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Novel technologies: study for application to fresh dairy sector

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Preface

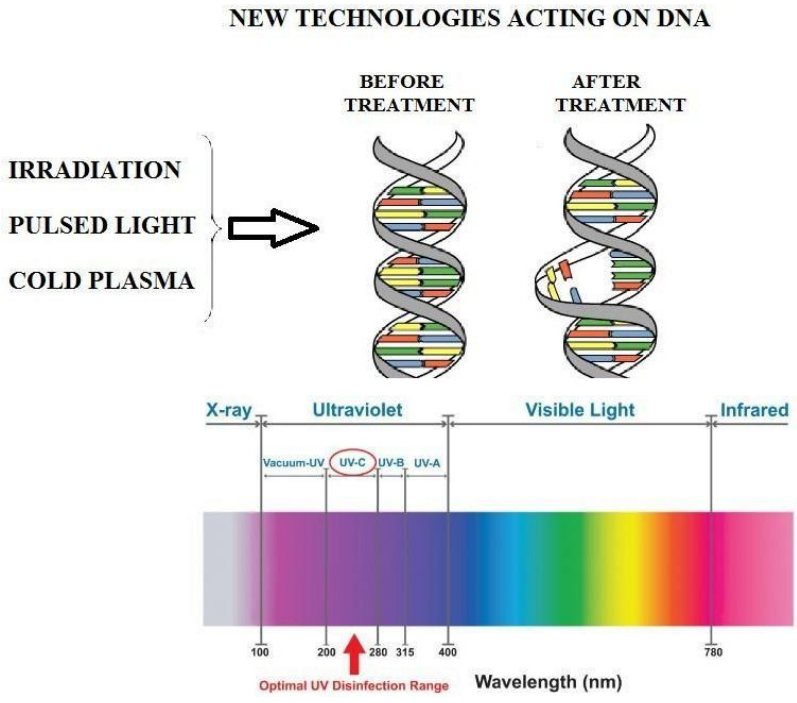
There are currently modern trends for food consumption around the world and these new trends are based on five main categories: sensory and pleasure, healthiness and well-being, comfort and practicality, quality and reliability, sustainability and ethics. Therefore, a food product that aims to participate in the new trend market must respect these categories. Some researchers have set out to develop new products and technologies that give consumers a sensorially pleasing food, which provides wholesomeness and convenience, and a reliable product that features an environmentally friendly manufacturing process. If food waste is always regrettable, from an ethycal point of view, the waste of animal-based food is even worse, from an evironmental sustainability perspective, due to the higher impacts generally related with the production of animal-based products. Modern consumers are now very aware of what they are consuming and want to know more and more how food is produced and the origin of its ingredients (Marcuta, Marcuta and Marza, 2014). In this sense, some non-thermal technologies were studied on ricotta cheese to meet the needs of new trends for food production. Selected non-thermal techniques act on the DNA of microorganisms by inactivating their vital functions and causing cell death (Kalaiselvan, Sugumar and Radhakrishnan, 2018; Lung et al., 2015; Jaiswal, 2016; Delorme et al., 2020; Ziuzina et al., 2014; McDonald et al., 2002). In general, the mechanisms of spoilage vary according to the type of microorganism, but typically include: (1) the production of extracellular enzymes that break down components including proteins, lipids and lactose, producing unpleasant odors, unpleasant taste and body defects; (2) visually detectable growth; (3) production of pigments from bacterial and fungal contaminants; (4) other metabolic processes. When spoilage occurs, it renders all or part of the product inedible and results in a waste of dairy products. Shelf life prolongation of fresh dairy food by mild approaches represents a striking issue for food industry.

Abstract

Ricotta is a potential growth medium for a wide range of microorganisms and it is an easily perishable food, characterized by a short shelf life (2-3 days), even under refrigerated conditions. Heat-based treatments can provoke microbial reduction, but alteration of the chemical and sensory profile can also occur. Therefore, in this Ph-D research alternative non-thermal technologies were tested on artisanal and industrial ricotta cheese to optimize parameters that promote quality preservation. The technologies tested were summarized in the figure 1 reported below (X-rays, Ultraviolet (UV-C) and Near Ultraviolet visible light (NUVL), Pulsed Light (PL) and Cold plasma). Specifically, artisanal and/or industrial ricotta were adopted for testing the effects of the above-mentioned techniques. In the first test artisanal and industrial ricotta samples were irradiated at 0.5, 2.0 and 3.0 kGy. At the two highest intensities (2.0 and 3.0 kGy), the artisanal product remained acceptable for more than 20 days, while the untreated samples became unacceptable after only 3 days of storage. The shelf life of the product irradiated at 0.5 kGy was limited to 14 days, due to the appearance of sensory defects. The industrial product irradiated at all X-rays intensities recorded a significant extension of the shelf life up to 84 days compared to the control which was discarded after 40 days due to sensory defects. As regard PL treatments at increasing fluence (1.3, 3.1, 7.5, 15.0 J/cm^2) it was observed that PL at 1.3 and 3.1 J/cm^2 allowed to delay microbial spoilage during storage, but higher fluences favored microbial growth and odor formation, possibly due to the surface nature of the PL technology. The analyses carried out on the product demonstrated that PL induced the formation of small protein particles, capable of interacting with lipids and carbohydrates and reorganizing into larger aggregates. Aggregation reduced protein solubility and occurred after exposure to hydrophobic protein groups. The photoreaction of proteins was confirmed by the formation of melanoidins and

carbonyls. Ricotta cheese artificially inoculated with *Pseudomonas fluorescens* was also tested by UV-C and NUVL. Results highlighted that the control samples became unacceptable after less than 5 days, while treated samples remained acceptable for more than 6 days. Finally, two different plasma-assisted approaches were assessed to extend the shelf life of artisanal ricotta cheese. In one test the plasma is applied indirectly, with plasma ignited in a controlled atmosphere (91% of N_2 plus 9% of O_2 supply gas) and the gaseous effluents (no plasma) directed to flow through small sterile tubes containing the cheese. In the other test product was directly exposed to the effects of the plasma. Treated and untreated samples were stored at 4 °C for a period of 8 days, during which microbiological, sensory and pH measurements were carried out. The results of the two approaches were similar: the concentrations of viable cells in the treated samples remained lower than those recorded in the control cheese, thus making the product more stable. From a sensorial point of view, the properties of ricotta in treated cheeses have been better preserved. Therefore, also cold plasma was effective in prolonging ricotta cheese shelf life.

Summary picture: non-thermal techniques tested on ricotta cheese in this Ph-D thesis



Chapter 1

Introduction

1.1 Thermal technologies in dairy sector

Milk is a product obtained from the complete, natural and uninterrupted milking of animals in good health, nutrition and in complete lactation. However, this food, from a chemical and physical point of view, is an emulsion of fats in whey. In addition to the lipid fraction, there are proteins (including enzymes), carbohydrates, mineral salts and vitamins (water-soluble and fat-soluble). The chemical compositional structure of milk depends on multiple factors: breed of the animal, age, individual (each animal has a different genetic makeup), state of health, lactation stage, environment, nutrition etc. In table 1.1 the composition of milk in percentage terms is shown (Corradini 1995).

COMPONENTS OF MILK	COW	BUFFALO	GOAT	SHEEP
WATER	87.3 %	82.2 %	87.2 %	81.4 %
SUGAR	4.7 %	4.7 %	4.5 %	4.5 %
FAT	3.8 %	7.5 %	3.8 %	7.4 %
PROTEIN (N x 6.38)	3.3 %	4.8 %	3.6 %	5.8 %
MINERAL SALTS	0.9 %	0.8 %	0.9 %	0.9 %

Table 1.1: shows the variation of milk constituents expressed in percentage terms of four different animals

From a nutritional point of view, milk offers many benefits. One of the main arguments of raw milk advocates is that heating reduces the nutritional value of milk, as milk is a good source of protein (essential amino acids), fats (unsaturated fatty acids), vitamins and minerals. The nutritional value of food depends not only on nutrient content, but also on the bioavailability and contribution of these nutrients to the recommended daily allowance (RDI) (Claeys et al., 2013).

Raw milk has a wide range of microorganisms and it is possible to find bacteria and fungi, including the microorganisms responsible for spoilage. In general, a large number of lactic bacteria such as *Lactococcus*, *Streptococcus* and *Leuconostoc* can be

found in fresh milk as well as many other Gram-positive such as *Bacillus*, *Microbacterium*, *Micrococcus* and *Staphylococcus*. Gram-negative bacteria belonging to the genera *Pseudomonas, Aeromonas, Acinetobacter, Stenotrophomonas* and *Chryseobacterium*, as well as numerous *Enterobacteriaceae* such as *Enterobacter, Hafnia and Klebsiella* are frequently found in raw milk. Same thing is also valid for the presence of some vievites such as *Candida, Kluyveromyces* and *Pichia* (Von Neubeck et at., 2015).

The risks associated with the consumption of raw milk are mainly microbiological in nature and raw cow's milk does not really present any nutritional risks. Among the pathogenic microorganisms for humans, bacteria are particularly interesting (*Salmonella spp., Brucella abortus, Mycobacterium bovis, Coxiella burnetii, Mycobacterium avium subsp. Paratuberculosis, Listeria monocytogenes, E. coli O157: H7, Campylobacter coli* and *jejuni, Yersinia enteroclolitica, Bacillus cereus*, Enterotoxin-producing *Staphylococcus aureus, Arcanobacter pyogenes, Streptococcus zooepidemicus* and *Leptospira*) and viruses (Rift Valley fever virus and Tick-borne encephalitis complex virus (TBE), of which the 'Central Europe), but the possible presence of the parasite *Cryptosporidium parvum*, in addition to type B toxins synthesized by *Clostridium botulinum*, can also be dangerous. Regarding the impact on public health, the development of a disease after the consumption of raw (contaminated) milk depends on number of factors, such as the pathogenicity of the microorganism (or the toxicity of the toxin), the number of microorganisms ingested (or quantity of toxins), the human infectious dose and the state of health of the consumer (D'Aoust, 1989; Lund and O'Brien, 2011). The people most at risk are the very young, the elderly, pregnant women and immune-compromised people (YOPI), although anyone can be affected, including healthy young adults (Griffiths, 2010). The consequences of a milk infection may be limited to the common symptoms of diarrhea, vomiting, nausea, fever, abdominal cramps, etc., but a certain percentage of people may develop more severe clinical symptoms such as Guillain-Barré syndrome (*Campylobacter spp*.) and hemolytic uremic syndrome (HUS) (*E. coli O157: H7*) or

long-term and sometimes chronic complications, such as reactive arthritis or even death. Assuming a severity score on a scale of 1 to 4 (with 4 for the most severe effect) for pathogens frequently found in raw milk-related outbreaks, a score of 3, 3, 4 and 4 can be assigned to *Campylobacter spp*., *Salmonella spp*., Human pathogenic *E. coli* and *L. monocytogenes*, respectively (Claeys et al., 2013).

The main objective of pasteurization of milk for cheese making is the elimination of pathogens that may be present in milk. Regardless of post-pasteurization contamination, the growth of milk pathogens is highly dependent on the type of cheese and the technology involved. It is well documented that pathogens grow more readily in cheeses with high humidity, high pH and low salt content, compared to long-aged cooked cheeses. For hygienic reasons, most cheeses are made with pasteurized milk; however, with a production of 700,000 tons per year, raw milk represents a significant share of the mature cheeses produced in Europe, particularly in Italy, France and Switzerland. In the context of trade and international regulation, where hygiene aspects are predominant (Grappin and Beuvier, 1997).

In the dairy industry, pasteurization and sterilization methods are applied for heat treatment. Pasteurization is a heat treatment applied to destroy harmful microorganisms in milk (Tamime, 2009). Milk must also show a negative alkaline phosphatase reaction after pasteurization treatment (Ritota et al., 2017). Pasteurized milk must be refrigerated immediately after heat treatment. Pasteurization methods include long-term low-temperature pasteurization (LTLT) (63 °C for 30 min) and short-term high-temperature pasteurization (HTST) (72 °C for 15 s) (Tiamine, 2009). Correctly applied pasteurization (i.e. 71-74 °C/15-40 s for "short period at high temperature" or HTST pasteurization) eliminates all vegetative microorganisms present in the milk (i.e. their probability of survival is reduced by a factor 10^6), including vegetative pathogens such as human pathogenic verocytotoxic *E. coli*, *Salmonella spp., L. monocytogenes, Y. enterocolitica, C. jejuni / E. coli*, enterotoxin producing *S. aureus* and vegetative *C. botulinum*. Already formed and heat-resistant

enterotoxins from *S. aureus* and the B toxin of *C. botulinum* as well as the emetic (cereulide) toxins of *B. cereus* are not destroyed by pasteurization. Pasteurization does not destroy either the heat resistant spores of *C. botulinum* or *B. cereus*. On the contrary, it can induce the germination of these spores, which are subsequently able to grow and produce toxins during the storage of pasteurized milk (Claeys et al., 2013).

Sterilization allows the milk to be kept at room temperature for several months. Milk can be sterilized using UHT (Ultra Hight Temperature) processing and bottle sterilization (Tamime, 2009). The purpose of the UHT processing plant is to heat the product to the sterilization temperature (between 135 and 150 °C), hold it there for a few seconds and then cool it to a suitable filling temperature. There are two main technologies that are distinguished by the medium used for heating to UHT systems, direct and indirect. Steam, hot water and electricity are heating methods for UHT equipment. Sterilizers that use steam or hot water can be sub-categorized as direct or indirect heating systems. In the indirect system, the product and the heating medium have no contact, as there is a barrier (stainless steel). Direct heating modes include steam injection, steam infusion and scraped surface. Direct heating systems include steam injection (steam into milk) and steam infusion (milk into steam). The culinary steam must be of high quality and must not impart unpleasant flavors to the dairy product. The temperature of the product rises almost instantly due to the latent heat of vaporization. The condensed vapor that dilutes the milk is removed later as the heated milk is cooled in a vacuum chamber (Chavan et al., 2011). Sterilization (110-120 °C/10-20 min) and UHT (135-140 °C/6-10 s for indirect and 140-150 °C/2-4 s for direct UHT) treatments destroy vegetative pathogens and most sporulating pathogens (including the spores of *C. botulinum* and *B. cereus*, but with the exception of the spores of some very heat-resistant non-pathogenic bacilli such as *Bacillus thermodurans*), thus offering in most cases a so-called commercially sterile product (minimum one log reduction of 12). *S. aureus* and *C. botulinum* toxins and *B. cereus* enterotoxins are also destroyed. The emetic toxin of *B. cereus* is very heat-resistant,

but has never been shown to be present in raw milk (Claeys et al., 2013). The main purpose is to inactivate heat resistant spores, thus producing a commercially sterile product with a long shelf life. Although these heat treatment methods can kill or control microorganisms, they can also induce the Maillard reaction, which generates hazardous substances (Boitz and Mayer, 2015). Thermisation (57-68 °C/15-20 s), which is mainly a pre-treatment performed for technological reasons only to extend the shelf life of the refrigerated milk, results in a 3-4 log reduction of the commensal vegetative flora (i.e. *Micrococcus, Coliforms, Pseudomonas, Flavobacterium, Enterobacter, Aeromonas, Alcaligenes*, etc.), but does not guarantee the inactivation of all vegetative pathogens (Clayes et al., 2013).

1.1.1 Effects of thermal technologies

Heating affects the quality and technological properties of milk have suggested that this group of effects includes the degradation of lactose to organic acids and the formation of lactulose, the denaturation of whey proteins, the destruction of vitamins and enzymes, protein and lipid hydrolysis and calcium/phosphorus balance disturbance (Pestana, et al., 2015), decreased coagulation capacity, changes in the structure of the fatty globule membrane.

1.1.2 Formation of lactulose

The disaccharide lactulose, consisting of 2 monomers galactose and fructose, is formed by isomerization of lactose only during the heating of milk (figure 1.1) and can help to evaluate the severity of the thermal process (Olano and Calvo, 1989). The International Dairy Federation (IDF) suggests a lactulose range of 100-600 mg/L as a feature of the UHT process. Values below 100 mg/L are related to pasteurization and above 600 mg/L to the sterilization process (Mortier et al., 2000).

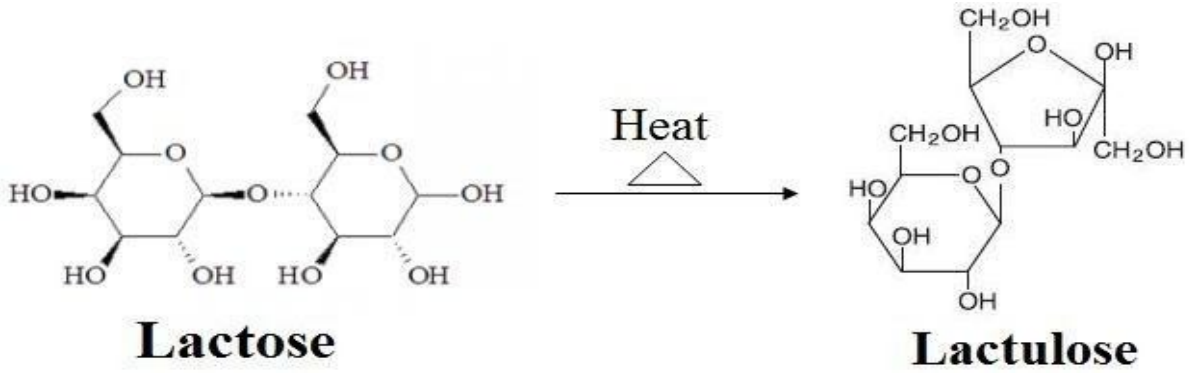


Figure 1.1: formation of lactose after heat treatment of milk

1.1.3 Denaturation of whey proteins and reduction of the coagulation capacity of caseins

Denatured whey proteins form complexes with other whey proteins, caseins and fat globules (Dunkley and Stevenson, 1987). The amount of β-lactoglobulin associated with micelle casein increases with heating time and the trend is similar for α-lactalbumin but to a lesser extent. A-Lactalbumin can interact with k-casein only in the presence of β-lactoglobulin, possibly through the initial formation of α-lactalbumin/β-lactoglobulin aggregates, which then interact with k-casein (Elfagm and Wheelock, 1978). The stability of casein micelles increases with the concentration of k-casein and the presence of colloidal calcium phosphate. Better stability conditions are found in smaller micelles. Thanks to the help of heat, the breakage of peptide bonds and de-phosphorylation phenomena occurs, while due to an increase in acidity the "Calcium-Phosphorus" balance is shifted between casein sub micelles and therefore the fraction of casein increases soluble and micellar, due to heat, the interaction between k-casein and αs_2-casein with β-lactoglobulin occurs. Furthermore, hydrophobic interactions occur which lead to the formation of a whole series of molecular complexes "denatured calcium phosphocaseinate/whey protein" which modify the rheological and gelling properties of milk. If the milk is heat treated instantly (by direct heating), all whey protein begins to unfold at the same

time and this gives a greater opportunity for the deployment of monomers to aggregate and consequently the attachment to casein micelles will be less efficient (Oldfield et al., 1998). The association with casein micelles depends on the pH and decreases with increasing pH (Skelte and Yuming, 2003). Whey proteins, due to their molecular structure, can denature themselves even at low temperatures and the heat stability is strictly dependent on the presence of cysteine in the primary structure of these proteins. Due to the heat there is a conformational change of these polypeptides, breaking of intramolecular sulphide bridges between cysteine residues with release of H_2S (with production of reactive sulfhydryl groups in the liquid medium, which have a direct correlation with the appearance of the cooked taste) and the formation of intermolecular sulphide bridges, thus leading to the formation of polymers between different protein structures, which, having reached a certain size, tend to precipitate. Due to a free thiol group, β-lactoglobulin is the most thermolabile serum protein, manifesting an irreversible state of denaturation already at 70 °C.

1.1.4 Changes in the structure of fat globules

The milk heating processes cause changes in the membranes of the fat globule and this leads to a reduction in the diameter of the latter and the transfer of triglycerides into the external medium with consequent rancidity. Even mild heat treatments such as a treatment performed at a temperature of 80 °C for a time ranging from 2 to 20 minutes generates changes in the structure of the membrane with the incorporation of serum proteins, in particular β-lactoglobulin. In addition to heat, mechanical stresses occurring at high temperatures can also affect the stability of the membrane.

1.1.5 Maillard reaction

The Maillard reaction (Fig. 1.2) comprises a series of complex reactions between reducing sugars and amino acids or proteins and can be divided into early, intermediate and advanced stages. A commonly accepted path of progress/evolution of the Maillard reaction in milk is shown by various authors (Chavez-Servin et al., 2005, Sunds et al., 2018). Important stages of the Maillard reaction can be identified by indicators. For example, Nε-(2-furoylmethyl)-L-lysine (furosine) and furfurals (5-(hydroxymethyl) furfural (HMF) and 2-Furaldehyde (F)) are the products of the initial and intermediate stage of the Maillard reaction, respectively. Their presence can be used to evaluate heat treatment in milk (Ritota et al., 2017). Furosine is often used as a marker to assess protein damage following over-processing and has been used in the control of dairy product processing (Schmidt, Boitz and Mayer, 2017). Furthermore, furosin has been one of the key indicators for the discrimination of reconstituted milk (Liu et al., 2020). HMF can be generated by the fragmentation of sugars and the degradation of Amadori products. As an indicator for evaluating the intermediate steps of the Maillard reaction in processed foods, HMF can be classified as free and potential HMF (protein-bound: lactulosyl-lysine, 1-2 enolysed products, etc.) (Ritota et al., 2017). The end products of advanced glycation (AGE), such as Nε-(carboxymethyl) lysine (CML) and Nε-(carboxyethyl) lysine (CEL), are generally considered products of the advanced stage of the Maillard reaction (Erbersdobler and Somoza, 2007), and have been reported in UHT milk during storage (Zhang et al., 2019). These comounds are commonly used as markers to indicate the extent of Maillard reactions (Dittrich et al., 2006). Furthermore, HTST and UHT milks exhibit different levels of warm flavors, which reflect different degrees of Maillard reaction during milk processing (Gandy et al., 2008).

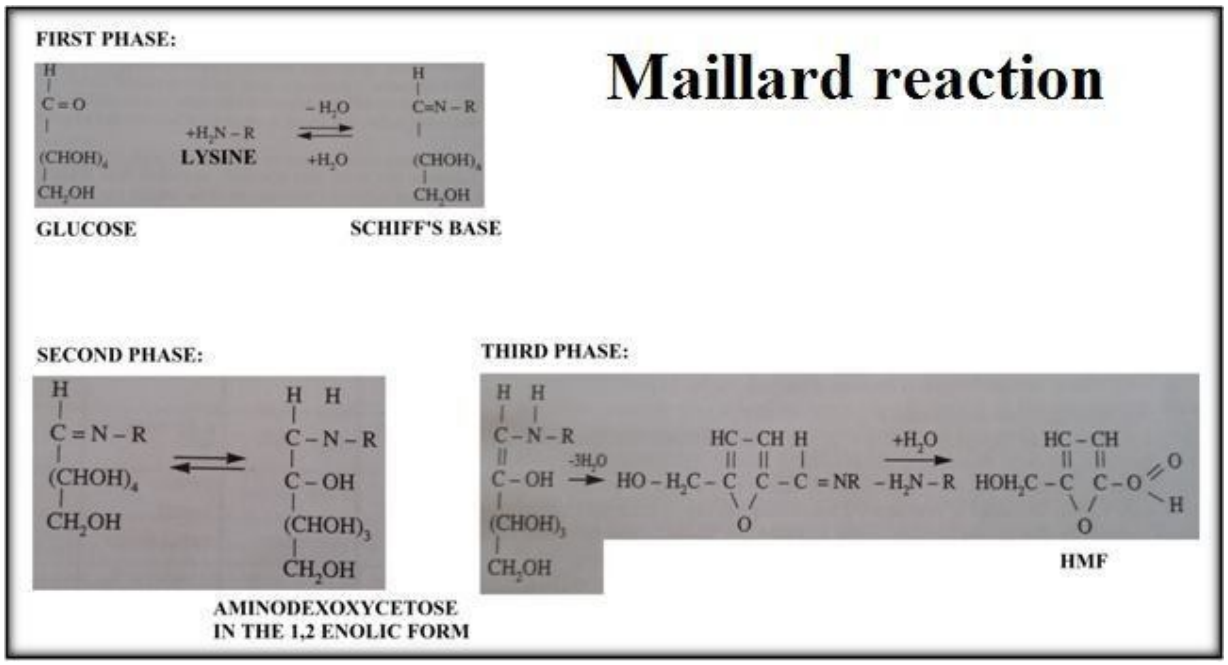


Figure 1.2: Maillard reaction divided into its 3 steps, based on the intensity of the heat treatment

1.1.6 Decreased coagulation ability

When a milk is subjected to a heat treatment, the loss of the ability to coagulation occurs precisely following the formation of casein /whey protein systems. The action of the rennet proteases on milk caseins is not completely inhibited, but it is strictly dependent on the severity of the heat treatment undergone and this leads to an increase in the coagulation time and formation of clots with poor rheological properties, precisely because it increases its consistency and decreases the act of syneresis for the expulsion of the serum. Therefore, when a milk must be destined for a dairy processing act, in order not to severely damage the characteristics of the curd, it must be limited to the thermal treatment of thermisation. Other phenomena that contribute to curd coagulation are the formation of complex micelles, the reduction of Ca^{2+} in ionic form and the deposit action of the whey proteins on the surface of the casein micelles, generating a steric hindrance to the enzymatic action of the rennet proteins (fig. 1.3).

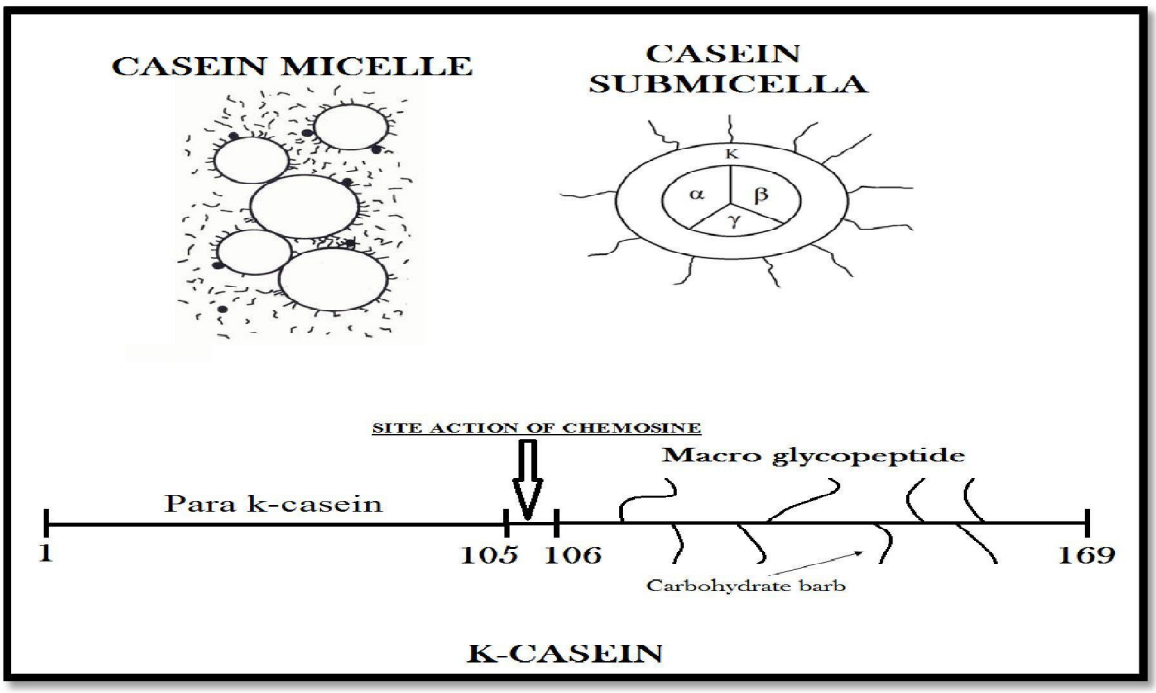


Figure 1.3: structure of a casein micelle made up of many subunits called submicells. Each submicella has a central core where α, β e γ casein intersect, while the k-casein envelops this central structure with the carbohydrate beards facing outwards. The steric hindrance by denatured whey proteins occurs at the level of amino acid 105-106 where it hinders the action of chymosin.

1.2 Non-thermal technologies

The term "emerging technologies or non-thermal technologies" can define all those technologies that are currently being developed by researchers in the near future (after 5 or 10 years), or already developed and marketed. These new technologies offer an interesting alternative to normal heat-based treatments because, in addition to food safety and quality, they offer the possibility of shortening treatment times and reducing both energy consumption and carbon dioxide emissions. The effectiveness of these technologies, in compliance with the quality of the treated product, depends on the type of food and treatment parameters (treatment time, treatment intensity, etc.). This has entailed a continuous and accurate study in the different areas of application of these methods. Below, some details for the most common non-themal processes was given.

1.2.1 Irradiation: X-rays, γ-rays and electron beam

Irradiation can be defined as non-thermal process in which the foods subjected to treatment record a clear reduction in the altering and pathogenic microflora due to the penetrating power of the radiation, with a consequent shelf life increase. Changes in food after irradiation, including nutritional effects, are generally acceptable (Ravindran and Jaiswal, 2019). Among the new technologies, ionizing radiation shows a remarkable ability to reduce the microbial load in food (Lacivita et al., 2019). Technologies based on ionizing radiation are basically represented by X-rays (≤7.5 MeV in some countries and ≤5 MeV in others), electron beams (≤10 MeV) and gamma radiation (γ-rays, generated using radionuclides such as ^{60}Co or ^{137}Cs, but this latter isotope is not taken into consideration in the food sector because it is soluble in water) (Lung et al., 2015). The γ-rays are high-energy photons produced by the disintegration of radioactive isotopes. The isotopes ^{60}Co and ^{137}Cs are commonly used in food. The disintegration process (Co^{60}) begins with the production of an unstable intermediate isotope (^{60}Ni) which emits beta radiation and further

disintegrates into a stable isotope (^{60}Ni) by emitting two gamma rays. Due to the high energy and low initial cost, ^{60}Co is preferred over ^{137}Ce for the irradiation process. ^{60}Co irradiators require frequent refills with fresh isotopes due to the short half-life and an overdose of treatment can damage the food matrix making it unacceptable for consumption, while an inadequate dose will fail to achieve the desired effects. Dosimetry is the method of measuring the amount of radiation dose absorbed by food and the quality of the irradiated product depends on the quality of the dosimetry. First, you need to: (a) develop the appropriate dose for the food product being researched, (b) obtain the data for putting the food into service via the regulatory agency, and (c) establish quality control procedures in the food manufacturing plant. A distinction must be made between terminologies such as: radiation, rooting and recappertization:

- Radiation is defined as the process of reducing the population of organisms and delaying the onset of deterioration by exposing food to ionizing radiation. It is also known as radiation pasteurization. The dosage range is 75-250 krad.
- Radicidation is the process of exposing food to ionizing radiation to kill all non-spore-forming pathogens and parasites. It is generally used for the sanitization of frozen products. The dose range is 250-1000 krad.
- Radappertization is also known as radiation sterilization, defined as exposing food to the necessary dosage of ionizing radiation to hermetically kill all sealed-packaged food spoilage organisms. Doses are greater than 1 Mrad (generally 3.5 Mrad) (Kalaiselvan, Sugumar and Radhakrishnan, 2018).

Electron beam irradiation is a process of generating electrons from a cathode in a vacuum environment from commercial electricity. The electrons are then activated or pulsed by an electronic gum in sequence, creating a beam of electrons. The pulsed electron beam is carried across a radiofrequency wavelength in the linear accelerator, which has positively and negatively charged cavities that increase the velocity of the beam as it travels through the radiofrequency waveform through the accelerator. The

speed of the electron is increased to 99.99% of the speed of light at energies not exceeding 10 MeV, which are capable of breaking molecular or atomic bonds by releasing free electrons and ions that react with additional particles, charged molecules or atoms, to release secondary ions. At the end of the 1950s, accelerator technology matured, with lower equipment production costs; therefore, this technology could be used for the purposes of food irradiation. Electron accelerators appear to be more successful than γ-rays due to the following advantages: (1) source (machine) which can suspend irradiation at any time; (2) non-nuclear energy which can accelerate the generation of radiation when needed; (3) little risk of accidents at work; and (4) applicability in high-flux and high-dose irradiation. In recent years, electron beam technology has been used in the food industry (for decontamination and disinfection), pharmaceutical (disinfection of disposable medical equipment) and chemical (crosslinking and polymerization of polyethylene and polypropylene), hospital and medical applications (radiotherapy and brachytherapy) and environmental applications in various sectors (sludge disinfection and pollution monitoring and control). However, the main disadvantage is the problematic low penetrability of the electron beam. The decontamination effect can be influenced by size, thickness, direction (one or two-sided exposure) and packaging of the food. The treatment is particularly effective in low density and evenly packaged foods. The effect on low density polyethylene (LDPE)/polyamide films was also evaluated and a dose of 3 kGy was observed to cause small or negligible changes in the functionality of the films, but did not alter the inhibitory effect on *Listeria harmless* and *Escherichia coli* (Lung et al., 2015).

X-rays are generated inside a system when a high-energy electron beam is fired at a target (usually tungsten), once the electrons reach this metal structure the atoms that constitute it are excited and emit an electron, it follows that an electron from the upper orbital of the atom immediately falls into the lower energy level releasing energy (photon). There are two fundamental factors for the working mechanism of X-rays on which penetration depends, energy and current. The current (expressed in

mA) is strictly related to the number of photons generated as a result of the treatment, and the energy refers to that of the photon as it exits the tube (Haff and Toyofuku, 2008). When a food is subjected to the radiating action of X-rays, a phase of ionization of the atoms in the food matrix occurs when the latter reacts with photons. This ionization strictly depends on the chemical and physical properties of the irradiated food and on the intensity of irradiation that is used to sanitize the product. Two different energy transfer processes can occur: photoelectric absorption and Compton dispersion. Photoelectric absorption occurs when low-energy photons are used, in this case all the energy of the photon is completely absorbed by the target electron, which in turn can interact with the other atoms. Compton scattering occurs when at high energy levels the electrons are targeted on the outer orbit, in this case the same photon and the ions (anions and cations) generated by the interaction (hence the term ionizing radiation), can continue to interact with other atoms that make up food. The activity of water in food is a fundamental element for obtaining a good efficacy of X-ray irradiation treatment because its presence favors the formation of highly reactive free radicals such as hydrogen and hydroxide (without the hydrogen bond). These excited water molecules generate further reactions up to the production of stable products from the water radiolysis process, which are hydrogen peroxide and hydrogen (Moosekian et al., 2012). Therefore, based on the principles mentioned above, the sanitizing activity of this technology can be divided into 2 effects: primary effect and secondary effects. The primary effect is when the electrons that are released following the interaction with photons stimulate other nearby atoms favoring the formation of ions. Side effects occur when these ions generated as result of primary reactions react with other molecules to form highly reactive components such as free radicals, electrons and ions (Miller, 2005). The effects of radiolysis depend on the dose of adsorbed energy per unit of mass. The unit of measurement that defines the adsorbed dose is the Gray (Gy), which corresponds to the unit value of 1 J/Kg, even if the unit of measurement is expressed in "adsorbed radiation dose" (rda): 1 rad = 0.01 Gy (Miller 2005). In many cases the shelf life of a food is closely

related to microbial inactivation. X-rays cause damage to cellular structure including DNA, killing the cell and rendering it unable to replicate and exert its biological function in food. In percentage terms, 20% of the attack occurs on the sugars that make up the genetic material, and the rest occurs on nitrogenous bases. Due to the orientation of the DNA in the cell, double-strand damage is more difficult (Jaiswal, 2016). During an ionizing treatment, most DNA strand breaks are due to the cleavage of the C-3 phosphodiester bond, which produces 5-PO_4 and 3-PO_4 (ratio 3:1) (Johnston and Stevenson 1990). In addition to DNA, harmful effects on proteins and membranes can occur, compromising the life of the microorganism (Ravindran and Jaiswal, 2019). After an irradiating treatment, the potential survival of microorganisms depends on many factors, such as the chemical compositional structure of the food in which the microorganisms are present, the temperature, the pH and the intensity of the treatment. Spores are generally more resistant than vegetative cells, although greater resistance to radiation treatment is demonstrated by yeasts, molds and viruses (Monk et al., 1994). However, microbial species able to resist the action of ionizing radiation treatments have been described, an example is *Deinococcus radiodurans*, which, through its proteomic system, is able to repair damaged DNA following radiation treatment. This microorganism is able to withstand up to a treatment intensity of 30 kGy (Ravindran and Jaiswal, 2019). X-rays have the ability to pass through thick materials (about 30-40 cm) and this feature makes them suitable for the treatment of pre-packaged foods, avoiding recontamination of the product. X-ray application should never exceed the recommended dose of 10 kGy (Ricciardi et al., 2019).

1.2.2 UV rays: UV-A, UV-B and UV-C

Ultraviolet light refers to a band that exists between the X-rays (200 nm) and the visible light region (400 nm) in the electromagnetic spectrum. It is usually classified

into long wavelength UV-A (320-400 nm), medium wavelength UV-B (280-320 nm) and short wavelength UV-C (200-280 nm). The sun beam contains all the UV light, but only UV-A and UV-B can reach the surface of the earth, while the UV-C is completely absorbed by the ozone layer and reaches the earth without any dose (Urban et al., 2016).

The inhibitory effect of UV rays on microorganisms has long been elucidated (Bintsis et al., 2000). The mode of action of UVs has been attributed to the photochemical transformation of pyrimidine bases that produce bonds between successive pyrimidines on a DNA strand to form dimers (McDonald et al., 2000). The resulting effect is that DNA transcription and replication are blocked, compromising cell functions and ultimately leading to cell death (Guerrero-Beltran and Barbosa-Canovas, 2004). UV-C radiation is a non-thermal, non-chemical intervention technology that uses physical energy and is considered safe and non-toxic. It is a technique that requires low investment and maintenance costs compared to other conservation methods and is environmentally friendly as it does not cause environmental impacts. Commercial ultraviolet equipment was mainly produced for the pharmaceutical and aquaculture industries, which could not tolerate chemical disinfection and interest in use by the food industries, its use subsequently emerged for the purpose to prevent negative effects of heat in the processing of food matrices. The treatment process consists in the application of UV light at short wavelengths, in the range of 200-280 nm, which is capable of destroying the DNA of microorganisms, altering their metabolism and reproduction, leading to cell death. Choosing the right UV source is extremely important as it can increase the efficiency of microbial inactivation by increasing the penetration of UV light into food. Commercially available UV sources include low and medium pressure mercury lamps (LPM and MPM), pulsed light (PL) and light emitting diodes (LEDs). According to the FDA for germicidal applications, the most used sources are the discharges of mercury vapors. Of these, low pressure sources are generally the most favored for most applications (Delorme et al., 2020). Anderson et al. (2000) and

Rowan et al. (1999) reported the following susceptibility trend in descending order: Gram-negative bacteria, Gram-positive bacteria and fungal spores. Spore color can play a significant role in susceptibility to fungal spores. *Aspergillus niger* spores are more resistant than *Fusarium culmorum* spores, this can be explained because the pigment of *A. niger* spore absorbs more in the UV-C region than that of *F. culmorum* spores, protecting the spores from UV rays (Anderson et al., 2000). In contrast, Gómez-López et al. (2005) did not observe any pattern of sensitivity among different groups of microorganisms, after studying 27 species of bacteria, yeasts and molds. Xenon flash lamps have an emission spectrum ranging from ultraviolet to infrared light. The UV-C part of the spectrum is the most important for microbial inactivation. Rowan et al. (1999) reported that inactivation of food-related microorganisms (*Bacillus cereus, Listeria monocytogenes, Staphylococcus aureus, Escherichia coli, Salmonella Enteriditis, Pseudomonas aureginosa,* and *Saccharomyces cerevisiae*) was 5-6 log CFU/plate using high UV flash, while with low UV light only 1-2 log CFU/plate was obtained. Using a monochromator, Wang, MacGregor, Anderson and Woolsey (2005) determined the germicidal efficiency against *E. coli* as function of wavelength in the 230-360 nm range using approximately 6 mJ/cm^2. The results showed a maximum inactivation around 270 nm and no measurable inactivation was observed above 300 nm. Furthermore, the authors concluded that the rich UV content of 220 to 290 nm in the UV spectrum provides the greatest contribution to inactivation, whatever the type of UV source used.

1.2.3 Pulsed Light (PL)

Pulsed UV light is a modified and enhanced version of UV-C light, which uses devices containing lamps that emit high-powered ultraviolet light at regular intervals, using extremely short times (1 μs - 0.1 s) in the range of 200 - 1100 nm (Delorma et al., 2020). The technique used to produce the flashes results, in addition to a high

peak power, a greater relative production of light with shorter bactericidal wavelengths. Considerable research has been conducted on the mechanism of microbial inactivation by pulsed light. The lethal action of PL may be due to a photothermal and/or photochemical mechanism. It's possible that both mechanisms coexist and the relative importance of each would depend on the fluence and the target organism. Most authors explain their results based on the photochemical effect. For example, since Rowan et al. (1999) achieved inactivation with a temperature rise below 1 °C, concluded that lethality can be attributed to the photochemical action of the shorter UV wavelengths. Compared to continuous ultraviolet (UV) light, this pulsed light technology is able to inactivate bacteria, fungi and viruses more quickly and effectively than continuous UV treatment (Cheigh et al., 2013). In fact, it has been shown that PL, besides being more efficient, acts differently from continuous UV irradiation, due to the high peak power and short treatment duration of PL (Kramer, Wunderlich and Muranyi, 2015).

Xenon lamps can produce flashes several times per second. The following units are commonly used to characterize a PL treatment:

- Fluence rate: it is measured in Watt/meter^2 (W/m^2) and it is the energy received from the lamp by the sample per unit area per second.
- Fluence: it is measured in Joule/meter^2 (J/m^2) and is the energy received from the lamp by the sample per unit area during the treatment.
- Dose: it is used sometimes as a synonym of fluence.
- Exposure time: length in time (seconds) of the treatment.
- Pulse width: time interval (fractions of seconds) during which energy is delivered.
- Pulse-repetition-rate (prr): number of pulses per second (Hertz [Hz]) or commonly expressed as pps (pulses per second).
- Peak power: it is measured in Watt (W) and it is the pulse energy divided by the pulse duration (Gomez-Lopez et al., 2007).

The mechanism of microbial inactivation of PL is often explained on the basis of studies using UV, in which the inactivation is photochemical. Although the inactivation mechanism by PL may have similarities to that of UV light, there may be some differences. The germicidal effect of UV light on bacteria is mainly due to the formation of pyrimidine dimers, mainly thymine dimers (Giese and Darby, 2000). The dimer inhibits the formation of new DNA chains in the process of cell replication, thus leading to the inactivation (inability to replicate, called clonogenic death) of microorganisms affected by UV (Bolton and Linden, 2003). On bacterial spores, UV-C treatment mainly results in the formation of the "spore photoproduct" 5-tminyl-5,6-dihydrotimine, and in single strand breaks, double strand breaks and pyrimidine dimers of cyclobutane (Slieman and Nicholson, 2000).

Comparing the wavelength sensitivity for *E. coli* inactivation with previously reported absorption spectra of purine and pyrimidine bases of DNA, Wang et al. (2005) supported the hypothesis that the photochemical effect produced as a consequence of UV absorption by DNA is the main cause of inactivation of microorganisms by PL. However, there is evidence that a photo-thermal effect can also occur. Literature explained the inactivation of microorganisms with PL by both mechanisms, pointing out that the pulses of light heat a surface layer of food in such a way that the heat produced on the surface is eventually conducted into the product. However, the total amount of heat produced may be small compared to the amount of heat that would be required to substantially raise the temperature of the entire product. Wekhof (2000) has proposed that with a fluence greater than 0.5 J/cm^2, disinfection is achieved by breaking down bacteria during their epochal overheating caused by the absorption of all UV light by a flash lamp. Subsequently, Wekhof, Trompeter and Franken (2001) provided evidence for this hypothesis by showing electron microscope photographs of spores belonging to *A. Niger* flashing with PL, which exhibit severe deformation and rupture. The rupture of the top of a spore was presented as evidence of its superheated contents leaking.

1.2.4 Near Ultraviolet Visible Light (NUVL)

An alternative approach to kill topical bacteria is lethal photosensitization (photodynamic therapy - PDT). It has been found that reactive oxygen species (ROS), mainly singlet oxygen ($^1O_2$), generated by the illuminated photosensitizer introduced into the bacteria, cause lethal effects. This photochemical treatment is used successfully in vitro and however, its main drawback is the difficulty of introducing photosensitizers into some bacteria. It should also be noted that the introduction of photosensitizers into the body is a dangerous procedure, as it sensitizes not only the pathogens but also the surrounding mammalian cells. Hence, light in the visible field generates ROS including singlet oxygen, in living cells following its uptake by endogenous cellular photosensitizers such as flavins, cytochromes (porphyrins) and NADH + H^+. Endogenous cellular photosensitizers have wide absorption bands over the entire visible range, especially in the blue region, and bacteria also possess endogenous photosensitizers. Previous studies have shown that the low fluences of argon laser irradiation (488-514 nm) exert a phototoxic effect on *Porphyromonas* and *Prevotella spp*. Both are gram-negative anaerobic bacteria that produce porphyrins (Lipovsky et al., 2009). The observation that visible light alone has bactericidal properties has previously been documented for bacteria, particularly the acne-associated bacterium *Propionibacterium acnes*. Research on *P. acnes* has found that irradiation of this organism with blue light leads to photosensitization of intracellular porphyrins, the stimulation of which leads to the production of reactive species, mainly delta oxygen singlet and, consequently, to cell death. Other bacteria that have been found susceptible to inactivation solely through exposure to visible light include *Helicobacter pylori* and some oral bacteria. NUVLhas been used to study the bactericidal properties of high intensity, narrow band wavelengths of visible light against *Staphilococcus aureus* and other bacterial pathogens and it has been shown that the *S. aureus* bacterium can be inactivated using super bright diodes, using the visible light without the use of exogenous photosensitizers or δ-ALA-induced porphyrins. Studies show that blue light between 400 and 420 nm, but optimally 405

nm (± 5 nm), has bactericidal effects against *S. aureus* (Maclean et al., 2008). Although the germicidal efficiency may be lower than UV, visible light at a wavelength of 400 nm is considered much safer alternative. While UV light affects DNA, the mechanism of inactivation by visible light is from an oxygen-dependent process credited to photostimulation of exogenous porphyrin molecules that cause energy transfer and production of ROS that are bactericidal. To date, only a single study has investigated the effect of high intensity light at 405 nm on *Campylobacter jejuni*, which was reported to be significantly more sensitive than *Escherichia coli O157: H7* and which required up to 16 times the dose applied to *C jejuni* to achieve a reduction of 5 log CFU/mL when suspended in buffered peptone water. The apparent susceptibility of *C. jejuni* to visible light requires further research on its potential applications for the control of this organism (Haughton et al., 2012).

1.2.5 Cold Plasma (CP)

The term "plasma" has been described as the fourth state of matter and it refers to an almost neutral ionized gas, containing a mixture of electrons, ions, atomic species, UV photons and charged particles (Li and Farid, 2016; Niemira, 2012). The activation of food pathogens by cold plasma is not based on thermal killing as traditional techniques (Niemira, 2012). The atmospheric plasma process consists in the generation of reactive molecular species from electrical discharges to ionize a given gas. The mechanisms by which the cold plasma inactive microorganisms are three: one) direct chemical interaction of cells with reactive species and charged particles; (2) UV damage of cellular components and membranes; (3) UV-mediated DNA strand break. The microbial inactivation does not occur thanks to the help of heat (room temperature processes) and only a negligible part can be attributed to UV-light produced by the glow discharge (Hong et al., 2009). The effectiveness of cold plasma inactivation depends on the composition of the supply gas, voltage, treatment

time and relative humidity (Li and Farid, 2016; Ziuzina et al., 2014). The most part of the bacterial inactivation is attributed rather to the generation of reactive species of oxygen and nitrogen generated in the pure gases or in air feeding the electrical discharges, with hydroxyl radicals, singlet oxygen and hydrogen peroxide being the main agents involved (Surowsky et al., 2014). There is a growing body of literature that recognizes the importance of reactive oxygen species (i.e. H_2O_2, O_2^-, OH) for inactivation of microorganisms. However, very little attention has been paid to the role of reactive nitrogen species (RNS) in deactivation of bacteria (i.e. NO, NO_2^-, NO_3^-) that in principle can rapidly and easily diffuse into the cells, even when the membrane is intact, and oxidize many macromolecules (Joshi et al., 2011). As reported in the literature, CP was applied to foods with effects that are strictly dependent on the matrix to be considered (Coutinho et al., 2018). The specific applications of CP to dairy products are still limited (Lee et al., 2012; Young et al., 2015a; 2015b), but the results recorded to date are noteworthy and encourage a deep investigation on other fresh perishable products such as artisanal ricotta cheese. The efficacy/efficiency expressed in terms of microbial inactivation depends not only on the gas composition, but also on the treatment time, on the initial conditions of relative humidity and on the voltage. The microbial inactivation phase basically takes place by direct contact of the microbial cells with the reactive components generated and also by damage to cell membranes and endo-cellular components (enzymes and other proteins), that compromise the cell vitality, including genetic material (Hernàndez-Hernàndez et al., 2019). The advantages of plasma over the heat treatment are lower water consumption, operating temperatures and lower costs compared to heat treatment (Li and Farid, 2016). Cold plasma research has focused on sanitizing surfaces in food products that have a complex surface, as chicken pieces with skin, porous nut and scar on the stem or flowered ends of the fruit (Niemira, 2012). The exact mechanism of spore inactivation by cold plasma is attributed to several synergistic mechanisms of surface erosion, UV radiation, oxidation caused by ozone and reactive species and charged particles (Veen et al., 2015; Ziuzina et al.,

2015). Cold plasma treatment has also been used for decontamination of packaging as it prevents post-processing contamination (Misra et al., 2014; Misra et al., 2019). Cold plasma processing has been used for sterilization and disinfection functionalization, enzyme inactivation, alteration of hydrophilic function/hydrophobic properties, etching or thin film deposition (Jayasena et al., 2015; Korachi et al., 2014). Meat products are not considered for plasma treatment because some of the reactive species trigger the oxidation of high lipid-producing aromatics-producing food products that cause rancidity (Awad et al., 2012; Priyadarshini et al., 2018). There are three different modalities to use the plasma:

Direct exposure - This approach, as the name suggests, is to expose the food to the plasma discharge itself. This could be the plume from a plasma jet or the field between two electrodes. Direct plasma exposure maximizes the food interactions with the short-lived reactive gas species, UV and electric field. However, it can be difficult to ensure uniform exposure of complex surfaces, particularly pores, such as those of food products.

Indirect or remote exposure - This approach involves placing the target some distance from the plasma discharge. Only relatively long-lived species interact with target because of the recombination of many of the induced species. The approach can be useful in limiting potential detrimental effects from direct exposure for fragile or susceptible tissue. Reactive gas species can be delivered in a more uniform manner for treatment of certain produce.

Plasma Activity Water - This approach used a liquid (typically water) as a delivery media for plasma-generated reactive species. Following exposure to a plasma discharge (usually direct) the water is activated for a period with concentrations of metastable species. Solution exposed to plasma result in the generation of relatively long-lived secondary products such as hydrogen peroxide, nitrates and nitrite, which may react to from further cell toxic compounds such as peroxynitrous acid. During

this active window, the plasma can be used as active wash water for food decontamination (Sarangapani et al., 2018).

1.2.6 Pulsed Electric Fields (PEF)

Pulsed electric fields (PEF) are an emerging non-thermal technique capable of ensuring good product quality and efficiency in energy use (Yang, Huang, Lyu and Wang, 2016). Potential applications of PEF for processing a wide range of food products have been extensively studied around the world (Bhat et al., 2019, Gabrić et al., 2018, Giteru et al., 2018, Zhang et al., 2011). A typical PEF food processing system is mainly composed of a high voltage pulse power supply, a treatment chamber, a fluid management system, and control and monitoring systems. Among them, the high voltage pulse power supply and the treatment chamber are the two most important subsystems. Exponential decaying waves and square waves (monopolar or bipolar) are the commonly used waveforms, and square wave pulses are considered superior to exponential decaying pulses because the former can be treated with sustained and constant intensity for the total duration of the pulse (Wan et al., 2009). Compared to monopolar pulses, bipolar pulses are more effective for cell membrane permeability, which can be attributed to structural membrane fatigue caused by alternating stress generated by the high frequency inversion of the pulse polarity (Wang et al., 2018). Furthermore, an appropriate design for a treatment chamber is important to maximize the treatment capacity of the PEF, the intensity of the peak electric field and the uniformity of the treatment. Therefore, the structural design of the treatment chamber must fully consider the electric field distribution and the distortion of the electric field strength of the treatment chamber, as well as the field distribution of the fluid and the temperature of the food in the treatment chamber. Typical types of treatment chambers are mainly planar, coaxial and axial, and the commonly used materials of the treatment chamber currently are corrosion-

resistant stainless-steel electrodes and polytetrafluoroethylene (PTFE) shells (Zhang et al., 2011). Furthermore, depending on the design of the treatment chamber, the PEF process can be performed in continuous or batch mode (Liu et al., 2018, Yin et al., 2006). In recent years, PEF processing has attracted strong attention in the food industry. In industrial applications, different commercial-scale systems for processing liquid and solid food PEF have been developed in the United States and European countries (Blahovec et al., 2017). PEF technology for food processing is based on a pulsating power delivered to the product placed between a series of electrodes that delimit the treatment space of the PEF chamber (Góngora-Nieto et al., 2002, Wang et al., 2018). In the space between the electrodes, food is subjected to a force per charge, the so-called electric field (Góngora-Nieto et al., 2002). It is worth emphasizing that foods are complex multi-component / multiphase systems and many useful chemical reactions occur during processing. When exposed to a specific electric field, certain electrochemical and physicochemical properties of electrically sensitive components in food change reactively (Li et al., 2007, Ma et al., 2012). This mainly involves PEF-induced permeabilization of biomembranes, the occurrence of electrochemical and electrolytic reactions, the polarization and realignment of molecules, and the reduction of the activation energy of chemical reactions. All these PEF processing performances are expected to play an effective role in food processing, such as inactivating microorganisms, extracting active constituents, modifying biomacromolecules, enhancing chemical reactions and accelerating the process of aging in fermented foods. The efficiency of PEF for food processing depends on many factors, which can essentially be classified as process parameters, (medium) parameters of the food product and characteristics of the processing target. The main process parameters of PEF are electric field strength, number of pulses, pulse shape, time/length of pulse and initial temperature (Gabrić et al., 2018, Wouters et al., 2001). Among them, the electric field strength and the treatment time (t = number of pulses × pulse duration) are central processing factors and increasing the intensity of these parameters, generally improving the processing efficiency of the PE

(Gabrić et al., 2018). Furthermore, foods are very complex materials, and the content of various chemical components in foods (for example, fats and proteins) has erratic effects on conductivity. Many previous studies have shown that food product parameters such as conductivity and pH influenced the efficiency of PEF (Buckow et al., 2013, Gabrić et al., 2018), making it difficult to implement "one size fits all" in processed foods. In this case, each food may need to be tested separately to identify the appropriate PEF parameters. The mechanism of inactivation of microorganisms by PEF is still under discussion. At present, the main acceptable mechanism for inactivating microorganisms by PEF treatment is that PEF induces electrical rupture of cell membranes (known as electroporation), leading to destruction of cell membranes, increased cell permeability and cell death. (Wang et al., 2018, Wouters et al., 2001). In the electrical failure theory, the cell membrane is considered as a capacitor filled with a dielectric medium (Buckow et al., 2013). Under an externally applied electric field, a transmembrane potential difference is formed on the cell membrane. When the transmembrane potential exceeds a critical threshold value (about 1 V), electroporation of the membrane is induced (Zhang, Li, et al., 2019). Depending on the intensity of the PEF treatment, electroporation can be reversible or irreversible. Strong intensities of PEF treatment cause irreversible effects, which ultimately lead to cell death (Wouters et al., 2001). Based on the above mechanism, many studies by Chinese researchers have explored the application of PEF to inactivate microorganisms in liquid food products (Zhang et al., 1994, Zhang et al., 2010). However, these studies suggest that applications of PEF for the inactivation of microorganisms are limited to low-conductivity, bubble-free products, such as milk (Zhao et al., 2013), milk soy (Li et al., 2013), fruit juices (Zhang et al., 2010), beverages (Zhao et al., 2008) and wine (Huang et al., 2013) to avoid dielectric breakdown.

1.2.7 Dense-CO_2

High Pressure Carbon Dioxide (HPCD), a collective term for liquid CO_2, supercritical CO_2, and dense phase carbon dioxide, is one of the alternative non-thermal pasteurization techniques for food and is also attracting a lot of interest in the food industry (Damar and Balaban, 2006). The HPCD has been shown to have a significant lethal effect on microorganisms and enzymes, especially in liquid foods, while maintaining the sensory quality, color, aroma, nutritional and physical quality of some liquid foods, avoiding the effects thermals of pasteurization (Zhou et al., 2009). In general, most of the liquid foods that have HPCD processing applied are relatively acidic foods, such as fruit juices, vegetable juices and beverages. Microbial inactivation, including artificially inoculated spoilage or pathogenic microorganisms, and naturally occurring microbes, usually became the major concerns. Inactivating enzymes and maintaining quality were also important aspects of interest to researchers and the food industry. However, the use of HPCD as an alternative and innovative technique for low-acid foods, especially the processing of milk and dairy products, was still limited. In addition, further studies should be conducted to elucidate the effects of HPCD on the main intrinsic indicative parameters involved in dairy processing and quality maintenance/improvement, thus contributing to its full application for the dairy industry dairy (Amaral et al., 2017). Enzymes in foods and enzyme solutions, including polyphenol oxidase, peroxidase, lipoxygenase, and pectin esterase, have been reported to be inactivated by HPCD (Balaban et al., 1991; Gui et al., 2007; Liu et al., 2008). The resistance of some enzymes makes them suitable indicators for assessing the severity or effectiveness of heat treatment of milk and dairy products. Alkaline phosphatase (ALP) has been used as an indicator of the adequacy of pasteurization (Fadiloglu, Erkmen, and Sekeroglu, 2004). A high enzymatic activity of alkaline phosphatase (ALP) indicates an inadequate pasteurization process. Inactivation and kinetic analyzes of ALP in raw cow's milk (Fadiloglu et al., 2004; Levieux, Geneix, and Levieux, 2007), in goat's milk (Wilińska et al., 2007) and in horse milk (Marchand et al., 2009) following heat

treatments and high intensity pulsed electric fields have been extensively studied. However, few studies have been conducted on the inactivation of ALP by HPCD. ALP activity in whole milk was reported to be correlated with HPCD treatment conditions using a continuous instrument, which were characterized with an increase at 30 °C and 8-18 MPa while with a 98.2% reduction at 70 °C and 8 MPa for 30 minutes (Ceni et al., 2016). The result revealed the potential application of continuous HPCD in the inactivation of ALP in milk, especially at higher temperatures. Given that the activity of a certain enzyme typically decreases with increasing CO_2 pressure levels (Hu et al., 2013), it's worth trying to inactivate alkaline phosphatase in milk at higher pressure. In addition to inactivating microbes and enzymes, maintaining/improving quality was an important aspect of evaluating an emerging technology for food processing and HPCD was also not exceptional. Several studies had reported the effect of HPCD on the physico-chemical, sensory and/or nutritional properties of milk (Ceni et al., 2016; Di Giacomo, Taglieri, and Carozza, 2009). Since the protein and fat content and characteristics of milk are important properties for milk and dairy products, lipolysis and proteolysis have been well studied (Tisi, 2004). The sensory properties of skimmed milk processed both by supercritical CO_2 and by the short term at high temperature were evaluated by Di Giacomo et al. (2009). Although higher CO_2 pressure resulted in greater extraction of flavor compounds from milk and followed by undesirable changes in sensory characteristics, higher acceptance of processed milk by supercritical CO_2 was observed compared to short-term high-temperature pasteurized milk under the same conditions conservation. In addition, the physico-chemical characteristics of whole milk processed with HPCD compared to untreated milk were evaluated and no difference was observed between them (Ceni et al., 2016). In general, information regarding the modification of the quality indices of raw cow's milk with HPCD technology is still limited.

1.2.8 High hydrostatic pressures (HHP)

High-pressure (HPP) or high hydrostatic pressure (HHP) processing is a promising food processing technology used to effectively inactivate food enzymes at room temperature, thus maintaining the flavor, aroma and nutritional value of foods without compromise the shelf life of final products (Andrés et al., 2016; Terefe et al., 2015; Zheng et al., 2014). The technique of high-pressure processing was first reported as being used in 1900 for the preservation of milk by a famous scientist Hite and his team (Hite, 1989). This method was followed on different types of fruit and vegetables by the same scientist (Hite et al., 1994). However, after the 1990s, this technique has been studied in an interesting way for many food products to discover the various aspects of this method (Buckow et al., 2009; Terefe et al., 2015; Thakur and Nelson, 1998). packaged products (liquid or solid) are placed inside a pressure chamber containing water. The desired pressure is applied for a specific time. After depressurization, the transformed product is removed to be stored for final use (Balasubramaniam and Farkas, 2008; Briongos et al., 2016; Terefe et al., 2015). HHP is considered a better alternative to thermal processing as it can effectively destroy spoilage enzymes at mild temperatures, thus preserving the quality of fresh foods (Buckow et al., 2009; Gómez-López et al., 2010; Liu et al., 2014; Marszałek et al., 2017c) However, it's noted that some quality associated enzymes have limited inactivation after treatment with HHP in food (Swami Hulle and Srinivasa Rao, 2016). Many studies have revealed that HHP can only break non-covalent hydrogen bonds between enzymes but does not have the ability to break peptide bonds in the enzyme (Jiménez-Aguilar et al., 2015; Lopes and Paz, 2010; Zhang et al., 2016). Since the weak hydrogen bonds of the enzyme are affected, this method can cause the structural changes (primary, secondary and tertiary) of the enzyme. During HHP processing, high pressure secondary and tertiary structure breakdown of > 600 MPa at due to the interruption of the electrostatic and hydrophobic interaction (Lopes and Paz, 2010). Hence, this structural change may be related to the phenomenon of activation or inactivation of polyphenol oxidase (PPO) (Queirozet al., 2008), since

inactivation of the enzyme requires a certain pressure which can cause the breakdown of the enzyme structure (Lopes et al., 2010; Picouet et al., 2015). Several factors such as the type of food, the origin of the enzyme, the duration of the treatment, the initial pH and the presence of some additives in food are the key factors that determine the efficiency and validity of this technique (Chen et al., 2015).

1.2.9 Ultrasound (US)

Over the past decades, ultrasound processing has developed as a non-thermal alternative food processing technique file (Troy et al., 2016; Valdramidis and Koutsoumanis, 2016). Applying ultrasound to a food matrix file causes acoustic cavitation, which is the phenomenon of bubble generation, growth and eventual collapse that causes mechanical and chemical effects on matrix foods. Mechanically turbulence, significant pressure variations (up to 1000 atm) and shear stress occur (Liu et al., 2016; Majid, Nayik, and Nanda, 2015). Depending on the frequency of the ultrasound, locally produced resulting in variations in pressure, expansion or compression of the material in the rupture of the cells. The ultrasonic frequency range is from 20 kHz to > 1 MHz (Chandrapala, Oliver, Kentish and Ashokkumar, 2013; Li and Farid, 2016; Valdramidis and Koutsoumanis, 2016). Ultrasound applications and commercial perspectives can be classified as low intensity sonication (frequencies above 100 kHz with energies below 1 W/cm^2) and high intensity sonication (frequencies below 100 kHz with energies above 10 W/cm^2) (Jermann et al., 2015). High intensity or power ultrasound is used to induce physical, mechanical, biochemical and chemical changes due to biological matrices to cavitational effects (Kadam et al., 2015; Valdramidis and Koutsoumanis, 2016). In this way, the mechanisms through which the United States acts can be divided into three:

- mechanical damage to the cell wall induced by pressure gradients generated during the collapse of the cavitation bubbles inside or near the cells.
- the micro-streaming that takes place inside the cell.
- the formation of chemical compounds during cavitation that attack the cell wall structure leading to their disintegration (Tiwari and Mason, 2012).

There is large number of high-intensity ultrasound applications that rely on three main methods such as direct application to the product, adapting to the device and immersed in an ultrasonic bath (Chemat, Zill, and Khan, 2011; Zenker, Heinz and Knorr, 2003). There is a great deal of interest in the United States due to the resulting benefits including reduced processing times, higher productivity and lower energy consumption such as the fact that industries can be supplied with practical and reliable ultrasonic equipment. Furthermore, this technology attracts attention because it is considered an environmentally friendly green technology (Rawson et al., 2011; Zenker et al., 2003). Furthermore, ultrasound is said to be economically feasible and meets the process requirements such as simplicity, energy efficiency and scale up (Arvanitoyannis, Kotsanopoulos, and Savva, 2017; Chemat et al., 2011; Kadam et al., 2015). US use has emerged as an alternative processing option with applications in many areas of the food industry, food processing, and food analysis (Mukhopadhyay and Ramaswamy, 2012; Rawson et al., 2011). The application of ultrasound has contributed to the improvement drying process by facilitating the improvement of moisture and vapor migration, changes in the structure and properties (density, viscosity, etc.) of the material and others. The application of the ultrasonic process in industry may depend on the simplicity of design, use of localized reaction zones, the uniqueness of capabilities offered and operational simplicity (Musielak, Mierzwa and Kroehnke, 2016). The well-known commercial use in the United States is for beverages and beverages, however it has been an efficient tool for other applications, such as emulsification, homogenization, extraction, crystallization, dehydration, degassing, and defoamer (Jermann et al., 2015). Due to the versatility of the US

process, one of the most important challenges for the US application industry is the optimization of parameters in all processes and products as well as the study of qualitative, nutritional and sensory aspects, in order to scale the process at an industrial level (Knorr et al., 2013; Tiwari and Mason, 2012). However, although ultrasonic processing offers advantages, it too produces negative effects such as degradation of food properties, including flavor, color or nutritional value (Farkas and Mohácsi-Farkas, 2011; Harder, Arthur and Arthur, 2016; Priyadarshini et al., 2018). Thus, a better understanding of the complex mechanism of ultrasound and its effect on the functional properties of foods and on the overall composition will promote the industry adoption of this technology. Hence, for its adoption in the industry high power process design must be improved, energy efficiency, ease of installation, competitive power consumption and low maintenance cost must be considered to make it feasible for large scale- up industrial with useful economic advantages (Alarcon-Rojo et al., 2015; Priyadarshini et al., 2018; Zinoviadou et al., 2015).

1.3 Ricotta cheese

Fresh ricotta has a high moisture content, high residual sugar concentration, initial pH above 6.0 and does not require the addition of starter cultures in production. Consequently, fresh ricotta has a limited shelf life (2-3 days) even in the refrigerator (Hough et al., 1999; Martins et al., 2010). The quality and yield in ricotta strictly depend on the coagulation of the whey proteins following heating. Regards this process, various variables come into play, such as: milk protein concentration (whey proteins), process temperature, pH and ionic strength of the medium. This last aspect is fundamental for the generation of protein aggregation. In an environment with ionic strength <20 mM, particularly in the absence of Ca^{2+} ions and with pH>6.5, there is charge repulsion between proteins and consequently protein aggregation is prevented. The serum, on the other hand, has an ionic strength >20 mM and, at higher levels of ionic strength (>20<100 mM), the protein aggregation phase does not depend on the ions involved in the process. For a concentration >100 mM the nature of the ions (especially calcium and sodium) significantly influences the aggregation phase between sieroproteins, especially the Ca^{2+} ion is able to neutralize part of the negative charges and consequently a reduction in repulsion charge between whey proteins occurs, favoring the protein aggregation phase (Mucchetti, Carminati and Pirisi, 2002). However, the added Ca^{2+} in the form of calcium chloride is able to bring about a reduction in the pH of the whey and this requires further neutralization to favor the protein aggregation phase (Weatheurp, 1986). The pH value is essential for determining the physical structure of the protein clot. In the event that the coagulation phase should take place at neutral pH, the structure has a more elastic consistency; on the contrary, at pH<6 and as we approach the isoelectric point (pH 4.6) the structure becomes "particulate" and deprived of elasticity (Mucchetti, Carminati and Pirisi, 2002).

Due to the naturally poor competitive microflora (Pintado, Macedo, and Malcata, 2001), composition, intrinsic physical and chemical properties and absence of preservatives, fresh ricotta is an excellent substrate for the growth of deteriorating

microorganisms mainly represented by *Pseudomonas spp*., Yeasts, molds and enterobacteria (De Santis and Mazzette, 2002; Pala et al., 2016; Pintado et al., 2001). *Pseudomonas spp*. represent the main obstacle to the shelf life of fresh ricotta; deterioration could cause significant discoloration resulting from the secretion of yellow-green pyroverdine and blue pigment (Spanu et al., 2018). Whey-based products processed at high temperatures and subsequently cooled and stored in refrigerated conditions are particularly exposed to the risk of growth of *Bacillus cereus* (Heyndrickx and Scheldeman, 2002). Endospores are activated by heat treatment applied to denature whey proteins (T> 80 °C) and their growth is facilitated by the absence of competing microorganisms, inactivated by heat treatment (Scheldeman et al., 2006). Psychrotropic strains of *B. cereus* can grow at temperatures up to 4-5 °C and the risk of growth is increased by the slow cooling of the product (Huck et al., 2007). *B. cereus* can enter the dairy supply chain mainly through contaminated raw milk at farm level (Heyndrickx, 2011). However, contamination can also result from the food processing environment (da Silva et al., 2014). All of these bacteria negatively impact texture, appearance, color, smell and taste of cheese (Baruzzi et al., 2012; Cantoni et al., 2003; Caputo et al., 2015).

Improving hygiene practices is a measure that could certainly reduce the initial contamination level of the ricotta surface. However, the shelf life of artisanal ricotta is very short (much less than one week). For this reason, at industrial level, ricotta is generally thermally treated. The industrial process includes a final pasteurization phase, which ensures a shelf life of between 20 and 40 days (Mucchetti and Neviani, 2006). Depending on the food business operator, fresh ricotta can be packaged in atmospheric air or modified atmosphere (MAP) conditions (30% CO_2 and 70% N_2) (Spanu et al., 2018).

To the best of the current knowledge, ricotta was never treated by non-thermal processes, thus giving rise to explore the possibility to improve its shelf life by using novel approach than the common ones.

Aim and outline of this Ph-D thesis

The aim of this Ph-D was to test some of the most common non-thermal technologies on artisanal and industrial ricotta cheese. Tests were also carried out on cheese previously contaminated by specific strains of *Pseudomonas* spp. to optimize the parameters to be adopted during effective treatments. X-rays, UV and NUVL, Pulsed Light and Cold plasma were assessed on ricotta cheese quality. To the aim, shelf life tests were carried out on both treated and untreated samples and during porper refrigerated storage period under refrigerated conditions, main spoilage microorganisms were analyzed (*Mesophiles*, *Enterobacteriaceae*, *Pseudomonas spp*. yeasts and *Bacillus cereus*). Furthermore, the pH and the sensory quality were also motitored during the entire storage period. The fitting procedure of experimental data allowed to calculate the precise day when the product became unacceptable from the microbiological and/or sensory point of view. Comparing the fitting parameters, it was possible to calculate for each technique the shelf life of control and treated ricotta samples, thus suggesting that more than one tested non-thermal approach could found great interest at industrial level, above all for perishable food as artisanal ricotta cheese.

Chapter 2

X-ray treatments applied to ricotta cheese

2.1 Aim of the study

The main objective of this study was to test the ricotta under x-rays in order to explore its evolutionary behavior over time and then translate it in terms of shelf life compared to the untreated product. To the aim, the study was carried out on both artisanal ricotta and industrial ricotta, using 3 different X-ray doses, 0.5-2 and 3 KGy. Both microbiological and sensory quality of treated and untreated samples were monitored during a refrigerated storage period.

2.2 Materials and Methods

2.2.1 X-ray treatment

The X-ray treatments on fresh ricotta cheese samples were performed using the RS-2400 system (Rad Source, Brentwood, TN, USA) installed at the IZS Institute (Foggia, Italy) shown in figure 2.1.

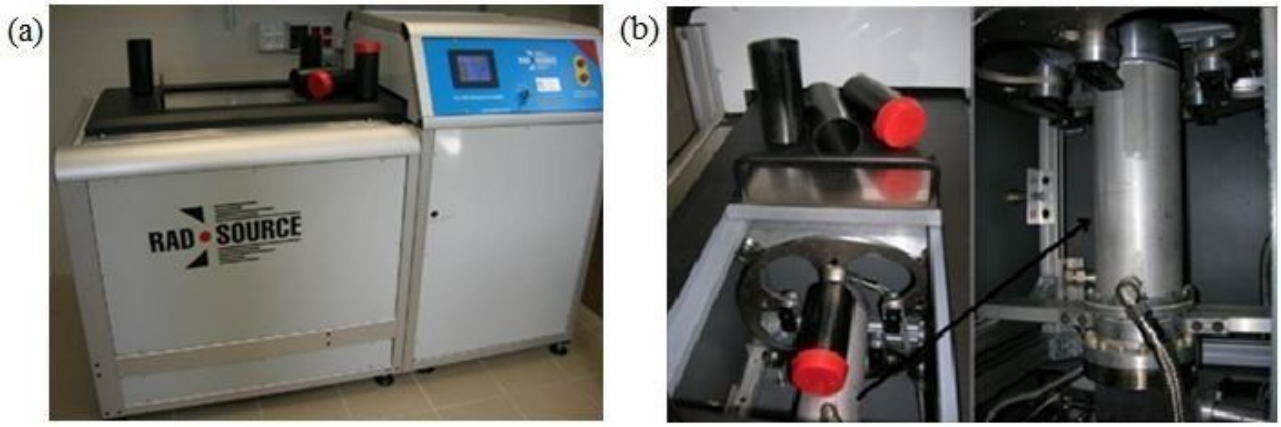


Figure 2.1: RS-2400 system used for the treatments (photo a). In photo b, you can see the cylinders where the samples are inserted for the treatment and the cylindrical re-rotation system, which turns when the machine is activated.

The first test was performed on 50 samples of fresh artisanal ricotta cheese (50 g), purchased from the company "Caseificio della Daunia" in Foggia (Italy). On the same day of production, the samples were packed individually (PA/PE type 95 bags) and transported to the IZS Institute under refrigeration conditions. The second test was carried out on 80 pasteurised industrial packaged ricotta samples (100 g), kindly provided by Granarolo S.p.A. (Bologna, Italy). All the samples, one day after production, underwent X-ray irradiation treatment at 0.5, 2.0 and 3.0 kGy, respectively. Untreated control samples and treated samples in both tests were stored at 4 °C for 24 and 84 days, for artisanal and industrial samples, respectively.

2.2.2 Microbiological analyses

In each test, the microbiological quality of control and treated samples was analysed by monitoring microbial growth of mesophilic bacteria, *Pseudomonas spp*., *Enterobacteriaceae*, yeasts and *B. cereus*. For artisanal ricotta cheese, the sampling was carried out at time 0, 1 and every 2/3 days up to 24 days of storage, while the industrial samples were analysed at time 0, 1, every 7 days up to 56 days and at 84 days of storage. To this aim, twenty grams of ricotta cheese were removed from each pack, diluted with 180 mL of 0.9% NaCl solution and thoroughly homogenised (Bag Mixer Interscience, St Nom, France) before making decimal dilutions of homogenates. The dilutions were plated on appropriate media in Petri dishes. The classic plate count technique was applied. Culture media and incubating conditions for spoilage microorganisms were reported in the study of Del Nobile, Gammariello, Conte, and Attanasio (2009), also dealing with ricotta cheese. *B. cereus* counts were determined using MYP culture medium (Oxoid, Milan, Italy), adding, after sterilisation, egg yolk emulsion (50 mL, Oxoid) and Polymyxin B (1 bottle, SR009E, Oxoid) as supplements. The fitting of experimental data allowed quantifying the microbiological acceptability limit (MAL), calculated according to the same mathematical approach adopted by Angiolillo, Conte, Faccia, Zambrini, and Del Nobile (2014). The pH value was assessed on homogenates of ricotta cheese, in duplicate, by a pH-meter (Crison GLP 21+, Barcelona, Spain), after appropriate calibration of the instrument (Lacivita et al., 2016).

2.2.3 Sensory analysis

Sensory analysis was performed by 7 trained panellists from the Food Packaging Laboratory of the University of Foggia. They had several years of experience in sensory evaluation, but before the analysis they were re-trained (2 sessions, 2 h each session) to better define sensory attributes and score evaluation. To this aim, odour,

colour, texture and global quality were taken into account, using properly evaluation grid ranged from 0 to 7, where 4 represented the cheese acceptability threshold (Chen et al., 2009, Lacivita et al., 2016). The fitting of experimental data allowed quantifying the sensory acceptability limit (SAL), as reported in Angiolillo et al. (2014).

2.2.4 Statistical analysis

Both microbiological, pH determination and sensory analyses were carried out twice, on two different samples treated and packaged separately. Mean and standard deviation of experimental data were calculated. Fitting of experimental data gave us microbial acceptability limit (MAL = day when ricotta became unacceptable for microbial proliferation) and sensory acceptability limit (SAL = day when ricotta became unacceptable for defects in sensory quality). These fitting parameters were compared by one-way ANOVA test (STATISTICA 7.1 for Windows, StatSoft Inc., Tulsa, OK, USA). To determine significant differences among fitting parameters the Duncan's multiple range test, with the option of homogeneous groups ($p < 0.05$), was used. The lowest value between the MAL and SAL parameters gave us the final shelf life.

2.3 Results and Discussion

This study investigated the effect of three X-ray irradiation doses (0.5, 2 and 3 kGy) on microbial spoilage and sensory quality of fresh ricotta cheese over time when kept under refrigeration. In particular, two tests were carried out to evaluate the effectiveness of X-rays on both artisanal and commercial fresh products.

2.3.1 Effects of X-rays on microbiological quality of artisanal and industrial fresh ricotta cheese

To evaluate the X-ray effects on microbial population of fresh ricotta, *Pseudomonas spp.*, *Enterobacteriaceae*, yeasts, total mesophilic count and *B. cereus* were monitored during time, as main responsible factors for product stability (Pala et al., 2016, Sattin et al., 2016, Spanu et al., 2017, Spanu et al., 2016). Fig. 2.2 shows the evolution of *Pseudomonas spp*. in treated samples of artisanal ricotta compared with non-irradiated control cheese (Ctrl). As can be seen, for the irradiated samples the applied treatment exhibited different effects on microbial counts. In particular, for both samples irradiated at 2.0 and 3.0 kGy *Pseudomonas spp*. growth was completely inhibited by the X-ray treatment. On the contrary, the samples of artisanal ricotta irradiated at 0.5 kGy showed a long lag phase, little more than one week, and then slightly increased, without ever reaching the microbiological acceptability limit set to 10^6 CFU/g. The microbial evolution of irradiated samples was significantly different compared with non-irradiated control cheese. In fact, as can be seen in Fig. 2.2, a gradual increase of *Pseudomonas spp*. population was noticed during the 10 days of storage monitored, overcoming the microbiological acceptability limit after only 3 days.

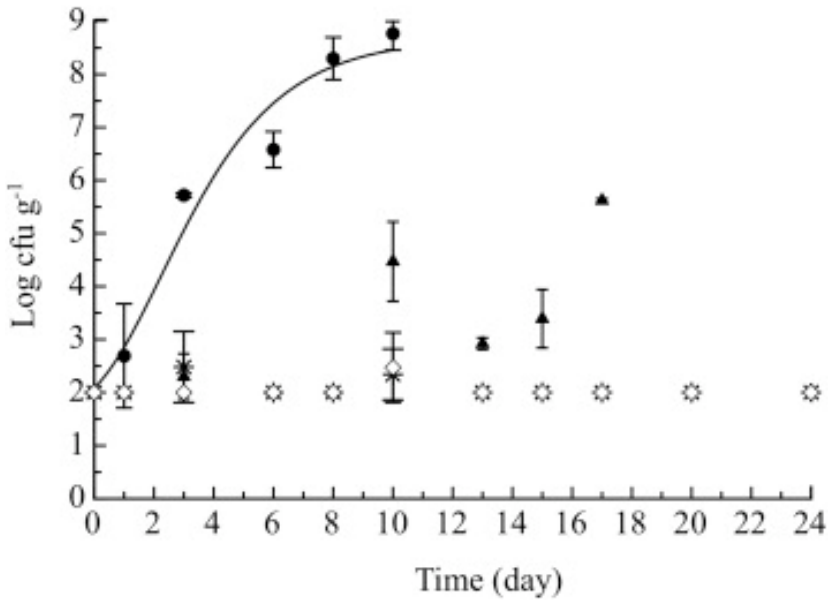


Figure 2.2: Evolution of *Pseudomonas spp*. in X-ray treated artisanal Ricotta cheese (▲0.5 kGy; *2 kGy; ◊3 kGy) compared with non-irradiated control (•); symbols are the experimental data, line is the best fit to control data.

It is well known that *Pseudomonas spp*. are psychrotrophic bacteria with a short generation time (only a few hours) at refrigeration temperature. As a consequence, microbial cell counts can exceed 10^6 CFU/g within a few days of chilled storage (Samaržija, Zamberlin, and Pogacic, 2012). This was, in fact, the behaviour observed in the control samples, whereas in the treated products the higher doses of treatment were able to keep the microbial count very low during the 24 days of monitoring. These results were well supported by an earlier study (Lacivita et al., 2019), where the effects of X-ray were assessed on Fior di latte cheese.

A very similar behaviour was also observed for *Enterobacteriaceae* and yeasts. Fig. 2.3 and 2.4, respectively, show the evolution of *Enterobacteriaceae* and yeasts of X-ray treated artisanal ricotta samples compared with the control cheese. As can be seen, for both microbial groups the X-ray treatment significantly reduced the microbial growth. Contrary to this trend, the microbial count of *Enterobacteriaceae* and yeasts in the control samples gradually increased, overtaking the microbial acceptability limit set to 10^4 and 10^6 CFU/g after 4 and 8 days, respectively. According to other studies also reported in the literature (Konteles et al., 2009,

Odueke et al., 2016), the initial microbial load of total mesophilic count (10^5 CFU/g) was significantly reduced immediately after irradiation by more than 3 log cycles in treated ricotta while it increased in the control cheese. These great differences between control and treated products were maintained during the entire monitoring period; samples treated at 2 and 3.0 kGy maintained a microbial count around 10^3 CFU/g, whereas samples treated at 0.5 kGy reached about 10^6 CFU/g, and control ricotta arrived to about 10^9 CFU/g. The irradiation treatment, even at low doses, exerted a great impact on total mesophilic counts. *B. cereus* was not detected in both non-irradiated and treated samples. In general, the experimental findings on artisanal ricotta cheese confirmed the effectiveness of irradiation already verified on other types of fresh dairy products (Badr, 2011, Huo et al., 2013, Lacivita et al., 2019, Velasco et al., 2019) and suggested that with 2 kGy treatment a total spoilage control of artisanal Ricotta can be reached.

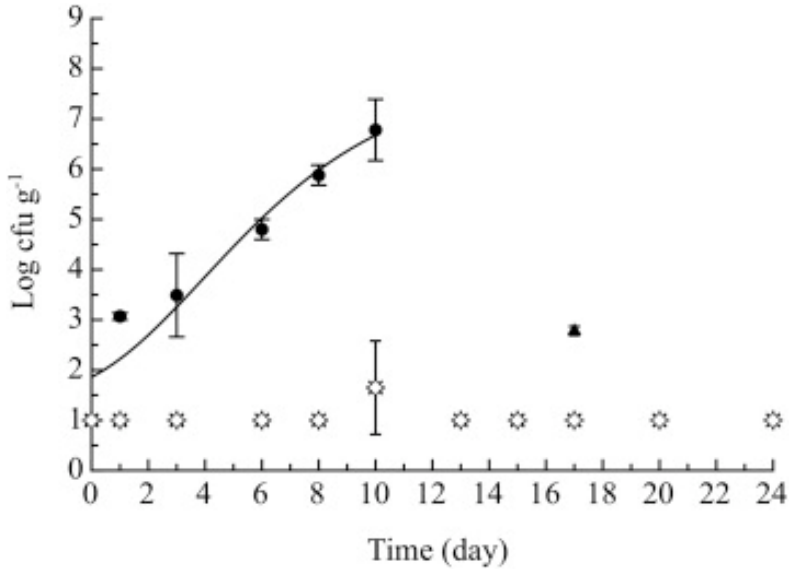


Figure 2.3: Evolution of *Enterobacteriaceae* of X-ray treated artisanal Ricotta cheese (▲0.5 kGy; *2 kGy; ◊3 kGy) compared with non-irradiated control (•); symbols are the experimental data, line is the best fit to control data.

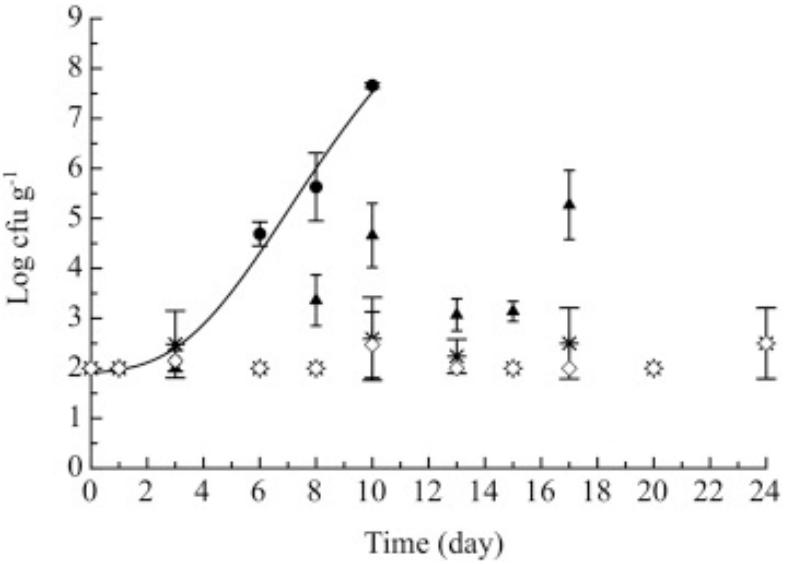


Figure 2.4: Evolution of yeasts of X-ray treated artisanal Ricotta cheese (▲0.5 kGy; *2 kGy; ◊3 kGy) compared with non-irradiated control (•); symbols are the experimental data, line is the best fit to control data.

Regarding the microbiological quality of industrial ricotta samples, it was striking to find no spoilage proliferation, in terms of *Pseudomonas spp., Enterobacteriaceae*, yeasts and *B. cereus*, in all the cheese samples examined during a long storage time. For mesophilic bacteria (data not shown) the same trends recorded for control and treated artisanal samples, respectively, were also found for industrial ricotta cheese. The lack of spoilage of these samples was due to the stabilising thermal treatment applied just before packaging that generally assures a shelf life of more than one month. Therefore, for the industrial ricotta, the effects of X-ray treatments were more evident in terms of sensory quality, as reported below.

No great pH variations in both artisanal and industrial treated ricotta samples were found, in line with other studies where irradiation was applied to fresh dairy cheese (Badr, 2011, Konteles et al., 2009). Specifically, in the artisanal products pH ranged around 7.20 with very similar trends among samples during the entire period. On the other side, a significant pH decline was recorded in the control cheese just after the first 3 days of storage (from 7.20 to 6.70). Likewise, in the industrial dairy products, a great difference between treated and control ricotta was found. In this case, the pH of treated samples ranged around 6.30, whereas, pH in the control cheese slightly

decreased reaching values that accounted for about 5.75 when the product became unacceptable (after circa 40 days).

2.3.2 Effects of X-rays on the sensory quality of artisanal and industrial fresh ricotta cheese

For the sensory evaluation the trained panel judged colour, odour and texture of control and irradiated artisanal fresh cheese. Trend of mean values of sensory scores were shown in Fig. 2.5 in terms of overall quality. As can be inferred from the figure, non-irradiated samples became unacceptable after 6 days of storage, cheese irradiated at 0.5 kGy after 14 days, whereas the two samples treated at higher doses remained acceptable for 24 days with a very similar trend. As expected, sensory evolution can be strictly correlated to microbial proliferation, thus confirming that in cheese samples where microbial growth was inhibited (ricotta treated at 2.0 and 3.0 kGy) also sensory properties were more appreciated (Lacivita et al., 2019).

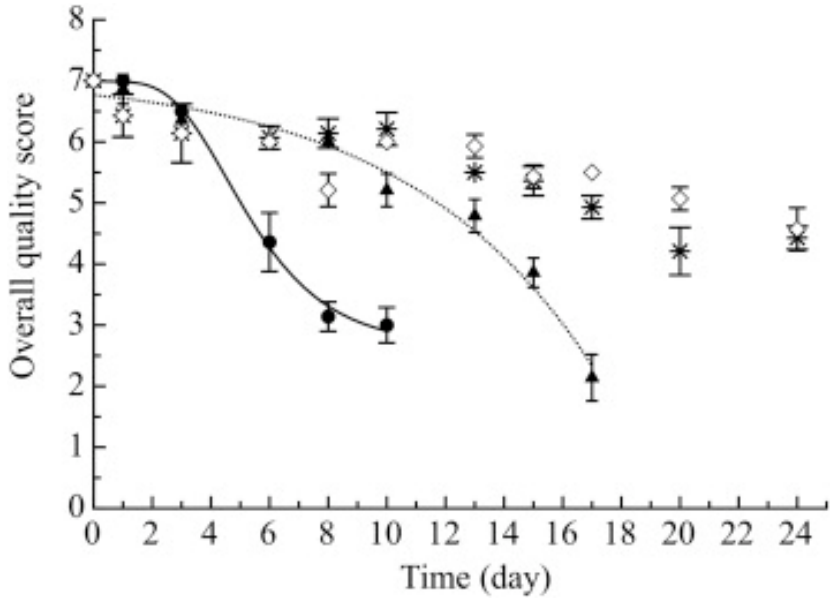


Figure 2.5: Evolution of overall quality of irradiated artisanal Ricotta cheese samples (▲0.5 kGy; *2 kGy; ◊3 kGy) compared with non-irradiated control (•); symbols are the experimental data, solid and dotted lines are the best fit to control and 0.5 kGy data, respectively.

As regards industrial ricotta sensory quality, important and interesting differences between control and treated samples were found, elongating the storage period to 84 days. As reported in Fig. 2.6, the kinetic decay of the overall quality referred to the control samples was very different from that related to treated cheese. In particular, while the control ricotta became unacceptable after 38 days of storage, all the X-ray treated samples remained acceptable for more than 80 days, without any substantial differences among the three irradiation-doses.

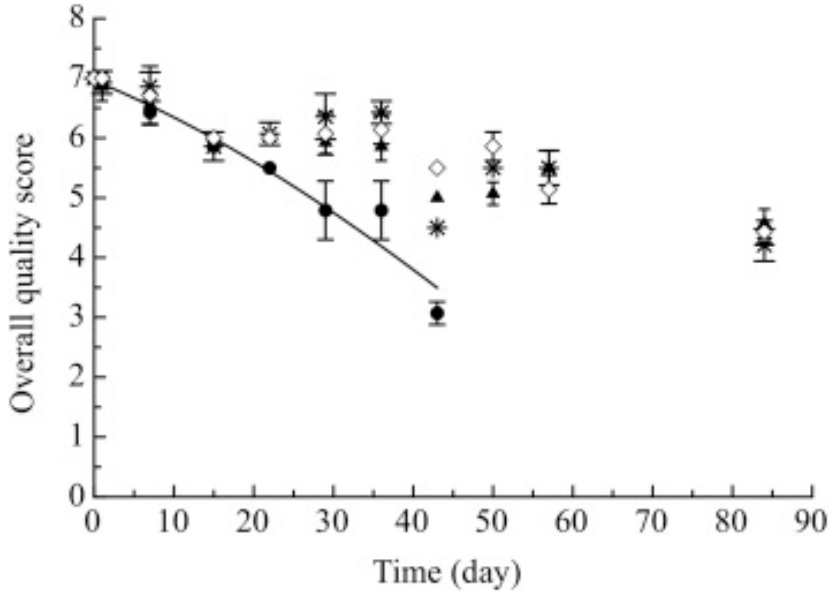


Figure 2.6: Evolution of overall quality of irradiated industrial Ricotta cheese samples (▲0.5 kGy; *2.0 kGy; ◊3.0 kGy) compared with non-irradiated control (•); symbols are the experimental data, line is the best fit to control data.

These experimental findings in terms of sensory quality on both artisanal and industrial ricotta also suggested that X-rays did not compromise main ricotta attributes, even when applied at high dose. The same result was also recorded by Aly, Farag, and Galal (2012), who found no differences between treated and untreated cheese samples after e-beam and γ-irradiation at different doses (from 1.0 to 5.0 kGy). However, in this context, data from scientific literature of irradiated food are dependent on the type of irradiation and on the nature of dairy cheese. Sometimes, some changes, especially in odour and colour, were perceived after ionising radiation

at dose up to 3.0 kGy (Konteles et al., 2009, Velasco et al., 2019, Velasco et al., 2016).

2.3.3 Shelf life of artisanal and industrial ricotta cheese

In general, the shelf life of food products is compromised by undesired spoilage microorganism proliferation and consequently by sensory deterioration (Lacivita et al., 2019). Therefore, also in the current study, the shelf life of tested ricotta cheese samples was reported in Table 2.1 as the lowest value among microbial acceptability limits, in terms of $MAL^{Pseudoonas\ spp.}$, $MAL^{Enterobacteriaceae}$ and MAL^{Yeasts}, and sensory acceptability limits in terms of overall quality (SAL). As expected, a substantial difference between artisanal and industrial ricotta was found, being that the industrial product was characterised by a longer shelf life than the artisanal ricotta (Mucchetti and Neviani, 2006). As a fact, the factors that affect ricotta quality are different between artisanal and industrial cheese and consequently the X-rays treatment influenced the two types of products differently. Specifically, control artisanal cheese remained acceptable for a few days (little more than 3), due to prompt *Pseudomonas spp*. proliferation. For artisanal samples treated at 0.5 kGy, a shelf life prolongation by about 5 times was recorded: the samples remained acceptable for about 2 weeks. X-rays significantly inhibited the cheese spoilage, but from the sensory point of view after 14 days some undesirable colour changes appeared on the product, thus determining the end of shelf life. Ricotta treated at 2.0 or 3.0 kGy recorded a shelf life 8 times longer than the control cheese, thus demonstrating the great effect of high doses of X-rays irradiation on both microbiological and sensory quality for a prolonged storage period. Shelf life of industrial product was mainly due to sensory deterioration because in both control and treated samples no significant spoilage growth was detected. Control cheese, even if acceptable from the microbiological point of view, became unacceptable after less than 40 days due to anomalous superficial colour appearance. On the contrary, treated industrial ricotta remained acceptable from both the microbiological and sensorial points of view for the entire

monitoring period, thus recording a shelf life that was doubled (84 days) compared with the corresponding control cheese. On the basis of our findings X-rays can represent a useful treatment to extend the shelf life of both artisanal and industrial fresh Ricotta cheese.

Table 2.1: Shelf-life (days) of artisanal and industrial Ricotta cheese as the lowest value between microbiological acceptability limit (MAL) of *Pseudomonas, Enterobacteriaceae* and yeasts and sensory acceptability limit (SAL).[a]

Samples	Microbial quality (days)			SAL (days)	Shelf life (days)
	$MAL^{Pseudomonas}$	$MAL^{Enterobacteriaceae}$	MAL^{Yeasts}		
Artisanal					
Ctrl	3.92 ± 0.61^{a}	4.23 ± 0.87^{a}	7.99 ± 0.41^{a}	6.42 ± 0.22^{a}	3.92 ± 0.61^{a}
0.5 kGy	>17	>17	>17	14.23 ± 0.44^{b}	14.23 ± 0.44^{b}
2 kGy	>24	>24	>24	>24	>24
3 kGy	>24	>24	>24	>24	>24
Industrial					
Ctrl	>43	>43	>43	37.94 ± 0.11^{a}	37.94 ± 0.11^{a}
0.5 kGy	>84	>84	>84	>84	>84
2 kGy	>84	>84	>84	>84	>84
3 kGy	>84	>84	>84	>84	>84

[a] Values in columns with different superscript letters are significantly different ($p < 0.05$).

The Figure 2.7 comparatively shows, by means of histograms, the shelf life levels of the artisanal and industrial ricotta of the treated samples with respect to the respective controls.

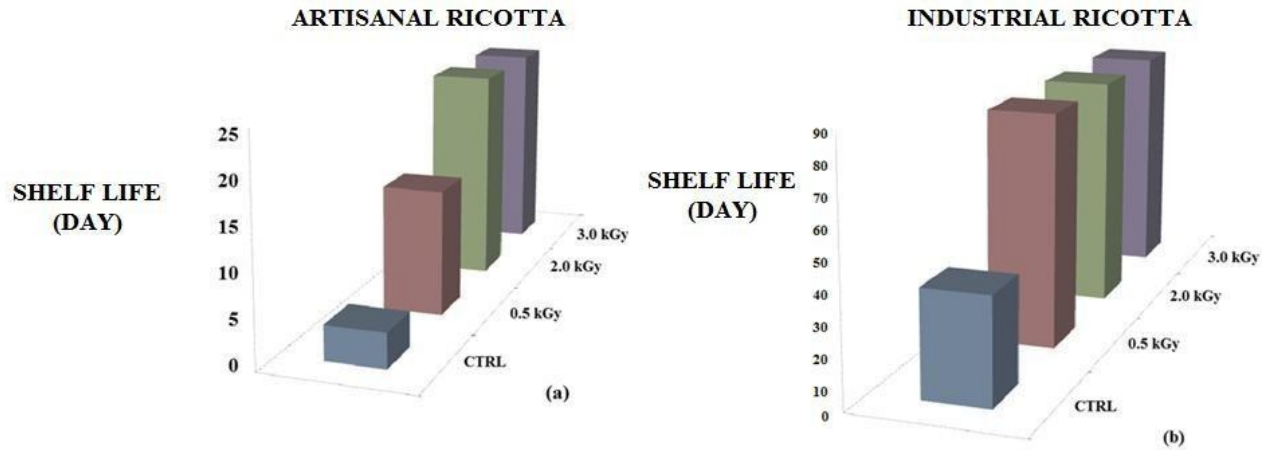


Figure 2.7: Shelf life of artisanal (a) and industrial (b) ricotta cheese treated at 0 (CTRL), 0.5, 2.0 and 3.0 kGy.

2.4 Conclusions

X-rays were successfully tested on artisanal and industrial ricotta. Specifically, for the artisanal product, the shelf life of the sample treated at 0.5 kGy reached 14 days of storage, compared with 3 days for the untreated sample. In comparison, samples treated at 2.0 and 3.0 kGy remained acceptable for more than 20 days. The industrial product, being subjected to thermal pasteurisation, reached a shelf life of about 40 days even without any treatment. Treated samples recorded a significant shelf life prolongation, up to 84 days without any defects from the microbiological and sensory point of view. This study highlights the great sanitising power of X-rays and the different effects according to the initial quality of the product. An intensity of 2.0 kGy, for both artisanal and industrial ricotta cheese, is enough to obtain excellent results.

Chapter 3

Pulsed Light applied to ricotta cheese

3.1 Aim of the study

This study was performed to test the action of pulsed light on fresh ricotta in order to verify the microbial breakdown and any sensory alteration compared to untreated product. In addition, a targeted study on protein analysis was also performed to also study the depth of penetration and the structural changes provoked to the whey proteins after treatment.

3.2 Matherials and Methods

3.2.1 Pulsed light treatments on ricotta cheese samples

Fresh cow's ricotta cheese was purchased from a local retailer “Caseificio Coderno” (Udine, Italy) and stored at 4 °C until the test. The samples were prepared by cutting the ricotta into cylinders presenting different surface/volume ratios. A 2.6 cm^{-1} surface/volume ratio was chosen to mimic the original geometry of commercial ricotta cheese available on the market. Thinner samples, presenting an increased surface/volume ratio of 3.1 and 5.6 cm^{-1}, were also prepared to emphasise PL surface effects. PL treatments were carried out at room temperature using a mobile PL decontamination unit (Claranor, Rouaine, France) equipped with 4 xenon lamps with maximum emission in the range 200–1000 nm (200–400 nm: 41%; 400–700 nm: 51%; 700–1000 nm: 8%), shown in fig. 3.1.

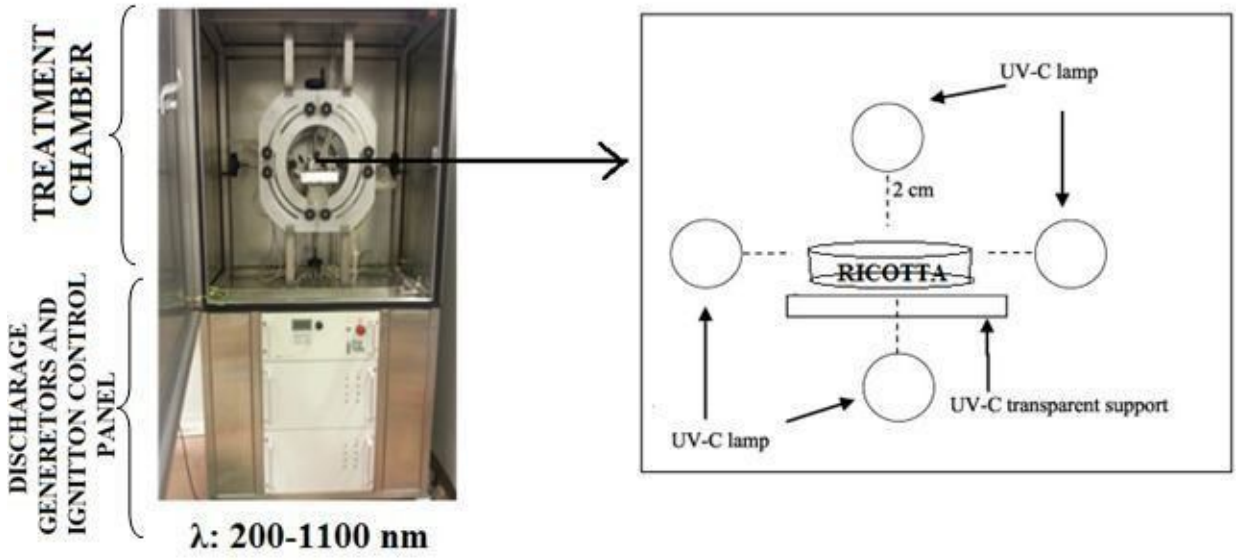


Figure 3.1: PL Claronor machine, with lamp system and physical space to place the sample for treatment.

The samples were placed on the quartz plate and exposed to an increasing fluence of light up to 15.0 J/cm^2, by increasing the voltage (0–3000 V) and the number of pulses up to 10. According to the manufacturer's instructions, each pulse at 1,000, 2000 and 3000 V delivered to the sample a light fluence of 0.26, 0.62 and 1.5 J/cm^2, respectively. Lamps were symmetrically positioned above, below and on the lateral sides of the sample at 2 cm distance. Pulse duration was 0.50 μs and the repetition rate was 0.50 Hz.

3.2.2 Temperature measurement and sample packaging

Surface sample temperature was measured immediately after the treatments by using a laboratory thermometer (Testo 805, Milano, Italy). The interval time between the end of the pulsed light treatment and the temperature measurement was less than 10 s. Then, samples were packed into PET/EVOH/PE (Savonitto, Italy) rigid food plastic trays, (13.5 × 9.6 × 4.4 cm) and sealed with a PET/PE film (0.064 mm, 26/70) (Profi2, Orved, VE, Italy). Ricotta cheese samples not exposed to any PL treatment were similarly packed and used as control. The ricotta cheese samples were stored at 4 °C up to more than 10 days. During storage, samples were removed from the refrigerated cell and subjected to the analyses.

3.2.3 Microbiological analyses

From each package, 10 g of ricotta cheese was taken, diluted with 90 mL of 0.9 g/100 mL NaCl solution in a stomacher bag and homogenized. Decimal dilutions of homogenates were prepared and plated on appropriate media in Petri dishes for the microbial count. For *Pseudomonas spp*. Enumeration, the Pseudomonas Agar Base (PAB, Oxoid), suitably modified with Pseudomonas C–F–C (SR0103E, Oxoid) selective supplement (Orion should, Milano, Italy) was used; the plates were

incubated for 48 h at 25 °C. The medium VRBG-Agar (Oxoid) was used for the enumeration of *Enterobacteriaceae*; the plates were incubated for 24 h at 37 °C; the evaluation of the mesophilic bacteria was carried out using the plate count agar APHA medium (Sacco), after incubating the plates for 48 h at 30 °C, while for the detection of yeasts the Sabouraud Agar (SAB) was adopted. *Bacillus cereus* was determined using the Agar Base culture medium PEMBA (Oxoid), adding the supplements Egg Yolk Emulsion (SR0047, Oxoid) and Polymyxin B (SR0099E, Oxoid). The pH of the homogenized samples was measured using a laboratory pH-meter (Crison BASIC 20, Barcelona, Spain).

3.2.4. Sensory analysis

Seven panellists, expert in the evaluation of dairy products, were asked to judge the sensory attributes of the ricotta cheese samples. At each sampling time, the panellists received ricotta cheese samples simultaneously and randomly identified with a three-digit code. Panellists were asked to judge colour, texture and odour using a 7-point scale, where score equal to 4 was identified as the threshold for sensory acceptability (Chen, Wolle, and Sommer, 2008).

3.2.5 UV-C light penetration depth

UV-C light penetration depth in ricotta cheese was determined photometrically using a luminometer (HD-2102.2 Delta Ohm, Padova, Italy) equipped with a UV-C light probe (LP471 UVC, Padova, Italy) as described by Manzocco et al. (2011). Ricotta cheese sections of increasing thickness up to 24 mm were manually cut by a sharp blade. Sample thickness was measured by a digital calibre (ABS Digimatic, Mitutoyo Corporation, Kawasaki, Japan). Ricotta cheese sections were positioned on the luminometer sensor and exposed to 15 W/m^2 UV-C light. The irradiance of the light

transmitted through ricotta was measured as previously reported for other food products (Lacivita et al., 2016; Manzocco et al., 2011). In particular, the ratio between transmitted light (I0) and incident light (I) was simply measured and fitted by the Beer-Lambert law (eq. (1)):

eq. 1: $I/I_0 = e^{-\alpha x}$

where x (mm) is the ricotta thickness and α (mm^{-1}) is an experimental parameter. The penetration depth (δ, mm) of UV-C light was quantified as the reciprocal of α.

3.2.6 Protein extraction

Ricotta cheese protein extracts were obtained according to the method described by Fernández et al. (2014) with minor adjustments. Ricotta cheese was mixed (30 min, 20 °C) with a 0.15 mol/L KCl solution (Sigma Aldrich, Milan, Italy) by applying a 1:5 sample:solvent ratio (Microstirrer magnetic stirrer, Velp scientifica, Usmate, Italy). The dispersion was then adjusted to pH 5.7 using HCl 0.1 mol/L (Carlo Erba, Milan, Italy). The protein pellet was collected by centrifugation at 13,000 g at 4 °C for 20 min (Beckman Avanti tm J-25, Beckman Instruments Inc., Palo Alto, California, USA), frozen at −80 °C for 24 h and freeze-dried for 72 h at 4053 Pa (Mini Fast 1700, Edwards Alto Vuoto, Milan, Italy). The obtained freeze-dried protein extracts were sealed in plastic trays and stored into a desiccator (P_2O_5, 5% RH) at room temperature until use.

3.2.7 Protein content

The protein content of protein extracts was determined using the bicinchoninic acid method (BCA) adapted to a 96-well microplate procedure. The working reagent (WR) was obtained by mixing “BCA Reagent A” (Sigma-Aldrich, Milan, Italy) and

"BCA-Reagent B" (Merck KGaA, Darmstadt, Germany) in a 50:1 ratio. Protein extracts were dispersed in deionized water (1.0 mg/mL) and 1 mL of dispersion was added with 200 μL NaOH 1 mol/L (Carlo Erba Reagent, Milan, Italy). Subsequently, 25 μL of the sample was mixed with 200 μL of WR and incubated at 37 °C for 30 min in the dark. Absorbance was measured at 562 nm using a microplate reader (Sunrise, Tecan, Männedorf, Switzerland). Water blanks were run in each assay. Protein concentration (g protein/100 g extract) was determined by comparison with a calibration curve built with bovine serum albumin (BSA) (Sigma-Aldrich, Milan, Italy) (0.0–2.0 mg/mL).

3.2.8 Protein extract solubility

The following gravimetric method was used. Protein extracts (100 mg) were suspended in 2 mL bi-distilled water and centrifuged at 10,000 g at 20 °C for 10 min (Mikro 120, Hettich Italia S. r.l. Milan, Italy). The pellet was dehydrated under vacuum (10 kPa, 8 h, 75 °C) (Vuotomatic 50, Bicasa, Milan, Italy) and exactly weighted. The difference between the initial extract weight and the pellet weight was used to quantify the amount of soluble material contained in 100 g of protein extract.

3.2.9 Particle size distribution

The extracts were dispersed in bi-distilled water (0.01 g/mL) and the particle size distribution was assessed, according to Fayaz, Plazzotta, Calligaris, Manzocco, and Nicoli (2019), by using the Zetasizer Nano ZS instrument (Malvern, Milan, Italy), by setting the observation angle at 173°. The solution refractive index and viscosity were set at 1.333 and 0.00088 Pa s, respectively, corresponding to the values of pure water at 25 °C. Mean particle size diameter and peak area corresponding to volume distribution were assessed.

3.2.10 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli (1970). An amount of extract containing 3 mg of proteins (based on BCA assay, paragraph 2.7) was mixed with 500 μL of Laemmli Sample Buffer 2 × (Bio-Rad Laboratories, Inc., Hercules, California, US) in the presence or absence of 10 μL of the reducing agent β-mercaptoethanol (Bio-Rad Laboratories, Inc., Hercules, California, US), incubated for 1 h at 20 °C, heated at 95 °C for 5 min and centrifuged 10 min at 10,000 g at 20 °C (Mikro 120, Hettich Italia S. r.l., Milan, Italy). Ten μL of the prepared sample was loaded into SDS-PAGE pre-stained gels (Mini-PROTEAN TGX Stain-Free Gels, Bio-Rad Laboratories, Inc., Hercules, California, US) and the electrophoresis was performed at 30 mA for 2 h. A bioanalytical imaging system (G:Box Chemi XX9, Syngene, Cambridge, UK) was used to see the protein lanes and the software GeneSys (Syngene, Cambridge, UK) was used to take the gel images. Protein identification was based on the comparison with protein standards in the molecular weight range 10–250 kDa (Precision Plus Protein Standards, Kaleidoscope, Bio-Rad Laboratories, Inc., Hercules, California, US).

3.2.11 Absorbance

Protein extract absorbance was measured according to the methods described by Manzocco, Panozzo, and Nicoli (2013), with the proper adjustments. In particular, the protein extracts were dispersed (5 mg/mL) in 0.1 M phosphate buffer at pH 12.0 and stirred for 20 min at 20 °C in a Microstirrer magnetic stirrer (VELP scientifica, Usmate, Italy). Absorbance was measured at 280 and 380 nm using a UV-2501 PC UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan).

3.2.12 Free SH groups

Free SH groups were determined according to the method described by Beveridge, Toma, and Nakai (1974), and Ellman (1959), and calculated as follows (eq. (2)) (Beveridge et al., 1974):

eq. 2: $\mu MSH/g = 73.53 * A_{412} * D/C$

where A412 is the absorbance at 412 nm, C is the sample concentration (mg extract/mL), D is the dilution factor (2) and 73.53 is derived from 106/(1.36 × 104), where 1.36 × 104 is the molar absorptivity of Ellman's reagent.

3.3.13 Fourier transform infrared spectroscopy (FTIR) measurement

FTIR spectra of protein extracts were recorded at 25 °C using an FTIR spectrometer equipped with an ART accessory and a Zn–Se crystal that allowed the collection of spectra directly on the sample, without sample preparation (Alpha-P, Bruker Optics, Milan, Italy). Background scan of the clean Zn–Se crystal was acquired before sample scanning. Spectra were obtained in the wavenumber range from 4000 to 400 cm^{-1} at a spectrum resolution of 4 cm^{-1} and with 32 co-added scans. The second derivative of the amide I band (1600-1700 cm^{-1}) was obtained by using OPUS software (version 7.0, Bruker Optics, Milan, Italy).

3.3.14 Carbonyl groups

Protein carbonyls were quantified according to the method described by Cui, Xiong, Kong, Zhao, and Liu (2012). Protein carbonyls were calculated according to the following equation: eq. 3.

eq.3: $C(nmol/mL) = A_{370}*45.45$

where 45.45 derives from the DNPH molar extinction coefficient (Reznick and Packer, 1994). The carbonyl content was expressed as nmol carbonyls/mg protein. To determine the protein content of each sample, the absorbance at 280 nm of the blank samples was compared to that of a calibration curve built with BSA in urea 6 mol/L (0.2–2.0 mg/mL).

3.3.15 Data analysis and MAL calculation

All determinations were expressed as the mean ± standard deviation (SD) of at least three repeated measurements from two experiment replicates. Statistical analysis was performed by one-way ANOVA test. A Duncan's multiple range test, with the option of homogeneous groups ($p < 0.05$), was used to determine significance among differences (STATISTICA v. 7.1 for Windows, StatSoft Inc., Tulsa, OK, USA). A modified version of the Gompertz equation was fitted to microbiological data of *Enterobacteriaceae*, *Pseudomonas spp*. and yeasts to estimate the microbial acceptability limit (MAL), representing the number of days necessary to reach the microbiological limit (Conte, Gammariello, Di Giulio, Attanasio, and Del Nobile, 2009). The threshold for *Enterobacteriaceae* was set equal to 10^4 CFU/g, while 10^6 CFU/g for the other two spoilage groups. The overall microbiological acceptability of the samples was calculated as the lowest MAL among those obtained for the different microorganisms (Gammariello et al., 2011; Gammariello et al., 2009; Lacivita et al., 2016). Experimental data of the ratio between transmitted light (I0) and incident light (I) were fitted to the Beer-Lambert equation (eq. (1)) by non-linear regression procedures using TableCurve2D software (Jandel Scientific, ver. 5.01). The fittings were calculated at a 95% significance level and the goodness of fit was evaluated based on statistical parameters of fitting (R^2).

3.3 Results and Discussion

3.3.1 Effect of pulsed light on microbial inactivation and sensory properties of fresh ricotta cheese

Ricotta cheese samples presented a surface/volume ratio analogous to that of the product available on the market (2.6 cm^{-1}). The samples were subjected to PL treatments at increasing fluence up to 15.0 J/cm^2. Sample temperature after the treatments increased with the increase of PL fluence but always remained below 23 °C. The inactivation effects of PL treatments on *Pseudomonas spp.*, *Enterobacteriaceae* and yeast load of ricotta cheese samples are reported in Fig. 3.2. By contrast, *B. cereus* always resulted below the detection limit (10^2 CFU/g). These spoilage groups were chosen as the main responsible factors for product deterioration (Ricciardi et al., 2019). Within the first days of storage, all the treated samples presented *Pseudomonas spp.*, *Enterobacteriaceae* and yeast loads lower than those of the untreated sample, thus confirming literature data on the decontamination effect of PL (Heinrich, Zunabovic, Bergmair, Kneifel, and Jäger, 2015). During storage, while the samples treated at 3.1 J/cm^2 and 7.5 J/cm^2 showed a microbial growth significantly lower than that of the untreated cheese, a further increase of PL fluence up to 15.0 J/cm^2 showed lower effectiveness on all the spoilage groups. Microbiological experimental data were fitted to calculate the microbial acceptability limit (MAL) of the ricotta cheese samples, considering a threshold value of 10^4 CFU/g and 10^6 CFU/g for *Enterobacteriaceae* and *Pseudomonas spp*. and yeasts, respectively. The fitting curves are also reported in Fig. 3.2.

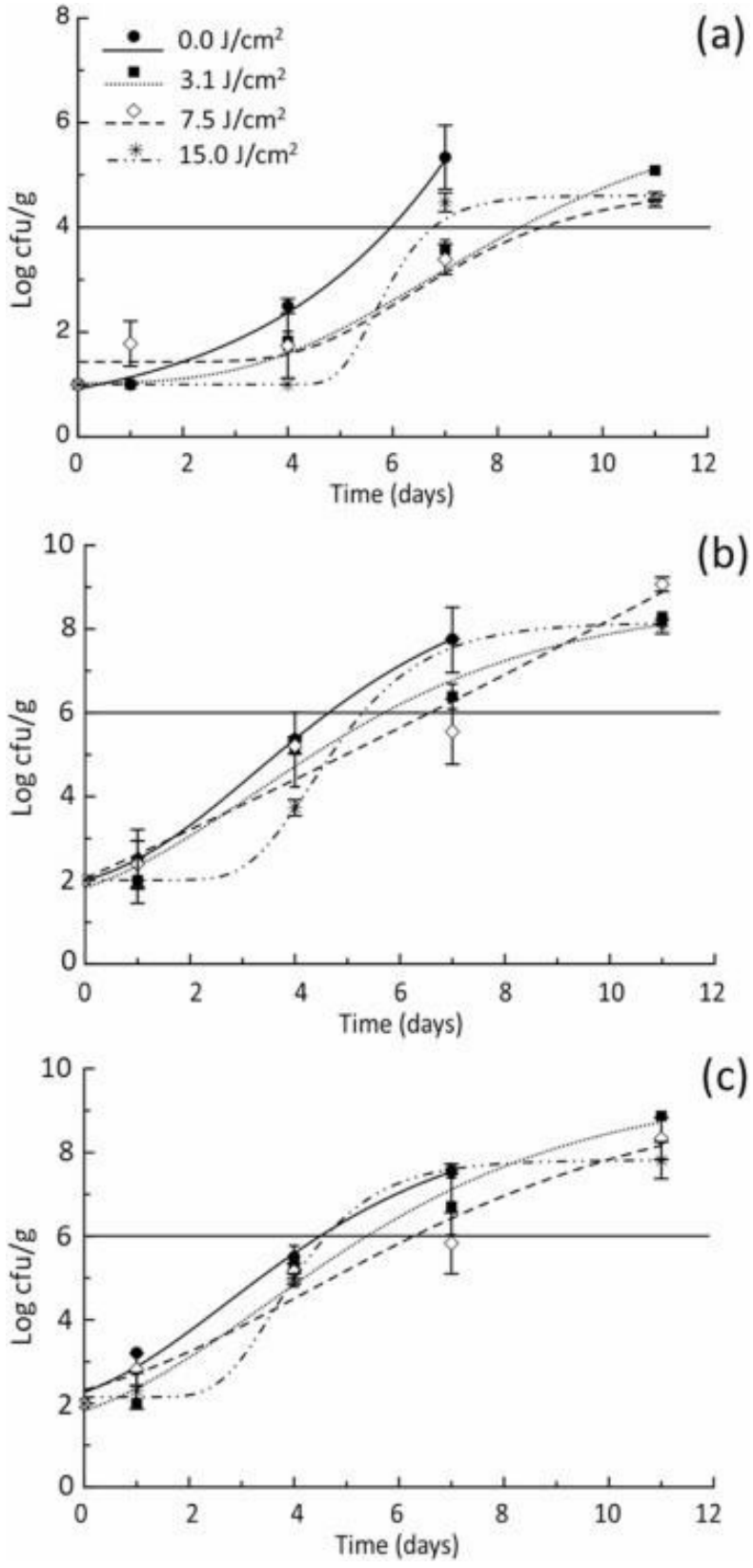


Fig. 3.2. Evolution of *Enterobacteriaceae* (a), *Pseudomonas spp.* (b) and yeasts (c) in ricotta cheese samples (surface/volume ratio = 2.6 cm^{-1}) subjected to pulsed light treatments at increasing fluence (0.0; 3.1; 7.5; 15.0 J/cm^2). Symbols: experimental data; Lines: best-fit of experimental data; Horizontal line: microbiological limit.

The chosen model well-fitted the experimental data, as indicated by the χ^2, which always resulted lower than 1. In terms of microbial stability, the untreated ricotta cheese and the samples treated at 15.0 J/cm^2 remained acceptable for about 4 days, while the other samples treated at 3.1 and 7.5 J/cm^2 reached the microbial acceptability limit at about 5 and 6 days, respectively, and yeast proliferation was the main factor affecting microbiological quality (Fig. 3.2c). The lack of a relationship between the increase of PL fluence and the decontamination efficacy can be explained considering that, at the microscopic level, the surface of ricotta cheese is characterized by local roughness, which is expected to obscure the microbial cells, compromising the germicidal effect of PL (Pedrós-Garrido et al., 2018). Moreover, PL probably presented a very low transmittance through ricotta cheese tissue (Lacivita et al., 2016). To confirm this hypothesis, measurements of light transmittance through ricotta cheese samples with increasing thickness were performed (Supplementary Figure S1). The attention was focused on the UV-C light component of PL, which is the one affecting microorganisms and biomolecules mostly. These measures allowed estimating a penetration depth of UV-C light into ricotta cheese equal to 1.5 mm (R2 = 0.991). It is therefore likely that the PL effect was limited to a thin surface layer of the treated product. To verify the role of sample thickness on microbial decontamination, an additional experimental step was performed on ricotta cheese samples presenting increasing surface/volume ratio (3.1, 5.6 cm−1) at a fixed PL fluence (3.1 J/cm2). Based on a 1.5 mm penetration depth, in a sample having a 5.6 cm−1 surface/volume ratio, the light was able to reach the sample core, thus ensuring the decontamination of the entire mass sample. As shown in Tab. 3.1, the decontamination efficacy of PL increased with the surface/volume ratio. In particular, data fitting allowed identifying MAL values that significantly increased ($p<0.05$) with the surface/volume ration for all the considered spoilage groups.

Table 3.1. Microbiological acceptability limit (MAL) of ricotta cheese samples of increasing surface/volume ratio (2.6; 3.1; 5.6 cm^{-1}) subjected to 3.1 J/cm^2-pulsed light treatments.

Surface/volume ratio (cm^{-1})	MAL ^{Enterobacteriaceae} (day)	MAL $^{Pseudomonas\ spp.}$ (day)	MAL ^{Yeasts} (day)
2.6	8.42 ± 0.55^{ab}	5.69 ± 0.58^{a}	5.39 ± 0.54^{a}
3.1	8.73 ± 0.88^{bc}	5.57 ± 0.25^{a}	5.57 ± 0.33^{a}
5.6	9.77 ± 0.77^{c}	6.97 ± 0.15^{b}	7.20 ± 0.60^{b}

Data points Means ± SD (n = 2); $^{a, b, c}$: in each column, means identified by different letters are statistically different ($p < 0.05$).

Ricotta cheese samples having the typical surface/volume ratio of the commercial product (2.6 cm^{-1}) were also subjected to sensory analysis, to assess the effect of increasing PL fluence on ricotta cheese quality. Judges did not highlight differences among samples in terms of colour and texture (data not shown). By contrast, a great worsening of the odour was perceived when PL fluence was increased above 3.1 J/cm^2 (Fig. 3.3).

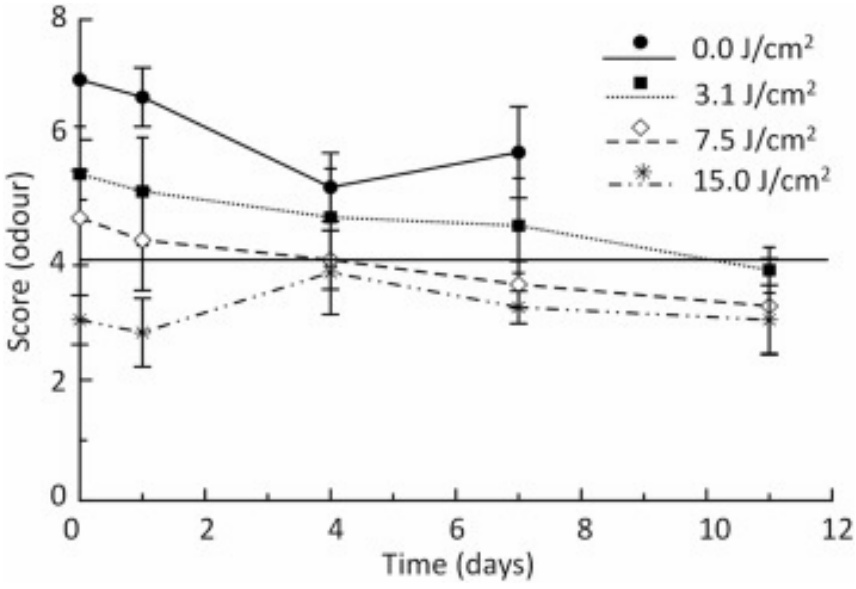


Fig. 3.3 Odour sensory score of ricotta cheese samples (surface/volume ratio = 2.6 cm^{-1}) subjected to pulsed light treatments at increasing fluence (0.0; 3.1; 7.5; 15.0 J/cm^2). The broken lines have been added as a guide for the eyes to evidence the trend; Horizontal line: acceptability limit.

These additional experimental findings, together with microbiological results (Fig. 2), underline that a PL fluence of 7.5 J/cm^2 or higher provoked significant changes in the product. These data agree with previous literature, in which these PL effects were attributed to the modification of the components of the treated matrix (Proulx et al., 2017). To this regard, Fernández et al. (2014) indicated that PL treatments with a fluence higher than 8.4 J/cm^2 induced protein modification in both bovine serum albumin solutions and cheese slices. In the case of ricotta cheese, PL-induced protein modifications could have led to both a higher availability of nutrients for microbial growth, possibly explaining data reported in Fig. 1, and to the development of off-odours (Fig. 3). It must be noted that the lack of abundant literature data on these specific aspects did not allow a deep understanding of the involved phenomena. For this reason, a further investigation of protein structure and composition was carried out, as described in paragraph 3.2.

3.3.2 Effect of pulse light on fresh ricotta proteins

The second part of the study aimed at understanding the effect of PL treatments on the protein fraction of ricotta cheese. Since PL effects have been demonstrated to be restricted to a thin surface layer of the product, treatments were applied on ricotta slices having the highest surface/volume ratio (5.6 cm^{-1}) to ensure that all the sample was reached by the light radiation. Table 3.2 shows the protein content of the protein extracts of ricotta cheese exposed to increasing PL fluence. As compared to paragraph 3.1, an additional fluence value (1.3 J/cm^2) was taken into considerations, to allow a more detailed evaluation of the effects of PL on ricotta cheese proteins.

Table 3.2. Protein content, free SH groups, absorbance at 280 nm, solubility, absorbance at 380 nm and carbonyl content of protein extracts obtained from ricotta cheese subjected to pulsed light treatments at increasing fluence (0.0; 1.3; 3.1; 7.5; 15.0 J/cm^2).

Fluence (J/cm^2)	Protein content (g/100 g extract)	Free SH (μM/g protein)	Absorbance at 280 nm/mg protein	Solubility (g soluble material/100 g extract)	Absorbance at 380 nm/mg protein	Carbonyls (nmol/mg protein)
0.0	84.2 ± 0.6^{a}	18.1 ± 0.6^{b}	0.038 ± 0.002^{c}	16.5 ± 0.5^{a}	0.106 ± 0.007^{d}	38.5 ± 0.8^{d}
1.3	70.8 ± 0.8^{b}	20.8 ± 0.3^{ab}	0.046 ± 0.002^{b}	13.4 ± 0.5^{b}	0.136 ± 0.006^{c}	47.0 ± 1.3^{c}
3.1	70.3 ± 1.0^{b}	20.6 ± 0.6^{ab}	0.044 ± 0.002^{bc}	13.5 ± 0.4^{b}	0.158 ± 0.004^{b}	51.9 ± 2.1^{ab}
7.5	70.0 ± 1.1^{b}	21.4 ± 0.2^{a}	0.039 ± 0.006^{c}	14.1 ± 0.6^{b}	0.168 ± 0.004^{a}	52.3 ± 1.2^{b}
15.0	64.5 ± 0.9^{c}	17.5 ± 0.4^{c}	0.059 ± 0.006^{a}	12.4 ± 0.3^{c}	0.163 ± 0.004^{ab}	55.4 ± 1.7^{a}

Data points Means ± SD (n = 2); $^{a, b, c, d}$: in each column, means identified by different letters are statistically different ($p < 0.05$).

A significant decrease in the protein content of the extracts was observed with the increase in the PL fluence. It can be inferred that PL favoured the formation of protein interactions with other ricotta components, such as lipids and carbohydrates, possibly hampering protein extraction. This hypothesis was confirmed by FTIR analysis (Fig. 3.4a). All samples showed the typical bands associated to the presence of proteins (1600-1700 cm^{-1}), but peaks at 600–1300, 1750 and 2800-3000 cm^{-1}, relevant to the presence of lipids and polysaccharides, were also detected (Gurdeniz, Tokatli and Ozen, 2007; Kačuráková and Wilson, 2001; Lei et al., 2010; Rohman and Che Man, 2012). The increase in PL fluence was associated with a progressive increase of the intensity of these peaks, confirming that PL favoured the interaction of proteins with other components.

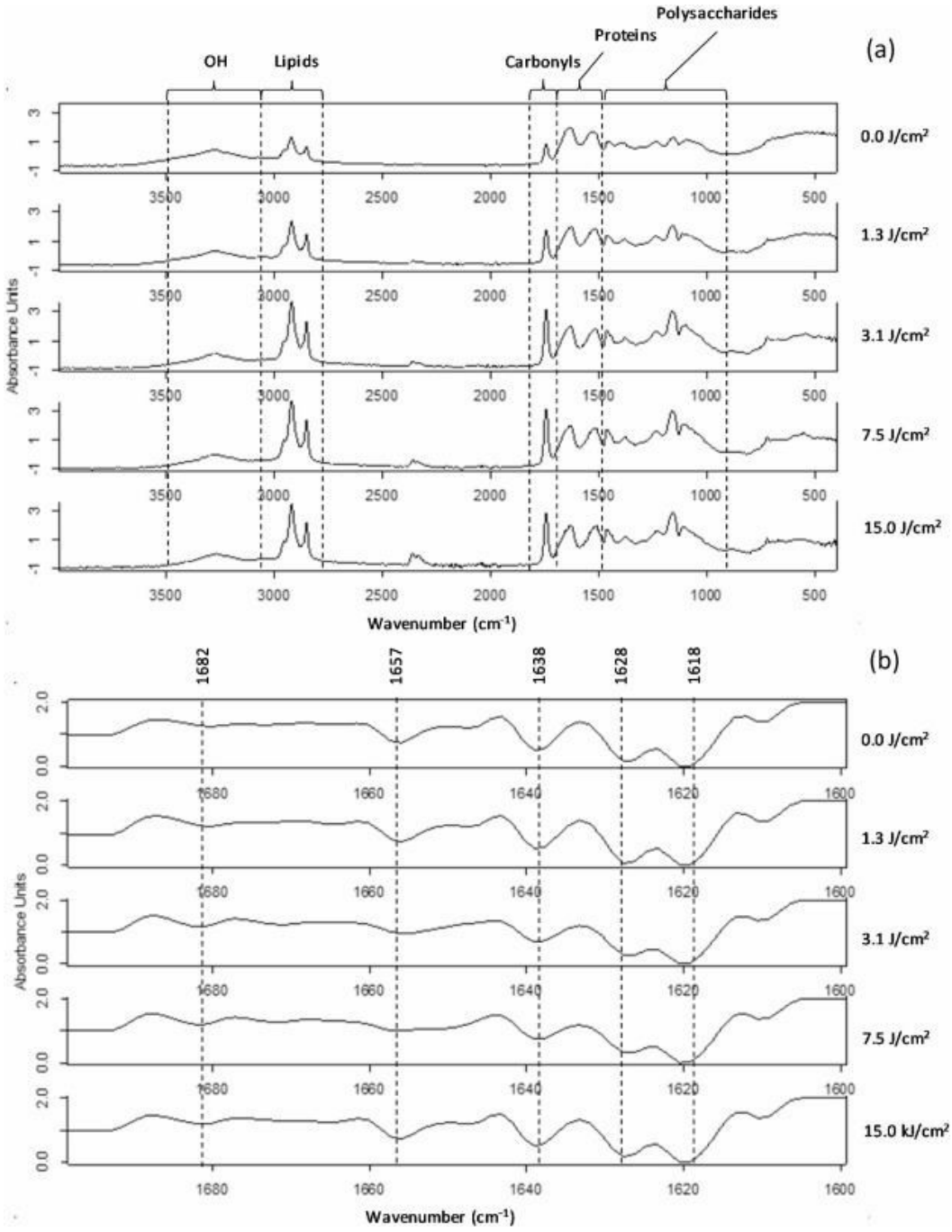


Figure 3.4. FTIR spectra (a) and second derivative of the Amide I band (b) of protein extracts obtained from ricotta cheese subjected to pulsed light treatments at increasing fluence (0.0; 1.3; 3.1; 7.5; 15.0 J/cm^2).

The presence of aggregates was confirmed by dynamic light scattering (DLS) (Fig. 4). DLS spectra of the untreated sample showed the presence of the typical whey protein aggregates, formed upon thermal treatment during ricotta cheese production, characterized by a diameter of about 1 μm (Fig. 3.5). Additional families of particles at about 300 nm and 10 μm were also detected. PL treatments at increasing fluence

reduced the occurrence of 1 μm particles and increased the frequency of small (<100 nm) and large (about 10 μm) aggregates. Thus, PL probably induced the breakage of the typical ricotta particles, forming small fragments, able to further interact and rearrange into large aggregates. In this regard, the increase in PL fluence from 4 to 16 J/cm^2 has been previously associated with aggregation phenomena in whey protein model solutions (Siddique, Maresca, Pataro, and Ferrari, 2017).

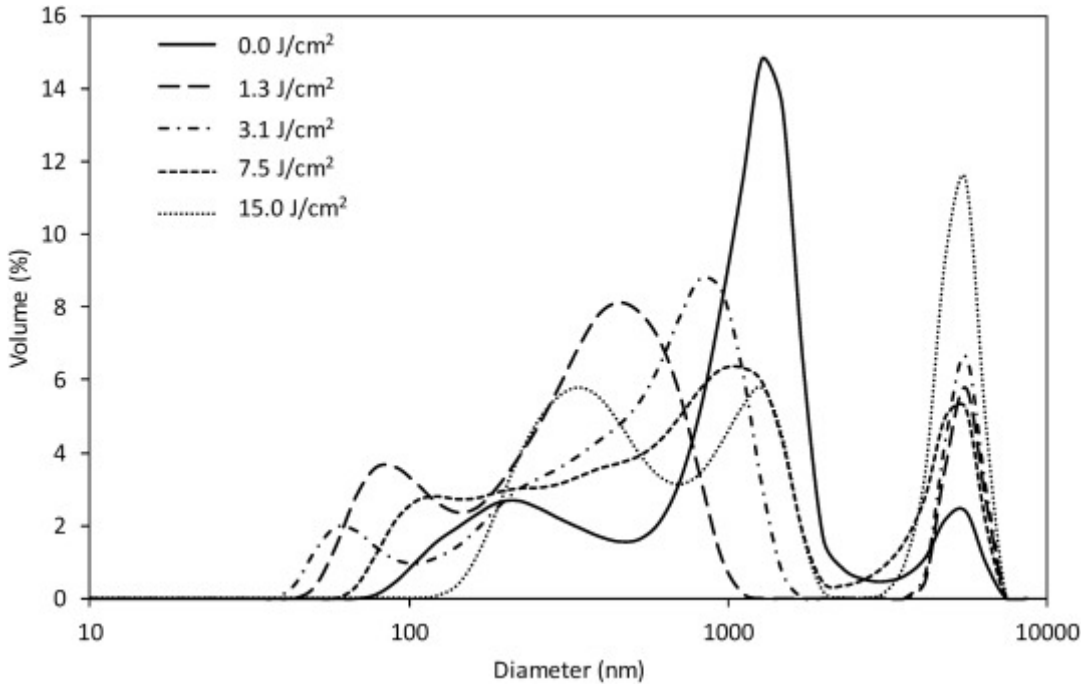


Figure 3.5. Size distribution of particles of protein extracts obtained from ricotta cheese subjected to pulsed light treatments at increasing fluence (0.0; 1.3; 3.1; 7.5; 15.0 J/cm^2).

To confirm the protein aggregation effect of PL, SDS-PAGE analysis was carried out (Fig. 3.6). The SDS-PAGE patterns in the presence of a reducing agent evidenced the presence of the typical milk proteins (Costa et al., 2014; Elmnasser et al., 2008; Jovanovic, Barac, Macej, Vucic, and Lacnjevac, 2007): lactoferrin (Lf), bovine serum albumin (BSA), immunoglobulin (Ig), α, β e k casein, β-lactoglobulin (β-Lg) e α-lactalbumin (α-La). Moreover, aggregates of about 150 kDa were observed in all the samples. Most of the protein bands were not visible in SDS-PAGE patterns obtained

in not-reducing conditions (Fig. 6). In this case, only the proteins not involved in the formation of S–S stabilized aggregates were visible. This confirms that most proteins in ricotta cheese are organized in aggregates stabilized by S–S bonds. Under non-reducing conditions, the bands relevant to BSA, caseins and β-Lg in the PL-treated sample resulted less intense than in the untreated cheese, thus suggesting their involvement into aggregates with a molecular weight exceeding 250 kDa (gel separation range).

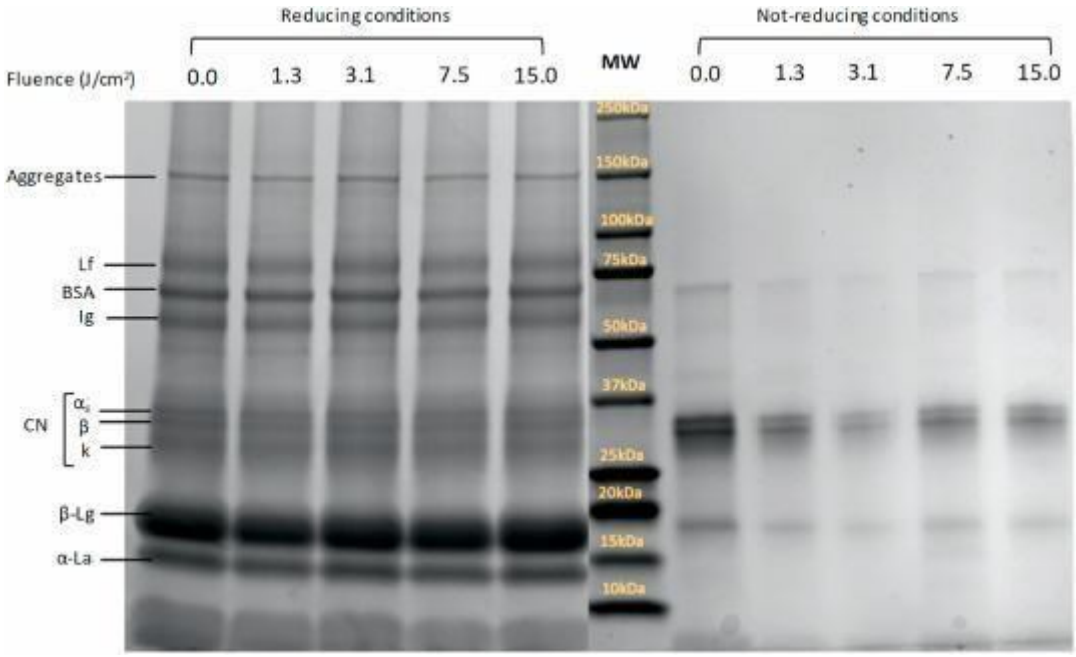


Figure 3.6. SDS-PAGE patterns of protein extracts obtained from ricotta cheese subjected to pulsed light treatments at increasing fluence (0.0; 1.3; 3.1; 7.5; 15.0 J/cm^2), under reducing and non-reducing conditions. MW = molecular weight standards, Lf = Lactoferrin, BSA=Bovine serum albumin, Ig = Immunoglobulin, CN = casein, β-Lg = β-lactoglobulin, α-La = α-lactalbumin.

To further investigate the effects of PL on ricotta cheese proteins, the extracts were analysed for their content in free SH groups and exposure of tyrosine and tryptophan residues through the measure of the absorbance at 3 nm (Table 3.2). Obtained results indicate that the most intense PL treatment was associated with a significant increase of absorbance at 280 nm and a concomitant decrease in SH groups. Such results suggest that PL promoted a rearrangement of protein structure, by exposing

tryptophan and tyrosine residues and increasing S–S bonds. In turn, this indicates a higher hydrophobicity and S–S stabilized aggregation of proteins, in agreement with the decrease of protein solubility observed with the increase of PL fluence (Table 3.2). Similar results were obtained by Siddique et al. (2017) and Elmnasser et al. (2008), who found that PL treatments of whey protein solutions and milk could induce protein aggregation, stabilized by S–S.

Protein aggregation was further confirmed by the analysis of the II derivative of FTIR spectra in the Amide I range (1600-1700 cm^{-1}) (Fig. 3.4b). The spectrum of untreated ricotta showed the typical bands of β-sheet (1618-1640 cm^{-1}) and α-helix (1648-1657 cm^{-1}) structures of whey proteins (Haque, Aldred, Chen, Barrow and Adhikari, 2014; O'Loughlin, Kelly, Murray, Fitzgerald, and Brodkorb, 2015). As compared to the untreated sample, the spectra of PL-treated protein extracts showed a visible change in the α-helix structures (1657 cm^{-1}), as well as a new peak at about 1680 cm^{-1}. The latter has been previously associated to the formation of aggregates through intermolecular β-sheets stabilized by hydrogen bonding (de Vries, Lopez Gomez, Jansen, van der Linden, and Scholten, 2017; Ngarize, Herman, Adams, and Howell, 2004).

Ricotta protein extracts were further analysed to evaluate if the effect of PL treatments on proteins could be associated with the observed alterations in ricotta quality. In particular, the absorbance at 380 nm was assessed, as an indicator of photo-induced browning (Manzocco et al., 2013) (Table 3.2). The increase in PL treatment fluence led to a significant increase in absorbance, possibly indicating the formation of melanoidins. This agrees with literature studies showing that the UV-C light component of PL can induce the formation of brown compounds in both model solutions (Sheldon, Shibamoto and Daniel Jones, 1988) and protein-rich food ingredients, such as egg white (Manzocco et al., 2013). Moreover, the increase of the peak at 1747 cm^{-1} in the FTIR spectra, which is due to the vibrations of the C=O bond (Gurdeniz et al., 2007; Lei et al., 2010), might suggest not only a higher lipid concentration in the extracts but also the development of protein oxidation. To

confirm this hypothesis, samples were analysed for the content in carbonyls through the DNPH assay (Table 3.2), which is commonly used as an indicator of oxidative degradation of proteins (Suzuki, Carini and Butterfield, 2010). Obtained results show a progressive increase of protein carbonyls with the increase of PL fluence, thus confirming the photo-induced oxidation of proteins. These results agree with the study of Scheidegger, Pecora, Radici, and Kivatinitz (2010) and Fernández et al. (2014), who observed an increase of protein carbonyls upon UV-C light treatment of milk and PL-treatment of cheese slices, respectively. As reported in the literature, protein oxidation is related to the development of off-flavours (Reinemann, Gouws, Cilliers, Houck, and Bishop, 2006), possibly explaining the results obtained by sensory evaluation of ricotta cheese samples subjected to PL-decontamination (Fig. 3.3).

3.4 Conclusions

Results demonstrate that PL could be used to decontaminate the surface of ricotta cheese. However, the successful implementation of PL technology for industrial use will depend on the identification of proper processing conditions, able to decontaminate the product and delay microbial growth without causing adverse quality changes. In the case of protein-rich foods, such as ricotta cheese, PL treatments exceeding a given fluence could promote intense quality depletion, mainly associated with photo-induced modification of proteins. In the literature the study of the effects of light on food protein structure and reactivity has been mostly focused on enzymatic inactivation or protein reactivity in model systems. The fundamental role of the food matrix in protein photoreactivity should, however, modify this paradigm, allowing a better understanding of the interactions among light and proteins in real foods.

Chapter 4

UV-C and NUVL applied to ricotta cheese

4.1 Aim of the study

This study was aimed to study the efficacy/efficiency of two surface technologies such as Ultraviolet light (UV-C) and Near-Ultraviolet-Visible-Light (NUVL) in a comparative mode, using similar intensities and comparing the results obtained with respect to the control sample. A preliminary test was also carried out on industrial ricotta samples inoculated at different concentrations with *Pseudomonas Fluorescens*.

4.2 Materials and Methods

4.2.1 Sample preparation

Ricotta cheese (2.5 kg) was purchased at a local supermarket (Tesco, Dublin, Ireland) and transported to the laboratory under refrigerated conditions. The ricotta was manipulated under aseptic conditions to prepare the experimental samples. Cheese was placed into small Petri dishes (diameter 5.5 cm, thickness 1.3 cm, surface 57 cm^2, volume 30.87 cm^3, surface/volume (S/V) ratio 1.84 cm^{-1}, net weight 24.24 g) to use a container with a high surface/volume ratio and increase the efficacy of the two non-thermal treatments to be applied. Two tests were made: a preliminary test to study the influence of bacterial concentration on the decontaminating effect of UV-C and NUVL and a subsequent shelf life test to assess the effect of the two technologies on the quality of bacteria-inoculated ricotta during storage. The preliminary test was performed with a total of 12 ricotta samples, differently inoculated and then treated by UV-C and NUV–vis, subjected to MAP (30% CO_2/70% N_2) using a tray sealer (Ilpra, Cheshire, UK), and stored at 4 °C. Microbial enumeration after treatments was carried out to evaluate the inactivation level. For the shelf life test, a total of 36 ricotta samples were inoculated at a specific microbial inoculum concentration, treated by UV-C and NUV–vis, subjected to MAP (30% CO_2/70% N_2), stored at 4 °C, and analyzed during the subsequent 9 days for microbiological and sensory quality; individual cheese samples were available at each time point. In both preliminary and shelf life tests, control samples of ricotta (inoculated but not treated) were also considered.

4.2.2 Sample Inoculation

To inoculate the ricotta samples, different concentrations of *P. fluorescens* were used.

To prepare the inoculum, a *P. fluorescens* (DSM 50090, type strain, DSMZ-German Collection of Microorganisms and Cell Cultures GmbH) culture was prepared in 10 mL of sterilized Tryptic Soy Broth (TSB, Oxoid, Basingstoke, UK) and then incubated at 25 °C for 24 h. Then, the culture was diluted to adjust the microbial concentration, which was measured with a spectrophotometer (UV-mini1240, Spectrophotometer, Shimadzu, Duisburg, Germany). In the preliminary test, samples of ricotta were inoculated with 10^5, 10^4, and 10^3 CFU/mL. For the shelf life test, the concentration selected for the inoculums was 10^3 CFU/mL. For the inoculation, 50 µL of microbial suspension was spread on the product surface, and the suspension was completely absorbed by the cheese. The time between inoculation and treatment was about 1 h for all samples.

4.2.3 UV-C and NUVL Treatment

The samples were treated once, on the same day; this corresponded to time zero of the storage period. In both preliminary and shelf life tests, only one light dose per technology was tested. The UV unit was custom-made, with internal dimensions of 790 × 390 × 345 mm (L×W×H) and consisted of four 95-W bulbs of 50 cm length (Baro Applied Technology Limited, Manchester, UK) as shown in fig. 4.1.

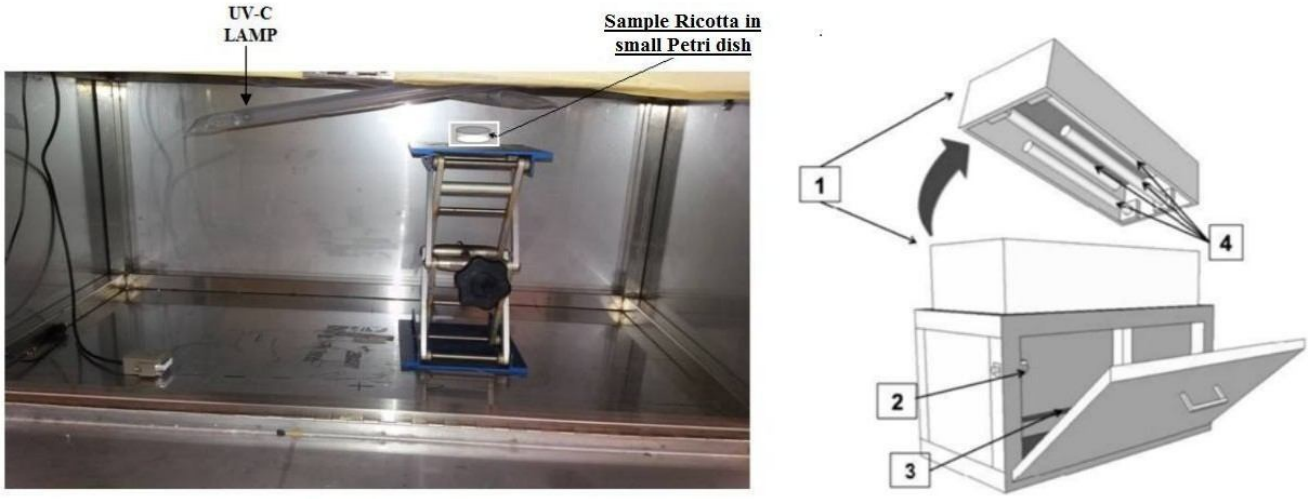


Figure 4.1: Layout of UV treatment unit. 1, using for UV light; 2, safety interlock; 3, treatment chamber with dimensions (length, width, and height) of 790 by 390 by 345 mm; 4, UV light (95 W) 500 mm in length.

An initial characterization of the UV chamber was performed at a total of 27 carefully selected locations within the chamber, using a radiometer ILT 1700 (International Light Technologies, Boston, MA, USA) connected to a solar blind vacuum photodiode detector (configuration SED240/NS254/W) for measuring the energy delivered at 254 nm. The aim of these measurements was to obtain the actual energy received by the sample, which may vary depending on the sample position within the chamber, its distance from the lamps, and the treatment time. To treat the samples, a specific position to reach a final dose of 6.54 J/cm^2 (3.5 cm from the lamp for 30 s) was selected.

The NUVL was produced by a LED array (OD-2049) (Opto Diode Corp, sourced from AP Technologies, Bath, UK) with a center wavelength of 395 ± 5 nm, a bandwidth of 12 nm full-width at half maximum (FWHM), and a half intensity beam angle of 30° (Haughton et al., 2012) as shown in fig. 4.2.

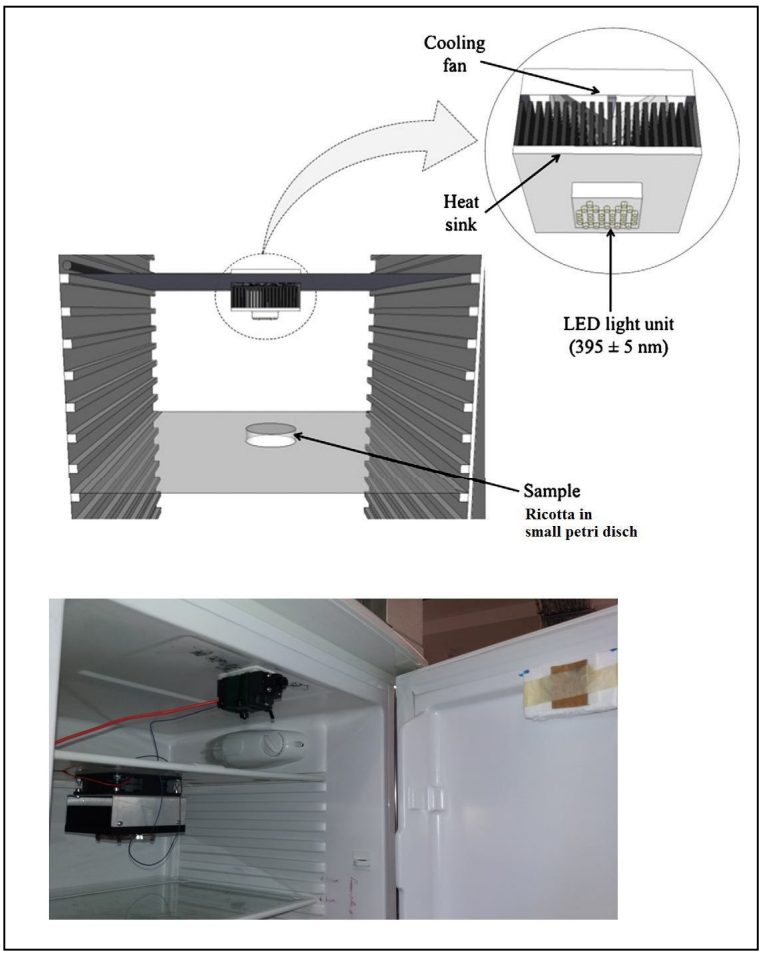


Figure 4.2: Hight intensity near ultraviolet/visible light unit (395 ±5 nm).

The irradiance of light emitted from the LED unit (J/cm^2) was measured using a UV–VIS radiometer (model no. RM12, Dr. Gröbel UV Electronik, GmbH, Ettlington, Germany) fitted with an RM UV-A sensor (part no. 811030, Dr. Gröbel UV Electronik) at different locations within the unit. Based on the equipment characterization results, all treatments of the samples were performed in a central position, at 6 cm from the LED light source (perpendicular light incidence) for 400 s, applying a total dose of 6.36 J/cm^2.

4.2.4 Shelf Life calculation

During shelf life, bacterial enumeration was carried out. To this aim, from each package, 10 g of ricotta was taken, diluted with 90 mL of a 0.9% NaCl solution in a

stomacher bag (Blander Blags, Cona, Ferrara, Italy), and homogenized (400 circulators—Seward, Hamilton, Islandia, New York, USA) for 60 s. Subsequently, appropriate decimal dilutions of the homogenates were made for microbial counts, using the same diluent. *Pseudomonas spp*. enumeration was performed on Pseudomonas CFC (Cetrimide, Fucidin, Cephalotin) selective agar (Oxoid), after incubating the plates for 48 h at 25 °C. *Enterobacteriaceae* counts were carried out using VRBGA (violet-red bile glucose agar) medium (Aucmedia Lab 088), after incubating the plates for 24 h at 37 °C (Lacivita et al., 2016), while Sabouraud Agar (SAB) (Nogen Culture Media NCM 2012A) was used for the detection of yeasts (Gammariello et al., 2014). *Mesophilic* bacteria were enumerated using PCA (plate count agar) medium (Scharlau), after incubating the plates were for 48 h at 30 °C. Finally, *B. cereus* counts were performed using B. cereus Agar Base (PEMBA, Oxoid) culture medium, adding egg yolk emulsion (SR0047, Oxoid) and polymyxin B supplement (SR0099E, Oxoid) (Ricciardi et al., 2019). All analyses were performed at least in duplicate for each sample.

To calculate the microbial acceptability limit, indicated as MAL, a modified version of the Gompertz equation was used to fit *Pseudomonas spp*. and yeast counts as limiting microbial groups for ricotta shelf life (Equation (1)):

$$\log(N(t)) = \log(N_{max}) - A\cdot\exp\left\{-\exp\left\{\left[(\mu_{max}\cdot 2.71)\cdot\frac{\lambda - MAL}{A}\right] + 1\right\}\right\} + A\cdot\exp\left\{-\exp\left\{\left[(\mu_{max}\cdot 2.71)\cdot\frac{\lambda - t}{A}\right] + 1\right\}\right\} \quad (1)$$

where N(t) is the viable cell concentration at time t, A is related to the difference between the decimal logarithm of maximum bacteria growth attained at the stationary phase and the decimal logarithm of the initial value of cell concentration, μ_{max} is the maximal specific growth rate, λ is the lag time, N_{max} is the microbial threshold value, MAL is the microbiological acceptability limit (i.e., the time at which N(t) is equal to N_{max}), and t is the storage time (Ricciardi et al., 2019; Gammariello et al., 2014). The value of N_{max} was set to 10^7 cfu/g for both *Pseudomonas spp*. and yeasts because literature data confirm that this level of contamination is compatible with a possible

alteration of the product (Spanu et al., 2018; Fleet, 1990). The goodness of fitting was evaluated by the χ^2 value.

A sensory screening of ricotta samples was also performed by a group consisting of seven people, members of the laboratory of the School of Agriculture and Food Science of the University College of Dublin. Panelists were asked to judge odor, color, consistency, and overall quality, using a 7-point scale. The score of 4 was set as the minimum threshold for product acceptance (Chen et al., 2009). Analyses were performed at least in duplicate for each sample. The sensory acceptability limit, indicated as SAL, was calculated to establish the remaining number of days the cheese was sensory-acceptable (Ricciardi et al., 2019; Lacivita et al., 2016). To this aim, the same modified version of the Gompertz equation was used to fit the sensory experimental data, by setting the score 4 as the threshold for product acceptability (Equation (2)):

$$OSQ(t) = OSQ_{min} - A^Q \cdot \exp\left\{-\exp\left\{\left[\left(\mu_{max}^Q \cdot 2.71\right) \cdot \frac{\lambda^Q - SAL}{A^Q}\right] + 1\right\}\right\} + A^Q \cdot \exp\left\{-\exp\left\{\left[\left(\mu_{max}^Q \cdot 2.71\right) \cdot \frac{\lambda^Q - t}{A^Q}\right] + 1\right\}\right\} \quad (2)$$

where OSQ(t) is the ricotta overall sensory quality at time t, A^Q is related to the difference between the ricotta overall quality attained at the stationary phase and the initial value of ricotta quality, μ_{max} is the maximal rate at which OSQ(t) decreases, λ^Q is the lag time, OSQ_{min} is the threshold for sensory acceptability, SAL is the sensory acceptability limit (i.e., the time at which OSQ(t) is equal to OSQ_{min}), and t is the storage time. The goodness of fitting was evaluated by the χ^2 value.

For the shelf life estimation, it was considered that wherever the global quality of a food product depends on several quality sub-indices, its shelf life is, by definition, the time at which one of the product quality sub-indices reaches the threshold. Therefore, in the current study, the shelf life of each tested sample was calculated as the lowest value among the three fitting parameters, i.e., the two MAL values and the SAL value.

4.2.5 Experimental Plan and Statistical Analysis

Tests were carried out on duplicate batches. Experimental data are the average of two replicates. Fitting parameters were compared by one-way ANOVA. A Duncan's multiple range test, with the option of homogeneous groups ($p < 0.05$), was used to determine significance among differences. All these analyses were performed with Statistica 7.1 for Windows 152 (StatSoft Inc., Tulsa, OK, USA).

4.3 Results and Discussion

4.3.1 Effects of UV-C and NUVL

In the preliminary test, prior to the shelf life study, ricotta samples inoculated with different concentrations of *P. fluorescens* (i.e., 10^3, 10^4, and 10^5 CFU/mL) were exposed to UV-C and NUV–vis treatments. Thus, the influence of the bacterial concentration on the decontaminating effect of both technologies was assessed. The inactivation levels of *Pseudomonas spp*. after these treatments are shown in Fig. 4.3. *Pseudomonas spp*. levels in cheese after inoculation with 10^3, 10^4, and 10^5 CFU/mL were 3.6 ±0.1, 4.4 ±0.1, and 5.7 ±0.03 CFU/g, respectively. Exposure to UV-C and NUV–vis light caused a decrease of the microbial population below the detection limits (<100 CFU/g) when 10^3 and 10^4 CFU/mL were used as inoculation levels. When 10^5 CFU/mL were inoculated, the inactivation levels were −1.03 ±0.02 and −2.26 ±0.04 CFU/g after UV-C and NUV–vis treatments, respectively. These results coincide with those of other authors who also found that the effectiveness of ultraviolet light depends on several factors, such as the characteristics of species and bacterial strains, growth rate, initial bacterial population density, method of inoculation, composition, and food type (Hyun et al., 2020). Specifically, the method of inoculation and the concentration of cells added can affect the inactivation efficiency when light treatments are used, due to the formation of agglomerates, clusters, or layers of bacterial cells that cause a shadowing effect, impeding light penetration and bacterial inactivation (Bottino et al., 2016; Pedrós-Garrido et al, 2018). Furthermore, it is also important to consider that ricotta is a food matrix with surface irregularities, which may act as physical protection against light, contributing to bacterial survival. At a microscopic level, the surface of ricotta is not smooth but characterized by roughness, which is expected to shade microbial cells, further compromising the germicidal effect of light (Lacivita et al., 2016).

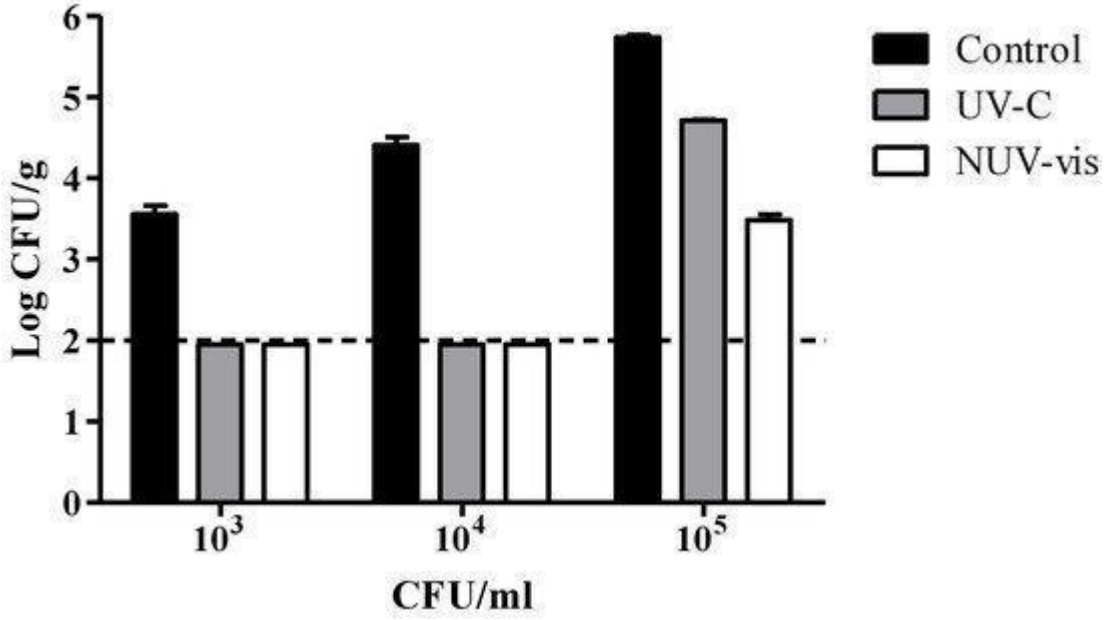


Figure 4.3: Mean log colony-forming units (CFU/g) *of Pseudomonas* spp. recorded in the preliminary test in ricotta cheese inoculated with three different concentrations (10^3, 10^4, and 10^5 CFU/mL) of *Pseudomonas fluorescens* (DSM 50090) immediately after treatments with ultraviolet light (UV-C) and near-UV–visible light (NUV–vis) at one light dose per technology, with their respective controls (inoculated and not treated). A total of 12 ricotta samples were treated once, on the same day, with UV-C and NUV–vis. The dashed horizontal line represents the detection limit (≥100 CFU/g). Each bar represents mean ± SD.

It has also been demonstrated that UV-C light can promote protein degradation, thus increasing nutrient bioavailability for the remaining bacteria (Koutchma, 2014; Guerrero-Beltran et al., 2004). All these reasons suggest that the application of doses (J/cm^2) higher than those required for microbial inactivation does not increase the decontamination efficiency. Therefore, considering this set of factors, the inoculum concentration for the subsequent shelf life test was that with the lowest initial bacterial population density.

4.3.2 Shelf Life of Ricotta Cheese Treated by UV-C and NUVL

During shelf life, *Enterobacteriaceae, Pseudomonas spp.*, yeasts, mesophilic bacteria, and *B. cereus* were monitored in all treated and untreated samples. *B. cereus* was not detected in any of the samples during shelf life, as also reported by Ricciardi

et al. (2019), who studied the effects of X-ray irradiation on ricotta cheese shelf life. *Enterobacteriaceae* counts increased from values below the detection limit to 3 log CFU/g in the control cheese and to 2 log CFU/g in the treated samples. The experimental findings recorded for treated cheese are supported by the literature, because also the study of Allende et al. (2006) confirmed that *Enterobacteriaceae* are more photosensitive than other bacteria.

Fig. 4.4 shows counts of *Pseudomonas spp*. recorded in ricotta samples treated with UV-C and NUVL with respect to controls (untreated) during shelf life. Similarly to the results found in the preliminary study, the initial level of *Pseudomonas* spp. before treatments was 3.74 ±0.08 log CFU/g, and after both treatments the microbial levels were below the detection limit. Over the shelf life, these values remained between 1 and 3 log below the control, and after 5 days the levels were 5.24 ±0.14 and 4.40 ±0.70 log CFU/g for UV-C and NUVL, respectively, while for the control, they were 7.55 ±0.10 log CFU/g. Therefore, while the untreated samples reached the threshold after 4 days, treated ricotta remained acceptable for more than 6 days. Manzocco at al. (2015) also proved the efficacy of UV-C light on *Pseudomonas spp.* in fresh-cut pineapple; they observed great effects of treatment during the first days of storage; however, over the shelf life, microbial counts of all treated samples approached those recorded in the control fruit.

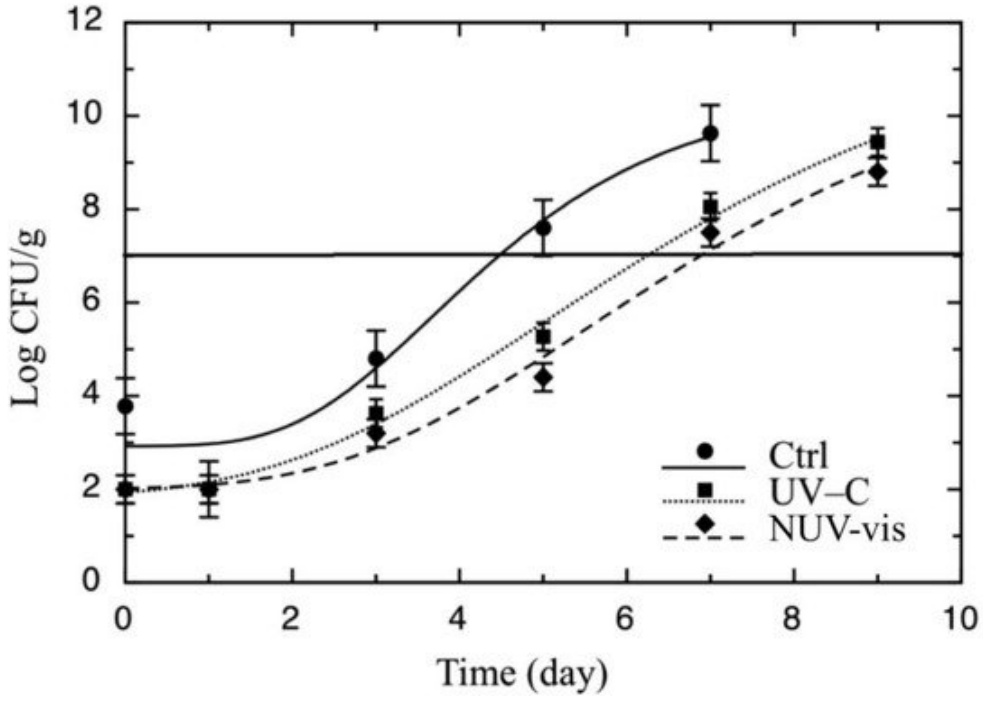


Figure 4.4: Evolution of *Pseudomonas* spp. during the shelf life test in ricotta cheese inoculated with *P. fluorescens* (DSM 50090) (10^3 CFU/mL), treated with UV-C and NUV–vis light, at one light dose per technology, with respective control samples (Ctrl, inoculated, and not treated). A total of 36 samples were treated once, on the same day, which corresponded to time zero of the storage period, and then analyzed for 9 days. The continuous line represents the best fit of Equation (1) to the experimental counts of *Pseudomonas* spp. The horizontal line represents the threshold for microbiological acceptability set to 7 log CFU/g.

As reported in the Materials and Methods section, microbiological data related to *Pseudomonas spp*. were fitted by a mathematical approach (Equation (1)) to calculate the remaining number of days the cheese was microbiologically acceptable (Ricciardi et al., 2019; Lacivita et al., 2016). The re-parameterized form of the Gompertz equation is useful for the purpose of the present study. It contains, among the parameters, the MAL value, which represents the number of days necessary to reach the limit that we established (10^7 CFU/g). Data of the fitting procedure are reported in Tab. 4.1. Comparing the results of MAL for *Pseudomonas spp*. of the treated samples reported in the Table, similar values were recorded for UV-C light and NUV–vis light, thus suggesting that both technologies exerted comparable effects against *Pseudomonas spp*., which was also found in our preliminary test. Other studies carried out with different dairy products treated by surface-acting technologies recorded similar effects on *Pseudomonas spp*. For example, Lacivita et al. (2016)

assessed the effect of UV-C light to control surface contamination and extend the shelf life of Fior di latte cheese. They inoculated the 'pasta filata' cheese with *Pseudomonas spp*. and then exposed it to 0.1, 0.6, 1.2, and 6.0 kJ/m^2 of UV-C light, packaged it with brine, stored it at 9 °C, and analyzed it for microbial growth and sensory quality. A germicidal effect between 1 and 2 log cycles was observed during storage. In this study, penetration depth was also measured by a luminometer equipped with a UV-C light probe. A very low UV-C light transmittance through the food tissue and an antimicrobial effect limited to a very thin surface layer of the product were found in that study. Similarly, in the current research a surface action of UV radiation was hypothesized.

Table 4.1. Microbiological acceptability limit (MAL) and sensory acceptability limit (SAL) measured on a day as fitting parameters of Equations (1) and (2) to microbiological and sensory experimental data, respectively. The goodness of fitting was evaluated by the χ^2 value. The values of shelf life are the lowest value among the two MAL and the SAL for each sample. Ultraviolet light-treated cheese = UV-C; Near-UV-visible light-treated cheese = NUV–vis; Control sample (inoculated and not treated) = Ctrl.

Samples	MAL^{Yeasts} (day)	$MAL^{Pseudomonas\ spp.}$ (day)	$SAL^{Overall\ Quality}$ (day)	Shelf Life (day)
Ctrl	5.82 ± 0.19^{b} ($\chi^2 = 0.06$)	4.46 ± 0.86^{b} ($\chi^2 = 1.75$)	4.50 ± 0.40^{b} ($\chi^2 = 0.20$)	4.46 ± 0.86^{b}
UV-C	6.53 ± 0.25^{a} ($\chi^2 = 0.42$)	6.23 ± 0.25^{a} ($\chi^2 = 0.24$)	8.02 ± 1.08^{a} ($\chi^2 = 0.78$)	6.23 ± 0.25^{a}
NUV–vis	6.81 ± 0.17^{a} ($\chi^2 = 0.15$)	6.87 ± 0.45^{a} ($\chi^2 = 0.45$)	6.78 ± 0.42^{a} ($\chi^2 = 0.30$)	6.78 ± 0.42^{a}

^{a,b} Data in each column with different letters are significantly different ($p < 0.05$).

Hyun and Lee (2020) investigated the effect of 460–470 nm light-emitting diode illumination (LED 460–470 nm) against pathogenic and spoilage bacteria on the surface of agar media and packaged sliced cheese. Among spoilage bacteria, inoculated *P. fluorescens* showed a high inhibition after treatments. LED 460–470 nm treatments were performed at 4 and 25 °C, and microbial reduction levels were higher at 4 °C than at 25 °C, because at lower temperatures cellular injury, cell membrane disruption, and loss of cytoplasmic components were observed.

Fig. 4.5 represents yeast counts over the shelf life for treated and untreated cheese samples. Yeast populations grew in all ricotta samples, and within the first week, all of them crossed the threshold, which was set to 7 log CFU/g because it was

previously observed that yeast proliferation above 6–7 CFU/g can produce important sensory defects (Fleet, 1990). Differences were found between control and treated cheese: control ricotta samples became unacceptable after 5 days, whereas treated cheese remained acceptable after 6–7 days (Table 1). Regarding mesophilic bacteria (data not shown), similar trends to those reported for *Pseudomonas spp*. were recorded.

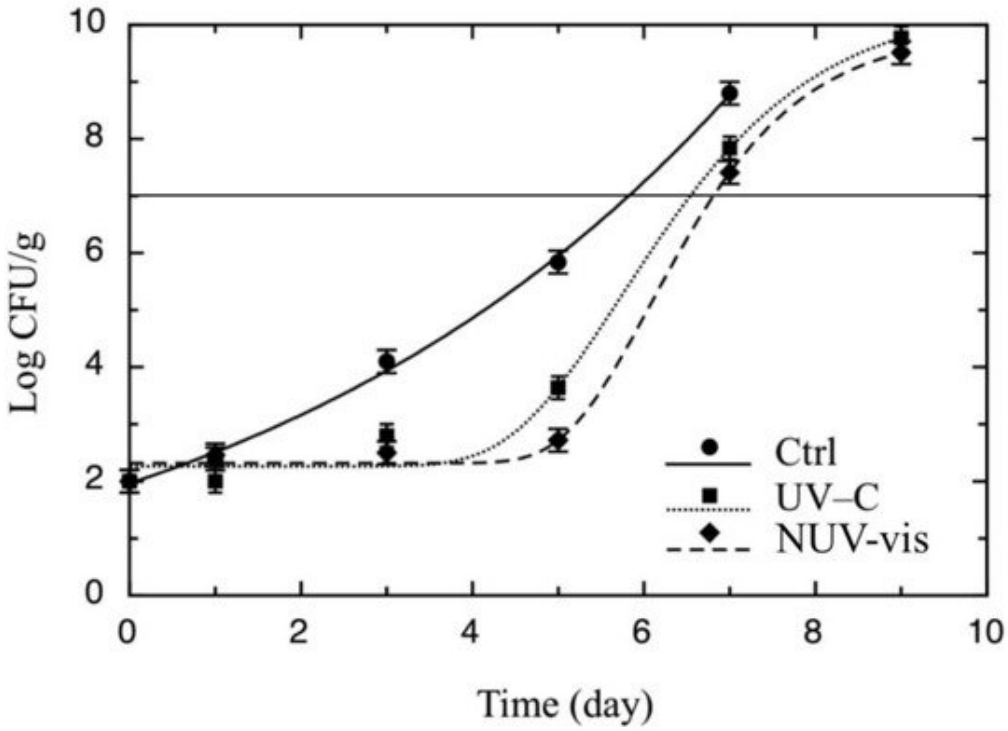


Figure 4.5: Evolution of yeasts during the shelf life test in ricotta cheese inoculated with *P. fluorescens* (DSM 50090) (10^3 CFU/mL), treated with UV-C and NUV–vis light, at one light dose per technology, with respective control samples (Ctrl). A total of 36 samples were treated once, on the same day, which corresponded to time zero of the storage period, and then analyzed for 9 days. The continuous line represents the best fit of Equation (1) to the experimental counts of yeasts. The horizontal line represents the threshold for microbiological acceptability, set to 7 Log CFU/g.

The effects of the two technologies on the sensory quality of cheese were also evaluated. Results of the panel referring to the overall quality, which represents the mean values of odor, color, and consistency of ricotta cheese, are presented in Figure 4.6. Two considerations are necessary to underline differences among samples, which are referred to the quality either just after the treatments or during storage. Cheese quality scores immediately after both treatments were slightly lower than for control

samples, because panelists detected some off-odours. This off-odor perception in samples treated with UV-C could be attributed to photo-induced changes, e.g., photo reactivity of proteins, which was also attested previously (Lacivita et al., 2016), being ricotta a food very rich in amino acids (Salvatore et al., 2014). For NUVL-treated samples, these detected off-odors may be due to the sample exposure for 400 s would increase the surface temperature, thus affecting the product characteristics and in particular the smell perceived just after the treatment (Reinemann et al., 2006). However, this initial apparent defect disappeared during storage, and it was possible to observe two different kinetics in terms of acceptability for the treated and untreated samples, over shelf life. Control cheese samples became unacceptable after ~5 days, due to a general product spoilage which mainly affected odor and color (Pintado et al., 2001). UV-C- and NUVL treated samples remained acceptable for 7–8 days, with a very similar trend. This would suggest that the application of these light technologies in combination with proper refrigeration and packaging conditions, could contribute to the color, odor, and texture preservation of ricotta cheese for more days. Hyun and Lee (2020) also reported that it may be important to find the appropriate conditions of irradiance and storage temperature to minimize sensory quality changes for each individual type of food.

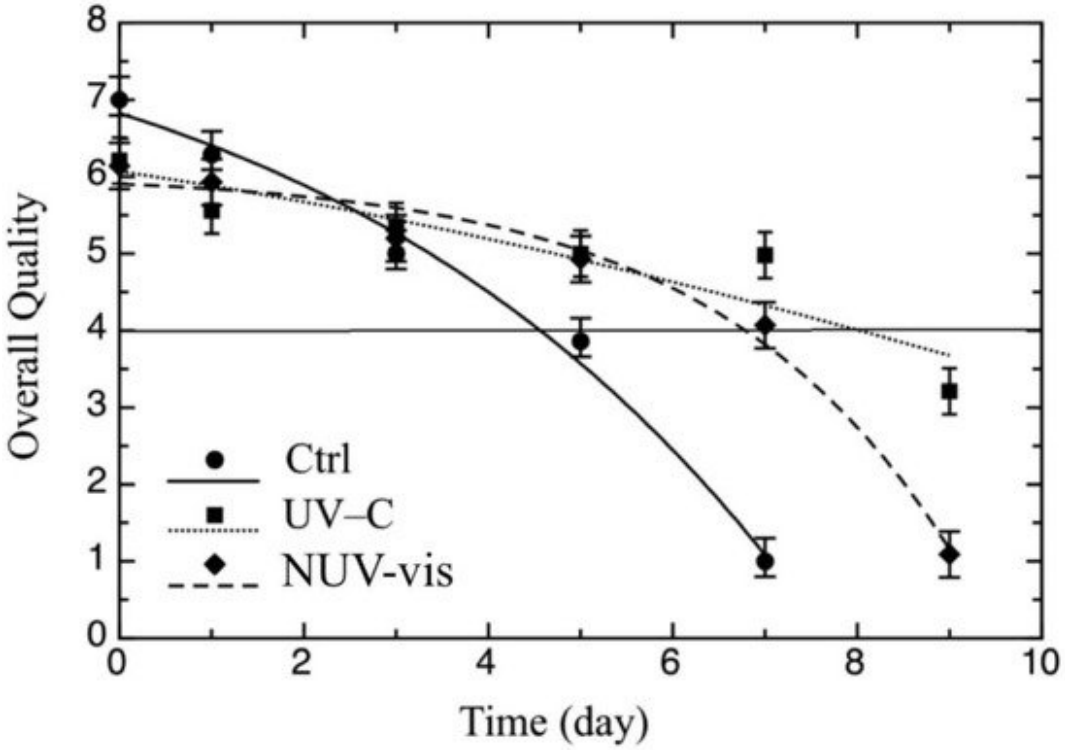


Figure 4.6: Evolution of sensory quality during shelf life test of ricotta inoculated with *P. fluorescens* (DSM 50090) (10^3 CFU/mL), treated with UV-C and NUV–vis light, at one light dose per technology, with respective control samples (Ctrl). A total of 36 samples were treated once, on the same day, which corresponded to time zero of the storage period, and then analyzed for 9 days. The continuous line represents the best fitting of Equation (2) to the experimental data of sensory quality. The horizontal line represents the threshold for overall quality acceptability set to 4.

Color is one of the most important quality parameters used to evaluate food quality with light-based technologies (Haunghton et al., 2011; Srimagal, Ramesh and Sahu, 2016). However, in the present study, the two types of treatments did not affect cheese color, which was detected sensorially during storage (data not shown). Data from sensory results were also fitted to calculate SAL. The re-parameterized Gompertz equation (equation 2) was used because one of the fitting parameters was SAL, i.e., the number of days necessary to reach the score of 4, which represents the threshold for product acceptability. Results of the fitting procedure of the sensory data are also reported in Table 1, as SAL values of the three samples. Treated cheese recorded general acceptability values higher than those of control samples; in particular, while the SAL value of untreated cheese was 4.5 ±0.4 days, the values recorded for treated cheese were 2 or 3 days higher.

Both microbiological and sensory fitting values (MAL and SAL) were taken into account for shelf life estimation (Conte et al., 2009). Results in terms of shelf life (Table 1) showed that the treated samples had a longer shelf life than the control, in agreement with other previous studies, where the effects of photo-sanitizing technologies on product quality were also evaluated (Xu et al., 2016; Loi et al., 2019; Donsingha and Assatarakul, 2018). In particular, the untreated product became unacceptable after 4 days of storage, whereas treated ricotta recorded a shelf life of more than 6 days. Data also evidenced that UV-C and NUVL are technologies with very comparable efficacy. The preservation effects of these two sanitizing technologies on microbial and sensory quality make them feasible to be used, also considering the intentional inoculation.

4.4 Conclusions

The effects of UV-C and NUVL were assessed on contaminated ricotta cheese. The effectiveness of these treatments was compared after similar treatment doses (J/cm^2). Both microbial and sensory quality were assessed during subsequent refrigerated storage. From the results, it is possible to conclude that both surface-decontaminating technologies affected microbial proliferation and controlled sensory deterioration with comparable efficacy. Therefore, the final shelf lives of inoculated control and treated cheese were significantly different, and in particular, a shelf life extension was recorded for both types of treated cheese. The efficacy recorded in the study would suggest further investigation on industrial ricotta cheese, with the final aim of substituting the traditional thermal treatment with appropriate combinations of novel mild sanitizing technologies. In addition, it is also worth considering that further research could be still carried out, because both techniques could be further optimized to extend ricotta shelf life. Different process parameters, such as distance of the sample from the lamps, sample position in the treatment cell, and treatment time,

could be experimentally changed so as to reach an even better shelf life extension. The current results are interesting not only for their novelty but also because the application of these optimized surface techniques is promising for the entire dairy sector and applicable to other fresh products with higher added value than ricotta cheese.

Chapter 5

Cold Plasma applied to fresh ricotta cheese

5.1 Aim of the study

The aim of this study was to explore the role of ROS (Reactive Oxygen Species) and RNS (Reactive Nitrogen Species) generated by cold plasma for bacterial killing in ricotta cheese. To reduce the potential loss of nutrients or the reduction of organoleptic quality, in our study the characteristics of ricotta directly exposed to plasma were compared with those indirectly exposed to the action of plasma. In the latter case, the gas supply was activated by the plasma, enriched with ROS and RNS and gently flowed over the food to be treated using a sealed system. In this way it is assumed that only long-lived species are involved in the potential inactivation of bacteria and the effects of UV radiation. The effect of dielectric barrier discharges fed with nitrogen and small quantities of oxygen used for direct application and with treated gas on artisanal ricotta was then evaluated. For this purpose, after an adequate treatment of the cheese, both the microbiological and sensorial quality were monitored during storage.

5.2. Materials and methods

5.2.1 Ricotta cheese sample preparation

Artisanal ricotta cheese (1.5 Kg) was purchased the same day of its production, at a local retailer "Fattoria Posta la Via" (Foggia, Italy) and transported to the laboratory. The ricotta was produced by thermal treatment and acidification of whey proteins. Then, it was slightly dehydrated and vacuum-packed. At the laboratory the ricotta was manipulated under aseptic conditions to prepare the experimental samples. To the aim, 40 g of cheese were placed into small sterile tubes (diameter 5.6 cm, volume 100 cm^3) (Kartell, Milano, Italy) to use a packaging suitable for plasma treatments. Two tests were made to assess the effects of plasma on cheese quality during storage, taking also into account the variability of artisanal ricotta in terms of initial microbial contamination level. Both tests were performed with a total of 42 ricotta samples, 28 of them plasma-treated and the rest used as control cheese.

5.2.2 Plasma treatments applied to ricotta cheese

In both tests, samples were treated once, in the same day, that corresponded to the time zero of the storage period. Two types of treatments were applied: plasma-treated gas (PTG) and direct plasma (DP), following the schematic overview reported respectively in Figure 5.1a and Figure 5.1b.

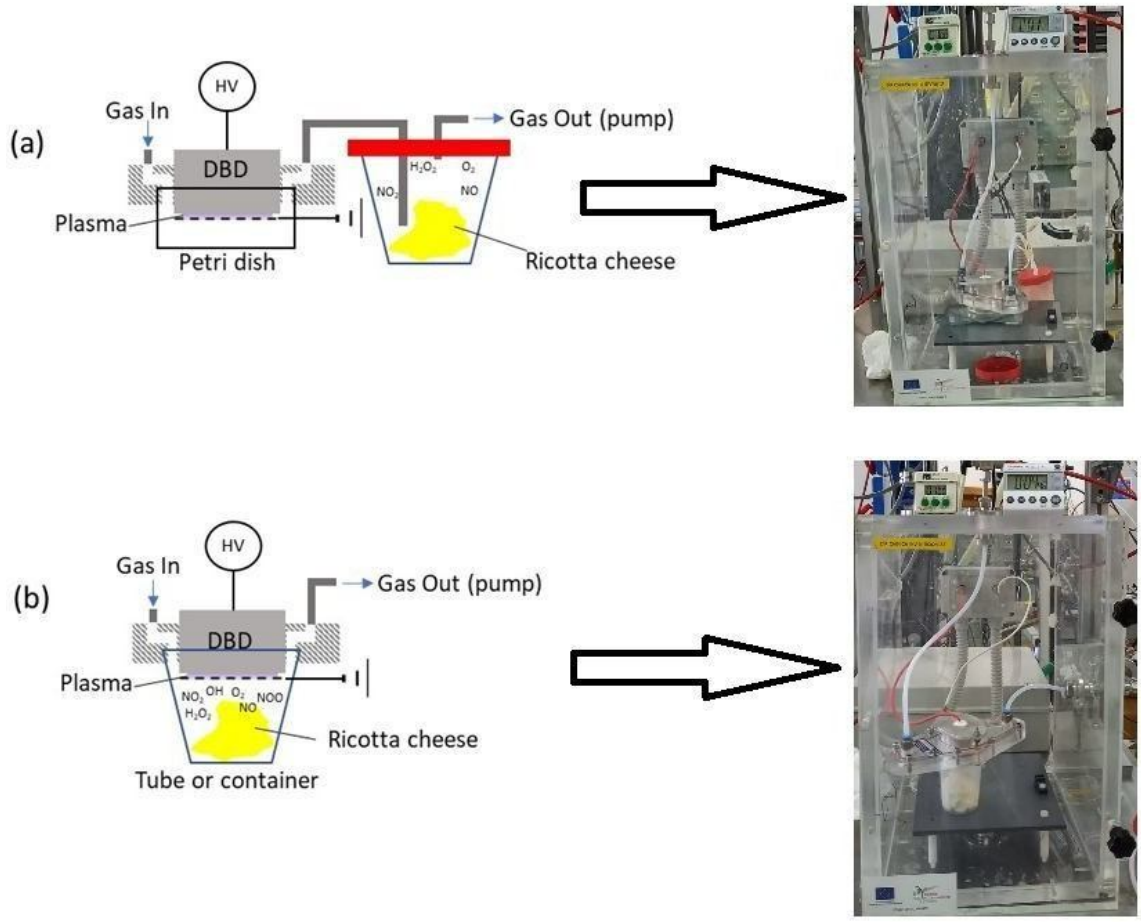


Figure 5.1. Schematic representation of plasma treatment of ricotta cheese (a) through the plasma treated gas (PTG) or (b) directly (DP) with a volume Dielectric Barrier Discharge used to generate plasma.

Plasma was generated by using a DBD PetriPlas+ plasma source, well described elsewhere (Sardella et al., 2020). The source consists of a Plexiglas flow unit, set with gas connections, and a discharge unit. A dry diaphragm pump (Pfeiffer Vacuum, Aßlar, GER) is connected or directly to the flow unit in case of DP configuration or to the sterile tube containing the ricotta cheese in case of PTG. The pump is necessary to evacuate the exhaust and keep the pressure constant (760 Torr), as measured with a MKS baratron. An electric field of 6-kHz frequency and 13.8 kV peak-to-peak voltages was applied with a power supply connected to a programmable 10 MHz DDS function generator (TG1010A, Aim-TTi, Huntingdon, UK) to ignite a volume discharge as wide as the HV electrode (3 cm diameter). The plasma discharges switched on for 5 min, were pulsed with a 50% duty cycle (D.C.), 50 ms of plasma on (ton) over a period (t= ton + toff) of 100 ms; the D.C. was kept constant

in all the experiments reported. In case of DP, the discharge unit is properly adapted for the remote treatment of ricotta cheese contained into small sterile tubes (diameter 5.6 cm, volume 100 cm^3) (Kartell, Milano, Italy) housed under the reactor by means of a slot of the same diameter on the flow unit.

The distance between the plasma source and the ricotta cheese was around 2 cm. In the case of PTG a glass Petri dish is placed underneath the plasma source and the exhaust gas forced to flow through the sterile tube containing the ricotta cheese (figure 5.1a). Both in cases of DP and of PTG, the process is carried out in a closed system (not exposed to surrounding atmosphere); by purging the gas feed before igniting the plasma for 1 min, it is possible to control the chemical composition of the area in which is flowing the gas during the discharge. The treatment of the ricotta cheese in both the configurations was accomplished by placing diffusion of ROS and RNS from plasma to the liquid through the ground grid (figure 5.1b). Pure oxygen and nitrogen were mixed in a ratio of 10% (i.e., 100 sccm O_2 and 1slm N_2) to treat the ricotta cheese. The ratio was chosen as the result of the analyses of nitrites contained in plasma treated double distilled water, used as control, and placed directly underneath the plasma source (DP) fed with different O_2/N_2 mixtures during plasma treatment performed in the same experimental conditions in which was treated the ricotta cheese (Figure 5.2). The gas flow rates were controlled with MKS mass flow controllers.

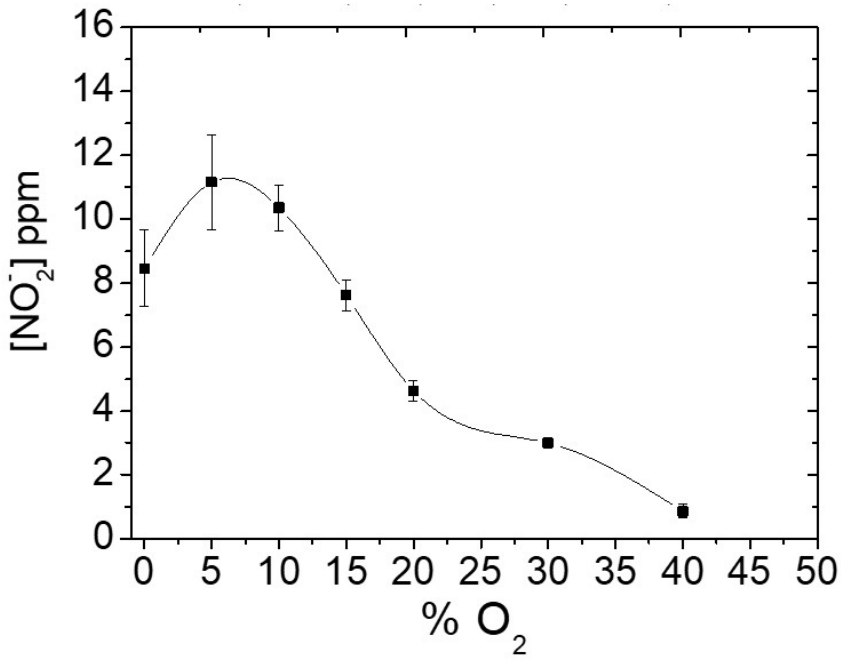


Figure 5.2. Percentage of nitrites produced in 2 mL of double distilled water contained in a Petri dish placed underneath the glow discharge.

5.2.3 Detection of NO_2^-

Nitrite ions were detected with the Griess assay (Spectroquant® 1.14776.001, Merck). To the aim, 13 mg of the reagent mixture were added to 1 mL of sample directly in a 1-mL-volume cuvette; the mixture was then vigorously pipetted till the complete dissolution of the reagent powder and left to react for 10 min (reaction time). Then, the measurement was performed spectrophotometrically (λmax: 525 nm). The color of the measurement solution remained stable for 60 min.

5.2.4 Shelf life test

For each shelf life test, both control and treated ricotta samples were stored at 4 °C, and analyzed during the subsequent 8 days for microbiological and sensory quality. During refrigerated storage, bacterial and fungal enumeration was carried out, according to the standard plate count technique. An amount of 10 g of ricotta was taken from each sample, diluted with 90 mL of 0.9% NaCl solution in a stomacher bag, homogenized for 90 s (400 circulators - Seward, Hamilton, NJ, USA) and finally

decimal diluted for microbial counts. Spoilage microorganisms and relative count conditions, in terms of medium and incubation temperatures to be adopted, were also reported in the study of Ricciardi et al. (2020b), also dealing with ricotta cheese. Specifically, *Pseudomonas* spp., *Enterobacteriaceae*, yeasts and mesophilic bacteria were enumerated on specific medium. All analyses were performed in duplicate on two different samples. The microbial acceptability limit, indicated as MAL, was calculated by using a modified version of the Gompertz equation. To the aim, the fitting procedure was carried out with experimental data of *Pseudomonas* spp., *Enterobacteriaceae* and yeast proliferation, being the limiting spoilage groups for ricotta cheese. The value of the maximum acceptable concentration was set to 10^6 CFU/g for both *Pseudomonas spp*. and yeasts, and 10^4 CFU/g for the *Enterobacteriaceae* (Ricciardi et al., 2020a). For the sensory evaluation, seven trained members of the laboratory of the University of Foggia, carried out a screening of ricotta samples. Panelists were asked to judge odour, colour, texture and overall quality, using a 7-point scale (Ricciardi et al., 2020a). The same modified version of the Gompertz equation was used to fit the sensory experimental data and calculate the sensory acceptability limit (SAL) (Ricciardi et al., 2020a). In the fitting equation the threshold for cheese sensory acceptability was set equal to 4.

The shelf life of each tested sample was calculated as the lowest value among the calculated MAL and the SAL values.

5.2.5 Experimental Plan and Statistical analysis

Tests were carried out on duplicate batches. Experimental data are the average of two replicates. To determine whether significant differences ($p < 0.05$) existed among the mean values of the fitting parameters, the one-way variance analysis (ANOVA) and Duncan's multiple range test with the option of homogeneous groups were used by means of STATISTICA 7.1 for Windows (StatSoft, Inc., Tulsa, OK, USA).

5.3 Results and discussion

In figure 5.2 is reported the analysis of 2 mL of double distilled water used as model to assess which are the experimental conditions suitable to produce high amount of nitric oxide derivatives (i.e. NO_2^-) by plasma fed with mixtures of O_2 and N_2 ranging from 0% of O_2 to 100%. The trend shows that the increasing of the percentage of O_2 up to 10% in the gas feed produces first an increasing of NO_2^- content and then a fast decrease when the percentages of oxygen exceed 10%. In order to promote the formation of reactive nitrogen species, supposed to be involved in the antimicrobial activity of the plasma assisted approach, the discharges were carried out with 10% of O_2 and 90% of N_2.

The microbial efficacy of plasma treatments on microbiological stability of ricotta was tested in terms of main spoilage groups, *Pseudomonas spp*., *Enterobacteria* and yeasts; in addition, the total viable count of mesophilic bacteria was assessed (Ricciardi et al., 2019). The evolution of viable cell concentration of *Pseudomonas spp*. during refrigerated storage is shown in Figure 5.3.

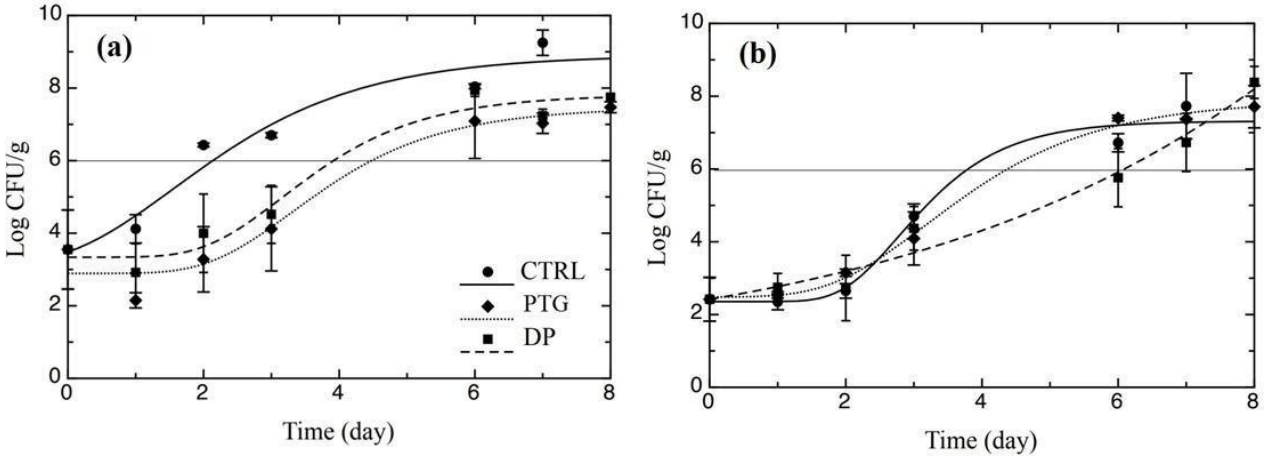


Figure 5.3. Evolution of *Pseudomonas spp*. viable cell concentration in artisanal treated and untreated ricotta cheese in both tests (a) and (b); (•) control cheese; (♦) PTG = cheese treated by indirect plasma; (▪) DP = cheese treated by direct plasma; Symbols are the experimental data, line is the best fit to the experimental data.

As can be inferred from both graphs, the initial microbial concentration in the cheese was quite different, being the product in the first test slightly more contaminated (Fig. 3a). This initial different level of contamination also promoted a different evolution of bacteria during time. In fact, control cheese in the first test rapidly reached the microbial threshold (within 2 days), whereas twice the time was necessary in the second test to reach the same *Pseudomonas spp*. proliferation level, after about 2 days of lag phase. Figure 3 also shows a remarkable difference between treated and untreated ricotta samples. Specifically, in both tests a relevant delay in microbial growth, compared to the control, can be observed for samples treated by direct plasma. As regards the ricotta treated by PTG, marked differences with control cheese can be observed only in the first test where ricotta was more contaminated (Fig.3a). As reported in M&M section, to quantitively determine the advantage in terms of microbial stability gained by the plasma treatment, the experimental data were fitted by a mathematical equation. The calculated MAL values were listed in Table 5.1.

Table 5.1. MAL, SAL and shelf life values (days) of artisanal treated and untreated ricotta cheese in both test (a) and (b). For each sample, the Shelf Life is the lowest value among its MAL and SAL values.

TEST	SAMPLES	MAL *Enterobacteriaceae* (day)	MAL Yeasts (day)	MAL *Pseudomonas spp.* (day)	SAL Overall Quality (day)	**SHELF LIFE (day)**
1ST TEST (a)	CTRL	2.58 ±0.25^{b}	2.87 ±0.17^{b}	2.13 ±0.32^{b}	6.61 ±0.60^{b}	**2.13 ±0.32^{b}**
	PTG	4.25 ±0.64^{a}	4.74 ±0.64^{a}	4.47 ±0.98^{a}	8.58 ±0.21^{a}	**4.25 ±0.64^{a}**
	DP	4.36 ±0.72^{a}	3.98 ±0.85^{ab}	3.91 ±0.68^{a}	8.83 ±0.21^{a}	**3.91 ±0.68^{a}**
2ND TEST (b)	CTRL	3.26 ±0.16^{b}	4.32 ±0.83^{b}	3.79 ±0.45^{b}	6.12 ±0.38^{b}	**3.26 ±0.16^{b}**
	PTG	5.54 ±0.34^{a}	5.30 ±0.64^{ab}	4.37 ±0.24^{b}	6.20 ±0.45^{b}	**4.37 ±0.24^{b}**
	DP	5.39 ±0.85^{a}	5.81 ±0.42^{a}	6.09 ±0.40^{a}	7.40 ±0.16^{a}	**5.39 ±0.85^{a}**

[a] Values in each column, for each test, with different superscript letters are significantly different (P< 0.05).

Data in Table 5.1 related to *Pseudomonas spp*. highlight that in the first test, statistically significant differences (P<0.05) were recorded between the control and the treated cheese samples, being both treated samples acceptable for about 4 days, whereas the control cheese became unacceptable after only 2 days of storage. In the

second test, both treatments were able to increase the MAL values, even though direct plasma was found the more effective. In case of DP, with high probability, the UV rays generated during plasma treatment are lethal on *Pseudomonas spp*., as also demonstrated by the study of Manzocco et al (2015), who applied UV-C on slices of ananas. However, in this paper for the first time in literature is demonstrated also the efficacy of PTG flowing on the food attesting for the involvement of chemical long-living species produced in the gas phase that can actively contribute in sterilizing the environment and the ricotta cheese.

As long as *Enterobacteriaceae* is concerned, Fig. 5.4 shows the recorded results.

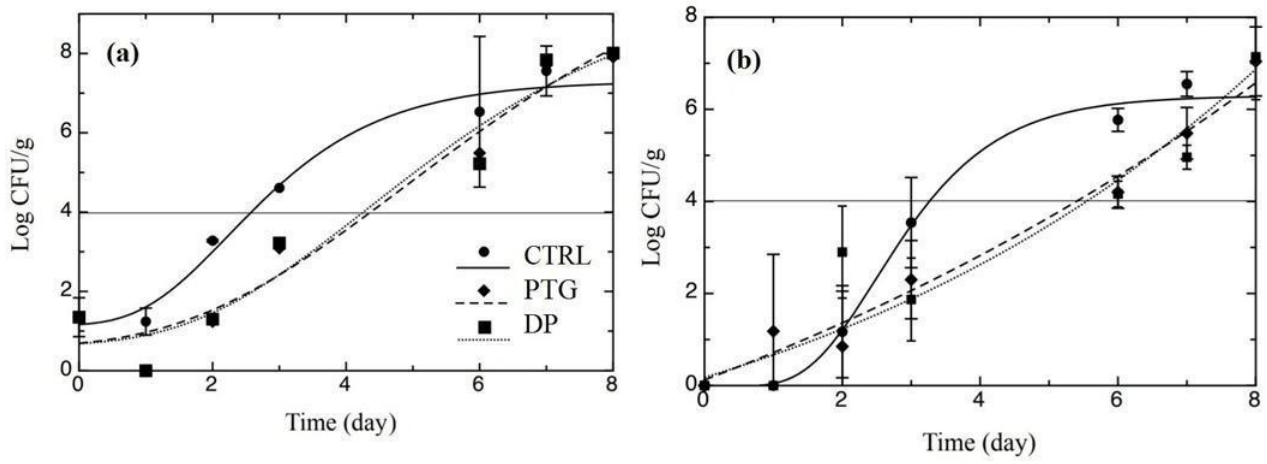


Figure 5.4. Evolution of *Enterobacteriaceae* viable cell concentration in in artisanal treated and untreated ricotta cheese in both tests (a) and (b); (•) control cheese; (♦) PTG = cheese treated by indirect plasma; (▪) DP = cheese treated by direct plasma; Symbols are the experimental data, line is the best fit to the experimental data

As one would expect, data shown in the Figure confirm that samples in the first test are slightly more contaminated than cheese used in the second test. Despite this initial different level of contamination, the control cheese became unacceptable in more or less 3 days, thus reaching 10^4 CFU/g very rapidly. In this case all the treated samples, regardless the plasma treatment adopted, delayed the microbial proliferation, showing a marked lag phase compared to the control samples. As observed for *Pseudomonas spp*., in treated cheese the time to reach the threshold was longer, this is particularly true for samples used in the second test. Most probably it can be due to the better

initial quality of cheese. As fact, initial viable cell concentration generally affects microbial proliferation (D'amico, 2014). Data in Table 1 for *Enterobacteria* also highlight statistically significant differences ($P<0.05$) between treated and untreated cheese samples. Comparing MAL values of the control and the treated ricotta samples, an extension of about 2 days was recorded in both tests. The effects of DP on *Enterobacteria* are not surprising because the scientific literature also reports interesting results dealing with plasma treatments applied to dairy products inoculated by microorganisms belonging to this spoilage group (Lee et al., 2012; Young et al., 2015a). During plasma treatment, ROS and UV are generally produced, thus promoting antimicrobial effects that are more marked on *Enterobacteria* because the microbial group is more photo-sensitive to UV radiation than other target bacteria (Allende et al., 2006). However, also in this case the novelty is represented by the indirect approach (i.e. PTG) in which no UV is present but only the action of chemical long-living species (i.e. RNS) is expected. Fig. 5.5 shows the yeast growth curves of investigated samples during refrigerated storage.

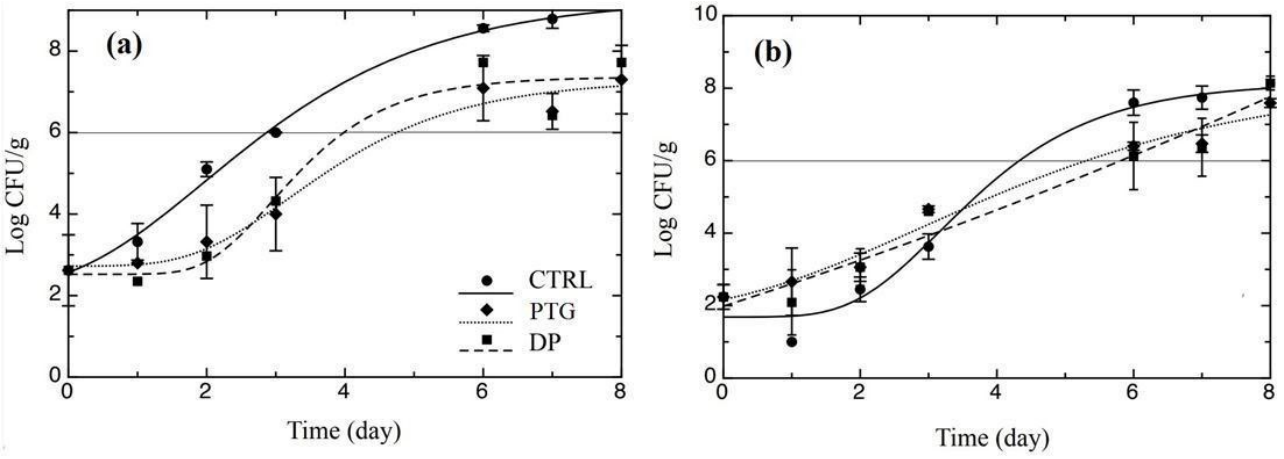


Figure 5.5. Evolution of yeast viable cell concentration in artisanal treated and untreated ricotta cheese in both tests (a) and (b); (•) control cheese; (♦) PTG = cheese treated by indirect plasma; (▪) DP = cheese treated by direct plasma; Symbols are the experimental data, line is the best fit to the experimental data.

It is well recognized that a state of marked yeast proliferation can lead to many sensory problems, that make the product unacceptable to consumer (Fleet et al.,

1990). Therefore, our experimental data were examined taking into account a proper fungal threshold (10^6 CFU/g). As can be seen in the Fig. 5a, the starting microbial load was higher than 10^2 CFU/g. The control sample reached the threshold within the third day of storage, while the treated samples showed a slower growth, reaching the same contamination level after the fourth day of storage, with a lengthier growth for the ricotta treated by the PTG approach. In the second test (Fig. 5b), even starting from a similar initial count, the control reached the limit within 4 days of storage, after a lag phase of about 2 days; whereas, for the treated samples, the threshold was reached after about 5 days of refrigerated storage. Slightly better results were obtained in the case of DP treated cheese. The anti-fungal properties of plasma can be also ascribed to the action of UV rays, as demonstrated in the studies dealing with ultraviolet and near-ultraviolet–visible light applied to ricotta (Ricciardi et al., 2020a; 2020b). Data in Table 1 underline that statistically significant differences ($P<0.05$) between the control and the treated cheese samples were also recorded in terms of MAL-yeast, thus demonstrating that plasma treatments prolonged, by at least 1 day, the time necessary to reach the fungal threshold. Table 5.2 reports data of mesophilic bacteria recorded in the samples during the entire observation period.

Table 5.2. The evolution of mesophilic viable cell concentration in the artisanal treated and untreated ricotta cheese.

TEST	TIME (day)	CTRL (Log CFU/g)	PTG (Log CFU/g)	DP (Log CFU/g)
1ST TEST	0	3.80 ±0.22[a]	3.80 ±0.22[a]	3.80 ±0.22[a]
	1	4.31 ±0.12[a]	3.57 ±0.07[ab]	3.33 ±0.63[b]
	2	4.33 ±0.12[a]	3.62 ±0.07[b]	3.45 ±0.50[b]
	3	7.14 ±0.23[a]	6.68 ±0.25[b]	6.61 ±0.19[b]
	6	8.52 ±0.11[a]	8.52 ±0.11[a]	8.65 ±0.24[a]
	7	8.98 ±0.05[a]	9.08 ±0.23[a]	8.98 ±0.02[a]
	8	-	9.61 ±0.12[a]	9.32 ±0.45[a]
2ND TEST	0	3.30 ±0.62[a]	3.30 ±0.62[a]	3.30 ±0.62[a]
	1	3.43 ±0.19[a]	4.23 ±0.21[a]	3.10 ±1.56[a]
	2	3.61 ±0.33[a]	3.98 ±0.90[a]	4.29 ±1.15[a]
	3	3.42 ±0.08[b]	5.10 ±0.40[a]	5.58 ±0.31[a]
	6	6.52 ±1.19[a]	7.16 ±0.18[a]	6.27 ±1.22[a]
	7	6.23 ±1.73[a]	7.55 ±0.29[a]	6.81 ±1.54[a]
	8	-	8.04 ±0.06[a]	8.49 ±0.12[b]

[a] *Values in each row with different superscript letters are significantly different (P<0.05).*

As can be inferred from the abovementioned table, similar trends were found in treated and untreated cheese samples. Specifically, in the first test, microbial load started from 10^3 CFU/g, increased more rapidly in the control cheese than in the treated samples and after 6 days of storage all dairy products reached about 10^8 CFU/g. In the second shelf life test the initial concentration, accounting for about 10^3 CFU/g, increased more rapidly in the treated cheese than in the control ricotta and after 6 days the concentrations of viable cells were around 10^7 CFU/g. The rapid microbial evolution of mesophilic bacteria, observed also in plasma treated cheese, is related not only to the concentration of many spoilage groups, but also to the growth of lactic acid bacteria, thus demonstrating that plasma did not compromise the activity of desired microorganisms (Mai-Prochonow at al., 2016).

The pH values recorded during storage were similar in all the samples (data not shown). Specifically, pH values ranged between 6.90-6.70, regardless the treatment, in both tests. For this reason, no influence can be attributed to the pH for the antimicrobial and antifungal effects observed in the plasma treated ricotta samples. This aspect is very important because quality of processed foods is closely attributed to pH and acidity. Any drastic change could lead to undesirable impact on consumer acceptability and product shelf life. Our results are in contrast with the most part of literature in which the presence of high amount of reactive nitrogen species generally promote a dramatic decreasing of the pH due to the formation of acid species like nitric acid or hydroperoxyl ions in water (Yokoyama et al., 2019; Oehmigen et al., 2010). On the contrary, in our case, a probable buffering effect of organic species present in the food can promote a certain stabilization of the pH (Xu et al., 2017).

As long as the effects of plasma on sensory quality are concerned, Table 5.3 reports data recorded from the panel test.

Table 5.3. Overall Quality (score) of artisanal treated and untreated ricotta cheese.

TEST	TIME (day)	CTRL	PTG	DP
1ST TEST	0	7.00 ±0.00[a]	7.00 ±0.00[a]	7.00 ±0.00[a]
	1	7.00 ±0.00[a]	7.00 ±0.00[a]	7.00 ±0.00[a]
	2	7.00 ±0.00[a]	7.00 ±0.00[a]	7.00 ±0.00[a]
	3	7.00 ±0.00[a]	6.86 ±0.24[a]	6.94 ±0.18[a]
	6	7.00 ±0.00[a]	5.64 ±0.24[a]	5.36 ±0.48[a]
	7	2.79 ±0.24[b]	4.93 ±0.19[a]	5.00 ±0.00[a]
	8	-	4.36 ±0.24[a]	4.50 ±0.29[a]
2ND TEST	0	7.00 ±0.00[a]	7.00 ±0.00[a]	7.00 ±0.00[a]
	1	7.00 ±0.00[a]	7.00 ±0.00[a]	7.00 ±0.00[a]
	2	7.00 ±0.00[a]	7.00 ±0.00[a]	7.00 ±0.00[a]
	3	7.00 ±0.00[a]	7.00 ±0.00[a]	7.00 ±0.00[a]
	6	6.43 ±0.59[a]	6.50 ±0.00[b]	6.50 ±0.00[a]
	7	2.57 ±0.53[b]	3.21 ±0.27[a]	5.36 ±0.24[a]
	8	-	3.14 ±0.24[a]	3.21 ±0.24[a]

[a] *Values in each row with different superscript letters are significantly different ($P < 0.05$).*

The judges were asked to give product evaluation in terms of overall quality, thus taking into account the following sensory attributes: odor, color and texture. All the products were considered at top quality (score = 7) at the begin of the shelf life test. As expected, during storage time a decrease in sensory quality was observed in all the samples. Data listed in Table 5.3 also indicate quite clearly that the investigated treatments are effective in preserving ricotta cheese. In fact, control cheese lost its acceptability after 6 days, whereas treated samples remained acceptable for longer time, above all in the first test. In particular, the control sample became unacceptable due to its odor. In fact, *Pseudomonas spp.*, *Enterobacteriaceae* and yeasts proliferation is reported to be responsible for unpleasant odor in ricotta cheese (Pala et al., 2016). It is worth noting that also in treated cheese the odor compromised the product acceptability. A possible explanation of these findings for DP can be ascribed to the action of UV produced during plasma treatments because these rays can promote photo-induced changes in polypeptides (Lacivita et al., 2016). Considering that ricotta composition is mainly constituted by whey proteins (Salvatore et al., 2014), photo-induced effects, as well as chemical one on proteins, cannot be excluded. However, similar results were obtained with indirect approach (i.e. PTG) where the effect of UV-radiation is absent, thus confirming once again that the role of

plasma produced chemical species is more important than the one of UV for the sterilization. After one week of storage, an anomalous color appeared in all the investigated ricotta cheese samples, which compromised product acceptability (Pintado et al., 2001). SAL values obtained according to the procedure reported in the M&M section are also reported in Table 1. The results highlight that statistically significant differences ($P<0.05$) were observed between the control and the plasma treated cheese samples in terms of sensory quality, thus confirming that plasma both in case of direct or indirect approach, not only is responsible for a slower microbial growth, but it also helped maintaining a good sensory quality during refrigerated storage.

In the last column of Table 5.1 are listed the ricotta sample shelf life. As reported beforehand, shelf life was defined as the lowest value among all the calculated MAL and SAL values, as each one of them represents a single food quality index. When one of them reaches its threshold, the product cannot be considered acceptable any longer (Conte et al., 2009). Looking at data in the last column it can be seen that in both tests, the control cheese has a shorter shelf life than the plasma treated samples, whatever the plasma assisted approach used. In particular, a shelf life extension ranging between 1 and 2 days was found, depending on the initial contamination level of cheese, as well as the type of plasma treatment applied to the product. The limited knowledge available in the literature on the effects of plasma applied to dairy products (Coutinho et al., 2018) can now be also supported by our experimental evidence recorded for ricotta cheese, also including indirect approach (PTG) that can be more easily integrated in the line production of such food. Further research is still necessary because plasma is a complex mix of active species and different experimental conditions than that adopted in the current study could promote different results (Sarangapani et al., 2018).

On the basis of the data expressed in terms of shelf life reported in Table 5.1, Figure 5.6 shows by means of two comparative graphs (a and b) the increase in the shelf life of the ricotta treated with PTG and DP compared to the control.

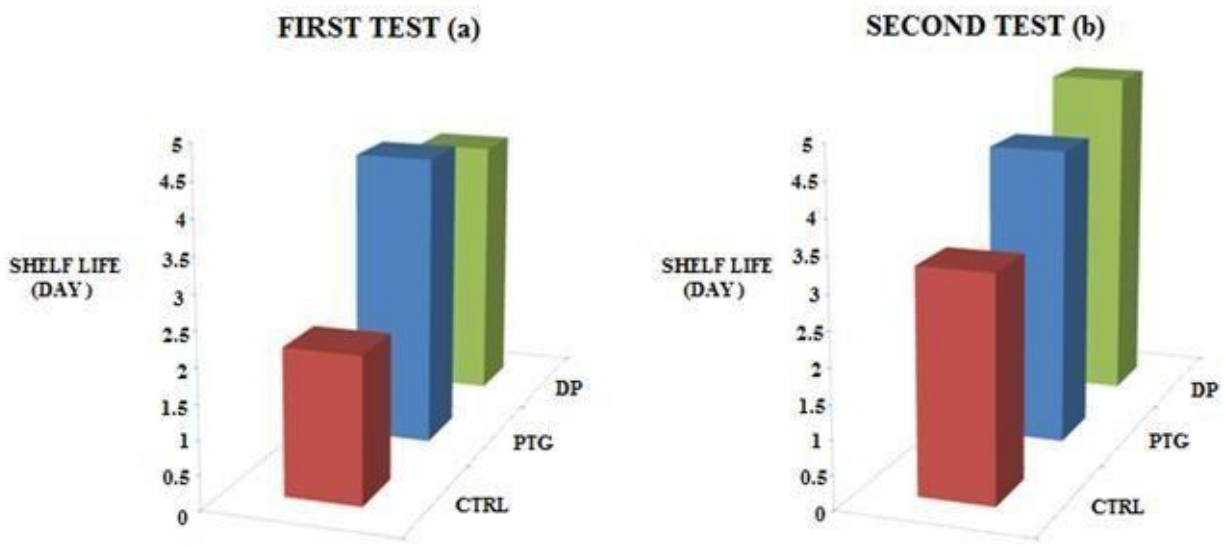


Figure 5.6. The shelf life (days) intended as the lowest value between MAL for *Pseudomonas spp.*, *Enterobacteriaceae* and yeasts and SAL, in both tests (a and b). CTRL = control cheese; PTG = cheese treated by indirect plasma; DP = cheese treated by direct plasma.

5.4 Conclusions

The effects of two distinct plasma assisted approaches, PTG and DP, on artisanal ricotta samples were assessed. Both approaches have led to positive results in terms of shelf life extension. In fact, the investigated plasma treatments have slowed down both the microbial growth and the sensory quality decay during refrigerated storage. In particular, an increase in shelf life ranging between 50% and 100%, compared to the untreated ricotta, was observed for all plasma treated samples. The differences depend both on the level of the initial contamination, and on the type of plasma treatment. Due to the efficacy of PTG approach, the involvement of long-living plasma produced Reactive Species has been assessed. However, the complexity of the mechanisms involved in plasma technology require further in-depth studies to better understand the phenomena involved and the interactions among the active species that are formed during the treatment and main food components.

General conclusions of the Ph-D thesis

The research study carried out during the Ph-D has brought positive results in terms of shelf life. In all the treatments carried out the potential effectiveness of alternative methods to heat on artisanal and industrial ricotta was tested. The experiments carried out showed interesting sanitizing effects against deteriorating bacteria and sensory decay. Among the surface technologies tested, pulsed light proved to be the most efficient compared to other continuous treatments such as UV-C and Near Ultraviolet Visible Light, although further studies are still needed to better investigate the process variables. Cold Plasma also recorded positive results in terms of shelf life, as both the direct and indirect plasma treatments tested in this research led to an increase in the duration of storage up to 2 days, a very promising result for a fresh product such as artisanal ricotta. For the first time, plasma gaseous effluents enriched with reactive oxygen or nitrogen species have been proposed as a gentle and effective treatment of perishable fresh dairy products. Furthermore, the simplicity of the industrial application of plasma could arouse great interest in the fresh dairy sector, even if the complexity of the composition of the plsma requires further investigation on the subject. Among the treatments analyzed in the last 3 years, X-rays have shown the best results, both on fresh and industrial ricotta, as X-rays are able to penetrate inside the food and inhibit microbial cells, thus guaranteeing a clear increase in shelf life compared to the untreated product. The technology of food irradiation deserves attention from public health authorities, industry and consumers around the world because it can significantly extend the shelf life of products by stimulating the market even far away from local production sites. Its full acceptance is slow and often controversial, despite documented safety evidence from decades of research. Further studies are needed for consumer education, without neglecting the standardization of process parameters and economic feasibility. Therefore, I hope that the research study carried out in this thesis will contribute significantly to add useful knowledge in the specific theme of the new approach to food sanitation.

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List of publications relevant to the Ph-D research:

- **Ricciardi E.F.**, Lacivita V., Conte A., Chiaravalle E., Zambrini A.V. and Del Nobile M.A. (2019). X-ray irradiation as a valid technique to prolong food shelf life: The case of ricotta cheese. *International Diary Jurnal*, 99, 104547.

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- **Ricciardi, E.F**., Sardella, E., Fracassi, F., Conte, A., Del Nobile, M.A. 2021. "Plasma treatments applied to dairy sector: advances on fresh ricotta cheese". Submitted to Innovative Food Science and Emerging Technology. *Under review.*

Participation to international conferences:

- **“IFT-EFFoST 2018, International Nonthermal Processing Workshop and Short course”**

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- **“International Centre for Mechanical Sciences (CISM) – Nutrient Delivery and Impact on Human Helth”**

  Udine, Italy – November 4-6, 2019.

- **CA19110 "Plasma applications for smart and sustainable agriculture"**

  On line international conference organized in Jahorina, Bosnia and Herzegovina – 17-19 March 2021.

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