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FACULDADE DE CIÊNCIAS DA NUTRIÇÃO E ALIMENTAÇÃO
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Safety and Quality

Assessment of Ultrasound Treated Lettuce

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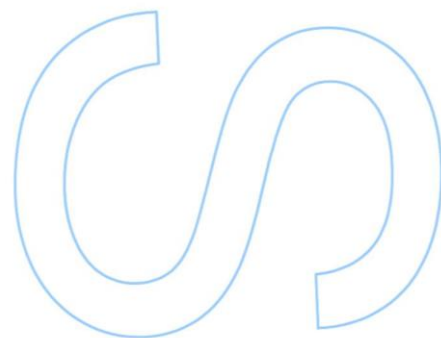
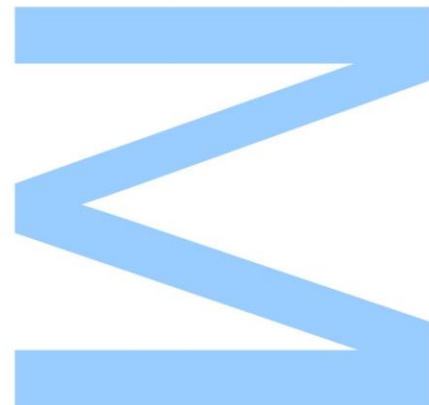
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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto,

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Dedicated to My Dear Family

***“Perseverance is the hard work you do after you get tired of
doing the hard work you already did”***

- Newton Gingrich

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ABSTRACT

There is an imminent concern about food safety due to increased notifications of food contamination by pathogenic microorganisms. Leafy green vegetables are presented as the priority food sector regarding safety and control is fundamental to reduce their contamination. It is evident, therefore, that the effective implementation of decontamination techniques and processing technologies in the production of food and ready-to-eat vegetables is a prerequisite to achieve the production of safe products. Although safety is a major factor to be ensured by the industry, more and more consumers are looking for good quality products. The green vegetables fall within the range of healthy products and even after treatment should remain their appealing sensory characteristics to consumers. This work assesses the efficacy of ultrasound technology as a decontamination process for maintaining the safety and sensory characteristics of lettuce. Therefore, this work evaluates the microbiological safety of ultrasound treated lettuce during storage and its quality properties based on sensory analysis.

Keywords: Ultrasound, sensory analysis, lettuce, quality, shelf life.

RESUMO

Existe uma preocupação eminente relativamente à segurança alimentar, devido ao aumento de notificações de contaminações alimentares por microrganismos patogénicos. Os vegetais de folha verde são apresentados como prioridade de ação em que é fundamental controlar e diminuir a sua contaminação. É evidente, portanto, que a aplicação eficaz de técnicas de descontaminação e tecnologias de processamento na produção de alimentos e na indústria de processamento de vegetais frescos é um pré-requisito para alcançar a produção de produtos seguros. No entanto o sucesso do produto é dependente de outros fatores além da segurança alimentar. Embora este seja o primeiro fator assegurado, cada vez mais os consumidores procuram produtos saudáveis e de qualidade. Os vegetais inserem-se na gama de produtos saudáveis que mesmo depois de tratados devem manter características sensoriais apelativas para os consumidores. Este trabalho procura analisar a tecnologia de Ultra-som como técnica de desinfeção mantendo a segurança e as características sensoriais da alface. Este trabalho divide-se portanto em duas partes sendo a primeira relativa à manutenção da segurança microbiológica da alface e a segunda relativa à preservação da qualidade sensorial da alface avaliada por um painel sensorial.

Palavras-chave: Ultra-som, análise sensorial, alface, qualidade, tempo de vida.

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ABBREVIATIONS LIST

µL – microliters

µm – micrometers

°C – degrees Celsius

CFU – Colony Forming Units

cm – centimeters

CO₂ – Carbon Dioxide

EOs – Essential Oils

EU – European Union

FAO – Food and Agriculture Organization for United Nations

g – relative centrifugal force

h – hour

Hz – Hertz

kGy – kilo Gray

kHz – kilo Hertz

MAP – Modified Atmosphere Packaging

min – minutes

mL – milliliters

NCTC – National Collection of Type Cultures

nm – nanometers

OPP – Oriented Polypropylene

PCA – Principal Component Analysis

PLS – Partial Least Squares

ppm – parts per million

RASFF – Rapid Alert System for Food and Feed

rpm – revolutions per minute

spp – species

TSA – Tryptone Soya Agar

TSB – Tryptone Soya Broth

TSP – Trisodium Phosphate

US – Ultrasound

UV – Ultraviolet

VRBDA – Violet Red Bile Dextrose Agar

WHO – World Health Organization

XLD – Xylose Lysine Deoxycholate

1. INTRODUCTION

1.1 Health and Nutritional Aspects of Fresh Produce

The portion of fresh vegetables in the human diet increased in the last decade due to the raising awareness of the consumers for a healthy diet and nutrition (Birmpa *et al.* 2013). Previous reports have shown that the consumption of fresh vegetables could be beneficial to reduce the risk of several diseases such as hypertension, coronary heart disease and some types of cancer (Boeing *et al.* 2012).

For a healthy diet and to avoid several health diseases World Health Organization (WHO) recommends an intake of 400g of fruits and vegetables per day. The low intake of fruits and vegetables is causing 31% of the ischaemic heart disease and 11% of stroke worldwide. It is worth to highlight that up to 2.7 million lives could be saved with the increase of fruit and vegetable consumption (WHO 2003). A greater intake of green leafy vegetables (cabbage, brussels sprouts, cauliflower, lettuce, parsley, dill, fennel, spinach) was associated with a 14% reduction in risk of type 2 diabetes (Esposito *et al.* 2011).

Other constituents present in fruits and vegetables might also help to prevent and control more diseases. Fiber intake contributes to control diabetes and high serum cholesterol level and might prevent diverticulosis. Antioxidants act on cataracts prevention and on the oxidation of cholesterol in the arteries.

The content of potassium in fruits and vegetables help to prevent or control hypertension, reducing the subsequent risk of stroke and heart disease. Additionally, the small content of fat and energy reduces the risk of obesity (Steinmetz *et al.* 1996).

An effort has been observed on public awareness of the benefits of the incorporation of vegetables and fruit in the alimentation. Campaigns such as “5 a day” have been carried out in many countries to promote the ingestion of at least 5 portions of fruits and vegetables per day (WHO 2003). Therefore, the consumption, production of green leafy vegetables and their ready-to-eat (RTE) salads, are expected to continue increasing (Mercanoglu *et al.* 2011).

1.2 Foodborne Outbreaks

The safety of fresh vegetable production is a global issue due to the fact that these products are part of a healthy diet. Therefore, the security of these products is vital (Birmipa *et al.* 2013). In 2006, the Codex Alimentarius Commission requested the WHO and Food and Agriculture Organization (FAO) the assessment of this issue to work towards the decrease of foodborne outbreaks. The aim was to look for scientific support that finally resulted in a report called “Code of Hygienic Practice for Fresh Fruits and Vegetables” for the Codex Alimentarius. The Codex Alimentarius Commission highlighted the need for this particular approach to fresh fruits and vegetables.

In 2007, FAO/WHO handled an experts meeting, where they agreed on the establishment of a six criteria to decide the severity of the outbreak cases. The defined criteria were:

- a) frequency and severity of the disease; size and scope of the production;
- b) diversity and complexity of the production chain/industry;
- c) potential for amplification of foodborne pathogens through the food chain;
- d) potential for control;
- e) extent of international trade and economic impact (FAO/WHO 2008).

Accordingly to these criteria three main food groups were created: 1. leafy green vegetables; 2. berries, green onions, melons, sprouted seeds and tomatoes; 3. mix of fruits and vegetables that have been involved in outbreaks but did not have a strong impact in the population has the others. From the above mentioned groups, it was clear that the first group (priority group) gathered the biggest concern due to the microbiological hazards. This group includes all the leafy vegetables (leaves regarded for consumption) such as lettuce, spinach, kale, chicory, fresh herbs (coriander, basil, parsley) and watercress (FAO/WHO 2008).

A consistent daily intake of leafy vegetables is beneficial to health, and it is expected to increase in consumption and therefore in the production. In contrast with this benefit, over the last two decades, the number of outbreaks of human gastroenteritis caused by foodborne pathogens related to green leafy vegetables also increased worldwide (Mercanoglu *et al.* 2011). The leafy green vegetables are linked to

several outbreaks and with a high number of reported cases in three different regions of the world. In the last ten years, there were, at least, thirty cases in the USA and five in Sweden. The severity of the diseases is commonly connected with the implicated hazards, and the range of hazards allied to these products is extensive (FAO/WHO 2008b). According to Rapid Alert System for Food and Feed (RASFF) report, the number of notifications associated with foodborne pathogens has increased in the last years reaching the 600 notifications in 2011 (RASFF 2011).

From 1998 to 2008 approximately 46% of foodborne illnesses in the USA were associated with fresh produce (Bhargava *e. al.* 2015). It is remarkable that *Salmonella* and *E. coli* O157: H7 consistently caused large outbreaks of foodborne illness associated with fresh produce. *Salmonella enterica* caused 76%, 60% and 30% of outbreaks related to fruits, seed sprouts and leafy vegetables, respectively (Olaimat *et al.* 2012). From 2000 to 2008 *Salmonella* spp. is estimated to cause annually in the United States 1 000 000 illnesses, 19 000 hospitalizations and 380 deaths (CDC, 2012). In 2013, 818 foodborne disease outbreaks were reported, resulting in 13,360 illnesses, 1,062 hospitalizations, 16 deaths, and 14 food recalls. In the United States, outbreaks caused by *Salmonella* increased 39% from 2012 (113) to 2013 (157), and outbreaks associated hospitalizations caused by *Salmonella* increased 38% from 2012 (454) to 2013 (628) (CDC, 2013).

A single confirmed or suspected etiologic agent was identified in 605 outbreaks (74%, with 439 confirmed and 166 suspected). Among the 439 outbreaks with a single confirmed etiologic agent, which were caused by: (i) bacteria (239 outbreaks, 54%), (ii) viruses (160, 36%), (iii) chemicals (33,8%) and (iv) parasites (7, 2%). Norovirus was the most common cause of confirmed, single-etiology outbreaks, accounting for 154 (35%) outbreaks and 3,758 (40%) illnesses. *Salmonella* was next, accounting for 149 (34%) outbreaks and 3,553 (38%) illnesses. Among the 147 confirmed *Salmonella* outbreaks with a serotype reported are: (i) Enteritidis (34 outbreaks, 23%), (ii) Typhimurium (24, 16%), (iii) Heidelberg (12, 8%), (iv) Newport (9,6%), (v) Javiana (8, 5%). Shiga toxin-producing *Escherichia coli* (STEC) caused 29 confirmed, single etiology outbreaks, of which 26 (90%) were caused by serogroup O157, 2 (7%) by O26, and 1 (3%) by O111 (CDC, 2013).

Several factors might be responsible for a foodborne outbreak (Table 1). To identify the source of contamination, scientific research has to be conducted. As soon as the implicated food is found as a common source of an outbreak, a review of the

production, processing and transportation steps is elaborated to disclose where and when the contamination took place. It is worth to notice that the prevention of an outbreak related illnesses is a difficult task due to the product short shelf life, fast distribution and consumption, along with the delay in the outbreak recognition (Lynch *et al.* 2009). For instance, *Salmonella* and other enteric bacterial pathogens present in food outbreaks were able to survive long transport or storage for prolonged periods of time without notice (Lynch *et al.* 2009). The growth of food poisoning organisms such as *Salmonella* species and *Listeria monocytogenes* will not necessarily be accompanied by changes in appearance, odour, flavour or texture that could be detected by the human senses, and consequently carries serious health concerns. The growth of spoilage organisms is often readily identified by sensory changes, for example, visual mould growth, generation of off-odours, flavours and changes in texture, frequently from the action of enzymes produced by microorganisms (Kilcast *et al.* 2000; Brandl *et al.* 2006).

Table 1: Factors involved in the emergence of produce-linked outbreaks Brandl *et al.* 2006

Changes in the fresh produce industry

- Intensification and centralization of production
- Wider distribution of produce over longer distances
- Introduction of minimally processed produce
- Increased importation of fresh produce

Changes in consumer habits

- Increased consumption of meals outside the home
- Increased popularity of salad bars
- Increased consumption of fresh fruits and vegetables, and fresh fruit juices

Increased size of at-risk population (elderly, immunocompromised)

Enhanced epidemiological surveillance

Improved methods to identify and track pathogens

Emerging pathogens with low infectious dose

1.3 Decontamination of Fresh Produce

Processing vegetables result in a faster physiological deterioration due to peeling, slicing, dicing or shredding before packaging and storage usually made in RTU vegetables. Microorganisms are natural contaminants of fresh produce and minimally processed fresh-cut products, and contamination arises from a number of sources, including postharvest handling and processing (Rico *et al.* 2007).

To control the microorganisms present in food products, and to avoid their continuous proliferation, an effective decontamination is required. Several techniques are applied to reduce the microbiological activity to harmless values for human health (Wirtanen *et al.* 2003) In the industry, the decontamination techniques are divided into two main groups which were proved to be moderately effective against pathogens and endogenous microflora: the chemical and the physical methods (Ramos *et al.* 2013).

The chemical sanitizers approved in the food industry are compounds based on alcohol, chlorine, quaternary ammonium, oxidants (peracetic acid, hydrogen peroxide and ozone), persulphates, surfactants and iodophors (Wirtanen *et al.* 2003). Belonging to the chemical methods group, chlorine is the sanitizer more commonly used. The rinses of chlorine vary between 50 to 200 ppm and the contact time is less than 5 min (Rico *et al.* 2007). In fact, to be effective it requires concentrations of 50-200 ppm, pH < 8, and to be in contact with the products for not less than 1 min (Goodburn *et al.* 2013). Nevertheless, the chlorine's activity is reduced by the organic material and can also lead to the release of chlorine vapours and formation of chlorinated by-products with potential adverse health effects (Parish *et al.* 2003). Therefore, in some countries like Germany, The Netherlands, Switzerland and Belgium, the use of chlorine in RTU products is forbidden (Rico *et al.* 2007). As reported in Goodburn *et al.* 2013, based on the commonly used chlorine concentrations and pH and immersion time, the usual log reduction was less than 2 logs for mesophilic bacteria and more than 2 logs following 7 days storage of ready to eat vegetables. Thus, alternatives to chlorine have been explored (Rico *et al.* 2007). Chlorine dioxide was also considered in two physical forms, i.e., gas and aqueous. Although it could stand as an alternative, the aqueous form is not efficient as the gaseous form. Its efficacy has been verified in green peppers surface decontamination against *E. coli* O157:H7 and *Listeria monocytogenes*. Nevertheless, chlorine gas form still needs further improvements in this field (Han *et al.* 2000).

Organic acids (lactic, citric, acetic, tartaric, ascorbic), unlike the other chemical methods, are allowed to be used also in organic products and are documented as simple to use. Nevertheless, the interferences with the sensory quality of the products are remarkable. Special requirements depending on the organic acid, the strain and the product has to be evaluated prior the treatment (Ramos *et al.* 2013). Trisodium phosphate (TSP) showed effectiveness in *Salmonella* inactivation for solutions of 15% of TSP. Nevertheless, concentrations higher than 10% TSP showed to be prejudicial to the sensory quality of lettuce (Ramos *et al.* 2013).

Quaternary ammonium compounds are also used for decontamination. The compounds are colorless, odorless and with good penetrating ability. Nevertheless, it is costly and ineffective against *Listeria monocytogenes* (Parish *et al.* 2003). Calcium based solutions have been widely used for delicate fruits with high senescence index like grapefruit or peaches. This method can increase the final calcium content presented in the product. Yet, some bitterness and off-flavours were associated with calcium chloride treatment. Calcium lactate, as a fresh-cut lettuce sanitizer revealed analogous effectiveness as chlorine reducing the microbial quantity (Rico *et al.* 2007).

Ozone has been widely studied as a chemical sanitizer and is well known for its good penetrability, high reactivity and spontaneous decomposition to non-toxic products. When dissolved in water it reaches concentrations between 0.03 to 20.0 ppm while in the gaseous form extend to higher doses such as 20,000 ppm. The decontamination with aqueous ozone requires high initial investment cost, on-site generation, and the monitoring in indoor applications. The gaseous form of ozone is more effective than the aqueous form. Nevertheless, regarding possible limitations of these two forms, gaseous is more toxic and reactive and some product changes might occur (Ramos *et al.* 2013).

Electrolyzed water is another studied method to replace chlorine as a disinfectant technology. It has a strong bactericidal effect against pathogens and spoilage microorganisms and is more effective than chlorine due to high oxidation-reduction potential. The employment of this technology has to be balanced to avoid the reduction of quality in fresh-cut vegetables (Rico *et al.* 2008).

Regarding the physical methods, modified atmosphere packaging (MAP) intends to extend the postharvest life of fresh produce. Minimizing metabolic activity, delaying enzymatic browning and retaining visual appearance are some of the ways to achieve this purpose. The modified atmosphere allows the slowing down of the

respiration rate, the reduction of the metabolism and maturation (Rico *et al.* 2007). Nevertheless and in some cases the rebalance of the atmosphere inside the package, with high levels of CO₂, might stimulate the growth of pathogens (Ramos *et al.* 2013).

The process of irradiation requires gamma-ray, X-ray, and electron beams to produce ions that ionize the water. The process is based on the production of free radicals responsible for reacting, destroy and deactivate the bacteria components. Irradiation was approved by FDA to be used in fresh fruit and vegetables to a maximum level of 1.0 kGy. It was also already reported that the dose of 2.0 kGy strongly inhibits the aerobic mesophilic and lactic flora in shredded carrots (Allende *et al.* 2006; Farkas 2006).

Ultraviolet light radiation is distinguished according to the wavelength of operation: UV-A ranges from 315 to 400 nm; UV-B from 280 to 315 nm and UV-C from 100 to 280 nm (Figure 1). UV light has different practical applications like decontamination of surfaces, liquids, air but also decontamination in food processing (Bintsis *et al.* 2000). UV-C is the most applied to fresh fruits and vegetables. It can cause direct bacterial DNA damage, induce different resistance mechanisms against pathogens (Ramos *et al.* 2013). It can have an impact at a nutritive level because of water soluble vitamins sensitive to the UV light (Goodburn *et al.* 2013).

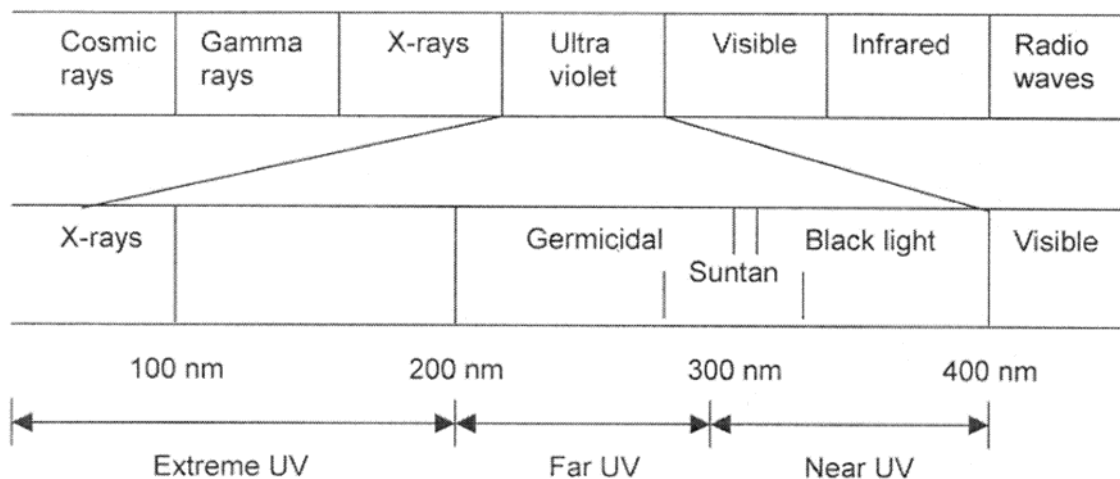


Figure 1: Electromagnetic spectrum (Guerrero-Beltrán *et al.* 2004)

Alternatively to UV radiation, pulsed light, also known as high-intensity light pulse (HILP), is effective in solid or liquid food for the bacteria inactivation. It has a wide spectrum of action from 100 to 1100 nm wavelength. This decontamination technique appears to be dependent on the microbial light absorption and effectiveness can be decreased if food components also absorb the effective wavelengths (Ramos *et al.*

2013). Pulsed light can have an impact to the nutritional level of the products similarly to the UV light (Goodburn *et al.* 2013).

Cold plasma is an emerging antimicrobial technology for decontamination. It consists of the use of non-thermal ionized gases constituted by photons, electrons, positive and negative ions, atoms, free radicals and excited or non-excited molecules. This method uses electricity and a carrier gas to inactivate the microorganisms, and it has been used in fresh produce such as lettuce, tomato and carrots (Bérmudez-Aguirre *et al.* 2013). Cold plasma revealed to be a highly effective against pathogens like *E. coli* O157:H7 and *Salmonella spp* (Ramos *et al.* 2013).

The combination of one or more of the above mentioned methods could be beneficial to avoid the use of severe conditions of one single technology. The combination might result in better decontamination and product quality. Still, further research on this topic is required. None of the methods applied nowadays in the industry have control over all the parameters necessary to achieve the extension of shelf-life, without compromising products quality (Ramos *et al.* 2013; Bermudez-Aguirre *et al.* 2013).

1.4 Ultrasound Technology

The introduction of new decontamination technologies could be beneficial to reduce the processing time and improve the decontamination conditions. The consumer's preferences are changing and the demand for safety and high-quality products, which retain their nutritional and natural characteristics, is increasing. This challenge requires on the one hand the development and use of decontamination technologies that preserve several aspects of food and on the other hand that they are also efficient against foodborne pathogens (Cárcel *et al.* 2012).

From the industry point of view, the reduction of energy in the process also has a positive impact in limiting the financial and environmental costs, leading ultimately to affordable products to the consumer. . In the last years, the ultrasound technology has been spotlighted and led to improvements in these areas (Birmpa *et al.* 2013; Cárcel *et al.* 2012). The energy used in the ultrasound technology can be optimized. Bauman *et al.* (2005) quantified the total energy needed for treatments in the ultrasound. They concluded that to process pineapple juice, the time that the ultrasound system took to reach the required conditions for an effective bacterial inactivation was long and consumed a large amount of the total energy. In the other hand, grape juice required

least energy to achieve microbial inactivation. The ultrasound technology consists of acoustic waves that diffuse through a material medium. Ultrasound waves are classified by the frequency above the audible by humans (20 hertz (Hz) and 20 kilohertz (kHz)) (Cárcel *et al.* 2012). There are two types of ultrasounds: low intensity (with an intensity range below $1\text{W}/\text{cm}^2$ and frequency superior to 100 kHz), and high intensity (with intensity superior $1\text{W}/\text{cm}^2$ and frequencies between 18 and 100 kHz) (Figure 2). The low intensity ultrasounds are applied as a non-invasive technology for emulsification, filtration, drying and freezing processes, stimulation of the activity of living cells and cleaning food surfaces. The high intensity ultrasounds are used in processes such as induction of oxidation/reduction, extraction of enzymes and proteins, inactivation of enzymes and proteins, degassing liquids, inactivation of enzymes and induction nucleation of crystallization (Knorr *et al.* 2004). The mechanism of action of ultrasound is related to the acoustic cavitation and acoustic streaming phenomena. In the course of the acoustic cavitation, longitudinal waves are created producing alternate compression and expansion areas that produce bubbles (Figure 2). The acoustic streaming generates acoustic energy dissipation that results in fluid flows. The contribution of all these actions causes bacterial disorders-disruptions. Furthermore, the gases in the bubbles with high temperatures and pressures are changed to reactive species and free radicals. These chemical reactions (free radicals) and physics (pressure gradient and heat) created during the ultrasound act at a cellular level. During treatment, ultrasound can originate some damage in the tissues of the product provoking mechanical damage, destroying the cellular walls and consequently the intracellular is set free causing cellular death (Cárcel *et al.* 2012; Sango *et al.* 2014).

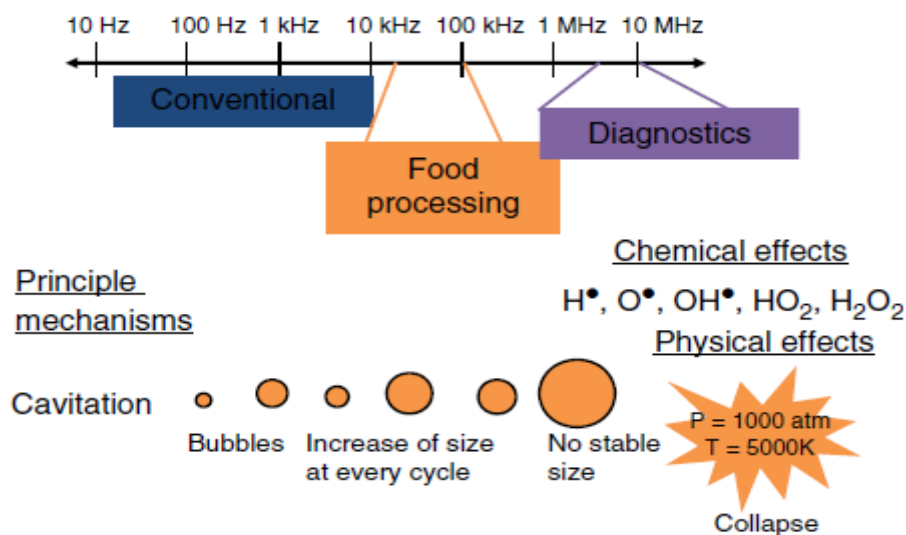


Figure 2: Ultrasound microbial mechanism of action Sango *et al.* 2014.

The combined use of ultrasound with other technologies like pressure, temperature, U.V., decontamination solutions and antimicrobial solutions is beneficial to the efficacy of the ultrasound since this technology requires more time and energy to inactivate microorganisms when compared with assisted ultrasound. The use of this complemented technologies are more energy efficient and have less impact on the properties of the food (Sango *et al.* 2014). The combination of temperature with the ultrasound technology helps the reduction of the technology requirements. The heat during the thermo-sonication contributes to the mechanical rupture of the cells, becoming more susceptible to the cavitation action. It presents several advantages like the reduction of the energy costs and the preservation of food properties when compared with heat treatment itself. Nevertheless, too high temperatures can originate an opposite effect and reduce the cavitation phenomena (Cárcel *et al.* 2012).

Pressure can also be conjugated with ultrasound technology to raise the microorganism's inactivation because it increases the production of free radicals and generates a major implosion of bubbles. For this combined technology is important to determine the level of critical pressure to reach the maximum synergetic effect (Cárcel *et al.* 2012). The combination of heat, pressure and ultrasound can be applied for a higher microbial inactivation. This treatment can inactivate sporulated bacteria. At lower temperatures, <50°C, the pressure has the primary effect on inactivation and the effect of heat is negligible. At higher temperatures, the rate of microbial reduction is a result of the synergistic effect of heat and pressure (Cárcel *et al.* 2012). The ultrasound is also used in combination with other technologies such as ozone and chlorine dioxide (Bhargava *et al.* 2015; Bermúdez-Aguirre *et al.* 2013).

1.5 Shelf-life and Sensory Maintenance

According to the Institute of Food Science and Technology (IFST) guidelines shelf-life is defined as “the time during which the food product will remain safe, be certain to retain desired sensory, chemical, physical and microbiological characteristics and comply with any label declaration of nutritional data when stored under the recommended conditions” (Kilcast *et al.* 2000). All food undergoes deterioration at some point which may include loss of nutritional value and texture, colour and organoleptic changes and most prominently the safety may be jeopardized (Tucker 2008).

Fruits and vegetables are among the most perishable foods in the market (Ramos *et al.* 2013). Specifically in leafy vegetables, the most common factors of deterioration are the enzymatic activity, growth of spoilage microorganisms, moisture loss and wilting (Watada *et al.* 1999). The shelf-life of fruits and vegetables is influenced not only by the water activity, the presence of pathogens or the prevalence of physical damage, but also for the specific pre-harvest characteristics like the position of a fruit in the tree, the exposure to environmental factors like sunlight, pests or diseases (Kilcast *et al.* 2000). General seasonal effects (particularly temperature during development), rootstock, cropping, pruning, irrigation, plant nutrition and the use of plant growth regulators during production all can impact the future sensory characteristics (Mattheis *et al.* 1999). Such factors have influence in the pre-harvest season and can lead to an early harvest time and, consequently, improper development of the desirable flavour (Tucker 2008).

The green vegetables fall within the range of healthy products and even after their treatment should remain their appealing sensory characteristics to consumers (Oliveira *et al.* 2013). The choice to purchase a product lies with the visual characteristics and differences (Ragaert *et al.* 2007). Colour has been shown to have a significant part in food choice, food preference and acceptability in addition to tasting thresholds, sweetness perception and pleasantness (Rico *et al.* 2007). Through appearance, it is possible to understand the deterioration level of a fresh produce product, perceiving the freshness and quality decline. Appearance and also texture are among the quality attributes that raise more interest improving as a way to maximize shelf-life (Toivonen *et al.* 2008).

Regarding fresh produce, lettuce and carrot can suffer colour loss due to different factors, mainly carotene degradation, whiteness and browning on carrots, and chlorophyll degradation and browning appearance in the case of lettuce (Rico *et al.* 2007). Many products have the sensory shelf-life determined by their browning rate (Jacxsens *et al.* 2002). Lettuce presents two types of browning, edge browning and russet spotting. Damaging the lettuce by cutting, cracking or breaking will produce a signal that migrates through tissue inducing the metabolic process of melanin production causing browning fragments (Rico *et al.* 2007). The rate of enzymatic browning can be slowed down by lowering the O₂ concentrations, becoming the shelf-life depending on other sensory quality attributes except from this one (Ragaert *et al.* 2007). Enzymatic browning is mainly caused by two plant enzymes PAL (phenylalanine

ammonium lyase), a major enzyme in phenolic biosynthesis and PPO (polyphenoloxidase), responsible for the conversion of phenols into quinones. The condensation of these quinones originates from the brown polymers (melanins). When the fresh produce has bruised, or cells are ruptured in damaged areas of tissue, the contact between cellular enzymes such as PAL and PPO with substrates results in phenol oxidation and ensuring melanin formation (Kilcast *et al.* 2000; Ragaert *et al.* 2007). A theoretical approach to modulate the PPO enzyme actions and subsequent browning of the tissues was the use of anti-browning formulations (Toivonen *et al.* 2008). Sulphites were once used for browning prevention, but their use was banned by FDA in 1986 because its potential threats to health (Buta *et al.* 1999).

Other alternatives to prevent browning were investigated such as citric acid, ascorbic acid, isoascorbic acid and sodium erythorbate, thiol containing amino acids such as N-acetylcysteine and glutathione, oxalic acid and 4-hexylresorcinol (Oms-Oliu *et al.* 2010). Calcium derivatives are as well used as an alternative and were proved to maintain or improve tissue firmness and crispness. The most frequently used is calcium chloride but it is reported to leave a residual taste in the product. Calcium salts such as calcium lactate, calcium propionate or calcium ascorbate have been investigated as alternative sources of calcium (Oms-Oliu *et al.* 2010).

Another important sensory aspect of fresh produce is texture. On a cellular level, the texture of plant products is determined by cell-wall structure and internal pressure within the cells (turgor). The firmness and intercellular adhesion is given by cell wall polysaccharides, the pectins. Pectins can be destroyed by enzymes that are released in the case of tissue damage (Ragaert *et al.* 2007). Next to this, the texture is also dependent on genetic, environmental, postharvest handling and storage conditions (Toivonen *et al.* 2008). The microbiological activity might have influence on texture breakdown and it is likely that the initiate decay will be originated from a complex mixture of bacteria rather than a single pathogenic (Ragaert *et al.* 2007).

Texture can be portrayed in different ways: crispness, hardness, mealiness, flouriness, grittiness among others (Harker *et al.* 1997). Two major factors have more influence on the consumer, i.e., the firmness and the juiciness. Firmness is determined largely by the physical anatomy of the tissue, particularly cell size, shape and packing, cell wall thickness and strength, and the extent of cell-to-cell adhesion, together with turgor status. These can be inter-related with juiciness once tissues with small cells tend to have a greater content of cell walls, a lower relative amount of cytoplasm and

vacuole (cell sap), a greater area of cell-to-cell contact, and low amounts of intercellular air spaces, making the tissue firmer and apparently less juicy (Toivonen *et al.* 2008).

For consumers, vegetables that maintain firm and crunchy texture are more desirable once they associate these textures with freshness and wholeness. Without a doubt, the appearance of a soft or limp product may result in prior rejection by the consumer before consumption (Rico *et al.* 2007). The off-flavours can also result in rejection by the consumer. The off-flavours can be detected by high performance liquid chromatography (HPLC), gas chromatography (GC), electronic nose systems or sensory analysis by a trained panel. Off-flavours can be the result of the microbial load. Often, bacteria count exceeding 8 log cfu/g or a yeast count exceeding 5 log cfu/g are indicators of off-flavours (Ragaert *et al.* 2007). Surface treatments using aqueous solutions of antimicrobial agents, antioxidants, calcium salts or functional ingredients such as minerals and vitamins have been used to dip fresh-cut fruits and improve the quality. Still, the efficiency of these compounds could be enhanced with the application of edible coatings in order to increase the shelf-life of fresh-cut produce (Oms-Oliu *et al.* 2010).

1.6 Sensory Analysis

Sensory analyses are the response for the sensory evaluation of products quality using three main criteria discrimination, description and preference (Meilgaard *et al.* 2006). Discrimination is of particular relevance in the context of product quality control and in the investigation of possible defects in products. It is dependent on the person's ability to detect and recognize differences. Descriptive tests are more suitable for the development of products or reformulate an existing one by changing the ingredients or processes. This test requires an extensive knowledge of the product's sensory characteristics so frequently to achieve high levels of sensory acuity trained, and expert panels are used (Carpenter *et al.* 2000). Preference and acceptability tests are aimed at establishing whether product differences are recognised by the consumer and are seen to be improving liking or acceptability. These methods lie on the border between sensory analysis and consumer research and have different panel recruitment criteria from those of discrimination or descriptive tests. These assessors need to be representative of the target consumer population and preferably to have little or no sensory training. The purpose of a shelf-life study is to find out how long a food product may be stored before there is an unacceptable deterioration in its sensory quality (Carpenter *et al.* 2000).

Sensory analysis is a tool widely used due to its simplicity and speed of implementation, and it can be used for quality control. It can be applied in different stages from raw materials up to finished products. The Qualitative Descriptive Analysis method (QDA) evaluates all the sensory attributes of food products, explicitly appearance, aroma, flavor, and texture (Oliveira *et al.* 2013). In the sensory evaluation of lettuce, appearance has a major influence in choosing the product since brown spots on the leaves (surface browning) and extremities (cut edge tissue browning) might be the cause for the rejection of the product. The sensory attributes are crucial for good consumer acceptance that lead to the purchase of the product (Oliveira *et al.* 2013).

AIM

The objective of this study is to evaluate the impact of ultrasound treatment in the lettuce leaves shelf-life and sensory parameters. On the one hand lettuce storage studies are performed in order to verify the impact of this technology in the shelf-life of lettuce and the behaviour of the microorganisms in time at different temperatures. On the other hand sensory studies are performed to assess the impact of ultrasound technology on the quality of lettuce. The more suitable sensory parameters are evaluated such as appearance, browning, texture and odours.

2. MATERIAL AND METHODS

2.1 Methodology Review

Initially a literature review of existing methodologies was performed. This research consisted of previous studies focusing on both microbiological and sensorial analysis. Therefore, this methodological review focused on microbial storage studies of fresh produce and decontamination treatments (particularly ultrasound treatment) and sensory studies in fresh produce and shelf-life of these products. As a result, two summary tables with the most important aspects were compiled and can be consulted in the annexes:

2.2 Microbiological Analysis

2.2.1 Preparation of fresh-cut lettuce

Romaine lettuce (*Lactuca sativa* L. var. longifolia) originated from a local farm in Rabat (Malta) was purchased from a local supermarket. The outer leaves were removed and discarded. The inner leaves were washed with tap water to remove any visual contamination and then cut in 4 cm x 4 cm square pieces with a knife scalpel.

2.2.2 Culture Preparation and Inoculation

Cells of *Salmonella enterica* Typhimurium, obtained from the National Collection of Types Cultures (Health Protection Agency, Salisbury, England), were prepared in beads and kept in vials in a freezer at -70°C. The stock cultures were re-activated by inoculation onto Tryptic Soy Agar (TSA) (Oxoid, UK) plates which were incubated for 24 ± 2 h at 37°C in order to obtain single colonies. The inoculum was prepared by selecting a single colony from the stock culture and incubating it in 100 mL of Tryptic Soy Broth without Dextrose (TSB-D; Scharlau, Spain) at 37°C for 24 ± 2 h. A subculture was also prepared in TSB-D at the same temperature for 17 h. After incubation, *Salmonella* reached their stationary phase. Late stationary phase culture of *Salmonella* (10^8 cfu/mL) was used because this is the phase where bacteria are most resistant and stable. The bacterial cells were collected by centrifugation (6400 g) for 20 min (Benchtop Centrifuge 2-16P, Sartorius, Goettingen, Germany) washed in Ringer's solution (Oxoid, UK) and vortexed. The final pellet was re-suspended in Ringer's solution to create a suspension with *Salmonella* (10^6 CFU/cm²). A spot inoculation method was used to inoculate the pathogenic bacteria onto lettuce surface. 100µL of

inoculum was spotted (approximately 8 drops) onto the surface of each piece. After 60 min (to allow the bacteria attachment) the same procedure was performed on the other side of the pieces. Each sample was composed of six pieces of lettuce.

2.2.3 Treatment Procedure and Storage

Lettuce samples were immersed in a glass beaker with 500 mL of sterile water. The ultrasound treatment was applied with an ultrasonic system (UP 200ST, Hielscher Ultrasonic, Teltow, Germany) operating at 26 kHz, 90 μm , 200 W attached to a probe of 14 mm \varnothing . The technology was set up to 100% amplitude, 3cm water depth in continuous mode for 5 min. The experiments were developed at room temperature. Operation conditions were selected based on previous studies proving the antimicrobial efficiency of ultrasound on *E. coli* (Sango *et al.* 2014). Four different samples were prepared: (i) a non-inoculated sample (negative control 1), (ii) a non-inoculated sample with ultrasound treatment (negative control 2), (iii) an inoculated sample without ultrasound treatment (negative control 3) and (iv) an inoculated sample with ultrasound treatment. All samples were dewatered with a manual salad spinner, packed in 40 μm OPP bags (Zamco, Malta), hot sealed and stored at different temperatures, i.e., 5°C, 10°C and 15°C for 11 days.

2.2.4 Bacteria Enumeration

For enumeration of bacteria, each lettuce sample was transferred into a sterile stomacher bag (BagFilter O, Interscience, Saint Nom, France) with 30 mL of Ringer's solution and homogenized in a stomacher (BagMixer 400P, Interscience, Saint Nom, France) for 2 min. Serial 10-fold dilutions were prepared in 9mL tubes of Ringer's solution. 100 μL of appropriated dilutions were spread-plated onto selective media. Total aerobic count and psychrotrophic bacteria were plated on Tryptic Soy Agar (TSA; Oxoid, UK) and incubated at 37°C for 24h and 4°C for 10 days, respectively. Salmonella was plated in Xylose Lysine Deoxycholate media (XLD; Scharlau, Spain) at 37°C for 24h. For the Enterobacteria, Violet Red Bile Dextrose Agar (VRBDA; Scharlau, Spain) was used and the plates were incubated at 37°C for 24h. Yeasts and moulds were determined by plating in Rose Bengal Chloramphenicol Agar (Oxoid, UK) supplemented with chloramphenicol and incubated at 25°C for 5 days. The bacterial enumeration was made at days 0, 2, 4, 7, 9 and 11 of storage.

2.3 Sensory Analysis

2.3.1 Preparation of the fresh-cut lettuce

The Romaine lettuce (*Lactuca sativa* L. var. longifolia) originated from a local farm in Rabat (Malta) was purchased from a local supermarket. The outer leaves were removed and discarded. The inner leaves were washed with tap water to remove any visual contamination and then cut into 4 cm x 4 cm square pieces with a knife scalpel in the same way as the microbiological analysis lettuce samples.

2.3.2 Packaging, Storage and Treatment Procedures

The samples were treated with ultrasound at the same mode of the microbial studies, i.e., 5 min, as well as shorter treatments that can be considered more feasible at industrially operation conditions, i.e., 2.5 min. Similarly, control samples were only emerged in water for a 2.5 treatment time. The samples were stored at the lowest and highest temperatures of the microbial analysis (5°C and 15°C) and evaluated at five different days (0, 2, 4, 7 and 10).

2.3.3 Panellists Training

The panel was selected from a group of volunteers who were submitted to training sessions. The volunteers with best scoring results in the screening tests were selected for the panel. These tests included: matching tests, discrimination tests and ranking/rating tests.

In this training sessions, the panel had to do the colour-blindness test, ranking sweet and sour solutions, ranking different samples according to the intensity of the off-odour, ranking lettuce images (alike the lettuce samples used in the study) according to the overall appearance. In the last training sessions, the panel had more specific tasks that comprised using the ranking scale that would be utilized in the study, evaluating the attributes that would be assessed in the study and get to know the sensory cabinet and all its compounds. In the training sessions, the answers and responses were discussed to create a possible uniform evaluating system, following the same criteria.

The sensory attributes were evaluated by a semi-trained six member panel. The panel was divided into groups of one or two elements for each evaluation, and no share of opinions was made. The sensory questionnaire can be seen in annexe 1.

2.3.4 Sensory Cabinet

All the sensory evaluations took place in a room equipped with two individual sensory cabinets: equipped and non-equipped (Figure 3a and 3B). The room was set at 20 ± 2 °C (room temperature) with natural and artificial white light.



Figure 3: Equipped sensory cabinet.

The material presented to the panel in each evaluation consisted in: the sample for analysis (Figure 3C); scissors to open the bag, sensory questionnaire and a pen (Figure 3F); the plate to place the sample outside the bag (Figure 3D) and a spittoon (Figure 3E).

2.3.5 Sensory Evaluation

Each evaluation day a fresh sample was presented to the panel in the first place as a blind control. Four different samples were presented to each member in each assessment. To minimize the positional bias during the evaluation, samples were assigned random three-digit codes and submitted to the panel in random order (except the fresh sample that was always presented in the first place).

Five different attributes were evaluated for the panel. Overall appearance was rated on a 9 to 1 scale: 9 = highly acceptable; 8 = acceptable; 7 = moderately acceptable; 6 = slightly acceptable; 5 = neither acceptable or unacceptable; 4 = slightly unacceptable; 3 = moderately unacceptable; 2 = unacceptable; 1 = highly unacceptable. Off-odour, cut edge tissue browning, surface browning and sogginess/watery were evaluated on a 1

to 5 scale: 1 = none; 2 = slight; 3 = moderate; 4 = moderately severe; 5 = severe. Texture was evaluated on a different 1 to 5 scale: 1 = very crisp; 2 = slightly crisp; 3 = moderate; 4 = moderately flaccid; 5 = very flaccid.

2.4 Statistical Analysis

In this experiments we had different variables that could influence the growth of the different type of bacteria. This analysis might be beneficial to have a main conception of all the experiments variables input (different bacteria, sensory scores and processes/preservation). To be able to analyse this behaviour, we choose multivariate analysis. Tests were performed in duplicate or triplicate. The results of the microbial analysis and the individual scores of the sensory evaluation given by the panel were analysed by Multivariate Analysis using R Program v. 3.2.1 for Windows (R Foundation for Statistical Computing, Vienna, Austria). The principal component analysis was the statistical procedure applied followed by a partial least squares that is a standard approach in a regression analysis.

3. RESULTS

3.1 Microbiological Analysis

3.1.1 Total Viable Count

The results show the bacterial growth during storage under the following 4 different conditions:

“No Ino” - non-inoculated sample: natural microflora presented in the lettuce after washed with water;

“No ino+US” - non-inoculated with ultrasound treatment;

“Ino” - inoculated with the pathogenic bacteria;

“US” - inoculated with the pathogenic bacteria with ultrasound treatment.

The total viable count results are presented in the three following graphs (Figure 4, 5 and 6) for all the tested temperatures, i.e., 5°C, 10°C and 15°C. At the lowest temperature, which simulates the refrigeration temperature commonly used, the ultrasound treated samples showed slower bacterial growth than at the others temperatures, until day 9.

Regarding natural microflora, the higher numbers were found at 15°C. After day 4, the non-inoculated treated samples resulted in a great amount of bacteria comparing with the untreated.

The analysis of the pathogenic bacteria has showed that treated samples from day 9, at 5°C, reached higher amounts than the inoculated without treatment. Comparing with the other temperatures, the same was observed earlier at day 4. The higher numbers related to the treated samples could be related to mechanical damaged in the ultrasound samples or because the bacteria reached their stationary phase in the untreated samples. The ultrasound treated samples had a faster microbial increase at high temperatures than at 5°C storage showing that the antimicrobial efficiency of ultrasound treatment is decreasing with the increase of the temperature. The ultrasound appeared more efficient against pathogenic bacteria than against the natural microflora. At 15°C, the bacterial growth was higher than at the other storage temperatures. Additionally, at day 2, the inoculated and treated samples had a higher

amount of bacteria than the others samples showing an early increase comparing with the day 2 from the others temperatures (5°C and 10°C).

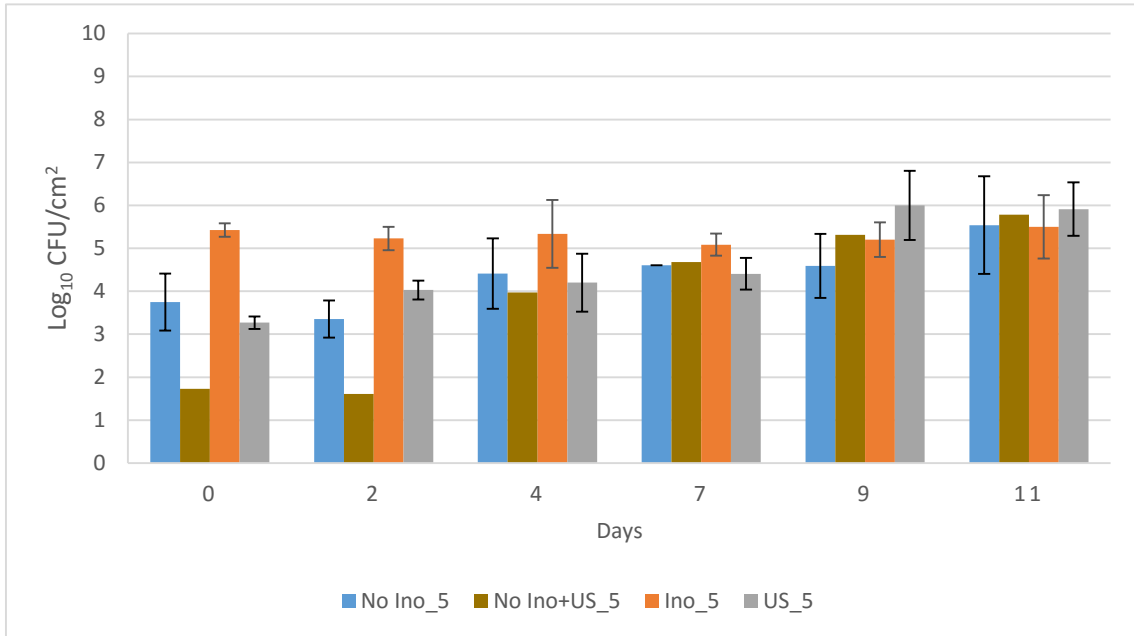


Figure 4: Effects of ultrasound treatment in Total Viable Count during storage for 11 days at 5°C.

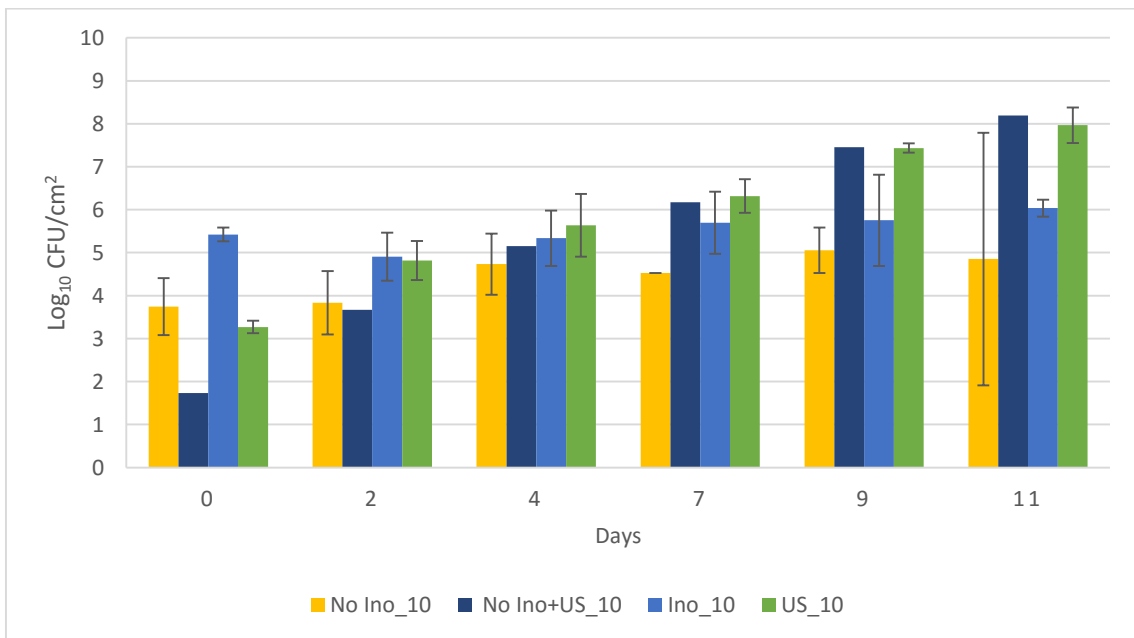


Figure 5: Effects of ultrasound treatment in Total Viable Count during storage for 11 days at 10°C.

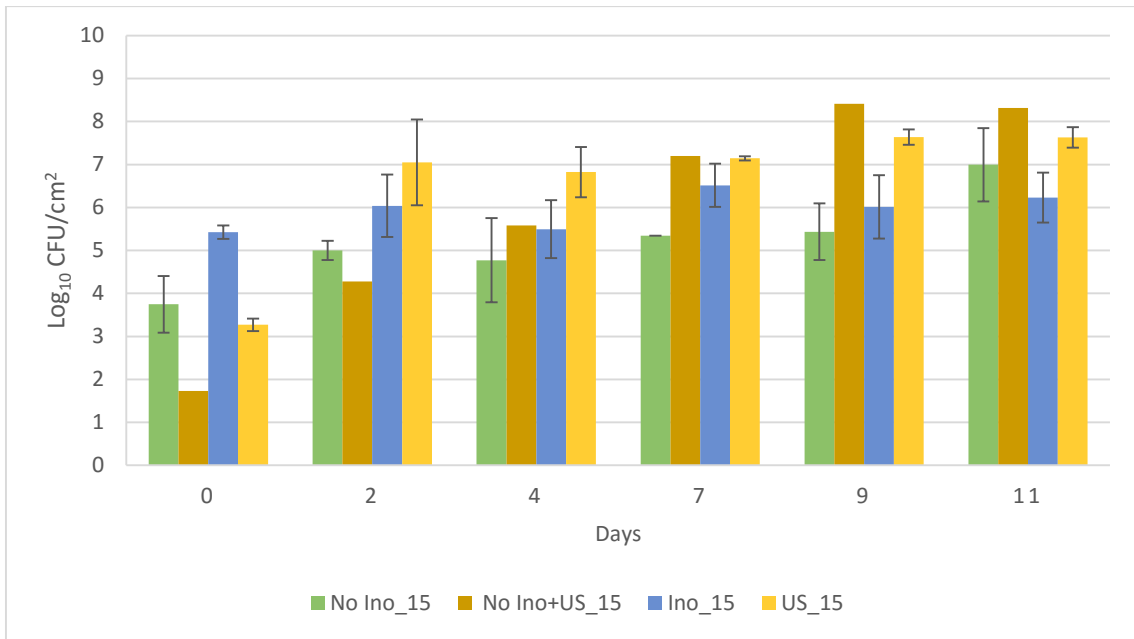


Figure 6: Effects of ultrasound treatment in Total Viable Count during storage for 11 days at 15°C.

PLS – Total Viable Count

In figure 7, PLS (partial least squares) indicates that the storage time had a higher influence (0.71) than the temperature (0.27) or ultrasound treatment (0.06) in the total viable count. The different lettuce samples (numbers in black) were organized accordingly the experiment variables influence (red coloured).

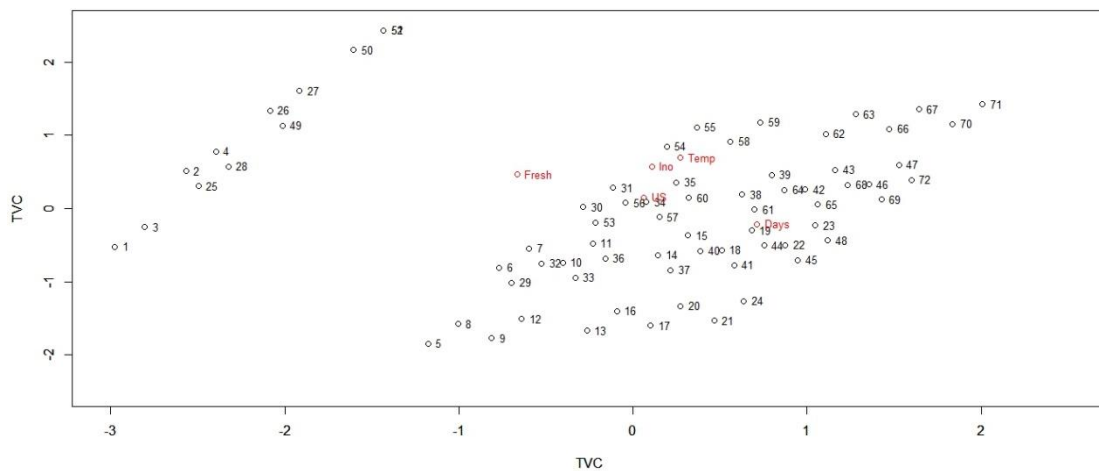


Figure 7: PLS for Total Viable Count

According to PLS, storage time showed to have a significant influence on the results. The linear correlation below shows the relation between storage time and total viable count (Figure 8). The r^2 equals 0.9722 (from 0 to 1) proving a high correlation between the observations and the storage time.

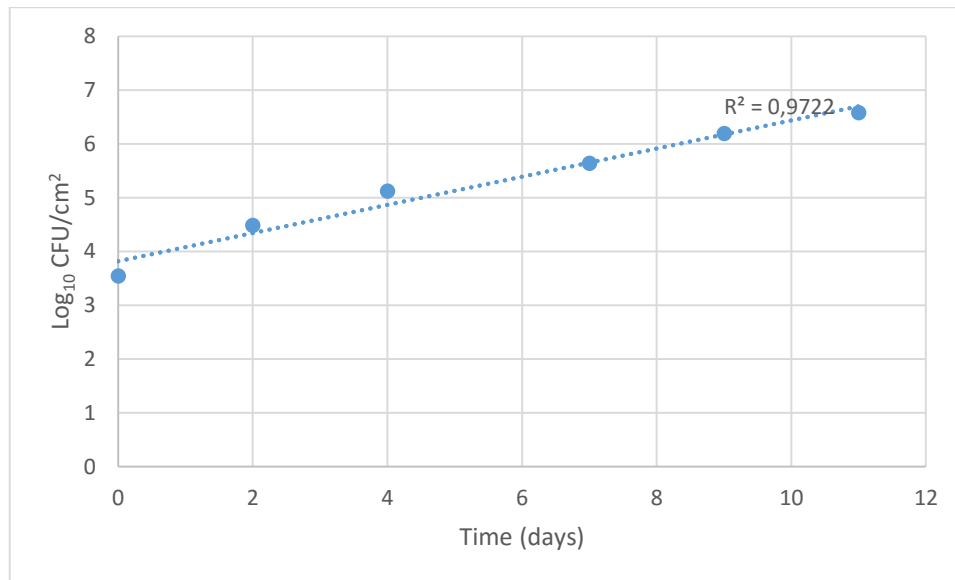


Figure 8: Linear correlation between total viable count amount and storage time.

3.1.2 Enterobacteria

Next to the total viable count analysis the Enterobacteria amount was also analysed and the results are depicted in the three following graphs (Figure 9, 10 and 11) at storage temperatures 5°C, 10°C and 15°C, respectively. It is evident from Figure 10 that Enterobacteria was successfully reduced by ultrasound treatment at 5°C.

The inoculated sample, which includes pathogenic bacteria and the natural microflora had the higher values until day 11, while non-inoculated and ultrasound treated samples had shown a gradual growing through time. This observations could be due to the mechanical damage in the leaves provoked by the ultrasound treatment that might allow better access to nutrients and easier attachment of the bacteria. At low storage temperatures ultrasound appeared to be more efficiency.

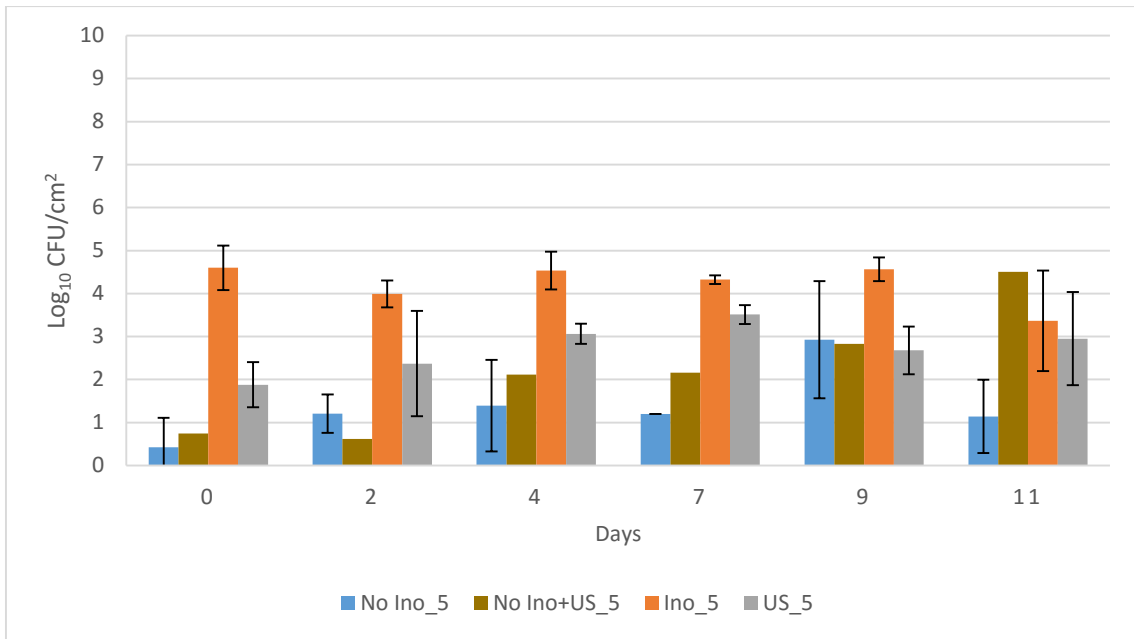


Figure 9: Effects of ultrasound treatment in Enterobacteria growth during storage for 11 days at 5°C.

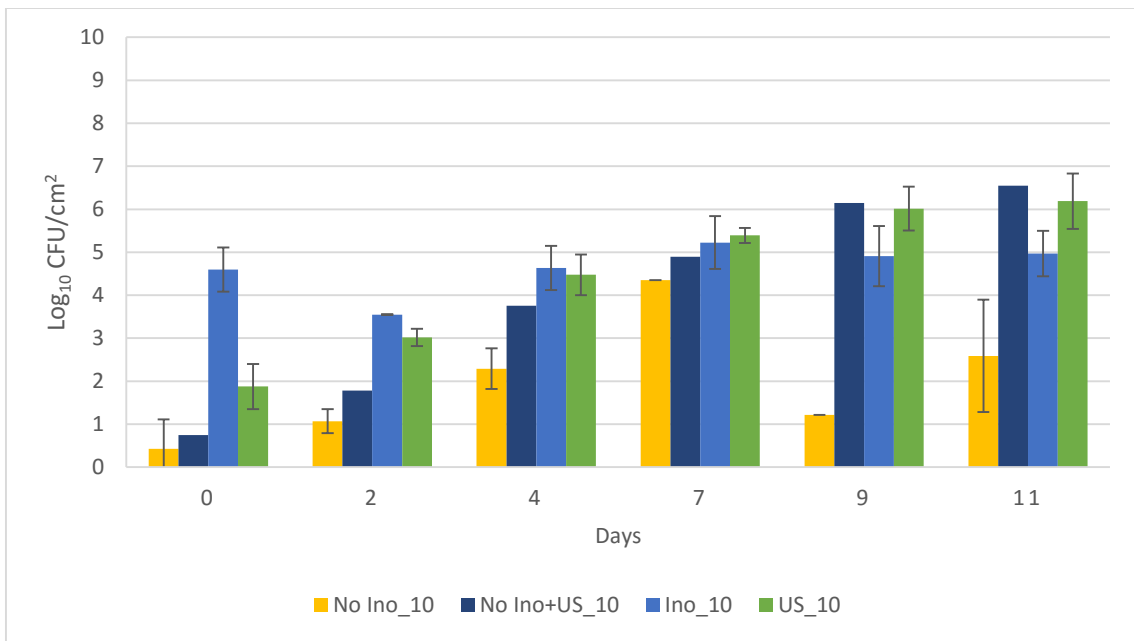


Figure 10: Effects of ultrasound treatment in Enterobacteria growth during storage for 11 days at 10°C.

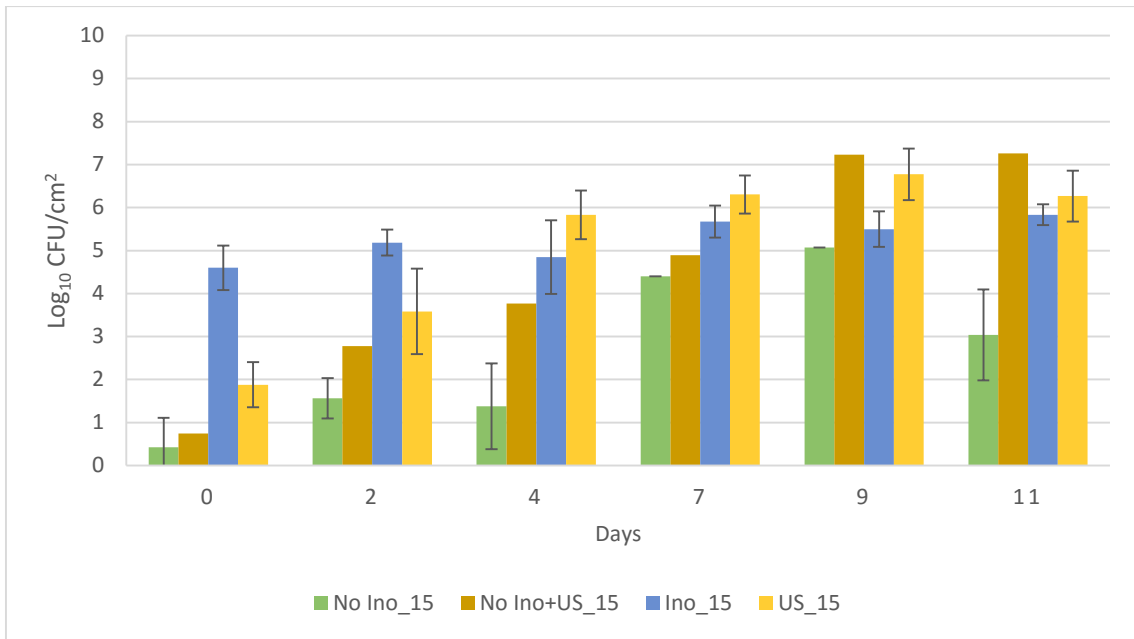


Figure 11: Effects of ultrasound treatment in Enterobacteria growth during storage for 11 days at 15°C

PLS – Enterobacteria

The PLS indicated that storage time had higher influence (0.66) than temperature (0.33) or ultrasound treatment (0.11) on Enterobacteria. The presence of pathogenic bacteria (0.31) had similar influence, as the temperature, in the amount of Enterobacteria indicating that there was not any observed competition on the growth of the studied microorganisms (Figure 12). The PLS for Enterobacteria showed a similar result comparing with total viable count PLS.

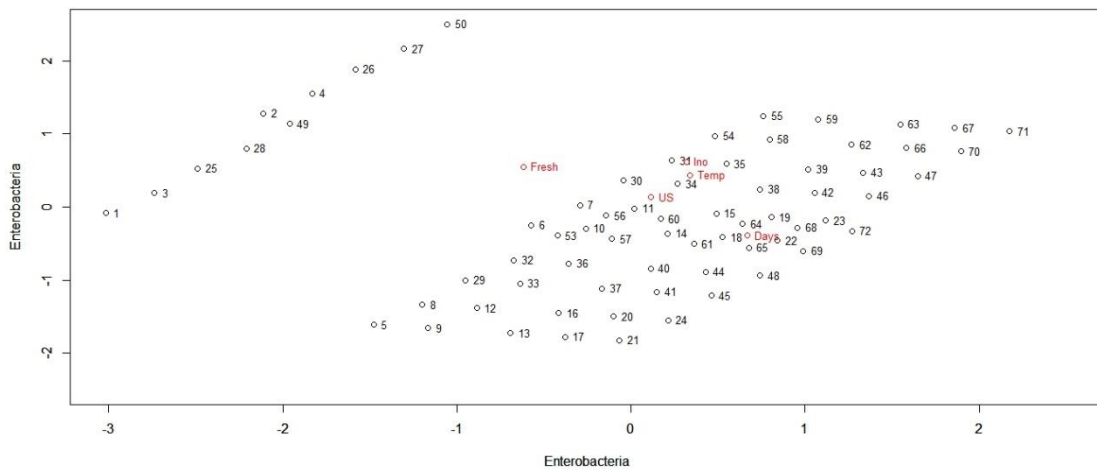


Figure 12: PLS for Enterobacteria.

In Figure 13 the correlation between the Enterobacteria amount and storage time was shown. According to PLS, exists a stronger correlation between storage time and Enterobacteria amount that can be observed through the high r^2 value of 0.915.

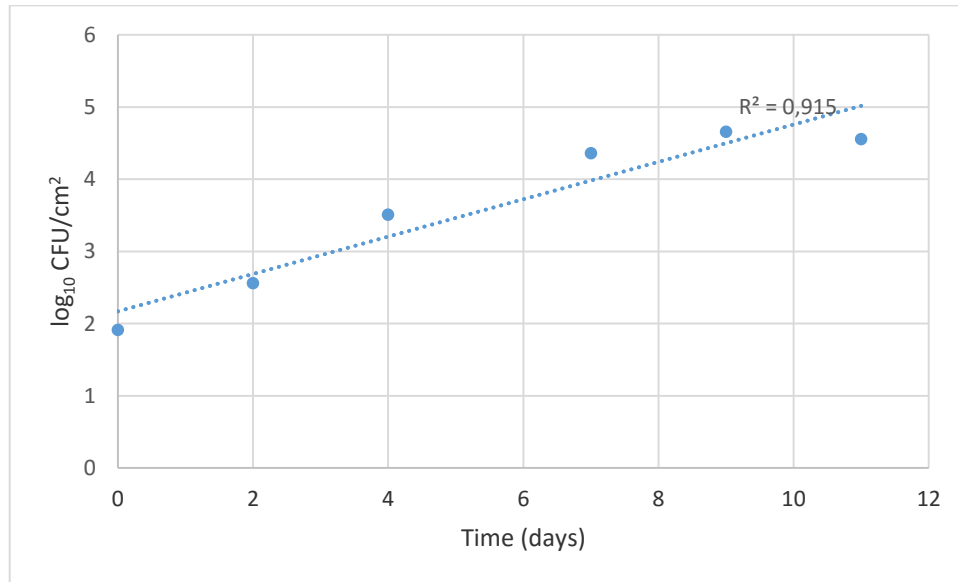


Figure 13: Linear correlation between Enterobacteria amount and storage time.

3.1.3 Psychrotrophic bacteria

Related to the psychrotrophic bacteria, the non-inoculated sample resulted in microbial levels of 4 and 5 \log_{10} CFU/cm² at 5°C and 10°C (Figure 14 and 15, respectively) while at 15°C (Figure 16) the values were higher (7 \log_{10} CFU/cm²). The non-inoculated US treated samples had the greatest increase during the storage. The inoculated sample appeared to have constant growth at the three different temperatures while the inoculated and ultrasound treated samples had a slow increase along with the increase of the temperature.

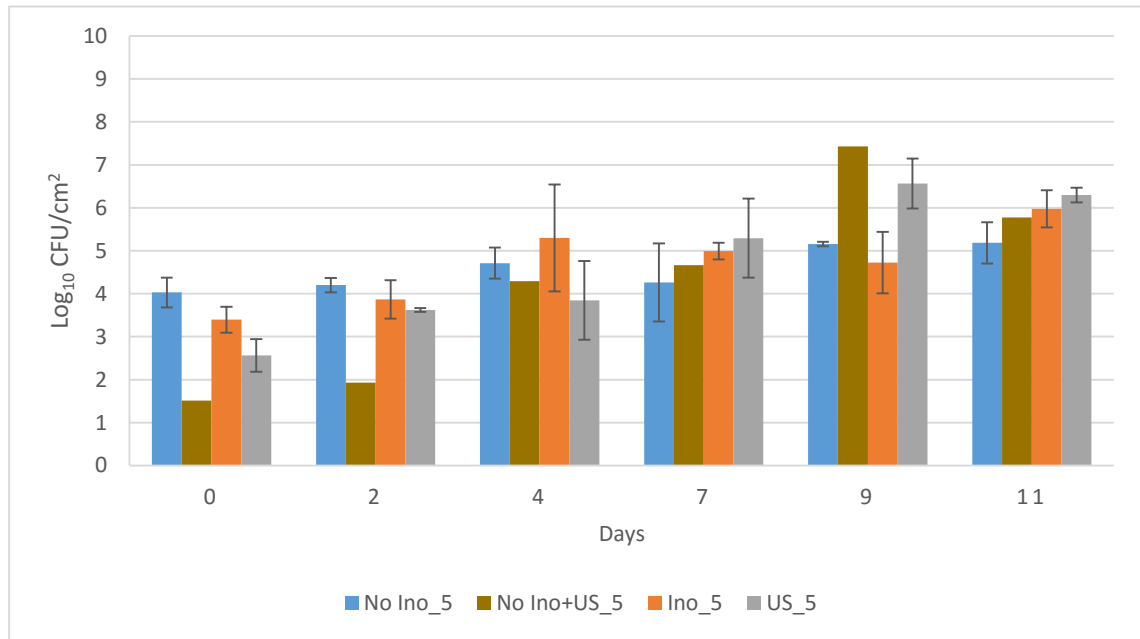


Figure 14: Effects of ultrasound treatment in Psychrotrophic bacteria growth during storage for 11 days at 5°C.

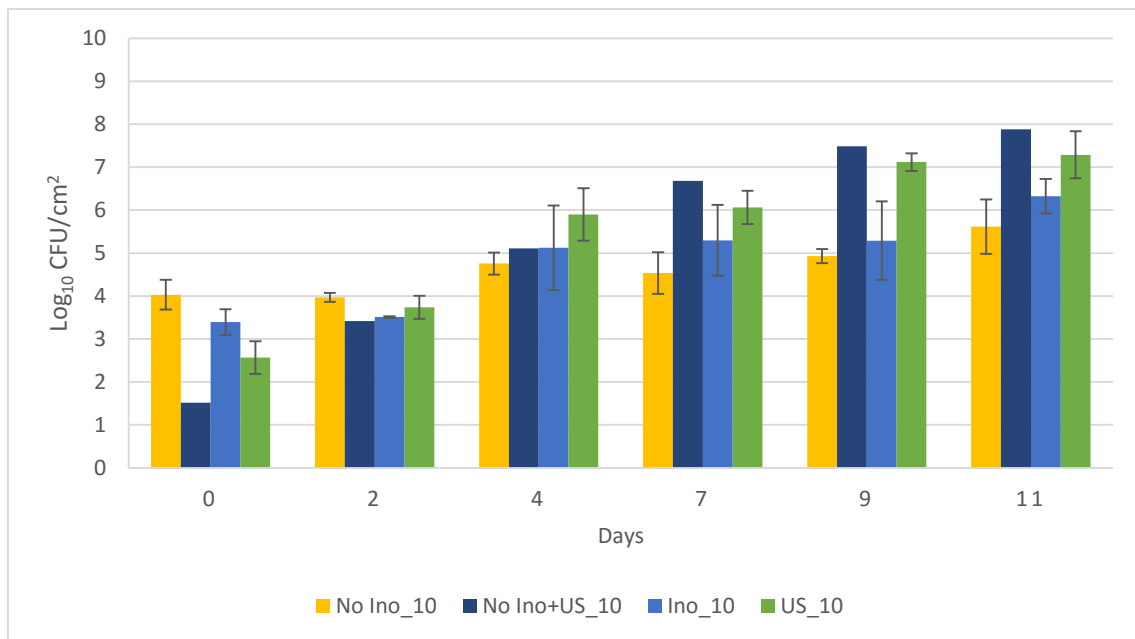


Figure 15: Effects of ultrasound treatment in Psychrotrophic bacteria growth during storage for 11 days at 10°C.

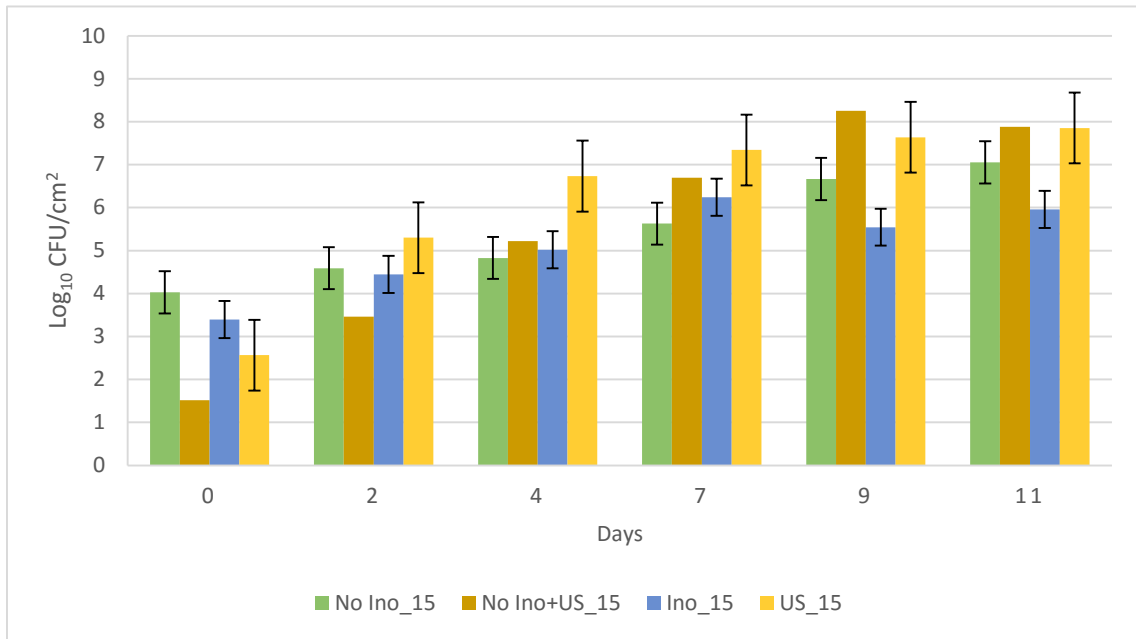


Figure 16: Effects of ultrasound treatment in Psychrotrophic bacteria growth during storage for 11 days at 15°C.

PLS – Psychrotrophic Bacteria

PLS indicates that the storage time had major influence (0.73) (Figure 17). Temperature and ultrasound treatment resulted in lower values, 0.13 and 0.06 respectively. Apparently, the temperature and ultrasound did not affect the bacterial growth considering their values.

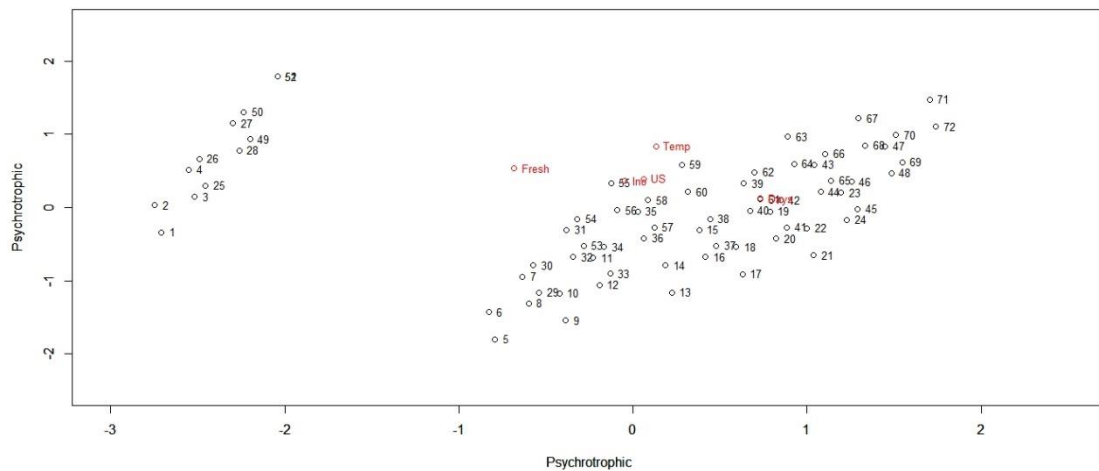


Figure 17: PLS for Psychrotrophic bacteria.

In Figure 18 the correlation between the Psychrotrophic bacteria and the storage time was shown. According to PLS, there is a stronger correlation between storage time and Psychrotrophic bacteria amount that can be seen through the high r^2 value of 0.9517.

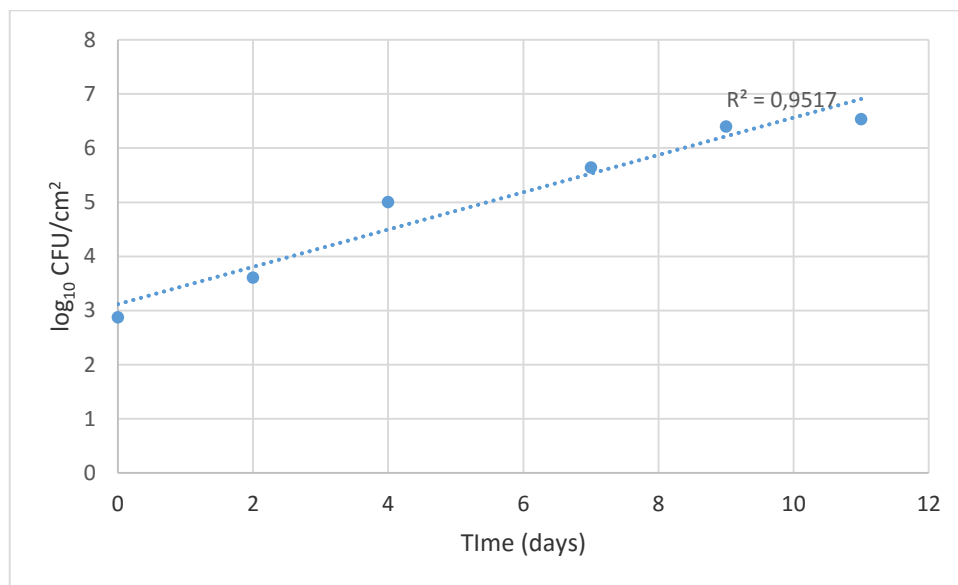


Figure 18: Linear correlation between psychrotrophic bacteria amount and storage time.

3.1.4 Pathogenic bacteria – *Salmonella enterica* Typhimurium

The non-inoculated samples had a bacterial level below the detection limit, and therefore were not presented in the following figures. The US treated inoculated sample had lower values during the 11 days of storage at 5°C (Figure 19) showing a higher efficiency of US against pathogenic bacteria at low temperatures. With the increase of the storage temperature (Figure 20 and 21), the ultrasound resulted in very similar or high amounts of bacteria.

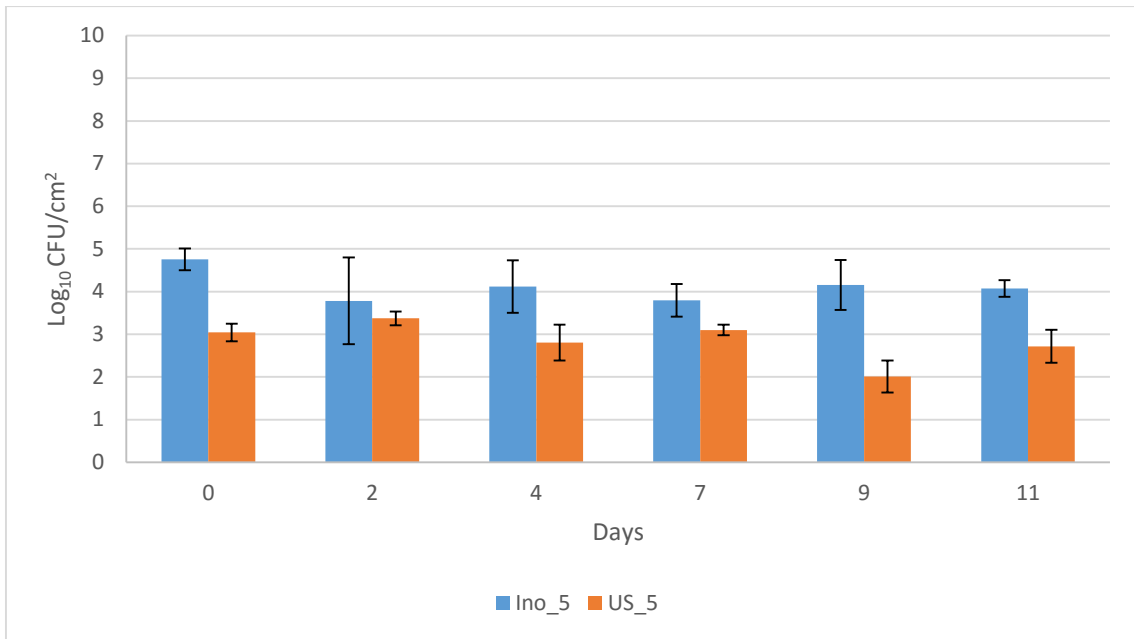


Figure 19: Effects of ultrasound treatment in Salmonella growth during storage for 11 days at 5°C.

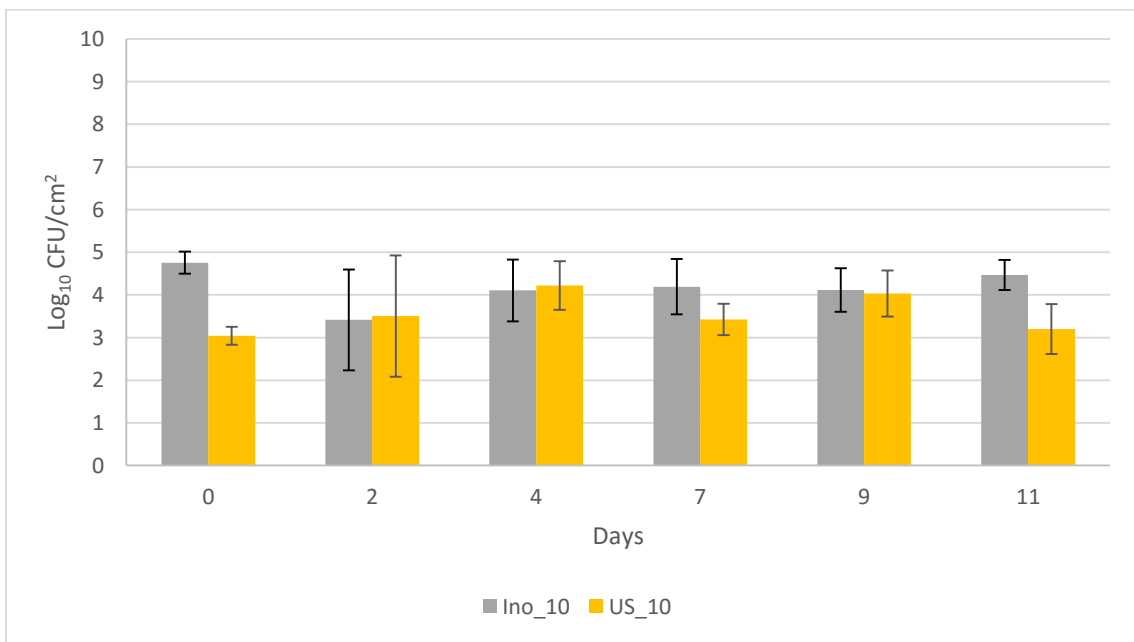


Figure 20: Effects of ultrasound treatment in Salmonella growth during storage for 11 days at 10°C

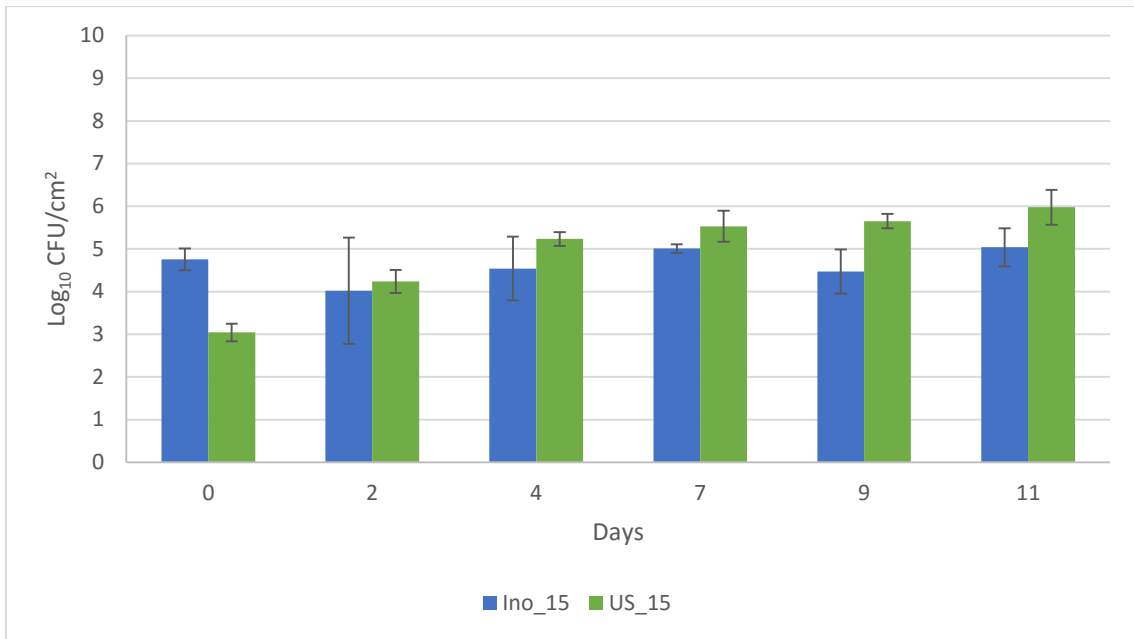


Figure 21: Effects of ultrasound treatment in Salmonella growth during storage for 11 days at 15°C

PLS – Pathogenic bacteria *Salmonella enterica* Typhimurium

The PLS indicated that temperature had major influence (0.84) on the growth of *Salmonella*. The storage time resulted in lower value (0.28) while ultrasound influenced the amount of bacteria in the opposite way (-0.41), suggesting that ultrasound contributed to the decrease of pathogenic bacteria amount (Figure 22).

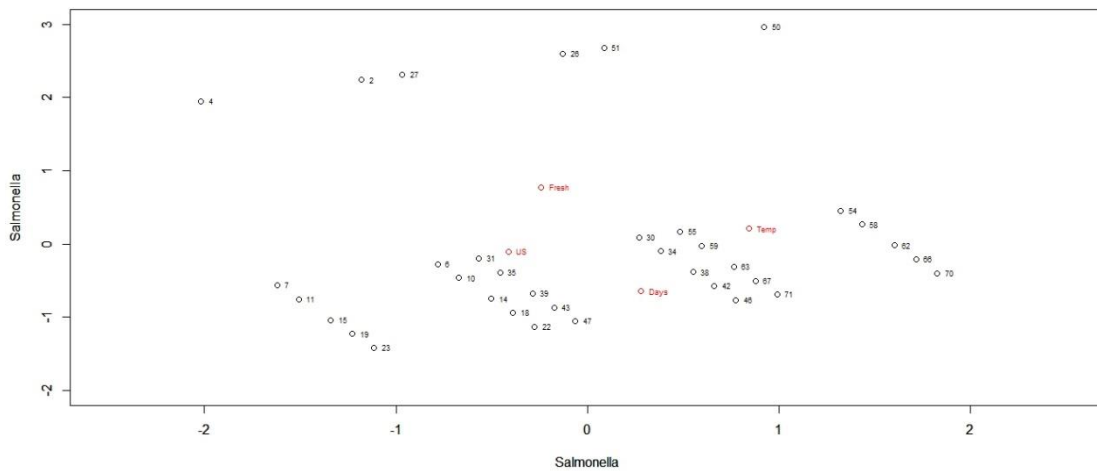


Figure 22: PLS for Pathogenic bacteria.

Figure 23 shows the correlation between the level of the pathogenic bacteria and the temperature.

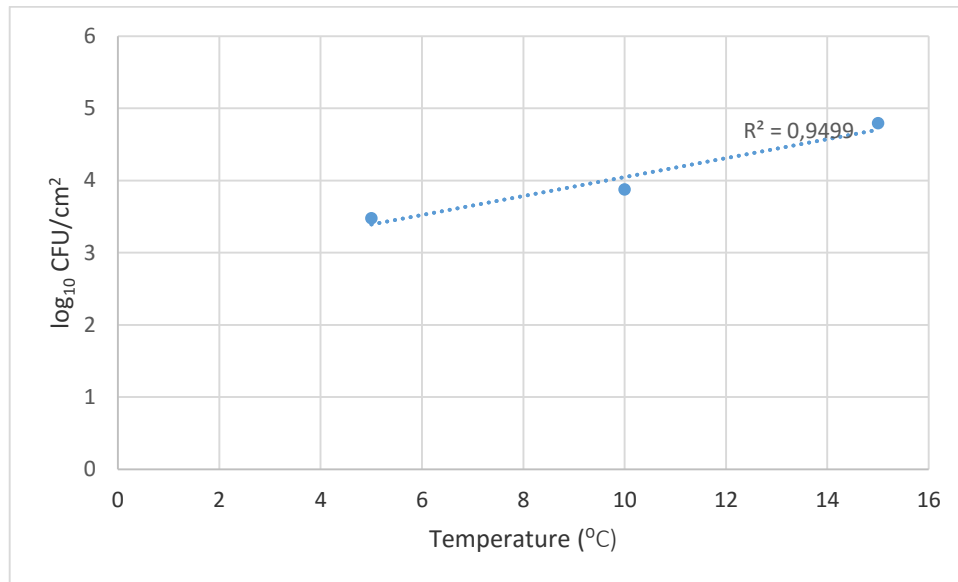


Figure 23: Linear correlation between pathogenic bacteria amount and temperature.

3.1.5 Yeasts and Moulds

In the present study, the number of yeasts and moulds in all samples and at the different temperatures did not exceed 3.5 log₁₀ CFU/cm² during the entire storage period.

3.1.6 Principal Component Analysis (PCA) in Microbiological Analysis

The PCA analysis showed how the different bacteria grew, vary and behave related to the other variables (Figure 24). From the statistics of the PCA was shown that PC1 explained 79.6% of the variance observed while PC2 explained 11.4%. For this reason only PC1 was considered for further analysis.

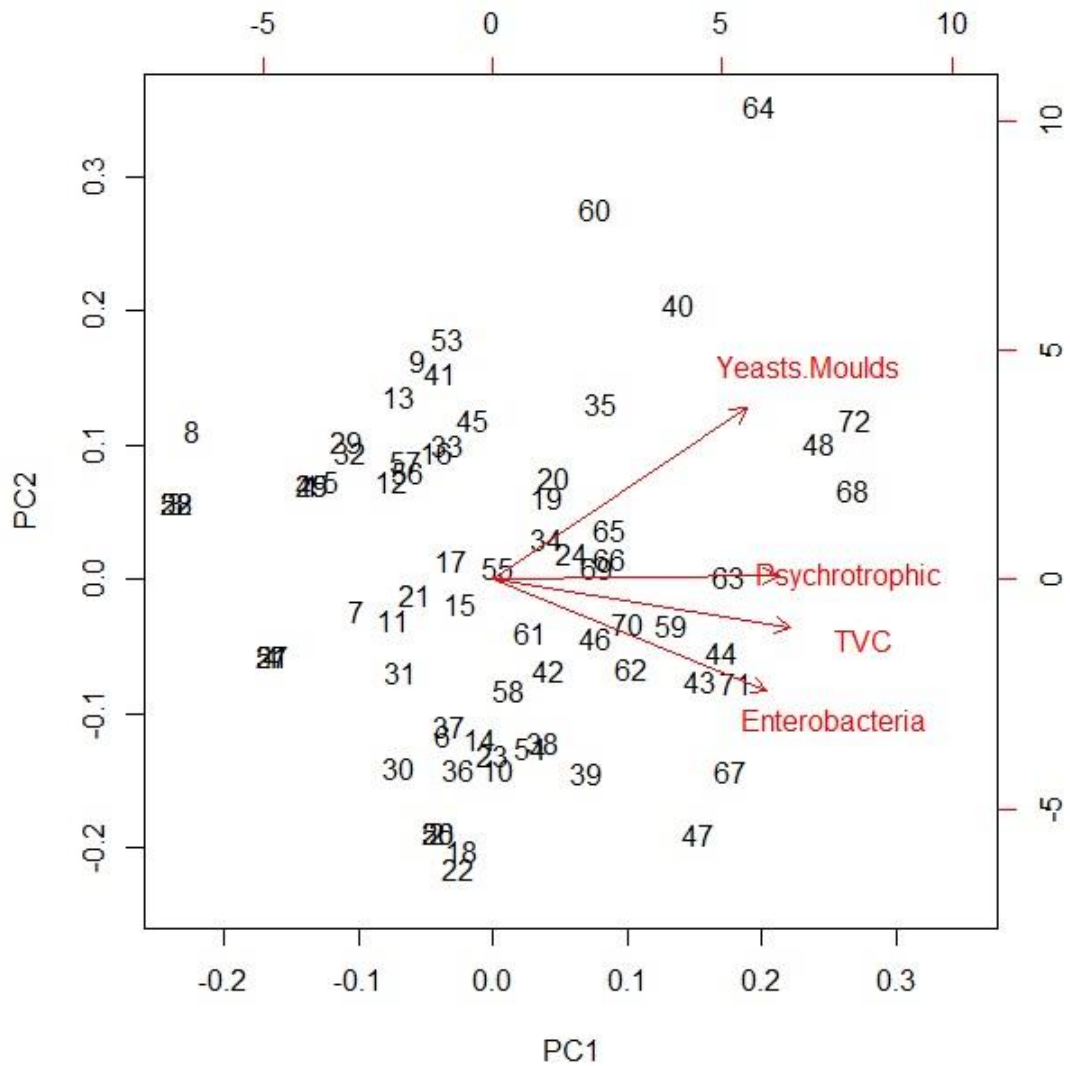


Figure 24: PCA of Microbiological Data. Red lines show the variables while the numbers represent the individual lettuce samples.

3.2 Sensory Analysis

3.2.1 Overall Appearance

In the Overall Appearance attribute (Figure 25), the fresh lettuce was always considered in the range of “acceptable” by the panel presenting the higher scores. The control sample showed a better score at 5°C even after 10 days of storage while, at 15°C, the decrease in quality occurred immediately after day 2.

Regarding the treated samples, ultrasound appeared to have an impact in the sensory quality of lettuce as these samples were the ones with lower scores. The ultrasound damaged the structure of the leaves causing an aspect less acceptable. Nevertheless, samples at 15°C still had lower values than those at 5°C suggesting that higher temperatures were not favourable for the storage of fresh produce at a sensory level.

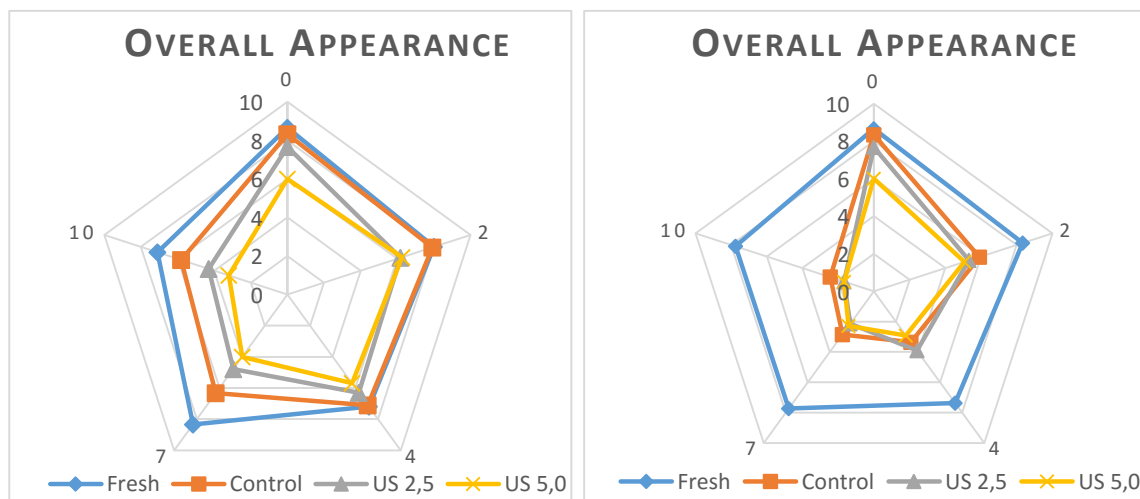


Figure 25 Scores attributed to Overall Appearance, after storage for 10 days at 5°C (left) and at 15°C (right).

PLS – Overall Appearance

The partial least squares model indicated that storage days (-0.77) had more influence on the sensory evaluation, followed by the ultrasound treatment (-0.58). The temperature (-0.22) was the less responsible as can be depicted in Figure 26. The overall appearance values followed an opposite relation with the other attributes due to the evaluation scale (10 for the high quality lettuce and 1 for the low quality). Based on this we can assume that lettuce stored at 5°C for 10 days can be considered of better quality than lettuce stored for 2 days at 15°C

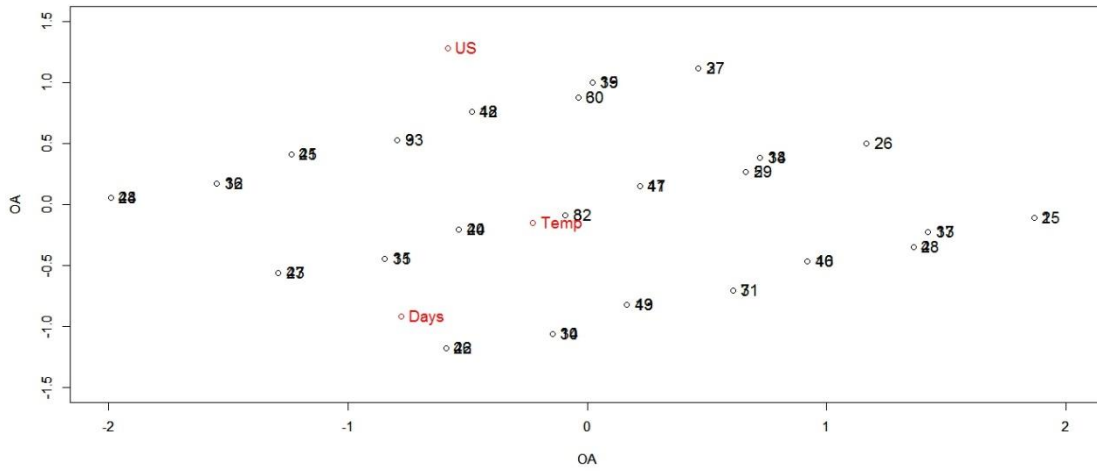


Figure 26: PLS for the Overall Appearance attribute.

The Figure 27 represents the linear correlation between overall appearance attribute and storage time. It can be observed that there was a decrease in the overall appearance with the increase of the storage time.

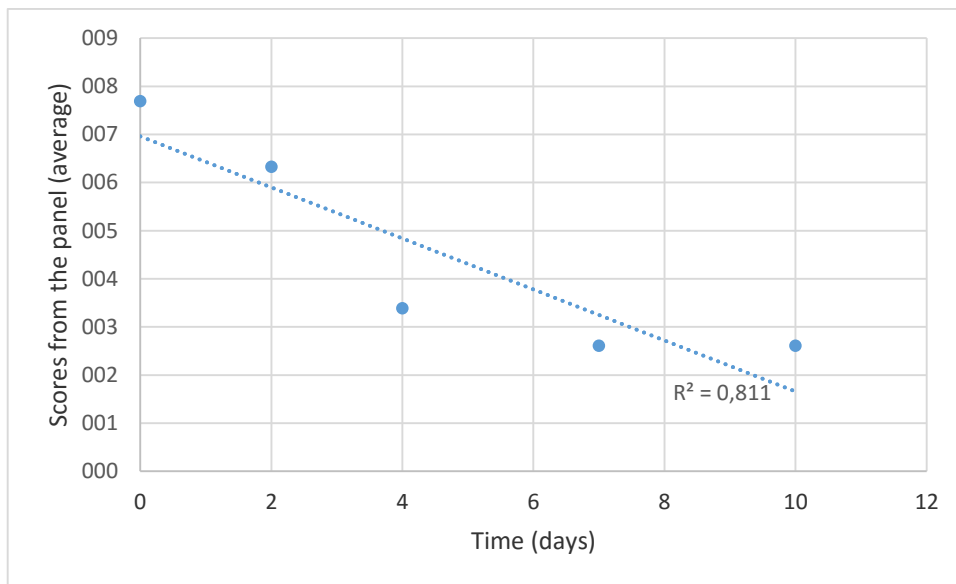


Figure 27: Linear correlation between overall appearance scores and storage time.

3.2.3 Off-Odour

Regarding the off-odour scores during storage (Figure 28), it was observed that the higher score was obtained at day 10 at 5°C while at 15°C the increase in off-odours was observed at day 4 onwards.

The treated samples had the lower quality scores. Nevertheless, the major time of storage between 4 and 7 days showed similar results comparing to the other attributes.

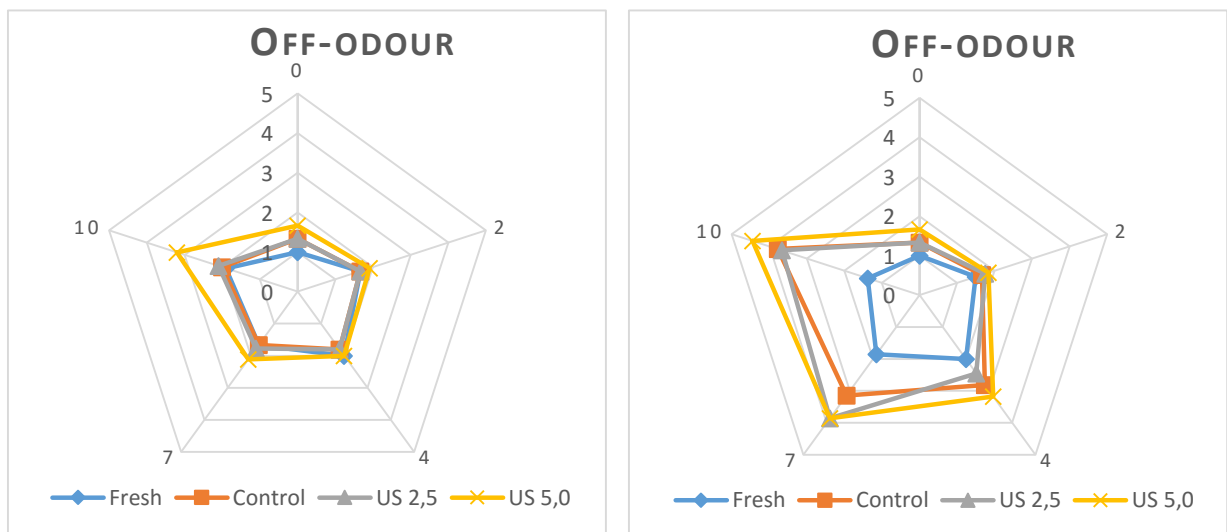


Figure 28: Scores attributed to Off-Odour, after storage for 10 days at 5°C (left) and at 15°C (right).

PLS – Off-Odour

PLS analysis indicated that temperature has a slightly greater influence on off-odour attribute (0.69) than storage time (0.68) followed by ultrasound (0.26) (Figure 29). A closer analysis of the results indicate that the samples with better sensory results were the US treated and stored at 5°C for 2 days. The worst results were observed for the US treated for 5 min when stored at 15°C for 10 days. Consequently, the off-odour attribute could be better controlled if samples are stored for more time but at a lower temperature.

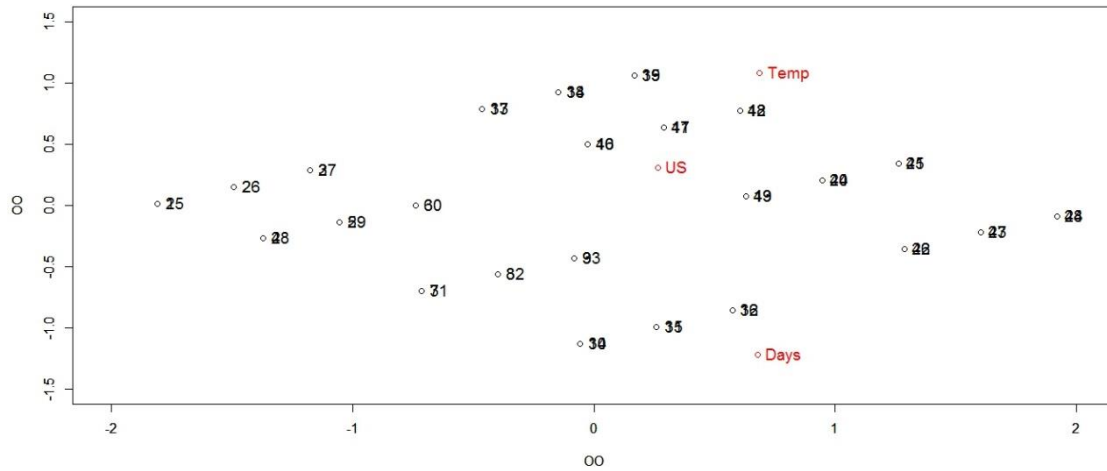


Figure 29: PLS for Off-Odour attribute.

3.2.4 Cut Edge Tissue Browning

The fresh lettuce resulted in the most acceptable scores (Figure 30). At 5°C, the samples maintain lower values until day 4 decreasing the sensory quality until day 10. At 15°C, the samples showed higher scores indicating greater browning in the cut edge of the lettuce. The US treated samples and the control had similar scores showing that US might not have a major influence in this attribute.

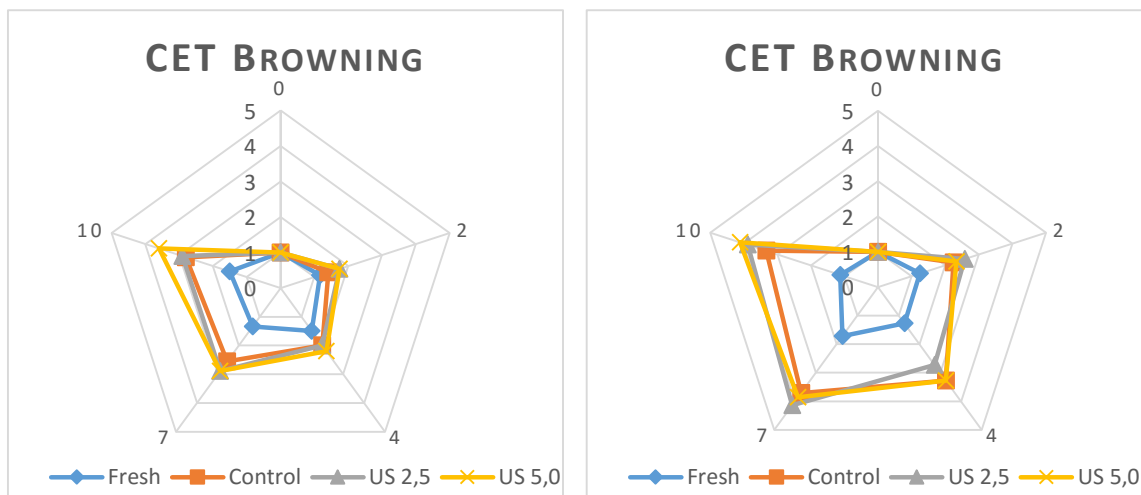


Figure 30: Scores attributed to Cut Edge Tissue Browning, after storage for 10 days at 5°C (left) and at 15°C (right).

PLS – Cut Edge Tissue Browning

The PLS (Figure 31) showed that the time of storage (0.76) had higher influence on the lettuce quality than the temperature (0.61) or ultrasound treatment (0.18).

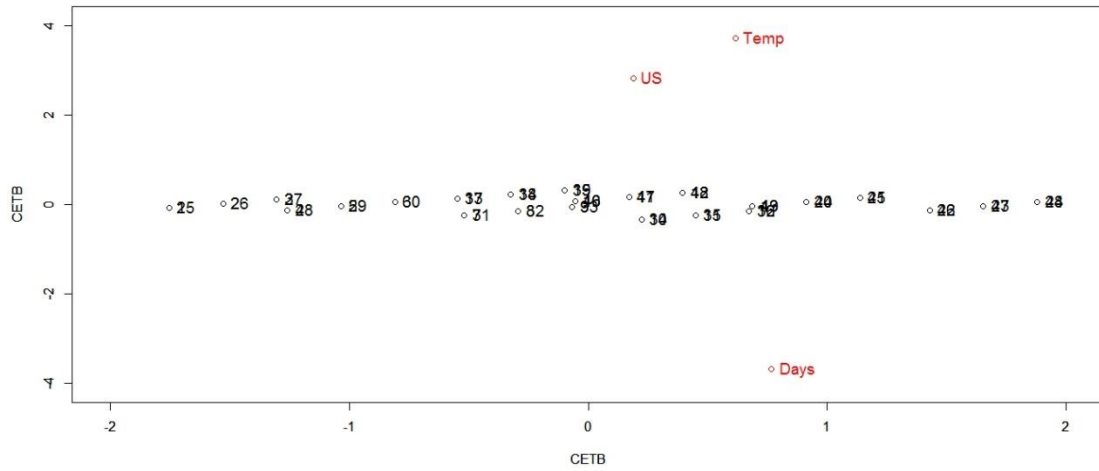


Figure 31: PLS for Cut Edge Tissue Browning attribute.

Figure 32 shows the correlation between cut edge tissue browning and storage time. According to PLS, there is a stronger correlation between storage time and cut edge tissue browning attribute that can be observed through the high r^2 value of 0.9227.

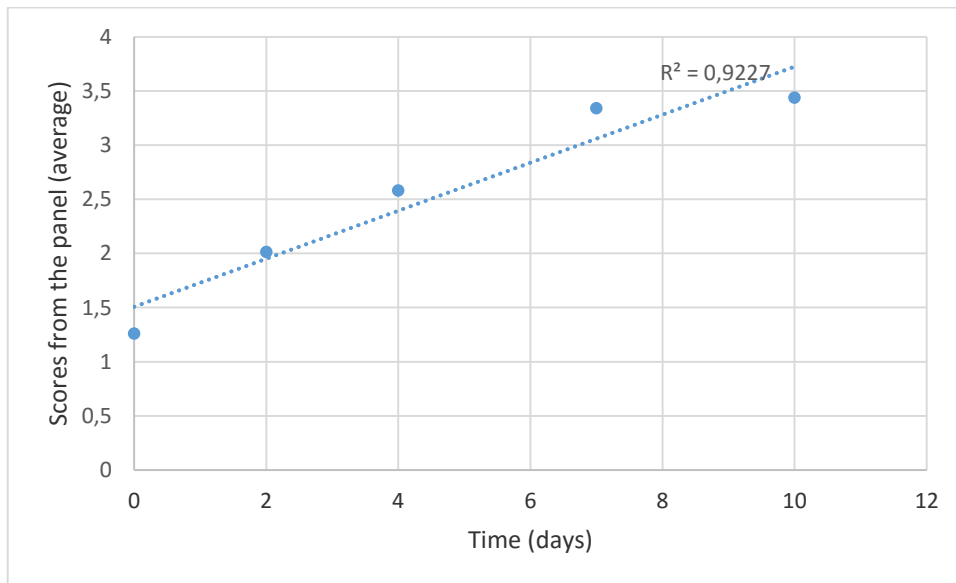


Figure 32: Linear correlation between cut edge tissue browning scores and storage time.

3.2.5 Surface Browning

The evaluation of surface browning (Figure 33) at 5°C showed differences between the samples. The ultrasound treated samples resulted in the highest scores. At 15°C, the treated and the control samples had the highest scores. Surface browning increased after day 2 and the maximum values were reached at the last day of storage.

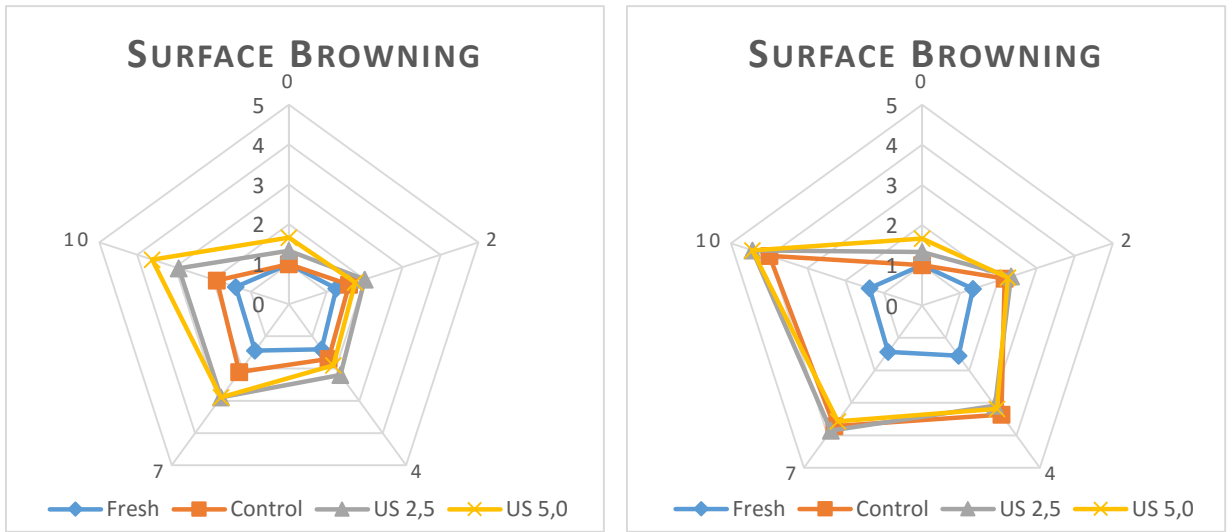


Figure 33: Scores attributed to Surface Browning, after storage for 10 days at 5°C (left) and at 15°C (right).

PLS – Surface Browning

PLS analysis showed that the storage time (0.71) has a slightly higher influence on this attribute than temperature (0.67) and ultrasound treatment (0.19) (figure 34). Both temperature and storage time affected the sensory quality due to the increase of the surface browning.

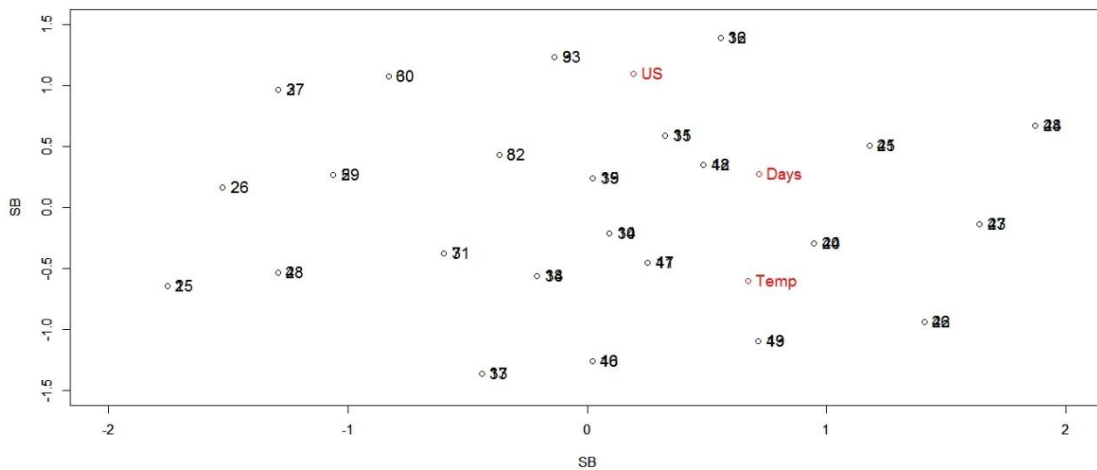


Figure 34: PLS for Surface Browning attribute.

The Figure 35 shows the correlation between surface browning and storage time. According to PLS, there is a stronger correlation between storage time and surface browning attribute that can be observed through the high r^2 value of 0.9693.

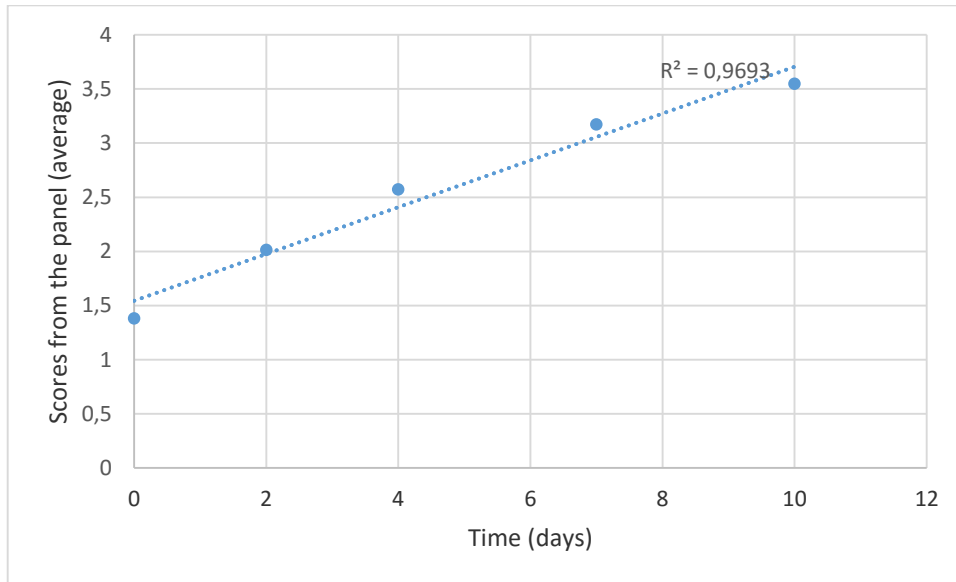


Figure 35: Linear correlation between surface browning scores and storage time.

3.2.6 Sogginess/Watery

At both temperatures the fresh sample stands from the other samples in the inner pentagon of the figure (in blue), having the better quality evaluation (Figure 36). At day 0, the ultrasound treated sample for 5 min has the higher score for this attribute at both temperatures. At 15°C the samples scored higher values faster than at 5°C.

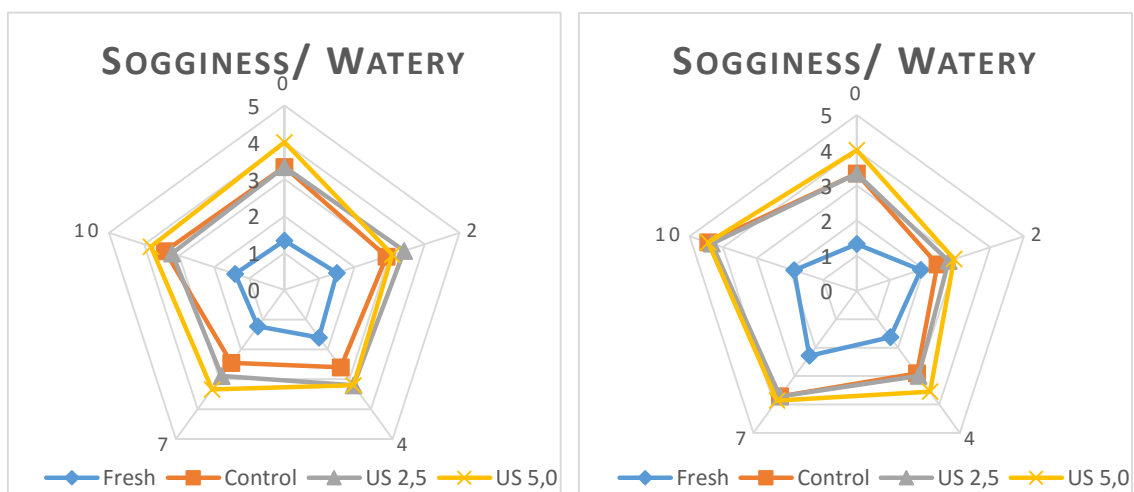


Figure 36: Scores attributed to Sogginess/ Watery, after storage for 10 days at 5°C (left) and at 15°C (right).

3.2.7 Texture

The texture attribute scores (Figure 39) show better results at 5 °C than at 15°C. Contrary of the other attributes the fresh sample did not stand from the other samples. At day 10, the ultrasound treated sample for 2.5 min has the higher score for this attribute at 15 °C. At 5°C and day 10, the control presented the poorest result.

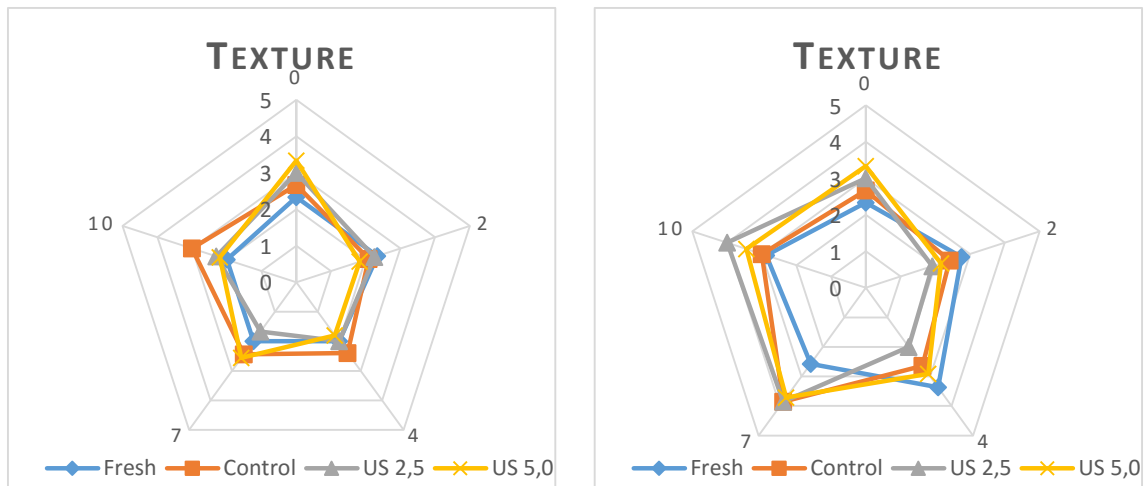


Figure 39: Scores attributed to Texture, after storage for 10 days at 5°C (left) and at 15°C (right).

PLS – Texture

In figure 40, PLS analysis showed that the temperature (0.71) has a slightly major influence in this attribute than storage time (0.68) and ultrasound treatment (-0.11). For this attribute, the ultrasound is represented with a faintly negative value, which means that ultrasound does not seem to affect this particular attribute. Although, a linear correlation should be done with more points, in this case (temperature) we only studied 3 different temperatures being each point related to one temperature

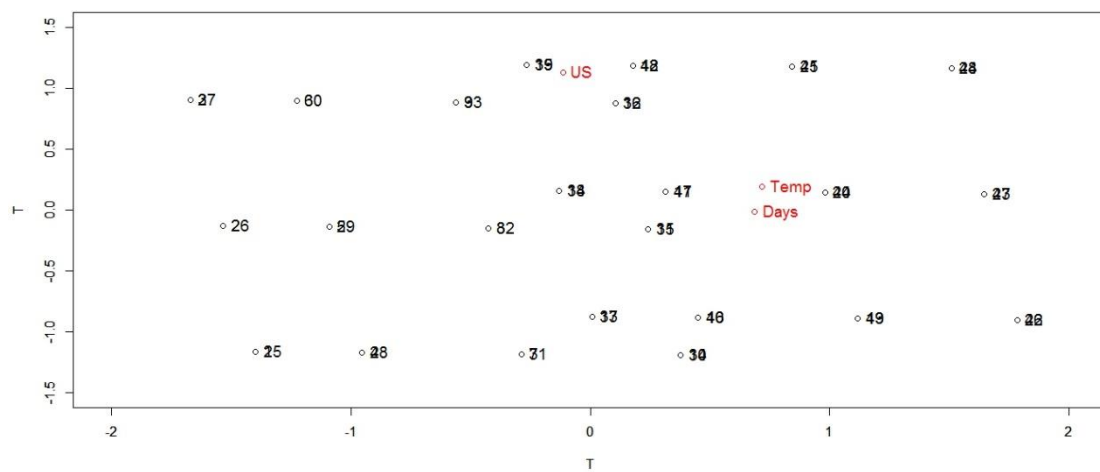


Figure 40: PLS for Texture attribute.

3.2.7 Principal Component Analysis (PCA) in Sensory Evaluation

The PCA analysis shows how the tested variables vary and behave in relation to each other (Figure 41). From the statistics of the PCA is shown that PC1 explains 77.2% of the variance observed while PC2 explains 12.5%. For this reason only PC1 will be considered.

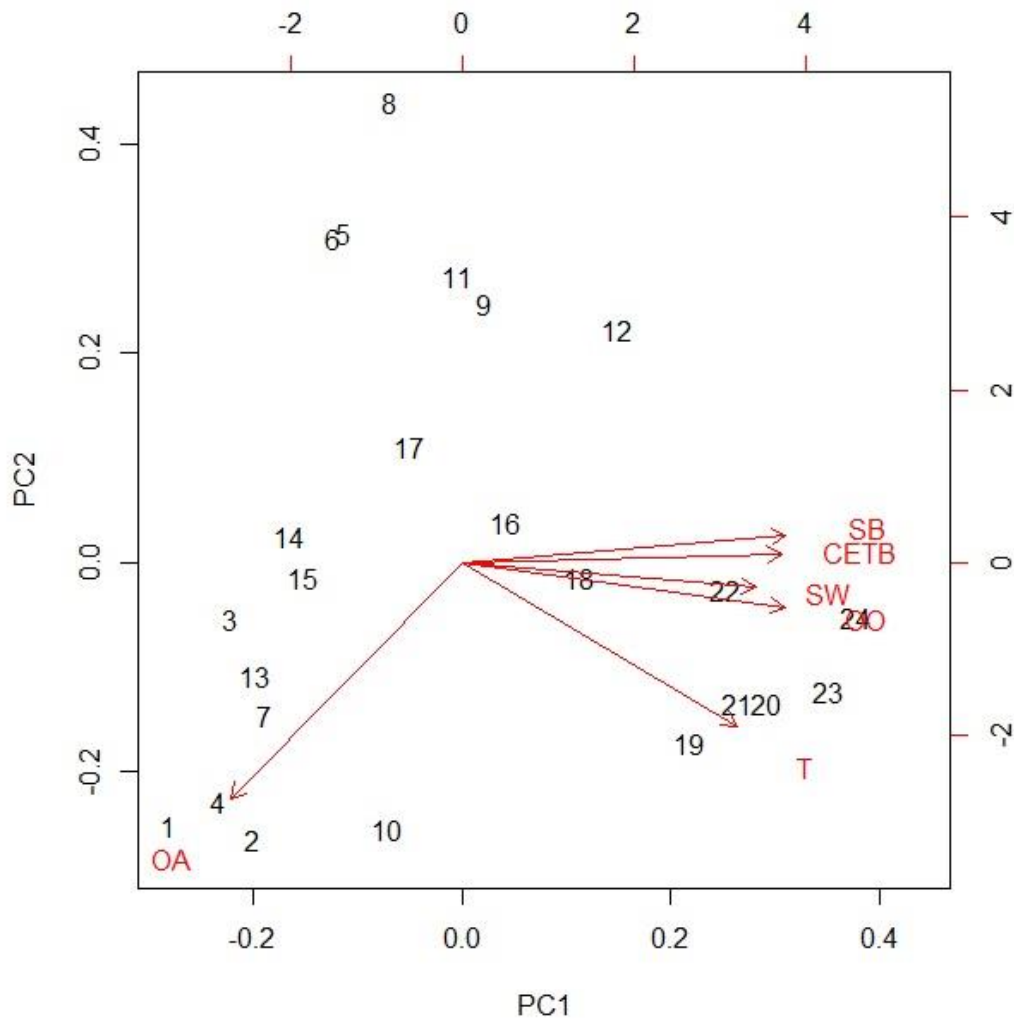


Figure 41: PCA of the Sensory Data In red are represented the variables and the black numbers are the individual lettuce samples. Surface Browning – “SB” -; Cut Edge Tissue Browning – “CETB” -; Sogginess/Watery – “SW” -; Off-Odour – “OO” and Texture – “T”- present the same tendency.

As is showed in figure 41 the attribute Overall Appearance – “OA” – varies in an opposite way when compared with the other variables. This means that when some of the other variables score high values the overall appearance scores lower values (explain by the scale used by the panel).

4. DISCUSSION AND CONCLUSIONS

Studying the effects of decontamination sanitizers through time in fresh produce is crucial in order to suggest strategies for increasing the shelf-life of the products. Vegetables including lettuce are generally colonized by a wide variety of microorganisms, such as bacteria, yeasts and fungi that cause spoilage (Lindow *et al.* 2003). Some microorganisms which contaminate lettuce surfaces are known to increase during storage, even under refrigeration (Li *et al.* 2001). In the current study levels of microorganisms increased as storage time increased, regardless of the applied treatments or temperatures. At day 0, the total microbial viable count of the ultrasound treated samples resulted in lower values than the non-treated samples, at the three different temperatures. After storage for 11 days, the differences between the bacterial levels for each sample decreased. At 5°C, and towards the end of the storage the lower bacterial concentrations were achieved in comparison with the other storage values at that time. There is a major growth increase of total viable count at the three temperatures during all the storage conditions. Palma-Salgado *et al.* 2014 reported a major intensification of the bacterial growth after day 7 of storage, at 4°C in samples treated with chlorine and ultrasound. These observations are also evident in the current study specifically for the ultrasound treated samples at the three temperatures and could be potentially explained by the tissue damage, the presence of moisture or nutrients on the produce which can support microbial growth.

High populations of *Enterobacteria* are present in lettuce as part of natural microflora. In the current study this population was reduced by ultrasound following storage at 5°C, when compared with the non-treated samples till day 9. Related to the population of psychrotrophic bacteria it reaches higher values at 15°C. The treated samples resulted in higher values after day 2 at 15°C, and the same was seen at 5°C but only after day 7. A limit of 8 log₁₀ CFU/g for psychrotrophic was reported by Gomez-López *et al.* 2008 to determine the end of the shelf-life. Total viable count and psychrotrophic bacteria increased from day 0, but remained constant after mid-point of storage. Similar results were reported by Akbas *et al.* 2007 who treated lettuce samples with organic acid and stored them at 4°C for 12 days.

Related to the pathogenic bacteria amounts, the treated samples showed better results than the non-treated. At 5°C the ultrasound treated samples had lower values during the 11 days of storage and at 10°C had very similar or lower values compared to the

non-treated sample at the same temperature. At 15°C, the ultrasound treated samples showed slightly bigger amounts than the untreated. The influence of the temperature is reaffirmed by the PLS studies, where we can see that this variable of temperature has major influence explaining 84% of the results. Ultrasound influences the amount of bacteria in the opposite way confirming the decontamination capacity. The lack of studies relating storage time and pathogenic bacteria limits a fair discussion.

Yeasts and moulds did not seem to have major influence in lettuce contamination. The growth of yeasts and moulds was below $3.5 \log_{10}$ CFU/cm². As seen in other works mould spoilage does not appear to be a major problem in ready-to-eat salads [29]. However, some authors (Tournas 2005 and Tournas *et al.* 2005) referred the possible health problems associated with the presence of moulds in vegetables, as some may produce mycotoxins and others are known to cause allergies when they are able to produce large numbers of conidia. Palma-Salgado *et al.* 2014 reported concentrations below $0.7 \log_{10}$ CFU/g in lettuce treated with chlorine and ultrasound after 14 days of storage. According to Mahmoud *et al.* 2010 the recommended limit for yeasts and moulds in vegetables is a $5 \log_{10}$ CFU/g to guarantee sensory quality.

The PCA for the microbiology data explains 79.6% of the variance observed and shows that the different studied bacteria vary in the same direction (have the same tendency). Analysing the different PLS for each time we can see that storage time is the main prompter of the bacterial growth. When analysing the correlation between the bacterial growth of each type of bacteria and the storage time, the correlation resulted in high r^2 values that varied between 0.91 and 0.97. Only for pathogenic bacteria, temperature has a major influence showing that a sample stored for more days at lower temperatures may present minor bacterial growth than samples stored at high temperatures for less time.

The sensory scores of lettuce decrease in quality with the increasing storage time for the different samples. The same was reported by Kou *et al.* 2014 where sensory scores of the lettuce and kale during storage decreased with increasing storage time for all treatments. Regarding overall appearance, the better scores were obtained for the fresh lettuce followed by the control sample. PLS indicates that although ultrasound has a negative effect on lettuce, storage time contributes for the major appearance decadence. Even though temperature presents a small influence it can be agreed that the samples stored at 5°C for more days kept the sensory scores higher than those stored at 15°C. Related to off-odour scores, both samples scored less till day 4 at 15°C

and day 7 at 5°C. Lopez-Galvez *et al.* 2010 reported that slight off-odours were detected after 10 days of storage but panellists also indicated that samples were acceptable for consumption. In the current study by day 10, both samples stored at temperatures 5°C and 15°C presented off-odours but the ultrasound treated sample for 5 min do not reached the acceptability levels. According to PLS, temperature has a slight major influence comparing with storage time. For lettuce, browning is considered to be the critical factor in perceived loss of quality. Cut edge tissue browning and surface browning had similar behaviour during storage. The ultrasound treated samples for 5 min showed the higher values corresponding to lower sensory quality. Samples stored at 15°C showed browning appearance at a higher rate. The PLS showed that both storage time and temperature influence the decreasing in sensory quality. In works with other decontaminations technologies Lopez-Galvez *et al.* 2010 is reported that after washing with aqueous chlorine dioxide and sodium hypochlorite no significant changes were notice related to browning. Olmez *et al.* 2009 stated that above 2.5 ppm ozone levels, there was a loss in colour, freshness and onset of browning, which in turn resulted in a reduction in the shelf-life of product. Sogginess and watery showed high scores from day 0 for the treated samples and the control sample, at both temperatures. PLS shows that storage time explains 82% of the results related to sogginess and watery of the samples. Palma-Salgado *et al.* 2014 reported no significant differences in sogginess during 14 days of storage for Iceberg or Romaine lettuce, both treated with chlorine and ultrasound. Sogginess and texture represent the crispness that is also an important quality parameter as consumers associate it with the freshness of the product (Kilcast *et al.* 2000). Related to texture, samples stored at 5°C showed better quality results than the ones stored at 15°C. For this parameter, all the samples present very similar scores. The PLS showed that temperature might have slightly major influence than storage time.

The PCA statistics for the sensory data explains 77.2% of the variance observed. The overall appearance attribute varies in an opposite way comparative to the others attributes meaning that when some of the other variables score high values, overall appearance scores lower values. This is also explained by the different scale used for the panel to score the overall appearance attribute comparative to the others. All the others attributes have the same tendency.

When analysing the correlation between the sensory scores for the different sensory parameters and storage time or temperature (depending on the PLS results for that

parameter), this resulted in high r^2 values between 0.81 and 1. In the results obtained from the sensory experiments, we can see that comparing the treatment times (2.5 min vs 5 min) the 5 minutes treatment has a more negative impact on lettuce characteristics than 2.5 minutes treatment. Due to this we can conclude that, on lettuce and under these conditions longer treatments are more aggressive and inflicts more damage on the product.

According to this results, ultrasound can be efficient specifically against pathogenic bacteria. As we can see on figure 19 and 20 ultrasound reduced the bacterial growth, having more efficiency at lower temperatures. Although, the sensory results showed that the ultrasound treatment has a negative effect in lettuce leaves. The samples treated during more time presented lower results related to quality. It would be beneficial to conjugate ultrasound with other technologies to formulate a mid-term between anti-microbial efficacy and sensory damage. Nevertheless, ultrasound parameters can be set to guarantee quality and safety for the consumers.

In a future work we propose to study also the influence of essential oils to improve the quality of lettuce by developing sensorial analysis studies including taste attribute. Combining ultrasound technology with other technologies or compounds might be a good way of improving quality of lettuce and in fresh produce.

Regarding my career and personal development, during these work had the opportunity to apply numerous concepts I had acquired during the curricular year. Moreover, I was able to solidify laboratorial techniques and learn new ones. I was able to plan my own experiments and manage my scheduler. I had the opportunity to train and lead a panel for the sensory tests. More importantly, I had the opportunity to work at an international laboratory of excellence and got to know a new culture and different ways of thinking. It was a very enriching experiment to me.

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6. ANNEXES

Annex 1 – Microbiological Studies Methodology Review

No	Year	Product	Technology	Microorganisms	Microbiological Analysis	Storage days/ temperatures	References
1	2014	Lettuce	Sanitizer, surfactant and ultrasound	Aerobic; yeasts; moulds	Total aerobic plate count: Tryptic Soy Agar (TSA, Difco Lab, Detroit, MI, USA) at 37 °C/48h. Yeasts and moulds: Acidified Potato Dextrose Agar, ph adjusted with tartaric acid (PDA, Difco Lab, Detroit, MI, USA) at 25 °C/5 d.	4 °C/ 14d • Day 0, 7 and 14	(Salgado, Pearlstein et al. 2014)
2	2013	Lettuce	Essential oils; thyme, oregano and rosemary	<i>Listeria innocua</i> *	<i>Listeria</i> : <i>Listeria</i> selective agar (LSA, Oxoid CM 856) containing a modified <i>Listeria</i> selective supplement (Oxoid SR0206) at 35 °C/48h. Total bacterial counts: Tryptone Soya Agar (TSA, Oxoid CM131) at 35 °C /48 h.	8 °C/ 10d and 4 °C/10d • Day 0, 2, 6 and 9	(Scollard, Francis et al. 2013)
		Coleslaw mix		<i>Listeria monocytogenes</i> *			
3	2010	Lettuce	Silver nitrate, chlorine, hydrogen peroxide and electrochemic ally generated Ag and H ₂ O ₂	Enterobacteriaceae; yeast, <i>Pseudomonas</i>	Total viable count: Plate Count Agar (PCA). <i>Pseudomonas</i> : <i>Pseudomonas</i> agar with CFC supplement. Enterobacteriaceae: Violet Red Bile Glucose Agar (VRBGA). Yeasts and moulds: Dichloran Rose-Bengal Chloroamphenicol with Chloroamphenicol supplement (DRBC).	12 °C/ 7d • Day 0, 2, 4 and 7	(Gopal, Coventry et al. 2010)

4	2010	Lettuce	X-ray	Mesophilic; moulds; psychrotrophic; yeast;	Mesophilic: TSA at 37 °C for 48 h. Psychrotrophic: TSA at 5 °C for 10 d. Yeast and moulds: acidified (with tartaric acid) potato dextrose agar (PDA) (Difco- Becton Dickinson) at 25 °C for 5 d.	4 °C/30d • Day 0, 3, 6, 9, 12, 20 and 30	(Mahmoud 2010)
				<i>O157:H7</i> ≠ strains* <div style="display: flex; align-items: center; margin-left: 20px;"> <div style="border-left: 1px dashed black; border-right: 1px dashed black; padding: 0 5px; margin-right: 5px;"><i>E. coli</i></div> <div style="border-left: 1px dashed black; border-right: 1px dashed black; padding: 0 5px; margin-right: 5px;"><i>L. monocyto</i></div> <div style="border-left: 1px dashed black; border-right: 1px dashed black; padding: 0 5px; margin-right: 5px;"><i>S. enterica</i></div> <div style="border-left: 1px dashed black; border-right: 1px dashed black; padding: 0 5px;"><i>S. flexneri</i></div> </div>	Tryptic soy agar for 6 h with selective medium <i>E. coli</i> O157:H7 (CT-SMAC agar); <i>L. monocytogenes</i> , (MOA); <i>S. enterica</i> and <i>S. flexneri</i> (XLD) All incubated for an additional 18h at 37 °C.	Inactivation studies	
5	2009	Lettuce	Ozone, chlorine, organic acids	Aerobic mesophilic; psychrotrophic; Enterobacteriaceae.	Aerobic mesophilic and psychrotrophic: Plate Count Agar (PCA, Oxoid, Basingstoke, UK), incubation at 30 °C/48 h and at 4 °C/10 days, respectively. Enterobacteriaceae: Violet Red Bile Glucose Agar (VRBGA, BioRad, Marnes la Coquette, France) incubated at 37 °C/24 h.	4° C/12d	(Olmez and Akbas 2009)
				<i>L. monocytogenes</i> *	<i>Listeria</i> : Listeria selective agar (PALCAM, Oxoid, Basingstoke, UK) with modified Listeria selective supplement (Oxoid) after incubation at 35 °C/48h.	Inactivation studies	
6	2009	Lettuce	Essential oils: oregano and thyme; chlorine	Lactic acid bacteria, Enterobacteria; <i>Pseudomonas</i>	Total Viable Count (TVC): Tryptic Soy Agar (TSA, Scharlau Chemie, Spain); Lactic Acid Bacteria (LAB): Man, Rogosa and Sharpe Agar (MRSA, Scharlau Chemie);	4 °C/ 7d • Day: 0, 2, 4 and 7	(Gutierrez, Bourke et al. 2009)

					Enterobacteria: Violet Red Bile Dextrose Agar (VRBDA, Scharlau Chemie); Pseudomonas: CN Selective Agar Base (CNA, Scharlau Chemie) For 48h at 30 °C (TSA, MRSA and CNA plates) or 37 °C (VRBDA).		
7	2008	Iceberg lettuce	UV and H ₂ O ₂	<i>Salmonella</i> *;	<i>Salmonella</i> P2: tryptic soy agar (TSA; Oxoid) supplemented with 32 µg ml ⁻¹ kanamycin (Fisher) incubated at 37 °C /24h.	4 °C and 25 °C/ 8d	(Hadjok, Mittal et al. 2008)
		<i>E.coli</i> O157:H7*;		<i>E. coli</i> O157:H7 ph1: tryptic soy agar (TSA; Oxoid) supplemented with 200 µg ml ⁻¹ ampicillin (Fisher) incubated at 37 °C /24h.			
		<i>Pseudomonas fluorescens</i> .			When no colonies were recovered on plates, the samples were enriched at 37 °C for 24 h using TSA containing the appropriate antibiotic. The enriched cultures were streaked onto TSAKM or TSAAMP and incubated at 37 °C for 24h.		
		<i>Salmonella</i> ;		<i>P. fluorescens</i> P30: TSAKM plates incubated at 30 °C/48h.			
		<i>E.coli</i> O15:H7;				<i>Pe. Carotovora</i> : PT (polygalacturonic acid tergitol) medium (Burr and Schroth 1977) incubated at 30 °C/48h.	
		<i>Pseudomonas fluorescens</i> ;					
		<i>Pectobacterium carotovora</i> .					
Baby spinach	<i>Salmonella</i> ;						
<i>E.coli</i> O157:H7.							
8	2008	Lettuce	Neutral electrolysed water (EW)	Mesophilic bacteria	Total Count: Plate count agar (PCA) incubated at 30 °C/72h.	4 °C /7d • Day: 1 and 7	(Rico, Martin-Diana et al. 2008)

9	2008	Lettuce	Gaseous chlorine dioxide	Total plate count; Lactic acid bacteria; psychrotrophicaerobic yeasts	<p>Total aerobic plate count (APC): Plate Count Agar (Oxoid, CM325, Basigstoke, Hampshire, England) and incubated at 30 °C for 3 days.</p> <p>Total aerobic psychrotrophic count: Plate Count Agar (Oxoid, CM325, Basigstoke, Hampshire, England) incubated at 22 °C for 5 days.</p> <p>Lactic acid bacteria (LAB): de Man-Rogosa-Sharpe medium (Oxoid, CM361) with 0.14% sorbic acid (Sigma, S-1626) incubated at 30 °C for 3 days;</p> <p>Yeasts: 15 g agar (Agar N° 1, Oxoid, LP0011), 5 g yeast extract (Oxoid, L21), and 20 g dextrose (Sigma–Aldrich, Steinheim, Germany) per litre with 50 mg/L (Tournas et al., 2001) chlortetracycline (Difco, 233331) incubated at 30 °C for 3 days.</p>	<p>7 °C /7d</p> <ul style="list-style-type: none"> • Day: 0, 3, 4, 5 and 7. 	(Gomez-Lopez, Ragaert et al. 2008)
10	2008	Lettuce	Gaseous chlorine dioxide	Psychrotrophic; yeasts; mesophilic, mould	<p>Mesophilic: TSA at 37 °C for 24 h.</p> <p>Psychrotrophic: TSA at 5 °C for 10 d.</p> <p>Yeast and moulds: potato dextrose agar (PDA) (Difco- Becton Dickinson) at 25 °C for 5 d.</p>	<p>4 °C /7d</p> <ul style="list-style-type: none"> • Day: 0, 1, 2, 3, 5 and 7. 	(Mahmoud and Linton 2008)
				<p>3 different Strains* } <i>E. coli</i>; <i>Salmonella</i></p>	<p>By a membrane transferring method using tryptic soy agar (TSA) (Difco- Becton Dickinson) for 6 h followed by transferring the membrane to:</p> <p><i>E. coli</i>: cefixme-tellurite sorbital MacConkey (CT-SMAC) (Difco- Becton Dickinson) agar;</p> <p><i>Salmonella</i>: xylose lysine desoxycholate (XLD) (Difco-Becton Dickinson).</p>	Inactivation studies	

					Incubated for an additional 18h at 37 °C.		
11	2007	Lettuce	Chlorine, citric acid, lactic acid, ozone	Mesophilic; psychrotrophic; enterobacteriaceae	Mesophilic: plate count agar (PCA, Oxoid, Basingstoke, UK) incubation at 35 °C /48h. Psychrotrophic: PCA (Oxoid) at 4 °C /7 days. The number of Enterobacteriaceae: Violet Red Bile Glucose Agar (VRBGA, BioRad, Marnes la Coquette, France) incubated at 37 °C/18–24 h.	4 °C/ 12d • Day: 0, 3, 6, 8, 10 and 12.	(Akbas and Olmez 2007)
12	2007	Lettuce	Ozone	Aerobic mesophilic; <i>E. coli</i> <i>Salmonella</i>	Aerobic mesophilic: spread plate procedure on DEV Nutrient agar (Merck, Darmstadt, Germany) incubated at 25 °C/72h.	4 °C/6d • Before and after storage	(Hassenberg, Idler et al. 2007)
				<i>Shigella</i> (not determined rare occurrence)	<i>E. coli</i> : two chromogenic growth media - Tryptone Bile X-Glucuronide medium (TBX, Oxoid, Basingstoke, UK) incubated at 30 °C/4h and 44 °C/18h. -Chromocult (Merck) incubated 37 °C/24h. Salmonella spp.: according to DIN EN ISO 6579:2002.		
13	2006	Red oak leaf lettuce	UV-C radiation	Aerobic, lactic acid and enteric bacteria	Aerobic and facultative aerobic bacteria: nutrient agar (Difco Lab, Sparks, Maryland, USA) incubated at 30 °C/24–48h in air and in modified atmospheres (5 kPa O ₂ and 20 kPa CO ₂) respectively. Yeast and fungi: PDA with the addition of chloramphenicol (100 µg ml ⁻¹) (Difco Lab, Sparks, Maryland, USA) incubated at 30°C/48h.	5 °C/10d • Day: 0, 3, 4, 5, 6, 7, 8 and 10.	(Allende, McEvoy et al. 2006)

					<p>Lactic acid bacteria: Lactobacilli MRS broth (Difco Lab, Sparks, Maryland, USA) with addition of Bacto™ agar (10 gL⁻¹) (Difco Lab, Sparks, Maryland, USA), incubated at 30 °C/72h under modified atmosphere (20 kPa CO₂ and 5 kPa O₂).</p> <p>Enteric bacteria: McConkey agar (Difco Lab, Sparks, Maryland, USA) incubated at 37 °C/24h.</p>		
14	2005	Lettuce	Temperature and chlorine	<p>Aerobic; <i>Pseudomonas</i>; <i>enterobacteriaceae</i></p>	<p>Total mesophilic aerobic bacteria: Plate Count Agar (PCA).</p> <p><i>Pseudomonadas</i>: Glutamate Starch Phenol Red Agar (GSP).</p> <p><i>Enterobacteriaceae</i>: Violet Red Bile Dextrose Agar (VRBD).</p> <p>All agars were obtained from VWR. PCA and GSP plates were incubated at 30 °C/24h and VRBD plates at 37 °C/18 h.</p>	<p>4 °C/10d</p> <ul style="list-style-type: none"> Day: 0, 2, 5, 7 and 9) 	(Baur, Klaiber et al. 2005)
15	2005	Lettuce	Intense light pulses; equilibrium modified atmosphere	<p>Aerobic psychrotrophic; yeasts</p>	<p>Aerobic psychrotrophic: Plate count Agar, incubated at 22 °C/5d.</p>	<p>7 °C/5d</p> <ul style="list-style-type: none"> Day: 0, 3 and 5. 	(Gomez-Lopez, Devlieghere et al. 2005)
				<p><i>Listeria monocytogenes</i></p>	<p><i>L. monocytogenes</i> and <i>P. phosphoreum</i>: in Brain Heart Infusion</p>	<p>Inactivation studies</p>	

			packaging	<i>Photobacterium phosphoreum</i> ;	<i>broth (CM 225, Oxoid) at 30 °C.</i>		
				<i>Candida lambica</i>	<i>C. lambica</i> : Sabouraud liquid medium (CM 147, Oxoid) at 30 °C.		
16	2002	Mixed lettuce	Temperature	<i>L. monocytogenes</i> st ;	<i>L. monocytogenes</i> : Trypton Soya Agar (TSA, Oxoid CM131), subcultured twice after 24h in Brain Heart Infusion (BHI, Oxoid CM225) at 37 °C. <i>Aer. Caviae</i> : Nutrient Agar (NA, Nutrient broth (Oxoid CM1) with 10 g/l agar (Oxoid L11)) subcultured twice after 24 h in Brain Heart Infusion (BHI, Oxoid CM225) at 30 °C. To enumerate <i>L. monocytogenes</i> , <i>Listeria</i> -selective agar base (Oxford formulation, Oxoid CM856+ <i>Listeria</i> selective supplement—Oxford formulation, Oxoid SR140E) incubated at 37 °C for 48 h. <i>Aer. caviae</i> was spread-plated on modified bile salts irgasan brilliant green agar (mBIBG) (pH 8.7) and incubated at 30 °C for 24 h. Psychrotrophic: pour-plated on Plate Count Agar (PCA, Oxoid CM325) incubated at 22 °C for 5 days.	2 °C, 4 °C, 7 °C and 10 °C/ 9d	(Jacxsens, Devlieghere et al. 2002)
				<i>Aer. Caviae</i> st ;			
				Psychrotrophic bacteria;			
17	2011	Lettuce	Ultrasound and organic acids	<i>E. coli O157:H7</i> st ; <i>S. Typhimurium</i> st ; <i>L. monocytogenes</i> st .	<i>E. coli O157:H7</i> : Sorbitol MacConkey Agar (SMAC), Difco; <i>S. Typhimurium</i> : Xylose Lysine Desoxycholate Agar (XLD), Difco; <i>L. monocytogenes</i> : Oxford Agar Base with antimicrobial Supplement Bacto™ (MOX), Difco. All incubated 37°C 24/48h.	Inactivation Studies	(Sagong, Lee et al. 2011)

Annex 2 – Sensory Studies Methodology Review

No	Year	Product	Treatment	Attributes	No of people in panel	Storage	Assessment	References
1	2014	Spinach	Temperature	<ul style="list-style-type: none"> - Visual appeal; - Purchase intent; - Off odor; - Decay extent; - Texture; - Overall quality. 	5 Trained panel	Temp: 1, 4, 8, 12, 16, 20 °C Days: 16	2 in 2 days For panel training were used photographs from preliminary studies then grouped by the panel.	(Kou, Luo et al. 2014)
2	2014	Lettuce	Sanitizer, surfactant and ultrasound	<ul style="list-style-type: none"> - Overall visual quality; - Cut edge tissue browning; - Surface browning; - Sogginess/watery. 	5 Trained panel	Temp: 4 °C Days: 14	In days: 0, 7 and 14	(Salgado, Pearlstein et al. 2014)
3	2013	Lettuce	Essential Oils of tea tree and clove	<ul style="list-style-type: none"> - Overall visual quality; - Leaf color; - Leaf texture; - Odor; - Brightness. 	5 Trained panel	-----	Immediately after harvest (the EOs were applied in preharvest stages)	(Goñi, Tomadoni et al. 2013)

4	2013	Lettuce, cabbage and carrots	Essential oils: thyme, oregano and rosemary	-Appearance of the samples	5 People with experience	Temp: 8 °C Days: 10	In days: 2, 6, 9.	(Scollard, Francis et al. 2013)
5	2011	Lamb's lettuce	Hydroponic and soil cultivation	- Overall acceptability; - Consumer acceptability.	200 Consumers (faculty staff and students)	Temp: 4 °C Days: 20	Images of very spoiled and fresh lettuce were used.	(Manzocco, Foschia et al. 2011)
6	2011	Leafy vegetables	Hydroponic and soil cultivation	- Appearance; - Flavour; - Texture; - Overall acceptability.	96 (Faculty staff and students)		Harvesting day	(Fouladkhan, Bunning et al. 2011)
7	2010	Lettuce	Sodium Acid Sulphate (SAS), Levulinic Acid (LA), Sodium dodecylsulfate (SDS)	- Overall visual quality; - Cut edge tissue browning; - Surface browning; - Sogginess/watery.	3 Trained panel	Temp: 4 °C Days: 14	In days: 1, 7 and 14	(Guan, Huang et al. 2010)
8	2010	Lettuce	Chlorine dioxide, sodium hypochlorite	- Overall quality; - Flavour; - Firmness; - Off-odors; - Browning;	4 Expert panel	3 days at 4 °C followed by 7 days at 7 °C	In days: 0, 7 and 10	(Lopez-Galvez, Allende et al. 2010)

				- Dehydration.				
9	2009	Lettuce and carrots	Essential oils of oregano and thyme	<ul style="list-style-type: none"> -Vegetable aroma; - Off-odor; - Color; - Browning; - Texture; - Vegetable taste; - Off-after taste; - Overall acceptability; - Overall appreciation. 	10 Trained panel	Temp: 4 °C Days: 7	In days: 1, 4 and 7 No tasting in day 7.	(Gutierrez, Bourke et al. 2009)
10	2009	Lettuce	Ozone, chlorine, organic acids	<ul style="list-style-type: none"> - Overall visual quality; - Cut edge tissue browning; - Firmness; - Aroma. 	8 Trained panel	Temp: 4 °C Days: 12	In days: 0, 5, 7, 9 and 12	(Olmez and Akbas 2009)
11	2009	Lettuce	Calcium- bio fortified	<ul style="list-style-type: none"> - Initial crispness; - Green overall; - Green peapod; - Green, grassy/leafy; - Green winey; - Celery; 	5 Highly trained descriptive panellists			(Park, Elless et al. 2009)

				<ul style="list-style-type: none"> - Lettuce; - Spinach; - Woody; - Water-like; - Musty/earthy - Tooth-etch - Sweet overall; - Sour. 		-----	60 days after germination (studies with trangenic lettuce)	
12	2009	Lettuce	Acidulants	- Overall appearance.	6 Trained panel	Temp: 5 °C and 20 °C Days: 21		(Samara and Koutsoumanis 2009)
13	2008	Lettuce	Natural electrolysed water (EW)	<ul style="list-style-type: none"> - Fresh appearance; - Vascular browning; - Photosynthetic browning; - General acceptability. 	12 Trained panel	Temp: 4 °C Days: 7 days	In days: 1 and 7	(Rico, Martin-Diana et al. 2008)

Annex 3 - Sensory Analysis Questionnaire

Sensory Evaluation of Lettuce Leaves Under Different Processing Conditions

A307 Date: _____ Sample Code: _____

1] Before opening the bag, evaluate the **Overall Appearance** according to the scale:

- (9) Highly acceptable
- (8) Acceptable
- (7) Moderately acceptable
- (6) Slightly acceptable
- (5) Neither acceptable or unacceptable
- (4) Slightly unacceptable
- (3) Moderately unacceptable
- (2) Unacceptable
- (1) Highly unacceptable

2] Open the top of the bag lengthwise with the help of a scissors and sniff inside.
Evaluate the **Off-odour** according to the scale:

- None Slight Moderate Moderately severe

3] Turn the bag and drop the leaves into a plate. Then evaluate the following attributes:

Cut edge tissue browning:

- None Slight Moderate Moderately severe

Surface Browning:

- None Slight Moderate Moderately severe

Sogginess/Watery:

- None Slight Moderate Moderately severe

4] Take a couple of leaves and fold them between your thumb and index finger.

Evaluate perceived **Texture** with the following scale:

- Very crisp Slightly crisp Moderate Moderately flaccid Very