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The Antimicrobial activity of Whey permeate (against *Escherichia coli* ATCC 25922)

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(M.Sc.)

Thesis submitted to Dublin Institute of Technology, Ireland, in fulfilment of the requirement for the award of the degree of

PgDip by research

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College of Sciences & Health,
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Ireland.

Under the supervision of

Dr. Catherine Barry-Ryan

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Abstract

This project evaluated the antimicrobial activity of whey samples and its potential as a new sanitising agent. Whey samples produced during the manufacture of various cheese types were tested. Different thermal treatments (65°C for 10, 20 and 30 minutes, 72°C for 15 sec and 121°C for 15 minutes) were applied to the whey samples. The impact of the heat treatment on mesophilic, psychrotrophic and lactic acid bacteria, yeast and moulds were monitored. The physio-chemical properties (pH, water activity, moisture content, ash content, soluble solids and turbidity), proximate analysis (protein content using the Bradford assay and peptide pattern using SDS-PAGE) of the various samples were determined. Their antimicrobial activity against *Escherichia coli* ATCC 25922 (microtiter plate assay) was investigated. The application of heat treatment (65°C for 20 minutes) after dialysis reduced initial microbiological load in all whey samples. Blue cheese whey sample non dialysed (ND) had the highest protein content (338.01 ± 3.79 mg/100ml) and had the highest percentage inhibition (93.29 ± 5.25%) against *E. coli* which is equal to the activity of sodium benzoate (60 mg/ml) a popular food preservative.
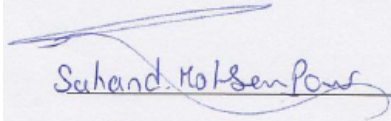
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Sahand Mohsen Pour (Candidate)

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Abbreviation list

α	Alpha
β	Beta
χ^2	Chi square
a*	Degree of red (+a*) to green (-a*)
ARS	Agricultural Research Services
b*	Degree of yellow (+b*) to blue (-b*)
Ca^{2+}	Calcium Chloride
CCP	Critical Control Points
CFU	Colony forming units
$CFUg^{-1}$	Colony Forming Units per gram
CIE	International Commission of Illumination
cm	Centimetre
FAO	Food and Agricultural Organisation
FDA	Food and Drugs Administration
FSA	Food Standard Agency
FSAI	Food Safety Authority Ireland
Fig	Figure
g	Gram
GAP	Good Agricultural Practice
GHP	Good Hygienic Practice
GMP	Good Manufacturing Practice
GRAS	Generally Recognise as Safe
HACCP	Hazard Analysis Critical Control Points
h	Hour
HCl	Hydrochloric acid
L	Litre
L*	Lightness index
LAB	Lactic Acid Bacteria
MA	Modified Atmosphere
MAP	Modified Atmosphere Packaging
mg	Milligram
mgl^{-1}	Milligram per litre

mg ml^{-1}	Milligram per millilitre
min	Minute
ml	Millilitre
MR	Moisture ratio
MRS	de Man, Rogosa and Sharpe Agar
NaOH	Sodium hydroxide
O$_3$	Ozone
OCl^{-1}	Hypochlorite ion
OH	Hydroperoxide radical
PCA	Plate count agar
R^2	Co-efficient of determination
rpm	Revolutions per minute
RTE	Ready-to-Eat
s	Second
SD	Standard deviation
Spp	Species
T	Temperature (Kelvin or ºC)
t	Time (h)
TSB	Tryptone Soya Broth
TVC	Total Viable Count
UV	Ultraviolet
WHO	World Health Organization
VTEC	Verocytotoxin-producing *Escherichia coli*
X (t)	Instantaneous moisture content (kg H$_2$O/kg dry matter)
X$_0$	Initial moisture content (kg H$_2$O/kg dry matter)
μg	Microgram
μl	Microlitre

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1. Introduction

1.1 Introduction

The retail fresh produce market has been growing rapidly during recent years. The Irish horticulture industry has increased with the annual value of the fresh produce market increased by 3% to €1.23 billion in March 2013 compared to March 2012. This increase started in 1990 as more households buy larger volumes of fresh produce regularly (Bord Bia, 2013). Fruits and vegetables are an important source of nutrition and sufficient consumption of fruits and vegetables (minimum of 400 g recommended) has been associated with a number of health benefits including the prevention of chronic disease such as heart disease, cancer, diabetes and obesity (WHO, 2003). Therefore fruits and vegetables are vital component of a healthy balanced diet and the Department of Health and Children with the Food Safety Authority of Ireland (FSAI) are involved in activity to increase the consumption of fruits and vegetables by the Irish population (FSAI, 2011).

Along with promoting the increase in consumption of fruits and vegetables by public health, it is also important that the microbiological safety of the products be secured. Ready-to-eat (RTE) fresh fruits and vegetables are consumed raw and it is critical that they are free of contamination, either chemical or microbiological.

Fresh vegetables are a rich source of vitamins and carbohydrates, but are associated with pH values conducive to the growth of spoilage bacteria, yeast and moulds. Fruits similarly can support microbial growth but have lower pH values (Beuchat, 2002). While spoilage bacteria, Lactic acid bacteria, yeast and moulds dominate the microflora of fresh fruits and vegetables, possible contamination can be from human or animal sources during harvesting, transportation, handling, processing or preparation (Beuchat and Ryu, 1997). Microorganisms impact the quality of fresh fruits and vegetables reducing their shelf life and also causing human illness (Rico *et al*., 2007).

Many outbreaks of human illness associated with the consumption of raw vegetables and fruits contaminated by pathogens, viruses and parasites have been reported across Europe and the United States (Heaton and Jones, 2008). The risk of infection disease associated to fruits and vegetables are low but the contamination and microbial growth of these products can be limited by good hygiene practice from farm to fork (Barth *et al*., 2010). The

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handling, processing and distribution stages are important, in terms of cross-contamination and quality control.

The recent appearance of pathogens such as *E. coli* O157:H7 and antibiotic resistant strains have attracted the attention of researchers. Moreover, the health risk associated with non-natural decontaminants, added to the current concerns, has lead to the investigation of natural antimicrobial agents from others sources (Stanford *et al*., 2012).

Several studies have confirmed the antimicrobial activity of proteins such as Lactoferrin which also exist in the whey obtained from dairy industry. The antimicrobial activity of whey peptides were reported against different gram-positive and gram-negative bacteria, yeast and filamentous fungi. Whey components such as lactoferrin and lactoperoxidase have been extensively studied as antimicrobial agents associated with human health and food preservatives. Lactoferrin is an 80 kDa iron binding glycoprotein, causing damage to the membrane of various bacteria and fungi by binding to the membrane and causing loss of cytoplasmic fluids. Another known protein which is lactoperoxidase, that causes damage to the bacterial and after cell membranes, is a strong oxidising agent (Rizzello *et al*., 2005; Fitzgerald and Murray, 2006).

1.2 Microbiology of fruits and vegetables

Microorganisms form part of the epiphytic flora of fruits and vegetables and some of them may be present at the time of consumption. Populations of bacteria present will vary depending on different variables such as seasonal or climatic and may vary from 10^5 to 10^7 colony forming unit per gram (CFUg^{-1}). Many of these organisms are non-pathogenic for humans. Gram-negative bacteria are dominant microorganisms on the surface of plants and belong either to the *Pseudomans* group or *Enterobacteriaceae* family (Beuchat, 2002).

The natural structures of fruits and vegetables usually make natural protection for inner tissue from spoilage microorganisms but processing technology such as slicing, peeling and cutting will affect this defence barrier and may also increase the risk of contamination with spoilage microorganisms (European Commission, 2002).

Most microorganisms that are present in fruits and vegetables are inhabitants of the soil. The microbial densities of fruits and vegetables can vary depending on the harvesting conditions or postharvest handling. Soil particles, airborne spores and irrigation water are vehicles for the distribution of these microorganisms (Nicholson *et al*., 2005; Heaton and Jones, 2008).

Understanding the ecosystem of the epithelial microorganisms of fruits and vegetables, and controlling their growth, can also reduce the risk of contamination (Beuchat, 2002). Potential sources of pre-harvest and post-harvest contamination include soil, irrigation water, faeces, dust, wild and domestic animals, insects, humans, harvesting equipment and transport containers. Other factors which influence the microbial load in fresh fruits and vegetables include intrinsic factors, which refer to the composition of food; these can vary greatly for different products, in relation to such features as: the pH of the products, water activity (a_w), nutritional content, biological structure, antimicrobial defences and wounding responses. For instance, high water activity and high nutritional value of fruits and vegetables make them suitable for microbial growth. The low pH value of fruits is favourable for growth of yeast and mould that are more acid tolerant than bacteria (Beuchat, 2002).

Extrinsic factors and environmental conditions that influence the microbial status of fruits and vegetables include storage temperature and humidity (Barth *et al*., 2010).

1.2.1 Food borne human infections associated with fresh produce

Postharvest source of contamination can result from cross contamination, equipment, water for washing and as well as storage processing and packaging (Beuchat, 2002).

In England and Wales during the years 1992 to 2003 about 7.7 percent of the outbreaks of intestinal disease reported were associated with the consumption of fruits and vegetables (Advisory Committee on the Microbiological Safety of Food, 2005). Different pathogens were reported in these outbreaks including *Salmonella* sp (21%), *Norovirus* (17%), *Shigella* (6%), *Campylobacter* (5%) and *E. coli* (3%). Cross contamination is the major problem associated with outbreaks that are linked to the consumption of fresh produce as these products are consumed raw.

1.2.2 Pathogens associated with RTE fruit and vegetable

It has been demonstrated that due to global distribution system of supplying produce in different seasons and diverse locations, there is risk fruits and vegetables of contaminated with pathogens (Heaton and Jones, 2008).

Most common pathogens associated with fruits and vegetables are presented (Table 1.1).

Table 1.1 Pathogens associated with fruits and vegetables

Bacterial	*Aeromonas* *Bacillus cereus* *Campylobacter* *Clostridium botulinum* *Clostridium perfringens* *Escherichia coli* O157 *Listeria monocytogenes* *Salmonella* sp *Shigella* *Staphylococcus aureus* *Vibrio cholera*
Viral	Hepatitis A Norovirus
Protozoan	*Cryptosporidium parvum* *Cyclospora cayetanesis* *Giardia*

Adapted from Heaton and Jones, 2008

A number of *E. coli* infections have been linked to the consumption of vegetables. One of the large largest outbreaks of pathogen *E. coli* O157:H7 that was reported occurred in Japan in 1996 which was linked to the consumption of raw vegetables. Approximately 6000 people were affected and 3 deaths resulted (European Commission, 2002).

1.2.3 Spoilage microorganisms on fresh fruits and vegetables

Spoilage of fresh fruits and vegetables occurs due to the activity of microorganisms such as fungi and bacteria. These precipitate changes in the colour, texture and odour of fruits and vegetables, a process known as rot. Factors such as the presence of wounds and damage to products during storage, in addition to high water content, will facilitate the spoiling process in fresh fruits and vegetables. The most common fungal infections of fruits are

Penicillium expansum, *Botrytis cinerea*, *Monilinia laxa* and *Rhizopus stolonifer*, while the most common bacteria are *Erwinia carotovora*, *Xanthomonas campestris* pv. *Vesicatoria, Lactic acid bacteria* and *Pseudomonas* spp have been isolated from fresh vegetables. *Pseudomonas* is the most common gram-negative and psychrotrophic spoilage microorganism of refrigerated fruit and vegetables. Pseudomonads are heat sensitive and disappear in heat processed food. They produce pectolytic enzymes which is contribute to spoilage of produce (Tournas, 2005; Barth *et al*., 2010).

1.3 Shelf life of F&V

The shelf life of fruits and vegetables is the length of time during which the quality of product remains intact, before food begins to develop undesirable characteristics which may be chemical, physical and microbiological. A period of 8-14 days has been considered as the average shelf life associated with whole and fresh-cut fruits and vegetables (Barth *et al*., 2010). Microbiological spoilage affects the shelf life and quality of fruits and vegetables, as it causes surface discoloration, moisture loss, unpleasant aromas, flavour changes, texture changes, soft rot and microbial colonies. Therefore, microbial spoilage can be a reliable indicator of quality loss of fruit and vegetables as microbiological shelf life and sensory shelf life are very often the same (Barth *et al*., 2010). Measuring shelf life can be carried out by analysing different parameter such as quality (headspace, dry matter, colour changes, pH, texture and sensory analysis), microbial enumeration (mesophilic, psychrotrophic and lactic acid bacteria) and nutritional marker throughout the storage time (Ahmed *et al*., 2011).

1.4 The detection and isolation of microorganisms

The methods adopted for the detection of microorganisms depend on the visible damage to a sample infected by spoilage microorganisms. If there is no visible sign of disease, sampling and enumeration will provide a viable count of microorganisms at or near the outer surface of the produce. The sample is added to the sterile diluents to achieve a serial dilution, phosphate-buffered saline or 1% buffered peptone water can be used as diluents. The stomacher is one of the most common and efficient mechanical methods available for sample preparation in the food industry. Serial dilution following sample preparation is

followed by the spread plating method (0.1ml), and, subsequently, incubation at a particular temperature. The incubation time and temperature varies among a wide range of different microorganisms (Barth *et al*., 2010).

Selective media for enumeration of microorganism are as follows: plate count agar (PCA) can be used for detection of mesophilic bacteria with incubation time at 30°C for 72 h. Enumeration of psychrotrophic bacteria will be carried out using plate count agar (PCA) at 4°C for 7 days and DeMan rogosa sharp agar (MRS) at 35°C for 48 h will be used for enumeration of lactic acid bacteria (Ahmed *et al*., 2011).

1.5 Preventing microbial contamination along the food chain

There are different sources of contamination which must be monitored in order to minimise the risk of microbial contamination of fresh produce.

- *Preventing microbial contamination in the field*

Many pathogens are living in the soil where the vegetables are grown either directly with their roots or close to the soil as leafy vegetables so there is potential of contamination during growing (Beuchat, 2002).

There are different factors that affect the survival of microorganism on soil such as: moisture content, temperature and the type of soil. *E. coli* O157:H7 and Salmonella sp may survive in soil from 7 to 25 weeks depending on these factors (Lang and Smith, 2007). A washing step in the packaging process remove the soil but it's difficult to eliminate the risk of soil-borne contamination from vegetables. Animals, insects and birds can also act as reservoirs for human pathogens which should be prevented from entering fields.

Animal waste is added to soil as a source of nutrients for developing plant. For minimising the risk of microbiological contamination of fruits and vegetables, the FSA has issued guidelines (FSA, 2005). The Food Safety Authority of Ireland (FSAI) has also produce guidelines on the use of manure and compost in the fresh produce supply chain in ROI (FSAI, 2001).

The other important potential source of microbial contamination is water as required during irrigation, pesticide application, washing step and cooling system that influence the microbial safety of fresh produce (Barth *et al.*, 2010; Warriner *et al.*, 2009). Critical factors in this contamination are related to amount of water that is applied which will affect the bacterial level, microbiological quality of water and length time of application that also affect the pathogen survival rate. FSAI have produced guideline to minimise the risk of contamination of water in fresh produce supply chain (FSAI, 2001).

- *Preventing microbial contamination during harvesting*

It is important to prevent microbial contamination during harvesting as hands are used in much of the harvesting process. Also preventing cross-contamination through harvesting equipment and transport vehicles are important (Chilled Food Association, 2002).

- *Preventing microbial contamination during processing steps*

There are different steps in the processing of minimally processed vegetables and it is critical to follow hygienic practices in order to eliminate risk of contamination and prevent damage from raw material to the end product. Temperature of processing is also important to prevent product spoilage and also to prevent the growth of microorganisms. In addition to that the prior quality of vegetables for minimal processing must be a good grade, easily washable and peelable (FSAI, 2001).

The first step in minimal processing of fresh fruits and vegetables is removal of outer surface contamination (Figure 1.1) by washing method to eliminate dirt, pesticide residues, soil and foreign bodies (Gil *et al.*, 2009). This is an essential step as most contaminants are on the surface and must be sufficient to reduce contamination. Many researchers have recommended using salt sanitiser such as sodium or calcium hypochlorite for surface sanitation of fruits and vegetate in order to extend the shelf life of product and to prevent the microbial growth (Gorny *et al.*, 2002).

The next step is cutting step which is important in terms of microbial growth which might occur due to physical damage (Figure 1.1). Therefore it has effects on the nutritional value and shelf life of minimally processed fruit and vegetables (Parish *et al.*, 2003). Many machines can slice, shred and chop fresh produce. It is critical to prevent cross contamination from surface to internal issue by disinfecting and washing process (Allende and Artés, 2003).

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Raw material

Manual trimming and preliminary washing
(Removal of outer layers, soil and dirt)

Slicing or shredding

Washing and/or disinfection
(e.g. 100 mg/l chlorine solution)

Moisture removal
(air or centrifugal drying)

Packaging
(Modified atmosphere packaging, ideally 2-5%
O_2, 3-10% CO_2)

Storage at refrigeration temperatures
(2-5°C)
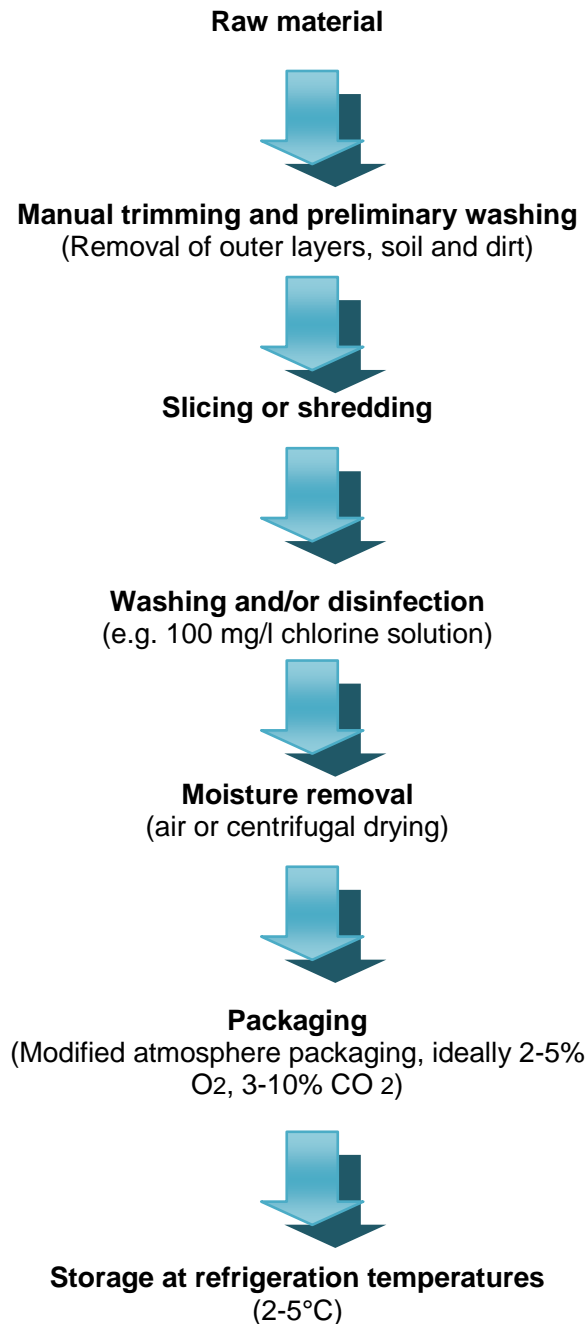
Figure 1.1 Flow diagram for the production of minimally processed vegetables

Source: Francis *et al*., 1999

1.5.1 Washing and disinfection process

Washing is a critical step in processing of fresh produce in order to remove the dirt from the surface, reduce microbiological and chemical load on the produce and enhance the shelf- life of the product (Kim *et al*., 1999).

Therefore an affective decontamination process is an essential step prior to packaging (Figure 1.1). The most common detergent for commercial disinfection of fresh produce is chlorine. Washing water containing 50-150 ppm of chlorine solution is frequently used and might be acidified to optimise chlorine efficacy with approximately 150-200 ppm of citric acid to pH value about 6.5 to 7.5 (Allende and Artés, 2003). Further to the decontamination process, the washing process should continue with a final tank stage using rinse water without chlorine which has been chilled to 1-2°C in order to remove traces of chlorine, reducing product temperature and increasing shelf life (FSAI, 2001).

1.5.2 Moisture removal

The next processing step is drying and removing water from fruits and vegetables (Figure 1.1). The water must be removed after the washing step as it may increase microbial growth and make the produce unsafe. This can be obtained by using spin dryers, racks and sieves. It is critical that to gently remove the water and try to avoid any physical damage that could lead to the quality loss of product (Heaton and Jones, 2008).

1.5.3 Packaging and storage

As fresh produce are highly perishable and have a limited shelf life at chill temperature, therefore using of advanced technologies to maximise the shelf life of products in fresh produce industry is important. In Modified Atmosphere Packaging (MAP), gases such oxygen, carbon dioxide and nitrogen are used for altering the normal composition of air surrounding the fresh cut produce in order to reduce respiration rate thus extending the shelf life of products (McMillin, 2008).

Temperature is also another important factor that influences the spoilage of harvested commodities. Generally most fresh produce are kept at refrigerator temperature 1-5 °C and will achieve maximum shelf life. The growth rate of bacteria is slow at temperatures below 5°C and below 0°C which is freezing temperature and might cause tissue damaging to the fresh products (FSAI, 2001).

However, MAP and refrigeration alone is not enough to prevent microbial growth. Psychrotrophic bacteria can remain constant and grow at refrigerated temperatures and this treatment may be less effective against *L. monocytogenes* (Parish *et al*., 2003). Hazard Analysis Critical Control Point (HACCP), Good Manufacturing Practice (GMP) and Good Agricultural Practice (GAP) should also be applied to avoid the contamination (FSAI, 2001).

1.6 Limitation of Minimally Processed Fruits and Vegetables

Fresh fruits and vegetables are eaten in their raw and uncooked form, therefore it is essential to be free of contamination. The need for decontamination strategies to maintain the safety of minimally processed vegetables is very important as there is no step in the processing of these products, such as heating, to kill the microorganisms (Parish *et al*., 2003). Furthermore there is growing customer demand for natural and additive-free products. So it's desirable to decontaminate and sanitise food products by natural disinfectants (Schuenzel and Harrison, 2002).

In order to meet customer demands food industry has produced different methods and strategies to reduce the population of microorganisms on the whole and fresh cut fruits and vegetables and extend the shelf life of products, but each one of these methods has distinct advantage and disadvantages (Parish *et al*., 2003).

For controlling microbial populations however, different methods such as chemical, physical and biological have been developed by industry, it's critical to ensure that water used for washing and sanitising process is free of contamination as well as equipment and facilities in order to prevent cross contamination (FSAI, 2001).

1.7 Chemical decontamination methods

1.7.1 Chlorine

Chlorine has traditionally been one of the most common sanitisers used by the food industry (Rico *et al*., 2007; Tirpanalan *et al*., 2011), in the form of sodium hypochlorite

(NaOCl). It is an effective sanitising agent with a broad spectrum of antimicrobial activity, it is easy to use and inexpensive. The concentration of added chlorine is typically 50-200 ppm, with 1-2 minutes' contact time. Sodium hydroxide (NaOH) and hypochlorous acid (HOCl) form with water, sodium hypochlorite and hypochlorite ions (OCl⁻) (Tirpanalan *et al*., 2011). The efficacy of chlorine is affected by pH, temperature, contact time, the quality of the water and the presence of organic material (Parish *et al*., 2003). A major disadvantage of this decontaminant is the formation of toxic by-products which affect the environment and human health, such as trihalomethans, haloacetic acid and haloketons (Gil *et al*., 2009). As a result, alternative methods are sought (Rico *et al*., 2007).

1.7.2 Chlorine dioxide

Chlorine dioxide (ClO_2) is another sanitising agent that is used because of its robust antimicrobial activity and its oxidising properties in water. It can be used in either aqueous or gas form, and, unlike chlorine, it does not produce environmentally damaging by-products (Singh *et al*., 2002). Chlorine dioxide interacts with the cell membranes of microorganisms via oxidation, removing an electron, which leads to cell damage and the disruption of the bacterial cell. It presents more advantages than chlorine, being active over a wide range of pH levels, and exhibiting less reactivity with organic material. Furthermore, it is effective in low concentrations and possesses greater oxidising power, in comparison to chlorine. However, it is an explosive gas, and must be produced on site (Singh *et al.*, 2002; Parish *et al.*, 2003; Rico *et al.*, 2007; Tirpanalan *et al.*, 2011). Its antimicrobial effectiveness has been studied in relation to *Escherichia coli* O157 H7 (Singh *et al.*, 2002), *Listeria monocytogenes* and *Salmonella typhimurium* (Lee *et al*., 2004), thus determining its capabilities in treating fresh produce. Wu and Kim. (2007) have studied the effect of aqueous chlorine dioxide in comparison to traditional gaseous chlorine dioxide, as a disinfection agent for blueberries. They revealed log reductions for *Listeria monocytogenes*, *Pseudomonas aeruginosa, Salmonella typhimurium*, *Staphylococcus aureus*, *Yersinia enterocolitica*, yeast and moulds, without the presence of negative effects on the visual quality of blueberries.

1.7.3 Hydrogen peroxide

Hydrogen peroxide (H_2O_2) has been generally recognised as safe (GRAS) for use as a bleaching agent during packaging and production processes. It exhibits oxidant and antimicrobial activity, and can be used for surface disinfection, sterilising and bleaching in the food industry. Its antimicrobial and sporicidal capacities are due to its production of oxidising agents, such as hydroxyl radicals, which damage the cell structure of microorganisms (Parish *et al.*, 2003). The primary advantage of hydrogen peroxide is it rapidly breaks down to non-toxic products. It has been recommended as a sanitiser in treating fruit surfaces prior to processing. However, it is not a perfect decontaminant for the treatment of shredded lettuce, as browning results at a swift rate (Parish *et al.*, 2003). Several studies have recommended the use of hydrogen peroxide for reducing microbial loads, while maintaining pleasant sensory properties when applied to bell peppers, cantaloupes, cucumbers, zucchinis and honeydew melons (Beuchat and Ryu, 1997). Its limitation is related to possible effect on the product colour (browning or bleaching) (Parish *et al.*, 2003).

1.7.4 Peroxyacetic acid

Peracetic acid, or peroxyacetic acid (PAA) (a mixture of acetic acid and hydrogen peroxide), possesses higher oxidising potential than chlorine or chlorine dioxide, with a wide spectrum of antimicrobial activity (Kitis, 2004). Its antimicrobial effectiveness has been studied in relation to *E. coli* O157:H7, *Salmonella typhimurium* and *Listeria monocytogenes*, for the decontamination of shredded lettuce (Tirpanalan *et al.*, 2011). The use of PAA in water processing or fresh produce, to reduce the risk of contamination, is very common (Fan *et al.*, 2009). Microbial studies have illustrated the efficacy of a peroxyacetic/octanoic mixture for improving the log reduction of yeast and moulds in recycled water processes (Hilgren and Salverda, 2000). Choosing PAA is associated with several advantages, including its effectiveness within a short contact time, its lower dependency on pH and temperature and its non-toxicity (it decomposes to acetic acid, oxygen and water). One drawback is that it is associated with an increase in organic material and effluent, in addition to the substantial costs required (Kitis, 2004).

1.7.5 Ozone

Ozone (O_3) has been recognised as a strong antimicrobial agent useful in the treatment of drinking water (WHO, 1998). It has been approved by the FDA (2001) as an antimicrobial decontaminant for minimally processed fruits and vegetables. Inactivating the decontaminants is accomplished either directly, via the reaction with molecular ozone (O_3), or indirectly, as free radicals (OH and H_2O), which are derived from ozone (Tirpanalan *et al.*, 2011). It has been recognised as safe (GRAS), from 1997, in the US for use in food processing. Ozone must be generated on site, as it decomposes quickly into water and oxygen. It does not form by-products, and exhibits greater oxidation activity than chlorine. As a decontaminant, it has limitations, due to its production of aldehydes, ketones and carboxylic acid in the presence of organic matter (Guzel-Seydim *et al.*, 2004; Tirpanalan *et al.*, 2011). Ölmez and Temur. (2010) and Kim *et al*. (1999) have studied the efficacy of ozone for combating *Escherichia coli* contamination on lettuce. They reported significant log reductions for mesophilic and psychotropic microorganisms on lettuce. Furthermore, they revealed that bubbling gaseous ozone in water is the most effective method of application.

Fan *et al.* (2012) demonstrated the effectiveness of ozone in the log reduction of *E. coli* O157:H7 and *Salmonella* sp on the surface of a packaged tomato, using the ozonation method as an alternative to a chemical sanitiser. A high concentration of ozone was formed over a short period, allowing the contact of ozone with the produce's surface. In this study, it was observed that no negative effects concerning colour and texture affected the tomato during the 22 days of storage and treatment. The major disadvantage of ozone application is related to the safety concerns of staff who are working with ozone, in addition to the high cost pertaining to its generation (Rico *et al*., 2007; Ölmez and Temur, 2010).

1.7.6 Electrolysed oxidising water

Electrolysed oxidising water (EOW), also known as electrolysed water, is generated via the electrolysis of diluted sodium chloride, producing electrolysed basic and acidic solution at the cathode and anode sites (Kim *et al*., 2000). Bari *et al*. (2003) studied the effectiveness of electrolysed acidic water in the log reduction of *Escherichia coli* O157:H7, *Salmonella*

sp and *Listeria monocytogenes*, in comparison with chlorine, and suggested that it can be used as a treatment agent for controlling pathogens in fresh produce. A shelf-life study was conducted by Gomez-Lopez *et al*. (2007), involving minimally processed cabbage under a modified equilibrium and atmospheric storage conditions. It was discovered that electrolysed water extends the shelf life of cabbage by at least 3 to 5 days. The effect of electrolysed water as a disinfectant was evaluated by Izumi (1999) on several fresh-cut vegetables. Electrolysed water containing 50 ppm chlorine illustrated a stronger bactericidal effect than that containing 15 or 30 ppm chlorine, in relation to spinach, fresh-cut carrot and cucumber. It was also reported in this study that electrolysed water did not affect the general appearance of fresh vegetables, including surface colour or tissue pH. Acidic electrolysed water (AEW) with a low pH value was associated with a high oxidation reduction capacity, and was more effective than chlorine in combating specific pathogens and spoilage microorganisms (Keskinen *et al*., 2009). Acidic electrolyzed water and neutral electrolyzed water have shown strong bactericidal effects on most known pathogenic bacteria, however, it has some disadvantages such as being corrosive for processing equipment, irritating for hands and short storage life due to chlorine loss (Len *et al*., 2002; Deza *et al*., 2005).

1.7.7 Essential oils treatment

Essential oils (EO) consist of concentrated aroma compounds, and are volatile or ethereal oils that are usually extracted from plant materials such as leaves, bark or fruit (Oussalah *et al*., 2007). Plant essential oils have GRAS status. They present a broad spectrum of antimicrobial activity useful for reducing the risk of contamination associated with food-borne pathogens linked to ready-to-eat vegetables (Gutierrez *et al*., 2008). The high efficacy of essential oils against spoilage microorganisms and specific pathogens has been reported in various studies (Hammer *et al*., 1999; Dorman and Deans, 2000; Elgayyar *et al*., 2001; Gutierrez *et al*., 2008). Gram-negative bacteria are slightly less susceptible to antimicrobials than gram-positive organisms because of lipopolysaccharide present within the outer membrane (Burt, 2004).

The antimicrobial activity of essential oils against *L. monocytogenes*, *S. typhimurium*, *E. coli* O157:H7, *Shigella dysenteria*, *B. cereus* and *S. aureus* have demonstrated log reductions. Gram-positive bacteria have demonstrated greater sensitivity than gram-negative organisms when washed with bergamot, linalool or citral (Fisher and Phillips,

2006). Lactic acid bacteria, among other gram-positive microorganisms, were revealed to be more resistant, according to Holley and Patel (2005). The *Pseudomonas* species have been identified as spoilage microorganisms in fresh produce, and are more resistant to decontaminants than other species (Holley and Patel, 2005). The *Origanum* genus recognised as more effective than *Pseudomonas* species, with the exception of *P. aeroginosa* (Bendahou *et al*., 2008). Among these, *Pseudomonas aeruginosa* is the least sensitive microorganism when treated with essential oils (Burt, 2004). The effect of essential oils on sensory properties should be considered when added to food. Their application might be limited at high concentration, due to the interaction of essential oils with food components which might affect the organoleptic quality of food (Devlieghere *et al*., 2004).

1.7.8 Edible films and coatings

Edible films and coatings are transparent layers that coat the food, and can be prepared either individually or from a combination of different components such as polysaccharide-based (cellulose, chitosan, alginate, starch, pectin and dextrin), protein-based (wheat gluten, collagen, corn zein, soy, casein and whey protein) and lipid-based components (waxes, acylglycerols and fatty acidswhich), typically located as a thin layer on the surface of food, or on different layers of food components (Baldwin *et al*., 1995; Debeaufort and Voilley, 2009). Edible coatings and films not only act as a barrier against moisture, gases and volatile substances, but can also be used as food additives, such as flavouring, antioxidants, vitamins and colourants. In recent years, their anti-browning, nutritional properties and antimicrobial activities have been demonstrated, indicating that they can affect the shelf life of fresh produce, reducing the risk of pathogen growth on a cut surface (Odriozola-Serrano *et al*., 2008).

Polysaccharide coating acts as a gas and moisture barrier, protecting fresh-cut commodities from dehydration, thus increasing the shelf life of such produce (Baldwin *et al*., 1995). Protein coatings and polysaccharides possess oxygen- and moisture-resistant properties because of their hydrogen-bound structure, but, due to their hydrophilic nature, are poor water barriers (Lin and Zhao, 2007). This can be improved via the incorporation of lipids in the film's formulation. As protein coatings are commonly fragile, with a risk of cracking, the addition of plasticisers (glycerol, mannitol, sorbitol, etc.) is essential to improve their

flexibility. Some of the edible coatings have been widely used due to their antimicrobial and shelf life extension properties in food commodities (Yang and Paulson, 2000).

Several studies have demonstrated that chitosan, which is a film coating based on polysaccharides, can successfully inhibit the growth of pathogenic bacteria and fungi (Romanazzi *et al*., 2002; No *et al*., 2007). Durango *et al*. (2006) demonstrated ability to control the growth of mesophilic, psychotropic microorganisms, in addition to yeast and moulds, during the storage period, with the use of edible coatings containing chiston and yam starch in minimally processed carrots. Recently, other antimicrobial edible coatings have been recognised as effective in relation to fresh produce, such as *Aloe vera*, which possesses antifungal properties (Martínez-Romero *et al*., 2006). The effectiveness of the antimicrobial activity of edible coatings and films can be measured by inhibition zone tests such as the agar diffusion method, also known as the disk diameter test (Sebti *et al*., 2002; Min and Krochta, 2005) The effectiveness of edible coatings against *Listeria monocytogenes* (Ponce *et al*., 2008), *E. coli* O157:H7 (Raybaudi-Massilia *et al*., 2008) and *Salmonella Montevideo* (Franssen *et al*., 2003) has been demonstrated throughout various studies. Edible coatings also harbour the potential to increase the nutritional value and antioxidant activity of fruits and vegetables (Lin and Zhao, 2007).

1.8 Biological decontamination strategies

1.8.1 Bacteriophages

Lytic bacteriophages which attack and lyse bacterial cells harbour the potential to function as natural methods for the control of the microorganism population in fresh produce. Bacteriophages are ubiquitous in the environment, and their specific targeting of food-borne pathogens can be useful in food preservation research, without changing the microbial ecology of produce. The phage particle structure is composed of the core nucleic acid, which may be double- or single-stranded DNA or RNA coated with a protein shell, which forms the capsid (Ackermann, 2007). A wide diversity of bacterial viruses or bacteriophages exists. These have been grouped into six basic phage types, based on morphology and nucleic acid composition (Bradley, 1967). Group A (*Myoviridae*) possess a contractile tail, with a double stranded DNA nucleic acid type; group B (*Siphoviridae*)

exhibit a long, non-contractile tail, with double-stranded DNA; group C (*Podoviridae*) display a short, non-contractile tail, with double-stranded DNA; group D (*Microviridae*) lack a tail, and are composed of a large capsomere, with single-stranded DNA; group E (*Leviviridae*) do not possess a tail, and use a small capsomere to contain single-stranded RNA; meanwhile, group F (*Inoviridae*) do not possess a head, but exhibit a flexible filament with single-stranded DNA (Bradley, 1967).

Some 5,500 phages have been characterised using electron microscopy, and most (96%) have been identified as tailed phages, with more than half of these (61%) belonging to group B, the *Siphoviridae* family, possessing long, non-contractile tails (Ackermann, 2007). For food-borne pathogens (*E. coli* O157:H7, *Salmonella* sp, *Listeria monocytogenes*), the use of a mixture of bacteriophages for reducing the chance of lytic phage infection resistance has been studied. It is unlikely that bacteria would develop resistance to an amalgamation of bacteriophages (Sharma *et al*., 2009; Boyacioglu *et al*. 2010). Sharma *et al.* (2009) demonstrated the effect of mixing bacteriophages by combining three *E. coli* O157:H7 lytic bactriophages in a mixture, which was sprayed on fresh-cut lettuce inoculated by *E. coli* O157:H7; it proved effective in their inactivation. Boyacioglu *et al.* (2010) investigated the effectiveness of lytic bacteriophage in reducing specific pathogens under modified atmosphere packaging (MAP), which was effective when compared to a control test (not infected by phages) on fresh-cut packaged leafy greens. Leverentz *et al*. (2001) revealed that a cocktail of four lytic bacteriophages was effective in the log reduction of specific *Salmonella enteritidis* in fresh-cut honeydew melons via spot treatment.

Another study involved a mix of 6 and 14 lytic bateriophages specific to *Listeria monocytogenes*, and demonstrated their effectiveness on honeydew melons, reducing pathogen levels (Leverentz *et al*., 2003). The cocktail of *Salmonella*-specific bacteriophages was applied to the population of *S. enteritidis* on an apple slice stored at 10°C; this achieved various log reductions across different pH levels, and indicated that the low activity of lytic phages against *S. enteritidis* might be related to pH (4.2) in sliced apple. These results indicated that selective bacteriophages used for the inactivation of a pathogen population should be evaluated via an *in vitro* study, in order to fulfil the customer demand for fresh produce (Leverentz *et al*., 2001).

1.8.2 Protective culture

Microbial antagonism has been recognised in food preservation for many years. Using microbial cells to control other populations of microorganisms involves a biological control which displays different rates of growth, competition for space and nutrition or creating antimicrobial substances between competitors (Cleveland *et al*., 2001). *Pseudomonas spp.* has been recognised as a biocontrol agent in the spoilage of fruit and vegetables. The industry has developed some biocontrol products based on *Pseudomonas*, such as the 'Biosave series' used to reduce the fungal blight of fruits (Mikani *et al*., 2008). In recent studies, some strains of *Pseudomonas* selected from apple and leaf surfaces demonstrated potential as a biocontrol agent of grey mould (Mikani *et al*., 2008).

1.8.3 Bacteriocins

Bacteriocins are antimicrobial peptides produced by a variety of bacteria that can reduce the microbial population when applied during washing treatment (Abriouel *et al*., 2011). Several studies indicate that the bacteriocins produced by lactic acid bacteria (LAB), or *Bacillus* species, are bio-protective in relation to fruits and vegetables (Cascales *et al*., 2007; Abriouel *et al.*, 2011). Bennik *et al*. (1997) suggested that the application of bacteriocinogenic lactic acid bacteria used in the inhibitory activity of *Listeria monocytogenes* and *Clostridium butulinum* might prove effective in minimally processed vegetables and in the inoculation of bacteriocin-producing lactic acid bacteria in ready-to-eat salad, reducing the microbial density in total mesophilic bacteria populations, particular *Coliforms* and *Enterococci*. LAB have historically been used as preservatives in the dairy industry (Stiles and Holzapfel, 1997), and are generally recognised as safe (GRAS), being approved by the FDA (FDA, 1998). Allende *et al*. (2007) have demonstrated the log reduction of *L. monocytogenes* using LAB on fresh-cut lettuce during storage. Another study has demonstrated the application of bacteriocins (nisin) in conjunction with sodium lactate and potassium sorbate, facilitating the log reduction of *Salmonella* sp on both whole and fresh-cut cantaloupe (Ukuku *et al*., 2005).

1.9 Miscellaneous decontaminants

1.9.1 Acidic sodium chloride

Acidic sodium chloride (ASC) is a low-pH sodium chloride, with GRAS status that has been approved by the FDA for fresh-cut produce (FDA, 2010). The log reduction of a microbial population has been demonstrated by ASC 1.2g/l against *E. coli* O157:H7 and *Salmonella* sp (Park and Beuchat, 1999). Gonzalez *et al*. (2004) reported the efficacy of ASC, at a concentration of 1.1g/l, in the treatment of *E. coli* O157:H7, in minimally processed carrots, compared with other decontaminants, such as chlorine, citric acid, and peroxyacetic acid which was effective.

The antimicrobial activity of ASC at a concentration of 1.2g/l was studied by (Stopforth *et al*., 2008) against *Salmonella* sp*, Listeria monocytogenes* and *E. coli* O157:H7. It was inoculated onto leafy greens. Log reduction of the microbial population was accomplished without affecting the physical appearance of the leaves. Ruiz-Cruz *et al*. (2007) demonstrated the effect of this sanitiser on the biochemical and nutritional properties of shredded carrots, rather than the microbiological aspects. In this study, the glucose, fructose, sucrose, carotene and antioxidant capacity in the carrot was retained at a higher level when treating with ASC sanitiser at a concentration of 0.5g/l, compared to unwashed controls.

1.9.2 Lactic acid

Lactic acid (LA), which has GRAS status (FDA, 2010) as an antimicrobial decontaminant, has been investigated across several studies. Sagong *et al*. (2011) revealed the effectiveness of washing with LA (1%) for the log reduction of *E. coli* O157:H7, *Salmonella typhimurium* and a *Listeria monocytogenes* population on iceberg lettuce, without any changes in colour or firmness. The efficacy of LA solution can be enhanced by increasing temperature (Huang and Chen, 2011).

1.9.3 Calcinated calcium

Calcinated calcium is the calcinated powder of oyster shells that is produced by incineration, mainly composed of calcium oxide. It has achieved GRAS status, similarly to calcium oxide (FDA, 2010), and has been reported as an antimicrobial decontaminant in several studies. Bari *et al.* (2002) studied the effectiveness of calcinated calcium solution in the log reduction of *E. coli* O157:H7, *Salmonella* sp and *L. monocytogenes* populations on the surface of a tomato. Another study conducted by Kim *et al*. (2011) demonstrated the effectiveness of this decontaminant in controlling microbial populations inhabiting minimally processed lettuce and broccoli, while retaining good quality during storage.

1.9.4 Levulinic acid

A mixture of 3% levulinic acid with 1% sodium dodecyl sulfate (SDS) has been identified as effective in the log reduction of microbial populations inoculated in romaine lettuce (Zhao *et al*., 2009). Levulinic acid and SDS have not yet been approved by the FDA for use in lettuce treatment, but the FDA has approved levulinic acid for other uses, such as food additives for human consumption (FDA, 2010). Another study conducted by Guan *et al*. (2010) demonstrated a low log reduction of *E. coli* O157:H7 following treatment with 0.5% levulinic acid mixed with 0.05% SDS on iceberg lettuce.

1.10 Physical methods for decontamination

Different physical methods are used in industry to obtain microbial decontamination such as heat treatment, radiation and filtration.

One of the physical methods for treatment of food is irradiation that can extend the shelf life of minimally processed fruits and vegetables. It uses a form of energy called ionising radiation that exposing the food to gamma and X-rays for a limited time. A low dose irradiation (0.25-1.0 KGy) is common for decontamination of fruits and vegetables extending the shelf life and delay ripening. The undesirable effect of irradiation is the formation of lipid oxides and also changing the chemical composition of food and affecting the flavour, odour and texture.

1.11 Potential Future Sanitiser – Whey & whey permeate

Whey is a liquid by-product and protein rich that is obtained during the production of cheese. It has been promoted as a functional food boasting a number of health benefits, due to its nutritional and biological properties (Ahmed *et al*., 2011). The components of whey include α-lactalbumin, β-lactoglobulin, lactoferrin, glycomacropeptide and immunoglobulins, which are associated with antimicrobial, antiviral and antitumour activities (Marshall, 2004). In the cheese-making process, whey after processing contains water, lactose, protein, lipids and minerals (Abboud *et al*., 2010). One of the problems associated with the process of cheese production is the generation of a large volume of whey. This can accumulate to the level of approximately 9 kg for every kilogram of cheese manufactured (Martin-Diana *et al*., 2006). Several techniques have been employed for exploiting this, such as feedstock fermentation, for the production of lactic acid, acetic acid, propionic acid, ethanol and single cell protein (Panesar *et al*., 2007; Ahmed *et al*., 2011). The problems associated with whey relate to its high lactose content, in addition to its high COD (chemical oxygen demand) and BOD (biological oxygen demand). With the evolution of separation technologies such as ultrafiltration, for concentrating protein, and diafilteration, to remove most of the lactose, the industry is able to produce whey protein concentrate. Therefore, whey permeate has been evaluated as a bio-preservative prolonging the shelf life of fresh-cut vegetables (Martin-Diana *et al*., 2006; Ahmed *et al*., 2011). Various concentrations of delactosed whey permeate treatment were examined by Ahmed *et al.* (2011), in comparison to traditional chlorine treatment for tomatoes during storage. They revealed a significant reduction of the total microbial count, in addition to yeast and moulds, without affecting the sensory properties of tomato, compared with chlorine. Minor antimicrobial peptides detected in whey (Kitts and Weiler, 2003) act against a wide spectrum of gram-positive and gram-negative microorganisms, yeast and fungi (Rizzello *et al*., 2005).

Several studies have demonstrated that lactoferrin plays a significant role in combating pathogens within the body (Breton-Gorius *et al*., 1980; Boxer *et al*., 1982), as lactoferrin chelates iron, therefore depriving microorganisms of access to this nutritional source (Shah, 2000). In a review by Shah (2000), the antimicrobial and antifungal activity of lactoferrin against a number of organisms, including *Escherichia coli, Salmonella typhimurium, Shigella dysenteriae, Listeria monocytogenes, Bacillus stearothermophilus, Bacillus*

subtilis, Micrococcus luteus and *Candida albicans* (Jones *et al.*, 1994; Rizzello *et al.*, 2005; Fitzgerald and Murray, 2006) was considered. Furthermore, lactoferrin, in combination with lysozyme, demonstrated higher efficacy against *Pseudomonas aeruginosa, Listeria monocytogenes, and E. coli* (Shah, 2000). In addition, the effect of whey protein concentrates in controlling *Helicobacter pylori* was studied by Early *et al.* (2001) and Di Mario *et al.* (2003) and highlighted the potential of whey protein concentrate in treatment of *Helicobacter pylori* infection. Ahmed *et al.* (2011) and Martin-Diana *et al.* (2006) suggested that whey could represent a promising natural bioactive alternative for the decontamination and preservation of fresh produce.

1.11.1 Whey processing

With the advancement of new technology in dairy industry membrane process technology has been developed. Different types of membranes are used in the industry for various purposes such as extending shelf life, increasing yield and quality of the dairy products. The composition and temperature of the whey make it suitable for microorganisms, thus whey obtained from industry should be either processed or cooled down to about 5 °C very quickly.
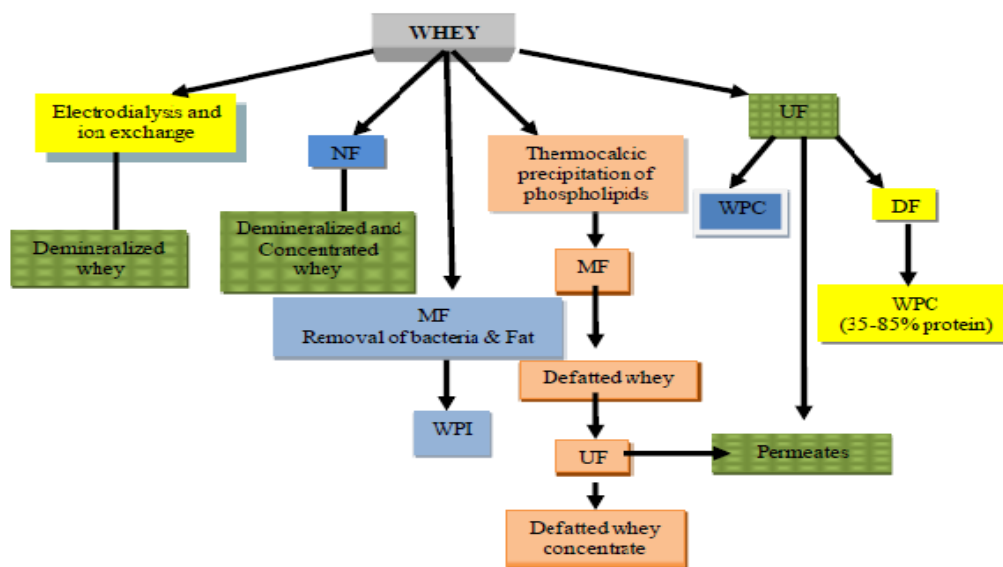
Figure 1.2 Colour scheme of membrane application in whey processing
Source: Kumar *et al.*, 2013

The colour scheme of whey processing is shown in the Figure 1.2. Separation mechanism is taking place through thin filters of a specific pore size and based on a sieving effect using

a hydrostatic pressure as a driving force. The membrane separation which including Microfiltration (MF) for removing bacterial cells and fat from the whey sample. Ultrafiltration (UF) is used for the fraction at, or of whey proteins and this separation is based on the molecular weight which usually proteins have a molecular weight cut-off range from 10-50 kDa. In this case proteins and fat can't pass and stay as retentate while water, minerals and lactose pass the membrane as permeate (Figure 1.3).
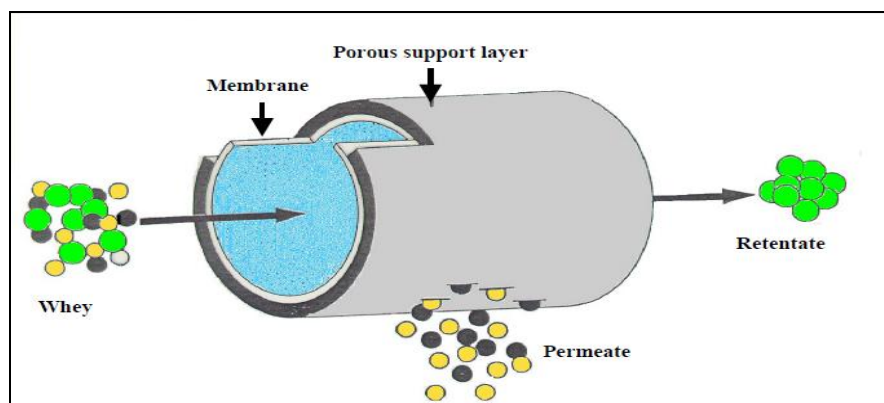
Figure 1.3 Schematic diagram of whey permeate and whey retentate
Source: De Wit, 2001

Adding more water to remove more salts and lactose is called Diafiltration. Nanofiltration (NF) is used for the removal of the salt, smaller molecules and demineralization of whey samples and it can be applied as a substitute for Electrodialysis of whey samples in the desalting processes. Reverse osmosis (RO) which is not a filtration process but it is applied to remove water against osmotic pressure. WPC could have low, medium or high protein content but whey protein isolates (WPI) have high protein content and fat is separated with microfiltration (MF) (Kumar *et al*., 2013).

1.12 Milk and milk proteins

Milk is a complex liquid secretion from the mammalian females, providing complete nutrition containing amino acids, essential fatty acids, vitamins and carbohydrates. Milk also has protein and peptides such as immunoglobulin, lactoferrin, peroxidase and lysozyme which play physiological and protective functions of the milk. The main composition of bovine milk is water, lactose, milk proteins and milk fat.

Commercially available milk from cow, goat, and sheep as well as human milk are well characterised. Genetic, environmental condition and physiological factors play important roles in the composition of different mammalian species.

1.12.1 Milk protein

Protein has been considered as the important nutrition for supporting human diet. Milk proteins are most likely the best characterised food protein system among dietary proteins and have been extensively studied since the early nineteenth century.

Milk proteins can be classified based on their solubility at pH 4.6 in to two type's casein which is about 80 % and insoluble and whey proteins about 20 % that remain soluble.

Caseins are classified into 4 groups according to their primary structure as α_{s1}, α_{s2}, β and κ-caseins. This group is composed of high molecular mass of about 10^8 Da in milk. Casein proteins are extremely stable when heated to about 100°C for 24 hours or 140°C for up to 20-25 min and they will not coagulate. The heat stability is due to the tertiary structure of caseins that result in making them insoluble in water. However, whey proteins which exist as a monomer or small quaternary structure in milk are heat sensitive. They are soluble at pH 4.6 and also completely denatured at 90°C for 10 min. Whey proteins are not phosphorylated but their sulphur content is higher than caseins.

Whey is the a by-product of cheese manufacture which remains after the removal of casein from milk and its composition varies depending on the different separation method of casein. For instance sweet whey with a pH > 5.6 contains different amounts of lactose, minerals and ash content compared to acid whey. It has been estimated that annually about 0.5 million whey by-products produced during the cheese making processes and disposal of it is an environmental problem but recently has been recognized as a valuable source of proteins (Fox and McSweeney, 2003; Walstra *et al*., 2006; Thompson *et al*., 2009).

1.12.2 Whey proteins

Whey proteins contain major proteins including β-lactoglobulin, α-lactalbumin, bovine serum albumin, immunoglobulins, proteose peptones and some other minor proteins including lactoperoxidase, lysosome and lactoferrin (Fitzsimons *et al*., 2007).

1.12.2.1 β-lactoglobulin

β-lactoglobulin is one of the major whey proteins with molecular weight of approximately 18.3 kDa and primary sequence composed of 162 amino acids. About 50 % of total whey protein is β-lactoglobulin and represents almost 12% of total milk proteins.

The molecule contains two disulfide bonds, which are located between cysteines (Cys_{66}-Cys_{160} and Cys_{106}-Cys_{119}) (Figure 1.4).

β-lactoglobulin is very acid stable and the denaturation temperature of it depends on the pH. It's most stable at pH 6.0 and heat sensitive at pH near 4.0. It is in the mainly dimer form in milk and at natural pH at room temperature but when temperature is increased above 65°C the monomer form appears.

Also β-lactoglobulin is one of those proteins in milk that are responsible for human allergy (Kontopidis *et al*., 2004).
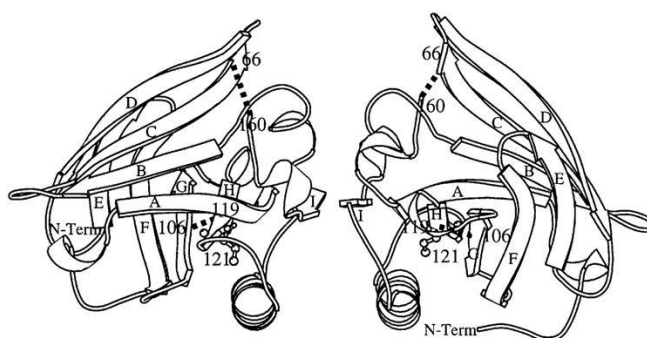
Figure 1.4 Structure of β-lactoglobulin Qi *et al*., 1997

1.12.2.2 α-lactalbumin

α-lactalbumin is a small protein in whey with a molecular weight of approximately 14 kDa and consists of 123 amino acid residues with an isoelectric point pH of 4.8. 20 % of total whey protein is α-lactalbumin and represents almost 3.5 % of total milk proteins. This globular protein has four disulfide bonds between cysteines (Cys_6-Cys_{120}, Cys_{28}-Cys_{111}, Cys_{61}-Cys_{77}, and Cys73-Cys_{91}) that make it relatively heat stable among whey proteins (Figure 1.5). Also it has a Ca^{2+} binding site that promotes the unfolding of α-lactalbumin and heat stability of it (Fox and McSweeney, 2003).

26
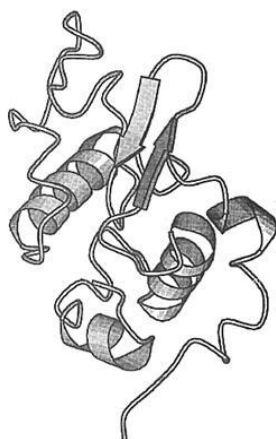
Figure 1.5 Structure of α-lactalbumin

1.12.2.3 Bovine serum albumin

Bovine serum albumin is another single polypeptide of whey with a molecular weight of approximately 66 kDa and consists of 582 amino acid residues. The isoelectric pH of it is about 5.3. It has a multi domain structure with 17 disulfide bridges and one free sulfhydryl group as a thiol group at residue 34. It can be bond to free fatty acids and flavor compounds due to its size and higher level of structure (Thompson *et al*., 2009).

1.12.2.4 Lactoferrin (LF)

Lactoferrin and Lactoperoxidase are minor whey proteins. It is an iron-binding glycoprotein with molecular weight of approximately 80 kDa which is capable of binding and transferring Fe^{3+} ions. The three-dimensional structure of human lactoferrin was first reported in 1987. It can be found on mucosal surfaces, in biological fluids, in milk, saliva, tears, nasal, intestinal secretion, pancreatic juice and seminal fluids. One of the most abundant sources of Lactoferrin is milk. The human early milk contains to 7g/l and the concentration varies in other human body fluid. Tears contain 2mg/ml and in blood only 1μg/ml, however, it can rise to a level of 200 μg/ml in the case of inflammatory condition. Bovine milk contains from 0.02 to 0.35 mg/ml of LF. At the tertiary level structure human and bovine lactoferrins are very similar sharing 69% sequence homology.

Many biological functions have been reported for LF which are more related to its iron-binding properties which make it one of the valuable proteins present in whey due to various therapeutic properties it shows (Farnaud and Evans, 2003).

1.12.2.5 Lactoperoxidase (LP)

Lactoperoxidase is a single polypeptide containing 162 amino acid residues which is characterised with a molecular mass of 80 kDa. LP is a member of mammalian peroxidases and appears in animal secretions such as tears, saliva and milk. It represents 1% (w/w) of the total protein in whey sample and has about 0.03 g/l concentration in whey (Krissansen, 2007).

1.12.2.6 Immunoglobulins (IG)

Immunoglobulins concentration in whey is about 0.7 g/l and contains a complex group that is produced by B-lymphocytes and contains three classes: IGG, IGA and IGM. IGG is divided to two subgroup, IGG_1 and IGG_2 and represent about 80% of immunoglobulins in milk or whey.

IG has either a monomer or polymer structure of two light chains and two heavy chains. Molecular weight of the light chain is about 25,000 kDa and molecular weight of the heavy chains is about 50,000 to 70,000 kDa. IG possesses immunological function (Krissansen, 2007).

1.12.2.7 Microbiology of milk

Milk provides a favorable condition for the growth of broad spectrum of bacteria, yeasts and moulds particularly at temperature above 16°C.

There are different sources of milk contamination such as the cow, air, feed stuff, equipments and personnel. Microorganisms can grow rapidly in the milk due to the nutritional content. The initial microbial count in milk may range from 10^3 to 10^6cfu/ml. As a result of poor hygienic conditions in processing, higher microbial loads will be observed in milk product (Pásztor-Huszár and Farkas, 2008).

Storage conditions of milk at low temperature will also result in a change of the number of microorganisms such as psychrotrophic bacteria that can grow at 7°C or below. The main psychrotrophic microfloras encountered in raw milk are *Pseudomonas* spp. *Pseudomonas* strains are usually proteolytic and lipolytic that can cause deterioration of milk even at low temperature after storage time (Table 1.2).

Table 1.2 Types of bacteria that are commonly associated with milk and whey

Bacterial types commonly associated with milk

Pseudomonas	Spoilage
Brucella	Pathogenic
Enterobacteriaceae	Pathogenic and spoilage
Staphylococci	
Staphylococcus aureus	Spoilage
Streptococci	
S. agalactiae	Spoilage
S. thermophilus	Acid fermentation
S. lactics	Acid fermentation
S. lactics-diacetylactis	Flavour production
S. cremoris	Acid fermentation
Leuconostoc lactis	Acid fermentation
Lactobacilli	
L. lactics	Acid production
L. bulgaricus	Acid production
L. acidophilus	Acid production
Propionibacterium	Acid production
Mycobacterium tuberculosis	Pathogenic

Source: Pásztor-Huszár and Farkas, 2008

1.13 Separation techniques and Antimicrobial activity assays

Proteins can be separated according to their properties such as: charge, hydrophobicity and molecular weight (Figure 1.6).

One of the main components of whey is Lactose which can be recovered by crystallization from whey.
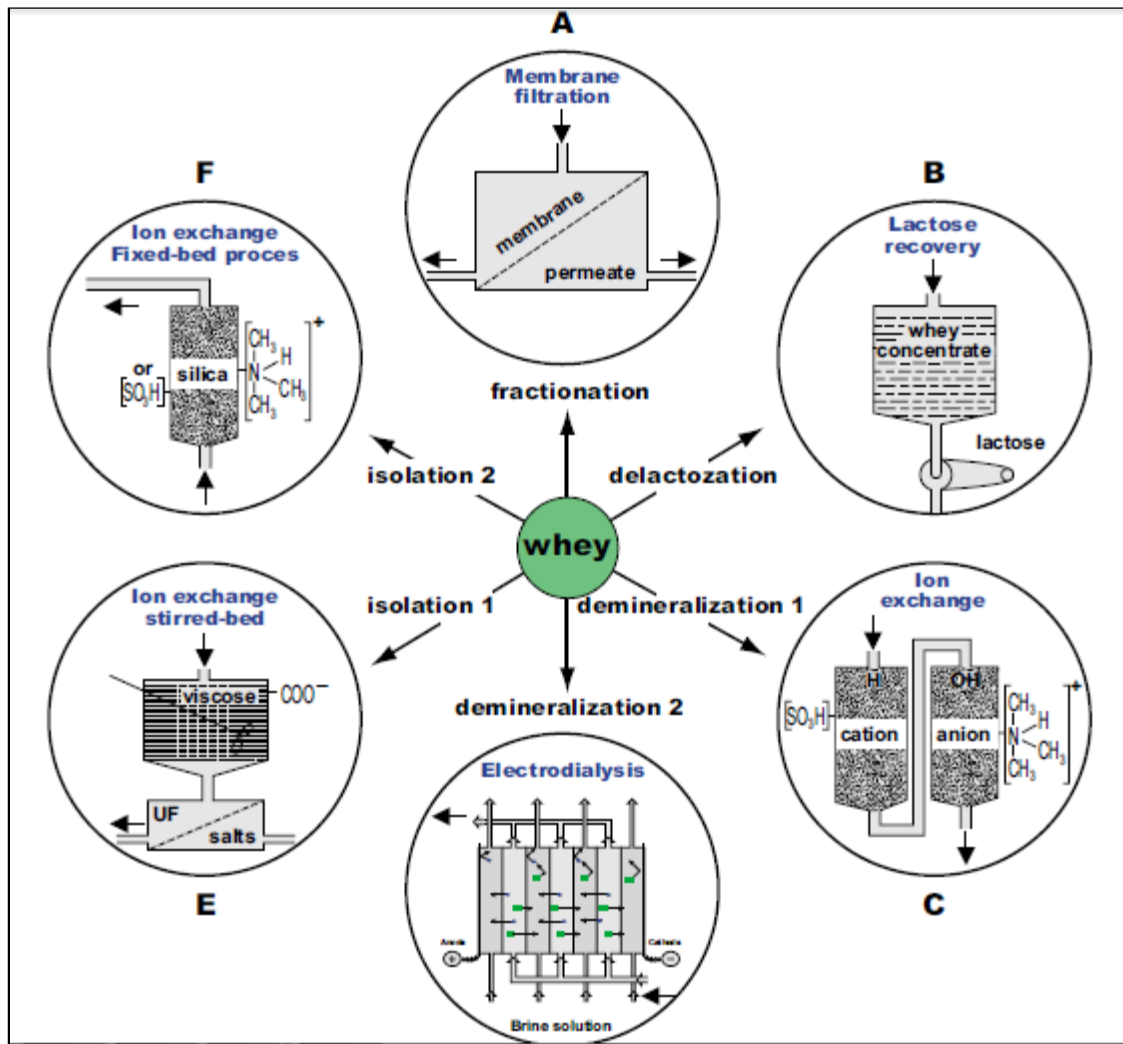
Figure 1.6 Schematic diagram of different membrane separation techniques
Source: De Wit, 2001

1.13.1 Polyacrylamide Gel Electrophoresis

Ions can be separated according to their total charge, size and shape. One of the separation techniques which is based on the mobility of ions in the electric field is Electrophoresis by migrating positively charged ions towards a negative electrode and negatively charged ions moving toward positively electrode.

Macromolecules can be separated based on their molecular weight. Mobility in an electrical field related to the molecular size and shape are directly proportional to the voltage and charge of the molecule. Proteins can be separated based on their molecular size, if at a set voltage these molecules are charged to the same degree.

In polyacrylamid gel electrophoresis (PAGE) proteins will be separated in an electrical field based on their molecular weight and they are charged negatively by binding to the sodium dodecyle sulfate (SDS).

Monomer molecule acrylamide and BIS will be polymerized by adding ammonium per sulfate (APS) and TEMED (-N,N,N',N'-tetramethylethylene diamine) as acrylamide and BIS are nonreactive just by themselves and the initial concentration of bis-acrylamide control the hardness of the gel.

High concentrations of acrylamide can cause hard gels and may cause difficulty for migration of high molecular weight components and loose gel is not suitable due to movement of some high molecular weight molecules that can migrate further (Pásztor-Huszár and Farkas, 2008).

1.13.2 SDS Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate (SDS) is an anionic surfactant that denatures the protein and prepares them for electrophoresis without breaking the peptide bonds. This denaturation of protein occurs by heating them in a buffer containing 2-mercaptoethanol as a soluble thiol reducing agent and SDS. Mercaptoethanol is used for reducing disulfide bonds and disrupting the structure of proteins. Therefore, denatured proteins can be separated based on the size in a buffered polyacrylamide gel which contains SDS and 2-mercaptoethanol (Pásztor-Huszár and Farkas, 2008).

1.13.3 Antimicrobial assay using Kinetic –reading microplate system

Chemical and physico-chemical methods, especially high performance liquid chromatography, have been introduced as a method for the analysis of antimicrobial agents' component.

The most commonly used techniques that determine the minimal inhibitory concentration (MIC) of antimicrobial agents are the agar dilution and broth dilution methods. Antimicrobial agents could be an antibiotic or any other substances that kill or inhibit the growth of bacteria.

For the agar dilution method, a solution with a defined number of bacterial cells will be spotted directly onto nutrient agar plates that have different concentrations of an antimicrobial agent.

The presence of bacterial colonies on the plate after incubation time indicates the growth of the microorganism. In broth dilution method, liquid growth medium containing an increasing concentration of the antimicrobial agent (usually a twofold dilution series) which is inoculated with a defined number of bacterial cells will be used.

The final volume of the test determines whether the assay is macrodilution or microdilution. For macrodilution assay the total volume is about 2 ml when using the test tubes and for microdilution assay the total volume is less than 500 µl per well if it is performed in the microtiter plates. After incubation time, the presence of turbidity or sediment indicates the growth of the microorganisms. The MIC is defined as the lowest concentration of the antimicrobial agents or substance that prevents the growth of the microorganisms under defined conditions (Lourenço and Pinto, 2011).

1.14 Objectives

The aim of this project is to investigate potential of whey as a new sanitising agent for fruits and vegetables by testing and optimizing the antimicrobial activity of different whey samples.

The specific objectives were:

- *Microbial assessment of various whey samples.*

- *Determining different treatments for the whey samples.*

- *Analysing the physio-chemical properties and proximate analysis of the whey samples.*

- *Examining the antimicrobial activity of whey against specific pathogens.*

- *Characterising the protein content and peptide pattern of different whey samples.*

2. Material and methods

2.1 Raw material (Whey samples)

Different whey samples in liquid form were kindly supplied by HFC Ltd (Highland Fine Cheese, Scotland) (Table 2.1). The samples were transported to the microbiology lab and stored at 4°C as chilled sample and food processing lab at -20°C as frozen samples (Table 2.1).

Table 2.1 List of different whey sample Batches received from the cheese industry

Batch	Number	Whey sample
1	1	Cheddar cheese whey non pasteurised
2	2	Cheddar cheese whey pasteurised
3	3	Cheddar cheese whey dialysed-pasteurised
4	5	Cheddar cheese whey pasteurised-ultrafiltrated-retentate
	6	Cheddar cheese whey pasteurised-ultrafiltrated-permeate
	7	Cheddar cheese whey pasteurised-dialysed
	8	Cheddar cheese whey pasteurised-dialysed-ultrafiltrated-retentate
	9	Blue cheese whey (original) pasteurised
	10	Blue cheese whey pasteurised- ultrafiltrated-permeate
	11	Blue cheese whey pasteurised-dialysed
	12	Brie cheese whey pasteurised-ultrafiltrated-retentate
	13	Brie cheese whey pasteurised-ultrafiltrated-permeate
	14	Brie cheese whey pasteurised-dialysed
	15	Skimmed milk whey pasteurised-ultrafiltrated-retentate
	16	Skimmed milk whey pasteurised-ultrafiltrated- permeate
	17	Skimmed milk whey pasteurised- dialysed
	18	Skimmed milk whey unpasteurised-ultrafiltrated-retentate
	19	Skimmed milk whey unpasteurised-ultrafiltrated- permeate
	20	Skimmed milk whey unpasteurised- dialysed
5	21	Blue cheese whey permeate
	22	Blue cheese whey retentate
6	23	Cheddar cheese whey
	24	Blue cheese whey
	25	Skimmed milk cheese whey

Figure 2.1 Different whey samples received from the cheese industry (Batch 4)
9: Blue cheese whey (original) pasteurised, **14:** Brie cheese whey pasteurised-dialysed, **6:** Cheddar cheese whey pasteurised-ultrafiltrated-permeate, **16:** Skimmed milk whey pasteurised-ultrafiltrated- permeate, **17**: Skimmed milk whey pasteurised- dialysed, **11:** Blue cheese whey pasteurised-dialysed, **13**: Brie cheese whey pasteurised-ultrafiltrated-permeate, **7:** Cheddar cheese whey pasteurised-dialysed, **8:** Cheddar cheese whey pasteurised-dialysed-ultrafiltrated-retentate, **15:** Skimmed milk whey pasteurised-ultrafiltrated-retentate

Example of different whey samples that were received from the cheese industry can be seen in Figure 2.1 and an overview of the experiments carried out in this thesis can be seen in Figure 2.2.
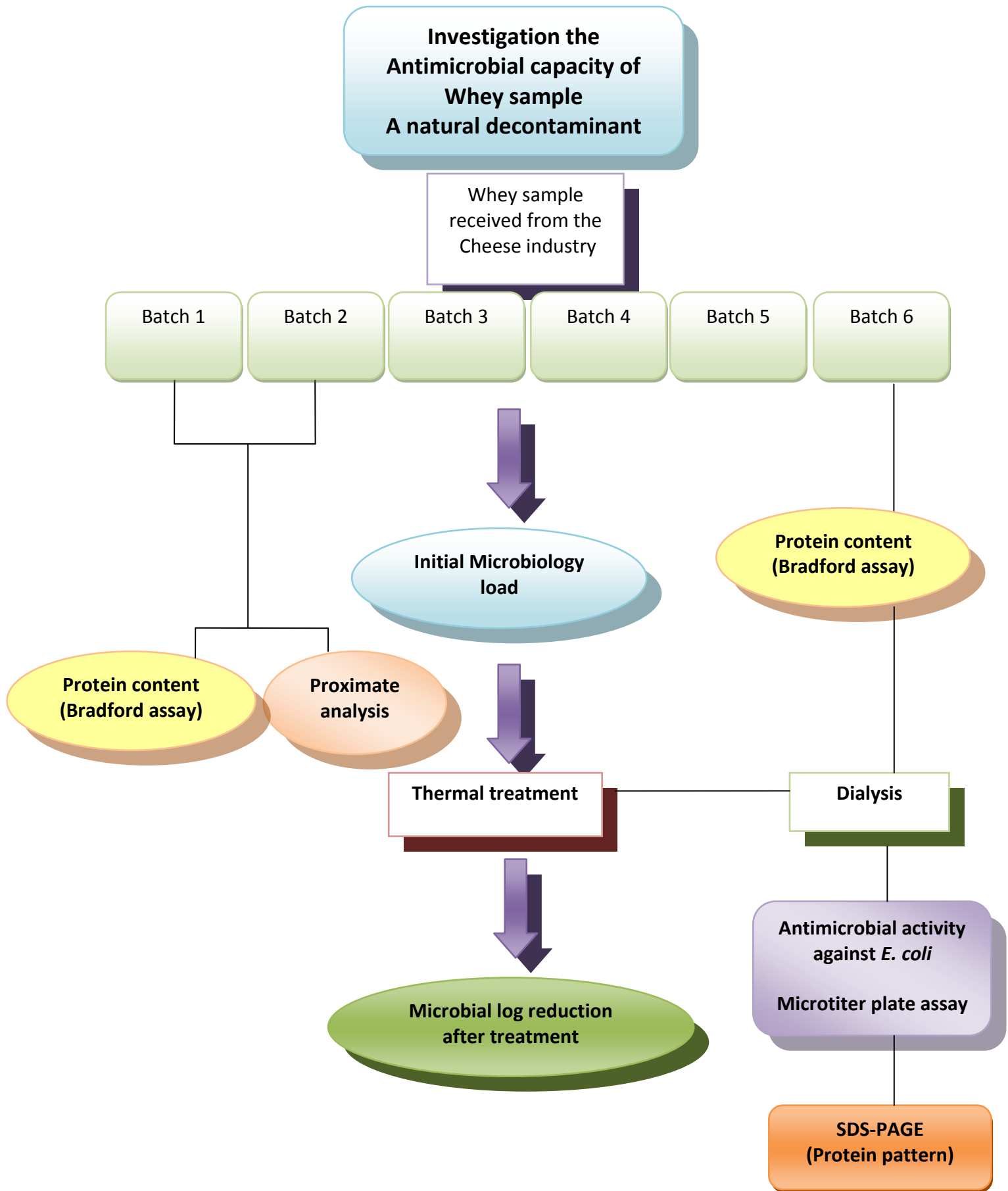
Figure 2.2 Overview of the experiments carried out

2.2 Proximate analysis of whey sample

2.2.1 *pH*

Ten grams of the samples were blended for 2 mins and the pH of whey samples were measured at room temperature using an Orion research pH-meter (Fisher Scientific, Dublin, Ireland).

2.2.2 *Total soluble solids*

Soluble solids of whey samples were determined using a digital refractometer (ATAGO, Tokyo, Japan). A drop of sample at 20°C was transferred onto the refractometer and results were expressed as Degree Brix (Sánchez-Moreno *et al*., 2006).

2.2.3 *Water activity*

The water activity of whey sample was measured with a water activity meter (Aqua Lab series 3 quick start, 3TE, Pullman WA, USA). Water activity was performed by filling half of the small plastic cup with sample, on to the base chamber. The measuring head enclosed the sample and formed an airtight seal with the base.

2.2.4 *Turbidity*

The turbidity of the whey samples was measured using a 2100QIS Turbidimeter (Hach Co, Loveland, CO, USA). Twenty ml of sample was transferred in to the transparent glass cell (dimensions 2.5 cm X 2.5 cm X 5 cm) and the absorption was read at 450 nm.

2.2.5 *Moisture content*

Moisture content was determined by the AOAC method (AOAC, 1990) (Method 925.098). Samples were weighed (4-6 g) and placed in an universal oven (Memmert, Schwabach, Germany) at 105°C overnight and then weighted again.

Equation 2.1 % moisture content = (weight of moisture / weight of sample) $\times$ 100

2.2.6 *Ash content*

Ash content was determined by the AOAC, (1990) method (method 923.098). The sample was returned to the furnace at 550°C after moisture content and left until a white ash resulted (about 4.5 hours). Cooled in a dessicator and reweighed.

Equation 2.2 % of ash = (weight of ash/original sample) × 100

2.2.7 *Colour analysis*

For colour analysis a Colour Quest XE colorimeter (HunterLab, Northants, UK) was used. Samples were placed directly on the colorimeter sensor and measured. Before measuring the instrument was calibrated using a white tile and a black tile standard. The L* parameter (lightness index scale) range from 0 (black) to 100 (white). The a* parameter measures degree of red (+a*) or green (-a*) colour and the b* parameter measures the degree of yellow (+b*) or blue (-b*) colour. The CIE*a*b*parameter was converted to Hue (arctan b*/a*) and chroma $(a*^2+b*^2)^{1/2}$.

2.3 Protein content of whey samples

Protein content of the whey samples were calculated according to Bradford, (1976). A standard curve with dependent variable (mg/ml) on the X axis and the independent variable (Abs at 595 nm) on the y axis was prepared and then the protein concentration of unknown samples was calculated using the liner regression according to Beer-Lambert Law.

2.4 Microbiological markers

Different microbial markers (mesophilic, psychrotrophic, lactic acid bacteria, yeast and moulds) were monitored for chilled whey samples stored at 4°C and some frozen sample stored at -20°C.

2.4.1 *Microbial Enumeration*

Microbiological analysis was carried out on the samples before and after thermal treatment of the whey samples. Serial dilutions were carried out using 1 ml of whey sample and 9 ml of peptone water. Test media was prepared and then 100µl of each dilution was spotted and a spread technique using a sterile spreader was used. Duplicate and control samples were taken for each sample and only counts of 30-300 log colony forming unit per millilitre were considered (Log cfu/ml).

2.4.2 *Total counts, Mesophilic and Psychrotrophic*

Plate count agar (PCA) from Biokar diagnostics (no.BK144HA) (Medical Supply Co. Ltd, Dublin, Ireland) was used for enumeration of viable microorganism. 25g of the medium were suspended in 1 litre of distilled water and then sterilised by autoclaving at 121°C for 15 minutes. The medium was cooled down to 50°C and poured into sterile Petri dishes. After spreading plates with the test sample, plates were incubated at 30°C for 72 hours for enumeration of mesophilic and at 4°C for 7 days for enumeration of psychrotrophic microorganisms.

2.4.3 *Lactic acid bacteria*

DeMan Rogosa (MRS) agar (Bioker, BK089HA) (Medical Supply Co. Ltd, Dublin, Ireland) was used for enumeration of lactic acid bacteria. 70.3g of the medium were suspended in 1 litre of distilled water. The medium was then sterilised by autoclaving at 121°C for 15 minutes and then cooled down to 50°C before pouring in to sterile petri dishes. After spreading plates with test samples, they were incubated at 35°C for 48 hours.

2.4.4 *Yeast and Moulds*

Potato dextrose agar (PDA) from Biokar (no.BK095HA) (Medical Supply Co. Ltd, Dublin, Ireland) was used for enumeration of yeast and moulds. 39g of the medium were suspended in 1 litre of distilled water. The medium was then sterilised by autoclaving at 121°C for 15 minutes. After cooling down to 50°C it was poured in to sterile Petri dishes. After

spreading with the test sample, the plates were incubated at 25°C for 72 hours. The results were expressed as Log colony forming units per millilitre (Log cfu/ml).

Data was presented as mean ± standard deviation of 2 replicates for two Batches.

2.5 Antimicrobial activity of whey sample

2.5.1 *Microbial culture*

The bacterial strain selected in this study was *Escherichia coli* ATCC 25922 (Oxoid, Dublin, Ireland) to analyse the antimicrobial activity of different whey samples. The culture was maintained at -70°C in 20% glycerol stocks and grown in Tryptic soy broth (TSB) from Sigma (no. 22092) (Sigma Aldrich, Dublin, Ireland) at 37°C for 24 hours in order to obtain sub-cultures. Working cultures were prepared for the bacterium from sub-cultures and grown for 18 hours under optical conditions. Working cultures were then adjusted to the required concentration by first making bacterial suspension in saline solution (NaCl 0.85%; BioMerieux, Marcy 1'Etoile, Paris, France) equivalent to a McFarland standard of 0.5, using the Densimat photometer (BioMerieux Inc.). This suspension was then diluted in TSB in order to obtain a working concentration of 10^6 colony forming unit per millilitre (CFU/ml).

2.5.2 *Antimicrobial activity assay*

The antimicrobial activity of whey samples were assessed against the specific pathogen using a 96-well micro titre plates (Sarstedt Ltd., Leicester, UK). A volume of 200 µl of whey sample was added to the first row of each plate. All other wells were filled with 100 µl of TSB and 100 µl from the first well was serial diluted two-fold along each column. Finally, 100 µl of bacterial suspension containing 10^6cfu/ml was added to the wells. Wells containing whey sample and sterile TSB were treated as sample blank, while control wells contained sterile TSB and bacteria suspension. The last column was used for bacterium *E. coli*, media control and samples blanks (Figure 2.3). After the plate was inoculated with bacterial culture absorbance readings were taken at 0 and 24 hours by a micro titre plate spectrophotometer (Powerwave, Bioteck, Vermont, USA) at 600 nm with 20 seconds

agitation before each optical density reading (OD) at 37°C. Sodium benzoate and sodium nitrite were used as positive controls.

Percentage inhibition was calculated according to Equation 2.3, where I is the percentage inhibition of growth, where C24 – C0 is (OD of the organism at 24 hours – OD of organism at 0 hours) and T24 – T0 is (OD of the sample at 24 hours – Blank at 24 hours) – (OD of the sample at 0hours – Blank at 0 hours). Results were interpreted by classification percentage inhibition criteria based on Table 2.2.

Equation 2.3 Bacterial inhibition I% = (C24-C0)-(T24-T0)/ (C24-C0) × 100

Table 2.2 Classification of growth inhibition in antimicrobial assays

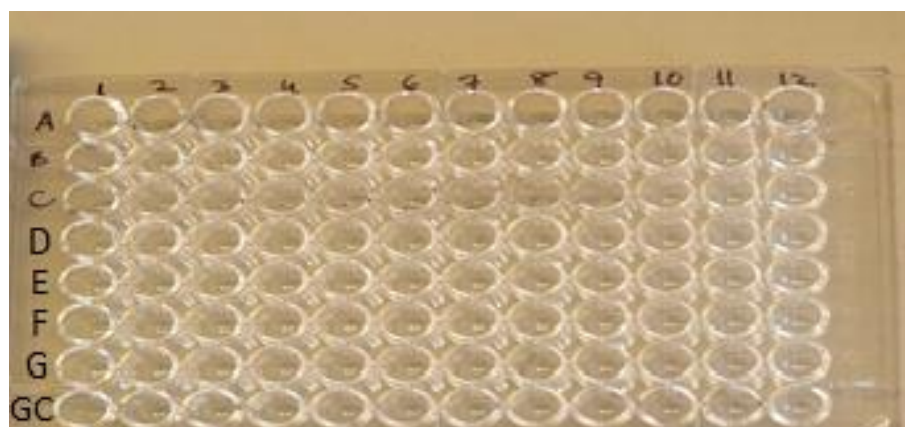| Classification criteria (% inhibition)	Classes (inhibition intensity)
100	Very strong
90-100	strong
50-90	moderate
> 50	weak

Source: Dubber and Harder, 2008

Figure 2.3 Schematic diagram of 96-well microtiter plate for antimicrobial assay

	Whey R1	Whey R2	Whey blank	Whey R1	Whey R2	Whey blank
	1	**2**	**3**	**4**	**5**	**6**
A	200 ul whey +100 ul of E. coli	200 ul whey +100 ul of E. coli	200 ul whey +100 ul of sterilize TSB	200 ul whey +100 ul of E. coli	200 ul whey +100 ul of E. coli	200 ul whey +100 ul of sterilize TSB
B	100 ul TSB+100 ul of whey from A1 +100 ul of E. coli	100 ul TSB+100 ul of whey from A2+100 ul of E. coli	100 ul TSB+100 ul of whey from A3+100 ul of sterilize TSB	100 ul TSB+100 ul of whey from A4+100 ul of E. coli	100 ul TSB+100 ul of whey from A5+100 ul of E. coli	100 ul TSB+100 ul of whey from A6+100 ul of sterilize TSB
C	100 ul TSB+100 ul of whey from B1 +100 ul of E. coli	100 ul TSB+100 ul of whey from B2+100 ul of E. coli	100 ul TSB+100 ul of whey from B3+ 100 ul of sterilize TSB	100 ul TSB+100 ul of whey from B4+100 ul of E. coli	100 ul TSB+100 ul of whey from B5+100 ul of E. coli	100 ul TSB+100 ul of whey from B6+100 ul of sterilize TSB
D	100 ul TSB+100 ul of whey from C1 +100 ul of E. coli	100 ul TSB+100 ul of whey from C2+100 ul of E. coli	100 ul TSB+100 ul of whey from C3+ 100 ul of sterilize TSB	100 ul TSB+100 ul of whey from C4+100 ul of E. coli	100 ul TSB+100 ul of whey from C5+100 ul of E. coli	100 ul TSB+100 ul of whey from C6+100 ul of sterilize TSB
E	100 ul TSB+100 ul of whey from D1 +100 ul of E. coli	100 ul TSB+100 ul of whey from D2+100 ul of E. coli	100 ul TSB+100 ul of whey from D3+ 100 ul of sterilize TSB	100 ul TSB+100 ul of whey from D4+100 ul of E. coli	100 ul TSB+100 ul of whey from D5+100 ul of E. coli	100 ul TSB+100 ul of whey from D6+100 ul of sterilize TSB
F	100 ul TSB+100 ul of whey from E1 +100 ul of E. coli	100 ul TSB+100 ul of whey from E2+100 ul of E. coli	100 ul TSB+100 ul of whey from E3+ 100 ul of sterilize TSB	100 ul TSB+100 ul of whey from E4+100 ul of E. coli	100 ul TSB+100 ul of whey from E5+100 ul of E. coli	100 ul TSB+100 ul of whey from E6+100 ul of sterilize TSB
G	100 ul TSB+100 ul of whey from F1 +100 ul of E. coli	100 ul TSB+100 ul of whey from F2+100 ul of E. coli	100 ul TSB+100 ul of whey from F3+100 ul of sterilize TSB	100 ul TSB+100 ul of whey from F4+100 ul of E. coli	100 ul TSB+100 ul of whey from F5+100 ul of E. coli	100 ul TSB+100 ul of whey from F6+100 ul of sterilize TSB
GC	100 ul TSB+100 ul of E. coli	100 ul TSB+100 ul of E. coli	200 ul TSB	100 ul TSB+100 ul of E. coli	100 ul TSB+100 ul of E. coli	200 ul TSB

	SB R1	SB R2	SB blank	SN R1	SN R2	SN blank
	7	**8**	**9**	**10**	**11**	**12**
A	200 ul SB + 100 ul of E. coli	200 ul SB + 100 ul of E. Coli	200 ul SB + 100 ul of sterilize TSB	200 ul SN + 100 ul of E. coli	200 ul SN + 100 ul of E. coli	200 ul SN + 100 ul of sterilize TSB
B	100 ul TSB + 100 ul of SB from A7 + 100 ul of E. coli	100 ul TSB + 100 ul of SB from A8 + 100 ul of E. coli	100 ul TSB + 100 ul of SB from A9 + 100 ul of sterilize TSB	100 ul TSB + 100 ul of SN from A10 + 100 ul of E. coli	100 ul TSB + 100 ul of SN from A11 + 100 ul of E. coli	100 ul TSB + 100 ul of SN from A12 + 100 ul of sterilize TSB
C	100 ul TSB + 100 ul of SB from B7 + 100 ul of E. coli	100 ul TSB + 100 ul of SB from B8 + 100 ul of E. coli	100 ul TSB + 100 ul of SB from B9 + 100 ul of sterilize TSB	100 ul TSB + 100 ul of SN from B10 + 100 ul of E. coli	100 ul TSB + 100 ul of SN from B11 + 100 ul of E. coli	100 ul TSB + 100 ul of SN from B12 + 100 ul of sterilize TSB
D	100 ul TSB + 100 ul of SB from C7+ 100 ul of E. coli	100 ul TSB + 100 ul of SB from C8+ 100 ul of E. coli	100 ul TSB + 100 ul of SB from C9 + 100 ul of sterilize TSB	100 ul TSB + 100 ul of SN from C10 + 100 ul of E. coli	100 ul TSB + 100 ul of SN from C11 + 100 ul of E. coli	100 ul TSB + 100 ul of SN from C12 + 100 ul of sterilize TSB
E	100 ul TSB + 100 ul of SB from D7+ 100 ul of E. coli	100 ul TSB + 100 ul of SB from D8+ 100 ul of E. coli	100 ul TSB + 100 ul of SB from D9 + 100 ul of sterilize TSB	100 ul TSB + 100 ul of SN from D10 + 100 ul of E. coli	100 ul TSB + 100 ul of SN from D11 + 100 ul of E. coli	100 ul TSB + 100 ul of SN from D12 + 100 ul of sterilize TSB
F	100 ul TSB + 100 ul of SB from E7+ 100 ul of E. coli	100 ul TSB + 100 ul of SB from E8+ 100 ul of E. coli	100 ul TSB + 100 ul of SB from E9 + 100 ul of sterilize TSB	100 ul TSB + 100 ul of SN from E10 + 100 ul of E. coli	100 ul TSB + 100 ul of SN from E11 + 100 ul of E. coli	100 ul TSB + 100 ul of SN from E12 + 100 ul of sterilize TSB
G	100 ul TSB + 100 ul of SB from F7 + 100 ul of E. coli	100 ul TSB + 100 ul of SB from F8 + 100 ul of E. coli	100 ul TSB + 100 ul of SB from F9 + 100 ul of sterilize TSB	100 ul TSB + 100 ul of SN from F10 + 100 ul of E. coli	100 ul TSB + 100 ul of SN from F11 + 100 ul of E. coli	100 ul TSB + 100 ul of SN from F12 + 100 ul of sterilize TSB
GC	100 ul TSB + 100 ul of E. coli	100 ul TSB + 100 ul of E. coli	200 ul TSB	100 ul TSB + 100 ul of SN from G10 + 100 ul of E. coli	100 ul TSB + 100 ul of SN from G11 + 100 ul of E. coli	200 ul TSB

Figure 2.4 Experimental design for the antimicrobial activity assay

R: Replication, **SB**: Sodium benzoate, **SN**: Sodium nitrite, **GC**: Growth control of *E. coli* without whey sample, **TSB**: Tryptic soy broth

2.5.3 *Relationship between turbidity and viable count*

A standard curve of *E. coli* (OD 600 nm versus log CFU/ml) was prepared. A bacterial suspension containing 10^6 CFU/ml was prepared as described in section 2.5.1. A volume of 200 µl from this was dispensed into the 96-well microtiter plate. Every hour the OD was read and an aliquout of 100 µl was transferred to 900 µl of diluent. By taking 100 µl of the relevant dilution on TSA a spreading plate was prepared to determine the viable count. Plates were incubated at 37°C for 24 h before determining the number of CFU/ml.

2.6 Treatment of whey sample

After receiving samples from the cheese industry and doing microbiology analysis for initial microbial load the samples were subject to heat treatment in order to reduce the microbial loads and dialysis to reduce lactose content.

2.6.1 *Thermal treatment*

Bottles of different whey samples were heated at 65°C for 10, 20 and 30 min, 72°C for 15 sec and 121°C for 15 min in order to reduce microbial loads. After which the bottles were cooled then stored at 4°C.

2.6.2 *Dialysis*

Whey samples were placed into a dialysis tube cellulose membrane from Sigma (no. d9652) (Sigma Aldrich, Dublin, Ireland). The sealed dialysis tube was placed in a container of distilled water for 24 h at 4°C. In this procedure lactose which is form of sugar tends to move out from the dialysis tube and the concentration will be decreased (Figure 2.5).
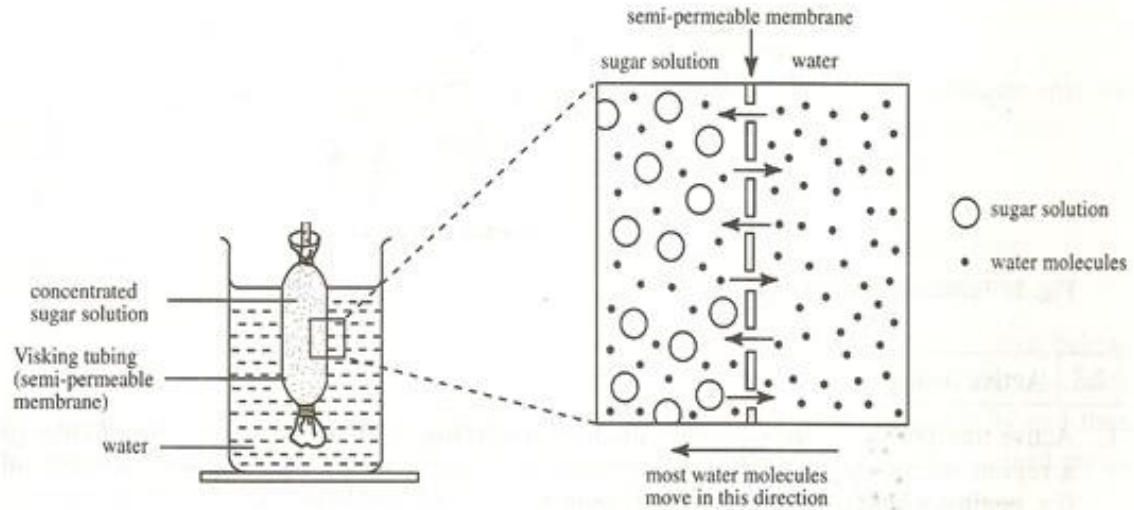
Figure 2.5 Schematic diagram of the dialysis procedure

2.7 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to analyse the protein of whey samples (Laemmli, 1970). Samples were prepared for running on the gel by adding 15 µl of the whey sample and 15 µl of the loading buffer containing β-Mercaptoethanol (Sigma, Dublin, Ireland) incubated at 99 °C for 4 min along with approximately 10 µl of pre strain protein marker, Broad range (6.5-200) kDa and (10-225) kDa (SigmaMarkerTM & Promega Marker, Dublin, Ireland).

The 4 X lower gel buffer containing 1.5 M Tris-HCl (pH 8.8) and 0.4 % SDS was prepared. Then the 12.5 % solution of lower gel was prepared by adding 5 ml of the 4 X lower gel buffer, 6.7 ml water, 8.3 ml Bis/acryl, 66 µl Ammonium Persulfate (0.1 g/ml) and 25 µl Temed (Table 2.3). The lower gel was poured and 200 µl of isopropanol was used to overlay the gel and allowed to set. The isopropanol was then removed from the gel.

The 4X upper gel buffer containing 0.5 M Tris-HCl (pH 6.8) and 0.4 % SDS was made. Then the 4.5 % solution of upper gel was prepared by adding 2.5 ml of the 4X upper gel buffer, 6.5 ml water, 1 ml Bis/acrylamide, 50 µl APS and 15 µl Temed (Table 2.3). The upper gel was added and the comb inserted and removed when the gel had polymerised fully (about 60 minutes).

Table 2.3 Standard method for making different percentages of SDS-PAGE gel

Solutions	Lower Gel (mL) for up to 8 gels				Upper Gel (mL)	
	7.5 %	10 %	12.5 %	15 %	3 %	4.5 %
Lower GB 4 X	10	10	10	10	-	-
Water	20	16.6	13.4	10	6.5	6
Bis/acryl	10	13.4	16.6	20	1	1.5
Upper GB 4 X	-	-	-	-	2.5	2.5
APS (µL)	120	120	120	120	50	50
Temed (µL)	25	25	25	25	15	15

Bis/acryl: Bisacrylamide   APS: Ammonium persulfate

The gel was run in 1 X running buffer at 180 V for 1 hour and continued until the tracking dye had reached the bottom of the gel. Proteins were stained with Coomassie Blue (2.5 g Coommassie brilliant blue, 454 ml water, 454 ml methanol and 92 ml acetic acid) for 1 hour and incubated for another hour with destain solution (454 ml methanol, 454 ml water and 92 ml acetic acid). When the background of the gel became clear it was scanned and recorded to analyse the protein profile of the samples (Schagger, 2006).

3. Results and discussion

3.1 Proximate analysis of whey sample

Two cheddar cheese whey samples from Batch 1 and 2 were tested for proximate analysis as described in section 2. 2. One sample was unpasteurised and the other one was pasteurised. In general, the composition of cheese whey is related to different factors such as: source of whey and type of cheese, ratio of whey to milk (if milk is added to producing cheese) and different process technologies such as heat treatment and filtration methods (Pintado *et al*., 2001).

The mean values of the proximate analysis of unpasteurised and pasteurised whey samples are expressed in Table 3.1. The findings indicated that variation of some parameters like total soluble solids and turbidity might be attributed to heat treatment and different processing methods that can influence the composition of different whey samples. The high value of water activity and moisture content in both samples can support growth of microorganisms and can be variable depending on the origin of whey and manufacturing processes. This data is in agreement with the data obtained from initial microbiology analysis. Most fresh foods have a_w values above 0.99 and this water can be removed by drying, adding salt or sugar. Another parameter which is pH 4.5 and it's a critical point in food processing. The pH value less than 4.6 is for high acid food and above of 4.6 is for low acid food that can be manipulated by adding acid and fermentation processes.

The values of the parameters measured (Table 3.1) were in accordance with the findings of Pereira *et al*. (2002) and the variation might be related to difference processing methods for the whey.

Pasteurised whey sample had lower value in *L**, *a**, *b** and Hue parameters than unpasteurised whey sample. This indicates that different processing and treatment influence the colour in whey samples. Results of colour analysis were similar to the results that observed by Croissant *et al*. (2009) and Listiyani *et al*. (2011).

Table 3.1 Proximate analysis of Cheddar cheese whey samples

Proximate analysis	Whey unpasteurised	Whey pasteurised
pH	4.5 ± 0.00	5 ± 0.00
Turbidity (FNU)	48.06 ± 2.30	87.41 ± 13.35
Water activity (a_w)	0.994 ± 0.003	0.995 ± 0.00
Total soluble solid (degree brix)	6 ± 0.00	4.55 ± 0.52
Moisture content (%)	94.35 ± 0.11	94.15 ± 0.09
Ash content (%)	0.477 ± 0.07	0.601 ± 0.46

Colour analysis	Whey unpasteurised	Whey pasteurised
L*	39.50 ± 0.46	27.92 ± 0.06
a*	-4.39 ± 0.42	-3.16 ± 0.17
b*	4.36 ± 1.15	1.45 ± 0.27
Hue	-44.16 ± 5.46	-24.52 ± 3.44
Chroma	6.21 ± 1.09	3.48 ± 0.25

Data are expressed as means $\pm$ SD (n=9)

3.2 Protein content of whey samples

3.2.1 *Protein content of cheddar cheese whey Batch 1 and 2, unpasteurised and pasteurised*

Protein content of the whey samples was determined by the Bradford assay, as described in section 2.3. Figure 3.1 shows the protein composition of pasteurised and unpasteurised cheddar cheese whey samples that was produced during the manufacture of cheddar cheese type, Batch 1 and 2. The protein content was higher in cheddar cheese whey unpasteurised sample than the samples that had thermal treatment. The results were in accordance with the finding of Tovar Jiménez *et al*. (2012) and the difference in the protein content among the various whey samples could be due to the heat treatment and the effect of it on the whey concentration. The denaturation of whey protein might be occur by heat treatment and cause either unfolding or aggregation steps. According to study that carried out by Kamizake *et al*. (2003) determination of total proteins in milk sample (without extraction of lipids) can be carried out by Bradford assay instead of the Kjeldahl method. Advantages

of Bradford assay could be for higher sensitivity for protein, shorter time for whole experiment, simpler assay and determination of only protein nitrogen.
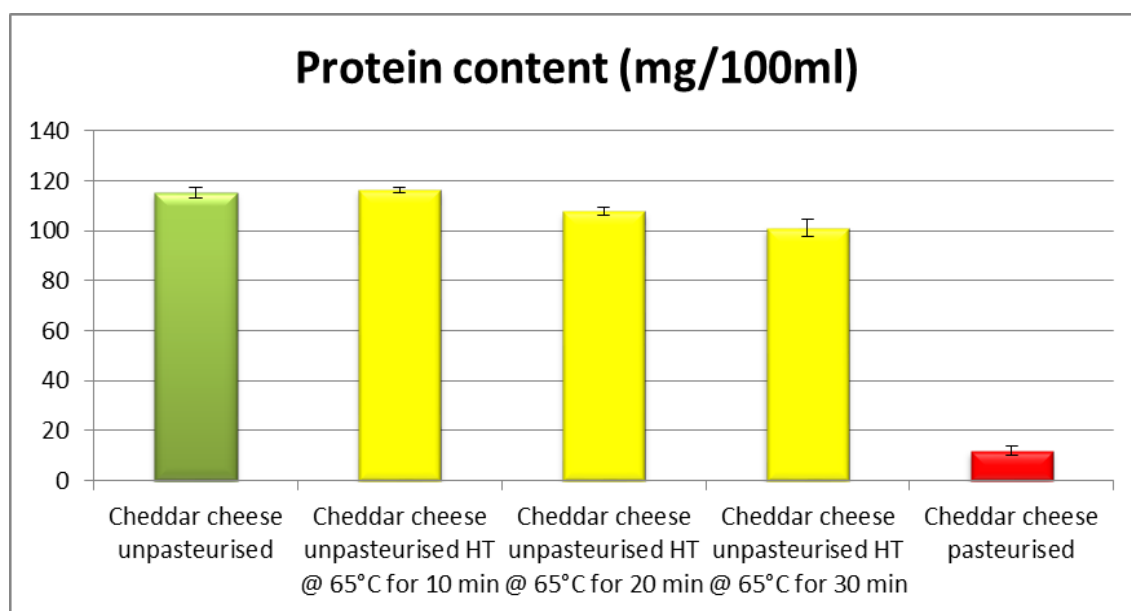

Figure 3.1 Total protein content of the cheddar cheese whey unpasteurised and pasteurised
Data are expressed as means ± SD (n=9)

3.2.2 *Protein content of whey samples Batch 6, Blue cheese whey, cheddar cheese whey and skimmed milk cheese whey*

Protein content of the whey samples in Batch 6 were determined by the Bradford assay, as described in section 2.3 (Figure 3.3). The protein content was higher in blue cheese whey samples and lower in skimmed milk cheese whey samples (Figure 3.2). Cheddar cheese whey samples had lower protein content than blue cheese whey samples and higher level of protein content than skimmed milk cheese whey samples. This result is in agreement with the result that obtained from antimicrobial activity of this Batch. The differences in the protein content among the various whey samples could be due to different sources of whey samples and difference in the starter culture for different cheeses. Culture type might influence the oxidative stability of liquid whey and whey flavour (Campbell *et al*., 2011). Method of treatment (heat treatment and dialysis) might also affect on the composition of whey samples.

Blue cheese non dialysed (ND) from the blue cheese whey samples had a higher protein content at level of approximately 338.01 ± 3.7 (mg/100ml) and blue cheese dialysed non heat treatment (DNHT) had lower protein content about 312.66 ± 4.14 (mg/100ml).

50

Cheddar cheese non dialysed (ND) from the cheddar cheese whey samples had a higher protein content approximately 307.25 ± 7.12 (mg/100ml) and cheddar cheese dialysed heat treatment (DHT) had a lower level of protein content approximately 289.46 ± 6.47 (mg/100ml).

Skimmed milk non dialysed (ND) from the skimmed milk cheese whey samples had a higher protein content at level of approximately 214.60 ± 5.51 (mg/100ml) and skimmed milk cheese dialysed heat treatment (DHT) had a lower level of protein content approximately 184.03 ± 2.12 (mg/100ml).

This data indicated that non dialysed (ND) whey samples had higher protein content and some of the difference in the protein content among the different whey samples could be due to dialysis treatment.
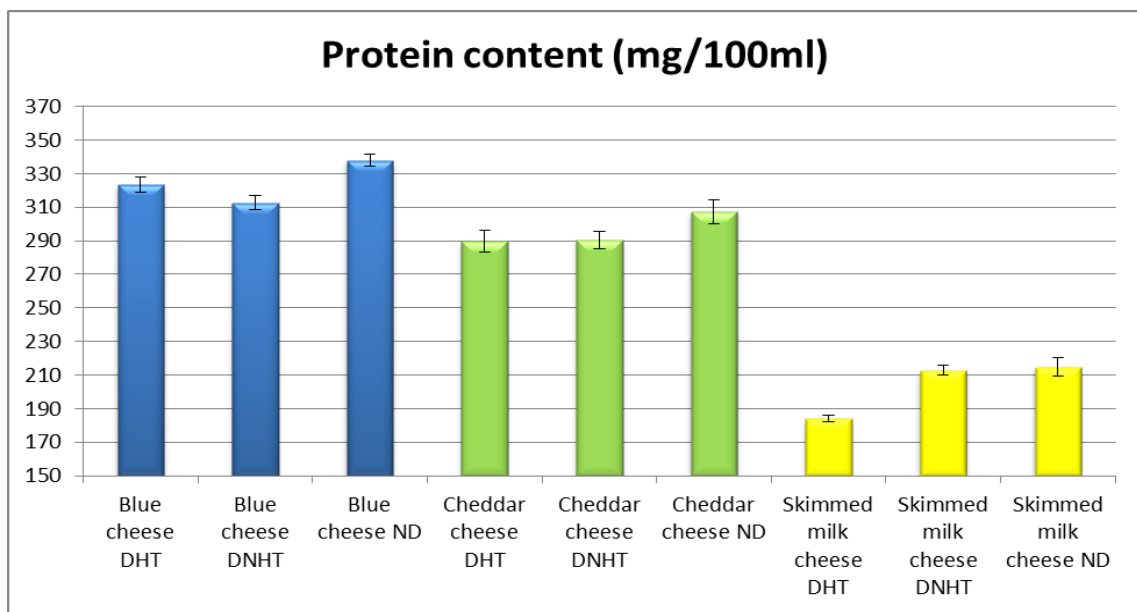

Figure 3.2 Protein content of whey samples, Blue cheese, Cheddar cheese and Skimmed milk cheese whey samples.
D: Dialysed, HT: Heat treated @ 65°C for 20 min, N: None, ND: Non-dialysed
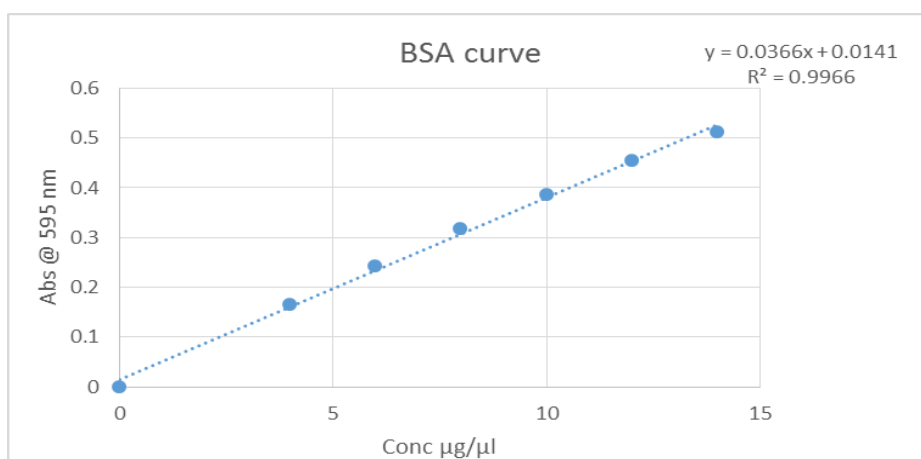Data are expressed as means ± SD (n=9)

Figure 3.3 Standard curve of Bradford assay for BSA. The Bradford assay was performed on different concentrations of a solution of BSA. The absorbance (at 595 nm) was measured to determine the concentration of protein using the equation y=0.0366x+0.0141 with the R^2 value of 0.9966, where y is absorbance at 595 nm and x is protein concentration.

3.3 Microbiological markers Mesophilic, Psychrotrophic, Lactic acid bacteria and Yeast & Moulds

The analysis of initial microflora of whey samples was carried out as described in section 2.4. In general different factors can affect the growth of microorganisms in whey samples such as availability of nutrients, water activity, pH and temperature. Fresh cheese whey samples usually have high pH and moisture content and low salt content which make them very susceptible to microbial spoilage, especially by yeast and moulds and this might refer to the influence of the starter culture. All microbial groups tend to grow in the first 24 h following production and later psychrotrophs at low temperature storage (Pintado *et al*., 2001).

3.3.1 *Batch 1, Cheddar cheese whey sample unpasteurised*

Cheddar cheese whey samples unpasteurised had an initial load of total mesophilic bacteria of 5.28 ± 0.13 Log cfu/ml. The growth of psychrotrophic counts in unpasteurised whey sample during 7 days were lower than other microorganisms and reached 1.79 ± 0.19 Log cfu/ml. Initial load of lactic acid bacteria (LAB) was 4.08 ± 0.08 Log cfu/ml. High LAB load can be linked to the fermentation process. However, these bacteria have shown positive anti-microbial effect due to their production of bacteriocins (Rico *et al*., 2007).

Cheddar cheese whey sample had initial loads of yeast and moulds 3.1 ± 0.05 Log cfu/ml (Figure 3.4). The results were in accordance with the finding of other authors (Pintado *et al.*, 2001; Broadbent *et al.*, 2013) and the difference in the total microbial count among the various whey samples could be due to the influence of the starter culture or source of the whey samples.
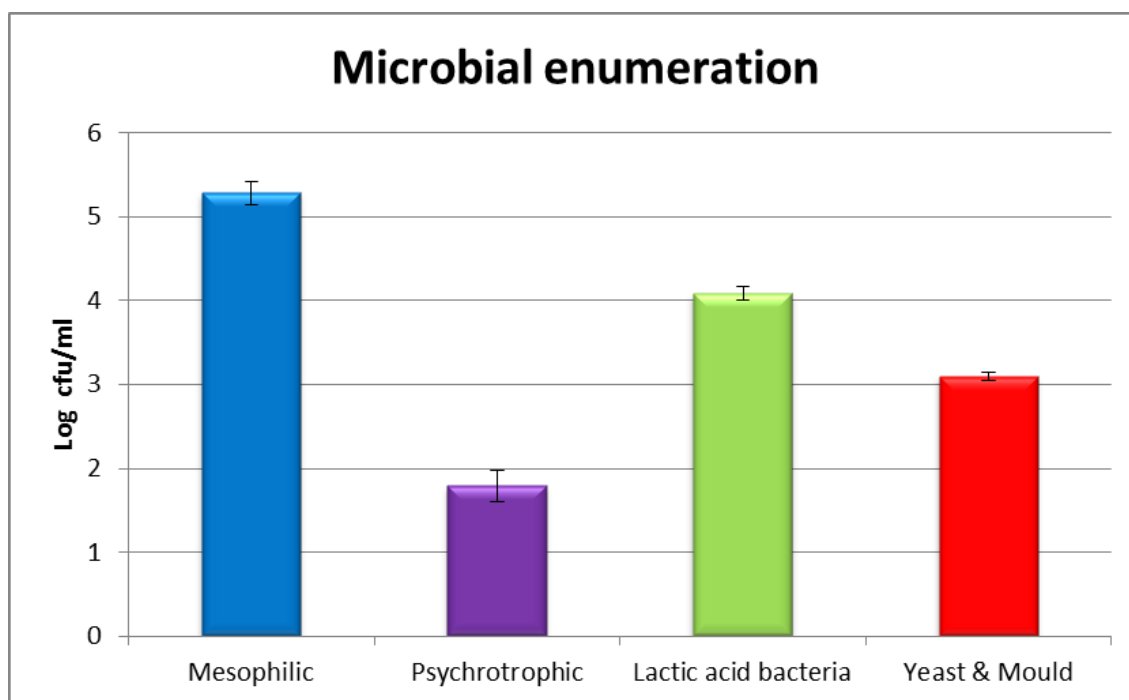
Figure 3.4 Batch 1, Cheddar cheese whey sample unpasteurised, Mesophilic, Psychrotrophic, Lactic acid bacteria, Yeast and Moulds. Two independent trials were carried out in duplicate.

3.3.2 *Thermal treatment of raw whey sample, Cheddar cheese from Batch 1*

Since the initial microbial loads of whey samples were comparatively high (~ 5 Log cfu/ml), raw whey samples were subjected to heat treatment for reducing the microbial loads. Among the various temperature and times that have been applied to the whey samples as described in section 2.6.1, the conventional 'low-temperature-long-time' (LTLT) pasteurisation technique, i.e. at 65°C for 30 minutes was found to be the most effective one to heat treat the whey samples and was suggested to the industry for future samples. Among other thermal treatment however, no viable counts were observed for 121°C for 15 minutes and 72°C for 15 sec, but there was high risk of protein denaturation and changing the main composition of whey samples. No viable counts for psychrotrophic, yeast and moulds were observed at 65°C thermal treatment of whey samples for 10, 20 and

30 minutes and approximately 3 Log reduction was achieved for LAB and mesophilic after 30 minutes treatment at 65°C (Figures 3.5 and 3.6). These results are in consistent with those observed by Gatti *et al.* (2006).

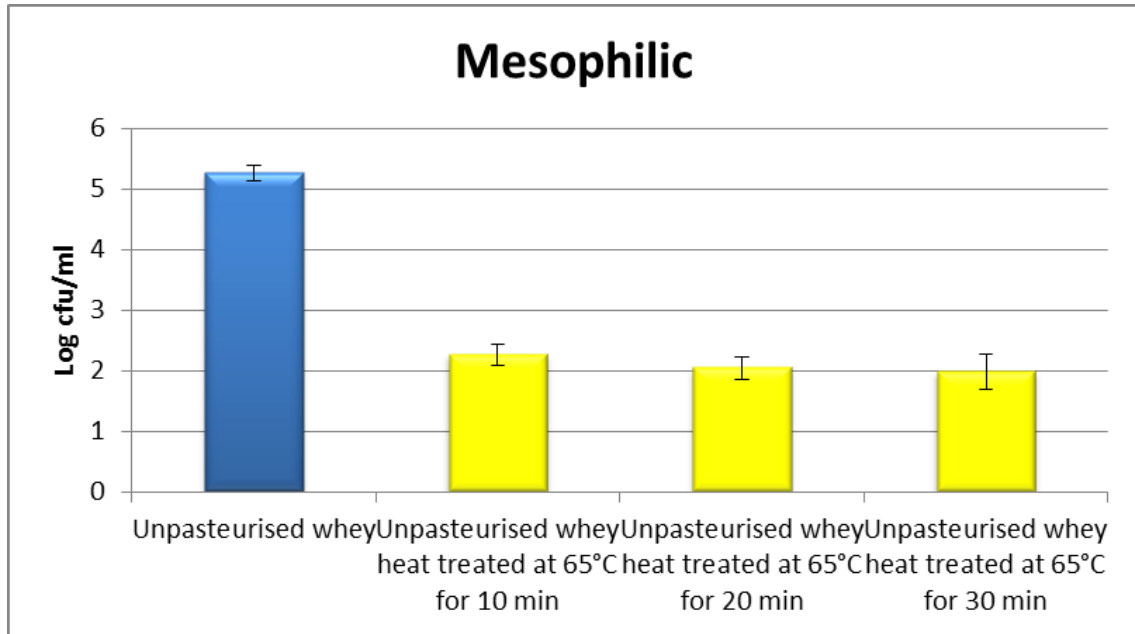**Figure 3.5** Unpasteurised cheddar cheese whey sample, Mesophilic bacteria before and after heat treatment. Two independent trials were carried out in duplicate.
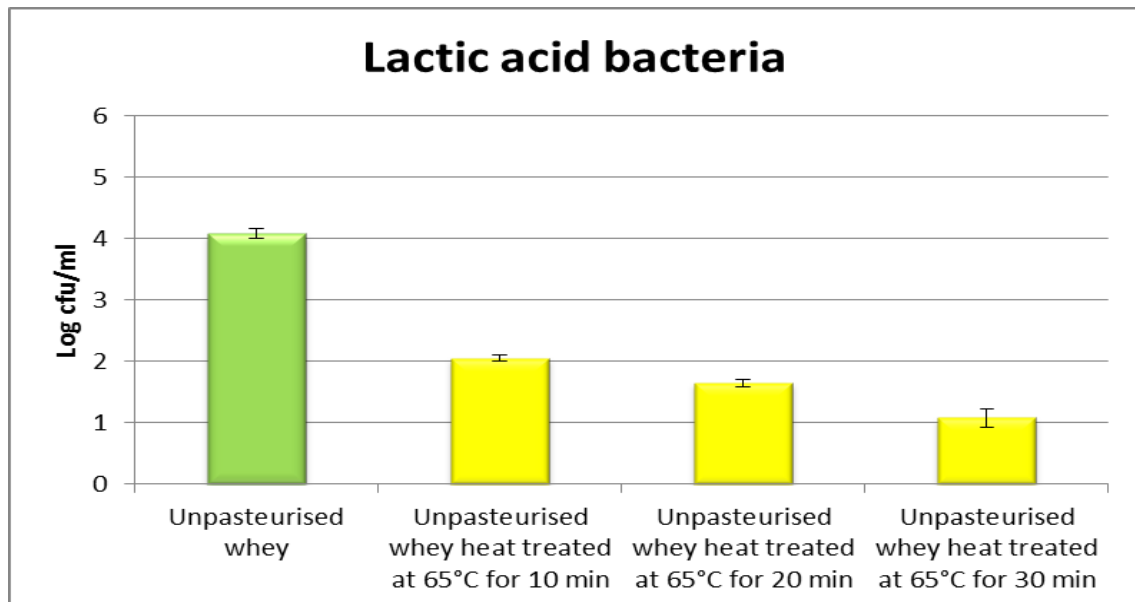

Figure 3.6 Unpasteurised cheddar cheese whey sample, LAB before and after heat treatment. Two independent trials were carried out in duplicate.

3.3.3 *Batch 2, Pasteurised cheddar cheese whey sample*

The microbial enumeration of the whey samples were carried out as described in section 2.4. The pasteurised fresh whey sample had initial loads of total mesophilic bacteria of 4.93 ± 0.04 Log cfu/ml which was higher than other microorganisms. The growth of psychrotrophic counts in pasteurised fresh sample during 7 days reached 4.48 ± 0.17 Log cfu/ml. Initial loads of lactic acid bacteria (LAB) was 3.03 ± 0.02 Log cfu/ml which was lower than other microorganisms. Pasteurised fresh whey samples had initial loads of yeast and moulds of 4.22 ± 0.06 Log cfu/ml (Figure 3.7). This Batch in comparison with Batch 1 had higher loads of psychrotrophic, yeast and moulds and lower level of LAB. These differences are related to temperature of pasteurisation and longer storage time.
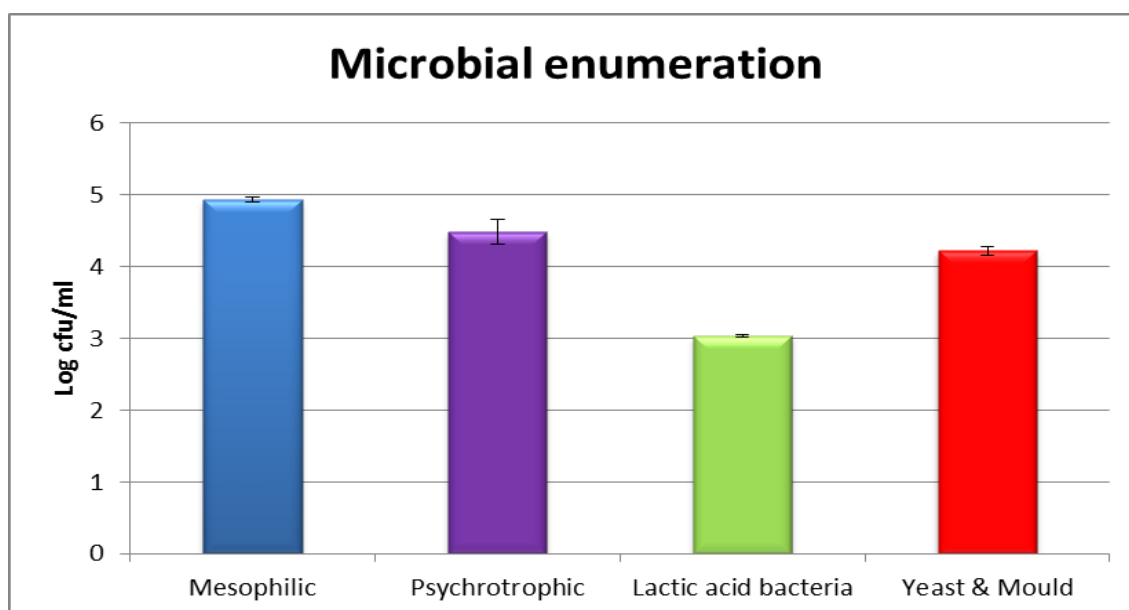
Figure 3.7 Pasteurised cheddar cheese whey sample, Mesophilic, Psychrotrophic, Lactic acid bacteria, Yeast and Moulds. Two independent trials were carried out in duplicate.

3.3.4 *Batch 3, Dialysed, pasteurised fresh cheddar cheese whey sample*

The microbial enumeration of whey samples were carried out as described in section 2.4. Dialysed-Pasteurised fresh whey samples had initial loads of total mesophilic bacteria of 7.36 ± 0.03 Log cfu/ml that was higher than other microorganisms. Initial loads of lactic acid bacteria were 4.27 ± 0.10 Log cfu/ml which was lower than other microorganisms. Pasteurised fresh whey samples had initial loads of yeast and moulds 7.57 ± 0.02 Log

cfu/ml (Figure 3.8). The number of mesophilic, yeast and moulds microorganisms was quite high in this sample in comparison with Batch 1 and 2. These differences could be due to different methods of processing of whey samples and the effect of temperature and time and increased number of processing steps (dialysis) on the number of viable count of microorganisms.
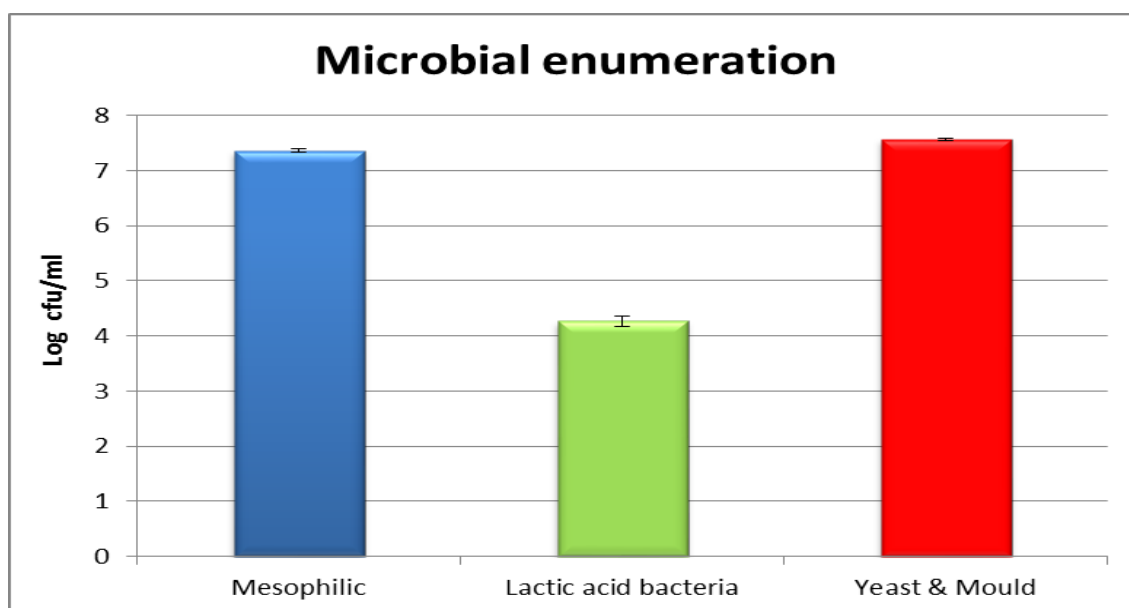
Figure 3.8 Dialysed pasteurised fresh whey sample from cheddar cheese, Mesophilic, Lactic acid bacteria, Yeast and Moulds counts. Two independent trials were carried out in duplicate.

3.3.5 *Batch 4, 16 different whey samples obtained from 4 different cheeses including Cheddar, Blue, Brie and Skimmed milk*

The microbial enumeration of whey samples were carried out as described in section 2.4. The whey samples were studied for microbiological enumeration from 4 cheeses (Cheddar, Blue, Brie and Skimmed milk) after different processing methods.

The cheddar cheese whey samples that were pasteurised and ultrafiltrated (permeate) had higher initial load of mesophilic counts 8.58 ± 0.08 Log cfu/ml, LAB counts 8.53 ± 0.03 and yeast and moulds counts 8.56 ± 0.02 Log cfu/ml. Cheddar cheese whey pasteurised dialysed one had lower initial load of mesophilic counts 7.67 ± 0.04 Log cfu/ml, LAB counts 7.55 ± 0.03 and yeast and moulds counts 7.77 ± 0.04 Log cfu/ml. In terms of

psychrotrophic, initial loads among different cheddar whey samples, cheddar pasteurised, ultrafiltrated permeate had higher counts 7.98 ± 0.05 Log cfu/ml and ultrafiltrated (retentate) had lower counts 6.03 ± 0.04 Log cfu/ml.

The blue cheese whey samples that were pasteurised and ultrafiltrated (permeate) had higher initial loads of mesophilic counts 8.53 ± 0.06 Log cfu/ml, LAB counts 7.54 ± 0.04, yeast and moulds counts 8.57 ± 0.05 Log cfu/ml and psychrotrophic counts 7.94 ± 0.03 Log cfu/ml. Blue pasteurised dialysed one had lower initial load of mesophilic counts 5.05 ± 0.05 Log cfu/ml, LAB counts 5.13 ± 0.04 Log cfu/ml and yeast and moulds counts 7.13 ± 0.02 Log cfu/ml.

The brie cheese whey samples that were pasteurised and ultrafiltrated (retentate) had higher initial load of mesophilic counts 8.19 ± 0.02 Log cfu/ml, yeast and moulds counts 7.92 ± 0.04 Log cfu/ml and lower initial loads of LAB 5.94 ± 0.04 Log cfu/ml and psychrotrophic counts 7.51 ± 0.05 Log cfu/ml. Among brie samples, ultrafiltrated permeate whey sample had lower initial counts of mesophilic and yeast & moulds 5.75 ± 0.05 and 5.7 ± 0.06 Log cfu/ml respectively. Also this sample had higher initial loads of psychrotrophic 8.09 ± 0.04 Log cfu/ml. The higher level of LAB approximately 7.79 ± 0.03 Log cfu/ml was observed in dialysed samples.

The skimmed milk cheddar pasteurised whey samples that were pasteurised and dialysed had higher initial loads of mesophilic 7.84 ± 0.04 Log cfu/ml, psychrotrophic 7.73 ± 0.04 Log cfu/ml, LAB 7.77 ± 0.06 Log cfu/ml, yeast and moulds 7.65 ± 0.04 Log cfu/ml. Permeate sample had lower initial loads of mesophilic and yeast & moulds approximately 6.56 ± 0.03 and 6.56 ± 0.05 Log cfu/ml, respectively. Also retentate sample had lower initial counts of psychrotrophic 5.66 ± 0.04 Log cfu/ml and LAB 6.68 ± 0.05 Log cfu/ml.

The skimmed milk cheddar unpasteurised whey samples that were unpasteurised (retentate) had higher initial loads of mesophilic 7.81 ± 0.04 Log cfu/ml, LAB 7.66 ± 0.04 Log cfu/ml, yeast and moulds 7.57 ± 0.04 Log cfu/ml and lower initial counts of psychrotrophic 6.02 ± 0.05 Log cfu/ml. Permeate sample had lower initial load of mesophilic 7.21 ± 0.01 Log cfu/ml and dialysed sample had lower initial counts of LAB 6.75 ± 0.06 Log cfu/ml (Figures 3.9, 3.10, 3.11 and 3.12). These differences in the number of microorganisms in different samples could be related to the different sources of whey and different starter

culture in different cheese. Different processing steps such as heat treatment, dialysis and filtration processes might also have an influence on the number of microorganisms.

Figure 3.9 Mesophilic bacteria counts of sixteen whey samples obtained from 4 different cheeses. Two independent trials were carried out in duplicate.
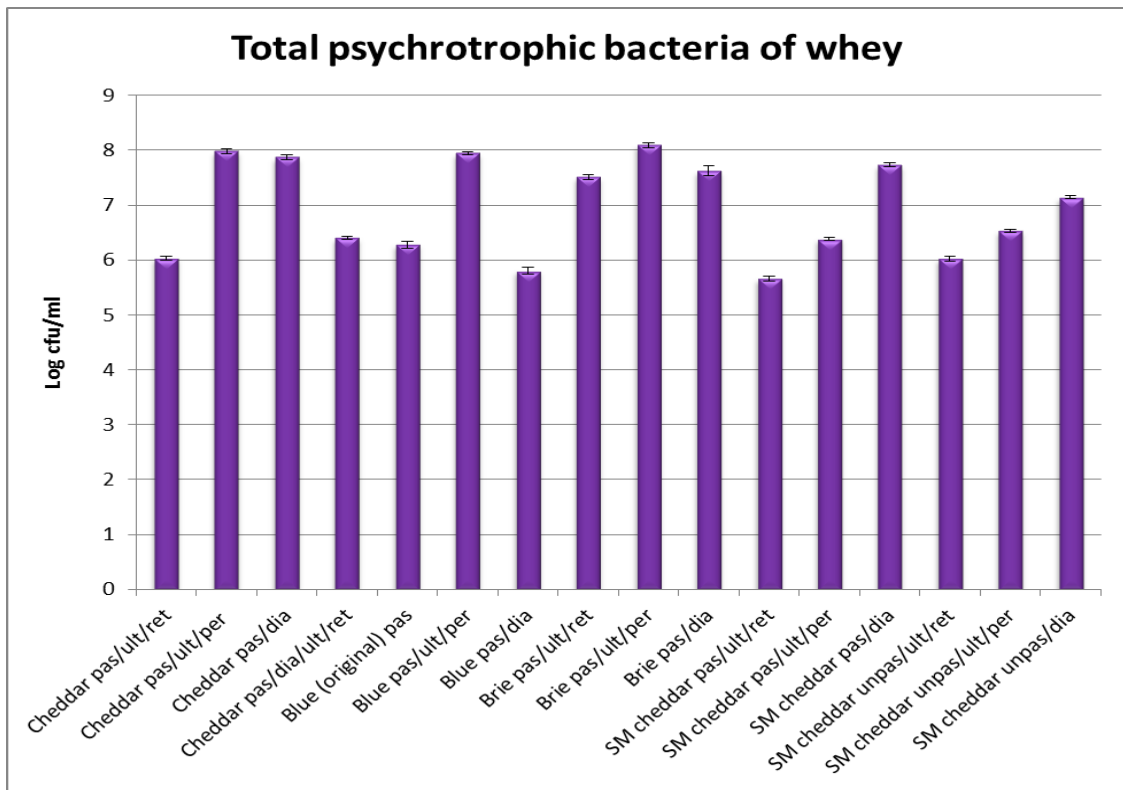
Figure 3.10 Psychrotrophic bacteria counts of sixteen whey samples obtained from 4 different cheeses. Two independent trials were carried out in duplicate.
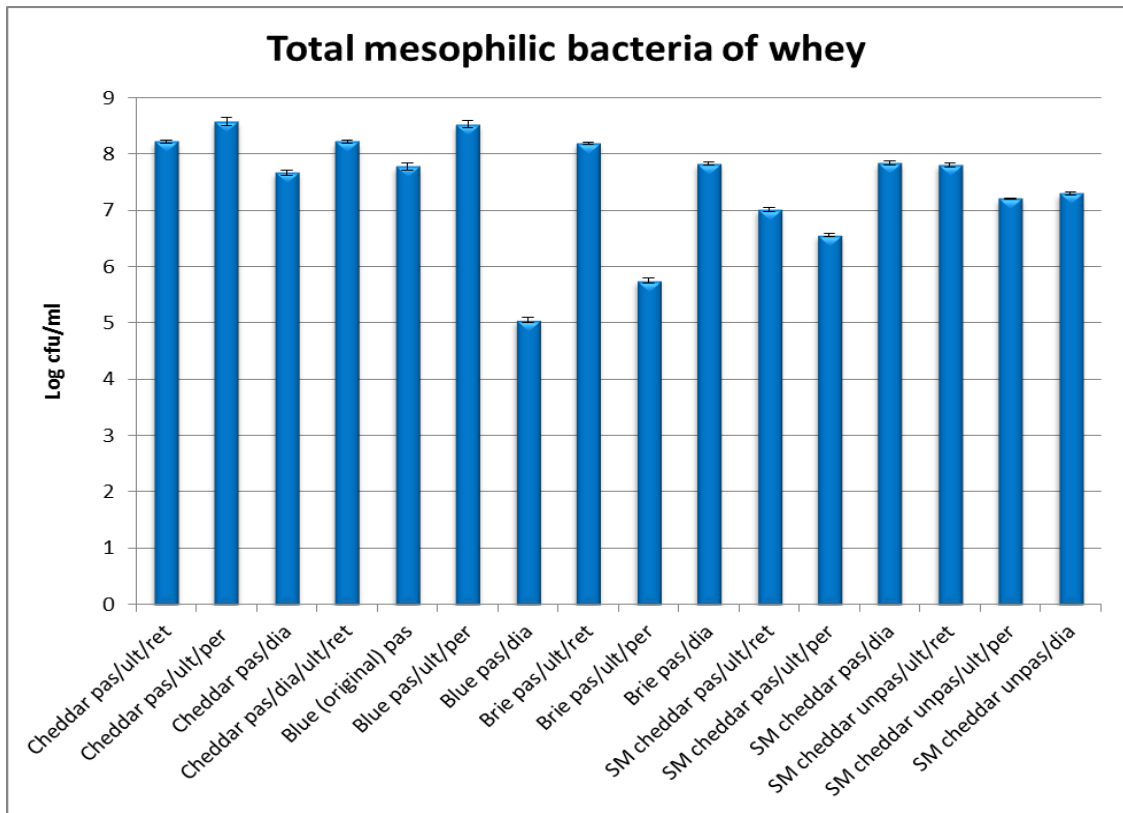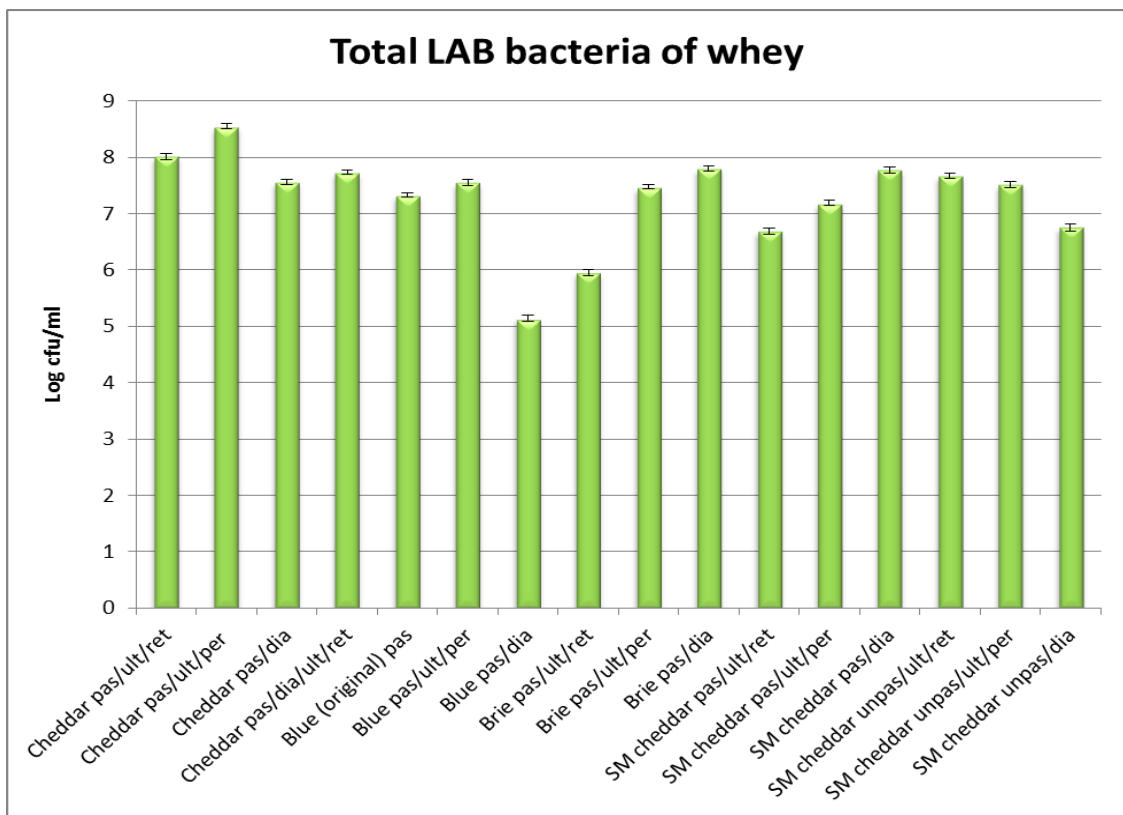

Figure 3.11 LAB bacteria counts of sixteen whey samples obtained from 4 different cheeses. Two independent trials were carried out in duplicate.
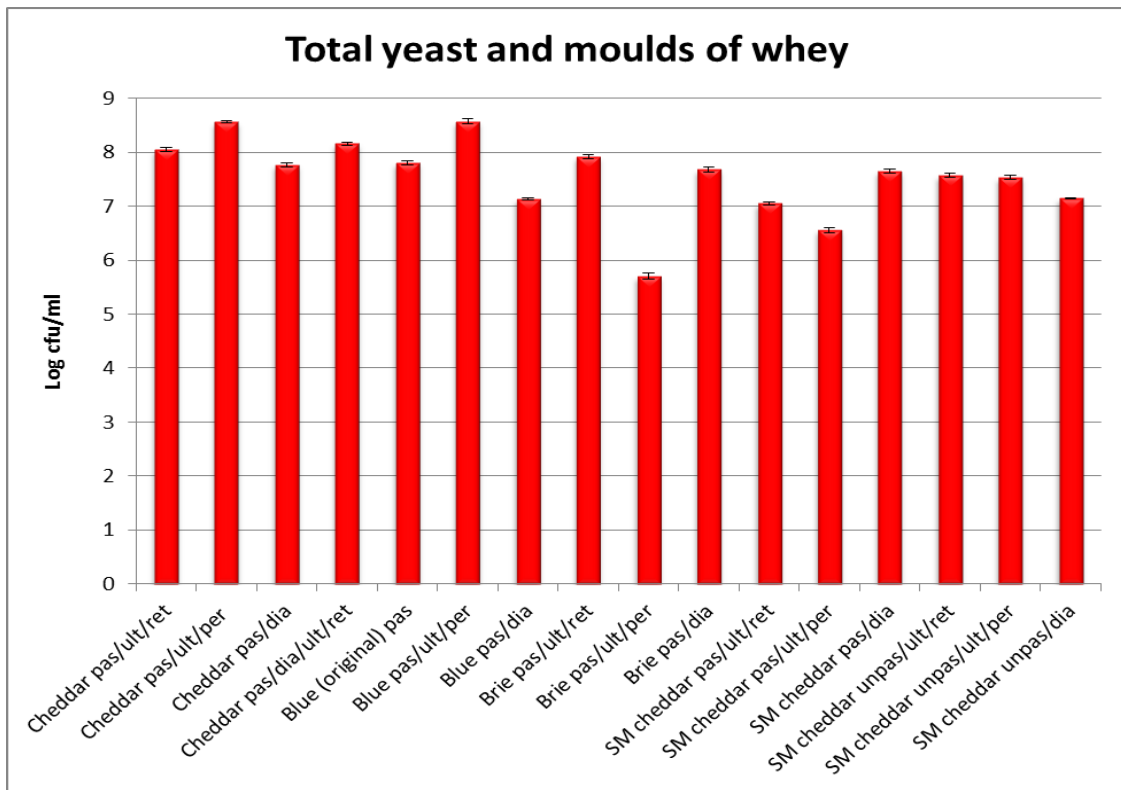
Figure 3.12 Yeast & Moulds counts of sixteen whey samples obtained from 4 different cheeses. Two independent trials were carried out in duplicate.

3.3.6 *Batch 5, Blue cheese whey samples permeate and retentate*

The microbial enumeration of whey samples were carried out as described in section 2.4 and heat treatment was carried out before transferring samples to the microbiology lab. Two samples were studied for microbiological markers from blue cheese whey samples before and after heat treatment at 65°C for 20 min. Blue cheese whey samples that were heat treated (retentate) had higher initial load of mesophilic counts 8.35 ± 0.02 Log cfu/ml, LAB counts 8.35 ± 0.02 and yeast and moulds counts 8.66 ± 0.05 Log cfu/ml. Non-heat treated permeate had lower initial loads of mesophilic 6.78 ± 0.05 Log cfu/ml, LAB counts 4.64 ± 0.03 and yeast and moulds counts 7.07 ± 0.05 Log cfu/ml. Initial counts of psychrotrophic was higher for non-heat treated permeate 7.91 ± 0.06 Log cfu/ml and lower for non-heat treated retentate 3.2 ± 0.02 Log cfu/ml (Figure 3.13). The overall result of microbial load of this Batch was quite high and heat treatment before transferring of sample wasn't effective to reduce the microbial load of whey samples. Whey sample permeate had lower microbial load in compare to retentate sample except psychrotrophic bacteria.

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Figure 3.13 Four fresh blue cheese whey samples, heat treatment at 65°C for 20 min, were examined for Mesophilic, Psychrotrophic, LAB, Yeast and Moulds counts. Two independent trials were carried out in duplicate. HT: Heat treated @ 65°C for 20 min, N: None,

3.3.7 *Batch 6, 3 different whey samples from blue chesses, cheddar cheeses and skimmed milk cheeses whey samples*

The initial microflora of whey samples were carried out as described in section 2.4 and treatments (dialysis and heat treatment) were carried out as described in section 2.6. Three different fresh whey samples were studied for microbiological markers from blue cheese whey, cheddar cheese whey and skimmed milk cheese whey after different processing methods. Among the whey samples blue cheese, dialysed heat treated had lower initial load of mesophilic counts 0.66 ± 0.56 Log cfu/ml, LAB counts 0.54 ± 0.58 and yeast and moulds counts 0.13 ± 0.35 Log cfu/ml. Skimmed milk cheese dialysed not heat treated whey sample had higher initial load of mesophilic counts 8.39 ± 0.04 Log cfu/ml, higher initial load of LAB counts 7.96 ± 0.04 and yeast and moulds counts 8.41 ± 0.03 Log cfu/ml.

Initial counts of psychrotrophic was at higher level for non-heat blue cheese dialysed 6.74 ± 0.06 Log cfu/ml and at lower level for whey sample skimmed milk cheese non dialysed 2.55 ± 0.06 Log cfu/ml. for all 3 whey samples all psychrotrophic microorganisms vanished after thermal treatment (Figure 3.14). The result of this Batch indicated that heat

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treatment at 65°C for 20 min was effective in reducing microbial population of whey samples and is in accordance with the data obtained from previous heat treated samples as described in section 3.3.2.

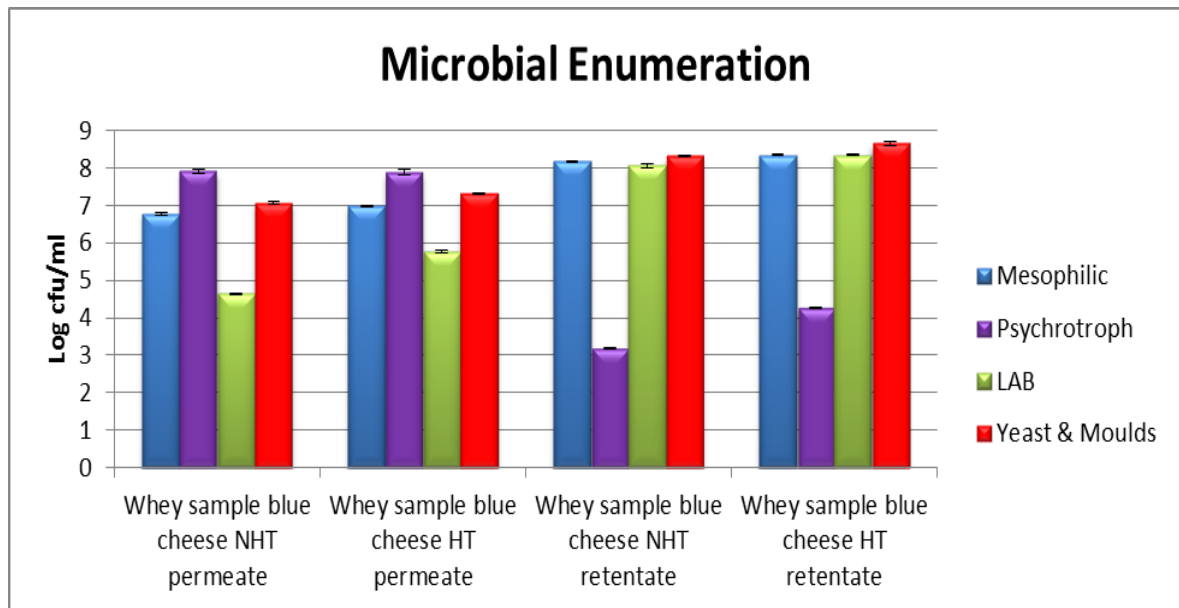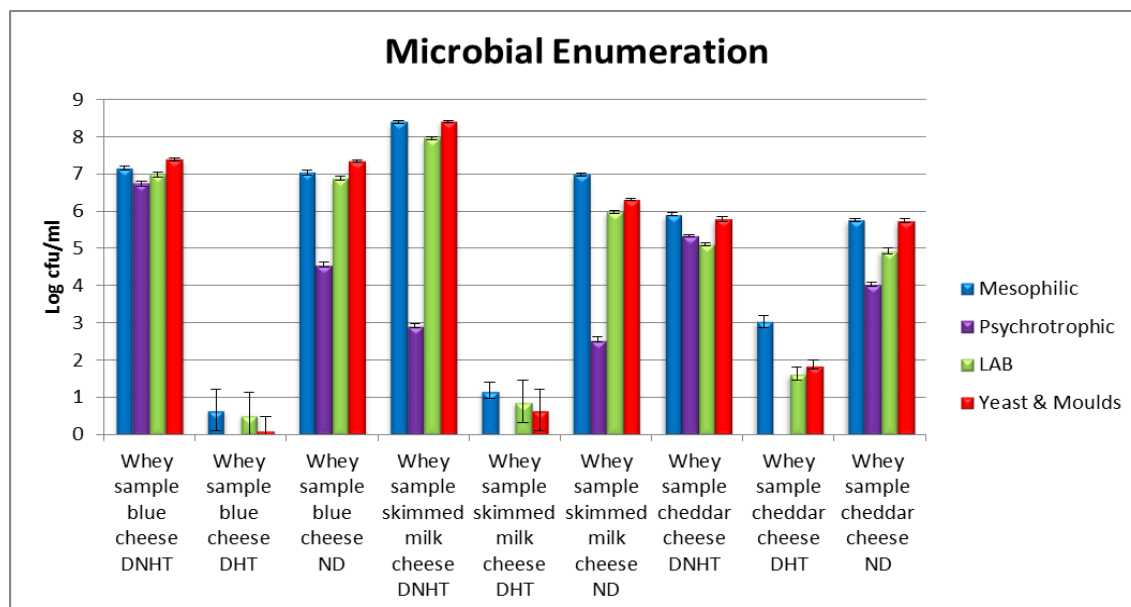**Figure 3.14** Three different fresh whey samples from blue cheese, cheddar cheese and skimmed milk cheese whey, heat treatment at 65°C for 20 min, were examined for Mesophilic, Psychrotrophic, LAB, Yeast and Moulds counts. Two independent trials were carried out in duplicate. D: Dialysed, HT: Heat treated @ 65°C for 20 min, N: None, ND: Non-dialysed.

3.4 Antimicrobial activity of whey

Percentage inhibition of each whey samples was calculated over 24 h period and the assay revealed different susceptibilities of *E. coli* under investigation to the whey samples.

Fresh whey samples, TSB, fresh overnight culture of *E. coli* was utilised in this assay. The percentage inhibition of the highest concentration of whey sample against specific pathogen/spoilage microorganism is presented in the Figures 3.16, 3.20 and 3.24.

Generally, the specific pathogen *E. coli* under investigation was susceptible to the whey samples. At the highest concentrations with highest protein content all whey samples presented antimicrobial activity.

The skimmed milk whey samples were the only sample that had no antimicrobial activity against the pathogen E. *coli* and in fact enhanced the growth of this bacterium. A possible explanation for this might be due to differences in the sources of this sample and starter culture and also the low level of protein might impact on the antimicrobial activity.

Sodium benzoate and sodium nitrite were used as controls. Sodium benzoate and sodium nitrite are common food preservatives used in salad, carbonated drink, meat and fish. According to European Food Directive 95/2/EC on "Food Additives other than colours and Sweeteners", the maximum level permitted of sodium benzoate is 0.15-2 g/Kg depending on the food product. Sodium benzoate and sodium nitrite had similar bacterial growth inhibition to the whey sample tested.

On a large scale food production, the presence of bacteria causes food spoilage and economical losses. There is interest in natural decontaminants with antimicrobial activity to prolong the shelf life of food products. In this context, the result of the present study show that whey samples could have the potential as a source for new antimicrobial agents equal to that of commercially applied synthetic antibacterial agents. The blue cheese whey samples non-dialysed (ND) with the highest protein content had the highest percentage inhibition against *E. coli* which is equal to the activity of sodium benzoate a popular food preservative. Results are presented in Figure 3.16.

These results indicate that the antimicrobial activity of whey samples were concentration dependent. At higher concentrations whey samples had the strongest activity compared to others. Analysis of the most effective concentration of whey samples against the specific pathogen *E. coli* was performed over a 24 h period.

The whey samples inhibited the growth of *E. coli* tested from the first hour resulting in lag phase extension. All whey samples displayed inhibition activity similar to that of the commercial controls. In previous reports (Madureira *et al*., 2007) the main biological activity of whey proteins was reviewed. Whey proteins contain bioactive antimicrobial peptide including lactoferrin (Lf), lactoperoxidase (LP) glycomacropeptide (GMP), immunoglobulins (Ig), etc. The antimicrobial activity of whey peptides reported against different types of bacteria either gram-positive or gram-negative, yeast and filamentous fungi. This potential might be due to low pH and presence of lactic acid, Lactoferrin (iron

binding protein to sequester iron from bacteria inhibiting its growth and metabolism), Lactoperoxidase (catalyse the oxidation of thiocyanate in hypothiocyanate ion which cause damage to bacterial cells) and Immunoglobulins (IGS). The antimicrobial activity of Lactoferrin has been reported in several studies against gram-positive and gram-negative bacteria due to interaction with LPS in gram-negative bacteria damaging cell walls and reduction of negative charge on the cell wall of gram-positive bacteria by binding to anionic molecules (González-Chávez *et al*., 2009).

Finally, this study utilised a substantially more sensitive detection method to quantify the antimicrobial effects of whey as compared to the less sensitive standard agar disc diffusion assay. The microtiter assay applied allowed the detection of antimicrobial effects of whey samples at low concentration levels which would otherwise have been undetected in the agar disc diffusion assay (Dubber and Harder, 2008).

3.4.1 *Antimicrobial activity of different blue chesses whey samples*

Percentage inhibition of three Blue cheese whey samples (DHT, DNHT, ND) were calculated over a 24 hours period against *Escherichia coli* as described in section 2.5. The assay revealed different susceptibilities of the bacteria under investigation to the whey samples.

The percentage inhibition of the highest concentration of blue cheese whey sample against food spoilage bacteria is presented in the Figure 3.16. The highest concentration of blue cheese whey which was non-dialysed (ND) sample with the peptide concentration of 338.01 ± 3.79 mg/100ml produced the highest antimicrobial activity achieving very strong percentage inhibition about 93.29 ± 5.25 against *E. coli* and dialysed heat treated (DHT) blue cheese whey sample with the peptide concentration of 323.31 ± 4.46 mg/100ml produced a percentage inhibition of 71.80 ± 2.08 against *E. coli* which is moderately high, but less than two other blue cheese whey samples.

Sodium benzoate and sodium nitrite at a concentration of 60 mg/ml achieved almost 100 % inhibition against *E. coli*. Sodium benzoate and sodium nitrite had similar bacterial growth inhibition to the blue cheese whey sample. This implies that the blue cheese whey sample

with a peptide concentration above 300 mg/100ml had similar activity to commercially applied antimicrobials.

The OD of bacterial culture was converted to Log CFU/ml by the standard curve as explained in section 2.5.3.
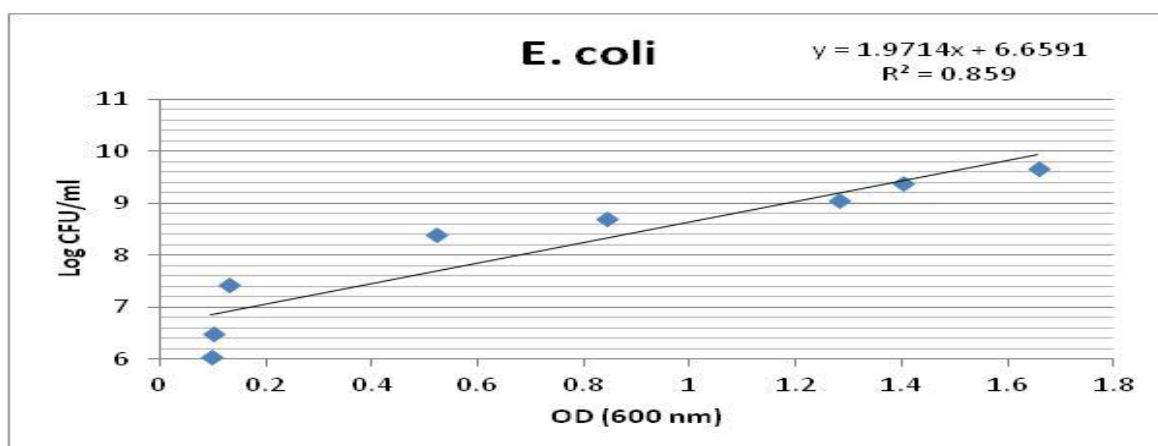
Figure 3.15 Growth curve of bacteria (OD) versus Log cfu/ml, relationship between turbidity and viable count.

The antimicrobial activity of the blue cheese whey samples was evaluated in the form of (I %) percentage inhibition of different concentration of whey samples and viable count (log cfu/ml) of *E. coli* in the presence of different concentrations of the whey samples (Figure 3.16).

The growth inhibition of *E. coli* in the presence of different concentrations of the blue cheese whey samples based on optical density (OD) of the samples over a 24 h period, red colour presented growth control of *E. coli* without whey sample (Figure 3.18).

Evaluating antimicrobial activity of the **Blue cheese whey** samples against *E. coli*

(a)

(b)

(c)

Figure 3.16 Percentage inhibition of different concentrations of the blue cheese whey samples dialysed heat treated (a), dialysed non heat treated (b) and non-dialysed (c) against *E. coli* over 24h & Viable count (log cfu/ml) of *E. coli* in the presence of different concentrations of blue cheese whey samples over 24 h, red colour representing growth control of *E. coli* without whey sample.

Two independent trials were carried out in duplicate. D: Dialysed, HT: Heat treated @ 65°C for 20 min, N: None, ND: Non-dialysed.

(a)

(b)
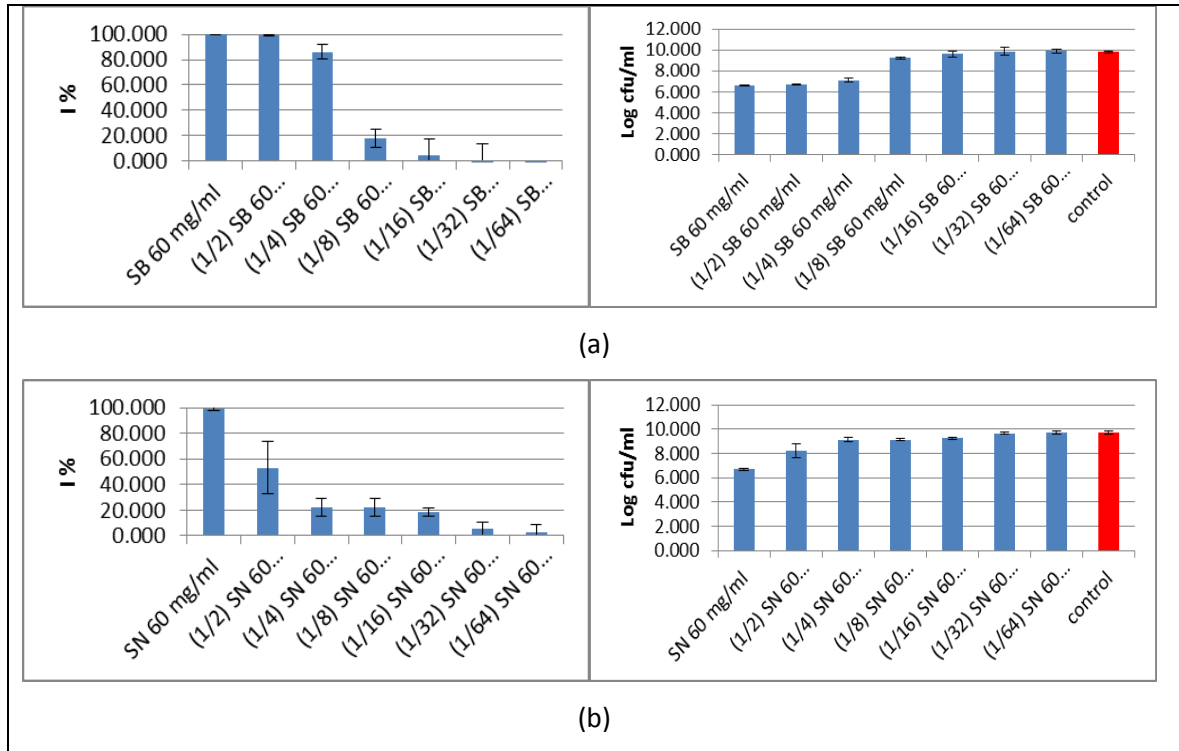
Figure 3.17 Percentage inhibition of different concentrations of sodium benzoate (a) and sodium nitrite (b) against *E. coli* over 24 h & Viable count (log cfu/ml) of *E. coli* in the presence of different concentrations of sodium benzoate and sodium nitrite over 24 h, red colour representing growth control of *E. coli* without SB.

Two independent trials were carried out in duplicate. SB: sodium benzoate and SN: sodium nitrite.
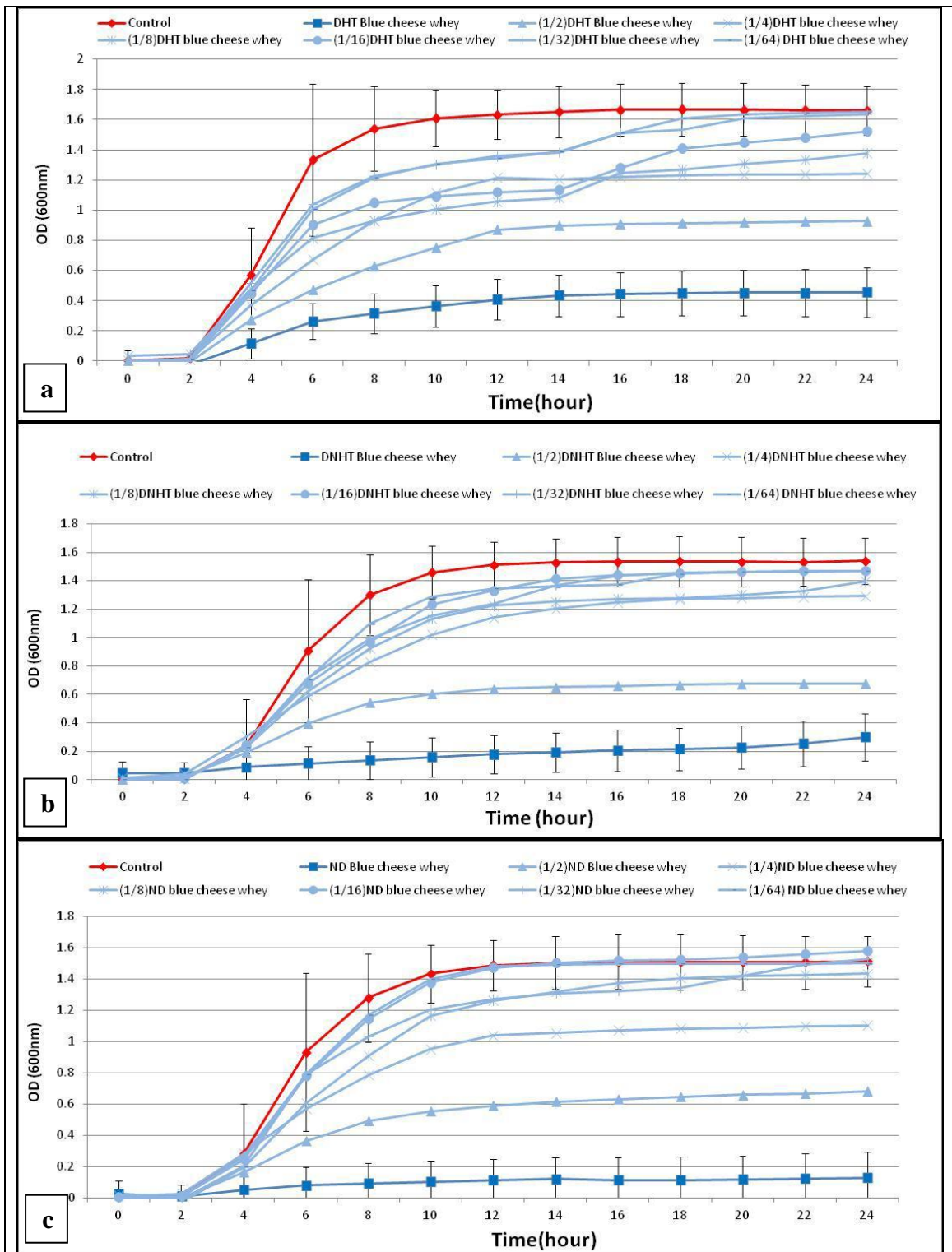
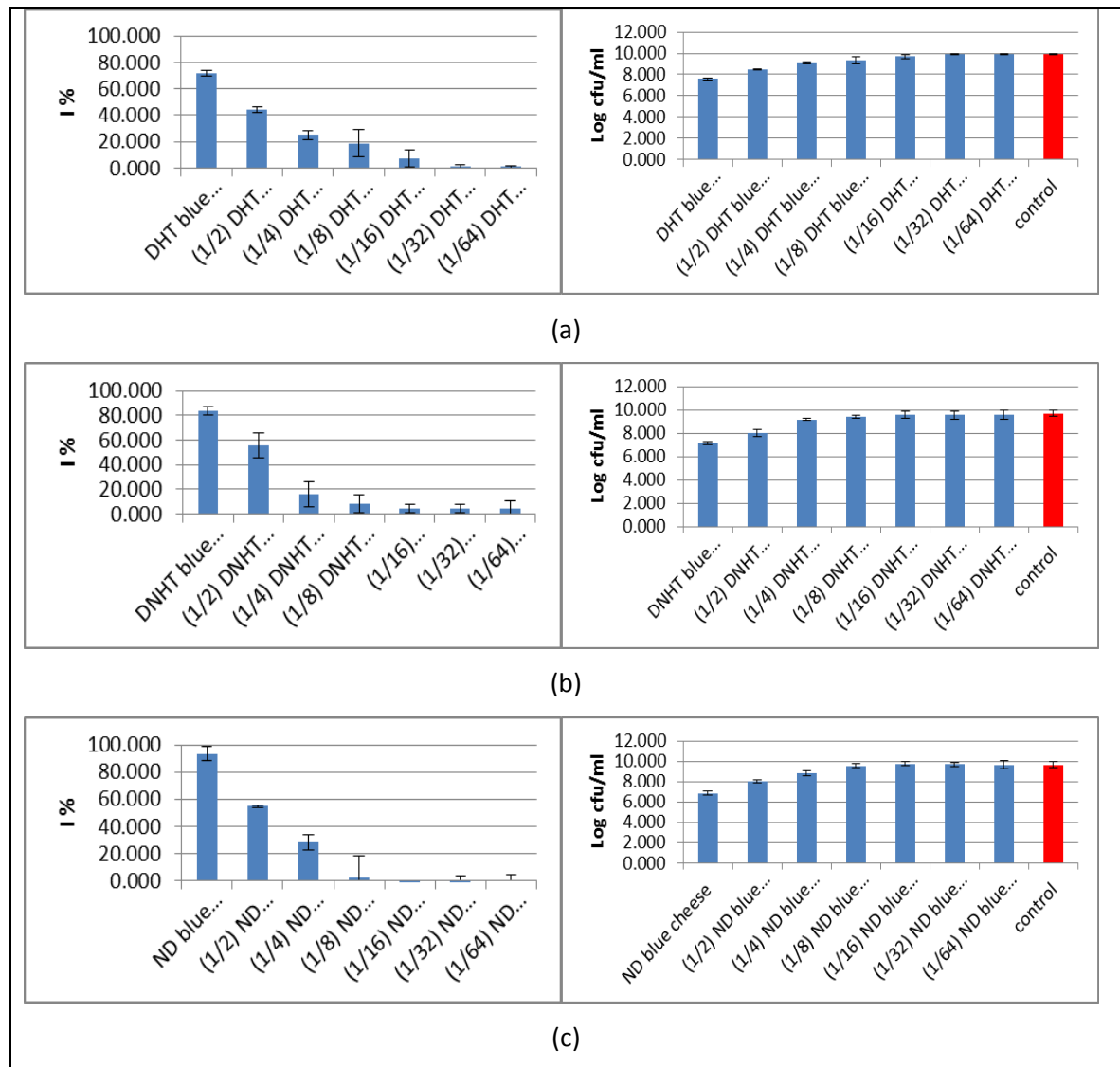**Figure 3.18** Growth inhibition analysis based on optical density (OD) of the different concentrations of blue cheese whey samples dialysed heat treated (a), dialysed non heat treated (b) and non-dialysed (c) against *E. coli* over a 24 h period, red colour representing growth control of *E. coli* without whey sample.

Two independent trials were carried out in duplicate. D: Dialysed, HT: Heat treated @ 65°C for 20 min, N: None, ND: Non-dialysed.

(a)

(b)

Figure 3.19 Growth inhibition analysis based on optical density (OD) of the different concentrations of sodium benzoate (a) and sodium nitrite (b) against *E. coli* over a 24 h period, red colour representing growth control of *E. coli* without SB.

Two independent trials were carried out in duplicate. SB: sodium benzoate and SN: sodium nitrite.
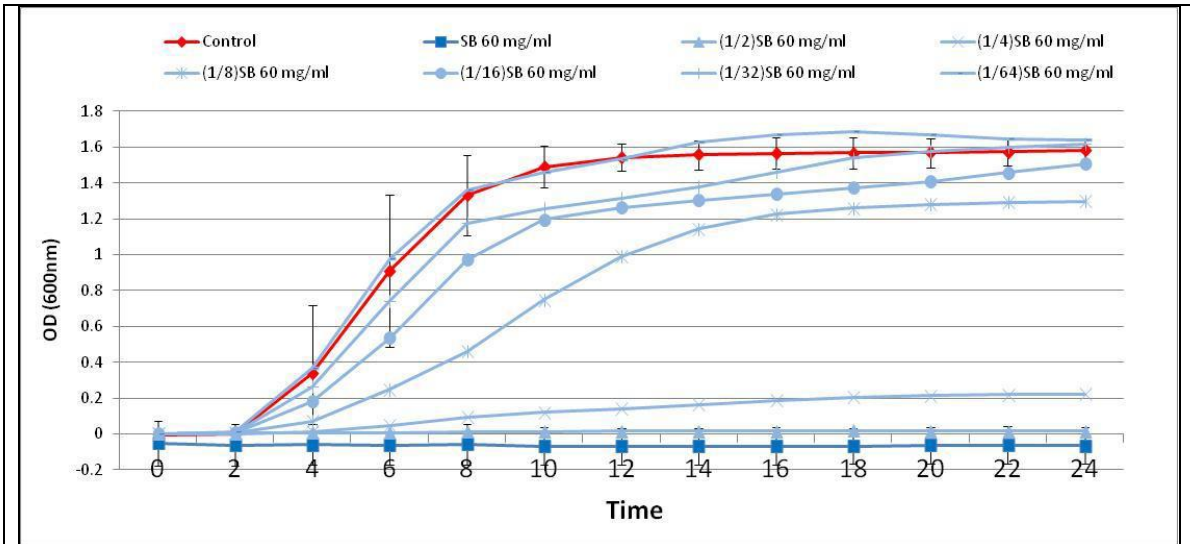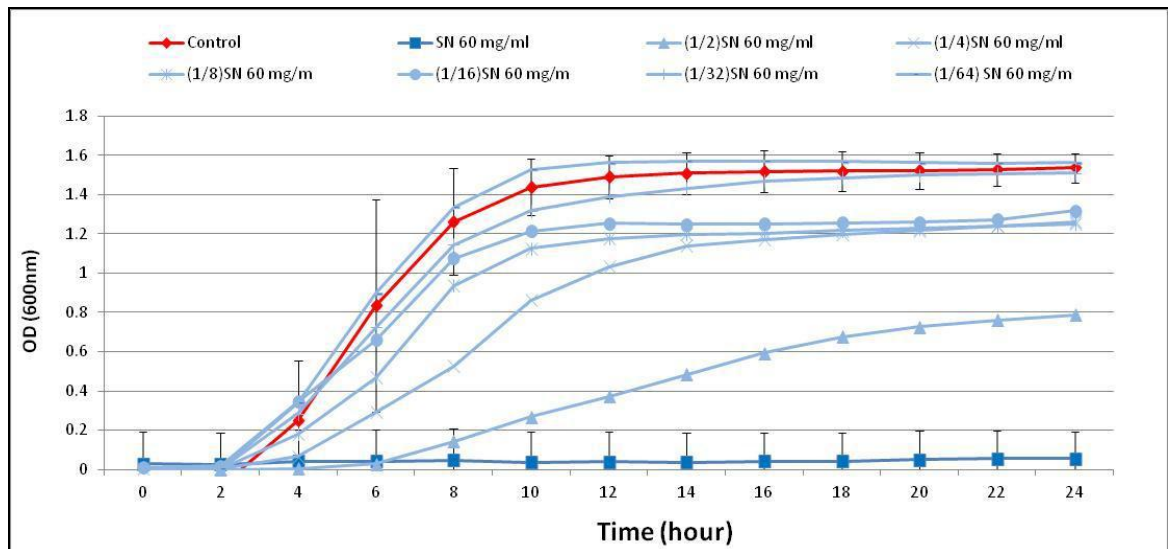
3.4.2 *Antimicrobial activity of different cheddar cheeses whey samples*

Percentage inhibition of three whey samples (DHT, DNHT, ND) was calculated over a 24 hours period against *Escherichia coli* as described in section 2.5. The assay revealed different susceptibilities of the bacteria under investigation to the whey samples.

The percentage inhibition of the highest concentration of cheddar cheese whey sample against food spoilage bacteria is presented in the Figure 3.20. At the highest concentration of whey sample, dialysed and heat treated (DHT) cheddar cheese whey sample with the peptide concentration of 289.46 ± 6.47 mg/100ml produced the highest antimicrobial activity achieving very strong percentage inhibition about 85.503 ± 1.53 against *E. coli* (Figure 3.20) and dialysed and non-heat treated (DNHT) cheddar cheese whey sample with the peptide concentration of 290.53 ± 5.12 mg/100ml produced lowest percentage inhibition about 80.75 ± 1.50 against *E. coli*.

Sodium benzoate and sodium nitrite at a concentration of 60 mg/ml achieved almost 100 % inhibition against *E. coli*. Sodium benzoate and sodium nitrite had similar bacterial growth inhibition to the cheddar cheese whey sample. This implies that cheddar cheese whey sample with a peptide concentration above 300 mg/100ml had similar activity to commercially applied antimicrobials.

The antimicrobial activity of cheddar cheese whey samples were evaluated in the form of (I %) percentage inhibition of different concentration of whey samples and viable count (log cfu/ml) of *E. coli* in the presence of different concentration of whey samples (Figure 3.20).

The growth inhibition of *E. coli* in the presence of different concentrations of cheddar cheese whey samples based on optical density (OD) of the samples over a 24 h period, red colour presented growth control of *E. coli* without whey sample (Figure 3.22).

Evaluating antimicrobial activity of the **Cheddar cheese whey** samples against *E. coli*

(a)

(b)

(c)
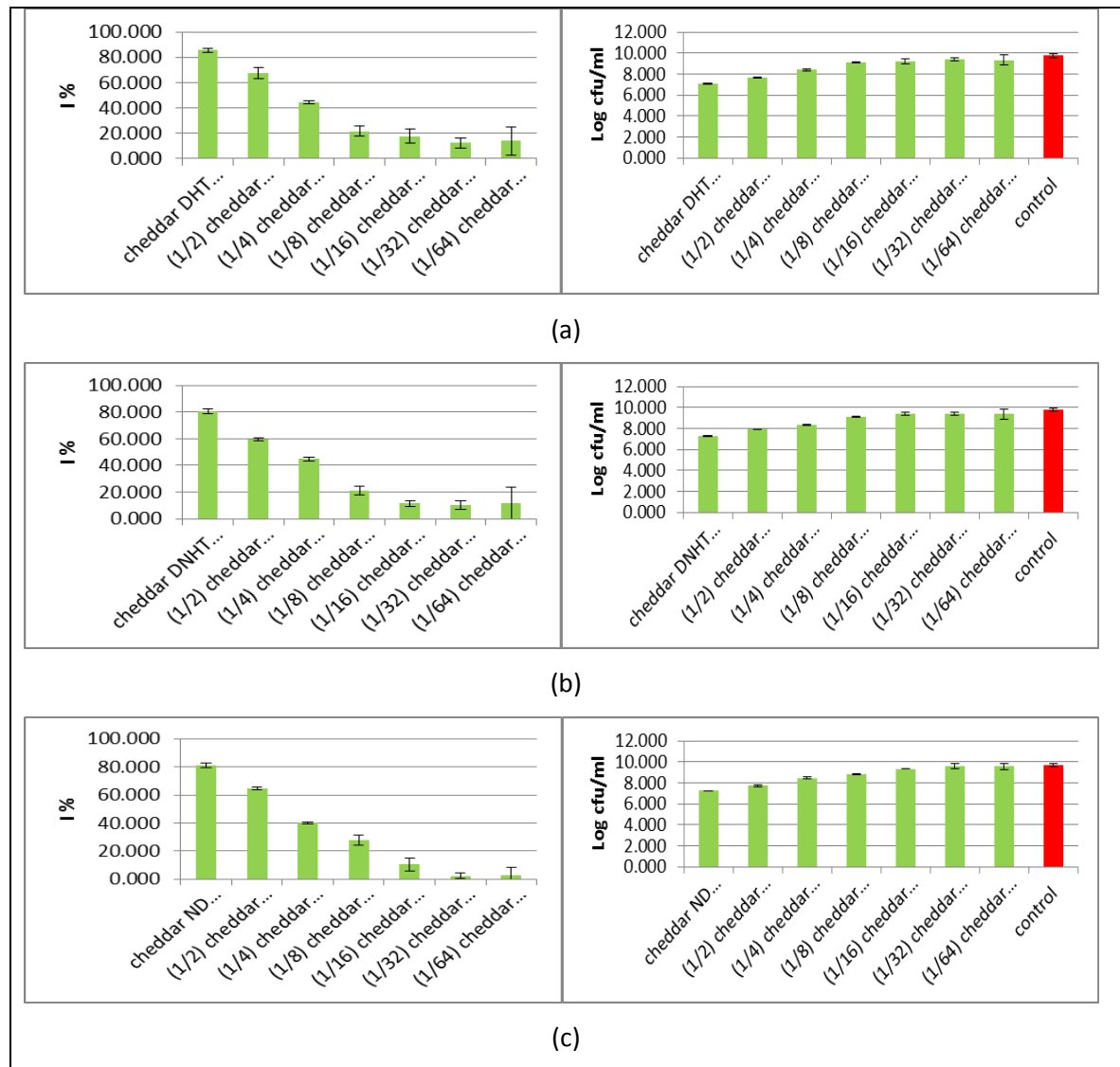
Figure 3.20 Percentage inhibition of different concentrations of cheddar cheese whey samples dialysed heat treated (a), dialysed non heat treated (b) and non-dialysed (c) against *E. coli* over 24h & Viable count (log cfu/ml) of *E. coli* in the presence of different concentrations of blue cheese whey samples over 24 h, red colour representing growth control of *E. coli* without whey sample.

Two independent trials were carried out in duplicate. D: Dialysed, HT: Heat treated @ 65°C for 20 min, N: None, ND: Non-dialysed.
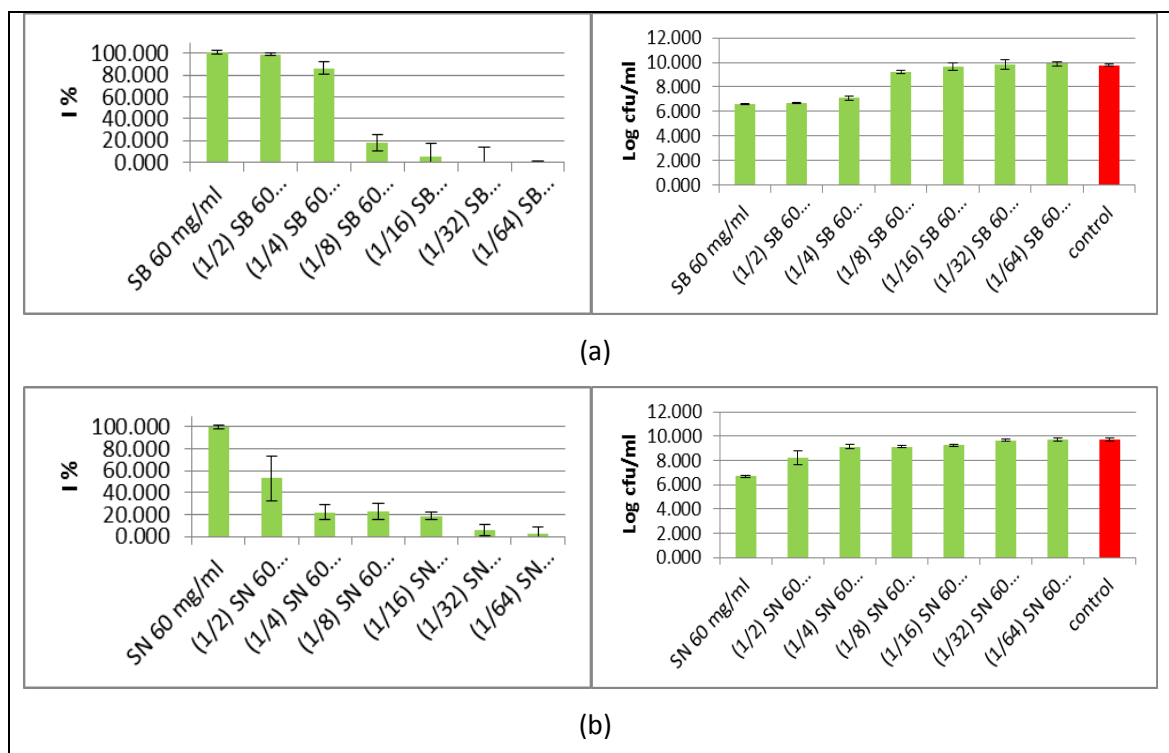
Figure 3.21 Percentage inhibition of different concentrations of sodium benzoate (a) and sodium nitrite (b) against *E. coli* over 24 h & Viable count (log cfu/ml) of *E. coli* in the presence of different concentrations of sodium benzoate and sodium nitrite over 24 h, red colour representing growth control of *E. coli* without SB.

Two independent trials were carried out in duplicate. SB: sodium benzoate and SN: sodium nitrite.
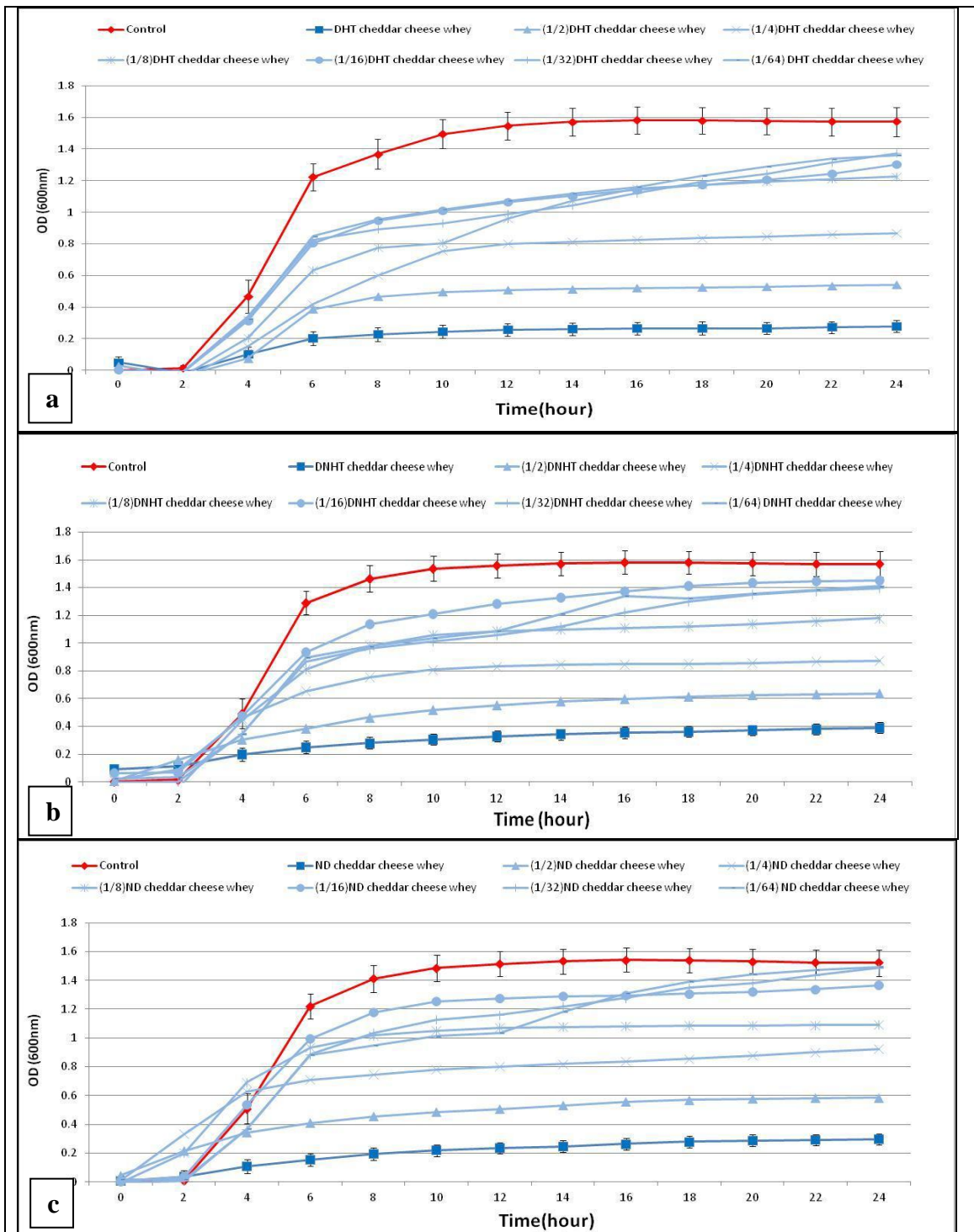
Figure 3.22 Growth inhibition analysis based on optical density (OD) of the different concentrations of cheddar cheese whey samples dialysed heat treated (a), dialysed non heat treated (b) and non-dialysed (c) against *E. coli* over a 24 h period, red colour representing growth control of *E. coli* without whey sample.

Two independent trials were carried out in duplicate. D: Dialysed, HT: Heat treated @ 65°C for 20 min, N: None, ND: Non-dialysed.

Figure 3.23 Growth inhibition analysis based on optical density (OD) of the different concentrations of sodium benzoate (a) and sodium nitrite (b) against *E. coli* over a 24 h period, red colour representing growth control of *E. coli* without SB.
Two independent trials were carried out in duplicate. SB: sodium benzoate and SN: sodium nitrite.

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3.4.3 *Antimicrobial activity of different skimmed milk cheeses whey samples*

Percentage inhibition of three whey samples (DHT, DNHT, ND) were calculated over a 24 hours period against *Escherichia coli* as described in section 2.5. The assay revealed different susceptibilities of the bacteria under investigation to the whey samples.

The percentage inhibition of the highest concentration of skimmed milk cheese whey sample against food spoilage bacteria is presented in the Figure 3.24. At the highest concentration of the whey sample, dialysed heat treatment (DHT) skimmed milk cheese whey sample with the peptide concentration of 184.03 ± 2.12 mg/100ml produced the highest antimicrobial activity achieving moderate percentage inhibition about 64.160 ± 9.18 against *E. coli* (Figure 3.24) and dialysed and non-heat treated (DNHT) skimmed milk cheese whey sample with the peptide concentration of 212.87 ± 2.74 mg/100ml produced lowest and weak percentage inhibition about 1.503 ± 2.29 against *E. coli*.

Sodium benzoate and sodium nitrite at a concentration of 60 mg/ml achieved almost 100 % inhibition against *E. coli*. Sodium benzoate and sodium nitrite had different bacterial growth inhibition to the skimmed milk cheese whey sample. This implies that skimmed milk cheese whey sample with a peptide concentration under 215 mg/100ml had not similar activity to commercially applied antimicrobials.

The antimicrobial activity of skimmed milk cheese whey samples were evaluated in the form of (I %) percentage inhibition of different concentration of whey samples and viable count (log cfu/ml) of *E. coli* in the presence of different concentration of whey samples (Figure 3.24).

The growth inhibition of *E. coli* in the presentence of different concentrations of skimmed milk cheese whey samples based on optical density (OD) of the samples over a 24 h period, red colour presented growth control of *E. coli* without whey sample (Figure 3.26).

Evaluating antimicrobial activity of **skimmed milk cheese whey** samples against *E. coli*

(a)

(b)

(c)
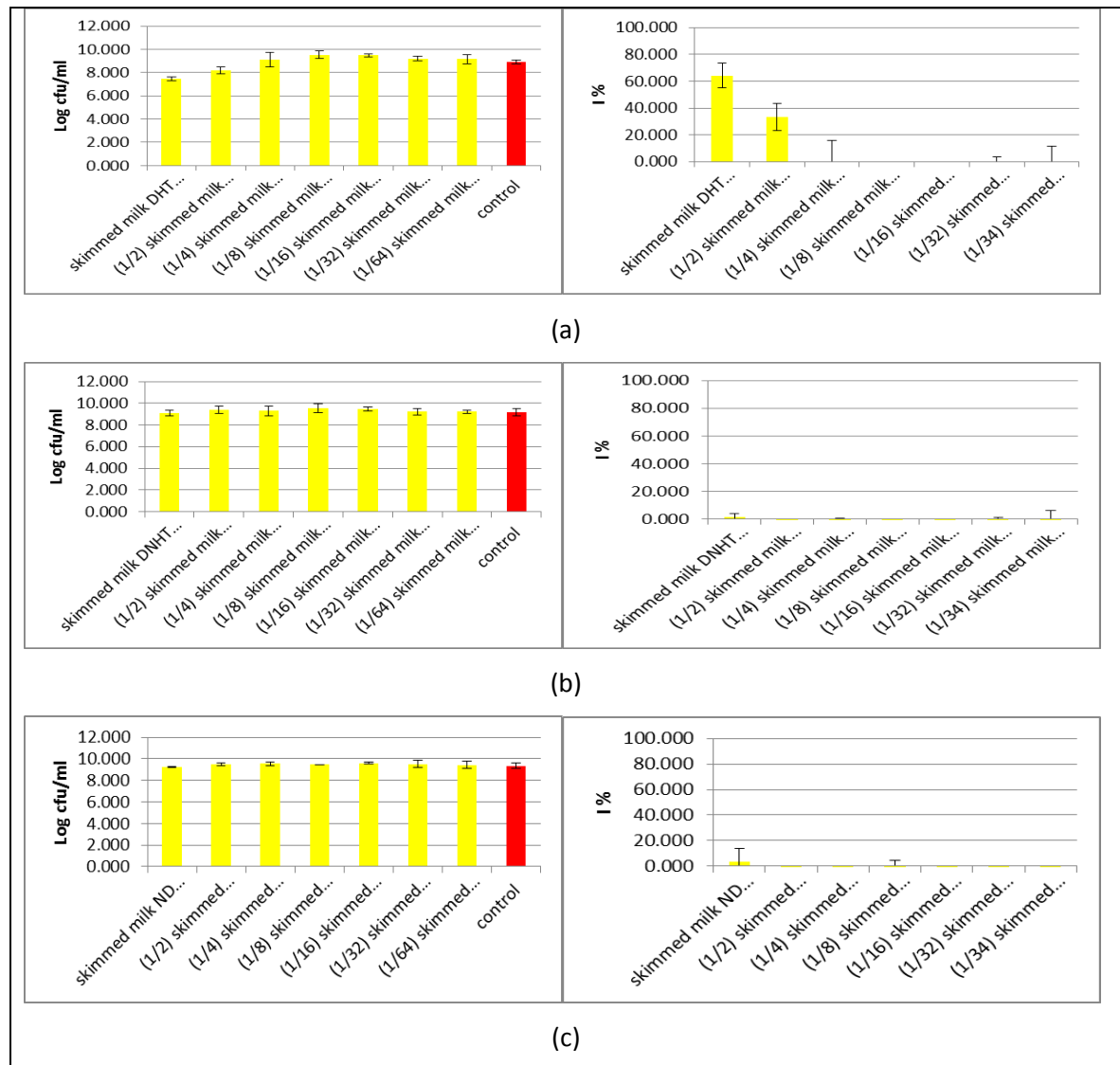
Figure 3.24 Percentage inhibition of different concentrations of skimmed milk cheese whey samples dialysed heat treated (a), dialysed non heat treated (b) and non-dialysed (c) against *E. coli* over 24h & Viable count (log cfu/ml) of *E. coli* in the presence of different concentrations of blue cheese whey samples over 24 h, red colour representing growth control of *E. coli* without whey sample.
Two independent trials were carried out in duplicate. D: Dialysed, HT: Heat treated @ 65°C for 20 min, N: None, ND: Non-dialysed.
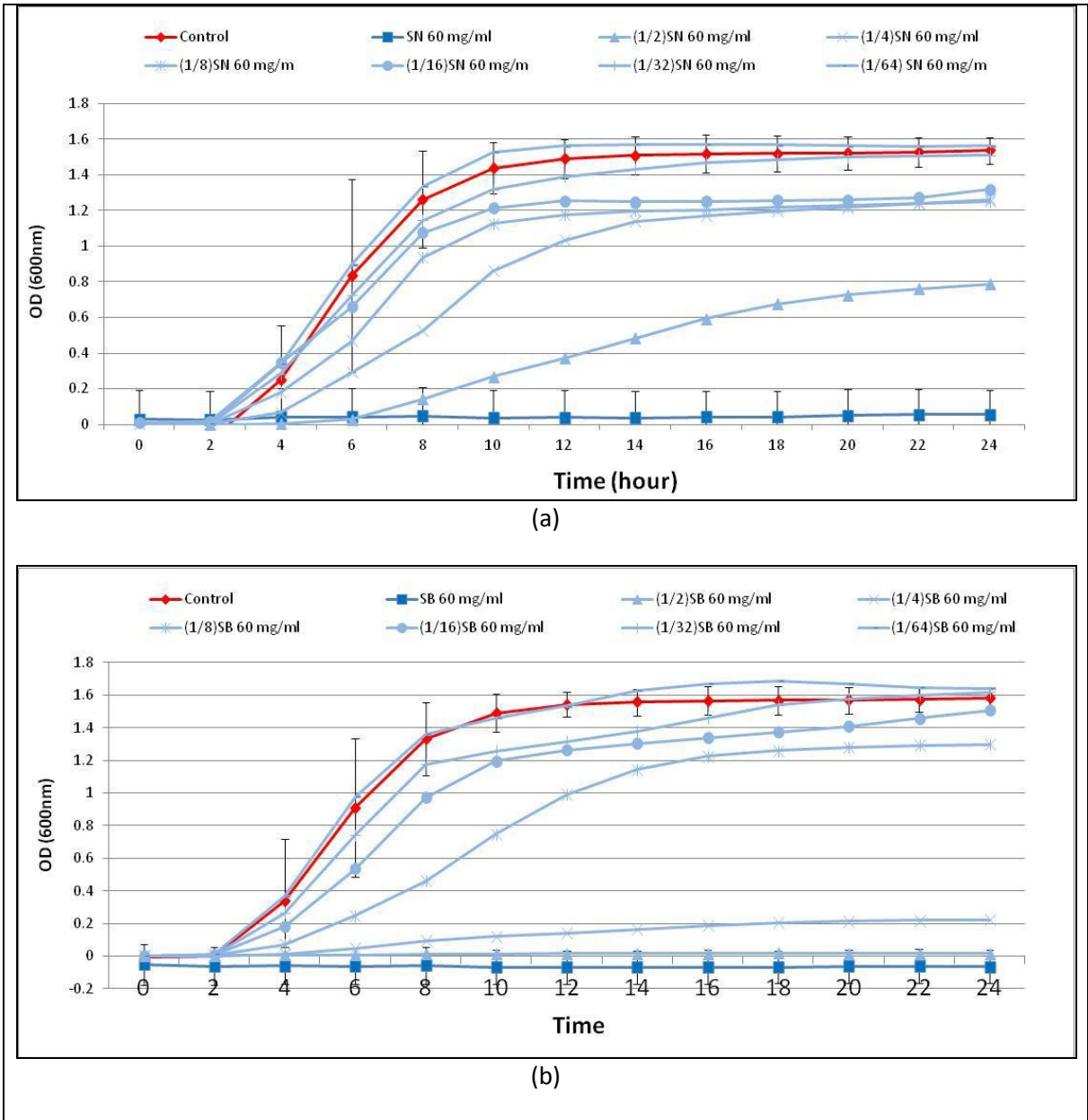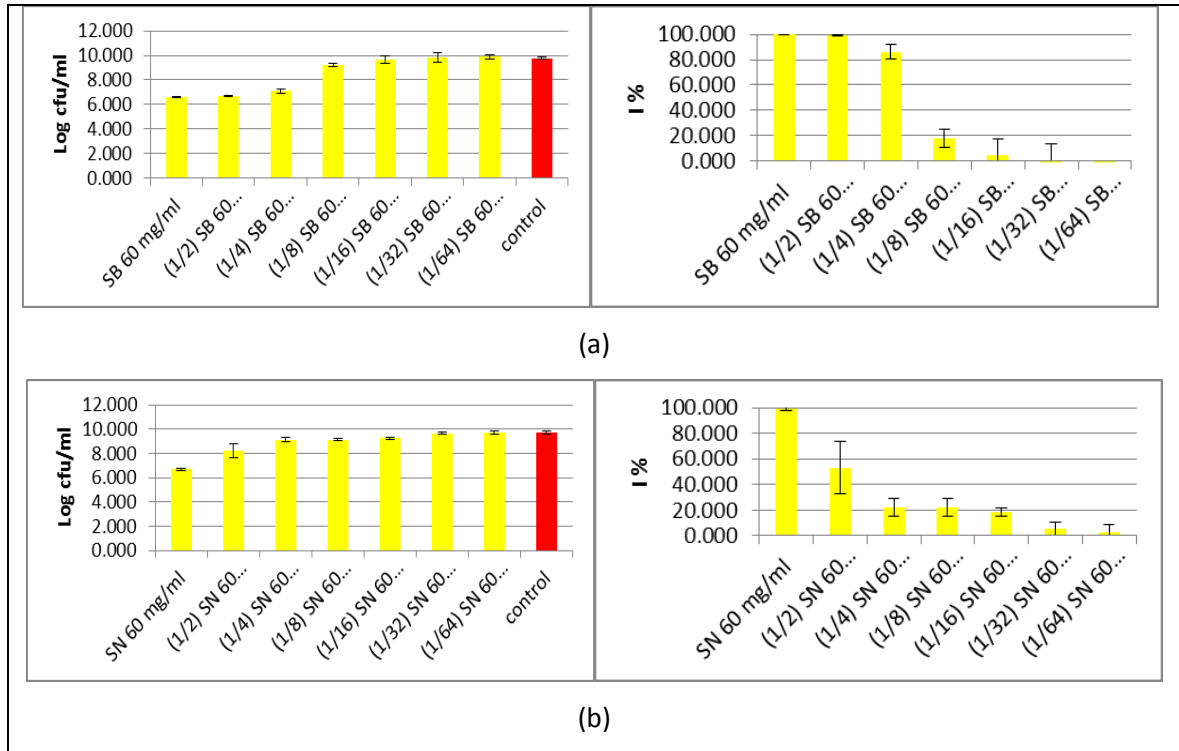
Figure 3.25 Percentage inhibition of different concentrations of sodium benzoate (a) and sodium nitrite (b) against *E. coli* over 24 h & Viable count (log cfu/ml) of *E. coli* in the presence of different concentrations of sodium benzoate and sodium nitrite over 24 h, red colour representing growth control of *E. coli* without SB.

Two independent trials were carried out in duplicate. SB: sodium benzoate and SN: sodium nitrite.
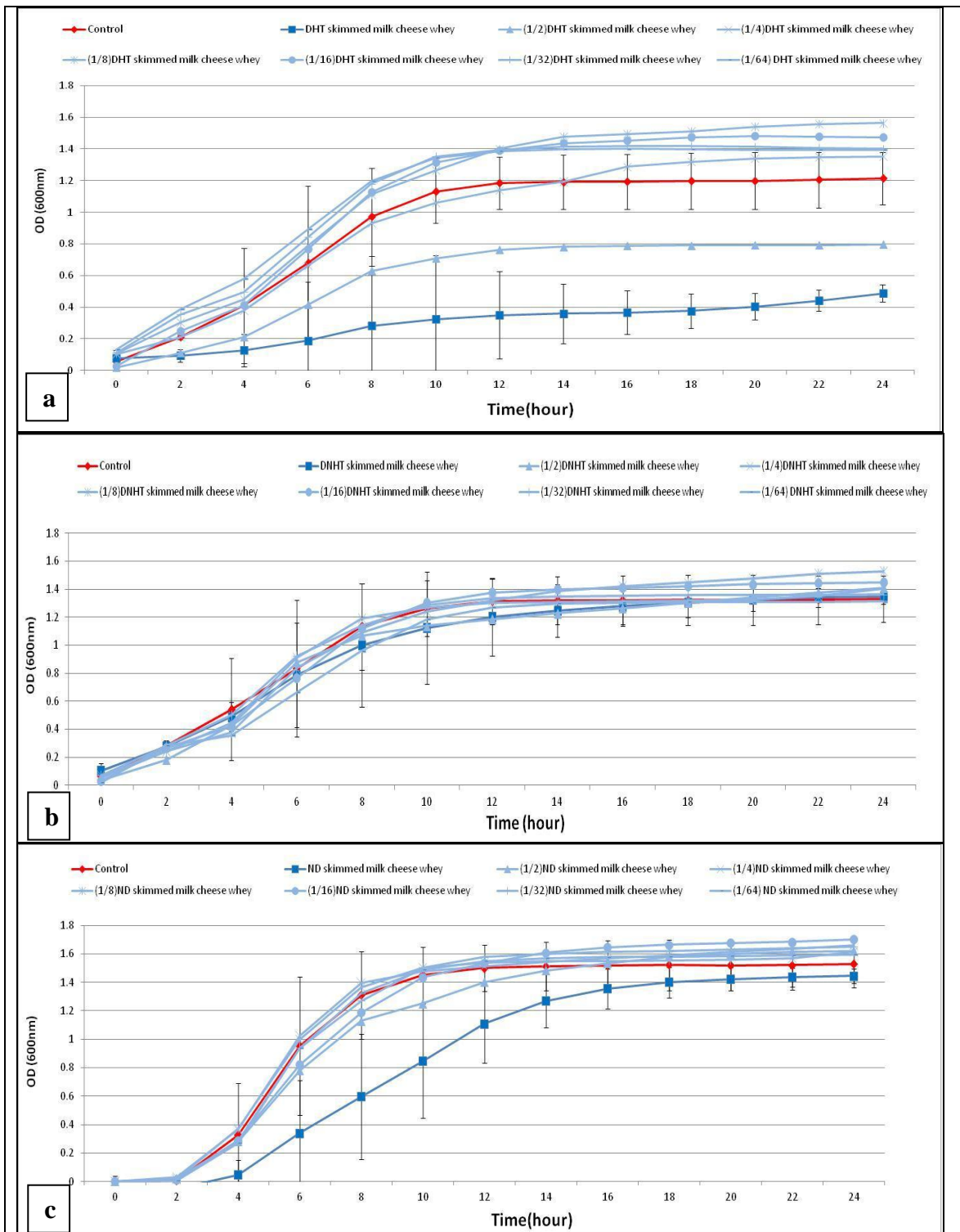
Figure 3.26 Growth inhibition analysis based on optical density (OD) of the different concentrations of skim milk cheese whey samples dialysed heat treated (a), dialysed non heat treated (b) and non-dialysed (c) against *E. coli* over a 24 h period, red colour representing growth control of *E. coli* without whey sample.

Two independent trials were carried out in duplicate. D: Dialysed, HT: Heat treated @ 65°C for 20 min, N: None, ND: Non-dialysed.
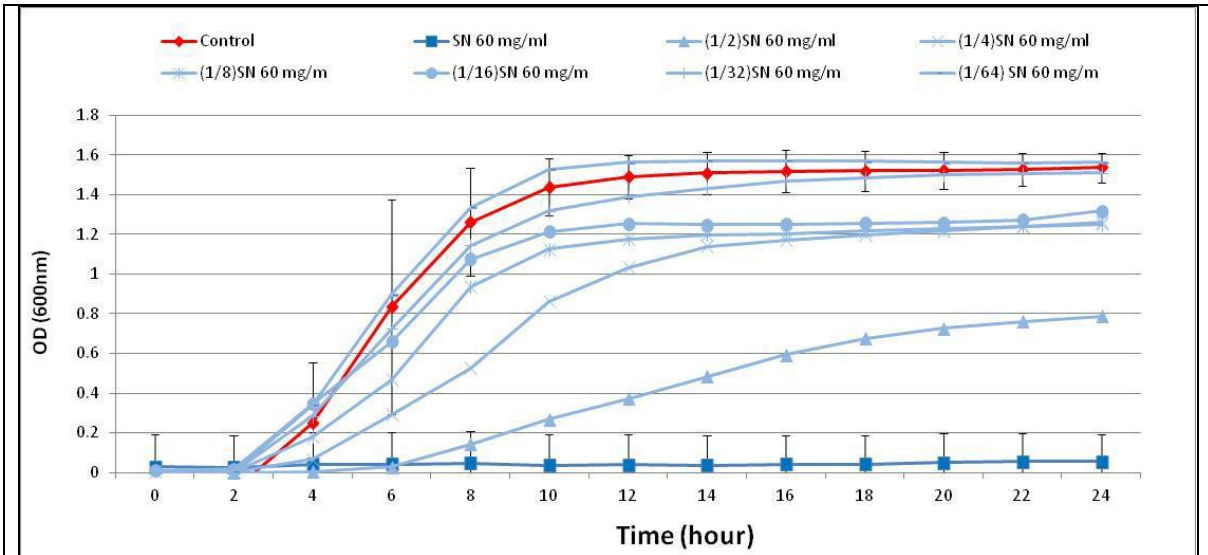
(a)
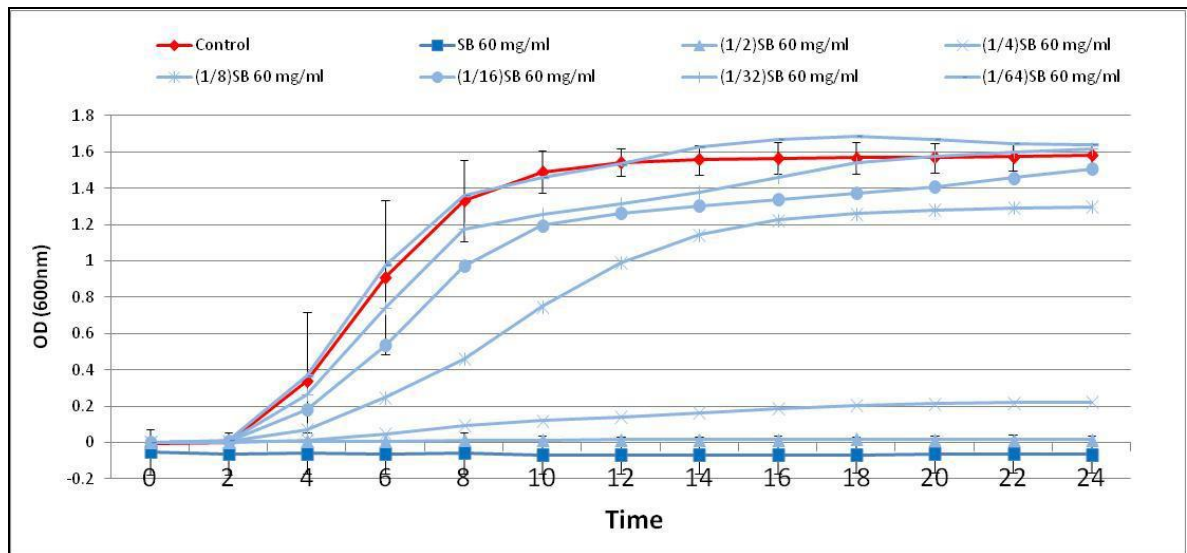
(b)

Figure 3.27 Growth inhibition analysis based on optical density (OD) of the different concentrations of sodium benzoate (a) and sodium nitrite (b) against *E. coli* over a 24 h period, red colour representing growth control of *E. coli* without SB.

Two independent trials were carried out in duplicate. SB: sodium benzoate and SN: sodium nitrite.

3.5 SDS-PAGE of whey samples for Peptide Molecular Weight Evaluation

The peptide pattern of the whey samples were observed by SDS-PAGE as described in section 2.7. Figures 3.28 and 3.29 show the peptide profile of three whey samples from Batch 6 after different processing steps. The SDS-PAGE was used to analysis the molecular weight distribution of peptides of the whey samples. The three whey samples investigated had different banding patterns because of the difference of the protein and peptide contents among the different whey samples. The SDS-PAGE showed that the whey samples had two visible bands in the molecular weight range of $10 - 25$ kDa (Figure 3.28). These two bands are α-Lactalbumin approximately 14 kDa and β-Lactoglobulin approximately 18 kDa. The blue cheese whey samples bands are more intense compared to others (Figure 3.29). The bands of skimmed milk cheese whey samples were the weakest implying the lowest amount of peptides. These results were in agreement with the finding of protein content in Bradford assay and microtiter plate in antimicrobial assay techniques. Low molecular weight standards and high molecular weight standards are shown as markers.

Minor bands are related to secretory components with molecular weight of approximately 63 kDa, Serum albumin with molecular weight of approximately 66 kDa and Lactoferrin with molecular weight of approximately 76 kDa. Other small bands with high molecular weight are related to Immunoglobulin G1 and Immunoglobulin G2 with molecular weight of approximately over 150 kDa. This data were in accordance with the other studies that were investigated whey proteins trough SDS-PAGE (Tovar Jiménez *et al*., 2012: Bonnaillie *et al*., 2014).

1: Blue cheese, DHT
2: Blue cheese, DNHT
3: Blue cheese, ND
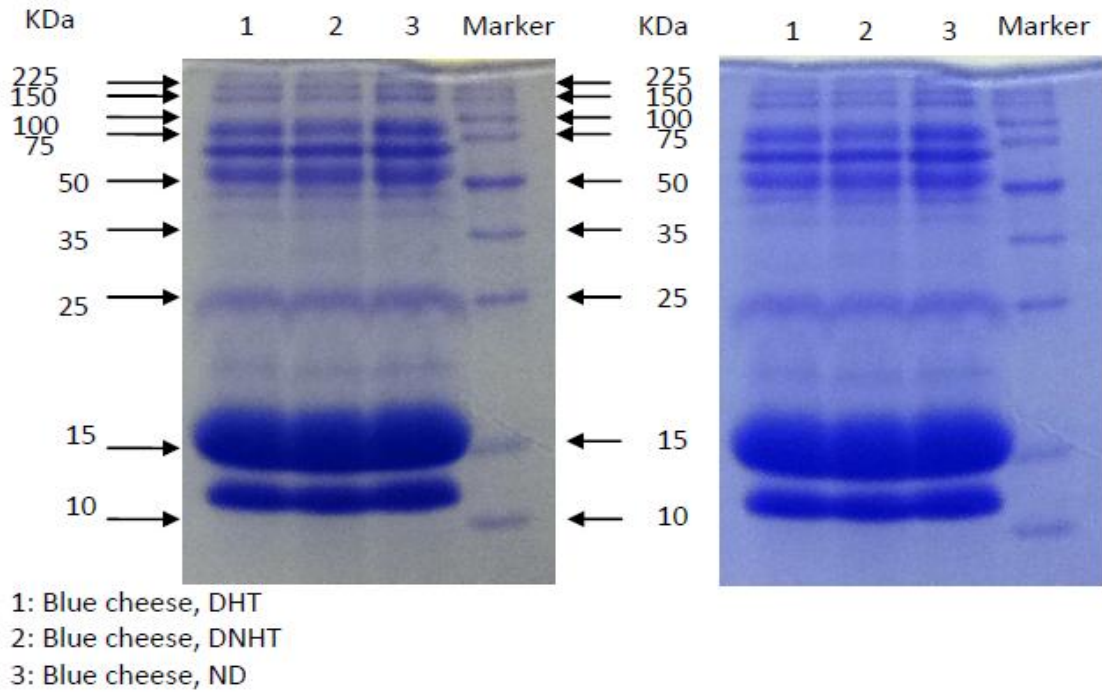
Figure 3.28 SDS-PAGE Blue cheese whey samples
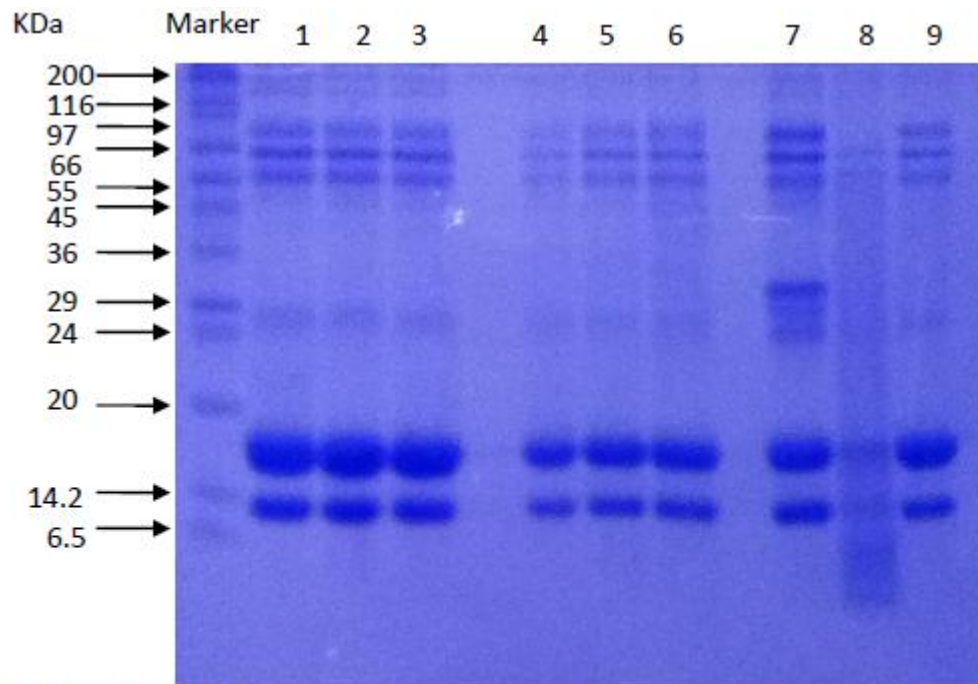
1: Blue cheese, DHT
2: Blue cheese, DNHT
3: Blue cheese, ND
4: Cheddar cheese, DHT
5: Cheddar cheese, DNHT
6: Cheddar cheese, ND
7: Skimmed milk cheese, DHT
8: Skimmed milk cheese, DNHT
9: Skimmed milk cheese, ND

Figure 3.29 SDS-PAGE Blue cheese, cheddar cheese and skimmed milk cheese whey samples.

4. Conclusion

A summary of the main conclusions arising from this work, including suggestions for future research

4.1 General conclusions

In conclusion, among all the whey samples the last three samples from Batch 6 were chosen for treatment and further characterisation as they had a higher protein content and they showed antimicrobial activity against the specific pathogen *Escherichia coli* ATCC 25922.

The result from the heat treatment (65°C at 20 minutes) showed significant log reduction approximately 6 log for the blue cheese whey samples and the skimmed milk cheese whey samples. Lower log reduction of about 3 log was observed after heat treatment in the cheddar cheese whey samples. Microorganisms were almost completely removed in the blue cheese whey samples after treatment but in the cheddar cheese whey samples and the skimmed milk cheese whey samples still remained after treatment and continued to grow over storage time.

The result from the antimicrobial activity assay were in agreement with Bradford assay as blue cheese non dialysed (ND) had the highest protein content about 338.01 ± 3.79 mg/100ml and showed the highest percentage inhibition rate about 93.29 ± 5.25 against specific pathogen *E. coli*.

Whey samples from Batch 6, Blue cheese and cheddar cheese whey samples successfully displayed antimicrobial activities. At higher concentrations whey samples, antimicrobial activity was the strongest, indicating that this activity was concentration dependent. Analysis of the most effective concentration of whey samples against specific pathogen *E. coli* was performed over a 24 h period using microtiter plate assay.

The blue cheese non-dialysed (ND) had the highest antimicrobial content of the other 9 studied whey samples from Batch 6 against *E. coli* which is equivalent to the activity of sodium benzoate (60mg/ml) a popular food preservative. The antimicrobial activity of other whey samples (blue cheese whey and cheddar cheese whey samples) did not vary significantly except skimmed milk cheese whey samples which showed weaker antimicrobial activity against *E. coli*.

This study utilised a substantially more sensitive detection method to quantify antimicrobial effects of whey, which mainly relied on rather insensitive standard agar disc diffusion assays. The microtiter assay applied allowed the detection of antimicrobial effects of whey sample at low concentration level which would otherwise have been undetected in the agar disc diffusion assay.

The microbial enumeration study revealed the microflora of the whey samples were dominated by mesophilic, lactic acid bacteria, yeast and moulds. The numbers of psychrotrophic microorganisms varied in different Batches. In general due to availability of nutrients, water activity, suitable pH and temperature in fresh whey cheeses, all microbial groups tend to grow in viable numbers within the first 24 h following production and later psychrotrophs even in low temperature grew. In this study average of microorganisms' population in different Batches were over 5 log (CFU/mL).

Bradford assay was carried out to determine the protein content of the whey samples instead of the Kjeldahl method with advantage of higher sensitivity for protein, shorter time for whole assay and simplicity of the experiment. The difference in the protein content among the various whey samples could be due to different methods for processing of whey samples and the effect of heat treatment and dialysis on the concentration of protein. The results of Batch 1 and 2 whey samples showed that heat treated samples had lower protein content than non-heat treated samples and this was also observed in the result of Batch 6 whey samples.

In general, the blue cheese non dialysed (ND) whey sample had higher protein content of 338.01 ± 3.7 (mg/100ml). This data was in agreement with the data that obtained from antimicrobial activity assay that was higher for the blue cheese non dialysed (ND) whey sample.

Peptide pattern of whey samples from Batch 6 were observed by SDS-PAGE. The blue cheese whey sample bands were more intense compared to others. Two visible bands were observed in the molecular weight of approximately 14 kDa (α-Lactoalbumin) and 18 kDa (β-Lactoglobulin). Other minor bands are related to molecular weight of approximately 63 kDa (Secretory components), 66 kDa (Serum albumin), 76 kDa (Lactoferrin) and over 150 kDa (Immunoglobulin).

The antimicrobial activity of whey protein can be attributed to the iron-binding property of α-Lactoalbumin, β-Lactoglobulin, Lactoferrin, Lactoperoxidase (Lp), BSA and Lysozyme. Antimicrobial activity of the whey peptides reported against different gram-positive, gram-negative bacteria, yeast and filamentous fungi. This potential might be particularly related to Lactoferrin (iron binding protein to sequester iron from bacteria inhibiting its growth and metabolism) which was observed in SDS-PAGE of whey samples, or other parameters that discussed in literature review. The antimicrobial activity of Lactoferrin has been reported in several studies against gram-positive and gram-negative bacteria due to interaction with LPS in gram-negative bacteria damaging cell wall and reduction of negative charge on the cell wall of gram-positive bacteria by binding to anionic molecules (Gonzalez-chavez et al., 2008).

The physio-chemical properties (pH, water activity, moisture content, ash content soluble solids, turbidity and colour analysis) of whey samples Batch 1 and 2 were determined. Variation of some parameters such as total soluble solids and turbidity might be attributed to different process technologies such as heat treatment and filtration methods that can influence the composition of different whey samples. The water activity and moisture content in both samples were high which can be variable depending on the origin of the whey samples and manufacturing processes. Another parameter which is pH value that was 4.5 and it's a critical point in food processing. The colour analysis of whey samples showed that heat treatment affected the colour of whey samples.

The blue cheese dialysed and heat treated (DHT) was considered the cleanest sample among other whey samples in terms of microorganisms due to high log reduction after treatment. The blue cheese (DHT) with a protein content of 323.31 ± 4.46 mg/100ml showed percentage inhibition rate of 71.80 ± 2.08 against *E. coli* which will be suggested for washing fruits and vegetables as a natural decontaminant for future investigation.

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