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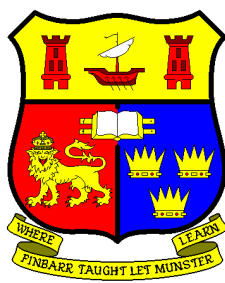Coláiste na hOllscoile Corcaigh

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University College Cork

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OXIDATION-REDUCTION POTENTIAL AND ITS INFLUENCE ON CHEDDAR CHEESE QUALITY

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TABLE OF CONTENT

Effect of Enterococcus faecium *adjunct on microbiological and physicochemical characteristics of Cheddar cheese*

Caldeo, V., & McSweeney, P.L.H. (2012). Changes in oxidation-reduction potential during the simulated manufacture of different cheese varieties. *International Dairy Journal*, 25, 16-20.

Parente, E., Patel, H., Caldeo, V., Piraino, P. & McSweeney, P.L.H. (2012). RP-HPLC peptide profiling of cheese extracts: A study of sources of variation, repeatability and reproducibility. *Food Chemistry*, 131, 1552-1560.

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Declaration

I hereby declare that the work submitted is entirely my own and has not been submitted to any other university or higher education institute, or for any other academic award in this university.

_____   Date: _____

Veronica Caldeo

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Abstract

Oxidation-reduction (redox) potential is a fundamental physicochemical parameter that affects the growth of microoganisms in dairy products and contributes to a balanced flavour development in cheese. Even though redox potential has an important impact on the quality of dairy products, it is not usually monitored in dairy industry. The aims of this thesis were to develop practical methods for measuring redox potential in cheese, to provide detailed information on changes in redox potential during the cheesemaking and cheese ripening and how this parameter is influenced by starter systems and to understand the relationship between redox potential and cheese quality. Methods were developed for monitoring redox potential during cheesemaking and early in ripening. Changes in redox potential during laboratory scale manufacture of Cheddar, Gouda, Emmental, and Camembert cheeses were determined. Distinctive kinetics of reduction in redox potential during cheesemakings were observed, and depended on the cheese technology and starter culture utilised. Redox potential was also measured early in ripening by embedding electrodes into Cheddar cheese at moulding together with the salted curd pieces. Using this approach it was possible to monitor redox potential during the pressing stage. The redox potential of Emmental cheese was also monitored during ripening. Moreover, since bacterial growth drives the reduction in redox potential during cheese manufacture and ripening, the ability of *Lactococcus lactis* strains to affect redox potential was studied. Redox potential of a Cheddar cheese extract was altered by bacterial growth and there were strain-specific differences in the nature of the redox potential/time curves obtained. Besides, strategies to control redox potential during cheesemaking and ripening were developed. Oxidizing or reducing agents

were added to the salted curd before pressing and results confirmed that a negative redox potential is essential for the development of sulfur compounds in Cheddar cheese. Overall, the studies described in this thesis gave an evidence of the importance of the redox potential on the quality of dairy products. Redox potential could become an additional parameter used to select microorganisms candidate as starters in fermented dairy products. Moreover, it has been demonstrated that the redox potential influences the development of flavour component. Thus, measuring continuously changes in redox potential of a product and controlling, and adjusting if necessary, the redox potential values during manufacture and ripening could be important in the future of the dairy industry.

Chapter 1

A review on oxidation-reduction potential and its role in dairy products

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1.1   Oxidation-reduction   potential: definition,   measurement   and applications

In the food industry parameters such as temperature, pH and water activity are taken into account to control fermentation processes and product quality. However, another important physicochemical parameter related to food stability, and not routinely measured, is oxidation-reduction (redox) potential (Brown and Emberger, 1980; Riondet et al., 2000b) and more attention should be paid to this parameter.

Oxidation and reduction processes are defined in terms of electron migration between chemical species. Oxidation is the loss of electrons by a substance while reduction is the gain of electrons. Furthermore, substances that cause the oxidation or the reduction of others are called oxidizing or reducing agents, respectively (Brown and Emberger, 1980; McCarthy and Singh, 2009). Oxidation-reduction potential of a system can be measured by using redox dyes or potentiometrically.

1.1.1   Measurement of redox potential by colorimetric methods

Chemical dyes can be used to characterize redox state and properties of a medium and microbial metabolism through reduction of colour by enzyme activity (Jacob, 1970). Redox dyes are usually coloured in the oxidized form and colourless in the reduced form and their colour should not be affected by changes in pH (Jacob, 1970). In dairy industry, an old method to verify raw milk quality consisted of using methylene blue (blue when is oxidized and colourless when reduced); dye reduction correlates with microbial growth (Thornton and Hastings, 1929b, 1930) and poor quality milks decolourise methylene blue in less than one hour. Another dye, that could be used to quantify bacteria in milk (Pesch and Simmert, 1929) and to measure

mastitis and tissue cells in milk (Morris, 2000), is resazurin. In a study by Davis (1932), redox dyes were added to milk and the cheese made therefrom was examined during ripening. Dyes were in reduced state inside the cheese and after cutting the cheese the colour of the reduced dyes reappeared quickly due to exposure of the cheese surface to air.

However, since dyes can modify reactions and inhibit enzymes by accepting or donating electrons (Jacob, 1970; Kjaergaard, 1977) and they can also be toxic to bacteria by oxidizing their cells (Dubos, 1929; Hewitt, 1930a), their use is obsolete. Moreover, dyes are efficient only over a small range of redox potential and for large changes in redox potential combinations of more than one dye must be used (Jacob, 1970). Ward (1938) assumed that potentiometric methods are better than colorimetric methods for studying the redox potential of microbiological cultures. Dyes were found to be unsuitable to measure redox potential in presence of coliform bacteria (Galesloot and Kooy, 1960).

It can be concluded that the use of dyes is not exact and reliable (Jenness and Patton, 1959; Galesloot, 1960a) and measurements of redox potential are now commonly carried out by potentiometric methods.

1.1.2   Measurement of redox potential by potentiometric method

In a potentiometric system, electrons migrate from a reference electrode (commonly Ag/AgCl or calomel electrode) to a working electrode (an inert metal like platinum or gold) connected throughout a sensitive potentiometer. The redox potential at the inert electrode is measured in volts or millivolts and requires the usage of a potentiometer with an input impedance higher than 10^{12} Ω to measure tiny potential

created by the circuit without loading errors (Kjaergaard, 1977; Skoog et al., 2004) and electrode polarisation (Jacob, 1970).

1.1.3   Determination of redox potential in biological systems

By convention, the redox potential measurements are referred to the standard hydrogen electrode (E_0) that is set to a value of 0 mV in a solution of unit activity (pH=0), and at -414 mV at pH 7, in equilibrium with hydrogen gas at 1 atm pressure (Jenness and Patton, 1959; Fox and McSweeney, 1998; Morris, 2000).

The redox potential of a redox process is described by the Nernst equation:

$$E_h = E_0 + 2.3RT/nF \log [Ox]/[Red] \qquad \qquad (1)$$

Where E_h is the oxidation-reduction potential where the letter "h" is used to indicate that measurements are related to the standard hydrogen electrode, E_0 is the standard redox potential (potential when reactant and products are at unit activity), [Ox] and [Red] are, respectively, the molar concentrations of the oxidized and reduced forms, R is the universal gas constant (8.314 J/mol x K), T is the temperature in Kelvin, F is the Faraday constant (96.5 kJ/ V x mol), n is the number of electrons involved in the reaction (Jenness and Patton 1959; Brown and Emberger, 1980; Sherbon, 1988; McCarthy and Singh 2009).

The Nernst equation shows the importance of temperature. At 25°C and for a one-electron transfer the value of 2.3RT/nF is 0.059 V and the equation can be simplified as:

$$E_h = E_0 + 0.059 \log ([Ox]/[Red]) \qquad \qquad (2)$$

pH influences many reaction (Schafer & Buettner, 2001) and redox potential is affected by pH (McCarthy and Singh, 2009; McSweeney et al., 2010):

$$E_h = E_0 + 0.059 \log ([Ox]/[Red]) - 0.059 \, pH \qquad (3)$$

0.059 V represent the change in E_h per pH unit when the ratio of protons to electrons is equal. This occurs in pure chemical solutions where the pH depends only on the redox couple (DeLaune and Reddy, 2005).

In natural environments, several redox couples are present at the same time and it is often difficult to take into consideration all the kinetics of redox reactions that occur in a system (Noyhouzer et al., 2009). All the species present in a system can transfer a certain number of electrons depending on their nature, their concentrations and their ability to interact with each other (Gillespie and Rettger, 1938; Bard and Faulkner, 2000; Morris, 2000), with the system and with the surface of the electrodes (Noyhouzer et al., 2009). Moreover, it could be hypothesed that chemical redox couples present in a system have an intrinsic redox potential and by modifying the external redox potential those chemical groups could be oxidized or reduced at a specific redox potential values (Tallec, 1985).

Therefore in complex systems, redox potential should be reported with the pH and temperature at which it is measured (Jacob, 1970; Scarinci et al., 1994; Riondet et al., 2000a,b; Morris, 2000; McSweeney et al., 2010). Figure 1.1 shows the influence of pH on different system that can be found in a natural environment like milk (Jenness and Patton, 1959; Sherbon, 1988; Fox and McSweeney, 1998).

Different values of redox potential can be measured depending on the electrode used (Saal and Heukelom, 1947); therefore, to compare data from different investigators, it

is important to convert the raw values to E_h. When conversion to E_h is not reported by investigators, it would be advisable to refer to changes in redox potential rather than to the potential values themselves (Saal and Heukelom, 1947).
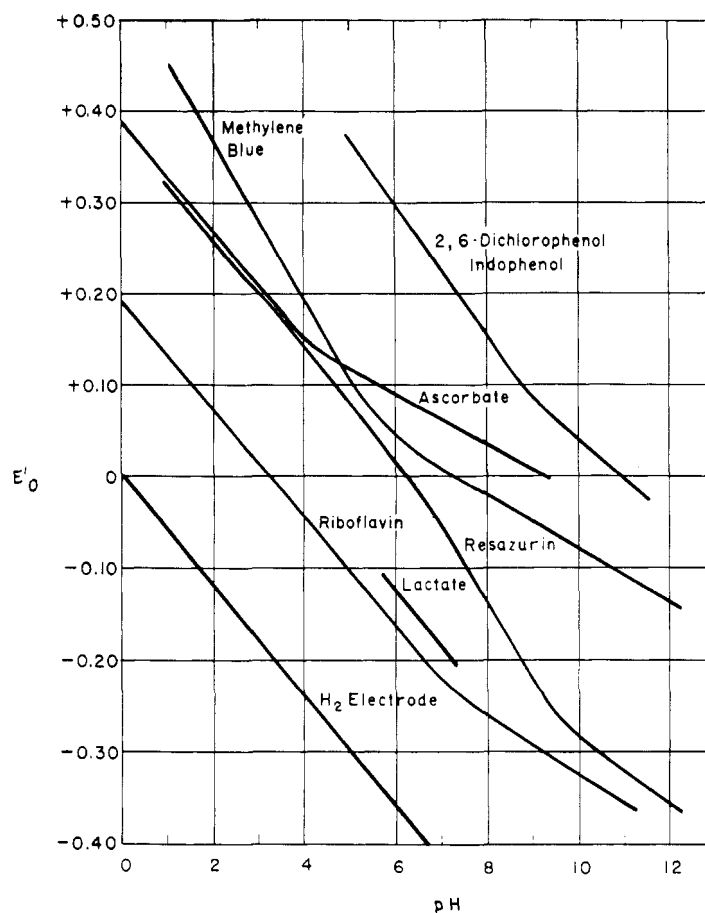
Figure 1.1   Effect of pH on the oxidation-reduction potential of various systems (from Sherbon, 1988).

The following equation is used to convert the redox potential measured, uncorrected for pH and without reference to the standard hydrogen electrode (ORP), to E_h (McSweeney et al., 2010; Abraham et al., 2013; Martin et al., 2013):

$$E_h = ORP + E_r \qquad\qquad (4)$$

Where E_r is the potential of the reference electrode relative to the standard hydrogen electrode at a certain temperature. Table 1.1 shows the E_r values according to the reference electrode used and the temperature measured (Skoog et al., 2004).

Table 1.1   Electrode potentials for reference electrodes as a function of electrode composition and temperature (from Skoog, 2004).

Temperature (°C)	Potentials vs Standard Hydrogen Electrode, mV				
	0.1 M Calomel	3.5 M Calomel	Saturated Calomel	3.5 M Ag/AgCl	Saturated Ag/AgCl
12	336		253		
15	336	254	251	212	209
20	336	252	248	208	204
25	336	250	244	205	199
30	335	248	241	201	194
35	334	246	238	197	189

The potentials of the reference electrodes change with temperature and it is possible to correct the redox readings using the temperature coefficients (Sawyer et al., 1995). For example, temperature compensation can be calculated using the following formula when Ag/AgCl reference electrode is used:

$$E_r = +199 - 1.01(T - 25\ °C) \qquad\qquad (5)$$

where T is the temperature recorded and 1.01 is the temperature coefficients in mV (Sawyer et al., 1995).

As shown in Equation 3, E_h depends on pH. In biological environments, like milk, where a large number of redox couples are involved in the redox equilibrium (Jenness and Patton, 1959), to overcome the pH dependency it is possible to calculate E_{h7} (E_h of a solution at pH 7) (Leistner and Mirna, 1959; McSweeney et al., 2010; Abraham et al., 2013; Martin et al., 2013):

$$E_{h7} = E_h - [\alpha\ (7\text{-}pH_m)] \tag{6}$$

where α is the pH correction factor and pH_m is the pH measured.

The pH correction factor can be calculated by measuring redox potential and pH changes in a solution (mV/pH unit) where the pH is modified by the addition of acid or base at a certain temperature (Jacob, 1970; Brown and Emberger, 1980). The slope of the curve obtained by plotting the redox potential against pH gives the variation in redox potential for unit of variation of pH (Morris, 2000). In the literature, different pH correction factors have been reported depending on the system analyzed; for example, authors reported a variation of 38 mV/pH unit at 26℃ and 40 mV/pH unit at 42℃ for sterilized skim milk (Cachon et al., 2002); 59 mV/ pH unit at 25℃ in a gelled model food (Ouvry et al., 2001); 34 mV/pH unit at room temperature in brain heart infusion broth culture medium (Ignatova et al., 2008, 2009); 38 mV/pH unit at 30°C in reconstituted skim milk (Jeanson et al., 2009); 53 mV/pH unit in milk at 30°C (Tachon et al., 2010); 30 mV/pH unit at 45°C in milk (Ebel et al., 2011); 60 mV/pH unit in starter culture (Galesloot and Kooy, 1960); 62 mV/pH unit at 37℃ in cell suspension buffer (Kieronczyk et al., 2006); 40 mV/pH unit at 45℃ in skim milk

(Martin et al., 2009, 2010, 2011); 38 mV/pH unit at 28°C in milk (Abraham et al., 2013). While it is not possible to calculate α in cheese, studies by Abraham et al. (2007, 2013) on Camembert cheese and cheesemaking used as correction factor the same value of 38 mV/pH unit determined in milk.

In the past, the term rH was used to remove the dependency of redox potential to pH when all H^+ were dissociated at all pH values (Brown and Emberger, 1980; Vahcic et al., 1992). rH is not used anymore since too many redox couples are present in a real systems (Morris, 2000) and they are often not at equilibrium (Kjaergaard, 1977).

Real systems can have the capacity to resist to changes in redox potential (Morris, 2000) and this buffering capacity, called "poising effect" (Bolduc et al., 2006a), depends on the total concentration of redox couples and the quantity of reduced and oxidized substances (Morris, 2000).

1.1.4   Consideration on the choice of the appropriate measurement conditions and care of the electrodes

Correct and reproducible redox potential readings are not easy to obtain in food systems (Hewitt, 1950; Tabatabai and Walker, 1970) and attention must be taken regarding the choice of the right electrodes, depending on the matrix analysed, and their maintenance. Therefore, it is important to standardize the method (Saal and Heukelom, 1947) to interpret the results correctly (Abraham et al., 2013).

Moreover, some of the studies done on redox potential in dairy products, especially in cheese, and referenced in this chapter are from 1930-1970 and the methodologies used may not have been accurate (Davis, 1932; Vos, 1948; Peltola and Antila, 1953; Kristoffersen and Gould, 1959; Galesloot, 1960a,b; Higginbottom and Taylor, 1960;

Kristoffersen et al., 1964b; Tabatabai and Walker, 1970; Langeveld and Galesloot, 1971).

Electrodes can be sensitive and attention must be taken in cleaning operations that should follow the suppliers' instructions. To obtain valid and reproducible readings (Lund et al., 1984), the accuracy of electrodes has to be checked against commercial standard solutions (Calligaris et al., 2004; Bolduc et al., 2006a; Schreyer et al., 2008; Jayamanne and Adams, 2009; Ignatova et al., 2009), that have a positive redox potential since reducing solutions are not chemically stable, or against tap water (Abraham at al., 2007, 2013; Jeanson et al., 2009; Martin et al., 2009, 2010, 2011; Ebel et al., 2011), saturated KCl solution (Kristoffersen et al., 1964b), buffer solution with hydroquinone (Hartman et al., 1943), solution of potassium ferrocyanide and potassium ferricyanide (Boldoc et al., 2006a; Bazinet, 2009; Haratifar et al., 2011; Bazinet et al., 2011), solution of 0.1 M $FeCl_2$ and 0.1 M $FeCl_3$ (Rubin and Vaughan, 1979) or phosphate buffer 0.066 M pH 7 (Lund et al., 1984), in aerated phosphate buffer (Lund et al., 1984) or in quinhydrone saturated buffers at pH 4 and 7 (Tabatabai and Walker, 1970; George et al., 1998). Moreover, electrodes can be checked by comparing redox potential reading of the same sample with each other and the measurements should give difference not greater than 10 mV between electrodes (Saal and Heukelom, 1947). In the care of interference with the signal transmitted by electrodes, especially if handmade, it is possible to carry out measurement in a Faraday cage (Ouvry et al. 2001; Abraham et al. 2007).

The choice of the appropriate reference electrode should take into consideration the exchange current density (Sawyer et al., 1995), a parameter that reflects the rate of

electron transfer between the solution and the electrode. It depends on the nature the electrode, including the material, the shape, and the structure of its surface.

Inert metal electrodes like noble metals, in particular gold and platinum, should be used as working electrodes since they have a high standard voltage (Galster, 2000). Platinum is a better material than gold since it provides a faster response and it melts with glass giving a most robust electrode (Sawyer et al., 1995). Gillespie and Rettger (1938) reported identical redox potential values using platinum or gold electrodes whereas Saal and Heukelom (1947) found their gold electrode unable to give reproducible values. The shape of the metal electrode should be large enough to guarantee exchange of charges and the surface should be smooth to avoid deposit of impurities (Higginbottom and Taylor, 1960). To avoid surface contamination, the electrode should be periodically cleaned mechanically or chemically and replaced when necessary (Danly, 1973). Ward (1938) created one of the first cleaning procedures and hot chromic acid, nitric acid and *aqua regia* were used. Also commercial standard solution can be used to clean the electrodes (Jayamanne and Adams, 2009). The polish of the metal electrode is important and Jacob (1974) found cerium oxide to be the best polisher. Good results are also given by using fine alumina powder (Cachon et al., 2002; Topcu et al., 2008) to restore the platinum surface (Jacob, 1970).

In liquid systems, redox potential can be measured by immersing the redox electrodes into the solution (Brown and Emberger, 1980) and for solid foods it is necessary to create a salt bridge into which the reference electrode can be placed (Skoog et al., 2004).

Time is required to obtain stable readings (Lund et al., 1984) depending on the shape and material electrode used, the metabolites present in the system (Gillespie and Rettger, 1938; Saal and Heukelom, 1947) and the temperature of the sample (Reichart et al., 2007; Topcu et al., 2008). In our laboratory, it was established that readings in tap water and in milk reach an equilibrium value within 10 min at room temperature.

In the past, difficulties to obtain reproducible measurements of redox potential were attributed to possible residues of chemical detergents on the apparatus (Tabatabai and Walker, 1970), to lack of knowledge of the chemical constituents of the medium (Gillespie and Rettger, 1938) and also to the possibility of keeping records of all the variations in redox potential since, as Gillespie and Rettger (1938) pointed out, even a scientist requires sleep. As mentioned by Davis (1932), one problem associated with handmade electrodes is the sealing of the metal to the plastic or glass, where the presence of very small cracks can alter the results. Moreover, in measurement of redox potential of solid foods, the design of the electrode should be less destructive towards the sample to avoid cracks in the food matrix which admit oxygen that can give false reading.

In a study on the growth of *Escherichia coli* in a synthetic medium conducted by Ward (1938), the difficulties on measuring reproducible redox potential values were attributed to the absence of a poisoning material in the synthetic medium compared to broth medium.

1.1.5   Effect of oxygen on redox potential

One variable that is supposed to affect the redox potential is the oxygen level of a medium and the relationship between redox potential and dissolved oxygen is still

not well understood (Bolduc et al., 2006a). Saal and Heukelom (1947) reported that oxygen does not disturb the measurement of the redox potential of many systems and the decrease in oxygen level not always results in a decrease in redox potential (Bolduc et al., 2006a). In biological systems, redox potential gives more information than the molecular oxygen tension (Davis, 1932). Lund et al. (1984) found that oxygen can influence the redox potential reading depending on the poising capacity of the solution. For example, salt solutions have a low poising capacity and therefore oxygen levels can influence the reading. Flushing nitrogen gas alone into milk to eliminate oxygen does not result in a decrease in the redox potential to negative values (Bolduc et al., 2006a; Jeanson et al., 2009; Martin et al., 2009; Larsen et al., 2015) probably due to the good poising capacity of the milk.

Redox potential can be successfully used when the partial pressure of oxygen is too low to be detected in a system by an oxygen electrode (Davis, 1932; Kjaergaard, 1976, 1977; Lund et al., 1984) and therefore E_h can be used to control aeration in media where the oxygen level is very low (Wimpenny, 1969a).

The incorporation of air into a buffer solution can cause an increase in the time required to the electrodes to reach equilibrium (Saal and Heukelom, 1947) and also in the case of measurement of redox potential in solid food like cheese, cracks at the surface can lead to penetration of air into the food (Davis, 1932; Vos, 1948) and cause false readings.

1.1.6   Strategies to control redox potential in biological systems

Redox potential can be a difficult parameter to take into consideration since in a biological system more than one chemical and biological reaction occurs

simultaneously and/or consequently to others (Tango and Ghaly, 1999). Therefore, modification of the redox potential of a medium has been done by some authors to study the effect of the redox potential on microbial survival and growth (Clifton, 1933; Gillespie and Rettger, 1938; Ward, 1938; Bagramyan et al., 2000; Martin et al., 2010, 2011, 2013; Michelon et al., 2010; Ebel et al., 2011) and on the organoleptic characteristics of dairy products (Webb and Hileman, 1937; Greenbank, 1940; Saal and Heukelom, 1947; Manning, 1979; Kristoffersen, 1985; Kieronczyk et al., 2006). Oxidation-reduction potential of a system can be modified by the addition of chemical compounds like dithiotreitol, potassium ferricyanide, sodium borohydride, cysteine (George and Peck, 1998; Riondet et al., 2000b; Bolduc, et al., 2006b; Kieronczyk et al., 2006; Ignatova et al., 2009) or flushing with gasses such as oxygen, nitrogen or hydrogen (Ignatova et al., 2009; Jeanson et al. 2009; Martin et al. 2009, 2010, 2011; Ebel et al., 2011) or by applying electro-reduction (Swanson and Sommer, 1940; Inoue and Kato, 2003; Bolduc et al., 2006a; Schreyer et al., 2006; Schreyer et al. 2008; Haratifar, 2009; Haratifar et al., 2011). It is important to take into consideration the fact that chemicals like cysteine or ascorbate might affect the pH whereas some gasses like nitrogen and hydrogen might not (Ebel et al., 2011).

1.2   Oxidation-reduction potential of milk

Several authors have measured the E_h of milk over the years and, in aerobic conditions, at 25°C it is between +250 and +350 mV at pH 6.6-6.7 (Thornton and Hastings, 1929a; Swanson and Sommer, 1940; Walstra and Jenness, 1984; Vahcic et al., 1992; Sherbon, 1988; Morris, 2000; McCarthy and Singh, 2009). In the literature,

different values of redox potential of milk have been reported depending on its composition and the mechanical or thermal treatments to which it is subjected; conversion to E_h is not always shown and pH and temperature at which the measurements were taken are not always reported.

Redox potential can vary depending on species' milk. The redox potential of fresh cow and sheep milk measured at 20°C by Pappas et al. (1989) was +144 mV (range +119 to +156 mV) and +124 mV (range +92 to +162 mV), respectively.

Matthes et al. (2000) found differences in E_h values depending on the season; January milk had an E_h of +217 mV, March milk +175 mV and September milk +143 mV and the milk collected in September showed the greatest poising capacity. Also Saal and Heukelom (1947) found lower E_h values in summer time (+180 mV) probably due to inadequate refrigeration conditions in the past.

Pastushenko et al. (2000) found that redox potential increased with increasing fat content of milk. They measured E_h of milk with fat concentration higher than 5%, between 4-5% and below 4% and they found redox potential values of +192 mV, +179 mV and +169 mV, respectively. An explanation of these differences was given by Schreyer et al. (2008) who asserted that milk fat could form a layer on the surface of the electrodes that could alter the data measured. Schreyer et al. (2008) reported that variation in redox potential of whole milk due to eletro-reduction were lower compared to skim milk but the authors also found that fat molecules do not have effect on redox potential values since they are not electro-reducible. Moreover, Saal and Heukelom (1947) found that the potential of cream, skim milk and whey made from whole milk showed the same redox potential values as the whole milk, although butter plasma had a higher value (E_h = +410 mV).

1.2.1   Milk constituents affecting the redox potential

Milk contains different redox couples that are present in equilibrium (Jacob, 1974; Brown and Emberger, 1980) and those redox couples can have low or high redox potentials. The global redox potential of milk is due to the balance of all those substances, their ratio and concentrations (Sherbon, 1988) and identification of redox couples that influence redox potential is not easy (Morris, 2000). Table 1.2 shows the principal oxidizing and reducing components present in milk. Components that have a redox capacity are ascorbate, riboflavin (a reducing compound and an enzyme cofactor), dissolved free oxygen (Saal and Heukelom, 1947; McCarthy and Singh, 2009; Haratifar et al., 2011), lactate-pyruvate (Saal and Heukelom, 1947), lactose (Saal and Heukelom, 1947), succinates and citrates that have reducing capacity (Thornton and Hastings, 1929a), glutathione and the associated cysteine-containing compounds in the milk proteins (Saal and Heukelom, 1947), other compounds with sulfhydryl groups that have reducing capacity (Saal and Heukelom, 1947), and free hydrogen ions (Saal and Heukelom, 1947; Jacob, 1974).

Table 1.2   Standard redox potentials (E_0) of the principal oxidizing and reducing components of milk (Walstra et al., 1999, 2006; Tachon, 2009).

Redox system	Standard redox potential (E_0) (mV) at pH 6.7	Quantity in the milk (mg/L)
Oxygen	+818	-
Fe^{2+}/Fe^{3+}	+772	0.3-0.6
Cu^+/Cu^{2+}	+150	0.1-0.2
Ascorbate	+58	10-20
Lactate/pyruvate	-160	*
Riboflavin	-200	1.5-2.0
Cysteine	-340	300-1000
Zn	-762	4.0-4.5

*concentration depends on bacterial activity

Ascorbic acid is a reducing compound in milk (Hartman et al., 1943) and some work showed its impact on redox potential (Saal and Heukelom, 1947; Walstra et al., 2006). In oxygen-free milk, redox potential depends mainly on ascorbic acid and ascorbic acid keeps the E_h around +50 mV (Walstra et al., 2006). After milking, ascorbate is reversibly oxidized to dehydroascorbate depending on temperature, oxygen, Cu and Fe concentrations (McCarthy and Singh, 2009). Moreover, ascorbic acid content was assumed as cause of difference in redox potential found in summer and winter time and lower ascorbic acid content were found in milk with higher redox potential (Saal and Heukelom, 1947). A study by Roine (1948) found that milk fermented by bacteria (low redox potential) had a stable ascorbic acid concentration during storage

independent of pH and, if dehydro-ascorbic acid was added, it was reduced to ascorbic acid.

The redox potential of milk can be influenced by the animal's diet (Sherbon, 1988), especially the intake of antioxidants and by the farming conditions. The chemical compounds that maintain the redox balance in milk are mainly tocopherols, β-carotene, ascorbic acid and their concentration in the diet vary depending on the seasonal food quality (Matthes et al., 2000). Other substances that are present in milk and have antioxidant capacity are caseins, β-lactoglobulin and L-cysteine (Taylor and Richardson, 1980) and they can influence redox potential according to their concentration (Schreyer et al., 2008).

Redox reactions can be responsible for the degradation quality of milk (Schreyer et al., 2006), especially the oxidation state (Noyhouzer et al., 2009). Lipid oxidation can be associated with redox potential (Bazinet et al, 2009), since polyunsaturated fatty acids present in milk fat globule membrane can be subjected to oxidation and cause production of oxidized flavour (Haratifar et al., 2011) and vitamins A and E can degrade (Bolduc et al., 2006a).

Redox potential can be also used to detect copper contamination in milk (Webb and Hileman, 1937) and Vahcic et al. (1992) found a relationship between E_h and level of copper, iron and ascorbic acid in milk.

Many authors (Greenbank, 1940; Jenness and Patton, 1959; Jacob, 1970; Schreyer et al., 2008; Haratifar et al., 2011) had attributed to milk a poising capacity, therefore, milk, like other biological systems, is able to counteract variations in redox potential. When milk has a good poise, E_h does not change and therefore oxidation reactions do

not easily occur (Greenbank, 1940). On the contrary, Noyhouzer et al. (2009) stated that milk is not a well-poised system since it contains compounds that can be oxidized.

1.2.2   *Effect of oxygen, pH and temperature on milk redox potential*

The relationship between redox potential and dissolved oxygen is still not well known in food systems. However, decreasing oxygen levels does not result in a decrease in redox potential to negative values (Bolduc et al., 2006a) and milk free from oxygen still has a positive redox potential (Saal and Heukelom, 1947; Jeanson et al., 2009; Larsen et al., 2015) due to ascorbate (Walstra et al., 2006; McCarthy and Singh, 2009).

When milk is deaerated and then heated, the redox potential is reduced and the reducing properties of the milk are increased (Harland et al., 1952). Milk powder produced from deaerated and high-temperature heated milk showed a longer storage stability, probably due to the formation of sulfhydryl compounds that prevent oxidation of fat (Greenbank and Wright, 1951). Higginbottom and Taylor (1960) studied the effect of heating temperature and presence of oxygen on the redox potential of milk. Milk in a vacuum bottle with N_2 in the headspace had an E_h value almost constant of -290 mV in a range of temperature between 104.5 and 117.5°C and a good poising capacity (E_h equilibrium value was achieved in 120 min). In the same temperature range, the E_h of the milk exposed to air was subjected to a slight decrease (from +10 mV to -30 mV) and when exposed to N_2, E_h decreased to -150 mV or lower values depending on the poising capacity of the milk. In milk without O_2, the production of reducing substances was higher than in milk in contact with air and a raise in temperature increased the production of reducing compounds.

Besides oxygen content, the other two factors that can have an effect on redox potential measurements are pH and temperature.

pH influences redox potential (Saal and Heukelom, 1947); the addition of an acid or base causes variation E_h and if the pH is restored to initial value E_h would also be restored to its initial value.

Redox potential can also differ as a function of temperature and duration of heat treatment (Greenbank, 1940). Pasteurized milk has an E_h slightly higher than raw milk (Webb and Hileman, 1937). Schreyer et al. (2008) measured the redox potential of pasteurized skim milk, raw skim milk, pasteurized whole milk, ultrafiltration permeate to be +225 mV (pH 6.69), +213 mV (pH 6.82), +237 mV (pH 6.74) and +233 mV (pH 6.53), respectively (data were not corrected to E_h). Vahcic et al. (1992) reported an E_h of raw, pasteurized and sterilized milk at 20°C of +154, +219 and +204 mV, respectively. Redox potential of pasteurized skim milk was found to be +120 mV by Bazinet (2009) and +182 mV by Bolduc et al. (2006a) (both data were not corrected to E_h) and the values were constant during 8 days storage at 4°C (pH 6.8) (Bolduc et al., 2006a). Chemical changes occur during heating and consequently influence redox potential (Greenbank, 1940) since heat can inactivate enzymes, decrease the oxygen content and degrade antioxidants present in the milk. Reducing sulphydryl groups generated by milk heat treatments (Adhikari and Singhal, 1992) and products of the Maillard reaction, produced in extreme heating conditions (Taylor and Richardson, 1980; McGookin, 1991), are correlated with redox potential and aroma (Webb and Hileman, 1937; Greenbank, 1940; Swanson and Sommer, 1940). Heating the milk to a temperature higher than the normal pasteurization temperature reduced the redox potential (Saal and Heukelom, 1947), oxidised less

ascorbic acid, retained iodosobenzoate-, acid ferricyanide- and thiamine disulfide-reducing substances (Harland et al., 1952) and liberated highly active sulfhydryl groups from proteins (Greenbank and Wright, 1951; Taylor and Richardson, 1980), probably by reduction of disulphide compounds (RSSR) into thiol compounds (RSH) (Saal and Heukelom, 1947).

Calligaris et al. (2004) heated milk at three different temperatures (80, 90 and 120°C) and measured the changes in redox potential over time. E_h increased from +213 mV to +297 mV in 24 h when heated at 80°C, when heated at 90°C E_h increased slightly at the beginning (+292 mV) and then decreased to +239 mV after 24 h and, when a temperature of 120°C was used, E_h decreased to +194 mV within 2 h. The slight increase in E_h at 80 and 90°C was associated to a degradation of antioxidant compounds (Taylor and Richardson, 1980), oxidation of sulfhydryl groups of proteins and liberation of free radicals from the oxidation of lipids.

1.2.3   Strategies to modify the redox potential of milk: electro-reduction, addition of chemical compounds or gasses

Over the years, some authors have used techniques like addition of chemical compounds (George and Peck, 1998; Riondet et al., 2000b; Bolduc, et al., 2006b; Kieronczyk et al., 2006; Ignatova et al., 2009) or gasses (Jeanson et al., 2009; Martin et al., 2009, 2010, 2011, 2013; Ebel et al., 2011; Ignatova et al., 2009) to modify the redox potential of milk and in an attempt to understand its influence on milk characteristics and, additionally, electro-reduction techniques have been used (Swanson and Sommer, 1940; Inoue and Kato, 2003; Bolduc et al., 2006a; Schreyer et al., 2006, 2008; Haratifar, 2009; Haratifar et al., 2011).

Electro-reduction is a technique used to reduce electroactive species by the generation of electrons (Haratifar et al., 2011) and it has been used in dairy research to study the effect of redox potential on milk (Swanson and Sommer, 1940; Inoue and Kato, 2003; Bolduc et al., 2006a; Schreyer et al. 2006, 2008; Haratifar, 2009; Haratifar et al., 2011) without altering the aroma of the product (Bazinet et al., 2009) and it is considered favourable instead of addition of chemicals compounds because this technique does not alter the milk composition (Schreyer et al., 2006).

For example, when a current of -1.15 V was applied, the redox potential of milk decreased to -209 mV within 10 min and the reduction in redox potential was greater with increasing voltage applied (Schreyer et al., 2008). Studies done on the maintenance of a constant negative redox potential value during storage (Bolduc et al., 2006a; Schreyer et al., 2008; Haratifar et al., 2011) showed that over time the redox potential of milk (Bazinet et al, 2009) or yoghurt (Dave and Shah, 1997b) increased and the oxygen content decreased (Schreyer, 2007). The dimension of the headspace of the jars influenced the increase of redox potential during storage (Haratifar, 2009; Haratifar et al., 2011) and even when air was excluded from the headspace (Schreyer et al., 2008), the redox potential increased. The use of a glass jar prevented the increase of redox potential of electroreduced milk stored at 4°C for 7 days (Bazinet et al., 2009) and the redox potential was kept to reduced values for 14 days (Haratifar, 2009). This phenomenon could be due to the presence of electro-active compounds that are unstable and reversible and therefore can be re-oxidized during storage (Bolduc et al., 2006a,b; Haratifar, 2009), even if an anaerobic ambient is maintained during the experiment (Schreyer et al. 2008; Haratifar et al., 2011). The oxygen content decreased during storage probably because it used by compounds

that try to come back to their original state before reduction (Schreyer et al., 2008). These trends in redox potential and oxygen content could be associated with lipid oxidation (Shekar and Bhat, 1983) and to the concentration of sulphydryl and carbonyl groups and ascorbic acid during storage (Fink and Kessler, 1986; Adhikari and Singhal, 1992) and also to the changes in the redox state of bovine serum albumin (Bazinet et al., 2011). Moreover, a study done on milk electroreduced and inoculated with bifidobacteria reported that after one week the redox potential values were positive and slightly decreased during storage probably due to microbial activity (Bolduc et al., 2006b). On the contrary, milk reduced by the use of gasses had an E_h value negative throughout storage (Ebel et al., 2011).

Bazinet et al. (2011) optimized earlier studies on electro-reduction of milk by using a hydrodynamic electro-reduction cell and determined that minimum 3 V are needed to reduce milk species and to maintain negative redox potential during storage up to 7 days.

Electro-reduction was applied to different types of milk and the ultrafiltrate (UF) permeate was less reduced (ORP = -314 mV) compared to raw and pasteurized milk (both ORP ~ -560 mV) (Schreyer et al., 2008). This difference can be attributed to the presence of fewer redox substances in UF permeate compared to raw milk (Bazinet et al., 1997).

Also the addition of chemical compounds (soluble metals, gasses, acids) can influence the redox potential in milk and can be used to study its redox potential. Addition of soluble copper and ferrous irons can increase the redox potential of the milk (Haratifar et al., 2011). Synthetic ascorbic acid added to the milk decreased the redox potential (Swanson and Sommer, 1940; Haratifar et al., 2011) and when added

together with copper the decrease was followed by a rise (Haratifar et al., 2011). The decrease or increase of redox potential whene chemicals are added and the speed at which the equilibrium values were reached are correlated to the concentrations of the compounds added to the milk (Hartman et al., 1943). However, when nickel sulfate and ferric, vanadium, aluminum, manganese, chromium, and stannous chlorides, were added in concentrations of 10^{-5}, 4×10^{-5}, and 10^{-4} mol/L milk, the redox potential was not influenced (Hartman et al., 1943).

In other studies, the addition of gasses to milk was used to modify the redox potential of dairy products (Ouvry et al., 2002; Ignatova et al. 2009; Martin et al., 2010). Ignatova et al. (2010) flushed air, nitrogen and a mixture containing 96% nitrogen and 4% hydrogen to obtain E_{h7} values of +380, 0 and -360 mV, respectively. Ouvry et al. (2002) adjusted the E_h values to +350 mV by flushing nitrogen and to -300 mV using hydrogen gas. Martin et al. (2010) obtained E_{h7} of +433 mV by flushing air, +283 mV by flushing nitrogen and -349 mV using a mixture containing 96% nitrogen and 4% hydrogen. Also vacuum can be used to decrease redox potential (Bolduc et al., 2006b) even if the decrease in oxygen concentration and redox potential is lower compared to the one caused by the use of gasses.

1.3   Oxidation-reduction potential and dairy microorganisms

Potter (1911) was the first scientist to observe that the oxidation-reduction potential of a sterile medium, measured potentiometrically, decreased during bacterial growth and Gillespie (1920) noticed that the reduction caused by aerobic bacteria differed from that of anaerobic bacteria. Later, many authors have described and interpreted

this phenomena. Tabatabai and Walker (1970) designed the first apparatus to measure E_h, pH and growth of microorganism (Figure 1.2).

A. ELECTRODE VESSEL   B. GAS INLET AND OUTLET   C. SALT BRIDGE
                             TUBE ASSEMBLY
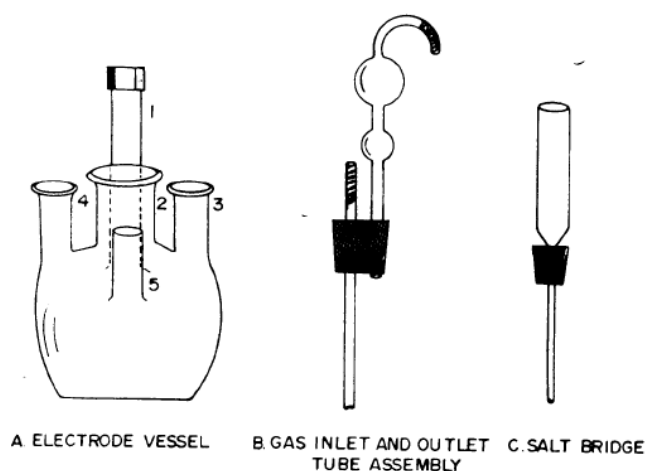
Figure 1.2   Schematic drawing of electrode vessel assembly for measuring E_h and pH of bacterial cultures. (A) electrode vessel; (1) screw-cap tube for introduction and removal of samples; (2) port for holding gas inlet and outlet tube assembly (B); (3 and 4) ports for holding glass and platinum electrodes; (5) port for holding salt bridge (C) (from Tabatabai and Walker, 1970).

Anaerobic bacteria cannot grow in conditions of high redox potential whereas aerobic bacteria can grow at positive redox potential values (Dubos, 1929; Messing, 1934; Brown and Emberger, 1980). For example, the interior of cheese is characterized by a low redox potential where only anaerobic bacteria are able to grow, the outside part is exposed to oxygen and therefore aerobic bacteria are predominant (Beresford et al., 2001). Anaerobic microorganisms have optimum development at E_h between -150 mV and -420 mV (Wimpenny, 1969b) and they can reduce E_h to lower values

(Wimpenny, 1969b; Michelon et al., 2010). On the other hand, aerobic microorganisms grow at positive E_h (around +300 mV) (Wimpenny, 1969b) and they can reduce E_h to moderate level of E_h (Hewitt, 1950).

1.3.1   Relationship between redox potential, microorganisms growth and acidification activity

A mutual dependency between redox potential and microorganism activity might exist (Boucher et al., 2006). Microorganisms, during their growth and acidification, adjust the redox potential of the media to a value depending on their metabolic activities (Clifton, 1933; Hewitt, 1950), characteristic of each species and strain. Moreover, the redox potential of the medium (Allyn and Baldwin, 1932; Tabatabai, 1970; Boucher et al., 2006) and the environmental conditions, like composition, pH, temperature, free O_2 and free H^+ (Jacob, 1974), can influence the growth and metabolism of microorganisms (Davis, 1932; Wood, 1935). Wood (1935) suggested that there is a "critical potential value" typical of each microorganisms and that bacteria bring the redox potential of a medium close to the "critical potential value" needed for their growth (Tammam et al., 2001).

It is interesting to observe that within closely related bacterial species there is a variety of reducing capacities (Hewitt, 1950; Brasca et al., 2007) even in the same medium and under identical conditions (Frazier and Whittier, 1931a, b; Gillespie and Rettger, 1938). Lactic acid bacteria (LAB) can usually reduce the redox potential of milk from positive to negative values (Davis, 1932; Keen, 1972; Adda at al., 1982; Cachon et al., 2002; Abraham at al., 2007) with a trend characteristic of the subspecies and strains (Boucher et al., 2006; Larsen et al., 2015). Although some lactococci have

the same acidification profiles, and they can be generally very reducing, whereas some LAB, like bifidobacteria (Bolduc et al., 2006b), can be non-reducing (Keen, 1972; Scarinci et al., 1994; Boucher et al., 2006). Cachon et al. (2002) studied the redox potential and acidification trends in nine strains of *Lactococcus* sp., six strains of *Streptococcus thermophilus* and five strains of *Lactobacillus helveticus*. *Lactococcus lactis* reduced E_h of milk six times faster than streptococci and lactobacilli even if their acidification rate was slower. Moreover, strains with slow acidification activity can be more reducing than others (Brasca et al., 2007). The time required to reduce milk and the equilibrium value reached, under the same conditions, can differ between strains (Scarinci et al., 1994; Carrasco et al., 2005; Boucher et al., 2006; Brasca et al., 2007). For example, Brasca et al. (2007) and Morandi et al. (2006) found that *Enterococcus faecalis* reduced milk and reached the minimum value in one hour (Brasca et al., 2007) and *Lactobacillus helveticus* had a positive redox potential value under the same conditions. Moreover, strains that belong to the same species showed differences on the minimum E_h reached (Brasca et al., 2007). Among eight strains of *Lb. helveticus* tested, the minimum E_h reached varies between +27 mV and +105 mV and among ten strains of *Streptococcus thermophilus*, the minimum E_h varies between -85 mV and +48 mV. Figure 1.3 shows the trend in redox potential, reported by Brasca et al. (2007), of different LAB under the same conditions. The shape of the redox potential curve over time was characteristic of each specie and could be used to characterize microorganisms (Reichart et al., 2007).
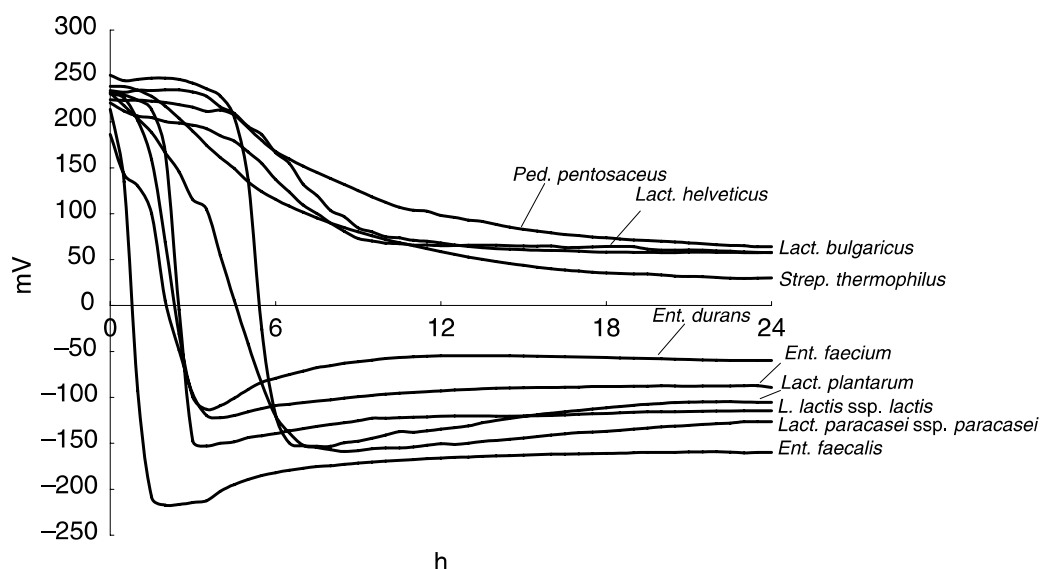
Figure 1.3   Evolution of the redox potential in sterile skimmed milk during the growth of different lactic acid bacteria (from Brasca et al., 2007).

Larsen et al. (2015) studied the trend in redox potential in milk acidified by LAB strains isolated from starter cultures containing citrate positive (cit+) strains of *L. lactis* and *Leuconostoc* species. The authors found variations between strains of the same subspecies in acidification and reduction activities; in particular, two of the strains studied kept the E_{h7} to positive values and their acidification activity was the same or higher compared to the other strains analysed in the study (Laesen et al., 2015). Carrasco et al. (2005) monitored the acidification and redox potential trends of different *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* strains isolated from cheese, in reconstituted skim milk at 37°C. *Streptococcus* strains showed a similar decrease of E_h reaching -100/-150 mV in 5 h, whereas *Lactobacillus* strains reached variable final values from -50 to -400 mV in 7 h. They also studied mixture of strains and they found trends in E_h different compared to the single culture

used (Carrasco et al., 2005). Tabatabai (1970) found that *Pseudomonas fluorescens* in the presence of *Clostridium perfringens* at E_{h7} of +40 mV grew one log cycle less compared to its absence under the same condition.

Some authors have studied the relationship between redox potential during bacterial growth and pH changes since the value of E_h depends on pH (Carrasco et al., 2005) and during bacterial acidification, changes in E_h and chemical reactions occur (Gillespie and Rettger, 1938). The first paper on a linear model for pH, ORP, water activity and temperature was published by Reichart and Mohàcsi-Farkas (1994) and no correlation was reported between pH and redox potential in synthetic media during heat destruction of foodborne microorganisms. Analysing the redox potential and acidification activities of several LAB strains, Brasca et al. (2007) found no correlation between them and the production of lactate by LAB seemed not to be responsible for changes in E_h (Tachon, 2009).

1.3.2   Mechanisms associated with the decrease of redox potential during bacterial growth

In the past, some workers had associated the drop in redox potential to the bacterial growth phase, in particular to the transition from logarithmic to stationary phases (Davis, 1932; Saal and Heukelom, 1947; Ouvry et al., 2002). However, other works have reported that when a bacterial culture is grown in a medium, the drop in redox potential, which might occur depending on the species, is not related to the growth phase (Messing, 1934; Bagramyan et al., 2000) since negative values could be reached even after the log phase (Bagrarnyan and Trchounkm, 1997).

The mechanisms by which redox potential of inoculated media decreases are still unclear (Bagrarnyan and Trchounkm, 1997) and it can be difficult identifying the redox couples involved directly in establishing the redox potential of a culture system (Wimpenny, 1969b) or identify the substances that could be indirectly the cause of unknown reversible, semi-reversible or irreversible reactions (Hewitt, 1950). Michelon et al. (2008) showed that *L. lactis* reduced the redox potential of a MRS medium and when the cells where separated by filtration, the redox potential of the medium was restored to its initial value. This finding demonstrate that the decreased in E_h is due by the whole cell and the authors also found that the reactive exofacial sulfhydryl-groups are responsible of the reduction of the medium. The redox balance inside the bacterial cell is maintained by the presence of regulatory sensors that may induce the activation of metabolic pathways adapted to a new environment in response to changes in E_h (Green and Paget, 2004).

Causes of the decreased in redox potential during bacterial growth can be attributed to:

a)   *Production of redox metabolites like metabolic end and intermediary products* (Bagrarnyan and Trchounkm, 1997; Riondet et al., 2000a).

b)   *Changes in the cell redox equilibrium.* Changes in the cell redox equilibrium are characterized by the presence of electron transport chains in the cell membrane of bacteria (Wimpenny, 1969b; Bagramyan et al., 2000) that consist of the presence of electron donors and acceptors able to transfer protons across the cell membrane (Tachon, 2009; Tachon et al., 2010). The exofacial thiol groups present on the membrane proteins, mainly cysteine and glutathione residues in proteins (Oktiabr'skiǐ and Smirnova, 2012), increase in

level during fermentation and are considered responsible for the decrease in E_h during the growth of *L. lactis* (Michelon et al., 2008, 2010). Tachon (2009) demonstrated that the reduction of milk depended on the different components of the electron transport chain like menaquinones and NADH dehydrogenases NoxA and NoxB. Bacteria including *L. lactis* and *Enterococcus faecalis* were found to produce menaquinone and therefore reduce the redox potential; others, like *Streptococcus termophilus*, did not produce menaquinone and did not decrease E_h to such negative values. However, one strain of *L. lactis* was found to reduce E_h without producing menaquinone (Tachon et al., 2010).

On the other hand, E_h can influence the redox state of proteins present at the membrane surface that contain thiol-disulfide groups (Bagrarnyan and Trchounkm, 1997) by acting on the gradient of electrochemical potential for H^+ (Bagramyan et al., 2000) and affecting the affinity for the carried molecules.

c)   *Production of reducing molecules like sulfhydryl compounds and hydrogen by facultatively anaerobic bacteria* (Hewitt, 1950; Jacob, 1974; Ouvry et al., 2001).

Sulfhydryl compounds influence the redox potential by being reduced or oxidised and catalyse enzymatic processes favouring bacterial growth (Messing, 1934; Oktiabr'skiĭ and Smirnova, 2012). In an aerobic environment, the growth of anaerobic bacteria in the presence of sulfhydryl compounds is possible thanks to the consumption of oxygen by those compounds and the reduction of redox potential (Messing, 1934; Oktiabr'skiĭ and Smirnova, 2012). Redox values reached a reducing equilibrium value when the thiol groups

arrived at their maximum and stable concentration even if the *L. lactis* fermentation was still not complete. Moreover, this redox equilibrium can be reversed by the addition of a thiol-reactive compound. In the work done by Michelon et al. (2010), the authors concluded that exofacial thiol groups are the principal groups responsible for the decrease in E_h and that milk and other broths can reach the same E_h reducing value when a sufficient cell density is present around the electrodes to guarantee adequate current transfer between thiol groups and platinum since thiol molecules concentration are related to cell bacteria concentration.

d)   *Modification of enzyme activities* (Riondet et al., 2000a; Bagramyan et al., 2000).

In the 1930s, a series of papers was published by Hewitt (1930a, b, c, d; 1931a, b, c, d, e, f; 1933) on the relationship between the oxidation-reduction conditions under which cells grew and their consequent biological behaviour. The main explanation of the differences encountered between different organisms seemed to lie in their possession of different enzymes (Hewitt, 1950). For instance, in cultures of haemolytic streptococci (Hewitt, 1930a) and of pneumococci (Hewitt, 1931b) that contain catalase the redox potential was negative even after the log phase of bacterial growth and after growth the redox increased due to the formation of hydrogen peroxide (Hewitt, 1931a). Moreover, aerated conditions positively affected the growth of aerobic microorganisms, i.e., *Micrococcus lysodeikticus* (Hewitt, 1931d), *Corynebacterium diphtheria* (Hewitt, 1930b) and *Staphylococcus aureus* (Hewitt, 1930c) resulting in a more reducing effect.

Hewitt (1930b) demonstrated that the reduction of redox potential is not only related to the growth of microorganisms. *C. diphtheria* (Hewitt, 1930b) and *St. aureus* (Hewitt, 1930c) have a low redox potential even after the proliferation probably due to the inability to form peroxide; an opposite characteristic was shown during the growth of haemolytic streptococci (Hewitt, 1930a) and pneumococcal cultures (Hewitt, 1930d) where the redox values increased after proliferation. However, *Shigella dysenteriae* is not a peroxide-forming bacterium, has no catalase and it has low reducing properties (Hewitt, 1931c). Among all the bacteria studied by Hewitt (1931e), *Balantidium coli* was the one reaching lower values of redox potential due to the capacity to produce hydrogen in the medium.

Another work by Wimpenny (1969a) related E_h to the formation of enzymes in presence of oxygen or nitrite or nitrate and the author found high production of cytochrome b_1 at +100 mV whereas the hydrogenase activity was maximum at E_h around -150 mV and fell at positive E_h values.

e)   *Consumption of oxygen and conversion to water* (Jacob, 1974; Ouvry et al., 2001).

Oxygen can be removed by bacteria thanks to their respiratory processes and also by fermentative reactions that lead to the production of reversible and/or non-reversible systems capable of reducing E_h (Davis, 1932). Some LAB have oxygen-eliminating enzymes such as NADH oxidase, pyruvate oxidase and lactate oxidase that can improve and/or modify the redox balance (Hols et al., 1999) and compounds like carbon dioxide and hydrogen can be produced throughout bacterial metabolism (Davis, 1932).

In milk, *L. lactis* can metabolise dissolved oxygen into water by a water-forming NADH oxidase (Lopez de Felipe and Hugenholtz, 2001) that is specific for each strain (Tachon, 2009; Tachon et al., 2010). However, some strains consume oxygen without reducing the E_h of milk; therefore other enzymes might be present that are able to use oxygen. Thus, the reducing activity of *L. lactis* differed due to a diversification in oxygen consumption. Studies on *L. lactis* showed that elimination of oxygen seems to be required prior to the reduction of the E_h of the medium (Tachon, 2009) and, after reduction of the medium, acidification occurred (Jeanson et al., 2009), whereas streptococci reduced and acidified the media at the same time. These differences are rather due to the lower capacity of streptococci to remove oxygen, since oxygen remains in the medium at the end of the acidification (Cachon et al., 2002). In contrast, in aerobic conditions reduction and acidification of the medium by *L. lactis* were simultaneous after oxygen consumption (Jeanson et al., 2009) and without producing hydrogen or hydrogen sulfide (H_2S) (Michelon et al., 2010). However, the removal of oxygen is not the only mechanism involved in reducing activity. Oxygen consumption was assessed to represent only 15% of the reduction of MRS broth by *Lb. plantarum* since E_{h5} can be reduced to a value of -40 mV, whereas E_{h5} decreased to -340 mV under nitrogen saturation (Ouvry et al., 2002). Even if oxygen is removed, the medium can contain oxygen metabolites such as reactive oxygen species that are highly oxidizing (Ouvry et al., 2002) and the dissolved oxygen still present can be more-or-less used by bacteria depending on their capacity for scavenging oxygen resulting in different redox potentials (Boucher, 2008). Some LAB have antioxidant capacity probably due to antioxidant peptides generated by proteolysis.

However, the most highly antioxidant LAB are not the most reducing. Among the species studied, lactococci are not antioxidants, whereas lactobacilli and leuconostoc are strongly antioxidant (Virtanen et al., 2007). Therefore, the antioxidant capacity is not the main factor responsible for the reducing ability of bacteria (Tachon, 2009).

A positive correlation between redox potential and the concentration of dissolved oxygen was reported by Kjaergaard (1976) and Tango and Ghaly (1999). The decrease in redox potential in milk by *L. lactis* seems to be related to the consumption of oxygen caused by the action of NADH oxidase and the alkyl hydroperoxide reductase complex that reduce oxygen into water (Tachon, 2009). Whereas, a linear correlation between the redox potential and the logarithm of dissolved oxygen concentration was found by Tengerdy (1961) studying the growth of a strain of *Pseudomonas* in an aerated and nitrogen-flushed medium. The author suggested the introduction of the term "differential redoxymetry" to indicate the oxygen available measured by difference in redox potentials of aerated and deoxygenated systems. In contrast, Wimpenny (1969a) found that as oxygen concentration increased in culture medium of *E. coli*, the E_h increased reaching a plateau when the medium was saturated with oxygen.

1.3.3   *Relationship between microorganisms' redox potential and heat treatment of the growth medium*

Another important factor to take into consideration in redox potential studies on microbial growth is the temperature and time of the heat treatment (Reichart et al.,

2007). Ouvry et al. (2002) reported that the effect of redox potential on growth and acidification of *Lb. plantarum* was temperature-dependent. The authors found a slow grow of this bacterium in medium at -400 mV at 37°C but not a 10°C and attributed this to alteration of ATPase activity, membrane fluidity and surface properties. However, this condition can be considered extreme since fermented dairy products do not reach so negative E_h values. Differences in reduction potential and acidification activity of different strains in skim milk at 37°C and 45°C were found by Scarinci et al. (1994). Starting from a redox potential of +40 mV in skim milk at 37°C, *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris* and *St. thermophilus* reached in 3 h values of -35 to -50 mV, *L. helveticus* and *L. delbrueckii* subsp. *bulgaricus* were slower in redox potential reduction and reached values of -5 to -10 mV.

1.3.4   *Effect of redox potential on the growth of pathogenic bacteria*

Over the years, studies have been done to understand the relationship between redox potential and microbial contamination in food. Particular attention has been paid to *E. coli* that is a facultative anaerobe able to ferment in absence of oxygen and that can reduce E_h to values around -500 mV in aerobic or anaerobic conditions independently of growth phase (Bagramyan et al., 2000; Michelon et al., 2010). The changes in redox potential during *E. coli* growth are due to the increase in the number of SH-groups in the medium and on the cell surface (Oktiabr'skiĭ and Smirnova, 1988). In the presence of oxidizing compounds *E. coli* growth slowed due to the inhibition of K^+ and H^+ transportation through the membrane (Bagramyan et al., 2000). On the contrary, the presence of reducing compounds increases the permeability of protons (Riondet et al., 1999).

Redox potential affects the heat resistance of anaerobic (facultative and obligate) pathogens (George et al., 1998; Riondet et al., 2000b) more than oxygen concentration (George and Peck, 1998). In anaerobic conditions, sub-lethally heat-damaged cells of *E. coli*, *Salmonella enteritidis* and *Listeria monocyotgenes* could recover more easily in reducing conditions (-310 mV) at pH 6.4 than at +470 mV at pH 7. The recovery might also be possible in aerobic conditions in reducing environments (George and Peck, 1998; George et al. 1998). Riondet et al. (2000b), studying the heat resistance of *E. coli* (52°C for 30 min) in reducing and oxidizing conditions, found correlation between E_h, pH and survival. At pH 7 and 5 cells were more resistant in oxidative conditions than in reducing ones, but at pH 6 no differences were found. In low E_h medium (-100 mV), sublethally heat-damaged cells of *E. coli* could recover and grow slowly, probably due to the reduction in proton motive force that decreases pH and microbial growth (Riondet at al., 1999). Heat resistance of *L. monocytogenes*, like *E. coli*, decreased in reducing conditions (Ignatova et al., 2008) and even if *L. monocytogenes* could survive in extreme redox conditions, its growth was slowed down in reducing conditions (Ignatova et al., 2009).

To mimic a food matrix, Ouvry et al. (2001) studied the growth of *E. coli* and *L. pantarum* in a gelled medium. *L. plantarum* decreased uniformly the pH of the medium from 6.5 to 3.8 and E_h from +400 mV to +250 mV within 30 h. *E. coli* caused no uniform acidification and reduction. At the beginning of the growth, *E. coli* used O_2 (*L. pantarum* did not since it has a fermentative metabolism) to convert glucose into acids (acetic, lactic, formic and succinic) and after 7 h gas bubbles formed in the gel medium as formic acid was converted to H_2 and the E_{h7} decreased to -350 mV. At the end of the growth, the redox potential increased to +250 mV since H_2 diffused to the

atmosphere. The buffering capacity of the system produced by *L. plantarum* was more stable compared to *E. coli*.

Therefore, to preserve food in an anaerobic environment, it could be possible to add oxidants that would interfere with the membrane transport systems of bacteria and reduce the bacterial growth and activity.

Recently, Erdősi et al. (2012) and Reichart et al. (2007) developed a method to detect in 10 h the total and *Enterobacterium* counts in food and based on the principle that the reduction in redox potential is related to the numbers of microbes.

Also, the effect of bacteriophage on redox potential have been studied and Hewitt (1931c) found that bacteriophage did not have a direct effect on E_h but inhibited the bacterial growth. In a study on *Staphylococcus aureus*, Clifton (1933) found that *S. aureus* could reduce redox potential of a broth over time and when its growth reached maximum rate the redox potential rapidly decreased and the subsequent addition of an anti-*Staphylococcus* bacteriophage to the medium resulted in an increase in redox potential.

1.3.5   Strategies to control redox potential during microbial growth

Redox agents or gasses can be used to control the redox potential during bacterial growth. Clifton (1933) was one of the first authors to add an oxidizing agent to a sterile medium or a medium inoculated with *S. aureus* and the author observed that the addition of oxidising agents (potassium ferricyanide and potassium ferrocyanide) increased the redox potential of the media and, over time in the presence of *S. aureus*, the redox potential decreased probably due to bacterial activity. Therefore, in the presence of positive redox potential created by oxidizing agents, bacterial

development might not be influenced (Bagramyan et al., 2000). Whereas, flushing O_2 trough an anaerobic medium caused an increase in redox values in both sterilized and inoculated media (Ward, 1938) and inhibited the growth of anaerobic bacteria (Bagramyan et al., 2000).

Gillespie and Rettger (1938) were the first to flush nitrogen through a culture to eliminate the effect of metabolic hydrogen on the redox potential values and found a constant negative reading during all the treatment. However, nitrogen gas alone cannot reduce E_h to negative values (Clifton, 1933; Martin et al., 2010, 2011, 2013; Ebel at al., 2011; Larsen et al., 2015) and degassing the medium only decreases the E_h by 100-150 mV (Michelon et al., 2010). However, hydrogen can reduce E_h to negative values (Ebel et al., 2011).

Larsen et al. (2015) studied the effect of oxygen on E_h and acidification activity of LAB and found that flushing oxygen into milk inoculated with LAB can slow the acidification rate of the bacteria and delay the reduction in redox potential probably because more time was required for metabolism of oxygen by the bacteria studied.

1.4   Oxidation-reduction potential in yoghurt

Commercial yoghurts have a E_h from +100 to +150 mV (Jayamanne and Adams, 2009) and a E_{h7} from -150 to +410 mV (Aubert et al., 2002).

Studies have been done on the effect of redox potential on yoghurt during storage and on strategies to control redox potential.

The effect of redox potential on bifidobacteria was studied for the first time by

Jayamanne and Adams (2009). In general, deaeration, addition of reducing agents like potassium ferricyanide, ascorbic acid, L-cysteine-HCl, and dithiothreitol (DTT) (Brunner et al., 1993a,b), electro-reduction and the presence of LAB able to scavenger oxygen can promote the survival of *Bifidobacterium* in milk during storage (Bolduc et al., 2006b).

The redox potential of yoghurt can be lowered by the addition of cysteine (Dave and Shah, 1997a, 1998; Bolduc et al., 2006b) or ascorbic acid (Dave and Shah, 1997b) and yogurts had values more negative as the concentrations of the reducing agents increased. However, during storage the redox potential of yoghurts made with cysteine increased reaching positive values within 35 days and the pH dropped to values lower for the samples containing higher concentrations of cysteine (Dave and Shah, 1997a; Bolduc et al., 2006b) whereas, when ascorbic acid was used instead of cysteine, redox potential reached positive values within 15 days. However, redox agents can affect bacterial survival. The initial low redox potential caused by the addition of cysteine, lowered the count of *Streptococcus thermophilus* (Dave and Shah, 1997a, 1998) but the viability of *Lactobacillus acidophilus* was improved (Dave and Shah, 1998). Moreover, *Bifidobacterium* growth was ideal in conditions of low redox potential and the absence of oxygen (Lourens-Hattingh and Viljoen, 2001; Bolduc et al., 2006b; Ebel et al., 2011); reducing the oxygen content lessens the fermentation time at 43°C of yoghurt inoculated with *Lactobacillus delbruechii* subsp. *bulgaricus* and *St. thermophilus* (Horiuchi et al., 2009).

The level of dissolved oxygen during storage can be reduced also by the addition of enzymes like glucose oxidase, NADH-peroxidase or H^+-ATPase to milk and addition favoured probiotic viability in yoghurt without altering the aroma (Brunner et al.,

1993a, b; Cruz et al., 2012).

Martin et al. (2010) made yoghurt under different redox conditions produced by the use of gasses like air, N_2, N_2-H_2 (4%H_2) or ungassed. At the beginning of the fermentation (45°C), the E_{h7} values were +433 mV, +283 mV, -349 mV and +405 mV, respectively. During fermentation, the pH of all the yoghurts decreased, with the same trend, to 4.6 in 3.5 h and E_{h7} of the yoghurt treated with a reducing gas was constant during the all fermentation, whereas the other experimental yoghurts had a decrease in E_{h7} probably due to the utilization of oxidizing compounds present and production of exofacial thiol proteins in the cell surface.

The rheological and organoleptic characteristics of yoghurt can be influenced by its redox potential. In a study on the rheological characteristic of gels made from milk of different redox potentials (Martin et al., 2009), caused by addition of gasses, it was found that redox potential can influence gel structure. In particular, gel firmness was higher in reducing environment (Martin et al., 2009). Moreover, the whey separation and the viscosity of yogurt made under reducing condition were more appreciable (Martin et al., 2010). Analysis of the aroma compounds influenced by redox potential during yoghurt storage was carried out by Martin et al. (2011). During storage, a positive E_h favoured the stability of acetaldehyde and dimethyl sulphide. Negative E_h values decreased levels of acetaldehyde that was reduced to ethanol and increased dimethyl sulphide concentration. Levels of diketones decreased in positive or negative E_h during yoghurt storage.

1.5   Oxidation-reduction potential in cheese

The first method designed to measure the redox potential potentiometrically in Cheddar cheese was described by Davis (1932). The system consisted on placing a gold or platinum electrode into the cheese itself and an agar bridge with a saturated KCl reference electrode (Figure 1.4).
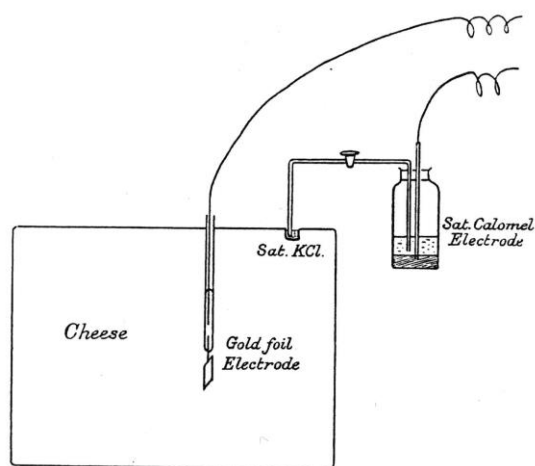
Figure 1.4   Measurement of redox potential in Cheddar cheese by Davis (1932).

However, results were not satisfactory since cracks in the cheese matrix altered the measurements (Davis, 1932) probably due to the shape of the working electrode (Sawyer at al., 1995) that did not ensure a good contact between the cheese matrix and the surface of the electrodes (Vos, 1948; Tammam et al., 2001) causing air to diffuse into the cheese. To prevent oxygen penetration, some authors had placed paraffin liquid or wax paste around the electrode (Vos, 1948; Kristoffersen et al., 1964b; Langeveld and Galesloot, 1971; Abraham et al., 2007); in another study by

Langeveld and Galesloot (1971), very young Cheddar cheese was placed in a tight beaker and covered with liquid paraffin to avoid oxygen penetration due to a weak cheese structure. Whereas, in a study on the poising capacity of Cheddar cheese, a slurry of cheese and phosphate buffer was used (Kristoffersen et al., 1966). Moreover, since Cheddar curd pieces are salted and pressed together, the internal structure of the cheese can have fissures in its structure that could interfere with the measurement and delay the time required to reach a redox equilibrium reading. Thus, the shape of the electrodes can influence E_h readings (Higginbottom and Taylor, 1960). To prevent the effects of cracks in the matrix, microelectrodes had been used by some authors (Ouvry et al., 2001; Abraham et al., 2007). In the study by Abraham et al. (2007), the use of microelectrodes (diameter 0.61 mm) allowed measurement of equilibrium redox potential value within 10 min to 1 h in Camembert cheese at 25°C. A redox potential gradient was found in the cheese from the surface to the centre and also during ripening (E_{h7} from +330 to -300 mV at 15 days of ripening and E_{h7} from +360 to -360 mV at 35 days). Due to the high moisture content of the Camembert cheese, it is possible to insert both working and reference electrodes directly into the cheese. In a work by Topcu et al. (2008), a self-assembled platinum electrode was used and the optimum conditions for redox measurements in hard cheeses were established (Figure 1.5). The platinum electrode was thin and flexible enough to be directly insert into the cheese 5 cm deep without penetration of air. The equilibration time needed can also be affected by the distance between the reference and working electrode. Topcu et al. (2008) placed the reference electrode 2.5 cm apart from the working electrode into a 4 cm deep hole filled with 3 M KCl solution to complete the electrochemical cell.
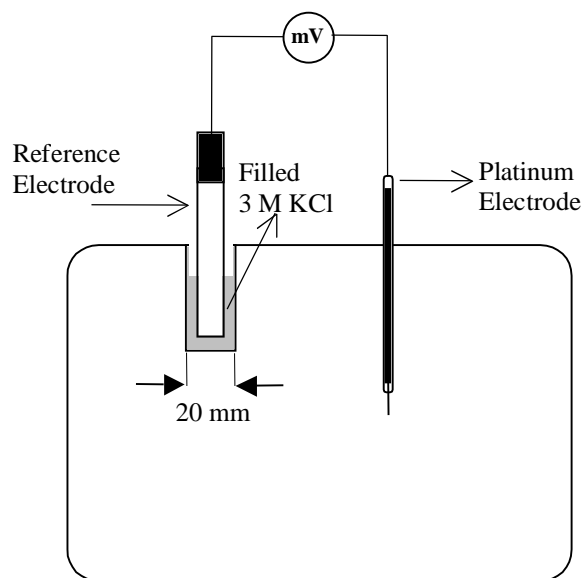
Figure 1.5   Measurement of redox potential in Cheddar cheese by Topcu et al. (2008).

The storage temperature of cheese can affect E_h; Broadbent et al. (2002) found that values of E_h in Cheddar cheese were lower at a higher temperature of storage. Similar results were found by Topcu et al. (2008) in Cheddar cheese where a significant lower redox potential value was found at 20°C compared to 4°C. This phenomenon is due to the ionic activity of the medium since interaction between ions increases with increasing temperature (Topcu et al., 2008). The time required to reach equilibrium is also temperature-dependent; at lower temperature longer time is required for the redox readings to stabilize. The E_h equilibrium values in Cheddar cheeses were measured in few studies. The equilibrium E_h value of -120 mV in a mild Cheddar

cheese at 12°C was reached within 2-3 days (Topcu et al., 2008) whereas an E_h of -110 mV was reached in 4-5 days at 10°C (Kristoffersen et al., 1964b).

1.5.1   Evolution of cheese redox potential from cheesemaking to ripening

Studies on redox potential trends during cheesemaking and at the beginning of ripening have been done to understand the mechanisms involved in the E_h changes from the positive values of milk to the negative values of cheese. Davis (1932) attempted to measure the redox potential during Cheddar cheesemaking by incorporating dyes (like Janus green, Natural red and Safranine) into the milk. At the whey drainage step of the manufacture, Janus green was already reduced, Natural red was reduced after cheddaring and Safranine was oxidised throughout the all manufacture. However, the reduction processes could have been accelerated by sunlight during the cheesemaking. The surfaces of the cheese and around cracks had a positive redox potential whereas the inside had a negative value and when the cheese was cut the colour rapidly reappeared due to the reversibility of the redox dyes (Davis, 1932; Galesloot, 1960b). For these reasons, and also because of the possible toxicity of the dyes (Higginbottom and Taylor, 1960), the use of dyes does not appear suitable for estimation of E_h in cheese (Galesloot, 1960a).

Changes in redox potential during Cheddar cheesemaking was also determined by Caldeo and McSweeney (2012). During the manufacture, the authors found a significant drop in redox potential when the whey was drained until the milling stage; during the overnight pressing the values of redox decreased again to values close to mature Cheddar. Results were in agreement with those of Green and Manning (1982). The trends in E_h during the cheesemaking of other cheeses like Camembert, Gouda

and Emmental were also studied by Caldeo and McSweeney (2012) and differed from each other due to the manufacturing steps and starter culture used.

The redox potential during Cheddar ripening was measured potentiometrically and colorimetrically by Kristoffersen and Gould (1959) and different values were found. At the beginning of the ripening the mean E_h of a good flavour Cheddar cheese was -104 mV (pH 5.3-5.8), -90 mV after 1 month, and -270 mV after from 5 to 6 months using electrodes. When measured by redox dyes, the initial E_h was -270 mV and E_h reached values of -225 mV and -157 mV, in raw and pasteurized cheeses, respectively, in about 6 months. Davis (1932) reported that in the first days of ripening the redox potential is low due to production of trioses (methyl glyoxal) and acetaldehyde by lactococci, after this period the redox potential slowly increases probably because of diffusion of oxygen in the cheese and due to the death of lactococci, then the redox values decrease again, possibly due to the growth of lactobacilli after one month of ripening. In a study (McSweeney et al., 2010) on redox potential during Cheddar ripening, the increase in redox potential at about one month was not found probably due to the cheeses being vacuum packed during ripening avoiding oxygen penetration into the block. E_h measurements were taken from the pressing stage to one month of ripening and the redox potential was measured electrometrically by embedding miniature electrodes into the mould and pressing them together with the cheese curd. Values were nearly constant (E_h ~ -120 mV) once the equilibrium was reached in the first hour of pressing (McSweeney et al., 2010).

Moreover, the redox potential is uniform inside Cheddar cheese (Davis, 1932) whereas Camembert cheese has a positive E_h on the surface (E_{h7} ~ +300 mV) and a negative E_{h7}, ~ -350 mV, in the cheese core at 35 days of ripening (Abraham et al.,

2007; 2013). In Camembert cheese, the changes in firmness of the cheese during ripening and in different locations across the cheese were correlated to the redox potential values (Abraham et al., 2007). Contradictorily opinions are present in the literature regarding the redox potential of Edam cheese; Galesloot (1960a) found a uniform redox potential in the cheese whereare Vos (1948) and Peltola and Antila (1953) supposed that redox potential in the cheese is not uniform.

Together with cheese structure and manufacturing procedure, another parameter that can influence the equilibrium E_h value and the time needed to reach it in mature cheeses, could be the internal microflora (Beresford et al., 2001).

The negative internal E_h characteristic of the majority of dairy products, excludes the development of obligate aerobic bacteria like *Pseudomonas*, *Brevibacterium*, *Bacillus* and *Micrococcus* spp. inside the product (Brasca et al., 2007; Cretenet, et al., 2011) that can populate the surface of the cheese (Abraham et al., 2007; Beresford et al., 2001). Mould-ripened cheeses, like Camembert, shown a positive redox potential on the surface due to the presence of mould (Abraham et al., 2007). The internal cheese has a negative redox potential due to the presence of obligatory or facultatively anaerobic microorganisms (Davis, 1932; Abrahan at al., 2007; Topcu et al. 2008) since the starter LAB ferment lactose to lactic acid (Fox et al., 2000; van Dijk et al., 2000; Aubert et al., 2002; Cachon et al., 2002) and convert the oxygen in milk into water by enzymes like NADH oxidase (Thomas, 1986) creating an anaerobic environment (Beresford et al., 2001; Crow et al., 1995). In addition, other work suggests NSLAB populations, types and numbers may vary in their ability to maintain a low E_h potential (Thomas, 1986; Broadbent et al., 2002). Broadbent et al. (2002) compared the E_h of Cheddar to Colby cheese (a curd washed cheese) that has a higher pH and

lower acid content since lactate and lactose are removed during manufacture. Colby cheese had a higher redox potential compared to Cheddar cheese at the beginning of the ripening, due to the low activity of oxidative metabolization of lactose by starter and at two months of ripening it had a lower E_h compared to Cheddar due to the higher number of NSLAB.

In the past, few studies were dedicated to the measurement of the redox potential in Edam cheese. Vos (1948) was the first to report a decline in redox potential during ripening of Edam cheeses. A value of -180 mV was reached after 24 h from pressing. In Edam cheese, Galesloot (1960a) found a $E_h \sim$ -130 mV at one day of ripening when the cheese was made from pasteurized, over pasteurized milk or raw milk under aseptic conditions. When coliform bacteria were found or when the cheese was made from raw milk in septic condition the E_h was $\sim$ -270 mV. During ripening, a sharp reduction in E_h (from -247 to -269 mV at pH 5.0) was observed when butyric acid fermentation developed in the cheese producing CO_2 and H_2 (Peltola and Antila, 1953; Galesloot, 1960a) and therefore, nisin-producing starter could be used to avoid this phenomenon (Galesloot, 1960a).

Aubert et al. (2002) measured E_{h7} of Camembert and Comté cheeses and found values from -259 to -350 mV in Camembert and -156 mV in Comté cheese.

1.5.2   Strategies to control redox potential in cheese

The reactions that occur in cheese and that are related to the redox potential are still unclear (Kristoffersen, 1985) and it is interesting to understand how to control redox potential. Only few studies have been done on strategies to control redox potential in cheese during ripening. Davies et al. (1934) made Cheddar cheeses with the addition

of redox agents and protein degradation was determined. The oxidants used (KNO_3, $KClO_3$, $KClO_4$) were able to maintain the redox potential, measured colorimetrically, to values higher than the control and to decrease proteolysis. The reducing agent (KCN) did not affect the redox potential and protein breakdown. The paper did not specified how the chemical compounds were added to the cheese.

Addition of 0.03% of KNO_3 to the milk used for the production of Edam cheese by Vos (1948) increased the E_h to values ~ -70 mV (pH 5.03) at 8 weeks of ripening whereas the control Edam cheese had a value of -283 mV (pH 5.18).

Galesloot (1961a,b) measured redox potential potentiometrically in a study of the effects of oxidising salts such as potassium nitrate (KNO_3), potassium nitrite (KNO_2), potassium chlorate ($KClO_3$), potassium bromate ($KBrO_3$) and potassium persulphate ($K_2S_2O_8$) on the development of butyric acid bacteria in fermented milk and Edam cheese. Among those salts, only potassium nitrate and potassium nitrite were able to avoid the decrease of E_h due to coliform growth and the effect was more pronounced in acidified milk than in cheese (Galesloot 1960b), probably due to the higher presence of reducing component in cheese compared to milk. In Edam cheese, the E_h decreased to ~ -290 mV in 6 days while the addition of KNO_3 kept the E_h at -130/-140 mV for 10 days and after that the E_h decreased to values close to the control cheese (Galesloot, 1961a). When coliform bacteria were added in high quantity, the effect of potassium nitrate and potassium nitrite on the redox potential was counteracted (Galesloot, 1961b). However, the inhibitory effect of potassium nitrite against butyric acid bacteria is not a consequence of their effect on E_h (Galesloot, 1961b).

Peltola and Antila (1953) reported that the redox potential of Emmental cheese at one month of ripening (~ -270mV) increased by the addition of potassium nitrate to the

cheese milk but not by adding potassium chlorate. Moreover, if the nitrate was added in high quantity it delayed the deamination of some amino acids by propionic acid bacteria (Peltola and Antila, 1953).

Redox potential has been implicated in pink discoloration defect of Italian cheese (Shannon et al., 1977) and dyes were used to correlate the redox potential of the first stage of cheese ripening with pink discoloration of cheese caused by oxidative metabolism of strains of *Lactobacillus* (Shannon et al., 1969).

1.6   Oxidation-reduction potential and flavour development in dairy products

Redox potential has an important influence on the development of flavour in dairy products (Kristoffersen et al., 1964a, 1967; Lowrie et al., 1974; Law et al., 1976; Green and Manning, 1982; Kristoffersen, 1985; Olson, 1990; Kieronczyk et al., 2006; Ledon and Ibarra, 2009; Brasca et al., 2007; Topcu et al., 2008). Most of the oxidation-reduction reactions that occur in milk and cheese are related to the development of flavour compounds (Kristoffersen, 1985; Kieronczyk et al., 2006).

1.6.1   Influence of sulfur compounds

The negative E_h of cheese ensures the development of reactions necessary to the release of a good aroma (Kristoffersen et al., 1964a; Urbach, 1993; Topcu et al., 2008; Brasca et al., 2007).

Cheese flavour develops during ripening by proteolysis, lipolysis and breakdown of carbohydrate (Kristoffersen, 1985). The metabolism of intermediate carbon compounds, in particular, is through in redox reactions (Kristoffersen, 1985). During ripening, transfer of hydrogen, oxygen and electrons are involved in redox reactions.

The relation between the presence of sulfur compounds and the development of cheese flavour is well known (Kristoffersen et al., 1964a; Manning, 1974; Manning et al., 1976, Manning and Nursten, 1985; Urbach, 1993; Seefeldt and Weimer, 2000). Negative redox potential seems important for the development of Cheddar cheese flavour (Manning, 1979; Adda et al., 1982) and both chemical and enzymatic reactions guide the development of cheese aroma (Adda et al., 1982). Swiss cheese has a higher concentration of active sulfhydryl groups and a lower redox potential compared to Cheddar cheese (Kristoffersen, 1985), probably due to the presence of propionic acid bacteria.

However, even if the formation of active sulfhydryl groups is essential for flavour generation, not all the cheeses with a low content of active sulfhydryl groups have poor flavour development (Kristoffersen et al., 1966; Kristoffersen, 1985).

Few old publications (Law et al., 1976; Manning 1979; Green and Manning, 1982) suggested that redox potential was the only factor involved in the flavour development and that microorganisms are not responsible. Nowadays, it is actually difficult to think that flavour is only due to chemical reactions and Singh (2003), Kristoffersen (1973) Kristoffersen et al. (1964a) showed that both reducing conditions and microorganisms are essential for flavour development. Moreover, a decrease in redox potential by bacterial fermentation is essential for the formation of sulfur compounds (Green and Manning, 1982; Tammam et al., 2001).

A study by Kieronczyk et al. (2006) monitored the flavour compounds that are produced through amino acid catabolism by *Lactococcus lactis in vitro* under both oxidizing and reducing conditions made by using DTT and potassium ferricyanide, respectively. Most of the steps of the amino acid catabolic pathways in *L. lactis* (Figure 1.6) are oxidation-reduction reactions; therefore redox potential is expected to influence the production of aromatic compounds (Kristoffersen, 1985). Results from this study showed that reducing conditions stimulated the production of carboxylic acids and hydroxy acids, in particular phenyl acetate, while oxidative conditions stimulated the production of aldehydes from leucine and methionine and the production of alcohols derived from leucine. Results from the same study indicated that the production of volatile sulfur compounds by *L. lactis* was affected by redox conditions. In particular, dimethyltrisulfide (DMTS), resulting from the oxidation of methanethiol and diacetyl, was present in high level in reducing system compare to the oxidising system (Kieronczyk et al., 2006) whereas dimethyldisulfide (DMDS) production was favoured in an oxidising environment.
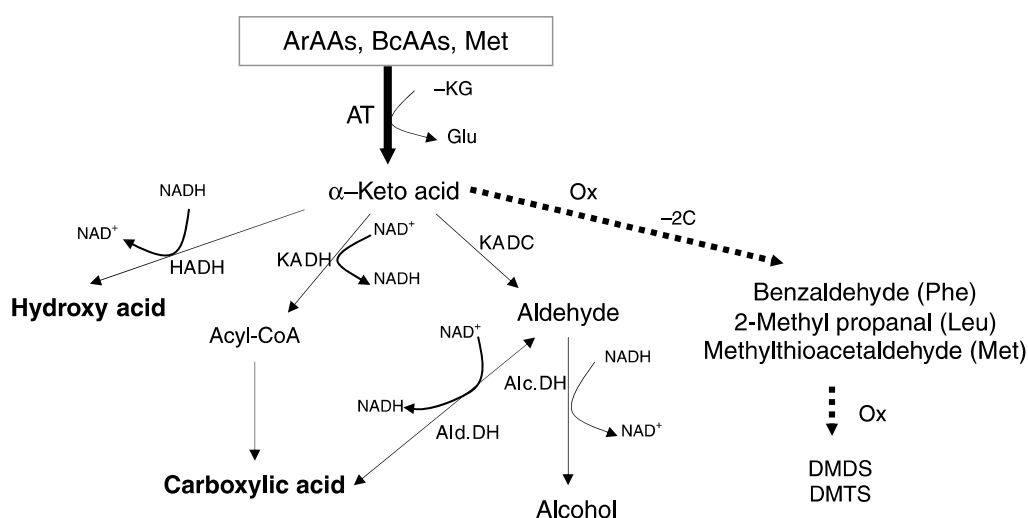
Figure 1.6   Amino acid catabolism pathways in *Lactococcus lactis*. ArAAs, aromatic amino acids; BcAAs, branched-chain amino acids, Met, methionine; α-KG, α-ketoglutarate; Glu, glutamate; AT, aminotransferases; HADH, hydroxyl acid dehydrogenase; KADH, keto acid dehydrogenase; KADC, keto acid decarboxylase; Alc.DH, alcohol dehydrogenase; Ald.DH, aldehyde dehydrogenase; Ox, chemical oxidation. The products whose production was enhanced under reducing conditions are in bold and on the left hand side and those mainly obtained under oxidative conditions are on the right hand side (from Kieronczyk, 2006).

Heating raw milk in the absence of air to produce dried milk generates a lower redox potential due to the production of reducing compounds (Greenbank, 1940) like sulfur compounds that prevent the development of tallow flavour by regenerating the natural antioxidants and using the residual oxygen faster than fat (Harland et al., 1949; Greenbank and Wright, 1951) and the product shelf life could be extended.

Methanethiol (CH_3SH) and H_2S are not produced in cheese made with chemical acidification using gluconic acid lactone (GAL) (redox potential of +300 mV, measured by indicators) as peptides are not converted to free amino acids by bacteria. However,

the addition of a reducing agent (such as cysteine or DTT) to an acidified cheese using GAL produced CH_3SH and H_2S (Green and Manning, 1982). Manning (1979) demonstrated that CH_3SH could be immediately oxidised to dimethyl disulphide (CH_3SSCH_3, a key aroma compound in Cheddar cheese) and dimethyl trisulphide since by adding a reducing agent to the cheese acidified by GAL, the DTT interacted with protein determining the production of H_2S and CH_3SH. In particular, CH_3SH was produced from reaction of methionine and its residues with reducing agents and H_2S was an intermediary molecule produced during the reaction (Manning, 1979).

1.6.2   Production of oxidized flavour

Redox reactions are the main changes that can occur during milk storage and processing causing formation of oxidized flavour primarily by phospholipid oxidation (Haratifar, 2009) and consequential aroma alteration. Moreover, the formation of oxidized flavour compounds depends on oxidation of ascorbic acid and further formation of oxidizing components (Greenbank, 1940; Saal and Heukelom, 1947). Those oxidizing components determine the redox potential of a medium. When oxidizing conditions are too strong, the development of oxidized flavor does not happen and reactions continued to a more complete stage (Greenbank, 1940).

The initial redox value is correlated with flavour development since a lower initial redox gives a higher change in potential compare to a higher initial value (Webb and Hileman, 1937; Swanson and Sommer, 1940; Saal and Heukelom, 1947).

Factors that favor high E_h also favor development of oxidized flavor. When ascorbic acid was added to milk, it could delay the development of oxidized flavor but not off flavor (Corbett and Tracy, 1941; Bell, 1948).

The development of oxidized flavour can be prevented by the presence of a low redox potential (Brown and Thurston, 1940) and electro-reduction of milk could be used to control redox potential and prevent reactions like lipid oxidation. Haratifar (2009) demonstrated a reduction on the fatty acids degradation in milk electro-reduced at 4 V stored in anaerobic glass jar in the dark.

Also homogenization of milk can prevent the development of oxidized flavor in milk without influencing its E_h (Larsen et al., 1941).

E_h could be used to detect metal contamination in milk since the presence of copper catalyses the production of oxidized flavour causing an increase in redox potential (Webb and Hileman, 1937; Greenbank, 1940). In milk, the addition of copper can alter flavour more than iron and by increasing copper concentration, the intensity of oxidized flavour detected increases (Greenbank, 1940).

It is not possible to relate the formation of oxidized flavour in butter to the change in redox potential (Saal and Heukelom, 1947) due to a higher disposition to oxidation of butter compare to the cream from which it was made and higher presence of oxidizing compounds formed via peroxides arising in the fat phase of the butter.

In cheese, the extent of oxidation reactions is limited since oxidizing components are promptly reduced due to the low redox potential (Delgado et al., 2009) and the presence of antioxidants like vitamin E or α-tocopherol (Singh et al., 2003) therefore substances like aromatic hydrocarbons are present due to the existence of oxidative processes (Adda et al., 1982).

1.7   Conclusions

Measurement of redox potential during manufacture and ripening of dairy products can be of fundamental importance to understand the dairy microflora and its metabolism and to control the physico-chemical and sensorial properties of fermented dairy products (Aubert et al., 2002; Broadbent at al., 2002; Cachon et al., 2002; Tachon, 2009; Abraham et al., 2013).

In order to obtain reproducible and accurate results in redox potential measurements it is of fundamental importance to:

a) Standardize the method used;

b) Pay attention on the care of electrodes;

c) Report all the conditions under which the experiments take place, including temperature and pH.

It has been demonstrated that microorganisms can have different abilities to modify redox potential during their growth and acidification (Cachon et al., 2002; Tachon, 2009; Brasca et al., 2007; Larsen et al., 2015). Bacteria cause different extents of reduction in redox potential and they can reach an equilibrium value at different times depending on the environmental conditions, such as composition of the growth medium, pH, temperature, oxygen, and their metabolic activities, that are characteristic of each microbial species and strain.

Therefore, it is important to select microorganisms as candidates to be used as starters in fermented dairy products (Scarinci et al., 1994; Brasca et al., 2007; Tachon,

2009) and study the ability of bacteria to influence redox potential lunder different conditions before their introduction into a dairy process (Scarinci et al., 1994).

The redox potential value of a product could be considered a discriminating factor to distinguish between bacteria (Brasca et al., 2007; Tachon, 2009) since their diversity in reducing activity results in different redox potentials between fermented products (Aubert et al., 2002). The redox potential values during the manufacture of a dairy product could influence the development of desired final characteristics.

Further studies will be necessary to develop the perfect blend of starter and adjunct cultures to use in the manufacture of dairy products that are able to control redox potential and that will prevent the growth of unwanted bacteria (Boucher et al., 2006).

The redox potential that develops in a system, by adjunct microorganisms or by physico-chemical actions, can be considered as a reason why some reactions occur and others do not. In dairy products, it has been demonstrated that the redox potential influences the development of flavour component (Kristoffersen et al., 1964a,b, 1967; Lowrie et al., 1974; Law et al., 1976; Green and Manning, 1982; Kristoffersen, 1985; Olson, 1990; Kieronczyk et al., 2006; Brasca et al., 2007; Martin et al., 2011) and there is also some evidence that redox potential could contribute to the rheological properties of dairy products like yoghurt (Martin et al., 2009, 2010).

In the food industry, understanding the behaviour of microorganisms in fermented products is gaining more relevance in the last few years (Carrasco et al., 2005) and the introduction of the redox potential as an additional tool to control the quality of

dairy products could help to predict and keep under control flavour development in the final product (Boucher et al., 2006).

The development of strategies to measure continuously the changes in redox potential of a product and to control, and adjust if necessary, the redox potential values during a manufacturing and ripening could be an important achievement for the future of the dairy industry.

1.8   References

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Chapter 2

Changes in oxidation-reduction potential during the simulated manufacture of different cheese varieties

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Abstract

Oxidation-reduction (redox) potential (E_h) is a fundamental physicochemical property of cheese that partly determines its microenvironment both during manufacture and ripening. In this study, changes in redox potential, pH and temperature during the simulated cheesemaking of Cheddar, Gouda, Emmental and Camembert cheeses were determined. The E_h of milk decreased from +349 mV to +236 mV during pasteurization. E_h became negative during the whey drainage step of cheese manufacture except for Emmental that remained positive. The E_h of Cheddar, Gouda and Camembert decreased to -117 mV during the pressing stage while the E_h of Emmental reached +230 mV during the pressing step. Variations in redox potential may be attributed to both the cheesemaking protocol used and starter culture added. Monitoring of the redox potential throughout the cheesemaking could allow its better control and standardization.

2.1   Introduction

Cheese is a fermented dairy product that is manufactured with the purpose of conserving the principal constituents of milk (Fox and Cogan, 2004). Fermented foods, in addition to being more shelf-stable, typically develop desirable sensory qualities. The sensory properties of fermented dairy products result from the metabolic activity of lactic acid bacteria (LAB) in converting lactose to lactic acid; fermentation and growth of LAB are monitored indirectly by pH determination. Taking into consideration the chemical, physical and microbiological changes that occur during the cheese manufacture and the effect of these changes on cheese quality, it is helpful to develop tools to monitor the progression of the various changes during cheesemaking.

Oxidation-reduction (redox) potential is an important physico-chemical parameter that, together with pH, temperature and ionic strength, determines the microenvironment in cheese. Redox potential can be defined as the measure of the tendency of a chemical/biochemical system to oxidize (lose electrons) or reduce (gain electrons).

The relationship between redox potential and the concentrations of the oxidized and reduced forms of a substance is given by Nernst equation (1), in ideal systems:

$$E_h = E_0 + 2.3RT/nF \log [Ox] / [Red] \qquad\qquad (1)$$

where E_h is the potential at the standard hydrogen electrode (V), E_0 is the standard potential of the system when the activities of all reactants are unity, R is the universal gas constant (8.314 J/K x mol), T is temperature (in Kelvin), F is the Faraday constant

(96.5 kJ/V x mol), n is the number of electrons involved in redox reaction (Brown and Emberger, 1980; McCarthy and Singh, 2009).

During redox measurement, it is essential to specify not only the measured potential value, but also the pH and the temperature at which it is determined (Jacob, 1970; Morris, 2000). Furthermore, in the same manner in which the pH is a measurement of the hydrogen ion activity in a system, redox potential is a measurement of the activity of electrons in a system (Kjaergaard, 1977) so it is affected by all oxidising and reducing agents present in a system. Therefore, the measurement of redox potential should be taken in consideration during fermentation processes.

Redox potential is an important selective factor for microbes in all environments (Brown and Emberger, 1980) and knowledge of the actual redox conditions is important for the interpretation of milk quality and freshness (Noyhouzer et al., 2009). Different studies have shown that growth of microorganisms in dairy products may be affected by the redox potential of the system (Beresford et al., 2001; Bolduc et al., 2006b; Boucher, et al., 2006; Brasca, et al., 2007; Morandi et al., 2006; Scarinci et al., 1994). Moreover, redox potential contributes to the conditions necessary for flavour development in fermented dairy products (Cachon et al., 2002; Carrasco et al., 2005; Morandi et al., 2006). In cheese, it is thought that a negative redox potential is required for the stability of aroma, especially that associated with volatile sulfur compounds (Green and Manning, 1982; Kristoffersen, 1967).

Milk at 25°C in equilibrium with air has usually a E_h value in a range from +250 to +350 mV at pH 6.6-6.7 (Fox and McSweeney, 1998) due to the presence of oxygen and oxidizing compounds; however, cheese has a negative E_h value. For example, values

reported for the E_h of Cheddar and Camembert are -120 mV (Topcu et al., 2008) and -300 to -360 mV (Abraham et al., 2007), respectively. It is well known that anaerobiosis and low redox potential contribute to the development of balanced flavour (Abraham et al., 2007) and different volatile flavour compounds are produced *in vitro* under oxidizing and reducing conditions (Kieronczyk et al., 2006). However, the exact mechanism by which the redox potential of cheese is reduced is not clear.

Although redox potential is believed to be an important physiochemical parameter of fermented dairy products, only one study by Green and Manning (1982) reported changes in redox potential at different stages of cheesemaking. The aim of this study was therefore to evaluate the changes in redox potential during the simulated manufacture of Cheddar, Gouda, Emmental and Camembert cheeses throughout the various stages of cheesemaking; pH and temperature were also measured.

2.2   *Materials and methods*

2.2.1   *Redox potential, pH and temperature measurements*

Measurements of redox potential were made using a platinum working electrode (Pt 1800; Schott, Mainz, Germany) and silver/silver chloride reference electrode (REF 201; Radiometer Analytical, Villeurbanne Cedex, Lyon, France). Before cheese manufacture, the reference and the platinum electrodes were cleaned using the method described by Topcu et al. (2008). The platinum electrodes surface was polished with fine alumina (Sigma-Aldrich, St. Louis, Mo., U.S.A.) powder and rinsed with distilled water and allowed to dry in air. Reference electrodes were cleaned

using pepsin in HCl solution and RENOVA-N (Radiometer Analytical) solution. The

accuracy of electrodes was checked against tap water (Abraham at al., 2007) and

using a 3 M KCl solution (Topcu et al., 2008) and the E_h value recorded in 3 M KCl was

≈ +479 mV at room temperature. At the end of each experiment the electrodes were

rinsed with water and checked again in 3 M KCl solution. During cheese manufacture,

pH was determined using a combined electrode (PHC3001-8; Radiometer Analytical)

connected to a pH meter (PHM210 Standard pH Meter; Radiometer, Copenhagen,

Denmark). The temperature was measured using a thermocouple. The redox

electrodes, the pH probe and the thermocouple were placed into the matrix during

the all experiment.

All the detectors were connected to a data logger (Squirrel Data Logger 2040-2F16

Series; Grant, Data Acquisition, Cambridge, UK) through an amplifier (PHTX-21,

Omega, USA) that enabled data acquisition on a computer during the simulated

manufacture of the cheeses. The measured data were recorded every minute and data

reported were averaged over 5 minutes.

Redox potential was reported as ORP, redox potential uncorrected for pH and without

reference to the standard hydrogen electrode without correction for pH and

temperature. ORP data were also converted to E_h (potential related to the standard

hydrogen electrode), using the following formula:

$$E_h = ORP + E_r \qquad\qquad\qquad\qquad (2)$$

Where E_r is the potential of the reference electrode, i.e., +199 mV at 25°C when a

Ag/AgCl reference electrode is used (McSweeney et al., 2010). Reference electrode

potentials change with temperature and it is possible to correct the redox readings
using the temperature coefficients (-1.01 mV at 25°C for Ag/AgCl reference electrode;
Sawyer et al., 1995). Temperature compensation was done using the following
formula:

$$Er = +199 - 1.01 \ (T-25°C) \hspace{3cm} (3)$$

Where T is the recorded temperature.

2.2.2   Simulated cheesemaking

Cheddar, Gouda, Emmental and Camembert were made in triplicate on a laboratory
scale with simultaneous measurement of pH, temperature and redox potential. For
each cheesemaking, raw milk (4 L) was obtained from a local dairy farm and
pasteurized at 63°C for 30 min. Cheese manufacture was performed in a glass beaker
(5 L) in water bath with temperature control and manual stirring was used for milk
and curd agitation. The major variables during the manufacture of Cheddar, Gouda,
Emmental and Camembert-type cheeses are summarized in Table 2.1.

2.2.2.1 Cheddar simulated manufacture

Pasteurized milk was cooled to 31°C and inoculated at a rate of 0.02% (w/v) with a
concentrated starter culture suitable for Cheddar cheese (DVS R604, Chr. Hansen,
Hørshom, Denmark) containing *Lactococcus lactis* subsp. *cremoris* and *Lactococcus
lactis* subsp. *lactis*. After a 30 min ripening period, the milk was supplemented with
1M $CaCl_2$ (0.9 mL/L). Coagulant (Chymax-180, Chr. Hansen, Hørshom, Denmark),
diluted 1:5 with distilled water, was added at a level of 0.3 mL/L. When the coagulum
reached the desired firmness after 40-50 min, the curd was cut and allowed to heal

for 10 min. The temperature was raised to 39°C and the cheese milk stirred at 39°C until a pH of 6.2 was obtained. The whey was then drained and the curd was matted. During the simulated cheddaring stage, the pieces of curd were not turned over in order to avoid removal of the electrodes from the curd. After reaching a pH of 5.4, the curd was milled and dry-salted with 2.5% NaCl of the theoretical curd yield for 20 min. Then the curd was moulded and pressed at 0.69 bar overnight at room temperature.

2.2.2.2 Gouda simulated manufacture

Pasteurized milk was adjusted to a fat content:protein content ratio of 0.9:1 using skim milk and cooled to 31°C. The milk was then inoculated with 0.013% (w/v) of a concentrated starter culture used for Gouda cheese (DVS B-11, Chr. Hansen, Hørshom, Denmark) containing *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteroides* subsp. *cremoris* and *Lactococcus lactis* subsp. *diacetylactis* and allowed to ripen. After 45 min, $NaNO_3$ (0.06 g/L) and 1M $CaCl_2$ (0.9 mL/L) were added. The milk was renneted using Chymax-180 diluted 1:8 with distilled water, at a level of 0.3 mL/L. After 40-50 min the coagulated milk was cut and after 20min of healing, part of the whey (1/3 of the volume of the milk) was removed and replaced with warm water (76-80°C). After 20 min the curd was washed again using the procedure described above. Thus, the temperature of the cheese milk was increased to 38°C followed by stirring for 90 min. The cheese milk was then transferred into mould and pressed under the whey for 30 min. The whey was then drained and the curd pressed overnight. The cheese was brine-salted (20% NaCl and 0.5% $CaCl_2$) at 10°C for 1 day and allowed to dry.

2.2.2.3 Emmental simulated manufacture

Pasteurized milk was standardized to 3% fat with skim milk and cooled to 32-33°C. The milk was inoculated for 45 min at 0.02% (w/v) with concentrated starter cultures suitable for Emmental cheese (DVS R604, Chr. Hansen and DVS LH-B02, Chr. Hansen) containing *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *Lactis* and *Lactobacillus helveticus*. After 45 min of ripening 1 M $CaCl_2$ (0.9 mL/L) and coagulant (Chymax-180), diluted 1:6 with distilled water, at a level of 0.3 mL/L, were added. When proper firmness was reached, after 35 min, the coagulum was cut and stirred for 20 min. The temperature of the curds-whey mixture was increased to 52-53°C over 40 min and stirred out for 45 min. The whey was then drained, the curd was pressed for 3h and placed in a thermostated oven for 20h at 36°C. The cheese was brine salted (23% NaCl) at 8°C for 4 h and allowed to dry.

2.2.2.4 Camembert simulated manufacture

Pasteurized milk was adjusted to a fat content:protein content ratio of 0.7:1 using skim milk and cooled to 30°C. The milk was than inoculated with 0.02% (w/v) of a starter culture suitable for Camembert cheese (F-DVS FLORA-DANICA, Chr. Hansen) containing *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteroides* subsp. *cremoris* and *Lactococcus lactis* subsp. *diacetylactis* and allowed to ripen for 35 min. The milk was supplemented with 1 M $CaCl_2$ (0.7 mL/L) and coagulant (Chymax-180, Chr. Hansen), diluted 1:5 with distilled water, was added at a level of 0.3 mL/L. After 50 min the coagulated milk was hooped without cutting and left to stand overnight at 25°C. The cheese was brine-salted (20% NaCl, 0.05% $CaCl_2$) for 40 min and allowed to dry at 14°C.

Table 2.1   Features of the simulated manufacture of Cheddar, Gouda, Emmental and Camembert cheeses.

Common steps	Details specific to each variety			
	Cheddar	**Gouda**	**Emmental**	**Camembert**
Standardization	Casein to fat ratio of 0.7:1	Casein to fat ratio of 0.9:1	Standardized to 3% fat	Casein to fat ratio of 0.7:1
Starter	0.02% (w/v) DVS R604 and ripened for 30 min	0.013% (w/v) DVS B-11 and ripened for 45 min	0.02% (w/v) DVS R604 and DVS LH-B02 and ripened for 45 min	0.02% (w/v) F-DVS FLORA-DANICA and ripened for 35 min
Coagulant The coagulum was allowed to reach adequate firmness	Addition of 1 M $CaCl_2$ (0.9 mL/L)	Addition of $NaNO_3$ (0.06 g/L), 1 M $CaCl_2$ (0.9 mL/L)	Addition of 1 M $CaCl_2$ (0.9 mL/L)	Addition of 1 M $CaCl_2$ (0.7 mL/L)
Cutting				Curd moulded without cutting
Cooking	Curds were cooked to 39°C	Curd washed two times by removing and replacing part of the whey (1/3 of the volume of the milk) with hot water (76-80°C) Curds were cooked at 38°C and stirred for 90 min. Curds were pressed under whey for 30 min	Curds were cooked to 52-53°C over 40 min and stirred out for 45 min	No cooking
	Whey drained at pH 6.2 followed by simulated Cheddaring and milling at pH 5.4	Whey drainage. Curds were moulded and pressed overnight	Curds were then moulded and pressed for 3 h and then held at 36°C for 17 h	Curds stood overnight in moulds at 25°C
Salting	Dry-salting (2.5% NaCl) for 20 min followed by moulding and pressing	Brine-salting (20% NaCl and 0.5% $CaCl_2$) at 10°C for 1 day	Brine-salting (23% NaCl) at 8°C for 4 h	Brine-salting (20% NaCl and 0.05% $CaCl_2$) for 40 min and ripened at 14°C

2.2.3 Compositional analysis

The composition (pH, protein, salt, moisture and fat) of 1 day-old cheeses was determined in triplicate. The pH was measured by probing the cheese directly with a combined glass electrode (PHC3001-8, Radiometer Analytical) connected to a pH meter (PHM210 Standard pH Meter, Radiometer Analytical). The protein content of the cheeses was determined by the macro-Kjeldahl method (IDF, 1986), salt by a potentiometric titration (Fox, 1963), moisture by oven drying at 102°C (IDF, 1982) and fat by the Gerber method (IIRS, 1955).

2.3 Results and discussion

Table 2.2 shows pH and composition of the four cheeses made at laboratory scale at one day of ripening. Values were generally within the range typical of each variety (Kocaoglu-Vurma, 2005; Lawrence et al., 2004; Sousa and McSweeney, 2001; Verachia, 2005).

Table 2.2   Composition and pH at one day of ripening of laboratory-scale Cheddar, Gouda, Emmental, and Camembert-type cheeses. Data are means (± standard deviations) of three independent cheesemaking trials.

	pH	Moisture (%)	Fat (%)	Protein (%)	Salt (%)	MNFS[a] (%)
Cheddar	5.12±0.01	37.33±0.76	32.00±0.58	31.89±0.77	1.29±0.03	54.62±0.43
Gouda	5.68±0.03	37.60±0.81	28.00±0.71	30.43±0.71	2.43±0.07	51.99±1.46
Emmental	5.37±0.04	37.98±0.10	30.00±1.61	32.19±0.48	1.92±0.13	54.13±1.10
Camembert	4.78±0.01	45.04±0.45	32.00±0.58	31.89±0.77	1.72±0.03	65.92±1.22

[a]Moisture in non-fat substances.

Changes in E_h (mV), ORP (mV), pH and temperature (°C) during the cheesemaking stages are shown in Fig. 1A-D for Cheddar, Gouda, Emmental and Camembert, respectively.

The milk utilised for all the cheese manufacture had a pH of 6.6±0.1 and a E_h of +349±4.8 mV (ORP = +150.4±5.2 mV) at 28.7±0.5°C. Redox potential value for raw milk was within the range reported by McCarthy and Singh (2009). The positive redox potential of the milk is mainly due to dissolved oxygen (McCarthy and Singh, 2009).

A

1
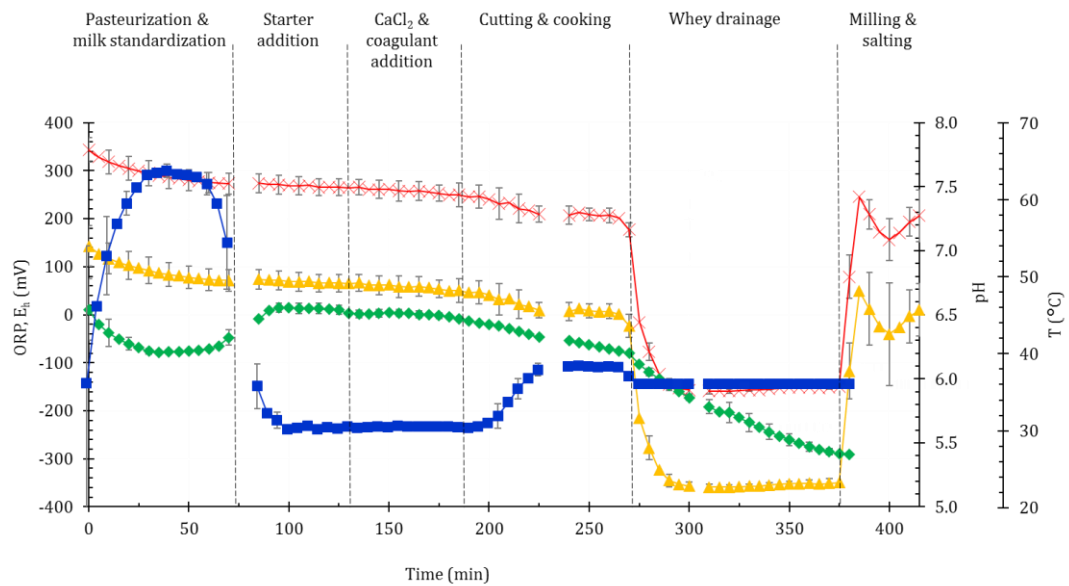
2
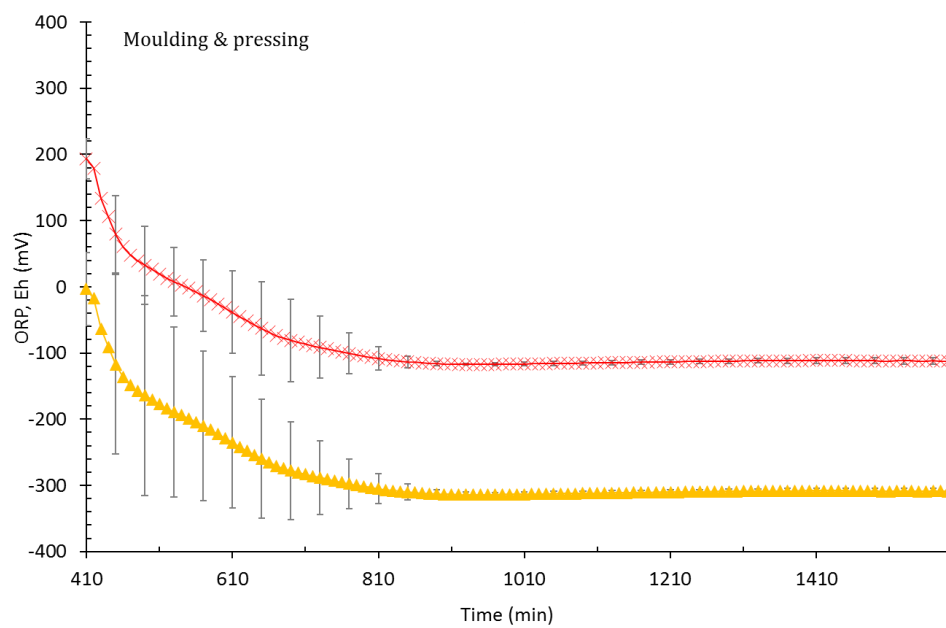
B

1
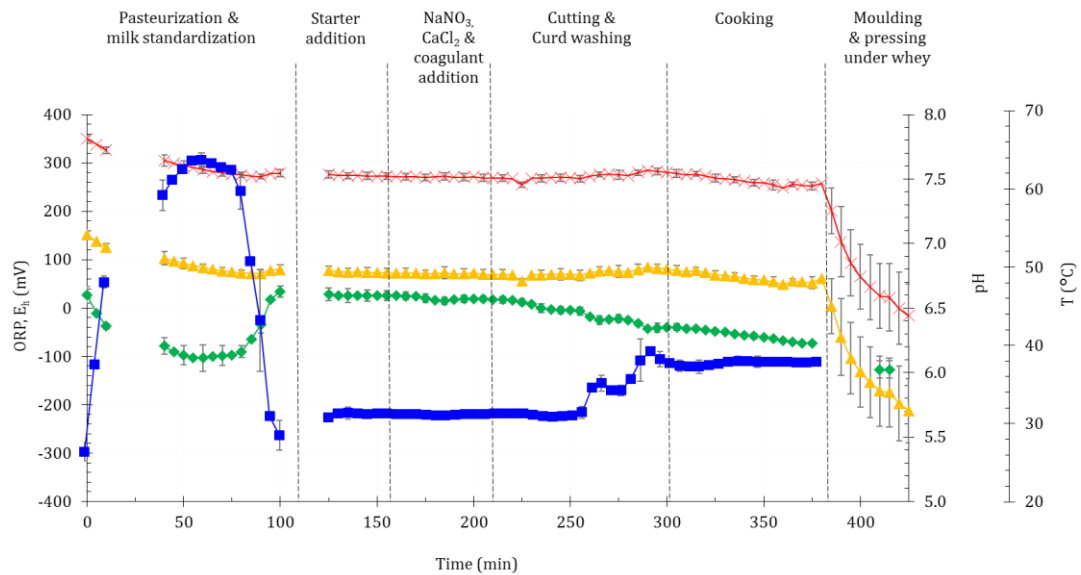
2
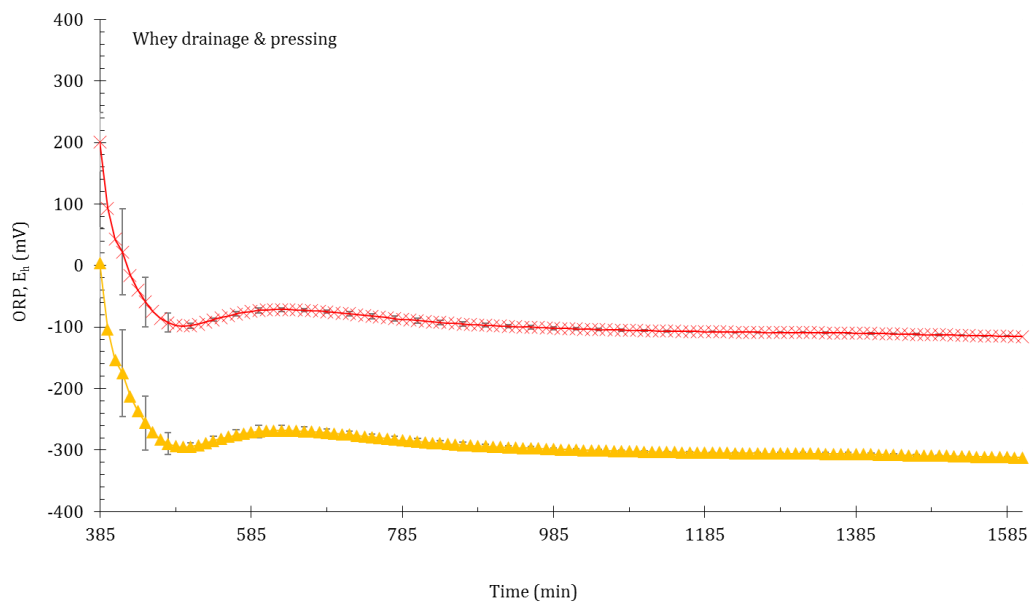
C

1
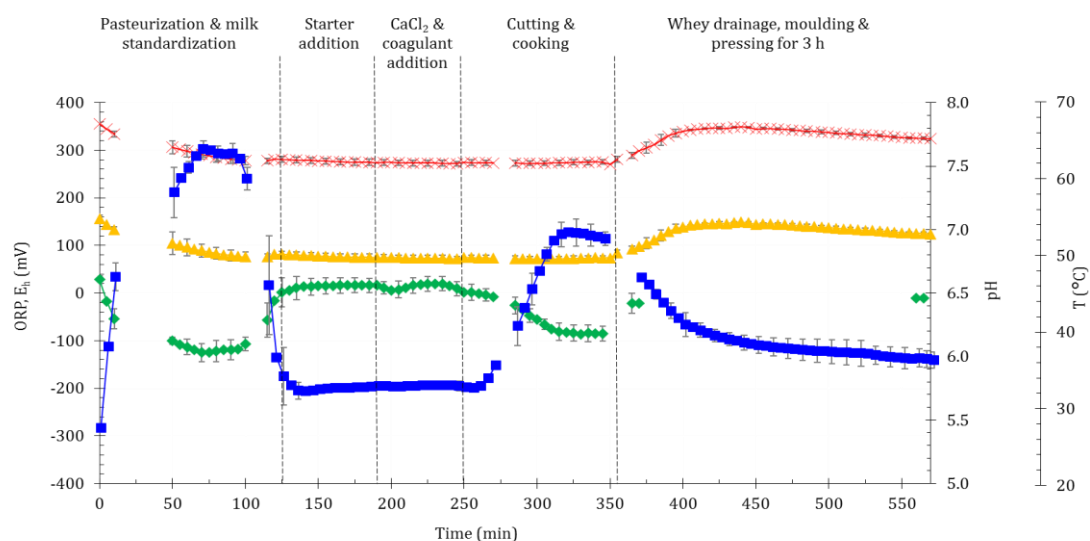
2
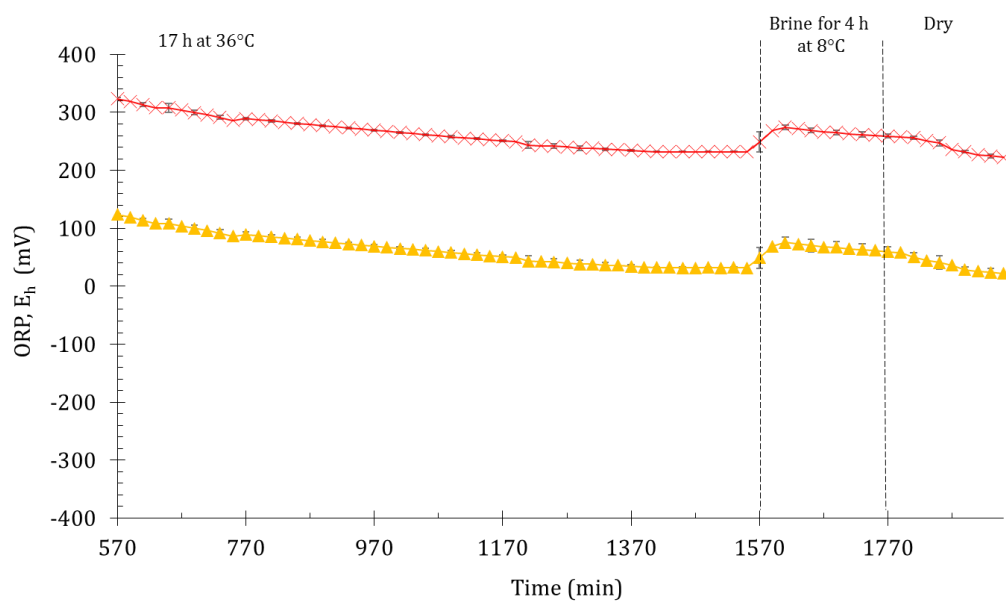
D

1
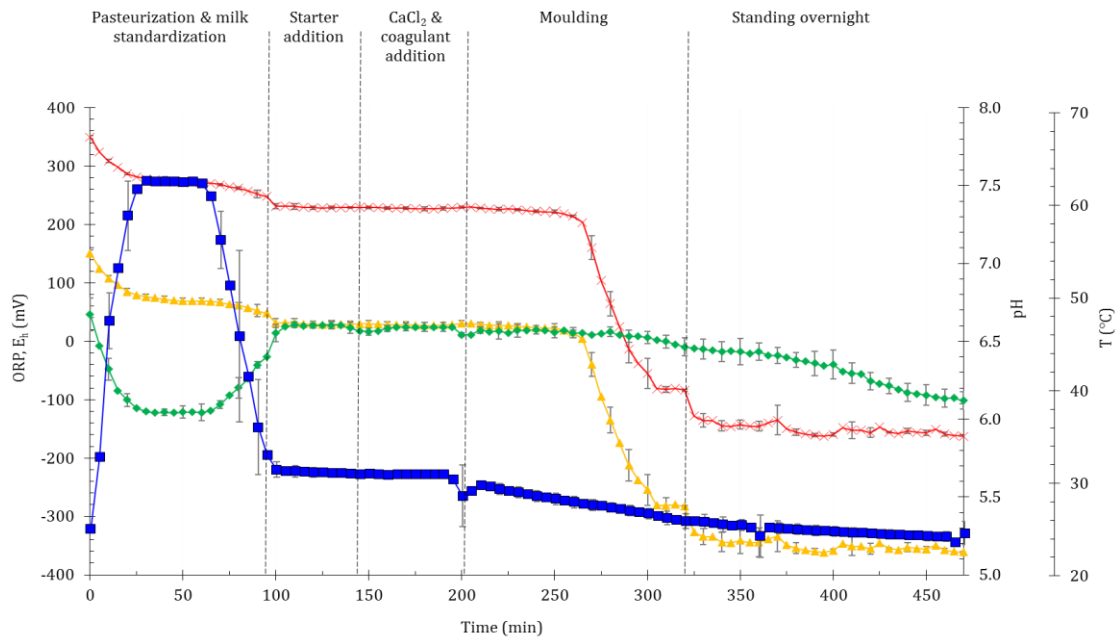
2
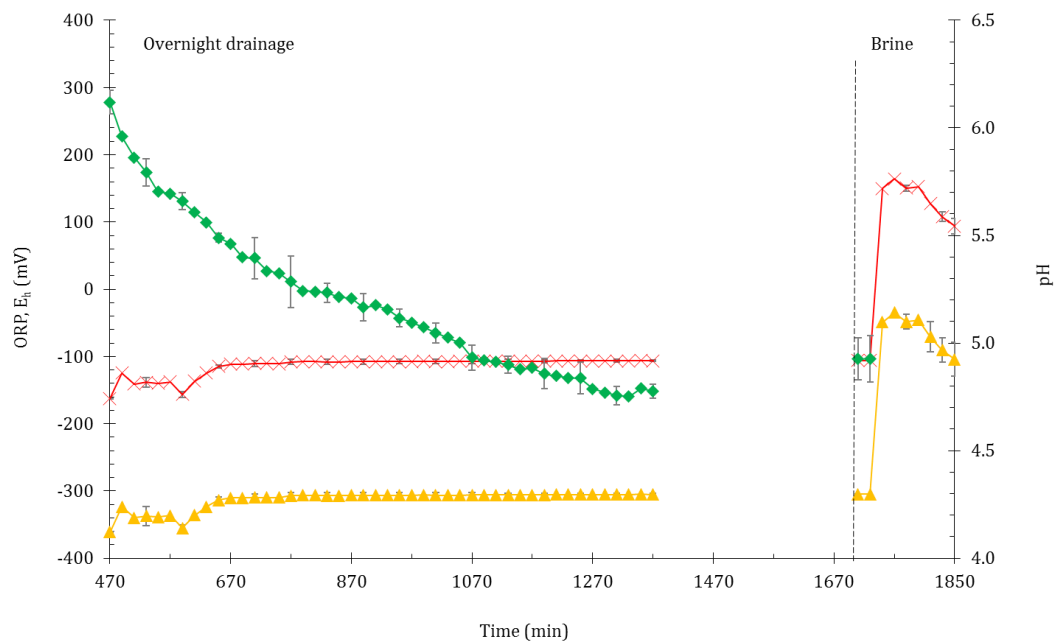
Figure 2.1   Changes oxidation-reduction potential (E_h, ✖ ; potential related to the standard hydrogen electrode and ORP, ▲; direct measurement with platinum electrode-Ag/AgCl reference electrode and without correction for pH), pH (♦) and temperature (T, ■) as a function of time and cheesemaking steps during the manufacture of laboratory-scale Cheddar (A), Gouda (B), Emmental (C) and Camembert (D) cheeses. Data are means (± standard deviations) of three independent cheesemaking trials. For each cheese, graph 1 refers to the cheesemaking and graph 2 to the pressing stage.

Milk was pasteurized (63°C for 30 min) and cooled to 30-31°C. During this stage of manufacture E_h values decreased to +263 mV (ORP +63.5 mV), presumably due to loss of oxygen (Bolduc et al., 2006a; Vahcic et al., 1992). Heat treatment caused a decrease in pH because of the heat-induced transfer of calcium and phosphate to the colloidal state (Jenness and Patton, 1959) with the concomitant production of H^+; these changes are reversible on cooling and when the temperature was decreased to 30-31°C, the pH increased.

In Cheddar cheese manufacture (Figure 2.1A), after addition of starter cultures, the temperature was kept constant at 30-31°C and the redox potential decreased slowly with pH, as LAB metabolized lactose to lactate. Moreover, *L. lactis* reduces, in addition to dissolved oxygen to water, other oxidizing compounds present in milk. In a recent study, oxygen was eliminated by treatment of the milk with various gases and the reduction of the redox potential occurred in the presence of *L. lactis* (Jeanson et al., 2009); the reducing capacity of LAB is due not only to removing molecular oxygen but mainly to the production of reducing molecules (e.g. trioses and cetaldehyde) during their growth (Davis, 1932; Ouvry et al., 2002).

In Cheddar cheese (Figure 2.1A), in the course of the cooking stage at 38-39°C the decrease in redox potential and pH were more marked, probably due to the heat treatment. The E_h reached values close to +200 mV (ORP ≈ 0 mV) at the whey drainage stage (pH 6.2) during which stage a large reduction in E_h to -159 mV (ORP -359 mV) occurred during the early stages of cheddaring and values remained low until the milling stage while the pH decreased rapidly to 5.4 when the curds were subdivided and exposed to the atmosphere. During salting and moulding stages the E_h values were still positive, while during the overnight pressing the values decreased again to -117 mV (ORP -314 mV).

Throughout the manufacturing process a progressive development of acidity occurred since lactic acid bacteria utilized in Cheddar manufacture have the ability to produce lactic acid quickly; a few studies have reported that *L. lactis* is one of the most reducing LAB (Cachon et al., 2002; Brasca et al., 2007). Moreover, the elimination of whey during the drainage and pressing stage allowed the determination of redox potential of the curd since the electrodes were surrounded by the pieces of curd at these stages. Generally similar changes in redox potential were described by Green and Manning (1982) during Cheddar cheese manufacture. Furthermore, the redox values determined at the pressing stage in this study are close to the values reported by Topcu et al. (2008) for the redox potential of mild Cheddar cheese.

In Gouda cheesemaking (Figure 2.1B), from the addition starter culture to the curd washing stage the E_h had constant values around +250 to +270 mV (ORP +50 to +70 mV). The pH started to decrease slightly after the cutting step. During the cooking stage the temperature was increased by removing some whey and replacing with

warm water and the temperature reached 38°C. During the cooking stage redox
potential decreased slightly while the pH reached the value of 6.2 at moulding. Then
the curd was pressed under the whey resulting in a rapid decrease in redox potential.
At whey drainage stage, E_h was around 0 mV (ORP ≈ -200 mV) and pH was 6.02. In a
study by Rukure and Bester (2001) on the affect of *Bacillus cereus* on the growth of
LAB during Gouda manufacture, a similar trend in pH was observed during
manufacture of the control cheese. During the pressing stage E_h continued to decrease
and it reached a value of -118 mV (ORP -315 mV).

In Gouda manufacture in addition to *L. lactis* spp., *Leuconostoc mesenteroides* subsp.
cremoris was utilized as a component of the starter. In a study by van Dijk et al.
(2000), the redox potential during sauerkraut fermentation was investigated; its
fermentation was characterized by an initial heterofermentative process caused
mainly by *Leuconostoc mesenteroides* that lead to acidification and decrease in redox
potential (from E_{h7} +200 to -240 mV).

In Emmental manufacture (Figure 2.1C), after the addition of starter culture, $CaCl_2$
and coagulant, the redox potential values were constant (E_h ranging from +278 to
+272 mV; ORP from +78 to +72 mV) until the cooking step; the pH also remained
largely unchanged. During the cooking stage as the temperature was increased to
52.5°C, the pH decreased while redox potential remained constant. After the cooking
stage, the whey was drained and the temperature was increased determining the
decrease of pH that reached 6.4 due to the increasing of temperature. When the whey
was drained, E_h increased and it even increased during the pressing stage, reaching a
value of +350 mV (ORP +150 mV) and thereafter decreased slightly. At the end of the

pressing stage, the pH was 6.48. In Swiss-type cheeses, the acidification of the curd
matrix occurs largely after drainage and during the pressing of the curd (Gagnaire et
al., 2002). The cheese was kept at 36°C for 17 hours, during this stage the E_h
decreased to +230 mV (ORP +30 mV) at the end of this period. The cheese was then
brine salted causing an increase in E_h to +280 mV (ORP +80 mV). During brine salting,
the redox potential decreased slightly and kept decreasing during the drying stage.
Unlike the other cheeses, the redox potential of Emmental cheese did not reach a
negative value as in this variety acidification is slow and mainly occurs during the
early stages of ripening. In addition, *Lactobacillus helveticus* is utilised as starter in
Emmental manufacture; in the study of Brasca (2007) on the ability of different LAB
to change the redox potential of milk the authors found that *L. helveticus* had the
lowest reducing capacity.

In Camembert manufacture (Figure 2.1D), after the addition of starter culture, $CaCl_2$
and coagulant, the redox potential values and pH ($\approx$ 6.6) remained constant until the
moulding step ($E_h \approx$ +230 mV; ORP $\approx$ +31 mV). After almost one hour from the
transfer of the coagulum in the mould a drop in E_h to -115 mV (ORP -314 mV)
occurred and remained at these values during the overnight drainage of the coagulum
while the pH decreased slightly reaching values of 4.9. A sharp increase in redox
potential happened at the brining stage and it can be considered an artefact due to the
exposure to the atmosphere; thereafter the values decreased again. Abraham et al.
(2007) measured redox potential and pH in Camembert cheese during ripening using
a microelectrode system and a E_h value of -300 mV was found at 15 days of
maturation. This value is lower than the values found in this study during
cheesemaking, perhaps due to further microbial action during the early stages of

ripening and/or the growth of the surface fungal flora.

2.4   Conclusion

Distinctive trends in redox potential during cheesemaking of Cheddar, Gouda, Emmental and Camembert were observed and depended on the cheese technology and on the starter culture utilised. Different studies have reported that the reducing activity of LAB is species dependent (Cachon et al., 2002; Brasca et al., 2007) therefore changes in the redox potential values are related to the starter utilized.

Cheese flavour and its development are affected by different factors including manufacture conditions and microrganisms activity. Redox potential can be considered an important parameter as it can provide more information on the microenvironment in cheese during manufacture and ripening. Redox potential plays an important role on flavour characteristics in dairy products (Green and Manning, 1982). Thus, monitoring redox potential all along cheesemaking and ripening could allow for better control of the development of aroma in cheese.

Further work could include the development of strategies to control the redox potential during cheesemaking and ripening by using oxidizing and reducing agents and/or microrganisms with different abilities to affect redox potential.

Continuous measurement of redox potential could give information regarding the progress and state of the cheese manufacture and therefore improve cheese quality.

2.5   Acknowledgments

2.6   References

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Chapter 3

Redox potential in fermented milk and its control during simulation of Cheddar cheesemaking and during ripening

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Abstract

Oxidation-reduction (redox) potential is a physicochemical parameter that can influence the quality of dairy products. Redox potential of milk and fermented dairy products can be controlled by the utilization of specific strains of lactic acid bacteria or by the addition of redox agents. To understand the influence of lactic acid bacteria on redox potential during Cheddar cheesemaking, single strains of lactococci and a commercially available lactic acid bacterial culture were inoculated into milk during simulation of the temperature profile of Cheddar cheesemaking. Redox potential of each bacterial culture was measured and pH and microbial growth were determined at the beginning and at the end of the experiment. Moreover, three single strains studied were added to cheese-milk to produce Cheddar cheeses. Redox potential was measured during ripening and the volatile compound produced at 4 months of ripening were analysed. To study the influence of redox agents on the redox potential of milk, redox agents were added to milk and redox potential and pH were monitored. Two of the chemicals tested, potassium iodate and sodium hydrosulfite (oxidising and reducing agents, respectively), were added to milk inoculated with a commercially available lactic acid bacterial culture to verify their toxicity towards the cells and their effect on the redox potential of milk. Potassium iodate and sodium hydrosulfite were added during simulated Cheddar cheesemaking to control redox potential during manufacture. The addition of an oxidising agent caused the presence of a positive redox potential during whey drainage and pressing stage and the addition of a reducing agent caused the decrease of redox potential to values lower than those of the control. Microbial growth and pH at the end of the pressing stage was not affected

by the presence of redox agents. From our findings, the decrease in redox potential of milk inoculated with lactic acid bacteria was characteristic of each strain tested. In addition, the redox agents used in milk could reduce or increase the redox potential to different values depending on the quantity added and their redox capacity. Moreover, when redox agents were added during cheesemaking, it was possible to control the redox potential of the curd for Cheddar cheese.

3.1   Introduction

Milk has an oxidation-reduction (redox) potential (E_h) that varies between +250 and +350 mV at 25°C in contact with the atmosphere (Thornton & Hastings, 1929; Swanson & Sommer, 1940; Walstra & Jenness, 1984; Sherbon, 1988; Vahcic et al., 1992; Morris, 2000; McCarthy & Singh, 2009). The E_h of milk can be influenced by the redox components that are present in the milk (Jacob, 1974; Morris, 2000) and their equilibrium status (Sherbon, 1988). Moreover, other factors such as oxygen content (Higginbottom and Taylor, 1960; Bolduc et al., 2006a), pH (Saal and Heukelom, 1947) and temperature (Greenbank, 1940; Calligaris et al., 2004; Schreyer et al., 2008) affect on the redox potential of milk.

Redox potential can be modified by flushing gasses into the milk (Ignatova et al., 2009; Jeanson et al., 2009; Martin et al., 2009, 2010, 2011, 2013; Ebel et al., 2011) or by electro-reduction techniques that consist on apply a certain voltage to the milk to reduce E_h (Swanson & Sommer, 1940; Inoue and Kato, 2003; Bolduc et al., 2006a; Schreyer et al., 2006, 2008; Abraham et al., 2007; Haratifar, 2009, Haratifar et al., 2011) or by the addition of chemical compounds that act as reducing or oxidising agents (George and Peck, 1998; Riondet et al., 2000b; Bolduc et al., 2006b; Kieronczyk et al., 2006; Ignatova et al., 2009). Reducing agents have the capacity to donate electrons to other substances, whereas oxidising agents accept electrons from other substances (Skoog et al., 2004).

One of the first works that reported the modification of the E_h of milk by the addition of metals was Gebhardt and Sommer (1931); the authors noticed that the addition of copper to milk increased its redox potential. Hartman et al. (1943) also studied the

effect of different chemical compounds like copper, iron, ascorbic acid, nickel, vanadium, aluminium, manganese and chromium on the redox potential of milk. In another study, Swanson and Sommer (1940) added redox reagents like copper sulphate solution and ascorbic acid to study the development of oxidised flavour in milk.

It has been demonstrated that E_h can influence intracellular metabolic fluxes and cell balances and regulations (Keen, 1972; Riondet et al., 2000a; Kieronczyk et al., 2006) and some authors designed experiments in which reducing and oxidizing reagents were used to modify the redox potential of a system to study some specific characteristics of a microorganism. For example, Kieronczyk et al. (2006) used potassium ferricyanide or dithiothreitol (DTT) to increase or reduce, respectively, the E_h of a medium containing strains of *Lactococcus lactis* and the effect of E_h on amino acid catabolism was studied. The authors found that in the presence of DTT the production of carboxylic acids, hydroxy acids, in particular phenyl acetate, and sulfur compounds like dimethyltrisulfide was favoured whereas the presence of potassium ferricyanide stimulated the production of aldehydes. Potassium ferricyanide and DTT were also used in a study by George et al. (1998) and Riondet et al. (2000b) to investigate the ability of heat damaged *Escherichia coli* cells to recover and they found that *E. coli* regained the capacity to grow in oxidising conditions. Bagramyan et al. (2000) added potassium ferricyanide to a culture of *Escherichia coli* under anaerobic conditions to delay the decrease in E_h due to their growth and to understand cell functions. The growth of bifidobacteria in milk reduced by the addition of cysteine during refrigeration was studied by Bolduc et al. (2006b) whereas Jayamanne and Adams (2009) used DTT and potassium ferricyanide to study the influence of E_h, pH

and temperature on the survival of bifidobacteria in milk. The authors found that negative redox potential favoured survival.

Moreover, the redox potential of a dairy product can be controlled by the microorganisms that are present in the system. Works have been published on the ability of lactic acid bacteria (LAB) to modify the redox potential during their growth in a medium (Clifton, 1933; Hewitt, 1950; Cachon et al., 2002; Brasca et al., 2007). LAB can adjust the E_h to their ideal value during growth (Boucher et al., 2006) and this ability is characteristic of each species and strain (Cachon et al., 2002; Boucher et al., 2006) and it can be influenced by the environmental conditions of the system (Jacob, 1974; Ouvry et al., 2002). In addition, strains belonging to the same species, when placed in a system under identical conditions, can modify the E_h to different extents that are not related to their acidification profiles (Keen, 1972; Scarinci et al., 1994; Boucher et al., 2006) or growth phase (Messing, 1934; Bagramyan et al., 2000).

In the literature, there are no studies where redox agents were added during Cheddar cheesemaking. Previous studies from our laboratory have been done on the measurement of redox potential during Cheddar cheesemaking (Caldeo and McSweeney, 2012). We reported that during Cheddar cheesemaking E_h dropped to values of -120 mV at the whey drainage stage until the milling stage which caused an increase in E_h and, after salting, the E_h decreased again to a negative value within the first hours of pressing (McSweeney et al., 2010; Caldeo and McSweeney, 2012) and this value is maintained during ripening (McSweeney et al., 2010).

The objectives of this work were to understand how the redox potential of milk and cheese could be influenced by the addition of strains of lactic acid bacteria and by the

addition of redox agents. The aims of the experiments performed in this work were to:

a.   Study the ability of different strains of lactic acid bacteria to modify the redox potential during their growth in milk heated to follow the typical Cheddar cheesemaking temperature profile;

b.   Evaluate the effect of the addition of redox agents to milk and to milk inoculated by a commercial starter mix culture of lactic acid bacteria typically used for Cheddar cheesemaking;

c.   Control redox potential during simulated Cheddar cheesemaking by adding redox agents at the end of the cooking stage of cheese manufacture.

d.   Study the ability of three lactic acid bacteria strains selected for their ability to influence the redox potential during Cheddar cheesemaking temperature profile (Point a, above) to influence E_h and flavour development during ripening of Cheddar cheese.

3.2   Materials and methods

3.2.1   Effect of lactic acid bacterial growth on E_h during Cheddar cheesemaking temperature profile simulation

3.2.1.1 Lactic acid bacteria used

Lactococcus lactis subsp. *lactis* 303 was obtained from MFRC collection (Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland). *Lactococcus lactis* subsp.

cremoris 223 and 229 were obtained from the culture collection of the Department of Microbiology, University College, Cork, Ireland. *Lactococcus lactis* subsp. *cremoris* C and S2 and *Lactococcus lactis* subsp. *lactis* C10 were obtained from the culture collection of the Department of Nutrition and Food Sciences, Utah State University, Logan, UT, USA.

Moreover, a commercial direct vat set (DVS) lactic acid bacteria starter, commonly used in Cheddar cheese manufacture, (R604, Chr. Hansen, Horsholm, Denmark) was also used in our experiments.

Other than the DVS culture, bacteria were prepared from frozen stock (-80°C) by one transfer (1% v/v) in M17 broth (Oxoid, Thermo Fisher Scientific, Basingstoke, Hampshire, UK) supplemented with 10% (w/v) lactose and incubated at 30°C for 16-20 h. Then a second transfer (1% v/v) to sterilized skim-milk (reconstituted skim-milk powder 10% (w/v) autoclaved at 110°C for 10 min) was done and bacteria were incubated at 30°C for 16-20 h corresponding to cell count 10^6-10^7 cfu/mL.

3.2.1.2 Experimental design

Skim milk powder (10% w/v) was hydrated overnight and sterilised at 110°C for 10 min the day of the experiment. Experiments were carried out in a water bath placed under a laminar air-flow hood using sterile utensils.

Each strain was inoculated at 1% in 200 mL of milk at 30°C and pH and redox potential were measured. Temperature was modified to simulate the temperature changes that occur during Cheddar cheesemaking. For 100 min the temperature was

kept at 30°C then it was increased to 38.5°C (1°C every 3 min), held at 38.5°C for 50

min, decreased to 36°C and kept at 36°C for 2 h and 30 min.

pH was measured using a combined electrode (PHC3001-8; Radiometer Analytical,

Villeurbanne Cedex, Lyon, France) connected to a pH meter (PHM210 Standard pH

Meter; Radiometer Analytical) at the beginning and at the end of the experiment.

Redox potential was measured using a platinum working electrode (XM120) and

silver/silver chloride reference electrode (REF 201, both from Radiometer

Analytical). Before each experiment, the reference and the platinum electrodes were

cleaned by the method reported by Topcu et al. (2008) and Abraham et al. (2013).

The accuracy of electrodes was checked against tap water (Abraham at al., 2007,

2013; Jeanson et al., 2009) and against a standard solution (470 mV, HI 7022, Hanna

Instruments, Szeged, Hungary) at 25°C before and at the end of each experiment.

The redox potential probes and the pH probe were sanitized by immersing them in

0.3% H_2O_2 for 15 min and then rinsed with sterile distilled water.

Redox potential electrodes were connected to a data logger (Squirrel Data Logger

2040-2F16 Series; Grant, Data Acquisition, Cambridge, UK) through an amplifier

(PHTX-21, Omega, Stamford, CT, USA) for data acquisition on a computer during

simulated cheesemaking temperature profile. The data were recorded every five

minutes.

Oxidation-reduction potential data were recorded without reference to a hydrogen

reference electrode and converted to E_h (potential related to the standard hydrogen

electrode) according to Caldeo and McSweeney (2012) with temperature compensation.

LAB were enumerated at the beginning and at the end of the experiment by decimal dilutions in sterile quarter-strength Ringer's solution tubes plated in duplicate on LM17 agar (Merck, Darmstadt, Germany) after incubation at 30°C for 3 days. The total colony forming units in milk were calculated by counting colonies on plates with between 30 and 300 colonies.

Experiments were carried out in triplicate.

3.2.2   Effect of the addition of redox agents to the E_h of skim milk and skim milk inoculated by Cheddar cheese starter culture

3.2.2.1 Addition of redox agent to skim milk

The effect of the addition of redox agents on redox potential and pH of milk was evaluated.

Skim milk powder (10% w/v) was hydrated overnight and supplemented by the reducing and oxidising agents. Sodium hydrosulfite ($Na_2S_2O_4$), sodium metabisulfite ($Na_2S_2O_5$), sodium erythrbate ($C_6H_7NaO_6$), dithiothreitol ($C_4H_{10}O_2S_2$), cysteine ($C_3H_7NO_2S$) and L-ascorbic acid ($C_6H_8O_6$) were used as reducing agents. Potassium iodate (KIO_3), ammonium persulfate (($NH_4)_2S_2O_8$), potassium nitrate (KNO_3), potassium dichromate ($K_2Cr_2O_7$) and potassium fericyanide ($K_3[Fe(CN)_6]$) were used as oxidising agents. The redox agents were added at different percentages to skim milk at 25°C and the E_h and pH of milk were measured following the methods described in Section 3.2.1.2.

3.2.2.2 Addition of redox agent to skim milk inoculated with LAB

To evaluate the effect of the addition of redox agents on the survival of lactic acid bacteria commonly used for Cheddar cheesemaking, sterile skim milk was supplemented with oxidising or reducing agents and inoculated with 0.02% (w/v) of Cheddar cheese starter culture (DVS R604, Chr. Hansen, Hørshom, Denmark) for 16 h at 30°C.

The redox agents used were selected from the previously described experiment (Section 3.2.2.1). Potassium iodate (0.005 mol in 1 g of KIO_3; Sigma-Aldrich, St. Louis, MO, USA) was used as oxidising agent and sodium hydrosulfite (0.070 mol in 1 g of $Na_2S_2O_4$; Sigma-Aldrich) was used at reducing agents. Redox agents were added at 0.05, 0.1 and 0.5% (w/v). As control to the treatment with redox agents, skim milk was inoculated with the cheese starter culture for 16 h at 30°C without addition of redox agents.

The E_h, pH and microbial growth were measured at the end of the 16 h of incubation using the methods described in Section 3.2.1.2.

3.2.3   Laboratory Cheddar cheesemaking simulation with the addition of redox agents

Cheddar cheese was made on a laboratory scale with measurement of redox potential during the simulation of the cheesemaking temperature profile. Microbial growth and pH were measured after overnight pressing. Pasteurized and non-homogenized milk (4 L) from the market was used in the experiments.

At the end of the cooking stage, redox agents were added to the cheesemilk. Potassium iodate (KIO_3) at 0.1% (w/v) was used as an oxidizing agent whereas

sodium hydrosulfite ($Na_2S_2O_4$) at 0.05% (w/v) was used as a reducing agent. To the
control cheese, no redox agents were added.

3.2.3.1 Measurement of oxidation-reduction potential during cheemaking simulation

The method used to measure the redox potential during cheesemaking was as
reported by Caldeo and McSweeney (2012).

3.2.3.2 Measurement of microbiological growth and pH in the laboratory Cheddar cheeses

After one day of ripening, 5 g of cheese were homogenised in 45 mL of 2% (w/v) tri-
sodium citrate solution for microbiological analysis and decimal dilutions were
prepared in tubes of sterile quarter-strength Ringer's solution. Starter lactic acid
bacteria (LAB) were enumerated as reported in Section 2.1.2. Non-starter LAB were
enumerated in duplicate under anaerobic conditions, in an anaerobic jar with a Merck
Anaerocult A gas pack (Merck, Darmstadt, Germany), on Rogosa agar (Merck) after
incubation at 30°C for 5 days. pH of the cheeses was measured at one day by probing
the cheese directly with a combined glass electrode (PHC3001-8, Radiometer
Analytical) connected to a pH meter (PHM210 Standard pH Meter, Radiometer
Analytical).

3.2.4   Effect of the utilization of three selected single strains LAB on redox potential during Cheddar cheese ripening

3.2.4.1 Lactic acid bacteria used and cheese manufacture

Lactococcus lactis subsp. *cremoris* C and S2 and *Lactococcus lactis* subsp. *lactis* C10
reported in Section 3.2.1.1 were used in this experiment. These three strains were

also used in Chapter 4 where the ability of strains of LAB to modify redox potential during simulation of the pressing stage of Cheddar cheese was studied in Cheddar cheese extract (CCE), produced by extracting the water-soluble portion of the cheese matrix.

In this study, Cheddar cheeses were made in the food processing facilities at University College Cork, Ireland, according to a standard Cheddar cheese-making protocol. Raw bovine milk was standardized to a casein to fat ratio of 0.7:1 and HTST-pasteurized (73.5 °C, 15 s). Three open vats were filled with 50 L of milk at 31°C and inoculated with *Lactococcus lactis* subsp. *cremoris* C and S2 and *Lactococcus lactis* subsp. *lactis* C10 to cell count corresponding 10^8-10^9 cfu/mL. After a 30 min ripening period, the milk was supplemented with 1 M $CaCl_2$ (0.9 ml/L). Coagulant (Chymax-180, Chr. Hansen), diluted 1:5 with distilled water, was added at a level of 0.3 mL/L. When the coagulum reached the desired firmness after 40-50 min, the curd was cut and allowed to heal for 10 min. The temperature was raised to 39 °C over 30 min and the cheese milk stirred at 39°C until a pH of 6.2 was obtained. The whey was then drained and the curd was cut into large blocks and these were inverted every 15 min until pH reached 5.8 when the blocks were stacked on top of each other. After reaching a pH of 5.4, the curd was milled and 2.5% (w/w) of NaCl was added. After 20 min, the curd was moulded and pressed at 490 kPa overnight at room temperature. Cheese blocks were vacuum packed and ripened at 8 °C for up to 4 months. Cheeses were manufactured in triplicate.

3.2.4.2 Analyses performed on the cheeses during ripening

Oxidation-reduction potential was measured following the method reported by Topcu et al. (2008). A platinum working electrode (R-XM110) and silver/silver chloride reference electrode (REF 201, Radiometer Analytical, Villeurbanne Cedex, Lyon, France) filled with saturated KCl solution (KCl-L-30, Radiometer Analytical) were used. Before each experiment the electrodes were cleaned as indicated by Topcu et al. (2008), Caldeo & McSweeney (2012) and Abraham et al. (2013). The accuracy of electrodes was checked against tap water (Abraham at al., 2007, 2013; Jeanson et al., 2009) and against a standard solution (470 mV, HI 7022, Hanna Instruments, Szeged, Hungary) at 25°C. The cheese block samples of about 10 x 10 cm were wrapped in transparent film to prevent loss of moisture. The Pt-electrode was inserted directly into a cheese block samples to a depth of 5 cm and the reference electrode was placed 2.5 cm apart in a hole of 4 cm deep and 1.5 cm wide filled with 3 M KCl solution as described by Topcu et al. (2008). The electrodes were connected to a data logger (Squirrel Data Logger 2040-2F16 Series, Grant, Data Acquisition, Cambridge, UK) through an amplifier (PHTX-21, Omega, USA) for data acquisition. The measured data were recorded every five minutes. The redox potential data recorded (without reference to a hydrogen reference electrode) were converted to E_h according to Caldeo & McSweeney (2012) and Abraham et al. (2013) with temperature compensation. For each cheese, single measurement of redox potential was taken at 1, 14, 30, 60 and 120 days of ripening.

Starter and non-starter lactic acid bacteria (LAB) counts were performed on 14, 30, 60, 120-days-old cheeses. Cheese (5 g) was homogenised in 45 mL of 2% (w/v) tri-sodium citrate solution for microbiological analysis and decimal dilutions were

prepared in tubes of sterile quarter-strength Ringer's solution tubes. Starter LAB were enumerated in duplicate on LM17 agar (Merck, Darmstadt, Germany) after incubation at 30°C for 3 days. Non-starter LAB were enumerated in duplicate under anaerobic conditions (Merck Anaerocult A gas pack; Merck), on Rogosa agar (Merck) after incubation at 30°C for 5 days. The total colony forming units in cheese were calculated by counting colonies on plates with between 30 and 300 colonies.

The composition (pH, protein, salt, moisture and fat) of 14-day-old cheeses was determined in triplicate. The protein content of the cheeses was determined by the macro-Kjeldahl method (IDF, 1986), salt by a potentiometric titration (Fox, 1963), moisture by oven drying at 103 ± 1°C (IDF, 1982) and fat by the Gerber method (IIRS, 1955).

The pH was measured in triplicate at 1, 14, 30, 60 and 120 days of ripening. pH of the cheeses were measured by probing the cheese directly with a combined glass electrode (PHC3001-8, Radiometer Analytical) connected to a pH meter (PHM210 Standard pH Meter, Radiometer Analytical).

Cheese samples at 4 months of ripening were wrapped in aluminium foil, vacuum packed and stored at -20°C until analysed. Volatile compounds were analysed by solid phase microextraction coupled to gas chromatography-mass spectrometry (SPME GC-MS) at Teagasc Food Research Centre as described by Hou et al. (2014).

3.2.5   Statistical analysis

Analysis of variance (one-way ANOVA) was conducted using SPSS Version 20.0 for Mac OS X (SPSS Inc., Chicago, IL, USA). When differences were significant ($P < 0.05$), the means were analysed using Tukey's test.

The data for the volatile compounds identified at 4 months of ripening for the cheeses made with the addition of *Lactococcus lactis* subsp. *cremoris* C and S2 and *Lactococcus lactis* subsp. *lactis* C10 were analysed by principal component analysis (PCA) by Unscrambler V 6.1 (CAMO AS, N-70421 Trondheim, Norway).

3.3   Results and discussion

3.3.1   Effect of lactic acid bacteria growth on E_h during temperature profile simulation of Cheddar cheesemaking

Two single strains *L. lactis* subsp. *lactis* (303 and C10), four single strains of *L. lactis* subsp. *cremoris* (223, 229, S2 and C) and a commercially available starter used in Cheddar cheese manufacture (DVS R604) were inoculated in 10% reconstituted skim milk powder and redox potential was measured during a simulation of Cheddar cheesemaking temperature profile.

The trends in E_h of the cultures studied in this experiment are reported in Figure 3.1 together with the temperature profile used to mimic the temperature during Cheddar cheesemaking. Microbial growth and pH measured at the beginning and at the end of the experiments are reported in Table 3.1.
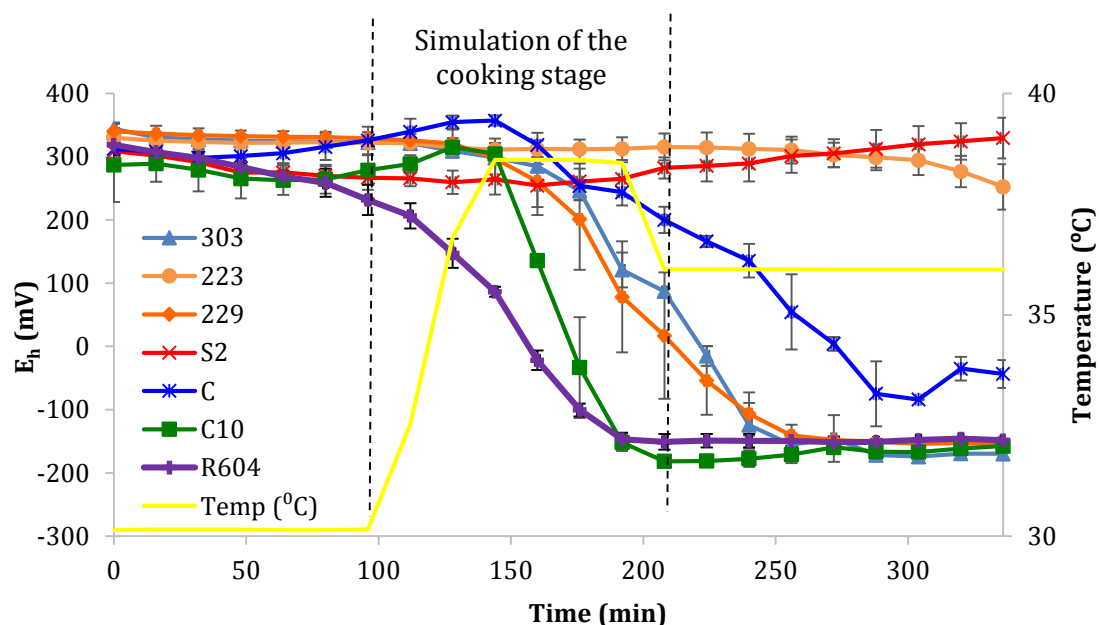
Figure 3.1 Trend in redox potential (E_h, mV) of skim milk inoculated with different lactic acid bacteria during simulation of the cheesemaking temperature profile. The yellow solid line corresponds to the temperature. The bacteria analysed were *Lactococcus lactis* subsp. *lactis* 303 (303, ▲), *Lactococcus lactis* subsp. *cremoris* 223 (223, ●), *Lactococcus lactis* subsp. *cremoris* 229 (229, ♦), *Lactococcus lactis* subsp. *cremoris* S2 (S2, x); *Lactococcus lactis* subsp. *cremoris* C (C, ∗), *Lactococcus lactis* subsp. *lactis* C10 (C10, ■) and a commercial lactic acid bacteria starter DVS R604 (R604, │). Values are mean of three independent trials with standard deviation indicated by the vertical bars.

Table 3.1   Microbial growth and pH measurements recorded at the beginning (T_0) and at the end (T_f) of the experiments on the effect of lactic acid bacteria growth on redox potential during cheesemaking temperature profile.

	pH at T_0	pH at T_f	Microbial growth (cfu/ml) at T_0	Microbial growth (cfu/ml) at T_f
L. lactis ssp. *lactis* 303	6.26[a] (0.02)	5.60[d,e] (0.04)	2.57×10^7 [a,b] (1.17)	8.35×10^8 [c,d] (1.08)
L. lactis ssp. *cremoris* 223	6.26[a] (0.02)	5.35[c,d] (0.10)	7.59×10^6 [a] (1.15)	1.19×10^8 [a] (1.14)
L. lactis ssp. *cremoris* 229	6.22[a] (0.02)	5.13[c] (0.07)	1.01×10^7 [a,b] (1.68)	5.41×10^8 [b,c,d] (1.51)
L. lactis ssp. *cremoris* S2	6.26[a] (0.01)	5.71[e] (0.10)	1.41×10^7 [a,b] (2.60)	1.61×10^8 [a,b] (1.12)
L. lactis ssp. *cremoris* C	6.26[a] (0.03)	5.06[b,c] (0.24)	2.85×10^7 [a,b] (4.85)	2.57×10^8 [a,b,c] (1.61)
L. lactis ssp. *lactis* C10	6.25[a] (0.02)	4.72[a] (0.05)	3.53×10^7 [a,b] (1.40)	1.81×10^9 [d,e] (1.13)
DVS R604	6.27[a] (0.01)	4.78[a,b] (0.09)	7.30×10^7 [b] (1.11)	2.93×10^9 [e] (2.61)

Numbers represent mean and standard deviation, with the latter in parentheses. Within a column, different superscript lowercase letters denote significant differences between lactic acid bacteria tested in the experiment (Tukey's, $P < 0.05$).

The initial redox potential (E_h) of skim milk was +320 (±12) mV at 30°C and its pH was 6.26 (±0.02). Bacteria after inoculation were counted and all bacteria reached $\sim$ 10^7 cfu/mL milk.

During the 100 min at 30°C, the E_h of the bacterial cultures studied did not differ; main changes occurred during the simulation of the cooking stage of Cheddar cheesemaking that consisted of an increase temperature to 38.5°C, holding it at 38.5°C for 50 min and then decreasing it to 36°C.

R604 was the first to show a rapid decrease in E_h to values of about -150 mV during

the simulated cooking stage and E_h remained at that value to the end of the

experiment (Figure 3.1). *L. lactis* subsp. *lactis* C10 reached an equilibrium E_h value

slightly lower than that of R604 (about -170 mV) at the same time (200 min). No

significant differences (P < 0.05) were found between the final pH values of R604 and

L. lactis subsp. *lactis* C10 (pH values of 4.78 and 4.72, respectively) and between their

final growth values (2.93 x 10^9 and 1.81 x 10^9 cfu/mL milk, respectively) (Table 3.1).

L. lactis subsp. *lactis* 303 and *L. lactis* subsp. *cremoris* 229 gave the same trend in

reduction of E_h during the experiments (Figure 3.1). Their E_h started to decrease

during the simulated cooking stage and reached equilibrium values at the end of this

stage (250 min). The equilibrium values of *L. lactis* subsp. *lactis* 303 and *L. lactis*

subsp. *cremoris* 229 were close to those obtained by R604 and *L. lactis* subsp. *lactis*

C10; values of about -170 and -150 mV were reached, respectively. *L. lactis* subsp.

lactis 303 and *L. lactis* subsp. *cremoris* 229 reached similar numbers at the end of the

experiment (8.35 x 10^8 and 5.41 x 10^8 cfu/mL milk, respectively); however, their final

pH values were significantly different (P < 0.05) (5.60 and 5.13, respectively) (Table

3.1). Their pH values also differed from the final pH values of R604 and *L. lactis* subsp.

lactis C10.

The trend in E_h of *L. lactis* subsp. *cremoris* C differed from R604, *L. lactis* subsp.

cremoris 229, *L. lactis* subsp. *lactis* C10 and 303 (Figure 3.1). The E_h started to

decrease at the same time as *L. lactis* subsp. *cremoris* 229, and *L. lactis* subsp. *lactis*

C10 and 303 but with a less pronounced slope and the final E_h value reached at the

end of the experiments was higher (-40 mV) compared to the other strains. However,

L. lactis subsp. *cremoris* C acidified milk to a pH value of 5.06, not significantly different (P < 0.05) to the final pH of R604 and *L. lactis* subsp. *cremoris* 229; and it grew to 2.57 x 10^8 cfu/mL milk that was significantly different (P < 0.05) to the values obtained for R604 and *L. lactis* subsp. *lactis* C10 (Table 3.1).

L. lactis subsp. *cremoris* 223 and S2 showed a positive and constant E_h (around +300 mV) at all times during the Cheddar cheesemaking simulation experiments (Figure 3.1). *L. lactis* subsp. *cremoris* 223 acidified the pH of the milk to 5.35 at the end of the experiments and this value was not significantly different (P < 0.05) to the final pH of cultures of *L. lactis* subsp. *lactis* 303 and *L. lactis* subsp. *cremoris* 229 and C; however, the final pH of *L. lactis* subsp. *cremoris* S2 was significantly higher (P < 0.05) compared to the final pH (5.71) of the other strains studied in these experiments (Table 3.1). Both strains *L. lactis* subsp. *cremoris* 223 and S2 grew slightly more than one log cycle at the end of the experiments. *L. lactis* subsp. *cremoris* S2 had value of 1.61 x 10^8 cfu/mL milk that was not significantly different from *L. lactis* subsp. *cremoris* C and 229 (P < 0.05). *L. lactis* subsp. *cremoris* 223 grew to a significantly lower count (P < 0.05) compared to the other bacteria; a value of 1.19 x 10^8 cfu/mL milk was measured.

From our findings, differences in E_h were evident within the same subspecies studied and between the single strains of *L. lactis*. However, all the strains were able to acidify milk to pH values from 5.71 to 4.72 and to grow to 10^8 - 10^9 cfu/mL milk at the end of the experiments.

Differences in redox potential of cultures of closely related bacterial species were found in previous studies (Gillespie and Rettger, 1938; Hewitt, 1950; Brasca et al.,

2007) and some studies have shown that there is no correlation between the E_h and the bacterial growth phase (Messing, 1934; Bagramyan et al., 2000) or between the E_h and the acidification capacity (Reichart and Mohàcsi-Farkas, 1994; Bresca et al., 2007; Tachon, 2009).

Generally, LAB reduce the E_h of a media to negative values (Davis, 1932; Keen, 1972; Adda et al., 1982; Cachon et al., 2002; Abraham at al., 2007; Jeanson et al., 2009) and, as we found in our experiments, the pathway taken to reach an equilibrium negative value and the final E_h value can be characteristic of the specific bacteria (Scarinci et al., 1994; Carrasco et al., 2005; Boucher et al., 2006; Reichart et al., 2007). In fact, four of the bacteria used in our study (R604, *L. lactis* subsp. *cremoris* 229 and *L. lactis* subsp. *lactis* 303 and C10) reduced the E_h to a similar value (around -160 mV) and the time needed to reach that value was different. Moreover, as we found in trend in the E_h values of *L. lactis* subsp. *cremoris* C, some strains can reduced the E_h to higher values compared to other stains (Carrasco et al., 2005).

When we tested a commercially available starter culture we found that its E_h was the first to decrease compared to the other bacteria studied, probably because it is a combination of different single strains of *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* (Carrasco et al., 2005).

One of the two stains that did not reduce E_h during Cheddar cheesemaking temperature profile in our experiment, *L. lactis* subsp. *cremoris* S2, was also used in a study by Boucher et al. (2006). The authors measured the redox potential of the strain over 32 h at 21°C in sterile broth together with pH and microbial growth and their results showed a positive E_h during bacterial growth and acidification.

Three of the strains used in this experiment (*L. lactis* subsp. *lactis* C10, *L. lactis* subsp. *cremoris* S2 and C) were studied in Chapter 4. In that chapter, the strains were added to a Cheddar cheese extract (CCE) to study their ability to modify redox potential during simulation of the pressing stage of Cheddar cheese and trend in redox potential were the same as the one reported in this Chapter.

3.3.2   Effect of the addition of redox agents to the E_h of skim milk and skim milk inoculated by Cheddar cheese starter culture

In this part of our study, we tested firstly the effect of the addition of different reducing agents or oxidising agents on the E_h and pH of skim milk and secondly the effect of two selected redox agents on pH and E_h of milk inoculated with lactic acid bacteria.

In the first experiment, the skim milk used had a redox potential of +360 mV and a pH of 6.63. Among the reducing agents tested, sodium hydrosulfite ($Na_2S_2O_4$) was the most reducing (Table 3.2). E_h decreased to -376 mV by adding 0.05% $Na_2S_2O_4$ and without affecting the pH of the milk. Other reagents like dithiothreitol ($C_4H_{10}O_2S_2$) at 0.05% and cysteine ($C_3H_7NO_2S$) at 0.1% reduced the E_h to negative values, -11 mV and -7 mV, respectively, but milk pH was also slightly influenced and values of 6.72 and 6.53, respectively, were recorded (Table 3.2).

On the other hand, sodium metabisulfite ($Na_2S_2O_5$), L-ascorbic acid ($C_6H_8O_6$) and sodium erythrbate ($C_6H_7NaO_6$) did not reduce the E_h to negative values (Table 3.2) even when added at higher concentrations and milk pH decreased when $Na_2S_2O_5$ or $C_6H_8O_6$ were added and pH increased when $C_6H_7NaO_6$ was added.

Table 3.2   Redox potential and pH values at 25°C of reconstituted skim milk supplemented with oxidising and reducing agents added at different percentages.

	% (w/v)	pH	E_h (mV)
Skim milk		6.63	360
Reducing agents			
Sodium hydrosulfite ($Na_2S_2O_4$)	0.05	6.64	-376
	0.1	6.62	-405
Sodium metabisulfite ($Na_2S_2O_5$)	0.05	6.56	271
	0.1	6.49	250
	0.5	6.2	199
	1	6.05	180
	2	5.88	167
	3	5.77	158
	5	5.62	151
Sodium erythrbate ($C_6H_7NaO_6$)	0.05	6.67	212
	0.1	6.68	184
	0.5	6.71	137
	1	6.74	115
	2	6.78	94
	3	6.81	79
	5	6.86	63
Dithiothreitol ($C_4H_{10}O_2S_2$)	0.05	6.72	-11
	0.1	6.7	-37
Cysteine ($C_3H_7NO_2S$)	0.05	6.6	121
	0.1	6.53	-7
	1	6.48	-51
	2	6.4	-78
	5	6.3	-89
	8	6.2	-113
	10	6.13	-128

L-ascorbic acid ($C_6H_8O_6$)	0.05	6.39	210
	0.1	5.47	186
	1	5	187
	2	4.14	205

Oxidizing agents

Potassium iodate (KIO_3)	0.05	6.64	376
	0.1	6.68	428
	0.5	6.70	439
	1	6.79	447
	2	7.22	413
	5	7.42	402
Ammonium persulfate (($NH_4)_2S_2O_8$)	0.05	6.57	422
	0.1	6.40	460
	0.5	6.1	523
Potassium nitrate (KNO_3)	0.05	6.67	390
	0.1	6.67	394
	0.5	6.71	401
	1	6.74	404
	2	6.77	408
	3	6.79	407
	5	6.82	410
Potassium dichromate ($K_2Cr_2O_7$)	0.05	6.31	404
	0.1	6.28	406
	0.5	6.13	427
	1	6.06	448
	2	5.97	486
Potassium fericyanide ($K_3[Fe(CN)_6]$)	0.05	6.69	408
	0.1	6.69	420
	0.5	6.76	463
	1	6.8	484
	2	6.86	505

Among the oxidising agents tested, potassium iodate (KIO_3) and potassium dichromate ($K_2Cr_2O_7$) at 0.1% increased E_h to about +420 mV; however, KIO_3 influenced less the pH of the milk compared to $K_2Cr_2O_7$ (Table 3.2). Potassium nitrate (KNO_3) had a slight effect on E_h whereas ammonium persulfate (($NH_4)_2S_2O_8$) at 0.1% increased the E_h to +460 mV but caused a decrease in pH to 6.4. Potassium fericyanide ($K_3[Fe(CN)_6]$) slightly increased E_h and dropped the pH of milk to 6.28 when added at 0.1% (Table 3.2).

The redox agents tested could reduce or increase the E_h to different values depending on the quantity added and their ability to influence the redox couples that are present in the milk (Sherbon, 1988).

From the results obtained on the addition of redox agents to skim milk, two of the chemicals tested were selected to evaluate the effect of adding redox agents to milk inoculated with lactic acid bacteria (LAB). A commercial direct-vat-set Cheddar cheese starter culture (DVS, R604) was inoculated at 0.02% (w/v) in skim milk in the presence of a reducing agent, $Na_2S_2O_4$, or an oxidising agent, KIO_3. E_h, pH and survival of bacteria were measured after 16 h at 30°C (Table 3.3). The two reagents were added at 0.05, 0.1 or 0.5% (w/v).

Table 3.3　　　Redox potential, pH and microbial growth of reconstituted skim milk supplemented by redox agents at different percentages and inoculated with 0.02% (w/v) of a commercial direct-vat-set Cheddar cheese starter culture (DVS R604) for 16 h at 30°C.

	Redox agent % (w/v)	Milk pH	Milk E_h (mV)	Microbial growth (cfu/ml)
Control		4.62 (±0.02)	-116 (±6)	8.00×10^8 (1.27)
Sodium hydrosulfite ($Na_2S_2O_4$)	0.05	5.75 (±0.03)	-172 (±3)	1.17×10^7 (1.61)
	0.1	6.07 (±0.02)	-265 (±6)	9.33×10^5 (1.15)
	0.5	5.94 (±0.02)	-359 (±19)	7.94×10^5 (1.11)
Potassium iodate (KIO_3)	0.05	5.16 (±0.02)	+314 (±6)	1.58×10^8 (1.84)
	0.1	5.72 (±0.02)	+321 (±13)	1.49×10^7 (2.37)
	0.5	6.63 (±0.03)	+361 (±6)	8.07×10^5 (1.29)

As control, skim milk with the sole addition of the starter culture was used and at the end of the 16 h at 30^{0}C the E_h reached -116 mV, the milk was acidified to a pH of 4.62 and the bacteria were present at 8.00×10^8 cfu/mL milk (Table 3.3). When $Na_2S_2O_4$ was added at 0.05, 0.1 and 0.5% (w/v) to milk inoculated with LAB, E_h decreased to -172, -265 and -359 mV, respectively, after 16 h of incubation at 30°C. Whereas the addition of KIO_3 at 0.05, 0.1 and 0.5% (w/v) to milk inoculated with LAB, E_h increased to 314, 321 and 361 mV, respectively.

The addition of a reducing or oxidising agent could successfully decrease or increase the E_h of the control milk. However, the addition of KIO_3 or $Na_2S_2O_4$ influenced also the pH and the survival of the microorganisms. The pH reached values between 5.75 and 6.07 when $Na_2S_2O_4$ was added and when KIO_3 was used values between 5.16 and

6.63 were measured. The number of LAB that survived at the end of the experiment was 1-3 log lower compared to the control milk (Table 3.3).

3.3.3   Control of oxidation-reduction potential during cheesemaking simulation

Oxidation-reduction potential during cheesemaking was previously measured in our laboratory (Caldeo and McSweeney, 2012) and in this study, the same method was used to follow the change in redox potential, pH and temperature during Cheddar manufacture. Figure 3.2 shows the trend in E_h during cheesemaking of control cheese and cheeses made with addition of reducing or oxidizing agents. Our control Cheddar cheese showed the same trend that was previously described (Caldeo and McSweeney, 2012).

Since the main changes in redox potential during cheesemaking occur during the whey drainage and pressing steps, in this study reducing or oxidising agents were added to the experimental cheese curds at the end of the cooking stage.

The quantity of redox agents used was decided based on results discussed in Section 3.2. KIO_3 and $Na_2S_2O_4$ were used at 0.1 and 0.05%, respectively, since when added at these levels in inoculated milk they gave similar pH (5.74) and microbial growth values (values around 7×10^7 cfu/mL) (Table 3.3).
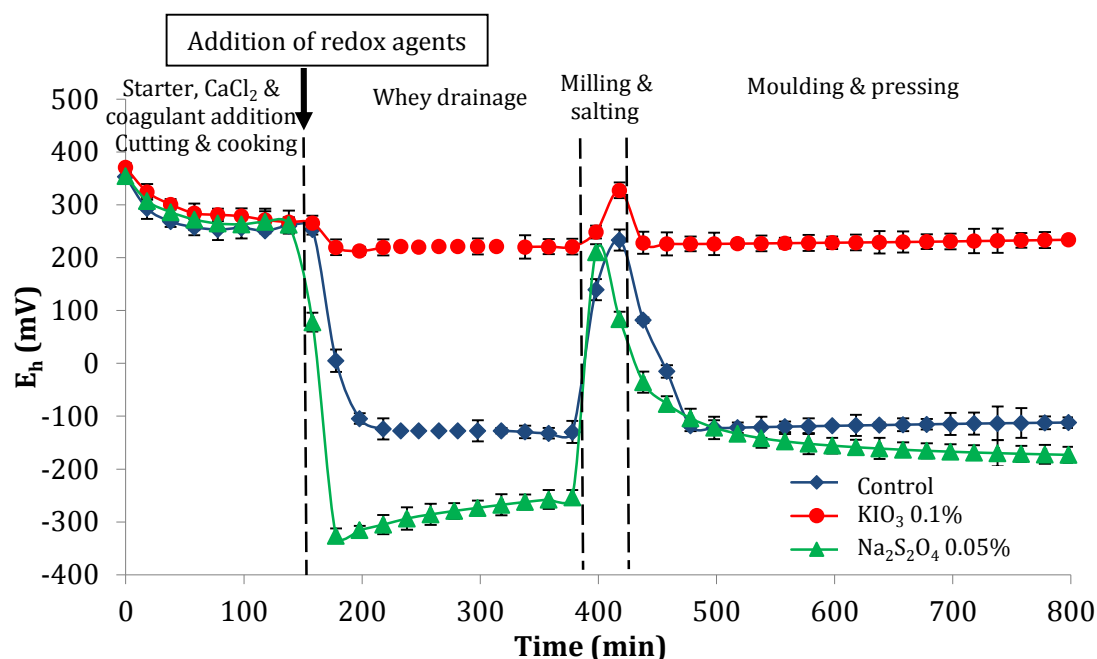
Figure 3.2   Redox potential (E_h, mV) during simulation of the cheesemaking of control cheese (♦) and cheeses made with the addition of 0.1% (w/w) KIO_3 (●) and 0.05% (w/w) $Na_2S_2O_4$ (▲). Values are mean of three independent trials with standard deviation indicated by the vertical bars.

The addition of KIO_3 maintained the E_h at a positive value of +200 mV during the whey drainage stage; at salting the redox potential slightly increased and during the pressing stage it was positive (+200 mV). The addition of $Na_2S_2O_4$ as reducing agent caused the drop of redox potential during the whey drainage to the values lower than the control cheese (-300 mV). During salting stage, the redox potential increased and it dropped again at the pressing stage at values of -200 mV.

The redox electrodes measured the redox potential during overnight pressing. The E_h of control cheese, cheese made with the addition of KIO_3 and cheese made with the addition of $Na_2S_2O_4$ were -111.89 mV, +233.89 mV and -173.17 mV, respectively. The

E_h value of the control cheese was comparable to the redox potential of mature Cheddar cheese (McSweeney et al., 2010; Topcu et al., 2008) and the addition of reducing or oxidizing agents was effective in maintaining the redox potential at values lower or higher than the E_h of the control cheese.

At one day of ripening, the pH and the LAB growth of the cheeses were measured. Control cheese, cheese made with KIO_3 and cheese made with $Na_2S_2O_4$ had a pH of 5.10 (±0.01), 5.07 (±0.03) and 5.05 (±0.05), respectively, and LAB counts corresponded to 1.03 x 10^{10} (±1.11), 4.07 x 10^9 (±1.06) and 4.68 x 10^9 (±1.05) cfu/g, respectively.

The addition of redox agents did not influence the pH of the cheeses and decrease one log the LAB counts.

To our knowledge, this is the first study where redox potential was followed during Cheddar manufacture in the presence of redox agents.

3.3.4   Effect of the utilization of three selected single strains LAB on redox potential during Cheddar cheese ripening

Three of the strains used in the study reported in Section 3.3.1 (*L. lactis* subsp. *lactis* C10, *L. lactis* subsp. *cremoris* S2 and C) were selected on the basis of their ability to drive E_h during temperature simulation of Cheddar cheesemaking.

The three strains showed different pattern during the experiment reported in Section 3.3.1: *L. lactis* subsp. *lactis* C10 was the first among the strains studied in Section 3.3.1 to reduce E_h to negative values during the simulation of the cooking stage, *L. lactis* subsp. *cremoris* C slowly decreased E_h during the experiment and the negative value

reached at the end of the measurement was higher compared to *L. lactis* subsp. *lactis* C10 and *L. lactis* subsp. *lactis* S2 kept a positive E_h during the experiment (Figure 3.1). In Chapter 4, *L. lactis* subsp. *lactis* C10, *L. lactis* subsp. *cremoris* S2 and C were added to CCE to study their ability to modify redox potential during simulation of the pressing stage of Cheddar cheese. Moreover, the volatile compounds produced at the end of the experiment in CCE were measured.

To evaluate the ability of the strains to control redox potential in Cheddar cheese during ripening, *L. lactis* subsp. *lactis* C10*, L. lactis* subsp. *cremoris* S2 and C were added to the cheesemilk to a cell count corresponding 10^8-10^9 cfu/mL and cheeses were ripened for 4 months.

Composition and pH were measured at 14 days of ripening and results are reported in Table 3.4. Values of moisture, salt, fat and protein contents of the cheeses were within the range of those typical of Cheddar cheese (Lawrence et al., 2004). One-way ANOVA on the compositional data did not find significant differences ($P < 0.05$) among cheeses within the same trial (Table 3.4). However, significant differences ($P < 0.05$) were found between the same chesses made in different trials as indicated in Table 3.4.

Table 3.4   Composition and pH of Cheddar cheeses made with the addition of *Lactococcus lactis* subsp. *cremoris* C, *Lactococcus lactis* subsp. *cremoris* S2, *Lactococcus lactis* subsp. *lactis* C10 at 14 days of ripening.

	Salt %		Moisture %		Protein %		Fat %		pH	
Trial 1										
L. lactis subsp. *cremoris* C	1.45 [Aa]	(0.01)	36.81 [Ba]	(0.08)	25.50 [Ba]	(0.09)	34.00 [Aa]	(0.00)	5.51 [Aa]	(0.01)
L. lactis subsp. *cremoris* S2	1.45 [Aa]	(0.03)	36.84 [Aa]	(0.15)	24.83 [Aa]	(0.07)	35.00 [Ba]	(0.00)	5.56 [Aa]	(0.02)
L. lactis subsp. *lactis* C10	1.44 [Aa]	(0.04)	37.12 [Aa]	(0.19)	25.19 [Aa]	(0.47)	34.00 [Aa]	(0.00)	5.54 [Aa]	(0.00)
Trial 2										
L. lactis subsp. *cremoris* C	1.50 [ABa]	(0.03)	36.54 [ABa]	(0.21)	24.84 [Aa]	(0.03)	34.00 [Aa]	(0.00)	5.49 [Aa]	(0.01)
L. lactis subsp. *cremoris* S2	1.46 [Aa]	(0.09)	36.94 [Aa]	(0.37)	25.37 [ABa]	(0.26)	34.33 [ABa]	(0.58)	5.74 [Aa]	(0.02)
L. lactis subsp. *lactis* C10	1.50 [ABa]	(0.02)	36.37 [Aa]	(0.47)	25.26 [Aa]	(0.35)	34.33 [Aa]	(0.58)	5.69 [Aa]	(0.00)
Trial 3										
L. lactis subsp. *cremoris* C	1.54 [Ba]	(0.00)	36.26 [Aa]	(0.18)	25.69 [Ba]	(0.32)	34.00 [Aa]	(0.00)	5.57 [Aa]	(0.01)
L. lactis subsp. *cremoris* S2	1.55 [Aa]	(0.05)	36.96 [Aa]	(0.10)	25.51 [Ba]	(0.32)	33.67 [Ba]	(0.58)	5.64 [Aa]	(0.03)
L. lactis subsp. *lactis* C10	1.56 [Ba]	(0.03)	36.93 [Aa]	(0.56)	25.17 [Aa]	(0.02)	34.00 [Aa]	(0.00)	5.59 [Aa]	(0.04)

Numbers represent mean and standard deviation, with the latter in parentheses. Within a column, different superscript capital letters denote significant differences between different trials for the same cheese. For each trial, different superscript lowercase letters denote significant differences between cheeses (Tukey's, $P < 0.05$).

The count of starter and non-starter LAB was done at 1, 14, 30, 60 and 120 days of ripening and results are reported in Figures 3.3A and B. The LAB strains used in this experiment grew to 10^9 cfu/g of cheese at 1 day of ripening, indicating their ability to survive in cheese (Figure 3.3A). During ripening, the starter microflora showed a similar trend in all the cheeses with the typical decline after the first month of ripening. Slightly lower values were measured during ripening time in cheese made with *L. lactis* subsp. *cremoris* S2 (Figure 3.3A). During ripening, non-starter LAB levels increased in all the cheeses and at 4 months they reached values of 10^7 cfu/g of cheeses in cheeses made with *L. lactis* subsp. *lactis* C10 and *L. lactis* subsp. *cremoris* C whereas cheese made with *L. lactis* subsp. *cremoris* S2 had counts of 10^6 cfu/g cheese (Figure 3.3B).

pH and redox potential were measured at 1, 14, 30, 60 and 120 days of ripening and results are shown in Figure 3.4A and B. pH was around 5.4-5.5 during ripening in all the cheeses and pH values were not significantly different ($P < 0.05$) among the cheeses at each time point of ripening (Figure 3.4A).

A
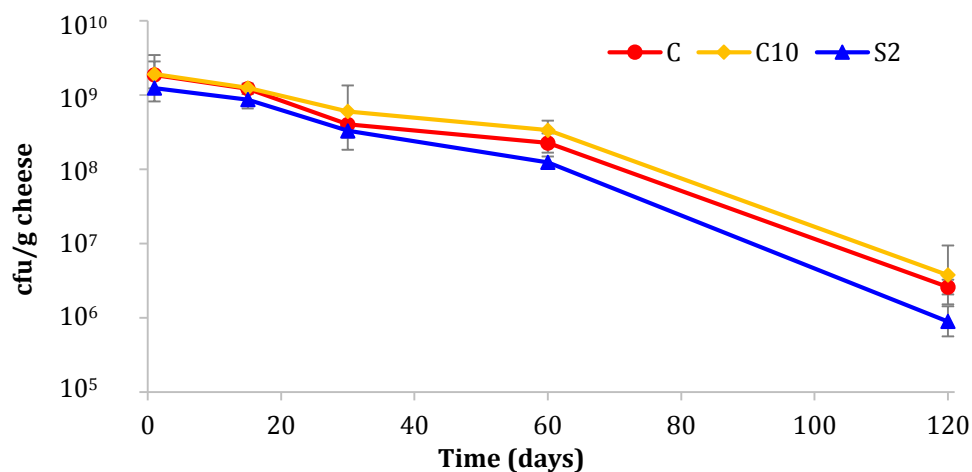
B
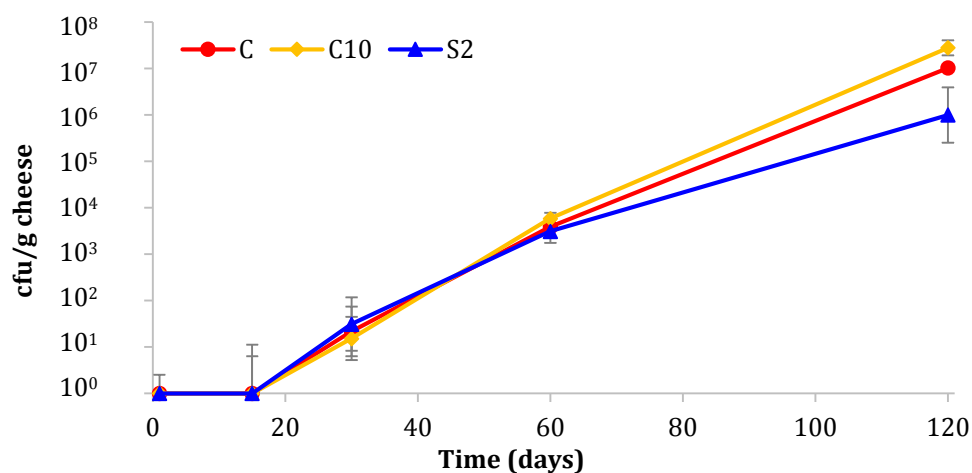
Figure 3.3   Counts of cheese starter (A) and non-starter (B) lactic acid bacteria in Cheddar cheese made with the addition of *Lactococcus lactis* subsp. *cremoris* C (C, ●), *Lactococcus lactis* subsp. *lactis* C10 (C10, ◆) and *Lactococcus lactis* subsp. *cremoris* S2 (S2, ▲). Values are mean of three independent trials with standard deviation indicated by the vertical bars.

A

B
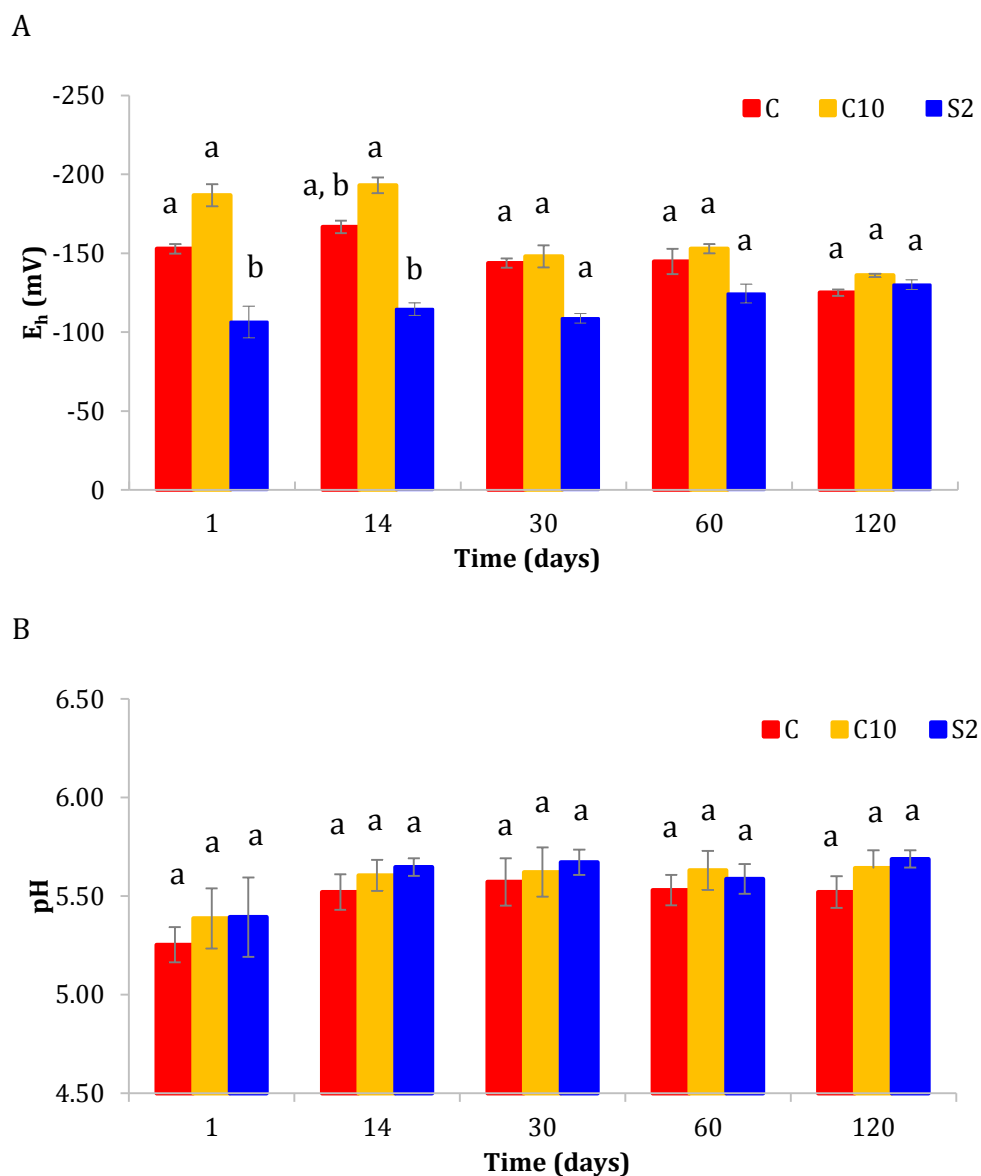
Figure 3.4   Oxidation reduction potential (E_h) (A) and pH (B) during ripening of Cheddar cheese made with the addition of *Lactococcus lactis* subsp. *cremoris* C (C, ■), *Lactococcus lactis* subsp. lactis C10 (C10, ■) and *Lactococcus lactis* subsp. *cremoris* S2 (S2, ■). Values are mean of three independent trials with standard deviation indicated by the vertical bars. At each time point, different superscript lowercase letters denote significant differences between the cheeses made with the addition of single strains of lactic acid bacteria (Tukey's, P < 0.05).

The redox potential of the cheeses was negative in all the cheeses during ripening. However, at one day of ripening, the redox potential of the cheese made with *L. lactis* subsp. *cremoris* S2 was significantly higher (P < 0.05) (E_h = -106 mV) compared to the E_h of the cheeses made with *L. lactis* subsp. *lactis* C10 (E_h = -186 mV) and *L. lactis* subsp. *cremoris* C (E_h = -152 mV) and at 14 days the E_h of the cheese made with *L. lactis* subsp. *cremoris* S2 was significantly higher (P < 0.05) (E_h = -114 mV) compared to the E_h of the cheese made with *L. lactis* subsp. *lactis* C10 (E_h = -193 mV). *L. lactis* subsp. *cremoris* S2 was able to keep positive E_h value in the experiment reported in Section 3.3.1 and in this study, even if positive E_h values were not measured, a significantly higher values (P < 0.05) was found supporting our results found in Section 3.3.1.

Moreover, at 1 day of ripening, E_h values of Cheddar cheese made with *L. lactis* subsp. *cremoris* S2 and *L. lactis* subsp. *lactis* C10 differed from E_h values measured in commercial Cheddar cheese at one day of ripening. In Chapter 5, the redox potential was measured during ripening of commercial Cheddar cheeses at different time points during ripening and E_h values of -150 mV was measured at one day of ripening. Therefore, at one day of ripening *L. lactis* subsp. *cremoris* S2 used as starter in Cheddar cheese manufacture was able to reduce E_h to negative values higher than the E_h value of normal Cheddar cheese whereas *L. lactis* subsp. *lactis* C10 reduced E_h to values lower than the E_h value of normal Cheddar cheese.

After 1 month of ripening, the E_h of the three cheeses did not differ and this could be attributed to the reduction of the number of the starter bacteria and to the

development of non-starter LAB that maintain a low E_h potential in mature Cheddar cheese (Broadbent et al., 2002; Abraham et al., 2007; Topcu et al. 2008).

At four month of ripening, analysis of the volatile profile was performed on the cheeses and 29 individual volatile compounds were identified and listed in Table 3.5. Table 3.5 also shows the respective peak areas of the identified compounds and the typical odour descriptors where known for each compound.

Three carboxylic acids were detected (acetic, butanoic and hexanoic acid). Acetic acid results from lactose metabolism and both butanoic and hexanoic acids are the result of lipolysis. The content of the acid compounds identified in the three cheeses was not significant different ($P < 0.05$).

The following hydrocarbons were detected and reported: pentane, hexane, heptane, toluene, 2,2,3-trimethyl-hexane, and octane. 2,2,3-Trimethyl-hexane was the most abundant hydrocarbon compound in the three cheeses analysed. Similar quantities of the hydrocarbons identified were found in the three cheeses.

One ester compound was identified (ethyl butanoate) and it was present in significantly higher concentration in the cheese made with *L. lactis* subsp. *cremoris* S2 compared to the other two cheeses analysed.

Table 3.5   Volatile compounds of Cheddar cheeses made with the addition of *Lactococcus lactis* subsp. *cremoris* C (C), *Lactococcus lactis* subsp. *cremoris* S2 (S2), *Lactococcus lactis* subsp. *lactis* C10 (C10) at 4 months of ripening. Units for concentrations of volatile compounds are averaged peak areas in arbitrary units.

Compound	Trial 1			Trial 2			Trial 3			Average Trial						Odour Descriptor
	C	S2	C10	C	S2	C10	C	S2	C10	C		S2		C10		
Acids																
Acetic acid	7.17E+06	1.86E+07	9.36E+06	4.07E+07	3.87E+06	8.53E+06	5.57E+06	4.00E+07	1.07E+07	1.78E+07 [a]	(1.98E+07)	2.08E+07 [a]	(1.82E+07)	9.53E+06 [a]	(1.09E+06)	vinegar, sour
Butanoic acid	3.44E+07	3.69E+07	1.99E+07	1.35E+07	1.56E+07	3.73E+05	4.26E+07	3.31E+06	2.64E+07	3.02E+07 [a]	(1.50E+07)	1.86E+07 [a]	(1.70E+07)	1.56E+07 [a]	(1.35E+07)	fecal, cheesy
Hexanoic acid	2.56E+07	2.84E+07	1.25E+07	8.15E+06	8.07E+06	1.21E+07	1.42E+07	9.00E+06	1.32E+07	1.60E+07 [a]	(8.86E+06)	1.52E+07 [a]	(1.15E+07)	1.26E+07 [a]	(5.57E+05)	goaty, fatty
Hydrocarbons																
Pentane	7.17E+05	4.51E+05	8.08E+05	5.69E+05	5.05E+05	7.74E+05	2.49E+05	9.89E+04	7.20E+05	5.12E+05 [a]	(2.39E+05)	3.52E+05 [a]	(2.21E+05)	7.67E+05 [a]	(4.44E+04)	mild gasoline type odour
Hexane	9.10E+06	1.14E+06	2.75E+06	1.35E+07	1.10E+06	1.47E+06	1.39E+06	1.23E+06	1.19E+06	8.00E+06 [a]	(6.13E+06)	1.16E+06 [a]	(6.66E+04)	1.80E+06 [a]	(8.32E+05)	*
Heptane	1.02E+07	4.31E+06	7.97E+06	8.37E+06	1.77E+06	5.57E+06	2.20E+06	2.04E+06	3.41E+06	6.92E+06 [a]	(4.19E+06)	2.71E+06 [a]	(1.40E+06)	5.65E+06 [a]	(2.28E+06)	mild gasoline type odour
Toluene	3.62E+06	2.97E+06	2.10E+06	1.76E+06	1.40E+06	1.21E+06	1.93E+06	1.58E+06	2.15E+06	2.44E+06 [a]	(1.03E+06)	1.98E+06 [a]	(8.59E+05)	1.82E+06 [a]	(5.29E+05)	sweet, pungent
2,2,3-trimethyl-hexane	7.45E+06	1.75E+07	6.82E+06	9.87E+06	5.72E+07	5.04E+07	4.74E+07	3.27E+07	3.00E+07	2.16E+07 [a]	(2.24E+07)	3.58E+07 [a]	(2.00E+07)	2.91E+07 [a]	(2.18E+07)	*
Octane	2.40E+06	2.79E+06	3.19E+06	1.72E+06	2.25E+06	2.36E+06	2.70E+06	3.11E+06	2.25E+06	2.27E+06 [a]	(5.02E+05)	2.72E+06 [a]	(4.35E+05)	2.60E+06 [a]	(5.14E+05)	*
Esters																
Ethyl butanoate	9.88E+06	2.36E+07	7.76E+06	1.10E+07	1.75E+07	7.01E+06	1.01E+07	1.30E+07	1.03E+07	1.03E+07 [a,b]	(5.93E+05)	1.80E+07 [b]	(5.32E+06)	8.36E+06 [a]	(1.72E+06)	fruity
Ketones																
Acetone	4.72E+07	3.44E+07	6.45E+06	2.20E+07	3.00E+07	3.59E+07	3.93E+07	4.96E+07	4.47E+07	3.62E+07 [a]	(1.29E+07)	3.80E+07 [a]	(1.03E+07)	2.90E+07 [a]	(2.00E+07)	Hay, wood
2,3-Butanedione	1.71E+07	5.76E+06	1.30E+06	1.52E+07	1.40E+06	2.60E+06	1.28E+07	5.78E+04	1.23E+06	1.50E+07 [b]	(2.15E+06)	2.41E+06 [a]	(2.98E+06)	1.71E+06 [a]	(7.72E+05)	buttery, carmel, sweet

2-Butanone	3.68E+07	2.94E+07	7.90E+07	7.14E+07	2.49E+07	2.94E+07	2.44E+07	3.45E+04	1.76E+07	4.42E+07[a]	(2.44E+07)	1.81E+07[a]	(1.58E+07)	4.20E+07[a]	(3.26E+07)	Butterscotch
2-Pentanone	5.56E+06	4.84E+06	1.92E+07	2.77E+07	4.28E+06	1.35E+07	5.65E+06	1.14E+07	7.83E+06	1.30E+07[a]	(1.28E+07)	6.84E+06[a]	(3.96E+06)	1.35E+07[a]	(5.69E+06)	sweet, fruity
Acetoin	7.62E+08	1.66E+08	2.48E+07	1.64E+09	3.46E+07	9.85E+07	1.78E+09	0.00E+00	3.56E+06	1.39E+09[b]	(5.52E+08)	6.69E+07[a]	(8.76E+07)	4.23E+07[a]	(4.98E+07)	sour milk
5 Methyl-2-hexanone	2.94E+07	2.97E+07	8.32E+07	3.41E+07	2.33E+07	2.80E+07	3.48E+07	2.39E+07	2.81E+07	3.28E+07[a]	(2.94E+06)	2.56E+07[a]	(3.53E+06)	4.64E+07[a]	(3.18E+07)	pleasant fruity
Alcohols																
Ethanol	2.50E+08	5.75E+08	1.40E+08	1.59E+08	7.43E+08	2.70E+08	2.66E+08	8.69E+08	6.43E+08	2.25E+08[a]	(5.77E+07)	7.29E+08[b]	(1.47E+08)	3.51E+08[a,b]	(2.61E+08)	Dry/dust
2-Butanol	3.33E+06	7.47E+06	5.85E+06	5.22E+06	2.28E+07	1.54E+07	3.57E+06	1.06E+06	1.11E+07	4.04E+06[a]	(1.03E+06)	1.04E+07[a]	(1.12E+07)	1.08E+07[a]	(4.78E+06)	sweet, alcohol
2-Methyl-1-propanol	3.75E+05	1.31E+08	6.28E+06	2.43E+06	9.67E+07	9.58E+05	1.52E+06	1.60E+08	4.91E+06	1.44E+06[a]	(1.03E+06)	1.29E+08[b]	(3.17E+07)	4.05E+06[a]	(2.76E+06)	plastic
3-Penten-1-ol	6.64E+05	1.33E+05	3.59E+04	2.73E+05	1.66E+04	1.18E+05	4.33E+05	2.31E+05	1.39E+05	4.57E+05[b]	(1.97E+05)	1.27E+05[a,b]	(1.07E+05)	9.76E+04[a]	(5.45E+04)	
3-Methyl-1-butanol	6.08E+06	2.42E+09	1.28E+08	1.30E+07	2.51E+09	1.11E+07	1.03E+07	1.84E+09	4.70E+07	9.79E+06[a]	(3.49E+06)	2.26E+09[b]	(3.64E+08)	6.20E+07[a]	(5.99E+07)	alcohol, green
2-Methyl-1-butanol	9.55E+04	3.17E+08	1.03E+06	3.65E+05	3.20E+08	5.39E+05	2.54E+05	2.08E+08	1.37E+06	2.38E+05[a]	(1.35E+05)	2.82E+08[b]	(6.38E+07)	9.80E+05[a]	(4.18E+05)	
1-Pentanol	1.62E+06	1.31E+06	1.13E+06	2.20E+06	2.23E+06	1.06E+06	1.20E+06	2.53E+05	5.67E+05	1.67E+06[a]	(5.02E+05)	1.26E+06[a]	(9.89E+05)	9.19E+05[a]	(3.07E+05)	fruity balsamic
2,3-Butanediol	0.00E+00	1.01E+08	5.75E+07	0.00E+00	7.67E+07	9.94E+07	0.00E+00	1.38E+07	1.34E+07	0.00E+00[a]	(0.00E+00)	6.38E+07[a]	(4.50E+07)	5.68E+07[a]	(4.30E+07)	fruity, creamy buttery
2-Hexanol	0.00E+00	5.90E+07	0.00E+00	0.00E+00	3.71E+07	0.00E+00	0.00E+00	2.11E+07	0.00E+00	0.00E+00[a]	(0.00E+00)	3.91E+07[b]	(1.90E+07)	0.00E+00[a]	(0.00E+00)	
Aldehydes																
3-Methyl-butanal	1.56E+06	6.91E+07	3.65E+05	3.01E+05	4.38E+06	8.68E+04	3.59E+05	8.66E+06	1.80E+05	7.40E+05[a]	(7.11E+05)	2.74E+07[a]	(3.62E+07)	2.11E+05[a]	(1.42E+05)	Green, malty
Pentanal	3.23E+06	1.10E+06	1.00E+06	1.21E+06	1.53E+05	3.53E+05	5.96E+05	5.34E+04	1.04E+05	1.68E+06[a]	(1.38E+06)	4.35E+05[a]	(5.78E+05)	4.86E+05[a]	(4.62E+05)	strong pungent odour
Sulfur																
Dimethyl sulfide	4.81E+06	8.26E+06	1.49E+07	2.36E+06	7.43E+06	1.09E+07	4.92E+06	3.49E+07	9.84E+06	4.03E+06[a]	(1.45E+06)	1.69E+07[a]	(1.56E+07)	1.19E+07[a]	(2.67E+06)	cabbage-like, sulfurous
Carbon disulfide	1.74E+08	2.03E+08	3.21E+08	1.08E+08	1.87E+08	3.10E+08	3.06E+08	4.11E+08	3.47E+08	1.96E+08[a]	(1.01E+08)	2.67E+08[a]	(1.25E+08)	3.26E+08[a]	(1.90E+07)	sweet 'ether-like'

* Compounds omitted from principal component analysis due to their lack of odour activity.

Different superscript lowercase letters denote significant differences between cheeses for each volatile component (Tukey's, $P < 0.05$).

Six ketones were detected in the cheese samples (acetone, 2,3-butanedione, 2-butanone, 2-pentanone, acetoin, 5-methyl-2-hexanone). Among the ketones identified, 2,3-butadione and acetoin were significantly higher in cheese made with *L. lactis* subsp. *cremoris* C compared to the cheeses made with *L. lactis* subsp. *cremoris* S2 and *L. lactis* subsp. *lactis* C10. The content of the other ketones compounds identified in the three cheeses was not significant different (P < 0.05).

A total of nine alcohol compounds were detected in all cheese samples (ethanol, 2-butanol, 2-methyl-1-propanol, 3-penten-1-ol, 3-methyl-1-butanol, 2-methyl-1-butanol, 1-pentanol, 2,3-butanediol and 2-hexanol). Ethanol was the alcohol present in highest concentration in all the cheeses. Cheese made with *L. lactis* subsp. *cremoris* S2 contained a significantly higher quantity of ethanol, 2-methyl-1-propanol, 2-methyl-1-butanol and 3-methyl-1-butanol compared to cheeses made with *L. lactis* subsp. *cremoris* C and *L. lactis* subsp. *lactis* C10. Significantly higher amount of 3-penten-1-ol was present in cheese made with *L. lactis* subsp. *cremoris* C compared to cheeses made with *L. lactis* subsp. *lactis* C10 and *L. lactis* subsp. *cremoris* S2. In cheese produced with *L. lactis* subsp. *cremoris* C, 2,3-butanediol was not detected and 2-hexanol was detected only in cheese made with *L. lactis* subsp. *cremoris* S2.

Two aldehydes were identified (3-methyl-butanal and pentanal) and the content of the aldehydes identified in the three cheeses was not significant different (P < 0.05).

Finally, the content of the two sulfur compounds (dimethyl sulphide and carbon disulphide) identified in the three cheeses was not significant different (P < 0.05).

Principal component analysis (PCA) of the volatile compounds listed in Table 3.5 for each trial and bacterial strain was performed (Figure 3.5). PCA analysis of the volatile profiles was undertaken to represent graphically optimal discrimination between the samples. Principal components (PC) 1 and 2, which accounted for 34 and 21% of the variation, respectively, clearly show a distinct difference between the cheeses produced using the three bacterial strains. It is apparent that the volatile profiles of the cheeses made with different bacterial strains are different to each other another. Some differences were noted within individual replicates, but in general a clear pattern was evident. In terms of its volatiles' profile, cheese produced with L. *lactis* subsp. *cremoris* C was closer to cheese produced with *L. lactis* subsp. *lactis* C10, with *L. lactis* subsp. *cremoris* S2 been different. Also in Chapter 4, PCA of the volatile compounds produced by the utilization of the three strains in CCE clearly separate *L. lactis* subsp. *cremoris* C and *L. lactis* subsp. *lactis* C10 from *L. lactis* subsp. *cremoris* S2.

L. lactis subsp. *cremoris* S2 was associated with more alcohol compounds but also with acid and aldehyde compounds and to a less extent to both sulfur compounds identified. *L. lactis* subsp. *cremoris* S2 was able to keep E_h to less reducing values than the other two strains in cheese at 1 and 14 days of ripening and this could explain the production of different volatile compounds compared to the volatiles produced by *L. lactis* subsp. *lactis* C10 and *L. lactis* subsp. *cremoris* C. *L. lactis* subsp. *cremoris* S2 was also able to keep redox potential to positive values in CCE (Chapter

4) and in milk (Section 3.3.1) and in Chapter 4 *L. lactis* subsp. *cremoris* S2 was associated to the presence aldehydes, ketones, amines and hydrocarbons compounds in CCE. In previous studies, the production of aldehydes was found to be favourite in presence of positive redox potential (Kieronczyk et al., 2006; Martin et al. 2013) therefore cheese with a less negative E_h at the beginning of the ripening could be characterized by a higher aldehyde volatiles compounds production during ripening.
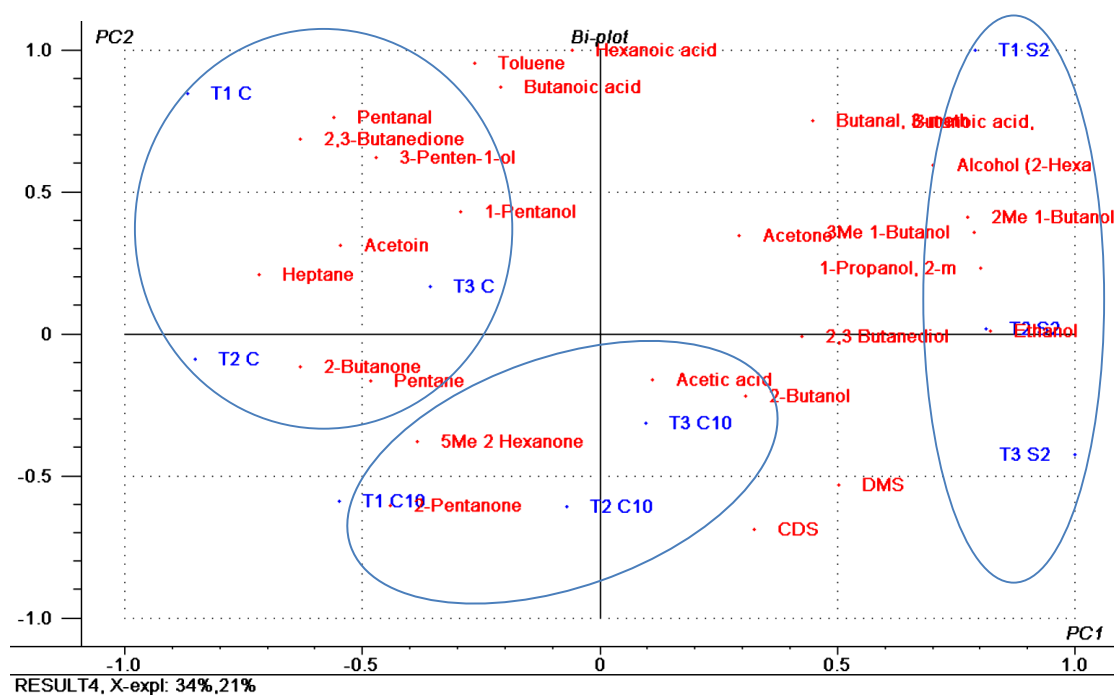
Figure 3.5   Principal component analysis of data of volatile compounds of Cheddar cheese samples at four months of ripening. Principal components (PC) 1 and 2, which accounted for 34 and 21% of the variation, respectively. The volatile compounds identified are written in red and the cheese analysed are in blue colour. T1C10, T2C10 and T3C10 correspond to the cheeses produced with *L. lactis* subsp. *lactis* C10 in Trial 1, 2 and 3, respectively. T1C, T2C and T3C correspond to the cheeses produced with *L. lactis* subsp. *cremoris* C in Trial 1, 2 and 3, respectively. T1S2, T2S2 and T3S2 correspond to the cheeses produced with *L. lactis* subsp. *cremoris* S2, in Trial 1, 2 and 3, respectively.

From the PCA (Figure 3.5), *L. lactis* subsp. *lactis* C10 was associated with sulfur compounds (dimethyl sulfide and carbon disulfide). Likewise, in Chapter 4 the addition of *L. lactis* subsp. *lactis* C10 to CCE was associated to the development of sulfur compounds. The presence of sulfur compounds is an index of good quality in Cheddar cheese (Singh et al., 2003) and they are associated with a negative redox

potential in Cheddar cheese (Green and Manning, 1982; Kristoffersen, 1985) and in non-fat yoghurt (Martin et al., 2011). *L. lactis* subsp. *lactis* C10 was able to reduce E_h to negative values in a short time in milk during the Cheddar cheesemaking temperature profile simulation as reported in Section 3.4 and when added to the cheesemilk during the Cheddar cheese manufacture, *L. lactis* subsp. *lactis* C10 decreased the E_h to values lower compare to normal Cheddar cheese at the start of ripening and this effect on E_h could be related to the development of sulfur compounds during ripening.

Therefore, selection of starter LAB based on the ability of drive E_h in milk or cheese model system could be useful to control the flavour development in Cheddar cheese during ripening.

3.4   Conclusion

This study is divided in four different sections.

In the first set of experiments, we inoculated milk with LAB during simulation of Cheddar cheesemaking temperature profile. The E_h and pH of the starting skim milk were the same in each test performed and bacteria were inoculated to similar values, $\sim 10^7$ cfu/mL milk. The bacteria studied differed at subspecies and strain level and did not show the same trend in redox potential, as reported by other authors (Gillespie and Rettger, 1938; Hewitt, 1950; Scarinci et al., 1994; Carrasco et al., 2005; Boucher et al., 2006; Brasca et al., 2007; Reichart et al., 2007), during the Cheddar cheesemaking temperature profile simulation probably due to differences

in the metabolic capabilities of these strains (Boucher et al., 2006). Moreover, milk acidification and bacterial growth seemed not to be correlated to the redox potential of the milk. Therefore, LAB could be classified on the basis of their ability to influence redox potential and they could be used to control the E_h in fermented dairy products.

The second set of experiments regarded the addition of redox agents to milk. This gave us information on the capacity of the redox agents tested to influence E_h and pH of milk. The redox agents tasted could reduce or increase the E_h and the pH of milk to different values depending on the quantity added and their ability to influence the redox couples that are present in the milk (Sherbon, 1988). Two of the redox agents tested (potassium iodate and sodium hydrosulfite) were selected and their effect on starter LAB and pH of milk was studied. The addition of KIO_3 or $Na_2S_2O_4$ could successfully increase or decrease the E_h of the milk, respectively. However, the pH and the survival of the microorganisms were also influenced. Therefore, when selecting redox agents to control the redox potential of a dairy products tests on their ability to influence the pH of the system and their toxicity on the bacteria used should be performed.

In the third set of experiments, the two redox agents tested in milk inoculated with starter LAB (KIO_3 and $Na_2S_2O_4$) were added to the cheese-curd at the end of the cooking stage of Cheddar cheesemaking simulation. In this experiment, we demonstrated the possibility of controlling the redox potential of Cheddar cheese during manufacture by the addition of 0.1 % KIO_3 or 0.05% $Na_2S_2O_4$ without affecting pH. This is the first study where redox potential was followed during

Cheddar manufacture in the presence of redox agents and it was demonstrated that the addition of redox agents could be used to control the redox potential in fermented dairy products.

In the fourth set of experiment, Cheddar cheeses were manufactured with the addition of three single strain starters selected from the first set of experiments. One strain, *L. lactis* subsp. *lactis* C10, was able to decrease E_h to values lower than the other two strains and its presence was associated with the development of sulfur compounds that characterize a good quality Cheddar cheese (Singh et al., 2003). At 1 and 14 days of ripening, single strain Cheddar cheese made with *L. lactis* subsp. *cremoris* S2 showed a higher E_h compared to the E_h of the cheeses made with the other two strains analysed. From the PCA analysis of the volatile compounds, this cheese was clearly separated from the other two and it was not associated with sulfur compounds. Therefore, the selection of strains able to control the redox potential at the early stages of ripening could be useful to control the production of volatile compounds and perhaps the quality of Cheddar cheese. Moreover, we hypnotise that cheese with a low redox potential at the early stages of ripening could be more likely to produce volatile compounds that are associated with a good quality Cheddar cheese.

3.5   Acknowledgments

3.6   References

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Chapter 4

Effect of lactic acid bacteria on changes to the oxidation-reduction potential during the simulated pressing stage of Cheddar cheese

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Abstract

The ability of 30 strains of *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* to modify redox potential (E_h) during simulation of the pressing stage of Cheddar cheese was studied in Cheddar cheese extract (CCE), produced by extracting the water-soluble portion of the cheese matrix. CCE was adjusted to pH 6 and 1.6% lactose and inoculated with 1% of various single strain cultures at 33-34°C; when pH 5.4 was reached salt solution was added to CCE and the temperature was decreased to 13°C over 18 h. Microbiological growth and trends in E_h, pH and temperature were measured over the simulated Cheddar pressing temperature profile. Strains were divided on the basis of their redox profiles into three groups. Some strains reduced the redox potential to values around -120 mV in about 3 h, other strains showed a slower reduction ability reaching equilibrium in more than 4 h. Other strains maintained a positive redox potential during the whole experiment. All the strains studied acidified the CCE over time to similar values and showed similar growth. Three of these strains were selected and analysed for volatile profiles at the end of the experiment. Principal component analysis of data from volatile compounds was able to separate the three strains chosen. Selection of lactic acid bacteria used for cheesemaking can be done on the basis of their aptitude to influence redox potential in CCE.

Keywords: oxidation-reduction potential, lactic acid bacteria, cheese model.

4.1   Introduction

Lactic acid bacteria (LAB) are vital for cheesemaking and ripening and include starter and adjunct cultures, as well as non-starter lactic acid bacteria (NSLAB) that gain access to the cheese through the ingredients used and from the environment. In Cheddar cheese manufacture, defined-strain cultures containing two or more strains of *Lactococcus lactis* are generally added to the cheesemilk. (Fox at al., 2000).

The fermentation characteristics of starter cultures are fundamental for the success of dairy industry. Starters play an important role in cheesemaking by converting lactose to lactic acid that assists in the rennet coagulation of the cheesemilk and prevents the growth of pathogens and spoilage microorganisms. Moreover, LAB are responsible for biochemical modifications during ripening (McSweeney and Sousa, 2000). In particular, their proteolytic and lipolytic activities contribute to the development of cheese flavour and texture. Starter LAB are added at the beginning of cheese manufacture and they grow during Cheddar cheesemaking from 1-5 x 10^6 cfu/mL to 1-10 x 10^8 cfu/mL at the moulding stage and their numbers decrease as ripening proceeds (Parente and Cogan, 2004).

LAB utilised as starter cultures are chosen on the basis of their technological performance based on parameters such as acidification activity, phage and antibiotic resistance, proteolytic activity and ability to metabolize citrate (Fox et al., 1993, 2000; Limsowtin at al., 1996; Yvon and Rijnen, 2001). Another important characteristic of LAB that is rarely taken in consideration in fermentation processes of dairy products is their ability to reduce the oxidation-reduction potential of the medium. Redox potential is an intrinsic parameter of all biological systems defined by the equilibrium

between oxidising and reducing couples in a medium. Several studies indicate the importance of redox potential to the development of desirable cheese aroma (Kristoffersen, 1967; Kristoffersen et al., 1967; Kieronczyk et al., 2006) and to the quality in dairy products (Jönsson and Pettersson, 1977; Dave and Shah, 1998).

L. lactis strains isolated from dairy environments differ in their reducing activity in milk (Brasca et al., 2007) and their ability to reduce redox potential could differ within the same species (Tachon et al., 2010).

Previous studies carried out in our laboratory found that redox potential in Cheddar cheese decreases rapidly during the pressing stage of the cheese manufacture (Caldeo and McSweeney, 2012) and it reaches equilibrium (E_h = -117 mV) within few hours (McSweeney et al., 2010).

In this study, we hypothesized that this major drop in redox potential could be attributed to the strain of LAB used and that different strains of LAB may be able to modulate redox potential in different or similar ways depending on strain-dependent characteristics. To validate this hypothesis and to investigate the strain-specific contribution of *L. lactis*, we did our study in a Cheddar cheese model (Cheddar cheese extract, CCE) which mimicked the natural environment of Cheddar cheese during pressing. Therefore, pH and lactose content and temperature of CCE were adjusted to values close to those of the cheese curd before pressing, LAB strains were added and when the pH reached 5.4, salt was added to CCE and the temperature was decreased to mimic salting and pressing.

Microbiological growth, redox potential, pH and temperature were measured in triplicate during the simulation of the Cheddar pressing temperature profile for 30

strains of *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*. Three of these strains were then selected and analysed for their volatile profiles at the end of the experiment.

4.2   Materials and methods

4.2.1   Production of Cheddar cheese extract

Cheddar cheese extract (CCE) was produced from 2 week old commercial Cheddar cheese to create a medium containing the water-soluble portion of the cheese matrix. The cheese was cut into 5 x 5 cm cubes using a double handled cheese knife and shredded by a food processor (Urschel Cutter, Model 3600, Urschel Laboratories Inc., Valparaiso, IN, US) using a 2.5/20-cm cutting head. The shredded cheese was added to distilled water (1:2 ratio (w/w) of cheese to water) in a steam jacketed vat. The temperature of the cheese-water mixture was raised to 50°C over 40 min and held for 20 min while continuously stirring. The cheese-water was separated from the cheese and processed by a filtration system (Sepratech, GPD 503 Pilot plant filtration system; Separation Technologies Inc., Valparaiso, IN, US). The mixture went through the ultrafiltration membranes (1-100 kDa MWCO), where the permeate (smaller particles) was kept and the retentate (large particles) was returned to the membranes with added water and continued to be filtered. The ultrafiltration permeate was then concentrated by reverse osmosis membranes, where the concentrate returned to the system until no more concentration could take place. The system concentrated the CCE 1.6 x fold.

Prior to each experiment, the lactose content and pH of CCE were adjusted to 1.6% (Fox et al., 1990) and 6.00 (Pandey, et al., 2003), respectively, to reflect the values of Cheddar cheese curd before the pressing stage.

4.2.2   Strain preparation

Thirty strains of *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*, listed in Table 4.1, were obtained from the culture collection of the Department of Food Science, Utah State University and were used in this study. They are common single strains of LAB from industry collections and isolated by universities that can be used as starter for the manufacture of dairy products.

Strains were prepared from frozen stock (-80°C) by one transfer (1% vol/vol) in M17 lactose broth (Difco Laboratories, Detroit, MI, USA) and incubated at 30°C for 16-20 h. Then a second transfer (1% vol/vol) of the M17 lactose broth to UHT milk was done and incubated at 30°C for 16-20 h corresponding to cell count 10^8-10^9 cfu/mL.

Table 4.1   Lactic acid bacteria used in the study.

Genus	Species	Subspecies	Strain	Source
Lactococcus	*lactis*	*lactis*	C10	University of Minnesota
Lactococcus	*lactis*	*lactis*	DL16	University of Minnesota
Lactococcus	*lactis*	*lactis*	ATCCII454	American Type Culture Collection
Lactococcus	*lactis*	*lactis*	ML3	University of Minnesota
Lactococcus	*lactis*	*lactis*	ATCC29146	American Type Culture Collection
Lactococcus	*lactis*	*lactis*	M70	Danisco (Copenhagen, Denmark)
Lactococcus	*lactis*	*lactis*	C2	University of Minnesota
Lactococcus	*lactis*	*lactis*	26-2	Ohio State University
Lactococcus	*lactis*	*cremoris*	Z8	University of Minnesota
Lactococcus	*lactis*	*cremoris*	HP	American Type Culture Collection
Lactococcus	*lactis*	*cremoris*	45P+	Utah State University
Lactococcus	*lactis*	*cremoris*	SW224	Utah State University
Lactococcus	*lactis*	*cremoris*	SK11 2004	University of Wisconsin
Lactococcus	*lactis*	*cremoris*	E	NORDICA
Lactococcus	*lactis*	*cremoris*	R	NORDICA
Lactococcus	*lactis*	*cremoris*	UC317	University College Cork
Lactococcus	*lactis*	*cremoris*	EB7	Ohio State University
Lactococcus	*lactis*	*cremoris*	D	NORDICA
Lactococcus	*lactis*	*cremoris*	W	NORDICA
Lactococcus	*lactis*	*cremoris*	G	NORDICA
Lactococcus	*lactis*	*cremoris*	S2	University of Wisconsin
Lactococcus	*lactis*	*cremoris*	A	NORDICA
Lactococcus	*lactis*	*cremoris*	B	NORDICA
Lactococcus	*lactis*	*cremoris*	C	NORDICA
Lactococcus	*lactis*	*cremoris*	F	NORDICA
Lactococcus	*lactis*	*cremoris*	I	NORDICA
Lactococcus	*lactis*	*cremoris*	J	NORDICA
Lactococcus	*lactis*	*cremoris*	Q	NORDICA
Lactococcus	*lactis*		MM510(akaS4)	Rhodia
Lactococcus	*lactis*		SCO213	University of Wisconsin

4.2.3   Experimental design

Experiments were performed in a 250 mL autoclaved reactor containing 210 mL of filter-sterilized CCE (0.45 μm filter; Thermo Fisher Scientific, Waltham, MA) previously adjusted to pH 6.0 with 1 N HCl and to 1.6% (w/v) lactose content (lactose monohydrate; Sigma-Aldrich, St. Louis, MO, USA). Sterilized redox electrodes were placed into the CCE during the experiment and redox data were recorded every minute. The headspace of the reactor was filled up with a mixture gas of N_2 and H_2 (96:4 vol/vol) to eliminate oxygen. CCE was inoculated with 1% vol/vol of culture and incubated at 33-34°C in water bath. Samples of CCE were taken using a sterile syringe to measure pH (pH meter 443i, Corning Inc., Corning, NY, USA). When pH 5.4 ± 0.02 was reached, a solution of filter sterilized CCE and salt was added to bring the salt concentration in the reactor to 4% (w/v). pH of the CCE salt solution had been previously adjusted to 5.4. pH of CCE was measured again at the end of the experiment. The temperature was then decreased following the temperature profile during the pressing stage of Cheddar cheese (from 33-34°C to 13°C over 18 h). Microbiological growth was monitored after about 1 h from the start of the fermentation and at the end of the experiment. Experiments were conducted in triplicate for each *L. lactis* strain. Moreover, as control was used CCE without bacteria inoculation and redox potential and pH were constant during the experiment.

Three of the strains were selected and analysed for volatile profiles at the end of the experiment.

4.2.4   Measurement of redox potential, pH and microbiological growth

Measurement of redox potential was made using a platinum working electrode (XM120) and silver/silver chloride reference electrode (REF 201, both from Radiometer Analytical, Villeurbanne Cedex, Lyon, France). Before each experiment, the reference and the platinum electrodes were cleaned by the method reported by Topcu et al. (2008), Caldeo and McSweeney (2012) and Abraham et al. (2013). The accuracy of electrodes was checked against tap water (Abraham at al., 2007, 2013; Jeanson et al., 2009) and against a standard solution (470 mV, HI 7022, Hanna Instruments, Szeged, Hungary) at 25°C.

The probes were sanitized by immersing them in 4% commercial bleach (Clorox® Regular Bleach, Clorox Company, Oakland, CA, USA) pH 5.5-6.0 for 2 min. Then the probes were rinsed with sterile distilled water, dipped in 95% ethanol and placed directly in the reactor. After the experiment, the probes were rinsed with distilled water, dipped in 95% ethanol and placed in 3M KCl.

Redox potential electrodes were connected to a data logger (Squirrel Data Logger 2040-2F16 Series; Grant, Data Acquisition, Cambridge, UK), through an amplifier (PHTX-21, Omega, Stamford, CT, USA), that enabled data acquisition on a computer during simulated pressing. The data were recorded every minute.

Oxidation-reduction potential data were recorded without reference to a hydrogen reference electrode and converted to E_h (potential related to the standard hydrogen electrode) according to Caldeo and McSweeney (2012) with temperature compensation.

Chapter 4

Effect of lactic acid bacteria on changes to the oxidation-reduction
potential during the simulated pressing stage of Cheddar cheese

L. lactis strains were enumerated by serial dilution with 0.1% BBL polypeptone peptone (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and plated in duplicate on M17 lactose agar. Plates were incubated aerobically at 30°C for 3 days. The total colony forming units in CCE were calculated by counting colonies on plates with between 30 and 300 colonies.

4.2.5 Headspace Solid Phase Micro-Extraction Gas Chromatography Mass Spectrometry (HS SPME GC-MS) Methods

The method and the equipment used to analyze the CCE headspace was the same described by Young (2011). 1,4-Diclorobenzene (2 µl) was used as internal standard.

Data files obtained from the GC-MS were exported in the netCDF format. Peak picking and deconvolution were performed using the Automated Mass Spectral Deconvolution and Identification System (AMDIS; version 2.62, 1999-2000) developed by the National Institute of Standards and Technology (NIST). Deconvoluted mass spectra were submitted to the online analysis tool Spectconnect (www.spectconnect.mit.edu) for the systematic detection of possible metabolites that were conserved across samples. Metabolites resulting from this analysis were identified by a database search against the NIST Mass Spectral (version 2.0, 2005) and Fiehn (Agilent Technologies Inc., Santa Clara, CA, USA) libraries, and by comparison with linear retention indices (LRI). The LRI were established by injection of standard n-alkanes from 7 to 40 carbons (Supelco, Bellefonte, PA, USA). Parent peak intensities were normalized to the internal standard in each run prior to statistical analysis. Data were expressed as a ratio relative to the internal standard.

4.2.6   Statistical analysis

Analysis of variance (ANOVA) of the growth of microorganisms at the beginning of the experiments and growth and pH values at the end of the fermentations were conducted using SPSS Version 20.0 for Mac OS X (SPSS Inc., Chicago, IL, USA). When differences were significant ($P < 0.05$), the means were analysed using Tukey's test.

The parameters calculated from the redox potential curves were analysed using multivariate statistical techniques to discriminate between strains with reducing ability.

Multivariate statistics were also used to analyse the concentrations of the volatile compounds determined by HS SPME GC-MS. In order quantitatively to compare peak areas of each volatile compound identified in each chromatogram for the three strains selected, the areas were normalized to the internal standard and converted into the percentage of the summed peak areas of all volatile compounds in a sample. Principal component analysis (PCA) was performed on the variables using a correlation matrix by SPSS.

4.3   Results and discussion

4.3.1   Redox potential, pH and microbiological growth of LAB strains in CCE

Our previous work (McSweeney et al., 2010; Caldeo and McSweeney, 2012) suggested that the major drop in E_h in Cheddar cheese manufacture and ripening occurs during the pressing stage and it is our hypothesis that this reducing activity could be

attributed to the strains of LAB used. Therefore, this study was designed to simulate the environment of the industrial pressing stage of Cheddar cheese manufacture in a cheese model (CCE). CCE has been used as model in other studies (Budinich, 2011; Tan et al., 2012) and it is essentially the aqueous phase of Cheddar cheese that should contain all the necessary constituents for the physiological growth of microorganisms. CCE was adjusted to temperature, pH and lactose content close to the typical values of the cheese curd before pressing and inoculated with 1% (vol/vol) single strains LAB. At pH 5.4, salt solution was added and the temperature was decreased to 13°C over 18 h to mimic the cooling stage of Cheddar cheese manufacture after pressing.

Microbiological growth of all the *L. lactis* strains, shown in Table 4.2, was measured after 1 h from the beginning of the fermentation (T1) and at the end of the experiment (T2) in triplicate. At T1, no significant differences ($P < 0.05$) were found between strains in terms of number of cells and the strains grown between 1.00×10^7 and 1.32×10^7 cfu/mL CCE. At the end of the fermentation, the strains had grown about one log cfu/mL CCE to values between 9.02×10^7 and 7.41×10^8 cfu/mL CCE and some differences had found between strains and reported in Table 4.2.

pH was measured during each experiment periodically until value of 5.4 was reached. All the strains studied in these experiments were able to acidify CCE and pH 5.4 was reached at different times depending on the strain. After the addition of the salt solution to CCE, all the strains were able to acidify the medium further to values not significantly different ($P < 0.05$) between 4.43 and 4.69 at the end of the experiment (Table 4.2). However, those pH values were lower compared to the pH of Cheddar cheese at the end of the pressing stage of manufacture.

Table 4.2   Microbiological growth of *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* measured in Cheddar cheese extract after 1 h from the start of the fermentation (T1) and at the end of the pressing stage simulation of Cheddar cheese manufacture (T2). pH values at the end of the experiment.

Strains	T1 (log cfu/mL CCE)	T2 (log cfu/mL CCE)	pH
L. l. lactis C10	8.05 [a] (0.78)	8.42 [a,b] (0.43)	4.59 [a] (0.06)
L. l. lactis DL16	7.96 [a] (0.48)	8.28 [a,b] (0.18)	4.48 [a] (0.05)
L. l. lactis ATCC11454	7.31 [a] (0.50)	8.73 [a,b] (0.32)	4.56 [a] (0.01)
L. l. lactis ML3	8.12 [a] (0.46)	8.65 [a,b] (0.46)	4.61 [a] (0.17)
L. l. lactis ATCC29146	8.01 [a] (0.25)	8.87 [b] (0.14)	4.55 [a] (0.07)
L. l. lactis M70	8.08 [a] (0.32)	8.87 [b] (0.39)	4.58 [a] (0.03)
L. l. lactis C2	7.36 [a] (0.16)	8.53 [a,b] (0.04)	4.62 [a] (0.02)
L. l. lactis 26-2	7.86 [a] (0.63)	8.81 [a,b] (0.09)	4.57 [a] (0.04)
L. l. cremoris Z8	7.57 [a] (0.02)	8.61 [a,b] (0.12)	4.63 [a] (0.11)
L. l. cremoris HP	7.75 [a] (0.41)	8.68 [a,b] (0.07)	4.58 [a] (0.04)
L. l. cremoris 45P+	7.46 [a] (0.06)	7.96 [a] (0.54)	4.66 [a] (0.10)
L. l. cremoris SW224	7.87 [a] (0.19)	8.40 [a,b] (0.38)	4.56 [a] (0.04)
L. l. cremoris SK11 2004	7.67 [a] (0.11)	8.86 [b] (0.08)	4.59 [a] (0.04)
L. l. cremoris E	7.64 [a] (0.19)	8.60 [a,b] (0.30)	4.66 [a] (0.16)
L. l. cremoris R	7.82 [a] (0.31)	8.30 [a,b] (0.44)	4.51 [a] (0.01)
L. l. cremoris UC317	8.07 [a] (0.17)	8.74 [a,b] (0.01)	4.61 [a] (0.04)
L. l. cremoris EB7	7.82 [a] (0.05)	8.65 [a,b] (0.22)	4.55 [a] (0.02)
L. l. cremoris D	7.64 [a] (0.44)	8.43 [a,b] (0.20)	4.46 [a] (0.07)
L. l. cremoris W	7.87 [a] (0.32)	8.38 [a,b] (0.18)	4.47 [a] (0.09)
L. l. cremoris G	7.44 [a] (0.92)	8.20 [a,b] (0.31)	4.50 [a] (0.08)
L. l. cremoris S2	7.00 [a] (0.51)	8.09 [a,b] (0.36)	4.59 [a] (0.08)
L. l. cremoris A	7.37 [a] (0.88)	8.55 [a,b] (0.24)	4.56 [a] (0.18)
L. l. cremoris B	7.59 [a] (0.25)	8.27 [a,b] (0.21)	4.67 [a] (0.22)

L. l. cremoris C	7.18 [a] (0.43)	8.18 [a,b] (0.51)	4.69 [a] (0.24)
L. l. cremoris F	7.49 [a] (0.09)	8.31 [a,b] (0.22)	4.64 [a] (0.04)
L. l. cremoris I	7.36 [a] (0.21)	7.98 [a,b] (0.01)	4.56 [a] (0.07)
L. l. cremoris J	7.29 [a] (0.19)	8.17 [a,b] (0.05)	4.43 [a] (0.09)
L. l. cremoris Q	7.37 [a] (0.45)	7.96 [a] (0.28)	4.53 [a] (0.04)
L. l. MM510(akaS4)	7.83 [a] (0.38)	8.44 [a,b] (0.25)	4.49 [a] (0.09)
L. l. SCO213	7.60 [a] (0.75)	8.65 [a,b] (0.15)	4.53 [a] (0.04)

Numbers represent mean and standard deviation in parenthesis. Mean in a column
with different superscripts are significantly different (Tukey, $P < 0.05$).

All the strains grown under conditions simulating the environment of cheese pressing
were able to acidify the CCE over time to similar values and showed similar growth
(Table 4.2).

Oxidation-reduction potential was measured during the experiments. For all the
microorganisms, the initial E_h value of the uninoculated CCE was $+378 \pm 29$ mV then
the redox potential varied depending on the strain and different values were recorded
at pH 5.4 for each strain. Since pH 5.4 was reached at different times depending on the
strain, data of redox potential were considered from the attainment of pH 5.4 to the
end of the simulation of the pressing stage. The average E_h values of each strain are
reported in Figure 4.1 where time 0 corresponds to the time at which the medium
reached pH 5.4.
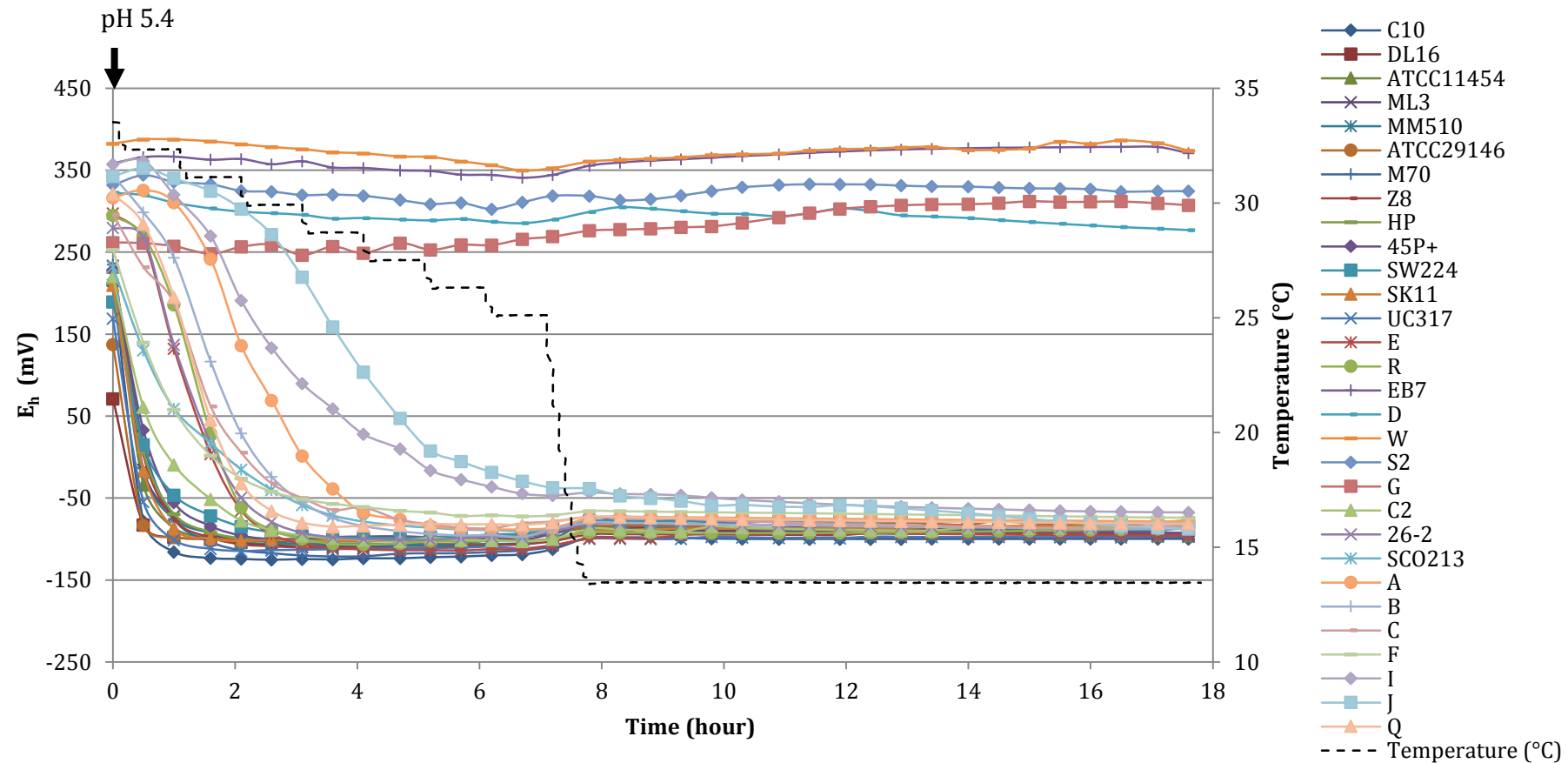
Figure 4.1 Trend in oxidation-reduction potential (E_h, mV) and temperature (°C) of the lactic acid bacteria strains from the salting stage during the simulation of the Cheddar cheese pressing step in Cheddar cheese extract (CCE). E_h data are means of tree independent trials.

Chapter 4

Effect of lactic acid bacteria on changes to the oxidation-reduction potential during the simulated pressing stage of Cheddar cheese

Despite growing to about 3.00×10^8 cfu/mL and reducing the pH, strains of *Lactococcus lactis* subsp. *cremoris* EB7, D, W, G, S2 maintained a positive redox potential during the whole experiment and they can be clearly separated from the others. All the other strains reduced the redox potential during the growth under condition simulating the environment of cheese pressing. To discriminate between strains able to reduce the redox potential faster than others, the E_h at pH 5.4 ($E_h t_i$), the E_h reached at equilibrium ($E_h t_{eq}$), the time at which $E_h t_{eq}$ occurred (t_{eq}), the differences between $E_h t_{eq}$ and $E_h t_i$ ($E_h t_{eq}$-t_i) and the maximum difference between two measures (D_{max}) were estimated (Figure 4.2). Table 4.3 shows these parameters for each strain and the differentiation of the strains into fast and slow groups was given by principal component analysis on the basis of their ability to reduce E_h over time.
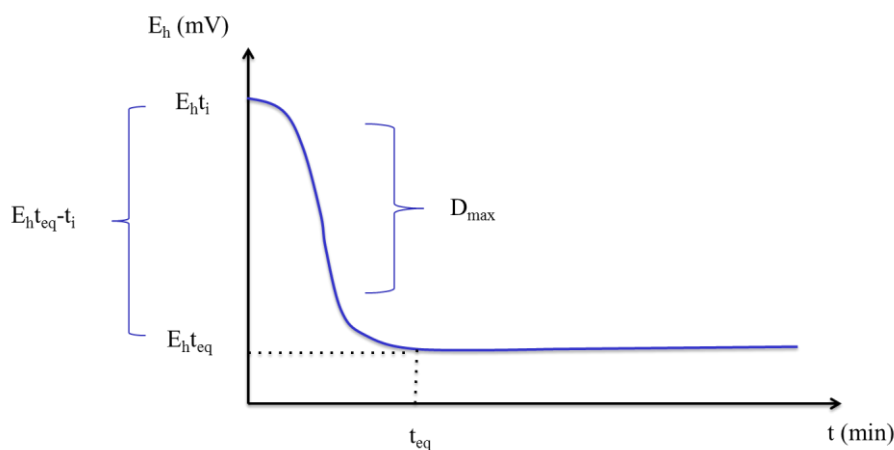
Figure 4.2 Example of redox potential curve of LAB in CCE during the simulation of the Cheddar cheese pressing stage and parameters extracted from the curve. $E_h t_i$, E_h at pH 5.4; $E_h t_{eq}$, E_h reached at equilibrium; t_{eq}, time at which $E_h t_{eq}$ occurred; $E_h t_{eq}$-t_i, differences between $E_h t_{eq}$ and $E_h t_i$; D_{max}, maximum difference between two measurements.

Table 4.3   Mean and standard deviations (sd) values of the parameters calculated from the redox potential curves of LAB in CCE during the simulation of Cheddar cheese pressing (Fig. 1). For each strain, the E_h at pH 5.4 (E_ht_i), the E_h reached at equilibrium (E_ht_{eq}), the time at which E_ht_{eq} occurred (t_{eq}), the differences between E_ht_{eq} and E_ht_i (E_ht_{eq}-t_i) and the maximum difference between two measures (D_{max}) are reported.

Strains	E_ht_i (mV)	sd	E_ht_{eq} (mV)	sd	E_ht_{eq-i} (mV)	sd	D_{max} (mV)	sd	t_{eq} (h)	sd
L. l. lactis C10	210.66	5.38	-125.24	6.76	-335.90	1.58	101.44	4.08	1.92	0.16
L. l. lactis DL16	69.74	10.16	-101.74	3.22	-171.48	11.44	66.20	33.02	1.92	0.32
L. l. lactis ATCCII454	231.68	6.75	-102.72	8.39	-334.40	1.89	98.63	5.64	1.77	0.07
L. l. lactis ML3	233.33	24.10	-107.51	6.92	-340.84	24.73	87.27	20.35	2.17	0.38
L. l. lactis ATCC29146	137.16	13.51	-97.70	6.37	-234.87	11.47	141.21	1.98	0.96	0.08
L. l. lactis M70	234.84	17.80	-113.74	4.39	-348.58	17.76	55.62	33.23	2.10	0.03
L. l. lactis C2	220.16	13.67	-101.19	2.46	-321.34	12.00	76.05	21.05	3.99	0.03
L. l. lactis 26-2	279.56	9.39	-100.41	12.52	-379.97	18.12	42.17	9.27	4.02	0.07
L. l. cremoris Z8	230.94	10.80	-106.64	15.93	-337.59	7.72	66.05	33.07	2.10	0.07
L. l. cremoris HP	212.06	5.59	-97.34	3.22	-309.40	2.56	60.03	30.92	1.87	0.07
L. l. cremoris 45P+	228.14	8.43	-100.68	13.34	-328.82	6.23	54.77	27.91	2.29	0.17
L. l. cremoris SW224	189.22	6.99	-93.08	13.52	-282.30	12.69	42.23	26.70	2.58	0.17

L. l. cremoris SK11 2004	209.64	2.44	-95.62	5.77	-305.27	7.87	96.45	35.21	1.23	0.08
L. l. cremoris E	297.35	15.91	-104.68	19.89	-402.03	31.78	65.83	22.22	3.02	0.07
L. l. cremoris R	295.15	24.23	-101.42	3.04	-396.56	24.95	61.03	32.21	3.11	0.02
L. l. cremoris UC317	168.98	8.69	-110.89	5.15	-279.86	9.90	87.16	19.00	1.44	0.05
L. l. cremoris EB7	358.30	30.44			-358.30	30.44				
L. l. cremoris D	322.62	11.32			-322.62	11.32				
L. l. cremoris W	382.47	30.26			-382.47	30.26				
L. l. cremoris G	261.87	7.99			-261.87	7.99				
L. l. cremoris S2	332.13	29.91			-332.13	29.91				
L. l. cremoris A	295.98	3.81	-83.42	4.57	-379.40	7.89	62.08	41.10	5.33	0.03
L. l. cremoris B	343.71	8.43	-92.89	1.90	-436.61	6.55	39.71	34.21	4.81	0.10
L. l. cremoris C	316.55	20.45	-82.65	5.78	-399.20	23.74	32.81	11.42	4.99	0.05
L. l. cremoris F	253.01	3.56	-71.37	26.86	-324.38	28.16	36.03	9.96	5.48	0.15
L. l. cremoris I	357.19	25.36	-43.87	34.17	-401.06	41.78	19.79	8.65	7.96	0.03
L. l. cremoris J	342.54	14.55	-60.50	14.44	-403.04	22.12	16.93	3.48	10.52	0.08
L. l. cremoris Q	321.18	7.67	-83.51	7.35	-404.69	15.01	31.62	7.62	4.27	0.00
L. l. MM510(akaS4)	222.74	20.56	-101.43	11.30	-324.17	19.98	96.75	34.69	2.21	0.19
L. l. SCO213	232.12	21.80	-85.02	2.45	-317.14	19.35	41.38	17.95	4.85	0.00

Multivariate factorial analysis was carried out on the parameters estimated from the reducing curves. Principal component analysis shows that the first component has an eigenvalue greater than 1 (3.69) and it explains the 64% of the variance between strains and the second component explains the 17% of the variance (Figure 4.3). On the first component, all the variables had an absolute loading value greater than 0.76 and $E_h t_i$ and D_{max} were negatively correlated with PC1. The first component separated the strains into two groups. Strains concentrated on the left side of the bi-plot are associated with a faster reduction activity, since they are placed where D_{max} is located. On the right side of the bi-plot are strains with a slower reducing activity. In particular, strains *L. lactis* subsp. *cremoris* J and *L. lactis* subsp. *cremoris* I were able to reach their equilibrium values in more than 6 h. Strain separation by PCA did not appear to be related to subspecies.
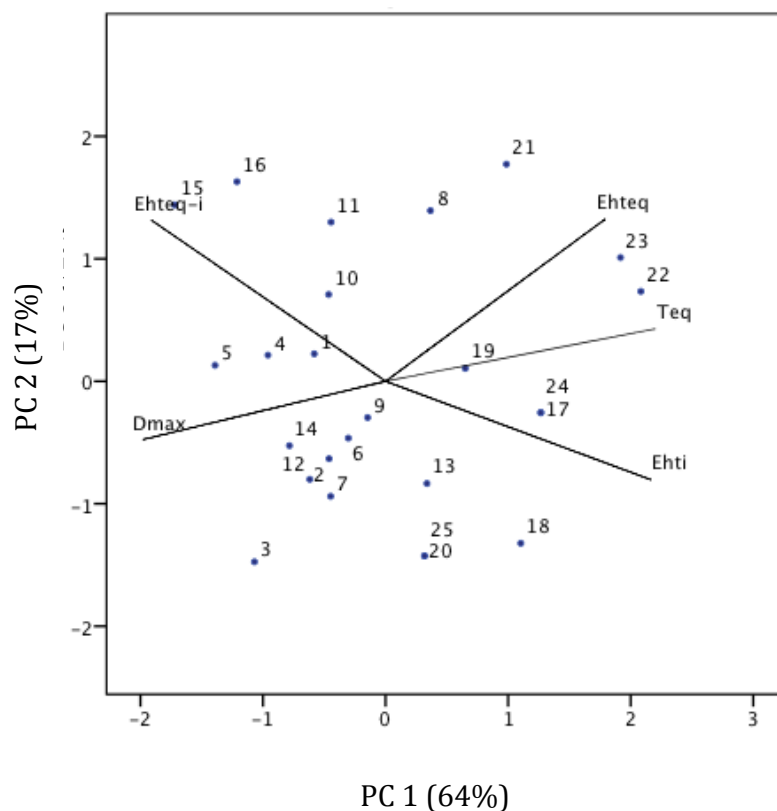
Figure 4.3   Bi-plot of the principal component 1 and 2 showing the lactic acid bacteria strains scores and the loadings of the parameters used to discriminate between strains with reducing activity during the simulation of the Cheddar cheese pressing stage in Cheddar cheese extract (CCE). LAB strains are indicated by numbers: 1, C2; 2, ML3; 3, C10; 4 SK11; 5, UC317; 6, Z8; 7, M70; 8, SCO213; 9, 45+; 10, HP; 11, SW224; 12, ATCC11454; 13, 26-2; 14, MM510; 15, ATCC29146; 16, DL16; 17, C; 18, B; 19, A; 20, E; 21, F; 22, I; 23, J; 24, Q; 25, R. The parameters extracted from the LAB redox curves are: $E_{h}t_i$, E_h at pH 5.4; $E_{h}t_{eq}$, E_h reached at equilibrium; t_{eq}, time at which $E_{h}t_{eq}$ occurred; $E_{h}t_{eq}$-t_i, differences between $E_{h}t_{eq}$ and $E_{h}t_i$; D_{max}, maximum difference between two measurements.

The E_h at pH 5.4 ($E_h t_i$) of the LAB studied was higher in the strains with slower ability to reduce E_h (values between +382 and +329 mV) except the strain *L. lactis* SCO213 that had a value of +232 mV. Strains with a faster reduction had $E_h t_i$ values between +69 and +234 mV. The strains with reducing activity reached their equilibrium values at different times. Strains with faster reducing activity reached their equilibrium value within 2.58 h, except the strain *L. lactis* subsp. *lactis* C2 that reached its equilibrium within 3.99 h. Strains with a slower ability to reduce E_h, reached their equilibrium in more than 3.02 h. The equilibrium value reached was different between strains with faster and slower E_h reduction. Slow strains had $E_h t_{eq}$ values between -43 and -92 mV, excluding strains *L. lactis* subsp. *lactis* 26-2, *L. lactis* subsp. *cremoris* R and *L. lactis* subsp. *cremoris* E that had $E_h t_{eq}$ values of -100.41, -101.42 and -104.68 mV, respectively.

The strains with a fast reduction of E_h were characterized by a high D_{max} values and they were able to reduce E_h in less than 3 h. Those strains had a D_{max} values between 42.23 and 141.21 mV. Strains *L. lactis* subsp. *cremoris* R, L. *lactis* subsp. *cremoris* A and *L. lactis* subsp. *cremoris* E were amongst the slow strains even if their D_{max} values were about 60 mV since they were characterized by a higher $E_h t_i$.

Therefore, strains were divided on the basis of their reducing profiles into three groups. After the pH 5.4 was reached, some strains reduced the redox potential to values around -120 mV in about 3 h, other strains showed a slower reduction reaching the equilibrium in more than 4 h. Other strains maintained a positive redox potential during the whole experiment despite their growth and acidification of CCE.

These results are in agreement with previous studies that have reported the ability of bacteria to modify redox potential of a medium. In particular, difference in redox capacity among strains of *Lactobaciulls delbrueckii* subsp. *bulgaricus* in skim milk at 37°C over 7 h were studied by Carrasco et al. (2005). Another study by Brasca et al. (2007) on the evolution of redox potential over time in milk fermented by LAB, found differences in redox potential at species level and also within the same species.

Three of the strains analysed in this study (*L. lactis* subsp. *lactis* C10, *L. lactis* subsp. *cremoris* S2 and C) were also studied in Chapter 3 where their influence on the milk E_h during Cheddar cheesemaking temperature profile simulation was evaluated. The trend in redox potential of each strain were the same as the one reported in this Chapter. *L. lactis* subsp. *lactis* C10 decreased the E_h to values lower than *L. lactis* subsp. *cremoris* C and in a shorter time whereas *L. lactis* subsp. *cremoris* S2 kept the redox potential at positive values during the experiment. These strains were also used to produce single strains Cheddar cheeses and the redox potential during ripening and the volatile compounds produced at 4 month of ripening were measured (Chapter 3). The redox potential of the cheeses was negative in all the cheeses during ripening. However, at 1 and 14 days of ripening, E_h of the cheese made with *L. lactis* subsp. *cremoris* S2 was significantly higher compared to the E_h of the cheeses made with *L. lactis* subsp. *lactis* C10.

4.3.2   Volatile analysis

One strain from each group was chosen and analysed in triplicate for volatile profiles at the end of growth under simulation of cheese pressing. A total of 77

compounds were detected by GC-MS and Table 4.4 shows the volatile compounds involved with the aroma profiles of strains analysed and their peak intensities normalized to the internal standard, the retention index (RI) and the odour associated with each compound.

Among the acids compounds identified by GC-MS, hexanoic and octanoic acids were the most abundant in all the strain. There were not significant differences among the quantities of each acid compound identified in each strain. 2,3-Butanediol and 2-ethyl-4-penten-2-ol were the alcohol compounds with a significant higher concentration in the strain C10 then S2 and C. 2,3-Butanediol is one of the products of citrate metabolism by LAB and it is an important flavour compound in cheese (Wilkinson and Kilcawley, 2007). Strain S2 had a significantly higher content of 2,5-dimethyl-2,5-hexanediol compared to strains C and C10. Among the aldehydes compounds identified, benzeneacetaldehyde was present in strain S2 in a significantly higher concentration compared to the other two strains. The quantity of the two amines identified did not differ among the strains. Between the ketone compounds identified, 2-pentanone was present in significantly higher concentration in CCE containing strain S2 compared to strain C and C10. Moreover, strain S2 had a significantly higher content of 2-hexanone and 3-ethyl-2-pentanone compared to strain C10. Hydrocarbon components like undecane and 3-methyl-decane were present in high quantity in the three strains. Strain S2 was significantly richer in prophyl-cyclopropane compared to strain C and undecane and 3-methyl-decane were present in significantly higher content in strain C 10 compared to strain S2. The volatile profile of the strain C10 was characterized by a significantly high content of 1-butanol-2-methyl propanoate, 3-octyl acetate, ethyl propionate and

methyl butanoate compared to strain S2. Two sulfur compounds were identified in

the volatile profile of the three strains and their contents were no significant.

Table 4.4   Volatile compounds produced by *Lactococcus lactis* subsp. *lactis* C10, *Lactococcus lactis* subsp. *cremoris* S2, *Lactococcus lactis* subsp. *cremoris* C in CCE at the end of growth under simulated Cheddar cheese pressing conditions identified by SPME GC-MS. Compound odour description, Kovats retention index (RI), averaged peak area normalized to the internal standard and standard deviation are reported in the table.

Volatiles compounds	RI	C		C10		S2		Odour description
Acid compounds								
hexanoic acid	974	0.054 [a]	(0.047)	0.113 [a]	(0.023)	0.030 [a]	(0.044)	Cheese rind, grated cheese, sweaty, goaty [ς]
benzoic acid	1150	0.010 [a]	(0.011)	0.015 [a]	(0.007)	0.002 [a]	(0.003)	Balsamic [φ]
octanoic acid	1173	0.055 [a]	(0.057)	0.124 [a]	(0.019)	0.050 [a]	(0.021)	Body odour, sweat [ς]
2-methyl-hexanoic acid	1009	0.005 [a]	(0.007)	0.000 [a]	(0.000)	0.000 [a]	(0.000)	Cheesy [φ]
2-methyl-butanoic acid	811	0.002 [a]	(0.002)	0.001 [a]	(0.002)	0.002 [a]	(0.003)	Acidic, fruity, dirty, cheesy with a fermented nuance [φ]
butanoic acid	775	0.000 [a]	(0.000)	0.013 [a]	(0.023)	0.000 [a]	(0.000)	Sharp, dairy-like, cheesy, buttery with a fruity nuance [φ,ς]
2-methyl-heptanoic acid	1109	0.000 [a]	(0.000)	0.000 [a]	(0.000)	0.001 [a]	(0.002)	Waxy, green, cheesy and sweaty [φ]
Alcohol compounds								
2,3-butanediol	743	0.087 [a]	(0.032)	0.337 [b]	(0.134)	0.114 [a]	(0.046)	Fruity creamy buttery [φ]
3,4-dimethyl-1-pentanol	832	0.006 [a]	(0.005)	0.001 [a]	(0.002)	0.006 [a]	(0.010)	-
3-methyl-4-penten-2-ol	706	0.004 [a]	(0.004)	0.009 [a]	(0.015)	0.002 [a]	(0.003)	-

2-ethyl-1-hexanol	995	0.184 [a]	(0.063)	0.380 [b]	(0.062)	0.186 [a]	(0.023)	Sweet fatty fruity [φ]
1-dodecanol	1457	0.005 [a]	(0.005)	0.000 [a]	(0.000)	0.000 [a]	(0.000)	Earthy soapy waxy fatty honey coconut [φ]
2,3-hexanediol	942	0.000 [a]	(0.000)	0.003 [a]	(0.003)	0.000 [a]	(0.000)	-
1-nonanol	1159	0.019 [a]	(0.017)	0.063 [a]	(0.031)	0.021 [a]	(0.019)	Fatty, green [ʃ]
4,5-octanediol	1141	0.000 [a]	(0.000)	0.003 [a]	(0.003)	0.002 [a]	(0.002)	-
2,6-dimethyl-4-heptanol	950	0.000 [a]	(0.000)	0.000 [a]	(0.000)	0.019 [a]	(0.018)	mild fresh ethereal fermented yeasty [φ]
2,5-dimethyl-2,5-hexanediol	1000	0.000 [a]	(0.000)	0.000 [a]	(0.000)	0.012 [b]	(0.006)	-
Aldehydes								
Hexanal	806	0.061 [a]	(0.024)	0.044 [a]	(0.016)	0.077 [a]	(0.081)	Green, slightly fruity [ʃ]
Nonanal	1104	0.147 [a]	(0.008)	0.092 [a]	(0.007)	0.147 [a]	(0.059)	Green, tallowy, animals [ʃ]
2,4-dimethyl-pentanal	777	0.000 [a]	(0.000)	0.000 [a]	(0.000)	0.004 [a]	(0.005)	
benzeneacetaldehyde	1081	0.000 [a]	(0.000)	0.000 [a]	(0.000)	0.076 [b]	(0.022)	Honey, sweet, floral, chocolate and cocoa, with a spicy nuance [φ]
Amines								
2-octanamine	995	0.051 [a]	(0.037)	0.006 [a]	(0.011)	0.054 [a]	(0.049)	-
isopropylamine	498	0.000 [a]	(0.000)	0.000 [a]	(0.000)	0.001 [a]	(0.002)	Fishy [φ]
Esters								
propyl propanoate	2085	0.283 [a]	(0.080)	0.672 [a]	(0.311)	0.185 [a]	(0.098)	Mild fatty [φ]
1-butanol 2-methyl- propanoate	785	0.672 [a,b]	(0.122)	0.952 [b]	(0.200)	0.263 [a]	(0.124)	Sharp, chemical, pungent with sweet fruity lift notes [φ]
3-octyl acetate	920	0.072 [b]	(0.034)	0.135 [b]	(0.041)	0.048 [a]	(0.016)	-
ethyl propionate	1118	1.417 [a,b]	(0.148)	0.134 [b]	(0.078)	0.037 [a]	(0.006)	Earthy [φ]
methyl tetradecanoate	709	0.066 [b]	(0.058)	0.112 [a]	(0.043)	0.079 [a]	(0.025)	Fruity [φ]
methyl hexadecanoate	1680	0.393 [a]	(0.105)	0.518 [a]	(0.125)	0.369 [a]	(0.136)	Fatty waxy peta [φ]

methyl decanoate	1878	0.001 [a]	(0.001)	0.000 [a]	(0.000)	0.000 [a]	(0.000)	Oily waxy fatty [φ]
methyl oleate	1282	0.130 [a]	(0.016)	0.116 [a]	(0.103)	0.084 [a]	(0.097)	Oily wine fruity floral [φ]
2-methylpropyl propanoate	820	0.002 [a]	(0.002)	0.010 [a]	(0.009)	0.002 [a]	(0.003)	Fruity green ether sweet tutti frutti banana [φ]
methyl butanoate	686	0.005 [a,b]	(0.000)	0.010 [b]	(0.004)	0.001 [a]	(0.002)	Fruity [φ]
propyl acetate	686	0.002 [a]	(0.003)	0.001 [a]	(0.002)	0.001 [a]	(0.001)	Fruity [φ]
propane-1,1-diol diacetate	988	0.002 [a]	(0.003)	0.013 [a]	(0.013)	0.001 [a]	(0.002)	-
butyl butyrate	920	0.004 [a]	(0.007)	0.013 [a]	(0.013)	0.002 [a]	(0.003)	Fruity [φ]
methyl tridecanoate	1580	0.009 [a]	(0.008)	0.024 [a]	(0.007)	0.008 [a]	(0.008)	-
tert-butyl formate	698	0.005 [a]	(0.009)	0.014 [a]	(0.013)	0.009 [a]	(0.008)	-
2-propyn-1-ol acetate	683	0.000 [a]	(0.000)	0.000 [a]	(0.000)	0.003 [a]	(0.003)	-
Hydrocarbons								
D-limonene		0.015 [a]	(0.013)	0.051 [a]	(0.010)	0.010 [a]	(0.053)	Sweet, citrus and peely [φ]
3-methyl-decane	1051	0.077 [a,b]	(0.007)	0.122 [a]	(0.045)	0.052 [b]	(0.017)	-
2,4,4-trimethyl-hexane	767	0.011 [a]	(0.010)	0.000 [a]	(0.006)	0.004 [a]	(0.000)	-
4-methyl-decane	1051	0.017 [a]	(0.015)	0.000 [a]	(0.012)	0.007 [a]	(0.000)	-
Undecane	1115	0.046 [a,b]	(0.013)	0.068 [a]	(0.003)	0.038 [b]	(0.011)	-
propyl-cyclopropane	620	0.004 [a]	(0.008)	0.029 [a,b]	(0.005)	0.016 [b]	(0.013)	-
3-ethyl-4-methyl-hexane	788	0.000 [a]	(0.000)	0.005 [a]	(0.005)	0.003 [a]	(0.009)	-
Hexane	788	0.000 [a]	(0.000)	0.020 [a]	(0.000)	0.000 [a]	(0.019)	-
2,2,6-trimethyl-octane	966	0.000 [a]	(0.000)	0.021 [a]	(0.000)	0.000 [a]	(0.024)	-
2,2-dimethyl-decane	1130	0.000 [a]	(0.000)	0.001 [a]	(0.000)	0.000 [a]	(0.001)	-
2,5,9-trimethyl-decane	1121	0.000 [a]	(0.000)	0.014 [a]	(0.010)	0.010 [a]	(0.024)	-
1,3-dimethyl-cyclopentane	722	0.000 [a]	(0.000)	0.000 [a]	(0.003)	0.004 [a]	(0.000)	-
3-ethyl-octane	951	0.006 [a]	(0.010)	0.000 [a]	(0.012)	0.014 [a]	(0.000)	-

Decane	1015	0.010 [a]	(0.009)	0.000 [a]	(0.010)	0.012 [a]	(0.000)	-
3,3-dimethyl-hexane	732	0.005 [a]	(0.003)	0.006 [a]	(0.005)	0.005 [a]	(0.006)	-
Ketones								
2-hexanone	754	0.032 [a,b]	(0.010)	0.027 [a]	(0.014)	0.063 [b]	(0.014)	Ethereal [φ]
2-methyl-1-penten-1-one		0.040 [a]	(0.017)	0.023 [a]	(0.011)	0.077 [a]	(0.032)	-
3-ethyl-2-pentanone	789	0.018 [a,b]	(0.003)	0.006 [a]	(0.004)	0.028 [b]	(0.008)	-
4-heptanone	853	0.017 [a]	(0.007)	0.000 [a]	(0.000)	0.017 [a]	(0.015)	fruity cheese sweet cognac pineapple [φ]
3-heptanone	853	0.090 [a]	(0.014)	0.099 [a]	(0.039)	0.150 [a]	(0.104)	Fruity ketonic, sweet with a musty cheese-like note [φ]
2-heptanone	853	0.043 [a]	(0.019)	0.031 [a]	(0.008)	0.054 [a]	(0.019)	Fruity, fatty, spicy, herbaceous, animals, blue cheese [ς]
Cyclohexanone	891	0.058 [a]	(0.022)	0.047 [a]	(0.013)	0.079 [a]	(0.017)	Minty [φ]
Butyrolactone	825	0.006 [a]	(0.004)	0.000 [a]	(0.000)	0.010 [a]	(0.009)	Creamy, oily with fatty nuances [φ]
2-nonanone	1052	0.031 [a]	(0.022)	0.154 [a]	(0.087)	0.083 [a]	(0.119)	Malty, fruity, hot milk [ς]
6-methyl-3-heptanone	888	0.035 [a]	(0.031)	0.012 [a]	(0.020)	0.042 [a]	(0.072)	-
Acetone	455	0.005 [a]	(0.005)	0.000 [a]	(0.000)	0.003 [a]	(0.005)	Wood pulp, hay [ς]
3,4-hexanedione	1039	0.008 [a]	(0.003)	0.004 [a]	(0.003)	0.005 [a]	(0.004)	Buttery toasted almond nutty caramellic [φ]
2-octanone	952	0.004 [a]	(0.005)	0.007 [a]	(0.006)	0.002 [a]	(0.004)	Fruity, hearty [ς]
2-butanone	555	0.000 [a]	(0.000)	0.000 [a]	(0.000)	0.001 [a]	(0.002)	Butterscotch [ς]
2-ethyl-cyclohexanone	1051	0.000 [a]	(0.000)	0.000 [a]	(0.000)	0.000 [a]	(0.000)	-
3,3-dimethyl-cyclobutanone	786	0.000 [a]	(0.000)	0.001 [a]	(0.001)	0.010 [a]	(0.013)	-
2-pentanone	845	0.000 [a]	(0.000)	0.003 [a]	(0.003)	0.014 [b]	(0.004)	Orange peel, sweet, fruity [ς]
3-hexanone	754	0.004 [a]	(0.007)	0.005 [a]	(0.009)	0.036 [a]	(0.020)	Sweet, fruity, waxy and diffusive [φ]
2,3-heptanedione	989	0.000 [a]	(0.000)	0.005 [a]	(0.005)	0.000 [a]	(0.000)	Butter cheese oily [φ]

3-octanone	952	0.000 [a]	(0.000)	0.016 [a]	(0.027)	0.000 [a]	(0.000)	Musty, mushroom, ketonic, moldy and cheesy fermented with a green, vegetative nuance Φ
Sulfur compounds								
dimethyl sulfide	727	0.011 [a]	(0.010)	0.024 [a]	(0.007)	0.042 [a]	(0.058)	Sulfurous Φ
methyl thiocyanate	915	0.071 [a]	(0.065)	0.150 [a]	(0.021)	0.038 [a]	(0.066)	-

Φ Odour descriptions from www.thegoodscentscompany.com

ς Odour descriptions from Curioni and Bosset (2002)

Different superscript lowercase letters denote significant differences between strains for each volatile compound (Tukey's, $P < 0.05$).

PCA was performed on the concentration of compounds reported in Table 4 and it separated the three strains selected. The first and the second component explained 42.44 and 34.56% of the variation in the data, respectively (Figure 4.4).
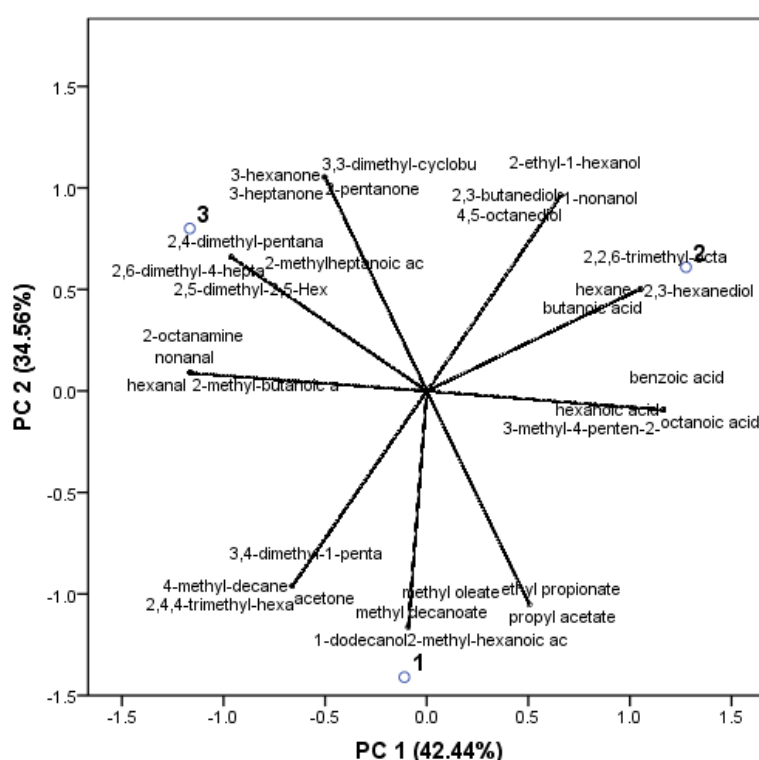
Figure 4.4   Principal component analysis of the volatile compounds measured at the end of the simulation of Cheddar cheese pressing stage in Cheddar cheese extract (CCE) produced by three strains of LAB showing different trends in redox potential. Bi-plot showing the strain scores and volatile compound loadings. 1, *Lactococcus lactis* subsp. *cremoris* C; 2, *Lactococcus lactis* subsp. *lactis* C10; 3, *Lactococcus lactis* subsp. *cremoris* S2.

To correlate the volatile profiles of the strains C, C10 and S2 to the classes of compounds identified (alcohols, acids, aldehydes, ketones, amines, hydrocarbons, esters and sulfur compounds) a second PCA (Figure 4.5) was performed on the total percentage of the volatile of each class of compounds for each strain. The first and the second component explained 52.02 and 30.12% of the variation in the data, respectively.
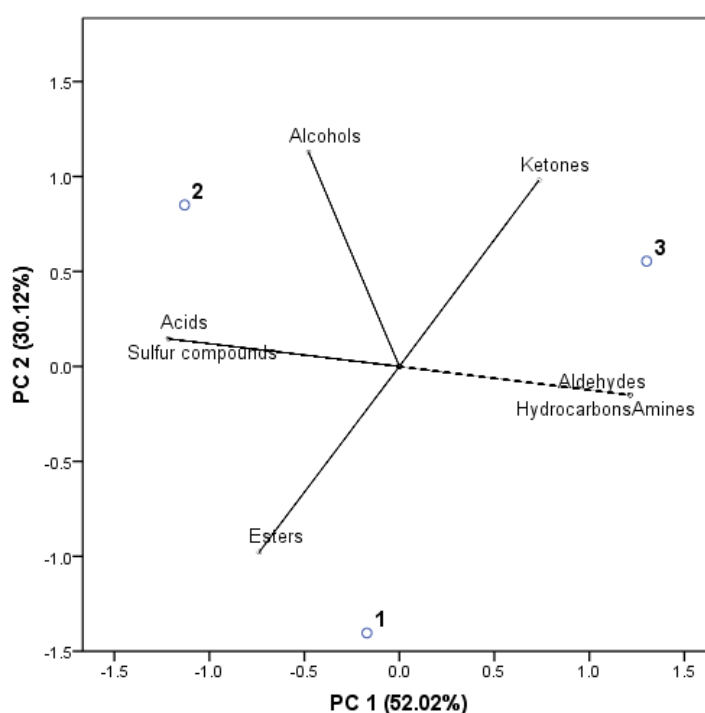
Figure 4.5   Principal component analysis of the volatile classes of compounds measured at the end of the simulation of Cheddar cheese pressing stage in Cheddar cheese extract (CCE) produced by three strains of LAB showing different trends in redox potential. Bi-plot showing the strain scores and volatile classes of compounds loadings. 1, *Lactococcus lactis* subsp. *cremoris* C; 2, *Lactococcus lactis* subsp. *lactis* C10; 3, *Lactococcus lactis* subsp. *cremoris* S2.

Strain S2 maintained the redox potential at positive values during the cheese pressing stage temperature profile in CCE and it was correlated with aldehyde, ketone and hydrocarbon compounds. Aldehyde compounds have been previously associated with oxidative conditions in an *in vitro* study by Kieronczyk et al. (2006) on the influence of redox potential on amino acid conversion to volatile compounds by *Lactococcus lactis*. Moreover, in a study by Martin et al. (2011) the production of aldehydes and ketones (like acetaldehyde and diacetyl) was associated to positive redox potential condition in yogurts and low level of dimethylsulfide were found.

Strain C10 reduced the redox potential to negative values in a short time in CCE during the experiments and it was mainly associated with sulfur compounds and to a lesser extent with acid and alcohol compounds. The production of sulfur compounds have been linked to a negative redox potential in good quality Cheddar cheese (Green and Manning, 1982; Kristoffersen, 1985) and in non-fat yoghurt (Martin et al., 2011).

Moreover, PC1 in Figure 4.5 clearly separate strains C and C10 that reduced the redox potential of CCE to negative values from strain S2 that kept the redox potential of CCE to positive values during the experiment indicating that different volatile compounds could be produced under different redox potential conditions produced by the utilization of different strains of LAB.

The three strains analysed in this study for volatile compounds were also studied in Chapter 3 where single strains Cheddar cheeses were produced and

analysed for redox potential during ripening and the volatile compounds produced at 4 month of ripening. Also in Chapter 3, PCA of the volatile compounds produced in Cheddar cheese clearly separated strains C and C10 from S2. In Cheddar cheese, strain S2 was associated with more alcohol compounds but also acids, aldehydes and to a less extent to sulfur compounds. Whereas strain C10 was associated with sulfur compounds in cheese (Chapter 3) as found in CEE in this study.

4.4   Conclusion

Lactic acid bacteria used for dairy fermentations are the most significant component of the final quality of the products therefore improvements in the knowledge of their characteristics will help optimize starter formulations.

Previous studies had demonstrated that redox potential is a characteristic parameter of a species and typical trends in redox potential have been found for different strains grown in various media (Aubert et al., 2002; Boucher et al., 2006; Brasca et al., 2007; Cachon et al., 2002; Carrasco et al., 2005).

In this study, attention was focused on *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* because of their importance to cheesemaking and a Cheddar cheese extract was used model to simulate as close as possible the conditions in Cheddar cheese at the pressing stage. Previous work from our laboratory demonstrated that significant changes in redox potential occurs throughout the manufacture steps of Cheddar cheese (Caldeo and McSweeney, 2012) and in particular during the pressing stage (McSweeney, 2010) and that redox potential remains constant during much of ripening. Thus, we hypothesised that the drop in redox potential can be due to the starter used during manufacture and we designed our experiment to mimic the pressing stage of Cheddar in the CCE. Moreover, the volatile profile of CCE inoculated with three strains with different redox activity was investigated.

The strains of LAB selected for this study were able to acidify CCE over time to similar values and they showed similar growth rates. Therefore, modification of redox potential during microorganism growth is not only related to the fermentation of lactose by starters. Our results demonstrate that redox potential is a strain-specific characteristic (Cachon et al., 2002; Tachon, 2009; Brasca et al., 2007) and starting from the same redox potential conditions it is possible to modify the redox of the media by the addition of different strains.

Moreover, the development specific flavour compounds seems to be associated with the redox potential of the medium. The three stains selected from each of the group identity in this study based on the redox potential trend in CCE and analysed for flavour development were associated with different flavour compounds. Therefore, the selection of strains with a specific ability to influence redox potential could be used to control the aroma of dairy products and gives desired flavour. However, we cannot state that the strains selected for volatile analysis were representative of each redox potential group identified in our experiments.

4.5   Acknowledgments

4.6   References

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Chapter 5

Changes in oxidation-reduction potential in cheese during ripening

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Abstract

In Cheddar cheese, the main changes in redox potential occurs during the pressing stage of manufacture. In this work, a strategy to follow the changes in redox potential during the pressing stage and during the first month of ripening was developed. Redox potential reached an equilibrium value within two hours of pressing and values were constant during the first month of ripening. Redox potential was also measured in blocks of Cheddar cheese at 1, 30, 90 and 180 days of ripening in different locations of the block. Redox potential was significantly lower at day 1 than at day 180 of ripening and the values were uniform across the block. In addition, the redox potential of Emmental cheese was followed from the salting stage throughout the first stages of ripening. Redox potential slowly decreased to negative values in the first days of ripening and an equilibrium value was reached at about 20 days of ripening. Redox potential of commercial cheeses was also measured in this study and differences in redox potential and in the time needed to reach an equilibrium value were found between the varieties analysed. From our results, it was found that measurement of redox potential in cheese could be used to give information on the quality of the product at different stages of ripening.

Keywords: oxidation-reduction potential, cheese, ripening.

5.1   Introduction

Oxidation-reduction (redox) potential (E_h) is an important physicochemical parameter in dairy products that deserves more attention. Redox potential could be useful to select the microflora used in dairy product manufacture (Keen, 1972; Scarinci et al., 1994; Bolduc et al., 2006; Boucher et al., 2006; Morandi et al., 2006; Brasca et al., 2007) and to help to control the physico-chemical and sensorial qualities of fermented dairy products (Aubert et al., 2002; Broadbent at al., 2002; Cachon et al., 2002).

Measurement of redox potential in cheese is not easy. Redox potential can be measured potentiometrically by using a reference electrode and a metal electrode connected through a sensitive potentiometer (Skoog et al., 2004). Care of the electrodes and accuracy on the measurements are fundamental to obtain reproducible results.

In cheese the electrodes can be inserted directly into the matrix (Davis, 1932; Topcu et al., 2008). However, the measurement could be altered by the presence of cracks in the cheese (Davis, 1932) or due to the shape of the electrodes (Sawyer et al., 1995) that could cause air penetration and consequently alter the readings. Some authors (Ouvry et al., 2001; Abraham et al., 2007) have used microelectrodes to prevent air penetration into the cheese. Topcu et al. (2008) established the optimum conditions for redox measurements in hard cheeses and the authors found that in Cheddar cheese about 2-3 days are needed to reach a E_h equilibrium value (-120 mV) and that the equilibration time needed can also be affected by the distance between the reference and working electrodes. The redox potential of cheese is very reducing largely due to the presence of obligatory or facultatively anaerobic microbes in the interior part of cheese (Beresford et al., 2001). On the contrary, the redox potential of milk is positive and,

depending on the oxygen content of milk, values between +250 and +350 mV are reported in literature (Walstra and Jenness, 1984; Sherbon, 1988; Vahcic et al., 1992; Morris, 2000; McCarthy and Singh, 2009). In our previous study (Caldeo and McSweeney, 2012), we followed E_h during cheesemaking to understand the mechanisms involved in the E_h changes from the positive values of milk to the negative values of cheese. During simulated manufacture of Cheddar, a significant drop in redox potential occurred when the whey was drained until the milling stage where the cheese-curd was cut causing an increase in E_h due to air penetration into the matrix. After salting the cheese-curd was pressed and during the overnight pressing the values of E_h decreased again to values close to those of mature Cheddar cheese. In the same study, E_h during simulated cheesemaking of Gouda, Emmental and Camembert cheeses was followed and differences were found due to the difference in manufacture steps and starter lactic acid bacteria used. Negative E_h values were found at the end of simulated manufacture for Cheddar, Gouda and Camembert cheeses, whereas Emmental had a positive E_h, probably due to the slow reducing capacity of *Lactobacillus helveticus* (Brasca et al., 2007) and slow acidification during cheesemaking.

Together with cheese structure and manufacture procedures, another parameter that can influence the redox potential of cheese could be the internal microflora (Beresford et al., 2001). Starter bacteria reduces the internal redox potential of cheeses by fermenting residual lactose (Fox et al., 2000; van Dijk et al., 2000; Beresford et al., 2001; Aubert et al., 2002; Cachon et al., 2002), by consuming oxygen (Ouvry et al., 2001) and by producing reducing groups like sulfhhydryl compounds (Ouvry et al., 2001). Moreover, the growth of non-starter lactic acid bacteria maintains a reducing environment in cheese during ripening (Boucher et al., 2006) and a low redox potential

seems fundamental to provide the anaerobic conditions required for a balanced flavour development in cheese (Singh et al., 2003; Carrasco et al., 2005; Morandi et al., 2006).

The objective of this study was to investigate the changes in redox potential during cheese ripening and the redox potential of mature cheeses. Therefore, strategies to measure E_h in early stages of Cheddar cheese ripening were developed. Furthermore, the redox potential of commercial Cheddar cheeses was followed during ripening to evaluate the changes due to the aging of the cheese and within the different location in the same cheese block and the redox potential of a commercial Emmental cheese was followed from the pressing stage throughout the cheese ripening.

In addition, redox potential of four commercial cheeses, Cheddar, Gouda, Emmental and Camembert, was measured to evaluate differences between cheese varieties.

5.2   Materials and methods

5.2.1   Measurement of oxidation-reduction potential in Cheddar cheese in early stage of ripening

Cheddar cheese was made in the food processing facilities at University College of Cork, Ireland, according to standard Cheddar cheese-making procedures. Raw bovine milk was standardized to a casein to fat ratio of 0.7:1 and HTST-pasteurized (73.5°C, 15 s). One open vat was filled with approximately 50 L of milk at 31°C and inoculated at a rate of 0.02% (w/v) with a concentrated starter culture (DVS R604, Chr. Hansen, Hørshom, Denmark). After a 30 min ripening period, the milk was supplemented with 1 M CaCl$_2$ (0.9 mL/L). Coagulant (Chymax-180, Chr. Hansen, Hørshom, Denmark), diluted 1:5

with distilled water, was added at a level of 0.3 mL/L. When the coagulum reached the desired firmness after 40-50 min, the curd was cut and allowed to heal for 10 min. The temperature was raised to 39°C over 30 min and the cheese milk stirred at 39°C until a pH of 6.2 was obtained. The whey was then drained and the curd was cut into large blocks and these were inverted every 15 min until pH reached 5.8 when the blocks were stacked on top of each other. After reaching a pH of 5.4, the curd was milled. The curd pieces were salted with 2.5% (w/w) of NaCl and after 20 min, the curd was moulded and the redox potential measurement started.

To measure the redox potential during the pressing stage of Cheddar cheese manufacture, miniature platinum electrodes were manufactured in our laboratory using 1 mm diameter platinum wire (Sigma-Aldrich, St. Louis, MO, USA). Bright platinum wire was soldered with silver to copper wire, and it was coated with tube that adhere to the wire when heated (FP-301, 3M™, St. Paul, MN, USA). Two tubes of different diameters were used to coat the electrodes and epoxy glue was used to secure the junction between the tube and the wire. The final diameter of the platinum electrode was 0.5 cm (Figure 5.1A). As reference a commercially available miniature Ag/AgCl reference electrode (DRIREF-5SH, World Precision Instruments Inc., Sarasota, FL, USA) was placed in agar containing saturated KCl gelled in plastic tubes with holes drilled in the sides (Figure 5.1A). Electrodes were cleaned following the procedure reported by Topcu et al. (2008), Caldeo & McSweeney (2012) and Abraham et al. (2013) and the accuracy was measured against a standard solution (470 mV, HI 7022, Hanna Instruments, Szeged, Hungary) and against tap water (Abraham at al., 2007; Jeanson et al., 2009; Martin et al., 2011) at 25°C before and at the end of each experiment.

The reference electrode and three manufactured platinum electrodes were embedded into the cheese by placing them into the mould (Figure 5.1B) and pressing them together with the milled and salted cheese pieces (Figure 5.1C) at 490 kPa overnight at room temperature. After pressing, the 5 kg block of cheese was vacuum packed by passing the leads from the electrodes through the vacuum packing bag and then sealing (Figure 5.1D). Redox potential was recorded during about one month of ripening at 8°C and the experiment was performed in triplicate. Redox potential electrodes were connected to a pH meter (PHM210 Standard pH Meter, Radiometer Analytical) and data were recorded without reference to a hydrogen reference electrode and converted to E_h (potential related to the standard hydrogen electrode) according to Sawyer et al. (1995) and Caldeo and McSweeney (2012) with temperature compensation.

A

B

C

D
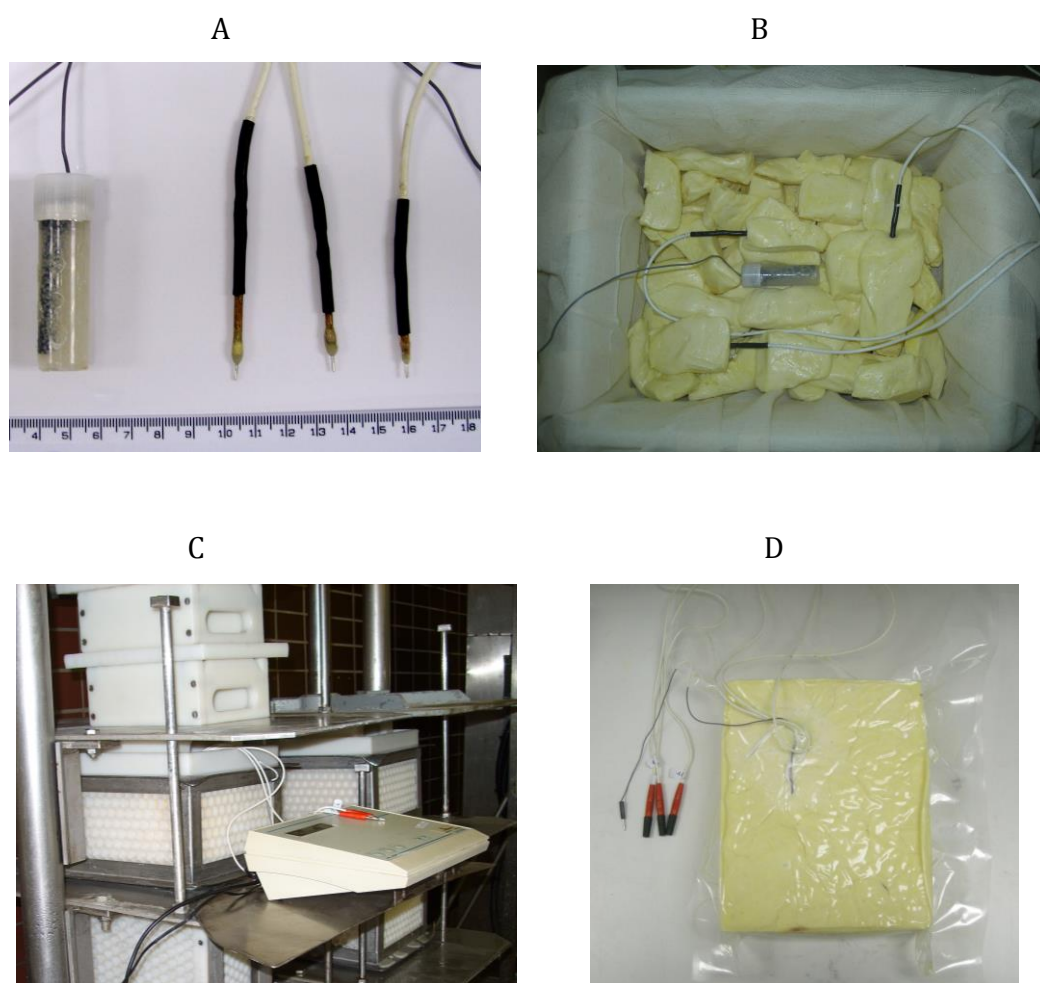
Figure 5.1 Apparatus used to measure the redox potential of Cheddar cheese during early stages of ripening. A. A perforated plastic tube (left) containing miniature Ag/AgCl reference electrodes surrounded by KCl gelled in agar as an electrolyte and (right) three miniature platinum electrodes. B. Electrodes embedded into the salted cheese curd. C. Redox potential measurement during the pressing stage of manufacture. D. Block of Cheddar vacuum sealed after overnight pressing.

5.2.2   Oxidation-reduction potential in cheese

5.2.2.1 Measurement of oxidation-reduction potential in Cheddar cheese during ripening

Redox potential and pH were measured on four blocks of commercial Cheddar cheese of mass 20 kg each (36.5 cm (L), 28 cm (W), 18 cm (H)) at 1, 30, 90 and 180 days of ripening. At each sampling point, a block was trimmed and a 10 cm slice was cut diagonally across the block (Figure 5.2A). Five different locations across this diagonal slice were taken for E_h measurement such that the exterior and interior locations of the block were represented (Figure 5.2B).

A   B
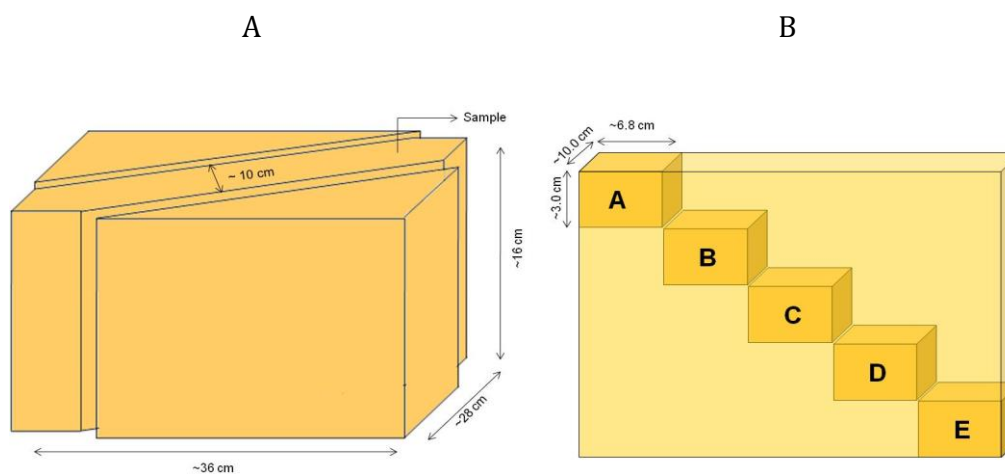
Figure 5.2   Schematic representation of a cheese block showing a 10 cm slice cut diagonally across the block (A). Five different locations across this diagonal slice were taken for redox potential measurements and the five blocks are indicated with letters A, B, C, D and E (B).

Oxidation-reduction potential was measured in cheese following the method reported by Topcu et al. (2008). A platinum working electrode (R-XM110) and silver/silver chloride reference electrode (REF 201, Radiometer Analytical, Villeurbanne Cedex, Lyon, France) filled with saturated KCl solution (KCl-L-30, Radiometer Analytical) were used. Electrodes were cleaned and the accuracy checked by following the methods reported in Section 5.2.1. The cheese blocks were wrapped in transparent film to prevent loss of moisture. The electrodes were connected to a data logger (Squirrel Data Logger 2040-2F16 Series, Grant, Data Acquisition, Cambridge, UK) through an amplifier (PHTX-21, Omega, Stamford, CT, USA) for data acquisition. The measured data were recorded every five minutes and converted to E_h according the indications reported in Section 5.2.1.

pH of the cheeses was measured by probing the cheese directly with a combined glass electrode (PHC3001-8, Radiometer Analytical) connected to a pH meter (PHM210 Standard pH Meter, Radiometer Analytical).

5.2.2.2 Measurement of oxidation-reduction potential in Emmental cheese during ripening

One block (about 20 kg) of commercial Emmental cheese was collected from a local manufacturer (Tipperary-Coop, Tipperary Town) immediately after the brining stage of manufacture. At our laboratory, the ripening temperature profile used for Emmental cheese was mimiced and the E_h was measured. Cheese were stored at 8-10°C for 7-10 days, transferred to 24°C for 24-28 days and then ripened at 13°C. The block was then divided into three smaller blocks, vacuum packed and ripened at 10°C. Redox potential was measured on one block during the first stage of ripening, 10°C for 8 days. During

this time the cheese block was covered by a plastic coat (Paracoat Natural 0.0.70, Paramelt, Rosemead, CA, USA) to prevent loss of moisture and contamination with mould. After 10 days, the two remaining blocks were transferred to 24°C and one block, removed from the vacuum bag and covered with plastic coat, was used to measured redox potential for 24 days. After this period, the third block of Emmental cheese was transferred to 13°C, covered by plastic coat and the redox potential was measured over 5 days.

E_h measurements were done in triplicate by using three separate couples of reference and platinum electrodes placed in three different locations on each block and the method used was as in Section 5.2.2.1.

pH and microbial count were measured at the beginning of each redox potential measurement on each block. For microbial counts, cheese (5 g) was homogenised in 45 mL of 2% (w/v) tri-sodium citrate solution and decimal dilutions were prepared in tubes of sterile quarter-strength Ringer's solution tubes. Thermophilic lactic acid bacteria (LAB) were enumerated in duplicate on LM17 agar (Merck, Darmstadt, Germany) after incubation at 30°C for 3 days. Non-starter LAB were enumerated in duplicate under anaerobic conditions (Merck Anaerocult A gas pack; Merck, Darmstadt, Germany), on Rogosa agar (Merck) after incubation at 30°C for 5 days. And propionic acid bacteria were enumerated in duplicate on Yeastrel lactate agar under anaerobic conditions after incubation at 30°C for 6 days. The total colony forming units in cheese were calculated by counting colonies on plates with between 30 and 300 colonies.

5.2.2.3 Measurement of oxidation-reduction potential, pH, salt and moisture in commercial cheeses

Redox potential and pH of four commercial cheeses, Cheddar, Gouda, Emmental and Camembert, was measured in triplicate at room temperature using the method described in Section 5.2.2.1.

Figure 5.3 shows the settings used to measure the redox potential in cheese blocks mature Cheddar, Gouda, Emmental and Camembert. The Pt-electrode was inserted directly into a cheese block samples to a depth of 5 cm and the reference electrode (red in Figure 5.3) was placed 2.5 cm apart. In Camembert cheese the reference electrode was placed directly into the cheese block whereas, in the other cheeses, the reference electrode was placed in a hole of 4 cm deep and 1.5 cm wide filled with 3 M KCl solution as described by Topcu et al. (2008).

A   B   C   D
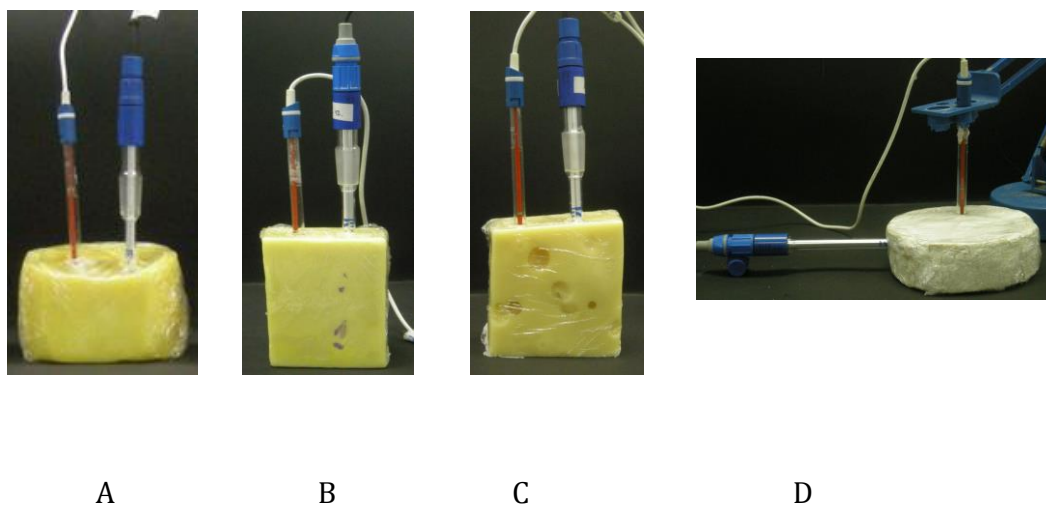
Figure 5.3   Measurement of redox potenital in commercial Cheddar (A), Gouda (B), Emmental (C) and Camembert (D) cheeses. Reference electrode (red) and platinum electrode (silver) were used to perform the measurements.

5.2.3   Statistical analysis

Analysis of variance (one-way ANOVA) was conducted using SPSS Version 20.0 for Mac OS X (SPSS Inc., Chicago, IL, USA). When differences were significant ($P < 0.05$), the means were analysed using Tukey's test.

5.3   Results and discussion

5.3.1   Oxidation-reduction potential in early Cheddar cheese ripening

Redox potential was measured during the laboratory manufacture of Cheddar cheese (cheese of about 500 g) in our previous study (Caldeo and McSweeney, 2012) and E_h

decrease during the pressing stage and reached an equilibrium value of -120 mV after 10-12 hour of pressing. The same value was found in mild Cheddar cheese by Topcu et al. (2010) after 2-3 days of measurement. Therefore, measuring changes in the redox potential of intact Cheddar cheese early in ripening is hampered by the long equilibration time necessary for platinum electrodes coupled with reference electrodes placed in a KCl salt bridge. To overcome this problem and to measure redox potential in Cheddar cheese at the early stage of ripening, miniature platinum electrodes were manufactured in our laboratory (Figure 5.1A). As reference, a miniature commercial electrode was used and the reference electrode was placed into a plastic tube with holes containing gelled KCl solution to complete the salt bridge and increase the electrodes transfer surface between electrodes.

Cheddar cheese was made and before pressing the electrodes were embedded into the salted curd pieces in the mould (Figure 5.1B) and pressed together. Redox potential was measured during the pressing stage (Figure 5.1C) thanks to the presence of holes in the cheese-mould that allowed the passages of the wires and their connection to the data logger. After overnight pressing, the cheese was vacuum sealed (Figure 5.1D) and reading of E_h continued during ripening at 8°C.

Results obtained using this approach (Figure 5.4) showed that E_h reached its equilibrium value of ca. -120 mV during overnight pressing within two hours, presumably due to continuing starter activity fermenting residual lactose to lactate and that there was relatively little change in this parameter during the early stages of ripening (to day 40). Redox potential measurements continued until 3 months of ripening, however results were erratic and not reproducible probably because the electrodes were left for too long into the cheese and the electrode transfer signal

decreased over time; therefore Figure 5.4 shows values obtained up to 40 days of ripening. Only in one study, published by Kristoffersen and Gould (1959), redox potential was measured in Cheddar cheese from the pressing stage by embedding platinum electrode into the curd; however, the work did not give details on the measurement procedure, electrodes used and on the time needed to reach an equilibrium value. Results reported by the authors showed that at the beginning of the ripening the E_h was -104 mV and E_h slightly increased to   -90 mV after 1 month. The E_h values measured in our study were slightly lower than those of Kristoffersen and Gould (1959) and did not increase as much. In a study from Davis (1932) redox potential was measured during ripening using dye indicators and the author found an initial decrease in redox potential to negative values followed by a slow increase in redox potential probably due to diffusion of oxygen in the cheese and then the redox values decreased again, possibly due to the growth of lactobacilli after one month of ripening. On the contrary to Davis (1932), in our study E_h did not increase in the first month of Cheddar cheese ripening probably because our cheeses were vacuum packed during ripening avoiding oxygen penetration into the block.
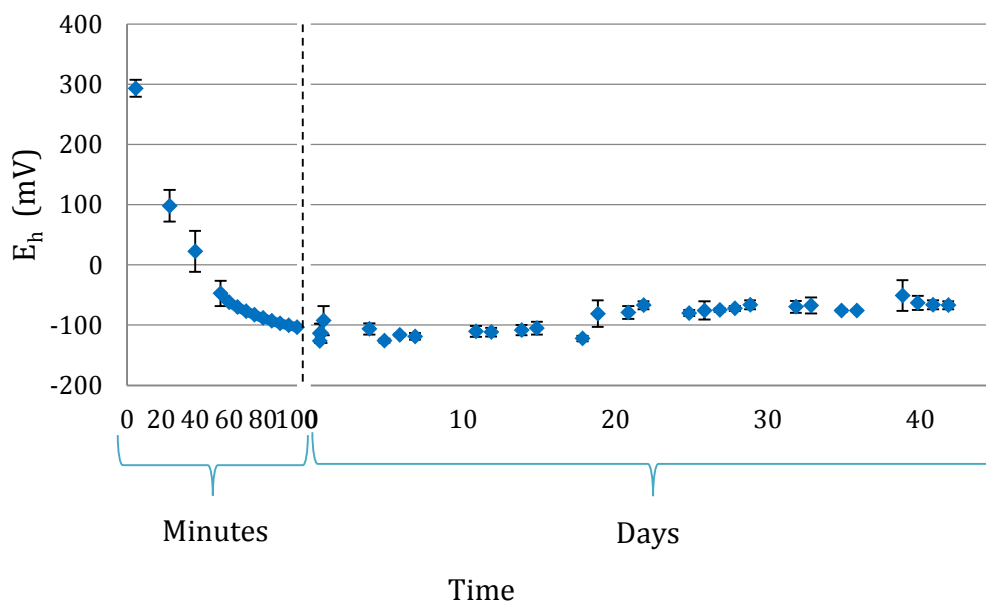
Figure 5.4   Changes in the oxidation-reduction potential (E_h) of pilot- scale cheddar cheese during pressing overnight at room temperature and the early stages of cheese ripening (8°C) (means ± standard deviations of three independent working electrodes in a single cheese block).

5.3.2   Oxidation-reduction potential during Cheddar cheese ripening

Four blocks of 20 kg each were analysed for changes in redox potential during ripening. At day 1, one block was trimmed and a 10 cm slice was cut diagonally across the block (Figure 5.1A) and redox potential was measured in five different locations across this diagonal slice (Figure 5.1B). The same procedure was repeated at 30, 90 and 180 days of ripening on each of the other three blocks. The pHs of the blocks were 5.34 (±0.06),

5.36 (±0.09), 5.38 (±0.06), 5.39 (±0.11) at day 1, 30, 90 and 180 of ripening, respectively.

Figure 5.5 shows the trends in E_h measurement recorded in the four Cheddar cheese blocks, 1, 2, 3 and 4, at day 1, 30, 90 and 180, respectively, on the five different locations indicated as A, B, C, D and E. The time reported in the graphs indicates the minutes from which the electrodes were placed into the cheese blocks. One-way anova was performed on the readings recorded at the end of the measurements (after 60 hours) and there were no significant differences between the E_h values recorded in location A, B, C, D and E during ripening. Therefore, the redox potential in Cheddar cheese was uniform throughout a commercial block and our result is in agreement with Davis (1932).
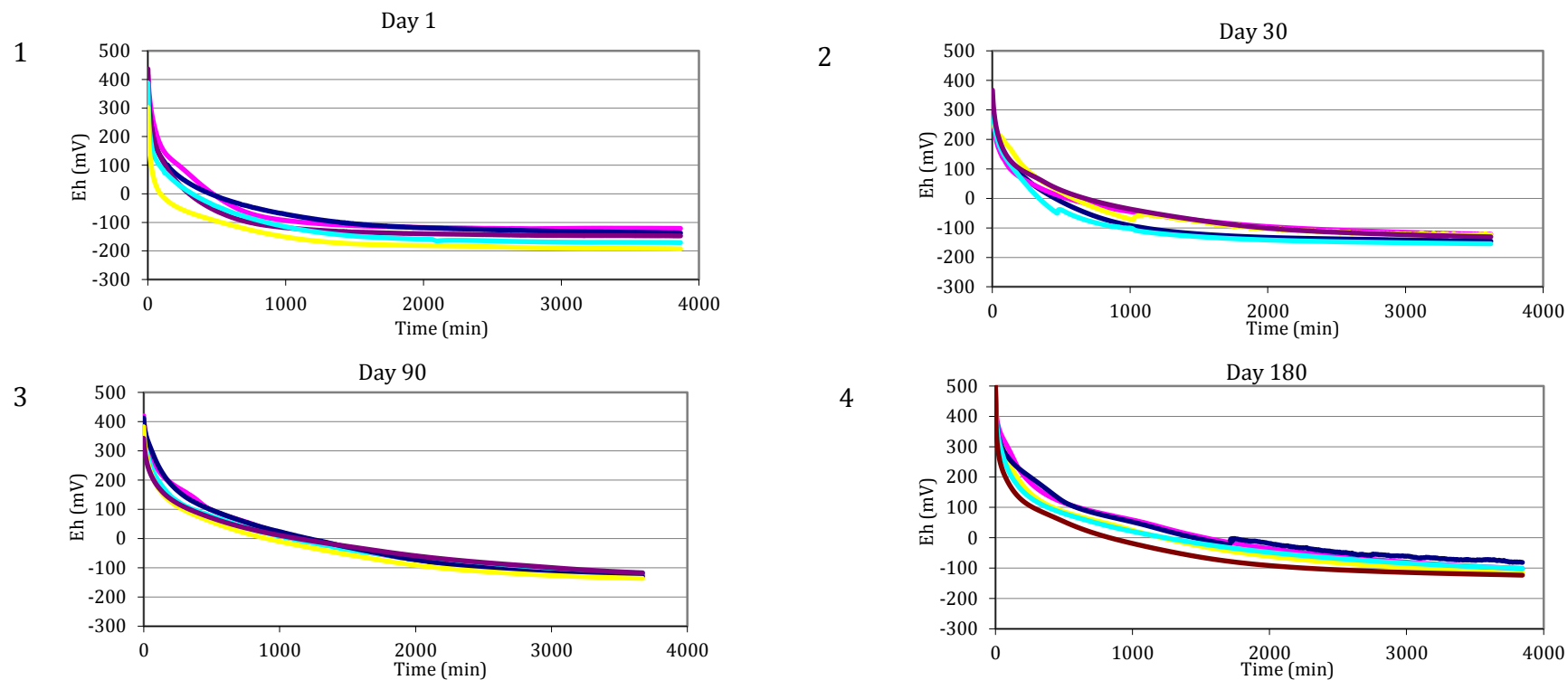
Figure 5.5　　Redox potential (E_h) measured in in four blocks of Cheddar cheese (1, 2, 3 and 4). E_h was measured in block 1 at day 1 of ripening, in block 2 at day 30 of ripening, in block 3 at day 90 of ripening and in block 4 at day 180 of ripening. Measurements in each block were done in five different position, A ▬ , B ▬ , C ▬ ,D ▬ , and E ▬ .

The five readings at each time points were averaged after 60 hours of readings and values are reported in Table 5.1. Results shown that redox potential at day 1 (-153 mV) was significantly lower compared to the values recorded at day 180 (-104 mV).

Table 5.1   Average of the five readings of redox potential (E_h) measured at equilibrium in locations A, B, C, D and E in cheese blocks at 1, 30, 90 and 180 days of ripening after 60 hours of measurements.

Ripening time (days)	E_h (mV) at 60 hours of measurement
1	-153[a] (28)
30	-136[a,b] (13)
90	-127[a,b] (8)
180	-104[b] (15)

Numbers represent mean and standard deviation, with the latter in parentheses. Different superscript lowercase letters denote significant differences between E_h measurements (Tukey's, $P < 0$).

Moreover, considering at the graphs in Figure 5.5, it is evident that the time needed to reach equilibrium values increased during ripening. At day 1, the E_h equilibrium values were reached within 33 hours whereas at day 180, 50 hours were needed before the E_h equilibrium values were achieved. One possible explanation to this differences in the time needed to reach the E_h equilibrium values could be the differences in water activity (a_w) during ripening. The cheeses analysed in this study were from the same study pubished by Hickey et al. (2013) and the authors reported that a_w decreased

significantly during ripening. The authors reported α_w values of 0.964 at day 1 and 0.958 at day 180 of ripening. The water activity could affect the interactions between ions in cheese and a decrease in α_w could delay the movement of ions between the electrodes; however, there are no studies published on the possible relationship between α_w and E_h in cheese.

In the literature, as reported in Section 5.3.1, there is only one study that followed the E_h of Cheddar cheese during ripening (Kristoffersen and Gould, 1959). The authors reported values of -104 mV at day 1, -90 mV after 1 month, and -270 mV after from 5 to 6 months. Those values differed from our findings; at 1 month of ripening E_h of our Cheddar cheese was lower (-136 mV) and at 6 months of ripening E_h of our Cheddar cheese was higher (-104 mV). Our results suggest that redox potential in Cheddar cheese is significantly lower at 1 day than at 180 days of ripening.

5.3.3   Oxidation-reduction potential during Emmental cheese ripening

The redox potential of a commercial block of Emmental cheese was followed after the brining stage of manufacture throughout the ripening stages (Figure 5.6). A big block (about 20 kg) of Emmental cheese collected from Tipperary-Coop immediately after the brining stage of manufacture was divided into three smaller blocks and the temperature during ripening was mimicked at our laboratory. The first stage of ripening consisted of keeping the cheese blocks at 8-10°C for 7-10 days, during this stage the E_h was measured in one block by using three couples of reference and platinum electrodes directly inserted into the cheese in three different location of the block.
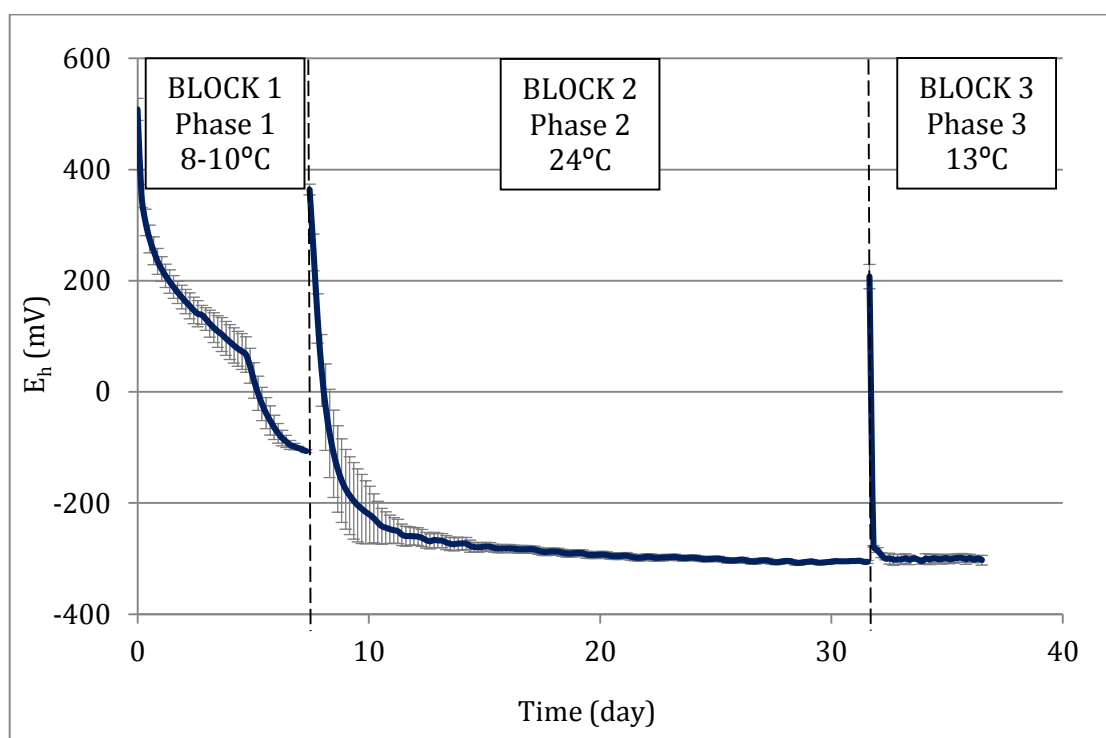
Figure 5.6   Redox potential (E_h) measured in a block of Emmental cheese collected from Tipperary-Coop immediately after the brining stage of manufacture. Measurement were performed during the mimicked ripening stages of the cheese that consisted on ripening the cheese at 8-10°C for 7-10 day (phase 1) then at 24°C for 24-28 days (phase 2) and finally at 13°C (phase 3). The block of Emmental cheese was divided into three blocks and one block at the time was used to measure E_h in each phase.

At the beginning of the E_h measurement, the pH of the block was 5.20 (0.06). E_h slowly decreased and negative values were reached after 5 days. At day 8 of ripening, values of -106 (±2) mV were reached. After 8 days, the remaining two cheese blocks were transfer to 24°C for 24-28 days for the second stage of Emmental ripening. At the start of this stage, the pH value was 5.23 (±0.02), and the starter thermophilic bacteria reached values of 7.48 log cfu/g of cheese and the non-starter bacteria were 7.85 log

cfu/g of cheese. During this second stage, the E_h decreased within one day to negative values. At day 11 of ripening, E_h reached -249 (±22) mV and E_h slightly decreased to -306 (±2) mV after 32 days of ripening. The third block of Emmental cheese was transfer to 13°C. The value of pH was 5.42 (±0.01), the starter thermophilic bacteria reached values of 5.13 log cfu/g of cheese, the non-starter bacteria were 6.50 log cfu/g of cheese and the propionic acid bacteria were 7.98 log cfu/g of cheese. E_h reached a negative value of -302 (±9) mV within 10 hours of measurement, E_h was measured for the following 5 days and not changes were found.

In our previous study (Caldeo and McSweeney, 2012), the redox potential of Emmental cheese made at laboratory scale was measured during manufacture and at the end of the brine-salting stage E_h was positive and values of about +200 mV were reached after the brining stage. Also in this study the E_h was positive for about 5 days of ripening and this could be due to the slow acidification of the cheese (Caldeo and McSweeney, 2012) and to the type of starter used in Emmental cheese manufacture. We are not aware of the starter used during Emmental cheesesmaking by the company that provided us the cheese block. However, *Lactobacillus helveticus*, *Lactobacillus delbrueckii* ssp. *lactis* and *Streptococcus thermophilus* are commonly used in Emmental cheesemaking and they have a slow reducing capacity (Brasca et al., 2007).

The negative E_h reached in the second and third stages of Emmental ripening, could be due to the development of propionic bacteria that caused propionic acid fermentation and consequence generation of the carbon dioxide from lactate that leads to the formation of the characteristic eyes in the cheese. In a previous study by Peltola and Antila (1953) the redox potential of Emmental cheese was measured during ripening

and values of about -250 mV were recorded in the first 30 days of ripening. However, after 30 days of ripening E_h increased and reached -180 mV at 90 days of ripening.

Also, Vos (1948) monitored E_h in Emmental cheese during ripening. In contrast to our results, at 5 days of ripening the author found a negative E_h (-190 mV) whereas in our measurement the E_h reached values ~ 0 mV after 5 days of ripening. The author recorded E_h also after 18 and 60 days of ripening and found values of -278 mV and -283 mV, respectively.

The E_h values recorded by Peltola and Antila (1953) at 30 days of ripening and Vos (1948) at 18 and 60 days of ripening were higher than our results and this is probably due to differences in measuring the redox potential, for example Vos (1948) used liquid paraffin around the electrodes to prevent oxygen penetration and this might have altered the results. In our measurements, the use of liquid paraffin was not necessary since the electrodes penetrate inside the cheese block without generating cracks.

A further observation that emerged from our results was that redox potential appeared to be uniform in Emmental cheese since no significant differences were observed in E_h values recorded using electrodes in different locations in the block. This theory is in contrast with Vos (1948) and Peltola and Antila (1953) who suggested that the redox potential within Emmental cheese was not uniform.

5.3.4   Oxidation-reduction potential in mature cheeses

Four commercial cheeses (Cheddar, Gouda, Camembert, and Emmental) were obtained from a local market. Three cheeses were analysed for each variety for pH and redox potential measurement.

The pH values recorded were 5.37 (±0.18), 5.80 (±0.10), 6.81 (±0.12), and 5.61 (±0.09), for Cheddar, Gouda, Camembert, and Emmental, respectively. Figure 5.7 shows the redox potential measured in each cheese from the moment at which the electrodes were placed into the cheeses over time. E_h of Cheddar, Gouda, Camembert, and Emmental cheese were -127, -153, -252, and -277 mV, respectively, and the equilibrium value was reached after approximately 60, 30, 20, and 5 hours of measurements, respectively.

The redox potential of mature Cheddar cheese was measured in previous studies (Kristoffersen et al., 1964, 1966; Topcu et al., 2008) and values similar to our measurements were found. Kristoffersen et al. (1964) measured values of -105 mV and the equilibrium was reached after 5 days of measurement, the same authors found in a later work (Kristoffersen et al., 1966) values of -116 and -136 mV in mature Cheddar cheese. Topcu et al. (2008) studied the importance of distances between electrodes to measured E_h in Cheddar cheese and the authors found E_h values of -118/-126 mV and that 2-3 days were needed to reach equilibrium values. Moreover, the values of mature Cheddar cheese were close to those obtained in Sections 5.3.1 and 5.3.2 and supported our hypothesis that the E_h in Cheddar cheese is constant after 30 days from the pressing stage throughout ripening.
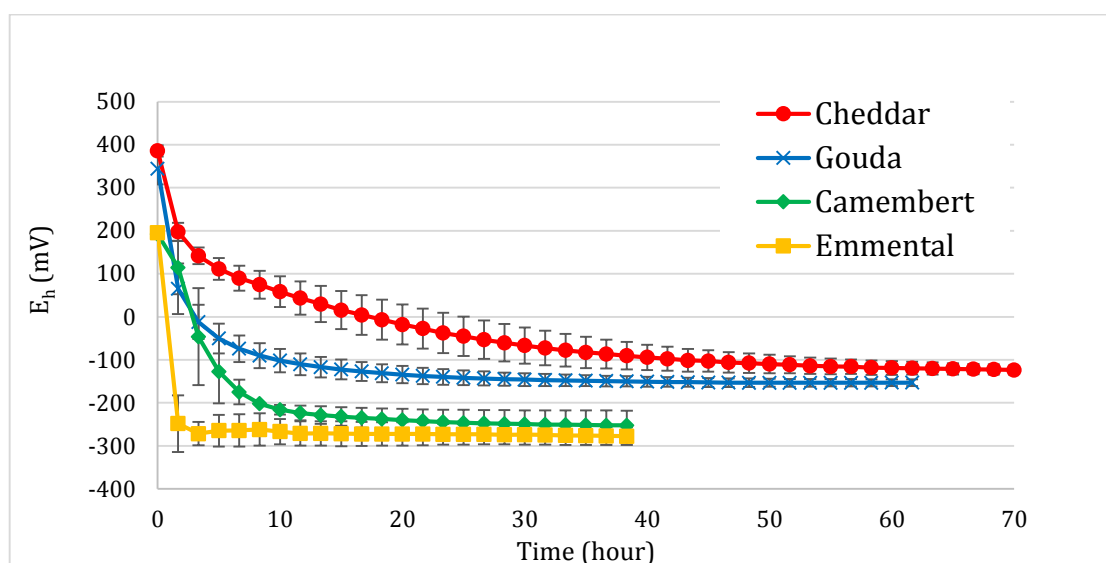
Figure 5.7   Redox potential (E_h) measured in commercial Cheddar (●), Gouda (x), Camembert (♦) and Emmental (■) cheeses.

Redox potential of commercial Gouda cheese has not been measured previously. The time needed to reach the equilibrium value was shorter compared to Cheddar cheese; however, a similar final E_h value were measured. Moreover, the E_h value measured was closed to the one reported in our previous study (Caldeo and McSweeney, 2012) on the changes in E_h during simulated Gouda manufacture; at the end of the pressing stage, values of -118 mV were reported.

Redox potential of mature Camembert cheese was studied by Abraham et al. (2007); microelectrodes were used and readings of about -300 mV were recorded within 10 min in a 15 days old Camembert whereas a 45 days old Camembert had values of -350 mV. Our readings were about 50-100 mV higher and the time needed to reach the equilibrium was longer compared to the results obtained by Abraham et al. (2007); this difference could be due to the electrodes used in the experiments. Microelectrodes are

able to reach an equilibrium value in a short time and can be considered a good tool in measuring the E_h of soft cheeses since the matrix is soft enough to prevent damage of the electrode. Abraham et al. (2013) published another study on redox potential in Camembert cheese; redox potential was measured in a commercial Camembert cheese and the value found were higher (E_{h7} around -100 mV) compared to our results. This difference could be probably due to differences in the electrodes used and in the cheeses.

Redox potential of commercial Emmental cheese was -277 mV and value was reached within 5 hours of measurement and a similar value was found in Section 5.3.3 on the third block of Emmental cheese analysed where value of -302 mV was measured within 10 hours. Emmental was the fastest to reach an equilibrium values compared to the other commercial cheeses analysed and some possible explanations to this event are reported below.

Differences in redox potential values between cheese varieties could be due to the different manufacture technologies and starter microorganisms used (Caldeo and McSweeney, 2012; Abraham et al., 2013) and to the internal microflora that develops during ripening (Thomas, 1986; Broadbent et al., 2002) together with the formation of redox compounds. Moreover, the time needed to reach an equilibrium value could be due to the structure of the cheese. For example, in Cheddar cheese internal micro fissures might be present since the curd is cut in pieces and pressed together and therefore delay in the time needed to reach the equilibrium might be possible.

Another aspect that could delay the time required to achieve an equilibrium is the water activity of the cheese. Gouda and Cheddar cheese were the slower to reach an equilibrium time and their water activity is about 0.95 whereas Emmental and

Camembert have a higher value (0.97) (Guinee and Fox, 2004) and their E_h equilibrium value was reached in a shorter time. However, the water activity of the cheeses studied in this work was not measured and further studies are needed to validate the hypothesized relationship between the time required to measure an equilibrium redox potential value and water activity.

5.4   Conclusion

The oxidation-reduction potential of cheese was measured in this study. The methods used were able to overcome difficulties experienced by some authors in obtaining reproducible results in cheese. Redox potential was followed during ripening of Cheddar cheese from the pressing stage of manufacture and in Emmental cheese from the end of the salting stage. Moreover, redox potential of commercially available cheeses were measured.

We developed a method to measure redox potential during the pressing stage of Cheddar cheesemaking and we found that redox potential reached negative values within two hours of pressing and the values reached were rather constant for 30 days of ripening. While, in Emmental cheese the reduction in redox potential occurred after about 5 days of ripening and E_h kept decreasing during the first 20 days of ripening reaching values in E_h lower than these obtained in Cheddar cheese.

Furthermore, we found that cheeses that belong to different varieties differed in the time required to reach their equilibrium E_h value during redox measurement. Also, analysing the E_h of Cheddar cheese at different time during ripening, we found that the

time needed to reach equilibrium E_h values increase during ripening. We hypnotize that those differences in time could be associated to the water activity of the cheeses; however further studies are necessary to confirm this possible relationship.

The determination of the redox potential of cheese should become a routine measurement in dairy industry. Measuring the redox potential in cheese could became a useful tool in monitoring the quality of cheese at different stages of ripening and in standardizing cheesemaking and ripening of dairy products.

5.5   Acknowledgments

5.6   References

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Chapter 6

Control of oxidation-reduction potential during Cheddar cheese ripening and its effect on the production of volatile flavour compounds

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Abstract

Oxidation-reduction (redox) potential is a fundamental physicochemical parameter
that affects the production of volatile flavour compounds derived from the growth
and activity of starter and non-starter lactic acid bacteria. In cheese, a negative redox
potential is required for the stability of aroma, especially that associated with volatile
sulfur compounds. To control the redox potential during ripening, redox agents were
added to the salted curd of Cheddar cheese before pressing. The control cheese
contained only salt, while different oxidizing or reducing agents were added with the
NaCl to the experimental cheeses. KIO_3 (at 0.05%, 0.1% and 1%, w/w) was used as
the oxidizing agent while cysteine (at 2%, w/w) and $Na_2S_2O_4$ (at 0.05 and 0.1%, w/w)
were used as reducing agents. Cheeses were analysed during ripening for redox
potential, pH, composition, microbial counts, and volatile compounds. During ripening
the redox potential of the cheeses made with the reducing agents did not differ
significantly from the control cheese ($E_h \approx$ -120 mV) while the cheeses made with
0.1% and 0.05% KIO_3 had a significantly higher (P < 0.05) and positive redox
potential in the first month of ripening. After 2 months the redox potential of most
experimental cheeses reached values close to that of the control cheese. Cheese made
with 1% KIO_3 had positive values of redox potential throughout ripening but no
starter lactic acid bacteria survived in this cheese; however, numbers of starter
organisms in all other cheeses were similar. Principal component analysis (PCA) of
the data obtained from GC-MS analysis of volatile compounds clearly separated the
cheeses made with the reducing agents from cheeses made with the oxidising agents
at 2 month of ripening. Cheeses with reducing agents were characterized by the
presence of sulfur compounds whereas cheeses made with KIO_3 were characterized

mainly by aldehydes. At 6 month of ripening, separation by PCA was less evident. These findings support the hypothesis that redox potential could be controlled during ripening and that this parameter has an influence on the development of cheese flavour.

Keywords: oxidation-reduction potential, redox agents, Cheddar cheese, volatile flavour compounds.

6.1   Introduction

Development of cheese flavour can be influenced by the cheese microflora (Broadbent et al., 2002; Boucher et al., 2006; Ledon and Ibarra, 2009), manufacturing and ripening conditions (Kristoffersen, 1985; Fox, 1989, 1993; McSweeney, 2007) and also by physicochemical parameters like temperature, pH, water activity, the ratio of salt to moisture (Singh et al., 2003; Hill, 2007). A fundamental parameter that can influence cheese ripening, and that is not largely taken into consideration, is oxidation-reduction (redox) potential (E_h). Therefore, monitoring and control of E_h during manufacture and ripening of cheese could be important to understand the effect of this parameter on growth and survival of microorganisms and on its organoleptic characteristics. Redox potential can be modified by the addition of redox agents. Substances that can oxidize other substances are called oxidizing agents and substances that can reduce other substances are called reducing agents (Brown and Emberger, 1980; McCarthy and Singh, 2009).

Redox potential could affect the levels of volatile flavour compounds in dairy products produced by the growth and activity of starter and non-starter lactic acid bacteria (Boucher et al., 2006; Kieronczyk et al., 2006) and could influence their metabolic pathways of flavour generation (Martin et al., 2011). In a study by Kieronczyk et al., (2006) on the effect of extracellular E_h on *in vitro* amino acid catabolism by two strains of *Lactococcus lactis*, differences were found in the flavour compounds produced under conditions of different redox potentials. In particular, oxidative E_h stimulated the production of aldehydes from leucine and methionine, it also promoted the production of alcohols derived from leucine. In contrast, reducing E_h

promoted the production of carboxylic and hydroxy acids by both strains analysed. Sulfur compounds were influenced by the E_h and production was favoured in an oxidising environment; however dimethyltrisulfide was mainly produced in reducing condition by one of the strains analysed.

The development of a characteristic cheese aroma is influenced by redox potential (Davis, 1932; Galesloot, 1960a; Kristoffersen, 1967; Manning, 1979) and stable aroma is thought to be due to a negative E_h (-150 to -300 mV) (Adda et al., 1982; Beresford et al., 2001). Cheddar cheese has an E_h of about -120 mV and this negative value (Kristoffersen et al., 1964; Green and Manning, 1982; Urbach, 1993) is associated with the production of volatile sulfur compounds (Green and Manning, 1982; Kristoffersen, 1985).

In the literature, addition of chemical compounds like dithiotreitol, potassium ferricyanide, sodium borohydride, cysteine (George and Peck, 1998; Riondet et al., 2000; Bolduc et al., 2006b; Kieronczyk et al., 2006; Ignatova et al., 2009) or the usage of gasses like oxygen, nitrogen and hydrogen (Ignatova et al., 2009; Jeanson et al., 2009; Martin et al., 2009, 2010, 2011; Ebel et al., 2011) or the application of electro-reduction (Swanson and Sommer, 1940; Inoue and Kato, 2003; Bolduc et al., 2006a; Schreyer et al., 2006, 2008; Abraham et al., 2007; Haratifar et al., 2011) have been used to control the redox potential of dairy products.

However, only a few studies have been conducted on the addition of redox agents to cheese (Galesloot, 1961a; Green and Manning, 1982). Oxidizing agents, like nitrate, nitrite or chlorate, were added to milk destined to the production of Edam cheese to prevent butyric acid fermentation and, as a consequence, the decrease in E_h was

delayed (Vos, 1948; Galesloot, 1961a); however, in the absence of butyric acid bacteria, nitrate had no effect on the redox potential of Edam cheese (Galesloot, 1960b).

Only in one study (Green and Manning, 1982) reducing agents (dithiothreitol, glutathione or cysteine) were added to cheese curd before the pressing stage of the manufacture of Cheddar cheese acidified with a single strain starter. The addition of reducing compounds caused a decrease in redox potential to values 20-40 mV lower than the control cheese and led to the production of higher concentrations of hydrogen sulphide and methanethiol at 3 months of ripening.

Studies from our laboratory have measured redox potential during Cheddar cheesemaking and ripening (Topcu et al., 2008; McSweeney et al., 2010; Caldeo and McSweeney, 2012). We reported that during Cheddar cheesemaking a significant drop ($P < 0.05$) in redox potential (E_h around -120 mV) occurs at the whey drainage stage until the milling stage (Caldeo and McSweeney, 2012); the salting stage causes an increase in redox potential due to oxygen penetration. After salting, the redox potential decreases again to negative value within the first hours of pressing (McSweeney et al., 2010) and this value is maintained during ripening (Topcu et al., 2008; McSweeney et al., 2010).

The objective of this work is to control the redox potential during Cheddar ripening through the addition of oxidizing or reducing agents to the salted curd before pressing. Cheese were analysed to study the effect of the addition of redox agents on the ripening of Cheddar cheese and the development of flavour compounds.

6.2   Materials and methods

6.2.1   Cheddar cheese manufacture

Cheddar cheeses were made in the food processing facilities at University College Cork, Ireland, according to a standard Cheddar cheese-making protocol. Raw bovine milk was standardized to a casein to fat ratio of 0.7:1 and HTST-pasteurized (73.5 °C, 15 s). Four open vats were filled with approximately 100 L of milk at 31°C and inoculated at a rate of 0.02% (w/v) with a concentrated starter culture (DVS R604, Chr. Hansen, Hørshom, Denmark). After a 30 min ripening period, the milk was supplemented with 1 M $CaCl_2$ (0.9 mL/L). Coagulant (Chymax-180, Chr. Hansen), diluted 1:5 with distilled water, was added at a level of 0.3 mL/L. When the coagulum reached the desired firmness after 40-50 min, the curd was cut and allowed to heal for 10 min. The temperature was raised to 39°C over 30 min and the cheese milk stirred at 39°C until a pH of 6.2 was obtained. The whey was then drained and the curd was cut into large blocks and these were inverted every 15 min until pH reached 5.8 when the blocks were stacked on top of each other. After reaching a pH of 5.4, the curd was milled. The curd pieces obtained from the four vats were mixed and then separated into batches of 8 kg. To each batch 2.5% (w/w) of NaCl and oxidizing or reducing agents were added. Four trials were manufactured. Four trials were manufactured and Table 6.1 lists the cheeses made for each trial.

Table 6.1   Summary of the trials made in this study. In each trial, a control cheese made without addition of redox agents was manufactured.

	Trial 1		Trial 2		Trial 3		Trial 4	
	Control		Control		Control		Control	
Oxidizing agents	KIO_3	1%	KIO_3	0.1%	KIO_3	0.1%	KIO_3	0.05%
	KIO_3	0.1%	KIO_3	0.05%	KIO_3	0.05%		
Reducing agents	$Na_2S_2O_4$	0.05%	$Na_2S_2O_4$	0.1%	$Na_2S_2O_4$	0.1%	$Na_2S_2O_4$	0.1%
			Cys	2%	Cys	2%	Cys	2%

In the first trial, potassium iodate (0.005 mol in 1 g of KIO_3; Sigma-Aldrich, Steinheim, Germany) at 1% and 0.1% (w/w) were used as the oxidizing agents while sodium hydrosulfite (0.070 mol in 1 g of $Na_2S_2O_4$; Sigma-Aldrich) at 0.05% (w/w) was used as the reducing agent. In the second and third trials, KIO_3 at 0.1% and 0.05% (w/w) were used as the oxidizing agent while $Na_2S_2O_4$ at 0.1% (w/w) and cysteine (Cys, 0.008 mol in 1 g of $C_3H_7NO_2S$; Sigma-Aldrich) at 2% (w/w) were used as reducing agents. In the fourth trial, KIO_3 at 0.1% (w/w) was used as the oxidizing agent while $Na_2S_2O_4$ at 0.1% (w/w) and Cys at 2% (w/w) were used as reducing agents. In each trial, a control cheese was made without the addition of redox agents. After 20 min, the curd was moulded and pressed at 490 kPa overnight at room temperature. Cheese blocks were vacuum packed and ripened at 8°C for up to 6 months.

6.2.2   Measurement of oxidation-reduction potential during ripening

Oxidation-reduction potential was measured following the method reported by Topcu et al., (2008). A platinum working electrode (R-XM110) and silver/silver chloride reference electrode (REF 201, Radiometer Analytical, Villeurbanne Cedex, Lyon,

France) filled with saturated KCl solution (KCl-L-30, Radiometer Analytical) were used. Before each experiments the electrodes were cleaned following the indication reported by Caldeo and McSweeney (2012) and Abraham et al., (2013). The accuracy of electrodes was checked against a 3 M KCl solution (Topcu et al., 2008) and tap water (Abraham et al., 2007; Jeanson et al., 2009; Martin et al., 2011) at 25°C.

The cheese block samples of about 10 x 10 cm were wrapped in transparent film to prevent loss of moisture. The Pt-electrode was inserted directly into a cheese block samples to a depth of 5 cm and the reference electrode was placed 2.5 cm apart in a hole of 4 cm deep and 1.5 cm wide filled with 3 M KCl solution as described by Topcu et al. (2008).

The electrodes were connected to a data logger (Squirrel Data Logger 2040-2F16 Series, Grant, Data Acquisition, Cambridge, UK) through an amplifier (PHTX-21, Omega, Stamford, CT, USA) for data acquisition. The measured data were recorded every five minutes.

The redox potential data recorded (without reference to a hydrogen reference electrode) were converted to E_h according to Caldeo and McSweeney (2012) and Abraham et al., (2013) with temperature compensation.

For each cheese, single measurement of redox potential was taken at 1, 14, 30, 60, 120 and 180 days of ripening.

6.2.3   Measurement of microbial growth

Starter and non-starter lactic acid bacteria (LAB) counts were performed on 14, 30, 60, 120 and 180-days-old cheeses.

Cheese (5 g) was homogenised in 45 mL of 2% (w/v) tri-sodium citrate solution for microbiological analysis and decimal dilutions were prepared in tubes of sterile quarter-strength Ringer's solution tubes. Starter LAB were enumerated in duplicate on LM17 agar (Merck, Darmstadt, Germany) after incubation at 30°C for 3 days. Non-starter LAB were enumerated in duplicate under anaerobic conditions (Merck Anaerocult A gas pack; Merck), on Rogosa agar (Merck) after incubation at 30°C for 5 days. The total colony forming units in cheese were calculated by counting colonies on plates with between 30 and 300 colonies.

6.2.4   Compositional analysis and pH

The composition (pH, protein, salt, moisture and fat) of 14-day-old cheeses was determined in triplicate. The protein content of the cheeses was determined by the macro-Kjeldahl method (IDF, 1986), salt by a potentiometric titration (Fox, 1963), moisture by oven drying at 103 ± 1°C (IDF, 1982) and fat by the Gerber method (IIRS, 1955).

The pH was measured in triplicate at 1, 14, 30, 60, 120 and 180 days of ripening. pH of the cheeses were measured by probing the cheese directly with a combined glass electrode (PHC3001-8, Radiometer Analytical) connected to a pH meter (PHM210 Standard pH Meter, Radiometer Analytical).

6.2.5   Determination of volatile compounds by SPME-GC-MS

Cheese samples at 2 and 6 months of ripening were wrapped in aluminium foil, vacuum packed and stored at -20°C until analysed. Volatile compounds were analysed by solid phase microextraction coupled to gas chromatography-mass spectrometry (SPME GC-MS) at Teagasc Food Research Centre as described by Hou et al. (2014).

6.2.6   Flow cytometry

Flow cytometry (FCM) was employed to monitor the profile of live, dead or permeabilised/damaged bacterial cells in control cheeses and cheeses made with the addition of 0.1% KIO_3 at 2 month of ripening. The bacterial cells were recovered from 5 g grated cheese, stained using the Live/Dead®BacLight™ reagent kit (Molecular Probes, Life Technologies, Carlsbad, CA, USA) and cytometric analysis was performed using a Becton Dickinson LSR I flow cytometer (BD Biosciences, San Jose, CA, USA) as described by Kilcawley et al., (2012).   Reference control populations of live, permeabilised/damaged, or dead cells were identified as described by Sheehan et al. (2005) and Doolan and Wilkinson (2009).

6.2.7   Statistical analysis

Analysis of variance (one-way ANOVA) of redox potential measurements, microbiological counts, composition and pH of the cheeses were conducted using SPSS Version 20.0 for Mac OS X (SPSS Inc., Chicago, IL, USA). When differences were significant ($P < 0.05$), the means were analysed using Tukey's test.

The data for the volatile compounds identified at 2 and 6 months of ripening for all the cheeses were averaged and analysed by principal component analysis (PCA) by Unscrambler V 6.1 (CAMO AS, N-70421 Trondheim, Norway).

6.3   Results and discussion

Cheeses made with the addition of redox agents at the salting stage of the manufacture and control cheeses were analysed for composition at 14 days of ripening. Redox potential, microbial growth and pH were monitored during ripening at 1, 14, 30, 60, 120 and 180 days. The data reported for E_h, microbial growth and pH are averaged values of three independent trials for all the cheeses, except for cheeses made with the addition of 1% KIO_3 and 0.05% $Na_2S_2O_4$ that were made only in Trial 1. Furthermore, cell viability of control cheeses and cheeses made with the addition of 0.1% KIO_3 was measured at 60 days of ripening.

6.3.1   Chemical composition, pH and microbiological growth of the cheeses

Results for the composition and pH of control and experimental cheeses at 14 days of ripening are reported in Table 6.2.

The value of moisture, salt, fat and protein contents of the cheeses were within the range of those typical of Cheddar cheese (Lawrence et al., 2004). One-way ANOVA on all the compositional data found significant differences (Tukey's, $P < 0.05$) among cheeses within the same trial and between different trials is indicated in Table 6.2.

Table 6.2 Composition and pH of control cheeses and Cheddar cheeses made with the addition of redox agents at the salting stage of manufacture at 14 days of ripening. Potassium iodate (KIO_3) at 1, 0.1 and 0.05% (w/w) were used as the oxidizing agents while sodium hydrosulfite ($Na_2S_2O_4$) at 0.1 and 0.05% (w/w) and cysteine (Cys) at 2% (w/w) were added as the reducing agents.

	Salt %	Moisture %	Protein %	Fat %	pH
Trial 1					
Control	1.31 [Cab] (0.02)	36.24 [Aa] (0.44)	25.58 [BCb] (0.51)	31.50 [Aa] (0.71)	5.31 [Ba] (0.01)
KIO_3 1%	1.31 [ab] (0.01)	37.27 [a] (1.47)	22.48 [a] (0.33)	30.50 [a] (0.71)	5.59 [b] (0.01)
KIO_3 0.1%	1.29 [Ca] (0.00)	36.14 [Aa] (0.20)	23.52 [Ba] (0.00)	32.00 [Aa] (0.00)	5.43 [Aa] (0.01)
$Na_2S_2O_4$ 0.05%	1.36 [b] (0.00)	37.90 [a] (0.12)	22.22 [a] (0.62)	30.00 [a] (0.00)	5.35 [a] (0.07)
Trial 2					
Control	1.15 [ABa] (0.04)	36.18 [Aa] (0.14)	22.48 [Aa] (0.33)	32.00 [Abc] (0.00)	5.21 [Aa] (0.01)
KIO_3 0.1%	1.11 [Aa] (0.00)	38.81 [Cb] (0.00)	21.86 [Aa] (0.37)	31.50 [Aabc] (0.71)	5.51 [Ac] (0.02)
KIO_3 0.05%	1.07 [Aa] (0.02)	37.65 [Aab] (0.83)	21.61 [Aa] (0.24)	33.00 [Ac] (0.00)	5.26 [Aa] (0.02)
$Na_2S_2O_4$ 0.1%	1.22 [Aa] (0.01)	38.18 [Ab] (0.15)	21.87 [Aa] (0.03)	30.50 [Aab] (0.71)	5.37 [Ab] (0.01)
Cys 2%	1.32 [Aa] (0.16)	37.86 [Aab] (0.39)	22.59 [Aa] (1.08)	30.00 [Aa] (0.00)	5.34 [Ab] (0.00)
Trial 3					
Control	1.12 [Aa] (0.02)	36.97 [Aa] (0.78)	23.37 [ABa] (0.15)	32.50 [Ab] (0.71)	5.23 [Aa] (0.01)
KIO_3 0.1%	1.25 [Bb] (0.01)	37.35 [Ba] (0.16)	25.50 [Cb] (0.54)	33.00 [Ab] (0.00)	5.44 [Ac] (0.04)
KIO_3 0.05%	1.14 [Aa] (0.00)	36.35 [Aa] (0.05)	23.52 [Ba] (0.32)	32.00 [Aab] (0.00)	5.27 [Aab] (0.01)
$Na_2S_2O_4$ 0.1%	1.25 [Ab] (0.01)	37.35 [Aa] (0.16)	25.50 [Bb] (0.54)	32.00 [Aab] (0.00)	5.44 [Ac] (0.04)
Cys 2%	1.37 [Ac] (0.04)	37.57 [Aa] (0.42)	26.51 [Bb] (0.12)	31.00 [Aa] (0.00)	5.39 [Abc] (0.03)
Trial 4					
Control	1.25 [BCa] (0.02)	36.23 [Aa] (0.39)	26.11 [Ca] (0.94)	33.00 [Aa] (0.00)	5.30 [Ba] (0.01)
KIO_3 0.05%	1.36 [Ba] (0.02)	35.76 [Aa] (1.43)	26.54 [Ca] (0.46)	31.00 [Aa] (1.41)	5.31 [Aa] (0.00)
$Na_2S_2O_4$ 0.1%	1.32 [Ba] (0.03)	36.69 [Aa] (1.59)	25.62 [Ba] (0.38)	32.50 [Aa] (0.71)	5.43 [Ab] (0.01)
Cys 2%	1.35 [Aa] (0.08)	36.91 [Aa] (1.39)	25.79 [Ba] (0.05)	30.50 [Aa] (0.71)	5.40 [Ab] (0.01)

Numbers represent mean and standard deviation, with the latter in parentheses. Within a column, different superscript capital letters denote significant differences between different trials for the same cheese. For each trial, different superscript lowercase letters denote significant differences between cheeses (Tukey's, $P < 0.05$).

Figure 6.1 shows changes in pH of control and experimental cheeses during ripening. The pH of the cheese made with the addition of 1% KIO_3 significantly differed (P < 0.05) from the control cheese and the other experimental cheeses and had values around 5.8 throughout ripening. During ripening, the pH of control cheese and cheese made with the addition of 0.05% KIO_3 was 5.2-5.3 and the other experimental cheeses had a pH significantly slightly higher (P < 0.05) (pH around 5.4) than the control. At the end of the ripening, the pH of all the cheeses, except the cheese made with the addition of 1% KIO_3, had values between 5.3 and 5.4.
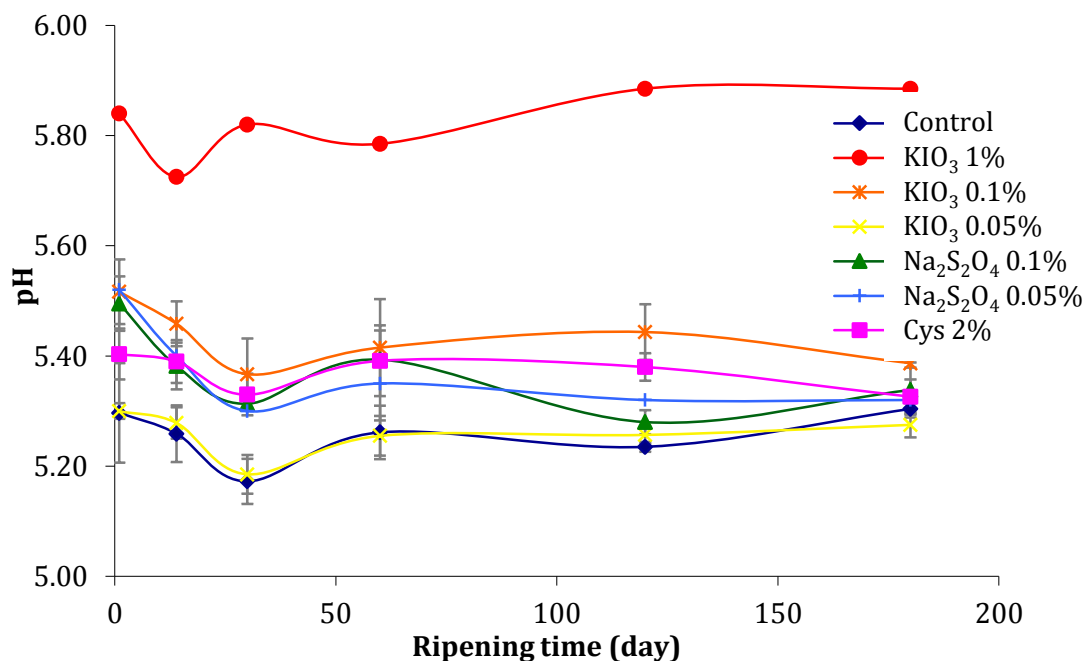
Figure 6.1   Changes in pH during ripening of control Cheese cheese (♦) and cheeses made with the addition of redox agents at the salting stage of manufacture. Potassium iodate (KIO$_3$) at 1 (●), 0.1 (✳) and 0.05% (x) (w/w) was used as the oxidizing agents while sodium hydrosulfite (Na$_2$S$_2$O$_4$) at 0.1 (▲) and 0.05% (❙) (w/w) and cysteine (Cys) at 2% (■) (w/w) were added as the reducing agents. Values are average of pH values of three independent trials, except for cheeses made with the addition of 1% KIO$_3$ and 0.05% Na$_2$S$_2$O$_4$ that were made only in Trial 1. pH of each cheese was measured in triplicate.

Growth of starter and non-starter LAB are shown in Figure 6.2 A and B, respectively. At 14 day of ripening, starter LAB grew to similar values (9-10 log cfu/g cheese) in control cheeses and cheese made with the addition of 0.1 and 0.05% KIO$_3$, 0.1 and 0.05% Na$_2$S$_2$O$_4$ and 2% Cys. During ripening, those cheeses showed a typical decline in starter LAB number. Cheese made with 1% KIO$_3$ did not contain any viable starter

LAB during the all ripening, suggesting that the quantity of KIO_3 used might have been toxic for the bacteria. Therefore, the cheese made with the addition of KIO_3 at 1% was produced only in the first trial.

During ripening, non-starter LAB levels increased in all the cheeses. However, at 120 and 180 days of ripening, the cheeses made with the addition of 1% KIO_3 and 0.1% $Na_2S_2O_4$ had non-starter LAB numbers lower by five and three log cycles, respectively, compared to the control cheese. Cheese made with the addition of 0.05% $Na_2S_2O_4$ had counts lower by three log cycles than the control cheese at 120 days of ripening. Other experimental cheeses showed a growth of non-starter LAB similar to the control cheese.
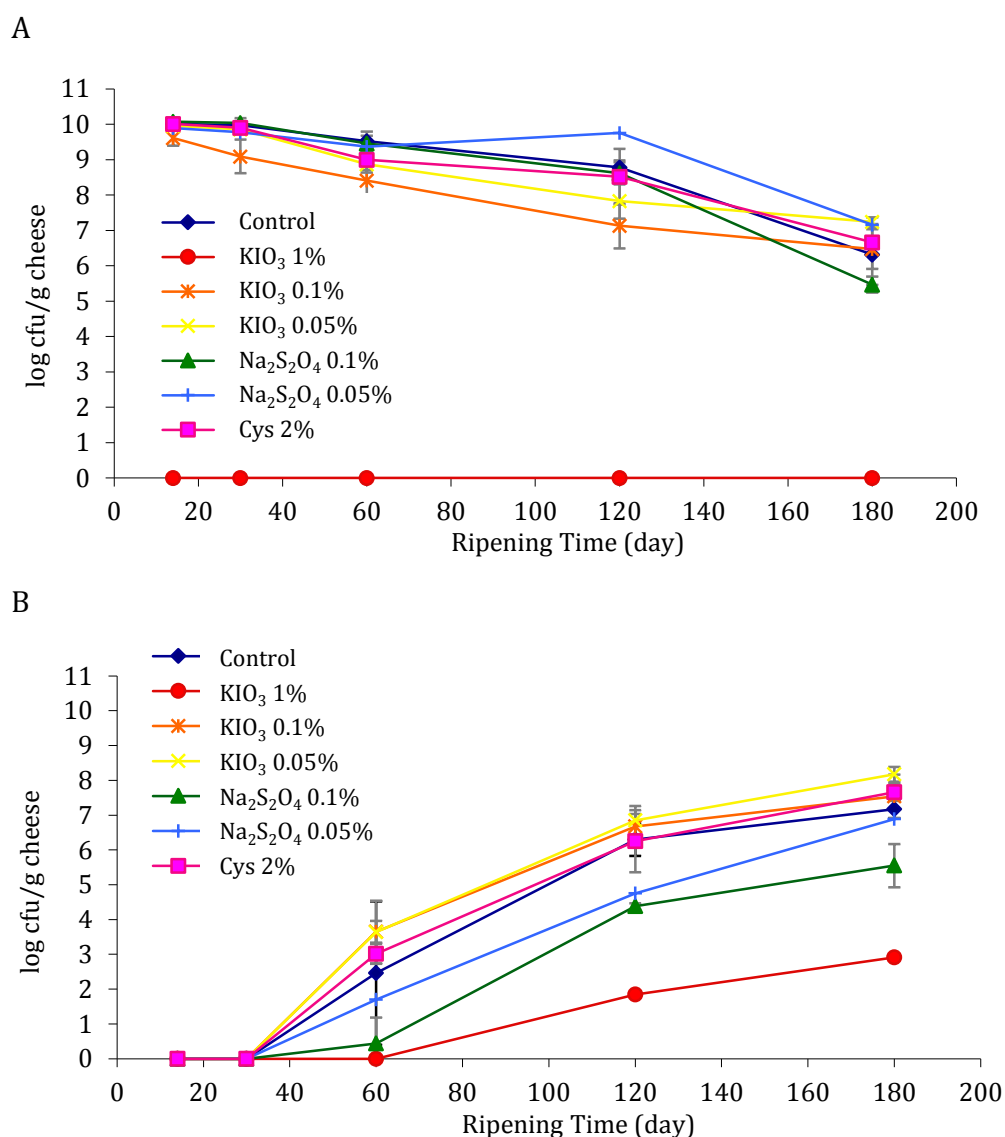
Figure 6.2   Counts of cheese starter (A) and non-starter (B) lactic acid bacteria in control cheese Cheddar (♦) and cheeses containing different redox agents during six months of ripening. Potassium iodate (KIO_3) at 1 (●), 0.1 (∗) and 0.05% (×) (w/w) was used as the oxidizing agents while sodium hydrosulfite ($Na_2S_2O_4$) at 0.1 (▲) and 0.05% (❙) (w/w) and cysteine (Cys) at 2% (■) (w/w) were added as the reducing agents. Values are average of microbial counts of three independent trials, except for cheeses made with the addition of 1% KIO_3 and 0.05% $Na_2S_2O_4$ that were made only in Trial 1. Counts were measured in duplicate in each cheese.

6.3.2   Cell viability

Since KIO$_3$ at 1% appeared to be toxic for starter LAB growth in Trial 1, lower concentrations of KIO$_3$ were used (0.1 and 0.05%) in subsequent trials. FCM was used to identify bacteria cells that are active and non-culturable on LM17 agar in control cheese and cheese made with 0.1% KIO$_3$. Figure 6.3 shows the FCM plots of bacterial populations harvested. Lower left quadrant (LL) is mainly debris while lower right (LR) quadrant is normally live intact cells. Upper left quadrant (UL) usually contains highly damaged/permeabilized/dead cells.   Upper right quadrant (UR) usually contains damaged/permeabilized cells which may also be viable.
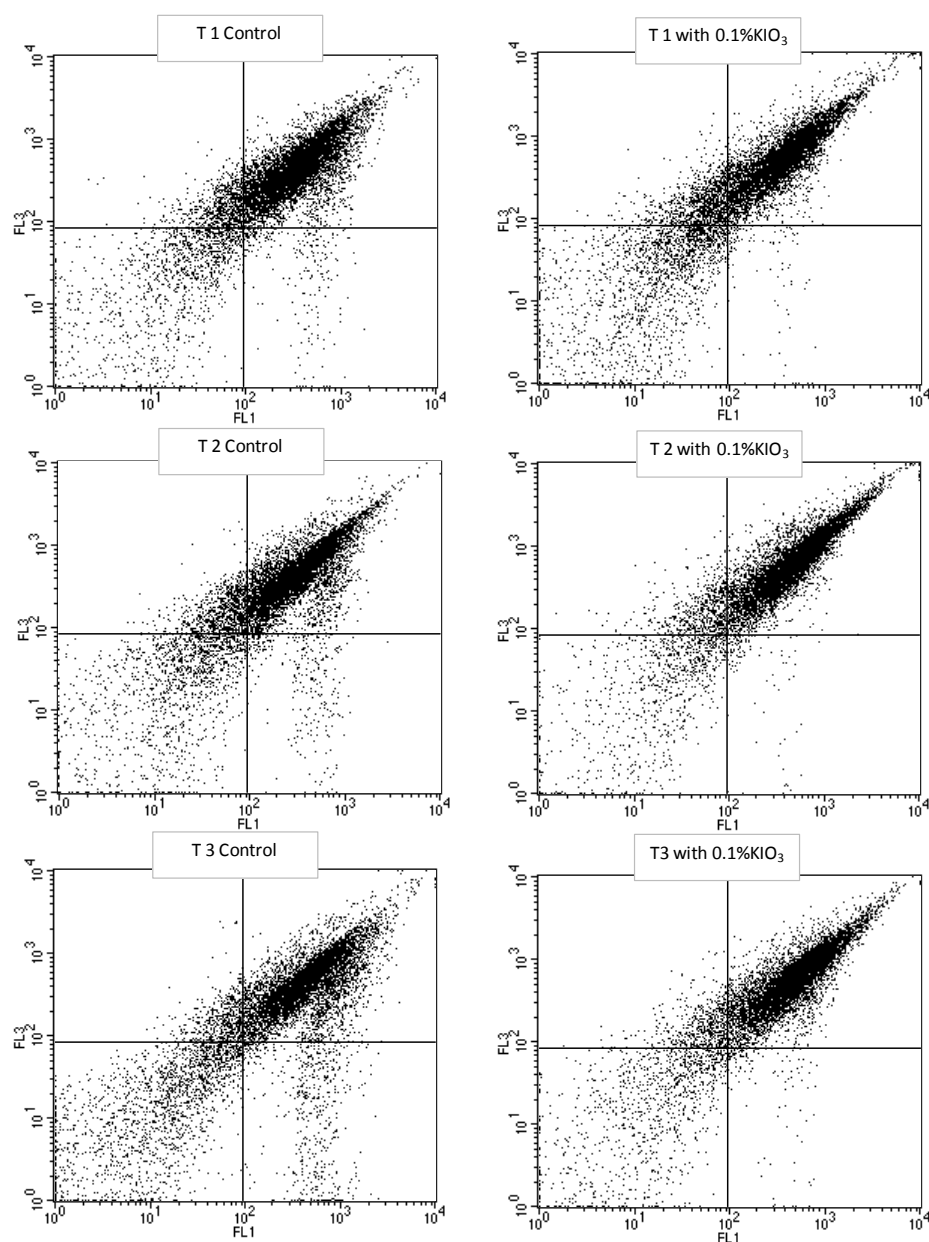
Figure 6.3   Flow cytometer mulitparameter dot plots of SYTO 9 fluorescence (FL1) vs propidium iodide fluorescence (FL3) of cells harvested from control cheeses and from cheeses with 0.1% KIO3 of trial 1 (T1), 2 (T2) and 3 (T3).

In all cheeses distinct populations of permeabilized cells were observed in the UR regions of the dot plots. In the control cheeses there was also a distinct population of

intact cells (LR), whereas fewer intact cells were present in the cheeses made with 0.1% KIO_3. This population of cells could be starter or non-starter LAB.

FCM results indicate that the addition of 0.1% KIO_3 caused a decrease in the population of intact cells and did not affect the damaged/permeabilized and perhaps viable cells.

6.3.3   Control of oxidation-reduction potential during ripening

Oxidation-reduction potential was measured at 1, 14, 30, 60, 120 and 180 days of ripening. Figure 6.4 shows the E_h equilibrium values reached at each time point during cheese ripening.

The redox potential of control cheese was around -130 mV during ripening. This value is in agreement with previous studies where E_h of mature Cheddar cheese was measured (Kristoffersen et al., 1964; Topcu et al., 2008, McSweeney et al., 2010). The E_h of cheeses made with the addition of reducing agents did not differ significantly (P < 0.05) from the control cheese. However, cheese made with the addition of Cys was 30 mV lower compared to the control cheese.

Cheese made with the addition of 1% KIO_3 had positive redox potential values, around +400 mV, throughout ripening, probably due to the absence of LAB and the higher pH. Starter LAB were not able to survive during ripening and non-starter LAB only grew slowly at the end of the ripening. In a study by Green and Manning (1982), Cheddar cheese made under aseptic conditions and in the absence of starter LAB, had a positive redox potential (+315 mV) at 42 days of ripening. Indeed, as demonstrated

by Jeanson et al. (2009), the redox potential of milk treated with different gasses was constant over time in absence of bacterial growth.
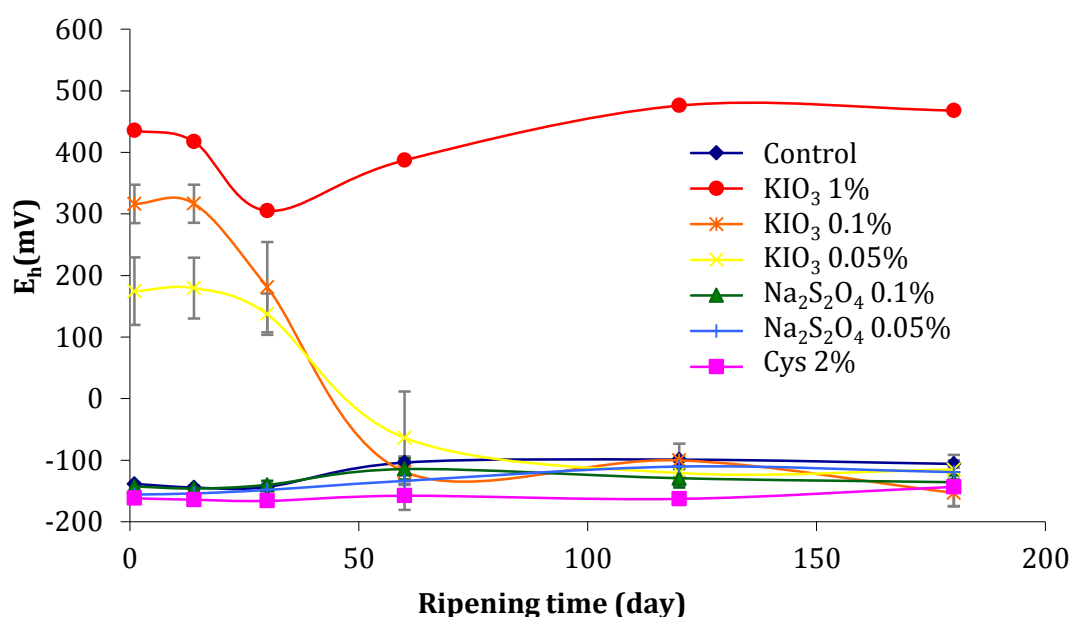
Figure 6.4   Equilibrium values of oxidation-reduction potential (E_h) during ripening of control Cheddar cheese (♦) and cheeses made with the addition of redox agents. Potassium iodate (KIO_3) at 1 (●), 0.1 (✳) and 0.05% (✕) (w/w) was used as the oxidizing agents while sodium hydrosulfite ($Na_2S_2O_4$) at 0.1 (▲) and 0.05% (❙) (w/w) and cysteine (Cys) at 2% (■) (w/w) were added as the reducing agents. Values are average of equilibrium E_h values of three independent trials, except for cheeses made with the addition of 1% KIO_3 and 0.05% $Na_2S_2O_4$ that were made only in Trial 1.

In a second trial, lower quantities of KIO_3 (0.05 and 0.1%) were added to the cheeses in order to have an effect on the redox potential without influencing microbial growth. Cheeses made with the addition of 0.1% and 0.05% KIO_3 had a significantly

higher ($P < 0.05$) redox potential compared to the control cheese and positive values of +316 and +179 mV were maintained for the first two weeks of ripening, respectively (Figure 6.4). At one month of ripening, E_h of cheeses made with 0.1 and 0.05% KIO_3 decreased slightly and after 2 months the E_h reached values closed to that of the control cheese (Figure 6.4). In the past, studies on the addition of oxidising salts to the milk designed for Edam cheese manufacture have been conducted in order to prevent the development of anaerobic butyric acid bacteria (Vos, 1948; Peltola and Antila, 1953; Galesloot, 1960b, 1961a, b) and a trend in redox potential similar to our results was found. Among the salts studied by Galesloot (1961a), KNO_3 was able to keep the E_h at values about 100 mV higher than the control cheese for 10 days and after that period the E_h decreased to values close to those of the control cheese. The inability to keep the E_h at constant positive values throughout ripening when oxidising agents were added to the cheese could be due to the bacterial growth in the cheese. The oxidising agent added might be reduced by bacterial redox systems present in the cheese (Jacob, 1970; Martin et al., 2010). In our study, the decrease in E_h of the experimental cheeses containing oxidising agents (KIO_3 at 0.05 and 0.01%) occurred in conjunction with the growth of non-starter LAB.

6.3.4   *Volatile analysis*

Study of the volatile compounds in the cheeses analysed at 2 and 6 months of ripening by SPME GC-MS identified 40 compounds. Table 6.3 shows the averaged peak areas values and standard deviation of separate trials. Cheeses made with the addition of 1% KIO_3 and 0.05% of $Na_2S_2O_4$ that were manufactured only in Trial 1 were excluded

from the analysis. The compounds identified are typical of Cheddar cheese (Singh et al., 2003; Hannon et al., 2007).

Table 6.3   Volatile compounds of control Cheddar cheese and cheeses made with the addition of reducing or oxidising agents at 2 (A) and 6 (B) month of ripening. Potassium iodate (KIO_3) at 0.1 and 0.05% (w/w) were used as the oxidizing agents while sodium hydrosulfite ($Na_2S_2O_4$) at 0.1 (w/w) and cysteine (Cys) at 2% (w/w) were added as the reducing agents. Units for concentrations of volatile compounds are averaged peak areas in arbitrary units, results are presented as the mean values of separate trials with standard deviation in parenthesis.

A

Compound	Control		KIO_3 0.1%		KIO_3 0.05%		$Na_2S_2O_4$ 0.1 %		Cys 2%	
Acids										
Acetic acid	4.80E+08 [a]	(4.15E+08)	1.72E+09 [a]	(1.04E+09)	1.04E+09 [a]	(8.14E+08)	1.50E+09 [a]	(3.20E+08)	4.02E+08 [a]	(4.29E+08)
Butanoic acid	7.57E+08 [a]	(3.33E+08)	4.56E+08 [a]	(1.59E+08)	6.98E+08 [a]	(4.41E+08)	5.42E+08 [a]	(4.25E+08)	3.57E+08 [a]	(1.33E+08)
Hexanoic acid	9.44E+08 [a]	(5.22E+08)	5.85E+08 [a]	(6.42E+08)	9.66E+08 [a]	(1.15E+09)	6.70E+08 [a]	(4.78E+08)	4.99E+08 [a]	(9.95E+07)
Octanoic Acid	1.25E+08 [a]	(7.08E+07)	4.23E+07 [a]	(3.51E+07)	1.22E+08 [a]	(1.07E+08)	1.05E+08 [a]	(8.48E+07)	9.03E+07 [a]	(8.00E+07)
Alcohols										
Ethanol	9.29E+09 [a]	(1.73E+09)	6.74E+09 [a]	(3.18E+09)	7.82E+09 [a]	(1.75E+09)	5.09E+09 [a]	(8.78E+08)	6.40E+09 [a]	(1.49E+09)
2-Propanol	8.34E+06 [a]	(3.63E+06)	1.08E+07 [a]	(1.01E+07)	1.18E+07 [a]	(7.48E+06)	1.05E+07 [a]	(5.72E+06)	2.71E+07 [a]	(2.18E+07)
2-Butanol	1.64E+08 [a]	(2.84E+08)	8.70E+06 [a]	(9.00E+06)	7.59E+07 [a]	(1.24E+08)	1.41E+07 [a]	(5.79E+06)	2.02E+07 [a]	(1.28E+07)
3Me-3-Buten-1-ol	6.01E+07 [a]	(4.08E+07)	4.32E+07 [a]	(1.22E+07)	4.41E+07 [a]	(3.65E+07)	4.53E+07 [a]	(1.56E+07)	1.59E+07 [a]	(1.76E+07)
3Me-1-Butanol	5.47E+06 [a]	(4.19E+06)	1.12E+07 [a]	(6.54E+06)	1.10E+07 [a]	(6.70E+06)	9.89E+06 [a]	(2.41E+06)	3.05E+06 [a]	(1.64E+06)
1-Pentanol	9.82E+06 [a]	(6.69E+06)	5.66E+06 [a]	(3.23E+06)	1.00E+07 [a]	(3.81E+06)	1.27E+07 [a]	(1.48E+07)	3.70E+06 [a]	(9.76E+05)

2Me-2-Buten-1-ol	1.07E+07 [a]	(4.20E+06)	8.68E+06 [a]	(4.45E+06)	1.64E+07 [a]	(5.58E+06)	6.52E+06 [a]	(1.68E+06)	7.62E+06 [a]	(7.80E+06)
2,3-Butanediol	1.76E+08 [a]	(1.24E+08)	5.04E+08 [a]	(5.52E+08)	5.53E+08 [a]	(3.19E+08)	1.50E+08 [a]	(9.15E+07)	1.08E+08 [a]	(4.82E+07)
1 Hexanol	7.60E+06 [a]	(3.05E+06)	6.60E+06 [a]	(4.10E+06)	9.83E+06 [a]	(3.11E+06)	8.76E+06 [a]	(2.63E+06)	4.51E+06 [a]	(1.54E+06)
Aldehydes										
Acetaldehyde	1.74E+07 [a]	(9.04E+06)	5.53E+07 [a]	(3.89E+07)	2.66E+07 [a]	(3.43E+07)	3.41E+07 [a]	(3.92E+07)	4.39E+07 [a]	(2.69E+07)
3Me-Butanal	1.14E+07 [a,b]	(1.03E+06)	2.03E+07 [b,c]	(8.20E+06)	2.56E+07 [c]	(3.11E+06)	1.33E+07 [a,b]	(9.46E+05)	4.99E+06 [a]	(5.33E+06)
2Me-Butanal	3.50E+06 [a]	(5.46E+06)	7.51E+05 [a]	(3.96E+05)	8.55E+05 [a]	(5.29E+05)	1.06E+06 [a]	(9.36E+04)	6.28E+05 [a]	(4.14E+05)
Benzaldehyde	4.88E+06 [a]	(7.69E+05)	1.64E+07 [b]	(9.87E+06)	7.18E+06 [a,b]	(1.02E+06)	3.15E+06 [a]	(4.18E+05)	5.16E+06 [a,b]	(4.45E+06)
Octanal	6.88E+07 [b]	(1.28E+07)	5.99E+07 [a,b]	(4.04E+07)	8.23E+07 [a,b]	(3.86E+07)	7.33E+07 [b]	(1.60E+07)	6.96E+07 [a]	(4.90E+07)
Nonanal	6.40E+06 [a]	(4.82E+06)	4.36E+06 [a]	(4.10E+06)	3.28E+06 [a]	(1.77E+06)	4.53E+06 [a]	(3.32E+06)	4.76E+06 [a]	(4.08E+06)
Esters										
Ethyl acetate	4.58E+07 [b]	(2.60E+07)	1.86E+06 [a]	(1.45E+06)	1.87E+07 [a,b]	(2.53E+07)	1.41E+07 [a,b]	(9.08E+06)	2.50E+07 [a,b]	(8.37E+06
Ethyl propanoate	1.16E+06 [a]	(1.21E+06)	7.62E+05 [a]	(6.01E+05)	1.71E+06 [a]	(3.76E+05)	9.71E+05 [a]	(4.42E+05)	1.31E+06 [a]	(1.08E+06
Methyl buyanoate	1.34E+07 [a]	(7.85E+06)	6.72E+06 [a]	(2.54E+06)	1.02E+07 [a]	(2.86E+06)	7.21E+06 [a]	(1.77E+06)	1.02E+07 [a]	(5.41E+06
Ethyl butanoate	5.18E+08 [a]	(1.70E+08)	2.65E+08 [a]	(1.17E+08)	5.00E+08 [a]	(1.71E+08)	1.87E+08 [a]	(8.99E+07)	4.19E+08 [a]	(1.04E+08
Ethyl hexanoate	5.79E+07 [a]	(1.91E+07)	2.83E+07 [a]	(1.55E+07)	4.67E+07 [a]	(1.44E+07)	2.80E+07 [a]	(7.16E+06)	4.68E+07 [a]	(1.42E+07
Hydrocarbons										
Toluene	1.49E+07 [a]	(2.15E+06)	1.45E+07 [a]	(4.63E+06)	1.95E+07 [a]	(5.81E+06)	1.83E+07 [a]	(4.36E+06)	1.88E+07 [a]	(2.24E+06
Ethylbenzene	5.19E+07 [a]	(3.22E+07)	4.54E+07 [a]	(3.00E+07)	3.97E+07 [a]	(2.71E+07)	3.33E+07 [a]	(1.98E+07)	3.70E+07 [a]	(2.07E+07
p-Xylene	9.73E+07 [a]	(6.13E+07)	8.37E+07 [a]	(5.64E+07)	7.26E+07 [a]	(5.15E+07)	6.27E+07 [a]	(4.12E+07)	6.70E+07 [a]	(4.02E+07
o-Xylene	8.09E+07 [a]	(4.61E+07)	6.56E+07 [a]	(4.10E+07)	6.29E+07 [a]	(3.77E+07)	5.58E+07 [a]	(3.18E+07)	5.72E+07 [a]	(2.81E+07
Ketones										
Acetone	1.02E+09 [a]	(3.15E+08)	3.61E+08 [a]	(3.60E+08)	6.96E+08 [a]	(2.06E+08)	9.44E+08 [a]	(2.34E+08)	9.38E+08 [a]	(2.67E+08

Compound	Control		KIO₃ 0.1%		KIO₃ 0.05%		Na₂S₂O₄ 0.1%		Cys 2%	
Diacetyl	8.73E+07 [a]	(1.32E+08)	3.56E+08 [a]	(2.70E+08)	9.44E+07 [a]	(5.89E+07)	4.43E+07 [a]	(4.70E+07)	4.57E+07 [a]	(6.98E+07
2-Butanone	3.03E+08 [a]	(2.01E+08)	4.37E+08 [a]	(4.03E+08)	4.83E+08 [a]	(2.81E+08)	4.14E+08 [a]	(6.83E+07)	6.14E+08 [a]	(3.07E+08
2-Pentanone	2.17E+08 [a]	(9.79E+07)	1.56E+08 [a]	(6.57E+07)	1.84E+08 [a]	(4.98E+07	2.18E+08 [a]	(5.87E+07)	2.22E+08 [a]	(5.52E+07
2,3-Pentanedione	7.98E+07 [a]	(4.57E+07)	2.85E+07 [a]	(2.68E+07)	8.37E+07 [a]	(2.26E+07)	7.29E+07 [a]	(1.24E+07)	6.60E+07 [a]	(2.19E+07)
Acetoin	1.70E+08 [a]	(2.04E+08)	8.68E+09 [a]	(7.33E+09)	3.56E+09 [a]	(5.30E+09)	3.76E+08 [a]	(3.61E+08)	1.19E+07 [a]	(7.09E+06)
2-Heptanone	3.56E+08 [a,b]	(7.15E+07)	1.77E+08 [a]	(7.68E+07)	3.14E+08 [b]	(5.11E+07)	4.17E+08 [b]	(8.83E+07)	3.38E+08 [a,b]	(9.58E+07)
2-Nonanone	2.95E+07 [a]	(7.98E+06)	1.85E+07 [a]	(9.88E+06)	2.63E+07 [a]	(3.68E+06)	3.33E+07 [a]	(6.32E+06)	2.50E+07 [a]	(4.20E+06)
Sulfurs										
Methanethiol	1.05E+07 [a]	(8.58E+06)	2.14E+06 [a]	(9.45E+06)	2.33E+06 [a]	(1.31E+07)	6.97E+07 [b]	(3.82E+07)	5.52E+07 [b]	(2.75E+07)
Dimethylsulfide	1.82E+07 [a]	(8.97E+08)	6.87E+06 [a]	(7.80E+07)	1.49E+07 [a]	(5.33E+08)	4.75E+07 [a]	(7.61E+08)	5.23E+07 [a]	(2.71E+08)
Carbon disulfide	2.32E+09 [a]	(1.11E+06)	8.96E+07 [a]	(6.75E+05)	7.09E+08 [a]	(7.08E+05)	2.53E+09 [a]	(7.86E+06)	3.42E+09 [a]	(1.31E+06)
Dimethyltrisulfide	3.92E+06 [a]	(8.36E+06)	8.09E+05 [a]	(1.60E+06)	1.28E+06 [a]	(9.30E+05)	3.81E+07 [b]	(1.32E+07)	4.58E+06 [b]	(1.98E+07)

B

Compound	Control		KIO₃ 0.1%		KIO₃ 0.05%		Na₂S₂O₄ 0.1%		Cys 2%	
Acids										
Acetic acid	2.07E+09 [a]	(1.12E+09)	1.01E+09 [a]	(5.53E+08)	1.52E+09 [a]	(1.26E+09)	1.36E+09 [a]	(7.65E+08)	8.75E+08 [a]	(9.07E+08)
Butanoic acid	8.08E+08 [a]	(4.09E+08)	5.85E+08 [a]	(6.45E+08)	6.58E+08 [a]	(5.90E+08)	6.86E+08 [a]	(3.37E+08)	9.07E+08 [a]	(2.28E+08)
Hexanoic acid	7.43E+08 [a]	(2.76E+08)	1.78E+09 [a]	(2.07E+09)	8.88E+08 [a]	(2.13E+08)	1.11E+09 [a]	(4.05E+08)	8.73E+08 [a]	(4.05E+08)
Octanoic Acid	1.98E+08 [a]	(9.52E+07)	1.24E+08 [a]	(1.10E+08)	1.12E+08 [a]	(1.07E+08)	1.64E+08 [a]	(6.24E+07)	1.20E+08 [a]	(9.16E+07)
Alcohols										
Ethanol	3.29E+09 [a]	(4.60E+08)	3.93E+09 [a]	(5.47E+08)	4.28E+09 [a]	(1.49E+09)	4.03E+09 [a]	(2.22E+09)	4.51E+09 [a]	(1.00E+09)
2-Propanol	3.27E+08 [a]	(2.76E+08)	3.99E+08 [a]	(1.82E+08)	5.37E+08 [a]	(1.24E+08)	1.27E+08 [a]	(3.34E+07)	6.74E+08 [a]	(1.03E+09)

2-Butanol	2.38E+09 [a]	(1.87E+09)	2.03E+09 [a]	(1.30E+09)	6.19E+09 [a]	(4.26E+09)	5.05E+09 [a]	(2.40E+09)	3.86E+08 [a] (3.11E+08)
3Me-3-Buten-1-ol	2.40E+07 [a]	(7.08E+06)	5.70E+07 [a]	(7.06E+07)	2.06E+07 [a]	(7.53E+06)	1.28E+07 [a]	(5.87E+06)	1.14E+07 [a] (8.17E+06)
3Me-1-Butanol	1.62E+08 [a]	(1.41E+08)	3.44E+08 [a]	(3.30E+08)	2.48E+08 [a]	(1.23E+08)	9.39E+08 [a]	(1.27E+09)	4.05E+06 [a] (3.26E+06)
1-Pentanol	1.88E+07 [a]	(4.04E+06)	7.60E+06 [a]	(3.59E+06)	9.47E+06 [a]	(1.81E+06)	1.50E+07 [a]	(1.94E+07)	6.73E+06 [a] (2.88E+06)
2Me-2-Buten-1-ol	7.15E+06 [a]	(3.49E+06)	3.94E+06 [a]	(2.00E+06)	3.75E+06 [a]	(2.08E+05)	1.95E+06 [a]	(1.82E+06)	5.26E+06 [a] (2.36E+06)
2,3-Butanediol	2.15E+08 [a]	(2.17E+08)	4.08E+08 [a]	(2.56E+08)	1.21E+08 [a]	(9.60E+07)	8.30E+07 [a]	(9.22E+07)	2.03E+08 [a] (1.80E+08)
1 Hexanol	3.89E+07 [a]	(2.68E+07)	3.97E+07 [a]	(3.14E+07)	6.00E+07 [a]	(5.42E+07)	1.67E+09 [a]	(2.87E+09)	6.25E+07 [a] (1.49E+07)
Aldehydes									
Acetaldehyde	5.99E+07 [a]	(9.50E+07)	1.59E+07 [a]	(7.35E+06)	4.89E+07 [a]	6.06E+07)	2.08E+07 [a]	(3.01E+07)	5.66E+07 [a] (4.63E+07)
3Me-Butanal	1.92E+07 [a]	(9.27E+06)	3.10E+07 [a]	(9.04E+06)	3.40E+07 [a]	(8.79E+06)	5.71E+07 [a]	(1.97E+06)	4.10E+07 [a] (7.94E+07)
2Me-Butanal	8.22E+06 [a]	(1.46E+07)	1.58E+08 [a]	(2.55E+08)	1.49E+07 [a]	(1.13E+07)	2.82E+07 [a]	(2.53E+07)	2.02E+06 [a] (2.19E+06)
Benzaldehyde	4.93E+06 [a]	(1.35E+06)	1.68E+07 [b]	(3.76E+06)	5.55E+06 [a]	(9.71E+05)	2.73E+06 [a]	(1.97E+05)	4.58E+06 [a] (1.26E+06)
Octanal	4.82E+07 [a]	(6.11E+07)	3.97E+07 [a]	(3.33E+07)	3.55E+07 [a]	(4.74E+07)	3.46E+07 [a]	(5.17E+07)	7.96E+07 [a] (9.79E+07)
Nonanal	3.11E+06 [a]	(4.19E+06)	3.99E+06 [a]	(4.28E+06)	3.63E+06 [a]	(3.33E+06)	5.86E+06 [a]	(4.99E+06)	7.87E+06 [a] (6.53E+06)
Esters									
Ethyl acetate	1.56E+07 [a]	(6.18E+06)	2.60E+07 [a]	(9.18E+06)	9.90E+06 [a]	(5.08E+06)	4.06E+07 [a]	(5.74E+07)	1.32E+07 [a] (7.71E+06)
Ethyl propanoate	6.25E+05 [a]	(2.41E+05)	3.68E+05 [a]	(3.18E+05)	7.86E+05 [a]	(2.66E+05)	1.33E+06 [a]	(1.74E+06)	8.08E+05 [a] (2.32E+05)
Methyl buyanoate	2.58E+07 [a]	(3.36E+07)	5.14E+06 [a]	(5.17E+06)	9.19E+06 [a]	(4.06E+06)	4.99E+06 [a]	(3.62E+06)	3.82E+06 [a] (2.88E+06)
Ethyl butanoate	3.03E+08 [a]	(1.01E+08)	1.93E+08 [a]	(3.13E+07)	2.19E+08 [a]	(5.42E+07)	3.13E+08 [a]	(2.22E+08)	3.09E+08 [a] (8.41E+07)
Ethyl hexanoate	1.34E+08 [a]	(4.05E+07)	6.11E+07 [a]	(2.02E+07)	9.28E+07 [a]	(1.03E+07)	1.59E+08 [a]	(1.15E+08)	1.49E+08 [a] (4.83E+07)
Hydrocarbons									
Toluene	1.77E+07 [a]	(1.11E+07)	2.26E+07 [a]	(8.15E+06)	1.35E+07 [a]	(4.63E+06)	1.21E+07 [a]	(3.83E+06)	2.45E+07 [a] (7.22E+06)
Ethylbenzene	6.10E+06 [a]	(3.86E+06)	6.27E+06 [a]	(3.94E+06)	4.34E+06 [a]	(1.22E+06)	3.79E+06 [a]	(1.54E+06)	5.44E+06 [a] (1.69E+06)
p-Xylene	9.61E+06 [a]	(9.01E+06)	1.20E+07 [a]	(7.67E+06)	5.54E+06 [a]	(2.50E+06)	4.12E+06 [a]	(1.69E+06)	6.61E+06 [a] (4.07E+06)

o-Xylene	4.00E+06 [a]	(2.42E+06)	4.35E+06 [a]	(2.35E+06)	2.05E+06 [a]	(6.23E+05)	2.44E+06 [a]	(2.74E+05)	4.05E+06 [a] (6.06E+05)
Ketones									
Acetone	4.76E+07 [a]	(1.86E+07)	6.98E+08 [a]	(9.03E+08)	1.74E+08 [a]	(1.82E+08)	2.33E+08 [a]	(1.21E+08)	6.78E+07 [a] (5.99E+07)
Diacetyl	9.39E+06 [a]	(8.40E+05)	7.32E+07 [a]	(2.20E+07)	6.70E+07 [a]	(7.16E+07)	1.66E+07 [a]	(3.87E+06)	2.01E+06 [a] (4.44E+05)
2-Butanone	2.42E+08 [a]	(1.41E+08)	2.46E+09 [b,c]	(3.82E+08)	1.08E+09 [a,b]	(9.37E+08)	3.03E+09 [c]	(1.00E+09)	7.35E+07 [a] (7.38E+07)
2-Pentanone	1.95E+08 [a,b]	(1.02E+08)	6.99E+08 [a,b]	(6.73E+08)	6.18E+08 [a,b]	(3.54E+07)	1.04E+09 [b]	(5.51E+08)	5.99E+07 [a] (3.34E+07)
2,3-Pentanedione	2.25E+07 [a,b]	(5.63E+06)	1.68E+07 [a,b]	(1.17E+07)	1.13E+07 [a]	(4.83E+06)	1.70E+07 [a,b]	(7.98E+06)	3.24E+07 [b] (8.58E+06)
Acetoin	2.29E+09 [a]	(2.30E+09)	1.36E+09 [a]	(8.56E+08)	4.38E+09 [a]	(5.24E+09)	1.86E+09 [a]	(1.85E+09)	1.21E+09 [a] (2.16E+09)
2-Heptanone	1.43E+09 [a,b,c]	(7.48E+08)	1.15E+09 [a,b]	(9.79E+08)	2.76E+09 [b,c]	(9.69E+08)	3.21E+09 [c]	(8.98E+08)	6.20E+08 [a] (2.05E+08)
2-Nonanone	6.04E+07 [a]	(1.58E+07)	6.67E+07 [a]	(2.88E+07)	1.56E+08 [a]	(1.41E+08)	1.75E+08 [a]	(1.42E+08)	6.43E+07 [a] (2.14E+07)
Sulfurs									
Methanethiol	1.55E+07 [a]	(3.27E+06)	1.55E+06 [a]	(1.43E+06)	1.24E+07 [a]	(3.37E+06)	2.88E+07 [a]	(5.07E+06)	3.04E+07 [a] (7.29E+06)
Dimethylsulfide	8.01E+06 [a]	(3.34E+08)	5.65E+06 [a]	(8.71E+07)	5.30E+06 [a]	(7.68E+07)	7.92E+06 [a]	(2.67E+08)	1.39E+07 [a] (4.71E+08)
Carbon disulfide	8.32E+08 [a]	(3.86E+06)	2.34E+08 [a]	(2.35E+05)	2.74E+08 [a]	(8.42E+04)	7.13E+08 [a]	(7.13E+06)	1.05E+09 [a] (4.29E+05)
Dimethyltrisulfide	3.84E+06 [a]	(1.32E+07)	5.06E+05 [a]	(1.28E+06)	3.13E+05 [a]	(2.03E+07)	7.76E+06 [a]	(3.12E+07)	1.31E+06 [a] (2.06E+07)

Different superscript lowercase letters denote significant differences between cheeses for each volatile component (Tukey's, $P < 0.05$).

Four acid compounds (acetic acid, butanoic acid, hexanoic acid and octanoic acid) were identified and octanoic acid was the acid present in low concentration in all the cheeses at 2 and 6 months of ripening. There were no significant differences among the quantity of acids identified.

Among the alcohol compounds identified (ethanol, 2-propanol, 2-butanol, 3-methyl-3-buten-1-ol, 3-methyl-1-butanol, 1-pentanol, 2-methyl-2-buten-1-ol, 2,3-butanediol and 1-hexanol), at 2 month of ripening, ethanol was the most abundant in all the cheeses. During ripening, the quantities of ethanol, 2-propanol, 2-butanol, 3-methyl-1-butanol and 1-hexanol increased. At 2 or 6 months of ripening there were no significant differences among the cheeses in the quantity of each alcohol compound identified.

Six aldehydes were identified (acetaldehyde, 3-methyl-butanal, 2-methyl-butanal, benzaldehyde, octanal and nonanal) and at 2 months of ripening cheeses made with the addition of 0.1 and 0.05% KIO_3 were significantly richer in 3-methyl-butanal than the other cheeses. The content of benzaldehyde was significantly higher in cheese made with the addition of 0.1% KIO_3 compared to the control cheese and the cheese made with 0.1% $Na_2S_2O_4$. Cheese made with the addition of Cys had a significantly lower content of octanal than the other cheeses. At 6 month of ripening, quantities of 3-methyl-butanal and 2-methyl-butanal increased in all the cheeses compared to the quantity at 2 month of ripening and the concentration of benzaldehyde was significantly more abundant in cheese made with 0.1% KIO_3 compared to the other cheeses.

Five esters (ethyl acetate, ethyl propanoate, methyl buyanoate, ethyl butanoate and ethyl hexanoate) were identified. Ethyl butanoate and ethyl hexanoate were the most abundant in all the cheeses at 2 and 6 months of ripening. At 2 months, ethyl acetate was present in significantly lower quantities in the cheese made with the addition of KIO_3 at 0.1% compared to the control cheese. At 6 month of ripening, quantities of ethyl butanoate decreased in all the cheeses whereas ethyl hexanoate slightly increased in all the cheeses at 6 months of ripening.

Four hydrocarbon compounds were identified (toluene, ethylbenzene, *p*-xylene and *o*-xylene) and there were no significant differences in the quantities of each compound at 2 and 6 months of ripening. In all the cheeses, during ripening, the concentrations of the hydrocarbons decreased except for toluene that slightly increased.

Eight ketones (acetone, diacetyl, 2-butanone, 2-pentanone, 2,3-pentanedione, acetoin, 2-heptanone and 2-nonanone) were identified. Diacetyl, 2,3-pentanedione and 2-nonanone were present in low quantities at 2 and 6 months of ripening. At 2 months of ripening, cheese made with the addition of 0.05% KIO_3 had a content of 2-heptanone significantly lower than the other cheeses. At 6 months of ripening, cheese made with the addition of 0.1% Na_2S_2O had a content of 2-butanone significantly higher than control cheese and cheeses made with Cys and KIO_3 0.1%. Moreover, the cheese made with $Na_2S_2O_4$ had a significantly higher content of 2-pentanone and 2-heptanone than the cheese made with the addition of Cys. 2,3-Pentanedione was present in significantly higher quantity in the cheese made with Cys than in the cheese containing 0.05% KIO_3.

Four sulfur compounds (methanethiol; dimethylsulfide, DMS; carbon disulfide, CDS; and dimethyltrisulfide, DMTS) were identified. Methanethiol, DMS and DMTS are important flavour compound in Cheddar cheese (Singh et al., 2003) and their presence is favoured by a low redox potential. In our study at 2 months of ripening, methanethiol and DMTS were significantly higher in cheeses made with reducing agents compared to the control cheese and the cheeses made with oxidising agents. Sulfur aroma in high quality Cheddar cheese is associated with the presence of methanethiol (Aston and Dulley, 1982) and a reducing environment favour the production of methanethiol. As demonstrated by Manning (1979), in cheese made using chemical acidification the addition of reducing compounds favours the formation of methanethiol. Moreover, LAB have enzymatic pathways that lead to the formation of methanethiol from methionine (Dias and Weimer, 1998).

At 6 month of ripening, the quantities of the sulfur compounds identified did not differ significantly between the cheeses.

Principal component analysis (PCA) was performed to assess the relationship within and among the cheeses and the volatile compounds identified. PCA of the volatile data was performed at 2 (Figure 6.5A) and 6 (Figure 6.5B) months on control cheeses and cheeses made with the addition of 0.1 and 0.05% KIO_3, 0.1% $Na_2S_2O_4$ and 2% Cys on the averaged peak areas of separate trials. Cheeses made with 1% KIO_3 and 0.05% $Na_2S_2O_4$ were excluded from the PCA analysis since they were produced only in Trial 1.

A

RESULT3, X-expl: 37%,31%

B

RESULT4, X-expl: 35%,34%

Figure 6.5 Principal component analysis of data of volatile compounds of Cheddar cheese samples at two (A) and six (B) months of ripening. Values are mean of the different trials. At 2 months of ripening, principal components (PC) 1 and 2, which accounted for 37 and 31% of the variation, respectively, and at 6 months, PC 1 and 2 accounted for 35 and 34%, respectively. The volatile compounds identified as written in red and the cheese analysed as in blue colour. Data of volatile compounds identified in control cheeses (Control), cheeses made with the addition of potassium iodate at 0.1 (KIO_3 0.1%) and 0.05% (KIO_3 0.05%), cheeses made with the addition of sodium hydrosulfite at 0.1% ($Na_2S_2O_4$ 0.1%) and cheeses made with the addition of 2% cysteine (Cys 2%) were analysed by PCA. Cheeses made with the addition of 1% KIO_3 and 0.05% $Na_2S_2O_4$ were excluded from the analysis as produced only in one trial. Plots are loadings and scores of the first two components. Volatile compounds are reported in red colour and cheese samples in blue.

At 2 months of ripening, principal components (PC) 1 and 2, which accounted for 37 and 31% of the variation, respectively, clearly separated the cheeses made with the reducing agent from cheeses made with the oxidising agents (Figure 6.5A). Cheeses made with the addition of reducing agents were characterized by the presence of sulfur and ketone compounds. Sulfur compounds like DMTS and DMS are considered important aroma compounds that characterize mature Cheddar cheese (Singh et al., 2003) and the addition of reducing agents favoured the production of sulfur compounds already at two months of ripening. Similarly, a study by Green and Manning (1982) reported that at 6 weeks of ripening concentrations of hydrogen sulphide and methanethiol were higher in the cheeses made with the addition of a reducing compound (Cys). Moreover, Martin et al.

(2011) found an increase in DMS in yogurt made under reducing conditions throughout 28 days of storage. Not in agreement with our results, Kieronczyk et al. (2006), in a study on the flavour compounds produced by amino acid catabolism *in vitro* of two strains of *Lactococcus* under reducing and oxidising conditions, found that methanethiol and DMDS were mainly produced under oxidising conditions. However, among the volatile sulfur compounds identified by the authors, DMTS was present in higher level in reducing system than in oxidizing system.

Moreover, from the PCA of volatiles data at 2 month of ripening suggests that cheeses made with oxidising agents were characterized mainly by aldehydes, in particular benzaldehyde and acetaldehyde (Figure 6.5A). These results are in agreement with previous studies. Kieronczyk et al. (2006) reported a higher quantity of benzaldehyde when LAB were grown in vitro at positive redox potential. A study by Martin et al. (2013) analysed the volatile compounds produced by yogurts under reducing or oxidising conditions, made by the usage of gasses. The authors found that yogurts made under positive redox potential had higher quantities of acetaldehyde and diacetyl and lower level of DMS.

Furthermore, the PCA at 2 month of ripening showed that other volatile compounds like esters and hydrocarbons were present at higher level in the control cheese and cheese made with 0.05% KIO_3 than in other cheeses (Figure 6.5A).

At 6 month of ripening, PC 1 and 2 accounted for 35 and 34%, respectively, and separation between the cheeses was less pronounced (Figure 6.5B). Control cheese and cheese made with the addition of Cys were separated by PC2 from the cheeses made with the addition of oxidising agents and $Na_2S_2O_4$.

6.4   Conclusion

In this study addition of oxidising and reducing agents was done at the salting stage of Cheddar cheese manufacture in order to understand the influence of oxidation-reduction potential on Cheddar cheese ripening.

Our findings support the hypothesis that redox potential has an influence on the development of cheese flavour during ripening. This study confirms that a negative redox potential is essential for the development of sulfur compounds (Green and Manning, 1982; Kristoffersen, 1985) and those compounds were present already at two months of ripening when reducing agents were added to the cheese at the salting stage.

Moreover, from our findings it seems that the cheese microflora has in important effect on the redox potential of Cheddar cheese. In absence of LAB, caused by the addition of a high concentration of KIO_3, the redox potential of our experimental cheese was positive over 6 months of ripening. When KIO_3 was added at 0.1 and 0.05% to the cheese, the E_h was positive for about 2 months of ripening and it decreased to negative values when the number of non-starter LAB increased. This suggests that LAB might be able to use the oxidising agents added and produce reducing metabolites and drive the environmental redox potential to values close to the E_h of the control cheese.

Redox potential can modify microbial activity and metabolic bacteria paths and as consequence act on the flavour development (Ledon and Ibarra, 2009).

In conclusion, understanding and controlling redox potential can be useful to guide

aroma formation in dairy products.

6.5   Acknowledgments

6.6   References

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Chapter 7

Effect of *Enterococcus faecium* adjunct on microbiological and physicochemical characteristics of Cheddar cheese

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Abstract

Enterococcus is a lactic acid bacterium found widely in nature and has different technological applications including in cheese production. The aim of this work was to study the influence of *Enterococcus faecium* EF031 as adjunct culture on the quality of Cheddar cheese. Four batches of Cheddar cheeses were manufactured in triplicate. The control batch contained starter culture only, while the experimental batches contained starter culture and different levels (10^6, 10^7, 10^8 cfu/mL cheesemilk) of *E. faecium* EF031. During aging the number of enterococci decreased by one log cycle in all three experimental cheeses while the control cheese contained a low level of enterococci. No differences in levels of starter and non-starter lactic acid bacteria between the control and experimental cheeses were evident and typical trends were found for all the cheeses. Cheese composition was not influenced by the addition of *E. faecium*. Redox potential was measured during cheesemaking and at four months of ripening; redox potential of the cheese made with the highest level of *E. faecium* had a lower value compared to the control cheese. Assessment of proteolysis was investigated by principal component analysis (PCA) of RP-HPLC peptide profiles of pH4.6-SN fractions of cheese samples at 15, 60 and 180 days of ripening. Cheeses were separated by ripening period and cheeses made with different level of adjunct cultures had different peptide profiles. Moreover, volatile compounds were assessed by SPME-GC-MS and PCA found quantitative differences between the control cheese and the cheeses made with *E. faecium*. These findings support the technological utility of *E. faecium* as an adjunct culture for Cheddar cheese.

Keywords: *Enterococcus faecium*, Cheddar cheese, oxidation-reduction potential.

7.1   Introduction

Enterococci are ubiquitous lactic acid bacteria likely to be found in plants, vegetables and other foods (Franz et al., 2003; Giraffa, 2003; Lopes et al., 2006) and they are naturally present in dairy products (Giraffa et al., 1997; Ogier and Serror, 2008). The two most common species isolated in dairy products are *Enterococcus faecium* and *Enterococcus faecalis* and in certain varieties they can range from 10^5 to 10^7 cfu/g of cheese (Ogier and Serror, 2008). Despite the natural presence of enterococci in several cheeses, their utilization in dairy fermentations is a controversial topic. Enterococci are associated with human infections and can transfer antibiotic resistance (Ogier and Serror, 2008). Moreover, enterococci can contribute to the production of biogenic amines (Kučerová, et al., 2009). On the other hand, enterococci have been shown to produce bacteriocins (Nascimento et al., 2008), to inhibit the growth of food pathogens (Gardiner et al., 1999; Ogier and Serror, 2008) and to have probiotic properties (Gardiner et al., 1999).

In cheese, enterococci can contribute to ripening (Ogier and Serror, 2008) and to the development of flavour and aroma (Giraffa, 2003; Rasouli Pirouzian et al., 2010), therefore, they can be used as starter or adjunct culture in cheese manufacture (Gardiner at al., 1999; Giraffa, 2003; Ogier and Serror, 2008, Rasouli Pirouzian et al., 2010).

Addition of enterococci to raw-milk cheeses starters seems to be important to preserve the characteristics of traditional varieties like Cebreiro cheese (Centeno et al., 1999) or Mediterranean traditional cheeses where enterococci are often present at high numbers (Giraffa, 2003; Sarantinopoulos et al., 2002). Enterococci can be isolated from

traditional cheeses and classified according to their biochemical characteristics (Giraffa et al., 1997; Jamaly et al., 2010; Serio et al., 2010). Serio et al. (2010) isolated several strains of *Enterococcus* from Pecorino Abruzzese cheese and their metabolic activities were evaluated at ripening temperature. After 15 days at 10°C, the *Enterococcus* strains isolated maintained their metabolic activities indicating their important influence on the ripening of Pecorino Abruzzese cheese. Jamaly et al. (2010) isolated 23 strains of *E. durans* from Moroccan dairy products and reported the potential contribution of the strains to cheese ripening and aroma. The *Enterococcus* strains isolated by Jamaly et al. (2010) and Serio et al. (2010) demonstrated slow acidification of milk indicating their inability to be used as starter culture in cheesemaking. However, the use of enterococci as adjunct culture was evaluated in some studies where enterococci were added to the cheese-milk during cheesesmaking and the quality of the cheese was assessed. As reported by Gardiner et al. (1999), enterococci added as adjunct culture during Cheddar cheese manufacture can survive during ripening and contribute to cheese quality by accelerating cheese ripening and contributing to the development of cheese aroma. In a study by Gursoy and Kinik (2010), *E. faecium* was added as adjunct culture to the cheesemilk destined to the production of white cheese and the cheese quality was investigated; *E. faecium* survived during cheese ripening and an improvement of cheese taste. *E. faecium* was also used as adjunct culture in the production of Feta cheese (Sarantinopoulous et al., 2002) resulting in an improvement in Feta cheese quality. *E. faecium* influenced proteolysis in Feta cheese; moreover, flavour and texture of the cheese were favourably improved. In a study by Rasouli Pirouzian et al. (2012), *E. faecium* was used in the manufacture of Iranian UF white cheese and positive effect on flavour development

were observed; *E. faecium* increased lipolysis and secondary proteolysis in the Iranian white cheese. *Enterococcus faecium* was also added to a cheese model system in a study published by Tavaria et al. (2006) and the authors found a production of volatile compounds increased compared to the production of volatile in the model system inoculated with *Lactobacillus plantarum* or *Lactococcus lactis*.

The aim of this study is to investigate the contribution of *Enterococcus faecium* to the quality of Cheddar cheese. Therefore, a control Cheddar cheese was manufactured with only traditional starter culture whereas experimental cheeses contained the starter culture and different levels (10^6, 10^7 or 10^8 cfu/mL cheesemilk) of *E. faecium* EF031 (Bioprox, Levallois, France). Three independent trials were manufactured and during ripening composition, pH, microbial growth, redox potential, proteolysis, lipolysis, production of biogenic amine and flavour development were monitored.

7.2   Materials and methods

7.2.1   Bacterial strain

The food-grade commercial culture of *Enterococcus faecium* EF 031 utilized in this study was obtained from Aroma-Prox® (Bioprox, Levallois-Perret, France) and used as a direct vat set culture. The culture was stored at -80°C until utilization.

7.2.2 Cheddar cheese manufacture

Pilot-scale Cheddar cheeses were manufactured in triplicate, with each vat containing 10 L of pasteurized milk. Milk at 30-31°C was inoculated at a rate of 0.02% (w/v) with a concentrated starter culture (DVS R604, Chr. Hansen, Hørshom, Denmark). The control vats contained starter culture only, to the experimental vats started culture and different numbers (10^6, 10^7, 10^8 cfu/mL) of *Enterococcus faecium* were added. After a 30 min ripening period, the milk was supplemented with 1 M $CaCl_2$ (0.9 mL/L) and coagulated with Chymax-180 (Chr. Hansen, Hørshom, Denmark), diluted 1:5 with distilled water, added at a level of 0.3 mL/L. The curd was cut after 40-50 min and allowed to set for 10 min. The temperature was increased to 39°C (at a rate of 1°C every 3 min) and the curd was stirred until a pH of 6.2 was reached. The whey was drained off and the curd was left for continued acidification and matting. During this period, the curd was formed into blocks that were turned upside down and stacked (cheddaring stage). When the pH reached 5.4, the curd was milled and salted at the rate of 2.5% (w/w). Following moulding and pressing overnight at 1.5 bar the cheeses were removed from the mould, vacuum-packed, and ripened for 6 months at 8°C.

Cheddar cheeses were sampled at different time points during the ripening for microbiological and physicochemical analysis.

7.2.3 Bacteriological analysis

Enterococcus, starter and non-starter lactic acid bacteria (LAB) counts were performed on 1, 15, 30, 60, 120 and 180 day-old cheeses.

Cheese (5 g) was homogenised in 45 mL of 2% (w/v) tri-sodium citrate solution for microbiological analysis and decimal dilutions were prepared in tubes of sterile quarter-strength Ringer's solution tubes. Starter LAB were enumerated in duplicate on LM17 agar (Merck, Darmstadt, Germany) after incubation at 30°C for 3 days. Non-starter LAB were enumerated in duplicate under anaerobic conditions (Merck Anaerocult A gas pack; Merck, Darmstadt, Germany), on LBS agar (Becton, Le Pont de Claix, France) after incubation at 30°C for 5 days. Enterococci were enumerated in duplicate on kanamycin esculin azide agar (Fluka, Buchs, Switzerland) after incubation at 37°C for 2 days. Since enterococci can grow on LM17 agar, the number of starter LAB was calculated by subtracting the number of colonies counted on kanamycin esculin azide agar in the control cheese from the number of colonies counted on LM17 agar. The total colony forming units in cheese were calculated by counting colonies on plates with between 30 and 300 colonies.

7.2.4 Cheese compositional analysis, pH and oxidation-reduction potential measurements

The composition (protein, salt, moisture and fat) of grated samples was determined in triplicate on 15 day-old cheeses.

The protein content of the cheeses was determined by the macro-Kjeldahl method (IDF, 1986), salt by a potentiometric titration (Fox, 1963), moisture by oven drying at 102°C (IDF, 1982) and fat by the Gerber method (IIRS, 1955).

The pH was measured throughout ripening by direct insertion of a pH probe (PHC3001-8, Radiometer Analytical, Villeurbanne Cedex, Lyon, France) connected to a

pH meter (PHM210 Standar pH Meter, Radiometer Copenhagen, Denmark) into a cheese-water slurry (1:10 cheese in water dilution).

The oxidation reduction potential (E_h) was monitored during cheesemaking until the whey drainage stage of manufacture using the method described by Caldeo and McSweeney (2012) and at four months of ripening E_h measurement were performed using the method reported by Topcu et al. (2008). Measurements were taken using a platinum working electrode platinum working electrode (XM120) and silver/silver chloride reference electrode (REF 201, both from Radiometer Analytical, Villeurbanne Cedex, Lyon, France). Before each experiment, the reference and the platinum electrodes were cleaned by the method reported by Topcu et al. (2008), Caldeo and McSweeney (2012) and Abraham et al. (2013). The accuracy was measured against a standard solution (470 mV, HI 7022, Hanna Instruments, Szeged, Hungary) and against tap water (Abraham at al., 2007; Jeanson et al., 2009; Martin et al., 2011) at 25°C. Oxidation-reduction potential data were recorded through a data logger (Squirrel Data Logger 2040-2F16 Series; Grant, Data Acquisition, Cambridge, UK) without reference to a hydrogen reference electrode and converted to E_h (potential related to the standard hydrogen electrode) according to Caldeo and McSweeney (2012) with temperature compensation.

7.2.5   Assessment of proteolysis

7.2.5.1 Protein fractionation

The pH 4.6-soluble and -insoluble extracts of the Cheddar cheese samples were prepared according to the procedure of Kuchroo and Fox (1982) with modifications by

homogenization of grated cheese with 5-fold the amount of water for 15 min in a stomacher bag (Stomacher 400, Seward Limited, Worthing, West Sussex, UK). The homogenate was adjusted to pH 4.6, and left to stand at room temperature for 30 min. pH was readjusted to pH 4.6, if necessary. After pH adjustment, the homogenate was held in a water-bath at 40°C for 1 h and centrifuged at 3000 x g for 30 min at 4°C (Sorvall RC 5C Plus centrifuge, Beckman Coulter, Ramsey, MN, USA) and then paced at 4°C for 30 min. The supernatant was filtered through glass wool and filter paper (113, Whatman, GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK). Portions of the soluble part was freeze of freeze dried for further analysis, while the insoluble fraction was freeze dried.

The nitrogen content of the pH 4.6-soluble fractions was determined by the macro-Kjeldahl method (IDF, 1986) and expressed as a % of the total nitrogen content of the cheeses.

7.2.5.2 One-dimensional gel electrophoresis

Cheeses were analysed by urea-polyacrylamide gel electrophoresis (urea-PAGE, 12.5% total monomer concentration (T); 4% cross-linking monomer (C), pH 8.9) on 1, 15, 30, 60, 120, 180-day-old cheeses using a Protean II XI vertical slab-gel unit (Bio-Rad Laboratories Ltd., Watford, Herts, UK) according to the method of Andrews (1983) with modifications. Cheese samples and sodium caseinate were prepared by dissolving in sample buffer (10 mg/mL) and heated few minutes at 50°C to dissolve the samples completely; 3.5 μL of sodium caseinate and 6 μl of cheese samples were applied to the gel. For visualization of proteins, gels were stained with Coomassie Brilliant Blue G250 using the method of Blakesley and Boezi (1977) with gentle agitation at 20 rpm on an

orbital platform shaker (Heidolph Rotamix 120, Heidolph Canada Biotech Edge, Toronto, Canada) overnight. Gels were destained in distilled water until the background was clear, and scanned on a flatbed scanner (Epson Perfection 4180 Photo, Seiko Epson Corporation, Nagano, Japan).

7.2.5.3 Total and individual free amino acid analysis

Total free amino acids were quantified on the pH 4.6-soluble extracts of the cheeses at by using the trinitrobenzene-sulphonic acid method (TNBS) (Adler-Nissen, 1979). A calibration curve was prepared using leucine (Leu, Sigma) as standard (range 0.0-1.0 mmol/L of Leu), and results were expressed as mg Leu/g of cheese.

Individual free amino acids were determined on the pH 4.6-soluble extracts of the cheeses at 180 days of ripening. Samples were deproteinised by mixing with equal volumes of 24% (w/v) trichloroacetic acid (TCA) and allowed to stand for 10 min before centrifugation at 14,400 g for 10 min (Microcentaur, MSE, London, UK). The supernatant was removed and diluted with 0.2 M sodium citrate buffer (pH 2.2) to give approximately 250 nmol of each amino acid residue per mL. The samples were then diluted 1:2 with the internal standard norleucine to give an approximate final concentration of 125 nmol of each amino acid residue in 1 mL of injection solution. Samples were then analysed using a Jeol JLC-500/V amino acid analyser (Jeol Ltd., Garden City, Herts, UK) fitted with a Jeol Na$^+$ high performance cation-exchange column. The individual free amino acids were separated by ion-exchange chromatography with post-column ninhydrin derivatization and visible colorimetric detection at 570 nm. Results were recovered using an Aminotaq data handling system (Joel Ltd).

7.2.5.4 Reverse phase-high performance liquid chromatography (RP-HPLC)

Peptide profiles of 4.6-soluble extracts on 15 and 180-day-old cheeses were performed at Hacettepe University (Ankara, Turkey). Peptides were detected by ThermoFinnigan HPLC system (ThermoFinnigan Inc., San Francisco, USA) integrated with an auto sampler including temperature control for the column (SpectraSystem AS3000), a degasser system (SpectraSystem SCM1000), a quaternary gradient pump (SpectraSystem P4000), a photodiode-array detector (SpectraSystem UV6000LP). Peptides separation was achieved using a Jupiter C18 Widepore column (5 μm, 300 Å, 250 x 4.6 mm, Phenomenex, Torrance, CA, USA) equipped with SecurityGuard Ultra Widepore guard column for 4.6 mm ID (Phenomenex). Separations were conducted at 30°C using a mobile phase of two solvents at eluent flow rate of 0.8 mL/min. Buffer A was 0.1% (v/v) trifluoroacetic acid (TFA, HPLC grade, J.T.Baker, Center Valley, PA, USA) in deionized water, and buffer B was 0.1% (v/v) TFA in acetonitrile (ultragradient grade; Merck, Darmstadt, Germany), starting with 100% buffer A for 10 min, and continuing with a linear gradient to 50% B over 80 min, a linear gradient to 60% B over 5 min, and 60% B for 5 min. The column was finally eluted with 100% buffer B for 2 min. Samples (10 mg/mL) of freeze-dried 4.6-soluble extracts were dissolved in buffer A and filtered through 0.45 μm cellulose acetate filter, and an aliquot (100 μl) of the filtrate was injected to the HPLC system. The absorbance was monitored at 214 nm (Topcu et al., 2006).

The retention times of three aromatic amino acids (tyrosine, phenylalanine and tryptophan) were used to define hydrophilic and hydrophobic zone, at a detection wavelength of 214 nm. These amino acids were identified by comparison of their

retention time with that of standard solutions that were injected separately under the same conditions. The hydrophilic peptide portion consisted of the peptides that eluted between Tyr and Trp (from 20 to 37.5 min). The group of hydrophobic peptides consisted of peptides with retention times from 37.5 to 80 min.

Data from the RP-HPLC chromatograms of the pH4.6-SN fractions of cheese were pre-processed as described by Piraino et al. (2004). Briefly, chromatograms (height and retention time of peaks) were exported in a data sheet; 111 classes of 0.75 min were defined over the retention time axis of the chromatogram from 7 to 90 min; Quantity was not expressed as %, and positions were not logarithmically transformed. No noise for attribute was set. Flat range and membership in the flat range were 95 and 99.9% respectively. Class width was 0.75 min that is 0.9% of the selected range. Flat range width was 0.72 min. The shape parameter was 303.9 and classes of retention time were used as variables for principal component analysis (PCA), which was performed using the covariance matrix.

7.2.6   *Free fatty acid extraction and analysis*

Levels of free fatty acids on 60 and 180-day-old cheeses were measured by gas chromatograpy (GC) as described by De Jong and Badings (1990). Extraction of free individual fatty acids (FFA) for GC analysis was carried out by using a solid-phase extraction technique; 500 mg Varian Bond Elut-NH_2 cartridges were used. FFAs were quantified using a gas chromatograph (Varian Star 3400 CX with Varian 8200 CX autosampler and flame ionization detector (300ºC) interfaced with Star Chromatography Workstation 5.0 software for data acquisition; Varian Analytical Instruments, Harbor City, CA, USA). A wall-coated open tubular fused silica capillary

column (25 m length x 0.32 mm internal diameter) coated with FFAP-CB was used.

FFAs were separated and identified by reference to known standards and quantified by

peak area. A standard calibration mix of 17 fatty acids (including internal standards,

valeric acid C5:0; pelargonic acid C9:0; margaric acid C17:0) was prepared at

concentrations 1000, 500, 300, 200, 100 and 50 ppm.

7.2.7   Analysis of volatile compounds

Analysis of volatile compounds in cheese was performed by SPME-GC-MS on 14, 60 and

180 day-old cheeses at Inonu University (Malatya, Turkey). Vacuum-packed cheese

samples were frozen-sliced into small granules of 1-2 mm in size and immediately

placed in glass vials [15-mL (Supelco, Bellefonte, PA, USA)] in a freezer at -20°C. On the

day of analysis, 81 ppm of the internal standard (2-methyl-3-heptanone in methanol

(Sigma-Aldrich Co. USA)) was added to 3.00 g of the sample and allowed to equilibrate

at 40°C for 30 min. Extraction of volatiles was carried out using a solventless extraction

technique as reported by Pawliszyn (1997). The method is suitable for the isolation of

volatile compounds from the sample matrix and has been used in various studies for

the characterization of cheese samples. Essentially, extraction is achieved by

introducing a 75 μm carboxen-polydimethylsiloxane (CAR/PDMS) fibre (Supelco,

Bellefonte, PA, USA) through the septum into the vial and exposing it to the headspace

for 30 min at 40°C. Desorption of the extracted volatiles was performed using a

Shimadzu GC-2010 gas chromatography-QP-2010 mass spectrometry system

(Shimadzu Corporation, Kyoto, Japan) and run in split (ratio was 1:20) mode. During

desorption, the SPME fibre remained in the injector for 2 min at a temperature of 250

°C, with helium as the carrier gas at a flow rate of 1.0 mL/min. The volatile compounds

were separated on a DB-Wax column (60 m × 0.25 mm × 0.25 um; J&W Scientific, Folsom, CA, USA). The oven was held at 40°C for 2 min (desorption period), then increased at 5°C per min to 240°C, where it was held for 5 min to give a total run time of 47 min. The mass spectrometer was set to scan from 33 to 450 Da (threshold 1,000 Da) at a sampling rate of 1.11 scans/s. The identification of the volatile compounds was performed by calculation of retention indices (RI) of each compound by using *n*-alkane series (C8 to C20 supplied from Sigma Chemical Co., St. Louis, MO) under the same conditions. The tentative identifications were based on comparing mass spectra of unknown compounds with those in Wiley 7 (7th edition) and NIST/EPA/NIH mass spectral library with Search Program (Data Version: NIST 14, Software Version 2.2g). Identifications were also confirmed by comparing retention times with reference standards when available. A total of 33 authentic standard compounds (Sigma Chemical Co., St. Louis, MO, USA) were used to confirm the identities of volatile compounds in the cheese samples. The RI values were also compared with those described in the literature as determined under the same conditions for matching the compounds. The concentrations were calculated by the comparison of the peak areas of the internal standard and unknown compounds. Each compound was expressed as µg/100 g of cheese.

7.2.8   Detection of biogenic amines

Biogenic amines (BA) were detected in cheeses at 180 days of ripening at Tomas Bata University in Zlin (Czech Republic).

Lyophilized 4.6-soluble fractions samples (obtained from 1 mL of extract) were diluted using perchloric acid (0.6 mol/L) and 0.5 mL of obtained mixtures (vigorously

vortexted) were subjected to derivatisation with dansyl chloride according to Dadáková et al. (2009); 1,7-heptanediamine was used as an internal standard. The derivatised samples were filtered (porosity 0.22 μm) and applied on a column (Zorbax Eclipse XDB-C18, 150 x 4.6 mm, 3.5μm, Agilent Technologies, Santa Clara, CA, USA). The concentration of eight biogenic amines (histamine, tyramine, phenylethylamine, tryptamine, putrescine, cadaverine, spermidine, spermine) was monitored by an high performance liquid chromatography system equipped with a binary pump, an autosampler (LabAlliance, State College, MD, USA), a column thermostat, a UV/VIS DAD detector (λ= 254 nm), and a degasser (1260 Infinity, Agilent Technologies, Santa Clara, CA, USA). The conditions for separation of the monitored BA are described by Smelá et al. (2004). Each of the lyophilized samples was derivatised three times. Standards, reagents and eluents were obtained from Sigma Aldrich (St. Louis, MO, USA).

7.2.9   Statistical analysis

Analysis of variance (one-way ANOVA) was conducted using SPSS Version 20.0 for Mac OS X (SPSS Inc., Chicago, IL, USA) on compositional data, pH values, level of pH 4.6-soluble nitrogen, level of total free amino acids and level of free fatty acids. When differences were significant ($P < 0.05$), the means were analysed using Tukey's test.

PCA of the processed peak height data from RP-HPLC of the pH 4.6 soluble extracts for the Cheddar cheeses and of the volatile compounds identified for the Cheddar cheeses were performed using SPSS software, version 20.0 (SPSS Inc., Chicago, IL, USA).

7.3   Results and discussion

7.3.1   Chemical composition, pH, E_h and microbiological growth of the cheeses

At 15 days of ripening, cheeses were analysed for protein, fat, moisture and salt content and pH. Results for each trial are reported in Table 7.1. The composition of the control and experimental cheeses were generally found to be within the range typical for Cheddar (Lawrence et al., 2004). One-way ANOVA was performed on the compositional data within each trial and significant differences (Tukey's, $P < 0.05$) among cheeses are indicated in Table 7.1.

Table 7.2 shows changes in pH of control and experimental cheeses during ripening as average of three independent trials. Results showed no differences between the pH of control cheeses and cheeses containing *E. faecium* as adjunct culture (Table 7.2). The addition of *E. faecium* did not affect the conversion of residual lactose to lactic acid that occurring during ripening (Madkor et al., 2000).

Table 7.1   Composition and pH of control Cheddar cheese and cheeses made from milk containing different levels (10^6, 10^7, 10^8 cfu/mL milk) of *Enterococcus faecium* EF031 at 15 days of ripening.

Composition	pH	Moisture (%)	Fat (%)	Protein (%)	Salt (%)
Trial A					
Control	4.96^b (0.01)	40.95^d (0.10)	32.50^a (0.71)	22.55^a (0.32)	1.46^b (0.01)
E. faecium 10^6 cfu/ml	4.91^a (0.00)	39.50^c (0.28)	33.00^a (0.00)	22.78^a (0.63)	1.38^a (0.01)
E. faecium 10^7 cfu/ml	$4.95^{a,b}$ (0.01)	38.47^b (0.19)	34.00^a (0.00)	23.89^a (0.32)	1.54^c (0.01)
E. faecium 10^8 cfu/ml	4.96^b (0.00)	37.72^a (0.09)	34.50^a (0.71)	23.89^a (0.32)	1.62^d (0.02)
Trial B					
Control	4.86^a (0.01)	43.33^c (0.23)	32.50^a (0.71)	22.55^a (0.32)	1.13^a (0.04)
E. faecium 10^6 cfu/ml	4.84^a (0.00)	$42.51^{b,c}$ (0.39)	32.00^a (0.00)	22.78^a (0.63)	1.23^a (0.03)
E. faecium 10^7 cfu/ml	4.90^b (0.01)	40.5^a (0.01)	33.00^a (0.00)	23.89^a (0.32)	1.19^a (0.08)
E. faecium 10^8 cfu/ml	4.90^b (0.01)	$41.68^{a,b}$ (0.51)	32.50^a (0.71)	23.89^a (0.32)	1.22^a (0.01)
Trial C					
Control	4.93^a (0.01)	39.98^a (0.76)	32.75^a (1.06)	$22.13^{a,b}$ (0.29)	1.36^b (0.06)
E. faecium 10^6 cfu/ml	4.89^a (0.01)	40.60^a (0.32)	32.25^a (0.35)	$22.22^{a,b}$ (0.15)	1.21^a (0.02)
E. faecium 10^7 cfu/ml	5.00^b (0.01)	39.41^a (0.18)	32.50^a (0.71)	22.44^b (0.16)	1.37^b (0.04)
E. faecium 10^8 cfu/ml	4.91^a (0.00)	40.84^a (0.22)	32.50^a (0.71)	21.54^a (0.17)	$1.30^{a,b}$ (0.01)

Numbers represent mean and standard deviation, with the latter in parentheses. In each trial, mean in a column with different superscripts are significantly different (Tukey's, P ≤ 0.05).

Table 7.2   Changes in pH during ripening of control Cheese cheese (A), Cheddar cheese made from milk containing 10^6 (B), 10^7 (C), or 10^8 (D) cfu/mL of *Enterococcus faecium*.

	Ripening time (day)					
	1	**15**	**30**	**60**	**120**	**180**
A	4.96[a] (0.03)	4.91[a] (0.05)	4.94[a] (0.05)	4.93[a] (0.06)	4.96[a] (0.05)	4.99[a] (0.04)
B	4.91[a] (0.08)	4.88[a] (0.04)	4.91[a] (0.04)	4.94[a] (0.04)	4.95[a] (0.05)	5.01[a] (0.09)
C	4.95[a] (0.04)	4.95[a] (0.05)	4.96[a] (0.05)	4.99[a] (0.04)	5.01[a] (0.05)	5.06[a] (0.05)
D	4.95[a] (0.08)	4.92[a] (0.03)	4.96[a] (0.03)	4.97[a] (0.02)	4.99[a] (0.00)	5.02[a] (0.02)

Numbers represent mean and standard deviation, with the latter in parentheses. Mean in a column with different superscripts are significantly different (Tukey's, $P \leq 0.05$).

Counts of cheese starter, non-starter and *Enterococcus* bacteria in Cheddar control cheese and cheeses containing *E. faecium* as adjunct culture at level of 10^6, 10^7 and 10^8 cfu/g cheese during 6 months of ripening are shown in Figure 7.1. Numbers of starter LAB were similar in all the cheeses indicating that the addition of *E. faecium* did not affect the starter population (Figure 7.1A). In all the cheeses, starter LAB decreased during ripening as reported from other authors (McSweeney et al., 1993a; Milesi et al., 2008). During ripening, non-starter LAB levels increased in all the cheeses and values of 3.0 x 10^6 cfu/g of cheese were found at the end of the ripening (Figure 7.1B). Adjunct *Enterococcus* bacteria were enumerated during ripening (Figure 7.1C). *E. faecium* were added to the cheese milk at 10^6, 10^7 and 10^8 cfu/mL milk and the experimental cheeses showed the survival of the *Enterococcus* and values of 2.67 x 10^6, 2.15 x 10^7 and 2.13 x 10^8 cfu/g cheeses were found, respectively. However, during ripening one log cycle

reduction in *Enterococcus* population was observed in all the experimental cheeses. Control cheese had numbers of *Enterococcus* below 7.0 x 10^1 cfu/g cheese during ripening and those levels were lower compared to the numbers reported by Gardiner et al. (1999).
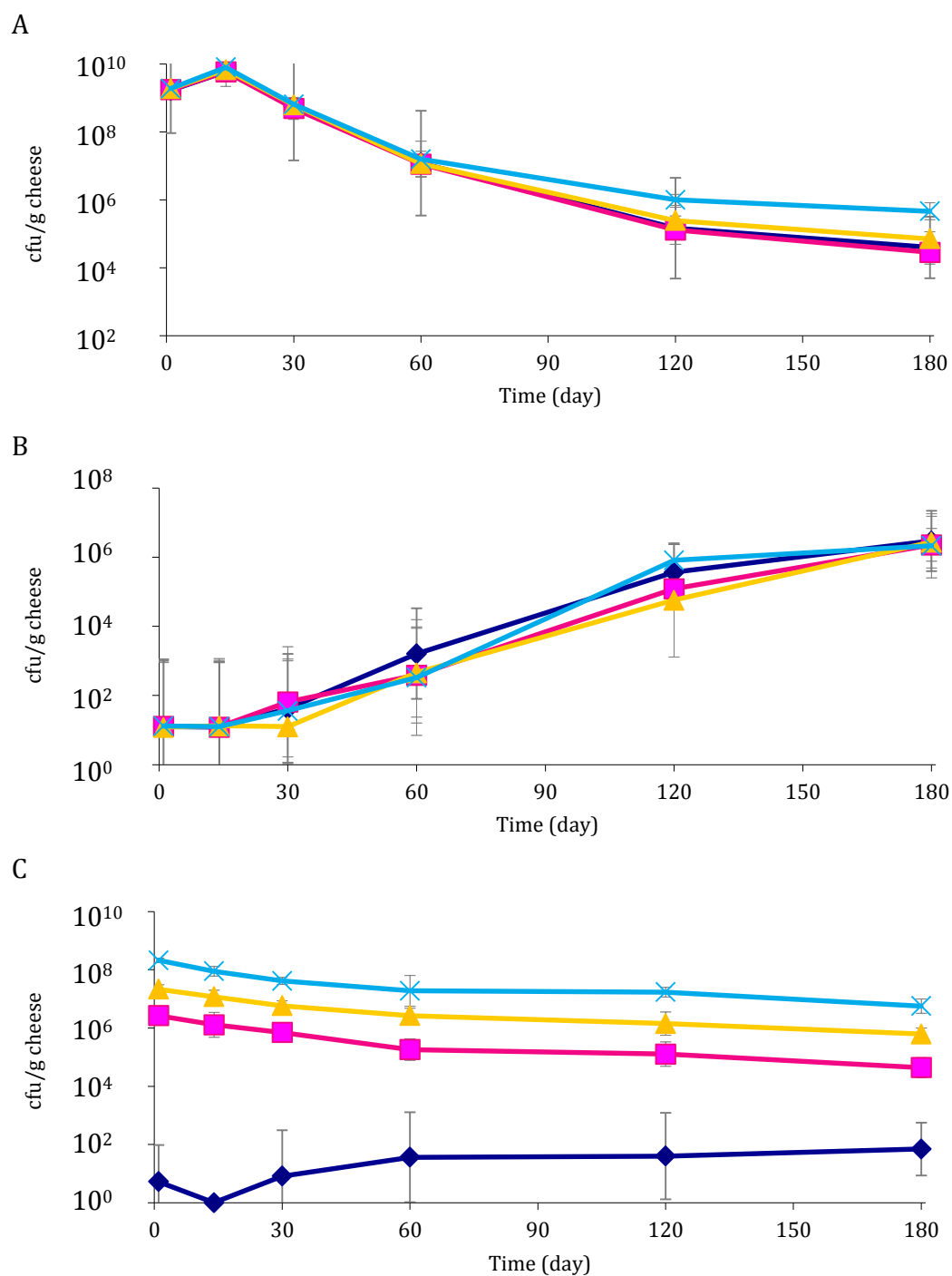
Figure 7.1 Counts of starter lactic acid bacteria (A), non-starter lactic acid bacteria (B) and Enterococcus (C) during ripening of control Cheddar cheese (♦), Cheddar cheese made from milk containing 10^6 (■), 10^7 (▲), or 10^8 (x) cfu/mL of *Enterococcus faecium*. Count are average of three independent trials, with standard deviations indicated by vertical bars.

Oxidation-reduction potential (E_h) was measured during cheesemaking until the whey drainage stage. Results are shown in Figure 7.2A and the trend in E_h values of the cheeses made with the addiction of *E. faecium* showed a faster decrease in E_h compared to the control cheese, especially in the cheese made with the addiction of 10^8 cfu/mL of *E. faecium*. Moreover, the values reached by the cheese made with 10^8 cfu/mL of *E. faecium* was lower compared to the other cheeses.

E_h was measured at 4 months of ripening in control cheese and in the cheese made with the higher level of *E. faecium* and results are shown in Figure 7.2B. During measurements, the experimental cheese made from milk containing 10^8 cfu/mL milk of *Enterococcus faecium* had lower E_h and the equilibrium value was reached faster compared to the control cheese. At equilibrium, the E_h of the control cheese did not differ from the value of the cheese made with the addition of *E. faecium*.

A
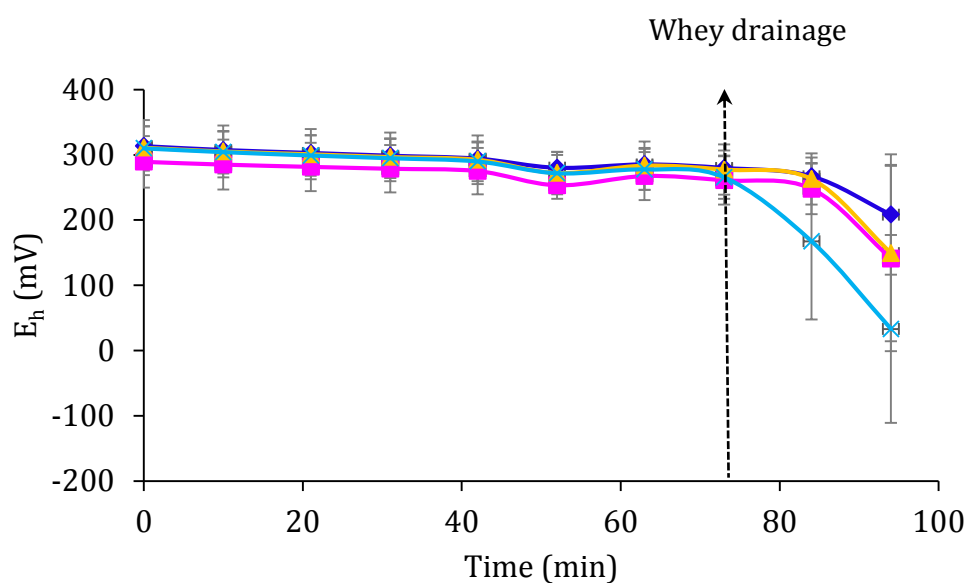
B
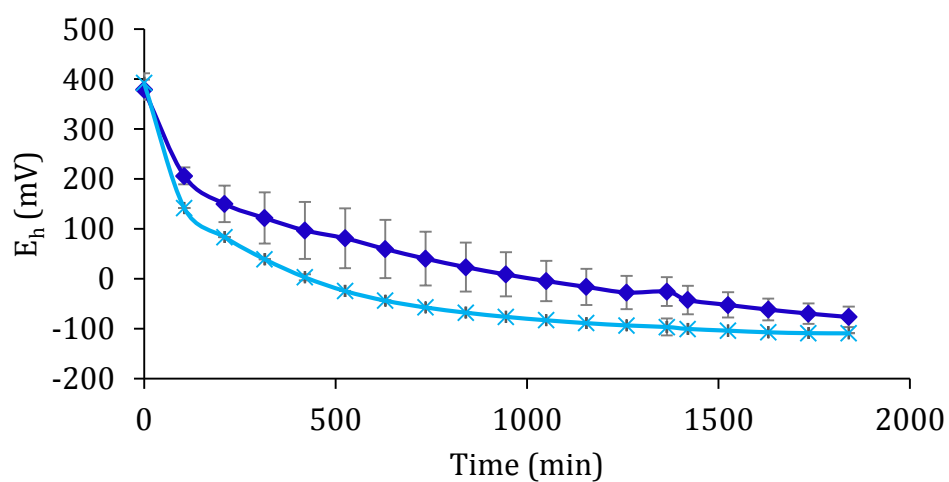
Figure 7.2   Oxidation-reduction potential (E_h) of control Cheddar cheese (♦), Cheddar cheese made from milk containing 10^6 (■), 10^7 (▲), or 10^8 (x) cfu/mL of *Enterococcus faecium* (A) during cheesemaking and E_h of Cheddar cheese (♦) and Cheddar cheese made from milk containing 10^8 (x) cfu/mL of *Enterococcus faecium* at 4 months of ripening (B). Values are average of three independent trials, with standard deviations indicated by vertical bars.

Therefore, the presence of *E. faecium* during cheesemaking and ripening seems to contribute to a faster reduction in E_h. Enterococci were found to reduce the redox potential to negative values in a shorter time compare to other microorganisms in a study by Brasca et al. (2007) where different species were incubated in skim milk and E_h was measured.

7.3.2   *Assessment of proteolysis*

7.3.2.1 *Urea-PAGE*

Electrophoretograms of the control cheese and cheeses made with the addition of *E. faecium* as adjunct culture at level of 10^6, 10^7 and 10^8 cfu/mL during 6 months of ripening are shown in Figures 7.3 A and B (one trial is shown). No differences were found between the profile patterns of casein breakdown in control and experimental cheeses indicating that the addition of *E. faecium* did not contribute to the primary proteolysis in Cheddar cheese. Primary proteolysis is mainly due by the action of chymosin and plasmin (McSweeney et al., 1993b; Sousa et al., 2001) and other authors reported that primary proteolysis is generally not influenced by the addiction of adjunct cultures (Lynch et al., 1998; Michaelidou et al., 2003; McSweeney et al., 1994; Milesi et al., 2008; Gursoy and Kinik, 2010).

However, Sarantinopoulos et al. (2002) found that the addition of *Enterococcus faecium* during the manufacture of Feta type cheeses promoted the degradation of α_{s1}- and β-caseins during ripening; similar results were found by Centeno et al. (1999) during ripening of Cebreiro cheese made with the addiction of *Enterococcus faecalis*.

A
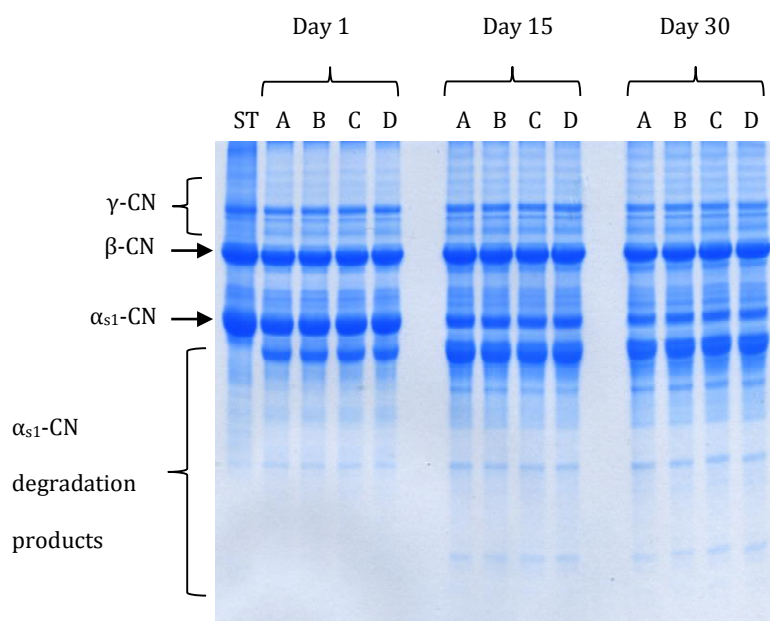
B
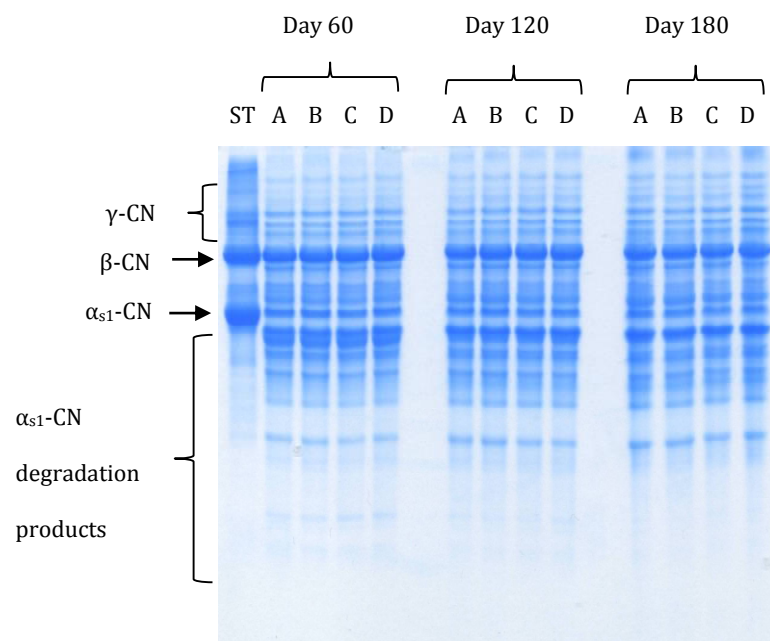
Figure 7.3   Urea- polyacrylamide gel electrophoretograms of sodium caseinate (ST), control Cheddar cheese (A), Cheddar cheese made from milk containing 10^6 (B), 10^7 (C), or 10^8 (D) cfu/mL of *Enterococcus faecium* at 1, 15, 30, 60, 120 and 180 days of ripening. (CN: casein).

7.3.2.2 pH 4.6 soluble nitrogen

Mean levels of pH 4.6-soluble nitrogen as percentage of total nitrogen at 1, 15, 30, 60, 120 and 180 days of ripening are shown in Table 7.3.

The level of pH 4.6-solube nitrogen increased in all cheeses as a result of ripening indicating the progression of proteolysis. The trends of pH 4.6-soluble nitrogen found are typical of Cheddar cheese and at the end of the ripening levels were below 30% of the total nitrogen (Fox et al., 2000). Within each trial, slight differences were found between the control cheese and the experimental cheeses. At each time point, there were no significant differences in levels of pH 4.6-soluble nitrogen between the control and experimental cheeses in Trial 3. Whereas in Trial 1 control cheese had a significantly higher (P < 0.05) value compare to the experimental cheeses at 180 days of ripening. In Trial 2 at 1, 30 and 120 days of ripening the experimental cheeses had levels of pH 4.6-soluble nitrogen significantly higher compared to the values of the control cheese.

Table 7.3   Levels of pH 4.6-soluble nitrogen as percentage of total nitrogen in control Cheese cheese (A), Cheddar cheese made from milk containing 10^6 (B), 10^7 (C), or 10^8 (D) cfu/mL of *Enterococcus faecium* at 1, 15, 30, 60, 120 and 180 days of ripening.

	Ripening time (day)					
	1	**15**	**30**	**60**	**120**	**180**
Trial 1						
A	4.88[a] (0.17)	9.48[a] (0.26)	11.04[a] (0.18)	15.49[a] (0.08)	20.47[a] (1.39)	26.35[b] (0.49)
B	5.61[a] (0.69)	9.88[a] (0.17)	10.93[a] (0.03)	15.77[a] (0.45)	20.10[a] (1.35)	24.16[a] (0.14)
C	5.28[a] (0.49)	9.71[a] (0.05)	11.27[a] (0.35)	15.67[a] (0.55)	21.54[a] (2.39)	24.99[a,b] (0.24)
D	5.95[a] (0.44)	9.97[a] (0.07)	11.21[a] (0.20)	15.43[a] (0.67)	20.61[a] (0.47)	24.34[a] (0.80)
Trial 2						
A	5.47[a] (0.04)	10.34[a] (0.12)	11.84[a] (0.08)	15.43[a] (0.20)	19.24[a] (0.09)	28.44[a] (1.28)
B	5.47[a] (0.20)	10.35[a] (0.53)	12.36[a,b] (0.09)	15.54[a] (0.18)	19.44[a] (0.40)	24.27[a] (0.02)
C	5.72[a,b] (0.04)	10.50[a] (0.38)	11.92[a,b] (0.39)	15.23[a] (0.12)	21.27[b] (0.79)	26.34[a] (3.83)
D	5.98[b] (0.01)	10.77[a] (0.37)	12.76[b] (0.17)	15.30[a] (0.04)	21.67[b] (0.07)	24.33[a] (0.17)
Trial 3						
A	5.95[a] (0.16)	10.19[a] (0.27)	11.94[a] (0.34)	15.31[a] (0.51)	19.79[a] (1.23)	24.25[a] (1.61)
B	5.57[a] (0.10)	10.01[a] (0.08)	12.23[a] (0.20)	15.68[a] (0.02)	23.22[a] (0.29)	23.19[a] (0.10)
C	5.53[a] (0.16)	10.17[a] (0.05)	12.07[a] (0.19)	15.40[a] (0.58)	21.04[a] (0.21)	23.64[a] (0.60)
D	5.56[a] (0.21)	10.79[b] (0.08)	12.81[a] (0.01)	15.66[a] (0.11)	22.40[a] (1.94)	23.54[a] (0.68)

Numbers represent mean and standard deviation, with the latter in parentheses. In each trial, mean in a column with different superscripts are significantly different (Tukey's, $P \leq 0.05$).

These findings support our results obtained by urea-PAGE and are in agreement with previous studies where *Enterococcus* was used as an adjunct culture in cheese manufacture (Gardiner et al., 1999; Michaelidou et al., 2003; Gursoy and Kinik, 2010)

indicating that *E. faecium* has little or no effect on primary proteolysis. The main agent responsible for the increase of pH 4.6-soluble nitrogen is the proteolytic activity of rennet (Fox et al., 2000). In contrast to our results, in a study on the effect of the addition of *Enterococcus* to the manufacture of Cebreiro cheese (Centeno et al., 1999), the authors found a significantly higher level of soluble nitrogen in cheeses made with the adjunct cultures.

7.3.2.3 *Reverse phase-high performance liquid chromatography (RP-HPLC)*

Reverse phase-high performance liquid chromatography (RP-HPLC) was performed on the pH 4.6-soluble fraction of the 15, 60 and 180 days old control cheeses and cheeses made with the addition of *E. faecium* as adjunct culture at level of 10^6, 10^7 and 10^8 cfu/mL.

The RP-HPLC profiles of the pH 4.6-soluble fraction at 15 and 180 days of ripening are given in Figure 7.4. The profiles are characteristic of the proteolytic activity of enzymes naturally present in milk, in the rennet and derived from starter, adjunct and non-starter bacteria (Sousa et al., 2001). In the chromatograms, peptides eluted at lower retention time are considered hydrophilic, whereas peaks eluted at higher retention time correspond to hydrophobic peptides (Hynes et al., 2003). Peptides in Cheddar cheese are derived mainly from the N-terminal half of β-casein and also from the N-terminal half of α_{s1}- and α_{s2}- caseins and they are produced by the action of LAB proteinase or chymosin or plasmin (Singh et al., 1997; Sousa et al., 2001).
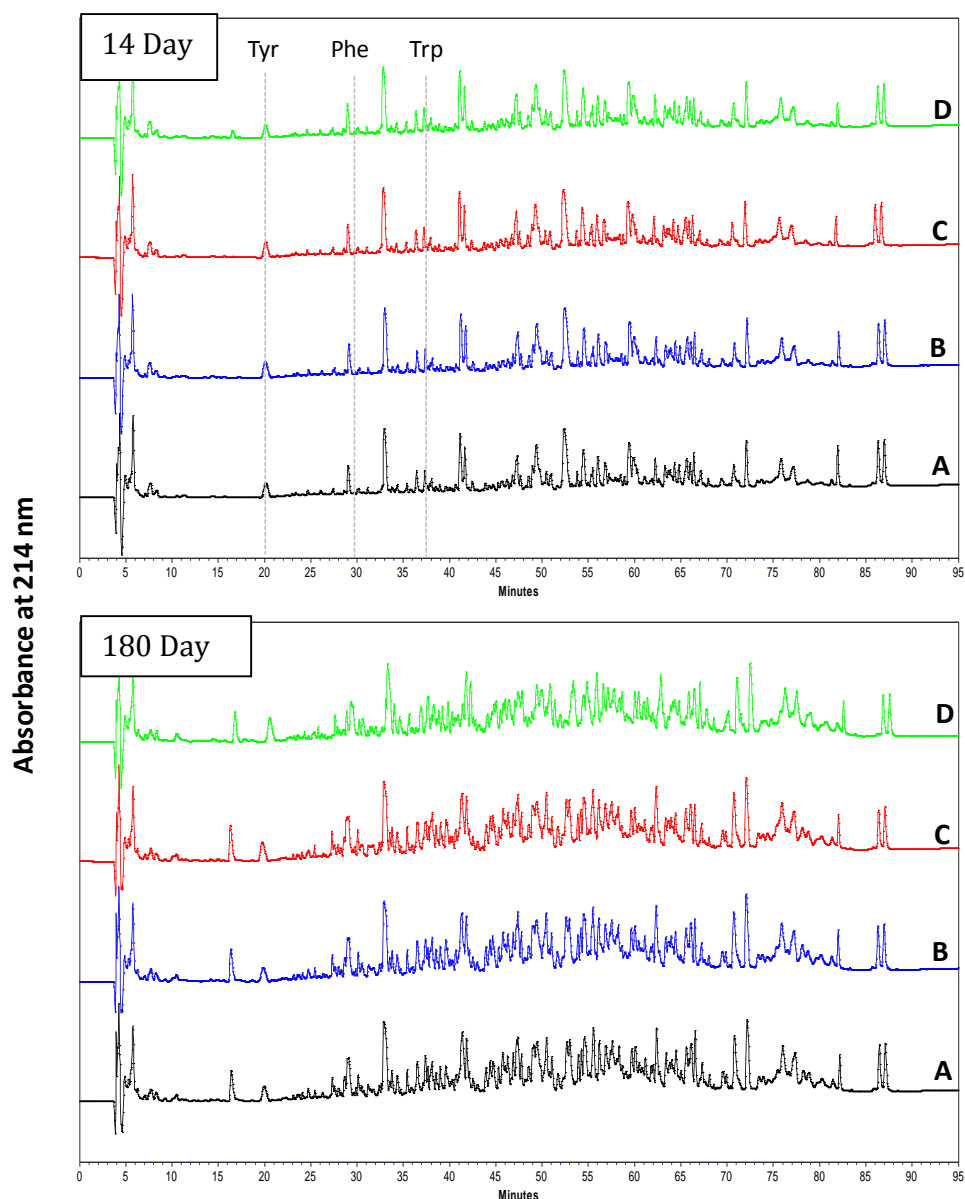
Figure 7.4   Reverse-phase HPLC profiles (at 214 nm) of the pH4.6 soluble fractions of of Cheddar cheese samples at 15 and 180 days of ripening (Trial 1 is shown). A: Control Cheddar cheese; B: Cheddar cheese made from milk containing 10^6 cfu/mL of *Enterococcus faecium,* C: Cheddar cheese made from milk containing 10^7 cfu/mL of *Enterococcus faecium,* D: Cheddar cheese made from milk containing 10^8 cfu/mL of *Enterococcus faecium,* Tyr: Tyrosine; Phe: Phenylalanine; Trp: Tryptophan.

In our study, RP-HPLC profiles at 15 or 180 days of ripening were quite similar in all the cheeses and comparable to the profiles reported by Law et al. (1992; 1993). An increase of the peak height and number of peaks during the ripening period was evident in all the cheeses during ripening (Figure 7.4).

Three peaks eluting at 82, 87 and 88 min were identified as α-lactalbumin and the A and B genetic variants of β-lactoglobulin, respectively (Figure 7.4). Levels of peptides eluting at 42 and 43 min increased during ripening and according to Sarantinopoulos et al. (2002) they could be identified as α_{s1}-casein (f1-13) and α_{s1}-casein (f1-14), respectively (Exterkate and Alting, 1995; Madsen and Ardö, 2001; Ardö et al., 2007). α_{s1}-casein (f1-13) and α_{s1}-casein (f1-14) derive from the activity of starter proteinases on rennet-hydrolysed α_{s1}-casein (Ardö et al., 2007) and they might contribute to bitter taste in cheese (Madsen and Ardö, 2001). As shown in Figure 7.4, Tyrosine (Tyr), Phenylalanine (Phe), and Tryptophan (Trp) peaks where identified by separated injection of standard solutions (with retention time of 20, 30, and 37.5 minutes, respectively) and their concentrations increased during the ripening period. These amino acids also have a bitter taste and have 2870, 2650, and 3000 hydrophobicity (Δf) values, respectively (Ney, 1979). In many cheese varieties it has been found that the Tyr, Trp, and also hydrophobic peptide levels are highly related to β-casein hydrolysis (Lemieux and Simard, 1992).

To investigate differences between the chromatographic profiles of pH 4.6-soluble fraction of cheese control samples and cheese samples manufactured different level of adjunct culture of *E. faecium*, PCA was performed on peak height data and results are shown in Figures 7.5A and B. Cheeses were separated by ripening period (PC1, 47.6%

of variance explained for Trial 1) and the control and experimental cheeses were separated by on the second component (PC2, 28.8% of variance explained for trial) (Figure 7.5A). Cheeses were cluster into three groups depending on the ripening time (Figure 7.5A). At 15 days of ripening, all the cheeses had very similar peptide profiles. At 60 and 180 days of ripening, according to PC2, cheeses made with different levels of adjunct cultures had different peptide profiles. Cheeses made with the higher quantities of *E. faecium* were clearly separated from the control cheeses (Figure 7.5A). The differences in the proteolytic system of the adjunct culture may have had an influence on the peptide profiles obtained. In other studies, PCA was successful in discriminating among the contribution of starter and adjuncts to the secondary proteolysis. In a study of Shakeel-Ur-Rehman et al. (1999) the peptide profiles were used to differentiate the contribution of different individual strains of *Lactococcus* to cheese ripening in miniature Cheddar. Moreover, Pripp et al. (1999) classified single strains of *Lactococcus* used during Cheddar cheesemaking into groups according to their chromatographic profile.

A

B

Figure 7.5   Plot of scores (A) and loadings (B) for the first and second principal components following principal component analysis (PCA) of the processed peak height data from RP-HPLC of the pH 4.6 soluble extracts for the Cheddar cheeses at 15 (15d), 60 (2m) and 180 (6m) days of ripening (Trial 1). In the plot of scores (A), letters A, B, C and D refers to control Cheddar cheese and Cheddar cheeses made from milk containing 10^6, 10^7 and 10^8 cfu/mL of *Enterococcus faecium*, respectively. In the plot of loadings (B), C is the category values obtained following pre-processing of chromatographic data using a logistic function. Numbers refer to chromatographic retention time.

7.3.2.4　Total and individual free amino acids

The levels of total free amino acids at 1, 15, 30, 60, 120, and 180 days of ripening were determined in cheeses by TNBS assay and were expressed as mg leucine/g cheese (Table 7.4). Concentration of free amino acids increased in all the cheeses during ripening as reported from other studies where the free amino acids were monitored during Cheddar cheese ripening (Lynch et al. 1996, 1998; Madkor et al., 2000). Significant differences among the cheeses were found at 120 and 180 days of ripening in trial 1 and 3; cheeses made with the highest quantity of adjunct culture had significantly higher ($P < 0.05$) level of total free amino acids compared to the control cheese. In trial 2, at 120 days of ripening there were no significant differences among the cheeses whereas at 180 days the cheese made with 10^8 cfu/mL of *E. faecium* had a significantly higher level of total free amino acids than control cheese. Those results are in agreement with a study by Gardiner et al. (1999) were a strain of *E. faecium* was used as adjunct culture during the manufacture of Cheddar cheese and higher level of total free amino acids were found. Moreover, other studies reported the ability of adjunct culture to increase the peptidase activity and therefore liberate higher quantity of amino acids (Broome et al., 1990; Lynch et al., 1996).

Table 7.4   Level of total free amino acids (mg Leu/g cheese) in control Cheese
cheese (A), Cheddar cheese made from milk containing 10^6 (B), 10^7 (C), or 10^8 (D)
cfu/mL of *Enterococcus faecium* at 1, 15, 30, 60, 120 and 180 days of ripening.

			Ripening time (day)			
	1	**15**	**30**	**60**	**120**	**180**
Trial 1						
A	2.25[a] (0.34)	3.24[a] (0.11)	3.75[a] (0.12)	5.42[a] (0.06)	8.04[a] (0.10)	11.18[a] (1.18)
B	2.18[a] (0.13)	3.47[a] (0.04)	3.94[a] (0.25)	5.45[a] (0.30)	7.78[a] (1.02)	11.85[a] (0.83)
C	2.11[a] (0.05)	3.25[a] (0.17)	3.51[a] (0.01)	6.06[a] (0.09)	9.17[b] (0.05)	13.14[a,b] (2.01)
D	2.26[a] (0.10)	3.50[a] (0.05)	3.93[a] (0.32)	5.82[a] (0.21)	8.72[a,b] (1.43)	13.98[b] (2.41)
Trial 2						
A	2.40[a] (0.05)	3.47[a] (0.04)	4.03[a] (0.12)	5.59[a] (0.27)	7.42[a] (0.25)	11.72[a] (1.75)
B	2.30[a] (0.04)	3.40[a] (0.01)	4.05[a] (0.32)	5.70[a] (0.10)	7.60[a] (0.14)	11.22[a] (1.12)
C	2.23[a] (0.10)	3.74[a] (0.19)	4.25[a] (0.09)	5.82[a] (0.07)	8.78[a] (0.06)	12.36[a,b] (0.58)
D	2.15[a] (0.08)	3.92[a] (0.31)	4.43[a] (0.12)	5.85[a] (0.17)	8.97[a] (0.26)	14.46[b] (0.23)
Trial 3						
A	2.26[a] (0.08)	3.35[a] (0.12)	4.24[a] (0.11)	6.07[a] (0.04)	8.49[a] (0.08)	11.98[a] (3.02)
B	2.08[a] (0.13)	3.27[a] (0.06)	3.98[a] (0.16)	5.64[a] (0.05)	8.96[a,b] (1.11)	12.47[a] (0.91)
C	2.25[a] (0.07)	3.23[a] (0.11)	4.46[a] (0.17)	5.75[a] (0.14)	8.85[a,b] (0.23)	11.56[a] (1.03)
D	2.18[a] (0.05)	3.43[a] (0.43)	4.32[a] (0.04)	5.99[a] (0.14)	9.48[b] (0.22)	13.63[b] (0.15)

Numbers represent mean and standard deviation, with the latter in parentheses. In
each trial, mean in a column with different superscripts are significantly different
(Tukey's, $P \leq 0.05$).

Concentrations of individual free amino acids at 180 days of ripening are reported in
Table 7.5 and Figure 7.6. Table 7.5 indicates the values measured in the cheeses in each
trials and Figure 7.6 reports the concentrations of individual free amino acids as

average of three separate trials. Leu and Phe were the most abundant amino acids in
all the cheeses and this result is in agreement with O'Mahony et al. (2003) that found
Leu and Phe, together with Glu, to be the most abundant amino acids in Cheddar cheese
ripened at 8°C for 180 days. There were no significant differences among the
concentration of each amino acids between the cheeses. However, concentration of all
the amino acids, except Cys, Ile, Thr, Tyr, Arg and Pro, was higher in the experimental
cheeses compare to the control cheeses. Whereas, concentration of Arg was lower in
experimental cheeses than in control cheeses and values decreased by increasing the
concentration of *E. faecium* added to the cheese. The ability of *E. faecium* to degrade
arginine was reported by previous studies on the use of *Enterococcus* as adjunct culture
in cheese (Broome et al., 1991; Gardiner et al., 1999; Gursoy and Kinik, 2010) and the
degradation of this amino acid can reduce bitter taste in Cheddar cheese.

Table 7.5   Level of individual free amino acids (mg/g cheese) in control Cheese cheese (A), Cheddar cheese made from milk containing 10^6 (B), 10^7 (C), or 10^8 (D) cfu/mL of *Enterococcus faecium* at 180 days of ripening.

	Trial 1				Trial 2				Trial 3			
	A	**B**	**C**	**D**	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
Asp	0.08	0.09	0.11	0.11	0.07	0.07	0.08	0.10	0.09	0.06	0.09	0.09
Thr	0.03	0.02	0.04	0.05	0.02	0.02	0.03	0.03	0.03	0.02	0.02	0.02
Ser	0.05	0.06	0.07	0.09	0.05	0.05	0.06	0.06	0.05	0.04	0.04	0.04
Glu	0.03	0.14	0.22	0.21	0.13	0.13	0.17	0.20	0.02	0.13	0.15	0.09
Gly	0.03	0.04	0.04	0.05	0.04	0.03	0.04	0.04	0.03	0.03	0.04	0.03
Ala	0.08	0.10	0.10	0.11	0.08	0.07	0.09	0.09	0.08	0.07	0.08	0.08
Cys	0.16	0.17	0.16	0.15	0.17	0.15	0.13	0.16	0.16	0.16	0.17	0.15
Val	0.17	0.20	0.23	0.20	0.15	0.11	0.15	0.20	0.16	0.14	0.18	0.16
Met	0.10	0.15	0.17	0.16	0.12	0.11	0.12	0.15	0.11	0.11	0.10	0.12
Ile	0.02	0.04	0.05	0.03	0.00	0.00	0.00	0.04	0.03	0.02	0.00	0.00
Leu	0.79	0.94	1.04	0.94	0.81	0.71	0.85	0.94	0.89	0.79	0.71	0.85
Tyr	0.35	0.40	0.37	0.40	0.33	0.35	0.37	0.35	0.31	0.25	0.24	0.28
Phe	0.73	0.83	0.88	0.86	0.86	0.79	0.86	0.86	0.80	0.75	0.81	0.84
Gaba	0.29	0.34	0.38	0.38	0.32	0.31	0.34	0.37	0.31	0.30	0.27	0.32
His	0.36	0.48	0.46	0.44	0.37	0.40	0.40	0.37	0.40	0.41	0.39	0.33
Lys	0.19	0.22	0.26	0.22	0.13	0.14	0.17	0.22	0.19	0.16	0.17	0.18
Arg	0.04	0.03	0.05	0.04	0.07	0.07	0.06	0.04	0.10	0.05	0.02	0.00
Pro	0.04	0.10	0.02	0.04	0.06	0.02	0.00	0.07	0.09	0.04	0.08	0.08
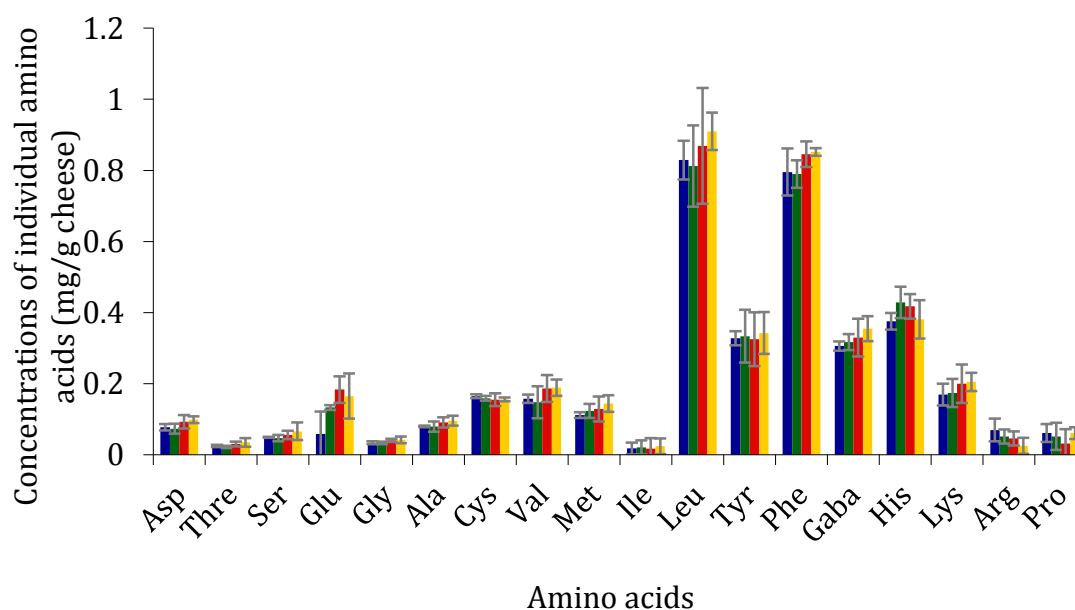
Figure 7.6   Concentration of individual free amino acids in Cheddar control cheese (■) and experimental cheeses made from milk containing *Enterococcus faecium* as adjunct culture at level of 10^6 (■), 10^7 (■) and 10^8 (■) cfu/g at 180 days of ripening. Values are average of three independent trials and standard deviation is indicated by the vertical bars.

7.3.3   Assessment of lipolysis

7.3.3.1 Free fatty acids

Enzymes naturally presented in milk and the action of microorganisms in the cheese generate free fatty acids that contribute to the flavour development in mature cheese (Collins et al., 2003).

Free fatty acids were extracted from the cheeses and quantified at 60 and 180 days of ripening; results are reported in Table 7.6.

Table 7.6   Level of free fatty acids (mg/kg cheese) in control Cheese cheese (A), Cheddar cheese made from milk containing 10^6 (B), 10^7 (C), or 10^8 (D) cfu/mL of *Enterococcus faecium* at 60 (A) and 180 (B) days of ripening.

A

FFA (mg/Kg cheese)	60 days ripening			
	A	B	C	D
C4:0	44.35 [a] (0.52)	43.15 [a] (2.89)	42.03 [a] (0.29)	168.78 [b] (3.82)
C6:0	26.96 [a] (1.13)	25.72 [a] (0.37)	26.34 [a] (0.01)	83.41 [b] (0.29)
C8:0	30.19 [a] (0.63)	29.28 [a] (0.73)	28.55 [a] (0.80)	86.04 [b] (0.03)
C10:0	58.06 [a] (0.43)	56.51 [a] (2.61)	55.89 [a] (1.09)	173.91 [b] (0.21)
C12:0	90.28 [a] (0.76)	85.95 [a] (4.95)	86.84 [a] (3.86)	238.13 [b] (3.89)
C14:0	269.50 [a] (3.92)	260.88 [a] (10.15)	274.18 [a] (13.55)	794.37 [b] (18.94)
C16:0	788.54 [a] (14.29)	744.16 [a] (42.40)	792.73 [a] (42.90)	2053.89 [b] (52.55)
C18:0	255.43 [a] (4.58)	247.13 [a] (11.34)	240.89 [a] (6.44)	562.27 [b] (12.16)
C18:1	336.31 [a] (3.94)	341.85 [a] (11.81)	347.70 [a] (16.56)	2059.18 [b] (17.59)
C18:2	36.09 [a] (1.82)	33.70 [a] (0.59)	35.07 [a] (2.02)	121.71 [b] (1.39)
C18:3	41.39 [a] (0.55)	35.44 [a] (13.03)	42.70 [a] (0.66)	125.95 [b] (8.15)
SC FA	249.85 [a] (3.46)	240.60 [a] (11.56)	239.65 [a] (5.45)	750.27 [b] (8.23)
LC FA	1727.27 [a] (10.95)	1663.17 [a] (39.64)	1733.27 [a] (80.81)	5717.36 [b] (110.79)
Total FA	1977.12 [a] (7.49)	1903.77 [a] (51.20)	1972.92 [a] (86.27)	6467.63 [b] (119.01)

B

FFA	180 days ripening			
(mg/Kg cheese)	A	B	C	D
C4:0	139.33 [a] (0.64)	138.05 [a] (2.11)	141.16 [a] (4.60)	145.19 [a] (9.75)
C6:0	49.52 [a] (0.00)	51.11 [a,b] (1.10)	51.34 [a,b] (1.77)	55.25 [b] (0.59)
C8:0	48.43 [a] (0.14)	49.14 [a] (1.20)	49.81 [a] (1.38)	51.72 [a] (2.79)
C10:0	126.70 [a] (1.14)	128.33 [a] (3.13)	134.53 [a] (3.22)	137.51 [a] (10.73)
C12:0	196.24 [a] (1.73)	205.50 [a] (11.61)	213.59 [a] (1.23)	218.96 [a] (12.52)
C14:0	667.45 [a] (9.79)	706.18 [a] (56.52)	731.74 [a] (21.57)	762.03 [a] (53.99)
C16:0	1737.20 [a] (14.87)	1821.86 [a] (172.99)	1931.84 [a] (57.87)	1989.92 [a] (126.30)
C18:0	511.36 [a] (12.98)	520.51 [a] (10.65)	538.86 [a] (10.12)	547.94 [a] (48.76)
C18:1	1186.13 [a] (4.94)	1272.76 [a] (0.99)	1236.95 [a] (4.75)	1236.19 [a] (133.26)
C18:2	74.51 [a] (0.75)	77.76 [a] (0.41)	77.94 [a] (0.87)	77.66 [a] (8.53)
C18:3	86.82 [a] (0.55)	91.71 [a] (0.68)	88.79 [a] (0.03)	88.28 [a] (9.05)
SC FA	560.22 [a] (2.38)	572.13 [a] (12.53)	590.42 [a] (9.74)	608.62 [a] (36.39)
LC FA	4263.46 [a] (6.53)	4490.79 [a] (239.45)	4606.11 [a] (74.97)	4702.01 [a] (379.89)
Total FA	4823.68 [a] (8.91)	5062.92 [a] (251.98)	5196.53 [a] (65.23)	5310.63 [a] (416.28)

Numbers represent mean and standard deviation, with the latter in parentheses, of three independent trials. (SC FA: short and intermediate-chain fatty acids; $C_{4:0}$-$C_{12:0}$; LC FA: long-chain fatty acids; > 12 carbons atoms). In each table, mean in a column with different superscripts are significantly different (Tukey's, $P \leq 0.05$).

The concentration of free fatty acid in control cheese and experimental cheeses made with *E. faecium* as adjunct culture at level of 10^6, 10^7 and 10^8 cfu/mL of cheese-milk was higher at 180 days than at 60 days except for the cheese made with the higher numbers of adjunct that had a higher concentration of free fatty acid values at 60 days

than at 180 days of ripening (Table 7.6A). At 60 days of ripening, level of all the acids

identified were present at significantly higher (P < 0.05) concentrations in cheese made

with 10^8 cfu/mL of *E. faecium* compared to the other cheeses. The total values of short

and intermediate-chain fatty acids (C4:0-C12:0) and long-chain fatty acids were

significantly higher in cheese containing 10^8 cfu/mL of *E. faecium* (Table 7.6 A). At 180

days of ripening, the total values of short, intermediate and long-chain fatty acids were

not significantly different among the cheeses (Table 7.6 B). No significant differences

in the concentration of each fatty acid were present among the cheeses except for

butyric acid that was present at significantly higher (P < 0.05) concentration in the

cheese containing 10^8 cfu/mL of *E. faecium* compared to the other cheeses. The general

increase in fatty acids concentration observed in control Cheddar cheese and cheeses

made with 10^6 and 10^7 cfu/mL of *E. faecium* is in agreement with previous study on the

analysis of fatty acids during Cheddar cheese ripening (Woo et al., 1984; Ikens et al.,

1988).

7.3.4. Volatile compounds

Volatile compounds were analysed by SPME-GC-MS in control Cheddar cheeses (A),

Cheddar cheeses made with the addition of 10^6 (B), 10^7 (C), 10^8 (D) cfu/mL of

Enterococcus faecium at 15, 60 and 180 days of ripening and average values of three

independent trials are reported in Table 7.7.

Table 7.7   Concentrations of volatile compounds (µg/100 g cheese) in the headspace gas of control Cheese cheese (A), Cheddar cheese made from milk containing 10^6 (B), 10^7 (C), or 10^8 (D) cfu/mL of *Enterococcus faecium* at 15, 60 and 180 days of ripening. Numbers represent mean and standard deviation of three independent trials.

Chemical groups				Cheese samples			
	RI	ID Method	Age (day)	A	B	C	D
Acids (3)							
Acetic acid	1456	MS,RI,AS	**15**	18.03±3.43	46.10±11.80	45.94±28.73	40.24±13.41
			60	55.79±34.06	29.62±12.27	49.96±22.45	42.18±28.37
			180	73.37±30.48	78.17±25.86	72.54±18.73	86.71±26.84
Butanoic acid	1636	MS,RI,AS	**15**	4.78±1.55	13.74±1.05	13.04±5.78	17.46±2.49
			60	45.83±25.94	26.68±12.09	54.22±16.12	40.00±24.74
			180	48.16±21.60	67.40±14.66	49.52±26.85	55.15±19.34
Hexanoic acid	1866	MS,RI, AS	**15**	ND	ND	ND	ND
			60	ND	ND	ND	ND
			180	2.40±0.40	2.85±0.00	1.03±1.46	1.07±0.46
Esters (5)							
Methyl acetate	827	MS,RI	**15**	ND	ND	ND	ND
			60	ND	ND	ND	ND
			180	0.18±0.31	0.43±0.50	0.32±0.00	0.26±0.36

Ethyl acetate	887	MS,RI	**15**	0.45±0.23	1.41±0.68	2.20±0.29	1.86±0.62
			60	0.77±0.04	0.61±0.33	5.48±5.27	2.07±0.89
			180	1.44±0.48	1.64±1.20	5.82±1.75	7.78±3.90
Methyl butanoate	985	MS,RI	**15**	0.35±0.24	1.21±0.65	9.68±5.01	1.13±0.38
			60	0.87±1.23	0.38±0.54	0.55±0.00	1.56±0.00
			180	0.55±0.00	0.47±0.54	1.09±1.12	1.04±0.78
Ethyl butanoate	1035	MS,RI	**15**	1.60±0.00	5.02±0.00	5.96±1.27	2.34±0.78
			60	13.88±7.52	ND	7.51±0.00	4.41±0.89
			180	ND	3.92±1.85	ND	12.65±9.01
Ethyl hexanoate	1235	MS,RI	**15**	ND	ND	ND	ND
			60	ND	ND	ND	ND
			180	0.22±0.00	0.30±0.03	0.39±0.20	0.58±0.64
***Alcohols* (15)**							
2-propanol	927	MS,RI	**15**	4.50±2.52	18.28±12.10	9.53±8.48	13.58±4.53
			60	12.00±2.36	6.89±1.25	10.32±7.13	12.26±10.00
			180	13.71±6.66	33.09±12.42	7.53±3.72	13.08±10.38
Ethanol	933	MS,RI, AS	**15**	40.95±2.97	100.17±31.30	123.83±26.84	108.80±36.27
			60	66.63±21.84	46.81±5.71	127.91±41.66	58.16±32.78
			180	31.02±7.89	62.85±22.02	30.05±17.81	79.29±34.53
2-butanol	1024	MS,RI, AS	**15**	ND	ND	ND	ND
			60	ND	ND	ND	ND
			180	14.90±14.91	27.98±5.04	48.60±11.09	68.14±31.30

1-propanol	1040	MS,RI	**15**	8.01±4.16	32.83±31.60	43.00±13.08	57.26±19.09
			60	ND	10.13±0.22	49.10±11.08	34.26±11.65
			180	ND	9.76±0.00	ND	ND
2-methyl-1-propanol	1090	MS,RI	**15**	0.34±0.10	0.88±0.33	1.03±0.25	1.02±0.34
			60	2.89±1.77	3.11±0.24	13.43±6.39	3.32±2.21
			180	4.42±3.76	10.46±1.62	3.26±2.40	5.69±5.10
2-propen-1-ol	1043	MS,RI	**15**	ND	ND	ND	ND
			60	ND	ND	ND	ND
			180	0.41±0.17	0.81±0.00	0.27±0.24	0.71±0.62
2-pentanol	1123		**15**	ND	ND	ND	0.29±0.10
			60	ND	ND	3.47±0.00	1.61±0.00
			180	12.10±14.32	8.92±3.62	3.85±4.11	4.61±2.79
1-butanol	1144	MS,RI	**15**	0.14±0.09	0.71±0.00	0.47±0.22	0.57±0.19
			60	1.20±0.55	0.49±0.13	0.70±0.56	1.11±0.88
			180	1.77±1.48	18.80±3.35	0.90±0.51	1.92±1.24
1-penten-3-ol	1157	MS,RI	**15**	0.46±0.30	1.58±1.10	1.80±0.37	1.35±0.45
			60	0.91±0.37	0.73±0.25	1.03±1.04	0.58±0.27
			180	ND	ND	ND	ND
3-methyl-1-butanol	1208	MS,RI	**15**	0.93±0.88	1.11±0.45	1.67±1.06	2.32±0.77
			60	11.43±7.94	22.29±6.31	44.16±26.77	15.86±8.53
			180	22.80±15.97	67.02±7.38	22.45±2.70	32.10±20.40
1-pentanol	1249	MS,RI, AS	**15**	11.74±1.89	35.78±10.56	24.10±14.71	22.72±7.57

			60	30.23±9.26	26.01±9.82	42.45±28.47	26.21±6.82
			180	8.58±4.48	18.75±9.88	7.99±5.82	8.48±4.65
2-heptanol	1318	MS,RI,AS	**15**	ND	ND	ND	ND
			60	ND	ND	ND	ND
			180	0.81±0.49	2.77±2.71	0.51±0.26	0.66±0.33
3-penten-2-ol	1321	MS,RI,	**15**	0.61±0.06	2.01±0.77	1.30±0.74	1.46±0.49
			60	1.05±0.57	0.97±0.47	0.73±0.69	0.95±0.55
			180	0.60±0.08	1.48±1.80	0.62±0.56	0.59±0.28
1-hexanol	1353	MS,RI,AS	**15**	0.09±0.12	0.53±0.22	0.63±0.00	0.71±0.24
			60	0.88±0.60	0.72±0.20	4.38±3.01	3.06±2.27
			180	0.93±0.87	1.67±0.49	1.16±0.79	1.62±1.23
2-ethyl-1-hexanol	1489	MS,RI,	**15**	ND	ND	ND	ND
			60	ND	ND	ND	ND
			180	0.55±0.14	1.48±1.54	8.57±1.24	5.56±3.42
***Ketones* (8)**							
2-propanone	812	MS,RI,	**15**	39.62±9.44	65.83±2.38	79.65±43.16	98.06±32.69
			60	55.88±17.19	44.56±5.31	67.36±26.46	49.92±12.52
			180	5.98±4.28	14.32±2.37	10.25±1.67	11.03±1.65
2-butanone	901	MS,RI,AS	**15**	13.07±2.74	33.35±6.85	23.93±14.31	25.64±8.55
			60	18.33±3.55	15.06±1.48	28.57±8.33	14.46±4.40
			180	18.06±9.81	45.90±6.63	15.13±2.28	22.67±16.19
Diacetyl	972	MS,RI,AS	**15**	2.11±1.22	3.92±1.65	8.34±5.90	4.31±1.44

			60	ND	ND	ND	ND
			180	ND	ND	ND	ND
2-pentanone	977	MS,RI,AS	**15**	6.24±0.77	10.04±4.13	12.72±8.15	11.37±3.79
			60	7.51±6.69	2.18±0.41	8.25±7.75	3.53±1.34
			180	37.56±29.96	25.78±17.39	29.92±13.38	41.88±21.89
2-heptanone	1183	MS,RI,AS	**15**	ND	ND	ND	ND
			60	ND	ND	ND	ND
			180	17.38±4.00	6.26±2.68	11.00±9.38	21.04±16.37
4-octanone	1185	MS,RI,	**15**	3.31±2.35	2.27±0.00	3.56±0.00	3.43±1.14
			60	14.71±4.27	9.50±5.50	46.20±8.25	10.71±3.13
			180	ND	ND	ND	ND
3-hydroxy-2-butanone (acetoin)	1291	MS,RI,AS	**15**	25.36±3.12	21.70±7.30	51.96±37.08	34.43±11.48
			60	54.25±26.26	14.59±3.48	68.17±45.24	23.64±9.83
			180	4.33±3.34	11.18±2.03	7.84±5.73	11.06±3.37
2-nonanone	1394	MS,RI,AS	**15**	ND	ND	ND	0.49±0.16
			60	0.97±0.00	ND	3.17±0.00	1.29±0.00
			180	1.06±0.54	0.99±0.09	1.02±0.37	0.82±0.29
Aldehydes **(5)**							
Acetaldehyde		MS,AS	**15**	ND	1.09±0.82	1.55±0.00	1.05±0.35
			60	0.76±1.00	ND	4.87±0.00	ND
			180	ND	ND	ND	0.37±0.00
3-methylbutanal	919	MS,RI,	**15**	ND	ND	ND	0.64±0.21

			60	2.65±1.67	2.52±1.08	9.08±4.43	1.94±1.11
			180	0.68±0.39	0.49±0.52	0.39±0.10	0.71±0.38
Pentanal	980	MS,RI,	15	7.59±2.83	16.69±14.47	14.04±7.12	13.51±4.50
			60	53.68±14.44	93.89±12.40	69.25±33.81	71.67±53.26
			180	ND	ND	ND	ND
Hexanal	1083	MS,RI, AS	15	1.14±0.88	6.49±4.16	2.78±1.79	3.30±1.10
			60	3.67±3.64	1.64±0.20	3.09±2.45	1.50±1.14
			180	0.51±0.19	N.D.	1.22±1.64	0.69±0.47
Benzaldehyde	1489	MS,RI,	15	ND	ND	ND	ND
			60	ND	ND	ND	ND
			180	0.26±0.13	0.34±0.11	0.35±0.06	0.43±0.00
***Miscellaneous* (6)**							
Heptane, 2,2,4,6,6-pentamethyl-	915	MS,RI,	15	5.27±3.87	24.28±8.60	13.45±10.53	7.74±2.58
			60	15.16±5.08	15.37±9.06	34.78±28.07	14.68±5.19
			180	7.76±5.00	15.25±9.40	5.11±1.40	11.35±9.87
3,5-dimethyl heptane	1015	MS,RI,	15	0.47±0.24	2.67±0.00	0.62±0.08	0.69±0.23
			60	0.65±0.10	0.61±0.24	1.11±1.13	1.38±0.10
			180	0.30±0.02	0.33±0.00	0.17±0.12	0.95±0.51
Trichloromethane (Chloroform)	1019	MS,RI,	15	0.26±0.39	3.76±0.00	1.49±0.66	2.30±0.77
			60	2.62±2.96	2.74±1.68	1.25±0.00	4.24±0.00
			180	0.52±0.46	0.11±0.15	0.51±0.47	0.77±0.28

Toluene	1042	MS,RI,	**15**	13.29±0.79	24.42±9.16	29.12±12.72	12.55±4.18
			60	27.74±26.18	17.75±10.61	29.91±13.57	28.79±11.71
			180	20.46±4.53	43.76±39.79	15.58±10.21	19.89±11.57
2,3-pentadione	1055	MS,RI,	**15**	0.65±0.00	1.37±0.00	1.35±0.00	1.39±0.46
			60	ND	ND	ND	ND
			180	ND	ND	ND	ND
2,3-butanediol	1576	MS,RI,	**15**	2.26±1.71	7.33±1.56	6.69±6.04	7.99±2.00
			60	2.73±1.46	1.14±0.38	1.73±1.23	2.64±2.37
			180	0.64±0.40	3.57±3.42	1.43±1.92	2.64±2.37
Concentration of the total volatile compounds detected			**15**	215.75±9.61	493.45±20.17	542.75±24.21	507.74±23.86
			60	511.63±19.26	400.76±17.80	799.31±27.94	490.66±17.76
			180	355.81±15.02	589.3±21.55	366.39±15.66	533.99±21.79

5

MS: Mass **S**pectral identification using NIST 07 and Wiley 7 library; **RI:** Identification using alkan series (C8-C20) by calculation of **R**etention **I**ndices at the same chromatographic conditions; **AS,** identification by using **A**uthentic **S**tandard at the same chromatographic conditions.**Values were given as mean of 3 trials. ND:** Not dedected

A total of 43 volatile compounds were identified including 3 acids, 5 esters, 15 alcohols, 8 ketones, 5 aldehydes and 6 miscellaneous compounds (Table 7.7) and all of them, except for the miscellaneous compounds identified in this study, were previously found in good quality Cheddar cheese (Singh et al., 2003). The concentration of the total volatile compounds detected were higher in the cheeses containing *E. faecium* than in the control cheese at 15 and 180 days of ripening.

Levels of acetic acid and butanoic acid increased in all the cheeses during ripening and at 180 days of ripening they were more abundant in the experimental cheeses compared to the control cheese. Hexanoic acid was not present at 15 and 60 days of ripening and it was measured at 180 days of ripening in all the cheeses and it was in lower concentrations in the cheeses made with the addition of 10^7 and 10^8 cfu/mL of *E. faecium*. Among the acids identified, acetic acid was the one present at highest concentration and within the cheeses, cheese made with 10^8 cfu/mL of *E. faecium* was the richest one in acetic acid.

Among the esters identified, methyl acetate and ethyl hexanoate developed in cheeses at 180 days of ripening and they were both present in higher concentrations in the experimental cheeses. Ethyl acetate concentration increased during ripening in all the cheeses and cheeses made from milk containing 10^7 and 10^8 cfu/mL of *E. faecium* had the highest concentration of ethyl acetate at 180 days of ripening. Methyl butanoate was present in higher concentration in cheese made with 10^7 cfu/mL of *E. faecium* at 15 days of ripening whereas at 60 days of ripening of ripening it was more abundant in cheese made from milk with 10^8 cfu/mL of *E. faecium* and at 180 days of ripening higher concentration was found in cheeses made with the addition of 10^7

and 10^8 cfu/mL of *E. faecium*. Ethyl butanoate was present in higher concentrations in the experimental cheeses at 15 and 180 days of ripening whereas at 60 days control cheese was the richest in ethyl butanoate.

Fifteen alcohols were identified in the cheeses during ripening. Ethanol was the most abundant during ripening and highest concentrations were present in experimental cheeses. 2-Propanol developed in control cheese only at 180 days of ripening whereas it was present also at 15 and 60 days of ripening in the experimental cheeses. 2-Butanol was present in all the cheeses only at 180 days of ripening and higher concentrations were measured in the experimental cheeses compared to the control cheese. Concentration of 1-propanol decreased during ripening in all the cheeses and at 180 days of ripening it was detected only in cheese made with the addition of 10^6 cfu/mL of *E. faecium*. Levels of 3-methyl-1-butanol increased in all the cheeses during ripening and higher concentrations were measured in the experimental cheeses compared to the control cheese. 1-Pentanol was present in higher concentrations at 60 days in all the cheeses compared to the measurements done at 15 and 180 days of ripening. The other alcohol compounds identified (2-methyl-1-propanol, 2-propen-1-ol, 2-pentanol, 1-butanol, 1-penten-3-ol, 2-heptanol, 3-penten-2-ol, 1-hexanol and 2-ethyl-1-hexanol) were present at low concentrations (values lower than 10 µg/100 g cheese).

Among the ketones identified, 2-propanone was the most abundant at 15 days of ripening and its concentration decreased in all the cheeses during ripening. However, the experimental cheeses had a higher concentration compared to the control cheese at 180 days of ripening. 2-Butanone was present in all the cheeses and

the concentrations detected were quite constant during ripening (around 10-30 µg/100 g cheese). Diacetyl was present in all the cheeses only at 15 days of ripening whereas 2-nonanone was detected only at 60 and 180 days of ripening at concentrations lower than 10 µg/100 g cheese. 2-Pentanone increased in concentration during ripening and higher values were measured in control cheese and cheese made from milk containing 10^8 cfu/mL of *E. faecium* compared to the cheeses made with 10^6 and 10^7 cfu/mL of *E. faecium*. 2-Heptanone was detected only at 180 days of ripening and higher values were measured in control cheese and cheese made with the addition of 10^8 cfu/mL of *E. faecium* compared to the other two experimental cheeses. 4-Octanone was present only at 15 and 60 days of ripening and higher concentrations were present at 60 days in all the cheeses compared to the value measured at 15 days of ripening. Acetoin decreased in concentration in all the cheeses during ripening and at 180 days of ripening, experimental cheeses presented higher concentrations compared to the control cheese.

Five aldehydes were identified and pentanal was the one present in higher concentrations at 15 and 60 days of ripening with the highest quantity detected in the experimental cheeses compared to the control cheese. However at 180 days of ripening, pentanal was not present in the cheeses. Hexanal concentrations decreased on all the cheeses during ripening and at 180 days of ripening it was present at higher values in the cheeses made with the addition of 10^7 and 10^8 cfu/mL of *E. faecium* compared to the control cheese and cheese made with 10^6 cfu/mL of *E. faecium*. Benzaldehyde was detected only at 180 days of ripening in all the cheeses at similar values. Acetaldehyde was detected only at 60 days in control cheese and in all the

experimental cheeses at 15 days of ripening. At 180 days acetaldehyde was present only in the cheese made with the addition of 10^8 cfu/mL of *E. faecium*. 3-Methylbutanal was measured only in the cheese made with the highest quantity of *E. faecium* at 15 days, at 60 and 180 days it was present in all the cheeses, however, in lower concentrations at 180 days of ripening.

We can conclude that at 180 days of ripening, the concentrations of each individual volatile compound in cheeses made with the addition of the highest concentration of *E. faecium* were higher compared to control cheeses with the exception of hexanoic acid, 2-pentanol, 2-heptanol, and 2-nonanone. In agreement to our results, a study by Tavaria et al. (2006) that evaluated the volatile profile of microorganisms inoculated in a model system found that *E. faecium* produced the highest quantity of volatile compounds compared to the other bacteria studied. Among the volatile compounds present in higher concentration in the experimental cheeses, acetic acid, ethyl acetate, ethanol and 3-methylbutanal were found to be present in higher concentration in another study, published by Gardiner et al. (1999), where a strain of *E. faecium* was added as adjunct culture in Cheddar cheese.

To investigate differences between the volatile compounds identified in the cheeses samples PCA was performed and results are shown in Figures 7.7A and B. Cheeses were separated by ripening period (PC1, 59.5% of variance explained) and control and experimental cheeses were separated by the second component (PC2, 19.2% of variance explained) (Figure 7.7A). Cheeses were clustered into three groups depending on the ripening time (Figure 7.7A). At 15 days of ripening, control cheese had a volatile profile different from the experimental cheeses. Moreover, cheese

made from milk containing 10^6 cfu/mL of *E. faecium* was separated from the cheeses made with the addition of 10^7 and 10^8 cfu/mL of *E. faecium* (Figure 7.7A). At 60 days of ripening, control cheeses and cheeses made with the addition of 10^7 cfu/mL of *E. faecium* had a volatile profile different from cheeses made with the addition of 10^6 and 10^8 cfu/mL of *E. faecium*. At 180 days of ripening, control cheeses and cheeses made from milk containing 10^6 cfu/mL of *E. faecium* had a volatile profile different from cheeses made with the addition of 10^7 and 10^8 cfu/mL of *E. faecium*. Therefore, according to PC2, cheeses made from different level of adjunct cultures had different concentration of volatile compounds. In particular at 15 and 180 days of ripening, cheeses made with the addition of *E. faecium* were clearly separated from the control cheeses.

A

B
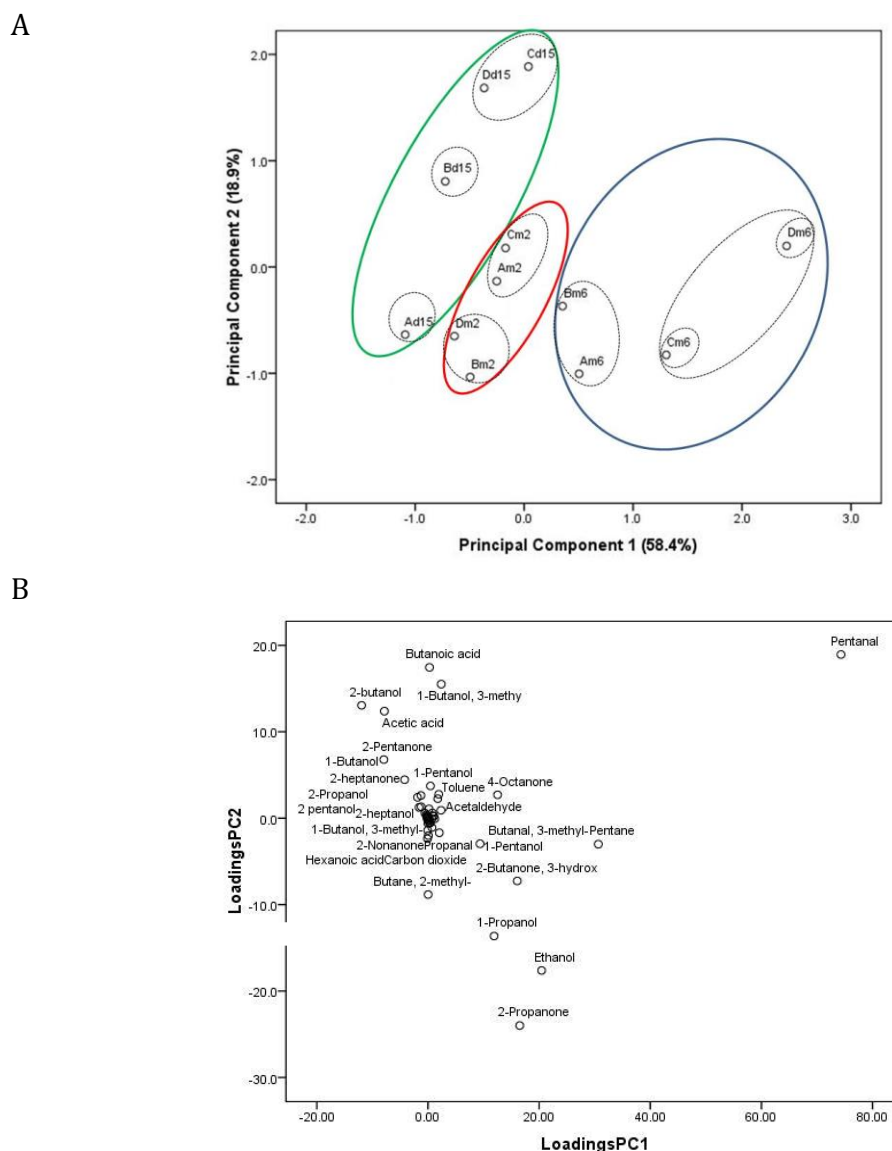
Figure 7.7   Plot of scores (A) and loadings (B) for the first and second principal components following principal component analysis (PCA) of the volatile compounds identified for the Cheddar cheeses at 15 (15d), 60 (2m) and 180 (6m) days of ripening. In the plot of scores (A), letters A, B, C and D refers to control Cheddar cheese and Cheddar cheeses made from milk containing 10^6, 10^7 and 10^8 cfu/mL of *Enterococcus faecium*, respectively.

7.3.5   Biogenic amines

Levels of biogenic amines (BA) measured at 180 days of ripening are shown in Table 7.8 as average of three separate trials and expressed as µg of BA per g of cheese. Presence of BA can cause toxic reactions in humans; histamine is the most toxic amine detected in foods (Santos, 1996) and the Food and Drug Administration consider level equal to 500 mg/kg to be danger to humans (Ladero et al., 2008). Limits of 100-800 mg/kg of tyramine and 30 mg/kg of phenylethylamine have been recommended (Linares et al., 2011). In cheese the levels of BA vary as a function of ripening period and microorganisms present in the product; usually highest levels are found in cheeses contaminated with spoilage microorganisms (Fox et. al, 2000). In our study, tryptamine, cadaverine and histamine were not detected in any of the cheese manufactured.

No significant differences were found between the control cheese and the experimental cheeses between the concentrations of the BA detected in the cheeses (Table 7.8).

Table 7.8   Level of biogenic amines (BA) in control Cheese cheese (A), Cheddar cheese made from milk containing 10^6 (B), 10^7 (C), 10^8 (D) cfu/mL of *Enterococcus faecium* at 180 days of ripening.

BA (µg/1g cheese)	A	B	C	D
tryptamine	ND	ND	ND	ND
phenylethylamine	2.18[a] (0.99)	2.08[a] (0.44)	3.34[a] (0.62)	2.29[a] (0.66)
putrescine	18.15[a] (0.70)	29.32[a] (18.89)	17.70[a] (0.90)	29.55[a] (11.53)
cadaverine	ND	ND	ND	ND
histamine	ND	ND	ND	ND
tyramine	12.98[a] (2.33)	11.52[a] (4.51)	10.00[a] (6.86)	10.97[a] (3.37)
spermidine	34.25[a] (3.48)	33.94[a] (0.97)	33.23[a] (3.29)	31.52[a] (10.04)
spermine	6.01[a] (2.53)	7.62[a] (2.69)	8.00[a] (2.78)	7.88[a] (2.86)

Numbers represent mean and standard deviation, with the latter in parentheses, of three independent trials. Mean in a row with different superscripts are significantly different (Tukey's, $P \leq 0.05$). (ND: not detected)

Enterococcus has been found to be responsible of the production of tyramine by tyrosine decarboxylase activity (Kučerová, et al. 2009) and in a study by Rea et al. (2004), levels of tyramine in Cheddar cheeses made with enterococci reached values of 197 mg/kg at 9 months of ripening. Whereas, in our control and experimental cheeses the concentration of tyramine was considerably lower.

7.4   Conclusion

To investigate the contribution to Cheddar cheese ripening of *E. faecium* EF031, cheeses was manufactured from milk containing three different quantities (10^6, 10^7 or 10^8 cfu/mL) of *E. faecium* in addition to a lactic starter culture. As control, a Cheddar cheese containing starter culture was made.

E. faecium did not affect the Cheddar cheese composition and pH. Redox potential was reduced to lower level and in a faster time in the cheese containing the higher concentration of adjunct culture compared to the control cheese.

Results of the assessment of proteolysis in cheeses during ripening demonstrated that secondary proteolysis was increased in cheeses made with the addition of 10^8 cfu/mL of *E. faecium*. Moreover, the addition of 10^8 cfu/mL of *E. faecium* contributed to lipolysis during ripening. The addition of *E. faecium* did not cause the production of biogenic amines to harmful values. Quantitative differences in flavour compounds were found between the control cheese and the cheeses made with *E. faecium*.

These findings suggest that the quality of Cheddar cheese is not negatively influenced by the presence of *E. faecium* EF031 and support the technological utility of *E. faecium* as an adjunct culture for Cheddar cheese.

7.5   Acknowledgments

7.6   References

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Chapter 8

Conclusions and recommendations

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8.1   Recommendations on oxidation-reduction potential measurement

Oxidation-reduction (redox) potential is an important physicochemical parameter in the food industry (Brown and Emberger, 1980; Riondet et al., 2000); however, it is not routinely measured during manufacture and ripening of dairy products.

Difficulties in obtaining correct and reproducible redox potential measurements have been reported by several authors (Davis, 1932; Gillespie, 1920; Gillespie and Rettger, 1938; Ward, 1938; Hewitt, 1950; Tabatabai and Walker, 1970). From our experience, the following recommendations should be taken into consideration when redox potential measurements are performed in dairy products:

- Standardize the method used and record all the conditions under which the experiments take place like temperature and pH.

- Convert the redox potential measured, uncorrected for pH and without reference to the standard hydrogen electrode to the oxidation-reduction potential related to the standard hydrogen electrode (E_h) or to E_h of a solution at pH 7 (E_{h7}) in order to be able to compare data from different investigators. In Chapter 1 formulas to perform these conversions are reported.

- Use a data logger to collect measurements during experiments to avoid omission of data. Ensure that the potentiometer used has an input impedance higher than 10^{12} Ω to measure tiny potential created by the circuit without loading errors (Kjaergaard, 1976; Skoog et al., 2004) and electrode polarisation (Jacob, 1970).

- Choose the best electrode depending on the nature of the product. The shape of the metal electrode should be selected depending on the product. The metal surface should be large enough to guarantee exchange of charges and smooth to avoid deposit of impurities (Higginbottom and Taylor, 1960). In our experiments, three different commercial platinum electrodes were used depending on the products analysed (Table 8.1). The electrode Pt 1800 (Schott, Mainz, Germany) was used to measured redox potential during cheesemaking (Chapter 2) since the ring shape guarantees a good exchange surface and it is robust enough to insure readings during cheesemaking steps where the cheese curd was subjected to vigorous stirring, mixing and pressing. Electrodes R-XM110 and R-XM120 (Radiometer Analytical, Villeurbanne Cedex, Lyon, France) have a platinum wire and plate surface, respectively. They were both used successfully to measure the redox potential in cheeses whereas in liquid products, like milk and Cheddar cheese extract, the electrode R-XM120 gave more stable and reproducible readings, probably due to the larger plate exchange surface compared to the wire surface.

  During our experiments, the platinum working electrodes were connected to a silver/silver chloride reference electrode (REF 201, Radiometer Analytical, Villeurbanne Cedex, Lyon, France) filled with saturated KCl solution (KCl-L-30, Radiometer Analytical).

  Combined electrodes could be also used to measured redox potential; however, they were not tested in our laboratory.

Table 8.1   Working electrodes used in our laboratory

	Working electrodes		
	R-XM120 Radiometer Analytical, Villeurbanne Cedex, Lyon, France	R-XM110 Radiometer Analytical, Villeurbanne Cedex, Lyon, France	Pt 1800 Schott, Mainz, Germany
Picture			
Diameter	8 mm	8 mm	12 mm
Length	120 mm	120 mm	120 mm
Sensing element	Platinum Plate, 5 x 5 mm	Platinum Wire, Ø 1 mm	Platinum Ring, Ø 6 mm

- Electrodes can also be manufactured. It is important to ensure a good seal of the metal to the plastic or glass (Davis, 1932, Topcu et al., 2008) since the presence of small cracks can alter the results. In our laboratory, two different platinum electrodes were manufactured (Figure 8.1A and B). One electrode was made following the design reported by Topcu et al. (2008) (Figure 8.1A).

And a smaller and more robust electrode that could resist to the pressure applied to the cheese curd during the pressing stage of Cheddar cheesemaking (Chapter 5) was manufactured (Figure 8.1B). Both electrodes were tested in tap water, standard solutions, milk and mature cheese and results were the same as the one obtained using commercial platinum electrodes (XM110 and XM120).

Figure 8.1   Handmade self-sealing platinum electrode made following the design reported by Topcu et al. (2008) (A) and made in our laboratory (B).

- Maintenance of the electrodes is important. When commercial reference and working electrodes are used it is important to follow the suppliers' instructions.

- To obtain valid and reproducible results, the accuracy of the electrodes can be checked against commercial standard solutions (Calligaris et al., 2004;

Bolduc et al., 2006; Schreyer et al., 2008; Jayamanne and Adams, 2009; Ignatova et al., 2009) or tap water (Abraham at al., 2007, 2013; Jeanson et al., 2009; Martin et al., 2009, 2010, 2011; Ebel et al., 2011) or buffer solutions (Tabatabai and Walker, 1970; Lund et al., 1984; George et al., 1998b).

Moreover, electrodes can be checked by comparing redox potential reading of the same sample with each other and the measurements should give a difference not greater than 10 mV between electrodes (Saal and Heukelom, 1947).

When readings are not reproducible, reference and working electrodes should be cleaned and tested again in solutions of known redox potential.

It is good practice to check the accuracy of the electrodes before and at the end of each experiment.

- The surface of the platinum electrode has to be smooth and it can be checked using a microscope. In our laboratory, metal microelectrode (PTM123B20KT; World Precision Instruments, Inc., Sarasota, FL, U.S.A.) (Figure 8.2A and B) were tested to measured redox potential in mature Cheddar cheese by inserting the electrode directly into the cheese and results were poor and not reproducible. The metal surface was examined under a microscope and the metal tip was damaged (Figure 8.2C). Therefore, the electrode was not robust enough to be inserted into the Cheddar cheese block. However, this type of electrode could be successfully used to measured redox potential in soft cheeses like Camembert as reported by Abraham et al. (2007, 2013).

A
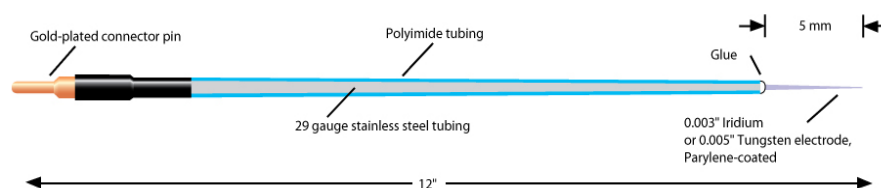
B   C

Figure 8.2 Schematic representation of the microelectrode electrode (www.wpionc.com) (A), picture of the electrode (B) and microscope picture of the tip of the electrode (C).

- In liquid products like milk or cheese extract redox potential can be measured by immersing the redox electrodes into the solution (Brown and Emberger, 1980).

- It is recommended to avoid the use of metal tools during experiments to avoid altering redox potential readings in case of contact of the metal tool and the working electrode. In Chapter 2, where redox potential was followed during cheeses manufacture, plastic and glass tools were used.

- In soft cheeses like Camembert the reference and working electrodes can be inserted directly into the cheese (Chapter 5) (Abraham et al., 2007, 2013).

- When redox potential is measured in semi-hard and hard cheeses it is necessary to create a salt bridge into which the reference electrode can be placed (Davis, 1932; Skoog et al., 2004). Moreover, the design of the electrode should ensure good contact between the cheese matrix and the surface of the electrode (Vos, 1948; Tammam et al., 2001) and the insertion of the probe should cause minimum damage to the matrix of the sample in order to avoid the formation of cracks which allows oxygen to penetrate the sample and give false readings. In our experiments, redox potential was measured in mature cheeses following the settings reported by Topcu et al. (2008). Silver/silver chloride reference electrode was placed in a hole of 4 cm deep and 1.5 cm wide filled with 3 M KCl to complete the salt bridge with the working electrode that was inserted directly into the cheese at 2.5 cm distance and to 5 cm deep (Figure 8.3).

Figure 8.3   Measurement of redox potential in Cheddar cheeses. Silver/silver cloride reference electrode (red electrode) placed in a hole wide filled with 3 M KCl to complete the salt bridge with the working electrode that was inserted directly into the cheese.

- When redox potential is measured in cheese, it is recommended to have a block big enough to avoid cracks of the cheeses during the insertion of the electrodes (block of about 8 x 10 x 10 cm). Moreover, the cheese block should be wrapped in transparent film to prevent loss of moisture and measurement should be performed until an equilibrium E_h value is reached. In case of experiments where the redox potential is measured for more than few days, it is advised to coat the cheese with a plastic coat to prevent loss of moisture and the growth of yeasts and moulds.

- At the end of the measurement of redox potential in hard or semi-hard cheeses, it is advisable to check the cheese surface for cracks and to remove the electrodes and cut through the holes where the electrodes were placed

to check for the presence of cracks inside the cheese matrix and to check the

diffusion of the 3 M KCl solution into the matrix (Figure 8.4) that could have

alter the readings. Moreover, it often happened after redox potential

measurement in cheese that when the working electrode is removed a sound

resembling the popping of a cork was produced indicating that good

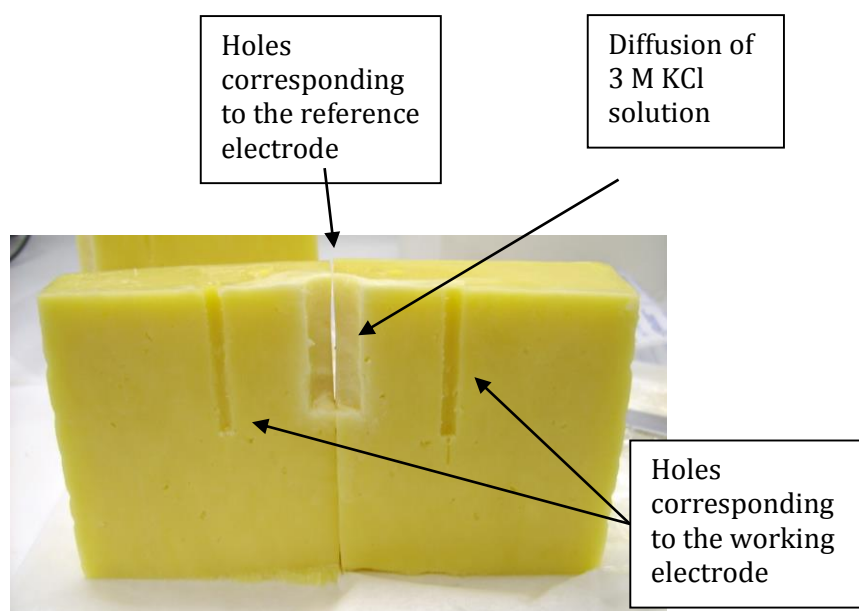cohesion between the electrode and the cheese matrix was present.

Figure 8.4   Block of Cheddar cheese cut at the end of the measurement of redox potential.

8.2   *Main conclusions from experimental Chapters*

In Chapter 2, the oxidation-reduction potential during the simulated manufacture of Cheddar, Gouda, Emmental and Camembert cheese was measured and we can conclude that:

- During cheesemaking of Cheddar, Gouda, and Camembert, redox potential decreased to negative values during the whey drainage steps whereas during Emmental manufacture the redox potential remained positive at that stage. During the pressing stage of manufacture, E_h of Cheddar, Gouda and Camembert decreased to values ∼ -115 mV while the E_h of Emmental reached +230 mV.

- Trends in redox potential during the manufacture of the cheeses made in this study differed depending on the cheesemaking technology used.

- The starter culture used in the cheesemaking could also be responsible of the different trends in redox potential among the cheeses manufactured.

- Redox potential measurement could be performed in dairy industry during the manufacture of dairy products to add information on the changes occurring during processing.

  If routinely measured E_h could contribute to the standardization of dairy products manufacture with positive effects on the quality of the final products.

In Chapter 3, the redox potential was measured in milk inoculated with lactic acid bacteria (LAB) cultures and we can conclude that:

- The six single stains LAB and a commercially available starter culture (R604) tested in this experiment during Cheddar cheesemaking temperature profile simulation were inoculated at the same level in skim milk and they were able to acidify milk to pH values from 5.60 to 4.72 and to grow to 10^8 - 10^9 cfu/mL milk at the end of the experiments. Differences in E_h were evident between the single strains of *Lactococcus lactis* and the commercial starter culture studied. R604 was the first to show a rapid decrease in E_h to an equilibrium values of about -150 mV during the simulated cooking stage. *L. lactis* subsp. *lactis* C10 reached an equilibrium E_h value slightly lower (-170 mV) than that of R604 at the same time. Two of the strains (*L. lactis* subsp. *cremoris* 223 and *L. lactis* subsp. *cremoris* S2) showed a positive and constant E_h during the experiments. *L. lactis* subsp. *lactis* 303 and *L. lactis* subsp. *cremoris* 229 showed a decrease in E_h during the simulated cooking stage and reached equilibrium values at the end of this stage of about -160 mV. The trend in E_h of *L. lactis* subsp. *cremoris* C differed from the other LAB and the final E_h value was higher (-40 mV) compared to the other strains.

- LAB even when closely related have different capacity to influence redox potential. Therefore, the ability of LAB to influence the redox potential in milk could be used to classified strains of LAB and to control the E_h in fermented dairy products.

- Three of the single strains studied that differed in the ability to drive E_h during Cheddar cheesemaking temperature profile simulation (*L. lactis* subsp. *cremoris* C, *L. lactis* subsp. *lactis* C10 and S2) were selected to produce Cheddar cheese. Their capacity to influence redox potential during ripening and the volatile compounds produced were measured.

The redox potential of the cheeses was negative in all the cheeses during ripening and differences between the E_h values were found at 1 and 14 days of ripening. At day 1, the redox potential of the cheese made with *L. lactis* subsp. *cremoris* S2 was significantly higher (P < 0.05) (E_h = -106 mV) compared to the E_h of the cheeses made with *L. lactis* subsp. *lactis* C10 (E_h = -186 mV) and *L. lactis* subsp. *cremoris* C (E_h = -152 mV) and at 14 days the E_h of the cheese made with *L. lactis* subsp. *cremoris* S2 was significantly higher (P < 0.05) (E_h = -114 mV) compared to the E_h of the cheese made with *L. lactis* subsp. *lactis* C10 (E_h = -193 mV).

Moreover, the strains used were able to give an E_h values different compared to commercial Cheddar cheese measured in Chapter 5 at 1 day of ripening; Cheese made with the addition of *L. lactis* subsp. *cremoris* S2 had a E_h higher compared to the E_h value of normal Cheddar cheese whereas *L. lactis* subsp. *lactis* C10 gave a lower value compared to the E_h value of normal Cheddar cheese.

Principal component analysis (PCA) of the volatile compounds data produced at 4 month of ripening clearly separated the cheese made with the addition of *L. lactis* subsp. *cremoris* S2 from the other two cheeses and it was

associated with alcohol, acid and aldehyde compounds. *L. lactis* subsp. *lactis* C10 was able to decrease E_h to values lower than the other two strains and its presence was associated with the development of sulfur compounds.

- Therefore, the selection of strains able to control the redox potential at the early stages of ripening could be useful to control the production of volatile compounds and perhaps the quality of Cheddar cheese. Moreover, we could hypothesize that cheese with a low redox potential at the early stages of ripening could be more likely to develop volatile compounds that are associated with a good quality Cheddar cheese.

- Further studies on the ability of single strains of LAB to influence E_h at early stages of ripening and on the volatiles produced should be performed to investigate the correlation between E_h and flavour development in cheese.

Moreover, in Chapter 3, strategies to control the redox potential during simulation of Cheddar cheesemaking were developed. The effect of the addition of different reducing and oxidising agents on the E_h and pH of skim milk and on milk inoculated with commercial starter culture (R604) was studied. Furthermore, the ability of controlling redox potential during simulation of Cheddar cheesemaking of two selected redox agents was evaluated. From this study we can conclude that:

- The redox agents tested in skim milk could reduce or increase the E_h to different values depending on the quantity added and their ability to influence the redox couples that are present in the milk (Sherbon, 1999). pH was also influenced by the redox agents used.

- Two of the redox agents tested (potassium iodate and sodium hydrosulfite) were selected and their effect on LAB survival and pH of milk was studied. The addition of KIO_3 or $Na_2S_2O_4$ could successfully increase or decrease the E_h of the milk, respectively. However, the pH and the survival of the microorganisms were also influenced depending on the quantity of the redox agents used.

- The ability of KIO_3 or $Na_2S_2O_4$ to increase or decrease, respectively, the E_h was tested during simulation of Cheddar cheesemaking. The redox agents were added at the end of the cooking stage in order to avoid interference with the starter LAB growth. The addition of KIO_3 maintained the E_h at a positive value during cheesemaking and pressing stage whereas the addition of $Na_2S_2O_4$ dropped of redox potential during the whey drainage to values lower than the control cheese. The addition of redox agents did not influence the microbial development and pH of the cheeses.

- Therefore, the addition of redox agents could be used to control the redox potential in fermented dairy products. It is important to find redox agents that are able to influence the redox potential of the product without altering the natural pH and killing the starter LAB.

- When selecting redox agents, it is advised to perform preliminary tests on their ability to influence E_h and also the pH of the system and their toxicity on the bacteria used.

In Chapter 4, the ability of 30 strains of *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* to modify redox potential (E_h) during simulation of the pressing

stage of Cheddar cheese was studied in Cheddar cheese extract (CCE). From this study we can conclude that:

- During the experiment, all the strains acidified CCE to similar values and showed similar growth. The strains could be divided on the basis of their redox profiles into three groups. Some strains reduced the redox potential to values around -120 mV in about 3 h, other strains showed a slower reduction ability reaching equilibrium in more than 4 h. And other strains maintained a positive redox potential during the whole experiment.

  Therefore, different strains are able to modify the redox potential of CCE following different pattern and redox potential can be considered a parameter specific of each strain.

- The development of specific flavour compounds seems to be associated with the redox potential of the medium. One strain from each group was selected and the volatile compounds produced at the end of the experiments were measured. PCA was performed and the strain that maintained the redox potential at positive values (*L. lactis* subsp. *cremoris* S2) was correlated to aldehyde, ketone and hydrocarbon compounds. Whereas the strain that reduced the redox potential to negative values in a short time (*L. lactis* subsp. *lactis* C10) was mainly associated sulfur compounds and the strain that showed a slower reduction ability (*L. lactis* subsp. *cremoris* C) was associated with ester compounds.

- The three strains selected in this study were also studied in Chapter 3. The same trends of E_h were found in skim milk and CCE. Moreover, in Chapter 3,

these strains were used as starter in Cheddar cheese manufacture and the volatile compounds produced were evaluated. Results in skim milk and in CCE reported that *L. lactis* subsp. *lactis* C10 was able to reduce the redox potential faster compared to the other strains analysed and in Cheddar cheese the redox potential at the beginning of the ripening was lower compared to commercial Cheddar cheese. Moreover, *L. lactis* subsp. *lactis* C10 was associated with the presence of sulfur compounds in CEE and in Cheddar cheese.

This finding supports the hypothesis of a correlation between the production of sulfur compounds and the negative redox potential in good quality Cheddar cheese (Green and Manning, 1982; Kristoffersen, 1985) and in yoghurt (Martin et al., 2011).

As reported in the conclusions related to Chapter 3, further investigations on the relationship between E_h and flavour compounds should be performed.

- CCE can be considered a good medium to study the ability of LAB to influence redox potential and it can be used to select strains with a specific effect on E_h.
- Moreover, selection of LAB should be also influenced on the ability of the bacteria to control the redox potential in order to produce the perfect blend of starter and adjunct cultures to use in dairy products.

In Chapter 5, changes in oxidation-reduction potential in cheese during ripening were studied and we can conclude that:

- Reproducible measurement of redox potential in cheese were obtained in our laboratory. Our method overcomes difficulties experienced by some authors (Davis, 1932; Vos, 1948; Tabatabai and Walker, 1970; Sawyer at al., 1995) in obtaining reproducible results in cheese.

- A method to follow the redox potential changes at early stages of ripening was developed. Robust miniature platinum electrodes were manufactured in our laboratory and they were embedded into the cheese curd and pressed together with the curd pieces into the mould.

  Redox potential of Cheddar cheese reached negative values of ca. -120 mV within two hours of pressing and the values reached were rather constant during the first month of ripening.

- The redox potential of a commercial block of Emmental cheese was followed after the brining stage of manufacture throughout the ripening stages.

  The reduction in redox potential to negative values occurred after about 5 days of ripening. This results was in agreement with our study in Chapter 2 were a positive E_h in Emmental cheese at the pressing stage was measured. The starter used in the cheesemaking could be responsible of the slow reduction in E_h in early stage of ripening in Emmental cheese. The E_h kept decreasing during the first 20 days of ripening reaching values in E_h lower than the one obtained in Cheddar cheese probably due to the development of propionic bacteria.

Moreover, redox potential in Emmental cheese resulted to be uniform since there were no differences between readings reported by electrodes located in different positions of the block.

- The redox potential of Cheddar cheese was followed during ripening (at day 1, 30, 90 and 180) and in five different locations across a 20 kg cheese block. The redox potential of Cheddar cheese in uniform since there were no significant differences between the E_h values recorded in the five locations at each time point. The E_h at day 1 was significantly lower compared to the values recorded at day 180. Moreover, the time needed to reach an equilibrium value increased during ripening and a relationship between the water activity and the time needed to reach the E_h equilibrium value could be hypothesized since water activity decrease significantly during ripening (Hickey et al., 2013). However, further studies are necessary to investigate this possible relation.

- The redox potential of commercial Cheddar, Gouda, Emmental and Camembert cheeses was measured and differences in redox potential values were recorded. These differences could be due to the different manufacturing technology and starter used, to the development of non-starter microorganisms during ripening and to the formation of redox compounds.

  Furthermore, time is needed to reach an equilibrium value during redox measurement in cheese and it is important to ensure that the equilibrium potential is reached before measurements are taken. This time needed to

reach an equilibrium value could be due to the structure of the cheese, to the microorganisms present in the cheese and also to the water activity of the cheese.

Further studies are necessary to confirm this possible relationship between redox potential and water activity in cheese.

- Redox potential could be a useful tool for monitoring the quality of cheese at different stages of ripening and for standardizing ripening of dairy products.

In Chapter 6, strategies to control the redox potential of Cheddar cheese during ripening were developed. Moreover, the flavour volatile compounds produced during ripening were measured. From this study we can conclude that:

- Redox agents were added at the salting stage of Cheddar cheese manufacture and during ripening the E_h of the cheeses made with the reducing agents (cysteine at 2%, w/w and $Na_2S_2O_4$ at 0.05 and 0.1%, w/w) did not differ significantly from the control cheese ($E_h \approx$ -120 mV) while the cheeses made with 0.1% and 0.05% KIO_3 had a significantly higher (P < 0.05) and positive E_h in the first month of ripening. After 2 months the E_h of most experimental cheeses reached values close to that of the control cheese. Cheese made with 1% KIO_3 had positive values of E_h throughout ripening but no starter LAB survived in this cheese; however, numbers of starter LAB in all other cheeses were similar. The volatile compounds produced at 2 and 4 month were analysed by PCA and at 2 months of ripening cheeses made with the addition of reducing agents were characterized by the presence of sulfur and ketone compounds and cheeses made with oxidising agents were characterized

mainly by aldehydes. At 6 month of ripening, cheese made with the addition of Cys were separated from the cheeses made with the addition of oxidising agents and $Na_2S_2O_4$.

- The addition of reducing agents favoured the development of sulfur compounds early in ripening and these compounds are associated to a good quality Cheddar cheese (Green and Manning, 1982; Kristoffersen, 1985). This finding supports the hypothesis of a correlation between redox potential and flavour development.

- The addition of KIO_3 at 1% kept the E_h at positive values during ripening; however, the starter LAB did not survive. When KIO_3 was added at 0.1 and 0.05% to the cheese, the E_h decreased to negative values when the number of non-starter LAB increased suggesting that LAB might be able to use the oxidising agents added and produce reducing metabolites. From these results it seems that cheese microflora has in important role on the redox potential of Cheddar cheese. Further investigations on this finding could be done in order to understand better the correlation between redox potential and LAB in cheese.

- Redox agents can modify microbial activity and metabolic pathways and as consequence act on the flavour development. Therefore, understanding and controlling redox potential can be useful to guide aroma formation in dairy products.

In Chapter 7 the contribution of *Enterococcus faecium* EF031 to Cheddar cheese ripening was investigated and we can conclude that:

- The presence of 10^8 cfu/mL of *E. faecium* as adjunct culture reduced the E_h to lower level and in a faster time compare to the control Cheddar cheese.

- Secondary proteolysis was increased in cheeses made with the addition of *E. faecium*. Moreover, the addition of *E. faecium* contribute to the lipolysis during ripening.

- Quantitative differences in flavour compounds were found between the control cheese and the cheeses made with *E. faecium*.

- *E. faecium* EF031 could be added as adjunct culture during cheesemaking and contribute to the quality of Cheddar cheese.

8.3   *Proposed future research work*

This study provides new knowledge on the importance of oxidation-reduction potential for the dairy industry and the research community in the area and it opens new areas for future research.

The biodiversity of reducing capacity within *Lactococcus lactis* strains is revealed in this study and further work dedicated in understanding the mechanisms involved in the reduction activity of lactococci is needed. Investigation on the relationship between the bacteria present in a dairy product and the final quality of a product could be relevant to provide further information for producing the perfect blend of bacteria for dairy product.

Our study shows that a low redox potential at the early stages of ripening could be more beneficial to the development of volatile sulfur compounds that are associated

with a good quality Cheddar cheese. Therefore, further studies on the ability of lactococci to influence redox potential at early stages of ripening and the correlation with the flavour development are necessary.

We found that redox potential can be controlled by the bacteria present in the product and by the addition of redox agents and an important aspect of future research work is the investigation of the relationship among redox potential and sensory quality of the product. Sensory analysis could be done on dairy products where food grade redox agents or microorganisms with specific ability to modify the redox potential are added.

Our study hypothesized a relationship between the water activity and the time needed to reach the redox potential equilibrium value in cheese and further studies are necessary to investigate this possible relation.

While cheesemakers pay close attention to pH, composition, ionic strength (NaCl) and temperature; it is possible that equally close attention to redox potential, which have to date received little attention may allow much more precise control of cheese quality and avoid specific defects. We develop methods to follow redox potential during cheesemaking and ripening and demonstrate that redox potential during the manufacture and ripening of the cheeses studied differed depending on the cheesemaking and ripening technologies used and on the starter and non-starter bacteria present. Practical method for measuring and/or implementing redox potential of Cheese in industry should be studied in order to optimize the Cheddar cheesemaking process and reduce variation in quality. Thus, the development of strategies to measure continuously changes in redox potential of a product and to

control, and adjust if necessary, the redox potential values during a manufacturing

and ripening could be an important achievement for the future of the dairy industry.

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Appendix

Changes in oxidation-reduction potential during the simulated manufacture of different cheese varieties

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ABSTRACT

Oxidation-reduction (redox) potential is a fundamental physicochemical property of cheese that partly determines its microenvironment both during manufacture and ripening. In this study, changes in redox potential (E_h), pH and temperature during the simulated cheesemaking of Cheddar, Gouda, Emmental and Camembert cheeses were determined. The E_h of milk decreased from +349 mV to +236 mV during pasteurization. E_h became negative during the whey drainage step of cheese manufacture except for Emmental that remained positive. The E_h of Cheddar, Gouda and Camembert decreased to −117 mV during the pressing stage while the E_h of Emmental reached +230 mV during the pressing step. Variations in redox potential may be attributed to both the cheesemaking protocol used and starter culture added. Monitoring of the redox potential throughout the cheesemaking could allow its better control and standardization.

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1. Introduction

Cheese is a fermented dairy product that is manufactured with the purpose of conserving the principal constituents of milk (Fox & Cogan, 2004). Fermented foods, in addition to being more shelf-stable, typically develop desirable sensory qualities. The sensory properties of fermented dairy products result from the metabolic activity of lactic acid bacteria (LAB) in converting lactose to lactic acid; fermentation and growth of LAB are monitored indirectly by pH determination. Taking into consideration the chemical, physical and microbiological changes that occur during the cheese manufacture and the effect of these changes on cheese quality, it is helpful to develop tools to monitor the progression of the various changes during cheesemaking.

Oxidation-reduction (redox) potential is an important physicochemical parameter that, together with pH, temperature and ionic strength, determines the microenvironment in cheese. Redox potential can be defined as the measure of the tendency of a chemical/biochemical system to oxidize (lose electrons) or reduce (gain electrons).

The relationship between redox potential and the concentrations of the oxidized and reduced forms of a substance is given by Nernst equation (1), in ideal systems:

$$E_h = E_0 + 2.3RT/nF \log[\text{Ox}]/[\text{Red}] \tag{1}$$

where E_h is the potential at the standard hydrogen electrode (V), E_0 is the standard potential of the system when the activities of all reactants are unity, R is the universal gas constant (8.314 J K^{-1} mol^{-1}), T is temperature (in Kelvin), F is the Faraday constant (96.5 kJ V^{-1} mol^{-1}), n is the number of electrons involved in redox reaction (Brown & Emberger, 1980; McCarthy & Singh, 2009).

During redox measurement, it is essential to specify not only the measured potential value, but also the pH and the temperature at which it is determined (Jacob, 1970; Morris, 2000). Furthermore, in the same manner in which the pH is a measurement of the hydrogen ion activity in a system, redox potential is a measurement of the activity of electrons in a system (Kjaergaard, 1977) so it is affected by all oxidizing and reducing agents. Therefore, the measurement of redox potential should be taken in consideration during fermentation processes.

Redox potential is an important selective factor for microbes in all environments (Brown & Emberger, 1980) and knowledge of the actual redox conditions is important for the interpretation of milk quality and freshness (Noyhouzer, Kohen, & Mandler, 2009). Different studies have shown that growth of microorganisms in dairy products may be affected by the redox potential of the system (Beresford, Fitzsimons, Brennan, & Cogan, 2001; Bolduc, Raymond, Fustier, Champagne, & Vuillemard, 2006b; Boucher, Brothersen, & Broadbent, 2006; Brasca, Morandi, Lodi, & Tamburini, 2007; Morandi, Brasca, Andrighetto, Lombardi, & Lodi, 2006; Scarinci,

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Carrasco, & Simonetta, 1994). Moreover, redox potential contributes to the conditions necessary for flavour development in fermented dairy products (Cachon, Jeanson, Aldarf, & Divies, 2002; Carrasco, Scarinci, & Simonetta, 2005; Morandi et al., 2006). In cheese, it is thought that a negative redox potential is required for the stability of aroma, especially that associated with volatile sulphur compounds (Green & Manning, 1982; Kristoffersen, 1967).

Milk at 25 °C in equilibrium with air has usually a E_h value in a range from $+250$ to $+350$ mV at pH 6.6—6.7 (Fox & McSweeney, 1998) due to the presence of oxygen and oxidizing compounds; however, cheese has a negative E_h value. For example, values reported for the E_h of Cheddar and Camembert are -120 mV (Topcu, McKinnon, & McSweeney, 2008) and -300 to -360 mV (Abraham, Cachon, Colas, Feron, & De Coninck, 2007), respectively. It is well known that anaerobiosis and low redox potential contribute to the development of balanced flavour (Abraham et al., 2007) and different volatile flavour compounds are produced in vitro under oxidizing and reducing conditions (Kieronczyk, Cachon, Feron, & Yvon, 2006). However, the exact mechanism by which the redox potential of cheese is reduced is not clear.

Although redox potential is believed to be an important physiochemical parameter of fermented dairy products, only one study by Green and Manning (1982) reported changes in redox potential at different stages of cheesemaking. The aim of this study was therefore to evaluate the changes in redox potential during the simulated manufacture of Cheddar, Gouda, Emmental and Camembert cheeses throughout the various stages of cheesemaking; pH and temperature were also measured.

2. Materials and methods

2.1. Redox potential, pH and temperature measurements

Measurements of redox potential were made using a platinum working electrode (Pt 1800; Schott, Mainz, Germany) and silver/silver chloride reference electrode (REF 201; Radiometer Analytical, Villeurbanne Cedex, Lyon, France). Before cheese manufacture, the reference and the platinum electrodes were cleaned using the method described by Topcu et al. (2008). The platinum electrodes surface was polished with fine alumina (Sigma—Aldrich, St. Louis, Mo., U.S.A.) powder and rinsed with distilled water and allowed to dry in air. Reference electrodes were cleaned using pepsin in HCl solution and RENOVA-N (Radiometer Analytical) solution. The accuracy of electrodes was checked using a 3 M KCl solution (Topcu et al., 2008) and the E_h value recorded in 3 M KCl was $\approx +479$ mV at room temperature. At the end of each experiment the electrodes were rinsed with water and checked again in 3 M KCl solution. During manufacture pH was determined using a combined electrode (PHC3001-8; Radiometer Analytical) connected to a pH meter (PHM210 Standard pH Meter; Radiometer, Copenhagen, Denmark). The temperature was measured using a thermocouple. The redox electrodes, the pH probe and the thermocouple were placed into the milk/curds-whey mixture in all experiments.

All the detectors were connected to a data logger (Squirrel Data Logger 2040-2F16 Series; Grant, Data Acquisition, Cambridge, UK) that enabled data acquisition on a computer during the simulated manufacture of the cheeses. The measured data were recorded every minute and data reported were averaged over 5 min.

Redox potential was reported as ORP, redox potential uncorrected for pH and without reference to the standard hydrogen electrode. ORP data were also converted to E_h (potential related to the standard hydrogen electrode), using the following formula:

$$E_h = ORP + E_r \tag{2}$$

where E_r is the potential of the reference electrode, i.e., $+199$ mV at 25 °C when an Ag/AgCl reference electrode is used (Sawyer, Sobkowiak, & Roberts, 1995; McSweeney, Caldeo, Topcu, & Cooke, 2010). Reference electrode potentials change with temperature and it is possible to correct the redox readings using the temperature coefficients (-1.01 mV °C^{-1} at 25 °C for Ag/AgCl reference electrode; Sawyer et al., 1995). Temperature compensation can be done using the following formula:

$$E_r = +199 - 1.01(T - 25\ °C) \tag{3}$$

where T is the temperature recorded.

2.2. Simulated cheesemaking

Cheddar, Gouda, Emmental and Camembert were made in triplicate on a laboratory scale with simultaneous measurement of pH, temperature and redox potential. For each cheesemaking, raw milk (4 L) was obtained from a local dairy farm and pasteurized at 63 °C for 30 min. Cheese manufacture was performed in a glass beaker (5 L) in water bath with temperature control and manual stirring was used for milk and curd agitation. The major variables during the manufacture of Cheddar, Gouda, Emmental and Camembert-type cheeses are summarized in Table 1.

2.3. Compositional analysis

The composition (pH, protein, salt, moisture and fat) of 1 day-old cheeses was determined in triplicate. The pH was measured by probing the cheese directly with a combined glass electrode (PHC3001-8, Radiometer Analytical) connected to a pH meter (PHM210 Standard pH Meter, Radiometer Analytical). The protein content of the cheeses was determined by the macro-Kjeldahl method (IDF, 1986), salt by a potentiometric titration (Fox, 1963), moisture by oven drying at 102 °C (IDF, 1982) and fat by the Gerber method (IIRS, 1955).

3. Results and discussion

Table 2 shows pH and composition of the four cheeses made at laboratory scale at one day of ripening. Values were generally within the range typical of each variety (Kocaoglu-Vurma, 2005; Lawrence et al., 2004; Sousa & McSweeney, 2001; Verachia, 2005), although the final pH of Gouda was higher than normal for this variety. Changes in E_h (mV), ORP (mV), pH and temperature (°C) during the cheesemaking stages are shown in Fig. 1A—D for Cheddar, Gouda, Emmental and Camembert, respectively.

The milk utilized for all the cheese manufacture had a pH of 6.6 ± 0.1 and an E_h of $+349 \pm 4.8$ mV (ORP $= +150.4 \pm 5.2$ mV) at 28.7 ± 0.5 °C. Redox potential value for raw milk was within the range reported by McCarthy and Singh (2009). The positive redox potential of the milk is mainly due to dissolved oxygen (McCarthy & Singh, 2009). Milk was pasteurized (63 °C for 30 min) and cooled to 30—31 °C. During this stage of manufacture E_h values decreased to $+263$ mV (ORP $+63.5$ mV), presumably due to loss of oxygen (Bolduc, Bazinet, Lessard, Chapuzet, & Vuillemard, 2006a; Vahcic, Palic, & Ritz, 1992). Heat treatment caused a decrease in pH because of the heat-induced transfer of calcium and phosphate to the colloidal state (Jenness & Patton, 1959) with the concomitant production of H$^+$; these changes are reversible on cooling and when the temperature was decreased to 30—31 °C, the pH increased.

In Cheddar cheese manufacture (Fig. 1A), after addition of starter cultures, the temperature was kept constant at 30—31 °C and the redox potential decreased slowly with pH, as LAB metabolized lactose to lactate. Moreover, *Lactococcus lactis* reduces, in addition

Table 1
Features of the simulated manufacture of Cheddar, Gouda, Emmental and Camembert cheeses.

Common steps	Details specific to each variety			
	Cheddar	Gouda	Emmental	Camembert
Standardization	Casein to fat ratio of 0.7:1	Casein to fat ratio of 0.9:1	Standardized to 3% fat	Casein to fat ratio of 0.7:1
Starter (Chr. Hansen, Horsholm, Denmark)	0.02% (w/v) DVS R604 and ripened for 30 min	0.013% (w/v) DVS B-11and ripened for 45 min	0.02% (w/v) DVS R604 and DVS LH-B02 and ripened for 45 min	0.02% (w/v) F-DVS FLORA-DANICA and ripened for 35 min
Coagulant (Chymax-180, Chr. Hansen; 0.3 mL L^{-1}). The coagulum was allowed to reach adequate firmness (35—50 min)	Addition of 1 M $CaCl_2$ (0.9 mL L^{-1})	Addition of $NaNO_3$ (0.06 g L^{-1}), 1 M $CaCl_2$ (0.9 mL L^{-1})	Addition of 1 M $CaCl_2$ (0.9 mL L^{-1})	Addition of 1 M $CaCl_2$ (0.7 mL L^{-1})
Cutting				Curd moulded without cutting
Cooking	Curds were cooked to 39 °C	Curd washed two times by removing and replacing part of the whey (1/3 of the volume of the milk) with hot water (76—80 °C) Curds were cooked at 38 °C and stirred for 90 min. Curds were pressed under whey for 30 min.	Curds were cooked to 52—53 °C over 40 min and stirred out for 45 min	No cooking
	Whey drained at pH 6.2 followed by simulated cheddaring and milling at pH 5.4	Whey drainage. Curds were moulded and pressed overnight	Curds were then moulded and pressed for 3 h and then held at 36 °C for 17 h	Curds stood overnight in moulds at 25 °C
Salting	Dry-salting (2.5% NaCl) for 20 min followed by moulding and pressing	Brine-salting (20% NaCl and 0.5% $CaCl_2$) at 10 °C for 1 day	Brine-salting (23% NaCl) at 8 °C for 4 h	Brine-salting (20% NaCl and 0.05% $CaCl_2$) for 40 min and ripened at 14 °C

to dissolved oxygen to water, other oxidizing compounds present in milk. In a recent study, oxygen was eliminated by treatment of the milk with various gases and its reduction occurred only in the presence of *L. lactis* (Jeanson et al., 2009); the reducing capacity of LAB is due not only to removing molecular oxygen but mainly to the production of reducing molecules (e.g., trioses and acetaldehyde) during their growth (Davis, 1932; Ouvry Waché, Tourdot-Maréchal, Diviès, & Cachon, 2002).

In Cheddar cheese (Fig. 1A), in the course of the cooking stage at 38—39 °C the decrease in redox potential and pH were more marked, probably due to the heat treatment. The E_h reached values close to +200 mV (ORP ≈ 0 mV) at the whey drainage stage (pH 6.2) during which stage a large reduction in E_h to −159 mV (ORP −359 mV) occurred during the early stages of cheddaring and values remained low until the milling stage while the pH decreased rapidly to 5.4 when the curds were subdivided and exposed to the atmosphere. During salting and moulding stages the E_h values were still positive, while during the overnight pressing the values decreased again to −117 mV (ORP −314 mV).

Throughout the manufacturing process a progressive development of acidity occurred since lactic acid bacteria utilized in Cheddar manufacture have the ability to produce lactic acid quickly; a few studies have reported that *L. lactis* is one of the most reducing LAB (Brasca et al., 2007; Cachon et al., 2002). Moreover,

the elimination of whey during the drainage and pressing stage allowed the determination of redox potential of the curd since the electrodes were surrounded by the pieces of curd at these stages. Generally similar changes in redox potential were described by Green and Manning (1982) during Cheddar cheese manufacture. Furthermore, the redox values determined at the pressing stage in this study are close to the values reported by Topcu et al. (2008) for the redox potential of mild Cheddar cheese.

In Gouda cheesemaking (Fig. 1B), from the addition starter culture to the curd washing stage the E_h had constant values around +250 to +270 mV (ORP +50 to +70 mV). The pH started to decrease slightly after the cutting step. During the cooking stage the temperature was increased by removing some whey and replacing with hot water and the temperature reached 38 °C (temperature of curds/whey mixture never exceeded 40 °C). During the cooking stage redox potential decreased slightly while the pH reached the value of 6.2 at moulding. Then the curd was pressed under the whey resulting in a rapid decrease in redox potential. At whey drainage stage, E_h was around 0 mV (ORP ≈ −200 mV) and pH was 6.02. In a study by Rukure and Bester (2001) on the effect of *Bacillus cereus* on the growth of LAB during Gouda manufacture, a similar trend in pH was observed during manufacture of the control cheese. During the pressing stage E_h continued to decrease and it reached a value of −118 mV (ORP −315 mV).

Table 2
Composition and pH at one day of ripening of laboratory-scale Cheddar, Gouda, Emmental and Camembert-type cheeses.[a]

Cheese	pH	Moisture (%)	Fat (%)	Protein (%)	Salt (%)	MNFS (%)
Cheddar	5.12 ± 0.01	37.33 ± 0.76	32.00 ± 0.58	31.89 ± 0.77	1.29 ± 0.03	54.62 ± 0.43
Gouda	5.68 ± 0.03	37.60 ± 0.81	28.00 ± 0.71	30.43 ± 0.71	2.43 ± 0.07	51.99 ± 1.46
Emmental	5.37 ± 0.04	37.98 ± 0.10	30.00 ± 1.61	32.19 ± 0.48	1.92 ± 0.13	54.13 ± 1.10
Camembert	4.78 ± 0.01	45.04 ± 0.45	32.00 ± 0.58	31.89 ± 0.77	1.72 ± 0.03	65.92 ± 1.22

[a] Data are means (±standard deviations) of three independent cheesemaking trials; MNFS, moisture in non-fat substances.
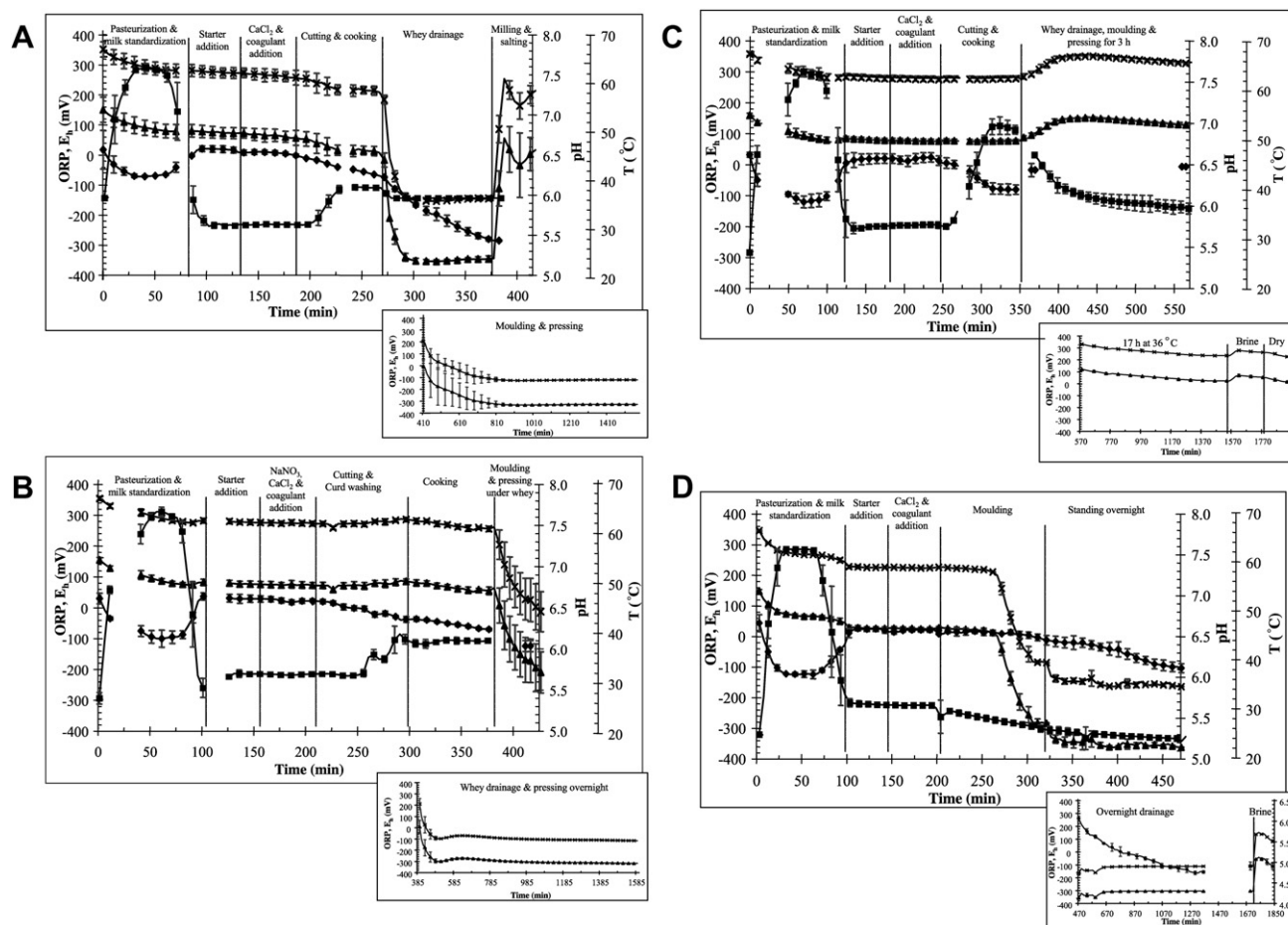
Fig. 1. Changes oxidation-reduction potential (E_h, ×; potential related to the standard hydrogen electrode and ORP, ▲; direct measurement with platinum electrode-Ag/AgCl reference electrode and without correction for pH), pH (◆) and temperature (T, ■) as a function of time and cheesemaking steps during the manufacture of laboratory-scale Cheddar (A), Gouda (B), Emmental (C) and Camembert (D) cheeses. Data are means (±standard deviations) of three independent cheesemaking trials.

In Gouda manufacture in addition to *L. lactis* spp., *Leuconostoc mesenteroides* subsp. *cremoris* was utilized as a component of the starter. In a study by van Dijk et al. (2000), the redox potential during sauerkraut fermentation was investigated; its fermentation was characterized by an initial heterofermentative process caused mainly by *Leu. mesenteroides* that lead to acidification and decrease in redox potential (from E_{h7} +200 to −240 mV).

In Emmental manufacture (Fig. 1C), after the addition of starter culture, CaCl₂ and coagulant, the redox potential values were constant (E_h ranging from +278 to +272 mV; ORP from +78 to +72 mV) until the cooking step; the pH also remained largely unchanged. During the cooking stage as the temperature was increased to 52.5 °C, the pH decreased while redox potential remained constant. After the cooking stage, the whey was drained and pH reached 6.4. When the whey was drained, E_h increased and it even increased during the pressing stage, reaching a value of +350 mV (ORP +150 mV) and thereafter decreased slightly. At the end of the pressing stage, the pH was 6.48. In Swiss-type cheeses, the acidification of the curd matrix occurs largely after drainage and during the pressing of the curd (Gagnaire, Trotel, Le Graët, & Léonil, 2002). The cheese was kept at 36 °C for 17 h, during this stage the E_h decreased to +230 mV (ORP +30 mV) at the end of this period. The cheese was then brine salted causing an increase in E_h to +280 mV (ORP +80 mV). During brine salting, the redox potential decreased slightly and kept decreasing during the drying stage. Unlike the other cheeses, the redox potential of Emmental cheese did not reach a negative value as in this variety

acidification is slow and mainly occurs during the early stages of ripening. In addition, *Lactobacillus helveticus* is utilized as starter in Emmental manufacture; in the study of Brasca et al. (2007) on the ability of different LAB to change the redox potential of milk the authors found that *Lb. helveticus* had the lowest reducing capacity.

In Camembert manufacture (Fig. 1D), after the addition of starter culture, CaCl₂ and coagulant, the redox potential values and pH (≈6.6) remained constant until the moulding step (E_h ≈ +230 mV; ORP ≈ +31 mV). After almost 1 h from the transfer of the coagulum in the mould a drop in E_h to −115 mV (ORP −314 mV) occurred and remained at these values during the overnight drainage of the coagulum while the pH decreased slightly reaching values of 4.9. A sharp increase in redox potential happened at the brining stage and it can be considered an artefact due to the exposure to the atmosphere; thereafter the values decreased again. Abraham et al. (2007) measured redox potential and pH in Camembert cheese during ripening using a microelectrode system and a E_h value of −300 mV was found at 15 days of maturation. This value is lower than the values found in this study during cheesemaking, perhaps due to further microbial action during the early stages of ripening and/or the growth of the surface fungal flora.

4. Conclusion

Distinctive trends in redox potential during cheesemaking of Cheddar, Gouda, Emmental and Camembert were observed and depended on the cheese technology and on the starter culture

utilized. Different studies have reported that the reducing activity of LAB is species dependent (Brasca et al., 2007; Cachon et al., 2002) therefore changes in the redox potential values are related to the starter utilized.

Cheese flavour and its development are affected by different factors including manufacturing conditions and activity of various microorganisms. Redox potential is an important parameter as it can provide more information on the microenvironment in cheese during manufacture and ripening. Redox potential also plays an important role in flavour development in dairy products (Green & Manning, 1982). Thus, monitoring redox potential all along cheesemaking and ripening could assist control of the development of aroma in cheese.

Acknowledgements

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Analytical Methods

RP-HPLC peptide profiling of cheese extracts: A study of sources of variation, repeatability and reproducibility

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ABSTRACT

Peptide profiling of cheese extracts by RP-HPLC is widely used to study cheese quality and authenticity. Repeatability, reproducibility and the contribution of differences in sample/treatment, extraction, and the effect of the chromatographic run was studied. Chromatograms (108) of pH 4.6-soluble extracts from 180 d Cheddar cheeses were used to evaluate the precision of the method and to quantify the effect of treatment and replicate cheese trial. Repeatability and reproducibility coefficient of variations of peaks/RT classes varied widely and no clear correlation was found between peak size and reproducibility. ANOVA of Principal Component Scores identified significant differences amongst cheeses and a trial effect. Partial Least Square Discriminant Analysis identified variables related to cheese treatment. Statistical process control methods were used to study the variability of replicate injections over the life of a single column showing the discriminatory power of RP-HPLC and giving possibilities to improve both process control and process optimisation.

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1. Introduction

Proteolysis is the degradation of the casein matrix in cheese to a range of peptides and free amino acids and is recognised as the most complex and important biochemical events leading to the development of texture and flavour of mature cheese (Upadhyay, McSweeney, Magboul, & Fox, 2006). Proteolysis contributes to the textural changes of the curd due to breakdown of the casein, decrease in a_w through water binding by liberated carboxyl and amino groups. Moreover, the formation of peptides and free amino acids is associated directly with development of desirable or undesirable taste and aroma and contributes to the liberation of substrates for secondary catabolic changes (Sousa, Ardö, & McSweeney, 2001). Because of its importance in determining cheese properties, the assessment of proteolysis has a great relevance in issues related to quality, authenticity and process control (Coker, Crawford, Johnston, Singh, & Creamer, 2005).

Analytical techniques for the assessment of proteolysis in cheese have been reviewed (Bansal, Piraino, & McSweeney, 2009). Non-specific methods give information about the extent of proteolysis and the general contribution of each proteolytic agent.

Specific techniques (i.e. electrophoresis and chromatography, which may be coupled to mass spectrometry) resolve, isolate and identify the peptides that are produced or degraded during cheese ripening (McSweeney & Fox, 1997). Most of the methods used for assessing proteolysis in cheese are based on the principle that peptides of different size produced from caseins may be soluble in different solvents (Bansal et al., 2009). The pH 4.6-soluble extract of Cheddar cheese contains numerous small and medium sized peptides, free amino acids and their degradation products, and organic acids and their salts (Bansal et al., 2009). RP-HPLC profiling of pH 4.6- or water-soluble extracts is amongst the methods most frequently used for the characterisation of secondary proteolysis in cheese. This technique is considered to be highly discriminant and it is considered amongst the most valuable techniques for assessing authenticity and quality of cheese (Arvanitoyannis & Tzouros, 2005; Coker et al., 2005; Karoui & De Baerdemaeker, 2007), although proteomic approaches and mass-spectrometry based techniques, which provide better resolution and allow the identification of peptides, are increasingly being used (Masotti, Hogenboom, Rosi, De Noni, & Pellegrino, 2010; Pappa et al., 2008).

RP-HPLC profiles of pH 4.6- or water-soluble extracts are multivariate in nature. The raw data are ordered couples of peak position (retention time) and peak intensity (as area or height). Extraction of the data from the raw chromatographic profile has been performed by a variety of methods. Visual matching of all peaks in different chromatograms is the method most frequently

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used (Hannon et al., 2004; Pripp, Shakeel-Ur-Rehman, McSweeney, & Fox, 1999; Pripp, Shakeel-Ur-Rehman, McSweeney, Sørhaug, & Fox, 2000; Pripp, Stepaniak, & Sørhaug, 2000). Alternatively, only few peaks of the chromatogram may be visually matched and used for analysis (Hynes, Bergamini, Suárez, & Zalazar, 2003; Milesi, McSweeney, & Hynes, 2008a, 2008b) or the chromatogram may be divided in classes of retention time and peaks in each class summed (Barile, 2006). Recently we proposed the use of a fuzzy logistic function to accumulate value of peaks in an automated way in classes of retention time (Piraino, Parente, & McSweeney, 2004).

The processed data are usually analysed using descriptive techniques. Principal Component Analysis (PCA) is the most frequently used (Hannon et al., 2004; Milesi et al., 2008a, 2008b; O'Shea, Uniacke-Lowe, & Fox, 1996; Piraino et al., 2004; Pripp et al., 1999, 2000; Upadhyay et al., 2006). This technique has the advantage of reducing the dimensionality of the data set (a typical RP-HPLC chromatogram contains typically more than 100 peaks of different heights/areas) by identifying a number of linear combinations of the original variables (the Principal Components) which are (i) uncorrelated, i.e. orthogonal and (ii) summarise decreasing amounts of the variance. A detailed description of application of PCA in the evaluation of cheese quality and authenticity can be found in Pripp, Stepaniak, and Sørhaug (2000), and Coker et al. (2005). Unfortunately, PCA is a descriptive, not an inferential technique, and differences amongst treatments are usually analysed visually on the basis of distance of points in the score plots. Variability related to treatment or sample (i.e. different cheeses belonging to the same variety in authenticity studies, cheeses obtained in replicate cheesemaking trials) and variability due to the analytical technique (sampling, extraction, chromatographic separation) may significantly affect the HPLC profiles obtained (Coker et al., 2005), and visual assessment of small differences to evaluate the effect of treatment or ripening time may be difficult. Linear Discriminant Analysis has been used on Principal Component Scores (Hynes et al., 2003; O'Shea et al., 1996) in order to assess the significance of difference amongst treatments or ripening time and to identify the variables associated with differences. Partial Least Squares Regression (PLSR) and Partial Least Squares Discriminant Analysis (PLSDA) have become the techniques of choice when there is the need to model the relationships between a multivariate X data matrix and an univariate or multivariate Y data matrix (Arvanitoyannis & Tzouros, 2005; Coker et al., 2005; Wold, Sjöström, & Eriksson, 2001) because they allow datasets with many correlated X variables to be handled and provide excellent results even when the number of observations is lower than the number of predictor (X) variables, and produces parsimonious models by extracting only the portion of the X variance which is related to the Y variance. PLSR and PLSDA have been frequently used to relate spectroscopic or chromatographic data to quality parameters and sensory properties of cheese (Arvanitoyannis & Tzouros, 2005; Coker et al., 2005), but they have never been used, to our knowledge, to explore systematically the relative importance of sources of variation in RP-HPLC of extracts from cheese.

Because of the importance of peptide profiling by RP-HPLC there is a need to characterise this measurement process in terms of repeatability and reproducibility, and to identify the major sources of variation which affect the discriminatory ability of this method. Measurement process characterisation includes a variety of statistical techniques which address the issues which affect the measurement of one or more variables in process control (NIST/ SEMATECH, 2008): bias (i.e. differences between the measurements on a given object and its true value), short-term variability or precision (i.e. the variability of measurements on the same sample caused by short-term effects, also called repeatability, measured by the repeatability or level 1 standard deviation), long-term variability (i.e. the variability of measurements on

the same sample caused by more long-term effects , measured by level 2 standard deviation), and uncertainty (i.e. the dispersion of the measurements around the mean, which defines the precision of the measurement process). The measurement process of peptide profiling in cheese extracts by RP-HPLC requires several steps: sampling, extraction and storage of the extracts, chromatography, primary analysis of chromatographic data (removal of background, identification of peaks, measurement of peak height/area), all of which may affect accuracy, repeatability and reproducibility. One special issue in the analysis of peptide profiles from cheese extracts is that internal or external standards are not included in the analysis and the analysis is, at least in part, destructive (little is known on the stability of cheese extract over time). This prevents the use of some techniques used in measurement process characterisation, like check standard methodology (NIST/SEMATECH, 2008) which requires repeated measurement on a suitable standard.

The objectives of this work were the evaluation of the repeatability and reproducibility of the measurement of RP-HPLC peptide profiles of pH 4.6-soluble extracts from Cheddar cheese and the identification of the relative amount of variability contributed by the different steps of the analysis (including primary data processing) and how this variability affects the ability to discriminate different cheese samples, and to derive from this information guidelines to improve the design and analysis of experiments for profiling cheese proteolysis. For this purpose two approaches were used: (i) data from a pilot plant experiment in which four treatments were used in the production of Cheddar cheese and for which replicate cheese trials, with replicate extraction for each sample and replicate injections for each extract were available were analysed by univariate and multivariate methods to assess repeatability and reproducibility and evaluate the relative importance of different sources of variation and (ii) replicate injections of the same extract of a single cheese were used to evaluate the performance of the chromatographic method over time, in order to evaluate the potential impact of column ageing.

2. Materials and methods

2.1. Cheese manufacture and analysis

Four different Cheddar cheeses were made in three replicate trials in 20 L vats using a standard procedure: Control (K), added Lac⁻ adjunct (L), added coagulant (R), and cheese added with Lac⁻ adjunct and coagulant (LR). DVS starter (R-604, Chr-Hansen, Hørsholm, Denmark) was added at the rate of 0.016% to all treatments. Cheese milk was standardised to casein to fat ratio of 0.7:1, pasteurised (72 °C × 15 s) and cooled to 30–31 °C. Starter (R-604; Chr. Hansen Ltd., Little Island, Cork) was added (0.15 g) to 20 L milk and allowed to ripen for 40 min. *Lactococcus lactis* L57156 adjunct was added to cheese vats L and LR. Rennet (Maxiren 180, DSM Food Specialties, Delft, Netherlands) was diluted five-fold with water before addition the milk (0.3 ml/L for treatments K and L, 0.9 ml (L for treatments R and LR). Cheese blocks were vacuum packaged and ripened at 8 °C. Samples were taken for analysis at 180 days. pH 4.6-Soluble extracts were prepared according to the method of Kuchroo and Fox (1982). Three independent extractions were carried out for each cheese sample.

Peptide profiles of the pH 4.6-soluble fractions were determined by RP-HPLC by the method described by Sousa and McSweeney (2001) using a Varian HPLC system (Varian Associates Inc., Walnut Creek, CA, USA) and a Nucleosil RP-8 (250 × 4.6 mm, 5 µm particle size, 300 Å pore size) analytical and guard columns (4.6 × 10 mm) (JVA Analytical, Dublin, Ireland) and an

acetonitrile–water gradient with trifluoroacetic acid as the ion pair reagent and spectrophotometric detection at 214 nm. Three independent injections were performed for each extract. Raw chromatograms were processed using Varian Star software (version 6.2) for baseline removal and peak identification. Processed data file were imported in Microsoft Excel for further processing.

2.2. Primary data processing

A total of 108 chromatograms (four treatments × three replicate cheese trials × 3 replicate extractions × three replicate injections), each with approximately 80 identified peaks was available for analysis.

Chromatograms were pre-processed using the fuzzy approach described by Piraino et al. (2004). Briefly, the following equation was used to accumulate peaks for each chromatogram in 61 classes of retention time (each class had a width of 1.1 min, with the first class entered at 4 min)

$$C_c = \sum_{j=1}^{n} ph_j \frac{1}{1 - e^{a\left(|rt_j - rt_c| - \frac{w_c}{2}\right)}} \qquad (1)$$

where C is the accumulated value for variable (class, or interval of time defined over the elution time axis) c; ph is peak height for peak j in the chromatogram; n is the number of peaks in the chromatogram; a is a shape parameter of the function; rt_c and rt_j are retention time for the centre of class c and for peak j, respectively; w_c is class width. The second term of Eq. (1) is the weight (0–1) with which ph is attributed to class c: the closer the peak to class centre, the higher the value. Due to the nature of the function a peak will be attributed with the highest weight to the class to whose centre it is closer, and a peak which is close to the border of two classes will be attributed to both classes with almost equal weights.

The shape parameter can be adjusted by choosing appropriate values for two further parameters: flat range (FR) and membership in the flat range (MFR). MFR is the minimum weight (in percent) for ph when peak position was within a specific distance d from the class centre ($d = |rt_c - rt_x|$). FR was defined as the range around the class centre given by $rt_c \pm d$ (expressed as percent of w_c).

$$a = \ln\left(\frac{100}{MFR} - 1\right) \frac{2}{w_c^{\left(\frac{FR}{100} - 100\right)}} \qquad (2)$$

A low value of FR and a high value of MFR would result in high weights attributed only to peaks which are close to the class centre. Since technical replicates were available for each cheese sample (replicate extractions and injections) the choice of the parameters FR and MFR was based on their effect of the repeatability and reproducibility coefficient of variation for the replicates, in order to choose the combination of parameters which minimised the contribution of the fuzzy processing to the variance of the experiment. All combinations of two values of FR (30% and 50%) and three values for MFR (90%, 95% and 99%) were used for processing the data.

2.3. Multivariate data analysis

A Principal Component Analysis was carried out on the covariance matrix of the data. Analysis of variance was performed on scores for the first four components to calculate the contribution to the variance of treatment, replicated cheese trial, extraction. PCA and ANOVA were performed using Systat 11 (Systat Inc., Chicago, IL). Partial Least Square Regression Discriminant Analysis (PLS-DA) was performed on the same data using "pls" package of R. Variance decomposition was performed using a mixed-effect model and the proportion of variance explained by each factor

was estimated. Such model assumes the grand mean as unique fixed effect whilst all the other factors are included as random.

The model for a complete randomised block design is given by:

$$y_{irjt} = \mu + \tau_i + \rho_r + \beta_j + \omega_t + \varepsilon_{irjt} \qquad (3)$$

where y_{irjt} = the measure in the jth extraction, under r^{th} injection (chromatographic) for the ith cheese from the tth trial, μ = overall mean, τ_i = i^{th} cheese – random, ρ_r = effect of the rth injection (chromatographic run) – random, β_j = effect of the jth extraction – random, ω = effect of the tth trial – random, ε_{irj} = random error using package "lme4" (http://lme4.r-forge.r-project.org/, Pinheiro & Bates, 2000) of R (R Development Core Team, 2010). R output (x-scores and weights, y-loadings, variance proportions were post-processed using Systat 11 to obtain graphs. Data were centred and scaled and cross-validation was performed by bootstrapping.

2.4. Use of quality control methods to evaluate the performance of the chromatographic column over time

A single pH 4.6-soluble extract from a mature commercial Cheddar cheese (10 months old) was prepared and aliquots (1 mL) of pH 4.6-soluble nitrogen were freeze-dried (Lyovac GT2, Amsco Finn-Aqua Gmbh, Hürth, Germany) and stored at −20 °C. Over the life of a single chromatographic column (approximately 900 injections), two replicate injections were performed at approximately weekly intervals. Chromatographic conditions were those described above and raw data were processed as described above and used as an input for PCA. The PCA scores were used to obtain lag-plots and Exponentially Weighted Moving Average (EWMA) plots and to describe the change in performance of the column.

3. Results and discussion

RP-HPLC profiles of peptides extracted from cheese are commonly used and are an extremely valuable tool in dairy research, but can also be used in authenticity studies and for process control and optimisation (Arvanitoyannis & Tzouros, 2005; Coker et al., 2005; Karoui & De Baerdemaeker, 2007; Upadhyay et al., 2006). Given the importance of this analytical procedure, it is rather surprising that data on its reproducibility and accuracy are lacking in the literature.

The sources of variation which affect this analysis are numerous. In process control (i.e. when the variability of a given cheese in different production lots is of interest), authenticity studies (i.e. when the variability of quality of a given cheese type is of interest) and in process optimisation experiments (i.e. when replicates of a single treatment used for cheese production are compared), biological variation due to a large number of factors (milk quality, variations in cheese-making procedure, ripening, etc.) may be relatively high (Fox & Cogan, 2004). Further variability may be introduced by technical factors: sampling, variability due to the extraction step, variability due to column performance, to the chromatographic run, to peak selection and matching, to measurement of peak area/height (Coker et al., 2005).

In this work we used two different data sets in an attempt to estimate the relative importance of different sources of variability.

The first data set was obtained from a pilot plant experiment which may be considered as representative of situations encountered in process control and process optimisation: four treatments were compared in a randomised block design with three replicates. Sufficient replication to estimate both biological (in cheese milk, in starter and coagulant: replicate cheese making trials) and technical (in sampling, extraction, chromatographic runs: replicate extractions, replicate injections) variability was available and this in principle allows the estimation of both repeatability and

reproducibility. This data set was used to calculate repeatability and reproducibility measures and to compare the effect of different sources of variation. A second data set included a number of replicate injections of a single freeze-dried extract over the life of a chromatographic column, and was used to assess the effect of column degradation on the RP-HPLC profile.

3.1. Optimisation of primary data processing and evaluation of repeatability and reproducibility of chromatographic runs

Raw RP-HPLC chromatograms (108) from a pilot plant cheese-making trial were pre-processed using a fuzzy approach (Piraino et al., 2004).

Four treatments were compared in a randomised block design with three replicates, for which chromatograms derived from replicate extractions and injections were available. Since the method used for primary data processing may introduce a bias we carried out a preliminary data analysis to identify the optimal values for the shape parameters of the fuzzy logistic function. For each chromatogram, peaks were accumulated in 61 retention time (RT) classes of 1.1 min using six different combinations of the parameters FR (flat range 30% or 50%) and MFR (membership in the flat range, 90%, 95%, or 99%). For each of the cheese samples repeatability and reproducibility coefficient of variations (CV) were calculated, and average, minimum and maximum values of CV were computed. In addition, to evaluate how the fuzzy approach compares with visual matching, repeatability and reproducibility of both peak retention time and peak height were calculated for selected peaks which had been visually matched.

Measurement precision is defined as "closeness of agreement between indications or measured quantity values obtained by replicate measurements on the same or similar objects under specified conditions" and is usually measured by standard error (JCGM, 2008). Measurement repeatability is defined as "measurement precision under a set of repeatability conditions of measurement, i.e. condition of measurement, out of a set of conditions that includes the same measurement procedure, same operators, same measuring system, same operating conditions and same location, and replicate measurements on the same or similar objects over a short period of time" (JCGM, 2008). Therefore, for the purpose of evaluation of primary data processing, repeatability was assessed over replicate injections for the same cheese extract.

Measurement reproducibility is defined as "measurement precision under reproducibility conditions of measurement, i.e. condition of measurement, out of a set of conditions that includes different locations, operators, measuring systems, and replicate measurements on the same or similar objects". Therefore, reproducibility was measured on replicate extractions for the same cheese sample.

The formulas used to calculate the appropriate standard deviations were derived from check standard methodology (NIST/SEMATECH, 2008). Given I replicate injections for J replicate extractions of each of the 12 cheese samples, the J within-extractions mean and standard deviations for each class of each of the six processed chromatograms (FR $\times$ MFR combinations were being tested for each of the raw chromatograms) were calculated as follows:

$$\bar{x}_{j\cdot} = \frac{1}{I} \sum_{i=1}^{I} x_{ij} \tag{4}$$

$$s_j = \sqrt{\frac{1}{I-1} \sum_{i=1}^{I} (x_{ij} - \bar{x}_{j\cdot})^2} \tag{5}$$

Level-1 standard deviations, appropriate to measure repeatability (i.e. variability due to replicate injections of the same extract) were calculated as follows:

$$s_1 = \sqrt{\frac{1}{J} \sum_{j=1}^{J} s_j^2} \tag{6}$$

The grand-mean, which is an estimate of the true value of each class and the level-2 standard deviation, which is appropriate to estimate the variability due to extraction were calculated as follows:

$$\bar{x}_{\cdot\cdot} = \frac{1}{J} \sum_{j=1}^{J} \bar{x}_{j\cdot} \tag{7}$$

$$s_2 = \sqrt{\frac{1}{J-1} \sum_{j=1}^{J} (\bar{x}|_{j\cdot} - \bar{x}_{\cdot\cdot})^2} \tag{8}$$

An appropriate measure of reproducibility was calculated as follows:

$$s_R = \sqrt{\frac{1}{J-1} \sum_{j=1}^{J} (\bar{x}_{j\cdot} - \bar{x}_{\cdot\cdot})^2 + \frac{I-1}{I} \frac{1}{J} \sum_{i=1}^{I} (x_{ij} - x_{j\cdot})^2}$$
$$= \sqrt{s_2^2 + \frac{I-1}{I} s_1^2} \tag{9}$$

Since class heights (expressed as % of total height in a chromatogram) within each chromatogram varied between 0% and 7.7%, repeatability and reproducibility were expressed as coefficient of variations:

$$CV_1 = \frac{s_1}{\bar{x}_{\cdot\cdot}} \tag{10}$$

$$CV_R = \frac{s_R}{\bar{x}_{\cdot\cdot}} \tag{11}$$

The combination FR = 30% and MFR = 90% resulted in the lowest value for mean, minimum and maximum CV and was selected for the treatment of the chromatograms.

The repeatability and reproducibility coefficient of variations ranged from 0.002 to 3 and from 0.014 to 24.34, respectively, with median values of 0.16 and 0.25 (for 50% of the classes repeatability standard deviation was 16% of the mean class height or less, whilst the reproducibility standard deviation was 25% or less). Although extreme values were found for classes with low height (corresponding to small peaks) there was no clear correlation between class height and repeatability and reproducibility.

Since both repeatability and reproducibility measures were available, processed chromatograms were plotted as pseudo-chromatograms (bar plots in which each bar corresponds to the class height and the repeatability or reproducibility coefficient of variations are shown as the error bars). Fig. 1 shows an example for treatment K.

Although the chromatograms are strikingly similar in shape clear difference in the replicate cheese trials are evident and small peaks between 10 and 20 min or at the end of the chromatogram (>60 min) show poor repeatability and reproducibility.

In order to compare the reproducibility of classes generated with the fuzzy approach with those of chromatographic peaks, peaks closest to the centre of classes at 5.1, 21.6, 31.5, 32.6, 33.7, 38.1, 40.3 and 49.1 min were visually matched and the repeatability and reproducibility of peak retention time, peak height and relative peak height were calculated. Whilst the repeatability and reproducibility of retention time were excellent (minimum, median and maximum values for s_1 coefficient of variation were 0.001, 0.005 and 0.027, respectively; minimum, median and maximum values for s_R coefficient of variation were 0.001, 0.006 and 0.029, respectively), median repeatability and reproducibility of relative peak heights were comparable to those of the corresponding classes (minimum, median and maximum values for s_1 coeffi-
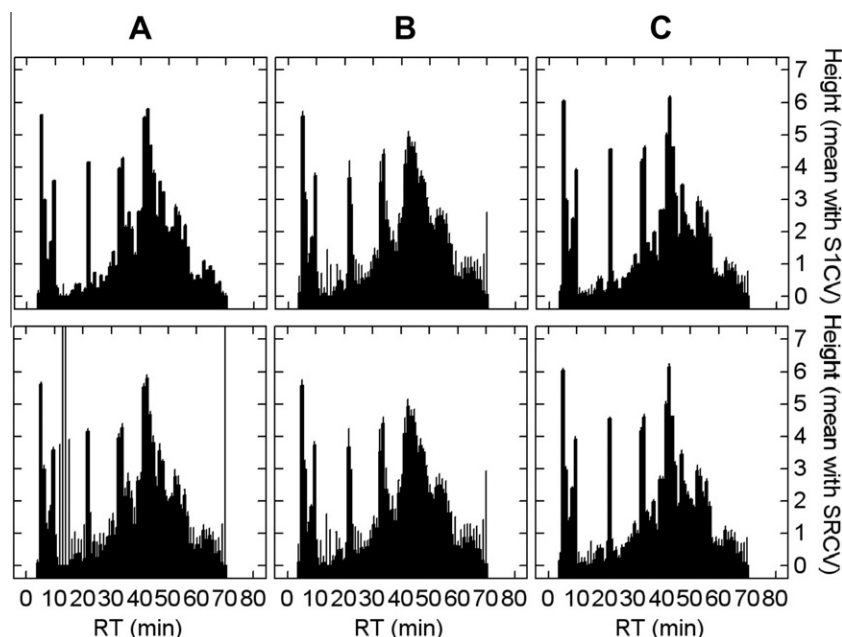
Fig. 1. RP-HPLC pseudo-chromatograms of pH 4.6-soluble extracts from 180 d old Cheddar cheeses for treatment K (control). Bars show the mean of class values. Error bars show the repeatability ($S1CV$) and reproducibility ($SRCV$) coefficient of variation of each class. Each pseudo-chromatogram is the mean of three (replicate cheese trials) × three (replicate extractions) × three (replicate injections) chromatograms.

cient of variation for peaks were 0.018, 0.131 and 1.165, whilst those for class height were 0.006, 0.116 and 0.784, respectively; minimum, median and maximum values for s_R coefficient of variation for peaks were 0.025, 0.168 and 1.346, whilst those for class height were 0.034, 0.188 and 0.939, respectively) and no significant differences were found using a non-parametric sign test although maximum values for s_R coefficient of variation were higher for relative peak heights compared to classes.

The usual approach for extraction of variables from RP-HPLC profiles is visual matching of peaks (Pripp et al., 1999). Recently, we showed that a faster, more objective approach based on accumulation of peaks in classes of retention time using a fuzzy approach produced equivalent results when descriptive multivariate statistical methods were used (Piraino et al., 2004). Here, we have shown that, when technical replicates are available, the parameters of the logistic function (FR, flat range and MFR, membership in the flat range) can be optimised in a deterministic way to minimise the repeatability coefficient of variation of peaks and that the repeatability and reproducibility coefficient of variation of variables extracted using the fuzzy approach compare favourably with those obtained using visual matching.

The results obtained on the repeatability and reproducibility of RP-HPLC profiles were far from encouraging. Using the fuzzy approach, although the minimum repeatability (s_1) and reproducibility (s_R) coefficient of variations were low, median and maximum values were high. The values estimated for selected peaks obtained by visual matching were of the same order of magnitude as those estimated with the fuzzy approach. Unfortunately there are no data in the literature on this subject. Although instrumental measurements of single analyte pose different problems compared to determination of RP-HPLC profiles (in which calibration cannot be performed) the precision of instruments used for monitoring other biological fermentation processes is significantly higher: 0.1–0.3% for pH and temperature measurements, but as high as 10% for dissolved oxygen measurement in fermentation broths (Schügerl, 1991); <0.5% for mass spectrometric analysis of gas composition (Heinzle & Dunn, 1991).

Since many peaks have a poor signal (peak or class height) to noise (repeatability or reproducibility) ratio (see Fig. 1) prior to fur-

ther analysis of the data all variables which show poor precision should be discarded.

3.2. Multivariate analysis and assessment of the relative impact of sources of variations on precision

In order to evaluate the relative importance of the main sources of variation (treatment, replicate cheese trial, technical variability due to sampling, extraction and injection) which affect precision in typical experiments in cheese proteolysis profiling by RP-HPLC of soluble extracts three multivariate approaches were used for the analysis of the pre-processed chromatograms.

Processed chromatograms were subjected to Principal Component Analysis using the covariance matrix. When all 61 classes were used, the first eight components explained 78% of the variance, but most classes had very low loadings on all factors. The analysis was repeated using only the 20 variables which had loadings >0.4 in absolute value on any of the factors. In the resulting model the first four components explained 67% of the variance, whilst the first two components accounted for 46.8% of the variance. The score and loading plots for the first two components are shown in Fig. 2.

A significant variability is evident amongst technical (replicate injections, replicate extractions) and biological replicates (replicate cheese trial) of the same treatment. However, the availability of replicates allowed the use of Analysis of Variance on Principal Component Scores to pinpoint sources of variations. The results are summarised in Table 1. Even with the relatively high variability found in the data set, the inferential analysis on principal components allowed evaluation of the statistical significance of the differences amongst treatments and cheesemaking trials, whilst the effect of extraction was never significant. The effect of treatment explained from 43% to 87% of the variance whilst the effect of replicate cheese trial explained from 7% to 51% of the variance. (Table 1)

Results obtained when all the factors where combined in a multivariate analysis of variance model were similar (data not shown).

Descriptive multivariate statistical techniques (Principal Component Analysis, Multidimensional Scaling, Cluster Analysis) are
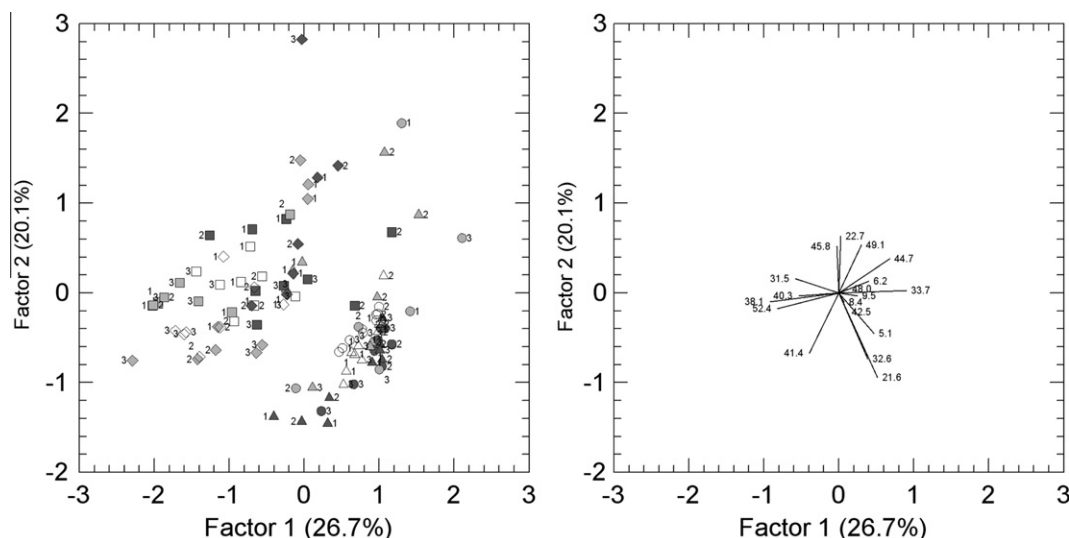
Fig. 2. Score (left) and loading plots (right) for the first two components of Principal Component Analysis performed on pre-processed RP-HPLC chromatograms of pH 4.6-soluble extracts. Treatments are ○K, △L, □LR and ◇R; shading is used differentiate replicate cheese trials (A = white; B = grey; C = black) whilst replicate extractions for each trial are indicated by numbers close to the symbols.

Table 1
Analysis of variance on Principal Component Scores for pre-processed RP-HPLC chromatograms of pH 4.6-soluble extracts from Cheddar cheese.

Component (% of variance explained)	Treatment[a] (K, L, LR, R) (%)	Cheesemaking trial (A–C) (%)	Comment
1 (26.7)	***87.1[b]	**10.0	Contrasts K and L vs LR and R; C vs A and B
2 (20.1)	***64.2	ns 7.4	Contrasts K and L vs LR and R;
3 (9.7)	***45.8	***51.3	Contrasts L or LR vs K and R; B and C vs A
4 (9.4)	***44.8	***47.2	Contrasts K or L vs LR and R; B vs A and C
5 (6.4)	***43.4	***47.2	Contrasts L and K vs LR and R; B and C vs A

[a] **$p < 0.01$; ***$p < 0.001$; ns not significant. The effect of extraction was never significant.
[b] % of total variance explained.

the techniques of choice for presenting and comparing RP-HPLC profiles of cheese extracts (Arvanitoyannis & Tzouros, 2005; Coker et al., 2005; Karoui & De Baerdemaeker, 2007; Pripp et al., 1999, 2000). However, the data presented here clearly show that visually judging differences amongst samples or treatments on the basis of their distance on MDS or PCA score plots may be complicated or lead to wrong conclusions. PCA on the pilot cheese data set presented in this study explained a relatively low amount of the variance. This is due both to the fact that the treatments were relatively similar and to the analysis of a single ripening time. This implies that the data lack the clear variance/covariance structure which is frequently encountered in studies in which several ripening times are compared. In fact, the data used in this study were a subset extracted from a large experiment in which samples were taken at several ripening time (3, 45, 75, 120 and 180 d): when the whole data set was analysed, ripening time was, quite obviously the main source of variation (data not shown), and this made difficult to evaluate the effect of other sources of variability. However, ANOVA on Principal Component Scores allowed identification of variables related to different sources of variability and showed that the contribution of extraction is relatively small. Although Linear Discriminant Analysis on component scores could have been

used instead of ANOVA, this technique has a relatively strict requirement for homogeneity of variances.

PLS-Discriminant analysis was carried out on the same data set using four dummy variables to code factor treatment. After cross-validation, a model with 10 components explained 70.6% of the X variance and 90–91%% of the Y-variance. The X-score plot and the weight and loading plots for the first two components are shown in Fig. 3. By comparing the score plots in Figs. 2 and 3 the superiority of PLS-DA over PCA in extracting components which are better related to the relationships between the X-data set (processed RP-HPLC chromatograms) and the Y-data set (design matrix) and the ability to produce a more parsimonious model was evident with the effect of coagulant and adjunct addition well separated in the first two PLS components, whilst several PCA components were needed to separate all treatments. A significant scatter due to replicate cheese trials, extraction and injection remained evident.

An additional single mean linear mixed-effect model allowed partitioning the variance contributed by the different factors, all used as random. The results are shown in Fig. 4. Treatment (addition of adjunct or extra rennet) was the main source of variance. Because of the high variability, X-variables contributed little to explain differences due to treatment which were related to small hydrophilic peptides eluting from 5 to 10 min, some peptides eluting around 24.9, 33.7 and 38.1 min and between 51.3 and 52.4 min. The differences amongst cheeses in this region are apparent in the top panel of Fig. 4. The second significant source of variation was cheese trial, whose contribution was even larger than that of treatment for peaks eluting around 18.1 min and 48 min. The contribution of replicate extraction and injection was always very low.

PLS-DA has less strict assumption than LDA (Coker et al., 2005; Wold et al., 2001) and was able to select a parsimonious set of variables which related to differences amongst the cheeses. It also allowed the variance to be partitioned and to confirm that only a limited number of peaks were related to differences in cheeses. Replicate cheese trials were the second most important source of variation; replicate cheese-making trials are the equivalent of lot to lot variation in statistical process control. The relatively high contribution of this source of variability even in controlled pilot plant experiments makes it difficult to assess small differences between lots and may reduce the power of comparisons amongst dif-
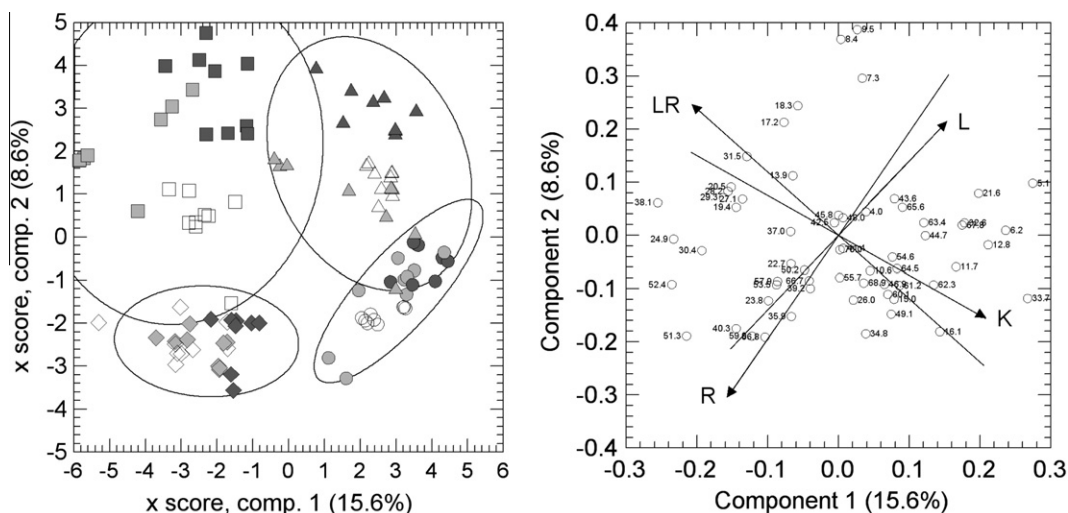
Fig. 3. X-scores (left) and weight and loading plot (right) for the first two components of a PLS-Discriminant Analysis carried out on pre-processed RP-HPLC chromatograms of pH 4.6-soluble extracts from Cheddar cheese. Treatments are ○K, △L, □LR and ◇R; shading is used differentiate replicate cheese trials (A = white; B = grey; C = black) whilst replicate extractions for each trial are indicated by numbers close to the symbols. Retention time of the class centres is shown in the weight and loading plot whilst the y-loadings for cheeses are shown as vectors. Bivariate confidence ellipses for each cheese type (*p* = 0.95) are shown in the X-score plot.
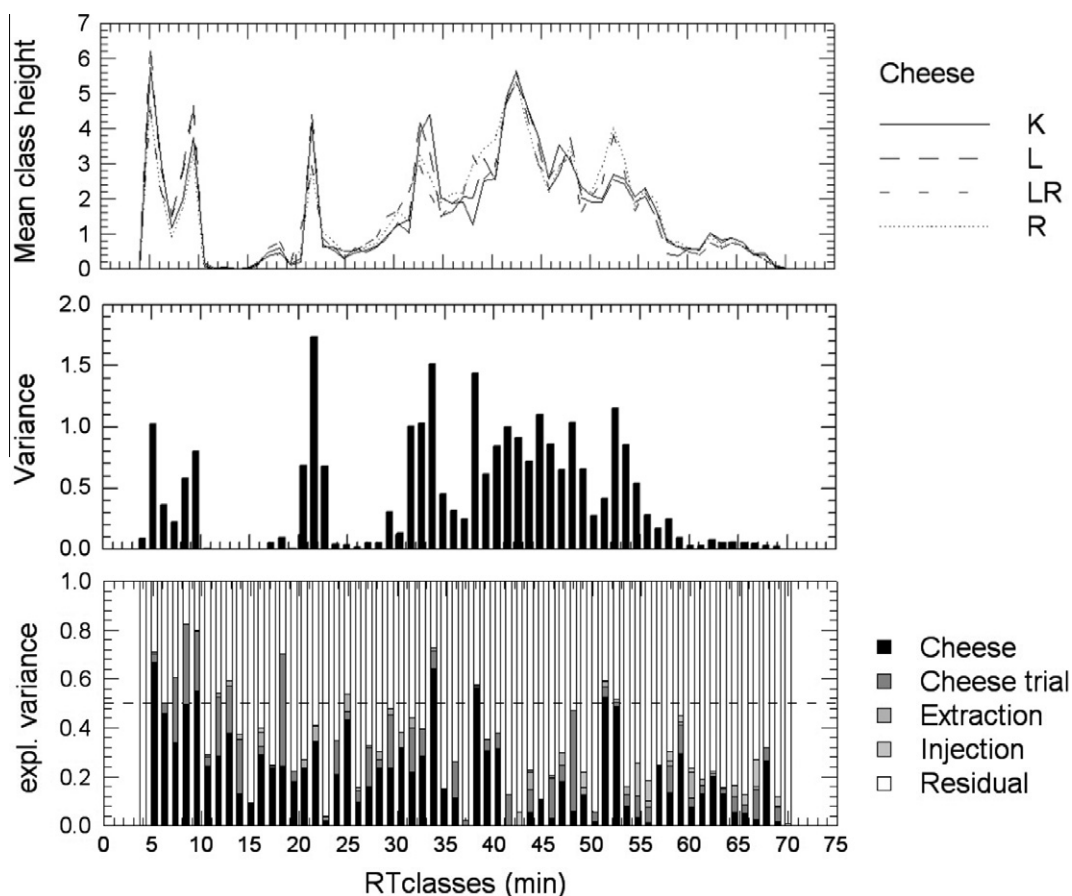

Fig. 4. Variance components, explained variance and mean profiles for a single mean mixed effect model carried out on an pre-processed RP-HPLC chromatograms of pH 4.6 soluble nitrogen. The bottom panel shows the proportion of variance explained by each factor, for each of the variables (height of retention time classes extracted from the raw chromatograms), the middle panel shows the total variance explained by each of the variables whilst the top panel shows the mean profile of each cheese.

ferent treatments, resulting in the need for a high number of replicates. Although extraction and injection had little systematic effect, the relatively poor reproducibility of many classes of RT would prevent the use of RP-HPLC of cheese extracts in statistical process control, unless one focuses on a limited number of peaks with clear relationships with cheese quality or specific properties.
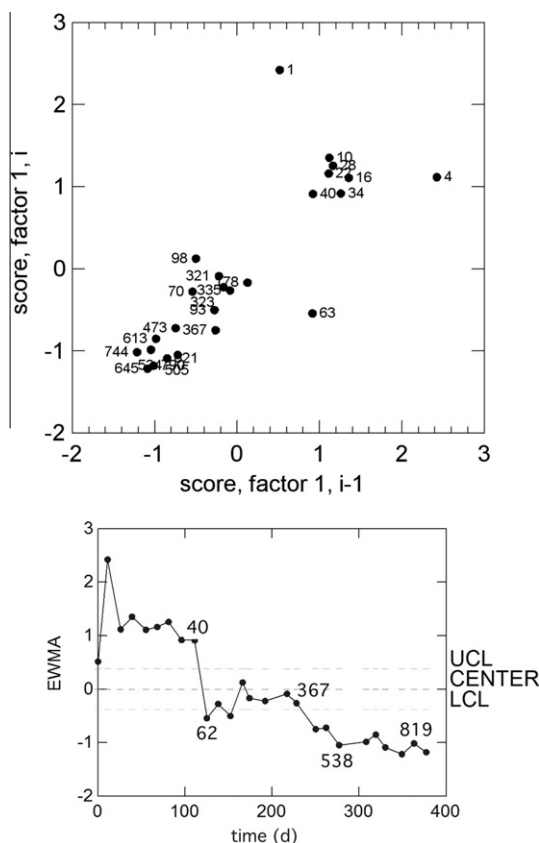
Fig. 5. Top: Lag plot of the average score (for two replicate injection) of the pre-processed RP-HPLC chromatograms first component extracted from a data set of 26 injections of a single pH 4.6-soluble extract from a single cheese performed over 377 days during which the chromatographic column was used for 818 injections. The points are labelled with the number of injections. Bottom: Exponentially Weighted Moving Average control chart for the mean score of component 1. Number of total injections are shown for some relevant points.

3.3. Use of quality control methods to evaluate the performance of a C_8 chromatographic column over time

In a large experiment several (>100) extracts are analysed by RP-HLPC, often over several days. The degradation of column performance may potentially affect the results especially if injections of different samples are performed sequentially and not randomised: in fact, this would lead to confounding of the effect of treatments or ripening time with column degradation. To evaluate this contribution a further experiment was performed by injecting the same freeze-dried extract twice at intervals over 377 days. The column was changed after the analysis of the first two replicates and then used for the analysis of 819 samples. Overall 26 injections of the same sample were performed in replicate. The chromatograms were pre-processed and the processed data were submitted to PCA as described above.

By comparing pseudo-chromatograms obtained at the beginning (0 days), in the middle (96 days, 40 injections) and at the end of the column time life (377 days, 819 injections) both shift in classes and differences in height were indentified (data not shown). It is impossible to determine if this was due to sample deterioration or to changes in column performance, or to both (the former is perhaps less likely as the sample was stored freeze-dried at −20 °C and reconstituted immediately before analysis). However, the results clearly show that comparing chromatograms of a single sample subjected to RP-HPLC over a long period of time and of column use is difficult. To represent the variation graphically over time during which chromatograms were obtained,

Principal Component Analysis was carried out on the data set. A model with seven components explained 82.9% of the variance and time-related patterns were clearly evident in the score plots of the first two components. The occurrence of non-random patterns over time can be shown by several methods. Fig. 5 shows a lag plot of the average scores for each replicate injection for the first component (top) and an Exponentially Weighted Moving Average control chart for the mean scores (bottom). In a lag plot a measurement taken at time i (in this case the index of the replicate injection was used) is plotted against the same measurement at time $i − 1$. If no systematic patterns occur the points will be randomly scattered around the centre of the graph. Here a clear pattern was evident. The column was changed after injection 1 and injection 4 marks the first sample injected in the new column. Three groups of injections, with a single outlier (injection 63) are evident. This is confirmed in the EWMA control charts. Control charts are used evaluate if systematic pattern exist in a parameter (in this case the mean score for the first component of two replicate adjacent injections). The centre line shows the mean (μ) whilst the upper and lower confidence limits in the graph mark the region of the chart for the null hypothesis H_0: $m_i = \mu$ with $\alpha = 0.01$, where m_i is the mean for sample i. The chart shows the occurrence of systematic patterns with three groups of samples with relatively close means.

The time-series experiment showed the effect of the degradation of column performance on the results. Although the effect of sample storage and that of the performance of the column are confounded (and thus cannot be evaluated separately) it is clear from lag-plots and EWMA plots that there are discrete events which separate group of replicates which show only random variation (in Fig. 5 apparently groups of 100 injection behave in a similar way but are separated by gaps).

4. Conclusions

This is the first study in which a systematic approach was used to assess the contribution of different sources of variability to the precision of RP-HPLC profiles of soluble extracts used for the evaluation of cheese quality or authenticity. We feel that this study has several important consequences on the design and technical execution of experiments for the profiling of cheese proteolysis. We confirmed that a fast and objective approach based on accumulation of peaks in classes of retention using logistic weighting is at least as effective as visual matching and provided measures for average repeatability and reproducibility of the chromatograms of single samples. Due to the relatively poor reproducibility of small peaks, the use of information from the whole chromatogram in statistical analysis is probably of little value, and selection of peaks/classes which contribute most to systematic variability (due to differences in samples or treatments, rather than to random effects) is likely to decrease the power of statistical tests. After optimising the data pre-processing stage we used multivariate descriptive and inferential statistical techniques to estimate the contribution of different sources of variability to the results. The largest source was the treatment, followed by replicate cheese making trials. The latter includes sources of variability related to differences in milk quality, inconsistencies in the cheese making technique etc. Both extraction and chromatographic run had a small effect. Variability in replicate extracts may be due to both lack of sample homogeneity and to variability due to the different phases of extraction (correction of pH, extraction and precipitation time, freeze-drying, etc.). Unless there is a need to assess the effect of lack of sample homogeneity it is probably more convenient to pool replicate samples from a single cheese before extraction. This would also reduce the number of injections and the effect of

column deterioration. We did not assess the variability de to different operators or RP-HPLC equipment: this variability is probably important in inter-laboratory studies and should specifically be measured using gauge repeatability and reproducibility studies (NIST/SEMATECH, 2008).

Part of the variability in large experiments which require that a large number of injections are performed on a single column is due to degradation of column performance. Randomisation of samples injection is essential to avoid that this systematic effect becomes confounded with design variables. Although this problem may be circumvented by warping techniques (van Nederkassel, Daszykowski, Eilers, & Heyden, 2006), it is doubtful that this would substantially contribute to the improvement in reproducibility of the results: in fact, when visual matching was used, the variability of peak retention time was very small when compared to the variability of peak height/intensity. We showed that purely descriptive multivariate statistical techniques may lead to overestimation of true differences amongst samples and should be used with caution. On the other hand, even when there is a relatively high biological and technical variability, the use of appropriate statistical design and of inferential statistical techniques still allows identification of differences amongst treatments and exploration of relationships between design variables and proteolysis.

Acknowledgements

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Ripening of cheese: oxidation-reduction potential and calcium phosphate

Rennet-coagulated cheeses are generally ripened (matured) from about two weeks (e.g. mozzarella) to two or more years (e.g. parmigiano reggiano or extra-mature cheddar) during which the flavour and texture characteristic of the variety develop. Ripening usually involves changes to the microflora of the cheese, including death and lysis of the starter cells, development of an adventitious non-starter microflora and, in many cheeses, growth of a secondary microflora. The flavour of cheese curd immediately after manufacture is rather bland and it is during ripening that cheese flavour develops due to the production of a wide range of flavour compounds by a number of biochemical pathways.

Biochemical reactions through which cheese ripens are conventionally grouped into four major categories: (1) glycolysis of residual lactose and catabolism of lactate; (2) catabolism of citrate, which is very important in certain varieties; (3) lipolysis and the catabolism of free fatty acids; and (4) proteolysis and the catabolism of amino acids. Most aspects of the primary proteolytic, glycolytic and lipolytic pathways of ripening have been reviewed extensively over recent decades (e.g. Rank *et al.* 1985; Grappin *et al.* 1985; Fox 1989; Fox and Law 1991; Fox *et al.* 1990, 1993, 1994, 1995, 1996; Fox and McSweeney 1996, 1997; Christensen 1999; Sousa *et al.* 2001; Yvon and Rijnen 2001; Smit *et al.* 2002; Ardo 2004; Upadhyay *et al.* 2004; Collins *et al.* 2003, 2004; McSweeney and Fox 2004) and will not be discussed further here.

Increasing attention is being paid to the secondary pathways of ripening through which fatty acids and amino acids are converted to volatile flavour compounds. In addition to their direct role in cheese flavour, fatty acids are important precursors for the production of other volatile flavour compounds during ripening. As discussed by Collins *et al.* (2003, 2004), fatty acid esters are produced by reaction of fatty acids with an alcohol; ethyl esters are most common in cheese. Thioesters are formed by reaction of a fatty acid with a thiol compound formed through catabolism of methionine. Fatty acid lactones are cyclic compounds formed by the intramolecular esterification of hydroxyacids; γ- and δ-lactones contribute to the flavour of a number of cheese varieties. The most important class of volatile flavour compounds in blue cheese are *n*-methyl ketones (alkan-2-ones) which are produced from fatty acids by partial β-oxidation. *n*-Methyl ketones may be reduced to the corresponding secondary alcohols.

The products of proteolysis are medium and small-sized peptides and amino acids and these contribute to a brothy background flavour in many cheese varieties. In addition, short, hydrophobic peptides are bitter. Amino acids contribute directly to cheese flavour as some amino acids have tastes. However, it has now become apparent that accelerating proteolysis does

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Abstract

Rennet-coagulated cheeses are ripened for periods varying from two weeks to two or more years during which the flavour and texture characteristics of the variety develop. Microbial changes during ripening include a decline in starter organisms and an increase in numbers of adventitious non-starter bacteria and, often, the development of a desirable bacterial or fungal secondary flora. Biochemical changes during ripening have been studied in considerable detail and include lipolysis and proteolysis, together with the metabolism of residual lactose, and of lactate and citrate. Lipolysis and proteolysis result ultimately in the liberation of fatty acids and amino acids, respectively, which may be metabolised to a range of volatile flavour compounds. In particular, amino acid catabolism is of considerable importance to flavour development in cheddar cheese. The effects of a number of parameters including pH, moisture, NaCl and temperature on the rate of ripening have been studied thoroughly. Oxidation-reduction (redox) potential is another fundamental physicochemical parameter that has been shown to affect the rate of formation of a number of volatile flavour compounds *in vitro*. Methods have been developed to monitor redox potential during cheese manufacture and ripening, and avenues are being explored to alter ripening by controlling this parameter which has heretofore been largely ignored. In addition to the production of flavour compounds, cheese ripening usually involves considerable changes to its texture due to changes to moisture, cleavage of the casein matrix of the cheese by proteolysis and alteration to the calcium equilibrium. Recent work has shown that in cheeses such as cheddar, changes to the equilibrium between casein-bound and soluble calcium phosphate affect texture development which can be modified by alteration of this equilibrium.

Aust. J. Dairy Technol. **65**, 178-184

not necessarily accelerate flavour development (Upadhyay *et al.* 2003), suggesting that the production of amino acids is not the rate-limiting step in the development of cheese flavour. It is now generally believed that the principal role of proteolysis in the production of flavour compounds is the liberation of amino acids as precursors for a complex series of catabolic reactions that produce many important volatile flavour compounds. Amino acid catabolism was reviewed by Yvon and Rijnen (2001), Curtin and McSweeney (2004) and Ardo (2006) and appears to proceed principally *via* transaminase action catalysed by

aminotransferases which transfer the α-amino group from an amino acid to an α-keto acid (usually α-ketoglutarate) with the production of an amino acid and an α-keto acid corresponding to the amino acid substrate. The catabolism of some amino acids (e.g. production of volatile sulphur compounds from the side-chain of Met) may also be initiated by elimination reactions. In addition, decarboxylases remove the carboxylic acid group of amino acids to produce amines, some of which have adverse physiological effects. Decarboxylases may also act on α-keto acids to produce aldehydes, which in turn may be oxidised to carboxylic acids or reduced to primary alcohols. The α-amino group of amino acids may be removed by the action of deaminases, with the formation of a carboxylic acid and ammonia.

This review will focus principally on two aspects of cheese ripening: (i) oxidation-reduction potential and its influence on cheese ripening; and (ii) the equilibrium between soluble and casein-bound calcium phosphate and its role in the changes to cheese texture which occur during ripening.

Oxidation-reduction potential and cheese ripening

Basic considerations

Oxidation-reduction (redox) potential is an important physicochemical parameter that determines the tendency of systems to oxidise or reduce. The relationship between E_h, the potential at a standard hydrogen electrode, and the concentrations of the oxidised and reduced forms of compounds is described by the Nernst equation:

$$E_h = E_o + 2.3RT/nF \log [Ox] / [Red] \qquad (1)$$

Where E_o is the standard electrode potential which is characteristic for each half-reaction, R is the universal gas constant (8.314 J K^{-1} mol^{-1}), T is temperature (in Kelvin), F is the Faraday constant (96.5 kJ V^{-1} mol^{-1}), n is the number of electrons involved in redox reaction. At 25°C, the value of $2.3RT/nF$ is 59 mV for a one-electron transfer and Equation 1 can be simplified as follows:

$$E_h = E_o + 59 \log [Ox] / [Red] \qquad (2)$$

The E_h values are dependent on pH according to:

$$E_h = E_o + 59 \log [Ox] / [Red] - 59 \text{ pH} \qquad (3)$$

That is, the E_h will change by approximately 59 mV for every unit of pH change. However, applying the 59 mV correction factor is only valid for simple solutions. Deviation from the predicted values in biological systems is due to mixed potential or various redox couples. For such biological systems, pH correction must be determined experimentally by the measurement of E_h variation with pH (mV/pH unit) using acid or base addition at a certain temperature (Jacob 1970; Morris 2000). If E_h is plotted against pH, the slope of the resultant line will give the value which will represent the changes in E_h for unit change of pH (Brown 1980). E_h variation of 38 mV/pH unit has been determined experimentally at 26°C for sterilised skim milk (Cachon et al. 2002). Abraham et al. (2007) also used the same correction factor for camembert cheese. However, in a gelled medium (as a model food), a correction factor of 59 mV/ pH unit at 25°C has been used (Ouvry et al. 2001). For cheddar cheese, Kristoffersen et al. (1964) used a correction factor of

56.5 mV/pH unit at 10°C. Thus, it is important that when E_h values are quoted, the pH value at which they were measured should always be given together with the nature of the reference electrode and measurement temperature.

In general, potentials are referred to the hydrogen reference electrode and expressed as E_h at a defined pH and temperature. Since calomel or Ag/AgCl electrodes are used as a reference, the potential of the reference electrode (E_r) should added to the measured potential (E_m) as given in Equation 4:

$$E_h = E_m + E_r \qquad (4)$$

Typical electrode values for saturated calomel and Ag/AgCl reference electrodes are 244 mV and 199 mV, respectively, at 25°C (Skoog et al. 2004). It is necessary to consult the manufacturer's instructions for the electrode values at different temperatures. E_h (mV) of a solution at pH 7 measured using a Ag/AgCl reference electrode at 25°C can be calculated from:

$$E_h \text{ (pH 7)} = E_m + 199 + \alpha \text{ (pH}_m \text{ -7)} \qquad (5)$$

Where α is the pH correction factor, pH_m is the measured pH at E_m reading.

In principle, redox measurements should not require calibration unlike pH measurements. The condition of the measuring electrode is important for the redox values obtained. In practice, it may be necessary to check the system against standards of known potential. However, it has been concluded that solutions with a strong redox character should not be used to test electrodes since the measured redox values are independent of the condition of the surface and its preparation (Jacob 1974). In other studies, the redox electrodes were verified against each other in tap water (Abraham et al. 2007; Jeanson et al. 2009; Martin et al. 2009), or in 0.066 M pH 7 phosphate buffer (Jacob 1970; Lund et al. 1984), or in saturated KCl (Kristofersen et al. 1964), or in 3 M KCl (Topcu et al. 2008).

Measurement of redox potential

There are two commonly used methods for measuring the E_h of a system: by potentiometry or using redox dyes. The gain or loss of electrons induces changes in colour of redox dyes at their characteristic E_h. A well-known example of this is the methylene blue reduction test which is an old method to determine the microbial quality of raw milk based on the assumption that there is a general correlation between the reduction time of methylene blue and number of bacteria (Morris 2000). Dyes were also used to determine redox potential of cheddar cheese (Davis 1932). However, measurement of redox potential by dyes is not exact and requires a number of different dyes to obtain semi-quantitative measurement; furthermore many of these dyes may be toxic to the cells or may inhibit enzymes (Jenness and Patton 1959; Jacob 1970). The use of redox indicator dyes is now largely obsolete. E_h has usually been determined in most recent investigations (Cachon et al. 2002; Abraham et al. 2007; Topcu et al. 2008; Schreyer et al. 2008; Martin et al. 2009) with the aid of an inert metal (platinum, gold, or less often iridium or palladium) and calomel (mercury/mercurous chloride) or Ag/AgCl reference electrodes coupled with a sensitive potentiometer. Carbon, on which redox reactions are typically fast, may also be used as the inert electrode. The drawback of

carbon electrodes is that electron transfer is not always reversible resulting in non-reproducible potentials (Hutchins and Bachas 1997). The potentiometer itself must have a very high input impedance (>10^{12} Ω) in order to measure the very tiny voltages generated by the circuit without polarisation of electrodes (Jacob 1970; Skoog *et al.* 2004).

The measurement of E_h in biological systems such as milk and cheese is not always accomplished easily. Difficulty often is encountered in reproducing results between electrodes and in achieving a steady potential. Frequently, careful cleaning of electrodes according to manufacturer's instructions will overcome difficulties. According to Jacob (1974), polishing the metal electrode surface (e.g. by cerium oxide or aluminium oxide to restore the surface of platinum) has an important effect for reproducible redox measurement.

For potentiometric measurement of the redox potential of liquid food samples such as milk, inert metallic and reference or combined electrodes can be used directly (Brown 1980). For measurement of solid foods such as cheese with a low moisture content it is necessary to use a KCl salt bridge (KCl used as salt bridge because of the equal mobility of K^+ and Cl^- ions so junction potential is small enough to be neglected; Skoog *et al*. 2004) to help the E_h measurement (Topcu *et al.* 2008). Despite being a fundamental physicochemical parameter, redox potential has been neglected in cheese largely due to difficulties in methodology for its measurement. However, recently (Topcu *et al*. 2008) a novel self-sealing platinum electrode suitable for measurement of the redox potential of cheddar cheese was developed and has provided reproducible readings. Unlike pH electrodes, redox electrodes require a certain time to equilibrate depending on the nature of the sample. Redox measurement in cheddar cheese has a considerable time dependency because of low moisture content (equilibrium can take up to 2-3 days) (Topcu *et al*. 2008). However, Abraham *et al.* (2007) used microelectrodes to measure directly the redox potential of camembert cheese (full fat soft cheese) by inserting platinum and reference electrodes into cheese; redox equilibrium is achieved rapidly (between 10 min and 1 h) in this soft cheese.

Redox potential of milk

The E_h of milk in equilibrium with air is usually in the range + 250 to + 350 mV at pH 6.6 to 6.7 (Fox and McSweeney 1998) whereas values reported in the literature for cheddar cheese are -118 to -126 mV (Topcu *et al.* 2008) and -90 to -210 mV (Kristoffersen and Gould 1959), and -300 to -360 mV for camembert (Abraham *et al.* 2007). It has been stated that the major components of milk other than water, i.e. fat, lactose and protein, have relatively little direct effect on its redox potential; the principal redox couples in milk involve lactate-pyruvate, ascorbate and riboflavin (McCarthy and Singh 2009). However, thiol groups liberated on thermal denaturation of milk proteins can influence its redox potential (Schreyer *et al*. 2008).

According to Schreyer *et al.* (2008), redox potential could be used to assess organoleptic quality of milk; the authors demonstrated that the concentrations of copper and iron (ferrous) in milk are linked to its redox potential and the development of oxidised flavour.

Heat treatment of milk is associated with a decrease in redox potential related to loss of oxygen and, depending on severity of heating, exposure of thiol groups on protein denaturation (Vahcic *et al.* 1992; Bolduc *et al.* 1996). A study conducted by Calligaris *et al.* (2004) on the influence of different heat treatments on the antioxidant and pro-oxidant properties of cheese, reported that heat treatment of milk was associated with an increase in its antioxidant activity. This conclusion was in agreement with previous data from Taylor and Richardson (1980). In particular, thiol groups in milk, through their antioxidant capacity, reduce the redox potential in proportion to their concentration. Moreover, during severe heat treatment of milk, products of the Maillard reaction may also reduce the redox potential (Schreyer *et al.* 2008).

Oxygen can affect the measured redox potential of culture medium or a food by affecting the balance of oxidising and reducing agents present and by direct interaction with the redox electrode (Lund *et al.* 1984). Jeanson *et al.* (2009) demonstrated a very strong relationship between concentration of dissolved oxygen and the redox potential of milk. Under anaerobiosis, *Lactococcus lactis* immediately decreased the redox potential of milk and acidified only afterwards. In aerobic conditions, acidification and reduction of milk occurred at the same time (Jeanson *et al.* 2009). However, redox potential is considered to be a valuable measurement when the concentration of dissolved oxygen cannot be measured (Kjaergaard 1977; Lund *et al.* 1984).

Influence of redox potential on cheese ripening

Studies have shown that redox potential could be a useful tool in determining the type of micro-organisms that grow in dairy products (Keen 1972; Scarinci *et al.* 1994; Bolduc *et al.* 1996; Boucher *et al.* 2006; Morandi *et al.* 2006; Brasca *et al.* 2007). The interior of cheese is an anaerobic system, able to support growth only of obligatory or facultatively anaerobic microbes (Beresford *et al*. 2001). During bacterial growth, the redox potential of the medium decreases and the nature of the redox potential/time curve is determined by the type of micro-organism growing (Reichart *et al.* 2007; Brasca *et al.* 2007). A reduced environment is created in cheese by growth of starter bacteria by fermentation of residual lactose (Beresford *et al*. 2001) and by O_2 consumption by cell metabolism (Ouvry *et al.* 2001). Moreover, LAB produce reducing groups like sulphhydryl compounds (Ouvry *et al*. 2001). It seems that NSLAB growth is mainly responsible for the decrease in oxidation-reduction potential during ageing, and the oxidation-reduction potential of cheese plays a significant role in determining which species or strains are predominant at a given time in the ripening (Boucher *et al*. 2006). Oxidation-reduction potential at any given time affects both the production of microbiologically derived flavour compounds (i.e. the flavour compounds produced through the growth and metabolism of the starter and/or the NSLAB) (Boucher *et al*. 2006; Kieronczyk *et al.* 2006) and chemically derived flavour compounds in cheese (especially volatile sulphur compounds) (Manning 1979).

Redox potential contributes to the creation of conditions necessary for balanced flavour development (Cachon *et al.*

180

The Australian Journal of Dairy Technology. Vol. 65, No. 3 – November 2010

2002; Carrasco *et al.* 2005; Morandi *et al.* 2006). Good-quality cheeses have a low redox potential that provides the anaerobic conditions required for the flavour formation (Urbach 1995). A chemical relationship between redox potential changes in cheese and active thiol groups was found by Kristoffersen *et al.* (1967). Sulphydryl oxidase oxidises and protects the sulphydryl groups of proteins and this may affect the redox potential of cheese and the stability of thiol compounds and hence the quality and stability of cheese (Fox and Cogan 2004).

Changes in redox potential cause variations in cell regulation by different groups of enzymes and alter the production of a number of fermentation products (Keen 1972). Fatty acids, in particular polyunsaturated fatty acids, are susceptible to lipid oxidation. Almost uniquely for high fat foods, lipid oxidation is generally not of great significance during cheese ripening. This is partly due to the low level of polyunsaturated fatty acids in milk fat. However, the low redox potential of the cheese together with presence of natural antioxidants prevents or reduces this process (Singh *et al.* 2003).

A study by Kieronczyk *et al.* (2006) monitored *in vitro* the end products of amino acid catabolism for two different strains of *L. lactis* with different catabolism profiles for amino acids (Phe, Leu and Met), under both reducing and oxidising extracellular conditions. The results of this study indicated that reducing conditions (E_h » -200mV) stimulated the production of carboxylic and hydroxy acids from all three amino acid substrates and for both strains. On the contrary, oxidising conditions (E_h ³ +300mV) stimulated the production of aldehydes from Leu and Met. Results from the same study (Kieronczyk *et al.* 2006) indicated that both strains of *L. lactis* produced methanethiol that is readily oxidised to dimethyl disulphide and dimethyl trisulphide. These compounds are produced directly from methanethiol under the low redox potential of cheese (Singh *et al.* 2003).

In a recent study (Caldeo, unpublished), changes in redox potential during laboratory-scale cheese manufacture were determined. This parameter was measured during the simulated manufacture of cheddar, gouda, emmental and camembert cheese; data for cheddar are shown in Figure 1). Redox potential, pH and temperature were monitored by using a commercially available platinum electrode and an Ag/AgCl reference electrode, a combined electrode and a thermocouple, respectively, and all were connected to a data logger. Initially, the redox potential in milk was above 0 mV, due to diffusion of molecular oxygen from the atmosphere into the milk. Pasteurisation (63°C x 30 min) caused a slight decrease in redox potential, presumably due to loss of O_2 (Fox and McSweeney 1998). During the heat treatment the pH decreased because of the transfer of calcium and phosphate to the colloidal state with concomitant production of H^+ (Jenness and Patton 1959). After addition of the starter culture, redox potential decreased slowly with pH, as LAB metabolised lactose to lactate. During whey drainage a big drop in redox potential occurred and continued until the milling stage, while the pH decreased slowly to 5.4. A large increase in the redox potential occurred during milling when the curd mass was cut into small pieces exposed to the

air, while during the overnight pressing the redox potential decreased again to about -320 mV.

Work in the authors' laboratory (Caldeo and Topcu, unpublished) into changes in the redox potential of vacuum-sealed cheddar cheese during ripening has found that oxidation-reduction potential reaches equilibrium at *ca.* -300 mV relatively quickly after manufacture and that the oxidation-reduction potential remains at this value for much of ripening. However, measuring changes in the redox potential of intact cheddar cheeses early in ripening is hampered by the long equilibration time necessary for platinum electrodes coupled with Ag/AgCl reference electrodes placed in a KCl salt bridge. To overcome this problem and to measure changes in oxidation-reduction potential during pressing, recently, we used the approach of embedding electrodes into the cheese by placing them into the mould and pressing them together with the milled and salted cheese pieces. Miniature platinum electrodes were developed and commercially available miniature Ag/AgCl reference electrodes were placed in agar containing saturated KCl gelled in plastic tubes with holes drilled in the sides (Figure 2A). Preliminary results obtained using this approach (Figure 2C) suggest that oxidation-reduction potential reaches its equilibrium value of *ca.* -300 mV during overnight pressing, presumably due to continuing starter activity fermenting residual lactose to lactate and that there is relatively little change in this parameter during the early stages of ripening.

Future work in this area will concentrate on study of the ability of commercially available starter stains to reduce the oxidation-reduction potential of milk during simulated cheddar cheesemaking and to control the oxidation-reduction potential of cheddar cheese by the addition of foodgrade oxidising and
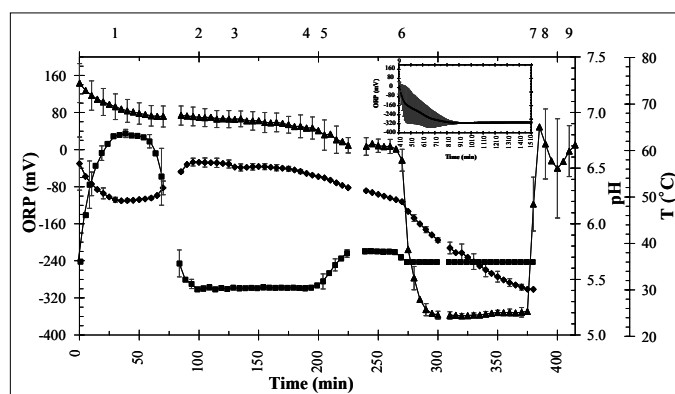

Figure 1: Changes in oxidation-reduction potential (ORP, ▲; direct measurement with platinum electrode-Ag/AgCl reference electrode and data recorded using a data logger and without correction of pH), pH (♦) and temperature (T, ■) as a function of time and cheesemaking steps (1, pasteurisation; 2, addition of starter DVS R604; 3, addition of CaCl$_2$ and coagulant; 4, cutting; 5, cooking; 6, whey drainage; 7, milling; 8, dry salting; 9, moulding) during the manufacture of laboratory-scale cheddar cheese. Inset graph shows changes in oxidation-reduction potential during overnight pressing. Data (uncorrected for pH and without reference to the hydrogen reference electrode in the case of oxidation-reduction potential) are means (± standard deviations) of three independent cheesemaking trials (V. Caldeo, unpublished).

reducing agents to the salted curd before pressing, and through the use of starters with differing abilities to reduce redox potential during manufacture.

Calcium equilibrium and its effect on cheese texture and functionality

Casein-bound calcium phosphate (CCP) is an important structural component in the *para*-casein network of cheese (Lucey and Fox 1993; Horne 1998). The role of calcium phosphate gradients in camembert cheese has been studied thoroughly (see McSweeney and Fox 2004). In addition, many studies have shown that changes in the Ca equilibrium (the distribution of calcium between CCP and serum phase of cheese) during ripening have a major impact on rheological and functional properties of other cheese varieties also (Lucey *et al*. 2005; O'Mahony *et al*. 2005; Choi *et al*. 2008; Brickley *et al*. 2009). Functional properties such as meltability and stretch are of particular importance in cheeses such as mozzarella and cheddar, which have applications as ingredient cheeses. Lucey and Fox (1993) reported that up to 80-90% of the total CCP content of milk is retained in cheddar cheese curd immediately after manufacture. These authors also suggested that the CCP in cheese plays a much more significant role in modulating its textural properties than the total Ca content. Lucey *et al*. (2003) suggested that electrostatic repulsion, cross-linking by CCP and attractive hydrophobic interactions are the main interactions between caseins that control cheese melting and the behaviour of cheeses at elevated temperatures. It is thought that solubilisation of CCP decreases the number of CCP cross-links between caseins and increases localised repulsion by exposing phosphoserine residues, decreasing the structural integrity of the *para*-casein matrix leading to increased meltability of cheese (Lucey *et al*. 2003). Dynamic small amplitude oscillatory rheometry is widely used by cheese researchers to study the rheological properties of cheese. When cheddar cheese is heated, the storage modulus decreases considerably and the loss tangent increases ≥40°C (Lucey *et al*. 2003), and these values decrease and increase respectively during the first month of ripening.

The distribution of total calcium between the soluble and insoluble forms in cheese during ripening can be quantified accurately by two methods. One method involves determination by atomic absorption spectrophotometry of the calcium concentration in the cheese aqueous phase ('cheese juice') extracted from grated cheese with the use of hydraulic pressure (Morris *et al*. 1988; Hassan *et al*. 2004). The other approach, based on the buffering action of CCP, uses an acid-base titration method to quantify the amount of calcium associated with the caseins in milk and cheese (Lucey and Fox 1993; Hassan *et al*. 2004; Choi *et al*. 2008). During acidification of a cheese slurry, there is a strong buffering peak at pH ~4.8 due to solubilisation of CCP (Lucey and Fox 1993; Hassan *et al*. 2004); this buffering peak is absent when the slurry is re-titrated upwards from a low
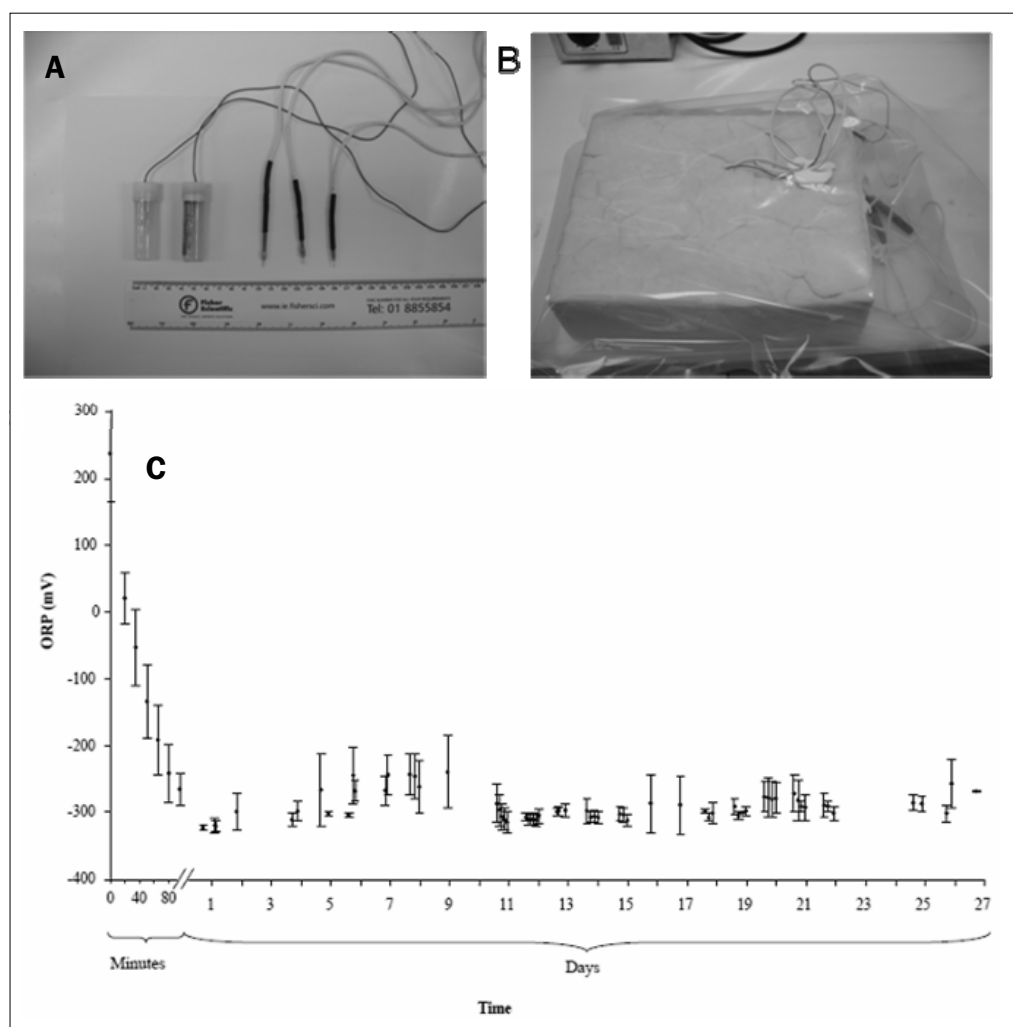

Figure 2: Apparatus used to measure the redox potential of cheddar cheese during ripening. A. Two perforated plastic tubes (left) containing miniature Ag/AgCl reference electrodes surrounded by KCl gelled in agar as an electrolyte and (right) three miniature platinum electrodes. B. After the electrodes were embedded into the cheese and pressing, the block (5 kg) of cheddar cheese was vacuum sealed. Leads from the electrodes were sealed as they passed through the vacuum packing bag. C. Changes in the oxidation-reduction potential (ORP) of pilot-scale cheddar cheese during pressing overnight at room temperature and the early stages of cheese ripening (8°C) (means ± standard deviations of three independent working electrodes in a single cheese block). (A. Topcu and V. Caldeo, unpublished). Oxidation-reduction potential: Direct measurement of oxidation-reduction potential by data-logger expressed as mV without referring to the hydrogen reference electrode, and without pH correction.

pH. By comparing the areas under the buffering curves obtained by titration to a low pH with acid and then in the opposite direction with alkali, it is possible to obtain a useful index of the amount of residual CCP present in cheese (Lucey and Fox 1993). Using both of these methods, Hassan *et al.* (2004) reported that the proportions of CCP in cheddar cheese decreased from ~73 to ~58% between day 1 and 4 months of ripening and that there were no significant differences between the two methods. The partial solubilisation of CCP in cheddar cheese appears to occur most rapidly during the first month of ripening (Hassan *et al.* 2004; O'Mahony *et al.* 2005; Lucey *et al.* 2005).

It is likely that the combined effects of both the solubilisation of CCP and of proteolysis determine the textural development of cheddar cheese during ripening (Lucey, *et al.* 2003). Lucey *et al.* (2005) reported that the rheological properties of cheddar cheese during early ripening were more significantly correlated to CCP content than with the extent of primary proteolysis in cheddar cheese (as monitored by levels of pH 4.6-soluble nitrogen). By inhibiting the chymosin-mediated hydrolysis of α_{S1}-casein at Phe$_{23}$-Phe$_{24}$ using pepstatin, O'Mahony *et al.* (2005) found the softening of cheddar cheese to be more highly correlated with the level of CCP during the first 21 days of ripening. However, plasmin-mediated proteolysis and secondary proteolytic events were not inhibited.

A number of researchers have altered the total Ca content of cheese in an effort to study its effect on cheese texture. Stretch and flow of mozzarella-type cheeses increase with a reduction in total Ca content when heated (Joshi *et al.* 2003; Sheehan and Guinee 2004). Increasing the total Ca content promotes casein-casein interactions through CCP bridging and charge neutralisation leading to increased hardness in cheese (Pastorino *et al.* 2003a). However, O'Mahony *et al.* (2006) developed a novel model system to alter the level of CCP in cheese rather than just its total Ca concentration using a synthetic cheddar cheese aqueous phase (SCCAP) to study the effects of CCP concentration on the rheological properties of cheddar cheese independent of proteolysis. In this study, the CCP content of four-month-old cheddar cheese slices was modified by incubating them in SCCAP solutions containing a range of calcium concentrations. The storage modulus at 70°C of the samples increased while the maximum loss tangent decreased with increasing level of CCP. Increasing the total Ca content of cheeses by addition of calcium chloride at the salting stage (Brickley *et al.* 2009) or post-manufacture (Pastorino *et al.* 2003a) has been found to increase hardness and decrease the meltability of cheese, presumably by shifting the equilibrium between soluble and casein-bound calcium phosphate towards the latter. Conversely, reducing the CCP content of cheddar cheese by addition of trisodium citrate (TSC) during manufacture (Brickley *et al.* 2009) has the effect of increasing the softness of cheese, due to the Ca sequestering ability of TSC removing Ca from CCP cross-links. Choi *et al.* (2008) made directly acidified cheeses using skim milk with EDTA added at a range of concentrations. The storage modulus decreased and loss tangent increased at 70°C with increasing EDTA concentration. The increased meltability of these cheeses was attributed to a reduction in insoluble Ca levels due to Ca

chelation by EDTA. Pastorino *et al.* (2003b) reported that post-manufacture injection of sodium citrate into cheddar cheese had no effect on CCP concentration.

It is well recognised that the pH and Ca content of cheese during manufacture are inter-related, as Ca is lost from the CCP as pH decreases (Lucey and Fox 1993). Preacidification of cheese milk results in a reduction in the calcium content of cheese, a less dense protein matrix, and higher meltability (McMahon *et al.* 2005). Choi *et al.* (2008) studied the effect of pH on CCP levels in directly acidified cheeses with the same gross composition made from skim milk at various pH values (6.0, 5.8, 5.6, 5.4). It was found that CCP levels decreased with decreasing pH, leading to increased meltability. Lee *et al.* (2005) found that cheddar cheeses with a pH <5.0 had limited meltability compared to cheeses with the same gross composition at higher pH, even though the low pH cheeses had much lower CCP levels. As the pH of cheese is reduced towards the isoelectric point of caseins (pH 4.6), there is a contraction of the casein matrix brought about by increasing hydrophobic interactions between caseins (Everett and Auty 2008). This suggests that the modulation of cheese texture due to CCP level is highly dependent on pH. Evidently, manipulating the Ca equilibrium in cheese is a useful method for producing cheeses with tailored functional properties, which is of interest commercially for ingredient applications.

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