Growth of *E. coli* in reduced salt cheddar cheese

By

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DECLARATION

I declare that the dissertation herewith submitted for the degree MSc Food Science at the University of Pretoria has not previously been submitted by me for a degree at any other university or institution of higher education.

Richard James Beardsley

March 2017

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DEDICATION

This work is dedicated to my parents, Cheryl and Robert, for always believing in me and for supporting me throughout the duration of my studies. I am incredibly thankful for you both.

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ABSTRACT

Growth of *E. coli* in reduced salt cheddar cheese

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Department: Food Science

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Modern day consumers have become more health conscious and there has been a movement towards reducing sodium intake in their diets. This is due to the risk of the development of hypertension and cardiovascular diseases, as well as other diet related non-communicable diseases associated with excessive sodium intake. Cheddar cheese is one of the most popular cheeses consumed globally and has a relatively high sodium content (2% w/w). A possible way of reducing the sodium content is by making use of replacement salts such as KCl and $MgCl_2$. Partial substitution of NaCl with KCl and $MgCl_2$ has been shown to be possible without compromising on key quality parameters, however very little work has been conducted on the effects of partial salt replacement on the growth of pathogenic bacteria such as *E. coli*. The first phase of the study focused on replicating the model employed by Grummer & Schoenfuss (2011), to determine equivalent water activities amongst the cheese samples made with different partial salt replacers. The model was adjusted accordingly, and any deviations were noted and taken into account for the second phase of the study. The second phase of the study involved the manufacture of reduced salt cheeses and their inoculation with three different serotypes of non-O157:H7 shiga toxin-producing *E. coli*. The effect that the alternative sources

of salt, as well as reduced NaCl levels had on the growth of *E. coli* were studied. Physicochemical analyses for the water activity, moisture content and salt-in-moisture (S/M) content of all cheeses were carried out. All three *E. coli* serotypes were able to grow at water activities greater than 0.95, irrespective of the type of salt treatment used. Even though the Full NaCl control cheeses (2% NaCl) were salted to bring about water activities of less than 0.95, *E. coli* was still able to grow and increased for 14 days. No differences were found between *E. coli* growth in the different salt treated cheeses. A correlation was found between the S/M ratio and *E. coli* growth, with a higher S/M ratio resulting in less *E. coli* growth. Although water activity is a critical parameter with respect to the inhibition of *E. coli* growth in cheddar cheese, the S/M ratio was found to be just as crucial a consideration. A combination of hurdle technology is therefore required for ensuring the safety of cheddar cheese products. Salt content, in addition to low pH and low storage temperature work synergistically to exclude the growth of pathogenic bacteria, however much care must be taken when reducing the salt content of cheddar cheese. Reduction of the salt content may interfere with the balance of inhibition of other currently non-problematic bacteria, which may result in the necessity for the replacement of its antimicrobial action. It is therefore apparent that further research on the effects of salt reduction as well as its partial replacement on the growth of *E. coli* and other pathogens is required, before the implementation of potential salt reduction regulations in cheese products is considered.

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CHAPTER 1: INTRODUCTION AND PROBLEM STATEMENT

The manufacture of cheese originally evolved from a need to extend the shelf life as well as to conserve the nutritional components of milk. These activities were achieved through either acid production and/or whey removal (Beresford, 2001). Cheddar cheese originated in the town of Cheddar in England during the 16th century, and has since become one of the most widely consumed cheeses globally (Robinson, 1995). Cheddar cheese can be prepared either by a traditional cheddaring process or by any other process that results in a finished cheese product with identical physical and chemical properties (D'amico, et al., 2010). The process often used is the stirred-curd method, which is convenient to implement on a small scale.

Salt is a vital ingredient in cheese manufacture as it reduces the water activity (a_w) and acts as a preservative by minimising spoilage and preventing the growth of pathogenic microorganisms, contributing to the desired flavour of the cheese, as well as affecting the texture and body of the cheese and biochemical changes that occur during ripening (Guinee, 2004). Salt, acting together with both the pH and calcium level, affects the extent of *para*-casein hydration in the cheese. This consequently affects the water-binding capacity of the casein matrix, as well as its tendency for syneresis, rheological and textural characteristics, and cooking properties (Guinee, 2004). The addition of sodium chloride (NaCl) to cheese has a preservative function by lowering the a_w and thereby restricting microbial growth (Taormina, 2010). Salt content and more specifically salt-in-moisture (S/M) level is one of the most important factors that affect the overall acceptability of cheese products and it is therefore considered to be a key contributor to cheese diversity, quality and safety (Guinee, et al., 2013).

In recent years, consumers have become more health conscious and there has been a drive towards reducing the sodium intake in their diets. Excessive sodium intake has been linked to the development of hypertension and cardiovascular disease (Taormina, 2010). According to Guinee (2004) the average sodium intake in the modern western diet is excessive, being two to three times more than the recommended dietary intake (RDA for NaCl being 6g/day). The World Health Organization (WHO) recommends a reduction of sodium intake in adults to <2g/day (5g salt/day) with the primary goal to reduce the occurrence of high blood pressure and other associated diseases (WHO, 2012) . In 2013, the Department of Health announced new legislation with respect to the amount of sodium allowed in processed food products in an

effort to reduce the salt intake of the population, however, cheese was not included in the legislation (Department of Health, 2012). Although cheese only comprises a relatively small portion of their diets, many consumers are conscious of the high salt content of cheese and the demand for cheeses with reduced sodium content has grown.

Coliform bacteria such as *Escherichia coli* (*E. coli*) can cause spoilage of cheese products by inducing a condition known as early blowing (Robinson, 1981). Strains of *E. coli* are also responsible for numerous gastrointestinal infections when consumed. Sufficient pasteurisation of milk prior to cheese manufacture should destroy all *E. coli* organisms present in the milk. The presence of *E. coli* in cheese would indicate that the pasteurisation process is not running efficiently, or alternatively that HACCP procedures are not being followed strictly enough due to the cheese becoming contaminated with *E. coli* post pasteurisation. The elimination or reduction of salt levels from cheese without sufficient research into its implications may lead to enhanced pathogenic growth and survival as well as accelerated spoilage of the product (Taormina, 2010).

Although the issue of reducing the salt content of food may seem a simple one to address, when focussing on reducing the salt content of complex food products like cheese, many parameters and considerations need to be taken into account (Paquin, 2013). Due to the wide range of uses of salt in cheese, much care must be taken when reducing the salt content in terms of monitoring its effects on the functional and safety properties of the cheese product. When the salt content (NaCl concentration) of cheese is just simply reduced without the addition of an alternative, a number of undesirable changes occur. There is a significant increase in proteolysis, water activity, acidity and bitterness, while there is also a decrease in firmness and saltiness (KatsiariI, et al., 2001). Research has been conducted into the possible use of alternatives for NaCl as a source of salt in cheese. KCl, $MgCl_2$ and $CaCl_2$ have been shown to be the most widely used and successful partial replacements for NaCl as the cheese maintains the desired salty taste, and has enzymatic and microbial stability (Reddy & Marth, 1991; KatsiariI, et al., 2001). A model has been developed by Grummer and Schoenfuss (2011) that can assist in determining the correct balance of NaCl and partial replacement salt to impart an equivalent a_w among cheeses made by using different partial replacement salts.

This project investigated the effect that alternative sources of salt in cheddar cheese (partial replacers KCl, $MgCl_2$), as well as reducing the NaCl levels had on the growth of *E. coli*. The

model developed by Grummer and Schoenfuss (2011) was used to determine equivalent a_w's amongst the cheese samples made with different partial salt replacers.

CHAPTER 2: LITERATURE REVIEW

2.1 FUNCTIONAL PROPERTIES OF SODIUM CHLORIDE IN CHEESE

Salt, which is the common name for sodium chloride (NaCl), is composed of approximately 40% sodium and 60% chloride (w/w) (Paquin, 2013). Salt in the form of NaCl is an essential ingredient in cheese manufacture and is responsible for inferring a range of functional properties on the cheese product. Such functional properties include the role it plays during the biochemical reactions of glycolysis, lipolysis and proteolysis, its effect on textural and rheological properties, as well as its effect on the flavour and aroma of the cheese, as is illustrated in Figure 2.1 (Guinee & Fox, 2004). The most important role of salt in cheese, is however to act as a preservative and ensure the safety of the product. It acts by killing or limiting the growth of foodborne pathogens and spoilage organisms by decreasing a_w to a level that excludes bacterial growth (Doyle & Glass, 2010).

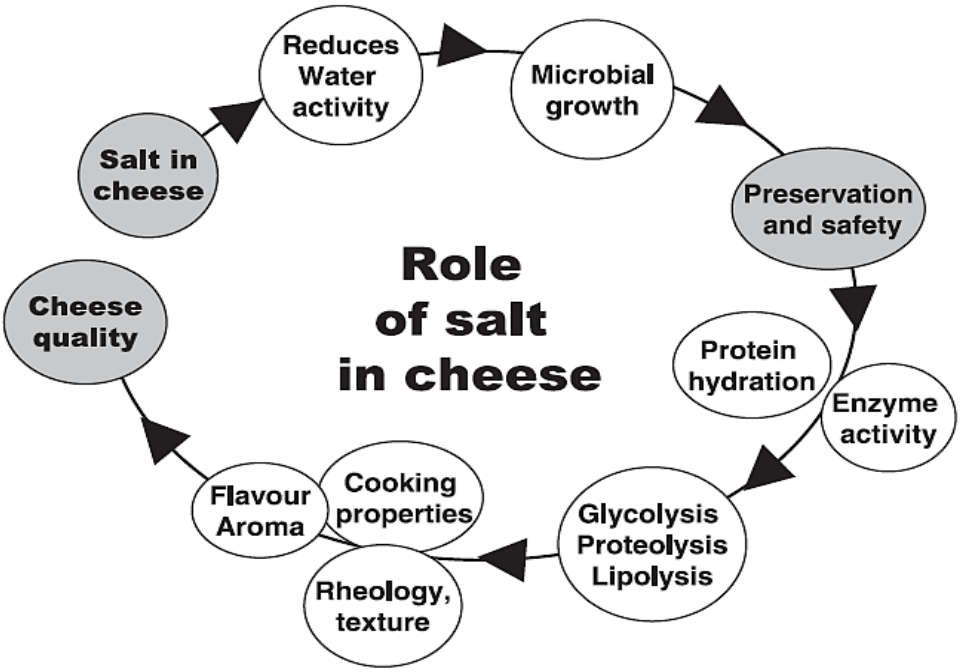


Figure 2.1: The various roles that salt plays during cheese manufacture (Guinee & Fox, 2004)

The primary mode of preservation by NaCl is its ability to lower the a_w to a level that excludes the growth of spoilage and pathogenic microorganisms, however Taormina (2010) indicated that it also infers certain bacteriostatic properties due to the direct toxic effect of the Cl^- ion,

by removing oxygen from the medium as well as interfering with the action of proteolytic enzymes.

NaCl imparts a preservative effect on the cheese by exerting a drying effect, drawing water out of cells of both the cheese and microorganisms via the process of osmosis (Doyle & Glass, 2010). Water activity in foods is dependent on two factors: the level of moisture present, as well as the concentration of solutes of low molecular weight. It is therefore evident that salt has a preservative effect not only by reducing the a_w of the cheese, but also by disturbing the mass transfer through the microbial cell membranes, as well as by releasing extracellular-bound enzymes (McMahon, 2010; Cruz, et al., 2011).

Salt also has a direct effect on starter bacteria activity as it helps to control the growth and survival of the desirable starter lactic acid bacteria (LAB) as well as secondary microorganisms known as non-starter lactic acid bacteria (NSLAB) which are responsible for flavour and aroma production during storage and maturation (McMahon, et al., 2014) . By reducing the salt content, growth of starter bacteria is stimulated, allowing them to dominate over NSLAB for a longer amount of time than in cheeses salted with a regular amount of salt (Ardo, et al., 2013). The reduction in salt content results in a higher a_w, which means that several bacterial species that are inhibited in cheese salted with a regular amount of salt are able to grow. Reducing the salt content of cheese without compromising product quality therefore requires a high level of control by the cheese-maker over the microbial composition of the milk and cheese, including starter as well as non-starter LAB and potentially detrimental microorganisms too (Skeie, et al., 2013). Studies have also shown that decreasing the salt content in cheese results in an increase in chymosin activity on casein, which increases the amount of peptides released, including bitter peptides which have a negative effect on the consumer acceptability of the cheese product (Ardo, et al., 2013).

E. coli require a minimum a_w of 0.95 to grow (Marshall, et al., 1971; Doyle & Glass, 2010). Hence, in order to preclude the growth of *E. coli* in cheese, salt should be added in a concentration large enough to impart the reduction of a_w in the food system to below that of 0.95.

2.2 POTASSIUM CHLORIDE AND OTHER MINERAL SALT REPLACERS

A number of partial replacement salts have been investigated in an attempt to produce cheeses with reduced sodium content due to the negative perceptions regarding the adverse health effects caused by excessive NaCl intake (Grummer, et al., 2012). The function of these mineral salt replacers is to impart the desired salty taste as well as to ensure enzymatic and microbial stability of the cheese by maintaining a a_w that precludes microbial growth (Grummer, et al., 2012). The most widely investigated partial replacement salt is potassium chloride (Hoffmann, 2013). Like sodium, potassium is also a Group 1 metallic element and its metal halide salt, potassium chloride (KCl), is odourless and has a white vitreous crystal appearance (Hoffmann, 2013). KCl is an approved food additive and flavour enhancer in the European Union, as well as being approved as an additive in cheese manufacture by Codex Alimentarius (CODEX STAN 283, 1978; Hoffmann, 2013). Magnesium chloride ($MgCl_2$) has also been investigated as a potential partial salt replacer, but with less success than KCl.

Partial substitution of NaCl with KCl seems to be possible without compromising on key quality parameters. Investigations have shown that substituting up to 30% of the NaCl may be possible for some cheddar-style cheeses, with a maximum of 0.5-0.7% KCl in the cheese (Hoffmann, 2013). Most studies indicated that the concentration of the partial replacers were not used in as high concentration as necessary to impart the same reduction in a_w that was imparted by the use of NaCl. The reason for using a lowered concentration is because when used in higher concentrations, an undesirable bitter, metallic taste was detected in the cheese (Guinee & Fox, 2004). However, in order to compare the effectiveness of different partial salt replacers, equivalent a_w's need to be reached in the various cheese samples. Grummer & Schoenfuss (2011) developed a model system that can be used to calculate the exact concentration of NaCl and partial salt replacer required to achieve the equivalent a_w to that of the Full NaCl control cheese. The model is based on Raoult's Law and states that:

$$a_w = \gamma_s X_{water} = \gamma_s \frac{n_{water}}{n_{water} + n_{solutes}},$$

Where: a_w = water activity

: γ_s = activity coefficient

: X = mole fraction

: n = number of moles

Shoenfuss (2010) indicated that the use of KCl as a partial replacer, when used in conjunction with NaCl produced a final pH in cheddar cheese that was most similar to the pH produced by using just NaCl, as can be seen in Table 2.1.

Table 2.1: Effect that different replacer salts have on the final pH of cheddar cheese (Schoenfuss, 2010)

	pH
NaCl	5.17
NaCl + KCl	5.08
NaCl + modified KCl	4.96
NaCl + $MgCl_2$	4.95
NaCl + $CaCl_2$	4.97

KCl has a molecular weight of 74 g/mol, which is the closest to the that of NaCl (58g/mol), with $MgCl_2$ having a molecular weight of 94g/mol. The molecular weight of the partial salt replacer has a direct effect on a_w due to the phenomenon of molality (Grummer & Schoenfuss, 2011). At equivalent mass, solutes with lower molecular mass contain more molecules than those with higher molecular weights, the former resulting in a greater a_w -lowering effect (Grummer & Schoenfuss, 2011). NaCl has the lowest molecular weight when compared to the other salts and therefore has the greatest a_w -lowering effect. When salts dissociate, they split into the individual ions that they are composed of (Schoenfuss, 2010). Hence, NaCl dissociates into individual Na^+ and Cl^- ions. Part of the preservative effect of the salts can be attributed to the action of the Cl^- ion (Maris, 1995). Chlorine is an electronegative ion, and therefore has the ability to oxidise peptide links and denature proteins. Chlorine dioxide has an effect on the

permeability of the external membrane of *E. coli* by means of a primary lethal phenomenon which results in a substantial leakage of K^+ ions. Sub-lethal doses of chlorine dioxide inhibit cellular respiration due to a nonspecific oxidising effect (Maris, 1995).

2.3 THE IMPORTANCE OF WATER ACTIVITY IN CHEESE

Water in foods is dependent on interactions which not only alter the properties of the water itself, but also the properties of the components with which it interacts (Seow, et al., 1988). Many different water related criteria have been studied with respect to the stability and safety of food products, including water content, solute concentration, osmotic pressure, equilibrium relative humidity (ERH) and a_w (Troller & Christian, 1978). Water content as well as solute concentration do not give us insight into the specific properties of the water in the food system. The other criteria mentioned are measurements of related colligative or osmotic properties and are generally good indicators of the availability of water to participate in reactions. However, not all of these criteria are equally appropriate or useful when studying the availability of water in certain food systems - one can only make use of osmotic pressure in systems that contain membranes of suitable permeability. The use of ERH measurements strictly refer to the equilibrium between the food product and the atmosphere with which the food product interacts, not specifically to the food product itself (Troller & Christian, 1978).

The primary factors that influence the ERH are the interactions between water molecules and solutes in the food system (Grummer & Schoenfuss, 2011). Due to the complex nature of cheese and because of the various processes that it undergoes during manufacture such as heating and drying, it can be considered a multi-component, multi-phasic food stuff (Grummer & Schoenfuss, 2011). In many situations in the food industry, the water in the food system may not be in equilibrium with the water vapour in the atmosphere surrounding the food product. It is for these reasons that a_w is the most useful expression for understanding the requirements and relationship between microbial growth and enzyme activity with water in food systems (Scott, 1957; Troller & Christian, 1978).

According to Potter (1998) a_w can be defined in a number of ways: qualitatively it is a measure of the free, unbound water in a system that is available to support biological and chemical reactions. Essentially it is this a_w and not the water content which bacteria, enzymes and other chemical reactants encounter and interact with at the micro-environmental level in food

systems. Potter and Hotchkiss (1998) further defines a_w, in accordance with Raoult's Law as: quantitatively it is equal to the ratio of the vapour pressure in the food system to that of pure water.

$$a_w = \frac{P}{Po}$$

Where: a_w = water activity

: P = partial pressure of water in the food at a given temperature

: P_o = vapour pressure of pure water at the same temperature

When a salt dissolves in water, it interacts with the solvent through dipolar, ionic and hydrogen bonds. This consequently results in a decrease in the escaping tendency or free energy of the water and hence there is a lowering in the a_w (Bell & Labuza, 2000).

2.4 THE RELATIONSHIP BETWEEN WATER ACTIVITY AND WATER CONTENT

As mentioned previously, Raoult's Law and the model developed by Grummer and Schoenfuss (2011) was used to manufacture cheddar cheeses of varying water activities. These calculations are also based on an assumed moisture content in the final cheese product of 40%. This was based on pilot studies, data from analyses of commercial cheddar cheese as well as moisture sorption isotherms. Moisture sorption isotherms plot the relationship between moisture content and a_w under conditions of equilibrium and as such are useful in predicting the a_w in a food system at a certain moisture content and vice versa, as can be seen in Figure 2.2. These isotherms are plotted by measuring the a_w of a food product by certain objective methods at varying moisture contents. Isotherms are not only useful in showing at what specific water content (moisture level) certain desirable or undesirable a_w levels are achieved but also how significant an effect a particular change in water content of the food system will have on the subsequent a_w (Troller & Christian, 1978).

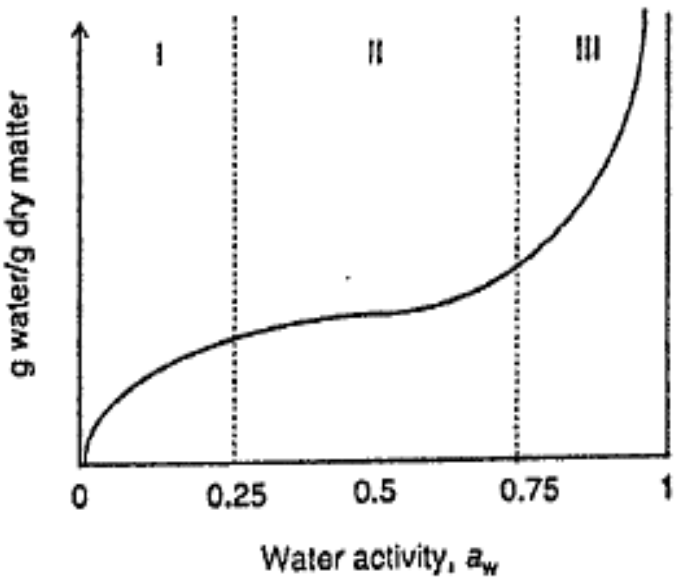


Figure 2.2: A generalised moisture sorption isotherm of a typical food material (Guinee & Fox, 2004)

In the above figure, region I indicates monolayer water that is tightly bound to polar groups of macromolecules such as proteins and carbohydrates in the food system (Guinee & Fox, 2004). The water that is found in this monolayer is very stable and is thought to behave as part of the solid food, thus being non-freezable at any temperature (Duckworth, 1974).

Region II comprises a combination of both monolayer water and multilayer water. The water in this region of the isotherm is less tightly bound than in the monolayer of Region I. This is due to adsorption in the multilayer as well as interactions between soluble components and insoluble solids present in this region (Duckworth, 1974).

Region III contains a mixture of bulk phase water as well as monolayer and multilayer water (Guinee & Fox, 2004). This bulk phase water is also referred to as free water, even though it is mechanically trapped in the food system, it is only exposed to very weak restrictive forces. The steepness of the curve in Region III is indicative of these weak interactions (Duckworth, 1974).

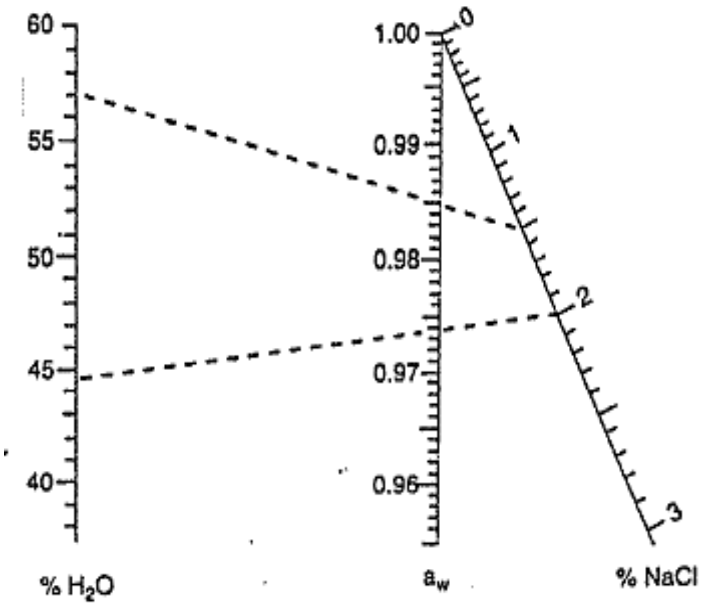


Figure 2.3: Nomograph for the approximation of water activity of fresh cheese from known moisture and salt percentages (Marcos & Esteban, 1982)

The nomograph depicted in Figure 2.3 is also a useful tool for predicting the a_w of fresh cheese at varying salt and moisture concentrations. The a_w of foods are dependent on two primary factors: the moisture content of the food as well as the concentration of solutes of low molecular mass. The a_w of a young cheddar cheese is almost exclusively determined based on the concentration of NaCl in the aqueous phase (Guinee & Fox, 2004). The nomograph was constructed by making use of the following equation, with relates a_w to the molality of NaCl:

$$\text{Water activity } (a_w) = 1 - 0.033\ [NaCl_m] = 1 - 0.00565\ [NaCl]$$

The example illustrated in the graph (Figure 2.3) shows that a moisture content of 44.5% (w/w) and a salt content of 2% would result in a a_w of 0.974 (Marcos & Esteban, 1982; Guinee & Fox, 2004).

2.5 THE RELATIONSHIP BETWEEN WATER ACTIVITY AND MICROBIAL GROWTH

E. coli require a minimum a_w of 0.95 to grow (Marshall, et al., 1971; Doyle & Glass, 2010). By controlling the a_w, the cheesemaker can minimise bacterial growth, due to the effect that this parameter has on the various growth cycle phases of the bacteria namely: germination time,

growth rate, size of the stationary population, as well as the death rate (Troller & Christian, 1978). The parameters that will be studied in this project will be the rate of growth, the extent of growth and presence or absence of growth of *E. coli* at varying a_w's. In a complex food system such as cheese, it is difficult to say whether the reduction of the a_w alone is responsible for the inhibition of *E. coli* growth, and therefore ensuring the safety of the product. The addition of salt, which reduces the a_w considerably, as well as the low pH of the final cheese product may work together to impart a synergistic inhibition of microbial growth (Troller & Christian, 1978).

2.6 *ESCHERICHIA COLI*

E. coli are Gram negative rods which belong to the family of microorganisms known as Enterobacteriaceae (Neidhardt, et al., 1987). They are asporogenous, aerobic coliforms and can contain numerous flagella when motile (Foster, et al., 1957). *E. coli* have complex cell walls, consisting of about 10% peptidoglycan and the rest consisting of an outer membrane of phospholipids, proteins and lipopolysaccharides (LPS), as can be seen in Figure 2.4 below. (Neidhardt, et al., 1987).

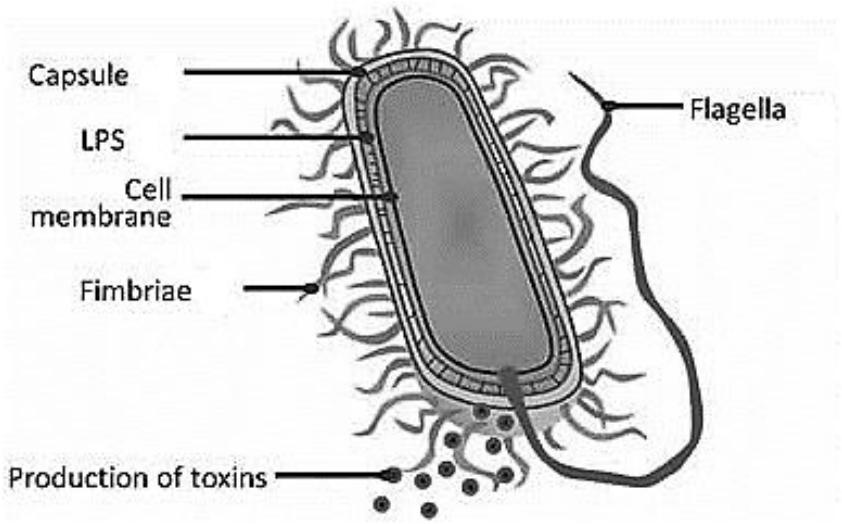


Figure 2.4: The *E. coli* bacterium (University of Montreal, 2004) (Accessed 22 April 2016)

E. coli are found in the small intestine of most warm-blooded animals, hence the use of the term 'coliforms' to describe them (Robinson, 1981). Its presence in milk is therefore an indication of faecal contamination and is also an indication of mastitis in cows. *E. coli* is

responsible for spoiling cheese by inducing a condition known as 'early blowing'. This is due to its ability to ferment lactose rapidly and the consequent production of lactic acid, ethanol, acetic and succinic acids, carbon dioxide and hydrogen (Foster, et al., 1957; Walstra, et al., 1999).

E. coli are not only undesirable due to their potential to spoil cheese, they can also be pathogenic and can cause serious illness in humans if consumed. There are a number of strains of pathogenic *E. coli*. Enteropathogenic strains are responsible for causing gastroenteritis, along with fever and nausea (Zottola & Smith, 1991).The other classes of pathogenic *E. coli* include enterohaemorrhagic (EHEC), enterotoxigenic (ETEC) and enteroinvasive (EIEC). *E. coli* O157:H7 is a particularly virulent strain of enterohaemorrhagic *E. coli* and is characterised by its ability to produce verotoxins (Marek, et al., 2004). Epidemiological studies have shown that dairy cattle are the primary reservoir of *E. coli* O157:H7, with subsequent faecal contamination being the major route of transmission of the pathogen to humans (Marek, et al., 2004).

Shiga toxin-producing *E. coli* (STEC) are a class of EHEC and are a group of highly pathogenic bacteria, characterised by their ability to produce one or more shiga toxins (Stx1 and Stx2) (Elhadidy & Mohammed, 2012). These shiga toxins or verotoxins consist of powerful phage-encoded cytotoxins that are responsible for tissue damage in humans and can cause severe intestinal damage through their ability to attach to the epithelial cells in the digestive tract (Elhadidy & Mohammed, 2012). STEC are responsible for causing a number of debilitating diseases including haemolytic-uremic syndrome (HUS) and hemorrhagic colitis (HC) (Stephan, et al., 2008). HUS is characterised by a number of symptoms including: the breakdown of the red blood cells (haemolytic anemia), low platelet count (thrombocytopenia) and uremia (acute kidney failure) (Noris & Remuzzi, 2005). Children are more susceptible to infection and the disease generally manifests in the form of watery or bloody diarrhoea (Noris & Remuzzi, 2005). Most cases of STEC-based infections have been attributed to strains of STEC O157:H7, however there is increasing awareness of the danger of non-O157:H7 STEC strains in the proliferation of HUS, HC and other serious gastrointestinal diseases (Stephan, et al., 2008).

STEC are of great concern in cheddar cheeses made with altered compositions, i.e. cheeses salted with alternative salts (Oh, et al., 2014). In a study to test the effect of various microbial

hurdles on the growth of STEC in cheddar cheese, it was found that pH was the hurdle primarily responsible for controlling STEC growth. Both NaCl as well as lactic acid concentration were found to have insignificant effects on the growth of STEC in cheddar cheese (Oh, et al., 2014).

Salt has a lowering effect on the a_w of the cheese, and together with milk heat treatment (pasteurisation, thermisation etc.), starter activity, cheese pH and the ripening or storage temperature, help to control microbial growth in cheese (Labrie, et al., 2014). Reducing the salt content of cheese will have an effect on the microbial behaviour, including that of pathogenic bacteria that may be present in the cheese. As most pathogens are destroyed by heat treatment, the behaviour of pathogens in cheese is a major concern in cheeses produced from unpasteurised milk (raw milk cheeses) and situations in which there is a possibility of post-pasteurisation contamination (Labrie, et al., 2014). The temperatures that the milk and consequently the curd are processed at during cheese manufacture are not sufficient to destroy pathogenic microorganisms (Zottola & Smith, 1991). *E. coli* are however heat sensitive and are destroyed with sufficient heat treatment, i.e. pasteurisation (Robinson, 1981). Therefore it is most probable that the presence of *E. coli* in the cheese product is due to contamination in the stages following pasteurisation, either due to poor hygiene from the cheese-makers, or through the use of contaminated equipment, as can be seen in Figure 2.5. There have also been cases where malfunctioning filtration systems responsible for sterilising river water to be used in the cleaning of the cheese making equipment have been suspected as sources of contamination (Zottola & Smith, 1991). Tests can be carried out on the finished product, or samples can be taken at various stages throughout processing (post pasteurisation) in an attempt to isolate the stage in the production line where contamination is occurring. It has also been documented that if the raw milk contains an excessive number of coliform bacteria, a few viable cells may be able to survive the pasteurisation process. This is, however, unlikely and is avoided by using high quality milk from a properly controlled milk supply (Robinson, 1981). In extreme cases, certain pathogenic bacterial species have been shown to have the ability to genetically transfer virulence factors to non-pathogenic bacteria, which can potentially lead to the presence of antibiotic resistant strains that are capable of surviving the pasteurisation process (Johnson, et al., 1990).

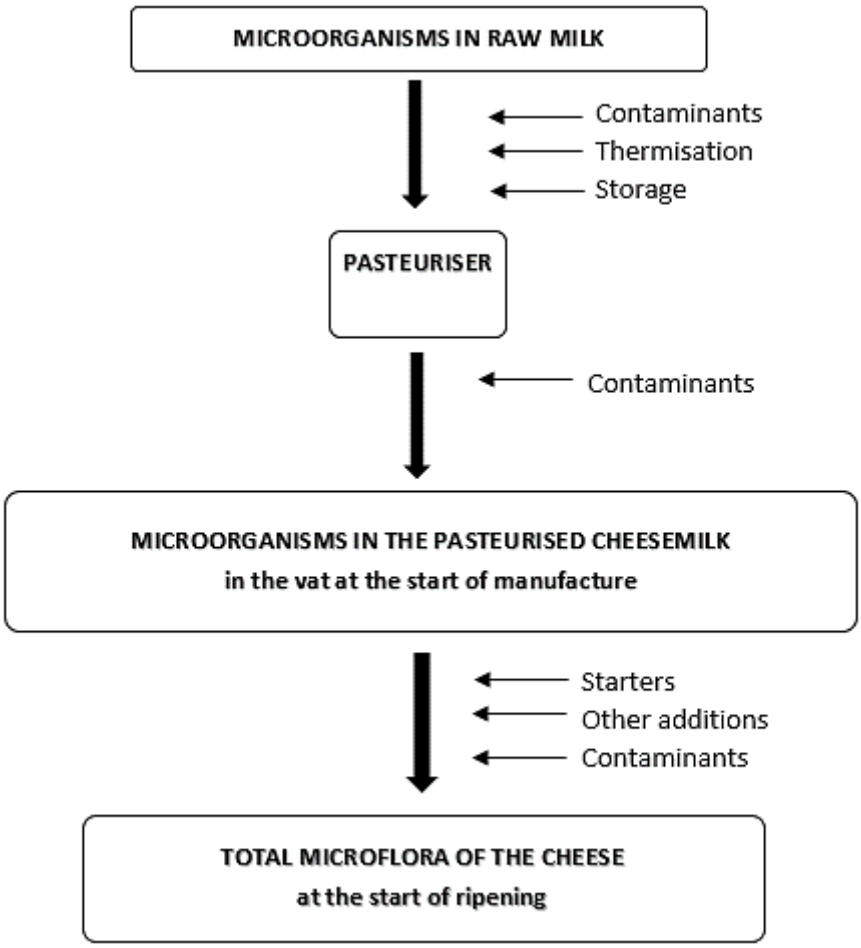


Figure 2.5: Flow diagram indicating possible contributors to total microflora in cheese manufacture (Martley & Crow, 1993)

2.7 STARTER AND NON-STARTER LACTIC ACID BACTERIA

Starter cultures are an essential additive in cheese-making and play a very important role in the conversion of milk to a cheese-like product. Starter cultures typically comprise a mixture of various lactic acid producing bacteria, whose primary role is to lower the pH of the milk through lactic acid fermentation to assist with the coagulation of the curd. Depending on the cheese variety, starter LAB are generally capable of producing sufficient acid to lower the pH below 5.3 when milk is heated between 30-37°C over a 6 hour period (Beresford & Williams, 2004). LAB are characterized by being Gram-positive, catalase-negative, asporogenous, and facultative anaerobic (Kandler & Weiss, 1986; Settanni & Moschetti, 2010). These bacteria constitute a heterogeneous group of genera which share common physiological and biochemical features, the most universal being their capacity to ferment sugars primarily into

lactic acid via homo- or heterofermentative metabolism (Salminen & von Wright, 1998; Settanni & Moschetti, 2010). Starter bacteria are the most prominent contributor of microbial biomass in young curd, typically achieving cell densities of $\geq 10^8$ cfu/g (Beresford & Williams, 2004).

In cheddar cheese manufacture, the typical cultures used are *Lactococcus lactis* and *Lactococcus cremoris*, either on their own or in combinations of the two (Callanan & Ross, 2004). These microorganisms ferment the lactose in the milk and the consequent lactic acid production influences the coagulation of the milk, firming of the curd, conferring of the desired textural properties, as well as affecting the flavour characteristics of the cheese. The lactic acid also helps to establish certain conditions that directly influence the nature and degree of the activities of all other organisms in the cheese, and thereby it has a preservative effect (Leroy & De Vuyst, 2004).

NSLAB refer to a group of secondary microflora that have no active role during cheese manufacture but are vital role-players along with the starter bacteria in the ripening process (Beresford & Williams, 2004). The types of NSLAB identified include homofermentative and heterofermentative mesophilic lactobacilli and pediococci as well as *Enterococcus* and *Leuconostoc* (Crow, et al., 2001; Beresford & Williams, 2004). The predominant species of NSLAB found in cheddar cheese include *Lactobacillus casei*, *Lactobacillus plantarum* and *Lactobacillus brevis*, typically attaining cell densities of 10^4 cfu/g during the first 10 days, to approximately 10^8 cfu/g within a few weeks of maturation (Peterson & Marshall, 1990). NSLAB are not added to the milk deliberately, but are considered to be ‘adventitious contaminants’, which proliferate during ripening and contribute to flavour development (Beresford & Williams, 2004). It is probable that the milk is the primary source of NSLAB in cheese, due to the ability of certain strains to survive pasteurisation conditions. However, it has been suggested that the equipment used during cheese manufacture could be a possible reservoir for the growth of NSLAB and that these organisms can enter the cheese milk post pasteurisation in this manner (Martley & Crow, 1993; Beresford & Williams, 2004).

2.8 EFFECT OF SALT CONCENTRATION ON LACTIC ACID BACTERIAL GROWTH

LAB play an important role in food preservation, due to their ability to inhibit the growth of both spoilage and pathogenic microorganisms, thereby leading to improved shelf life and quality of food products (Henderson, et al., 1992).

The reduction of salt levels in cheese, as well as the substitution of NaCl with replacement cations has been shown to affect the salt-related response of bacteria (McMahon, et al., 2014). The use of salt along with buffering capacity to regulate final cheese pH is a unique characteristic of British type cheeses such as dry-salted cheddar cheese (Guinee & Fox, 2004). A salt level of >1.5% w/w has been shown to inhibit starter activity (Guinee & Fox, 2004). In cheddar cheese, the pH has almost reached its final value at the stage of hooping and thus salt is added to maintain this ultimate pH level (5.25-5.35). Cheddar curd contains 0.6-1.0% w/w lactose at hooping (Turner & Thomas, 1980; Guinee & Fox, 2004), this residual lactose is fermented by starter bacteria activity during the early stages of ripening. This is however dependent on both the salt in moisture ratio of the curd, as well as the salt tolerance of the starter culture (Guinee & Fox, 2004).

2.9 EFFECT OF ORGANIC ACIDS ON MICROBIAL GROWTH

The addition of acids to reduce the pH, as well as fermentations in which organic acids and other inhibitory metabolites are produced as by-products of LAB activity, are essential food preservation techniques (Presser, et al., 1998). Inhibition of microbial growth by organic acids has been used in fermented food products as a way to extend shelf life and improve food safety for approximately 10 000 years (Oh, et al., 2014). Weak organic acids are widely used in food preservation due to their effectiveness at relatively low concentrations and their ability to sufficiently lower the pH to preclude growth of spoilage microorganisms. The combined effect of weak organic acids, reduced pH and reduced a_w are key parameters in ensuring the stability of many shelf-stable foods such as cheddar cheese (Presser, et al., 1998).

The exact mechanisms by which microbial inhibition is achieved through the use of weak organic acids are not yet fully understood. However, it has been hypothesised that the diffusion of protonated acids across the cytosolic membrane and the consequent dissociation of the acids

due to an elevated intracellular pH, results in the release of both protons and anions within the cell (Oh, et al., 2014). These released protons lead to acidification of the cytoplasm, resulting in the reduction of internal pH which causes essential enzyme inhibition and subsequent cessation of cellular function and cell death, as can be seen in Figure 2.6 (Adams & Moss, 2000). Oh et.al. (2014) showed that there was an increase in the death of shiga-toxin producing *E. coli* with an increased concentration of protonated lactic acid, hence indicating that the primary cause of cell death was intracellular acidification.

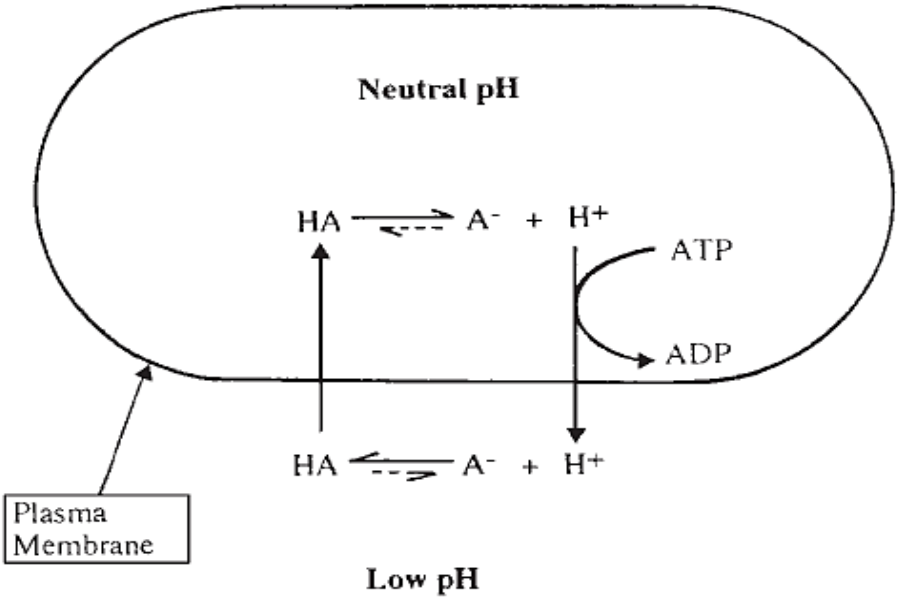


Figure 2.6: Microbial inhibition by weak organic acids (Adams & Moss, 2000)

2.10 CASEIN MICELLE MICROSTRUCTURE, EFFECT OF CALCIUM ADDITION AND CHANGES DURING COAGULATION

The first step in cheese manufacture is the conversion of liquid milk to solid cheese curd (Horne & Banks, 2004). This is achieved by the addition of a highly specific proteolytic enzyme, chymosin, which destabilises the milk proteins and causes the consequent precipitation of the largest group of milk proteins. The two types of protein found in milk are whey proteins, which are soluble in the serum phase and constitute approximately 20% of bovine milk protein, and casein proteins, which are a family of phosphoproteins comprising four distinct gene products namely αs1-, αs2-, β- and κ- caseins (Horne & Banks, 2004). These casein proteins are arranged in a colloidal suspension of aggregates known as casein micelles (Horne & Banks, 2004). Casein micelles consist of casein proteins as well as calcium phosphate and are spherical

particles, dispersed in a continuous phase that consists of water, salt, lactose and serum proteins. Casein micelles form a stable colloid with their stabilising properties being conferred by the action of the externally situated κ- casein molecules (de Kruif, 1999). These κ-casein molecules form a 'hairy' layer on the outer surface of the micelle, also known as a polyelectrolyte brush.

The brush is considered to be a salted polyelectrolyte brush due to the fact that part of the κ-casein molecule that extends into the surrounding liquid carries a number of charged groups which are highly screened by the salt ions in the solution (de Kruif, 1999). The stability of the κ-casein brush depends on a number of factors, namely brush density, pH, (divalent) salt concentration, as well as ethanol level. Brush stability is reduced by lowering brush density, lowering charge density (pH), adding divalent cations and lowering solvent quality by adding ethanol. Casein micelles are an association colloid, which means that the proteins and calcium phosphate are not covalently bound, and when the pH is lowered, calcium phosphate is solubilised leaving just the four casein proteins present in the micelle (de Kruif, 1999).

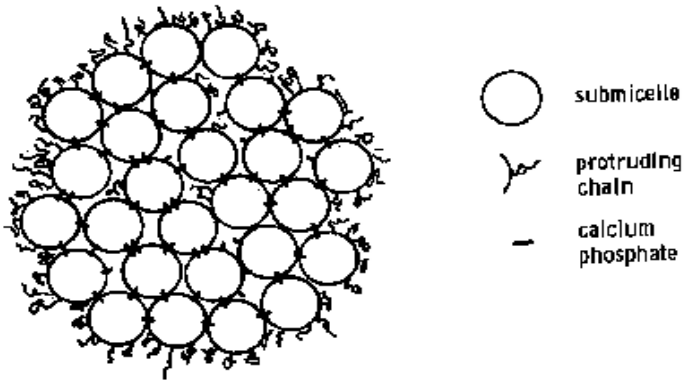


Figure 2.7: Model of casein micelle (Walstra & Jenness, 1984; van Hooydonk, 1987)

Rennet is added during cheese making to assist with the coagulation of the curd. Rennet contains the enzyme chymosin which is responsible for cleaving off the glyco-macro-peptide (GMP) from the κ-casein brush on the outer surface of the micelles. This results in the loss of steric stability of the casein micelles and the consequent flocculation of the particles, which is essential to curd coagulation. The enzymatic hydrolysis of the κ-casein layer increases the surface hydrophobicity of the casein micelles, which result in shrinkage and loss of water from the *para*-casein matrix (Everett, et al., 2012). The addition of the lactic acid-producing starter

culture assists in the reduction of the pH which also helps to destabilise the κ-casein polyelectrolyte brush (de Kruif, 1999).

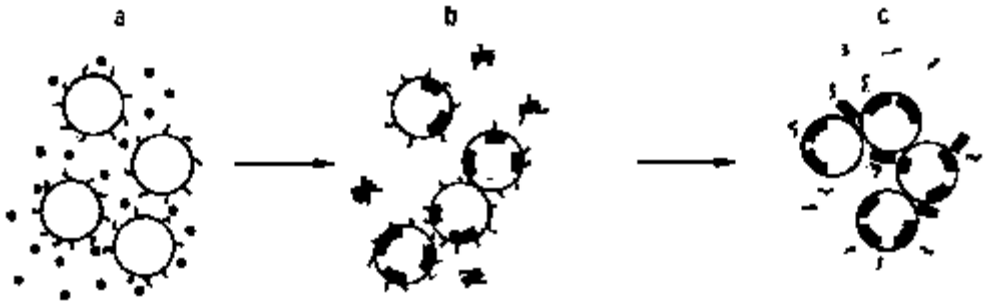


Figure 2.8: Schematic depiction of changes taking place after heating and renneting of (a) the casein micelles with CMP protrusions and native whey protein in unheated milk; (b) after heating; denatured whey protein complexed with casein micelles and dissociated casein; (c) after renneting of heated milk (van Hooydonk, 1987)

$CaCl_2$ is added during cheese manufacture to ensure a firm coagulum. The mechanism by which this phenomenon takes place is syneresis, as the calcium molecules help to expel water from the casein matrix. However, if added in excess amounts, $CaCl_2$ can impart negative effects on the quality of the cheese. Increasing the calcium content of cheese affects how the proteins in the cheese matrix interact with each other. It is due to this reason that $CaCl_2$ was not considered as a potential partial salt replacer in this project. By using $CaCl_2$ in the concentration necessary to impart the desired a_w -lowering effect, an unpalatable, brittle curd would be produced due to excess syneresis and calcium bridging in the protein matrix. Calcium promotes protein-to-protein interactions via the mechanisms of calcium bridging and charge neutralization, resulting in the contraction of the protein matrix and expulsion of serum from the matrix. As the protein matrix becomes less hydrated, and protein-to-protein interactions become more prominent, it results in a firmer curd (Pastorino, et al., 2003).

2.11 INTERACTIVE EFFECTS OF NACL, RATE OF ACIDIFICATION AND CA^{2+} LEVEL ON MOISTURE CONTENT

The moisture content of cheddar cheese is affected by numerous interrelated elements, including the extent of cutting of the gel, degree of acidification as well as scald temperature

(Guinee, et al., 2013). Salting of the curd generally causes a reduction in moisture content by approximately 2% (w/w) for every 1% w/w increase in salt. However, the ratio of moisture loss-to-salt uptake is dependent on a number of factors, including pH of curd at salting, moisture content of curd at salting, calcium phosphate content of curd, the effect of absorbed salt on further acidification, pH decrease and the temperature and fat content of the curd (Guinee, et al., 2013). After salting, the curd is pressed, resulting in further loss of moisture and dissolved solutes i.e. residual lactose, lactic acid, soluble calcium and phosphate and some fat. This moisture loss leads to a consequent concentration of fat and protein (calcium phosphate *para*-casein) (Guinee, et al., 2013).

Cheese proteins are subject to a large number of interactions, not only amongst themselves but with other bulk phase constituents of the cheese product, namely the water, fat and salt. The exact nature and degree of these interactions are dependent on both the ionic environment of the cheese as well as specific processing parameters such as levels of chymosin added, processing temperatures and pressing conditions (Floury, et al., 2009; Cruz, et al., 2011). It is thought that the degree of protein hydration is a vital factor for determining the rheological and textural properties of the final cheese product as it directly influences the physico-chemical stability and physical properties of the cheese (Guinee & O'Kennedy, 2007). The amount of salt present, pH, and calcium content of the cheese product have a direct impact on *para*-casein hydration which consequently has an effect on the hydration capacity of the protein matrix and its related propensity to syneresis (Guinee & Fox, 2004; Cruz, et al., 2011).

Syneresis in cheese manufacture refers to the expulsion of the liquid whey component of the milk from the solid curd (Calvo & Balcones, 2000). Essentially it is the expulsion of moisture from the calcium-phosphate *para*-casein network which was formed after rennet addition (Everett, et al., 2012). As the coagulum forms and becomes firmer, its tendency to contract decreases. It is therefore essential to cut the curd to encourage syneresis. The cutting of the curd and resultant syneresis is a critical step in cheese manufacture because the amount of whey expelled influences the moisture content of the cheese, which consequently affects the sensorial, safety and functional properties of the cheese product (Calvo & Balcones, 2000). The temperature at which the coagulum is cut is a crucial parameter that influences syneresis rate, with an increase in temperature resulting in a concomitant increase in syneresis (Calvo & Balcones, 2000).

There are a number of factors that can affect the level of syneresis experienced by cheese. These factors include the composition and source of the cheese milk (the fat, protein and calcium levels), the quantity of chymosin used to bring about coagulation of the casein micelles, and the specific treatment of the curd during cheese manufacture. Such treatments that can affect the degree of syneresis include the degree of acidification of the cheese milk prior to cutting, the temperature of the curd at cutting, the time left for the curd to 'heal', the extent of dry stirring of the curd, as well as the size of pressed cheese (Everett, et al., 2012). Additional syneresis is experienced by the cheese curd during the dry-salting process as well as during the pressing of the cheese overnight to knit the individual blocks of curd together, forming the green, unripened cheese (Guinee & Fox, 2004).

CHAPTER 3: HYPOTHESES AND OBJECTIVES

3.1 HYPOTHESES

E. coli grows at water activities greater than 0.95 (Marshall, et al., 1971; Doyle & Glass, 2010), regardless of the type of salt replacer used. Reduced salt cheese, substituted with salt replacers to produce an equivalent water activity as a Full sodium control (2% NaCl w/w) will have a similar effect on *E. coli* growth. This is because water activity is the most important factor with respect to allowing for, or inhibiting *E. coli* growth (Grummer & Schoenfuss, 2011).

E. coli will not be able to grow at water activities lower than 0.95 due to growth inhibition resulting from the drying effect in which water is drawn out of the *E. coli* cells via osmosis (Doyle & Glass, 2010). This will result in a disruption of the microbial cell membranes, culminating in the death of *E. coli* cells (McMahon, 2010; Cruz, et al., 2011). *E. coli* growth will also be inhibited due to the direct toxic effect of the Cl^- ion, by its removal of oxygen from the medium as well as its interference with the action of proteolytic enzymes (Taormina, 2010) .

3.2 OBJECTIVES

To determine the effect of water activity on the growth of *E. coli* in reduced NaCl cheese.

To determine the effect of salt replacers (KCl, $MgCl_2$) and reduced NaCl levels on the growth of *E. coli* in stirred-curd cheddar cheese.

CHAPTER 4: RESEARCH

The Research component of the project was divided in to two separate phases: Phase I and Phase II. Each phase was reported in terms of its Materials and Methods, Results, Discussions and Conclusions. In Phase I, a pilot study was conducted in order to replicate the model developed by Grummer & Schoenfuss (2011), to determine equivalent a_w's amongst the cheese samples made with different partial salt replacers. This was absolutely vital work for the success of the project, as one of the objectives of the study was to test the ability of *E. coli* to grow in cheeses with specific salt concentrations and a_w's. Phase II of the study involved the manufacture of reduced salt cheeses and their inoculation with three serotypes of non-O157:H7 shiga toxin-producing *E. coli*. The effect that the alternative sources of salt, as well as reduced NaCl levels had on the growth of *E. coli* were studied.

PHASE I

4.1 MATERIALS AND METHODS

Four different cheddar cheese samples were prepared (in triplicate) with varying salt compositions and concentrations. The samples were as follows:

1. Full NaCl control;
2. Reduced NaCl;
3. KCl Partially Replaced;
4. $MgCl_2$ Partially Replaced.

Cheddar cheese samples were prepared in the University of Pretoria's Pilot Plant, using a stirred-curd method, in which cheese samples were prepared in 6 litre (L) aluminium pots placed in a water bath, such that the temperature could be closely controlled during manufacture. The method was based on the procedure used by the ARC (Agricultural Research Council, Irene, personal communication with Jacobus Strydom, Researcher: Dairy Science and Technology) and is as follows:

- The milk (not standardised) or pasteurised was obtained from the experimental farm of the University of Pretoria and refrigerated at 5°C.

- The milk was pasteurised at 72°C for 15 seconds.
- The milk was then poured into 6L aluminium pots and placed in a water bath at a temperature of 31°C.
- The milk was then inoculated with a 1% starter culture CH-N22 (a mixture of *Lactococcus lactis* and *Lactococcus lactis* subsp. *cremoris*). The stock cultures were supplied in freeze-dried form by Dairy Cheret (Midrand, South Africa).
- 25ml of a 0.02% $CaCl_2$ solution was added to the milk.
- Approximately ten drops of annatto extract were added to the milk to give a light yellow colour.
- The milk was then renneted by adding 0.3ml of Maxiren Liquid Rennet, supplied by Dairy Cheret (Midrand, South Africa).
- The milk was stirred thoroughly after each addition, after which it was allowed to stand undisturbed for 40 minutes to coagulate.
- The stiffness of the coagulum was tested and if the desired consistency had been reached, the coagulum was cut into blocks of approximately 10cm × 10cm, after which the contents of the aluminium pots were left undisturbed for 10-15 minutes such that the curd could heal and firm up slightly prior to the commencement of stirring. The pH and titratable acidity was measured at this stage and was 6.5-6.6 and 0.09-0.12% respectively.
- Heating was started, initially at a rate of 1°C every 8 minutes until 34°C had been reached and then at a rate of 1°C every 6 minutes until the final cooking temperature of 39°C had been reached. The cooking temperature of 39°C was reached approximately 1 hour after the cutting of the curd into blocks.
- The titratable acidity and pH were determined again at this stage, and slight deviations are desirable which was indicative of the activity of the starter cultures.
- After the cooking temperature had been reached, stirring was stopped and the curd particles were allowed to settle to the bottom of the aluminium pot. Approximately 50% of the whey (half whey) was removed from the pot.
- The contents of the aluminium pot were stirred for a further one hour while the cooking temperature was maintained at 39°C.
- The remaining whey was then removed (final removal), when the curd was judged to have firmed sufficiently and an increase of 0.04-0.06% in titratable acidity from the measurement taken after cutting was observed. The pH was 6.1-6.2 at this point.

- Small residual amounts of whey were poured out of the pot over a period of 20 minutes. The curd had matted together and the titratable acidity was at least 0.2% and the pH less than 6.0.
- The curd was then cut into blocks of approximately 5cm × 5cm and turned every 15-20 minutes over a period of 1.5 hours after the final drainage of whey.
- The curd was adjudged to be milled when the titratable acidity had increased to 0.5-0.6% or alternatively when the pH had decreased to 5.3-5.2.
- The curd was allowed to cool to below 32°C after which salting was commenced (the amount of each salt added to the different cheese samples are detailed below).
- The salted curds were then placed into moulds and pressed for 18 hours overnight to expel any residual moisture and mat the curds together tightly.
- The cheese samples were then taken out of their moulds, vacuum packaged and refrigerated at 4°C for 4 weeks.

4.2 GUIDELINES BASED ON THE MANUFACTURE OF NUMEROUS COMMERCIAL CHEDDAR CHEESES

A number of commercial cheddar cheeses were analysed in terms of their moisture content, a_w and salt content.

Table 4.1: Table indicating the water activity, moisture content and salt content of various commercial cheddar cheeses

Commercial cheese	Water activity (a_w)	Moisture content (%)	Salt content (%)
ARC cheddar[2]	0.935 (0.007)[1]	39.82 (0.354)	2.15 (0.071)
Spar cheddar	0.93	40.205 (0.94)	1.95 (0.071)
Parmalat cheddar	0.935 (0.007)	36.715 (0.007)	2.1
Lancewood cheddar	0.945 (0.007)	37.275 (0.078)	1.95 (0.071)

[1]Standard Deviations in parenthesis, n = 2

[2]Agricultural Research Council

The amount of salt to be added to each of the samples was based on a target of 2% salt content in the Full NaCl control and a subsequent 30% reduction in NaCl in the Reduced NaCl cheddar.

The amount of salt to be added to the KCl and $MgCl_2$ Partial Replaced cheeses was based on a 30% reduction in the NaCl content, with the amount of partial replacement salt to be added being calculated by making use of Raoult's Law which states:

$$a_w = \gamma_s X_{water} = \gamma_s \frac{n_{water}}{n_{water} + n_{solutes}},$$

Where: a_w = water activity

: γ_s = activity coefficient[1]

: X = mole fraction

: n = number of moles

[1] γs assumed to be 1 (Bell & Labuza, 2000)

By carrying out a few basic calculations, and assuming that the cheddar cheese will have a final moisture content of 40%, Raoult's Law was used to determine the approximate a_w of the cheddar cheeses.

Numerous pilot studies determined the approximate yield (from 5L of milk) to be around 500g. Assuming a 40% moisture content in the cheddar cheese, then:
500g × 40% = 200g water.

Number of moles of water in the cheese is therefore:

n = m/Mm

= 200/18.01

= 11.1 mol H_2O

The amount of salt added to the Full NaCl control cheddar was based on a target salt content of 2%, therefore, 500g × 2% = 10g NaCl

Number of moles of salt (NaCl) in the cheese is therefore:

n = m/Mm

= 10/58.44

= 0.17 mol NaCl

The approximate a_w can be calculated as follows:

$$a_w = \gamma s \times \frac{n\,(H2O)}{n\,(H2O)+n\,(solutes)}$$

$$= 1 \times \frac{11.1}{11.1+ 0.171}$$

= 0.985

In an ideal situation, the Full NaCl control cheddar would therefore have 10g of NaCl added to it during the cheddaring process, and have an approximate a_w of 0.985. In the above equation, the activity coefficient γs, which is a measure of the non-ideality of the solution, was assumed to be 1 (Bell & Labuza, 2000). In certain instances the activity coefficient can deviate from 1 when particular solutes form unique interactions with water via dipole-dipole, hydrogen bonds, and ion-dipole interactions. However, for dilute solutions of low molecular weight solutes, an activity coefficient of 1 (hence the assumption of an ideal solution) can adequately predict the a_w of the solution (Bell & Labuza, 2000). Another factor that may influence the a_w calculations is the assumption that all salt molecules are undergoing complete dissociation into the individual ions, Na^+ and Cl^- . Some of the NaCl molecules may not completely dissociate into the individual Na^+ and Cl^- ions, thus resulting in fewer ions for the water molecules to bind with, and hence a higher than expected a_w due to the presence of more free water than predicted by the equation.

4.3 PREPARATION OF CHEDDAR CHEESE SAMPLES

All cheddar cheese samples were prepared in triplicate. Analyses were conducted 24 hours after manufacture, as well as 28 days after manufacture.

4.3.1. Full NaCl control

NaCl (10g) was added to the cheese (based on a target salt content of 2%).

4.3.2 Reduced NaCl cheddar

NaCl (7g) was added. This was based on a 30% reduction in the NaCl content which was shown by Hoffmann (2013) to be the maximum possible reduction in NaCl to still produce a cheese with acceptable quality characteristics.

4.3.3 KCl Partially Replaced cheddar

NaCl (6.91g) and KCl (3.78g) was added to the cheese. The amount of NaCl added was based on the target of a 30% reduction in the NaCl content (hence constituting 70% of the added salt), whereas the KCl constituted the remaining 30% of the salt required to impart an equivalent a_w to the control cheese. Using Raoult's Law, and assuming a target a_w of 0.985 and moisture content of 40%, the total number of moles of solute needed would be 0.169 mol. Through the use of mole fractions, we can then calculate the amount of each salt that needs to be added to impart the desired final a_w. NaCl will constitute 70% of the salt added, therefore it will constitute 70% of the number of moles required: 0.169×70% = 0.118 mol. The number of moles of KCl that would need to be added would then be: 0.169-0.1183 = 0.057 mol. The mass of KCl was then calculated using the formula:

$$n = \frac{m}{Mm}$$

$$m = 0.057 \times 74.55$$

$$= 3.78\text{g KCl}$$

4.3.4 $MgCl_2$ Partially Replaced cheddar

NaCl (6.91g) and $MgCl_2$ (4.83g) was added to the cheese. As with the KCl Partially Replaced cheddar, the NaCl content was based on a 30% reduction. Using the same mole fractions as with the KCl Partially Replaced cheddar, the mass of $MgCl_2$ was calculated as follows:

$$n = \frac{m}{Mm}$$

$$m = 0.057 \times 95.211$$

$$= 4.83\text{g } MgCl_2$$

4.4 SAMPLE ANALYSES

Measurements were taken in quadruplicate on each cheese, 1cm on either side of a vertical cut dividing the cheese wheel in half, followed by a 1cm trans-section back to the central cut-line (see Fig. 4.1). This was done to ensure that a representative sample was taken and to ensure repeatability and reliability of results.

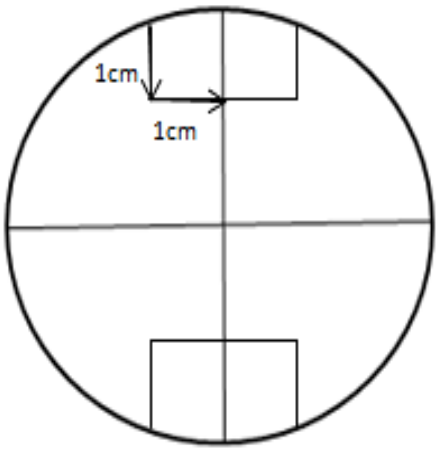


Figure 4.1: Schematic representation of sampling points taken from each cheese sample

4.4.1 Physico-chemical analyses

4.4.1.1 pH

pH was measured with a Hanna pH211 Microprocessor pH Meter (Hanna Instruments, Johannesburg, South Africa) at various stages throughout the cheese manufacturing process as well as at the stages of pre-pressing, directly after pressing as well as two weeks and four weeks after pressing

4.4.1.2 Moisture content

Moisture content was determined by oven drying overnight at 103°C as described by James (1995).

4.4.1.3 Water activity

Water activity of the various cheeses was measured with a Pawkit Portable water activity meter (Decagon devices, Inc. Wyoming, USA).

4.4.1.4 Salt content

The salt content was determined according to a quick Australian factory method as described by the Australian Society of Dairy Technology (1966).

4.5 RESULTS

Three replicates of the four cheddar cheeses were tested for moisture content, a_w, salt content and pH and were measured 24 hours after pressing, as well as 28 days after pressing.

Table 4.2: Moisture content, water activity and salt content of various salt treated cheddars, measured 1 day and 28 days after manufacture

Treatment	Time	Moisture content (%)	a_w	Salt content (%)	pH
Full NaCl	1 day	41.47 (0.83) [1]	0.96 (0.01)	1.89 (0.03)	5.27 (0.02)
	28 days	40.43 (1.25)	0.95	1.87 (0.05)	5.26 (0.02)
Reduced NaCl	1 day	43.46 (2.38)	0.96 (0.01)	1.27 (0.03)	5.25 (0.01)
	28 days	42.30 (1.52)	0.96	1.31 (0.01)	5.24(0.01)
KCl Partial Replaced	1 day	43.28 (1.30)	0.96 (0.01)	1.58 (0.03)	5.24 (0.01)
	28 days	42.02 (1.46)	0.96	1.6 (0.03)	5.23 (0.01)
$MgCl_2$ Partial Replaced	1 day	41.57 (2.21)	0.96 (0.01)	1.65 (0.03)	5.25 (0.01)
	28 days	41.13 (1.20)	0.97	1.66 (0.01)	5.24 (0.01)

[1]Standard deviation in parenthesis, n =3

4.6 DISCUSSION

After 28 days of refrigeration at 4°C, the Full NaCl samples had a mean moisture content of 40.43% and a mean a_w of 0.95. The cheese had a mean salt content of 1.87% and mean pH of 5.25.

For the Reduced NaCl cheese, a mean moisture content of 42.3% and mean a_w of 0.96 was recorded. This was expected as cheeses with lower salt content tend to have both higher moisture contents and a_w's than cheeses salted with higher concentrations of salt. The a_w of cheese is largely dependent on the concentration of solids in water, hence the moisture content, pH, salt concentration and non-protein nitrogen (NPN) are crucial parameters (Marcos, et al., 1981). The Reduced NaCl cheddar cheese had a mean salt content of 1.3% and mean pH of 5.23.

The KCl Partially Replaced cheddar had a mean moisture content of 42.02% and mean a_w of 0.96. This cheese had a mean salt content of 1.6% as well as a mean pH of 5.23. The $MgCl_2$ Partially Replaced cheese had a mean moisture content of 41.13% and a mean a_w of 0.97. It had a mean salt content of 1.66% and a mean pH of 5.24.

The cheeses all had lower a_w than what was predicted by the model (0.985). There are a few possible reasons for this discrepancy. The commercial cheeses that were tested are matured for a significantly longer period than the test samples were, possibly well over 6 months. The test samples were analysed after only one month of maturation. Over an extended maturation period of a few months, a decrease in a_w is expected due to the increased concentration of low molecular mass solutes such as non-protein nitrogen released by proteolysis (Marcos, et al., 1981). There was an observed decrease in the a_w of the Full NaCl over the one month maturation period, while the other samples maintained the same a_w from day 1 to 28, with the exception of the $MgCl_2$ Partial Replaced which had a slight increase in a_w. The model used to predict the a_w of the cheeses only takes the concentration of the salts into account, it does not accommodate the inclusion of other solutes of low molecular mass which will have a a_w - lowering effect on the cheese (Marcos, et al., 1981). It is also possible that the assumption of an ideal solution, and hence use of the activity coefficient of 1, contributed to the lower than predicted a_w in the cheese samples (Bell & Labuza, 2000).

There was also a slightly lower than targeted salt content in all of the test samples. This was possibly due to incomplete dissociation of the salts into their individual ions as well as losses during the pressing process. Another possible reason for an underestimation of the salt content could be because the titration method used is specific for measuring NaCl content, it may therefore not accurately account for the entire salt content of the cheese in terms of the partial replacers KCl and $MgCl_2$. There was a general trend for the moisture content and pH of the cheeses to decrease over the 28 day maturation period.

4.7 CONCLUSIONS

Phase I of the experimental work was vital as it indicated a number of important discrepancies that could be expected, most notably a lower than predicted a_w in all samples. It also highlighted some important trends such as the general decrease in both moisture content and pH of all samples over the 28 day period. The lower than targeted salt content due to losses on salting emphasised the need for precise measuring and application to the curd in the experimental work to come. In Phase II of the project, the proposed scaling down from a starting milk volume of 5L to 1.5L could further increase the margin for error so much care would need to be taken to ensure accurate results.

PHASE II

4.8 MATERIALS AND METHODS

4.8.1 Experimental design

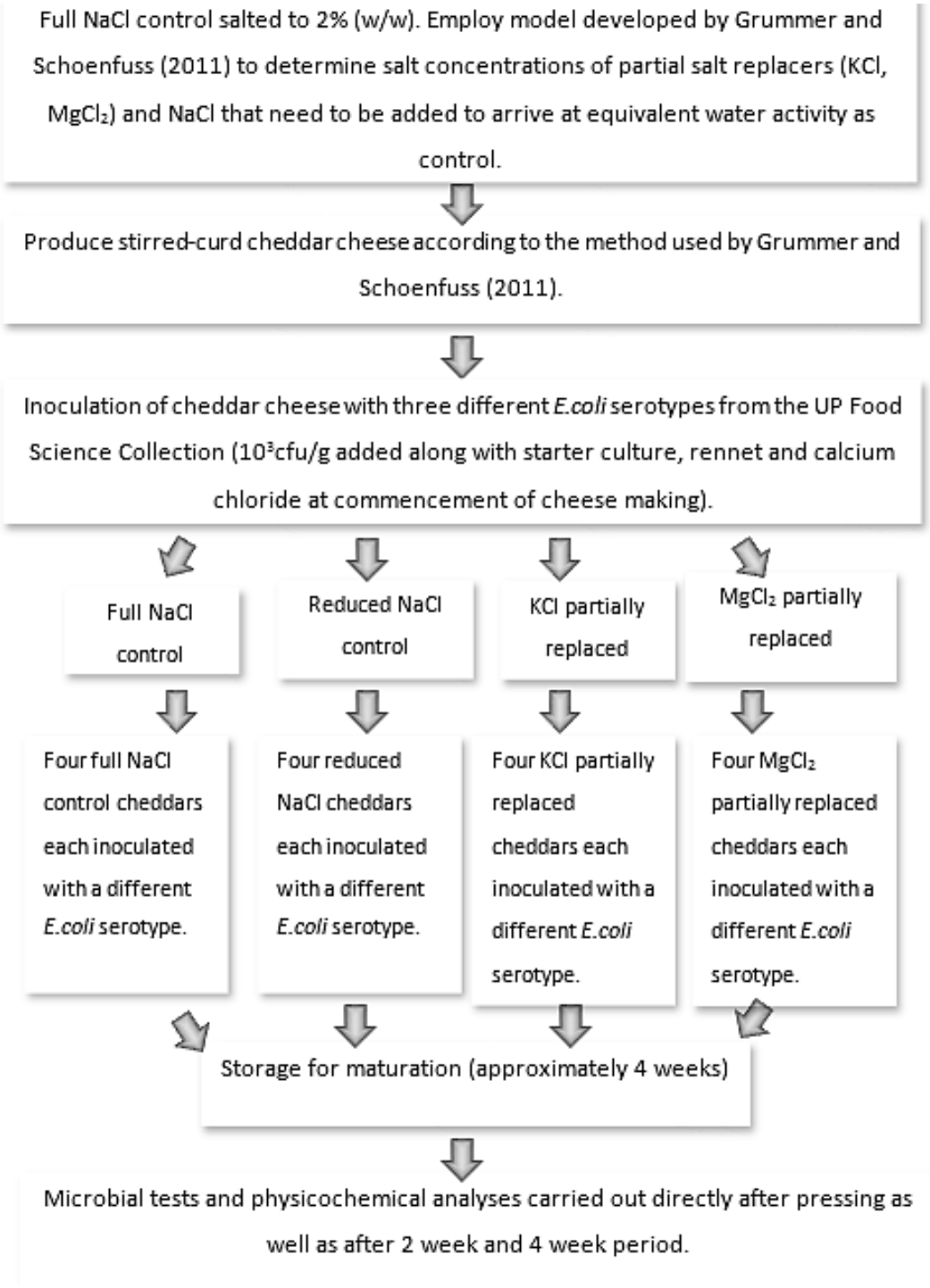


Figure 4.2: Procedure for cheese manufacture and inoculation with *E. coli* serotypes

4.8.2 Manufacture of cheddar cheese

Cheddar cheese samples were prepared in a similar, stirred-curd manner to the samples prepared in Phase I of the experimental work. There were however a few key differences in the cheese making process. The operations were restricted to the Microbiology Laboratory of the University of Pretoria, the volume of milk used was reduced from 5L to 1.5L, the pots and instruments used were sterilised in an autoclave at 121°C for 15 minutes prior to cheese manufacture, and the milk was inoculated with *E. coli* serotypes at the commencement of cheese-making.

4.8.3 *E. coli* preparation and standardisation

E. coli serotypes that were isolated from milk and which came from the UP Food Science Collection were used.

E. coli cells were inoculated into tubes containing Tryptone Soy Broth (TSB) (Oxoid, Cambridge, UK) and incubated overnight at 37°C. These were conditions that encourage rapid multiplication of *E. coli* cells. The tubes containing the *E. coli* cells were then centrifuged multiple times at 2400 rpm for 15 minutes at 4°C, with the supernatant being decanted and consequently being substituted with peptone water and vortexed to ensure homogeneity. This process was repeated until a milky white liquid containing only *E. coli* cells and peptone water remained. The samples were then ready for standardisation.

The concentration of *E. coli* to be added to the cheese samples at the initiation of cheese manufacture was decided to be 1×10^3 cfu/ml, based on the maximum possible numbers expected to be found in cheese samples in industry. The method used to ensure that this approximate number of viable *E. coli* bacteria were present in the cheese milk at the commencement of cheese making was by use of McFarland Standards. A standard is prepared with a known quantity of barium chloride and sulphuric acid to produce a barium sulphate precipitate with a milky, turbid appearance. The combination of these two liquids has a known absorbance when analysed at a specific wavelength in a spectrophotometer. *E.coli* cells were pipetted from the *E.coli*/peptone water mixture into a tube containing peptone water alone, until such a point at which the turbidity of the latter tube matched that of the prepared McFarland Standard 0.5.

The McFarland Standard chosen was number 0.5, corresponding to an approximate cell density of 1.5×10^8 cfu/ml. A serial dilution was then done, whereby 1ml of the standard was pipetted into 9ml sterile 0.1% peptone water, producing a solution with an approximate cell density of 1.5×10^7 cfu/ml. This solution was vortexed before a further 1ml of this solution was pipetted into 9ml sterile 0.1% peptone water, producing a solution with an approximate cell density of 1.5×10^6 cfu/ml. 1ml of this solution was inoculated into each of the pots containing 1.5L of milk, at the commencement of cheese making, resulting in an approximate starting cell density of 1.0×10^3 cfu/ml. Samples were taken directly after inoculation of the cheese milk and plated on both Sorbitol MacConkey and Nutrient agar. The McFarland Standard was also further serially diluted down to 1.5×10^2 and plated on Nutrient Agar to test the accuracy of the standard.

4.8.4 Microbiological analyses

4.8.4.1 Enumeration and microbiological analysis

Ten gram (10g) quantities of the various cheese samples were weighed and macerated in 90ml of sterile peptone water with the aid of a Stomacher Lab Blender (Seward Laboratory, London, UK) to achieve an initial 10^{-1} dilution. Further decimal dilutions were prepared in 9ml test tubes containing peptone water and 0.1ml portions were plated on the following agar plates by use of the spread plate method:

E. coli were enumerated on Sorbitol MacConkey Agar plates (Oxoid, Cambridge, UK). The plates were incubated at 37°C. LAB were enumerated on MRS agar (De Man, Rogosa and Sharp, 1960). The plates were incubated at 30°C. An APC was conducted using Nutrient Agar plates (Oxoid, Cambridge, UK). The plates were incubated at 37°C.

Microbial counts were taken directly after addition of *E. coli* to starter milk, pre-pressing, directly after pressing, two weeks and four weeks after pressing with fresh samples being taken from the cheese samples and plated at the respective time intervals.

4.8.5 Physico-chemical analyses

4.8.5.1 Moisture Content

Moisture content was determined by oven drying overnight at 103°C as described by James (1995).

4.8.5.2 Water Activity

Water activity of the various cheeses was measured with a Pawkit Portable water activity meter (Decagon devices, Inc. Wyoming, USA).

4.8.5.3 Salt content

The salt content was determined according to a quick Australian factory method as described by the Australian Society of Dairy Technology (1996).

4.8.5.4 Salt-in-moisture

The salt-in-moisture ratio was measured as follows:

% S/M = (% NaCl ÷ % Moisture) × 100

4.9 STATISTICAL ANALYSES

Analysis of variance was done by using Statistica Software for Windows Version 7 (Tulsa, Oklahoma, USA, 2003). This was done to determine whether there were any significant differences with respect to growth of various *E. coli* serotypes in the Full NaCl cheese when compared to cheeses made with partial salt replacers. Analysis of variance was also done to test for the effect of treatment (Full NaCl/Reduced NaCl/KCl Partial Replaced/$MgCl_2$ Partial Replaced), *E.coli* serotype and time on the physico-chemical parameters a_w, moisture content, salt content and S/M content of cheddar cheeses. Analyses were carried out in quadruplicate for each *E. coli* serotype.

4.10 RESULTS

Tables 4.3 and 4.4 indicate that there were significant interactions ($p<0.05$) observed between the main factors *E. coli* serotype and time on the growth of *E. coli*, APC and LAB during manufacture, as well as a significant interaction ($p<0.05$) between *E. coli* serotype and time on the growth of LAB during storage.

Table 4.3: ANOVA of salt treatment (Full NaCl/Reduced NaCl/KCl Partial Replaced/$MgCl_2$ Partial Replaced), *E.coli* serotype (O2, O4, O9) and time on the *E. coli*, APC and LAB growth of Cheddar Cheeses through manufacture

Independent variables	Dependent variables		
	E. coli	APC[1]	LAB[2]
Treatments (Full NaCl, Reduced NaCl, KCl Partial Replaced, $MgCl_2$ Partial Replaced)	0.989	0.994	0.845
E. coli serotype (O2,O4,O9)	0.001	0.000	0.0002
Time (0, 0.5, 1 days)	0.000	0.000	0.000
Treatments * *E. coli* serotype	0.892	0.916	0.994
Treatments * Time (days)	0.916	0.959	0.886
E. coli serotype * Time (days)	0.000	0.000	0.000
Treatments**E. coli* serotype *Time (days)	0.998	0.926	0.791

[1] Aerobic Plate Count
[2] Lactic Acid Bacteria

Table 4.4: ANOVA of salt treatment (Full NaCl/Reduced NaCl/KCl Partial Replaced/$MgCl_2$ Partial Replaced), *E.coli* serotype (O2, O4, O9) and time on the *E. coli*, APC and LAB growth of Cheddar Cheeses through storage

Independent variables	Dependent variables		
	E. coli	APC	LAB
Treatments (Full NaCl, Reduced NaCl, KCl Partial Replaced, $MgCl_2$ Partial Replaced)	0.861	0.965	0.939
E. coli serotype (O2,O4,O9)	0.000	0.000	0.000
Time (1, 14, 28 days)	0.061	0.007	0.000
Treatments * *E. coli* serotype	0.973	0.919	0.447
Treatments * Time (days)	0.986	0.993	0.831
E. coli serotype * Time (days)	0.437	0.678	0.0005
Treatments**E. coli* serotype *Time (days)	0.999	0.993	0.999

For serotypes O2 and O4, a similar trend was observed for all four salt treatments, as can be seen in Figures 4.3 and 4.4. There was a significant ($p< 0.05$) increase in *E. coli* numbers through manufacture, reaching their highest level at day 14 of storage. Between days 14 and day 28 there was however a slight decrease in *E. coli* numbers. For serotype O9 a slight increase in *E. coli* numbers was observed between inoculation (day 0) and prior to pressing (day 0.5). When counts were taken 1 day after pressing, *E. coli* numbers had decreased, irrespective of salt treatment. There was then a slight increase in *E. coli* growth, reaching their highest level at day 14, after which a slight decrease in *E. coli* numbers was observed between days 14 and day 28 of storage. The only exception to this trend was observed in the $MgCl_2$ Partial Replaced cheeses inoculated with serotype O2, which reached its highest level after day 28. The discrepancy in growth between serotypes O2 and O4, and serotype O9 is highlighted in Figure 4.4. Irrespective of salt treatment, *E. coli* growth in cheeses inoculated with serotype O9 was approximately 2 Log cfu/g less than *E. coli* growth in cheeses inoculated with serotypes O2 and O4 ($p<0.05$).

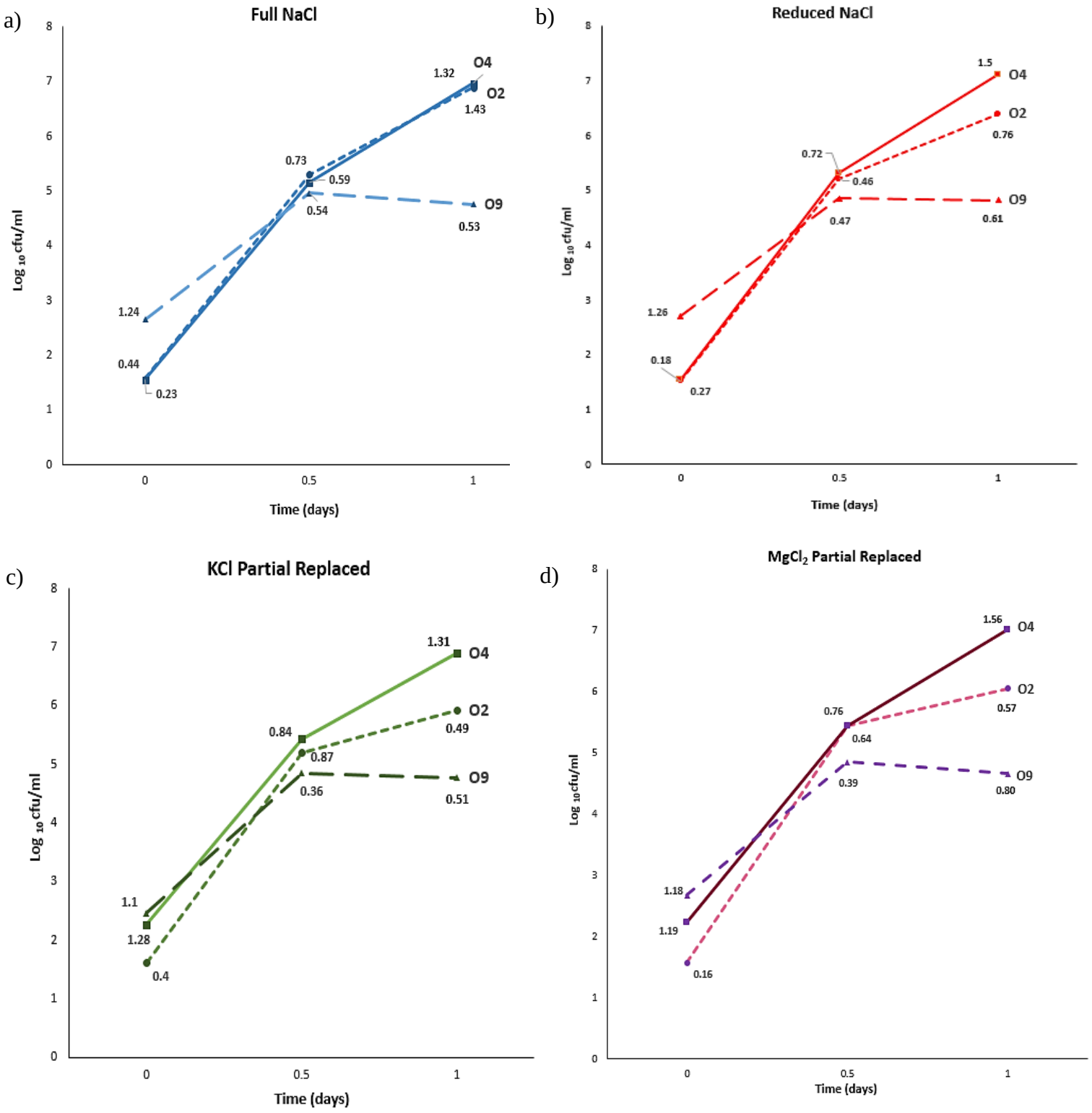


Figure 4.3: *E. coli* growth of three serotypes (O2, O4, O9) in (a) Full NaCl control, (b) Reduced NaCl, (c) KCl Partial Replaced and (d) $MgCl_2$ Partial Replaced cheeses, during manufacture. Results expressed are mean ± SD (illustrated for each value), n = 4.

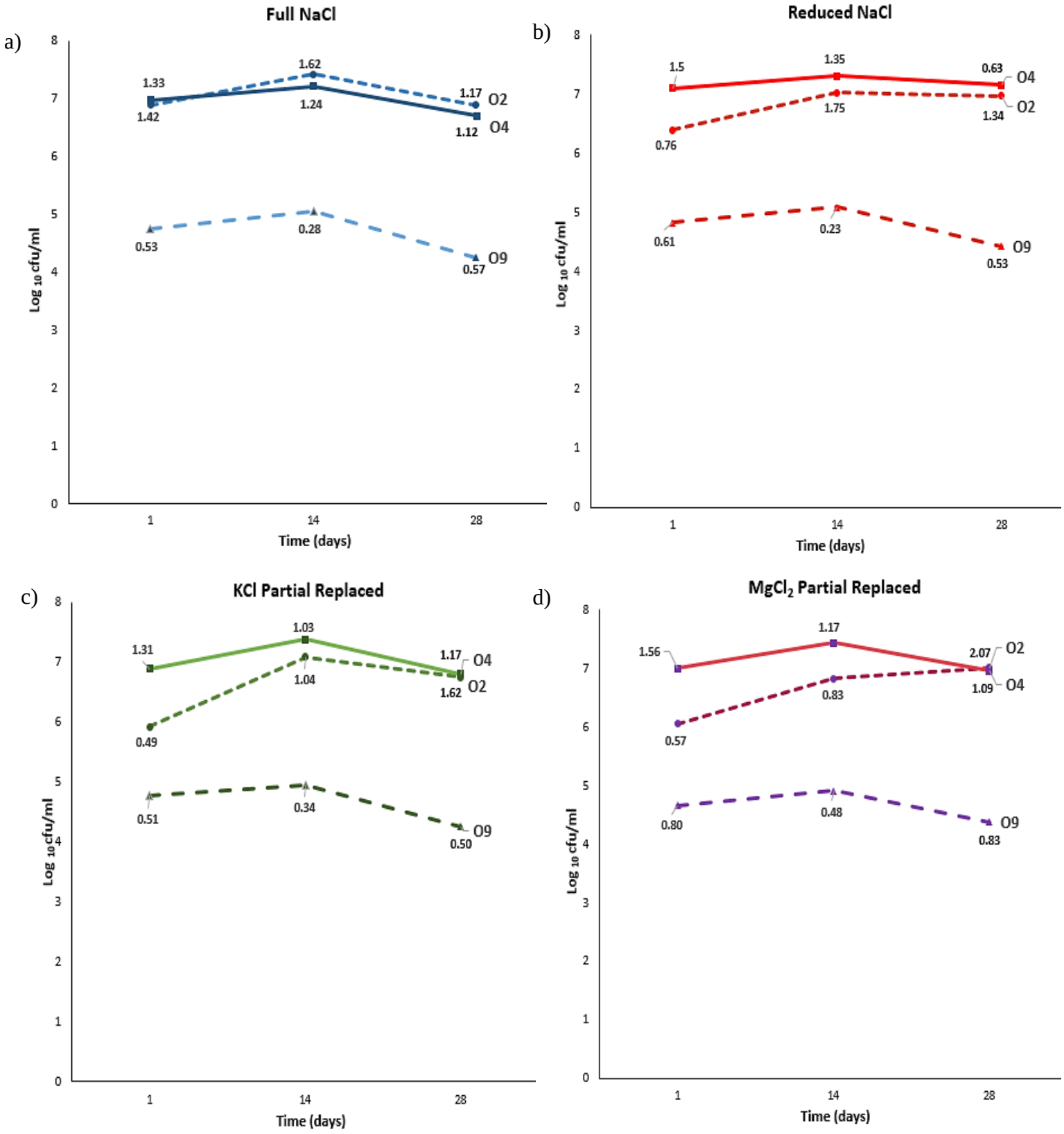


Figure 4.4: *E. coli* growth of three serotypes (O2, O4, O9) in (a) Full NaCl control, (b) Reduced NaCl, (c) KCl Partial Replaced and (d) $MgCl_2$ Partial Replaced cheeses, during storage. Results expressed are mean ± SD (illustrated for each value), n = 4

Table 4.5 indicates the initial level of inoculation of the different serotypes of *E. coli* in the reduced salt treated cheeses.

Table 4.5: *E. coli* growth (log10 cfu/g) in reduced salt cheeses after inoculation (day 0)

Treatment	*E. coli* Serotype[1]		
	O2	O4	O9
Full NaCl	1.58 (0.44)[2] [a]	1.55 (0.23) [a]	2.65 (1.24) [a]
Reduced NaCl	1.52 (0.27) [a]	1.55 (0.18) [a]	2.70 (1.26) [a]
KCl Partially Replaced	1.62 (0.40) [a]	2.27 (1.28) [a]	2.46 (1.10) [a]
$MgCl_2$ Partially Replaced	1.57 (0.16) [a]	2.23 (1.19) [a]	2.67 (1.18) [a]

[1] Mean values followed by same letters in column do not differ significantly at a level of $p<0.05$
[2] Standard deviations in parenthesis, n = 4

Table 4.6 indicates that there were significant differences in growth of *E. coli* between the three different serotypes tested ($p<0.05$). Serotype O2 and O4 exhibited no differences between any of the four reduced salt treatments, however serotype O9 differed significantly from serotypes O2 and O4 at all four reduced salt treatments ($p<0.05$). A similar trend was observed in Figures 4.3 and 4.4, with the growth of *E. coli* of serotypes O2 and O4 being similar but the *E. coli* growth of serotype O9 differing significantly ($p<0.05$) from the other two serotypes. There was a general trend for the Full NaCl treated cheeses to exhibit the lowest numbers of *E. coli* growth after 28 days, and in the case of serotypes O4 and O9 the Reduced NaCl cheeses having the highest numbers of *E. coli* growth, as was expected. In the case of serotype O2 the highest numbers of growth were found in the $MgCl_2$ Partially Replaced cheese samples. Figure 4.9 illustrates that $MgCl_2$ Partially Replaced cheese had a higher a_w than its Reduced NaCl counterpart, which explains this discrepancy.

Table 4.6*: E. coli* growth (log10 cfu/g) in reduced salt cheeses after 28 days

Treatment	*E. coli* Serotype[1]		
	02	04	09
Full NaCl	6.87 (1.17)[2] ghi	6.70 (1.16) fghi	4.24(0.57) b
Reduced NaCl	6.96 (1.34) ghi	7.16 (0.63) ghi	4.42 (0.53) b
KCl Partially Replaced	6.75 (1.62) fghi	6.81 (1.17) ghi	4.26 (0.50) b
$MgCl_2$ Partially Replaced	7.01 (2.07) ghi	6.95 (1.09) ghi	4.38 (0.83) b

[1] Mean values followed by same letters in column do not differ significantly at a level of $p<0.05$
[2] Standard deviations in parenthesis, n= 4

Figures 4.5 and 4.6 indicate the APC in the various cheese samples through manufacture and storage. There was a similar trend in the growth, irrespective of the treatment or serotype for the APC numbers to increase over the 28 day period. For serotypes O2 and O4, APC numbers reached their highest level at day 14, after which there was a slight decrease in numbers to day 28. The only exceptions to this trend for serotype O4 were observed in the $MgCl_2$ Partially Replaced cheeses and the Full NaCl cheeses, which exhibited their highest levels at day 28. The initial bacterial numbers of cheese samples inoculated with *E. coli* serotype O9 were significantly higher than the initial numbers for the other two serotypes ($p<0.05$). APC numbers were still increasing when counts were taken after 28 days, even though the rate of growth of bacteria in these samples was a lot more gradual in comparison to serotypes O2 and O4.

There was a similar trend in the growth, irrespective of the treatments or serotype for the LAB levels to increase over the 28 day period, as can be seen in Figures 4.7 and 4.8. Serotypes O2 and O4 once again exhibited a very similar trend with LAB numbers increasing rapidly over the 28 day period , with final numbers upwards of $Log_{10}10$, regardless of treatment. Although the initial LAB numbers for all salt treatments of serotype O9 were significantly ($p<0.05$) higher than the initial numbers for serotypes O2 and O4 (approximately $Log_{10}6$ compared to $Log_{10}3$ and $Log_{10}5$ for serotypes O2 and O4, respectively), the final counts after 28 days for all salt treatments for cheeses inoculated with serotype O9 were in the range of $Log_{10}9$. This was significantly ($p<0.05$) lower than the final counts of the other two *E. coli* serotypes. The rate of LAB growth observed in all cheeses inoculated with serotype O9 was visibly lower than for serotypes O2 and O4.

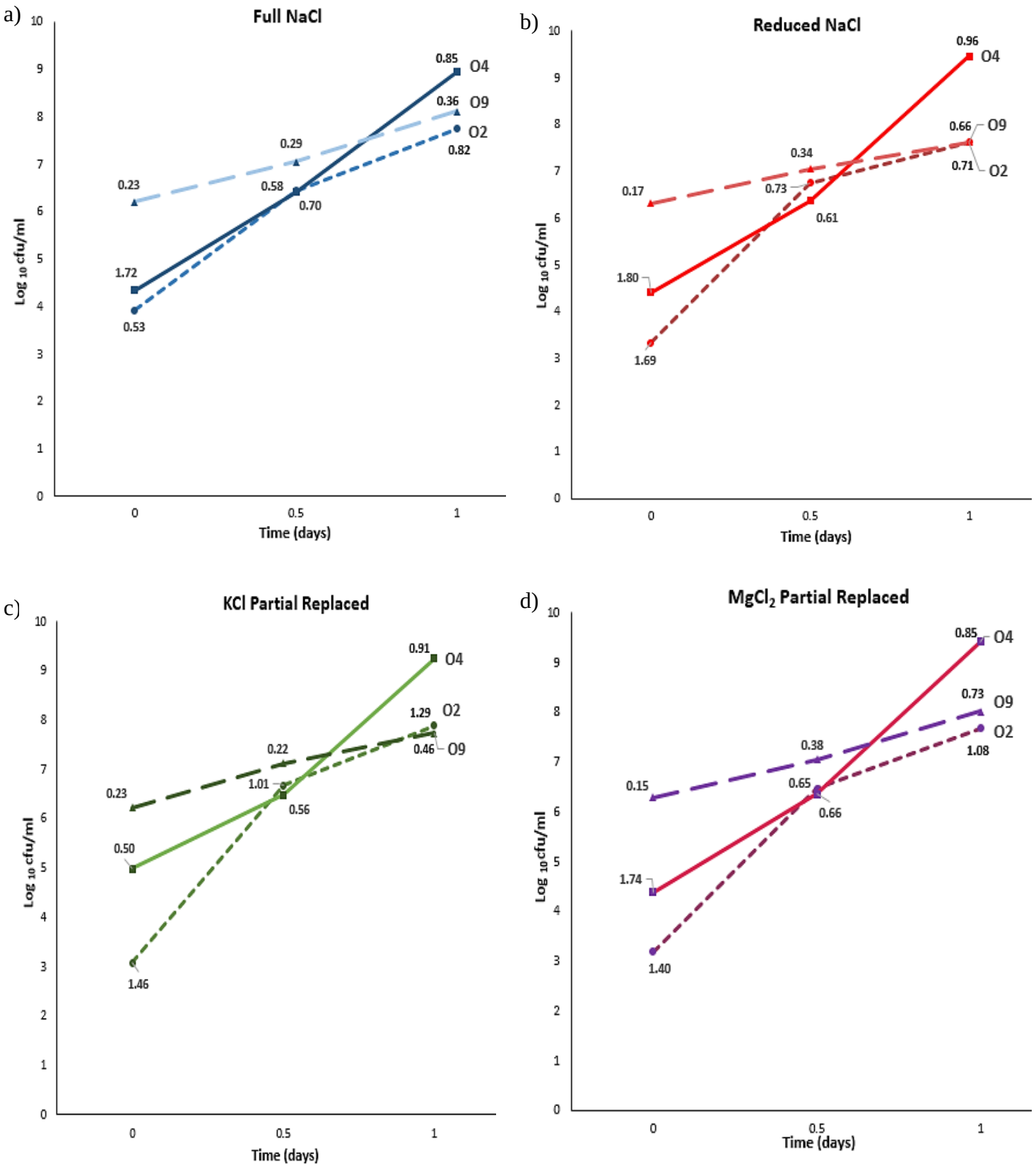


Figure 4.5: APC of a) Full NaCl control, (b) Reduced NaCl, (c) KCl Partial Replaced and (d) $MgCl_2$ Partial Replaced cheeses inoculated with three *E. coli* serotypes (O2, O4, O9), during manufacture. Results expressed are mean ± SD (illustrated for each value), n = 4

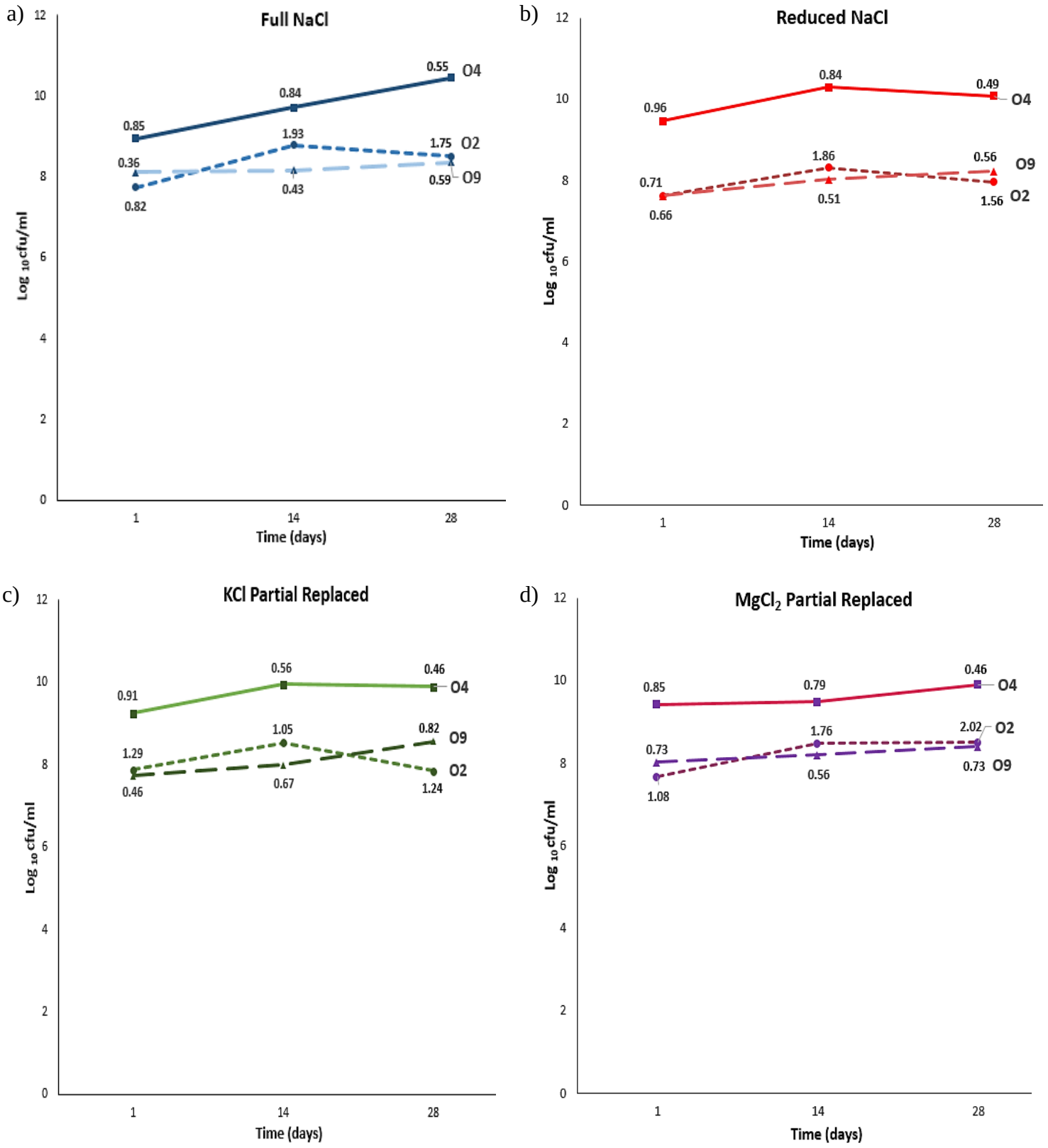


Figure 4.6: APC of a) Full NaCl control, (b) Reduced NaCl, (c) KCl Partial Replaced and (d) $MgCl_2$ Partial Replaced cheeses inoculated with three *E. coli* serotypes (O2, O4, O9), during storage. Results expressed are mean ± SD (illustrated for each value), n = 4

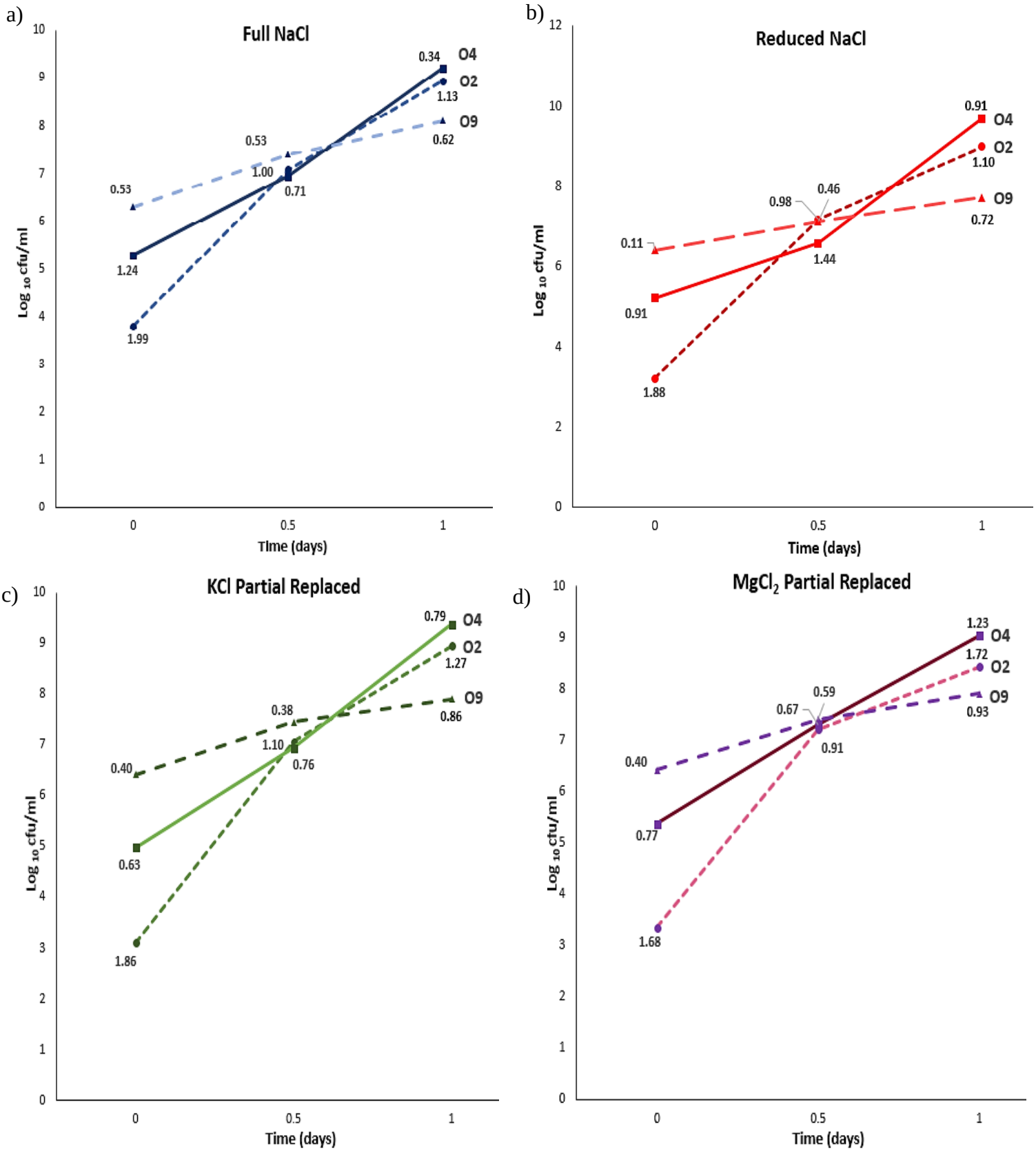


Figure 4.7: LAB growth in a) Full NaCl control, (b) Reduced NaCl, (c) KCl Partial Replaced and (d) $MgCl_2$ Partial Replaced cheeses inoculated with three *E. coli* serotypes (O2, O4, O9), during manufacture. Results expressed are mean ± SD (illustrated for each value), n = 4

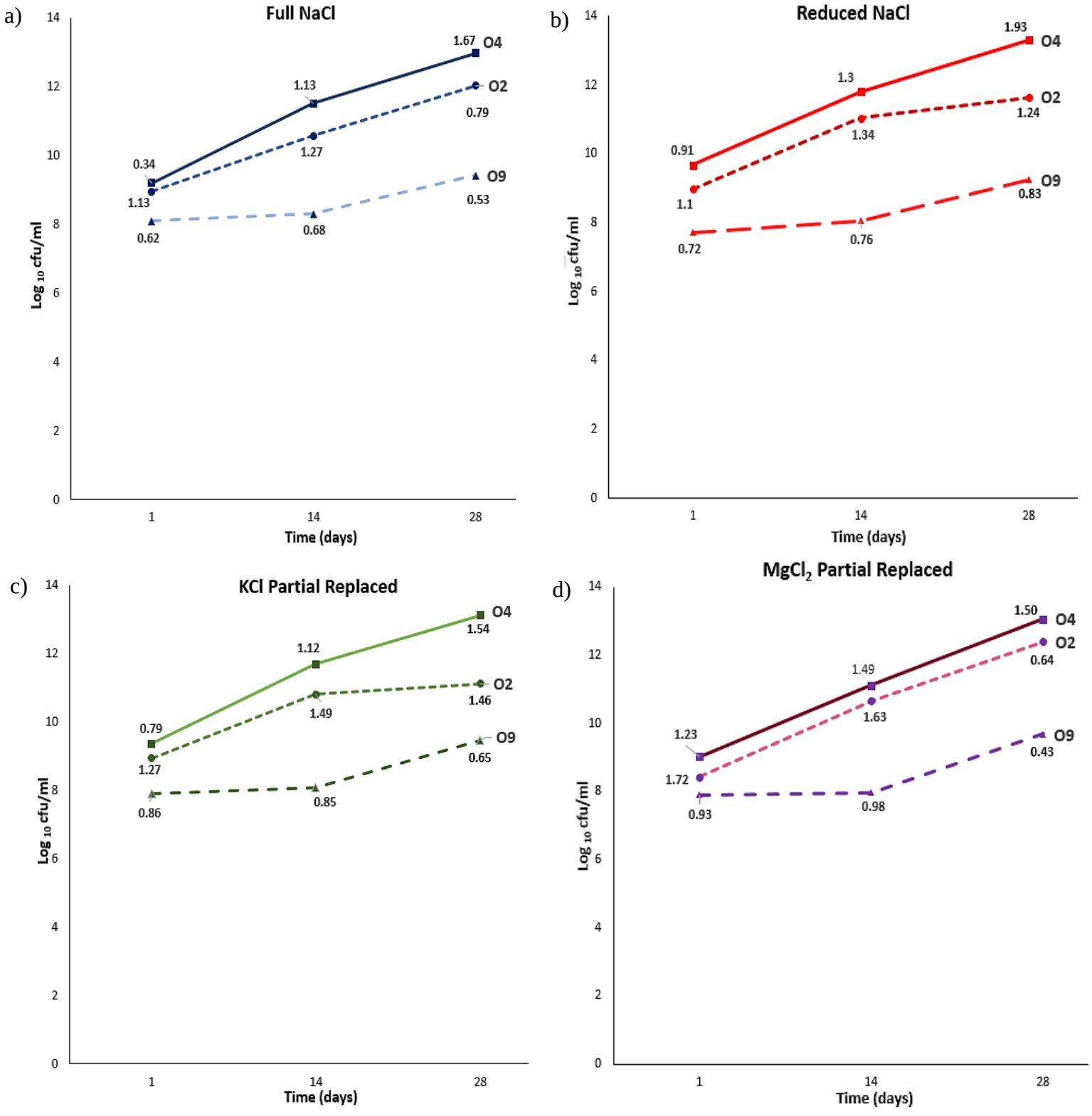


Figure 4.8: LAB growth in a) Full NaCl control, (b) Reduced NaCl, (c) KCl Partial Replaced and (d) $MgCl_2$ Partial Replaced cheeses inoculated with three *E. coli* serotypes (O2, O4, O9), during storage. Results expressed are mean ± SD (illustrated for each value), n = 4

Table 4.7 indicates that there were no significant interactions observed between the main factors *E. coli* serotype, time and treatment on the physico-chemical parameters a_w, moisture content, salt content and S/M ratio of the chees samples.

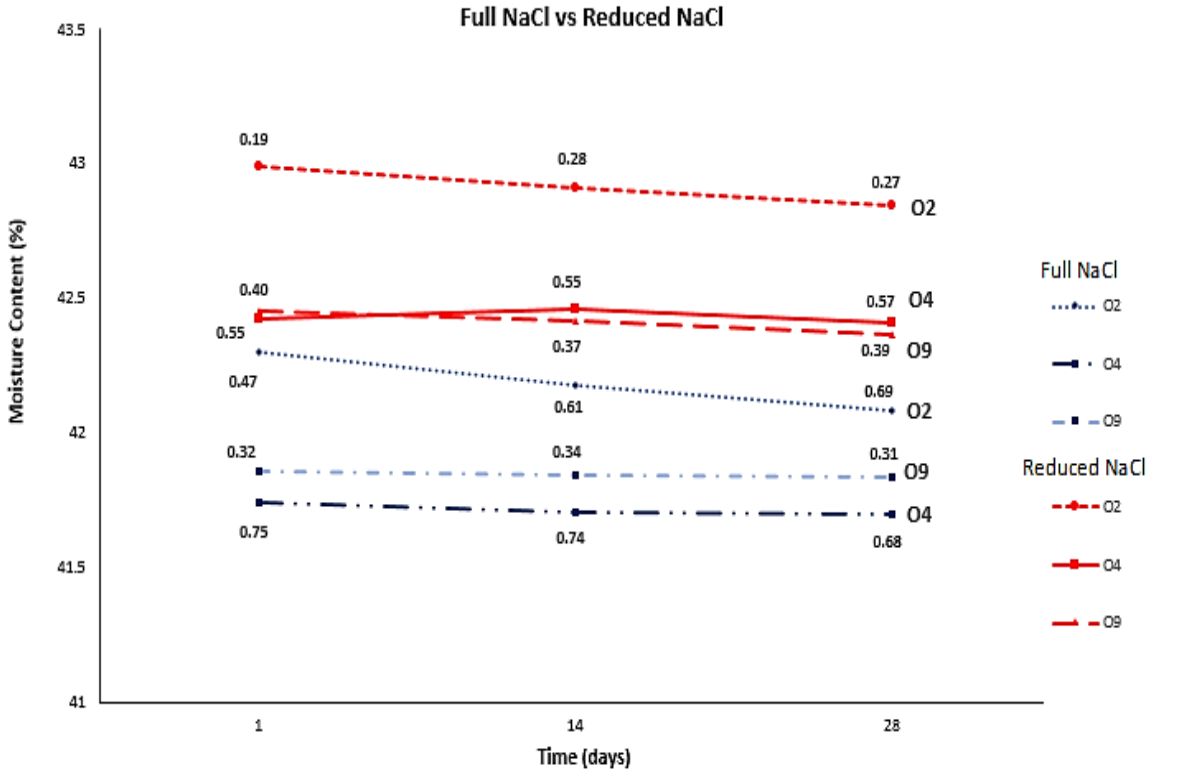
Table 4.7: ANOVA of salt treatment (Full NaCl/Reduced NaCl/KCl Partial Replaced/$MgCl_2$ Partial Replaced), *E.coli* serotype (O2, O4, O9) and time on the Water Activity (a_w), Moisture Content, Salt Content and Salt-in-Moisture (S/M) Content of Cheddar Cheeses

Independent variables	Dependent variables			
	a_w	Moisture Content	Salt Content	S/M
Treatments (Full NaCl, Reduced NaCl, KCl Partial Replaced, $MgCl_2$ Partial Replaced)	0.000	0.000	0.000	0.000
E. coli serotype (O2, O4, O9)	0.000	0.000	0.000	0.000
Time (1, 14, 28 days)	0.000	0.665	0.429	0.408
Treatments * *E. coli* serotype	0.848	0.923	0.756	0.774
Treatments * Time (days)	0.996	1.000	0.999	0.999
E. coli serotype * Time (days)	0.711	0.995	0.308	0.304
Treatments**E. coli* serotype *Time (days)	0.949	1.000	1.000	1.000

Figure 4.9 (a) and (b) indicates the moisture content of the reduced salt cheeses, inoculated with the three *E. coli* serotypes. For serotype O2, a similar trend was observed over the 28 day period. The Reduced NaCl cheeses had the highest moisture content when measured 1 day after pressing (42.9%), with the Full NaCl cheeses having the lowest moisture content (42.3%). The $MgCl_2$ Partially Replaced cheeses had the second highest moisture content (42.7%), followed by the KCl Partially Replaced cheeses with a moisture content of 42.5%.There was a common trend amongst all four reduced salt cheeses to decrease in moisture content over the 28 day period, with the Full NaCl cheeses having the largest decrease (0.3%) over this period. Similar trends were observed for both serotypes O4 and O9, with the Reduced NaCl cheeses having the highest moisture contents, the Full NaCl cheeses having the lowest moisture contents and a general trend of decrease in the moisture contents, irrespective of salt treatment or serotype, over the 28 day period.

Figure 4.10 (a) and (b) indicates the a_w of the reduced salt cheeses, inoculated with the three *E. coli* serotypes. From the graph it can be seen that the Full NaCl cheeses exhibited the lowest a_w irrespective of *E. coli* serotype. It was expected that the Reduced NaCl cheeses would have the highest a_w's due to their low salt contents, however this wasn't the case in all three serotypes. Although there was a similar trend, irrespective of the treatment or serotype for the a_w to decrease over the 28 day period, for all 3 *E. coli* serotypes, the $MgCl_2$ Partially Replaced cheeses exhibited the highest a_w after 28 days.

a)



b)

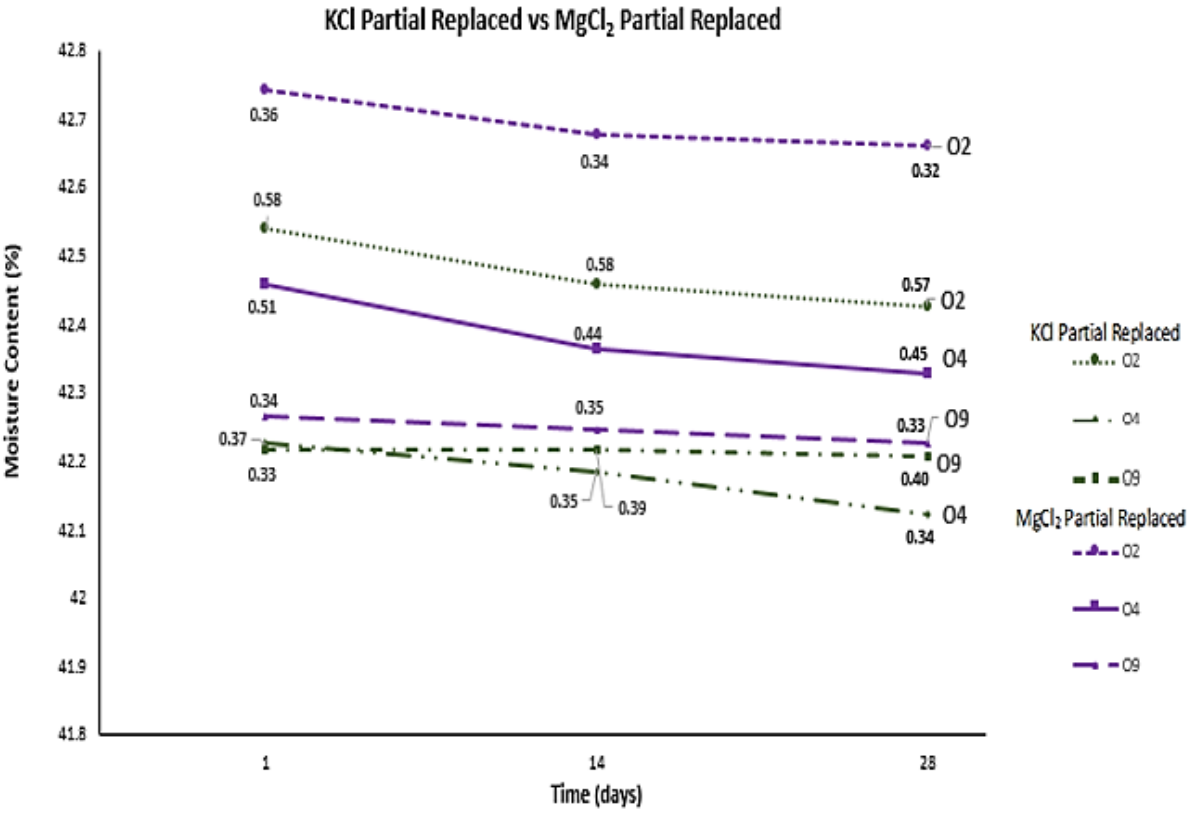
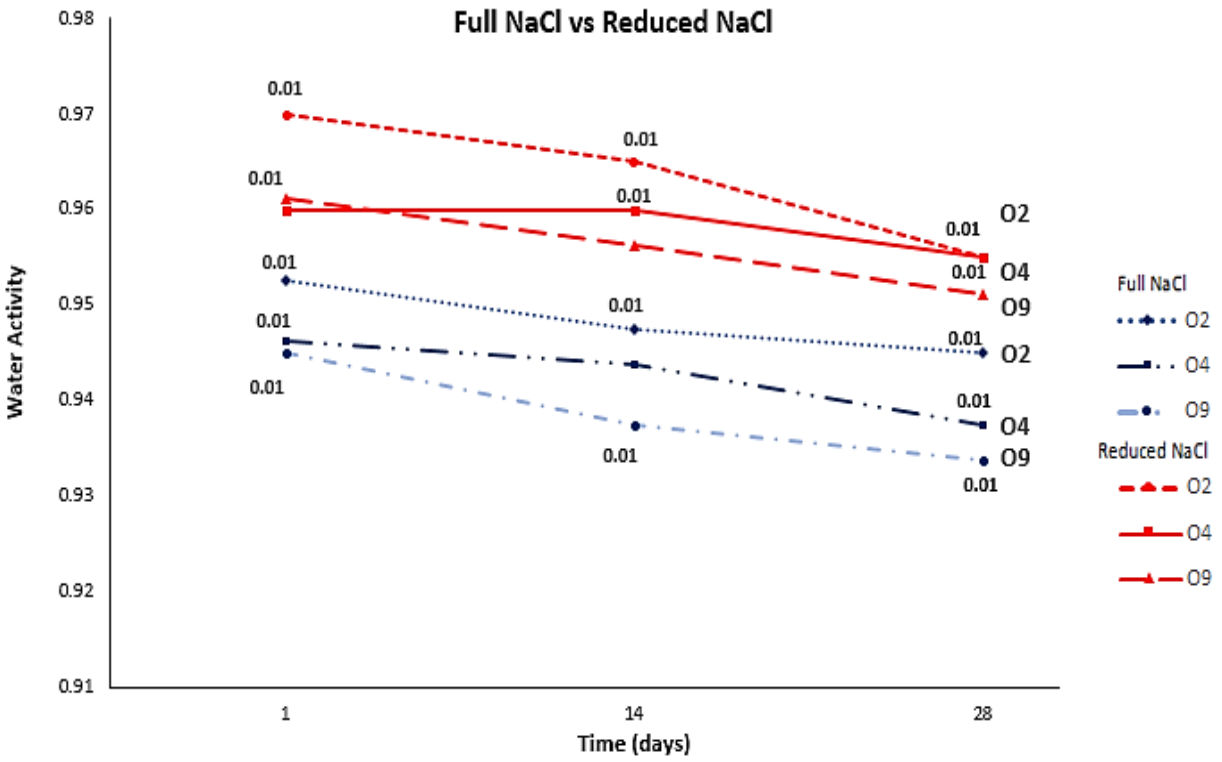


Figure 4.9: Moisture Content of (a) Full NaCl vs Reduced NaCl cheeses and (b) KCl Partial Replaced vs $MgCl_2$ Partial Replaced cheeses inoculated with three *E. coli* serotypes (O2, O4, O9) over 28 days. Results expressed are mean ± SD (illustrated for each value), n = 4

a)



b)

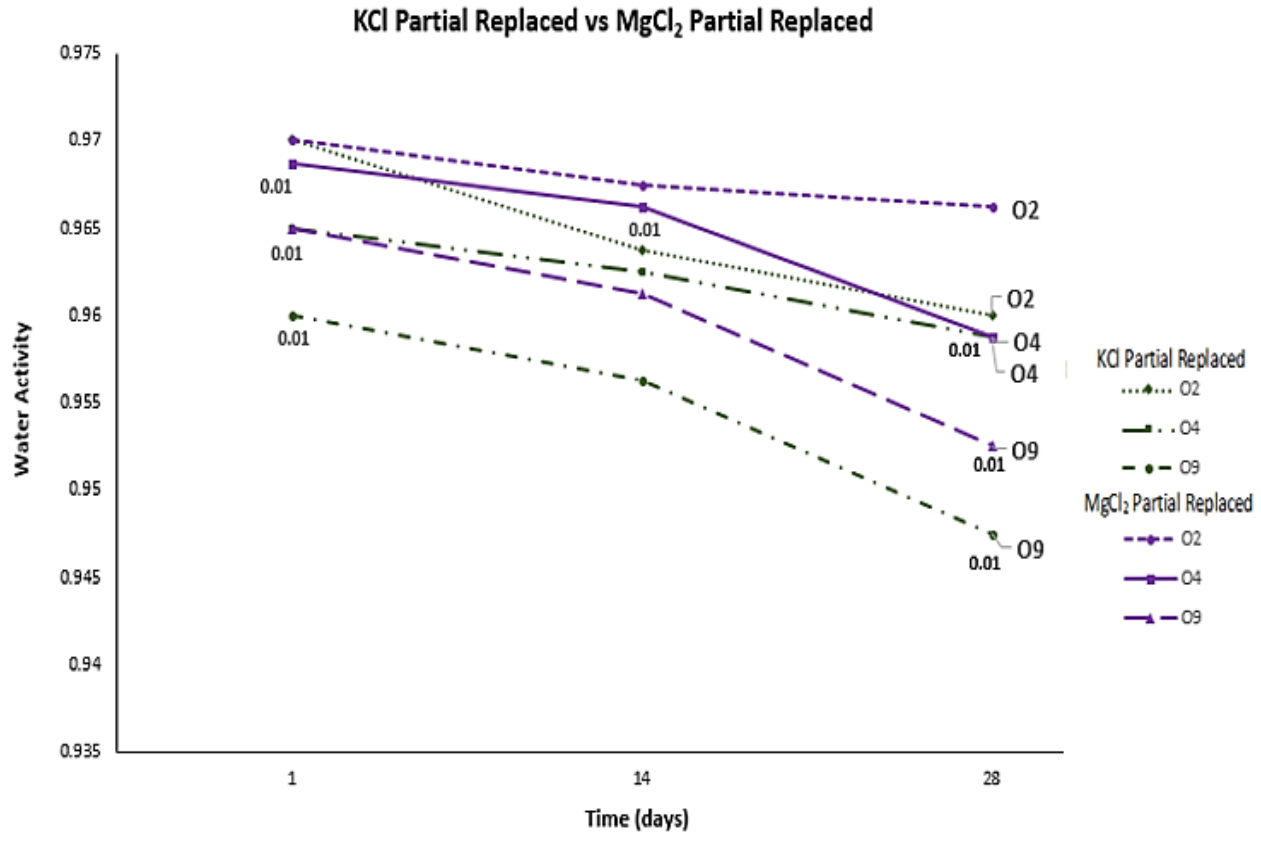


Figure 4.10: Water Activity of (a) Full NaCl vs Reduced NaCl cheeses and (b) KCl Partial Replaced vs $MgCl_2$ Partial Replaced cheeses inoculated with three E. *coli* serotypes (O2, O4, O9) over 28 days. Results expressed are mean ± SD (illustrated for each value), n = 4

4.11 DISCUSSION

E. coli and aerobic plate count growth through manufacture and storage

Irrespective of salt treatment, *E. coli* numbers from serotypes O2 and O4 increased until day 14, after which numbers began to decline to day 28. The only exception to this was seen in the $MgCl_2$ Partial Replaced cheeses, inoculated with serotype O2, in which numbers increased slightly from day 14 to day 28. This phenomenon was unexpected and when the individual trials for this treatment were closely scrutinised, three out of the four trials indicated a decrease in numbers from day 14 to day 28 as expected, with only one trial indicating an increase in numbers from day 14 to day 28 of storage. The high standard deviation associated with this particular treatment accounted for this discrepancy.

Populations of *E.coli* serotype O2 increased to approximately 5 $\log_{10}$cfu/g in the drained curd (day 0.5) and to 6 $\log_{10}$cfu/g in the pressed curd (day 1) for all salt treatments. These observed increases may have been the result of possible concentration of *E. coli* cells during the cheese making process, due to growth near its optimum temperature (Schlesser, et al., 2006). The increases could be due to entrapment of bacterial cells in the curd, and a subsequent population growth after a lag phase experienced during the heating of the curd to encourage syneresis (Maher, et al., 2001).

Populations of serotype O4 behaved similarly throughout all four treatments to serotype O2. However, counts taken after day 1 were approximately 1 $\log_{10}$cfu/g higher for all treatments, with the exception of the Full NaCl treatment which indicated approximately equal numbers to serotype O2. This could indicate that this strain possessed a slightly higher salt tolerance than the other serotypes tested, as it displayed the highest growth numbers, across all four salt treatments after 1 day (Abdulkarim, et al., 2009). It is also possible that serotype O4 was more acid tolerant and was able to withstand lower pH's than serotypes O2 and O9.

These differences exhibited by the serotypes tested can be attributed to possible physiological differences between them, as well as variance in the manufactured cheeses with regard to the level of nutrients available, redox potential and oxygen content in the cheese curd (Johnston, et al., 2000). In a study conducted to ascertain which microbial hurdle had the greatest effect on STEC growth in cheddar cheese, it was found that pH was the parameter predominantly responsible (Oh, et al., 2014). More specifically, the interaction between pH and protonated

lactic acid levels was found to have a significant effect on STEC survival (Oh, et al., 2014). Certain bacteria have the ability to adapt to stress conditions such as heat, acidity and high salt levels (Abdulkarim, et al., 2009). Enteric Gram negative pathogens such as *E. coli* are typically very acid tolerant (Oh, et al., 2014).

The method of adaptation to survival under such harsh conditions is by signal transduction, which is responsible for coordinating the expression of very specific genes crucial in cellular defence mechanisms (Abdulkarim, et al., 2009). *E. coli* and other species belonging to the family Enterobacteriaceae are not known to be able to tolerate high levels of NaCl, however certain strains are halotolerant and are able to survive in conditions of high salt concentrations (Abdulkarim, et al., 2009). It has been hypothesised that this ability to survive in conditions of high salt concentration is due to production of proline in the cells. When *E. coli* and other enteric bacteria are liberated from their hosts into the environment, they encounter numerous high stress conditions including oxidative stress, osmotic and temperature shock and nutrient starvation, to name a few. Some of these pathogenic bacteria are adapting successfully to survive in such stressed conditions and hence more research needs to be conducted into understanding fully the mechanisms deployed by the microorganisms to achieve this (Abdulkarim, et al., 2009; How, et al., 2013).

E. coli serotype O9 behaved slightly differently to serotypes O2 and O4. Instead of *E. coli* numbers steadily increasing to maximum numbers at day 14, there was a decrease in numbers from the drained curd (day 0.5) to the pressed curd (day 1), across all four salt treatments. This could indicate that this particular *E. coli* serotype was most susceptible to salt i.e it displayed the lowest salt tolerance as there was a clear decrease in numbers after the addition of salt, alternatively it was least resistant to the drop in pH.

Cheeses inoculated with *E. coli* serotypes O2 and O4 exhibited a trend of steadily increased growth of APC until day 14, after which numbers began to decline to day 28. The only exception to this was seen in the $MgCl_2$ Partial Replaced cheeses, inoculated with serotype O2, in which numbers increased marginally from day 14 to day 28, and in the Full NaCl and $MgCl_2$ Partial Replaced cheeses inoculated with serotype O4, which also increased slightly from day 14 to day 28. The high standard deviation associated with the $MgCl_2$ Partial Replaced treatment inoculated with serotype O2 accounted for this discrepancy.

When microbial cells are exposed to NaCl, they can experience a condition known as hyperosmotic shock, which leads to a shrinkage of the bacterial cell's cytoplasm, also known as plasmolysis (Taormina, 2010). *E. coli* growth in all four treatments reached their maximum at day 14, and then began to decline to day 28. *E. coli* numbers for serotype O9 were however approximately 2 $\log_{10}$cfu/g lower than for serotypes O2 and O4 across all four treatments after day 28, even though the initial level of inoculation in serotype O9 was approximately 1 $\log_{10}$cfu/g higher on day 0. There were no differences with respect to the initial level of inoculation of the three different *E. coli* serotypes in the various salt treated cheeses, indicating that the standardisation of *E. coli* was carried out satisfactorily.

In terms of the APC numbers exhibited in cheeses inoculated with *E. coli* serotype O9, a similar trend was observed in all four salt treatments. Growth of APC increased steadily over the 28 day period and was still on the rise when counts were taken on day 28. Similarly to the trends seen in the *E. coli* growth, the initial bacterial numbers taken at day 0 were on average 2-3 $\log_{10}$cfu/g higher than for both serotypes O2 and O4. Even though all cheeses inoculated with serotype O9 exhibited a steady increase in bacterial numbers over the 28 day period, the rate of growth was far slower than that of serotypes O2 and O4. These differences in growth rates could be due to physiological differences between the serotypes, resulting in differences in the lengths of the lag and exponential growth phases experienced by the different serotypes (Johnston, et al., 2000). As with the trend seen in the *E. coli* growth, serotype O4 once again exhibited the highest bacterial numbers after day 1, adding further evidence that this particular serotype was least affected by the initial addition of salt (Abdulkarim, et al., 2009). All treatments inoculated with serotype O4 showed the highest APC numbers after 28 days of storage, when compared to serotypes O2 and O9.

There were no differences in *E. coli* growth observed between the four different salt treated cheeses after 28 days. There were, however significant ($p<0.05$) differences in *E. coli* growth between serotypes O2 and O4, and O9. These differences in response of the three serotypes may be explained by strain variation which is a feature of *E. coli* O157:H7 as well as other food-borne pathogens (Mackey & Gibson, 1997; Rowe & Kirk, 1999). This was indicative of serotype O9 being less halotolerant than the other two *E. coli* serotypes. Although all the various salt treated cheeses inoculated with *E. coli* serotype O9 did indicate an increase in *E. coli* numbers up to day 14, the rate of growth was visibly lower than that of the other two serotypes.

Lactic acid bacteria growth through manufacture and storage

The LAB numbers increased throughout the 28 day storage period for all *E. coli* serotypes and for all treatments. Once again, the discrepancy between the rate of growth of LAB in cheeses inoculated with serotypes O2 and O4 differed largely to that of the growth of LAB in cheeses inoculated with serotype O9. Even though the initial numbers of LAB in all cheeses inoculated with serotype O9 were approximately 3 $\log_{10}$cfu/g and 1 $\log_{10}$cfu/g higher than the initial counts of LAB in cheeses inoculated with serotypes O2 and O4 respectively, the final LAB counts taken on day 28 were on average 3 $\log_{10}$cfu/g lower than the LAB growth in cheeses inoculated with serotypes O2 and O4. Some variation in starting LAB numbers was expected, due to intrinsic factors such as differences in bacterial content of milk with seasonal changes in the milk composition, as well as extrinsic factors such as the presence of NSLAB on the surfaces of the cheese making equipment used (McMahon, et al., 2014).

For serotypes O2 and O4 the highest LAB counts after day 1 were observed in the Reduced NaCl treated cheeses. This was expected as cheeses with lower salt contents tend to support higher LAB populations (Schroeder, et al., 1988). The reduction of salt levels in cheese, or the substitution of sodium with replacement cations has been shown to affect the salt-related response of bacteria. This is likely to redirect the starter bacteria as well as affect NSLAB survival and metabolism, consequently affecting flavour and aroma production during storage and maturation (McMahon, et al., 2014). It was expected that the Full NaCl treated cheeses would exhibit the least amount of LAB growth over the 28 day period due to those samples being treated with the highest amount of salt. This was only true in the cheeses inoculated with *E. coli* serotype O4, as the Full NaCl treated cheeses showed slightly lower LAB growth than its Reduced NaCl counterpart. In the case of the cheeses inoculated with serotypes O2 and O9, the Reduced NaCl samples exhibited less LAB growth than the Full NaCl treated cheeses.

Although dry salting of curd will inhibit starter activity, it must be remembered that the cheddar cheese curd is milled into relatively large particles (blocks) of approximately 2cm × 2cm. It therefore may take a substantial amount of time for the salt solution to diffuse through to the centres of the curd blocks, thus resulting in the possible continued growth of starter LAB's at the centre of the milled curd blocks (Guinee & Fox, 2004). There were relatively high standard deviations associated with the LAB numbers for both serotypes O2 and O4 for all treatments, which may explain some of these discrepancies.

Physicochemical changes through storage

For all *E. coli* serotypes, a similar trend was observed over the 28 day period with respect to the moisture contents of the cheeses. The Full NaCl treated cheeses had the lowest moisture content, whereas the Reduced NaCl cheeses had the highest moisture content. This was expected as the more salt that is added to a cheese, the lower the moisture content will become as more water is expelled via syneresis, as well as bound to the salt present (Tzanetakis, et al., 1991). Syneresis refers to the expulsion of the liquid whey component of the milk from the solid curd (Calvo & Balcones, 2000). When cheddar cheese is salted, the salt dissolves in the moisture phase of the curd, and becomes constrained within the network of *para*-casein micelles (Everett, et al., 2012). The addition of salt to the curd increases the hydrophobicity of the casein, which leads to shrinkage of the *para*-casein micelle network due to water loss via the process of osmosis (Doyle & Glass, 2010; Everett, et al., 2012). There was a similar trend, irrespective of the treatment or serotype for the moisture content of all cheeses to decrease over the 28 day period. This was also expected, as the cheeses experienced a concentration in solutes during storage which assists with the expelling of residual water. The gelled network is subjected to increased moisture loss over time, resulting in a more concentrated curd, reduced moisture content, culminating in the subsequent formation of a finished cheese product with the desired moisture content and textural properties (Everett, et al., 2012).

The S/M ratio which is the ratio of salt to moisture in the cheeses displayed a similar trend, with the Full NaCl treated cheeses having the highest S/M ratio and the Reduced NaCl treated cheeses having the lowest S/M ratio. The S/M ratio tended to increase over the 28 day period, which is indicative of the increased concentration of dissolved solutes, in combination with the concomitant decrease in moisture content of cheese samples. Salt content and more specifically salt-in-moisture level is one of the most important factors that affect the overall acceptability of cheese products and it is therefore considered to be a key contributor to cheese diversity, quality and safety (Guinee, et al., 2013). Cheesemakers usually aim for a S/M ratio of between 4.5% and 5.5% (Fox, 1987). This was the targeted S/M for the Full NaCl treated cheeses in this study, but it was not achieved in any of the samples. There was a correlation between S/M and *E. coli* growth, with the cheese samples having the lowest S/M exhibiting the highest *E. coli* growth. The Reduced NaCl treated cheeses inoculated with serotype O4 experienced the most *E. coli* growth after 28 days, and coincidentally had the lowest S/M ratio after 28 days (2.79%). This was followed by the Reduced NaCl treated cheeses inoculated with serotype O2 (S/M:

2.80%), and finally Reduced NaCl treated cheeses inoculated with *E. coli* serotype O9 indicating the highest S/M after 28 days of storage (3.78%).

The Full NaCl treated cheeses exhibited the lowest a_w for all 3 *E. coli* serotypes tested. This was expected as Raoult's Law tells us that by adding more solutes into a system, a larger decrease in the free energy of the water is achieved and hence a decrease in a_w is expected. Water activity in foods is dependent on two factors, namely: the level of moisture present as well the concentration of solutes of low molecular weight (McMahon, 2010; Cruz, et al., 2011). It was therefore expected that the cheeses with the lowest moisture content and highest salt content would exhibit the lowest a_w. The opposite would therefore be expected, in terms of the Reduced NaCl treated cheeses showing the highest a_w due to being treated with the least amount of salt. This however was not the case, as the $MgCl_2$ Partial Replaced samples exhibited the highest a_w for all 3 *E. coli* serotypes tested. This did not in any way make logical sense, as the Reduced NaCl treated cheeses contained 30% less NaCl than the Full NaCl treated cheeses, whereas the $MgCl_2$ Partial Replaced cheeses contained the 30% reduction in NaCl in addition to 30% $MgCl_2$ to replace the reduced NaCl.

Upon close perusal of all salt calculations and scrutinising of raw materials used, an error was found in terms of the molecular mass used for $MgCl_2$. The anhydrous molecular mass of 95.211g/mol was used for the $MgCl_2$ calculations. The form of salt that was used was however $MgCl_2.6H_2O$ (Magnesium Chloride Hexahydrate). The correct molecular mass therefore should have been 203.211g/mol. Due to this error in calculation, an incorrect amount of $MgCl_2$ was weighed out and was therefore not able to bring about the desired a_w -lowering effect expected. The addition of extra water into the system in the form of the hexahydrate could also account for an increased a_w than what was originally expected.

Due to the divalent nature of magnesium, it is possible that some of the salt may have acted in a similar manner to calcium in terms of its interactions with the casein micelles. Calcium is present in cheese in two distinct phases: insoluble casein-bound calcium-phosphate, or colloidal calcium phosphate (CCP), as well soluble calcium which is found in the aqueous phase (Cooke & McSweeney, 2013). There is the possibility that some of the added Mg^{2+} formed complexes with inorganic phosphates, thereby reducing the amount of $MgCl_2$ available in the aqueous phase to bind with water and reduce the a_w (Cooke & McSweeney, 2013). This could also account for the higher than expected a_w in the $MgCl_2$ Partial Replaced cheeses.

The KCl Partial Replaced samples showed a slightly lower a_w than their $MgCl_2$ counterparts, but were only lower than the Reduced NaCl cheeses in the samples inoculated with *E. coli* serotype O9. This was unexpected, as the KCl Partial Replaced samples contained the same amount of NaCl as the Reduced NaCl cheeses, in addition to having enough KCl to replace the 30% reduction in NaCl. It's possible that due to the small scale of testing and diminutive amount of salt added to the cheeses that uniform distribution of the salt was not achieved and hence the desired a_w -lowering effect not accomplished.

CHAPTER 5: GENERAL DISCUSSION

5.1 CRITICAL REVIEW OF METHODOLOGY

5.1.1 Limitations of the study

There were numerous limiting factors that could have affected the results of this study. Phase I of the experimental work, regarding the testing of the model developed by Grummer and Schoenfuss (2011) was conducted on a starting volume of 5L of milk, to render an approximate yield of 500g of cheese curd. All additions and calculations were based on this assumed yield. At the commencement of Phase II of the experimental work however, the initial volume of milk was reduced to 1.5L of milk, with an expected yield of approximately 150g cheese curd. All calculations were adjusted accordingly. Numerous factors can however affect the yield of curd during cheese making, some environmental and dependent on the milk composition and others that are the responsibility of the cheese maker.

5.1.1.1 Seasonal changes affecting milk quality

Due to the nature of the study, as well as the large sample size (a total of 48 cheeses were prepared), Phase II of the project spanned a period of 8 months (April to November). Milk was used only from the Experimental Farm at the University of Pretoria. It is possible, that due to the project spanning this extended period of time that the composition of the milk varied from the start to the end due to the changing diets of the cows on the farm. The milk may have varied significantly in protein and fat content, which may have had an effect on the final yield of cheese. Cheese yield is defined as the mass of cheese (kg) produced from 100 kg of milk of a defined protein and fat content, alternatively it can be defined as the volume of milk (L) required to produce 1 tonne of cheese (Lucey & Kelly, 1994). It is approximated that 10000L of milk is needed to produce 1 tonne of cheddar cheese.

Environmental factors which can affect milk composition can be divided into two distinct categories: 1) environmental factors which the dairy farmer has little to no influence over; and 2) factors which the dairy farmer can control (Covington, 1993). The primary environmental factor that the dairy farmer has control over is through the nutrition they provide to their cows. The factors that they cannot control include: seasonal changes, changes to milk composition

during lactation and gestation, age of the cow as well as ambient temperature (Covington, 1993). The milk composition can vary throughout the year, especially during lactation periods, where the concentration of milk constituents can be exceptionally low during early lactation phases and significantly higher towards later lactation periods, where the maximum yields will be attained (Lucey & Kelly, 1994). The concentration of both milk proteins and fat content vary greatly with the nutritional intake of the cows and the quality of feed consumed. Fat and protein content were not parameters that were measured, however, so one can only speculate as to the effect and magnitude that these possible seasonal changes in milk composition may have had on the yield of cheese produced throughout the experiment.

5.1.1.2 Salting of the curd

The amount of salt added to the curd was targeted to be around 2% for the Full NaCl control cheese. Cheese samples of approximately 150g were prepared. Hence a mass of 3g of salt was added to the curd during milling. Due to the salt content of the cheese being such a critical parameter in this project, even distribution of the salt throughout the curd was vital, and hence effective and uniform mixing of the salt into the curd was crucial (Robinson, 1995). Cheese samples were salted over a 30 minute period, during which small amounts of salt were sprinkled on to the milled curd blocks. Due to the exceptionally small amount of salt added to the curd, great care was taken during the weighing of the salt as well as application to the curd. It is however possible that a small amount of the salt was lost during the salting operation, or dissolved in residual water being expelled from the curds. These losses could explain a slightly lower than expected salt content in the cheese samples.

There was a slightly lower than targeted salt content in all of the cheese samples. This was possibly due to incomplete dissociation of the salts into their individual ions as well as losses during the pressing process. Another possible reason for an underestimation of the salt content could be because the titration method used is specific for measuring NaCl content, it may therefore not accurately account for the entire salt content of the cheese in terms of the partial replacers KCl and $MgCl_2$. For more precise results in terms of quantifying the salt content of the cheese samples, a chloride analyser could be used.

5.1.1.3 Pressing

The cheese samples prepared in Phase I of the experimental work were pressed with a proper cheese press in the Pilot Plant of the University of Pretoria. Cheese curds were placed into moulds and stacked on top of each other, two by two, and pressed to expel the residual whey from the curds. This pressing process also resulted in losses of salt, as some of the salt would have been dissolved in the whey and consequently been expelled during pressing.

However, at the commencement of Phase II of the experimental work, all operations were restricted to the Microbiology Laboratory at the University of Pretoria. Due to the pathogenicity of the cheeses, the cheese samples were prepared, pressed and stored in the Microbiology Laboratory. The samples were placed into small plastic moulds and pressed with the weight of stainless steel weights and bricks. Although effective at expelling residual whey from the cheese samples and knitting the curds together in the desired manner, the lack of a true cheese press made it difficult to ensure completely uniform pressure application to cheese samples, replicating the conditions that cheese samples were subjected to in Phase I of the experimental work. This may have resulted in cheese samples with varying levels of moisture as well as salt content.

5.2 RESEARCH FINDINGS AND FUTURE WORK

In June 2012, new legislation was announced by government in terms of reducing the sodium content of a number of food commodities including bread, butter and fat spreads, breakfast cereals, processed meats, savoury snacks, potato crisps, gravy powders and stock (Department of Health, 2012). Companies were given four years to adjust their formulations in order to comply with the new legislation. Cheese was however not one of the commodities included in the new regulations. Much research has been conducted into the reduction of salt in cheese, as well as the partial replacement of NaCl with salts such as KCl and $MgCl_2$. Hoffman (2013) showed that cheddar cheese can be substituted by up to 30% with KCl, without compromising on key quality parameters. This was the basis for the salt reduction executed in this study, and no differences were found with respect to growth of various serotypes of STEC non-O157 *E. coli* in Full NaCl cheeses vs Reduced NaCl and Partial Replaced cheeses. Tests were however carried out on a very small scale and more testing will be needed to confirm these results, on an industrial processing scale. It is however evident from the results obtained that

the salt content of cheddar cheese can be reduced and partially replaced with either KCl or $MgCl_2$ without significantly affecting the quality and microbiological integrity of the product. Salt content alone is not a sufficient hurdle for inhibition of bacterial growth in cheese. Salt content, along with low pH and low storage temperature work synergistically to exclude the growth of pathogenic bacteria, however much care must be taken when reducing the salt content of cheddar cheese. Reducing the salt content may disrupt the balance of inhibition of other currently non-problematic bacteria, resulting in the necessity for the replacement of its antimicrobial action (Labrie, et al., 2014). It is for this reason that further conclusive research on the effects of salt reduction as well as its partial replacement on the growth of *E. coli* and other pathogens is required.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

The *E. coli* serotypes used in this study were able to grow at a_w's greater than 0.95, irrespective of the type of salt treatment used. Even though the Full NaCl control cheeses were salted to bring about a_w's of less than 0.95, *E. coli* bacteria was still able to grow and increase in numbers for 14 days. No differences were found between *E. coli* growth in the different salt treated cheeses, regardless of differences in a_w of the various cheese samples. There were however significant differences found between the growth of the different *E. coli* serotypes used in this study. These could be due to strain variation as well as physiological differences between the *E. coli* serotypes used.

The Reduced salt cheeses substituted with salt replacers exhibited a similar effect on *E. coli* growth as the Full NaCl control. All three *E. coli* serotypes increased to a maximum after 14 days irrespective of salt treatment, after which *E. coli* numbers began to decline to day 28. Equivalent a_w's however were not achieved as intended with the use of the model developed by Grummer and Schoenfuss (2011).

A correlation was found between the S/M ratio and *E. coli* growth, with the cheese samples containing the highest S/M ratio exhibiting the least growth over the 28 day period, and those cheese samples containing the lowest S/M ratio exhibiting the highest *E. coli* growth.

The hypothesis that a_w is the most important factor with respect to allowing for or inhibiting *E. coli* growth must be rejected. It is evident that the S/M ratio is just as important, if not more so than a_w with respect to the inhibition of *E. coli* growth in cheddar cheese. A multi-hurdle approach is therefore absolutely vital for ensuring the safety of cheddar cheese products.

Although a_w is a crucial parameter in insuring the safety of cheese products, when attempting to produce cheeses with reduced sodium content, along with partial salt replacers, cheese makers should be very vigilant of the S/M ratio achieved in their final product and its effect on the growth of pathogenic bacteria such as *E. coli.*

CHAPTER 7: REFERENCES

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