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MICROBIAL QUALITY, VOLATILE FLAVOUR COMPOUNDS AND GLUCOSINOLATES OF READY-TO-EAT ROCKET SALADS (*Diplotaxis tenuifolia* and *Eruca sativa*) IN THE CONTEXT OF THE SUPPLY CHAIN

Thesis submitted for the degree of Doctor of Philosophy

Department of Food and Nutritional Sciences

By

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Declaration

I confirm that the work presented in this thesis is my own and that the use of all the literature from other source has been properly and fully acknowledge.

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ABSTRACT

Bagged fresh ready-to-eat (RTE) vegetables are rich in nutrients, contain high moisture content, have nearly neutral pH and have exposed wounded tissues, making the produce a perfect media for microbes to proliferate. Breaches of cold temperature along the supply chain, could aggravate the situation, thus increasing the risk of foodborne illness and accelerate the rate of food spoilage. A study to evaluate the effects of handling and storage conditions on microbial loads, volatile organic compounds, distribution of naturally occurring bacteria and formation of biofilms, and types of bacteria and moulds present in fresh RTE rocket was conducted. Antimicrobial capability of rocket leaves obtained from different varieties with different glucosinolate (GLS) profiles in rocket leaves (*Eruca sativa* and *Diplotaxis tenuifolia*) was additionally evaluated.

The abundance of bacteria was also highly dependent on storage temperature and whether the bag was kept sealed (closed) or if it was opened. The produce kept continuously at 4 ^{o}C contained low bacterial loads until the end of the study period (day 6 of storage). As the storage temperature was increased from 4 to 13 ^{o}C, the bacterial loads remained low up to 4 days of storage when the bags were kept closed. When the produce are kept at an ambient temperature (20 ^{o}C), regardless of bag condition (opened or closed), the produce contained low bacterial loads only up to two days of storage.

Fresh RTE rocket leaves exposed to 25 ^{o}C for 4 h and 'consumed' at 24 h after 'purchase' did not cause any significant increase in bacterial loads. In contrast, the bacterial loads in leaves that have never been exposed to high temperature but instead kept at 'display' temperature (13 ^{o}C) for relatively long duration until the date until display and beyond contained high loads of microbes. Heat shock treatment on winter grown rocket increased respiration rate that occurred concurrently with reduced O_2 level in the bagged fresh RTE rocket leaves.

Organic volatile compounds (VOCs) associated with off-odour of rocket, such as sulphur containing compounds (dimethyl sulfide, dimethyl trisulfide and dimethyl sulfoxide), alcohols (1-butanol, 1-penten-3-ol, (z)-2-penten-1-ol, 1-hexenol and phenethyl alcohol), acetic acid and furan were detected in bagged fresh RTE rocket leaves held at 13 ^{o}C. The

abundance of the VOCs increased with time duration. Rocket leaves that were purchased early and kept under 4 ºC, regardless of whether the bag is opened or closed, and 'consumed' on date until display contained the highest concentration of total GLS.

Scanning electron microscopy (SEM) images revealed the abundance of bacteria on the rocket leaves was higher on dented areas and stomata, especially along the leaf vein. Long term storage leads to the formation of bacterial biofilms.

Whole genome and 16S metagenomic DNA sequencing of the bacteria revealed that the types of bacteria that live on bagged fresh RTE rocket were very diverse. DNA sequencing analysis detected 267 types of bacteria present in RTE rocket leaves. *Salmonella enterica* was detected in leaves that were heat shocked (4h at 25 ºC) followed by keeping at moderate temperature (13 ºC) up date until display and in leaves that were continuously kept at 13 ºC till date until display. *Pseudomonas viriflava* and *Erwnia* sp., and lactic acid bacteria (*Lactococcus lactis*) were detected in fresh RTE rocket held at 13 ºC until date until display. Six strains of toxigenic moulds, *Fusarium equiseti, F. chlamydosporum, Alternaria alternata, A. tenuissima,* and other two species of *Alternaria* were present in the RTE rocket leaves exposed to moderate (13 ºC) and high (25 ºC) temperature.

There was no clear relationships exist between GLS content and abundance of naturally occurring bacteria present on leaves of rocket varieties with different GLS profiles. The abundance of *E.coli* K12 was reduced when the bacteria were cultured in extract of leaves obtained from rocket varieties containing high concentration of GLS. The reduction of bacterial loads was correlated negatively with the concentration of dimeric-4-mercaptobutyl (DMB) in the leaf extracts.

The results of the study clearly show that bacterial and mould counts, VOC and GLS abundance, distribution of naturally occurrence bacteria and formation of biofilms in bagged fresh RTE rocket leaves were significantly affected by handling and storage/display conditions of the produce. Analysis of DNA sequencing of the microbes revealed that the species and strains of bacteria and moulds detected residing in the RTE rocket leaves were very diverse, ranging from epiphytes, food spoilage, plant pathogens, as well as human pathogen microbes.

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ABBREVIATIONS AND SYMBOLS

AFP	Aspergillus flavus and parasiticus agar
ANOVA	Analysis of variance
APHA	Animal and Plants Health Agency
BLASTN	Nucleotide Basic Local Alignment Search Tool
C_2H_4	Ethylene
CAS Number	Chemical Abstracts Services Number
CASAVA base calling	Consensus Assessment of Sequence And Variation base calling
cfu	Colony forming unit
contigs	Contiguous sequences
CYA	Czapek yeast extract agar
CZID	Czapek-dox iprodione dichloran agar
DEPC	Diethyl pyrocarbonate buffer
DMB	Dimeric 4-mercaptobutyl-glucosinolate
DRBC	Dichloran rose bengal chloramphenicol agar
DRYS	Rose bengal yeast extract sucrose agar
DUD	Display until date
DVB	Divinylbenzene
DW	Dry weight
EP	Epidermis
EPA	Environmental Protection Agency
EPEC	Enteropathogenic *Escherichia coli*
EPS	Exopolyssacharides
ESI	Electrospray ionization
GC	Guard cell
GC-FID	Gas chromatography-flame ionization detector
GC-MS	Gas chromatography-mass spectromwtry
GH3	Glucoside hydrolase family 3
GLS	Glucosinolate
GMP	Good Manufacturing Practices

HACCP	Hazard Analysis and Critical Control Point
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HMMER	Profile Hidden Markov Models
HPLC	High performance liquid chromatography
I.C.M.S.F	Internal Commission on Microbiology Specification for Food
ITC	Isothiocynate
ITS	Internal transcribed spacer
ITS	Isothiocynate
kPa	kiloPascal
LAB	Lactic acid bacteria
$LCMS^2$	Liquid chromatograpy- mass spectrometry^2
LOL	Low oxygen limit
LRI	Linear retention indices
MA	Modified atmosphere
MAP	Modified atmosphere packaging
ML	Maximum Likehood
MRD	Maximum recovery diluent
MYRS	Myrosinase
na	Not applicable
ND	Not detected
NIH	National Institute of Health
NIST	National Institute of Standards and Technology
nl	Nanoliter
OD	Optical density
OTR	Oxygen transmission rate
OTUs	Operational taxonomic units
PC1	Principal Component 1
PCA	Plate count agar
PCoA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
PCR-ARDRA method	Polymerase Chain Reaction-Amplified Ribosomal DNA Restriction Analysis

PDMS	Polydimethylsiloxane
ppm	Parts per million
psi	Pound per square inch
PVDF	Durapore polyvinylidene fluoride
rpm	Rotation per minute
rRNA	Ribosomal ribonucleic acid
RTE	Ready-to-eat
SEM	Scanning electron microscopy
SPME	Solid-phase microextraction
STEC	Shiga toxin-producing *Escherichia coli*
T3SS	Type 3 secretion system
TETRA	Tetra-nucleotide Usage Patterns
TGG	Thioglucosidase glucohydrolase
TNTC	Too numerous to count
TPC	Total plate count
tRNA	Transfer ribonucleic acid
TSB	Triptic soy broth
TWC	The Watercress Company
VOC	Volatile organic compounds
VTEC	Verotoxin
w/v	Weight/volume

CHAPTER 1

INTRODUCTION

1.1 Background

Ready-to-eat (RTE) food is any food that is suitable for consumption without further treatment or processing. RTE food covers both open- and pre-packed products, it can either be eaten hot or cold, purchased in the store and is normally consumed elsewhere. Depending on the food, it is normally kept under refrigeration or at room temperature (Robbins, 2013).

Fresh-cut, also known as minimally processed, fruits and vegetables, including rocket (*Eruca sativa* and *Diplotaxis tenuifolia*) are one of the RTE foods which are rapidly gaining in popularity (Rekhy and Mc Conchie, 2014). Most of the fresh RTE produce is known to have high nutritional content, high water activity and near neutral pH, making the foods ideal media for microbial growth. If not handled and stored properly, the fresh RTE food can become a source of food-borne illness (Beauchat, 2002; Harris et al., 2003; Nygard et al., 2008). Therefore, the microbiological quality and safety of the products has become a major concern among those involved in the food supply chain. Much effort has been put into identifying the source of microbial contaminants in these foods, as well as to understand the characteristics of the foods and the conditions that would appear to affect the shelf-life of the food, in view of minimizing the growth of microbes associated with food-borne diseases and food spoilage organisms (Alwi and Ali, 2014; De Giusti et al., 2014; Koukkidis et al., 2016; McCann et al., 2003; Tournas 2005a, 2005b).

Microbial contamination on fresh RTE produce is closely associated with postharvest quality management, which is generally aimed at providing favourable conditions for retention of quality such as physical appearance, texture and flavour as well as nutritional attributes. During postharvest quality management, proper product handling and appropriate environmental conditions must be provided, including factors such as temperature, relative humidity, and time of storage (Kader and Rolle, 2004; Lokke et al., 2012).

Preparation of fresh RTE fruits and vegetables involves washing, cutting and packing, before being kept at an appropriate storage temperature. The cutting leads to the release of tissue exudates which make the RTE product become a suitable media for bacterial growth (Jablasone et al., 2005). Furthermore, without proper hygiene, cross contamination among the hands of food handlers, equipment used and the foods may occur, thus increasing the risk of food poisoning (Williamson et al., 1992). The situation can be worsened when the foods are stored or kept at inappropriate temperature during processing, transportation display or storage and/or consumed after the recommended date of expiry (Sela and Fallik, 2009). In this thesis the growth and diversity of bacterial populations will be examined on fresh RTE rocket which will be subjected to a number of different supply chain scenarios, in order to assess the microbiological risk present within the present best practice, and to assess the level to which risk is increased if best practice is not adhered to.

To avoid contamination by any pathogenic or spoilage bacteria, the RTE foods must be prepared with extra care. One way is by using effective bacterial inactivation techniques that may involve the use of radiation, chemical treatment, heat treatment, bio-control agents, and modified or control atmosphere storage (Koseki et al., 2002; Palou et al., 2007). However,

the inactivation of microbes is seldom successful and the remaining bacteria still survive (Alexopoulus et al., 2013). This phenomenon could be linked to internalization of bacteria into stomata and other internal spaces of the leaves, thus protecting bacteria from sanitization processes and/or antimicrobial treatments (Saldana et. al., 2011). The presence of biofilms on leaf surfaces may also contribute to this problem. Bacterial biofilms have been observed and reported to exist on food and food-processing surfaces and can increase the risk of product quality and food safety (Alhede et al., 2012; Carmichael, 1999; Koukkidis et al., 2016). Formation of a bacterial biofilm could provide a protective condition for microbes against antimicrobial agents and non-conducive environment such as water stress condition. Therefore, a thorough understanding of the residing sites of bacteria and the possible role of biofilms in affecting the effectiveness of washing and sanitization procedures needs to be investigated. Furthermore, the nature of microbes present in fresh RTE food or salads are very diverse (Tournas, 2005a, 2005b) and therefore reaction of the microbial community towards inactivation treatments and variation in handling, transportation and storage conditions could be different. In view of such scenarios, efforts to determine the diversity of microbes (bacteria and fungi) were made in this study and information generated were discussed in relation to their possible role as human and plant pathogens, as well as food spoilage agents.

Along the supply chain of fresh RTE products are a series of packing house operations (trimming, grading, washing and packaging), transportation from the factory to warehouse or retailer premises, display and store at retail premises, before being bought, eaten, or kept by customers for later use. At certain phases within the supply chain, the produce may be subjected to a degree of temperature abuse. Ideally, the products must be kept in a continuous

cool-chain environment (usually around 4 ºC) but this is not always possible. Exposure to higher temperatures would shorten product shelf-life, and reduce its quality. Reduction in quality is normally seen as changes in physical appearance, and the changes are accompanied by an increase in microbial loads, reduced nutritional quality and reduction of beneficial phytochemical contents and release of off-odour volatiles (Bell et al., 2017a; Deza-Durand and Petersen, 2014; Khalil, 2016; Lokke et al., 2012; Luca et al., 2016). Due to its unique role in determining the taste of rocket (Jahangir et al., 2009), and the potent antioxidant and anticancer properties of rocket (Ombra et al., 2017), as well as its possible role as an antimicrobial agent (Khoobchandani et al., 2010), special emphasis was given to evaluate the impact of temperature abuse on compounds within the group of glucosinolates. Due to the diversity of rocket species and varieties available in the market, which are known to contain different levels of phytochemicals and hence give different taste to consumer (Bell et al., 2017a; Taranto et al., 2016), efforts were made to elucidate possible relationships between rocket varieties with varying GLS contents and its ability to reduce microbial loads.

1.2 Aims of the study

A. To evaluate the effect of handling and storage conditions of rocket on the microbial load, quality attributes and release of volatile organic compounds in the context of the supply chain;

B. To discover the natural distribution of bacteria and formation of biofilms in fresh RTE rocket salads;

C. To elucidate the diversity and identify of naturally occurring microbes (human and plant pathogens, and food spoilage microbes) that exist in fresh RTE rocket salads at various phases of a simulated supply chain; and

D. To examine the antimicrobial properties of glucosinolates found in rocket leaves on naturally occurring bacteria and a cultured species of *E .coli.*

1.3 Objectives of the study

A1. To determine the effects of varying storage temperature and packaging conditions (closed/opened bags) on the abundance of naturally occurring bacteria present on fresh RTE rockets leaves at critical points along the supply chain;

A2. To determine the effects of simulated temperature abuse and storage conditions during transit, display and storage on the abundance of naturally occurring bacteria present on fresh RTE rocket leaves;

A3. To determine the effects of simulated temperature abuse and storage conditions during transit, display and storage on bacteria loads, gas composition and greenness of fresh RTE rocket leaves produced in two contrasting growing seasons;

A4. To determine the effects of simulated temperature abuse and storage conditions during transit, display and storage on the composition of volatile organic compounds of fresh RTE rocket leaves;

A5. To determine the effects of simulated temperature abuse and storage conditions during transit, display and storage on the composition of glucosinolates of fresh RTE rocket leaves;

B1. To discover the distribution, visual relative abundance and formation of biofilms of naturally occurring bacteria present on and in rocket leaves as affected by storage temperature and duration;

C1. To determine the abundance and identity of naturally occurring bacteria present on rocket leaves at different points of sampling along the simulated supply chains using 16S rDNA and whole genome sequencing analyses;

C2. To determine the abundance and identify naturally occurring moulds present on rocket leaves at different points of sampling along simulated supply chains by sequencing the non-coding internal transcribed spacer (ITS) region of the ribosomal operon;

D1. To determine the loads of naturally occurring bacteria in and on leaves of rocket with different contents of glucosinolates; and

D2. To determine the effects of extracts of rocket leaves obtained from different varieties of rocket with known concentrations of glucosinolates on the growth of *E. coli* K12.

1.4 Structure of the thesis

To achieve the above aims and objectives, various aspects of experiments involving salad rocket salad (*Eruca sativa*) and wild rocket (*Diplotaxis tenuifolia*) were carried out. In this thesis, the reports of the study are divided into seven chapters.

***Chapter 1*:** This is an introductory chapter. The chapter outlined the background of the subject of interests, especially background information of fresh ready-to-eat vegetables, brief information about rocket as a vegetables and salad, and problems associated with fresh RTE vegetables, especially in relations to microbial quality. Also included in this chapter are aims and objectives of the study.

***Chapter 2*:** In this chapter, the literature review was mainly focussed on microbial growth on fresh RTE vegetables, internalization of bacteria in the leaves and the effect of product handling and storage conditions on microbial growth and bacterial loads in fresh RTE vegetables. In the review, a special emphasis was given to the changes in the generation of volatile compounds from fresh RTE vegetables especially those that give off-odour aroma to the produce. The review also covered various aspects of glucosinolates, a group of volatiles that give hot and peppery taste of brassicas, including rocket, which also might contribute to antimicrobial properties of rocket leaves following the changes in the growing environment, postharvest handling and storage conditions.

***Chapter 3*:** This chapter reports the changes in bacterial loads, gas composition and volatile organic compounds of fresh bagged ready-to-eat rocket in the context of the supply chain of

fresh RTE vegetables. Among others, this chapter reports the effects of storage temperature and conditions of the packaging (opened/closed) over a period of time during storage. The variation in bacterial loads, gas composition (O_2, CO_2 and C_2H_4) sampled from the bags and greenness of the rockets leaves produced in two contrasting seasons (summer and winter) were also reported. The impacts of varying handling and storage/display conditions and practices commonly adopted either by suppliers, retailers and consumers on generation of volatile compounds and glucosinolates were included.

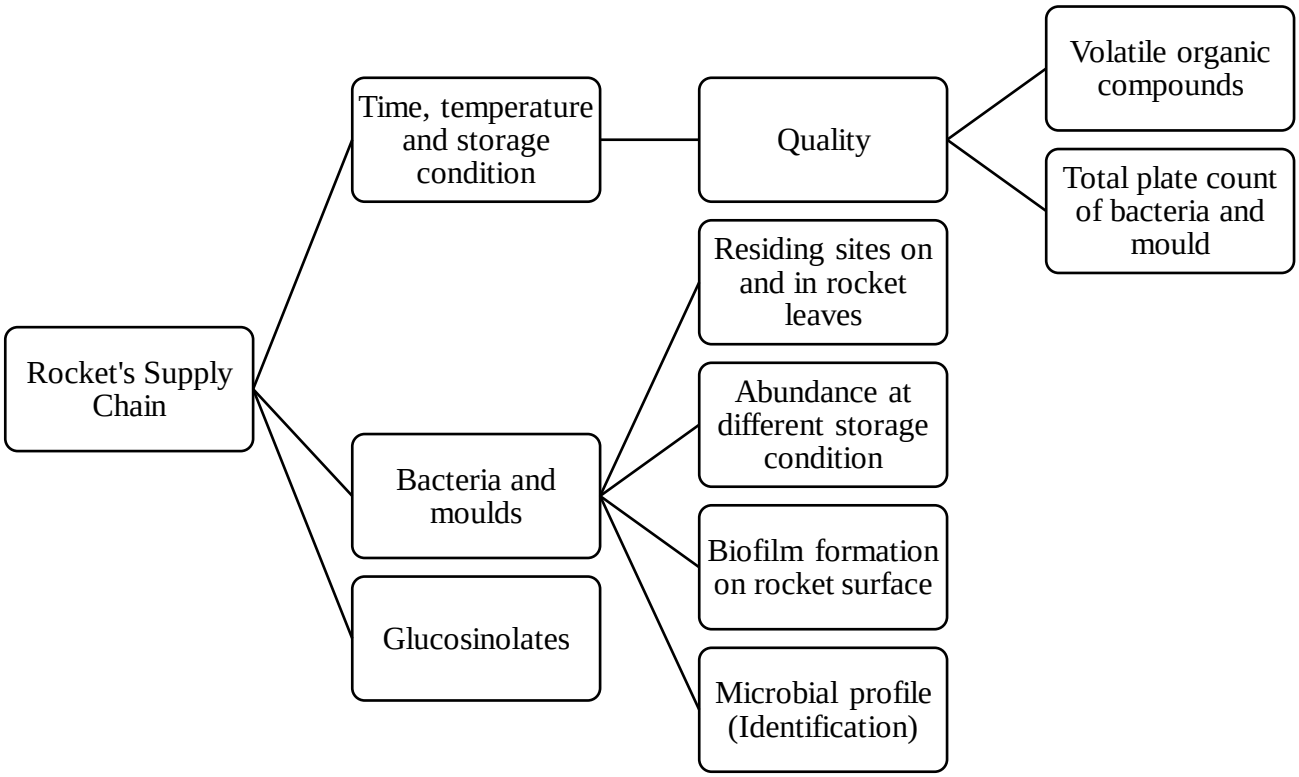
***Chapter 4*:** This chapter dealt with occurrence and distribution of naturally occurring bacteria and formation of biofilms on rocket leaves stored under different temperature and duration using scanning electron microscopy. This is done in view of understanding the difficulty of the removal of the bacteria (and other microbes) that internalize within the leaves during washing and sanitization processes. Two different sample preparation techniques were used, conventional chemical fixation and Cryo-SEM methods, before viewing under a scanning electron microscope.

Chapter 5: A study aimed at elucidating the abundance and identification of bacteria and mould species, reflecting the diversity of the microbes is reported in this chapter. Identification of bacteria that exist at various points of sampling in the context of the supply chain was performed using 16S rDNA and whole genome sequencing techniques, while the mould was determined by sequencing the non-coding internal transcribed spacer (ITS) region of the ribosomal operon. Besides identification, phylogenetic trees of bacteria and mould were constructed in order to evaluate the relationship between species detected.

***Chapter 6*:** In this chapter, the results of a study to evaluate the impact of glucosinolate profiles of six varieties of rocket on the loads of naturally occurring bacteria that live on the leaves were reported. Also included in this chapter is a report on the effects of leaf extract obtained from the six varieties of rocket with distinct glucosinolate profiles on the growth of *E. coli* K12, a strain of *E. coli* with resemblance to *E. coli* 0157:H7, but which does not express Shiga and Shiga-like toxins.

***Chapter 7*:** This is the General Discussion and Conclusion chapter. This chapter discusses the main results generated from this study. Suggestions on future work were also outlined.

In summary, the work flow is illustrated as below.



CHAPTER 2

LITERATURE REVIEW

2.1 Rocket

Rocket salad is one of the vegetables that gaining in popularity and widely consumed as fresh and ready-to-eat salads. Rocket plants can be divided in two genera, *Eruca* and *Diplotaxis* (Figure 2.1; Pasini et al., 2011). Rocket is also known as arugula, rucola and roquette and is from the *Brassicaceae* family (Martinez-Sanchez et al., 2006). Rocket is an annual or biannual herb that originated in the Mediterranean regions (Zeven and de Wet, 1982). The plant is now widely grown all over the world including USA, UK, Italy, Spain, Morocco, Israel, India and Australia (Bozokalfa et al., 2011). Rocket salads from both species have been regarded as healthy herbs as the leaves are reported to contain high concentrations of health promoting phytochemicals such as vitamin C, glucosinolates and flavonols (Martinez-Sanchez et al, 2008a; Bell et al., 2014). Rocket has a strong peppery taste and can be used in various culinary ways such as in soups, salads and pizza toppings.

The rocket plants grows 20–100 cm in height. The leaves are deeply pinnately lobed with four to ten small lateral lobes and a large terminal lobe. The flowers are 2–4 cm in diameter, arranged in a corymb with creamy white petals veined with purple, and with yellow stamens; the sepals are shed soon after the flower opens. The fruit is a pod, 12–35 mm long with an apical beak, and containing several seeds.

In the UK, the plant can be grown between April to early September and the leaves can be harvested beginning at four weeks after sowing (Figure 2.2 (https://www.rhs.org.uk/advice/grow-your-own/vegetables/rocket). A typical processing flow chart of rocket leaves are presented in Figure 2.3.

Figure 2.1. Image of wild rocket (*Diplotaxis tenuifolia*-left) and rocket salad (*Eruca sativa*-right) leaves. Source from http://www.naturallifeenergy.com/wild-arugula-vs-rocket-salad-arugula.

Figure 2.2. General image of rocket plant. Source from https://www.rhs.org.uk/advice/grow-your-own/vegetables/rocket.

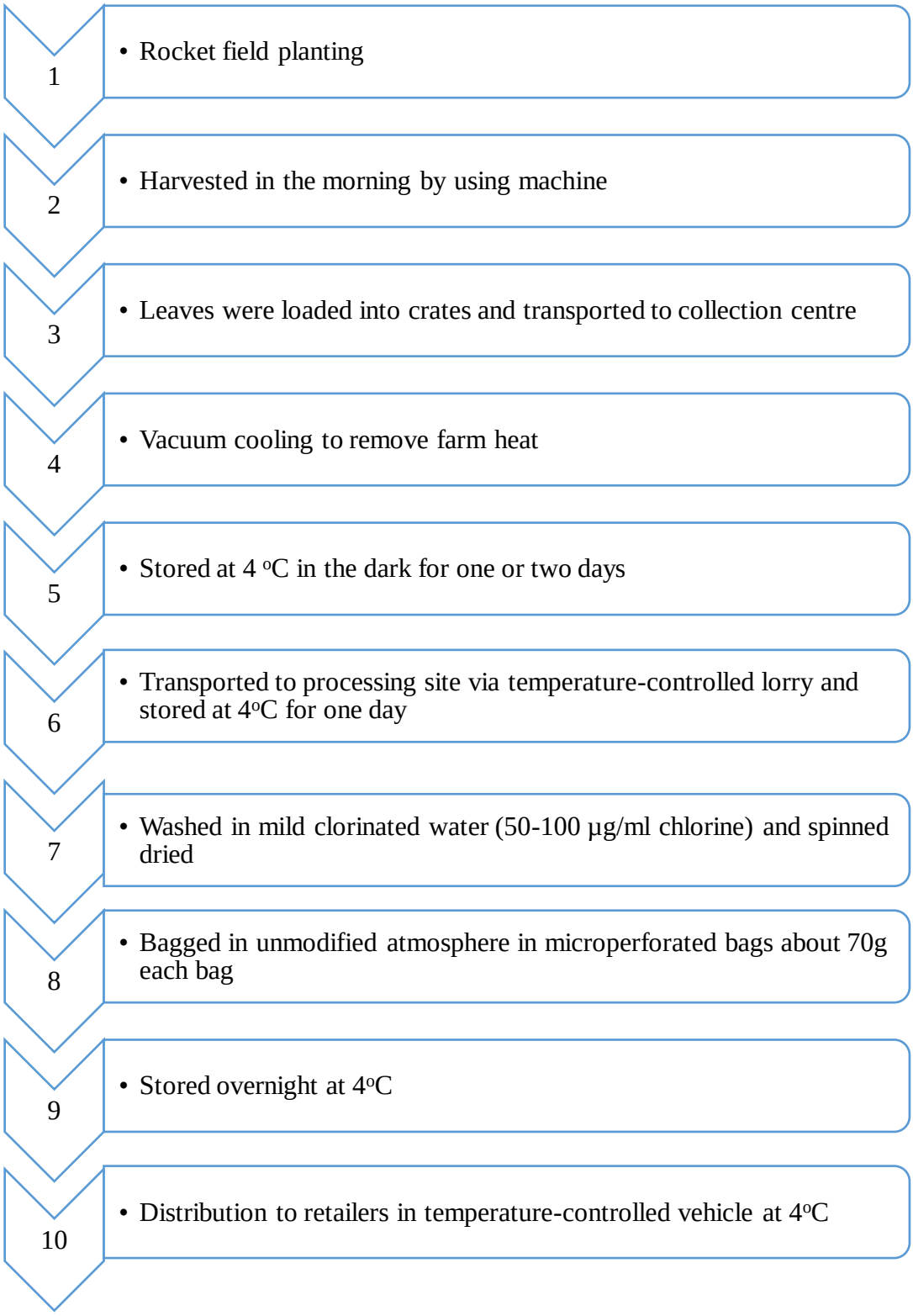


Figure 2.3 Typical processing flow chart of rocket leaves from farm to retailers (Adapted from Bell et al., 2017)

2.2 Microbial growth and contamination of fresh RTE vegetables

Uncooked or raw fresh vegetables and greens are increasingly being noted as important vehicles for the transmission of pathogenic bacteria to human beings (Berger et al., 2010). Due to this problem, the industry has responded to the threat of bacterial contamination by practicing good manufacturing practices and adopting risk management procedures which include worker sanitation in the field, high care environment and sanitation of wash water. However, despite the steps taken, the number of foodborne-related illness is increasing worldwide (Mead et al., 1999; Soon et al., 2011) and in most cases, the outbreak is linked with fresh RTE vegetables (Gibbons et al., 2006; Gilbreth et al., 2005).

Goulet et al. (2008) reported that there was an increase in the incidence of listeriosis in European countries during the past few years and the increase could be resulted from the increase in the consumption of *Listeria monocytogenes* infested RTE foods (Garrodo et al., 2009). Similar cases involving other foodborne pathogens, such as Staphylococcal food poisoning resulted from ingestion of enterotoxins in food contaminated by certain strains of *Staphylococcus aureus* (Notermans and van Otterdijk, 1985). The problem was also reported for *Escherichia coli* O15:H7 that would lead to hemorrhagic colitis and consequently become a more serious problem as haemolytic uremic syndrome (Meng et al., 1998; Blanco et al., 2003). Nygard et al. (2008) reported that consumption of rocket salad imported from Italy in pre-prepared salad mix in pre-packed plastic bags had caused a salmonellosis outbreak (*Salmonella enterica*) in Norway, Denmark and UK in 2004.

The initial microbial load in RTE vegetables is a crucial factor in determining the risk associated with microbial presence in the food system. However, other factors including processing, transportation, storage and display conditions throughout the supply chain may affect the microbial presence at the point of consumption (Legnani and Leoni, 2004; De Giusti et al., 2014).

There are various sources that may contribute to the contamination in rocket salad throughout the whole production chain. It may begin at the farm level. Among the factors that may influence the occurrence of pathogen contamination in vegetables include use of organic fertilizers, poor quality of water and incorrect application of Good Manufacturing Practice (GMP) and Hazard Analysis and Critical Control Point (HACCP) system during production, processing, packaging and distributions (De Guisti et al., 2010; Heaton and Jones, 2008).

RTE vegetables are usually colonized by plant pathogenic bacteria. These bacteria do not cause illness to human beings, but they are capable of affecting physical appearance of the product and reducing its shelf life. The predominant microbiological populations in RTE salads comprises psychrotrophs (such as *Pseudomonas* spp. and *Erwinia* spp.) and lactic acid bacteria (such as *Leuconostoc mesenteroids*) (Willocx et al., 1993). In general, bacterial spoilage on leafy vegetables appeares to be watery and slimy (Barth et al., 2009). Most bacteria that cause spoilage of fresh RTE vegetables are capable of propagating and growing at low temperatures, including under refrigeration temperatures (Abadias et al., 2007). Fresh produce is high in sugar content which may undergo microbial fermentation caused by lactic acid bacteria or yeasts, while other microorganisms could generate soft rot symptoms which are associated with the growth of pectinolytic bacteria (Barth et al., 2009). Spoilage of pre-

packed fresh RTE vegetables if frequently reported as being linked to many species of microbes (Brackett, 1992; Nguyen-the and Carlin, 1994; Pingulkar et al., 2001; Tournas, 2005a, 2005b). Table 2.1 shows the major species of bacteria involved in the spoilage of common vegetables (Tournas, 2005a).

Fungal spoilage can be distinguished from bacterial decay by the presence of a mycelial network and characteristic spore-forming bodies. Frequently, fungi cause spoilage by penetrating areas of wounded leaf surfaces. Leaf injury could be initated through mechanical damage, freezing or chilling injury or enzymatic activity (Tournas, 2005b). Table 2.2 presented a list of common fungi that cause spoilage on a variety of vegetables, while the range of yeast and mould counts and their frequency of occurence for some type of vegetables as reported by Tournas (2005b) are given in Table 2.3.

Table 2.1 . Common spoilage bacteria found on different types of fresh vegetables.

Bacterial species	Types of spoilage	Vegetables
Corynebacterium michiganense	Canker and fruit spot	Tomatoes, others
C. sepedonicum	Tuber rot	White potatoes
Erwinia carotovora	Bacterial soft rot	Crucifers, lettuce, endives, parsley, celery, carrots, onions, garlic, tomatoes, beets, peppers, cucumbers
Pseudomonas chicorii	Bacterial zonate spot	Cabbage and lettuce
P. marginalis group	Soft rot of vegetables	Lettuce, other
P. morsprunorum group	Halo blight	Beans
P. tomato group	Bacterial specks	Tomatoes
P. syringae group	Diseases in soybeans	Soybeans
Xanthomonas campestris	Black rot	Cabbage, cauliflower

Source: Tournas (2005a)

Table 2.2. Common fungi causing spoilage of common vegetables.

Fungal species	Type of spoilage	Vegetables
Alternaria alternate	Alternaria rot	Tomatoes, peppers, cucurbits
A. brassicola and A. oleracea	Alternaria rot	Leafy crucifers
Aspergillus alliaceus	Black rot	Onions, garlic
Botrytis allii	Neck rot	Onions
B. cinerea	Gray mould rot	Leafy crucifers, lettuce, onions, garlic, asparagus, pumpkin, squash, carrots, celery, sweet potatoes and most other vegetables
Bremia lactucae	Downy mildew	Lettuce
Ceratocystis fimbriata	Black rot	Sweet potatoes
Cladosporium cucumerinum	Scab	Cucumber, pumpkin
Colletotrichum coccodes	Anthracnose	Cucumbers, squash, pumpkin, peppers, tomatoes
Diaporthe batatis		Sweet potato
D. vexans		Eggplant fruit
Fusarium spp.	Dry rot	Potatoes
Geotrichum candidum	Sour rot	Asparagus, crucifers, onions, garlic, beans, carrots, parsley, parsnips, lettuce, endives tomatoes, globe artichokes, various vegetables

Source: Tournas (2005b)

Table 2.3. Fungi and yeast and their counts commonly found in selected fresh RTE vegetables.

Organisms	Counts ($cfu.g^{-1}$)	Frequency (% of contaminated samples)
Salads		
Alternaria	$<100-5.0\times10^3$	10.3
Cladosporium	$<100-5.9\times10^3$	23.3
Penicillium	$<100-2.0\times10^3$	5.1
Yeasts	$<100-9.2\times10^6$	94.9
Tomatoes		
Alternaria	$<100-2.0\times10^3$	12.5
Cladosporium	$<100-1.4\times10^4$	50.0
Geotrichum	$<100-2.4\times10^5$	12.5
Penicillium	$<100-3.2\times10^4$	25.0
Yeasts	$<100-1.5\times10^6$	62.5
Cucumbers		
Alternaria	$<100-2.1\times10^3$	33.3
Cladosporium	$<100-2.0\times10^2$	66.7
Yeasts	$<100-1.5\times10^3$	66.7
Green onions		
Cladosporium	$<100-1.9\times10^3$	66.7
Yeasts	$<100-2.1\times10^3$	66.7
Lettuce		
Alternaria	$<100-6.0\times10^2$	20.0
Cladosporium	$<100-8.2\times10^3$	40.0
Penicillium	$<100-1.0\times10^2$	20.0
Yeasts	$1.9\times10^3-9.5\times10^4$	100.0
Green peppers		
Alternaria	$<100-1.0\times10^4$	66.7
Cladosporium	$<100-6.4\times10^3$	33.3
Yeasts	$<100-2.0\times10^3$	33.3
Spinach		
Cladosporium	$<100-1.0\times10^3$	50.0
Yeasts	$2.0\times10^3-3.7\times10^4$	100.0
Radishes		
Alternaria	$<100-2.0\times10^3$	33.3
Cladosporium	$<100-1.3\times10^3$	66.7
Yeasts	$6.8\times10^3-1.1\times10^4$	100.0

Source: Tournas (2005b).

There are several effective methods available for inactivation of microorganism and enzymes produced by the microbes which responsible for quality decay in fruits and vegetables. These methods include thermal and non-thermal technologies such as blanching, UV-C radiation, ultrasounds and ozone treatment (Alexandre et al., 2011). Some of the technologies involving thermal treatment such as blanching may be the most effective way to reduce microbial load and also enzyme activity. Chlorine is the most frequently used sanitising agent in the food industry (Gil et al., 2009). Kim et al. (1999) proposed that an effective sanitizing agent should reduce the microbial population by at least five logs during the first 30 sec of exposure, while Torlak et al. (2013) defined an anti-microbial agent as a compound that leads to a minimum of two logs reduction in the microbial load. These discrepancies may contribute to the disparities in the interpretation of the results found in the research.

2.3 Effect of storage temperature and duration on microbial growth on RTE vegetables

Storage temperature plays an important role in keeping the food safe beside some other intrinsic and extrinsic parameters. Ridgwell and Winson (2001) stated the refrigeration temperature should be kept at 5 ^{o}C or below to inhibit bacterial growth. However, each bacterium requires a different optimum temperature to grow and therefore their reaction towards refrigeration or storage temperatures and conditions may vary between species. Based on their capacity to growth and survive under a specific range of temperatures, microorganisms could be divided into several groups. Psychrophiles or cold-loving microbes can grow well at refrigerated temperature of 7 ^{o}C and below (Olson and Nottingham, 1980; Kraft, 1992), and they are not usually founds at temperatures above 20 ^{o}C. Another

group of bacteria, known as mesophiles, prefer a moderate temperature and grow well under a wide range of temperatures (20–45 °C) with optimum growth generally occuring between 30 – 40 ^{o}C. Mesophiles could survive at refrigeration temperatures (Jay, 1992), even if they do not proliferate under these conditions. Some other microbes, belonging to the group of psychrotrophs, can grow at low temperatures as do psychrophiles, while their optimum growth rate is at the temperature of mesophiles. The major group of psychrotrophs that are commonly involved in food spoilage during storage are listed in Table 2.4.

Normally, vegetables are naturally colonized with microbes belonging to mesophiles and psychrophiles (Tournas, 2005a, 2005b). Mesophiles could be the predominant microbes in the field and during storage at ambient or room temperatures, while the psychrophiles could flourish and constitute the dominant flora on produce during storage at low temperatures. Due to differences in their preferred growing temperature and poor adaptation of some species of microbes, a proportion of the microbes colonizing the produce will be killed or injured during low temperature storage due to cold shock (Ridgwell and Winson, 2011). The others will survive the chilling and resume growth once the produce is transferred to its suitable or preferred temperature.

Table 2.4. Psychotrophic spoilage microorganisms.

Bacteria	*Acinetobacter, Aeromonas, Alcaligenes, Arthrobacter, Bacillus, Chromobacterium,Citrobacter, Clostridium,Corynebacterium, Enterobacter, Erwinia, Escherichia,Flavobacterium, Klebsiella, Lactobacillus, Leuconostoc, Listeria,Microbacterium, Micrococcus, Moraxella,Proteus, Pseudomonas, Serratia, Streptococcus, Strepto-myces, Vibrio, Yersinia*

Moulds	*Aspergillus*, *Penicillum*
Yeast	*Candida*, *Cryptococcus*, *Torulopsis*

Source: Brackett (1992).

Among the psychrophilic human pathogens, *Listeria monocytogenes* receives special attention as this pathogen can grow at refrigeration temperatures. It is known to survive in the processing environment, and has the potential to cause human mortalities (Fain, 1996). Some other human pathogens that are commonly found in RTE vegetables are *Escherichia coli, Salmonella enterica* and *Shigella sonei* which usually grow at room to body temperature (20 – 37 ^{o}C).

Time taken between a series of events is also a critical factor associated with microbial growth, especially when it is coupled with a break in the cool chain. While technologies in extending the shelf life of fresh RTE vegetables, such as modified atmosphere packaging, active packaging, washing with chlorine, ozone and acidic electrolyzed water are widely available, with the added time factor microbes are able to replicate, even at a slow rate, and to colonize within or on the surface of fresh produce. This happens when the viable inoculums are still present in fresh vegetables if they have not been totally removed or inactivated throughout the sanitizing treatment. Thus, it is likely that microbial loads present on fresh vegetables will increase with increasing storage or shelf life time.

2.4 Microbial colonization on leaves

Despite the increased importance of fresh RTE vegetables as a route for human pathogen infection, currently there is scarce body of knowledge on the mechanism by which the microbes internalize and survive on or in fruits and vegetables (Berger et al., 2010). Plant surfaces, or the phyllospheres, provide an important and suitable habitat for large and diverse communities of bacterial species (Lindow and Brandl, 2003).

There is some evidence showing that some plant pathogen have evolved to overcome plant defence mechanisms, and to colonize leaf surfaces and other inner tissues of the plants. These pathogens use wounds and natural openings like stomata, hydathodes and lenticels as natural openings for them to get into internal plant tissues (Hirano and Upper, 2000; Melotto et al., 2006). Previous studies have shown Shiga-toxigenic *E. coli* (STEC) colonize leaf surfaces, on and around stomata, and between cellular spaces and xylem (Warriner et al., 2003; Franz et al., 2007). Saldana et al. (2011) have also shown that STEC are capable of residing within the stomata internal cavity and internal tissues. In the study, Saldana et al. used scanning electron microscopy (SEM) analysis of spinach leaves after 1 to 24 h of incubation with *E.coli* strain EDL933. Their results revealed that the number of the bacteria on the stomata and within its internal cavity increased with incubation time (Figure 2.4). They also reported the presence of the *E.coli* in intercellular spaces of the spongy mesophyll, as well as in the vascular conducting tissues (xylem and phloem) (Saldana et al., 2011).

Different types of bacteria may use different methods in plant colonization. *Pseudomonas syringae,* a plant pathogen, utilizes its filamentous type III secretion system (T3SS) and an array of effector molecules, to override inherent plant defence mechanisms and to eliminate the host (Chang et al., 2005; Chisholm et al., 2006). A similar mechanism of colonization was also reported for *E. coli* O157:H7, as well as the related enteropathogenic *E. coli* (EPEC), to a variety of salad leaves (Shaw et al., 2008). However, the precise mechanisms of adherence of these organisms to plant surfaces and the types of physical interactions participating in this interplay are largely unknown. *Listeria monocytogenes*, a human pathogen, uses flagella for attachment to plant tissue, while *Klebsiella, Enterobacter* and *Pseudomonas* use fimbriae to mediate attachment to plant surfaces (Gorski et al., 2003).

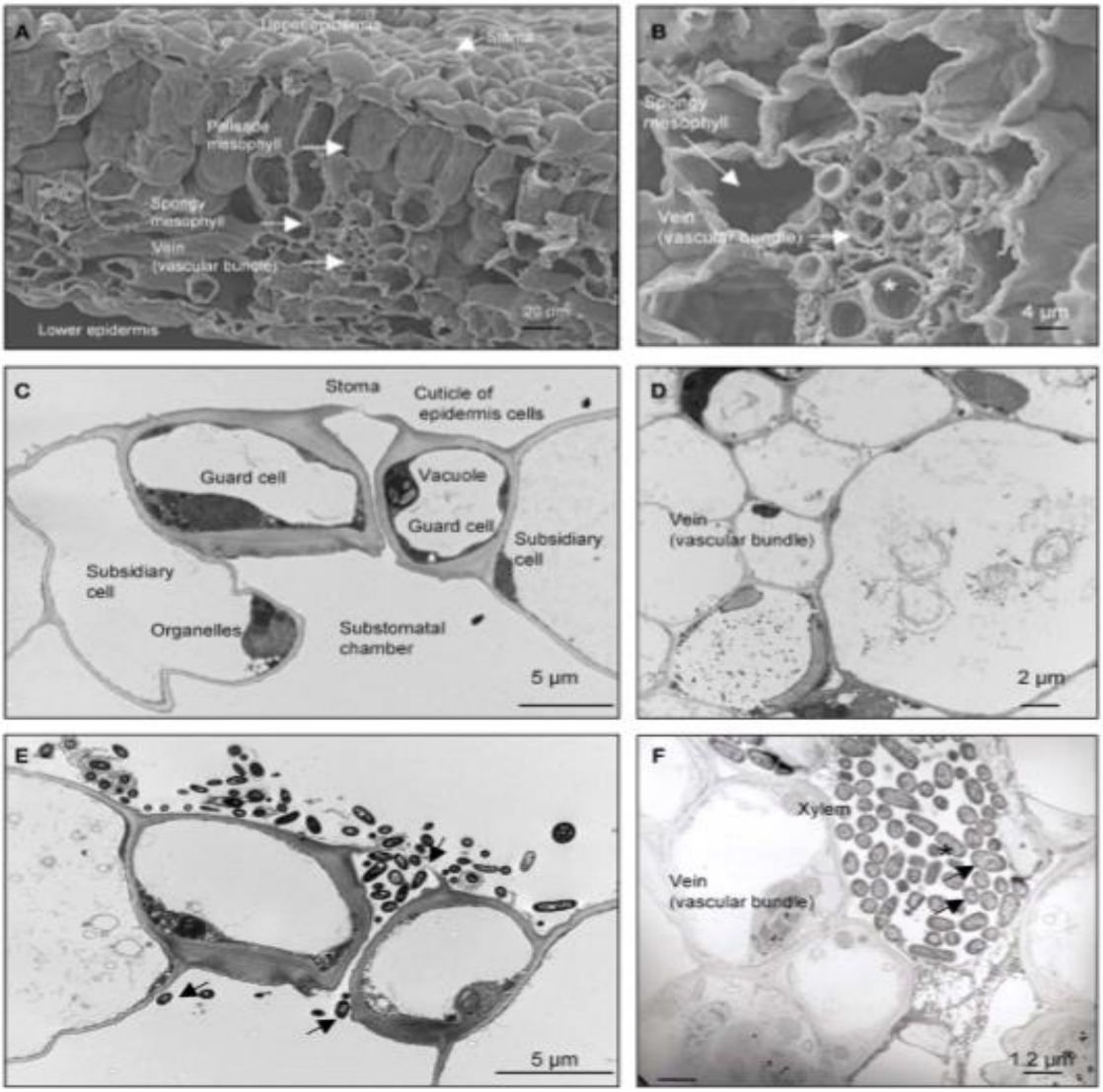


Figure 2.4. Images of scanning electron microscopy (SEM) of spinach leaves showing the presence of STEC in stomata chambers and in leaf vein. A. A cross section of uninfected leaf. B. Close up of the internal tissue of the leaf showing the vascular bundle and spongy mesophyll structures. C and D. Cross section of uninfected leaf after 24 h. E. Cross section of leaf infected with *E.coli* for 24 h showing colonization on the cuticle of the epidermis, stomata and the substomatal cavity. F. Cross section showing internal structures of the leaf and colonization of the xylem with *E.coli* (Saldana et al., 2011).

E. coli O157:H7 was reported to be able to survive as a component of the microbial community, both as epiphytes on leaf surfaces and endophytes within leaf tissues (Jablasone

et al., 2005). Some researchers have suggested that *E. coli* O157:H7 could be absorbed from soil and transported into internal plant tissues through plant roots (Franz et al., 2007; Sharma et al., 2009; Xicohtencati-Cortes et al., 2009).

A study was performed by Saldana et al. (2010) to determine whether or not bacteria have to capacity to avoid decontamination procedures in fresh produce. In the study, they subjected the STEC-inoculated spinach leaves to 0.1–10% of hypochlorite solutions. For comparison, the leaves were also washed with ozonated water. They found that 10% sodium hypochlorite (equivalent to 6,500 ppm) and 125 ppm of ozonated water killed 100 and 99.9% of the bacteria, respectively. However, they noticed that the bacteria could still be recovered from the leaves later on, suggesting that a significant number of bacteria could have been residing in the stomata and other inner parts of the leaves that could have served as protective sites for the bacteria, and survived these sodium hypochlorite and ozone treatments.

Microbial aggregation on leaf surfaces causes production of sufficient quantity of substrates, normally in the form of polysaccharide or other matrix materials. These substrates could protect the microorganism from dehydration and other environmental stresses (Romantschuk et al., 1996). The polysaccharide formed, generally termed exopolyssacharides (EPS), are carbohydrate polymers secreted by bacteria forming a bound capsule layer when associated with the cell wall, or that are released by the cell creating the protective structure for the bacteria (Leight and Coplin, 1992). Bacterial aggregates that exist within a matrix of EPS have been observed on plant surfaces, and these structures are known as biofilms (Morris et al., 1997). The role of biofilms as protective environments for pathogens against the efficacy

of sanitizing agents has been observed for *Salmonella*, *Listeria monocytogenes* and *E. coli* (Drenkard and Ausubel, 2002; Sheffield and Crippen, 2012).

The formation of bacterial biofilm is a function of time (Alhede et al., 2012) and it can be observed normally when cryo-scanning electron microscopy is employed. Figure 2.5 shows images on *Pseudomonas aeruginosa* biofilm development using Cryo-SEM examination. The presence of bacterial biofilms on food and food-processing surfaces can contribute to the increased risk in food safety (Carmichael, 1999). Using staining procedures with confocal imaging techniques Carmichael (1999) revealed that both clusters of bacterial biofilm and individual bacteria existed on lettuce leaf surfaces before processing. However, after processing and four days of storage, the surface colonization including EPS matrix was reduced compared to those in the unprocessed lettuce. Less EPS matrix observed in processed lettuce could be linked to the effect of washing the leaves. However, as the duration of storage was extended, the growth of the bacterial biofilm progressed and it became more extensive and multi-layered. Formation of biofilm could be enhanced by the leaf exudate released by the wounded tissues of RTE lettuce. Koukkidis et al. (2014) observed that the growth and formation of biofilm of salmonella were positively associated with the release of exudate from lettuce leaves. This result suggests that chopping the leafy greens or other fresh produce into smaller pieces would enhance bacterial growth in RTE foods, as smaller pieces of vegetables means more wounds created, resulting in more exudate production.

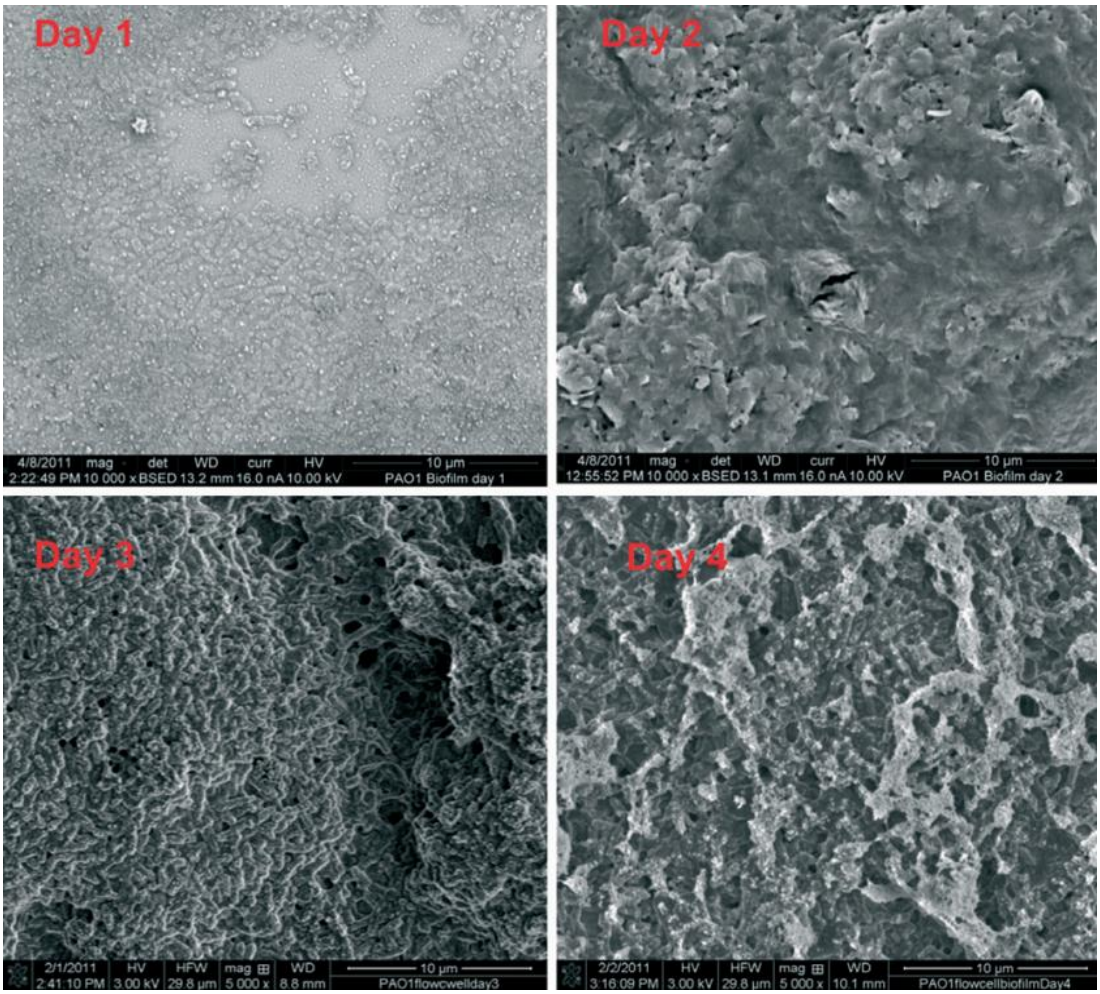


Figure 2.5. Images of *Pseudomonas aeruginosa* biofilm development (Day 1-4) viewed using Cryo-Scanning electron microscopy. Source: Alhede et al. (2012).

2.5 Flavour compounds released from fresh RTE bagged rocket salads

Flavour is an important component in determining the quality and perception of consumer in purchasing fresh RTE rocket salad. Alteration in flavour profiles and concentration generated during storage affects the sensory attributes perceived by consumers when the product is consumed (Bell et al., 2017a; Lokke et al., 2012; Ragert et al., 2004) and this influences repeated buying of the produce.

Perhaps the most important flavour compounds associated with rockets are glucosinolates (GLSs) and their hydrolysis products isothiocyanates (ITCs). Glucosinolates are a group of compounds that serve as aroma pre-cursor in the formation of isothyocyanates after an enzymatic reaction involving myrosinase enzyme. ITCs contribute to the pungent flavour of rocket leaves when the leaves are crushed or chewed. Apart from glucosinolates, other volatile compounds that would have significant impact on the aroma of the rocket are sulphur and nitrogen compounds, esters, alcohols and carbonyls (Blaževic and Mastelić, 2008; Miyazawa et al., 2002). Bell et al. (2016) working with rocket salad (*Eruca sativa*) produced under controlled environment (16 h light, 200 $\mu mol.m^{-2}.s^{-1}$, 20 ^{o}C daytime, 14^{o}C night) identified 42 volatile compounds from 16 chemical groups. The details of the compounds detected by Bell et al. (2016) are presented in Table 2.5.

Table 2.5. Volatile compounds putatively identified in rocket salad grown under controlled environment.

Compounds	LRI	CAS No.	Chemical groups
(E)-4-oxohex-2-enal	989	EPA-374042	Aldehyde
4-Isothiocyanato-1-butene	1021	3386-97-8	Isothiocyanate
1-Penten-3-ol	677	616-25-1	Alcohol
1-Penten-3-one	683	1629-58-9	Ketone
2-(1,1-Dimethylethyl)-1H-indole	1582	1805-65-8	Aromatic compound
2-Methyl-2-butenal	702	1115-11-3	Aldehyde
2-Hexenal	865	505-57-7	Aldehyde
(E)-2-hexenal	858	6728-26-3	Aldehyde

Compounds	LRI	CAS No.	Chemical groups
(Z)-2-penten-1-ol	782	1576-95-0	Alcohol
(E)-2-pentenal	760	1576-87-0	Aldehyde
(E,E)-2,4-hexadienal	933	142-83-6	Aldehyde
5-Ethyl-2(5H)-furanone	1003	2407-43-4	Furanone
3-Ethyl-1,5-octadiene	961	EPA-114877	Alkene
3-Hexen-1-ol	872	3681-71-8	Alcohol
3-Hexenal	810	4440-65-7	Aldehyde
(Z)-3-hexenal	898	6789-80-6	Aldehyde
3-Octyne	921	15232-76-5	Alkyne
3-Pentanone	691	96-22-0	Ketone
5-Methyl-4-hexen-3-one	1073	13905-10-7	Ketone
4-Methylpentyl isothiocyanate	1184	17608-07-0	Isothiocyanate
5-Nonanone oxime	791	14475-42-4	Imine
O-methyloxime-butanal	672	31376-98-4	Imine
1-Isothiocyanato-3-methyl-butane	1082	628-03-5	Isothiocyanate
Ethylidene-cyclopropane	564	18631-83-9	Cycloalkane
Dimethyl-sulphide	570	75-18-3	Sulphur compound
2-Ethyl-furan	696	3208-16-0	Aromatic compound
3-Methyl-furan	605	930-27-8	Aromatic compound
3-Methyl-hexadecane	1658	6418-43-5	Alkane
n-Hexyl-isothiocyanate	1223	4404-45-9	Isothiocyanate

Compounds	LRI	CAS No.	Chemical groups
2-Oxo-hexanoic acid methyl ester	1085	6395-83-1	Ester
n-Pentyl isothiocyanate	1120	629-12-9	Isothiocyanate
Oxalic acid diallyl ester	808	EPA-309229	Ester
Iberverin	1355	505-79-3	Isothiocyanate
Propanoic acid anhydride	1086	123-62-6	Acid anhydride
4-Methyl-2-(2-methyl-propenyl)-pyridine	1424	104188-16-1	Pyridine derivative
Pyrrolidine-1-dithiocarboxylic acid 2-oxocyclopentyl ester	1391	147723-50-0	Sulphur aromatic compound
3-Ethyl-thiophene	885	1795-01-3	Sulphur aromatic compound
Tetrahydrothiophene	826	110-01-0	Sulphur aromatic compound
[Unknown 2]	693	-	–
[Unknown 8]	1129	-	–
[Unknown 9]	991	-	–
Vinylfuran	726	1487-18-9	Aromatic compound

Source: Bell et al. (2016).

Along the supply chain, the aroma of the fresh produce could change due to deterioration of the leaves during postharvest handling and storage, which is highly dependent on time and

storage temperature (Spadafora et al., 2016). Beside storage conditions, pre-harvest factors such as cultivar, growing conditions and cultural practices adopted at farm level also greatly affect flavour of fresh RTE vegetables (Deza-Durand and Petersen et al., 2014, Bell et al., 2016; Bell et al., 2017b).

Storage conditions affect the physiological processes and visual quality of leaves from harvest to consumption (Kader, 2002; Khalil, 2016; Lokke, 2012; Luca, 2016), hence altering the aroma and flavour of the produce. To keep the changes in aroma to a minimum, keeping the fresh produce at low temperature could be the most important practice in order to slow down the overall metabolism that directly extending shelf life (Spinard and Ferrante, 2012). Cantwell and Kasmire (2002) suggested keeping rocket salad at 0 ^{o}C would be necessary to prolong rocket shelf life by 5-6 days when compared to 7 ^{o}C. In the case of wild salad (*Diplotaxis tenuifolia*), Løkke et al. (2012) suggested that under a situation whereby storage temperature cannot be controlled and kept low during distribution and retailing, the leaves must be packed in film that permits sufficient levels of gas exchange for aerobic respiration to occur. Similar with other leafy vegetables, rocket has a short shelf life and the leaves will senesce within four to eight days even when stored at refrigeration temperature of 5 ^{o}C (Ferrante et al., 2004). Other than leaf senescence, an increase of storage temperature reduced leaf vitamin C content and hastened the release of off-odours.

In the supply chain, the requirement of a continuous cold chain is sometimes compromised and therefore off-odour may develop faster than expected, especially at the end of shelf life (Peneau et al., 2007). Rocket is usually transported and stored at 10 ^{o}C. However,

temperature abuse or breach of cold-chain could occur during transportation and display at the retailer's premises (Koukounaras et al., 2007) and this would accelerate the deterioration process of the produce. Figure 2.6 shows a typical temperature profile experienced by most field-grown green vegetables from harvesting to the retailer's premises.

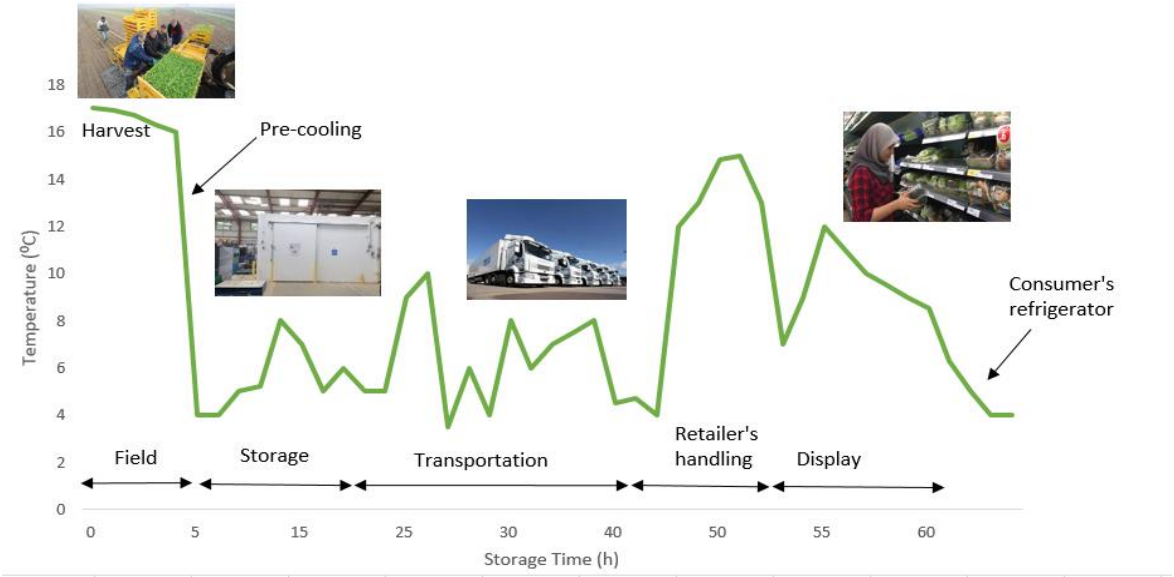


Figure 2.6. A typical temperature profile of green vegetables from the field harvesting to the retailer (Adapted from Koseki and Isobe, 2005).

Rocket displayed and sold at retailers' premises are usually packed in small bags, made of laser perforated film that allows certain levels of gas exchange creating a passive modified atmosphere (MA) to maintain post-harvest quality. Maintaining the quality during the postharvest phase is a concern in industry to reduce losses due to customer rejection. The rejection of the produce may link to both physical appearance such as colour, as well as due to development of off-odour aroma. To elucidate the role of various factors that determine flavour generation in fresh produce, Deza-Durand and Petersen (2014) followed the changes in profile of volatile compounds in RTE iceberg lettuce as affected by growing season,

varieties, packaging and storage duration at 5 ^{o}C. Three different cultivars of minimally processed lettuce grown between June and September were tested and the lettuce were stored at three different conditions: two in passive MA packaging with two different film permeabilities, and another one was left unpacked. Twenty-one potent volatiles including cis-3-hexanol, 2,3-butanedione, elemene and β-selinene were identified from the samples. Other volatiles emitted by lettuce identified were aldehydes, alcohol, terpenes, ketones, acids, sulphur compounds, acetate esters, pyrazine and furan. A higher abundance of cis-3-hexenol was found in MA packages and air-stored samples after day 1 of storage, which could have been associated with the aerobic conditions. On the other hand, the concentrations of 2,3-butanedione, elemene and β-selinene were significantly enhanced under anaerobic conditions after 11 days of storage. Cultivar did not give any significant effects on the volatiles released. They also found that compounds associated with off-odour of lettuce such as 2,3 butanedione hexanal, β-selinene and some other compounds which were characterized as sweet, unpleasant and spoiled vegetables aroma notes were detected after day 11 of storage. Some compounds such as elemene or caryophyllene initially could be regarded as desirable aroma but after a long storage that caused accumulation of the volatiles at a higher concentration, the compounds could then contribute to off-flavour to the produce (Belitz et al., 2009).

Vegetables grown in warmer condition are likely to produce more potent flavour compounds compared to those grown in cooler environment. Lettuce grown in summer months contained high concentration of 2,3 butanedione, caryophyllene, β-selene and elemene due to acceleration of deterioration during storage (Deza-Durand and Petersen, 2014). However, some flavour compounds may appear to be more dominant in lower temperature seasons with long days, resulted from a more conducive environment for the synthesis of amino acids such

as valine and s-methylmethionine which could have acted as precursor for formation of 2-methoxy-3-isopropylpyrazine and dimethylsulfide.

It is well acknowledged that changes of the gas composition of bagged-fresh produce is a result of a complex interaction between permeability properties of the packaging film, respiratory behaviour of the produce, nature of products, quantity of the products in the bags, atmospheric composition of the bags and activity of the microbes (Caleb et al., 2013; Khalil, 2016; Lokke et al., 2012; Martinez-Sanchez et al., 2006). Utilization of packaging film with low gas permeability for produce with higher respiration rate leads to a low oxygen (O_2) condition, thus creates an anaerobic condition (Løkke et al., 2012). Anaerobic respiration usually begins at a low oxygen limit (LOL) of 2 kPa O_2 in most commodities (Kader and Saltveit, 2003; Luca et al., 2016). Under LOL condition, anaerobic respiration begins to dominate over aerobic respiration, and such conditions should be avoided for fresh produce as low O_2 would cause the formation of unpleasant off-odours (Kim et al., 2004; Løkke et al., 2012; Luca et al., 2016; Nielsen et al., 2008). Results of Løkke et al. (2012) shows that the colour of rocket leaf packed in low oxygen transmission rate (OTR) film and stored for 4 days at 10 ^{0}C did not change during storage. However, higher scores for acidity, rotten and smoked odour of the rocket leaves was noted upon the opening of packages as compared to control packages, suggesting that odour of the leaves can change without affecting its colour.

Due to its significance in determining the quality and acceptance of the produce by consumers, research to identify, monitor and reduce the generation of volatiles that cause off-odour of the fresh produce draws attention of research scientists around the globe.

Research results of previous studies clearly showed that availability of O_2 in the packaging markedly affected the generation of VOCs; the release of the VOCs varies between compounds and interacts strongly with storage temperature and time of storage. Luca et al. (2016) performed a detailed study on VOCs produced from wild rocket kept under modified atmosphere, which were stored for 14 days at 5 ^oC and 10 ^oC with two oxygen (O_2) concentration (restricted-O_2 , < 2.1 kPa and moderate O_2 , ≥ 2.1 kPa). They found that initially the VOC abundance in the headspace was low, but later the content increased and 23 VOCs were identified. Among the VOCs identified eight were sulphur compounds, five esters, four alkanes, two alcohols, two furans, one ketone and one nitrogen-containing compound. All 23 volatiles were detected in the headspace of wild rocket stored under restricted O_2 conditions.

Under O_2 restricted conditions, the abundance of nitromethane, pentane, 3-methylfuran, 2-ethylfuran and dimethyl sulfoxide did not change, but the abundance of sulphur compounds, i.e. carbonyl sulfide, methanethiol, carbon disulfide, methyl isothiocyanate and dimethyl trisulfide increased as the partial pressure of O_2 dropped below <2.1 kPa. A similar trend of sulphur compound generation was reported to occur in other brassicas under LOL condition (Di Pentima et al., 1995; Forney and Jordan, 1999; Lokke et al., 2012).

Sulphur compounds, carbonyl sulfide, carbon disulfide and methyl isothiocyanate have previously been reported produced in wounded rocket (Bell et al., 2016), as the wounding would lead to formation of volatile compounds *via* enzymatic reaction. Upon wounding, the enzymes will be in contact with the substrates in plant cells that caused the reactions

(Christensen et al., 2007). Detection of volatiles such as methyl acetate and ethyl acetate in packed salad, especially under moderate O_2 at 10 ^oC, could explain the significance of wounding in affecting flavour compound generation. Upon wounding pentane, a product of lipid peroxidation that is often associated with wounding of plant tissues (Belitz et al., 2009), is normally detected in rocket leaves (Bell et al., 2016). Formation of methyl- and ethyl acetate esters during prolonged storage of wild rocket was due to a loss of cell integrity and mixing of enzymes and substrates (Luca et al., 2016). Loss of cell integrity, and hence texture during or after storage of wild rocket, was recorded occurred under restricted O_2 conditions ($O_2 \leq 0.5$ kPa) (Lokke et al., 2012). Compared to those kept under restricted O_2 levels, the abundance of some compounds, methyl acetate, ethyl acetate, methyl propionate, methyl 3-methylbutanoate, and ethyl 3-methylbutanoate esters produced by wild rocket was very low during storage at 10 °C under moderate O_2 conditions.

Ethanol, an alcohol, is often increasing during storage of bagged RTE vegetables (Luca et al. (2016). This phenomenon may provide explanation on the effect of LOL condition and a shift from aerobic to anaerobic respiration resulting in the release of acetaldehyde and ethanol (Kays and Paull, 2004).

Besides being released by the fresh produce through enzyme and substrate reaction, VOCs detected in the flavor headspace of bagged green vegetables could also be generated by the microbes. As an example, acetone has also been reported as a compound of microbial origin (Borjesson et al., 1992; Sunesson et al., 1995). The presence of microbes in the system would exaggerate the generation of VOCs. In rocket, Nielsen et al. (2008) observed an increase in the concentration of dimethyl sulfide and dimethyl disulfide from rocket salad (*Eruca sativa*)

inoculated with aerobic microorganisms and stored under moderate O_2 conditions. Microbes, both fungi and bacteria, are naturally occurring in the salads, even though the produce are thoroughly washed before packaging. If the produce are not kept under low temperature, beside high respiration and degradation of chemical compounds *via* enzymatic reaction, rapid growth of these microbes could accelerate the generation of off-odour VOC. Luca et al. (2016) noticed that storage temperature had great impact in affecting the formation of carbon disulfide, nitromethane, 3-methylfuran, and 2-ethylfuran concentration in rocket. Bell et al. (2017b) observed that breaks in the cold-chain, coupled with high temperature as experienced in summer in the UK, have caused high bacterial load in bagged RTE rocket salads. Similar observations on the effects of high temperature on bacterial growth was also reported by Spadafora et al. (2016).

Furans (2-ethylfuran and 3-methylfuran) generation from wild rocket were found to be associated with wounding in response to cell damage during long storage under moderate O_2 conditions (Luca et al., 2016). These furans may also be produced by fungi (Börjesson et al., 1992) following oxidation of isoprene (Gu et al., 1985; Helmig et al., 1998) or as degradation products of catechol and phenol (Huber et al., 2010).

High abundance of dimethyl sulfide and dimethyl disulfide in bagged green vegetables under high temperature and after long storage could also be associated with microorganisms present on the leaves (Nielsen et al., 2008). It is well acknowledged that dimethyl sulfide and dimethyl disulfide have low values of odour threshold, giving off-odour, unpleasant aroma that are described as intense onion, wild radish, decayed- or cabbage-like (Bell et al., 2017a; Nagata, 2003). Such odours can negatively influence product quality. Lokke et al. (2012)

reported that the intensity of rotten odour was increased in packaged wild rocket (*Diplotaxis tenuifolia*) during storage at high temperature for prolonged period of time. Beside bacteria, mould is known to be involved in the transformation of organic compounds in leafy vegetables, thus contributing to development of off-odour of fresh produce. For example, Demyttenaere and De Pooter (1996) revealed that *Penicilium digitatum* was involved in transformation of geraniol and nerol to 6-methyl-5-hepten-2-one. Other moulds that have contributed to the development of off-odour volatiles in fresh produce include *Aspergillus niger* and *Penicillium roqueforti* which degrade triglycerides to methyl ketones by spores (Demyttenaere and De Pooter, 1996).

The release of S-compounds arises from the action of the enzyme L-cystein sulfoxide lyase or alliinase on alk(en)ylcysteine sulfoxide. As mentioned earlier, the enzyme and substrate are normally separated in plant cells; however, when the cell structure was damaged or collapsed during processing or handling, or by microbial activity, the enzyme mixes with the substrate leading to the flavour-forming reactions. The main substrate is S-methyl-L-cysteine sulfoxide with the primary product of methyl sulfenic acid, which is unstable then decomposes to produce a range of compounds including methyl thiosulfinate, methyl disulfide, methyl thiosulphonate, methyl sulfide and methyl trisulfide (Saxby, 1993).

2.6 Glucosinolates detection and changes throughout the supply chain

Rocket is often considered as a good source of antioxidant and anticancer molecules, including glucosinolates (GLS) and other compounds that belong to sulphur compounds and polyphenols (Cavaiuolo and Ferrante, 2014; Ombra et al., 2017). GLSs are derived from

amino acid biosynthesis (Bellostas et al., 2007; Chen and Andreasson, 2001; Podsedek, 2007). Typically, flavour and odour of brassicas are related to their GLS contents (Martinez-Ballesta et al., 2013; Padilla et al., 2007). Glucosinolates are nitrogen and sulphur rich anionic secondary metabolites. GLSs are also known as b-thioglucoside-*N*-hydroxysulfates, *cis*-*N*-hydroximinosulphate esters or *S*-glucopyranosyl thiohydroximates (Cataldi et al., 2007). Based on the R-group, GLSs are divided into three groups: aliphatic, aromatic and indolyl (Wallsgrove et al., 1999).

In the plant cell, glucosinolates are situated in the vacuole (Helmlinger et al., 1983). When they are exposed to the myrosinase enzyme (β-thioglucoside glucohydrolase, TGG) upon tissue disruption and cell damage, GLS are hydrolysed to produce isothiocyanates (ITCs), thiocyanates, nitriles and sulfates (Figure 2.7). These GLS derivatives together with GLSs are responsible for the sharp pungent taste and smell of brassicas (Bell et al., 2017a; Chin and Lindsey, 1994).

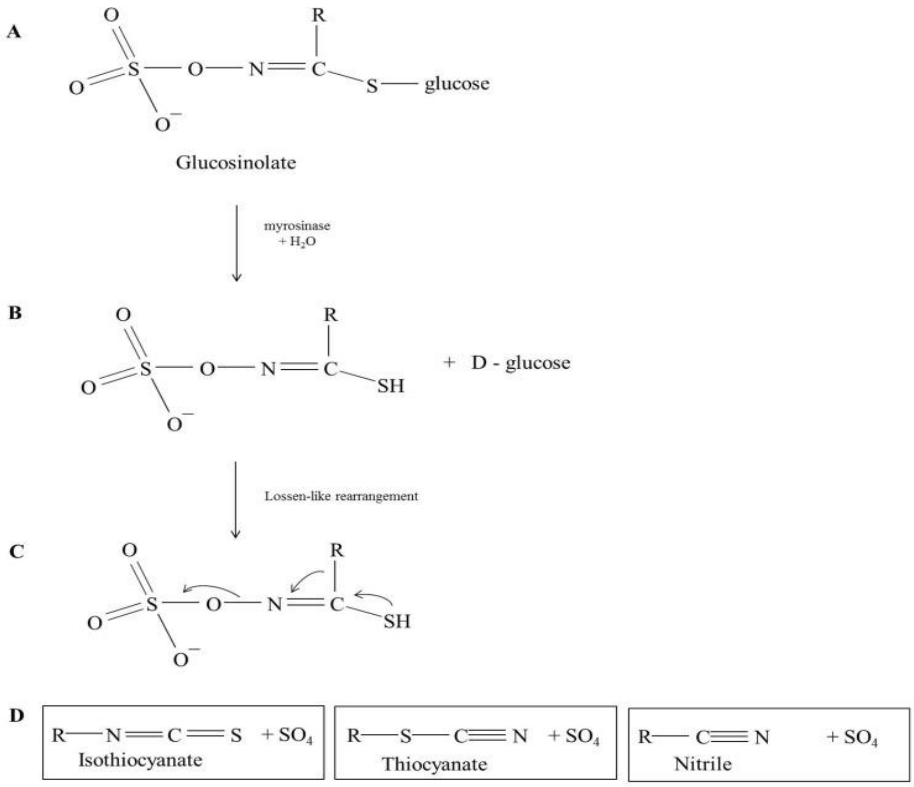


Figure 2.7. Hydrolysis of glucosinolates by myrosinase (**A**) producing D-glucose and aglycone (**B**); Aglycone undergoes a Lossen-like rearrangement (**C**) releasing isothyocianate, thyocianate or nitrile and sulphate (**D**). Source: Cavaiuolo and Ferrante (2014).

Commonly found GLS in *E. sativa* and *D. tenuifolia* leaves are presented in Table 2.6 (Bell et al., 2015). The glucosinolate profiles of rocket are highly variable but in both rocket species, aliphatic GSLs normally contribute to more than the 80% of total GSLs. Glucoraphanin, glucoerucin and dimeric 4-mercaptobutyl-GLS (DMB) are consistently present in relatively high concentration in rocket leaves (Cataldi and Rubino, 2007; D' Antuono et al., 2008), while glucoerucin is the predominant GLS found in seeds and roots, and glucosativin is the GLS mostly found in flowers (Bennette et al, 2006). The glucosinalbin is limited to the roots of wild rocket *(D. tenuifolia*) and it is not detected in rocket salad (*E. sativa*) (Bennette et al., 2006).

Table 2.6. Glucosinolates detected in different varieties and accessions of *Eruca* and *Diplotaxis*.

Common name	R-group
4-Hydroxyglucobrassicin	4-Hydroxy-3-indolylmethyl
Glucotropaeolin	Benzyl
Glucolepiidin	Ethyl
Glucoraphanin	4-(Methylsulfinyl)-butyl
Glucoiberverin	3-(Methylthio)-propyl
Glucosativin	4-Mercaptobutyl
DMB	Dimeric-4-mercaptobutyl
Glucoalyssin	5-(Methylsulfinyl)-pentyl
Glucoerucin	4-(Methylthio)-butyl
Glucoraphenin	4-Methylsulfinyl-3-butenyl
Diglucothiobeinin	4-(β-D-Glucopyranosyldisulfanyl)-butyl
Glucoibarin	7-(Methylsulfinyl)-heptyl

Source: Bell et al. (2015).

The concentration and the chemical groups of GLSs are very dependent on the plant species and variety, growing conditions and cultural practices (Bell et al., 2015; Bennette et al, 2006; Verkerk et al., 2009). Previous studies also revealed that the contents of glucosinolates is altered during storage and this is dependent on postharvest conditions, especially temperature and period of storage (Force et al., 2007; Selma et al, 2010).

During storage, Force et al. (2007) reported that the total GLSs in leaves of *E. sativa* increased up to the third day of storage and this occurred at either 4 °C or 15 °C, but the GLS then decreased thereafter. Selma et al. (2010) observed a similar trend of changes in glucorapahnin and glucoerucin in 7-days old rocket sprouts whereby both GLS were found to decline after 7-days of storage at 4 °C. Glucoerucin and glucoiberverin have been shown to decrease significantly during shelf life, while glucoraphanin increased over the storage period of *E. sativa* and *D. tenuifolia* (Cavaiuolo and Ferrante, 2014).

As highlighted earlier, hydrolysis of GLSs by myrosinase will give rise to a variety of isothiocyanates (ITCs) (Cavaiuolo and Ferrante, 2014). Hydrolysis of glucotropaeolin yields benzyl isothiocyanate, gluconasturtin to phenethyl isothiocyanate and sinigrin to allyl isothiocyanate. Indole glucosinolates give rise to 3-indolemethanol, 3-indoleacetonitrile, 3, 3′-diindolylmethane and indole-3-carbinol (Labague et al., 1991). Erucin or 4-methylthiobutylisothiocyanate which is formed from the hydrolysis of glucoerucin, is one the main ITC found in rocket. In addition, erucin is also produced through the reduction of 4-(methylsulfinyl) butyl isothiocyanate (also called sulforaphane). Sulforaphane is originated from glucoraphanin. Iberin, formed from glucoiberin, was detected in *E. sativa* but not in *D. tenufolia* (Villatoro-Pulido et al., 2013). Goitrin is an oxazolidine-2-thione that derives through a spontaneous cyclizing of ITCs containing a hydroxy group from 2-hydroxybut-3-enyl glucosinolate (Hanley et al., 1990).

Using Gas Chromatography-flame Ionization Detector (GC-FID) and Gas Chromatography-mass Spectrometry (GC-MS) analyses, Jirovetz et al. (2002) identified up to 50 volatile constituents from fresh leaves of *E. sativa*. Among them 4-methylthiobutyl

isothiocyanate, *cis*-3-hexen-1-ol, *cis*-3-hexenyl butanoate, 5-methylthiopentyl isothiocyanate, *cis*-3-hexenyl 2-methylbutanoate, and 5-methylthiopentanenitrile were the most abundant ITCs detected. Similar study was performed by Pasini et al. (2012) where glucosinolate and phenolic profiles of 37 rocket salad accessions (32 *E. sativa* and five *D. tenuifolia*) were detected by Liquid Chromatography–Mass Spectrometry (LC-MS). They isolated seven disulpho-glucosinolates and reported the GLS profiles did not differ between species, but GLS concentration varied greatly among accessions. As an example, Pasini et al. (2012) reported the concentration of total GLS ranged from 756.0 to 2459.0 $mg.kg^{-1}$ DW and from 1164.2 to 3031.1 $mg.kg^{-1}$ DW present in *E. sativa* and *D. tenuifolia* accessions, respectively.

Pasini et al. (2012) revealed that three types of GLS, glucoraphanin, DMB and glucoerucin were present in consistently high concentration in all rocket accessions tested, with glucoraphanin contributed up to 17.6 to 63.0% of the total GLS. DMB together with diglucothiobeinin present in the range of 12% to 49.5%. Glucoerucin content ranged from 7.7% to 28.1%. Indole 4-OH-glucobrassicin and glucobrassicin, and aromatic glucosinalbin derived compounds represented a lower proportion in total glucosinolates. Glucosinalbin was the only aromatic glucosinolate detected. Although it was a less frequently detected compound, it was the only compound that could be used to differentiate between the two species. The relative content of glucosinalbin in *Eruca* ranged from 0.2 to 1.5 $mg.kg^{-1}$ DW, and the respective values for *Diplotaxis* ranged from 6.4 to 27.3 $mg.kg^{-1}$ DW.

Most of GLSs identified in rocket were found also in other brassicas (Cartea and Velasco, 2008; Vig et al., 2009). Glucoraphanin, glucoerucin, progoitrin, 4-OH-glucobrassicin and

glucobrassicin were identified in relatively high concentration in cabbage, cauliflower, broccoli, brussels sprouts (Pasini et al., 2012), *Brassica rapa* and *B. napus* (Cartea and Velasco, 2008), and in Chinese cabbage (Vig et al., 2009). In detecting GLS in brassicas, Pasini et al. (2012) noticed that differences in results reported is greatly influenced by the analytical methods adopted by the individual laboratory.

Bell at al. (2017b) measured the concentration of GLSs in of six underutilised accessions and one commercial variety of rocket in a commercial supply chain with details accounts on pre-harvest practices, harvesting, postharvest transportation (from farm to factory), postharvest handling and storage condition and duration. The changes in GLS concentration of rocket leaves kept at 4 ^{o}C were monitored on day 0, 2, 5, 7 and day 9. Overall, Bell et al. (2017b) found that total GLS concentration in rocket leaves increases with storage time. They found glucoerucin and glucoraphanin, which are among the most abundance types of glucosinolates in rocket were not reduced by processing, suggesting that GLSs are not lost due to leaching or affected by reaction of myrosinase in wash water. Lack of any significant changes in glucoraphanin concentration observed by Bell et al. (2017b) is in agreement with the result previously reported for broccoli florets (Winkler et al., 2007). This GLS seems to be more stable than other GLSs found in rocket. The increase in GLS concentration observed during storage could be a positive reaction of the leaves in response to both abiotic (wounding, environmental changes – such as storage temperature) and biotic (microbial/pathogen attacks) stresses exerted on the leaves and the conversion of pre-existing precursors to GSL continued in the harvested leaves.

GLSs are often regarded as metabolites that normally produced by plants as part of defence mechanism (Jahangir et al., 2009).

Extracts of *E. sativa* were shown to have antimicrobial activity both on Gram negative bacteria *(Escherichia coli*, *Pseudomonas aeruginosa* and *Shigella flexneri*) and Gram positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) (Khoobchandani, et al., 2010). Similarly, extracts obtained from flowers of *E. sativa* was found effective against many bacterial species (*Bacillus subtilis*, *Staphylococcu aureus*, *Enterococcus faecalis*, *B. thuringiensis*, *Micrococcus luteus Klebsiella pneumoni*, *E. coli*, *Salmonella typhimurium*, *Enterobacte*r sp. and *Pseudomonas aeruginosa*) (Koubaa et al., 2015). The antimicrobial activity of the *Eruca* extract could have been associated with the presence of high concentration of GLSs and isothiocyanates.

Among GLS derivatives, sulforaphane which is derived from 4-methyl-sulfinylbutyl-glucosinolates (glucopharanin), has been the focus of many researches because of its potential to serve as anti-carcinogenic agent (Hayes et al., 2008; Juge et al., 2007; Ombra et al., 2017), as well as for its functional properties as an antimicrobial agent to depress the growth of *Helicobacter pylori*, a potential causal agent of stomach cancer (Fahey et al., 2002; Haristoy et al., 2003). Another glucosinolate, sinigrin, has been reported to have little effect on the growth of microorganisms, but its hydrolysis products resulted in inhibition of growth of *E. coli* (Barbban and Edward, 1995). Therefore the efficacy of the glucosinolates in reducing the growth of bacteria is well acknowledged, but variation among rocket varieties in affecting the growth of bacteria may exist due to their differences in GLS content.

Furthermore, association between individual GLS found in rocket and their possible antimicrobial properties has never been reported before.

2.7 Concluding remarks

Due to the nature of the produce, consumption of fresh RTE vegetables is often regarded as a potential risk factor for infection with pathogenic microbes. Source of contamination of the RTE vegetables are numerous which include use of organic fertilizers, use of contaminated water or due to cross contamination that link to poor worker hygiene. The growth of bacteria could be aggravated to the levels that would cause illness to human when the produce are not kept continuously under a cold-chain. The effect of temperature abuse during postharvest handling and storage on fresh RTE vegetables is well established, but not on RTE rocket salads, especially in view of its complex supply chain.

Besides looking at the impact of different temperature and duration of storage on abundance of microbes, their effect on the formation of biofilms and internalization of the microbes in phyllosphere is important in view of the effectiveness of the sanitation processes to deactivate or remove the microbes. Understanding of microbe-leaf surface interactions will lead to the formulation of better control strategies to limit the survival of pathogen in the phyllosphere. Besides, it is also critical to identify types of microbes present in the leaves. Information on species of microbes would indicate the potential risk to human health from the produce.

Increase in microbes on packed fresh RTE vegetables alters both physical and chemical properties of the produce. This would produce great impacts on generation of volatile flavour

compounds, which affects consumer perception, especially when it involves generation of off-odour compounds. In brassicas, including rocket, one of the phytochemical typical for the members of this family is glucosinolate. GLS is always being associated with hot peppery taste of rocket (Martinez-Sanchez et al., 2007). GLS was reported to have strong antioxidant, anticancer and antimicrobial properties (Fahey et al., 2002; Koubaa et al., 2015; Ombra et al., 2017). Change in GLS composition in the leaves would affect the above-mentioned properties. Therefore, the impact of inappropriate handling and storage condition on flavour compounds, including GLS that occur along the supply chain will be elucidated in this study.

In view of above scenarios, a study presented in this thesis aiming at evaluating the effects of handling and storage conditions of rocket on the microbial load, quality attributes and release of volatile organic compounds in the context of the supply chain; discovery of the natural distribution of bacteria and formation of biofilms in fresh RTE rocket salads; to elucidate the diversity and identify naturally occurring microbes (human and plant pathogens, and food spoilage microbes) that exist in fresh RTE rocket salads at various phases of a simulated supply chain; and to examine the antimicrobial properties of glucosinolates found in rocket leaves on naturally occurring bacteria and on a cultured species of *E .coli* was performed.

CHAPTER 3

CHANGES IN BACTERIAL LOADS, GAS COMPOSITION AND VOLATILE ORGANIC COMPOUNDS OF FRESH BAGGED READY-TO-EAT ROCKET IN THE SUPPLY CHAIN

The works in this chapter are presented in three subchapters which were divided according to their sample materials and treatments.

3.1 Introduction

Fresh-cut fruits and vegetables, including rocket (*Eruca sativa* and *Diploxtaxis tenuifolia*) are one of the RTE foods which are rapidly gaining in popularity; most of them are considered nutritious, healthy, convenient and ready to eat. Fresh RTE foods are prepared without heat treatment. With its high water activity, rich nutritive values and near neutral pH, the foods can become ideal media for microbial growth.

Processing of fresh RTE foods involves many steps including those that may cause tissue to wound and collapse. Wounds inflicted during the preparation of fresh-cut promote many physical and physiological changes that hasten loss of product quality (Brecht 1995, Saltveit 1997), among others leading to the removal of the protective epidermal layer and exposure of internal cells. These changes would provide easy access for microbial pathogens.

Wounding and increased microbial population would alter physiological processes, including increased respiration and ethylene production, and also affect synthesis and accumulation of

certain phytochemicals that eventually change the biochemical profile of the leaf (Barth et al., 2009). All these will affect physical appearance, flavour and the concentration of beneficial phytochemicals of the RTE products.

The microbiological quality and safety of the produce have become major concerns to both service provider and consumer. The US Food and Drug Administration Food Code recommend that packaged fresh-cut leafy green vegetables be kept at 5 ^{o}C or below at in transport, storage and retail display (Kou et al., 2014). However, in the real supply chain situations, a continuous cold-chain to keep the fresh RTE food would be difficult to achieve (Kou et al., 2014; Koseki and Isobe, 2005). Slightly higher temperatures will be experienced during transportation and handling from the factory to the retailers, display at the retailer's premises and during transport home after purchase by the consumer. Some of the packages with same expiry date on the display shelf may be purchased later than the others. Once bought, the consumer may not consume all of the product in one siting and may keep it their home refrigerator for later consumption. All these practices certainly would impact the microbial loads, freshness, nutritive values and aroma of the products.

The effect of temperature on microbial load, quality and generation of flavour compounds of fresh RTE foods and vegetables, include rocket salads, are well studied. However, most of the studies are conducted under constant temperature regimes. In contrast, the studies reported in this chapter involved treatments that simulate various phases that normally occur along the supply chain of fresh RTE rocket and their impacts on microbial loads, quality, physiological processes and volatile organic compounds. A special emphasis is given to evaluate the effects of the treatments on glucosinolates due to their role in determining the

peppery taste of the rocket (Pasini et al., 2011; Luke et al., 2016) , and their anticancer (Hayes et al., 2008; Ombra et al., 2017) and antimicrobial properties (Khoobchandani et al., 2010; Koubaa et al., 2015).

High temperature would normally enhance multiplication of mesophiles and psychrotrophs thus increasing the abundance of bacteria present on the leaves, especially when the situation is prolonged. As the growth of bacteria is functional to time of storage, brief exposition to high or abused temperature is expected to affect microbial loads. Leaves in opened bag and that have been disturbed by hands could have caused cross-contamination and therefore the leaves would contain a higher bacterial load. Due to the role of temperature in affecting bacterial growth, summer grown is expected to contain relatively higher bacterial loads compared to those produced in winter. However, winter grown rocket is expected to have lower respiration rate and ethylene production. Higher bacterial loads will speed up the spoilage of the leaves, thus leaves with high bacterial counts would contain higher concentration of off-odour volatile organic compounds. Variation in bacterial load is expected to affect glucosinolate contents in leaves.

3.2 Subchapter 3a: Growth of bacteria at different storage conditions

3.2.1 Materials and Methods

These experiments are considered a preliminary study to inform future supply chain simulation experiments (Section 3.3). Two experiments were carried out to determine the impact of different temperature and storage conditions on the fresh RTE wild rocket.

3.2.1.1 *Plant materials and experimental treatments*

In the first experiment, bagged fresh RTE wild rocket (*Diplotaxis tenuifolia*) was obtained from Sainsbury's, Reading. Each bag contained 70 g wild rocket. Based on the labels on the bags, the rocket was grown in Portugal. The leaves were then subjected to three different storage temperatures: 4 ^{o}C (standard refrigeration temperature), 13 ^{o}C (display temperature at retail market) and 20 ^{o}C (room temperature). According to Koseki and Isobe (2005), although the display temperature was set at 7 ^{o}C, but the temperature history of the lettuce in his study from farm to the retail store (Figure 2.3) showed that the display temperature varies roughly from 7-13 ^{o}C. Therefore, 13 ^{o}C were selected as the retail's display temperature. 20 ^{o}C temperature was used as a room temperature based on Bell et al. (2017) where the temperature of rocket samples they received from the supplier was 21.7 ^{o}C. Samples were kept in the respective temperature treatment for six days and total plate count were evaluated for every two days intervals. Each experimental unit was comprised of one bag (70 g).

In the second experiment, 57 bags for fresh RTE wild rocket samples purchased from Sainsbury's, Reading, also produced in Portugal, were subjected to a two-factors factorial experiment: three storage temperatures (4, 13 and 20 ^{o}C which represent refrigeration, display temperature at retail stores and room temperature, respectively), and two storage packaging conditions (opened and closed bag) for six days. The rocket stored in opened bags was given a contact with un-gloved hands to simulate the situation of a consumer's prior handling of the product before further consumption. To avoid biasness, six persons that were available around the Department of Food and Nutritional Sciences, University of Reading

were asked to disturbed the samples. The microbial analyses were done at two-day intervals beginning from day 0 until day 6.

Both experiments were run with three replicate bags at each sampling point.

3.2.1.2 *Microbial count analysis*

Apparatus

Walk-in 30 ^{o}C incubator, stomacher (400 Circular Seward), pipettes (Gilson 1.0 ml), weighing scale (Mettler, Sartorius BL 310), pipette tips, 500 ml Scott Duran bottle, autoclave (Tactrol, Priorclave), petri dishes, colony counter (Gallen Kamp), 13 ^{o}C refrigerator (LMS cooler incubator), 4 ^{o}C cold room, 50 ml falcon tube, magnetic stirrer, measuring cylinder, stomacher bag, universal bottle.

Preparation of nutrient agar for total plate count

11.75 g of Standard plate count agar (Oxoid Ltd, Basingstoke, Hampshire, UK) was diluted in 500 ml of distilled water in 500 ml Scott Duran bottle and was stirred on a hot plate using a magnetic stirrer until boiling giving the concentration of 2.4% (w/v). Then, it was sterilized for 15 min at 121 ^{o}C. The media were kept in 45 °C water bath to maintain the liquid state. The media were transferred in Eppendorf tube prior of usage and temperature were taken using sterile thermometer to confirm the temperature did not exceed 45 °C.

Preparation of maximum recovery diluent for sampling preparation

9.5 g of maximum recovery diluent (MRD) (Sigma Aldrich, Missouri, USA) was diluted in 1 L of distilled water (0.95%) (w/v) in 1 L Scott Duran bottle and stirred using magnetic stirrer until completely dissolved. MRD diluents were poured into 100 ml Scott Duran bottle with 90 ml in each bottle. Then, it was sterilized for 15 min at 121 ^{o}C. The mixture was cooled down in laminar flow hood before being used or was kept in 4 ^{o}C cold room for longer term storage.

Preparation of maximum recovery diluent for enumeration

9.5 g of MRD was diluted in 1 L of distilled water in 1 L Scott Duran bottle and stirred using magnetic stirrer until completely dissolved. MRD were transferred in bottle with each bottle containing 9 ml of MRD. Then, it was sterilized for 15 min at 121 ^{o}C. The mixture was cooled down in laminar flow before being used kept in 4 ^{o}C cold room for longer storage.

Total plate count

10 g of leaves were added to 90 ml of MRD in a stomacher bag and shaken for 120 sec, and this will create 10^{-1} dilution (w/v). 0.1 ml of the homogenized/inoculum was sampled from the bag and it was serially diluted to obtain 10^{-2}, 10^{-3}, 10^{-4} until 10^{-7}. The homogenized (1 ml) was sampled from the bag and it was serially diluted to obtain 10^{-1}, 10^{-2} and 10^{-3} dilution. Then 1 ml of the respective solution was placed on the nutrient agar plates using pour plate

technique under temperature <45 ^{o}C and the plates were swirled to mix evenly. The inoculated plates were allowed to cool at room temperature until the liquid solidified. The plates then were incubated at 30 ^{o}C in inverted condition. After 72 ± 3 h of incubation, number of colonies per plate were counted using a colony counter. Plates with colonies more than 300 colonies are labelled with TNTC (too numerous to count) and plates with colonies less than 30 colonies were discarded.

3.2.1.3 *Data analysis*

Data on total plate counts were subjected to analysis of variance (ANOVA) using Statistical Analysis System ver. 9.3. (SAS Institute, Cary, NC, USA). Mean differences between treatment were compared using Duncan's Multiple Range Test at $P<0.05$. Graphs were then plotted using Excel spread sheet.

3.2.2. Results

3.2.2.1 **Growth of bacteria at different storage conditions**

Differences in storage temperatures significantly affected the bacteria growth as shown in their total plate counts (TPC) in rocket salads (Figure 3.1). Rocket kept at 4 ^{o}C contained the least amount of bacteria and demonstrated the slowest growth rate. The amount of microbes increased from 7.23E+08 to 3.08E+09 cfu.g^{-1} as the storage period was extended from 0 to day 6. The growth rate at 4 ^{o}C was extremely slow compared to the growth of bacteria on

rocket kept at 13 and 20 ^{o}C. At 13 ^{o}C, number of counts increased from day 0 to day 4 which has increased from 7.23E+08 to 9.48E+09 cfu.g^{-1}. However, from day 4 to day 6, there was a slight decrease in counts which was dropped by about 13.3% to 8.22E+09 cfu.g^{-1}.

Total plate counts for rocket kept at 20 ^{o}C have a similar growth pattern to rocket kept at 13 ^{o}C but with a higher growth rate. The TPC of bacteria from rocket kept at 20 ^{o}C increased from 7.23E+07 cfu.g^{-1} at day 0 to 1.10 E+10 cfu.g^{-1} at day 2, then to a maximum high of 2.13E+10 cfu.g^{-1} before declining to 1.32E+10 cfu.g^{-1} at day 6. Results recorded in this experiment is as expected as the leaves were kept at a higher temperature, the range used here would promote the growth of the bacteria.

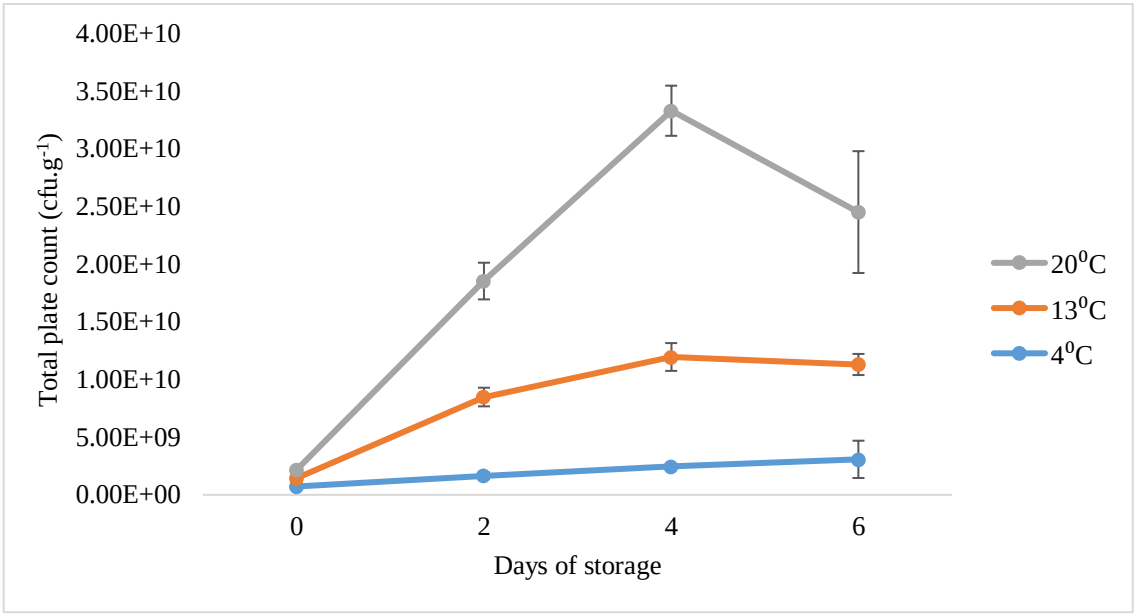


Figure 3.1. Total plate count in rocket salad during storage at 4, 13 and 20 ^{0}C within six days of storage. (n=30; bars indicate standard error of means)

Results of the second experiment show that storage temperature and duration, and packaging conditions (opened/closed bags) markedly affected the growth of bacteria on the fresh RTE rocket salads ($P<0.0001$). The results also clearly show that all three factors interacted significantly in affecting the bacterial growth ($P<0.0001$, Table 3.1).

For leaves kept at 4 ^{o}C, the TPC with different bag conditions did not change significantly from day 0 to day 4. However at day 6, the counts for rocket in closed and opened bags were significantly different as shown by significant interaction between packaging conditions and days of storage. The counts for opened bags were lower compared to the closed bags at day 6. Although the counts of microbes in rocket kept in opened bags was relatively low compared to those in closed bags, the difference in the count was not large enough to prove any significant different between treatments. Overall, the TPC for the rocket kept at 4 ^{o}C were relatively lower compared to the value of TPC in rocket with closed bags stored at 13 ^{o}C and 20 ^{o}C.

The results in Figure 3.2b show the relationship between bacterial counts in rocket salad in both closed and opened bags within 0 to 6 days stored at 13 °C. Initially, the increase in bacteria counts was not significant from day 0 to day 2 for both closed and opened bags. Then the growth trend changed after day 2 where the counts of the bacteria increased from 2.05E+09 on day 2 to 1.87E+10 cfu.g^{-1} on day 4, for rocket salads kept in closed bags. The corresponding TPC for rocket kept in opened bag were 1.55E+09 to 2.08E+10 cfu.g^{-1}, respectively, but the difference in TPC of the two bags at day 4 was not differed significantly. The TPC for rocket kept in opened bags has increased markedly to 7.30E+09 cfu.g^{-1} at day 6, as compared to only 1.66E+09 cfu.g^{-1} for rocket in closed bags. The result of bacterial

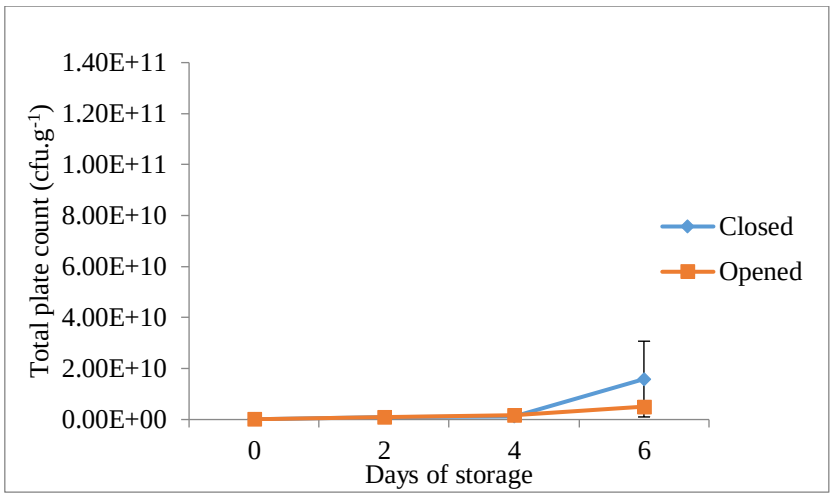
counts of rocket during six days of storage at 20 °C showed that bacteria growth in both opened and closed bag condition increased exponentially from day 0 to day 6 (Figure 3.2c). The TPC for the opened bags was significantly higher compared to those in the closed bag. At day 6, TPC for closed bag was 5.29E+10 cfu.g^{-1} whereas the respective value for opened bag was 1.18E+11 cfu.g^{-1}.

Result recorded for samples kept at 20 ^{o}C were as expected where the overall bacterial counts in samples stored with opened bags were higher than those with closed bags. As expected, the results clearly show that the growth of bacteria were significantly affected by all factors imposed in the study, i.e. storage temperature and duration, as well packaging conditions. However, the changes in bacteria growth pattern in response to one factor are interdependent with the other two factors as shown by significant interaction among them. At 4 ^{o}C and 13 ^{o}C, TPC values for the opened bags was not necessarily higher compared to those in closed bags, but samples in opened bags stored at 20 ^{o}C had higher TPC value compared to TPC in closed bags at any day within the storage period.

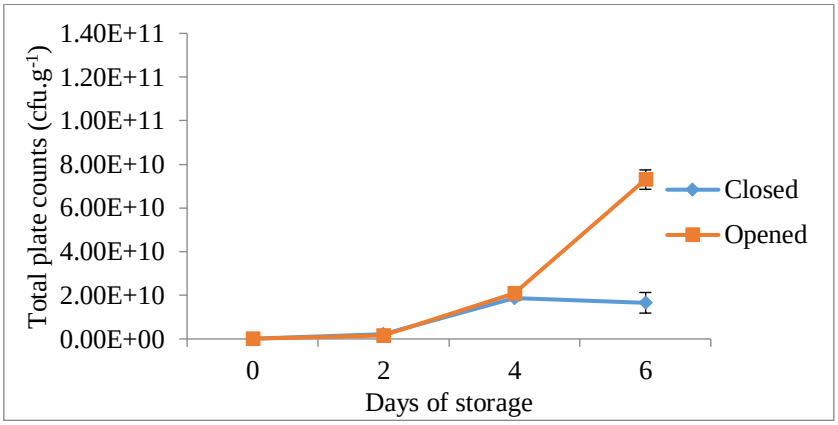
Table 3.1. Changes in total plate count in closed and opened bag at storage duration at 4°, 13° and 20 °C.

Temperature	Packaging	Days	Total plate counts ($cfu.g^{-1}$)
4°C	Closed	0	1.20E+07
		2	1.04E+08
		4	1.43E+08
		6	1.76E+09
	Opened	0	1.20E+07
		2	1.04E+08
		4	2.01E+08
		6	5.62E+08
13°C	Closed	0	1.20E+07
		2	2.27E+08
		4	2.08E+09
		6	1.84E+09
	Opened	0	1.20E+07
		2	1.72E+08
		4	2.31E+09
		6	8.11E+09
20°C	Closed	0	1.20E+07
		2	1.07E+09
		4	1.92E+09
		6	5.88E+09
	Opened	0	1.20E+07
		2	2.12E+09
		4	5.09E+09
		6	1.31E+10
F-test (significant level)			
Temperature (T)			P<0.0001
Packaging (P)			P<0.0001
Days (D)			P<0.0001
T x P			P<0.0001
P x D			P<0.0001
T x D			P<0.0001
T x P x D			P<0.0001

a.



b.



c.

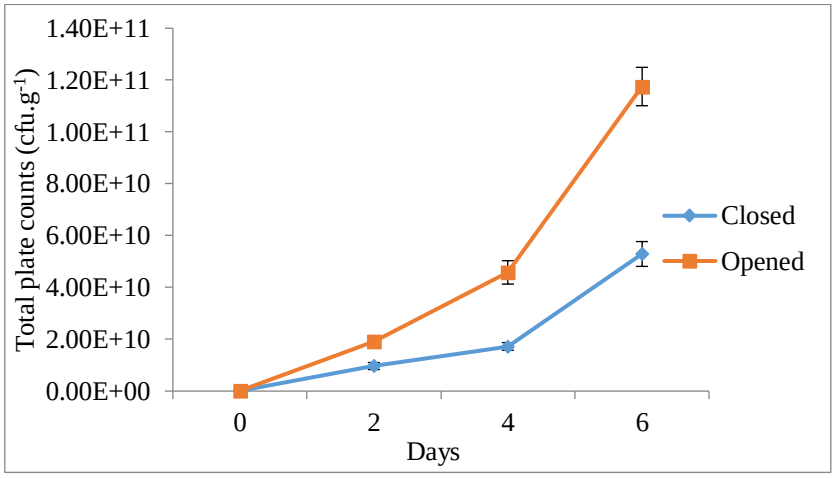


Figure 3.2. Total plate count in rocket salad during storage at (a) 4 ^{o}C (b) 13 ^{o}C and (c) 20 ^{o}C within six days of storage both in closed and opened bag condition (n=21; bars indicate standard error of means).

3.3 Subchapter 3b: Growth of bacteria and volatiles generation from bagged fresh RTE wild rocket in the context of the supply chain

3.3.1 Material and Methods

3.3.1.1 *Plant materials and experimental treatments*

Samples of wild rocket used in this study were obtained from Alresford Salads (Alresford, Hampshire, UK) which were transported to the Department of Food and Nutritional Sciences, University of Reading on the day of arrival into the factory. The rocket variety was *Reset*, and was a second cut crop grown in Italy. The cold chain was maintained at below 5 ^{o}C during transportation from Italy to UK, as well as from Alresford Salads to University of Reading in temperature controlled lorry. More details on the processing of the leaves used in this experiment are as described in section 2.2 in Bell et al. (2017). The materials were then subjected to different handling and storage condition with three replicates. The treatments used are summarized as shown Table 3.2. Each experimental unit is comprised of one bag (70g). The time lines of sampling are presented in Figure 3.1.

Table 3.2. Sampling points and descriptions of activity for determination of microbial loads and volatile organic compounds at different handling and storage condition.

Treatment	Code	Description
Control	Control (Day 0)	The day samples arrived at Food and Nutritional Sciences, University of Reading from Alresford Salads.
Heat shock by supplier for 4 h	HSS24	The samples were kept at 25 ^{o}C for 4 h and later were transferred at 13 ^{0}C for 24 h before TPC were counted. This condition is to simulate the condition where the temperature was abused by supplier or retailer while the delivering and receiving process. The samples were kept at 13 ^{o}C for as for the display temperature at the market. The 25 ^{o}C was used based on Koseki and Isobe (2005) representing temperature on hot summer temperature.
Heat shock by consumer for 4 h	HSC24	The samples were kept at 25 ^{o}C for 4 h and later were transferred at 4 ^{o}C for 24 h before TPC were counted. This condition is to mimic a worst case scenario where temperature was abused by consumer after purchasing rocket and perhaps leaving rocket in the car for few hours in summer before keeping it in the home refrigerator.
Heat shock by supplier for 4 h on DUD	HSSDUD	This treatment was similar as in Treatment HSS24 but the duration of the sample stored at 13 ^{o}C were prolonged until display until date (DUD). This scenario were to mimic the abused temperature by supplier at earlier stage and keep selling and displaying the products at 13 ^{o}C until DUD which later purchased and consumed immediately after purchased.
Heat shock by consumer for 4 h on DUD	HSCDUD	This treatment was the similar as in Treatment HSC24 but the duration of the sample stored at 4 ^{o}C were prolonged until DUD. This is to mimic the condition where early purchased was done but the temperature was abused in earlier stage after purchased and later were kept in home refrigerator. The consumer only consumed the rocket on the day of expiry.

Purchased and consumed on DUD	NPDUD	This treatment is to mimic where the samples were displayed at 13 ^{o}C, purchased on the DUD and consumed on the same day.
Early purchased, closed and consumed on DUD	EPCDUD	This scenario is to mimic where the samples were early purchased (long before DUD date), kept at 4^{o}C home refrigerator and were consumed on the day of DUD.
Early purchased, opened and consumed until DUD	EPODUD	This scenario is to mimic where the samples were early purchased (long before DUD) and were consumed partly and kept later for further consumption until day of its expiry. The rockets were given a contact with hand to simulate the situation of consumer's handling prior to further consumptions and the last consumption were on the day of expiry. Different hands were used to avoid any cross contamination from each bag of rocket.
Heat shock by supplier for 4 h on 2 days after DUD	HSSDUD+2	This treatment was similar as Treatment HSS 4H but the duration of the storage at 13 ^{o}C were prolonged until DUD and kept at 4 ^{o}C for two more days. This scenario were to mimic the abused temperature by supplier at earlier stage and keep selling and displaying the rocket at 13 ^{o}C until DUD and purchased by consumer later and keep for another two days at 4 ^{o}C before consumption (DUD+2)
Heat shock by consumer for 4h, consumed 2 days after DUD	HSCDUD+2	This treatment was similar as in Treatment HSC 4H but the duration of the storage at 4 ^{o}C was prolonged until two days after DUD. This is to simulate the condition of early purchased but the temperature was abused in earlier stage after purchased and later were kept in home refrigerator. However, the consumer only consumed the rocket two days after DUD.
Purchased and consumed on 2 days after DUD	NPDUD+ 2	This treatment is to simulate where the sample is purchased on the day of DUD and kept for 2 more days at 4 ^{o}C in home refrigerator prior consumption.

Early purchased, closed and consumed on 2 days after DUD	EPCDUD +2	This scenario is to mimic early purchase (long before DUD), kept at 4 ^{o}C in home refrigerator and were only consumed on 2 days after DUD.
Early purchased, opened and consumed until two days after DUD	EPODUD+2	This scenario is to simulate early purchased, kept at 4 ^{o}C home refrigerator and were consumed partly and kept later for further consumption until 2 days after expiry date. The rockets were given a contact with hand to simulate the situation of consumer's handling prior to further consumption. Different hands were used to avoid any cross contamination from each bag of rocket.

*Rocket leaves used were processed as descibed in section 2.2 Bell et al. (2017)

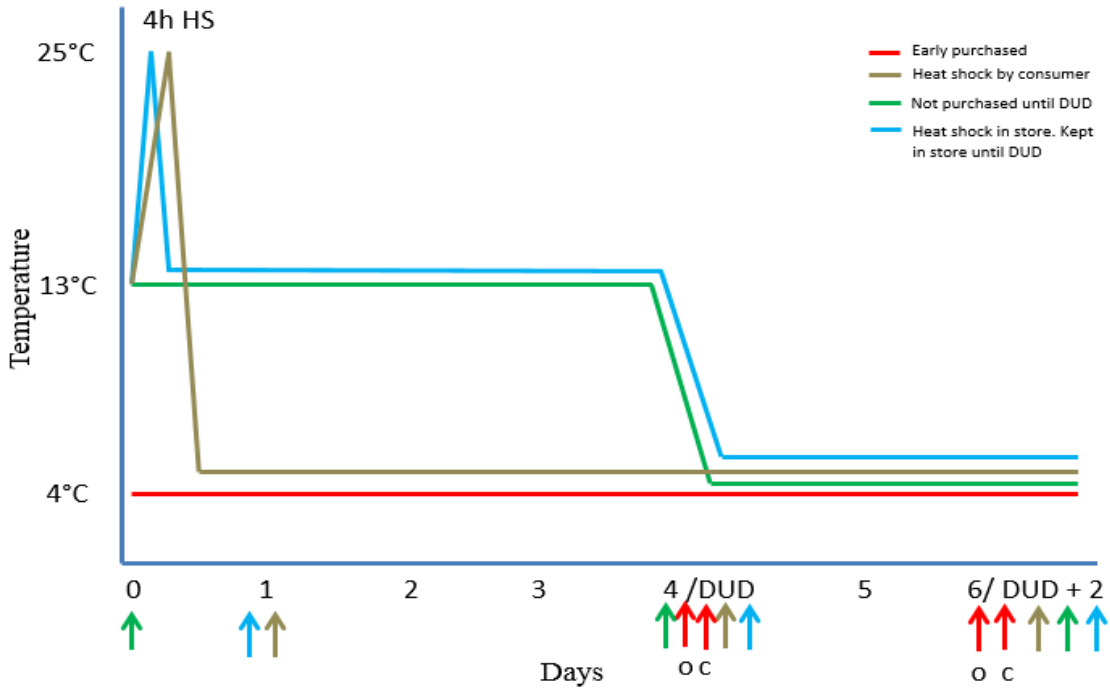


Figure 3.1. Graph shows the timeline of the sampling points of activity for determination of microbial loads and volatile organic compounds at different handling condition. Arrows - day of sampling/ microbial count; O-opened packaging; C-closed packaging.

3.3.1.2 *Microbial count analysis*

As described in Section 3.2.1.2 with addition of 25 ºC incubator.

3.3.1.3 *Data analysis for microbial counts*

As described in Section 3.2.1.3

3.3.1.4 *Solid phase microextraction for volatile organic compounds*

An SPME device (Supelco, Bellefonte, PA, USA), containing a 1 cm Stable-flex fibre coated with 50/30 μm DVB/Carboxen on PDMS, was used. The fibre was conditioned before use by heating it in a gas chromatograph injection port at 250 °C for 30 min.

Bagged rocket salad was analysed by SPME. A steel needle was used to make a hole in the top of the bag, through which to insert the SPME syringe needle. The needle was inserted into the bag and the fibre was exposed to the headspace above the sample for 30 min. The bag and syringe were supported with clamps so that the fibre did not touch the rocket or the bag. After extraction the SPME device was removed from the sample bag and inserted into the injection port of the GC-MS system. 33 bags of rocket were sampled.

3.3.1.5 *Gas chromatography-mass spectrometry (GC-MS)*

All analyses were performed on a Hewlett-Packard (Palo Alto, CA, USA) 5972 mass spectrometer, coupled to a 5890 Series II gas chromatograph and a G1034C Chemstation. The volatile compounds on each SPME fibre were desorbed for 3 min in a split/splitless injection port, held at 250 °C, onto the front of a Stabilwax DA fused silica capillary column (30 m × 0.25 mm i.d., 0.50 μm film thickness; Restek Corporation, Bellefonte, PA, USA). The front of the column was shaped into 5 small loops in a coil, which were cooled in solid carbon dioxide, contained within a 250-ml beaker. The injection port was in splitless mode, the splitter opening after 3 min.

During desorption the oven was at 40 °C. After desorption, the beaker containing the solid carbon dioxide was immediately removed from the oven. The oven was maintained at 40 °C for a further 2 min and then the temperature was raised at 5 °C min^{-1} to 250 °C and hold for 5 min. Helium at 12.7 psi was used as the carrier gas, resulting in a flow of 1.5 ml min^{-1} at 40 °C. *n*-Alkanes (C_5-C_{25}) were run under the same conditions to obtain linear retention index (LRI) values for the components.

The mass spectrometer was operated in electron impact mode with an electron energy of 70 eV and an emission current of 50 μA. The ion source was maintained at 170 °C. The mass spectrometer scanned from *m/z* 29 to *m/z* 450 at 1.81 scans.s^{-1}. Compounds were identified by first comparing their mass spectra with those contained in the NIST/EPA/NIH Mass Spectral Database or in previously published literature, followed by comparing LRI values

with either those of authentic standards or published values. The relative amounts of the compounds in the samples were compared by measuring their peaks areas obtained by integration using ChemStation integrator. Relative amounts of the compounds in the samples were compared by measuring their peak areas obtained by integration using the ChemStation integrator.

3.3.1.6 *Data analysis for volatile organic compounds*

Analysis of variance (ANOVA) was carried out on the quantitative data for each compound identified in the GC-MS analyses. For those compounds exhibiting significant difference in the ANOVA, Duncan multiple range test was applied to determine which sample means differed significantly ($P<0.05$).

3.3.2 Results

3.3.2.1 Growth of bacteria in bagged fresh RTE wild rocket in the context of the supply chain

TPC in RTE rocket varied along the various points of the supply chain as shown by the chosen treatments in Table 3.3, indicating that the increase in TPC varies depending on the temperature, handling and storage condition. Rocket kept under the conditions of HSSDUD contained the highest TPC (1.50E+10 cfu.g^{-1}). This was followed the TPC of samples in NP DUD+2, NPDUD and HSS DUD+2 with their respective TPC of 1.16E+10, 1.06E+10 and 9.60E+09 cfu.g^{-1}. Rocket leaves subjected to other treatments (Control, HSS24, HSC24,

HSCDUD, EPCDUD, EPODUD, HSCDUD+2, EPCDUD+2 and EPODUD+2) were not differed significantly amongst each other but markedly lower than those for treatment HSSDUD, NPDUD, HSSDUD+2 and NPDUD+2.

The results recorded here clearly show the importance of temperature in determining the growth of bacteria in RTE salads. The high bacteria counts in HSS DUD was linked to the prolonged exposure to high temperatures (25 and 13 °C). Surprisingly the TPC of treatment HSS DUD was significantly higher than those in treatment HSS DUD+2 by 5.40E+09 cfu.g^{-1}, whereas those in treatment HSS DUD+2, although it had been subjected to similar conditions plus two extra days in storage at 4 °C. Lower TPC counts in treatment HSSDUD+2 could be the results of microbial death due to insufficient nutritional supply and low temperature for a certain type of bacteria. Exposure to high temperature for 4 h, followed by keeping the RTE rocket at 13 °C produced a significant effect on microbial growth.

However, confirming the hypothesis, TPC did not significantly increase when rocket were kept at 4 °C although the samples has been exposed to 25 °C for 4 h. Similar results were shown by rocket stored at 4 °C for both opened and closed bags (EPCDUD and EPODUD). Storing rocket at 4 °C for seven days either in closed or opened bags until DUD (EPCDUD+2 and EPODUD+2) did not increase the growth of bacteria, and the TPC of the leaves of these treatments were similar (non-significant) from the leaves in control. On the other hand, rocket that was been kept under display temperature (13 °C) shows a high TPC on the DUD even it was not exposed to heat shock and hand contamination. The results of this study

reaffirmed the importance of proper handling and storage of rockets in inhibiting or slowing down the microbial growth**.**

Table 3.3. Total plate counts of RTE rocket salads subject to different handling and storage conditions.

Code for treatment	Total plate count ($cfu.g^{-1}$)	Standard error ($cfu.g^{-1}$)
Control	6.11E+07c	2.35E+07
HSS24	8.31E+08c	1.39E+08
HSC24	6.06E+07c	1.50E+07
HSSDUD	1.50E+10a	2.21E+09
HSCDUD	3.78E+08c	9.00E+06
NPDUD	1.06E+10b	1.36E+09
EPCDUD	2.16E+08c	2.00E+07
EPODUD	3.25E+08c	1.03E+08
HSSDUD+2	9.60E+09b	1.91E+09
HSCDUD+2	8.68E+08c	4.12E+08
NPDUD+2	1.16E+10b	1.97E+09
EPCDUD+2	5.01E+08c	7.94E+07
EPODUD+2	1.84E+09c	9.04E+08

Means within column followed by similar letter are not significantly different according to Duncan Multiple Range Test at $P<0.05$. (Refer Table 3.2 for treatments)

3.3.2.2 Changes in the concentration of volatile organic compounds of bagged fresh RTE wild rocket in the context of the supply chain

Flavour compounds emitted from the rocket leaves during their shelf life were markedly affected by the handling and storage condition as shown in the results presented in Table 3.4, volatiles from nine different groups of compounds (sulphur compounds, alcohols, ketones, furans, aldehydes, esters, alkanes carboxylic acids, phenols and indoles). The grouping of the compounds detected followed the schemes as outlined by Bell et al. (2016).

Four sulphur compounds were detected in the samples: dimethyl sulfide, dimethyl disulfide, dimethyl trisulfide and dimethyl sulfoxide. The pattern of generation of S-compounds differed between compounds. Dimethyl sulfide was not detected in the first four treatments; fresh leaves (Day 0), HSC24 (heat shocked by supplier for 4h), HSS24 (heat shocked for 4 h by consumers) and EPCDUD (early purchased and consumed on display until date). The results show that the abundance of dimethyl sulfide in HSCDUD (heat shocked by consumer for 4h and consumed on display until date), HSSDUD (heat shocked by supplier for 4h and consumed on display until date), EPCDUD+2 (early purchased and consumed on two days after display until date), HSCDUD+2 (heat shocked for 4 h by consumer and consumed on two days after display until date) are not differed significantly among them. The abundance of the compound was highest in leaves that were purchased and consumed on display until date (NPDUD), heat shocked by supplier and consumed on two days after display until date (HSSDUD+2) and those that were purchased and consumed on two days after display until date (NPDUD+2).

Except for the control (Day 0), dimethyl disulfide was detected in all other treatments. Leaves that were experienced heat shock (25 ^{o}C) for 4 h before being transferred at 4^{o}C for 24 h (HSC24) contained the lowest abundance of dimethyl disulfide but the value did not differ significantly from the leaves under HSS24, HSSDUD, HSSDUD+2, NPDUD+2. Leaves of EPCDUD (early purchased, closed, and kept at 4 ^{o}C and consumed on display until date) generated less abundance dimethyl disulfide compared to those that were in EPCDUD+2 treatment (early purchased, closed, and kept at 4 ^{o}C and consumed on two days after display until date). Amongst treatments, leaves that were early purchased and consumed on two days after display until date (EPCDUD+2) generated the greatest abundance of dimethyl disulfide. Comparatively, leaves that were heat shocked by supplier for 4h and consumed on display until date (HSSDUD), heat shocked by supplier for 4h and consumed on two days after display until date (HSSDUD+2) and leaves that were purchased and consumed on two days after display until date (NPDUD+2) produced lower abundance of dimethyl disulfide than EPCDUD and EPCDUD+2 but no significant different with other treatments. This results suggest that short term temperature abuse or heat shock (at 25 ^{o}C) did not significantly affect the generation of dimethyl disulfide.

Table 3.4. Volatiles organic compounds of bagged fresh RTE wild rocket at different point of the supply chain.

Group	Compounds	LRI	Approximate abundance										
			Day 0 (Control)	HSC24	HSS24	EPCDUD	HSCDUD	HSSDUD	NPDUD	EPCDUD+2	HSCDUD+2	HSSDUD+2	NPDUD+2
Sulfur compound	Dimethyl sulfide	723	ND	ND	ND	ND	48751bc	45882bc	58275ab	49485bc	34860c	72648a	72171a
	Dimethyl disulphide	1074	ND	36177f	100473ef	994226b	850534bc	285459def	613920bcd	1925900a	850534bc	547133bcde	488204cdef
	Dimethyl trisulfide	1383	ND	ND	ND	ND	ND	95234a	ND	ND	ND	103294a	ND
	Dimethyl sulfoxide	1575	41850cd	66841cd	24851d	58011cd	68552cd	167636bc	674793a	106528cd	58154cd	148632bcd	270731b
Alcohol	1-butanol	1141	ND	ND	ND	ND	ND	ND	104638a	ND	ND	ND	47017b
	1-penten-3-ol	1167	ND	ND	ND	ND	ND	161008a	169796a	ND	ND	240394a	182107a
	(Z)-2-penten-1-ol	1325	ND	ND	ND	ND	ND	60392b	56969b	ND	ND	189387a	21001bc
	1-hexanol	1356	ND	ND	ND	ND	ND	19291bc	25741b	ND	ND	191233a	21456bc
	(Z)-3-hexen-1-ol	1387	ND	ND	ND	ND	ND	28316c	110713b	ND	ND	674664a	128690b
	Phenethyl alcohol	1917	ND	ND	ND	ND	ND	20559c	95639ab	ND	ND	123894a	45721bc
Ketone	3-pentanone	977	ND	ND	ND	ND	ND	490619a	494765a	ND	ND	748950a	515684a
	3-methyl 2-butanone	983	ND	ND	ND	ND	ND	146954ab	92696b	ND	ND	119768ab	183878a
	Dihydro-2(3H)thiopheno-ne	1650	ND	ND	ND	ND	7465b	26590ab	41451a	20127ab	39656a	30019ab	39181a
	Acetoin	1293	ND	ND	ND	ND	ND	ND	ND	ND	ND	238609a	ND
Furan	2-methylfuran	886	48829d	126524dc	98980d	165694dc	140764dc	418299bc	631652ab	19073 cd	18216d	750423a	724294a
	3-ethylfuran	951	ND	ND	ND	38706bc	ND	155254a	146589a	ND	ND-C	109640ab	115037ab
Aldehyde	Benzaldehyde	1529	ND	ND	ND	ND	ND	301795a	368037a	ND	24369a	437493a	376229a
	2-thiophenecarboxaldehyde	1703	ND	ND	ND	ND	ND	21853c	65241a	ND	ND	46514b	22288c

Ester	(Z)-3-hexenyl 3-methylbutanoate	1490	ND	ND	ND	ND	ND	71100bc	518177a	ND	ND	152931b	181271b
Alkane	2,4-dithiapentane	1289	ND	ND	ND	26303de	24226de	50432cd	113681a	19397de	12712e	71018bc	89307ab
Carboxylic acid	Acetic acid	1465	ND	46584bc	26699bc	36617bc	36512bc	59389bc	268439a	ND	30856bc	109740b	119162b
Phenol		2013	16912b	26111b	19093b	41054b	29301b	21309b	67068ab	14791b	19294b	479523a	109653a
Indole		2462	ND	ND	ND	ND	ND	ND	ND	21183b	42497b	135492a	14365b

Note: ND – not detected; Values within row with similar letters are not differed significantly based on DMRT at P<0.05.

Dimethyl trisulfide was only detected in leaves of two treatments, i.e. HSSDUD (heat shocked at 25 ºC by supplier for 4 h and consumed on display until date) and HSSDUD+2, the leaves that were experienced heat shocked at 25 ºC for 4h before being kept at 13ºC until display until date and consumed 2 days after the display until date.

As with other S-compounds, the occurrence of dimethyl sulfoxide differed among treatments, whereby rocket in treatment NPDUD (those purchased and consumed on display until date) generated the highest amount of dimethyl sulfoxide, suggesting that leaves that were displayed at relatively high temperature at the retailer premises (13 ºC, NPDUD) generated significantly high abundance of dimethyl sulfoxide compared to those kept continuously at 4 ºC. (EPCDUD). The amount of the compound produced by rocket leaves in Day 0, HSC24 (heat shocked by consumer for 4h), HSS24 (heat shocked by supplier for 4 h), EPCDUD (early purchased and consumed on display until date), HSCDUD (heat shocked by consumer and consumed on display until date) and HSCDUD+2 (heat shocked by consumer and consumed on two days after display until date) were similar as indicated by non-significant differences among their mean values.

Based on the results, different sulphur compounds responded differently to storage temperature and duration of storage. Dimethyl sulfoxide is the least sensitive towards temperature and the days of storage, with most the changes in the compound were not affected by storage temperature except for NPDUD and NPDUD+2. However, the dimethyl sulfoxide abundance seems to be increasing after longer duration of storage although the values were not significantly differed. Dimethyl sulfide and dimethyl disulfide both were influenced by the changing temperature and duration of storage. The abundance of both

compounds were positively associated with days of storage as seen leaves of HSC24 and HSCDUD treatments. Storing at higher temperature also significantly increased the compounds abundance but it was not affected by the heat shock treatment as seen in leaves of HSSDUD+2 and NPDUD+2. Conversely, dimethyl trisulfide was only affected by the heat shock treatment when the heat treatment was followed by long exposure to high storage temperature. In the present study, dimethyl trisulfide was only detected in leaves of HSSDUD and HSSDUD+2.

There are five types of alcohols detected in the study: 1-butanol, 1-penten-3-ol, (Z)-2-penten-1-ol, 1-hexanol, (Z)-3-hexen-1-ol and phenethyl alcohol. The generation pattern of these compounds were similar, except for 1-butanol. The alcohols were only detected in leaves that were exposed to long storage durations at high temperature. Overall, rocket leaves generated high amount of alcohols under HSSDUD+2 treatment (heat shocked at 25 ^{o}C by supplier for 4h and consumed two days after display until date) and this is followed by leaves in treatments NPDUD (purchased and consumed on display until date) and NPDUD+2 (purchased and consumed on two days after display until date). The alcohol in leaves of Day 0 and those that were subjected to treatment HSS24 (heat shocked by supplier), HSC24 (heat shocked by consumer), EPCDUD (early purchased and consumed on display until date), EPCDUD+2 (early purchased and consumed on two days after display until date) and HSCDUD+2 (heat shocked by consumer and consumed on two days after display until date) were undetectable. The results clearly shown that short term heat shocked 25^{o}C for 4 and exposure to 13^{o}C aggravated alcohol production in rocket leaves.

The abundance of 1 penten-3-ol in samples with or without heat shock did not significantly different between each other. However, this compound was affected by the duration of storage. At the earlier days of storage as observed in Day 0, HSC24 and HSS24, 1 penten-3-ol was not detected but later, it was found at the HSSDUD and HSSDUD+2 which were on 5 and 7 days of storage, respectively. In contrast to 1 penten-3-ol, (Z) 2 penten-1-ol abundance was influenced by the heat shock treatment but the significant different was only seen in leaves stored up to two days after the expiry date (HSSDUD+2 and NPDUD+2). This alcohol was also affected by the duration of storage particularly in leaves that were kept at high temperature, whereby it was not detected in HSS24H, but it was found in HSSDUD and then increased markedly in HSSDUD+2 leaves. The changes in phenethyl alcohol was found to be in similar pattern with 1-hexanol, (Z) 3 hexen-1-ol, but the abundance of phenethyl alcohol was not affected by duration of storage.

The generation of ketones among rocket differed markedly by treatments and the patterns of responses varied depending on the compounds. The generation patterns of 3-pentanone and 3-methyl 2-butanone were almost similar where these two compounds were detected in leaves that were subjected to treatment HSSDUD (heat shocked at 25 ^{o}C by supplier for 4h and consumed on display until date), NPDUD (purchased and consumed on display until date), HSSDUD+2 (heat shocked at 25 ^{o}C for 4h before being kept at 13^{o}C until display until date and consumed on 2 days after the display until date) and NPDUD+2 (purchased and consumed on two days after display until date). NPDUD (purchased and consumed on display until date). Between the two compounds, the amount of 3-pentanone released was much higher compared to 3-methyl 2-butanone. 3-pentanone and 3-methyl 2-butanone were detected in leaves stored at 13 ^{o}C. Heat shocked did not affect the abundance of the two

compounds. Duration of storage affected the abundance of the compounds but only until DUD. Extending storage two days after DUD did not influence the abundance of 3-pentanone and 3-methyl 2-butanone.

In contrast to 3-pentanone and 3-methyl 2-butanone, dihydro-2(3H) thiophenone was detected more frequently, but it appeared in smaller amount. Dihydro-2(3H) thiophenone was also detected in leaves kept at low temperature but only in those that experienced heat shocked treatment and have been exposed kept to high temperature (HSCDUD and HSCDUD+2). The results suggests that exposure to 25 ^{o}C for 4 h produced significant effect on the abundance of dihydro-2(3H) thiophenone in rocket leaves. The results also show that long storage duration affected the abundance of dihydro-2(3H) thiophenone but this phenomenon only occurred in leaves that have been kept at low temperature as seen between HSCDUD and HSCDUD+2.

Acetoin was only detected in one treatment, i.e. in HSSDUD+2 (heat shocked at 25 ^{o}C for 4h, displayed at 13^{o}C and consumed two days after the display until date). The results clearly showed that exposing the leaves to high temperature as heat shock treatments, coupled with long period of storage, triggered the formation of acetoin.

There are two compounds detected that belong to the furan family, 2-metylfuran and 3-ethylfuran. Between the two, 2-methylfuran was found in leaves of all treatments while 3-ethylfuran was only detected in five treatments. Among the treatments, leaves subjected to treatments NPDUD (purchased and consumed on display until date), HSSDUD+2 (heat shocked at 25 ^{o}C for 4h, displayed at 13^{o}C and consumed two days after the display until

date) and NPDUD+2 (purchased and consumed on two days after display until date) produced the highest amount of 2-methylfuran. In contrast, beside NPDUD (purchased and consumed on display until date), HSSDUD+2 (heat shocked by supplier and consumed on two days after display until date) and NPDUD+2 (purchased and consumed on two days after display until date), similar abundance of 3-ethylfuran was generated from leaves of HSSDUD (heat shocked by supplier and consumed on display until date). 2-methylfuran generation was affected by temperature and storage duration whereby the compound has been found increased significantly at high temperature under long storage duration. 3-ethylfuran was detected in rocket that have been kept mainly at high temperature.

Between the two aldehydes detected, benzaldehyde was found to be more abundantly produced compared to the other aldehyde (2-thiophenecarboxaldehyde). The occurrence of these two compounds was similar except that, beside detected in HSSDUD (heat shocked by supplier and consumed on display until date), NPDUD (purchased and consumed on display until date) and HSSDUD +2 (heat shocked by supplier and consumed on two days after display until date) and NPDUD +2 (purchased on display until date and consumed on two days after display until date), benzaldehyde was also detected in leaves that were heat shocked at 25 ^{o}C for 4h, displayed at 13^{o}C and consumed on 2 days after the display until date (HSCDUD+2). Among the treatments in which benzyaldehyde was detected, the difference among them are not significant. In contrast, among the leaves of treatments that contained 2-thiophenecarboxaldehyde, the abundance between them was differed significantly. Leaves that were purchased and consumed on the display until date (NPDUD) contained the highest abundance of 2-thiophenecarboxaldehyde and this was followed by leaves that were experienced heat shocked by supplier and consumer on two days after date

of display (HSSDUD +2), purchased and consumed on two days after display until date (NPDUD +2) and the leaves that were heat shocked by supplier, purchased and consumed on day until display (HSSDUD). The results show that exposing rocket to high temperature affected the abundance significantly of both aldehyde compounds. Keeping the leaves for a longer duration of storage at 13 ^{o}C also influenced the presence of benzaldehyde and 2-thiophencarboxaldehyde. Both compounds increased significantly with increasing temperature and duration of storage.

The ester generated, (Z)-3-hexenyl 3-methylbutanoate was detected in four out of 11 treatments (HSSDUD, NPDUD, HSSDUD+2 and in NPDUD+2). Among the treatments, leaves that were purchased and consumed on display until date (NPDUD) generated the highest amount of (Z)-3-hexenyl 3-methylbutanoate, followed by those that were purchased and consumed on two days after display until date (NPDUD+2), heat shocked by supplier and consumed on two days after display until date (HSSDUD+2) and leaves that heat shocked, then displayed at 13^{o}C and consumed on display until date (HSSDUD). The difference between the amount of the compound from NPDUD+2 (purchased on display until date and consumed on two days after display until date) and HSSDUD+2 (heat shocked for 4 h, kept at 13 ^{o}C and consumed on two days after display until date) was not significant. Apparently, (Z)-3-hexenyl 3-methylbutanoate was positively affected by both storage duration and temperature as the compound was only detected in leaves that were kept at high temperature until display until date (DUD) and DUD+2 days.

The alkane, 2,4-dithiapentane, was not detected in fresh rocket (Day 0) and the leaves that were heat shocked by consumer for 4 h (HSC24) and those treated with heat shocked by

supplier (HSS24). Like furan, aldehyde and ester, the highest amount of 2,4-dithiapentane was detected in leaves that were subjected to NPDUD (purchased and consumed on display until date), and this was similar to those treated under NPDUD+2 (purchased and consumed on two days after display until date) as shown non-significant different between the means of the two treatments. It was proven that the generation of 2,4 dithiapentane was high in leaves that have been kept at high temperature regardless of exposure to heat shock but only at the point of DUD and later.

Acetic acid was found to be released and detected in nine out of 11 treatments. Leaves of treatment NPDUD (purchased and consumed on display until date) generated the highest amount of acetic acid. Although there was no acetic detected in leaves on Day 0 and in leaves of EPCDUD+2 (early purchased, kept at 4^{o}C and consumed on two days after display until date), but the amount of acetic acid detected was not differed with the values for other treatments except for HSSDUD+2 (heat shocked by supplier, kept at 13^{o}C and purchased and consumed on display until date) and NPDUD+2 (purchased and consumed on two days after display until date). Acetic acid was mostly detected in leaves that were exposed to high temperature (13 ^{o}C and 25 ^{o}C) for a long duration of storage, but the relationship between duration of storage and temperature was not clear.

Phenol was detected in samples for all treatments. The amount of phenol generated from leaves of NPDUD (purchased and consumed on display until date), HSSDUD+2 (heat shocked by supplier and consumed on two days after display until date) and NPDUD+2 (purchased and consumed on two days after display until date) were similar as shown by non-

significant different between the mean values. The means of these treatments were significantly higher than the means of the other eight treatments.

The presence of indole in rocket leaves was detected in four treatments (EPCDUD+2, HSCDUD+2, HSSDUD+2 and NPDDUD+2). Indole generated from samples of HSSDUD+2 were the highest and differed significantly from the others. The difference among the mean values of EPCDUD+2, HSCDUD+2, HSSDUD+2 and NPDDUD+2 was not significant.

Phenol and indole were both significantly affected with duration of storage and temperature exposure. Leaves kept at high temperature and long duration of storage had more abundance of the two compounds especially at two days after expiry date. However, exposing to 25 ^{0}C for 4 h only affected indole where it abundance was higher than those released by leaves in non-heat shocked treatments as seen in treatments HSSDUD and HSSDUD+2.

3.4 Subchapter 3c: Bacterial abundance, gas composition and greenness of bagged fresh RTE wild rocket produced in two different seasons and changes in the concentration of glucosinolates in bagged fresh RTE wild rocket in the context of the supply chain

3.4.1 Material and Methods

3.4.1.1 *Plant materials and treatments*

Plant materials obtained from Alresford Salads (Alresford, Hampshire, UK) were produced in Italy. For winter grown leaves, the crop was produced during January to February using wild rocket var. *Reset*, which was second cut, with 38 days growth cycle after sowing, while the summer crop was produced in July to August, using var. *Extrema*, also second cut leaves with 32 days growth cycle after sowing. The treatments used in this study were similar treatment as described in Section 3.3.1.1 (Table 3.2).

3.4.1.2 *Microbial count analysis*

As described in Section 3.3.1.2.

3.4.1.3 *Analysis of oxygen, carbon dioxide and ethylene*

Oxygen and carbon dioxide gas analyser for MAP packages (Oxybaby, Witt, Germany) was used to measure oxygen and carbon dioxide content (%) in rocket packages. Ethylene was measured from head space of the bag using Hewlett Packard 5890 series 2 Gas Chromatography (Hewlett Packard, Reading, UK) with a build-in integrator and equipped with flame ionisation detector set at 150 °C, a 30 m GS-Q column with an internal diameter of 0.53 mm and helium carrier gas at a flow rate of 15 ml/min. The column and injector volume of 100 µl was used and quantified using a 1 $\mu l.l^{-1}$ sample of ethylene as a standard.

Retention time was confirmed using pure ethylene, and quantification was calibrated using standard gas mixture with 1 ppm ethylene. Three replicates were performed.

3.4.1.4 *Determination of relative chlorophyll content*

Relative chlorophyll content of treated leaves were measured using chlorophyll content meter (Hansatech CL-01, Hansatech, Kings, Lynn, UK). Reading for each treatment were taken nine times using three leaves (three measurements per leaf).

3.4.1.5 Changes in the concentration of glucosinolates in bagged fresh RTE wild rocket in the context of the supply chain

Glucosinolate extraction

Glucosinolate extraction and LC-MS^2 analysis employed by Bell et al. (2015) were followed. Three experimental replicates were prepared as follows: 40 mg of ground rocket powder was heated in a dry-block at 75 °C for 2 min to inactivate as much myrosinase enzyme before liquid extraction (Pasini et al., 2012). One ml of pre-heated 70% methanol (v/v) (70 °C) was added to each sample and placed in a water bath at 70 °C for 20 min. Samples were then centrifuged for 5 min (6000 rpm, 18 °C) to collect loose material into a pellet. The supernatant was then taken and put into fresh Eppendorf tubes. The volume was adjusted to 1 ml with 70% methanol and frozen at −80 °C until analysis.

LC-MS^2 analysis

Immediately before LC-MS analysis, the samples were filtered using 0.25 µm filter discs with a low protein binding Durapore polyvinylidene fluoride (PVDF) membrane (Millex, EMD Millipore, Billerica, MA, USA) and diluted with 9 ml of HPLC-grade water. Samples were then run in a random order with QC samples (Dunn et al., 2012). An external reference standard of sinigrin hydrate was also prepared for quantification of glucosinolates (GLSs), using the flowing procedure: 12 mM sinigrin hydrate solution was prepared in 70% (v/v) methanol. A dilution series of concentrations was prepared to obtained an external calibration curve with HPLC-grade water (5.6, 14, 28, 42, 56, 100, 150, 200 ng.µl^{-1}; sinigrin regression equation: $y = 12.496x - 15.012$; $R^2 = 0.993$). Standard response factors were used in the calculation of GLS concentrations where available (Wathelet, 2004). Where such data could not be found for intact GLSs, response factors were assumed to be 1.00 (Lewis and Fenwick, 1987). LC-MS analysis was performed in the negative ion mode on an Agilent 1200 Series LC system equipped with a binary pump, degasser, auto-sampler, thermostat, column heater, photodiode array detector and Agilent 1100 Series LC/MSD mass trap spectrometer. Separation of samples was achieved on a Zorbax SB C18 column (2.1×100 mm; 1.8 µm; Agilent, Santa Clara, CA, USA) with pre-column filter. Mobile phases consisted of ammonium formate (0.1%) and acetonitrile with a gradient of 95% and 5% respectively at a flow rate of 0.3 ml.min^{-1}, with a column temperature of 30 °C. 5 µl of sample was injected. MS analysis settings were as follows: Electrospray ionization (ESI) was carried out at atmospheric pressure in negative ion mode (scan range *m/z* 50–1050 Da). Nebulizer pressure was set at 50 psi, gas-drying temperature at 350 °C, and capillary voltage at 20,000 V. The compounds were identified using their nominal mass and characteristic fragment ions, and

by comparing with published data in the literature. GLSs were quantified at a wavelength of 229 nm and all data were analysed using Bruker Daltronics software.

3.4.2 Results

3.4.2.1 Bacterial abundance, gas composition and greenness of bagged fresh RTE wild rocket produced in different seasons in the context of the supply chain

3.4.2.1.1 *Bacterial abundance*

Total plate counts in fresh RTE rocket produced in different growing seasons varied along the various points of the supply chain and the results are shown in Table 3.5. As expected, rocket produced in summer contained significantly ($P<0.0001$) higher loads of bacteria than that produced in winter. However, there was a clear interaction effect between growing season and postharvest handling and storage treatment ($P<0.0001$) on microbial loads, suggesting that the trends of change in bacterial population for each season varied according to the treatments.

For rocket produced in summer, rocket that was subjected to HSSDUD contained the highest bacterial loads, and this was followed by those treated under NPDUD, HSSDUD+2 and NPDUD+2. The differences among them were not significantly different. Variations in handling and storage condition of rocket in other treatments, include a short temperature abuse (4 h) at 25 ^{o}C did not affect bacterial loads in leaves as shown by non-significant differences of HSS24 and HSC24 compared to those in the control. Consistent with the earlier

finding, the results recorded here show that exposure of rocket leaves briefly at higher temperature (4 h) and analysed at 24 h after treatment began did not affect bacterial loads in rocket, as shown by no difference in TPC of HSS24 and HSC24, which is similar to the leaves in control (Day 0).

Among the treatments of winter rockets, leaves of treatment HSSDUD+2 contained the highest bacterial loads. Leaves treated under HSCDUD, NPDUD and NPDUD+2 contained similar loads of bacteria as HSSDUD+2 as shown by non-significant different among their means. Bacterial population of leaves in treatments HSS24, HSC24, HSCDUD+2, EPCDUD, EPCDUD+2, EPODUD and EPODUD+2 were low and did not differ from the bacteria in the control leaves. Results obtained in this study were consistent with the results reported in Section 3.3.2.1

Table 3.5. Total plate count, concentration of carbon dioxide, oxygen and ethylene, and chlorophyll index of summer and winter grown rocket leaves subjected to varying handling and storage condition in the context of their supply chain after harvest.

Treatment	Total plate counts ($cfu.g^{-1}$)		Carbon dioxide (%)		Oxygen (%)		Ethylene ($nl.kg^{-1}.h^{-1}$)		Chlorophyll index	
	Summer	Winter	Summer	Winter	Summer	Winter	Summer	Winter	Summer	Winter
Control	6.105E+07c	2.211E+07c	2.73d	1.13g	17.90a	18.23a	7.27a	5.29a	7.84a	12.64a
HSS24	8.310E+07c	6.356E+07c	6.37bc	7.36e	13.07b	12.20c	3.11bc	3.65a	5.36a	9.32a
HSSDUD	1.494E+10a	2.211E+0b	9.77a	14.17a	7.87c	0.33h	1.56cd	3.27b	6.15a	10.57a
HSSDUD+2	9.600+E09b	4.589E+08a	9.93a	13.13b	2.47e	0.50h	1.68cd	2.43bcd	6.82a	9.71a
HSC24	6.060E+07c	5.400E+07c	4.23cd	4.74f	16.27a	14.7b	4.05b	5.12a	5.76a	10.57a
HSCDUD	3.780E+08c	1.100E+08bc	5.40bc	8.56d	13.94b	9.30e	1.26cd	1.48cd	6.20a	10.03a
HSCDUD+2	8.664E+08c	9.289E+07bc	5.70bc	9.50c	13.37b	7.30g	0.62d	1.65bcd	6.82a	9.49a
NPDUD	1.062E+10b	4.272E+08a	10.43a	14.23a	6.87cd	0.33h	1.40cd	3.13bc	6.51a	8.35a
NPDUD+2	1.158E+10b	3.878E+08a	10.63a	12.73b	5.63d	1.23h	1.20cd	2.44bcd	6.17a	9.12a
EPCDUD	2.160E+08c	8.778E+07bc	5.37bc	7.73e	13.47b	10.47d	0.68d	1.91bcd	6.57a	9.31a
EPCDUD+2	5.010E+08c	9.289E+07bc	6.87b	8.70d	12.27b	8.33f	0.61d	1.41d	6.00a	9.20a
EPODUD	3.249E+08c	1.334E+08bc	na*	na	na	na	na	na	6.57a	9.78a
EPODUD+2	1.842E+09c	1.153E+08bc	na	na	na	na	na	na	6.78a	9.50a

Notes: na* – not applicable; Means values within column followed with similar letter are not significantly different using DMRT at $P<0.05$. All parameters among seasons are differed significantly at $P<0.0001$.

3.4.2.1.2 *Gas composition*

The composition of oxygen, carbon dioxide and ethylene generated in bagged fresh RTE rocket salads subjected to different treatments were monitored. For both seasons, handling and storage treatments and interaction effects between the two factors significantly affected the CO_2, O_2 and C_2H_4 content of the gas sampled (Table 3.5).

Surprisingly, except for the gas sampled from the control, rocket produced in winter contained higher percentage of CO_2 suggesting that the leaves produced in winter have a higher respiration rate. Among the summer leaves, the gas sampled from HSSDUD (9.77%), HSSDUD+2 (9.93%), NPDUD (10.43%) and NPDUD+2 (10.63%) contained significantly higher concentration of CO_2 than the other treatment, suggesting that leaves that were exposure to higher temperature (25 ^oC) during heat shock/temperature abuse and 13 ^oC during storage/display) for a long period of time had generated more CO_2. As expected, the gas sampled from the bags immediately after the arrival of the leaves in our laboratory (Day 0) contained the lowest percentage of CO_2 (2.73%).

Similar trends on the release of CO_2 were observed for winter rocket, whereby exposure to higher temperature led to higher concentration of the gas detected in the bagged fresh RTE rockets as seen in HSSDUD (14.17%), HSSDUD+2 (13.13%), NPDUD (14.23%) and NPDUD+2 (12.73%) treatments. As for the summer grown rocket, gas of the control samples contained the lowest composition of CO_2 (1.13%).

For summer grown rocket, oxygen in the gas sampled from the control was the highest (17.90%) and this was followed by O_2 in HSC24 (16.27%). Among the treatments, those leaves experienced heat shock at 25 ^oC, followed by long term storage/display condition at 13 ^oC before being consumed on two days after expiry date (DUD) contained the lowest O_2 content (2.47%). Overall, the composition of O_2 of summer rocket was higher than those contained in winter grown rocket, except for the control, HSS24 and HSC24. The means for O_2 composition for summer and winter rockets are 11.19 and 7.54%, respectively.

Consistent with the summer rocket, the gas immediately sampled from bags upon their arrival (Day 0) contained the highest O_2 (18.23%), while those that experienced heat shock followed by storage at 13 ^oC until expiry date (HSSDUD, 0.33% and HSSDUD+2, 0.50%) or continuously kept under storage/display temperature (13^oC) (NPDDUD, 0.33% and NPDUD+2, 1.23%) contained the lowest O_2 concentration. Differences in O_2 concentration in HSSDUD, HSSDUD+2, NPDDUD and NPDUD+2 were not significant.

The concentration of C_2H_4 also markedly varied among seasons ($P<0.001$), handling and storage conditions ($P<0.0001$) and the concentration of C_2H_4 subjected to different postharvest treatments varied among seasons (interaction between season x treatment significant at $P<0.01$). Results in Table 3.4 shows that bagged summer RTE rocket leaves contained lower C_2H_4 concentration than the winter rocket with their respective means concentrations of 2.14 and 3.16 $nl.kg^{-1}.h^{-1}$. Leaves of the control treatment generated the highest rate of C_2H_4 (7.27$nl.kg^{-1}.h^{-1}$) and this was followed by leaves of HSC24 (4.05 $nl.kg^{-1}.h^{-1}$) and HSS24 (3.11 $nl.kg^{-1}.h^{-1}$). The different between HSC24 and HSS24 was not

significant. The rate of C_2H_4 generated from leaves under HSC24 and HSS24 treatments were significantly higher than the rates of C_2H_4 generated by leaves of other treatments.

In winter grown rocket the C_2H_4 generated from leaves in control, HSS24 and HSC24 were similar, but the rate of C_2H_4 produced here was higher than the rate of C_2H_4 generated from leaves of other treatments, indicating that the leaves exposed to higher temperature would have a higher release rate of C_2H_4. As reported for summer rocket, the rate of C_2H_4 generated by winter grown rocket was lower in leaves kept at low temperature (4 ^oC), including those that previously have been exposed to heat shock as seen the treatments HSCDUD, HSCDUD+2, EPCDUD, EPCDUD+2.

Comparing the seasons, leaves of winter grown rocket seem to be more sensitive to heat shock as shown by marked differences in C_2H_4 generated in HSS24 and HSC24 treatments. Relatively high generation of C_2H_4 in response to heat shock or temperature abuse of winter rocket are perhaps associated with poor adaption to high temperature as the crops in winter were grown under low field temperature in winter. As an example, the C_2H_4 in leaves subjected to heat shock for 4 h followed by storage at 13 ^oC when the gas was sampled at 24 h after the treatment begun (HSS24), showed a reduction of C_2H_4 by 57.2% compared to the control, whereas the similar treatment of the winter rocket led to 25.7% increase in the rate of C_2H_4 released.

3.4.2.1.3 *Chlorophyll index*

Unlike other parameters showed in Table 3.5, the greenness of the leaves shown by chlorophyll index was only affected by the growing season and not by postharvest treatments. Winter rocket was greener than the leaves grown in summer, with their respective chlorophyll index of 9.88 and 6.32.

3.4.2.1.4 *Changes in the concentration of glucosinolates in bagged fresh RTE wild rocket in the context of the supply chain*

Six types of GLSs were detected in the bagged fresh RTE wild rocket used in the study (Table 3.6) and the concentration of all types of GLSs varied significantly among treatments. Surprisingly, no individual GLS and hence no total GLS was detected in the leaves analyzed on Day 0. The individual GLS detected at later postharvest stages were glucosativin, glucotropaeolin, glucoerucin, glucoraphanin, diglucothiobeinin and DMB. Among the treatments, leaves that were experiencing heat shock at 25 ^{o}C but analysed after 24 h (HSS24 and HSC24) and leaves that stored at low temperature (4 ^{o}C) (EPCDUD, EPODUD, HSCDUD and HSCDUD+2) contained highest glucosativin, and the means values of this compound did not differ significantly among each other. On the average, glucosativin content in these leaves was 4.90 mg g^{-1} DW. Beside the control leaves (Day 0), glucosativin was also not detected in leaves of NPDUD+2.

Similar to glucosativin, glucotropaeolin was not detected in leaves analysed on the day of arrival (Day 0). Glucotropaeolin was also undetected in leaves of treatments HSC24, HSS24,

HSCDUD, HSSDUD and NDPDUD+2. In contrast to glucosativin, the leaves subjected to HSCDUD+2 contained the highest concentration of glucotropaelin (4.41 $mg.g^{-1}$) and this was followed by those in treatments EPCDUD (3.92), EPODUD+2 (3.72) and EPODUD (3.59). The content of glucotropaeolin in leaves under NPDUD, EPCDUD+2 and HSSDUD+2 conditions were similar (non-significant), which were ranged between 3.33-3.49 mg g^{-1} DW.

Early purchased rocket and kept at low storage temperature either in open or close conditions until date of expiry leaves (EPCDUD and EPODUD) contained the highest concentration of glucoerucin (4.91 and 4.54 $mg.g^{-1}$, respectively) and this was followed by those treated with EPODUD+2, HSS24 and HSCDUD+2. The compound was not detected in other treatments.

Glucoraphanin was not detected in leaves analysed immediately their arrival (Day 0) and in HSC24 samples. The concentration of glucoraphanin was highest (4.26 $mg.g^{-1}$) in leaves that experienced heat shock for 4 h followed a low temperature storage at 4 ^{o}C and analysed on 2 days after DUD (HSCDUD+2) and this concentration was significantly higher than those recorded for other treatments.

Diglucothiobeinin was not detected in leaves of Day 0, HSS24, HSCDUD, HSSDUD, EPODUD+2 and HSCDUD+2. Among the samples that contained diglucothiobeinin, leaves in treatments HSC24, NPDUD, HSSDUD+2 and NPDUD+2 contained significantly high concentration of diglucothiobeinin. Leaves from NPDUD, EPODUD+2 and NPDUD+2 contained the highest concentration of DMB and the difference among them was not significant with their respective values of 6.52, 5.89 and 7.10 $m.g^{-1}$.

Overall, EPODUD contained the highest concentration of total GLS, and this is followed by the content of GLS in leaves of EPODUD+2.

Table 3.6. Concentration of glucosinolates in bagged fresh ready-to-eat wild rocket at different point of the simulated supply chain.

Treatments	Concentration of glucosinolates ($mg.g^{-1}$ of sinigrin hydrate equivalent, DW)						
	Glucosativin	Glucotropaeolin	Glucoerucin	Glucoraphanin	Diglucothiobeinin	DMB	Total GLS
Day 0 (Control)	ND	ND	ND	ND	ND	ND	ND
HSC24	6.34a	ND	ND	ND	3.56a	ND	9.90de
HSS24	6.15a	ND	1.81c	3.48b	ND	1.22cd	12.66c
EPCDUD	4.67abc	3.92b	4.91a	3.36bc	2.32bc	1.64c	20.82b
EPODUD	6.05ab	3.59cd	4.54a	3.47b	1.67c	4.85b	24.17a
HSCDUD	6.03ab	ND	ND	3.53b	ND	ND	9.56de
HSSDUD	2.89def	ND	ND	3.13bc	ND	1.58c	7.60e
NPDUD	1.92f	3.33e	ND	3.08bc	3.16ab	6.52a	18.01bc
EPCDUD+2	4.41bcd	3.49de	ND	3.19bc	1.82c	2.54c	16.05bcd
EPODUD+2	4.02cde	3.72c	3.05b	3.51b	ND	5.89ab	20.19b
HSCDUD+2	5.14abc	4.40a	1.51c	4.26a	ND	ND	15.31bcd
HSSDUD+2	2.65ef	3.43e	ND	2.78c	3.55a	ND	12.41cd
NPDUD+2	ND	ND	2.88b	3.05bc	3.08ab	7.10a	16.11bcd

Note: ND-not detected; Values within columns followed by with similar letters are differed significantly according to DMRT at $P<0.05$.

3.5 Discussion

3.5.1 Temperature regulates bacterial growth

Results of the study clearly show that temperature plays an important role in keeping food safe in addition to several other factors such as pH, water activity, nutrient content and other intrinsic and extrinsic parameters. Ridgwell and Winson (2001) stated the refrigerator should be functioning at 5 ^{o}C or below to inhibit bacterial growth. This explains the lower count of bacteria for the samples kept at low temperature (4 ^{o}C) and *vice-versa*. However, different species of bacteria requires different temperatures for optimum growth. Some bacteria are able to grow at temperatures even lower than those commonly used for the storage of fresh produce.

This correlates with result reported in Section 3.3.1 where the number of bacterial counts was still increasing through postharvest storage, although by a small amount when the rocket is kept under refrigeration. A study conducted by Babic et al. (1996) showed a similar trend of increase in microbial population during cold storage. They reported that total populations of mesophilic and psychrotrophic aerobic microflora in fresh cut spinach stored at 10 ^{o}C with initial loads of 10^{6}-10^{7} cfu.g^{-1} had increased to 10^{10} cfu.g^{-1} on day 8 of storage, then the counts were remained almost constant up to 12 days. The bacterial population reported by Babic et al. (1996) were within the range of the population of bacteria detected in the present study which ranges from 10^{7}-10^{10} cfu.g^{-1} depending on handling, storage conditions as well as growing season (Table 3.5).

The main species isolated and identified were *Pseudomonas fluorescens, Citrobacter freundii, Serratia liquefasciens, Aeromonas caviea, Klebsiella pneumoniae* and *Staphylococcus xylosus*.

There was some reduction of bacterial counts occurring during the storage of rocket observed at six days of storage at 13 ^{o}C and 20 ^{o}C. There were a few possibilities that may influence the results. It is a nature of living organism to die off after some time and, therefore, the reduction in TPC was perhaps due to the death of the bacteria. Another attributable factor could be the biological variation of the samples which could include the phenological age of the plants (young or mature), environmental conditions where the plants are grown and biotic or abiotic stresses faced by the plants. Such conditions could influence the microbial preference to colonize and become internalized within the plants.

3.5.2 Adverse handling of rocket is protected by the cold chain

Rapid increase in bacterial counts in opened bags (Figure 3.2) that occurred at higher temperatures could be associated with contamination. However, results obtained in study showed that when the rocket was put in contact with the hands of a food handler, and possibly had caused a crossed-contamination, and followed by low temperature storage (4 ^{o}C), this situation did not result in increase in the number of the bacteria, even though the rocket was kept for up to two days after DUD (Table 3.3, treatments EPODUD and EPODUD+2). A similar result was also obtained for rocket that was exposed to high temperatures for 4 h, then kept at 4 ^{o}C, suggesting that even though the storage temperature was abused for a few hours the subsequent maintenance of 4 ^{o}C will retard the growth of the microbes.

Disruption of the cold chain for a short period of time (4 h) can result in significantly higher bacterial growth than if the cold chain is maintained, therefore the shelf life and possibly the health of consumers (if the bacteria happen to be pathogenic bacteria) is at risk. The indications are that a bacterial inoculum is usually present at the point of packing.

Result of a study by Kou et al. (2015) showed that there was an increase of aerobic mesophilic bacteria populations in packed RTE baby spinach stored in open refrigerated display shelves at supermarket and groceries stores. They also reported that the shelf position where packed baby spinach was displayed had some impact on the amount of bacteria in the sample. Samples located in outer part of the shelf contained higher microbial counts which was perhaps due to higher temperatures from the surrounding as warm air coming from the aisle compared to those in samples located in the inner shelf which is exposed to cooler air. However, as mentioned earlier, if the cold chain is maintained, introduction of new inoculum, for example through opening the bag as shown in our study, the microbial growth in the fresh RTE salads can be checked effectively.

There are various sources that may contribute to the contamination of the rocket salad as it may occur throughout the whole production chain. It may begin at the farm levels. Potential sources of contamination include water used for irrigation and in the phases during production of the rocket salad. Other factors include the use of manure as fertilizers, and incorrect application of Good Manufacturing Practice (GMP) and Hazard Analysis and Critical Control Point (HACCP) system during production, processing, packaging and distributions (De Giusti et al., 2010). This also includes cross contaminations from the staff handling the products and also the processing equipment.

Although minimally processed or RTE vegetables are usually colonized by harmless saprophytic Gram-negative flora, they are capable of affecting the shelf life of food products. Incorrect irrigation and fertilization may contribute to the presence of pathogenic micro-organisms of human origin in minimally processed vegetables (Legnani and Leoni, 2004).

3.5.3 Handling and storage condition in the supply chain affected the occurrence and abundance of volatile organic compounds

Volatile organic compounds produced by rocket is largely dependent on temperature and duration of storage. At Day 0, only three VOCs were detected which were dimethyl sulfoxide, 2-methylfuran and phenol. In the following 24 h of storage, an additional compound, dimethyl disulfide was detected apart from the compounds detected at Day 0 and more compounds were produced as the storage time was extended making 23 compounds in total. Some VOCs identified at later points of storage in this study were from sulfur group such as dimethyl sulfide and dimethyl trisulfide. Other groups of compounds detected at the later stage of storage are alcohol, ketone, aldehyde, ester, alkane, carboxylic acid and indole. As expected, the abundance of each compound was found to be associated with days of storage and temperature as reflected in variation of the handling and storage condition treatments, but the trends were different between compounds. Similar results were reported by Luca et al. (2016) who found that the initial abundance of VOC released by wild rocket were low, but then the overall abundance of the most of the VOCs were higher with the time of storage.

The significance of storage temperature in affecting generation of VOCs in rocket was also recorded before by Spadafora et al. (2016). Spadafora et al. reported that four types of aldehydes, 2,4-hexadienal, E and Z-2-hexenal, E/Z-3-hexenal seems to positively correlated with temperature when the bouquet of head space of bagged of rocket salad stored at 0, 5 and 10 ^{o}C were analysed. Increasing temperature enhanced the activity of enzymes, thus accelerating metabolic processes leading to generation of more species of VOCs and increasing their abundance.

Sulphur compounds are important group of compounds commonly detected in the stored product of brassicas. This include dimethyl sulfide, dimethyl sulfoxide and dimethyl disulfide detected here is in line with the report of Spadafora et al. (2016). In green produce, the increase in the abundance of sulphur compounds is normally associated with leaf aging, wounding, product deterioration and microbial activity (Christensen et al., 2007). It was claimed that sulphur-containing compounds could associate with off-odour aroma and it is commonly occur in line with the increase in microbial load that occurred under long storage duration (Section 3.2.2.1 and 3.3.2.1).

While dimethyl sulfoxide is almost odourless with a faint smell of garlic, dimethyl sulfide has a distinctive smell described as rotten cabbage. It has also been associated with the odour of cooked cauliflower (Engel et al., 2002). Dimethyl sulfoxide could break down to dimethyl sulfide (Spadafora et al., 2016). Dimethyl disulfide was also reported as having a very unpleasant odour with a low odour threshold (Zinder and Brock, 1978). Nielsen et al. (2008) reported that abundance of dimethyl sulfide and dimethyl disulfide were higher when the samples were kept at 8 ^{o}C than those kept at 4 ^{o}C. These two sulfides increased when were inoculated with *Psedomonas* spp. and *Xanthomonas* spp. and at

oxygen levels below 1% (Spadafora et al., 2016). This low level of O_2 was reached in their study of *D. tenuifolia* when leaves were stored at 10 °C for 6 days and at 5 °C for nine days, that occurred concurrently with the rise in bacterial counts. He increased in bacteria count coincided with lower aroma score noted by the sensorial analysis at day nine of storage. Similar anaerobic conditions could be experienced in some handling and storage condition, especially those that were under HSSDUD, HSSDUD+2, NPDUD and NPDUD treatments. The microbial counts were highest under these conditions (Table 3.3), supporting our hypothesis on the possible positive relationship between number of bacteria and generation of a certain species of VOCs in fresh RTE rocket salad system. Furthermore, leaves of the treatments also were found to contain high abundance of alcohols, which could be associated with anaerobic condition.

Despite of the increase in abundance of some compounds belong to sulphur compounds, some members of furan and carboxylic acid were found to decrease, but these were seen only on the expiry date and two days after. We found that 2-thiophenecarboxaldehyde (aldehyde), and 1-butanol (an alcohol) had decreased throughout days of storage. Similar observation was also recorded by Spadafora et al. (2016) where 21 VOCs were negatively correlated with days of storage, including members of aldehydes, isothiocynates and esters.

Rocket that was kept at display temperature until display until date (13 ^{o}C, NPDUD) seem to have higher abundance of (Z)-3-hexenyl 3-methylbutanoate abundance compared to the leaves kept until 2 days after expiry date (NPDUD+2). In addition, dimethyl disulfide, 3-ethylfuran and acetic acid were at their highest concentration on the expiry date but then decreased on two days after expiry date. The decrease in the abundance of these

compounds could be due their degradation leading to the formation of other new volatile compounds.

2-ethylfuran and 3-ethylfuran are associated with earthy and savory flavour (Bell et al., 2017a). Acetic acid give pungent, savory and sourness (Tandon et al., 2000) and astringency characteristics (Hufnagel and Hofmann, 2008). It was the most abundant in samples with long storage and stored at high temperature and therefore leaves subjected to such treatment will give a stronger pungency and sourness aroma of the rocket bag towards the end its shelf life.

Amongst the chemical groups identified, sulphur compounds were reported to have a strong relationship between their abundance and hot, peppery, mustard and warming attributes of rockets (Bell et al., 2017a). These compounds could be aroma precursors as they will change with growing season. As seen in this study, sulphur compounds were seen more abundance in samples subjected to high temperatures such as those under HSSDUD and NPDUD conditions for dimethyl sulfide. For this reason, sulphur compounds could play important role in determining the taste and aroma of rocket following differences in handling and storage conditions (Nielsen et al., 2008).

Relatively, alcohol and esters has been shown to have a lower contribution to the total aroma composition in brassica leaves (De Pinho et al., 2009). These two groups of compounds were detected in rocket leaves only in samples stored at high temperature on DUD. In general, alcohol compounds are usually act as a defensive mechanism (Ruther and Kleier, 2005) where it is always produced when the leaves are being cut or chewed which often provide cut-grass aroma in vegetables. 1-penten-3-ol in particular linked with

burnt and pungent attributes after storage of brassicas (Buttery et al., 1990), explaining the pungent and sharp smell of the samples after a long storage period compared to the fresh leaves.

Under modified atmosphere conditions, short chain methyl-branched alcohols and esters were normally found to predominate the gas in the bouquet after long periods of storage which could be as results of severe fermentation (Smyth et al., 1998; Deza-Durand et al., 2014). In this study, esters, (Z)-3-hexenyl 3-methylbutanoate were detected in samples that were either heat shocked followed by storage at 13 ^{o}C until DUD, (HSSDUD), those that were not purchased, and stored at 13 ^{o}C until DUD (NPDUD), as well as samples that experienced under similar conditions with two more days (HSSDUD+2 and NPDDUD+2) before being analysed. VOCs generated at the later stage of the storage normally contribute to off-odour aroma of the fresh produce. It is interesting to note that indole was only detected in all treatments with extra two days after DUD, with the leaves experiencing heat shock contained the highest abundance of the compound. This is expected as indole is normally produced by bacteria as a degradation products of amino acid tryptophan. At low concentration, indole would have flowery aroma but it will turn to a faecal smell when present at higher concentrations (Mahmoud and Buettner, 2016).

Phenol is a secondary plant metabolite that has a strong antioxidant capability. Phenol may also act as a defense system upon disturbance of pathogens and insect and other form of stresses (Lattanzio et al., 2006). In this study, the most abundance of phenol was detected occurred in leaves that were continuously kept for a long storage periods under relatively high temperature such as those in NPDUD, NPDUD+2 and HSSDUD+2,

which coincided with high bacterial counts, emphasizing the role of phenols in responding to the stresses induced by high load of bacteria.

Apart from the VOCs detected and reported in this section, another group of important volatiles that attract a lot interest of scientists of various disciplines found in rocket and other types of brassicas is glucosinolates. Due to its key role in contributing to rocket flavour and other biological functions, GLS is discussed separately in Section 3.5.5.

3.5.4 Variation in bacterial load, gas composition and appearance of RTE wild rocket in the supply chain are affected by handling, storage conditions and growing season

As expected we found that rocket produced in warmer field condition in summer contained higher bacterial loads compared to the rocket produced in winter (Table 3.5). Similar observations were reported by other researchers (Caponigro et al., 2010; Garrido et al., 2015). Garrido et al. (2015) reported that higher counts of psychrotroph bacteria in baby spinach were found in samples grown in spring (April, mean temperature 13.4 ^{o}C) compared to baby spinach grown in winter (January, mean temperature 10.2 ^{o}C) in Spain. They also found that leaves harvested in the evening of spring crop contained higher number of bacteria, but this phenomenon was not observed in winter crop. However, this was only true for psychrotrophs and not with Pseudomonas. Similarly, Caponigro et al. (2010) reported that bacterial loads in several species of ready-to-eat salads (lettuce, rocket, spinach and lamb's lettuce) produced in summer and autumn were higher than those produced in winter and spring. They reported that *E. coli* were found

in 27% of the samples. No salmonella or *Listeria monocytogenes* was found. Overall, total viable bacterial counts varied from $5.01x10^1$ cfu.g^{-1} in December to $7.94x10^3$ cfu.g^{-1} in April. Lactic acid bacteria (LAB) were not detected in December but the counts increased to $7.94x10^5$ cfu.g^{-1} in July and September (Capgnigro et al., 2010). Overall, the counts reported in their study were lower compared to our data. This could be due to difference in handling procedures which related to distance from farm to retailers. The study conducted by Capgnigro et al. (2010), was carried out in Italy using locally grown fresh produce whereas in our case, rocket leaves imported from Italy were used. Long distance transportation which would take a longer time allows bacteria to proliferate.

The increase in bacterial counts reported by previous researchers were similar to the findings in this thesis, but at this point of time our results were based on total plate counts of a pool of bacteria without being sorted by different species or genera of bacteria. A more detailed analysis of the genera and species of bacteria found in fresh RTE rocket is be reported and discussed in Chapter 5.

Results presented in Table 3.5 clearly show that leaves in both seasons subjected to high (25 ^oC) and moderate (13 ^oC) temperatures encouraged the growth of bacteria resulting higher bacterial loads as seen in the samples HSSDUD, HSSDUD+2, NPDUD and NPDUD+2. Brief exposure of the rocket to high temperature followed by long term storage at appropriate cold temperature keeps the bacterial loads low. This result implies that temperature abuse by consumer during transit before being stored under domestic refrigeration would not risk the increase in bacteria loads in rocket. In contrast, temperature abuse by supply could be dangerous if the leaves contain pathogenic bacteria

because after exposing the produce at higher temperature, the leaves will be displayed at moderate temperature (13 ^{o}C) that would not stop the bacteria from growing.

The variation in bacterial counts normally correlated well with gas composition sampled from the headspace of the bags. The significance of the gas composition in bagged fresh RTE produce and its relationship with bacterial loads, VOCs and its quality is well acknowledged. Generally, after long storage, especially at an abused temperature, the production of O_2 will be reduced and at the same time the production of CO_2 will be elevated (Table 3.5; Deza-Durand et al., 2014). Differences in growing environment as experienced by crops grown in different seasons, markedly affected all gas composition measured, CO_2, O_2 and ethylene. Summer grown rocket was found to have a lower CO_2 and ethylene concentrations, but have a higher O_2 in their head space than those in the bags of winter rockets. The results recorded here seem to be surprising if we relate them to the overall bacterial loads. High overall CO_2 and low O_2 are actually associated with high concentration of the gases in four out of 11 treatments, i.e. in HSSDUD, HSSDUD+2, NPDUD and NPDUD+2 of winter rocket. In these four treatments, at a certain stage, the leaves were exposed to high (25 ^{o}C, HSSDUD, HSSDUD+2) and moderate temperature (13 ^{o}C, NPDUD, NPDUD+2). High CO_2 of the leaves in these treatments would attributable to high respiration suggesting that the winter grown crops that have been acclimatized to low field temperature might have poor adaptability to relatively high temperature during handling and storage phases, causing high respiration rates (Amodio et al., 2015).

In some treatments of winter rocket, the O_2 level have dropped to a very low level that could have reached to a limiting O_2 level, as seen in treatments that involved heat shock,

followed by storage at moderate temperature (13 ^{o}C) (HSSDUD, HSSDUD+2) and the samples that continuously stored at 13 ^{o}C (NPDUD, NPDUD+2). The results indicate that the condition in the respective bags could have reached an anaerobic condition, thus affecting the aroma of rocket following the generation of off-flavour volatiles (Deza-Durand et al., 2014; Section 3.5.3).

The release of ethylene from rocket leaves was higher at higher storage temperature, but this response normally can only be observed in the first days in storage (Koukounaras et al., 2007). Koukounaras et al. also reported that, during storage, the concentration of ethylene was found to peak on day one. Similar results were obtained in our study whereby the highest ethylene concentration were recorded in control samples, as well as in gas in bags containing leaves of treatments HSS24 and HSC24, regardless of growing seasons. It is generally known that ethylene is linked to the loss of chlorophyll resulting in the senescence of leaves (Able et al., 2003). Even though the concentration of ethylene was higher in the above treatments, our results did not show any marked differences in chlorophyll index of the leaves across treatments. In the study, measurement of chlorophyll was performed immediately after the treatments ended (within 24 h) and therefore there could be insufficient time for the chlorophyll to degrade.

3.5.5 Handling and storage condition in the supply chain affected the occurrence and abundance of glucosinolates

Six types of individual GLS were detected in rocket in this study and all of the six GLS recorded here were also recorded by Bell et al. (2015). The behaviour of selected rocket accessions and varieties in accumulating individual GLS was also documented by Bell et

al. (2015). Beside the pre-harvest factors, the occurrence and concentration of individual GLS are affected by post-harvest factors such as storage conditions including storage duration, temperature and atmospheric conditions of the storage environments. In addition, metabolic profiles of the produce vary according to its growth stage, such as leaf age in the case of rocket (Koukounaras et al., 2007). After harvest, metabolic processes will still continue to take place and hence continue to alter the phytochemical compositions of the plant tissue (Jahangir et al., 2009; King and Morris, 1994).

Continuation of metabolic process after harvest are reflected in changes of GLSs in rocket leaves during storage, and their changes varied according to handling and storage conditions (Table 3.6). In general, there is no clear patterns of response which could be used to describe the changes in individual GLS in relation to changing handling and storage conditions detected, suggesting that the synthesis of individual GLS could have been synthesized through different specific pathways, or triggered by different signals. Among the signals that could be present in bagged fresh RTE rocket used in the study might come from variation in temperature, microbial activity and gas compositions. Furthermore, there are a lot of different microbes present in system (Chapter 5); mould and yeast, and different types of bacteria that could be producing different biochemical signals for plants that trigger the generation of different types of glucosinolates.

Our results clearly show that the concentration of glucosativin in leaves subjected to different treatments varied significantly with the concentration in glucosativin seems to be lower when stored at 13 ^{o}C compared to those stored at 4 ^{o}C. The negative effects of high temperature on glucosativin are clearly seen for leaves in HSSDUD, NPDUD, HSSDUD+2 and NPDUD+2 treatments. In all these treatments, the leaves were subjected

to high temperature, either at 13 º or 25 ºC or both temperatures, at at least one point of their handling and/or storage.

Conversely, results in Table 3.6 show that glucoerucin was not detected in control, HSC24, HSCDUD and EPCDUD+2 leaves and this could be associated with its low formation of the compound as the synthesis of GLS in generally triggered by stress factors, as discussed earlier. Under such situation, the leaves may not experience sufficient stress at postharvest stage. Furthermore, the rocket used in this study were grown in winter that unlikely to experience heat and drought stress in months of January and February under Mediterranean Italian weather conditions. In addition, the leaves of all these treatments contained relatively low microbial loads, following low naturally infection at farm level as well as slow growth of bacteria at shelf life stage due to low storage temperature (Table 3.5).

Anaerobic conditions, formed after long storage under closed conditions at 4 ºC (EPCDUD+2), could have responsible for reduced glucoerucin content, indicating that the stability of glucoerucin is affected either by $O_{2//}CO_2$ balance in the packaging atmosphere. The influence of bacterial activity on the stability of glucoerucin could be ruled out as the TPC of EPCDUD+2 and EPODUD+2 were similar. The biosynthesis of GLS involves a very complex process that link to biochemical reaction including oxidation (Grubb and Abel, 2005). Lack of sufficient level of O_2 would alter the pathway thus affecting the type and abundance of individual GLS produced. Martinez-Sanchez et al. (2006) found that reduction of GLS in rocket leaves was higher when it was stored under low O_2 and high CO_2 conditions which could be up to 60-100% losses.

In contrast to other individual GLS, formation of DMB was enhanced by moderate temperature (13 ^{o}C) but exposure to briefly to high temperature (25 ^{o}C) reduced its content. Such effects were seen in samples of NPDUD and NPDUD+2 for moderate temperature, and in samples of HSC24 and HSS24 for high temperature exposure or heat shock treatments. Undetectable or low level of DMB were also noticed in leaves of other treatments involves high temperature treatment (HSCDUD, HSSDUD, HSCDUD+2 and HSSDUS+2). The formation of DMB is important in term of GLS's nutritional and biological functions as DMB was found to negatively correlate to antimicrobial load induced by rocket leaves (Chapter 6).

Compared to other GLSs, glucoraphanin was detected in most of the treatments, except for control (Day 0) and HSC24 samples, suggesting that the synthesis of glucoraphanin could occur in many situations. However, the reason behind the variation in its contents among handling and storage condition is difficult to explain as there is no clear pattern. An unclear pattern of response to various treatments also occurred for diglucothiobeinin.

GLSs were reported to be among the most dynamic constituents in rocket leaves during storage, whereby their concentration could be reduced up to 33% when leaves were stored in air (Martinez-Sanchez et al., 2006). The losses could be higher (60-100%) when the atmosphere in the packaging reaches to an anaerobic condition. Similar trends were also observed in this study except for leaves that received heat shock before being kept at 4 ^{o}C as seen in treatment HSC24 and HSCDUD+2, causing an increase of 54.6% in total GLS, increasing from 9.90 to 15.31 mg.g^{-1} sinigrin hydrate equivalent GLS. Overall, leaves that were kept in low temperature and 'consumed' on DUD especially with the

bags opened contained the highest content of total GLS and this is also true even for the leaves that were 'used' or 'eaten' on two days after DUD.

3.6 Conclusion

The growth of bacteria in bagged fresh RTE rocket (*Eruca sativa* and *Diplotaxis tenuifolia*) was significantly affected by temperature and duration of storage. The effects of the factors however are modified by other interacting factors that may occur simultaneously or along the supply chain of fresh RTE rocket. Hand contact onto the leaves that possibly caused crossed contamination, followed by storing them at high temperature (13 and 20 ^{o}C) had caused dramatic increases in bacterial loads.

In the supply chain of RTE fresh produce, the products may be subjected to varying handling and storage conditions that could involve various parties: supplier, retailers and consumers. Temperature abuse, whether caused by supplier, retailer or consumer during transit, display or storage played important role in determining the bacteria loads, shelf life, appearance, flavour and nutritional values of the rocket. When the produce are purchased and consumed within 24 h after their arrival and displayed at the retailers' premises, exposure to high temperature (25 ^{o}C) did not increase the bacterial loads in the leaves. Similarly, heat shock at 25 ^{o}C did not affect the bacterial loads if the produce are then stored under low temperature (4 ^{o}C) as practiced by consumer by keeping them in domestic refrigerator. Keeping the produce under display temperature, normally at 13 ^{o}C at the retailer premises, regardless of heat shock or not, increased bacterial counts. An additional two days after the expiry date at 4 ^{o}C did not produce a significant impact on bacterial loads.

RTE rocket prepared using leaves produced in summer contained higher counts of bacteria. In general, the effects of temperature abuse during transit and storage of the produce were similar as described earlier, except that additional two days of storage at 13 ^{o}C of previously heat shocked at 25 ^{o}C (HSSDUD+2) caused a significant increase in bacterial loads for winter leaves. During storage, heat shock of winter leaves followed by storage at 13 ^{o}C induced anaerobic condition as shown by high CO_2 and low O_2 concentration in the packaging. Similar condition was also experienced by the leaves that continuously kept at 13 ^{o}C until expiry date. Compared to summer grown rocket, winter leaves generated higher rate of ethylene that may be associated with poor adaptation to relatively high storage temperature compared to the field temperature. However, this did not cause any differences in leaf appearance.

Results of the study to evaluate the effects of handling and storage conditions on volatiles revealed that short term storage (up to 24 h) and storage under low temperature (at 4 ^{o}C) managed to reduce the generation of off-flavour VOCs. As expected, heat shock and long term storage at 13 ^{o}C resulted in the release of high concentration of off-flavour compounds. Among important volatiles that contribute to off-flavour aroma detected include sulphur containing compounds (such as dimethyl sulfide, dimethyl sulfide, dimethyl trisulfide and dimethyl sulfoxide), and alcohols (such as 1-butanol, 1-penten-3-ol, (z)-2-penten-1-ol, I-hexenol and phenethyl alcohol). The presence of acetic acid and furan could also contribute to off-flavour aroma.

Six individual GLSs glucosativin, glucotropaeolin, glucoerucin, glucoraphanin, diglucothiobeinin and DMB were detected. The occurrence and concentration of these GLSs in RTE rocket varied among treatments and therefore a general trend cannot be

derived to explain the generation pattern of glusinolates across treatments. Leaves that experienced heat shock at 25 ^{o}C but analysed immediately after 24 h, and leaves that were stored at low temperature (4 ^{o}C), contained high concentrations of glucosativin. In contrast glucotropaeolin was not detected when the heat shocked leaves were analysed after 24 h. Glucoerucin was found to be highest in leaves that have been kept at low temperature from the beginning. Differences in the occurrence and concentration of individual GLS detected in rocket leaves in response to varying handling and storage conditions could be associated with the complexity of the biosynthesis pathways of glucosinolates (Grubb and Abel, 2005). Changes in GLS content in rocket is considered as an important event as this would have impact on nutritional values of the produce in view of its anticancer and antimicrobial capabilities. The relationships between GLS and its antimicrobial properties will be discussed further in Chapter 6.

CHAPTER 4

DISTRIBUTION OF NATURALLY OCCURING BACTERIA AND FORMATION OF BIOFILMS ON ROCKET LEAVES VIEWED USING SCANNING ELECTRON MICROSCOPY

4.1 Introduction

Sanitation strategies deployed to limit microbial presence on plant tissue are generally not as effective as expected and the remaining bacteria may recover if a continuous cold chain is not maintained (Section 3.3). This evidence could be associated with internalization of bacteria into stomata and internal compartments of the leaves, thus protecting bacteria from being fully exposed to washing and contact with sanitizing agents (Karaca et al., 2014; Saldana et al., 2011; Selma et al., 2008). Crevices, cracks and small fissures in the product, along with the hydrophobic nature of the leaf surfaces may prevent sanitizers from reaching the target organisms (Burnett and Beuchat, 2000). Apart from these residing places, resistance of the bacteria to sanitation has been linked with the presence of biofilms on leaf surfaces. A scanning electron microscopic analysis by Saldana et al. (2011) on spinach leaves incubated from 1 to 24 h with *E. coli* confirmed the presence of the *E.coli* within internal cavities, intercellular spaces of the spongy mesophyll, and notably in the vascular tissue (xylem and phloem).

Biofilms are complex communities of microorganisms that are attached to a surface and to each other, and which are embedded in a self-produced matrix of extracellular polymeric substances. The major composition of biofilms is water and bacterial cells, besides a variety of secreted compounds such as polysaccharides, proteins, lipopolysaccharides, DNA and lipids, in addition to minerals and other components from

dead cells of the host components (Costerton et al., 1999). Bacterial biofilms may develop on food and food-processing surfaces, which could increase the risk of food poisoning (Carmichael et al., 1999). Therefore, thorough understanding of the residing sites of bacteria and the possible role of biofilm in affecting the effectiveness of ozone treatment and other sanitizing agent needs to be investigated.

The present study was conducted to discover the natural distribution of bacteria and formation of biofilms on leaves of rocket during different storage conditions. In some cases, non-commercially relevant storage conditions were used in order to establish the 'worst case scenario' and to ensure that bacteria were present on the leaves during the development of the analytical methods. Information generated in this study will be useful as a guide to analysing the efficacy of sanitising compounds that are deployed in an effort to remove or deactivate the microbes residing on the leaf surfaces. It was hyphothesized that higher abundance of bacteria could be seen around the dented area and stomata, as well as along the vein of the leaves as these areas would provide protection and nutrients to the bacteria. Also, it is expected that rapid multiplication of bacteria under higher temperature would to the formation of biofilms in the leaves.

4.2 Materials and Methods

The scanning electron microscopy was performed using Quanta 600 Environmental Scanning Electron microscope (FEI, Hillsboro, OR, USA) at the Centre for Advanced Microscopy, University of Reading. Two methods of sample preparation were employed which were chemical fixation method (Conventional SEM) and cryo-method (Cryo-SEM).

4.2.2 Conventional SEM method

4.2.2.1 *Plant materials*

The washed fresh RTE rocket samples used for this study were obtained from Sainsbury's, Reading, UK. At the retailer's premise, the leaves were displayed in gas permeable bags normally at 10-13 ^{o}C. The leaves were subjected to different storage and handling conditions, before being prepared for SEM viewing as follows:

i. Prepared and fixed immediately upon arrival at the Department of Food and Nutritional Sciences, University of Reading, designated as Day 0;
ii. Stored at either 4, 13 or 20 ^{o}C (room temperature) and prepared for SEM viewing on day six after purchase;
iii. Stored at 13 ^{o}C in either opened or closed bag conditions and prepared for SEM viewing on day six after purchase. In the 'opened bag' treatment, the bags were cut opened on the day of arrival and the samples were mixed with bare hand. The bags then were left open afterwards;
iv. Stored at 20 ^{o}C in a closed bag and fixed on day six after purchase for SEM viewing; and
v. Leaves were kept at 4 ^{o}C until DUD followed by storage for another six days at 20 ^{o}C in closed bag for SEM viewing.

4.2.2.2 *Chemical fixation and SEM viewing*

Samples were cut into small squares (3 mm x 3 mm) to facilitate penetration and were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 25 mM HEPES buffer. The samples were kept in fixative overnight at 5 ^{o}C. The samples were washed in HEPES buffer for 15 min and were dehydrated through a serial dilution of acetone starting with 30%, 50%, 70%, 80%, 90%, 100%, and twice with 100% dry solvent with molecular sieve with 15 min interval for every dilution. The samples were critical point-dried using CO_2 and sputter coated with gold for 2.5 min before the images were viewed with Quanta 600 Environmental Scanning Electron microscope operated at 20kV.

4.2.3 Cryo-SEM method

4.2.3.1 *Plant materials*

For Cryo-SEM method, leaves of two different storage and handling conditions were used:

i. Leaves of Day 0 (prepared immediately upon arrival at the Department of Food and Nutritional Sciences, University of Reading); and
ii. Leaves were kept at 4 ^{o}C until DUD followed by storage for another six days at 20 ^{o}C in closed bag for SEM viewing.

4.2.3.2 *Preparation of sample and SEM viewing*

Leaf pieces were cut into small squares (3 mm x 3 mm) and were mounted and fixed on sample shuttle using colloidal graphite and OCT low temperature adhesive. The samples were then plunge-frozen in slushed liquid nitrogen at -210 ^{o}C until they stopped boiling, before being transferred into the chamber, by raising the shuttle and sample out of the nitrogen and into the chamber, which was under vacuum. Each sample was placed into the cryo-preparation chamber which is attached to the Quanta 600 Environmental Scanning Electron microscope. The temperature of the chamber was then raised to -90 ^{o}C for 5-10 min to sublimate any condensed ice from the surface gained during sample transfer. The temperature of the sample was then reduced to -135 ^{o}C. To avoid charging problems while searching for suitable site, the sample was sputter coated with thin layer of platinum for 30 sec. Afterwards, the samples were loaded on the cryo-stage, which was maintained at -135 ^{o}C using nitrogen liquid. Imaging was performed using an acceleration voltage of 5-10kV.

4.3 Results

4.3.1 Distribution of bacteria on leaves

Representative of SEM images taken from abaxial surfaces of rocket leaves with different treatments demonstrated the presence of bacteria both inside stomata and on the leaf surfaces. Leaves condition prior to processing for SEM viewing are shown in Figure 4.1, Figures 4.3 - 4.8, and Figure 4.18.

Figure 4.1. Rocket leaves on Day 0 (Control)

Based on electron scanning micrographs obtained, the bacteria are predominantly found on the abaxial surface of the leaves, especially on the indented surface area along the veins of the leaves and around the stomata/guard cells (Figures 4.2 A and B).

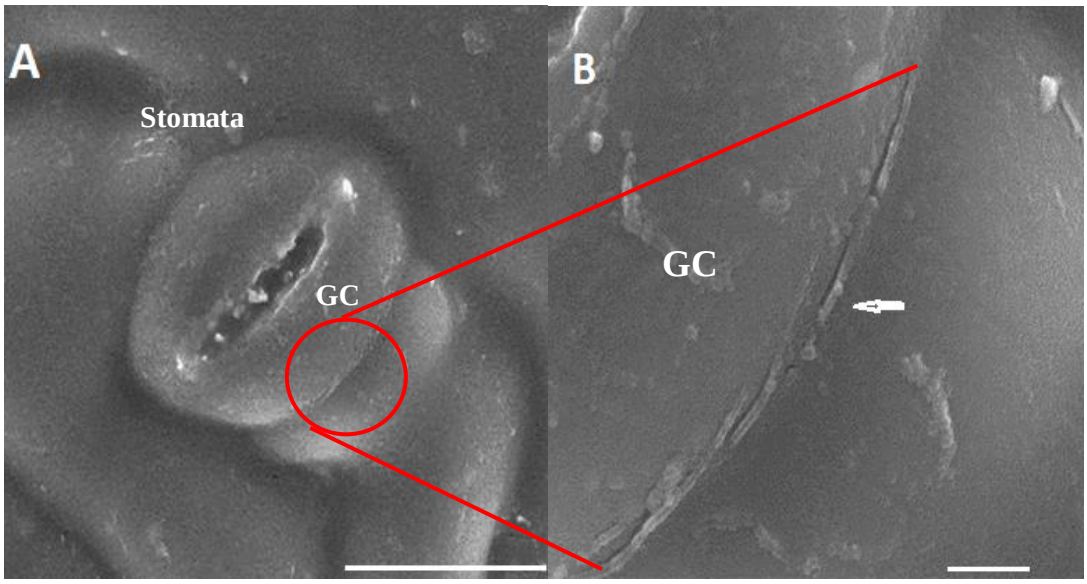


Figure 4.2. Images taken from abaxial surface of a rocket leaf epidermis in the region of a stoma on day 0 on sample prepared using Cryo-SEM method. The images show the bacteria adherence on the leaf surfaces surrounding the guard cell (arrow). Scale bars = 20µm (A), 2µm (B), guard cell (GC).

Information depicted in the micrographs (Figures 4.9-4.12, 4.15 and 4.16) indicated that the abundance of bacteria varied markedly, depending on storage duration, storage temperature and openness of the packaging. As expected, the rocket stored at higher temperatures contained more extensive distribution of microbes. The number of bacteria increased markedly when the samples were kept at higher temperatures (13 and 20 ^{o}C) for six days as shown in Figures 4.11 and 4.12 compared to those kept at 4 ^{o}C (Figure 4.10). The respective bacterial counts for each case are available in Table 3.1. The visual difference observed in bacterial distribution and density seen on these micrographs were consistent with the result of the total plate counts reported in Chapter 3.

Figure 4.3. Rocket leaves stored at 4 ^{o}C on Day 6 with opened bag.

Figure 4.4. Rocket leaves stored at 4 ^{o}C on Day 6 with closed bag.

Figure 4.5. Rocket leaves stored at 13 ºC on Day 6 with closed bag.

Figure 4.6. Rocket leaves stored at 13 ºC on Day 6 with opened bag.

Figure 4.7. Rocket leaves stored at 20 ºC on Day 6 with closed bag.

Figure 4.8. Rocket leaves stored at 20 ºC on Day 6 with opened bag.

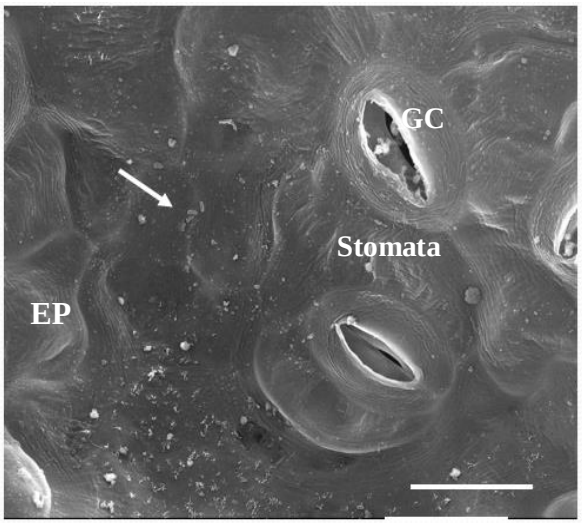


Figure 4.9. Image taken on day 0 (control sample) from sample prepared using chemical fixation method. Arrow shows the bacteria. Scale bar = 20µm, leaf epidermis (EP), guard cell (GC).

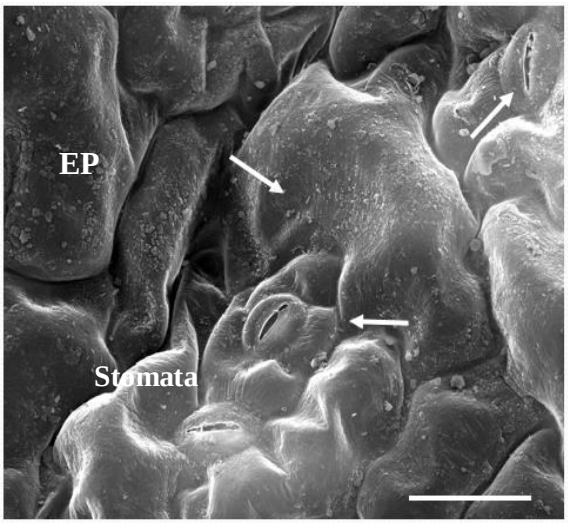


Figure 4.10. Image taken on day six from sample prepared using chemical fixation method stored at 4 °C. Arrows show the presence of bacteria on leaf surfaces. Scale bar = 50µm, leaf epidermis (EP).

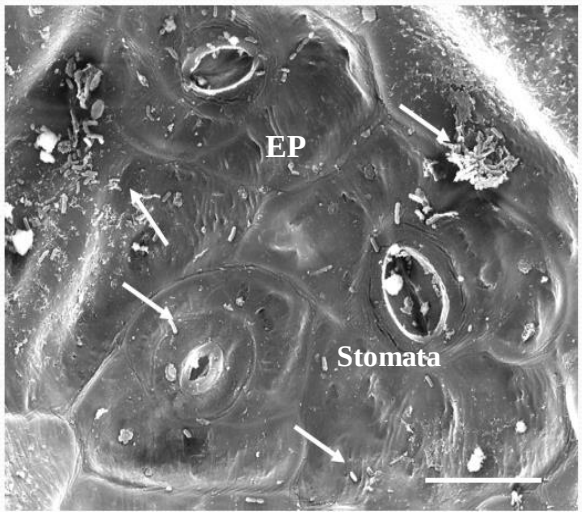


Figure 4.11. Image taken on day six from sample stored at 13 °C prepared using chemical fixation method. Arrows show the bacteria. Scale bar = 20µm, leaf epidermis (EP).

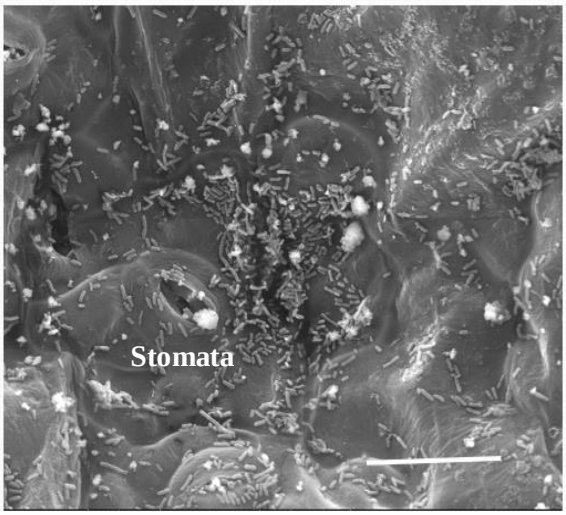


Figure 4.12: Image taken on day six from sample stored at 20 °C prepared using chemical fixation method. Bacteria can be seen everywhere. Scale bar = 20µm, leaf epidermis (EP).

Differences in the numbers of bacteria in samples kept in closed and opened bags are clearly shown in Figures 4.15 and 4.16. The micrograph in Figure 4.16 clearly revealed that rocket kept in opened bags contained higher counts of bacteria compared to the rocket kept in closed bags (Figure 4.15) and the results shown here are consistent with the results of numerical bacterial counts reported in Section 3.2.2.1 (Table 3.1 and Figure 3.2). However, this observation was only implicated for samples kept at temperatures higher than 4 ^{o}C. Samples stored at 4 ^{o}C had almost similar microbial abundance regardless of the packaging condition (Figures 4. 13 and 4.14, Table 3.1). Apart from residing on the leaf surface, bacteria were also found inside the stomata (Figure 4.16).

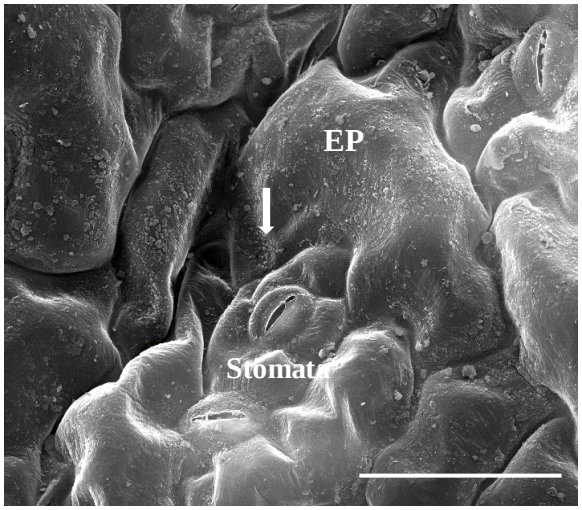


Figure 4.13. Image taken on day six from a sample stored at 4 ^{o}C in a closed bag prepared using chemical fixation method. Arrows show the bacteria. Scale bar = 50µm, leaf epidermis (EP).

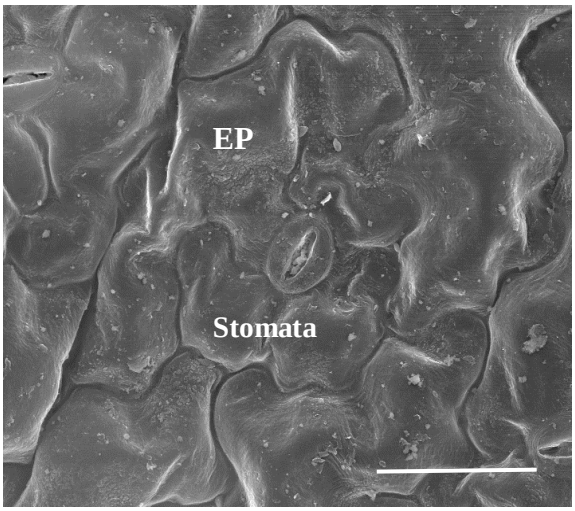


Figure 4.14. Image taken on day six from a sample stored at 4 ^{o}C in an opened bag prepared using chemical fixation method. Arrows show the bacteria. Scale bar = 50µm, leaf epidermis (EP).

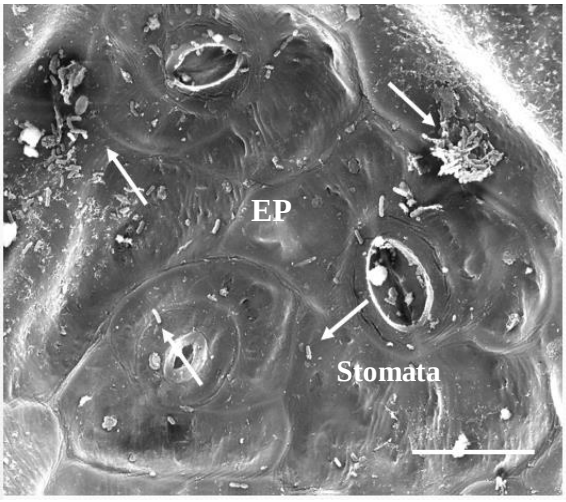


Figure 4.15. Image taken on day six from a sample stored at 13 ^{o}C in a closed bag prepared using chemical fixation method. Arrows show the bacteria. Scale bar = 20µm, leaf epidermis (EP).

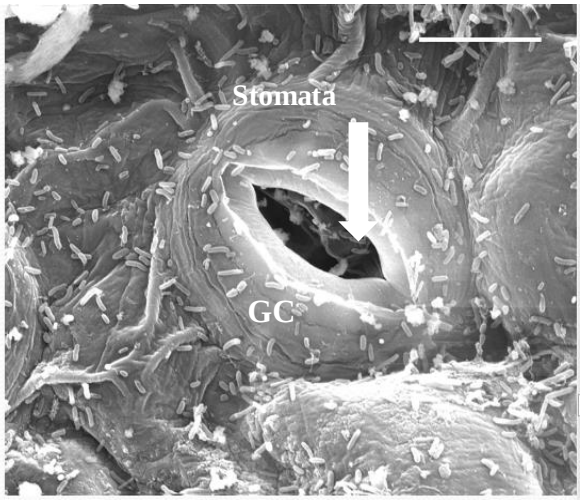


Figure 4.16. Image taken on day six from a sample stored at 13 ^{o}C in a previously opened bag prepared using chemical fixation method. Bacteria can be seen everywhere. Arrow shows microbes reside in stomata. Scale bar = 10µm, leaf epidermis (EP), guard cell (GC).

Micrographs obtained from samples using the conventional chemical SEM fixation method did not show formation of biofilms (Figure 4.19) whereas the biofilms were clearly shown in micrograph produced by Cryo-electron microscopy (Cryo-EM method) (Figure 4.20). This is because sample preparation in conventional methods involved dehydration, which could have destroyed the biofilms. From the observation, formation of biofilm was only seen on rocket leaves which kept at high temperature for a long storage. No biofilm detected on sample kept at 4 ^{o}C on display until date (Figure 4.17). Formation of biofilm was only observed on rocket leaves on day six after display until date on a sample kept at 20 ^{o}C (Figure 4.20). The condition of the rocket leaves covering with biofilm viewed on day six after display until date were far beyond consumption (Figure 4.18).

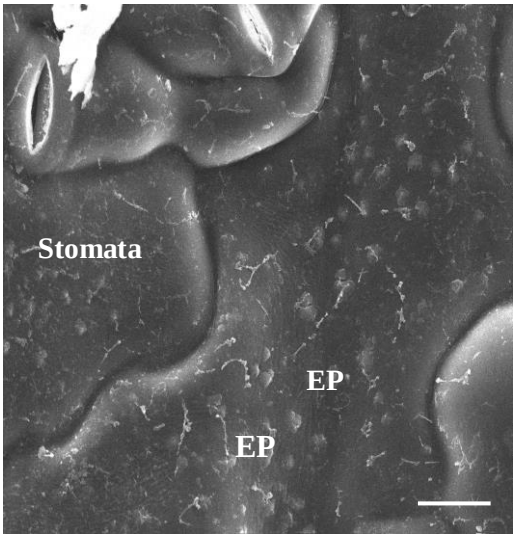


Figure 4.17. Image taken on rocket leaves kept at 4 ºC on display until date using Cryo-SEM method. No biofilm formation was detected. Scale bar = 20µm, leaf epidermis (EP).

Figure 4.18. Rocket leaves taken on day six after display until date on a sample kept at 20 ºC.

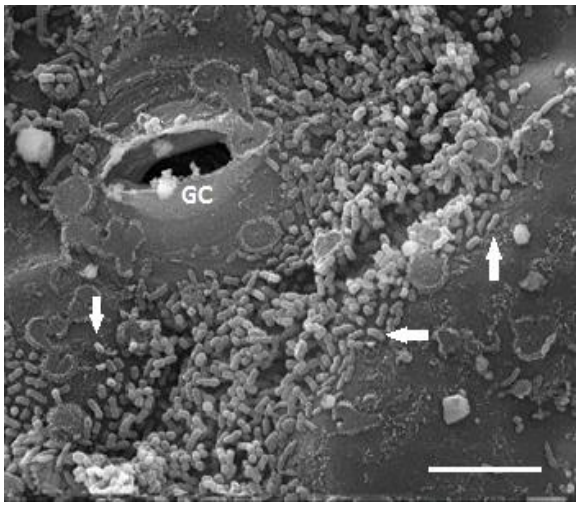


Figure 4.19. Image taken on day six after display until date on a sample kept at 20 ºC prepared using chemical fixation method. Arrows show that bacteria adhere on leaf epidermis and also on the guard cell of the stomata (GC). No biofilm can be seen. Scale bar = 10µm

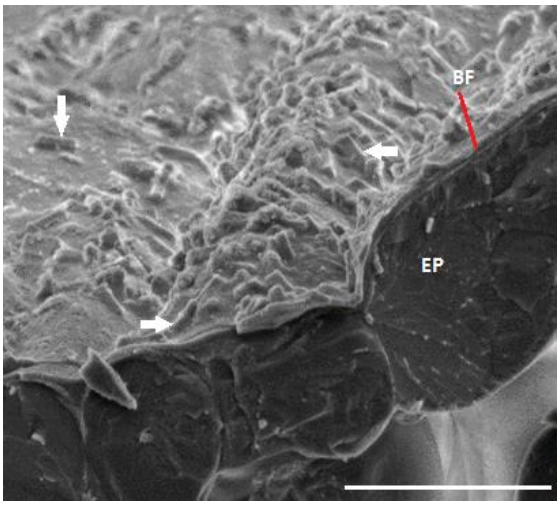


Figure 4.20. Image taken on day six after display until date on a sample kept at 20 ºC prepared using Cryo-SEM method. Arrows show bacteria reside on leaf epidermis (EP) and covered with biofilm (BF). Scale bar = 20µm

4.4 Discussion

4.4.1 Leaf surface anatomy impacts on bacterial distribution

The above-ground parts of plants are normally colonized by a variety of bacteria, yeasts, and fungi (Lindow and Brandl, 2003; Tournas 2005a). While a few microbial species can be isolated from endogenous plant tissues, many more are recovered from the exogenous surfaces of healthy plants. A study by Warner et al. (2008) revealed that bacteria are predominantly found in epidermal cell margins, around the stomata and around the leaf vein as these areas provide shelter against harsh environment and potentially provide nutrients to the microbes (Jablasone et al., 2005). Similarly, this can be seen in the present study as bacteria were distributed on the leaf surface, including inside the stomata. Similar observations were obtained from other studies (Kroupitski et al., 2009; Leben et al. 1988; Shaw et al., 2008). Warner et al. (2008) presented a visual description of adherence of microbial cells on spinach to quantitatively track the colonization of salad leaves by the human pathogen, *Salmonella enterica*, in view of determining how the microbes interact with the leaves surface and biofilms. Their results indicated that characteristics of leaf surfaces affect microbial colonization. For instance, spinach leaves which have greater proportion of the surfaces covered by organic matter appeared to have higher appearance of individual microbial cells and clusters, whereas for watercress the contamination was confined almost exclusively to the margin between epidermal cells. The presence of debris on spinach leaves represent the unwashed or dirty product.

Bacteria are by far the most numerous colonists of leaves, often being found in numbers averaging from 10^6 to 10^7 cells.cm^{-2} (and can go up to 10^8 cells.cm^{-2}) (Hirano and Upper, 1995; Andrew and Harris, 2000; Bettie and Lindow, 2000). As leaf surfaces are the most dominant aerial plant structure compared to other parts (Lindow and Brandl, 2003), most researchers have focused mainly on leaves to understand the distribution of microbes in plants. The physical environment surrounding phyllosphere is known to affect and change microbe populations continuously, with daily cycles in temperature, radiation, relative humidity, wind velocity, and leaf wetness affecting microbial growth (Hirano and Upper, 2000). Plant exudates that are rich in nutrition that could promote the growth of microbes are also thought to be a contributory factor (Jablasone et al., 2005). In the present study, the abundance of bacteria was modified according to the storage temperature, days of storage and whether the samples were kept in opened or closed packaging. Samples kept in opened-packaging clearly contained higher population of bacteria, as the leaves were disturbed with bare and unsanitised human hands, mimicking the handling that normally occurred during food preparation by the consumer.

Apart from the environmental conditions, the physical and physiological characteristics of leaves may also impact on the residing sites of microbes as the plants develop, mature, and senesce and as host phenology changes (Lindow and Brandl, 2003). These factors can partially contribute to elevated microbial populations observed after long storage, especially at high temperatures that could occur during product transit whereby raised temperature promotes faster senescence processes of leaf tissue that may make it more susceptible to bacterial colonization and provides a more conducive environment for bacteria growth.

Examination of the micrographs also revealed that the bacteria were also more likely to reside at invaginated areas within the surface of the leaves where it can be seen in images that contained small population of bacteria (Figures 4.2A and B, 4.9 and 4.10). Such observations may be associated with suitability of the micro-environment of the sites, which may include high availability of nutrients. The dented area in leaves normally exist along the leaf veins that may form of sites of exudate release. In a related study, Jablasone et al. (2005) observed that *E.coli* O157:H7 preferentially colonize epidermal root junctions for the same reason. The root junction, and perhaps also the dented leaf area, potentially offer openings for bacterial internalization into plant tissues (James and Olivares, 1997; Lugtenberg et al., 2001). Other common sites for microbial aggregation on leafy surfaces include leaf vein (Leben, 1988), trichomes (Timmer et al., 1989), stomata (Franz et al., 2007; Timmer et al., 1989), cell wall junctions (Romantschuk et al., 1996) and inter cellular spaces and xylem (Franz et al., 2007; Warriner et al., 2003).

4.4.2 Biofilm formation occurs towards the end of shelf life

Biofilms are a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface including plants (Costerton et al., 1999). Biofilms were detected on leaves that were kept at 20 ^{o}C examined on day 6 after the date until display (Figure 4.20). There was no formation of bacterial biofilm detected in samples that were kept at 4 ^{o}C at display until date (DUD) (Figure 4.17).

Clearly, in aggregate, these bacteria are sufficiently numerous to contribute too many important processes such as by giving protection in hostile environment and against sanitization. Biofilms were observed to form around the solute-conducting vascular tissue

(phloem), as shown in Figure 4.20. Phloem is the structure involved in the transportation of sugars and other solutes which become the last area of the leaves to undergo senescence (Lim et al., 2007). This would make sugar more concentrated in vascular region compared to other parts of the leaf and is potentially a source of carbon and energy for bacteria. Carbon sources would be more easily accessible for bacteria as the leaf structure degrades during storage life, potentially promoting more replication of bacteria towards the end of rocket shelf life.

Biofilms contain channels that allow nutrients to circulate and offer protection from fluctuations in environmental conditions (Molina et al., 2003; Morris and Monier, 2003) as the leaf surface is exposed to a dynamic and hostile environment. The biofilm communities can provide a condition that is suitable for the bacteria to rapidly multiply and disperse (De Beer et al., 1994). Biofilms also provide a protection layer against antimicrobial agents or sanitizers, most likely by inhibiting the sanitizing agent's ability to penetrate the full depth of the biofilm (Costerton et al., 1999).

Polymeric substances like those that make up the matrix of a biofilm are known to retard the diffusion of antibiotics (Costerton et al., 1999). For instance, biofilm-induced bacterial resistance to antibiotics is enhanced up to 1000-fold over the level observed when bacteria are grown under planktonic or free-living conditions (Cortes et al., 2011). In addition, presence of biofilms is able to increase the opportunity for gene transfer between bacteria and may be significant for the transfer of resistance genes to associated susceptible bacteria (Cortes et al., 2011) which may convert an avirulent strain into a virulent pathogen (Wuertz et al., 2001). Biofilms could therefore promote genetic diversity of bacteria and, at the same time, maintain the high cell density needed for

efficient genetic exchange that may lead to expression of new virulent microbial phenotypes (Wuertz et al., 2001). Several bacteria have plasmids and these substances can be transferred horizontally by conjugation to different species in a biofilm (Molin and Tolker-Nielson, 2003). Thus, the community provides microbes protection from many forms of abiotic stresses, such as chemical stress that may come in the form of antibiotics or disinfectants. If such a scenario occurred in the food chain system, it will expose consumers to a higher risk of getting disease as the microbes are more resistance to sanitizers and there exists the potential of having more virulent microbes present on the leaf.

4.5 Conclusion

As hypothesized, bacteria were mainly found both on and inside the leaves, especially around the stomata. The bacteria also preferentially colonized the 'sheltered' areas of leaves, such as at the dented areas along the leaf vein that may also form of sites of exudate release which are rich with nutrients. Biofilm formation was clearly detected on day 6 after date until display of leaves that were kept at higher temperature (20 ^{o}C).

CHAPTER 5

DIVERSITY OF BACTERIA AND MOULDS FOUND IN FRESH READY-TO-EAT ROCKET LEAVES AT CRITICAL PHASES OF THE SUPPLY CHAIN

5.1 Introduction

The incidence of foodborne illness arising from the consumption of fresh-cut produce has increased markedly in the last two decades (Koseki and Isobe, 2005; Olaimat and Holley, 2012). The outbreaks are usually associated with illness caused by consumption of the produce contaminated by diverse types of bacteria and other microbes such as *Salmonella enterica* serovars, *Listeria monocytogenes*, *Escherichia coli* pathovars, *Yersinia enterocolitica*, *Bacillus cereus*, *Vibrio cholerae*, *Shigella* spp., *Campylobacter* spp., *Aeromonas hydrophila*, *Yersinia enterocolitica* and *Clostridium* spp.; viruses such as norovirus and hepatitis A; and protozoa such as *Cyclospora cayetanensis* and *Cryptosporidium parvum* (Yaron and Römling, 2014). Since fresh RTE produce are typically consumed without thermal treatment, outbreaks following ingestion of such foods normally involve a large number of individuals.

Testing for the presence of pathogens in ready-to-eat food products contributes to maintaining food safety. Detection of *Escherichia coli* 0157 (and other verocytotoxin producing *E.coli*, VTEC), *Salmonella* spp., *Campybacter* spp., *Shigella* spp. and *Vibrio cholera* in fresh RTE food represents an unacceptable risk to health regardless of the number of bacteria present in the food samples (Health Protection Agency, 2009).

Beside detection of foodborne pathogens that impose health risks, the presence of food spoilage microorganisms are also important to all parties involved in the food supply chain. Among the groups of microbes, those belonging to bacteria, fungi (moulds) and yeasts are the most important as these groups of microbes could dramatically reduce the quality and shelf life of the produce and hence affect the marketability and consumer preference of the product. In RTE fresh salads, results of Tournas (2005b) showed that the most common moulds found in 39 samples of fresh and minimally processed vegetables purchased around Washington DC were *Cladosporium*, *Alternaria* and *Penicillium* with their counts ranged from less than 100 up to 4.0×10^4 cfu.g^{-1}.

Three experiments were conducted and are reported in this chapter, with the objective to identify the natural occurring bacteria and mould present at critical phases in the supply chain of fresh RTE rocket salads. Since the samples used for DNA sequencing were derived from leaves that have been subjected to a wide range of temperature and handling conditions, the expected species/strain of microbes identified would be very diverse.

5.2 Materials and Methods

Two methods were employed to identify bacteria colonising rocket salad. This study started with isolation of culturable bacteria on plate count agar followed by identification using whole genome sequencing. However, this method has its limitation where the identification made was very selective and only applied to culturable bacteria. Hence, 16S metagenomics was employed to identify bacteria profile in rocket leaves. 16S method

was able to identify all types of bacteria present on the rocket leaves together with their relative abundance.

5.2.1 Identification of bacteria using whole genome sequencing

5.2.1.1 *Sample preparation*

Culture stock

Rocket culture stocks from samples of winter grown rocket (Section 3.3.1.1) were kept in 30% glycerol stock and kept at -80 ^{o}C for further usage. Only four samples as listed in Table 5.1 were used for bacterial identification.

Bacteria isolation and stock culture

Single colonies with different morphologies were isolated and grown on PCA plates at 30 ^{o}C for 24 h. The single colony bacteria were grown in TSB for overnight and kept at in 30% glycerol for further usage.

DNA extraction

Rocket culture stocks from -80 ^{o}C were grown overnight at 30 ^{o}C in triptic soy broth (TSB). DNA of bacteria was extracted using DNA purification from Gram negative and Gram positive bacteria using Gentra Puregene yeast/bacteria Qiagen kit according to manufacturer's instruction (Qiagen House, West Sussex, UK). DNA of gram positive and

negative bacteria was combined. PCR and gel electrophoresis was done before sending for sequencing

5.2.1.2 *DNA identification screening for whole genome sequencing*

Isolated bacteria were sent to Eurofins Genomic (Ebersberg, Germany) for 16S sequencing to further select samples for whole genome sequencing. Screening was needed to avoid sending the same type of sample species multiple times for genome sequencing.

PCR amplification

The 16S primer pair with forward primer is 5'GTGYCAGCMGCCGCGGTA 3' and reverse 5' CCCGYCAATTCMTTTRAGT 3'. The amplification reaction for the DNA of all bacterial isolates, together with a negative control without template, was performed in a 25 µl PCR reaction using the GeneAmp PCR system 2700. The PCR reaction was 1 cycle at 94 °C for 3 min followed by 30 cycles with a denaturation step at 94 °C for 30 sec, then an annealing step at 55 °C for 45 sec and an extension step at 72 °C for 1 min, and finally, with 1 cycle at 72 °C for 8 min (Ellis, 2013). The 25 µl PCR mix containing 12.5 µl of PCR Bio taq mix red (PCR Biosystems Ltd., London, UK). One µl each of the primers forward and reverse primers DNA of bacteria and made-up with sterile water to the volume. The DNA amount was adjusted to make final concentration to 150ng/ µl. The DNA concentration were measured using NanoDrop spectrophotometer (Model ND1000, NanoDrop Products, Wilmington, DE USA).

The PCR products were sequenced by Eurofins Genomics, Germany. The results were then compared with the online DNA database of Nucleotide Basic Local Alignment Search Tool (BLASTN) programme of the NCBI. The nucleotide sequence generated using the reverse primer of each bacteria genomic DNA was converted to its forward strand equivalent by using the online reverse software programme of the NCBI. The sequence then were aligned with online multiple sequence alignment software programme (Clustal Omega, Hinxton, Cambridgeshire, UK) using forward and reverse primers with each other. Sections of complete complementarity for each sample were used to compare with the database in the NCBI using BLASTN to form matches that statistically significance in identifying of bacteria.

5.2.1.3 *Whole genome sequencing*

DNA of individual cultures were selected using the outcomes of the preliminary identification, which was based on percentage of similarity among isolates. Isolates with similar identification hits in NCBI database were excluded. The samples were sent to Novogen (HK) Company Ltd. (Wan Chai, Hong Kong) on dry ice for whole genome sequencing. After receiving the DNA samples, their quality in term of purity and degradation were checked. Then the quantified DNA samples were used to construct a library. DNA samples were then sheared into smaller fragments with a desired size before adding an ‘A’ base to the 3' end of the blunt phosphorylated DNA fragments. Adapters were ligated to the ends of the DNA fragments, before the quantified DNA libraries were used for sequencing. The original optic data obtained by high-throughput sequencing (Illumina HiSeq platform) were transformed into raw sequenced reads (raw data, or raw reads) by CASAVA base calling (San Diego, California, USA) and stored in FASTQ (fq)

format which contained reads' sequences and corresponding sequencing quality. Quality control was performed and the adapter and low quality sequences were removed. The clean data obtained were used for subsequent analysis.

Sequencing analysis

Sequencing was executed using the Illumina HiSeq 2500 system on rapid run mode. Sequencing reads obtained from the sequencer were assessed and filtered to retain only good quality bases. Good quality reads were then assembled to obtain contiguous sequences (contigs). Genes were predicted from the contig sequences, and the functions of protein-coding genes were predicted by looking for sequence or domain similarity with known proteins.

Sequencing reads quality control

Raw sequences were later subjected to the trimming of adapters and low quality reads based on Q30 using Skewer (Jiang et al., 2014). Reads that is below 25 bp were excluded in the *de novo* assembly. Sequencing quality of the reads was examined using FastQC (Andrews, 2010). During preparation for sequencing, the bacteria genomic DNA fragments were attached to the Illumina sequencing adapters, which contains the anchoring site of the sequencing primers. As the adapters are attached at both ends and if the sequencing length is longer than the bacteria genomic DNA fragment, the sequencing will proceed onto the sequencing adapter, resulting in sequencing reads containing the bacteria genomic DNA and the Illumina sequencing adapter sequences. Therefore, all sequencing reads were scanned for presence of Illumina sequencing adapters, and for the

adapter sequences to be removed while retaining the portion containing the bacteria genomic DNA. In summary, the sequencing reads obtained from the Illumina sequencer were scanned for adapter sequences and low quality sequences (where part of the reads containing these sequences were removed). Short sequencing reads were discarded after adapter and quality trimming.

Genome assembly

The data were then *de novo* assembled using SPAdes version 3.9.1 (Prjibelski et al, 2014*)*. Only contigs greater than 200bp were used for downstream analysis. Due to the different sequence profiles, bioinformatic gene prediction for bacteria genomes are divided according to the types of genes. Gene prediction algorithms for predicting transfer RNA (tRNA) and ribosomal RNA (rRNA) genes can predict the gene sequences and assign the gene function (e.g. a predicted rRNA gene codes for the 16S rRNA or a predicted tRNA gene codes for a tRNA which is aminoacylated by Alanine), but most protein-coding genes prediction algorithms only predict the gene sequences. The gene function (e.g. what protein does a protein-coding gene code for) prediction currently relies heavily on identifying sequence or pattern similarity with proteins of known function. The rRNA genes were predicted using RNAmmer (Lagesen et al. 2007) while the tRNA genes were predicted using ARAGORN (Laslett and Canback, 2004). The protein-coding genes were first predicted using Prodigal (Hyatt et al. 2010), and the predicted sequences were used to predict their function by using BLASTN (Camacho et al. 2009) and HMMER (Eddy, 1998) to search against various sequence or domain databases.

5.2.1.4 *Phylogenetic and phylogenomic analyses*

Data were analyzed by Bioeasy (M) Sdn Bhd Shah Alam, Malaysia. The phylogenetic tree was constructed by using the Maximum Likelihood technique following the Kimura 2-parameter model (Kimura, 1980). The bootstrap consensus tree inferred from 50 replicates (Felsenstein, 1985) was considered to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa bundled together in the bootstrap test (50 replicates) are shown next to the branches.

Table 5.1. Samples of fresh ready-to eat rocket leaves used for identification of bacteria and moulds.

Samples selected for sequencing	Sample description	Justification
Control	The day sample arrived at Food and Nutritional Sciences, University of Reading from Alresford Salad.	Control
Purchased and consumed on display until date (DUD) (NPDUD)	This treatment was to simulate where the samples were kept on supermarket display shelf (13 ^{o}C) until DUD and were purchased and consumed on the day of DUD.	The samples were selected to identify the type of microbes that have been growing in samples kept at display temperature, purchased and consumed by consumers on the same day. This scenario becomes a main concern as foods are always sold at reduced price in supermarket when it almost reach its expiry date to speed up sales.
Heat shock by supplier for 4h on DUD (HSSDUD)	The samples were kept at 25 ^{o}C for four hours and later were transferred to 13 ^{o}C until DUD. This condition is to simulate the condition where the temperature was abused by supplier	The samples were selected to detect the type of microbes that might grow and present if the samples were temperature abused for

	or retailer while delivering and receiving the products. The samples were kept at 13 ^{o}C, mimicking the display temperature at the supermarket and the samples were purchased and consumed on the same day.	a few hours, before being kept at display temperature in market until expiry date. This indicates the worst case scenario that could happen in the food supply chain.
Early purchased, opened and consumed until DUD (EPODUD)	This scenario is to simulate early purchased, consumed partly and kept later at 4 ^{o}C home refrigerator for further consumption until DUD. The rockets were given a contact with hand to simulate the situation of consumer's handling prior to further. Different hands were used to avoid any cross contamination from each bag of rocket.	The samples were selected to know the types of microbes being introduced from hand-held contamination and present during the storage.

Initial trees for the heuristic search were attained automatically by applying the Maximum Parsimony method. A discrete Gamma distribution was employed to model evolutionary rate differences amongst sites (13 categories (+G, parameter = 0.2440)). The rate variation model permitted for some sites to be evolutionarily invariable ([+I], 38.7053% sites). The analysis involved 71 nucleotide sequences. Codon position included were 1st+2nd+3rd+noncoding. There were a total of 1566 codon positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

In order to build the phylogenetic tree, top five hits from the blast results were selected to build the Maximum Likehood (ML) tree. By default, blast will sort result based on maximum score, which is the score of single best aligned sequence. This maximum score is inaccurate if the subject has length shorter than the query, giving the chance of it to get better alignment. Therefore, the results generated in the ML tree showing the relationship between the samples and blast results against the reference 16S rRNA sequence might be

different. To further investigate, tetra-nucleotide usage patterns (TETRA) analysis was performed on the samples that showed different results between the ML tree and blast results, namely HSS1, HSS3, EPO3, NP4, NP8. The analysis uses the whole genome sequence information. Tetra-nucleotide usage patterns were performed using Jspecies1.2.1 (Richter and Rosselló-Móra, 2009). TETRA analyzes tetra-nucleotide usage patterns in genomic fragments (Kim et al., 2004; Teeling et al., 2004). A correlation coefficient value (r) above 0.99 suggests the probability of two strains being from the same species (Takahashi et al., 2009).

5.2.2 Identification of bacteria using 16S metagenomics

5.2.2.1 *Sample preparation*

Culture stock

Rocket culture stocks from samples of winter grown rocket (similar to those used for whole genome analysis, Section 5.2.1.1) were used in this study. The leaves were kept in 30% glycerol stock and stored at -80 ºC for further usage. Only four samples as listed in Table 5.1 were used for identification.

DNA extraction

As described in Section 5.2.1.1.

PCR amplification

The 16s primer pair with primer forward is 5'GTGYCAGCMGCCGCGGTA 3' and reverse 5' CCCGYCAATTCMTTTRAGT 3'. The amplification reaction for the DNA of all bacteria isolates, together with a negative control without template, was performed in a 25 µl PCR reaction using the GeneAmp PCR system 2700. The PCR reaction was 1 cycle at 94 °C for 3 min followed by 30 cycles with a denaturation step at 94 °C for 30 sec, an annealing step at 55 °C for 45 sec and an extension step at 72 °C for 1 min and 1 cycle at 72 °C for 8 min (Ellis, 2013). The 25 µl PCR mix containing 12.5 µl of PCR Bio taq mix red (PCR Biosystems Ltd., London, UK). One µl each of the primers forward and reverse primers DNA of bacteria and made-up with sterile water to the volume. The amount of DNA was adjusted to make final concentration to 150ng.μl^{-1}. The DNA concentration was measured using NanoDrop spectrophotometer (Model ND1000, NanoDrop Products, Wilmington, DE USA).

5.2.2.2 Detection of amplified products and sequencing

Amplified DNA in the PCR products was separated by gel electrophoresis using ethidium bromide stained 1% agarose gel in 20 x TAE buffer. The latter was prepared from 20 ml of 20x TAE buffer in 980 ml of sterile diethyl pyrocarbonate buffer (DEPC water). Five µl aliquots of each amplified PCR product were loaded into separate wells of the agarose gel. Five µl of a 200 – 10,037 base pair molecular weight marker (Hyperladder 1kb, 100 lanes, Bioline, London, UK) was run alongside the PCR products to determine the approximate sizes of the amplified PCR products. The run time was 90 min at 80 volts. DNA detected by electrophoresis was imaged under ultraviolet light using the Syngene

G:Box and its GeneSys automatic control software (Beacon House, Cambridge, UK). Sizes of amplified bands of DNA were determined.

5.2.2.3 Data analysis

454 sequencing of DNA products were carried out by Animal and Plants Health Agency (APHA), Surrey, UK. The sequences then later were analyzed using 8.0 CLC benchwork done by Professor Simon Andrews (School of Biological Sciences, University of Reading, UK), as those described by Zhu et al. (2017). Bioinformatic analyses on raw data were performed using CLC Workbench software (V.8.0.2) with Microbial genomics module plug in (QIAGEN). Quality and chimera crossover filtering were executed using default parameters. Operational taxonomic units (OTUs) clustering and taxonomic assignment were carried out using Greengenes v13_5 database as reference (clustered at 97%) with multiple sequence comparison by log-expectation on fixed length trimmed (240 bp) sequences. New OTUs were indicated when similarity percentage was lower than 80% with minimum occurrence of five reads. Low abundant OTUs were discarded from further analyses (less 10 reads, less 0.01% relative abundance). The relative abundance of taxa at multiple levels of resolution (phylum, order, family, etc.) was determined for each sample. MUSCLE software (Edgar, 2004) was used for sequence alignment in order to create a maximum likelihood phylogeny with neighbour joining as construction method and Jukes Cantor as nucleotide substitution model and 100 bootstrap replicates. Alpha diversity was calculated using number of OTUs, Chao 1 bias-corrected and phylogenetic diversity; Beta diversity was generated using Bray-Curtis matrix and represented as Principal Coordinate Analysis (PCoA). Sequencing results were compared with the DNA greengenes database.

5.2.3 Abundance and identification of moulds

5.2.3.1 Mould abundance

Plant materials and experimental treatments

Samples of wild rocket obtained from Alresford Salads (Alresford, Hampshire, UK) were transported to the Department of Food and Nutritional Sciences, University of Reading using temperature controlled lorry (< 5 ^{o}C). The rocket var. *Bellezia*, second cut came from North Italy. It was harvested on the 28 days after drilling date. The materials were then subjected to different handling and storage as in Table 3.2. Each experimental unit is comprised of one bag (70g). The time lines of sampling are presented in Figure 3.1.

Mould count analysis

Two counting techniques, direct plating and dilution plating techniques were employed to determine the microbial counts. The direct planting was used to determine the percentage of mould occurrence while dilution plating was used to determine the abundance of the moulds.

Preparation of nutrient agar for mould

Universal medium, dichloran rose bengal chloramphenicol agar (DRBC, Oxoid, Basingstoke, United Kingdom) was prepared following protocols given by the manufacturer. The media were autoclaved at 121 ^{o}C for 15 min (Pitt and Hocking, 1997).

Media were then poured on petri dishes, cooled to solidify before being kept at 4 ^{o}C for future use.

Analysis of mould occurrence using direct plating method

The direct plating technique was used in mould enumeration (Tournas, 2005b). 10 cuts of leaves (2-3 mm) from 50 g samples for each treatment were inoculated onto each of five sterile, dried DRBC, DRYS, CZID and AFP agar plates, making a total of 50 pieces of leaves per sample. Inoculated plates were then incubated at 25 ± 1°C for 5 days. At the end of the incubation period, the percentage of mould occurrence was determined.

Analysis of mould abundance using dilution plating method

The mould abundance determined using dilution planting method (International Standards Organization, ISO, 21527-1: 2008). Serial dilution was performed and 100 µl of each dilutions were inoculated onto DRBC, DRYS, AFP and CZID media in petri dishes (90 x 15 mm) and spread evenly. The cultures were incubated under aerobic conditions at 25 ± 1°C for 5 days. Colonies of moulds growing on the agars were counted.

Data analysis

Means and standard error of data obtained were calculated using Excel. Bar graphs were plotted and used to compare differences among means of treatments.

5.2.3.2 Mould identification

Plant materials and experimental treatments

Plant materials and treatments as described in Section 5.3.1.1.

DNA sequencing

The DNA sequencing of non-coding internal transcribed spacer (ITS) region in the ribosomal operon was used to identify the mould present in the rocket leaves (Seifert, 2009).

DNA extraction

140 mg of four day culture of mould isolate grown on Czapek yeast extract agar (CYA) was used for DNA extraction. Genomic DNA was extracted using the plant-fungi DNA isolation kit (Norgen Biotek Corp.) according to protocol outlined by manufacturer. Extracted DNA was quantified using a NanoDrop spectrophotometer (Model ND1000, NanoDrop Products, Wilmington, DE, USA).

PCR amplification and detection

The ITS1 (forward) and ITS4 (reverse) primer pair with primer sequence 5'TCCGTAGGTGAACCTGCGG 3' and 5' TCCGCTTATTGATATGC 3', were used to amplify ITS region of moulds (Op De Beeck et al., 2014). The amplification of DNA

including negative control was performed in a 40 µl PCR reaction volume using the GeneAmp PCR system 2700. The PCR reaction was 1 cycle at 95 °C for 15 min followed by 30 cycles with a denaturation step at 95 °C for 30 sec, an annealing step at 55 °C for 30 sec and an extension step at 72 °C for 30 sec and finally, 1 cycle at 72 °C for 6 min (Ferrer et al., 2001). The 40 µl PCR mix contained 12.5 µl of PCR Bio taq mix red (PCR Biosystems), 1 ng each of the primers ITS1 and ITS4 primers, DNA fungal and DEPC-treated water to make up the volume. Amount of DNA was adjusted to a final concentration of 5ng.μl^{-1}.

Detection of amplified products and sequencing

The detection of products and sequencing of DNA employed here was similar as described earlier in Section 5.2.2.2.

Analysis of sequencing results

Sequencing of PCR products were outsourced to Eurofins Genomics, Germany. The results were compared with the DNA database of the NCBI using online BLASTN programme. The sequence obtained using the ITS4 primer was converted to its forward strand equivalent using the online reverse compliment software. The Clustal Omega software (Hinxton, Cambridge, UK) was used to align the sequences obtained using ITS1 and ITS4 primers with each other. Regions of complete complementary were compared with the NCBI database using BLASTN. A phylogenic tree was generated using Clustal Omega software to establish taxonomic relationships amongst the isolated fungi.

5.3 Results

5.3.1 Bacteria identified using whole genome sequencing analysis

Based on a 97% sequencing similarity (Stackebrandt and Goebel, 1994 ; Koike at al., 2003), 18 colonies from 4 samples with three replicates, all of the bacteria colonies were considered to represent known bacterial species. 16 colonies of the bacteria had more than 97% similarity with known bacterial species. They were *Enterobacter aerogenes* strain KCTC 2190, *Pseudomonas fluorescens* strain A506, *Citrobacter gillenii* strain CDC 4693-86 (2 isolates), *Enterobacter aerogenes* strain KCTC 2190, *Obesumbacterium prote*us strain 42, *Pseudomonas putida* F1 strain F1, *Aeromonas hydrophila* strain ATCC 7966, *Enterobacter xiangfangensis* strain 10-17, *Pseudomonas graminis* strain DSM 11363, *Salmonella enterica* subsp. enterica serovar Typhimurium strain LT2 (2 isolates), *Citrobacter rodentium* ICC168 strain ICC168, *Lactococcus lactis* subsp. lactis IO-1, *Morganella morganii* subsp. morganii KT (3 isolates), and *Providencia vermicola* strain OP1. Another two isolates, *Pseudomonas fluorescens* strain A506 and *Lactococcus lactis* subsp. lactis IO-1 have similarity below 97%. The bacteria identified and their match percentage are listed in Table 5.2. The phylogenetic representation of a microbial community present in RTE rocket leaves are shown in Figure 5.1.

However, there was a contradictory identification from the sequencing results generated in the ML tree that shows the relationship among the samples and blast results against the reference 16S rRNA sequence. Therefore, tetranucleotide usage patterns (TETRA) analysis was performed on the samples that showed different results between the ML tree and blast results, namely isolate HSS1, HSS3, EPO3, NP4, NP8. The analysis uses

the whole genome sequence information. Only five isolates were analyzed as isolate EPO1 constantly gave the same bacteria species, *Enterobacter aerogenes.* The correlation coefficient obtained are presented in Table 5.3. A correlation coefficient (r) value of above 0.99 suggests the probability of two strains being from the same species. Unfortunately, TETRA is only able to identify up to species level as there is no software that is available to determine with more detailed identification. Isolate HSS1 was identified as *Enterobacter xianfangensis* with r=0.998. HSS1 was closely related with HSS3 with r=0.999 and NP4 (r=0.999). EPO3 was closely matched with *Obesumbacterium proteus* (r=0.996) whereby NP8 was detected as *Providencia rettgeri* (r =0.999).

Table 5.2. Known bacteria sharing more than 97% similarity in 16s rDNA sequence present in rocket culture with different treatments. (* indicates similarity below than 97%).

Isolate	Subject Description	% identity
DO2	*Enterobacter aerogenes* strain KCTC 2190	100
DO8	*Pseudomonas fluorescens* strain A506	91.85*
DO9	*Citrobacter gillenii* strain CDC 4693-86	99.80
EPO1	*Enterobacter aerogenes* strain KCTC 2190	99.80
EPO3	*Obesumbacterium proteus* strain 42	99.73
EPO7	*Pseudomonas putida* F1 strain F1	99.80
EPO8	*Aeromonas hydrophila* strain ATCC 7966	99.54
HSS1	*Enterobacter xiangfangensis* strain 10-17	99.89
HSS2	*Pseudomonas graminis* strain DSM 11363	98.75
HSS3	*Salmonella enterica* subsp. enterica serovar Typhimurium strain LT2	98.43
HSS4	*Citrobacter rodentium* ICC168 strain ICC168	98.56
HSS6	*Citrobacter gillenii* strain CDC 4693-86	99.41
HSS9	*Lactococcus lactis* subsp. lactis IO-1	91.65*
NP2	*Morganella morganii* subsp. morganii KT	99.80
NP3	*Morganella morganii* subsp. morganii KT	99.80
NP5	*Morganella morganii* subsp. morganii KT	99.80
NP8	*Providencia vermicola* strain OP1	99.54
NP4	*Salmonella enterica* subsp. enterica serovar Typhimurium strain LT2	98.43

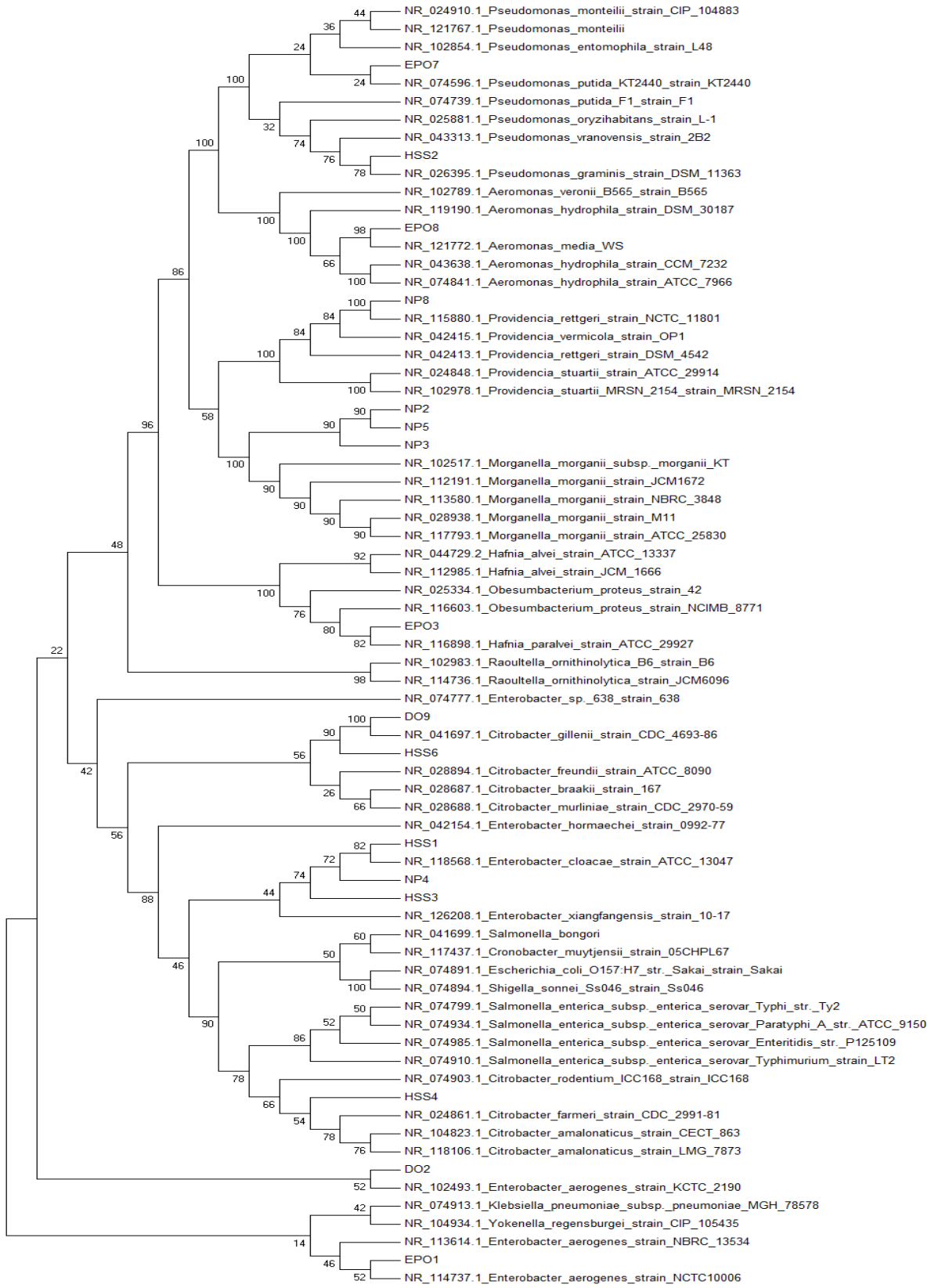


Figure 5.1. Molecular phylogenetic analysis by Maximum Likelihood method.

Table 5.3.Tetranucleotide usage patterns (TETRA) analysis in genomic fragments of five bacteria species. Correlation coefficients (r) above 0.99 suggest the probability of two strains being from the same species.

Bacteria	*E. xiangfangensis* strain NS19	*H. paralvei* ATCC 29927	HSS1	*O. proteus* ATCC 12841	*S. enterica Typhi* strain LT2	*P. rettgeri* strain RB15	HSS3	*O. proteus strain* DSM 2777	*E. cloacae* ATCC 13047	*S. enterica* Typhi Ty2	EPO3	*E. xiangfangensis* strain LMG27195	NP4	NP8	*P. rettgeri* Dmel1
E. xiangfangensis strain NS19	---	0.681	0.998	0.732	0.917	0.505	0.999	0.721	0.987	0.916	0.708	0.999	0.999	0.502	0.484
H. paralvei ATCC 29927		---	0.684	0.979	0.769	0.848	0.681	0.978	0.724	0.772	0.976	0.690	0.682	0.846	0.843
HSS1			---	0.736	0.912	0.511	0.999	0.725	0.985	0.910	0.712	0.998	0.999	0.507	0.489
O. proteus ATCC 12841				---	0.777	0.810	0.733	0.999	0.760	0.778	0.996	0.739	0.733	0.810	0.805
S. enterica Typhi strain LT2					---	0.605	0.913	0.766	0.933	0.999	0.751	0.921	0.913	0.598	0.587
P. rettgeri strain RB151						---	0.506	0.807	0.551	0.608	0.800	0.513	0.508	0.990	0.988
HSS3							---	0.722	0.985	0.911	0.709	0.999	0.999	0.503	0.484
O. proteus strain DSM 2777								---	0.748	0.767	0.997	0.728	0.722	0.807	0.803
E. cloacae ATCC 13047									---	0.932	0.733	0.988	0.985	0.554	0.539
S. *enterica* Typhi Ty2										---	0.753	0.920	0.912	0.601	0.589
EPO3											---	0.714	0.710	0.799	0.795
E. xiangfangensis strain LMG27195												---	0.999	0.51046	0.492
NP4													---	0.505	0.486
NP8														---	0.998
P. rettgeri Dmel1															---

5.3.2 Bacteria distribution and abundance using 16S metagenomics

Following all the denosing and filtering steps in CLC microbial metagenomics (V.8.0.2) (Redwood City, CA, US), a total of 72,790 sequences from bacteria isolated from four rocket samples with three replicates each were gained in final analysis. Analysis of beta diversity analysis of bacterial culture from the four treatments based on principal coordinate analysis (PCoA) based on Jaccard's similarities shown in Figure 5.2 suggesting high uniformity among samples within the treatments. Moreover, all rarefaction curves generated from analysis reached near plateau indicated that the sequencing depth was sufficient to cover the microbial species richness (Figure 5.3).

249 types of bacteria were identified, from which the combined abundance varied from 16,854 for Enterobacteriacae to two. The latter consisted of 34 types of bacteria which were comprised of different genus background. Bacteria identified using 16S metagenomics could only be identified at different taxonomic levels since there is a limitation of the database. Most of the bacteria could be identified only down to family level whereby only 33 bacteria were identified down to species level (Table 5.4).

Overall, the bacteria identified were from Bacteroides, Myroides, Exiguobacterium, Carnobacterium, Enterococcus, Vagococcus, Lactococcus, Streptococcus, Clostridium, Ruminococcus, Microvirgula, Shewanella, Citrobacter, Enterobacter, Erwinia, Klebsiella, Morganella, Providencia, Serratia, Trabulsiella, Acinetobacter, Pseudomonas and Stenotrophomonas genus. However, 124 bacteria were identified to family level and two were identified to order level only. More detailed information on relative abundance of dominant bacterial families are available in Appendix 5.1.

Table 5.4. Bacterial species identified and their relative abundance in rocket with different treatments.

Name in Greengenes database	Bacteria species	Rocket samples and abundance of bacteria identified	Combined abundance
510899	*Enterococcus casseliflavus*	Day 0 (23) EPODUD (1) NPDUD (43) HSSDUD (9)	76
360402	*Lactococcus garvieae*	Day 0 (148) NPDUD (85)	233
JJLHQ1M03DA0DZ	*Lactococcus garvieae*	Day 0 (1) NPDUD (1)	2
JJLHQ1M03DCKIJ	*Lactococcus garvieae*	Day 0 (2)	2
583562	*Ruminococcus bromii*	Day 0 (4)	4
JJLHQ1M03DH49P	*Microvirgula aerodenitrificans*	NPDUD (2)	2
859279	*Erwinia soli*	Day 0 (3)	3
233981	*Morganella morganii*	NPDUD (2722) HSSDUD (385)	3107
1890229	*Morganella morganii*	NPDUD (5)	5
JJLHQ1M03C7RIY	*Morganella morganii*	NPDUD (5)	5
JJLHQ1M03DH7YD	*Morganella morganii*	NPDUD (4)	4
JJLHQ1M03C0Z13	*Morganella morganii*	NPDUD (2)	2
JJLHQ1M03C5DUR	*Morganella morganii*	NPDUD (2)	2
JJLHQ1M03CXN01	*Morganella morganii*	NPDUD (2)	2
JJLHQ1M03C28OE	*Providencia stuartii*	HSSDUD (6)	6
810238	*Serratia marcescens*	EPODUD (89) NPDUD (35) HSSDUD (74)	198
818462	*Trabulsiella farmeri*	Day 0 (49)	77

		EPODUD (12) NPDUD (10) HSSDUD (6)	
JJLHQ1M03C4PO6	*Trabulsiella farmeri*	EPODUD (2) NPDUD (2)	4
839479	*Acinetobacter rhizosphaerae*	Day 0 (70) NPDUD (6)	76
670210	*Pseudomonas fragi*	Day 0 (16)	16
538344	*Pseudomonas veronii*	Day 0 (5)	5
1109916	*Pseudomonas veronii*	Day 0 (2)	2
553180	*Pseudomonas viridiflava*	HSSDUD (4658)	4658
734337	*Pseudomonas viridiflava*	HSSDUD (38)	38
JJLHQ1M03DNN19	*Pseudomonas viridiflava*	HSSDUD (17)	17
569729	*Pseudomonas viridiflava*	HSSDUD (10)	10
JJLHQ1M03DKM2S	*Pseudomonas viridiflava*	HSSDUD (15)	5
JJLHQ1M03C5C1T	*Pseudomonas viridiflava*	HSSDUD (4)	4
JJLHQ1M03C4Q5A	*Pseudomonas viridiflava*	HSSDUD (2)	2
JJLHQ1M03C7TN9	*Pseudomonas viridiflava*	HSSDUD (2)	2
JJLHQ1M03DEG7A	*Pseudomonas viridiflava*	HSSDUD (2)	2
JJLHQ1M03DO68T	*Pseudomonas viridiflava*	HSSDUD (2)	2
967275	*Stenotrophomonas geniculate*	Day 0 (3) NPDUD (1) HSSDUD (7)	11

Based on the findings, the bacteria identified up to species level were distributed differently based on treatments applied. However, two bacteria, which were *Enterococcus casseliflavus* (510899) and *Trabulsiella farmeri* (818462), were present in all four samples. Both have combined abundance of 76 and 77 reads, respectively. *Enterococcus casseliflavus* (510899) was the most abundance in NPDUD, meanwhile *Trabulsiella farmeri* (818462) was the most abundance in Day 0 with 49 reads, followed by EPODUD (12), NPDUD (10) and HSSDUD (6). This shows *Trabulsiella farmeri* (818462) was present at earlier point of shelf life and did not survive with storage. Another species of *Trabulsiella farmeri* (JJLHQ1M03C4PO6) was detected in EPODUD and NPDUD but in a low abundance (4). Three types of *Lactococcus garvieae* were detected which two of them (*Lactococcus garvieae* 360402 and JJLHQ1M03DA0DZ) were present in Day 0 and NPDUD and the other one was found only in Day 0. Only one type of *Ruminococcus bromii* was identified in this study, which was found in NPDUD and in a very low abundance (4 reads).

As expected, plant pathogens or food spoilage bacteria were also detected in the samples of rocket leaves. This includes *Erwinia soli* (859279) and 13 species of Pseudomonas. *Erwinia soli* was detected in Day 0 sample and among the 13 species of Pseudomonas, *Pseudomonas fragi* and *Pseudomonas veronii* were found also on Day 0 samples. All types of *Pseudomonas viridiflava* were detected in sample HSSDUD indicating that *Pseudomonas viridiflava* prefer to proliferate at relatively higher temperature (13 ^{o}C) compared to the other species of *Pseudomonas*.

Seven types of *Morganella morganii* were identified within the same sample which was NPDUD except for one *Morganella morganii* (233981) which was detected in NPDUD

and HSSDUD samples. It shows that long storage at 13 ºC provides a suitable growing environment for *Morganella morganii* (233981). *Providencia stuartii* were detected in sample that experienced heat shock conditions (HSSDUD) only. Keeping RTE rocket leaves at 25 ºC was found to promote the growth of this bacterial species.

Serratia marcescens was found present at later time of the storage regardless of temperature and was detected on all samples on their display until date (DUD). However, their abundance were highest in EPODUD followed by HSSDUD and NPDUD. No *Serratia marcescens* was detected on Day 0. *Stenotrophomonas geniculate* was detected in Day 0, NPDUD and HSSDUD samples.

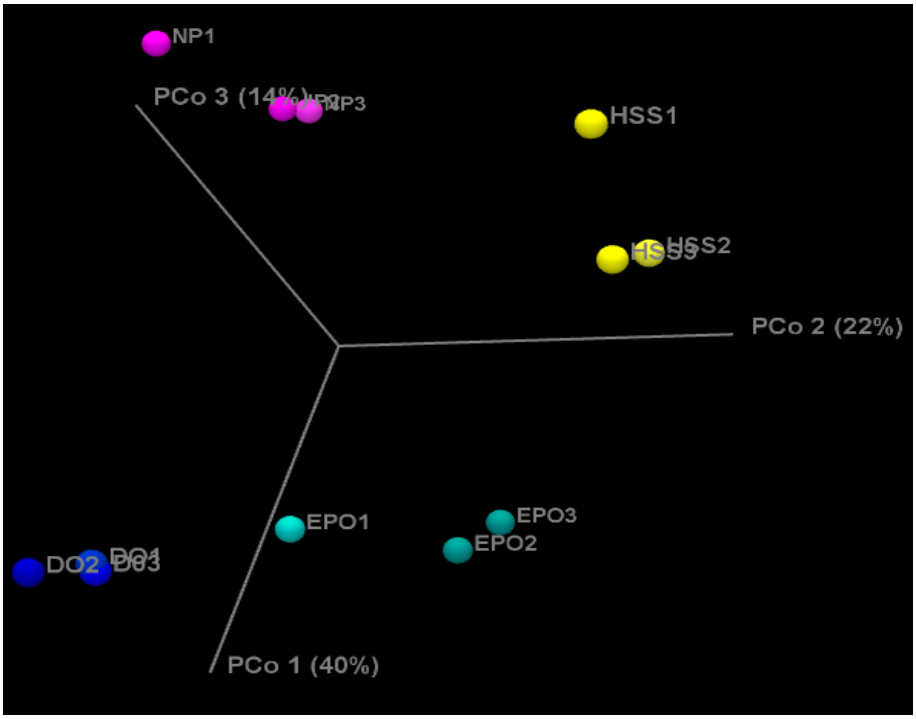


Figure 5.2. Results of the beta diversity analysis. Grouping of samples of bacterial culture from four treatments using Principal coordinate analysis (PCoA) based on Jaccard's similarities shows high uniformity among samples within treatments. Bullets with similar colour are replicates of the same treatments. Legends: NP1-NP3=NPDUD; HSS1-HSS3=HSSDUD; EPO1-EPO3=EPODUD and DO1-DO3 (Control) (Table 5.1).

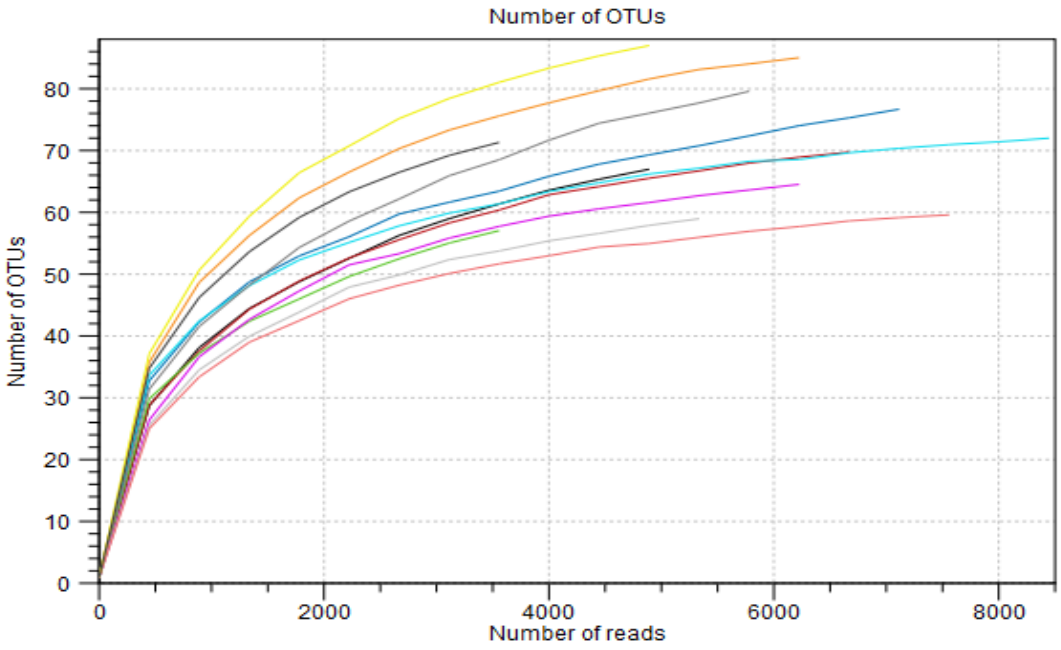


Figure 5.3. Alpha diversity of samples based on number of Operational Taxonomic Unit (OTUs).

5.3.3 Mould abundance and identification

The abundance of moulds present in rocket leaves at various phases of the simulated supply chain of RTE rocket is presented in Figure 5.4. Images of the mould isolated and identified are presented in Appendix 5.4. Results obtained clearly show that heat shock at 25 ^{o}C markedly increased the abundance of mould as seen the counts of treatments HSC24, HSS24, HSCDUD and HSSDUD, regardless of their storage temperature (either at 4 ^{o}C for HSC24 and HSCDUD, or at 13 ^{o}C for HSS24 and HSSDUD) after the heat shock treatments. Keeping the leaves at low temperature at 4 ^{o}C, whether the leaves have been disturbed or not, right from the beginning was found effective in maintaining low counts of mould (EPODUD and EPCDUD). Leaves samples that constantly kept at 13 ^{o}C also had low count of moulds (NPDUD). Apparently, the growth of moulds was very dramatic after expiry date where their counts were considered as TNTC (*too numerous to*

count) as shown by counts in EPODUD+2, EPODUD+2, NPDDUD+2, HSCDUD+2 and HSSDUD+2.

Moulds were found to exist in all samples with the occurrence rate of 92% for the control sample and 100% for samples of other treatments as shown by results from the direct plating technique (Figure 5.5).

A total of 26 species of moulds were isolated and identified present in rocket leaves used in the study. The identities of the isolates as shown in Table 5.5. The phylogenic tree showing relationship between the fungal isolates are shown in Figure 5.6.

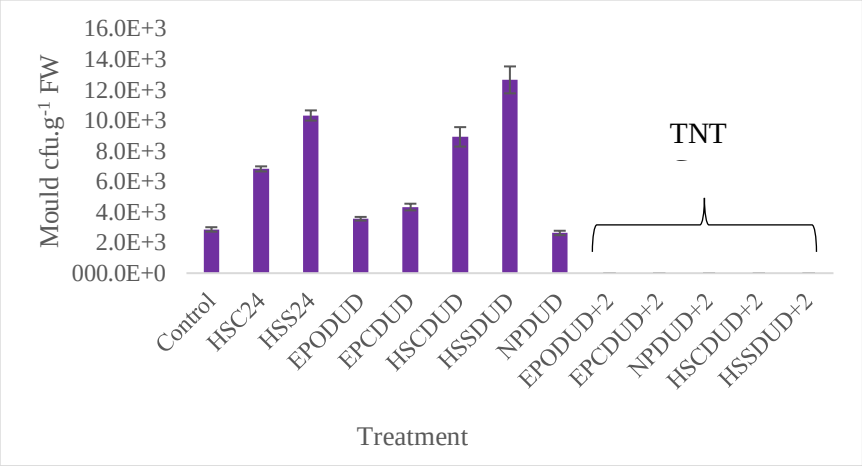


Figure 5.4. Mould abundance in ready-to-eat rocket leaves subjected to different handling and storage conditions. TNTC – too numerous to count.

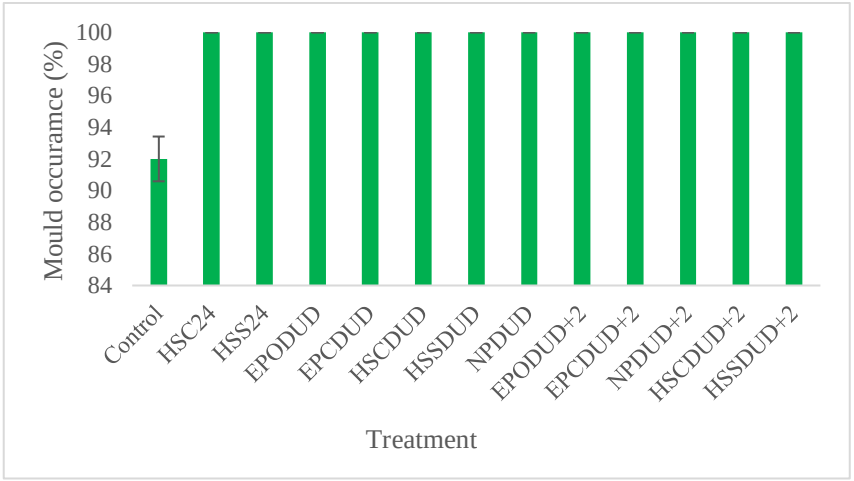


Figure 5.5. Mould occurrence in ready-to-eat rocket leaves subjected to different handling and storage conditions.

Table 5.5. Species of moulds found in ready-to-eat rocket leaves based on closest match from NCBI database intergenic spacer sequences.

Isolate number	**Sample code**	**Identity of closest match**	**Gene bank accession no.**
1	AHY38	*Fusarium equiseti*	KU361582
2	AHY02	*Fusarium equiseti*	KT362203
3	AHY03	*Alternaria infectoria*	KX928829
4	AHY04	*Fusarium equiseti*	KU361582
5	AHY39	*Alternaria cesenica*	KP711383
6	AHY06	Alternaria gasein	JX391937
7	AHY07	*Fusarium equiseti*	KU361582
8	AHY08	*Alternaria infectoria*	KX928829
9	AHY10	**Rhizopus*	-
10	AHY12	*Alternaria tenuissima*	KX196414
11	AHY13	*Alternaria tenuissima*	KX196414
12	AHY41	*Epicoccum nigrum*	KR909153
13	AHY15	*Alternaria tenuissima*	KX196414
14	AHY16	*Penicillium oxalicum*	LT558936
15	AHY17	*Cladosporium* sp.	KY369146
16	AHY18	*Alternaria tenuissima*	KX196414
17	AHY19	*Stemphylium vesicarium*	KX8322961
18	AHY21	*Alternaria tenuissima*	KX065045
19	AHY22	*Fusarium equisetti*	KR709055
20	AHY23	*Alternaria tenuissima*	KX196414
21	AHY24	*Fusarium equiseti*	KU361582
22	AHY26	*Alternaria tenuissima*	KX196414
23	AHY27	*Fusarium chlamydosporum*	KF998978
24	AHY28	*Alternaria tenuissima*	KC568287
25	AHY29	*Fusarium chlamydosporum*	KU878096
26	AHY30	*Alternaria alternate*	KF876820

NCBI – National Centre for Biotechnology Information.

*Low signal (Not sequenced). Identification based on physical appearance.

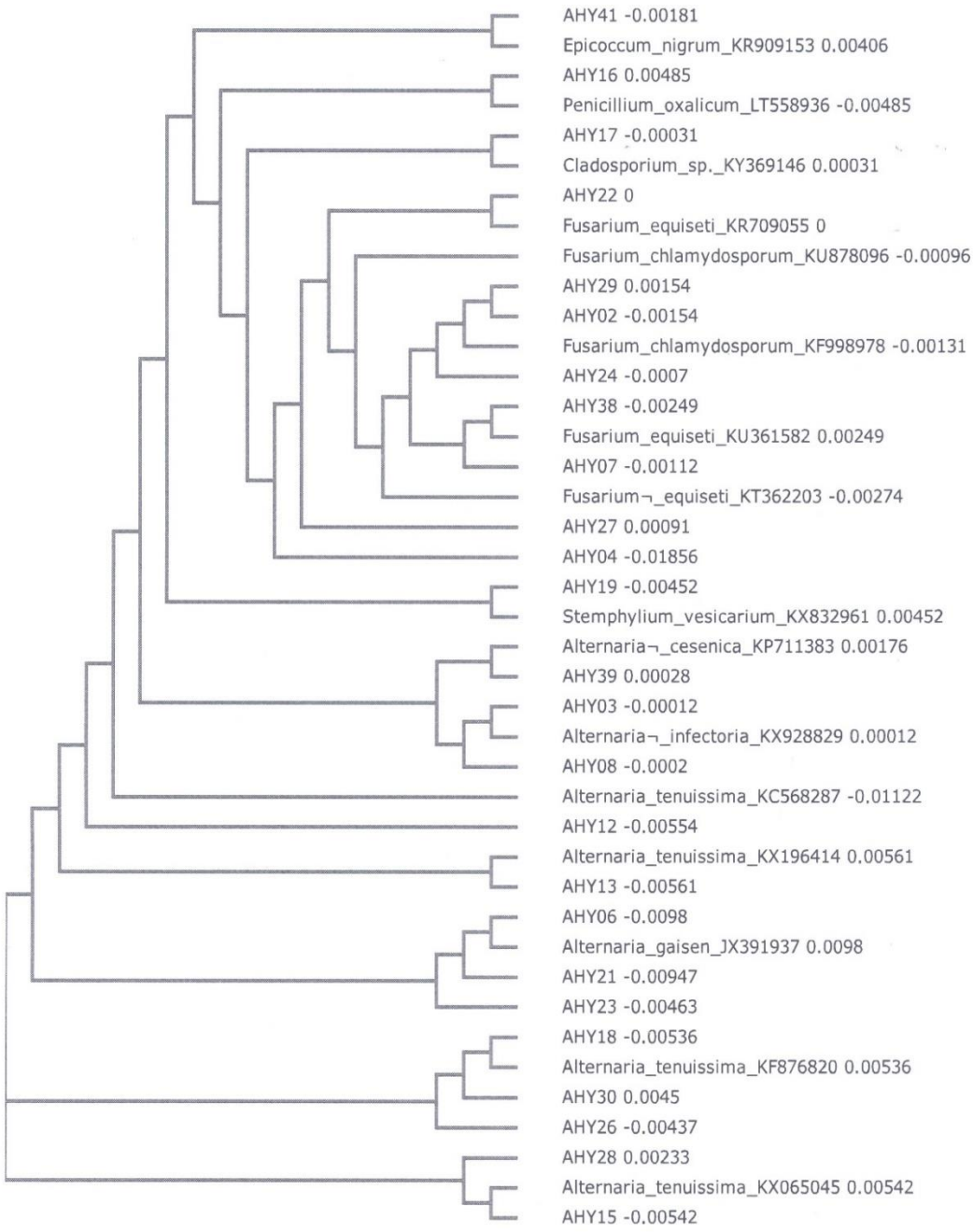


Figure 5.6. Phylogenetic tree of moulds present in ready-to-eat rocket leaves identified using DNA sequencing in the internal non-coding internal transcribed spacer (ITS) region.

5.4 Discussion

5.4.1 Diversity of bacteria found in ready-to-eat rocket leaves

In this study, a very diverse background of bacteria had been identified. An opportunist human pathogenic bacterium was detected along with along with other bacteria that are more likely to be associated with food spoilage. Presence of the pathogenic bacteria in ready-to-eat salad could be an alarming sign where their outbreak could occur and this will give a huge impact especially in large scale production facilities would affect a wide customer population.

During June 2011, there was an *E. coli* outbreak in Germany causing several deaths. This led to the destruction of all cucumbers produced surrounds Europe. (Retrieved from http://www.boston.com/bigpicture/2011/06/e_coli_outbreak_in_europe.html, 2017). Other examples were Salmonella outbreak linked to alfalfa sprouts and Listeria associated with packed salad produced at Springfield, Ohio, which both occur in year 2016 in United State (Centers for Disease Control and Prevention, 2016). This kind of incident would need a product recall damaging consumer confidence and the industry (Heaton and Jones, 2008).

In this study, whole genome and 16S metagenomics analyses were employed to identify bacteria in RTE rocket leaves. Initially, whole genome bacterial sequencing was employed. However, this method was only able to identify culturable bacteria, but with a detailed strain. In order to determine both culture and unculturable bacteria, 16S metagenomics analysis was used. Apart from its ability to identify both culture and

unculturable bacteria, results generated from 16S metagenomic analysis could also be used to determine the relative abundance of bacteria present in each sample.

A human pathogenic bacteria species, *Salmonella enterica* subsp. enterica serovar Typhimurium strain LT2 was detected in RTE rocket leaves. Some serovars of *Salmonella enterica* can cause systemic infections and typhoid fever whereas *Salmonella typhimurium* can cause gastroenteritis (McClelland et al., 2001). It is unacceptable or potentially hazardous if Salmonella serovars is present in 25 g of RTE food (Forsythe, 2010). The existence of the bacteria most likely caused by irrigation of the crop using contaminated water (Nygard et al., 2008). This finding was not suprising as other research shows Salmonella poses as number one rank foodborne illness. Based on a model which developed using seven criteria (strength of associations between food and pathogen based on the foodborne outbreak data from EU Zoonoses Monitoring (2007-11), incidence of illness, burden of disease, dose-response relationship, consumption, prevalence of contamination and pathogen growth potential during shelf life) by the European Food Safety Authority to identify and rank specific food/pathogen combination in non-animal origin in the EU (FoNAO) which linked most to human cases, the top ranking food/pathogen combination was Salmonella spp. and leafy greens eaten raw followed by (in equal rank) Salmonella spp. and bulb and stem vegetables, Salmonella spp. and tomatoes, Salmonella spp. and melons, and pathogenic Escherichia coli and fresh pods, legumes or grain (Anonymous, 2013)

There were also a lot of opportunistic pathogenic bacteria detected in the sample (Table 5.2 and Table 5.4). They were *Enterobacter aerogenes* strain KCTC 2190, *Citrobacter gillenii* strain CDC 4693-86, *Morganella morganii* subsp. morganii KT, *Enterococcus*

casseliflavus, Lactococcus garvieae, Microvirgula aerodenitrificans, and *Serratia marcescens*.

Enterobacter aerogenes and Morganella morganii are capable of causing a wide variety of nosocomial infections (Miller 2015; Sanders and Sanders, 1997). *Citrobacter gillenii spp.* could cause infections of the urinary tract and infant meningitis and sepsis (Badger et al., 1999). *Enterococcus casseliflavus* is a rare pathogen, usually associated with urinary and intra-abdominal infections. However, this pathogen is easily treated since it is sensitive to ampicillin or sulbactam, penicillin, tetracycline, quinolones and teicoplanin (Pappas et al., 2004). *Lactococcus garvieae* is the etiological agent of Lactococcosis, an emergent disease which affects many fish species and causes important economic losses both in marine and freshwater aquaculture when water temperature increases over 16 °C in summer months (Vendrell et al., 2006). Normally, it causes a hyperacute and haemorrhagic septicemia. *Lactococcus garvieae* is also known human pathogen, which causes lumbar osteomyelitis, hepatic abscess and infective endocarditis (Vendrell et al., 2006). *Microvirgula aerodenitrificans* has been isolated globally but has never been described as causative of clinical infection until 2012 due to the infection of a vascular access device in an infant with Pompe's disease (Murphy et al., 2012). S*erratia marcescens* is a widely distributed saprophytic bacterium, and has been found in food, particularly in starchy variants which provide an excellent growth environment (Yang et al., 2012). S*erratia marcescens* is implicated in a wide range of serious infections including pneumonia, lower respiratory tract infection, urinary tract infection, bloodstream infection, wound infection and meningitis (Hejazi and Falkiner, 1997). The organism has also been described as an important cause of ocular infection with high incidence in contact lens-related keratitis (Atlee et al., 1970). Infections caused by

S*erratia marcescens* may be difficult to treat because of resistance to a variety of antibiotics, including ampicillin and first and second generation cephalosporins (Carlesimo et al., 2014). More information on the pathogenicity of bacteria found in fresh RTE rockets are presented in Appendix 5.2. Among of the human pathogenic bacteria found, there was one bacteria identified which was beneficial for human gut, *Ruminococcus bromii* which was reportedly involved in the degradation of resistant starch in humans (Ze et al., 2012).

Apart from human pathogens, plant pathogens are also important as their presence affects quality and shelf life of the fresh produce. Few types of *Pseudomonas viridflava* and *Erwinia* sp. were identified. *Pseudomonas viridflava* have the ability to cause foliar and stem necrotic lesions, and basal stem rots (Tournas, 2005b; Gonzalez et al., 2012).

On the other hand, there were also bacteria present that are believed to be beneficial to plant growth. *Pseudomonas putida* is a plant growth promoting bacteria and has the capacity to protect the host plant from plant pathogenic bacteria (Espinosa-Urgel et al., 2000). Due to these properties, *Pseudomonas putida* could become a research focus in bioengineering research to develop environmental friendly biopesticides in promoting healthy plant growth. *Pseudomonas fluorescens strain A506* was also reported could be used as a biological control agent for *Erwinia amylovora* in pear's blossom (Wilson and Lindow, 1992). In addition, certain *Acinetobacter rhizosphaerae* strain was found to exhibit the plant growth-promoting attributes of inorganic and organic phosphate solubilization, auxin production, 1-aminocyclopropane-1-carboxylate deaminase activity, ammonia generation, and siderophore production in Himalaya's areas (Gulati et al., 2009). Unfortunately, the strain of *Acinetobacter rhizosphaerae* in this study cannot

be identified due to limitation of the database. Other than functioning as a plant growth promoting bacteria, *Pseudomonas graminis* has the ability to protect the host plant by naturally infect and kill insects upon ingestion of bacteria (Ruffner et al., 2013). Similar with *Pseudomonas fluorescens, Pseudomonas graminis* also could protect plants against *Erwinia amylovora* which could cause fire blight (Mikiciński et al., 2016).

Being psychrotrophs, *Pseudomonas spp*. are motile when live at a medium temperature range (15 and 25 ^{o}C) but not at 30 ^{o}C. At low refrigeration, the bacteria still grow but at a slower rate (Behrendt et al., 1999; Willocx et al., 1993). This phenomenon was also observed in this study whereby *Pseudomonas graminis* strain DSM 11363 was found in sample HSSDUD and *Pseudomonas fragii* was found in control sample where the leaves were kept at 4 ^{o}C. For similar reason, apart from *Pseudomonas*, other types of psychrotrophs bacteria, such as Erwinia and lactic acid bacteria predominantly colonised the RTE salads (Willocx et al., 1993). In the present study, *Pseudomona*s spp. were detected in Day 0, EPODUD and HSSDUD samples and *Erwinia* spp. were detected present in sample Day 0.

RTE salads including rocket are a good source of carbon for the bacteria. Keeping the produce at relatively high temperature at display shelves in the supermarket at 10-13 ^{o}C, or exposing the produce at higher temperature as experience in the heat shock treatment in this study, will accelerate the degradation and mobilization of carbon and other sources of nutrients that can be utilised by the microbes. Availability of sugar would speed up the fermentation process by lactic acid bacteria. This hypothesis was proven in our findings where lactic acid bacteria were found in HSSDUD sample. Few strains of lactic acid bacteria (LAB) also were found by Kruger and her colleagues (2013) in rocket leaves.

Using the PCR-ARDRA method, *Lactococcus lactis* subsp. lactis MK02R was identified and characterized as the only bacteriocinogenic among 12 strains isolated. The bacteriocin MK02R produced by the bacteria is heat stable for 1 h at 60 ^{o}C and 100 ^{o}C and it was inactivated by treatment with proteolytic enzymes. Bacteriocin MK02R inhibited the growth of other bacteria such as *Enterococcus faecium, Lactobacillus sakei, Lactobacillus delbrueckii, Lactobacillus sakei subsp. sakei, Listeria innocua* and *Listeria monocytogenes* from different serological groups (Kruge et al., 2013).

In this study, the matching percentage of the LAB found is less than 97% similarity and the contig is very high (4019). A very diverse of genome sequence could have led to high contig value. This could be due to the diversity of the strain and genome of this LAB bacteria. In a study by Passerini et al. (2010), based on 16S rDNA sequencing, the data revealed that the strains of *Lactoccus lactis* subsp. lactis in their study could belong to various subspecies or to other genera other than *Lactococcus* which could be *Enterococcus faecalis, Enterococcus pseudoavium, Lactobacillus casei,* and *Leuconostoc citreum.*

The matching rate of *Pseudomonas florescence* identified is 91.85%. Similarly to LAB, low rate of matching could be due genomic and genetic diversity within *Pseudomonas fluorescens* genera. Pseudomonas was reported as one of the most diverse genera, which is depicted in more than 100 species (Parte, 2004). The taxonomic classification of *Pseudomonas* has undergone many changes. Several studies have evaluated the presence of groups and subgroups within the genera (Gomila et al., 2015; Mulet et al., 2010) and one of which is the *Pseudomonas fluorescens* group, which comprises of more than 50 strains. The species then has been divided into subgroups that differ in multilocus

sequence analysis (MLSA) and phylogenomic analysis (Gomila et al., 2015; Mulet et al., 2012; Rodendo-Nieto et al., 2013).

Morganella spp. were identified in samples that were kept at display temperatures (NPDUD and HSSDUD). The results suggest that display shelf temperature (13 ^{o}C in our case) and heat shock (20 ^{o}C) for several hours provide suitable environment for *Morganella morganii* to proliferate. According to previous finding (Miller, 2015) this species of bacteria were found in environment and in the intestinal tracts of humans, mammals and reptiles as normal flora.

Aeromonas spp. have been isolated from a wide range of fresh produce including sprouted seeds, broccoli, cauliflower, celery, lettuce, mushroom and watercress (Merino et al., 1995). Watercress was found to contain higher counts of *Aeromonas* than lettuce or escarole (chicory) samples (Saad et al., 1995). Callister and Aggar (1987) found that 48% *Aeromonas* isolates from fresh vegetables were *A. hydrophila,* the species most often linked to disease in humans. Food isolates of *Aeromonas* have been shown to tolerate low pH and to grow at the refrigeration temperatures due to its ability to adapt to low temperature throughout the supply chain (Merino et al., 1995). It was reported that *Aeromonas* may be responsible for the many gastrointestinal infections (McMahon and Wilson, 2001). However, results of a study reported by Isonhood and Drake (2002) failed to establish conclusive relationship between a foodborne outbreaks with *Aeromonas* as the causative agent. An association between the increased number of *Aeromonas* in human stools and on fresh vegetable samples during the summer months was established by Saad et al. (1995) which aligned with our data in Chapter 3 where we found higher count of total bacteria in rocket leaves produced in summer months. Neyts

et al. (2001) suggested that *Aeromonas* could have posed a risk to immune compromised groups. They also discovered among the 12 species of Aeromonas isolated, *Aeromonas jandaei* ATCC 49568 was the most pathogenic, and this was followed by *Aeromonas hydrophila* ATCC 7966. Symptoms of *Aeromonas sp.* infections vary from gastroenteritis to wound infections (cellulitis, ecthyma gangrenosum, and myonecrosis) and septicaemia (Seshadri et al., 2006).

There are few other bacteria that were detected in this study where their pathogenicity is not known or not much information was found. *Enterobacter xiangfangensis* strain 10-17 is a *g*ram-stain-negative which phylogenetically related to *Enterobacter hormaechei* CIP 103441T, *Enterobacter cancerogenus* LMG 2693T, *Enterobacter asburiae* JCM 6051T, *Enterobacter mori* LMG 25706T, *Enterobacter ludwigii* EN-119T and *Leclercia adecarboxylata LMG 2803T. Enterobacter xiangfangensis* strain 10-1 was isolated in Chinese traditional sourdough (Gu et al., 2014). In our study, based on the molecular phylogenetic analysis by maximum likelihood method, *Enterobacter xiangfangensis* strain 10-1 detected was closely related to *Enterobacter hormaechei* strain 099277 *and Enterobacter cloacae* strain 13047. Another bacteria, *Stenotrophomonas geniculate* which was reported to be closely related to *Stenotrophomonas maltophila* (Svensson et al., 2012). *Providencia rettgeri* was also identified in this study which is a ubiquitous organism that is infrequently associated with human disease. Naturally, it can be found in diverse locations as fresh water sources, run-off wastewater, and explosive-contaminated soil (Washington et al., 2015).

5.4.2 Diversity of moulds found in ready-to-eat rocket leaves

Previous studies have shown that temperature and other environmental factors have a strong effect on growth of various fungi (Marin et al., 1998; Schubert et al., 2010). At temperatures ranging from 20 – 27.5°C, spores of mesophiles such as *Penicillium expansum* germinated at a faster rate compared to those subjected to lower temperature (Gougouli and Koutsoumanis, 2012).

This factor may have contributed to the higher mould counts observed with heat shocked samples after the temperature shift from low temperature during storage and transportation by supplier to the heat shock treatment at 25 °C for 4 h.

This shift may have stimulated spore germination resulting in increased in microbial counts observed in HSC24, HSS24, HSCDUD and HSSDUD samples compared to samples that did not experience heat shocked treatment (EPODUD, EPCDUD and NPDUD).

After processing, usually the RTE vegetables are stored at low temperature (4-5 °C) before being displayed in open refrigerated cabinets/shelves at retailers' premises with 10-13 °C. Under low storage temperature, psychrophiles are expected to dominate the produce. After transferring from storage temperature to display conditions, psychrophiles would usually display a lag time to enable them to adjust to their new environment prior to growth, thus explaining the lowered fungal counts observed at 13°C (NPDUD) compared to 4 °C (EPODUD and EPCDUD, Figure 5.5).

Like bacteria, it is possible that the activity of spoilage fungi may have been markedly reduced by the effect of a lowered temperature. The heat shock treatment may also have facilitated the growth of some spoilage fungi leading to the early onset of microbial spoilage in these samples. Significant increases in fungal counts during and beyond shelf life have implications for the safety of RTE vegetables as low storage temperatures are known to extend the shelf life of many leafy vegetables (Martinez-Sanchez et al., 2008) due to the reduced microbial spoilage and respiration rate (Medina et al., 2012).

The mould identified in this study were previously found in crops and some of them are toxin producing fungi. A summary of the characteristics for mould identified in this study are given in Appendix 5.3.

5.5 Conclusion

A total of 267 bacteria were detected in this study but only 51 bacteria were identified to species level which comes from 17 different genera. As expected, bacteria identified are very diverse ranging from human pathogens, plant pathogens, beneficial bacteria to both human and plant to epiphytic bacteria which just colonizing plant's surface without causing any good or harm. More bacteria was found in rocket leaves which were kept at relatively higher temperature, emphasizing the importance of keeping the RTE produce in low temperature in preventing the proliferation of bacteria. A pathogenic bacteria, *Salmonella enterica* was found at sample HSSDUD and NPDUD. Both samples were kept at display temperature (13 ^{o}C). Under long display duration condition at 13 ^{o}C, lactic acid bacteria were seems to proliferate and causing fermentation. Fermentation process produces off flavour volatiles (Chapter 3). Similar findings was found for *Morganella morgani* which were detected in samples kept at display temperature. On the other hand,

Pseudomonas spp. were found in all temperature abused, display temperature and refrigeration temperature. Whole genome sequencing and 16s metagenomics analyses gave the same conclusion where *Pseudomonas* spp. were found to survive at any handling and storage temperature.

The results also show that mould counts were also increased during and the beyond the shelf life of the bagged fresh RTE rocket with the highest counts observed beyond DUD. Samples subjected to temperature abuse (25 ^{o}C) contained higher mould counts compared to those did not receive heat shocked. Such situation, together with the impact of high population of bacteria discussed earlier would increase the risk of foodborne illness and fasten the rate food spoilage. Molecular identification of the moulds revealed that there were six strains of toxin producing fungi were detected present in bagged RTE rocket leaves: *Fusarium* equiseti, *Fusarium chlamydosporum* (2), *Alternaria alternata* (5), *Alternaria tenuissima* (4) and two Alternaria species.

The results of this study clearly show temperature plays an important role on the presence and proliferation of bacteria and moulds in fresh RTE rocket leaves. Keeping rocket leaves at the correct temperature along the supply chain able to reduce the proliferation of both types of microbes but this is only applicable to those with mesophilic characteristics. Psychrophiles and psychotrophs microbes could still able to grow at refrigeration temperature but in a slow rate since they still have optimal growth temperature around 15 ^{o}C, including at the display temperature of the open refrigerated shelves at the retailers' premises.

CHAPTER 6

IMPACT OF GLUCOSINOLATE PROFILE IN ROCKET AND PROCESSING ON MICROBIAL LOADS

The work in this chapter contributed to the following publication which is bound into the back of this thesis:

Luke Bell, **Hanis Nadia Yahya**, Omobolanle Oluwadamilola Oloyede, Lisa Methven and Carol Wagstaff. 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.

6.1 Introduction

Members of *Brassicaceae* including rocket are known to contain high concentrations of glucosinolates (GLSs). Apart from being responsible for the hot and peppery flavour of rocket, glucosinates were reported to possess biological activity against the growth of cancer cells (Ombra et al., 2017), bacteria and fungal pathogens (Blazevic et al., 2010; Khoobchandani et al., 2010; Ombra et al., 2017) and insects (Wittstock et al., 2004). The formation of GLS in plants is triggered by stress factors and influenced by cultural and environmental factors such as fertilizers and salts, temperature and radiation, as well as by postharvest handling and storage conditions (Jahangir et al., 2009).

To be converted into an active form, GLS must be hydrolysed in the presence of the enzyme myrosinase. Under intact cellular conditions, GLS and myrosinase are physically

separated into different cellular compartments; both GLS and myrosinase are stored in vacuole but they are physical separated. Myrosinase is stored as myrosin grains while glucosinolates are stored in adjacent separate 'cells' *(*Koroleva et al., 2000).

The two substances are mixed when the cells are disrupted upon various physical and mechanical processes such as wounding, chewing and cutting, as well as after exposure to high temperature (Blazevic et al., 2010). Following hydrolysis of GLS products such as isothiocyanates, nitriles, oxazolidinethiones, thiocyanates and epithionitriles are formed. These newly formed products are actually responsible for the complex aroma of brassicas and various biological activities that are linked to GLS described earlier.

Within the same genus and species, the content of total GLS and its individual GLS or their derivatives varied among accessions, as reported by Bell et al. (2016) for rocket and Charron et al. (2004) for broccoli. In rocket, variation in individual and total GLS content of six varieties of rocket used in this study are given in Table 6.1. Therefore, the biological activity against bacterial growth amongst varieties of rocket containing different concentration and composition of GLS was expected to differ. Ludwig-Muller et al. (1997) for example noticed that cabbage with a high content of aliphatic glucosinolates were more sensitive to club root disease caused by *Plasmodiophora brassicae*, whereas those resistant to the disease contained high concentrations of aromatic glucosinolates. Charron et al. (2004) evaluated the impact of glucosinolate content in broccoli on the growth of *Pseudomonas marginalis*, a causal agent of bacterial soft rot. They reported that in an *in vitro* assay, there was a linear relationship between total GLS and the percentage of suppression of *P. marginalis* with a R^2 of 0.48. Based on this finding, it is

hypothesized that the extracts of rocket leaves containing high concentration of GLS would have lower bacteria loads.

The study reported in this chapter examined the changes in the naturally occurring bacterial population residing in leaves of different varieties of rockets with varying compositions of GLS and subjected to different handling and storage conditions. In addition, the antimicrobial activity of the leaf extracts obtained from different varieties of rocket was also examined.

6.2 Materials and Methods

6.2.1 Microbial loads in fresh RTE rocket of different varieties containing varying concentrations of glucosinolates

6.2.1.1 *Plant materials and experimental treatments*

Five gene bank accessions of *Eruca sativa* and one commercial variety (*Diplotaxis tenuifolia* var. Torino) with different glucosinolate contents (Bell et al., 2017a, Table 6.1) were used in this study. Total plate count (TPC) of the plant materials were determined at nine different processing points. The details of the processing points are given in Table 6.2. TPC analysis for commercial rocket (wild rocket) were only determined at the point of prewashed until two days after display until date. The wild rocket samples were commercially produced in Italy and supplied in the UK by Alresford Salads.

Table 6.1. Rocket varieties with different concentrations of individual and total glucosinolates (mg.g^{-1} DW sinigrin hydrate equivalents).

Variety	Gluco-erucin	Gluco-raphanin	Digluco-thiobeinin	Gluco-sativin	Gluco-iberverin	DMB	Total GLS
ERU16	0.938	1.447	0.021	5.314	0	1.76	9.658
ERU18	0	1.424	0.011	3.113	0.011	0.397	4.956
ERU140	0	0.736	0	3.083	0	2.172	5.991
ERU154	1.665	2.045	0.011	2.898	0	0.698	7.315
CGN24247	0.357	1.26	0.012	6.09	0.011	0.021	7.751
Torino	0.48	1.275	0	7.568	0	2.201	11.524

Source: Bell et al. (2017a).

Table 6.2. Sampling points and descriptions of activity for determination of microbial loads of six varieties of rocket.

Date of activities	Processing points	Code	Descriptions
16 July 2014	10-day-old plants	10 days	Leaves of 10-day old, field grown rocket was sampled at The Watercress Company farm (TWC), UK. (During sampling, the temperature was 23 °C). About 30g for each variety were sampled at random and put in two different zip-lock plastic bags. Each bag is considered as one replicate.
25 July 2014	Harvest Day	Harvest day	The rocket was harvested as normal farm practices and the leaves were stored at TWC for three days at 4°C
28 July 2014	Alresford Salad Factory	Alresford	After three days of storage at TWC, the rocket was transported to factory at Alresford in a refrigerated truck (5 °C). Journey took around two hours. The leaves were sampled immediately upon arrival at the factory.
29 July 2014	Prewash	Prewash	The rocket was kept at 4 °C overnight before processing began. The samples were taken just prior to washing.
29 July 2014	Post wash	Post wash	The rocket was washed using potable water and spun in a salad spinner to remove excess water. The working room temperature was between 10 -12 °C. The rocket was sampled prior to packaging.
30 July 2014	Samples were transported to University of Reading (Reading/Day 0).	Reading	Samples were transported from Alresford Salad Factory in a refrigerated truck (4 °C) to Reading University. The journey took around one hour. Samples from each variety were taken in three replicates for microbial analysis and kept at 4 °C.
1 Aug. 2014	Day 2	D2	TPC was performed on day two on samples stored at 4 °C
6 Aug. 2014	Display until date (DUD)	DUD	TPC was performed on DUD date (on 6 days after Day 0)
8 Aug. 2014	Two days after DUD	DUD2	TPC was performed on two days after DUD date (eight days after day 0)

Samples were taken in three biological replicates.

6.2.1.2 *Microbial count analysis*

As described in section 3.2.1.2 but without the 13 and 25 ^{o}C incubator in apparatus section.

6.2.1.3 *Data analysis*

As described in Section 3.2.1. 3

6.2.2 Antimicrobial properties of extracts of different of rocket variety

6.2.2.1 *Plant Materials*

Leaves of five rocket salads (*Eruca sativa*) (coded as CGN 24247, ERU 16, ERU 18, ERU 140, and ERU 154 and one variety of *Diplotaxis tenuifolia* var. Torino) were used in the study. The leaves were sampled on eight days of storage (DUD2) as the leaves were found to contain the concentration of glucosinolates at the stage.

6.2.2.2 *Extraction of glucosinolates*

As described in section 3.4. Rocket extracts were sterilised using 0.22 µm filter disc with a low protein binding Durapore polyvinylidene fluoride (PVDF) membrane (Millex, EMD Millipore, Billerica, MA, USA).

6.2.2.3 *Treatments*

The treatments used in this study are as outlined in Table 6.3. *E.coli* K12, a strain of *E.coli* with resemblance of *E.coli* 0157:H7, but which does not express Shiga and Shiga-like toxins, obtained from Department of Food and Nutritional Sciences, University of Reading was used. The bacteria were grown overnight at 37 ^{0}C in LB broth and were adjusted to 0.02 optical density (OD) measured at 600 nm using a spectrophotometer. 12.5 units of myrosinase were also added into the mixture to test the role of myrosinase on the inhibitory effect on the growth of bacteria. Positive control for myrosinase was also included.

Table 6.3. Treatment structure used to assess the antimicrobial properties of extracts of different of rocket variety.

Rocket variety/myrosinase	Leaf extract (µl)	Bacteria suspension(µl)	Myrosinase (unit)	LB broth (µl)
CGN 24247	200	25	none	None
CGN 24247+myrosinase	180	25	20	None
Torino	200	25	none	None
Torino+ myrosinase	180	25	20	None
ERU16	200	25	none	None
ERU16+ myrosinase	180	25	20	None
ERU18	200	25	none	None
ERU18+ myrosinase	180	25	20	None
ERU140	200	25	none	None
ERU140+ myrosinase	180	25	20	None
ERU154	200	25	none	None
ERU154+ myrosinase	180	25	20	None
Positive control	None	25	none	200
Myrosinase positive control	none	25	20	180

Note: The experiments were conducted in three replicates.

6.3 Results

6.3.1 Bacterial loads in fresh RTE rocket of different variety containing varying concentration of glucosinolates

Results presented in Figure 6.1 shows the interaction of rocket variety with different points of the supply chain on total plate counts (TPC). The results clearly showed that all factors produced marked effects on TPC, but the effects of one factor on the growth of bacteria were highly dependent both on rocket variety and points of supply chain. Total plate count ranged from 7.65E+06 cfu.g^{-1} (ERU 140 at 10 days old) to 2.39E+09 cfu.g^{-1} (Torino at D2).

Torino contained the highest bacterial load compared to loads in other rocket varieties. Among the points of sampling, TPC of wild rocket at day 2 of storage (processing point, D2) had the highest TPC value (2.39E+09 cfu.g^{-1}). The bacterial count was significantly reduced from D2 to DUD (1.53E+09 cfu.g^{-1}), indicating that washing had removed some of the surface bacterial load, but was raised again at the last point of supply and processing included in the study (i.e. at 2 days after DUD (DUD2), 2.20E+09 cfu.g^{-1}). The TPC values at D2 were not significantly different from those counted at DUD2.

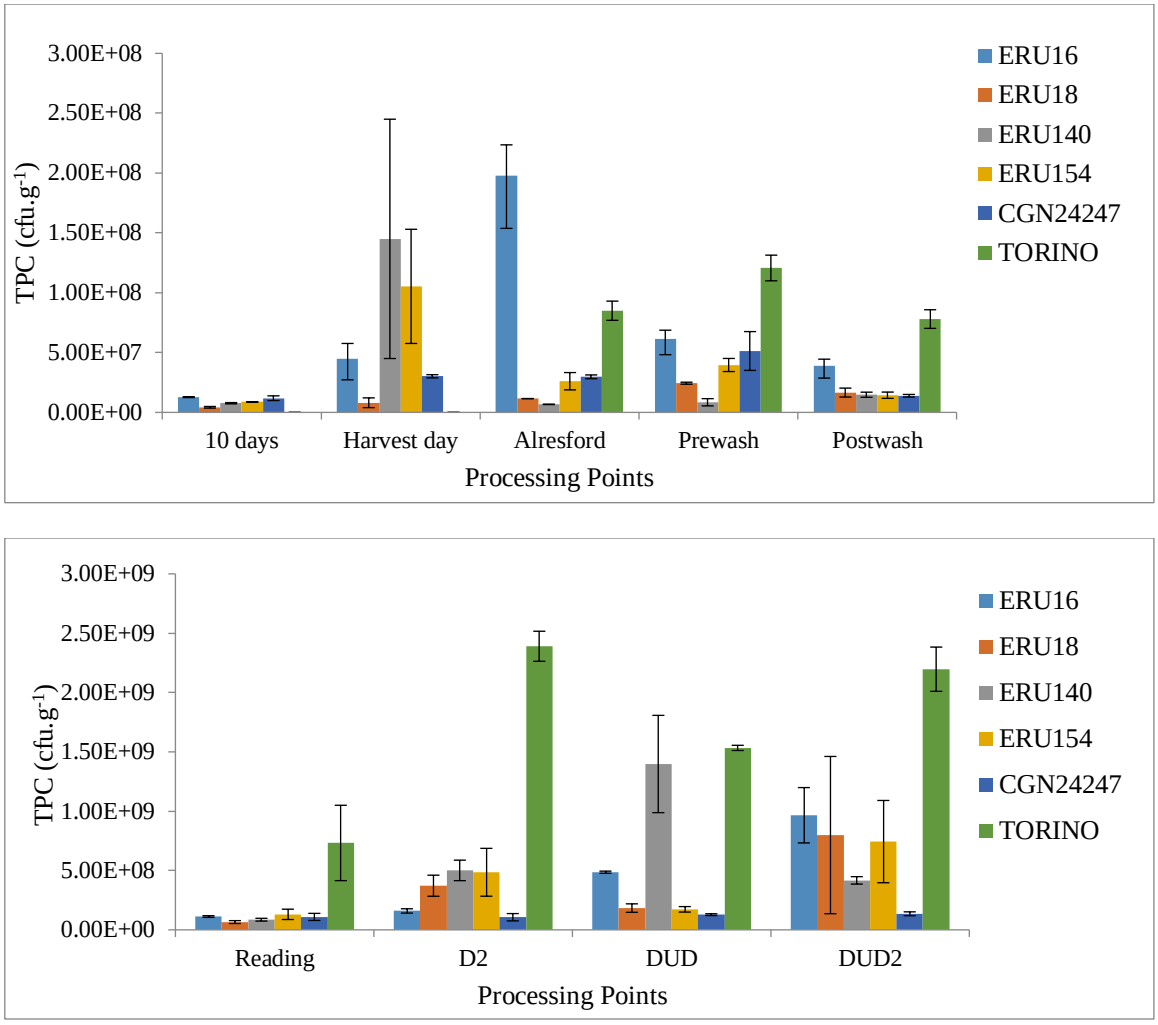


Figure 6.1. Total plate counts in six rocket varieties determined on 10 days after sowing (10 days), on the harvest day (Harvest day), on arrival at Alresford factory from the farm (Alresford), before washing (prewash), after washing (postwash), on arrival at University of Reading from Alresford (Reading), on day 2 after start of storage at 4 ^{o}C (D2), stored at 4 ^{o}C until display until date (DUD) and stored at 4 ^{o}C until 2 days after display until date (DUD2). Wild rocket was excluded in the point of 10 days and Harvest day. Bars indicate standard error of means, n=156.

During storage at the factory, the TPC value was 8.5E +07 cfu.g^{-1} but it has increased to 1.21E +08 cfu.g^{-1} immediately before washing, suggesting the increase in the microbial growth while waiting for the subsequent processing procedure. Results also show that washing was effective in reducing the microbial loads in rocket, whereby the TPC in washed rocket was markedly reduced from 1.21E+08 to 7.80E+07cfu.g^{-1}when the samples were washed using potable water, a reduction of 35.5%. The TPC of washed rocket was similar to those rocket sampled upon it arrival at our laboratory in Department

of Food and Nutritional Sciences, University of Reading (Reading) with the value of 6.57E+07cfu.g^{-1}. However, washing only gave a temporary reduction in microbial population in rocket as the values were seems to markedly increased again as the points of processing and handling advanced. Result also revealed that the increase in TPC in Wild rocket was faster than the increase in salad rocket.

As for CGN 24247 variety, TPC value increased slightly from 10 days old rocket to the point where the samples being transported to University of Reading except for the post-washed samples whereby the TPC of CGN 24247 was reduced from 5.13E+07 cfu.g^{-1} in pre-washed samples to 1.39E+07 cfu.g-^{1} in post-washed samples. Bacterial loads in variety CGN 24247 increased from 1.09E+08 cfu.g^{-1} in rocket upon its arrival at the University of Reading (Reading) to 1.35E+08 cfu.g^{-1} at two days after DUD (DUD2), an increase of 2.60E+07 cfu.g^{-1}. TPC of ERU 18 also shows the same growth pattern but with different TPC values. Overall, relative to other varieties, variety CGN 24247 and ERU 18 have lower microbial loads.

Initially, TPC for rocket varieties coded as ERU 16, ERU 18, ERU 140 and ERU 154 did not vary markedly among each other at processing points of 10 days old. However, the TPC values among them then varied distinctively at the later point of processing and handling. TPC of ERU 16 was noticed to increase at the point of harvest and drastically increased at the point where the samples arrives at Alresford from 1.28E+07 cfu.g^{-1} on 10 day old rocket, 4.50E+07 cfu.g^{-1} on harvest day and to 1.98E+08 cfu.g^{-1}on the day the arrival day at Alresford. However, TPC values decreased from the samples that arrived at Alresford to point of pre-washed and the TPC was decreased further after washing at post-washed rocket from 1.98E+08 to 6.12E+07 to 3.21E+07 cfu.g^{-1}, respectively. The

values increased during the storage at 4 ^{o}C until DUD before dropped again at two days after DUD.

For ERU 154 and ERU 140, TPCs for both varieties have a same pattern of bacterial growth and started to differ from each other during storage at 4 ^{o}C. The TPCs for these rocket varieties was also quite high at harvest, which were 1.05E+08 and 1.45E+08 cfu.g^{-1} respectively for ERU 154 and ERU 140, compared to other varieties. The growth of bacteria for ERU 154 declined at DUD from 4.85E+08 (D2) to 1.73E+08 cfu.g^{-1}. However, the counts started to increase again on day two after display until date (DUD2) which increased to 7.44E+08 cfu.g^{-1}. On the other hand, TPC for ERU 140 increased linearly from the arrival day at University of Reading (Reading) to DUD, then decreased on the DUD2 with their respective values of 8.57E+07 cfu.g^{-1} (Reading), 5.01E+08 cfu.g^{-1} (D2), 1.40E+09 cfu.g^{-1} (DUD) and 4.17E+08 cfu.g^{-1} on DUD2.

6.3.2 Antimicrobial properties of leaf extracts from different varieties of rocket salads

The growth of *E.coli K12* over 24h cultured on LB broth added with extracts of six different variety of rocket with and without myrosinase are presented in Figure 6.2. The growth of the bacteria was measured in term of optical density: higher OD represents a higher bacterial population and *vice-versa*.

The effects of rocket leaf extract on the growth of *E.coli K12* are clearly demonstrated in this study ($P<0.001$). The results also show that the inhibitory effects of the extract of rocket leaves on the growth of bacteria was dramatically enhanced with the addition of

myrosinase into the culture media. The effect also differed markedly between rocket varieties. Amongst the variety tested, with the addition of myrosinase, the extract of Torino was found to be most effective in inhibiting bacterial growth with 71.7% reduction compared with the control. This was then followed by CGN 24247 (66.5%), ERU 18 (66.3%), ERU 140 (63.5%), ERU 16 (60.1%) and ERU 154 (57.1%). The corresponding values for the leaf extracts without myrosinase were 46.5, 56.8, 36.7, 41.9, 47.1 and 46.5%.

As mentioned earlier, addition of myrosinase enhanced the antibacterial capability of the rocket extract as seen as reduction OD for all varieties. However, the magnitude of the inhibitory effect of rocket extract + myrosinase on bacterial growth varied significantly among rocket varieties measurement (variety x myrosinase significant at $P < 0.001$). Compared to the extract without myrosinase, extract of leaves for variety ERU 18 with myrosinase increased the antimicrobial activity by 80.7%. The increase in antimicrobial activity following the addition of myrosinase into the extract of CGN 24247 was the lowest (17.1%) (Table 6.4).

Results shown in Figure 6.2 also revealed that the trend of bacterial growth over time differed among rocket varieties (time x variety significant at $P<0.0001$). The growth of bacteria peaked at about 7h of incubation for CGN 24247, Torino, ERU 16 and ERU 154, whereas the bacteria populations are kept on growing in ERU 18 and ERU 140 till the end of the study period (24h).

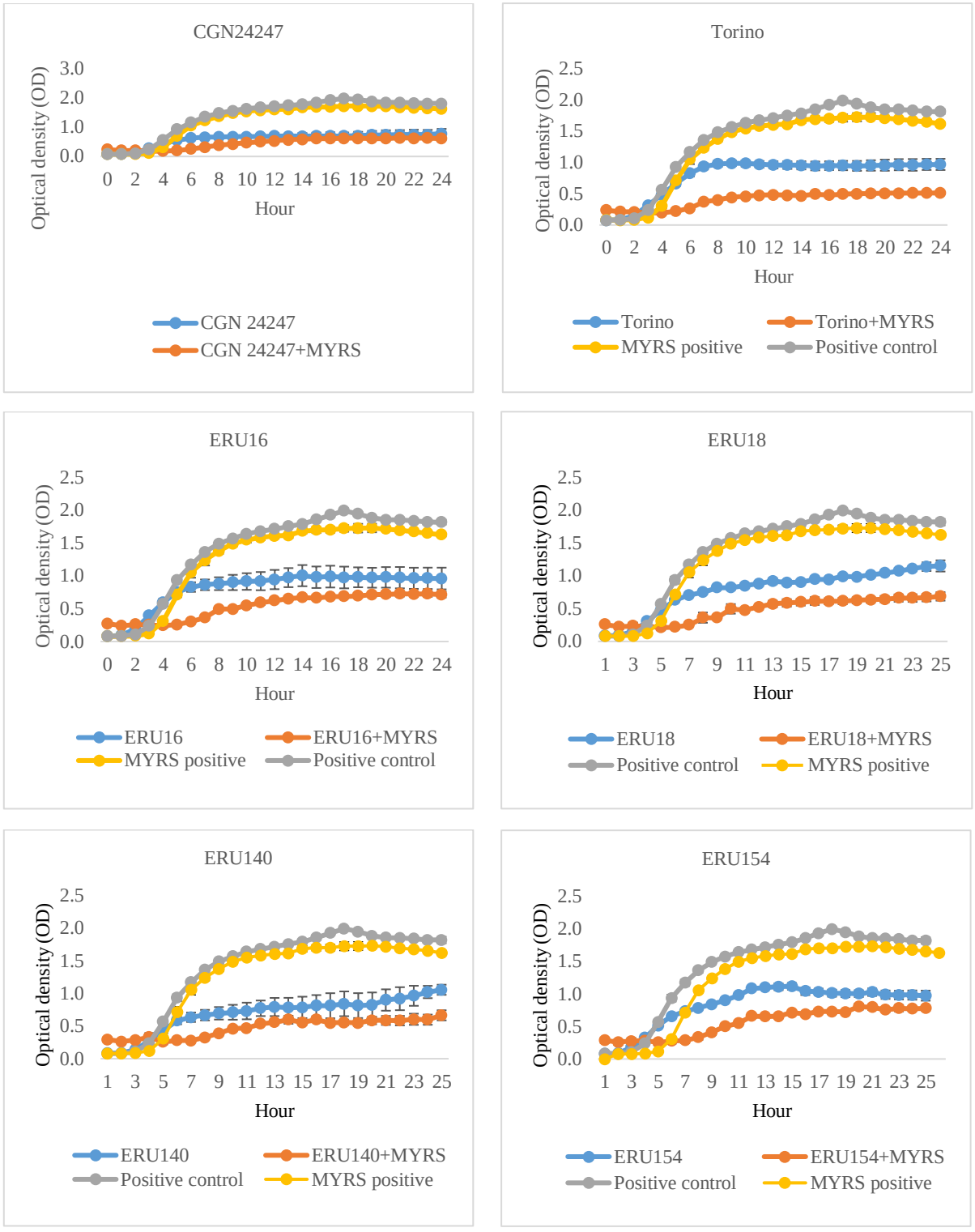


Figure 6.2. Changes in optical density of the bacterial suspension measured over a time course of 24 h for six variety of rockets. Higher ODs indicate higher bacterial loads present in the suspension.

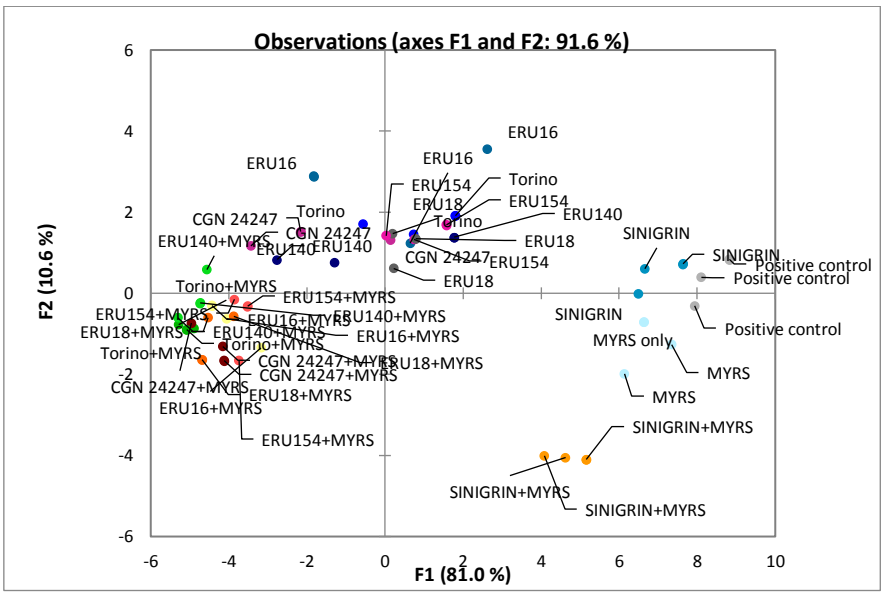
Table 6.4.Changes in bacterial population in the rocket leaf extract as affected by the presence of myrosinase at 24h after incubation.

Rocket variety	Reduction of bacterial population (%)		Changes in antimicrobial activity with addition of myrosinase (%)
	Extract with myrosinase	Extract without myrosinase	
CGN24247	66.5	56.8	+17.1
Torino	71.7	46.5	+54.2
ERU16	60.1	47.1	+28.9
ERI18	66.3	36.7	+80.7
ERU140	63.5	41.9	+51.6
ERU154	57.1	46.5	+22.8

The antimicrobial properties of the six varieties of rocket were also examined using principal component analysis (PCA) and results generated are presented in Figure 6.3. The loading plot and score plot from the PCA showed the overall trends in the antimicrobial activity of leaf extract of each variety. Principal component 1 (PC1) accounted for 81.0% of the variance in the data. This component represents the effectiveness of a cluster of extracts which mainly have been mixed with myrosinase (MYRS) (except for CGN 24247, r=0.434) in inhibiting the growth of the bacteria, which include Torino+MYRS (r=0.953), CGN 24247+MYRS (r=0.916), ERU 16+MYRS (r=0.902), ERU 18+MYRS (r=0.944), ERU 140+MYRS (r= 0.944) and ERU 154+MYRS (r=0.822), which are placed in the bottom-left side of the plots. All the correlation coefficient are significant at $P<0.05$. PC2 accounted for 10.6% of the variance in the data. The PC2 represents another cluster of the extract which are less effective in inhibiting the growth of the bacteria: the extract that are correlated with PC2 include Torino (r=0.683), ERU 16 (r=0.933) and ERU 154 (r=0.622). All scores belong to 'treatments' without the leaf extract are located one side (right hand-side) validated the

effectiveness of the extract (with and without out myrosinase) used in this antimicrobial test.

a



b

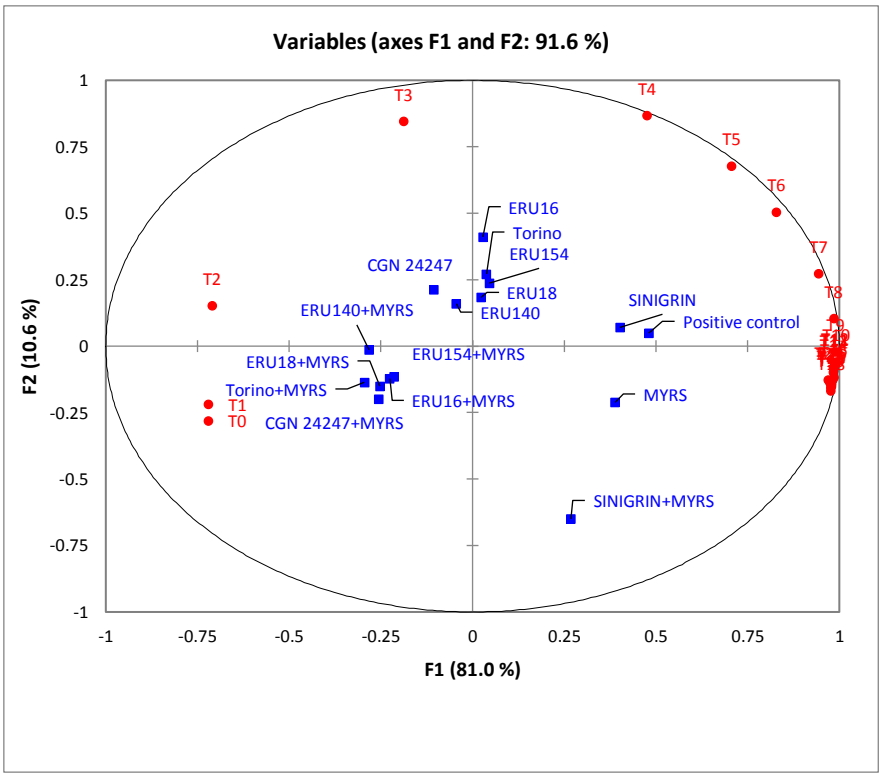


Figure 6.3. Loading plot (a) and score plot (b) from principal component analysis of leaf extracts of six varieties of rockets in relations to microbial loads. PC1 vs. PC2 (F1 and F2) accounted for 91.6% of the total variation within data. T0 to T24 indicate time of sampling with one hour interval.

In order to examine the role of individual GLS as an antimicrobial agent, correlation analysis among bacterial load (as OD) and all types of GLSs including total GLS was performed and the results obtained are presented in Table 6.5. Amongst the GLS, DMB was found to be the only GLS that significantly correlated with the OD (r=-0.507, P=0.0379). Negative significant correlation between DMB and OD indicated that increasing DMB contents in rocket leaves would couple with reduced bacterial loads, in this case *E.coli* K12. As hypothesized, total GLS was also negatively correlated with the OD (r=-0.500, P=0.0412). Results in Table 6.5 also show that glucoerucin and glucosativin was correlated positively with total GLS, giving an indication that increase in these two GLSs could contribute directly to the antimicrobial properties of the rocket extracts.

Table 6.5. Pearson correlation coefficients among bacterial population (*E.coli* K12) (measured as optical density, individual glucosinolates and total glucosinolate.

	Optical density	Glucoerucin	Glucoraphanin	Diglucothiobeinin	Glucosativin	Glucoiberverin	DMB[+]	Total GLS
Optical density	1	-0.170	0.164	0.158	-0.369	-0.038	-0.507	-0.500
		P=0.5000	P=0.5150	P=0.5300	P=0.1322	P=0.8796	P=0.0379	P=0.0412
Glucoerucin		1	0.493	0.460	0.236	-0.016	-0.249	0.480
			P=0.0378	P=0.0550	P=0.3456	P=0.9509	P=0.3358	P=0.0512
Glucoraphanin			1	0.546	0.019	-0.024	-0.296	0.299
				P=0.019	P=0.9411	P=0.9257	P=0.2479	P=0.2437
Diglucothiobeinin				1	0.114	0.135	-0.347	0.232
					P=0.6528	P=0.5923	P=0.1718	P=0.3713
Glucosativiv					1	0.105	0.180	0.833
						P=0.6781	P=0.4897	P<0.0001
Glucoiberverin						1	-0.294	-0.083
							P=0.2518	P=0.7529
DMB							1	0.453
								P=0.0678
Total GLS								1

[+]Note: DMB – Dimeric-4-mercaptobutyl

6.4 Discussion

The general trend in the data matched our hypothesis that bacterial populations would increase during shelf life, which is in agreement with Martinez-Shancez et al. (2006). With the exception of ERU140 and Torino, all other cultivar TPC numbers peaked on day 6 (DUD); and with the exception of ERU 140 and ERU 154. Torino had significantly greater numbers of bacteria present from Prewash through to DUD2; possibly due to the difference in leaf morphology of *D. tenuifolia*.

The breaches in the cool-chain, combined with high summer field temperatures, likely contributed to the high bacterial counts. Previous data presented under pseudo-commercial conditions for rocket *D. tenuifolia* (Spadafora et al., 2016) showed that produce stored above 10 °C for 14 days (~4.0 $cfu.g^{-1}$ FW) has significantly more bacteria on the leaves than those stored at 5 °C and 0 °C. The samples in this study were stored for only eight days, and bacterial counts were highest on D2 of shelf life, and much higher in abundance (Torino; Figure 6.1). Conversely, the cultivar ERU 154 saw no significant changes in bacterial load throughout the entire supply chain. This indicates that there may be a genotypic component imparted by each cultivar on the endemic leaf bacteria that determines their proliferation.

Bacteria continued to multiply during shelf life on all varieties, possibly due to the high relative humidity within bags (Watada et al., 1996) and sufficient availability of nutrients for the microbes. The aforementioned factors likely permitted the natural bacterial populations present within/on leaves to proliferate. Non-pathogenic field bacteria are likely to be resistant to extremes of temperature due to the variable climate of the UK,

and so are likely to grow even under cold-chain conditions. Further study is needed on commercial produce in order to properly elucidate these effects.

Glucosinolates (GLS) are known to possess some degree of antimicrobial properties (Khoobchandani et al., 2010). However, this relationship was not observed in this study. In contrast, we found that the increase in bacterial load in the samples coincided with high GLS contents in leaves. Our hypothesis was that higher GLSs/ITCs would reduce bacterial load, but instead the opposite results was observed. More specifically, we like to highlight that variety Torino, the variety with the highest GLS contents (Table 6.1) contained the highest number of bacteria numbers (Figure 6.1). It has been previously shown by Schreiner et al. (2009) that under nutrient limited conditions some bacterial strains may use GLSs as a source of carbon, which is not the case in the present study, whereby the leaves should provide sufficient amount of free sugar concentrations to support bacterial growth.

The failure to establish a positive correlation between reduction of microbial counts and the increased GLS contents in leaves of the six varieties of rocket tested here could be due to the possibility of microbes live on rocket leaves are highly adapted to the variable UK weather in the field, and have evolved a tolerance for high GLS/ITC concentrations, or a way to avoid the GLS-myrosinase system. There will also be a possibility that the bacteria have evolved to make use of GLS/ITCs as their source of nutrients. It has been documented that *Citrobacter* spp., a soil bacteria, possess a glucoside hydrolase family 3 (GH3) β-glucosidase enzyme, which may be useful for them in extracting carbon from GLSs (Albaser et al., 2016).

The same could be true of bacteria that live on rocket leaves. Mechanism of adaptation of insects to the GLS-myrosinase system is well established (Alan and Renwick, 2002) but adaptation of bacteria to a similar system is not well documented.

Results of Bell et al. (2017b) revealed that there were positive correlations between bacteria growth and some types of amino acids such as valine, isoleucine, threonine, asparagine, phenylalanine, glutamine, lysine, histidine, tyrosine, tryptophan, including total amino acid were observed. Coupled with the trends seen for GLSs as a potential carbon source for the microbes, free amino acids could have acted as important nutrient source for them to survive on leaves with potentially high contents of antimicrobial compounds.

Lack of positive correlation between the growth of naturally bacteria live on/within rocket leaves and the contents of GLS of the leaves, has led us to conduct another study. To exclude the possibility of adaptability of the microbes to the rocket leaves, we used a cultured *E.coli* K12, a strain of *E. coli* resemblance of *E.coli* 0157: H from the Department of Food and Nutritional Sciences, University of Reading. As hypothesized, results of the study clearly show that increasing total GLS in leaves was negatively correlated with the population growth of *E.coli* K12 (Table 6.5). Amongst the individual GLS in rocket extract, DMB was the only GLS that associated negatively with the population of the bacteria. To test our hypothesis on possible adaptation of naturally occurring bacteria to rocket leaf phyllosphere and its biochemical composition, further experiment could be performed by using bacteria isolated from the rocket leaves on extract of rocket leaves and spike with myrosinase as practiced in the present study.

As discussed above, the impact of frequently reported antimicrobial compounds detected in plants may vary between bacterial species and bacteria-food/media systems (Savoia, 2012; Schreiner et al., 2009). Using *Enterobacter radicincitans* DSM 16656, a plant growth promoting strain, Schreiner et al. (2009) reported that both morphological and phytochemicals, including aliphatic and aromatic glucosinolates, affected the colonization efficiency of the bacteria.

Our results also revealed that inclusion of myrosinase enhanced the effectiveness of the leaf extract in inhibiting the growth of the bacteria. The results strengthen the fact that the functional antimicrobial compounds were actually the compounds that belong to the group of isothiocyanates (ITCs) (Lin et al., 2000; Blazevic et al., 2010), which are formed after hydrolysis of glucosinolates that occurs in the presence of myrosinase. With the addition of myrosinase into the extract, the system had caused up to 71.7% reduction in bacterial load (in Torino+myrosinase), ranging from 57.1 to 71.7%; depending on variety. Without myrosinase, the percentage of the inhibition of the bacteria growth ranged from 36.7 to 46.1%, compared to the control. The highest inhibitory effects shown by Torino extract in the presence of myrosinase is consistent with our expectation as Torino contains the highest concentration of total GLS (Table 6.1). Furthermore, DMB was detected to be positively correlated with the antimicrobial properties of rocket, therefore the results of this study offers an opportunity for breeders alike to look for the possibility to use an individual GLS as the biochemical markers for selection criteria of the rocket to be used in developing new variety of rocket with high antimicrobial capability.

6.5 Conclusion

In the study reported in this chapter, we attempted to establish relationship between GLS content and loads of naturally occurring bacteria live on/within the leaves of rocket varieties with varying GLS contents at different processing and storage points. We hypothesize that due to the possible role of GLS and their derivatives, we expect varieties with high GLS content would contain lower bacterial counts. However, our study showed the opposite result. The actual explanation to this phenomenon is not known but it is possibly associated with a few possibilities: adaptation of the bacteria to the variable UK weather in the field enable them to be more adaptable in the changing conditions along the supply chain and evolve to survive on GLS-producing plants; or perhaps the bacteria are capable of utilizing GLS as a carbon source and free amino acid as a nitrogen source (Bell et al., 2017b). The species of bacteria colonize the leaves may be non-pathogenic, but their presence could enhance the rate of leaf deterioration and emission of sulphur-containing compounds as reported in section 3.3.

To further examine the possible relationship between GLS and bacterial loads, *E.coli* K12 were cultured in the extract of six rocket varieties. As expected, the growth of this strain of *E.coli* was lowest in leaf extract of variety Torino, the variety with the highest GLS content. Addition of myrosinase enhanced the inhibitory effects of the extract strengthening the fact that the actual compounds that are biological active against the bacteria are belong to isothiocyanates.

Amongst the individual GLS, DMB was found to be positively correlated with the antimicrobial properties of rocket extract, offering an opportunity for breeders to look for the possibility to use an individual GLS as the biochemical markers for selection criteria of the rocket to be used in developing new variety of rocket with high antimicrobial capability.

CHAPTER 7

GENERAL DISCUSSION

7.1 Discussion

RTE vegetables are a perfect media for microbes to proliferate, leading to food spoilage and increasing the risk of foodborne illness outbreaks (Beauchat, 2002; Harris et al., 2003; Nygard et al., 2008). The conditions are worsened when the fresh produce is unintentionally exposed to high (ambient) temperatures due to breaks in the cold chain that may occur during processing, transportation, storage, display at retailers' premises or consumers. Realising the potential risk that may be incurred by contaminated fresh RTE vegetables entering the food chain, a study was conducted with the following interrelated aims on rocket salads (*Eruca sativa* and *Diplotaxis tenuifolia*):

- To evaluate the effects of handling and storage conditions on rocket microbial loads, quality and volatile organic compounds in the context of the supply chain;
- To discover the natural distribution of bacteria and formation of biofilms in fresh RTE rocket salads using scanning electron microscopy;
- To identify naturally occurring microbes (both human and plant pathogens, and food spoilage microbes) that exist in fresh RTE rocket salads sold in national retailers; and
- To examine the antimicrobial properties of glucosinates found in rocket leaves on naturally occurring bacteria and on a cultured species of *E.coli.*

Microbes, like any other any living organisms, will grow at their best when they are given a conducive environment for them to propagate. Although the microbes come in different

categories based on their preferred growing temperature, apparently those present in fresh RTE rocket leaves grow best in relatively high temperatures (Figure 3.1 and 3.2). The results suggest that most of the bacteria present in the samples are belong to either mesophiles or psychrotrophs. The results presented here are consistent with the finding of Willocx et al. (1993), who reported that psychrotrophs such as *Pseudomonas* spp. and *Erwinia* spp. predominate in the population of microbes found in ready-to-eat salads. Similar findings were also reported by Brackett (1992) and Tournas (2005a, 2005b). These findings provide an indication that storing the fresh RTE vegetables in continuously low temperatures throughout the supply chain (less than 5 ^{o}C, Ridgwell and Winson, 2001) would be appropriate to inhibit bacterial growth.

The loads or abundance of bacteria on rocket leaves is a function of storage temperature, storage duration and whether the bag is opened or closed (which may determine the initial loads of bacteria as well as O_2 availability). The results clearly suggest that if the produce is kept continuously under 4 ^{o}C, the risk of contracting a food-borne illness associated with the consumption of fresh RTE vegetables remains relatively low until day 6 of storage. As the storage temperature was increased from 4 to 13 ^{o}C, the risk remained low up to 4 days of storage if the bags were kept closed.

At high temperature (13 and 20 ^{o}C), leaves in closed, uncontaminated samples continued to have low loads of bacteria for up to four days of storage. The results also suggest that once the bag of RTE vegetables is opened and the produce is disturbed, that may cause cross-contaminated by the intervention of human hands, the produce must be either consumed within two days if low storage facilities are not available. The need to keep the produce under continuous cold-chain conditions is not only critical from the view point

of food safety, but also in relation to food spoilage. The presence of food microflora, either in the form of bacteria, fungi or yeasts would alter the physical appearance (such as colour and texture) and composition of flavour compounds found in the rocket.

Along the supply chain from the production field to the dining table, the breach of cold-chain of the fresh RTE vegetables may occur at many stages: i.e. during storage, transportation, transit and display. Breaches of cold-chain would cause the increase in microbial abundance as seen when the fresh RTE rockets were subjected to various treatments.

High bacterial loads in leaves that have never been exposed to high temperature, but instead kept at 'display' temperature (13 ^{o}C) for relatively long durations (up to DUD or DUD+2 as in treatments NPDUD and NPDUD+2) emphasizing the role of time in determining the abundance of bacteria on the produce. The effects of time on the abundance of bacteria was also seen in leaves that were previously being exposed to higher temperatures and consumed on DUD as seen in HSSDUD and HSSDUD+2. The effectiveness of low temperature storage (4 ^{o}C) in inhibiting the growth of bacteria, regardless of product history, including the leaves 'consumed' on two days after expiry date, as shown by TPC in leaves of treatments HSCDUD, EPCDUD, EPODUD, HSCDUD+2, EPCDUD+2 and EPODUD+2.

Pre-harvest field conditions experienced by crop plants influenced their postharvest performance (Deza-Durand et al., 2014; Martinez-Ballesta et al., 2013). During storage, amongst the most common physiological parameters affected by the preharvest factors are respiration rate and ethylene generation (Deza-Durand et al., 2014; Koukounaras et

al., 2007; Licciardello et al., 2016). Abiotic stress imposed in the form of heat shock to the winter grown rocket increased its respiration rate, as seen in the increase of CO_2 that occurred concurrently with reduced O_2 level in the bagged fresh RTE rocket leaves (HSSDUD, HSSDUD+2). Similar effects was also observed in leaves that were kept for a relatively long duration at 'display temperature' (13 ^oC) (NPDUD and NPDUD+2). This phenomenon reflects poor adaptation of winter grown rocket to higher temperatures experienced by the plant materials at postharvest stages (Amodio et al., 2015). A generally low sugar content in the crop when it is grown in low temperature conditions, coupled with its high respiration rate (Licciardello et al., 2016), leads to a rapid depletion of carbon reserves and shortens its shelf life.

High CO_2 and low O_2 in the gas bouquet sampled from the bag of fresh RTE rocket creates anaerobic conditions inside the bag. Alteration of the bag's atmospheric conditions affects leaf physiological processes and biochemical pathways, and this would influence the type of microbes living on the leaves (Chapter 5) and leaf chemical compounds (Chapter 3). Anaerobic conditions promote the growth of lactic acid bacteria or yeast, and this leads to microbial fermentation yielding various types of volatiles, which is normally indicated by the surge of alcohol abundance (Kays and Paull, 2004). Luca et al. (2016) reported that more volatile compounds were detected under restricted O_2 (<2.1 kPa O_2 pressure) conditions of modified atmosphere packed wild rocket. Among the predominant VOCs detected under restricted O_2 are sulphur-containing compounds such as carbonyl sulphide, methanethiol, carbon disulphide, methyl isothicyanate and dimethyl trisulfide. The correlation between microbial loads and abundance of sulphur compounds in bagged vegetables is well acknowledged (Christensen et al., 2007; Lokke et al., 2012; Nielsen et al., 2008; Spadofora et al., 2016). In our case, anaerobic conditions could have occurred

in bags of HSSDUD, HSSDUD+2, NPDUD and NPDUD+2 treatments as the gas sampled from these bags contained high abundance of alcohols (Table 3.4).

Changes in product handling practices, storage and display conditions of bagged fresh RTE rocket significantly affected the abundance and types of VOC produced. To get the best flavour out of the rocket leaves, it must be purchased as early as possible and consumed immediately. Alternatively, consumers should purchase the fresh RTE vegetables as early as possible and keep the produce under refrigeration and consume it before DUD. They should avoid purchasing the RTE rocket near to the DUD as displaying the produce at retailer's premises at moderate temperature (13 ^{o}C as used in this study) would result in the release of a high abundance of off-odour compounds.

Amongst the phytochemicals found in rocket, and other members of brassicaceae, glucosinolates (GLS) are perhaps the most researched group of compounds (Bellostas et al., 2007; Cataldi et al., 2007; Cavaiuolo and Ferrante, 2014; Ombra et al., 2017). This is due to the potential role of GLS as antioxidants, anticancer and antimicrobial compounds, apart from the association of these compounds with the peppery and hot taste of many brassicas. In rocket, the number of detectable individual GLSs varies between reports as the abundance of GLS-compounds is sensitive to change according to plant materials (variety, age of leaves, plant part), growing conditions, agricultural practices as well postharvest handling and storage (Force et al., 2007; Selma et al., 2010; Verkerk et al., 2009). Methods of analysis perhaps also influence the number and abundance of GLS detected. Bell et al. (2015), using similar analytical techniques as used in this study, but with a different nature of plant materials, detected 12 individual GLSs (Table 2.6). In the present study, only six individual GLS were detected. The formation of GLS involves a

complex biochemical pathways (Grubb and Abel, 2005). Synthesis of each individual GLS begin with a specific pre-cursor, before it is converted into isothiocyanate in a hydrolytic reaction mediated by myrosinase (Cavaiuolo and Ferrante, 2014). For these reasons, generalization on the trends of change in the concentration of GLS is impossible (Section 3.4.2.1.4, Table 3.6). The results of this study revealed that leaves of EPODUD contained the highest concentration of total GLS and this is followed by those of EPCDUD and EPODUD+2 treatments. The results suggest that early purchase of fresh RTE rocket and keeping them at 4 ^{o}C would be advantageous in GLS retention, which then would retain their peppery taste and possible biological roles as an antimicrobial and anticancer agent.

Analysis of SEM images revealed the presence of bacteria on the rocket leaves and that the concentration of the microbes was higher on dented areas and stomata, especially along the leaf vein. High concentrations of microbes live within these areas as they provide a better protection site, as well as being nearer to the source of plant exudates that are normally emitted from the vascular bundles (xylem and phloem) when the cell collapses, resulting in an elevated abundance of bacteria present in/on the leaves. The results shown by SEM images (Chapter 4) were consistent with those obtained through numerical counts. Long term storage (days after display until date) leads to the formation of bacterial film, making any effort to deactivate the bacteria becoming more difficult. Formation of biofilm, coupled with high loads of bacteria would increase the health risk upon the consumption of the leaves purchased or kept until or beyond display until date.

Compared to loose leaves, consumption of bagged leaves may impose a higher health risk. This is because in the bagged fresh RTE vegetables, leaf exudates are collected at

the bottom of the bag and this would become a very rich source of nutrients for bacteria and promote the formation of bacterial biofilms (Koukkidis et al., 2014). Understanding the internalization process of bacteria in phyllosphere is an important aspect of food safety. Improved knowledge on the dynamics of bacterial-leaf surfaces interaction in fresh RTE vegetables could lead to a better control strategies to reduce their population. DNA sequencing of the bacteria and moulds found in bagged fresh RTE rocket leaves proved that the leaves contained very diverse composition of microbes. As far as fresh RTE food is concerned, human pathogens and food spoilage microbes are regarded as more important that the other groups of microbes.

Amongst the microbes identified, *Salmonella enterica* was found in leaves that experienced heat shock (four hours at 25 ^{o}C) followed by keeping at relatively high temperature (in HSSDUD and NPDUD). The presence of *Salmonella enterica* in food imposes a high risk and are potentially dangerous to health. The food contaminated with Salmonella would become unfit for human consumption (Health Protection Agency UK, 2009). Following detection, immediate investigation to track the origin of the sample, its production process and environment needs to be monitored. The infectious dose for Salmonella is usually quite large, but records of the outbreaks have shown that ingestion of low numbers of Salmonella to the gastrointestinal tract can cause infection (Kothary et al., 2001).

Beside human pathogenic bacteria, food spoilage bacteria were detected in bagged fresh RTE rocket after long storage, especially at display temperature. This include bacteria that cause leaf necrosis, and stem and basal rot such as *Pseudomonas viriflava* and *Erwnia* sp., and lactic acid bacteria such as *Lactococcus lactis*. As these microbes are

psychrotrophs, their presence is expected to be detected in a wide range of temperature. For this, while cold-storage could slow-down their growth, action should be taken to reduce the number of initial inoculum by practicing good manufacturing processes (GMP) which is equally important to avoid contamination of these bacteria on the produce.

The significant increases in mould abundance were also observed for the RTE rocket leaves held at moderate and high temperature, suggesting that product safety is at stake as six strains of toxigenic moulds, *Fusarium equiseti*, *F. chlamydosporum*, *Alternaria alternata*, *A. tenuissima*, and two species of *Alternaria* spp. were detected present in the produce. As for bacteria, this finding emphasizes the need for the continuous cold chain and sanitation processes at all stages along the supply chain.

The relationships between GLS content and abundance of naturally occurring bacteria that live on/within the leaves of rocket varieties was examined. It was expected that the varieties with high GLS content would contain lower bacterial counts, but the results showed otherwise. Unexpected results could be linked to a few possibilities; either because of adaptation of the bacteria to the variable UK weather during production phase making them to be more adaptable in the changing conditions along the supply chain, including against high level of GLS in rocket plants; or GLS found in the leaves could have become carbon and nitrogen sources for the bacteria (Bell et al., 2017b).

Such an expectated relationship between bacterial abundance and GLS content in the leaves may be associated with a very complex system involving hundreds of species ad strains of microflora including bacteria (Table 5.3), moulds (Tables 5.4) and yeasts

(Ofori-antwi, 2015) that may have different growth requirements and growth dynamics. Using a much more simple system, we have found a more conclusive result whereby the abundance of *E.coli* K12 was reduced under high concentration of GLS (correlation between total GLS *vs.* optical density, r=-0.500; P=0.0412) and apparently the reduction was associated with the concentration of DMB (correlation between DMB *vs.* optical density, r=-0.507; P=0.0379). The results of the latter type of experiment to underpin the antimicrobial property of any botanical material suggest that results generated from the study involving a complex food-microbial interaction may have a little practical values.

The results of the present study clearly show that bacterial and mould counts, volatile organic compound and glucosinolate abundance, distribution of naturally occurrence bacteria and formation of biofilms in bagged fresh RTE rocket leaves were significantly affected by handling and storage/display conditions of the produce. Molecular identification of the microbes revealed that the species and strains of bacteria and moulds detected residing in the RTE rocket leaves were very diverse, ranging from plant growth promoting bacteria, food spoilage organisms, plant pathogens, as well as human pathogen microbes. Except for the plant growth promoting bacteria, the latter three categories of microbes are all have economic importance in fresh RTE food supply chain and industry. Food spoilage microbes and plant pathogens will reduce product quality (causes changes in colour, texture and aroma), thus reduce consumer acceptability. The presence of human pathogens would increase the risk of fresh RTE related food poisoning. The pathogenicity of these microbes and their preferred growth environment differ among each other and therefore managing the products to inactivate or reduce the proliferation of a specific or a group of microbes may not be effective in reducing the other groups of microbes. In cases where a mixture of hazardous microbes are found in fresh RTE

vegetables as shown by the present study, microbial inactivation efforts should be targeted to low tolerance type of microbes. In our case, the most hazardous microbe detected was *Salmonella entrica.*

7.2 Contribution to society and industry

Information generated from this study could be useful to growers, manufacturers, retailers and consumers as the information could be used to guide them to extend the quality of the fresh produce, as well as to avoid food poisoning. Among the most relevant informations in this regard include differences in physiological behaviour of rocket grown under different growing conditions, insignificant effect of temperature abuse for 4 h on quality of rocket leaves, and the interactive impact of temperature and storage duration on bacterial abundance. Generation of off-odour volatile organic compounds in leaves kept at abuse temperature for relatively long duration will reduce consumer acceptance. This would lead economic losses to the growers and all parties involve along the supply chain.

Knowing the residing area and identification of species/strain of microbes in the leaves would help us in strategizing to inactivate them. Information on the antibacterial properties of glucosinolates found in rocket leaves could be useful in respect to drug discovery. Such information is also useful to plant breeder in developing new plant variety that contain high specific individual glucosinolates.

7.3 Future studies

1. As shown in Table 3.6, the concentration of glucosativin was reduced as the storage duration was extended but the trend was not observed for other types of GLSs. Reduction of glucosativin could be due to the consumption of the substance by microbes as the reduction of glucosativin concentration was coupled with the increase in microbial abundance. Similar assumption was also hypothesized by Bell et al. (2017b). The evidence observed here could become an interesting phenomenon for further experimentation to evaluate the effects of individual GLS present in rocket leaves in affecting the growth of bacteria, especially when dealing with individual species or strains of bacteria and fungi. Both commonly detected human pathogens (such as *Salmonella enterica, Escherichia coli* and *Enterobacter aerogenes* and plant pathogens (such as *Pseudomonas* spp., *Alternaria* spp., *Cladosporium* spp. and *Fusarium* spp., could be included in the study. To verify the possibility of adaptation of naturally occurring bacteria that reside on/in the rocket leaves that could have given negative responses against extracts of leaves obtained from different rocket varieties with varying levels of GLS, bacteria isolated from the leaves should also be included in the study. Results generated would affirm the possible role of GLS as an antimicrobial agent in a more specific manner at individual GLS levels on specific species and strains of microbes. This may in turn lead to research into the use of specific GLS as botanical fungicides that could be used in organic farming.

2. Our study in examining the impact on bacterial growth of glucosinolate contents in rocket leaves obtained from different varieties of rocket with known composition of glucosinolates was not conclusive. Variety ERU118 for example, with the lowest GLS

content, had the lowest antimicrobial activity in the absence of myrosinase but this turned to produce the highest antimicrobial effects when myrosinase was added into the system (Table 6.4). In a real life situation, in human's intestines, some gut microflora are able to perform the same hydrolysis reaction (Li et al., 2009). Therefore varieties such as ERU118, which did not show high antimicrobial capability in a study where myrosinase had been inactivated, could produce high antimicrobial activity *in vivo*. For this reason, further study to evaluate the effectiveness of rocket varieties with different GLS contents, involving intact leaves without deactivation of the enzyme is needed. The study could be more useful when it is conducted under simulated human digestive conditions.

3. Stress stimuli received by plants trigger accumulation of secondary metabolites (Martínez-Ballesta et al., 2013) as a reaction which is thought to increase the plant defence system (Jahangir et al., 2009). Glucosinolates are perhaps amongst the chemicals produced in large quantites under this situation and, when this occurs, it could increase the abundance of GLS, thereby enhancing the anticancer and antimicrobial potential of the crop. Therefore, research on the possible impact of stresses, for examples that induced by limited supply of water, high salinity and high temperature, on rocket plants during production level on glucosinolates, and its effects on microbial activity, is suggested.

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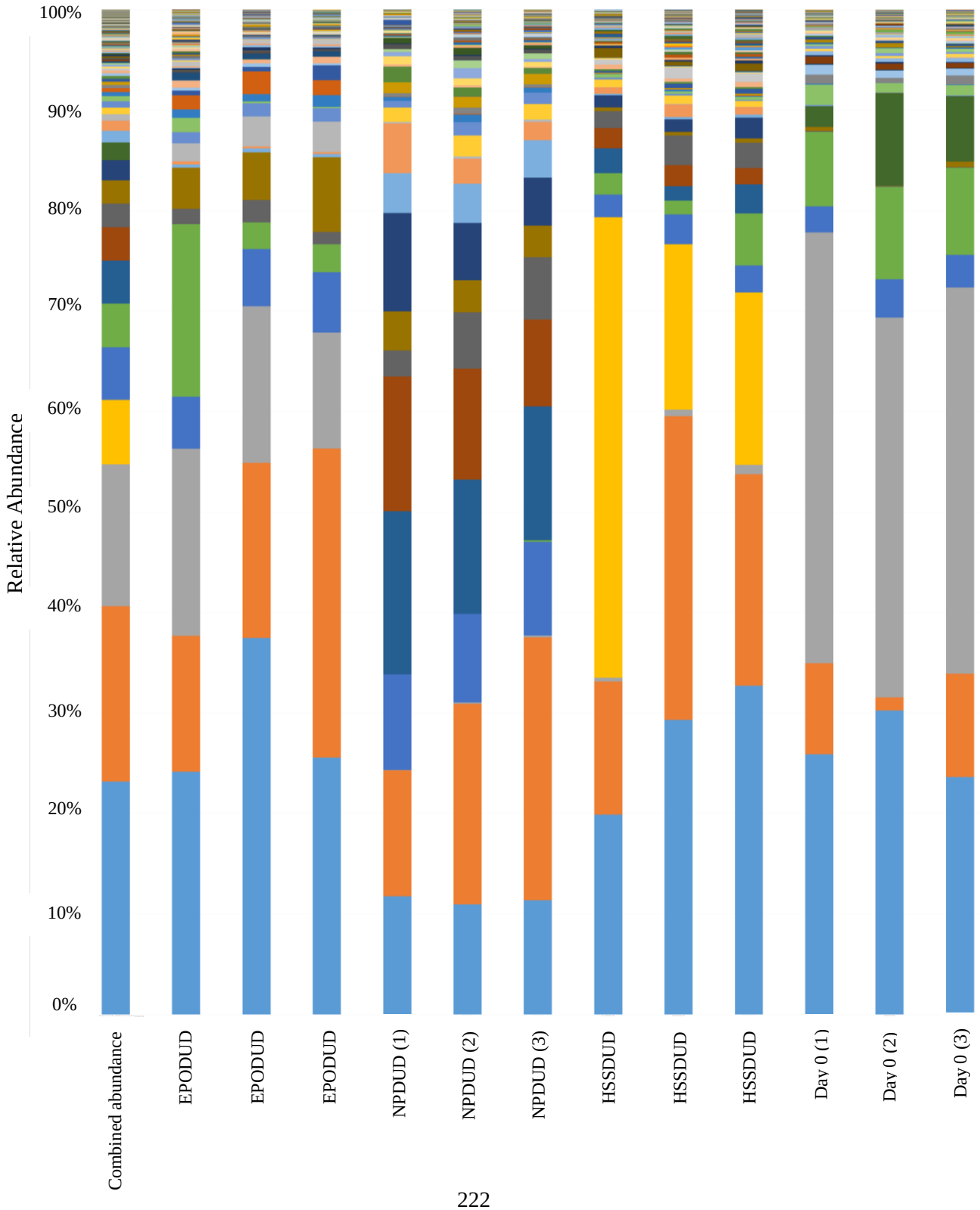
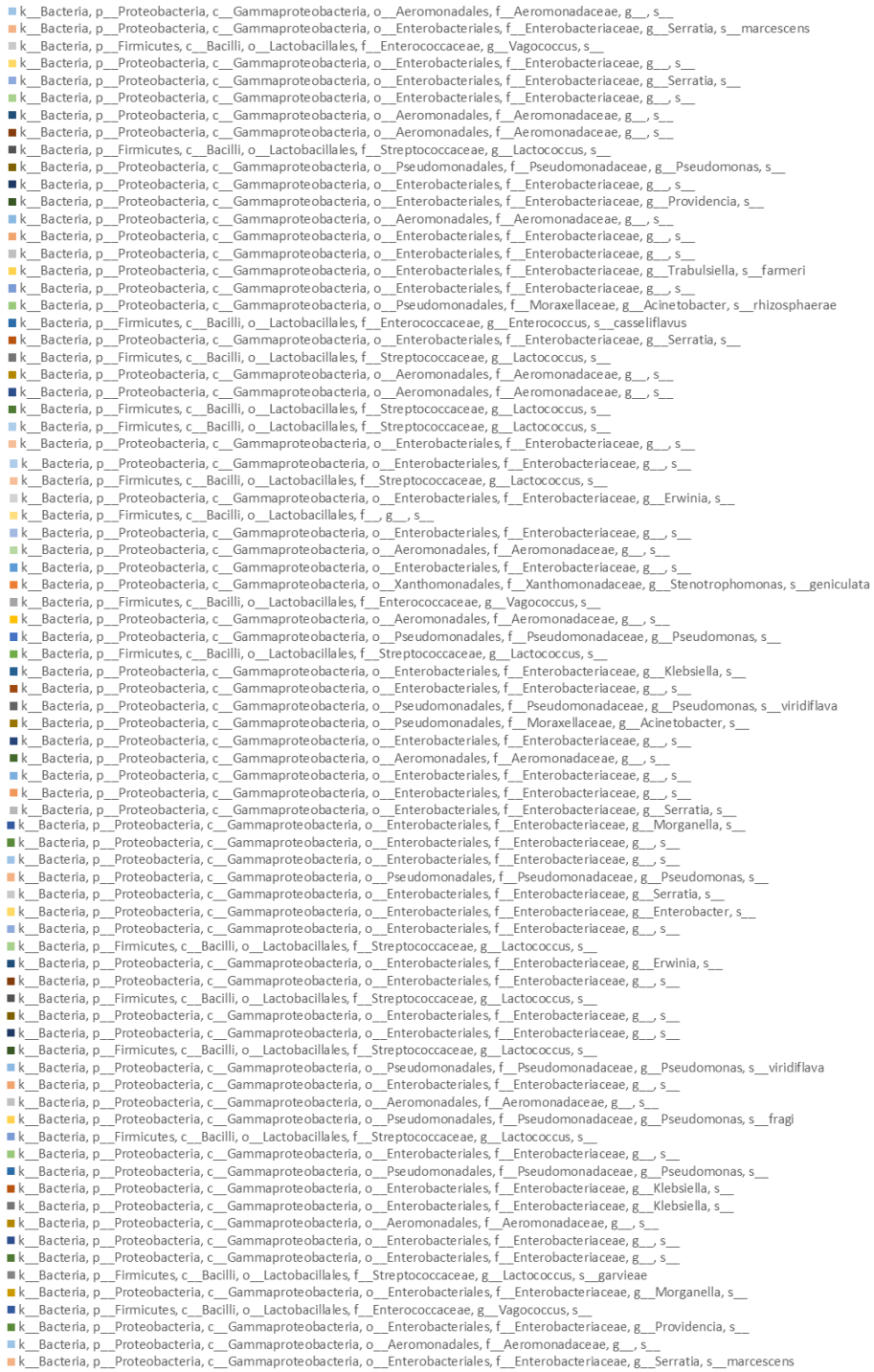
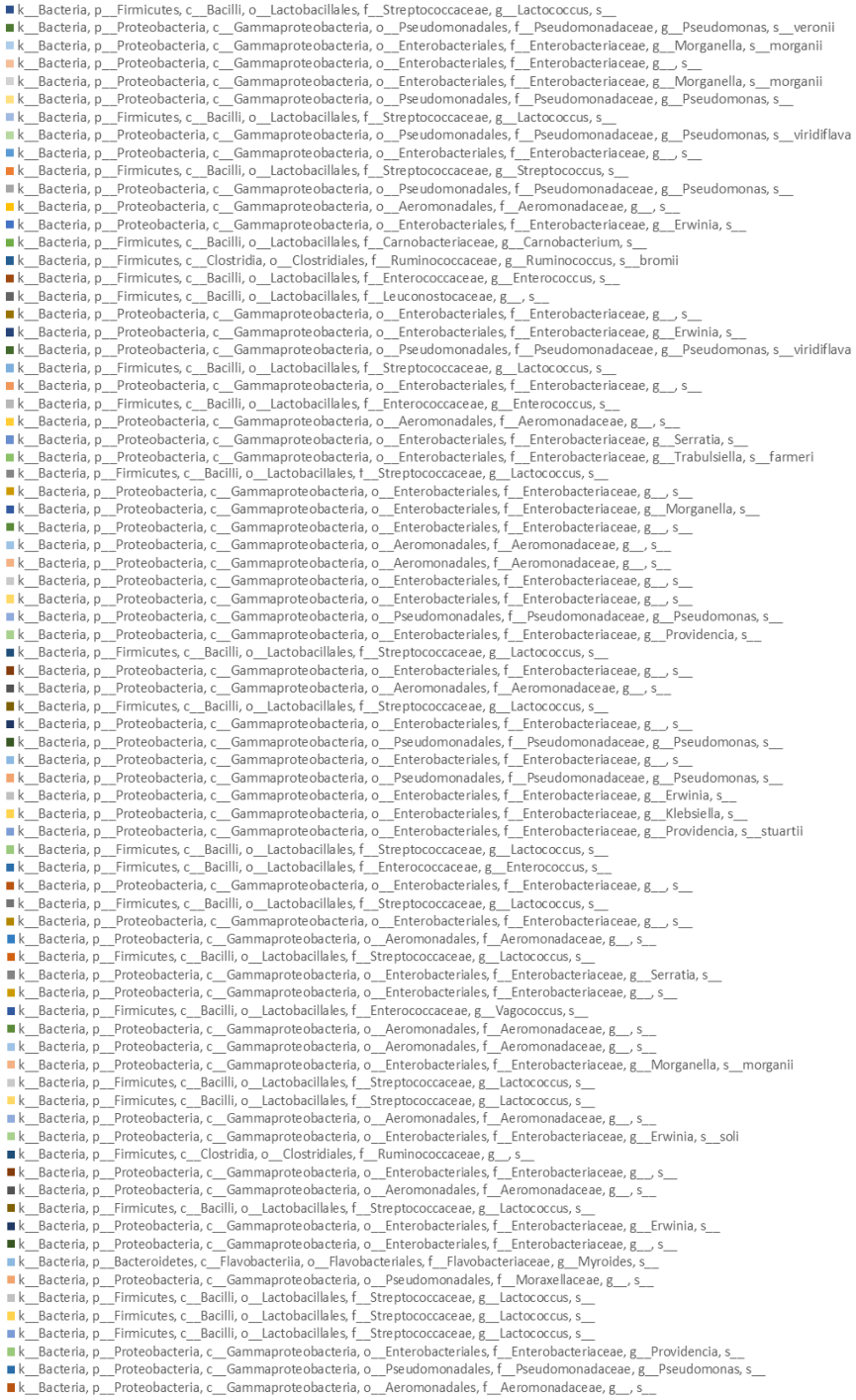
APPENDICES

Appendix 5.1. The relative abundance of dominant bacterial families identified in rocket leaves under 12 handling and storage conditions.



Legends for Appendix 5.1




k__Bacteria, p__Firmicutes, c__Bacilli, o__Lactobacillales, f__Streptococcaceae, g__Lactococcus, s__
k__Bacteria, p__Proteobacteria, c__Gammaproteobacteria, o__Pseudomonadales, f__Pseudomonadaceae, g__Pseudomonas, s__veronii
k__Bacteria, p__Proteobacteria, c__Gammaproteobacteria, o__Enterobacteriales, f__Enterobacteriaceae, g__Morganella, s__morganii
k__Bacteria, p__Proteobacteria, c__Gammaproteobacteria, o__Enterobacteriales, f__Enterobacteriaceae, g__, s__
k__Bacteria, p__Proteobacteria, c__Gammaproteobacteria, o__Enterobacteriales, f__Enterobacteriaceae, g__Morganella, s__morganii
k__Bacteria, p__Proteobacteria, c__Gammaproteobacteria, o__Pseudomonadales, f__Pseudomonadaceae, g__Pseudomonas, s__
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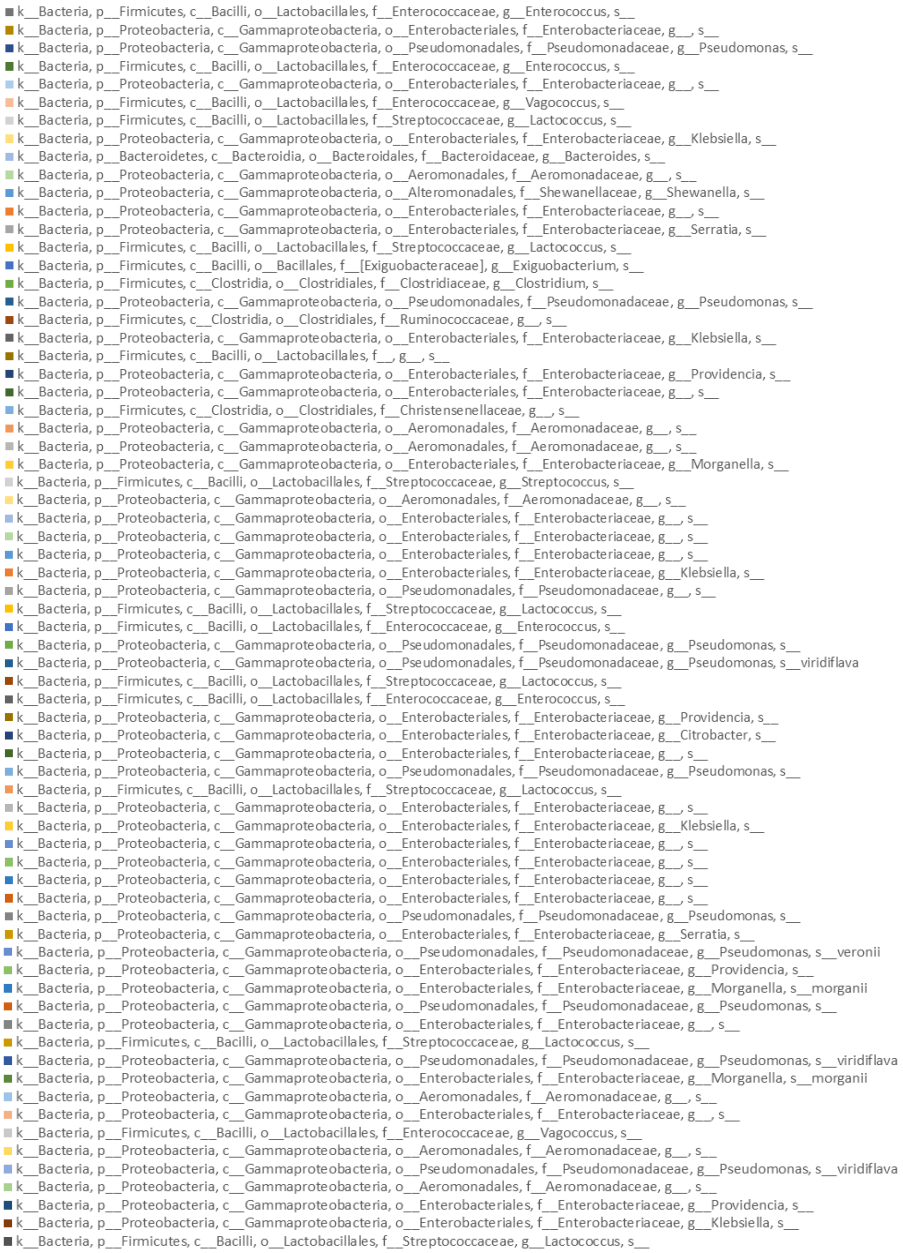
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k__Bacteria, p__Firmicutes, c__Bacilli, o__Lactobacillales, f__Streptococcaceae, g__Lactococcus, s__

Appendix 5.2. Characteristics of bacteria identified at different storage and handling condition in the supply chain context.

Bacteria	Characteristics	References
Enterobacter aerogenes strain KCTC 2190	Enterobacter aerogenes is a Gram-negative, oxidase negative, catalse positive citrate positive, indole negative, rod shape bacterium. It is a nosocomial and pathogenic bacterium that causes opportunistic infections including most types of infections. The majority are sensitive to most antibiotics designed for this bacteria class, but this is complicated by their inducible resistance mechanism, particularly lactamase, which means that they quickly become resistant to standard antibiotics during treatment, requiring a change in antibiotic to avoid worsening of the sepsis.	Sanders and Sanders (1997).
Pseudomonas fluorescens strain A506	Act as biological control agent for *Erwinia amylovora*.	Wilson and Lindow (1992).
Citrobacter gillenii strain CDC 4693-86	*Citrobacter gillenii* is a species of Gram-negative bacteria, but not much information available specifically for this bacterial strain but this strain was isolated from human stool in France. *Citrobacter* spp. are differentiated by their ability to convert tryptophan to indole. These bacteria can be found almost everywhere in soil and water. They can also be found in the human intestine. They are rarely the source of illnesses, except for infections of the urinary tract and infant meningitis and sepsis.	Brenner et al. (1999); Lipsky et al. (1980); Drelichman and Band (1985); Badger et al., (1999).
Obesumbacterium proteus strain 42	Generally, *Obesumbacterium proteus strain 42 is a* Gram-negative bacteria associated with brewery yeasts. Occurs as a brewery contaminant which can survive and grow in the presence of live yeasts during beer production.	Priest and Barker (2010); Shimwell (1963).
Pseudomonas putida F1 strain F1	*Pseudomonas putida* is a rod-shaped, flagellated, gram-negative bacterium that is found in most soil and water habitats where there is oxygen. They utilizes toluene as a sole source of carbon and energy for growth. It grows optimally at 25-30 ^{o}C and can be easily isolated. *Pseudomonas putida* induces plant growth and protects the plants from pathogens. Because *Pseudomonas putida* assist in promoting plant development, researchers use it in bioengineering research to develop biopesticides and to the improve plant health.	Espinosa-Urgel et al. (2000); Zylstra and Gibson (1989).

Aeromonas hydrophila strain ATCC 7966	*Aeromonas*, a ubiquitous waterborne bacterium, has been placed by the Environmental Protection Agency on the Contaminant Candidate list because of its potential to cause human disease. Based on a study of the pathogenicities of 12 species of the genus Aeromonas, the most pathogenic species by measuring 50% lethal doses in Swiss-Webster mice was *Aeromonas jandaei* ATCC 49568, followed by *Aeromonas hydrophila* ATCC 7966. Symptoms of Aeromonas sp. infections vary from gastroenteritis to wound infections (cellulitis, ecthyma gangrenosum, and myonecrosis) and septicaemia.	Galindo et al., (2006); Seshadri et al. (2006); Janda and Kokka (1991).
Enterobacter xiangfangensis strain 10-17	A Gram-stain-negative bacterial strain *Enterobacter xiangfangensis* 10-17 phylogenetically related to *Enterobacter hormaechei CIP 103441T, Enterobacter cancerogenus LMG 2693T, Enterobacter asburiae JCM 6051T, Enterobacter mori LMG 25706T, Enterobacter ludwigii EN-119T and Leclercia adecarboxylata LMG 2803T*. The pathogenicity is not known.	Gu et al. (2014).
Pseudomonas graminis strain DSM 11363	Generally *Pseudomonas* spp. motile when grown at 15 and 20 ^{o}C but not at 30 ^{o}C. The optimum growth temperature was 25 ^{0}C, still grow slowly at 4 ^{o}C. The best matches were found to the 16S rDNA sequence of *Pseudomonas oryzihabitans* (97.7 %), *Pseudomonas putida* (97-6 %), *Pseudomonas agarici* (97.2 %), and *Pseudomonas pavonaceae* (97.1 %).	Behrendt et al. (1999).
Salmonella enterica subsp. enterica serovar Typhimurium strain LT2	Some serovars of *Salmonella enterica*, such as *Salmonella typhi* could cause systemic infections and typhoid fever, whereas others, such as *Salmonella typhimurium* can cause gastroenteritis. *Salmonella typhimurium* LT2 is in subspecies of *Salmonella enterica*, which colonizes mammals and birds and causes 99% of Salmonella infections in humans.	McClelland et al. (2001); Liu and Sanderson. (1998).
Citrobacter rodentium ICC168 strain ICC168	Citrobacter *rodentium* strain ICC168 was found to having many features in common with other bacterial pathogens that have recently adapted to live in a new environment and colonise new hosts. Generally, the pathogenic bacterium *Citrobacter rodentium* naturally infects mice using a mechanism similar to those employed by certain strains of *E. coli* that cause severe gastro-intestinal infections in humans.	Petty et al. (2011).
Lactococcus lactis subsp. lactis IO-1	*Lactococcus lactis* subsp. lactis IO-1 has high similarity to three *L. lactis subsp. lactis* strains IL1403, KF147, and CV56 (97%), while two *Lactococcus lactis* subsp. cremoris strains, MG1363 and SK11, showing a homology of 90%.	Kato et al. (2012).

Morganella morganii subsp. morganii KT	*Morganella morganii* is a Gram-negative rod commonly found in the environment and in the intestinal tracts of humans, mammals, and reptiles as normal flora. Despite its wide distribution, it is an uncommon cause of community-acquired infection and is most often encountered in postoperative and other nosocomial settings. However, if happens, *M. morganii* infections respond well to appropriate antibiotic therapy. It is an opportunistic pathogens capable of causing a wide variety of nosocomial infections. In the late 1930s, *M. morganii* was identified as a cause of urinary tract infections. The KT strain was found to be susceptible to amikacin, ertapenem, gentamicin, meropenem, and cefepime but resistant to ampicillin, amoxicillin-clavulanate, cefazolin, cefuroxime, cefmetazole, flomoxef, and cefotaxime.	Miller (2015); Chen et al. (2012).
Providencia vermicola strain OP1	*Providencia vermicola* strain OP1 is closely related to *Providencia stuartii* strain DSM 4359 and *Providencia retregii* strain DSM 4542. *Providencia vermicola* strain OP1 could ferment D-glucose and grow in temperature up to 41^{o}C. It is an opportunistic pathogen in fish.	Somvanshi et al. (2006); Ramkumar et al. (2014).
Enterococcus casseliflavus	*Enterococcus casseliflavus* are rare pathogens, usually associated with urinary and intra-abdominal infections. *E. casseliflavus* was sensitive to ampicillin/sulbactam, penicillin, tetracycline, quinolones and teicoplanin, but exhibited intermediate sensitivity to vancomycin.	Pappas et al. (2004).
Lactococcus garvieae	*Lactococcus garvieae* is the etiological agent of Lactococcosis, an emergent disease which affects many fish species and causes important economic losses both in marine and freshwater aquaculture when water temperature increases over 16 °C in summer months. Normally, it causes a hyperacute and haemorrhagic septicemia. *L. garvieae* is also known human pathogen, which causes lumbar osteomyelitis, hepatic abscess and infective endocarditis.	Vendrell et al. (2006); Vinh et al. (2006).
Ruminococcus bromii	*Ruminococcus* bromii helps for the degradation of resistant starch in the human.	Ze et al. (2012).
Microvirgula aerodenitrificans	*Microvirgula aerodenitrificans* is a denitrifying Gram-negative organism. The organism has been isolated globally but has never been described as causative of clinical infection until 2012 by Murphy et al., 2012) due to the infection of a vascular access device in an infant with Pompe's disease.	Murphy et al. (2012).

Erwinia soli	Not much information regarding this bacteria was found. Usually, *Erwinia* spp. is a soft rot bacteria.	Tournas (2005).
Providencia stuartii	The long-term-catheterized urinary tract may offer a particular niche to Provi*dencia stuartii*, which is otherwise an uncommon clinical isolate.	Warren (1986).
S*erratia marcescens*	It is a widely distributed saprophytic bacterium, and has been found in food, particularly in starchy variants which provide, an excellent growth environment. *S. marcescens* is implicated in a wide range of serious infections including pneumonia, lower respiratory tract infection, urinary tract infection, bloodstream infection, wound infection and meningitis. The organism has also been described as an important cause of ocular infection with high incidence in contact lens-related keratitis. Infections caused by *S. marcescens* may be difficult to treat because of resistance to a variety of antibiotics, including ampicillin and first and second generation cephalosporins.	Hejazi and Falkiner (1997); Jones 2010); Kawecki et al. (2011; Merkier (2013); van der Vorm and Woldring-Zwaan (2002).
Trabulsiella farmeri	Not much information regarding this bacteria was found.	
Acinetobacter rhizosphaerae	*Acinetobacter rhizosphaerae* strain BIHB 723 is a plant growth promoting bacteria. It was found to exhibit the plant growth-promoting attributes of inorganic and organic phosphate solubilization, auxin production, 1-aminocyclopropane-1-carboxylate deaminase activity, ammonia generation, and siderophore production in Himalayas areas.	Gulati et al. (2009).
Pseudomonas fragi	It is a psychrophilic organism implicated in spoilage of dairy products (fruity aroma).	Omelianski (1923).
Pseudomonas veronii	This bacterium is nutritionally versatile since prior studies found that it degraded chloroaromatic compounds and alkyl methyl ketones. It may be used for bioremediation of contaminated soils, as it has been shown to degrade a variety of simple aromatic organic compounds. *P. veronii* has been implicated in human pathogenicity because the microorganism was isolated from human intestinal inflammatory pseudotumors.	Nam et al. (2003); Onaca (et al. (2007); Cheuk et al. (2000).
Pseudomonas viridiflava	The pectinolytic species *Pseudomonas viridiflava* has a wide host range among plants, causing foliar and stem necrotic lesions and basal stem and root rots.	Gonzalez et al. (2012); Sarris et al. (2012).
Stenotrophomonas geniculata	No information on this bacteria was found. It is closely related to *S. maltophilia*	Svensson et al. (2012).

Providencia rettgeri	*Providencia rettgeri* is a ubiquitous organism that is infrequently associated with human disease. It can be found in diverse locations as fresh water sources, run-off wastewater, and explosive-contaminated soil.	Washington et al. (2015); Kitts et al. (1994); Abo-Amer et al. (2013).

Appendix 5.3. Characteristics of moulds identified at different storage and handling condition in the supply chain context.

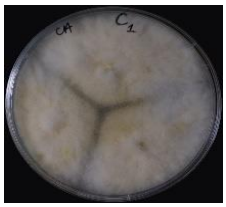
Moulds identified based on top match in NCBI	Characteristics	References
Alternaria alternata	Many species of *Alternaria* are plant pathogens. *Alternaria alternata* is a common species and is found in a wide variety of cereal crops and vegetables. This mould produces the mycotoxins, tenuazoic acid, alternariol, alternariol monomethyl ether, altenuene, altertoxin1 and tentoxins.	Pitt and Hocking (1997); Snowdon (1991); Pitt et al. (1994); Noser et al. (2011).
Alternaria infectoria	This *Alternaria* is commonly isolated from wheat, barley, rye and other small grains and produces the same toxins produced by *Alternaria alternata* but it produce toxins in smaller amount.	Andersen and Thrane (1996).
Alternata tenuisssima	*Alternata tenuisssima* is of common occurrence in the UK, residing on leaf surfaces, or as a secondary pathogen living within the plant tissue or thriving on dead or decaying matter. Similar with *Alternaria alternata*, *Alternata tenuisssima* produces the tenuazonic acid mycotoxins and is closely related to *Alternaria infectoria*.	Pitt and Hocking (1997).
Alternaria gasein	Information on their pathogenicity of this fungus is not known as results of its recent discovery.	
Fusarium equisetti	*Fusarium equisetti* is a soil saprophyte, commonly associated with decaying plant materials. It is a pathogen of a wide range of crops plants, especially in grain. It has the ability to grow in low oxygen tensions and has been implicated in the spoilage of UHT and processed fruit juices. It produces several mycotoxins including nivalenol, zearalenone, butennolide, fusarenon-X,T2 and diacetoxyscirpenol. Ingestion of diacetoxyscirpenol could induce liver disorders.	Marin et al. (2012); Hocking (1990), Lautraite et al. (1997).
Fusarium chlamydosporum	*Fusarium chlamydosporum* is a soil inhabitant usually found in warmer ecosystems, but it is not a plant pathogen. It however produces the trimycotoxins, T-2, HT-2 toxin and monoacetoxycirpenol, neosolaniol, iso neosolanial and chlamydosporol. The T-2 toxin could lead to vomiting, diarrhea, skin irritation, itching, rash, blisters, bleeding and shortness of breath in the acute stages of the diseases.	I.C.M.S.F. (1998).

Stemphylium vesicarium	*Stemphylium vesicarium* is a pathogen for asparagus, alfafa and mango. Not much information is recorded about this mould.	
Epicoccum nigrum	*Epicoccum nigrum* is widely distributed, commonly found in soil, dead plant parts, seeds of grains, mouldy paper, textile, human skin and sputum. It is not a plant pathogen and mycotoxin production has not been recorded for this mould.	Samson and Reenen-Hoekstra (1988).
Penicillium oxalicum	*Penicillium oxalicum* able to grow rapidly at warm temperature and was found to cause plant diseases as found in freshly harvested maize, yam and cassava.	Hesseltine et al. (1981), Adeniji (1970).
Aspergillus flavus	It produces the mycotoxin secalonic acid D as a major metabolite which is known to be significantly toxic to animals. *Aspergillus flavus* able to thrive in low water activity environments.	Ciegler et al. (1980).
Cladosporium cladosporioides	*Cladosporium cladosporioides* is a global occurrence, commonly found in a wide range of foods, on plant materials and soils. It has the ability to grow at refrigeration temperatures causing spoilage of many refrigerated foods. It is not known to produce mycotoxins and found associated with various South East Asian foods such as mungbeans.	Samson and Reenen-Hoekstra (1988), Northolt et al. (1980), Pitt and Hocking (1997).

Appendix 5.4. Images of mould isolated from rocket leaves at different storage and handling condition in the supply chain context on different plate agar.

1. *Fusarium equiseti* (KU361582)

MEA



CYA

GNA

(Colony reverse)

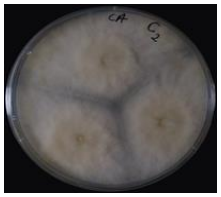
MEA

CYA

GNA

2. *Fusarium. equiseti* (KT362203)

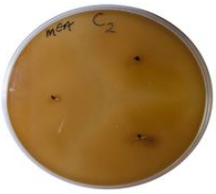
MEA



CYA

GNA

(Colony reverse)

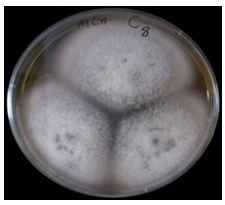


MEA

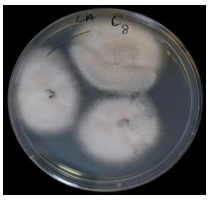
CYA

GNA

3. Alternaria infectoria (KX928829)



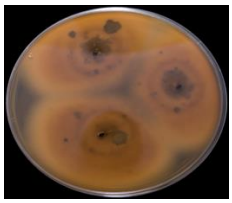
MEA



CYA

GNA

(Colony Reverse)

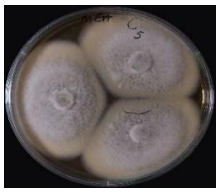


MEA

CYA

GNA

4. *Alternaria cesenica* (KP711383)



MEA

CYA

GNA

(Colony Reverse)

MEA

CYA

GNA

5. *Alternaria gasien* (JX391937)

MEA

CYA

GNA

(Colony Reverse)

MEA

CYA

GNA

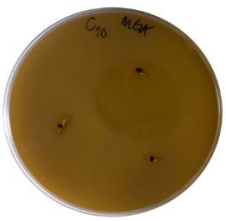
6. Rhizopus

MEA

CYA

GNA

(Colony reverse)



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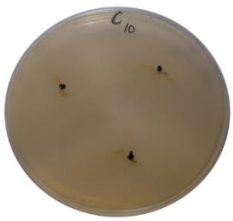


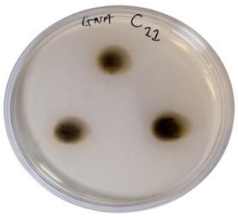
CYA

GNA

7. *Alternaria tenuissima* (KX196414)

MEA

CYA



GNA

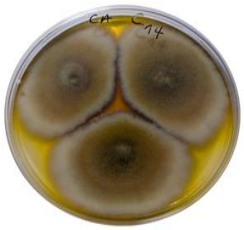
(Colony Reverse)

MEA

CYA

GNA

8. *Epicoccum nigrum* (KR909153)



MEA

CYA

GNA

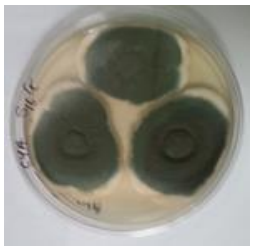
(Colony Reverse)

MEA

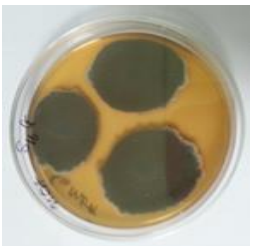
CYA

GNA

9. *Penicillium oxalicum* (LT558936)



MEA



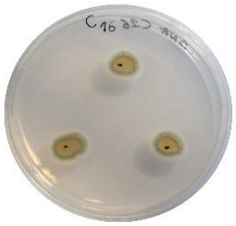
CYA

GNA

(Colony reverse)

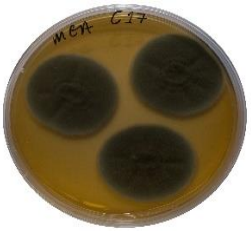
MEA

CYA



GNA

10. *Cladosporium cladosporioides* (KY369146)



MEA

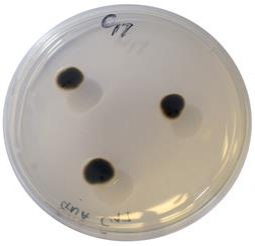
CYA

GNA

(Colony reverse)

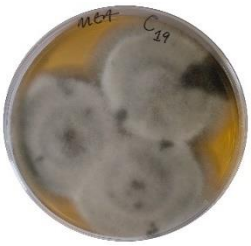
MEA

CYA



GNA

11. *Stemphylium vesicarium* (KX8322961)



MEA

CYA

GNA

(Colony reverse)

MEA

Not available

GNA

12. *Alternaria tenuissima* (KX065045)

MEA

CYA

GNA

(Colony reverse)

MEA

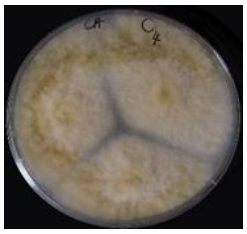
CYA

Not available

GNA

13. *Fusarium equiseti* (KR709055)

MEA



CYA

GNA

(Colony Reverse)

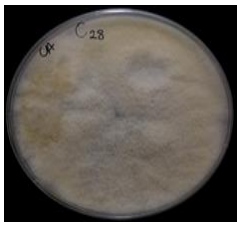
MEA

CYA

GNA

14. *Fusarium chlamydosporum* (KF998978)

MEA



CYA

GNA

(Colony reverse)

MEA

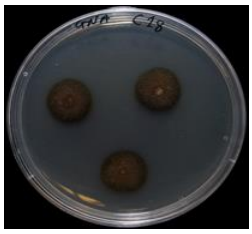
CYA

GNA

15. *Alternaria tenuissima* (KC568287)

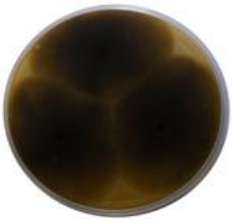
MEA

CYA



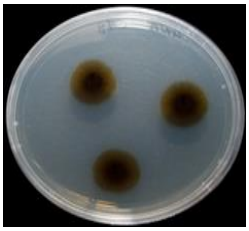
GNA

(Colony reverse)



MEA

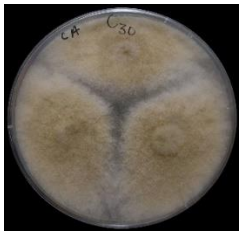
CYA



GNA

16. *Fusarium chlamydosporium* (KU878096)

MEA



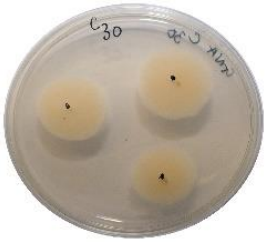
CYA

GNA

(Colony reverse)

MEA

CYA

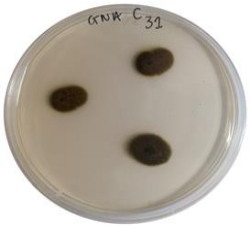


GNA

17. *Alternaria alternaria* (KF876820)

MEA

CYA

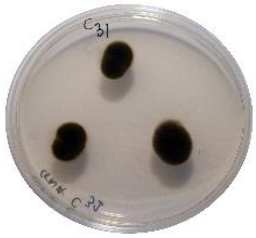


GNA

(Colony reverse)

MEA

CYA



GNA

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