ELIMINATION OF BACTERIA IN MILK BY PLASMA CORONA DISCHARGE

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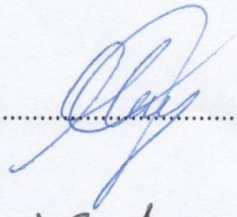
Assist. Prof. May Korachi ..... 
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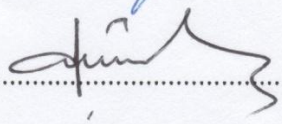
Assoc. Prof. F. Yeşim Ekinci ..... 
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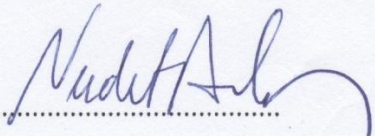
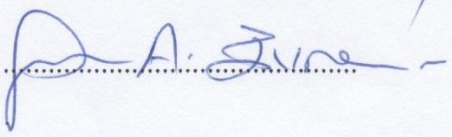
Prof. Necdet Aslan ..... 

Assoc. Prof. Gülsün Akdemir Evrendilek ..... 

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ABSTRACT

ELIMINATION OF BACTERIA IN MILK BY PLASMA CORONA DISCHARGE

Consumption of raw milk can be dangerous due to pathogenic microorganisms which may lead to serious diseases such as diarrhea, salmonellosis, gastroentritis and septicaemia. Growing demands towards safe, natural and fresh-like foods have led to the exploration of milder processing techniques for milk decontamination. Plasma technology is currently being applied in many different fields, including the medical and textile industry for sterilization and surface modification purposes. Plasma has several key benefits, since it is relatively cheap, works at low temperatures, and operates at atmospheric pressure. The work done in this M.S. report is part of a joint effort to develop a novel plasma system and test its feasibility on milk decontamination that involves microbiological, physical, chemical, shelf-life properties, and killing mechanism. Sterile milk was inoculated with *E. coli* ATCC 25922, *S. aureus* ATCC 25921, and *S.* Typhimurium ATCC 14028. AC power supplies operate at 9 and 15 kV were tested on antimicrobial activities at time intervals of 0, 3, 6, 9, 12, 15, and 20 min. The optimization of the plasma system and the determination of effects on milk and microorganisms are the subjects of this M.S. report. The pH, color, proteins, fatty acids, and volatile compounds of milk were monitored during treatment. Microbiological and chemical analyses were further investigated after plasma-treated milk samples were stored at 4°C for 6 weeks. For the determination of inactivation mechanism of plasma, SEM pictures were taken and bacterial DNA and cell wall fatty acid structure were analyzed. Following plasma 4, 2, and 2.6 log reductions of *E. coli*, *S. aureus,* and *S.* Typhimurium, respectively were obtained in milk. The pH, color, and proteins of the milk were not affected while fatty acid and volatile compound concentrations slightly changed after treatment. Storage microbiological results showed sterilization after 1 week, however no changes were observed in the protein, fatty acid, and volatile compound composition of the milk. Plasma causes destruction of the cells and DNA, however the total fatty acid profile was not affected.

ÖZET

PLAZMA KORONA DEŞARJ SİSTEMİ KULLANILARAK SÜTTEKİ BAKTERİLERİN ELİMİNASYONU

Çiğ süt tüketimi, patojen mikroorganizmalar nedeniyle ishal, salmonellosis, gastroentritis ve kan zehirlenmesi gibi tehlikeli hastalıklara neden olabilmektedir. Tüketici ve üreticilerin, daha güvenli, doğal ve taze gıdalara olan talepleri daha hafif işleme teknolojilerinin incelenmesini ve geliştirilmesini beraberinde getirmiştir. Plazma teknolojisi günümüzde tıbbi malzeme ve tekstil endüstrisinde sterilizasyon ve yüzey modifikasyonu gibi birçok alanda uygulama alanı bulunmaktadır. Plazma teknolojisi daha ucuz olması, düşük sıcaklıklarda ve atmosferik basınçta çalışması nedeniyle ümit vadeden bir teknolojidir. Bu M.S. raporu, özgün bir plazma sisteminin geliştirilmesi ve sütün dekontaminasyonu sırasında mikrobiyolojik, fiziksel, kimyasal, depolama ömrü özellikleri ile öldürme mekanizması analizlerini kapsayan ortak bir çalışmadır. Steril süt *E. coli* ATCC 25922, *S. aureus* ATCC 25921, ve *S.* Typhimurium ATCC 14028 ile inoküle edildi. AC güç kaynakları 9 ve 15 kV'da çalıştırılarak 0, 3, 6, 9, 12, 15 ve 20. dakikalarda antimikrobiyal aktivite tayini yapıldı. Bu çalışmanın amacı plazma sisteminin optimizasyonu ile süt ve mikroorganizmalar üzerindeki etkilerinin araştırılmasıdır. Sütün pH'ı, rengi, proteinleri, yağ asidi ve uçucu bileşikleri üzerindeki etkileri uygulama boyunca gözlemlendi. Mikrobiyolojik ve kimyasal analizler, plazma uygulanmış süt 4°C'de 6 hafta bekletildikten sonra tekrarlandı. Plazmanın inaktivasyon mekanizmasını daha iyi anlayabilmek için SEM fotoğrafları alındı ve bakterilerin DNA ve hücre duvarı yağ asidi yapısı incelendi. Sonuçlar, plazmanın sütte *E. coli, S. aureus*, ve *S.* Typhimurium için sırasıyla 4, 2 ve 2.6 log azalmaya sebep olduğunu gösterdi. Plazma uygulaması sütün pH, renk ve protein değerlerine etki etmezken, yağ aside ve uçucu bileşik konsentrasyonlarında bazı değişikliklere sebep oldu. Depolama sonrası mikrobiyolojik sonuçlarda, plazma uygulanan süt 1 hafta bekletildikten sonra üreme olmadığı görüldü. Ancak protein, yağ asidi ve uçucu bileşiklerde bir değişiklik gözlemlenmedi. SEM sonuçları, hücrelerin morfolojisinin ve DNA'nın plazmadan etkilendiğini, fakat hücre yağ asidi profilinin etkilenmediğini gösterdi.

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LIST OF SYMBOLS/ABBREVIATIONS

AC	Alternative current
CFU	Colony forming units
DC	Direct current
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleatide triphosphate
EDTA	Ethylene diamine tetra acetic acid
FAME	Fatty acid methyl ester
GC/MS	Gas Chromatograpgy/Mass Spectroscopy
h	Hour
L	Liter
M	Molar
min	Minute
mL	Mililiter
MUFA	Monounsaturated fatty acid
NTP	Non-thermal plasma
PCR	Polymerase chain reaction
PEF	Pulsed electric field
PUFA	Polyunsaturated fatty acid
rDNA	Ribosomal DNA
SDS-PAGE	Sodium dodecyl sulfate – Polyacrylamide gel electrophoresis
sec	Second
SEM	Scanning electron microscope
SFA	Saturated fatty acid
SPME	Solid phase microextraction
TBE	Tris/Borate/EDTA buffer
UHT	Ultra high temperature
VOC	Volatile organic compounds
µL	Mikroliter

1. INTRODUCTION

As the world population grows so does the need for more sustainable technologies that can provide maximum nutrition, non perishable products that have sustainable use at low costs to the consumer and cheaper productivity. Technologies that can positively address these requirements would aid in reducing economic losses inherent in the current techniques by improving the quality and shelf life of a product destined for both local and overseas markets, while simultaneously answering food safety criteria.

Food safety is a major concern for public health issue because of the emerging food-borne diseases and rise in the population of the world with the increasing demands. These expectations for fresher, more natural foods, less severely processed, contain less preservatives, free from artificial additives, have a sufficient shelf-life, nutritionally more advantageous and posing no microbiological or chemical health hazards brought up with the technological aspects of being convenient to handle, time efficient, cost effective and relevant for food safety and exportation criteria. These requirements along with the effort to increase the global economy, interest for both innovative and feasible novel technologies in food processing that provides sustainable solutions for both producers and industry is rising.

The benefits of the milk and its importance in human dietary life is evident for ages, however drinking raw and/or improperly treated milk can be infectious and cause severe illnesses including bloody diarrhea, fatal hemolytic uremic syndrome, hemorrhagic colitis and thrombotic thrombocytopaenic purpura [1, 2, 3]. Dairy related outbreaks are mostly related with pathogenic bacteria [4] such as *campylobacter* [5], *salmonella* [6], *yersinia* [7], *listeria* [8], M. *tuberculosis* [9], *brucella* [10], *staphylococcus* [11], *streptococcus* [12, 13] and *Escherichia coli* 0157:H7 [14].

Therefore, current legislation calls for milk before it is consumed to be properly treated either by pasteurization or ultra high temperature (UHT) which are general methods being used worldwide for decontamination of raw milk. Raw milk is heated up to 72°C and 138°C in pasteurization and UHT, respectively. The common process of pasteurization of

food, especially dairy products, is not intended to kill all microorganisms. The objective of pasteurization is only to achieve a "log reduction" in the total number of viable organisms. Subsequent refrigeration or freezing is intended to maintain the microorganism population at low numbers for long enough to allow consumption without causing disease, but over time the numbers of microorganisms and their metabolic byproducts, some of which are toxic, will inexorably increase. Furthermore, the heating process induces some changes that take place in the chemical and physiological composition of the milk and milk products. These changes have been widely discussed in the literature regarding browning [15], flavor changes [16], serum protein denaturation resulting in whiter appearance [17, 18], fat agglomeration and solids destabilization, freezing point depression, possibility of adulteration with condensate and boiler compounds and partial loss of some vitamins [15]. However an advantage of the higher temperature processes (UHT) is that it kills more microorganisms and thus increases the shelf life of the milk significantly. Although once the package is opened milk should be consumed within approximately a week, regardless of the type of pasteurization. The advantage of UHT pasteurization is that it facilitates cross-country shipment of dairy products simply because of the increased shelf life. Opponents of UHT pasteurization, mainly consumers of organic foods, point out that the UHT process is potentially damaging to the quality of the milk, and promotes national food distribution practices rather than local/regional food production and consumption.

The lack of technology that enhances microbial food safety and increases shelf life, while protecting the nutritional, functional and sensory characteristics of foods, is a challenge which this project was addressed and endeavours to fulfill with our novel non-thermal plasma (NTP) system.

Plasma is matter that contains partially or wholly ionised gas molecules and is the fourth state of matter. NTP systems were developed for use in delicate electronics and have been applied to many fields such as heat-sensitive medical devices and in biomedical, textile and polymerization applications [19, 20]. More recently, NTP's are being investigated for their application to foods and beverages [21, 22, 23]. However, comprehensive studies on food systems have yet to be carried out in the area of food processing. Limited studies on NTP's have shown it to injure and inactivate pathogenic bacteria on inert food contact surfaces, on some solid foods and in liquids [21, 22, 23].

Recent investigations about bactericidal effects of NTP include the evaluation of the inactivation of food-borne pathogens seeded onto thin films of agar, treated with a LTP [24] and also sprayed onto the surface of heat sensitive polyethylene terephthalate (PET) foils, exposed to a dielectric barrier discharge [25]. Deng *et al.* [22] reported up to a 5 log reduction of *E. coli* adsorbed to the surfaces of almonds, using NTP. The antimicrobial efficacy of NTP is related to the specific technology used, the power level used to generate the plasma, the gas mixture used in the plasma emitter and the intensity and length of exposure. Several studies to date, including those carried out by our research group, have shown NTP is able to inactivate a variety of microorganisms, including spores on solid surfaces and liquids.

NTP's can be used successfully against microbial cells and spores on surfaces with more than 4-log reductions. There are three basic mechanisms that have been attributed to the inactivation of microorganisms by plasma. These include destruction of DNA, volatilization of compounds and etching of the cell surface by adsorption of reactive species like free radicals [26]. These effects may vary due to the operational conditions and the design of the plasma generator.

The subject of this multidisciplinary study as well as its objectives, reaches out to the wider community and has a strong social element as they are aimed at tackling important food safety and commercial problems. Specifically, optimization of a novel and cheap atmospheric, non-thermal plasma corona discharge system for use in decontamination of milk.

2. THEORETICAL BACKGROUND

2.1. MILK AS A NUTRITION SOURCE

Sufficient and balanced food intake helps a person protect and improve his/her health as well as helping for a more quality life. Sufficient and balanced food intake is the concept of taking the amount of energy and dietary nutrients that is necessary for the body needs, every day. These requirements are fulfilled via four different food groups that are; meat and meat products, milk and milk products, vegetables and fruits and bread and wheat.

Milk and milk products such as cheese, yoghurt and milk powder have very important contributions in meeting human dietary requirements. Raw milk contains many nutrients such as carbohydrates, proteins, fatty acids, amino acids, vitamins and minerals. Because of its rich nutritional value it is a quick and easy way of supplying human dietary requirements for energy, protein and several key minerals and vitamins. On the other hand, milk is crucial for a human being during growth and structural development because of the contents like immunoglobulin, enzymes, inhibitors, growth hormones and factors, antibacterial agents, proteins, peptides, fatty acids, vitamins and minerals which are crucial in every period of human life [27].Therefore, it is highly recommended and encouraged to consume milk every day by clinicians especially to women, children and young people.

Milk is very a very important sector, because of its part in human diet in every stage and also due to added value to economy of the country. Milk and milk products cover 16% of the whole food industry. Turkey is one of the countries with highest milk production which was ranked 15th with 10 million tons of production every year, from which 90% is cow milk. Suggested amount of daily milk consumption varies according to age and it is 0.5 liters per day for children (age 1-8), 1.08 liters per day for young people (age 9-18) and 0.9 liters per day for adults (age 19+). However, it is a problem in developing countries to find sufficient and quality milk. The collection and quality control processes increases the costs up to 15 % which results in high consumer prices of processed milk. Turkey ranks the 15th in the whole world for milk production. It produces 10 million tons of milk every

year from which 90 % is cow milk. The market for dairy food in Turkey increased at a compound annual growth rate of 4.7 % between 2004 and 2009. However, companies are unable to obtain any profits due to the structure of small enterprises, lack of proper farming controls, inefficiency of existing cooperatives, cyclical (seasonal) changes in milk quality, problems in milk collection and the high production costs of EU standards. Furthermore, the milk produced in Turkey is not suitable for food sanitation due to problems such as antibiotic addition, uncontrolled structure of plants and high temperatures during harvesting and processing. Because of these facts, the FAO prepared a report for milk activity plan for Turkey in collaboration with the European Commision and Ministry of Agricultural and Rural Affairs.

2.2. COMPOSITIONAL PROPERTIES OF MILK

Sheep, goat, cow, camel and buffalo can be milked and used for dairy production. The nutritional compounds differ according to the type of animal being milked. The greatest yield from milking and production process is obtained from cow milk. Nutritional value of cow milk is affected by seasons, physiological aspects, diseases and so on [28, 29]. Needs to be explained little more

2.2.1. Energy

Raw milk energy content is composed of macro molecules such as carbohydrates, fat and protein [30, 31] as well as organic acids and alcohol [32, 33]. The amount of energy is 70, 50 and 38 kcal in 100 g of whole, semi-skimmed and skimmed milk, respectively.

2.2.2. Carbohydrates

Lactose is the main carbohydrate in raw milk. Approximately 4.7% of the milk is composed of lactose [34] which is 54% of dry weight except the fat. Also galactose, glucose and oligosaccharides are present. Glucose and galactose are produced by hydrolytic properties of lactase enzyme. It is possible to produce lactose-free milks using this enzyme [30, 32].

2.2.3. Fat

Fat content of the milk is very important due its effects on properties of milk. These properties are appearance, taste, flavor and resistance. On the other hand, fat is a source for essential fatty acids, vitamins (A, D, E, K) and energy. Fat forms a globular structure in water suspension. These fats are found in forms of tri-glyceraldehydes (97%), phospholipids (1%), free sterols such as cholesterol and wax (0.3%), free fatty acids and derivatives and vitamins (A, D, E, K) [30, 32]. While normal raw milk contains 3% fat, whole milk can be changed by processes into semi-skimmed and skimmed milk with 1.5% and 0.1% fats, respectively.

Total milk fat is made up of triglycerides (97-98% of total milk fat) and the rest containing phospholipids, cholesterol, diacylglycerol, monoacylglycerol and free fatty acids. Milk contains three kinds of fatty acids which are saturated fatty acids, mono- and poly-unsaturated fatty acids. Saturated fatty acids (SFA) represent the greatest amount (64%) of all fatty acids. Approximately 12% of SFA are short-chain fatty acids ranging from C4:0 to C10:0. Stearic acid (C18:0) makes up a further 12% and the remaining SFA contain lauric acid (C12:0), myristic acid (C14:0) and palmitic acid (C16:0). Monounsaturated fatty acids (MUFA) represents approximately 30% of the fatty acids and 4% are poly-unsaturated fatty acids (PUFA). Most of these unsaturated fatty acids are found in cis- double bond form and a smaller proportion with 4%, are trans- fatty acids. From these fat contents of the milk, saturated fat, conjugated linoleic acid, spingomyelin, butiric acid and miristic acid were described to be beneficial for health [32, 35].

2.2.4. Protein and Amino Acids

2.2.4.1. Properties of Proteins

Amino group of a amino acid and carboxyl group of another reacts to form a peptide bond and a mole of water is produced [36]. Therefore, 20 different amino acids with different radical groups come together to form different kinds of proteins.

Raw milk contains approximately 3% high quality proteins including; casein, whey proteins, enzymes and low amount of nitrogen-compounds [27]. Casein and whey proteins

are found in very high amounts and compose 80 and 20 %, respectively. These proteins made up of essential amino acids such as leusin, isoleusin, valine, methionine, phenilalanine, threonine, thryptophane, and lysine. These essential amino acids refer to the key factors for high quality proteins [32, 35, 37].

2.2.4.2. Properties of Amino Acids

Amino acids are found in important amounts in milk and milk products as proteins. Eight amino acids are defined as essential for humans since they cannot be synthesized in human body which are phenylalanine, valine, threonine, tryptophan, isoleucine, methionine, leucine, and lysine [38] and partially histidine and arginine. Additionally, alanine, glycine, glutamine, proline, serine, aspartate, cysteine and tyrosine are found in milk structure [32, 37, 39, 40].

The importance and functions of essential amino acids can be given as follows [27, 35, 41, 42]:

Methionine: Contains sulfur in its structure and is found in 3-6% in proteins. Methionine serves as a methyl donor in biochemical reactions. Methionine is found in inadequate amounts in plant proteins.

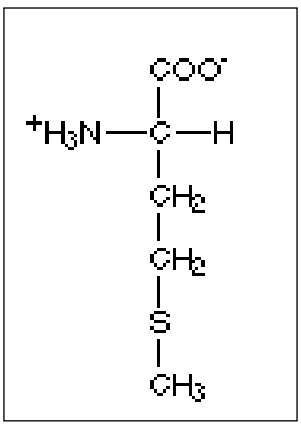

Figure 2.1. Molecular structure of methionine

Tryptophan: Zein, elastine, gelatin and collagen contains high amount of tryptophan, however the level in wheat is insufficient. Niacin which is a vitamin essential for human

life can be synthesized by tryptophan. Also it serves as an initiator of an important neurotransmitter, serotonin.

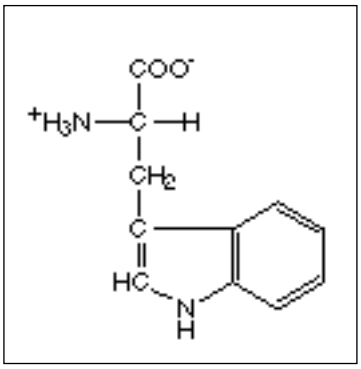

Figure 2.2. Molecular structure of tryptophan

Threonine: is present in great amounts (3.5-5%) in proteins and serves as a phosphate group carrier in phospho-proteins. Threonine is effective in collagen and elastin formation.

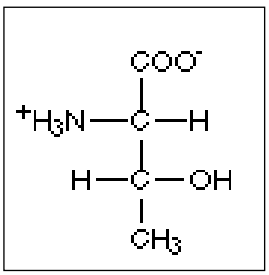

Figure 2.3. Molecular structure of threonine

Phenylalanine: is an aromatic amino acid that consists of 4-5% of proteins. It is converted to tyrosine in organisms and reacts in nor-epinephrine synthesis therefore playing an important role in central nervous system.

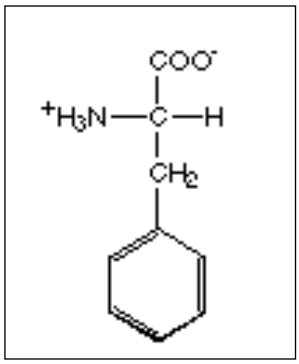

Figure 2.4. Molecular structure of phenylalanine

Leucine: is a branched-chain amino acid which can be found in most of the proteins such as gelatin in low amounts and wheat proteins in higher amounts. Leucine functions in nitrogen cycle in infants during growth and has effects on the blood glucose level.

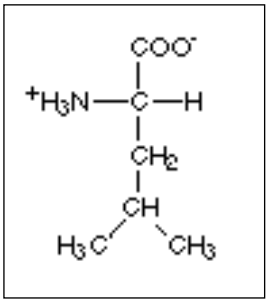

Figure 2.5. Molecular structure of leucine

Isoleucine: is a branched-chained amino acid and can be found in meat, milk and egg proteins with a percentage of 5-6.5%. Isoleucine can be formed by digestion of fibrin and hemoglobin synthesis. Also has beneficial effects regarding nitrogen cycle and blood glucose level.

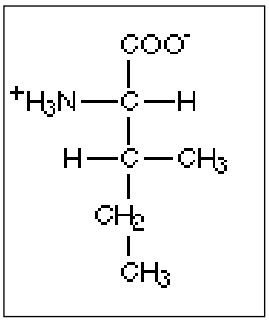

Figure 2.6. Molecular structure of isoleucine

Valine: is a branched-chain amino acid with an aliphatic radical group. Valine is a constituent of fibrous protein in the body and participates in hydrophobic interactions. The molar rate of valine is 6.9% and regulates biochemical reactions in muscle and nervous systems.

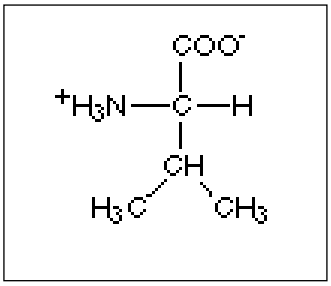

Figure 2.7. Molecular structure of valine

Lysine: rates 6-8% of milk, egg and meat proteins. It is an essential component for growth, tissue repair, immune system and bone development in children. Lysine plays an important role in absorption and conservation of calcium and collagen formation.

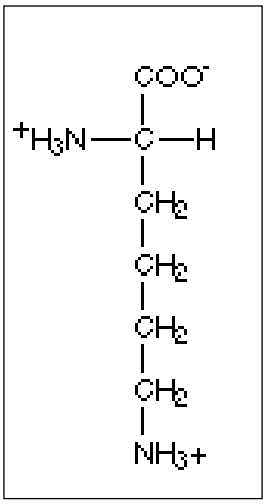

Figure 2.8. Molecular structure of lysine

2.2.5. Vitamins

Almost every vitamin necessary for human health are present in raw milk. Some of these vitamins are A, D, E, K, C, B1, B2 and folic acid [34]. The amount of fat soluble vitamins (A, D, E, K) varies regarding the type of milk (whole, semi-skimmed and skimmed). Whole milk contains higher amount of vitamin A is required for vision, immune system and growth and development of body tissues. Vitamin D plays an important role in the absorption of calcium and phosphorus. Vitamin E serves as an anti-oxidant and reduces the risk of diseases such as cancer. Vitamin K is essential for blood clotting, however it is found in very little amounts in milk.

Milk also contains water soluble vitamins B1, B2, B3, B5, B6, B12, vitamin C, and folate. Thiamin (vitamin B1), riboflavin (vitamin B2) and vitamin B12 are the most predominant vitamins in milk and are found in higher amounts compared to niacin (vitamin B3), pantothenic acid (vitamin B5), vitamin B6, vitamin C, and folate. Despite being an important source, vitamin content is decreased because of heat processing [32]. It was demonstrated that boiling milk for 10 minutes results in 60, 25, 12, 21 and 32% loss, respectively in thiamin, riboflavin, niacin, vitamin B12 and folate content [43].

2.2.6. Minerals

Milk is a good source of minerals including calcium, phosphorus, potassium, magnesium, selenium, and zinc [34]. Minerals are essential for enzyme functions, bone health, water balance maintenance and oxygen transport. Mineral content of milk is related with the physiological situation of cow, lactation stage, environmental factors, genetics and processing techniques [32, 35].

2.3. CHEMICAL, PHYSICAL AND SENSORY PROPERTIES OF MILK

Outstanding points of milk are the color, odor, taste, appearance and texture. Milk can specified according to these properties and the quality, physical, chemical and microbiological aspects can be determined. Milk has a viscose, homogenous structure and white or creamy in color. The calcium caseinate and fat molecules cause milk to appear white while the color changes to greeny yellow when casein is isolated and to blueish white after fat content is disregarded [44, 45, 46].

Due to lactose, fat and mineral content of milk, the taste is mildly sweet and pleasant. Thermal processing and/or feeding condition of animal may result in bad taste [27, 36].

Fat molecules in milk might absorb scents [44] hence affected by environmental factors and animal hormonal defects and bacterial infections [28, 45].

Milk, as a solution of a lot of components including lactose and minerals, has a lower freezing point than distilled water, which is approximately -0.55°C. Boiling the raw milk results in degradation of the most of the components so that leading to increased freezing point [44]. Since the freezing point is a characteristic property of milk, it can be used as a marker of artificial additives [41, 44, 46]. Also the same components (lactose and minerals) increase the degree for boiling to 100.16°C which can also be an indicative of additives to milk [45].

2.4. BENEFICIAL HEALTH EFFECTS OF MILK

Milk and milk products may be given as functional foods with regards to the number of bioactive components that have been described above. The growing interest and knowledge about foods that are beneficial to the prevention of chronic human diseases is growing rapidly. Some of the advances of milk as a functional food have been related to fat content of the milk from which the most significant is conjugated linoleic acid (CLA) [47]. CLA is a potent, natural anti-carcinogen, moreover possessing anti-atherogenic and immunomodulatory properties [47]. Also, vaccenic acid and rumenic acid have been showed to have anti-carcinogenic and anti-atherogenic effects [48, 49]. On the other hand, rumenic acid was demonstrated to improve the plasma cholesterol profile and reduce coronary heart disease risk [48].

The most abundant beneficial effect of milk is on bone growth and development and maintenance of healthy teeth. But it also serves as a modulator and regulator for blood pressure, cardiovascular disease, levels of cholesterol in the blood, obesity, type 2 diabetes, cancer (colorectal and breast cancer) and hydration of the body.

Milk contains calcium, phosphor and vitamin D which are essential for bone health and lack of these nutrition results in bone related diseases such as osteoporosis [50]. Inadequate calcium intake should be supplemented especially in adults in order to decrease the age related bone tissue degradation. Also, calcium, magnesium and phosphor were stated to be beneficially related with blood pressure and hypertension with a mechanism of inverse proportion between calcium intake and blood pressure [51, 52]. This mechanism is described via parathyroid hormone and vitamin D concentrations [53]. Obesity is a growing chronic health problem which also may lead to hypertension and type 2 diabetes. Dealing with this disease is carried out by targeting adipose tissue which can be evacuated with the help of calcium [53, 54]. This mechanism is described by inhibition pathways between vitamin D and parathyroid hormone like in blood pressure [53]. Cancer is the most contradicting of them all has been studied by several researches. Results from these studies shows that a higher intake of calcium can protect against bowel cancer. Although, milk has been claimed to be linked with risk of developing prostate and ovarian cancers,

in-depth studies related to milk fat compounds and cancer stated anticarcinogenic properties of milk [47, 48, 49].

2.5. MILK-BORNE PATHOGENS

Nutritious value of milk is described above but this property also makes it a good source for many microorganisms as a rich medium for the growth. Fresh raw milk normally has a low microbial load [55], however the load may rise up to more than 100 fold at normal temperatures [56]. When the milk is produced at farms first it contacts with the dairy cattle. During handling and processing, some adverse changes in the milk may occur due to lactic acid bacteria growth and/or coliform bacteria. On the other hand, a cow with mastitis may contaminate fresh clean milk at high microbial load concentrations [57]. The presence of pathogens in milk is a possible marker of contamination from the udder, milk utensils or water supply used [58, 59]. The etiological agents in milk-borne diseases change dramatically, however, up to 90% of all dairy-related cases are of bacterial origin. Milk-borne outbreaks have been associated with the consumption of milk contaminated with *Listeria monocytogenes, Salmonella, Campylobacter, Staphylococcus aureus, Bacillus cereus* and *Clostridium botulinum*. Presence of these pathogenic bacteria in milk has been a major public health concern since milk is very susceptible to both spoilage and pathogenic microorganisms. Therefore, current regulations are being based on thermal processing of raw milk for the inactivation of microorganisms before being consumed.

2.5.1. Escherichia coli and Eschericial Infections

Escherichia coli presence in food samples evidences the risk of contamination with enteric pathogens, therefore it is used as an indicator microorganism in food systems [60]. Determination of *E. coli* in milk, generally results from fecal contamination of the milk. *E. coli* 0157:H7 is a highly pathogenic strain of coliform bacteria and has always been a serious problem for the dairy industry. Several outbreaks have been reported in diseases developed by *E. coli* O157:H7 infection, including mild diarrhea, fatal hemolytic uremic syndrome (HUS), hemorrhagic colitis and thrombotic thrombocytopenic purpura [1, 2, 3, 61, 62, 63]. Generally, insufficient cooking process fails to eliminate *E. coli* O157:H7 in food and leads to infection by ingestion of contaminated raw milk. Unfortunately, dairy

cattle reserve great amounts of this pathogen, being an important source for this pathogen [1, 64]. The dairy cattle harbor *E. coli* O157:H7 in their intestinal tract which makes the treatment of milk before consumption more crucial.

2.5.2. Staphylococcus aureus and Staphylococcal Infections

Infections with methicillin-resistant strains of *Staphylococcus aureus* (MRSA) is the lead health care associated infection [65] and increased 10 fold in the last decade with 368,000 hospitalizations [66]. Staphylococcal infections are usually associated with low hygiene and sanitation practices. Common items used in daily life such as badges, beepers and cell phones are potential carriers for *S. aureus* [67].

Many outbreaks and illnesses are reported caused by *S. aureus* [68]. A study showed that, among the other food-borne pathogens, *S. aureus* represents the largest proportions for outbreaks with 66% in England and Wales [69].

Once a food is consumed which was contaminated with Staphylococcus, intoxication occurs due to staphylococcal enterotoxins. Milk has been implicated of staphylococcal infections with one of the largest outbreaks which have occurred in 2000 in Japan with 13,420 hospitilizations. The cause of the infection was enterotoxin-producing strain of *S. aureus* [70]. Also it was reported in Spain that 75% of dairy related outbreaks are of Staphylococcal infection [71].

2.5.3. Salmonella Typhimurium and Salmonellosis

Salmonella spp. are major pathogenic bacteria which can be most frequently found in milk and milk products [70, 72]. Salmonella spp. can cause serious health problems in milk and milk products since its presence even at low levels can pose a health risk [73, 74]. Several outbreaks and cases reported in association with the consumption of contaminated dairy products [70, 75, 76].

The need of the hour for the food industry is a rapid, simple, sensitive, specific, online and affordable technique for detection of pathogens including *Salmonella*

Typhimurium in foods. There is an increase in the *S.*Typhimurium cases because of contamination in pasteurized milk which makes milk an important carrier for Salmonella from infected farm animals to humans [77].

On the other hand, Salmonella has species such as enterica serovar typhimurium Definitive Phage Type 104 (DT 104) which is resistant to some antibiotics including ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline [78, 79]. Food-borne outbreaks due to *Salmonella* Typhimurium DT 104 transmission were documented in the UK, with possible transmission vehicles such as roast beef, ham, pork sausage, salami sticks, chicken legs, and raw milk [80]. Also it was reported in the United States that this serotype of Salmonella ranked second in the most frequently detected in food-borne salmonellosis [81].

2.6. DECONTAMINATION TECHNIQUES

During the last centuries, the food processing technology and food-borne illnesses has changed dramatically all over the world. Though technologies have been legislated such as pasteurization and proper canning in order to decrease food-borne diseases, new agents have been detected as causative for food-borne illnesses. Outbreaks were limited to a number of cases in a definite area because of high contamination doses due to personnel errors. However, with the increased rate of export/import and consumption of ready to eat foods, outbreaks have emerged to a huge extent and spread to a wide area also involving different countries resulting in higher number of patients. The technologies that are being used are based on heat treatments to kill the germs for food sterilization and preservation. However these technologies can be detrimental in damaging other sensory and nutritional properties. Such factors have motivated scientists to explore alternative treatments that are more effective, cheaper, and less disruptive to nutritional quality than traditional thermal processing while also eliminating pathogenic microorganisms in food. In the search for less severe or 'minimal processing' technologies new, 'non-thermal' methods of food preservation are taking the lead. Mild preservation technologies enhance the shelf life of foods, are usually applied at room temperature and have a minor impact on the quality and fresh appearance of food products. They are referred to as mild processing since they pose little stress on foods. This, on the other hand, increases the importance of food safety

considerations. Extended shelf life and a fresh-like product presentation emphasize the need to take full account of food safety risks, alongside possible health benefits to consumers.

Application of the technologies listed above offers various opportunities for mildly processed products by preserving their sensory quality, nutritional value and appearance. However, the application potential of any new technologies, which are coming from research laboratories and not 'sanctioned' by centuries of empirical use, is influenced by many factors:

1. Technological feasibility
2. Technical possibility
3. Health impact
    a. Wholesomeness of the product
    b. Occupational safety
4. Environmental friendliness
5. Economic feasibility (including their energy demand)
6. Infrastructural conditions/requirements
7. Investment need and availability of investment power
8. Political attitude
9. Social consequences
10. Psychological aspects/ risk-benefit perception.

Current popular, non thermal technologies under investigation include: high pressure processing (HPP), high voltage electric field pulses (HELP) and pulsed electric field treatment (PEF) [82]. Possibilities to use these technologies for preservation of food products are reported and effects on quality of food products are being studied. Although, it is hoped that some of these products will be marketable in the near future, these techniques do hold some disadvantages. Success of these technologies for further development depends on effective control and careful establishment of their performance criteria for the reduction of relevant pathogens to the required safety standards [83].

2.6.1 Pasteurization

Pasteurization is a thermal decontamination technique, first designed in 1857 to inactivate tubercle bacillus. Milk is heated up to 72 - 80ºC in pasteurization process for 12 - 16 seconds. This process eliminates the major pathogenic and spoilage bacteria in raw milk further increases the shelf-life if pasteurized milk is kept at refrigerated conditions. The common process of pasteurization of milk is not intended to kill all microorganisms. The objective of pasteurization is only to achieve a "log reduction" in the total number of viable organisms. Subsequent refrigeration or freezing is intended to maintain the microorganism population at low numbers for long enough to allow consumption without causing disease, but over time the numbers of microorganisms and their metabolic byproducts, some of which are toxic, will inexorably increase.

However, several bacterial infections including salmonellosis have been reported due to consumption of pasteurized milk [84, 85]. So, it is necessary to further increase the shelf-life of pasteurized milk, in order to increase the convenience of the consumers and protect the products against temperature abuse. Thus, it is important to avoid the onset of cooked flavor, which would result from more severe pasteurization temperatures.

2.6.2. Ultra High Temperature

In ultra-high temperature (UHT) process the milk is heated to 135-150 ºC for 2-6 seconds which sterilizes the milk. This increased temperature levels yields more microbial inhibition hence increasing the shelf life of the milk significantly. While the shelf-life of pasteurized milk varies within 14 to 21 days after processing, UHT milk has a shelf life between 45 to 55 days and after aseptic packaging sealed boxes have a shelf-life of six months. Therefore making cross-country shipments possible for UHT treated dairy products since the shelf life is increased. However UHT process potentially damages the quality of the milk. When milk is heated during this process, enzymes are inactivated, chemical reactions take place and some changes may occur in the physical properties of milk. These changes can include decrease in pH, precipitation of calcium phosphate, denaturation of whey proteins and interaction with casein, maillard browning and modifications to the casein micelle. These changes have been widely discussed in the

literature regarding browning [15], flavor changes [16], serum protein denaturation resulting in whiter appearance [17, 18], fat agglomeration and solids destabilization, freezing point depression, possibility of adulteration with condensate and boiler compounds and partial loss of some vitamins [15]. However an advantage of the higher temperature processes (UHT) is that it kills more microorganisms and thus increases the shelf life of the milk significantly. UHT milk has a shelf life of 45-55 days in standard packaging and up to six months in sealed aseptic boxes. Although once the package is opened milk should be consumed within approximately a week, regardless of the type of pasteurization.

Although, thermal processes such as pasteurization and ultra high temperature (UHT) are the predominant techniques currently used, they have undesirable effects on the nutritional, sensory, organoleptic, probiotic and immunopotency properties of milk. Furthermore, pasteurization is ineffective against spores and has a short shelf life, while UHT inhibits further processing of products (e.g. cheese) and decreases milk yield. Therefore, many producers in developing countries prefer the use of low processing temperatures and time to provide higher yields resulting in poor quality, and un-exportable products.

Although well-established traditional processing options, such as thermal processing answers the calls for microbial safety in foods, it has the disadvantage of degrading the quality of foods to some extent and can be detrimental in damaging other sensory and nutritional properties [15, 16, 17, 18]. In contrast, non thermal emerging techniques that either use new 'protective cultures' utilizing their antagonism against pathogenic microorganisms [86] or new 'biopreservatives' such as bacteriocins [87, 88] or are predominantly reliant on physical processes that include, ionizing radiation treatment; high hydrostatic pressure treatment; pulsed electric fields and low-temperature plasma that inactivate microorganisms at ambient or moderately elevated temperatures and short treatment times [82]. At this moment, novel technologies such as high pressure, pulsed electric fields and the use of biopreservatives are beyond the first development phase.

2.6.3. Ionizing Radiation

The most popular non thermal treatments that are currently under investigation include ionizing radiation for sterilization of medical products and pasteurization of food. These applications are usually in one of the following forms: (1) gamma radiation, (2) beta radiation, (3) electron beam, and (4) X-ray. These forms of energy allow cold decontamination because heat is not directly applied to the material being sterilized. Due to the ambient temperatures and the penetrating nature of ionizing radiation, it is often possible to sterilize food, drugs, medical materials, equipment, and supplies after they have been packaged and thermally sealed, thereby minimizing the chances of recontamination before use. Ionizing radiation is a versatile form of processing energy used already in a wide range of non-food applications [89, 90]. Its slow implementation can be explained by; the long time which was needed to demonstrate adequately the safety and wholesomeness of irradiated food, the lack of readily available radiation facilities, their investment-demanding character, as well as an inadequate awareness of problems which justify the use of this technology. The safety and nutritional adequacy of irradiated food have been well established [91, 92], the technology is ready to use technically, and the need to improve the microbial and parasitic safety of food became a major driving force behind the implementation of food irradiation in both developed and developing countries. Irradiation treatment has significantly reduced the colonies of *E. coli* and *Salmonella* in vegetable juice [93].

The mechanisms of all forms of ionizing radiation are thought to be the same during sterilization process. The mechanism occurs in direct and indirect routes. While direct mechanism of ionizing radiation targets directly the cellular components, indirect mechanism destructs the microbial structure via radiolysis products such as H^+ and OH^- radicals which are produced by hydrolysis of water during ionizing radiation. During the indirect act mechanism, the major structural component of the cell, chromosomal DNA, is damaged which in turn renders the cells non-viable, also with the cell membrane therefore leading immediately deactivation of microorganisms.

This radiation process brought out a variety of concerns regarding the safety of irradiation-treated food products. Several studies have been carried out on the effects of

radiation sterilization on foods, and on humans and demonstrated no evidence to date, that this technology has potential health risks to humans. However, this treatment technology causes some degradation to the quality of foods [94, 95]. These changes in the quality can be given as undesirable flavor formation in some dairy products and textural deformations in some fruits such as nectarines and peaches after ionizing radiation treatment [96].

2.6.4. High Pressure

The studies about development of high pressure processing (HPP) for milk and vegetable products treatment have a background of more than 100 years when the first investigations of Hite [97] and Hite *et al.* [98] were established. The HPP involves using high pressures from 300 to 700 MPa and can be modified for selective commercial applications in food processing. HPP is being used in 120 industries with batch processing volumes [99, 100]. The HPP inactivates bacterial endospores using these pressures while the temperature can be modified from 0 to 100ºC depending on the structure of process [101]. This process is being conditions is tested on spore inactivation using high pressure in combination with moderate heat treatments in order to achieve inactivation of bacterial spores at temperatures lower than that normally associated with heat sterilization processes [102].

Although HPP results in significant reductions of microorganisms, it also has some serious limitations, including 1) changes in the pH, 2) the inability of inactivating pressure resistant vegetative bacteria after successive pressure treatments, 3) large technological requirements and costs, and 4) non-continuous nature of the process [103, 104].

Due to these facts, feasibility of this technology for further development depends on effective control by appropriate combination of different treatments and additional research for required safety factor while carefully established performance criteria for reduction of the number of relevant pathogens [83].

2.6.5. High Voltage Electric Pulses

High voltage electric field pulses (HELP) to effect non-thermal inactivation of microorganisms in foods was also explored, and these studies led to the development of prototype and industrial scale devices recently [101]. HELP treatment is the application of pulses of very high field strength (2-5 V.um-1) for a very short time (microseconds) to foods between two electrodes. The treatment requires fairly complex electronic and fluid handling systems. However, the application of HELP will probably be limited to liquid foods or liquids containing small particulates [83]. There are still, however, considerable knowledge gaps that will need to be addressed, and regulatory hurdles to be overcome, before commercialization of the technology.

The efficacy of the HELP technology has been tested on a variety of microorganisms, including bacteria (vegetative cells and spores), molds and fungi, viruses, and protozoa. Studies on combination processes have shown potentially useful synergies because "electroporated" bacterial cells become much more sensitive than untreated cells (e.g. to bacteriocins). The process parameters depends on the type of application and some wavelengths, such as UV, are known to be more effective than others regarding ability of inactivating microorganisms. However, UV light can cause undesirable photochemical effects while longer wavelengths are more likely to cause photothermal (heating) effects.

Mechanism of the light in the UV spectrum depends on the destruction of DNA molecule. Also, the inhibition effect of HELP treatment on many microorganisms is observed due to direct destructive effects on cell membranes, motor proteins, enzymes and other cellular structures in addition to the structural changes in DNA.

2.6.6. Pulsed-Electric Field

A typical PEF system consists of a high voltage pulse generator, a treatment chamber, a fluid handling system and monitoring devices. The typical PEF treatment parameters differ depending on the particular PEF systems used, including pulsed field intensity of 15-50 kV /cm, pulse width of 1-5 ms, and pulse frequency of 200-400 Hz (pulses/s).

Floury *et al.* [105] have demonstrated a pilot PEF system, and its capabilities on inactivation of total viable count in raw skim milk and the number of *Salmonella* Enteritidis inoculated into milk at moderate temperatures (<50°C). Studies on PEF disinfection were performed in wide range of microorganisms and supporting media including Gram-positive (*Bacillus cereus, Listeria monocytogenes* NCTC 11994) and Gram-negative (*Escherichia coli* NCTC 9001) bacteria in peptone solution [106, 107], and *E. coli* O157:H7 inoculated into apple juice [108].

There are some hypotheses in inactivation process and the generally accepted one is the electroporation theory by Zimmermann [109] and Tsong [110]. This theory describes the electric potential produced by PEF treatment is greater than then the microbial trans-membrane potential therefore leading to irreversible cell membrane destruction and inhibition of the cell permeability [111, 112, 113]. Another study on mechanism of cell death post-PEF treatment stated that membrane permeabilisation was associated with the species of microorganisms and the pH of the treatment medium [114].

Also reversible permeabilisation of bacterial cells was observed which was responsible for the sub-lethal injury of the cells caused by PEF, and this was stated to be related with the target microorganism, pH of the treatment medium, and the field intensity used [114]. Gram-positive bacteria (*Bacillus subtilis, Listeria monocytogenes*) showed greater susceptibility by means of sub lethally injury to PEF treatment at pH 7.0 than at pH 4.0, and adversely Gram-negative bacteria (*E. coli, E. coli* O157:H7, *Pseudomonas aeruginosa, Salmonella* Senftenberg, *S.* Typhimurium, *Yersinia enterocolitica*) were more susceptible to treatment at pH 4.0 than at pH 7.0.

PEF treatment was tested on several microorganisms and different foods for its bacteria inactivation potential and storage properties. These studies were carried out in beverages such as blended orange and carrot juice [115], apple juice and cider [116], cranberry juice and chocolate milk [116], tomato juice [117, 118], and orange juice [119, 120, 121, 122]. Among these studies Grahl and Markl [123] reported that the microbial inactivation potential of PEF was negatively affected by the fat content of milk. However, another study showed no significant differences in the reduction rates of *L. monocytogenes* in whole, semi-skimmed and skimmed milk by PEF treatment [124].

The most recent advances in non thermal processing technology have been the use of PEF. Conversely, PEF has several disadvantages in that application is restricted to liquid food products with no air bubbles and low electrical conductivity. Furthermore, PEF is a continuous processing method, which is not suitable for solid food products that are not pumpable. The maximum particle size in the liquid must be smaller than the gap of the treatment region in the chamber in order to ensure proper treatment. In spite of the large body of research on PEF, many questions remain unclear with many contradictory research results. Moreover, PEF studies have shown this technique to have problems inactivating microorganisms in products such as milk that contain a high fat percentage (e.g. cream with 18% fat). This is believed to act as a protective shield, restricting the effects of PEF [125]. Moreover, the studied variables, polarity, pulse number, pulse width, electric field intensity, and the combined action of pulse number with pulse width or electric field intensity significantly affect the microbial death meaning that a variety of parameters should be considered and optimized before the system is adjusted for a specific use [126].

2.7. PLASMA

Perhaps the most novel of all non-thermal technologies being investigated as a possible food processing tool is non thermal plasma (NTP) also referred to as cold atmospheric plasma. Plasma is the fourth state of matter and is a gaseous mixture of both negatively and positively charged particles with a net zero electrical charge. High energy radiation or electric field can ionize gas molecules and particles resulting in the formation of released electrons, and charged ions and heavy particles. These species are produced during the ionization by collision of electrons or photons with neutral atoms and/or other molecules when sufficient energy is provided.

Plasma was first described by Sir William Crookes in 1879 and electron beams were identified by Sir J.J. Thomson in 1897 [127]. The name “plasma” was first used by Irving Langmuir in 1928 who described this state of matter as: “Except near the electrodes, where there are *sheaths* containing very few electrons, the ionized gas contains ions and electrons in about equal numbers so that the resultant space charge is very small. We shall use the name *plasma* to describe this region containing balanced charges of ions and electrons” [128].

Plasma was considered as a distinct state of matter due to its properties. Plasma does not have a regular shape or volume and it may form filament, beams and double layers under magnetic field. Some very common examples to plasmas can be given as stars and neon lamps.

Plasma is being used in fluorescent and neon lights and developed for use in industries such as microelectronics and automotive. The research areas of plasma technology are growing every day and has been particularly used for bio-medical materials and devices [129, 130, 131], surface modification of textiles [20], removal of chemicals on surfaces of medical devices manufactured from heat sensitive plastics [19], water sterilization [21] and most recently food sanitation systems [23]. Studies to date on plasmas have shown its capability to injure and inactivate pathogenic bacteria on inert food contact surfaces, on some solid foods and in liquids [21, 22, 23]. The antimicrobial efficacy of NTP is related to the specific technology used, the power level used to generate the plasma, the gas mixture in the plasma emitter, the intensity and length of exposure, design of the system, flow rate and pressure.

Plasmas can be divided into two groups depending on the method of generation as Non-Thermal Plasma (NTP), and Thermal Plasma (TP). NTP consists of gas molecules with moderate temperature and electrons with higher temperature whereas in TP the electrons and gas temperatures found in equilibrium [132]. NTP is also called as cold and non-equilibrium plasma with regards to the energy level, temperature and ionic density.

NTPs were mostly operated at low pressure with expensive vacuum systems and could be used in batch processes, however with the latest developments they can be generated at atmospheric pressure. Atmospheric pressure NTPs have some advantages because of the reduced costs and high throughput studies [133].

2.7.1. Non-Thermal Atmospheric Plasmas

NTPs are associated with lower degrees of ionization (10 %) when compared to hot plasmas where the ionization level reaches 100 %. In NTPs charged and neutral molecurlar

and atomic species are present at low temperature degrees along with the electrons with relatively high temperatures.

There is an increasing interest on non-thermal plasmas since they can be operated at low temperatures and atmospheric pressures. NTPs have been adopted for their use in material processing industry, sterilization [134], disinfection, surface treatment and plasma displays [135, 136].

NTPs may vary according to the type of plasma generation, energy source and electrode geometries [137]:

i. Glow discharge: plasma is generated at low pressure in between flat electrodes.
ii. Silent discharge: one of the electrodes is covered with a dielectric layer.
iii. Radio frequency (RF) discharge: occurs at both low and atmospheric pressures by use of radio frequency power supply.
iv. Microwave discharge: plasmas are created in a wavelength structure or resonant cavity.
v. The corona discharge: is initiated at atmospheric pressure using inhomogeneous electrode geometries like a pointed electrode and a plane or a thin wire or on the liquid surface.

Other classifications and statements are available on the literature including one atmosphere uniform glow discarges (OAUGDP), atmospheric-pressure plasma jets (APPJ), resistive barrier discharges (RBD) [138].

2.7.2. Plasma Corona Discharges

Discharges can be divided into groups as negative, positive, AC, DC, high frequency and pulsed discharges depending on the behavior of sustained electric energy which is chosen for a specific use. In corona discharges high voltages are applied between two wire electrodes at atmospheric pressure and the plasma discharge occurs around the tip of the wire as lighting. During this process ions are accelerated towards the wire while electrons are accelerated into the plasma. These high energy electrons in plasma collide with the

heavy particles in corona by ionization, excitation and dissociation resulting in the formation of radicals.

As shown in Figure 2.9, in atmospheric pressure plasma corona discharge, one of the electrodes is shaped in definite geometry and placed in a conductive liquid, plasma discharge occurs between the electrode and liquid surface. On the other hand, other electrode has a sharp point and the gradient of the air is much higher around this pointed electrode. High voltage subjected to a small area gives rise to high energy and ionizes the gas molecules. These ions interact with the liquid surface and penetrate because of the high electric field.

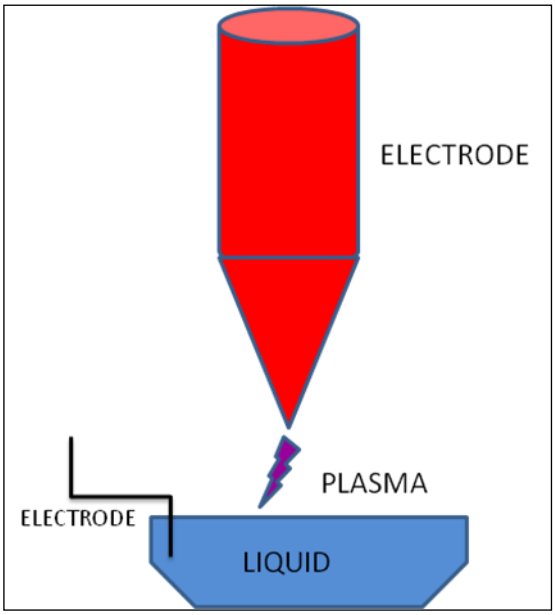

Figure 2.9. Schematic view of plasma corona discharge electrodes

The first use of a plasma corona discharge in purpose of water disinfection water was reported by Siemens [139] which was the first statement of plasma technology being used for inactivation of microorganisms.

2.7.3. Plasma Chemistry

The high electric field applied between two electrodes initiates the ionization of carrier gas resulting in the partial discharge activity in the gas molecules. Non-thermal

plasmas are characterized as non-equilibrium because of the large temperature differences between the electrons and the ions. The electrons are extremely light and they move quickly which is altering their heat capacity. These high energy electrons react with the gas molecules in cold plasma to form reactive species, free radicals and/or atoms. The complex reactions occur in plasma discharge neither predictable nor controllable due to non-equilibrium kinetics.

Atmospheric pressure discharges structure contains predominantly neutral reactive species such as oxygen atoms (O or O•-), singlet oxygen and peroxide (O_2^{-2} and H_2O_2) and ozone (O_3) [140, 141]. Ozone, from these species is found to be the most abundant form with a concentration of more than five times of singlet and atomic oxygen. This proportion was associated with the longer existence of O_3 [142] since the other oxygen species rapidly disappear in milliseconds [143] to form ozone.

Hydroxyl radicals (OH•) are other type of reactive species found in plasmas and they play an important role by means of plasma chemistry and mechanism [144, 145, 146]. Water vapor in the plasma discharge gas induces OH• formation meanwhile inhibiting the ozone production [147].

These reactions can be schematized as follows [137]:

$$e + O_2 \rightarrow 2O + e \quad (2.1)$$

$$O + O_2 \rightarrow O_3 \quad (2.2)$$

$$O_3 + H_2O \rightarrow O_2 + H_2O_2 \quad (2.3)$$

$$H_2O_2 \rightarrow 2\ OH\cdot \quad (2.4)$$

$$e + H_2O \rightarrow H\cdot + OH\cdot \quad + e \quad (2.5)$$

$$e + N_2 \rightarrow N\cdot + N\cdot + e \quad (2.6)$$

$$e + N_2 \rightarrow N_2\cdot + e \quad (2.7)$$

$$e + NO \rightarrow N\cdot + O\cdot + e \quad (2.8)$$

$$2H_2O \rightarrow H_2O_2 + H_2 \quad (2.9)$$

$$OH\cdot, O_3 + M \rightarrow [\text{decomposed M}] \quad (2.10)$$

where M is a macromolecular compound.

As described above, the chemical composition of cold plasmas compromised of nitrogen and oxygen active species, free radicals and highly reactive intermediate species [131]. As schematically described in Figure 2.10., these reactive species along with the generation of UV radiation are responsible for anti-microbial effects of plasma discharges [129].

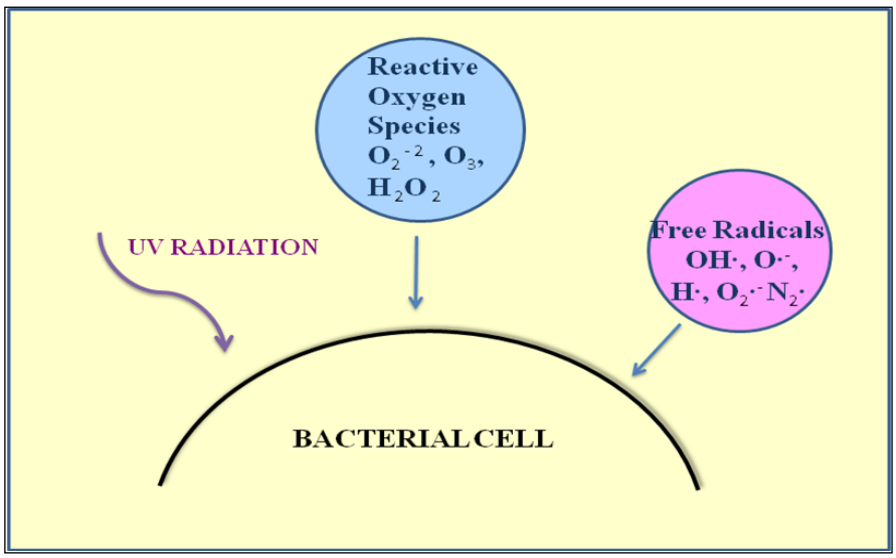

Figure 2.10. Schematic view of active species formed during plasma discharge

2.7.4. Plasma Decontamination of Foods

Plasma-based processing techniques for use in sterilization and decontamination of medical devices, pharmaceutical products and packaging material modifications are being developed worldwide.

The adoption of mild preservation technologies under European legislation is an ongoing process, as shown by the Novel Food Regulation [148]. Application of these non thermal technologies offers various opportunities for mildly processed products by preserving their sensory quality, nutritional value and appearance. However, the application potential of any new technology is influenced by many factors these include: technological feasibility, technical possibility, health impact, occupational safety, environmental friendliness, economic feasibility, infrastructural conditions/requirements, investment need and availability of investment power, political attitude, social consequences, psychological aspects and risk-benefit perception. Although, there is no current technology that answers all these requirements, plasma technology seems to have potential to positively address the majority.

Furthermore since plasma operates at atmospheric pressure, it eliminates the need for complex and expensive vacuum systems and can achieve sterilization in as little as 5 min [21]. Limited studies to date, including those carried out by our research group, have shown NTP is able to inactivate a variety of microorganisms, including spores on solid surfaces and liquids [21, 146]. However, the actual application of NTP to food products still needs to be studied in depth, in order to supply a basis for the use of this technology in food processing.

Because of the reactive species described above, plasma has potent antimicrobial effects [149, 150]. These species have higher sterilization and chemical potentials than conventional chemical agents. Several researches have shown the ability of plasma at inactivating microorganisms on material surfaces [145, 151, 152, 153]. However, plasma application targeting food-borne pathogens and inactivation of microorganisms in complex food specimens is yet to be studied.

Processing of foods with non-thermal plasma is being studied for decontamination of fruits [22, 154], vegetables [155, 156] and water [21]. It was found that the plasma corona discharge system was able to efficiently sterilize water from the majority of microorganisms tested including bacteria (*E. coli, S. aureus, Pseudomonas aeruginosa, Streptococcus mutans, Bacillus subtilis*), yeast (*Candida albicans*), fungi (*Aspergillus niger*) and green algae [21].

2.7.5. Sterilization Mechanism of Plasma

The use of plasma technology for sterilization purposes is a relatively new research area, while the advantages of this method are various, including relatively low temperatures during process, its non-toxic nature in comparison to more traditional methods such as chemical biocides, killing a wide range of microorganisms within a short time, running at atmospheric pressure therefore lacking expensive equipments, and ease of operation.

To date plasma systems are not commercially available as a sterilizing tool in medical and food industry, mainly due to lack of researches on the biochemical effects of plasma discharge on biological components. Plasma sterilization works in a synergistic and complex behavior of specific active agents that have a biocidal effect on microorganisms. Although the biocidal mechanisms of plasma systems are still being investigated [19], there are some explanations on the possible reasons for cell death. Mechanisms differ according to the operational parameters such as pressure, temperature, energy consumption, and design of the plasma system.

Moisan *et al.* [157] suggested three basic mechanisms attributing to the cell death by plasma. These are 1) Etching of the cell surface by reactive species formed during plasma, 2) Volatilization of compounds and intrinsic photodesorption of UV photons and 3) Destruction of genetic material. These deactivation mechanisms cause several reactions in bacterial cell including lipid peroxidation of poly-unsaturated fatty acids, oxidation of amino acids and DNA oxidation [145].

Plasma sterilization works through UV irradiation, charged particles and reactive species described in Plasma Chemistry Section (2.7.3). The sterilization effect of UV radiation acts through disturbing the genetic material of microorganisms by causing thymine dimers in the DNA [157] therefore, inhibiting the reproduction of bacteria. However, it is stated that UV radiation does not have a big impact on inactivation of microorganisms by atmospheric pressure cold plasmas [158, 159, 160, 161]. An explanation for these results can be inactivation of microorganisms only occur within a biocidal wavelength [162]. Hence, if an appreciable dose of UV radiation is not emitted, the inactivation of microorganism occurs due to different agents.

Effects of reactive species were found to be more recognizable via studies on the germicidal effect of oxygen containing (air) plasmas. The reactive species in non-thermal atmospheric air plasmas are nitrogen– and oxygen–based species such as atomic oxygen, ozone, nitrogen oxide and hydroxyl. These active species play a crucial role in the microbial inactivation mechanism, since they directly interact with the bacterial membranes. These active species have short lifes in gas phase and they can dissolve in liquid [163]. Radicals are destroyed after chemical reactions, so that disappearing after plasma treatment.

Plasma discharges with a higher oxygen concentration were associated with increased levels of microbial survival inhibition due to oxygen based active species, atomic oxygen and ozone [159, 161]. Damage to membrane proteins and/or lipids occur because of oxidative stress. The active species formed during plasma discharge, OH, singlet oxygen, ozone, and radicals can initiate lipid peroxidation thus producing shorter chain fatty acyl compounds. These products include alkanes, ketones, epoxides, and aldehydes [164, 165, 166]. The short chained of charged fatty acids have a lower ability to rotate within membrane and increase the fluidity of the membrane [166, 167] which results in distraction of structural membrane integrity.

Reactive oxygen species were associated with the lipid peroxidation of the unsaturated fatty acids in the cell membrane [168]. These reactive oxygen species are produced during normal respiratory process, as well. Therefore, some defense mechanisms and repair systems are enhanced by living organisms to those oxidizing agents that are

formed by plasma [168, 169]. Because of these mechanisms, oxidative damage is formed when the counteract capacity of the cell is overwhelmed. This situation initiates a cascade of chemical reactions in cell leading to disruption of the structure of cellular molecules such as lipids, proteins, carbohydrates and genetic material, and subsequently inactivation of the survival activities [170]. The reactions of ozone with dienes, amines, and thiols [168] as well as organic molecules such as aldehydes, poly-unsaturated fatty acids [171, 172] and sulfhydryl groups of amino acids are widely discussed in the literature [169, 173].

Charged particles are described to destruct the outer membrane of bacterial cells [174]. This reaction occurs through the electrostatic forces of the charged particles, as they can overcome the strong tensile force of the membrane and cause rupture due to charge accumulation on the surface [174].

As the rates and susceptibilities differ for the sterilization techniques according to the bacterial strains and species, vegetative cells tent to be more susceptible to plasma application than spores. Also, some differences have been demonstrated regarding capability of plasma on inactivating gram-positive and gram-negative bacteria [144, 145]. This can be explained as the gram-negative bacteria have a thinner murein layer (approx. 2 nm) than gram-positive bacteria (approx. 15 nm). Therefore, it gives more possibilities to disruption by plasma reactive oxygen species.

3. MATERIALS

3.1. BACTERIAL STRAINS

Test microorganisms *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25921 were provided from Yeditepe University, Genetic and Bioengineering Department Culture Collection, Turkey. *Salmonella typhimurium* ATCC 14028 was obtained from Oxoid, UK.

3.2. BACTERIAL GROWTH MEDIA AND SOLUTIONS

3.2.1. Nutrient Broth

Nutrient broth was purchase from Merck, Germany and prepared according to companies instructions. Sterile broth was distributed into 50 ml sterile falcon tubes and stored at 4 °C for further use.

3.2.2. Tryptic Soy Agar

Tryptic soy agar was purchased from Merck, Germany and prepared according to companies instructions. After sterilization at 121°C for 15 minutes in autoclave (Hirayama, Japan) the medium was distributed into sterile, 90 mm radius petri dishes and stored at 4°C throughout the experiments.

3.2.3. Violet Red Bile Agar

Violet red bile agar was purchased from Merck, Germany and prepared according to instructions of company and then the medium was poured into sterile, 90 mm radius petri dishes and stored at 4°C.

3.2.4. Mannitol Salt Agar

3.2.4.1. Composition

Proteose Peptone ..... 10.0 g/L

Beef Extract..... 1.0 g/L

D-Mannitol ..... 10.0 g/L

Sodium Chloride..... 75.0 g/L

Agar ..... 15.0 g/L

Phenol Red ..... 25.0 mg/L

3.2.4.2. Method of Preparation

111 g were suspended in 1 liter of distilled water and mixed thoroughly. The medium was heated with frequent agitation and boiled for 1 minute. After the medium was autoclaved at 121°C for 15 min, it was poured into sterile, 90 mm radius petri dishes and stored at 4°C.

3.2.5. Xylose Lysine Deoxycholate Agar

Xylose lysine deocycholate agar was purcahesed from Merck, Germany and prepared according to instructions of the company. Then the medium was poured into sterile, 90 mm radius petri dishes and stored at 4°C.

3.2.6. Saline Solution

8.5 g of sodium cloride was dispersed in 1 L of distilled water and sterilized at 121°C for 15 min.

3.2.7. Milk

Commercial UHT milk samples were obtained from local market in Istanbul, Turkey. The samples included whole (3.5 % fat), semi-skimmed (1.5 % fat), and skimmed milk (0.3 % fat).

3.3. BUFFERS, CHEMICALS AND REAGENTS

3.3.1. 40% SDS-PAGE Gel Preparation

3.3.1.1. Seperating Gel

Five per cent running gel was prepared by mixing 11.25 mL of 30 % acrylamide/bis solution, 37.5:1 (BioRad, Canada), 7.5 mL of pH 8.8 Tris-HCl buffer, 300 µL of 10% sodium dodecylsulfate, 300 µL of 10% ammonium persulfate, 10.65 mL of distilled water and 15 µL of TEMED. The pH of this gel was higher than the stack gel therefore allowing the mobility of protein-SDS complex and separation of protein molecules depending on the sizes.

Tris in this gel was used as a buffer because of its innocuous property to most proteins. Acrylamide was used for polymerization purposes. SDS is used for the denaturation of native proteins to polypeptides. After treating with SDS, polypeptides have a shape like a rod and negatively charged structure per unit length so that the protein motilities are a linear function of their molecular weights. Ammonium persulfate was used as a catalyst for formation of the gel. TEMED (N, N, N', N'-tetramethylethylenediamine) with ammonium persulfate were used as initiators.

3.3.1.2. Stacking Gel

15% stacking gel was prepared by mixing 1.275 mL of 30% acrylamide/bis solution, 37.5:1 (Biorad, Canada), 1.25 mL of pH 6.8 Tris-HCl buffer, 100 µL of 10% sodium dodecylsulfate, 100 µL of 10% ammonium persulfate, 7.28 mL of distilled water and 10 µL of TEMED. The stacking gel has low acrylamide concentration and contains ions in which the protein molecules are trapped so the proteins were concentrated at this gel.

3.3.2. Buffers, Chemicals and Solutions for SDS-PAGE

3.3.2.1. Tris-HCl Buffer

1.5 M, pH 8.8 Tris-HCl buffer was prepared by weighing 181.71 g of Tris and dissolving in 1 L of distilled water. The pH was adjusted to 8.8 with 1 N hydrochloric acid solution.

0.5 M, pH 6.8 Tris-HCl buffer was prepared by dissolving 30.3 g of Tris base in 500 mL of distilled water. Then the pH was adjusted to 6.8 with 1 N hydrochloric acid solution.

3.3.2.2. Laemmli Buffer

Laemmli buffer (2X) containing 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M, pH 6.8 Tris HCl was purchased from Sigma Aldrich (USA). The SDS detergent binds to all positively charged molecules of proteins and denatures the proteins and subunits at a regular interval. SDS binds to proteins at rate of approximately 1.3 g of SDS per gram protein. Therefore all proteins have the same overall negative charge so that the proteins are separated based on size and not by charge. Glycerol in the Laemmli buffer was used for increasing the density of the sample so that it fell to the bottom of the well, minimizing puffing or loss of protein sample in the buffer, and layer in the sample well. The beta-mercaptoethanol reduces intra and inter-molecular disulfide bonds of the proteins to allow proper separation by size. Bromophenol blue is a dye which indicates the level of migration of the proteins allowing them to be observed during electrophoresis since the migration of bromophenol blue is faster than the proteins.

3.3.2.3. Lower Buffer

10X lower buffer was prepared by dissolving 15 g of Tris and 5 g of SDS in 500 mL of distilled water and the pH of the solution was adjusted to 8.4 with 1 N hydrochloric acid.

3.3.2.4. Upper Buffer

10X upper buffer was prepared by weighing 7.6 g of Tris, 2.5 g of SDS and 36 g of glycine and dissolving in 250 mL of water. The mixture was mixed and used for filling the wells of the gel.

3.3.2.5. Molecular Mass Markers

Sharp Mass V (Abnova, Taiwan) and SigmaMarker Wide Range (Sigma, USA) were used as molecular mass markers. Sharp Mass V Protein MultiColor Marker consists of prestained eight proteins with molecular weightes of ranging from approximately 16 kDa to 230 kDa. This marker was chosen because of its ability of visualizing proteins without staining during electrophoresis. The marker was purchased in gel loading buffer containing

10mM TrisHCl (pH 7.0), 2 mM EDTA, 1% SDS, 10 mM dithiothreitol and 10% glycerol. Marker was stored at -20°C.

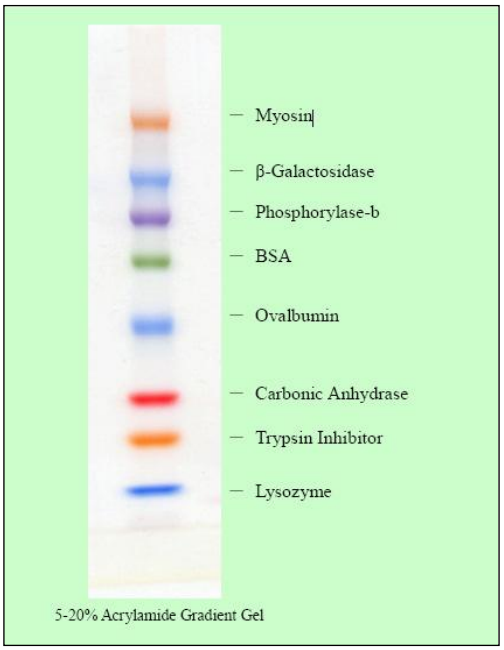

Figure 3.1. Sharp Mass V Protein MultiColor Marker pre-stained protein bands in 5-20% acrylamide gel

Table 3.1. Colors and molecular weights of pre-stained proteins in Sharp Mass V Protein MultiColor Marker

Protein	**Color**	**Apparent Molecular Weight (kDa)**
Myosin	Orange	229.3
Beta-Galactosidase	Blue	140.4
Phosphorylase-b	Purple	95.5
Bovine Serum Albumin (BSA)	Green	64.9
Ovalbumin	Blue	44.5

Table 3.1. Colors and molecular weights of pre-stained proteins in Sharp Mass V Protein MultiColor Marker (Contuniue)

Protein	Color	Apparent Molecular Weight (kDa)
Carbonic Anhydrase	Red	32.2
Soybean Trypsin Inhibitor	Orange	24.9
Lysozyme	Dark Blue	14.7

The dyes in Sharp Mass V Protein MultiColor Marker are covalently bonded to proteins which affects the protein mobility. Therefore, this pre-stained protein marker was used for estimation of protein molecular weights and an unstained Sigma Wide Range Marker was used for precise molecular weight determination.

Sigma Marker Wide Range was purchased from Sigma, USA as lyophilized form with sample buffer containing 62 mM, pH 6.8 Tris-HCl, 1 mM EDTA, 4% sucrose, 0.5% dithiothreitol, 2% SDS, and 0.0005% bromophenol blue. Hence the marker was reconstituted with 100 µL of distilled water before use and stored at -20°C.

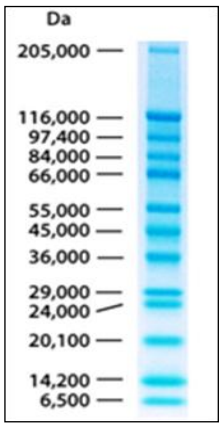

Figure 3.2. Molecular weight and patterns of Sigma Wide Range Protein Marker

3.3.2.6. Colorizing Solution

Colorizing solution consisted of 0.1% Coomassie Brilliant Blue R250, 40% of methanol and 7% glacial acetic acid. The solution was prepared by mixing defined amounts of chemicals and used for staining the poly-acrylamide gel.

3.3.2.7. Decolorizing Solution

The gels were decolorized with a solution prepared with 7% glacial acetic acid and 5% methanol.

3.3.3. Fatty Acid Extraction of Milk and Chemical Esterification

3.3.3.1. Sulfochromic Acid

Sulfochromic acid solution containing 7.5% chromic acid and 25% sulfuric acid was used for washing the glassware before use. This solution was used in order to eliminate possible fatty acid contaminations because of its high capacity to react with organic acids and clean glass surfaces.

3.3.3.2. Dichloromethane-Methanol (2:1)

Dichloromethane-methanol solution was prepared by mixing 2 units of dichloromethane and 1 unit of HPLC grade methanol and mixed thoroughly.

3.3.3.3. Saturated Solution of Sodium Chloride

More than 36 g of sodium chloride was dissolved in 100 mL of distilled water in order to prepare saturated sodium chloride solution.

3.3.3.4. Hexane

Fatty acids were extracted by adding n-hexane (Merck, Germany) which is a non-polar, hydrocarbon solvent consisting of 6 carbon atoms.

3.3.3.5. 2N Potassium Hydroxade

28.05 g of potassium hydroxade (KOH) was prepared in 250 mL of HPLC grade methanol. KOH is a strong base which serves as hydroxyl source and reacts with the polar bonds in fatty acids therefore saponifies the fatty acid methyl esters.

3.3.3.6. Diazomethane

Diazomethane, which was used as a methylating agent, is a flammable yellow gas at room temperature, so it was kept at -20°C and handled very carefully during experiments.

3.3.4. Chemicals for Identification of Volatile Compounds in Milk

3.3.4.1. Divinylbenzene-Carboxen-Polydimethylsiloxane-Coated fiber

Polydimethylsiloxane (PDMS) fiber is a non-polar polymer used for non-polar compounds. PDMS is preferred because it can withstand high injector temperatures up to 300°C. Coating the fibers with a combination of polymers such as divinylbenzene and carboxen increases the retention capacity via effecting the adsorption and distribution to the stationary phase. The thickness of the fiber polymer coating was 65 μm since the volatile compounds require a thick polymer coat.

3.3.4.2. Internal Standard

The internal standard, used in gas chromatography analysis, was 4-methyl-2-penthanol. The concentration of the internal standard was 100,000 ppm and was used for the determination of the amount of the organic acids in the specimen.

3.3.5. Solutions Used in DNA Isolation of Bacterial Strains

3.3.5.1. Tris-EDTA Buffer

The Tris-EDTA solution was used to break the protein cross-links and contained 10mM Tris Base, 1mM EDTA Solution and 0.05% Tween 20. 1.21 g of Tris base and 0.37 g of EDTA was dispersed in 100 ml of distilled water and the pH was adjusted to 9.0. Then 0.5 ml of Tween 20 was added and mixed. 10X Tris-EDTA buffer was stored at room temperature and diluted to 1X before use.

3.3.5.2. Proteinase K

Proteinase K was used for cleaving peptide bonds at the carboxylic sides of aliphatic, aromatic or hydrophobic amino acids. 28.9 kDa monomer Proteinase K was stored in 10 mM Tris-HCl (pH 7.5), containing calcium acetate and 50% (v/v) glycerol supplied by the company (Fermentas, Canada).

3.3.6. PCR and Gel Electrophoresis Buffers and Chemicals

3.3.6.1. Taq polymerase and Taq polymerase buffer

Taq DNA polymerase was used which catalyzes the addition of a single adenosine at the 3' end of the PCR products. The enzyme is supplied in 20mM Tris-HCl (pH8.0), 1mM DTT, 0.1mM EDTA, 100mM KCl, 0.5 per cent (v/v) Nonidet P40, 0.5 per cent (v/v) Tween 20 and 50 per cent (v/v) glycerol by Fermentas, Canada.

10X Taq buffer with KCl (Fermentas, Canada) composed of 100mM Tris-HCl (pH8.8 at 25°C), 500 mM KCl and 0.8% (v/v) Nonidet P40 was used which did not contain $MgCl_2$. Taq polymerase and buffer were stored at -20 °C.

3.3.6.2. $MgCl_2$ Solution

0.22 μm membrane-filtered 25mM magnesium chloride was used for optimizating ion concentration in PCR with Taq DNA Polymerase. Solution was purchased from Fermentas, Canada and stored at -20 °C.

3.3.6.3. Deoxyribo Nucleotide Triphosphate (dNTP) Mix

dNTP Mix of dATP, dCTP, dGTP and dTTP with 10 mM each was purchased from Promega, USA and in titrated solution to pH 7.5 with NaOH.

3.3.6.4. Primers

PCR amplification was performed using 27F forward primer and 1492R reverse primer were purchased from Sigma-Aldrich, Germany which are complementary to the 5' end and 3' end of the prokaryotic 16S rDNA, respectively. Ten times diluted stock solutions of both primers were used in PCR reaction.

3.3.6.5. Tris-Boric Acid-EDTA (TBE) Buffers

10X stock solution of TBE buffer was prepared by dissolving 108 g of Tris base, 55 g of boric acid and 40 mL of 0.5 M, pH 8.0 EDTA in one liter of distilled water. TBE stock solution was diluted 10-folds with distilled water in order to obtain 1X TBE buffer and used in agarose gel preparation. 20-fold diluted TBE stock solution with distilled water, 0.5X TBE buffer, was used in gel tank.

3.3.6.6. One Per Cent Agarose Gel

PCR products were run in agarose gel electrophoresis. For 1% agarose solution 0.8 g of agarose (Sigma-Aldrich, Germany) was dissolved in 80 mL of 1X TBE buffer. Agarose solution was boiled in microwave at 600°C for a homogenized solution.

3.3.6.7. Ethidium bromide

Ethidium bromide (Sigma-Aldrich, Germany) was used to intercalate into and stain the DNA strands amplified with PCR. The fluorescent complexes formed by interaction of ethidium bromide with nucleic acids were observed under UV light.

3.3.6.8. Loading Dye (Buffer)

6X loading dye (buffer) (Takara, Japan) with a composition of 30% glycerol, 30 mM EDTA, 0.03% Bromophenol Blue and 0.03% Xylene Cylanol, was used for loading of the DNA samples on agarose gel. The two dyes, bromophenol blue and xylene cyanol, provide the visual tracking of DNA migration during electrophoresis. Glycerol makes DNA heavy which ensures sitting down of DNA into wells of the gel. On the other hand, EDTA included in the buffer binds divalent metal ions and inhibits metal-dependent nucleases.

For gel electrophoresis, 6X loading dye was diluted to 1X by mixing 1 µL of dye with 5 µL of DNA sample.

3.3.6.9. DNA Marker

Wide Range DNA ladder containing 16 DNA fragments with different sizes ranging from 50 bp to 10000 bp as shown in Figure 3.3 was purchased from Takara, Japan. DNA marker (ladder) was used to determine the size of the DNA samples detected in agarose gel electrophoresis.

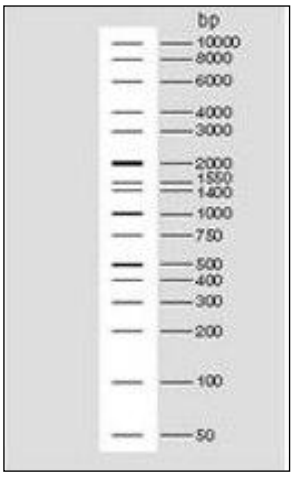

Figure 3.3. Takara wide range DNA ladder (50 – 10000 bp)

3.3.7. Genomic DNA Isolation Buffers and Chemicals

3.3.7.1. Sodium Acetate Buffer

3M, pH 5.2 sodium acetate buffer was prepared by dissolving 408.24 g of sodium acetate (tri-hydrate) in 800 mL of distilled and adjusting the pH to 5.2 with glacial acetic acid. Total volume was completed to 1 L with distilled water.

3.3.7.2. Absolute and Seventy Per Cent Ethanol

70 mL of absolute ethanol (99.5 per cent) (Merck, Germany) was mixed with 30 mL of distilled water in order to obtain 70% ethanol solution. Absolute and 70% ethanols were used in genomic bacterial DNA extraction.

3.3.7.3. TE Buffer

10 mL of 10 mM, pH 7.5 Tris-Cl and 2 ml of 0.5 mM, pH 8.0 EDTA were mixed and the final volume was completed to 1 L.

3.3.8. Chemicals for Fatty Acid Extraction of Bacteria

3.3.8.1. Reagent One

Reagent 1 was used for the first step of fatty acid extraction which is saponification. 45 g of sodium hydroxide (Merck, Germany) was dissolved in distilled water and 150 mL

of HPLC grade methanol (Merck, Germany) was added to 150 mL distilled water. The solution was stirred thoroughly for a homogenized solution.

3.3.8.2. Reagent Two

Second solution was used as a methylating reagent. Reagent 2 was prepared by mixing 325 mL of 6 N hydrochloric acid (Merck, Germany) (161 mL of 37% hydrochloric acid and 164 ml of distilled water) with 275 mL of HPLC grade methanol.

3.3.8.3. Reagent Three

This solvent extracts the FAMEs into organic phase and stands for the extraction solvent. Reagent 3 was prepared by mixing 200 mL of HPLC grade hexane with 200 mL of HPLC grade methyl tert-butyl ether .

3.3.8.4. Reagent Four

Sample was cleaned up with Reagent 4 by base wash. This reagent was prepared by dissolving 10.8 g of sodium hydroxide (Merck, Germany) in 900 mL of distilled water.

3.4. LABORATORY EQUIPMENTS

A variety of laboratory equipments were used in this study including:

Laminar flow cabinet

Spectrophotometer

Waterbath

Incubator

Autoclave

Light emission intensity spectrometer

Gel electrophoresis

Color measurement spectrophotometer

Rotatory evaporator

GC/MS

Nanophotospectrometer

Automatic thermocycler

Agarose gel electrophoresis

Poly-acrylamide gel electrophoresis

Polymerase Chain Reactor Machine

Scanning Electron Microscopy

Gel visualization systems

Heat Block

Microwave

-80ºC refrigerator

Centrifuge

4. METHODS

4.1. BACTERIAL CULTIVATIONS AND PREPARATIONS

Pure colonies of *E. coli* ATCC 25922, *S. aureus* ATCC 25921 and *S.* Typhimurium ATCC 14028 were grown on Tryptic Soy Agar (TSA) (Merck, Germany) and in Nutrient Broth (NB) (Merck, Germany) at 37°C for 48h. The identity of the strains were confirmed by Gram staining and biochemical assays. Stock cultures of pure colonies were prepared by adding one loopful (approximately 20 mg) of bacteria into 1 mL 1:1 solution of 30% glycerol and 8% NB and stored at -80 ^{0}C. Viable counts were performed according to standard methods on TSA and expressed in log CFU/mL.

4.1.1. Milk Samples

UHT milk samples were obtained from a local market in Istanbul, Turkey. The samples included whole (3.5 % fat), semi-skimmed (1.5 % fat), and skimmed milk (0.3% fat).

4.1.2. Growth Curves

100 µL of stock bacterial solution was inoculated to 900 µL of NB and incubated at 37°C. Cultures were incubated overnight and 0.56 mL of bacterial solution was inoculated into 25 mL of NB in sterile 50 mL falcon tubes. The cultures were incubated in a shaking waterbath at 37°C (6*g*). The growth curves of ATCC cultures were observed photometrically by reading the optical density (OD) in a spectrophotometer (Thermo Labsystems, UK). The concentrations of bacterial cultures were determined by measuring the absorbance every 30 min at 600 nm for *S. aureus* and *S.* Typhimirium and at 660 nm for *E. coli*. The absorbance were monitored for a total of 6 h until they reached stationary phase. The bacteria in mid-exponential phase were used in the experiments.

4.1.3. Preparation of Starting Culture

Hundred µL of the overnight bacterial solution was resuspended in 4.5 mL of NB and incubated in waterbath at 37°C with 200 rpm agitation. All cultures were cultivated until they reached the exponential growth phase of the test microorganism. The absorbance was then adjusted to an OD of 0.2 with sterile saline solution. Hundred µL of the culture was pipetted into 15 mL of sterile UHT milk in a sterile 50 ml falcon tube and vortexed for 10 sec for homogenization.

The number of cells in starting culture was calculated by preparing serial dilutions and counting the colonies on TSA plates after 48 h of incubation at 37°C. Starting concentrations in 15 mL of milk samples were observed as $6x10^7$, $2.3x10^5$, and $4x10^5$ CFU/mL for *E. coli*, *S. aureus,* and *S.*Typhimurium, respectively. Hundred µL of these bacterial suspensions were used as a control sample before plasma treatment and plated onto the TSA and appropriate selective agar at time zero and incubated at 37°C for 48 h.

4.2. PLASMA CORONA DISCHARGE SYSTEM

4.2.1. Apparatus Design

Figure 4.1 illustrates the atmospheric plasma corona discharge model system used in this study. This model system included an isolation transformer, a high voltage AC power supply, a circuit for impedance matching, serially connected electrodes (Nickel–Chrome, 1 mm radius), a stirrer with a DC motor and a thermometer as shown in Figure 4.1. One of the electrodes was immersed in liquid (milk) at a fixed location while the other one was kept 0.5 cm above the liquid and rotated by a wheel.

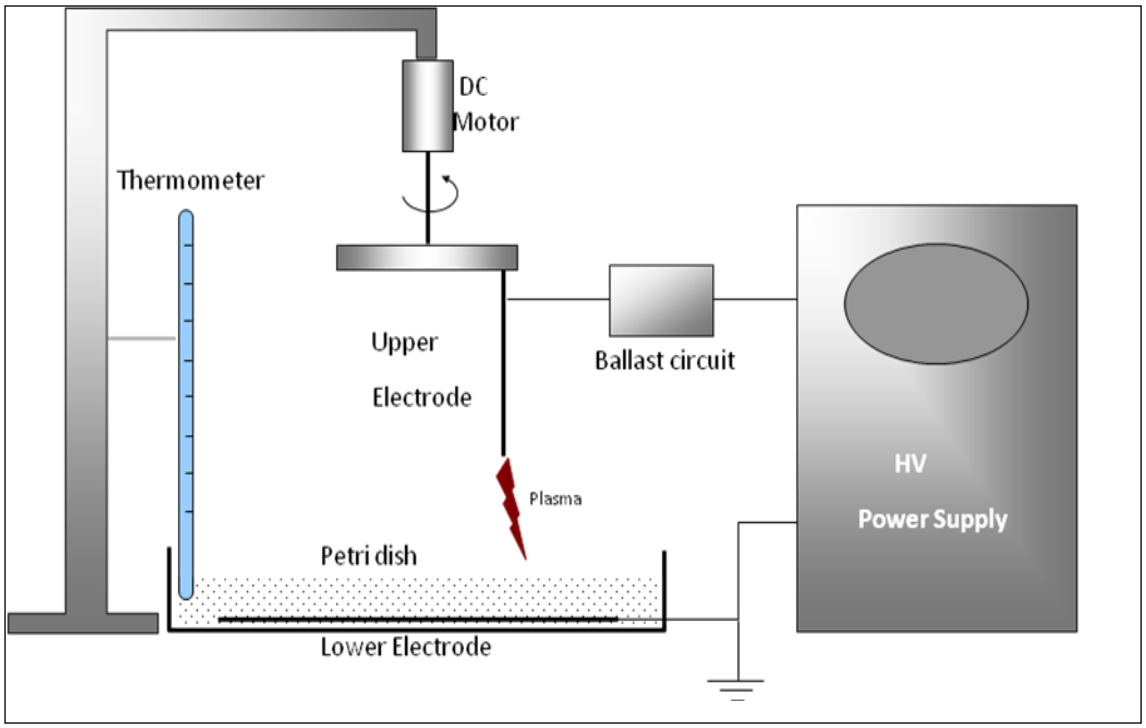

Figure 4.1. Schematic view of atmospheric plasma corona discharge experimental setup

The electrode systems, impedance circuit, on-off circuit, electrical isolation and the system were designed and developed by Prof. Dr. Necdet Aslan from Physics Department, Yeditepe University, Turkey.

The plasma discharge was created by applying high voltage between upper electrode and liquid surface at atmospheric pressure. The shape of the electrode causes very high electric field in a small region resulting in the ionization of the air. When the second electrode is immersed in the liquid, the liquid surface serves as another electrode so that the plasma discharge can occur between upper electrode and the liquid surface. The temperature of the milk was monitored with a thermometer during the experiments in order to prevent thermal decontamination of the microorganisms.

4.2.2. Test Parameters of Plasma System

4.2.2.1 Power Supply

This system can use both AC and DC power supplies. DC causes a unidirectional continuous flow of the electrons and ions produced and the flow directions of electrons and

ions do not change, thus producing a continuous bombardment of ions on the cell. Comparatively, when an AC power supply is utilized, the flow direction changes according to the supply's frequency. In this case, the ions and electrons move in back and forth motion rather than in one direction, causing, a decrease in the amount of energy transfer on the average.

Since in previous studies [21] DC power supply was observed to be more potent to the study media, AC power supply was decided to be used in milk studies.

4.2.2.2. Voltage

The experiments were started by using 9 kV of power supply and for those that could not be killed with 9 kV, the power was increased to 15 kV.

4.2.2.3. Exposure Time

Also, effects of plasma exposure time on decontamination properties of test bacteria were detected. Plasma was applied to milk samples at time points of 0, 3, 6, 9, 12, 15 and 20 min.

4.2.3. Light Emission Spectroscopy of Plasma Corona Discharge System

The light emission intensities of the ions generated in AC plasma discharge were determined by a UV-visible emission spectrometer (Baki, Turkey) developed in the Laser Technologies Laboratory of Kocaeli University, Turkey. The visible light falling onto the collector lens was transferred through a fiber cable to the spectrometer and the emissions were recorded between wavelengths of 200 and 500 nm for AC supply. Peaks indicating the ionization levels of the molecules were identified using the NIST Atomic Spectra Database [175].

The diameter of the discharge "D" occurring between the tip of the positive electrode and water surface was determined to be nearly D=1.0 mm by the dark room light intensity observations. When a current of nearly 90 mA was applied, the current density was measured to be nearly 10 A/cm^2.

4.3. THE EFFECTS OF PLASMA APPLICATION ON MILK

4.3.1. Microbiological Analysis

The plasma corona discharge system was placed in laminar flow cabinet in order to prevent contamination and the milk suspension including the test bacteria was poured into a sterile empty petri dish. Starting concentrations of *E. coli, S. aureu,* and *S.* Typhimurium were 7.8, 5.4, and 5.6 log CFU/mL, respectively The petri dish was placed into the shelf holder of the plasma apparatus as shown in Figure 4.1 and a negative electrode was placed into the milk while the positive electrode was kept 0.5 cm above the milk surface. The upper electrode was connected to a wheel which rotated during plasma corona discharge exposure in order to increase plasma discharge contact surface area and homogenize plasma discharge. Both of the electrodes were sterilized before each experiments.

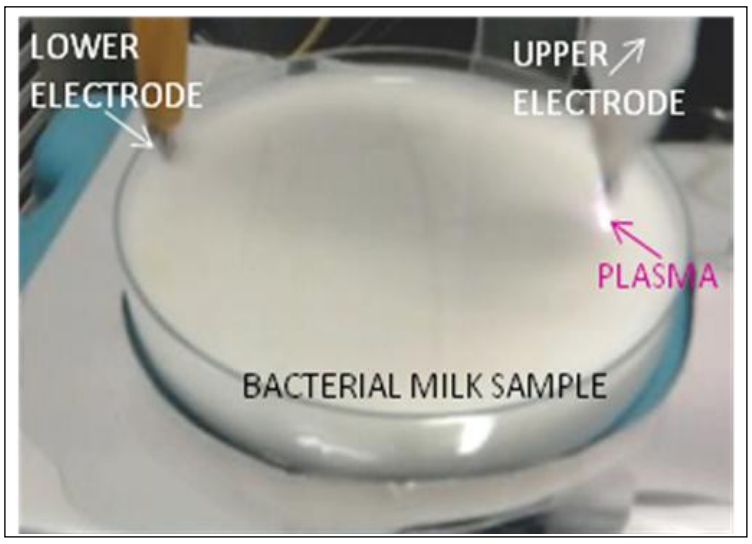

Figure 4.2. Plasma discharge occurring on the milk surface by high voltage (9 kV) applied between upper (on the right) and lower electrodes (on the left)

Plasma was applied to the milk surface for 20 min. Hundred µL of plasma treated milk sample were taken and inoculated every 3 min onto TSA and Violet Red Bile (VRB) agar, Mannitol Salt Agar (MSA) and Xylose Lysine Deoxycholate (XLD) agar for *E. coli*, *S. aureus* and *S.*Typhimurium, respectively and incubated at 37ºC for 48h. The temperature of the milk suspension was controlled at 37 ºC throughout the treatments.

4.3.2. Physical and Chemical Analysis of Treated and Untreated Samples

4.3.2.1. pH Analysis

The pH of the milk was measured with a pH meter (Mettler Toledo, USA) throughout the treatment time. The pH of post-plasma treated and control milk was compared to detect any changes in the hydrogen concentration due to plasma discharge.

4.3.2.2. Color Measurement Tests

A portable spectrophotometer, CM-600d (Konica Minolta, USA) was used for the color analysis in milk. In this system, an illuminator with 8° diffused illumination and 8° visioning angle was used. The light source used was a pulsed xenon lamb with UV cut filter and the wavelength ranged from 400 nm to 700 nm pitching every 10 nm. The light was detected with a silicon photodiode array.

Since the temperature affects the color values all samples were warmed to room temperature before color measurement. Results were shown according to the CIE-LAB system which includes L*: luminance or lightness component (ranging from 0 to 100), a*: green to red (ranging from -60 to +60) and the b* component: blue to yellow (ranging from -60 to +60) as shown in Figure 4.3.

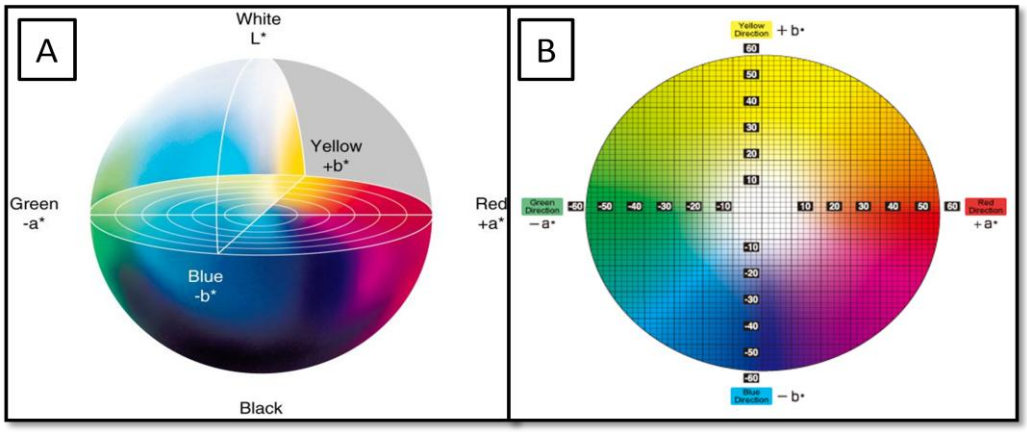

Figure 4.3. Chromatic diag and representations of colorimetric measurement
A) Representation of color density for color space. B) Chromaticity diagram of a* and b*.

The overall color difference was expressed by means of Delta-E (ΔE) which indicates the distance between two colors. Using control L^*_1, a^*_1 and b^*_1 measurements and the sample L^*_2, a^*_2, b^*_2 values, ΔE was calculated using the following equation:

$$\Delta E = [\ (L^*_2 - L^*_1)^2 + (a^*_2 - a^*_1)^2 + (b^*_2 - b^*_1)^2\ ]^{1/2} \qquad (2)$$

Depending on the dE values, the color difference was evaluated as not noticeable (0 – 0.5), slightly noticeable (0.5 – 1.5), noticeable (1.5 – 3) and well visible (3 – 6).

4.3.2.3. Proteolysis Assessment

The effect of plasma treatment on the protein content of milk was evaluated by Sodium Dodecyl Sulfate – PolyAcrylamide Gel Electrophoresis (SDS-PAGE). Twenty μL of milk sample was added to 20 μL of reducing buffer (Laemmli sample buffer) (Sigma Aldrich, USA). The 1:1 solution was heated in waterbath to 100°C for 10 min in order to denature the milk proteins. The samples were stored at +4°C until SDS-PAGE analysis.

15 μL of sample was loaded to 5% and 15% SDS-polyacrylamide gels after the wells were loaded with upper buffer. Two broad range molecular mass markers (Sigma, USA and Abnova, Taiwan) were used as standards. Following the addition of lower buffer, gels were run at 90 V for 1 hour and then at 250 V for 3.5 h. The temperature was kept constant at 10°C. A Vertical System Hoefer SE 600 SERIES (Amersham Pharmacia Biotech, UK) was used for the gel electrophoresis as shown in Figure 4.4.

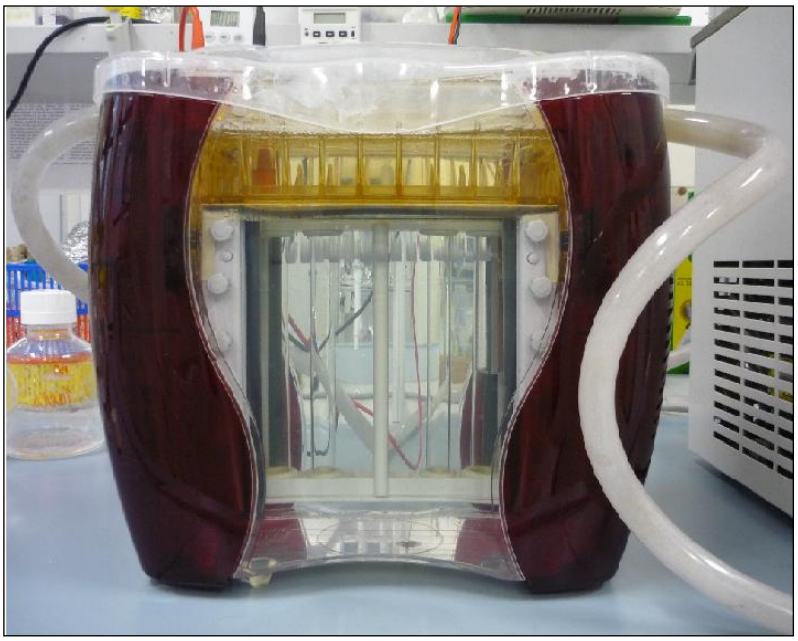

Figure 4.4. The vertical SDS-PAGE electrophoresis system

After the electrophoresis was completed, protein bands were stained with a colorizing solution containing Coomassie Brilliant Blue R-250 (Thermo Scientific, USA). Gels were then destained by washing with decolorizing solution until bands could be observed.

4.3.2.4. Detection of Fatty Acids in Milk

Lipid extraction was carried out according to the method described by Lopez-Lopez *et al.* [176] with some modifications. 25 mL of dichloromethane-methanol (2:1) was added to 1.5 mL of milk. The mixture was mechanically agitated in a shaker (Hotech, Taiwan) for 15 min at 6*g* and centrifuged at 3000*g* for 8 min. Eight mL of distilled water was added into the tube and the solution was shaken for a further 15 min at 6*g*. The sample was re-centrifuged at 3000*g* for 8 min. The upper (aqueous) phase was removed with a pipette. The organic phase was washed with saturated sodium chloride solution and agitated at 6*g* for 15 min. Then the solution was centrifuged at 3000*g* for 8 min. The organic phase was filtered through filter paper (Whatman, Maidstone, UK) by way of a separating funnel.

Following filtration, the lower phase was transferred to a round bottom flask and the solvent was removed in a rotatory evaporator (Heidolph, Germany). The lipid extract was resolved in hexane and stored in a glass tube at -20°C until methylation.

Methylation was carried out by adding 1 mL of n-hexane and 1 mL of potassium hydroxide in methanol (2N) to 50 mg of extracted lipids in test tube. Following vortexing for 2 min, 1 mL of n-hexane was added and the upper phase with n-hexane was transferred into another glass tube. After evaporating the sample under N_2 flux, 4-5 droplets of diazomethane were added and the solvent was removed by N_2 flux. The weight of the total fatty acid ester was measured by weighing the glass tube.

As an internal standard C11:0 was added to the samples prior to addition of hexane to the test tube with a concentration of 100 µL per 2.5 mg of fatty acid esters. Fatty acid methyl ester analysis was carried out using an Agilent Hewlett-Packard 7890GC gas chromatograph equipped with a MS detector (Hewlett-Packard 5970 MSD, CA) (GC/MS) and a 30 m x 0.32 i.d. fused silica capillary column coated with a 0.2 µm film of Carbowax (Supelco, Bellefonte, PA, United State) as the stationary phase.

The identification of the individual FFAs of milk samples was based on the comparison of the retention times of the unknown FFAs with those obtained from the known FFA standards (Sigma-Aldrich, Germany). The identification of free fatty acids was carried out by computer matching of their mass spectral data with those of the compounds contained in the Agilent Hewlett-Packard NIST 98 and Wiley vers.6 Mass spectral data base.

4.3.2.5. Identification of Volatile Compounds in Milk

Volatile compounds associated to each milk sample were evaluated by gas chromatography (GC)-mass spectrometry (MS)/solid-phase micro-extraction (SPME) analysis. A divinylbenzene-carboxen-polydimethylsiloxane-coated fiber (65 µm) and a manual SPME holder (Supelco Inc., Bellefonte, PA) were used for the SPME of volatile compounds in milk. Before the injection of samples, the fiber was exposed to the GC inlet for 10 min at 250°C for thermal desorption purposes. Five mL of each sample was transferred into sterile 10 mL vials and capped with poly-tetrafloroethylene/silicon septa.

Fifty ppm of internal standart (4-methyl-2-penthanole) was pipetted into the samples and equilibrated at 40°C for 10 min as shown in Figure 4.5.

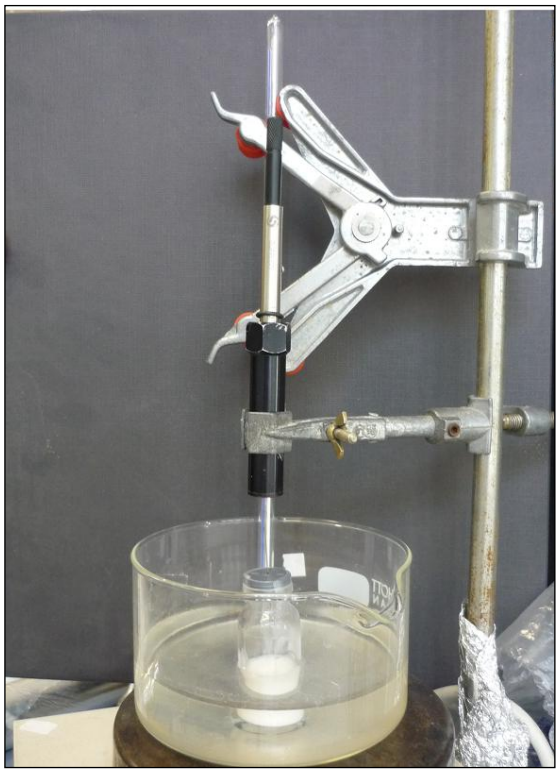

Figure 4.5. Transaction of divinylbenzene-carboxen-polydimethylsiloxane-coated fiber with the headspace of milk sample

The fiber was placed into the headspace of the sample for 40 min as shown in Figure 4.5 and then the fiber was inserted into the injection port of the GC/MS as shown in Figure 4.6. After a sample desorption of 10 min the fiber was unsettled. The GC/MS analyses were performed with an Agilent 7890 gas chromatograph (Agilent Technologies, CA) coupled to an Agilent 5970 mass selective detector which is illustrated in Figure 5.6. This system was operated in electron impact mode with an ionization voltage of 70 eV. The column used was a Chrompack CP-Wax 52 CB capillary column which is 50-m in length and 0.32-mm in internal diameter (Chrompack, The Netherlands). The temperature was adjusted to 50°C for 2 min and then rised 1°C every minute up to 65°C and after that 5°C per minute to 220°C. The temperatures of the injector, interface, and ion source were 250, 250, and 230°C, respectively. Injections were carried out in splitless mode, and the carrier

gas was helium with a ratio of 1 mL per minute. Volatile compounds were identified using mass spectra databases (NIST/EPA/NIH version 2005).

Figure 4.6. Agilent 7890 gas chromatograph and divinylbenzene-carboxen-polydimethylsiloxane coated fiber injected to the port

The quantification of the main volatile compounds was performed on the basis of calibration curves obtained by adding pure standards to 5 mL of milk samples and prepared as previously described for aroma compound analysis.

4.4. DETERMINATION OF MICROBIOLOGICAL AND CHEMICAL COMPOSITION OF PLASMA APPLIED MILK DURING STORAGE

4.4.1. Microbiological Analysis

The same methodology was carried for storage samples as described in 4.3.1. Whole milk was inoculated with test microorganisms and samples were treated with plasma for 20 min at 9 kV for *E. coli* and 15 kV for *S. aureus* and *S.* Typhimurium. Microbiological composition of 20 min plasma treated whole milk inoculated with *E. coli*, *S. aureus*, and *S.* Typhimurium was monitored during storage at 4°C by determining the colony counts,

periodically up to 42 days. Hundred μL of milk samples were taken every 7 days and inoculated onto TSA and incubated at 37ºC for 48h. The results were expressed as log CFU/mL.

4.4.2. Proteolysis Assessment

The methodology that was used for plasma treated milk samples, as shown in Section 4.3.2.3, was repeated for the milk samples which were stored at 4°C for 6 weeks.

4.4.3. Detection of Fatty Acids in Milk

Fatty acid analysis of the milk samples which were stored at 4°C for 42 days were performed for according to the methodologe explained in 4.3.2.4.

4.4.4. Identification of Volatile Compounds in Milk

Previously described methodology in 4.3.2.5 was used for the detection of volatile compounds in samples which were stored at 4°C for 42 days.

4.5. DETERINATION OF KILLING MECHANISM OF PLASMA ON BACTERIAL CELLS

4.5.1. Observation of Morphology of Bacterial Cells

The morphologies of the test microorganisms were observed before and after plasma application under a scanning electron microscope (SEM) (Carl Zeiss, USA) in order to determine any morphological change due to plasma discharge. Before the observation the cells were washed with distilled water and 5 μL of the suspension was dried on a sterile strip. In SEM imaging, the sample surface is scanned with a high-energy electron beam so the specimens must be electrically conductive. Therefore the cells were coated with gold by low vacuum sputter coating or by high vacuum evaporation in order to prevent the specimen from static electric accumulation during irradiation. The SEM images were taken at high vacuum and 10 kV EHT.

4.5.2. Plasma Effect on DNA

4.5.2.1. DNA Extraction

The genetic material of bacteria of tested bacterial strains post plasma application were extracted and analyzed according to the methodology described by Jimenez (Jimenez *et al.* [177] with some modifications. Bacterial cells were obtained from the bacterial solutions by centrifugation at 550*g* for 10 min. One ml of Tris-EDTA buffer was added to the bacterial pellet and transferred into a 2 mL micro-centrifuge tube and centrifuged at 14000 rpm for 2 min. The supernatant was discarded and pellet was re-suspended in 1mL of Tris-EDTA buffer and vortexed. Then the suspension was centrifuged at 27000*g* for 2 min and this process was repeated 3 times. The pellets were dissolved in 300 µL of Tris-EDTA buffer and 20 µL of Proteinase K was added to the gram positive bacteria (*S. aureus*). The solution was incubated at 56°C for 10 min in heat block (Bioer, China) and heated to 95°C for 30 min. DNA was obtained from the supernatant following centrifugation at 27000*g* for 2 min. The extracted DNA was measured for purity in nanophotometer and was stored at -20°C until required for PCR applications.

4.5.2.2. 16S rDNA PCR Amplification

16S rDNA genes of the bacterial DNA isolates (*E. coli* and *S. aureus*) were amplified by PCR (BIORAD, Italy) using purified DNA. Reaction master mixture was containing 0.2 mM 27F forward primer and 1492R reverse primer, 1 U Taq DNA polymerase (Fermentas, USA), 0.2mM deoxynucleoside triphosphate (dNTP), 2 mM $MgCl_2$, 10X Taq buffer and 1 µL of template DNA. PCR amplifications were carried out in a total volume of 50 µL.

Table 4.1. Forward and reverse primer sequences

Primer	Sequence
27F	5'-AGAGTTTGATCCTGGCTCAG-3'
1492R	5'-GGTTACCTTGTTACGACTT-3'

PCR conditions were as follows : Pre-amplification 94°C for 5 min, denaturation at 94°C for 30s, annealing at 55°C for 40s, elongation at 72°C for 2 min repeated 34 cycles

and then post amplification for final extension for 10 min at 72°C. The amplified DNA products were detected in a 1% agarose gel stained with ethidium bromide and run 1 h at 80V and visualized using a Biorad image analysing system (BIORAD, Italy).

4.5.2.3. Ethanol Precipitation of DNA

Sodium acetate buffer (3M, pH 5.2) was added to the isolated DNA in order to equalize ion concentrations. 100% cold ethanol was then added (up to 2X volume) and stored at -20°C for 1 h. Samples were then centrifuged for 15 min at optimum speed in a 4°C microcentrifuge, the supernatant removed and resuspended in 70% cold ethanol. DNA was centrifuged again for 5 min at 4°C, and the supernatant was removed. The remaining ethanol was evaporated. Finally the genomic DNA pellet was resuspended in 50 µL of TE buffer. The precipitated genomic DNA was detected by using Biorad image analysing system (BIORAD, Italy) in a 1% agarose gel stained with ethidium bromide for 1 h at 80V.

4.5.3. Fatty Acid Structure Analysis of the Bacterial Cells (FAME-GC)

Fatty acids from cell walls of test bacteria were isolated in order to test plasma effect on fatty acid molecules present in membrane. The total fatty acids were extracted and methylated using a standard protocol described by MIDI [178] and analyzed in gas chromatography (GC). The bacteria were quadrant streaked on TSA culture media and incubated for 24 h at 37°C. Microorganisms were harvested from the fourth quadrant of the plate in control samples and plasma applied colonies were washed twice with saline and centrifuged at 4000 rpm for 10 min.

The fatty acid extraction process was performed by saponification, methylation, extraction and base wash steps. 1.0 mL of Reagent 1 was added to each test tube containing ~40mg of cells. The tubes were vortexed briefly and placed in the waterbath pre-heated to 100°C for 5 min then the tubes were vortexed for 10 sec and replaced to the waterbath for 25 min. The fatty acids were methylated by adding 2 mL of Reagent 2 when the tubes were cool. After vortexing the tubes for 5 seconds they were heated to 80°C for 10 min. Extraction was carried out with the addition of 1.25 mL of Reagent 3. The tubes were capped and rotated using a clinical rotator for 10 min.

After the extraction organic (upper) phase was pipetted into another sterile tube and 3mL of Reagent 4 was added. The tubes were rotated for 5 min. Following rotation 2 mL of organic phase was pipetted into a GC vial, capped and stored at 4°C until GC analyze. The relative proportions of fatty acid methyl esters between C10:0 and C20:0 were identified with a model 58 microbial identification system (Microbial ID, Newark, Del.) equipped with a Hewlett Packard model 5980 GC. A 5% phenyl-methyl silicone capillary column (0.2mm by 25m) with a flame ionization detector and auto sampler (Hewlett-Packard models 3392 and 7673) were used. The carrier gas flow was 80 mL/min and the injector and detector temperatures were adjusted to 250°C and 300°C respectively. 2 µL of sample was injected automatically in the split mode and the column temperature was raised from 170°C to 270°C at a rate of 5°C /min. Peaks were automatically integrated by comparison of retention times and percentages were calculated. Calibration standards, a negative control blank, and a positive control culture preparation were run with each batch of samples, as recommended by MIDI [178]. Experiments were repeated 3 times for reproducibility of the fatty acid profiles.

4.6. STATISTICAL ANALYSIS

Each treatment was performed three times. Minitab software version 16 (Minitab, Inc., State College, PA) was used for data analysis. Kruskal-Wallis was used to compare the mean values of the response variable and treatment conditions. Evaluations were based on a significance level of $P < 0.05$.

5. RESULTS

5.1. GROWTH CURVES OF BACTERIAL SPECIES

The spectrophotometric growth curves of control cultures represented in Figures 5.1-5.3 with the OD along the *y*-axis and time along the *x*-axis. According to the absorbance measured at 600 nm, exponential phases of the microorganisms were determined and cells in mid-exponential phase were used during the experiments.

The log phase of *E. coli* was observed between 0 and 90 min of cultivation in NB. *E. coli* exponential phase was detected to be between 90 and 270 min regarding optical densities observed at 660 nm. The cells at same growth phase and same absorbance were used throughout the experiments which were in their mid-exponential phase.

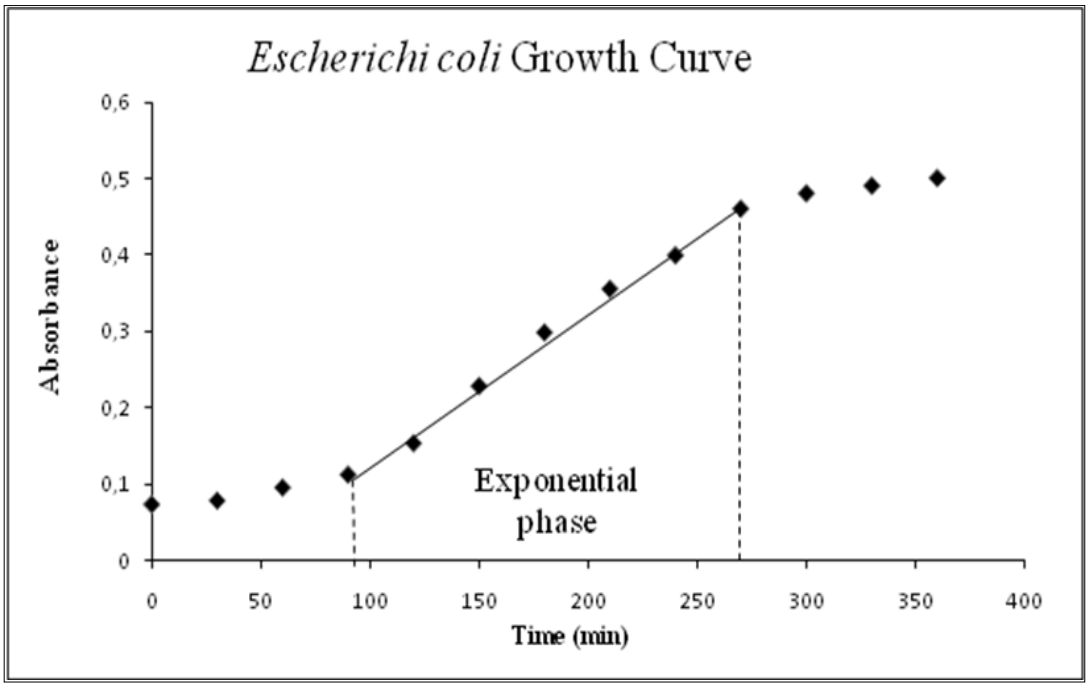

Figure 5.1. Growth curve of *E. coli* ATCC 25922 in NB at 37°C

The log phase of *S. aureus* was observed between 0 and 90 min of cultivation in NB. *S. aureus* exponential phase was detected to be between 90 and 330 min regarding optical

densities observed at 600 nm. The cells at same growth phase and same absorbance were used throughout the experiments which were in their mid-exponential phase.

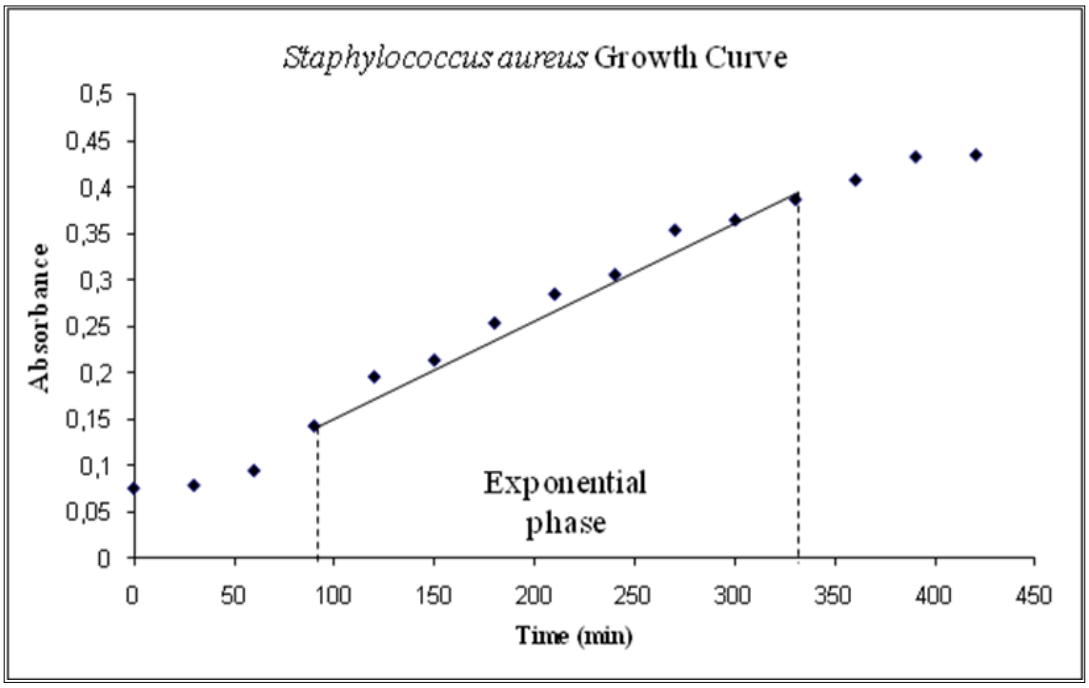

Figure 5.2. Growth curve of *S. aureus* ATCC 25921 in NB at 37°C

Also same observations were carried out for *S.* Typhimurium. The log phase of *S.* Typhimurium was observed between 0 and 90 min of cultivation in NB. *S.* Typhimurium exponential phase was detected to be between 90 and 330 min regarding optical densities observed at 600 nm. The cells at same growth phase and same absorbance were used throughout the experiments which were in their mid-exponential phase.

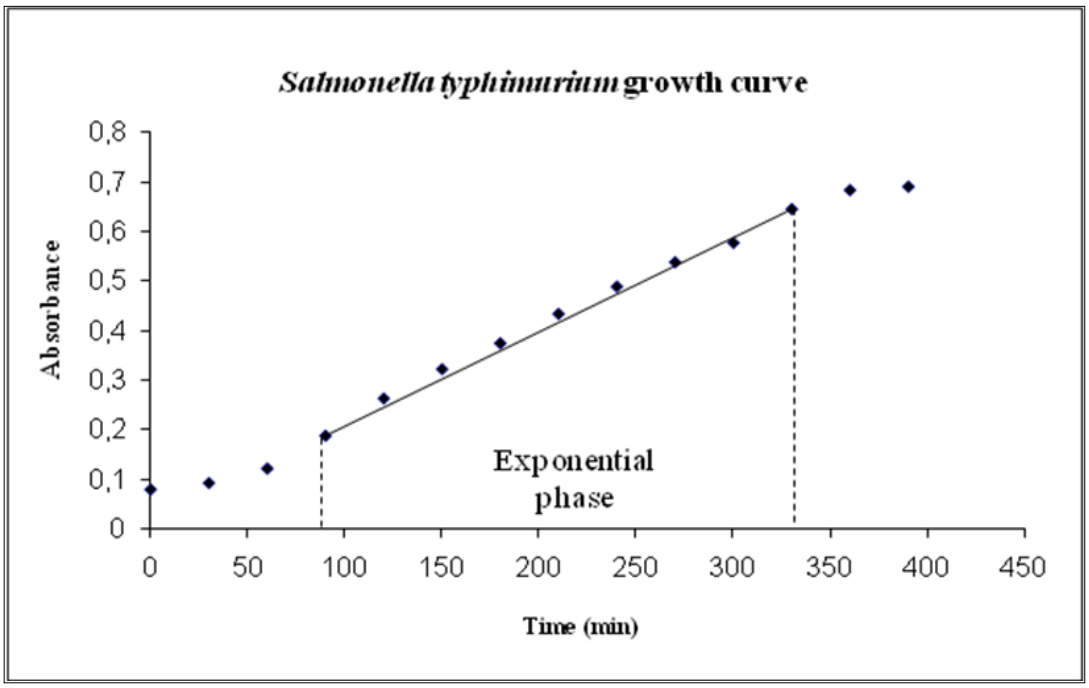

Figure 5.3. Growth curve of *S.* Typhimurium ATCC 14028 in NB at 37°C

5.2. PLASMA SYSTEM TEST PARAMETERS RESULTS

5.2.1. Voltage Results

Plasma system utilized with a 9 kV power supply was tested on *E. coli*, *S. aureus* and *S.* Typhimurium in milk. *E. coli* was killed after 3 min of plasma application. *S. aureus* and *S.* Typhimurium were not killed by the plasma system using 9 kV power supply even after 20 min of application (data not shown). However, *S. aureus* and *S.* Typhimurium were killed after 3 min of plasma treatment at 15 kV. It was seen that higher voltage played a significant role in reducing the inactivation time of *S. aureus* and *S.* Typhimurium in milk.

5.2.2. Exposure Time

The exposure time was an efficiency factor in inactivation of microorganisms in milk. The first 3 min of application had the highest log reductions regardless of the type of microorganism. Following 15 min of treatment slower inactivation activities were observed. Detailed information is given in Section 5.4.1.

5.3. LIGHT EMISSION SPECTROSCOPY RESULTS

The emission intensity spectrum of atmospheric pressure plasma with a frequency of 1 kHz was shown in Figure 5.4. The peaks indicating the ionization levels of the ions in the corona discharges were compared and identified according to NIST Atomic Spectra Database available online [175].

The spectra from the plasma system which is illustrated in Figure 5.4 showed that oxygen (O) and nitrogen (N) were the most abundant molecules excited during plasma discharge. This is due to the air which was used as the carrier gas for plasma. Nickel (Ni) and Chrome (Cr) molecules were also detected in plasma spectra, those were evaporated from the upper Ni–Cr electrode. The peak near λ=310 nm gave plasma discharge the characteristic dark violet color.

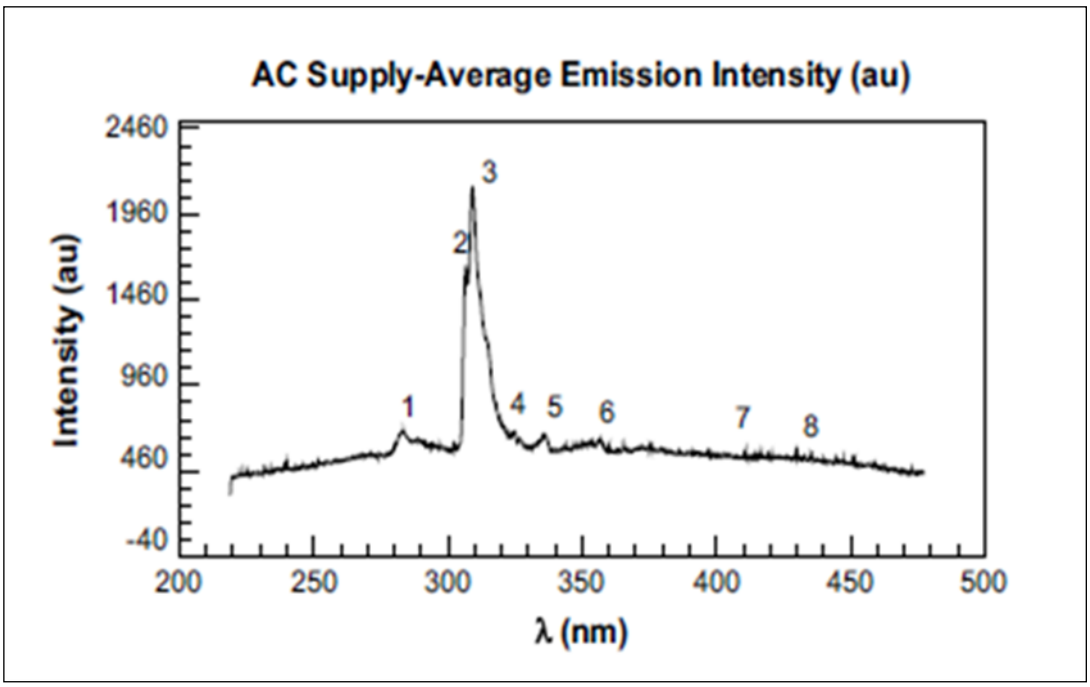

Figure 5.4. The emission intensity spectra of atmospheric pressure AC corona discharge

Peaks 1–8 in Figure 5.4 were as follows: Peak 1: λ=282.858 nm 4th ionization level of oxygen (OIII), Peak 2: λ=306.763 nm 3rd ionization level of nitrogen (NIV), Peak 3: λ=309.154 nm 2nd ionization level of oxygen (OII), Peak 4: λ=324.306 nm 1st ionization

level of nickel (NiI), Peak 5: λ=335.878 nm 3rd ionization level of nitrogen (NIII), Peak 6: λ=356.213 nm 2nd ionization level of oxygen (OII), Peak 7: λ=410.487 nm 1st ionization level of chrome (CrI), and Peak 8: λ=435.459 nm 2nd ionization level of oxygen (OII).

5.4. THE EFFECTS OF PLASMA APPLICATION ON MILK

5.4.1. Microbiological Analysis

The reduction of viable cells during plasma treatment was calculated by counting the colonies on TSA plates as shown in Figure 5.5. The mean values of triplicate experiments were used to calculate log reductions of *E. coli* cells. Biphasic survival curves were obtained after plasma discharge treatment. The highest reduction rate was observed during the first treatment period (0-3 min) and followed by lower levels of bactericidal action.

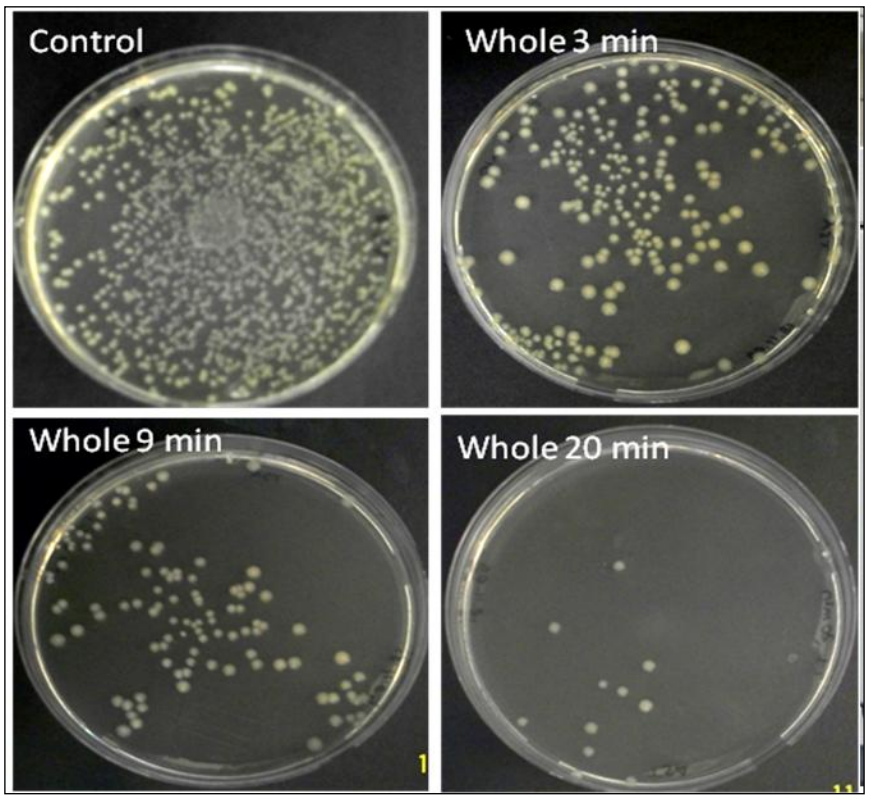

Figure 5.5. *E. coli* colonies in whole milk cultivated and incubated on TSA at 37°C for 48 h after plasma treatment for 3, 9 and 20 min

The effects of plasma corona discharge system on the viable cells of *E. coli* ATCC 25922 inoculated into whole, semi-skimmed, and skimmed milk during 20 min of plasma application with 9 kV power supply is shown in Figure 5.6. Plasma application on milk inoculated with *E. coli* showed a bactericidal effect after only 3 min with a 3 log CFU/mL at 9 kV. The beginning bacterial concentration of 7.78 log CFU/mL and significantly decreased to 4.23 (p=0.02), 4.14 (p=0.07), and 4.23 (p=0.09) log CFU/mL in whole, semi-skimmed, and skimmed milk, respectively. The slowest inhibition acitivity was observed between 15 and 20 min. The bacterial concentrations were 3.84, 3.80, and 3.64 log CFU/mL at 15 min in whole, semi-skimmed, and skimmed milk, respectively which decreased to 3.63, 3.40, and 3.34 log CFU/mL, respectively after 20 min of plasma application. Total log reductions following 20 min of plasma application were 4.38, 4.15, 4.44 in whole, semi-skimmed and skimmed milk, respectively.

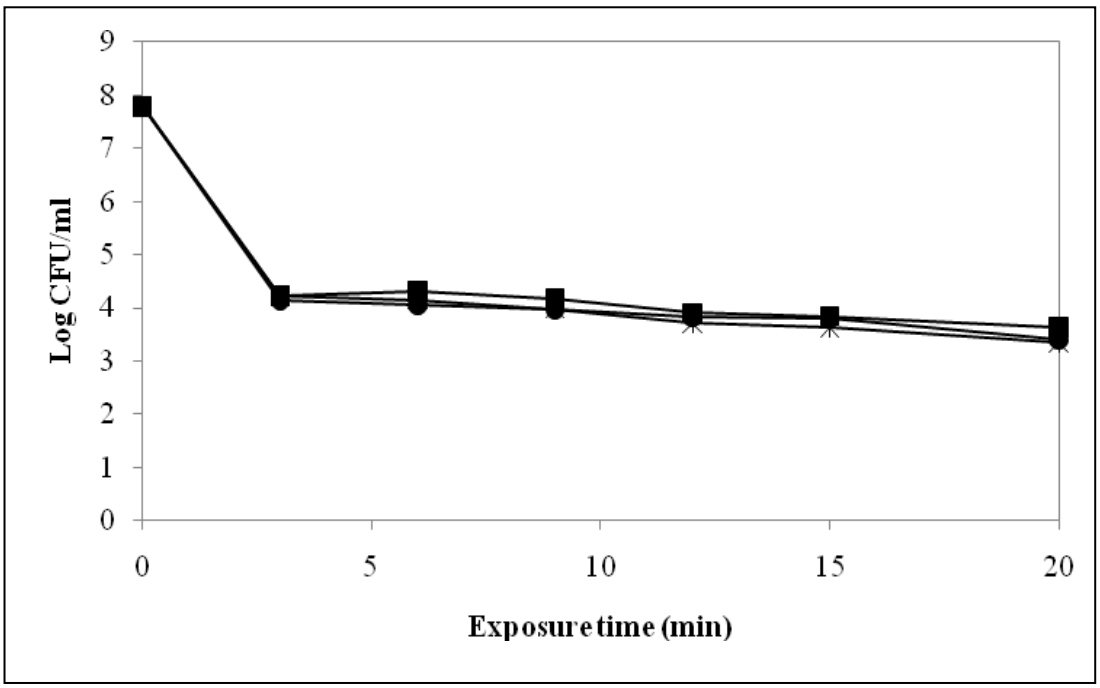

Figure 5.6. Survival curves for treatments of atmospheric pressure plasma on *E. coli* ATCC 25922 inoculated in whole, semi-skimmed and skimmed milk

Symbols are; ■, whole milk; ●, semi-skimmed milk, and *, skimmed milk

Since no significant difference in log reductions and killing time was observed between all 3 milk types, further experiments were carried out on whole milk only. The

plasma application for 20 min, generated with 9 kV, AC power supply did not show significant difference in the number of viable cells of *S. aureus* inoculated into whole milk (data not shown). Therefore, the following experiment was carried out with 15 kV, AC power supply utilized to the plasma system.

Figure 5.7 shows the effects of plasma corona discharge system on the viable cells of *S. aureus* inoculated into whole milk during plasma application for 20 min with 15 kV power supply. The highest inactivation activity was observed in first 3 min of treatment. The beginning concentration of 5.36 log CFU/mL decreased to 4.52 log CFU/mL when the milk was exposed to the plasma corona discharge system for 3 min. In the following treatment mins a gradual decrease was observed in viable cells of *S. aureus*. The bacterial concentration was 3.84 log CFU/mL in 15 min and decreased to 3.44 log CFU/mL Therefore, as a result of 20 min plasma corona discharge application with 15 kV, AC power supply significant inactivation of *S. aureus* inoculated into whole milk was obtained with (p=0.037).

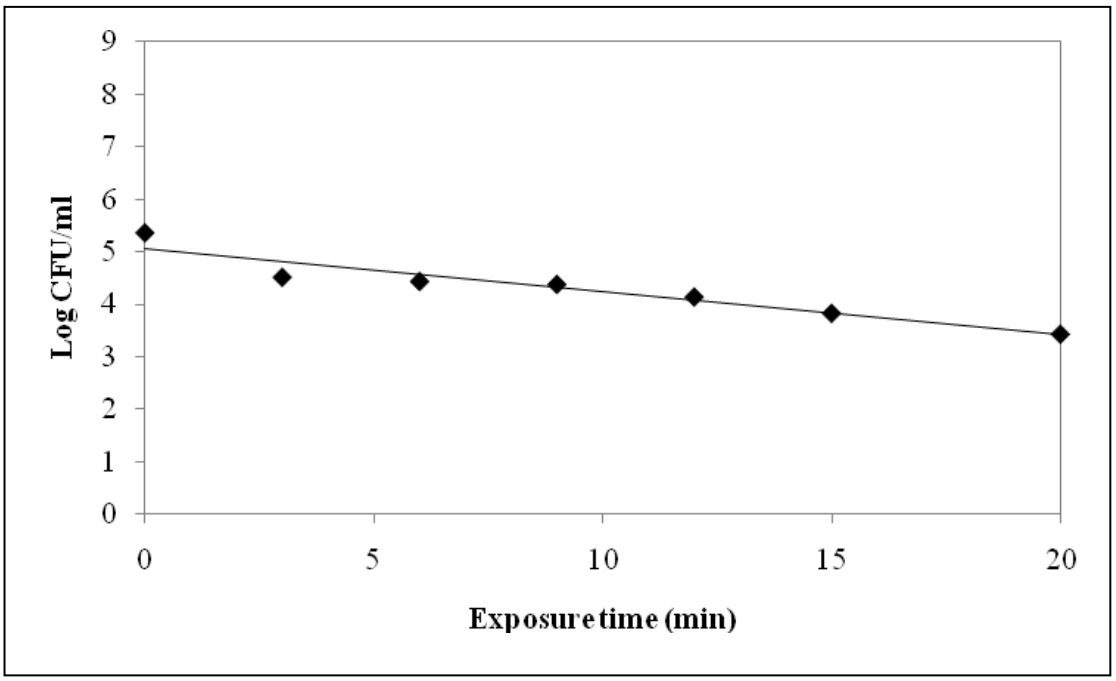

Figure 5.7. Survival curve for treatment of atmospheric pressure plasma on *S. aureus* ATCC 25921 inoculated in whole milk (♦, *S. aureus*)

Similar results were observed when the 9 kV plasma system was applied for 20 min, to the *S.* Typhimurium cells inoculated into whole milk (data not shown). Therefore, the plasma system utilized with 15 kV, AC power supply was used for the following experiment.

While the bactericidal activity was similar in plasma both bacterial species, slightly higher reduction was observed in the viable cells of *S.* Typhimurium inoculated into whole milk than *S. aureus* cells.

Figure 5.8 shows the effects of plasma corona discharge system on the viable cells of *S.* Typhimurium inoculated into whole milk during plasma application for 20 min with 15 kV power supply. Simirlarly, the first 3 min of plasma treatment with 15 kV power supply had the highest inactivation activity. *S.* Typhimurium under the same conditions showed highly significant inactivation with 2.62 log CFU/mL (p=0.001) as shown in Figure 5.8. The survival curve of *S.* Typhimurium was linear as shown in Figure 5.8 with increased inactivation potential every 3 min of plasma application.

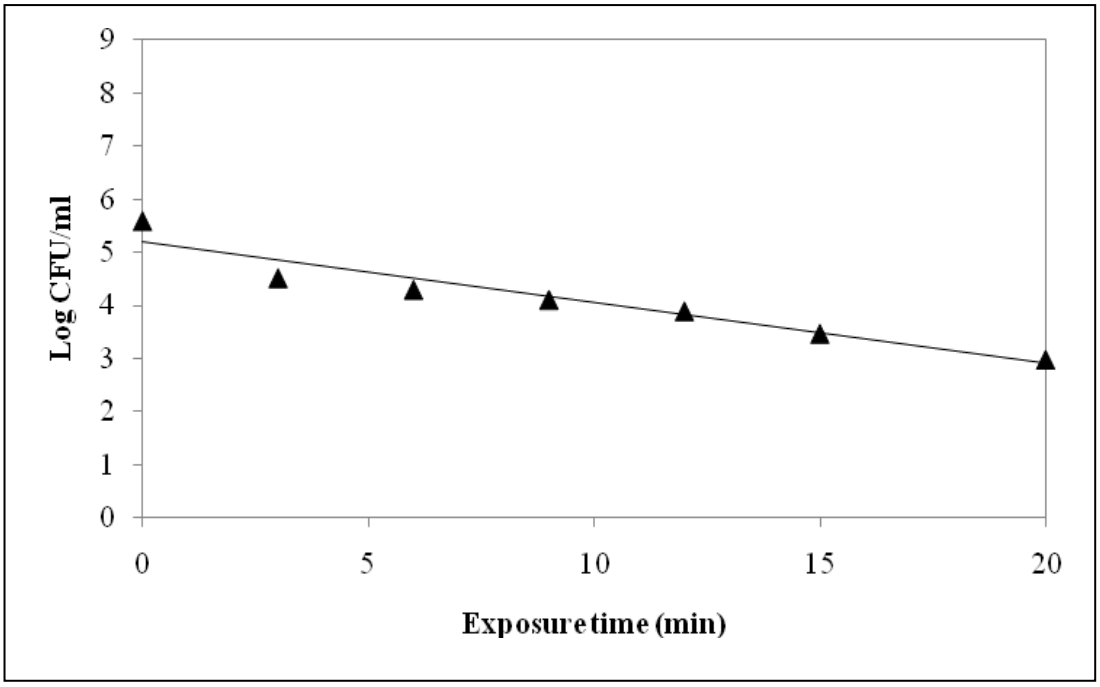

Figure 5.8. Survival curve for treatment of atmospheric pressure plasma on *S.* Typhimurium ATCC 14028 inoculated in whole milk (▲, *S.* Typhimurium)

The colony numbers of *E. coli*, *S. aureus*, and *S.* Typhimurium in 1 mL of whole milk after 0, 3, 6, 9, 12, 15, and 20 min of treatment were shown in Table 5.1. For all 3 samples, the number of cells decreased simultaneously with plasma treatment time. The number of *E. coli* cells in milk was decreased from $6.0x10^7$ to $2.1\ x10^4$ after 3 min of 9 kV plasma application. When the plasma treatment was further carried on rather slow inactivation kinetics were observed. The number of *E. coli* cells in 6 min plasma treated milk was $1.7\ x10^4$ and reduced to $4.2\ x10^3$ after a total of 20 min application. The highest inactivation rate of *S. aureus* was observed during first 3 min with a decrease of colonies from $2.3\ x10^5$ to $3.3\ x10^4$. After 9 min of treatment a linear 2-fold reduction rate was observed and the number of the cells were reduced from $2.4\ x10^4$ to $2.8\ x10^3$. *S.* Typhimurium was killed from $4.0\ x10^5$ to $3.4\ x10^4$ cell numbers within 3 min of application. Between 12 and 15 min the reduction rate was higher and the number of cells in 1 mL was $8.0\ x10^3$ and $3.0\ x10^3$ respectively. When the milk was exposed to plasma for 20 min, the final cell concentration was observed to be $9.7\ x10^2$.

Table 5.1. The effects of plasma system on CFU/ml of *E. coli*, *S. aureus*, and *S.* Typhimurium in whole milk

*Milk inoculated with *E. coli* was treated with 9 kV power supply, while milk samples inoculated *S. aureus,* and *S.*Typhimurium were treated with 15 kV plasma systems

^{xy}Means in the same column followed by different superscript letters are significantly different ($p<0.05$)

Exposure Time (min)	***E. coli****	***S. aureus****	***S.* Typhimurium***
0	$6.0x10^{7x}$	$2.3\ x10^{5x}$	$4.0\ x10^{5x}$
3	$2.1\ x10^{4y}$	$3.3\ x10^{4y}$	$3.4\ x10^{4y}$
6	$1.7\ x10^{4y}$	$2.8\ x10^{4y}$	$2.0\ x10^{4y}$
9	$1.5\ x10^{4y}$	$2.4\ x10^{4y}$	$1.3\ x10^{4y}$
12	$8.0\ x10^{3y}$	$1.4\ x10^{4y}$	$8.0\ x10^{3y}$
15	$7.0\ x10^{3y}$	$6.9\ x10^{3y}$	$3.0\ x10^{3y}$
20	$4.2\ x10^{3y}$	$2.8\ x10^{3y}$	$9.7\ x10^{2y}$

The total log reductions of *E. coli*, *S. aureus* and *S.* Typhimurium in whole milk after 20 min of plasma application were shown in Figure 5.9 log reductions of *E. coli*, *S. aureus* and *S. Typhimurium* were 4.15, 1.92 and 2.62, respectively. *E. coli* in whole milk was killed more than 4 logs after 20 min plasma treatment while *S. aureus* and *S.* Typhimurium showed lower total reductions. A total treatment of plasma utilized with 15 kV power supply showed approximately 2 log reductions in the numbers of *S. aureus* inoculated into whole milk. Also, *S.* Typhimurium in whole milk was remarkable reduced following plasma application with the plasma system using 15 kV power supply. A total reduction of more than 2.6 log was detected in milk sample inoculated with *S.* Typhimurium.

The decontamination activity of plasma system on milk previously inoculated with test microorganisms changed between bacterial strains. Highest activity was observed on inactivation of *E. coli* in milk, which was followed by *S.* Typhimurium and *S. aureus*. However, voltages which were 9 kV for *E. coli* and 15 kV for *S. aureus* and *S.* Typhimurium should be taken into consideration upon comparison of milk decontamination with plasma system.

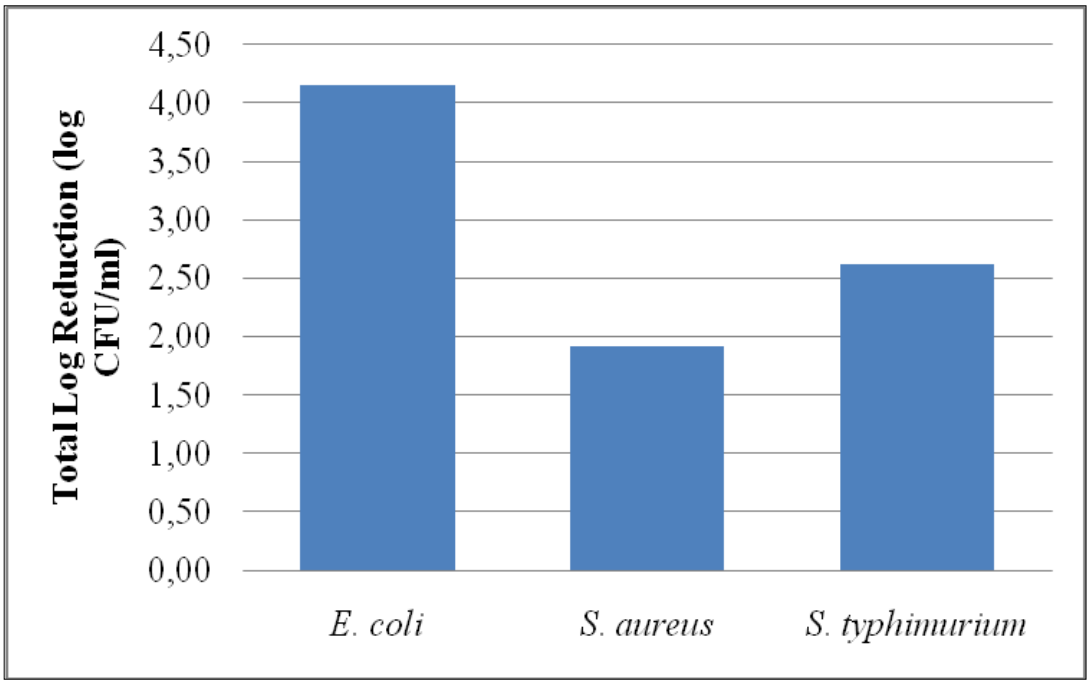

Figure 5.9. The effect of 20 min plasma application on *E. coli* ATCC 25922, *S. aureus* ATCC 25921 and *S.* Typhimurium ATCC 14028 cells in whole milk

5.4.2. Physical and Chemical Analysis of Treated and Untreated Milk

5.4.2.1. pH Analysis

Atmospheric pressure plasma application with 15 kV, AC power supply did not cause significant change in pH values of UHT whole milk samples inoculated with *E. coli*, *S. aureus* and *S.* Typhimurium compared to the controls. pH values of the samples were approximately 6.7 ± 0.05 after 0, 3, 6, 9, 12, 15 and 20 min of plasma corona discharge application.

Since similar results were observed for all 3 test microorganisms, a representative graph was shown in *E. coli*.

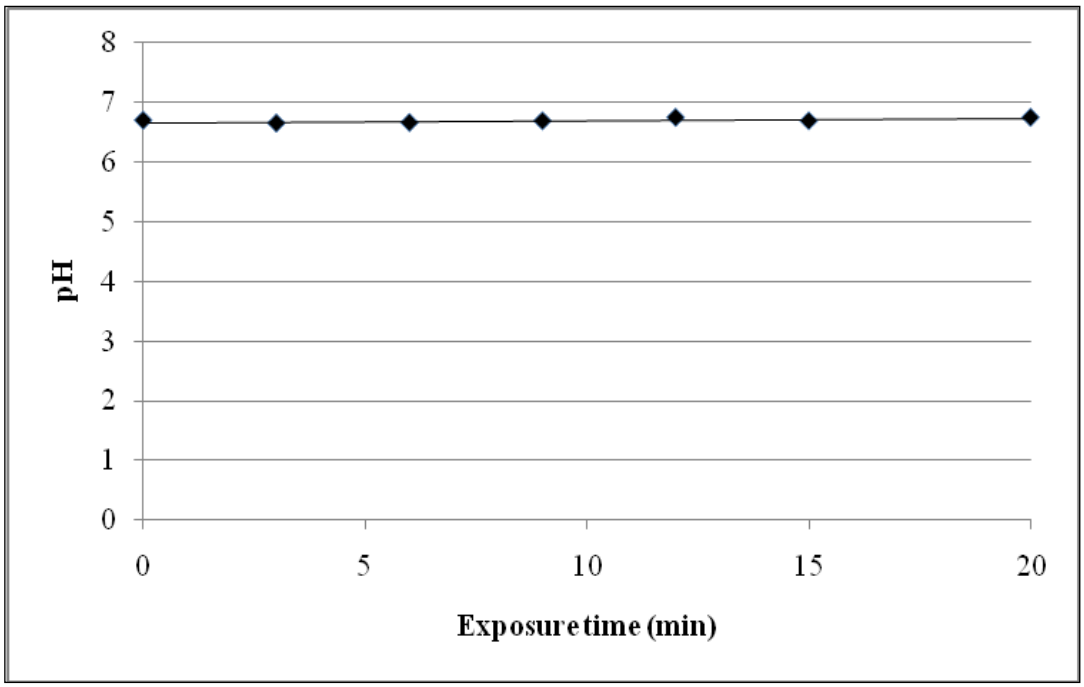

Figure 5.10. The effects of plasma system on pH of milk (♦, pH)

5.4.2.2. Color Measurements

Milk color measurements of plasma treated samples and untreated controls were shown in Table 5.2. The color values (L*, a*, and b*) and the total color difference (ΔE) of whole milk samples treated for 9 min showed no changes in color measurements ΔE and milk treated at 15 kV for 20 min showed only slightly noticeable differences.

Table 5.2 shows the results of instrumental determination of color of UHT whole milk after 9 and 20 min of 15 kV plasma treatment. After 9 min of plasma treatment L* value increased from 84.60 to 84.78, while a value increased from -1.53 to -1.51. Plasma treatment for 9 min caused a slight increase in the value of b* which was 5.52 in control milk and 5.84 in plasma-treated sample. However 20 min of plasma application caused significant reduction of L* value which decreased from 84.78 to 84.09 and the a* and b* values significantly increased from -1.53 and 5.52, to -1.44 and 6.18, respectively (p=0.01). Total ΔE values were calculated for 9 and 20 min plasma-treated samples by the equation given in section 4.3.2.2. The total color difference for milk after 9 min of plasma treatment with 15 kV was 0.37 while longer exposure to plasma (20 min) caused slightly higher color differences with a ΔE value of 0.84. Therefore, plasma application resulting in no or slightly noticeable color differences in milk samples.

Table 5.2. Results of instrumental determination of color of UHT whole milk after 9 and 20 min of 15 kV plasma treatment

L*: lightness (ranging from 0 to 100), a*: green to red (ranging from -60 to +60) and the b*: blue to yellow (ranging from -60 to +60), ΔE: calculated color differences ^{xy}Means in the same row followed by different superscript letters are significantly different (p<0.05)

	Untreated Control	**9 min**	**20 min**
L*	84.60^{x}	84.78^{x}	84.09^{y}
a*	-1.53^{x}	-1.51^{x}	-1.44^{y}
b*	5.52^{x}	5.84^{x}	6.18^{y}
ΔE		0.37	0.84

As shown in Table 5.2 milk samples tested had slight green and yellow components. The untreated control milk samples were slightly whiter than 20 min plasma treated samples and less greeny and more yellowish color.

5.4.2.3. Protein Analysis

Dark smears were observed after SDS-PAGE was performed for all samples due to high fat concentration of whole milk. Even though the bands were not very clear, it was

observed that plasma discharge did not cause any significant change to the milk quality by means of proteolysis. Atmospheric plasma application for 20 min at 37°C on whole milk inoculated with *E. coli*, *S. aureus* and *S.* Typhimurium did not affect the protein composition.

Although, SDS-PAGE results were not very clear because of the high fat concentration of whole milk, some dense, darker bands could be observed around 65 kDa, 50 kDa, and 30 kDa. The milk is composed of numerous proteins from which the primary groups are caseins and whey proteins including beta-lactoglobulin and alpha-lactalbumin. The relative sizes of some proteins are; alpha-lactalbumin 14 kDa, beta-lactoglobulin 18 kDa, caseins 25-35 kDa, serum albumin 66 kDa and lactoferrin 80 kDa. Therefore, the bands, observed in all samples regardless of the plasma application and storage, may correspond to the serum albumin and casein proteins naturally found in milk.

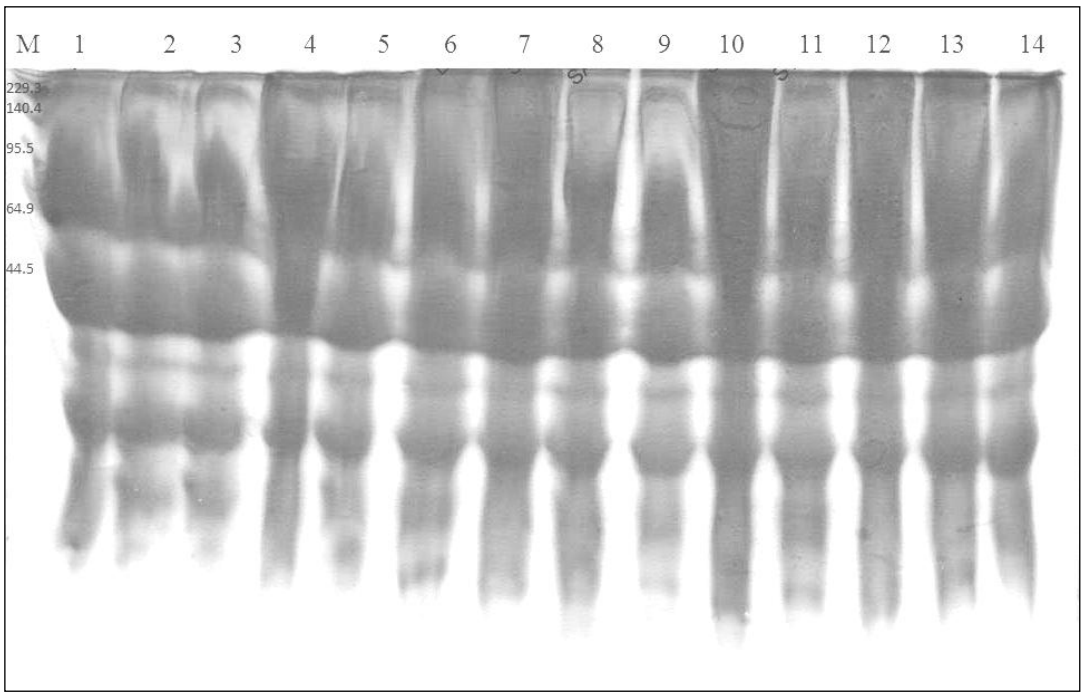

Figure 5.11. SDS-PAGE results after atmospheric pressure plasma treatment and storage M: MultiColor marker (Abnova, Taiwan), 1: *E. coli* control, 2: *E. coli* 6 min, 3: *E. coli* 9 min, 4: *S.* Typhimurium 3 min, 5: *S.* Typhimurium 15 min, 6: *E. coli* after storage, 7: *S.* Typhimurium after storage, 8: *S. aureus* storage control, 9: *E. coli* 3 min, 10: *S.*

Typhimurium control, 11: *S.* Typhimurium storage control,12: *S.* Typhimurium 9 min, 13: *S.* Typhimurium 12 min, 14: *E. coli* 12 min

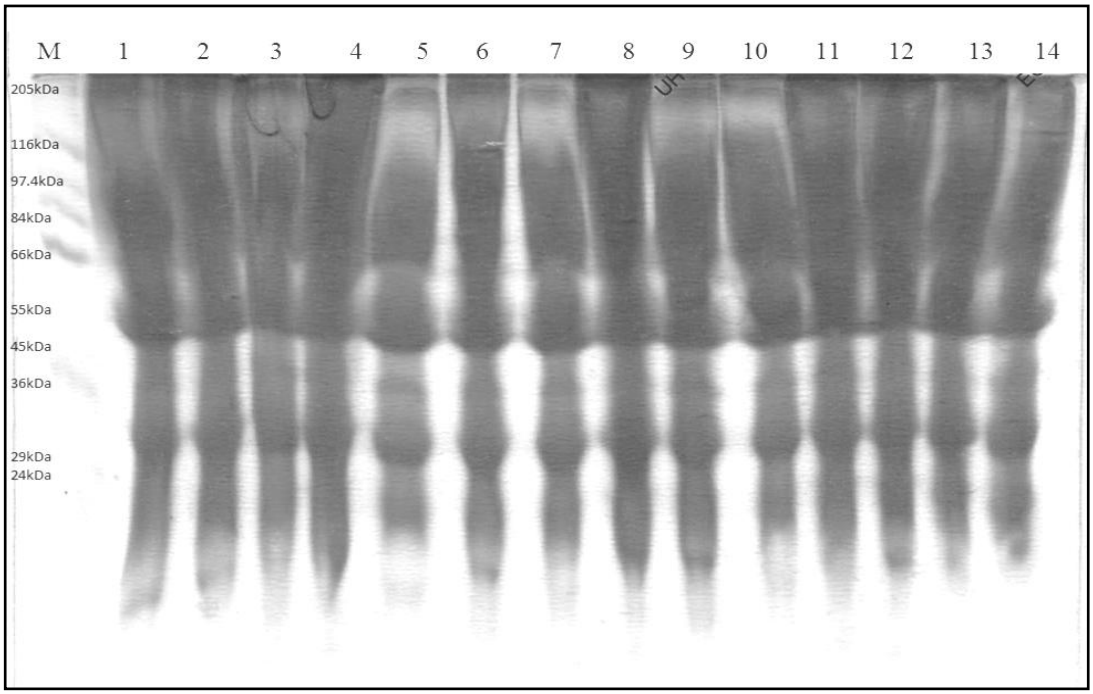

Figure 5.12. SDS-PAGE results after atmospheric pressure plasma treatment and storage M: Sigma wide range marker, 1: *S. aureus* 20 min after storage, 2: *S. aureus* 12 min, 3: *E. coli* 20 min, 4: *S. aureus* control, 5: *E. coli* 15 min, 6: *S. aureus* 9 min, 7: *S. aureus* 3 min, 8: *S.* Typhimurium 20 min, 9: Whole milk control, 10: *S. aureus* 6 min, 11: *S. aureus* 20 min, 12: *S. aureus* 15 min, 13: *S.* Typhimurium 6 min, 14: *E. coli* storage control

5.4.2.4. Fatty Acid Results of Milk

Free fatty acid (FFA) profiles of plasma treated milk samples were detected using GC/MS (Agilent, USA). FFAs composition of milk inoculated with *E. coli* was summarized in Figure 5.13 as percentages of total saturated, monounsaturated, and polyunsaturated fatty acids after each time interval of 9 kV plasma application. No significant differences were observed upon total and indivial fatty acid analysis. About 64 % of FFAs were saturated fatty acids (SFAs) and the rest was monounsaturated and polyunsaturated fatty acids with percentages of about 29 and 3, respectively. The SFAs concentration decreased from 63.95 to 59.75 % within first 3 min of plasma application which also showed the highest inactivation activity on *E. coli*. However, after 3 min, the

total SFAs increased gradually up to 66.32 % during 20 min of plasma application. The initial total monounsaturated fatty acids (MUFAs) concentration in untreated milk sample inoculated with *E. coli* was 27.53 % which increased to 31.70 and 31.94 %, respectively after 3 and 6 min of plasma application. After 9 min, the total MUFA level decreased to 27.11 % and further treatment time did not cause any major change in total MUFA amount. The 9 kV plasma application was most effective on polyunsaturated fatty acids (PUFAs) which was decreased from 3.06 to 2.63 % after 3min and descended to 1.72 % following 20 min of treatment.

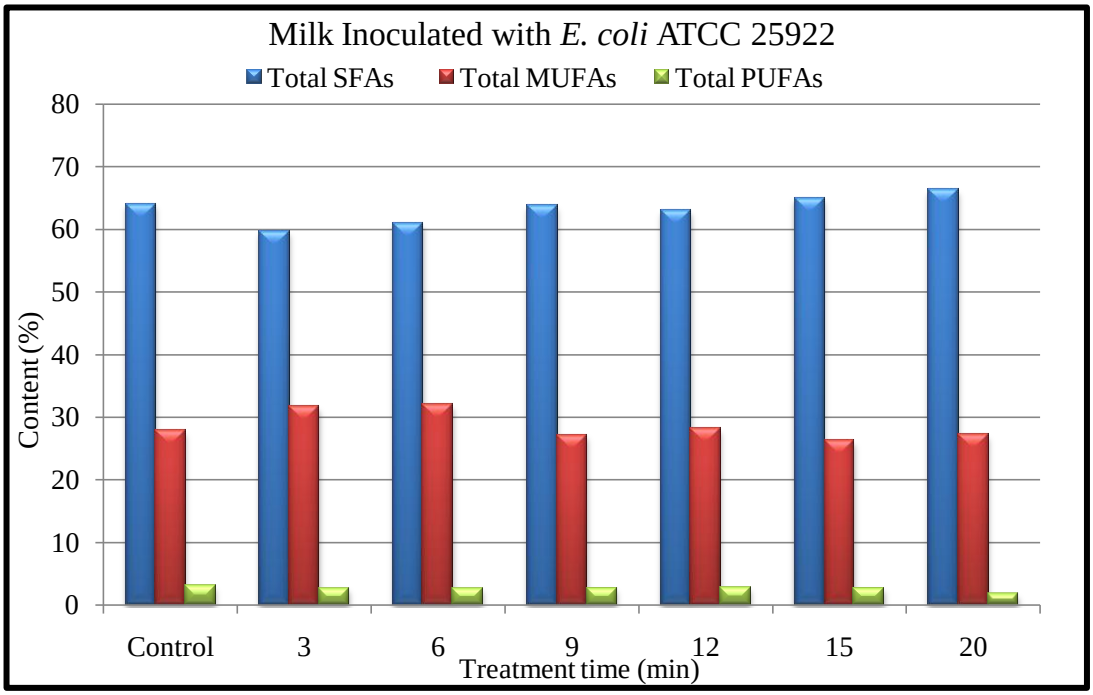

Figure 5.13. Total saturated, monosaturated and polyunsaturated fatty acid profiles of milk inoculated with *E. coli* ATCC 25922 after 9 kV plasma application
SFAs: Saturated fatty acids, MUFAs: Monounsaturated fatty acids, PUFAs: Polyunsaturated fatty acids

The fatty acid profiles of whole milk inoculated with *E. coli* after 9 kV plasma treatment were shown in Table 5.3. Total SFAs detected were between C8:0 – C20:0, MUFAs were C14:1, C16:1 cis and trans, C18:1, C19:1, C20:1 cis and trans, and PUFAs were C18:2, C20:4 and C20:3. The results showed that some fatty acids that were present less than 1 % before plasma such as C13:0, C16:1 Δ^{11}, C19:1, C19:0, C20:0, C20:1, C20:3,

and C20:4 were eliminated after plasma application while more abundant fatty acids showed slight quantitative changes. The most predominant fatty acids observed in milk sample before plasma application were hexadecanoic acid (C16:0), oleic acid (C18:1) and stearic acid (C18:0) which made up approximately 33 %, 25 % and 16 % of the fatty acid content of the whole milk, respectively. The concentration of hexadecanoic acid decreased from 33.43 to 29.17 % after 3 min of application and then increased up to 34.70 % when 20 min of plasma was applied. The amount of oleic acid increased from 24.69 to 28.10 and 28.27 % following 3 and 6 min of plasma application, respectively which was then decreased to 24.47 % after 20 min. The plasma application for 20 min caused a reduction in the amount of stearic acid which decreased from 15.62 to 12.74 %. Therefore, the overall percentages of the major fatty acids in whole milk were not affected by 20 min of 9 kV plasma application, except stearic acid which was reduced.

Table 5.3. Fatty acid composition of milk inoculated with *E. coli* ATCC 25922 after treatment with 9 kV plasma system

[a] 5,8,11,14-Eicosatetraenoic acid, [b] 7,10,13-Eicosatrienoic acid, [c] SFAs: Saturated fatty acids, MUFAs: Monounsaturated fatty acids, PUFAs: Polyunsaturated fatty acids

Fatty Acid	**Percent in Milk Inoculated with *E. coli* ATCC 25922 After Different Treatment Times**						
	Control	**3 min**	**6 min**	**9 min**	**12 min**	**15 min**	**20 min**
C8:0					0.00	0.49	0.00
C10:0	0.00	0.15	0.66	1.39	0.00	2.23	1.00
C11:0	1.36	0.14	0.55	0.16	1.05	0.32	0.00
C12:0	0.62	2.33	2.65	3.31	1.33	3.38	2.94
C13:0	0.06	0.10	0.11	0.12	0.08	0.13	0.00
C13 iso	0.16	0.24	0.26	0.28	0.21	0.27	0.19
C14:1	0.85	1.18	1.19	1.33	1.06	1.27	1.00
C14:0	9.86	10.95	10.88	11.97	10.95	11.53	12.51
i-C14:0	0.36	0.48	0.49	0.53	0.46	0.50	0.37
a-C14:0	0.80	1.03	1.06	1.14	1.02	1.09	0.85
C15:0	1.68	1.87	1.90	0.00	1.89	1.93	1.58

Table 5.3. Fatty acid composition of milk inoculated with *E. coli* ATCC 25922 after treatment with 9 kV plasma system (Continue)

Fatty Acid	**Percent in Milk Inoculated with *E. coli* ATCC 25922 After Different Treatment Times**						
	Control	**3 min**	**6 min**	**9 min**	**12 min**	**15 min**	**20 min**
C15:0 iso	0.50	0.61	0.62	0.66	0.59	0.63	0.45
C16:1 trans	0.12	0.16	0.15	0.16	0.15	0.16	0.09
C16:1 cis	1.90	1.89	1.94	2.07	1.96	1.94	1.61
C16:1 (Δ^{11})	0.04	0.03	0.00	0.02	0.02	0.02	0.00
C16:0	33.43	29.17	29.01	31.13	32.41	29.76	34.70
C16:0 iso	0.55	0.67	0.68	0.73	0.66	0.69	0.49
C16:0 anteiso	0.81	1.02	1.04	1.11	1.03	1.04	0.76
C17:0	1.01	1.16	1.19	1.25	1.14	1.19	0.85
C18:2	2.63	2.14	2.10	2.24	2.36	2.21	1.72
C18:1 (cis-Δ^{9})	24.69	28.10	28.27	23.15	24.56	22.42	24.47
C18:1 (trans-Δ^{9})	0.39	0.36	0.00	0.32	0.40	0.32	0.33
C18:1 (Δ^{11})	1.28	1.03	0.00	1.08	1.11	1.00	1.02
C18:0	15.62	13.42	13.49	14.03	13.66	13.55	12.74
C19:1	0.10	0.17	0.17	0.17	0.15	0.17	0.00
C19:0	0.07	0.18	0.12	0.13	0.11	0.13	0.00
C20:4	0.14	0.18	0.19	0.18	0.15	0.18	0.00
C20:3	0.12	0.12	0.12	0.09	0.09	0.12	0.00
C20:1 n9 (cis 11)	0.14	0.20	0.19	0.19	0.18	0.21	0.00
C20:0	0.24	0.27	0.31	0.30	0.25	0.31	0.00
Total SFA	63.953	59.751	60.860	63.787	62.876	64.942	66.322
Total MUFA	27.826	31.704	31.940	27.110	28.061	26.201	27.168
Total PUFA	3.065	2.631	2.631	2.736	2.790	2.748	1.716

Detectable quantitative differences were observed for the linear-chain FAs C10:0, C12:0 and C18:0 of milk previously inoculated with *E. coli* after exposure to 9 kV plasma

system. In particular as the exposure time increased, an increase in the percentages of C10:0 and C12:0 and a reduction in that of C18:0 were evidenced as shown in Figure 5.14. The amount of C10:0 increased from 0 to 1.39 % in 9 min and was not detected in sample treated for 12 min. After 15 and 20 min the concentrations were 2.32 and 1.0 %, respectively. The C12:0 concentration in control milk sample was 0.62 which was dramatically increased to 2.33 % in 3 min and gradually went up to 3.38 % in 9 min of application. A decrease in the concentration of C12:0 was observed in 12 min, however the amount was high in 15 and 20 min of application. Exposure to 9 kV plasma system caused a reduction from of total C18:0 concentration. Although an increase was detected in 9 min, the initial concentration of 15.62 was decreased to 12.74 % after treatment for 20 min.

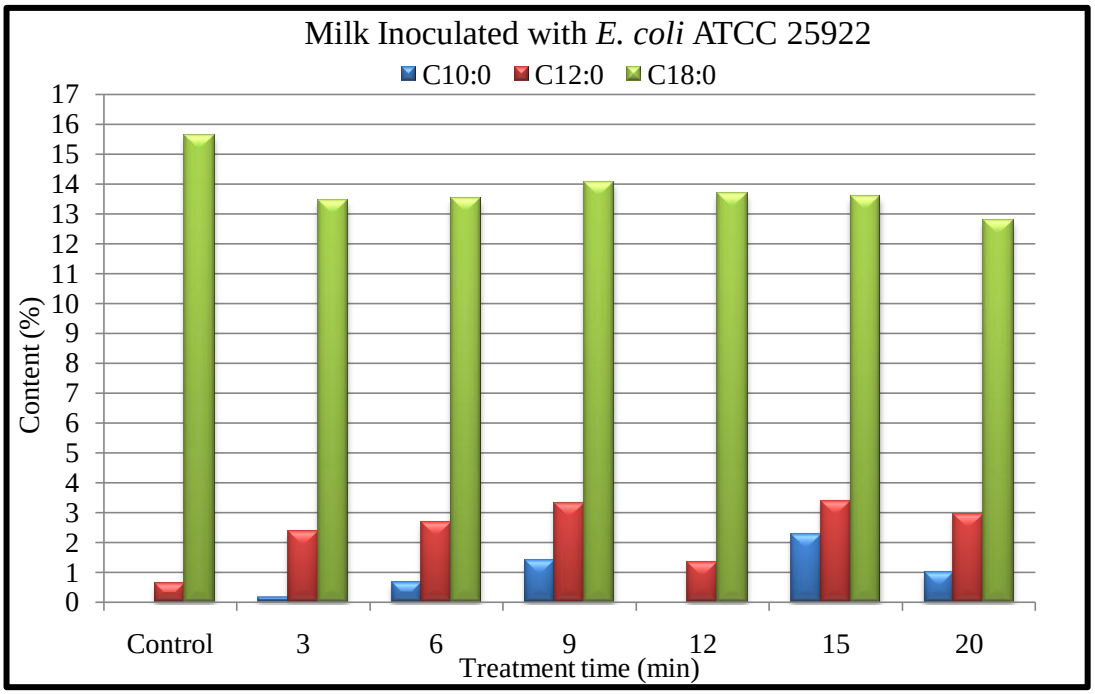

Figure 5.14. 9 kV plasma effects on C10:0, C12:0 and C:18:0 fatty acid profiles of milk inoculated with *E. coli*

Examination of the amount C18:0, C12:0, and C10:0 showed that long-chain fatty acid (C18:0) was decreased while short-chain fatty acids (C10:0 and C12:0) were increased during plasma.

FFAs composition of milk inoculated with *S. aureus* was summarized in Figure 5.15 as percentages of total SFA, MUFA, and PUFA after each time interval of 15 kV plasma

application. No significant differences were observed in total fatty acid concentrations regarding treatment time. About 66, 26 and 3% of total fatty acid content was SFAs, MUFAs, and PUFAs, respectively. The total fatty acid concentrations did not change even after 20 min of plasma application with 15 kV. Only a slight decrease was observed in total MUFA concentration which was 26.76 % in control whole milk sample and 24.45 % in 20 min plasma-treated milk.

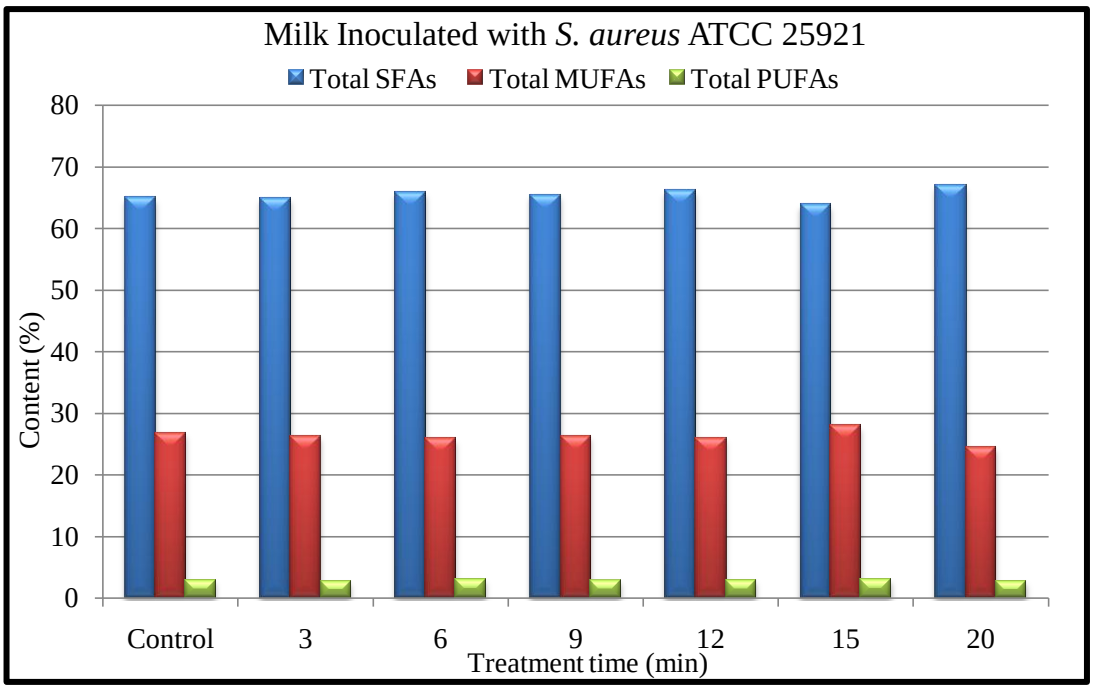

Figure 5.15. Total saturated, monosaturated and polyunsaturated fatty acid profiles of milk inoculated with *S. aureus* ATCC 25921 after 15 kV plasma application

The fatty acid profiles of whole milk inoculated with *S. aureus* after 15 kV plasma treatment were shown in Table 5.4. Total SFAs detected were between C8:0 – C20:0, MUFAs were C14:1, C16:1 cis and trans, C18:1, C19:1, C20:1 cis and trans, and PUFAs were C18:2, C20:4 and C20:3. The most predominant fatty acids observed in milk sample before plasma application were hexadecanoic acid (C16:0), oleic acid (C18:1) and stearic acid (C18:0) which made up approximately 31 %, 23 % and 15 % of the fatty acid content of the whole milk, respectively. The concentration of hexadecanoic acid was not changed during the experiment of 20 min plasma treatment at 15 kV. The amount of oleic acid decreased from 23.35 to 22.45 following 3 of plasma application, and then increased to

24.68 % in 15 min while the concentration decreased to 20.58 % when 20 min of plasma was applied to the sample. The stearic acid concentration was not significantly changed during 20 min of plasma exposure. Therefore, the overall percentages of the major fatty acids in whole milk were not affected by 20 min of 15 kV plasma application, except oleic acid which was slightly reduced. Another predominant fatty acid in profile of whole milk inoculated with *S. aureus* was C14:0 with 11.17 %. This component was constant during treatment by 15 kV plasma and an increase from 11.17 to 12.34 % was observed, only after 20 min application. Results showed that the fatty acid profiles of different exposure times of whole milk inoculated with *S .aureus* did not change significantly, revealing the FFAs content of milk unaffected even after 20 min by the plasma system using 15 kV power supply.

Table 5.4. Fatty acid composition of milk inoculated with *S. aureus* ATCC 25921 after treatment with 15 kV plasma system

[a] 5,8,11,14-Eicosatetraenoic acid, [b] 7,10,13-Eicosatrienoic acid, [c] SFAs: Saturated fatty acids, MUFAs: Monounsaturated fatty acids, PUFAs: Polyunsaturated fatty acids

Fatty Acid	**Percent in Milk Inoculated with *S. aureus* ATCC 25921 After Different Treatment Times**						
	Control	**3 min**	**6 min**	**9 min**	**12 min**	**15 min**	**20 min**
C8:0	0.00	0.15	0.00	0.00	0.00	0.00	0.00
C10:0	1.33	1.99	0.89	1.45	1.69	0.45	2.02
C11:0	0.40	0.24	0.30	0.23	0.63	0.40	0.57
C12:0	3.03	3.41	3.11	3.16	3.42	2.11	3.69
C13:0	0.11	0.13	0.13	0.13	0.12	0.09	0.15
C13 iso	0.22	0.26	0.18	0.22	0.18	0.19	0.27
C14:1	1.14	1.28	1.22	1.19	1.31	1.03	1.37
C14:0	11.17	11.76	11.76	11.50	11.96	10.70	12.34
i-C14:0	0.40	0.48	0.40	0.43	0.41	0.39	0.49
a-C14:0	0.86	1.04	0.87	0.91	0.91	0.81	1.04
C15:0	1.71	1.91	1.81	1.78	1.65	1.63	1.98
C15:0 iso	0.50	0.59	0.47	0.52	0.48	0.49	0.59

Table 5.4. Fatty acid composition of milk inoculated with *S. aureus* ATCC 25921 after treatment with 15 kV plasma system (Continue)

Fatty Acid	**Percent in Milk Inoculated with *S. aureus* ATCC 25921 After Different Treatment Times**						
	Control	**3 min**	**6 min**	**9 min**	**12 min**	**15 min**	**20 min**
C16:1 trans	0.11	0.14	0.13	0.12	0.12	0.12	0.15
C16:1 cis	1.87	1.97	1.98	1.91	1.86	1.83	2.04
C16:1 (Δ^{11})	0.03	0.02	0.03	0.03	0.03	0.03	0.03
C16:0	30.99	30.34	31.80	30.95	30.66	31.69	30.94
C16:0 iso	0.56	0.65	0.57	0.57	0.56	0.54	0.61
C16:0 anteiso	0.81	0.98	0.83	0.84	0.85	0.78	0.87
C17:0	1.03	1.14	1.05	1.03	0.98	0.99	1.08
C18:2	2.41	2.24	2.58	2.42	2.44	2.59	2.28
C18:1 (cis-Δ^{9})	23.35	22.45	22.26	22.75	22.40	24.68	20.58
C18:1 (trans-Δ^{9})	0.34	0.32	0.41	0.34	0.31	0.37	0.31
C18:1 (Δ^{11})	1.27	0.98	1.00	1.25	0.96	1.34	1.17
C18:0	14.88	13.53	14.65	14.90	14.72	15.40	13.84
C19:1	0.13	0.15	0.12	0.13	0.15	0.13	0.13
C19:0	0.09	0.11	0.10	0.09	0.08	0.08	0.10
C20:4[a]	0.17	0.17	0.16	0.14	0.16	0.18	0.16
C20:3[b]	0.13	0.12	0.11	0.11	0.11	0.12	0.11
C20:1n9 (cis11)	0.16	0.18	0.16	0.15	0.16	0.17	0.15
C20:0	0.25	0.28	0.25	0.23	0.24	0.26	0.24
Total SFAs[c]	65.00	64.98	65.83	65.45	66.15	63.79	66.95
Total MUFAs[c]	26.76	26.18	25.87	26.26	26.00	27.97	24.45
Total PUFAs[c]	2.87	2.71	3.01	2.82	2.87	3.06	2.72

Although the total SFA, MUFA and PUFA concentrations of milk inoculated with *S. aureus* did not change significantly, some quantitative differences were observed upon examination of the linear-chain FAs of C10:0, C12:0 and C18:0 after exposure to 15 kV

plasma system. Figure 5.16 shows the individual percentages of these FAs according to the treatment time. After 20 min of exposure, asignificant increase in the percentages of C10:0 and C12:0 and a reduction in that of C18:0 were evidenced. The amount of C10:0 increased from 1.33 to 1.99 % in 3 min and increased to 2.02 % following 20 min application. Moreover, the C12:0 concentration in control milk sample was 3.03 which was slightly increased to 3.41 % in 3 min and increased up to 3.69 % after 20 min of application. Plasma system using 15 kV power supply caused a reduction of total C18:0 concentration which was 14.88 and 13.53 % in control and 3-min sample, respectively. Conversely, 15 min of plasma application caused a decrease in C10:0 and C12:0 with percentages of 0.45 and 2.11, respectively while increasing the amount of C18:0 from 14.88 to 15.40 %.

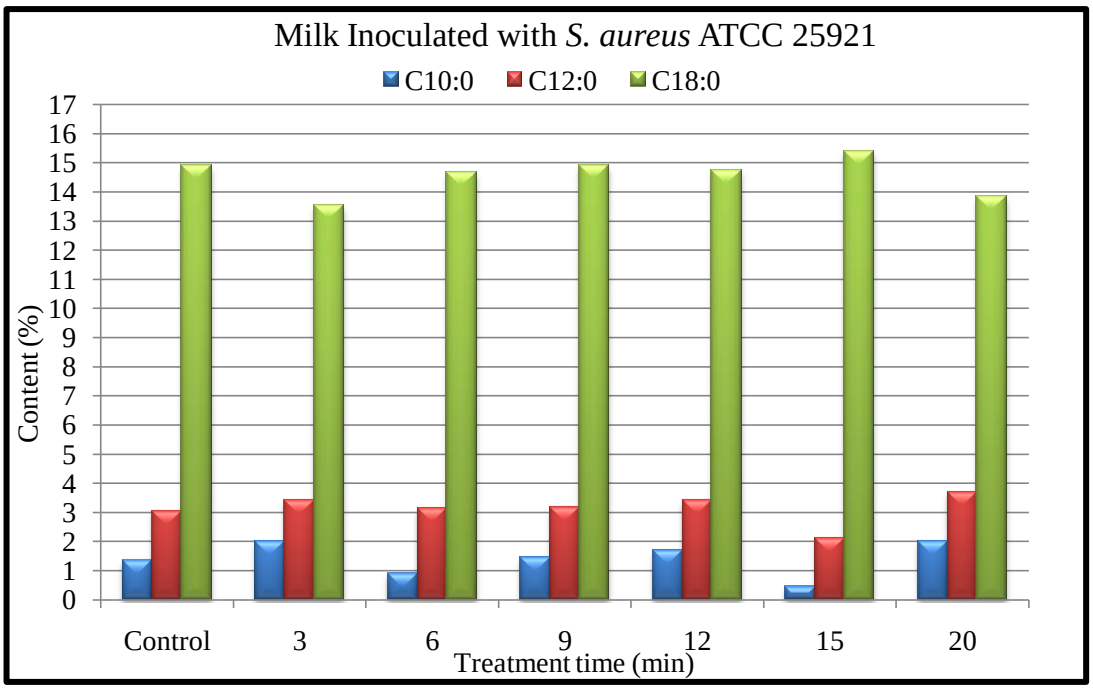

Figure 5.16. 15 kV plasma system effects on C10:0, C12:0 and C:18:0 fatty acid concentrations of whole milk inoculated with *S. aureus* ATCC 25921

FFAs composition of milk inoculated with *S.* Typhimurium was shown in Figure 5.17 as percentages of total SFA, MUFA, and PUFA after each time interval of 15 kV plasma application. About 65, 26, and 3 % of total fatty acid content of whole milk was SFAs, MUFAs, and PUFAs, respectively. The total fatty acid concentrations did not

change during the whole experiment of 20 min plasma application with 15 kV. Only a slight increase in the percentage of SFAs from 64.15 to 66.02 was observed after 3 min which was constant until SFAs showed decrease to 63.98 in 20min plasma treated sample. Total MUFA concentration did not change significantly during 20 min plasma treatment of milk inoculated with *S.* Typhimurium. On the other hand, a slight decrease was observed in the amount of PUFAs during 20 min which decreased to 2.98 % while the control sample contained 3.07 % of PUFAs. However, none of these changes were significant, resulting that total SFAs, MUFAs, and PUFAs profiles of whole milk inoculated with *S.* Typhimurium were unaffected by plasma treatment utilized with 15 kV power supply.

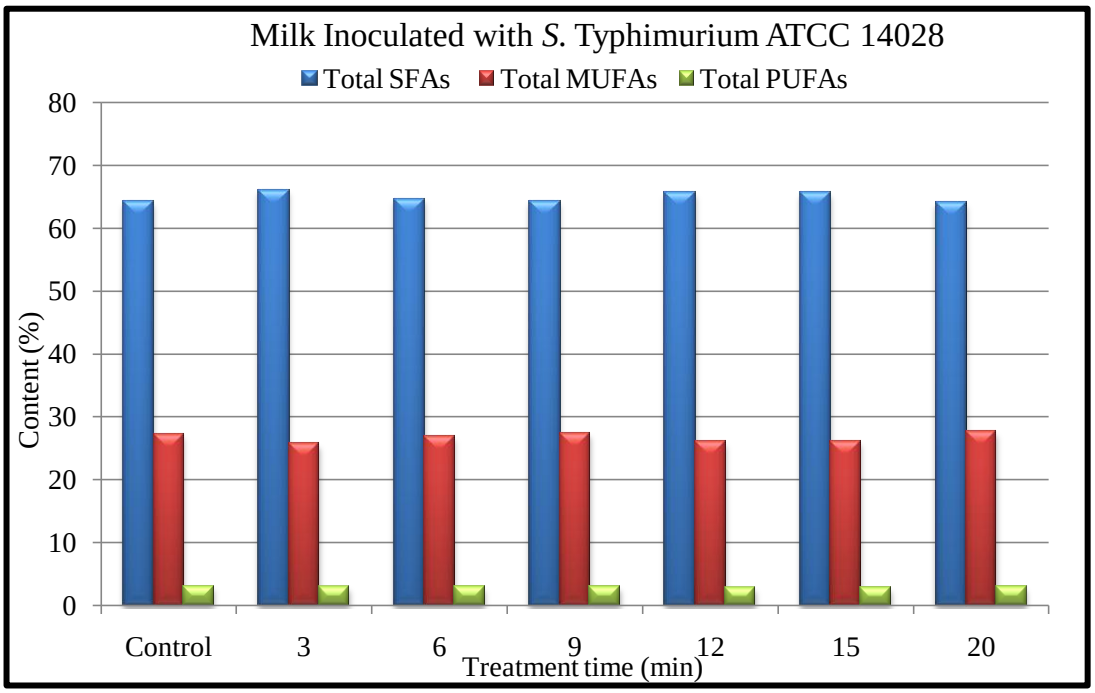

Figure 5.17. Total saturated, monosaturated and polyunsaturated fatty acid profiles of milk inoculated with *S.* Typhimurium ATCC 14028 post-plasma application

The highest killing activity on *S.* Typhimurium in whole milk was observed in 3 min of plasma application and more than 2.5 log reductions were achieved following 20 min application. However, the total FFAs of milk inoculated with *S.* Typhimurium were uneffected by the treatment of 15 kV plasma during 20 min of experiment.

The fatty acid profiles of whole milk inoculated with *S.* Typhimurium after 15 kV plasma treatment were shown in Table 5.5. Total SFAs detected were between C8:0 – C20:0, MUFAs were C14:1, C16:1 cis and trans, C18:1, C19:1, C20:1 cis and trans, and PUFAs were C18:2, C20:4 and C20:3. The most predominant fatty acids observed in milk sample before plasma application were hexadecanoic acid (C16:0), oleic acid (C18:1) and stearic acid (C18:0) which made up approximately 31 %, 24 % and 15 % of the fatty acid content of the whole milk, respectively. The concentration of hexadecanoic acid was slightly lower in the samples treated with 3 and 15 min plasma at 15 kV. Six, 9 and 20 min of application showed slightly increased percentages of 31.94, 31.83, and 31.91, respectively. The amount of oleic acid decreased from 23.68 to 21.99 following 3 of plasma application, and then increased back to 23.46 % in 6 min. A decrease in the concentration of oleic acid was observed after 12 min application while in 20 min it increased from 22.69 to 24.30 %. The reduction of stearic acid concentration started after 3 min of plasma application with a decrease of initial concentration (15.44 %) to 15.01 % and lowest levels of stearic acid were detected in 12 and 15 min of plasma exposure which were 14.77 and 14.90, respectively. However, the level increased after 20 min of 15 kV plasma application to 15.60 %.

Another predominant fatty acid in profile of whole milk inoculated with *S.* Typhimurium was C14:0 with a beginning concentration of 11.31 %. This fatty acid decreased to 10.88 % after 9 min plasma application with 15 kV power supply. The following treatment times showed similar results with beginning concentration while the level was 10.93 % after 20 min application.

Although some differences between control and plasma treated samples were detected, these changes were not significant. Therefore, the fatty acid profiles of whole milk inoculated with *S.* Typhimurium after different exposure times were not effected even after 20 min by the plasma system using 15 kV power supply.

Table 5.5. Fatty acid composition of milk inoculated with *S.* Typhimurium ATCC 14028 after treatment with 15 kV plasma system

[a] 5,8,11,14-Eicosatetraenoic acid, [b] 7,10,13-Eicosatrienoic acid, [c] SFAs: Saturated fatty acids, MUFAs: Monounsaturated fatty acids, PUFAs: Polyunsaturated fatty acids

Fatty Acid	**Percent in Milk Inoculated with *S.* Typhimurium ATCC 14028 After Different Treatment Times**						
	Control	**3 min**	**6 min**	**9 min**	**12 min**	**15 min**	**20 min**
C8:0	0.00	0.00	0.00	0.00	0.00	0.29	0.00
C10:0	0.069	1.947	0.045	0.368	0.964	1.984	0.08
C11:0	0.37	0.32	0.60	0.50	0.24	0.34	0.21
C12:0	2.10	3.38	1.93	2.10	3.05	3.21	2.04
C13:0	0.11	0.13	0.10	0.10	0.13	0.10	0.10
C13 iso	0.22	0.24	0.21	0.20	0.23	0.22	0.21
C14:1	1.16	1.24	1.12	1.06	1.23	1.16	1.07
C14:0	11.32	11.51	11.20	10.88	11.81	11.24	10.93
i-C14:0	0.42	0.45	0.43	0.40	0.45	0.41	0.40
a-C14:0	0.90	0.94	0.91	0.84	0.95	0.88	0.86
C15:0	1.80	1.83	1.80	1.74	1.86	1.72	1.71
C15:0 iso	0.54	0.55	0.54	0.52	0.54	0.50	0.52
C16:1 trans	0.13	0.13	0.13	0.12	0.13	0.12	0.13
C16:1 cis	1.95	1.96	1.94	1.90	1.96	1.88	1.89
C16:1 (Δ^{11})	0.03	0.03	0.03	0.03	0.03	0.03	0.03
C16:0	31.49	30.44	31.94	31.83	31.42	30.47	31.91
C16:0 iso	0.58	0.60	0.59	0.58	0.58	0.54	0.55
C16:0 ante	0.84	0.86	0.86	0.84	0.84	0.78	0.780
C17:0	1.07	1.08	1.08	1.06	1.05	1.00	1.03
C18:2	2.54	2.41	2.50	2.46	2.40	2.42	2.49
C18:1 (cis-Δ^{9})	23.67	21.99	23.46	24.00	22.36	22.69	24.30
C18:1 (trans-Δ^{9})	0.36	0.32	0.35	0.37	0.29	0.36	0.38
C18:1 (Δ^{11})	1.27	1.11	1.13	1.19	1.28	1.18	1.29
C18:0	15.44	15.01	15.60	15.37	14.77	14.90	15.60

Table 5.5. Fatty acid composition of milk inoculated with *S.* Typhimurium ATCC 14028 after treatment with 15 kV plasma system (Continue)

Fatty Acid	**Percent in Milk Inoculated with *S.* Typhimurium ATCC 14028 After Different Treatment Times**						
	Control	**3 min**	**6 min**	**9 min**	**12 min**	**15 min**	**20 min**
C19:1	0.13	0.14	0.137	0.14	0.13	0.13	0.13
C19:0	0.09	0.11	0.103	0.10	0.09	0.10	0.10
C20:4[a]	0.20	0.18	0.181	0.18	0.15	0.16	0.17
C20:3[b]	0.14	0.14	0.133	0.13	0.09	0.13	0.12
C20:1n9 (cis11)	0.19	0.18	0.181	0.18	0.15	0.15	0.19
C20:0	0.29	0.27	0.274	0.26	0.24	0.30	0.28
Total SFAs[c]	64.15	66.02	64.680	64.28	65.62	65.66	63.98
Total MUFAs[c]	27.24	25.64	26.978	27.40	25.97	26.13	27.71
Total PUFAs[c]	3.07	2.92	2.999	2.96	2.79	2.87	2.98

The total SFA, MUFA and PUFA concentrations of milk inoculated with *S.* Typhimurium were not affected by 15 kV plasma treatment, however slightly significant differences (p=0.046) were observed upon comparison of C10:0, C12:0 and C18:0 concentrations in untreated and 20 min treated samples with 15 kV plasma system. Figure 5.18 shows the individual percentages of C10:0, C12:0 and C18:0 FAs in 0, 3, 6, 9, 12, 15, and 20 min of plasma treated milk inoculated with *S.* Typhimurium.. After 3 min of exposure, an increase in the percentages of C10:0 and C12:0 and a reduction in that of C18:0 were detected. The amount of C10:0 increased from 0.07 to 1.95 % in 3 min. Also an increase in the concentration of C12:0 fatty acid was observed in the same plasma exposure time, from 2.10 to 3.38 %. While these contents increased in 3 min C18:0 decreased from 15.44 to 15.01 %. The C10:0 fatty acid level decreased to 0.05 % after 6 min of plasma application and gradually increased until 15 min. Twenty min of plasma caused the C10:0 amount decrease to 0.08 %. The fatty acid C12:0 slightly changed with the exposure time which was 3.05 and 2.04 % in 15 and 20 min plasma treatment, respectively. After 12 min of exposure to plasma system using 15 kV power supply a

reduction of total C18:0 concentration was observed from 15.37 to 14.77 %. Conversely, the amount of C18:0 increased after 20 min from 14.90 to 15.60 %.

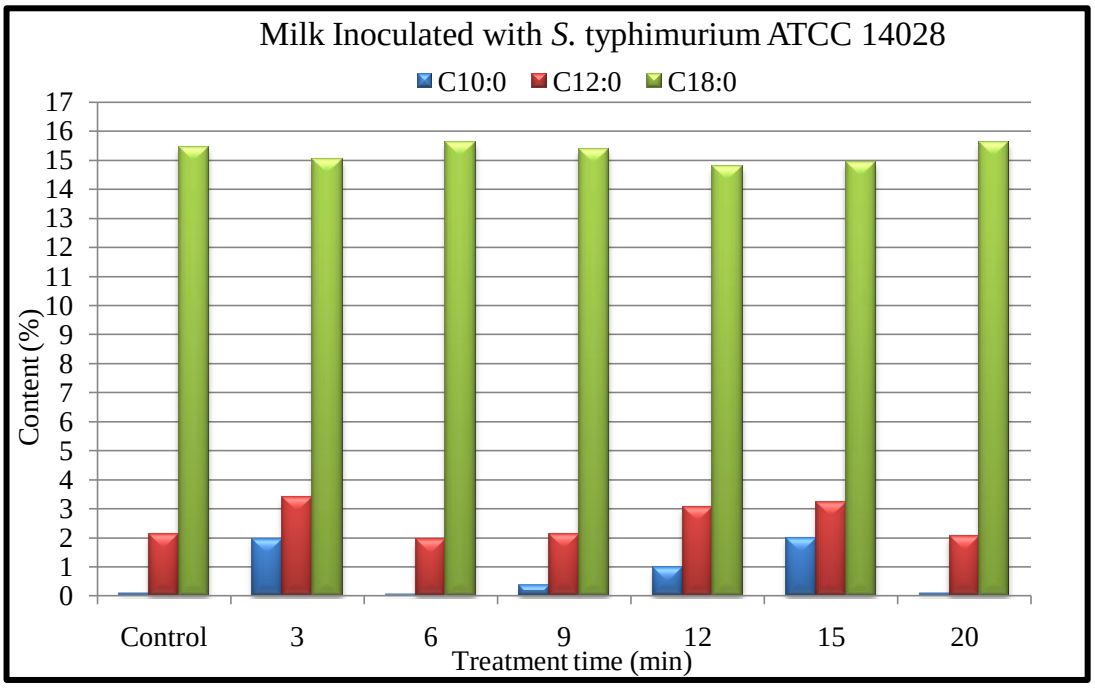

Figure 5.18. 15 kV plasma effects on C10:0, C12:0 and C:18:0 fatty acid profiles of whole milk inoculated with *S.* Typhimurium ATCC 14028

The values of C10:0, C12:0, and C18:0 in milk inoculated with *E. coli*, *S. aureus*, and *S.* Typhimurium following 20 min of treatment with 9 kV power supply for *E. coli* and 15 kV power supply for *S. aureus*, and *S.* Typhimurium were shown in Figure 5.19. Detectable qualitative differences were observed for the linear-chain FAs C10:0, C12:0 and C18:0 of milk previously contaminated with *E. coli*. An increase in the percentages of C10:0 and C12:0 and a reduction in that of C18:0 were evidenced as shown in Figure 5.19. On the contrary, samples inoculated with the other 2 microorganisms resulted to be completely unaffected by the plasma treatment regardless the exposure time although a high variability in the values was detected.

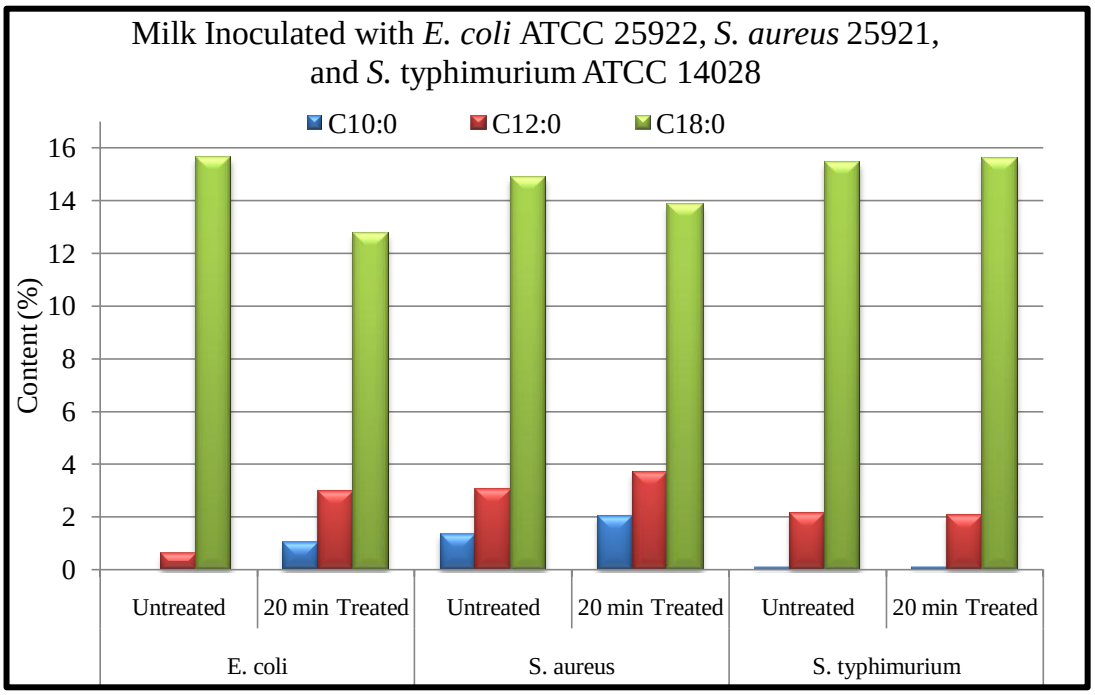

Figure 5.19. The comparison of final C10:0, C12:0, and C18:0 concentrations in 20 min plasma treated milk inoculated with *E. coli* ATCC 25922, *S. aureus* ATCC 25921, and *S.* Typhimurium ATCC 14028

The comparison C10:0, C12:0, and C18:0 concentrations in milk inoculated with *E. coli*, *S. aureus*, and *S.* Typhimurium showed that milk inoculated with *E. coli* was more effected by plasma treatment, although higher voltages were used in milk samples inoculated with *S. aureus* and *S.* Typhimurium. However, higher inactivation rates were obtained in milk inoculated with *E. coli* where nearly 4 log reductions were observed.

5.4.2.5. Effect of Plasma Treatment on Organic and Volatile Compounds of Milk

Solid phase microextraction-gas chromatography/mass spectrometry was used for the analysis of volatile compounds as a result of plasma corona discharge system exposure. More than 50 volatile organic compounds (VOCs) have been separated and identified. Ketones, aldehydes, alcohols and to a lesser extent also hydrocarbons were regularly detected in all milk samples. In particular, the list of the VOC found in all the control samples included acetone, 2-pentanone, 5-methyl-3-hexanone, 4-methyl-2-hexanone, 4-methyl-3-penten-2-one, 2-heptanone, 2-nonanone, 2-undecanone (ketones), hexanal, octanal, nonanal, benzaldehyde (aldehydes), ethanol, hexanol, 1-octanol (alcohols).

The total ketone, aldehyde and alcohol concentrations of 3, 6, 9, 12, 15 and 20 min of 9 kV plasma treated milk inoculated with *E. coli* were compared with the control samples and shown in Figure 5.20. The total ketone concentrations were approximately 10 ppm in control and dramatically decreased to 4.79 ppm after 3 min of 9 kV plasma application. Following 6 min treatment, the total ketone concentration increased to 10.25 ppm and kept constant at nearly 10 ppm, except 9 min which showed a decrease from 10.25 to 6.63. In general the exposure to 9 kV plasma system resulted in a decrease of the content (expressed as ppm) of the total aldehydes for milk samples inoculated with *E. coli*, however a significant increase from 4.65 to 24.42 ppm was observed in 15 min plasma treated sample. Total alcohol concentration was detected to be mostly effected by 9 kV plasma treatment. An initial concentration of total alcohols of 3.54 ppm was reduced during 20 min of total exposure with a final value of 1.5 ppm, while 15 min treatment caused an increase of total alcohols from 1.33 to 2.67 ppm which was also lower than the initial concentration.

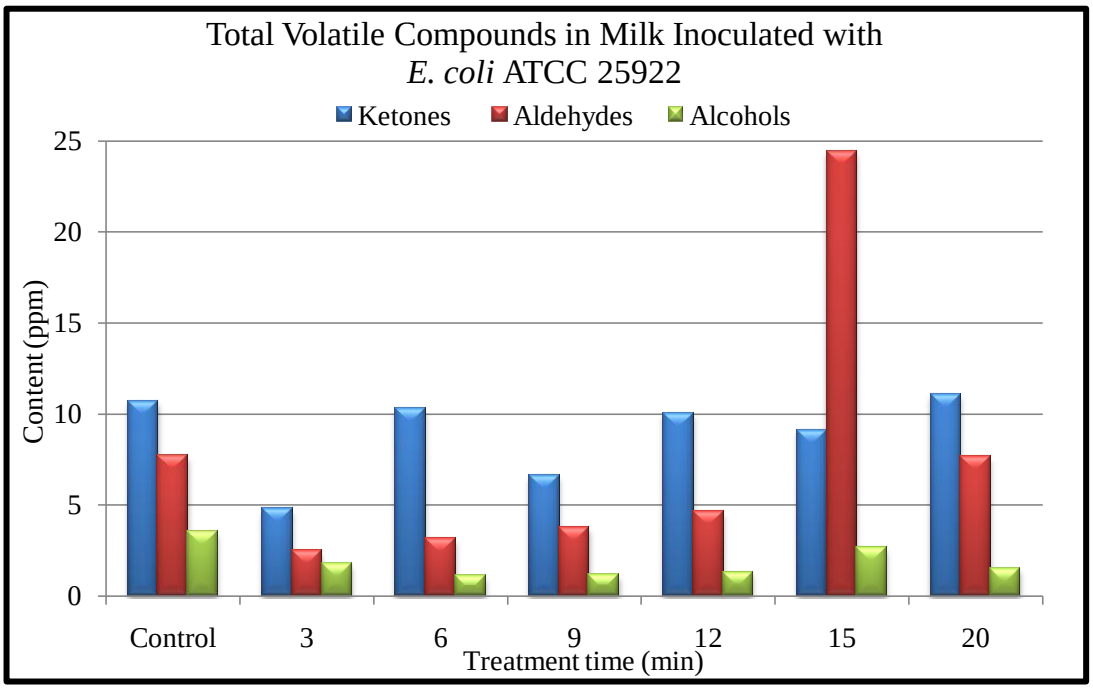

Figure 5.20. Total ketone, aldehyde and alcohol profiles of milk inoculated with *E. coli* ATCC 25922 after 9 kV plasma treatment

The individual ketone, aldehyde, and alcohol concentrations of milk inoculated with *E. coli* according to the 9 kV plasma treatment times were shown in Table 5.6. The ketones detected were, acetone, 2 butanone, 2 pentanone, methyl isobutyl ketone, 5 methyl 3 hexanone, 4 methyl 2 hexanone, 4 methyl 3 penten 2 one, 2,6 dimethyl 4 heptanone, 2 heptanone, 2 octanone, 2,5 octanedione, 2 nonanone, 3,5 octadien 2 one, and 2 undecanone. 2-heptanone was the most abundant ketone with 2.63 ppm in control whole milk inoculated with *E. coli*. Whereas the concentration of 2-heptanone decreased gradually, following increased amounts of exposure time. The concentration of 2-heptanone was decreased to 1.09 ppm in 3 min plasma-treated sample and a gradual reduction was observed by following plasma exposure times with a final value of 0.16 ppm after 20 min. Another component that decreased dramatically was 2-undecanone with a ppm value from 0.32 to 0.07 in 20 min. The 2-nonanone concentration decreased from 1.19 to 0.32 ppm in 3 min and after 20 min treatment a reduction from 0.33 to 0.19 ppm was detected. On the other hand, 2,6 dimethyl 4 heptanone was not detected in control milk samples inoclulated with *E. coli*. However after 3 min of plasma application the concentration was 0.03 ppm and increased to 4.05 ppm following 20 min of 9 kV plasma application.

The aldehydes detected by GC/MS in whole milk inoculated with *E. coli* were butanal, 2 butenal, hexanal, heptanal, 2 hexenal, octanal, 2 heptanal, nonanal, 2 octenal, 3 furaldehyde, 3-cyclohexene-1-carboxaldehyde, decanal, 2 nonenal, and benzaldeyde. Some of these aldehydes were not detected in untreated control samples for example butanal, 2-butenal, heptanal, 2-heptanal, 3-cyclohexane-1-carboxaldehyde, and 2-nonenal. These compounds were especially found in higher amounts in 15-min 9 kV plasma treated sample. The most abundant aldehyde in control sample was hexanal with 4.01 ppm. The amount of hexanal decreased to 1.01 ppm after plasma treatment for 3 min which also showed the highest decontamination activity on *E. coli* in milk. However, after 15 min, 9 kV plasma application hexanal increased from 1.30 to 3.73 ppm. After 3 min, 2-hexenal decreased from 0.97 to 0.28 ppm and then increased during the exposure of 20 min up to 0.78 ppm, except in 15 min which showed the highest 2-hexanal concentration with a ppm value of 2.31. The majority of aldehyde components in whole milk inoculated with *E. coli* decreased following 9 kV plasma application. Examples can be given as hexanal, 2 hexenal, octanal, 2 octenal, 3 furaldehyde, decanal and benzaldeyde.

The amount of alcohols in whole milk inoculated with *E. coli* before and after treament times were also determined by GC/MS. Alcohols were found in lower amounts compared to ketones and aldehydes. Detected alcohols were ethanol, 2 hexanol, 5 methyl 3 hexanol, 1 pentanol, 1 octen 3 ol, 1 octanol, and 2 furanmethanol. 2-hexanol was found to be the most predominant alcohol in control whole milk sample inoculated with *E. coli*, however regarding the longer treatment times ethanol took its place with accelerating percentage rates as shown in Figure 5.21. The levels of 2 hexanol and 1 octanol were decreased from 2.69 and 0.46 to 1.41 and 0.05 ppm, respectively after 3 min of plasma treatment. The lowest amount of 2-hexanol was detected in 15 min with a value of 0.14 ppm and increased to 0.34 ppm after 20 min plasma application. 1-octanol increased to 0.75 ppm in 15 min sample, however 0.21 ppm was detected in 20 min sample. The untreated control did not contain 5-methyl-3-hexanol, while after 3 min of 9 kV treatment 0.09 ppm was observed and the concentration increased during the plasma treatment experiments of whole milk inoculated with *E. coli.* Also 1-pentanol was only present in plasma treated samples which was detected after 12 min of treatment with a very low amount (0.05 ppm) also in 20 min (0.03 ppm), however 0.27 ppm was detected in 15 min.

Table 5.6. VOCs (amounts expressed as ppm) detected in whole milk inoculated with *E. coli* ATCC 25922 after plasma treatment with 9 kV power supply

Volatile Compound	**Retention time (min)**	**Amount in Milk Inoculated with *E. coli* ATCC 25922 After Different Treatment Times**						
		Control	3 min	6 min	9 min	12 min	15 min	20 min
octane	**4.278**	0.33					0.11	0.09
acetone	**4.761**	0.50	0.47	0.42		0.29	0.45	0.30
1 octene	**5.036**	0.18						
butanal	**5.915**		0.03					
2 butanone	**6.463**		0.16	0.15		0.23	0.17	0.25
Ethanol	**7.173**	0.38	0.22	0.42	0.60	0.60	0.65	0.71
2 pentanone	**8.333**	1.03	0.63	0.55	0.30	0.64	1.79	0.35
methyl isobutyl ketone	**8.973**	0.17	0.13	0.57	0.36	0.44	0.29	0.30

Table 5.6. VOCs (amounts expressed as ppm) detected in whole milk inoculated with *E. coli* ATCC 25922 after plasma treatment with 9 kV power supply (Continue)

Volatile Compound	**Retention time (min)**	**Amount in Milk Inoculated with *E. coli* ATCC 25922 After Different Treatment Times**						
		Control	3 min	6 min	9 min	12 min	15 min	20 min
2 butenal	**9.869**		0.10	0.07	0.06	0.23	0.39	0.23
5 methyl 3 hexanone	**10.461**	0.62	0.61	0.34	0.30	0.26		0.31
hexanal	**10.623**	4.01	1.01	0.85	0.91	1.30	3.73	1.52
4 methyl 2 hexanone	**11.424**	1.55	0.65	0.85	0.72	0.69	0.29	0.74
Ethylbenzene	**11.634**	0.44						
4 methyl 3 penten 2 one	**11.783**	2.56	0.58	4.24	2.80	3.92	3.13	4.28
2,6 dimethyl 4 heptanone	**12.417**		0.03	2.33	1.38	2.49	2.21	4.05
2 heptanone	**12.664**	2.63	1.09	0.59	0.40	0.54		0.16
heptanal	**12.724**			0.03		0.05	1.11	0.25
2 hexanol	**13.098**	2.69	1.41	0.46	0.28	0.43	0.14	0.34
5 methyl 3 hexanol	**13.35**		0.09	0.16	0.13	0.17	0.10	0.20
2 hexenal	**13.464**	0.97	0.28	0.30	0.40	0.54	2.31	0.78
1 pentanol	**13.651**					0.05	0.27	0.03
cloro 1 octane	**13.808**	1.13		0.10		0.02	0.29	0.11
styrene	**14.157**	0.24			0.02		0.14	
2,2,4,6,6 pentamethyl 3 heptene	**14.231**	1.49						
2 octanone	**14.478**					0.07	0.17	0.06
octanal	**14.558**	0.94	0.11	0.15	0.14	0.19	2.09	0.40
2,5 octanedione	**14.974**		0.04				0.20	
2 heptanal	**15.27**		0.01			0.04	0.81	0.06
tetradecane	**15.904**	0.19		0.03	0.06		0.10	0.08

Table 5.6. VOCs (amounts expressed as ppm) detected in whole milk inoculated with *E. coli* ATCC 25922 after plasma treatment with 9 kV power supply (Continue)

Volatile Compound	**Retention time (min)**	**Amount in Milk Inoculated with *E. coli* ATCC 25922 After Different Treatment Times**						
		Control	3 min	6 min	9 min	12 min	15 min	20 min
2 nonanone	**16.127**	1.19	0.32	0.19	0.30	0.33	0.33	0.19
nonanal	**16.223**	0.27	0.48	1.23	1.40	0.86	9.23	2.40
1 nitro pentane	**16.352**					0.03		
1 octen 3 ol	**16.748**						0.43	
2 octenal	**16.914**	0.11	0.03		0.11	0.29	1.35	0.34
3 furaldehyde	**17.377**	0.65		0.08	0.08	0.06	1.37	0.38
3-Cyclohexene-1-carboxaldehyde	**17.558**		0.03	0.05	0.06	0.16	0.16	0.14
decanal	**17.748**	0.34	0.05	0.12	0.19	0.08	0.45	0.16
3,5 octadien 2 one	**18.131**	0.11				0.04		
1 octanol	**18.297**	0.46	0.05	0.06	0.14	0.07	0.75	0.21
2 nonenal	**18.386**				0.03	0.02	0.43	0.04
benzaldeyde	**18.446**	0.40	0.31	0.28	0.35	0.82	0.97	0.92
nonadecane/hexadecane	**18.736**	1.74	0.19	0.02	0.23	0.09	0.69	0.96
2 undecanone	**19.082**	0.32	0.09	0.04	0.06	0.08	0.09	0.07
2 furanmethanol	**19.763**						0.34	
Total Ketones		10.67	4.79	10.25	6.63	10.03	9.10	11.06
Total Aldehydes		7.69	2.46	3.13	3.73	4.65	24.42	7.61
Total Alcohols		3.54	1.77	1.09	1.15	1.33	2.67	1.50

The comparison of concentrations of volatile compounds which significantly differed after 20 min of plasma treatment that were ethanol, 2-butenal, nonanal, decanal, and benzaldehyde are schematized in Figure 5.21. Nearly 2 fold increase was observed during

20-min plasma application in the concentrations of benzaldehyde. A gradual increase of benzaldeyde concentration from 0.40 ppm to 0.92 ppm was observed for control and 20 min samples, respectively; while 15 min of plasma application showed the highest amount (0.97 ppm). Approximately two fold increase in the ethanol concentration was detected after plasma application for 20 min. A slight decrease was observed in first 3 min and followed by higher amounts and the final concentration was 0.71 ppm after 20 min of plasma treatment. 2-butenal was only detected plasma treated samples which was observed after 3 min with a low concentration of 0.10 ppm, and increased to 0.39 and 0.23 ppm in 15 min and 20 min application, respectively. The nonanal levels in milk samples increased from 0.27 to 0.48, 1.23, 1.40, and 2.40 ppm, respectively in 3, 6, 9, and 20 min of treatment, whereas, a dramatical increase was observed in 15 min sample which contained 9.23 ppm of nonanal. Conversely, decanal concentration decreased during plasma treatment of 20 min from 0.34 to 0.16 ppm while an increase with a concentration of 0.45 ppm was detected in 15 min.

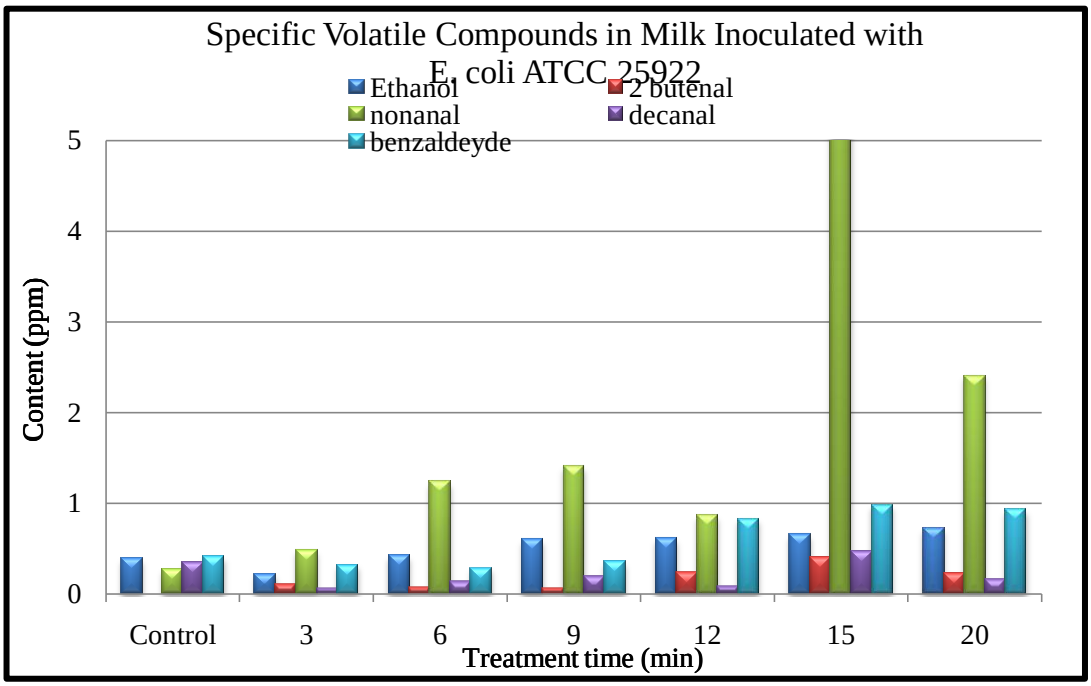

Figure 5.21. Specific volatile compound results of milk inoculated with *E. coli* ATCC 25922 after 9 kV plasma application

The total ketones, aldehydes, and alcohols were detected post plasma treatment of whole milk inoculated with *S. aureus*. Figure 5.22 shows the variations of the total amounts of ketones, aldehydes and alcohols depending on the 15 kV, plasma exposure time in milk inoculated with *S. aureus*. The total ketone concentration in milk samples inoculated with *S. aureus* were similar except that of post-plasma treatment for 12 min. Mild deviations were observed in the concentrations of alcohols, whereas, aldehyde levels changed dramatically over time of exposure. An increase of initial total ketone concentration (10.76 ppm) was observed following 12 min of plasma application with a value of 15.09 ppm. Total aldehyde concentration dramatically changed over time. A huge increase was observed after 3 min of application from 1.88 to 22.69 ppm, followed by rather small amounts after 6, 9, and 12 min with concentrations of 8.89, 7.01, and 6.68, respectively. The amount of total aldehydes were significantly higher (17.16 ppm) in plasma treated samples of 15 min with 15 kV, though the final concentration was 7.59 ppm. Total alcohol profiles of plasma treated samples evidenced an increase of concentration which was 0.64 ppm before plasma and around 1.7 ppm in milk samples post plasma.

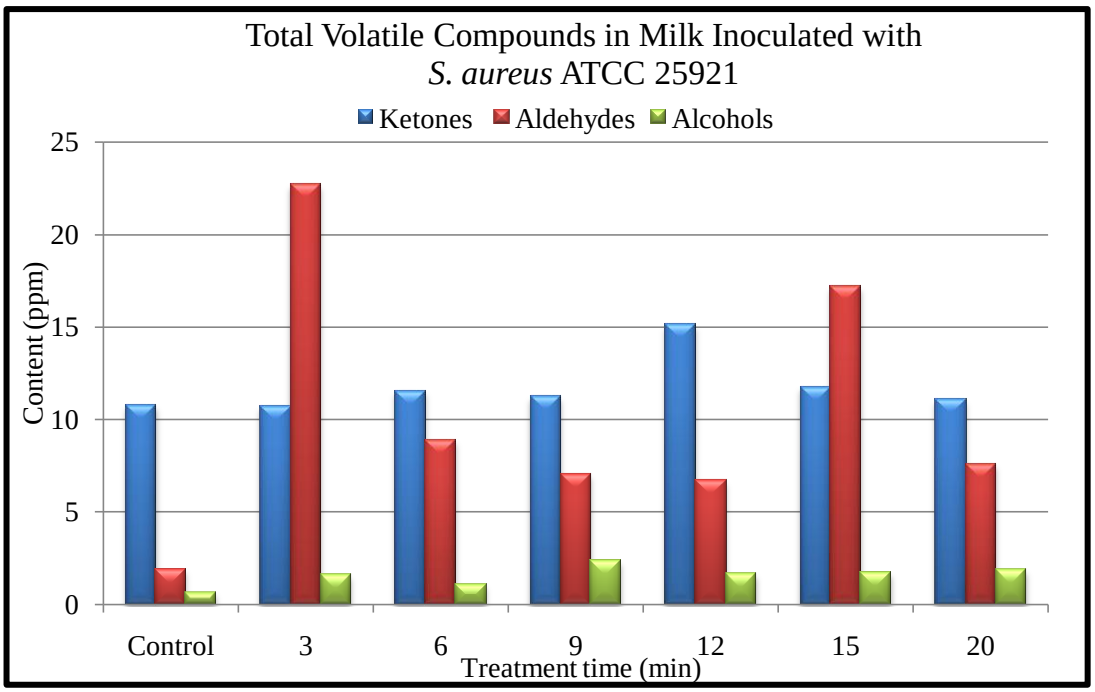

Figure 5.22. Total ketone, aldehyde and alcohol profiles of milk inoculated with *S. aureus* ATCC 25921 after plasma treatment

Detailed information about the ketones, aldehydes, and alcohols that were detected was shown in Table 5.7. According to the peaks that were identified following ketones were present; acetone, 2 butanone, 2 pentanone, methyl isobutyl ketone, 5 methyl 3 hexanone, 4 methyl 2 hexanone, 4 methyl 3 penten 2 one, 2,6 dimethyl 4 heptanone, 2 heptanone2 octanone, 2,5 octanedione, 2 nonanone, and 2 undecanone. The majority of the ketones did not show any change during plasma treatment with 15 kV. Although slight increases were detected in concentrations of methyl-isobutyl-ketone, 4-methyl-3-penten-2-one, and 2,6-dimethyl-4-heptanone while some contents decreased such as 2-peptanone and 2-heptanone. The most abundant ketone in control sample was 4 methyl 3 penten 2 one which was followed by 2,6 dimethyl 4 heptanone and 2 heptanone with ppm values of 3.34, 2.28, and 2.21, respectively. Higher amounts of 4 methyl 3 penten 2 one and 2,6 dimethyl 4 heptanone were detected after plasma treatments regardless of the exposure time. However, 2 heptanone decreased due to application of 15 kV plasma.

The aldehydes detected were butanal, 2-methylpropenal, 2 butenal, hexanal, heptanal, 2 hexenal, octanal, 2 heptanal, nonanal, 2 octenal, 3 furaldehyde, 3-cyclohexene-1-carboxaldehyde, decanal, 2 nonenal, and benzaldeyde. The major aldehyde in control was nonanal with a concentration of 0.78 ppm which increased to 4.94 following 3 min plasma and reached the greatest amount in 15 min. 2-methylpropenal, 2-butenal, heptanal, 2-hexenal, 2-heptanal, and 2-nonenal were detectable only after plasma application on whole milk inoculated with *S. aureus*. After 3 min of plasma exposure on milk, heptanal concentration increased to 3.52 ppm which was not detected in control and the concentration was rather low in the following treatment min.

The alcohols detected were 2-Propanol, ethanol, 2 hexanol, 5 methyl 3 hexanol, 1 pentanol, 1 octen 3 ol, 1 octanol, and 2 furanmethanol. The ethanol concentration increased gradually from 0.11 to 1.01 ppm during 20 min of plasma treatment with 15 kV power supply with a rapid increase in 9 min. 5-methyl-3-hexanol was found in high amounts with an avarage value of 0.2 ppm whose concentration was 0.07 ppm in control milk. Another alcohol that increased after plasma application was 1-octanol which was found in higher levels in 3 and 15 min samples.

Table 5.7. VOCs (amounts expressed as ppm) detected in whole milk inoculated with *S. aureus* ATCC 25921 after plasma treatment with 15 kV power supply

Volatile Compound	**Retention time (min)**	**Amount in Milk Inoculated with *S. aureus* ATCC 25921 After Different Treatment Times**						
		Control	3 min	6 min	9 min	12 min	15 min	20 min
octane	**4.278**	0.16		0.18	0.31	0.37	0.15	0.08
acetone	**4.761**	0.46	0.28		0.16	0.11	0.22	0.30
1 octene	**5.036**	0.05				0.14	0.23	0.22
butanal	**5.915**					0.03		
2-Methylpropenal	**6.062**				0.15	0.13	0.18	0.22
2 butanone	**6.463**	0.08			0.06	0.05	0.10	0.12
2-Propanol	**6.98**				0.06			
Ethanol	**7.173**	0.11	0.56	0.35	1.44	0.65	0.86	1.01
2 pentanone	**8.333**	0.52	1.63	0.27	0.34	0.20	0.36	0.25
methyl isobutyl ketone	**8.973**	0.3	0.27	0.33	0.43	0.40	0.44	0.54
1 decene	**9.515**						0.22	0.30
2 butenal	**9.869**		0.08	0.09	0.09	0.04	0.10	0.10
5 methyl 3 hexanone	**10.461**	0.2	0.19	0.29		0.37	0.25	0.21
hexanal	**10.623**	0.37	2.02	2.14	1.80	1.43	2.97	1.15
4 methyl 2 hexanone	**11.424**	0.71	0.51	0.74	0.66	1.27	0.91	0.53
ethylbenzene	**11.634**	0.15		0.21	0.15	0.21	0.28	0.45
4 methyl 3 penten 2 one	**11.783**	3.34	3.00	4.48	4.56	5.98	3.95	4.03
2,6 dimethyl 4 heptanone	**12.417**	2.28	3.74	4.76	3.89	6.21	5.21	4.50
2 heptanone	**12.664**	2.21	0.25	0.24	0.50	0.15		0.13
Heptanal	**12.724**		3.52	0.42	0.16	0.27	0.74	0.38
2 hexanol	**13.098**	0.36	0.21	0.32	0.49	0.62	0.38	0.48

Table 5.7. VOCs (amounts expressed as ppm) detected in whole milk inoculated with *S. aureus* ATCC 25921 after plasma treatment with 15 kV power supply (Continue)

Volatile Compound	**Retention time (min)**	**Amount in Milk Inoculated with *S. aureus* ATCC 25921 After Different Treatment Times**						
		Control	3 min	6 min	9 min	12 min	15 min	20 min
5 methyl 3 hexanol	**13.35**	0.07	0.23	0.16	0.18	0.22		0.23
2 hexenal	**13.464**		0.85	0.27	0.57	0.63	1.45	0.58
1 pentanol	**13.651**		0.13					
cloro 1 octane	**13.808**	0.05	0.27	0.20	0.15	0.16	0.57	0.28
Styrene	**14.157**		0.20	0.20	0.39	0.49		1.11
2,2,4,6,6 pentamethyl 3 heptene	**14.231**	0.26						
2 octanone	**14.478**		0.12					0.07
Octanal	**14.558**	0.14	1.53	1.03	0.40	0.51	1.18	0.66
2,5 octanedione	**14.974**		0.25					
2 heptanal	**15.27**		0.64	0.06	0.08	0.05	0.08	0.08
Tetradecane	**15.904**	0.05	0.18	0.05	0.08	0.08		0.09
2 nonanone	**16.127**	0.55	0.30	0.33	0.46	0.29	0.31	0.32
Nonanal	**16.223**	0.78	4.94	3.81	1.96	2.25	8.37	1.94
1 nitro pentane	**16.352**				0.04			
1 octen 3 ol	**16.748**		0.09					
2 octenal	**16.914**	0.03	6.68		0.34	0.31	0.24	0.14
3 furaldehyde	**17.377**	0.25	1.22	0.41	0.47	0.30	0.52	0.51
3-Cyclohexene-1-carboxaldehyde	**17.558**	0.06		0.04	0.09	0.05	0.11	0.13
Decanal	**17.748**	0.11	0.38	0.21	0.18	0.19	0.21	0.19
1 octanol	**18.297**	0.06	0.36	0.26	0.14	0.14	0.49	0.13
2 nonenal	**18.386**		0.51	0.04	0.02			

Table 5.7. VOCs (amounts expressed as ppm) detected in whole milk inoculated with *S. aureus* ATCC 25921 after plasma treatment with 15 kV power supply (Continue)

Volatile Compound	**Retention time (min)**	**Amount in Milk Inoculated with *S. aureus* ATCC 25921 After Different Treatment Times**						
		Control	3 min	6 min	9 min	12 min	15 min	20 min
Benzaldeyde	**18.446**	0.13	0.34	0.39	0.68	0.51	1.01	1.52
nonadecane/hexadecane	**18.736**	1.66	13.23	0.54	0.46	0.42	0.04	0.11
2 undecanone	**19.082**	0.12	0.14	0.08	0.14	0.06		0.05
2 furanmethanol	**19.763**	0.04						
Total Ketones		10.76	10.67	11.52	11.21	15.09	11.74	11.04
Total Aldehydes		1.88	22.69	8.89	7.01	6.68	17.16	7.59
Total Alcohols		0.64	1.58	1.10	2.32	1.63	1.72	1.85

When further detailed observations were carried out, the significant compounds were hexanal, 2-butenal, nonanal, benzaldehyde and ethanol as shown in Figure 5.23. The most abundant aldehyde detected in control was hexanal which was found in 0.37 ppm. Hexanal significantly increased during plasma exposure reaching a maximum level of 2.97 ppm in 15 min plasma treated sample. 2-butenal was only detectable after plasma application on whole milk inoculated with *S. aureus*. Among all the alcohols detected in milk samples, ethanol was the most significant and predominant one and the concentration increased following plasma application regardless of the exposure time. Nonanal was the dominant aldehyde in all of the samples and the amount of nonanal was quiet higher in the plasma treated samples compared to control. While, benzaldehyde concentration gradually increased in correlation with the exposure minutes.

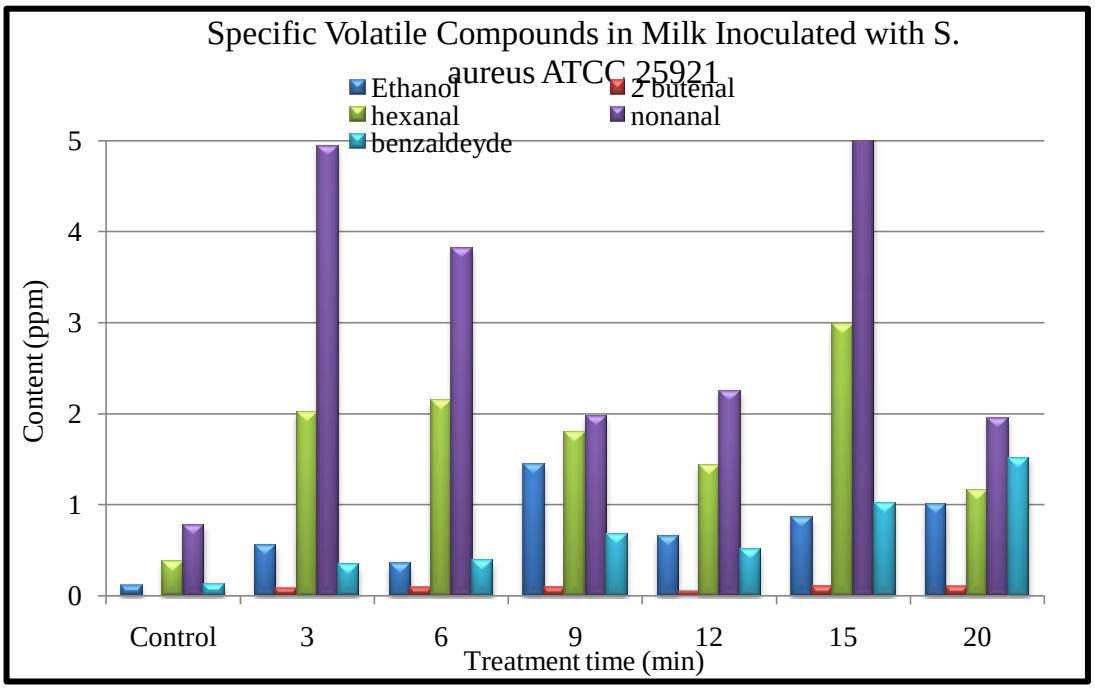

Figure 5.23. Specific volatile compound results of whole milk inoculated with *S. aureus* ATCC 25921 after plasma application with 15 kV power supply

The same methodology was carried for whole milk inoculated with *S.* Typhimurium and the results were shown in Figure 5.24. The atmospheric pressure plasma treatment at 15 kV, was seen to be degrading the ketone molecules which decreased from 14.63 to 12.17 and 9.71 ppm after 3 and 20 min, respectively as the aldehyde concentrations gradually increased. The initial concentration of aldehyde was decrease from 6.90 to 4.22 ppm however, after total application of 20 min a rapid increase of 15.65 ppm was observed. Total alcohols increased approximately 3 folds when 20 min of plasma was applied to whole milk inoculated with *S.* Typhimurium. Gradual increase was detected in the concentration of alcohols which increased from 0.96 to 3.69 ppm.

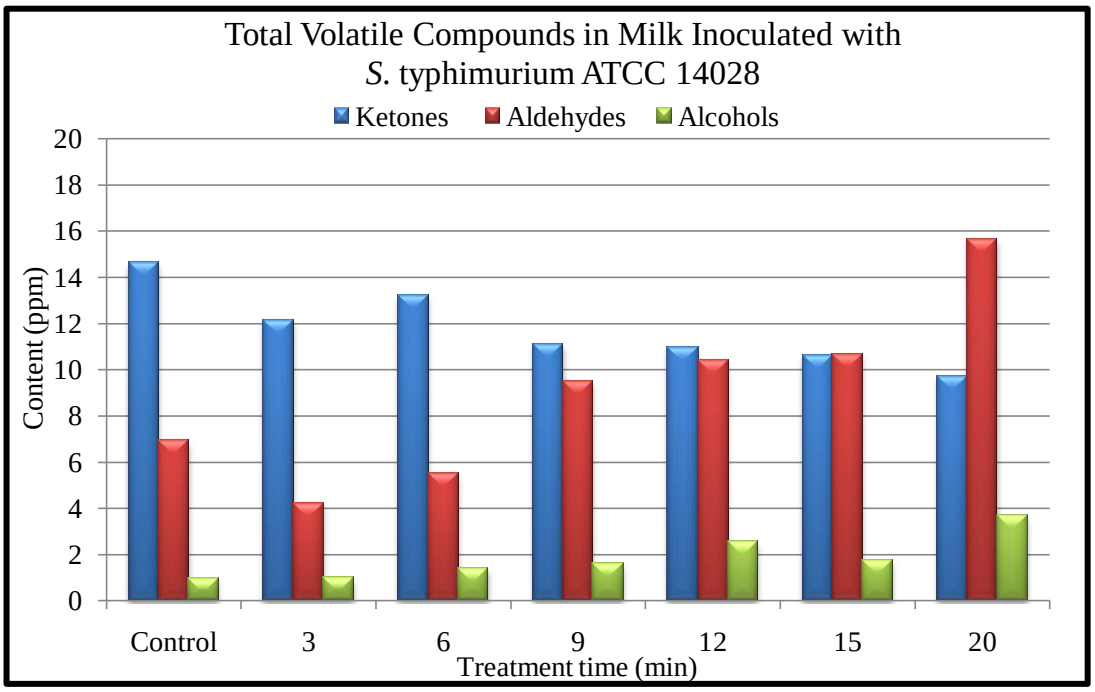

Figure 5.24. Total ketone, aldehyde and alcohol profiles of milk inoculated with *S.* Typhimurium ATCC 14028 after 15 kV plasma treatment

The concentrations of individual volatile compounds were shown in Table 5.8. Ketones that were detected in whole milk samples inoculated with *S.* Typhimurium were acetone, 2 butanone, 2 pentanone, methyl isobutyl ketone, 5 methyl 3 hexanone, 4 methyl 2 hexanone, 4 methyl 3 penten 2 one, 2,6 dimethyl 4 heptanone, 2 heptanone, 2 octanone, 2 nonanone, 3,5 octadien 2 one, and 2 undecanone. Higher amounts of acetone, 2-pentanone, 5-methyl-3-hexanone, 2,6 dimethyl 4 heptanone, 2 heptanone, and 2 nonanone were detected in controls when compared to the plasma treated samples. While the amounts of 2 heptanone. 2 butanone, methyl isobutyl ketone, and 2 undecanone increased over exposure time. The concentration of 2,6 dimethyl 4 heptanone in the control sample was 5.72 ppm which was the greatest value among the ketone profile. The 2,6 dimethyl 4 heptanone decreased to a final concentration of 3.76 ppm after 20 min of 15 kV plasma treatment.

Identified aldehydes by GC/MS were butanal, 2-Methylpropenal, 2 butenal, hexanal, heptanal, 2 hexenal, octanal, 2 heptanal, nonanal, 2 octenal, 3 furaldehyde, 3-cyclohexene-1-carboxaldehyde, decanal, 2 nonenal, and benzaldeyde. Most of the aldehydes were increased with plasma exposure time which also resulted in the higher total aldehyde

concentrations. However, 2-hexanal concentration slightly decreased while a rapid increase from 0.36 to 0.94 ppm was observed. 2 butenal and 2 nonenal were detected just after 3 min of plasma application on whole milk inoculated with *S.* Typhimurium with starting concentrations of 0.02 ppm which increased during treatment. The most predominant aldehyde was nonanal with 3.08 ppm. During the first 6 min of application a decrease was observed in the level of nonanal while it was 6.32 ppm after application of 20 min.

The alcohols 2-propanol, ethanol, 2 hexanol, 5 methyl 3 hexanol, 1 pentanol, 1 octen 3 ol, 1 octanol, 2 furanmethanol were identified using GC/MS. All of the alcohol compounds showed a tendency to increase in the amount of plasma treated milk samples. The major alcohol that was detected in untreated whole milk samples inoculated with *S.* Typhimurium was 2-hexanol. The concentration of 2-hexanol was constant after 3 min and increased from 0.47 to 0.56 ppm after 20 min of treatment.

Table 5.8. VOCs (amounts expressed as ppm) detected in whole milk inoculated with *S.* Typhimurium ATCC 14028 after plasma treatment with 15 kV power supply

Volatile Compound	**Retention time (min)**	**Amount in Milk Inoculated with *S.* Typhimurium ATCC 14028 After Different Treatment Times**						
		Control	3 min	6 min	9 min	12 min	15 min	20 min
octane	**4.278**	0.38	0.10	0.13	0.14	0.11	0.12	0.11
acetone	**4.761**	0.34	0.16	0.19	0.10	0.16	0.14	0.12
1 octene	**5.036**		0.04	0.08	0.20	0.23	0.17	0.35
butanal	**5.915**					0.05		
2-Methylpropenal	**6.062**	0.07		0.14		0.15	0.22	0.34
2 butanone	**6.463**	0.04	0.05	0.06		0.08	0.08	0.12
2-Propanol	**6.98**							1.02
Ethanol	**7.173**	0.13	0.32	0.57	0.66	1.61	0.88	1.50
2 pentanone	**8.333**	0.56	0.41		0.31	0.35	0.18	0.28
methyl isobutyl ketone	**8.973**	0.34	0.37	0.09	0.42	0.47	0.47	0.56

Table 5.8. VOCs (amounts expressed as ppm) detected in whole milk inoculated with *S.* Typhimurium ATCC 14028 after plasma treatment with 15 kV power supply (Continue)

Volatile Compound	**Retention time (min)**	**Amount in Milk Inoculated with *S.* Typhimurium ATCC 14028 After Different Treatment Times**						
		Control	3 min	6 min	9 min	12 min	15 min	20 min
1 decene	**9.515**			0.11	0.16	0.28	0.39	0.69
2 butenal	**9.869**		0.02	0.71		0.05	0.07	0.08
5 methyl 3 hexanone	**10.461**	0.48	0.36	0.34	0.28	0.21		0.15
hexanal	**10.623**	1.99	0.73	1.40	1.95	1.80	1.86	2.06
4 methyl 2 hexanone	**11.424**	1.13	1.01	0.78	0.64	0.59	1.29	
Ethylbenzene	**11.634**		0.04	0.26	0.35	0.52	0.76	1.12
4 methyl 3 penten 2 one	**11.783**	4.45	4.22	5.10	4.22	4.42	3.27	4.01
2,6 dimethyl 4 heptanone	**12.417**	5.72	3.85	4.55	4.25	3.91	3.86	3.76
dodecane	**12.511**					0.55		
2 heptanone	**12.664**	1.05	1.19	1.07	0.36	0.13	0.74	
heptanal	**12.724**	0.05		0.11	0.34	0.84		1.13
2 hexanol	**13.098**	0.47	0.47	0.53	0.48	0.50	0.55	0.56
5 methyl 3 hexanol	**13.35**	0.19	0.12	0.22	0.15	0.19	0.30	0.21
2 hexenal	**13.464**	0.58	0.44	0.36	0.94	0.47	0.40	0.49
1 pentanol	**13.651**				0.03			
cloro 1 octane	**13.808**	0.26	0.14		0.24	0.62	0.79	0.48
styrene	**14.157**	0.10	0.11	0.73	0.47	0.89	1.63	2.38
2,2,4,6,6 pentamethyl 3 heptene	**14.231**	0.04	0.02				0.35	0.16
2 octanone	**14.478**			0.09	0.06	0.06	0.12	0.13
octanal	**14.558**	0.55	0.28	0.35	0.74	0.93	0.60	1.25

Table 5.8. VOCs (amounts expressed as ppm) detected in whole milk inoculated with *S.* Typhimurium ATCC 14028 after plasma treatment with 15 kV power supply (Continue)

Volatile Compound	**Retention time (min)**	**Amount in Milk Inoculated with *S.* Typhimurium ATCC 14028 After Different Treatment Times**						
		Control	3 min	6 min	9 min	12 min	15 min	20 min
2 heptanal	**15.27**			0.08	0.08	0.09	0.11	0.19
tetradecane	**15.904**		0.06		0.06	0.13	0.13	0.11
2 nonanone	**16.127**	0.41	0.42	0.73	0.40	0.33	0.39	0.37
nonanal	**16.223**	3.08	2.04	1.05	4.09	4.26	5.39	6.32
1 nitro pentane	**16.352**			0.12	0.08	0.11	0.14	0.15
1 octen 3 ol	**16.748**				0.04			0.08
2 octenal	**16.914**	0.08	0.06	0.12	0.28	0.18	0.09	0.28
3 furaldehyde	**17.377**	0.24	0.25	0.23	0.33	0.66	0.39	0.80
3-Cyclohexene-1-carboxaldehyde	**17.558**			0.06	0.07		0.12	0.27
decanal	**17.748**	0.14	0.16	0.12	0.22	0.24	0.21	0.45
3,5 octadien 2 one	**18.131**			0.03		0.14		
1 octanol	**18.297**	0.17	0.09	0.08	0.26	0.23	0.06	0.26
2 nonenal	**18.386**		0.02	0.03	0.06	0.05	0.05	0.07
benzaldeyde	**18.446**	0.11	0.22	0.72	0.41	0.65	1.13	1.92
nonadecane/hexadecane	**18.736**	0.36	0.07	0.21	0.22	46.33	0.11	0.21
2 undecanone	**19.082**	0.10	0.12	0.19	0.07	0.10	0.09	0.21
Unknown	**19.31**			0.05	0.07	0.10	0.11	0.47
Total Ketones		14.63	12.17	13.22	11.11	10.95	10.64	9.71
Total Aldehydes		6.90	4.22	5.49	9.51	10.43	10.64	15.65
Total Alcohols		0.96	1.00	1.39	1.62	2.54	1.78	3.69

The molecules that significantly affected by plasma treatment included ethanol, 2-butenal, nonanal, decanal, benzaldehyde and 2-heptanal which showed a marked increase correlated with the exposure to the plasma. Such a behavior was observed also in milk samples subjected to a 20-min treatment and then stored in refrigerator as shown in Figure 5.25. The ethanol concentration raised from 0.13 to 1.50 ppm during total exposure of 20 min while the highest ethanol concentration was observed in 12 min plasma treated sample. Ethylbenzene was not detected in whole milk control sample, however the concentration reached up to 1.12 ppm after 20 min plasma application with 15 kV power supply. The amount of styrene rapidly increased from 0.10 to 0.73 and 2.38 ppm after 6 min and 20 min plasma application, respectively.

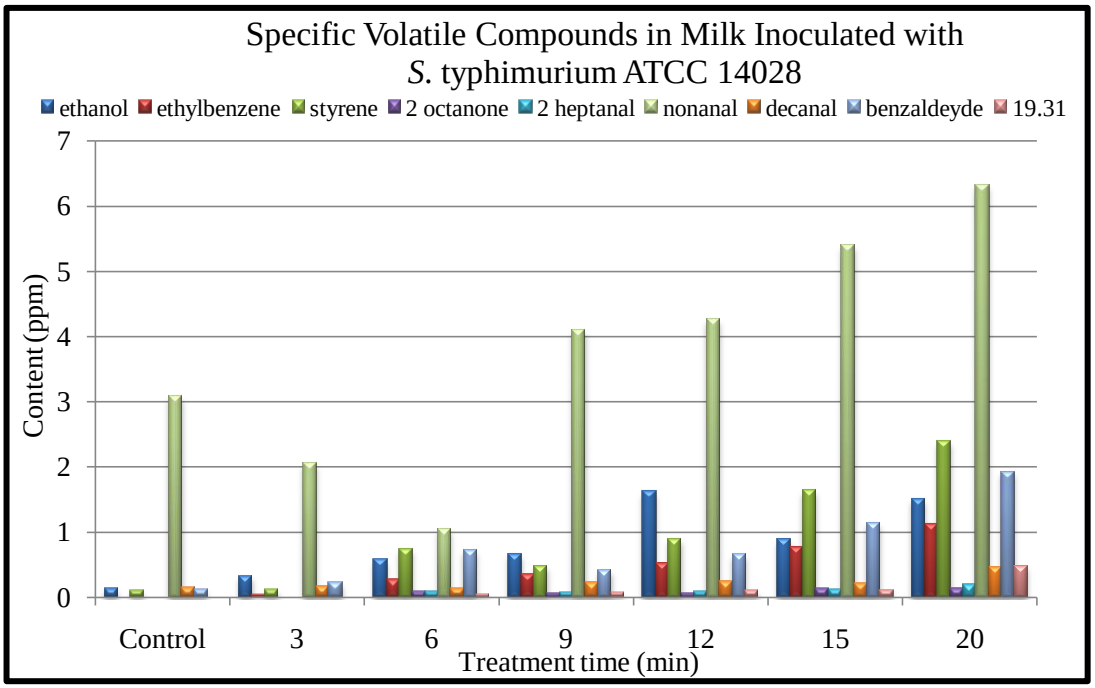

Figure 5.25. Specific volatile compounds results of milk inoculated with *S.* Typhimurium ATCC 14028 after 20 min of 15 kV plasma application

Figure 5.26 shows the comparison of total ketone, aldehyde, and alcohol concentrations in milk inoculated with 3 different bacterial species treated with 2 different voltages (9 and 15 kV). The results showed that the total concentrations of ketones slightly increased in milk samples inoculated with *E.coli* and *S. aureus* while a decrease was observed in milk inoculated with *S.* Typhimurium from 14.63 to 9.21 ppm. Total aldehydes

increased from 1.88 to 7.59 ppm in *S. aureus* milk sample and from 6.90 to 15.65 ppm in *S.* Typhimurium milk sample while the total aldehyde concentration remained the same in milk inoculated with *E. coli*. Similar results were obtained with alcohol concentrations of *S. aureus* and *S.* Typhimurium milk samples which increased from 0.64 to 1.85 and 0.96 to 3.69 ppm, respectively. Conversely, a reduction was observed in the total alcohol concentration of milk sample inoculated with *E. coli* which decreased from 3.54 to 1.50 ppm.

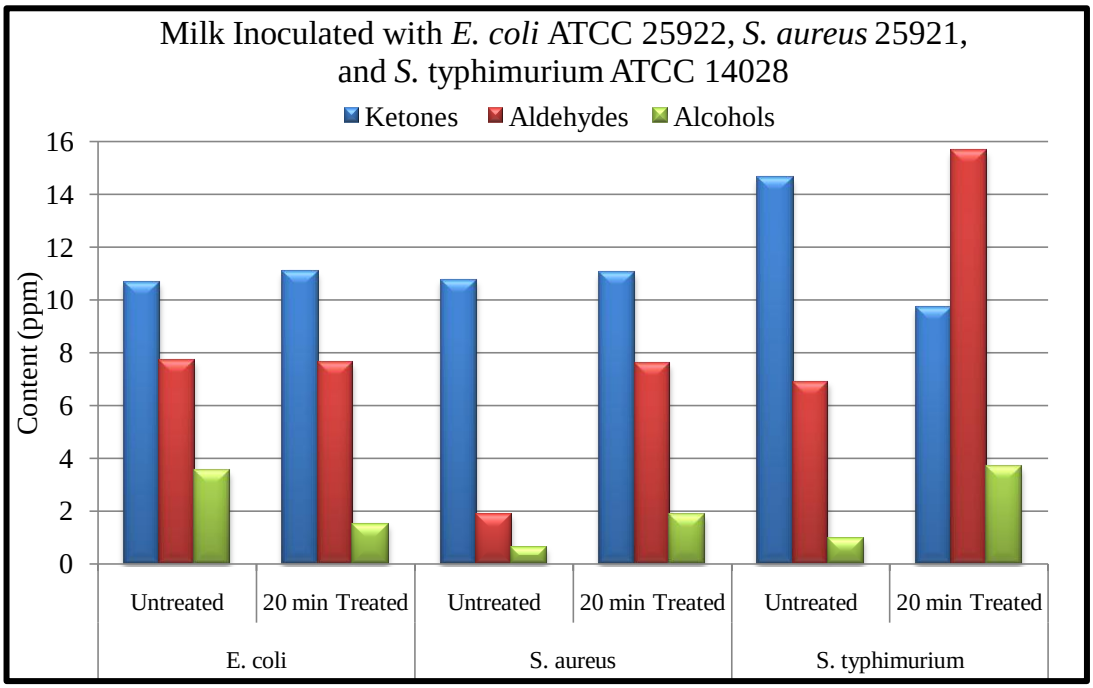

Figure 5.26. Total ketone, aldehyde and alcohol concentrations in whole milk inoculated with different test bacteria of *E. coli* ATCC 25922, *S. aureus* ATCC 25921 and *S.* Typhimurium ATCC 14028, after 20 min of plasma application

5.5. DETERMINATION OF MICROBIOLOGICAL AND CHEMICAL COMPOSITION OF PLASMA APPLIED MILK DURING STORAGE

5.5.1. Microbiological Results

Table 5.9 shows the long-term effects of plasma treatment on colony numbers of *E. coli*, *S. aureus*, and *S.* Typhimurium in whole milk during storage at 4°C for 0-6 weeks.

The population of test microorganisms in the plasma treated samples decreased during the storage periods while control milk samples inoculated with microorganisms decreased.only after 6 weeks of storage at 4°C. The cell concentration of 20 min, 9 kV plasma treated milk inoculated with *E. coli* was 3.3 log CFU/ml and after 1 week of storage the milk sample was totally sterilized. When the analyses were carried on for 6 weeks no bacterial growth was observed. Also similar results were observed for *S. aureus* and *S.* Typhimurium colonies in whole milk post plasma treatment. Following plasma application with 15 kV power supply for 20 min the number of *S. aureus* and *S.* Typhimurium colonies in whole milk were 3.44 and 2.98 log CFU/ml, respectively. However, no colonies were detected after storage for 1 week at 4°C and the results did not change even after 6 weeks.

Table 5.9. Effects of the plasma corona discharge on *E. coli*, *S. aureus* and *S.* Typhimurium bacterial cell viability in whole milk during storage at 4°C (Log CFU/mL)
*ND. No viable cells were detected

Test Microorganism	Plasma Treatment	Storage (week)						
		0	1	2	3	4	5	6
E. coli ATCC 25922	Untreated	7.78	7.78	7.78	7.78	7.78	7.78	3.23
	20 min	3.3	ND*	ND	ND	ND	ND	ND
S. aureus ATCC 25921	Untreated	5.36	5.36	5.36	5.36	5.36	5.36	3.6
	20 min	3.44	ND	ND	ND	ND	ND	ND
S. Typhimurium ATCC 14028	Untreated	5.6	5.6	5.6	5.6	5.6	5.6	ND
	20 min	2.98	ND	ND	ND	ND	ND	ND

The total sterilization effect of plasma corona discharge generated with 9 and 15 kV power supply was observed after storage in refrigerator for 1 week and no viable cells were detected since then as shown in Table 5.9. More than 2 log inhibition of bacteria was observed in control samples after 6 weeks of storage at refrigerator, regardless of the bacterial strain and application.

5.5.2. Proteolysis Assessment

Figures 5.27 and 5.28 revealed dark smears after SDS-PAGE was performed for all samples due to high fat concentration of whole milk. Even though the bands were not very clear, it could be seen that the protein content was higher around similar positions. Although, SDS-PAGE results were not very clear because of the high fat concentration of whole milk, some dense, darker bands could be observed around 65 kDa, 50 kDa, and 30 kDa. The milk is composed of numerous proteins from which the primary groups are caseins and whey proteins including beta-lactoglobulin and alpha-lactalbumin. The relative sizes of some proteins are; alpha-lactalbumin 14 kDa, beta-lactoglobulin 18 kDa, caseins 25-35 kDa, serum albumin 66 kDa and lactoferrin 80 kDa. Therefore, the bands, observed in all samples regardless of the plasma application and storage, may correspond to the serum albumin and casein proteins naturally found in milk.

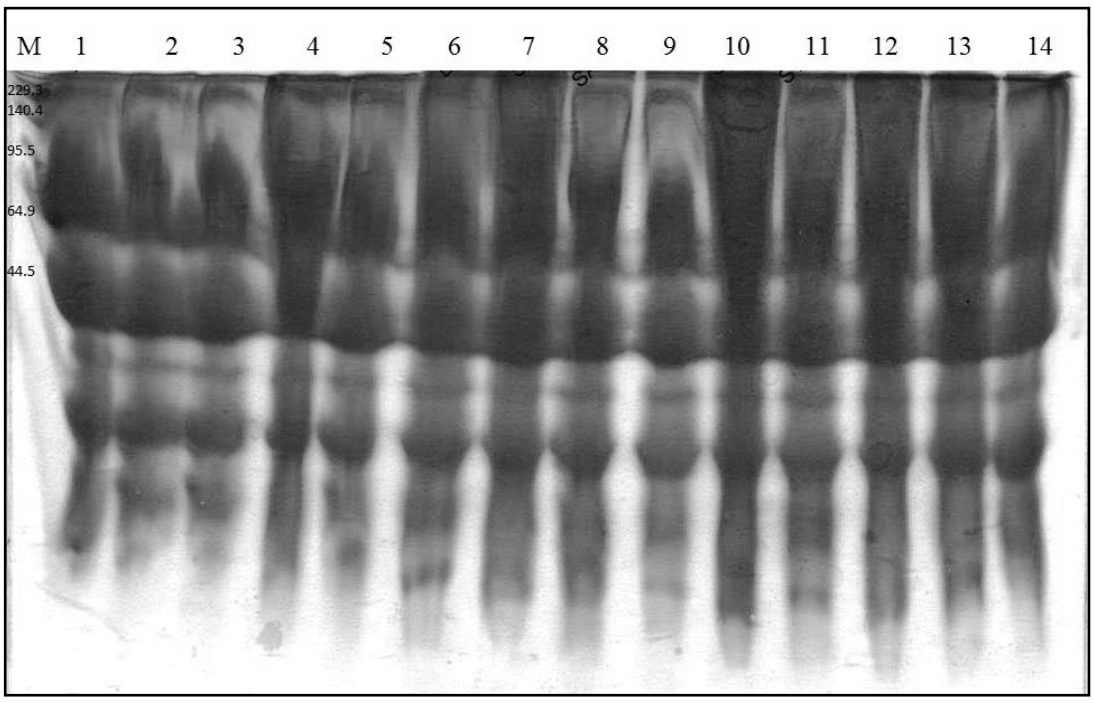

Figure 5.27. SDS-PAGE results after atmospheric pressure plasma treatment of whole milk inoculated with *E. coli* ATCC 25922, *S. aureus* ATCC 25921, and *S.* Typhimurium ATCC 14028 and 6 weeks of storage at 4°C

M: MultiColor marker (Abnova, Taiwan), 1: *E. coli* control, 2: *E. coli* 6 min, 3: *E. coli* 9 min, 4: *S.* Typhimurium 3 min, 5: *S.* Typhimurium 15 min, 6: *E. coli* after storage, 7: *S.*

Typhimurium after storage, 8: *S. aureus* storage control, 9: *E. coli* 3 min, 10: *S.* Typhimurium control, 11: *S.* Typhimurium storage control,12: *S.* Typhimurium 9 min, 13: *S.* Typhimurium 12 min, 14: *E. coli* 12 min.

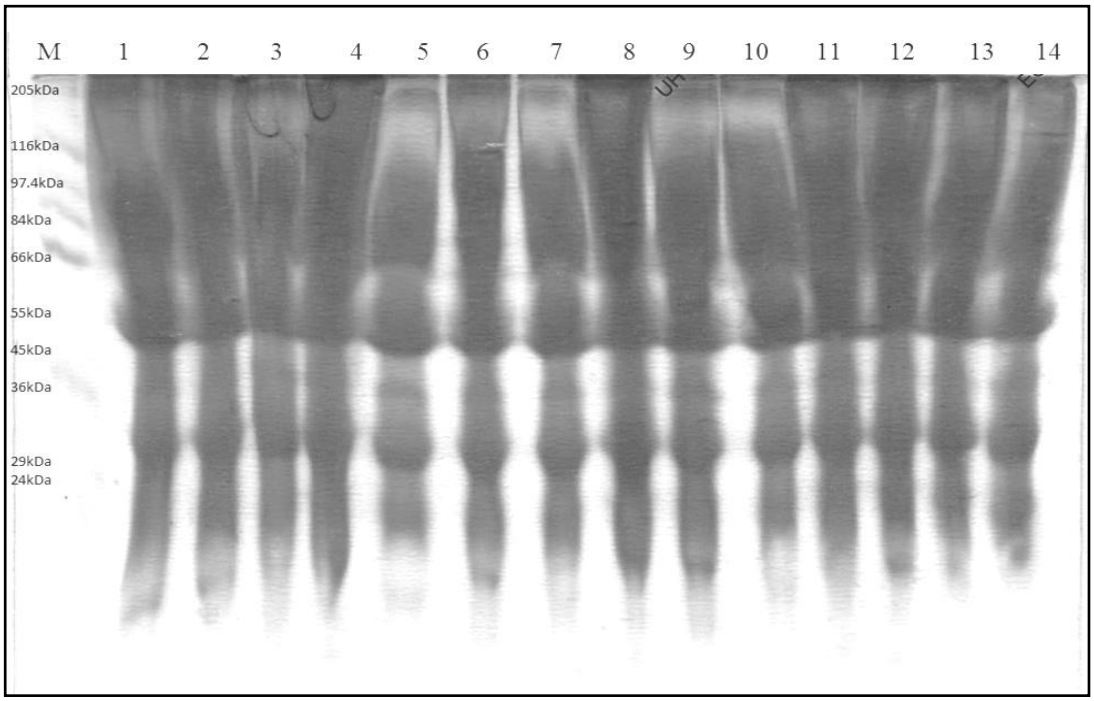

Figure 5.28. SDS-PAGE results after atmospheric pressure plasma treatment of whole milk inoculated with *E. coli* ATCC 25922, *S. aureus* ATCC 25921, and *S.* Typhimurium ATCC 14028 and 6 weeks of storage at 4°C

M: Sigma wide range marker, 1: *S. aureus* 20 min after storage, 2: *S. aureus* 12 min, 3: *E. coli* 20 min, 4: *S. aureus* control, 5: *E. coli* 15 min, 6: *S. aureus* 9 min, 7: *S. aureus* 3 min, 8: *S.* Typhimurium 20 min, 9: Whole milk control, 10: *S. aureus* 6 min, 11: *S. aureus* 20 min, 12: *S. aureus* 15 min, 13: *S.* Typhimurium 6 min, 14: *E. coli* storage control.

5.5.3. Detection of Fatty Acids in Milk

Free fatty acid (FFA) profiles of 20 min plasma treated milk samples after 6 weeks storage were detected using GC/MS (Agilent, USA). FFAs compositions of plasma applied milk samples inoculated with *E. coli*, *S. aureus* and *S.* Typhimurium were shown in Figures 5.29-5.31 as percentages of total saturated, monounsaturated, and polyunsaturated fatty acids after storage at 4°C for 6 weeks. Statistical analysis showed no significant

differences in the concentrations of fatty acids between the untreated and treated milk samples, regardless of the inoculated bacteria.

Figure 5.29 shows the total FA composition after storage (4°C) of uninoculated whole milk, whole milk inoculated with *E. coli* and 20 min 9 kV plasma applied milk inoculated with *E. coli*. Total SFAs, MUFAs, and PUFAs were found in percentages of 66, 26, and 3 in control milk samples after storage. The SFAs concentration increased from 65.98 to 66.29 % post plasma application in storage samples. Also the total MUFAs increased from 25.74 to 25.82 % following plasma appliecation. However, PUFAs were present with a percentage of 2.91 in control samples which decreased to 2.74 % following 20 min of treatment with 9 kV plasma system.

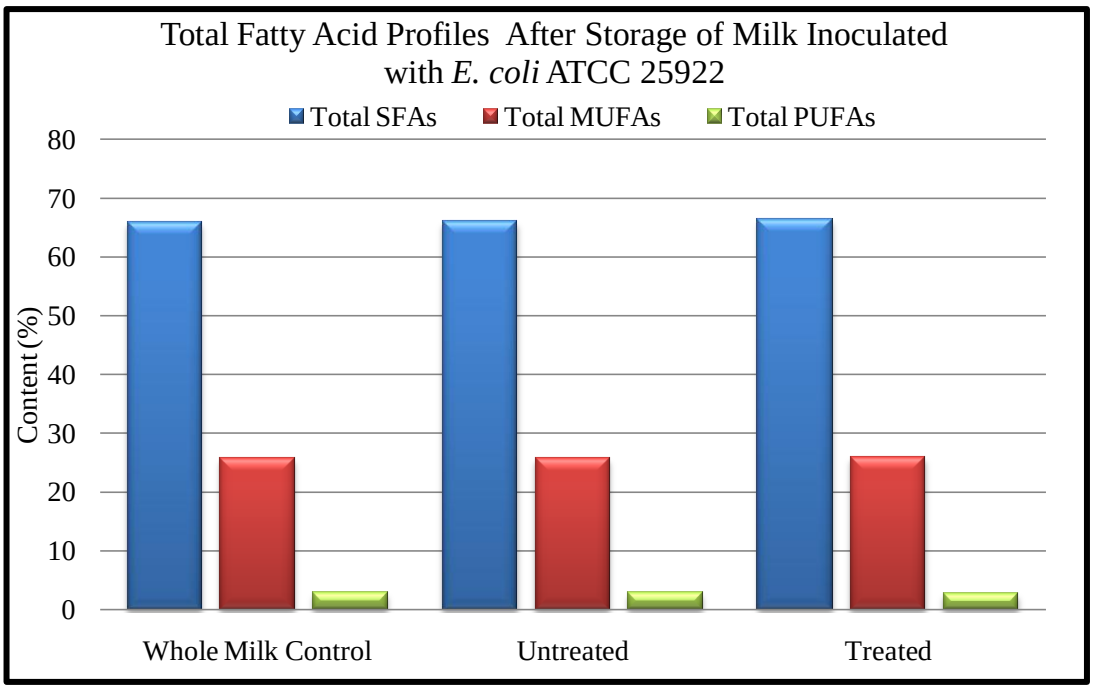

Figure 5.29. Total fatty acid profiles of whole milk, whole milk inoculated with *E. coli* ATCC 25922, and 20 min of 9 kV plasma treated sample after 6 weeks of storage (4°C) SFAs: Saturated fatty acids, MUFAs: monounsaturated fatty acids, PUFAs: polyunsaturated fatty acids

The fatty acid profiles of whole milk controls and 20 min of 9 kV plasma treated milk inoculated with *E. coli* were detected following storage of 6 weeks at 4°C which were

given in Table 5.10. Total SFAs detected were between C8:0 – C20:0, MUFAs were C14:1, C16:1 cis and trans, C18:1, C19:1, C20:1 cis and trans, and PUFAs were C18:2, C20:4 and C20:3. The results showed that the most predominant fatty acids observed in control milk samples were hexadecanoic acid (C16:0), oleic acid (C18:1) and stearic acid (C18:0) with the percentages of approximately 30, 22, and 15, respectively. The percentages of most abundant fatty acids were similar in fatty acid profiles of plasma treated samples before storage. The concentration of hexadecanoic acid increased during storage from 30.16 to 32.93 % after 20 min of application. Also, the amount of oleic acid increased from 22.15 to 22.24 % after storage following plasma treatment. The plasma application for 20 min caused a reduction in the amount of stearic acid which decreased from 14.59 to 14.13 %. The percentages of C10:0 and C12:0 decreased from 1.84 to 0.31 and 3.55 to 2.49 following plasma application. Although C10:0, C12:0 and C18:0 were affected by plasma treatment before storage, no significant changes were observed in storage results, regarding these fatty acids.

Table 5.10. Fatty acid composition of milk stored at 4°C for 6 weeks inoculated with *E. coli* ATCC 25922 after 20 min treatment with 9 kV plasma system

[a] 5,8,11,14-Eicosatetraenoic acid, [b] 7,10,13-Eicosatrienoic acid, [c] SFAs: Saturated fatty acids, MUFAs: Monounsaturated fatty acids, PUFAs: Polyunsaturated fatty acids.

Fatty Acid	**Percent in Milk After 6 weeks at 4°C**		
	Whole milk	***E. coli* ATCC 25922**	
		Control	**20 min Treatment**
C8:0	0.000	0.102	0.000
C10:0	1.178	1.835	0.312
C11:0	0.251	0.605	0.506
C12:0	3.470	3.551	2.493
C13:0	0.128	0.129	0.116
C13 iso	0.202	0.196	0.186
C14:1	1.349	1.303	1.294
C14:0	12.258	11.991	12.811

Table 5.10. Fatty acid composition of milk stored at 4°C for 6 weeks inoculated with *E. coli* ATCC 25922 after 20 min treatment with 9 kV plasma system (Continue)

Fatty Acid	Percent in Milk After 6 weeks at 4°C		
	Whole milk	*E. coli* ATCC 25922	
		Control	20 min Treatment
i-C14:0	0.434	0.405	0.431
a-C14:0	0.935	0.895	0.931
C15:0	1.741	1.670	1.762
C15:0 iso	0.518	0.492	0.499
C16:1 trans	0.123	0.127	0.128
C16:1 cis	1.938	1.866	1.931
C16:1 (delta 11)	0.020	0.029	0.028
C16:0	30.710	30.165	32.929
C16:0 iso	0.606	0.572	0.566
C16:0 ante	0.882	0.850	0.828
C17:0	1.031	0.982	0.952
C18:2	2.480	2.440	2.405
C18:1 (cis-Δ^9)	22.02	22.146	22.239
C18:1 (trans-Δ^9)	0.347	0.312	0.354
C18:1 (Δ^{11})	0.944	1.025	0.931
C18:0	14.726	14.592	14.131
C19:1	0.127	0.127	0.109
C19:0	0.100	0.095	0.082
C20:4[a]	0.160	0.171	0.133
C20:3[b]	0.113	0.129	0.083
C20:1n9 (cis11)	0.161	0.168	0.123
C20:0	0.243	0.262	0.198
Total SFAs[c]	65.836	65.978	66.291
Total MUFAs[c]	25.719	25.736	25.824
Total PUFAs[c]	2.914	2.907	2.745

Same procedure was carried for detection of free fatty acids (FFAs) of 20 min plasma treated milk samples inoculated with *S. aureus* and given as percentages of total SFAs, MUFAs, and PUFAs after storage at 4°C for 6 weeks. Figure 5.30 shows the results for compositions of milk inoculated with *S. aureus* after 6 weeks of storage (4°C) after 20 min of 15 kV plasma application. About 65, 26, and 3,% of total fatty acid content was SFAs, MUFAs, and PUFAs, respectively. Only slight decreases were observed in total MUFA and PUFA concentrations which were respectively, 26.87 and 2.85 % in control whole milk sample and respectively, 26.10 and 2.85 % in 20 min plasma-treated milk.

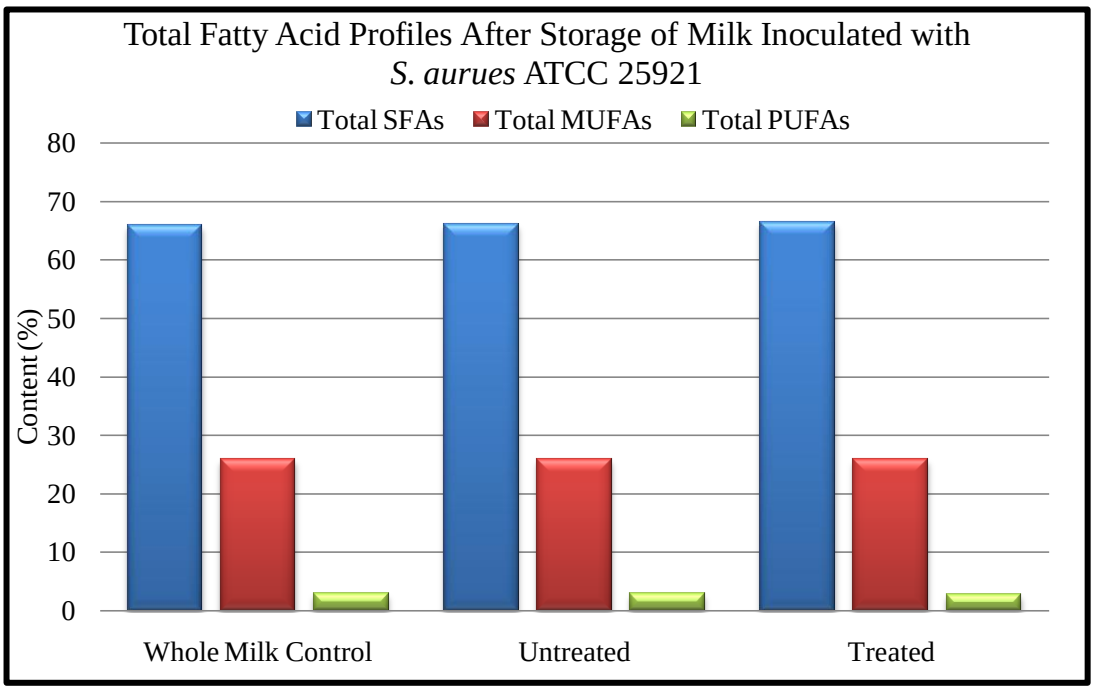

Figure 5.30. Total fatty acid profiles of whole milk, whole milk inoculated with *S. aureus* ATCC 25921, and 20 min of 15 kV plasma treated sample after 6 weeks of storage (4°C) SFAs: Saturated fatty acids, MUFAs: monounsaturated fatty acids, PUFAs: polyunsaturated fatty acids

Twenty min of plasma application showed 2 log reduction of *S. aureus* in whole milk. However, the total FFAs of milk inoculated with *S. aureus* were not effected by the treatment of 15 kV plasma even after 6 weeks of storage.

Storage results of the fatty acid profiles of whole milk inoculated with *S. aureus* after 15 kV plasma treatment for 20 min were shown in Table 5.11. Total SFAs detected were between C8:0 – C20:0, MUFAs were C14:1, C16:1 cis and trans, C18:1, C19:1, C20:1 cis and trans, and PUFAs were C18:2, C20:4 and C20:3. The most predominant fatty acids observed in milk sample before plasma application were hexadecanoic acid (C16:0), oleic acid (C18:1) and stearic acid (C18:0) which made up approximately 31, 23, and 15 % of the fatty acid content of the whole milk, respectively. The concentrations of these major fatty acids did not change during storage of 20 min 15 kV plasma treated samples. Another predominant fatty acid in profile of whole milk inoculated with *S. aureus* was C14:0 with 12 %. This component was constant during storage in all samples while C14:0 slightly increased in 20 min treated samples before storage. Results showed slight increases in the percentages of C10:0 and C11:0 which were 0.43 and 2.31 before treatment and 1.71 and 0.64, respectively following 20 min of 15 kV plasma treatment.

Table 5.11. Fatty acid composition of milk stored at 4°C for 6 weeks inoculated with *S. aureus* ATCC 25922 after 20 min treatment with 9 kV plasma system

[a] 5,8,11,14-Eicosatetraenoic acid, [b] 7,10,13-Eicosatrienoic acid, [c] SFAs: Saturated fatty acids, MUFAs: Monounsaturated fatty acids, PUFAs: Polyunsaturated fatty acids

Fatty Acid	**Percent in Milk After 6 weeks at 4°C**		
	Whole milk	***S. aureus* ATCC 25922**	
		Control	**20 min Treatment**
C8:0	0.000	0.000	0.148
C10:0	1.178	0.156	1.709
C11:0	0.251	0.431	0.644
C12:0	3.470	2.309	3.163
C13:0	0.128	0.110	0.120
C13 iso	0.202	0.181	0.217
C14:1	1.349	1.234	1.152
C14:0	12.258	11.732	11.334
i-C14:0	0.434	0.430	0.406
a-C14:0	0.935	0.920	0.869

Table 5.11. Fatty acid composition of milk stored at 4°C for 6 weeks inoculated with *S. aureus* ATCC 25922 after 20 min treatment with 9 kV plasma system (Continue)

Fatty Acid	**Percent in Milk After 6 weeks at 4°C**		
	Whole milk	***S. aureus* ATCC 25922**	
		Control	**20 min Treatment**
C15:0	1.741	1.727	1.710
C15:0 iso	0.518	0.519	0.503
C16:1 trans	0.123	0.129	0.119
C16:1 cis	1.938	1.968	1.837
C16:1 (Δ^{11})	0.020	0.029	0.031
C16:0	30.710	31.112	30.862
C16:0 iso	0.606	0.628	0.539
C16:0 ante	0.882	0.912	0.794
C17:0	1.031	1.065	0.997
C18:2	2.480	2.578	2.416
C18:1 (cis-Δ^{9})	22.02	23.172	22.697
C18:1 (trans-Δ^{9})	0.347	0.348	0.341
C18:1 (Δ^{11})	0.944	1.088	1.077
C18:0	14.726	15.440	14.821
C19:1	0.127	0.152	0.135
C19:0	0.100	0.112	0.097
C20:4[a]	0.160	0.191	0.159
C20:3[b]	0.113	0.129	0.112
C20:1n9 (cis11)	0.161	0.193	0.160
C20:0	0.243	0.286	0.251
Total SFAs[c]	65.836	64.480	65.855
Total MUFAs[c]	25.719	26.874	26.099
Total PUFAs[c]	2.914	3.117	2.847

Total FFAs compositions of milk inoculated with *S.* Typhimurium and stored for 6 weeks at 4°C were shown in Figure 5.31 as percentages of total SFA, MUFA, and PUFA after 20 min 15 kV plasma treatment. About 66, 26, and 3 % of total fatty acid content of whole milk was SFAs, MUFAs, and PUFAs, respectively. The total SFAs decreased from 66.29 to 65.50 % during storage of 20 min plasma treated sample with 15 kV. While slight increases were evidenced in the percentages of MUFAs and PUFAs which were 25.73 and 2.77 % in controls, respectively. MUFAs and PUFAs increased to 26.72 and 3.02 %, respectively after plasma treatment. However, none of these changes were significant, resulting that total SFAs, MUFAs, and PUFAs profiles of whole milk inoculated with *S.* Typhimurium were unaffected by plasma treatment utilized with 15 kV power supply during storage.

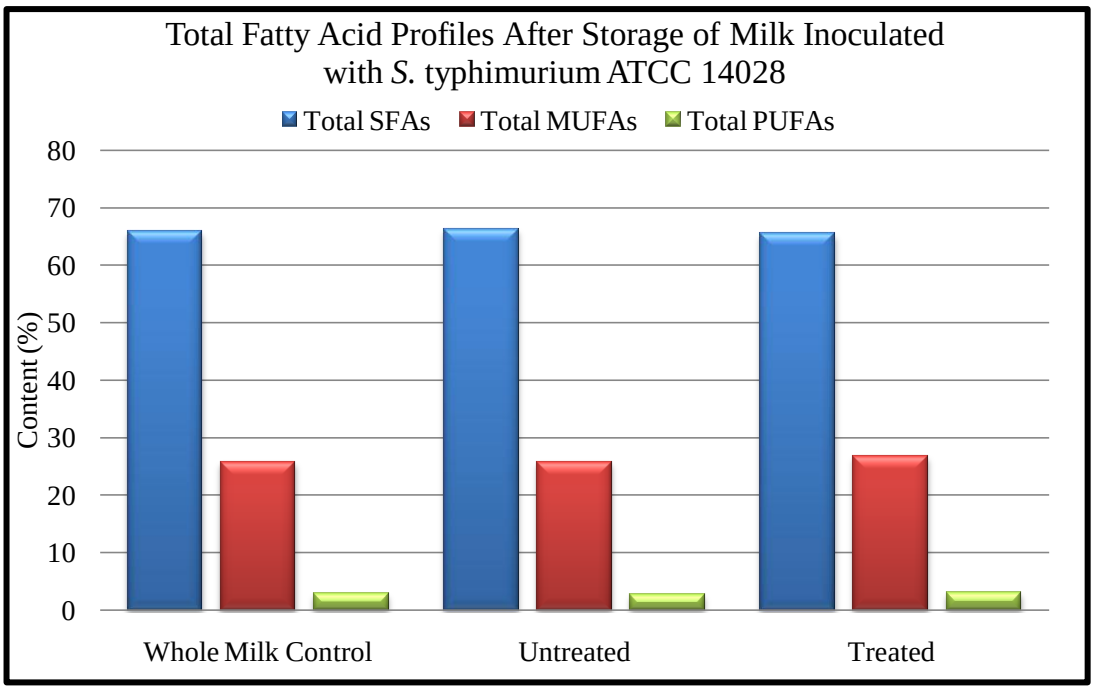

Figure 5.31. Total fatty acid profiles of whole milk, whole milk inoculated with *S.* Typhimurium ATCC 14028, and 20 min of 15 kV plasma treated sample after 6 weeks of storage

SFAs: Saturated fatty acids, MUFAs: monounsaturated fatty acids, PUFAs: polyunsaturated fatty acids

Storage results of the fatty acid profiles of whole milk inoculated with *S.* Typhimurium after 15 kV plasma treatment for 20 min were shown in Table 5.12. Total SFAs detected were between C8:0 – C20:0, MUFAs were C14:1, C16:1 cis and trans, C18:1, C19:1, C20:1 cis and trans, and PUFAs were C18:2, C20:4 and C20:3. The most predominant fatty acids observed in milk sample before plasma application were hexadecanoic acid (C16:0), oleic acid (C18:1) and stearic acid (C18:0) which made up approximately 31, 22, and 15 % of the fatty acid content of the whole milk, respectively. Hexadecanoic acid and oleic acid concentrations slightly changed during storage of 20 min 15 kV plasma treated samples with an increase from 31.45 to 32.12 % and 22.09 to 23.27 %, respectively while stearic acid concentration remained the same during this period. C14:0 decreased during storage of 20 min plasma treated sample from 12.47 to 11.53 % which was also decreased in 20 min treated samples of *S.* Typhimurium before storage. Results of storage showed slight decrease in the percentage of C10:0 which was 1.04 % before treatment and 0.94 % following 20 min of 15 kV plasma treatment.

Table 5.12. Fatty acid composition of milk stored at 4°C for 6 weeks inoculated with *S.* Typhimurium ATCC 14028 after 20 min treatment with 9 kV plasma system

[a] 5,8,11,14-Eicosatetraenoic acid, [b] 7,10,13-Eicosatrienoic acid, [c] SFAs: Saturated fatty acids, MUFAs: Monounsaturated fatty acids, PUFAs: Polyunsaturated fatty acids

Fatty Acid	**Percent in Milk After 6 weeks at 4°C**		
	Whole milk	***S.* Typhimurium ATCC 14028**	
		Control	**20 min Treatment**
C8:0	0.000	0.000	0.000
C10:0	1.178	1.042	0.944
C11:0	0.251	0.354	0.257
C12:0	3.470	3.294	3.014
C13:0	0.128	0.126	0.122
C13 iso	0.202	0.190	0.174
C14:1	1.349	1.324	1.163
C14:0	12.258	12.475	11.526
i-C14:0	0.434	0.433	0.370

Table 5.12. Fatty acid composition of milk stored at 4°C for 6 weeks inoculated with *S.* Typhimurium ATCC 14028 after 20 min treatment with 9 kV plasma system (Continue)

Fatty Acid	**Percent in Milk After 6 weeks at 4°C**		
	Whole milk	***S.* Typhimurium ATCC 25922**	
		Control	**20 min Treatment**
a-C14:0	0.935	0.943	0.815
C15:0	1.741	1.744	1.692
C15:0 iso	0.518	0.513	0.436
C16:1 trans	0.123	0.122	0.102
C16:1 cis	1.938	1.937	1.937
C16:1 (Δ^{11})	0.020	0.026	0.024
C16:0	30.710	31.451	32.121
C16:0 iso	0.606	0.585	0.537
C16:0 ante	0.882	0.867	0.783
C17:0	1.031	1.006	0.986
C18:2	2.480	2.411	2.644
C18:1 (cis-Δ^{9})	22.02	22.089	23.272
C18:1 (trans-Δ^{9})	0.347	0.297	0.407
C18:1 (Δ^{11})	0.944	0.971	0.860
C18:0	14.726	14.492	14.531
C19:1	0.127	0.118	0.115
C19:0	0.100	0.086	0.081
C20:4[a]	0.160	0.133	0.146
C20:3[b]	0.113	0.086	0.100
C20:1n9 (cis11)	0.161	0.137	0.134
C20:0	0.243	0.225	0.220
Total SFAs[c]	65.836	66.294	65.492
Total MUFAs[c]	25.719	25.727	26.723
Total PUFAs[c]	2.914	2.767	3.024

In addition to the relevant determinations of plasma exposure time on the lipid properties of milk, the changes on the fatty acid compositions after storage of 6 weeks was also discovered and shown above. Although the microbial quantifications during storage changed dramatically with a total sterilization after 1 week, the fatty acid profiles showed insignificant qualitative differences. After 6 weeks of storage, slight deviations occurred in the concentrations of C14:0 and C18:1, however the proportions of SFAs, MUFAs, and PUFAs were similar in all of the samples.

Figure 5.32 shows the total SFA, MUFA, and PUFA concentrations of whole milk, untreated whole milk inoculated with *E. coli, S. aureus*, and *S.* Typhimurium, respectively and 20 min plasma treated samples. The plasma system with 9 kV power supply was used for *E. coli* experiments whereas the system was utilized with 15 kV power supply for treatments of milk inoculated with *S. aureus* and *S.* Typhimurium. Upon comparison to the different species of microorganisms, no significant changes were observed regarding total fatty acid profiles. Although some insignificant deviations were observed in plasma treated milk samples inoculated with 3 test microorganisms, the concentration of total SFAs in control whole milk was 65.84 % which slightly increased in milk samples inoculated with *E. coli,* slightly decreased in milk samples inoculated with *S. aureus,* and did not change in *S.* Typhimurium milk samples. While total MUFAs slightly increased in bacteria inoculated milk samples compared to whole milk which was 25.72 % in control and 25.82, 26.01, and 26.72 % in 20 min plasma treated samples inoculated with *E. coli, S. aureus*, and *S.* Typhimurium, respectively. The control samples of whole milk and test microorganisms contained similar amounts of PUFAs (approximately 2.9 %) while some deviations were observed when the amounts were compared individually in different bacteria. The PUFA content decreased from 2.91 to 2.74 % in *E. coli* samples, 3.12 to 2.85 in *S. aureus* samples, and 2.77 to 3.02 % in *S.* Typhimurium samples.

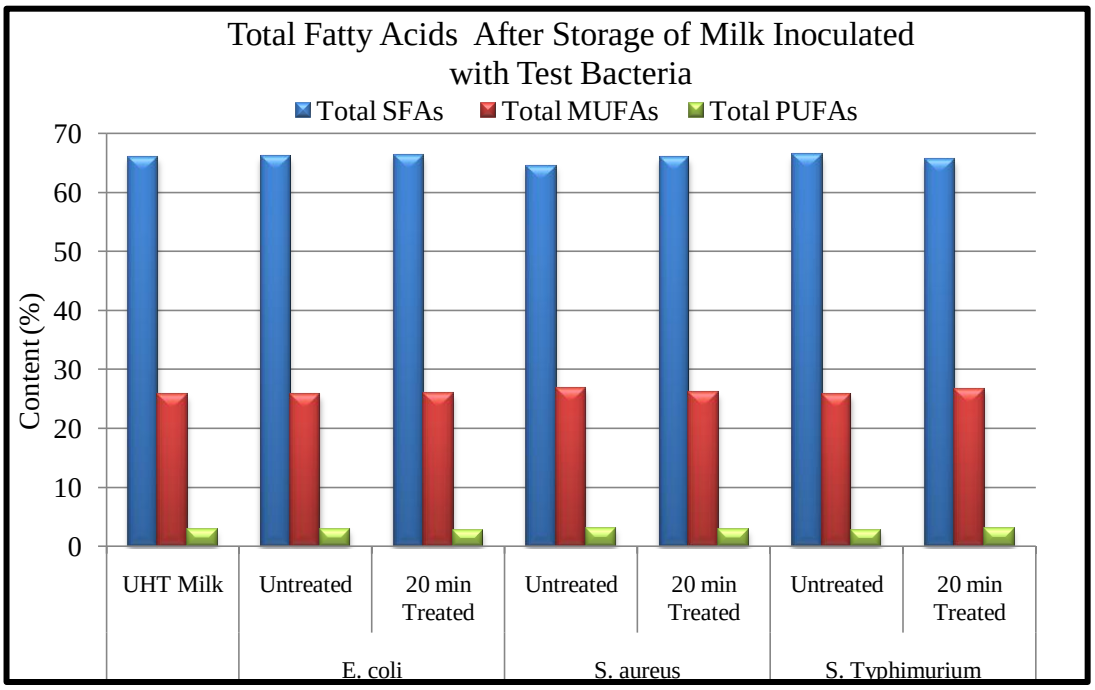

Figure 5.32. Total saturated, monounsaturated, and polyunsaturated fatty acid profiles milk samples inoculated with *E. coli* ATCC 25922, *S. aureus* ATCC 25921 and *S.* Typhimurium ATCC 14028 after storage for 6 weeks at 4°C following 20 min plasma treatment
SFAs: Saturated fatty acids, MUFAs: monounsaturated fatty acids, PUFAs: polyunsaturated fatty acids

As a result, no side effects on fatty acids profiles of milk samples due to the atmospheric pressure plasma exposure to a 20-min treatment were observed in samples stored at refrigeration temperatures for 6 weeks regardless of the microorganism inoculated before the treatment as shown in Figure 5.32.

5.5.4. Identification of Volatile Compounds in Milk

GC/MS-SPME was also perfomed for the samples stored in refrigerator for 6 weeks in order to further investigate the effects of plasma exposure on milk's compositional quality assessments during storage.

The total ketone, aldehyde and alcohol concentrations of plasma treatments of milk inoculated with *E. coli*, *S. aureus* and *S.* Typhimurium, respectively, were compared with

the control UHT whole milk as shown in Figure 5.33. The total ketones, aldehydes and alcohols were not affected by inoculation bacteria after storage for 6 weeks which means that there were no difference between the UHT whole milk and control samples inoculated with microorganisms by means of total ketones, aldehydes and alcohols as shown in Figure 5.33. The total ketone concentrations were kept nearly constant during plasma treatment and storage where total aldehydes increased in all samples. On the other hand, alcohol content increased with an average of 3 folds post-plasma application during storage, regardless of the type of microorganism.

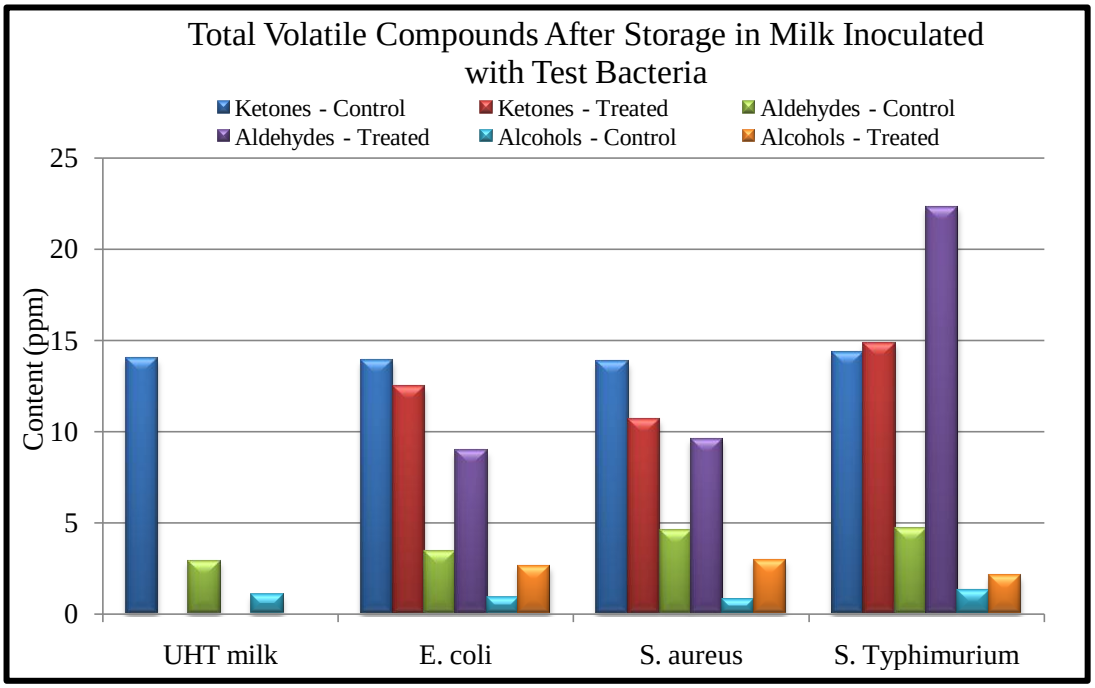

Figure 5.33. Ketone, aldehyde and alcohol concentrations in control, *E. coli*, *S. aureus* and *S.* Typhimurium milk samples after 6 weeks of storage

The individual ketone, aldehyde, and alcohol concentrations of milk stored at refrigerator following plasma treatments for 20 min were shown in Table 5.13. The ketones detected were, acetone, 2 butanone, 2 pentanone, methyl isobutyl ketone, 5 methyl 3 hexanone, 4 methyl 2 hexanone, 4 methyl 3 penten 2 one, 2,6 dimethyl 4 heptanone, 2 heptanone, 2 octanone, 2,5 octanedione, 2 nonanone, 3,5 octadien 2 one, and 2 undecanone. Among these ketones 4 methyl 3 penten 2 one was the most abundant one with 5.45 ppm in control whole milk and approximately 5 ppm in inoculated milk samples.

The concentration of 4-methyl-3-penten-2-one decreased following exposure to plasma in all samples, regardless of the bacterial strain while the greatest reduction was observed in milk inoculated with *S. aureus*. The concentration of 4-methyl-3-penten-2-one decreased from 4.99 to 4.15 ppm, 5.16 to 3.47 ppm, 5.47 to 4.37 ppm, respectively in *E. coli*, *S. aureus* and *S.* Typhimurium samples after 20 min plasma treatment. Another ketone that was found in great amounts was 2,6-dimethyl-4-heptanone. The amount of 2,6-dimethyl-4-heptanone in untreated samples of *E. coli* and *S.* Typhimurium were 3.83 and 5.16 ppm, respectively which decreased to 3.00 and 4.01 ppm, respectively in 20 min plasma-treated samples. The 2-heptanone concentration decreased from 2.10 to 0.55 ppm in *E. coli* sample after 20 min treatment and a total reduction was observed in 20 min 15 kV plasma treated *S. aureus* sample. However the concentration of 2-nonanone did not changed between the control and plasma treated samples.

The aldehydes detected in whole milk samples stored for 6 weeks were butanal, 2 butenal, hexanal, heptanal, 2 hexenal, octanal, 2 heptanal, nonanal, 2 octenal, 3 furaldehyde, 3-Cyclohexene-1-carboxaldehyde, decanal, 2 nonenal, and benzaldeyde. The most abundant aldehyde in all samples was nonanal with an average value of 2.5 ppm. However, nonanal concentration increased dramatically from 3.24 to 10.62 ppm in *S.* Typhimurium sample after treatment. Although, the nonanal concentrations in *E. coli* and *S. aureus* samples were slightly affected by plasma applications.

The amounts of alcohols in stored whole milk samples inoculated with *E. coli*, *S. aureus*, and *S.* Typhimurium before and after treaments were also determined by GC/MS. Alcohols were found in lower amounts compared to ketones and aldehydes. Detected alcohols were ethanol, 2 hexanol, 5 methyl 3 hexanol, 1 pentanol, 1 octen 3 ol, 1 octanol, and 2 furanmethanol. 2-hexanol was found to be the most predominant alcohol in control whole milk sample which was also the most abundant alcohol in milk inoculated with *E. coli* before storage. 2-hexanol concentrations decreased after plasma treatments in storage samples. On the other hand, most of the alcohol components were not detected in storage samples.

Table 5.13. The concentrations of volatile compounds in milk inoculated with *E. coli* ATCC 25922, *S. aureus* ATCC 25921 and *S.* Typhimurium ATCC 14028 after storage at 4°C for 6 weeks following 20 min plasma treatments

Volatile Compound	**Amount in Milk Samples (ppm)**						
	UHT Milk	*E. coli*		*S. aureus*		*S.* Typhimurium	
		Untreated	Treated	Untreated	Treated	Untreated	Treated
acetone	0.39	0.33	0.70	0.24	0.24	0.23	0.47
butanal			0.05				
2 butanone	0.07	0.06	0.42				
ethanol	0.21	0.21	1.76		2.21	0.42	1.66
2 pentanone	0.29	0.35	1.12	0.24	0.31	0.17	0.72
methyl isobutyl ketone	0.38	0.38	0.66	0.35	0.56	0.34	0.53
toluene		0.07	0.39	0.03	1.04	0.03	1.93
2 butenal	0.02	0.01		0.04	1.31	0.03	
5 methyl 3 hexanone	0.44	0.32	0.32	0.41	0.34	0.48	
hexanal	0.46	0.51	2.52	0.43	2.11	0.48	5.07
4 methyl 2 hexanone	0.97	0.98	0.74	1.05	0.70	1.15	1.06
ethylbenzene	0.06	0.07			0.87	0.08	0.95
4 methyl 3 penten 2 one	5.45	4.99	4.15	5.16	3.47	5.47	4.37
2,6 dimethyl 4 heptanone	4.26	3.83	3.00	4.61	4.66	5.16	4.01
2 heptanone	1.34	2.10	0.55	1.32		1.00	2.63
heptanal			0.28		0.88		
2 hexanol	0.51	0.47	0.42	0.47	0.37	0.58	0.42
5 methyl 3 hexanol	0.22	0.11	0.18	0.20	0.22	0.20	
2 hexenal	0.08	0.13	0.59	0.08	0.42	0.19	0.84
cloro 1 octane	0.20	0.18	0.14	0.41		0.32	1.07

Table 5.13. The concentrations of volatile compounds in milk inoculated with *E. coli* ATCC 25922, *S. aureus* ATCC 25921 and *S.* Typhimurium ATCC 14028 after storage at 4°C for 6 weeks following 20 min plasma treatments (Continue)

Volatile Compound	**Amount in Milk Samples (ppm)**						
	UHT Milk	*E. coli*		*S. aureus*		*S.* Typhimurium	
		Untreated	Treated	Untreated	Treated	Untreated	Treated
styrene	0.07	0.10	0.10		0.52	0.10	0.83
2,2,4,6,6 pentamethyl 3 heptene		0.36					0.45
2 octanone			0.18				0.20
octanal	0.12	0.18	0.45	0.41	0.61	0.26	1.59
2,5 octanedione			0.07				0.11
2 heptanal		0.08	0.04				0.19
tetradecane	0.06	0.05	0.05				
2 nonanone	0.32	0.44	0.37	0.36	0.31	0.27	0.28
nonanal	1.87	2.05	2.65	3.24	2.47	3.24	10.62
1 nitro pentane			0.16		0.25		0.55
2 octenal					0.28	0.05	0.40
3 furaldehyde	0.15	0.18	0.25	0.13	0.48	0.16	0.67
3-Cyclohexene-1-carboxaldehyde			0.35		0.30		0.43
decanal	0.10	0.12	0.09	0.13	0.18	0.13	0.31
3,5 octadien 2 one			0.04		0.10		0.45
1 octanol	0.07	0.07	0.17	0.11	0.13	0.12	
benzaldeyde	0.09	0.15	1.52	0.12	1.86	0.11	2.20
nonadecane/hexadecane	0.03	0.05	0.05	0.85		0.04	
2 undecanone	0.06	0.10	0.12	0.08		0.06	

Table 5.13. The concentrations of volatile compounds in milk inoculated with *E. coli* ATCC 25922, *S. aureus* ATCC 25921 and *S.* Typhimurium ATCC 14028 after storage at 4°C for 6 weeks following 20 min plasma treatments (Continue)

Volatile Compound	**Amount in Milk Samples (ppm)**						
	UHT Milk	*E. coli*		*S. aureus*		*S.* Typhimurium	
		Untreated	Treated	Untreated	Treated	Untreated	Treated
Total Ketones	13.99	13.89	12.43	13.81	10.68	14.32	14.83
Total Aldehydes	2.88	3.41	8.90	4.57	9.58	4.66	22.33
Total Alcohols	1.01	0.86	2.62	0.78	2.94	1.31	2.08

The volatile compounds that significantly changed after 20 min of plasma treatment under storage conditions were ethanol, hexanal, 2-hexenal, nonanal, and benzaldehyde. The amounts of these compounds were given in Figure 5.34 as comparison to control samples. All of these VOCs showed a tendency to increase in whole milk inoculated with microorganisms, regardless of the type of microorganism and treatment. However, the most dramatical change occurred in hexanal concentration of milk inoculated with S. Typhimurium, following plasma treatment and storage. The initial concentration of hexanal was 0.48 ppm in control sample of *S.* Typhimurium and increased to 5.07 ppm post-treatment.

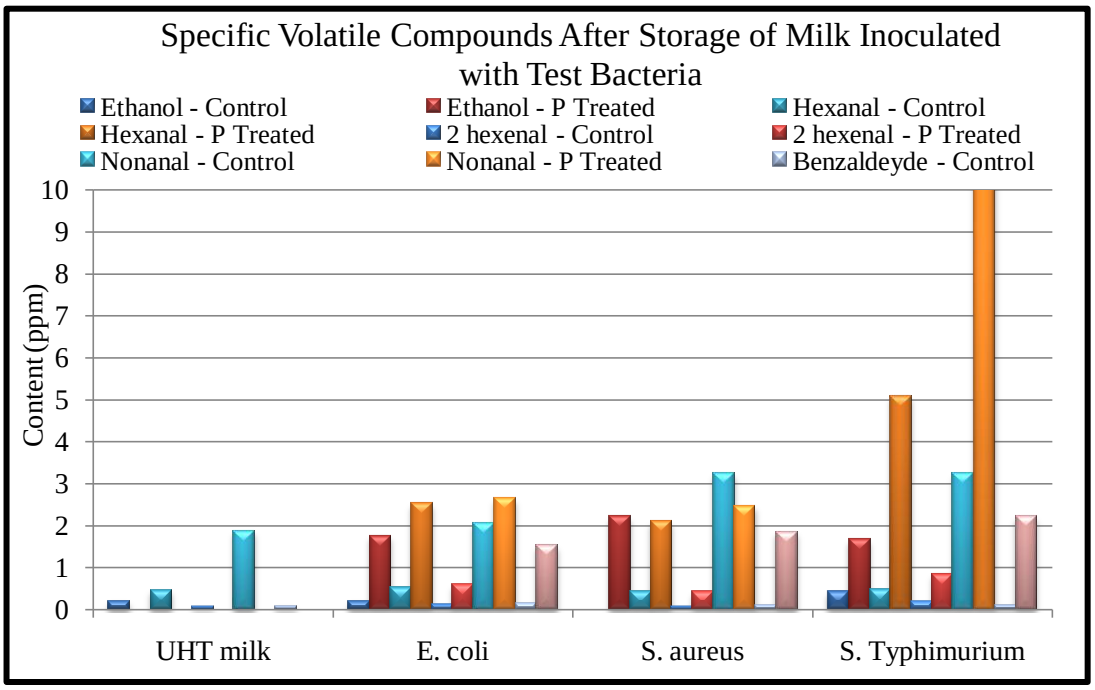

Figure 5.34. Concentrations of specific volatile compounds in control, *E. coli*, *S. aureus* and *S.* Typhimurium milk samples after 6 weeks of storage
P Treated: Plasma treated

In addition to the results from storage of the milk in refrigeration temperature after plasma corona discharge treatment which caused changes in the concentrations of some specific chemicals such as; ethanol, methyl-isobutyl-ketone, hexanal, 2-hexanal, nonanal, 3-furaldehyde, benzaldehyde, an unknown compound (19.31) occurred after 6 weeks of storage following 20 min of plasma application, regardless of the bacterial species used as shown in Figure 5.34.

5.6. DETERMINATION OF KILLING MECHANISM OF PLASMA ON BACTERIAL CELLS

5.6.1. Scanning Electron Micrographs After Plasma Application

The effects of plasma application on the cell morphologies of *E. coli* and *S. aureus* were shown in Figure 5.35 and Figure 5.37. SEM pictures revealed a considerable change in the morphology after plasma application. It was observed that *E. coli*, changed in shape

following plasma treatment. The cellular material spilled out to the cell surrounding as shown in Figure 5.35.

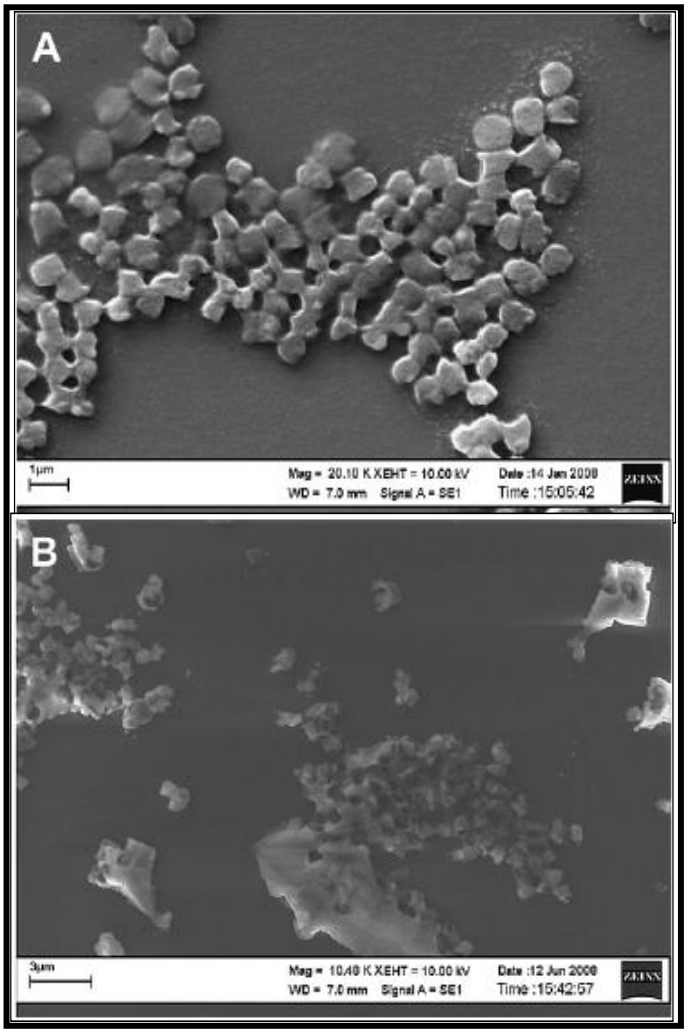

Figure 5.35. Scanning electron micrographs of untreated *E. coli* cells (A) and after 15 kV plasma treatment for 20 min (B)
Magnitude 20.10 KX (A) and 10.48 KX (B)

Similar changes in cell morphology was also observed for *S. aureus* cell after 20 min. Damage to the cell and cell contents was again observed compared to intact control cells. *S. aureus* cells were also affected by the application of plasma corona discharge. As shown in Figure 5.36, a trail of bacterial contents was detected.

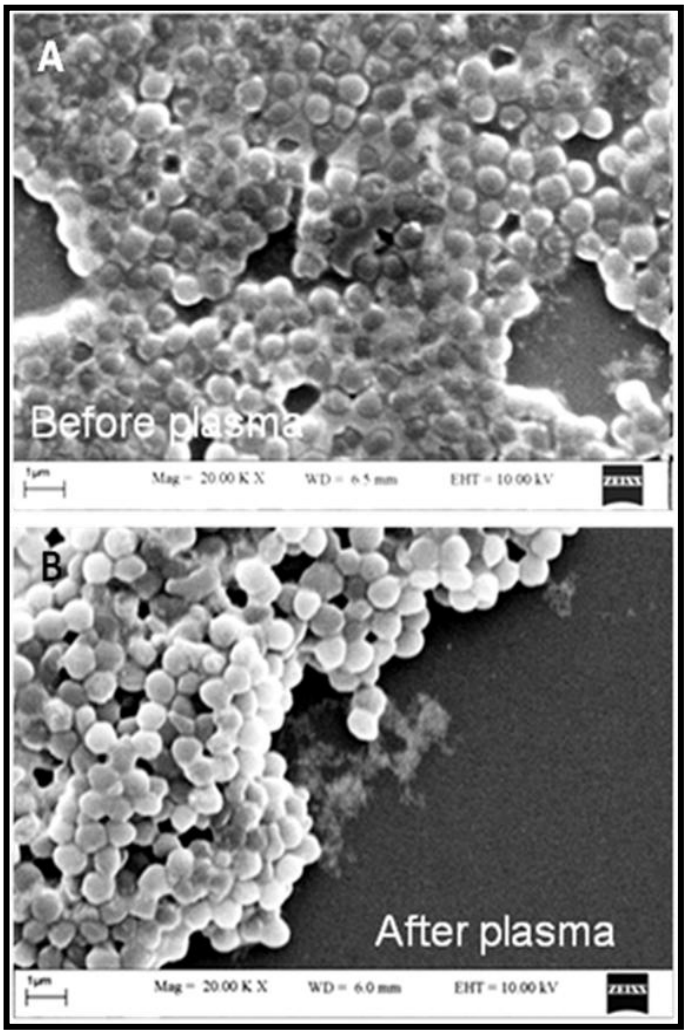

Figure 5.36. Scanning electron micrographs of untreated *S. aureus* cells (A) and after 15 kV plasma treatment for 20 min (B)
Magnitude 20000 KX

5.6.2. Effects of Plasma on DNA

Further experiments were carried out regarding cell structures in order to better understand the inactivation mechanism of plasma system. 16S PCR was performed for *E. coli* and *S. aureus* cells before and after plasma application.

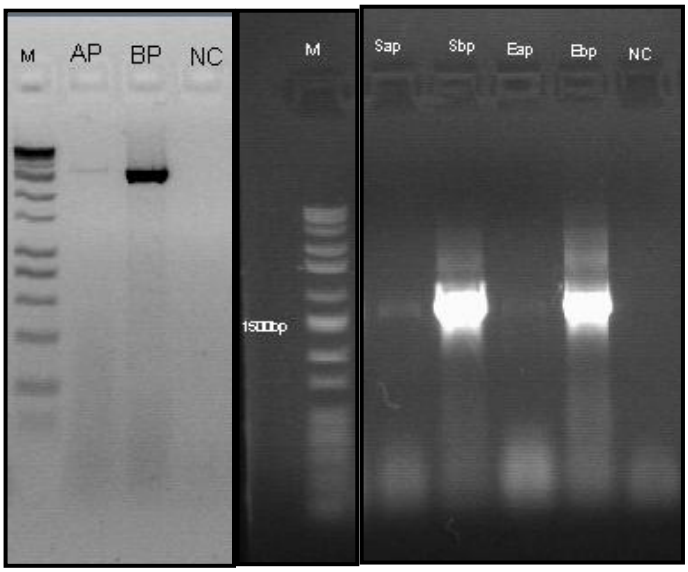

Figure 5.37. Whole DNA results of before and after plasma *E. coli* cells (on the left) and *S. aureus* cells (on the right)

M: Marker, AP: After plasma, BP: Before plasma, NC: Negative control, Sap: *S. aureus* after plasma, Sbp: *S. aureus* before plasma, Eap: *E. coli* after plasma, Ebp: *E. coli* before plasma.

Initial running of the extracted DNA onto a 1% agarose gel stained with ethidium bromide revealed no bands post-plasma treatment in comparison to the clear band observed in the control untreated bacterial DNA as shown in Figure 5.36. This result was observed three times and therefore amplification of 16S rDNA genes of *E. coli* was carried out, in order to increase the product yield. The amplified (34 cycles) 16S rDNA genes of *E. coli* after plasma and before plasma samples were run on 1% agarose gel stained with ethidium bromide. Figure 5.28 shows gel electrophoresis results for control samples of *E. coli*, before and after plasma application. A very faint band was observed following plasma treatment in comparison to the control pre-treated plasma DNA under the same conditions, indicating that DNA has been affected by the plasma, possibly causing its fragmentation and/or degradation.

Experiments were repeated and other bacteria was studied. Amplified 16S rDNA genes of *S. aureus* before and after plasma were investigated as shown in Figure 5.36. Gel

electrophoresis result again revealed fainter bands after plasma application for the bacteria investigated, compared to control samples under the same conditions.

In order to observe whether there was damage to DNA over time plasma was applied to the bacterial cell solution after 20 min plasma treated. Following DNA extraction and 16S rDNA amplification results showed a gradual decrease in the DNA band after 15 minutes of plasma application (data not shown). Ethanol precipitation was performed on the genomic DNA to try and gain a higher concentration and samples run on 1% agarose gel as shown in Figure 5.38. Precipitated total genomic DNA from *E. coli* was clearly seen as a band before plasma and after plasma revealed a second band with lower molecular weight which is indicative of DNA degradation.

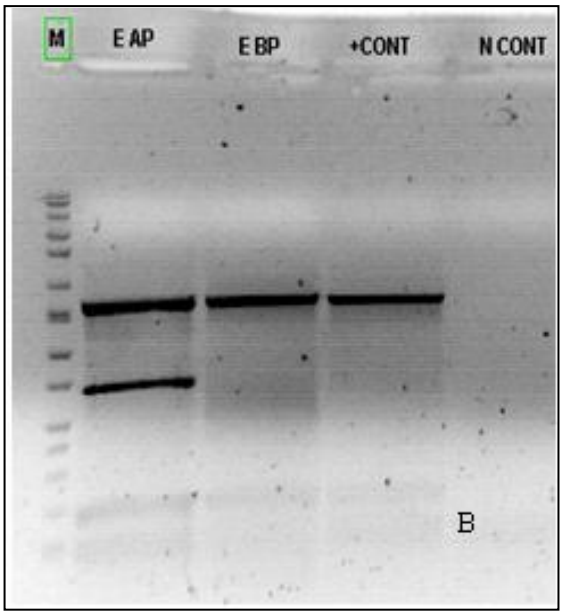

Figure 5.38. 16S rDNA gel results of *E. coli* cells before and after plasma
M: Marker, E AP: *E. coli* after plasma, E BP: *E. coli* before plasma, + Cont: Positive control, N Cont: Negative control.

5.6.3. Fatty Acid Profiles of Bacterial Cells

E. coli is known to predominantly, contain straight chain saturated fatty acids, with minor amounts of mono-unsaturated and branched (iso or anteiso) fatty acids in comparison to Gram-positive bacteria *S. aureus* which has mostly branched chain,

saturated fatty acids. The most abundant fatty acids observed in this study, in the *E. coli* profile, before plasma application, was hexadecanoic acid (C16:0) and heptadecanoic acid (C17:0) which made up approximately 35% and 20% of the fatty acid content of the cell respectively (see peaks D and E in Figure 5.39 A), while the predominant fatty acids found in *S. aureus* was C15:0 (anteiso) making up nearly 46% of the whole cell fatty acids and C17:0 (anteiso) present in the approximate amounts of 23%, (see peaks A and C in Figure 5.39 A). The *x*-axis of Figures 5.39 and 5.30 represent the retention time in minutes while the *y*-axis denotes peak intensity of ion extracts of the gas chromatograph.

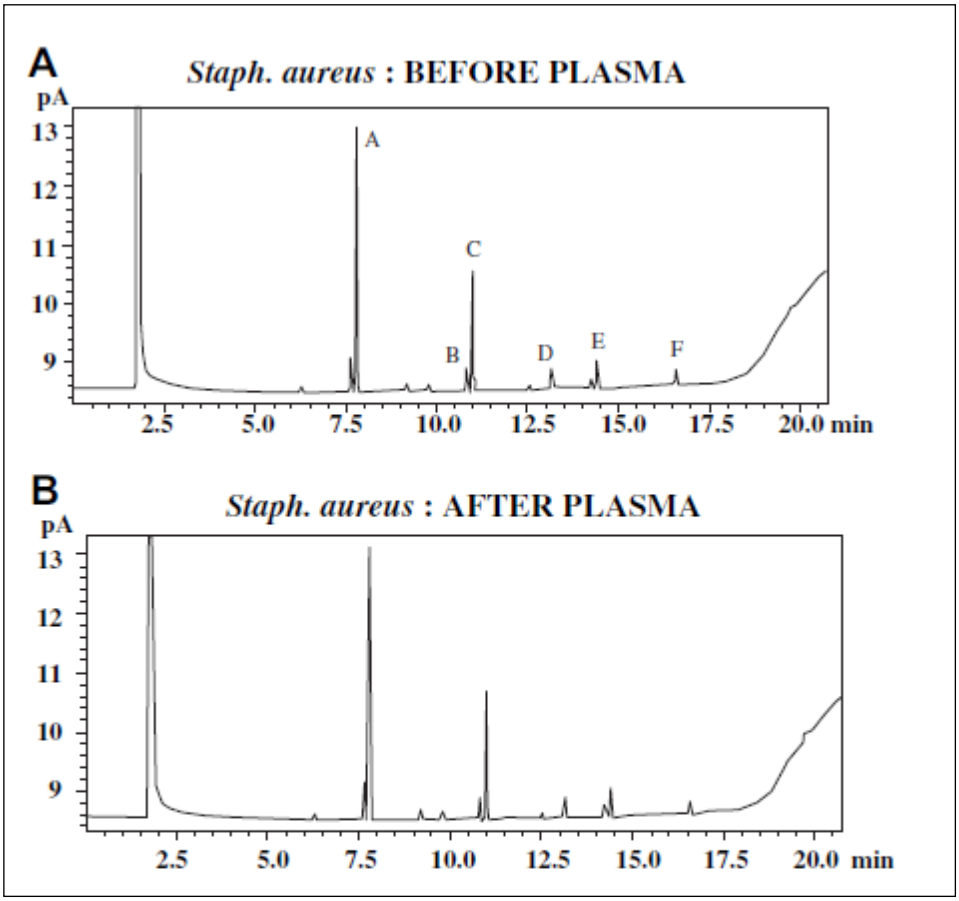

Figure 5.39. Peak intensity from gas chromatography as a function of retention time (in minutes) for *S. aureus* before and after plasma application

(A) BEFORE PLASMA: A=C15:0-anteiso, 46.37%; B=C17:0-iso, 4.58%; C=C17:0-anteiso, 23.23%; D=C18:0, 4.48%; E=C19:0-anteiso, 6.3%; F=C20:0, 3.25%; (B) AFTER PLASMA: A=C15:0-anteiso, 47.45%; B=C17:0-iso, 5.38%; C=C17:0-anteiso, 24.88%; D=C18:0, 3.92%; E=C19:0-anteiso, 4.66%; F=C20:0, 1.90%.

Comparison of the whole cell, fatty acid profiles of both *E. coli* and *S. aureus*, before and after plasma treatment (Figure 5.39 A, B and Figure 5.40 A, B, respectively) showed no qualitative changes in the fatty acid profiles following plasma application were observed. Upon closer examination it was found that some fatty acids present as less than 1% before plasma were eliminated after plasma application while more abundant fatty acids showed slight quantitative changes.

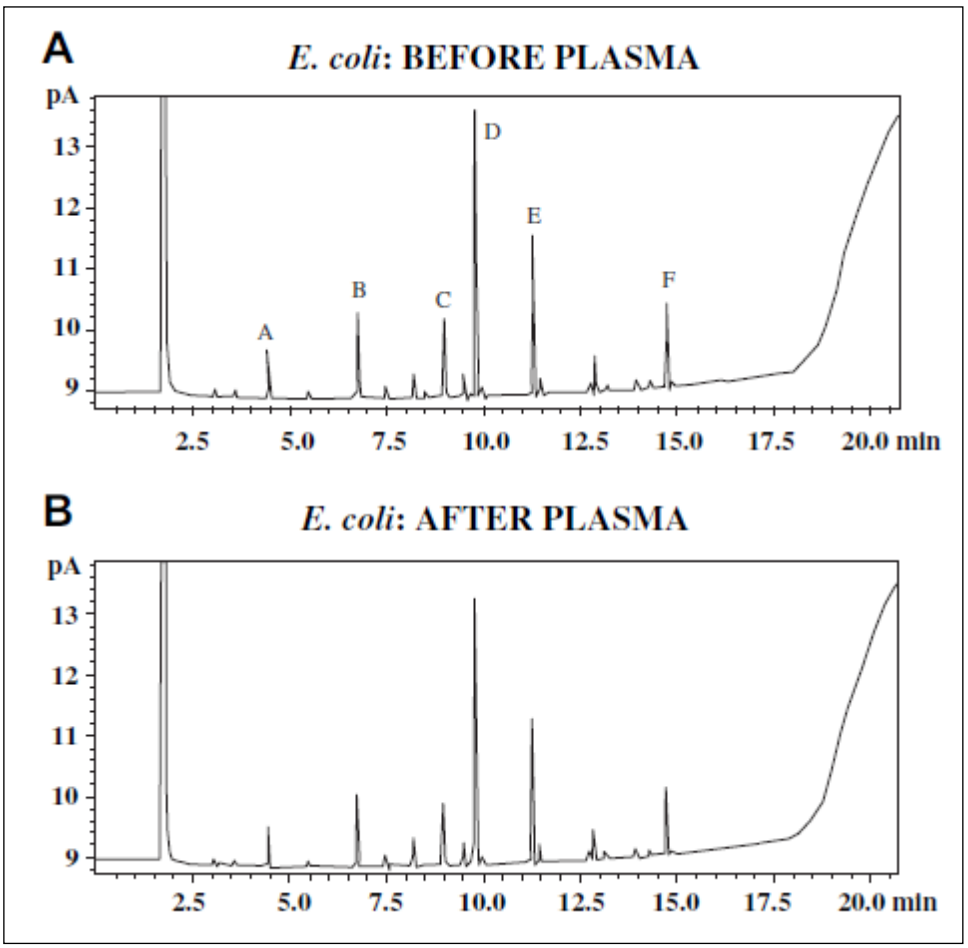

Figure 5.40. Peak intensity from gas chromatography as a function of retention time (in minutes) for *E. coli* before and after plasma application

(A) BEFORE PLASMA: A=C12:0, 4.0%; B=C14:0, 9.25%; C=C15:0, 8.71%; D=C16:0, 35.39%; E=C17:0, 20.91%; F=C19:0, 10.96%; (B) AFTER PLASMA: A=C12:0, 4.29%; B=C14:0, 9.6%; C=C15:0, 9.57%; D=C16:0, 35.19%; E=C17:0, 20.71%; F=C19:0, 11.33%

6. DISCUSSION

6.1. PLASMA SYSTEM

The need for milder decontamination techniques that keep the nutritional value, taste and color of food while producing safer and fresh-like characteristics also prolonging the shelf-life has led to the exploration of interdisciplinary novel approaches. Currently traditional milk disinfection techniques are mostly based on thermal processing which can cause damage to the nutritional and sensory properties. Therefore, some non-thermal, physical technologies are being modified for food processing such as ionizing radiation [89], high hydrostatic pressure treatment [99], high voltage electric field pulses [101], pulsed electric fields [105] and low-temperature plasma [21]. These systems are capable of inactivating microorganisms at ambient or moderately elevated temperatures within short treatment times [82]. These systems do however have disadvantages and are specialized for certain food mediums. For example, some disadvantages of ionizing radiation were established regarding undesirable flavor formation in some dairy products and textural deformations in some fruits [96]. HPP has also its own limitations such as changes in the pH, the inability of inactivating pressure resistant vegetative bacteria after successive pressure treatments, large technological requirements and costs [103, 104]. The high voltage electric field pulses treatment requires fairly complex electronic and fluid handling systems and the application is limited to liquids [83]. Studies of bacteria inactivation by pulsed electric field have been carried out for more than 14 years [111] and results have been showed that a variety of parameters should be considered and optimized before the system is adjusted for a specific use [126]. Non-thermal plasma is the most recent and novel technology that can be tested in food decontamination systems. Plasma systems have a lot of advantages including high anti-microbial activity, low cost, operating at relatively low temperatures and atmospheric pressure. Therefore, the feasibility of a pilot corona discharge system [21] was tested on decontamination of milk.

While testing the feasibility of the plasma system optimizations in the test parameters were also carried out to increase the microorganism inactivation and minimize the effects on the quality of the milk. One of the test parameters was power supply which is an

important factor on sterilization efficiency. Both AC and DC power supplies can be used with the plasma system in this study. When an AC power supply is utilized, the flow direction changes according to the supply's frequency. In this case the ions and electrons move in a back and forth motion rather than in one direction, causing, a decrease in the amount of energy transfer on the average. However, in the direct current (DC) a unidirectional continuous flow of the electrons and ions are produced thus producing a continuous bombardment of ions on the cell. In our previous studies we have demonstrated that continuous flow of electrons and ions (as described in DC) penetrate the bacterial cell walls and membranes more efficiently and cause destruction of the cells more rapidly [21]. However, the studies carried out with water showed that DC power supplies are highly potent to the supporting media due to the intense bombardment of electrons and ions [21, 147]. Thus, AC power supply was decided to be more suitable for the milk experiments because of the complex chemical structure of milk.

Voltage is another efficiency factor in sterilization of microorganisms [21, 224]. 9 kV of AC power supply was tested on the microorganisms inoculated into milk. This system significantly reduced the numbers of *E. coli* in milk with 4 log reductions, however no significant reductions were observed in *S. aureus* and *S.* Typhimurium studies with 9 kV power supply. Utilizing the system with an increased voltage of 15 kV resulted in the inactivation of *S. aureus* and *S.* Typhimurium after treatment for 20 min and resulted in 2 and 2.6 log reductions, respectively. This result is to be expected since an increase in voltage means an increase in energy of free ions. Song *et al.* [224] found similar results using atmospheric plasma system to decontaminate bacteria on cheese which showed an increase in the microbial log reductions from 2 to 8 log CFU/mL when the power input is increased 2 folds. The effect of different bacterial species on the sterilization efficiency of plasma system is discussed in Section 6.3.1.

Treatment time plays a crucial role in the inactivation capacity of plasma system and longer exposure time results in higher log reductions. In this study, the inactivation of test bacteria using the plasma system was continuous during the experiment which was monitored for a total of 20 min. The first 3 min of application showed the highest significant log reduction regardless of the type of microorganism and milk used in the study and was followed by insignificant reduction. This can be explained by the

inactivation mechanism of plasma, which is believed to be due to damage of the bacterial cell membrane, due to high electric potential and the adsorption of active species such as ions, electrons, free radicals [26]. These active species found using emission spectroscopy. The major ionizing molecules detected in the plasma discharge were oxygen and nitrogen. Previous studies on observation of emission spectra of atmospheric pressure plasmas showed similar excited species which are O, $O_{2,}$ and N [179, 180]. The oxygen and nitrogen molecules have been reported to react with the electrons to form radicals and reactive oxygen species as shown in Section 2.7.3. equation 2.1-2.2. These target the outer layer of bacterial cells causing chemical reactions with the bacterial molecules [19]. This is followed by UV destruction of the DNA which is a milder and slower reaction, thus playing the most crucial part in plasma sterilization process as described in Section 2.7.5.

6.2. THE EFFECTS OF PLASMA APPLICATION ON MILK

6.2.1. Microbiological Analysis

To date, no studies have been carried out regarding decontamination of milk by non-thermal plasma processing technologies. Studies using other non thermal processing techniques have shown that reducing the number of microorganisms in milk is more difficult than model buffer solutions because of the complex structure of milk [182, 183].

The plasma corona discharge system generated with 9 and 15 kV power supplies was tested on gram-positive (*S. aureus*) and gram-negative (*E. coli* and *S.* Typhimurium) bacteria in milk. Plasma system utilized with 9 kV power supply significantly killed *E. coli* in milk with more than 4 log reductions, however the number of *S. aureus* and *S.* Typhimurium in milk did not significantly decrease within 20 min of total application at this voltage. The population of *S. aureus* and *S.* Typhimurium were remarkably reduced in milk when the plasma was generated with a 15 kV power supply and caused 2 and 2.6 log respective reductions, following 20 min treatment. Although, there are no studies regarding inactivation of microorganisms in milk using NTP, studies with PEF processing of milk resulted in similar decontamination capabilities of *E. coli* O157:H7 dispersed in skim milk and *E. coli* ATCC 8739 cells in orange and milk mixed beverage [108, 181]. Furthermore,

less than 2 log reductions were achieved using HPP treatment in UHT milk inoculated with *L. monocytogenes* and *S. aureus* [185, 186]. Therefore plasma was seen to be an efficient decontamination method for milk.

There are a number of factors affecting the microbial inactivation mechanism of the plasma which can be listed as the initial concentration of the microorganism, the treatment duration, the type of the microorganism, and the structure of the supporting medium [189, 190]. Firstly, the different starting concentrations of bacteria may have resulted in a greater log reductions due to the higher microbial loads before application. In this study it was observed that the higher the beginning concentration, higher the total log reduction of test microorganisms. However, this theory has been challenged as some studies showed a converse result, in that the higher the initial load the lower the decontamination activity. This was attributed to the tendency of microorganisms to form protective structures against inactivating agents formed during plasma [187, 188]. For better understanding of the effect of initial microbial load on the efficiency of plasma inactivation mechanism, experiments with same beginning concentrations should be carried out.

A second obvious reason could time dependance. The plasma treatment duration was compared with the survival curves of *E. coli, S. aureus* and *S.* Typhimurium inoculated into milk. The results showed a highly significant decrease in the number of colonies during the first 3 min of application. *E. coli* had the highest decrease in the number of viable cells with 3 log reductions within 3 min, which is followed by *S.* Typhimurium and *S. aureus* with 1 log reductions. After 3 min a shouldering pattern was observed with linear (one-line curve) and insignificant reductions. Similar time dependent survival curves were reported by Hermann *et al.* [159] and Yamamoto *et al.* [160] after treatment with athmospheric pressure plasma discharges as well as high pressure plasma systems which reduced the concentration of microorganisms linearly during the treatment time [184].

Thirdly, different bacterial species are differently affected by the decontamination technique that is being used. There are some explanations on the killing mechanism of plasma regarding the differences in the cell wall structures of both types (Gram +ve/ Gram –ve) of microorganisms. Our results showed that gram-negative bacteria (*E. coli* and *S.* Typhimurium) were more susceptible to plasma application compared to the gram-positive

bacteria (*S. aureus*). Montie *et al*. [145] found similar results with an atmospheric pressure plasma system tested on gram-positive and gram-negative bacteria. Although contraversial results were published by Marsili *et al.* [153] in which the gram-positive *B. cereus* was the most susceptible microorganism to the plasma discharge in liquid, the majority of the literature shows that the cell wall structure of gram-negative bacteria makes it more susceptible to the charged particles formed during plasma than gram-positives [145, 159, 174, 184].

Finally, the supporting medium affects the the survival rate of the test microorganism [129]. Plasma inactivation of the microorganisms inoculated onto smooth solid surfaces for example thin agar films [24] and heat sensitive polyethylene terephthalate (PET) foils [25] have higher log reductions compared to the inactivation of microorganisms in complex surrounding materials [152, 21]. Our findings with whole, semi-skimmed and skimmed milk revealed significantly similar microbial reductions which were 4.23, 4.14, and 4.23, respectively. Therefore, the fat content of the milk did not affect the inactivation activity of plasma corona discharge system. Thus making this an advantage of this system for use in fatty substrates. Although there are no studies on the inactivation of food-borne pathogens in milk by plasma corona discharge for comparison, other techniques such as PEF showed that the fat content of the milk inversely effected the microbial inactivation via protection against electric pulses [123]. On the other hand, Reina *et al.* [124] and El-Hag *et al.* [126] found similar results with our findings who used PEF to treat whole and skim milk inoculated with *S. aureus* and *L. monocytogenes*.

6.2.2. Physical and Chemical Analysis of Treated and Untreated Milk

6.2.2.1. pH Analysis

The level of acidity in milk is a criterion of milk quality and is an indicative of poor hygienic properties of milk [191]. On the other hand, the hydrogen concentration in a solution is typically important while determining a system's decontamination capabilities. Low pH has an inactivation effect on most of the microorganisms including *E. coli*, *S. aureus* and *S.* Typhimurium. It was explained in section 2.7.3 that during plasma discharge hydrogen and hydroxyl radicals are formed. Thus, the pH of the milk samples treated with

plasma corona discharge system was monitored during the experiments. Results showed that the pH remained the same throughout the treatment, suggesting that cell death was not caused by acidity or alkalinity of the milk following the plasma treatment. Conversely, our previous study using the DC plasma system showed a dramatical change in the pH value of water from 7 to 1 [229]. Therefore, this AC plasma system has an advantage over DC plasma system which can also be used in compex liquids such as milk. In another study, the pH of the orange juice-milk beverage was measured after thermal processing and PEF treatment and no significant differences were obtained comparing to the controls [181].

6.2.2.2. Color Measurements

Color is an indicative of many physico-chemical quality factors in the milk. The precision of the color measurement tests were obtained by repeatedly determining the color of whole milk before and after plasma applications. The color measurements were carried out following 9 and 20 min of plasma applications with 15 kV power supply and compared to the UHT whole milk samples. The color difference was not noticeable and insignificant even after 9 min of plasma application. However, total application of 20 min resulted in significant color changes. L* value which indicates the lightness in the observed material which did not change after 9 min of treatment, however decreased from 84.60 to 84.09 in 20 min treated sample and this value is related to the browning reaction [192, 193]. Cserhalmi *et al.* [194] found similar results when grapefruit and tangerine juices were treated with PEF. Sampedro *et al.* [181] found similar results with the L* values which significantly decreased following thermal and PEF treatment of orange juice-milk beverage. Our a* value results of 9 min plasma application were insignificant while a significant reduction was observed following 20 min. Conversely, PEF treatment of juices and orange juice-milk beverage resulted in increased a* values meaning a more greeny color [181, 194]. The b* value indicates the yellow color and our results showed an increase in b* value, though it was only significant after 20 min of application. Conversely, decreased b* values were observed in PEF studies using different juices and orange juice-milk beverage [181, 194]. Therefore use of this system for milk decontamination should be restricted to below 20 min.

Calculations of overall color difference (ΔE) permits evaluation of severity of atmospheric pressure plasma treatment on milk. It was seen that time is an efficiency factor

in ΔE values of milk treated with AC power plasma discharge. The color difference (ΔE) results of 9 and 20 min of plasma treatment were 0.37 and 0.84, respectively. Even though, significant differences were observed upon comparison of instrumental color measurements of L*, a*, and b* values which could be due to the interaction of reactive species formed by plasma and fat content of the milk, ΔE values were lower than the visual perception of milk color difference (1.05). On the contrary, the traditional thermal processing techniques are known for their adverse effects on the color of milk [15, 17, 18]. Similar to the results of thermal treatment significant color differences were detected after PEF treatment of fruit juices and beverages [181, 194, 195, 196].

6.2.2.3. Protein Analysis

Proteolysis in milk is such an important factor in milk and dairy products since it has undesirable effects on dairy industry. Natural milk proteinase and rennet has crucial activities in the process of cheese ripening. On the other hand, oxygen radicals produced during plasma can cause protein oxidation [19] by reacting with the amino acids in the milk which leads to degradation of the proteins [202, 203, 204]. Therefore, proteolysis assessments were carried out for plasma treated milk samples inoculated with *E. coli*, *S. aureus* and *S.* Typhimurium for different exposure times and voltages. The results showed that all the samples have similar molecular weight proteins regardless of the inoculation bacteria, treatment time, and voltage. This is an advantage of plasma system compared to heat and PEF treatments of milk which cause serum protein denaturation [17, 18, 197, 198].

On the other hand, one of the major problems in milk spoilage is bacterial protease enzymes. These enzymes can overcome the milk decontamination process and allow for protein reaction during storage, therefore reducing the shelf life of milk. In this study no signs of protein degradation were observed in SDS-PAGE results. This could be an indication of bacterial enzyme inactivation due to NTP application [199, 200]. Because of these heat resistant proteases bitter flavor, coagulation and gelation can occur after thermal processing of milk [201].

6.2.2.4. Fatty Acid Analysis of Milk

There are several types of fatty acids in milk which are beneficial to health because of their anti-carcinogenic, anti-atherogenic and immunomodulatory properties [47, 48, 49]. Therefore, the fatty acid profiles of milk samples inoculated with *E. coli*, *S. aureus* and *S.* Typhimurium were investigated after plasma treatment for different exposure times and voltages. No significant differences were observed in plasma treated milk samples inoculated with *E. coli* while slightly significant changes (p=0.049) were observed in plasma treated milk samples inoculated with *S. aureus* and *S.* Typhimurium. This can be explained with the potency of voltages used for the different bacteria species which was 9 kV for *E. coli* and 15 kV for *S. aureus* and *S.* Typhimurium. This result can be explained as the plasma discharges with a higher oxygen concentration were associated with damage to the lipids due to oxygen based active species, atomic oxygen and ozone [159, 161].

Palmitic acid (hexadecanoic acid) was found to be the most predominant fatty acid in all samples. These results are in agreement with the previous studies who found C16:0 is the major fatty acid in milk [205, 206]. The concentrations of SFA were higher than unsaturated fatty acids, regardless of the time of exposure, type of microorganism and storage duration. Similar results were found by Guler *et al.* [205] and Prandini *et al.* [206] in milk samples. Oleic acid was the most abundant MUFA with an approximate percentage of 24 in *E. coli*, *S. aureus* and *S.* Typhimurium samples before and after plasma. Oleic acid was also found to be predominant MUFA in dairy products of Turkey [205, 207]. It has been reported by [207] that PUFA found in Turkish dairy products with an amount of 0.34 – 2.58 per cent. In our study the concentration of PUFA in all samples ranged from 1.7 to 3.1 per cent. Also Prandini *et al.* [206] detected a PUFA content between the range of our findings while higher concentrations were observed by Guler *et al.* [205].

Upon comparison to the concentrations of fatty acids C10:0, C12:0, and C18:0 a slightly significant increase in the percentages of C10:0 and C12:0 and a slightly significant reduction in that of C18:0 were observed in milk inoculated with *S. aureus* and *S.* Typhimurium which were treated with plasma using 15 kV of power supply (p=0.047). Such a tendency may suggest that the plasma corona discharge treatment with higher electrical potential results in hydrolytic mechanisms of long chain saturated fatty acids. Conversely, the free radicals such as hydroperoxyl radicals, superoxide radicals, singlet

oxygen are described to attack the poly-unsaturated fatty acids [171, 172] which generate shorter fatty acids [168]. On the other hand, the active species formed during plasma discharge can initiate lipid peroxidation and produce shorter chain fatty acyl compounds including alkanes, ketones, epoxides, and aldehydes [164, 165, 166].

6.2.2.5. Volatile and Organic Compounds Detected in Milk

The increasing consumer demand for nutritious, natural and tasty products has led to the exploration of alternative non-thermal processing technologies in milk industry to obtain microbial shelf-life similar to that of UHT milk and protecting the flavor. The total ketone, aldehyde, and alcohol concentrations increased with the treatment times in milk samples inoculated with test microorganisms. These results are similar with the findings of Vazquez-Landaverde [213] who used HPP for milk treatment and showed change in the volatile compounds of milk which favored the formation of aldehydes and methyl ketones.

In particular, the list of the VOC found in all samples included acetone, 2-pentanone, 5-methyl-3-hexanone, 4-methyl-2-hexanone, 4-methyl-3-penten-2-one, 2-heptanone, 2-nonanone, 2-undecanone (ketones), hexanal, octanal, nonanal, benzaldehyde (aldehydes), ethanol, hexanol, 1-octanol (alcohols). The comparison of concentrations of volatile compounds which significantly differed after 20 min of plasma treatment that were ethanol, 2-butenal, nonanal, decanal, and benzaldehyde regardless of the inoculation bacteria and power supply (9-15 kV). Upon comparison to the off-flavors and volatile compounds, 2-butanone, dimethyl sulphide, ethanol and 2-propanone are shown to be significant compounds causing degeneration of the natural taste of the milk [208, 215, 216, 217, 218]. Therefore, it can be said that plasma application results in slight denaturation of the flavor due to significantly increased ethanol concentrations. Similarly, Mounchili *et al.* [212] studied the volatile compounds characteristics of raw milk in relation to off-flavor defects who found significant changes in the 2-butanone and dimethyl sulphide concentrations in relation to the off-flavor of milk. Ketones that are detected in milk might be from different sources. For example: acetone and 2-butanone are thought to be derived from cow feed [208] and they have been detected in raw milk [209]. Ketones are naturally components of raw milk, however they can also be produced by beta-oxidation of saturated fatty acids [210] or by decarboxilation of beta-ketoacids found in milk fat [211].

The auto-oxidation of unsaturated fatty acids in milk and spontaneous decomposition of hydroperoxides can result in production of aldehydes [213]. Hexanal can also be present in raw milk [211], which was found to be most abundant aldehyte in control samples. This compound can be produced by light-induced lipid oxidation [214]. Two major unsaturated fatty acids found in milk are oleic and linoleic which may undergo degradation and form volatile compounds including 2-undecenal, 2-decenal, nonanal, octanal, decanal, nonanal, octanal, heptanol, 1-decene, 1-nonene, octane and heptanes, which derive from oleic acid, and 2,4-decadienal, hexanal, 3-nonenal, pentanol, 1,3-nonadiene and pentane deriving from linoleic acid [164, 165, 166].

On the other hand, significant increases in the contents of styrene and ethylbenzene have been detected particularly in samples inoculated with *S.* Typhimurium. Clearly, such molecules are not related to the pathogens but rather they are reported to be residues of the chemicals used for the packaging material production.

6.3. MICROBIOLOGICAL AND CHEMICAL COMPOSITION OF PLASMA APPLIED MILK DURING STORAGE

The decontamination technologies for milk aim reducing the number of bacteria and achieving the microbial safety during the shelf-life. Therefore, the microbiological analysis were carried out for 6 weeks during storage at 4°C for the 20 min plasma treated milk samples inoculated with *E. coli*, *S. aureus* and *S.* Typhimurium and which showed total sterilization of the test microorganisms in milk after one week. This result may be due to the assimilated cascade mechanisms in the cytoplasm of bacteria initiated by the reactive species and free radicals formed by plasma. The results of Song *et al.* [224] were similar to our findings, in which the number of *L. monocytogenes* on cheese significantly decreased during storage following low temperature plasma treatment. However, some reactions due to the reactive species formed in plasma may not cause a definite destruction to the cell membrane and cell death, while altering the biochemical pathways inside the cells [222, 223]. This situation is attributed as the “sub-lethal injury” which leads to recovery of the cells after the damaging factor is abrogated. The cells that undergo sub-lethal injury from the treatment process are unable to recover and grow under the incubations conditions

used. Therefore, further studies need to be carried out using suitable resuscitating environment for the test microorganisms.

Milk proteins can undergo proteolysis during storage which causes off-flavor of the milk. Therefore, the proteins of milk samples inoculated with *E. coli*, *S. aureus* and *S.* Typhimurium after 20 min of plasma application were observed with SDS-PAGE. The results of our studies showed no proteolysis due to plasma discharge treatment after the milk samples were stored for 6 weeks at 4ºC. Conversely, in a previous study, an extremely bitter flavour was detected in UHT milk sample at 180 days storage [226]. In another study, undesirable bitter flavours in UHT milk have been associated with late-eluted, hydrophobic peptides [225]. The casein degradation level can be attributed to the activity of heat-stable proteinases of native and/or bacterial origin [227]. On the other hand, in a study investigating the proteolysis of milk proteins due to thermal processings, defects were reduced by using higher temperature (150 rather than 145°C) however a more intense cooked flavour in the UHT milk while the overall acceptability was not affected [226].

Also, the fatty acid compositions of 20 min plasma-treated milk samples inoculated with *E. coli*, *S. aureus* and *S.* Typhimurium were investigated. Some changes on the fatty acid compositions after storage of 6 weeks were discovered. After 6 weeks slight deviations occurred in the concentrations of C14:0 and C18:1, though these changes were insignificant. Therefore, no side effects on fatty acids due to the exposure up to 20-min plasma treatment were observed in samples stored at refrigeration temperatures for 6 weeks regardless of the microorganisms inoculated before the treatment. On the contrary, significant changes in the C10:0, C12:0, and C18:0 concentrations in milk inoculated with *S. aureus* and *S.* Typhimurium were observed before storage. The effects of plasma treatment on fatty acid profiles of milk are widely discussed in section 6.2.2.4.

The results of volatile compounds in plasma-treated milk were of great importance to determine the acceptability of the product to the consumer since the shelf-life of the milk is not only determined by the growth of microorganisms but also its flavour. Therefore, the volatile compounds in plasma-treated milk samples inoculated with *E. coli*, *S. aureus* and *S.* Typhimurium were determined. The molecules that were significantly affected by

plasma treatments were ethanol, 2-butenal, nonanal, decanal, benzaldehyde and 2-heptanal which showed a marked increase correlated with the exposure to the plasma in milk samples subjected to a 20-min treatment and then stored under refrigeration. Also significant increases in the contents of styrene and ethylbenzene have been detected particularly in samples inoculated with *S.* Typhimurium. However, these molecules are not related to the pathogens but rather they are reported to be residues of the chemicals used for the packaging material production. The compositions of ketones, aldehydes, and alcohols were widely discussed in section 6.3.2.5. Although, there is lack of studies with non-thermal processing technologies, it was established that storage results in decreased amount of aldehydes in UHT milk stored at 4°C and room temperature [219]. However, our study revealed an increase in the total concentration of aldehytes which is in correlation with the findings of Rerkrai *et al.* [220]. The observed increase in the content of aldehydes in treated samples can be related to the plasma application and particularly to the reactive species such as the positive ion N^{2+} and OH and NO radicals which are produced during the treatments [221]. Another hypothesis is that the long-chain aldehydes and methyl ketones are produced via hydrolytic rather than oxidative mechanisms.

6.4. THE KILLING MECHANISM OF PLASMA ON BACTERIAL CELLS

To gain a better understanding of the killing mechanism of plasma corona discharge, SEM, DNA, and FAME studies have been carried out with gram-positive *S. aureus* and gram-negative *E. coli*. The findings of these studies will give us more insight in order to optimize the plasma corona discharge system for decontamination of milk. Morphological changes in the cells of *E. coli* and *S. aureus* after plasma corona discharge observed under SEM revealed that the cell membrane was destructed and caused spilling out of the cytoplasm. Similar observations were also reported by Laroussi *et al.* [223] and Montie *et al.* [145]. Similarly, other studies have been reported regarding cell injury due the disruption of the cell membrane following exposure to atmospheric pressure plasma discharge [19, 189].

To see if DNA was effected 16S PCR analysis were performed for the bacterial species. Results showed a gradual decrease in the DNA band after 15 minutes of plasma application and precipitated total genomic DNA from *E. coli* was clearly seen as a band

before plasma however after plasma revealed a smear and no DNA band which is indicative of DNA degradation. These results can be explained as the electroporation of microbial cells due to high electric potentials and allowing the free radicals to pass through and interefere with the nucleid acids. The UV radiation formed by plasma disturbes the genetic material of microorganisms by causing thymine dimers in the DNA [157] therefore, inhibiting the reproduction of bacteria. On the contrary, it is stated that UV radiation does not have a big impact on inactivation of microorganisms by atmospheric pressure cold plasmas [158–161]. An explanation for the finding of these studies can be given as the inactivation of microorganisms only occurs within a biocidal wavelength [162]. Hence, if an appreciable dose of UV radiation is not emitted, the inactivation of microorganism occurs due to different agents.

The effects of hydroxyl radicals that are formed during plasma have been widely discussed regarding lipid peroxidation of the fatty acids, unsaturated ones in particular [19, 158]. Upon this point view, it is not surprising that fatty acid profiles of *E. coli* and *S. aureus* fatty acid profiles were not disturbed due to plasma discharge, since both microorganisms had saturated fatty acids leaving no opportunity for the hydroxyl radicals to attach. Generally, straight chain fatty acids are characteristic of *E. coli* membrane and *S. aureus* tents to include branched chain fatty acids [228]. These specific fatty acid compositions play a crucial role in constructing the cell fluidity. Therefore any change in the composition of these fatty acids may lead to disruption of membrane fluidity and subsequently inhibition of the cell activity. The results from fatty acid profiles of *E. coli* and *S. aureus* showed that plasma radicals and active species are not major factors affecting the membrane fatty acids, although other actions may not be ruled out.

7. CONCLUSION

Plasma technology, represented here is non-toxic, safe, and easy to operate and currently applied in different fields, including the medical and textile industries for sterilization and surface modification. Corona discharges cause the formation of short lived, active species which are biocidal to microorganisms yet safe to humans. A novel plasma system was developed, tested and optimized for use as an alternative method for milk decontamination. This relatively cheap technology addressed the disadvantages of other techniques while complying with current consumer and producer demands.

This unique, NTP system has several advantages. Our system uses low temperatures (37°C) and natural air instead of other gases. These natural properties provide a natural alternative to other plasma systems and decreases complications related to the use of heat and gases in food products. The system also works at atmospheric pressure thereby, eliminating the need for expensive high pressure systems. Moreover this system was developed and validated by a collaborative combination of experts, which gave the opportunity to modify the system design in order to optimize the system during testing.

The system was assessed for decontamination properties while retaining milk's beneficial values. The findings of this work established the feasibility of using plasma technology for milk decontamination with minor effects on the nutritional and sensory quality, which will provide both health and economical impacts.

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