for effect of drain pH.

[3]Abbreviations: S/M, salt in moisture; MNFS, moisture in non-fat substances; FDM, fat in dry matter.

7.3.2. Composition at 14 d

The gross composition of the cheese was affected by pH but not by starter culture type (Table 7.1). The moisture content of the LDpH cheeses was below the maximum level (39 %, w/w) specified for Cheddar cheese (HMSO, 1996), while that of the HDpH cheeses was higher. Despite the difference in moisture content between the LDpH and HDpH cheeses (1.6 - 2.3 %, depending on starter culture system used), the higher moisture content of the HDpH cheeses was significant only in the case of HDpHGal$^-$ cheese. A similar trend was noted for content of moisture-in-fat substances (MNFS).

7.3.3. Changes in sugars during ripening

7.3.3.1. Lactose and galactose

The effects of drain pH and starter culture system on the changes in lactose and galactose over the course of ripening are shown in Figure 7.1 a to d and Table 7.2. The mean level of residual lactose in the LDpH cheeses over the 270-d ripening period was significantly affected by starter culture system, ripening time and their interaction (Figure 7.1a, Table 7.2). The mean lactose content in the HDpH cheeses was, similarly, influenced by ripening time, but not by starter culture (Figure 7.1b, Table 7.2).

Lactose content decreased during maturation (Figure 7.1a, b), and was, essentially, fully metabolized in all cheeses by 90 d, apart from the LDpHC cheese which had a significantly higher content than that of the corresponding LDpHGal$^+$ and LDpHGal$^-$ cheeses at this time. The mean lactose level over the 270-d ripening period in the LDpHC cheese was significantly higher than that in the corresponding LDpHGal$^+$ and LDpHGal$^-$ cheeses. The results indicate that residual lactose content in Cheddar cheese (< 180 d-old) can be reduced by the use of *Sc. thermophilus* (Gal$^+$

or Gal$^-$) as a culture adjunct when the pH at whey drainage is low, or by increasing the pH at whey drainage when the cheese is made using the control starter culture.

The galactose content at 1 d varied from ~ 0.2 – 0.025 %, remained relatively constant between d 1 and 14, and thereafter decreased to ≤ 0.05 % in all cheeses at 180 d (Figure. 7.1 c, d). Starter culture had a significant effect on the mean galactose level over the 270 d ripening period in the HDpH cheeses, with the mean concentration in the HDpHGal$^+$ cheese being higher than that of the HDPHC or HDpHGal$^-$ cheeses (Table 7.2). While a similar overall pattern was observed in the LDpH cheeses, the effect of starter culture was not significant, probably because of the relatively large inter-trial variation in galactose content. Overall, the results indicate that the use of the Gal$^+$ *Sc. thermophilus* led to higher residual galactose content in young Cheddar cheese (≤ 30 d), especially where the pH at whey drainage is high, as frequently is the case in large modern cheese manufacturing facilities using direct-vat starter cultures (DVS).

The mean levels of reducing sugars (lactose plus galactose) over ripening were unaffected by the addition of *Sc. thermophilus* (Table 7.2), indicating that the associated increase in galactose was offset by the concomitant decrease in lactose content; similarly, the drain pH did not significantly affect the level of reducing sugars for any of the cultures used.

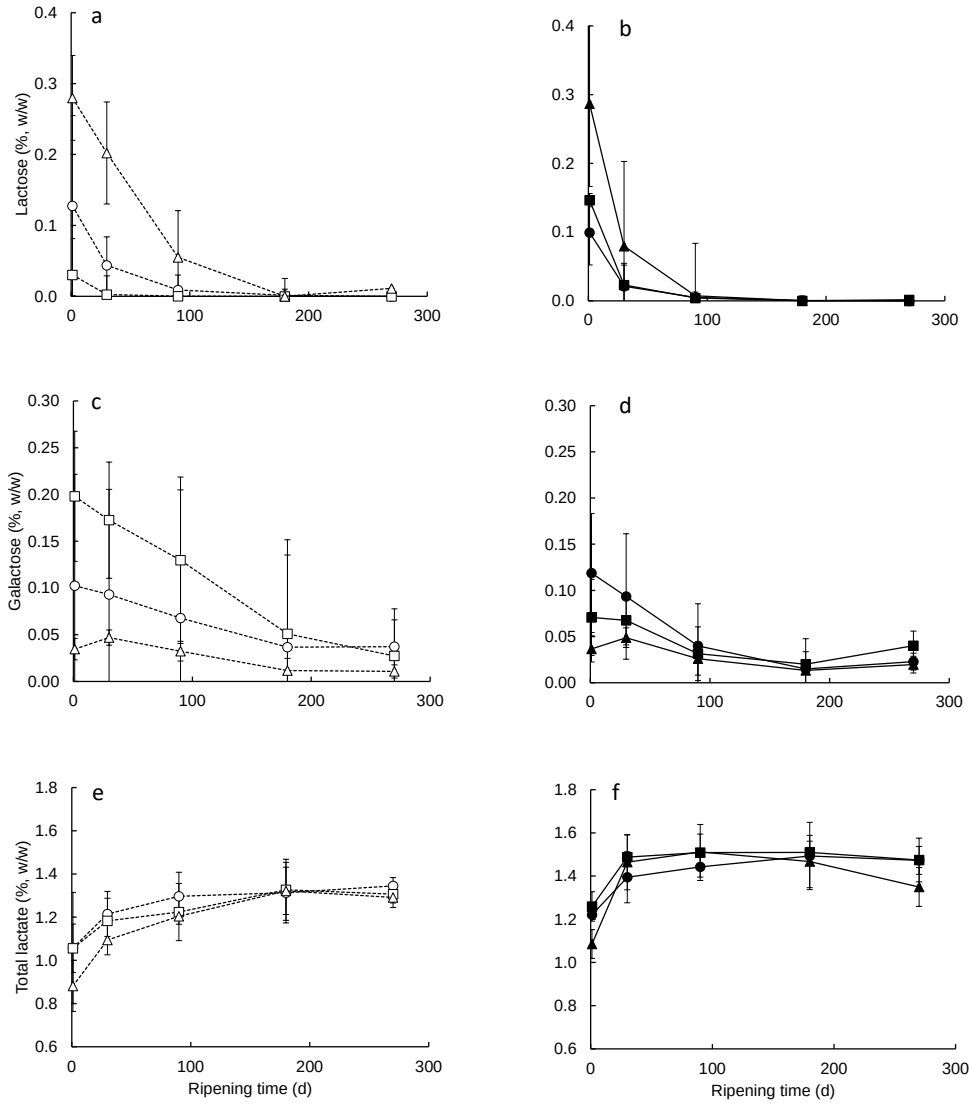

Figure 7.1. Changes in the level of residual lactose (a and b), galactose (c and d) and total lactate (e and f) during ripening in low drain pH, LDpH (broken line, open symbol) and high drain pH, HDpH (solid line, closed symbol) Cheddar cheeses made with control starter culture (LDpHC, △; HDpHC, ▲), control starter culture with a galactose metabolizing *Sc. thermophilus* culture adjunct (LDpHGal$^+$, ○; HDpHGal$^+$, ●) or control starter culture with a galactose non-metabolizing *Sc. thermophilus* culture adjunct (LDpHGal$^-$, □; HDpHGal$^-$, ■). Presented values are the means of three replicate trials; error bars represent standard deviations of the mean.

Table 7.2. Statistical significances (P values) for effects of starter culture and ripening time on concentrations of lactose, galactose, total lactate and pH in Cheddar cheeses made using low- or high-drain pH [1, 2]

Factor	Lactose		Galactose		Lactose + Galactose		Total lactate		pH	
	df	*P*	df	*P*	df	*P*	df	*P*	df	*P*
Low drain pH cheese										
Main plot										
Starter system	2	0.005	2	0.158	2	0.869	2	0.227	2	0.583
Sub-plot										
Ripening time	5	<.001	5	<.001	5	<.001	5	<.001	5	0.250
Interaction (starter system x ripening time)	10	0.0002	10	0.110	10	0.210	10	0.696	10	0.475
High drain pH cheese										
Main plot										
Starter system	2	0.073	2	0.036	2	0.394	2	0.371	2	0.207
Sub-plot										
Ripening time	5	<.001	5	<.001	5	<.001	5	<.001	5	0.554
Interaction (starter system x ripening time)	10	0.004	10	0.048	10	0.136	10	0.206	10	0.820

[1]Cheeses were made with different starter cultures (C, Gal^+ or Gal^-, as described in Materials and Methods) at low or high drain pH.
[2]Analysis of variance was carried using the general linear model (GLM) procedure of SAS, where the effects of treatment, ripening time and their interaction were estimated; there were '2' degrees of freedom (df) for starter system and '5' for ripening time.

7.3.3.2. Total lactate

The metabolization of lactose and galactose resulted in a significant increase in lactate content during the first 30 d, from ~ 1 to 1.3 % in the LDpH cheeses and 1.2 – 1.45 % in the HDpH cheeses; thereafter lactate levels remained relatively constant. The mean concentration over the 270 d maturation period was significantly higher in the HDpH cheeses than that in the corresponding LDpH cheeses for each culture type ($P < 0.05$). This trend is consistent with the higher level of moisture (which is the solvent for lactose) and higher enzyme activity in the HDpH cheeses.

The mean level of total lactate in the LDpH or HDpH cheeses over the 270 d ripening period was unaffected by the starter culture type (Figure 7.1e and f, Table 7.2).

7.3.4. pH changes during ripening

The mean pH of the LDpH and HDpH cheeses over the 270 d ripening period was not significantly affected by the starter culture, ripening time or their interaction (Table 7.2). In contrast, the pH at whey drainage had a significant effect, with the pH of the LDpH cheeses being slightly (~ 0.07 pH units), but significantly ($P < 0.05$), higher than that of the corresponding HDpH cheeses at all times. The slightly higher pH of the LDpH cheeses concurs with their lower contents of moisture and lactic acid.

7.3.5. Microbial counts of starter and non-starter lactic acid bacteria (NSLAB) in cheese

7.3.5.1. Starter bacteria (Lactococcus)

The mean count of starter lactococci significantly decreased in all cheeses during ripening, from ~ 1 x 10^{10} cfu g^{-1} at 1 d to ~ 3.2 x 10^{7} at 270 d (Table 7.3, Figure

7.2a, b). The inclusion of Gal^+ or Gal^- *Sc. thermophilus* strains in the starter culture did not influence the mean of count over the ripening period.

The lactococci count in the LDpHC cheese at 270 d was slightly, but significantly, higher than that of the corresponding HDpHC cheese ($P < 0.05$). As the counts in both cheeses (LDpHC, HDpHC) were similar at 1 d, the higher count in the 270 d-old LDpHC cheese suggests a lower degree of starter cell autolysis, which could be associated with its lower mean level of lactic acid over the 270 d ripening period (Nájera-Domínguez & Gutiérrez-Méndez, 2013) and would also due to the slightly higher S/M levels compared to HDpHC .

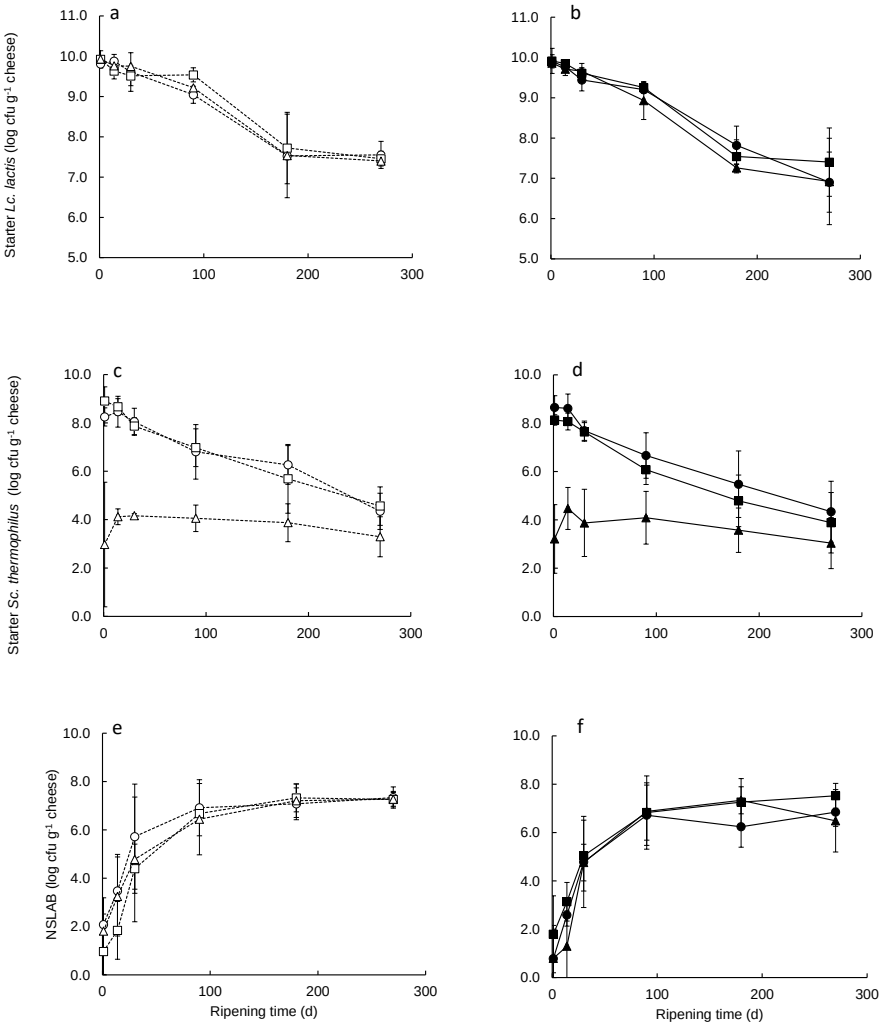

Figure 7.2. Changes in the counts of starter *Lactococcus* (a and b), *Sc. thermophilus* (c and d) and non-starter lactic acid bacteria (e and f) during ripening in low drain pH, LDpH (broken line, open symbol) and high drain pH, HDpH (solid line, closed symbol) Cheddar cheeses made with control starter culture (LDpHC, △; HDpHC, ▲), control starter culture with a galactose metabolizing *Sc. thermophilus* culture adjunct (LDpHGal^{+}, ○; HDpHGal^{+}, ●) or control starter culture with galactose non-metabolizing *Sc. thermophilus* culture adjunct (LDpHGal^{-}, □; HDpHGal^{-}, ■). Presented values are the means of three replicate trials; error bars represent standard deviations of the mean.

Table 7.3. Statistical significances (P values) for effects of starter culture and ripening time on counts of *Lactococcus*, *Sc. thermophilus* and non-starter lactic acid bacteria (NSLAB) in Cheddar cheeses made using low- or high-drain pH[1, 2].

Factor	*Lactococcus*[3]		*Sc. thermophilus*[3]		NSLAB[3]	
	df	*P*	df	*P*	df	*P*
Low drain pH cheese						
Main plot						
Starter system	2	0.883	2	0.028	2	0.002
Sub-plot						
Ripening time	5	<.001	5	<.001	5	<.001
Interaction (starter system x ripening time)	10	0.759	10	<.001	10	0.337
High drain pH cheese						
Main plot						
Starter system	2	0.120	2	<.001	2	0.284
Sub-plot						
Ripening time	5	<.001	5	<.001	5	<.001
Interaction (starter system x ripening time)	10	0.337	10	<.001	10	0.145

[1]Cheeses were made with different starter cultures (C, Gal^+ or Gal^-, as described in Materials and Methods) at high – or low drain pH.
[2]Analysis of variance was carried using the general linear model (GLM) procedure of SAS, where the effects of treatment, ripening time and their interaction were estimated; there were '2' degrees of freedom (df) for starter system and '5' for ripening time.

7.3.5.2. Sc. thermophilus

The mean count of *Sc. thermophilus* over the 270 d ripening period was significantly affected by starter system and ripening time in both the LDpH and HDpH cheeses (Table 7.3, Figure 7.2c, d).

Sc. thermophilus grew (from ~ 1 x 10^6 cfu g^{-1} in the milk following inoculation) during cheese manufacture and pressing to reach counts of ~1 x 10^9 cfu g^{-1} in the Gal^+ and Gal^- cheeses at 1 d (Figure. 7.2c, d). The population in the Gal^+ and Gal^- cheese decreased significantly during ripening to ~1 x 10^5 cfu g^{-1} at 270 d (Figure 7.2c, d). While the mean count of *Sc. thermophilus* over the 270 ripening period were similar

in the LDpHGal$^+$ and LDpHGal$^-$ cheeses, that in the HDpHGal$^-$ cheese was slightly, but significantly, lower than that in the HDpHGal$^+$ cheese.

The mean count of *Sc. thermophilus* in the control cheeses (LDpHC, HDpHC) was significantly lower than that of the corresponding Gal$^+$ and Gal$^-$ cheeses, which had similar counts at 1 d (1 x 10^9 cfu g^{-1}). Nevertheless, *Sc. thermophilus* was present in the control HDpHC and LDpHC cheeses at ~ 10^3 cfu g^{-1} cheese on 1 d, grew to ~ 1 x 10^4 cfu g^{-1} between 1 and 5 d, and remained essentially constant at this level through the remainder of ripening. The low *Sc. thermophilus* count in the control cheeses probably reflects cross-contamination during cheese manufacture, even though care was taken to avoid this.

7.3.5.3. Non-starter lactic acid bacteria (NSLAB)

NSLAB were present in all cheeses at ≤ 3.2 x 10^2 cfu g^{-1} at 1 d and grew during ripening, reaching counts of ~ 3.2 x 10^6 – 10^7 cfu g^{-1} at 180 d (Table 7.3, Figure 7.2e, f). The mean population in the LDpH cheeses over the 270 d ripening period was significantly affected by starter culture system, with the mean count in the LDpHGal$^+$ cheese being significantly higher than that in the LDpHGal$^-$ cheese, and numerically, though not significantly, higher than that in the LDpHC (Figure 7.2c, Table 7.3). Post-hoc analysis showed that the counts in the LDpHGal$^+$ were significantly higher than that in the LDpHGal$^-$ cheese at 1, 14 and 30 d but similar at all other times.

7.3.6. Proteolysis

The mean level of pH4.6-SN, which is indicative of hydrolysis of the insoluble intact calcium phosphate *para*-casein into water soluble peptides by residual chymosin, increased significantly in all cheeses during ripening from ~ 5 % of total nitrogen at 1

d to ~ 26 - 29 % at 270 d (Figure 7.3). The mean level over the 270-d ripening was significantly affected by starter culture system in the HDpH cheeses (Table 7.4), with the mean level in the HDpHC cheese being significantly lower than that in the HDpHGal$^+$ or HDpHGal$^-$ cheeses for which it was similar; no such difference was found between the LDpH cheeses. However, these differences were quite small (0.6 – 1.3 %) and are unlikely to have had a notable effect on the physical or sensory properties of the cheese. The pH at whey drainage did not affect the content of pH4.6-SN.

The concentration of FAA increased significantly during ripening (Figure 7.4), with glutamic acid, leucine, phenylalanine and valine being the major FAAs present in all cheeses (Figure 7.5). The mean concentration of FAA in the LDpH or HDpH cheeses over the 270-d ripening period was not affected by the starter culture (Table 7.4). In contrast, pH at whey drainage had a significant effect, with the LDpH cheeses having significantly higher mean levels of FAA than the corresponding HDpH cheeses over the 270 d ripening period. The 270 d-old LDpH cheeses had significantly higher levels of total FAA, glutamic acid, valine, leucine, phenylalanine, proline and lysine than the corresponding HDpH cheeses (Figure 7.5). The differences in FAA concentration between the cheeses may reflect inter-cheese differences in peptidase activities as affected by pH, NSLAB species (Gobbetti et al, 1999), and degrees of autolysis and permeability of starter and non-starter bacteria (Doolan &Wilkinson, 2009).

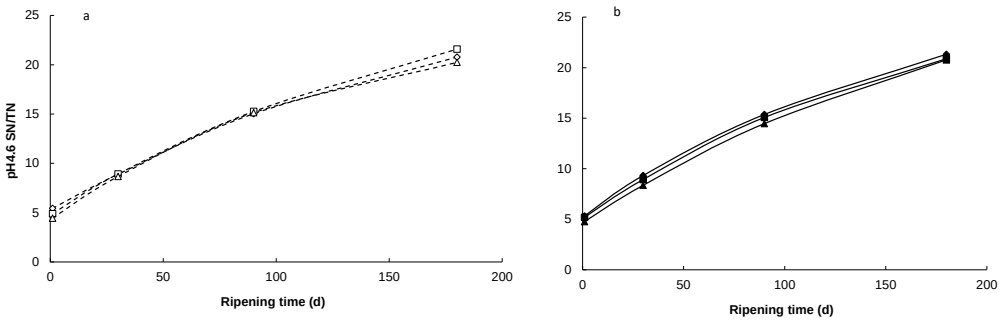

Figure 7.3. Changes in the concentrations of pH4.6-SN during ripening in low drain pH, LDpH (a, broken line, open symbol) and high drain pH, HDpH (b, solid line, closed symbol) Cheddar cheeses made with control starter culture (LDpHC,△; HDpHC,▲), control starter culture with a galactose metabolizing *Sc. thermophilus* culture adjunct (LDpHGal^{+},○; HDpHGal^{+},●) or a control starter culture with galactose non-metabolizing *Sc. thermophilus* as culture adjunct (LDpHGal^{-},□; HDpHGal^{-},■). Presented values are the means of three replicate trials; error bars represent standard deviations of the mean.

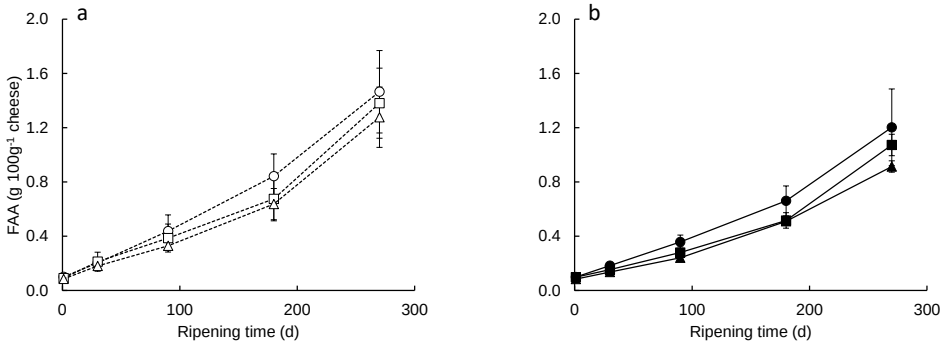

Figure 7.4. Changes in the concentrations of total free amino acids (FAA) during ripening in low drain pH, LDpH (a, broken line, open symbol) and high drain pH, HDpH (b, solid line, closed symbol) Cheddar cheeses made with control starter culture (LDpHC,△; HDpHC,▲), control starter culture with a galactose metabolizing *Sc. thermophilus* culture adjunct (LDpHGal^{+},○; HDpHGal^{+},●) or a control starter culture with galactose non-metabolizing *Sc. thermophilus* as culture adjunct (LDpHGal^{-},□; HDpHGal^{-},■). Presented values are the means of three replicate trials; error bars represent standard deviations of the mean.

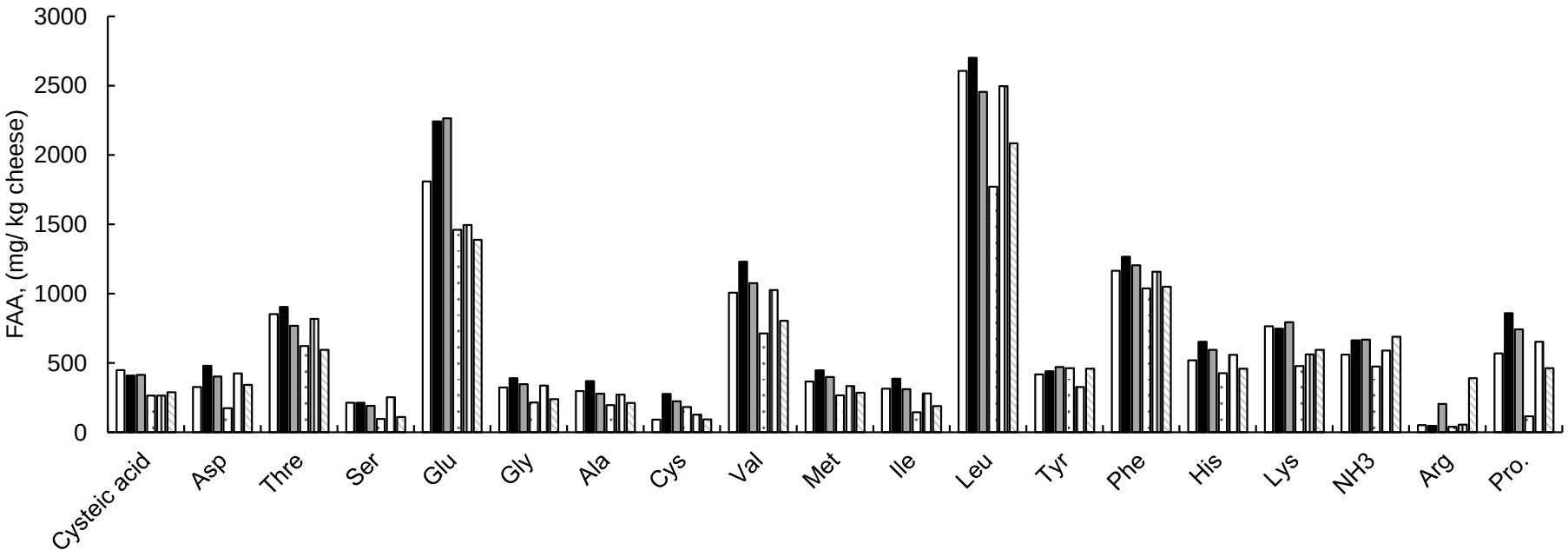

Figure 7.5. The concentrations of individual free amino acids (FAA) at day 270 in low drain pH, LDpH and high drain pH, HDpHCheddar cheeses made with control starter culture (LDpHC, □; HDpHC, ▨), control starter culture with a galactose metabolizing *Sc. thermophilus* culture adjunct (LDpHGal^{+},■; HDpHGal^{+}, ▥) or a control starter culture with galactose non-metabolizing *Sc. thermophilus* as culture adjunct (LDpHGal^{-}, ▩; HDpHGal^{-}, ▨). Presented values are the means of three replicate trials; error bars represent standard deviations of the mean.

Table 7.4. Statistical significances (*P* values) for effects of starter culture and ripening time on levels of pH 4.6-soluble N (pH4.6-SN) and free amino acids (FAA) in Cheddar cheeses made using low- or high-drain pH[1, 2].

Factor	pH4.6-SN		FAA	
	df	*P*	df	*P*
Low drain pH cheese				
Main plot				
Starter system	2	0.883	2	0.499
Sub-plot				
Ripening time	4	<.001	4	<.001
Interaction (starter system x ripening time)	8	0.96	8	0.754
High drain pH cheese				
Main plot				
Starter system	2	<.001	2	0.081
Sub-plot				
Ripening time	4	<.001	4	<.001
Interaction (starter system x ripening time)	8	0.978	8	0.122

[1]Cheeses were made with different starter cultures (C, Gal^+ or Gal^-, as described in Materials and Methods) at high or low drain pH.
[2]Analysis of variance was carried using the general linear model (GLM) procedure of SAS, where the effects of treatment, ripening time and their interaction were estimated; there were '2' for degrees of freedom (df) for starter system and '4' for ripening time.

7.3.7. Rheological properties

The mean values of firmness, fracture stress and fracture strain of all cheeses decreased significantly during ripening (Table 7.5). The decreases are consistent with the increase in primary proteolysis of calcium phosphate *para*-casein network (Figure 7.6a, b and c), which is the main structural component of the cheese matrix controlling the level of stress in response to applied deformation, e.g., during compression (Guinee, 2016). Starter culture had no effect on rheological properties of either the LDpH or HDpH cheeses (Table 7.5), a trend compatible with the very small differences in pH4.6-SN between the LDpH cheeses or HDpH cheeses. In contrast, the pH at drainage had a significant effect on firmness, with that of the LDpH cheeses, which had lower moisture content (Table 7.1), being significantly higher than that of the HDpH cheeses (Figure 7.6a).

Table 7.5. Statistical significances (*P* values) for effects of starter culture and ripening time on the firmness, facture stress and fracture strain of Cheddar cheese made using low- or high-drain pH[1, 2].

Factor	Firmness		Facture stress		Fracture strain	
	df	*P*	df	*P*	df	*P*
Low drain pH cheese						
Main plot						
Starter system	2	0.240	2	0.346	2	0.340
Sub-plot						
Ripening time	4	<.001	4	<.001	4	<.001
Interaction (starter system x ripening time)	8	0.949	8	0.453	8	0.294
High drain pH cheese						
Main plot						
Starter system	2	0.066	2	0.163	2	0.773
Sub-plot						
Ripening time	4	<.001	4	<.001	4	<.001
Interaction (starter system x ripening time)	8	0.923	8	0.483	8	0.949

[1]Cheeses were made with different starter cultures (C, Gal^+ or Gal^-, as described in Materials and Methods) at high – or low drain pH.
[2]Analysis of variance was carried using the general linear model (GLM) procedure of SAS, where the effects of treatment, ripening time and their interaction were estimated; there were ‘2’ for degrees of freedom (df) for starter system and ‘4’ for ripening time.

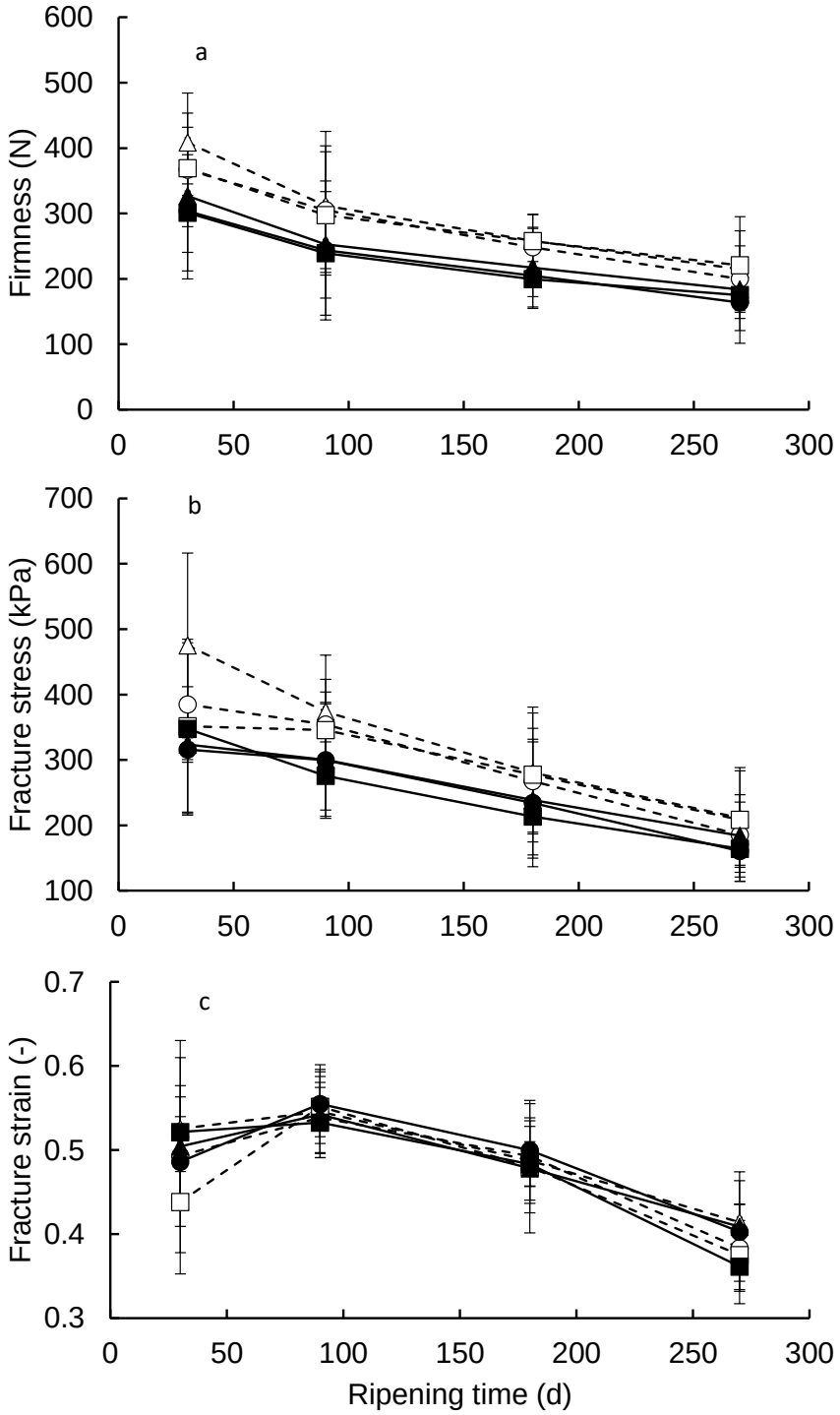

Figure 7.6. Changes in the cheese (a) firmness, (b) fracture stress and (c) fracture strain during ripening in low drain pH, LDpH (broken line, open symbol) and high drain pH, HDpH (solid line, closed symbol) Cheddar cheeses made with control starter culture (LDpHC,△; HDpHC,▲), control starter culture with a galactose metabolizing *Sc. thermophilus* culture adjunct (LDpHGal^{+},○; HDpHGal^{+},●) or control starter culture with a galactose non-metabolizing *Sc. thermophilus* as culture adjunct (LDpHGal^{-},□; HDpHGal^{-},■). Presented values are the means of three replicate trials; error bars represent standard deviations of the mean.

7.3.8. Volatile compounds at 270 d

Thirty six different volatile compounds were identified in the 270-d old cheese. These comprised 10 alcohols, 8 ketones, 3 esters, 3 aldehydes, 6 acids, 2 sulphur compounds, 2 alakanes, 1 alkene (Octene) and 1 terpene (limonene) were identified in all of the cheeses.

PCA was undertaken to establish if the different cheeses could be separated by the types and concentrations of volatile compounds; a biplot of the volatile compounds is presented in Figure 7.7. Principal components PC1 and PC2 accounted for 47 % of and 25 % of explained variance between the cheeses, respectively. Three cheeses including the control (LDpHC and HDpHC) and HDpHGal^{+} cheeses scored positively on PC1, and three (LDpHGal$^{+,}$ LDpHGal^{-} and HDpHGal^{-}) scored negatively. In contrast, all cheeses, apart from LDpHC and LDpHGal^{+}, scored positively on PC2. Two groupings of cheeses were identifiable based on their proximity on both PC1 and PC2, namely the cheeses made using *Sc. thermophilus* at high drain pH (HDpHGal^{+} and HDpHGal^{-}) or at low drain pH (LDpHGal^{+} and LDpHGal^{-}). The HDpHGal^{+} and HDpHGal^{-} cheeses were associated principally with ketones (butanone, acetoin), acetic acid, and dimethyl sulfone. The LDpHGal^{+} and HDpHGal^{-} cheeses were aligned with a large range of volatile compounds including fatty acids (butanoic, pentanoic, heptanoic and octanoic acid), alcohols (butanol, 3 methyl-1-butanol, 2 heptanol, 1 hexanol), ethyl esters of fatty acids (butanoic, octanoic, hexanoic), ketones (2 nonanone and 2 heptanone), aldehydes (benzeneacetaldehyde, nonanal) and limonene.

The control cheeses (LDpHC and HDpHC) differed from the adjunct-containing cheeses and from each other with respect to volatile compounds. The LDpHC cheese was characterized by the presence of an array of volatiles including alcohols (ethanol, 1- and 2-pentanol, 3-methyl-2-buten-1-ol and 3-methyl-3-buten-1-

ol), ketones (acetone, 2-butanone), hydrocarbons (octane, pentane, heptane), acids (butanoic, hexanoic) and carbon disulfide; few volatile compounds were identified in the HDpHC cheese.

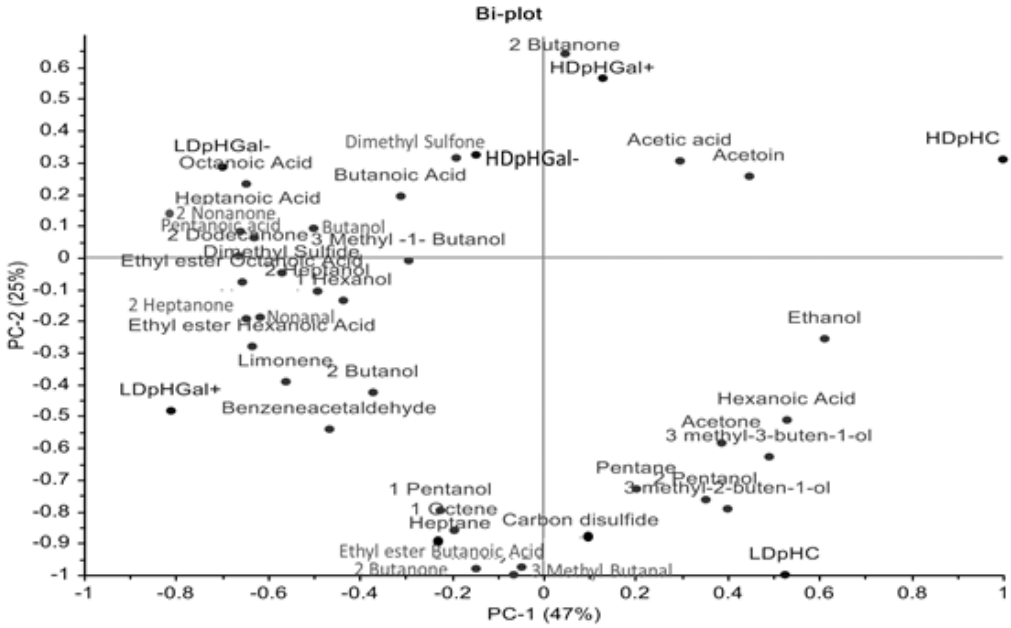

Figure 7.7. Principal component analysis showing the first two principal components (PC) of volatile compounds in 270 d-old low drain pH (LDpH) and high drain pH (HDpH) Cheddar cheeses made with control starter culture (LDpHC; HDpHC), control starter culture with galactose metabolizing *Sc. thermophilus* culture adjunct (LDpHGal$^+$; HDpHGal$^+$) or control starter culture with galactose non-metabolizing *Sc. thermophilus* as culture adjunct (LDpHGal$^-$; HDpHGal$^-$) Presented values are the means of three replicate trials.

7.3.9. Descriptive sensory analysis at 270 d

The PCA biplot for the different odour and flavour attributes of the 270 d-old cheeses is shown in Figure 7.8. The first two PCs discriminated significantly between the cheeses and accounted for a cumulative explained variance of 77 %. Two distinct groupings were evident based on proximity on PC1 and PC2, namely the HDpHGal$^+$ and HDpHGal$^-$ cheeses which had a sweaty and rancid flavour, a rancid and sweaty odour, and acid taste, and the LDpHC and LDpHGal$^+$ cheeses which had buttery flavour, caramel odour and sweet taste. In contrast, the HDpHC and LDpHGal$^-$

cheeses were separated from the above groupings and from each other with respect to sensory characteristics. The former had a fruity and creamy flavour, and a fruity buttery odour, while the LDpHGal^{-} cheese had a pungent and farmyard flavour, pungent odour, pungent throat and astringent sensations, and bitter taste (Figure 7.8).

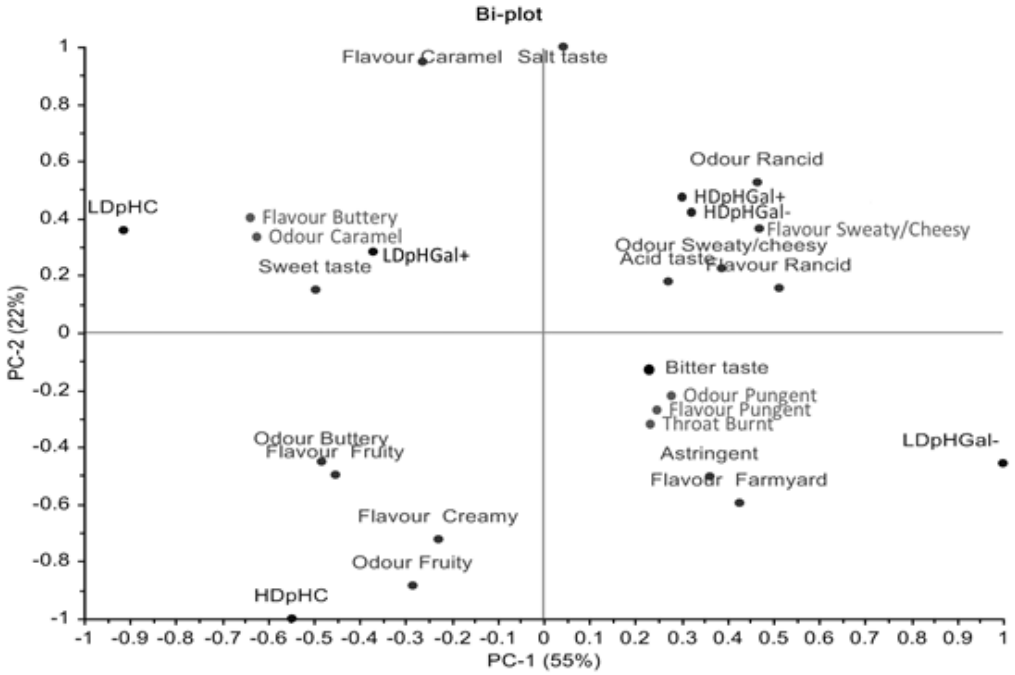

Figure 7.8. Principal component analysis showing the first two principal components (PC) of descriptive sensory odour and flavour attributes in 270 d-old in low drain pH (LDpH) and high drain pH (HDpH) Cheddar cheeses made with control starter culture (LDpHC; HDpHC), control starter culture with a galactose metabolizing *Sc. thermophilus* culture adjunct (LDpHGal$^+$; HDpHGal$^+$) or control starter culture with a galactose non-metabolizing *Sc. thermophilus* as culture adjunct (LDpHGal$^-$; HDpHGal$^-$) Presented values are the means of three replicate trials.

7.4. Discussion

The current study investigated the effects of adding either a galactose metabolising (Gal$^+$) or galactose non-metabolising (Gal$^-$) strain of *Sc. thermophilus* on the properties of Cheddar cheese made using either a low (pH 6.15) or high (pH 6.45) pH at whey drainage. The study is of significance because of the growing use of *Sc. thermophilus* as an adjunct culture and the increase in pH at whey drainage, accompanying the transition from bulk mesophilic starter culture (*Lc. lactis* subsp. *cremoris* or *lactis*) to direct-vat starter mesophilic culture in large modern Cheddar cheese factories. *Sc. thermophilus* was inoculated at a level of 10^6 cfu mL^{-1} milk, resulting in counts of 10^9 cfu g^{-1} cheese at 1 d, decreasing gradually to ~ 10^5 cfu g^{-1} at

270 d. The use of the *Sc. thermophilus* adjuncts had significant effects on levels of residual sugars (lactose plus galactose), volatile compounds and sensory properties of the 270 d-old cheese to a degree dependent on its galactose-metabolising ability and pH, and altering the pH at whey drainage directly affected cheese composition (contents of moisture, MNFS), lactic acid level, pH and firmness irrespective of the culture systems used.

While lowering the pH at whey drainage resulted in lower contents of moisture (1.6 - 2.3 %) and MNFS (~ 1.4 - 2.0 %) in all cheeses, the effect was significant only in cheeses made with the Gal^- *Sc. thermophilus* strain. The lower moisture content of the LDpH cheeses was consistent with the longer residence time of the curd particle-whey mixture in the cheese vat prior to whey drainage (Everard, O'Callaghan, Mateo, Castillo, Payne & O'Donnell, 2011). Whey expulsion in the cheese vat is more intense than that which occurs after whey drainage (e.g., during cheddaring, salting and moulding) because the higher surface area of the curd particles (compared to slabs of fused curd particles) and the higher temperature (~3 °C) compared to that during curd cheddaring (Dejmek & Walstra, 2004). The numerically higher moisture content of the HDpH cheeses, though non-significant in the case of the HDpHC and $HDpHGal^+$ cheeses, could have practical implications, in terms of compliance to compositional specification, quality and yield. However, normalization of moisture content in cheese produced at different pH could easily be achieved through process intervention, whereby factors such as firmness of gel at cutting, curd particle size and rate of cooking are altered (Guinee & O'Callaghan, 2010).

Owing to their lower moisture content, the LDpH cheeses had a lower mean level of lactic acid, higher pH and higher firmness than the corresponding HDpH cheeses. This trend concurs with the findings of other studies (Rynne, Beresford, Kelly,

& Guinee, 2004; Chevanan, Muthukumarappan, Upreti & Metzger, 2006; Upreti, Bühlmann & Metzger, 2006; McCarthy, Wilkinson, Kelly & Guinee, 2015, 2016). In contrast to the current results, Lee, Johnson & Lucey (2005) found that a reduction in the pH at whey drainage from 6.17 to 5.82 led to a reduction in pH of Cheddar cheese (from ~ 5.0 to 4.8), while Tunick, Guinee, van Hekken, Beresford and Malin (2007) reported no change in in the pH of half-fat Cheddar cheese when reducing the drain pH from 6.3 to 5.85. The inter-study discepancy on the effect of drain pH on cheese pH probably relates to differences in manufacaturing conditions, such as the pH at set and the range of pH investiagated, that influence the concentration of calcium phosphate which buffers the pH of cheese upwards (Lucey & Fox, 1993). Hence, reducing drain pH by lowering the set pH (e.g., by pre-acidication of the cheese milk) is conducive to a reduction in clacium phosphate content (Table 1), a lower buffering capacity and a lower cheese pH (Lee et al., 2005), while lowring drain pH by extending the curd residence time of the curd/whey mixture in the cheese vat is conducive to lower moisture content with liitle, or no, effect on calcium content and a slighly higher pH (as in the current results).

Lactose was present in all cheeses at 1 d, with levels in the control LDpHC and HDpHC cheeses (~ 0.25- 0.3%) being higher than that (< 0.15%) in the corresponding cheeses (LDpHGal$^+$, LDpHGal$^-$, HDpHGal$^+$, HDpHGal$^-$) made using the Gal$^+$ or Gal$^-$ strains of *Sc. thermophilus*. Moreover, the use of the *Sc. thermophilus* adjuncts led to faster depletion of lactose, especially in the LDpH cheeses, in which lactose was fully metabolised at 90 d in the LDpHGal$^+$ and LDpHGal$^-$ cheeses compared to 180 d in the LDpHC cheese. High residual lactose content in Cheddar cheeses has been previously reported by others (Shakeel-Ur-Rehman, Waldron & Fox, 2004; Upreti & Metzger, 2006; Hou et al., 2012). The current study indicated that increasing the pH at whey

drainage in the control Cheddar cheese (made with mesophilic culture) and the use of Gal^+ culture are effective means of reducing the residual lactose content in Cheddar cheese. In contrast to the trend noted for lactose, cheeses made using *Sc. thermophilus* ($LDpHGal^+$, $LDpHGal^-$, $HDpHGal^+$ and $HDpHGal^-$) had relatively high levels of residual galactose (~ 0.075 - 0.2 % or 4.2 - 11.34 mM at 1 d) compared to control LDpHC or HDpHC cheeses (~ 0.025 % or 1.3 mM at 1 d), especially at times ≤ 30 d. While the combined concentration of reducing sugars (lactose and galactose) were unaffected by *Sc. thermophilus*, the *Sc. thermophilus*-containing cheese may have a greater propensity to browning on cooking because of the higher concentration of galactose, which has lower molecular mass than lactose and, hence, higher number of reducing groups per unit weight of reducing sugars.

The addition of *Sc. thermophilus* had a notable effect on both the type of volatile compounds and sensory properties of the 270 d-old cheeses, with the effect dependent on culture phenotype and pH. At high drain pH, the addition of *Sc. thermophilus* increased the range of volatile compounds found in the cheeses ($HDpHGal^+$, $HDpHGal^-$), including butanoic acid, butanone, acetoin, dimethyl sulfone, and acetic acid; by comparison, the control HDpHC cheese had relatively few volatile compounds, apart from acetic acid and acetoin. The change in profile of volatile compounds coincided with a marked transition in sensory properties, from a buttery/fruity odour and creamy/fruity flavour in the HDpHC cheese to a sweaty, cheesy and rancid odour and sweaty, cheesy, rancid flavour. Such a trend is consistent with the strong sweaty, cheesy aroma of butanoic acid, and the sour aroma of acetic acid (Singh, Drake & Cadwallader, 2003; Kilcawley, 2016). Similarly, the addition of *Sc. thermophilus* to the low drain pH cheeses resulted in a major shift in the profile of volatile compounds, from a predominance of short-chain alcohols (ethanol, methyl-

butene-ols, pentanol), hydrocarbons and ketones in the control LDpHC cheese to short chain fatty acids (butanoic, pentanoic, heptanoic and octanoic acid), ethyl esters of fatty acids, alcohols, ketones and aldehydes (benzene acetaldehyde, nonanal), in the LDpHGal$^+$ and LDpHGal$^-$ cheeses; nevertheless, the latter cheeses varied with the short chain fatty acids being more closely aligned with the LDpHGal$^-$ cheese while aldehydes and ketones with the HDpHGal$^+$ cheeses. Unlike the trend observed in the HDpH cheeses, the effect of *Sc. thermophilus* of the sensory properties on the LDpH cheeses was dependent on its galactose metabolizing ability. Based on their closeness on both PC1 and PC2, the LDpHGal$^+$ and LDpHC cheese were distinguished as a group and characterised as having a caramel odour, sweet taste and buttery flavour, with the intensity of these attributes being higher in the former than the latter. In contrast, the LDpHGal$^-$ had a pungent odour, with a bitter, throat burn, astringent and farmyard flavour. Hence, despite the LDpHC and LDpHGal$^+$ cheeses belonging to the same PCA grouping for volatile compounds, they belonged to a different grouping for the corresponding descriptive sensory analyses. This confirms that sensory perception of cheese at any time is complex, being determined by volatile compounds, taste compounds, texture and their interaction (Szczesniak, 2002).

7.5. Conclusion

The use of *Sc. thermophilus* as an adjunct culture (to *Lc. lactis* subsp. *lactis*) affected the levels of residual lactose and galactose, the profile of volatile compounds and sensory properties of Cheddar cheese to an extent dependent on the drain pH and *Sc. thermophilus* phenotype (Gal$^+$ or Gal$^-$). At high drainage pH (6.45), the use of both Gal$^-$ or Gal$^+$ strains of *Sc. thermophilus* gave Cheddar cheese which at 270 d had a sweaty, rancid flavour, a rancid and sweaty odour, and acid taste, compared to control

cheese (without adjunct) which had a fruity, creamy flavour, and a fruity butter odour. This difference was associated with changes in the profile of volatile compounds. Conversely, at low drain pH (6.15), the control cheese and cheese made using Gal^+ strain of *Sc. thermophilus* were closer in sensory properties (buttery flavour, caramel odour and sweet taste) than the cheese made using the Gal^- strain of *Sc. thermophilus*, which had a pungent and farmyard flavour, pungent odour, pungent throat and astringent sensations, and bitter taste. For both the control culture and adjunct-containing cultures, reducing the pH at whey drainage from 6.45 to 6.15 resulted in cheese that had lower levels of moisture and FAA, and was firmer. The results suggest the use of *Sc. thermophilus* as a starter culture adjunct may be used as a means of creating Cheddar cheese variants with distinctive flavour profiles; but when using Gal^- variant of *Sc. thermophilus*, the pH at whey drainage should be increased to avoid the accumulation of high levels of residual galactose during ripening.

7.6. Acknowledgements

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Chapter 8: GENERAL DISCUSSION

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Irish cheese production and consumption, mostly Cheddar, are increasing annually from a pool of milk drawn from a small geographical area, using starters and coagulants obtained from a limited number of commercial sources and often with identical technology. However, intra- and inter-factory variations in quality beset cheesemakers and have defied their best attempts to produce consistently premium-grade Cheddar cheese. While previous research paid close attention to pH, composition, ionic strength (NaCl) and temperature, it is possible that equally close attention can be paid to to physicochemical and biochemical parameters which to date have received little attention, including milk composition (levels of lactose, protein and fat), processing parameters, residual lactose/lactate and galactose (in cheeses made with starter systems containing *Streptococcus thermophilus* as adjunct) and CaP, may allow much more precise control of cheese quality and avoid specific defects. The parameters studied in this project could easily be measured and/or implemented in industry and could form part of future strategies for optimization of the Cheddar cheesemaking process and reducing variation in quality. Routine determination of some of these parameters will lead to the generation of new sets of quality parameters giving a competitive advantage to the Irish Cheddar cheese industry. The objective of this study is to investigate the parameters likely to cause variation in quality but which could also be determined easily in industry within a quality assurance programme with a view to developing further process and product-specific strategies for maximizing Cheddar quality.

Existing research on Cheddar cheese quality has concentrated on a very limited number of attributes and industrial quality strategies typically focus on pH, moisture, fat-in-dry-matter salt-in-moisture and moisture-in-non-fat-substances, the importance of which were established by research in New Zealand up to nearly four decades ago

(Gilles & Lawrence, 1973; Pearce & Gilles, 1979; Lelievre & Gilles, 1982). This thesis investigated new approaches to quality assurance in Cheddar by investigating heretofore neglected parameters known to influence cheese ripening.

Chapter 2 assessed varying degrees of curding washing on Cheddar cheese composition, pH and bacterial growth. This study indicated that increasing levels of curd washing during the manufacture of Cheddar cheese significantly reduced levels of residual lactose and lactic acid and, hence, the concentration of LLAMc in the cheese. During maturation, L (+)-lactate was increasingly converted to D (-)-lactate while cheese pH was inversely related to concentration of total lactate and increased with degree of curd washing especially at later stage of ripening. However, curd washing had no effect on the populations of starter bacteria and NSLAB. The results showed that curd washing can be used as a method of reducing both the levels of total lactate and unfermented lactose content in Cheddar cheese which is important to reduce the incidence of calcium lactate crystals on cheese surfaces. Due to the effects on cheese pH during ripening, curd washing is also likely to influence casein hydration, proteolysis, texture and, and hence quality which was studied in Chapter 3.

Chapter 3 showed that curd washing significantly influenced the texture, sensory and volatile profiles of Cheddar cheese. During cheese ripening, the mean levels of proteolysis were not affected by curd washing; however, some free amino acids (glutamic acid, leucine, phenylalanine and proline) were lowest in the cheese with highest level of curd washing. The cheese texture was harder, less crumbly with curd washing. The effect of curd washing also showed a reduction of the concentration of most volatile compounds in the 270 day-old cheese. Nevertheless, descriptive sensory analysis indicated that curd washing contributed to intensity of cheese flavours described as ‘fruity’, ‘buttery’ and ‘sweet’, while non-washed cheese led to

more ‘rancid’ and ‘farmyard’ aroma. The results from Chapter 2 and 3 indicated that curd washing can be applied during Cheddar cheese manufacture as a means of reduction in the risk of lactose intolerance among sensitive populations and creating distinct cheese variants with subtle flavour and texture difference.

The calcium content of commercial Cheddar cheese varies significantly, which is influenced by cheese pH and can be controlled principally by the pH during manufacture, and has a major impact on cheese texture and functionality. Chapter 4 investigates the effect of curd washing on the composition, yield, rheology, volatile compounds and sensory properties of Cheddar cheeses with the range of calcium levels as found in retail Cheddar cheeses. In agreement to Chapter 2, applying curd washing on the standard calcium cheese significantly reduced the mean concentration of residual lactose and lactic acid and increased the mean values of pH and fracture stress of the cheese over the 270-day ripening period; however, this effect was not apparent in the reduced calcium cheese. Moreover, curd washing did not significantly affect gross composition, microbiology, proteolysis, or colour of Cheddar cheese. Descriptive sensory analysis of the 270 day-old cheeses showed that curd washing on the standard calcium cheese contributed a waxier flavour, a more ‘estery’, less fruity, less sweaty cheesy odour, nuttier and sweeter taste than the corresponding unwashed cheese and also showed a more buttery and caramel flavour in the reduced calcium cheeses. Reducing the calcium level in the cheese showed the 270 day-old reduced calcium cheeses had more pungent, onion, rancid, buttery and caramel flavours, more caramel and buttery odours, and a more bitter taste than the corresponding standard calcium cheeses. This study showed that curd washing is a very effective means at reducing and controlling the levels of residual lactose in Cheddar cheeses with

different calcium levels and also to differentiate the sensory properties of aged (270 day-old) Cheddar cheese to an extent that varies with calcium content.

Milk composition, especially its lactose and protein content, is one of the most important factors for production of high quality cheese. To ensure the consistent quality of cheese, milk is standardised to certain protein to fat ratio. Chapter 5 investigated the effect of applying different levels of curd washing to cheeses which made with milk at two different milk protein levels (low and high) using UF technology on the composition, sugar metabolism, pH, microbiology, proteolysis, rheology, volatile compounds and sensory properties of Cheddar cheeses. Three different curd washing levels were applied to two different protein level milka. Similarly, high wash levels affected residual lactose and lactic acid levels sias discussed in Chapter 2 and 4 and also significantly reduced the concentration of most of the volatile compounds in the 270 day-old cheese. Descriptive sensory analysis on 270 day-old cheeses showed similar results as Chapter 3 and 4 that higher levels of curd washing coincided with a higher intensity of ‘fruity’, ‘buttery’ and ‘sweet’ aroma, while non-washed cheeses had more ‘rancid’ and ‘farmyard’ aroma. Otherwise, curd washing did not significantly affect gross composition, microbiology, proteolysis or texture of cheese. As expected, increasing the milk protein levels from 3.3 % (w/w) to 4.0 % (w/w) by UF significantly increased cheese yields, cheese firmness and fracture stress while decreased the cheese moisture and primary proteolysis at later stage of ripening. Descriptive sensory analysis of the 270 day-old cheeses indicated that cheeses made with UF milk (protein-fortified milk) was fruity, buttery and caramel than the corresponding standardized milk cheeses which suggested that washing cheese curds made from protein-fortified milk may be used as a means of creating

Cheddar cheese variants with distinctive flavour profiles (from more savoury to sweeter) with low risk of residual lactose accumulation.

In modern Cheddar cheese practise, the starter culture system changed which is common inclusion of *Sc. thermophilus* for its thermo- and phage resistance properties and also as fast acid producers. However, most of *Sc. thermophilus* strains are galactose negative which may leading to problems such as browning during cooking of cheeses. Hence Chapter 6 evaluated 51 *Sc. thermophilus* strains for their sugar metabolism ability, salt sensitivity and fermentation rate for further Cheddar cheesemaking trials. Besides, 103 *Lactobacillus* strains from dairy origin were also evaluated as an adjunct culture for Cheddar cheese making due to their contributions to cheese flavour development. The combination of those adjunct cultures were also tested on their ability to reduce milk pH. Based on this study, one galactose-positive *Sc. thermophilus* strain, DPC1796 and one galactose-negative *Sc. thermophilus* strain, DPC5095 were selected together with one galactose-positive *Lactobacillus* strain, DPC4818 for further cheesemaking trials in Chapter 7.

Direct vat starter cultures (DVS) are getting more popular to be used in Cheddar cheese manufacture with a higher pH at whey drainage, for example ~ 6.4-6.5 compared to ~ 6.1-6.2 in traditional Cheddar cheese made using bulk starter culture. Chapter 7 compared the effect of Gal^+ and Gal^- strains of *Sc. thermophilus* selected in Chapter 6 as an adjunct culture on the composition, sugar metabolism, pH, proteolysis, volatile compounds, texture, microbiology and sensory properties of Cheddar cheeses made with a high drain pH (6.45), as in modern manufacture, or a low drain pH (6.15), as in more traditional manufacture. The results showed that the uses of Gal^+ and Gal^- *Sc. thermophilus* strains as adjunct cultures (to *Lc. lactis* subsp. *lactis*) affected the levels of residual sugars, the profile of volatile compounds and sensory properties of

Cheddar cheese due to their phenotypes. Reducing the pH at whey drainage from 6.45 to 6.15 resulted in cheeses that had lower levels of moisture and FAA, and was firmer. At high drainage pH (6.45), the use of both Gal^- or Gal^+ strains of *Sc. thermophilus* contributed a sweaty, rancid flavour, a rancid and sweaty odour, and acid taste, compared to control cheese (without adjunct) which had a fruity, creamy flavour, and a fruity butter odour. Conversely, at low drain pH (6.15), the control cheese and cheese made using Gal^+ strain of *Sc. thermophilus* were closer in sensory properties (buttery flavour, caramel odour and sweet taste) than the cheese made using the Gal^- strain of *Sc. thermophilus*, which had a pungent and farmyard flavour, pungent odour, pungent throat and astringent sensations, and bitter taste. This study indicated that the use of *Sc. thermophilus* as an adjunct culture may be used to create distinctive flavour profiles of Cheddar cheese. Moreover, increasing the drain pH can be used to avoid the accumulation of high levels of residual galactose during ripening when Cheddar cheese was made using Gal^- variant of S*c. thermophilu*s.

More work was done on using galactose-positive *Lactobacillus* strain in combination of Gal^+ and Gal^- *Sc. thermophilus* strains at different salt in moisture levels of cheese. This study was not included in this thesis but will be presented as publications later outlined below:

- Effect of S/M level in cheese on survival of galactose positive/negative *Sc. thermophilus* strains on Cheddar cheese quality.
- Interactive effect of use of galactose positive/negative *Sc. thermophilus* strains and NSLAB strains as adjuncts on Cheddar cheese quality.

In conclusion, the results of this project indicated that Cheddar cheese quality can be controlled by milk treatments/standardisation, varying processing method and by using adjunct cultures. The knowledge from this study can contribute to

commercial cheese producers the methods to control Cheddar cheese quality and minimise defects during manufacturing and ripening for different market needs.

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APPENDIX

CONFERENCES

May 2012	IDF Cheese Ripening Symposium, US. Title: Effect of altering milk protein content and the levels of lactose plus lactic acid on the quality of **Cheddar cheese** (Poster Presentation)
Apr 2012	Annual Food Conference, UCC. Title: Effects of variations in curd washing and levels of total sugar-to-protein ration on quality of **Cheddar cheese** (Poster Presentation)
Nov 2012	Walsh Fellowship Seminar, Dublin. Title: Effect of altering curd washing on the biochemistry, quality and yield of C**heddar cheese** (Oral Presentation)
Sep 2011	8th Cheese Symposium, Ireland. Title: Effect of altering curd washing on the biochemistry, sensory and quality of **Cheddar cheese** (Oral Presentation)
Mar 2011	Annual Food Conference, UCC. Title: Effects of variations in curd washing and levels of lactose / lactic acid in cheese moisture on quality of C**heddar cheese** (Oral Presentation)

PUBLICATIONS

Hou. J., Hannon. J.A., McSweeney. P.L.H., Beresford. T.P., Guinee. T.P. (2012). Effect of curd washing on composition, lactose metabolism, pH, and the growth of non-starter lactic acid bacteria in full fat **Cheddar cheese**. International Dairy Journal. 25: 21-28.

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