Electrical Conductivity as an Indicator of Milk Spoilage for Use in Biosensor Technology

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Abstract

Milk is characterised as a perishable food. It is vulnerable to microbial contamination and has a limited shelf life, even when stored in a cold environment. Rapid milk spoilage is a sustained problem that restrains the shelf life of milk, and it consistently burdens the global food waste. Thus, there is a continuous interest in seeking better means of milk quality control and management. Recently, the development of biosensing technology offers a potential solution for better managing strategies of milk quality. Biosensors have been developed from growing demand for a reliable, cost-effective and rapid chemical detection tool. Many disciplines including clinical medicine, food industry, and environment monitoring employ biosensors as analytical tools. In particular, the use of electrical conductivity (EC) as a biosensing approach has frequently been studied in the dairy sector. However, its application to milk spoilage has yet to be fully explored.

The scope of this study was to investigate the use of EC as a parameter to aid in the prediction of milk spoilage. A portable conductivity meter was used to measure the EC in milk; the total bacterial count (TBC), lactic acid (LA) concentration and pH were assessed using standard plate count methods, titratable acidity and digital pH meter, respectively. Commercial pasteurized skim and whole milk were used in the study. The variations of EC, TBC, LA concentration and pH were measured over an extended storage of milk that held at either 4 or 8℃ in the trial experiment. The change in EC was comparatively examined with the change of other measured parameters, and the interrelationship between EC and parameters was analysed by correlation analysis. In addition, several laboratory-controlled model systems were used to assess the impacts of every individual parameter on the change of EC. The results of trial and model systems were compared with each other.

The trial experiment showed that EC progressively increases with an increase in TBC, LA concentration and pH during spoilage of skim and whole milk under storing at 4 and 8℃. The change in EC was found to have moderate to strong correlations with the measured parameters in spoilt milk. A statistically significant difference in EC value was observed before the complete spoilage of milk, when either the flavour defects or textural changes occurred. Moreover, the model systems revealed that the increase in EC is proportional linear to an increased LA concentration and decreased pH. By comparing the results between trial experiment and model systems, it showed that LA approximately contributed one-quarter of the total proportion of changed EC in spoiled milk. Furthermore, a number of bacteria present in milk with more than 10^7 colony forming units (CFU)/ml significantly decreased the mean

EC value of milk. In addition, the 'best before date' (BBD) underestimated the correct shelf life of milk at both 4 and 8℃.

The fixed nature of BBD restrains its use as a suitable indicator. In comparison, EC can be a potential alternative to predict milk spoilage. Since it is a direct measurement of spoilage of milk, and changes simultaneously with the growth of bacteria, production of LA and acidity in milk held at either the optimal (4℃) or the inappropriate (8℃) temperatures. Further investigations are needed to obtain a better understanding of the interrelationship between EC and milk spoilage preceding the valid application of biosensing technology.

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me up with love, patience, encouragement, and supports. Without their support, this accomplishment would never have been successfully achieved.

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List of abbreviations

H_2O_2	Hydrogen peroxide
R^2	R square
℃	Degree Celsius
<	Less than
BBD	'Best before date'
Ca	Calcium
CFU	Colony forming unit
Cl	Chloride
EC	Electrical conductivity
GAL	β-galactosidase
GOx	Glucose oxidase
HCl	Hydrochloric acid
HDPE	High-density polyethylene
HTST	High-temperature short-time treatment
K	Potassium
KCl	Potassium chloride
LA	Lactic acid
MFGM	Milk fat globule membrane
Mg	Magnesium
mS/cm	milliSiemens per centimetre
Na	Sodium

NaOH	Sodium hydroxide
NIR	Near-infrared spectroscopy
P	Phosphate
PA2576	*Pseudomonas aeruginosa 2576*
PCA	Plate count agar
qPCR	Quantitative real-time polymerase chain reaction
SPC	Standard plate count
TBC	Total bacterial count
TDS	Total dissolved solids
TSA	Tryptic soy agar
UF	Ultrafiltration
UHT	Ultra-high temperature treatment

Chapter 1 Introduction

1.1. Trend in biosensors

Over the past decades, there has been an increasing demand for reliable and cost-effective analytic tools to meet the challenges in fields requiring compound detection (Turner, 2013). In response to this demand, biosensors have been developed as a promising tool to offer precise detection over a wide range of chemical components (Kirsch, Siltanen, Zhou, Revzin and Simonian, 2013). As one of the most advanced inventions, biosensing technology has initialised a revolutionary breakthrough and rapidly developed with a vital role in many applications. Their applications span disciplines as diverse as clinical medicine, food testing, environmental sensing, and process control monitoring (Goode, Rushworth and Millner, 2015). One of the major roles played by biosensors is to be an alternative with the most potential to replace the traditional procedures. The traditional methods regularly used in current industrial, clinical, and research laboratories are time and labour intensive, and also require specialised expertise (Zhang, 2000; Fraser, 1997; Goode et al., 2015). The advantageous features of biosensors overcome the weakness of traditional methods. These features include rapid detection, fast response, capabilities of miniaturization, low cost, robustness and high specificity and sensitivity (Zhang, 2000; Chaubey and Malhotra, 2002). Moreover, a growing volume of capital input and research effort have been made in the field of biosensors, and this leads to an exponential expansion in the application of biosensors among many industrial manufacturers and societies (Kirsch et al., 2013; Turner, 2013; Verma and Singh, 2003; Herrinngton, 1948). Among different categories of biosensors, electrochemical biosensors have attracted the most attention due to their overwhelmingly advantageous features over other biosensors. Electrochemical biosensors provide a mean to convert the biological events directly to electrical signals, and the measurable electrical signals are proportionally amplified with the biological reaction that is taking place in the testing solution. In addition, the most potential of electrochemical biosensors to be miniaturized at low cost, high sensitivity and specificity makes it the best choice of tools in many research areas (Escamilla-Gomez, Susana, Maria and Jose, 2008). In the extensive practical application of biosensors, except that medical diagnostics predominate the majority of research activities, food safety, quality control and agricultural and environmental monitoring rank the second major marketplace of biosensors (McMeekin and Ross, 1996).

1.2. Economic motivation and food waste issues in milk industry

In 2013, the total estimated milk production was greater than 644 million tons and valued at approximately USD $69 billion (Driehuis, 2013). A large amount of production is likely due to expansion in global wealth, increase in population and a healthier diet aided by milk as a key nutritional source (Tajammal, Yu, Young and Wilson, 2015). New Zealand contributes 3% of the world dairy market, which ranks ninth among the largest milk producing countries in the world (Foote, Joy and Death, 2015). In 2014, a total of 21 billion litres of milk were produced in New Zealand and exported all over the world, mainly to Australia, the United States and Asian countries (Foote et al., 2015). Dairy farming earns NZD $18 billion annual revenue and this plays a major role in the domestic economy (Foote et al., 2015).

Quality control of food, particularly perishable food is one of the most critical issues. Among the perishable food types, milk is of the greatest concern. The rapid spoilage and limited shelf life of milk frequently challenges the level of consumers' acceptance and contributes to global food waste and monetary diminishment during transportation, storage and post-consumption (Muir, 1996). Food loss that is caused by bacterial contamination of dairy products is of equal concern to the vast quantity of milk production. In 2008, the estimated total food loss at retail and consumer levels in the United States was 166 billion tons (Koester, 2013). Dairy product ranked third in the nine most frequent purchased food categories, and it accounted for 14% of total food loss and valued at USD $15 billion (Koester, 2013). In addition, developing countries sustain a substantial percentage of the global food loss due to the lack of suitable sanitation, transportation and food storage facilities (Koester, 2013). The shortage in appropriate dairy handling equipment leads to significantly lower transportation distance, shelf life stability and quality of fresh liquid milk. This issue causes additional food loss in dairy products, which is not only a burden to environmental sustainability but also leads to depleted economic growth and global consumption demands (Ledenbach and Marshall, 2009).

1.3. Problems associated with milk shelf life

Milk is a complex fluid containing rich nutritional sources of calcium (Ca), essential proteins, fatty acids, lactose, and a variety of vitamins and minerals (Eskin, 2012; O'Mahony and Fox, 2014). The freshly drawn raw milk is a great seeding bed for the growth of a variety of pathogenic and spoilage bacteria, and therefore, milk in its natural state is a delicate and

perishable material. Typically, to maintain the quality and extend the shelf life of milk to a period of 14-21 days of storage, a minimum thermal pasteurization often is employed to eliminate the growth of bacteria in fresh raw milk (McSweeney and Fox, 2008). In spite of this strategy, the freshly drawn milk remains susceptible to rapid spoilage by bacterial contamination. Microbial activity in milk is a leading cause of rapid milk spoilage (Fu and Labuza, 1993). Spoilage bacteria including but not limited to *Pseudomonas* and *Bacillus* are constantly present and worsen the quality in minimally processed refrigerated liquid milk during storage. Their presence not only carries the potential for infectious disease in humans, but is also the main detrimental contributor that limits the shelf life, quality stability, and the level of consumers' acceptance of milk (McMeekin and Ross, 1996; Muir, 1996).

Moreover, customers rely heavily on the 'best before date' (BBD) to predict the milk shelf life in the post-consumption stage. However, the use of BBD can be less than an accurate method. The correct prediction of shelf life by BBD is under certain circumstances when the milk is stored at the optimal storage temperature of 0-4℃ (McMeekin and Ross, 1996). In most conditions in which enormous variations in domestic refrigerating systems are not meeting the required cooling standards, therefore, the BBD is an inaccurate prediction mean of milk spoilage under this situation. The extensive variations in cooling system lead to a wide difference in the actual expiry time for every individual bottle of milk. The overall effect of the external factors result in a failure in the non-precise prediction of milk spoilage by BBD (McMeekin and Ross, 1996).

1.4. Research gaps

The issues related to the limited shelf life and rapid spoilage of freshly chilled pasteurized milk frequently challenges food scientists and technical engineers to look for reliable detection tools for on-the-spot inspection of milk quality. Typically, the inspection of milk quality during industrial production and laboratory testing is accomplished using traditional methods, the standard plate count (SPC) technique. SPC provides a quantitative evaluation of the total content of microorganisms by culturing, counting and biochemical assays (Marth, 1978). Although these methods are reliable, they are time-consuming and must be performed in microbiology laboratories with the aid of qualified personnel (Marth, 1978). Study evidenced that none of the direct count methods such as SPC, psychrotrophic bacterial count (PBC), modified psychrotrophic bacteria count (mPBC) and Moseley test can significantly predict the

shelf-life of post-pasteurized whole milk (Bishop, White and Firsrwnbergedenf, 1984). In contrast, their experimental results have shown that the method of using electrical resistance can provide a strong positive relationship to the shelf life of 100 tested whole milk specimens (Bishop et al., 1984). Therefore, the concept of using electrics as a faster and accurate detecting method appears to be a better solution than traditional analytic approaches.

In request of a reliable analytical mean for fast chemical detection, scientists and engineers in recent decades have regained insight into the application of biosensors. The use of biosensors in milk quality inspection have been frequently researched in the aspects of milk composition analysis (Willem, Olieman, Cazemier and Verheijen, 2001), milk safety and pathogenic bacteria detection (Velusamy, Arshak, Korostynska, Oliwa and Adley, 2010), evaluation of antibiotic contamination (Reder-Christ and Bendas, 2011), water adulteration (Durante, Becari, Lima and Peres, 2016), detection of allergenic contents (Eshkenazi, Maltz, Zion and Rishpon, 2000), and evaluation of progesterone concentration (Gillis, James, Joseph and Marian, 2002) in milk and milk products. However, the field of maintaining freshness and extending the limited shelf life of dairy products is a less attracted research area.

There are few devices that are capable of offering functions for freshness monitoring and shelf life inspection of liquid milk. Recently, a study by Wu et al. (2015) demonstrated a 3D-printed microelectronics integrated smart milk cap biosensor prototype that provided an on-package and real-time detection of the degree of freshness in the milk. Their biosensor model showed good characteristics to periodically monitor the freshness of liquid milk and juice (Wu, Yang, Hsu and Lin, 2015). Apart from this prototype, the number of biosensors that can offer such functionality in the marketplace or laboratories is inadequate. Therefore, there is a research and commercial gap in the assessment of quality deterioration of milk at the on-shelf storage and post-consumption stages. An emerging demand exists for rapid quality detection devices that would meet the requirement of dairy manufacturers and consumers' desire.

1.5. Research hypothesis and questions

The conductometric biosensor is a type of electrochemical biosensor that uses a conductivity meter as a transducer system to connect the electrical biorecognition element and the targeting analytes in testing environment. Conductometric biosensors measure the electrical conducting capacity of the tested material (Velusamy et al., 2010). Monitoring the change in electrical conductivity (EC) was proposed as a valid method to study the growth and metabolic activity

of many bacterial strains, including *Streptococcus salivarius*, *Streptococcus lactis*, *Lactobacillus delbrueckii*, *Lactobacillus bulgaricus*, and *Escherichia coli* in milk and milk products (Richards, Jason, Hobbs, Gibson and Christie, 1978; Liu, Settu, Tsai and Chen, 2015). However, there is very little known about how the growth of spoilage bacteria influences the change in EC in milk. Milk is intrinsically electrically conductive due to the presence of dissolved free state mineral salt and other soluble electrical conducting compounds in the fluid (Lanzanova, Mucchetti and Neviani, 1993). The total amount of dissolved inorganic and organic materials in a liquid solution is defined as the total dissolved solids (TDS). It has been illustrated that the balance of TDS in milk dynamically changes during an extended storage. Mabrook and Petty (2003) used laboratory-constructed biosensors to investigate the effect of milk composition on electrical conductance in skim and whole milk (Mabrook and Petty, 2003b). The investigator reported that the electrical conductance is mutually influenced by the interaction with various milk compositions during an extended storage, and the relationship associated more significant with increased storage time. Among the milk compositions, it was suggested that the electrical conductance is predominately associated with the mineral salt fraction in milk. Besides, the investigator further found that a decrease in conductance associated with increase in milk fat content, and not relevant to lactose content of milk (Mabrook and Petty, 2002).

Microbial activity is a critical causation agent of milk spoilage. The action of microbial metabolism was proposed to catabolize and degrade uncharged milk substrates (such as lactose, proteins and lipids) to charged electrical conductive species during the deterioration process of milk (**Figure 1**) (Lanzanova et al., 1993). The charged conductive species compose of lactic acid (LA), charged amino acid, hydrogen ions, organic acid, ionic minerals and so on (Lanzanova et al., 1993). Thus, the growth of microorganisms and their associated metabolism were proposed to be closely associated with the accumulation of charged ionic species in milk (Borch and Wallentin, 1993; Lanzanova et al., 1993; Allison, Anderson and Cole, 1938). Therefore, the microbial activity increase electrical current could own to the fact that they promote the concentration of TDS in milk.

The development of acidity during milk fermentation also affects EC in milk. Several investigations into the effect of milk acidification have demonstrated the influences of LA production and pH drop on EC. EC was found to be a function of acidification in fermented milk (Mucchetti, Gatti and Neviani, 1994; Diaz, Romero, Muelas, Sendra, Antoja and Paredes, 2011). It was explained that during milk fermentation, mainly but not exclusively the lactic acid producing bacteria utilise lactose as an energy source for growth. The lactose was

converted to LA and other organic acids as by-products of microbial metabolism. The production of LA contributes the majority of the acidity in the milk. Therefore, milk acidification is often treated as one of the determinant parameters for the quality of fermented milk (Lanzanova et al., 1993). Nevertheless, milk spoilage is a different biological process from milk fermentation, as the growth of spoilage microorganisms is not limited to lactic acid producing bacteria. Spoilage milk presents a diverse range of bacterial flora, and thus the subsequent impacts of metabolic activity on milk compositions and EC differ widely to fermented milk. The variation of EC in spoilage milk is therefore worthy of investigation.

This study proposes that the milk EC could be a potential indicative parameter to reflect the level of biological and physical changes of milk over the extent of spoilage. This prospection is based on the principle that milk EC change relates to several proposed spoilage associating factors, the factors including microbial activity, acid production and alteration in milk compositions.

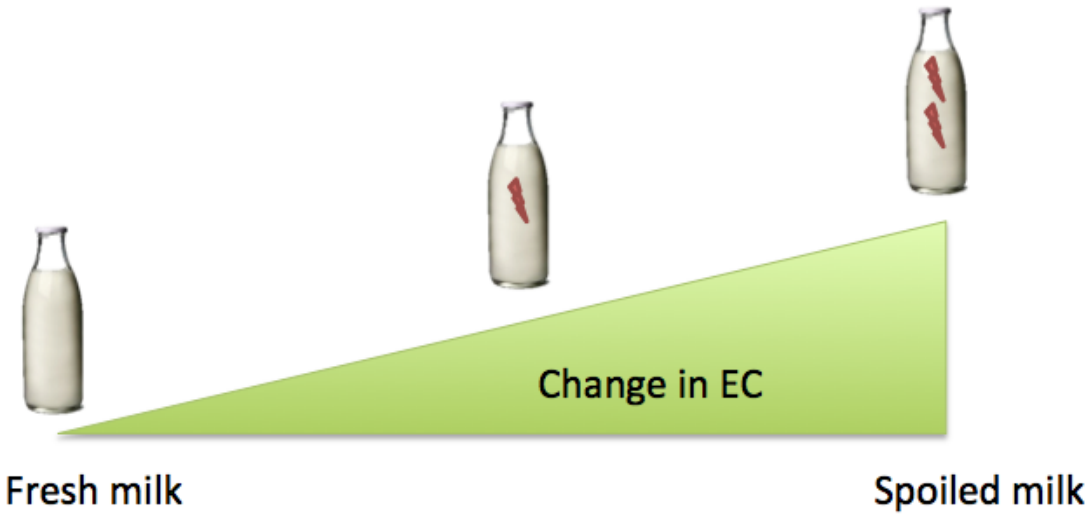


Figure 1. Schematic diagram illustrates the relationship between EC with other potential of changing during milk spoilage.

1.6. Aim and objectives

The aim of the present work is to conduct a preliminary study that investigates the use of EC as an indicative parameter for milk spoilage that can be potentially incorporated in a biosensor system. To this end, this study will examine the variations and relationship of EC and several milk spoilage associated factors in liquid fresh chilled pasteurized milk. This aim will be achieved by completion of the following objectives:

1. Quantify variations in EC, growth of bacteria, LA concentration and pH value in milk held at either 4 or 8°C for an extended period of time.
2. Analyse the relationship between EC and the measured milk spoilage associating factors and investigate the primary contribution of the change in EC during milk spoilage.
3. Justify and discuss the use of milk EC as a potential indicative parameter for milk spoilage indication compared to BBD.

1.7. Thesis outline

This thesis is presented in six chapters:

Chapter 1—Introduction

This chapter provides a brief background on the current trend in biosensing technology, economic motivation in the milk industry and current challenges in relation to milk quality control and management. This is followed by state research gaps and questions, and a proposal of aims and objectives of this thesis. A thesis outline is also present to give concise general insights to the scope of this thesis.

Chapter 2 – Literature review

This chapter contains a comprehensive literature review of biosensor technology and their applications in milk. It covers several aspects of general characteristics, classifications and advantages of biosensing technology. This is followed by illustrating the principle of measurement of EC. This chapter also discusses the physical, chemical and biological properties of milk and the utilising of individual milk compositions in current biosensor detections. In addition, the chapter includes the common contaminating agents that cause milk spoilage and a brief understanding of pasteurization in eliminating microbial contamination.

Chapter 3 – Material and methods

This chapter outlines the experimental design and the materials and methods that are used to conduct the experiments.

Chapter 4 – Results

This chapter displays and describes the findings of the study.

Chapter 5 – Discussion

This chapter discusses the experimental results and major findings in relation to the objectives of this study. It also discusses several limitations and challenges in the current study.

Chapter 6 – Conclusion and further work

This chapter summarises the main findings and suggests recommendations for further work.

Chapter 2 Literature review

2.1. The market of biosensor

In recent decades, biosensors have developed into one of the most significant technologies. They have a wide range of applications in areas consisting of medical diagnostics, food safety, quality control, and environmental monitoring (Patel, 2002). Since the first establishment of the concept of biosensor by Clark, biosensor-based analytic tools have attracted much attention and become an increasingly researched area in many fields (Clark and Lyons, 1962; Nakamura and Karube, 2003). A variety type of biosensor has been constructed with different biorecognition elements and transducers to target specific analytical chemicals (Cetó, Voelcker and Prieto-Simón, 2016). According to Web of Science (*Thomson Reuters*), more than 106,895 papers are containing the keyword of 'biosensor' that has been published during 2000 to 2015. This revealed a substantial growth of research input in the biosensor discipline in recent decades (**Figure 2**). In addition, a similar achievement has also been accomplished in the capital investment market. According to databases that gathered by Global Industry Analysts Inc., the global biosensor market underwent an exponential growth (**Figure 3**). The total capital investment has raised from USD $4.6 billion in 2004 to USD $8.2 billion in 2009. This has been followed by a continuous annual growth rate of 6.3% from 2010 onwards. It is expected to achieve an estimated sale in biosensor of over USD $17 billion worldwide by 2015 (Scognamiglio, Pezzotti, Pezzotti, Cano, Buonasera, Giannini and Giardi, 2010).

Medical diagnosis dominates the global biosensor market due to the rising incidences in diabetes, obesity, heart disease, cancer and respiratory disease. The application of biosensors in the field accounts for 99% of the global capital values, and it is perceived as the predominant driving force for current development of biosensors (Kirsch et al., 2013). This is followed by safety and quality control in food industry as the second largest source of capital input in the global biosensor market. Food safety detection is the most investigated area in food industry, because it provides a more lucrative market for biosensor companies. The application of biosensor in food safety is concentrated in the detection purpose of toxins, pathogenic and spoilage bacteria, antibiotics, and allergenic food compositions (Kirsch et al., 2013; Warriner, Reddy, Namvar and Neethirajan, 2014; Scognamiglio et al., 2010). Among a variety of food, the processed and perishable food are the main investigating food target, because they have most potential of microbial contamination and lack of long shelf life standards (Mello and Kubota,

2002). Thus, the identification and detection of microorganisms and virus offer a great market potential for biosensors in food testing area. The expenditures in pathogen and spoilage bacteria testing in food have grew to USD $192 million in 2005. Among the tests, dairy industry constitutes the second largest microbial testing market, it accounts for 32% of total

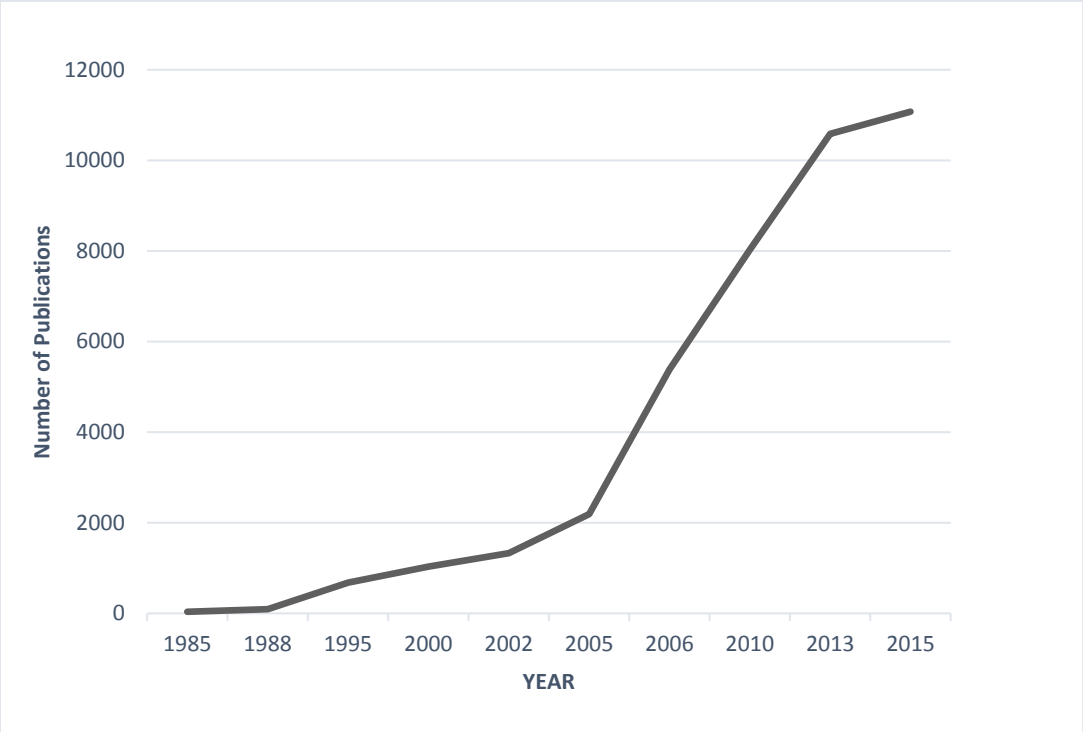


Figure 2. The number of publications involving biosensors during the period of 1985-2015. Data were gathered from ISI Web of Knowledge database, Thomson Reuters.

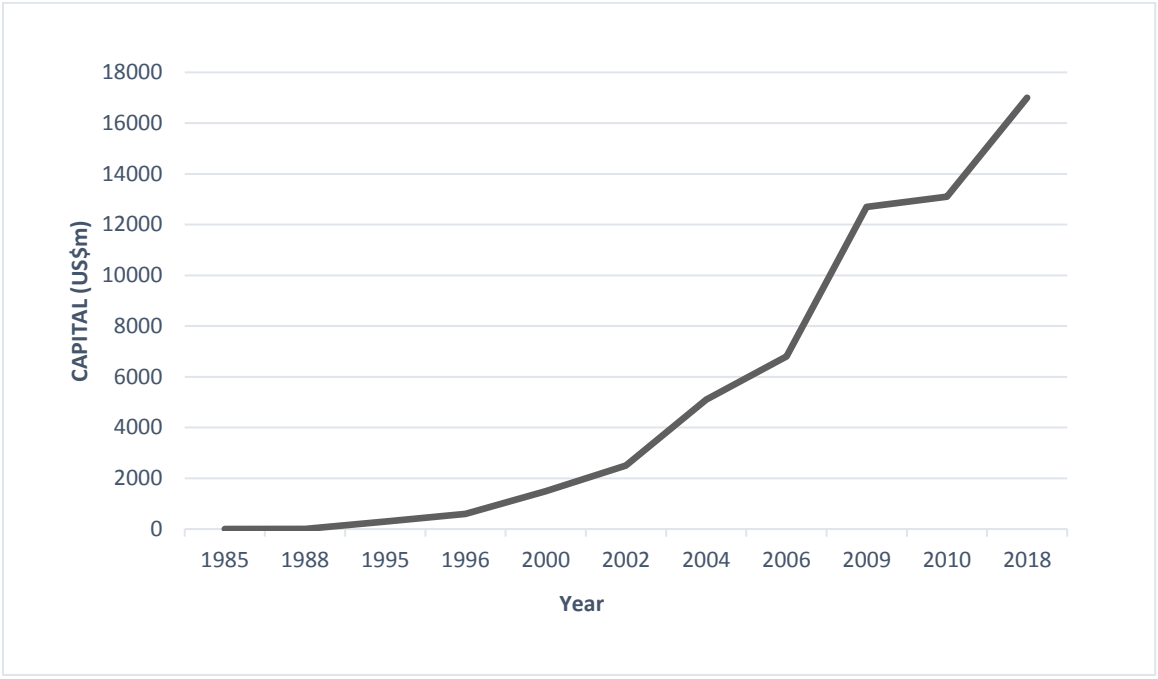


Figure 3. The estimation of global biosensor market during 1985-2018**.** Chart adapted from Turner, 2013.

testing in food industry with over 630 microbial tests per plant per week (**Figure 4**).

2.2. Advantages of biosensors over conventional procedures

Conventional approaches for identifying food pathogens and sanitary microbes are heavily reliant on bench top methods. The methods include enrichment culture, bacterial colony counting methods, biochemical assay, genetic interpretation and quantitative real-time polymerase chain reaction (qPCR). All of these laboratory procedures are time-consuming and require expensive equipment and reagents, and highly trained personnel (Adley and Ryan, 2015). In particular, each performance of qPCR requires 4-6 hours with an attaining detection limit of $10^1 - 10^6$ colony forming unit (CFU)/ml, and the colony counting method requires at least 72 hours for a detection limit of $10^1 - 10^{10}$ CFU/ml (Adley and Ryan, 2015). These methods are unsuitable for use in food quality control at post-consumption stages, because after 72-hours incubation of microbial testing, the product has been transported to the retailer, supermarket and domestic consumers. As a consequence, although conventional methods have high specificity and sensitivity, they are greatly restricted by assay time, equipment and sophisticated protocols (Adley and Ryan, 2015). The drawbacks of conventional procedure have led to a search for better detection alternatives in the food industry (Adley and Ryan, 2015; Mello and Kubota, 2002). Biosensors are a relatively newly emerged analytic technique, and it is a promising alternative to traditional methods due to the possibility of fulfilling demands that traditional analysis methods do not process (Mello and Kubota, 2002; Rasooly and Herold, 2011). Biosensor is a highly requested tool that satisfies the expanding demand for fast response, easy-to-use, and reliable chemical analytic devices (Turner, 2013). It offers good detection towards various target analytes, and the detection complete in a time and cost-effective manner. Several important advantageous features of biosensors over conventional bench top analytical procedures are listed below:

1. High sensitivity and specificity. In most cases, biosensor couples biological components in the biological recognition compartment to facilitate the detection. The biological components could be enzymes, antibodies or other biological elements. The mechanism of using these elements are similar to the biochemical assays used in conventional methods, but with improved effectiveness of detection time. Biosensor can target specific compounds and substrates in biological environment and convert the biochemical signals proportionally to quantified electrical signals (Mello and Kubota, 2002).

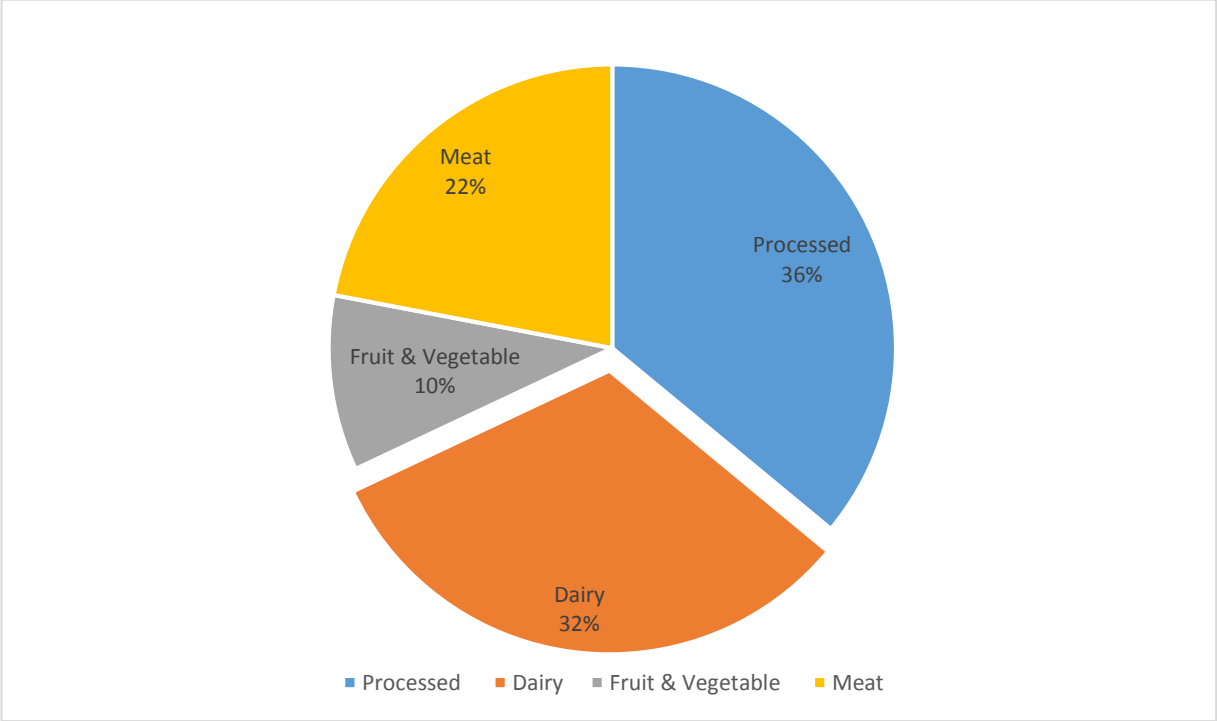


Figure 4. The percentage of total proportion of microbial test per sector in meat, processed, fruit, vegetable and dairy industry. Chart adapted from Alocilja and Radke, 2003.

2. Fast response and real-time analysis. One of the most appreciable features of biosensors is the fast rate of response. The targeting chemical reaction in the interface of biosensor and biological environments often occurs within minutes or seconds. This feature gives the potential to perform on-the-spot of detections (Chaubey and Malhotra, 2002).
3. Miniaturization. Biosensors have inestimable benefits of being miniaturized. Due to the miniaturization of transducer systems, many electrochemical biosensors can be fabricated with a thin layer of interdigitated electrical conducting material (Webster, Sismaet, Conte, Chan, and Goluch 2014; Sarkar, Tothill, Setford and Turner, 1999). Miniaturization facilitates the integration with various devices and equipment such as milking systems, dairy tanks, food packaging, and other dairy machinery for continuous monitoring (Rasooly and Herold, 2011).
4. Additional advantages include requiring minimum sample preparation, less training for operation, low cost of construction and storage space. The dairy industry is particularly in need of rapid and affordable instruments to monitor chemical compounds and microbial contamination during the course of production and shelf life maintenance with faster and reliable methods that can replace with existing one (Mello and Kubota, 2002).

2.3. Biosensor detection techniques

Biosensor is an analytical device that comprise of two components: the biological recognition element and the transducer system (Yunus, Jonas and Lakard, 2013). The biological recognition element recognises selective chemical targets in a biological environment and launches electrical signals to transducers (Rasooly and Herold, 2011). The chemical targets can be a wide range of substances. The substances are as diverse as ions, gases, enzymes, antibodies, and relative larger molecules such as biological active metabolites, organelles, whole cells and cellular tissues (Rasooly and Herold, 2011). The main function of transducer system is to further convert the biochemical changes that result from the interaction with chemical analytes in biological environment to detectable electrical signals (Chaubey and Malhotra, 2002; Warriner et al., 2014). Based on the type of transducer that used in biosensor, it can be divided into four categories: optical, calorimetric, piezoelectric and electrochemical biosensor (**Figure 5**).

2.3.1. Non-electrochemical Biosensors

There are three kinds of non-electrochemical biosensors; optical, calorimetric and piezoelectric biosensor. Optical biosensors measure light signals in the form of luminescent, fluorescent, colorimetric or other optics (**Figure 6**). The device is activated by the absorption and emission of light as a consequence of the chemical interaction with target analytes in the biorecognition element (Singh, Srivastava, Oh, Ahn, Choi and Asthana, 2012). In such sensors, the light waves are often guided by optical fibres to suitable detectors (Peterson and Vurek, 1984; Seitz, 1987). It provides a highly efficient and sensitive approach with near-immediate detection of a wide range of analytes (Singh et al., 2012). One example of the commercial optical biosensor is the hybrid electrochemical/optical light addressable potentiometric sensor (LAPS) (Tiefenthaler, 1993). In addition, Infrared (IR) and near-infrared (NIR) spectroscopy are the most widely used optical biosensors. They are often used in on-line analysis in the dairy industry, in particularly, it is frequently used in cheese making (Tajammal et al., 2015). They have advantages in measuring biological molecule components of dairy protein, carbohydrates, and lipids. However, the disadvantages of optical biosensor comprise high initial capital investment, hardly handle by non-professionals, and it cannot operate in environment with extreme low concentrations (Tajammal et al., 2015).

Calorimetric biosensors detect analytes on the basis of heat evolved or absorbed by the recognition element during a biochemical reaction (Chaubey and Malhotra, 2002). A variety of different substrates including enzymes, vitamins, and antigens have been detected in the biological settings using this biosensor (Danielsson and Mosbach, 1987). However, this approach has relatively limited use in systems with little heat exchange (Chaubey and Malhotra, 2002). Calorimetric biosensors are less sensitive and specific to targeting analytes than other types, because the majority of the heat evolved in the biochemical reaction was lost to the surrounding solution without being detected by the thermistor (Chaubey and Malhotra, 2002). However, the detection of calorimetric biosensor can be improved by co-immobilising enzymes to enhance the sensitivity and specificity.

Piezoelectric biosensors operate on a principle of generating electric dipoles and subjecting an anisotropic natural crystal to mechanical stress. The adsorption of the analyte increases the mass of the crystal and subsequently alters the basic frequency of oscillation (Chaubey and Malhotra, 2002). They are often used for the measurement of chemical elements such as ammonia, nitrous oxide, carbon monoxide, hydrogen, methane and other specific organophosphorus compounds (Chaubey and Malhotra, 2002). Although piezoelectric

biosensors are sensitive, they have limited use in turbid media, such as milk, and are difficult for non-professional personnel to handle (Chaubey and Malhotra, 2002).

In contrast, electrochemical biosensor has emerged as one of the most commonly used types of biosensors because it overcomes most of the disadvantages of other biosensors and provides additional advantages of rapid response, easy-to-handle, miniaturization and low-cost fabrication (Chaubey and Malhotra, 2002).

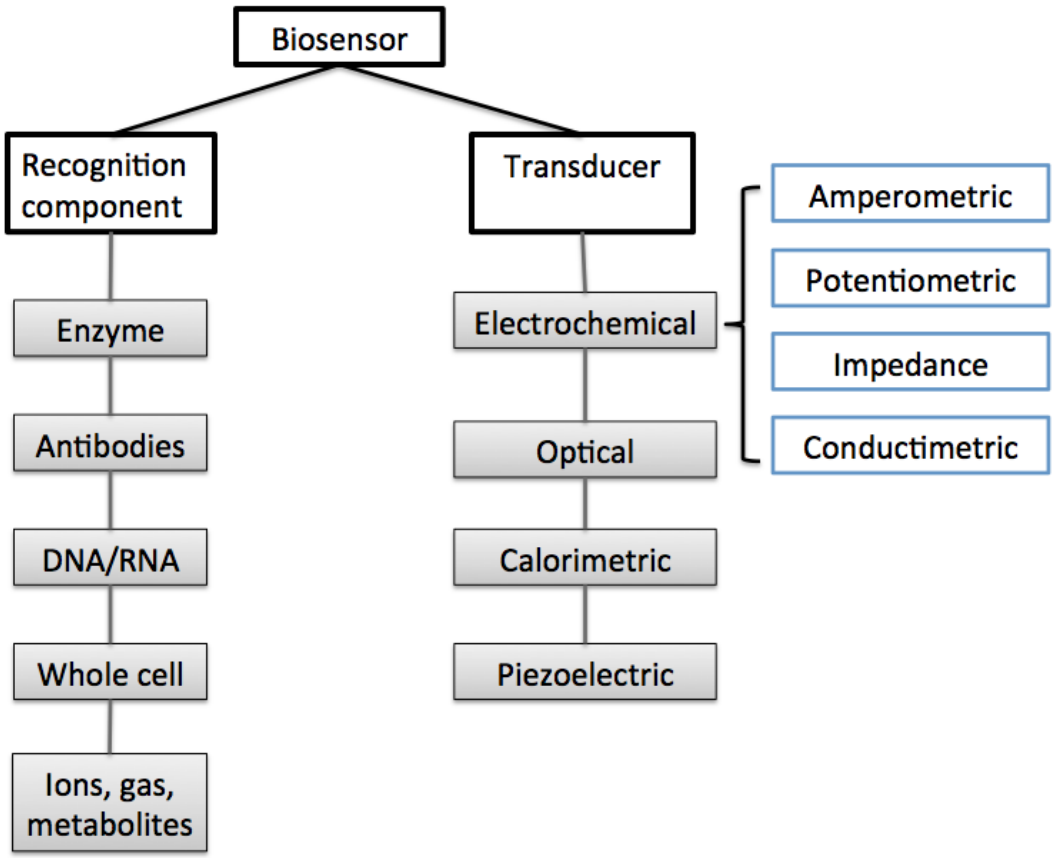


Figure 5. Biosensor categories. Diagram adapted from Chaubey and Malhotra, 2002.

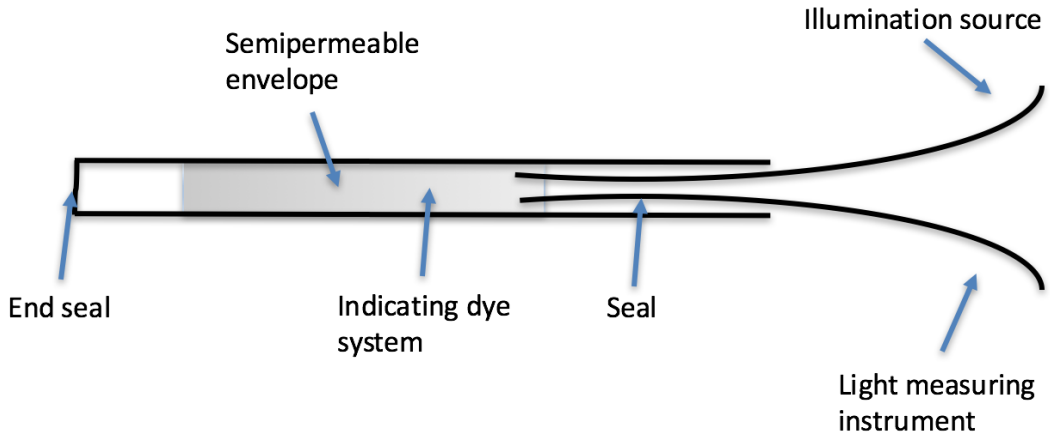


Figure 6. The concept of optical biosensor. Diagram adapted from Peterson and Vurek, 1984.

2.3.2. Electrochemical Biosensors

With the development of biosensing technology, the electrochemical biosensor is a prime focus of research among all biosensors. The total number of publications containing the keyword electrochemical biosensors during the period from 1994 to 2016 are shown in **Figure 7**. Since 2004, electrochemical biosensors have a considerable progression. The relative proportion of publications on electrochemical biosensors have significantly increased from approximately 12% in 2004 to 41% in 2016. The electrochemical biosensor measures electrochemical signals that have generated from a biochemical reaction during a recognition process. The production of electrochemical signals is subsequently measured by electrochemical transducers (Chaubey and Malhotra, 2002). In a general three-electrode configuration, the electrochemical biosensor is composed of a working electrode, a referencing electrode and a counter electrode (**Figure 8**). The working electrode is used to perform the electrochemical analysis. The measurable biochemical reaction is valued at the close proximity of working electrode. The reference electrode serves as a reference point against working electrodes. The potential difference on the working electrode is measured relative to a known potential that is obtained from the reference electrode. The counter electrode is used to apply current to the working electrodes (Rasooly and Herold, 2011). Depending on which of the electrochemical properties is measured by a detecting system, an electrochemical biosensor can be configured as amperometric, potentiometric, impedance or conductometric (**Figure 5**).

An amperometric biosensor measures the flow change in electrical current on a working electrode that is triggered by a biochemical redox reaction of the biorecognition system (Chaubey and Malhotra, 2002). Amperometric biosensors have relatively faster, more sensitive and accurate responses, and great potential for miniaturization. These features make it a better approach to be used in portable devices than other types of electrochemical biosensors (Chaubey and Malhotra, 2002). However, the narrow range of recognition specificity is a severe weakness of an amperometric biosensor because the detection only covers redox chemical reactions in a biological solution (Chaubey and Malhotra, 2002).

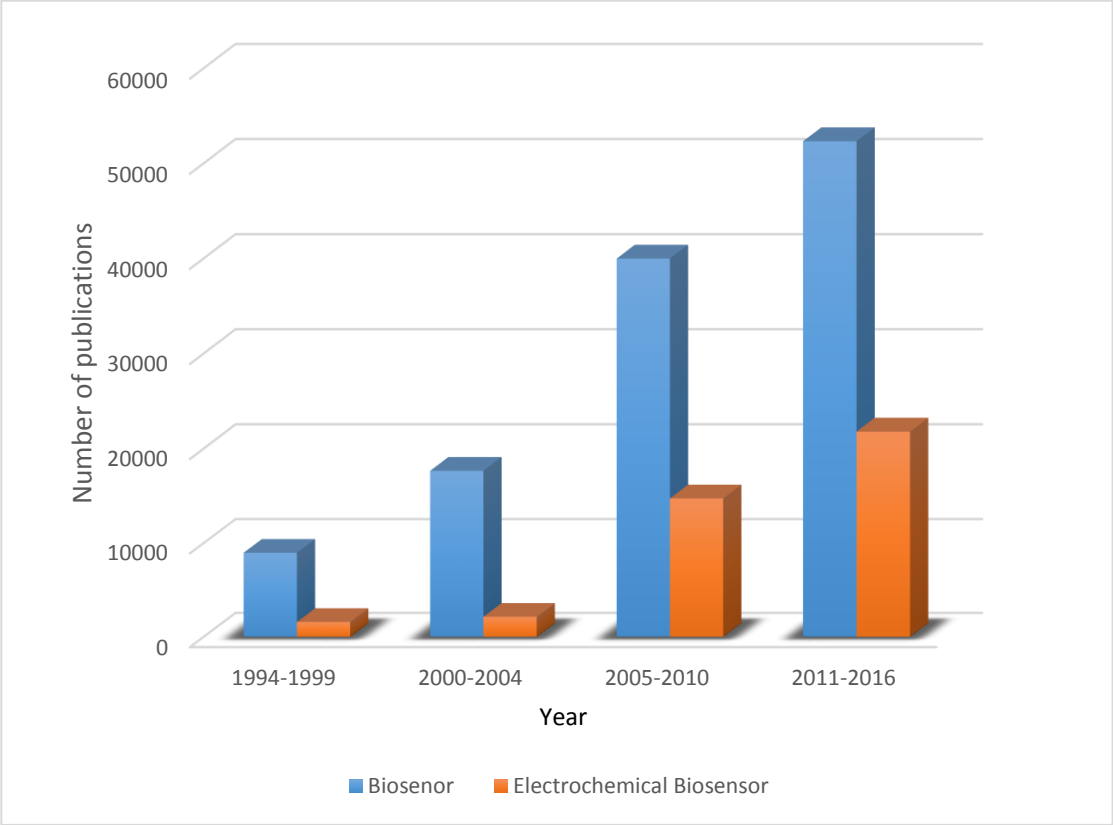
Similarly, the potentiometric biosensors measure differences in electric potential between electrodes that are generated by the production of desired analytical species in a solution (Yunus et al., 2013). The difference in the electric potential is a linear function of the concentration of electrical-active analytes in the solution of interest (Chaubey and Malhotra, 2002). Membrane-based ion-selective electrodes (ISE) and ion-selective field effect transistors (ISFET) are the most common uses of this biosensor. One classical example of a potentiometric

biosensor is the pH meter (Yunus et al., 2013). Purely relying on working electrodes alone cannot provide sufficient sensitivity and specificity in compared to amperometric biosensors. Instead, it is often fabricated with an additional sensitive membrane that is coupled with immobilised enzymes, antigens, antibodies, or conductive polymers to modify the surface of electrodes and enhance detection limits (Chaubey and Malhotra, 2002; Yunus et al., 2013). A number of such biosensors have been developed and used for the detection of ion concentration of sodium (Na), potassium (K) and Ca, pH level, and oxygen, glucose, urea, lactate, and creatinine content in clinical trials. It is also constructed for monitoring acidity, salinity, phosphate and nitrate content, organic molecules, and heavy metal content in water solution and other environmental conditions (Mello and Kubota, 2002; Yunus et al., 2013; Chaubey and Malhotra, 2002).

Impedance biosensors measure the electrical impedance at the electrode interface using steady state alternative current (AC) (Daniels and Pourmand, 2007). The impedance biosensor has application in monitoring the quality and freshness of food through detection of the presence of food-borne pathogens and sanitary microbes (Nieuwenhof and Hoolwerf, 1987). Impedance biosensors have been used in evaluation of viable bacteria content and their associated metabolic activities in milk and other food products (Felice, Madrid, Olivera, Rotger and Valentinuzzi, 1999). Several studies reported that microbial metabolism causes an increase in both conductance and capacitance, and a decrease in impedance (Bülte and Reuter, 1984; Nieuwenhof and Hoolwerf, 1987; Ur and Brown, 1975). The changes in impedance of a culture medium is quantitatively associated with the growth of inoculating microbes (Mello and Kubota, 2002). The Bactometer and the Malthus are the two most commonly studied commercial impedance biosensors for food quality assessment.

With a good similarity to impedance biosensors, conductometric biosensors measure the change in EC between a pair of electrodes in the solution of interest using steady state direct current (DC) (Mello and Kubota, 2002). A conductometric biosensor is often used as an approach to study the enzymatic activity, ionic strength, and conductivity properties of a solution (Grieshaber, Robert, Janos and Erik, 2008). Studies of conductometric biosensors in relation to dairy products are predominantly associated with the growth and metabolism of microorganisms (Chaubey and Malhotra, 2002). Changes in EC that are caused by the growth of microorganisms were already documented late in last century (Bülte and Reuter, 1984). Oker-blom (1912) was firstly determined the relationship between bacterial growth and the ionic concentration of a culture solution (Bülte and Reuter, 1984). It has been proposed that the

microbial metabolism can change uncharged substrates in dairy products to electrical conductive intermediates (Mello and Kubota, 2002). The amount of charged metabolites is directly proportional to the growth of microorganisms in the solution of interest (Mello and Kubota, 2002; Chaubey and Malhotra, 2002). However, several major drawbacks that prevent conductometric biosensors from being widely promoted are the non-specificity of targeting analytes, high background noise ratio, and highly restriction requirements of consistent operational temperatures (Mabrook and Petty, 2003a). In addition, it is suggested that the conductometric biosensor is unsuitable to measure small changes of electrical current in media



with high ionic strength. High ionic strength generates high background noises of dissolving.

Figure 7. The number of publications on biosensors and electrochemical biosensors during the period from 1994 to 2016. Data were obtained from ISI Web of Knowledge Database, Thomson Reuters. The electrochemical biosensor is a combination of searching results of keywords comprising of 'impedance', 'potentiometric', 'conductometric', and 'amperometric' biosensors.

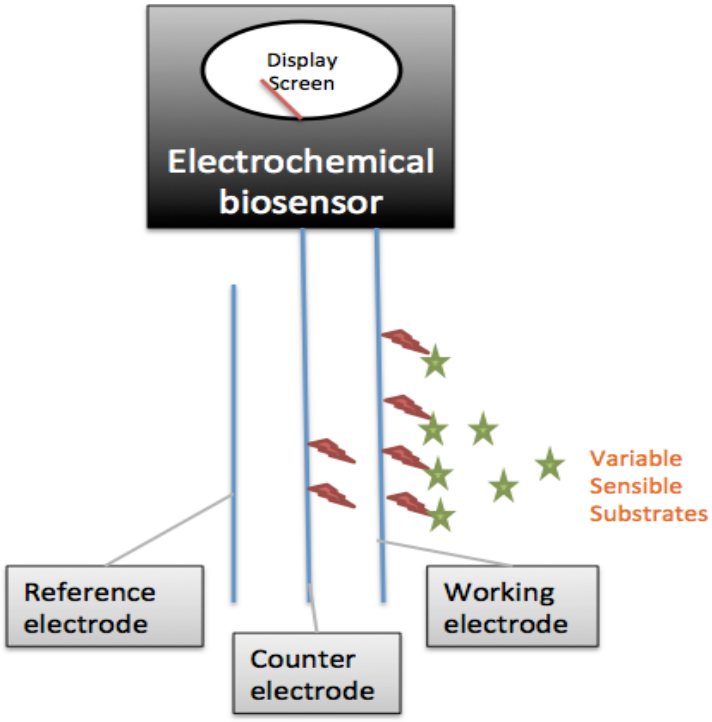


Figure 8. Electrochemical biosensor configuration. Diagram developed from Rasooly and Herold, 2011.

ionic species, which interfere with the transmission of electrical signals (Grieshaber et al., 2008)

2.4. Milk compositions and their applications in biosensors

Milk is a very complex fluid containing hundreds of molecular substances. It is an important nutritional source of animal protein, vitamins, minerals and essential fatty acids for infants and young adults (Eskin, 2012). The major constituents of bovine milk are water (86-88%), milk fat (3-6%), protein (3-4%), lactose (5%), and minerals (0.7%). The solids occupy 11-14% of the total milk weight (**Figure 9**). Milk composition shows large inter- and intra-breeding differences in the fat and protein contents (O'Mahony and Fox, 2014). This difference is known to be affected by a variety of factors, including breed, stage of lactation, season, genetics, and nutrition and health status of cows (McSweeney and Fox, 2008). The milk composition provides valuable information in determining the quality and market values of milk (Eskin, 2012). Indeed, a comprehensive understanding of the constituents of milk has a substantial impact on the physiological, biological, physical and chemical properties of milk and thus its appropriate applications in biosensors for quality control. This section discusses the diversity of bovine milk constituents and the applications of every individual constituent in biosensor and the physical chemistry properties of milk associated to conductivity measurement.

2.4.1. Non-salt compositions

Lactose

Milk is the single known source of lactose, and it is the principle carbohydrate in milk. Lactose is a disaccharide of galactose and glucose that linked by an α- (1-4) glycosidic bond (Eskin, 2012). It occupies 4.8-5.2% of the total weight volume of bovine milk. Apart from lactose, there are other types of free saccharides, mainly oligosaccharides, but in relative low concentrations (McSweeney and Fox, 2008; O'Mahony and Fox, 2014). Lactose serves two essential functions in milk. Firstly, it is a primary source of energy for bovine neonates, which provides 30% of the calories in milk, and secondly, it is responsible for about 50% of the osmotic pressure of milk (O'Mahony and Fox, 2014). Previous studies revealed that milk with a low level of lactose has a higher concentration of inorganic salts to maintain the osmotic pressure at the desired level. Thus, the concentration of lactose shows an inverse relationship with the inorganic salt concentration in milk (McSweeney and Fox, 2008).

Bacteria are dependent on lactose content in milk as primary carbohydrate source for growth. Therefore, it is an imperative indicator in which can be used in biosensor fabrication to estimate the growth of bacteria during milk deterioration (Velasco-Garcia and Mottram, 2003; Rasooly and Herold, 2011). Most lactose biosensors developed with electrodes have immobilizing enzymes that can catalyse the redox reaction of glucose. Glucose oxidase and dehydrogenase are the most frequently used catalytic enzymes. They catalyse the reaction that converts glucose and lactose into D-glucono-1,5-lactone, and produces detectable electrochemical signals including hydrogen peroxide (H_2O_2) and oxygen (**Figure 10**). A study proposed the use of an amperometric biosensor with platinum electrodes to analyse the lactose concentration in raw milk. The platinum electrodes were surface masked by a cellulose acetate film that co-immobilized two enzymes, β-galactosidase (GAL) and glucose oxidase (GOx) (Pilloton, Mascini, Casella, Festa and Bottari, 1987). GAL can catalyse the cleavage of the disaccharide lactose to glucose, while GOx sequentially breaks down glucose to produce H_2O_2, which is measurable by the electrodes (Pilloton et al., 1987). This biosensor exhibits good detection limits that showed a positive linear response at lactose concentrations between 0.02-3 mmol/L. Subsequently, Marrakchi et al. (2008) developed a conductometric biosensor using the same enzymes. The response of this biosensor showed a positive regression relationship between conductance and lactose concentration with R^2 equal to 0.988. This biosensor exhibited a more effective detection limit at lactose concentrations between 0.03-0.3 g/L (Marrakchi, Dzyadevych, Lagarde, Martelet and Jaffrezic-Renault, 2008). Another study proposed a similar instrument, but one using oxygen electrodes to detect dissolved oxygen concentration and estimate the level of lactose in milk. The biosensor was fabricated by immobilizing two enzymes, lactase and GOx, in a polyvinyl formal membrane and attached to the oxygen electrodes. This biosensor had a linear response to lactose concentration in a range between 30-2000 mmol/L (Rasooly and Herold, 2011). In addition, Eshkenazi et al. (2000) developed an amperometric biosensor based on a sequential reaction of three enzymes, GAL, GOx, and horseradish peroxidase (HRP), that was immobilized on glassy carbon electrodes (Eshkenazi et al., 2000). Multi-enzyme coupling reaction increases the selectivity and sensitivity of the sensor. The response of this sensor showed a positive linearity to a restricted lactose concentrations between 0.027-1 mmol/L (Eshkenazi et al., 2000). Another study by Amamcharla and Metzger (2011) evaluated the potential of using a blood glucose meter to determine the concentration of lactose in commercial milk. This study proposed a simple and rapid alternative, based on the principle of hydrolysis of lactose using GAL and subsequently measuring glucose using a blood glucose meter. It was found that the blood glucose meter is a valid approach to measurement and it showed no significant differences from the traditional

chemical compounds analytical tool, which is the high-performance liquid chromatography (HPLC) system (Amamcharla and Metzger, 2011). The investigators established a positive linear correlation between blood glucose meter readings and lactose concentration in solution between 1.9-6.5% (Amamcharla and Metzger, 2011). These studies proposed various potential methods of rapid and low-cost routine measurement of lactose in milk and other dairy products using portable devices.

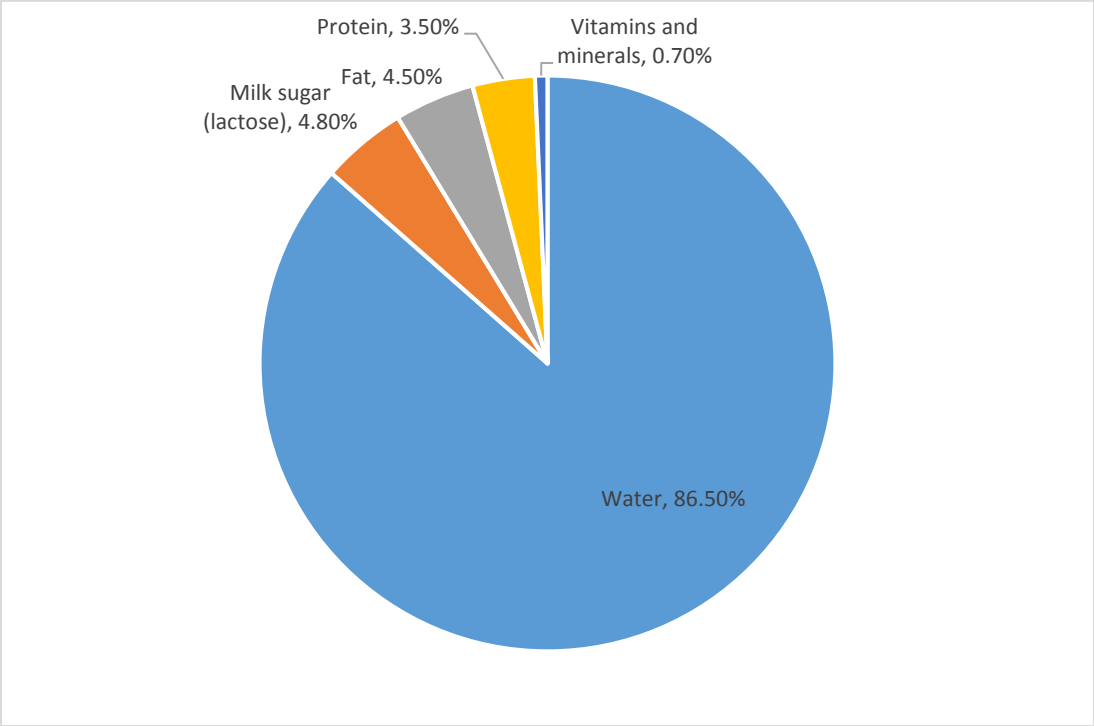


Figure 9. Composition of cow's milk. Chart adapted from Eskin, 2012.

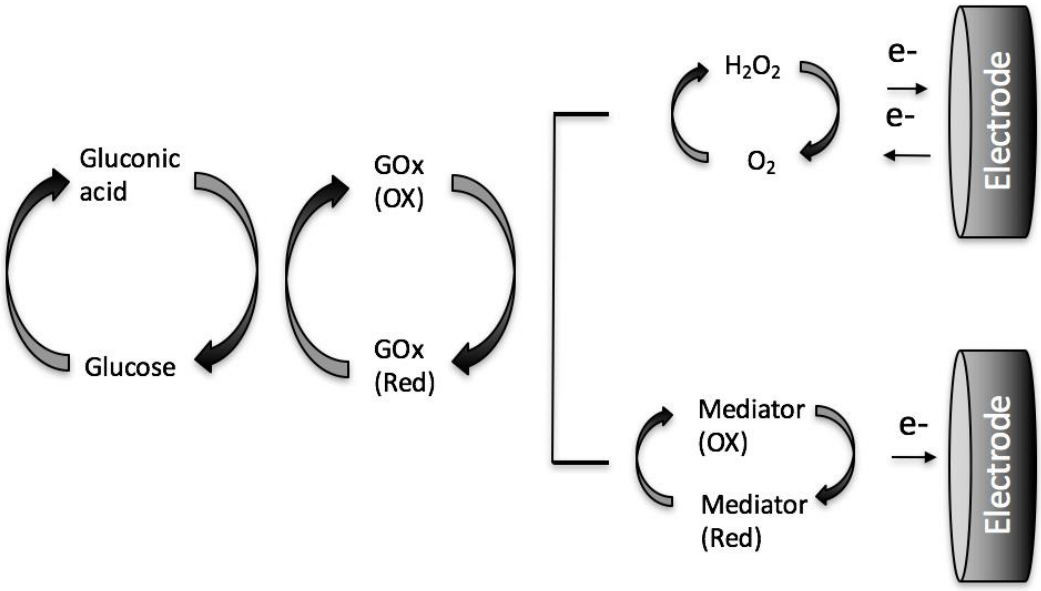


Figure 10. The sequence of reaction events in glucose biosensor. GOx(OX) represents the oxidation reaction of glucose oxidase; GOx(Red) represents the redox reaction of glucose oxidase; Mediator (OX) represents the oxidation reaction of mediator; Mediator (Red) represents the redox reaction of mediator. Diagram adapted from Wang, 2008.

Fats

Milk fats occupy 2.4-5.5% of net weight volume of liquid cow's milk. Milk fat is secreted by a lactating cow as small fat globules that emulsified in the aqueous phase of milk, ranging in size from 1-10 μm in diameter. Fat globules contain mostly 98% of triacylglycerides and other types of fatty acids such as neutral lipids, fat-soluble vitamins and pigments, and sterols and waxes (Rasooly and Herold, 2011). The majority of triacylglyceride is attributed to the surrounding interfacial membrane layer, called the milk fat globule membrane (MFGM) (Eskin, 2012). MFGM acts as an emulsion stabiliser, and it prevents the fat globules from aggregating with each other and ensures dispersion in the aqueous of milk (Srivastava, 2002). Similar to the lactose level of milk, the lipid content shows large interspecies differences in cow due to variations in breeding, nutrients and diet, genetic, season and stage of lactation (Srivastava, 2002). Milk fats are treated as the most valuable constituents in the history of dairy products, in which the price formulae of milk used to be a function of the fat content (Rasooly and Herold, 2011). There are an inadequate number of articles articulating the use of fat or fatty acid as an analytical target for the use of quality control in dairy products. Only one approach was found to measure the content of MFGM in cow's milk. It is proposed that the deconstruction of MFGM and release of fatty acid from fat globules has a potential to predict the aging effects of milk. Thus, the detection of free short chain fatty acids in the aqueous of milk is an effective way of evaluating milk quality. Schmidt et al. (1996) developed a microbial biosensor using the bacterial strain, *Arthrobacter nicotianae,* immobilized in calcium alginate on an electrode surface to determine the concentration of free fatty acids in milk (Rasooly and Herold, 2011). This instrument monitored the respiratory activity of the bacteria. It showed linearity between bacterial concentration with the fatty acid concentration in the aqueous phase of milk over the ranging between 10-160 μmol/L, with a minimum response time of approximately 3 minutes (Rasooly and Herold, 2011).

Protein

The physical property of milk and most dairy products are affected more by the protein content than other constituents. Milk protein is a heterogeneous mixture of two main groups, caseins and whey proteins (**Figure 11**). Caseins account for approximately 80% of the total protein content in cow's milk. They are mainly present as casein micelles which are suspension of particles ranging in size from 50-400 nm in diameter (Eskin, 2012; McSweeney and Fox, 2008). The casein micelles and fat globules comprise the dispersed phase of milk, and it is surrounded by the serum phase of milk. The serum phase consists the dissolving constituents

of lactose, whey protein, minerals and vitamins (Eskin, 2012). The major casein fraction of α_{s1}-, α_{s2}-, κ-, β- and γ- caseins account for 38%, 10%, 36% 13% and 3% of all caseins, respectively (Eskin, 2012). A key difference between individual casein protein is their chemical behaviours of binding with Ca ions (Eskin, 2012; Fox and Mulvihill, 1982). The whey protein was considered at one time to be a waste by-product in cheese making industry. However, after more research has been conducted, the whey proteins became to be a functional food composition (Eskin, 2012; Smithers, 2008). Whey proteins occupy approximately 20% of the total protein content of bovine milk. The major whey proteins are β-lactoglobulin (β-LG) and α-lactalbumin (α-LA). They are globular proteins and account for 70-80% of the net whey protein content. Other minor fractions including lactoferrin (LF), immunoglobulin (Ig) and serum albumin (SA) are also present in milk (O'Mahony and Fox, 2014). Overall, the milk protein fraction of α_{s1} -casein, α_{s2} -casein, β -casein, β -lactoglobulin, and α -lactalbumin comprises 89% of the total protein content of cow's milk. Other protein fractions are also present but at very low levels. The protein content heavily influences the nutritional value of milk and are responsible for the physical, chemical, biochemical, and physiological behaviour of milk (Eskin, 2012; Wedholm, Hallén, Larsen, Lindmark-Månsson, Karlsson and Allmere, 2007).

It has been proposed that the freshness and quality of milk can be determined by protein degradation in milk. The concentration of dissolving amino acids can be treated as an indicator of milk aging because it has been proposed that the occurrence of gradual decline in the concentration of both L- and D-amino acids in milk is associated with storage duration and condition (Mello 2002). The principle for amino acid detection is similar to that of lactose. It is utilised by the redox reaction of enzymes, amino acid oxidase and dehydrogenase, to generate oxygen, that are electrical measurable by electrodes. The amino acid detecting biosensor was firstly developed by Romette et al. (1983), who has constructed biosensor that immobilized with L-lysine α-oxidase in a gelatin support and fixed on an oxygen sensor to determine the concentration of L-lysine in a media fermenter. The sensor had a supportive function with good response to lysine concentration between range of 0.2-4 mM (Romette, Yang, Kusakabe and Thomas, 1983). Similarly, Sarkar et al. (1999) described an amino acid enzyme incorporating amperometric biosensor to measure L- and D-amino acid concentration in fresh milk. The device responded well to twenty common L-amino acids and all of the D-amino acids. Biosensor expressed a linear response to three common L-amino acids with a detection limit of 0.15-0.47 mM (Sarkar et al., 1999). In addition, Kelly et al. (2000) fabricated a highly sensitive, novel and fast-responding amperometric biosensor for detecting L-lysine using

glassy carbon electrodes immobilized with L-lysine oxidase. The biosensor showed a high specific response to L-amino acids with a precise detection limit ranging from 2-125 μM (Kelly, O'Sullivan and Guilbault, 2000).

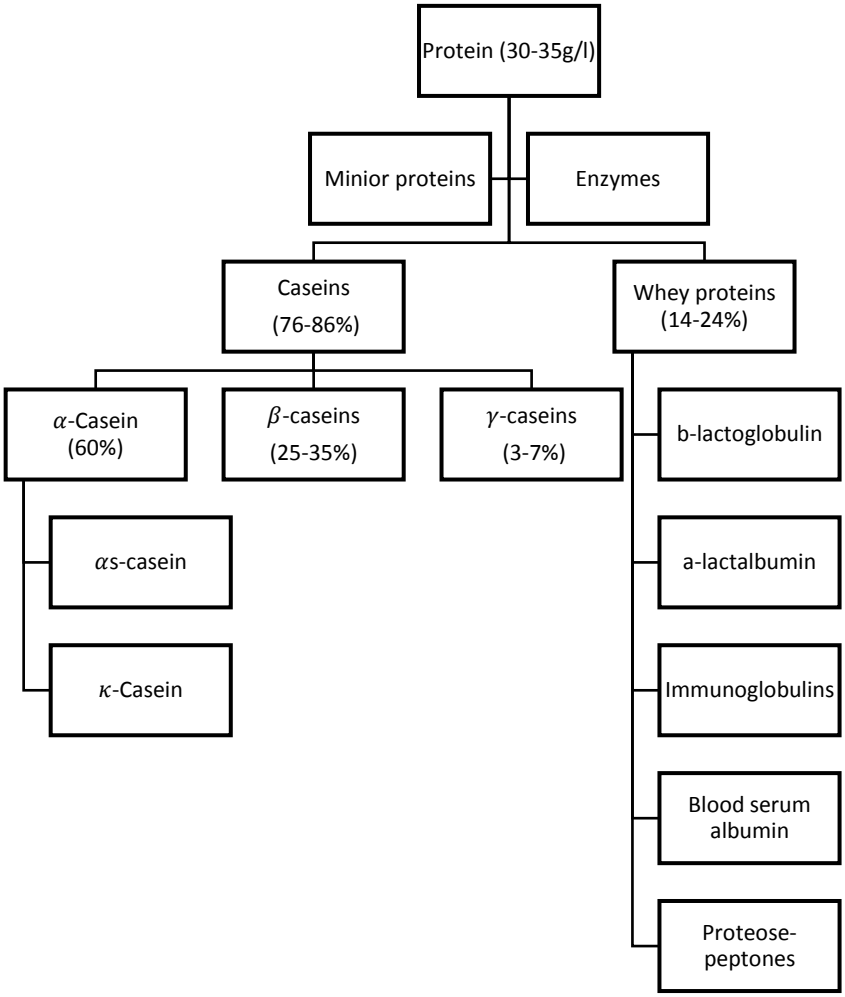


Figure 11. Milk protein fractions. Diagram developed from Eskin, 2012**.**

2.4.2. Salt composition

Inorganic salt accounts for 0.7% of total weight volume of bovine milk (O'Mahony and Fox, 2014). The major ionic constituents of bovine milk consist of Na, K, and chloride (Cl), which are also prevalently known as the monovalent ions of milk. They are the most prevalent kinds of mineral that present in the state of free suspension ions in the aqueous of milk. These salt ions collectively contribute more than 25% of the total osmolarity of bovine milk (Srivastava, 2002). Divalent ions, namely Ca, magnesium (Mg), citrate, phosphate (P) and sulphate, are the second most abundant mineral components in bovine milk. In addition, approximately 20 types of trace elements are naturally found in milk. the trace element includes copper, iron, silicon, zinc and iodine (Mutlu, 2010). The distribution of major ionic species in bovine milk is shown in **Table 1**. Ca was found to be present either in the free state in aqueous compartment or associated with casein protein to form casein micelles (**Figure 12**). Similarly, P is present as insoluble ions that associate with milk protein. Evidence showed that the concentration of both P and Ca is proportional correlated to the concentration of caseins (Nollet and Toldra, 2009; Srivastava, 2002). The high concentration of protein, and its ability to bind large quantities of P and Ca ensures the nutritional requirement of milk. In contrast, Mg was a critical ion that mainly found in the aqueous compartment of milk. The significant amount of Mg is associated with fats and membranous compartment of fat globules (Srivastava, 2002; Jenness, 1979). There are many factors influence the net change in ion concentration during lactation of cows. The main contributor that dramatically increases the concentration of monovalent ions (i.e., Na, Cl and K) in milk associate with physiological conditions in which promote the opening of tight junctions between epithelial cells in mammary glands. The condition occurs during pregnancy, local inflammation and infectious mastitis disease (Srivastava, 2002). Regardless of this, the major monovalent ion concentration remains at a relatively stable level and does not appear to be influenced by factors of nutrition, breeding, illness and other environmental factors (Srivastava, 2002).

2.5. Physical chemistry of milk

2.5.1. pH

The acidity of a solution is typically expressed as the pH. The neutral pH of bovine milk is range in 6.5-6.8, with pH of 6.6 being the standard value (**Table 2**). pH indicates the state of

equilibria of acid and base. The distribution of dissolving protons represents an important physical properties of dairy products. The measurement of pH used to be the simplest traditional method to assess the keeping quality of milk and milk products. Experimental evidence showed that the occurrence in depletion of acidity in milk accompany with extended

Table 1. Concentration and distribution of the principal milk salts. Table adapted from O'Mahony and Fox, 2014; Srivastava, 2002.

Species	Concentration (mg/L)	Soluble (%)	Form	Colloidal (%)
Na	500	92	Completely ionized	8
K	1,450	92	Completely ionized	8
Cl	1,200	100	Completely ionized	-
Sulphate	100	100	Completely ionized	-
Ca	1,200	34	35% Ca^{2+}; 55% bound to citrate; 10% bound to phosphate	66
Citrate	1,750	94	85% bound to Ca and Mg (undissociated); 15% $Citrate^{3+}$	6
Phosphate	750	43	10% bound to Ca and Mg; 54% $H_2PO_4^-$; 36% $HPO4^{2-}$	57
Mg	130	67	Probably similar to calcium	33

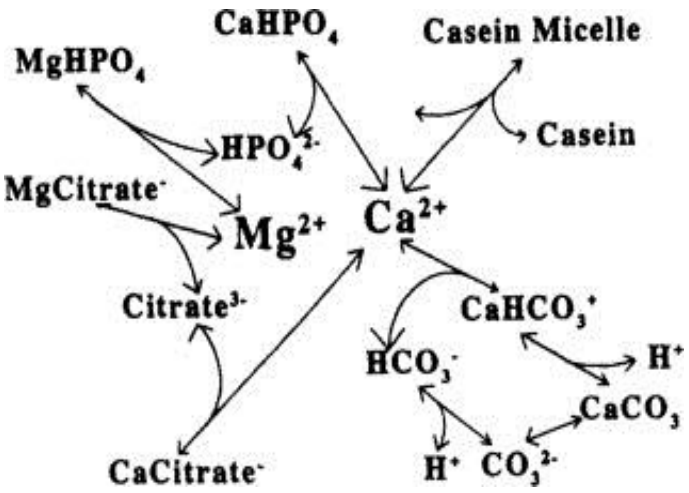


Figure 12. Principle interactions of Ca, P and Mg in the aqueous compartment of milk Nollet and Toldra, 2009.

Table 2. Summary of the principal physical properties of bovine milk. Table adapted from Sadler and Murphy, 2010.

Physical Property	Typical value
pH	6.5-6.8
Titratable acidity (%)	0.1-0.26%
Ionic strength	0.075 M
Electrical conductivity	4.6 -5.7 mS/cm

storage duration (Cooledge and Wyant, 1920). A variety of factors can alter the pH of milk. The certain thermal treatment that is used to sterilise liquid milk can alter the level of dissolving protons and thus pH value, because the present state of dispersing proteins and solubility of salt ions are changed by heating (McSweeney and Fox, 2008). The pH of milk is also influenced by temperature because the solubility of calcium phosphate is dependent on temperature change. Higher the temperature results in higher the solubility of salt ions in the aqueous of milk (Fox and McSweeney, 1998). In addition, exponential growth of contaminating bacterial flora in milk lead to increased production of acidity and lowers the pH of milk (Liu et al., 2015). Overall, fluctuation of unstable level of pH is multivariable-dependent. It depends on the production of acidity, equilibria state of soluble and insoluble salt ions, temperature, milk composition, breeding and other external factors (Sadler and Murphy, 2010).

2.5.2. Titratable acidity

Titratable acidity is a widely spread method used for the determination of organic acid, particularly the concentration of LA, in milk (Sadler and Murphy, 2010). The purposes of measuring titratable acidity are mainly due to two reasons, firstly, to comply with cleanliness standards and ensure the quality and freshness of milk, secondly, to control the production of acid by bacteria during fermentation manufacturing (Herrinngton, 1948). The main advantage of using titratable acidity as an index for quality assessment is the simplicity and fast measurement (McCarthy and Singh, 2009). Titratable acidity is usually determined by titration with standard sodium hydroxide (NaOH) solution to the phenolphthalein end-point at pH 8.35 (McCarthy and Singh, 2009). The development of titratable acidity can be categorised as natural or developed acidity. In general, freshly drawn bovine milk contains a small amount of LA as a natural constituent. The normal degrade of LA of bovine milk ranges from 0.10 to 0.26% (**Table 2**). The LA content of milk varies slightly among milk bottles and the variations in LA are due to differences in breeding, genetic and physiological condition of the udder of milking cows (Bergmann, Abel and Giffey, 1999). On the other hand, developed acidity refers to the development of LA during fermentation or deterioration, in which the microorganisms multiply in milk and convert lactose and other carbon sources to LA. This can result in an increase in acidity and thus a decrease in pH (Walstra and Jenness, 1984). In addition, the liberation of fatty acid from milk fats by the action of microbial enzymes can further result in more LA developed in cream or high-fat dairy products (McCarthy and Singh, 2009).

2.5.3. Ionic strength

Ionic strength measures the concentration of ionic compounds in a solution. The ionic strength of milk is mainly maintained by the counter ions on casein micelles and the free suspension of ionic salts (Wong, 2012). The ionic strength of cow's milk serum estimates at 0.075 M (**Table 2**). The presence of ionised and ionisable salt components of milk is in a very delicate physical balance. Among all the salt ions, the counter ions of Ca and Mg present in small amount on the surface of casein micelles, they are of great importance to the ionic strength of milk (Wong, 2012). This is because the Ca and Mg are 60 times as active as monovalent ions of Na and K in the flocculation of hydrophobic colloid particles (Wong, 2012). The electrical charge of casein micelle is mainly screened by the surface decorated counter ions (i.e. Ca, Mg and P) thus it is proposed that the electrostatic potential of casein micelles is proportional linear to the net charge potential of the surface counter ions. Casein micelle is the predominated protein kind of milk and its electric potential of great influences the net ionic strength of milk.

2.5.4. Electrical conductivity (EC)

Milk is an electrolyte and an intrinsic electrical conductor, because it contains 0.7% of inorganic salt and charged protein fractions (Fox and McSweeney, 1998). TDS refers to the overall dissolved inorganic elements in a solution, and TDS of milk carries electrical current and reflects changes in electrical conductance. EC is a measurement of a solution's ability to carry an electrical current, and it is a reflection of the net concentration of the presence of TDS of milk (McCarthy and Singh, 2009). EC of milk is initially employed as an indicative trait to predict the incidence of mastitis-related disease in the udder health of cows. The application of EC subsequently evolved to apply as a mean for milk quality control, in regard to water adulteration, LA production, and milk composition analysis during processing (Norberg, Hogeveen, Korsgaard, Friggens, Sloth and Løvendahl, 2004). Healthy milk has a normal EC range from 4.6-5.7 mS/cm (**Table 2**). When the value of EC is failed to read in this range, the milk is regarded as abnormal (McCarthy and Singh, 2009; McSweeney and Fox, 2008). The distribution of salt fractions in either the soluble or insoluble states have a large impact on the net milk conductivity (Mabrook and Petty, 2003b). A subtle change in ionic composition of a culture medium affects the EC and the electrical potential of milk solution. Schulz and Sydow (1957) reported that the Na, K, and Cl ions are the most greatest contributors to electrical

conductance change of milk, because they present in the highest concentration among all salt ions (Schulz and Sydow, 1957).

The salt fraction is proposed to be tightly associated with changes of EC of milk (Srivastava, 2002). A study showed that the changes in ionic composition due to an increase in bacterial density or metabolism in a culture medium increase the electrical conductance and decrease the electrical capacitance of milk (Wu et al., 2015). A study by Mabrook and Petty (2003) evaluated the changes in EC as an indicator for microbial growth and milk composition alternation in raw milk using laboratory-made gold electrodes. The author reported that bacterial growth lead to a change in milk compositions and a formation of new metabolites. These metabolites increase the concentration of charged ionic species in milk aqueous and alter the electrical conductance of milk (Mabrook and Petty, 2003a). The uncharged substances in a culture medium are metabolised by bacteria and subsequently transformed to ionic metabolites that can increase the electric conductivity of the medium. Thus, EC is proposed to be a useful property for monitoring bacterial growth in milk, because a correlation between the ionic compositions and the bacterial metabolism of a medium solution is potentially linked by EC (Noble, 1999).

2.6. Principle of measurement of electrical conductivity

EC measures the ability of a solution to conduct an electrical current between two electrodes, and it is usually measured in milliSiemens per centimetre (mS/cm) (McCarthy and Singh, 2009; Fox and McSweeney, 1998). If apply a known voltage across a pair of parallel electrodes immersed in a sample of interest, an electric field is generated at the interface of electrodes which can influence the direction of movement of anions and cations toward the oppositely charged electrodes in the solution (**Figure 13**). This phenomenon is responsible for EC change in milk, and it gives milk the ability to conduct electric current (Zaninelli, Agazzi, Costa, Tangorra, Rossi and Savoini, 2015; Adley and Ryan, 2015). Since the voltage (V) and flowing current (I) that applied across the pair of parallel electrodes is known, the electrical resistance (R) generated in the specimen is calculated according to Ohm's law. EC is inversely proportional to electrical resistance (Application Data Sheet, 2010). Thus, EC value can be calculated as reciprocal of electrical resistance and the equation states below: (Brown, 2006; Kaiser, 2004):

$$\mathrm{EC} = \frac{1}{R} = \frac{I}{V} \quad (1)$$

The strength of EC depends on the total concentration of ionic components in a solution, the length of a distance that ion travels, and the cross-sectional area of the specimen the current flows. The length and the cross-sectional area of a specimen determine the path of electrical current that is travelled, and it is defined by the sensor geometry, or called cell constant. The cell constant in most cases has a unit of 1/cm (length/area). EC of a given specimen is calculated by multiplying the electrical conductance (G) by the cell constant as implied in equation (2) (Application Data Sheet, 2010). According to the equation, EC is positively proportional to electrical conductance and inversely proportional to the cross-sectional area

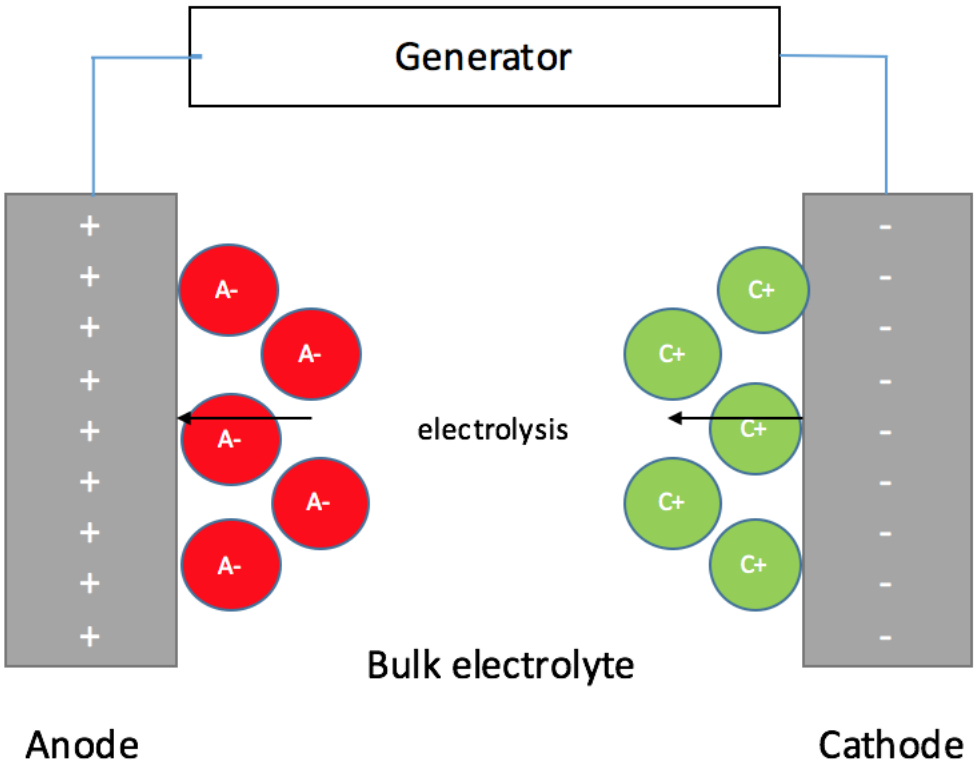


Figure 13. Principle of conductometric biosensor. Diagram adapted from Adley and Ryan, 2015.

(A) and the length (ℓ) of specimens (Kaiser, 2004; Brown, 2006; McCarthy and Singh, 2009):

$$\mathrm{EC} = \frac{1}{R} \times \frac{l}{A} = \mathrm{G} \times \frac{l}{A} \ (\mathrm{Sm}^{-1}) \quad (2)$$

Furthermore, temperature control is an essential parameter to sustain a consistent measurement of EC. The temperature coefficient, or called the temperature dependence degree of conductivity fluctuates between the range of 1.5-5.0% per degree Celsius depending on the testing materials (Down and Lehr, 2005). The most commercial conductivity sensors have integrated temperature sensors that allow analysis of processing temperatures, and correct and compensate the temperature change accordingly to calibration temperature. Although a single temperature coefficient can be used with reasonable accurate measurement over a range of 30-40℃, accuracy can be improved by calculating the temperature coefficient that is specific to the sampled material (Down and Lehr, 2005). EC of most material changes with temperature changed. Increasing the temperature of an electrolyte always enhances the value of EC, as the mobility of ions increases, the ability of ions to conduct electrical current increases. If the temperature of a specimen does not vary dramatically, the linear approximation of predicting the temperature coefficient of EC is typically used. The linear prediction is based on the observation that the EC of electrolyte changes by about the same percentage for every unit (℃) change in temperature, the implied equation states as (Down and Lehr, 2005):

$$\sigma(\mathrm{T}) = \sigma_{\mathrm{T_{cal}}}[1 + \alpha(\mathrm{T} - \mathrm{T_{cal}})] \quad (3)$$

where α is the temperature coefficient of electrical resistivity; T_{cal} is the calibration temperature (typical calibration temperature is 25 ℃ or room temperature); $\sigma(T)$ is the EC at the temperature T; $\sigma_{T_{cal}}$ is the EC at the calibration temperature.

2.7. Milk shelf life and spoilage

The highly nutritious nature of fresh liquid milk makes it a seeding bed for microbial growth. Milk is categorised as perishable foods, and it is susceptible to rapid spoilage (Ledenbach and Marshall, 2009). The limited shelf life of milk remains a sustainable issue in global food waste. It challenges microbiologists, food scientists and technologists to find better solutions to extend the shelf life and prevent rapid spoilage (Ledenbach and Marshall, 2009). Milk shelf life is best

defined as the time during which the product exhibits no physical or sensory defects and remains wholesome. It is related to product safety, quality and nutritional values (Muir, 1996). The estimations of freshness and quality of milk by permitted shelf life is usually determined by the rate of deterioration of milk composition and the growth of spoilage microbes (Rasooly and Herold, 2011).

Milk spoilage is characterised as a deterioration process in which both the chemical and physical (smell, texture, taste and colour) properties of milk proceed to a point at which a dramatic loss of nutritional values is resulted. This leads to reach a grade of quality that is unacceptable to customers (Ledenbach and Marshall, 2009). A number of bacteria ranging from 10^6 to 10^7 CFU/ml is statistically considered as complete spoilage of liquid pasteurized milk (Ledenbach and Marshall, 2009). A variety of internal and external forces are responsible for the spoilage of milk. Internal force includes self-decomposition of milk constituents by the intrinsic enzymatic activities during storage, but, this has a minor adverse impact on keeping quality and chemical stability of milk (McMeekin and Ross, 1996; Evrendilek, 2014). The external force, in particular, the growth of bacteria and their metabolic activity, are main determinant factors responsible for spoilage. The types of spoilage microorganisms differ widely among dairy foods. Due to the variations in entry pathways that bacteria escape into milk during production, formulation, processing, packaging, handling, storage and distribution (Ledenbach and Marshall, 2009). A variety of potential contaminating bacteria in milk products are shown in **Table 3.**

2.8. Milk pasteurization

Raw milk contains a vast number of pathogens and spoilage bacteria. Consumption of raw milk might result in serious life-threatening diseases. According to the New Zealand Food Safety Authority (NZFSA), under the governing New Zealand legislation (Food Act 1981), all raw milk and related milk products used for human consumption, commercial trading purposes, or as a food ingredient claims to have undergone a standard thermal treatment, namely pasteurization (Herrinngton, 1948). Pasteurization is an essential commercial disinfectant strategy used in milk sterilization. The goal of pasteurization of raw milk is to achieve an effective elimination of the majority of pathogens, spoilage microorganisms, and undesirable microbial-borne enzymatic activity in milk, without affecting its nutritional or sensory characteristics (Herrinngton, 1948; Moatsou and Moschopoulou, 2014; Reis, Coimbra

and Teixeira, 2009). Several thermal treatment strategies exist to serve variable purposes of milk sterilization. The most widely used thermal approaches are the high-temperature short-time treatment (HTST) and the ultrahigh-temperature treatment (UHT) (Reis et al., 2009). The primary differences between these thermal approaches are the heating temperature and duration time. As a consequence, their effects on eliminating types of bacteria in milk are distinguishable. HTST is also known as flash pasteurization, it involves heating fresh liquid raw milk to a minimum temperature of 71-74℃ for 15-30 seconds, and then milk is filled with sterilised plastic bottle (Sørhaug and Stepaniak, 1997; Moatsou and Moschopoulou, 2014). The HTST milk is also known as freshly chilled pasteurized milk. The minimum heat treatment of HTST was designed to achieve a reduction of 99.999% in total number of viable microorganisms in fresh liquid raw milk (Stabel and Lambertz, 2004). This method is considered adequate for destroying almost all yeasts, molds and common pathogens, including but not limited to *Salmonella*, *Yersinia*, *Listeria*, *Streptococcus*, *E.coli*, and *Micrococcus*, and common spoilage bacteria, such as *Pseudomonas*, *Lactobacillus*, *Alcaligenes*, *Enterococcus*, and *Flavobacterium*. It effectively aids in the elimination of all gram-negative bacteria and some heat-sensitive gram-positive bacteria, but it has no detrimental effects on thermoduric and spore-forming bacteria, such as *Bacillus*, *Clostridium*, *Mycobacterium* and *Tuberculosis*, because these bacteria have the capability to manage the surviving under high temperature and pressure (Ray and Bhunia, 2007). HTST is able to extend the shelf life of milk to 14-21 days of post-production under optimal processing and refrigerated storage (Ledenbach and Marshall, 2009).

2.9. Milk contamination

Milk is loaded up with various species of bacteria from where it is secreted from the mammary glands of cows. During secretion, milk is in contact with a variety of contaminating sources of udder and teats, dairy workers, farm environment and utensils (**Table 4**). The udder is one of the primary contaminating sources that impacts the initial bacteria load in raw milk. The number of bacteria in milk can increase to approximately 10^4 CFU/ml immediately after milking a healthy cow with poor cleanliness (Moatsou and Moschopoulou, 2014; Walstra and Jenness, 1984). One study reported that the total bacteria counts of milk positively correlate with the amount of soil on the udder and teats prior to milking (Elmoslemany, Keefe and Dohoo, 2010). After secretion, the milk liquid is constantly exposed to a variety of environmental contaminants during harvesting, processing, packaging, handling, transporting

and post-purchasing (Roberts and Skinner, 1983). A significant percentage of contamination comes from the soil, water, animal faeces and feed, dairy workers, equipment utensils and water sources (Moatsou and Moschopoulou, 2014; Ledenbach and Marshall, 2009).

Milking is one of the essential steps with a high risk of milk contamination. Workers with mastitis or infectious diseases have the potential to transfer pathogenic bacteria between themselves and the cows. Today's dairy farms are equipped with automatic milking machines. Nevertheless, milking machines including pipelines and milk filters might also act as reservoirs of bacterial contamination (Moatsou and Moschopoulou, 2014). Studies have revealed that poorly cleaned milk equipment contain milk resides, which often with a bacterial load approach 10^9 CFU/ml. 1 ml of this milk residue increases the bacterial load of 100 litres of

Table 3. Dairy product and associated common types of spoilage microorganisms or microbial activity (Ledenbach and Marshall, 2009)**.**

Milk Types	Spoilage microorganism or microbial activity
Raw milk	A wide variety of different microbes
Pasteurized milk	Psychrotrophs, sporeformers, lactic acid producing bacteria, microbial enzymatic degradation
Concentrated milk	Spore-forming bacteria, osmophilic fungi
Dried milk	Microbial enzymatic degradation
Yogurt	Yeasts
Other fermented dairy foods	Fungi, coliforms

Table 4. Predominant contamination sources and spoilage microorganisms that associated with different types of milk (Moatsou and Moschopoulou, 2014).

Contamination sources	Microorganism	Milk type
Milking utensils, cold-stored milk	Psychrotrophs, Pseudomonas spp., Achrimobacter spp., Aeromonas spp., Alcaligenes spp., Chromobacterium spp., Flavobacterium spp.	Cold-stored milk
Milking utensils, faeces	Lactic acid bacteria, Lactobacillus spp., Lactococcus spp., Leuconostoc spp., Streptococcus, thermophilus, Enterococcus spp.	Sour milk
Faeces, milking utensils, contaminated water	Coliforms, Escherichia coli, Klebsiella aerogenes.	Spoil milk and cheese
Feed, faeces, soil	Spore-forming bacteria, Bacillus cereus, Bacillus subtilis, Geobacillus, steanrothermophilus, Clostridium tyrobutyricum.	Pasteurized milk, spoil milk, cheese and cream.

newly collected milk to 10^4 CFU/ml (Walstra and Jenness, 1984). Circumstances in which poor cleanliness is applied to milking utensils can also cause formation of bacterial biofilm on the surfaces of utensils that are detrimental for milk quality (Moatsou and Moschopoulou, 2014). The management of hygiene control in milk production is crucial to sustain good quality milk for customer consumption (Moatsou and Moschopoulou, 2014). Once the milk has contaminated, it is almost impossible to trace back the original contaminating sources due to the vast diversity in animal management, farm environment and hygiene practices during milk production (Roberts and Skinner, 1983).

2.10. Major milk spoilage microorganisms

A sufficient strategy is essential to control the growth of bacteria in manufacture and handling process of dairy products. Currently, milk contamination is mainly prevented by pasteurization and cooling. Cooling is an effective way to manage and preserve the quality of dairy products by storing milk at the temperature of 3-7℃. It is an efficient way of dormancy the growth of some major pathogenic and spoilage bacteria. However, it has no detrimental effect on psychrotrophic bacteria. The growth of psychrotrophs remains active under cooling conditions (Moatsou and Moschopoulou, 2014). Psychrotrophs, also known as cold-tolerant bacteria, are characterised as having capability of growing at low temperatures (0−7℃). Post-pasteurization recontamination with psychrotrophs is a major issue of fresh pasteurized milk products (Ledenbach and Marshall, 2009). The most common psychrotrophic organisms implicated in spoilage of milk fluid primarily belong to pseudomonads (Murphy, 2007). Other important psychrotrophs include members of the genera *Bacillus*, *Micrococcus*, *Aerococcus*, and *Lactococcus* and of the family Enterobacteriaceae (Ledenbach and Marshall, 2009). Careful managing the bacterial contamination during production, processing and storage is necessary to maintain good milk quality. The section below discusses three major types of spoilage bacteria: pseudomonads, spore-forming bacteria and lactic acid bacteria.

2.10.1. Pseudomonads

Pseudomonads are aerobic rod-shaped gram-negative bacteria. They are considered to be the most important causative agent of spoilage of pasteurized milk in New Zealand (Richardson, 1981). Typically, pseudomonads represent a substantial percentage of 65-70% of total

psychrotrophic isolates from raw milk (Ledenbach and Marshall, 2009). It initially represents less than 10% of the total bacterial population in freshly pasteurized milk. However, studies revealed that after 10-day growth at 7℃, psychrotrophs could exceed 10^7 CFU/ml and account for over 99.99% of the total bacterial population in milk. With their ability to grow at low temperature with shortened doubling time (doubling every 8-10 hours at 4℃), they developed into the most important bacteria species among psychrotrophs and dominated the microflora of chilled pasteurized milk (Sørhaug and Stepaniak, 1997). In addition, when grow in milk at low temperature, many strains of Pseudomonads, and some other common psychrotrophs, including *Micrococcus*, *Bacillus*, *Staphylococcus*, *Flavobacterium* and *coliforms* can produce proteinases, lipases and phospholipases to hydrolyse milk caseins and lipids (Sørhaug and Stepaniak, 1997). Even after bacteria have been killed by pasteurization, these hydrolytic enzymes remain stable in milk (Eskin, 2012; Ledenbach and Marshall, 2009). Hydrolysis of milk composition results in change in quality and nutritional value and defects in flavour of milk (Hill and Kethireddipalli, 2013). However, at storage temperature ≥10℃, thermoduric bacteria, fungi, sporeformers, coliforms, and lactic acid bacteria overwhelm the growth of Pseudomonads and dominate the microbial flora in milk (Sørhaug and Stepaniak, 1997). Therefore, post-pasteurization re-contamination by Pseudomonads is more detrimental to the quality of pasteurized milk that stored at lower temperatures. Detection and quantification of number of Pseudomonads is useful to estimate the grade of contamination and spoilage, because it correlates better with the shelf life of chilled pasteurized milk (Sørhaug and Stepaniak, 1997).

2.10.2. Spore-forming bacteria

Spore-forming bacteria are another common causative agent of contamination in both raw and processed dairy products. The total number of spore-forming bacteria seldom exceeds 5×10^3 CFU/ml before pasteurization. However, they are often introduced for re-contamination after the milk has been processed (Ledenbach and Marshall, 2009). The genus of *Bacillus* is one of the most important spore-forming bacteria. Several bacterial strains of *Bacillus* such as *B. cereus, B. subtilis, B. licheniformis*, *B. mycoides and B. megaterium*, are commonly found in milk and milk products. A study reported that *B. cereus* is isolated in more than 80% of raw milk samples (Ledenbach and Marshall, 2009). The *Bacillus* was found to survive pasteurization and grow in cold temperatures, and they also can ferment lactose in many dairy products. Pasteurization fails in destroying them and the heating effects of pasteurization activates the surviving spores

to germinate at a favourable growth temperature after pasteurization (Ledenbach and Marshall, 2009). Cromie et al. (1989) reported that *B. circulus* was the dominant spoilage microbe found in UHT treated milk with spoilage occurred at low temperature (Ledenbach and Marshall, 2009).

2.10.3. Lactic acid bacteria

Lactic acid bacteria are gram-positive, non-motile, non-sporeforming bacteria. They produce LA as the major end-product of the fermentation of lactose in milk (Saris, 2014). Lactic acid bacteria are mainly thermoduric or haemophilic and prefer to grow at the temperatures ≥ 30℃. Depletion in storage temperature can dramatically suppress growth of lactic acid bacteria and encourage the outgrowth of psychrotrophic spoilage bacteria (Eskin, 2012). Their growth frequently results in an increase in acidity and a decrease in pH of milk. The growth of lactic acid bacteria found in spoilt milk but does not necessarily diminish the quality of milk, as some members of lactic acid bacteria, such as *lactobacillus*, *leuconostoc*, *lactococcus*, *pediococcus*, *streptococcus*, and *vagococcus*, can also be found in the fermentation process of dairy products (Saris, 2014).

2.11. Conclusion

Growing volume of research activity and capital investment in biosensor discipline support the constant growth of biosensing technology. A variety type of biosensors has been fabricated and studied in milk and other dairy products. Many milk compositions are being looked for a way to be incorporated in biosensors to perform a beneficial use in pathogenic detection, safety assessment, and quality control of milk products. However, the prediction of milk spoilage through the monitoring of change in milk composition using EC has yet to be fully explored. Biosensor is classified into a diverse kind, and each kind has their advantages and disadvantages in the aspects of robustness, sensitivity, specificity, and feasibility of specific applications. The variation in EC is evidenced to be affected by the equilibrium of salt content, microbial growth, and the level of acidity in fermented milk, but yet to be understood in spoilage milk. Due to the restraints in the availability of equipment, the EC was determined to be the most suitable mean to aid in the detection of milk spoilage in this study. EC offers opportunities in fast detection, high sensitivity and specificity, and non-discriminate

recognition towards a variety of electrical conducting components in milk. Most importantly, the examination can be accomplished with a minimum requirement of facilities and procedures, which provide it a suitable approach to meet the requirement of being a simple and portable device used in this study.

Chapter 3 Material and methods

This chapter demonstrates the materials and equipment used in the study, and the designing of experiments to conducted the study. The research is mainly carried out in two parts: the time trial experiment and the model systems. The time trial experiment was performed to quantify the values of EC, total bacterial count (TBC), LA concentration and pH in skim and whole milk during an extended storage at either 4 or 8℃. Two laboratory controlled model systems were separately performed to assess the effects of TBC, LA concentration and pH on EC changes in milk, respectively. The interrelationship between EC and other parameters was subsequently analysed by comparing results between trial and model systems. Lastly, experiment 4 was conducted to assess the temperature effect of EC in milk samples.

3.1. Milk

3 x 3L high-density polyethylene (HDPE) bottles of freshly chilled pasteurized skim and whole milk were purchased from a local supermarket (*Homebrand*, Countdown, New Zealand). The milk was used in the time trial experiment, namely experiment 1. The sampling was performed on the same day of purchase. All samples were kept at 4 ± 0.5℃ priors to use. In addition, 16 cartons of 1L UHT long life skim and whole milk were purchased from a local supermarket *(Anchor*, Countdown, New Zealand) one day before the performance of the experiment. This milk was used in experiments 2-4. All samples were kept at 4 ± 0.5℃ before being opened, and at either 4 or 8℃ for incubation after being opened. Each milk sample was taken freshly and directly from the incubating milk bottles on the measuring occasions.

The freshly chilled pasteurized skim and whole milk were purchased from the same batch series. The legitimated shelf life of skim and whole milk in New Zealand is 14 post-production days (Ministry for Primary Industries, 2014). The skim milk was purchased on day 4 of post-production, while the whole milk was purchased on day 3 of post-production. The first sampling was taken on the same day as milk was purchased. **Table 5** lists the date of purchase, batch series number and the BBD of all milk samples. The UHT long life milk was purchased from a variety of batch series. To control for and minimise interval variations between milk samples, the bottles of UHT milk with the most closely related BBDs were used to conduct the same experiment. The detailed information on UHT skim and whole milk is listed in **Table 6**. In addition, **Table 7** lists the nutritional values of pasteurized and UHT skim and whole milk.

To control and minimise the bacterial contamination of milk samples, the transformation of milk liquid between storing bottle and individual sampling tubes was performed in the biological laminar flow cabinet on each sampling occasions.

Milk samples	Purchase date	Best before date	Batch series number
Skim #1	3. Mar. 2016	13. Mar. 2016	CA059 07:14
Skim #2	3. Mar. 2016	13. Mar. 2016	CA059 07:14
Skim #3	3. Mar. 2016	13. Mar. 2016	CA059 07:14
Whole #1	3. Mar. 2016	14. Mar. 2016	CA060 08:08
Whole #2	3. Mar. 2016	14. Mar. 2016	CA060 08:08
Whole #3	3. Mar. 2016	14. Mar. 2016	CA060 08:08

Table 5. A list of purchase dates, best before dates and batch series numbers of freshly chilled pasteurized skim and whole milk (used for experiment 1).

Table 6. A list of the batch series numbers, best before dates and the experiments used of UHT skim and whole milk (used for experiments 2-4).

UHT long life milk	Batch series number	Best before date	Experiment used
Skim #1	1524281201 18:18	26. May. 2016	Experiment 2
Skim #2	1524281201 18:19	26. May. 2016	Experiment 2
Skim #3	1536181203 17:14	22. Sep. 2016	Experiment 3
Skim #4	1536181203 17:12	22. Sep. 2016	Experiment 3
Skim #5	1536181203 17:12	22. Sep. 2016	Experiment 3
Skim #6	1607381300 07:22	08. Dec. 2016	Experiment 4
Skim #7	1607381300 07:22	08. Dec. 2016	Experiment 4
Skim #8	1607381300 08:22	09. Dec. 2016	Experiment 4
Whole #1	1534081101 22:46	02. Sep. 2016	Experiment 2
Whole #2	1534081101 22:49	02. Sep. 2016	Experiment 2
Whole #3	1601281200 01:15	08. Oct. 2016	Experiment 3
Whole #4	1601381200 00:55	09. Oct. 2016	Experiment 3
Whole #5	1601381200 00:55	09. Oct. 2016	Experiment 3
Whole #6	1607581201 22:36	10. Dec. 2016	Experiment 4
Whole #7	1607581201 22:36	10. Dec. 2016	Experiment 4
Whole #8	1607581201 22:36	12. Dec. 2016	Experiment 4

Table 7. Nutritional values of pasteurized and UHT long life skim and whole milk.

The nutritional content	**Pasteurized milk (g / 100ml)**		**UHT long life milk (g / 100ml)**	
	Skim	Whole	Skim	Whole
Protein	3.7	3.1	3.7	3.2
Fat	0.6	3.3	0.1	3.3
Carbohydrate	4.9	4.7	5.1	4.9
Na	0.045	0.040	0.046	0.043
Ca	0.130	0.115	0.133	0.116

3.2. Measurement of electrical conductivity

On each sampling occasion, the EC of skim and whole milk was measured using a portable handheld conductivity meter (**Figure 14**) (Eutech Cond6+, *Thermo Fisher Scientific, New Zealand*). Priority to each measurement, the conductivity meter was calibrated against a provided calibration solution with a standardised conductance value of 1413 μS/cm at 25℃. To measure EC, the two-terminal electrode was entirely submerged in the milk. The total volume of the milk solution that in contact with the two-terminal electrodes was constrained by the built-in plastic cover guard. The digital display showed the readings of measurements, and the results were recorded. After each measurement, the electrodes were rinsed thoroughly with distilled water and dried prior to next use. This conductivity meter has $\pm$ 1% accuracy over a wide range of measurements with the resolution corrected to 0.05%. Apart from that, the conductivity meter has a built-in temperature sensor that automatically compensates for temperature fluctuations within the range of -10 to 110℃. The temperatures were also measured at each measurement of EC to facilitated the maintenance of consistent temperature of samples throughout the entire experiment. The temperature sensor has a resolution and accuracy of 0.1 and $\pm$ 0.5℃, respectively. A list of features of specifications of the conductivity meter is listed in **Table 8**.

Figure 14. Conductivity meter, the two-terminal electrode and the plastic cover guard.

Table 8. A list of specifications of the conductivity meter (Instruction manual, 2014).

Specifications	Values
Model	Eutech Cond 6+
Resolution	0.05% full scale
Accuracy	± 1% full scale + 1 digit
Temperature compensation	Build-in temperature sensor
Temperature range	-10 to 110℃
Temperature resolution and accuracy	0.1°C and ± 0.5 °C
Electrode constant (K)	1.0
Fabrication material of electrodes	Stainless steel
Power source	4 'AAA' x1.4V batteries

3.3. Measurement of total bacterial count

3.3.1. Preparation of microbial growing plate

The agar was prepared by dissolving 40 g of tryptic soy agar (TSA; *Difco*™) powder in 1 L of distilled water. The solution was thoroughly mixed by inversion and then subjected to autoclaving at 121℃ for 30mins. After autoclaving, the liquid TSA was cooled down by placing it in a 55℃ water bath. Approximately 10 - 15 ml of liquid TSA were poured into a plastic Petri dish. The performance was done in a biological laminar flow cabinet to minimise sample contamination. The plates were then allowed to set at ambient temperature and then stored at 4℃ until use.

3.3.2. Preparation of dilution media

Peptone (*Bacto*™) was purchased from Becton Dickinson (BD) and used as diluting blank for bacterial serial dilutions. 0.1% peptone was prepared by dissolving 1g peptone in 1L of distilled water. Aliquots of 9ml were dispensed to glass universal bottles, and the bottles were subjected to autoclaving at 121℃ for 30 minutes. After autoclaving, the dilution blank was stored at room temperature until use.

3.3.3. Calculating the total bacterial count

The total viable microbial numbers in the milk samples were determined using standard bacteria counting method (Frank, Christen and Bullerman, 1992). Approximately 5 ml of milk was transferred to a sterile glassware container at each sampling occasion. Aliquot of 1 ml of this milk was collected and diluted with 9 ml of 0.1% peptone to produce milk dilutions. A series of one in ten dilutions from 10^3, 10^4, 10^5, 10^6 10^7, 10^8, to 10^9 were generated accordingly to the countable range of numbers of colonies formed on the agar plate. Aliquot of 0.1 ml of the diluted milk was then spread onto a prepared TSA plate in triplicates (n=3). The plate was incubated at 25℃ for 72h until visible colonies were developed on the agar plates. The TBC as CFU/ml was recorded and calculated according to the equation:

$$\text{CFU/ml} = \frac{\text{the number of colonies per volume(ml) plated}}{\text{total dilution factors}} \quad (4)$$

3.4. Measurement of acidification

3.4.1. Titratable acidity

Titratable acidity is a traditional method used to determine the proportion of organic acid, particularly LA developed in the milk during fermentation (McCarthy and Singh, 2009; Sadler and Murphy, 2010). To calculate the LA concentration in each sample, a titration with 0.1 M NaOH solutions was performed. Milk samples (9ml) were diluted with an equal amount of deionised water in a measuring cup. Drops of 0.1 M NaOH were slowly titrated with the milk dilution using a burette. Meanwhile, a pH probe was also used to determine the pH of solution. The percentage of LA concentration was determined when 0.1 M N_aOH titrated up to pH of 8.35. The percentage was calculated according to the following equation:

$$\text{LA\%} = \text{titration volume of NaOH (mL)} \times \text{molarity of NaOH} \quad (5)$$

3.4.2. pH

pH measures the strength of acidity in the milk solution. The pH was measured using a conventional digital pH meter (pH 209, *Hanna Instruments, USA*). The pH meter has a wide detectable range of pH from 0 to 14. It has $\pm$ 0.01 accuracy of measurement at 20℃ with the resolution corrected to 0.01. The meter was calibrated against standard buffer solutions of pH 7 and pH 4 prior to each sampling. The display was also calibrated to the correct pH reading. At each measurement, the electrodes were entirely submerged in the milk. The digital display showed the reading of measurement within seconds and the results were recorded. After each measurement, the probe was rinsed thoroughly with distilled water and restored in 3.5 M potassium chloride (KCl) electrolyte solution.

3.5. Experiment 1: time trial experimental design

Six bottles of commercially purchased freshly chilled pasteurized milk (3 × skim milk, 3 × whole milk) were collected for an extended storage. A pre-processing separation process was carried out by evenly poured each bottle of milk into 6 individual sterilised Schott sub-bottle on the same day of purchase (**Figure 15**). The purpose of the separation process was to achieve

an even distribution of milk compositions and total initial bacterial population in each sub-bottle, thus minimizing the variations that were introduced to samples due to material, human or technique-dependent errors. Three bottles of milk were stored in a temperature of 4℃, and the other three were stored at a temperature of 8 ℃. This pre-processing procedure standardised to all bottles of skim and whole milk. The milk was stored at two different temperatures to investigate whether temperature variations affect EC of milk. Milk held at 4℃ was sampled on 11 occasions in 72-hour intervals over a 30-day incubation period. Due to skim and whole milk were bought on the same day, but different post-production day, the skim milk was sampled on the post-production days of 4, 7, 10, 13, 16, 19, 22, 25, 28, 31 and 34, while the whole milk was sampled on the post-production days of 3, 6, 9, 12, 15, 18, 21, 24, 27, 30 and 33 (**Table 9**). Milk held at 8℃ was sampled on 6 occasions at 48-hour intervals over a 10 or 12-day period. Skim milk was sampled at 4, 6, 8, 10, 12, 14, and 16 post-production day, while the whole milk was sampled at 3, 5, 7, 9, 11, and 13 post-production day. The sampling was terminated when the absolute spoilage was identified by either the change in flavour or curdling of milk was physically detected by olfaction or observation (Lu, Shiau, Wong, Lin, Kravis and Blackmon, Tanya, Jen, Cheng, Chang, Ong, Sarfaraz and Wang, 2013). On each occasion, the EC, TBC, LA concentration and pH of each sub-bottle were measured and the experimental data were recorded. The EC was measured using a portable handheld conductivity meter (for details refer to section 3.2), the TBC was determined by the standard microbiological plate count techniques (for details refer to section 3.3), and the development of acidity in milk was evaluated by titratable acidity and conventional digital pH meter, respectively (for details refer to section 3.4).

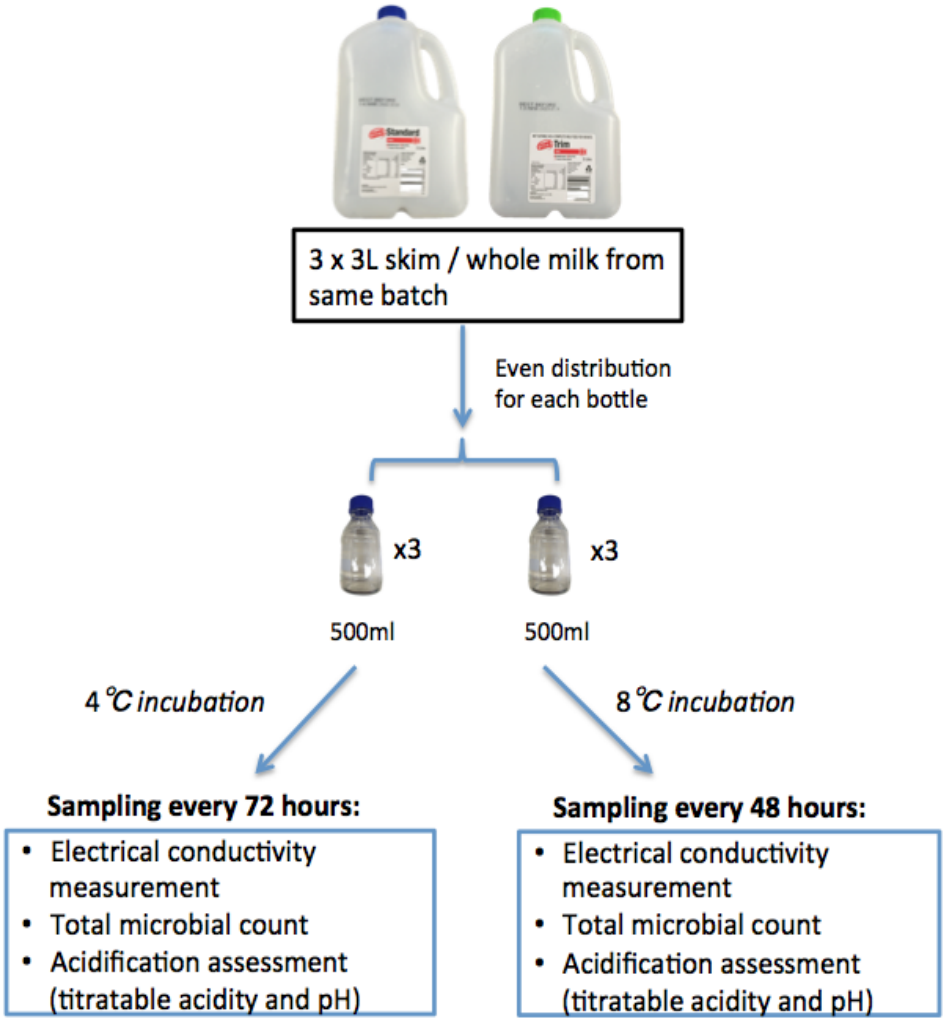


Figure 15. Schematic diagram of the design of time trial experiment.

Table 9. The sampling days of skim and whole milk stored at 4 and 8℃

Incubation temperature	Milk types	Sampling day (post-production day)
4℃	Skim milk	4, 7, 10,13, 16, 19, 22, 25, 28, 31, 34
	Whole milk	3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33
8℃	Skim milk	4, 6, 8, 10, 12, 14,16
	Whole milk	3, 5, 7, 9, 11,13

3.6. Experiment 2: determination of the effect of acidification on EC in model systems

3.6.1. Determining the effect of LA on EC

To quantitatively determine the effect of LA concentration on EC in milk. 200 μl aliquots of a stock solution containing 10% of LA (*Sigma-Aldrich*) was progressively added to 15 ml of UHT skim and whole milk. The EC and pH were determined after each addition of LA solution. Milk was held at either 4 or 20℃ during each measurement. The EC and pH were measured by conductivity meter and conventional pH meter, respectively. The measurements were performed as same as that described in section 3.2 and 3.4.

3.6.2. Determining the effect of pH on EC

To determine the correlation between pH and EC in milk. 1M hydrochloric acid (HCl; *Sigma-Aldrich*) stock solution was prepared by diluting 9.854 ml of 37% HCl in 90.146 ml of distilled water. The 1 M HCl stock solution was progressively added with 100 μl aliquots to 20 ml of skim and whole milk, respectively, to progressively reduce the pH of milk. The EC and pH were measured after each addition of HCl using the same procedures has listed in sections 3.2 and 3.4.

3.7. Experiment 3: determination of the effect of spoilage bacteria on EC in model systems

3.7.1. Determining the effect of presence of bacteria on EC

The bacterial strain, *Pseudomonas aeruginosa* 2576 (PA2576), was used in this experiment (provided by Professor Phil Bremer). The milk that used in this experiment was UHT long life skim and whole milk. Bacterial cells were harvested from an overnight culture by centrifugation (at 3000 rpm for 20 minutes). The cells were washed twice with 0.1% peptone to remove excess cell debris. The bacterial pellet was then re-suspended in 20 ml of either skim or whole milk. The drop-plate method was used to estimate the number of bacteria in the original inoculum (Herigstad, Hamilton and Heersink, 2001). The plates were then incubated in 37℃

overnight. The total number of presence of bacteria were counted and recorded the following day.

To perform a serial bacterial dilution, 2 ml of the original bacterial inoculum was diluted in 18 ml of UHT milk blank to produce 1 in 10 dilutions. The same method was followed to successively generate a series of 10^7, 10^6, 10^5, 10^4 and 10^3 CFU/ml bacterial dilutions in skim and whole milk samples. The EC values of each inoculum were measured and recorded at either 4 or 20℃.

3.7.2. Determining the effect of bacterial metabolism on EC

10 ml of PA2576 overnight culture were used in this experiment. The bacteria stock solution was prepared by centrifugation and washing, as same as the techniques were employed in the section above. The total bacteria concentration of the initial stock solution was determined using the drop-plate method. Aliquots of original bacterial stock solution (0.2 ml) were diluted in 19.8 ml of either skim or whole milk blank to produce a serial dilution of 10^8, 10^6, 10^4, 10^2 and 10^0 CFU/ml bacterial inoculum. The bacterial inoculums were incubated at 37℃ for 10 hours. The EC of the series of bacterial inoculum was measured on 5 occasions with 2-hour intervals, and the results were recorded.

3.8. Experiment 4: determination of the temperature-dependent effect of EC

To determine the effects of temperature on EC in milk, 60 ml of UHT long life skim milk, UHT long life whole milk, freshly chilled pasteurized skim milk and freshly chilled pasteurized whole milk were used. The milk was added to a beaker and placed on ice prior to measurements. Table salt was sprinkled on the surface of the ice to reduce the freezing point of ice if the temperature was difficult to maintain as low as 0℃. A pre-heated 50℃ water bath was employed for heating up the milk samples. The EC of milk samples was measured at every 5℃ while milk samples heated up from 0 to 50℃. At each measurement, milk was constantly stirred with a measuring probe to ensure even heating of samples. The results were recorded.

3.9. Statistical analysis

The results from experiment 1 were analysed by multivariate one-way ANOVA using Prism 6 (*GraphPad Software Inc*, California, USA). Six bottles of milk from the same batch were used in the experiment and sampling was performed in triplicates (n=3). The milk spoilage was investigated by measuring TBC, LA concentration and pH over time. The storage times for each milk sample were used as the independent variable factors. The change in EC, LA concentration, and pH were the dependent variables of storage time and milk variety. A Dunnett's post-test was performed for multiple comparisons among samples. P-value equal to or less than 0.05 were considered to be significant. The interrelationship between EC and TBC, LA concentration and pH were evaluated by Pearson's r correlation (or Pearson product-moment correlation coefficient). Any correlation coefficient equal to 0.1 was considered as weak correlation, 0.1-0.3 represent moderate correlations, and equal to or greater than 0.5 represents strong correlation (Cohen, 1988).

The effects of acidity, bacteria numbers and temperature on EC were also analysed with a multivariate one-way ANOVA using Prism 6. In experiment 2, the concentration of acidity was the independent variable, the changes in EC were the dependent variables upon increased concentration of acidity. In addition, a linear regression analysis was performed to establish the linear correlation between EC and LA and pH in milk. In experiment 3, the number of bacteria that added to milk was the independent variable, the changes in EC were the dependent variables of the number of bacteria and bacterial inoculums present in milk. In experiment 4, temperature was the independent variable, the changes in EC were the dependent variables of changed temperature in milk. The statistical analysis was gathered from triplicate sampling (n=3) in each experiment. A Dunnett's post-test was employed to perform the multiple comparisons among samples. P-value equal to or less than 0.05 were the significance level.

Chapter 4 Results

4.1. Experiment 1

The aim of this experiment was to determine the variations in EC, TBC, LA concentration and pH in skim and whole milk over an extended storage at either 4 or 8℃.

4.1.1. Quantify the values of EC, TBC, LA concentration and pH in skim and whole milk over an extended storage at 4℃

Three bottles of same batch produced commercial skim and whole milk were collected on post-production day 4 and 3, respectively. The milks were incubated at 4℃ for 30 days. The last sampling is considered when complete spoilage was physically detected with signs of curdling or off-flavour resulted in milk. On 10 occasions, the milk was sampled for EC, TBC, titratable acidity and pH (**Figure 16**).

The initial mean values of EC were 5.37 and 5.07 mS/cm for skim and whole milk, respectively (**Figure 16A**). With a single exception, in which one bottle of whole milk was 4.94 mS/cm. The mean EC values for all milk samples remained stable for 28 post-production days. An increase in EC occurred after day 28 for skim milk and a statistically significant increase ($p<0.05$) in EC was found on day 34, compared to that on the first sampling day (as the control group) (Appendix 1, Table 11). Eventually, the mean EC values of skim milk reached 5.54 mS/cm. The mean EC value of whole milk showed a statistically significant increase ($p<0.05$) from day 24 onwards, with an exception of day 30. This earlier significant increase of EC in whole milk is likely due to the lower value of initial mean EC value in one of the bottles of whole milk, which decreased the mean EC value on the first sampling day (Appendix 1, Table 12). Eventually, the mean EC values of whole milk reached 5.24 mS/cm. The BBD was due on post-production day 14 for both skim and whole milk. A statistically significant increase in EC did not occur before the BBD.

The growth of bacteria often is used as an indicator of spoilage in milk. Skim and whole milk had an initial TBC of 2.0×10^2 and 3.9×10^2 CFU/ml, respectively (**Figure 16B**). Skim and whole milk exhibited an initial lag phase before the growth of bacteria on post-production day 13 and 12, respectively. This was followed by an exponential growth in the number of bacteria to 7×10^7 CFU/ml for skim milk on day 34, and 2.8×10^7 CFU/ml for whole milk on day 33

(Appendix 1, Table 12). Interestingly, the milk bottles were from the same batch, but a wide variation in the number of bacteria was observed in the milk. There was an insignificant increase in the TBC was observed before the BBD.

The development of acidification in milk during storage was evaluated in two forms: titratable acidity and pH (**Figures 16C and D**). Skim milk contained an initial LA concentration of 0.13% and a final concentration of 0.158%. A subtle increase in LA concentration was observed on day 7 for skim milk, and day 6 for whole milk, which was followed by a progressive increase until the last sampling day. Skim milk samples showed a significant increase ($p<0.05$) in the mean concentration of LA on post-production day 34 (Appendix 1, Table 11). The variation in the measurements of LA was relatively large, as the maximum and minimum levels of LA concentration varied throughout the experiment. The trending of whole milk was relatively more inconsistent than the skim milk. The whole milk had an initial LA concentration of 0.13%. A sharp decrease in the mean LA concentration was observed in all samples on post-production day 12, which was followed by increasing concentration with a peak at 0.158% on post-production day 33. Whole milk samples showed a statistically significant increase ($p<0.05$) in the development of LA on post-production day 33 (Appendix 1, Table 12).

The increase in the production of LA and other organic acid decreased the pH of milk (**Figure 16D**)**.** At the start of the trial, the skim and whole milk had a near neutral pH of 6.77 and 6.75, respectively. A brief plateau in pH was observed before post-production day 14 and 13 for skim and whole milk, respectively. This was followed by a steady decrease to 6.62 and 6.51 on the last sampling day for skim and whole milk, respectively. Skim milk had a significant decrease ($p<0.05$) in pH from day 31 onwards, while whole milk had a significant decrease ($p<0.05$) in pH from day 24 onwards (Appendix 1, Tables 11 and 12). Increasing variations in pH developed among the triplicate milk bottles over the incubation period. The variation in the range of fluctuation of pH was scarcely noticeable at the beginning of the trial, which then progressed to an appreciable range on the last sampling day. Similarly, there was not a statistically significant decrease in pH before the BBD.

Overall, skim and whole milk showed a significant increase in mean EC, TBC and LA concentration and a decrease in pH values prior to the occurrence of sensory and/or texture changes in milk. However, statistically significant changes in EC, TBC, LA concentration and pH did not occur before the BBD, regardless of milk types.

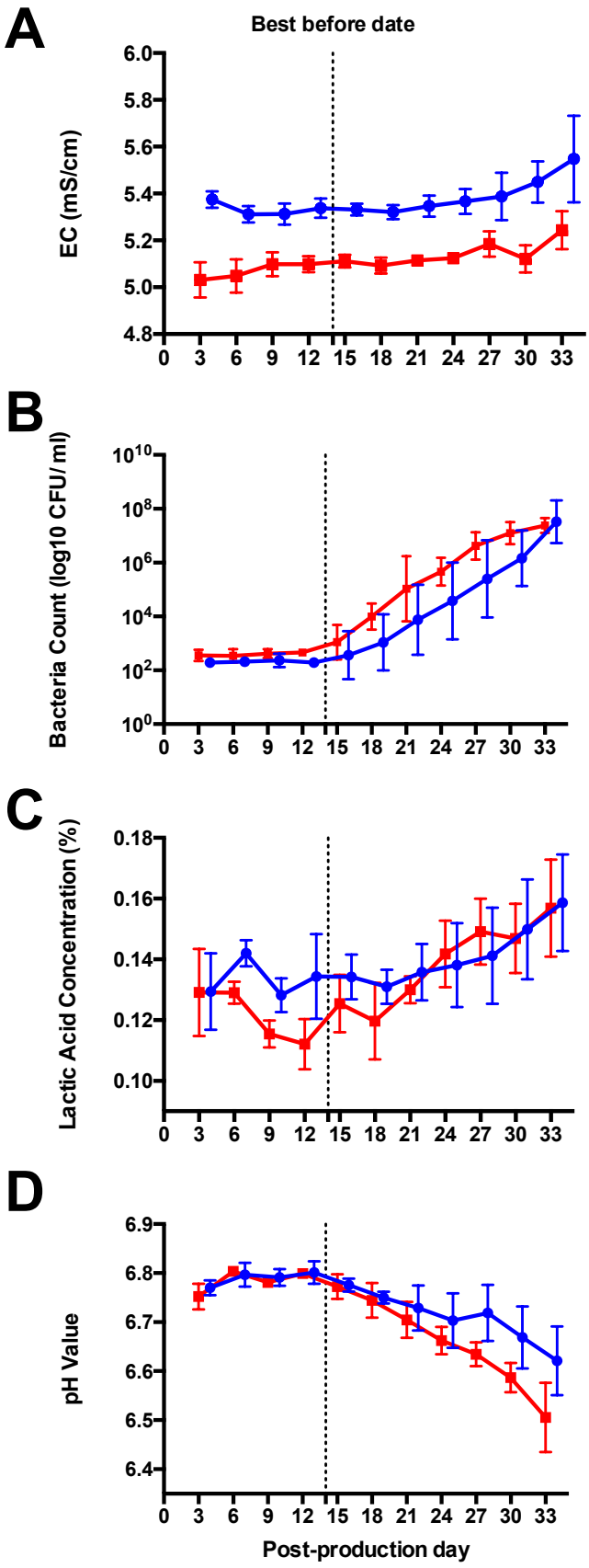


Figure 16. The value of EC **(A)**, TBC **(B)**, LA concentration **(C)** and pH **(D)** of skim (☻-blue) and whole (◙ -red) milk during an extended storage at 4℃. Each data point represents triplicate readings from three individual milk bottles (n=3). The line trend joins the means of each measurement. The vertical error bars represent the standard deviation of mean of each data point. The vertical dash line indicates the BBD for milk on post-production day 14.

4.1.2. Quantify the values of EC, TBC, LA concentration and pH in skim and whole milk over an extended storage at 8℃

To further investigate the interrelationship between EC and milk spoilage, milk was held at 8 ℃. The skim and whole milk samples each lasted 16 and 13 post-production days, respectively, until in least one or more of the triplicates were absolutely spoiled. The measurements of EC, TBC, LA concentration and pH for skim and whole milk are shown in **Figure 17**.

The initial mean EC value of skim milk was 5.19 mS/cm, which stayed consistent during the first 10 post-production days (**Figure 17A**). This was followed by a statistically significant increase ($p<0.05$) from day 12 onwards (Appendix 1, Table 14). The overall trend for skim milk was constant, except for a single data point from one bottle of the skim milk, which showed a sudden decrease in EC on day 14. The initial mean EC value of whole milk (4.98 mS/cm) was lower than that of skim milk, but it was followed a similar trend over time. A statistically significant increase ($p<0.05$) in the mean EC value of whole milk was established before the BBD and from day 9 onwards (Appendix 1, Table 15).

Skim and whole milk had a TBC of 1.9×10^2 and 3.5×10^2 CFU/ml, respectively, on the first sampling day (**Figure 17B**). A short lag phase of bacterial growth was observed on day 6 for skim milk, and day 5 for whole milk followed by the exponential growth. A sharp increase in TBC was observed from post-production day 10 and 7 onwards for skim and whole milk, respectively. Skim milk had a statistically significant increase ($p<0.05$) in TBC from day 10 onwards. Whole milk had a similar bacterial growth pattern to skim milk. A statistically significant increase ($p<0.05$) in TBC was observed on day 7 for whole milk, and the total number of bacteria reached 2.88×10^8 CFU/ml (Appendix 1, Tables 14 and 15). The significant levels ($p<0.05$) of TBC were showed before the BBD for both skim and whole milk.

All milk samples developed significant levels of LA during storage at 8℃ (**Figure 17C**). The initial LA concentration was 0.138 and 0.120% for skim and whole milk, respectively. Skim milk had a significant increase ($p<0.05$) in LA on post-production day 16, whereas a significant increase ($p<0.05$) in LA was observed in whole milk on post-production day 11 (Appendix 1, Tables 14 and 15).

All skim milk samples showed a steady decrease in pH **(Figure 17D**). With the exception of two data points from one bottle, which deviated from the general trend and shifted the mean

pH value lower than the rest of values on post-production day 6 for skim milk. The mean pH values of skim and whole milk changed from 6.81 to 6.6, and 6.82 to 6.63 over time, respectively. There was a significant reduction ($p<0.05$) in pH on day 6 for skim milk, and day

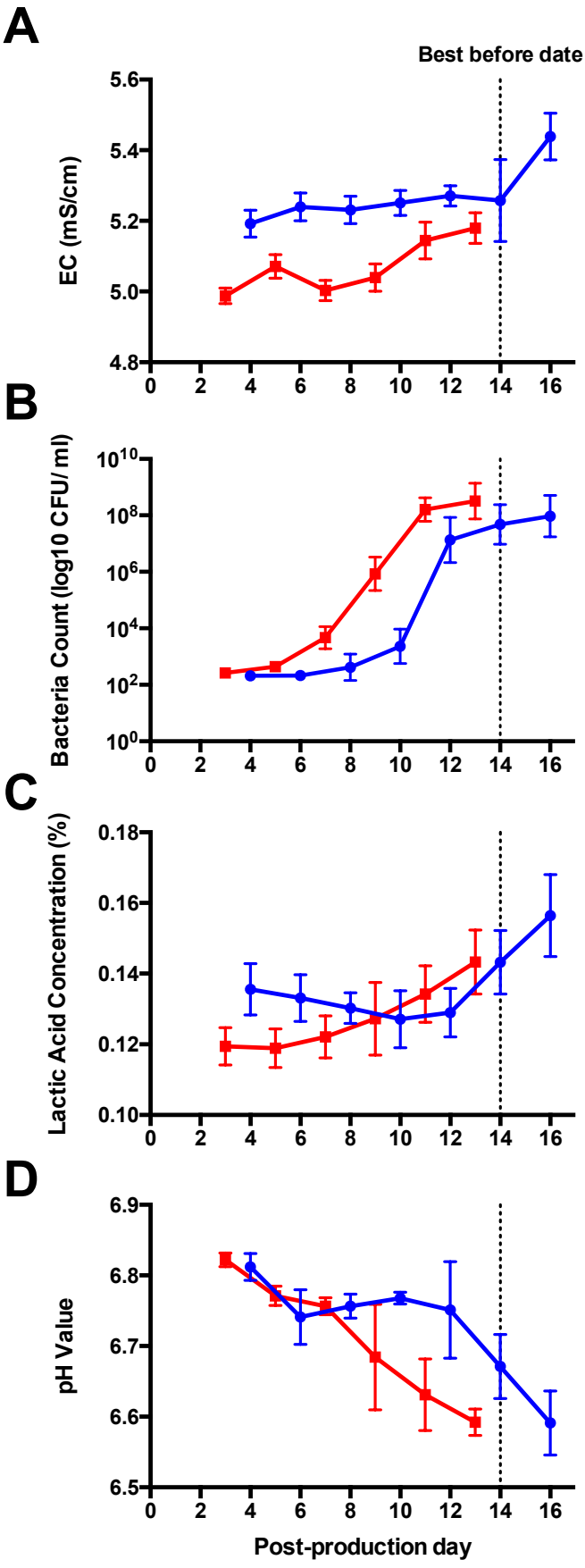


Figure 17. The value of EC **(A)**, TBC **(B)**, LA concentration **(C)** and pH **(D)** of (☻-blue) and whole (◙ -red) milk during an extended storage at 8℃. Each data point represents triplicate readings from three individual milk bottles (n=3). The line trend joins the mean of each measurement. The vertical error bars represent the standard deviation of mean of each data point. The vertical dash line indicates the BBD for milk at post-production day 14.

5 for whole milk, respectively. The significant levels ($p<0.05$) in pH were also observed before the BBD in skim and whole milk (Appendix 1, Tables 14 and 15).

Overall, storing milk at an improper temperature resulted in both skim and whole milk developing significant increases in EC, TBC, and LA concentration and decrease in pH, before the perception of the occurrence of spoilage according to olfactory sensation or texture observation. EC changed correspondingly with TBC, LA concentration and pH in milk. Skim milk spoiled at two days after BBD, and whole milk spoiled one day earlier the BBD. The variations in the end points of spoilage in milk led to a situation in which the BBD is invalid to predict the shelf life correctly at improper storing temperature.

4.1.3. Determination of percentage change in EC

To further investigate the extension of variations in EC during milk spoilage, the percentage change in EC was calculated using the EC value at each day (EC_T) minus the initial EC value at first sampling day (EC_0) and divided by the initial EC values (EC_0). The calculation was applied according to the equation stated in below:

$$\text{Percentage change of EC } (\%) = \frac{\text{EC}_T - \text{EC}_0}{\text{EC}_0} \times 100\% \quad (6)$$

Storing skim milk at 4℃ resulted in a 3.68% increase in EC on the last sampling day (**Figure 18A**). It was observed that one bottle of skim milk has scarcely changed its EC (not shown in figure), which pulled down the average EC value for all skim milk samples. This resulted in the mean percentage changes of EC remained at low value around the baseline before post-production day 28 in skim milk. Whilst a constant increase in EC values were observed in whole milk and the maximum threshold reached 4.02% on the last sampling day. The percentage change in EC of skim and whole milk stored at 8℃was 4.5% and 3.9% over the incubation period, respectively (**Figure 18B**). Skim and whole milk showed initial increases in EC on day 6 and 5, followed by plateaus until day 14 and 9, respectively. A sharp increase in percentage changes of EC was observed from day 14 onwards for skim milk and day 9 onwards for whole milk.

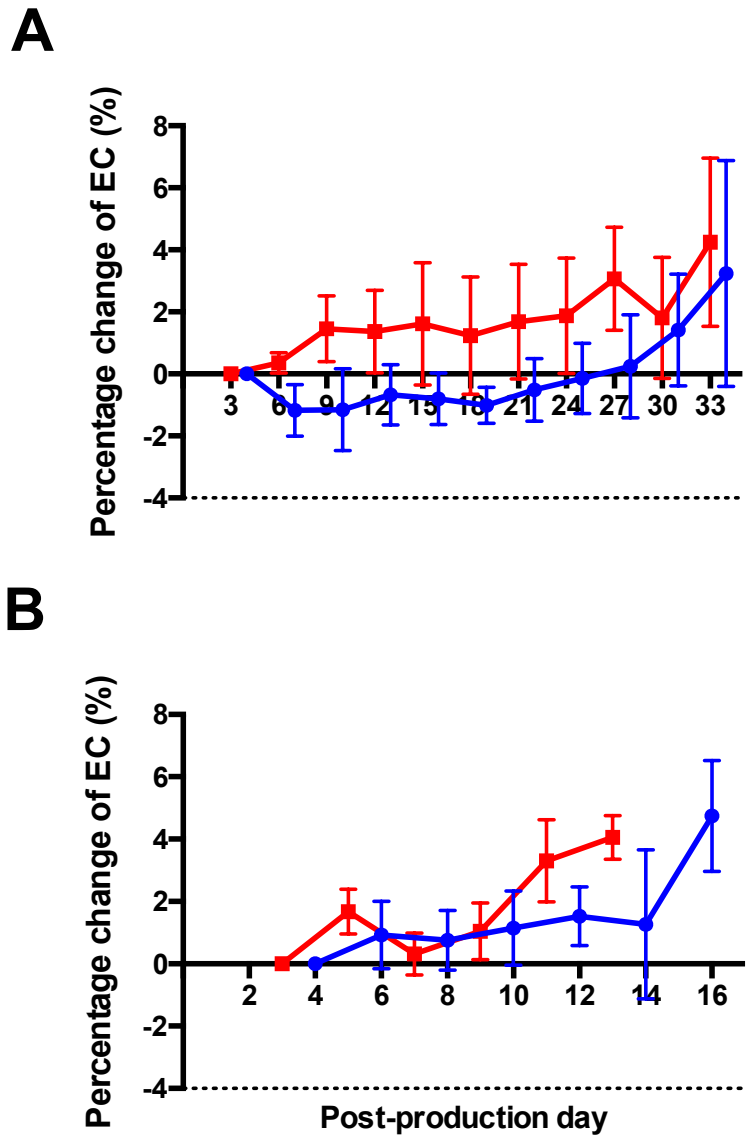


Figure 18. Percentage change of EC in skim (☻ -blue) and whole (◙ -red) milk held at 4℃ **(A)** and 8℃ **(B)**. The values were calculated from the data obtained from Figure 16A and 17A. The initial percentage change of milk EC for skim and whole milk were normalized to 0, and the following values were calculated according to formula (6). Each data point represents triplicate readings from three individual milk bottles (n=3). The line trend joins the mean of each measurement. The vertical

4.1.4. Analysis of the interrelationship between EC, acidification and growth of bacteria in milk

To further understand the interrelationship between EC, growth of bacteria and acidification in milk. The values of EC against the values of TBC, LA concentration and pH (i.e. acidity) were re-plotted for skim and whole milk (**Figures 19-22**). The correlation coefficient between EC and measured parameters was estimated using Pearson's correlation. Statistical analysis is presented in Appendix 1 (Tables 16-19).

At 4℃, a moderately strong positive correlation was showed between EC and TBC, LA concentration and acidity for skim milk, and the estimated Pearson's r was 0.6306, 0.5624 and 0.6645, respectively. The results showed a statistically significant correlation ($p<0.05$) between EC and TBC, LA concentration and acidity in skim milk (**Figure 19**). Similar results were found for whole milk, where a strong positive correlation was seen between EC and TBC, and EC and acidity. The Pearson's r was 0.5266 for EC and TBC, and 0.6047 for EC and acidity in whole milk (**Figure 20**). A relatively moderate correlation was estimated between EC and LA concentration in whole milk with Pearson's r equal to 0.3916.

At 8℃, the correlations between EC and TBC, and EC and LA concentration for skim milk were relatively moderate with Pearson's r equals to 0.5761 and 0.4931, respectively. A strong correlation was established between EC and acidity with a Pearson's r equal to 0.7221 (**Figure 21**). For whole milk, a strong correlation was seen between EC and TBC, LA concentration and acidity. The Pearson's r was 0.7460, 0.7014 and 0.8019, respectively (**Figure 22**). A statistically significant correlation ($p<0.05$) was established between EC and TBC, LA concentration and acidity in skim and whole milk.

Overall, all milk showed a moderate to strong positive correlation between the evaluated parameters of EC and TBC, LA concentration and acidity, regardless of milk type and storage conditions. The change in EC is not a solitary consequence of the change in any one of the parameters. It is a collective impact instigated by the growth of bacteria, production of LA and acidity during spoilage. The results suggested a positive interrelationship between EC and TBC, LA concentration and acidity in milk.

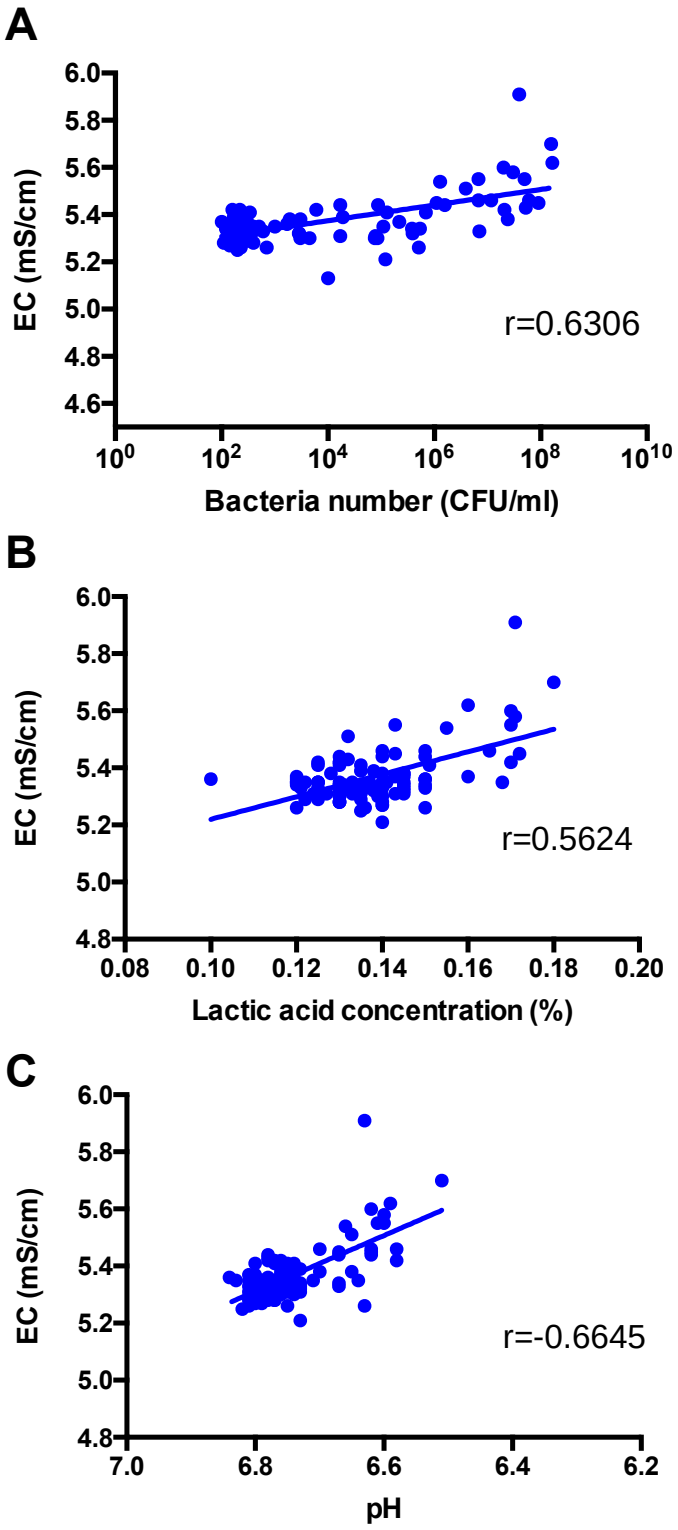


Figure 19. The interrelationship between the mean EC value with TBC **(A)**, LA concentration **(B)** and pH **(C)** in skim milk at 4°C. Each data point represents a single measurement. The values obtained from Figure 16. The straight line represents the estimated linear relationship between evaluated X and Y values.

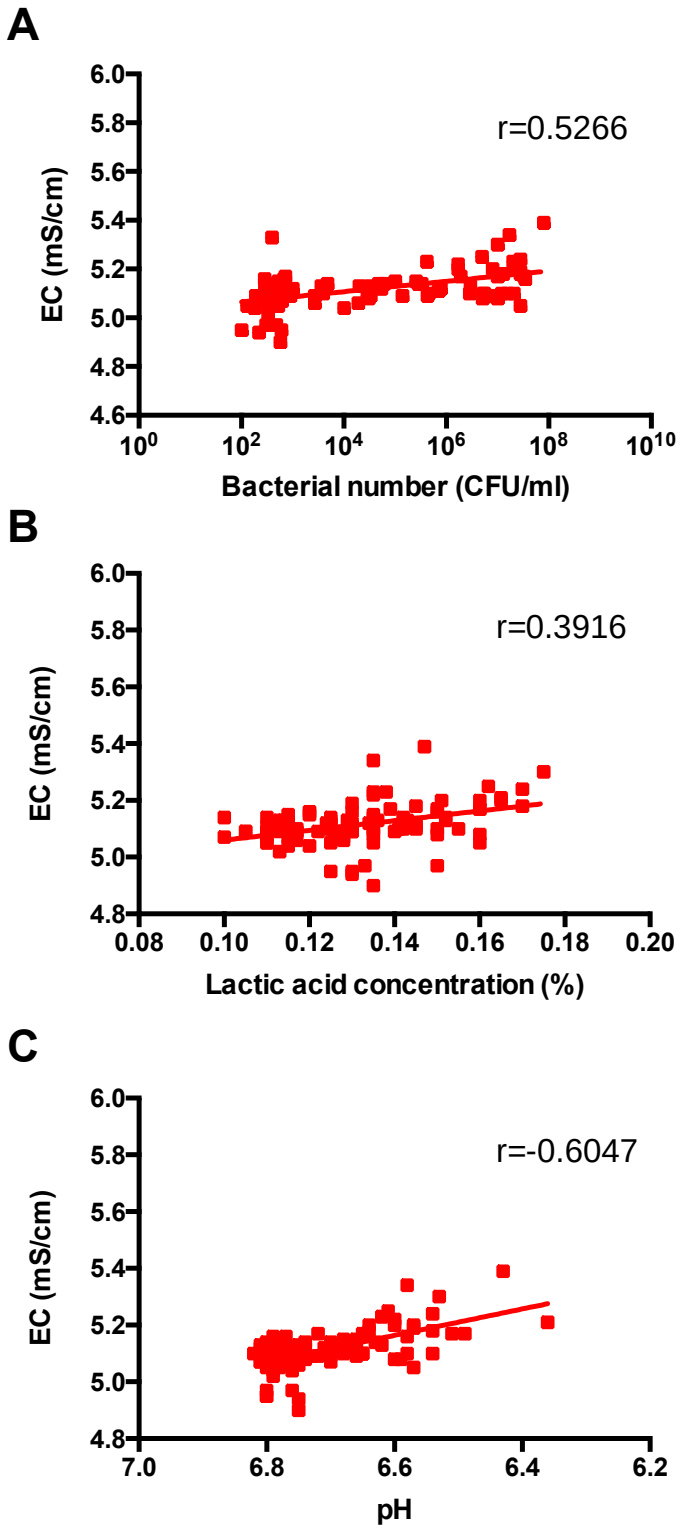


Figure 20. The interrelationship between the mean EC value with TBC **(A),** LA concentration **(B)** and pH **(C)** in whole milk at 4°C. Each data point represents a single measurement. The values obtained from Figure 16. The straight line represents the estimated linear relationship between evaluated X and Y values.

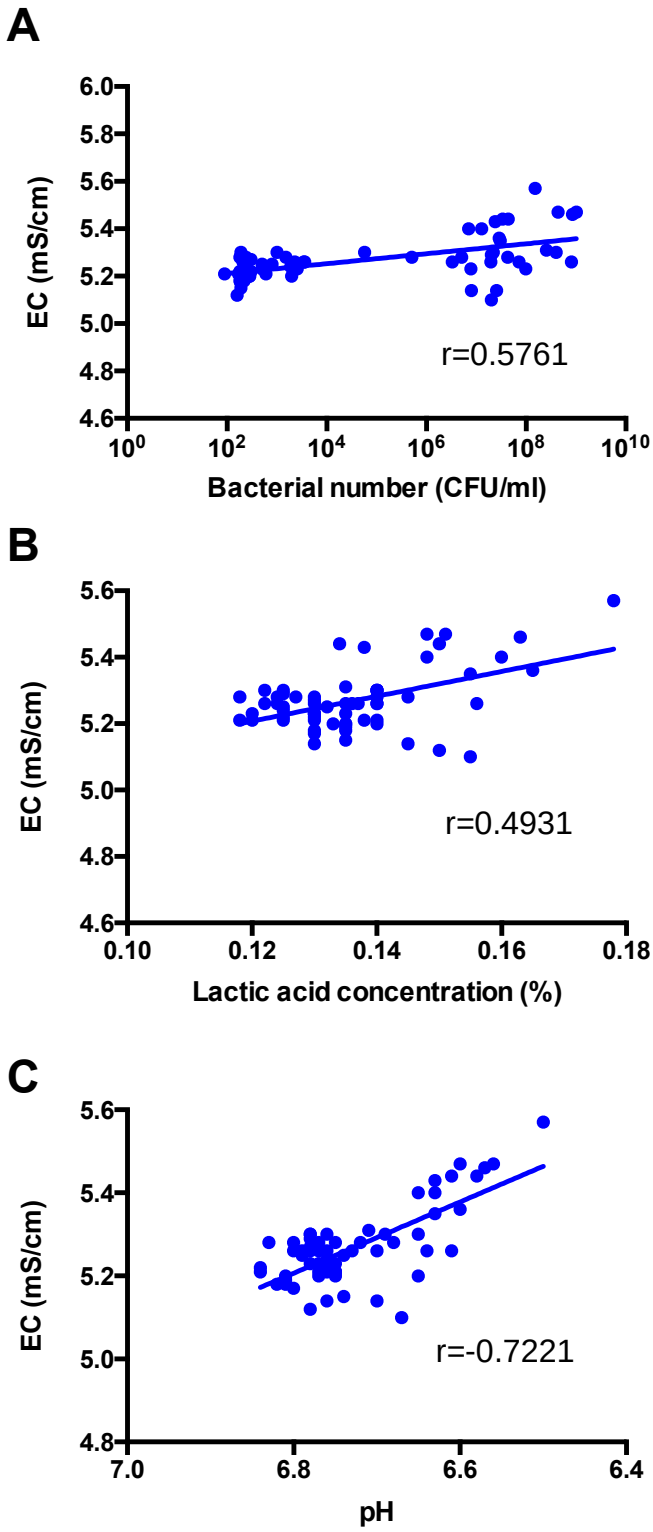


Figure 21. The interrelationship between the mean EC value with TBC **(A),** LA concentration **(B)** and pH **(C)** in skim milk at 8℃. Each data point represents a single measurement. The values obtained from Figure 17. The straight line represents the estimated linear relationship between evaluated X and Y values.

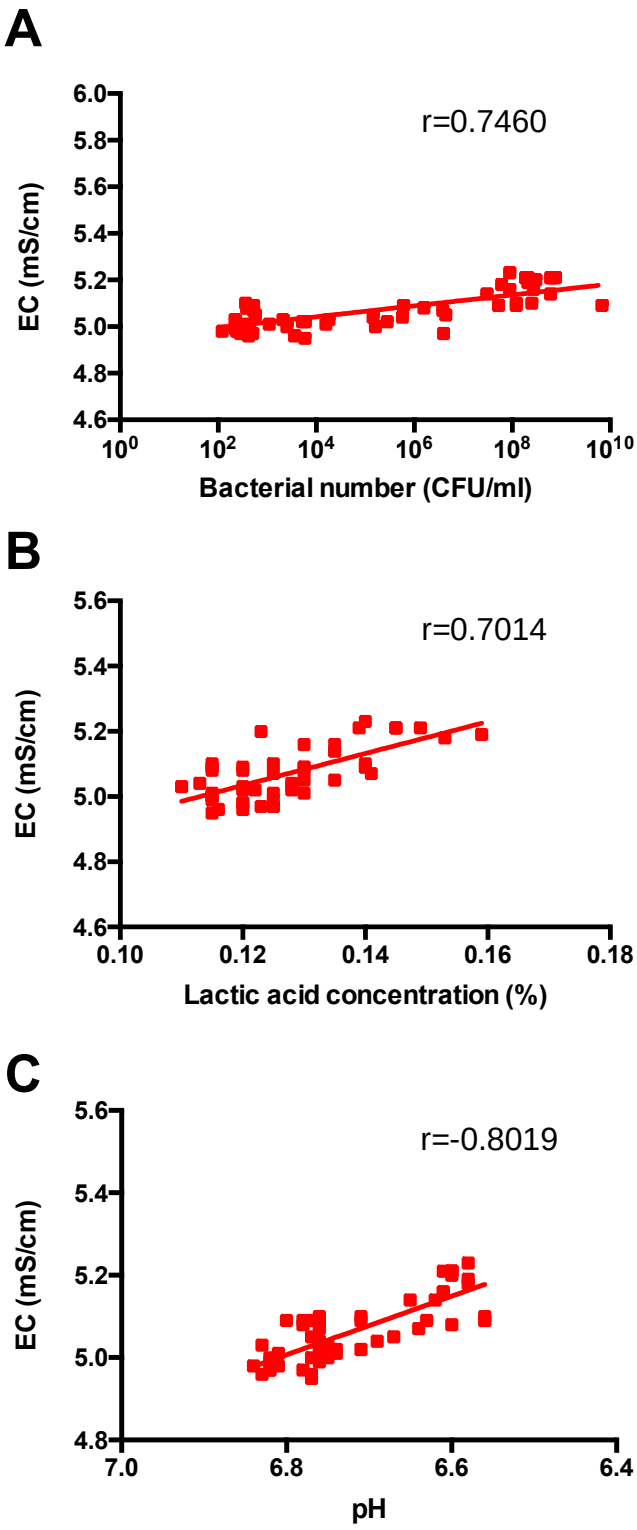


Figure 22. The interrelationship between the mean EC value with TBC **(A)**, LA concentration **(B)** and pH **(C)** in whole milk at 8°C. Each data point represents a single measurement. The values obtained from Figure 17. The straight line represents the estimated linear relationship between evaluated X and Y values.

4.2. Experiment 2

To determine which among the growth of bacteria, LA concentration and pH was the primary driving force for the changed EC during milk spoilage, two model systems were investigated using UHT long life skim and whole milk. The milk was added with either concentrated LA or HCl solutions to reduce the acidity. EC was measured in a temperature-controlled environment at either 4 or 20℃ (**Figures 23 and 24**). Statistical analysis is presented in Appendix 2.

4.2.1. The effect of LA on EC

LA free UHT skim and whole milk had initial mean EC values of 5.42 and 5.15 mS/cm at 4℃ (**Figure 23A**), and 5.25 and 4.98 mS/cm at 20℃, repsectively (**Figure 23C**). A 0.2 mS/cm difference in the initial mean EC values was observed among milk samples due to different storage temperatures. EC proportionally increased as the concentration of LA increased in both milk types. Linear regression analysis suggested a positive linear relationship between LA concentration and EC with R-square of 0.9324 and 0.9550 at 4℃, and 0.9895 and 0.9713 at 20℃ for skim and whole milk, respectively (Appendix 2, Tables 20 and 21). In addition, as shown in **Figures 23B and D,** the pH value correspondingly decreased as the LA concentration increased in both milk types under both temperature conditions.

The equation of linear regression between EC and LA concentration was computed as following formula: $EC_{skim} = 0.9192 \times LA_{skim} + 5.314$ and $EC_{whole} = 1.145 \times LA_{whole} + 5.037$ at 4 ℃ ; $EC_{skim} = 1.386 \times LA_{skim} + 5.146$ and $EC_{whole} = 1.314 \times LA_{whole} + 4.946$ at 20 ℃ (Appendix 2). In experiment 1, it was observed that a total increase of 0.05% and 0.06% LA was produced in the 30-day incubation experiment for skim and whole milk stored at 4℃, respectively (**Figure 16**). This correlates with a net increase in mean EC value by 0.2 and 0.3 mS/cm in skim and whole milk, respectively. In the model system, according to the formula listed above, an equal amount of increase in LA only caused a net increase in the EC value by 0.045 and 0.08 mS/cm in skim and whole milk, respectively. By comparing the results obtained from the experiment 1 with the model system, the total change of EC attributed to LA was estimated approximately one-quarter of the total portion of changed EC in milk.

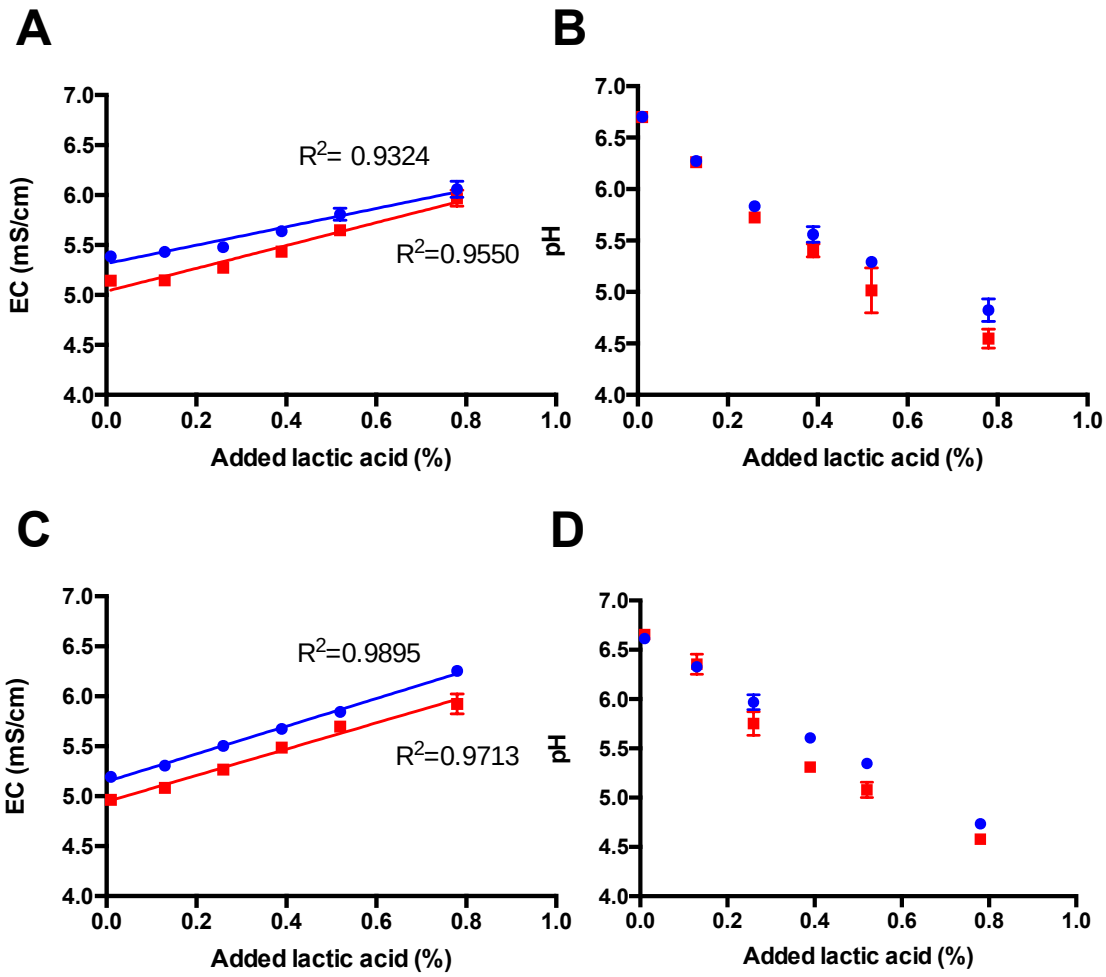


Figure 23. The effect of LA on EC in skim (● -blue) and whole (◙ -red) milk held at 4℃ **(A)** and 20℃ **(C)**. The corresponding pH shows in **(B)** and **(D)**. Each data point is the mean value of triplicate readings (n=3). Vertical error bars indicate the standard deviation of mean of each data point. The linear trend represents the linear regression with analysis based on three repeat experiments.

4.2.2. The effect of pH on EC

The effect of pH, which is independently changed from the effect of LA, was investigated on EC in skim and whole milk. The pH of milk was progressively reduced by adding 1M HCl (**Figure 24**). EC values of skim and whole milk with neutral pH were 5.46 and 5.62 mS/cm at 4℃ (**Figure 24A**), and 5.19 and 5.44 mS/cm at 20℃, respectively (**Figure 24B**). EC values were a function of acidity, as the average EC value was proportionally increased as acidity increased (i.e. pH value decreased) in milk held at both temperatures. Linear regression established a positive correlation between EC and acidity with R-square of 0.9632 and 0.9580 at 4℃, and 0.9605 and 0.9650 at 20℃, for skim and whole milk, respectively (Appendix 2, Tables 22 and 23).

The equation of linear regression between EC and pH was computed as following formula: $EC_{skim} = -1.587 \times pH_{skim} + 15.69$ and $EC_{whole} = -1.390 \times pH_{whole} + 14.25$ at 4℃; $EC_{skim} = -1.517 \times pH_{skim} + 15.11$ and $EC_{whole} = -1.261 \times pH_{whole} + 13.33$ at 20℃ (Appendix 2). In experiment 1, it was observed that 0.3 unit of decreases in pH correlated to an increase in EC by 0.3 mS/cm in both milk types (**Figure 16**). Whereas, according to the linear correlation established in the model system, a similar decrease in pH increased the mean EC value by 0.47 and 0.4 mS/cm for skim and whole milk, respectively. The model system showed same amount of decrease in pH resulted in a greater increase in EC value than in experiment 1. The results suggest that pH plays a role in EC change in milk.

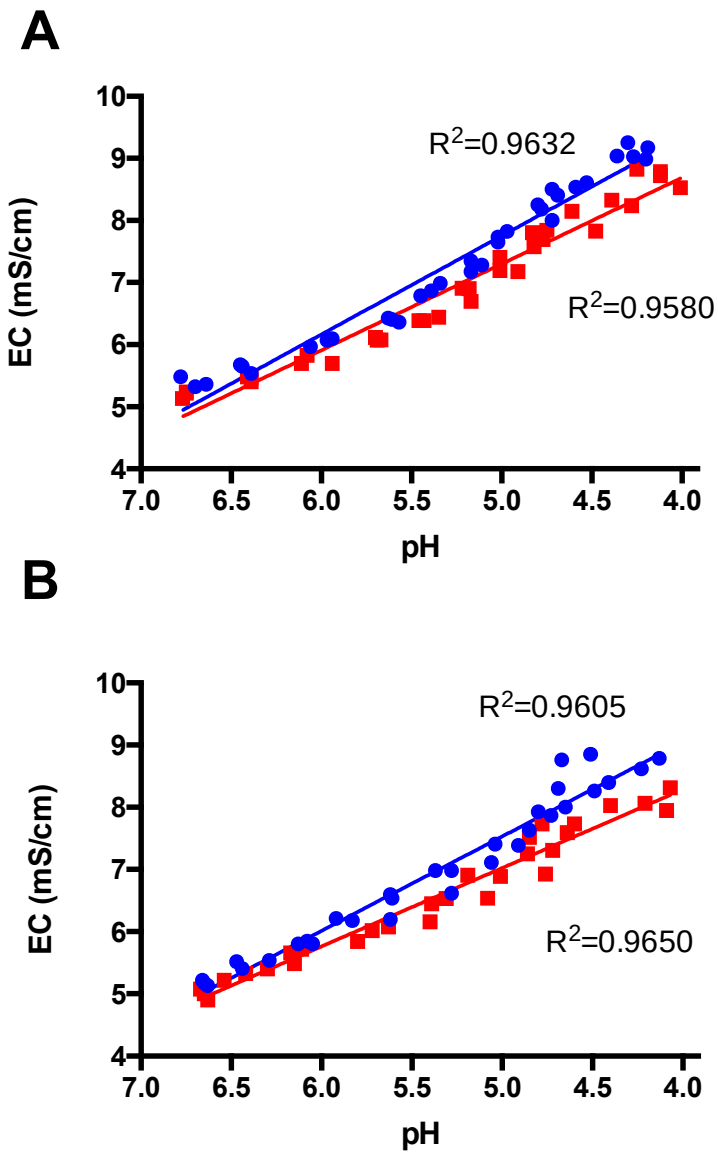


Figure 24. The effects of pH on EC in skim (☻ -blue) and whole (◙ -red) milk held at 4℃ **(A)** and 20℃ **(B)**. Each data point is a value of one reading. The linear trend represents the linear regression with analysis based on three measurements.

4.3. Experiment 3

To determine the impact of the growth of bacteria on EC in milk, an increasing number of bacteria were added to skim and whole milk. EC was measured either immediately (bacteria only condition) or at varying time intervals (bacterial growth and metabolism) after adding the bacteria. Statistical analysis is presented in Appendix 3.

4.3.1. The effect of presence of bacterial particles on EC

EC values were measured immediately after the inoculum of bacteria into milk without growth of bacteria or after the occurrence of bacterial metabolism. The average EC values of skim and whole milk samples with 7.2 x 10^4 CFU/ml were 5.36 and 5.06 mS/cm at 4℃ (**Figure 25A**), and 5.31 and 5.02 mS/cm at 20℃, repsectively (**Figure 25B**). The average EC value of all milk samples remained unchanged for the bacterial number less than 7.2 x 10^7 CFU/ml. A statistically significant decrease ($p<0.05$) in EC was observed when bacterial number exceeded 7.2 x 10^7 CFU/ml for both milk types and temperature conditions (Appendix 3, Tables 25 and 27). The average EC values of skim and whole milk with 7.2 x 10^7 CFU/ml were reduced to 5.04 and 4.98 at 4℃, and 4.8 and 4.82 mS/cm at 20℃, respectively. The results suggested that the presence of bacteria reduced the net EC of milk, and the number of bacteria present in the milk required to cause a change in EC was approximately 7.2 $\times$ 10^7 CFU/ml of cells.

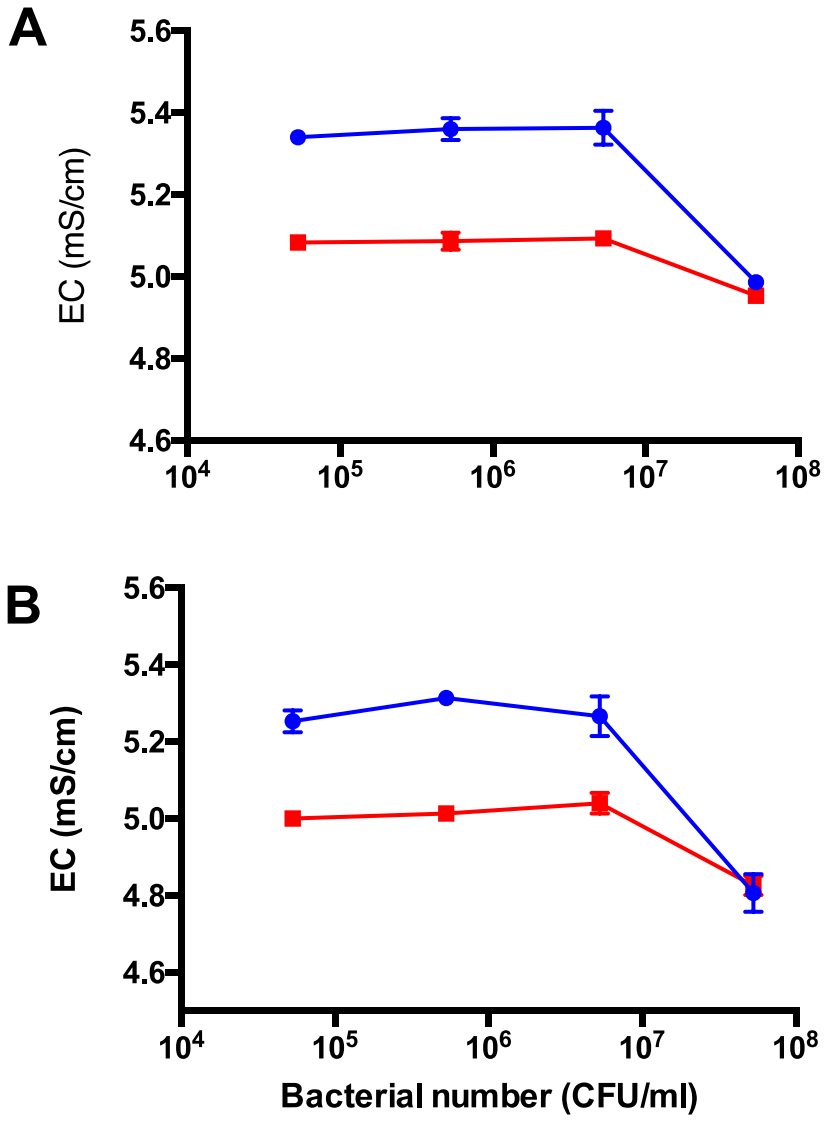


Figure 25. The effect of the presence of bacteria on EC in skim (●-blue) and whole (◙-red) milk held 4℃ **(A)** and 20℃ **(B)**. Each data point represents three measurements (n=3). The vertical error bar represents the standard deviation of mean of three measurements.

4.3.2. The effect of bacterial metabolism on EC

To investigate the effects of bacterial metabolism on EC, an experiment was designed in which an increasing number of bacteria was inoculated into milk and permitted to growth for 10 hours at 37℃ with EC being measured at two-hour intervals. The result is presented in **Figure 26**.

The values of EC in milk with and without bacterial inoculums decreased over the course of the experiment. Skim milk had an average EC value of 5.41 mS/cm, while the whole milk had an average EC value of 5.12 mS/cm. All bacterial inoculums with difference initial cell numbers showed a statistically significant decrease ($p<0.05$) in EC in all milk samples over the incubation period (Appendix 3, Tables 29 and 31). However, the milk with different initial inoculums showed no statistical difference with each other over the incubation period. This result suggested that over the 10-hour incubation period, the bacterial metabolism with different initial inoculums have a limited impact in EC.

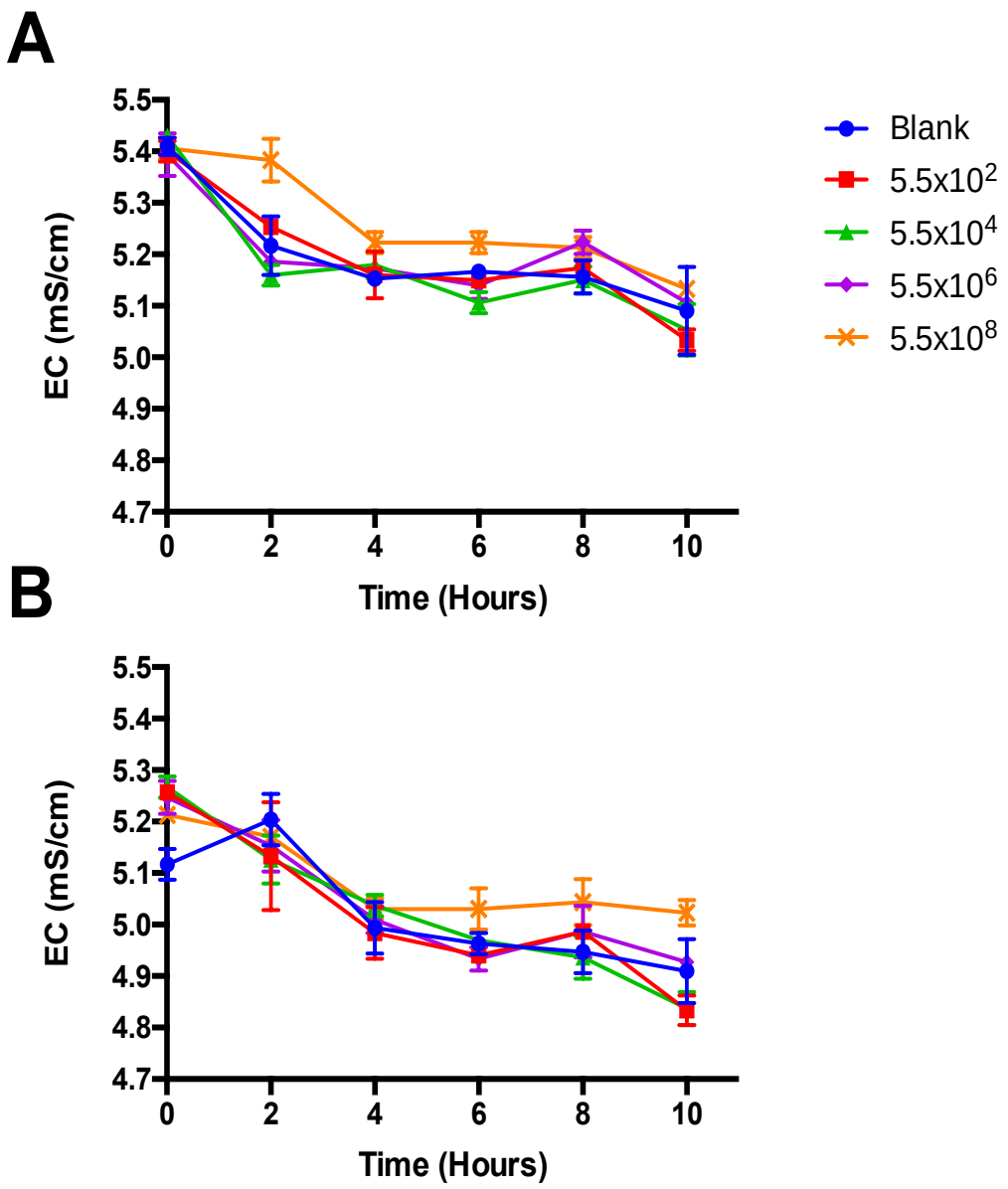


Figure 26. The effects of bacterial metabolism on EC change in skim **(A)** and whole milk **(B)**. Milk was initially inoculated with a number of bacteria of $\mathbf{10^0}$ (**blank**), $\mathbf{10^2}$, $\mathbf{10^4}$, $\mathbf{10^6}$ **and** $\mathbf{10^8}$ CFU/ml, respectively. Each data point represents the mean of three measurements (n=3). The vertical error bar represents the standard deviation of the mean of each data point.

4.4. Experiment 4: the effects of temperature on EC in milk

The influence of temperature on EC was determined for UHT skim milk, UHT whole milk, chilled pasteurized skim milk and chilled pasteurized whole milk, respectively. The average EC values were 5.71 mS/cm for UHT skim milk and 5.88 mS/cm for chilled pasteurized skim milk; the average EC values of whole milk were 5.51 and 5.59 mS/cm for UHT and chilled pasteurized milk at 0℃, respectively (**Figure 27**). The average EC values decreased steadily with increasing temperature from 0 to 20℃, this is followed by a plateau between the range from 20 to 50℃ for all tested milk samples. Statistical analysis of the data showed that the incubation temperature significantly influenced the EC of all milk samples ($p<0.05$) (Appendix 4). The results suggested that mean EC values influenced by temperature change in milk. EC decreased as temperature increase in range between 0-20℃, and remained steady in range between 20-50℃

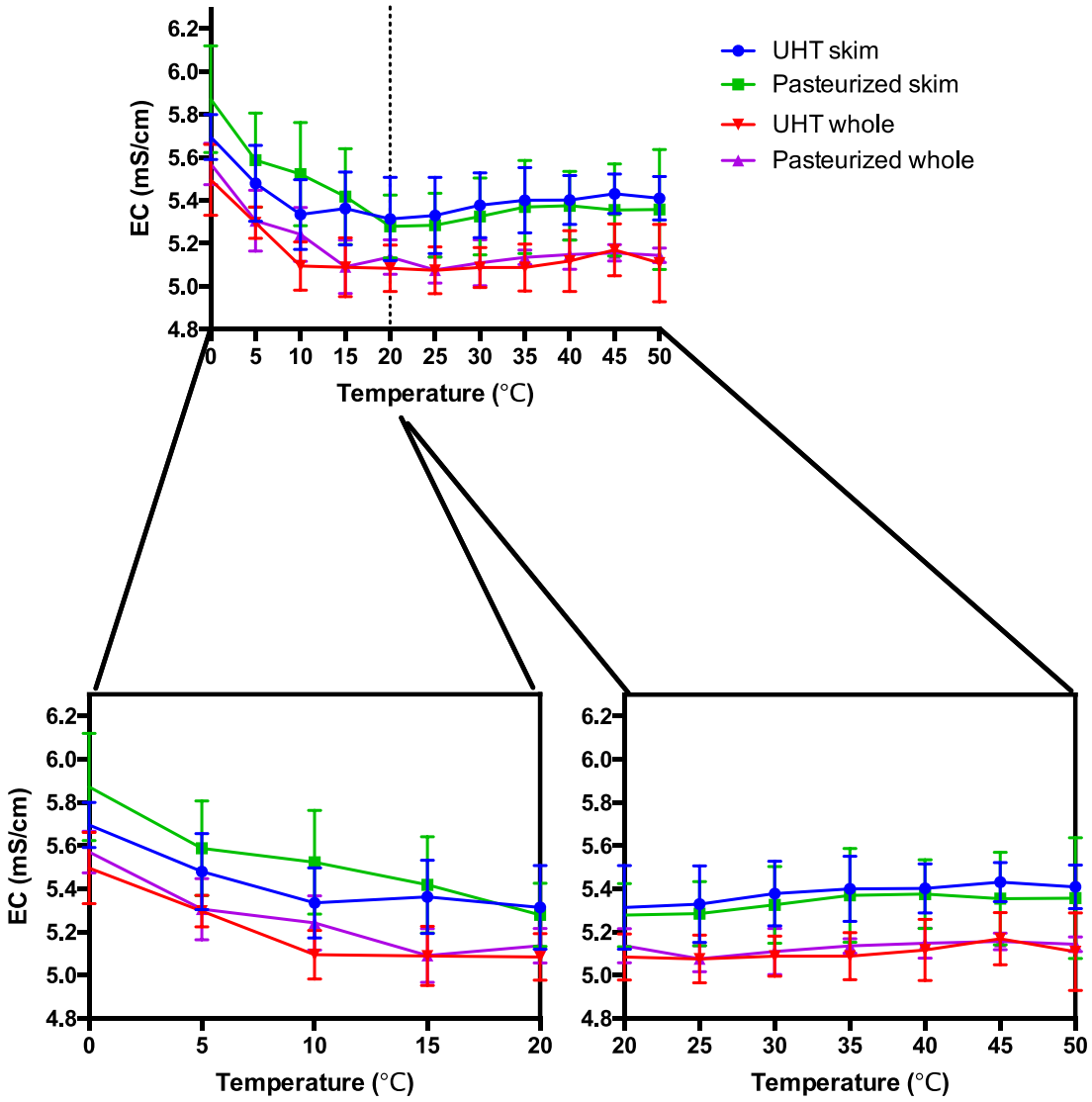


Figure 27. The effects of incubation temperature on EC in UHT skim milk, UHT whole milk, chilled pasteurized skim milk, and chilled pasteurized whole milk. Each data point represents the mean of three measurements (n=3). The vertical error bar represents the standard deviation of mean for each data point. The vertical dash line represents the segregation point of two temperature ranges.

Chapter 5 Discussion

The purpose of this study was to investigate whether there was a correlation between EC and milk spoilage and whether EC could be used by a biosensor as a potential indicator for milk spoilage. To achieve this aim, the study was conducted to measure and evaluate the variations in EC, TBC, LA concentration and pH of milk over an extended storage. In experiment 1, the value of EC, TBC, LA concentration and pH were simultaneously measured in milk held at either 4 or 8℃ (**Figures 16 and 17**). The interrelationship between EC with each of TBC, LA concentration and pH was analysed (**Figures 19-22**). In addition, to further determine which parameters are predominately contributing to the changes in EC, three independent model systems were consequently investigated. In experiment 2, concentrated LA and HCl were artificially added to milk to examine the impact of acidity on EC (**Figures 23 and 24**). In experiment 3, increasing numbers of PA2576 were added to milk to examine the impact of the presence of bacteria on EC (**Figures 25 and 26**). By comparing model systems with time trial experiments, the interrelationships between EC and measured parameters in milk spoilage can be assessed. Lastly, experiment 4 evaluated the dependent effects of temperature on change in EC in the used milk types (**Figure 27**).

5.1. Objective 1: EC changes during milk spoilage

Experiment 1 showed that the EC of milk gradually changes in both skim and whole milk during spoilage at either 4 or 8℃. The value of EC increased with increase in the growth of bacteria, LA concentration and acidity in milk. Under the optimal storing temperature of 4℃, a statistically significant increase in EC was found at the last sampling day, at where the complete spoilage of milk was physically sensed as the occurrence of flavour defects or curdling. Non-significant changes in EC were observed before the BBD within the legitimate shelf life in skim or whole milk (**Figure 16**).

During milk production, same milk containers may contain a higher total number of bacteria than others due to unpredictable and uncontrolled milk filling and packaging environments. A strategy was used in milk preparation to minimise the effects of internal variations among milk samples that caused by the uneven growth of bacteria. The equivalent amount of milk was divided into three sub-bottles. This step assists the even distribution of the net bacteria numbers in each sub-bottle in milk preparation. However, the number of bacteria remains

varied from sample to sample, in particular, the milk stored at the temperature of 4℃ showed significant differences between samples. The discrepancy in bacterial growth pattern was observed from the starting point of the exponential growth phase in both milk types (**Figure 16**). As a consequence, when bacteria grow differently, the gradually increased diversity in the range of LA and acidity was observed over the incubation time. A previous study reproted that the amount of variations in total viable bacterial count is relatively large from sample to samples during the growth within shelf life of milk (Tajammal et al., 2015). In addition, another study showed that increasing the incubation time of storage will promote large changes in differences in the number of bacteria, particularly psychrotrophs, and cause an unpredictable change in the growth profile and density during proliferation in milk (Böhmer and Hildebrandt, 1998).

When milk was held at a temperature of 8℃, both skim and whole milk showed significant changes in EC, along with increases in TBC, LA concentration and pH two days before the last sampling day, when off-flavour and/or change in texture of milks were physical detected (**Figure 17**). The last sampling day was two days after the BBD for skim milk and one day earlier the BBD for whole milk. It is noted that the BBD is no longer relevant when the milk is stored at an inappropriate temperature, because the prediction of shelf life by BBD is used for milk stored under optimal temperature (i.e. 4℃ or lower). Compared to the milk that stored at 4℃, this milk showed relatively high consistency and less variation in fluctuations of all measured parameters among milk bottles.

In a similar study, Kaptan (2012) examined the relationship between EC, TBC, LA concentration and pH in fresh milk samples held at an ambient room temperature of 25℃. They reported that the EC correlates significantly with the storage time during an 8-hour incubation period. In addition, EC strongly correlates to the presence of LA and increasing acidity in milk, and suggested the use of EC as a potential criterion for milk quality assessment (Kaptan, 2012). Similarly, a study by Borch and Wallentin (1993) used the measurement of conductance as mean to generate data and establish predictive modelling on the growth of *Yersinia enterocolotica* in minced pork. The proposed polynomial model describes the effects of temperature, pH and L-lactate level as parameters in conductance response curve that is able to predict the growth rate of *Y. enterocolitica*. Overall, studies suggested that the change in EC is associated with the growth of bacteria, LA concentration and acidity. Predictive models were established for individual bacterial strain to predict the growth pattern, which in line with the results of the current study.

5.2. Objective 2: the interrelationship between EC, TBC, LA concentration and pH

5.2.1. EC has a strong correlation with LA concentration and pH

The interrelationship between EC, TBC, LA concentration and pH was evaluated from the data obtained from the time trial experiment (experiment 1). The EC value was plotted against spoilage-dependent variables of TBC, LA concentration and pH. The change in EC was found to associate with the sum effect of spoilage-dependent variables (**Figures 19-22**). Each of the variable parameter dynamically increased with an increased EC for both skim and whole milk. In addition, the change in EC was found to significantly correlate with the presence of LA and acidity in milk. The EC value had moderate to strong correlation with the concentration of LA and pH in milk, regardless of the milk types and storage temperatures (**Figures 19-22, B and C**).

To further determine which factors were driving the changes in EC, milks added with progressively increased quantity of LA and HCl solution were carried out in model systems at either 4 or 20℃ (experiment 2). The EC value was found to have a strong positive linear correlation with an increase in LA and a decrease in pH in model systems for skim and whole milk at both temperatures. Linear regression analysis revealed that LA concentration and pH are functions of EC (**Figures 23 and 24**). In addition, according to the linear equations revealed in model system, LA concentration was identified to contribute an estimation of one-quarter of total increase in EC in milk trials. Căpriță et al. (2014) conducted a similar study that investigate the changes in pH, LA percentage and EC in relation to storage temperate and storing period in raw and pasteurized milks. They found that the LA concentration increased by 0.15% and pH decreased by 1 unit after a two-day incubation at 20℃. The study suggested a strong correlation between EC and LA concentration and pH (Căpriță, Căpriță and Crețescu, 2014). The overall effects of EC owning to the change in LA percentage and pH were found in line with the results obtained from the current study.

The contribution of pH to EC change was unable to identify because the equal amount of decrease in pH triggered a greater increase in EC in the model system than it did in the trial experiment **(Figure 24)**. This leads to an assumption that the change in EC might also be caused by the presence of other factors rather than pH change. The addition of HCl to milk did not only increase the H+ ion concentration, but at the same time, also increase the concentration of ionised Cl- in the milk that may have contributed to the rise in EC. A study by

Diaz et al. (2011) reported that the salt content contributes partial portion of the variance in EC (with R^2=0.91), in particular by the Cl content that originate from the milk serum. A later study by the same investigators further reported that the increase in EC was determined by both Cl content and the ratio of Na and K content that presented in both bovine and goat's milk (Diaz, Romero, Muelas, Alejandro and Peris, 2012). Studies supported the inferences that the addition of Cl ions might played a partial role in the changed EC and explained the more than expected increase in EC that resulted in milks in model systems.

5.2.2. EC decrease by the presence of bacteria particles but increase by bacterial growth

In time trial experiment, EC increased with an increase in TBC in milk during spoilage (**Figures 16 and 17**). By re-plotting the values of EC against the values of TBC, it revealed a moderate to strong correlation between EC and the growth of bacteria (**Figures 19-22**). To further investigate the effects of bacteria on change of EC, milks were added with an increasing number of bacteria (i.e. PA2576), and the EC values were assessed with and without the occurrence of bacterial metabolism in two model systems, respectively (experiment 3, **Figures 25 and 26**). It was found that, without bacterial metabolism, the threshold of number of bacteria to trigger a significant decrease in EC value was 7.6 x 10^7 CFU/ml of cells. The EC value maintained unchanged when the TBC did not exceed this threshold. Moreover, with bacterial metabolism, the experiment reported that the milks, which inoculated with different amount of initial bacterial cells, had the same level of decrease in EC in skim and whole milk, and the un-inoculated milk control exhibited same trending as the inoculated ones. The results showed no significant difference in the impacts of bacterial metabolism on EC in milk. Overall, the presence of bacteria decreased the mean EC values in milk. However, the bacterial growth and metabolism resulted in limited change in EC in model systems.

It has been reported that the optimal growth rate of *Pseudomonas aeruginosa* to reach a high population density without competing for nutritional sources with other bacterial floras in bottle water was doubling at every 3.6 hours at 37℃. However, the fastest doubling time significantly prolonged to 26 hours under the presence of competing bacterial flora (Tamagnini and González, 1997). In addition, the specific growth rate of a related bacterial strain, *Pseudomonas fluorescens,* in sterile milk that incubated at 29℃ was 2.8 ± 0.3 hours without competing bacterial floras (Lin, Havezipur, Yousef and Maleky, 2016). In experiment 3, the total incubation time that allowed bacteria to growth only lasted for ten hours, which did not, provided sufficient time for bacterial growth in a competitive environment. Competition with

the growth of other existing bacteria in milk during incubation might potentially increase the lag phase and delay the exponential growth of bacteria in milk. Hence, the variety bacterial metabolites that produced during incubation of milk, which may cause variations to EC values did not adequately studied in this experiments, therefore, insufficient experimental data of growth and metabolism of bacteria was observed in the experiment. Thus, the effects of bacterial metabolism on EC were unable to show in this study.

The inspection of the use of electrical conductance as an aid in monitoring bacterial growth and metabolism has frequently been studied. However, the impedance of electrical conductance by the presence of bacterial particles has less attracted for research (Lanzanova et al., 1993; Mabrook and Petty, 2002; Visser and de Groote, 1984). One study by Green and Larson (1922) demonstrated that the medium solution containing bacterial cells seem have a higher electrical resistance than the same solution without cells. Higher electrical resistances in solution impede the flow of electrical current because electrical resistance is the reciprocal of electrical conductance. In addition, the presences of bacteria spatially occupy the total volume of solution and increase the viscosity of solution to an extent that impede the movement of ionic species. Consequently, the presences of bacteria decrease the EC of milk (Green and Larson, 1922). Another study has also demonstrated that, a simple culture solution in which bacteria are living and dying is a heterogeneous system. The compositions and constituents of the solution are consistently changing during bacterial proliferation. The initial and final composition of the solution is known, but the intermediate reactions and metabolites that produced by the presence and growth of bacteria in many cases remain incomprehensible. As a result, their impact on EC is difficult to define (Allison et al., 1938). Several mechanisms having potential of changing milk conductivity are discussed in the next section.

5.3. Potential factors change milk conductivity

EC is frequently proposed as a biophysical parameter to be used for quality assessment of milk and milk products (Ostan, Gogoaşă, Rada, Baul, Fericean, Cazacu, Petcu and Creţescu, 2015). The greatest contributors to EC are consistently associated with TDS, primarily the salt content of milk (Henningsson, Ostergren and Dejmek, 2005). The balance of salt content is maintained at a stable equilibrium, unless the incidence of deterioration or contamination interrupts the equilibria in milk. Several possible mechanisms explain the change in salt content and composition in milk during spoilage are discussed below:

5.3.1. Salt ions

Changes in EC due to unbalanced equilibria of salt content in the occurrence of milk spoilage have been reported by many studies (McSweeney and Fox, 2008; Diaz et al., 2011; Borch and Wallentin, 1993; Mabrook and Petty, 2003b). The change in EC is influenced by the equilibrium state of milk compositions. The milk compositions encompass charged proteins, dissolved Ca, P and Mg from casein micelles, and readily dissolved K, Na, Cl, hydrogen ions and citrate. In addition, minor contributions of EC change are responsible by trace elements of iron, zinc, carbonate and sulphate that presented in the milk (Bazinet, Pouliot and Castaigne, 2010). Other major molecular components such as proteins, lactose and fat influence the EC through their effects on viscosity or by the action of microbial metabolism (Henningsson et al., 2005).

Salts are partially dissolved in milk serum as ions, while others are in existence in a temperature and pH dependent equilibria that might associated with casein micelles or other milk proteins (Henningsson et al., 2005). The transition equilibrium between the dissolved and non-dissolved states of major salt species such as K, Na, Ca, Mg and Cl is dynamically maintained in the biological atmosphere in milk during storage. Not all of the dissolved salt can be fully ionised at the pH of milk and contributed to changes in EC. Partial of the ionised salts associated with organic acids and bases such as phosphoric acid, carbonic acid, and secondary amines in the milk serum, which from substances that do not carry electrical currents (Walstra and Jenness, 1984; Fox and McSweeney, 1998). Therefore, their absolute contribution to the EC is difficult to ascertain. A study by Mucchetti et al. (1994) reported that commercial skim milk has a nearly identical EC value as ultrafiltration (UF) permeate milk. UF permeate milk is a derivative of milk that only contains salts, lactose, soluble nitrogen and vitamins (Mucchetti et al., 1994). The EC of raw milk has also measured and compared with pure solutions of salt components present in milk in several studies. Studies reported a similarity in values of EC between raw milk and the combination of solution of sodium chloride (NaCl), KCl, calcium chloride ($CaCl_2$) and dipotassium phosphate (K_2HPO_4) (Mucchetti et al., 1994). However, a complete understanding and clear contribution of individual salt content in milk to EC change has yet to be fully unveiled (Lanzanova et al., 1993; Henningsson et al., 2005). Owing to the complex nature of milk and mix effects of heating, cooling and pH changing during storage and spoilage, these factors influence the equilibria of salt content. When sum of the effects become dominant in milk, the change of EC in milk is difficult to predict (Henningsson et al., 2005).

5.3.2. Microbial growth and metabolism

Previous studies suggested that the increase in EC value of milk can cause by the growth of various microorganisms and their associated metabolic activities (Noble, 1999; Felice et al., 1999; Richards et al., 1978; Gómez, Bashir and Bhunia, 2002). The growth of bacteria causes successive consuming of lactose, lipid and protein during milk spoilage. This bacterial growth results in the transformation of milk constituents to catabolic end-products that might be electrical conductive (Sims, Hull and Chandler, 1991). Lactose as the primary carbon energy source is used for growth of most of the spoilage bacteria in milk. Fermentation of lactose to lactic acid is one of the key metabolic pathways for spoilage bacteria. During a fermentation at a relatively higher temperature, the production of LA concentration could reach 1-2% (Walstra and Jenness, 1984; Early, 1998). An increase in LA concentration in milk will certainly trigger a dramatic increase in EC, as seen that similar to experiment 1 of this study. In New Zealand, the genus of bacteria, *Pseudomonas* and *Bacillus,* are the most frequently found spoilage bacterial strains in milk and milk products (Richardson 1981; Chen, Daniel and Coolbear, 2003). In addition to producing LA, other key roles played by these bacteria in the process of milk deterioration are the production of lipases and proteases (Stead, 1986; Roberts and Skinner, 1983). Study has shown that *Pseudomonas fluorescens* can produce and accumulate more lipase in shaken skim milk than it did in nutrient broth under the same storage conditions (Richardson, 1981). Studies have also reported that when psychrotrophs had grown to $10^6 - 10^7$ CFU/ml, they can produce highly concentrated level of lipases and proteases at the late exponential phase of bacterial growth in refrigerated raw milk. When this quantity of bacteria was reached, it led to flavour defects and curdling in milk (Chen et al., 2003; Stead, 1986). The occurrence of a critical biochemical reaction at this time point is the degradation of casein micelles and fat globules by the enzymatic action of proteases and lipases. The degradation of protein and lipid led to release of micelle-bound salt ions and free fatty acids into the milk serum (Sørhaug and Stepaniak, 1997). An increase in dissolved salt and charged free fatty acid in milk serum is likely to change the EC of milk.

5.3.3. Charged milk proteins

All proteins including milk proteins carry a pH dependent net charge. Their mobility in an electric field can be measured by electrophoresis (Henningsson et al., 2005). It has been previously proposed that the milk protein affects the net change in EC via three ways: by carrying charged side chain amino acids, associating with ionized counter ions, or by influencing the viscosity of milk (Henningsson et al., 2005). Casein as one of the most

important milk protein, it is structurally embedded in a micelle and surface decorate with a mixture of protein and counter ions to from casein micelles. Several salts such as Mg, citrate, Na and K are contained inside of the casein micelle (Kanekanian, 2005; Walstra, 2013). The structural stability of casein micelles is maintained by the chemical bond between casein and decorated cations, primarily Ca and Mg (Kanekanian, 2005). The stability of casein micelles is a critical parameter of assessing the quality and freshness of milk because temperature and pH changes of milk influence the association and dissociation of casein and counter ions in casein micelles. The changes in stability of casein micelles consequently affect the change of EC in milk through the influences of content of dissolving ions in milk serum. Study showed that bacterial strain of *Pseudomonas* growing to 10^7 CFU/ml can produce sufficient amount of proteinase to degrade caseins (Law, 1979). Another study also reported that casein degradation was detected even before the bacterial population had reached 10^4 CFU/ml in milk (Barach, Adams and Speck, 1976). The degradation of casein micelles lead to a release of electrical negatively charged sialic acid into milk serum (Law, 1979). The presences of both additional protein and amino acid that produced by the transformation of milk compositions by the action of microbial catabolism have potential to contribute to a change in EC of milk.

5.4. Temperature-dependent effect of EC

An experiment was conducted to investigate the temperature-dependent effect of EC in skim and whole milk. In this study, EC was found to decrease steeply as temperature increase in the range between 0-20°C. This is followed by a stable trend with a plateau in EC between temperature range of 20-50°C for all tested pasteurized and UHT milk samples (**Figure 27**). The observation of temperature-dependent effects on EC is conflicting with existing literature. Prentice (1972) studied the temperature coefficient of electrolytic conductivity of normal milk. Author reported that within the temperature interval between 15-40°C, the temperature coefficient decreased from 2.41% at 15°C to 1.73% at 40°C. The temperature dependence of EC below 15°C was not found to fit in the suggested index (Prentice, 1972). Henningsson et al. (2005) further illustrated that at the temperature range lower than 20°C, milk has a low consistency and a high variation in EC values, and the protein content is likely to play a role in interfering with the value of EC at the lower temperatures. Their results in line agreed with Prentice's results that showed a consistent temperature coefficient of EC within the temperature interval of 20-40°C. However, the temperature coefficient of EC was yet to be conclusively shown at the temperatures lower than 20°C (Henningsson et al., 2005). On the

other hand, the current study showed different temperature-dependent effects of EC to that proposed by Prentice in all tested temperature intervals between 0-50℃.

Initially, the disparity was thought to caused by human- or device-dependent errors, but multiple attempts at repetition continued to yield same results. One of the critical issues discovered during measurement is that the time intervals between each measurement were too brief. The measurements need to be implemented at every 5℃ as temperature rising from 0 to 50℃. The temperature increased rapidly in the lower temperature range of 0-20℃. As a result, occasionally, no rinsing and drying procedures could be applied to the electrodes in between of some measurements. A common problem with electronic probes that exposed to long length monitoring in natural biological solutions is to develop unreliability with continuous reading, if there is no proper cleaning management that have applied to the interface of electrodes (Christy, Eriksson, Feinberg, Hermens, Hobert, Hopke, Kvalheim, McDowall, Scott and Webster, 2013). The purpose of regular cleaning of electrodes is to avoid build-up of biological and chemical deposits (Christy et al., 2013). The discovery of the issues might be a possible reason why the results were obtained differently from existing ones. In addition, the non-conformity of results might due to the differences in milk types used in current study. However, a study investigating the components of Swedish dairy milk (the milk used in the study by Henningsson et al.) showed no significant differences in milk compositions to New Zealand's dairy milk (Lindmark-Månsson, Fondén and Pettersson, 2003). The reasons for the different temperature-dependent effect of EC in current study remain unclear, and further investigation is certainly needed to understand the phenomenon.

5.5. Presence of milk fat decrease EC

In this study, the pasteurized whole milk was found to have a lower average EC value than pasteurized skim milk by 0.20 and 0.38 mS/cm at 4 and 8℃, respectively (**Figures 16 and 17**). Prentice (1962) was the first investigator to report that the presence of fat reduces the EC by an index power of 1.5 proportional to the fat-free volume in milk. They also established a defined mathematical equation to illustrate that EC is a function of both the fat content and non-fat-solid content in milk and cream (Prentice, 1962). In addition, Lawton and Pethig (1993) further confirmed that the presence of fat reduced EC with a more accurate index power of 1.7 proportional to the fat-free fraction of milk. The same phenomenon was also observed in study that performed by Binnur and Serep (2016), and authors reported that the EC decreased as fat content increase in milk kefir. The average EC values of non-fat milk kefir were lower than that

of whole milk kefir by 0.23 mS/cm at 25℃ (Binnur and Serap, 2016). In line with another study was conducted by Mabrook and Petty (2003), who found that the presence of fat reduced the EC of milk by 0.30 mS (Mabrook and Petty, 2003a). All studies agree with the experimental results from the current study. A possible reason why the presence of fat reduces EC due to the fact that more than 97% of the total milk fats exist in the form of fat globules, which are covered by a thin layer of nonconductive membrane. These fat globules hinder the conductance of milk by occupying the volume of conducting medium and impede the mobility of conducting ions (Mabrook and Petty, 2003a).

5.6. Objective 3: the prospect of using EC aid in predicting milk spoilage

Many studies have employed EC as a tool to aid in the quality monitoring and detection of compositions and toxic chemical inspections in milk and milk products (Borch and Wallentin, 1993; Mabrook and Petty, 2003b; Milner et al., 1996; Singh et al., 2012). This is because EC provides good sensitivity and selectivity in majority of detections (Diaz et al., 2011). However, the use of EC as a tool to predict milk spoilage and bacterial contamination has received less interest by researchers. Further study is required to ascertain whether EC is an optimal indicator of predicting milk spoilage. Nevertheless, this study provides experimental evidence to show that EC is a feasible approach to use in prediction of shelf life of milk during storage. This is primarily because that an increase in EC was seen as incubation period of milk was increased, this change was coincided with an increase in other measured spoilage associating factors, which reflects that the change of EC have combined effect of not only the microbial growth and metabolism but also the evolving biochemical reaction of milk deterioration. This could provide an advantage over the BBD. Moreover, the cooling systems in domestic settings, in most cases, do not quite meet the standard requirements for optimal food storage. When milk is stored outside of the functional range of BBD, which is at 4℃ or below, the use of BBD is no longer appropriate. In contrast with BBD, EC offers a relatively precise and accurate solution for indicating the correct use time of milk according to storage temperatures, because EC estimates the appropriate shelf life adjusting to temperature change. The rate of change in EC varies according to the rate of growth and metabolism of bacteria at different storing temperatures. This solution can possibly overcome the limitation of using the inflexible BBD.

However, the use of EC as an indicator for milk spoilage detection remains challenging, primarily due to the change in EC in all experiments was relatively small, which could be potentially generated variably by a number of spoilage associating factors that cannot be differentiated by measuring EC alone, even though it provides some advantage over BBD. The other reason is due to that the milk EC value is vary by the distribution of milk constituents

and growth profile of spoilage bacteria in every individual milk bottle. Spoilage bacteria, particularly psychrotrophs, has unpredictable growth pattern during the extended storage. These factors certainly enhance difficulties in quantitative correlating of the number of bacteria with the change in EC of milk, and also further challenge the proceeding of using EC as indicators for milk spoilage predicting.

In conclusion, this study provides evidence to articulate that the measurement of EC could be a more precise and accurate mean than BBD for indicating the correct shelf life of milk. Obviously, the fabrication and development of EC-facilitated monitoring devices for milk spoilage require further consolidate studies and evidence to prove the usability. This study has gained insights into the concept of a quality on-package detection biosensor for milk spoilage. The idea of constructing and fabricating biosensors with inbuilt EC transducers to provide on-the-spot detection of milk quality is not far away from being achieved.

5.7. Summary

This project proposed a preliminary study that investigates whether there was a correlation between EC and milk spoilage and whether it could be used as an indicative parameter for milk spoilage detection, that could be potentially interfaced in a biosensor system. To achieve this aim, we conducted a series of experiments to prove the concept that EC is a valuable parameter that can reflect the level of spoilage of milk. The experimental results shown that the EC incrementally changed in both skim and whole milk during spoilage at either 4 or 8℃. A significant increase in EC was observed at the point where absolute spoilage of milk was physically detected. The change in EC coincided with changes in the associated factors of TBC, LA and pH. This study also disclosed that EC correlates with the measured spoilage associating factors with a variety of correlation indexes, which help to gain a better insight to the potential feasibility of indicative function of EC in milk spoilage detection. In addition, the study provided evidence to demonstrate that EC offers a temperature-specific advantage over BBD in indicating the correct shelf life of milk. EC provides additional useful information in conjugation with the use of BBD of milk held at an appropriate storage temperature of 4℃, while it also provides advantages over BBD in milk held at an inappropriate storage temperature at 8℃, at which condition the BBD is not valid. Therefore, this study provided a comprehensive preliminarily investigation on the correlation between EC and milk spoilage, and analysis of the relationship between EC and measured spoilage associating factors of TBC, LA and pH.

5.8. Limitations and challenges of this study

This study presented several limitations. Firstly, the variation in growth profile and accumulation of bacteria numbers in milk is heterogeneous. The differences in bacterial growth within and among individual bottles of milk were considered in advance of the study. Corresponding management was also applied in the milk preparation procedure by separating well-mixed milk into individual sub-bottles. This step is used to ensure an even distribution of initial bacterial numbers in each sub-bottle. Even so, a considerable difference in growth of bacteria remains occurred among samples.

Moreover, the currently used conductivity meter has an inadequate level of accuracy to precisely detect the change in EC in milk. The decision was made to use the current conductivity meter due to limited availability of equipment in laboratory. This meter was the most suitable equipment to meet the demands and serve the purpose of investigating a fast-response and miniaturized device for on-the-spot detection. The conductivity meter has an accuracy of $\pm 1\%$ over a range of measurement. The low accuracy causes substantial fluctuation in each measurement as seen in experiment 1. The percentage change in EC varies increasingly as the progress of spoilage, which is eventually limiting the accuracy of prediction in milk. The study suggests that it is essential to employ meter with improved measure precision that is able to sense small changes in EC in further work.

In addition, the capability of accurate reading depends on the placement of electrodes in the solution, and also depends on if the electrodes were held stable while reading values. In most conditions, it is required to stir the solution with electrodes to ensure homogenous distribution of temperature and composition of milk before reading values. It has been noticed that the inconsistent EC readings were picked up in the same solution that held at the same environments. Besides that, the electrodes require adequate rinsing with distilled water to remove biomolecules or chemical contaminates and then drying prior to each single use. When, occasionally, no rinsing and drying steps is permitted before some uses due to lack of sufficient time intervals available in rapid measurements, such as in experiment 4. The meter tends to give unstable and inconsistent readings. It is likely due to the deposition of milk protein, salt compositions and other charged elements in the interface of electrodes, which impede the flow of electrical current between electrodes.

When transferring milk between storage and sampling locations, the temperature was unable to consistently maintained due to the large quantity of samples and constraints on laboratory

construction. Maintaining milk samples at exact temperatures (i.e. 4 or 8℃) were impossible to achieve during the transportation and sampling. Furthermore, it was also observed that, although milk samples were stored at 4 and 8℃ during incubation, the actual temperature at the time of sampling was slightly higher than that it should be in incubators. The temperature dependent coefficient of EC is 0.0309/℃ at 5℃, as 1℃ increase in temperature will result in 0.0309 mS/cm increase in EC in normal cow milk (Sharma and Roy, 1976). The net change in EC was intrinsically minor during spoilage, and the temperature-dependent errors introduced by storing milk at improper temperature might cause an unexpected change in EC of milk. Thus, this limitation suggests improving the temperature management strategies in future by employing better cooling systems.

Chapter 6 Conclusion and further work

6.1. Thesis summary

EC has frequently been studied as a tool to aid in monitoring and inspection of quality of milk and milk products. Their uses are prevalent in the area of milk composition detection, toxic compounds analysis and udder health inspection of cows (Prentice, 1977; Norberg et al., 2004; Fernando, Rindsig and Spahr, 1982; Kaptan, 2012). However, fewer studies have examined the feasibility and suitability of EC as a device-incorporated function for milk spoilage detection. Herein, the aim of this study was to conduct a preliminary study that investigate the possible use of EC as a potential indicative parameter to aid in the detection of milk spoilage that could be integrated in a biosensor. The aim is claimed through several objectives:

1. To examine the change in EC during milk spoilage.
2. To investigate the interrelationship between EC and milk spoilage, and to determine if the parameters of TBC, LA concentration and pH are main driving forces responsible for the changed EC.
3. To justify and discuss the use of EC as a feasible parameter compared to BBD in milk spoilage prediction.

To answer these questions, both time trial experiments and laboratory-controlled model systems were conducted using commercial pasteurized and UHT skim and whole milks. The measurements of EC, TBC, LA concentration and pH of milk were statistically analysed and then compared between experiments. Several conclusions are drawn from this study:

1. **The mean EC values gradually increase during the extended storage of skim and whole milk at 4 and 8℃.** At 4℃, bottles of both skim and whole milk showed a statistically significant increase in EC at the last sampling day, where the occurrence of off-flavour or curdling was detected. Along with an increase in TBC, LA concentration and decrease in pH were observed during the extended storage. At 8℃, all tested skim and whole milk showed a statistically significant increase in EC two days before the last sampling day. Corresponding increases in TBC, LA concentration and pH were also observed in skim and whole milks.

2. **The net change in EC is influenced by TBC, LA concentration and pH in spoilt milk.** The time trial experiment provided evidence to show that EC increases as TBC, LA concentration and acidity increase, regardless of milk types and storing temperatures. Several moderate to strong relationships between EC and TBC, LA concentration and pH were identified in skim and whole milk.

3. **EC is a function of LA concentration and acidity.** A positive linear correlation was identified between EC and LA concentration and acidity in both milk types in model systems. As increasing the production of LA and acidity lead to a proportional increase in EC values.

4. **EC increases by the growth of bacteria but decreases by the presence of bacteria.** The time trial experiment reported that the growth of bacteria leads to an increase in mean EC value over the extended storage in both skim and whole milks. The model systems reported that with the presence of bacteria numbers exceeds 7.6×10^7 CFU/ml of cells, it decreases the mean EC value of milk.

5. **Whole milk has lower EC value than skim milk.** The differences in mean EC value between skim and whole milk were observed over the extended storage at both 4 and 8℃. The differences in mean value of EC between milk types were suggested by other studies owing to the presence of milk fat content.

6. **EC offers a more accurate mean than BBD in predicting the shelf life of milk at either 4 or 8℃.** The time trial experiment showed that a statistically significant change in EC occurred before flavour defects or curdling of milks. However, no statistical significant change in EC, TBC, LA concentration or acidity was observed at BBD. The change in EC is accordingly adapted to differ storing temperatures as it reflects the progress of growth and metabolism of bacteria and evolution of deterioration of milk. Hence, EC offers a better solution for shelf life prediction than BBD.

6.2. Recommendations for further work

This study examined the use of EC as a feasible alternative for milk spoilage prediction. Further experimental improvements are required to fully understand the principle. The experiments can be further improved in a number of ways:

6.2.1. Improve the limitations and issues in current study

1. The incubation temperature in model systems was set at either 4 or 20℃. All of the temperature controls that conducted in the experiments should have been performed in 4 or 8℃ rather than 4 or 20℃ in order to compare with corresponding results from time trial experiments. In further work, the model systems should be repeated at a temperature of 8℃.

2. The model systems that were designed to assess the effects of acidity on EC should be improved by adding alternative acids to obtain sufficient data. Artificially increasing the acidity of milk using HCl solution not only increase the acidity but also increase the concentration of Cl- ions in milk. The dissolving of Cl- ions in milk was evidenced to have strong contribution to EC change. Thus, the contribution of acidity to EC change is masked by the presence of Cl- ions in model systems. Any future experiment should be tailored by adding an alternative strong acid that contains anions with a minor contribution to change of EC. The absolute contribution of changed EC is attributed only from the added H+ ions, which will possible to determine the impacts of acidity on EC. In addition, it would be useful to conduct a control experiment in which varying amount of acid solution were added to an equivalent volume of saline solution, which can provide comparative information about the buffering capacity of milk in relation to any development of acidity in spoilt milk.

3. The model systems that used to investigate the effects of bacterial metabolism on EC should be repeated with longer incubation time. Ten-hour incubation did not permit sufficient time for growth of bacteria. As the bacteria of PA2576 has a relative longer replication time at 3.6 hours without competition, and 26 hours with competing bacterial flora in a growth medium. The further experiment should be tailored to permit extended incubation period for bacterial growth. In addition, the kinds of inoculated bacteria should

not be limited to PA2576. Further improvements can be applied by employing multiple spoilage bacterial strains from milk. Bacterial strains including the most commonly found type of *Pseudomonas* and *Bacillus*, or the direct milk-isolated strains should be tested to examine their effects on EC change in milk.

4. Several attempts were made to identify the problematic issues associated with the temperature-dependent effects of EC in this study. Whether human, material, resource or equipment-dependent errors that had caused the problematic results to differ from existing literature were unidentified. In the future, the temperature-dependent effects of EC should be further determined using more advanced electrical conductance monitoring equipment, such as Malthus impedance analyser or Bactometer to check this problem and revalidated the findings.

6.2.2. Expand milk sample size and types

Milk has a different initial number of containing bacteria among milk bottles. The differences in the growth pattern of spoilage bacteria lead to produce variable metabolic products and alter EC value differently. This fact exacerbates difficulties in establishing a general predictive model that associates the change in EC with TBC in milk within a small sampling size. One of the approaches to increase the accuracy and precision of the correlation between milk EC and spoilage is to expand the experimental sample size by employing increased number and variety of milk. A further comprehensive comparative evaluation of more milk types including goat's and buffalo milk could be subjected. The predictive model that developed based on a larger sampling size of milk will effectively help to reduce variations in growth rate of spoilage bacteria and thus variations of milk samples, which will provide more confident results to predict accurate progression of spoilage in milk.

6.2.3. Improve the sensitivity of the electrodes

The range of change in EC is relatively small during milk spoilage. As it can be seen from the results of time trial experiment, the estimated total increase in EC was 0.2 mS/cm at the end of detections, at where the defects in flavour or curdling of milk were physical detected. The ultimate achievement of the alternative use of EC is to provide an early indication of milk spoilage than physical sensations and BBD. However, the currently used conductivity meter

has the inadequate sensitivity of detection, and it does not support sufficient evidence to prove that EC is a better detection mean to replace conventional BBD and physical sensations. Additional experiments are needed to investigate the use of EC in milk. In the future, the sensitivity and specificity of conductivity meter can be improved by modifying the conductometric electrodes. The improvements can be achieved by immobilising the surface of electrodes with enzymes or antibodies that can detect specific milk composition which reflects the progress of spoilage. Alternatively, modifying the surface of electrodes use high electrical conducting polymers to amplify sensing signals and to enhance detection limit of EC in milk.

References

Adley, C. C., and M. P. Ryan. (2015) "Conductometric biosensors for high throughput screening of pathogens in food." In high throughput screening for food safety assessment. pp. 315–326. doi: 10.1016/B978-0-85709-801-6.00014-9

Allison, J. B., J. A. Anderson, and W. H. Cole. (1938) "The method of electrical conductivity in studies on bacterial metabolism." Journal of Bacteriology 36 (6): 571–586. doi: 10.1111/j.1365-2672.1982.tb05065

Alocilja, E. C., and S. M. Radke. (2003). "Market analysis of biosensors for food safety." Biosensors and Bioelectronics 18 (5-6): 841–46. doi:10.1016/S0956-5663(03)00009-5.

Amamcharla, J. K., and L. E. Metzger. (2011). "Development of a rapid method for the measurement of lactose in milk using a blood glucose biosensor." Journal of Dairy Science 94 (10): 4800–4809. doi:10.3168/jds.2011-4416.

Application data sheet, "The theory and application of electrolytic conductivity." http://www2.emersonprocess.com/siteadmincenter/PM%20Rosemount%20Analytical%20Documents/Liq_ADS_43-018.pdf (Assessed May 2016)

Barach, J. T., D. M. Adams, and M. L. Speck. (1976). "Stabilization of a psychrotrophic pseudomonas protease by calcium against thermal inactivation in milk at ultrahigh temperature." Applied and Environmental Microbiology 31, no. 6: 875-879.

Bazinet, L., Y. Pouliot, and F. Castaigne. (2010). "Relative contributions of charged species to conductivity changes in skim milk during electrochemical acidification." Journal of Membrane Science 352 (1-2): 32–40. doi:10.1016/j.memsci.2010.01.056.

Bergmann, T., P. Abel, and K. Giffey. (1999). "Concentration of lactic acid in carcasses and fresh meat - estimation with an enzymatic-biosensor measuring system." Food and Agriculture Organization of the United Unions.

Binnur, K., and K. Serap. (2016). "The effects of temperature and milk fat content on the electrical conductivity of kefir during the incubation." American Journal of Food Science and Technology 4 (1): 25–28. doi:10.12691/ajfst-4-1-5.

Bishop, J. R., C. H. White, and R. Firsrwnbergedenf. (1984). "Rapid impedimetric method for determining the potential shelf-life of pasteurized whole milk." Journal of Food Protection 47 (6): 471–75.

Borch, E., and C. Wallentin. (1993). "Conductance measurements for data generation in predictive modeling." Journal of Industrial Microbiology 12 (3-5): 286–90. doi:10.1007/BF01584204.

Böhmer, L., and G. Hildebrandt. (1998). "Analysis of variance of bacterial counts in milk. 1.

characterization of total variance and the components of variance random sampling error, methodologic error and variation between parallel errors during storage." Berliner Und Munchener Tierarztliche Wochenschrift 111 (1): 13–20.

Brown, F. T. "Engineering system dynamics: a unified graph-centered approach." CRC press, 2006

Bülte, M., and G. Reuter. (1984). "Impedance measurement as a rapid method for the determination of the microbial contamination of meat surfaces, testing two different instruments." International Journal of Food Microbiology 1 (3): 113–25. doi:10.1016/0168-1605(84)90001-1.

Căpriță, A., R. Căpriță, and I. Crețescu. (2014). "The effects of storage conditions on some physicochemical properties of raw and pasteurized milk." Journal of agroalimentary processes and technologies 20 (2): 198-202

Cetó, X., N. H. Voelcker, and B. Prieto-Simón. (2016). "Bioelectronic tongues: new trends and applications in water and food analysis." Biosensors and Bioelectronics 79 (May): 608–26. doi:10.1016/j.bios.2015.12.075.

Chaubey, A., and B. D. Malhotra. (2002). "Mediated Biosensors." Biosensors and Bioelectronics 17 (6-7): 441–56. doi:10.1016/S0956-5663(01)00313-X.

Chen, L., R. M. Daniel, and T. Coolbear. (2003). "Detection and impact of protease and lipase activities in milk and milk powders." International Dairy Journal 13 (4): 255–75. doi:10.1016/S0958-6946(02)00171-1.

Christy, A. A., L. Eriksson, M. Feinberg, J. L. M. Hermens, H. Hobert, P. K. Hopke, O. M. Kvalheim, R. D. McDowall, D. R. Scott, and J. Webster. "Chemometrics in Environmental Chemistry-Applications" Edited by Jürgen Einax. Vol. 2. New York: 2013.

Clark, L. C., and C. Lyons. (1962). "Electrode systems for continuous monitoring in cardiovascular surgery." Annals of the New York Academy of Sciences 102 (1): 29–45. doi:10.1111/j.1749-6632.1962.tb13623.x.

Cohen, J. "Statistical Power Analysis for the Behavioral Sciences." 2nd edn. Hillsdale, New Jersey: 1988.

Cooledge, L. H., and R. W. Wyant. 1920. "Jugding the keeping quality of milk by a pH method." American Journal of Public Health 10 (12): 978–79.

Daniels, J. S., and N. Pourmand. (2007). "Label-free impedance biosensors: opportunities and challenges." Electroanalysis 19 (12): 1239–57. doi:10.1002/elan.200603855.

Danielsson, B., and K. Mosbach. "Theory and application of calorimetric sensors." In Biosensors: Fundamentals and applications pp. 575-595. Oxford University Press Oxford: 1987.

Diaz, J. R., G. Romero, R. Muelas, E. Sendra, J. C. F. Pantoja, and C. Paredes. (2011). "Analysis of the influence of variation factors on electrical conductivity of milk in murciano-granadina goats." Journal of Dairy Science 94 (8): 3885–94. doi:10.3168/jds.2011-4187.

Diaz, J. R., G. Romero, R. Muelas, M. Alejandro, and C. Peris. (2012). "Effect of intramammary infection on milk electrical conductivity in murciano-granadina goats." Journal of Dairy Science 95 (2): 718–26. doi:10.3168/jds.2011-4698.

Down, R. D., and J. H. Lehr. "Environmental instrumentation and analysis handbook." John Wiley & Sons: 2005.

Driehuis, F. (2013). "Silage and the safety and quality of dairy foods: a review." Agricultural and Food Science 22 (1): 16–34.

Durante, G., W. Becari, F. A. S. Lima, and H. E. M. Peres. (2016). "Electrical impedance sensor for real-time detection of bovine milk adulteration." IEEE Sensors Journal 16 (4): 861–65. doi:10.1109/JSEN.2015.2494624.

Early, R. "Technology of Dairy Products." Springer Science & Business Media: 1998.

Elmoslemany, A. M., G. P. Keefe, I. R. Dohoo, J. J. Wichtel, H. Stryhn, and R. T. Dingwell. (2010) "The association between bulk tank milk analysis for raw milk quality and on-farm management practices." Preventive veterinary medicine 95:1: 32-40.

Escamilla-Gomez, V., S. Campuzano, M. Pedrero, and J. M. Pingarron. (2008). "Electrochemical immunosensor designs for the determination of staphylococcus aureus using 3,3-dithiodipropionic acid di(N-succinimidyl ester)-modified gold electrodes." Talanta 77 (2): 876–81. doi:10.1016/j.talanta.2008.07.045.

Eshkenazi, I., E. Maltz, B. Zion, and J. Rishpon. (2000). "A three-cascaded-enzymes biosensor to determine lactose concentration in raw milk." Journal of Dairy Science 83 (9): 1939–45. doi:10.3168/jds.S0022-0302(00)75069-7.

Eskin, N. A. M. "Biochemistry of foods". Academic Press: 2012.

Evrendilek, G. (2014). "Non-thermal processing of milk and milk products for microbial safety." In Dairy Microbiology and Biochemistry. pp. 322–55. doi:10.1201/b17297-14.

Felice, C. J., R. E. Madrid, J. M. Olivera, V. I. Rotger, and M. E. Valentinuzzi. (1999). "Impedance microbiology: quantification of bacterial content in milk by means of capacitance growth curves." Journal of Microbiological Methods 35 (1): 37–42. doi:10.1016/S0167-7012(98)00098-0.

Fernando, R. S., R. B. Rindsig, and S. L. Spahr. (1982). "Electrical conductivity of milk for detection of mastitis." Journal of Dairy Science 65 (4): 659–64. doi:10.3168/jds.S0022-0302(82)82245-5.

Foote, K. J., M. K. Joy, and R. G. Death. (2015). "New Zealand dairy farming: milking our

environment for all Its worth." Environmental Management 56 (3): 709–20.

Fox, P. F., and P. L. H. McSweeney. "Dairy chemistry and biochemistry." New York Springer: 1998.

Fox, P. F., and D. M. Mulvihill. (1982). "Milk proteins: molecular, colloidal and functional properties." Journal of Dairy Research 49 (4): 679–93. doi:10.1017/S0022029900022822.

Frank, J. F., G. L. Christen, and L. B. Bullerman. "Tests for groups of microorganisms." Standard methods for the microbiological examination of dairy products. 16th Edn., Ed., RT Marshal. Washington, DC (1992): 271–86.

Fraser, D. M. "An introduction to in vivo biosensing: progress and problems." Biosensors in the body: continuous in vivo monitoring Edited by D. Fraser. Chichester: Wiley (1997), 1–56.

Fu, B., and T. P. Labuza. (1993). "Shelf-life prediction: theory and application." Food Control 4 (3): 125–33. doi:10.1016/0956-7135(93)90298-3.

Gillis, E. H., J. P. Gosling, J. M. Sreenan, and M. Kane. (2002). "Development and validation of a biosensor-based immunoassay for progesterone in bovine milk." Journal of Immunological Methods 267 (2): 131–38. doi:10.1016/S0022-1759(02)00166-7.

Goode, J. A., J. V. H. Rushworth, and P. A. Millner. (2015). "Biosensor regeneration: a review of common techniques and outcomes." Langmuir: the ACS Journal of Surfaces and Colloids 31 (23): 6276. doi:10.1021/la503533g.

Gómez, R., R. Bashir, and A. K. Bhunia. (2002). "Microscale electronic detection of bacterial metabolism." Sensors and Actuators B: Chemical 86 (2): 198–208.

Green, R. G., and W. P. Larson. (1922). "Conductivity of bacterial cells." The Journal of Infectious Diseases doi:10.2307/30080561.

Grieshaber, D., R. MacKenzie, J. Vörös, and E. Reimhult. (2008). "Electrochemical biosensors - sensor principles and architectures." Sensors 8 (3): 1400–1458. doi:10.3390/s8031400.

Henningsson, M., K. Ostergren, and P. Dejmek. (2005). "The electrical conductivity of milk - the effect of dilution and temperature." International Journal of Food Properties 8 (1): 15–22. doi:10.1081/JFP-200048143.

Herigstad, B., M. Hamilton, and J. Heersink. (2001). "How to optimize the drop plate method for enumerating bacteria." Journal of Microbiological Methods 44 (2): 121–29.

Herrinngton, B. L. "Milk and milk processing." Mcgraw-Hill Bok Company, Inc.; New York; Toronto; London: 1948.

Hill, A. R., and P. Kethireddipalli. "Chapter 8 - dairy products: cheese and yogurt." In Biochemistry of Foods (Third Edition), edited by N A Michael Eskin and Fereidoon Shahidi, 319–62. San Diego: Academic Press: 2013.

Jenness, R. (1979). "The composition of human milk." In Seminars in perinatology 3 (3): 225-239.

Kaiser, K. L. "Electromagnetic compatibility handbook." CRC press:2004.

Kanekanian, A. (2005). "Dairy technology—principles of milk properties and processes." International Journal of Dairy Technology 58 (4): 237–37. doi:10.1111/j.1471-0307.2005.00175.x.

Kaptan, B. (2012). "The relationship between some physico-chemical, microbiological characteristics and electrical conductivity of milk stored at different temperature." Journal of Tekirdag Agricultural Faculty 8 (2).

Kelly, S. C., P. J. O'Connell, C. K. O'Sullivan, and G. G. Guilbault. (2000). "Development of an interferent free amperometric biosensor for determination of L-lysine in food." Analytica Chimica Acta 412 (1-2): 111–19. doi:10.1016/S0003-2670(00)00767-4.

Kirsch, J., C. Siltanen, Q. Zhou, A. Revzin, and A. Simonian. (2013). "Biosensor technology: recent advances in threat agent detection and medicine." Chemical Society Reviews 42 (22): 8733–68. doi:10.1039/c3cs60141b.

Koester, U. (2013). "Total and per capita value of food loss in the United States - comments." Food Policy 41 (August): 63–64. doi: 10.1016/j.foodpol.2013.04.003.

Lanzanova, M., G. Mucchetti, and E. Neviani. (1993). "Analysis of conductance changes as a growth index of lactic acid bacteria in milk." Journal of Dairy Science 76 (1): 20–28. doi:10.3168/jds. S0022-0302(93)77319-1.

Law, B. A. (1979). "Enzymes of psychrotrophic bacteria and their effects on milk and milk products." Journal of Dairy Research 46 (03): 573–88. doi:10.1017/S0022029900017611.

Lawton, B. A., and R. Pethig. (1993). "Determining the fat content of milk and cream using AC conductivity measurements." Measurement Science and Technology 4 (1): 38–41. doi:10.1088/0957-0233/4/1/007.

Ledenbach, L. H., and R. T. Marshall. (2009). "Microbiological spoilage of dairy products." In Compendium of the Microbiological Spoilage of Foods and Beverages 41–67. Springer New York. doi:10.1007/978-1-4419-0826-1_2.

Lin, H., M. Shavezipur, A. Yousef, and Maleky. (2016). "Prediction of growth of pseudomonas fluorescens in milk during storage under fluctuating temperature." Journal of Dairy Science 99 (3): 1822–30. doi:10.3168/jds.2015-10179.

Lindmark-Månsson, H., R. Fondén, and H. Pettersson. (2003). "Composition of swedish dairy milk." International Dairy Journal 13 (6): 409–25. doi:10.1016/S0958-6946(03)00032-3.

Liu, J-T., K. Settu, J-Z. Tsai, and C-J. Chen. (2015). "Impedance sensor for rapid enumeration of E. coli in milk samples." Electrochimica Acta 182 (November): 89–95. doi:

10.1016/j.electacta.2015.09.029.

Lu, M., Y. Shiau, J. Wong, R. Lin, H. Kravis, T. Blackmon, P. Tanya, T. Jen, A. Cheng, J. Chang, E. Ong, N. Sarfaraz, N. S. Wang. (2013). "Milk spoilage: methods and practices of detecting milk quality. Food and Nutrition Sciences, 04(07), 113–123. http://doi.org/10.4236/fns.2013.47A014

Mabrook, M. F., and M. C. Petty. (2002). "Application of electrical admittance measurements to the quality control of milk." Sensors and Actuators B: Chemical 84 (2-3): 136–41. doi:10.1016/S0925-4005(02)00014-X.

Mabrook, M. F., and M. C. Petty. (2003a). "A novel technique for the detection of added water to full fat milk using single frequency admittance measurements." Sensors and Actuators B: Chemical 96 (1-2): 215–18. doi:10.1016/S0925-4005(03)00527-6.

Mabrook, M. F., and M. C. Petty. (2003b). "Effect of composition on the electrical conductance of milk." Journal of Food Engineering 60 (3): 321–25. doi:10.1016/S0260-8774(03)00054-2.

Marrakchi, M., S. V. Dzyadevych, F. Lagarde, C. Martelet, and N. Jaffrezic-Renault. (2008). "Conductometric biosensor based on glucose oxidase and beta-galactosidase for specific lactose determination in milk." Materials Science and Engineering: *C* 28 (5-6): 872–75. doi: 10.1016/j.msec.2007.10.046.

Marth, E. H. "Standard methods for the examination of dairy products." American Public Health Association: 1978.

McCarthy, O. J., and H. Singh. (2009). "Physico-chemical properties of milk." In Advanced Dairy Chemistry 691–758. Springer New York. doi:10.1007/978-0-387-84865-5_15.

McMeekin, T. A., and T. Ross. (1996). "Shelf life prediction: status and future possibilities." International Journal of Food Microbiology 33 (1): 65–83. doi:10.1016/0168-1605(96)01138-5.

McSweeney, P. L. H., and P. F. Fox. (2008). "Advanced dairy chemistry: lactose, water, salts and minor components". Edited by PF Fox. Chapman & Hall, London 3 (5): 316-317.

Mello, L. D., and L. T. Kubota. (2002). "Review of the use of biosensors as analytical tools in the food and drink industries." Food Chemistry 77 (2): 237–56. doi:10.1016/S0308-8146(02)00104-8.

Milner, P., K. L. Page, A. W. Walton, and J. E. Hillerton. (1996). "Detection of clinical mastitis by changes in electrical conductivity of foremilk before visible changes in milk." Journal of Dairy Science 79 (1): 83–86. doi:10.3168/jds. S0022-0302(96)76337-3.

Ministry for primary industries. New Zealand government. (July) 2014 "How to determine the shelf life of food: a guidance document" https://www.mpi.govt.nz/document-vault/3414 (Assessed July 2016).

Moatsou, G., and E. Moschopoulou. (2014). “Microbiology of raw milk.” In Dairy Microbiology and Biochemistry 1–38. CRC Press. doi:10.1201/b17297-2.

Mucchetti, G., M. Gatti, and E. Neviani. (1994). “Electrical conductivity changes in milk caused by acidification: determining factors.” Journal of Dairy Science 77 (4): 940–44. doi:10.3168/jds. S0022-0302(94)77029-6.

Muir, D. D. (1996). “The shelf-life of dairy products: 1. factors influencing raw milk and fresh products.” International Journal of Dairy Technology 49 (1): 24–32. doi:10.1111/j.1471-0307.1996.tb02616.x.

Murphy, S. C. (2007). "Shelf-life of fluid milk products–microbial spoilage: the evaluation of shelf-life." Dairy Foods Science Notes : 1-4.

Mutlu, M. “Biosensors in food processing, safety, and quality control.” CRC Press: 2010.

Nakamura, H., and I. Karube. (2003). “Current research activity in biosensors.” Analytical and Bioanalytical Chemistry 377 (3): 446–68. doi:10.1007/s00216-003-1947-5.

Nieuwenhof, F. F. J., and J. D. Hoolwerf. (1987). "Impedance measurement as an alternative to the plate count method for estimating the total count of bacteria in raw milk." Journal of Food Protection 50 (8): 665-668.

Noble, P. A. (1999). “Hypothetical model for monitoring microbial growth by using capacitance measurements--a minireview.” Journal of Microbiological Methods 37 (1): 45–49.

Nollet, L. M. L., and F. Toldra. "Handbook of dairy foods analysis". CRC Press: 2009.

Norberg, E., H. Hogeveen, I. R. Korsgaard, N. C. Friggens, K. H. M. N. Sloth, and P. Løvendahl. (2004). “Electrical conductivity of milk: ability to predict mastitis status.” Journal of Dairy Science 87 (4): 1099–1107. doi:10.3168/jds.S0022-0302(04)73256-7.

Ostan, M., I. Gogoaşă, O. Rada, B. Baul, M. Fericean, M. Cazacu, M. Petcu, and I. Crețescu. (2015). "The effect of boiling on the pH, electrical conductivity and lactose content of cow milk." Research Journal of Agricultural Science 47 (2): 130-133.

O’Mahony, J. A., and P. F. Fox. (2014). “Milk: an overview.” In Milk Proteins 19–73. doi:10.1016/B978-0-12-405171-3.00002-7.

Patel, P. D. (2002). “(Bio)sensors for measurement of analytes implicated in food safety: a review.” Trends in analytical chemistry 21 (2): 96–115. doi:10.1016/S0165-9936(01)00136-4.

Peterson, J. I., and G. G. Vurek. (1984). “Fiber-optic sensors for biomedical applications.” Science 224 (4645): 123–27. doi:10.1126/science.6422554.

Pilloton, R., M. Mascini, I. G. Casella, M. R. Festa, and E. Bottari. (1987). “Lactose determination in raw milk with a two-enzyme based electrochemical sensor.” Analytical Letters 20 (11): 1803–14. doi:10.1080/00032718708082597.

Prentice, J. H. (1962). "The conductivity of milk—the effect of the volume and degree of dispersion of the fat." Journal of Dairy Research 29 (02): 131–39. doi:10.1017/S0022029900017738.

Prentice, J. H. (1972). "The temperature coefficient of electrolytic conductivity of milk." Journal of Dairy Research 39 (02): 275–78. doi: 10.1017/S0022029900014102.

Prentice, J. H. (1977). "Evaluation of some coated titanium electrodes for measurements of conductivity of milk and milk products." Journal of Dairy Research 44 (03): 615–19. doi:10.1017/S0022029900020598.

Rasooly, A., and K. E. Herold. (2011). "Analytical methods | biosensors." In Encyclopedia of Dairy Sciences 235–47. doi:10.1016/B978-0-12-374407-4.00024-8.

Ray, B., and A. Bhunia. "Fundamental food microbiology" CRC press, 2007.

Reder C. K., and G. Bendas. (2011). "Biosensor applications in the field of antibiotic research—a review of recent developments." Sensors 11 (12): 9450–66. doi:10.3390/s111009450.

Reis C. D., J. Selia, and J. A. Teixeira. (2009). "Engineering aspects of milk and dairy products." Edited by Jane Coimbra and Jose Teixeira. CRC Press. doi:10.1201/9781420090390.

Richards, J. C. S., A. C. Jason, G. Hobbs, D. M. Gibson, and R. H. Christie. (1978). "Electronic measurement of bacterial growth" Journal of Physics E: Scientific Instruments 11 (6): 560–68. doi:10.1088/0022-3735/11/6/017.

Richardson, B. C. (1981) "purification and characterization of a heat-stable protease from Pseudomonas fluorescens B52." New Zealand journal of dairy science and technology.

Roberts, T. A., and F. A. Skinner. (1983). Food microbiology: advances and prospects 28 (4): 45.

Romette, J. L., J. S. Yang, H. Kusakabe, and D. Thomas. (1983). "Enzyme electrode for specific determination of L-lysine." Biotechnology and Bioengineering 25 (11): 2557–66.

Sadler, G. D., and P. A. Murphy. (2010). "pH and titratable acidity." In Food Analysis 219–38. Boston, MA: Springer. doi:10.1007/978-1-4419-1478-1_13.

Saris, P. (2014). "Biopreservation by lactic acid bacteria." In Dairy Microbiology and Biochemistry 86–94. CRC Press. doi:10.1201/b17297-5.

Sarkar, P., I. E. Tothill, S. J. Setford, and A. P. F. Turner. (1999). "Screen-printed amperometric biosensors for the rapid measurement of L - and D -amino acids." Analyst 124 (6): 865–70. doi:10.1039/A901404G.

Schulz, M. E., and G. Sydow. (1957). "The electrical conductivity (chloride-free) of milk and dairy products. Milchwissenschaft 12: 174-184.

Scognamiglio, V., G. Pezzotti, I. Pezzotti, J. Cano, K. Buonasera, D. Giannini, and M. T. Giardi. (2010). "Biosensors for effective environmental and agrifood protection and commercialization: from research to market." Microchimica Acta 170 (3-4): 215–25.

doi:10.1007/s00604-010-0313-5.

Seitz, W. R. (1987). "Optical sensors for clinical applications." Journal of Clinical Laboratory Analysis 1 (3): 313–16. doi:10.1002/jcla.1860010312.

Sharma, G. S., and N. K. Roy. (1976). "Influence of temperature on the electrical conductivity of buffalo Milk." Journal of Dairy Research 43 (02): 321–23. doi:10.1017/S0022029900015880.

Sims, L. A., P. C. Vasavada, R. R. Hull, and R. A. Chandler. (1991). "Impedimetric analysis of quality and shelf-life of milk pasteurized by a continuous microwave treatment." Journal of Dairy Science 74 (Suppl. 1) : 139.

Singh, S., A. Srivastava, H. Oh, C. Ahn, G. Choi, and R. K. Asthana. (2012). "Recent trends in development of biosensors for detection of microcystin." Toxicon 60 (5): 878–94. doi:10.1016/j.toxicon.2012.06.005.

Smithers, G. W. (2008). "Whey and whey proteins—from 'gutter-to-gold'." International Dairy Journal 18 (7): 695–704. doi:10.1016/j.idairyj.2008.03.008.

Srivastava, M. "Handbook of Milk Microbiology." Daya Books: 2002.

Stabel, J. R., and A. Lambertz. (2004). "Efficacy of pasteurization conditions for the inactivation of Mycobacterium avium subsp. paratuberculosis in milk."Journal of Food Protection 67 (12): 2719-2726.

Stead, D. (1986). "Microbial lipases: their characteristics, role in food spoilage and industrial uses." Journal of Dairy Research 53 (3): 481–505. doi:10.1017/S0022029900025103.

Sørhaug, T., and L. Stepaniak. (1997). "Psychrotrophs and their enzymes in milk and dairy products: quality aspects." Trends in Food Science & Technology 8 (2): 35–41. doi:10.1016/S0924-2244(97)01006-6.

Tajammal M. M., W. Yu, B. R. Young, and D. I. Wilson. (2015). "The current status of process analytical technologies in the dairy industry." Trends in Food Science & Technology 43 (2): 205–18. doi: 10.1016/j.tifs.2015.02.010.

Tamagnini, L. M., and R. D. González. (1997). "Bacteriological stability and growth kinetics of pseudomonas aeruginosa in bottled water." Journal of Applied Microbiology 83 (1): 91–94.

Tiefenthaler, K. (1993). "Grating couplers as label-free biochemical waveguide sensors." Biosensors and Bioelectronic 8 (7-8): xxxv–xxxvii. doi:10.1016/0956-5663(93)80070-6.

Turner, A. P. F. (2013). "Biosensors: sense and sensibility." Chemical Society Reviews 42 (8): 3184–96. doi:10.1039/C3CS35528D.

Thermo Fisher Scientific. "Instruction Manual Cond6+." http://www.eutechinst.com/manuals/english/economy_handheld/contdssalt6plus_Rev1.pdf. (Assessed June 2016)

Ur, A., and D. F. J. Brown. (1975). "Impedance monitoring of bacterial activity." Journal of

Medical Microbiology 8 (1): 19–28. doi:10.1099/00222615-8-1-19.

Velasco-Garcia, M. N., and T. Mottram. (2003). "Biosensor technology addressing agricultural problems." Biosystems Engineering 84 (1): 1–12. doi:10.1016/S1537-5110(02)00236-2.

Velusamy, V., KArshak, O. Korostynska, K. Oliwa, and C. Adley. (2010). "An overview of foodborne pathogen detection: in the perspective of biosensors." Biotechnology Advances 28 (2): 232–54. doi: 10.1016/j.biotechadv.2009.12.004.

Verma, N., and M. Singh. (2003). "A disposable microbial based biosensor for quality control in milk." Biosensors and Bioelectronics 18 (10): 1219–24. doi:10.1016/S0956-5663(03)00085-X.

Visser, I. J. R., and J. D. Groote. (1984). "Prospects for the use of conductometry as an aid in the bacteriological monitoring of pasteurized milk." Antonie Van Leeuwenhoek 50 (2): 202–6. doi:10.1007/BF00400187.

Walstra, P. "Dairy Technology: Principles of Milk Properties and Processes." CRC Press: 2013.

Walstra, P., and R. Jenness. "Dairy Chemistry & Physics." John Wiley & Sons: 1984.

Wang, J. (2008). "Electrochemical glucose biosensors." Chemical Reviews 108 (2): 814–25. doi:10.1021/cr068123a.

Warriner, K., S. M. Reddy, A. Namvar, and S. Neethirajan. (2014). "Developments in nanoparticles for use in biosensors to assess food safety and quality." Trends in Food Science & Technology 40 (2): 183–99. doi: 10.1016/j.tifs.2014.07.008.

Webster, T. A., H. J. Sismaet, J. L. Conte, I. J. Chan, and E. D. Goluch. (2014). "Electrochemical detection of pseudomonas aeruginosa in human fluid samples via pyocyanin." Biosensors and Bioelectronic 60 (c): 265–70. doi: 10.1016/j.bios.2014.04.028.

Wedholm, A., E. Hallén, L. B. Larsen, H. Lindmark-Månsson, A. H. Karlsson, and T. Allmere. (2007). "Comparison of milk protein composition in a swedish and a danish dairy herd using reversed phase HPLC." Acta Agriculturae Scand Section A 56 (1): 8–15. doi:10.1080/09064700600836745.

Willem H., K. Olieman, G. Cazemier, and R. Verheijen. (2001). "Direct biosensor immunoassays for the detection of nonmilk proteins in milk powder." Journal of Agricultural and Food Chemistry 49 (11): 5201–6. doi:10.1021/jf010440p.

Wong, N. P. (2012). "Fundamentals of Dairy Chemistry" Edited by Noble P Wong, Robert Jenness, Mark Keeney, and Elmer H Marth. Springer Science Business Media. Boston, MA: Springer Science & Business Media. doi:10.1007/978-1-4615-7050-9.

Wu, S-Y., C. Yang, W. Hsu, and L. Lin. (2015). "3D-printed microelectronics for integrated circuitry and passive wireless sensors." Microsystems & Nanoengineering 1 (July): 15013. doi:10.1038/micronano.2015.13.

Wu, W., L. Liu, Z. Dai, J. Liu, S. Yang, L. Zhou, X. Xiao, C. Jiang, and V. A. L. Roy. (2015).

"Low-cost, disposable, flexible and highly reproducible screen printed SERS substrates for the detection of various chemicals." Scientific Reports 5 (May): 10208. doi:10.1038/srep10208.

Yunus, S., A. M. Jonas, and B. Lakard. (2013). "Potentiometric biosensors." Encyclopedia of Biophysics. Springer Berlin Heidelberg. doi:10.1007/978-3-642-16712-6_714.

Zaninelli, M., A. Agazzi, A. Costa, F. M. Tangorra, L. Rossi, and G. Savoini. (2015). "Evaluation of the fourier frequency spectrum peaks of milk electrical conductivity signals as indexes to monitor the dairy goats' health status by on-line sensors." Sensors 15 (8): 20698–716. doi:10.3390/s150820698.

Zhang, S. (2000). "Materials and techniques for electrochemical biosensor design and construction." Biosensors and Bioelectronics 15 (5-6): 273–82. doi:10.1016/S0956-5663(00)00076-2.

Appendix 1

Table 10. One-way ANOVA of skim and whole milk stored at 4℃

			Sum of Squares	DF	Mean Square	F	Sig.
Skim milk	EC	Between Groups	0.46	10	0.05	7.04	.000
		Within Groups	0.57	87	0.01		
		Total	1.03	97			
	LA	Between Groups	0.01	10	0.01	5.35	.000
		Within Groups	0.01	87	0.00		
		Total	0.02	97			
	pH	Between Groups	0.30	10	0.03	16.80	.000
		Within Groups	0.16	87	0.0		
		Total	0.46	97			
	TBC (Log 10)	Between Groups	301.46	10	30.15	35.30	.000
		Within Groups	74.30	87	0.85		
		Total	375.76	97			
Whole milk	EC	Between groups	0.31	10	0.03	9.50	.000
		Within groups	0.29	88	0.00		
		Total	0.60	98			
	LA	Between groups	0.02	10	0.00	17.55	.000
		Within groups	0.01	88	0.00		
		Total	0.03	98			
	pH	Between groups	0.84	10	0.08	80.71	.000
		Within groups	0.10	88	0.00		
		Total	0.93	98			
	TBC (Log 10)	Between groups	342.49	10	34.249	124.13	.000
		Within groups	24.28	88	0.276		
		Total	366.77	98			

Table 11. Dunnett's post-test compares the values of EC, TBC, LA concentration with the values on first sampling day in skim milk

Dependent Variables	(I) Day	(J) Day	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
EC	4	7	0.06	0.02	0.07	-0.00	0.13
		10	0.06	0.02	0.18	-0.01	0.14
		13	0.04	0.02	0.85	-0.03	0.1
		16	0.04	0.01	0.28	-0.01	0.10
		19	0.05	0.02	0.11	-0.01	0.12
		22	0.03	0.02	0.99	-0.06	0.11
		25	0.03	0.03	1.00	-0.11	0.18

		28	-0.01	0.04	1.00	-0.17	0.14
		31	-0.08	0.03	0.62	-0.21	0.06
		34	-0.18*	0.06	0.05	-0.46	0.11
LA	4	7	-0.01	0.00	0.40	-0.03	0.01
		10	0.00	0.00	1.00	-0.02	0.02
		13	0.00	0.01	1.00	-0.03	0.02
		16	0.00	0.00	1.00	-0.02	0.02
		19	0.00	0.00	1.00	-0.02	0.02
		22	-0.01	0.01	1.00	-0.03	0.01
		25	-0.01	0.01	1.00	-0.03	0.02
		28	-0.01	0.01	0.95	-0.04	0.01
		31	-0.02	0.01	0.29	-0.05	0.01
		34	-.029*	0.01	0.03	-0.06	0.00
pH	4	7	-0.03	0.01	0.39	-0.07	0.01
		10	-0.02	0.01	0.36	-0.05	0.01
		13	-0.03	0.01	0.15	-0.07	0.01
		16	-0.01	0.01	1.00	-0.03	0.02
		19	0.02	0.01	0.23	-0.01	0.05
		22	0.05	0.02	0.53	-0.03	0.12
		25	0.07	0.02	0.14	-0.02	0.16
		28	0.05	0.02	0.52	-0.04	0.14
		31	0.10*	0.02	0.04	0.00	0.20
		34	0.14*	0.02	0.01	0.04	0.26
TBC (Log10)	4	7	-0.03	0.06	1.00	-0.28	0.21
		10	-0.09	0.09	1.00	-0.48	0.31
		13	0.00	0.07	1.00	-0.26	0.26
		16	-0.28	0.30	1.00	-1.64	1.09
		19	-0.75	0.35	0.76	-2.35	0.85
		22	-1.60	0.46	0.22	-3.81	0.62
		25	-2.29*	0.48	0.04	-4.49	-0.11
		28	-3.11*	0.48	0.01	-5.30	-0.91
		31	-3.87*	0.35	0.00	-5.45	-2.30
		34	-5.23*	0.27	0.00	-6.45	-4.02

*. The mean difference is significant at the 0.05 levels.

Table 12. Dunnett's post-test compares the values of EC, TBC, LA concentration with the values on first sampling day in whole milk

Dependent Variables	(I) Day	(J) Day	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
EC	3	6	-0.02	0.03	1.00	-0.15	0.12
		9	-0.07	0.03	0.73	-0.19	0.05
		12	-0.07	0.03	0.59	-0.18	0.05
		15	-0.08	0.03	0.32	-0.20	0.04

		18	-0.06	0.03	0.73	-0.18	0.06
		21	-0.11	0.04	0.29	-0.25	0.03
		24	-0.09	0.03	0.16	-0.21	0.02
		27	-0.15*	0.03	0.01	-0.28	-0.03
		30	-0.09	0.03	0.36	-0.22	0.04
		33	-0.21*	0.04	0.00	-0.36	-0.07
LA	3	6	0.00	0.00	1.00	-0.02	0.02
		9	0.01	0.00	0.44	-0.01	0.04
		12	0.02	0.01	0.25	-0.01	0.04
		15	0.00	0.01	1.00	-0.02	0.03
		18	0.01	0.01	0.99	-0.02	0.03
		21	0.00	0.00	1.00	-0.02	0.02
		24	-0.01	0.01	0.80	-0.04	0.01
		27	-0.02	0.01	0.16	-0.04	0.00
		30	-0.02	0.01	0.31	-0.04	0.01
		33	-0.02*	0.01	0.05	-0.06	0.00
pH	3	6	-0.05*	0.01	0.01	-0.09	-0.01
		9	-0.03	0.01	0.27	-0.07	0.01
		12	-0.04*	0.01	0.02	-0.09	-0.01
		15	-0.02	0.01	0.98	-0.07	0.03
		18	0.01	0.01	1.00	-0.05	0.07
		21	0.04	0.02	0.59	-0.02	0.10
		24	0.09*	0.01	0.00	0.04	0.14
		27	0.11*	0.01	0.00	0.07	0.17
		30	0.16*	0.01	0.00	0.11	0.22
		33	0.24*	0.03	0.00	0.14	0.35
TBC (Log10)	3	6	0.02	0.11	1.00	-0.41	0.45
		9	-0.06	0.09	1.00	-0.41	0.29
		12	-0.10	0.08	1.00	-0.42	0.22
		15	-0.48	0.22	0.75	-1.46	0.49
		18	-1.43*	0.18	0.00	-2.18	-0.69
		21	-2.47*	0.41	0.01	-4.32	-0.62
		24	-3.10*	0.19	0.00	-3.91	-2.31
		27	-4.06*	0.18	0.00	-4.85	-3.28
		30	-4.53*	0.15	0.00	-5.17	-3.90
		33	-4.81*	0.12	0.00	-5.28	-4.36

*. The mean difference is significant at the 0.05 levels.

Table 13. One-way ANOVA of skim and whole milk stored at 8℃

			Sum of Squares	DF	Mean Square	F	Sig.
Skim milk	EC	Between groups	0.34	6	0.06	16.03	0.00
		Within groups	0.20	56	0.00		
		Total	0.53	62			
	LA	Between groups	0.01	6	0.00	15.10	0.00

		Within groups	0.00	56	0.00		
		Total	0.01	62			
	pH	Between groups	0.29	6	0.05	30.55	0.00
		Within groups	0.09	56	0.00		
		Total	0.38	62			
	TBC (log10)	Between groups	385.07	6	64.18	198.24	0.00
		Within groups	18.13	56	0.32		
		Total	403.20	62			
Whole milk	EC	Between Groups	0.27	5	0.05	37.21	0.00
		Within Groups	0.07	48	0.00		
		Total	0.34	53			
	LA	Between Groups	0.00	5	0.00	14.51	0.00
		Within Groups	0.00	48	0.00		
		Total	0.01	53			
	pH	Between Groups	0.35	5	0.07	47.43	0.00
		Within Groups	0.07	48	0.00		
		Total	0.43	53			
	TBC (Log 10)	Between Groups	333.08	5	66.62	353.85	0.00
		Within Groups	9.04	48	0.19		
		Total	342.12	53			

Table 14. Dunnett's post-test compares the values of EC, TBC, LA concentration with the values on first sampling day in skim milk

Dependent Variables	(I) Day	(J) Day	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
EC	4	6	-0.05	0.02	0.28	-0.11	0.02
		8	-0.04	0.02	0.54	-0.10	0.03
		10	-0.06	0.02	0.06	-0.12	0.00
		12	-0.08*	0.02	0.00	-0.14	-0.02
		14	-0.07	0.04	0.86	-0.22	0.09
		16	-.25*	0.03	0.00	-0.34	-0.15
LA	4	6	0.00	0.00	1.00	-0.01	0.01
		8	0.01	0.00	0.71	0.00	0.02
		10	0.01	0.00	0.42	0.00	0.02
		12	0.01	0.00	0.66	-0.01	0.02
		14	-0.01	0.00	0.64	-0.02	0.01
		16	-0.02*	0.00	0.01	-0.04	0.00
pH	4	6	0.07*	0.01	0.01	0.02	0.12
		8	0.05*	0.01	0.00	0.03	0.09
		10	0.04*	0.01	0.00	0.02	0.07
		12	0.06	0.02	0.33	-0.03	0.15
		14	0.14*	0.02	0.00	0.08	0.20
		16	0.22*	0.02	0.00	0.16	0.28
TBC	4	6	0.00	0.04	1.00	-0.14	0.13

(Log10)		8	-0.30	0.16	0.70	-0.93	0.33
		10	-1.04*	0.20	0.01	-1.87	-0.21
		12	-4.81*	0.27	0.00	-5.89	-3.72
		14	-5.35*	0.24	0.00	-6.31	-4.40
		16	-5.65*	0.25	0.00	-6.64	-4.65

*. The mean difference is significant at the 0.05 levels.

Table 15. Dunnett's post-test compares the values of EC, TBC, LA concentration with the values on first sampling day in whole milk

Dependent Variables	(I) Day	(J) Day	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
EC	3	5	-0.08*	0.01	0.00	-0.13	-0.04
		7	-0.02	0.01	0.95	-0.06	0.03
		9	-0.05*	0.01	0.05	-0.10	0.00
		11	-0.15*	0.02	0.00	-0.23	-0.09
		13	-0.19*	0.02	0.00	-0.25	-0.13
LA	3	5	0.00	0.00	1.00	-0.01	0.01
		7	0.00	0.00	0.99	-0.01	0.01
		9	-0.01	0.00	0.53	-0.02	0.01
		11	-0.01*	0.00	0.01	-0.03	0.00
		13	-0.02*	0.00	0.00	-0.04	-0.01
pH	3	5	0.05*	0.01	0.00	0.03	0.07
		7	0.06*	0.01	0.00	0.05	0.08
		9	0.13*	0.03	0.01	0.04	0.23
		11	0.19*	0.02	0.00	0.13	0.26
		13	0.23*	0.01	0.00	0.21	0.25
TBC (Log10)	3	5	-0.22	0.07	0.08	-0.46	0.02
		7	-1.24*	0.15	0.00	-1.77	-0.72
		9	-3.50*	0.21	0.00	-4.28	-2.73
		11	-5.77*	0.15	0.00	-6.33	-5.22
		13	-6.07*	0.22	0.00	-6.90	-5.26

*. The mean difference is significant at the 0.05 levels.

Table 16. Pearson's r correlation between EC and each of TBC, LA concentration and pH in skim milk stored at 4℃

	EC vs. TBC	EC vs. LA	EC vs. pH
Pearson r	0.63	0.56	-0.66

95% confidence interval	0.49 to 0.73	0.41 to 0.68	-0.76 to -0.53
R squared	0.39	0.32	0.44
P value	< 0.0001	< 0.0001	< 0.0001
P value summary	****	****	****
Significant? (Alpha = 0.05)	Yes	Yes	Yes
Number of XY Pairs	98	99	99

Table 17. Pearson's r correlation between EC and each of TBC, LA concentration and pH in whole milk stored at 4℃

	EC vs. TBC	EC vs. LA	EC vs. pH
Pearson r	0.53	0.39	-0.60
95% confidence interval	0.36 to 0.65	0.21 to 0.54	-0.71 to -0.46
R squared	0.27	0.15	0.37
P value (two-tailed)	< 0.0001	< 0.0001	< 0.0001
P value summary	****	****	****
Significant? (Alpha = 0.05)	Yes	Yes	Yes
Number of XY Pairs	99	99	99

Table 18. Pearson's r correlation between EC and each of TBC, LA and pH in skim milk stored at 8℃

	EC vs. TBC	EC vs. LA	EC vs. pH
Pearson r	0.58	0.49	-0.72
95% confidence interval	0.38 to 0.72	0.27 to 0.66	-0.82 to -0.57
R squared	0.33	0.24	0.52
P value (two-tailed)	< 0.0001	< 0.0001	< 0.0001
P value summary	****	****	****
Significant? (Alpha = 0.05)	Yes	Yes	Yes
Number of XY Pairs	63	63	63

Table 19. Pearson's r correlation between EC and each of TBC, LA and pH in whole milk stored at 8℃

	EC vs. TBC	EC vs. LA	EC vs. pH
Pearson r	0.75	0.70	-0.80
95% confidence interval	0.59 to 0.84	0.53 to 0.81	-0.88 to - 0.68
R squared	0.55	0.49	0.64
P value (two-tailed)	< 0.0001	< 0.0001	< 0.0001

P value summary	****	****	****
Significant? (alpha = 0.05)	Yes	Yes	Yes
Number of XY Pairs	54	54	54

Appendix 2

Table 20. Linear regression analysis of effects of LA on EC in skim and whole milk at temperature of 4℃

	Skim Milk	Whole Milk
Best-fit values		
Slope	0.92 ± 0.06	1.15 ± 0.06
Y-intercept when X=0.0	5.31 ± 0.02	5.03 ± 0.03
X-intercept when Y=0.0	-5.78	-4.39
1/slope	1.08	0.87
95% Confidence Intervals		
Slope	0.78 to 1.05	1.01 to 1.27
Y-intercept when X=0.0	5.25 to 5.37	4.98 to 5.09
X-intercept when Y=0.0	-6.80 to -5.01	-5.01 to -3.91
Goodness of Fit		
R square	0.93	0.95
Sy.x	0.06	0.07
Is slope significantly non-zero?		
F	220.6	339.4
DFn, DFd	1.000, 16	1.000, 16
P value	< 0.0001	< 0.0001
Deviation from zero?	Significant	Significant
Equation	Y = 0.92*X + 5.31	Y = 1.14*X + 5.03

Table 21. Linear regression analysis of effects of LA on EC in skim and whole milk at temperature of 20℃

	Skim Milk	Whole Milk
Best-fit values		
Slope	1.39 ± 0.04	1.31 ± 0.05
Y-intercept when X=0.0	5.15 ± 0.02	4.95 ± 0.02
X-intercept when Y=0.0	-3.71	-3.76
1/slope	0.72	0.761
95% Confidence Intervals		
Slope	1.31 to 1.46	1.19 to 1.43
Y-intercept when X=0.0	5.11 to 5.18	4.89 to 4.99
X-intercept when Y=0.0	-3.95 to -3.50	-4.17 to -3.42
Goodness of Fit		
R square	0.99	0.97
Sy.x	0.04	0.06
Is slope significantly non-zero?		
F	1503	541.1
DFn, DFd	1.000, 16	1.000, 16
P value	< 0.0001	< 0.0001

Deviation from zero?	Significant	Significant
Equation	Y = 1.34*X + 5.15	Y = 1.34*X + 4.95

Table 22. Linear regression analysis of effects of pH on EC in skim and whole milk at temperature of 4℃

	Skim milk	Whole milk
Best-fit values		
Slope	-1.59 ± 0.05	-1.39 ± 0.05
Y-intercept when X=0.0	15.69 ± 0.29	14.25 ± 0.28
X-intercept when Y=0.0	9.88	10.25
1/slope	-0.63	-0.72
95% Confidence Intervals		
Slope	-1.70 to -1.47	-1.496to -1.28
Y-intercept when X=0.0	15.08 to 16.29	13.68 to 14.82
X-intercept when Y=0.0	9.57 to 10.24	9.89 to 10.67
Goodness of Fit		
R square	0.96	0.96
Sy.x	0.23	0.25
Is slope significantly non-zero?		
F	811.6	706.3
DFn, DFd	1.000, 31	1.000, 31
P value	< 0.0001	< 0.0001
Deviation from zero?	Significant	Significant
Equation	Y = -1.59*X + 15.69	Y = -1.40*X + 14.25

Table 23. Linear regression analysis of effects of pH on EC in skim and whole milk at temperature of 20℃

	Skim milk	Whole milk
Best-fit values		
Slope	-1.52 ± 0.06	-1.26 ± 0.05
Y-intercept when X=0.0	15.11 ± 0.31	13.33 ± 0.25
X-intercept when Y=0.0	9.97	10.57
1/slope	-0.66	-0.80
95% Confidence Intervals		
Slope	-1.63 to -1.40	-1.36 to -1.12
Y-intercept when X=0.0	14.48 to 15.75	12.81 to 13.84
X-intercept when Y=0.0	9.63 to 10.35	10.20 to 11.00
Goodness of Fit		
R square	0.96	0.97
Sy.x	0.25	0.20
Is slope significantly non-zero?		
F	704.9	745.4

DFn, DFd	1.000, 29	1.000, 27
P value	< 0.0001	< 0.0001
Deviation from zero?	Significant	Significant
Equation	Y = -1.52*X + 15.11	Y = -1.26*X + 13.33

Appendix 3

Table 24. One-way ANOVA of the effect of the number of bacteria on EC at 4℃

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value	Significant ?
Interaction	0.06	3	0.02	F (3, 12) = 68.63	P < 0.0001	Yes
Time	0.28	3	0.09	F (3, 12) = 317.7	P < 0.0001	Yes
Bacterial number	0.26	1	0.26	F (1, 4) = 304.9	P < 0.0001	Yes
Subjects (matching)	0.00	4	0.00	F (4, 12) = 2.860	P = 0.0707	No
Residual	0.00	12	0.00			

Table 25. Dunnett's multiple comparisons test of compares the EC values of different number of bacteria at 4℃

Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	SE of diff.	Significant ?
Skim Milk				
10^4 vs. 10^7	-0.38	-0.41 to -0.33	0.01	Yes
10^5 vs. 10^7	-0.37	-0.41 to -0.33	0.02	Yes
10^6 vs. 10^7	-0.35	-0.39 to -0.31	0.04	Yes
Whole Milk				
10^4 vs. 10^7	-0.14	-0.17 to -0.10	0.01	Yes
10^5 vs. 10^7	-0.13	-0.17 to -0.09	0.02	Yes
10^6 vs. 10^7	-0.13	-0.16 to -0.09	0.01	Yes

Table 26. One-way ANOVA of the effect of the number of bacteria at 20℃

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value	Significant ?
Interaction	**0.09**	**3**	**0.03**	**F (3, 12) = 30.27**	**P < 0.0001**	Yes
Time	**0.50**	3	0.17	F (3, 12) = 163.2	P < 0.0001	Yes
Column Factor	**0.22**	1	0.22	F (1, 4) = 228.0	P = 0.0001	Yes
Subjects (matching)	**0.00**	4	0.00	F (4, 12) = 0.9344	P = 0.4766	No
Residual	**0.01**	12	0.00			

Table 27. Dunnett's multiple comparisons test compares the EC values of different number of bacteria at 20℃

Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	SE of diff.	Significant?
Skim Milk				
$\mathbf{10^4}$ **vs.** $\mathbf{10^7}$	-0.06	-0.14 to 0.02	0.03	No
$\mathbf{10^5}$ **vs.** $\mathbf{10^7}$	-0.01	-0.09 to 0.06	0.01	No
$\mathbf{10^6}$ **vs.** $\mathbf{10^7}$	0.45	0.36 to 0.52	0.04	Yes
Whole Milk				
$\mathbf{10^4}$ **vs.** $\mathbf{10^7}$	-0.01	-0.09 to 0.06	0.02	No
$\mathbf{10^5}$ **vs.** $\mathbf{10^7}$	-0.04	-0.12 to 0.04	0.01	No
$\mathbf{10^6}$ **vs.** $\mathbf{10^7}$	0.17	0.09 to 0.25	0.02	Yes

Table 28. One-way ANOVA of the effect of bacterial metabolism on EC in skim milk

Source of Variation	SS	DF	MS	F (DFn, DFd)	P value	Significant?
Interaction	0.08	20	0.00	F (20, 50) = 3.96	P < 0.0001	Yes
Time	0.91	5	0.18	F (5, 50) = 174.8	P < 0.0001	Yes
Bacterial inoculum	0.08	4	0.02	F (4, 10) = 81.70	P < 0.0001	Yes
Subjects (matching)	0.00	10	0.00	F (10, 50) = 0.22	P = 0.9931	No
Residual	0.05	50	0.00			

Table 29. Dunnett's multiple comparisons test compares the EC values of different initial bacterial inoculums in skim milk

Dunnett's multiple comparisons test	Mean Diff.	SE of diff.	95% CI of diff.	Significant?
Blank				
0 vs. 2	0.19	0.05	0.12 to 0.26	Yes
0 vs. 4	0.26	0.00	0.19 to 0.32	Yes
0 vs. 6	0.24	0.00	0.17 to 0.31	Yes
0 vs. 8	0.25	0.02	0.18 to 0.32	Yes
0 vs. 10	0.32	0.07	0.25 to 0.38	Yes
5.8x$\mathbf{10^2}$				
0 vs. 2	0.15	0.02	0.07 to 0.21	Yes
0 vs. 4	0.24	0.01	0.17 to 0.30	Yes
0 vs. 6	0.25	0.03	0.18 to 0.31	Yes

0 vs. 8	0.23	0.03	0.15 to 0.29	Yes
0 vs. 10	0.37	0.00	0.29 to 0.43	Yes
5.8x$\mathbf{10^4}$				
0 vs. 2	0.27	0.00	0.20 to 0.33	Yes
0 vs. 4	0.25	0.00	0.18 to 0.31	Yes
0 vs. 6	0.32	0.00	0.25 to 0.39	Yes
0 vs. 8	0.28	0.00	0.21 to 0.34	Yes
0 vs. 10	0.38	0.00	0.30 to 0.44	Yes
5.8x$\mathbf{10^6}$				
0 vs. 2	0.21	0.00	0.13 to 0.27	Yes
0 vs. 4	0.22	0.00	0.15 to 0.28	Yes
0 vs. 6	0.25	0.00	0.18 to 0.32	Yes
0 vs. 8	0.17	0.00	0.10 to 0.23	Yes
0 vs. 10	0.29	0.02	0.21 to 0.35	Yes
5.8x$\mathbf{10^8}$				
0 vs. 2	0.02	0.03	-0.04 to 0.09	No
0 vs. 4	0.1833	0.02	0.11 to 0.25	Yes
0 vs. 6	0.18	0.02	0.11 to 0.25	Yes
0 vs. 8	0.19	0.01	0.12 to 0.26	Yes
0 vs. 10	0.27	0.00	0.20 to 0.34	Yes

Table 30. One-way ANOVA of the effect of bacterial metabolism on EC in whole milk

Source of Variation	SS	DF	MS	F (DFn, DFd)	P value	Significant?
Interaction	**0.12**	**20**	**0.01**	**F (20, 50) = 4.293**	P < 0.0001	Yes
Time	**1.11**	5	0.22	F (5, 50) = 153.3	P < 0.0001	Yes
Bacterial inoculum	**0.05**	4	0.01	F (4, 10) = 6.953	P = 0.0061	Yes
Subjects (matching)	**0.02**	10	0.00	F (10, 50) = 1.248	P = 0.2851	No
Residual	**0.07**	50	0.00			

Table 31. Dunnett's multiple comparisons test compares the EC values of different initial bacterial inoculums in whole milk

Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	SE of diff.	Significant?
Blank				
0 vs. 2	-0.09	-0.17 to -0.01	0.03	Yes
0 vs. 4	0.12	0.04 to 0.20	0.04	Yes
0 vs. 6	0.15	0.07 to 0.23	0.00	Yes
0 vs. 8	0.17	0.089 to 0.25	0.03	Yes
0 vs. 10	0.21	0.12 to 0.28	0.05	Yes
5.8x$\mathbf{10^2}$				
0 vs. 2	0.12	0.042 to 0.20	0.06	Yes

0 vs. 4	0.27	0.19 to 0.35	0.00	Yes
0 vs. 6	0.31	0.23 to 0.39	0.00	Yes
0 vs. 8	0.27	0.18 to 0.35	0.06	Yes
0 vs. 10	0.42	0.34 to 0.50	0.03	Yes
5.8x$\mathbf{10^4}$				
0 vs. 2	0.14	0.06 to 0.22	0.03	Yes
0 vs. 4	0.23	0.14 to 0.31	0.04	Yes
0 vs. 6	0.30	0.21 to 0.37	0.04	Yes
0 vs. 8	0.33	0.24 to 0.41	0.03	Yes
0 vs. 10	0.43	0.34 to 0.51	0.03	Yes
5.8x$\mathbf{10^6}$				
0 vs. 2	0.09	0.01 to 0.17	0.06	Yes
0 vs. 4	0.24	0.15 to 0.31	0.04	Yes
0 vs. 6	0.31	0.23 to 0.39	0.03	Yes
0 vs. 8	0.26	0.17 to 0.34	0.03	Yes
0 vs. 10	0.32	0.23 to 0.40	0.05	Yes
5.8x$\mathbf{10^8}$				
0 vs. 2	0.04	-0.03 to 0.12	0.05	No
0 vs. 4	0.18	0.10 to 0.26	0.00	Yes
0 vs. 6	0.18	0.10 to 0.26	0.04	Yes
0 vs. 8	0.17	0.09 to 0.25	0.03	Yes
0 vs. 10	0.19	0.10 to 0.27	0.00	Yes

Appendix 4

Table 32. One-way ANOVA of effects of temperature on EC in all used milk types

Source of variation	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.58	30	0.02	F (30, 320) = 2.043	P = 0.0014
Time	6.44	10	0.64	F (10, 320) = 67.58	P < 0.0001
Milk types	6.19	3	2.06	F (3, 32) = 13.36	P < 0.0001
Subjects (matching)	4.94	32	0.15	F (32, 320) = 16.20	P < 0.0001
Residual	3.05	320	0.01		