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Faculty of Geotechnical Sciences and Environmental Management

Doctoral Dissertation

Alternative ways of managing fresh produce and effects on safety and quality

Panayiota Xylia

Limassol, December 2020

CYPRUS UNIVERSITY OF TECHNOLOGY

FACULTY OF GEOTECHNICAL SCIENCES AND ENVIRONMENTAL MANAGEMENT

DEPARTMENT OF AGRICULTURAL SCIENCES, BIOTECHNOLOGY AND FOOD SCIENCE

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Approval Form

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The approval of the dissertation by the Department of Agricultural Sciences, Biotechnology and Food Science does not imply necessarily the approval by the Department of the views of the writer.

Dedicated to,

my family

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'It adds to the joy of discovery to know that your work may make a difference in people's lives.'

Flossie Wong-Staal

Abstract

Vegetables are an important component of a balanced diet and dietary guidelines suggest the increase consumption of vegetables as an important source of phytonutrients. However food poisoning outbreaks have been linked with the consumption of these commodities, since the risk of contamination of fresh produce with foodborne pathogens throughout the food chain is high if sanitary and precautionary measures are not taken. Moreover, there is a current trend towards the use of natural, eco-friendly products for the preservation of fresh produce quality and safety, as alternatives to synthetic compounds (i.e. chlorine) commonly used in the food industry. The purpose of this thesis was to investigate i) the microbiological and physicochemical attributes of ready-to-eat salads as affected by season, producer, type of salad and expiring date, ii) the effects of plant age, inoculum level and nutrient solution pH of hydroponically grown lettuce inoculated with *Salmonella* Enteritidis and iii) the effects of natural products on the quality and safety of ready-to-eat vegetables (minimally processed lettuce and shredded carrot). Results indicate that season, type of vegetable and expiring date greatly affected the microbial load and plant-tissue related parameters of ready-to-eat salads. More specific, higher microbial load of samples was observed during spring. Interestingly *Salmonella enterica* was not found in any of the tested samples, whilst 3.70% of samples were found to harbor *Listeria monocytogenes*. A correlation of phenolics and antioxidants with the presence of *Escherichia coli*, *Staphylococcus* spp., *Pseudomonas* spp. and *Bacillus cereus* was reported. Furthermore, spoilage microorganisms (i.e. *Pseudomonas* spp., yeasts and molds), CO_2 production and damage index were found in increased levels at the end of products shelf-life (expiring date). Furthermore, during hydroponic cultivation of lettuce (one of the main vegetables consumed and used for ready-to-eat salad preparations), plant age, greatly affected the internalization of *S.* Enteritidis on plant tissues, whilst the presence of the bacterium initiated plant defense mechanisms and damage index markers. Interestingly, the colonization and internalization of *S.* Enteritidis in root was more frequent in younger plants compared to older plants at higher pH values. It was evident that the presence of *S.* Enteritidis in nutrient solution, root rinse and internally of roots increased plant defenses and damage index. Examining different natural products (i.e. marjoram essential oil-EO and hydrosol, ascorbic acid-AA, and chitosan) but also their combination in postharvest management, seem to be remarkable alternatives for the preservation of minimally processed vegetables safety and quality. It is noteworthy, that the combination of marjoram EO+AA application enhanced nutritional attributes (phenols, carotenoids) on both commodities examined, while chitosan, EO, chitosan+EO and chitosan+AA resulted to decreased Total viable count (TVC) and yeast and molds counts on minimally processed lettuce. Further exploitation of different products and optimized methods of application (i.e. EOs encapsulation) are important to be considered for safer and more nutritive fresh products that will meet consumer's demands and acceptability.

Keywords: Food safety, foodborne pathogens, food quality, hydroponics, natural products, vegetables, processed products

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LIST OF ABBREVIATIONS

ABTS:	2,2'-Azino bis-3-ethylbenzothiazoline-6-sulfonic acid
a_w:	Water activity
CAT:	Catalase
CDC:	Centers for Disease Control and Prevention
cm:	Centimeter
cfu/g:	Colony forming units per gram
cfu:	Colony forming units
Chl:	Chlorophyll
DCPIP:	2,6-Dichlorophenolindophenol
DNA:	Deoxyribonucleic acid
dpi:	Days post inoculation
DPPH:	2,2-diphenyl-1-picrylhydrazyl
EC:	Electrical conductivity
EFSA:	European Food Safety Authority
EO:	Essential oil
ESBL:	extended spectrum β-lactamase
et *al*:	and others
etc:	et cetera (the rest and so on)
FAO:	Food and Agriculture Organization
FRAP:	Ferric reducing antioxidant power
Fw:	Fresh weight
g:	gravitational acceleration
g:	gram
GA:	Gallic acid
GAE:	Gallic acid equivalents
GRAS:	Generally Recognized As Safe
h:	hour
HPA:	Health Protection Agency
i.e.:	id est (that is)
ISO:	International Organization for Standardization
log:	Logarithm with base 10
MAP:	Modified atmosphere packaging
MDA:	Malondialdeyde
mg/g:	milligram per gram
mg:	milligram
min:	minute
mL:	milliliter
mM:	milli-molarity
mS/cm:	mili-Siemens per centimeter
NCTC:	National Collection of Type Cultures
nm:	nanometer

nmol/g:	nanomol per gram
NS:	Nutrient solution
^{o}C:	degrees Celsius
PCR:	Polymerase chain reaction
PE:	Polyethylene
PET:	Polyethylene terephthalate
pH:	Power of Hydrogen
POD:	Peroxidase
ppm:	parts per million
RH:	Relative humidity
SOD:	Superoxidase
TA:	Total acidity
TBARS:	Thiobarbituric Acid Reactive Substances
TSS:	Total soluble solids
TVC:	Total viable counts
USDA:	United States Department of Agriculture
UV:	Ultra Violet
v/v:	volume/volume
WHO:	World Health Organisation
η^2:	Eta squared
µg/mL:	micrograms per millilitre
µL:	microliter
µmol/g:	micromole per gram

Chapter 1. Literature review

1.1. Food safety and quality nowadays, challenges and needs for further research

The importance of a balanced diet for the promotion of human health has led to the establishment of dietary guidelines (i.e. food wheel, MyPyramid, MyPlate) which aimed to present healthy eating habits with increased serving sizes of fruits and vegetables (USDA, 2011; Cömert et al., 2020). National organizations encourage people to increase their fruits and vegetables intake (EFSA, 2010; USDA, 2015). However, increased consumption of fresh produce has been linked with the increase of food poisoning outbreaks (Balali et al., 2020; Carstens et al., 2019; Henriquez et al., 2020). Fruits and vegetables have been implicated in various outbreaks regarding the consumption of contaminated fresh produce especially leafy vegetables i.e. lettuce, spinach, cabbage and parsley (EFSA, 2013; Alegbeleye et al., 2018; Iwu and Okoh, 2019; Xylia et al., 2019a).

According to recent recommendations, the consumption of 400 g (or five portions) of fruits and vegetables per day (minimum) is an essential component of a healthy diet (FAO, 2015; FAO/WHO, 2019). The results from the European Health Interview Survey (EHIS) (issued by Eurostat) reveal that one in seven persons (≥ 15 years old) in the EU consumes daily at least five portions of fruits and vegetables (Eurostat, 2016). In this essence, vegetables are consumed in various ways i.e. fresh, cooked, minimally processed, frozen, canned and dried (Wells and Buzby, 2008; Lisiewska et al., 2009; Blistein et al., 2012; Miller and Knudson, 2014). Indeed, a large part of vegetables are consumed raw or slightly processed, and this increases the possibility of human contamination with spoilage and foodborne pathogen microorganisms that will eventually result to food poisoning diseases (Balali et al., 2020; Faour-Klingbeil et al., 2016a). The consumption of raw vegetables preserves the nutritional value of these products and at the same time it lowers the losses of nutrients such as vitamins that might degrade during processing (Balali et al., 2020; Carstens et al., 2019). From these findings, quality issues arise regarding safety, nutritional value and shelf-life of fresh produce and minimally-processed vegetables. It is essential for the food industry to ensure safe, highly nutritious products eliminating the risk of food poisoning outbreaks.

1.2. Vegetables – leafy vegetables and health benefits

Vegetables as well as fruits are products with high nutritional value, as they are a rich source of phytochemicals, micronutrients, minerals, vitamins (C, K, B complex), dietary fibers, antioxidants and phenolic compounds (Al-Kharousi et al., 2016; Balali et al., 2020; Cömert et al., 2020). Many studies have indicated the protective action of diets rich in antioxidants against reactive oxygen species (oxidative stress) and its correlation with prevention of chronic diseases i.e. cancer, cardiovascular diseases, diabetes, obesity, inflammation (Trichopoulou et al., 2003; Pereira et al., 2004; Alissa and Ferns, 2017; Balali et al., 2020; Cömert et al., 2020). Further studies have demonstrated that vitamins

(vitamin C and B complex) as well as minerals (K, Ca, Mg and Zn) abundantly found in vegetables play an important role in mental and emotional health (Huskisson et al., 2007; Kaplan et al., 2007; Boehm et al., 2018; Brookie et al., 2018). The consumption of food rich in dietary fibers (i.e. vegetables) seems to reduce the risk of cardiovascular disease, obesity and type II diabetes (FAO, 2015).

1.3. Microorganisms and outbreaks linked with fresh produce and ready-to-eat vegetable salads

The main microflora of fruits and vegetables consists of spoilage bacteria, yeasts and molds accompanied by human pathogenic bacteria due to possible contamination thought production (from cultivation to consumption (Al-Kharousi et al., 2016). The main foodborne pathogens associated with fresh produce include *Escherichia coli*, *Salmonella* spp., *Listeria monocytogenes*, *Bacillus cereus*, *Campylobacter* spp., *Yersinia enterocolitica*, *Staphylococcus aureus* and *Clostridium botulinum* (FAO/WHO, 2008; Al-Kharousi et al., 2016; Carstens et al., 2019; Dankwa et al., 2020). An infection with these pathogens could result to mild clinical symptoms such as fever, headache, diarrhea, vomiting, abdominal pain and muscle cramps. However, more complex diseases/syndromes might arise including haemorrhagic colitis, haemolytic uremic syndrome, dysentery, septicaemia, meningitis and even miscarriage (Yeni et al., 2016; Iwu and Okoh, 2019).

Non-typhoidal *Salmonella* and shiga-toxigenic *E. coli* were implicated in recent gastroenteritis outbreaks regarding the consumption of vegetables, sprouts, fruits and nuts (Brandl et al., 2013; Hernández-Reyes and Schikora, 2013; Cox et al., 2018; CDC, 2020; Henriquez et al., 2020). This evidence show that vegetables can act as vehicles of foodborne pathogens increasing the likelihood for human health risks. In 2008 a multistate outbreak regarding the consumption of fresh produce contaminated with *S.* Saintpaul was reported which lead to 1500 cases and 2 deaths (Barton Behravesh et al., 2011). A listeriosis outbreak in the USA was reported during 2016 and has been linked with packaged salads harboring *L. monocytogenes* (CDC, 2016). Two more multistate outbreaks were announced during 2018 and 2020 at the USA concerning the consumption of *E. coli* O157:H7 contaminated romaine lettuce (FDA, 2018; 2020). Among food poisonings, *Salmonella* outbreaks might be less severe than others, however numbers they seem to be increasing (Henriquez et al., 2020). During the last years increased prevalence of *S. enterica* on fresh produce has been observed and associated with food poisoning outbreaks (Carstens et al., 2019; Gu et al., 2019). It has been mentioned that incidences of *S. enterica* infection due to consumption of contaminated raw, fresh produce are the second most common after norovirus (Bennett et al., 2018; Jacob and Melotto, 2020).

Previous studies have shown that foodborne pathogens differ in susceptibility against antimicrobial agents currently used in the food industry (i.e. chlorine) or novel natural substances (i.e. essential oils and their constitutes) (Parish et al., 2003; Niemira, 2007; Corcoran et al., 2014; Xylia et al., 2017). The prevalence of human pathogens on fresh produce shows that they can survive on vegetables and the

processing environment. According to these findings Gram positive bacteria (i.e. *L. monocytogenes*, *B. cereus*, *S. aureus*) were found to be more susceptible to antimicrobial agents applied during postharvest processing compared to Gram negative bacteria (i.e. *E. coli*, *Salmonella* spp.). This may be attributed to the structural differences on their cell wall, with Gram negative bacteria possessing an external capsule that can prevent the penetration of the cell wall and membrane. Among foodborne pathogens associated with fresh produce *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* spp. have been linked with the majority of food poisoning outbreaks during the last years (Cox et al., 2018; CDC, 2020).

Escherichia coli

E. coli is a Gram-negative, non-spore forming, rod shaped, facultative anaerobic, motile (some cells) bacterium, member of the *Enterobaceriaceae* family, with optimal growth at 37 ^{o}C. Based on their virulence factors and the diseases they cause pathogenic *E. coli* can be classified in six main groups: enterohemorrhagic *E. coli* (EHEC) – which can cause hemorrhagic colitis and hemolytic-uremic syndrome (HUS). Additional terms for these groups are Vero toxin-producing *E. coli* (VTEC) and Shiga toxin-producing *E. coli* (STEC). Other groups of pathogenic *E. coli* include enterotoxogenic *E. coli* (ETEC), which causes traveler's diarrhea, enteropathogenic *E. coli* (EPEC) which leads to childhood diarrhea, enteroinvasive *E. coli* (EIEC) that can cause Shigella-like dysentery, *enteroadherent* E. coli (EAEC), which has been associated with childhood diarrhea and enteroaggregative *E. coli* (EAggEC) which can cause diarrhea (Meng and Schroeder, 2007).

The enterotoxinogenic *E. coli* strains have the ability to secrete both heat sensitive and resistant toxins in the small intestine. Heat resistant toxin cannot be inactivated by boiling at 100°C for one hour. Infection with enterotoxinogenic *E. coli* (including *E. coli* O157:H7) can cause watery diarrhea accompanied with abdominal pain, nausea and vomiting. Bloody diarrhea might develop 24 – 48 h after the watery diarrhea's appearance and can stop within 5 to 7 days after. The infectious dose of this pathogen is very low as it can be transmitted from human to human. Less than 1000 cells (maybe 10 cells) are able to cause disease (Meng and Schroeder, 2007).

Listeria monocytogenes

L. monocytogenes is one of the six species in the genus of *Listeria*, along with *L. seeligeri*, *L. ivanovii*, *L. innocua*, *L. welshimeri* and *L. grayi*. It is a psychrotrophic, Gram-positive, non-spore forming, aerobic or facultative anaerobic bacillus. This bacterium is able to grow and survive at environmental conditions with 5-10% CO_2, temperatures ranging from 0 up to 45 ^{o}C and pH value up to 4.4 (Allerberger, 2007).

Infection with *L. monocytogenes* can cause listeriosis, a rare, sporadic, yet severe disease with quite high mortality rate (20-30%). This disease mainly occurs among the pregnant, the elderly and the immunocompromised. The main symptoms of listeriosis include meningitis (headache, drowsiness and coma), perinatal infection, encephalitis, psychosis, infectious mononucleosis, septicemia and low-grade

infection like flu (not severe) except in pregnant women where it can cause miscarriage. The infectious dose is high up to 5 to 9 log cfu/mL of food and symptoms will occur 18 to 27 h after ingestion. It is noteworthy that healthy individuals when infected might not show any symptoms but can eliminate cells through their feces. Preventive vaccine does not exist (Allerberger, 2007).

Salmonella spp.

Salmonella is a Gram negative, rod-shaped, non-spore forming, facultative anaerobic, motile (most strains) bacterium, member of the *Enterobaceriaceae* family, that can grow at temperatures ranging from 8 up to 45 ^{o}C (optimum growth at 37 ^{o}C), pH values between 4.0 to 9.5 (optimum 6.5-7.5) and water activity (a_w) as low as 0.94 (optimum 0.995) (Cox and Pavic, 2014; Chlebicz and Śliżewska, 2018; Carstens et al., 2019). The genus of *Salmonella* spp. is divided in two species: i) *S. enterica* and ii) *S. bongori*. Moreover, *S. enterica* consists of six biochemically defined subspecies: *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV), and *S. enterica* subsp. *indica* (VI). Each species and subspecies is further divided into serogroups and each serogroup is determined by a particular O antigen (somatic antigen for serological purposes) (Wang and Hammack, 2014). It is worth mentioning that most of the *Salmonella* incidences of human salmonellosis reported have been linked to *S. enterica* subsp. *enterica* which includes more than 2600 serovars (i.e. *S.* Enteritidis, *S.* Typhimurium, *S.* Montevideo) (Ricke and Cast, 2014).

Clinical symptoms of salmonellosis include gastroenteritis, abdominal pain, fever, nausea and fever. Salmonellosis is considered a self-limiting disease (usually) which can last from 2 to 7 days, with 12-72 h incubation period (Cox and Pavic, 2014). It is proposed that the infectious dose of *Salmonella* ranges between 6 and 8 log cfu, however according to epidemiological data even a few cells are sufficient to colonize the gastrointestinal track and cause food poisoning (Cox and Pavic, 2014).

1.4. Sources of contamination of fresh produce

Intensive cultivation of vegetables over the years for higher fresh produce yields has led to the appearance of increased food poisoning outbreaks lined with fresh produce consumption (Alegbeleye et al., 2018). The probability of fresh produce contamination with foodborne pathogens is present along the food chain (from farm to consumer) and pre-harvest hazards play an important role in the prevalence of foodborne pathogens on fresh produce. Numerous routes have been previously reported including water (of many sources), use of manure (poorly treated or even raw), insects, livestock and/or wild animals (Mei Soon et al., 2012; Uyttendaele et al., 2015; Alegbeleye et al., 2018). Concerns and challenges regarding food safety arise once pathogens are established in the environment.

Irrigation water has been identified as one of the main routes of fresh produce contamination (Uyttendaele et al., 2015; Faour-Klingbeil et al., 2016b). Water is mainly being used for irrigation and preparation of nutrient solution (for hydroponic cultivation). The frequency of human pathogens presence and contamination of fresh produce via water depends on the irrigation regime (time and application method), irrigation water source, type of crop and land use practices, pathogen strain and population, (Olaimat and Holley, 2012; Uyttendaele et al., 2015; Alegbeleye et al., 2018; Dankwa e al., 2020). Previous studies have classified irrigation waters sources (from low to high contamination risk) as follows: potable/rainwater, deep groundwater, shallow groundwater, wells, surface and raw/inadequately treated wastewater (Pachepsky et al., 2011; Uyttendaele et al., 2015; Alegbeleye et al., 2018). It is noteworthy that the reuse of untreated or partially untreated wastewater poses a risk of health due to the potential presence of microbiological (i.e. foodborne pathogens) and chemical hazards (pesticides, pharmaceutical compounds) (Marcussen et al., 2007; Christou et al., 2014; Adegoke et al., 2017a,b; 2018, Dankwa e al., 2020).

The ability of human pathogens to colonize leafy vegetables has been linked to their ability to survive outside animal hosts (Martínez-Vaz et al, 2014). Many studies have examined the survival and proliferation of foodborne pathogens in soil and soil amended with contaminated compost. The use of non-properly or un-amended manure as a fertilizer on has been proven to serve as a vehicle and increase the possibility of microbial contamination of fresh produce (Martínez-Vaz et al, 2014). It has been shown that *S.* Thompson, *S.* Typhimurium and *E. coli* O157:H7 can survive in soil amended with contaminated compost for more than 200 days (Islam et al., 2004; García et al., 2010; Brandl et al., 2013). It is worth mentioning that the proliferation of human pathogens in compost and mixture of manure and compost, is also affected by soil moisture, topography and proximity to water sources (Martínez-Vaz et al, 2014). Moreover livestock, wild animals as well as insects (especially houseflies) can be vectors of human pathogens and mediate to the transmission of these microorganisms in leafy and other vegetables increasing the risk of food poisonings (Martínez-Vaz et al, 2014).

Postharvest practices also provide sources of human pathogens that can possibly contaminate fresh produce, increasing the likelihood of food poisoning risks. During postharvest management, fresh produce such as leafy vegetables undergo processes i.e. washing, shredding, chopping, slicing, peeling, which aim to reduce the microbial load of minimally processed vegetables and prepare them as ready-to-eat food (Gorny et al., 2006). However, along with mishandling and injured (surface damage), they can serve as sources of fresh produce contamination with foodborne pathogens lurking in the processing environment (Martínez-Vaz et al, 2014).

1.5. Internalization of foodborne pathogens in leafy vegetables

Internalization of foodborne pathogens in plants is influenced by the ability of the pathogen's to attach its self on a plant surface (i.e. rhizosphere, phyllosphere), survive, grow and proliferate on fresh produce (Alegbeleye et al., 2018). It has been previously mentioned that the internalization of human pathogens on plants occurs with the aid of stomata, lenticels, bruises and cracks of plant surfaces (including broken trichomes) (Alegbeleye et al., 2018).

It is thought that internalization is a pathogen-plant interaction and its occurrence depends on the pathogen (Alegbeleye et al., 2018). Moreover, a number of factors affecting foodborne pathogens internalization and proliferation in leafy vegetables has been reported that include among others, the route of contamination, plant type, age and morphology, type of cultivation system (open field, greenhouse, soil, hydroponics), strain and serovar of the pathogen (Hirneisen et al., 2012; Brandl et al., 2013; Lim et al., 2014; Alegbeleye et al., 2018). The internalization of human pathogen in plants could be a passive or an active process (Sant'Ana et al., 2014). As it has been reported, passive internalization takes places mainly through the roots and seeds, however the risk of contamination is considerably low compared to leaf contamination (Alegbeleye et al., 2018). This might be attributed to the fact that pathogens can be found in lower numbers in plant areas that are less prone to contamination, whereas human pathogens are more able to colonize more susceptible/contaminated areas and transmit to edible plant parts (Solomon et al., 2002; Cooley et al., 2007; Alegbeleye et al., 2018). Numerous studies have investigated the parameters that affect the internalization of human pathogens in leafy vegetables grown in soil (**Table 1.1**) and soilless culture-hydroponic system (**Table 1.2**).

Immersion of cilantro and lettuce leafy parts in solution containing *S.* Thompson, *P. agglomerans*, *P. chlororaphis*, *E. coli* O157:H7 (at a level of 5 log cfu/g) resulted to bacterial internalization mainly in the vein leaf area (Brandl and Mandrell, 2002; Brandl and Amundson, 2008). In another study, Alam et al. (2014) reported internalization of *E. coli* O157:H7 in spinach and rocket leaves when sprayed with inoculation (5-7 log cfu/g) in plants grown in soil. When lettuce plants were inoculated via irrigation with water contaminated with *E.* coli (7 log cfu/g) internalization of the bacterium was reported in the leafy parts (Guprta et al., 2016). Interestingly, no internalization was observed when lettuce plants were inoculated with a strain mixture of *E.* coli O157:H7 (4 and 6 log cfu/g) via root (soil inoculation) and exposed at heat and drought stress (Zhang et al., 2009a). Moreover, Klerks et al. (2007a) supported that different *Salmonella* serovars (*S.* Dublin, *S.* Typhimurium, *S.* Enteritidis, *S.* Newport, *S.* Montevideo) might establish and internalize different in various lettuce plants when inoculated at a high level (7 log cfu/g) in soil. At this study it was evident that among serovars *S.* Dublin was able to colonize lettuce endophytically as well as epiphytically, while lettuce cultivars Cancan and Nelly were less susceptible to bacterial contamination compared to cultivar Tamburo (Klerks et al., 2007a). These findings suggest that human pathogen internalization is affected by bacterial strain and serovar, type of vegetable and method of contamination.

Table 1.1. Internalization of foodborne pathogens in leafy vegetables grown in soil.

Plant	Bacteria tested	Plant part inoculated	Inoculum (log cfu/g)	Surface sanitation	Internalization	Internalization occurrence	Reference
Lettuce	*S.* Newport	root	6, 8	-	±	plant tissue	Bernstein et al., 2007
Cilantro	*S.* Thompson, *P. agglomerans*, *P. chlororaphis*	upper plant part (immersion)	5	-	+	vein area	Brandl and Mandrell, 2002
Lettuce	*E. coli* O157:H7, *S.* Thompson	upper plant part (immersion)	5	-	+	veins and in the areas between the veins	Brandl and Amundson, 2008
Spinach, green lettuce, parsley	*E. coli* O157:H7	root	2, 3, 4, 5, 6	+	+	no internalization via roots	Erickson et al., 2010a
Spinach, lettuce	*E. coli* O157:H7	upper plant part (spray)	2, 4, 6, 8	+	+	lettuce leaves	Erickson et al., 2010b
Spinach	*E.* O157:H7	root (root disruption + irrigation)	6	+	+	root (not in leaves) 7d post inoculation	Hora et al., 2005
Spinach, rocket	*E. coli* O157:H7	upper plant part (spray)	5-7	+	+	leaves	Alam eta al., 2014
Lettuce	*S.* Dublin, *S.* Typhimurium, *S.* Enteritidis, *S.* Newport, *S.* Montevideo	root	7	+	+	leaves	Klerks et al., 2007a
Spinach	*E. coli* O157:H7	leaves and roots	6	+	+	leaves	Mitra et al., 2009
Lettuce	*E. coli* O157:H7	root	1, 2, 3, 4	+	+	leaves	Mootian et al., 2009

Spinach	*E. coli* O157:H7	root	3, 7	+	+	leaves	Pu et al., 2009
Lettuce	*E. coli* O157:H7	root	4, 6, 8	+	+	in edible tissue	Solomon et al., 2002
Lettuce	*Escherichia coli* O157:H7	root (irrigation water)	8,9	+	+	in edible tissue and root	Solomon and Matthews, 2005
Lettuce	*E. coli* O157:H7	root	4, 6	+	-	-	Zhang et al., 2009a
Lettuce	*E. coli* O157:H7	leaves and roots	3, 4, 6	+	+	in inoculated leaves	Zhang et al., 2009b
Lettuce	*E. coli* O157:H7	roots, seeds	4	+	+	leaves and root	Cooley et al., 2006
Lettuce	*E. coli*	root	7	+	+	leaves	Guprta and Madramootoo, 2016
Lettuce	*E. coli*	root	3-4	-	+	leaves	Jensen et al., 2013
Crisphead lettuce	*E. coli* O157:H7	root	4	+	+	seedlings and leaves/root	Johannessen et al., 2005
Lettuce	*E. coli* O157:H7	leaves	5, 7	-	+	leaves	Moyne et al., 2011
Lettuce	*E. coli* O157:H7, *L. monocytogenes*, *S.* Senftenberd	root	5	+	+	leaves and root	Murphy et al., 2016
Lettuce	*E. coli* O157:H7, *S.* Typhimurium	root	8	+	+	root and shoot	Nicholson et al., 2015
Lettuce	*L. innocua*	root	7	-	+	leaves and root	Oliveira et al., 2011
Lettuce	*E. coli* O157:H7	root	7	-	+	leaves and root	Oliveira et al., 2012

Cabbage	*E. coli* O157:H7, *S.* Typhimurium	root	4, 7	+	+	leaves	Ongeng, 2011a
Cabbage	*E. coli* O157:H7, *S.* Typhimurium	root	4, 7	+	+	leaves	Ongeng, 2011b
Lettuce, cilantro	*S.* Montevideo, *S.*Enteritidis	leaves	8	-	not studied	not studied	Poza-Carrion et al., 2013
Cress, oats	*E. coli* O157:H7, *S.* Typhimurium	root	10	-	+	root and shoot	Semenov et al., 2010
Mizuna, tatsoi, red chard	generic *E. coli* , *E. coli* O157:H7	leaves (spray)	4	+	+	leaves	Tomás-Callejas et al., 2011
Spinach, lettuce	*E. coli* O157:H7	root	7	+	+	root and leaves	Wright et al., 2013
Lettuce	*S.* Enteritidis	root	1, 5, 6, 7, 8	+	+	whole plant and leaves	Honjoh et al., 2014
Rocket, basil	*L. monocytogenes*	root	9	-	-	-	Settanni et al., 2012
Cantaloupe, iceberg lettuce, bell peppers	*E. coli*, *S.* Typhimurium	root	5, 6, 7, 8	-	+	leaves	Stine et al., 2005a
Lettuce	*E. coli* O157:H7	leaves (spot method)	6, 7, 9	-	not studied	not studied	Dinu and Bach, 2011
Lettuce, spinach	*E. coli* O157:H7	leaves (abaxial-underside surface)	6, 8	+	+	leaves	Erickson et al., 2010c

Parsley	*S.* Typhimurium	root	7	+	+	leaves	Lapidot and Yaron, 2009
Lettuce	*E. coli* O157:H7	root	7	+	+	root and leaves	Quilliam et al., 2012
Lettuce, cabbage	*E. coli*	leaves and roots	4	-	not studied	not studied	Seidu et al., 2013
Lettuce	*E. coli* O157:H7	leaves (spray)	2, 4	-	not studied	not studied	Solomon et al., 2003
Lettuce (cultivars: 'Green Star', 'Muir', 'New Red Fire', 'Starfighter', 'Tropicana', and 'Two Star')	*S.* Enteritidis, *E. coli* O157:H7	leaves (spray)	5	+	+	leaves	Erickson et al., 2019
Spinach, lettuce	*E. coli* O157:H7	roots (compost and soil)	2, 3, 4, 5,7	+	-	-	Erickson et al., 2014
Spinach	*S.* Typhimurium, *E. coli* O157:H7	leaves (spray)	5	+	+	leaves	Erickson et al., 2018
Spinach, lettuce, tomato, *Nicotiana benthamiana*	*E. coli* O157:H7	leaves and roots	2	+	+	leaves	Wright et al., 2017

For surface sterilization column: +: performed, -: not performed. For internalization column: +: presence internally, -: no presence internally, ±: both presence and absence inside plant tissue.

As the need for renewable energy sources increases, soil to be more contaminated and less fertile, and intense weather phenomena are more evident, a turn towards hydroponic vegetable production was made. Hydroponic cultivation of vegetables is considered to be a clean, safe and environmentally friendly growing technique; however, incidence of microbial contamination might occur endangering human health (Treftz and Omaye, 2016; Dankwa et al., 2020; Lenzi et al., 2021). Macarisin et al. (2014) reported that *E. coli* O157:H7 (5, 6, 7 log cfu/mL) was isolated from hydroponically grown spinach roots, stems and leaves (nutrient solution inoculation). In the same study it is suggested that high inoculum levels resulted to increased internalization possibility (Macarisin et al., 2014). When cress, radish, spinach, lettuce, mustard, carrots, and tomatoes seeds grown in solidified hydroponic nutrient solution that subjected to inoculation with *E. coli* O157:H7, *S.* Typhimurium and *L. monocytogenes* (2 log cfu/mL), bacteria were isolated from leaf surfaces and at the same time *E. coli* colonization was influences by plant type and plant age (Jablasone et al., 2005). Moreover, Koseki et al. (2011) reported internalization of *E. coli* O157:H7, *S.* Enteritidis, *S.* Typhymurium and *L. monocytogenes* on hydroponically grown lettuce at both inoculums' levels investigated (3 and 6 log cfu/mL).

Table 1.2. Internalization of foodborne pathogens in leafy vegetables hydroponically grown.

Plant	Bacteria tested	Growth conditions	Plant part inoculated	Inoculum (log cfu/mL)	Surface sanitation	Internalization	Internalization occurrence	Reference
Lettuce	*E. coli* O157:H7, *S.* Typhimurium	hydroponic and soil	root	9	+	+	leaves and root	Franz et al., 2007
Spinach	*E. coli*	hydroponic and soil	root	2, 3	+	+	leaves	Warriner et al., 2003
Spinach	*E. coli* O157:H7	hydroponic medium and soil	root	3, 4, 5, 6, 7, 8	-	+	shoot and root	Sharma et al., 2009
Spinach	*E. coli*	hydroponic and soil	upper plant part (spraying)	2, 3, 4, 5	-	not studied	not studied	Gutierrez-Rodriguez et al., 2011
Spinach	*E. coli* O157:H7	hydroponic and soil	root	5, 6, 7	+	+	root, stem and leaves	Macarisin et al., 2014
Cress, radish, spinach, lettuce, mustard, carrots, tomatoes	*E. coli* O157:H7, *S.* Typhimurium, *L. monocytogenes*	solidified hydroponic nutrient solution	seeds	2	+	+	leaf surface	Jablasone et al., 2005
Lettuce	*S.* Dublin	soil and Hoagland's agar	root	7	+	+	leaves	Klerks et al., 2007b
Cabbage	*E. coli*	hydroponic model system	root	5	-	not studied	not studied	Wachtel et al., 2009a
Lettuce	*E. coli* O157:H7	soil and hydroponic model system	root	2, 4, 6, 8	-	not studied	not studied	Wachtel et al., 2009b
Lettuce	*E. coli* O157:H7	hydroponics	root	7	+	±	root and leaves	Hou et al., 2013

radish sprouts	*E. coli* O157:H7	hydroponics	root	3, 4, 7	-	+	cotyledon and hypocotyl	Hara-Kudo et al., 1997
radish sprouts	*E. coli* O157:H7	hydroponics	root	3	+	+	hypocotyl	Itoh et al., 1998
Spinach	*E. coli* O157:H7, *S.* Enteritidis, *S.* Typhimurium, *L. monocytogenes*	hydroponics	root, leaves and cut leaves	3, 6	-	+	root and leaves	Koseki et al., 2011
Basil	*S.* Typhimurium, *S.* Thompson, human NoV surrogates [murine norovirus 1 (MNV-1) and Tulane virus (TV)]	hydroponics	root and leaves	8	-	+	shoot and leaves	Li and Uyttendaele, 2018
Lettuce	*E. coli* O157:H7	hydroponics	root	7	+	+	root and leaves	Moriarty et al., 2019
Radish	*C. freundii, Enterobacter spp., E. coli, K. oxytoca, S. grimesii, P. putida, S. maltophilia*	hydroponics	root	6	+	+	hypocotyls	Settanni et al., 2013

For surface sterilization column: +: performed, -: not performed. For internalization column: +: presence internally, -: no presence internally, ±: both presence and absence inside plant tissue.

1.6. Pre- and post-harvest management and quality of fresh produce

1.6.1. Pre-harvest management

Throughout the production chain of fresh produce and minimally processed vegetables the risk for microbial contamination is lurking. Once human pathogen establish in an environment including plant tissue (biofilm formation and/or internalization) their elimination is challenging in the subsequent steps of the food chain (processing, distribution and consumption). Thus, preventive measures should be taken in order to minimize the probabilities of fresh produce contamination.

Use of clean and good source water is essential since water is one of the major factors that can lead to fresh produce contamination with foodborne pathogens either at conventional or hydroponic cultivation (Uyttendaele et al., 2015; Faour-Klingbeil et al., 2016b). According to Codex Alimentarus (2003), the water used in close hydroponic systems (close loop systems) is recycled or changed frequently since the composition of the recirculated nutrient solution is constantly changing due to the absorption of nutrients and water from plants, and in most cases, the nutrient solution is decontaminated against plant pathogens (Tzortzakis et al., 2020). Moreover, decontamination of seeds prior sowing has been proven able to lower the risk of pathogen contamination and the microbial load of the seed's microflora which could possible mediate to the internalization of human pathogens at the early stages of plant growth (Lenzi et al., 2021). Moreover, the use of protective nets against pest could be applied in order to eliminate the access of invaders (i.e. insects, animals) to fresh produce. Interestingly it has been suggested that the use of biodegradable nets with incorporated cinnamon essential oil, not only inhibited the growth of *Alternaria alternata* but also increased antioxidant activity of tomato fruits (Black-Solis et al., 2019).

1.6.2. Postharvest management

During processing of fresh produce, contamination might take place due to unsanitary or non-properly sanitized surfaces (i.e. people, water, soil, dust) (EFSA, 2011). The main decontamination during minimal processing of vegetables is washing and the use of sanitizing agents at this step has been proven to essential for the preservation of low microbial load. Sanitizing processes can be categorized as follows: i) chemical sanitizers (i.e. chlorine, chlorine dioxide, hydrogen peroxide, ozone), ii) physical methods (i.e. modified atmosphere packaging, irradiation, ultraviolet treatment) and ii) natural products (i.e. organic acids, essential oils and other plant extracts, edible films and coatings) (Ramos et al., 2013; Xylia et al., 2017).

1.6.2.1. Chemical means

The most common sanitizing agent used in the food industry is sodium hypochlorite (chlorine) and the concentration and time of its application can vary from 50 to 200 mg/L and 1 to 10 minutes depending on the product being processed. Many factors can influence its efficacy including the application and washing time, the temperature and the pH of the solution and the amount of the organic matter that is present (Francis et al. 1999). However, many studies have shown that the applied concentration might not be able to eliminate microbial load of fresh produce (including foodborne pathogens) and at the same time could result to the formation of trihalomethanes, carcinogenic substances that can be absorbed by vegetables and potentially harm human health (Akbas and Ölmez, 2007a; Coroneo et al., 2017). These findings have led the food industry to investigate the use of other more eco-friendly sanitizing agents that could not risk human health.

Chlorine dioxide (ClO_2) and hydrogen peroxide (H_2O_2) have been previously mentioned as alternative, safe decontamination agents (use up to 3 and 80 ppm, respectively), since they cannot react with organic matter despite their oxidizing nature (Joshi et al., 2013; Ramos et al., 2013) on the other hand H_2O_2 was found to cause severe browning to shredded lettuce (Parish et al., 2003). Ozone (O_3) has also been suggested and applied for the preservation of fresh produce (Ramos et al., 2013; Tzortzakis and Chrysargyris, 2017). It is an agent that presents increased antimicrobial activity due to its high reactivity and penetrability and these results are more evident at its gaseous compared to aqueous solutions (Ramos et al., 2013).

1.6.2.2. Physical means

The reduction of fresh produce microbial load could also be achieved with the use of physical methods such as the application of modified atmosphere packaging (MAP), irradiation and ultraviolet treatment (UV-C) (Ramos et al., 2013). The modification of the packaging atmosphere (O_2 and CO_2 levels) has been proposed as one method for preserve minimally processed vegetables due to its ability to inhibit the growth of aerobic spoilage microorganisms as well as foodborne pathogens (Rodriguez-Aguilera and Oliveira, 2009). In addition, irradiation of fresh produce has been approved by the FDA with maximum application level of 1 kGy, whilst UV-C has also been proposed for application to fruits and vegetables due to its direct and indirect antimicrobial activity (Parish et al., 2003; Ramos et al., 2013).

1.6.2.3. Natural products

Nowadays, a turn towards the use of less chemical and more natural products for the preservation of fruits and vegetables has been observed by the food industry as increasing consumer. This has resulted

to the investigation of natural products with numerous properties such as antioxidant, antimicrobial, antifungal, anti-browning and more properties (Altunkaya and Gökmen, 2009; Chrysargyris et al., 2016a,b; Yuan et al., 2016). These natural products include: organic acids (ascorbic, lactic, acetic, tartaric and oxalic acid), essential oils from aromatic plants and other plant extracts, their combinations and incorporation to edible films and coatings (Martin-Diana et al., 2006; Akbas and Ölmez, 2007a,b; Chrysargyris et al., 2016b; Yuan et al., 2016; Viacava et al., 2018; Tzortzakis et al., 2019; Xylia et al., 2019b; Nair et al., 2020).

Organic acids have been used by the food industry due to their ability to inhibit microbial load by decreasing the environmental and/or intracellular pH (Parish et al., 2003; Ramos et al., 2013). Moreover studies have also proven than organic acids such as ascorbic acid could prevent or minimize browning evidence when applied to cut surfaces (Akbas and Ölmez, 2007a,b; Altunkaya and Gökmen, 2009; Park et al., 2011; Xylia et al., 2019b).

Essential oils from aromatic plants and other plant extracts have also been considered as alternative fresh produce and minimally processed vegetables. Many studies have reported the benefits essential oils and plant extracts use in minimally processed food as well as fresh produce (Tzortzakis, 2009; Chen et al., 2017; Chrysargyris et al., 2017; Xylia et al., 2017; Xylia et al., 2018; Tzortzakis et al., 2019; Viacava et al., 2018; Xylia et al., 2019b). These effects are attributed to the numerous properties of the essential oils and their aromatic origin and include: antimicrobial, antifungal, antioxidant, antiparasitic, insecticide and many more (Burt, 2004). During the last years the incorporation of essential oils in edible films and coatings (i.e. chitosan) has gained great interest as is seems that these formulations can enhance the properties of the natural compounds and in some cases act synergistically prolonging the shelf-life of fresh produce and minimally processed vegetables (Yuan et al., 2016; Romanazzi et al., 2018; Nair et al., 2020).

It is worth mentioning that the nutritional value of minimally processed vegetables during processing might be adversely affected. For instance, oxidation of phenolic content, degradation of vitamin C (ascorbic acid) loss of dietary fibers might take place due to preparing practices (i.e. cutting, shredding, washing) (Favell et al., 1998; Vallejo et al., 2003; McDowell et al., 2007). Thus, it is essential for the food industry to investigate the use of compounds and processes that not only ensure the microbial quality of fresh produce and minimally processed vegetables but also preserve and/or enhance the nutritional of these products.

1.7. Objectives of the present studies

The present thesis aims to assess i) the microbiological and physicochemical attributes of ready-to-eat salads as affected by season, producer, type of salad in the whole districts of Cyprus, ii) the microbiological and physicochemical attributes of ready-to-eat salads on their expiring date comparing

with the date of purchase and how the season, the producer, and the type of salad could have a role on that, iii) the effects of pH of the nutrient solution, the plant age (small, medium and old plants) and inoculum levels (low and high levels) of *S.* Enteritidis on hydroponically grown lettuce and iv) the efficacy of natural products (essential oil, hydrosol, ascorbic acid and chitosan) and their combinations on the quality and safety of ready-to-eat vegetables (minimally processed lettuce and shredded carrot).

Chapter 2. Methodology and protocols

2.1. Media

Solutions and media used, were prepared with deionized water, reverse osmosis and sterilized by autoclaving (SANYO, MLS-3781L) at 121ºC for 15 minutes (or as otherwise indicated by the manufacturer.

2.1.1. BAIRD-PARKER Agar

The BAIRD-PARKER agar (BPA (Merck, Darmstadt, Germany) was prepared according to the manufacturer's instructions (Merck-105406) and then an aliquot of Egg yolk tellurite emulsion (Merck-103785) was added. The medium consists of peptone from casein (10.0 g/L), meat extract (5.0 g/L), yeast extract (1.0 g/L), sodium pyruvate (10.0 g/L), glycine (12.0 g/L), lithium chlorite (5.0 g/L) and agar (15.0 g/L). The Egg yolk tellurite emulsion consists of sterile egg-yolk (200 mL/L), sodium chloride (4.25 g/L), potassium tellurite (2.1 g/L) and distilled water (to give a final volume of 1000 mL).

2.1.2. Brain Heart Infusion Agar

The Brain Heart Infusion broth (BHI Broth) (Biolab, Hungary) was prepared according to the manufacturer's instructions (Biolab-BHI 291113044). The broth consists of nutrient substrate (brain and heart infusion, peptones) (27.5 g/L), glucose (2.0 g/L), sodium chlorite (5.0 g/L) and buffers (2.5 g/L). The BHI agar was prepared as the BHI broth with the addition of 1.5% agar (Sigma-BCBL0578V).

2.1.3. Cereus selective agar acc. to MOSSEL

The Cereus selective agar base acc. to MOSSEL (MYP agar)(Merck, Darmstadt, Germany) was prepared according to the manufacturer's instructions (MERCK-105267) and then an aliquot of Egg yolk emulsion (Merck-103784) and the content of one vial Bacillus cereus Selective Supplement (Merck-109875) were added. The medium consists of peptone from casein (10.0 g/L), meat extract (1.0 g/L), D-(-)-mannitol (10.0 g/L), sodium chloride (10.0 g/L), phenol red (0.025 g/L) and agar-agar (12.0 g/L). The Egg yolk emulsion consists of sterile egg yolk (500 mL/L), sodium chloride (4.25 g/L) and distilled water (to dive a final volume of 1000 mL). The selective supplement consists of polymyxin B (50000 IU/vial).

2.1.4. Chromocult Listeria Selective Agar

The Chromocult® Listeria Selective Agar Base acc. OTTAVIANI and AGOSTI (ISO 11290) (Merck, Darmstadt, Germany) was prepared according to the manufacturer's instructions (Merck-100427) and then an aliquot (according to the manufacturer's instructions) of Chromocult Listeria Agar Selective –Supplement (Merck-100432) and one bottle of ChromoCult Listeria Agar Enrichment Supplement (Merck-100439) were added. The base consists of peptone from meat (18.0 g/L), peptone from casein (6.0 g/L), yeast extract (10.0 g/L), sodium pyruvate (2.0 g/L), glucose (2.0 g/L), magnesium glycerophosphate (1.0 g/L), magnesium sulphate (0.5 g/L), sodium chlorite (5.0 g/L), lithium chlorite (10.0 g/L), disodium hydrogen phosphate (2.5 g/L), 5-bromo-4-chloro-3-indolyl-β-D-glycopyranoside (0.05 g/L) and agar (13.0 g/L). The selective supplement consists of amphotericin B (0.005 g/vial), ceftazidime (0.010 g/vial), nalidixic acid sodium salt (0.010 g/vial) and polymyxin B sulfate (38350 IU/vial). The enrichment supplement consists of L-α-phosphatidylinositol (raw extract from soy lecithin) (1g/vial).

2.1.5. CN agar for Pseudomonas

The CN agar for Pseudomonas base (Biokar diagnostics, France) was prepared according to the manufacturer's instructions (Biokar diagnostics-BK165HA). The medium consists of pancreatic digest of gelatin (16.0 g/L), acid hydrolysate of casein (10 g/L), potassium sulfate (10.0 g/L), magnesium chloride (1.4 g/L), cetrimide (0.2 g/L), nalidixic acid (0.015 g/L), bacteriological agar (11.0 g/L).

2.1.6. Coliform agar

The ChromoBio COLIFORM agar (Biolab, Hungary) was prepared according to the manufacturer's instructions (Biolab-COF20500). The medium consists of peptones (3.5 g/L), sorbitol 1 g/L), sodium chloride (5 g/L), sodium pyruvate (1 g/L), tryptophane (1 g/L), Tergitol 7 (0.15 g/L), chromogenic substrate (0.4 g/L), buffers (4.9 g/L) and agar (13 g/L).

2.1.7. ESBL agar

The Chromatic ESBL agar base (Liofilchem s.r.l, Italy) was prepared according to the manufacturer's instructions (Liofilchem s.r.l-610629) and then two vials of Chromatic ESBL Supplement (Liofilchem s.r.l-81089) or Chromatic ESBL + Amp Supplement (Liofilchem

s.r.l-81090) were added. The medium consists of peptone mix (43.2 g/L), chromogenic mix (1.0 g/L) and agar (15.0 g/L). The supplement consists of Selective Mix (0.5 g/vial).

2.1.8. FRASER Listeria Selective Enrichment Broth

The FRASER Listeria Selective Enrichment Broth base acc. ISO 11290 (Merck, Darmstadt, Germany) was prepared according to the manufacturer's instructions (Merck-110398). Half FRASER broth was prepared with the addition of one vial of ammonium iron (III) supplement (Merck-100092) and one selective supplement (Merck-100093). Full FRASER broth was prepared with the addition of another bottle of selective supplement (cat. No. 100093) to the half-concentrated FRASER broth. The base consists of proteosepeptone (5.0 g/L), peptone from casein (5.0 g/L), yeast extract (5.0 g/L), meat extract (5.0 g/L), sodium chloride (20.0 g/L), di-sodium hydrogen phosphate (9.6 g/L), potassium dihydrogen phosphate (1.35 g/L), esculin (1.0 g/L) and lithium chloride (3.0 g/L). The ammonium iron (III) supplement consists of ammonium iron (III) citrate (0.250g/vial). The selective supplement consists of acriflavine (0.00625 g/vial) and nalidixic acid (0.005 g/vial).

2.1.9. Maximum Recovery Diluent

The Maximum Recovery Diluent (MRD) (Merck, Darmstadt, Germany) solution was prepared according to the manufacturer's instructions (Merck-112535). The solution consists of peptone (1.0 g/L) and sodium chlorite (8.5 g/L).

2.1.10. MRS agar

The MRS broth ISO 15214 (Liofilchem s.r.l, Italy) was prepared according to the manufacturer's instructions (Liofilchem s.r.l-610025). The broth consists of peptospecial (10.0 g/L), beef extract (10.0 g/L), yeast extract (5.0 g/L), glucose (20.0 g/L), triammonium citrate (2.0 g/L), sodium acetate (5.0 g/L), magnesium sulphate (0.2 g/L), manganese sulphate (0.05 g/L) and di-potassium phosphate (2.0 g/L). The MRS agar was prepared as the MRS broth with the addition of 1.5% agar (Sigma BCBL0578V).

2.1.11. Plate Count Agar

The GranuCult Plate Count Agar acc. to ISO 4833, ISO 17410 and FDA-BAM (PCA agar) (Merck, Darmstadt, Germany) was prepared according to the manufacturer's instructions

(MERCK-1105463). The medium consists of enzymatic digest of casein (5.0 g/L), yeast extract (2.5 g/L), D-(+)-glucose (1.0 g/L) and agar-agar (14.0 g/L).

2.1.12. Peptone Water (buffered)

The Peptone Water (buffered) (BPW) acc. to ISO 6579(Merck, Darmstadt, Germany) was prepared according to the manufacturer's instructions (Merck-107228). The solution consists of peptone from casein (10 g/L), sodium chloride (5 g/L), potassium dihydrogen phosphate (1.5 g/L) and di-sodium hydrogen phosphate dodecahydrate (9.0 g/L).

2.1.13. Rose Bengal CAF agar

The Rose Bengal CAF agar (Liofilchem vs.r.l, Italy) was prepared according to the manufacturer's instructions (Liofilchems.r.l-610090). The medium consists of enzymatic digest of soybean meal (5.0 g/L), glucose (10.0 g/L), monopotassium phosphate (1.0 g/L), magnesium sulfate (0.5 g/L), Rose Bengal (0.05 g/L), chloramphenicol (0.1 g/L) and agar (15.0 g/L).

2.1.14. Salmonella enrichment broth acc. to RAPPAPORT and VASSILIADIS

The Salmonella enrichment broth acc. to RAPPAPORT and VASSILIADIS (RVS broth) (Merck, Darmstadt, Germany) was prepared according to the manufacturer's instructions (Merck-107700). The broth consists of peptone from soymeal (4.5 g/L), magnesium chloride-hexahydrate (28.6 g/L), sodium chloride (7.2 g/L), di-potassium hydrogen phosphate (0.18 g/L), potassium dihydrogen phosphate (1.26 g/L) and malachite green oxalate (0.036 g/L).

2.1.15. Tryptone Bile Glucuronic Agar

The TBX agar (Himedia, India) was prepared according to the manufacturer's instructions (Himedia-M1591). The medium consists of enzymatic digest of casein (20.0 g/L), bile salts mixture (1.5 g/L), X-β-D-glucuronic acid (0.075 g/L), dimethyl sulfoxide (3.0 g/L) and agar (15.0 g/L).

2.1.16. Violet Red Bile Dextrose Agar

The Violet Red Bile Dextrose (VRBD) agar acc. EP, USP, JP and ISO 21528 (Merck, Darmstadt, Germany) was prepared according to the manufacturer's instructions (Merck-110275).The medium consists of pancreatic digest of gelatin (enzymatic digest of animal tissue) (7.0 g/L), yeast extract (3.0 g/L), sodium chloride (5.0 g/L), D-(+)-glucose (10.0 g/L), Bile salts (1.5 g/L), neutral red (0.03 g/L), crystal violet (0.002 g/L), agar-agar (13.0 g/L).

2.1.17. Xylose-Lysine Deoxycholate Agar

The Xylose-Lysine Deoxycholate Modified Agar (XLD Agar) (Sharlau, Spain) was prepared according to the manufacturer's instructions (Sharlau-01552).The medium consists of xylose (3.75 g/L), L-lysine (5.0 g/L), lactose (7.5 g/L), sucrose (7.5 g/L), sodium chloride (5.0 g/L), yeast extract (3.0 g/L), phenol red (0.08 g/L), sodium deoxycholate (1.0 g/L), sodium thiosulphate (6.8 g/L), ammonium ferric citrate (0.8 g/L) and agar (15.0 g/L).

2.2. Protocols

2.2.1. Microbiological analyses

Plant tissue was homogenized with MRD (or BPW) in a 1:10 ration (w/v) (dilution 10^{-1}). Serial decimal dilutions were carried out (from 10^{-1} to 10^{-7}). 100 µL were taken from each bag and transferred into 900 µL of MRD (dilution 10^{-2}). The same procedure was repeated until the dilution 10^{-7}. 100 µL from each dilution were dispersed into a Petri dish with the appropriate medium for each microorganism tested and incubated at the appropriate conditions (temperature and time).

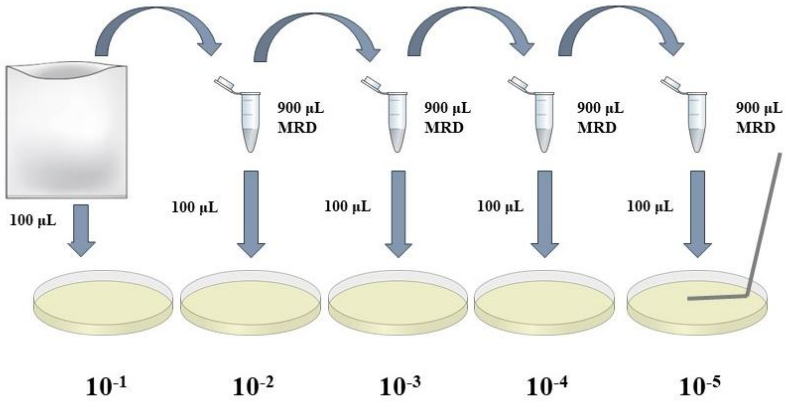


Figure 2.1. Serial decimal dilution procedure.

2.2.1.1. Total Viable Count (TVC)

For the determination of TVC 100 μL from each dilution (10^{-3}-10^{-5}) were dispersed on PCA medium and plates were incubated at 30ºC for 48 h and results were expressed as log cfu/g of sample.

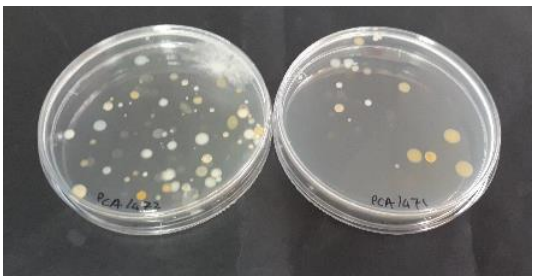


Figure 2.2. Total viable count on PCA.

2.2.1.2. *Enterobateriaceae* family

For the determination of *Enterobacteriaceae* family counts 100 μL from each dilution (10^{-2}-10^{-4}) were dispersed on VRBDA medium (with a covering layer of the same medium) and plates were incubated at 37ºC for 24 h. Characteristic pink to red or purple colonies with or without precipitation halo were counted and results were expressed as log cfu/g of sample.

Figure 2.3. Members of *Enterobacteriaceae* family on VRBDA.

2.2.1.3. Coliforms, *E. coli* and antibiotic resistant *E. coli* isolates

For the determination of coliforms and *E. coli* counts were determined by dispersing 100 μL from each dilution (10^{-1}-10^{-3}) on COLIFORMS agar and plates were incubated at 37ºC for 24 h. Characteristic pink (coliforms) and blue (*E. coli*) colonies were counted and results were expressed as log cfu/g of salad. One typical blue colony isolated from COLIFORMS agar was streaked on TBX agar and plates incubated at 37ºC for 24 h. After incubation typical blue-green colonies were further tested for antibiotic resistance (extended spectrum *β*-lactamaces-ESBL). The presence of antibiotic resistant *E. coli* isolates was tested by streaking one typical

blue-green colony from TBX on ESBL agar. Plates were incubated at 37ºC for 24 h and then examined for typical pink-reddish-mauve colonies.

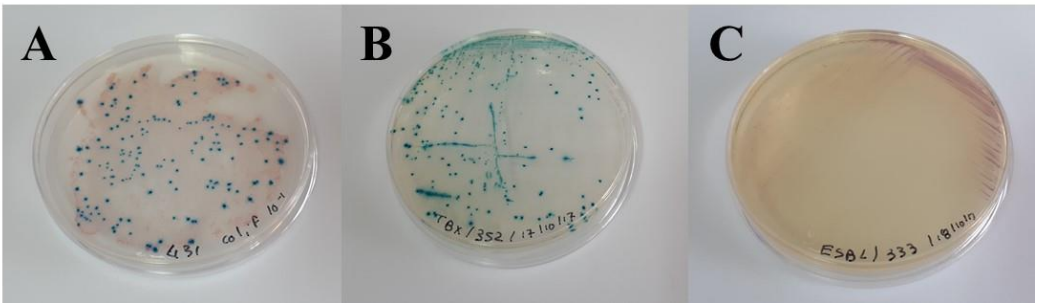


Figure 2.4. Coliforms and *E. coli* on COLIFORMS agar (**A**). *E. coli* isolates on TBX agar (B). *E. coli* isolates exhibiting antibiotic resistance on ESBL agar (**C**).

2.2.1.4. *Staphylococcus* spp.

For the determination of *Staphylococcus* spp. 100 µL from each dilution (10^{-1}-10^{-2}) were dispersed on BPA medium and plates were incubated at 37ºC for 24 h. Typical black or grey colonies with and/or without a clear halo were counted and results were expressed as log cfu/g of sample.

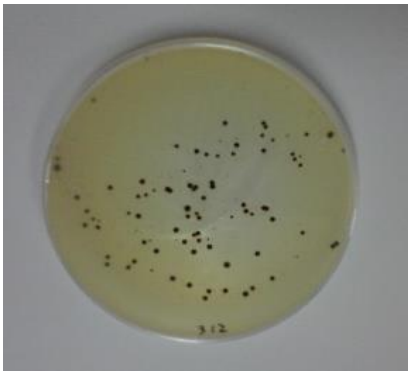


Figure 2.5. *Staphylococcus* spp. on BPA.

2.2.1.5. *B. cereus*

B. cereus counts were determined by dispersing 100 µL from each dilution (10^{-1}-10^{-2}) on MYP medium and plates were incubated at 30ºC for 24 h. Typical pink colonies with precipitation halo were counted and results were expressed as log cfu/g of sample.

Figure 2.6. *B. cereus* on MYP agar.

2.2.1.6. Lactic acid bacteria (LAB)

For the determination of lactic acid bacteria population 100 µL from each dilution (10^{-1}-10^{-3}) were dispersed on MRS medium and plates were incubated at 30°C for 48 h and results were expressed as log cfu/g of sample.

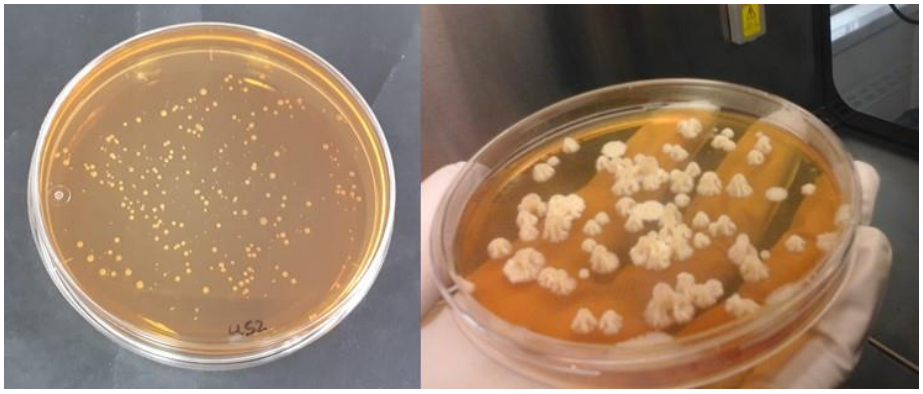


Figure 2.7. Lactic acid bacteria on MRS agar.

2.2.1.7. *Pseudomonas* spp.

Pseudomonas spp. population was determined by dispersing 100 µL from each dilution (10^{-1}-10^{-4}) on Pseudomonas agar and plates were incubated at 37°C for 24-48 h. Colonies producing a blue-green (pyocyanin) pigmentation (or presenting a fluorescence under UV light at 365 nm) were counted and results were expressed as log cfu/g of sample.

Figure 2.8. *Pseudomonas* spp. on Pseudomonas agar, under UV light.

2.2.1.8. Yeasts and molds

For the determination of yeasts and molds counts 100 μL from each dilution (10^{-1}-10^{-2}) were dispersed on Rose Bengal CAF medium and plates were incubated at 25°C for 5-7 days and results were expressed as log cfu/g of sample.

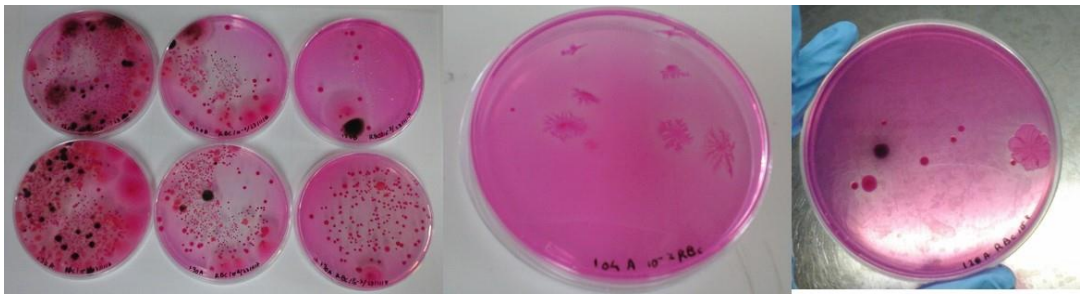


Figure 2.9. Yeasts and molds on Rose Bengal CAF agar.

2.2.1.9. Detection and isolation of *Listeria* spp.

For the detection and isolation of *Listeria* spp. the ISO 11290 (ISO 11290-1, 2004) method was used with modifications. The method consists of three steps: **i) pre-enrichment:** Five grams of plant tissue were homogenized with 45 mL of Half FRASER broth and samples were incubated at 30°C for 24 h, **ii) enrichment:** 100 μL from Half FRASER broth were transferred in 10 mL of Full FRASER broth and incubated at 37°C for 48 h and **iii) isolation:** A loopful from Full FRASER broth was streaked on ALOA medium and plates were incubated at 37°C. After 48 h plates were examined for the presence of typical blue-green colonies with opaque halo (typical Listeria-like colonies). Typical Listeria-like colonies were isolated, subcultured on BHI agar and incubated at 37 °C for 24 h.

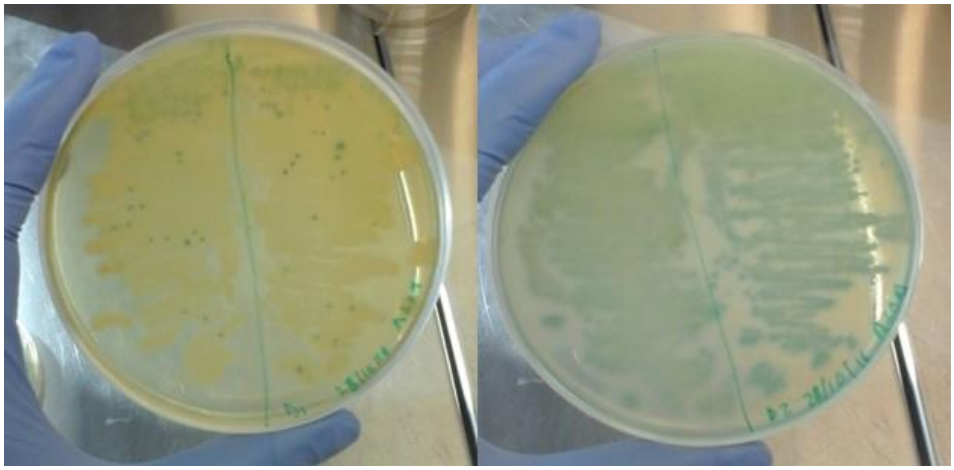


Figure 2.10. *Listeria* spp. on ALOA.

2.2.1.10. Detection and isolation of *Salmonella* spp.

For the detection and isolation of *Salmonella* spp. the ISO 6579 (ISO 6579, 2002) method was used with modifications. The method consists of three steps: **i) pre-enrichment:** Five grams of plant tissue were homogenized with 45 mL of BPW and samples were incubated at 37ºC for 24 h., **ii) enrichment:** 100 µL from BPW were transferred in 10 mL of RVS broth and incubated at 37ºC for 24 h and **iii) isolation:** A loopful from RVS broth was streaked on XLD medium and plates were incubated at 37ºC. After 48 h plates were examined for the presence of typical red colonies with black center. Typical colonies were isolated, subcultured on BHI agar and incubated at 37 ºC for 24 h.

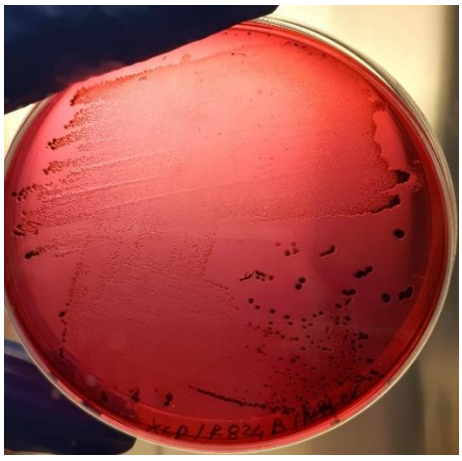


Figure 2.11. *Salmonella* spp. on XLD agar.

2.2.1.11. Molecular confirmation of *Listeria monocytogenes* and *Salmonella enterica*

DNA was extracted with heat lysis. For cell lysis and DNA extraction, colonies grown on BHI agar were selected and transferred in 200 μL of sterile dH_2O and heated at 100°C for 20 min. After that samples were centrifuged at 1500 x *g* for 5 min and supernatant was stored at -20°C until molecular analysis.

Salmonella spp. isolates were identified with real-time polymerase chain reaction (real-time PCR) using iCycler (Bio-Rad, USA) and a set of forward primer *inv*139 (5-´ GTGAAATTATCGCCACGTTCGGGCAA-3)´ and reverse primer *inv*141 (5-´ TCATCGCACCGTCAAAGGAACC-3)´ for the amplification of a 284 bp fragment of the *inv*A gene (Malorny et al., 2003). The incubation conditions were 95 °C for 1 min, followed by 35 cycles of 95 °C for 15 s, 64 °C for 30 s and 72 °C for 30 s (Malorny et al., 2003). A final extension of 72 °C for 4 min and a melting curve at 55 °C were also employed. *Listeria* spp. isolates were identified using real-time PCR for the amplification of a 274 bp fragment of the *prf*A gene with forward primer *prf*A LIP1 (5-´ GATACAGAAACATCGGTTGGC-3)´ and reverse primer *prf*A LIP2 (5-´ GTGTAATCTTGATGCCATCAGG-3)´ (Rossmanith et al., 2006). The incubation conditions were 94 °C for 2 min, followed by 45 cycles of 94 °C for 15 s and 64 °C for 1 min (Rossmanith et al., 2006). A melting curve at 55 °C was also employed.

In both protocols, the template for real-time PCR assays (for 1 reaction-20 μL final volume) was genomic DNA from heat lysed cells (2 μL) mixed with forward and reverse primers (1 μL each), sterile distilled water (6 μL) and KAPA CYBR FAST qPCR Master mix (KAPA Biosystems, USA) (10 μL).

2.2.2. Weight loss and color

Weight loss was monitored throughout storage and results were presented as percentage of total weight loss. Color was evaluated with a colorimeter (Chroma meter CR400Konica Minolta, Japan) where the L^* (lightness), a^* (redness), and b^*(yellowness) value were recorded. Chroma value (C), hue (h) and whiteness index (WI) were calculated by the following equations $C = (a^{*2} + b^{*2})^{1/2}$, $h = \tan^{-1}(b^*/a^*)$ and $WI=100 - [(100 - L^*)^2 + a^{*2} + b^{*2}]^{1/2}$(Bolin and Huxsoll, 1991; McGuire, 1992). Color index (CI) was calculated with the following equation: $CI = (a^* \times 1000)/(L^* \times b^*)$ (Goyeneche et al., 2014). Browning index (BI) was calculated with the following equation: $BI = 100 \times (X - 0.31)/0.17$, where $X = (a^* + 1.75 \times L^*)/(5.645 \times L^* + a^* - 3.012 \times b^*)$ (Palou et al., 1999).

2.2.3. Respiration rate and ethylene emission

For the determination of sample's respiration rate, the atmosphere of each package was withdrawing by a dual gas analyser (International Control Analyser Ltd, UK) as previously reported (Chrysargyris et al., 2016b; Xylia et al., 2019b). Briefly, enclosed samples container's air was withdrawing for appropriate time through a hole on the lid whilst recording the $\%CO_2$. Ethylene flow rate was estimated by measuring the ethylene concentration of the packages as in the determination of respiration rate, using an ethylene analyser (ICA 56 Analyser, International Control Analyser Ltd, UK) as described previously by Chrysargyris et al. (2016b). Briefly, samples container's air was withdrawing for appropriate time through a hole on the lid recording the ppm of ethylene produced.

2.2.4. pH, total soluble solids, total acidity, sweetness

Plant tissue was grinded/pressed in order to extract juice. The pH of the plant juice was measured with a pH-meter (HANNA HI2211, Cluj-Napoca, Romania). Total soluble solids (TSS) content was determined using a digital portable refractometer (Atago, Tokyo, Japan) and results were expressed in ^{o}Brix. Titratable acidity (TA) was determined by titration with 0.1 N NaOH as previously reported (Rocha et al., 2007; Scuderi et al., 2011) and results were expressed as percentage of acid grams per 100 g of fresh weight (% TA). Sweetness was evaluated by the ratio of TSS/TA (Picouet et al., 2015).

2.2.5. Ascorbic acid (Vitamin C)

The presence of ascorbic acid (reducing properties) during titration of a sample, results in the reduction of the redox dark blue dye 2,6-dichlorophenol-indophenol (DCPIP) used to a colorless compound and its subsequent oxidation to dehydroascorbic acid. The end-point of this reaction is indicated with the development of a pink color of sample's solution due to addition of excess unreduced dye (presents pink color in acid solution) (Shivembe and Ojinnaka, 2017).

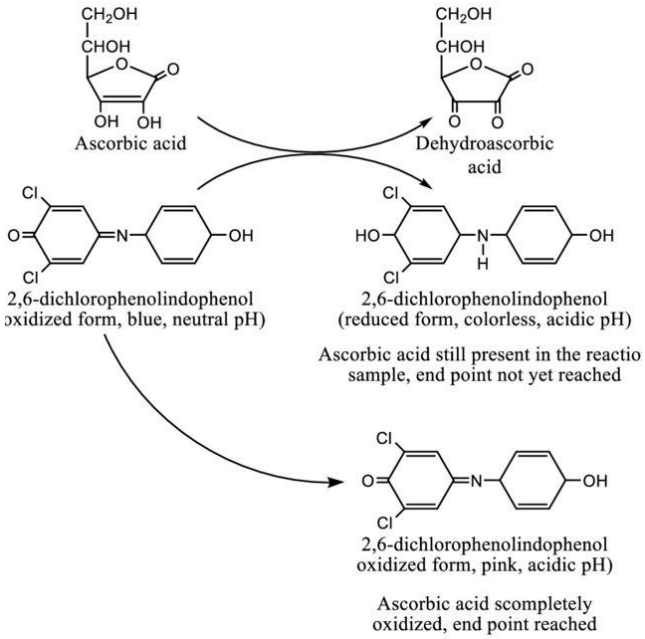


Figure 2.12. Reaction of ascorbic acid with DCPIP.

Ascorbic acid content was quantified by titration with 2,6-dichlorophenol-indophenol (DCPIP) (AOAC International, 2007) until the development of pink colored sample's solution and results were expressed as g of ascorbic acid per 100 g of fresh weight (g AA/ 100 g Fw).

2.2.6. Total carotenoids

Total carotenoid content was determined as described by Rocha et al. (2007) using 80% acetone (v/v); absorbance was measured at 480 nm and results were expressed as g of carotenoids per 100 g of fresh weight (g carotenoids/ 100 g Fw).

2.2.7. Leaf chlorophyll and carotenoid content

For leaf pigment extraction, plant tissue (three biological replicates/treatment/day; 0.1 g) was incubated with 10 mL dimethyl sulfoxide (DMSO) at 65 ^{o}C for 30 min and absorbance was measured at 480, 649 and 665 nm. Photosynthetic pigments (chlorophyll a, chlorophyll b and total chlorophyll) and total carotenoids content were calculated using the equations as previously reported by Wellburn (1994):

Chlorophyll a (Chl a) = $12.19 \times A_{665} - 3.45 \times A_{649}$

Chlorophyll b (Chl b) = $21.99 \times A_{649} - 5.32 \times A_{665}$

Total chlorophyll (Tot Chl) = Chl a + Chl b

Total carotenoids (Tot Carot) = (1000 x A_{480} – 2.14 x Chl a – 70.16 x Chl b)/220

Results were expressed as mg chlorophyll (or carotenoids) per g of fresh weight (mg chlorophyll/g Fw).

2.2.8. Determination of total phenolic content and antioxidant activity

2.2.8.1. Polyphenol extraction and analyses

Plant tissue was homogenized with methanol (50% v/v) for 60 seconds. Extraction was further assisted with a sonication bath and by centrifuging the samples for 15 minutes at 4000 x *g* at 4ºC. The supernatant was transferred in a 15 mL falcon tube and stored at -20 ºC until analysis.

2.2.8.2. Determination of total phenols using the Folin-Ciocalteu method

This method is based on the chemical reduction of the Folin-Ciocalteu reagent (a mixture of tungsten and molybdenum oxides). The products of this reduction are blue colored compounds that can absorb at 755 nm. The intensity of the absorption is proportional to the concentration of the phenolic compounds of the sample tested (Singleton and Rossi, 1965).

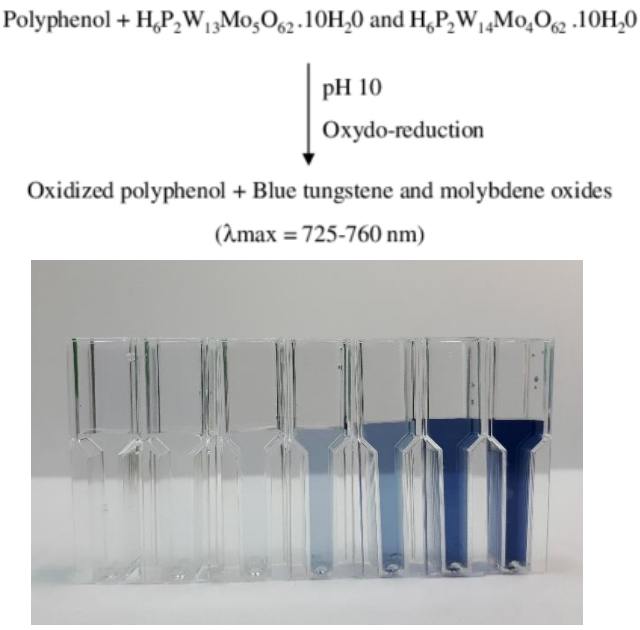


Figure 2.13. Reaction of polyphenols with Folin-Ciocalteu reagent (Callemien, D. and Collin, 2009).

The procedure was carried out as previously described by Tzortzakis et al. (2007). Briefly, a volume of sample (methanolic extract) were mixed with 125 µL of Folin-Ciocalteu reagent

(Merck, Darmstadt, Germany) and 1.25 mL of 7% (w/v) sodium carbonate (Sigma-Aldrich, Germany) (final volume 3 mL). After 1 h and 30 min incubation in the dark (room temperature), the absorbance was measured at 755 nm against a blank sample. For the quantification of total phenolic compounds, a standard curve was prepared from gallic acid stock solution (1 mmol/L) (Scharlau, Spain). The results were expressed as equivalents of gallic acid per g of fresh weight (mg of GAE/g Fw).

2.2.8.3. Determination of the antioxidant activity using the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) method

This assay is based on the capacity of antioxidants to reduce the DPPH• radical (violet color), leading to the formation of hydrazine. At 515-528 nm, the reduction of the red color is proportional to the concentration of the antioxidants in a DPPH• methanolic/ethanolic solution (Boligon et al., 2014).

Figure 2.14. Reaction of DPPH radical with antioxidants (adapted from Sekar et al., 2016).

The antioxidant capacity using the DPPH method was determined as previously described by Wojdylo et al. (2007) with slight modifications. Briefly, 1 mL of 0.3 mM DPPH 0.3 mM (Sigma-Aldrich, Germany) and a volume of methanolic extract were mixed (final volume 3 mL), the mixture was incubated for 30 minutes in the dark (room temperature) and the absorbance was measured at 517 nm. In order to calculate the amount of antioxidants in the

samples, a standard curve was prepared with trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) (Sigma-Aldrich, Germany) and results were expressed as mg of trolox per g of fresh weight (mg of trolox/g Fw).

2.2.8.4. Determination of the Ferric Reducing Antioxidant Power (FRAP)

The FRAP method is based on the reduction of Fe^{3+} to Fe^{2+} in the presence of antioxidants and the subsequent formation of $[Fe(TPTZ)_2]^{2+}$ complex. This blue colored complex can absorb at 593 nm and the intensity of the absorption is proportional to the concentration of antioxidants of the tested sample (Bezić and Strain, 1996).

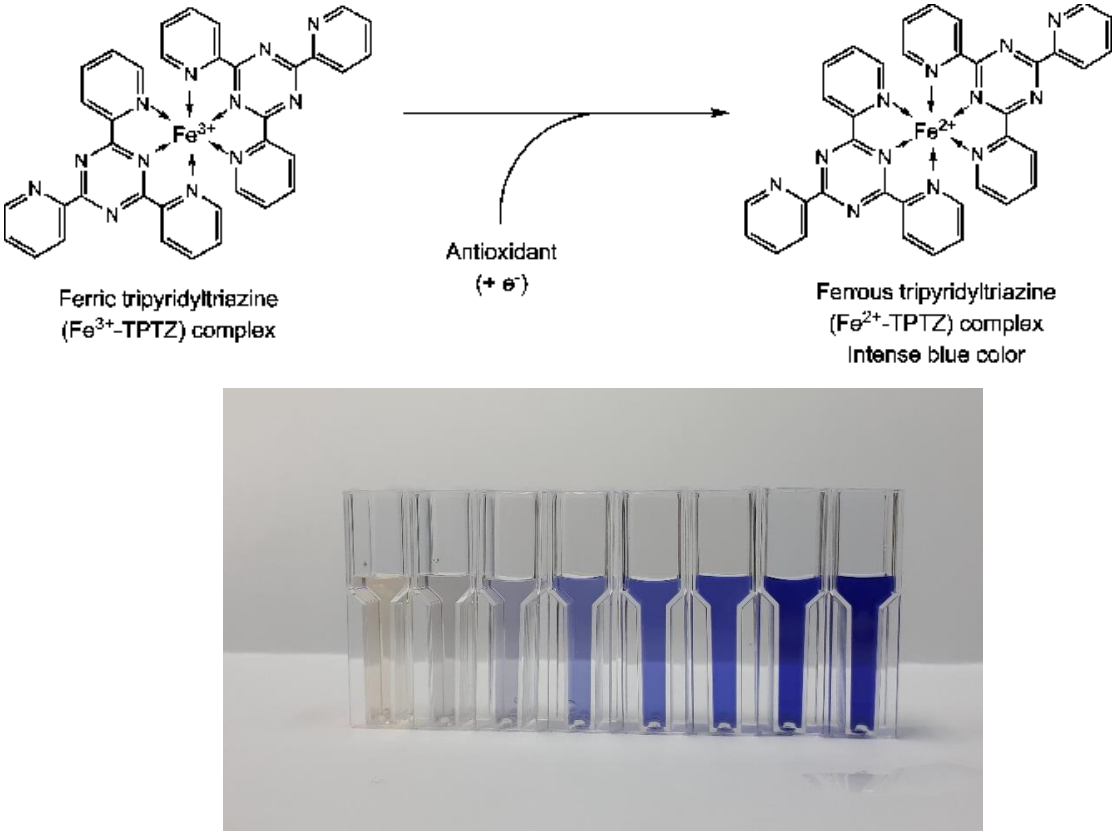


Figure 2.15. Reduction of Fe^{3+} to Fe^{2+} and formation of $[Fe(TPTZ)_2]^{2+}$ complex (adapted from Moon and Shibamoto, 2009).

The procedure was carried out as previously described by Chrysargyris et al. (2016a). Briefly, 2.5 mL of 0.3 M sodium acetate (Sigma-Aldrich, Germany) (pH=3.6), 0.25 mL of 10 mM TPTZ (Sigma-Aldrich, Germany), 0.25 mL of 20 mM $FeCl_3$ (Scharlab, Spain) and a volume of methanolic extract were mixed, incubated at 37°C for 4 minutes and the absorbance was measured at 593 nm against a blank sample. For the quantification of

antioxidants in samples, a standard curve was prepared with trolox and results were expressed as mg of trolox per g of fresh weight (mg of TROLOX/g Fw).

2.2.8.5. Determination of the antioxidant activity using the 2,2'-azinobis-(ethylbenzothiazoline-6-sulfonic acid) (ABTS) method

The assay is based on the oxidation of the ABTS reagent with ammonium persulfate to form $ABTS^{\bullet+}$. $ABTS^{\bullet+}$ is a blue-green radical with high absorbance at 734 nm. In the presence of antioxidants the radical is reduced (loss of blue-green color) and this reduction/discoloration is proportional to the concentration of the antioxidants in the sample tested (Villaño et al., 2004).

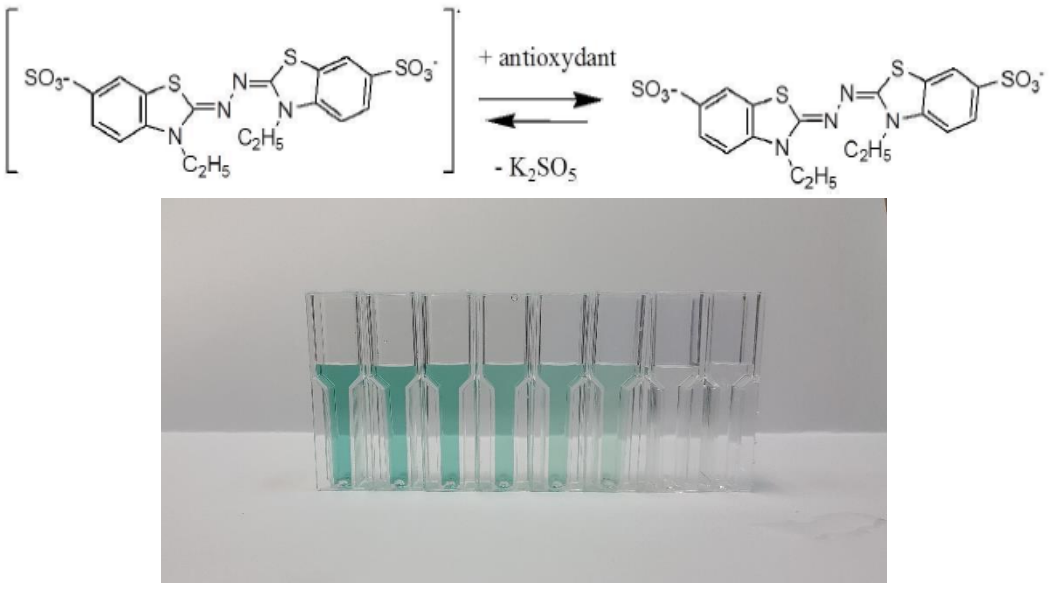


Figure 2.16. Reaction of $ABTS^{\bullet+}$ radical with antioxidants (adapted from El Rayess et al., 2014).

The procedure was carried out as previously described by Wojdylo et al. (2007). Briefly, 3 mL of ABTS solution (Sigma-Aldrich, Germany) and a volume of sample were mixed and after incubation for 6 minutes (room temperature) the absorbance was measured at 734 nm against a blank sample. In order to calculate the amount of antioxidants in the samples, a standard curve was prepared with trolox and results were expressed as mg of trolox per g of fresh weight (mg of trolox/g Fw).

2.2.9. Damage index: Determination of hydrogen peroxide and lipid peroxidation

2.2.9.1. Determination of hydrogen peroxide

Hydrogen peroxide (H_2O_2) is a compound that is produced in plants during normal physiological processes as well as in response to biotic and abiotic stress. The quantification of H_2O_2 is based on the oxidation of potassium iodide (KI) by the presence of H_2O_2 in an acidic solution. This reaction leads to the formation of iodine which can be further oxidized with iodide ions (I^-) present in the solution resulting to the production of triiodide (I_3^+), a compound with yellow color that can absorb at 390 nm. The intensity of the absorption is proportional to the concentration of H_2O_2 of the sample tested (Junglee et al., 2014).

$$\textbf{i) } 2I^- + 2H^+ + H_2O_2 \rightarrow I_2 + 2H_2O$$

$$\textbf{ii) } I_2 + I^- \rightarrow I_3^+$$

The H_2O_2 concentration was determined according to the method described previously by Loreto and Velikova (2001). Plant tissue (frozen grinded powder) was homogenized with 3 mL of 0.1% (w/v) trichloroacetic acid (TCA) (Scharlau, Spain) and centrifuged at 15000 x *g* at 4 ^oC for 15 min. An aliquot of the supernatant (0.5 mL) was mixed with 0.5 mL of 10 mM potassium phosphate buffer (pH=7.0) and 1 mL of 1 M potassium iodide (Merck, Darmstadt, Germany) and after 1 min the absorbance was measured at 390 nm. For the quantification of H_2O_2 content in samples, a standard curve was prepared with H_2O_2.

2.2.9.2. Determination of lipid peroxidation using the 2-thiobarbituric acid reactive substances (TBARS)

Lipid peroxidation is a universal indicator used for the assessment of plant membranes stress. The basic protocol for the determination of lipid peroxidation has been suggested by Heath and Packer (1968) and the method is based on the reaction of malondialdehyde (MDA) (one of the main oxidation product of polyunsaturated fatty acids) with 2-thiobarbituric acid (TBA) and the production of a red colored compound that can absorb at 532 nm. However, with this method other compounds such as anthocyanins and carbohydrates can interfere with the absorbance at this wavelength and thus their non-specific absorbance at 600 nm should be discarded (Taulavuori et al., 2001).

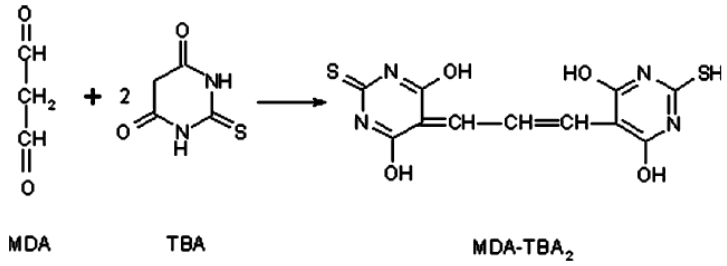


Figure 2.17. Formation of MDA-TBA_2 pigment (adapted from Ligor et al., 2012).

Lipid peroxidation was estimated according to the 2-thiobarbituric acid reactive substances (TBARS) method as previously described by de Azevedo Neto et al. (2006). The extraction procedure was carried out as mentioned above (Section **2.2.8.6.1**). An aliquot of the supernatant (0.5 mL) was mixed with 1.5 mL of 0.5% (w/v) 2-thiobarbituric acid (TBA) (Sigma-Aldrich, Germany) in 20% (w/v) TCA, the reaction was incubated at 95 ^{o}C for 25 min and then quickly cooled. The absorbance was measured at 532 and corrected by discarding the non-specific absorbance at 600 nm. In order to calculate the concentration of MDA the extinction coefficient of MDA was used ((ε) = 155 mM/cm).

Chapter 3. Investigation of safety and quality of ready-to-eat salads. A survey

3.1. Introduction

Dietary guidelines suggest the daily consumption of vegetables as an important source of vitamins, mineral, dietary fiber and phytonutrients. There is a general consensus that a diet rich in vegetables may reduce the risk of heart disease and protect against certain types of cancer (FAO, 2015). More and more people are changing their eating habits, increasing their daily intake of vegetables. Ready-to-eat salads are a healthy, low calorie and convenient option for a contemporary and busy lifestyle.

Ready-to-eat salads are characterized minimally processed foods as their processing includes washing, peeling, cutting, drying, and packaging and not any heat treatment (de Oliveira et al., 2011). Minimally processed fruits and vegetables are more susceptible to microbial contamination and proliferation, due to procedures such as cutting and peeling that can damage their outer natural protection, release plant juices favoring microbial growth (Abadias et al., 2008). Additionally spoilage bacteria, including several species of Pseudomonas and mesophilic organisms (e.g., lactic acid bacteria and *Enterobacteriaceae*) may be present in fresh produce and their activity (i.e., production of enzymes with lytic activity) may lead to great losses of quality, due to texture break down, or release of off-odors (Remenant et al., 2015). Vegetables have also been considered as vehicles of foodborne pathogen including enterohaemorrhagic strains of *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp. and *Campylobacter* spp. (Park et al., 2012). These pathogens may contaminate vegetables throughout the food chain either pre- (water, soil, manure, insects, handling) and/or post-harvest (water, peeling, cutting, packaging, handling) (EFSA, 2011).

Consumption of fresh produce and minimally processed fruits and vegetables has been linked to an increasing number of outbreaks of foodborne illnesses (Jeddi et al., 2014). It is noteworthy that the presence of extended spectrum β-lactamase (ESBL) - producing bacteria, especially from *Enterobacteriaceae* family (*E. coli* and *Klebsiella pneumoniae*) have been reported in leafy vegetables (Blaak et al., 2014; Kim et al., 2015). It is thus important to monitor the microbiological quality of fresh-cut packaged salads.

Health Protection Agency (HPA; United Kingdom) has given some guidelines regarding the assessment of the microbiological safety of ready-to-eat foods. According to these guidelines total aerobic counts of ready-to-eat salads usually range between 6 and 8 log cfu/g; *Enterobacteriaceae* counts are separated in three categories (> 4 log cfu/ g: unsatisfactory, 2–4 log cfu/g: borderline and<2 log cfu/g: satisfactory) and similarly *E. coli* presence is categorized (> 2 log cfu/g: unsatisfactory, 1.3–2 log cfu/g: borderline and<1.3 log cfu/g: satisfactory) (HPA, 2009).

Previous microbiological surveys of ready-to eat salads have investigated the presence of total aerobic counts, coliforms, *E. coli*, *Salmonella* spp., *Listeria* spp., yeasts and molds (Tournas, 2005; Caponigro et al., 2010; Santos et al., 2012; Gurler et al., 2015; Losio et al., 2015). The occurrence of

main foodborne pathogens such as *E. coli* O157:H7, *Listeria* spp. and *Salmonella* spp. has only been occasionally reported (Jeddi et al., 2014). Despite the extensive focus on hygiene and safety of fresh cut salads, to our knowledge, there are a few studies that have related the variability in the microbial load of ready-to- eat salads with their visual quality and % CO_2 production, which directly affect the acceptability of the products by the consumers (Caponigro et al., 2010; Santos et al., 2012; Nousiainen et al., 2016). For instance, Caponigro et al. (2010) studied the microbial populations and visual quality of ready-to-eat salads in Italy. Another study conducted in Portugal by Santos et al. (2012) assessed the microbial quality and sensory attributes (differences in taste) of ready-to-eat salads; while Nousiainen et al. (2016) studied the bacterial quality and safety as well as the O_2/CO_2 composition of ready-to-eat salads in Finland. Moreover, none of them have studied the possible correlations between the microbial load, quality attributes and plant tissue response to stress (both biotic and abiotic), including the non-proper handling and aversive storage conditions of ready-to-eat salads.

The aims of this study were: (i) to assess the variability in the microbiological quality of ready-to-eat salads in Cyprus, capturing the combined effect of collecting season, salad producer and type of salad and (ii) to assess the correlation between microbial load, antioxidant activity and respiration (in terms of % CO_2 production) of the samples tested. Furthermore, the possible relation between the antioxidant activity of salads and their microbial load will be investigated. The second part of this chapter aims to evaluate the effects of expiring date (OR "estimated expiring day") on microbial load and plant associated parameters (phenolic content, antioxidants, CO_2 production, damage indexes) of samples collected during two seasons.

3.2. Experiment 1. Variation of microbial load, visual quality and antioxidative capacity of ready-to-eat salads by vegetable type, season, and producer

3.2.1. Materials and methods

3.2.1.1. Sampling

In this study, a total of 216 samples (ready-to-eat salads) were randomly collected from different supermarkets in four cities of Cyprus (Larnaca, Limassol, Nicosia, Paphos) during one year period, in four sampling periods, namely autumn (October–November 2016), winter (January-February 2017), spring (April-May 2017) and summer (July- August 2017). For each period, samples were collected once a week, transported to the laboratory within 2 h, in a cool box, and stored at 7 °C for further analysis. Samples were examined for their production of CO_2 due to the respiration process, total phenolic content and antioxidant activity as well as for the presence of *Listeria* spp. and *Salmonella* spp. and the levels of the following microbial groups: total viable (aerobic) count (TVC), *Enterobacteriaceae*, coliforms, *E. coli*, *Staphylococcus* spp., *B. cereus*, lactic acid bacteria, *Pseudomonas* spp., yeasts and molds.

In each sample, appropriate amount of fresh tissue for microbiological analysis (*Salmonella* spp., *Listeria* spp. and others) and extraction of polyphenols and antioxidants was collected and stored at −20 °C (**Figure 3.S1**). The majority of salad producers used modified atmosphere packaging (MAP) with single-layer orientated polypropylene (OPP) or double-layer polyethelene (PE) material. Fresh produce sanitation was taken place during the washing steps with chlorine-based products (~2–3 ppm of free chlorine on the washing water), while treatment with sanitation prior to processing was not a common application.

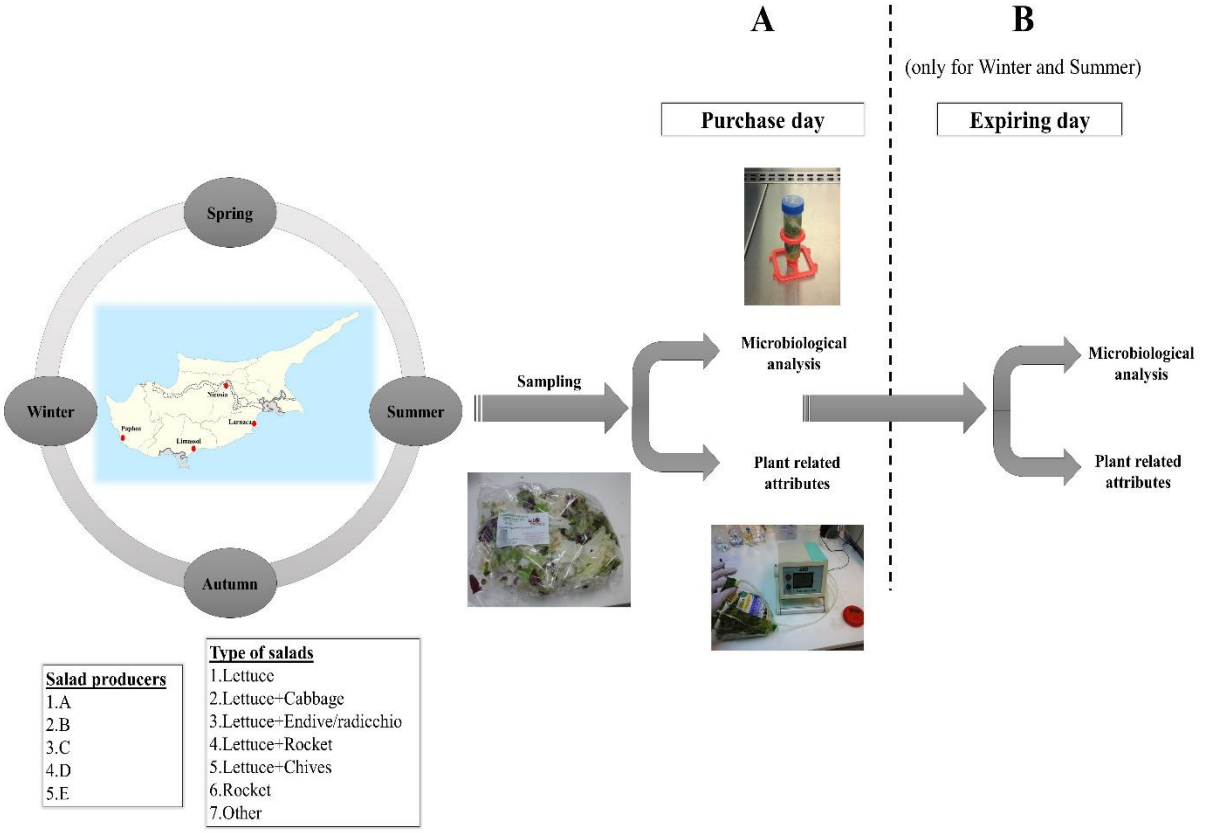


Figure 3.1. Experimental layout.

3.2.1.2. Microbiological analyses

Microbiological assessment of each sample was performed as previously described in section **2.2.1**. Briefly, 1 g of salad (the sampling weight was based on preliminary tests and previous reports of Xylia et al., 2017) was homogenized in stomacher for 1 min with 9 mL of MRD and after serial decimal dilutions were plated on each respective medium (sections **2.2.1.1**-**2.2.1.8**). ESBL producing *E. coli isolates* were identified as mentioned at section **2.2.1.3**.

Assessment of the microbiological safety of ready-to-eat salads were made according to HPA guidelines (HPA, 2009) while total aerobic counts were further categorized in three levels as i)

satisfactory (< 6 log cfu/g), ii) borderline (6–8 log cfu/g; based on HPA 2009) and iii) unsatisfactory (> 8 log cfu/g).

3.2.1.2.1. Isolation and identification of *Salmonella* spp. and *Listeria* spp.

For the isolation of *Salmonella* spp. and *Listeria* spp., 5 g of each sample were analyzed as described at sections **2.2.1.9** and **2.2.1.10**.

3.2.1.2.2. Molecular confirmation of *Salmonella enterica* and *Listeria monocytogenes*

DNA extraction and molecular confirmation of *S. entrerica* and *L. monocytogenes* from *Salmonella* spp. and *Listeria* spp. isolates were performed as mentioned at section **2.2.1.11 (Figure 3.S2-3S3)**.

3.2.1.3. Respiration production

The procedure for the determination of the metabolic respiration process of salads was performed as previously mentioned at section **2.2.3**, for 60 s. Results were expressed as percentage of CO_2 production (% CO_2) (the outcome of the initially flushed CO_2 and that produced by respiration) **Figure 3.S1**.

3.2.1.4. Polyphenol content and antioxidant activity of ready-to-eat salads

Pooled plant tissue (1 g) from each sample was homogenized with 10 mL 50% (v/v) methanol and extraction procedure was carried out as described at section **2.2.8.1**. Total phenols were determined with the Folin-Ciocalteu method (section **2.2.8.2**) and results were expressed as equivalents of gallic acid (Scharlau, Spain) per g of fresh weight (mg of GAE/g Fw). The antioxidant activity of each sample was evaluated with three different methods as 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay, ferric reducing antioxidant power (FRAP) assay and 2.2′-azinobis- (ethylbenzothiazoline-6-sulfonic acid) radical cation discoloration (ABTS) assay. The ability of the samples to scavenge the DPPH (Sigma-Aldrich, Germany) radical was evaluated as previously described at section **2.2.8.3** and results were expressed as mg of trolox per g of fresh weight (mg of trolox/g Fw).The capacity of the extracts to reduce Fe^{3+} was evaluated by measuring the absorbance of the $[Fe\ (TPTZ)^{2}]^{2+}$ complex at 593 nm, as previously described at section **2.2.8.4** and results were expressed as mg of trolox per g of fresh weight (mg of trolox/g Fw). The capacity of the extracts to scavenge the ABTS radical was determined according to procedure presented in section **2.2.8.5** and results were expressed as mg of trolox per g of fresh tissue (mg of trolox/g Fw).

3.2.1.5. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics version 25 where the effect of season, salad producer and type of salad as well as their interactions on the phenolic content, antioxidant activity and % CO_2 production of samples was assessed with three-way ANOVA. Data means were also compared with one-way analysis of variance (ANOVA) and Tukey's multiple range tests were calculated for the significant data $P < 0.05$. All measurements were done in duplicates. The confidence intervals (CI) for the proportions were also calculated using the adjusted Wald confidence interval (Agresti and Coull, 1998). The association between total viable counts and the presence of other microorganisms tested was assessed by Pearson's correlation coefficient (Cohen, 1988). Differences between positive and negative samples were analyzed by the independent-samples t-test and the magnitude of size effect was evaluated based on Eta squared (η^2) (Cohen, 1988).

3.2.2. Results

3.2.2.1. Microbiological analysis

3.2.2.1.1. Variability in microbial load associated with sampling period

Samples collected during spring were found to have higher microbial load, in most of the microorganisms, tested compared to samples from other seasons. Total viable counts ranged between 5.12 and 9.75 log cfu/g with an average of 7.73 log cfu/g (**Figure 3.2**). The majority of the samples 113 out of 216 (52.31%) had a viable count between 6 and 8 log cfu/g, which categorizes them as borderline, according to HPA guidelines (see **Figure 3.2**). Spring showed the highest value (8.18 log cfu/ g). During autumn, 7 out of 72 samples (9.72%) were characterized as satisfactory (< 6 log cfu/g), whereas in summer 53 out of 72 samples (73.61%) were characterized as unsatisfactory (> 8 log cfu/g). Spring also showed the highest value for *Enterobacteriaceae* (7.05 log cfu/g). Only a small fraction of the samples falls into the satisfactory category (< 2 log cfu/g) and these were observed only during autumn and winter (1.38 and 2.77%, respectively).

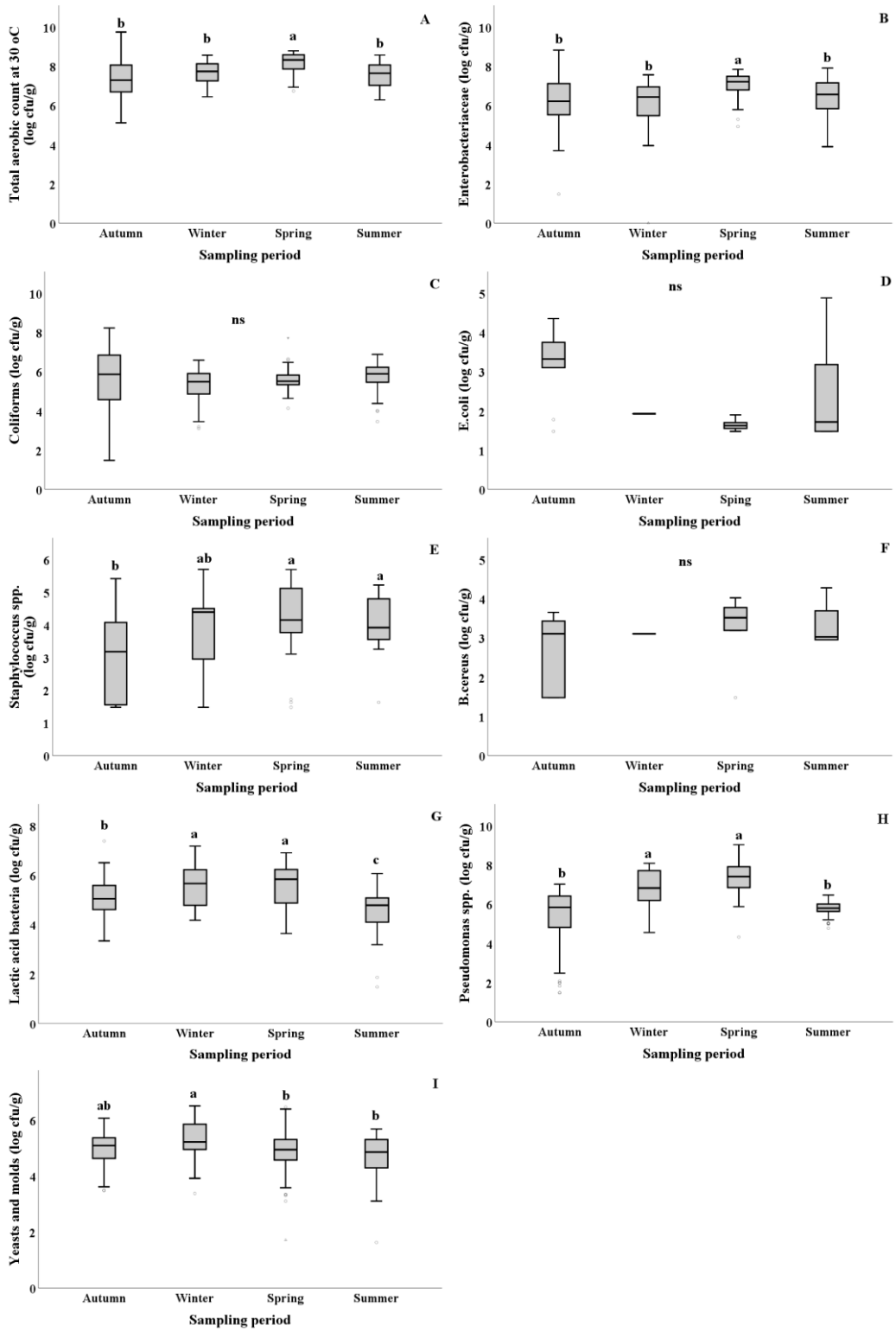


Figure 3.2. Microbiological quality of ready to eat salads collected during one year period from supermarkets in Cyprus as affected by season. Results include only positive samples for each microorganism tested and are the mean value ± standard deviation. Each box contains 50 per cent of cases and whiskers represent the rest. The line across the inside of the box represents the median value.

The majority of samples, 191 out of 216 (88.43%) were negative for *E. coli*, whereas counts for positive samples (11.57%; 95% CI: 7.91–16.6%) ranged between 1.48 and 4.88 log cfu/g. A total of 15 samples (6.94%), were characterized as borderline (cfu/g range: 1.3–2 log), whereas 10 (4.63%) were

unsatisfactory, with cfu/g numbers exceeding 2 log. When comparing seasons, the highest average value (3.17 log cfu/g) was recorded during autumn. All positive *E coli* samples were tested for antibiotic resistance. ESBL testing on the positive *E. coli* isolates revealed that five samples (2.62%) were positive with three of them were isolated during summer and two over spring.

The lowest value for *Staphylococcus* spp. was recorded in autumn (3.01 log cfu/g) and the highest in spring and summer (4.21 and 4.02 log cfu/g, respectively). Twenty samples (9.26%) were found positive for *B. cereus* and with values ranging from 1.48 to 4.28 log cfu/g. Positive samples were found in all seasons [9 (12.5%), 1 (2.78%), 6 (8.33%) and 4 (11.11%) in autumn, winter, spring and summer, respectively]. Additionally, lactic acid bacteria were present in 198 samples (91.67%) and values ranged between 1.48 and 7.39 log cfu/g. The lowest average value was observed during summer (4.52 log cfu/ g), whereas the highest values during winter and spring (5.61 and 5.66 log cfu/g, respectively). The presence of *Pseudomonas* spp. was observed in 165 samples (76.39%) and values ranged between 1.48 and 9.04, log cfu/g. The lowest values were observed during autumn and summer (5.15 and 5.76 log cfu/g, respectively), whereas the highest values during the winter and spring (6.85and 7.34 log cfu/g). Yeasts and molds were present in 197 samples (91.20%) and values ranged between 1.63 and 6.68 log cfu/g. The lowest values were observed during summer and spring (4.66 and 4.87 log cfu/g, respectively), whereas the highest value during winter (5.30 log cfu/g).

The majority of samples, 212 out of 216 (98.15%) were found negative for *Salmonella* spp. via the enrichment and culture-based steps of the detection, whereas four samples (collected during autumn) which were showed presumptive positive colonies after enrichment were negative for *S. enterica*, according to the PCR-tested samples for the presence of *inv*A gene. The presence of *L. monocytogenes* was detected in eight samples (3.70%; 95% CI: 1.8–7.3%) that were collected during spring.

3.2.2.1.2. Variability in microbial load associated with salad producer

The highest average value for total viable count was observed in samples from producer B (8.32 log cfu/g), whereas samples from producer E showed the lowest TVC levels (7.10 log cfu/g). *Enterobacteriaceae* family counts ranged between 1.48 and 8.82 log cfu/ g, whereas only one sample (combination of lettuce with two or more ingredients) was found negative and came from salad producer A on the second sampling period. Totally 208 out of 216 (96.30%) samples were characterized as unsatisfactory (> 4 log cfu/g), according to HPA guidelines (HPA, 2009). The lowest average value was observed by salad producer E (5.46 log cfu/g), whereas salad producer B showed the highest (7.27 log cfu/g) levels in their samples (**Figure 3.3**). The majority of samples tested (207 out of 216) (95.8%) were also positive for coliforms. During spring, the lowest value for coliforms was observed in samples by salad producer E (4.63 log cfu/g), whereas samples of salad producer B showed the highest (6.47 log cfu/g) value. Five out of the nine negative samples (55.56%) that did not harbor coliforms came

from salad producer A, whereas the rest came from producer D (4 samples). Additionally, the nine negative samples were found in autumn (7 samples) and in winter (2 samples) sampling period.

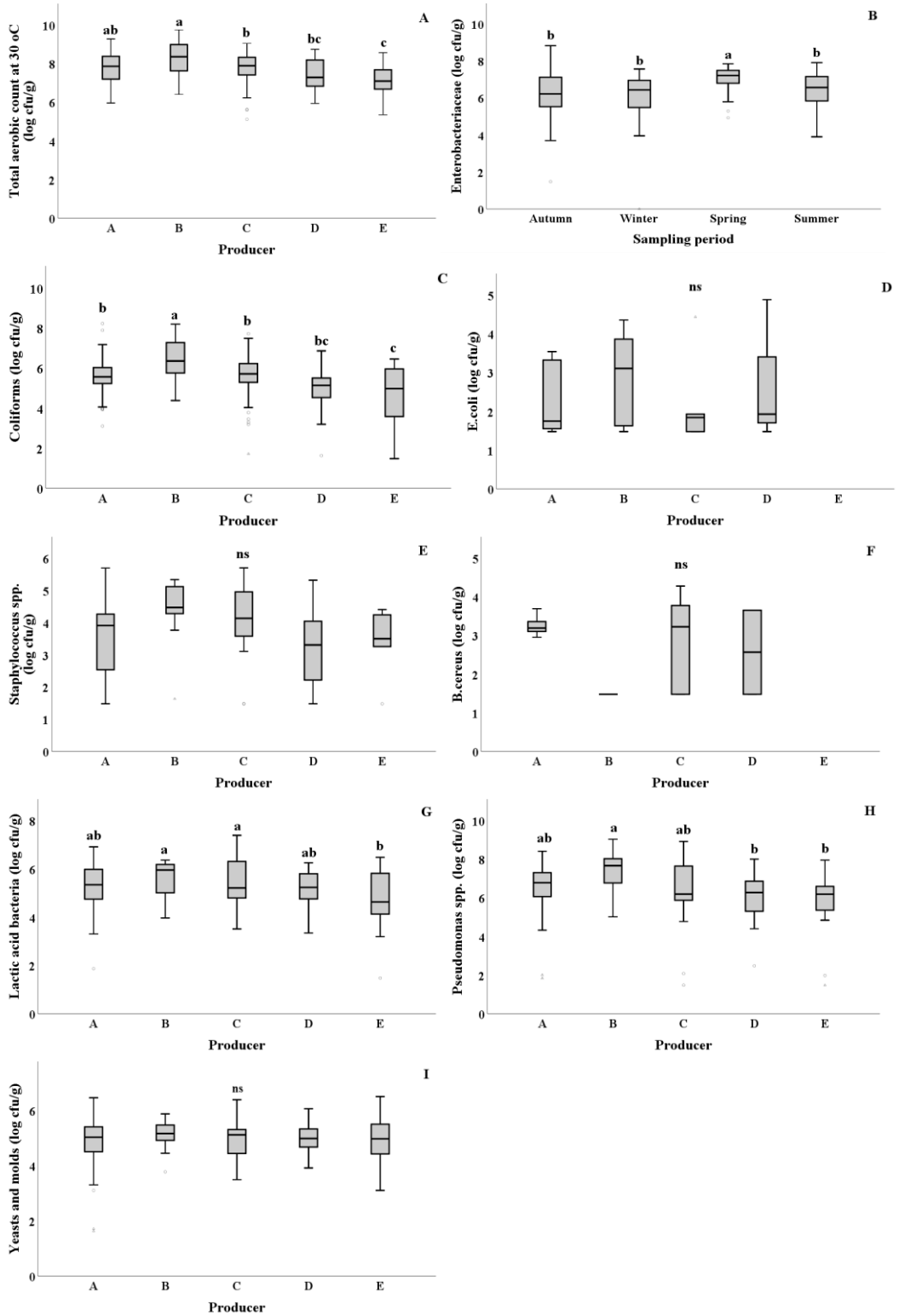


Figure 3.3. Effect of salad producer on the microbiological quality of ready to eat salads collected during one year period from supermarkets in Cyprus. Results include only positive samples for each microorganism tested and are the mean value ± standard deviation. Each box contains 50 per cent of cases and whiskers represent the rest. The line across the inside of the box represents the median value.

Salad producer E was the only one with negative for *E. coli* samples throughout the four sampling periods. *Staphylococcus* spp. was present in almost half of the samples (105 out of 216) (48.11%; 95% CI: 42.03–55.24%) and *S. aureus* was isolated from only two samples (0.93%; 95% CI: 0.04–3.53%) (1.48 log cfu/g each) that came from salad producer A (plain rocket) and C (combination of lettuce and chives). Only salad producer E did not had any positive samples for *B. cereus*, whereas samples from salad producer A, B, C, D were found positive [7 (7.61%), 1 (4.17%), 10 (17.86%) and 2 (7.69%), respectively]. Furthermore, among salad producers, producer E had the lowest average value for lactic acid bacteria counts and producer B the highest (4.67 and 5.63 log cfu/g, respectively). Similarly, salad producer E and D had the lowest values for *Pseudomonas* spp. (5.63 and 6.09 log cfu/g, respectively), whereas producer B the highest (7.36 log cfu/g). No significant difference was observed among yeasts and molds counts of the tested samples from the five salad producers (P=0.831). Samples found positive for *L. monocytogenes* derived from salad producer C (2.78%), A and D (0.46% and 0.46%, respectively).

3.2.2.1.3. Variability in microbial load associated with the type of salad

Salads with combination of lettuce+two or more ingredients were found to have the highest average counts of *E. coli* (3.37 log cfu/g) (**Figure 3.4**). Additionally, lettuce+cabbage had the highest value for *Staphylococcus* spp. (5.22 log cfu/g) among the different types of salad. The combination of lettuce+rocket had the lowest average value for *Pseudomonas* spp. (5.35 log cfu/g), contrary to lettuce+cabbage or lettuce+endive/radicchio which showed the highest levels (i.e., 6.70 and 6.71 log cfu/g, respectively). No significant differences between types of salads was observed for total viable counts (P=0.277), *Enterobacteriaceae* (P=0.235), coliforms (P=0.189), lactic acid bacteria (P=0.405) and yeasts and molds counts (P=0.218). The majority of samples positive for *L. monocytogenes* came from salads containing plain lettuce and lettuce+cabbage (1.38% and 0.92%, respectively).

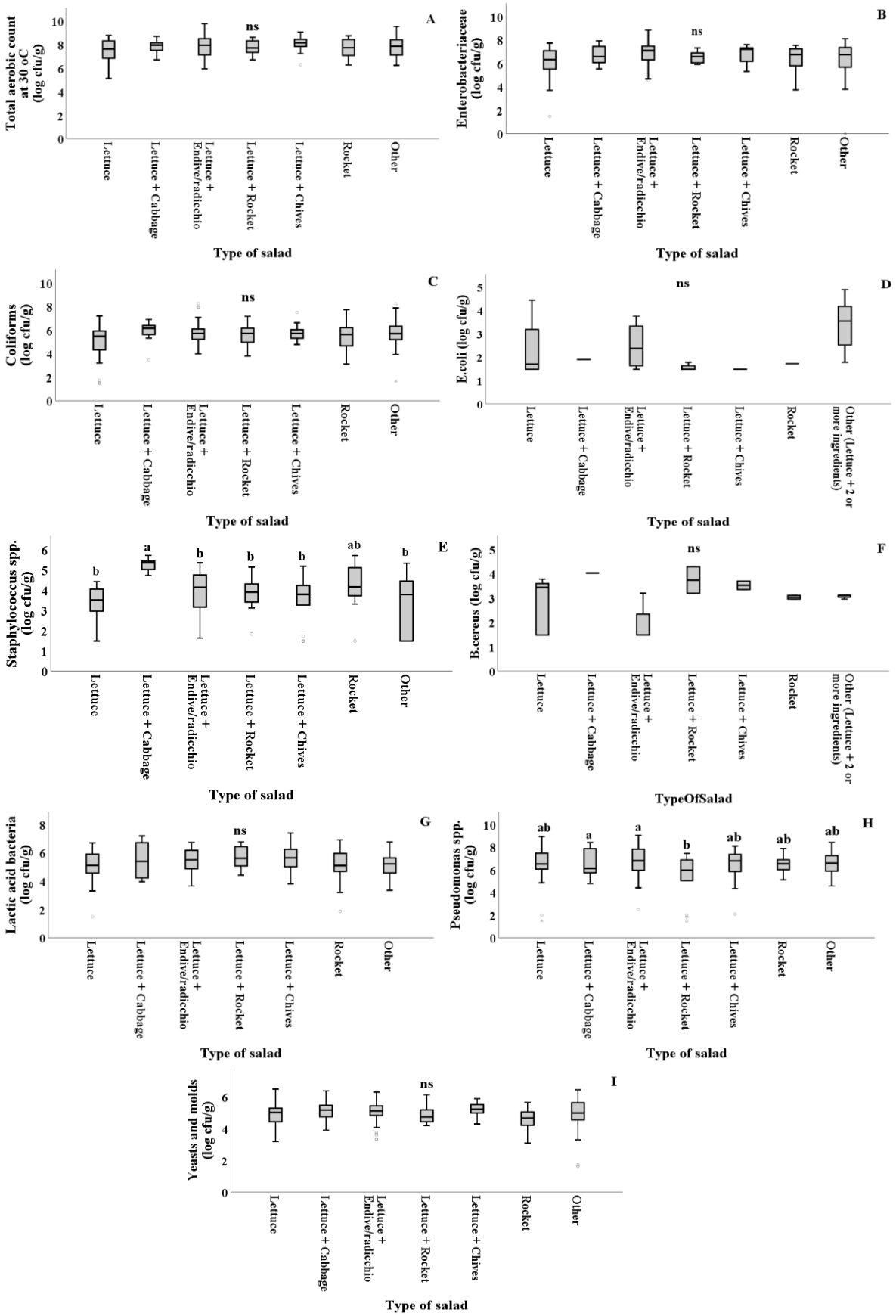


Figure 3.4. Microbiological quality of different types of ready to eat salads collected during one year period from supermarkets in Cyprus. Results include only positive samples for each microorganism tested and are the mean value ± standard deviation. Other = Lettuce +2 or more ingredients. Each box contains 50 per cent of cases and whiskers represent the rest. The line across the inside of the box represents the median value.

3.2.2.2. Total phenolic content, antioxidants, and CO_2 production

3.2.2.2.1. Sampling period

Total phenolic content ranged between 0.73 and 1.40 mg GAE/g Fw (**Table 3.1**). Among sampling periods, the highest value was observed during autumn (1.40 mg GAE/g Fw), whereas spring and summer showed the lowest values (1.04 and 1.05 mg GAE/g Fw, respectively). The antioxidant levels of the samples were tested with three different assays (DPPH, FRAP and ABTS). All three assays showed that the lowest value was observed during summer ($P < 0.05$). The production of CO_2 ranged between 0.25 and 20.49%. Fresh cut salad respiration was increased (8.22% CO_2) during spring whereas autumn and winter showed the lowest values (4.79 and 5.10% of CO_2, respectively).

3.2.2.2.2. Salad producer

The content of phenolics was varied among producers as the highest values were observed by salad producer E and A (1.33 and 1.34 mg GAE/g Fw, respectively) and the lowest by salad producer C (0.85 mg GAE/g Fw) (**Table 3.1**). All three antioxidant assays showed that the lowest antioxidant levels were observed by salad producer C ($P < 0.05$). Among salad producers, producer E had the lowest % CO_2 value (3.46%) and producer B the highest (9.83%). No differences were found on CO_2 production among producers A, E, and D.

3.2.2.2.3. Type of salad

Salads with rocket, lettuce and lettuce+two or more ingredients were found to have the highest phenolic content (1.40 and 1.37 mg GAE/g Fw, respectively), whereas lettuce+cabbage revealed the lowest (0.73 mg GAE/g Fw) (**Table 3.1**). Low antioxidant capacity was found (by FRAP and ABTS assays) in salads with lettuce+cabbage combination, whereas lettuce+two or more ingredients had the highest ($P < 0.05$) antioxidants. Salads with only rocket or lettuce showed the lowest % CO_2 values (4.10 and 4.73%, respectively), whereas lettuce+cabbage had the highest value (13.73%).

Three way ANOVA analysis shown in **Table 3.1** revealed that sampling period affected the antioxidant activity (DPPH and ABTS) of salads ($P < 0.001$) and FRAP ($P < 0.01$), whereas phenols and % CO_2 production were not significantly affected ($P \geq 0.05$). Salad producer significantly affected FRAP, ABTS ($P < 0.001$), DPPH and phenols ($P < 0.01$); however, % CO_2 was not significantly affected ($P \geq 0.05$). The % CO_2 production was significantly ($P < 0.001$) affected by the type of salad. Phenols, DPPH ($P < 0.01$), FRAP and ABTS ($P < 0.05$) were also affected. Total phenolic content and antioxidant activity (DPPH, FRAP and ABTS) were significantly ($P < 0.001$) impacted by the interaction of producer*salad. The interactions of period*producer and period*salad did not significantly ($P \geq 0.05$) affect total phenols, antioxidant activity (DPPH, FRAP and ABTS) or % CO_2

production of salads. Finally, the interaction of period*producer*salad only influenced ABTS ($P < 0.05$).

Table 3.1. Effect of sampling period, salad producer and type on total phenolic content (mg GAE/g Fw), antioxidants (mg trolox/g Fw) and %CO_2 of ready to eat salads collected during one year period from supermarkets in Cyprus.

	Phenols	**DPPH**	**FRAP**	**ABTS**	**% CO_2**
Sampling period					
Autumn	1.40 ± 0.74 a	1.56 ± 0.92 a	0.97 ± 0.74 a	1.31 ± 0.53 a	4.79 ± 1.98 b
Winter	1.14 ± 0.59 ab	1.56 ± 0.44 a	0.72 ± 0.59 ab	0.58 ± 0.22 b	5.10 ± 3.85 b
Spring	1.04 ± 0.52 b	1.45 ± 0.69 a	0.46 ± 0.37 bc	1.39 ± 0.38 a	8.22 ± 4.19 a
Summer	1.05 ± 0.65 b	0.42 ± 0.37 b	0.33 ± 0.39 c	0.54 ± 0.26 b	6.40 ± 4.39 ab
Salad producer					
A	1.34 ± 0.75 a	1.55 ± 0.83 a	0.80 ± 0.75 a	1.18 ± 0.56 ab	5.91 ± 3.91 bc
B	1.26 ± 0.54 ab	1.58 ± 0.97 a	0.74 ± 0.48 ab	1.41 ± 0.60 a	9.83 ± 6.34 a
C	0.85 ± 0.27 b	0.88 ± 0.27 b	0.36 ± 0.27 b	0.84 ± 0.41 b	8.19 ± 4.33 ab
D	1.12 ± 0.64 ab	1.33 ± 0.74 a	0.64 ± 0.53 ab	1.03 ± 0.50 b	4.91 ± 1.78 bc
E	1.33 ± 0.74 a	1.35 ± 0.86 a	0.70 ± 0.57 ab	1.03 ± 0.53 b	3.46 ± 1.92 c
Type of salad					
Lettuce	1.40 ± 0.63 a	1.83 ± 0.75 a	0.80 ± 0.54 a	1.16 ± 0.60 ab	4.73 ± 3.21 c
Lettuce+Cabbage	0.73 ± 0.28 b	1.11 ± 0.35 a	0.28 ± 0.17 b	0.76 ± 0.43 b	13.73 ± 3.66 a
Lettuce+Endive/radicchio	0.89 ± 0.42 ab	1.22 ± 0.45 a	0.41 ± 0.38 ab	0.94 ± 0.44 ab	7.94 ± 3.62 b
Lettuce+Rocket	1.26 ± 1.14 ab	1.90 ± 1.63 a	0.83 ± 1.32 a	0.94 ± 0.59 ab	5.68 ± 2.73 bc
Lettuce+Chives	0.86 ± 0.25 ab	1.12 ± 0.25 a	0.41 ± 0.32 ab	0.99 ± 0.45 ab	7.06 ± 1.85 bc
Rocket	1.35 ± 0.39 a	1.16 ± 0.24 a	0.50 ± 0.25 ab	1.16 ± 0.42 ab	4.10 ± 3.78 c
Other	1.37 ± 0.74 a	1.73 ± 0.79 a	0.92 ± 0.65 a	1.29 ± 0.59 a	5.77 ± 3.35 bc

Three-way Anova	**Phenols**	**DPPH**	**FRAP**	**ABTS**	**% CO_2**
Period	ns	***	**	***	ns
Producer	**	**	***	***	ns
Salad	**	**	*	*	***
Period*Producer	ns	ns	ns	ns	ns
Period*Salad	ns	ns	ns	ns	ns
Producer*Salad	***	***	***	***	ns
Period*Producer*Salad	ns	ns	ns	*	ns

Results shown are the mean value ± standard deviation and values followed by the same letter in each column are not significantly different ($P \geq 0.05$). .ns, *, **, and *** indicate non-significant or significant differences at $P \leq$ 5%, 1% and 0.1%, respectively, following two-way ANOVA. Other= Lettuce +2 or more ingredients.

3.2.3. Discussion

Safety and quality of fresh produce are of great concern as both are demanded by the food industry and the consumers. To our knowledge there is limited information regarding the microbiological safety of ready-to-eat salads with leafy vegetables in Cyprus. In a previous study by Eleftheriadou et al. (2002)

it was observed that 1.70% and 27.21% of mixed salads and vegetables in Cyprus were found positive for the presence of *B. cereus* (> 4 log cfu/g), *E. coli* (> 2 log cfu/g) and *L. monocytogenes*. The survival of *L. monocytogenes* and *Salmonella enterica* in fresh-cut salads even in low numbers has been previously mentioned by Manios et al. (2013).

Our findings regarding TVC are in accordance with several studies that have investigated the aerobic microbial counts of leafy vegetables, which ranged between 3 and 8 log cfu/g (Ailes et al., 2008; Korir et al., 2016; Nousiainen et al., 2016). However, high TVC counts (> 6 log cfu/g) do not necessarily indicate low microbiological safety of food, as it may be due to harmless (e.g., spoilage) epiphytic (predominant) microorganisms (Faour-Klingbeil et al., 2016a). Furthermore, high *Enterobacteriaceae* and coliforms levels in ready-to-eat salads have been linked with the preharvest application of untreated manure and immoderate postharvest handling (Faour-Klingbeil et al., 2016b). For instance, Jeddi et al. (2014) have mentioned coliforms levels ranging between 1.9 and 6.0 log cfu/g with the majority of fresh-cut vegetables having > 5 log cfu/g. It is worth mentioning that increased numbers of coliforms and *E. coli* in leafy vegetables from field to the marked has been observed in another study by Faour-Klingbeil et al. (2016b). It has been previously mentioned the environmental contamination with the use of untreated manure, contaminated water or soil during preharvest, as well as the excessive handling during postharvest might result to high loads of coliforms in ready-to-eat vegetables (Aycicek et al., 2006). Therefore, this confirms the possibility of contamination with faecal matter throughout the food supply or microbial proliferation due to improper chilling or inappropriate packaging conditions (e.g., failure in flushing or maintenance of a modified atmosphere in the package). Nousiainen et al. (2016) reported a positive correlation (Pearson correlation; r=0.661, $P < 0.001$) between TVC and coliforms counts and this is also evidence in our results with r=0.583 and $P < 0.001$. Coliforms have been used as sanitary indicators in foods for their presence in high numbers (higher than most foodborne pathogens) and their relatively quick and easy identification (HPA, 2009). However, coliforms presence in ready-to-eat salads may vary along the different processing steps and the end product (Faour-Klingbeil et al., 2016a). This complicates the efforts for obtaining useful information about the contamination occurrence and the adequacy of processing fresh produce for ready-to-eat salads production.

It has been mentioned in previous studies that the prevalence of *E. coli* on leafy green vegetables can be below 5% (Sagoo et al., 2003; Ailes et al., 2008; Santos et al., 2012; Cardamone et al., 2015), whereas some others reported levels around 15% and higher (de Oliveira et al., 2011; Campos et al., 2013; Nousiainen et al., 2016). According to our findings *E. coli* was found in 11.57% of samples and these are in accordance with a study by Abadias et al. (2008) that mentioned the presence of *E. coli* on 11.4% of fresh-cut vegetables including rocket, carrot, corn salad, endive, lettuce, spinach and mixed salads. The highest *E. coli* levels in our study were observed during autumn similarly to the results of another study in Italy where *E. coli* was detected in 27% of ready-to-eat salads and high levels were reported in autumn for both lettuce and arugula (Caponigro et al., 2010). In that study, Caponigro et al.

(2010) reported the short periods of concentrated rain often happen in autumn in Italy. This phenomenon is also observed in Cyprus as in other Mediterranean countries resulting to high moisture levels in field. Additionally, relatively high temperatures for the season are also common in Cyprus and thus the presence of *E. coli* on fresh produce might be favored.

The assessment of *E. coli* isolates in our study showed that 2.62% of samples harbored ESBL resistant genes and this has also been reported by Chau et al. (2017) in a microbiological survey of ready-to-eat salads in Singapore, where the corresponding values were of 2.3%. Additionally, Campos et al. (2013) have isolated *β*-lactamase producing bacteria (other than *E. coli*) from ready-to-eat salads in Portugal including *Rahnella aquatilis*, *Citrobacter freundii*, *Raoultella terrigena*, *Hafnia alvei*, *Enterobacter cloacae*, *Enterobacter aerogenes* and *Cronobacter sakazakii*. It is worth mentioning that in a study in Switzerland, 5% of ready-to-eat salads were found to harbor *β*-lactamase producing *Enterobacteriaceae* and environmental bacteria (minor ESBLs) and this suggests the possible presence of these bacteria throughout the food supply chain (Nüesch-Inderbinen et al., 2015). These findings suggest that food safety in Cyprus is within ranges found in developed countries as Portugal and Switzerland.

Survival and growth of *L. monocytogenes* and *Salmonella* spp. in RTE salads is influenced by storage and packaging conditions and one of the most important factors is storage temperature (Nousiainen et al., 2016). It has been shown that *L. monocytogenes* can survive and multiply at 7 and 15 °C on leafy vegetables (Sant'Ana et al., 2012a). Storage of fresh produce at abusive conditions i.e. temperatures above 4 °C have shown increased microbial growth and metabolic activities of plant tissue leading to pathogen growth and food spoilage (Caleb et al., 2013). Furthermore Poimenidou et al. (2016) found that antimicrobial treatments might not be effective against *L. monocytogenes* cells once fresh produce (i.e. cherry tomatoes and lettuce leaves) has been contaminated with cells incubated at low temperatures (5 °C) as the bacterium cells might be able to resist and survive the treatments applied after fresh produce contamination. In our study, 3.7% of samples stored at 7 °C were found to harbor *L. monocytogenes* and these results resemble those of other studies conducted in the UK and Brazil with prevalence of *L. monocytogenes* of 4.8% and 3.1%, respectively (Little et al., 2007; Sant'Ana et al., 2012b). On the other hand, other studies have reported lower (0.66%) or higher (6%) prevalence of *L. monocytogenes* in ready-to- eat salads (Santos et al., 2012; Ssemanda et al., 2017). However, it should be noted that as it has been mentioned by Angelidis et al. (2015) not all *Listeria* spp. isolates might actually be *Listeria* spp. and thus further molecular identification is essential.

Caponigro et al. (2010) reported the presence of *Salmonella* spp. in postharvest washing areas (6.7%) suggesting possible contamination of fresh produce during processing, whereas similar (5%) and lower (0.3%) prevalence has also been mentioned by others (Jeddi et al., 2014; Losio et al., 2015). Our results showed the absence of *S. enterica* which is in accordance with HPA guidelines for ready-to-eat food (HPA, 2009). These findings imply that improved preventive measures have been taken by the food

industry in Cyprus regarding this pathogen. This is further supported by a previous study of Eleftheriadou et al. (2002) who reported 0.68% prevalence of *Salmonella* spp. in mixed salads and vegetables. *L. monocytogenes* was found on some vegetable samples due to possible mishandling or contamination pre (irrigation water, soil, handling) and/or postharvest (handling, cutting, prior washing) and since it is able to survive at low temperatures (4 °C) during cold storage. As it has been previously mentioned, the disinfectants used in the food industry (mainly sodium hypochloride) are not able, in the currently used concentrations, to completely remove all pathogen and spoilage microorganisms from fresh produce (Losio et al., 2015). It is of great significance that the vegetable industry implement and control Listeria inside processing plants as RTE salads are widely consumed and vulnerable groups (children, pregnant women and elderly) might be exposed to great risk as mortality rate for these groups is quite high and ranges from 20 to 30% (Farber and Peterkin, 1991).

The correlation of microbial load of vegetables and the antioxidant activity of plant tissue is not commonly studied. However, it should be considered and investigated further as it will possibly give insights on how the plant tissue reacts to the presence of foodborne pathogens as well as spoilage microorganisms and prevents or enhances bacterial growth. The correlation matrix of microbial and physiological responses is presented in **Figure 3.5**. Phenols and DPPH are positive correlated with *Staphylococcus* spp., *Pseudomonas* spp., *E. coli*, and *B. cereus*. This may suggest that excessive handling increases microbial load as well as plant stress. However, the above microbes are negatively correlated to the ABTS antioxidant activity. It should be mentioned that several Pseudomonas have been associated with spoilage. For example, the presence of *P. chlororaphis* on lettuce, onions, potatoes, and carrots may lead to plant cell wall degradation and inevitably in spoilage of fresh produce (Lee et al., 2013). This might explain the higher antioxidant levels and phenolic content as a part of plant defense mechanisms against biotic stress. Furthermore, it can be assumed that the presence of *B. cereus* and *Pseudomonas* spp. (at high levels) may have contributed to the low prevalence of *L. monocytogenes* and *S. enterica* in our study as it has been previously reported by Santos et al. (2012) that those bacteria antagonize each other.

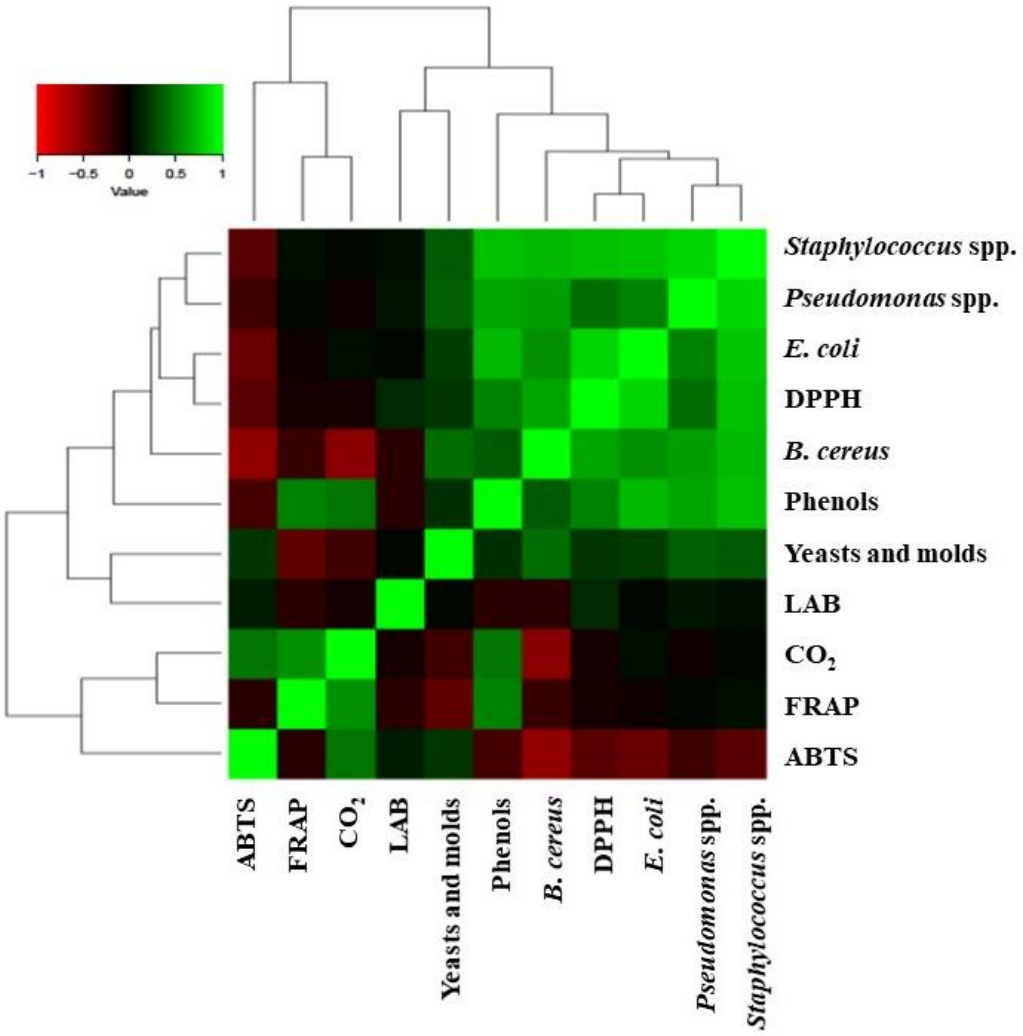


Figure 3.5. Heat-map matrices of the correlation between microbial and physiological responses in fresh cut salads. Each square indicates r (Pearson's correlation coefficient of a pair of responses).

The relative expression in microbial and physiological responses in fresh cut salads was examined in relation to the season, the salad producer (supplier) and type of salads, as presented by heat map in **Figure 3.6**. In spring, *Staphylococcus* spp., *Pseudomonas* spp., LAB, *Enterobactereaceae*, and TVC but also CO_2 and ABTS activity were relatively increased (**Figure 3.6A**). In summer, *E. coli* and coliforms were relatively increased, while LAB, yeasts and molds as well as antioxidants (DPPH, FRAP, ABTS) were decreased. Our findings are in accordance with Caponigro et al. (2010), who also reported LAB in ready-to-eat salads in Italy during spring and summer. Yeasts and molds were relatively increased during winter, whereas antioxidants and phenols were increased in autumn. A decrease of coliforms and *Pseudomonas* spp. and CO_2 was also observed during autumn. Our results are partly confirmed by the observations of Caponigro et al. (2010) where they reported higher average visual quality and lower microbial load in winter and spring compared to summer and autumn.

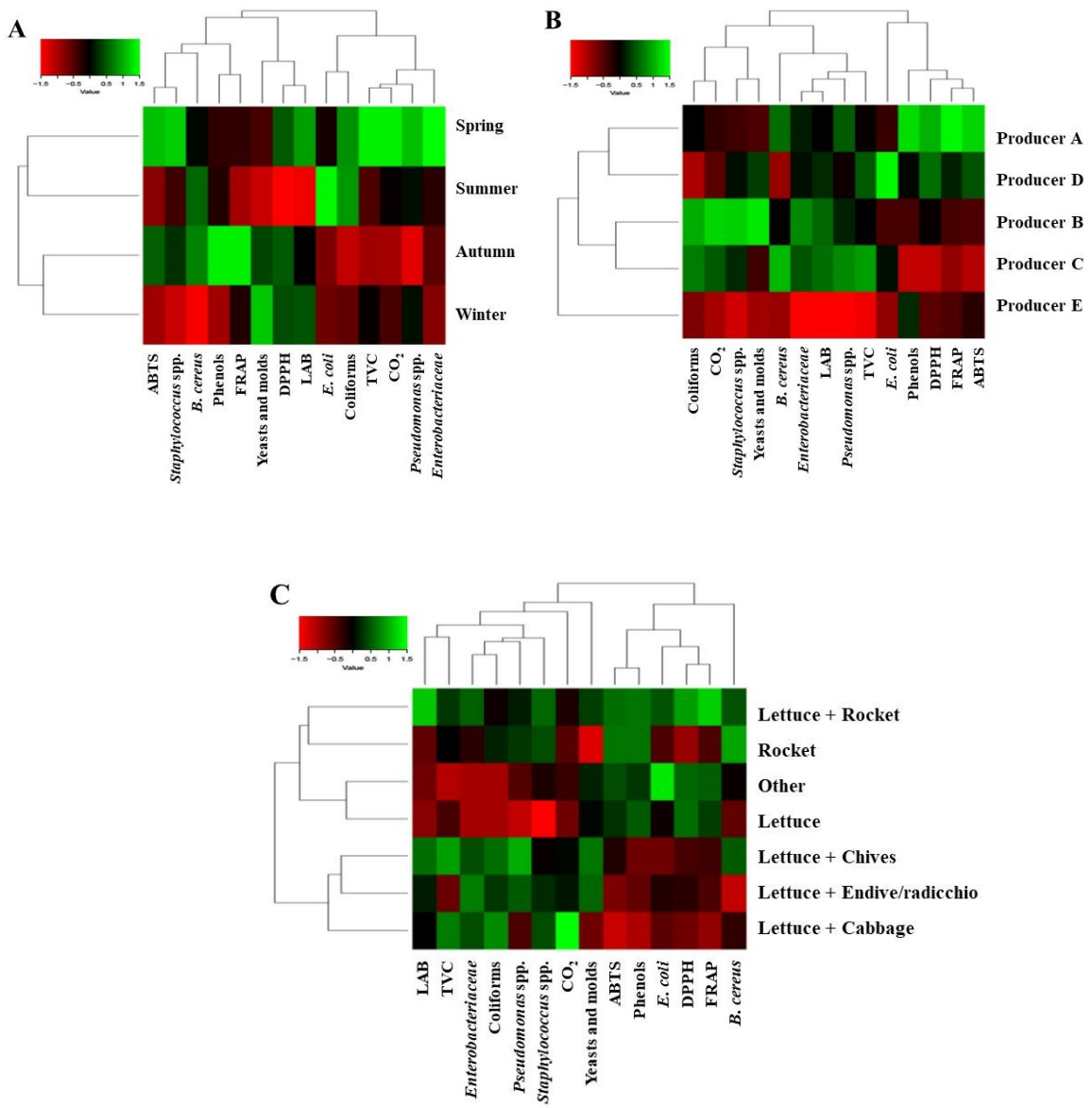


Figure 3.6. Microbial and physiological changes in fresh cut salads. Heat map representing relative expression of responses in (A) season (B) producer/supplier and (C) salad type.

Differences among salad producers might be due to different processing procedures and possible excessive handling (Sant'Ana et al., 2012a). Salad producing factories in Cyprus consist of small and big companies (exports to more than 25 countries) and the current packaging practices include the use of modified packaging atmosphere (some of them), different packaging materials (single-layer OPP and double-layer PE), as well as the use of chlorine-based disinfectants. Samples from producer A were found to have increased antioxidant levels and phenolic content, while samples from producer B had relatively increased CO_2 and microbial load (*Enterobacteriaceae*, coliforms, *Staphylococcus* spp. and yeasts and molds) (**Figure 3.6B**). Producer C samples had relatively increased microbial load (*B. cereus*, *Pseudomonas* spp. and TVC) and low antioxidant levels. *E. coli* was increased in producer's D

samples, whereas samples from producer E had relatively low microbial load, meaning safer produce, and CO_2 production.

The combination of lettuce+rocket had increased microbial load, while yeasts and molds were decreased in plain rocket salads. Low TVC and yeasts and molds counts in rocket salads have also been reported by Spadafora et al. (2016). *E. coli* and antioxidants (DPPH, FRAP) were increased in lettuce+two or more ingredients, whereas TVC, *Enterobacteriaceae* and coliforms were low (**Figure 3.6C**). Samples with plain lettuce had decreased microbial numbers (LAB, TVC, *Enterobacteriaceae*, coliforms, *Pseudomonas* spp. and *Staphylococcus* spp.). Increased microbial load was observed by lettuce+chives (LAB, TVC, *Pseudomonas* spp., coliforms, *Enterobacteriaceae* and yeasts and molds) and lettuce+endive/radicchio (*Enterobacteriaceae*, coliforms, *Staphylococcus* spp. and *Pseudomonas* spp.). The combination of lettuce+cabbage had increased microbial load (TVC, *Enterobacteriaceae*, coliforms, *Staphylococcus* spp.) and CO_2. Nousiainen et al. (2016) reported CO_2 production ranging between 0.10 and 22.20% and these findings are similar to our results (0.20–20.49%) suggesting that microbial load and the type of vegetable affect respiration of salads and may contribute to spoilage.

The study of TVC and *Enterobacteriaceae* as part of the normal microflora of vegetables and their correlation with the presence of pathogens may contribute to the establishment of preventive microbiological criteria and less time consuming analysis of ready-to-eat salads (i.e. 24 h instead of 3–4 days). Furthermore, the combination of these analyses with physicochemical attributes of RTE leafy vegetables might provide useful insights for assessing and preventing microbiological contamination of these products, as well as for preserving their nutritional value.

Microbiological quality and safety and physicochemical attributes of fresh produce are of great importance. The microbiological quality of ready-to-eat salads has been previously studied in many countries e.g. Brazil (de Oliveira et al., 2011), Italy (Cardamone et al., 2015), Portugal (Campos et al., 2013), and United States of America (Korir et al., 2016); whilst a number of them further assessed organoleptic attributes (Caponigro et al., 2010; Santos et al., 2012) or physiological parameters such as O_2/CO_2 packaging atmosphere composition (Nousiainen et al., 2016) of these products. The presence of foodborne pathogens in RTE salads arises consumer safety concerns and the ongoing need for understanding the prevalence and persistence of pathogens in fresh produce, towards better management practices and safety policies development. Future studies are required to fully understand the factors affecting the entrance, presence and survival of foodborne spoilage and pathogenic organisms in ready-to-eat salads, as well as the physiological attributes of vegetables in order to identify the gaps of the food supply chain that allow their establishment in the food industry.

3.3. Experiment 2. Effects of ready-to-eat salads expiring date (OR "estimated expiring day") on their microbial load and biochemical attributes

3.3.1 Materials and methods

3.3.1.1. Sampling

A total of 144 ready-to eat salads (samples) were randomly obtained from retail markets in four cities of Cyprus (Larnaca, Limassol, Nicosia, Paphos) in two sampling periods during any year period: winter (January-February 2017) and summer (July-August 2017). For each period, sampling was performed once a week and the collected samples were transferred in cool boxes to the laboratory within 2 h and immediately stored at a laboratory refrigerator (7 ^{o}C) for further analysis. In order to study the fresh produce susceptibility during storage in both food borne pathogens and preservation/nutritive value, double samples were collected in each season and half of them were directly analyzed as mentioned below, while the other half were stored at 7 ^{o}C until expiration date (as indicated on each package).

Analyses were performed as described above in section **2.2.1** with further determination of plant oxidation indexes (hydrogen peroxide production and lipid peroxidation).

3.3.1.2. Damage index (hydrogen peroxide production, lipid peroxidation)

The estimation of hydrogen peroxide (H_2O_2) production was carried out as described at section **2.2.9.1** and results were expressed as μmol of H_2O_2 per g of fresh weight (μmol H_2O_2/g Fw).

The 2-thiobarbituric acid reactive substances (TBARS) method was performed at 532 and 600 nm for the determination of samples lipid peroxidation (section **2.2.9.2**) and results were expressed as nmol of malondialdeyde (MDA) per g of fresh weight (nmol MDA/g Fw).

3.3.1.3. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics version 25 where the effect of season, salad producer and type of salad on the phenolic content, antioxidant activity, %CO_2 production and damage index of samples was assessed. Data means were compared with one way analysis of variance (ANOVA) and Tukey's multiple range tests were calculated for the significant data $P < 0.05$. All measurements were done in duplicates. Differences between seasons were analyzed by the independent-samples t-test, while paired-samples t-test was performed for the determination of differences among purchase and expiring day.

3.3.2. Results

3.3.2.1. Effects of season

3.3.2.1.1. Microbiological analysis

Salad producer

Significant differences of *E. coli* were reported from salad producers A and C (P = 0.022 and P = 0.001, respectively), where both producers showed higher values during summer (0.32 and 0.68 log cfu/g, respectively) compared to winter (0.00 log cfu/g, both) (**Figure 3.7D** and **Table 3.2**). Higher *B. cereus* values were observed during summer compared to winter for salad producer C (0.39 and 0.00 log cfu/g, respectively) (P = 0.047) (**Figure 3.7F**), whilst salad producer B showed higher *Pseudomonas* spp. counts (P = 0.002) during winter compared to summer (8.00 and 5.47 log cfu/g, respectively) (**Figure 3.7H**). Salad producers A, C and E presented significantly lower LAB values (P = 0.000, P = 0.000 and P = 0.010, respectively) during summer (4.13, 4.84 and 2.67 log cfu/g, respectively) compared to winter (5.66, 6.27 and 5.20 log cfu/g, respectively) (**Figure 3.7G**). Yeasts and molds were found to be significantly higher (P = 0.002) during winter for salad producer A compared to summer (5.47 and 4.73 log cfu/g) (**Figure 3.7I**). Similarly, producer B showed higher yeasts and molds counts during winter (P = 0.033) (5.99 and 4.20 log cfu/g). No significant differences (P > 0.05) for TVC, *Enterobacteriaceae*, coliforms and *Staphylococcus* spp. were observed among samples collected during winter and summer for all salad producers (**Figure 3.7A**, **3.7B**, **3.7C**, **3.7E** and **Table 3.2**).

Table 3.2. Effect of sampling period (winter-summer) on microbiological load (log cfu/g), total phenolic content (mg GAE/g Fw), antioxidants (mg trolox/g Fw), %CO_2 and stress markers- H_2O_2 (μmol/g Fw) and MDA (nmol/g Fw) of ready to eat salads according to producer.

	Producer				
	A	**B**	**C**	**D**	**E**
TVC	0.081	0.322	0.470	0.662	0.080
Enterobacteriaceae	0.750	0.706	0.858	0.492	0.608
Coliforms	0.105	0.280	0.080	0.527	0.829
E. coli	**0.022**	ni	**0.001**	0.485	ni
***Staphylococcus* spp.**	0.399	0.937	0.114	0.285	ni
B. cereus	0.717	ni	**0.047**	ni	ni
Lactic acid bacteria	**0.000**	0.079	**0.000**	0.225	**0.010**
***Pseudomonas* spp.**	0.100	**0.002**	0.568	0.107	0.817
Yeasts and molds	**0.002**	**0.033**	0.171	0.147	0.236
Phenols	0.786	0.327	**0.040**	0.853	0.479
DPPH	**0.000**	**0.001**	**0.000**	**0.017**	**0.000**
FRAP	**0.010**	0.277	0.093	0.324	**0.015**
ABTS	0.662	0.734	**0.020**	0.880	0.837
CO_2	0.365	0.837	0.690	0.992	0.605
H_2O_2	0.708	0.877	0.297	0.838	0.284
MDA	**0.001**	0.871	0.436	0.139	0.930

Results shown are the p values following independent samples t-test and bold values suggest significant differences ($P < 5\%$). Other= Lettuce +2 or more ingredients. ni = the correlation and t-test could not be computed because the standard error of the difference was 0.

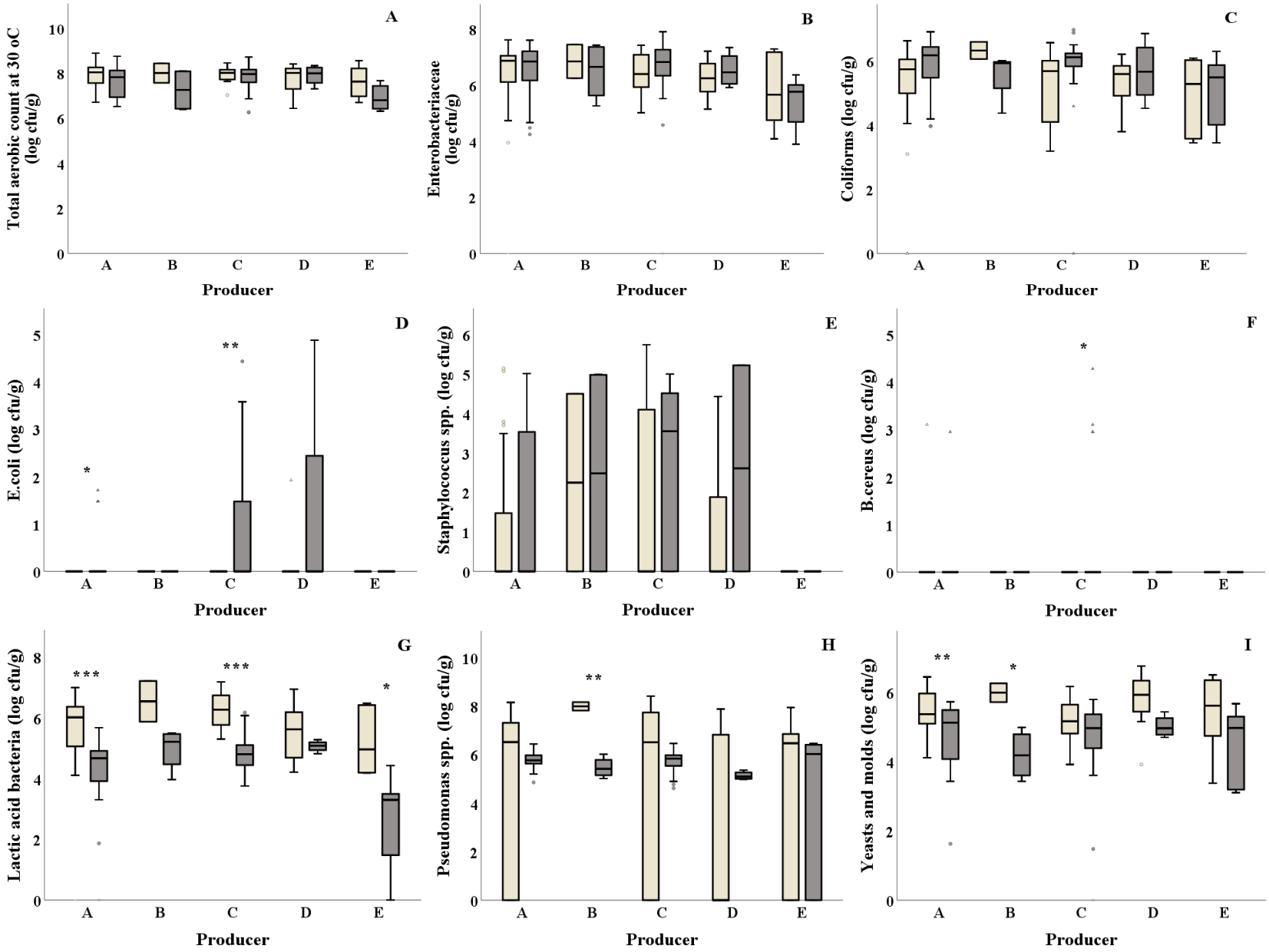


Figure 3.7. Effect of season on the microbiological quality of ready to eat salads collected during winter (□) and summer (■) among salad producers. Results include all samples for each microorganism tested and are the mean value ± standard deviation. Each box contains 50 per cent of cases and whiskers represent the rest. The line across the inside of the box represents the median value. *, ** and *** indicate significant differences at $P \leq 5\%$, 1% and 0.1%.

Type of salad

As shown in **Figure 3.8D** and **Table 3.3**, *E. coli* counts were reported significantly (P = 0.044) higher during summer for salads containing plain lettuce (0.00 and 0.67 log cfu/g). The combinations of lettuce with other types of leafy vegetables (lettuce+endive/radicchio, lettuce+rocket and lettuce+chives) showed significantly higher *Staphylococcus* spp. counts (P = 0.016, P = 0.012 and P = 0.028, respectively) during summer (2.10, 2.55 and 2.67 log cfu/g, respectively) compared to winter (0.32, 0.00 and 0.00 log cfu/g, respectively), while rocket presented higher values (P = 0.032) during winter compared to summer (3.69 and 1.44 log cfu/g) (**Figure 3.8E**). LAB on all types of salads were found in decreased numbers during summer (ranging from 3.98 to 5.02 log cfu/g) compared to winter (ranging from 5.21 to 6.63 log cfu/g) (**Figure 3.8G**). Samples of lettuce+cabbage collected during summer showed significantly higher *Pseudomonas* spp. values (P = 0.035) compared to those collected during winter (5.61 and 2.00 log cfu/g, respectively), while the opposite was evidenced for the lettuce+rocket samples that showed higher values (P = 0.003) on winter compared to summer (7.47 and 5.78 log cfu/g) (**Figure 3.8H**). Moreover, yeasts and molds were found in significantly decreased numbers in samples of lettuce+endive/radicchio, lettuce+rocket and other (lettuce+two or more ingredients) (P = 0.000, P = 0.000 and P = 0.046, respectively), collected in winter (4.74, 4.91 and 4.39 log cfu/g, respectively) compared to the ones collected in summer (5.84, 6.16 and 5.58 log cfu/g, respectively) (**Figure 3.8I**). TVC, *Enterobacteriaceae*, coliforms and *B. cereus* counts were not significantly different (P > 0.05) among samples collected during winter and summer for all types of salad as shown in **Figure 3.8A**, **3.8B**, **3.8C**, **3.8F** and **Table 3.3**.

Table 3.3. Effect of sampling period (winter-summer) on microbiological load (log cfu/g), total phenolic content (mg GAE/g Fw), antioxidants (mg trolox/g Fw), $\%CO_2$ and stress markers- H_2O_2 (µmol/g Fw) and MDA (nmol/g Fw) of ready to eat salads according to type of salad.

	Type of salad						
	Lettuce	**Lettuce+Cabbage**	**Lettuce+Endive/radicchio**	**Lettuce+Rocket**	**Lettuce+Chives**	**Rocket**	**Other**
TVC	0.131	0.244	0.373	0.949	0.827	0.343	0.511
Enterobacteriaceae	0.605	0.941	0.599	0.221	0.391	0.692	0.480
Coliforms	0.705	0.681	0.430	0.480	0.210	0.344	0.147
E. coli	**0.044**	0.168	0.336	0.408	0.178	0.082	0.432
***Staphylococcus* spp.**	0.465	0.483	**0.016**	**0.012**	**0.028**	**0.032**	0.589
B. cereus	ni	ni	ni	0.645	ni	0.081	0.530
Lactic acid bacteria	**0.029**	**0.001**	**0.000**	**0.004**	**0.011**	**0.037**	**0.001**
***Pseudomonas* spp.**	0.291	**0.035**	0.656	**0.003**	0.793	0.201	0.078
Yeasts and molds	0.254	0.092	**0.000**	**0.000**	0.887	0.089	**0.046**
Phenols	0.279	**0.026**	0.662	0.760	**0.005**	0.389	0.376
DPPH	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.001**
FRAP	**0.002**	0.510	0.085	0.060	0.777	**0.004**	0.396
ABTS	0.230	0.177	0.353	0.589	**0.021**	0.184	0.320
CO_2	0.771	0.989	0.464	0.745	0.179	0.955	0.897
H_2O_2	0.531	0.470	0.228	0.933	0.939	0.171	0.647
MDA	**0.002**	0.531	0.315	0.948	0.155	0.607	**0.003**

Results shown are the *p* values following independent samples t-test and bold values suggest significant differences ($P \leq 5\%$). Other= Lettuce +2 or more ingredients. ni = the correlation and t-test could not be computed because the standard error of the difference was 0.

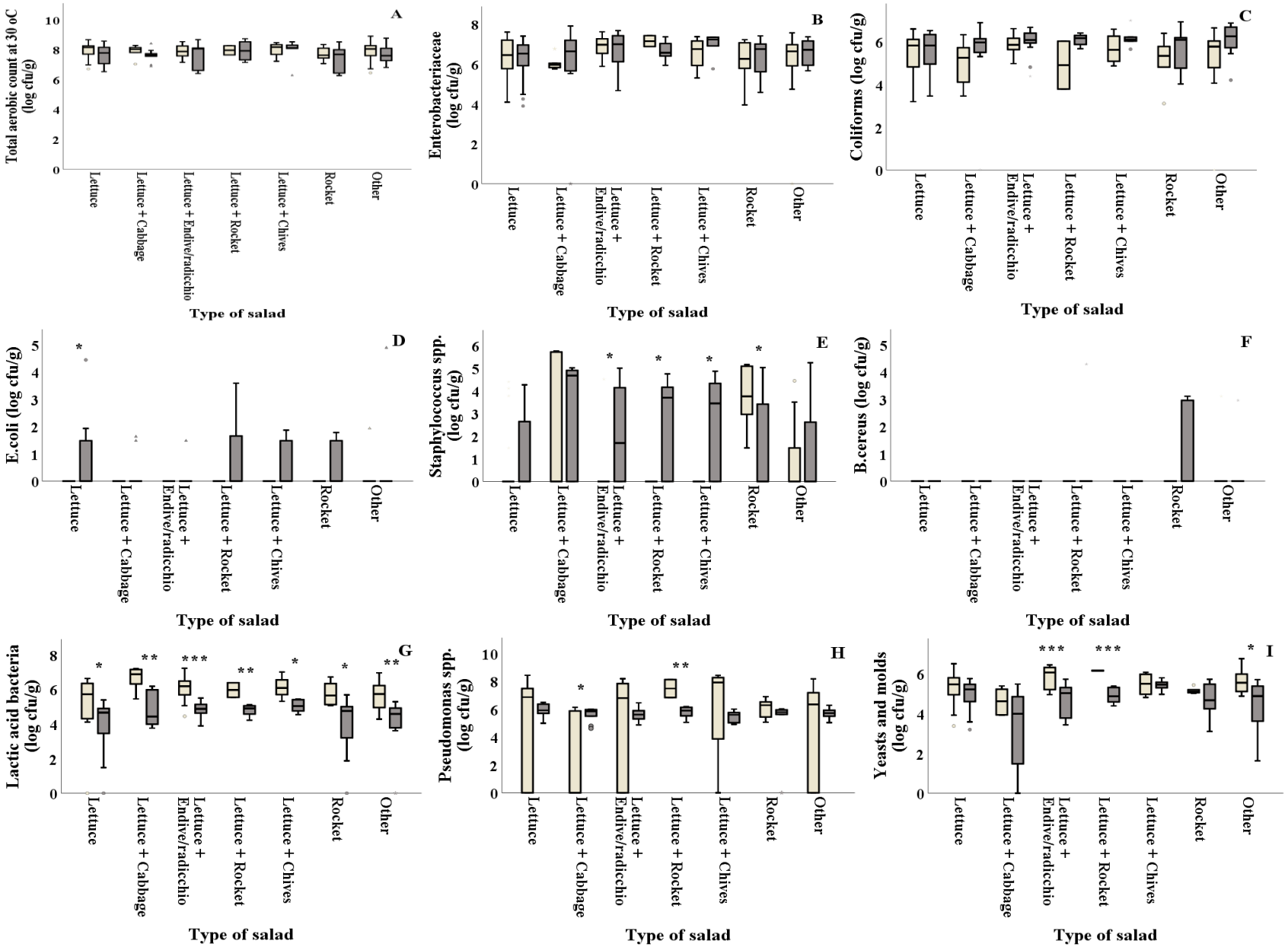


Figure 3.8. Microbiological quality of different types of ready to eat salads collected during winter (□) and summer (■). Results include only positive samples for each microorganism tested and are the mean value ± standard deviation. Other = Lettuce +2 or more ingredients. Each box contains 50 per cent of cases and whiskers represent the rest. The line across the inside of the box represents the median value. *, ** and *** indicate significant differences at $P \leq 5\%$, 1% and 0.1%.

3.3.2.1.2. Total phenols content, antioxidants, CO_2, H_2O_2 and lipid peroxidation

Salad producer

Total phenols content was found higher for producer C during summer compared to winter (0.86 and 0.69 mg GAE/g Fw) (**Figure 3.9A** and **Table 3.2**). Samples from all salad producers collected in winter showed significantly higher DPPH antioxidant values ($P < 0.01$) (DPPH: ranging from 1.22, to 1.60 mg trolox/g Fw, respectively) compared to the ones collected in summer (DPPH: ranging from 0.23 to 0.51 mg trolox/g Fw, respectively) (**Figure 3.9B** and **Table 3.2**). Producer E samples showed significantly lower FRAP activity ($P = 0.015$) during summer compared to winter (0.24 and 0.61 mg trolox/g Fw, respectively) (**Figure 3.9C**). Similarly, samples from producer A presented lower FRAP activity ($P = 0.010$) during summer compared to the ones collected in winter (FRAP: 0.40 and 0.80 mg trolox/g Fw). Samples from producer C presented higher ABTS activity ($P = 0.020$) in summer compared to winter (ABTS: 0.49 and 0.41 mg trolox/g Fw, respectively) (**Figure 3.9D**). Higher lipid peroxidation ($P = 0.000$) was observed during winter for producer A samples compared to summer (10.56 and 6.21 nmol MDA/g Fw, respectively) (**Figure 3.9G**). The H_2O_2 production and % CO_2 did not differ among the examined producers for both seasons (**Figure 3.9E, 3.9F** and **Table 3.2)**.

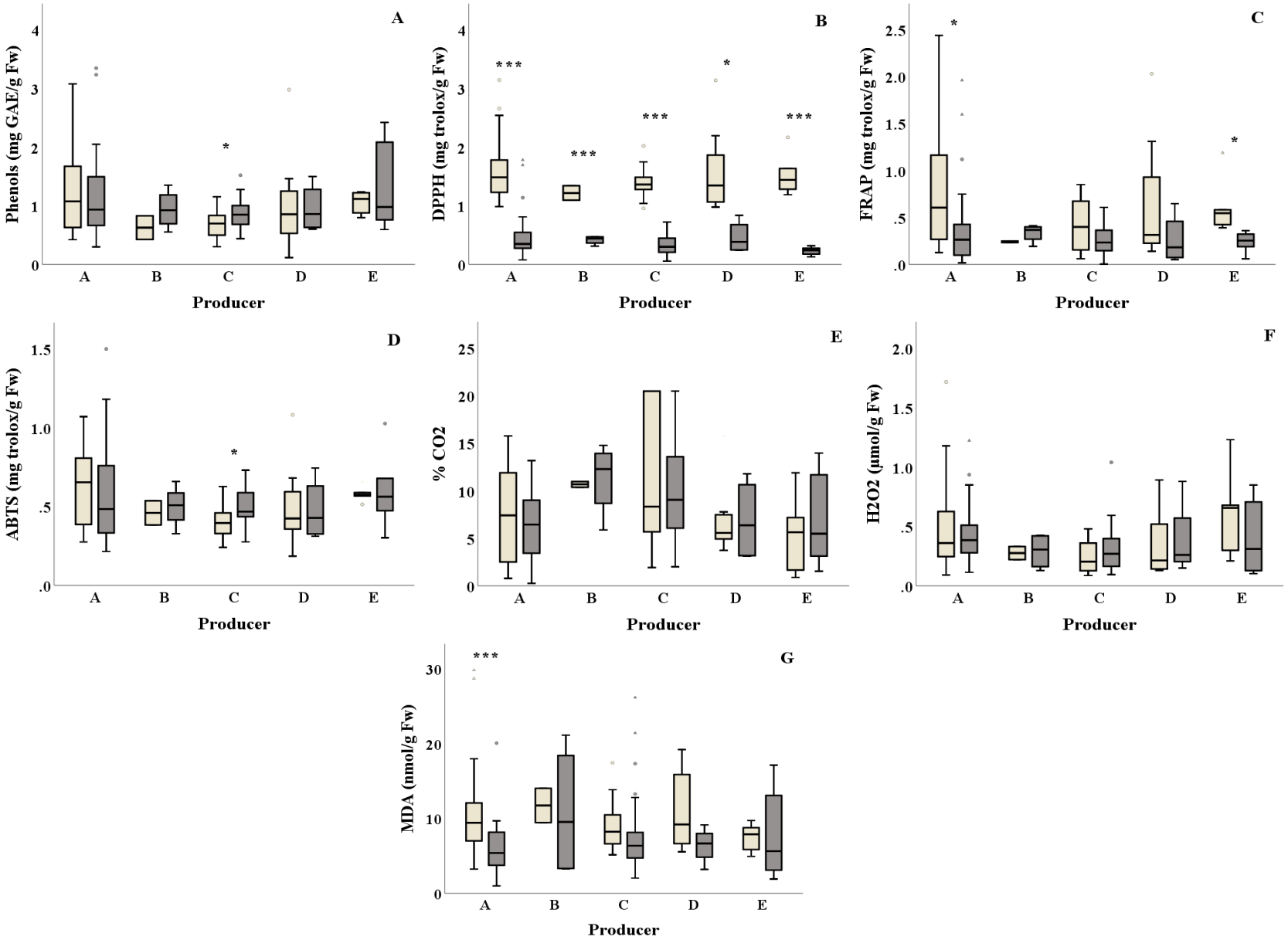


Figure 3.9. Effect of season on the total phenolic content, antioxidants, % CO_2 and damage index (H_2O_2 and lipid peroxidation) of ready to eat salads collected during winter (□) and summer (■) among salad producers. Results include all samples for each microorganism tested and are the mean value ± standard deviation. Each box contains 50 per cent of cases and whiskers represent the rest. The line across the inside of the box represents the median value. *, ** and *** indicate significant differences at $P \leq 5\%$, 1% and 0.1%.

Type of salad

Salad types of lettuce+cabbage and lettuce+chives revealed higher phenolic content ($P = 0.032$ and $P = 0.005$, respectively) during summer (0.85 and 0.84 mg GAE/g Fw, respectively) compared to winter (0.62 and 0.49 mg GAE/g Fw, respectively) (**Figure 3.10A**, **Table 3.3**). The DPPH assay revealed that antioxidant content of all types of salad significantly differed between the two seasons with summer (ranging from 0.27 to 0.89 mg trolox/g Fw, respectively) showing lower values than winter (ranging from 1.17 to 1.77 mg trolox/g Fw, respectively) (**Figure 3.10B, Table 3.3**). Plain lettuce and plain rocket presented higher antioxidant activity ($P = 0.002$ and $P = 0.004$, respectively) during winter (FRAP: 0.64 and 0.88 mg trolox/g Fw, respectively) compared to summer (FRAP: 0.25 and 0.33 mg trolox/g Fw, respectively) (**Figure 3.10C**). On the other hand, lettuce+chives significantly decreased the ABTS antioxidant activity ($P = 0.021$) in winter compared to summer (ABTS: 0.34 and 0.51 mg trolox/g Fw, respectively) (**Figure 3.10D**). Lipid peroxidation was found to be significantly higher during winter for plain lettuce ($P = 0.002$) and lettuce+two or more ingredients (other) ($P = 0.003$) (7.49 and 13.50 nmol MDA/g Fw, respectively) compared to summer (5.01 and 5.85 nmol MDA/g Fw, respectively) (**Figure 3.10G**). No significant differences ($P > 0.05$) were reported for % CO_2 and H_2O_2 production among samples collected during winter and summer for all types of salad, as illustrated in **Figure 3.10E, 3.10F** and **Table 3.3**.

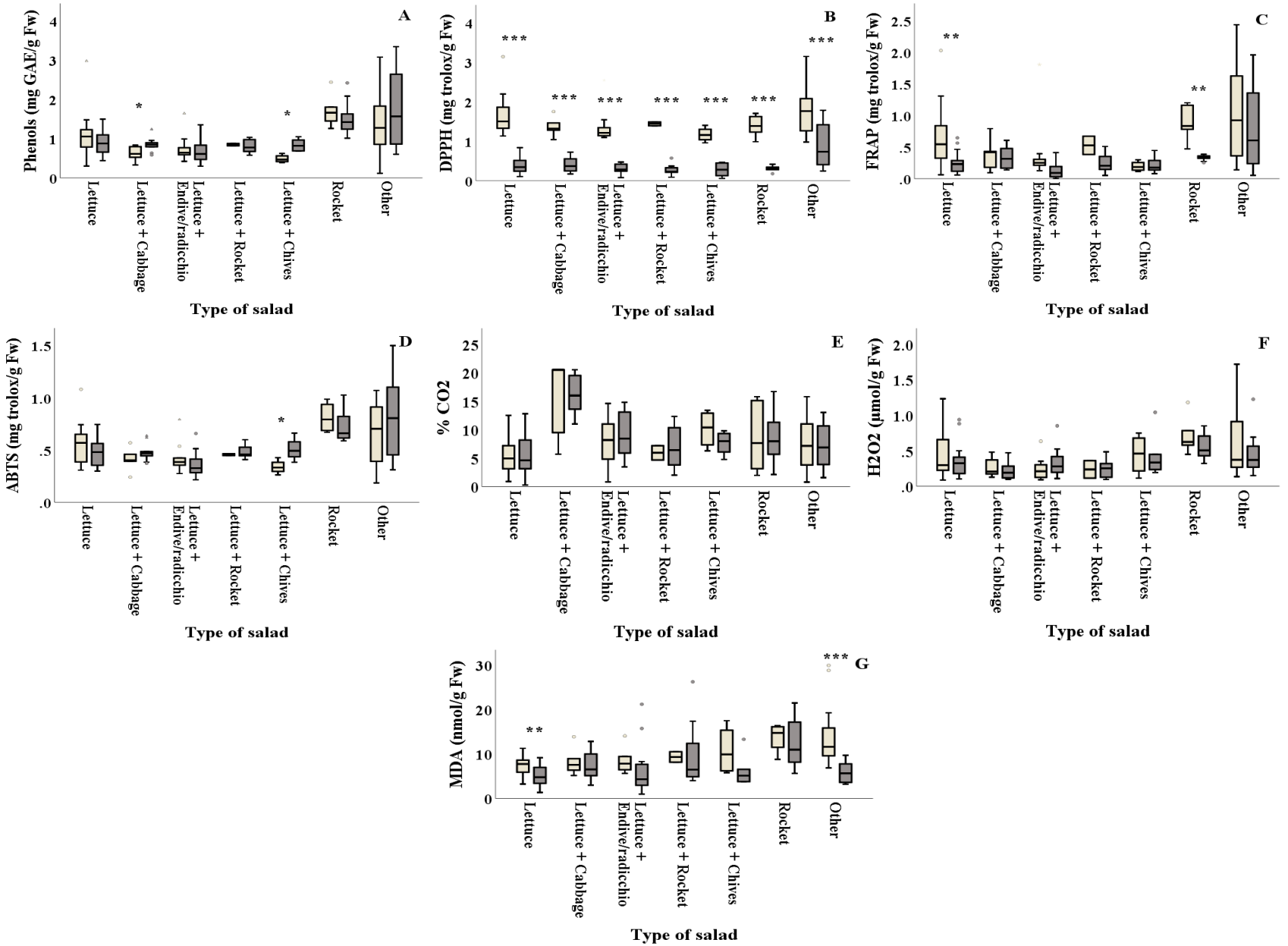


Figure 3.10. Effect of season on the total phenolic content, antioxidants, % CO_2 and damage index (H_2O_2 and lipid peroxidation) of ready to eat salads collected during winter (□) and summer (■) among type of salads. Results include all samples for each microorganism tested and are the mean value ± standard deviation. Other = Lettuce +2 or more ingredients. Each box contains 50 per cent of cases and whiskers represent the rest. The line across the inside of the box represents the median value. *, ** and *** indicate significant differences at $P \leq 5\%$, 1% and 0.1%.

3.3.2.2. Effects of shelf life

3.3.2.2.1 Microbiological analysis

Salad producer

Purchase (actual) vs Expiring day during winter and summer

The effect of storage period of purchase and expiring day on microbial load on different salad producers is presented in **Figures 3.11-3.19** and **Table 3.4.** Salads from producer A exhibited higher (*P*=0.003 for winter and *P*=0.036 for summer) TVC numbers at the end of their shelf life during winter (8.15 log cfu/g) than the expiring day during summer (7.93 log cfu/g) (**Figure 3.11A**). During summer, the polynomial curve with concave upward was described by $y=0.0556x^2-0.2409x+7.3881$; $R^2=0.92$, while the relevant curve during winter was $y=0.0065x^2+0.077x+7.5665$; $R^2=0.49$. Moreover, during winter, salads from producer A on their expiring day exhibited higher *Enterobacteriaceae* (*P*=0.025; 6.90 log cfu/g) with polynomial curve (concave downward) described by $y=-0.1334x^2+1.1601x+4.78$; $R^2=0.92$ (**Figure 3.12A**), and increased LAB (*P*=0.007; 6.04 log cfu/g) counts with polynomial curve and concave downward described by $y=-0.0995x^2+0.8922x+4.387$; $R^2=0.80$ (**Figure 3.17A**). Summer was the season in which increased *Staphylococcus* spp. (*P*=0.014; 2.00 log cfu/g) with polynomial curve (concave upward) described by $y=0.092x^2-0.3665x+0.7854$; $R^2=0.48$ for salads from producer A on the expiring day (**Figure 3.15A**). Additionally, during summer period, salads from producer B on the expiring day revealed decreased yeasts and molds numbers (*P*=0.045; 4.01 log cfu/g) with polynomial curve and concave downward being described by $y=-0.3454x^2+2.8219x-0.4864$; $R^2=1.00$ (**Figure 3.19B**). Samples from all salad producers collected throughout shelf life did not present significant differences ($P > 0.05$) among seasons for coliforms, *E. coli*, *B. cereus*, and *Pseudomonas* spp. (**Figure 3.13, 3.14, 3.16** and **3.18**, respectively). For both *S. enterica* and *L. monocytogenes* all samples were negative tested.

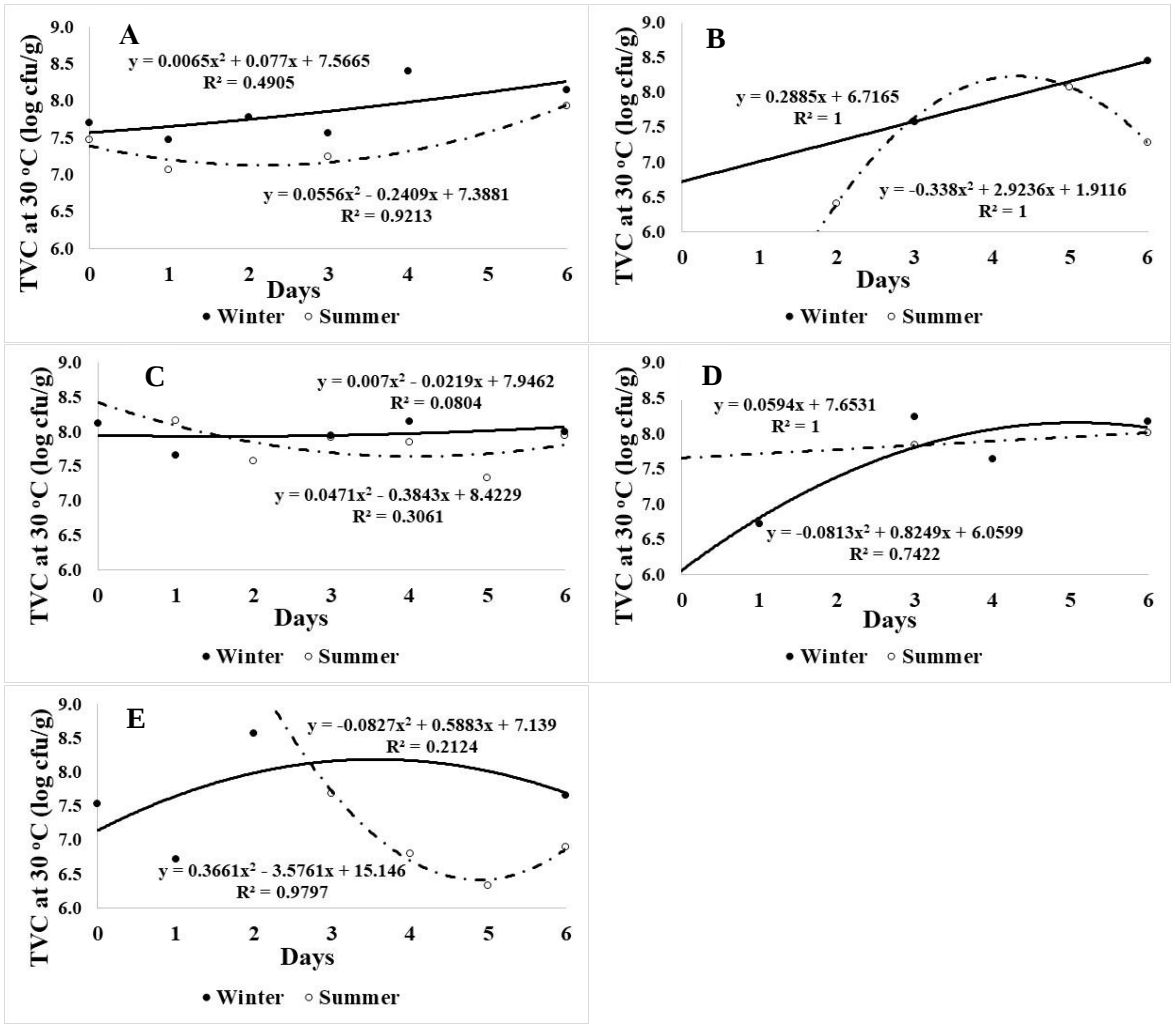


Figure 3.11. Effects of shelf life (days) on TVC per salad producer during winter and summer. A: producer A, B: producer B, C: producer C, D: producer D, E: producer E.

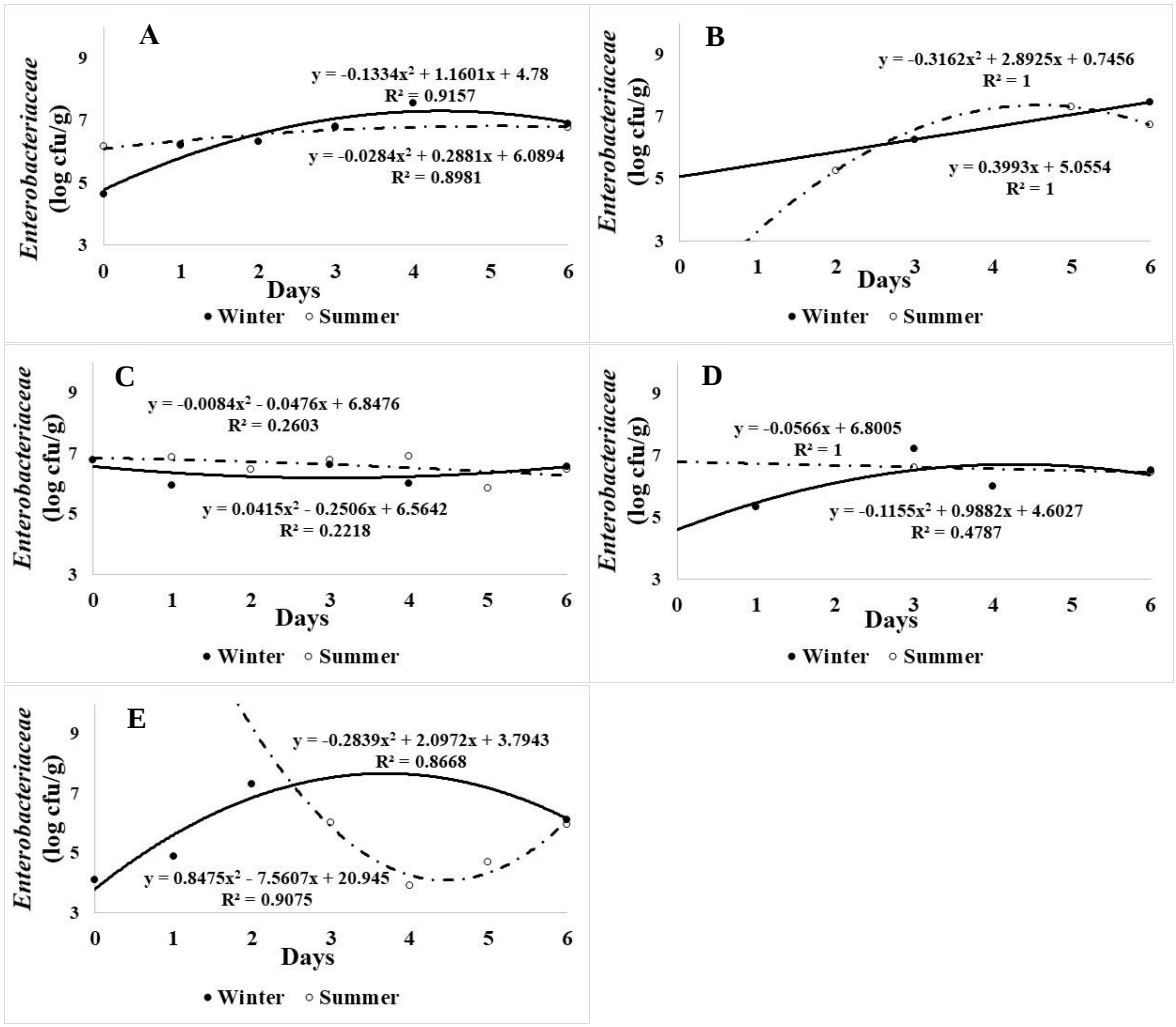


Figure 3.12. Effects of shelf life (days) on *Enterobacteriaceae* per salad producer during winter and summer. A: producer A, B: producer B, C: producer C, D: producer D, E: producer E.

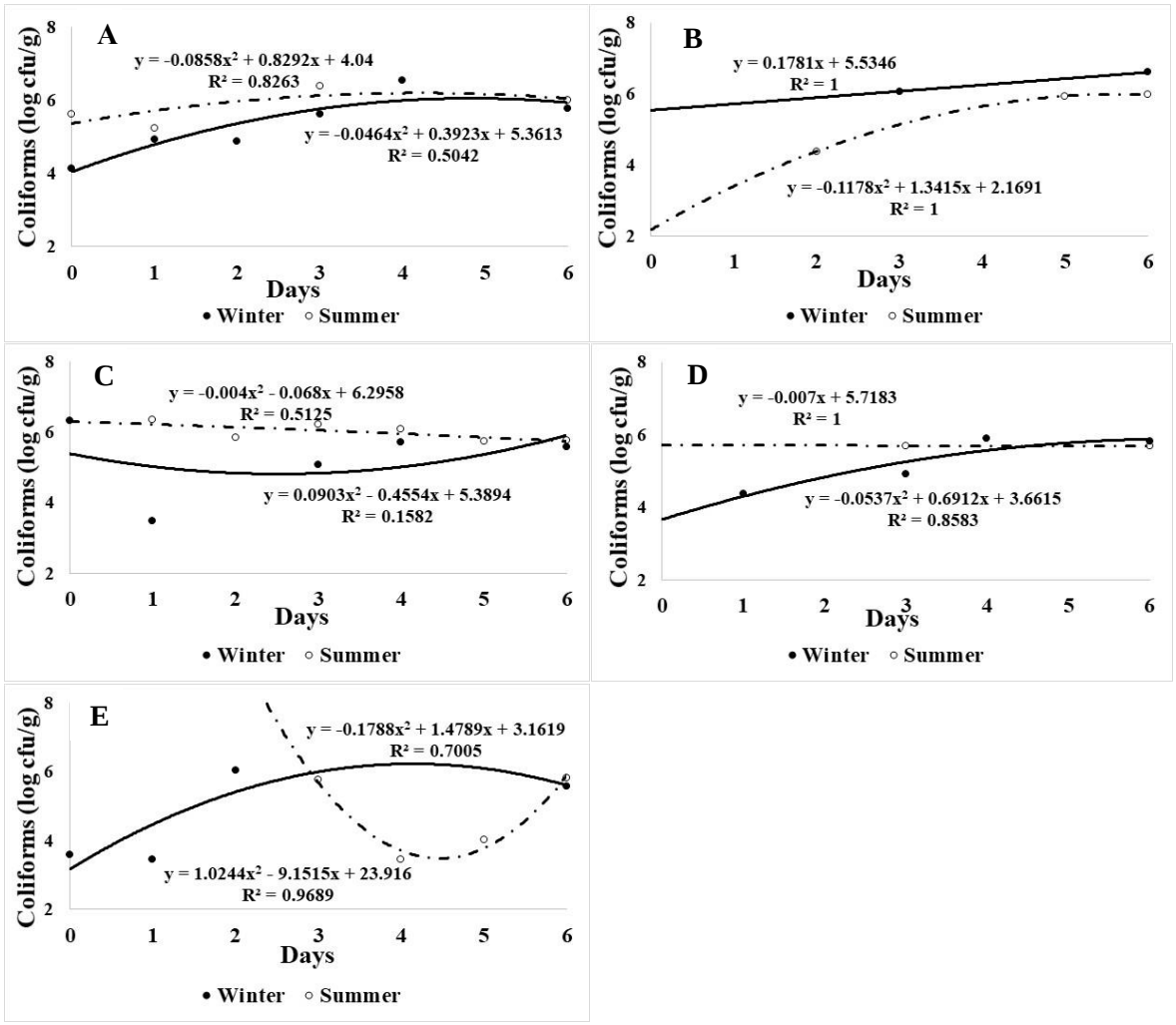


Figure 3.13. Effects of shelf life (days) on coliforms per salad producer during winter and summer. A: producer A, B: producer B, C: producer C, D: producer D, E: producer E.

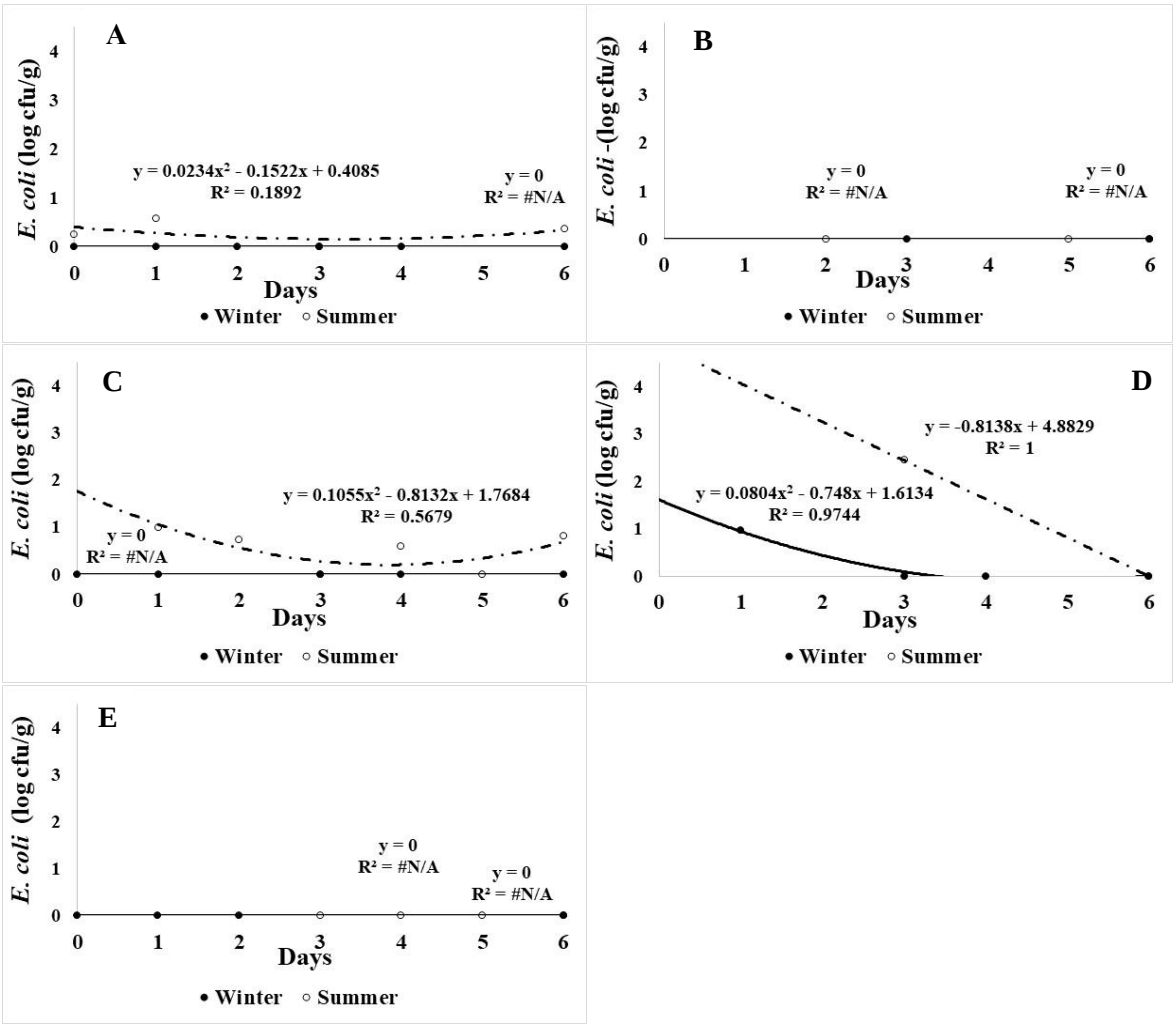


Figure 3.14. Effects of shelf life (days) on *E. coli* per salad producer during winter and summer. A: producer A, B: producer B, C: producer C, D: producer D, E: producer E.

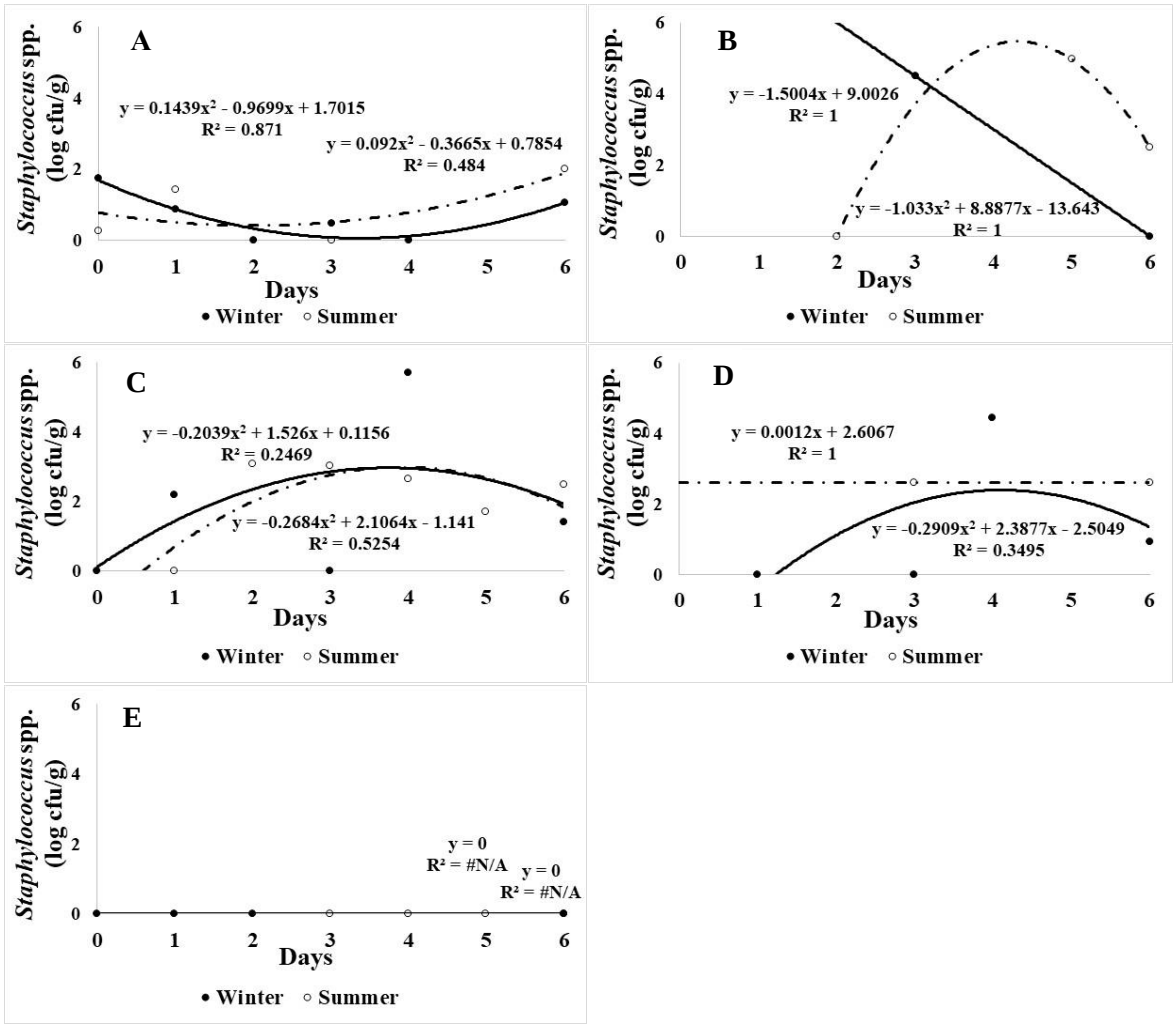


Figure 3.15. Effects of shelf life (days) on *Staphylococcus* spp. per salad producer during winter and summer. A: producer A, B: producer B, C: producer C, D: producer D, E: producer E.

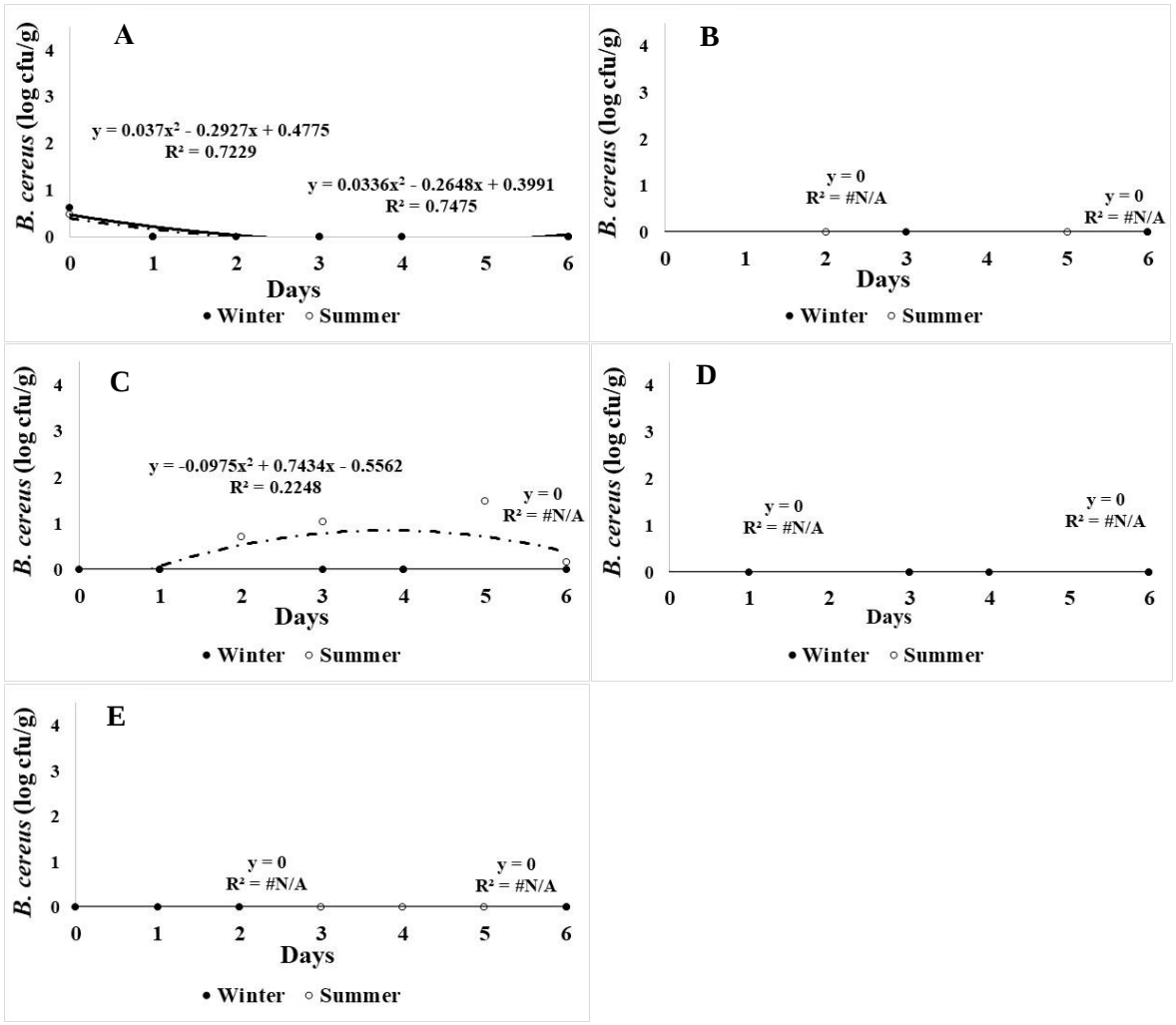


Figure 3.16. Effects of shelf life (days) on *B. cereus* per salad producer during winter and summer. A: producer A, B: producer B, C: producer C, D: producer D, E: producer E.

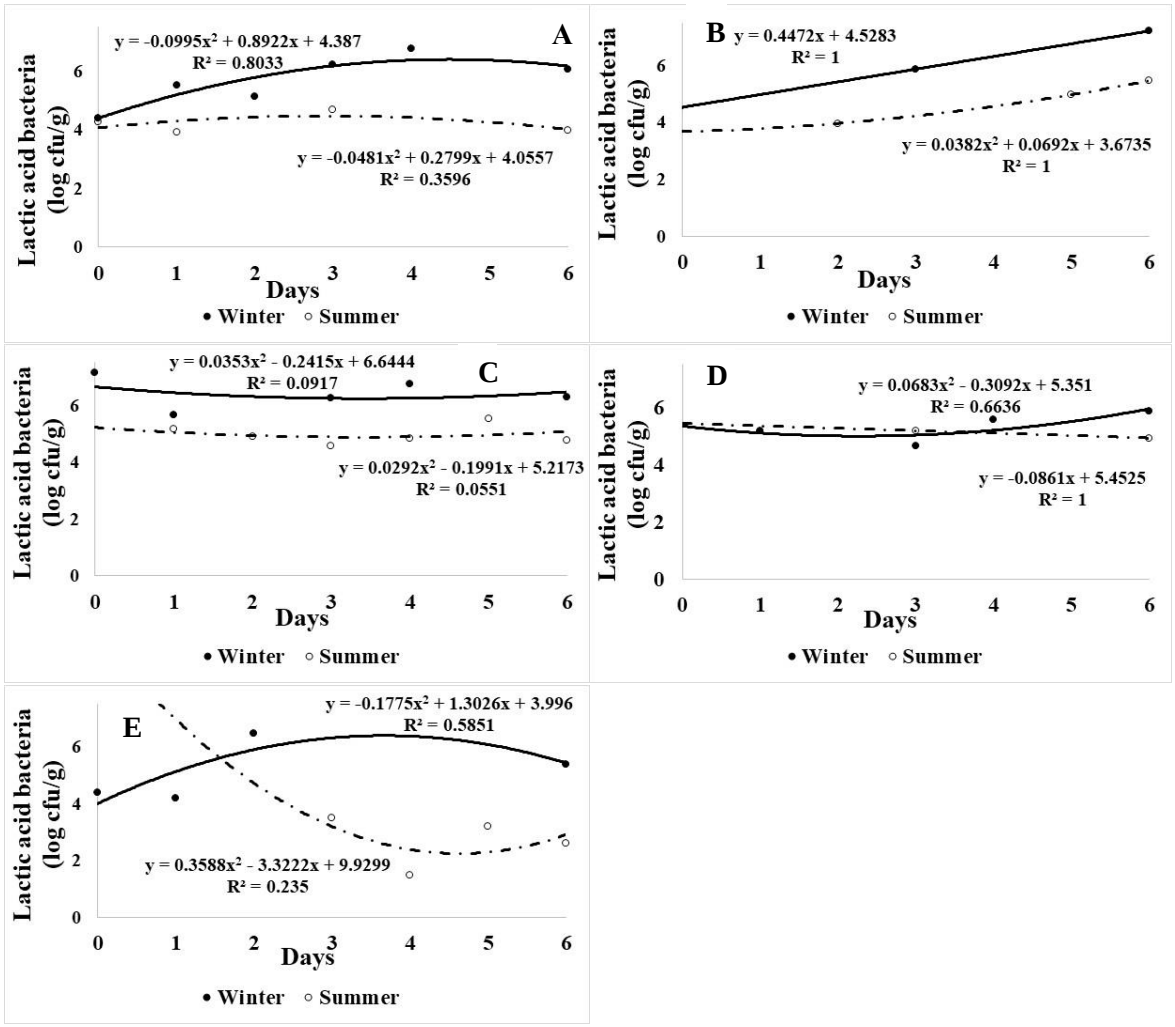


Figure 3.17. Effects of shelf life (days) on lactic acid bacteria per salad producer during winter and summer. A: producer A, B: producer B, C: producer C, D: producer D, E: producer E.

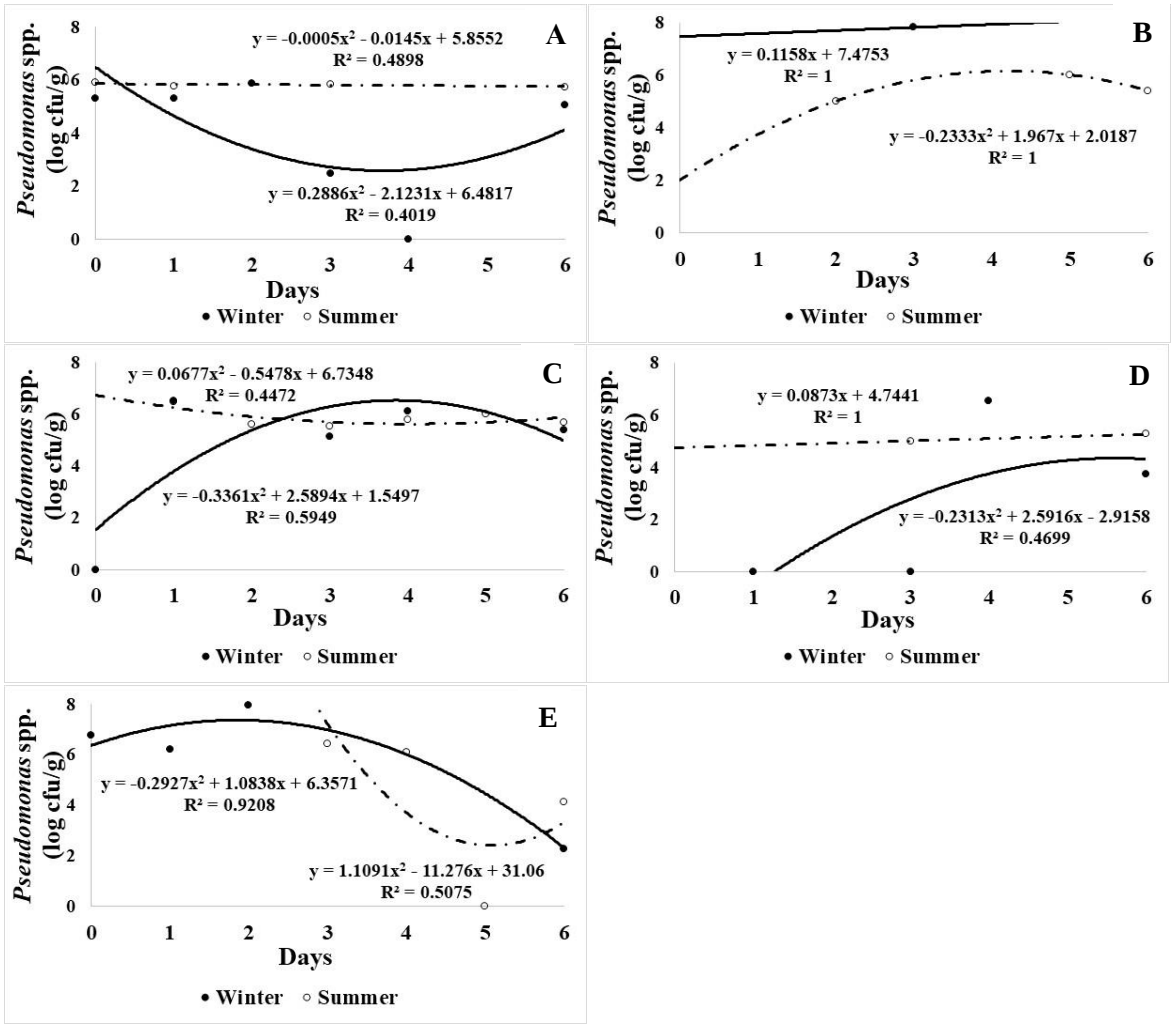


Figure 3.18. Effects of shelf life (days) on *Pseudomonas* spp. per salad producer during winter and summer. A: producer A, B: producer B, C: producer C, D: producer D, E: producer E.

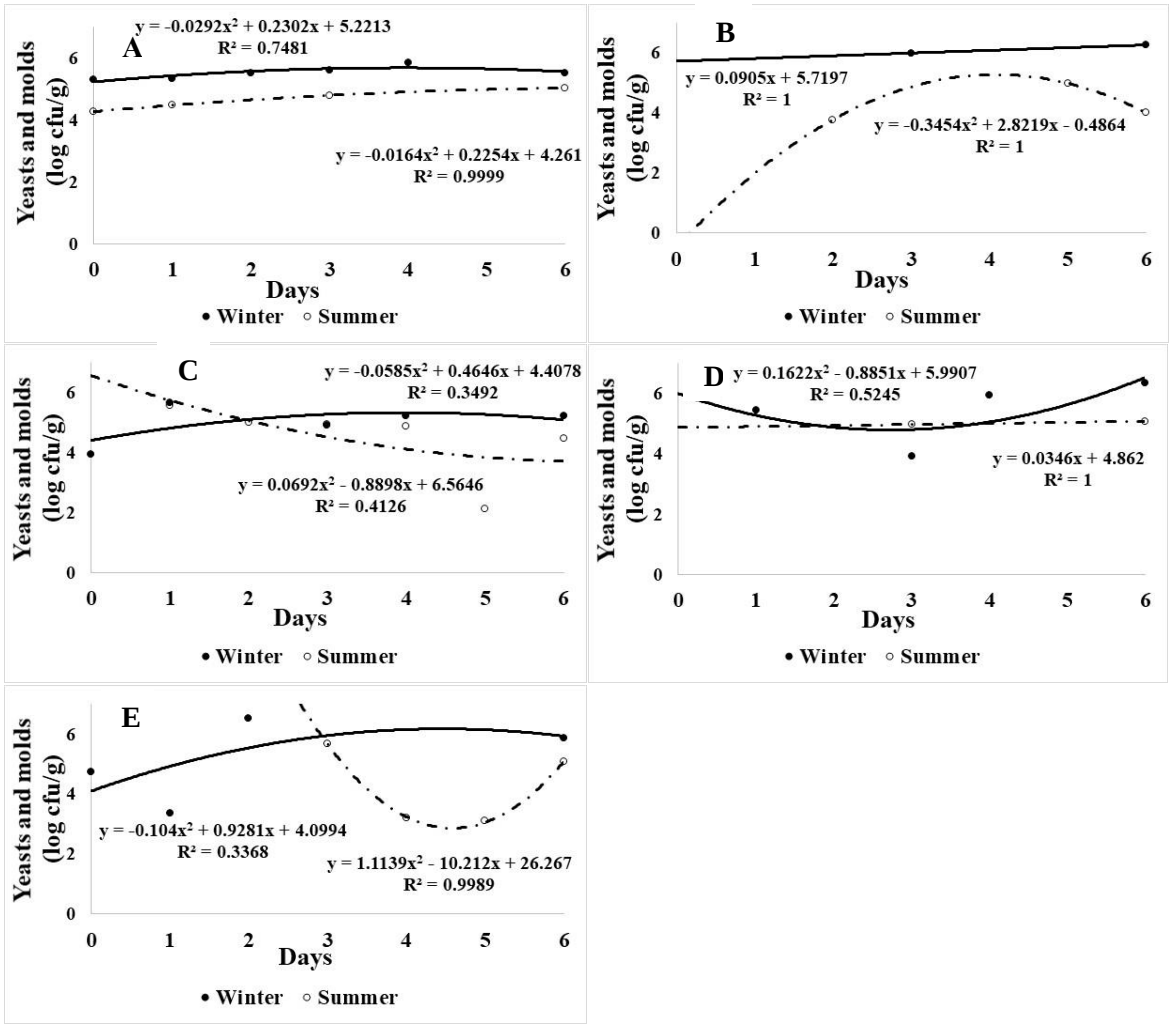


Figure 3.19. Effects of shelf life (days) on yeasts and molds per salad producer during winter and summer. A: producer A, B: producer B, C: producer C, D: producer D, E: producer E.

Type of salad

Purchase (actual) vs Expiring day during winter and summer

The effect of storage period of purchase and expiring day on microbial load on different types of salads is presented in **Figures 3.20-3.28** and **Table 3.5.** Expiring day TVC numbers were found higher during both seasons (P = 0.029 and P = 0.027 for winter and summer, respectively) for lettuce+endive/radicchio (8.13 and 8.01 log cfu/g for winter and summer, respectively) compared to purchase day (7.61 and 7.27 log cfu/g for winter and summer, respectively) (**Table 3.5**). During winter, lettuce+endive/radicchio the polynomial curve with concave upward is described by $y=0.0694x^2-0.4229x+8.156$; $R^2=0.68$, whilst the relevant curve (concave downward) during summer is described by $y=-0.0287x^2+0.5487x+5.8757$; $R^2=0.86$ (**Figure 3.20C**). During summer increased TVC numbers were observed for lettuce+rocket on products expiring day (P = 0.012; 8.45 log cfu/g), whereas rocket and lettuce+two or more ingredients (other) presented higher expiring TVC counts (P =0.036 and P = 0.019, respectively) during winter (7.87 and 8.22 log cfu/g, respectively) (**Table 3.5**). The polynomial curve with concave upward for lettuce+rocket is described by $y=0.1238x+7.5249$; $R^2=1.00$, while the relevant curves for rocket and other are described by $y=0.0384x^2-0.1831x+7.5846$; $R^2=1.00$ and $y=0.0091x^2+0.1099x+7.2934$; $R^2=0.85$, respectively (**Figure 3.20D**, **3.20F**, **3.20G**). Expiring *Enterobacteriaceae* numbers were found increased on lettuce+endive/radicchio on both seasons (7.26 and 7.09 log cfu/g for winter and summer, respectively) compared to purchase day (6.51 and 6.39 log cfu/g for winter and summer, respectively) and the polynomial curve with concave upward for winter is described by $y=0.032x^2-0.0589x+6.4607$; $R^2=0.99$, while the relevant curve (concave downward) for summer is described by $y=-0.1721x^2+1.7325x+2.9305$; $R^2=0.95$ (**Figure 3.21C**). Winter was the season in which plain lettuce and lettuce+endive/radicchio exhibited higher coliform counts (P = 0.039 and P = 0.001, respectively) on expiring day (5.82 and 6.07 log cfu/g, respectively) compared to purchase day (4.95 and 5.54 log cfu/g, respectively) (**Table 3.5**). The polynomial curve with concave downward for lettuce is described by $y=-0.0488x^2+0.5507x+4.3534$; $R^2=0.35$, while the respective curve for lettuce+endive/radicchio is described by $y=-0.033x^2+0.4141x+4.746$; $R^2=0.65$ (**Figure 3.22A**, **3.22C**). *Pseudomonas* spp. and yeasts and molds counts were found to be significantly higher on the expiring day for lettuce+rocket during summer (P = 0.040 and 0.001, respectively) (5.85 and 5.24 log cfu/g, respectively) compared to purchase day (5.71 and 5.24 log cfu/g, respectively), whilst on the same season increased LAB numbers (P = 0.025) were observed for lettuce+rocket on purchase day compared to expiring day (4.90 and 4.68 log cfu/g, respectively) (**Figure 3.26D**, **3.27D, 3.28D, Table 3.5**). During summer for lettuce+rocket, the polynomial curves for *Pseudomonas* spp. and yeasts and molds with concave upward are described by $y=0.0403x^2+5.6089x$; $R^2=1.00$ (**Figure 3.27D**) and $y=0.1393x^2-0.9671x+6.028$; $R^2=1.00$ (**Figure 3.28D**), respectively, whilst the relevant curve for LAB is described by $y=0.1141x^2-1.0079x+6.6222$; $R^2=1.00$ (**Figure 3.26D**). No significant differences ($P > 0.05$) were observed between the day of purchase and the expiring day of salads among seasons for *E. coli*,

Staphylococcus spp. and *B. cereus* (**Figure 3.23**, **3.24** and **3.25**, respectively). For both *S. enterica* and *L. monocytogenes* all samples were negative tested.

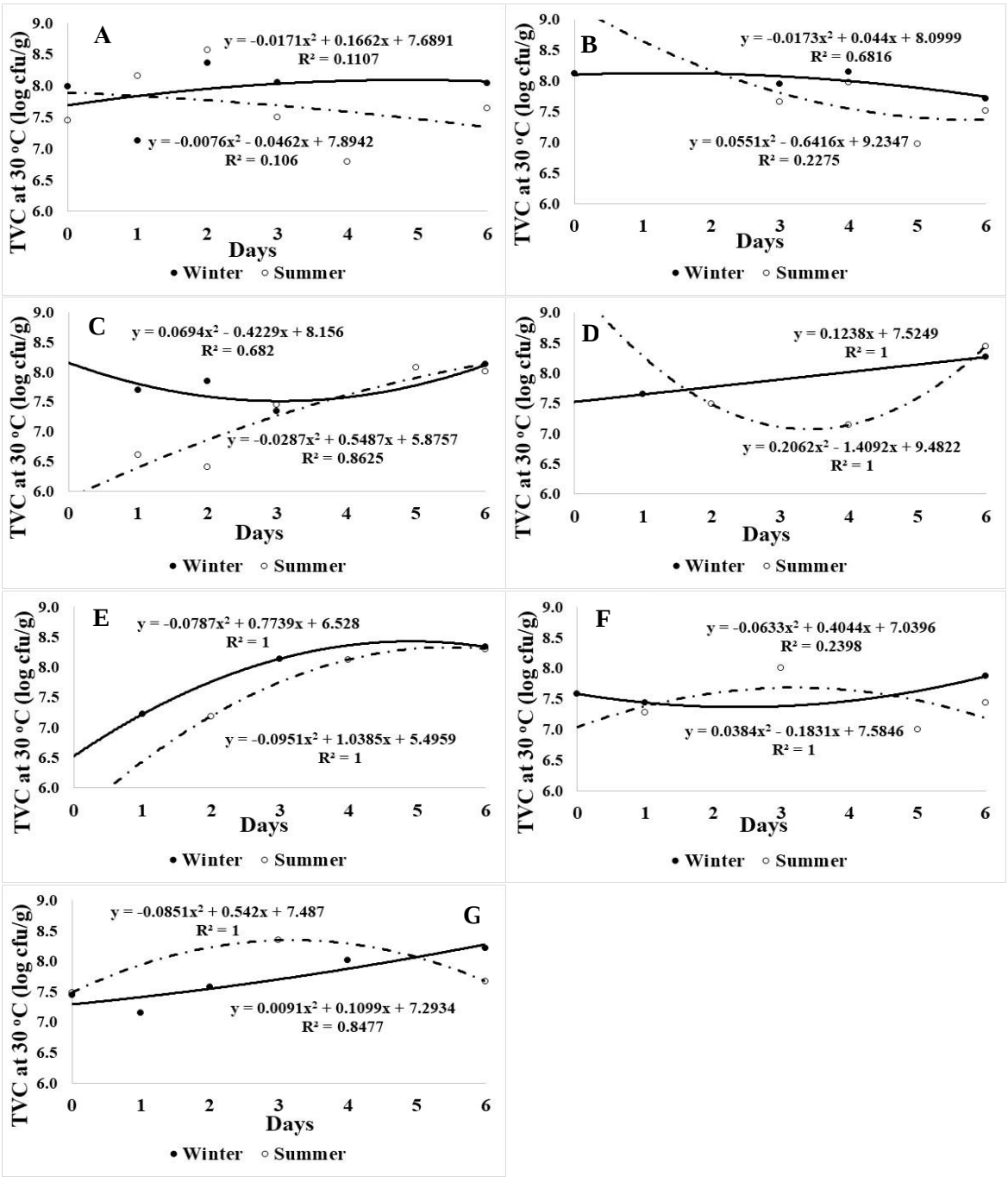


Figure 3.20. Effect of shelf life (days) on TVC per type of salad during winter and summer. A: lettuce, B: lettuce + cabbage, C: lettuce + radicchio/endive, D: lettuce + rocket, E: lettuce + chives, F: rocket, G: lettuce + two/more ingredients (other).

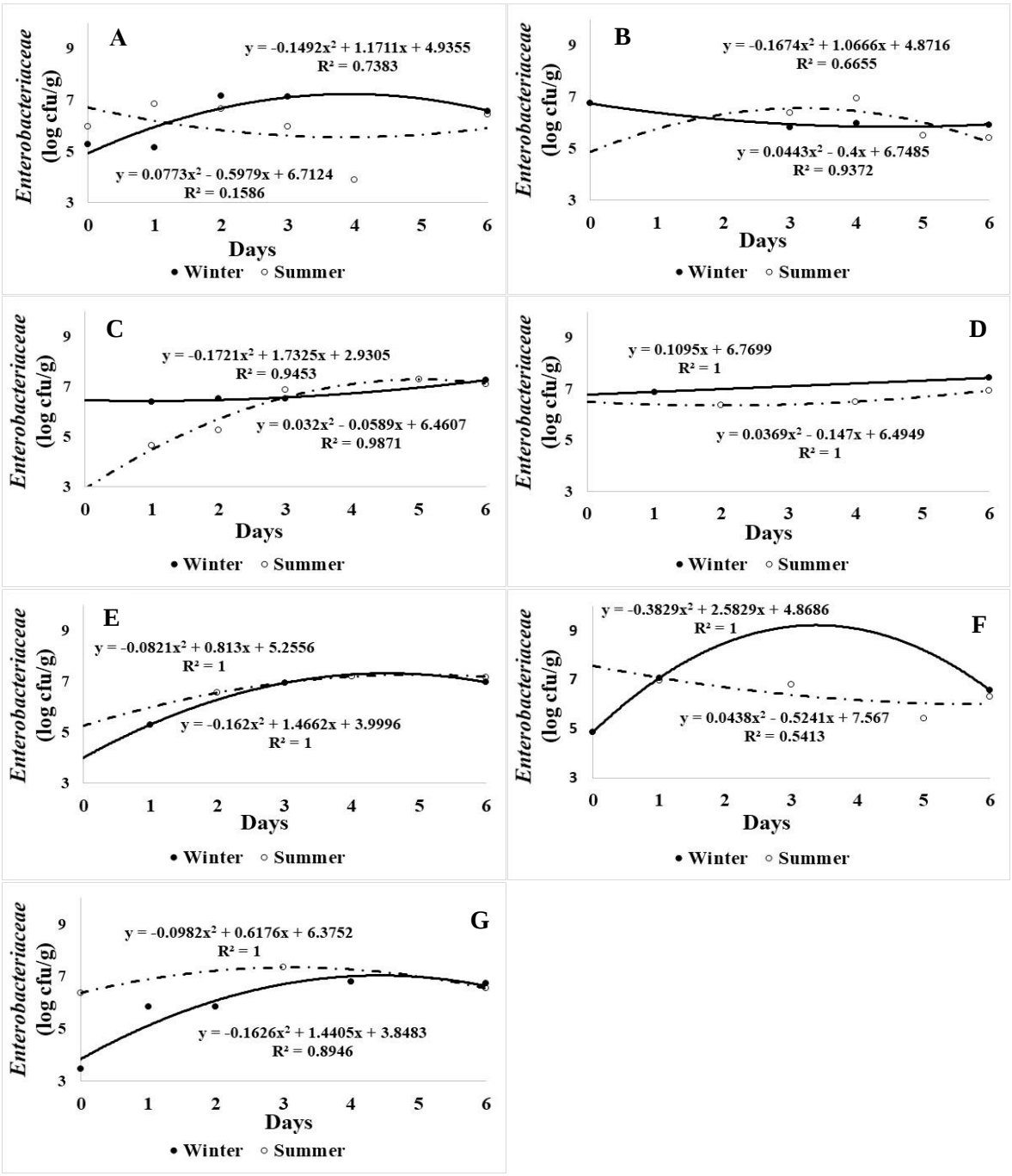


Figure 3.21. Effect of shelf life (days) on *Enterobacteriaceae* per type of salad during winter and summer. A: lettuce, B: lettuce + cabbage, C: lettuce + radicchio/endive, D: lettuce + rocket, E: lettuce + chives, F: rocket, G: lettuce + two/more ingredients (other).

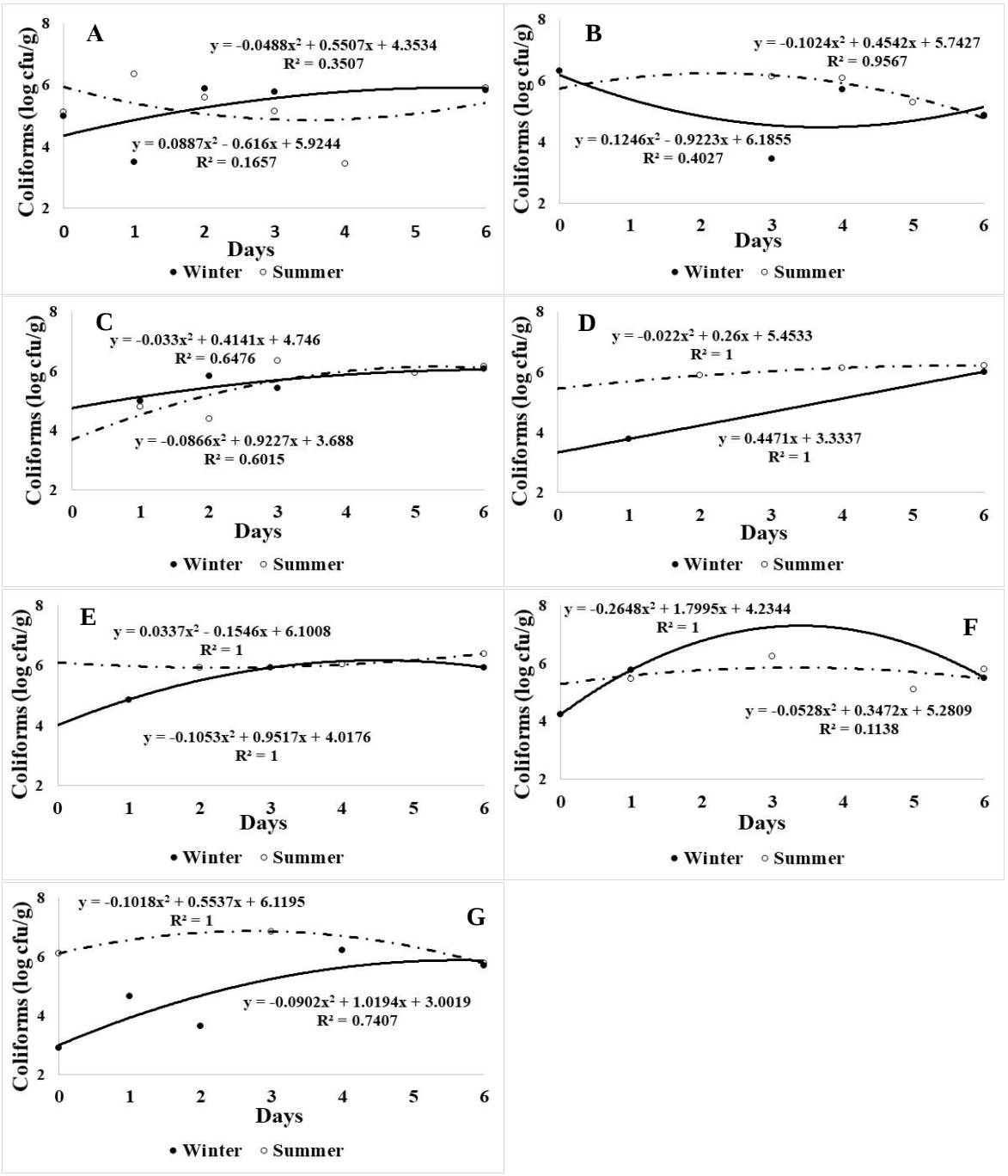


Figure 3.22. Effect of shelf life (days) on coliforms per type of salad during winter and summer. A: lettuce, B: lettuce + cabbage, C: lettuce + radicchio/endive, D: lettuce + rocket, E: lettuce + chives, F: rocket, G: lettuce + two/more ingredients (other).

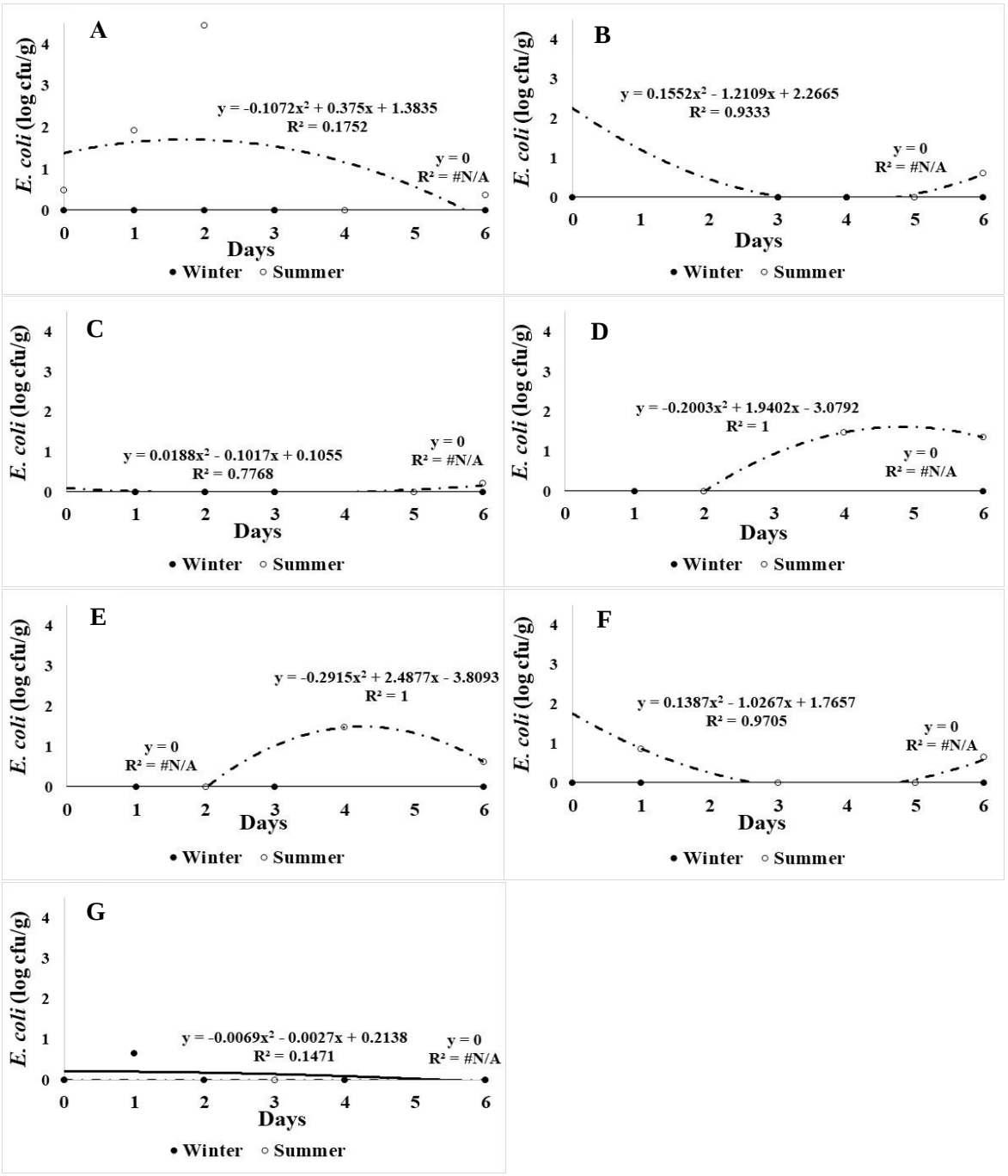


Figure 3.23. Effect of shelf life (days) on *E. coli* per type of salad during winter and summer. A: lettuce, B: lettuce + cabbage, C: lettuce + radicchio/endive, D: lettuce + rocket, E: lettuce + chives, F: rocket, G: lettuce + two/more ingredients (other).

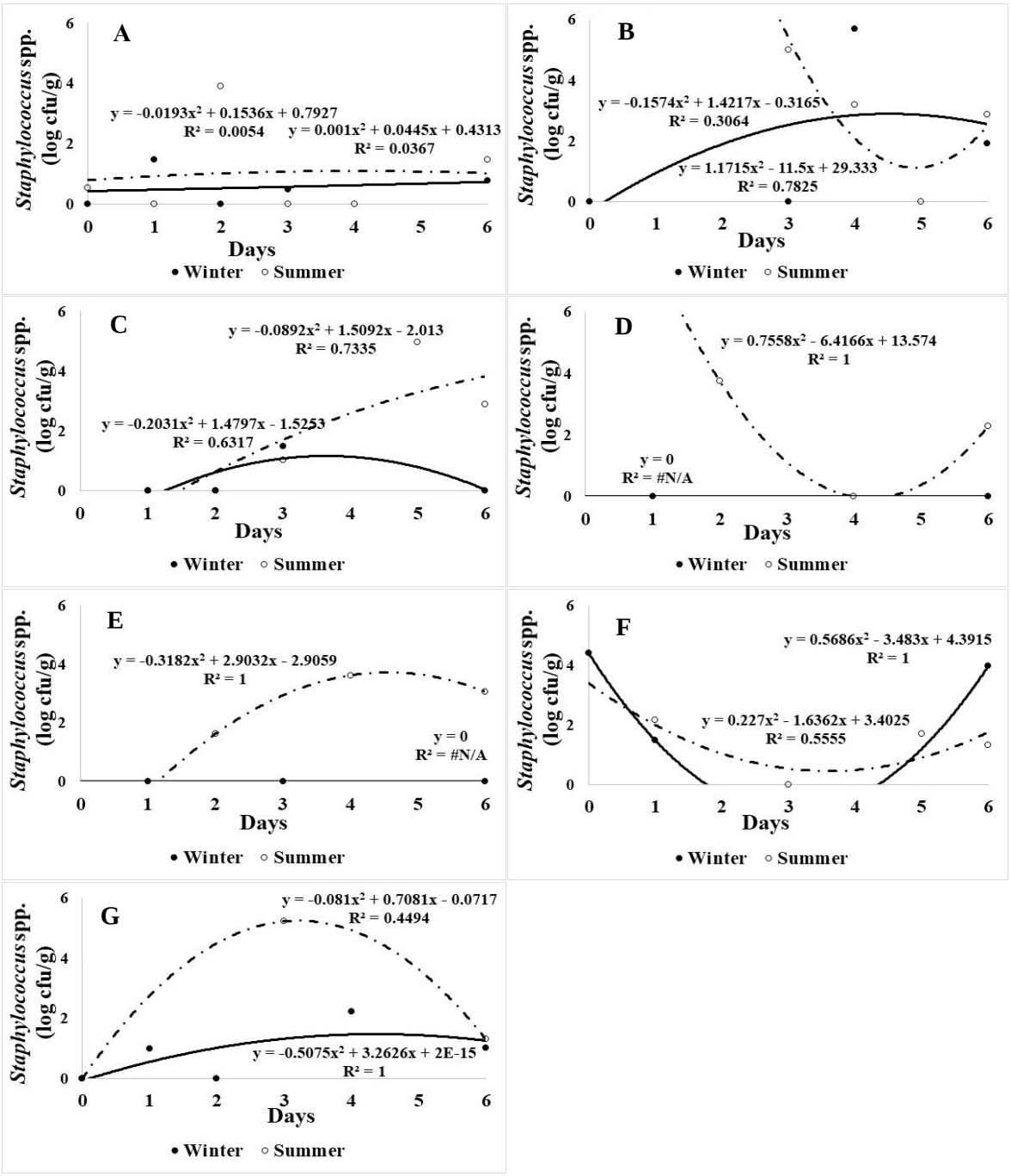


Figure 3.24. Effect of shelf life (days) on *Staphylococcus* spp. per type of salad during winter and summer. A: lettuce, B: lettuce + cabbage, C: lettuce + radicchio/endive, D: lettuce + rocket, E: lettuce + chives, F: rocket, G: lettuce + two/more ingredients (other).

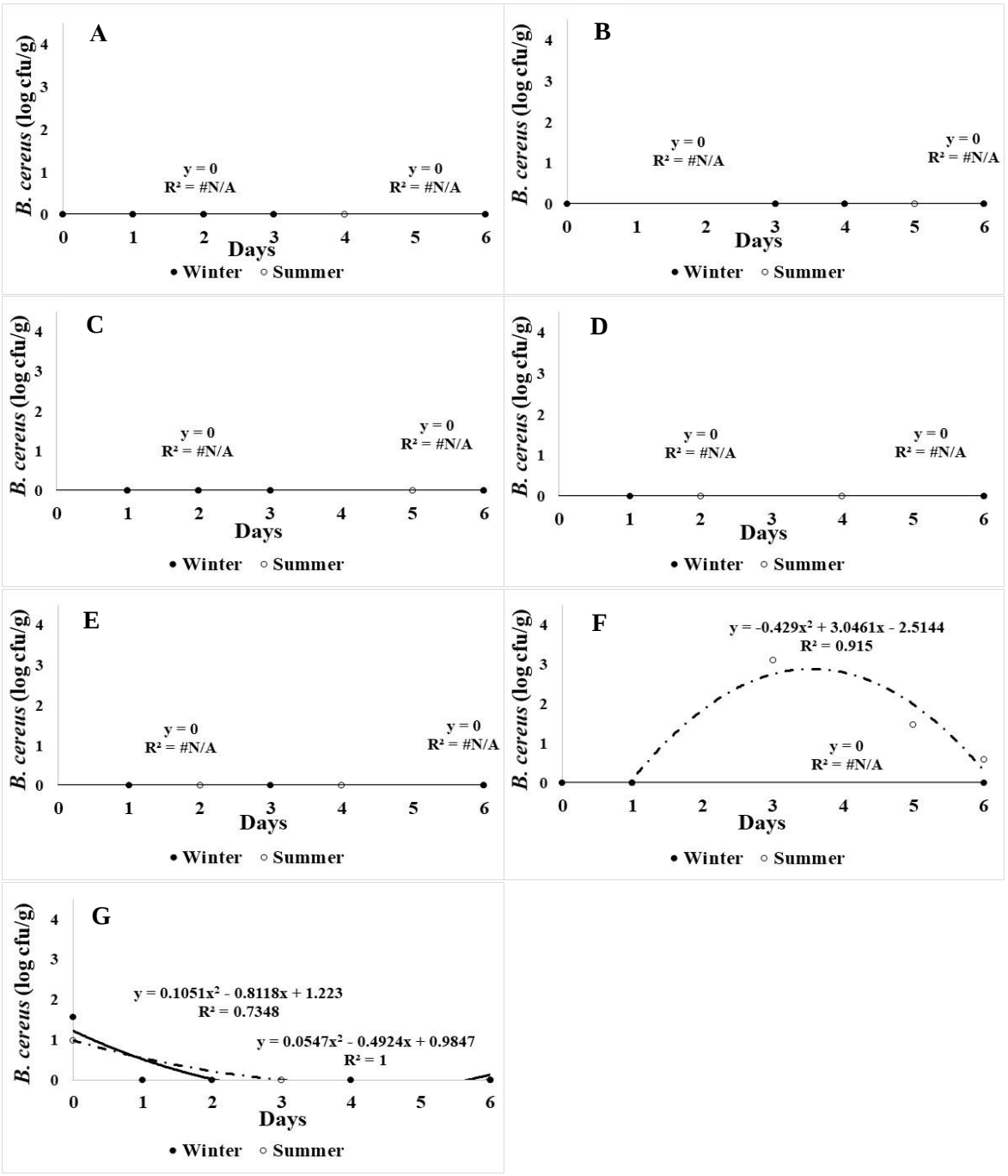


Figure 3.25. Effect of shelf life (days) on *B. cereus* per type of salad during winter and summer. A: lettuce, B: lettuce + cabbage, C: lettuce + radicchio/endive, D: lettuce + rocket, E: lettuce + chives, F: rocket, G: lettuce + two/more ingredients (other).

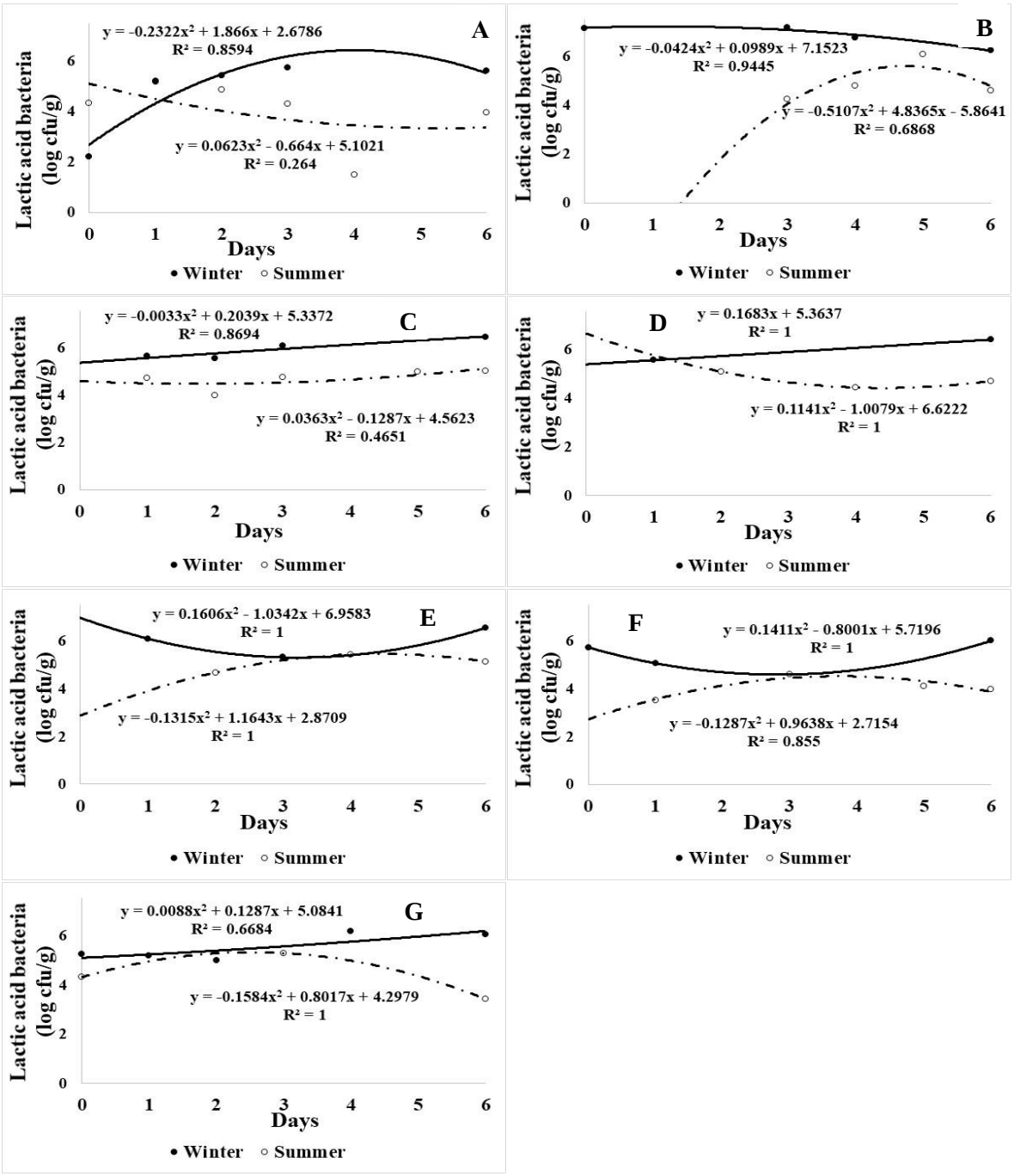


Figure 3.26. Effect of shelf life (days) on lactic acid bacteria per type of salad during winter and summer. A: lettuce, B: lettuce + cabbage, C: lettuce + radicchio/endive, D: lettuce + rocket, E: lettuce + chives, F: rocket, G: lettuce + two/more ingredients (other).

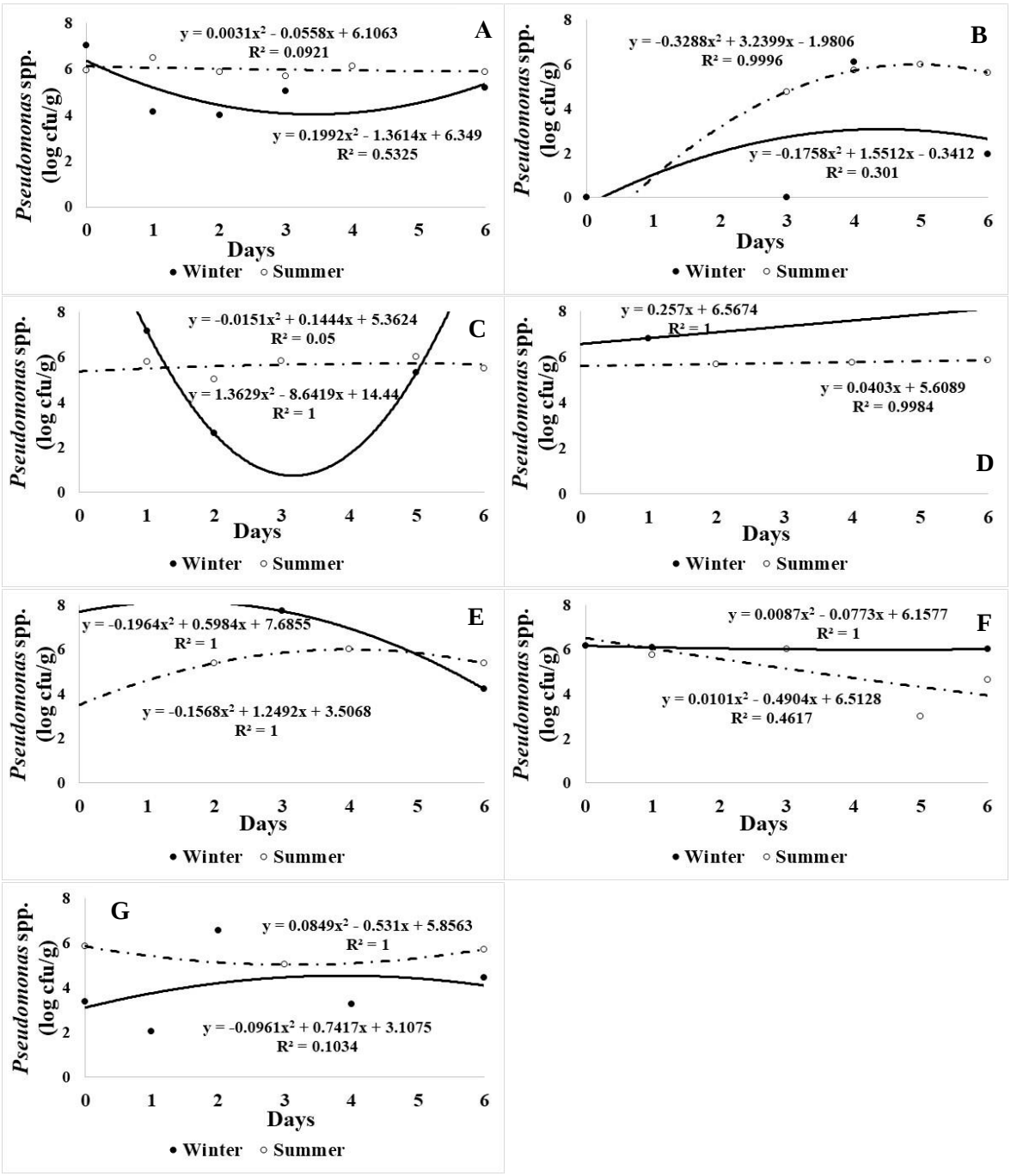


Figure 3.27. Effect of shelf life (days) on *Pseudomonas* spp. per type of salad during winter and summer. A: lettuce, B: lettuce + cabbage, C: lettuce + radicchio/endive, D: lettuce + rocket, E: lettuce + chives, F: rocket, G: lettuce + two/more ingredients (other).

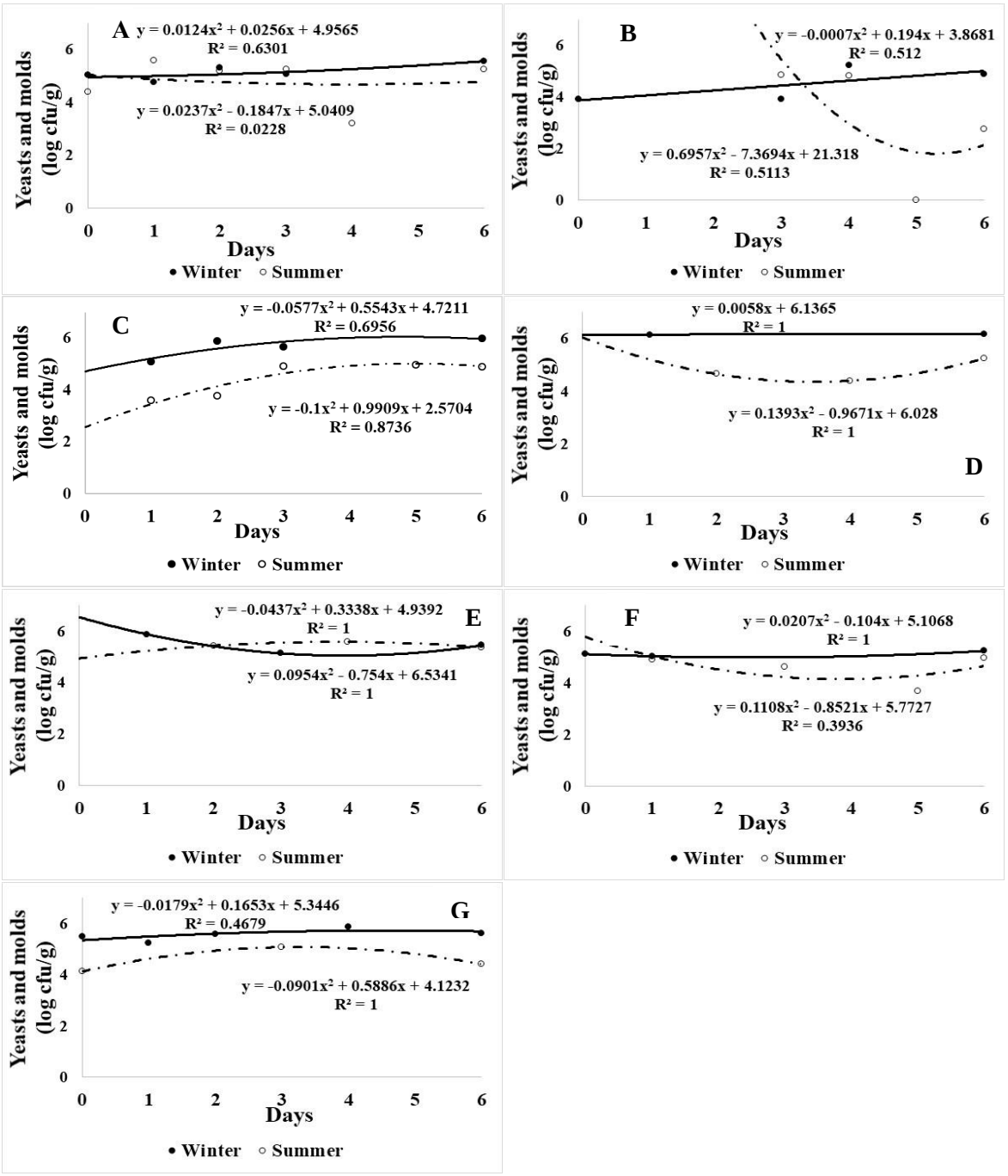


Figure 3.28. Effects of shelf life (days) on yeasts and molds per type of salad during winter and summer. A: lettuce, B: lettuce + cabbage, C: lettuce + radicchio/endive, D: lettuce + rocket, E: lettuce + chives, F: rocket, G: lettuce + two/more ingredients (other).

Table 3.4. Effect of shelf-life, salad producer and type on microbiological load (log cfu/g), total phenolic content (mg GAE/g Fw), antioxidants (mg trolox/g Fw) and $\%CO_2$ and stress markers- H_2O_2 (µmol/g Fw) and MDA (nmol/g Fw) of ready to eat salads according to salad producer during winter and summer.

	Producer									
	A		**B**		**C**		**D**		**E**	
	Winter	**Summer**	**Winter**	**Summer**	**Winter**	**Summer**	**Winter**	**Summer**	**Winter**	**Summer**
TVC	**0.003**	**0.036**	ni	0.119	0.691	0.303	0.058	0.837	0.953	0.777
Enterobacteriaceae	**0.025**	0.194	ni	0.391	0.418	0.702	0.397	0.892	0.522	0.153
Coliforms	0.061	0.432	ni	0.464	0.203	0.519	0.182	0.984	0.219	0.127
E. coli	ni	0.667	ni	ni	ni	0.506	0.391	0.500	ni	ni
***Staphylococcus* spp.**	0.167	**0.014**	ni	0.500	0.443	0.732	0.927	0.500	ni	ni
B. cereus	0.329	0.339	ni	ni	ni	0.162	ni	ni	ni	ni
Lactic acid bacteria	**0.007**	0.574	ni	0.313	0.999	0.359	0.394	0.086	0.813	0.956
***Pseudomonas* spp.**	0.692	0.237	ni	0.833	0.077	0.204	0.576	0.181	0.121	0.632
Yeasts and molds	0.682	0.093	ni	**0.045**	0.451	0.490	0.068	0.864	0.496	0.300
Phenols	0.062	0.665	ni	0.055	0.868	0.752	0.687	0.585	0.123	0.759
DPPH	0.446	0.444	ni	0.310	0.459	0.619	0.462	0.486	0.105	0.798
FRAP	0.203	0.312	ni	0.607	0.654	0.750	0.283	0.571	0.358	0.516
ABTS	0.091	0.952	ni	0.059	0.904	0.975	0.726	0.420	0.328	0.691
CO_2	**0.000**	**0.000**	ni	0.226	**0.016**	**0.000**	0.117	0.525	**0.018**	**0.048**
H_2O_2	**0.000**	**0.000**	ni	0.407	**0.000**	**0.000**	**0.011**	0.09	**0.044**	0.155
MDA	**0.000**	**0.001**	ni	0.366	**0.000**	**0.000**	**0.047**	0.224	**0.009**	0.204

Results shown are the p values following independent samples t-test and bold values suggest significant differences ($P < 5\%$). Other= Lettuce +2 or more ingredients. ni = the correlation and t-test could not be computed because the standard error of the difference was 0.

3.3.2.2.2. Total phenolic content, antioxidants, CO_2, H_2O_2 and lipid peroxidation

Salad producer

Purchase (actual) vs Expiring day during winter and summer

The effect of storage period of purchase and expiring day on plant-related parameters on different salad producers is presented in **Figures 3.29-3.35** and **Table 3.4.** Increased expiring CO_2 production was reported for producer A on both seasons (10.52 and 8.95% CO_2 for winter and summer, respectively). During winter, the polynomial curve with concave upward is described by $y=0.3216x^2-1.1189x+4.4455$; $R^2=0.44$, while the relevant curve (concave downward) during summer is described by $y=-0.2193x^2+2.5629x+1.5943$; $R^2=0.94$ (**Figure 3.33A**). For producer C, increased CO_2 production was also observed on expiring day for both seasons (14.25 and 12.09% CO_2 for winter and summer, respectively). During summer, the polynomial curve with concave downward is described by $y=-0.4411x^2+4.8788x-1.2666$; $R^2=0.88$, while the relevant curve (concave upward) during winter is described by $y=0.4674x^2-1.7828x+7.9173$; $R^2=0.50$ (**Figure 3.33C**). Moreover, salads from producer E presented higher CO_2 production on their expiring day on both seasons (7.73 and 10.31% CO_2 for winter and summer, respectively) compared to purchase day (3.24 and 3.44% CO_2 for winter and summer, respectively). The polynomial curve with concave upward for winter is described by $y=0.8554x^2-4.9645x+6.765$; $R^2=0.96$, while the relevant curve for summer is described by $y=1.56x^2-11.472x+23.244$; $R^2=0.97$ (**Figure 3.33E**). Higher expiring H_2O_2 levels were reported for samples from producer A during both seasons (10.04 and 5.86 μmol H_2O_2 /g Fw for winter and summer, respectively) and the polynomial curve with concave upward for winter is described by $y=0.0219x^2-0.1457x+0.5722$; $R^2=0.44$, while the relevant curve for summer is described by $y=0.0277x^2-0.1913x+0.5848$; $R^2=0.92$ (**Figure 3.34A**). Similarly, for producer C, increased H_2O_2 levels were observed on samples expiring day on both seasons (6.97 and 7.20 μmol H_2O_2 /g Fw for winter and summer, respectively). During winter, the polynomial curve with concave upward is described by $y=0.0209x^2-0.1735x+0.5001$; $R^2=0.98$, whilst the relevant curve (concave downward) during summer is described by $y=-0.0082x^2+0.0732x+0.1573$; $R^2=0.41$ (**Figure 3.34C**). Furthermore, during winter samples from producers D and E presented higher expiring H_2O_2 levels (9.58 and 7.81 μmol H_2O_2 /g Fw, respectively) compared to purchase day (0.33 and 0.40 μmol H_2O_2 /g Fw, respectively). During winter, the polynomial curve with concave upward for producer D is described by $y=0.0519x^2-0.3925x+0.8563$; $R^2=0.99$, while the relevant curve for producer E is described by $y=0.0207x^2-0.0554x+0.4204$; $R^2=0.52$ (**Figure 3.34D**, **3.34E**). Increased MDA levels were reported during expiring day for samples from producer A during both seasons (11.08 and 6.55 nmol MDA /g Fw for winter and summer, respectively). The polynomial curve with concave upward for winter is described by $y=0.138x^2-0.6603x+10.357$; $R^2=0.10$, while the relevant curve for summer is described by $y=0.0763x^2-0.4405x+6.3129$; $R^2=0.12$ (**Figure 3.35A**). Increased lipid peroxidation levels were also observed on samples expiring day for producer C on both seasons (10.89 and 8.26 nmol MDA /g Fw for winter and summer, respectively).

During winter, the polynomial curve with concave upward is described by $y=0.3184x^2-1.5166x+8.0841$; $R^2=0.52$, whereas the relevant curve (concave downward) during summer is described by $y=-0.3185x^2+3.0896x+0.661$; $R^2=0.58$ (**Figure 3.35C**). Samples collected during winter from producers D and E presented higher MDA levels on their expiring day (12.47 and 7.20 nmol MDA /g Fw, respectively) compared to purchase day (0.37 and 0.85 nmol MDA /g Fw, respectively) and the polynomial curve with concave upward for producer D on winter is described by $y=0.5684x^2-3.5204x+13.487$; $R^2=0.68$, while the relevant curve (concave downward) for producer E on the same season is described by $y=-0.0548x^2-0.2859x+7.5731$; $R^2=0.02$ (**Figure 3.35D**, **3.35E**). No significant differences ($P > 0.05$) were observed between the day of purchase and the expiring day of salads among producers during both seasons for their phenolic content and antioxidant capacity (with DPPH, FRAP and ABTS assays) (**Figure 3.29**, **3.30**, **3.31** and **3.32**, respectively). For both *S. enterica* and *L. monocytogenes* all samples were negative tested.

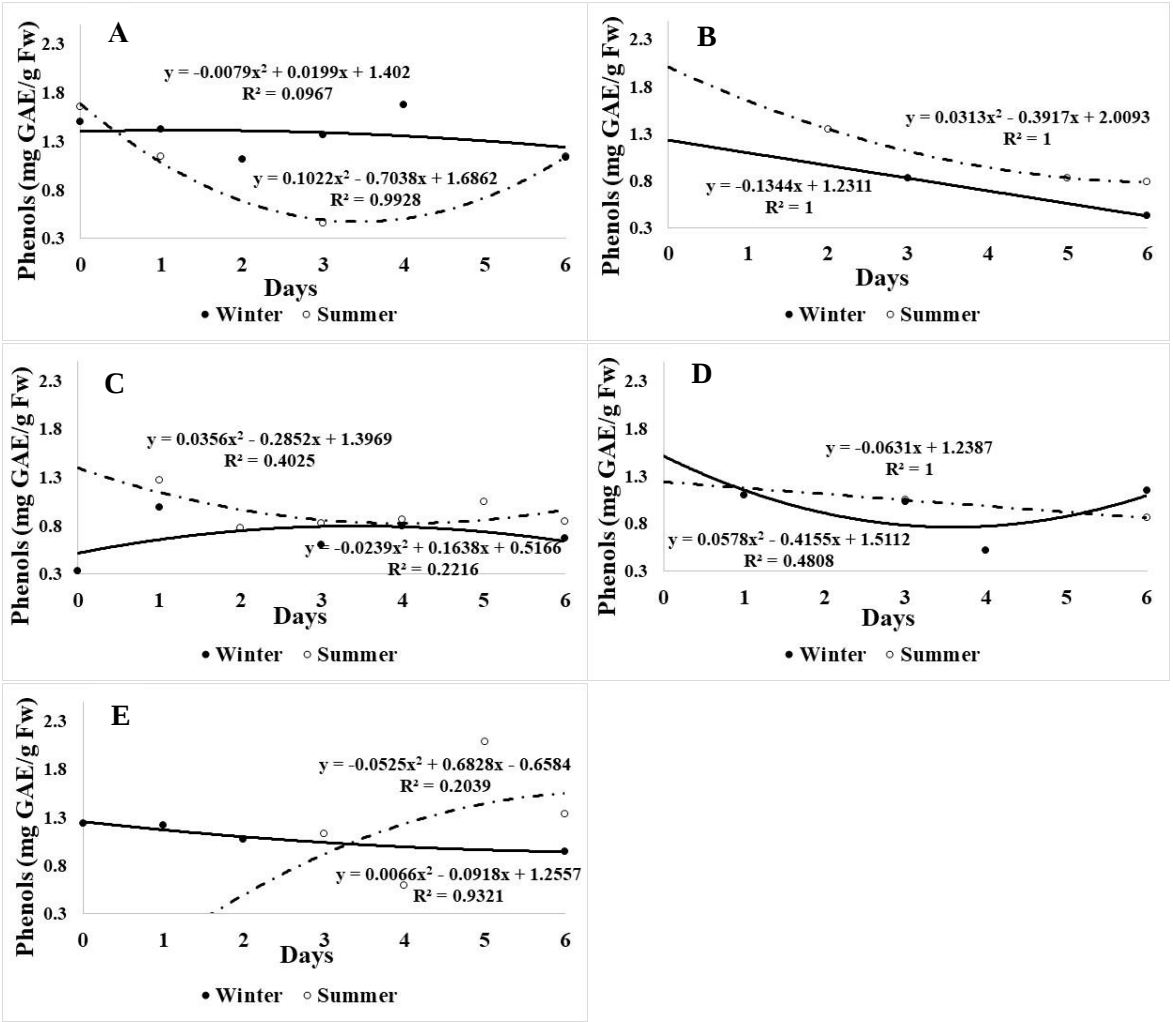


Figure 3.29. Effects of shelf life (days) on phenols per salad producer during winter and summer. A: producer A, B: producer B, C: producer C, D: producer D, E: producer E.

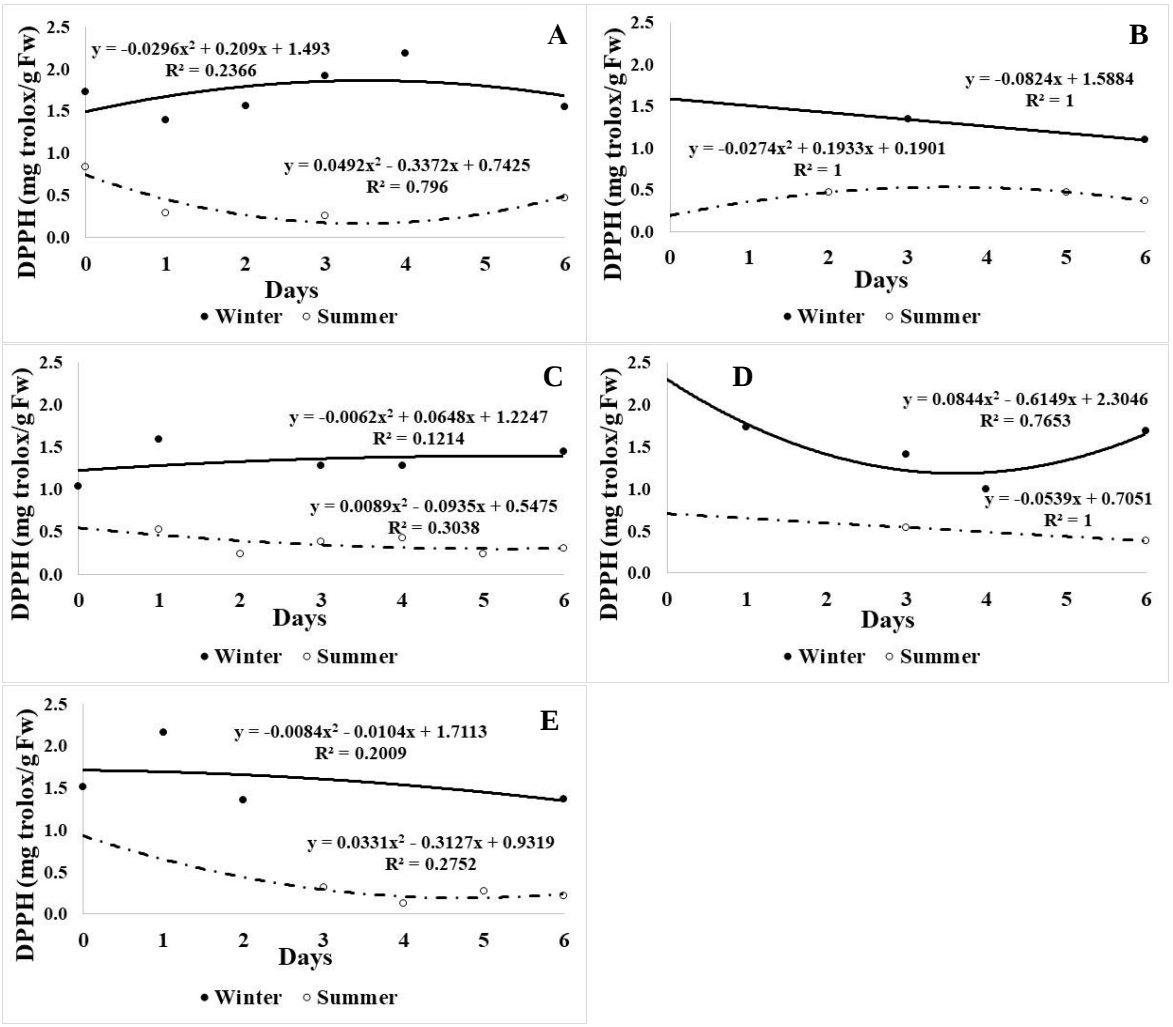


Figure 3.30. Effects of shelf life (days) on antioxidants (DPPH assay) per salad producer during winter and summer. A: producer A, B: producer B, C: producer C, D: producer D, E: producer E.

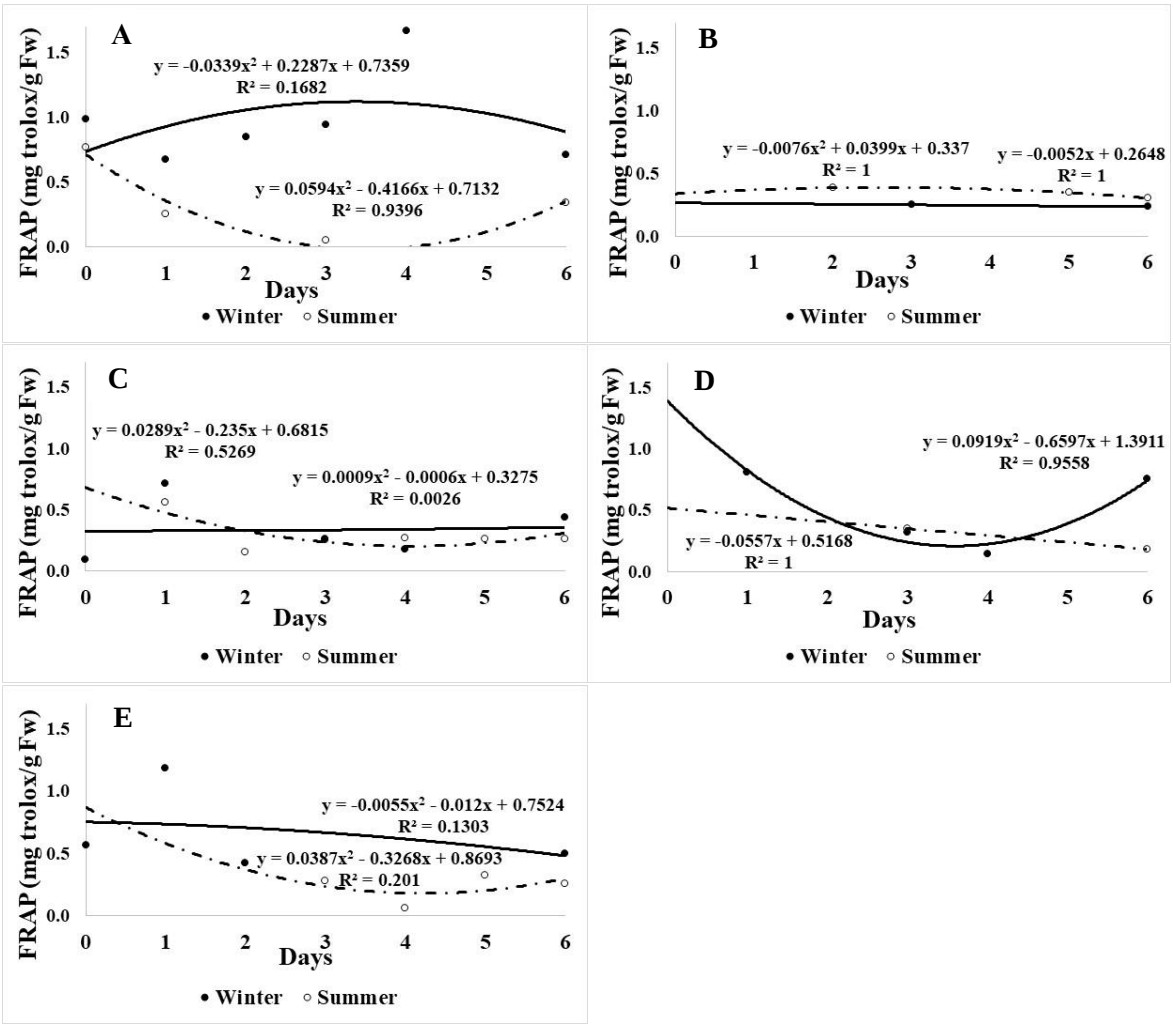


Figure 3.31. Effects of shelf life (days) on antioxidants (FRAP assay) per salad producer during winter and summer. A: producer A, B: producer B, C: producer C, D: producer D, E: producer E.

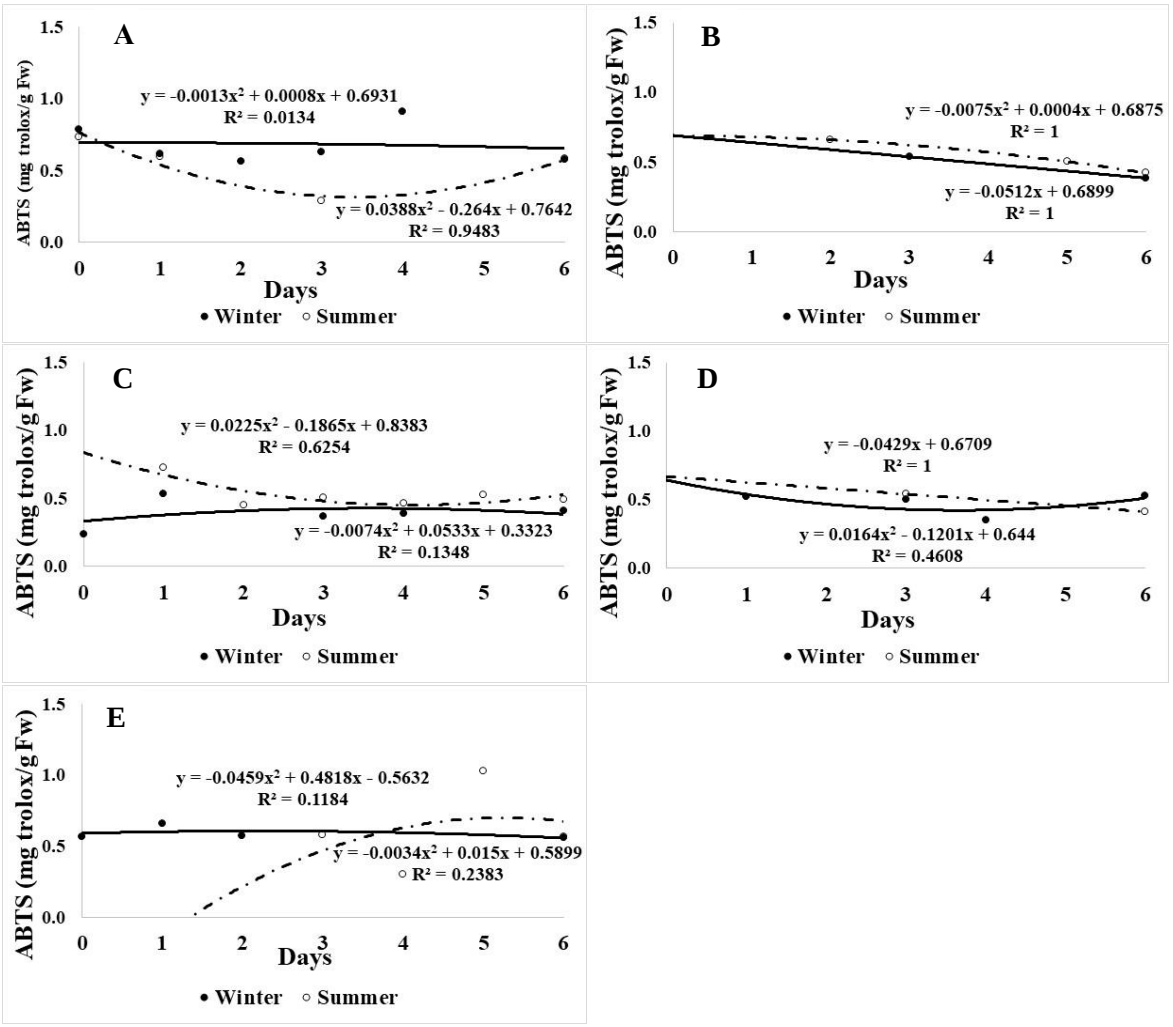


Figure 3.32. Effects of shelf life (days) on antioxidants (ABTS assay) per salad producer during winter and summer. A: producer A, B: producer B, C: producer C, D: producer D, E: producer E.

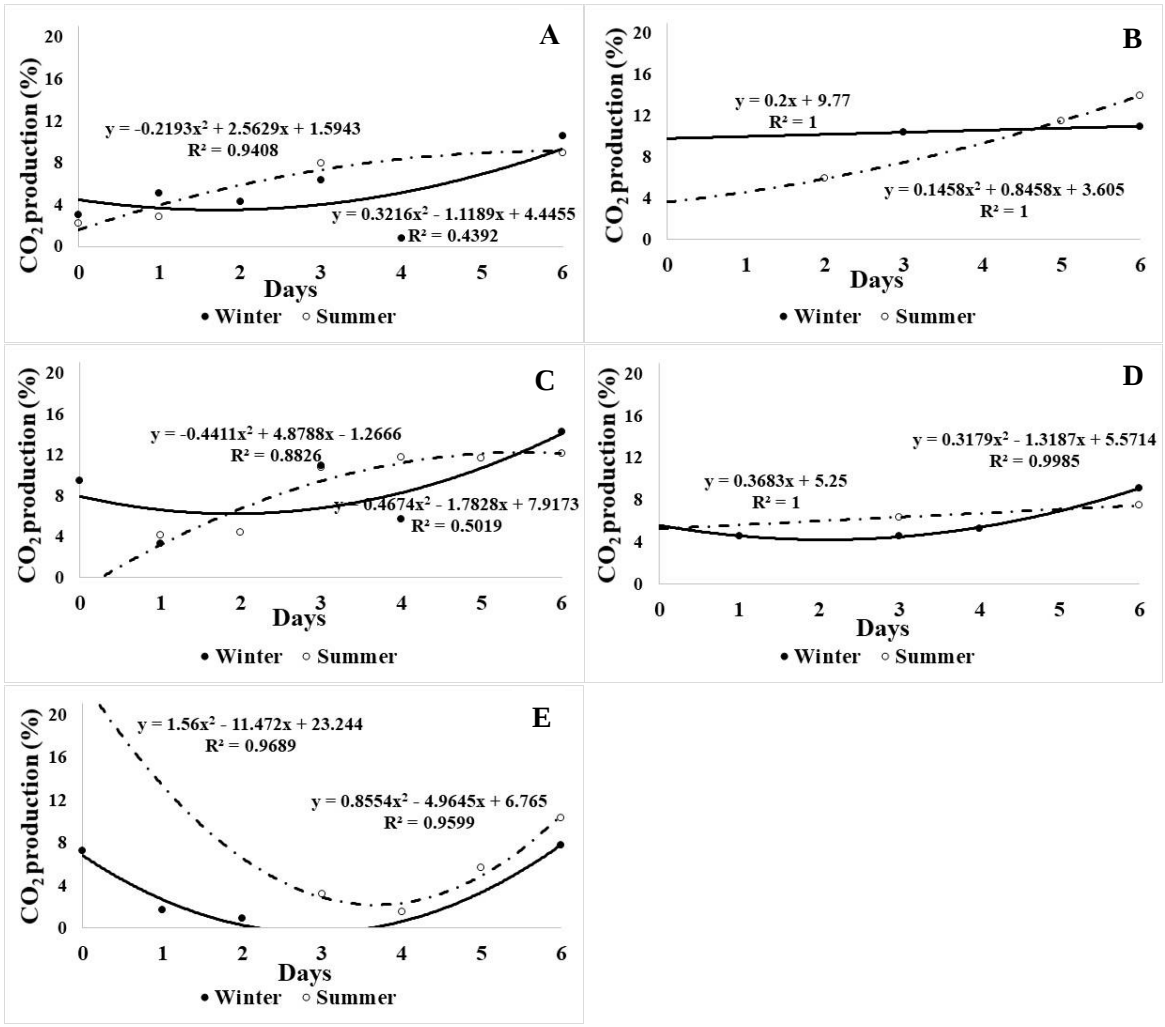


Figure 3.33. Effects of shelf life (days) on % CO_2 production per salad producer during winter and summer. A: producer A, B: producer B, C: producer C, D: producer D, E: producer E.

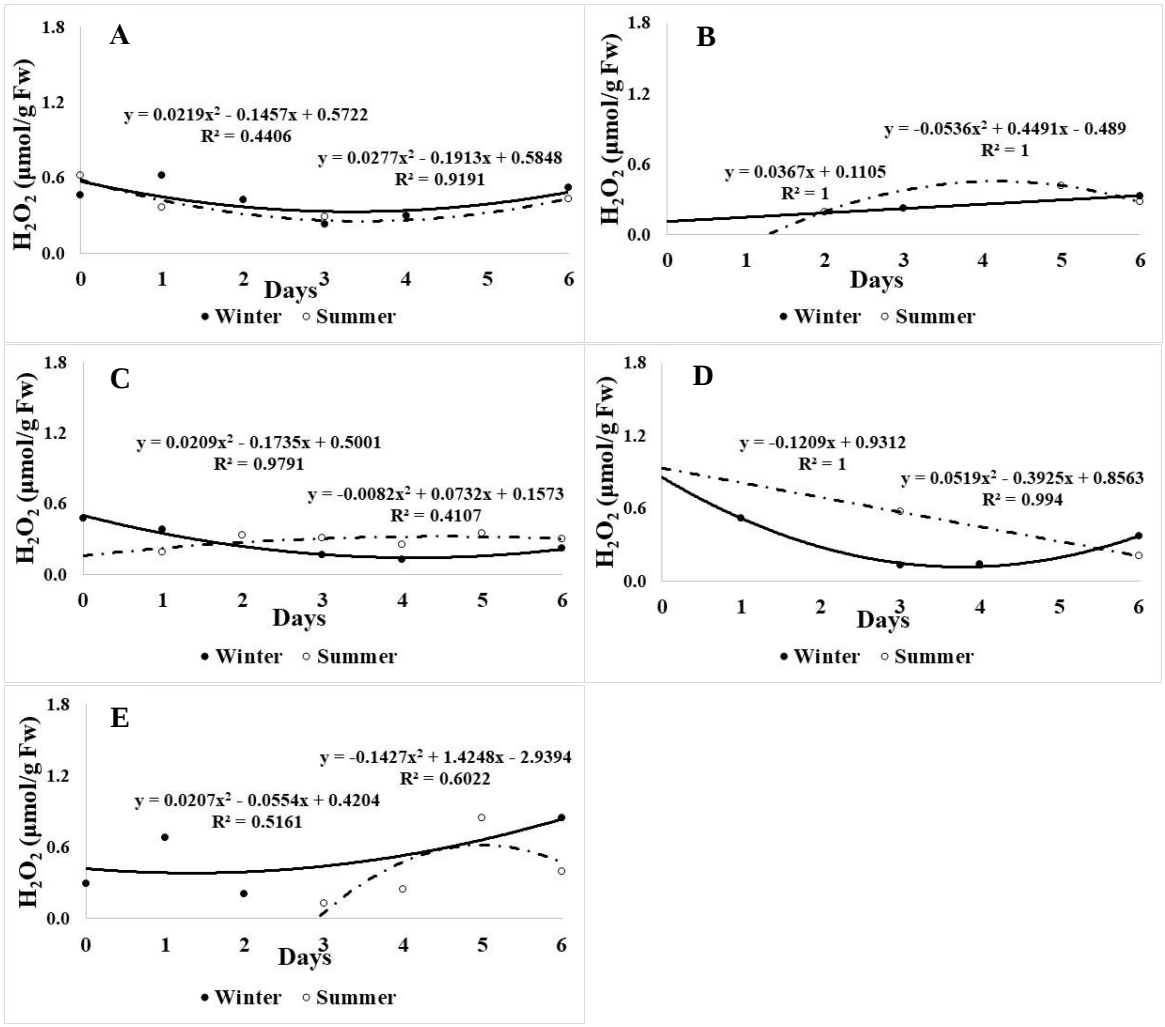


Figure 3.34. Effects of shelf life (days) on H_2O_2 levels per salad producer during winter and summer. A: producer A, B: producer B, C: producer C, D: producer D, E: producer E.

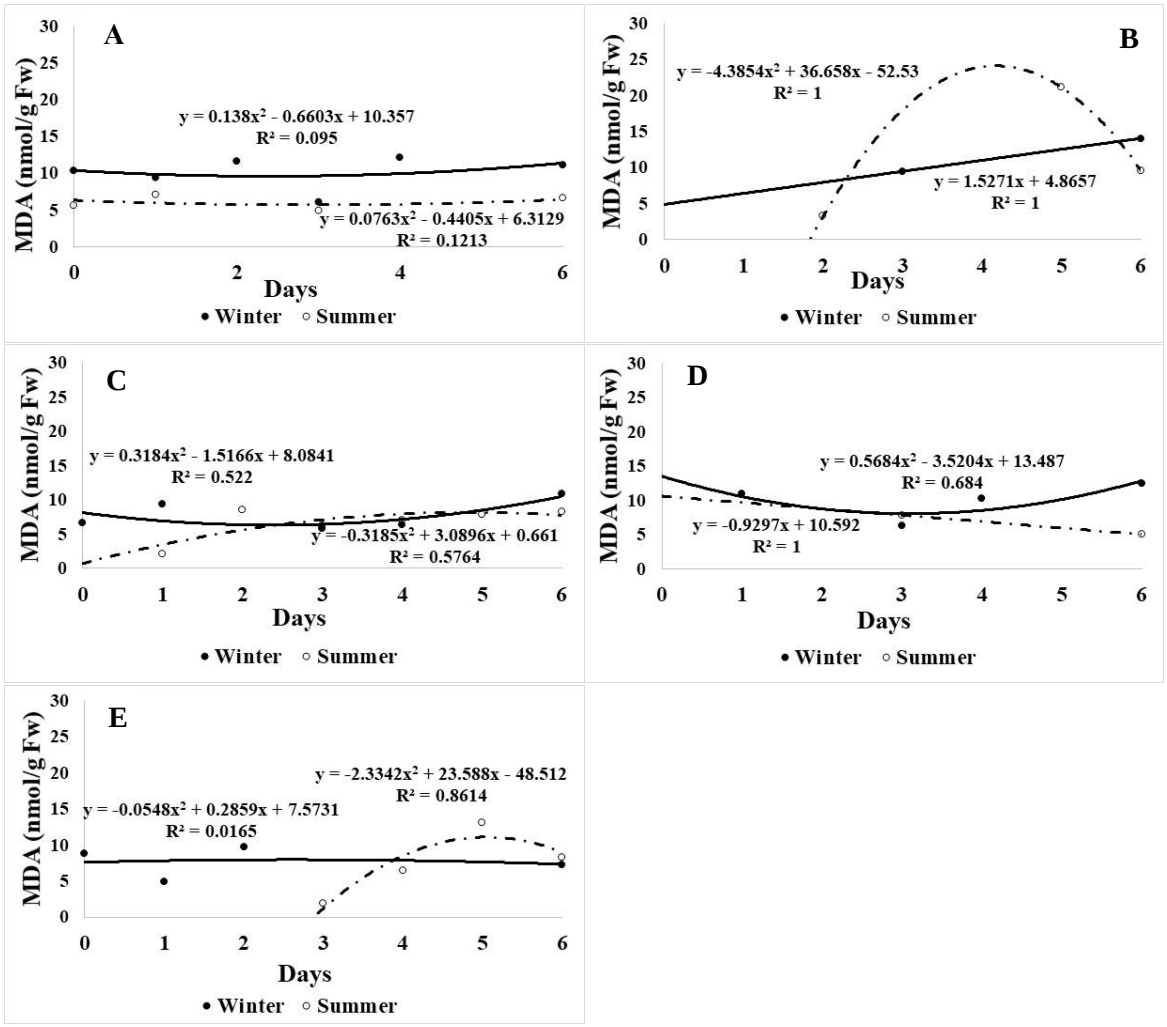


Figure 3.35. Effects of shelf life (days) on lipid peroxidation per salad producer during winter and summer. A: producer A, B: producer B, C: producer C, D: producer D, E: producer E.

Type of salad

Purchase (actual) vs Expiring day during winter and summer

The effect of storage period of purchase and expiring day on plant-related parameters on different types of salads is presented in **Figures 3.36-3.42** and **Table 3.5.** Increased phenolic content was observed on the expiring day during summer (P = 0.010) for lettuce+chives compared to purchase day (0.97 and 0.70 mg GAE/g Fw, respectively) and the polynomial curve with concave upward is described by $y=0.0398x^2-0.2518x+1.0533$; $R^2=1.00$ (**Figure 3.36E**, **Table 3.5**). During winter, increased FRAP antioxidant activity was reported on the expiring day for lettuce+cabbage compared to purchase day (FRAP: 0.55 and 0.23 mg trolox Fw/g, respectively), while plain rocket showed higher FRAP on the expiring day during summer compared to purchase day (FRAP: 0.34 and 0.32 mg trolox Fw/g, respectively) (**Figure 3.38B**, **3.38F**). The relevant polynomial curve with concave upward for

lettuce+cabbage is described by $y=0.0047x^2+0.0384x+0.1125$; $R^2=0.63$, while the respective curve for rocket is described by $y=0.0112x^2-0.0713x+0.3847$; $R^2=0.73$ (**Figure 3.38B**, **3.38F**).

Increased CO_2 production was observed for plain lettuce for both seasons on expiring day (7.87 and 7.89% CO_2 for winter and summer, respectively) compared to purchase day (3.45 and 2.72% CO_2 for winter and summer, respectively). During winter, the polynomial curve with concave upward is described by $y=0.3193x^2-1.293x+4.274$; $R^2=0.90$, whereas the respective curve during summer is described by $y=-0.1421x^2-0.203x+2.9354$; $R^2=0.36$ (**Figure 3.40A**). CO_2 production was higher during both seasons for lettuce+endive/radicchio on expiring day (10.35 and 10.94% CO_2 for winter and summer, respectively) compared to purchase day (5.78 and 7.42% CO_2 for winter and summer, respectively) and the polynomial curve with concave downward during winter is described by $y=-0.2781x^2+3.5527x-0.0031$; $R^2=0.98$, whereas the respective curve during summer is described by $y=-0.472x^2+5.0693x-2.9583$; $R^2=0.89$ (**Figure 3.40C**). Summer was the season in which lettuce+rocket and lettuce+chives presented higher CO_2 production on expiring day (9.25 and 9.26%CO_2, respectively) compared to purchase day (4.58 and 6.01% CO_2, respectively). During summer, the polynomial curve for lettuce+rocket with concave downward is described by $y=-0.5253x^2+5.6869x-5.9625$; $R^2=1.00$, whereas the relevant curve for lettuce+chives is described by $y=-0.0406x^2+0.6438x+3.965$; $R^2=1.00$ (**Figure 3.40D**, **3.40E**). Higher expiring CO_2 production was reported significantly higher on expiring day for plain rocket and lettuce+two or more ingredients (other) (P = 0.004 and 0.001, respectively) (14.25 and 10.31% CO_2, respectively) compared to purchase day (2.84 and 4.53% CO_2, respectively) (**Figure 3.40F**, **3.40G**, **Table 3.5**). During winter, the polynomial curve for rocket with concave upward is described by $y=0.2208x^2+0.6242x+2.555$; $R^2=1.00$, whereas the relevant curve for lettuce+two or more ingredients (other) is described by $y=0.343x^2-1.313x+5.0301$; $R^2=0.65$ (**Figure 3.40F**, **3.40G**).

Plain lettuce's expiring H_2O_2 levels were found significantly higher on both seasons (P = 0.000 and 0.002 for winter and summer, respectively) (6.91 and 5.11 μmol H_2O_2 /g Fw for winter and summer, respectively) compared to purchase day (0.34 and 0.45 μmol H_2O_2 /g Fw for winter and summer, respectively) (**Figure 3.41A**, **Table 3.5**). During winter, the polynomial curve with concave upward is described by $y=0.0292x^2-0.1805x+0.5055$; $R^2=0.37$, whereas the relevant curve for summer is described by $y=0.0048x^2-0.0596x+0.4689$; $R^2=0.20$ (**Figure 3.41A**). Expiring H_2O_2 levels for lettuce+cabbage were found higher on both seasons (6.04 and 7.32 μmol H_2O_2 /g Fw for winter and summer, respectively) compared to purchase day (0.28 and 0.0.23 μmol H_2O_2 /g Fw for winter and summer, respectively) and the polynomial curve for winter with concave upward is described by $y=0.0181x^2-0.1509x+0.4841$; $R^2=0.95$, whereas the relevant curve for summer is described by $y=0.0283x^2-0.2974x+0.94$; $R^2=0.67$ (**Figure 3.41B**). Increased H_2O_2 levels were found on both seasons for lettuce+endive/radicchio on expiring day (7.51 and 6.75 μmol H_2O_2 /g Fw for winter and summer, respectively) compared to purchase day (0.26 and 0.27 μmol H_2O_2 /g Fw for winter and summer, respectively) (**Figure 3.41C**). During winter, the polynomial curve with concave downward is

described by $y=-0.0111x^2+0.077x+0.1404$; $R^2=0.27$, whereas the relevant curve for summer (concave upward) is described by $y=0.0097x^2-0.0388x+0.2897$; $R^2=0.57$ (**Figure 3.41C**). H_2O_2 levels for lettuce+chives were increased on summer during expiring day compared to purchase day (4.67 and 0.30 μmol H_2O_2 /g Fw, respectively) and the polynomial curve with concave upward is described by $y=0.0532x^2-0.3693x+0.861$; $R^2=1.00$ (**Figure 3.41E**). Plain rocket's expiring H_2O_2 levels were found significantly increased on both seasons (P = 0.035 and 0.003 for winter and summer, respectively) (12.11 and 9.60 μmol H_2O_2 /g Fw for winter and summer, respectively) compared to purchase day (0.79 and 0.55 μmol H_2O_2 /g Fw for winter and summer, respectively) (**Figure 3.41F**, **Table 3.5**). During winter, the polynomial curve with concave downward is described by $y=-0.1148x^2+0.6913x+0.6017$; $R^2=1.00$, whereas the relevant curve for summer is described by $y=-0.0193x^2+0.1774x+0.2264$; $R^2=0.73$ (**Figure 3.41F**). Expiring H_2O_2 levels for lettuce+two or more ingredients (other) were found higher on both seasons (12.46 and 5.90 μmol H_2O_2 /g Fw for winter and summer, respectively) and the polynomial curve for winter with concave upward is described by $y=0.0101x^2-0.0294x+0.4121$; $R^2=0.20$, whereas the respective curve for summer is described by $y=0.0291x^2-0.22154x+0.6452$; $R^2=1.00$ (**Figure 3.41G**).

Plain lettuce's lipid peroxidation levels were found significantly increased on both seasons during expiring day (P = 0.000 for both) (8.08 and 4.91 nmol MDA /g Fw for winter and summer, respectively) compared to purchase day (0.49 and 0.27 nmol MDA /g Fw for winter and summer, respectively) (**Figure 3.42A**, **Table 3.5**). During winter, the polynomial curve with concave upward is described by $y=0.2601x^2-1.6429x+8.567$; $R^2=0.99$, whereas the respective curve for summer with concave downward is described by $y=-0.0423x^2+0.4921x+4.0235$; $R^2=0.13$ (**Figure 3.42A**). Expiring MDA levels for lettuce+cabbage were found higher on both seasons (10.39 and 7.14 nmol MDA /g Fw for winter and summer, respectively) and the polynomial curve for winter with concave upward is described by $y=0.3637x^2-1.5484x+6.615$; $R^2=1.00$, whereas the relevant curve for summer is described by $y=1.5875x^2-15.648x+43.292$; $R^2=0.74$ (**Figure 3.42B**). Increased expiring lipid peroxidation levels were found on both seasons for lettuce+endive/radicchio (8.54 and 5.95 nmol MDA /g Fw for winter and summer, respectively) compared to purchase day (0.21 and 0.36 nmol MDA /g Fw for winter and summer, respectively) (**Figure 3.42C**). During winter for lettuce+endive/radicchio, the polynomial curve with concave downward is described by $y=-0.052x^2+0.7069x+6.1172$; $R^2=0.59$, whereas the relevant curve for summer is described by $y=-0.5544x^2+5.7334x-3.2237$; $R^2=0.29$ (**Figure 3.42C**). Plain rocket's expiring MDA levels were found significantly increased on both seasons (P = 0.004 and 0.006 for winter and summer, respectively) (15.27 and 15.19 nmol MDA /g Fw for winter and summer, respectively) and the polynomial curve for winter with concave upward is described by $y=0.9881x^2-7.2301x+19.413$; $R^2=1.00$, whereas the relevant curve for summer is described by $y=0.1919x^2-0.6568x+4.8891$; $R^2=1.00$ (**Figure 3.42F**, **Table 3.5**). Lipid peroxidation levels for lettuce+two or more ingredients (other) were increased on expiring day for both seasons (14.54 and 5.80 nmol MDA /g Fw

for winter and summer, respectively) and the polynomial curve for winter with concave downward is described by $y=-0.3059x^2+2.4755x+9.6229$; $R^2=0.29$, whereas the relevant curve for summer is described by $y=-0.0791x^2+0.4891x+5.7124$; $R^2=1.00$ (**Figure 3.42G**).

No significant differences ($P > 0.05$) were observed between the day of purchase and the expiring day of salads among seasons for their antioxidant activity (with DPPH and ABTS assays) (**Figure 3.37** and **3.39**, respectively). For both *S. enterica* and *L. monocytogenes* all samples were negative tested.

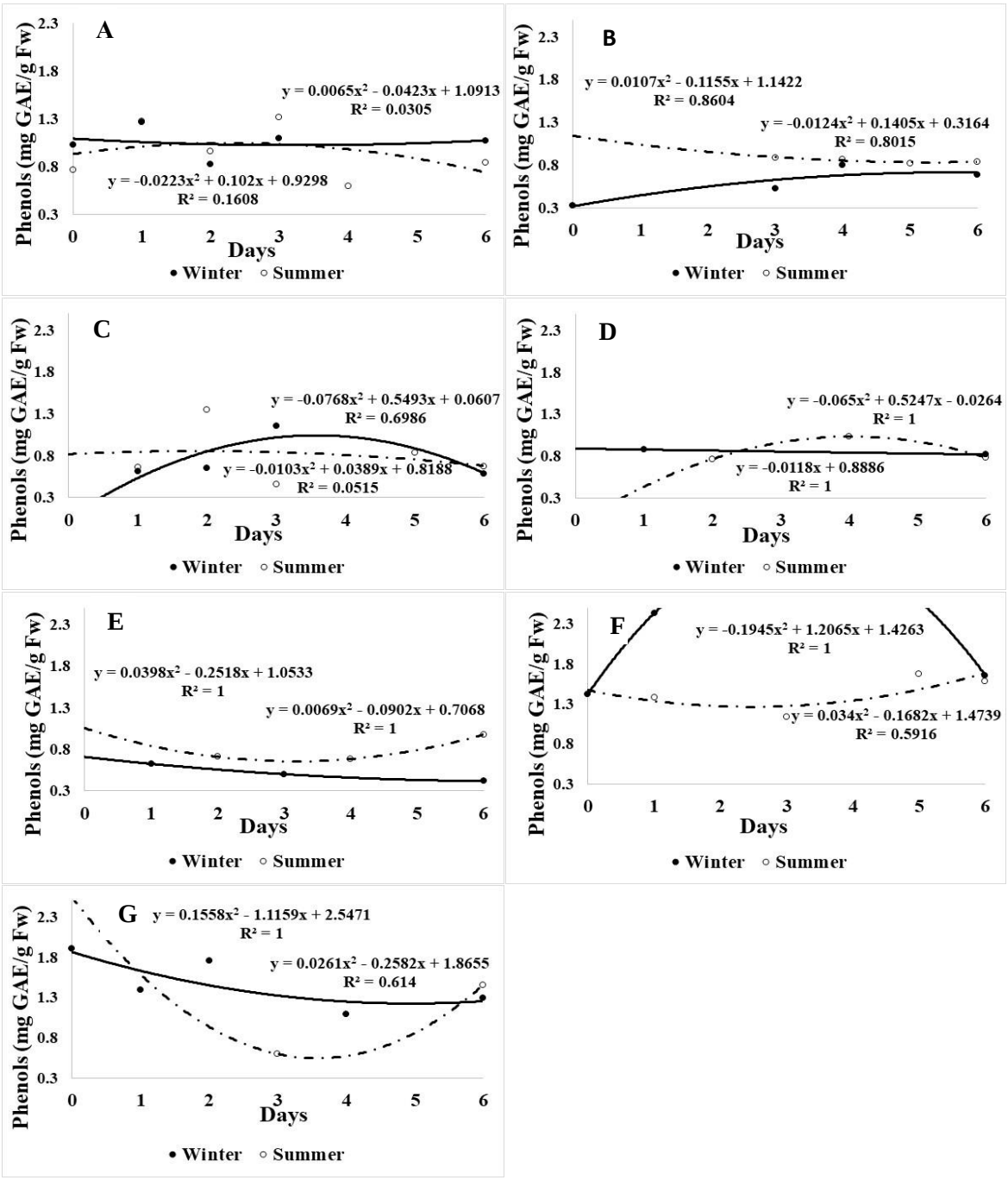


Figure 3.36. Effects of shelf life (days) on phenols per type of salad during winter and summer. A: lettuce, B: lettuce + cabbage, C: lettuce + radicchio/endive, D: lettuce + rocket, E: lettuce + chives, F: rocket, G: lettuce + two/more ingredients (other).

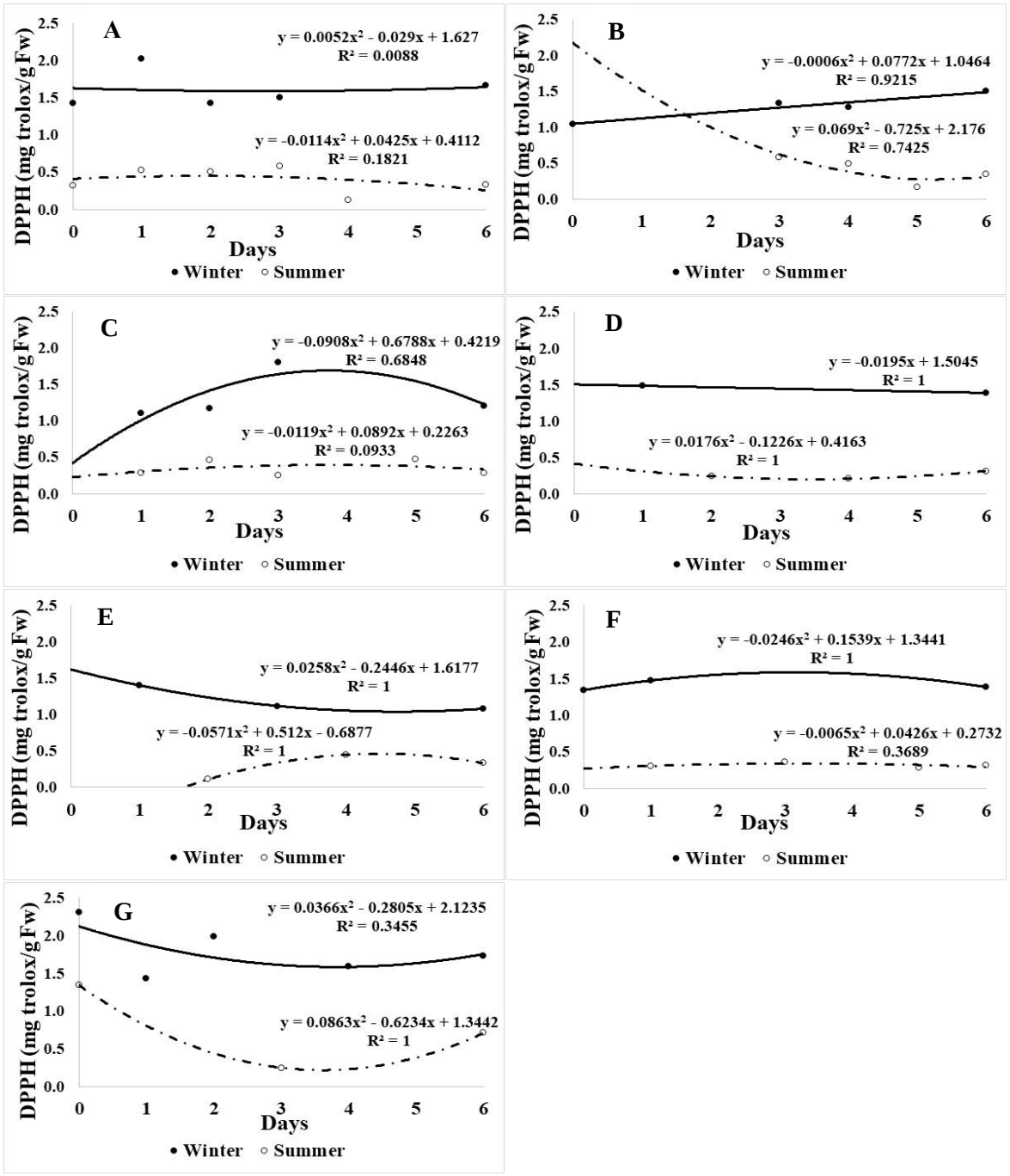


Figure 3.37. Effects of shelf life (days) on antioxidants (DPPH assay) per type of salad during winter and summer. A: lettuce, B: lettuce + cabbage, C: lettuce + radicchio/endive, D: lettuce + rocket, E: lettuce + chives, F: rocket, G: lettuce + two/more ingredients (other).

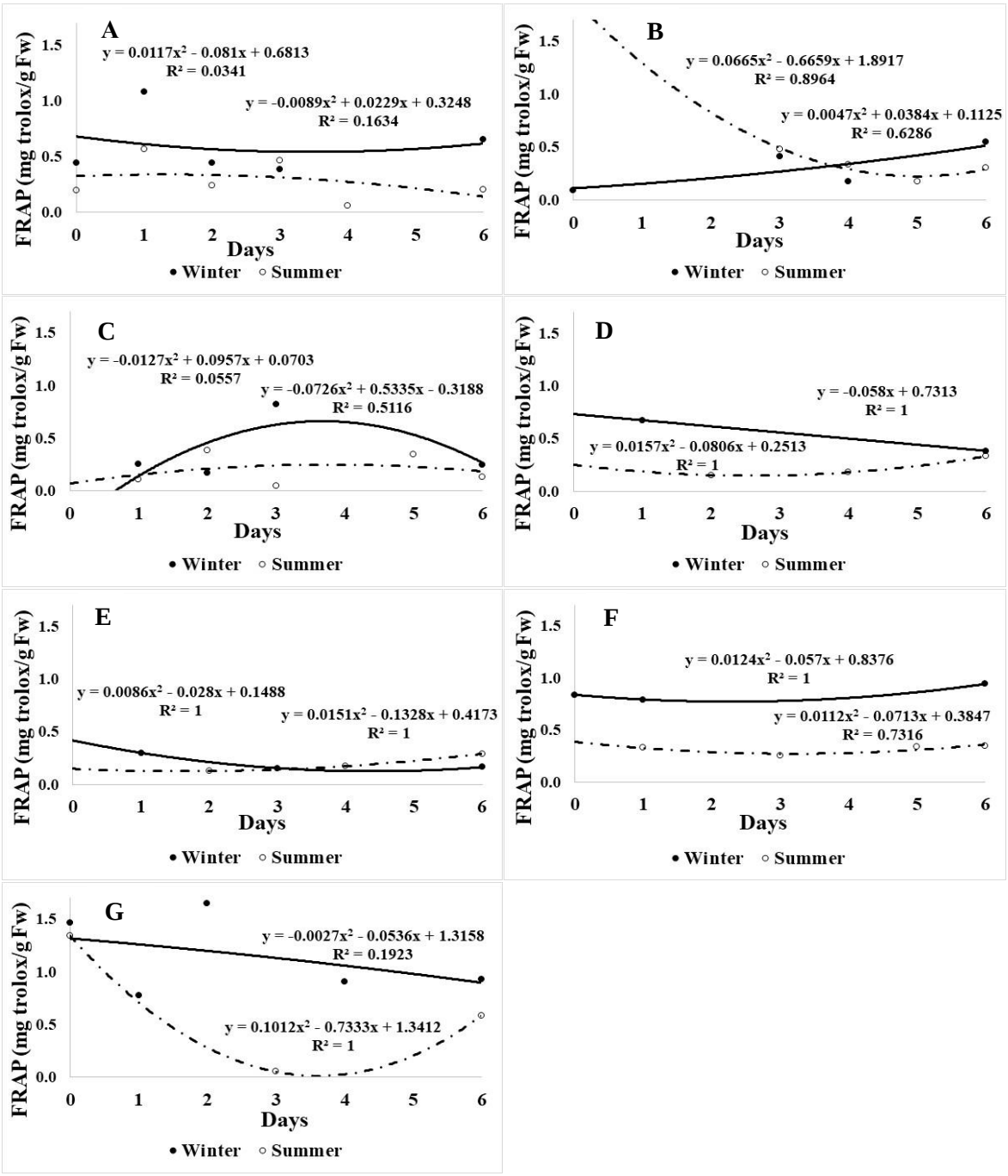


Figure 3.38. Effects of shelf life (days) on antioxidants (FRAP assay) per type of salad during winter and summer. A: lettuce, B: lettuce + cabbage, C: lettuce + radicchio/endive, D: lettuce + rocket, E: lettuce + chives, F: rocket, G: lettuce + two/more ingredients (other).

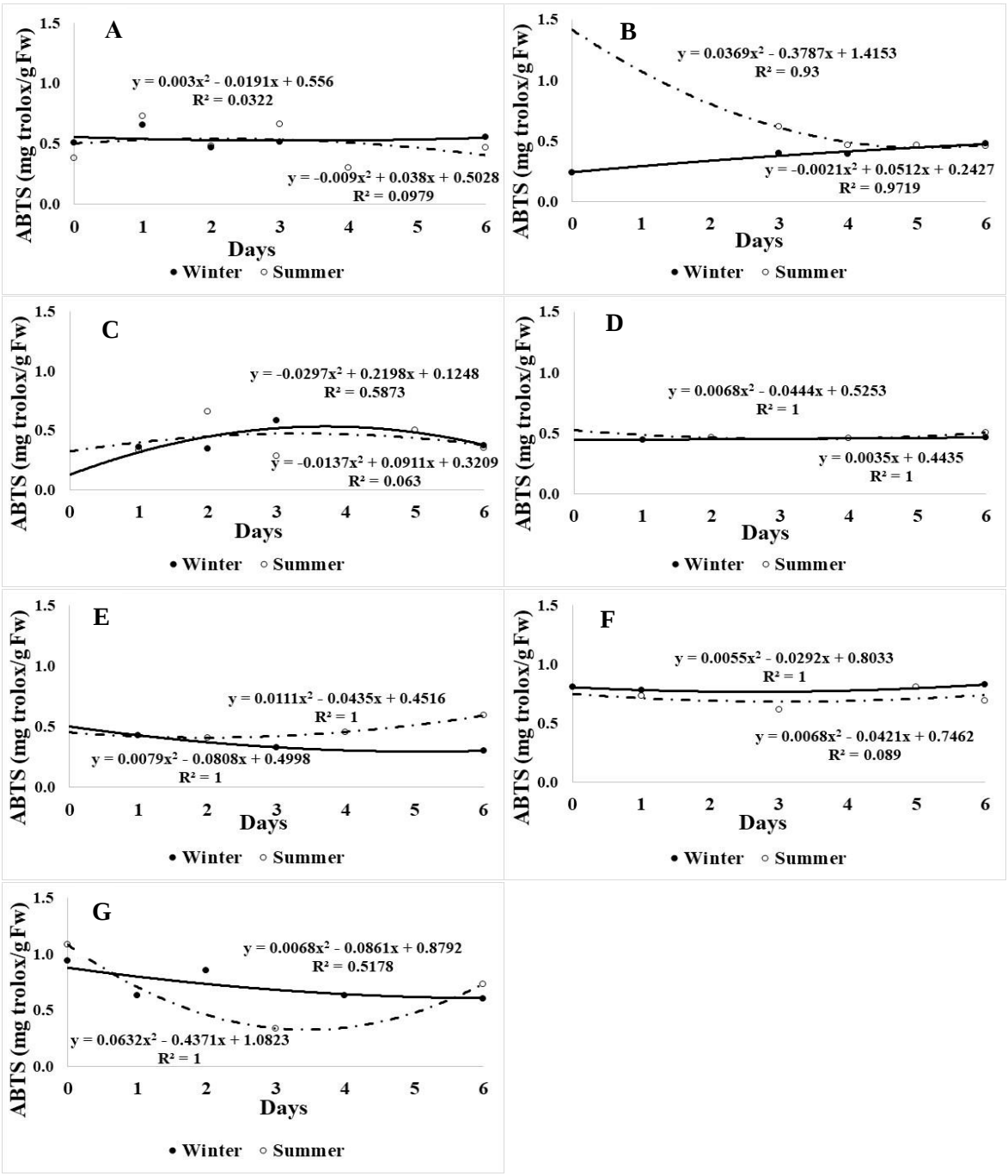


Figure 3.39. Effects of shelf life (days) on antioxidants (ABTS assay) per type of salad during winter and summer. A: lettuce, B: lettuce + cabbage, C: lettuce + radicchio/endive, D: lettuce + rocket, E: lettuce + chives, F: rocket, G: lettuce + two/more ingredients (other).

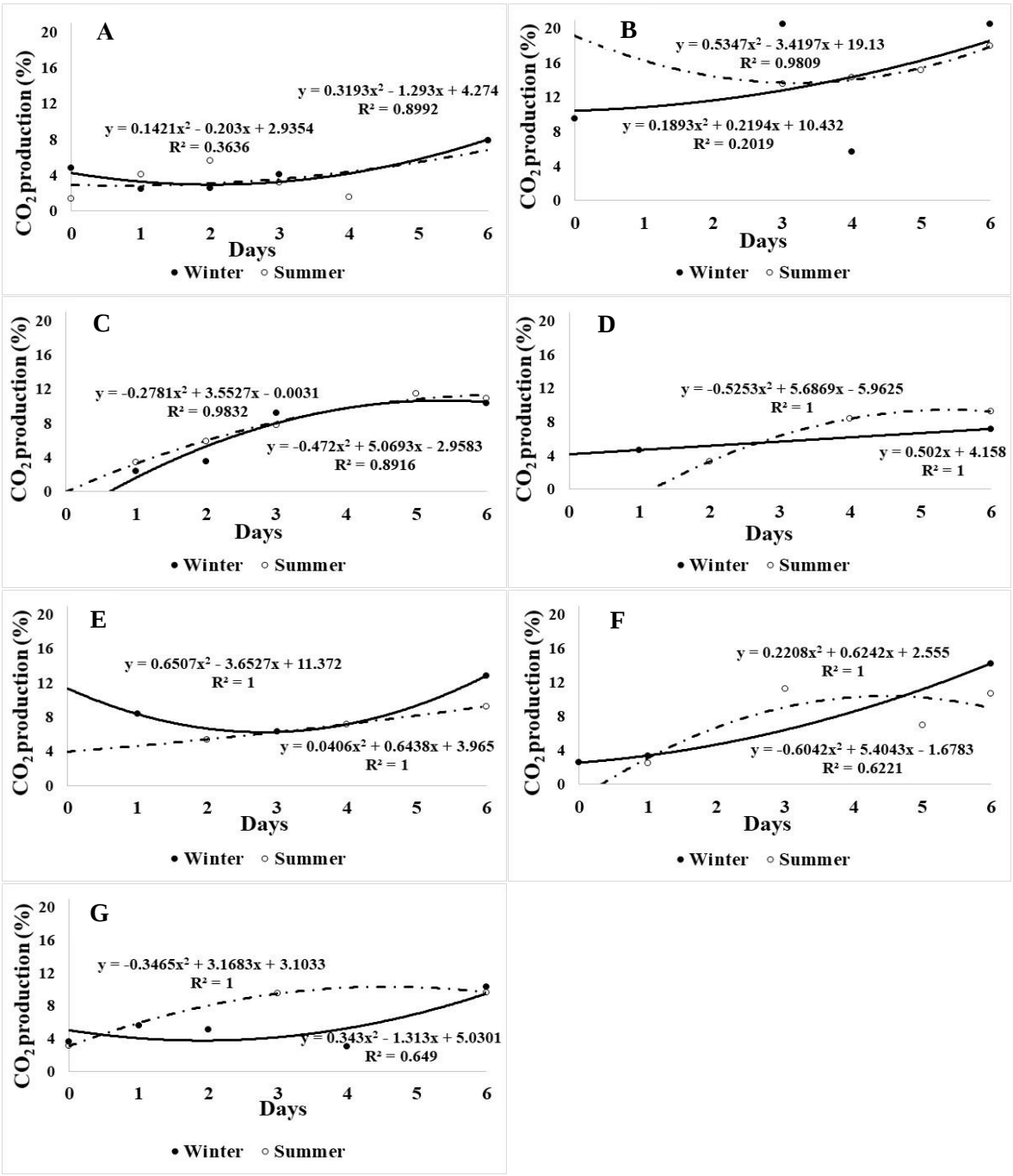


Figure 3.40. Effects of shelf life (days) on % CO_2 production per type of salad during winter and summer. A: lettuce, B: lettuce + cabbage, C: lettuce + radicchio/endive, D: lettuce + rocket, E: lettuce + chives, F: rocket, G: lettuce + two/more ingredients (other).

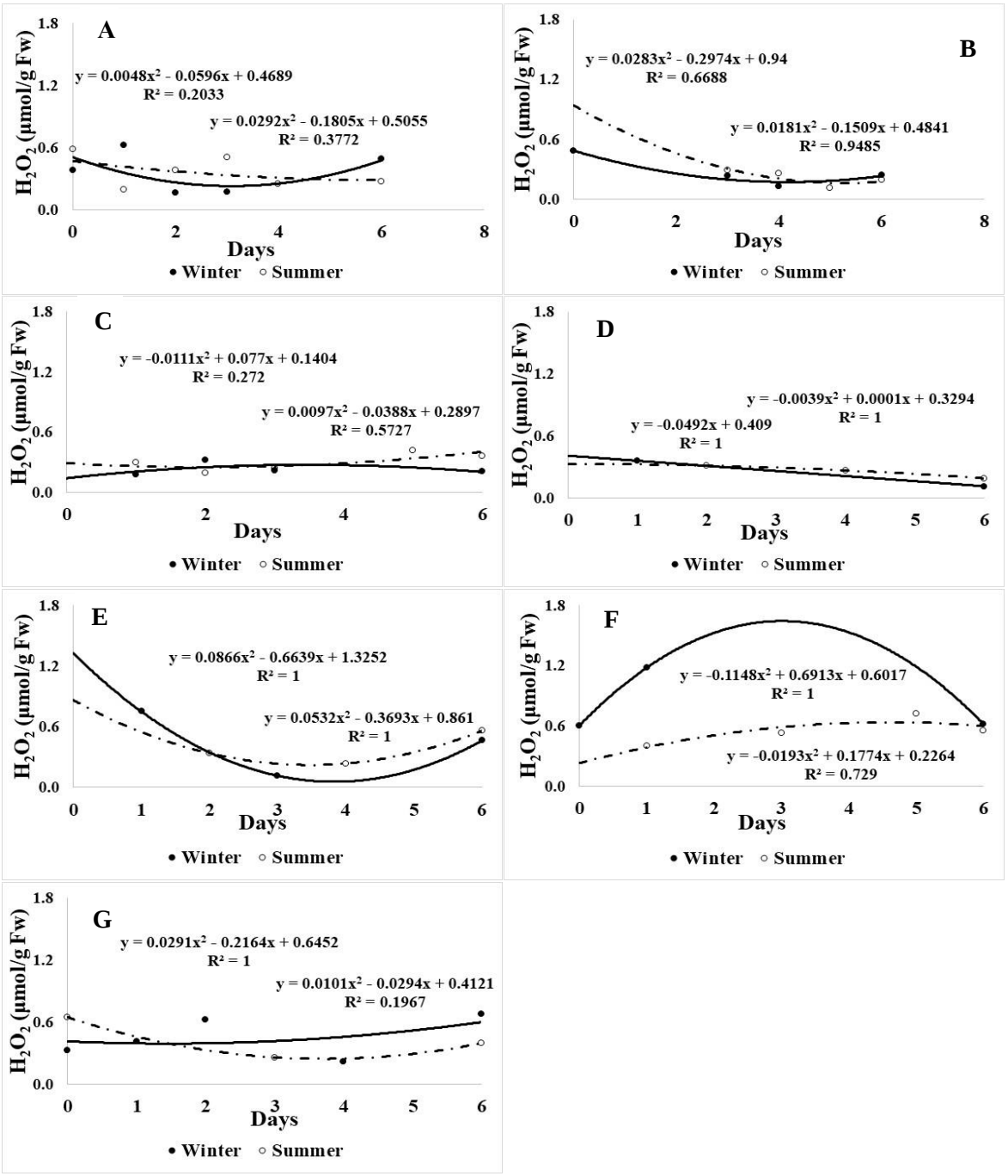


Figure 3.41. Effects of shelf life (days) on H_2O_2 levels per type of salad during winter and summer. A: lettuce, B: lettuce + cabbage, C: lettuce + radicchio/endive, D: lettuce + rocket, E: lettuce + chives, F: rocket, G: lettuce + two/more ingredients (other).

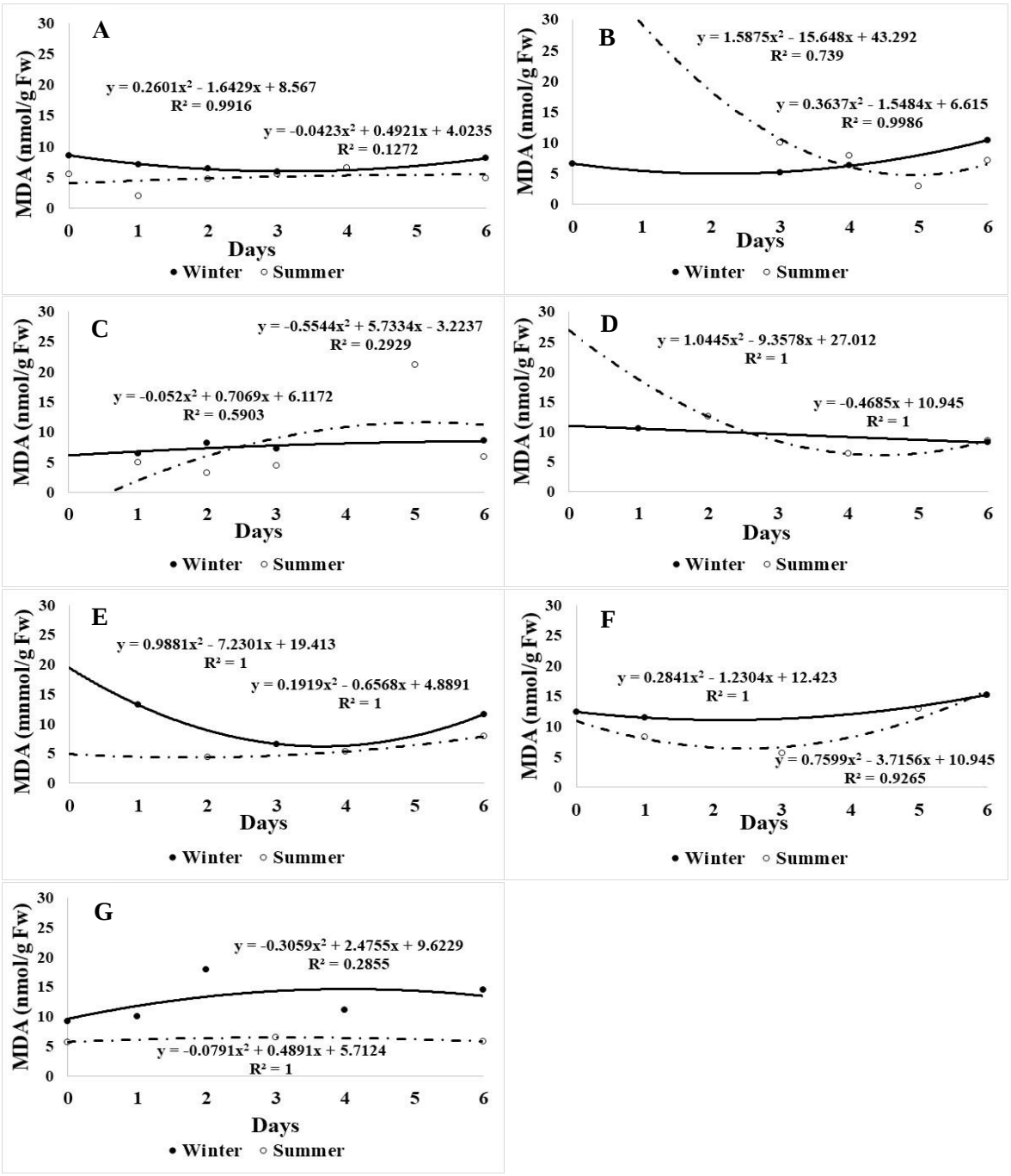


Figure 3.42. Effects of shelf life (days) on lipid peroxidation per type of salad during winter and summer. A: lettuce, B: lettuce + cabbage, C: lettuce + radicchio/endive, D: lettuce + rocket, E: lettuce + chives, F: rocket, G: lettuce + two/more ingredients (other).

Table 3.5. Effect of shelf-life on microbiological load (log cfu/g), total phenolic content (mg GAE/g Fw), antioxidants (mg trolox/g Fw), $\%CO_2$ and stress markers- H_2O_2 (µmol/g Fw) and MDA (nmol/g Fw) of ready to eat salads according to type of salad during winter and summer.

	Type of salad													
	Lettuce		**Lettuce+Cabbage**		**Lettuce+Endive/ radicchio**		**Lettuce+Rocket**		**Lettuce+Chives**		**Rocket**		**Other**	
	Winter	**Summer**	**Winter**	**Summer**	**Winter**	**Summer**	**Winter**	**Summer**	**Winter**	**Summer**	**Winter**	**Summer**	**Winter**	**Summer**
TVC	0.386	0.898	0.361	0.263	**0.029**	**0.027**	ni	**0.012**	0.294	0.384	**0.036**	0.810	**0.019**	0.943
Enterobacteriaceae	0.244	0.313	0.516	0.366	**0.011**	**0.024**	ni	0.206	0.286	0.389	0.482	0.999	0.121	0.871
Coliforms	**0.039**	0.145	0.686	0.364	**0.001**	0.276	ni	0.261	0.130	0.453	0.500	0.602	0.146	0.189
E. coli	ni	0.181	ni	0.178	ni	0.356	ni	0.437	ni	0.423	ni	0.648	0.343	0.391
***Staphylococcus* spp.**	0.647	0.213	0.423	0.698	0.356	0.065	ni	0.667	ni	0.186	0.363	0.632	0.686	0.391
B. cereus	ni	ni	ni	ni	ni	ni	ni	0.391	ni	ni	ni	0.348	0.343	0.391
Lactic acid bacteria	0.197	0.784	0.307	0.551	0.105	0.206	ni	**0.025**	0.051	0.469	0.223	0.985	0.060	0.391
***Pseudomonas* spp.**	0.880	0.397	0.423	0.778	0.298	0.135	ni	**0.040**	0.551	0.089	0.856	0.220	0.633	0.547
Yeasts and molds	0.188	0.159	0.406	0.180	0.329	0.309	ni	**0.001**	0.855	0.705	0.306	0.167	0.685	0.952
Phenols	0.982	0.496	0.487	0.884	0.168	0.988	ni	0.796	0.164	**0.010**	0.768	0.342	0.174	0.207
DPPH	0.797	0.419	0.161	0.512	0.234	0.583	ni	0.466	0.071	0.241	0.978	0.925	0.759	0.243
FRAP	0.821	0.355	**0.013**	0.843	0.384	0.696	ni	0.256	0.227	0.149	0.497	**0.044**	0.271	0.223
ABTS	0.883	0.725	0.059	0.563	0.293	0.660	ni	0.387	0.092	0.101	0.085	0.534	0.059	0.371
CO_2	**0.000**	**0.014**	0.192	0.098	**0.017**	**0.016**	ni	**0.016**	0.173	**0.035**	**0.004**	0.085	**0.001**	0.050
H_2O_2	**0.000**	**0.002**	**0.005**	**0.016**	**0.000**	**0.040**	ni	0.128	0.193	**0.014**	**0.035**	**0.003**	**0.000**	**0.020**
MDA	**0.000**	**0.000**	**0.029**	**0.001**	**0.000**	**0.023**	ni	0.069	0.314	0.105	**0.004**	**0.006**	**0.000**	**0.034**

Results shown are the *p* values following paired samples t-test and bold values suggest significant differences ($P \leq 5\%$). Other= Lettuce +2 or more ingredients. ni = the correlation and t-test could not be computed because the standard error of the difference was 0.

3.3.3. Discussion

Higher *E. coli* populations were observed for samples from producer A and C during summer compared to winter samples, while samples from producer C showed increased *B. cereus* counts during summer. It has been previously mentioned that *Bacillus* spp. and *Pseudomonas* spp. (including *Bacillus mojavensis*, *Bacillus megaterium* and *P. fluorescens*) have been isolated from ready-to-at salads (Santos et al., 2012). The presence of these bacteria may accelerate the degradation of vegetables or they can antagonize foodborne pathogens such as *Listeria monocytogenes* and *Salmonella enterica* in that environment (Santos et al., 2012). During winter higher levels of spoilage microorganisms such as yeasts and molds were reported for producers A and B. Furthermore, *Pseudomonas* spp. counts were increased for producer B during winter, whilst LAB were found higher for producers A, C and E on the same season. The presence of LAB was evident since the beginning of processing of ready-to-eat vegetables and increased numbers were reported after seven days of storage at 4 ^{o}C for sliced cabbage (air packaging), iceberg lettuce chopped (MAP), mixed endive, radicchio and "lollo rosso" lettuce (MAP) (Pothakos et al., 2014). This might suggest that LAB are part of the endogenous and epiphytic microflora of raw fresh produce. In a study conducted in Italy, no significant difference on yeasts and molds populations of ready-to-eat salads (rocket, baby leaf lettuce and lamb's lettuce) was reported among spring and summer (De Corato, 2012). No significant differences were observed for TVC, *Enterobacteriaceae*, coliforms and *Staphylococcus* spp. among seasons for all producers. On the other hand, aerobic psychotropic microorganisms were found in high numbers (up to 8.5 log cfu/g) in ready-to-eat salads collected during summer in Portugal (Santos et al., 2012).

Total phenolic content was increased during summer for samples from producer C, while antioxidants were increased for all salad producers during winter. Caponigro et al. (2010) reported higher average visual quality during winter and spring compared to summer and autumn. These findings might suggest less phenolic oxidation levels and other degradative processes that can compromise the nutritional value (phenols, antioxidants) of these products. Lipid peroxidation increased for samples collected from producer A on summer compared to winter. Kang and Saltveit (2002) have previously mentioned that wounding of plant tissue (i.e. from cutting) can induce increased antioxidant activity in romaine and iceberg lettuce. No differences were observed for CO_2 production and H_2O_2 levels among samples for all producers among seasons.

Summer was the season in which *E. coli* counts were found to be higher for plain lettuce. *Staphylococcus* spp. was found in increased numbers on summer for the lettuce+endive/radicchio, lettuce+rocket and lettuce+chives type of salads, whilst for plain rocket increased *Staphylococcus* spp. was reported during winter. Bell et al. (2017) reported significantly increased microbial load (total aerobic counts) of rocket salad during shelf-life. Decreased LAB populations were observed on summer for all types of salad, while *Pseudomonas* spp. was found in higher numbers for lettuce+cabbage during summer, while higher counts were also reported on winter for lettuce+rocket. High yeasts and molds

counts were observed on winter for lettuce+endive/radicchio, lettuce+rocket and lettuce+two or more ingredients (other). De Corato (2012) reported that lettuce salad presented lower yeasts and molds counts compared to rocket and lamb's lettuce. No significant differences were observed for TVC, *Enterobacteriaceae*, coliforms and *B. cereus* for all producers among seasons. Santos et al., (2012) reported increased aerobic psychotropic microorganisms for ready-to-eat salads (romaine lettuce and mixed vegetable salads) collected during summer compared to spinach samples during the same season.

Increased phenolic content of the lettuce+cabbage and lettuce+chives was observed on summer, while high antioxidant capacity of samples were observed on winter. Moreover, plain lettuce and rocket showed higher antioxidant activity during winter (as shown by the FRAP assay). Higher lipid peroxidation was reported on winter for plain lettuce and lettuce+two or more ingredients (other). Ferrante et al. (2009) reported higher lipid peroxidation values on fresh-cut lamb's lettuce leaves compared to intact ones when stored at 4 ^{o}C up to eight days (up to 51 nmol MDA/g Fw), suggesting that processing such as cutting along with storage duration and conditions induce plant stress. No differences were observed for CO_2 production and H_2O_2 levels among samples for all producers among seasons. On the other hand, in another study high CO_2 production was reported for rocket salads stored at 5 and 10 ^{o}C and this could be attributed to the high respiration rate of rocket as well as to the abusive storage temperatures (optimum storage conditions for rocket: 0 ^{o}C with 95–100% RH) (Amodio et al., 2015).

Expiring date of ready-to-eat salads is a matter of high importance since minimally processed vegetables reaching the maximum of their shelf life start to present defects such as wilting, browning (loss of green color) development of off-odors and off flavors that reduce product's acceptance from consumers (Nousiainen et al., 2016). Furthermore, increased spoilage (mostly) and pathogenic microorganisms have been reported when these products reach their expiring date (Caponigro et al., 2010; Cavaiuolo et al., 2015; Nousiainen et al., 2016). Higher TVC numbers were reported during expiring date on both seasons for producer A and high *Enterobacteriaceae* numbers were also reported for the same producer on expiring date during winter. A study by Fröder et al. (2007) revealed high *Enterobacteriaceae* and fecal coliforms populations (> 2 log cfu/g) in different types of one leafy vegetable salads (iceberg lettuce, watercress, spinach, rocket, chicories) and mixed salads collected during spring and summer. High total mesophilic counts were also reported during the end of self-life of ready-to-eat rocket salads (lower than 7 log cfu/g) (Giusti et al., 2014). Summer was the season in which samples from producer A showed increased *Staphylococcus* spp. during their expiring date compared to purchase date. On the other hand, samples from producer C presented low expiring *Staphylococcus* spp. numbers during summer. In our study, LAB counts were higher during winter for producer A on products expiring date. Expiring day during summer presented higher yeasts and molds populations for producer B compared to winter. It is worth mentioning that according to De Corato (2012) no significant variations of yeasts and molds counts were observed during the shelf-life of the

samples (rocket, baby leaf lettuce and lamb's lettuce), whilst a significant variation on these populations and high numbers of fungi were evident only at the first day of shelf-life. No significant differences were observed for coliforms, *E. coli* and *Pseudomonas* spp. in our study.

Higher CO_2 production and H_2O_2 levels were found during both seasons on expiring date of samples from producers A and C. This might be attributed to tissue wounding (due to processing, mishandling) in combination with storage and display conditions (i.e. temperature, shelf-life duration) that can accelerate respiration rate of lettuce (Deza-Durnand and Petersen, 2011). Lipid peroxidation and H_2O_2 levels were increased in winter at the last day of shelf life. Moreover, higher CO_2 production was observed for producer E during winter and summer. Increased respiration rate for wild rocket salad was reported in spring compare to summer (55.2 and 25.2 mL CO_2/kg/h, respectively) when samples were stored at 5 ^{o}C and at the same time rocket's green color was preserved better in spring compared to summer (Edelenbos et al., 2017). However, it has been previously mentioned that lipid peroxidation resulting from plant stress (including increased respiration) can affect negatively the green color vegetables due to pigment bleaching (chlorophylls, carotenoids) and the production of brown pigments (Hunter et al., 2017). In our study, no differences were reported for phenols and antioxidants among seasons and days of analysis for all producers.

Expiring date during summer showed high TVC numbers for the combinations of lettuce with radicchio/endive, and rocket, while during winter increased counts were found for plain rocket, the combinations of lettuce with radicchio/endive and two/more ingredients (other). In a study by Sant'Ana et al. (2012a) in which the microbial load of nine different ready-to-eat vegetables (escarole, collard green, spinach, watercress, arugula, grated carrot, green salad, and mix for yakisoba) was assessed it has been shown that total aerobic counts increased during the end of shelf-life of the products (ranging from 2 to 8 log cfu/g) and this resulted from different storage temperatures (the higher the temperature, the higher the populations) as well as the type of vegetable among other factors (Caponigro et al., 2010). Higher *Enterobacteriaceae* and coliforms populations were found on expiring day for the combination of lettuce and radicchio/endive on both seasons. Arvanitoyannis et al. (2011) reported that a decrease in *Enterobacteriaceae* populations was evident (up to 0.5 log cfu/g) on the tenth day of storage with or without MAP. Interestingly on the same study, psychrotrophic counts were not influenced with the combination of lettuce with rocket (Arvanitoyannis et al., 2011). However, in our study increased numbers of spoilage and psychrotrophic microorganisms (i.e. LAB, *Pseudomonas* spp., yeasts and molds) were observed on expiring day during summer for the combination of lettuce with rocket. It is noteworthy, that it has been previously mentioned that LAB have been isolated most from ready-to-eat vegetables under MAP (Abadias et al., 2008; Caponigro et al., 2010). Sant'Ana et al. (2012a) reported increased LAB populations on most ready-to-eat vegetables studied at the end of their shelf-life when stored at abusive temperatures (15 ^{o}C). De Corato (2012) reported that yeasts and molds counts were higher on the second day of shelf-life for rocket salad on both seasons assessed (spring and summer)

compared to lettuce and lamb's lettuce salads. In our study, no significant differences were reported for *E. coli, Staphylococcus* spp. and *B. cereus* between purchase and expiring day among seasons for all type of salads.

Increased total phenolic content was reported on expiring day of the combination of lettuce and chives during summer. On the other hand decreased phenolics were reported for baby lettuce, curly endive and iceberg lettuce after 4 days of storage at 4 ^{o}C, while no significant differences among phenolic content were reported for radicchio, rocket and lamb's lettuce (Preti and Vinci, 2016). This might be attributed to the packaging conditions in bagged samples due to modified atmosphere packaging of these vegetables. Higher antioxidants (by FRAP assay) on product expiring day were observed during winter for the combination of lettuce with cabbage and during summer for plain rocket. Preti and Vinci (2016) reported increased antioxidants compounds (by DPPH assay) on expiring date of baby lettuce, curly endive, lamb's lettuce, rocket and radicchio salads. The majority of the combinations of lettuce with other ingredients showed higher H_2O_2 and MDA levels on expiring day during both seasons. It is noteworthy to mention that Cavaiuolo et al. (2015) reported a relation between lipid peroxidation and storage temperature of rocket, suggesting that storage of minimally processed vegetables such as rocket at adverse (increased) temperatures increases respiration rate and affects negatively product quality due to plant stress and senescence. This is in accordance with our results since plain rocket showed higher CO_2 production and MDA levels on expiring day for both seasons. Arvanitoyannis et al. (2011) reported increased CO_2 levels of rocket with or without its combination with lettuce through storage at 5 ^{o}C for 10 days. Moreover, Nousiainen et al. (2016) suggested that the increased CO_2 production reported might have been attributed to the different types of vegetables as well as the microbial load of these products. These comes in accordance with the findings of our study, where lettuce+endive during winter showed increased microbial load (TVC, *Enterobacteriacea* and coliforms) and CO_2 production on expiring day. No significant differences were reported for antioxidant activity (by DPPH and ABTS assays) between purchase and expiring day among seasons for all type of salads.

3.4. Concluding marks

The present study was examining a number of metabolic variables (antioxidant activity, CO_2 production) besides the presence of spoilage and pathogen bacteria; in order to link the microbiological load of ready-to-eat salads and the response of plant tissue towards biotic (pathogens) and abiotic (storage) stress. Spring is the season that ready-to- eat salads are more pronounced on microbial load. *Salmonella enterica* was not found in any of the samples tested, whereas *Listeria monocytogenes* and ESBL *E. coli* were present in 3.70% and 2.62% of samples, respectively. The content of phenolics and the activity of antioxidants were positively correlated with the presence of *Staphylococcus* spp.,

Pseudomonas spp., *E. coli* and *Bacillus cereus*, whereas fresh produce processing accelerates microbial load and antioxidative mechanisms due to the plant stress. Various salad types are respiring differently through the respiration metabolic process. Furthermore, the interaction of salad producer*type of salad affected greatly the total phenolic content and the antioxidant activity of salads, as different processing practices may cause more or less plant stress and subsequently affecting the quality of fresh produce.

Regarding expiring date (OR "estimated expiring date"), it was evident that microbial load (mainly spoilage microorganisms such as *Pseudomonas* spp., yeasts and molds) increased during shelf-life. Moreover, CO_2 production and damage indexes (H_2O_2 and MDA) increased on expiring date on both seasons indicating plant stress at the end of shelf life. These results suggest that the investigation of shelf-life (form start to end) is essential for the understanding and development of novel technics monitoring the safety and quality of these products.

Chapter 4. *Salmonella enterica* performance in different temperatures and nutrient solution pH levels in hydroponically grown lettuce

4.1. Introduction

According to demographics it is estimated that by the year 2050 the world's population will reach 9.1 billion people, rising more environmental and economic issues (FAO, 2009; Treftz and Omaye, 2016). In an attempt to pursuit renewable energy sources and sustainable growing systems to meet the increasing needs for food production and the changing dietary guidelines for the consumption of vegetables as a part of a healthy and balanced diet, a turn towards the research on soilless culture-hydroponic cultivation was made over 70 years ago (Treftz and Omaye, 2016). The word hydroponic derives from the Greek words "hydro" (ὕδωρ) which means water and "ponos" (πόνος) which means labor (Treftz and Omaye, 2016). By definition soilless cultivation means the growing of plants under soilless conditions using water, nutrients essential for plant growth and a substrate (i.e. perlite, cocosoil, sand) (Resh, 2013; Treftz and Omaye, 2016; Jordan et al., 2018; Tzortzakis et al., 2020), while strictly hydroponic cultivation is referring to the aqua systems, excluding growing media. However, in most part of the word, soilless and hydroponic terms are used in common. It is considered to be an environmentally friendly food production system, producing high quality and safe fresh produce, with good agricultural practices such as the recirculation of the used water and minerals (Treftz and Omaye, 2016; Tzortzakis et al., 2020).

Lettuce is one of the most commonly hydroponically grown vegetables around the world due to its short cultivation period (varied from 1 to 1.5 months), versatile use in salads and other dishes, as well as its proven benefits to human health (good source of dietary fibers, folate-vitamin B_9 and vitamin C) (Kim et al., 2016; Manos and Xydis, 2019). Hydroponic cultivation of leafy and other vegetables is considered to be a safe and clean growing technique in which plants are grown in a controlled environment without soil and every essential component (water, fertilizers-nutrient solution, substrates) are closely monitored since sanitary practices are more commonly used compared to open field cultivation (Orozco et al., 2008; Resh, 2013; Dankwa et al., 2020; Lenzi et al., 2021). In this high-tech cultivation systems, it is essential that the pH, composition and level of the nutrient solution are being monitored and maintained throughout the cultivation period (Resh, 2013; Treftz and Omaye, 2016). These have resulted to the increased acceptance of hydroponically grown vegetables from both growers and consumers (Lee and Lee, 2015; Dankwa et al., 2020).

Safety of fresh produce is one of the main quality attributes that consumers take into account when buying. The consumption of vegetables (fresh and/or minimally processed) has been previously linked with food poisoning outbreaks associated with a variety of microorganisms including bacteria, viruses and protozoa (Whipps et al., 2008; Riggio et al., 2019). In recent years (2013-2016) fresh produce including lettuce, sprouts and ready-to eat salads has been implicated with foodborne outbreaks related

with the detection of *Salmonella* spp., *L. monocytogenes*, enteroinvasive and entrerohaemorragic strains of *E. coli* (Wadamori et al., 2017; Boqvist et al., 2018).

The most commonly reported foodborne bacteria present on fresh produce include *E. coli*, *Salmonella* spp., *L. monocytogenes*, *Shigella* spp., *Yersinia enterocolitica*, *Aeromonas hydrophila*, *Bacillus cereus*, *Campylobacter* spp., *Clostridium* spp. and *Vibrio choleae* (Whipps et al., 2008; Dankwa et al., 2020). Among these, *Salmonella* spp. has been occasionally reported on fresh produce as well as minimally processed vegetables and ready-to-eat salads (Jeddi et al., 2014; Wadamori et al., 2017). Furthermore, a number of these pathogens (i.e. *E. coli* O157:H7, *Salmonella* spp. and *L. monocytogenes*) has also been identified and reported on hydroponically grown fresh produce (Lopez-Galvez et al. 2014; Shaw et al. 2016). One of the main sources of contamination of fresh produce during hydroponic cultivation is the water used for the preparation of the nutrient solution, which could be either municipal water or surface water with the latter most likely to be contaminated if coming from an unsanitary source (Uyttendaele et al., 2015; Dankwa et al., 2020). Other sources of fresh produce contamination are through harvesting and postharvest handling and managements. During and after harvesting, produce might come in contact with contaminated surfaces (i.e. handling, washing water, marketing), however the current decontamination practices might not be able to eliminate the presence of foodborne pathogens (Aycicek et al., 2006; Jeddi et al. 2014; Faour-Klingbeil et al., 2016b; Nousiainen et al., 2016).

Previous studies have mentioned the presence and internalization of foodborne pathogens in leafy vegetables in soil and hydroponic cultivation in a variety of vegetables including lettuce, spinach, tomatoes, and radish sprouts and cabbage (Jablasone et al., 2005; Hintz et al., 2010; Koseki et al., 2011; Ongeng et al., 2011b; Standing et al., 2013; Macarisin et al., 2014). Entrance routes of pathogens include plant roots, leaf stomata, any niches caused by mechanical damage or even the use of contaminated seeds (Jablasone et al., 2005; Koseki et al., 2011; Erickson et al., 2018, 2019; Liu et al., 2019).

One of the main factors that might contribute to the internalization of foodborne pathogens in a plant tissue is the pathogen's surface attachment ability to fresh produce, since adhesion is the first step for infection (Schikora et al., 2012; Kyere et al., 2019). Interestingly, the presence of phytopathogens might also assist foodborne pathogen presence, survival and growth on vegetables (Aruscavage et al., 2010; Goudeau et al., 2013). It has been suggested that members of the *Enterobacteriaceae* family can be benefited from the damages caused by plant pathogens and utilize the sugars exudates of the damaged plant tissue (Aruscavage et al., 2010). It is well known that *Salmonella* possess the ability to form biofilms on many surfaces using a variety of adhesive structures (i.e. fimbrial and non-fimbrial structures, flagella and lipopolysaccharides) and this in combination with the presence of phytopathogens might enable plant colonization by *Salmonella* (Wiedemann et al., 2015).

The population of foodborne pathogens might also play a key role in plant colonization since studies have reported that high populations (up to 6-7 log cfu/mL) resulted to higher internalization frequencies (Sharma et al., 2009; Koseki et al., 2011; Macarisin et al., 2014). Indeed, hydroponically grown spinach inoculated with *S. enterica*, *E. coli* and *L. monocytogenes* at a level of 6 log cfu/mL showed higher probability of contamination compared to inoculation with 3 log cfu/mL (Koseki et al., 2011). It is noteworthy, that the high numbers of inoculum examined in many studies are unlikely to be found in the environment. Previous studies regarding the microbiological quality of hydroponically grown vegetables (lettuce and tomato) reported populations of < 1 log cfu/mL for hygiene indicators (coliforms and *E. coli*) (Orozco et al., 2008; Alcarraz et al., 2018).

The presence of phytopathogens in a plant tissue is evident with discoloration (chlorosis), necrosis or even death of the infected plant (Wiedemann et al., 2015). The fact that in most of the cases leafy vegetables can harbor human foodborne pathogens without exhibiting signs of spoilage is alarming (Barak and Schroeder, 2012; Lenzi et al., 2021). However, in some cases symptoms of chlorosis have also been reported on leaves during the colonization of lettuce plants with *Salmonella enterica* serovars (Klerks et al., 2007a,b).

Different cultivation conditions under hydroponics (with/without substrate, different nutrient solutions, hydroponic agar) have been assessed and results are not always in agreement (Jablasone et al., 2005; Franz et al., 2007; Klerks et al., 2007a; Koseki et al., 2011; Filho et al., 2018; Li and Uyttendaele, 2018; Liu et al., 2019; Riggio et al., 2019; Lenzi et al., 2021). Thus, it is essential to explore further and understand the mechanisms of internalization and survival of foodborne pathogens inside their plant hosts and further investigation of plant defense mechanisms and interaction of plant-foodborne pathogens as well as the cultivation conditions will give insights for safe fresh produce production.

The aims of this study were to assess i) the internalization of different populations of *S.* Enteritidis, ii) the effect of the different pH values of the nutrient solution, iii) the responses of different plant growth stage to bacteria and pH challenges, in lettuce plants cultivated in hydroponic nutrient solution and iv) the relationship among the possible internalization of the bacterium and plant related parameters.

4.2. Materials and methods

4.2.1. Bacterial strain and inoculum preparation

Salmonella enterica subsp. *enterica* serovar Enteritidis NCTC 5188 was obtained from the Agricultural Sciences, Biotechnology and Food Science Department (Lab of Food Microbiology), Cyprus University of Technology. Fresh cultures were prepared with overnight incubation (16-18 h at 37 ^{o}C) of pure culture (stored in -80 ^{o}C in 20% glycerol) in BHI broth.

4.2.2. *In vitro* performance of *S.* Enteritidis

The effect of pH, commonly found in soilless culture, on the survival and persistence of *S.* Enteritidis was assessed with bacterial growth kinetics and the generation of growth curves based on the changes of the turbidity of samples throughout incubation. Different pH values (5, 6, 7 and 8) of BHI broth, sterile hydroponic nutrient solution and their combination were examined at two incubation temperatures (21 ^{o}C and 37 ^{o}C). The hydroponic nutrient solution and its combination with BHI were sterilized via filtration with 0.45 μm filter. The pH values of each treatment solution were adjusted with the use of 5% HNO_3 and 10% KOH. The 96-well plates were prepared by dispersing 190 μL of treatment solution and 10 μL of inoculum (6 log cfu/mL). The final volume of each well was 200 μL. Negative controls were used for all treatments (200 μL), while the positive control contained 190 μL BHI broth (no pH adjusted- pH value 7.40) and 10 μL of inoculum. The plates were covered with a sterile plastic transparent membrane, placed for incubation at 21 ^{o}C and 37 ^{o}C for 21 h and bacterial growth was estimated by reading the absorbance at 600 nm every 30 min. The confirmation of the bacterial growth was performed at the end of incubation by plating 10 μL from each well on BHI agar plates and plates were placed for incubation at 37 ^{o}C for 24 h.

4.2.3. Preliminary experiment

4.2.3.1. Plant growth and cultivation conditions

4.2.3.1.1. Old plants

Lettuce seedlings (*Lactuca sativa* cv. Nogal) in potting mixture-substrate (peat-based media) at the stage of 2-3 true leaves were purchased from a commercial nursery and were transferred to 450 mL plastic (polyethylene terephthalate-PET) containers (72 plants in total; 3 replications per treatment per sampling day) filled with hydroponic nutrient solution (EC 2.02 mS/cm and pH 5.90) (**Figure 4.1**). The composition of nutrient solution (NS) was described previously (Chrysargyris et al., 2019) and was as follow: NO_3^--N = 14.29, K = 8.31, PO_4^{-3}-P = 1.61, Ca = 7.48, Mg = 5.76, SO_4^{-2}-S = 1.56 and Na = 1.91 mmol/L, respectively; and B = 18.21, Fe = 71.56, Mn = 18.21, Cu = 4.72, Zn = 1.53, and Mo = 0.52 μmol/L, respectively. Plants were placed in an experimental growth chamber (SANYO Versatile Environmental Test Chamber, MLR-351H, SANYO Electric Co. Ltd., Japan) with a photoperiod of 16 h at 21 ^{o}C and 8 h darkness at 19 ^{o}C. Plants were grown under hydroponic conditions with the basic NS for 3 days in order to adapt the new growing conditions in hydroponics, and then the NS was replaced with fresh NS of the appropriate pH level (pH values, 5, 6, 7 and 8 and EC 2.02 mS/cm). The initial pH values were adjusted with the use of 5% HNO_3 and 10% KOH.

4.2.3.1.2. Small-medium and medium plants

Lettuce seeds (*Lactuca sativa* cv. Verdede and Nogal) were aseptically germinated in sterile petri dishes with sterile filter paper and sterile dH_2O. Seeds were germinated for 7 days in the dark at 25 ^oC. Seedlings of approximately 3 cm height (at the 1st true leaf formation), were then transferred to 50 mL plastic falcon tubes. Each falcon tube was filled with hydroponic nutrient solution (EC 1.70 mS/cm and pH 6.10). Plants were placed in an experimental growth chamber with a photoperiod of 16 h at 21 ^oC and 8 h darkness at 19 ^oC. After approx. 7 days for small-medium and 12 days for medium plants grown in the hydroponic nutrient solution, plants were subjected to different pH levels, by replacing the NS with fresh NS of the relevant pH treatment (i.e. pH values of 5, 6, 7 and 8 and EC of 2.10 mS/cm). The initial pH values were adjusted with the use of 5% HNO_3 and 10% KOH. For small-medium plants, a total of 48 plants were used with two replications per treatment per sampling day. For medium plants, a total of 36 plants were used with four replications per treatment per sampling day.

Nutrient solution EC and pH were monitored throughout cultivation (from control plants) and the targeted pH and EC values of the nutrient solution was added to each treatment (pH) in order to maintain the solutions pH and EC.

4.2.3.2. Plant inoculation

In order to give the plants a chance to stabilize under the examined pH levels, inoculation took place 2-3 days after the pH change of the hydroponic nutrient solution. Plants were inoculated via roots (inoculum added into the hydroponic nutrient solution) with 3 and 6 log cfu/mL (low and high concentration, respectively), whilst control plants were not inoculated. Inoculation of older plants took place when plants were at the 11th day after transplanting in the plastic containers (inoculum: 3 and 6 log cfu/mL). Inoculation of medium plant (inoculum: 6 log cfu/mL) took place at the 15th day after transplanting in the falcon's tubes, and for small-medium plants (inoculum: 3 and 6 log cfu/mL) took place at the 10th day after transplanting in the falcon's tubes.

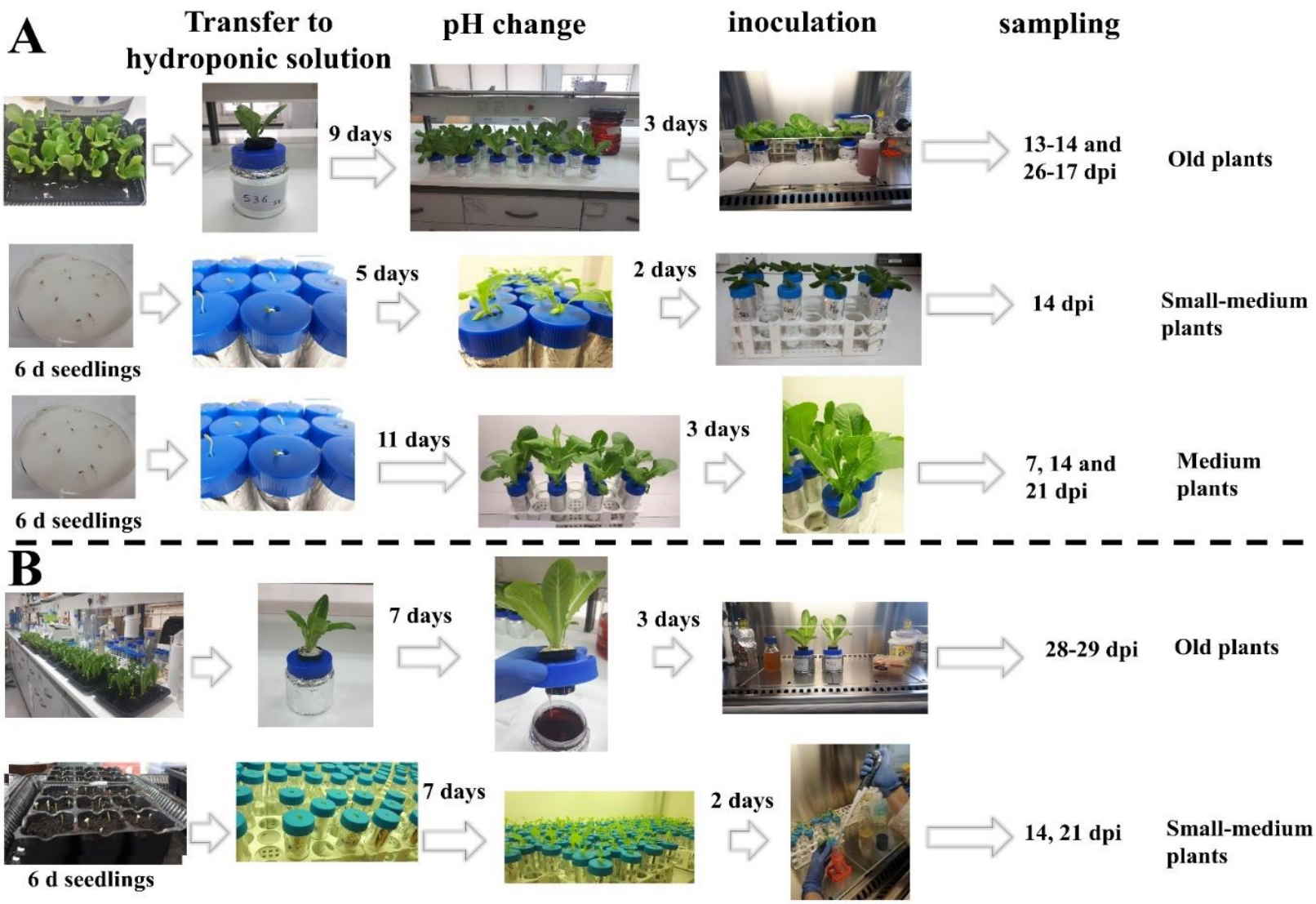


Figure 4.1. Schematic presentation of preliminary (A) and main experiments (B).

4.2.3.3. Sampling

Different sampling days were applied for the different growth stages of the examined plants. For old plants sampling was performed 13-14 and 26-27 days post inoculation (dpi). Sampling for medium plants took place 7, 14 and 21 dpi, while small-medium plants were sampled 14 dpi.

During sampling, the edible part of lettuce (leaves) was collected for microbiological analysis. For old plants 10 g of leaves were collected, while for small-medium and medium plants the whole lettuce head was used for analysis. Root sampling was performed first by rinsing the root with MRD and the root rinse was collected (from old and small-medium plants) for further analysis in order to determine possible bacterial attachment on the root surface. In order to assess the possible internalization of Salmonella in the roots, they were subsequently externally sterilized with 10% (v/v) sodium hypochloride for 60 sec, 80% (v/v) ethanol for 30 sec and three times with sterile dH_2O as previously described by Mitra et al. (2009). Moreover, hydroponic nutrient solution was also collected and stored for further analysis for the presence of Salmonella at the end of the cultivation period. All samples were stored at – 20 ^{o}C until analysis (**Figure 4.S1**).

4.2.3.4. Microbiological analysis

The presence of *S.* Enteritidis in lettuce leaves was assessed according to the standard microbiological analysis for Salmonella detection as described at section **2.2.1.10**. The same procedure was also followed for the analysis of roots in order to assess the possible internalization of *S.* Enteritidis in lettuce root samples. The surface drop method (Miles and Misra method) was used for the enumeration of control samples TVC (leaves and roots) and *S.* Entertidis in hydroponic nutrient solution. Briefly, serial decimal dilution of the samples were performed as previously described at section **2.2.1** and six drops (10 μL each) were placed on the appropriate medium (PCA for TVC and XLD for Salmonella) (**Figure 4.2**) (Miles et al., 1938).

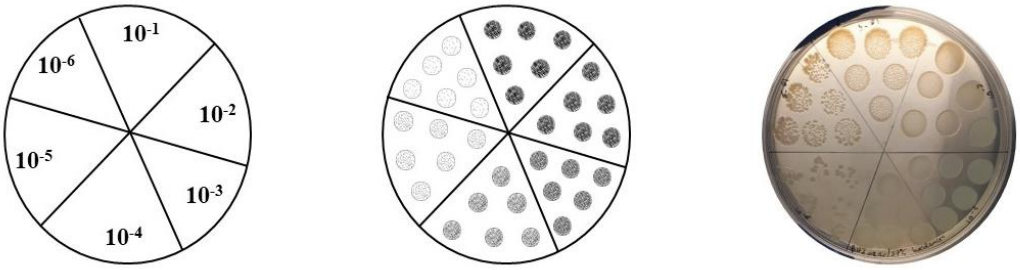


Figure 4.2. Surface drop method (Miles and Misra method).

Presumptive Salmonella isolates (pink colonies with black center) from leaves and roots were further assessed by the slide agglutination test with Poly-O antisera for the confirmation of *S.* Enteritidis. The

principle for this test is based on the agglutination (clump formation) of Salmonella in the presence of homologous antiserum (containing specific Salmonella antibodies). Briefly, enough biological material from presumptive Salmonella isolates (3-5 colonies) was emulsified with a loopful of MRD in a microscope slide and subsequently a drop of O poly A-S antisera (Polyvalent Somatic O Antisera – Polyvalent O A-S PL.6002, PRO-LAB DIAGNOSTICS, USA) was added to the emulsion. The slide was shaken back and forth for one min and afterwards observed for agglutination (**Figure 4.3**).

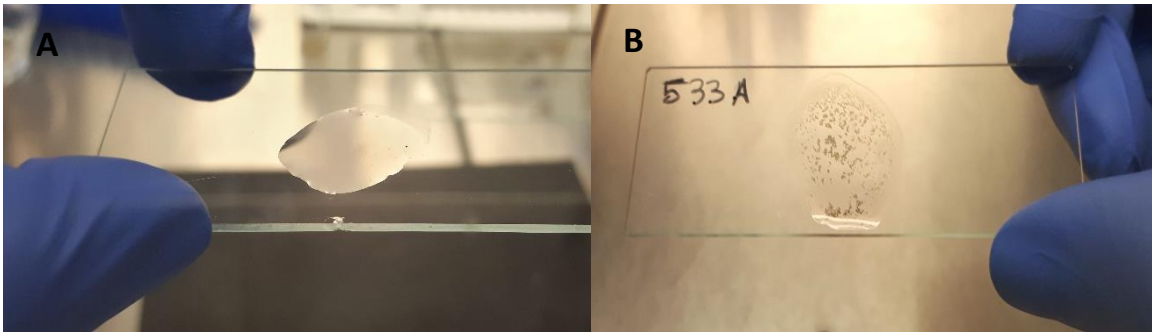


Figure 4.3. Slide agglutination test. A: negative sample, B: positive sample.

4.2.4. Main experiment

4.2.4.1. Plant growth and cultivation conditions

4.2.4.1.1. Old plants

Lettuce seedlings (*Lactuca sativa* cv. Nogal) in potting mixture-substrate (peat-based media) at the stage of 2-3 true leaves were purchased from a commercial nursery and were transferred to 450 mL plastic (polyethylene terephthalate-PET) containers (72 plants in total; 6 replications per treatment) filled with hydroponic nutrient solution (EC 1.70 mS/cm and pH 5.90). The composition of the nutrient solution was as previously described at section **4.2.3.1.1**. Plants were placed in an experimental growth chamber with a photoperiod of 16 h at 21 ^{o}C and 8 h darkness at 19 ^{o}C (**Figures 4.4-4.5**). In order for the plants to adapt the new growing conditions in hydroponics, plants were grown for 7 days in the basic NS, and afterwards the NS was replaced with fresh NS of the appropriate treatment (pH values, 5, 6, 7 and 8 and EC 1.70 mS/cm). The initial pH values were adjusted with the use of 5% HNO_3 and 10% KOH.

A

B

Figure 4.4. Plant transfer to nutrient solution and inoculation for old (A) and small-medium plants (B).

4.2.4.1.2. Small-medium plants

Lettuce seeds (*Lactuca sativa* cv. Nogal) were sown and germinated in potting mixture (commercial peat-based material) for 7 days. Seedlings were then transferred to 50 mL plastic falcon tubes filled with hydroponic nutrient solution (EC 1.45 mS/cm and pH 6.01) after the removal of the potting mixture-substrate around their roots. Plants were placed in an experimental growth chamber with a photoperiod of 16 h at 21 ^{o}C and 8 h darkness at 19 ^{o}C. After approx. 7 days the hydroponic nutrient solution was replaced with fresh solution of the appropriate treatment (pH values, 5, 6, 7 and 8 and EC 1.70 mS/cm). The initial pH values were adjusted with the use of 5% HNO_3 and 10% KOH (**Figure 4.5**).

Nutrient solution EC and pH were monitored throughout cultivation (from control plants) and appropriate pH and EC value nutrient solution was added to each treatment (pH) in order to maintain the solutions pH and EC, as previously mentioned at section **4.2.3.1.2.**

Figure 4.5. Plant growth in experimental growth chamber for old (A) and small-medium plants (B).

4.2.4.2. Plant inoculation

Plants were inoculated with 3 and 6 log cfu/mL (low and high concentration, respectively), as previously described at section **4.2.3.2**. Briefly, inoculation took place 2-3 days after the pH chance of the hydroponic nutrient solution, since plants were given a chance to stabilize (**Figure 4.4**). Plants were inoculated via roots (inoculum added into the hydroponic nutrient solution) with 3 and 6 log cfu/mL (low and high concentration, respectively). Control plants were also used and they were not inoculated. Inoculation of older plants took place when plants were at the 11^{th} day after transplanting in the plastic containers. Inoculation of small-medium plants took place at the 10^{th} day after transplanting in the falcon's tubes.

4.2.4.3. Sampling

Sampling for old plants took place 28-29 dpi (**Figure 4.S2**), while small-medium plants were sampled 14 and 21 dpi (**Figure 4.S3-4S4**). Sampling procedure was performed as previously described at section **4.2.3.3**. For old plants 25 g of leaves were collected, while for small-medium plants the whole lettuce head was used for analysis.

4.2.4.4. Microbiological analysis

Microbiological analyses for leaves, roots, root rinse and hydroponic nutrient solution were performed as previously mentioned at section **4.2.3.4**. Slide agglutination test with Poly O antisera was performed for the presumptive Salmonella isolates, as previously described at section **4.2.3.4**. Miles and Misra method (surface drop method) was performed for the enumeration of TVC on PCA for leaves and roots (control samples) and the enumeration of Salmonella in hydroponic nutrient solution on XLD, as previously described at section **4.2.3.4**.

4.2.4.5. Physicochemical analysis

For old plants, further plant related parameters were assessed. Leaf chlorophyll and carotenoid content were determined at 480, 649 and 665 nm, using the equations by Wellburn (1994) as previously described at section **2.2.7**. Polyphenol extraction and analysis were performed using the Folin-Ciocalteu method as previously mentioned at sections **2.2.8.1** and **2.2.8.2**. The antioxidant activity of lettuce plants was estimated using the DPPH, FRAP and ABTS protocols as mentioned in sections **2.2.8.3**, **2.2.8.4** and **2.2.8.5**, respectively. H_2O_2 levels and lipid peroxidation were also calculated in order to determine the damage index due to abiotic and abiotic stress, as mentioned in sections **2.2.9.1** and **2.2.9.2**, respectively.

4.2.5. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics version 25 where the effect of pH and inoculum level as well as their interactions on the phenolic content, antioxidant activity and damage index of samples was assessed with two-way ANOVA. One-way analysis of variance (ANOVA) was also performed for comparing data means of plate experiments and plant-related parameters and Tukey's multiple range tests were calculated for the significant data $P < 0.05$. For microbial analysis of inoculated plants, frequencies were calculated in order to assess the prevalence of *S.* Enteritidis in lettuce plants (leaves, roots), root rinse and nutrient hydroponic solution. Six replications were used for each measurement.

4.3. Results

4.3.1. Effects of different nutrient solution pH on the *in vitro* performance of *S.* Enteritidis

S. Enteritidis growth measured as OD value was affected by the pH of BHI broth and incubation temperature (**Figure 4.6**). Higher OD values were recorded in BHI broth with pH value 8 and followed by 7 compared to the lower ones (pH 5 and 6) at 21 ^{o}C (**Figure 4.6A**). Similarly, the Miles and Mirsa method showed higher bacterial counts with pH values 8 and 7 (9.55 and 9.21 log cfu/g, respectively) in comparison with pH values 5 and 6 which were lower but did not significantly ($P > 0.05$) differ between them at 21 ^{o}C (8.29 and 8.57 log cfu/g, respectively) (**Figure 4.6C**). At 37 ^{o}C *S.* Enteritidis presented lower OD values with BHI broth pH 5 compared to other pH values, while pH values 6 and 7 showed lower OD values than pH 8, they did not significantly ($P > 0.05$) differ between them (**Figure 4.6B**). Interestingly, bacterial counts at the end of incubation were found to decrease along with the decrease of the BHI broth pH value (**Figure 4.6D**).

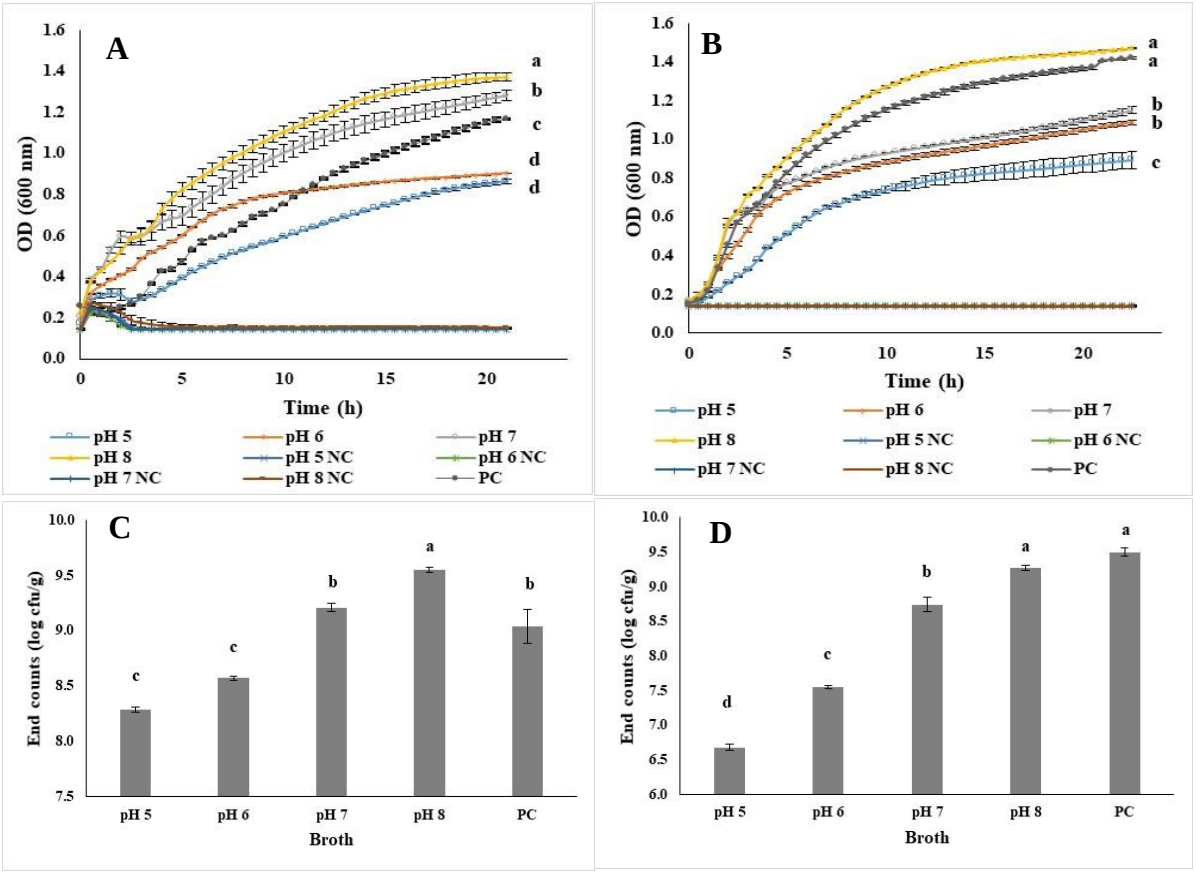


Figure 4.6. Effects of BHI broth's pH values on *S.* Enteritidis growth and survival at 21 ^{o}C (**A, C**) and 37 ^{o}C (**B, D**). Significant differences ($P < 0.05$) among pH values are indicated with different Latin letters. NC: negative control, PC: positive control.

The effects of hydroponic nutrient solution's pH and incubation temperature on *S.* Enteritidis are illustrated in **Figure 4.7**. At 21 ^{o}C all pH values showed lower OD values compared to the positive control (BHI broth- pH 7.40) and at the same time OD values from pH 6 and 5 were significantly lower to pH 7 (**Figure 4.7A**). Bacterial counts were reported lower for all pH values examined than positive control however, no significant ($P > 0.05$) differences among pH values (**Figure 4.7C**). Lower OD values were reported at 37 ^{o}C with all pH values compared to positive control and at the same time pH 5 presented lower OD values in comparison to pH 8 (**Figure 4.7B**). Bacterial counts were found to be significantly lower with pH 7 followed by pH 6 and 5 (8.51, 8.03 and 7.99 log cfu/g, respectively), compared to pH 8 and positive control which they did not significantly ($P > 0.05$) differ (9.47 and 9.50 log cfu/g, respectively) (**Figure 4.7D**).

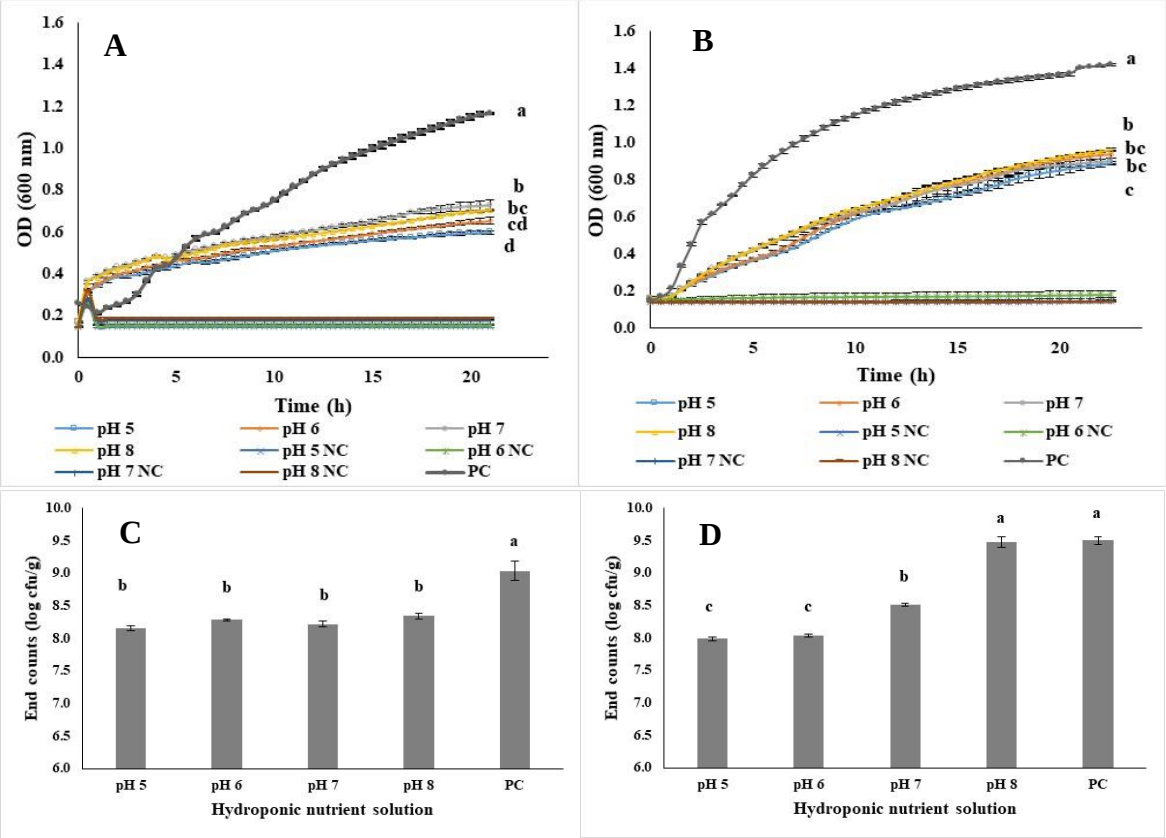


Figure 4.7. Effects of hydroponic nutrient solution's pH values on *S.* Enteritidis growth and survival at 21 ^{o}C (**A, C**) and 37 ^{o}C (**B, D**). Significant differences ($P < 0.05$) among pH values are indicated with different Latin letters. NC: negative control, PC: positive control.

The combination of BHI broth and the hydroponic nutrient solution has affected the growth of *S.* Enteritidis as presented in **Figure 4.8**. Higher OD value was observed with pH 8 compared to positive control and other pH values as OD values decreased with the decrease of pH at 21 ^{o}C (**Figure 4.8A**). It is noteworthy that bacterial counts were lower with pH 7, 6 and positive control followed by pH 5 (8.98,

9.04 and 8.40 log cfu/g, respectively) in comparison to pH 8 (9.48 log cfu/g) (**Figure 4.8C**). At 37 °C *S.* Enteritidis in the combination of broth+hydroponic nutrient solution showed decreased OD values with all pH values compared to the positive control (**Figure 4.8B**). Moreover, OD was significantly lower ($P < 0.05$) at pH 5 in comparison with other pH values. It is worth mentioning that bacterial counts at pH 5, 7 and 8 did not significantly ($P > 0.05$) differ (8.69, 8.66 and 8.72 log cfu/g, respectively) but values were lower compared to pH 6 and positive control (9.97 and 9.50 log cfu/g, respectively) (**Figure 4.8D**).

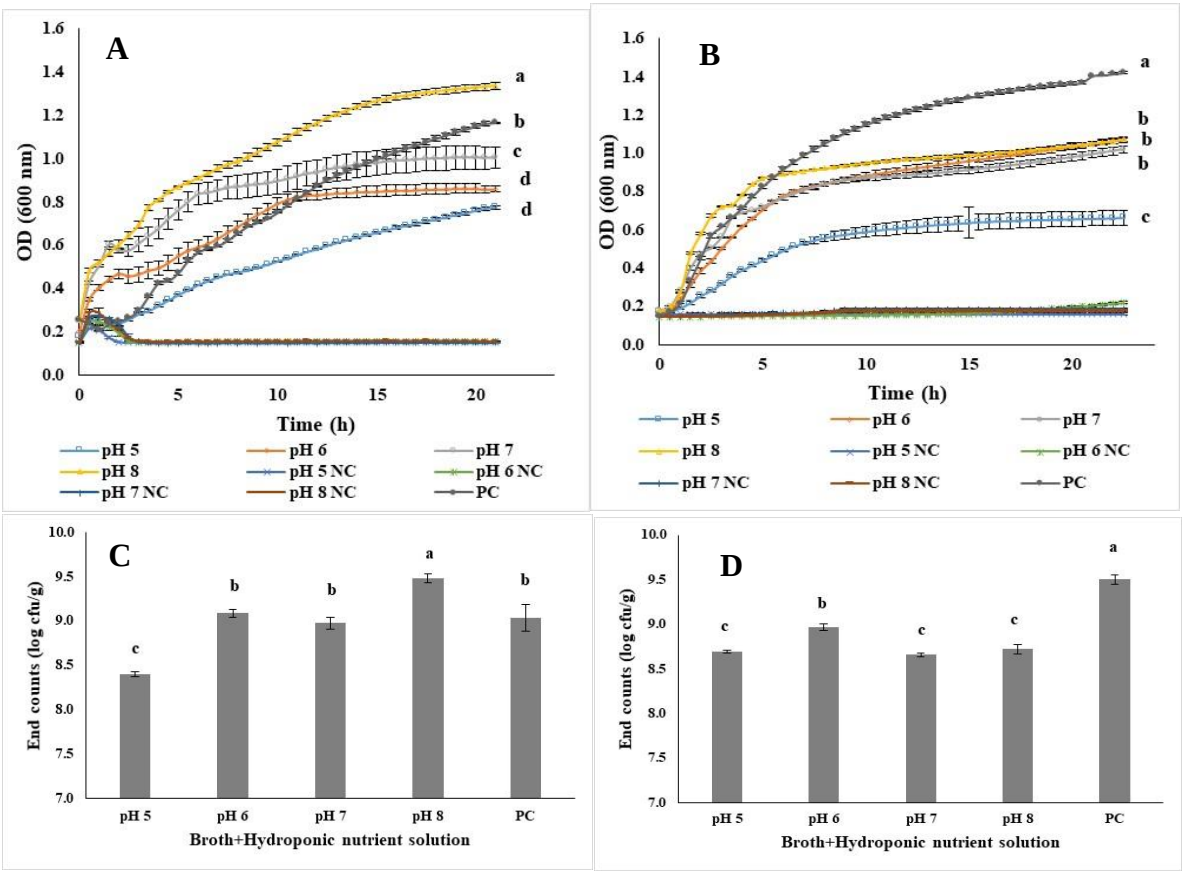


Figure 4.8. Effects of the combination of BHI broth and hydroponic nutrient solution's pH values on *S.* Enteritidis growth and survival at 21 °C (**A, C**) and 37 °C (**B, D**). Significant differences ($P < 0.05$) among pH values are indicated with different Latin letters. NC: negative control, PC: positive control.

4.3.2. Preliminary experiment

4.3.2.1. *S.* Enteritidis performance on different nutrient solution pH for small-medium, medium and old lettuce plants

The effects of pH and inoculation level on the presence of *S.* Enteritidis on lettuce (leaves, roots, root rinse) of different age and hydroponic nutrient solution are presented in **Tables 4.1-4.3**. The presence of *S.* Enteritidis on old lettuce plants was observed in roots 26 dpi at all pH values with high inoculum, whereas the bacterium was present around roots even 13 dpi (**Table 4.1**). No internalization

on leaves was reported at both sampling periods, while *S.* Enteritidis was found at the nutrient solution even after 26 dpi (**Table 4.1**). When small-medium plants were inoculated with *S.* Enteritidis internalization on roots was reported 14 dpi with high inoculum at all pH levels, while low inoculum resulted to root internalization at pH 7 and 8 (**Table 4.2**). Furthermore, no internalization on leaves was reported 14 dpi (**Table 4.2**). Internalization of *S.* Enteritidis on medium plants was observed 14 dpi at pH 7 in leaves, whist at the same time the bacterium was found internal in roots and hydroponic solution at all pH levels tested (5, 6, 7) (**Table 4.3**). Interestingly, within 7 dpi, no internalization of *S.* Enteritidis was observed in lettuce leaves, in all the examined pH values.

Table 4.1. Presence of *S.* Enteritidis on old lettuce plants (leaves, roots, root rinse) and hydroponic nutrient solution as affected by pH values and inoculum levels.

			Leaves		Roots		Root rinse		Hydroponic solution	
Code	pH	Treatment	13 dpi	26 dpi	13 dpi	26 dpi	13 dpi	26 dpi	13 dpi	26 dpi
511	5	Control								
512	5	Control								
513	5	Control								
521	5	Low								
522	5	Low								
523	5	Low								
531	5	High								
532	5	High								
533	5	High								
611	6	Control								
612	6	Control								
613	6	Control								
621	6	Low								
622	6	Low								
623	6	Low								
631	6	High								
632	6	High								
633	6	High								
711	7	Control								
712	7	Control								
713	7	Control								
721	7	Low								
722	7	Low								
723	7	Low								
731	7	High								
732	7	High								
733	7	High								
811	8	Control								
812	8	Control								
813	8	Control								
821	8	Low								
822	8	Low								
823	8	Low								
831	8	High								
832	8	High								
833	8	High								

Green: presence, Red: absence

Table 4.2. Prevalence (% presence) of *S.* Enteritidis on small-medium lettuce plants (leaves, roots, root rinse) and hydroponic nutrient solution as affected by pH values and inoculum levels.

Code	pH	Treatment	Leaves	Roots	Root rinse	Hydroponic solution
511	5	Control				
512	5	Control				
513	5	Control				
514	5	Control				
521	5	Low				
522	5	Low				
523	5	Low				
524	5	Low				
531	5	High				
532	5	High				
533	5	High				
534	5	High				
611	6	Control				
612	6	Control				
613	6	Control				
614	6	Control				
621	6	Low				
622	6	Low				
623	6	Low				
624	6	Low				
631	6	High				
632	6	High				
633	6	High				
634	6	High				
711	7	Control				
712	7	Control				
713	7	Control				
714	7	Control				
721	7	Low				
722	7	Low				
723	7	Low				
724	7	Low				
731	7	High				
732	7	High				
733	7	High				
734	7	High				
811	8	Control				
812	8	Control				
813	8	Control				
814	8	Control				
821	8	Low				
822	8	Low				
823	8	Low				
824	8	Low				
831	8	High				
832	8	High				
833	8	High				
834	8	High				

Green: presence, Red: absence

Table 4.3. Prevalence (% presence) of *S.* Enteritidis on medium lettuce plants (leaves, roots) and hydroponic nutrient solution as affected by pH values and inoculum levels.

			Leaves			Roots			Root rinse			Hydroponic solution		
Code	**pH**	**Treatment**	**7 dpi**	**14 dpi**	**21 dpi**	**7 dpi**	**14 dpi**	**21 dpi**	**7 dpi**	**14 dpi**	**21 dpi**	**7 dpi**	**14 dpi**	**21 dpi**
511	**5**	**Control**												
512	**5**	**Control**												
521	**5**	**Inoculated**												
522	**5**	**Inoculated**												
611	**6**	**Control**												
612	**6**	**Control**												
621	**6**	**Inoculated**												
622	**6**	**Inoculated**												
711	**7**	**Control**												
712	**7**	**Control**												
721	**7**	**Inoculated**												
722	**7**	**Inoculated**												

Green: presence, Red: absence

4.3.3. Main experiment

4.3.3.1. *S.* Enteritidis performance on different nutrient solution pH for small-medium lettuce plants

The prevalence of *S.* Enteritidis on small-medium lettuce plants is presented in **Table 4.4**. When lettuce plants were inoculated with low populations (3 log cfu/mL), no root internalization was observed at pH 5 on both sampling days, while at higher pH values (6, 7 and 8) *S.* Enteritidis was present at up to 33.33% inside roots. Not only in roots but also in root rinse the bacterium was absent at pH 5 at 14 and 21 dpi. High inoculum level (6 log cfu/mL) resulted to root internalization and presence in root rinse solution in all tested samples on both samplings and all pH values. However, low inoculum lead to lower accumulation of the bacterium around the roots (root rinse) (up to 66.67% at pH 8) 21 dpi. The bacterium was present in inoculated nutrient solution at both inoculum levels (low and high), however no internalization on leafy parts was observed on both sampling days regardless inoculum level and pH.

4.3.3.2. *S.* Enteritidis performance on different nutrient solution pH for old lettuce plants

4.3.3.2.1. Effects of nutrient solution pH on *S.* Enteritidis prevalence

The prevalence of *S.* Enteritidis on old lettuce plants is presented in **Table 4.5**. Inoculation with low population revealed no internalization of the bacterium in roots at pH 5, while it resulted up to 16.67% internalization in root tissues at pH $\geq$ 6 (i.e. pH of 6, 7 and 8), whilst high population resulted up to 83.33% at higher pH values (7 and 8). Interestingly, up to 50% of the samples inoculated with low population showed presence of *S.* Enteritidis around the rhizosphere, while the bacterium was found in all root rinse samples tested that have been inoculated with high population. No internalization on leaves was evident on samples from different pH levels and different inoculum levels, whist *S.* Enteritidis was present in the hydroponic nutrient solution on both inoculum levels.

Table 4.4. Prevalence (% presence) of *S.* Enteritidis on small-medium lettuce plants (leaves, roots, root rinse) and hydroponic nutrient solution as affected by pH values and inoculum levels.

		Leaves		Roots		Root rinse		Hydroponic solution	
pH	**Inoculum**	**14 dpi**	**21 dpi**	**14 dpi**	**21 dpi**	**14 dpi**	**21 dpi**	**14 dpi**	**21 dpi**
5	**Control**	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)
	Low	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	6/6 (100%)	6/6 (100%)
	High	0/6 (0%)	0/6 (0%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)
6	**Control**	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)
	Low	0/6 (0%)	0/6 (0%)	1/6 (16.70%)	2/6 (33.33%)	1/6 (16.70%)	3/6 (50%)	6/6 (100%)	6/6 (100%)
	High	0/6 (0%)	0/6 (0%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)
7	**Control**	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)
	Low	0/6 (0%)	0/6 (0%)	0/6 (0%)	1/6 (16.70%)	0/6 (0%)	3/6 (50%)	6/6 (100%)	6/6 (100%)
	High	0/6 (0%)	0/6 (0%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)
8	**Control**	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)
	Low	0/6 (0%)	0/6 (0%)	0/6 (0%)	2/6 (33.33%)	6/6 (100%)	4/6 (66.67%)	6/6 (100%)	6/6 (100%)
	High	0/6 (0%)	0/6 (0%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)

Table 4.5. Prevalence (% presence) of *S.* Enteritidis on old lettuce plants (leaves, roots, root rinse) and hydroponic nutrient solution as affected by pH values and inoculum levels.

		Leaves	Roots	Root rinse	Hydroponic solution
pH	**Inoculum**	**28 dpi**	**28 dpi**	**28 dpi**	**28 dpi**
5	**Control**	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)
	Low	0/6 (0%)	0/6 (0%)	3/6 (50%)	6/6 (100%)
	High	0/6 (0%)	2/6 (33.33%)	6/6 (100%)	6/6 (100%)
6	**Control**	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)
	Low	0/6 (0%)	1/6 (16.67%)	3/6 (50%)	6/6 (100%)
	High	0/6 (0%)	3/6 (50%)	6/6 (100%)	6/6 (100%)
7	**Control**	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)
	Low	0/6 (0%)	1/6 (16.67%)	3/6 (50%)	6/6 (100%)
	High	0/6 (0%)	5/6 (83.33%)	6/6 (100%)	6/6 (100%)
8	**Control**	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)
	Low	0/6 (0%)	1/6 (16.67%)	3/6 (50%)	6/6 (100%)
	High	0/6 (0%)	5/6 (83.33%)	6/6 (100%)	6/6 (100%)

4.3.3.2.2. Effects of nutrient solution pH and *S.* Enteritidis on lettuce physicochemical attributes

As presented in **Table 4.6**, the two-way ANOVA revealed that lettuce antioxidant activity (FRAP) and H_2O_2 production were significantly affected ($P < 0.01$) by the pH of the hydroponic solution. The different inoculum levels significantly affected chlorophylls and carotenoid content and antioxidant activity (DPPH, FRAP) ($P < 0.001$), as well as the phenolic content, H_2O_2 production and MDA levels of lettuce ($P < 0.01$). The antioxidant activity (FRAP), H_2O_2 production and MDA levels were significantly ($P < 0.05$, $P < 0.05$ and, $P < 0.01$, respectively) impacted by the interaction pH*inoculum.

Table 4.6. Effects of pH and inoculum on chlorophyll, carotenoid and phenolic content, antioxidant activity, H_2O_2 production and lipid peroxidation of lettuce.

	Chl a	Chl b	Total Chl	Total Car	Phenols	DPPH	FRAP	ABTS	H_2O_2	MDA
pH	ns	ns	ns	ns	ns	ns	**	ns	**	ns
Inoculum	***	***	***	***	**	***	***	ns	**	**
pH*Inoculum	ns	ns	ns	ns	ns	ns	*	ns	*	**

ns, *, **, and *** indicate non-significant or significant differences at $P \leq 5\%$, 1% and 0.1%, respectively, following two-way ANOVA.

Figure 4.9 illustrates the effects of pH and inoculum on pigment content of lettuce. High population of *S.* Enteritidis at pH 5 resulted to higher lettuce Chl a level than control on the same pH (0.64 and 0.42 mg Chl a/g Fw, respectively). Moreover, high population of *S.* Enteritidis increased Chl b, total Chl and total carotenoids content at pH 5, 6 and 7 of the hydroponic solution compared to the control at the same pH values. It is noteworthy that no significant differences were reported among pH on the same inoculum level ($P > 0.05$).

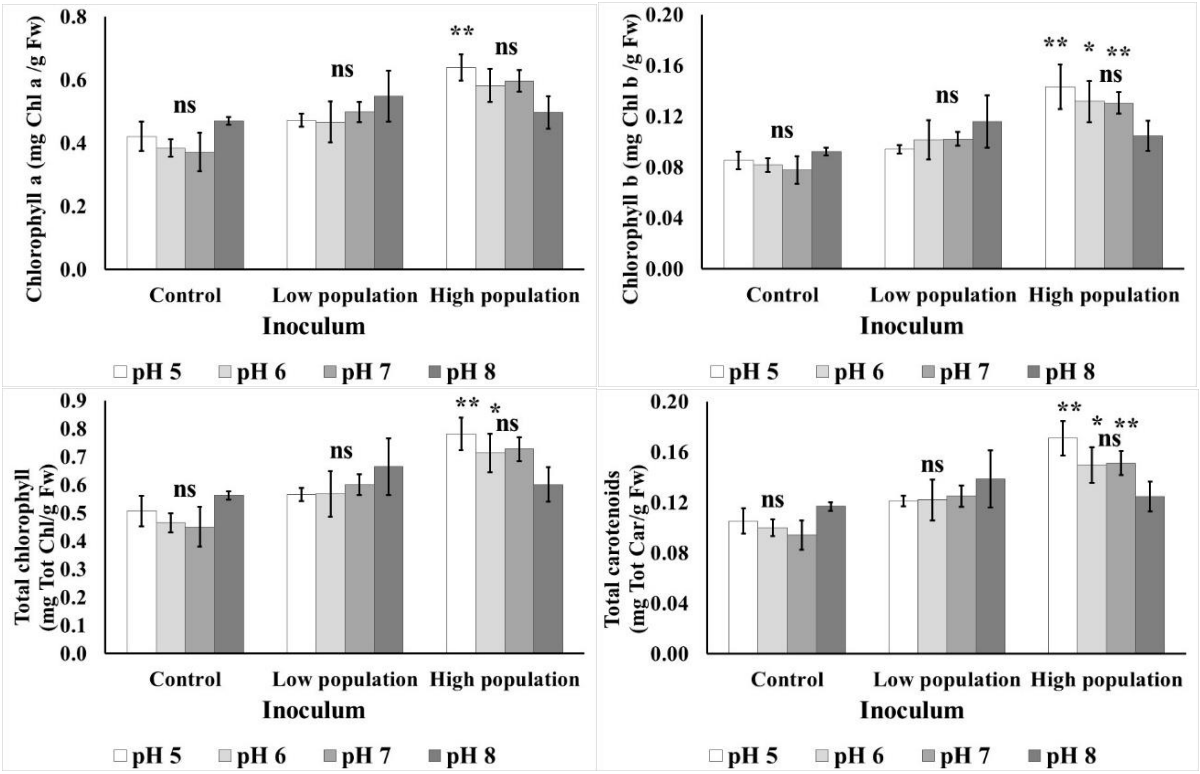


Figure 4.9. Effects of pH and inoculum on lettuce leaf pigments. Significant differences ($P < 0.05$) among pH on the same inoculum are indicated with different Latin letters. Asterisks (*) indicate the significant difference ($P < 0.05$) between inoculated and control plants on the same pH. ns, *, **, and *** indicate non-significant or significant differences among inoculum levels on the same pH value at $P \leq 5\%$, 1% and 0.1%, respectively, following one-way ANOVA.

As shown in **Figure 4.10** decreased phenolic content was observed at pH 6 of the hydroponic solution with high inoculum compared to pH 5 (0.11 and 0.15 mg GAE/g Fw, respectively). Low inoculum increased FRAP antioxidant activity of lettuce at pH 8 (0.12 mg trolox/g Fw) compared to pH 6 and 7 (0.08 and 0.06 mg trolox/g Fw, respectively), while decreased antioxidants were observed with high population at pH 6 compared to pH 5 (0.08 and 0.015 mg trolox/g Fw, respectively) (FRAP assay). Furthermore, the ABTS assay revealed decreased antioxidant activity with the presence of low population at pH 7 of the hydroponic solution compared to pH 6 on the same inoculum level (0.19 and 0.26 mg trolox/g Fw, respectively) (**Figure 4.10**). No significant differences ($P > 0.05$) were reported among pH on the same inoculum level with the DPPH assay, whilst no significant differences ($P > 0.05$) were reported among inoculum levels at the same pH values with the ABTS assay. Increased phenolic content was evident with high population at pH values 5 and 7 of the hydroponic solution (0.15 and 0.14 mg GAE/g Fw, respectively) compared to the control at the same pH values (0.10 and 0.08 mg GAE/g Fw, respectively). The DPPH assay showed that antioxidants increased with high *S.* Enteritidis population at pH values 5, 7 and 8 (0.11, 0.09 and 0.11 mg trolox/g Fw, respectively) in contrast to the control (0.05, 0.06 and 0.06 mg trolox/g Fw, respectively) (**Figure 4.10**). The presence of *S.* Enteritidis on the hydroponic solution at pH 8 (low and high population) (0.13 and 0.12 mg trolox/g

Fw, respectively) and pH 5 (high population) (0.15 mg trolox/g Fw) increased antioxidant activity of lettuce compared to the relevant control plants at the same pH values (0.08 and 0.07 mg trolox/g Fw, respectively) (FRAP assay).

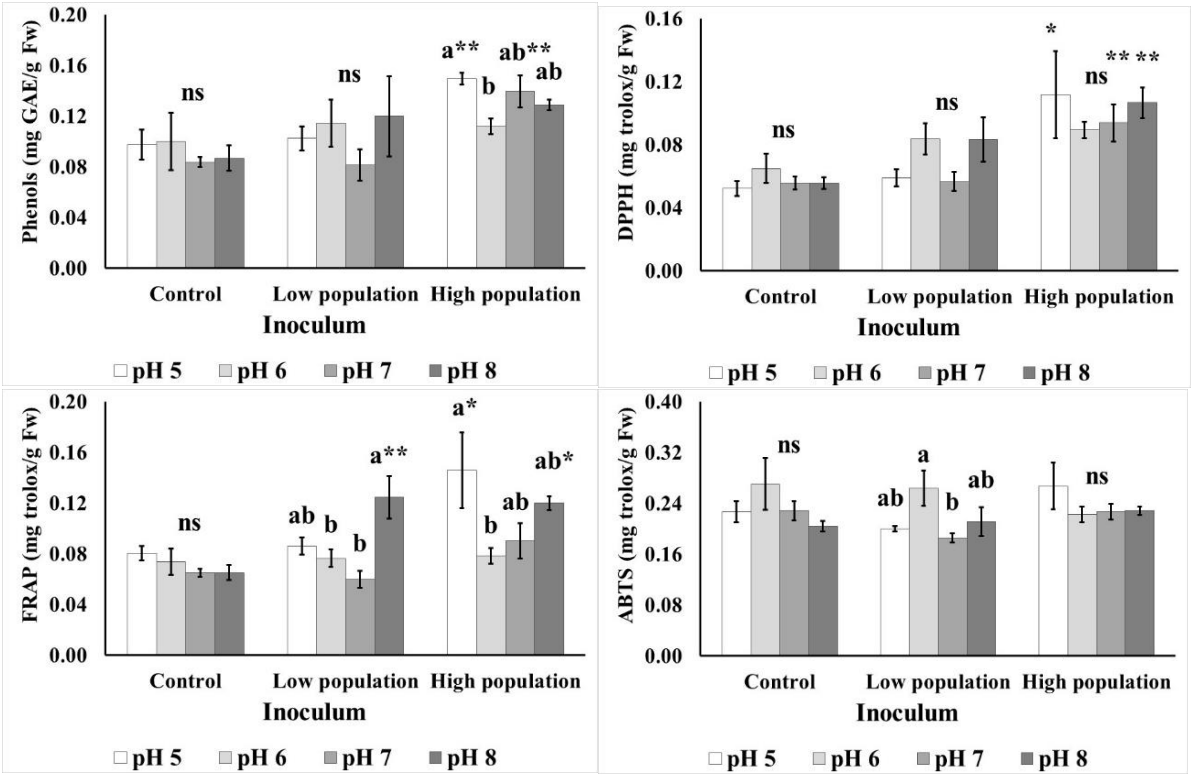


Figure 4.10. Effects of pH and inoculum on lettuce phenolic content and antioxidant activity (as assayed by DPPH, FRAP and ABTS methods). Significant differences ($P < 0.05$) among pH on the same inoculum are indicated with different Latin letters. Asterisks (*) indicate the significant difference ($P < 0.05$) between inoculated and control plants on the same pH. ns, *, **, and *** indicate non-significant or significant differences among inoculum levels on the same pH value at $P \leq 5\%$, 1% and 0.1%, respectively, following one-way ANOVA.

The effects of pH and inoculum on H_2O_2 production and lipid peroxidation of lettuce are illustrated in **Figure 4.11**. The presence of *S.* Enteritidis (low and high population) increased H_2O_2 production at pH 5 (0.43 and 0.36 μmol H_2O_2/g Fw, respectively) compared to all the other pH values of the hydroponic solution with both inoculum levels. MDA levels were increased at pH 8 with low population of *S.* Enteritidis (4.75 nmol MDA/g Fw) in comparison to pH values 5 and 7 on the low inoculum level (3.10 and 3.20 nmol MDA/g Fw, respectively) (**Figure 4.11**). The production of H_2O_2 at pH 5 significantly increased ($P < 0.001$) with the presence of *S.* Enteritidis on the hydroponic solution (low and high population) (0.43 and 0.36 μmol H_2O_2/g Fw, respectively) compared to the control plants at the same pH value (0.13 μmol H_2O_2/g Fw). The low inoculum of *S.* Enteritidis resulted to higher MDA levels in comparison to the control ones at pH 8 (4.75 and 2.80 nmol MDA/g Fw, respectively).

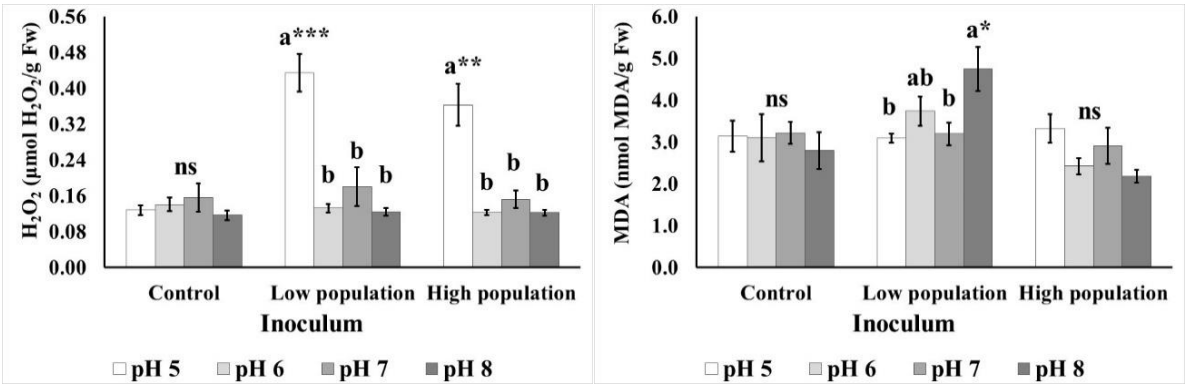


Figure 4.11. Effects of pH and inoculum on lettuce H_2O_2 production and lipid peroxidation (MDA levels). Significant differences ($P < 0.05$) among pH on the same inoculum are indicated with different Latin letters. Asterisks (*) indicate the significant difference ($P < 0.05$) between inoculated and control plants on the same pH. ns, *, **, and *** indicate non-significant or significant differences among inoculum levels on the same pH value at $P \leq 5\%$, 1% and 0.1%, respectively, following one-way ANOVA.

4.4. Discussion

In general, bacterial growth in food can be affected by i) intrinsic factors: moisture content/water activity (a_w), pH, nutrient content, natural occurring and/or added antimicrobial agents and redox potential (Eh), as well as ii) extrinsic factors: type of packaging/atmosphere, temperature, handling/storage conditions, processing. Vegetables can serve as a host for human foodborne pathogens such as *Salmonella* spp., *E. coli*, *L. monocytogenes* and *Shigella* spp. (Whipps et al., 2008; Balali et al., 2020; Lenzi et al., 2021). Leafy vegetable hydroponic production is considered to be a clean and safe procedure however incidence of microbial contamination may occur (Dankwa et al., 2019, Wang et al., 2020). Environmental factors that mediate microbial contamination of fresh produce by both conventional and hydroponic cultivation include pH (soil and water), temperature (field and greenhouse), nutrient form and availability among others (Machado-Moreira et al., 2019; Riggio et al., 2019).

In the present study, bacterial growth in BHI broth decreased with the decrease of pH value on both examined temperatures (21 and 37 ^{o}C) with lower counts at 21 ^{o}C and lower pH. It has been suggested that when *Salmonella* cells are first incubated at a less acidic environment prior inoculating a more acidic medium (i.e. pH 4.7) the bacterial cells are able to survive this pH change by the production of acid shock proteins (Eady and Park, 2019). Slower bacterial growth was observed with incubation of *S.* Enteritidis in nutrient solution and this was more evident at pH 5 and both examined temperatures (21 and 37 ^{o}C). When entering an aquatic environment (rich in nutrients), Salmonella serovars might face adverse conditions like variances in osmolarity, pH and environment, which ultimately will lead the bacterial cells to enter a dormancy or viable-but non culturable (VBNC) state and thus persist for long periods of time (Spector and Kenyon, 2012). Moreover, the assessment of the combination BHI broth and hydroponic nutrient solution revealed that bacterial growth decreased with the decreased of pH

values at both temperatures, with greater decrease at 37 ^{o}C. Bacterial survival in the environment is related to their ability to activate their temperature sensing mechanisms which allow them to respond to temperature changes (De Nisco et al., 2018). These results indicate that pH and temperature affect growth and survival of *S.* Enteritidis. Low temperature (i.e. 21 ^{o}C) and low pH values nutrient solution (i.e. pH 5) seem to slow down the growth of *S.* Enteritidis compared to the combination of BHI broth and nutrient hydroponic solution.

Persistence and survival of foodborne pathogens in plant tissues can be influenced by different factors including route and level of contamination, plant attributes (i.e. age, type, cultivar), bacterial species and even serovars (Franz et al., 2007; Koseki et al., 2011; Schikora et al., 2012; Standing et al., 2013; Macarisin et al., 2014). For instance, Jablasone et al. (2005) reported that *E. coli* O157:H7 counts dependent on plant type and stage of cultivation. At the same time *S.* Typhimurium was reported on lettuce seedlings after 21 dpi compared to *E. coli* O157:H7 and *S. aureus* which were recovered from seedling even from only 3 dpi and persisted up to 28 dpi and all bacteria tested were inoculated at a level of 5 log cfu/mL (Standing et al., 2013). Safety issues arise from the fate of foodborne pathogens once they enter a plant tissue (Erickson et al., 2012).

Hydroponic cultivation of leafy vegetables could possibly result to microbial contamination of fresh produce and pathogen internalization via contaminated nutrient hydroponic solution (Riggio et al., 2019). In the preliminary experiments of this study; no internalization in leaves was observed small-medium and old plants, while internalization of *S.* Enteritidis in leaves was observed 14 dpi on medium plants inoculated with 6 log cfu/mL at pH 7. Furthermore, the bacterium was present in roots of all tested ages plants at high pH values and high inoculum levels and managed to survive on the nutrient solution up to 26 days (at both inoculum levels). It is also noteworthy that high inoculum (6 log cfu/mL) resulted to internalization of the pathogen in roots at all pH values when inoculated to small-medium plants, while root internalization was also evident at pH 7 and 8 when inoculated at lower numbers (3 log cfu/mL). Franz et al. (2007) reported that when lettuce seedlings were inoculated with 7 log cfu/mL little to no internalization was occurred to the leafy part after 18 days cultivation in nutrient hydroponic solution and this also depended on *S.* Typhimurium morphotypes.

When small-medium plants were inoculated with *S.* Enteritidis, no internalization on leaves and roots and absence on root rinse were observed with low inoculum (3 log cfu/mL) at pH 5 up to 21 dpi, while higher inoculum (6 log cfu/mL) and pH values resulted to root internalization. According to Franz et al. (2007) internalization on lettuce leaves and roots was observed after 18 dpi with 7 log cfu/mL with *S.* Typhimurium. However, in that study the nutrient solution was 1/10 diluted compared to the solution used in our study, and that diluted nutrient solution was not at the optimum levels for appropriate plant growth. Moreover, in another study it has been shown that greater internalization incidence in hydroponically grown spinach inoculated with *E. coli* O157:H7 took place with 7 log cfu/mL compared to 5 log cfu/mL (Macarisin et al., 2014). It is worth mentioning that pH 8 resulted to

higher bacterial accumulation around roots of small-medium plants compared to other pH values. These findings suggest that higher inoculum levels increase the likelihood of foodborne pathogen internalization to leafy vegetables. Furthermore, a relationship between human pathogens internalization and chemotaxis has been previously suggested, indicating that foodborne pathogens such as *Salmonella* might activate flagella, move closer to plant tissues (i.e. leaves), attach themselves to surfaces and inevitably penetrate plant cell membranes and natural openings (Lim et al., 2014; Alegbeleye et al., 2018). No internalization on roots and absence on root rinse of old plants was reported with low inoculum (3 log cfu/mL) at pH 5 up to 28 dpi, while higher inoculum (6 log cfu/mL) and higher pH values resulted to greater root internalization. Low inoculum resulted to lower presence of the pathogen around roots. Koseki et al. (2011) also reported that low inoculum (3 log cfu/mL) resulted to lower contamination prevalence on hydroponically grown spinach plants compared to higher inoculum level (6 log cfu/mL) when inoculated through the roots.

Survival of *S.* Enteritidis was evident on nutrient hydroponic solution up to 28 days (both inoculum levels). It has been previously mentioned that foodborne pathogens such as *E. coli* and *Salmonella enterica* are able to adapt to environmental stress (i.e. low pH values) and thus survive in adverse environments for prolonged periods (Alegbeleye et al., 2018). *Salmonella* seems to be more resistant to environmental stress compared to *E. coli* due to the latter's inability to decrease its metabolic rate when organic carbon is not abundant in the environment (Alegbeleye et al., 2018).

It has been proposed that survival of pathogens concerning human health might be enhanced or compromised by temperature, humidity and the presence of other microorganisms (phytopathogens and/or microflora) present on the roots and leaves of plants (Johannessen et al., 2005). The isolation of *Klebsiella* spp. and *Serratia* spp. (members of the *Enterobacteriaceae* family and endophytes) from plant tissues, species closely related to *Salmonella* spp. and *E. coli*, justify the presence of the latter species on vegetables (Teplitski et al., 2011; Lenzi et al., 2021). Moreover, members of the *Enterobacteriaceae* i.e. *E. coli*, *S. enterica*, *Shigella* spp. and *Yersinia* spp. seem to be closely related to *Erwinia carotovora*, a plant pathogen (Whipps et al., 2008). These findings support the hypothesis that human pathogens have the ability to attach and enter a plant tissue as phytopathogens. Plant pathogens possess the ability to produce enzymes such as pectinases which can compromise plant cell walls enabling their internalization, while foodborne pathogens (i.e. *E. coli*, *Salmonella* spp.) are able to produce enzymes that can also lead to periplasmic pectin degradation (Abbott and Boraston., 2008; Lenzi et al., 2021). These evidences show that both plant microflora and foodborne pathogens might collaborate in their attempt to establish and survive in a plant tissue and at the same time support the findings of other studies that suggest the internalization of foodborne pathogens in vegetables without any signs of spoilage. In our study, variation in total viable counts (TVC) was observed in roots of old plants with pH 8 treated plants presenting decreased numbers (2.94 log cfu/g) compared to lower pH values, while TVC numbers on nutrient hydroponic solution and leaves did not differ among pH values

(**Table 4.7**). Interestingly, no significant differences were observed on leaves, roots and hydroponic nutrient solution TVC values for small-medium plants on both sampling days, whilst greater values were observed 21 dpi compared to 14 dpi (**Table 4.7**).

Inoculum level and pH values affected antioxidant activity, phenolic and pigment content and as well as stress indicators (H_2O_2 and MDA levels) of lettuce plants. High inoculum (6 log cfu/mL) increased pigment and phenolic content, while both inoculums increased antioxidants at pH 8 and 5. It is well known that plant defense mechanisms are activated in the presence of plant pathogens and this might be taking place with the presence of human pathogens as both can be interpreted as biotic stress and might be targeted by the same defense pathways (Erickson et al., 2012). Moreover, plants have the ability to detect bacterial flagella and interfere with human pathogen internalization by closing plant surface stomata (Lim et al., 2014). Plant defense mechanisms against abiotic and biotic stress include enzymatic (SOD, CAT, POD) and non-enzymatic processes (antioxidants, phenols, ascorbic acid). Lim et al. (2014) hypothesized that compounds derived from photosynthesis can be utilized by pathogens enabling their internalization in plant tissues. The interaction of pH*inoculum affected antioxidant activity, H_2O_2 and MDA levels. Low inoculum increased MDA levels at pH 8, whilst inoculated plants (both inoculums) presented higher H_2O_2 production at pH 5. Increased H_2O_2 and MDA levels suggest increased stress which might be due to the adverse pH levels (higher/lower than optimum pH value – 5.8- for hydroponics) and/or the presence of pathogens. The regulation of endophytic plant pathogens is regulated directly by the activation of plant defense mechanisms in order to fight the invasion (Teplitski et al., 2011; Erickson et al., 2012). It is believed that many of the mechanisms used from plants against plant pathogens are also activated during the presence and internalization of foodborne pathogens in plant tissues (Pieterse and Dicke, 2007; Erickson et al., 2012).

To fully understand the factors influencing the internalization and persistence of foodborne pathogens in leafy vegetables there is need for further investigation of good agricultural practices. Greater scale experiments are needed, with close monitoring of the plant growth conditions (i.e. pH, temperature, water quality) and a correlation of the pathogen's presence and internalization with plant related parameters could possible give more insights for safer fresh produce production.

Table 4.7. Total viable counts of old and small-medium lettuce plants (leaves, roots) and hydroponic nutrient solution as affected by pH values.

	Old plants			Small-medium plants					
	28 dpi			14 dpi			21 dpi		
pH	**Leaves**	**Roots**	**Hydroponic solution**	**Leaves**	**Roots**	**Hydroponic solution**	**Leaves**	**Roots**	**Hydroponic solution**
5	3.37 ± 0.31a	3.38 ± 0.16b	3.05 ± 0.36a	2.5 ± 0.49a	3.26 ± 0.23a	3.05 ± 0.56a	2.75 ± 0.67a	6.57 ± 0.31a	3.83 ± 0.10a
6	3.66 ± 0.70a	3.58 ± 0.15ab	2.87 ± 0.28a	2.62 ± 0.38a	3.17 ± 0.12a	2.69 ± 0.31a	2.96 ± 0.35a	6.74 ± 0.15a	3.37 ± 0.34a
7	3.5 ± 0.80a	3.62 ± 0.12a	2.89 ± 0.21a	2.48 ± 0.16a	3.24 ± 0.19a	2.95 ± 0.38a	2.91 ± 0.34a	6.25 ± 0.52a	4.1 ± 0.89a
8	3.57 ± 0.22a	2.94 ± 0.09c	2.98 ± 0.41a	2.89 ± 0.28a	3.19 ± 0.19a	2.93 ± 0.30a	2.64 ± 0.42a	6.98 ± 0.73a	3.99 ± 0.59a

Significant differences ($P < 0.05$) among pH on the same column are indicated with different Latin letters.

4.5. Concluding marks

Survival and growth of *S.* Enteritidis is affected by pH and temperature, where lower pH values and plant growth temperatures (21 ^{o}C) lead to decreased growth rates compared to optimum incubation conditions. Moreover, the growth of *S.* Enteritidis on nutrient hydroponic solution is slower and this was more evident at pH 5. This might suggest that human pathogens take more time to adapt to the new environmental conditions at lower pH values compared to higher values, where different nutrient availability is directly depended on the pH value. Higher inoculum levels increase the probability of internalization of foodborne pathogens in fresh produce and leafy vegetables.

It is noteworthy that plant age seems to affect the colonization of lettuce plants, since small-medium plants were more susceptible to root internalization compared to the older plants at higher pH values. The presence of *S.* Enteritidis in the nutrient solution and its internalization on plant tissues increases damage index (H_2O_2 production and MDA levels) and also enhances plant defense mechanisms such as the production of phenols and antioxidants. Further investigation of the factors affecting plant colonization and human pathogen internalization and persistence in relation to plant related parameters could lead to the development of guidelines for fresh produce hydroponic cultivation which will ensure the production of safe and nutritional products. Moreover, the role of individual nutrients in the form of cations and/or anions needs to be examined, as they might pose a targeted role on the bacterium performance (i.e. the level of potassium or calcium, etc.). In such a case, a Taylor-made nutrient solutions recipe could be adapted to balance the bacterium performance and plant growth conditions, ensuring safer and nutritional fresh produce.

Chapter 5. Effects of natural products on preservation and quality of fresh-cut vegetables

5.1. Introduction

Over the last few years the market of fresh-cut produce is growing dramatically as the increased consumer demand for fresh, safe, healthy (high nutritional value) and convenient food (Zhan et al., 2012). Vegetables (including leafy, roots and other plants' part) that are intended to be marketed as "fresh-cut" are subjected to washing, peeling, slicing, and shredding followed by packing and sealing in polymeric films (Akbas and Ölmez, 2007a). Among leafy vegetables, lettuce is quite popular due to its crispness, pleasant aroma, and high content of phytonutrients, including phenolic compounds and vitamins (C, K and folate). However, lettuce is a very perishable vegetable and when processed and/or stored at less favored conditions (i.e. high temperature, low humidity, improper packaging material) are susceptible to physiological disorders, enzymatic browning, loss of nutritional value, microbial contamination and spoilage (Allende et al., 2004; Zhan et al., 2012; Chen et al., 2017). Several studies assessed the microbial quality of ready-to eat vegetables and salads revealing a broad range of microorganisms (bacteria, fungi, viruses) that can possible adversely affect product quality (spoilage) and/or threaten human health (foodborne pathogens) (Gurler et al., 2015; Losio et al., 2015; Nousiainen et al., 2016; Xylia et al., 2019a).

The washing procedure during minimal processing is performed using chlorinated water which contains 50-200 mg/L sodium hypochloride (NaOCl) and the applied dose (concentration and time of application) depends on the product (fresh produce) (Francis et al., 1999). The main aim of washing is the reduction of the microbial load of vegetables (Akbas and Ölmez, 2007a; FAO/WHO, 2008). However, as it has been previously reported chlorine can result to the formation of undesirable and harmful by-products such as trihalomethanes, haloketones, chlorophorm and halloacetic acids with adverse effects in human health as well as the environment (Akbas and Ölmez, 2007a). This has led to increased concern and the demand for natural alternatives to replace chemicals used in the food industry and meet consumers' needs. These alternatives might include essential oils (EOs) from aromatic plants, organic acids (ascorbic, oxalic, lactic acid), peptides, reducing agents (ascorbic acid, cysteine) and more (Martin-Diana et al., 2006; Akbas and Ölmez, 2007a,b; Chrysargyris et al., 2016b; Viacava et al., 2018; Tzortzakis et al., 2019; Xylia et al., 2019b).

Chitosan is a naturally occurring polymer, delivered from crab shells and has been used as a stabilizer for ingredients in tablets in medicines. Chitosan was the first basic substance approved by the European Union for plant protection (Reg. EU 2014/563) due to its low toxicity and when applied to plants its effectiveness results from three mechanisms (film formation, antimicrobial activity and elicitation of host defense) (Romanazzi et al., 2018). It has been previously shown that edible chitosan coatings alone or incorporated with EOs presented enhanced antimicrobial activity against foodborne pathogens including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus cereus*, *Serratia*

marcescens, *Escherichia coli*, *Enterococcus faecalis* and *Salmonella* Enteritidis (Azevedo et al., 2014). Chitosan has been proven to act as an elicitor of plant defense mechanisms when applied to plant tissues and this forms a protection against pre- and postharvest microbial contamination and spoilage of strawberry fruit as reported by Landi et al. (2017). Chitosan and commercial chitosan formulations have been previously applied to fresh produce (dipping, spraying) with encouraging results (Romanazzi et al., 2013; Severino et al., 2014). However, little is to be known about the application on fresh-cut produce.

Ascorbic acid (AA) is being widely used in the food industry mainly as a reducing agent in order to prevent undesirable reactions such as enzymatic browning. As with other organic acids (i.e. citric, oxalic acid), AA has been used during processing in order to control the microbial load of fresh produce due to its antioxidant activity as well as its ability to lower the pH of washing water (Akbas and Ölmez, 2007a,b; Altunkaya and Gökmen, 2009; Park et al., 2011). Additionally, the application of AA on shredded carrots resulted to higher antioxidants, ascorbic acid and phenolic content, increasing the nutritional value of the product whilst preserving its visual quality throughout storage (Xylia et al., 2019b). The application of AA on fresh-cut lettuce has been previously reported with promising results (Rivera et al., 2006; Altunkaya and Gökmen, 2009; Altunkaya, 2011; Park et al., 2011). For instance, Altunkaya and Gökmen, (2009) reported that AA maintained high phenolic content of fresh-cut lettuce indicating lower phenol oxidation levels due to enzymatic activity and thus lower browning on cut surfaces. In another study the bright green color of lettuce was maintained with the application of AA (Rivera et al., 2006).

Essential oils are secondary metabolites of plants that have been used for prolonging food shelf life and improving organoleptic attributes (aroma, taste) of food since many of them are characterized as Generally Recognized as Safe (GRAS) (Yuan et al., 2016). They possess antioxidant and amicrobial activity among others and can be used in low concentrations that are safe for consumption (Sivakumar and Baños, 2014). Several studies have demonstrated the antioxidant activities of a plethora of EOs including lavender, spearmint, fennel, cumin, thyme (Viuda-Martos et al., 2011; Amorati et al., 2013; Chrysargyris et al., 2016a, 2017). Furthermore, EOs can exhibit antimicrobial activity against a wide range of microorganisms including spoilage and foodborne pathogenic bacteria and fungi (Arrebola et al., 2010; Viuda-Martos et al., 2011; Ma et al., 2016; Chrysargyris et al., 2017). For example Chrysargyris et al. (2017) reported the antibacterial activity of spearmint EO against foodborne pathogen bacteria from plants grown in different potassium levels, in hydroponics. The application of EOs on fresh produce has been previously mentioned and several studies have examined the effects of EO application on fresh-cut lettuce (Singh et al., 2002; Bagamboula et al., 2004; Scollard et al., 2016; Chen et al., 2017; Viacava et al., 2018). The application of EOs on minimally processed vegetables such as lamb's lettuce, shredded carrot, sliced cucumber and broccoli has been previously reported with encouraging results (Siroli et al., 2015; Xylia et al., 2019b; Zhao et al., 2020). For example, the

application of marjoram EO and hydrosol in combination with AA improved the quality of shredded carrot stored at 4 ^{o}C for nine days (Xylia et al., 2019b).

Hydrosols (hydrolates, aromatic waters) are another byproduct that derives from hydro/steam distillation of plants during EO production and started gaining attention recently (D'Amato et al., 2018). These products have been shown to possess many beneficial properties including antioxidant and antimicrobial activities. Due to their complex composition their properties and activity may vary. Many studies have assessed the antioxidant properties of hydrosol from various plants (Ćavar and Maksimović, 2012; Belabbes et al., 2017; Shen et al., 2017; Cid-Pérez et al., 2019). The antioxidant activity of hydrosols has been examined by many researchers on a wide range of microorganisms including bacteria and fungi (Sağdıç, 2003; Chorianopoulos et al., 2008; Ulusoy et al., 2009; Belabbes et al., 2017; Waller et al., 2017; Cid-Pérez et al., 2019). The application of hydrosols on fresh-cut leafy vegetables (i.e. lettuce, parsley) has been previously mentioned and the results are promising (Ozturk et al., 2016; Tornuk et al., 2014). The application of thyme, summer savory, rosemary, salvia, sideritis, oregano and bay leaf hydrosols presented antibacterial activity against *Salmonella* Typhimurium, *Listeria monocytogenes* and *Escherichia coli* O157:H7 inoculated on fresh-cut iceberg lettuce (Ozturk et al., 2016).

Hurdle technology could be applied during postharvest processing of fresh produce combining "hurdles" such as the use of preservatives, chilling storage temperatures for the improvement of microbial and sensory quality of ready-to eat vegetables (Leistner, 2000). In this sense the combination of EOs with anti-browning agents or edible coatings could possibly preserve or even improve fresh produce attributes during storage as fresh-cut products. For instance, Viacava et al. (2018) reported that the microencapsulation of thyme EO in β-cyclodextrin preserved the green color of fresh-cut lettuce compared to the EO when used alone. Moreover, the combination of *Lippia gracilis* with edible chitosan coating formulations showed encouraging results against several foodborne pathogens (Azevedo et al., 2014). However, EOs and their combinations should be used with precaution as toxicity or allergy issues might arise even though they are characterized as GRAS. Furthermore, the use of EOs could be considered as a costly procedure for the food industry but since their incorporation in edible coatings and the use of reduced concentrations (due to their intense aroma that can affect applied product's quality) this cost can be also reduced (Sánchez-González et al., 2011).

The purpose of this chapter was to assess the effects of natural products (marjoram EO and hydrosol, chitosan, ascorbic acid and their combinations) in the preservation of quality attributes of fresh-cut lettuce (Experiment 1) and shredded carrots (Experiment 2) stored under refrigerated conditions (7 and 4 ^{o}C, respectively).

5.2. Experiment 1. Effects of natural products on fresh-cut lettuce preservation

5.2.1. Material and methods

5.2.1.1. Plant material and EO extraction

Fresh lettuce (*Lactuca sativa* L.) was obtained from a local market in Limassol, Cyprus and samples were selected for uniformity in appearance and the absence of physical defects or injury and then stored at 4 ^{o}C and 90% RH until use (within 24 h).

Marjoram plants (*Origanum majorana* L.) were obtained from the experimental farm of Cyprus University of Technology, where they were cultivated in soil. Marjoram plant tissue was harvested, air-dried (in oven at 42 ^{o}C), chopped and hydrodistillation was performed using Clevenger apparatus, with 100 g of dried tissue for 3 h. The obtained EO was stored at -20 ^{o}C until use in amber glass bottles. The composition of marjoram EO was determined as described previously (Chrysargyris et al., 2016a), and the main components were terpinen4ol, γ-terpinene, trans sabinenehydrate, and α-terpinene.

5.2.1.2. Preliminary screening

After the removal of damaged exterior leaves, fresh lettuce leaves were cut into pieces (5 x 10 cm) and washed with tap water. Then, 70 g of fresh-cut leaves were dipped for 1 min (according to preliminary tests following evaluation for the dipping time of 1, 5 and 10 min during a 4-day storage, as presented in Figure 5.1S) into 500 mL of treatment solution. The following fourteen dipping solutions were studied: 1) distilled water (control), 2) marjoram EO (1:1000, 1:1500, 1:2000 and 1:2500 v/v), 3) chitosan (Chito Plant; ChiPro GmbH, Bremen, Germany) (0.1, 0.125, 0.25, 0.5 and 1% w/v) and 4) ascorbic acid (0.25%, 0.5%, 1%, and 2% w/v). Afterwards, leaf pieces were drained, and approx. 15 g were placed in a polypropylene (PP) plastic container (1 L) and enclosed into a polyethylene terephthalate (PET) plastic tray and stored at 7 ^{o}C and 90% RH until the day of sampling (Days 0, 2, 4 and 6). Three biological replicates per treatment/concentration were sampled and the appropriate amount of tissue was stored at -20 ^{o}C until analysis. For preliminary screening, aroma and marketability assessed by seven untrained panelists, while the weight loss and the total polyphenol content was determined and used for choosing the adequate dipping solutions for further assessment.

Lettuce weight loss was monitored every second day during storage at 7 ^{o}C for 6 days (Days 0, 2, 4 and 6) and results were presented as percentage of total weight loss. After storage for 6 days overall aroma and chroma/color (visual quality) were scored. Evaluation of aroma was assessed with a 5-point hedonic scale (0.5 interval) where 5 = not acceptable; 3 = not lettuce but acceptable; 1 = lettuce. Visual quality was evaluated with a 5-point hedonic scale (0.5 interval) where 5 = severe browning; 3 = light discoloration; 1 = green. Total phenolic content of methanolic extracts was determined as previously

described at section **2.2.8.2** and results were expressed as mg of gallic acid equivalents per g of fresh weight (mg GAE/g Fw).

5.2.1.3. Main study for the determination of quality and antioxidant activity

Following preliminary screening, lettuce were selected, prepared and processed as above. Then, approx. 70 g of fresh-cut lettuce were dipped for 1 min into 500 mL of treatment solution selected from the preliminary screening. The following eight dipping solutions/combinations were further assessed: 1) distilled water (control), 2) marjoram EO (1:1500 v/v), 3) chitosan (0.1% w/v), 4) ascorbic acid (1% w/v), 5) marjoram EO (1:1500) + chitosan (0.1%), 6) marjoram hydrosol (1:15) + ascorbic acid (1%), 7) chitosan (0.1%) + ascorbic acid (1%) and 8) chlorine (0.2 mL/L). Afterwards, lettuce pieces were drained, and approx. 70 g were placed into a polypropylene (PP) plastic tray and stored at 7 ^{o}C and 90% RH until the day of sampling (Days 0, 2, 4 and 6). Three biological replicates per treatment/day were sampled, while appropriate amount of tissue was stored at -20 ^{o}C until analysis.

5.2.1.3.1. Weight loss and color

Weight loss was determined as mentioned above and results were expressed as percentage of total weight loss. Minimally processed lettuce's color was evaluated using a colorimeter (Chroma meter CR400 Konica Minolta, Japan) and *L**, *a**, *b**, chroma value (*C*), color index (*CI*), hue (*h*), browning index (*BI*) and whiteness index (*WI*) color parameters were calculated as previously described at section **2.2.2**.

5.2.1.3.2. Respiration rate and ethylene emission

Respiration of the fresh-cut lettuce was evaluated by measuring the CO_2 concentration as described previously at section **2.2.3**. Briefly, samples were enclosed in a polypropylene (PP) plastic container (1 L) at room temperature for 1 hour, container's air was withdrawing for 40 seconds and respiration rate was expressed as mL of CO_2 per kg of fresh weight per h (mL CO_2/kg/h) according to the volume and weight of the processed lettuce. Ethylene flow rate was estimated by measuring the ethylene concentration of the packages as previously mentioned at section **2.2.3**. Briefly, samples container's air was withdrawing for 20 seconds through a hole on the lid recording the ppm of ethylene produced and ethylene emission was computed as μL of ethylene per kg of fresh weight per h (mL ethylene/kg/h).

5.2.1.3.3. Leaf chlorophyll and carotenoid content

For leaf pigment extraction and determination was performed as described at section **2.2.7** and results were expresses as mg chlorophyll (or carotenoids) per g of fresh weight (mg chl (or car)/g Fw).

5.2.1.3.4. Total soluble solids, total acidity, ascorbic acid

Lettuce tissue (three biological replicates/treatment/day; 1 g) was grinded/pressed to extract the juice with a domestic blender. Total soluble solids (TSS) content was determined as mentioned at section **2.2.4** and results were expressed in ^{o}Brix. Lettuce titratable acidity (TA) was assessed by as described section **2.2.4** and results were expressed as grams of malic acid per 100 g of fresh weight (% TA). Sweetness (TSS/TA) was also evaluated (section **2.2.4**).

The quantification of AA content was assessed by titration as described at section **2.2.5** and results were expressed as milligrams of AA per 100 g of fresh weight (mg AA/100 g Fw).

5.2.1.3.5. Total phenolic content and antioxidant activity

Total phenolic content was determined as mentioned at section **2.2.8.2** and results were expressed as mg GAE per g of fresh weight (mg GAE/g Fw). The 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assays were used for the determination of antioxidant activity of lettuce methanolic extracts according to the procedures described before at sections **2.2.8.3** and **2.2.8.5**. Results expressed as mg trolox per g of fresh weight (mg trolox/g Fw).

5.2.1.3.6. Hydrogen peroxide content and lipid peroxidation

The concentration of hydrogen peroxide (H_2O_2) was quantified according to the method described previously at section **2.2.9.1** and results were expressed as μmol of H_2O_2 per g of fresh weight (μmol H_2O_2/g Fw). Lipid peroxidation of minimally processed lettuce was evaluated using the 2-thiobarbituric acid reactive substances (TBARS) method as previously described at section **2.2.9.2** and results were expressed as nmol of malondialdeyde (MDA) per g of fresh weight (nmol MDA/g Fw).

5.2.1.3.7. Microbiological analysis

Total viable count (TVC) as well as yeast and filamentous fungi were determined as described at section **2.2.1**, **2.2.1.1** and **2.2.1.8** using Plate count agar (PCA, Merck, Darmstadt, Germany) and Rose Bengal Chloramphenicol Agar (Liofilchem s.r.l, Italy), respectively (Chen et al., 2010). Results were expressed as log cfu per g of fresh weight (log cfu/g).

5.2.1.3.8. Sensory evaluation

Sensory evaluation (aroma and visual quality) was performed on each sampling day, as described above (section **5.2.1.2**). Briefly, aroma was evaluated with a 5-point hedonic scale (0.5 interval) where 5 = not acceptable; 3 = not lettuce but acceptable; 1 = lettuce. Visual quality was evaluated with a 5-point hedonic scale (0.5 interval) where 5 = severe browning; 3 = light discoloration; 1 = green.

5.2.1.4. Statistical analysis

Statistical analysis was performed comparing data means with one way-analysis of variance (ANOVA) using IBM SPSS version 25 and Duncan's multiple range test was performed for $p = 0.05$. Four (n=4) biological replicates were used and values refereed to mean ±SE. Microbiological analysis was done with duplicate plates for each of the three replicates.

5.2.2. Results

5.2.2.1. Preliminary screening

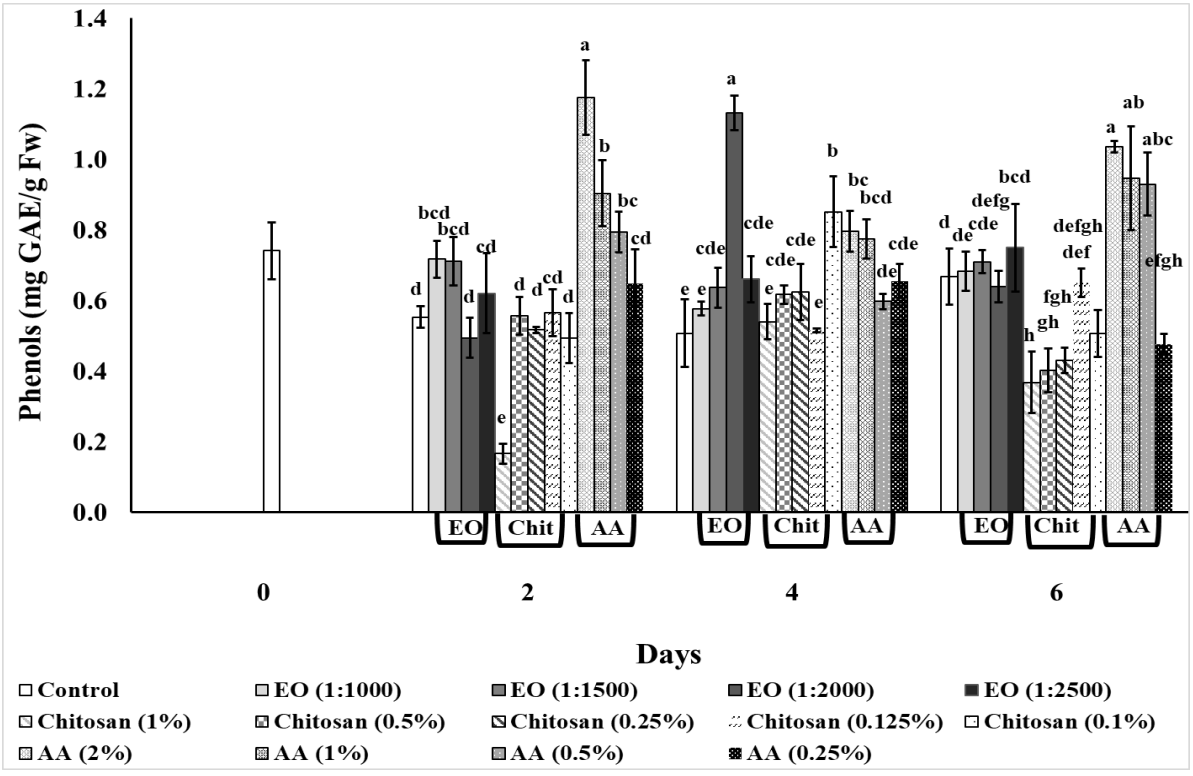
The effect of marjoram EO, Chitosan and AA application on fresh-cut lettuce during the preliminary screening is shown in **Figures 5.1** and **5.S2**. During storage, EO application (1:1500) reduced and AA of 0.5% accelerated weight loss after 2 days compared with the control treatment (**Figure 5.S2**). Following 6 d of storage, EO application (1:1500) and chitosan of 0.125% reveled lower weight losses while all the other applications had same weight losses as the control treatment.

Phenolic content was found to be higher with the application of AA 2%, 1% and 0.5% (1.18, 0.90 and 0.79 mg GAE/g Fw, respectively) compared to the control (0.55 mg GAE/g Fw) after 2 days of storage (**Figure 5.1**). On the same day, EO application (from 1:1000 to 1:2500) showed similar effects on lettuce phenolic content as the control treatment (0.55 mg GAE/g Fw), whilst the application of Chitosan 1% resulted to lower phenolics (0.17 mg GAE/g Fw). During the fourth day of storage an increase of phenols was observed with the application of EO 1:2000 (1.13 mg GAE/g Fw) and Chitosan 0.1% (0.85 mg GAE/g Fw) compared to the control treatment (0.51 mg GAE/g Fw). Increased phenolic content was also observed on the fourth day of storage with the application of AA 2%, 1% and 0.5% (0.80, 0.78 and 0.60 mg GAE/g Fw, respectively). Phenolic content was higher after six days with the application of AA 2%, 1% and 0.5% (1.04, 0.95 and 0.93 mg GAE/g Fw, respectively), while the application of AA 0.25% decreased the phenolic content (0.47mg GAE/g Fw) compared to the control treatment (0.67 mg GAE/g Fw). On the same day, decreased phenolics were reported with the

application of Chitosan 1%, 0.5% and 0.25% (0.37, 0.40 and 0.43 mg GAE/g Fw, respectively), whilst no significant difference was observed between the application of EO and the control treatment.

The application of Chitosan 0.5 and 1% after four and six days of storage resulted in a product marked with 3 out of a 5 scale, which indicated an acceptable aroma but not lettuce like (**Figure 5.1**). On the other hand, the application of AA and EO 1:1000 and 1:1500 after two and six days of storage revealed a lettuce like ("freshness") aroma scoring 1 and 2 out of a 5 scale.

The effects of the applied preservative means on the visual quality of fresh-cut lettuce are illustrated in **Figures 5.1** and **5.S3**. EO and AA (2% and 1%) application maintained the green color of fresh-cut lettuce throughout storage (up to six days). The application of Chitosan on higher concentrations (1% and 0.5%) resulted to higher scores on the fourth and sixth day of storage compared to other treatments. It is noteworthy that Chitosan 1% scored 4 out of a 5 scale reflecting the incidence of browning on fresh-cut lettuce from the second day of storage.



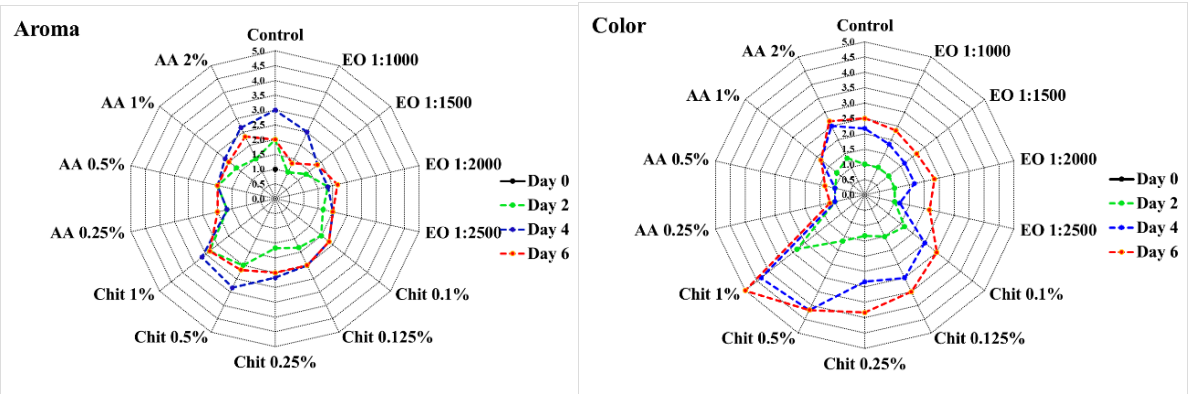


Figure 5.1. Screening of marjoram essential oil (EO), chitosan (Chit) and ascorbic acid (AA) on fresh-cut lettuce total phenols (top panel), aroma (bottom left, scale range from 1-lettuce like to 5-not lettuce, not acceptable) and color/visual quality (bottom right, scale: 1-green; 3-light discoloration; 5-severe browning) after 6 days of storage at 7 °C and 90% RH.

5.2.2.2. Main experiment

5.2.2.2.1. Effects on weight loss, respiration rate and ethylene emission

The application of AA increased (up to 5.53%) weight loss of fresh-cut lettuce on the second day of storage compared to control, Chitosan, Chitosan+AA, EO+AA and chlorine (1.90, 1.49, 2.16, 0.10 and 2.64%, respectively) (**Figure 5.2**). Similarly trend was observed after 4 days of storage but not at the sixth day, whereas all treatments revealed similar weight losses.

Respiration increased on the second day with the application of EO+AA, EO+Chitosan and AA (29.91, 15.71 and 22.25 mL CO_2/kg/h, respectively) in comparison to the control treatment (9.67 mL CO_2/kg/h) (**Figure 5.2**). Chitosan application resulted to lower respiration rate (9.03 mL CO_2/kg/h) on the fourth day compared to the control treatment and the combinations of EO+AA and Chitosan+AA (16.73, 19.55 and 16.61 mL CO_2/kg/h, respectively). Following that an increased respiration rate was also observed on the sixth day with the combination of Chitosan+AA that revealed the highest value (38.36 mL CO_2/kg/h) compared to all treatments.

Ethylene emission as affected by the application of the natural products is illustrated in **Figure 5.2**. The application of EO resulted to lower ethylene production (9.05 μL ethylene/kg/h) compared to the control and EO+Chitosan (18.46 and 18.03 μL ethylene/kg/h, respectively) treatments on the second day of storage. On the fourth day of storage increased ethylene emission was reported with the combination of EO+AA (18.68 μL ethylene/kg/h) as to the control treatment, EO and Chitosan+AA (11.30, 10.10 and 13.50 μL ethylene/kg/h, respectively). On the sixth day, the application of EO+Chitosan resulted to higher ethylene production (10.90 μL ethylene/kg/h) compared to all treatments.

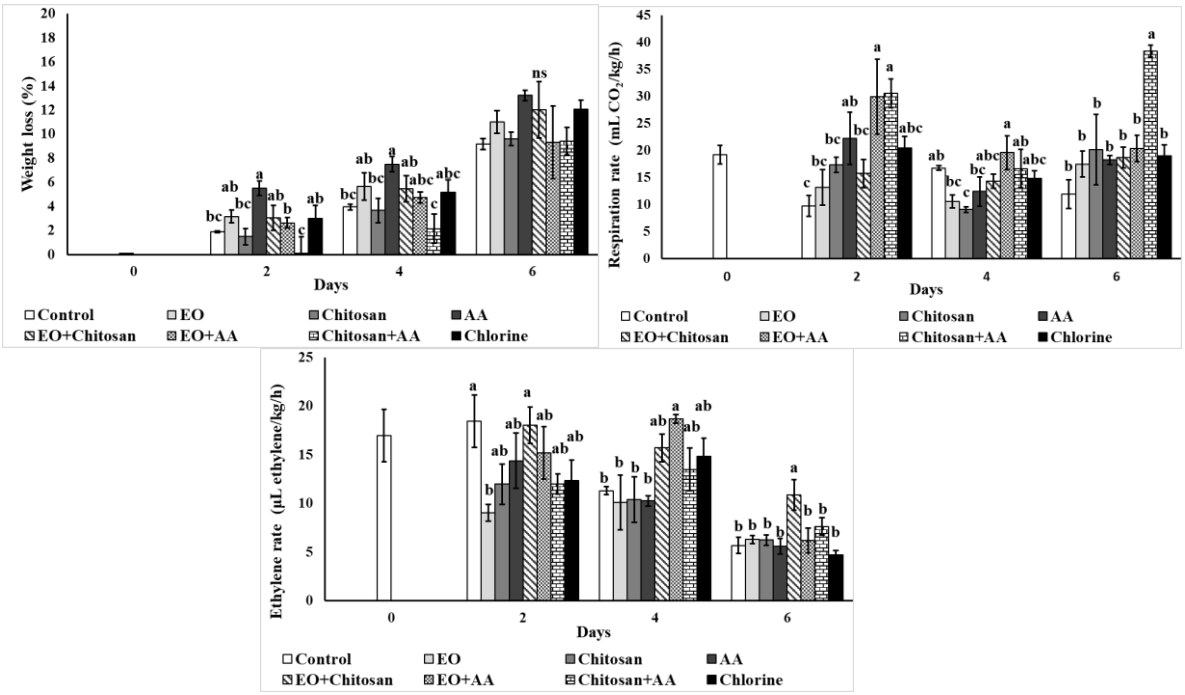


Figure 5.2. Effect of marjoram essential oil (EO), chitosan and ascorbic acid (AA) on fresh-cut lettuce weight loss, respiration and ethylene rate after treatment and 6 days of storage at 7 °C and 90% RH. On the columns, significant differences ($P < 0.05$) among treatments are indicated by different Latin letters; ns indicates no significant differences for different days.

5.2.2.2.2. Effects on color

The impact of the application of marjoram EO, Chitosan and AA and their combination on fresh-cut lettuce color is illustrated in **Figure 5.3**. The application of EO increased *L** value (58.98) compared to Chitosan, Chitosan+AA (50.97 and 51.18, respectively) after six days of storage. Lower *a** values (positive values are shown in **Figure 5.3**) were reported on the sixth day of storage with the application of Chitosan and Chitosan+AA (19.67 and 19.42, respectively). Similarly, decreased *b** values (positive values are shown in **Figure 5.3**) were observed with the application of Chitosan, EO+Chitosan and Chitosan+AA (33.10, 35.41 and 34.85, respectively). Chroma value decreased with the application of Chitosan, EO+Chitosan and Chitosan+AA on the sixth day of storage, while increased color index value (positive values are shown in **Figure 5.3**) was reported with the application of Chitosan (11.94) compared to EO, AA and chlorine (9.39, 9.34 and 9.59, respectively) on the same day. The application of Chitosan decreased Hue (59.16) compared to the application of AA and chlorine (61.92 and 61.80, respectively) on the sixth day of storage (positive values are shown in **Figure 5.3**). Browning index decreased on the sixth day of storage with the application of Chitosan (37.33) compared to the control treatment (35.32), whilst whiteness index increased with the application of AA (36.16) compared to EO+Chitosan and chlorine (32.27 and 32.44, respectively) on the second day. On the fourth day of storage AA increased whiteness index (37.08) in comparison to EO application (31.42).

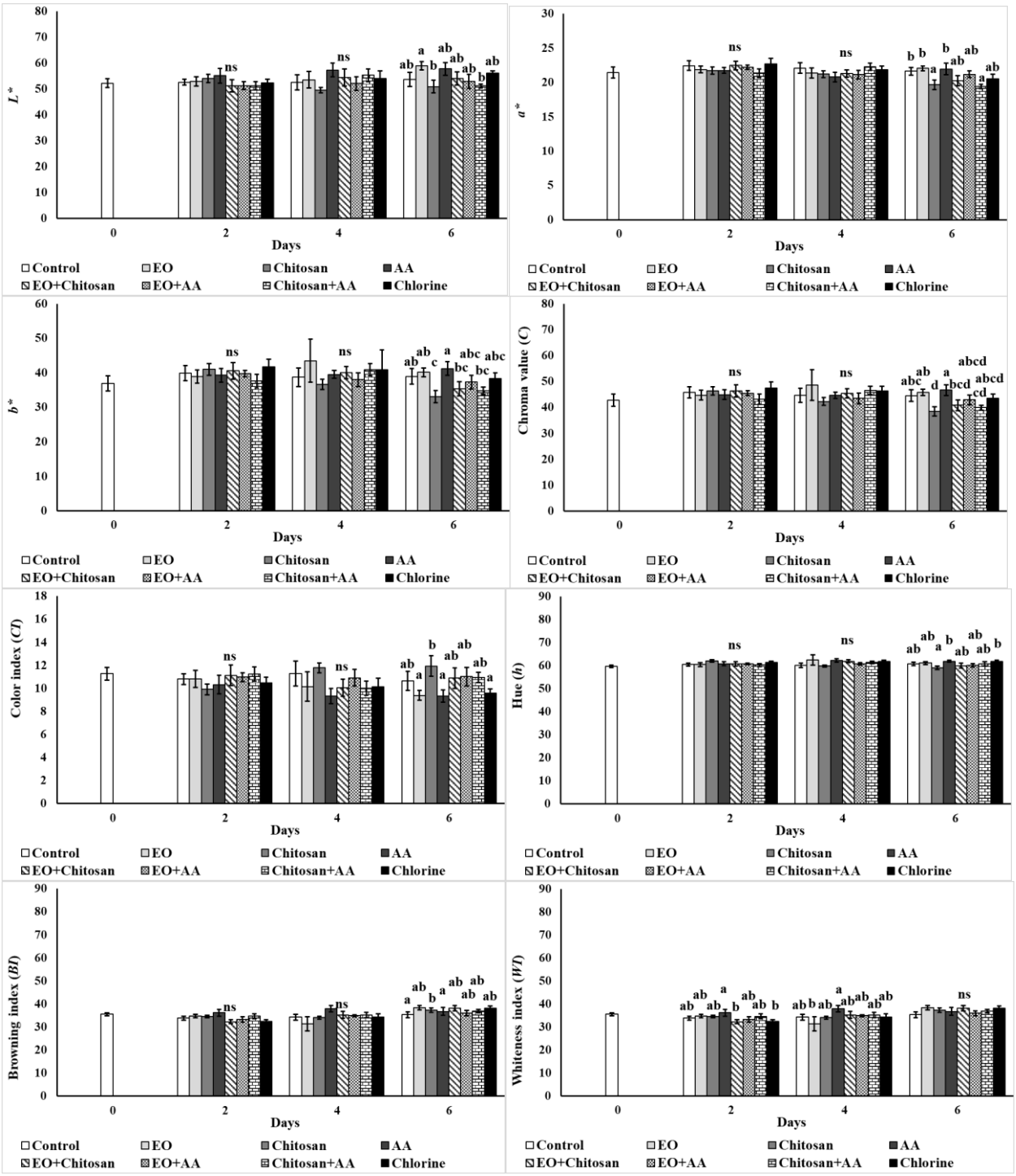


Figure 5.3. Effect of marjoram essential oil (EO), chitosan and ascorbic acid (AA) on fresh-cut lettuce on L^* value, a^* (greenness) (positive values), b^* (yellowness), chroma, color index (positive values), hue (positive values), browning index and whiteness index after treatment and 6 days of storage at 7 °C and 90% RH. On the columns, significant differences ($P < 0.05$) among treatments are indicated by different Latin letters; ns indicates no significant differences for different days.

5.2.2.2.3. Chlorophyll content (Chl a, Chl b, total Chl)

The effects of postharvest treatments applied on fresh-cut lettuce chlorophyll content are illustrated in **Figure 5.4**. All applied treatments except EO decreased chlorophyll content (Chl a, Chl b, Tot Chl) on the sixth day of storage. Chitosan application decreased Chl b levels (0.06 mg Chl b/g Fw) compared

to Chitosan+AA (0.13 mg Chl b/g Fw) on the second day, whilst the same trend on the same day was observed with Chl a content (0.44 and 0.64 mg Chl a/g Fw, respectively). In addition, Tot Chl levels decreased with the application of Chitosan+AA during the second day of storage.

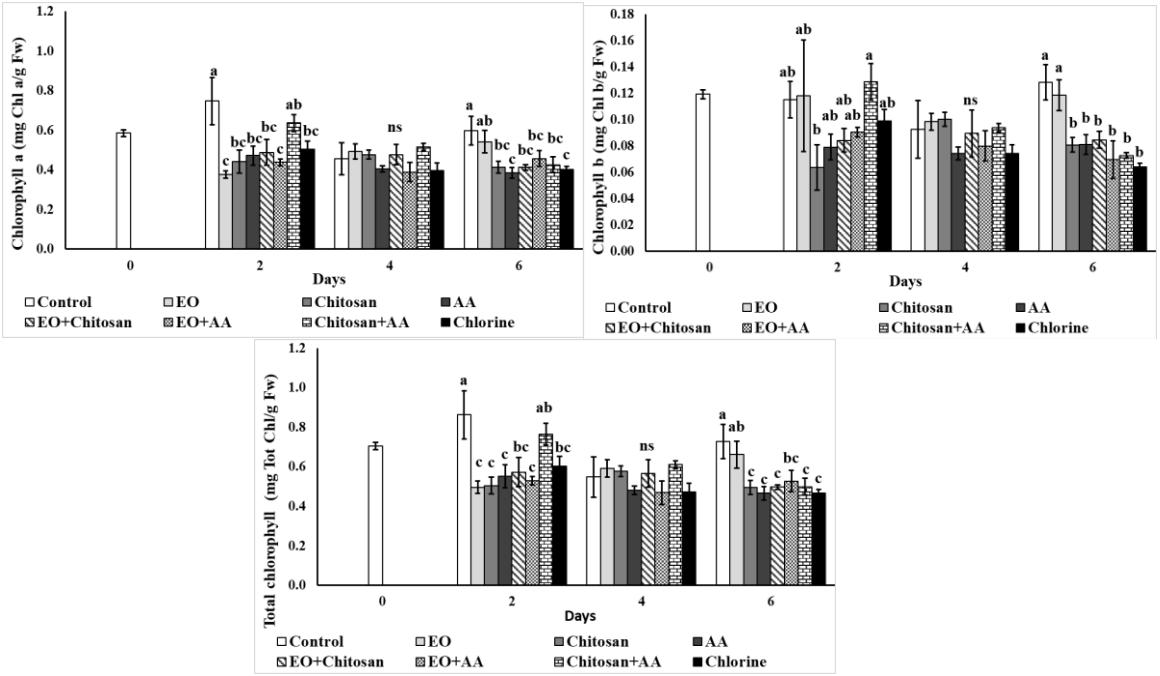


Figure 5.4. Effect of marjoram essential oil (EO), chitosan and ascorbic acid (AA) on fresh-cut lettuce chlorophyll a, chlorophyll b and total chlorophyll content after treatment and 6 days of storage at 7 °C and 90% RH. On the columns, significant differences ($P < 0.05$) among treatments are indicated by different Latin letters; ns indicates no significant differences for different days.

5.2.2.2.4. Effects on total soluble solids, total acidity and sweetness

Total soluble solids of fresh-cut lettuce decreased with the application of Chitosan on the second day of storage, while EO application increased TSS (2.07 ^{o}Brix) compared to Chitosan, Chitosan+AA and chlorine (1.60, 1.57 and 1.40 ^{o}Brix, respectively) on the fourth day (**Figure 5.5**). At the end of storage (sixth day), an increase of total acidity was observed with the application of EO and chlorine (1.01 and 1.00 g malic acid/kg Fw, respectively) compared to the control treatment (0.83 g malic acid/kg Fw) at day 6. As shown in **Figure 5.5** sweetness was increased with the combination of Chitosan+AA and chlorine (3.58 and 3.55, respectively) as to Chitosan (2.81) on the second day of storage. The application of chlorine decreased sweetness of fresh-cut lettuce (2.21) on the fourth day compared to the control treatment, EO and EO+AA (3.52, 3.64 and 3.49, respectively). Similarly, on the sixth day of storage chlorine lead to decreased sweetness (1.41) opposed to the control treatment and Chitosan (2.19 and 2.24, respectively).

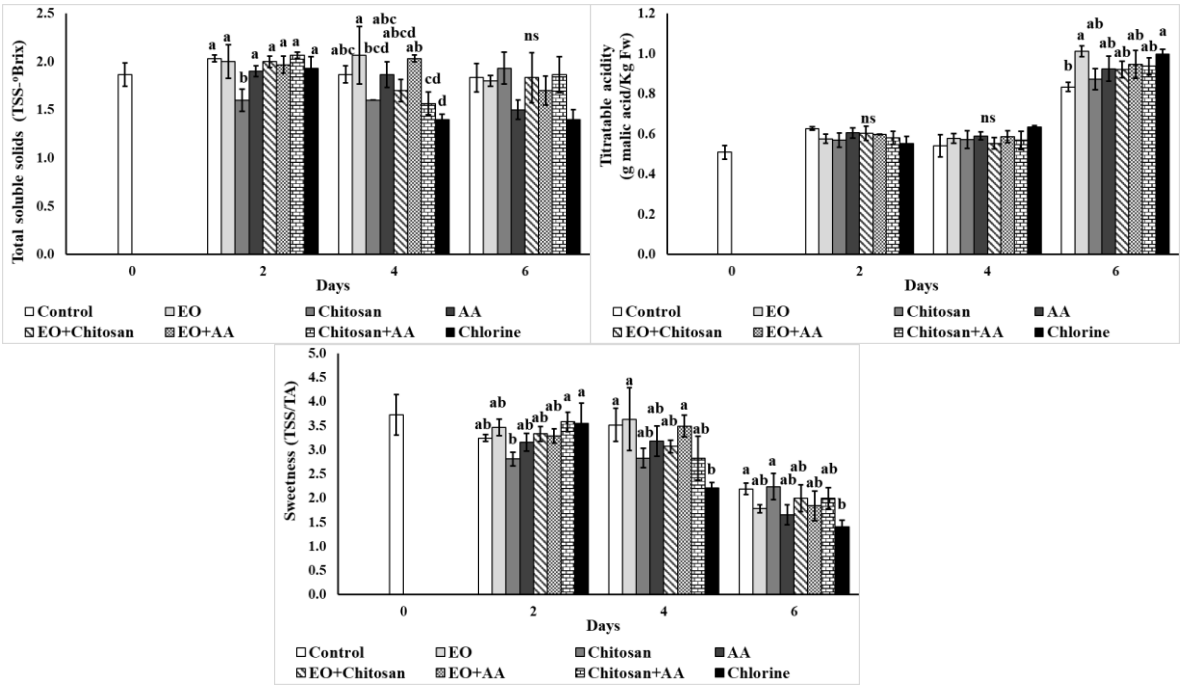


Figure 5.5. Effect of marjoram essential oil (EO), chitosan and ascorbic acid (AA) on fresh-cut lettuce total soluble solids, total acidity and sweetness after treatment and 6 days storage at 7 °C and 90% RH. On the columns, significant differences ($P < 0.05$) among treatments are indicated by different Latin letters; ns indicates no significant differences for different days.

5.2.2.2.5. Effects on total phenolics, antioxidants, ascorbic acid and total carotenoid content

Total phenolic content increased with the application of AA and Chitosan+AA (1.14 and 1.11 mg GAE/g Fw, respectively) on the second day as presented in **Figure 5.6**. On the fourth day of storage the combinations of EO+AA and Chitosan+AA increased phenolics (1.20 and 1.34 mg GAE/g Fw, respectively) and similarly EO+AA, Chitosan+AA and chlorine resulted to higher phenolic content (1.19, 0.96 and 0.92 mg GAE/g Fw, respectively) at the end of storage.

Ascorbic acid content increased with the application of AA and EO+AA (8.63 and 10.62 mg AA/100 g Fw, respectively) on the second day, while Chitosan application lowered ascorbic acid (5.02 mg AA/100 g Fw) of fresh-cut lettuce on the same day (**Figure 5.6**). Moreover, increased ascorbic acid content was observed with the application of Chitosan, chlorine, EO+AA and AA higher values (10.10, 10.12, 9.87 and 9.42 mg AA/100 g Fw, respectively) on the fourth day of storage. In a similar way the application of EO+AA and chlorine increased ascorbic acid (16.05 and 16.85 mg AA/100 g Fw, respectively) during the last day of storage.

Total carotenoid content decreased with the application of EO, Chitosan and EO+AA (0.10, 0.13 and 0.13 mg Car/g Fw, respectively) on the second day of storage, while the combination of Chitosan+AA, increased carotenoids (0.16 mg Car/g Fw) compared to EO+AA (0.11 mg Car/g Fw) during the fourth day of storage (**Figure 5.6**).

According to DPPH assay, the antioxidant activity of fresh-cut lettuce decreased with the application of Chitosan, EO+Chitosan and EO+AA (0.79, 0.86 and 0.89 mg trolox/g Fw, respectively) compared to the control treatment and AA (1.46 and 1.45 mg trolox/g Fw, respectively) on the second day (**Figure 5.6**). The combinations of EO+AA and Chitosan+AA resulted to higher antioxidant levels (1.42 and 1.40 mg trolox/g Fw, respectively) on the fourth day and in a similar way, the application of EO+AA, Chitosan+AA and chlorine increased antioxidants (1.28, 1.12 and 1.33 mg trolox/g Fw, respectively) during the sixth day. The ABTS assay revealed that AA application increased antioxidants (1.36 mg trolox/g Fw) on the second day compared to the application of Chitosan and EO+AA (0.88 and 0.90 mg trolox/g Fw, respectively) (**Figure 5.6**). The combinations of EO+AA and Chitosan+AA resulted to higher ABTS antioxidant levels (1.67 and 1.82 mg trolox/g Fw, respectively) compared to control, EO, EO+Chitosan, chlorine (1.18, 1.01 and 1.13 mg trolox/g Fw, respectively) during the fourth day. However, Chitosan and EO+Chitosan lowered lettuce ABTS antioxidants (1.01 and 0.96 mg trolox/g Fw, respectively) during the last day of storage compared to EO, EO+AA, Chitosan+AA and chlorine (1.33, 1.71 and 1.44 mg trolox/g Fw, respectively).

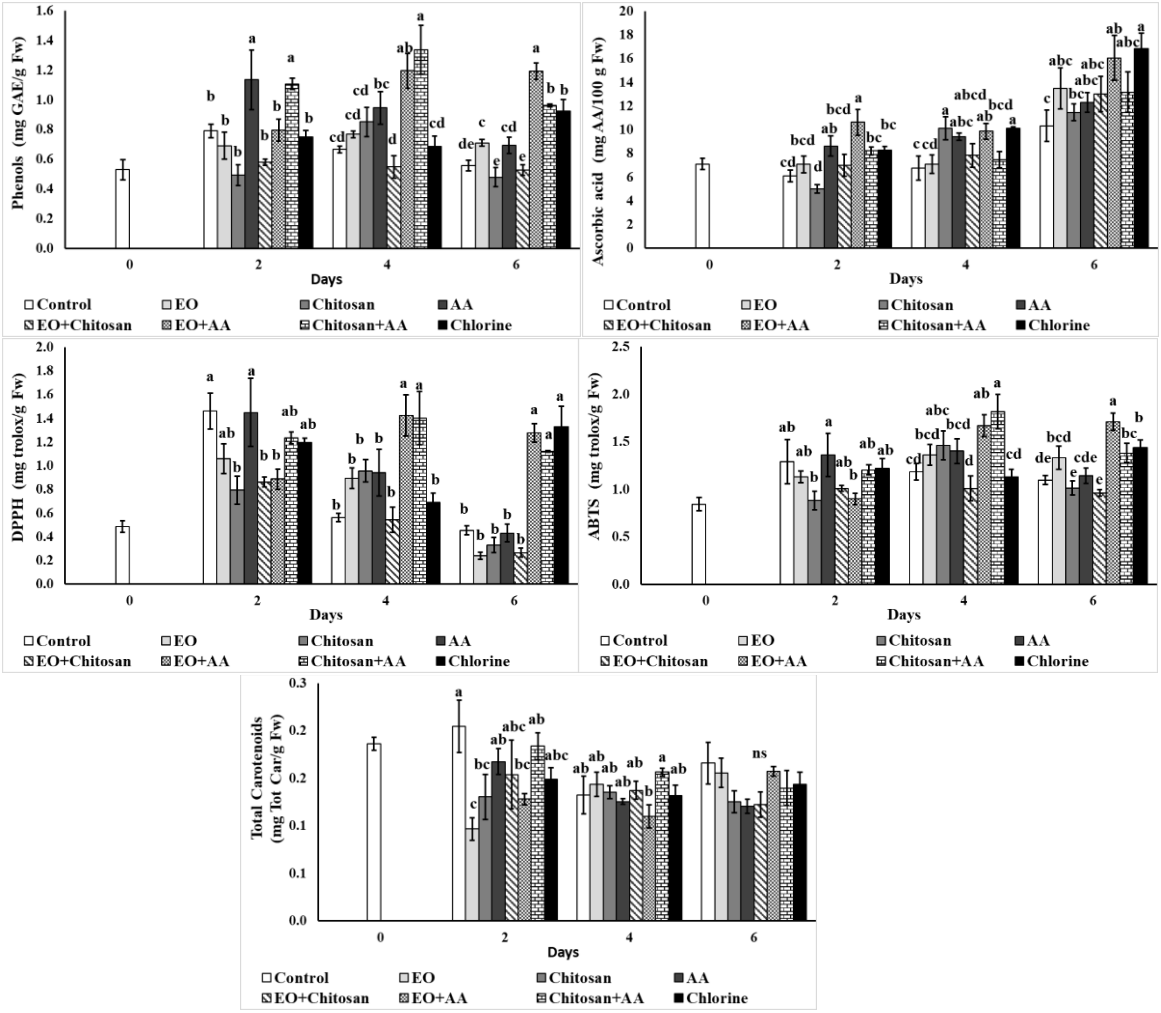


Figure 5.6. Effect of marjoram essential oil (EO), chitosan and ascorbic acid (AA) on fresh-cut lettuce total phenolics, ascorbic acid, antioxidant activity (DPPH, ABTS), and carotenoids content after treatment and 6 days

of storage at 7 °C and 90% RH. On the columns, significant differences ($P < 0.05$) among treatments are indicated by different Latin letters; ns indicates no significant differences for different days.

5.2.2.2.6. Effects on hydrogen peroxide and lipid peroxidation

The effect of marjoram EO, Chitosan and AA application on hydrogen peroxide and lipid peroxidation is illustrated in **Figure 5.7**. EO application, followed by AA and EO+AA increased H_2O_2 (0.11, 0.07 and 0.07 μmol H_2O_2/g Fw, respectively) on the second day of storage. Similarly, on the fourth day an increase in H_2O_2 was reported with the application of EO+AA, EO and AA (0.1, 0.08 and 0.08 μmol H_2O_2/g Fw, respectively). During the last day of storage, the application of AA, Chitosan+AA and chlorine lead to lower H_2O_2 levels (0.05, 0.04 and 0.05 μmol H_2O_2/g Fw, respectively), whilst EO application showed a higher value (0.11 μmol H_2O_2/g Fw).

Lipid peroxidation indicated by MDA, increased on the second day with the application of Chitosan+AA (6.56 nmol MDA/g Fw) compared to the control treatment and chlorine (5.22 and 4.62 nmol MDA/g Fw, respectively) (**Figure 5.7**). Increased MDA production was also reported with EO, EO+AA, and Chitosan+AA application (5.81, 5.66 and 5.81 nmol MDA/g Fw, respectively) on the fourth day. During the last day of storage EO and EO+AA lead to increased MDA production (9.29 and 8.51 nmol MDA/g Fw, respectively), while EO+Chitosan resulted to lower MDA levels (5.51 nmol MDA/g Fw).

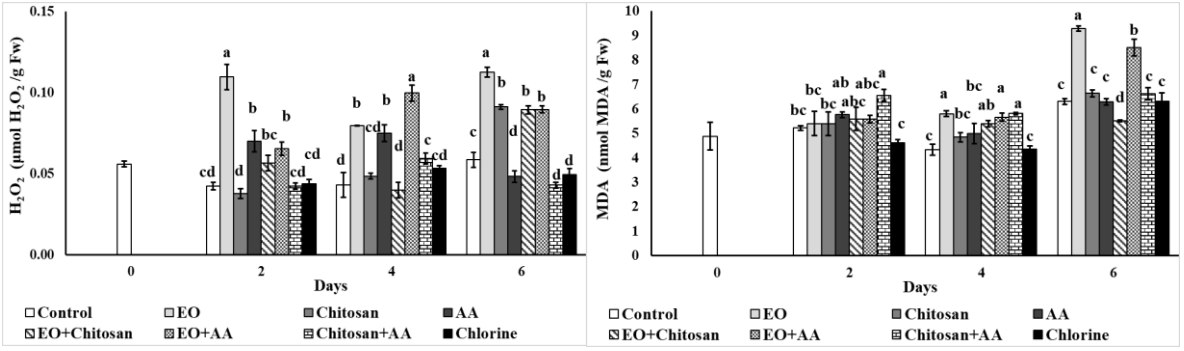


Figure 5.7. Effect of marjoram essential oil (EO), chitosan and ascorbic acid (AA) on fresh-cut lettuce on H_2O_2 production and lipid peroxidation after treatment and 6 days of storage at 7 °C and 90% RH. On the columns, significant differences ($P < 0.05$) among treatments are indicated by different Latin letters; ns indicates no significant differences for different days.

5.2.2.2.7. Effects on microbial load

Figure 5.8 shows the impacts of the preservative means applied on fresh-cut lettuce microbial quality. Chitosan application resulted to decreased total viable counts (TVC) (4.58 log cfu/g) during the last day of storage. The application of EO, EO+Chitosan, EO+AA, Chitosan+AA and Chlorine resulted to decreased yeast and molds (2.62, 2.62, 2.67 and 2.45 log cfu/g, respectively) on the fourth day of storage compared with the control treatment. Moreover, similar results were reported on the sixth day

where Chitosan and chlorine decreased yeast and molds (2.23 and 2.52 log cfu/g, respectively) compared to control, EO, AA, EO+Chitosan, EO+AA (3.49, 3.49, 3.50 and 3.42 log cfu/g, respectively).

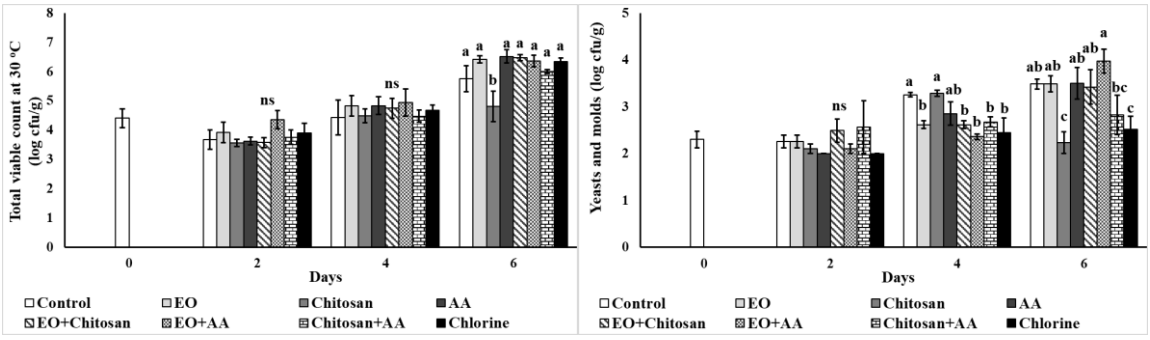


Figure 5.8. Effect of marjoram essential oil (EO), chitosan and ascorbic acid (AA) on fresh-cut lettuce on total viable count (TVC) and yeast and filamentous fungi after 6 days of storage at 7 °C and 90% RH. On the columns, significant differences ($P < 0.05$) among treatments are indicated by different Latin letters; ns indicates no significant differences for different days.

5.2.2.2.8. Effects on sensory characteristics

Figure 5.9 presents the impacts of the applied treatments on the aroma and visual quality of fresh-cut lettuce. The combination of Chitosan+AA scored higher values than all the applied treatments during the fourth and sixth days of storage. It is noteworthy that this combination scored 4 out of a 5 scale on the sixth day indicating a non-acceptable product. Furthermore, the application of EO scored lower values during all days suggesting an acceptable product with a pleasant/acceptable aroma (1-2 out of a 5 scale).

The visual quality of fresh-cut lettuce decreased with the application of Chitosan+AA, Chitosan as these treatments were scored higher with high values (3-4 out of a 5 scale) during all days of storage (Figures **5.9** and **5.S4**). Increased values were also observed with the application of AA during the fourth and sixth day (3 out of a 5 scale). On the other hand, EO application preserved the visual quality of fresh-cut lettuce during all days of storage (scores 1-2 out of a 5 scale).

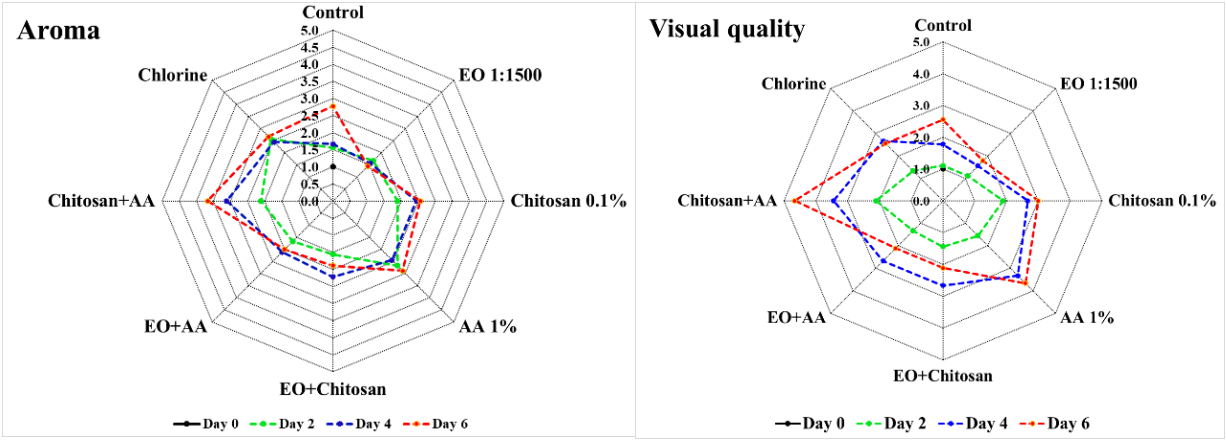


Figure 5.9. Effects of marjoram essential oil, chitosan, ascorbic acid alone and their combinations on fresh-cut lettuce aroma (left, scale range from 1-lettuce like to 5-not lettuce, not acceptable) and color/visual quality (right, scale: 1-green; 3-light discoloration; 5-severe browning) after 6 days of storage at 7 °C and 90% RH.

5.2.3. Discussion

Fresh vegetables consist one of the main components in a healthy and balanced diet, since they are high on dietary fibers and phytonutrients with low fat content and calories. However, when exposed to adverse handling and storage conditions; vegetables (especially minimally processed) tend to have short shelf life due to deterioration of their quality attributes. The use of natural products on fresh produce and minimally processed fruit and vegetables has been previously studied by many researchers and the results are promising (Ponce et al., 2011; Severino et al., 2014; Chrysargyris et al., 2016b; Landi et al., 2017; Xylia et al., 2017, 2018, 2019b; Tzortzakis et al., 2019), but most importantly are well appreciated by the consumers. In this study the effects of the application of marjoram EO, chitosan, AA and their combinations were assessed regarding the quality attributes of fresh-cut lettuce, indicating an alternative preservation postharvest management for quality and safe stored products.

Chitosan application affected negatively the aroma and visual quality of fresh-cut lettuce. A non-acceptable aroma and less attractive color was observed with the application of chitosan and chitosan+AA. On the other hand, EO application improved visual quality and acceptable (pleasant) aroma. Similarly, the use of AA maintained the visual quality of fresh-cut lettuce. The application of chitosan-based melatonin bilayer coating on ready-to eat cucumber and broccoli was assessed by Zhao et al. (2020) and their results supported the benefits observed by combining postharvest preservation means, and showed that the applied combinations of melatonin and chitosan maintained the fresh appearance of cucumber. On the same study, fresh-cut broccoli's bright green color was preserved with the combination of melatonin and chitosan, while the incorporation of 100 mg/L melatonin resulted to increased microbial spoilage and chlorophyll losses (yellowing) (Zhao et al., 2020). In another study, Viacava et al. (2018) mentioned thyme EO applied on fresh-cut lettuce resulted to lower quality product (visual quality, browning, texture, color and odor) compared to the microencapsulated EO in which no off-odors or severe browning were observed. The application of whey permeate (0.5%, 1.5% and 3%) on fresh cut lettuce and carrots did not affect negatively sensory attributes of lettuce, while the highest concentration resulted to higher surface whitening on carrots (Martin-Diana et al., 2006). Notably, Deza-Durand and Petersen (2011) reported that even cutting direction (transverse cutting) during processing of fresh-cut lettuce can influence aroma development and respiration rate due to severe tissue damage. These findings suggest that losses of fresh produce quality attributes may arise from processing, applied products (concentration, time of application, source) and the product itself.

Postharvest weight loss due to moisture loss can cause deterioration and shrinking of leaf tissue (Agüero et al., 2011). In this study up to 7.51% weight loss was observed with the application of AA, while chitosan and chitosan+AA resulted to lower weight losses. When chitosan is applied to a plant surface (via dipping or spraying) can form an edible coating that can act as a barrier for gas (O_2 and

CO_2) exchange and slow respiration rate (Sánchez-González et al., 2011; Romanazzi et al., 2018). It has also been suggested that edible coatings when applied to fresh produce might prevent moisture losses (Sánchez-González et al., 2011), and the combination of edible coating material with hydrophobic and hydrophilic properties is preferred on postharvest applications.

Among fresh produce, lettuce consists a commodity with moderate respiration rate and tissue wounding (due to mishandling or processing) might accelerate its respiration rate (Deza-Durnand and Petersen, 2011). Increased respiration was observed when the combinations of EO+AA and chitosan+AA were applied, whilst chitosan applied alone resulted to lower respiration rates. These findings are in agreement with Romanazzi et al. (2018) who suggested that application of chitosan on fruits and vegetables (film formation) can reduce respiration rate acting as a gas exchange barrier, but not as water barrier due its hydrophilic properties. Mechanical damage of leafy vegetables due to cutting, storage, transport; might induce ethylene release which in combination with increase weight loss can result to fibrosis and lignification on cut surfaces (Martínez-Romero et al., 2007; Chen et al., 2017). In this study the application of marjoram EO decreased ethylene emission, while increased ethylene levels was reported with the application of EO+chitosan. It has been suggested that a physiological disorder like russet spotting on lettuce can be induced by exposure to ethylene (Hyodo et al., 1978; Koukounaras et al., 2019). This might explain why the application of EO preserved the visual quality of fresh-cut lettuce and at the same time decreased its ethylene emission in our study. Ethylene has been linked with the induced production of plant defense compounds, such as antioxidants, phenols; as well as the activation of enzymes including chitinase, peroxidase, phenylalanine ammonia-lyase and polyphenol oxidase (Masia, 2003). It has also been mentioned that the production of ethylene in combination with moisture loss will result to fibrosis and lignification of cut tissue which will eventually lead to aging, senescence and quality losses of processed lettuce (Chen et al., 2017). Previous indications have shown that lettuce produces very little ethylene and at the same time it is sensitive to the presence of ethylene (Allende et al., 2004). Interestingly, Hamanaka and Izumi (2008) examining the effects of mustard and hop extracts on shredded cabbage and sliced cucumber reported lower respiration rates and ethylene production in both products.

Among fresh produce quality attribute, color and appearance are the most important ones, since they are the first attributes perceived by consumers and can affect their buying decision (Péneau et al., 2007). The degree of browning in vegetables can be assessed using the L^*, a^* and b^* chromaticity coordinates. As previously mentioned by Chen et al. (2017) browning might occur with lower L^* and higher a^* and b^* values. Decreased L^* values were observed with the application of chitosan and chitosan+AA, while high a^*, b^* and hue values were also noted suggesting a darker (less green) leaf and the formation of brown pigments. Similarly, color index and chroma values decreased with the application of chitosan and its combinations (EO+chitosan, chitosan+AA) and at the same time increased browning index was observed with chitosan. All these results indicated the development and the degradation of lettuce visual

quality. The application of AA increased whiteness index of fresh-cut lettuce. Chen et al. (2017) reported that the application of clove essential oil (0.05%) and eugenol (0.05%) on fresh-cut lettuce maintained its bright green color during 12 days of storage at 4 ^{o}C with high L^* and b^* values, as well as lower a^* values. On the same study lower browning index values were also observed the application of clove EO and eugenol compared to 5% ethanol treatment (Chen et al., 2017). In another study by Martin-Diana et al. (2006) reported that 3% whey permeate applied on fresh-cut lettuce resulted to high a^* values compared to lower concentrations; however no significant differences were observed for b^* value, hue and chroma value. Viacava et al. (2018) reported that thyme EO treatment resulted to lower L^* values, while the microencapsulation of thyme EO did not affect negatively fresh-cut lettuce green color throughout storage (up to 12 days) at 5 ^{o}C suggesting that the combination of film forming compounds can enhance/improve EOs attributes.

Total soluble solids decreased on the second and fourth day of storage with chitosan application. As it has been previously mentioned by Moreira et al. (2006) the decrease in TSS might be associated with respiration rate and the consumption of sugars during this process. In the present study little to no relation between respiration rate and TSS of fresh-cut lettuce was observed, since respiration rate slightly increased on the second day while it significantly decreased on the fourth day of storage with the application of chitosan. Lettuce's total acidity increased with EO and chlorine application after four days stored at 7 ^{o}C, whereas sweetness decreased with the application of chlorine on the fourth and sixth day of storage. According to Zhao et al. (2020) treatment with melatonin incorporated in a chitosan based bilayer coating (2.5% w/v) maintained the sugar-acid ratio (ripening index-sweetness) of fresh cut cucumber. In a previous study, the application of marjoram EO+AA and marjoram hydrosol+AA increased shredded carrots TSS compared to the hydrosol when applied alone, while increased TA was reported with the application of AA (Xylia et al., 2019b). It seems that natural products applied during minimal processing of fresh produce can affect differently quality parameters depending on their properties, the time of application or the product they are applied to, and tailor made investigations needed for each commodity.

One of the main issues on fresh-cut vegetables processing is the browning of the cut surface which is an undesirable process leading to reduced marketability products (darkening and softening), loss of nutritional value and the development of off-flavors (Altunkaya, 2011; Kim et al., 2014). Browning results from enzymatic and non-enzymatic reactions that are favored by the disruption of plant cell wall during processing and the exposure of phenolic compounds to oxygen and enzymes (de Rigal et al., 2000; Altunkaya and Gökmen, 2009). The effects of natural products as anti-browning agents applied on fresh produce have been previously assessed (Martin-Diana et al., 2008; Altunkaya and Gökmen, 2009; Altunkaya, 2011; Kim et al., 2014; Chen et al., 2017). The application of oxalic and ascorbic acid on lettuce aliquots maintained phenolic content on high levels compared to citric acid and cysteine application (Altunkaya and Gökmen, 2009). In this study, the application of EO+AA and chitosan+AA

combinations increased lettuce's phenolic content after four and six days of storage. Interestingly in a study by Viacava et al. (2018), it has been shown that the application of thyme EO on minimally processed lettuce increased total phenolic content, total flavonoids and antioxidant activity. Ascorbic acid and carotenoid content increased with the application of EO+AA (up to 10.62 mg AA/100 g Fw). According to Altunkaya and Gökmen (2009), color changes have been linked with the degradation of ascorbic acid and this might explain the positive effects of EO and EO+AA on the color of fresh-cut lettuce throughout storage time. Interestingly, fresh-cut lettuce's chlorophylls decreased during storage, though the application of chitosan+AA and EO maintained their levels up to the last day of storage. Another study reported that high green-tea extract concentration (up to 1% w/v) prevented ascorbic acid and carotenoid loss of fresh-cut lettuce (Martin-Diana et al., 2008).

Antioxidant activity of fresh-cut lettuce increased with the application of EO+AA. It has been shown that AA can react and scavenge radicals and regenerate polyphenols after their oxidation, due to its antioxidant activity (de Beer et al., 2005; Altunkaya and Gökmen, 2009). Furthermore, EOs possess antioxidant activity among others, thus alone or in combination EO and AA might have resulted to higher phenolic content and antioxidant levels when applied to fresh-cut lettuce. In a previous study, it has been shown that the application of spearmint and lavender EO and their mixture on endive leaves at concentrations as low as 0.001% resulted to lower antioxidant capacity compared to higher concentrations (up to 0.1%) where decreased antioxidants were reported (Xylia et al., 2017). As it has been previously mentioned by Kang and Saltveit (2002), wounding of plant tissue can result to increase antioxidant activity in romaine and iceberg lettuce. The application of eugenol, carvacrol and trans-anethole on cellulose sachets resulted to higher phenolic content as well as antioxidant capacity of fresh-cut iceberg lettuce when exposed to them (Wieczyńska and Cavoski, 2018). These findings suggest that the applied processing methods might induce defense mechanisms of plant tissue (i.e. increase in antioxidant capacity) that will lead to rapid deterioration and quality losses. This seems to be associated with the applied method, the time of application, the processing product, the storage conditions among other parameters.

The production of active oxygen species (i.e. H_2O_2, superoxide-$O^{2\bullet-}$, hydroxyl-$OH^{\bullet}$, singlet oxygen-$^1O_2$) has been linked with lipid peroxidation, production of brown pigments and core browning, pigment bleaching (chlorophyll, carotenoids) and other processes that lower postharvest quality of fruit and vegetables (Hodges, 2003; Hodges et al., 2004; Hodges and Toivonen, 2008; Hunter et al., 2017). In this study, H_2O_2 increased with the application of EO, AA and their combination (EO+AA) (0.11, 0.07 and 0.07 μmol H_2O_2/g Fw) on the second day, while decreased H_2O_2 levels were observed with the application of chitosan+AA (0.05 μmol H_2O_2/g Fw) at the end of storage. Increased lipid peroxidation was also observed with chitosan+AA (6.56 nmol MDA/g Fw) on the second day, whereas EO application showed lower MDA levels after six day of storage at 7 ^oC. An increase in H_2O_2 and MDA levels was reported during storage of fresh-cut rocket and melon at 4 and 20 ^oC with MDA levels lower

than 0.3 nmol/g Fw, which suggested a moderate lipid peroxidation level according to Cavaiuolo et al. (2015). It is noteworthy that Ferrante et al. (2009) reported higher lipid peroxidation values on fresh-cut lamb's lettuce leaves compared to intact ones when stored at 4 ^{o}C up to 8 days (values up to 41-51 nmol MDA/g Fw), confirming that processing (i.e. cutting) induces plant stress and increases lipid peroxidation that utterly result to low quality products presenting postharvest disorders such as tissue browning and leaf yellowing. Additionally, in another study the application of AA in combination with marjoram EO and hydrosol increased H_2O_2 levels and lipid peroxidation of shredded carrots suggesting that applied treatments might also induce oxidative stress and affect the quality of the end product (Xylia et al., 2019b).

Many studies have assessed the antimicrobial effects of EOs on the microbial load of fresh produce (Bagamboula et al., 2004; Viacava et al., 2018; Tzortzakis et al., 2019; Xylia et al., 2019b), indicating the importance of safe and high quality produce for the consumer. For instance, the application of thyme EO on minimally processed lettuce exhibited high bacteriostatic activity against mesophilic and psychtotrophic bacteria, *Enterobacteriaceae* and yeast and molds counts (Viacava et al., 2018). In another study thyme and tea tree EOs used alone or in combination resulted to great reduction of *E. coli* O157:H7 population (up to 2 log) inoculated on fresh lettuce leaves stored at 10 ^{o}C (Mouatcho et al., 2017). Bagamboula et al. (2004) reported that thyme and basil EOs at concentrations applied (0.5 and 1%) were able to reduce both inoculated (*S. sonnei* and *S. flexneri*) and indigenous flora populations on fresh-cut lettuce (up to 2 log reduction). On the other hand, one of the main activities' chitosan exhibits when applied to plants is the chitosan's antimicrobial activity and has been previously assessed by many researchers for a wide range of microorganisms (Romanazzi et al., 2018). Goñi et al. (2014) reported that the preharvest application of chitosan (0.1% w/v) during the last development stages controlled lettuce's native microflora as well as reduced *E. coli* survival inoculated on the edible plant parts. Another study reported lower microbial load of total bacteria and the presence of Salmonella in ready-to eat lettuce when treated with allyl- and benzyl-isothiocyanates and chitosan (Pablos et al., 2017). In the present study, chitosan decreased lettuce's TVC and yeasts and molds counts after six days of storage at 7 ^{o}C. In addition, EO, along with EO+chitosan, EO+AA and chitosan+AA resulted to lower yeast and molds counts on the fourth day of storage. These results are in accordance with studies that suggest that when chitosan is applied alone or in combination with antimicrobial compounds can inhibit microbial growth (Vu et al., 2011; Severino et al., 2014). It is noteworthy that the combination of citric and ascorbic acid (0.25 and 0.50 g/100g, respectively) was not able to remove *E. coli* and *L. monocytogenes* cells from lettuce leaves and prevent biofilm formation (Ölmez and Temur, 2010). However, the application of AA (1%) alone or in combination with marjoram EO and hydrosol resulted to reduction of TVC and yeast and molds counts of shredded carrots stored at 4 ^{o}C for up to nine days (Xylia et al., 2019b). Akbas and Ölmez (2007b) also reported that dipping of inoculated iceberg lettuce

leaves on ascorbic acid solutions (0.5 and 1%) were able to reduce *E. coli* population by almost 2 log and *L. monocytogenes* by 1 log.

5.3. Experiment 2. Effects of natural products on shredded carrots preservation

5.3.1. Material and methods

5.3.1.1. Plant material and EO extraction

Fresh carrots (*Daucus carota* L.) were obtained from a local market in Limassol, Cyprus and were selected for uniformity in appearance and the absence of physical defects or injury and then stored at 4 ^{o}C and 90% RH until use (within 24 h). Prior to processing, carrots were rinsed with running tap water for any soil residues removal and then paper dried.

Marjoram plants (*Origanum majorana* L.) were obtained from the experimental farm of Cyprus University of Technology, where they were cultivated in soil. Marjoram EO extraction was performed as previously mentioned at section **5.2.1.1**. Following EO hydrodistillation, the water solution (hydrosol) was collected and filtered to remove any plant residues. Freshly prepared hydrosol (Hyd) was used for all the sub studies. The composition of marjoram EO was determined as described previously (Chrysargyris et al., 2016a), and the main components were terpinen4ol, γ-terpinene, trans sabinenehydrate, and α-terpinene.

5.3.1.2. Preliminary screening

Fresh carrots were washed with tap and chlorinated water (100 mg/L), hand peeled and shredded (2 mm x 3 mm x 40-60 mm). Then, 100 g of shredded carrots were dipped for 10 min (according to preliminary tests) into 500 mL of treatment solution. The following thirteen dipping solutions were studied: 1) distilled water, 2) marjoram EO (1:500, 1:1000, 1:1500 and 1:2000 v/v), 3) marjoram hydrosol (1:5, 1:10, 1:15 and 1:20 v/v) and 4) ascorbic acid (0.25%, 0.5%, 1%, and 2% w/v). Afterwards, carrots were drained, and 25 g were placed into a 90 mm Petri dishes and stored at 4 ^{o}C and 90% RH until the day of sampling (Days 1, 3, 5 and 7). Four biological replicates per treatment/concentration were sampled and the appropriate amount of tissue was stored at -20 ^{o}C until analysis. For preliminary screening, aroma and marketability assessed by 7 untrained panelists, while the weight loss and the content of total polyphenols were measured and used for choosing the adequate dipping solutions.

Carrots weight loss was monitored every second day, as the weight of each Petri dish was registered before and during storage at 4 ^{o}C for 7 days (Days 1, 3, 5 and 7) and results were presented as percentage of weight loss. Overall aroma and chroma (visual quality) were evaluated after storage for 7 days.

Aroma evaluation was assessed with a 5-point hedonic scale (0.5 interval) where 5 = not acceptable; 3 = not carrot but acceptable; 1 = carrot. Visual quality was assessed with a 5-point hedonic scale (0.5 interval) where 5 = brown; 3 = orange; 1 = white. The content of total phenolics was determined from methanolic extracts as previously described at section **2.2.8.2** and results were expressed as mg of gallic acid equivalents per g of fresh weight (mg GAE/g Fw).

5.3.1.3. Main study for the determination of quality and antioxidant activity

Following preliminary screening, carrots were selected, prepared and shredded as above. Then, 150 g of shredded carrots were dipped for 10 min into 500 mL of treatment solution selected from the preliminary screening. The following six dipping solutions/combinations were further studied: 1) distilled water, 2) marjoram EO (1:1500 v/v), 3) marjoram hydrosol (1:15 v/v), 4) Ascorbic acid (1% w/v), 5) marjoram EO (1:1500) + Ascorbic acid (1%) and 6) marjoram hydrosol (1:15) + Ascorbic acid (1%). Afterwards, carrots were drained, and 25 g were placed into a 90 mm Petri dish at 4 ^{o}C and 90% RH until the day of sampling (Days 0, 3, 6 and 9). Four biological replicates per treatment/day were sampled and the appropriate amount of tissue was stored at -20 ^{o}C until analysis.

5.3.1.3.1. Weight loss and color

Carrots weight loss was determined as mentioned above (section **5.3.1.2**). Shredded carrot's color was evaluated with a colorimeter (Chroma meter CR400 Konica Minolta, Japan) and *L**, *a**, *b**, chroma value (*C*), whiteness index (*WI*) and hue (*h*) color parameters were calculated as previously described at section **2.2.2**.

5.3.1.3.2. Respiration rate

Respiration of the processed product was evaluated by measuring the CO_2 concentration as described previously at section **2.2.3**. Briefly, each sample (1 Petri dish) was enclosed in a polypropylene (PP) plastic container (1 L) at room temperature for 1 hour, container's air was withdrawing for 40 seconds and respiration rate was expressed as mL of CO_2 per kg of fresh weight per h (mL CO_2/kg/h) according to the volume and weight of the shredded carrot.

5.3.1.3.3. Total soluble solids, total acidity, pH, ascorbic acid, total carotenoid content

Carrot tissue (four biological replicates/treatment/day) was grinded/pressed to extract the juice with a domestic blender. The pH and total soluble solids (TSS) content was determined as described at section **2.2.4** and TSS results were expressed in ^{o}Brix. Titratable acidity (TA) was determined by

titration with 0.1 N NaOH as described in section **2.2.4** and results were expressed as grams of citric acid per 100 g of fresh weight (% TA). In addition, the ratio of TSS/TA was calculated as mentioned at section **2.2.4**.

AA content was quantified by titration with 2,6-dichlorophenol- indophenol (section **2.2.5**) and results were expressed as gram of AA per 100 g of fresh weight (mg AA/100 g Fw). Total carotenoid content was determined as described in section **2.2.6**, and results were expressed as mg of carotenoids per 100 g of fresh weight (mg of carotenoids/ 100 g Fw).

5.3.1.3.4. Total phenolic content and antioxidant activity

Total phenolics were determined as mentioned above (section **2.2.8.1**). The 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) scavenging activities were determined according to the procedures described before (section **2.2.8.3** and **2.2.8.5**). Results expressed as mg trolox per g of fresh weight (mg trolox/ g Fw).

5.3.1.3.5. Hydrogen peroxide content and lipid peroxidation

The hydrogen peroxide (H_2O_2) concentration was determined according to the method described previously at section **2.2.9.1** and results were expressed as μmol of H_2O_2 per g of fresh weight (μmol H_2O_2/g Fw).

Lipid peroxidation of shredded carrots was estimated according to the 2-thiobarbituric acid reactive substances (TBARS) method as previously mentioned (section **2.2.9.2**) and results were expressed as nmol of malondialdeyde (MDA) per g of fresh weight (nmol MDA/g Fw).

5.3.1.3.6. Microbiological analysis

Total viable count (TVC) as well as yeast and filamentous fungi were determined as described at section **2.2.1**, **2.2.1.1** and **2.2.1.8** by using Plate count agar (PCA, Merck, Darmstadt, Germany) and Rose Bengal Chloramphenicol Agar (Liofilchem s.r.l, Italy), respectively Alegria et al. (2010). Results were expressed as log cfu per g of fresh weight (log cfu/g).

5.3.1.4. Statistical analysis

Statistical analysis was performed comparing data means with one way-analysis of variance (ANOVA) using IBM SPSS version 22 and Duncan's multiple range test was performed for $p = 0.05$.

Four (n=4) biological replicates were used and values refereed to mean ±SE. Microbiological analysis was done with duplicate plates for each of the three replicates.

5.3.2. Results

5.3.2.1. Effectiveness of screening preservation means

The screening of the effects of marjoram EO, Hyd and AA on shredded carrots during storage is presented in **Figures 5.10** and **5.S5**. The content of total phenolics increased at treatments with 1-2% AA, even at the first day of storage, and this effect was maintained up to seven days of storage. The applications of Hyd (from 1:5 to 1:20), AA (from 0.25% to 2%) and EO at 1:500 and at 1:2000 reduced weight loss when compared to the control treatment (**Figure 5.S5**). The preservative applications affected the carrot aroma after seven days storage at 4 °C and 90% RH, whereas the EO marked with 3 out of 5 scale, which reflected an acceptable aroma but not carrot like. The application of AA marked as acceptable-carrot like aroma, whereas Hyd acceptability was marked among AA and EO acceptability (**Figure 5.10**). Carrot marketability, in terms of color, changes were also observed, and it was found that the both AA and EO maintained the orange carrot color whereas the application of Hyd decreased the marketability as the processed carrots had browner chroma (scored 4 out of 5 units), and this was evident even at the first day of storage (**Figure 5.S6**). Control treatment also revealed brown color on carrots after the fifth day (**Figure 5.10**).

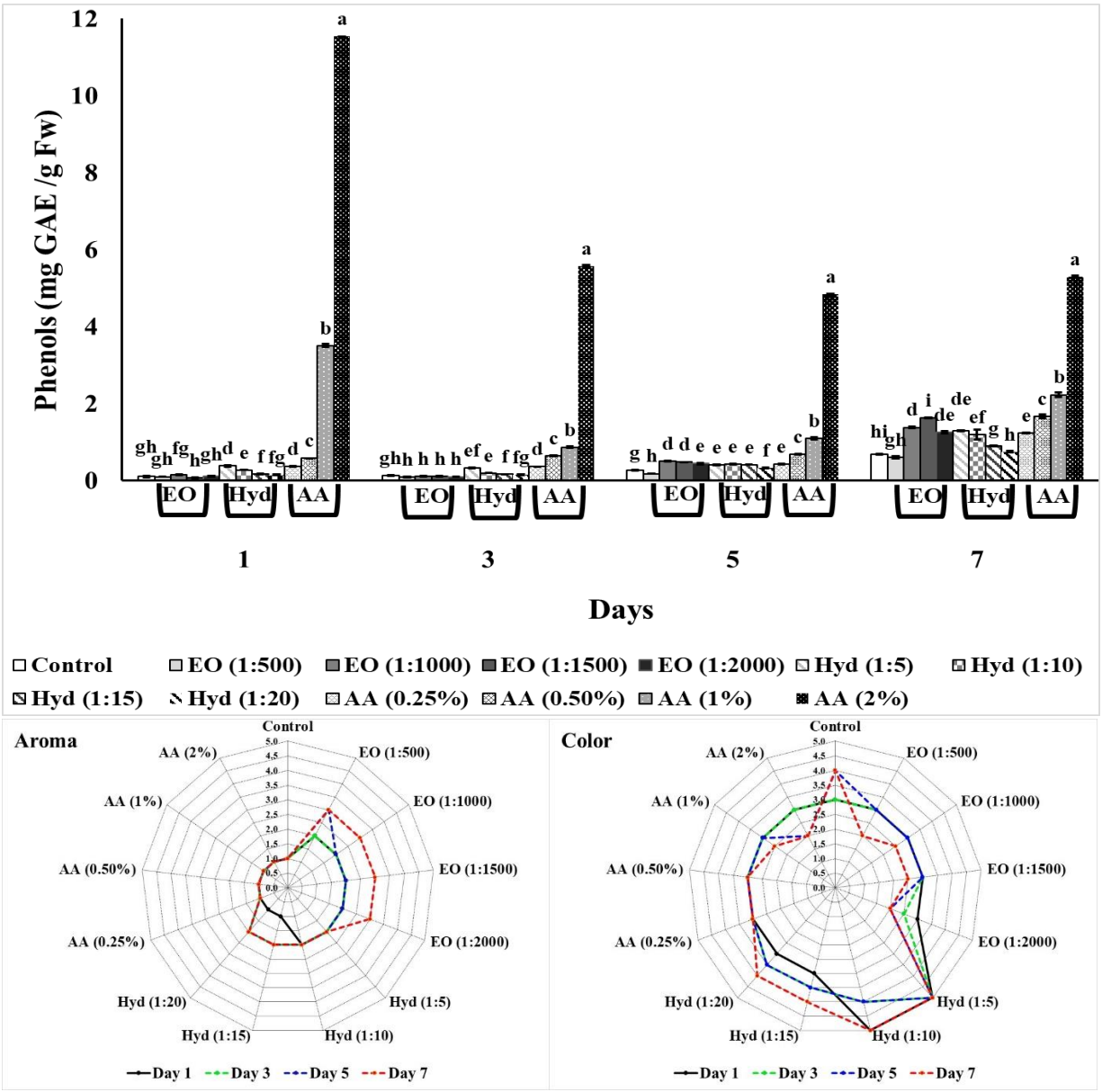


Figure 5.10. Screening of marjoram essential oil, marjoram hydrosol and ascorbic acid on shredded carrots total phenols (top panel), aroma (bottom left, scale range from 1-carrot like to 5-not acceptable) and color/marketability (bottom right, scale: 1-white; 3-orange; 5-brown) after 7 days of storage at 4 °C and 90% RH.

5.3.2.2. Impacts on quality and antioxidant activity

5.3.2.2.1. Effects on weight loss and respiration rates

The application of marjoram EO increased (up to 2.25%) weight loss on shredded carrots stored up to ninth day, while both Hyd or AA maintained weight losses to similar levels with the ones observed with the control treatment (**Figure 5.11**). Indeed, the combination of EO+AA and Hyd+AA resulted in higher weight losses compared to the individual effects of the AA or Hyd.

Respiration was increased with the combination of EO+AA on the sixth day (236.52 mL CO_2/kg/h), whereas Hyd maintained respiration rate to similar levels as the one observed by the control treatment,

on the same day (44.78 mL CO_2/kg/h) (**Figure 5.11**). Furthermore, the combinations of EO+AA and Hyd+AA increased respiration rate on the ninth day (128.92 and 145.77 mL CO_2/kg/h, respectively), while no differences were observed by the single applications of EO, Hyd and AA. Oxygen level was decreased slightly and averaged to 18% (data not presented) and remained almost constant throughout the storage period.

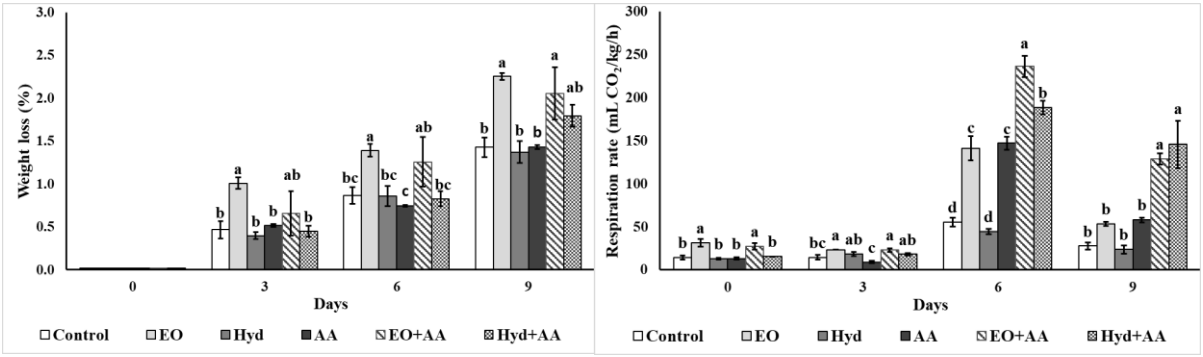
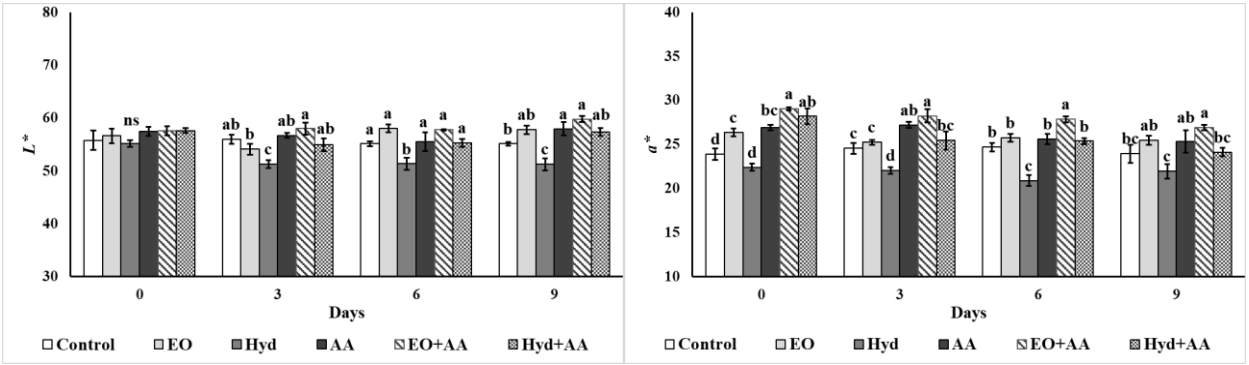


Figure 5.11. Effect of marjoram essential oil (EO), marjoram hydrosol (Hyd) and ascorbic acid (AA) on shredded carrots weight loss and respiration rate after treatment and 9 days of storage at 4 °C and 90% RH. On the columns, significant differences ($P < 0.05$) among treatments are indicated by different Latin letters; ns indicates no significant differences for different days.

5.3.2.2.2. Effects on color

The application of marjoram Hyd decreased L^* and a^* values up to the ninth day of storage, while b^* value was increased with the application of marjoram EO and the combination of EO+AA (46.38 and 47.12, respectively) (**Figure 5.12**). Moreover, the combination of EO+AA increased Chroma value of shredded carrots (54.27), whereas the application of AA (with or without marjoram Hyd) and the combination of EO+AA decreased Hue (1.77 and 1.78, respectively) and Whiteness index (32.45), respectively.



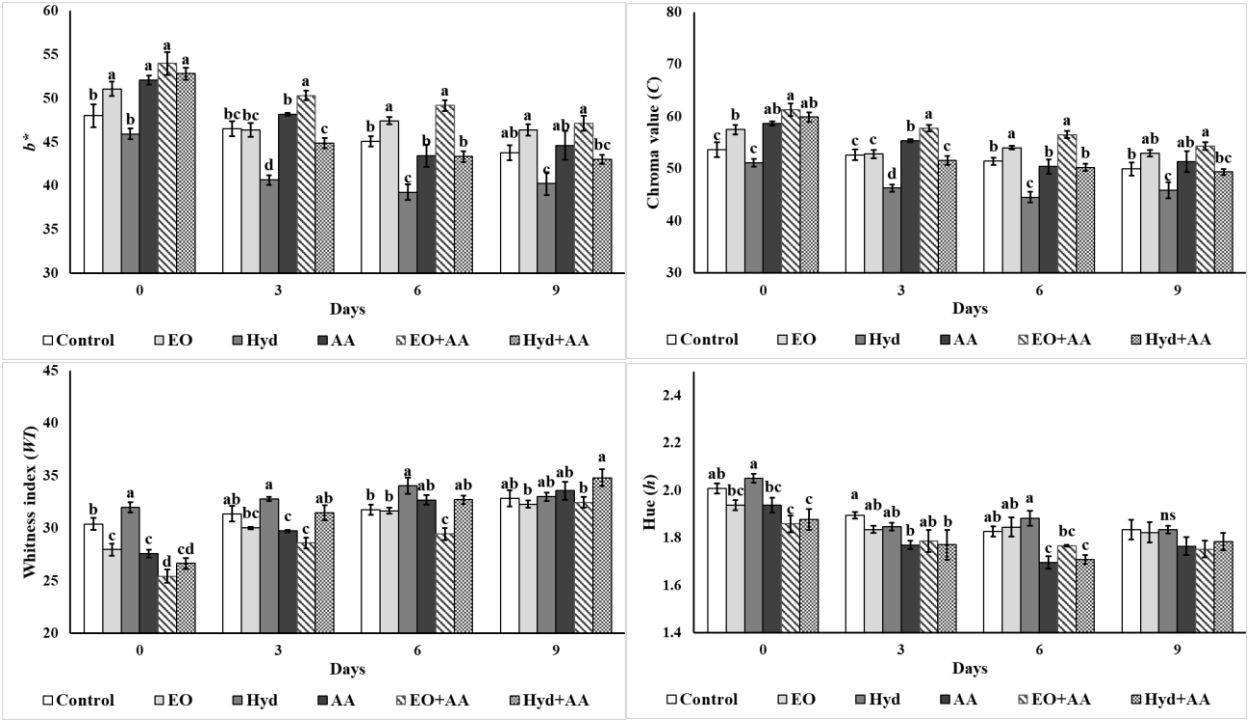


Figure 5.12. Effect of marjoram essential oil (EO), marjoram hydrosol (Hyd) and ascorbic acid (AA) on shredded carrots on *L** value, *a** (redness), *b** (yellowness), chroma, whiteness index and hue after treatment and 9 days of storage at 4 °C and 90% RH. On the columns, significant differences ($P < 0.05$) among treatments are indicated by different Latin letters; ns indicates no significant differences for different days.

5.3.2.2.3. Effects on total soluble solids, acidity, sweetness and pH

Total soluble solids (TSS) and sweetness (TSS/TA) of shredded carrots were decreased with the application of marjoram Hyd (4.97 ^{o}Brix and 1.25, respectively), whereas an increase of TSS was observed with the combination of EO+AA and Hyd+AA (6.80 and 7.00 ^{o}Brix, respectively) after the third day of storage and up to the ninth day (**Figure 5.13**). An increase in total acidity value was observed with the application of AA on the day of the application and after six and nine days (2.82, 2.63 and 3.12 %, respectively). The application of the combined treatments of EO+AA and Hyd+AA decreased pH values of the shredded carrots (pH 4.28 and 4.22, respectively), while AA acidified further the processed carrots and led to decreased pH values (pH 3.76) during the ninth day of storage at 4 °C and 90% RH.

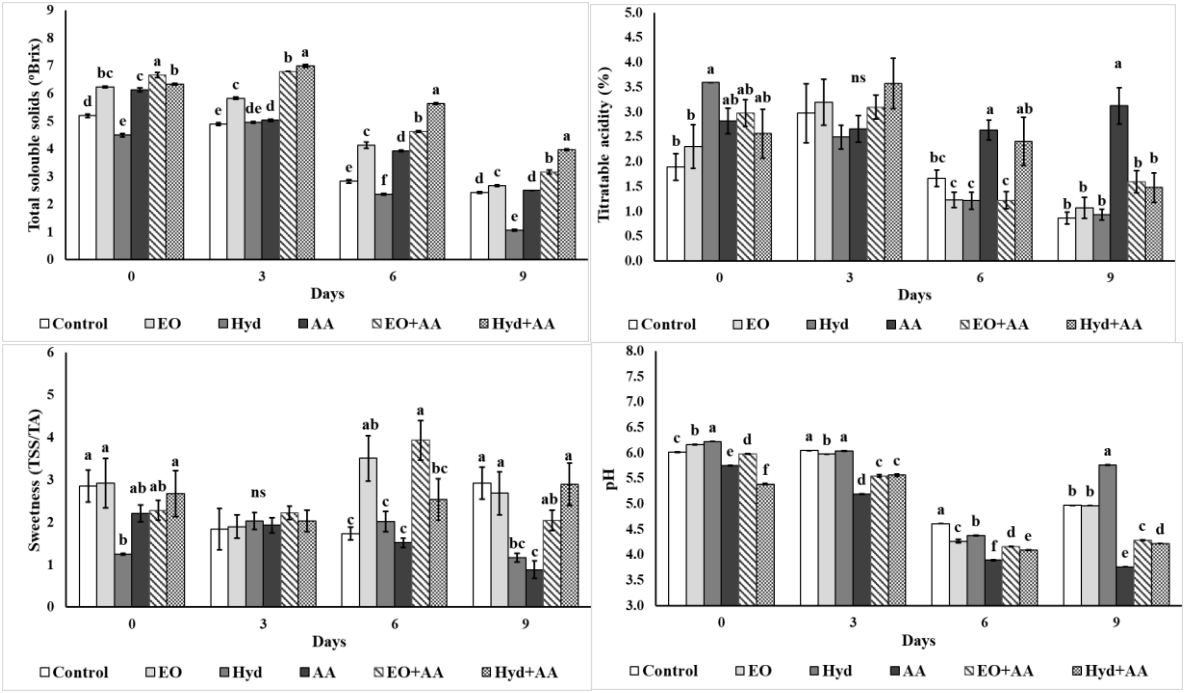


Figure 5.13. Effect of marjoram essential oil (EO), marjoram hydrosol (Hyd) and ascorbic acid (AA) on shredded carrots total soluble solids, total acidity, sweetness, and pH after treatment and 9 days storage at 4 °C and 90% RH. On the columns, significant differences ($P < 0.05$) among treatments are indicated by different Latin letters; ns indicates no significant differences for different days.

5.3.2.2.4. Effects on total phenolics, antioxidants, ascorbic acid and total carotenoids content

Total phenolic content was increased with the application of AA, and its combination with marjoram EO and Hyd as illustrated by **Figure 5.14**. The application of AA and the combination of Hyd+AA increased (up to 2.8-fold and 2.6-fold as assayed by DPPH and ABTS) antioxidant activity of shredded carrots on the day of the application, whereas the effects were almost exhausted following nine days of storage. Ascorbic acid content of shredded carrots was increased with the application of AA and the combination of Hyd+AA on the day of the application (118.27 and 309.55 g AA/100 g Fw, respectively), after six (26.63 and 22.35 g AA/100 g Fw, respectively) and nine days (44.96 and 43.18 g AA/100 g Fw, respectively). The combination of Hyd+AA maintained carotenoid content (11.31 mg carotenoids/100 g Fw) to similar levels like the relevant content found in control treatment, while a decrease of carotenoids was observed with the application of marjoram EO, marjoram Hyd and AA (8.44, 8.25 and 8.15 mg carotenoids/100 g Fw, respectively) on the 9th day at 4 °C and 90% RH.

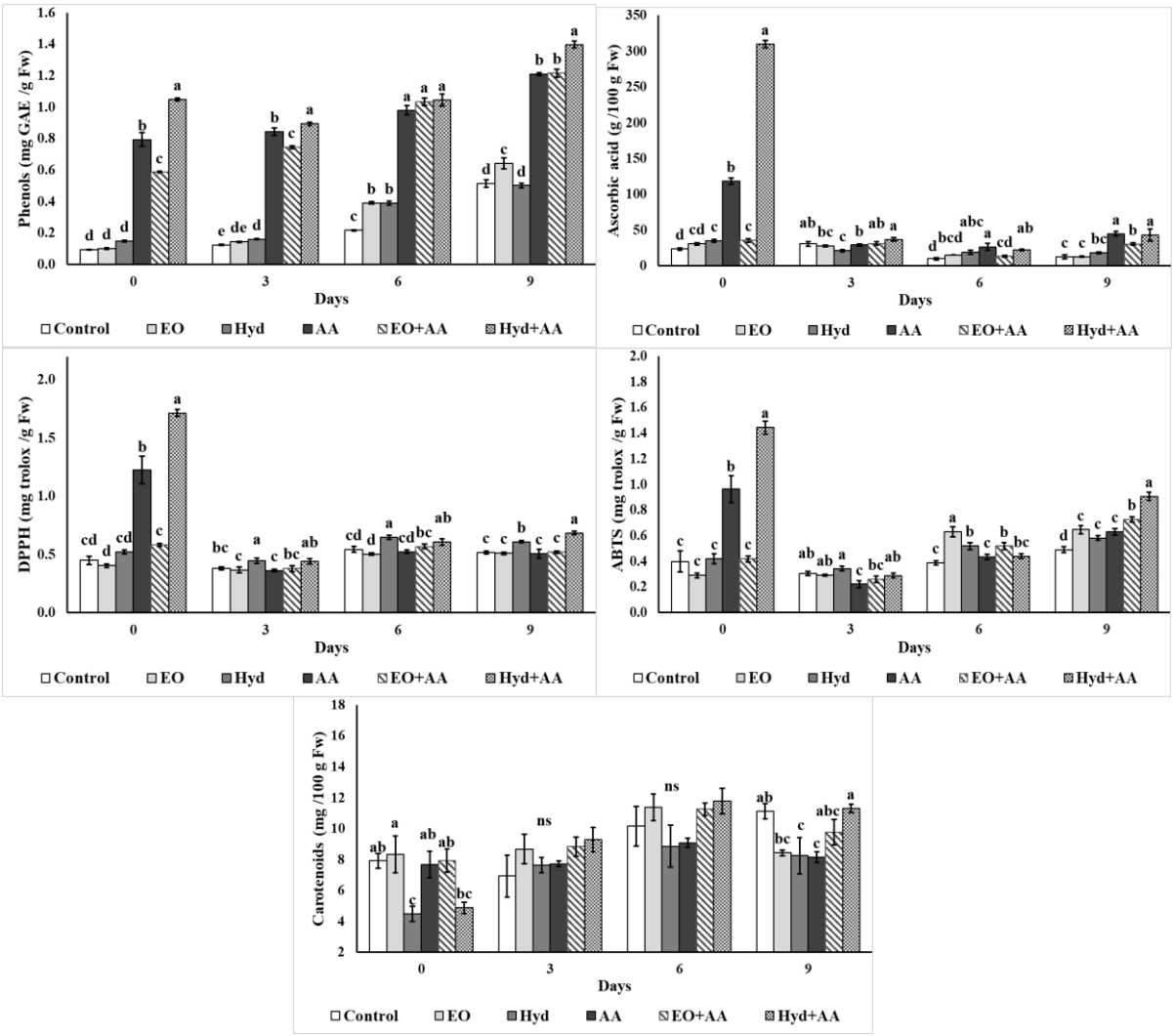


Figure 5.14. Effect of marjoram essential oil (EO), marjoram hydrosol (Hyd) and ascorbic acid (AA) on shredded carrots total phenolics, ascorbic acid, antioxidant activity (DPPH, ABTS), and carotenoids content after treatment and 9 days of storage at 4 °C and 90% RH. On the columns, significant differences ($P < 0.05$) among treatments are indicated by different Latin letters; ns indicates no significant differences for different days.

5.3.2.2.5. Effects on hydrogen peroxide and lipid peroxidation

The impacts of marjoram EO, marjoram Hyd and AA on hydrogen peroxide and lipid peroxidation are presented in **Figure 5.15**. The application of the combination of EO+AA followed by the EO treatment increased H_2O_2 while the application of AA decreased H_2O_2 on the ninth day of storage (43.46 nmol H_2O_2/g Fw). The MDA production was increased with the Hyd+AA and EO+AA during the ninth day of storage.

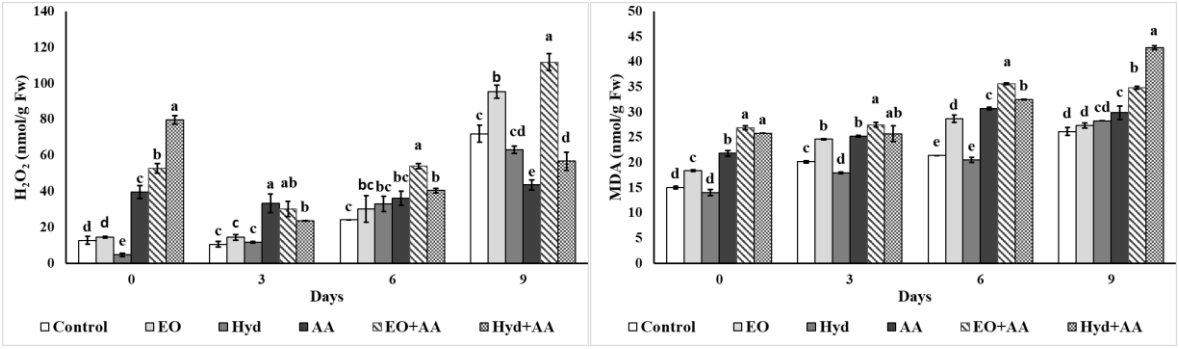


Figure 5.15. Effect of marjoram essential oil (EO), marjoram hydrosol (Hyd) and ascorbic acid (AA) on shredded carrots on H_2O_2 production and lipid peroxidation after treatment and 9 days of storage at 4 °C and 90% RH. On the columns, significant differences (P<0.05) among treatments are indicated by different Latin letters; ns indicates no significant differences for different days.

5.3.2.2.6. Microbiological analysis

Microbial quality of shredded carrot subjected to single or combine treatments are illustrated in **Figure 5.16**. A significant decrease in total viable counts (TVC) was detected on the sixth in all the examined treatments and on the ninth day with the application of AA and the combination of EO+AA and Hyd+AA (5.04, 5.27 and 4.79 log cfu/g Fw, respectively). No differences were found the first three days for the TVC. Yeast and filamentous fungi counts were decreased after 3 days for all the treatments (with exception the Hyd+AA) and the effects were more pronounced with the application of the combined treatment on the ninth day. Therefore, Hyd+AA revealed the greatest decrease on the yeasts and molds counts at the ninth day of application (2.30 log cfu/g Fw).

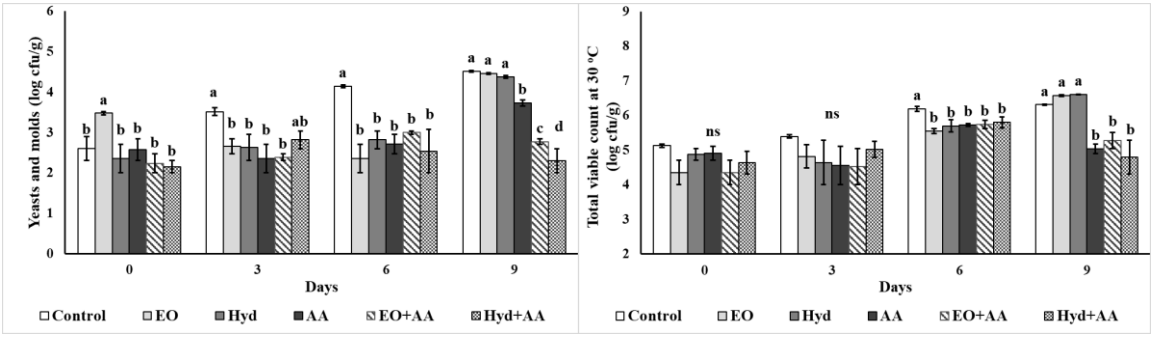


Figure 5.16. Effect of marjoram essential oil (EO), marjoram hydrosol (Hyd) and ascorbic acid (AA) on shredded carrots on yeast and filamentous fungi and total viable count (TVC) after 9 days of storage at 4 °C and 90% RH. On the columns, significant differences (P < 0.05) among treatments are indicated by different Latin letters; ns indicates no significant differences for different days.

5.3.3. Discussion

Minimally processed vegetables are quite popular nowadays as a source of phytochemicals, however they are perishable products as they tend to have short shelf life. The use of natural products such as essential oils and naturally occurring substances as an ecofriendly and healthier preservative method is rapidly growing, attracting scientific interest and consumer's acceptance (Song et al., 2017).

The antioxidant and antimicrobial activity of EOs from aromatic and medicinal plants have been previously mentioned (Lis-Balchin et al., 1998; Sellami et al., 2009, Tzortzakis, 2009; Xylia et al., 2017). It has been reported that marjoram EO possess high antioxidant and antimicrobial activity and its main components include 1,8-cineole, terpinolene, terpinen-4-ol (Lis-Balchin et al., 1998; Vera and Chane-Ming, 1999), and this is in line with the main components found in the examined marjoram EO. Marjoram EO has been applied to fresh cabbage, lettuce and radish sprouts with encouraging results (Hyun et al., 2015a, b). The use of AA as an anti-browning agent in fresh produce has been studied due to its high antioxidant activity (Akbas et al., 2007b; Altunkaya and Gökmen, 2009; Olmez and Temur, 2010; Park et al., 2011).

In this study no considerable weight losses (< 2.3%) were determined while the higher weight losses were observed with the application of EO+AA and Hyd+AA. The increased surface exposure to air of the minimally processed products can lead to moisture loss and furthermore surface whitening but also to decay presence (Li and Barth, 1998). It is noteworthy that Rocha et al. (2007) have reported up to 9% weight loss after storage at 2 ^{o}C for 10 days. Increased respiration was observed on the sixth day with the application of EO+AA whereas marjoram Hyd lead to a decrease of respiration rate. Heat treatment efficiently maintained shredded carrot quality by decrease in respiration rates (Algeria et al., 2010). As it has been previously mentioned by Rocha et al. (2007) increased fresh produce respiration can be caused by cutting which can result to protein, lipid and carbohydrates degradation and furthermore off-flavors and off-odors development.

The bright orange color of carrots is one of the most important attributes that can affect consumers buying decisions. The application of EO+AA increased Chroma value resulting in a lighter orange color throughout storage. In a previous study the application of mint hydrosol (1:10, v/v) on shredded carrots resulted in a less orange product on the sixth day of storage with lower Chroma value compared to the untreated samples (51.64 and 53.20, respectively) (Xylia et al., 2018). Rocha et al. (2007) reported that surface whitening might be attributed to increased moisture loss. Furthermore, Barry-Ryan and O'Breirne (1998) also pointed out that surface whitening and lignification of fresh cut carrots may be the results of moisture loss. The application of EO+AA decreased Whiteness index of shredded carrot exhibiting less surface whitening. Decreased Chroma and L^* values reflect in a less intense orange color and at the same time Whiteness index increases suggesting quality deterioration (Mastrocola and Lerici, 1991; Lavelli et al., 2006). Lavelli et al. (2006) correlated the visual appearance of whitening and the perception of off-odor on minimally processed carrots and concluded that WI can be regarded as the most sensible indicator of sensory quality. The increases in WI that were observed during storage are related to the reversible dehydration of outer tissue layers which can be related to formation of lignin and concomitant whitening of the shreds (Cisneros-Zevallos et al., 1995).

The application of marjoram Hyd decreased TSS and sweetness, whereas the application of EO+AA and Hyd+AA increased TSS. In the present study, TSS values ranged between 6.80-1.07 ^{o}Brix with the

application of marjoram EO, marjoram Hyd and AA. These values were lower to the values observed in our previous study with the application of mint EO and hydrosol in shredded carrot (TSS range: 8.26-9.33 ^{o}Brix), indicating the effectiveness of varied EO from different species (Xylia et al., 2018). Other studies have mentioned higher TSS values ranging between 8.3 and 9.6 (Koca and Karadeniz, 2008) or even up to 11.8 ^{o}Brix (Alegria et al., 2010). Total acidity was increased with the application of AA (up to 3.12% on the ninth day of storage) whereas in a previous study, the application of pomegranate juice on shredded carrot resulted in TA of 0.78% (Xylia et al., 2018). Moreover, Rodrigo et al. (2003) observed TA values up to 0.6% in carrot juice.

Decreased pH values were observed with the application of AA (up to 5.75), EO+AA and Hyd+AA (up to 4.09), and these findings are in accordance with previous reported studies (Alegria et al., 2009; 2010; Xylia et al., 2018). It is noteworthy that Rocha et al. (2007) mentioned that decreased pH values (pH 5.4–6.2) may be the result of CO_2 production that can react with water of the plant tissue and release H^+. In this sense, the increased respiration rates found in EO+AA and Hyd+AA could contribute to the H^+ release and acidified the processed carrots with pH decreases.

EOs can induce plant defense mechanisms, both directly and indirectly, by the production of phenols and antioxidants (Amorati et al., 2013). Total phenolic content was increased with the application of AA, EO+AA and Hyd+AA (0.98, 1.03 and 1.04 mg GAE/g Fw, respectively) after 6 d of storage. On the other hand, in another study the application of mint EO on shredded carrots decreased total phenols (1.65 mg GAE/g Fw), whereas mint Hyd increased total phenols (5.22 mg GAE/g Fw) after six days of storage (Xylia et al., 2018). Han et al. (2017) mentioned shredded carrots phenolic content ranging between 0.23 and 0.44 mg GAE/g Fw after storage at 4 ^{o}C, and in such levels control polyphenols were found. Increased antioxidant activity was observed with the application of marjoram Hyd and Hyd+AA whereas similar results were observed with the application of mint Hyd on shredded carrots in a previous study (Xylia et al., 2018).

The content of AA increased with the application of AA and Hyd+AA while single Hyd application did slightly increased AA content being in accordance with previous studies with mint Hyd on shredded carrots (Xylia et al., 2018). Carotenoid content varies between carrot cultivars (0.4-26 mg/100g Fw) (Nicolle et al., 2004). The application of Hyd+AA maintained carotenoid content whereas the application of marjoram EO, Hyd and AA decreased carotenoids. On the other hand, the application of mint EO and Hyd did not significantly affect carotenoids of shredded carrots (Xylia et al., 2018), indicating variation on the EO and Hyd effectiveness among the different plant species (marjoram versus mint). According to Rocha et al. (2007) the loss of carotenoids is related to longer storage period that can result in products with reduced nutritional value. The loss of carotenoids seems to be attributed to oxidation as minimally processing (peeling and shredding) exposes phloem (carotenoids mainly concentrated to that part of carrot tissue) to light, air and enzymes (Gross, 1991). On the other hand,

carotenoids are relatively stable in their natural environment, but postharvest treatments or processing operations may enhance the pigments' degradation.

Minimal processing induces wounds in plant tissue by causing a stress response of the wounded tissue. Han et al. (2017) reported levels of H_2O_2 ranging between 8.56 and 9.33 μmol/g Fw in shredded carrot stored at 4 ^oC. The same authors reported that the levels of H_2O_2 were affected by cutting style and storage temperature (Han et al., 2017). In our study EO+AA increased H_2O_2 levels and lipid peroxidation as measured by the MDA production, and this indicating an increased oxidative stress (reactive oxygen species-ROS) related to the applied treatment. Increased MDA was also found with the Hyd+AA treatment. The increased AA content in processed carrots seems not to be enough to detoxify the tissue, as MDA remained at high levels. Therefore, from the examined non-enzymatic antioxidants (ascorbic acid, polyphenols), the protection to oxidative stress was mainly related to the increased polyphenols. In the presence of free radicals and oxidative stress plant tissues have developed a series of mechanisms to scavenge these reactive compounds including phenols and antioxidant production as well as the action of antioxidant enzymes, such as superoxide dismutase-SOD, peroxidase-POD and catalase-CAT (Han et al., 2017). Alegria et al. (2010) observed a correlation of POD activity and quality attributes of carrots (color, flavor, texture and nutritional value) when treated with hot water at 100 ^oC for 45 s. For example, the inhibition of POD activity was correlated with preservation of the orange color of carrots (Alegria et al., 2010).

The application of AA, marjoram EO+AA and marjoram Hyd+AA decreased TVC values (5.04, 5.27 and 4.79 log cfu/g Fw, respectively) of shredded carrot compared to the untreated sample on the ninth day (6.31 log cfu/g Fw). In another study the application of ozonated-water, hot water and ultrasonication resulted in a TVC load ranging between 0.4 and 1.7 reduction depending on treatment (Alergia et al., 2009). Yeast and filamentous fungi counts have been decreased with the application of marjoram Hyd+AA and are in similar levels with another study in which their count was around 3.8 log cfu/g after the application of ozonated-water, hot water and ultrasonication (Alergia et al., 2009). In both studies yeast and filamentous fungi counts did not exceed 5 log cfu/g that could be characterized as the acceptable maximum limit for these microorganisms (Jacxsens et al., 2002). However, Lavelli et al. (2006) reported that the maximum limitation of microorganism can be obtained in higher storage temperature, for example 10 ^oC when compared to 4 ^oC, as took place in the present study.

Most naturally occurring substances and EOs are classified as GRAS and can be used for food preservation, however they should be used with caution as higher concentrations may be required in order to ensure food safety and quality and those concentrations may cause non-acceptable changes in the organoleptic characteristics of each product (Tzortzakis, 2007; Gündüz et al., 2010). The findings from this study are promising and thus, further studies are needed for the investigation of the conditions (time, concentrations) of the application of these and similar natural products in fresh produce.

5.4. Concluding marks

Despite the increased consumption of minimally processed products, including fresh-cut salads and shredded carrots, they are considered high perishable fresh produce and successful and eco-friendly preservation means are under investigations. In the present study, we examined natural products following initial screening and their combination on their performance as natural sanitizers and preservatives on fresh-cut lettuce and shredded carrots.

In case of lettuce, natural products used in the present study (marjoram EO, AA and chitosan) and the outcomes of their application on fresh-cut lettuce provide encouraging evidence for the use of natural compounds in the food industry. Marjoram EO improved aroma and visual quality of minimally processed lettuce, while chitosan used alone affected negatively lettuce color. The combination of EO+AA increased phenolic, ascorbic and carotenoid contend as well as the antioxidant capacity of fresh-cut lettuce, thus improving its nutritional value. Interestingly chitosan, EO, EO+chitosan and chitosan+AA presented antimicrobial activity against TVC and yeast and molds counts of minimally processed lettuce.

In case of carrots, marjoram EO and hydrosol alone were not as promising as AA application for the preservation and quality of processed carrots, while their combination with AA (Hyd+AA and EO+AA) provided increased quality attributes such as carotenoids. Additionally, AA increased polyphenols and antioxidant status and decreased pH of the shredded produce, providing valuable role in quality maintenance of the produce. During screening test, AA-treated carrots were marked an acceptable-carrot like aroma, while EO-treated carrots were marked with an acceptable aroma but not carrot like. Hyd acceptability was marked among AA and EO acceptability.

The findings from this study are promising since the combination of natural products with antioxidant and antimicrobial activities is gaining interest for the preservation of postharvest quality of fresh produce as well as minimally processed vegetables.

Chapter 6. General discussion and future prospects

6.1. General discussion

The present PhD thesis is divided into six chapters, including the current one and are outlined as follows:

Chapter 1 presents the introduction of this thesis, which is a literature review of the current perspectives regarding food safety and quality of fresh produce throughout the food chain (mainly on cultivation and processing). Fresh produce has been implicated in a series of food poisoning outbreaks that started either pre-or postharvest. Thus, it is essential to understand the possible routes and factors influencing the contamination of fresh produce with foodborne pathogens during cultivation as well as the investigation of alternative to chlorine and chlorine-based sanitizing agents (i.e. natural products among others) in order to ensure safety and preserve the quality of fresh produce and minimally processed vegetables after harvesting and during processing.

Chapter 2 presents the methods and protocols used for the experimental part of this thesis, including microbiological analyses and the respective media used, molecular identification of *S. enterica* and *L. monocytogenes*, postharvest related parameters (color, respiration, ethylene rate emission, pH, total soluble solids, total acidity, sweetness), ascorbic acid, photosynthetic pigments (chlorophylls, carotenoids), antioxidants and damage index (H_2O_2, MDA).

Chapter 3 examines on the first part the variability in the microbial quality (spoilage and pathogen microorganisms) of ready-to-eat salads as affected by season, produce, type of salad and their combination, while at the same time investigates the possible relation between physicochemical (antioxidants and CO_2 production) and the microbial load of these products. On the second part of this chapter the effects of the expiring date on the physicochemical (antioxidants and CO_2 production) and the microbial load of ready-to-eat salads during two seasons (winter and summer) is also evaluated. The results from this investigation indicate that season, type of vegetable and expiring date affected the microbial load and plant-tissue related parameters of ready-to-eat salads. Differences among total phenolic content and the antioxidant activity of salads were attributed to the interaction of salad producer*type of salad, since different processing practices influence the safety and quality of fresh produce by increasing/decreasing plant stress. As it was expected, plant stress was reported on expiring date and it was related to increased microbial (mainly spoilage microorganisms i.e. *Pseudomonas* spp., yeasts and molds), CO_2 production and damage index indicators.

Chapter 4 investigates the effects of different plant growth stages, pH and bacterial inoculum levels on hydroponically cultivated lettuce inoculated with *S.* Enteritidis. The results of this study the investigated parameters affected the internalization and survival of the pathogen in the hydroponic environment and plant tissue. Younger plants were found to be more susceptible to pathogen internalization compared to older ones. Under the current cultivation conditions no leaf internalization

was observed at all growth stages and even though the bacterium was present in the hydroponic solution. Moreover, these results possibly indicate that the presence of the bacterium initiates plant defense mechanisms i.e. increased phenols, antioxidants and damage index markers (H_2O_2, MDA) in order for the plant to resist contamination by the invader.

Chapter 5 examines the effects natural products (i.e. marjoram essential oil and hydrosol, ascorbic acid and chitosan) application on safety and quality of two minimally processed vegetables (fresh-cut lettuce and shredded carrot). According to the results of this chapter, the application seems to be a remarkable alternative for the preservation of minimally processed vegetables (fresh-cut lettuce and shredded carrot) safety and quality suggesting the reduction of chlorine and chlorine based chemical sanitizers in the food industry. The application of marjoram EO resulted to improved quality enhancing the aroma and visual quality of both minimally processed vegetables, reducing their microbial load. Furthermore in this study it has been shown that the combination of the examined products could be more efficient for the preservation of fresh produce compared to when used alone.

Chapter 6 (the current chapter) summarizes the work of this thesis and future prospects are also presented. Furthermore, in this thesis more details related to different chapters are presented in the appendix section.

A holistic approach for the investigation of fresh produce processing throughout the food chain (from field to consumer) could result to a better understanding of the current issues reported in the food industry and lead to the establishment of novel tools that will ensure safety and preserve the quality of these perishable products.

6.2. Future prospects

Based on the results of the present thesis regarding the pre- and post-harvest safety and quality of fresh produce and minimally processed vegetables, further studies could:

- Extensively assess the shelf-life of ready-to-eat salads (from packaging to expiring date) and correlation of their microbial and physicochemical attributes (including enzymatic activity) as well as the parameters from which it might be influenced for understanding and development of novel technics monitoring the safety and quality of these products.
- Investigate in a molecular level genes involved on plant colonization by *S.* Enteritidis, including genes associated with the attachment of *Salmonella* cells to surfaces and genes regarding the interaction of plant tissue with the presence of *Salmonella* (epi- and endophytically) for further understanding of bacterial survival and establishment in this environment.
- Examine with a different approach (than presented in this study-Chapter 4) for the visualization of internalization of *S.* Enteritidis alongside with cultures on Petri dishes (use

of GFP-labeled strain, bacterial staining i.e. SYTO9 stain) for better bacterial recovery results.

- Determine the role of individual nutrients (cations and/or anions forms) on the presence of human pathogens, which might lead to the development of guidelines for fresh produce hydroponic cultivation that will ensure the production of safe and high nutritional products.
- Investigate the possible inhibitory effects of the investigated natural products (reported on Chapter 5) on enzymatic and non-enzymatic browning reactions in minimally processed vegetables throughout storage time.
- Examine the possible application methods for the natural products (dipping, spraying) and their effects on fresh produce and its microbial load.
- Assess the antimicrobial activity of the applied concentrations of the natural products investigated (*in vitro* and in *vivo*) which will give insight to the food industry for eco-friendly, non-toxic agents that are able to ensure safer and of high nutritional products that meet the consumers demands.

6. References

Abadias, M., Usall, J., Anguera, M., Solsona, C. and Viñas, I., 2008. Microbiological quality of fresh, minimally-processed fruit and vegetables, and sprouts from retail establishments. *International Journal of Food Microbiology*, 123(1-2), 121–129.

Abbott, D.W. and Boraston, A.B., 2008. Structural biology of pectin degradation by *Enterobacteriaceae*. *Microbiology and Molecular Biology Reviews*, 72(2), 301-316.

Adegoke, A.A., Amoah, I.D., Stenström, T.A., Verbyla, M.E. and Mihelcic, J.R., 2018. Epidemiological evidence and health risks associated with agricultural reuse of partially treated and untreated wastewater: a review. *Frontiers in Public Health*, 6, 337.

Adegoke, A.A., Faleye, A.C., Singh, G. and Stenström, T.A., 2017a. Antibiotic resistant superbugs: assessment of the interrelationship of occurrence in clinical settings and environmental niches. *Molecules*, 22(1), 9.

Adegoke, A.A., Stenström, T.A. and Okoh, A.I., 2017b. *Stenotrophomonas maltophilia* as an emerging ubiquitous pathogen: looking beyond contemporary antibiotic therapy. *Frontiers in Microbiology*, 8, 2276.

Agresti, A. and Coull, B.A., 1998. Approximate is better than "exact" for interval estimation of binomial proportions. *The American Statistician*, 52, 119–126.

Agüero, M.V., Ponce, A.G., Moreira, M.R. and Roura, S.I., 2011. Lettuce quality loss under conditions that favor the wilting phenomenon. *Postharvest Biology and Technology*, 59, 124-131.

Ailes, E.C., Leon, J.S., Jaykus, L.A., Johnston, L.M., Clayton, H.A., Blanding, S., Kleinbaum, D.G., Backer, L.C. and Moe, C.L., 2008. Microbial concentrations on fresh produce are affected by postharvest processing, importation, and season. *Journal of Food Protection*, 71 (12), 2389–2397.

Akbas, M.Y. and Ölmez, H., 2007a. Effectiveness of organic acid, ozonated water and chlorine dippings on microbial reduction and storage quality of fresh-cut iceberg lettuce. *Journal of the Science of Food and Agriculture*, 87, 2609-2616.

Akbas, M.Y. and Ölmez, H., 2007b. Inactivation of *Escherichia coli* and *Listeria monocytogenes* on iceberg lettuce by dip wash treatments with organic acids. *Letters in Applied Microbiology*, 44(6), 619-624.

Alam, M., Ahlström, C., Burleigh, S., Olsson, C., Ahrné, S., El-Mogy, M., Molin, G., Jensén, P., Hultberg, M. and Alsanius, B.W., 2014. Prevalence of *Escherichia coli* O157: H7 on spinach and rocket as affected by inoculum and time to harvest. *Scientia Horticulturae*, 165, 235-241.

Alcarraz, E., Flores, M., Tapia, M.L., Bustamante, A., Wacyk, J. and Escalona, V., 2016, June. Quality of lettuce (*Lactuca sativa* L.) grown in aquaponic and hydroponic systems. In *VIII International Postharvest Symposium: Enhancing Supply Chain and Consumer Benefits-Ethical and Technological Issues*, 1194, 31-38.

Alegbeleye, O.O., Singleton, I. and Sant'Ana, A.S., 2018. Sources and contamination routes of microbial pathogens to fresh produce during field cultivation: a review. *Food Microbiology*, 73, 177-208.

Alegria, C., Pinheiro, J., Gonçalves, E.M., Fernandes, I., Moldão, M. and Abreu, M., 2010. Evaluation of a pre-cut heat treatment as an alternative to chlorine in minimally processed shredded carrot. *Innovative Food Science and Emerging Technologies*, 11, 155–161.

Alegria, C., Pinheiro, J., Gonçalves, E.M., Fernandes, I., Moldão, M. and Abreu, M., 2009. Quality attributes of shredded carrot (*Daucus carota* L. cv. Nantes) as affected by alternative decontamination processes to chlorine. *Innovative Food Science and Emerging Technologies*, 10, 61-69.

Alissa, E.M. and Ferns, G.A., 2017. Dietary fruits and vegetables and cardiovascular diseases risk. *Critical reviews in Food Science and Nutrition*, 57(9), 1950-1962.

Al-Kharousi, Z.S., Guizani, N., Al-Sadi, A.M., Al-Bulushi, I.M. and Shaharoona, B., 2016. Hiding in fresh fruits and vegetables: opportunistic pathogens may cross geographical barriers. *International Journal of Microbiology*, 2016, Article ID 4292417, 14 pages.

Allende, A., Aguayo, E. and Artés, F., 2004. Microbial and sensory quality of commercial fresh processed red lettuce throughout the production chain and shelf life. *International Journal of Food Microbiology*, 91(2), 109-117.

Allerberger, F., 2007. '*Listeria*', in Simjee S. (ed.), *Foodborne diseases*, Humana Press Inc., New Jersey, 27-39.

Altunkaya, A. and Gökmen, V., 2009. Effect of various anti-browning agents on phenolic compounds profile of fresh lettuce (*L. sativa*). *Food Chemistry*, 117, 122-126.

Altunkaya, A., 2011. Effect of whey protein concentrate on phenolic profile and browning of fresh-cut lettuce (*Lactuca sativa*). *Food Chemistry*, 128, 754-760.Altunkaya, A. and Gökmen, V., 2009. Effect of various anti-browning agents on phenolic compounds profile of fresh lettuce (*L. sativa*). *Food Chemistry*, 117, 122-126.

Amodio, M.L., Derossi, A., Mastrandrea, L. and Colelli, G., 2015. A study of the estimated shelf life of fresh rocket using a non-linear model. *Journal of Food Engineering*, 150, 19-28.

Amorati, R., Foti, M.C. and Valgimigli, L., 2013. Antioxidant activity of essential oils. Journal of *Agricultural and Food Chemistry*, 61(46), 10835-10847.

Angelidis, A.S., Kalamaki, M.S. and Georgiadou, S.S., 2015. Identification of non-*Listeria* spp. bacterial isolates yielding a *β*-D-glucosidase-positive phenotype on Agar Listeria according to Ottaviani and Agosti (ALOA). *International Journal of Food Microbiology*, 193, 114–129.

AOAC International, 2007. Official Methods of Analysis, 18th ed. Gaithersburg.

Arrebola, E., Sivakumar, D., Bacigalupo, R. and Korsten, L., 2010. Combined application of antagonist *Bacillus amyloliquefaciens* and essential oils for the control of peach postharvest diseases. *Crop Protection*, 29(4), 369-377.

Aruscavage, D., Phelan, P.L., Lee, K. and LeJeune, J.T., 2010. Impact of changes in sugar exudate created by biological damage to tomato plants on the persistence of *Escherichia coli* O157: H7. *Journal of Food Science*, 75(4), M187-M192.

Arvanitoyannis, I.S., Bouletis, A.D., Papa, E.A., Gkagtzis, D.C., Hadjichristodoulou, C. and Papaloucas, C., 2011. Microbial and sensory quality of "*Lollo verde*" lettuce and rocket salad stored under active atmosphere packaging. *Anaerobe*, 17(6), 307-309.

Aycicek, H., Oguz, U. and Karci, K., 2006. Determination of total aerobic and indicator bacteria on some raw eaten vegetables from wholesalers in Ankara, Turkey. *International Journal of Hygiene and Environmental Health*, 209(2), 197–201.

Azevedo, A.N., Buarque, P.R., Cruz, E.M.O., Blank, A.F., Alves, P.B., Nunes, M.L. and de Aquino Santana, L.C.L., 2014. Response surface methodology for optimisation of edible chitosan coating formulations incorporating essential oil against several foodborne pathogenic bacteria. *Food Control*, 43, 1-9.

Bagamboula, C.F., Uyttendaele, M. and Debevere, J., 2004. Inhibitory effect of thyme and basil essential oils, carvacrol, thymol, estragol, linalool and *p*-cymene towards *Shigella sonnei* and *S. flexneri*. *Food Microbiology*, 21, 33-42.

Balali, G.I., Yar, D.D., Afua Dela, V.G. and Adjei-Kusi, P., 2020. Microbial contamination, an increasing threat to the consumption of fresh fruits and vegetables in today's world. *International Journal of Microbiology*, 2020 (1), 1-13.

Barak, J.D. and Schroeder, B.K., 2012. Interrelationships of food safety and plant pathology: the life cycle of human pathogens on plants. *Annual Review of Phytopathology*, 50, 241-266.

Barry-Ryan, C. and O'Beirne, D., 1998. Quality and shelf-life of fresh cut carrot slices as affected by slicing method. *Journal of Food Science*, *63*, 851-856.

Barton Behravesh, C., Mody, R.K., Jungk, J., Gaul, L., Redd, J.T., Chen, S., Cosgrove, S., Hedican, E., Sweat, D., Chávez-Hauser, L. and Snow, S.L., 2011. 2008 outbreak of *Salmonella* Saintpaul infections associated with raw produce. *New England Journal of Medicine*, 364(10), 918-927.

Belabbes, R., Dib, M.E.A., Djabou, N., Ilias, F., Tabti, B., Costa, J. and Muselli, A., 2017. Chemical variability, antioxidant and antifungal activities of essential oils and hydrosol extract of *Calendula arvensis* L. from western Algeria. *Chemistry and Biodiversity*, 14(5), e1600482.

Bell, L., Yahya, H.N., Oloyede, O.O., Methven, L. and Wagstaff, C., 2017. Changes in rocket salad phytochemicals within the commercial supply chain: Glucosinolates, isothiocyanates, amino acids and bacterial load increase significantly after processing. *Food Chemistry*, 221, 521-534.

Bennett, S.D., Sodha, S.V., Ayers, T.L., Lynch, M.F., Gould, L.H. and Tauxe, R.V., 2018. Produce-associated foodborne disease outbreaks, USA, 1998–2013. *Epidemiology & Infection*, 146(11), 1397-1406.

Bernstein, N., Sela, S. and Neder-Lavon, S., 2007. Assessment of contamination potential of lettuce by *Salmonella enterica* serovar Newport added to the plant growing medium. *Journal of Food Protection*, 70(7), 1717-1722.

Bezić, N., Skočibušić, M, Dunkić, V and Radonić, A., 2003. Composition and antimicrobial activity of *Achillea clavennae* L. essential oil. *Phytotherapy Research*, 17(9), 1037–1040.
Blaak, H., van Hoek, A.H., Veenman, C., van Leeuwen, A.E.D., Lynch, G., van Overbeek, W.M. and de Roda Husman, A.M., 2014. Extended spectrum *β* -lactamase-and constitutively AmpC-producing *Enterobacteriaceae* on fresh produce and in the agricultural environment. *International Journal of Food Microbiology*, 168, 8–16.
Black-Solis, J., Ventura-Aguilar, R.I., Correa-Pacheco, Z., Corona-Rangel, M.L. and Bautista-Baños, S., 2019. Preharvest use of biodegradable polyester nets added with cinnamon essential oil and the effect on the storage life of tomatoes and the development of *Alternaria alternata*. *Scientia Horticulturae*, 245, 65-73.
Blitstein, J.L., Snider, J. and Evans, W.D., 2012. Perceptions of the food shopping environment are associated with greater consumption of fruits and vegetables. *Public Health Nutrition*, 15(6), 1124-1129.
Boehm, J.K., Soo, J., Zevon, E.S., Chen, Y., Kim, E.S. and Kubzansky, L.D., 2018. Longitudinal associations between psychological well-being and the consumption of fruits and vegetables. *Health Psychology*, 37(10), 959.
Boligon, A.A., Machado, M.M. and Athayde, M.L., 2014. Technical evaluation of antioxidant activity. *Medicinal Chemistry*, 4(7), 517–522.
Bolin, H.R. and Huxsoll, C.C., 1991. Effect of preparation procedures and storage parameters on quality retention of salad-cut lettuce. *Journal of Food Science*. 56, 60–62.
Boqvist, S., Söderqvist, K. and Vågsholm, I., 2018. Food safety challenges and One Health within Europe. *Acta Veterinaria Scandinavica*, 60(1), 1.
Brandl, M.T. and Amundson, R., 2008. Leaf age as a risk factor in contamination of lettuce with *Escherichia coli* O157: H7 and *Salmonella enterica*. *Applied and Environmental Microbiology*, 74(8), 2298-2306.
Brandl, M.T. and Mandrell, R.E., 2002. Fitness of *Salmonella enterica* serovar Thompson in the cilantro phyllosphere. *Applied and Environmental Microbiology*, 68(7), 3614-3621.
Brandl, M.T., Cox, C.E. and Teplitski, M., 2013. *Salmonella* interactions with plants and their associated microbiota. *Phytopathology*, 103(4), 316-325.
Brookie, K.L., Best, G.I. and Conner, T.S., 2018. Intake of raw fruits and vegetables is associated with better mental health than intake of processed fruits and vegetables. *Frontiers in Psychology*, 9, 487.
CAC (Codex Alimentarius Commission), 2003. Code of Hygienic Practice for Fresh Fruits and Vegetables. CXC 53-2003.
Caleb, O.J., Mahajan, P.V., Al-Said, F.A.J. and Opara, U.L., 2013. Modified atmosphere packaging technology of fresh and fresh-cut produce and the microbial consequences — a review. *Food Bioprocess Technology*, 6, 303–329.
Callemien, D. and Collin, S., 2009. Structure, organoleptic properties, quantification methods, and stability of phenolic compounds in beer—A review. *Food Reviews International*, 26(1), 1-84.
Campos, J., Mourão, J., Pestana, N., Peixe, L., Novais, C. and Antunes, P., 2013. Microbiological quality of ready-to-eat salads: an underestimated vehicle of bacteria and clinically relevant antibiotic resistance genes. *International Journal of Food Microbiology*, 166(3), 464–470.
Caponigro, V., Ventura, M., Chiancone, I., Amato, L., Parente, E. and Piro, F., 2010. Variation of microbial load and visual quality of ready-to-eat salads by vegetable type, season, processor and retailer. *Food Microbiology*, 27(8), 1071–1077.
Cardamone, C., Aleo, A., Mammina, C., Oliveri, G., Di Noto and A.M., 2015. Assessment of the microbiological quality of fresh produce on sale in Sicily, Italy: preliminary results. *Journal of Biological Research-Thessaloniki*, 22, 1–6.
Carstens, C., Salazar, J.K. and Darkoh, C., 2019. Multistate outbreaks of foodborne illness in the United States associated with fresh produce from 2010-2017. *Frontiers in Microbiology*, 10, 2667.
Cavaiuolo, M., Cocetta, G., Bulgari, R., Spinardi, A. and Ferrante, A., 2015. Identification of innovative potential quality markers in rocket and melon fresh-cut produce. *Food Chemistry*, 188, 225-233.
Ćavar, S. and Maksimović, M., 2012. Antioxidant activity of essential oil and aqueous extract of *Pelargonium graveolens* L'Her. *Food Control*, 23(1), 263-267.
CDC, 2020. Foodborne outbreaks, Available from https://www.cdc.gov/foodsafety/outbreaks/index.html. Accessed 11 November 2020.

CDC. 2016. Multistate outbreak of listeriosis linked to packaged salads produced at Springfield, Ohio Dole processing facility (Final Update), Available from: https://www.cdc.gov/listeria/outbreaks/bagged-salads-01-16/index.html. Accessed 28 May 2020. Accessed 2 December 2019.

Chau, M.L., Aung, K.T., Hapuarachchi, H.C., Lee, P.S.V., Lim, P.Y., Kang, J.S.L., Ng, Y., Yap, H.M., Yuk, H.G., Gutiérrez, R.A. and Ng, L.C., 2017. Microbial survey of ready-to-eat salad ingredients sold at retail reveals the occurrence and the persistence of *Listeria monocytogenes* Sequence Types 2 and 87 in pre-packed smoked salmon. *BMC Microbiology*, 17 (46), 1–13.

Chen, X., Ren, L., Li, M., Qian, J., Fan, J. and Du, B., 2017. Effects of clove essential oil and eugenol on quality and browning control of fresh-cut lettuce. *Food Chemistry*, 214, 432-439.

Chen, Z., Zhu, C., Zhang, Y., Niu, D. and Du, J., 2010. Effects of aqueous chlorine dioxide treatment on enzymatic browning and shelf-life of fresh-cut asparagus lettuce (*Lactuca sativa* L.). *Postharvest Biology and Technology*, 58, 232-238.

Chlebicz, A. and Śliżewska, K., 2018. Campylobacteriosis, salmonellosis, yersiniosis, and listeriosis as zoonotic foodborne diseases: a review. *International Journal of Environmental Research and Public Health*, 15(5), 863.

Chorianopoulos, N.G., Giaouris, E.D., Skandamis, P.N., Haroutounian, S.A. and Nychas, G.J., 2008. Disinfectant test against monoculture and mixed-culture biofilms composed of technological, spoilage and pathogenic bacteria: bactericidal effect of essential oil and hydrosol of *Satureja thymbra* and comparison with standard acid–base sanitizers. *Journal of Applied Microbiology*, 104(6), 1586-1596.

Christou, A., Maratheftis, G., Eliadou, E., Michael, C., Hapeshi, E. and Fatta-Kassinos, D., 2014. Impact assessment of the reuse of two discrete treated wastewaters for the irrigation of tomato crop on the soil geochemical properties, fruit safety and crop productivity. *Agriculture, Ecosystems and Environment*, 192, 105-114.

Chrysargyris A, Papakyriakou E, Petropoulos S.A. and Tzortzakis N. 2019. The combined and single effect of salinity and copper stress on growth and quality of *Mentha spicata* plants. *Journal of Hazardous Materials*, 368, 584-593.

Chrysargyris, A., Nikou, A. and Tzortzakis, N., 2016b. Effectiveness of *Aloe vera* gel coating for maintaining tomato fruit quality. *New Zealand Journal of Crop and Horticultural Science*, 44(3), 203-217.

Chrysargyris, A., Panayiotou, C. and Tzortzakis, N., 2016a. Nitrogen and phosphorus levels affected plant growth, essential oil composition and antioxidant status of lavender plant (*Lavandula angustifolia* Mill.). *Industrial Crops and Products*, 83, 577-586.

Chrysargyris, A., Xylia, P., Botsaris, G. and Tzortzakis, N., 2017. Antioxidant and antibacterial activities, mineral and essential oil composition of spearmint (*Mentha spicata* L.) affected by the potassium levels. *Industrial Crops and Products*, 103, 202-212.

Cid-Pérez, T.S., Ávila-Sosa, R., Ochoa-Velasco, C.E., Rivera-Chavira, B.E. and Nevárez-Moorillón, G.V., 2019. Antioxidant and antimicrobial activity of Mexican Oregano (*Poliomintha longiflora*) essential oil, hydrosol and extracts from waste solid residues. *Plants*, 8(1), 22.

Cisneros-Zevallos, L., Saltveit, M.E. and Krochta, J.M., 1995. Mechanism of surface discoloration of peeled (minimally processed) carrots during storage. *Journal of Food Science*, 60, 320–323.

Cohen, J.W., 1988. Statistical Power Analysis for the Behavioral Sciences, second ed. Lawrence Erlbaum Associates, Hillsdale, NJ.

Cömert, E.D., Mogol, B.A. and Gökmen, V., 2020. Relationship between color and antioxidant capacity of fruits and vegetables. *Current Research in Food Science*, 2, 1-10.

Cooley, M., Carychao, D., Crawford-Miksza, L., Jay, M.T., Myers, C., Rose, C., Keys, C., Farrar, J. and Mandrell, R.E., 2007. Incidence and tracking of *Escherichia coli* O157: H7 in a major produce production region in California. *PloS one*, 2(11), e1159.

Cooley, M.B., Chao, D. and Mandrell, R.E., 2006. *Escherichia coli* O157: H7 survival and growth on lettuce is altered by the presence of epiphytic bacteria. *Journal of Food Protection*, 69(10), 2329-2335.

Corcoran, M., Morris, D., De Lappe, N., O'Connor, J., Lalor, P., Dockery, P. and Cormican, M., 2014. Commonly used disinfectants fail to eradicate *Salmonella enterica* biofilms from food contact surface materials. *Applied and Environmental Microbiology*, 80(4), 1507–1514.

Coroneo, V., Carraro, V., Marras, B., Marrucci, A., Succa, S., Meloni, B., Pinna, A., Angioni, A., Sanna, A. and Schintu, M., 2017. Presence of trihalomethanes in ready-to-eat vegetables disinfected with chlorine. *Food Additives and Contaminants*: Part A, 34(12), 2111-2117.

Cox, J.M. and Pavic, A., 2014, 'Salmonella. Introduction', in Batt C.A and Tortorello M.L. (eds.), *Encyclopedia of food microbiology*, 2nd ed., Academic Press, London, 322-331.

D'Amato, S., Serio, A., López, C.C. and Paparella, A., 2018. Hydrosols: Biological activity and potential as antimicrobials for food applications. *Food Control*, 86, 126-137.

Dankwa, A.S., Machado, R.M. and Perry, J.J., 2020. Sources of food contamination in a closed hydroponic system. *Letters in Applied Microbiology*, 70, 55-62.

de Azevedo Neto, A.D., Prisco, J.T., Enéas-Filho, J., Abreu, C.E.B. and Gomes-Filho, E., 2006. Effect of salt stress on antioxidative enzymes and lipid peroxidation in leaves and roots of salt-tolerant and salt-sensitive maize genotypes. *Environmental and Experimental Botany*, 56, 87–94.

de Beer, D., Joubert, E., Gelderblom, W.C. and Manley, M., 2005. Antioxidant activity of South African red and white cultivar wines and selected phenolic compounds: In vitro inhibition of microsomal lipid peroxidation. *Food Chemistry*, 90, 569-577.

De Corato, U., 2012. Fungal population dynamics in ready-to-eat salads during a shelf-life in Italy. *Journal of Agricultural Science and Technology*, A2(4A), 569.

De Nisco, N.J., Rivera-Cancel, G. and Orth, K., 2018. The biochemistry of sensing: enteric pathogens regulate type III secretion in response to environmental and host cues. *MBio*, 9(1), e02122-17.

de Oliveira, M.A., De Souza, V.M., Bergamini, A.M.M. and De Martinis, E.C.P., 2011. Microbiological quality of ready-to-eat minimally processed vegetables consumed in Brazil. *Food Control*, 22, 1400–1403.

de Rigal, D., Gauillard, F. and Richard-Forget, F., 2000. Changes in the carotenoid content of apricot (*Prunus armeniaca*, var Bergeron) during enzymatic browning: *β*-carotene inhibition of chlorogenic acid degradation. *Journal of the Science of Food and Agriculture*, 80(6), 763-768.

Deza-Durand, K.M. and Petersen, M.A., 2011. The effect of cutting direction on aroma compounds and respiration rate of fresh-cut iceberg lettuce (*Lactuca sativa* L.). *Postharvest Biology and Technology*, 61, 83-90.

Dinu, L.D. and Bach, S., 2011. Induction of viable but nonculturable *Escherichia coli* O157: H7 in the phyllosphere of lettuce: a food safety risk factor. *Applied and Environmental Microbiology*, 77(23), 8295-8302.

Eady, M. and Park, B., 2019. The influence of environmental growth conditions on *Salmonella* spectra obtained from hyperspectral microscope images. *Food Analytical Methods*, 12, 2638-2646.

Edelenbos, M., Løkke, M.M. and Seefeldt, H.F., 2017. Seasonal variation in color and texture of packaged wild rocket (*Diplotaxis tenuifolia* L.). *Food Packaging and Shelf Life*, 14, 46-51.

EFSA Panel on Biological Hazards (BIOHAZ), 2013. Scientific Opinion on the risk posed by pathogens in food of non-animal origin. Part 1 (outbreak data analysis and risk ranking of food/pathogen combinations). *EFSA Journal*, 11(1), 3025, 1-138.

EFSA Panel on Dietetic Products, Nutrition, and Allergies (NDA), 2010. Scientific Opinion on establishing Food-Based Dietary Guidelines. *EFSA Journal*, 8(3), 1460, 1-42.

El Rayess, Y., Barbar, R., Wilson, E.A. and Bouajila, J., 2014. Analytical methods for wine polyphenols analysis and for their antioxidant activity evaluation. *Wine: Phenolic Composition, Classification and Health Benefits*, 71-101.

Eleftheriadou, M., Varnava-Tello, A., Metta-Loizidou, M., Nikolaou, A.S. and Akkelidou, D., 2002. The microbiological profile of foods in the Republic of Cyprus: 1991–2000. *Food Microbiology*, 19(5), 463–471.

Erickson, M.C., 2012. Internalization of fresh produce by foodborne pathogens. *Annual Review of Food Science and Technology*, 3, 283-310.

Erickson, M.C., Liao, J., Payton, A.S., Riley, D.G., Webb, C.C., Davey, L.E., Kimbrel, S., Ma, L., Zhang, G., Flitcroft, I. and Doyle, M.P., 2010c. Preharvest internalization of *Escherichia coli* O157: H7 into lettuce leaves, as affected by insect and physical damage. *Journal of Food Protection*, 73(10), 1809-1816.

Erickson, M.C., Liao, J.Y., Payton, A.S., Cook, P.W., Den Bakker, H.C., Bautista, J. and Pérez, J.C.D., 2019. Pre-harvest internalization and surface survival of *Salmonella* and *Escherichia coli* O157: H7

sprayed onto different lettuce cultivars under field and growth chamber conditions. *International Journal of Food Microbiology*, 291, 197-204.
Erickson, M.C., Liao, J.Y., Payton, A.S., Cook, P.W., Den Bakker, H.C., Bautista, J. and Pérez, J.C.D., 2018. Fate of enteric pathogens in different spinach cultivars cultivated in growth chamber and field systems. *Food Quality and Safety*, 2(4), 221-228.
Erickson, M.C., Webb, C.C., Diaz-Perez, J.C., Davey, L.E., Payton, A.S., Flitcroft, I.D., Phatak, S.C. and Doyle, M.P., 2014. Absence of internalization of *Escherichia coli* O157: H7 into germinating tissue of field-grown leafy greens. *Journal of Food Protection*, 77(2), 189-196.
Erickson, M.C., Webb, C.C., Diaz-Perez, J.C., Phatak, S.C., Silvoy, J.J., Davey, L., Payton, A.S., Liao, J., Ma, L. and Doyle, M.P., 2010a. Infrequent internalization of *Escherichia coli* O157: H7 into field-grown leafy greens. *Journal of Food Protection*, 73(3), 500-506.
Erickson, M.C., Webb, C.C., Diaz-Perez, J.C., Phatak, S.C., Silvoy, J.J., Davey, L., Payton, A.S., Liao, J., Ma, L. and Doyle, M.P., 2010b. Surface and internalized *Escherichia coli* O157: H7 on field-grown spinach and lettuce treated with spray-contaminated irrigation water. *Journal of Food Protection*, 73(6), 1023-1029.
European Food Safety Authority, 2011. Urgent advice on the public health risk of Shiga toxin producing. *European Food Safety Authority Journal,* 9 (6), 1–50, 2274.
Eurostat. 2016. Consumption of fruit and vegetables in the EU, *Eurostat Press Office*.
FAO/WHO Food and Agriculture Organization of the United Nations/World Health Organization, 2019. Sustainable healthy diets: guiding principles. Rome.
FAO/WHO-Food and Agriculture Organization of the United Nations/World Health Organization, 2008. Microbiological hazards in fresh leafy vegetables and herbs. Meeting report, Rome: Microbiological Risk Assessment Series No. 14.
FAO-Food and Agriculture Organization of the United Nations, 2015. Promotion of fruit and vegetables for health. Report of the Pacific regional workshop, Rome, 1-94.
Faour-Klingbeil, D., Murtada, M., Kuri, V. and Todd, E.C., 2016b. Understanding the routes of contamination of ready-to-eat vegetables in the Middle East. *Food Control*, 62, 125–133.
Faour-Klingbeil, D., Todd, E.C. and Kuri, V., 2016a. Microbiological quality of ready-to-eat fresh vegetables and their link to food safety environment and handling practices in restaurants. LWT - *Food Science and Technology*, 74, 224–233.
Farber, J.M. and Peterkin, P.I., 1991. *Listeria monocytogenes*, a food-borne pathogen. *Microbiology and Molecular Biology Reviews*, 55 (3), 476–511.
Favell, D.J., 1998. A comparison of the vitamin C content of fresh and frozen vegetables. *Food Chemistry*, 62(1), 59–64.
FDA, 2018. FDA Investigated multistate outbreak of *E. coli* O157:H7 infections linked to romaine lettuce from Yuma growing region. Available from: https://www.fda.gov/food/outbreaks-foodborne-illness/fda-investigated-multistate-outbreak-e-coli-o157h7-infections-linked-romaine-lettuce-yuma-growing#:~:text=On%20April%2019%2C%202018%2C%20Alaska,from%20the%20Yuma%20growing%20region. Accessed 28 May 2020.
FDA, 2020. Outbreak Investigation of *E. coli* O157:H7: Unknown Food (Fall 2020): Romaine sample from Michigan tests positive for *E. coli*, potentially linked to illnesses. Available from: https://www.fda.gov/food/outbreaks-foodborne-illness/outbreak-investigation-e-coli-o157h7-unknown-food-fall-2020#:~:text=Recall%20Information-,On%20November%206%2C%202020%2C%20Tanimura%20%26%20Antle%2C%20Inc.,%2D27918%2D20314%2D9. Accessed 11 November 2020.
Ferrante, A., Martinetti, L. and Maggiore, T., 2009. Biochemical changes in cut vs. intact lamb's lettuce (*Valerianella olitoria*) leaves during storage. *International Journal of Food Science and Technology*, 44, 1050-1056.
Filho, A.F.M., de Azevedo, C.A.V., de Queiroz Almeida Azevedo, M.R., Fernandes, J.D., Correa, E.B. and dos Santos, S.A., 2018. Microbiological and parasitological contamination of hydroponic grown curly lettuce under different optimized nutrient solutions. *Australian Journal of Crop Science*, 12(3), 400.
Food and Agriculture Organization of the United Nations (FAO) (2009) How to feed the world in 2050 Office of the Director, agricultural development economics division economic and social

development department Viale delle Terme di Caracalla 00153 Rome, Italy. 35 pp. http://www.fao.org/fileadmin/templates/wsfs/docs/expert_paper/How_to_Feed_the_World_in_2050.pdf. Accessed 29 October 2020.

Food and Agriculture Organization of the United Nations, 2015. Promotion of Fruit and Vegetables for Health. Report of the Pacific regional workshop, Rome.

Francis, G.A, Thomas, C. and O'Beirne, D., 1999. The microbiological safety of minimally processed vegetables. *International Journal of Food Science and Technology*, 34(1), 1–22.

Franz, E., Visser, A.A., Van Diepeningen, A.D., Klerks, M.M., Termorshuizen, A.J. and van Bruggen, A.H., 2007. Quantification of contamination of lettuce by GFP-expressing *Escherichia coli* O157: H7 and *Salmonella enterica* serovar Typhimurium. *Food Microbiology*, 24(1), 106-112.

Fröder, H., Martins, C.G., De Souza, K.L.O., Landgraf, M., Franco, B.D. and Destro, M.T., 2007. Minimally processed vegetable salads: microbial quality evaluation. *Journal of Food Protection*, 70(5), 1277-1280.

García, R., Bælum, J., Fredslund, L., Santorum, P. and Jacobsen, C.S., 2010. Influence of temperature and predation on survival of *Salmonella enterica* serovar Typhimurium and expression of *inv*A in soil and manure-amended soil. *Applied and Environmental Microbiology*, 76(15), 5025-5031.

Giusti, M.D., Solimini, A.G., Cottarelli, A., Vito, C.D., Aurigemma, C., Tufi, D., Piccinato, L., Boccia, A. and Marinelli, L., 2014. Temporal pattern of microbial indicators of ready-to-eat rocket salads during shelf life. *Annali dell'Istituto Superiore di Sanità*, 50, 90-95.

Goñi, M.G., Tomadoni, B., Roura, S.I. and Moreira, M.D.R., 2014. Effect of preharvest application of chitosan and tea tree essential oil on postharvest evolution of lettuce native microflora and exogenous *Escherichia coli* O 157: H 7. *Journal of Food Safety*, 34, 353-360.

Gorny, J.R., Giclas, H., Gombas, D. and Means, K., 2006. Commodity specific food safety guidelines for the lettuce and leafy greens supply chain, (April), 1-39.

Goudeau, D.M., Parker, C.T., Zhou, Y., Sela, S., Kroupitski, Y. and Brandl, M.T., 2013. The Salmonella transcriptome in lettuce and cilantro soft rot reveals a niche overlap with the animal host intestine. *Applied and Environmental Microbiology*, 79(1), 250-262.

Gross, J., 1991. *Pigments in vegetables*. New York: AVI, Van Nostrand Reinhold.

Gu, G., Strawn, L.K., Zheng, J., Reed, E.A. and Rideout, S.L., 2019. Diversity and dynamics of *Salmonella enterica* in water sources, poultry litters, and field soils amended with poultry litter in a major agricultural area of Virginia. *Frontiers in Microbiology*, 10, 2868.

Gündüz, G.T., Gönül, Ş.A. and Karapınar, M., 2010. Efficacy of oregano oil in the inactivation of *Salmonella* Typhimurium on lettuce. *Food Control*, 21, 513-517.

Gupta, D. and Madramootoo, C.A., 2017. *Escherichia coli* contamination on ready-to-eat (RTE), lettuce. *Exposure and Health*, 9(4), 249-259.

Gurler, Z., Pamuk, S., Yildirim, Y. and Ertas, N., 2015. The microbiological quality of ready-to- eat salads in Turkey: a focus on *Salmonella* spp. and *Listeria monocytogenes*. *International Journal of Food Microbiology*, 196, 79–83.

Gutiérrez-Rodríguez, E., Gundersen, A., Sbodio, A.O. and Suslow, T.V., 2012. Variable agronomic practices, cultivar, strain source and initial contamination dose differentially affect survival of *Escherichia coli* on spinach. *Journal of Applied Microbiology*, 112(1), 109-118.

Hamanaka, D. and Izumi, H., 2008. Combined effect of mustard and hop extract agents with emulsifier on microbial quality and physiology of fresh-cut vegetables. *Food Science and Technology Research*, 14(6), 565-565.

Han, C., Li, J., Jin, P., Li, X., Wang, L. and Zheng, Y., 2017. The effect of temperature on phenolic content in wounded carrots. *Food Chemistry*, 215, 116-123.

Hara-Kudo, Y., Konuma, H., Iwaki, M., Kasuga, F., Sugita-Konishi, Y., Ito, Y. and Kumagai, S., 1997. Potential hazard of radish sprouts as a vehicle of *Escherichia coli* O157: H7. *Journal of Food Protection*, 60(9), 1125-1127.

Health Protection Agency, 2009. Guidelines for Assessing the Microbiological Safety of Ready-To-Eat Foods. Health Protection Agency, London November 2009.

Heath, R.L. and Packer, L., 1968. Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives of Biochemistry and Biophysics*, 125, 189-198.

Henriquez, T., Lenzi, A., Baldi, A. and Marvasi, M., 2020. Frontiers in plant breeding: Perspectives for the selection of vegetables less susceptible to enteric pathogens. *Frontiers in Microbiology*, 11, 1087.

Hernández-Reyes, C. and Schikora, A., 2013. *Salmonella*, a cross-kingdom pathogen infecting humans and plants. *FEMS Microbiology Letters*, 343(1), 1-7.

Hintz, L.D., Boyer, R.R., Ponder, M.A., Williams, R.C. and Rideout, S.L., 2010. Recovery of *Salmonella enterica* Newport introduced through irrigation water from tomato (*Lycopersicum esculentum*) fruit, roots, stems, and leaves. *HortScience*, 45(4), 675-678.

Hirneisen, K.A., Sharma, M. and Kniel, K.E., 2012. Human enteric pathogen internalization by root uptake into food crops. *Foodborne Pathogens and Disease*, 9(5), 396-405.

Hodges, D.M. and Toivonen, P.M., 2008. Quality of fresh-cut fruits and vegetables as affected by exposure to abiotic stress. *Postharvest Biology and Technology*, 48(2), 155-162.

Hodges, D.M., 2003. Overview: oxidative stress and postharvest produce. In: Hodges, D.M. (Ed.), Postharvest Oxidative Stress in Horticultural Crops. The Haworth Press Inc., Binghamton, New York, pp. 1–12.

Hodges, D.M., Lester, G.E., Munro, K.D. and Toivonen, P.T.A., 2004. Oxidative stress: importance for postharvest quality. *HortScience*, 39(5), 924-929.

Honjoh, K.I., Mishima, T., Kido, N., Shimamoto, M. and Miyamoto, T., 2014. Investigation of routes of *Salmonella* contamination via soils and the use of mulch for contamination control during lettuce cultivation. *Food Science and Technology Research*, 20(5), 961-969.

Hora, R., Warriner, K., Shelp, B.J. and Griffiths, M.W., 2005. Internalization of *Escherichia coli* O157: H7 following biological and mechanical disruption of growing spinach plants. *Journal of Food Protection*, 68(12), 2506-2509.

Hou, Z., Fink, R.C., Sugawara, M., Diez-Gonzalez, F. and Sadowsky, M.J., 2013. Transcriptional and functional responses of *Escherichia coli* O157:H7 growing in the lettuce rhizoplane. *Food Microbiology*, 35(2), 136-142.

Hunter, P.J., Atkinson, L.D., Vickers, L., Lignou, S., Oruna-Concha, M.J., Pink, D., Hand, P., Barker, G., Wagstaff, C. and Monaghan, J.M., 2017. Oxidative discolouration in whole-head and cut lettuce: biochemical and environmental influences on a complex phenotype and potential breeding strategies to improve shelf-life. *Euphytica*, 213, 180.

Huskisson, E., Maggini, S and, Ruf, M., 2007. The role of vitamins and minerals in energy metabolism and well-being. *Journal of International Medical Research*, 35(3), 277-289.

Hyodo, H., Kuroda, H. and Yang, S.F., 1978. Induction of phenylalanine ammonia-lyase and increase in phenolics in lettuce leaves in relation to the development of russet spotting caused by ethylene. *Plant Physiology*, 62, 31-35.

Hyun, J.-E., Bae, Y.-M., Song, H., Yoon, J.-H. and Lee, S.-Y., 2015b. Antibacterial effect of various essential oils against pathogens and spoilage microorganisms in fresh produce. *Journal of Food Safety*, 35, 206–219.

Hyun, J.-E., Bae, Y.-M., Yoon, J.H. and Lee, S.Y., 2015a. Preservative effectiveness of essential oils in vapor phase combined with modified atmosphere packaging against spoilage bacteria on fresh cabbage. *Food Control*, 51, 307–313.

Islam, M., Doyle, M.P., Phatak, S.C., Millner, P. and Jiang, X., 2004. Persistence of enterohemorrhagic *Escherichia coli* O157: H7 in soil and on leaf lettuce and parsley grown in fields treated with contaminated manure composts or irrigation water. *Journal of Food Protection*, 67(7), 1365-1370.

Itoh, Y., Sugita-Konishi, Y., Kasuga, F., Iwaki, M., Hara-Kudo, Y., Saito, N., Noguchi, Y., Konuma, H. and Kumagai, S., 1998. Enterohemorrhagic *Escherichia coli* O157: H7 present in radish sprouts. *Applied and Environmental Microbiology*, 64(4), 1532-1535.

Iwu, C.D. and Okoh, A.I., 2019. Preharvest transmission routes of fresh produce associated bacterial pathogens with outbreak potentials: A review. *International Journal of Environmental Research and Public Health*, 16(22), 4407.

Jablasone, J., Warriner, K. and Griffiths, M., 2005. Interactions of *Escherichia coli* O157: H7, *Salmonella* Typhimurium and *Listeria monocytogenes* plants cultivated in a gnotobiotic system. *International Journal of Food Microbiology*, 99(1), 7-18.

Jacob, C. and Melotto, M., 2019. Human pathogen colonization of lettuce dependent upon plant genotype and defense response activation. *Frontiers in Plant Science*, 10, 1769.

Jacxsens, L., Devlieghere, F. and Debevere, J., 2002. Predictive modelling for packaging design: equilibrium modified atmosphere packages of fresh-cut vegetables subjected to a simulated distribution chain. *International Journal of Food Microbiology*, 73, 331-341.

Jeddi, M.Z., Yunesian, M., Gorji, M.E.H., Noori, N., Pourmand, M.R. and Khaniki, G.R.J., 2014. Microbial evaluation of fresh, minimally-processed vegetables and bagged sprouts from chain supermarkets. *Journal of Health, Population and Nutrition*, 32(3), 391–399.

Jensen, A.N., Storm, C., Forslund, A., Baggesen, D.L. and Dalsgaard, A., 2013. *Escherichia coli* contamination of lettuce grown in soils amended with animal slurry. *Journal of Food Protection*, 76(7), 1137-1144.

Johannessen, G.S., Bengtsson, G.B., Heier, B.T., Bredholt, S., Wasteson, Y. and Rørvik, L.M., 2005. Potential uptake of *Escherichia coli* O157: H7 from organic manure into crisphead lettuce. *Applied and Environmental Microbiology*, 71(5), 2221-2225.

Jordan, R.A., Ribeiro, E.F., Oliveira, F.C.D., Geisenhoff, L.O. and Martins, E.A., 2018. Yield of lettuce grown in hydroponic and aquaponic systems using different substrates. *Revista Brasileira de Engenharia Agrícola e Ambiental*, 22(8), 525-529.

Joshi, K., Mahendran, R., Alagusundaram, K., Norton, T. and Tiwari, B.K., 2013. Novel disinfectants for fresh produce. *Trends in Food Science and Technology*, 34, 54–61.

Junglee, S., Urban, L., Sallanon, H. and Lopez-Lauri, F., 2014. Optimized assay for hydrogen peroxide determination in plant tissue using potassium iodide. *American Journal of Analytical Chemistry*, 5, 730-736.

Kang, H.M. and Saltveit, M.E., 2002. Antioxidant capacity of lettuce leaf tissue increases after wounding. *Journal of Agricultural and Food Chemistry*, 50(26), 7536-7541.

Kaplan, B.J., Crawford, S.G., Field, C.J. and Simpson, J.S.A., 2007. Vitamins, minerals, and mood. *Psychological Bulletin*, 133(5), 747.

Kim, D.H., Kim, H.B., Chung, H.S. and Moon, K.D., 2014. Browning control of fresh-cut lettuce by phytoncide treatment. *Food Chemistry*, 159, 188-192.

Kim, H.S., Chon, J.W., Kim, Y.J., Kim, D.H., Kim, M.S. and Seo, K.H., 2015. Prevalence and characterization of extended-spectrum-*β*-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in ready-to-eat vegetables. *International Journal of Food Microbiology*, 207(17), 83–86.

Kim, M.J., Moon, Y., Tou, J.C., Mou, B. and Waterland, N.L., 2016. Nutritional value, bioactive compounds and health benefits of lettuce (*Lactuca sativa* L.). *Journal of Food Composition and Analysis*, 49, 19-34.

Klerks, M.M., Franz, E., van Gent-Pelzer, M., Zijlstra, C. and Van Bruggen, A.H., 2007a. Differential interaction of *Salmonella enterica* serovars with lettuce cultivars and plant-microbe factors influencing the colonization efficiency. *The ISME Journal*, 1(7), 620-631.

Klerks, M.M., van Gent-Pelzer, M., Franz, E., Zijlstra, C. and Van Bruggen, A.H.C., 2007b. Physiological and molecular responses of *Lactuca sativa* to colonization by *Salmonella enterica* serovar Dublin. *Applied and Environmental Microbiology*, 73(15), 4905-4914.

Koca, N. and Karadeniz, F., 2008. Changes of bioactive compounds and anti-oxidant activity during cold storage of carrots. *International Journal of Food Science and Technology*, 43, 2019–2025.

Korir, R.C., Parveen, S., Hashem, F. and Bowers, J., 2016. Microbiological quality of fresh produce obtained from retail stores on the Eastern Shore of Maryland, United States of America. *Food Microbiology*, 56, 29–34.

Koseki, S., Mizuno, Y. and Yamamoto, K., 2011. Comparison of two possible routes of pathogen contamination of spinach leaves in a hydroponic cultivation system. *Journal of Food Protection*, 74(9), 1536-1542.

Koukounaras, A., Siomos, A.S., Gerasopoulos, D. and Papachristodoulou, M., 2019. Active modified atmosphere package induced a new physiological disorder of minimally processed romaine lettuce leaves. *Food Packaging and Shelf Life*, 22, 100411.

Kyere, E.O., Foong, G., Palmer, J., Wargent, J.J., Fletcher, G.C. and Flint, S., 2019. Rapid attachment of *Listeria monocytogenes* to hydroponic and soil grown lettuce leaves. *Food Control*, 101, 77-80.

Landi, L., De Miccolis Angelini, R.M., Pollastro, S., Feliziani, E., Faretra, F. and Romanazzi, G., 2017. Global transcriptome analysis and identification of differentially expressed genes in strawberry after preharvest application of benzothiadiazole and chitosan. *Frontiers in Plant Science*, 8, 235.

Lapidot, A. and Yaron, S., 2009. Transfer of *Salmonella enterica* serovar Typhimurium from contaminated irrigation water to parsley is dependent on curli and cellulose, the biofilm matrix components. *Journal of Food Protection*, 72(3), 618-623.

Lavelli, V., Pagliarini, E., Ambrosoli, R., Minati, J.L. and Zanoni, B., 2006. Physicochemical, microbial, and sensory parameters as indices to evaluate the quality of minimally-processed carrots. *Postharvest Biology and Technology*, 40, 34-40.

Lee, D.H., Kim, J.B., Kim, M., Roh, E., Jung, K., Choi, M., Oh, C., Choi, J., Yun, J. and Heu, S., 2013. Microbiota on spoiled vegetables and their characterization. *Journal of. Food Protection*, 76(8), 1350–1358.

Lee, S. and Lee, J., 2015. Beneficial bacteria and fungi in hydroponic systems: Types and characteristics of hydroponic food production methods. *Scientia Horticulturae*, 195, 206-215.

Leistner, L., 2000. Basic aspects of food preservation by hurdle technology. *International Journal of Food Microbiology*, 55(1-3), 181-186.

Lenzi, A., Marvasi, M. and Baldi, A., 2021. Agronomic practices to limit pre-and post-harvest contamination and proliferation of human pathogenic *Enterobacteriaceae* in vegetable produce. *Food Control*, 107486.

Li, D. and Uyttendaele, M., 2018. Potential of human norovirus surrogates and *Salmonella enterica* contamination of pre-harvest basil (*Ocimum basilicum*) via leaf surface and plant substrate. *Frontiers in Microbiology*, 9, 1728.

Ligor, M., Olszowy, P. and Buszewski, B., 2012. Application of medical and analytical methods in *Lyme borreliosis* monitoring. *Analytical and Bioanalytical Chemistry*, 402(7), 2233-2248.

Lim, J.A., Lee, D.H. and Heu, S., 2014. The interaction of human enteric pathogens with plants. *The Plant Pathology Journal*, 30(2), 109-116.

Lis-Balchin, M., Deans, S.G. and Eaglesham, E., 1998. Relationship between bioactivity and chemical composition of commercial essential oils. *Flavour and Fragrance Journal*, 13, 98-104.

Lisiewska, Z., Gębczyński, P., Bernaś, E. and Kmiecik, W., 2009. Retention of mineral constituents in frozen leafy vegetables prepared for consumption. *Journal of Food Composition and Analysis*, 22(3), 218-223.

Little, C.L., Taylor, F.C., Sagoo, S.K., Gillespie, I.A., Grant, K. and McLauchlin, J., 2007. Prevalence and level of *Listeria monocytogenes* and other Listeria species in retail prepackaged mixed vegetable salads in the UK. *Food Microbiology*, 24(7-8), 711–717.

Liu, D., Cui, Y., Walcott, R., Díaz-Pérez, J., Tishchenko, V. and Chen, J., 2019. Transmission of human enteric pathogens from artificially-inoculated flowers to vegetable sprouts/seedlings developed via contaminated seeds. *Food Control*, 99, 21-27.

Lopez-Galvez, F., Allende, A., Pedrero-Salcedo, F., Alarcon, J.J. and Gil, M.I., 2014. Safety assessment of greenhouse hydroponic tomatoes irrigated with reclaimed and surface water. *International Journal of Food Microbiology*, 191, 97-102.

Loreto, F. and Velikova, V., 2001. Isoprene produced by leaves protects the photosynthetic apparatus against ozone damage, quenches ozone products, and reduces lipid peroxidation of cellular membranes. *Plant Physiology*. 127, 1781–1787.

Losio, M.N., Pavoni, E., Bilei, S., Bertasi, B., Bove, D., Capuano, F., Farneti, S., Blasi, G., Comin, D., Cardamone, C. and Decastelli, L., 2015. Microbiological survey of raw and ready-to-eat leafy green vegetables marketed in Italy. *International Journal of Food Microbiology*, 210, 88–91.

Ma, B.X., Ban, X.Q., He, J.S., Huang, B., Zeng, H., Tian, J., Chen, Y.X. and Wang, Y.W., 2016. Antifungal activity of *Ziziphora clinopodioides* Lam. essential oil against *Sclerotinia sclerotiorum* on rapeseed plants (*Brassica campestris* L.). *Crop Protection*, 89, 289-295.

Macarisin, D., Patel, J. and Sharma, V.K., 2014. Role of curli and plant cultivation conditions on *Escherichia coli* O157: H7 internalization into spinach grown on hydroponics and in soil. *International Journal of Food Microbiology*, 173, 48-53.

Machado-Moreira, B., Richards, K., Brennan, F., Abram, F. and Burgess, C.M., 2019. Microbial contamination of fresh produce: What, Where, and How?. *Comprehensive Reviews in Food Science and Food Safety*, 18(6), 1727-1750.

Malorny, B., Hoorfar, J., Bunge, C. and Helmuth, R., 2003. Multicenter validation of the analytical accuracy of Salmonella PCR: towards an international standard. *Applied Environmental Microbiology*, 69, 290–296.

Manios, S.G., Konstantinidis, N., Gounadaki, A.S. and Skandamis, P.N., 2013. Dynamics of low (1–4 cells) vs high populations of *Listeria monocytogenes* and *Salmonella* Typhimurium in fresh-cut salads and their sterile liquid or solidified extracts. *Food Control*, 29(2), 318–327.

Manos, D.P. and Xydis, G., 2019. Hydroponics: are we moving towards that direction only because of the environment? A discussion on forecasting and a systems review. *Environmental Science and Pollution Research*, 26, 12662-12672.

Marcussen, H., Holm, P.E., Ha, L.T. and Dalsgaard, A., 2007. Food safety aspects of toxic element accumulation in fish from wastewater-fed ponds in Hanoi, Vietnam. *Tropical Medicine and International Health*, 12, 34-39.

Martín-Diana, A.B., Rico, D. and Barry-Ryan, C., 2008. Green tea extract as a natural antioxidant to extend the shelf-life of fresh-cut lettuce. *Innovative Food Science and Emerging Technologies*, 9(4), 593-603.

Martin-Diana, A.B., Rico, D., Frias, J., Mulcahy, J., Henehan, G.T.M. and Barry-Ryan, C., 2006. Whey permeate as a bio-preservative for shelf life maintenance of fresh-cut vegetables. *Innovative Food Science and Emerging Technologies*, 7(1-2), 112-123.

Martínez-Romero, D., Serrano, M., Bailén, G., Guillén, F., Zapata, P.J., Valverde, J.M., Castillo, S., Fuentes, M. and Valero, D., 2008. The use of a natural fungicide as an alternative to preharvest synthetic fungicide treatments to control lettuce deterioration during postharvest storage. *Postharvest Biology and Technology*, 47(1), 4-60.

Martínez-Vaz, B.M., Fink, R.C., Diez-Gonzalez, F. and Sadowsky, M.J., 2014. Enteric pathogen-plant interactions: molecular connections leading to colonization and growth and implications for food safety. *Microbes and Environments*, ME13139.

Masia, A., 2003. Physiological effects of oxidative stress in relation to ethylene in postharvest produce. In: Hodges, D.M. (Ed.), Postharvest Oxidative Stress in Horticultural Crops. The Haworth Press Inc., Binghamton, New York, pp. 165–197.

Mastrocola, D. and Lerici, C.R., 1991. Colorimetric measurements of enzymatic and non enzymatic browning in apple purees. *Italian Journal of Food Science*, 3, 219–229.

McDowell, D. Maloney, M., Swan, L. and Erwin, P., 2007. A Review of the Fruit and Vegetable Food Chain., 1–102.

McGuire, R.G., 1992. Reporting of objective color measurements. *HortScience*, 27, 1254–1255.

Mei Soon, J., Manning, L., Paul Davies, W. and Baines, R., 2012. Fresh produce-associated outbreaks: a call for HACCP on farms? *British Food Journal*, 114 (4), 553-597.

Meng J. and Schroeder, C.M., 2007. '*Escherichia coli*', in Simjee S. (ed.), *Foodborne diseases*, Humana Press Inc., New Jersey, 1-25.

Miles, A.A., Misra, S.S. and Irwin, J.O., 1938. The estimation of the bactericidal power of the blood. *Epidemiology and Infection*, 38(6), 732-749.

Miller, S.R. and Knudson, W.A., 2014. Nutrition and cost comparisons of select canned, frozen, and fresh fruits and vegetables. *American Journal of Lifestyle Medicine*, 8(6), 430-437.

Mitra, R., Cuesta-Alonso, E., Wayadande, A., Talley, J., Gilliland, S. and Fletcher, J., 2009. Effect of route of introduction and host cultivar on the colonization, internalization, and movement of the human pathogen *Escherichia coli* O157: H7 in spinach. *Journal of Food Protection*, 72(7), 1521-1530.

Moon, J.K. and Shibamoto, T., 2009. Antioxidant assays for plant and food components. *Journal of Agricultural and Food Chemistry*, 57(5), 1655-1666.

Mootian, G., Wu, W.H. and Matthews, K.R., 2009. Transfer of *Escherichia coli* O157: H7 from soil, water, and manure contaminated with low numbers of the pathogen to lettuce plants. *Journal of Food Protection*, 72(11), 2308-2312.

Moreira, M.D.R., Ponce, A.G., del Valle, C.E., Ansorena, R. and Roura, S.I., 2006. Effects of abusive temperatures on the postharvest quality of lettuce leaves: ascorbic acid loss and microbial growth. *Journal of Applied Horticulture*, 8(2), 109-113.

Moriarty, M.J., Semmens, K., Bissonnette, G.K. and Jaczynski, J., 2019. Internalization assessment of *E. coli* O157: H7 in hydroponically grown lettuce. *LWT-Food Science and Technology*, 100, 183-188.

Mouatcho, J.C., Tzortzakis, N., Soundy, P. and Sivakumar, D., 2017. Bio-sanitation treatment using essential oils against *E. coli* O157: H7 on fresh lettuce. *New Zealand Journal of Crop and Horticultural Science*, 45(3), 165-174.

Moyne, A.L., Sudarshana, M.R., Blessington, T., Koike, S.T., Cahn, M.D. and Harris, L.J., 2011. Fate of *Escherichia coli* O157: H7 in field-inoculated lettuce. *Food Microbiology*, 28(8), 1417-1425.

Murphy, S., Gaffney, M.T., Fanning, S. and Burgess, C.M., 2016. Potential for transfer of *Escherichia coli* O157: H7, *Listeria monocytogenes* and *Salmonella* Senftenberg from contaminated food waste derived compost and anaerobic digestate liquid to lettuce plants. *Food Microbiology*, 59, 7-13.

Nair, M.S., Tomar, M., Punia, S., Kukula-Koch, W. and Kumar, M., 2020. Enhancing the functionality of chitosan-and alginate-based active edible coatings/films for the preservation of fruits and vegetables: A review. *International Journal of Biological Macromolecules*, 164(1), 304-320.

Nicholson, A.M., Gurtler, J.B., Bailey, R.B., Niemira, B.A. and Douds, D.D., 2015. Influence of mycorrhizal fungi on fate of *E. coli* O157: H7 and *Salmonella* in soil and internalization into Romaine lettuce plants. *International Journal of Food Microbiology*, 192, 95-102.

Nicolle, C., Simon, G., Rock, E., Amouroux, P. and Rémésy, C., 2004. Genetic variability influences carotenoid, vitamin, phenolic, and mineral content in white, yellow, purple, orange, and dark-orange carrot cultivars. *Journal of the American Society for Horticultural Science*, 129, 523-529.

Niemira, B.A., 2007. Relative efficacy of sodium hypochlorite wash versus irradiation to inactivate *Escherichia coli* O157:H7 internalized in leaves of romaine lettuce and baby spinach. *Journal of Food Protection*, 70(11), 2526–2532.

Nousiainen, L.L., Joutsen, S., Lunden, J., Hänninen, M.L. and Fredriksson-Ahomaa, M., 2016. Bacterial quality and safety of packaged fresh leafy vegetables at the retail level in Finland. *International Journal of Food Microbiology*, 232, 73–79.

Nüesch-Inderbinen, M., Zurfluh, K., Peterhans, S., Haechler, H. and Stephan, R., 2015. Assessment of the prevalence of extended-spectrum β-lactamase–producing *Enterobacteriaceae* in ready-to-eat salads, fresh-cut fruit, and sprouts from the Swiss market. *Journal of Food Protection*, 78 (6), 1178–1181.

Oliveira, M., Usall, J., Viñas, I., Solsona, C. and Abadias, M., 2011. Transfer of *Listeria innocua* from contaminated compost and irrigation water to lettuce leaves. *Food Microbiology*, 28(3), 590-596.

Oliveira, M., Vi, I., Usall, J., Anguera, M. and Abadias, M., 2012. Presence and survival of *Escherichia coli* O157: H7 on lettuce leaves and in soil treated with contaminated compost and irrigation water. *International Journal of Food Microbiology*, 156(2), 133-140.

Ölmez, H. and Temur, S.D., 2010. Effects of different sanitizing treatments on biofilms and attachment of *Escherichia coli* and *Listeria monocytogenes* on green leaf lettuce. *LWT-Food Science and Technology*, 43(6), 964-970.

Ongeng, D., Muyanja, C., Ryckeboer, J., Geeraerd, A.H. and Springael, D., 2011a. Rhizosphere effect on survival of *Escherichia coli* O157: H7 and *Salmonella enterica* serovar Typhimurium in manure-amended soil during cabbage (*Brassica oleracea*) cultivation under tropical field conditions in Sub-Saharan Africa. *International Journal of Food Microbiology*, 149(2), 133-142.

Ongeng, D., Vasquez, G.A., Muyanja, C., Ryckeboer, J., Geeraerd, A.H. and Springael, D., 2011b. Transfer and internalization of *Escherichia coli* O157: H7 and *Salmonella enterica* serovar Typhimurium in cabbage cultivated on contaminated manure-amended soil under tropical field conditions in Sub-Saharan Africa. *International Journal of Food Microbiology*, 145(1), 301-310.

Orozco, L., Rico-Romero, L. and Escartin, E.F., 2008. Microbiological profile of greenhouses in a farm producing hydroponic tomatoes. *Journal of Food Protection*, 71(1), 60-65.

Ozturk, I., Tornuk, F., Caliskan-Aydogan, O., Durak, M.Z. and Sagdic, O., 2016. Decontamination of iceberg lettuce by some plant hydrosols. *LWT-Food Science and Technology*, 74, 48-54.

Pablos, C., Fernández, A., Thackeray, A. and Marugán, J., 2017. Effects of natural antimicrobials on prevention and reduction of bacterial cross-contamination during the washing of ready-to-eat fresh-cut lettuce. *Food Science and Technology International*, 23(5), 403-414.

Pachepsky, Y., Shelton, D.R., McLain, J.E., Patel, J. and Mandrell, R.E., 2011. Irrigation waters as a source of pathogenic microorganisms in produce: A review. In *Advances in Agronomy*. Academic Press, London, 113, 75-141.

Parish, M.E., Beuchat, Suslow, T.V., L.R., Harris, L.J., Garrett, E.H., Farber, J.N. and Busta, F.F., 2003. Methods to reduce/eliminate pathogens from fresh and fresh-cut produce. *Comprehensive Reviews in Food Science and Food Safety*, 2, 161–173.

Park, S., Szonyi, B., Gautam, R., Nightingale, K., Anciso, J. and Ivanek, R., 2012. Risk factors for microbial contamination in fruits and vegetables at the preharvest level: a systematic review. *Journal of Food Protection*, 75(11), 2055–2081.

Park, S.H., Choi, M.R., Park, J.W., Park, K.H., Chung, M.S., Ryu, S. and Kang, D.H., 2011. Use of organic acids to inactivate *Escherichia coli* O157: H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* on organic fresh apples and lettuce. *Journal of Food Science*, 76(6), M293-M298.

Péneau, S., Brockhoff, P.B., Escher, F. and Nuessli, J., 2007. A comprehensive approach to evaluate the freshness of strawberries and carrots. *Postharvest Biology and Technology*, 45(1), 20-29.

Pereira, M.A., O'Reilly, E., Augustsson, K., Fraser, G.E., Goldbourt, U., Heitmann, B.L., Hallmans, G., Knekt, P., Liu, S., Pietinen, P. and Spiegelman, D., 2004. Dietary fiber and risk of coronary heart disease: a pooled analysis of cohort studies. *Archives of Internal Medicine*, 164(4), 370-376.

Picouet, P.A., Sárraga, C., Cofán, S., Belletti, N. and Guàrdia, M.D., 2015. Effects of thermal and high-pressure treatments on the carotene content, microbiological safety and sensory properties of acidified and of non-acidified carrot juice. *LWT-Food Science and Technology*, 62, 920–926.

Pieterse, C.M. and Dicke, M., 2007. Plant interactions with microbes and insects: from molecular mechanisms to ecology. *Trends in Plant Science*, 12(12), 564-569.

Poimenidou, S.V., Chatzithoma, D.N., Nychas, G.J. and Skandamis, P.N., 2016. Adaptive response of *Listeria monocytogenes* to heat, salinity and low pH, after habituation on cherry tomatoes and lettuce leaves. *PLoS One*, 11 (10), e0165746.

Ponce, A., Roura, S.I. and Moreira, M.D.R., 2011. Essential oils as biopreservatives: different methods for the technological application in lettuce leaves. *Journal of Food Science*, 76(1), M34-M40.

Pothakos, V., Snauwaert, C., De Vos, P., Huys, G. and Devlieghere, F., 2014. Monitoring psychrotrophic lactic acid bacteria contamination in a ready-to-eat vegetable salad production environment. *International Journal of Food Microbiology*, 185, 7-16.

Poza-Carrion, C., Suslow, T. and Lindow, S., 2013. Resident bacteria on leaves enhance survival of immigrant cells of *Salmonella enterica*. *Phytopathology*, 103(4), 341-351.

Preti, R. and Vinci, G., 2016. Nutritional and sensory evaluation of ready-to-eat salads during shelf life. *Agro FOOD Industry Hi Tech*, 27(1).

Pu, S., Beaulieu, J.C., Prinyawiwatkul, W. and Ge, B., 2009. Effects of plant maturity and growth media bacterial inoculum level on the surface contamination and internalization of *Escherichia coli* O157: H7 in growing spinach leaves. *Journal of Food Protection*, 72(11), 2313-2320.

Quilliam, R.S., Williams, A.P. and Jones, D.L., 2012. Lettuce cultivar mediates both phyllosphere and rhizosphere activity of *Escherichia coli* O157: H7. *PLoS One*, 7(3), e33842.

Ramos, B., Miller, F.A., Brandão, T.R., Teixeira, P. and Silva, C.L., 2013. Fresh fruits and vegetables—an overview on applied methodologies to improve its quality and safety. *Innovative Food Science and Emerging Technologies*, 20, 1-15.

Remenant, B., Jaffrès, E., Dousset, X., Pilet, M.F. and Zagorec, M., 2015. Bacterial spoilers of food: behavior, fitness and functional properties. *Food Microbiology*, 45, 45–53.

Resh, H.M., 2013. *Hydroponic Food Production: A Definitive Guidebook for the Advanced Home Gardener and the Commercial Hydroponic Grower*, 7th Ed., Florida: CRC Press.

Ricke, S.C. and Gast, R.K., 2014, 'Salmonella. *Salmonella* Enteritidis', in Batt C.A and Tortorello M.L. (eds.), *Encyclopedia of Food Microbiology*, 2nd ed., Academic Press, London, 343-348.

Riggio, G.M., Jones, S.L. and Gibson, K.E., 2019. Risk of human pathogen internalization in leafy vegetables during lab-scale hydroponic cultivation. *Horticulturae*, 5(1), 25.

Rivera, J.R.E., Stone, M.B., Stushnoff, C., Pilon-Smits, E. and Kendall, P.A., 2006. Effects of ascorbic acid applied by two hydrocooling methods on physical and chemical properties of green leaf lettuce stored at 5 °C. *Journal of Food Science*, 71(3), S270-S276.

Rocha, A.M., Ferreira, J.F., Silva, Â.M., Almeida, G.N. and Morais, A.M., 2007. Quality of grated carrot (var. Nantes) packed under vacuum. *Journal of the Science of Food and Agriculture*, 87, 447–451.

Rodrigo, D., Arranz, J.I., Koch, S., Frígola, A., Rodrigo, M.C., Esteve, M.J., Calvo, C. and Rodrigo, M., 2003. Physicochemical characteristics and quality of refrigerated Spanish orange-carrot juices and influence of storage conditions. *Journal of Food Science*, 68, 2111-2116.

Rodriguez-Aguilera, R. and Oliveira, J.C., 2009. Review of design engineering methods and applications of active and modified atmosphere packaging systems. *Food Engineering Reviews*, 1(1), 66-83.

Romanazzi, G., Feliziani, E. and Sivakumar, D., 2018. Chitosan, a biopolymer with triple action on postharvest decay of fruit and vegetables: Eliciting, antimicrobial and film-forming properties. *Frontiers in Microbiology*, 9, 2745.

Romanazzi, G., Feliziani, E., Santini, M. and Landi, L., 2013. Effectiveness of postharvest treatment with chitosan and other resistance inducers in the control of storage decay of strawberry. *Postharvest Biology and Technology*, 75, 24-27.

Rossmanith, P., Krassnig, M., Wagner, M. and Hein, I., 2006. Detection of *Listeria monocytogenes* in food using a combined enrichment/real-time PCR method targeting the *prf*A gene. *Research in Microbiology*, 157, 763–771.

Sağdıç, O., 2003. Sensitivity of four pathogenic bacteria to Turkish thyme and oregano hydrosols. *LWT-Food Science and Technology*, 36(5), 467-473.

Sagoo, S.K., Little, C.L., Ward, L., Gillespie, I.A. and Mitchell, R.T., 2003. Microbiological study of ready-to-eat salad vegetables from retail establishments uncovers a national outbreak of salmonellosis. *Journal of Food Protection*, 66(3), 403–409.

Sánchez-González, L., Vargas, M., González-Martínez, C., Chiralt, A. and Chafer, M., 2011. Use of essential oils in bioactive edible coatings: a review. *Food Engineering Reviews*, 3, 1-16.

Sant'Ana, A.S., Barbosa, M.S., Destro, M.T., Landgraf, M. and Franco, B.D., 2012a. Growth potential of *Salmonella* spp. and *Listeria monocytogenes* in nine types of ready-to-eat vegetables stored at variable temperature conditions during shelf-life. *International Journal of Food Microbiology*, 157, 52–58.

Sant'Ana, A.S., Igarashi, M.C., Landgraf, M., Destro, M.T. and Franco, B.D., 2012b. Prevalence, populations and pheno-and genotypic characteristics of *Listeria monocytogenes* isolated from ready-to-eat vegetables marketed in São Paulo, Brazil. *International Journal of Food Microbiology*, 155(1-2), 1–9.

Sant'Ana, A.S., Silva, F.F.P., Maffei, D.F. and Franco, B.D.G.M., 2014. 'Fruits and vegetables. Introduction', in Batt, C.A. and Tortorello, M.L. (eds.), *Encyclopedia of Food Microbiology*, 2nd ed., Academic Press, ISBN 9780123847300, 972-982.

Santos, M.I., Cavaco, A., Gouveia, J., Novais, M.R., Nogueira, P.J., Pedroso, L. and Ferreira, M.A.S.S., 2012. Evaluation of minimally processed salads commercialized in Portugal. *Food Control*, 23, 275–281.

Schikora, A., Garcia, A.V. and Hirt, H., 2012. Plants as alternative hosts for *Salmonella*. *Trends in Plant Science*, 17(5), 245-249.

Scollard, J., McManamon, O. and Schmalenberger, A., 2016. Inhibition of *Listeria monocytogenes* growth on fresh-cut produce with thyme essential oil and essential oil compound verbenone. *Postharvest Biology and Technology*, 120, 61-68.

Seidu, R., Sjølander, I., Abubakari, A., Amoah, D., Larbi, J.A. and Stenström, T.A., 2013. Modeling the die-off of *E. coli* and *Ascaris* in wastewater-irrigated vegetables: implications for microbial health risk reduction associated with irrigation cessation. *Water Science and Technology*, 68(5), 1013-1021.

Sekar, M., Zulkifli, N.F., Azman, N.A., Azhar, N.A.A., Norpi, A.S.M., Musa, H.I., Sahak, N.S. and Abdullah, M.S., 2016. Comparative antioxidant properties of methanolic extract of red and white dragon fruits. *International Journal of Current Pharmaceutical Research*, 8(3), 56-58.

Sellami, I.H., Maamouri, E., Chahed, T., Wannes, W.A., Kchouk, M.E. and Marzouk, B., 2009. Effect of growth stage on the content and composition of the essential oil and phenolic fraction of sweet marjoram (*Origanum majorana* L.). *Industrial Crops and Products*, 30, 395-402.

Semenov, A.M., Kuprianov, A.A. and Van Bruggen, A.H., 2010. Transfer of enteric pathogens to successive habitats as part of microbial cycles. *Microbial Ecology*, 60(1), 239-249.

Settanni, L., Miceli, A., Francesca, N. and Moschetti, G., 2012. Investigation of the hygienic safety of aromatic plants cultivated in soil contaminated with *Listeria monocytogenes*. *Food Control*, 26(2), 213-219.

Settanni, L., Miceli, A., Francesca, N., Cruciata, M. and Moschetti, G., 2013. Microbiological investigation of *Raphanus sativus* L. grown hydroponically in nutrient solutions contaminated with spoilage and pathogenic bacteria. *International Journal of Food Microbiology*, 160(3), 344-352.

Severino, R., Vu, K.D., Donsì, F., Salmieri, S., Ferrari, G. and Lacroix, M., 2014. Antibacterial and physical effects of modified chitosan based-coating containing nanoemulsion of mandarin essential oil and three non-thermal treatments against *Listeria innocua* in green beans. *International Journal of Food Microbiology*, 191, 82-88.

Sharma, M., Ingram, D.T., Patel, J.R., Millner, P.D., Wang, X., Hull, A.E. and Donnenberg, M.S., 2009. A novel approach to investigate the uptake and internalization of *Escherichia coli* O157: H7 in spinach cultivated in soil and hydroponic medium. *Journal of Food Protection*, 72(7), 1513-1520.

Shaw, A., Helterbran, K., Evans, M.R. and Currey, C., 2016. Growth of *Escherichia coli* O157: H7, non-O157 Shiga toxin–producing *Escherichia coli*, and *Salmonella* in water and hydroponic fertilizer solutions. *Journal of Food Protection*, 79(12), 2179-2183.

Shen, X., Chen, W., Zheng, Y., Lei, X., Tang, M., Wang, H. and Song, F., 2017. Chemical composition, antibacterial and antioxidant activities of hydrosols from different parts of *Areca catechu* L. and *Cocos nucifera* L. *Industrial Crops and Products*, 96, 110-119.

Shivembe, A. and Ojinnaka, D., 2017. Determination of vitamin C and total phenolic in fresh and freeze dried blueberries and the antioxidant capacity of their extracts. *Integrative Food, Nutrition and Metabolism*, 4(6), 1-5.

Singh, N., Singh, R.K., Bhunia, A.K. and Stroshine, R.L., 2002. Efficacy of chlorine dioxide, ozone, and thyme essential oil or a sequential washing in killing *Escherichia coli* O157: H7 on lettuce and baby carrots. *LWT-Food Science and Technology*, 35, 720-729.

Singleton, V.L. and Rossi, J.A., 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture*, 16(3), 144–158.

Siroli, L., Patrignani, F., Serrazanetti, D.I., Tappi, S., Rocculi, P., Gardini, F. and Lanciotti, R., 2015. Natural antimicrobials to prolong the shelf-life of minimally processed lamb's lettuce. *Postharvest Biology and Technology*, 103, 35-44.

Sivakumar, D. and Bautista-Baños, S., 2014. A review on the use of essential oils for postharvest decay control and maintenance of fruit quality during storage. *Crop Protection*, 64, 27-37.

Solomon, E.B. and Matthews, K.R., 2005. Use of fluorescent microspheres as a tool to investigate bacterial interactions with growing plants. *Journal of Food Protection*, 68(4), 870-873.

Solomon, E.B., Pang, H.J. and Matthews, K.R., 2003. Persistence of *Escherichia coli* O157: H7 on lettuce plants following spray irrigation with contaminated water. *Journal of Food Protection*, 66(12), 2198-2202.

Solomon, E.B., Yaron, S. and Matthews, K.R., 2002. Transmission of *Escherichia coli* O157: H7 from contaminated manure and irrigation water to lettuce plant tissue and its subsequent internalization. *Applied and Environmental Microbiology*, 68(1), 397-400.

Song, Z., Li, F., Guan, H., Xu, Y., Fu, Q. and Li, D., 2017. Combination of nisin and ε-polylysine with chitosan coating inhibits the white blush of fresh-cut carrots. *Food Control*, 74, 34-44.

Spadafora, N.D., Amaro, A.L., Pereira, M.J., Müller, C.T., Pintado, M. and Rogers, H.J., 2016. Multi-trait analysis of post-harvest storage in rocket salad (*Diplotaxis tenuifolia*) links sensorial, volatile and nutritional data. *Food Chemistry*, 211, 114–123.

Spector, M.P. and Kenyon, W.J., 2012. Resistance and survival strategies of *Salmonella enterica* to environmental stresses. *Food Research International*, 45(2), 455-481.

Ssemanda, J.N., Reij, M., Bagabe, M.C., Muvunyi, C.M., Joosten, H. and Zwietering, M.H., 2017. Indicator microorganisms in fresh vegetables from "farm to fork" in Rwanda. *Food Control*, 75, 126–133.

Standing, T.A., du Plessis, E., Duvenage, S. and Korsten, L., 2013. Internalisation potential of *Escherichia coli* O157: H7, *Listeria monocytogenes*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium and *Staphylococcus aureus* in lettuce seedlings and mature plants. *Journal of Water and Health*, 11(2), 210-223.

Stine, S.W., Song, I., Choi, C.Y. and Gerba, C.P., 2005a. Application of microbial risk assessment to the development of standards for enteric pathogens in water used to irrigate fresh produce. *Journal of Food Protection*, 68(5), 913-918.
Stine, S.W., Song, I., Choi, C.Y. and Gerba, C.P., 2005b. Effect of relative humidity on preharvest survival of bacterial and viral pathogens on the surface of cantaloupe, lettuce, and bell peppers. *Journal of Food Protection*, 68(7), 1352-1358.
Taulavuori, E., Hellström, E.K., Taulavuori, K. and Laine, K., 2001. Comparison of two methods used to analyse lipid peroxidation from *Vaccinium myrtillus* (L.) during snow removal, reacclimation and cold acclimation. *Journal of Experimental Botany*, 52(365), 2375-2380.
Teplitski, M., Warriner, K., Bartz, J. and Schneider, K.R., 2011. Untangling metabolic and communication networks: interactions of enterics with phytobacteria and their implications in produce safety. *Trends in Microbiology*, 19(3), 121-127.
Tomás-Callejas, A., López-Velasco, G., Camacho, A.B., Artés, F., Artés-Hernández, F. and Suslow, T.V., 2011. Survival and distribution of *Escherichia coli* on diverse fresh-cut baby leafy greens under preharvest through postharvest conditions. *International Journal of Food Microbiology*, 151(2), 216-222.
Tornuk, F., Ozturk, I., Sagdic, O., Yilmaz, A. and Erkmen, O., 2014. Application of predictive inactivation models to evaluate survival of *Staphylococcus aureus* in fresh-cut apples treated with different plant hydrosols. *International Journal of Food Properties*, 17(3), 587-598.
Tournas, V.H., 2005. Moulds and yeasts in fresh and minimally processed vegetables, and sprouts. *International Journal of Food Microbiology*, 99, 71–77.
Treftz, C. and Omaye, S.T., 2016. Hydroponics: Potential for augmenting sustainable food production in non-arable regions. *Nutrition & Food Science*, 46(5), 672-684.
Trichopoulou, A., Costacou, T., Bamia, C. and Trichopoulos, D., 2003. Adherence to a Mediterranean diet and survival in a Greek population. *New England Journal of Medicine*, 348(26), 2599-2608.
Tzortzakis, N. and Chrysargyris, A., 2017. Postharvest ozone application for the preservation of fruits and vegetables. *Food Reviews International*, 33(3), 270-315.
Tzortzakis, N., 2009. Impact of cinnamon oil-enrichment on microbial spoilage of fresh produce. *Innovative Food Science and Emerging Technologies*, 10, 97-102.
Tzortzakis, N., Borland, A., Singleton, I. and Barnes, J., 2007. Impact of atmospheric ozone-enrichment on quality-related attributes of tomato fruit. *Postharvest Biology and Technology*, 45(3), 317–325.
Tzortzakis, N., Nicola, S., Savvas, D. and Voogt, W., 2020. Soilless Cultivation Through an Intensive Crop Production Scheme. Management Strategies, Challenges and Future Directions. *Frontiers in Plant Science*, 11, 363.
Tzortzakis, N., Xylia, P. and Chrysargyris, A., 2019. Sage Essential Oil Improves the Effectiveness of *Aloe vera* gel on postharvest quality of tomato fruit. *Agronomy*, 9(10), 635.
Tzortzakis, N., 2007. Maintaining postharvest quality of fresh produce with volatile compounds. *Innovative Food Science and Emerging Technologies*, 8, 111–116.
Ulusoy, S., Boşgelmez-Tınaz, G. and Seçilmiş-Canbay, H., 2009. Tocopherol, carotene, phenolic contents and antibacterial properties of rose essential oil, hydrosol and absolute. *Current Microbiology*, 59(5), 554.
United States Department of Agriculture (USDA), 2011. A brief history of USDA food guides. https://www.choosemyplate.gov/eathealthy/brief-history-usda-food-guides. Accessed 28 June 2020.
Uyttendaele, M., Jaykus, L.A., Amoah, P., Chiodini, A., Cunliffe, D., Jacxsens, L., Holvoet, K., Korsten, L., Lau, M., McClure, P. and Medema, G., 2015. Microbial hazards in irrigation water: Standards, norms, and testing to manage use of water in fresh produce primary production. *Comprehensive Reviews in Food Science and Food Safety*, 14(4), 336-356.
Vallejo, F., Tomas-Barberan, F. and Garcia-Viguera, C., 2003. Health-promoting compounds in broccoli as influenced by refrigerated transport and retail sale period. *Journal of Agricultural and Food Chemistry*, 51(10), 3029–3034.
Vera, R.R. and Chane-Ming, J., 1999. Chemical composition of the essential oil of marjoram (*Origanum majorana* L.) from Reunion Island. *Food Chemistry*, 66, 143-145.

Viacava, G.E., Ayala-Zavala, J.F., González-Aguilar, G.A. and Ansorena, M.R., 2018. Effect of free and microencapsulated thyme essential oil on quality attributes of minimally processed lettuce. *Postharvest Biology and Technology*, 145, 125-133.

Villaño, D., Fernandez-Pachon, M.S., Troncoso, A.M. and Garcia-Parilla, M.C., 2004. The antioxidant activity of wines determined by the $ABTS^{\bullet+}$ method: influence of sample dilution and time. *Talanta*, 64(2), 501–509.

Viuda-Martos, M., Mohamady, M.A., Fernández-López, J., ElRazik, K.A., Omer, E.A., Pérez-Alvarez, J.A. and Sendra, E., 2011. In vitro antioxidant and antibacterial activities of essentials oils obtained from Egyptian aromatic plants. *Food Control*, 22(11), 1715-1722.

Vu, K.D., Hollingsworth, R.G., Leroux, E., Salmieri, S. and Lacroix, M., 2011. Development of edible bioactive coating based on modified chitosan for increasing the shelf life of strawberries. *Food Research International*, 44(1), 198-203.

Wachtel, M.R., Whitehand, L.C. and Mandrell, R.E., 2002a. Prevalence of *Escherichia coli* associated with a cabbage crop inadvertently irrigated with partially treated sewage wastewater. *Journal of Food Protection*, 65(3), 471-475.

Wachtel, M.R., Whitehand, L.C. and Mandrell, R.E., 2002b. Association of *Escherichia coli* O157: H7 with preharvest leaf lettuce upon exposure to contaminated irrigation water. *Journal of Food Protection*, 65(1), 18-25.

Wadamori, Y., Gooneratne, R. and Hussain, M.A., 2017. Outbreaks and factors influencing microbiological contamination of fresh produce. *Journal of the Science of Food and Agriculture*, 97(5), 1396-1403.

Waller, S.B., Cleff, M.B., Serra, E.F., Silva, A.L., dos Reis Gomes, A., de Mello, J.R.B., de Faria, R.O. and Meireles, M.C.A., 2017. Plants from Lamiaceae family as source of antifungal molecules in humane and veterinary medicine. *Microbial Pathogenesis*, 104, 232-237.

Wang, H. and Hammack, T.S., 2014, 'Salmonella. Detection by classical cultural techniques', in Batt C.A and Tortorello M.L. (eds.), *Encyclopedia of Food Microbiology*, 2nd ed., Academic Press, London, 332-338.

Wang, Y.J., Deering, A.J. and Kim, H.J., 2020. The occurrence of shiga toxin-producing *E. coli* in aquaponic and hydroponic systems. *Horticulturae*, 6(1), 1.

Warriner, K., Ibrahim, F., Dickinson, M., Wright, C. and Waites, W.M., 2003. Interaction of *Escherichia coli* with growing salad spinach plants. *Journal of Food Protection*, 66(10), 1790-1797.

Wells, H.F. and Buzby, J.C., 2008. Dietary assessment of major trends in US food consumption, 1970-2005. Economic Information Bulletin No. 33. Economic Research Service, U.S. Dept. of Agriculture. (No. 1476-2016-121022).

Whipps, J.M., Hand, P., Pink, D.A. and Bending, G.D., 2008. Human pathogens and the phyllosphere. *Advances in Applied Microbiology*, 64, 183-221.

Wieczyńska, J. and Cavoski, I., 2018. Antimicrobial, antioxidant and sensory features of eugenol, carvacrol and *trans*-anethole in active packaging for organic ready-to-eat iceberg lettuce. *Food Chemistry*, 259, 251-260.

Wiedemann, A., Virlogeux-Payant, I., Chaussé, A.M., Schikora, A. and Velge, P., 2015. Interactions of *Salmonella* with animals and plants. *Frontiers in Microbiology*, 5, 791.

Wojdylo, A., Oszmianski, J. and Czemerys, R., 2007. Antioxidant activity and phenolic compounds in 32 selected herbs. *Food Chemistry*, 105(3), 940–949.

Wright, K.M., Chapman, S., McGeachy, K., Humphris, S., Campbell, E., Toth, I.K. and Holden, N.J., 2013. The endophytic lifestyle of *Escherichia coli* O157: H7: quantification and internal localization in roots. *Phytopathology*, 103(4), 333-340.

Wright, K.M., Crozier, L., Marshall, J., Merget, B., Holmes, A. and Holden, N.J., 2017. Differences in internalization and growth of *Escherichia coli* O157: H7 within the apoplast of edible plants, spinach and lettuce, compared with the model species *Nicotiana benthamiana*. *Microbial Biotechnology*, 10(3), 555-569.

Xylia, P., Botsaris, G., Chrysargyris, A., Skandamis, P. and Tzortzakis, N., 2019a. Variation of microbial load and biochemical activity of ready-to-eat salads in Cyprus as affected by vegetable type, season, and producer. *Food Microbiology*, 83, 200-210.

Xylia, P., Chrysargyris, A., Botsaris, G. and Tzortzakis, N., 2017. Potential application of spearmint and lavender essential oils for assuring endive quality and safety. *Crop Protection*, 102, 94-103.

Xylia, P., Chrysargyris, A., Botsaris, G. and Tzortzakis, N., 2018. Mint and pomegranate extracts/oils as antibacterial agents against *Escherichia coli* O157: H7 and *Listeria monocytogenes* on shredded carrots. *Journal of Food Safety*, 38, 12423.

Xylia, P., Clark, A., Chrysargyris, A., Romanazzi, G. and Tzortzakis, N., 2019b. Quality and safety attributes on shredded carrots by using *Origanum majorana* and ascorbic acid. *Postharvest Biology and Technology*, 155, 120-129.

Yeni, F., Yavaş, S., Alpas, H.A.M.I. and Soyer, Y., 2016. Most common foodborne pathogens and mycotoxins on fresh produce: a review of recent outbreaks. *Critical Reviews in Food Science and Nutrition*, 56(9), 1532-1544.

Yuan, G., Chen, X. and Li, D., 2016. Chitosan films and coatings containing essential oils: The antioxidant and antimicrobial activity, and application in food systems. *Food Research International*, 89, 117-128.

Zhang, G., Ma, L., Beuchat, L.R., Erickson, M.C., Phelan, V.H. and Doyle, M.P., 2009a. Heat and drought stress during growth of lettuce (*Lactuca sativa* L.) does not promote internalization of *Escherichia coli* O157: H7. *Journal of Food Protection*, 72(12), 2471-2475.

Zhang, G., Ma, L., Beuchat, L.R., Erickson, M.C., Phelan, V.H. and Doyle, M.P., 2009b. Lack of internalization of *Escherichia coli* O157: H7 in lettuce (*Lactuca sativa* L.) after leaf surface and soil inoculation. *Journal of Food Protection*, 72(10), 2028-2037.

Zhan, L., Li, Y., Hu, J., Pang, L. and Fan, H., 2012. Browning inhibition and quality preservation of fresh-cut romaine lettuce exposed to high intensity light. *Innovative Food Science and Emerging Technologies*, 14, 70-76.

Zhao, H., Wang, L., Belwal, T., Jiang, Y., Li, D., Xu, Y., Luo, Z. and Li, L., 2020. Chitosan-based melatonin bilayer coating for maintaining quality of fresh-cut products. *Carbohydrate Polymers*, 235, 115973.

APPENDIX

Chapter 3.

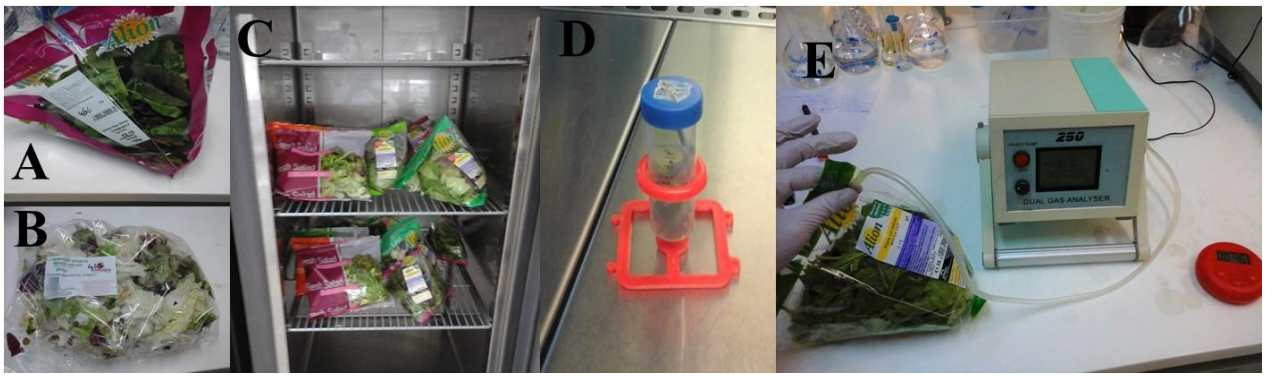


Figure 3.S1. Sample collection (A, B), storage (C), tissue storage (D) and determination of CO_2 production (E).

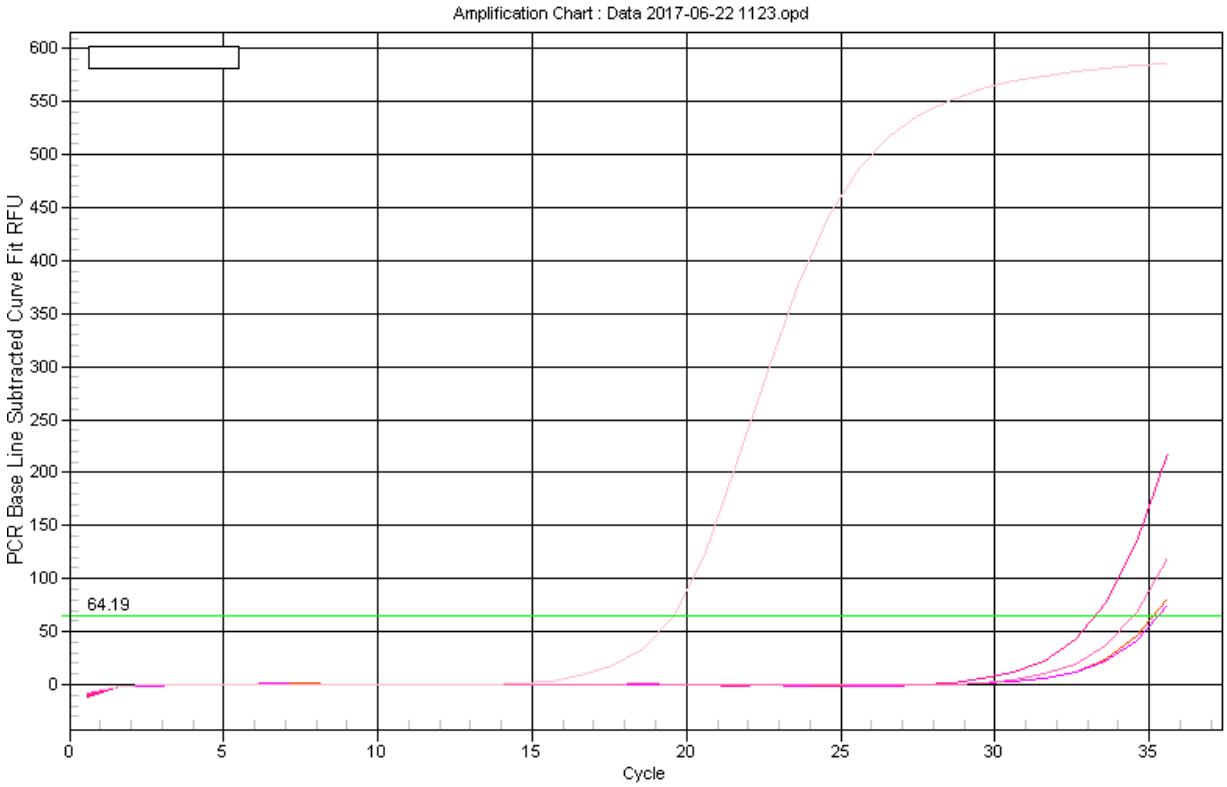


Figure 3.S2. Real-time PCR results for identification of *S. enterica* on ready-to-eat salads.

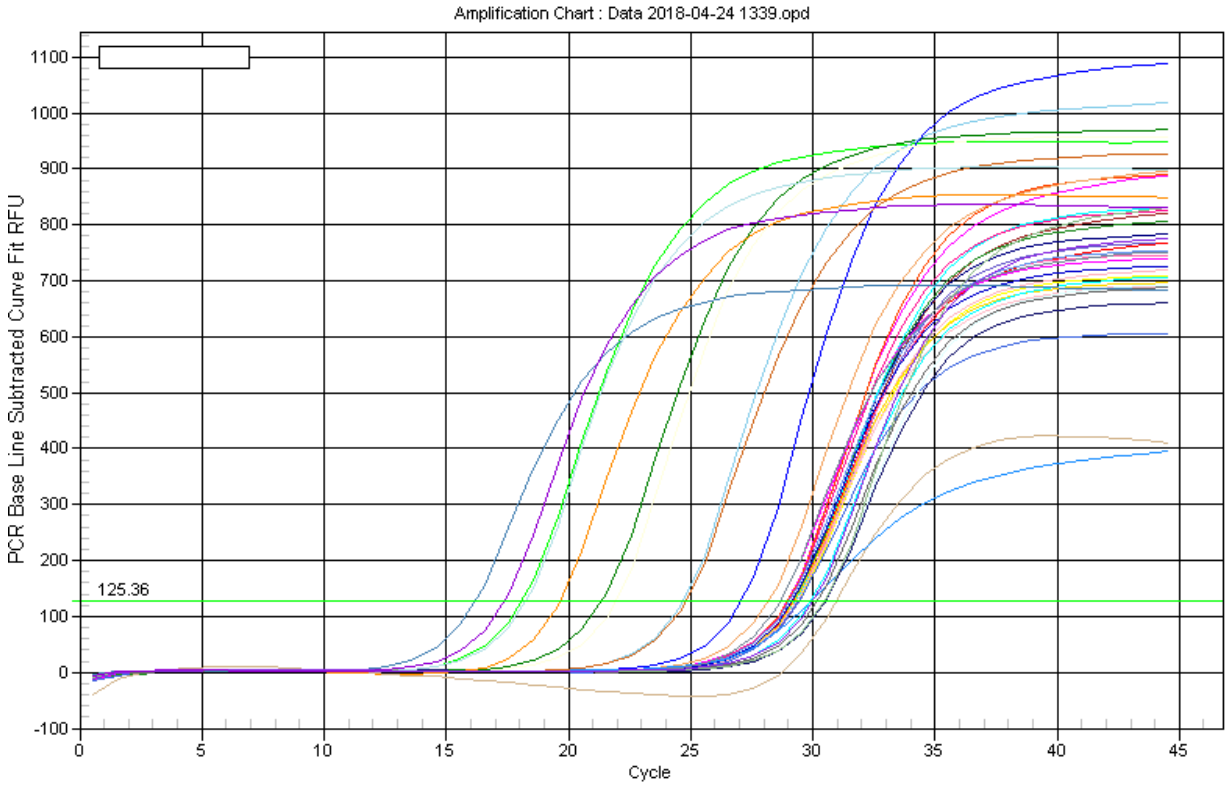


Figure 4.S3. Real-time PCR results for *L. monocytogenes* on ready-to-eat salads.

Figure 4.S1. Root rinse collection (A), root sterilization (B) and leaf sampling procedure (C, D).

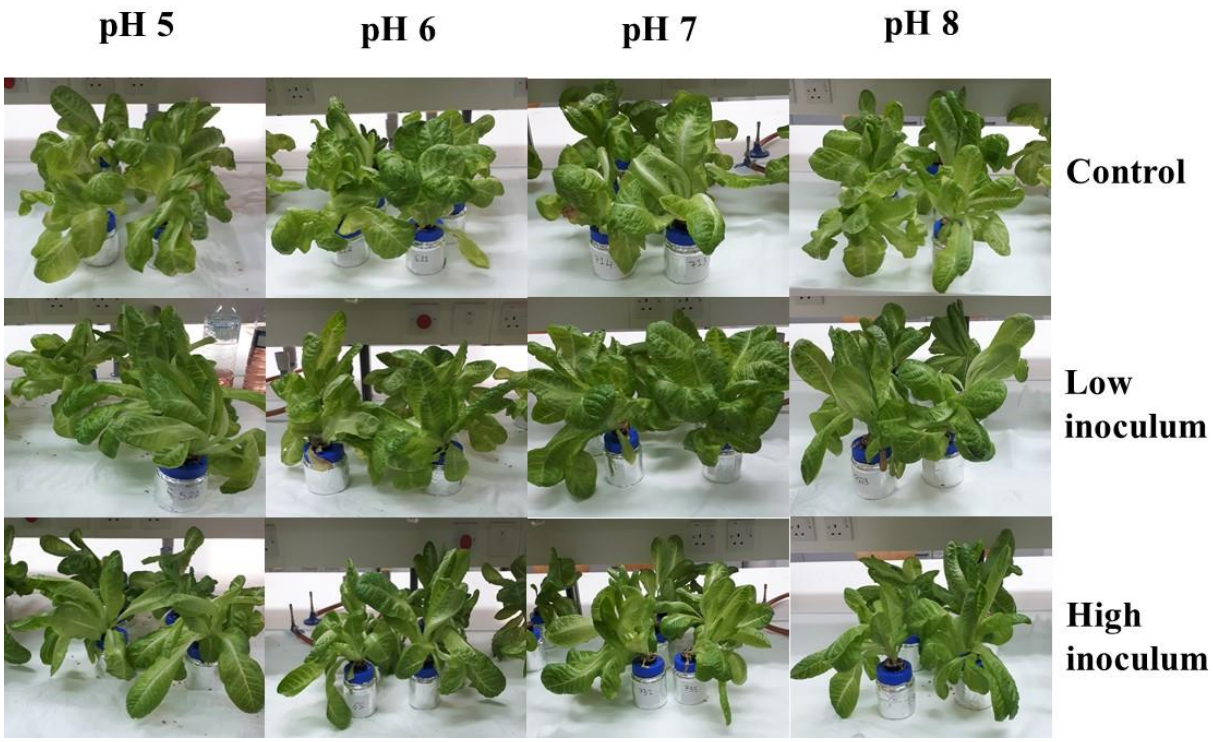


Figure 4.S2. Old plants 21 dpi.

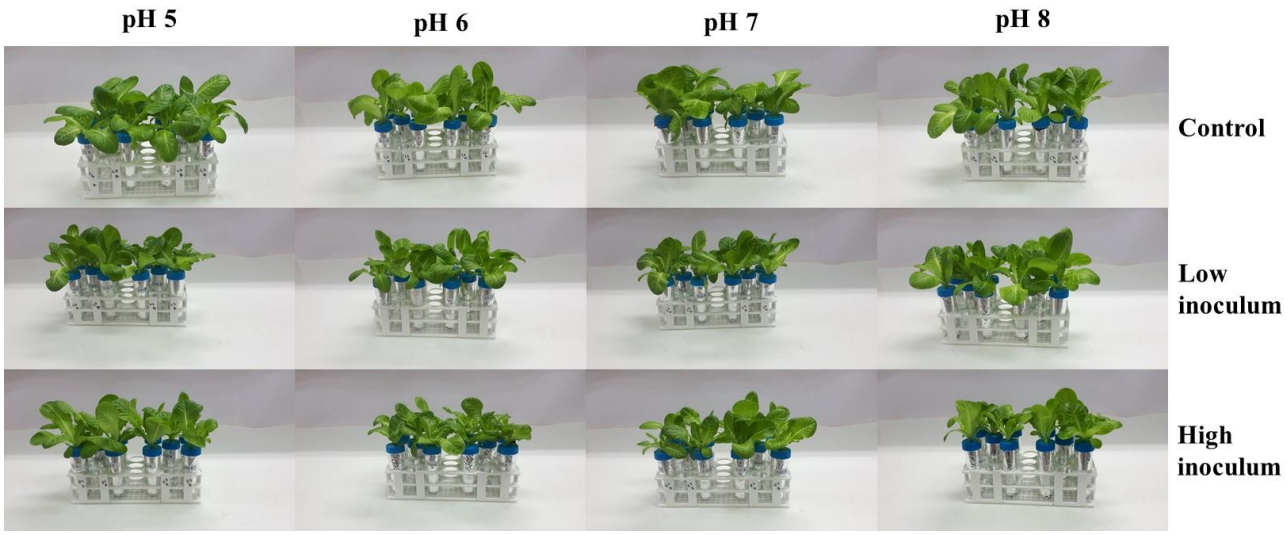


Figure 4S3. Small-medium plants 14 dpi.

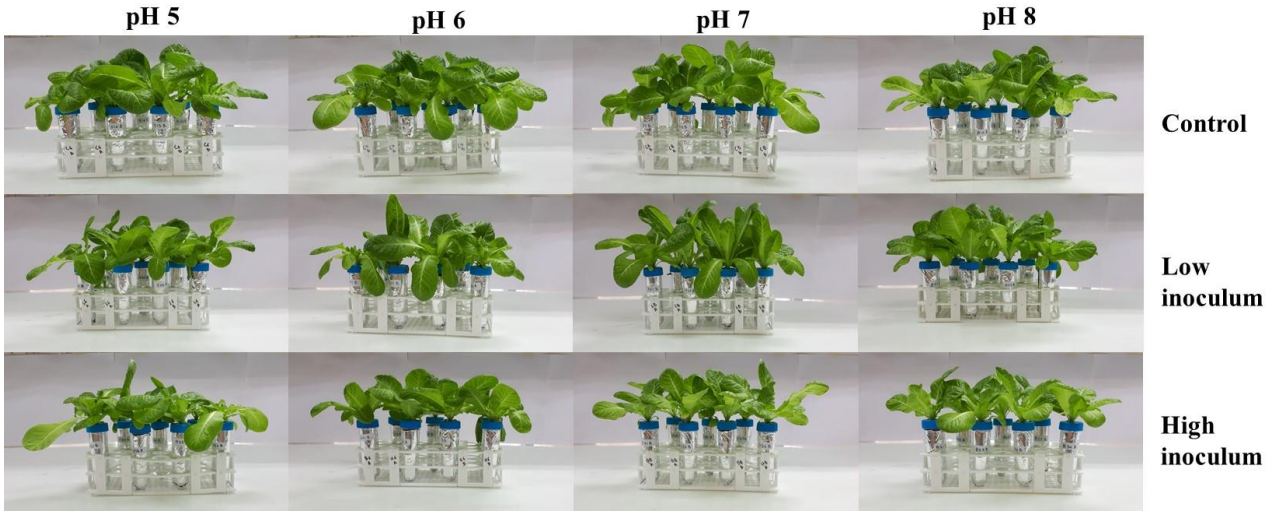


Figure 4.S4. Small-medium plants 21 dpi.

Chapter 5

Experiment 1: Lettuce

Materials and methods

Fresh lettuce leaves were cut into pieces (5 x 10 cm) and washed with tap water. Then, 50 g of fresh-cut leaves were dipped for 1, 5 and 10 min (according to preliminary tests) into 500 mL of chitosan solution (0.5 and 1% Chito Plant; ChiPro GmbH, Bremen, Germany). Leaf pieces were then drained, and placed and enclosed in a polypropylene (PP) and stored at 7 ^{o}C and 90% RH for four days. Samples were inspected (visually) on a daily basis for choosing the adequate dipping time for further assessment.

Figure 5.S1. Application of chitosan on fresh-cut lettuce (preliminary test for determining the time of application)

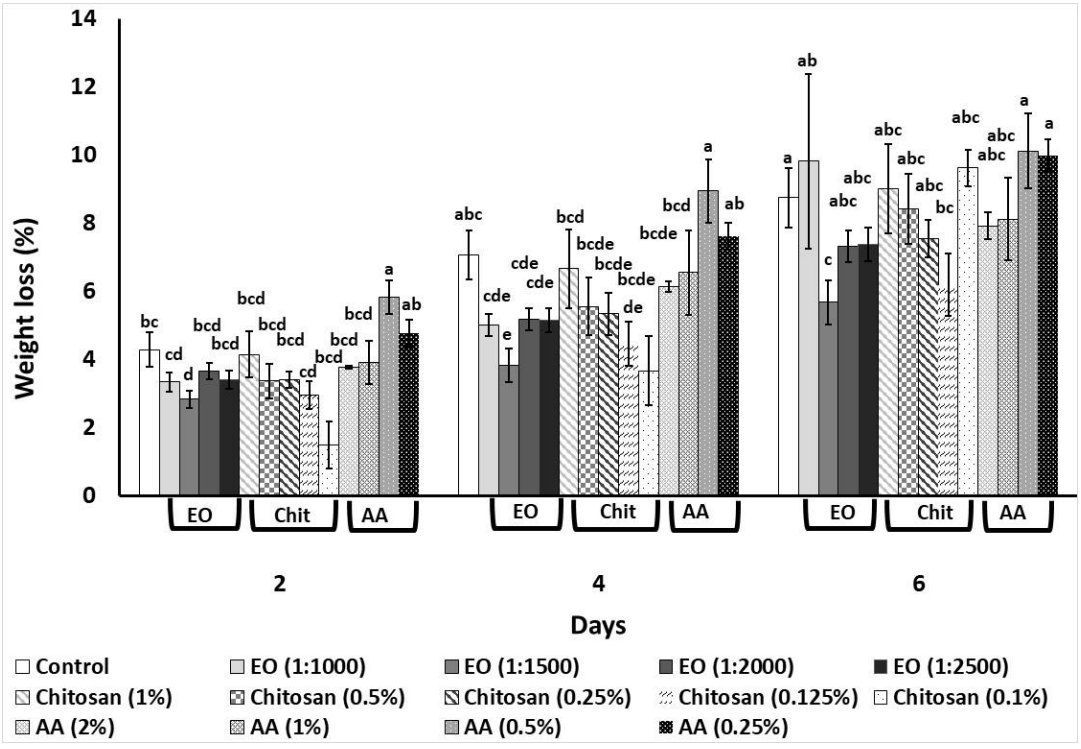


Figure 5.S2. Screening of marjoram essential oil, chitosan and ascorbic acid on fresh-cut lettuce weight loss (%) after 6 days storage at 7 °C and 90% RH. On the columns, significant differences (P<0.05) among treatments are indicated by different Latin letters for different days.

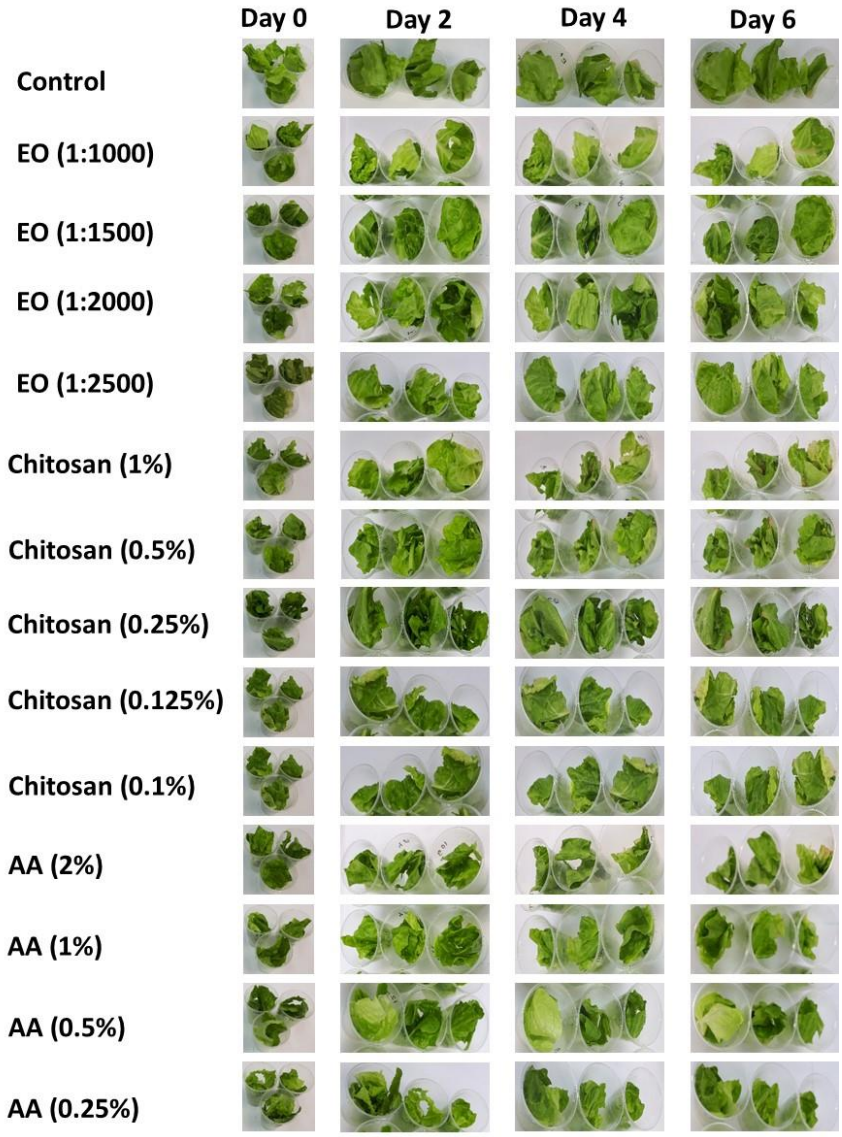


Figure 5.S3. Preliminary screening of marjoram essential oil (EO), chitosan and ascorbic acid (AA) on fresh-cut lettuce color after treatment and up to 6 days of storage at 7 °C and 90% RH. In each day, three representative samples for each treatment are shown.

Figure 5.S4. Impact of marjoram essential oil (EO), chitosan and ascorbic acid (AA) alone and their combinations on fresh-cut lettuce color after treatment and up to 6 days of storage at 7 °C and 90% RH. In each day, three representative samples for each treatment are shown.

Experiment 2: Carrots

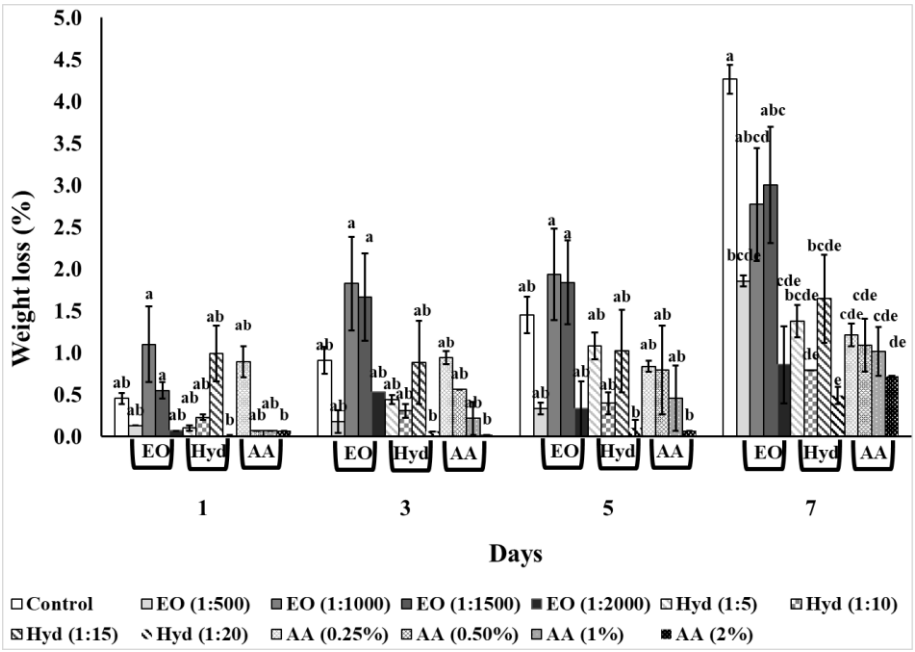


Figure 5.S5. Screening of marjoram essential oil, marjoram hydrosol and ascorbic acid on shredded carrots weight loss (%) after 7 days storage at 4 °C and 90% RH. On the columns, significant differences (P<0.05) among treatments are indicated by different Latin letters for different days.

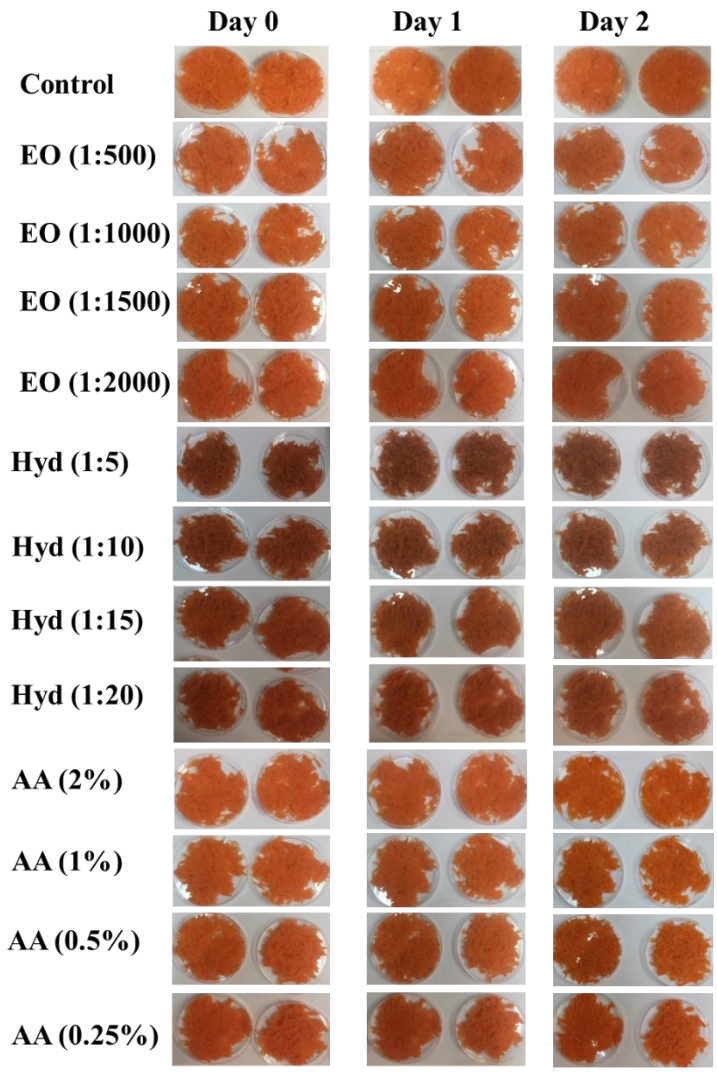


Figure 5.S6. Preliminary screening of marjoram essential oil (EO), marjoram hydrosol (Hyd) and ascorbic acid (AA) on shredded carrots color after treatment and up to 7 days of storage at 4 °C and 90 % RH. In each day, two representative samples (Petri dishes) for each treatment are shown.