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Improving the shelf-life of baby leafy salad vegetables

by

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Statements and declarations

Declaration of originality

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Vongai Dakwa conducted the experiments, data analysis and wrote the manuscript. Tom Ross, Alieta Eyles, Alistair Gracie and Mark Tamplin, contributed to the experimental design, interpretation of the results and editing of the manuscript.

Other manuscripts are in preparation, as follows:

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Based on data presented in Chapter 3

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Vongai Dakwa will be the primary author: she conducted the experimental work, data analysis and wrote the manuscript. Shane Powell provided advice and guidance on the analysis, graphical presentation and interpretation of the microbiome data as well and the editing of the manuscript. Alieta Eyles, Mark Tamplin, Tom Ross and Alistair Gracie contributed to the experimental design, shelf-life analysis, and editing of the manuscript.

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Abstract

Baby leafy salad vegetables have a limited shelf-life because they are fragile, have a high respiration rate, neutral pH, high a_w, and upon mechanical damage, release nutrients that support microbial growth. Sanitisation, handling (e.g. leading to damage), packaging systems, storage temperature, and high relative humidity (90-100%) are postharvest factors known to influence microbial loads and microbial growth potential and shelf-life. This thesis investigates how handling and processing of baby leafy salad vegetables influences shelf-life, quality and the composition of bacterial spoilage communities. This knowledge offers opportunities for longer shelf-life and, consequently, wider market access of leafy green vegetables.

Peroxyacetic acid (PAA) is a commercial organic sanitiser reported to extend shelf-life of some fresh produce including leafy salad vegetables. The effect of PAA sanitisation on the bacterial community of baby leafy salad vegetables during shelf-life has rarely been studied. Results showed that despite reducing total microbial load, PAA (80mg/L) did not influence bacterial diversity on intact baby spinach leaves on day-0 nor did washing with tap water only, and that spoilage bacteria were not eliminated, but were somewhat reduced. Sanitised baby spinach had lower bacterial diversity index (2.3) compared with water-washed leaves (2.8) at 4 °C storage. Relative abundance of *Pseudomonas* on PAA-treated intact (i.e. undamaged) baby spinach was >50% from day-6 until the end of shelf-life and was higher than in water-washed spinach. *Pseudomonas* ranged from 24-49% relative abundance from day-9 until the end of shelf-life on water-washed samples however, *Pantoea, Paenarthrobacter, Exiguobacterium,* and *Flavobacterium* were also prevalent. The shelf-life (23 d) of PAA-sanitised intact baby spinach was, similar to water-washed intact baby spinach. Thus, PAA treatment alone did not extend shelf-life of bagged baby-spinach leaves. Changes in microbiome composition did not appear to influence shelf-life either.

Mechanical damage (“bruising”) of leafy salad vegetables can occur during harvesting, transporting and processing, and is known to reduce shelf-life. However, the effect of ‘bruising’ (mechanical damage) on the bacterial community of leafy salad vegetables during shelf-life has not been rigorously explored. In this thesis, I studied the shelf-life and bacterial community of baby spinach of three ‘quality’ categories namely; 100% bruised leaves, 40% bruised + 60% intact (bruised + “intact”, i.e., undamaged leaves), and 100% intact leaves. All categories of leaves were sanitised with 80 mg/L PAA.

Bruising halved the shelf-life of baby spinach: intact leaves had a shelf-life of 23-d compared to 12-d for bruised or bruised + intact leaves. The relative abundance of *Pseudomonas, Sphingobacterium, Chryseobacterium Flavobacterium,* and *Janthinobacterium*, which are mostly recognised as spoilage bacteria, increased during shelf-life (days 1-15) on the bruised and ‘bruised + intact’ leaves. The bacterial diversity differences between the quality categories were not significant, though some differences in relative abundance of minor genera were observed. The bacterial community was dominated by *Pseudomonas* spp. and *Pantoea*, regardless of leaf quality and treatment, and similar to the observations in earlier experiments investigating the effects of sanitiser.

During commercial processing, most added water is removed from baby leafy salad vegetables after washing and partial sanitisation in disinfectant baths. Drying systems, are not completely efficient. Using leaves without surface moisture, this study demonstrated that addition of wash water as 1, 2 or 5 mL PAA (80 mg/L) to 60-g OPP bags (190 mm * 250 mm) of dry baby spinach leaves significantly reduced shelf-life. Two and five mL additions reduced shelf-life by 17 and 35%, respectively, in an initial trial (Trial 1). One mL added wash water reduced shelf-life by 13%, whereas 2 and 5 mL added wash water reduced shelf-life by 38% in a subsequent trial (Trial 2). Baby spinach leaves with no added wash water had the longest shelf-lives: 23-d in Trial 1 and 16-d in Trial 2 and retained normal quality attributes until the end of shelf-life.

The presence of grit reduces the eating quality of baby salad vegetables. The efficacy of a food grade anionic surfactant, sodium dodecyl sulphate (SDS), alone (0.025, 0.05, and 0.1% SDS), and in combination (0.05% SDS) with 40 mg/L PAA on grit removal, shelf-life, quality, and sensorial attributes of baby spinach was also studied. With SDS addition, grit levels were significantly reduced (21%) without reduction in quality attributes (colour, electrolyte leakage, visual quality and taste) nor reduction in shelf-life.

This research has provided new insights on postharvest factors that may influence the shelf-life and quality of leafy salad vegetables. Shelf-life studies demonstrated that excess wash water and bruising significantly reduced shelf-life of bagged baby spinach leaves. Although surfactants did not improve shelf-life, they could be used in industry to improve grit removal and product quality without compromising other quality attributes of baby spinach leaves or sanitiser efficacy. These studies also improved our understanding of the role of spoilage microorganisms in the shelf-life of leafy salad vegetables. Specifically, the survival and growth of the most dominant spoilage microorganism, *Pseudomonas*, was not affected by sanitiser treatment (PAA, 80 mg/L). Surprisingly, while bruising greatly reduced shelf-life, it did not influence bacterial diversity, although relative abundance of other spoilage microorganisms increased during storage. Future research should focus on optimising drying conditions for baby leafy salad vegetables and managing moisture accumulation in packages of leafy green vegetables during storage and distribution to extend shelf-life, and on reducing bruising during harvest and processing.

Explanatory note on thesis structure

This thesis contains a combination of peer reviewed publications, and articles undergoing peer-review or revision. Accordingly, some repetition may occur between chapters. Chapter 1 consists of a general introduction about the research topic and ends with the thesis objectives. Chapter 2 is the literature review which explores available information on factors

affecting the shelf-life of baby leafy focussing mainly on postharvest factors with emphasis on processing. Chapters 3 and 4 are experimental chapters written in the form of scientific publications and are being prepared for publication in the international refereed literature. Elements of Chapter 5 have already been published in the Journal of Food quality. Chapter 6 is a general discussion which ends with conclusions and recommendations for future work. The Appendices contains supplementary tables and figures and preliminary studies conducted as part of this research.

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List of abbreviations

AEW	acidified electrolysed water
ANOVA	One-way analysis of variance
BOPP	biaxially oriented polypropylene
ClO_2	chlorine dioxide
EOW	electrolyzed oxidizing water
FCC	free chlorine concentration
H_2O_2	hydrogen peroxide
HC	hydro cooling
HOCl	hypochlorous acid
LA	lactic acid
LDPE	low-density polyethylene
LeA	levulinic acid
MAP	modified atmosphere packaging
NaOCl	sodium hypochlorite
NEW	neutral electrolysed water
NNEW	near neutral electrolysed water
PAA	peroxyacetic acid
PP	polypropylene
SAEW	slightly acidified electrolysed water
SDS	sodium dodecyl sulfate
THM	trihalomethanes
TPC	total aerobic plate count
TW	tap water
UV	ultraviolet radiation
VC	vacuum cooling

Chapter 1: General introduction

1.1 Importance of leafy salad vegetables

Leafy salad vegetables are an important part of a healthy diet because they are highly nutritious, ready-to-eat (McMahon et al., 2013, Oms-Oliu and Soliva-Fortuny, 2010) and available throughout the year. In Australia, 54% of adults consume salad vegetables daily, with 41 % consuming them weekly (CSIRO, 2017). Baby leafy salad vegetables (Figure 1.1) contain ≥ 90% water but are rich in vitamins A, C, E and K, and minerals such as calcium, iron, phosphorous, potassium, magnesium, zinc and manganese and flavonoids, dietary fibre, folic acid (Bergquist et al., 2007, Butt and Sultan, 2011, Colonna et al., 2016, Hedges and Lister, 2005, Massa et al., 2015). A high consumption of vegetables lowers the risk of cardiovascular diseases (Rahal et al., 2014 , Wang et al., 2014) cancers, diabetes and other chronic diseases (Rahal et al., 2014). They are termed “baby leafy” salad vegetables because they are harvested at an early stage of growth and development, i.e., when they reach 50-120mm in height depending on leaf type (Saini et al., 2016). Garrido et al. (2015b) reported a growing cycle of 35 and 54 days, in spring and winter respectively in Spain, for baby spinach to reach commercial maturity stage.

Figure 1.1: Baby leafy salad vegetable mix consisting of baby spinach, baby lettuce (red and green), rocket and tatsoi

1.2 Leafy salad vegetable production in Australia

The production of leafy salad vegetables in Australia has increased by 15% since 2016-2018 (Table 1.1), while the fresh export volume has increased by 47% (Freshlogic, 2019). In the financial year 2017/2018, Australia exported leafy salad vegetables to Singapore (45%), Hong Kong (28%), Malaysia (5%), Indonesia (5%) and Thailand (4%) and 13% of the exports were to other countries (Freshlogic, 2019). The contribution to the production of leafy salad vegetables differs by Australian state: Victoria (45%), Queensland (28%), Tasmania (10%), South Australia (7%), New South Wales (7%), Western Australia (3%) for the year 2018/2019 (Freshlogic, 2019). During the year ending June 2019, 55% of households in Australia purchased leafy salad vegetables (Freshlogic, 2019).

The value of production for baby leafy salad vegetables in 2018 in Australia was $348.7 million and the value for fresh export was $9.4 million (Table 1.1) (Freshlogic, 2019). A longer shelf-life allows for a constant supply of fresh vegetables for the local and international market and increases profitability.

Table 1.1: Leafy salad vegetable production and export data in Australia for the year 2016-2018

	2016	**2017**	**2018**	**% change (2016-2018)**
Production (tonnes)	49,126	52,356	56,297	15%
Production ($ million)	271.9	304.3	348.7	28%
Fresh export volume (tonnes)	922	1, 313	1, 358	47%
Fresh export value ($ million)	5.1	8.4	9.4	84%

source: (Freshlogic, 2019)

1.3 Summary of factors influencing shelf-life of baby leafy salad vegetables and research considerations

Baby leafy salad vegetables have a high respiration rate (USDA, 2016) and their shelf-life mostly depends on storage temperature. Siomos and Koukounaras (2007) reported a shelf-life of 16 days at 0 °C, 13 days at 5 °C and 8 days at 10 °C for rocket. Mixed lettuce had a shelf-life of 9, 7, 5 and 3 days during storage at 2, 4, 7 and 10 °C respectively (Jacxsens et al., 2002). Produce properties including neutral pH, high water activity and initial microbial load, limit shelf-life (Brown et al., 2011, Rawat, 2015). The shelf-life of baby leafy salad vegetables is influenced by various preharvest and postharvest factors at different stages of production, processing, storage and distribution as illustrated in Figure 1.2. Baby leafy salad vegetables can be grown directly in the soil or in hydroponic systems (Sharma et al., 2018), however Manzocco et al. (2011), reported that hydroponically grown lettuce had a shorter shelf-life compared to soil cultivated lettuce. Contrastingly Lollo Rosso lettuce and Red Oak leaf lettuce grown in soilless system had better visual quality and higher vitamin C during shelf-life compared to soil grown lettuce (Selma et al., 2012).

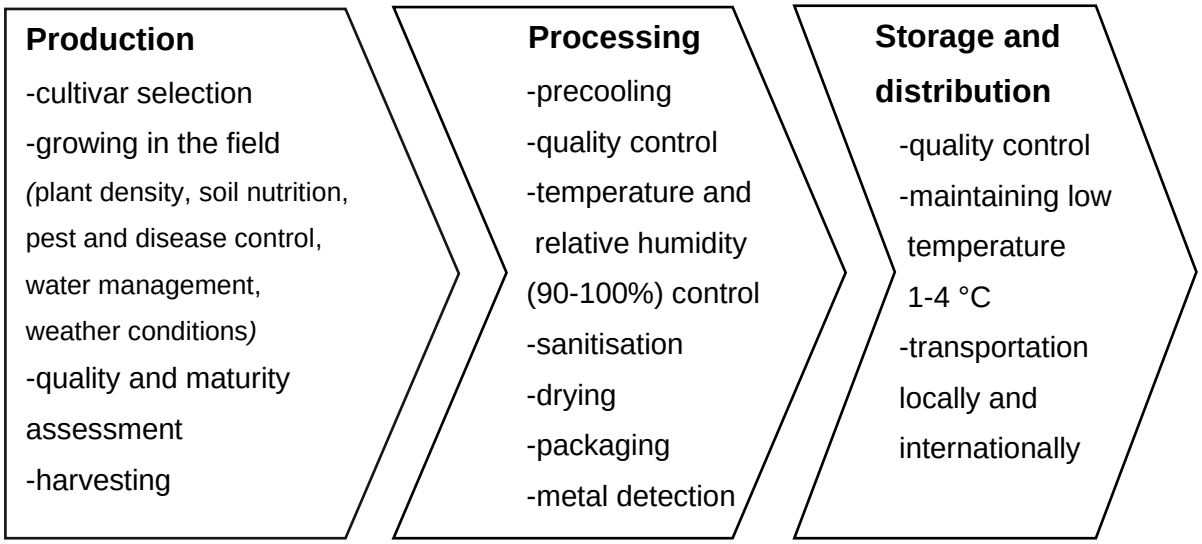

Figure 1.2: Summary of the factors that influence the shelf-life of baby leafy salad vegetables during production processing, storage and distribution (AHR, 2016, Gil, 2016 , Kou et al., 2014, Lee and Chandra, 2018, Manolopoulou et al., 2010, Marques, 2016, Nicola et al., 2006, Premier, 2013, Raju et al., 2011, Rodriguez-Hidalgo et al., 2010, Saini et al., 2016).

Although baby leafy salad vegetables can be manually harvested, they are generally mechanically harvested in large commercial operations (Twomey, 2006). Although these operations seek to minimise the impact on the leafy vegetables, physical damage ('bruising') can occur during harvesting, handling and processing leading to reduced shelf-life (Ariffin et al., 2017, Medina et al., 2012, Poonlarp et al., 2018). This leads to softening of vegetable tissue due to enzymatic action by pectinases such as polygalacturonase, pectin methyl esterase, pectic hydrolases, pectin lyases, pectate lyases (Barbagallo et al., 2009, Duvetter et al., 2008). After harvesting, baby leafy salad vegetables go through different processing steps within a food factory (Figure 1.2). The effect of bruising on the bacterial community of baby leafy salad vegetables has not been explored in detail.

Sanitisers such as chlorine, sodium hypochlorite (NaOCl), chlorine dioxide (ClO_2), electrolysed water, peroxyacetic acid (PAA), citric acid, lactic acid (LA), citric acid, acetic acid and nylate (Bachelli et al., 2013b, Lopez-Galvez et al., 2013, Nguyen et al., 2019, Premier, 2013) are used during processing to prevent cross contamination and reduce microbial load (Haute et al., 2015, Petri et al., 2015, Zhang et al., 2009). The effect of sanitisation on the changes in the bacterial community of baby leafy salad vegetables before and after wash and during shelf-life has received little attention. Gu et al. (2018) observed changes in relative abundance of bacterial species after washing baby spinach in chlorinated water and Lopez-Velasco et al. (2010) reported that disinfection with 12.5% (v/v) sodium hypochlorite for 10 min caused a decrease in bacteria species richness on ready-to-eat spinach. PAA is preferred over chlorine based sanitisers because it decomposes into environmentally friendly products namely; oxygen, water and acetic acid (Carrasco and Urrestaraz, 2010). Daddiego et al. (2018) reported differences in the distribution of bacterial populations/microbiome composition on pre-rinsed shredded lettuce treated with chlorinated water (20-30 mg/L) vs PAA (75 mg/L). There is also a need to understand the effect of sanitisation with PAA on the bacterial communities of baby leafy salad vegetables in

comparison to water-wash, after processing and during storage, which have yet to be examined, to be able to manipulate the microbiome to potentially, be able to extend the shelf life.

After sanitisation, produce undergoes drying steps during commercial processing. This often results in residual wash water on produce due to inefficient drying. Pirovani' et al. (2003) reported that centrifugation at 65.8 g-force resulted in less residual chlorine solution ≤ 1.5% on fresh-cut spinach. The amount of excess wash water had no influence on microbial growth and sensorial attributes including; colour and wilting during storage at 4 °C, although high excess wash water (≥24.6%) resulted in higher browning scores at the cut and damaged areas (Pirovani' et al., 2003). Nonetheless, the effect of residual wash water from other sanitisers on shelf-life and quality attributes of baby spinach still needs to be investigated.

A few studies have investigated the effect of sanitiser and surfactant treatment of leafy salad vegetables on microbial safety (Guan et al., 2010, Ho et al., 2011, Keskinen and Annous, 2011, Xiao et al., 2011, Zhao et al., 2009), however, the effect of the combination of sanitiser (PAA) and surfactant (SDS) on grit removal, shelf-life and other quality attributes has not been explored.

For continued industry success, productivity, and expansion (particularly into international markets) it will be important to develop innovative ways to extend the shelf-life of baby leafy salads to allow access to more distant markets as well as reducing waste and avoid economic loss.

Thesis objectives

This thesis focused on postharvest factors influencing shelf-life of baby leafy salad vegetables to explore opportunities for shelf life extension. The objectives were:

- to investigate the effect of peroxyacetic acid treatment and bruising on the microbial community on leaves to explore whether it strongly influences shelf-life of baby spinach (discussed in Chapter 3);
- to study the effect of different levels of residual wash water (peroxyacetic acid solution) on shelf-life and quality attributes of baby spinach during storage (discussed in Chapter 4);
- to explore the influence of combinations of sanitiser and surfactant treatment on grit removal, shelf-life and quality of baby spinach and coral lettuce (discussed in Chapter 5);

Chapter 2: Literature review

This literature review explores current knowledge on postharvest factors that influence the shelf-life of baby leafy salad vegetables. The postharvest period begins from just after harvest throughout the supply chain to the consumer (Irtwange, 2006). Shelf-life is the period when food remains safe to eat, and retains desired sensory, microbiological, physical and chemical characteristics and complies with labelling declarations (Institute of Food Science & Technology, 1993). At a given temperature, shelf-life is determined by the parameter which deteriorates fastest, in fresh-cut vegetables it can be growth of spoilage microorganisms, or sensorial spoilage due to chemical or biochemical reactions (Piagentini and Güemes, 2002).

2.1 Harvesting of baby leafy salad vegetables for optimal shelf-life

Baby leafy salad vegetables should be harvested at the optimum maturity stage, as this influences product quality, shelf-life, tolerance to processing and handling and profitability for producers (Ansah et al., 2018, Gil et al., 2012). Harvesting baby leafy salad vegetables early in the morning in summer and spring is recommended (Garrido et al., 2015b): spinach harvested at 6:00am had longer shelf-life by 3 days compared to spinach harvested at 9:00am or 12:00pm (Rogers, 2008). Garrido et al. (2015b) observed that mean photosynthetic and transpiration rates of baby spinach increased at midday corresponding to high temperature, radiation and water vapour pressure deficit resulting in a low relative water content and visual quality. In contrast, Clarkson et al. (2005) reported that the shelf-life of arugula rocket (*Eruca versicaria ssp. sativa*) and lollo rosso lettuce (*Lactuca sativa L "Rativa*") increased by 2-6 days when harvested at the end of the day (22:00 hrs) as compared to the morning 10:00 hrs, the improved shelf-life correlated with increased cell wall extensibility.

Mechanical damage ('bruising') can occur during harvesting and handling and can promote microbial growth at bruised surfaces, and increase the respiration rate and ethylene production thus reducing shelf-life (Kasso and Bekele, 2018, Opara and Pathare, 2014, Poonlarp et al., 2018, Thompson, 2003). Bruising is caused by compression, abrasion and

puncture damage and also results in cell damage, enzymatic oxidation and browning (Hodges et al., 2000, Li and Thomas, 2014). The severity of mechanical damage is influenced by leaf size, shape, maturity stage, leaf variety, season, texture, agronomic treatments, plant water status and the magnitude of exerted force (Ariffin et al., 2017, Opara, 2007). Medina et al. (2012) reported that the sanitisation process by washing resulted in an increase in the quantity of damaged leaves of baby spinach. To this candidate's knowledge, only two papers have studied the effect of bruising on shelf-life, produce loss and texture of leafy vegetables (Ariffin et al., 2017, Poonlarp et al., 2018). Poonlarp et al. (2018) reported that impact and compression damage occurred during packing and transportation of spinach from farm to pack house in plastic baskets. Minimising mechanical damage by avoiding hand pressure on produce, using foam boxes instead of plastic baskets and temperature management, gave three extra days of shelf-life for spinach (Poonlarp et al., 2018). Ariffin et al. (2017) observed that completely torn and half-torn ready-to-eat spinach leaves had a shelf-life of 8 days, whereas undamaged leaves and leaves with minor tears were still acceptable after 14 days of storage. The presence of cut/damaged leaves mixed with whole leaves within the same bag caused faster deterioration of all spinach leaves (Ariffin et al., 2017). Information is still lacking on the effect of bruising on the bacterial community of baby leafy salad vegetables during storage at low temperatures and whether that could provide opportunities to manipulate that community to extend product shelf life.

2.2 Influence of temperature and relative humidity on shelf-life

Cold chain management involves ensuring that the produce temperature is kept between 0-4 °C from after harvest (precooling), during storage, processing, transportation, retail to the point of consumption (Negi and Anand, 2015). After harvesting, leafy salad vegetables can be precooled to remove field heat by room cooling, forced air cooling, hydro cooling (HC) and vacuum cooling (VC) (Elansari et al., 2019, Ozturk and Ozturk, 2009). VC within 30 minutes of harvesting improved the shelf-life of lettuce by 3 days compared to VC after 2-4 hrs and forced air cooling within 30 mins (Rogers, 2008). Precooling methods can influence

leaf quality for example, Garrido et al. (2015a) reported that HC and VC reduced the respiration rate of baby spinach in spring and caused an increase in leaf water content in winter. Forced air cooling and VC reduced visual quality due to an increase in leaf damage (Garrido et al., 2015a). One concern of VC is the potential to influence food safety, VC increased the internalisation of *Escherichia. coli* O157:H7 in romaine lettuce (Li et al., 2008).

The shelf-life of baby leafy salad vegetables is highly dependent on storage temperature as high temperatures increase the rate of respiration, microbial growth, ethylene production, degradation of ascorbic acid and decrease in chlorophyll content (Irtwange, 2006, Jacxsens et al., 2002, Lopez-Velasco et al., 2010, Moreira et al., 2006, Zenoozian, 2011). Koukounaras et al. (2007) observed a shelf-life of 16, 13 and 8 days for rocket after storage at 0, 5 and 8 °C respectively. Baby leaf salad roquette/ arugula had a shelf-life of 11 days at 3 °C and 7 days at 11 °C, while baby lollo rosso lettuce had a shelf-life of 9 days at 3 °C and 6 days at 11 °C (Clarkson et al., 2005). Kou et al. (2014) observed that storing baby spinach at 1-4 °C gave a shelf-life of 18 days, whereas storage at > 8 °C caused faster deterioration of colour, membrane integrity and off-odour development.

Temperature abuse may occur along the supply chain during transportation and retail storage therefore, monitoring is important. Brown et al. (2016) reported that sensors in refrigerated truck trailers transporting bagged leafy greens recorded temperatures ranging from -0.7 - 8.1 °C with temperature abuse mainly occurring along the side walls. Poor temperature management can also occur in retail. For example, Nunes et al. (2009) reported temperatures of 1.1 to 19.2 °C in refrigerated display units for salad bags, 20% of produce loss was due to mechanical damage while 55% was caused by poor temperature management. Temperature fluctuations -0.8 to 6.5 °C in retail cabinets was shown to depend on defrost cycle interval, duration of defrost, thermostat setting and spatial location (i.e. position in retail display unit) of baby spinach bags (Kou et al., 2015).

Temperature management and relative humidity control are two of the key factors which determine the shelf-life of fresh produce. Moisture loss can occur due to high temperatures (>10 °C) and low relative humidity (≤90%) resulting in wilting and shrivelling and loss of saleable weight (Holcroft, 2015a). Medina et al. (2012) reported that storage of baby spinach at 15 °C for 36 hours at 72, 85 and 99 % relative humidity resulted in 19.7, 11.0 and 0.5% weight loss, respectively. The shelf-life of lettuce heads was reduced by 75% during storage at 0-2 °C at 70-72% relative humidity as compared to 95-98% (Agüero et al., 2011). Relative humidity of the atmosphere is not very important in bagged leafy salad vegetables since high relative humidity builds within the package.

2.3 Sanitisation of leafy salad vegetables

2.3.1 Importance of sanitisation

At a commercial scale, leafy salad vegetables pass through the conveyor belt and are sanitised by submersion with agitation, possibly followed by a rinsing step (Fig. 2.1) prior to drying. Sanitisation removes soil, dirt, debris, pesticide residues, cell exudates from cut surfaces and can extend shelf-life (Gil et al., 2010, Gil et al., 2009, Joshi et al., 2013, Premier, 2013, Qi et al., 2018, Siddiqui et al., 2011, Wang et al., 2019b).

Figure 2.1: Illustration of the sanitisation process of leafy salad vegetables for large scale processing. Symbols are explained as follows, green triangles ≈ leafy salad vegetables, grey rectangle ≈ conveyor belt, grey circles ≈ rotating rollers which cause movement of the conveyor belt, the pink rectangle ≈ wash bath containing sanitiser solution and lastly the water sprinkler is an optional rinsing step. (Allende et al., 2008b).

Sanitisation plays an important role in reducing microbial load on produce and maintaining the microbial quality of wash water, thus prevent cross-contamination (Petri et al., 2015, Tomás-Callejas et al., 2012, Zhang et al., 2009). For example, washing uninoculated cut iceberg lettuce for 1 min in artificially generated process wash water inoculated with 5.4 log CFU/g of *E. coli* for 1 min resulted in cross-contamination of the uninoculated lettuce pieces to 3.4 log CFU/g *E. coli* (Lopez-Galvez et al., 2010), thus emphasizing the need to use sanitisers. The ability of sanitisers to reduce microbial load differs as illustrated in Table 2.1. During storage and distribution microorganisms usually grow to levels exceeding initial values (Francis and O'Beirne, 2002, Gómez-López et al., 2013, Lopez-Velasco et al., 2010).

Leafy salad vegetables can be contaminated by pathogenic microorganisms along the supply chain from growing in the field, e.g., through faecal matter from domestic and wild animals, bird droppings, use of bovine and poultry manure, trash and contaminated irrigation water (FAO/WHO, 2008, Koukkidis and Freestone, 2019, Santos et al., 2010). Contamination may also occur through harvesting and processing equipment, wash water and poor hygiene/illness of workers (FAO/WHO, 2008, Koukkidis and Freestone, 2019, Santos et al., 2010). There have been many serious incidences of foodborne illness outbreaks that have been linked to the consumption of fresh produce including leafy salad vegetables globally as well as in Australia (Astridge et al., 2011, Carstens et al., 2019, Mercanoglu Taban and Halkman, 2011). In February 2016, *Salmonella* poisoning linked to the consumption of pre-packaged lettuce was reported in Victoria Australia, 54 people were diagnosed and two were hospitalised (ABCNews, 2016). *E. coli* food poisoning occurred in Britain in July 2016 linked to the consumption of mixed salad leaves containing rocket, 151 people were affected and 2 died (Meikle, 2016). Multi-state Listeriosis cases were reported from July 2015- January 2016 affecting 14 people in Canada and 19 in the US all associated with the consumption of packaged leafy green salads (Self et al., 2019).

Sanitisation in most cases can prevent cross-contamination of pathogenic bacteria when washing leafy salad vegetables. Jensen et al. (2015) demonstrated that the use of tap water only for washing caused cross-contamination of *E. coli* to uninoculated lettuce pieces. Similarly, tap water alone and ClO_2 (2 mg/L) did not prevent cross contamination of *E. coli* O157:H7 on cut iceberg lettuce whereas peroxyacetic acid (100 mg/L) and chlorinated water (65 mg/L) were effective (Petri et al., 2015). Tsunami 100 (PAA), Tsunami 200 (10-30 % PAA, 30-60 % (v/v) acetic acid, 10-30 % (v/v) octanoic acid, 4 % (v/v) hydrogen peroxide, 1-5 % (v/v) Peroxyoctanoic acid: Ecolab, USA) and NaOCl at 30 ppm for 90 sec prevented cross contamination of *E. coli* O157:H7 to uninoculated lettuce leaves unlike water wash (Zhang et al., 2009). In contrast, Tsunami 100 and Tsunami 200 at 50 ppm for 90 sec did not prevent cross-contamination of *E. coli* O157:H7 on lettuce (Davidson et al., 2017). ClO_2 at 3 mg/L treatment of red chard for 1 min prevented cross contamination of *E. coli* O157:H7 but not *Salmonella* (Tomás-Callejas et al., 2012).

2.3.2 Chlorine based sanitisers

Chlorine sanitisers, including NaOCl, ClO_2 and electrolysed water have been used for the sanitisation of produce for decades (Table 2.1). NaOCl generates hypochlorous acid (HOCl) in water, which is a strong oxidizing agent effective against microorganisms: when used within pH 6–7.5 (Artés et al., 2009, Gil et al., 2010). Several studies have shown that sanitisation of leafy salad vegetables with NaOCl at 30-200 ppm for 0.5-3 min effected a 0.8-2.5 log CFU/g decrease in pathogenic bacteria such as *L. innocua*, *E. coli* O157:H7, *E. coli* K-12, *Yersinia enterolitica* and *Salmonella* (Al-Nabulsi et al., 2014, Bermúdez-Aguirre and Barbosa-Cánovas, 2013, Ho et al., 2011, Keskinen et al., 2009, Kilonzo-Nthenge and Liu, 2019, Lopez-Galvez et al., 2009, Petri et al., 2015, Tomás-Callejas et al., 2012, Velázquez et al., 2009, Zhang et al., 2009). One study had a longer treatment time of 5 min (Al-Nabulsi et al., 2014), other studies are summarised in table 2.1.

Chlorine dioxide (ClO_2) is produced by the reaction of sodium chloride with acid or sodium chloride with chlorine gas (Ölmez and Kretzschmar, 2009). It is a powerful oxidant effective against bacteria and viruses (Artés et al., 2009, Joshi et al., 2013, Ölmez and Kretzschmar, 2009, Premier, 2013). ClO_2 acts by inhibiting metabolic function of the microorganism by entering through the cell wall (Joshi et al., 2013). It must be generated on site and is less reactive to organic matter (Gil et al., 2010). ClO_2 can be explosive and toxic at high concentrations (Praeger et al., 2018). Tomás-Callejas et al. (2012) reported that the concentration of ClO_2, decreased from 3 mg/L to 1 mg/L in 20 sec at 10-22 °C when added to water with turbidity of 160 Formazin Turbidity Unit (FAU) (Tomás-Callejas et al., 2012) whereas at 10 °C and 22 FAU ClO_2 concentration remained constant. ClO_2 treatment at 2-3 mg/L for 2 min resulted in 0.7-1.5 log CFU/g decrease in *E. coli* O157:H7 and *Salmonella* on lettuce and red chard.

Electrolysed water is produced by passing a current through a salt solution across a bipolar membrane (separating the anode and cathode), resulting in acidified electrolysed water (AEW) pH 2.3-2.7 with 1100-1150 mV Oxidation Reduction Potential (ORP) at the anode and alkaline electrolysed water with pH 11.4 and ORP of -795 mV ORP at the cathode due to NaOH production (Al-Haq and Gómez-López, 2012, Al-haq et al., 2005, Hati et al., 2012, Huang et al., 2008). Hypochlorous acid (HOCl) hydroxyl (OH-), hypochlorite ion (OCl^{-1}) and superoxide radicals (O2·-) are oxidisers with strong bactericidal effects which are produced in the process (Forghani and Oh, 2013, Hati et al., 2012, Huang et al., 2008, Pinto et al., 2016). Neutral Electrolysed water (NEW) can also be produced by electrolysis of a salt solution but in the absence of a membrane, it has a pH of 5–8.5 and ORP value of 500–700 mV (Al-haq et al., 2005, Ignat et al., 2016, Rahman et al., 2016).

AEW, slightly AEW (SAEW), low concentration electrolysed water, NEW and near NEW treatment resulted in 1-1.9 log CFU/g decrease in total bacterial count on leafy salad

vegetables (Forghani and Oh, 2013, Pinto et al., 2015, Zhao et al., 2019) as summarised in table 2.1. AEW, slightly AEW and low concentration AEW treatment at 5-50 mg/L FCC for 0.5-3 min caused 0.5-2.8 log CFU/g reduction of *E. coli* O157:H7 and *L monocytogenes* on leafy salad vegetables (Forghani and Oh, 2013, Keskinen et al., 2009, Pangloli and Hung, 2011, Rahman et al., 2010, Velázquez et al., 2009). Near NEW and AEW treatment at 100mg/L for 5 min resulted in 1.7-2.3 log CFU/g in *E. coli* O157:H7 and *L monocytogenes* on romaine lettuce (Singh et al., 2018). The available chlorine concentration (ACC) in electrolysed water decreases with time due to the decomposition of HOCl and volatilisation of Cl_2, ACC decline is slower under closed conditions and at lower temperature, 4 °C compared to 20 °C (Wang et al., 2019a).

Chlorine-based sanitisers are reported to react with organic matter to form trihalomethanes (THM) (Coroneo et al., 2017, López-Gálvez et al., 2010, Waters and Hung, 2014) which are potentially carcinogenic therefore, they are not permitted for use in some European countries (Ölmez and Kretzschmar, 2009). Gómez-López et al. (2013), reported that NaOCl and electrolysed oxidizing water (both 2-4 mg/L free chlorine) wash water had 194.0 and 50.2 µg/L of THM respectively. Baby spinach treated with NaOCl and electrolyzed oxidizing water (2-4 mg/L free chlorine) had $6.8\text{-}8.1*10^{-3}$ µg/g THM which is below legislative limits (80-100 µg/L) for drinking water for the European legislation and United States Environmental Protection Agency, after rinsing with water THM were undetectable (Gómez-López et al., 2013). López-Gálvez et al. (2010) reported that lettuce washed with sodium hypochlorite (100 mg/L)/ chlorine dioxide (3.7 mg/L) had < 5 µg/L of THM before and after the rinsing step. Therefore, it is important to consider the issue of THM or other chemical by-products when selecting sanitisers for shelf-life extension.

Table 2.1: Summary of studies on the sanitisation of leafy salad vegetables on microflora, leaf quality and shelf-life

Sanitiser and concentration	**Processing conditions**	**Leafy vegetable**	**Storage conditions**	**Antimicrobial effects on leafy vegetable**	**Other observations**	**Reference**
2000 ppm LA - 70 ppm PAA, NaOCl 15 ppm	30 sec, 4.4-7.2 °C	spinach and tender leaf mix	7 °C for 14-15-d	LA-PAA:1.5-1.7 log ↓, CW: 0.55 log ↓ in total aerobic plate count (TPC) on spinach and tender-leaf mix.	After storage, percentage of decayed leaves was 54% less for LA-PAA treated leaves than CW treated.	(Ho et al., 2011)
Acidified NaOCl 2–4 mg/L, PAA 80 mg/L, AEW 2-4 mg/L FCC, AEW 2-4 mg/L FCC + 1 g/L NaCl.	pH 5.6-6.8, 1 min, 7 °C, ratio 1:10	baby spinach	4 °C for 4-d in darkness followed by 7 °C for 7-d	PAA: 2.1 log ↓, NaOCl, AEW and AEW + NaCl: 1 log ↓ in psychrophilic bacteria, counts reached 8.1-8.6 log CFU/g for all treatments 11-d storage.	A decrease in visual quality was observed during shelf-life but no difference between treatments.	(Gómez-López et al., 2013)
Chriox 5 (4.6-5% PAA) at 0, 25, 80, 150 and 250 ppm	1, 5 and 10 min, 17 °C, ratio 1:10	cut iceberg lettuce		TPC: 0.4- 2.4 log ↓		(Vandekinderen et al., 2009)
ClO_2 3mg/L pH 7.1, NaOCl 100 mg/L	pH 6.5 - 7.1, 1 min then 1 min rinse, 4 °C	shredded iceberg lettuce	3-d 4 °C + 7-d at 8 °C	1.2-1.7 log ↓ in mesophilic bacteria, psychrophilic bacteria, *Pseudomonas* spp., *Enterobacteriaceae*, yeasts and moulds no differences between treatments.	Lettuce was still sensorially acceptable on day-10 however, LAB was higher for ClO_2 treated lettuce.	(López-Gálvez et al., 2010)
Prewashed for 1 min with TW, NaOCl 120 mg/L, acidified sodium chlorite (ASC) 100, 300, 500 mg/L, then TW rinse 1	NaOCl: pH 6.5, 120 sec ASC: pH 2.8, 60, 90, 120 sec,	tatsoi	8 °C for 11-d	ASC and NaOCl: 1 log ↓ in mesophilic bacteria, on day-11 similar counts between treatments.	Decrease in overall sensorial quality all samples still acceptable on day 11.	(Tomas-Callejas et al., 2012)

min	5 °C					
NaOCl 150 mg/L, NEW and AEW 30mg/L FCC	pH 6.5, 2 min, 8 °C, 1:10 ratio	lambs lettuce		1.0-1.5 log ↓ in total mesophilic count and *Pseudomonas* sp.		(Ignat et al., 2016)
Acidic electrolysed water (AEW) 4 mg/L FCC, levulinic acid (LeA, 3% v/v), AEW + LeA	pH 2.7 – 3.8 7 min, ratio 1:20	organic lettuce	7 °C for 7-d	1.02, 1.80 and 2.47 log ↓ in aerobic mesophilic counts by AEW, LeA and AEW + LeA respectively. A further 1.5 log ↓ during storage for AEW + LeA treated lettuce.	No differences in firmness and colour between treatments during storage, though electrolyte leakage was higher in AEW + LeA treated lettuce.	(Zhao et al., 2019)
SAEW 21-22 mg/L ACC, SAEW + ultrasonication (US) - water wash	pH 5.2-5.5, 3 min, 1:20 ratio, SAEW + US: 1 min	Spinach, lettuce		SAEW: 1.1-1.3 log ↓ in total bacterial count. SAEW + US: 2.1-2.4 log ↓ in total bacterial count		(Forghani and Oh, 2013)
sterile TW wash 2 min, then NEW 200 mg/L FCC	5 min, ratio 1:20, 5 °C	cut lettuce		1.86 - 1.91 log ↓ in mesophilic bacteria and Enterobacteriaceae	0.58-0.64 log ↓ during storage for 2-d at 4 °C.	(Pinto et al., 2015)
deionized water (DIW), low concentration electrolyzed water (LcEW) 5 mg/L ACC, strong acid electrolyzed water (SAEW) 50 mg/L ACC, aqueous ozone (AO) 5.2 mg O_3/L, 1% citric acid (CA) and NaOCl 100 mg/L available chlorine	pH: LcEW: 6.3, SAEW: .54, ozone: 6.6, CA: 2.6, NaOCl:10.6 3 min	spinach		0.47, 1.93, 1.94, 1.07, 1.39 and 1.61 log ↓ decrease in total bacteria after treatment with DIW, LcEW, SAEW, AO, 1% CA and NaOCl respectively.		(Rahman et al., 2010)

Irradiation doses (0.1 and 2.0 kGy)	22 °C and 55–60% relative humidity	spinach	4 °C, 90% RH, 30 d	0.1 kGy X-ray treatment resulted in 0.8 -1.4 log ↓ and 2.4-3.3 log ↑ during 30-d storage. 2.0 kGy treatment caused ~ 3.2-4 log ↓ in mesophilic bacteria, psychrotrophic bacteria, yeast and molds on spinach.	2.0 kGy treatment inhibited microbial growth for 6-12-d followed by 2.3-2.9 log ↑. X ray treatment did not influence spinach colour, both after treatment and during shelf-life.	(Mahmoud et al., 2010)
Ozonated water (2 ppm), chlorinated water (100 ppm), organic acid (0.25 g/100 g citric acid plus 0.50 g/100 g ascorbic acid), and cold water (control).	2 min, 1:20 ratio, 10 °C	Shredded green leaf lettuce	4 °C 12-d	Ozone, chlorinated water and organic acids gave 1.1-1.5 log ↓ in aerobic mesophilic bacteria, psychrotrophic bacteria and Enterobacteriaceae whereas water wash resulted in 0.5 log ↓ in aerobic mesophilic bacteria and psychrotrophic bacteria and no change in Enterobacteriaceae.	Good visual quality till day 7 for all treatments however control sample had inferior quality. Ozone treated lettuce had better visual quality on day-9 and retarded cut edge browning.	(Ölmez and Akbas, 2009)
Lactic acid (LA) 1 %, lemongrass oil (LO) 1%, LA 1% + LO 1%	5 min, 1:10 ratio	tatsoi	4 °C, 7-d	3.36-3.76, 3.47 and 4.98-5.18 log ↓ in aerobic mesophilic bacteria after treatment with, LA, LO and LA + LO respectively.	LA + LO treatment suppressed growth of aerobic mesophilic bacteria during storage.	(Jung and Song, 2015)
Essential oils: clove 10%, zataria 10%	0.8 mL/ 25g	baby leaf salad mix of lettuce spinach and	7 °C, 9-d	Zataria treated salad mix had 1-2.5 log lower total mesophilic bacteria during storage compared to the control,	Salad mix treated with essential oils maintained good visual quality for 7d,	(Azizkhani et al., 2013)

		rocket		whereas clove treated salad mix had similar counts to the control, 1.1 log ↓ occurred from day 7.	afterwards quality deteriorated.	
Oregano (essential oil) 250 ppm, 125 and 250 ppm oregano + thyme, respectively, 120 ppm chlorine		Cut iceberg lettuce	4 °C for 7-d	Essential oils were equally effective as chlorine against TVC, Enterobacteria and LAB.	Overall sensorial quality of lettuce washed with essential oils was unacceptable by day 7.	(Gutierrez et al., 2009)

2.3.3 Alternative organic sanitisers

2.3.3.1 Peroxyacetic acid

Peroxyacetic acid (PAA) is an aqueous quaternary equilibrium mixture comprising of hydrogen peroxide and acetic acid (Joshi et al., 2013, Ölmez and Kretzschmar, 2009, Premier, 2013) that is effective at temperatures as low as 2.2 °C. It is non foaming and decomposes into water, acetic acid and oxygen (Artés et al., 2009, Gawande et al., 2013, Premier, 2013). Its mechanism of action involves oxidation of bacterial cell contents by transfer of electrons through the cell wall and cell membrane (Gawande et al., 2013, Joshi et al., 2013). The efficacy of PAA (e.g. as Tsunami 200) at 30 ppm against *E. coli* O157:H7 on cut iceberg lettuce was not influenced by the presence of 10% organic matter whereas the efficacy of NaOCl (30 ppm) was significantly reduced (Zhang et al., 2009). Similarly, Davidson et al. (2017) reported that an organic load of 2.5-10% (wt/vol) did not influence the efficacy or PAA (as Tsunami 100) and mixed peracetic acid (as Tsunami 200) at 50 ppm against E. coli O157:H7 on iceberg lettuce.

PAA at 80 mg/L is often used commercially for sanitisation, and is preferred because of the challenges associated with the use of chlorine sanitisers mentioned earlier. PAA treatment at 30-80 ppm for 0.5-5 min resulted in 0.93-2.5 log CFU/g decrease in *E. coli* O157:H7, *E. coli* K-12, *Listeria innocua* and *Listeria monocytogenes* on inoculated leafy salad vegetables (Al-Nabulsi et al., 2014, Davidson et al., 2013, Davidson et al., 2017, Ho et al., 2011, Petri et al., 2015, Singh et al., 2018). Most studies focused on PAA efficacy on pathogenic microorganisms on leafy salad vegetables and a few on endogenous microflora and shelf-life (Table 2.1).

2.3.3.2 Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is produced by passing an electric discharge through a mixture of oxygen, hydrogen, and water vapor or electric oxidation of sulphuric acid (Ali et al., 2018). H_2O_2 is a strong oxidizing agent which has bactericidal and bacteriostatic activity, it is

effective at pH 6-10 (Artés et al., 2009, Joshi et al., 2013, Ölmez and Kretzschmar, 2009) H_2O_2 can be decomposed by the enzyme catalase into oxygen and water, (Joshi et al., 2013, Ölmez and Kretzschmar, 2009). 3% H_2O_2 treatment for 1 and 2 min gave 3.2 and 3.3 reductions of *E. coli* O157:H7, respectively on inoculated baby spinach (Litt et al., 2017). Huang and Chen (2011) reported 1.1 and 1.5 log CFU/g decrease in *E. coli* O157:H7 on inoculated baby spinach after treatment with 1% and 2% H_2O_2, respectively.

2.3.3.3 Other organic acids

Various organic acid treatments have been trialled in leafy salad vegetables including: LA, citric acid, malic acid, levulinic acid, caprylic acid, propionic acid, ascorbic acid and tartaric acid (Akbas and Olmez, 2007, Ferrante et al., 2004, Finten et al., 2017, Francis and O'Beirne, 2002, Ho et al., 2011, Huang and Chen, 2011, Jung and Song, 2015, Park et al., 2011, Singh et al., 2018, Velázquez et al., 2009, Zhao et al., 2019, Zhao et al., 2009). Most studies focused on the efficacy of organic acids sanitisers on food safety and a few on shelf life and microbiota (see table 2.1).

Organic acids have been shown to reduce the microbial load of pathogenic microorganisms. Finten et al. (2017) reported 2.3 - 3.1 log CFU/g reduction in *E.coli* and *L. innocua* on inoculated spinach after treatment with 0.5% citric acid (pH 2.3) for 2.5 min, whereas no growth was observed for *E. coli* during storage at 6.5 °C for 9 d, and a slight increase in *L innocua* was observed: however, log counts remained lower than the control. Akbas and Olmez (2007) found that lactic acid, citric acid, acetic acid, ascorbic acid all at 0.5% and NaOCl (100 mg/L free chlorine) for 2 min resulted in 1.9, 2.0, 1.3, 1.0 and 2.0 log CFU/g decrease in *E. coli* on cut iceberg lettuce respectively and 1.5, 0.9, 0.8, 1.0 and 1.0 log CFU/g decreases in *L. monocytogenes,* respectively. Conversely, citric acid treatment 0.5, 1.0 and 1.5 % (0,3, 6, 9, 12 and 15 min) had no influence on *E. coli* ATCC 11775 inoculated on cut romaine lettuce (Bermúdez-Aguirre and Barbosa-Cánovas, 2013). Organic acids

could potentially be used to sanitise organic produce as an alternative to chemical sanitisers (Park et al., 2011).

2.3.3.4 Essential oils

Some essential oils can be used for sanitisation and are produced by steam distillation of dried samples (Azizkhani et al., 2013, de Medeiros Barbosa et al., 2016, Mouatcho et al., 2017). Examples of essential oils that have been trialled in leafy salad vegetables include sage (*Salvia dolomitica*), thyme (*Thymus vulgaris*) tea tree (*Melaleuca alternifolia*), and ylang-ylang (*Cananga odorato*) oregano (*Origanum compactum*), oregano oil (*Oreganum onites*), clove (*Syzygium aromaticum*), rosemary (*Rosmarinus officinalis L.*), zataria (*Zataria multiflora Boiss*), myrtle leaves oil (*Myrtus communis L.*), mint (*Mentha*), basil (*Ocimum basilicum*) (Azizkhani et al., 2013, de Medeiros Barbosa et al., 2016, Gunduz et al., 2009, Gündüz et al., 2010, Gutierrez et al., 2009, Jung and Song, 2015, Karagözlü et al., 2011, Mouatcho et al., 2017, Ponce et al., 2011).

Essential oils have been shown to have strong antibacterial properties against pathogenic microorganisms during shelf-life. Treatment of loose lettuce leaves with 3% tea tree oil and 3% thyme oil separately and 1.5% tea tree oil + 1.5% thyme oil for 5 min gave 4.01, 4.01 and 6.09 log reduction of *E. coli* O157:H7 respectively, after 5 days of storage at 10 °C (Mouatcho et al., 2017). Treatment of baby leaf salad mix of lettuce spinach and rocket with essential oils (0.8ml/ 25g), 10% clove oil resulted in 2.8 log cfu/g reduction of *E. coli* O157:H7 in 7d at 7 ºC followed by slight growth for 2d, whereas treatment with 10% zataria oil gave 3.5 log CFU/g reduction of E. coli O157:H7 in 5d at 7 ºC and maintained this till the end of storage (Azizkhani et al., 2013). Treatment of shredded iceberg lettuce with 750 ppm myrtle leaf oil resulted in 1.42 log CFU/g reduction of *Salmonella Typhimurium (Gunduz et al., 2009).* 75 ppm oregano oil treatment was as equally effective as 50 ppm chlorine treatment against *Salmonella Typhimurium* on shredded lettuce (Gündüz et al., 2010).

Essential oils treatment also influences sensorial properties of leafy salad vegetables. Ponce et al. (2011) reported that romaine lettuce leaves treated with essential oils solutions extracted from clove, tea tree or rosemary at 1 minimum inhibitory concentration (MIC) by immersion had unacceptable sensorial quality in terms of texture, overall visual quality, browning and flavour by day-2 at 8 °C. On day-7 control (untreated) samples had unacceptable sensorial quality whereas lettuce treated with essential oils by spraying still had acceptable organoleptic properties (Ponce et al., 2011). Treatment of lettuce and spinach leaves with oregano essential oil resulted in darkening of spinach and yellowing of lettuce, this was confirmed by changes in L*, a*, b* parameters after 7-d storage at 5 °C (Poimenidou et al., 2016). Oregano + carvacrol at 120 mg/L treatment of lambs lettuce caused a decrease in colour L*, a*, b* parameters after 5-d storage at 6 °C compared to lettuce treated with 120 mg/L chlorine solution (Siroli et al., 2015).

2.3.3.5 Ozone (O3)

Ozone is generated by using ultraviolet radiation (188 nm wavelength) and corona discharge to split the oxygen molecule (O_2) into singlet oxygen which then reacts with O_2 (Guzel-Seydim et al., 2004, Ölmez and Akbas, 2009) to form O_3. Ozone must be generated onsite, since it is unstable at ambient temperature and pressure, having a half-life of 20-50 min (Wang et al., 2019). It is corrosive at concentrations > 4 ppm, can be toxic if inhaled and can cause stimulation of the respiratory system and mucous tissue of the eyes (Ölmez and Kretzschmar, 2009, Wang et al., 2019). Ozone in solution (aqueous ozone) decomposes into hydroxyl (HO^-), hydroperoxy ($\cdot HO_2$) and superoxide radicals ($\cdot O^{2-}$) radicals which have strong oxidising and power attack the DNA and RNA of bacterial cells, or sulfhydryl groups and amino acids of enzymes and proteins or oxidise polyunsaturated fatty acids to peroxides (Joshi et al., 2013, Guzel-Seydim et al., 2004). Ozone does not leave any residue on food since it decomposes into oxygen (Joshi et al., 2013, Candia et al., 2015, Wang et al., 2019). Treatment of wild rocket with aqueous ozone (10 mg L^{-1} total dose) for 1 min resulted in 1.8 log CFU/g reduction in mesophilic bacteria and did not influence the

respiration rate (Martínez-Sánchez et al., 2008). Other studies on ozone treatment of leafy salad vegetables are summarised in Table 2.1. The shelf-life of baby leaf samples treated with 0.5 ppm ozone during storage at 4 and 10 °C was similar to untreated samples however samples treated with 2 ppm ozone had unacceptable visual quality by day 3 (Candia et al., 2015).

2.3.4 Factors influencing choice and efficacy of sanitisers

The efficacy of the sanitisation process is influenced by various factors including produce to water ratio, pH, temperature, application method (spraying, dipping and agitation), concentration and contact time of the sanitiser, characteristics of the vegetable, initial bacterial load, physiological state of bacteria and whether the microorganisms on the product samples are naturally occurring or inoculated (Artés et al., 2009, Davidson et al., 2013, Gil et al., 2010, Gil et al., 2009, Gomez-Lopez et al., 2008, Ho et al., 2011, Huang and Chen, 2011, Keskinen et al., 2009, Luo, 2007, Ölmez and Akbas, 2009, Park et al., 2011, Pinto et al., 2015, Ponce et al., 2011, Singh et al., 2018, Vandekinderen et al., 2009, Velázquez et al., 2009). Aerosolised 40 ppm PAA treatments for 10, 30, and 60 min gave 0.8, 2.2, and 3.4 log CFU/g reductions of *E. coli* O157:H7, 0.3, 3.3, and 4.5 log CFU/g of *S. typhimurium* and 2.5, 2.7, and 3.8 log reduction of *L. monocytogenes*, respectively, on cut iceberg lettuce (Oh et al., 2005). Use of aerosolised sanitisers is impractical due to the sophisticated equipment required, exceptionally long treatment time and because process parameters must be optimised before use, though they could have higher penetrating power than aqueous sanitisers (Oh et al., 2005). The presence of soil particles can influence both bacterial attachment and removal from produce surfaces (Huang and Nitin, 2017).

Other factors to consider when selecting an ideal sanitiser are cost, regulatory approval, complexity, maintenance, corrosiveness, monitoring, logistics, environmental aspects, safety (*gaseous chemicals, danger of irradiation, electric shock*) and storage stability (Ali et al., 2018, Artés et al., 2009, Haute et al., 2015). Sanitisers should not have a negative effect on

produce quality (Guan et al., 2010). For example, reuse of sodium hypochlorite wash water causes a decrease in chlorine concentration and an increase in biological oxygen demand of the wash water, odour development and higher microbial populations in cut romaine lettuce (Luo, 2007).

2.3.5 Bacterial community of leafy salad vegetables

The microbial quality can be assessed by the conventional method of microbial enumeration, MALDI-TOF mass spectrometry (Frohling et al., 2018, Hausdorf et al., 2013) and culture-independent methods namely, fingerprinting techniques such as DGGE, TGGE and T-RFLP (Daddiego et al., 2018, Di Carli et al., 2016, Frohling et al., 2018, Lopez-Velasco et al., 2010, Nubling et al., 2016, Randazzo et al., 2009), and high throughput sequencing of the 16S rRNA gene for profiling bacterial communities (Ioannidis et al., 2018, Jackson et al., 2013, Leff and Fierer, 2013, Truchado et al., 2018). Sequencing provides detailed information on specific bacteria present as compared to conventional enumeration methods however, it is expensive, sample preparation data processing and analysis is complicated, time consuming and it does not differentiate between live and dead bacteria (Gorni et al., 2015, Hamady and Knight, 2009, Knight et al., 2018).

Table 2.2 gives a summary of studies on the effect of washing/ sanitisation, storage conditions and packaging on the bacterial communities of leafy salad vegetables. Only one study carried out by Daddiego et al. (2018) has compared the microbiome of lettuce treated with chlorinated water (20-30 mg/L free chlorine) vs lettuce treated with 75 mg/L PAA and the results showed differences in the microbiota. Gu et al. (2019) examined the bacterial community of spinach and lettuce rinse water containing 0.5-30 mg/L free chlorine and 0.5-50 mg/L PAA, results showed two distinct microbiomes especially at lower sanitiser concentrations. The bacterial community structure of baby spinach rinsed with sterile tap water vs disinfection with 12.5 % (v/v) NaOCl for 10 min stored at the same temperature had 30-40% similarity (Lopez-Velasco et al., 2010). Leafy salad vegetables harbour a wide range

of bacteria which include spoilage bacteria and in some cases pathogenic bacteria, see Appendix B.

Table 2.2: Studies on the bacterial community of leafy salad vegetables

Method	**Leafy vegetable**	**Conclusions**	**References**
Matrix-assisted laser desorption/ionization time-of light mass spectrometry (MALDI-TOF MS), terminal restriction fragment length polymorphism (TRFLP) analysis and 16S rRNA gene nucleotide sequence analysis	Endive lettuce during the processing line from raw material, cutting, washing, and spin-drying	Changes in the relative abundances of bacterial families and changes in the bacterial community occurred along the processing line	(Frohling et al., 2018)
16S rRNA gene amplification and T-RFLP analysis	Lettuce washed with chlorine solution 20-30 mg/L chlorine concentration vs 75 mg/L PAA	Differences in microbiota composition between chlorine treated vs PAA treated lettuce	(Daddiego et al., 2018)
MALDI-TOF MS 16S rRNAgene sequence analysis	Spinach washed in water 3 times consecutively	Bacterial diversity on spinach increased after the first wash then decreased with subsequent washing	(Hausdorf et al., 2013)
DNA extraction, 16S rRNA gene amplification, illumine sequencing	Baby spinach irrigated with ClO_2 100 ppm vs water only	No differences in bacterial diversity between treated vs untreated though, the relative abundance of some bacterial genera decreased	(Truchado et al., 2018)

DNA extraction and sequencing	Rocket and sliced RTE spinach washed with water vs 1% vinegar	The bacterial composition and diversity did not change after washing with vinegar or water for both spinach and rocket. The bacterial community composition of rocket was different from spinach	(Tatsika et al., 2019)
16S rDNA amplicon sequencing	Baby spinach grown in Arizona and California at different time periods	Bacterial diversity on spinach from California decreased after washing with chlorinated water. The relative abundance of some bacterial species changed after washing with chlorinated water and during storage at 4, 10 and 15 °C.	(Gu et al., 2018)
Pyrosequencing of the bacterial 16S rRNA gene	Baby spinach, romaine lettuce, green leaf lettuce, iceberg lettuce and red leaf lettuce	No differences in bacterial community composition between sterilised (with NaOCl and ethanol) vs unsterilised and conventionally grown vs organic farmed leafy vegetables.	(Jackson et al., 2013)
16 s rRNA gene amplicon sequencing analysis	Iceberg lettuce	Bacterial community profile of lettuce packaged under equilibrium modified atmosphere packaging and air was different from that of lettuce packaged under anaerobic conditions	(Ioannidis et al., 2018)
pyrosequencing of 16S rRNA amplicons	Baby spinach	Prolonged storage at 4 and 10 °C for 15 days resulted in a decrease in evenness, richness and diversity	(Lopez-Velasco et al., 2011)

DNA extraction, T-RFLP profiling of bacterial 16S rRNA	Lettuce	Type of packaging used and storage time at 8 °C influenced the bacterial community	(Di Carli et al., 2016)
Microbial profiling with illumina Miseq	Rocket lettuce	The bacterial community of lettuce and rocket at harvest was different. Leaf maturity and season also influenced bacterial community composition.	(Dees et al., 2015)

2.3.6 Innovative postharvest technologies influencing shelf-life

Physical treatments such as ultrasound, ultraviolet radiation (UV), ultrasonication and irradiation have been trialled for decontamination of leafy vegetables (Niemira, 2008, Bilek and Turantaş, 2013, Salgado et al., 2014, Neal et al., 2010, Petri et al., 2015, Escalona et al., 2010, Artés-Hernández et al., 2009, Martínez-Sánchez et al., 2008, Mahmoud et al., 2010, Huang et al., 2006). Despite a positive antimicrobial effect (Forghani and Oh, 2013, Mahmoud et al., 2010, Niemira, 2008), these treatments sometimes had negative effects on quality and structure of leafy vegetables. For example, ultraviolet light (UV-C) treatment (fluence 1.6 mW/ cm^2, at 31 cm working distance, 60 min) gave a 1.7 log reduction of *E. coli* however, it caused browning of lettuce, while ozone (5 ppm, 15 min) treatment resulted in loss of greenness of lettuce (Bermúdez-Aguirre and Barbosa-Cánovas, 2013). Application of positive (3 bar) and vacuum pressure (10 mbar) did not improve the efficacy of PAA (100 mg/L), ClO_2 (2 mg/L), chlorinated water (65 mg/L) and tap water treatment on cut iceberg lettuce (Petri et al., 2015). Instead, vacuum pressure resulted in removal of moisture and an increase in porosity while vacuum / negative pressure caused leaf tissue damage and changes in colour parameters (Petri et al., 2015). Lettuce treated with ultrasound (26kHz, 90µm, 200W,5 min) had lower sensorial scores, damaged structure and surface browning during shelf-life compared to untreated lettuce (Neto et al., 2019). Irradiation, UV, ultrasound

and ultrasonication are not commonly used for commercial leafy vegetable processing due to cost, complexity, legislation and safety issues.

Ethylene is a gaseous plant hormone which causes senescence and yellowing of leafy vegetables (Martínez-Romero et al., 2007, Saltveit, 1999) and therefore it may be important to manage ethylene in stored produce by the use of ethylene inhibitors and absorbers. Baby leafy salad vegetables are low producers of ethylene. Spinardi et al. (2010) reported values ranging from 1.67- 1.02 µl L^{-1} for lettuce and 0.11-0.02 µl L^{-1} for baby spinach during shelf-life. Treatment of rocket leaves with 0.5 µL/L 1-methylcyclopropene (1-MCP, SmartFresh™) (Agrofresh, 2019), an inhibitor of ethylene action, for 4 h at 10 °C before storage prevented yellowing (Koukounaras et al., 2006). Gergoff Grozeff et al. (2010) reported that treatment of spinach with 1.0 µL/L1-MCP (Smart Fresh^{SM}) delayed senescence. Treatment of shredded lettuce with 0.1 µL/L 1-MCP for 1 h increased its shelf-life by 50% (Wills et al., 2002).

2.3.7 Surfactants

Surfactants are surface active compounds which have an amphiphilic structure consisting of a hydrophobic and hydrophilic group (Castro et al., 2013, Singh et al., 2007) which can be used for washing leafy salad vegetables. Huang and Nitin (2017) showed that 0.1% sodium dodecyl sulfate (SDS), 0.1% Tween-20, and 0.1% lauric arginate (LAE) lowered the surface tension of water from 71.17 mN/m to 46.67, 36 and 36 mN/m respectively. Bioluminescence images of inoculated cut romaine lettuce showed the amount of bacteria adhering to the leaf surface decreased after treatment with the surfactants (Huang and Nitin, 2017). Table 2.3 contains a summary of studies on the effect of surfactant treatments on bacteria on leafy salad vegetables.

The combination of surfactant + sanitiser/ acid on microbial loads on leafy salad vegetables is variable. Predmore and Li (2011) reported that surfactants sodium dodecyl sulfate (SDS), Nonidet P-40 (NP-40), Triton X-100 at 50 ppm + chlorine solution 200 ppm improved the efficacy of chlorine sanitiser against MNV-1 on inoculated cut romaine lettuce by 1 log

PFU/ml. In contrast, Keskinen and Annous (2011) reported that the addition of surfactants 0.2% dodecylbenzenesulfonic acid (w/v) or 0.2% sodium 2-ethyl hexyl sulfate (v/v) did not improve the efficacy of ClO2 (100 ppm) or chlorine solution (100 and 200 ppm) against *E. coli* O157:H7 on cut romaine lettuce. The addition of 200-250 ppm sodium lauryl sulphate did not improve the efficacy of 4500 pmm lactic acid + 70 ppm PAA against *L. innocua* and *E. coli* K-12 on inoculated romaine lettuce and spinach and excessive foaming was observed (Ho et al., 2011). Increasing the concentration of levulinic aid from 0.5- 3% + 0.05% SDS (w/v) resulted in a decrease in texture, visual quality and an increase in sogginess in cut iceberg lettuce, samples were unacceptable by day 7 at 4 °C, however sodium acid sulfate + SDS treatment inhibited cut edge browning (Guan et al., 2010). Salgado et al. (2014) reported that PAA (80 mg/L) + 1 g/L SDS + ultrasound treatment gave similar log reductions to NaOCl (100 mg/L) + ultrasound and did not cause changes in colour and electrolyte leakage during shelf-life. Based on the advantages of PAA treatment mentioned earlier it would be of interest to explore the effect of PAA + surfactants only on microflora, quality and shelf-life of baby leafy salad vegetables.

Table 2.3: Surfactant studies on leafy salad vegetables

Surfactant and treatment conditions	**Leafy salad vegetable**	**Antimicrobial effects on leafy salad vegetables**	**Other observations**	**Reference**
Tween-20, sodium dodecyl sulfate (SDS), and lauric arginate (LAE), deionised water each at 0.1% w/w 20 min with 200rpm rotation	Cut romaine lettuce	Deionised water: 1.47 and 0.84 log ↓ Tween 20: 1.97 and 1.34 log ↓ SDS: 1.47 and 1.79 log ↓ LAE: 2.2 & 2.2 log ↓ in *E. coli* O157:H7-lux *L. innocua* respectively Surfactants were not effective against bacteria T7 phages virus	Surfactant treatment did not affect colour and electrolyte leakage but texture Loss in turgor after surfactant treatment	(Huang and Nitin, 2017)
sucrose monolaurate (SML) 0, 100, 250, or 10,000 ppm SML, 200 ppm NaOCl, ph 6, 3 min	Baby spinach	SML: 1.6-2.5 log ↓ NaOCl: 3.3 log ↓ NaOCL + 250 ppm SML 3.8 log ↓ NaOCL + 10,000 ppm SML: 4.3 log ↓ in E. coli O157:H7	SML at 250 and 10 000 ppm improved the efficacy of NaOCl against *E. coli* O157:H7	(Xiao et al., 2011)
0.3% levulinic acid + 0.05% SDS 0.5% levulinic acid + 0.05% SDS 0.5% levulinic acid + 0.05% SDS	Cut romaine lettuce	4.7 log ↓ in *Salmonella Enteridis* 4.5 log ↓ in *Salmonella Typhi* 4.2 log CFU/g ↓ in E.coli O157:H7		(Zhao et al., 2009)

3% levulinic acid + 1% SDS 2 min		7 log CFU/g ↓ in *Salmonellla Typhi* and *E. coli* O157:H7		
0.1% sodium lauryl sulfate (SLS) 0.1% tween 80 at 22 and 40 °C, 2 min	Green leaf lettuce	Salmonella 4.1-4.2 log ↓ Shigella 2.2-3.1 log ↓		(Raiden et al., 2003)
sodium hypochlorite (final chlorine concentration 0.01% (w/v) at pH 6.5, FIT 0.25% citric acid 0.5% Levulinic acid + 0.05% SDS 0.25% Sodium acid sulfate (SAS) + 0.05% SDS 5 min 1:5	Cut iceberg lettuce	NaOCl: 0.94 log ↓ FIT: 0.58 log ↓ LeA + SDS: 0.41 ↓ SAS + SDS: 0.87 log ↓ in *E. coli* O157:H7		(Guan et al., 2010)

2.4 Drying of leafy salad vegetables following the washing process

Excess wash water on the surface of bagged baby leafy salad vegetables after washing/ sanitisation creates an environment favourable for the growth of spoilage microorganisms during distribution and storage (Kader, 2013) and is therefore removed. This is achieved by centrifugation/spin drying immediately after washing (Davidson et al., 2013, Davidson et al., 2017) although it can cause bruising of leaves (Pirovani' et al., 2003). Infrared drying, forced air tunnel drying and vibration screens over a conveyor belt are also applied in some processing plants (Cantwell and Suslow, 2002, Moses et al., 2014). In most laboratory-based experimental studies, excess surface moisture after washing/sanitation is removed by the use of a salad spinner (Al-Nabulsi et al., 2014, Gómez-López et al., 2013, López-Gálvez et al., 2010, Lopez-Velasco et al., 2010, Medina et al., 2012, Rodriguez-Hidalgo et al., 2010), but a few studies have used paper towels (Randhawa et al., 2007), draining and passive air drying in a controlled environment (Oliveira et al., 2016).

The quantification of how the level of residual moisture affects shelf and quality of baby leafy salad vegetables has rarely been explored. Pirovani' et al. (2003) reported that use of higher centrifugal speed (≥ 39.2 g-force) when drying fresh-cut spinach resulted in less residual surface moisture (chlorinated water) 0.15-1.48%. The level of residual surface moisture (0.15% - 31.17%) did not influence microbial growth or sensorial quality except browning during shelf-life at 4 °C however, washing conditions were vigorous and longer (7.5 min + shaking 60 time/min) (Pirovani' et al., 2003). Thus, there is need for further, more systematic, research in this area.

2.5 Packaging

Packaging has a very important role in maintaining produce quality, convenience, ease of handling and distribution (Dainelli et al., 2008). The type of packaging films such as polypropylene (PP), polyethylene, bi-oriented PP (BOPP) and low-density polyethylene (LDPE), (Inestroza-Lizardo et al., 2016, Kaur et al., 2011, Lee and Chandra, 2018, Tomás-

Callejas et al., 2011, ViŠKelis et al., 2015) as modified by thickness and gas permeability (Islam et al., 2019, Kaur et al., 2011, Rodriguez-Hidalgo et al., 2010, ViŠKelis et al., 2015) have been shown to greatly influence the shelf-life of leafy salad vegetables (Table 2.4). Packaging films can be macro- or micro-perforated to influence permeability and shelf-life (Gontard and Guillaume, 2009, Kaur et al., 2011, Lee and Chandra, 2018) by manually pricking with a needle (Garrido et al., 2015b), punching holes (Zenoozian, 2011) or microperforations using laser perforation technology (Mampholo et al., 2015, Wieczyńska et al., 2016a).

Passive MAP is common for fruits and vegetables; the package is sealed with normal air inside, an equilibrium atmosphere (containing elevated CO_2) is established due to the respiration of the produce and gas permeability of packaging material (Gontard and Guillaume, 2009, Irtwange, 2006, Mudau et al., 2015). Modified atmosphere packaging (MAP) involves replacing air (N_2 78%, O_2 21%, CO_2 0.035%, water vapour and other gases 0.965%) inside a package with a gas mixture or single gas with the aim of improving shelf-life (Wilson et al., 2019, Zhang et al., 2016a). Gases such as argon (Ar), nitrogen (N_2), Helium (He), Oxygen (O_2), nitrous oxide (N_2O), carbon-dioxide (CO_2) (Inestroza-Lizardo et al., 2016, Mudau et al., 2015, Rodriguez-Hidalgo et al., 2010, Tomás-Callejas et al., 2011, Zenoozian, 2011) have been included in MAP systems when packaging leafy salad vegetables (Table 2.4) and maintained better quality compared to conventional MAP. Even in active MAP, the gas composition changes during storage due to produce respiration, microbial metabolism and package permeability (Inestroza-Lizardo et al., 2016, Rodriguez-Hidalgo et al., 2010, Salgado et al., 2014, Tomás-Callejas et al., 2011).

Active packaging is designed to extend shelf-life and involves including bioactive compounds which either release into or absorb from the packaging environment (Wilson et al., 2019) e.g., oxygen, moisture and ethylene absorbers, self-cooling packages, anti-fogging, gas permeable, antimicrobial releasing, carbon dioxide, sulphur dioxide and ethylene emitters (Dainelli et al., 2008, Ozdemir and Floros, 2004, Wieczyńska et al., 2016b). They can be in

the form of sachets, pads or incorporated into the packaging film (Gontard and Guillaume, 2009). Anti-fogging, non-perforated polypropylene gave the longest shelf-life of 16-d for red leaf lettuce compared to macroperforated polypropylene packaging 4-7-d and non-perforated packaging 13-d (Lee and Chandra, 2018). The inclusion of antimicrobial sachets of eugenol, carvacrol and trans-anethole, did not control growth of aerobic bacteria on organic wild rocket (*Diplotaxis tenuifolia L.*): for 100 g of product in 1.8 L polyethylene terephthalate trays (185*145*70 mm), wrapped with micro-perforated polypropylene film during storage at 5 °C for 6-d, 1-1.3 log CFU/g growth was reported (similar to the control). Sensory panellists detected higher characteristic odours of rotten cabbage and ammonia odours in control and carvacrol treatments compared to eugenol and trans-anethole treatments (Wieczyńska et al., 2016b). Use of biodegradable films incorporated with antimicrobial agents is also applicable for fresh foods (Zhang et al., 2016a).

Table 2.4: Packaging studies on leafy salad vegetables

Packaging type	**Leafy salad vegetable**	**Findings**	**Reference**
Polypropylene bags Control**:** perforated bags with normal air Modified atmosphere packaging (MAP): high Argon, Nitrogen, Oxygen, Helium, and Nitrous Oxide separately	40 g arugula rocket	Non-conventional modified atmosphere had higher appearance scores on day-11 compared to normal air. N_2O and helium had lower counts of psychrotrophic bacteria on d-11.	(Inestroza-Lizardo et al., 2016)
1500 mL polypropylene (PP) trays Passive MAP: trays thermally sealed with 40 µm thick bi-oriented PP (BOPP). Active MAP: Four nonconventional treatments initially composed of 100 kPa of O_2, He, N_2 and N_2O trays thermally sealed with 50 µm thick BOPP Storage at 5 °C for 8-d	40 g baby Red chard sanitised with chlorinated water	He maintained chlorophyll during shelf-life Chlorophyll loss was 16, 20, 26 and 21% of for N_2, N_2O, O_2 enriched MAPs and passive MAP 67% loss of Vit C for passive MAP after 8-d, whereas 50% loss for active MAP No differences in visual quality.	(Tomás-Callejas et al., 2011)
control (78% N_2; 21% O_2) MAP (5% O_2; 15% CO_2; balance N_2), Storage at 4, 10, and 20 °C for 12-d	Baby spinach	MAP at 4 °C had quality scores above acceptability limit on day-12 whereas packages with normal air were below the limit of acceptability from day-6. Total antioxidant activity and flavonoid content was higher for MAP. MAP at 4 °C had lower respiration rate, weight loss was	(Mudau et al., 2018)

		0.94 and 2.24% for the control. Weight loss and respiration rate were higher at 10 and 20 °C for both packaging types.	
22*20 cm polypropylene film, PPP-1320-hole (1 mm diameter), PPP-4-hole (6.5 mm diameter), Non-PPP non-perforated, Anti-Fog-PP polypropylene and control no packaging in a tray. Storage at 10 °C in a dark room	100 g red leaf lettuce	The shelf-life was 2, 4, 7, 13 and 16-d for control, PPP-1320-hole, PPP-4-hole, Non-PPP and Anti-Fog-PPP respectively based on overall visual quality assessment. Non-PPP and Anti-Fog-PPP lost 2.7% and 2.2% in 16-d, PPP-1320-hole lost 27% moisture in 12-d, PPP-4-hole lost 16.2% moisture in 16-d and control lost 20% moisture in 2-d. Anti-Fog-PPP inhibited chlorophyll degradation during shelf-life.	(Lee and Chandra, 2018)
60-g in 12.5×8.2×3.5 cm sized box sealed with 1,300, 20,000, 40,000 and 100,000 m^3/m^2/day/atm OTR MAP 50 µm polypropylene film and with a perforated film (100,000 cm^3/m^2/day/atm OTR film) with four 0.6-cm diameter holes. Storage at 8 °C, 90% relative humidity for 30-d.	Baby leaf red romaine lettuce	The perforated film and 1,300, 20,000, 40,000, 100,000 cm^3 OTR MAP films gave a shelf-life of 8, 15, 30, 25 and 20 days respectively. 20,000 cm^3 OTR MAP film had the lowest weight loss of < 1%, highest anthocyanin, flavonoid, vitamin C and total phenolic content and lowest browning and peroxidase activity.	(Islam et al., 2019)
Low-density polyethylene (LDPE) packaging film 40 µm thickness, polypropylene (PP) packaging film 36 µm thickness, 200, 400 and 600 g PP film packages. Macroperforations of 0.3 mm	Spinach	Chlorophyll loss was slower in LDPE compared to PP Higher retention of phenols, ascorbic acid and β-carotene in LDPE had compared to PP. No off-odour was observed for 200g samples both LDPE	(Kaur et al., 2011)

diameter 2 perforations per package. Stored at 15 °C for 4-d and 75% relative humidity.		and PP during 4-d of storage, slight off-odour for 400 and 600g PP, trace and strong off-odour for 400 and 600g packages of LDPE No water accumulation in 200g PP and all LDPE packages whereas, slight and prominent water accumulation in 400 and 600g PP respectively.	
30 µm polypropylene (PP), 35 and 40 µm polyethylene (PE) 3 and 9 days at 0, 4, 8 and 16 °C	Baby spinach 50 g	30 µm PP and 35 µm PE at 0-4 °C retained the best taste texture, and characteristic freshness odour for 9-day. 40 µm PE bags at 0 °C retained the highest amount of vitamin C and soluble solids. Quality was more dependent on storage temperature than packaging type.	(ViŠKelis et al., 2015)
Perforated packaging low density polyethylene (LDPE) pouch “20cm × 8 cm,” 5 mil thickness punched eighteen 6 mm diameter holes. MAP LDPE (7 - 10%, O_2, 7-10% CO_2, and 80-85% N_2) 5, 10, 20 and 25 °C 7-d	Spinach washed in sodium hypochlorite solution	Weight loss was the least in active MAP Vitamin C was better preserved in passive MAP Active MAP had higher total count.	(Zenoozian, 2011)
35 g in 1000 mL polypropylene (PP) baskets Passive MAP: Baskets thermally sealed with bioriented PP film (BPP) of 40 µm thickness Active MAP: N_2O-enriched high O_2 sealed with 50 µm thick BPP Storage for 10 days at 5 °C	Baby spinach grown under floating tray system with nutritive solution.	On day-8 of shelf-life, spinach fertilised with 8 and 16 mmol N L^{-1} and stored under N_2O-enriched MAP had the lowest microbial growth, good sensory quality and preserved antioxidant capacity.	(Rodriguez-Hidalgo et al., 2010)

2.6 Conclusion

This literature review revealed that the shelf-life of baby leafy salad vegetables is primarily influenced by harvesting produce at optimum quality, at the right time of day and removal of field heat. Maintaining the cold chain at 0-4 °C, storage at high relative humidity, controlled atmosphere, sanitisation, packaging, minimising bruising during handling and storage are key factors that significantly impact shelf-life. There is need to understand how excess wash water influences shelf-life of bagged baby leafy salad vegetables and to further study the use of sanitiser and surfactant on shelf-life. Though it is known that bruising reduces shelf-life and sanitisation with PAA can improve shelf-life, the influence of bruising and sanitisation with PAA on the bacterial community and shelf life of baby leafy salad vegetables needs further investigation.

Chapter 3: Effect of peroxyacetic acid treatment and bruising on the bacterial community and shelf-life of baby spinach.

This Chapter is being prepared for submission to the Food Microbiology journal and is presented in the format of the manuscript to be submitted to that journal.

Article title: Effect of peroxyacetic acid treatment and bruising on the bacterial community and shelf-life of baby spinach

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3.1 Abstract

Leafy salad vegetables are fragile, and therefore highly susceptible to bruising, which can occur during production, harvesting and postharvest processing and handling. The aim of this study was to investigate the combined effects of bruising and sanitisation with peroxyacetic acid (PAA) on shelf-life of baby spinach through possible changes in the bacterial community. Leaves were classified into three quality categories: all bruised (mechanically damaged), 40% bruised + 60% intact (undamaged), and 100% intact. All three leaf quality categories were treated with 80 mg/L peroxyacetic acid (PAA) separately, while half of the 100% intact leaves were washed with tap water only. Processed leaves were packaged, labelled and stored at 4 °C, and changes in total plate count and the bacterial communities were analysed during shelf-life. Bruised and bruised + intact leaves had a shelf-life of 12 d, whereas intact leaves had a shelf-life of 23 d, regardless of treatment, indicating that maintaining the integrity of baby spinach tissue can extend shelf-life. Bruising had no influence on bacterial diversity, though some differences in the relative abundance of minor genera were observed. *Pseudomonas* and *Pantoea* were the most dominant bacterial genera, regardless of leaf integrity and treatment. Washing with tap water and PAA on day 0 reduced the relative abundance of *Exiguobacterium*, however the bacterial diversity on baby spinach was not affected by washing. During shelf-life, the bacterial diversity index of sanitised baby spinach samples (2.3) was significantly lower than on water-washed leaves (2.8). The relative abundance of *Pseudomonas* on PAA-treated intact (i.e. undamaged) baby spinach was >50% from day-6 until the end of shelf-life and was higher than in water-washed spinach. Results showed that despite PAA (80mg/L) yielding a higher initial log reduction in TPC compared to tap water during washing, it does not lead to extension of shelf life, it is still essential to minimise potential cross-contamination via wash water.

3.2 Introduction

Vegetables are prone to microbial spoilage due to improper handling and storage practices (Rawat, 2015). Microbial spoilage of leafy green salad vegetables is thought to commence most often at sites on the leaves where tissue damage has occurred due to harvesting and handling, but there is a need to better understand and quantify the effect of mechanical damage on microbial quality and product shelf life. Baby leafy salad vegetables sold as pre-packaged salad mixes are fragile and highly susceptible to mechanical damage during harvesting and processing, facilitating microbial spoilage and resulting in up to 30% loss of spinach between harvest and retail sale/consumption (Poonlarp et al. 2018). Mechanical damage increases respiration rate, moisture loss, chlorophyll degradation and electrolyte leakage which promotes growth of spoilage microorganisms, and thus reduces shelf-life (Poonlarp et al., 2018, Roura et al., 2000). A range of factors affect the severity of mechanical damage including plant variety, leaf size, shape and texture, agronomic treatments, plant water status, magnitude of exerted force, maturity stage, and season (Ariffin et al., 2017, Opara, 2007). Ariffin et al. (2017) reported baby spinach was the least resistant to damage, as compared to “teen” (*a week older than baby spinach*) and salad spinach, with organic spinach showing the highest resistance.

Mechanical damage can occur from cutting, puncturing and tearing/splitting, abrasion, folding (Hodges et al., 2000), compression, and impact forces that cause bruising (Li and Thomas, 2014, Polat et al., 2012). Poonlarp et al. (2018) observed that compression and impact damage occurred during packing and transportation of spinach in plastic baskets from farm to pack house. Postharvest interventions to minimise mechanical damage such as the use of foam boxes instead of plastic baskets, avoiding hand pressure on produce, and temperature management, allowed three extra days of shelf-life (Poonlarp et al., 2018). Therefore, maintaining the integrity of the tissue appears to play a critical role in shelf-life extension of baby spinach. The extent of the consequences of mechanical damage on the

reduction of shelf life, and possible microbiome differences underlying the differences in shelf-life, are the subject of the work described here.

Pseudomonas has been identified as the most abundant bacterial genus causing spoilage of baby spinach and lettuce (Gu et al., 2018, Jackson et al., 2013, Nubling et al., 2016) however, the effect of leaf damage on potential for *Pseudomonas* growth and the composition of the total microbiome of leafy salad vegetables has not yet been reported.

Sanitisation treatments are required for minimally processed produce, such as baby leafy salad vegetables, primarily to reduce endogenous microflora, introduced microbial contaminants, and also dirt and pesticide residues (Artés et al., 2009, Gil et al., 2010, Joshi et al., 2013). A range of sanitisers have been evaluated including hydrogen peroxide, electrolysed water, citric acid, hypochlorite solution, peroxyacetic acid (PAA), ozonated water, and chlorine dioxide (Bachelli et al., 2013a, Barrera et al., 2012, Zhang and Yang, 2017). PAA has become a popular sanitiser because it decomposes to "environmentally-friendly" products, namely water, acetic acid, hydrogen peroxide, and oxygen (Carrasco and Urrestaraz, 2010). PAA is highly effective at low temperature such as 4°C (Premier, 2013) and its efficacy is less influenced by organic matter (Runmiao et al., 2017), as compared to chlorine (Gil et al., 2010). PAA is mainly effective against bacteria and viruses (Vandekinderen et al., 2009). Its mechanism of action against microorganisms involves the release of reactive oxygen species which oxidise, lipids, DNA bases, and cause enzyme inactivation and protein denaturation (González-Aguilar et al., 2012, Kitis, 2004). PAA has been successfully used to sanitise baby leafy salad vegetables (Gómez-López et al., 2013, Ho et al., 2011, Vandekinderen et al., 2009). For example, Gómez-López et al. (2013) observed 2.1 log CFU/g decrease in psychrophilic bacteria after washing baby spinach with 80 mg**/**L PAA for 1 min. During storage at 4 °C for 4 d, followed by 7 °C for 7 d, psychrophilic bacteria and *Pseudomonas* grew by 2.2 and 1.5 log CFU/g, respectively (Gómez-López et al., 2013).

Relatively few studies have examined the effect of sanitisation and storage time on microbial community structure and diversity of ready-to-eat leafy salad vegetables. Gu et al. (2018) observed a significant decrease in the relative abundance of *Pseudomonas* sp. '2' (-13.1%), *Acinetobacter* (-12.6%), *Flavobacterium succinicans* (-3.9%), *Psychrobacter* sp (-3.3%) and *Shewanella* sp (-3.0%), and an increase in the relative abundance of *Pseudomonas* sp '1' (12.53), *Erwinia* (21.9%) *Pseudomonas viridiflava (*4.3%*)*, *Paenibacillus* (3.8%), *Janthinobacterium* sp (2.7%) after washing California-grown baby spinach in chlorinated water for 20 sec. Hausdorf et al. (2013) observed that *Brachybacterium* sp. and *Comamonas* sp. were only identified on baby spinach after washing with tap water in the first wash bath during processing and were also detected in the wash water, however the bacteria were not detected in spinach samples before washing. Lopez-Velasco et al. (2010) reported a decrease in species richness after disinfection of ready-to-eat spinach with 12.5 (v/v) sodium hypochlorite for 10 min, although the time of storage at 4 and 10 °C had no influence on species richness. However, for spinach rinsed with sterile water only, an increase in species richness and abundance with storage time was observed at 4 and 10 °C (Lopez-Velasco et al., 2010). Tatsika et al. (2019) observed that the bacterial diversity of chopped spinach was not influenced by washing with 1% (v/v) vinegar for 1 min. Lopez-Velasco et al. (2011) observed that after storage of baby spinach for 15 d at 4 °C, the relative abundance of *Pseudomonas* and *Methylobacterium* increased, while *Sphingomonas*, *Brevundimonas*, *Naxibacter*, *Massilia*, and *Acinetobacter* decreased. Daddiego et al. (2018) observed differences between the microbiota of lettuce treated with 75 mg/L PAA chlorinated water vs (20-30 mg/L free chlorine).

This study investigated the effect of bruising and sanitisation with PAA on shelf-life, total plate count (TPC), and the bacterial community composition of baby spinach during storage at 4°C.

We hypothesized that;

- bruising favours growth of spoilage bacteria compared to intact leaves, and reduces bacterial diversity over shelf-life

- sanitisation with peroxyacetic acid reduces bacterial diversity on the day of processing and reduces growth of spoilage bacteria during storage.

3.2 Materials and methods

3.2.1 Plant material

Fresh baby spinach (40-100 mm length) was machine-harvested from a commercial farm near Richmond, south eastern Tasmania, in Australia in summer. Leaves were sorted into three quality categories while being maintained at 4 °C: 1) 100% leaves showing mechanical damage, hereafter referred to as ‘bruised’, 2) 100% intact leaves showing no visible signs of mechanical damage, hereafter referred to as ‘intact’, and 3) a combination of mechanically damaged leaves (40%) and intact leaves (60%), hereafter referred to as ‘bruised+intact’. Mechanical damage was defined as any cut, tear, fold, impact damage or bruising (Fig 3.1), which occurred during machine-harvesting or transportation. Samples were transported to the laboratory in an ice box within 40 min. The baby spinach leaves were stored for a maximum of 20 h at 4°C before experimentation.

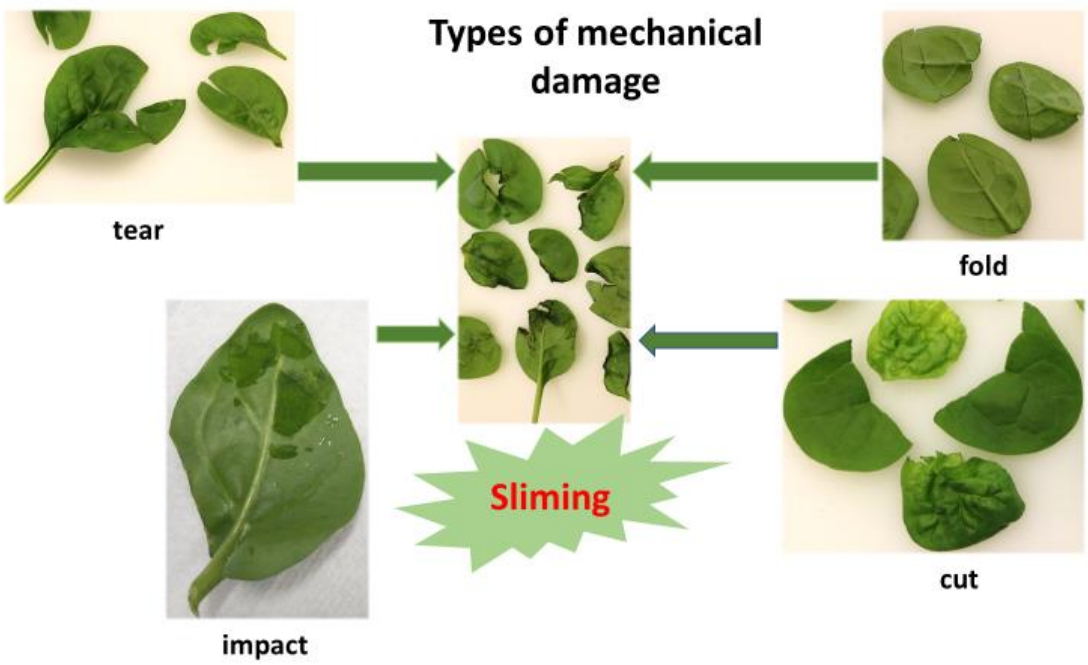


Figure 3. 1: Illustration of the types of mechanical damage on baby spinach considered for this study

3.2.2 Sanitising baby spinach

Each leaf quality category was sanitised separately by immersion in 80 mg/L Summit sanitiser (active compound peroxyacetic acid 10-30% (v/v) PAA: Sopura, Victoria, Australia) prepared in potable quality tap water at 1g produce per 30 ml of santiser solution for 45 sec. Additionally, half of the 100% intact leaves were washed with potable quality tap water only (Table 1), in order to study independently the effect of sanitisation with PAA vs water-wash.

Table 3.1: Treatment of the leaf quality categories

Leaf quality category	**Treatment**
bruised	PAA (80 mg/L)
Bruised (40%) + intact (60%)	PAA (80 mg/L)
intact	PAA (80 mg/L)
intact	potable tap water

A manual, domestic use, salad spinner was used to remove excess sanitiser solution/tap water by spinning leaves three times (~8 revolutions per ‘spin’, on average) in small batches. After washing, leaves were packaged manually in 30 g, 28x16 cm bags of oriented poly propylene (OPP) film (Apex films, Victoria, Australia) and stored at 4°C for shelf-life studies. Sampling for TPC and bacterial community analysis was conducted from triplicate samples on Day 0 (before and after wash), and again on days 3, 6, 9, 12, 15, 18, 21, 23 and 25 during storage in triplicate for each treatment.

3.2.3 Microbial analysis

10 g samples from each package were aseptically transferred to sterile 190 x 300 mm Whirl-Pack bags (Nasco, Fort Atkinson, Wisconsin), diluted 1:4 (wt/wt) in 0.1 % sterile buffered peptone water (Oxoid LP0037, UK), and homogenised using a Colworth Stomacher 400 (Seward, London, UK) for 120 sec. 10 mL of the 1:4 dilution was centrifuged (Eppendorf AG, 5810R, Germany) in 15 mL falcon tubes (Greiner Bio-one) for 35 min at 3900xg, to precipitate bacterial cells. Nine mL of supernatant was removed using sterile pipettes (Greiner Bio-one). The pellet was resuspended in the remaining 1 mL and transferred to a 1.5 mL-capacity eppendorf tube, and then stored at -80 °C for later (~1 month) DNA extraction.

The initial 1:4 dilution was further serially ten-fold diluted in 0.1 % sterile buffered peptone water (Oxoid LP0037, UK). 100 µL of appropriate dilutions were surface-plated on tryptone soya agar (Oxoid CM0129, Basingstoke, Hampshire, England) for enumeration of TPC (incubated for 72 h at 25 °C). Microbial counts were expressed as log CFU/g of spinach.

DNA extraction

Baby spinach rinsate 1 mL samples (three replicates per treatment for each sampling time) stored at -80°C containing bacterial cells were thawed at room temperature. Cells were centrifuged at 10,000 x g for 120 sec and the supernatant removed, followed by a second 60 sec spin to pellet remaining bacterial cells. Microbial DNA was extracted using DNeasy UltraClean Microbial Kit (Qiagen, Gaithersburg, MD) following the manufacturer's protocol from step 2. The concentration and quality of the extracted DNA was assessed using a Thermo Scientific Microvolume UV-Vis Nanodrop 8000 spectrophotometer (Thermo Fisher scientific, Inc, Wilmington, DE, USA) at 260/280 nm and 260/230 nm.

3.2.4 High throughput amplicon sequencing

Amplification and high throughput amplicon sequencing, including initial data processing, was conducted at the Ramaciotti Centre for Genomics (UNSW Sydney, Australia), using standard bacterial protocols, as follows. The master mix used for amplification of the 16S rRNA gene consisted of 1 U Immolase DNA Polymerase (Bioline), 2.5 mM MgCl2, 0.5 µM of each primer namely; 27F (5'-AGAGTTTGATCMTGGCTCAG-3') (Lane 1991) and 519R (5'-GWATTACCGCGGCKGCTG-3') (Lane et al. 1993), 0.2 µL dNTPs, 2.5 µL of ImmoBuffer (PCR buffer, Bioline), 1 µL of the template and water making a total volume of 25 µL. Thermal cycling involved initial denaturation at 95°C for 10 min, followed by 35 cycles of the sequential process of denaturation at 94°C for 30 sec, annealing at 55°C for 10 sec and elongation at 72°C for 45 sec, then final extension at 72°C for 10 min.

PCR products were cleaned, normalised and pooled by use of SequelPrep Normalisation kit (Invitrogen, Thermo Fisher scientific, Waltham, USA), following the manufacturer's instructions. The Axygen AxyPrep Mag PCR Clean-up Kit (Fisher Biotech) was used for library purification, according to the manufacturer's instructions. The pooled library concentration and quality was assessed using Qubit, and the Agilent 2200 Tapestation equipment was used to check library size. Primer dimers were removed by the Agencourt

AMPure XP Bead Clean-up kit. Sequencing of the library pool was conducted on MiSeq with a MiSeq reagent kit v3 with a 2*300bp run format; default run parameters included adaptor trimming. Addition of custom primers to reagent cartridge for read-1, index and read-2 was done for all runs.

3.2.5 Data processing and analysis

Mothur software package (v1.39.5, http://www.mothur.org/) (Schloss et al. 2009) was used to process reads following MiSeq SOP (https://www.mothur.org/wiki/MiSeq_SOP). The initial processes involved quality filtering, assigning reads to the samples, trimming samples to leave those ranging from 436–532 bp in length, and removing samples where homopolymers >8. The chimera.vsearch script (Rognes et al. 2016) was used in mothur to remove chimeric sequences. Remaining sequences were aligned and classified by comparing with the silva reference alignment (v132, http://www.arb-silva.de/) (Quast et al. 2013). Chloroplast, Archaea, Mitochondria, Eukaryota and unknown lineages were removed. OptiClust algorithm (Westcott and Schloss 2017) was employed to group sequences into OTU`s based on 97% similarity. For each sequencing run the sequencing error was assessed using the microbial community standard ZymoBIOMICS. OTU`s, which did not appear in 95 % of the samples, were not included for further analysis. Percentage relative abundance was calculated at phylum, class, order and genus level of bacteria, for each sample. Average relative abundance >1% for each leaf quality category at each time point (n=3) was plotted over time on a bar chart; only the 19 most abundant genera were included at the genus level.

3.2.6 Statistical analysis

TPC and relative abundance data were analysed using JMP statistical software (version 14, SAS Institute Inc, USA). A 2-way ANOVA assessed the significance of differences in TPC between leaf quality categories and storage time. One-way analysis of variance (ANOVA) analysed differences in relative abundance before and after washing with PAA or tap water,

for the four bacterial phyla and for each of the 19 most abundant bacterial genera. The differences in relative abundance of bacterial genera during shelf-life between sanitised versus unsanitised intact baby spinach, and also the effect of leaf quality (damaged, or intact) on bacterial genera in the community, was analysed using 2-way ANOVA. Calypso software (version 8.84) was used to analyse differences in microbial community structure using the Anosim (Bray-curtis test), and microbial alpha diversity using the Shannon index, for before and after wash and shelf-life data between leaf quality categories and treatments. P values < 0.05 were considered to represent a significant difference.

3.3 Results and discussion

3.3.1 Total plate count

Initial TPC values ranged from 5.9-6.3 log CFU/g before washing and were reduced by 0.9 log CFU/g for bruised leaves, 1.4 log CFU/g for bruised + intact leaves and intact baby spinach, respectively (Fig 3.2), following sanitisation with PAA. A 0.5 log CFU/g decrease was observed on intact leaves after washing with tap water (Fig 3.2). Nascimento et al. (2003) reported that sanitisation of lettuce with PAA (80 mg/L; Tsunami 100) produced a 1.85 log CFU/g reduction in TPC. Gu et al. (2018) observed a 0.8-1.6 log CFU/g reduction in total mesophilic aerobic bacteria on baby spinach after washing with chlorinated water and a 1.83-2.3 log CFU/g increase after storage at 4°C for one week. The treatment x time interaction effect for the intact leaves was significant (p=0.0002): microbial growth was faster on intact sanitised leaves compared to water washed leaves until Day 9, afterwards TPC was similar till day 23. Lopez-Velasco et al. (2010) also observed deterioration, e.g. sliminess, loss of turgidity and chlorosis after 23 days of storage of freshly harvested baby spinach stored at 4°C.

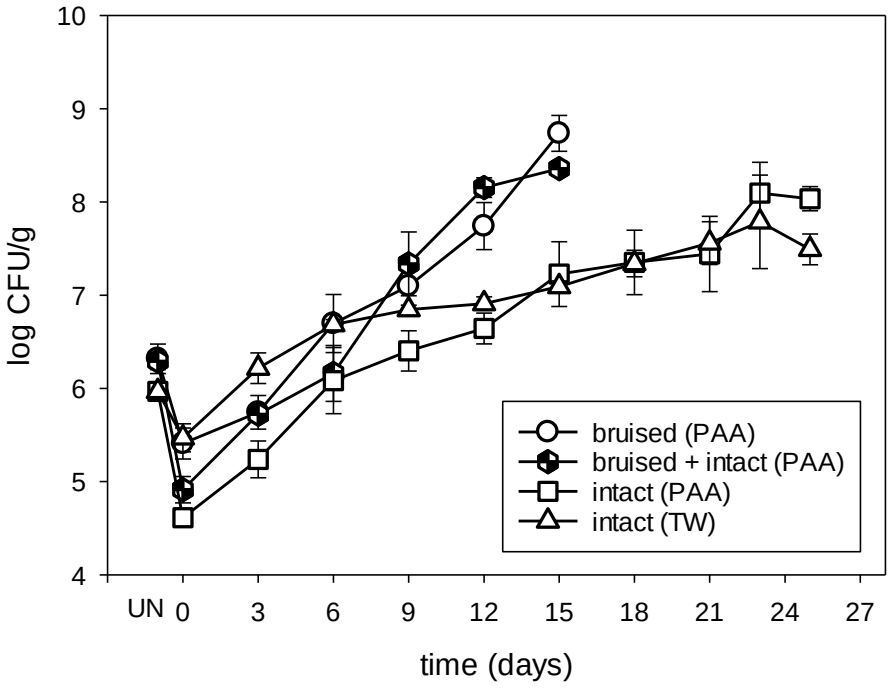


Figure 3. 2: Total aerobic plate count of sanitised bruised, bruised+intact, intact, and intact baby spinach washed with tap water before wash *(*UN - unwashed*)* and after wash, during storage at 4°C. Error bars represent the standard error of the mean (n=3). PAA: peroxyacetic acid, TW: water wash.

After Day 9, bruised and bruised+intact leaves had higher TPC compared to intact leaves, resulting in a shorter shelf-life of 12 days, compared to 23 days for intact leaves. This demonstrates that the presence of 40% of bruised leaves in bags reduced shelf-life by approximately 48%, sliming was evident on the bruised leaves. Slime indicates bacterial spoilage (Tournas, 2005), and developed faster on bruised leaves during shelf-life, compared to the wholly intact leaves. There was no significant difference in TPC between bruised and bruised+intact leaves during shelf-life ($p > 0.05$). Ariffin et al. (2017) observed that mixing whole leaves with cut/damaged leaves in the same bag caused faster deterioration of all spinach leaves and that partially and completely torn leaves had a shelf-

life of 8 days, whereas leaves with minor tears and undamaged leaves were still acceptable on day 14.

It has been reported that both the quantity and type of microorganisms explain the microbial quality of vegetables (Tournas, 2005), therefore it was considered important to understand the microbial community of bagged baby spinach.

3.3.2 Microbiome results

3.3.2.1 Bacterial phyla, classes, orders and families identified on baby spinach

Consistent with previous studies on leafy salad vegetables (Gu et al., 2018, Jackson et al., 2013, Leff and Fierer, 2013, Lopez-Velasco et al., 2010, Lopez-Velasco et al., 2011, Tatsika et al., 2019, Truchado et al., 2018) the four major bacterial phyla identified on baby spinach were Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria, regardless of leaf quality category or treatment. The relative abundance of Proteobacteria and Bacteroidetes increased during shelf-life (Fig 3.3) for all the leaf quality categories, whereas a decrease in relative abundance of Firmicutes and Actinobacteria (Fig 3.3) was observed during shelf-life. However, there was no change in the relative abundance of Firmicutes on the bruised leaves.

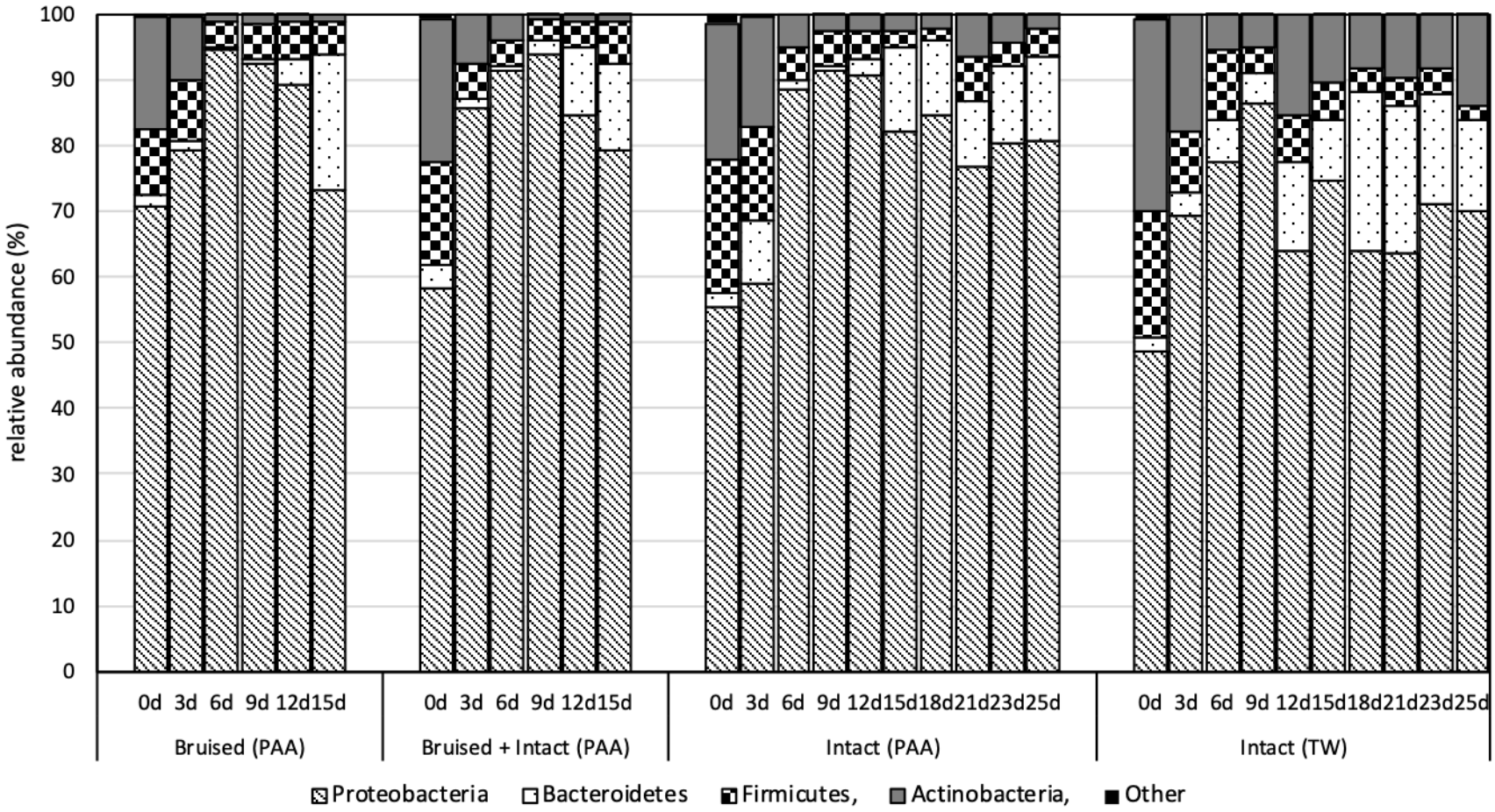


Figure 3. 3: Relative abundance of bacterial phyla on bruised, bruised+intact, and intact baby spinach leaves washed with PAA, and intact leaves washed with tap water. PAA: samples sanitised with peroxyacetic acid, TW: samples washed with tap water. N = 3 on each sampling day.

Among the bacterial classes listed in Table 3.2, Gammaproteobacteria were most abundant during storage (Supplementary Figure 1) on all baby spinach samples. Bacterial classes, orders and families identified in this study (Table 3.2) have been observed by other authors on leafy salad vegetables, with a few differences, as explained below. These include Gammaproteobacteria, Betaproteobacteria, Bacilli on lettuce and rocket (Leff and Fierer, 2013, Tatsika et al., 2019) and Actinobacteria and Alphaproteobacteria on baby spinach (Truchado et al., 2018).

Table 3.2: Summary of major bacterial classes, order and families with relative abundance >1%, identified on baby spinach.

Phylum	**Class**	**Order**	**Family**
Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae Moraxellaceae
		Enterobacteriales	Enterobacteriaceae
		Betaproteobacteriales	
		Xanthomonadales	Xanthomonadaceae
	Alphaproteobacteria	Rhizobiales	Rhizobiaceae
		Sphingomonadales	Sphingomonadaceae
			Burkholderiaceae
Bacteriodetes		Cytophagales	Hymenobacteriaceae
		Flavobacteriales	Flavobacteriaceae
			Weeksellaceae
		Sphingobacteriales	Sphingobacteriaceae
	Bacteroidia		
Actinobacteria	Actinobacteria	Micrococales	Microbacteriaceae Micrococcaceae
		Corynebacteriales	Nocardiaceae
Firmicutes	Bacilli	Bacillales	Bacillaceae
			Paenibacillaceae

After day 3 of storage at 4°C, Pseudomonadales had the highest relative abundance on sanitised leaves for all leaf quality categories (Supplementary Figure 2). Soderqvist et al. (2017) observed Enterobacteriales, Flavobacteriales, Bacillales, Sphingobacteriales, Xanthomonadales, with Pseudomonadales as the most abundant bacterial order on baby spinach washed with tap water during storage at 8°C on days 0 and 7, similar to these results. However, Tatsika et al. (2019) observed the trend of relative abundance of Enterobacteriales (39%) > Pseudomonadales (17.9%) > Betaproteobacteriales (10.6%) in baby spinach.

In this study, Pseudomonadaceae had the highest relative abundance among the bacterial families. Leff and Fierer (2013), however, found Flavobacteriaceae, Sphingobacteriaceae, Bacillaceae, Shewanellaceae, Exiguobacteraceae, Oxalobacteraceae, Enterobacteriaceae, Moraxellaceae, and Pseudomonadaceae at levels >1%, with Enterobacteriaceae being the most abundant on lettuce and rocket.

A total of 247 genera were identified from baby spinach; Table 3 lists the 19 most abundant genera. *Rathayibacter, Sphingomonas, Rahnella, Massilia, Hymenobacter* and *Herminiimonas* also had relative abundance >1%. *Pseudomonas* was the dominant genus identified for all spinach leaf quality categories and treatments during shelf-life, and *Pantoea* was the second most abundant (Fig 3.4). Some bacterial genera identified from this study are known to cause spoilage of vegetables and fruits. Previous studies have also identified *Pseudomonas* as the most dominant genus on baby spinach and lettuce (Jackson et al., 2013, Lopez-Velasco et al., 2011, Nubling et al., 2016, Soderqvist et al., 2017). *Pseudomonas marginalis, Pseudomonas chicorii* (Baylis, 2006, Tournas, 2005), *P. fluorescens, P. viridiflava,* and *Pseudomonas chlororaphis* SH36 cause spoilage by producing pectate lyases (Lee et al., 2013, Liao, 2006). Though the bacteria causing spoilage usually constitute a greater population of the spoiled product, other bacterial species can also significantly contribute to spoilage causing a synergistic effect (Remenant

et al., 2015). Lee et al. (2013) isolated *Chryseobacterium balustinum* SH43, *Pantoea agglomerans* SH58, *Pseudomonas chlororaphis* SH36, *Pseudomonas corrugata* SH50, *Pseudomonas fluorescens* SH55, *Pseudomonas putida* SH53 and *Stenotrophomonas maltophilia* SHG from spoiled lettuce and red mustard greens. Lee et al. (2011) observed *Bacillus sp, Chryseobacterium sp., Pantoea agglomerans, Pseudomonas* sp. and *Sphingobacterium* sp on spoiled green and red lettuce. *Erwinia carotovora* and other *Erwinia* species cause bacteria soft rot of vegetables such as spinach and lettuce in the field, or during storage during which it produces pectic enzymes (Liao, 2006, Saranraj et al., 2012). *Flavobacterium* also spoils dairy, fish, poultry, and other meat products (Betts, 2006). *Flavobacterium* was previously identified on baby spinach (Lopez-Velasco et al., 2010, Soderqvist et al., 2017). Other studies reported *Duganella* (Soderqvist et al., 2017), *Curtobacterium* (Lopez-Velasco et al., 2010) and *Janthinobacterium* on baby spinach (Jackson et al., 2013), though their spoilage abilities are unknown.

3.3.2.2 Effect of bruising on microbial community of baby spinach

The microbial communities profile of sanitised bruised, bruised+intact, and wholly intact sanitised leaves during shelf-life were significantly different (Anosim p=0.029; R=0.071), however differences were observed for minor genera as evidenced by the low mean values for relative abundance in table 3.3. The interaction effect between leaf quality and time was not significant for all the bacterial genera (table C2 – Appendix C2) except for Janthinobacterium (p=0.008), though leaf quality was significant for some of the genera (Table 3.3). Graphs illustrating changes in the relative abundance of bacterial genera during storage at 4°C for all leaf quality categories are presented in Appendix C1. Bruised and bruised+intact leaves had higher relative abundance of *Duganella,* compared to intact leaves, from day 9 to day 15 (Fig 3.4). At the start of shelf-life, bruised leaves had a higher relative abundance of *Pantoea* compared to intact leaves until day 6 though, overall, its relative abundance decreased during shelf-life. Bruised+intact leaves had higher relative abundance

of *Enterobacteriaceae*_unclassified as compared to bruised and intact leaves during shelf-life except on day 9 (Fig 3.4).

The relative abundance of *Bacillus, Micrococcaceae*_unclassified*, Pseudarthrobacter* and *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* was higher in intact leaves compared to bruised and bruised + intact leaves until day 6 (*see* Appendix C1) and subsequently continued to decrease to < 1% by day 15. This illustrates that some bacteria are less competitive during storage, regardless of leaf quality. In most cases, differences in relative abundance of bacterial genera between leaf quality types were not maintained during storage until the end of shelf-life. The relative abundance of the dominant genera (*Pseudomonas)* during storage until the end of shelf life was not influenced by bruising (Appendix C1).

Overall trends also observed during shelf-life among the leaf quality types were increases in *Pseudomonas, Duganella, Chryseobacterium, Sphingobacterium, Flavobacterium, Enterobacteriaceae-unclasified* and decreases in *Curtobacterium,* and *Rhodococcus* (Fig 3.4).

Table 3.3 The overall effect of leaf quality on relative abundance of bacterial genera during shelf-life. Values are means for each leaf quality type (N = 3 replicates).

Bacterial genus	Leaf quality p value	bruised leaves (mean)	bruised + intact leaves (mean)	Intact leaves (mean)
Pantoea	0.0500*	30.81 (±5.75)^{a}	21.20 (±4.77)^{ab}	16.83 (±2.78)^{b}
Duganella	0.0096**	3.57 (±0.89)^{a}	3.48 (±0.82)^{a}	1.11 (±0.27)^{b}
Bacillus	0.0003***	1.92 (±0.68)^{b}	2.53 (±0.97)^{b}	4.77 (±1.72)^{a}
Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium	<.0001***	1.38 (±0.93)^{c}	3.01 (±1.53)^{b}	5.30 (±1.71)^{a}
Paenibacilllus	0.0007***	2.31 (±0.36)^{a}	1.08 (±0.19)^{b}	1.24 (±0.47)^{b}
Sphingobacterium	0.0033**	1.05 (±0.56)^{ab}	2.04 (±0.43)^{a}	0.92 (±0.76)^{b}

Micrococcae_unclassified	<.0001***	0.68 (±0.30)[b]	0.84 (±0.30)[b]	1.40 (±0.36)[a]
Enterobacteriaceae_ unclassified	0.0019**	0.43 (±0.13)[b]	2.27 (±0.70)[a]	0.71 (±0.23)[b]
Pseudarthrobacter	0.0034**	0.47 (±0.16)[b]	0.53 (±0.15)[b]	0.92 (±0.23)[a]
Rhodococcus	0.0486*	0.58 (±0.28)[a]	0.32 (±0.12)[b]	0.44 (±0.14)[ab]

Different letter in a row signifies statistical differences

The Shannon index was 2.0, 2.3 and 2.2 for bruised, bruised+intact, and intact sanitised leaves, respectively, indicating that there was no significant difference in bacterial diversity (p=0.26) between leaf quality categories. Bruising did not select for the growth of specific spoilage bacteria as demonstrated in the relative abundance data. Koukkidis et al. (2017) reported that endogenous bacteria are less responsive to growth in the presence of salad leaf juices from leaf damage.

3.3.2.3 Effects of wash treatment on the bacterial community on day of processing

The relative abundance of Actinobacteria increased after washing with tap water on Day 0 (p=0.046) (Supplementary Table 1), however, no change in relative abundance of bacterial phyla was observed after sanitisation with PAA. This suggests that at higher taxonomic levels, sanitiser effects may not be profound. Gu et al. (2018) observed an increase in relative abundance of Proteobacteria after washing baby spinach with chlorinated water. At phylum and class levels, Truchado et al. (2018) observed no differences in relative abundance between baby spinach irrigated with water or chlorine dioxide solution. In my studies, during storage until the end of shelf-life, relative abundance of Actinobacteria remained higher for tap water washed samples compared to sanitised samples (Fig. 3.3), though the relative abundance for Proteobacteria was lower.

The microbial community profile before and after wash was significantly different (Anosim p =0.001; R=0.343) and changes in the relative abundance of some genera were observed. Sanitisation decreased the relative abundance of *Exiguobacterium, Enterobacteriaceae*

*(*unclassified*)*, and an increase in *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium, Bacillus and Curtobacterium* (Table 3.4)*.* Similarly, relative abundance of *Exiguobacterium* decreased after water wash, while *Curtobacterium* and *Flavobacterium* increased (Table 3.4). Therefore, the washing process only influenced minor genera with the exception of *Exiguobacterium* and *Bacillus*. Though washing with PAA and tap water decreased relative abundance of Exiguobacterium, common spoilage bacteria including Pseudomonas, Erwinia and Pantoea were not significantly affected by the wash treatment. Exiguobacterium are facultative anaerobes, capable of growing at a temperature range of -12 to 55 °C (Vishnivetskaya et al., 2009) and produce hydrolytic enzymes (Kasana and Pandey, 2018).

Gu et al. (2018) also observed a decrease in the relative abundance of Exiguobacterium (-3.43%) after washing Arizona-grown baby spinach with chlorinated water for 20 sec, and a decrease in other genera and species namely Sphingomonas sp (-25.46%), Microbacteriaceae sp. 1 (-4.95%), Sphingobacterium faecium (-4.91%) and Agrobacterium sp (-3.95%). Coriander washed with chlorine dioxide (60 mg/L) for 10 min at an 8:1 ratio of solution to leaf, reduced *Pseudomonas*, *Brevibacterium*, *Acinetobacter* and *Staphylococcus* to undetectable levels. Growth of these bacteria was not detected after storage at 2°C for 10 d (Jiang et al., 2017); however, ClO_2 treatment had no effect on *Deinococcus*, *Erwinia* or *Exiguobacterium*.

These data reveal that the relative abundance of some genera increased after washing, while other genera decreased. Gu et al. (2018) observed an increase in relative abundance of *Pseudomonas* ‘sp1’ (12.5%), *Erwinia* (21.9%) *Pseudomonas viridiflava* (4.3%), *Paenibacillus* (3.9%) and *Janthinobacterium* sp (2.7%) on California-grown spinach and *Pseudomonas* (18.2%), *Pedobacter* sp 1 (5.6%), *Pseudomonas* ‘sp 2’ (15.0%), *Erwinia* (3.4%) and *Cupriavidus* sp (2.9%) on Arizona-grown baby spinach after washing with chlorinated water.

Table 3.4: Changes in relative abundance (%) of the 19 most abundant bacterial genera on baby spinach before and after washing on the day of processing. PAA: samples sanitised with peroxyacetic acid.

Genera	**before wash**	**after sanitisation (PAA)**	**before wash**	**after water wash**
Pseudomonas	7.53 (±1.09)	7.39 (±1.03)	7.44 (±1.24)	4.36 (±0.85)
Pantoea	36.41 (±6.98)	31.77 (±7.72)	13.34 (±3.66)	27.94 (±8.43)
Duganella	0.73 (±0.32)	0.78 (±0.23)	0.15 (±0.04)	0.40 (±0.11)
Chryseobacterium	0.39 (±0.06)	0.66 (±0.14)	0.36 (±0.12)	0.41 (±0.14)
Exiguobacterium	13.44 (±2.57) [a]	2.00 (±0.32) [b]	21.60 (±1.31) [a]	7.37 (±1.33) [b]
Sphingobacterium	0.33 (±0.14)	0.29 (±0.06)	0.17 (±0.06)	0.24 (±0.15)
Erwinia	0.48 (±0.16)	0.42 (±0.1)	0.54 (±0.45)	0.22 (±0.1)
Flavobacterium	0.12 (±0.02)	0.19 (±0.04)	0.08 (±0.02) [a]	0.39 (±0.11) [b]
Enterobacteriaceae_unclassified	0.84 (±0.17) [a]	0.38 (±0.07) [b]	1.05 (±0.26)	0.95 (±0.5)
Curtobacterium	2.10 (±0.2) [a]	3.49 (±0.4) [b]	2.15 (±0.41) [a]	3.56 (±0.25) [b]
Stenotrophomonas	0.21 (±0.09)	0.76 (±0.4)	0.10 (±0.05)	1.07 (±0.68)
Paenibacillus	2.29 (±0.73)	1.17 (±0.23)	0.97 (±0.22)	1.16 (±0.19)
Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium	2.09 (±0.41) [a]	10.55 (±3.29) [b]	1.86 (±0.57)	3.61 (±0.92)

Janthinobacterium	0.02 (±0.01)	0.046 (±0.02)	0.02 (±0.01)	0.01 (±0.01)
Paenarthrobacter	2.54 (±0.62)	2.79 (±0.45)	3.47 (±1.55)	5.38 (±1.14)
Micrococcaceae_unclassified	2.13 (±0.39)	2.83 (±0.49)	3.04 (±0.87)	4.11 (±1.12)
Bacillus	4.77 (±1.01) [a]	10.77 (±2.52) [b]	7.88 (±1.58)	8.88 (±1.43)
Pseudarthrobacter	1.55 (±0.25)	1.74 (±0.32)	2.15 (±0.57)	2.67 (±0.30)
Rhodococcus	2.91 (±0.56)	1.80 (±0.37)	1.78 (±0.10)	5.57 (±1.58)

Numbers in brackets represent the standard error of the mean. A different letter before and after wash in the same row for each treatment represents a significant difference. (N=9 for column effect of sanitisation; N=3 for effect of washing only).

On Day 0, the Shannon index was 3.1 for the unwashed samples, 3.4 and 3.3 for sanitised and water-washed samples, respectively, however the diversity index was not significantly different among treatments (p=0.6). Therefore, washing with PAA or tap water did not eliminate specific bacterial groups or result in emergence of other species. Hausdorf et al. (2013) observed an increase in bacterial diversity after washing spinach with water in wash bath-1; washing with tap water in wash bath-2 and -3 decreased bacterial diversity. The bacterial community composition of baby spinach and lettuce treated with 1.3% NaOCl for 5 min + 70 % ethanol 2 min versus unsanitised product was not significantly different (Jackson et al., 2013).

3.3.2.4: Changes in microbial community of intact baby spinach during shelf-life

On Day 0, immediately after washing, the bacterial community of sanitised leaves was dominated by *Pantoea* (20.3%), *Bacillus* (16.1%) and *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (14.1%); water-washed baby spinach was also dominated by *Pantoea* 28.1%, *Bacillus* (8.9%) and Exiguobacterium (7.4%) (Fig. 3.4). *Pseudomonas* was the most prevalent genus after Day 6 (Fig 3.4 and Fig 3.5), with a relative abundance >50% for sanitised leaves. The interaction effect between treatment and time was significant ($p>0.05$) for *Pseudomonas, Micrococcaceae*_unclassified*, Paenarthrobacter, Stenotrophomonas, Paenibacillus, Enterobacteriaceae*_unclassified*, Janthinobacterium and Pseudarthrobacter* (Appendix C4). Though *Pseudomonas* was also most abundant (22-45%) after Day 6 on water washed samples, *Pantoea, Exiguobacterium, Chryseobacterium, Paenarthrobacter,* and *Flavobacterium* also dominated compared to sanitised samples (Fig 3.4, 3.6 and Fig 3.7).

An increase in the relative abundance of *Sphingobacterium, Duganella, Stenotrophomonas* and *Chryseobacterium* was observed during storage at 4°C (Appendix C3) and increases in Flavobacterium appear to be associated with end of shelf-life. The relative abundance of *Pantoea, Exiguobacterium, Paenarthrobacter, Bacillus, Micrococcaceae_unclassified,* and *Rhodococcus* decreased during shelf-life. Ioannidis et al. (2018) observed a significant increase in *Pseudomonas, Janthinobacterium, Rahnella, Flavobacterium,* and a decrease in *Mycoplasma* at the end of shelf-life of iceberg lettuce after storage in equilibrium MAP and perforated packaging for 10 d at 10°C. Gu et al. (2018) found that during storage at 4°C, the relative abundance of *Pseudomonas* 'sp2' (15.3%), *Flavobacterium succinicans* (10.3%), *Shewanella* sp (4.1%), *Chryseobacterium* sp (5.2%), *Janthinobacterium lividum* (2.9%), increased, while *Pseudomonas* 'sp1' (-8.8%), *Erwinia* sp. (-23.3%), *Pseudomonas viridiflava* (-4.9%), *Paenibacillus* (-2.7%) and *Janthinobacterium* sp (-1.8%) decreased on California-grown baby spinach. Arizona grown spinach showed different changes in relative abundance of bacterial genera compared to California grown spinach during shelf-life, this suggests that

growth conditions may influence bacterial community of baby spinach. As observed in this study of Tasmania-grown baby spinach, it is common that changes in relative abundance of bacterial genera on leafy salad vegetables occurs during storage at low temperature.

The relative abundance of *Curtobacterium* and *Bacillus* increased after washing, however a decrease in relative abundance during shelf-life was observed. Whereas though the relative abundance of *Exiguobacterium* decreased during washing, it also decreased during shelf-life on intact leaves. Gu et al. (2018) found that most bacterial species that decreased after chlorine washing increased during storage, while those which increased with chlorine washing decreased during storage. After seven days of storage the microbial community was comparable to that before washing (Gu et al., 2018), this suggests that the bacterial cells could be injured by the sanitisation process, and later recover and grow during cold storage.

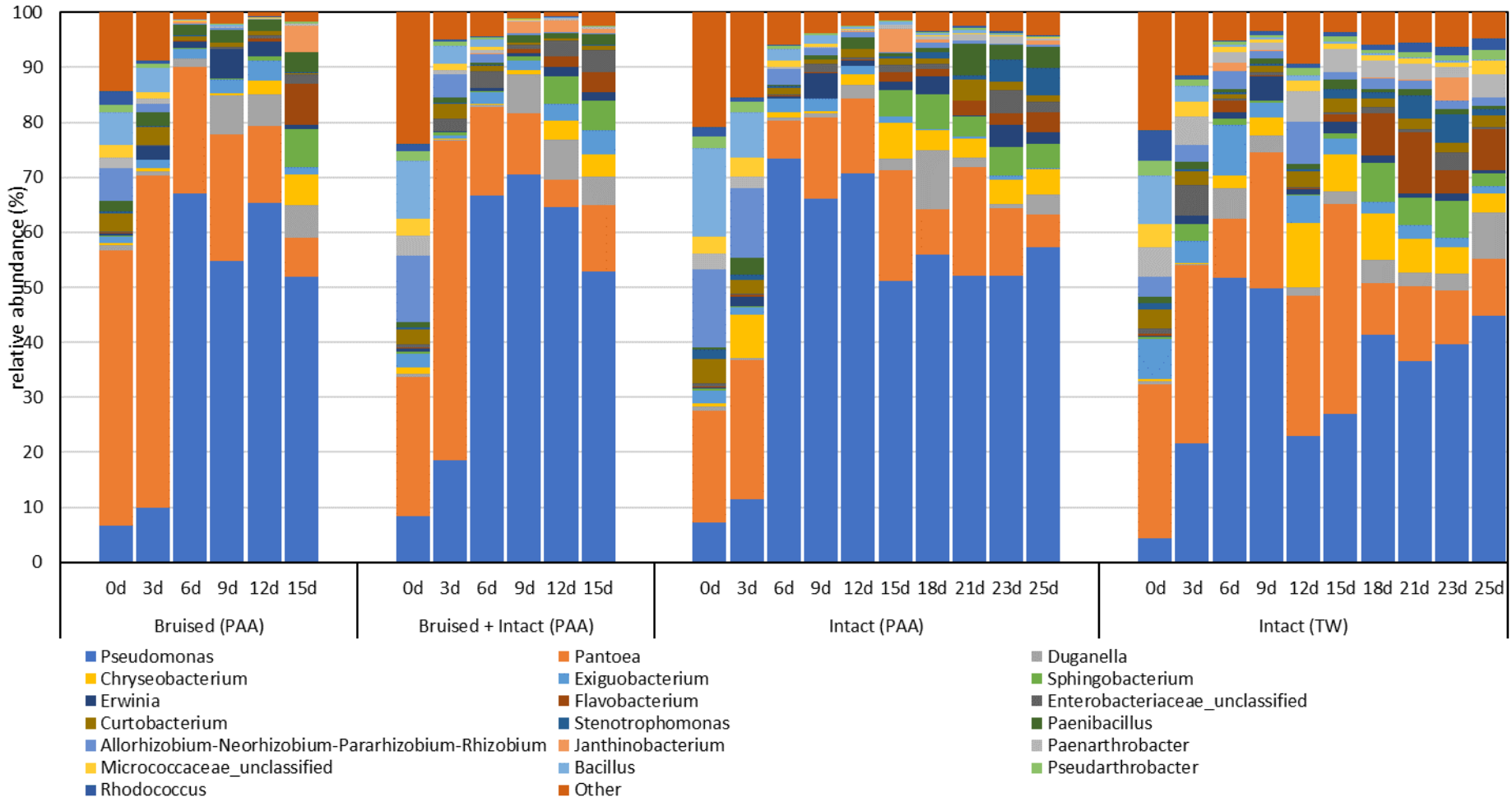


Figure 3. 4: Relative abundance of bacterial genera on bruised, bruised + intact and intact baby spinach leaves washed with PAA and intact leaves washed with tap water during storage at 4 °C. PAA: samples sanitised with peroxyacetic acid, TW: samples washed with tap water N=3.

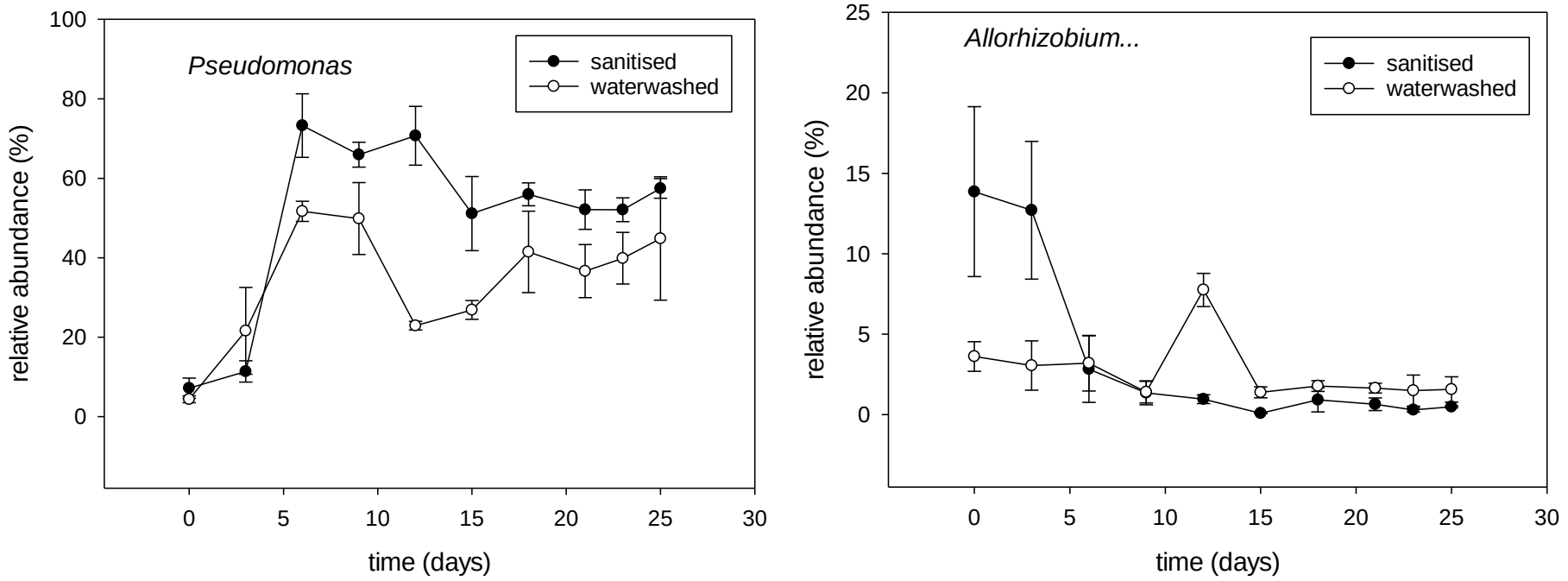


Figure 3. 5: Relative abundance of *Pseudomonas* (left) and *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (right) on intact baby spinach leaves sanitised with peroxyacetic acid and intact leaves washed with tap water during storage at 4 °C, N = 3 on each sampling day.

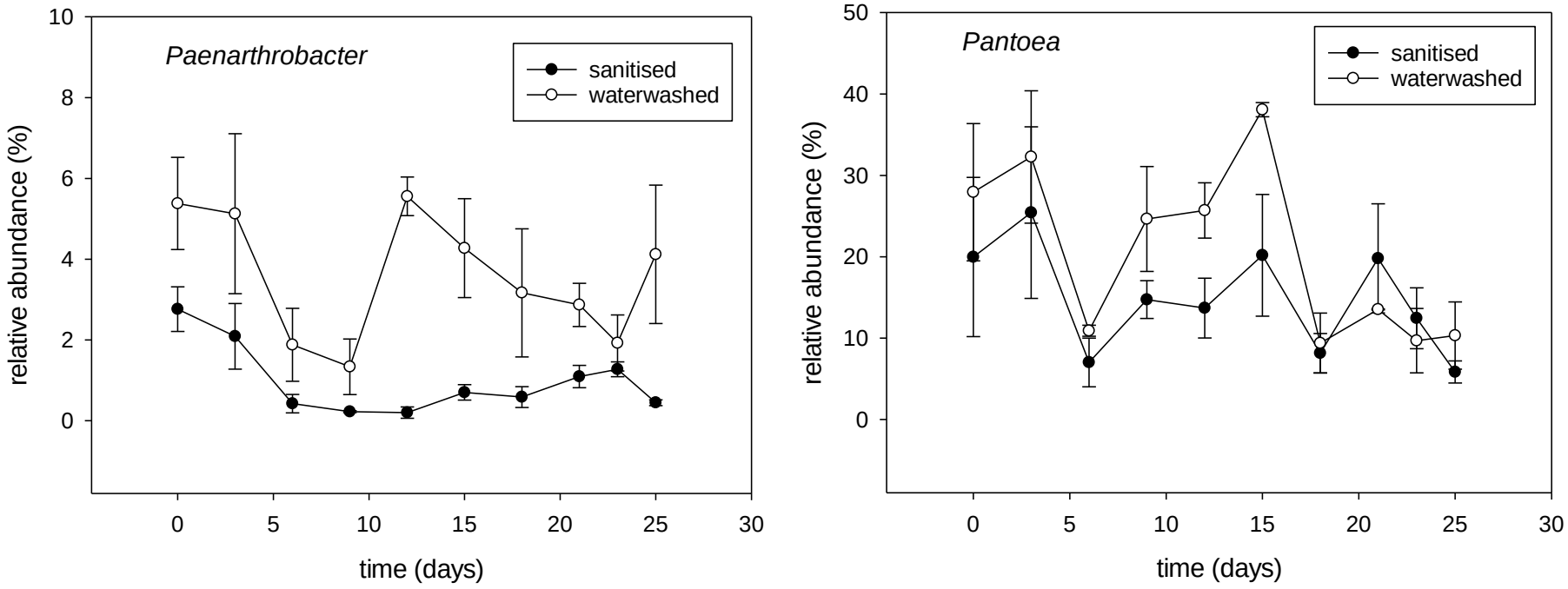


Figure 3. 6: Relative abundance of *Paenarthrobacter* (left) and *Pantoea* (right) on intact baby spinach leaves sanitised with peroxyacetic acid and intact leaves washed with tap water during storage at 4 °C, N = 3 on each sampling day.

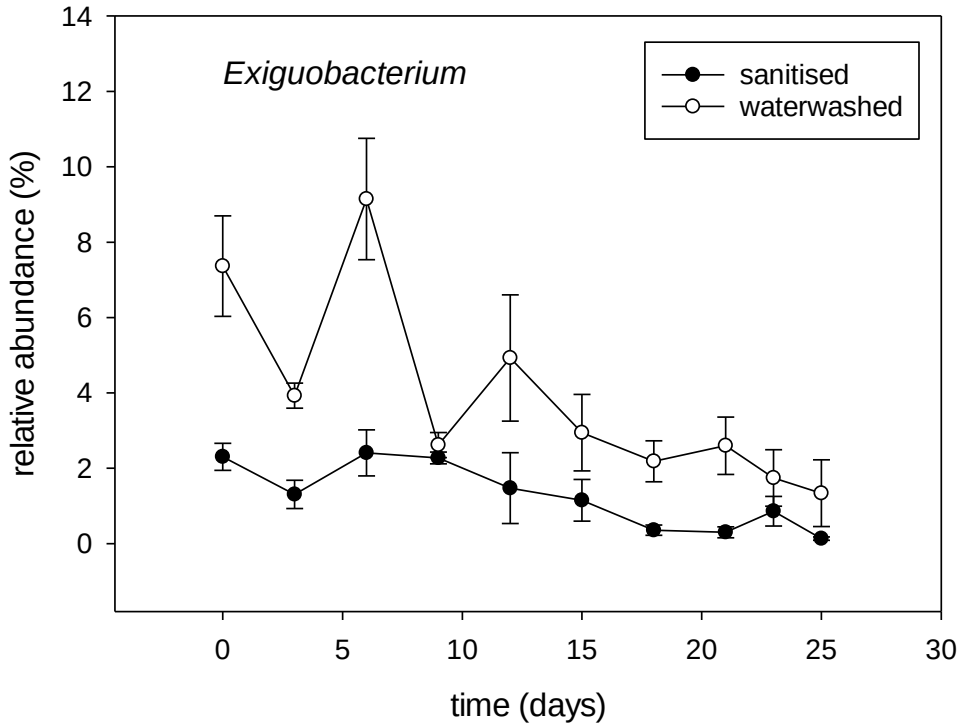


Figure 3. 7: Relative abundance of *Exiguobacterium* on intact baby spinach leaves sanitised with peroxyacetic acid and intact leaves washed with tap water during storage at 4 °C, N=3 on each sampling day.

Though these results demonstrate that sanitisation with PAA somewhat favours growth of *Pseudomonas* on baby spinach during storage at 4 °C compared to water washed samples, sanitisation had no influence on shelf-life. However, sanitisation of wash water is still an important step during processing to reduce the potential for cross-contamination of pathogens from contaminated produce to clean produce.

Conclusion

Bruising promoted the growth of total aerobic microorganisms and reduced the shelf-life of bagged baby spinach leaves as hypothesized, by 48% but had no influence on the bacterial diversity during storage at 4 °C contrary to the hypothesis.

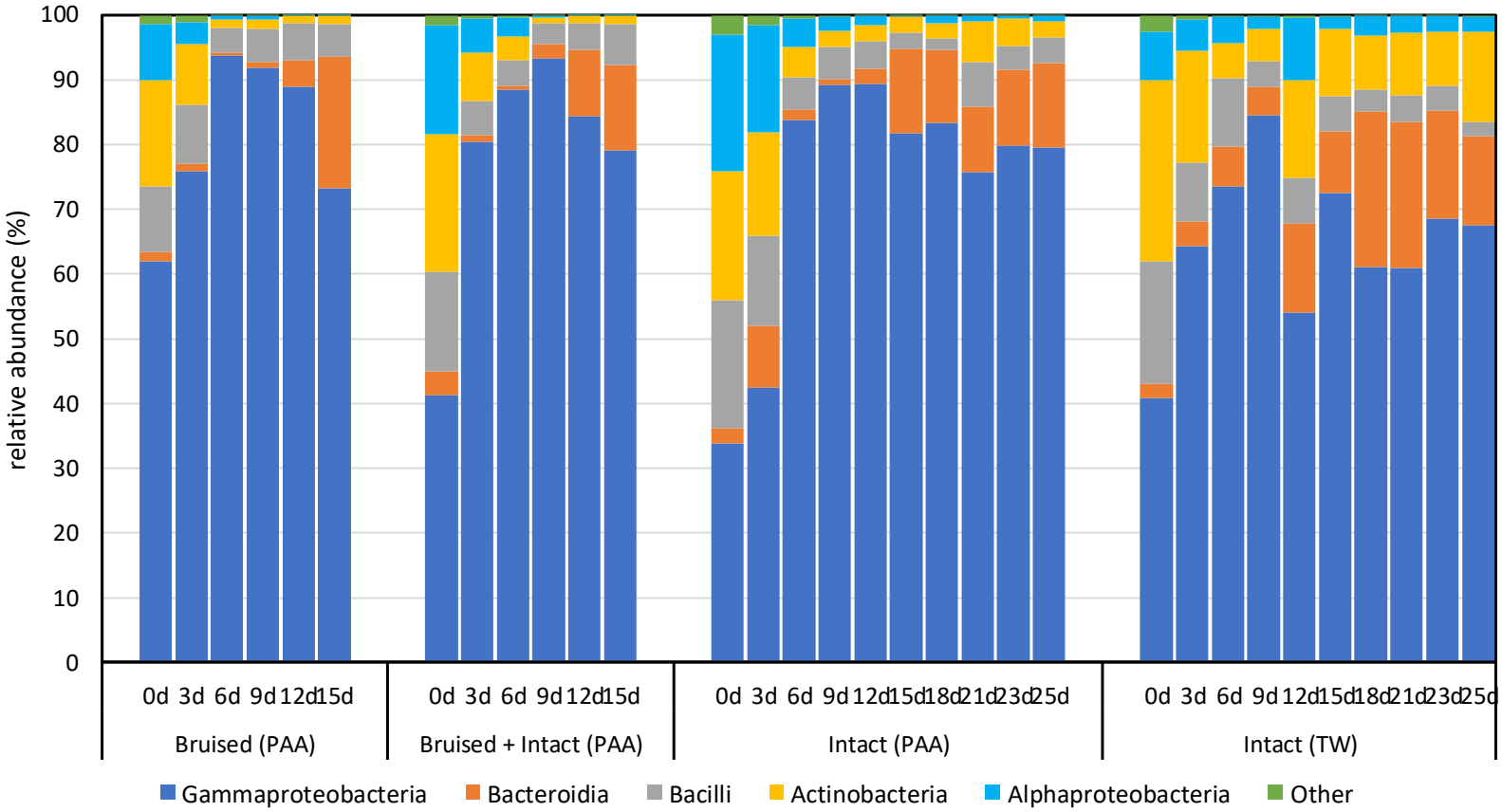
Sanitisation of baby spinach resulted in a decrease in the relative abundance of *Exiguobacterium* on day 0 although the bacterial diversity did not change . During cold storage the bacterial diversity of baby spinach sanitised with PAA was lower however, over time the relative abundance of *Pseudomonas* was higher compared to water-washed samples. This differed from our initial hypothesis that PAA treatment would reduce the growth of spoilage bacteria. The shelf-life (23 days) of intact sanitised baby spinach was comparable to that of water washed spinach. Therefore, shelf-life was not influenced by sanitisation with PAA (at 80 mg/L) and did not appear related to changes in bacterial community. Future work could focus on developing alternative sanitisation technologies which are effective against the dominant spoilage microorganisms (*Pseudomonas* and *Pantoea*) in effort to increase shelf-life.

3.4 Supplementary data

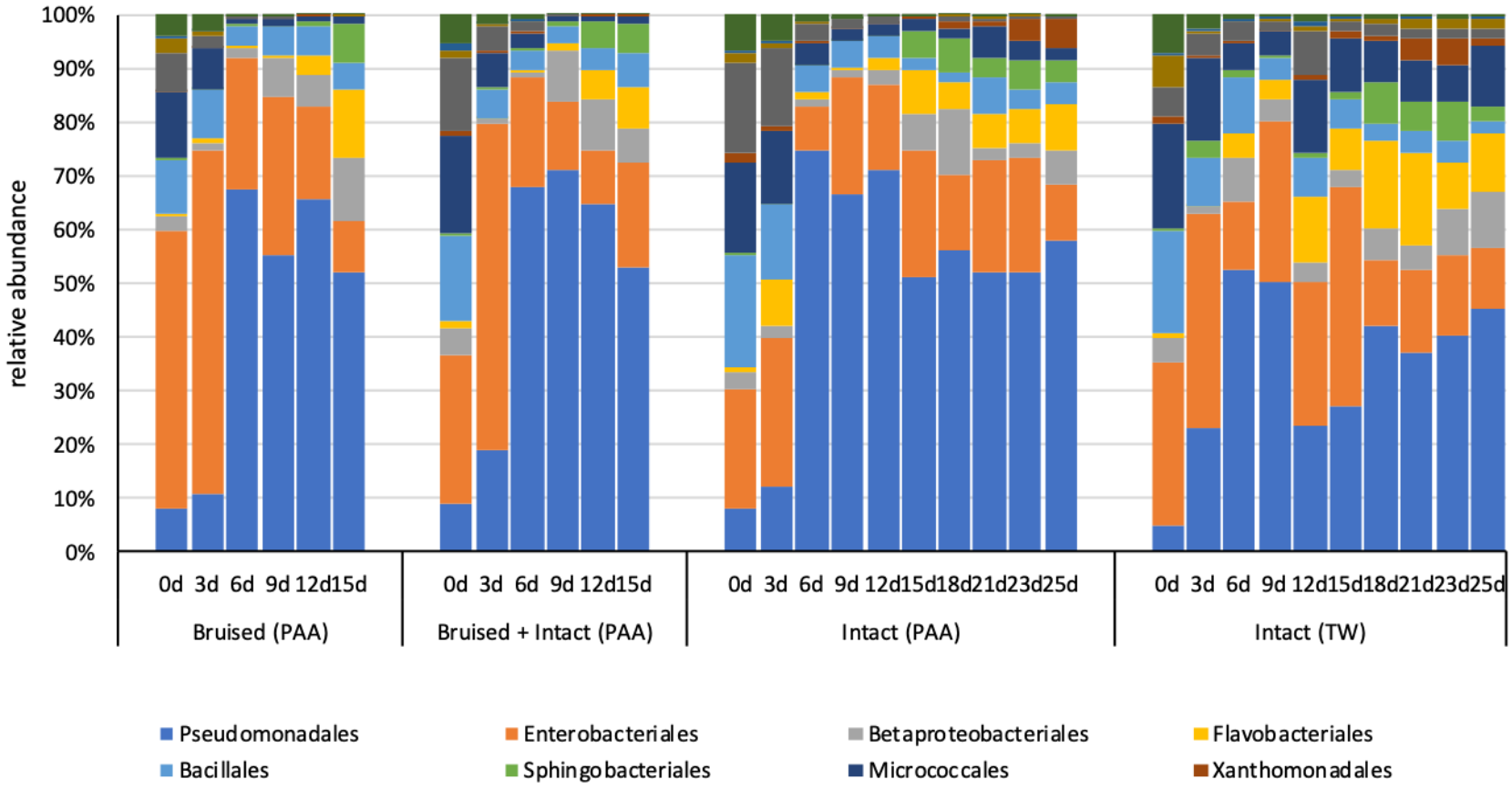
Supplementary Table 1: Changes in relative abundance of bacterial phyla on baby spinach before and after washing on the day of processing. TW: samples washed with tap water PAA: samples sanitised with peroxyacetic acid, N=9.

	Proteobacteria	Bacteroidetes	Firmicutes	Actinobacteria
before wash	54.83 (±6.33) [a]	3.87 (±1.32) [a]	21.91 (±3.35) [a]	17.03 (±2.30) [a]
PAA wash	61.02 (±4.25) [a]	2.46 (±0.43) [a]	15.15 (±2.56) [a]	19.63 (±2.17) [a]
TW wash	47.98 (±3.64) [a]	2.23 (±0.41) [a]	18.87 (±1.67) [a]	28.69 (±2.31) [b]

Numbers in brackets represent the standard error of the mean. Different letter in the same column represents a significant difference.



Supplementary Figure 1: Relative abundance of bacterial classes on bruised, bruised + intact and intact baby spinach leaves washed with PAA and intact leaves washed with tap water during storage at 4 °C. PAA: samples sanitised with peroxyacetic acid, TW: samples washed with tap water N=3.



Supplementary Figure 2: Relative abundance of bacterial orders on bruised, bruised + intact and intact baby spinach leaves washed with PAA and intact leaves washed with tap water during storage at 4 °C . PAA: samples sanitised with peroxyacetic acid, TW: samples washed with tap water N=3.

Chapter 4: Excess wash water significantly reduces the shelf-life of baby spinach

This Chapter is being prepared for submission to the Journal of Food Science and Technology and is presented in the format of the manuscript to be submitted to that journal.

Article title: Excess wash water significantly reduces the shelf-life of baby spinach

Proposed authors: Vongai Dakwa, Alieta Eyles, Alistair Gracie, Mark Tamplin, Tom Ross

4.1 Abstract

Processing of baby leafy salad vegetables typically includes a drying step to remove excess wash water but this step yields variable outcomes of residual moisture. This study sought to provide insight on the effect of excess wash water on the shelf-life and microbial quality of baby spinach. The effect of three levels of residual ('excess') wash water in bags (1, 2 or 5 mL) on the shelf-life and microbial quality of packaged baby spinach (60 g) following commercial sanitisation with peroxyacetic acid (80 mgL^{-1}) was assessed. Two and 5 mL residual wash water reduced shelf-life by 17 and 35%, respectively, in an initial trial. In a second trial, one mL excess wash water reduced shelf-life by 13%, whereas 2 and 5 mL excess wash water equally reduced shelf-life by 38%. Two and 5 mL of excess wash water in packages led to higher scores for bruising and sliming sooner, while chlorophyll content decreased during shelf-life regardless of the amount of excess wash water. Results from this study demonstrate the need to minimise residual wash water on the leaves after sanitisation to extend shelf-life.

4.2 Introduction

Postharvest handling of minimally processed fresh leafy salad vegetables involves the removal of residual wash water after washing (Francis et al., 1999). This ‘drying’ step is critical as wash water contains plant exudates that can promote microbial growth and reduce shelf-life (Holcroft, 2015b, Koukkidis et al., 2017).

In commercial processing, removal of residual wash water of leafy salad vegetables can be achieved by a range of methods such as continuous air-drying (Ilic et al., 2008), infrared drying (Moses et al., 2014) and centrifugal/spin drying in batches (Casquilho et al., 1994, Cefola and Pace, 2015, Davidson et al., 2013). However these methods are often inefficient and some can also result in bruising (Nicola et al., 2006, Varoquaux and Mazollier, 2002). Continuous air-drying of leafy vegetables can be made more efficient by applying rapid vibrations on a perforated conveyor belt with suction below, and also by blowing an air stream at an angle to turn the leaves during the drying process (Crosset, 1954).

To our knowledge, only one study has examined the effect of residual wash water on shelf-life of bagged leafy salad vegetables. Pirovani' et al. (2003) found spin-drying fresh-cut spinach at 39.2 – 156.8 g-force for 1-9 min reduced residual wash water from 31% to 5.46-0.15% (*calculated by expressing the difference in weight of spinach after washing as a percentage of the initial weight).* However, the amount of residual wash water had no influence on microbial growth and sensory attributes (Pirovani' et al., 2003). This is a surprising result, given the importance of the drying step in industry and requires further investigation because the impact of residual wash water on shelf-life of leafy salad vegetables remains unclear.

For packaged leafy salad vegetables, in addition to wash water, additional moisture can also originate from the leafy salad vegetables themselves via the physiological processes of respiration and transpiration (Bovi et al., 2016). The amount of moisture accumulated in the

package can vary with the physical properties of the packaging film such as its water vapour transmission rate (Aharoni et al., 2007) and permeability (Rodov et al., 2010), and the use of moisture absorbers (Gaikwad et al., 2018), and by managing temperature fluctuations that can result in condensation (Holcroft, 2015a, Rodov et al., 2010).

The aim of this study was to investigate the effect of 1, 2 and 5 mL of wash water treatments (comprising of sanitised wash water (PAA; 80 mgL^{-1})) on the shelf-life, microbial quality, and relative humidity of packaged baby spinach. We hypothesized that reducing the amount of residual wash water would improve shelf-life and maintain sensorial quality.

4.3 Materials and methods

4.3.1 Plant material

Baby spinach (*Spinacia oleracea L.*) (40-100 mm length) harvested from a commercial farm in south-eastern Tasmania, Australia was processed in a nearby local commercial processing factory. The processing involved sanitisation with Summit ® (active compound: peroxyacetic acid (PAA) at 10-30% (v/v); Sopura, Victoria, Australia (applied at 80 mg/L) for 45 sec followed by drying with a continuous air dryer to remove residual wash water. Residual wash water was removed by gentle patting the leaves with sterilised (121 °C for 15 min) and dried paper towels in a cold room at 4 °C. Baby spinach leaves were then packaged in 60 g OPP film bags (Apex films, Victoria, Australia) with different levels of wash water, namely 0 mL (dry leaves), 1 mL, 2 mL, or 5 mL of PAA (80 mg/L), thereafter referred to as 1 mL, 2 mL, or 5 mL treatments. The bags were transported to the laboratory (taking no longer than 30 min) in an ice box and stored in crates in a cold room at 4 °C for the subsequent shelf-life study.

4.3.2 Experimental design

In experiment 1 (preliminary study), the effect of two wash water treatments (PAA: 80 mg/L) i.e. 2 mL and 5 mL on microbial load and chlorophyll content (SPAD) were examined in a shelf-life study using 60 g bags of packaged baby spinach.

In Experiment 2 (main study), the effect of four wash water treatments comprising of 0 (dry leaves), 1, 2 and 5 mL of wash water (PAA: 80 mg/L) on microbial quality (TPC, *Pseudomonas* spp. and Enterobacteriaceae), chlorophyll content (SPAD), a_w, and visual quality were examined in a shelf-life study using 60-g bag of packaged baby spinach. Relative humidity inside the bags was measured every 30 min with an Emerson GO TH logger placed in 2 bags per treatment.

4.3.3 Microbial analysis

10-g baby spinach samples from each 60-g package was aseptically transferred to sterile filter bags (190 x 300 mm) and diluted 1:10 (wt/wt) with 0.1 % (v/v) sterile buffered peptone water (Oxoid LP0037, UK). The mixture was homogenised for 2 min using a stomacher (Colworth Stomacher 400, Seward, London, UK). Additional serial decimal dilutions were prepared with sterile peptone. 0.1 mL of the appropriate dilutions were surface-plated on tryptone soya agar (TSA; Oxoid CM0129, Basingstoke, Hampshire, England) and *Pseudomonas* agar (Oxoid CM0559, Basingstoke, Hants, UK) containing supplement SR0103 to enumerate total aerobic plate count (TPC) (72 h at 25 °C) and *Pseudomonas* spp. (48 h at 25 °C), respectively. Violet Red Bile Glucose Agar (Oxoid CM0485, Basingstoke, Hampshire, England) was used to enumerate Enterobacteriaceae for 24 h at 30 °C. Microbial populations were expressed as log CFU/g of fresh weight of spinach.

4.3.4 SPAD

The chlorophyll content of baby spinach leaves was measured using a chlorophyll meter (SPAD–502 plus; Konica Minolta Inc, Japan). 30 leaves per treatment (10 leaves/bag) were

randomly selected and assessed. For each leaf, a measurement was taken with the adaxial side facing the emitting window of the SPAD meter, on both the left and right side of the leaf, avoiding leaf veins, the average of the two measurements was considered the SPAD value for the leaf.

4.3.5 Water activity

The a_w of six baby spinach leaves per treatment (two per bag) was measured using an Aqualab CX-2 meter (Decagon devices Inc, Pullman, Washington, USA) at 24-25 °C. The meter was calibrated with water (1.000) and saturated salt (NaCl, 0.76) solution. Each leaf was cut with the adaxial side facing up, to fit the size of the meter cup, and carefully transferred to the cup in the same orientation to avoid loss of moisture.

4.3.6 Sensory evaluation

12 bags (three per treatment) were visually assessed for quality attributes, namely bruising, sliming and yellowing during shelf-life on day 1, 10, 14 and 16 on a scale of 1 to 5 by a trained panel of 6 to 11 individuals. A rating of 5 was the highest quality (no bruising/sliming/yellowing), 3 was the limit of consumer acceptability, and 1 was the lowest quality (high bruising, sliming, yellowing). This study was approved by the University of Tasmania Social Sciences Human Research Ethics Committee – ethics reference number H0016331. Written consent to participate was sought from the panellists, specifying that only the sensory evaluation data will be published without identifying individuals involved.

4.3.7 Data analysis

JMP statistical software (version 11, SAS Institute Inc, USA) was used to analyse data for both experiments. TPC, *Pseudomonas* spp., Enterobacteriaceae, SPAD and a_w were analysed using 2-way analysis of variance (ANOVA) with sampling day and treatment as the independent variables. Tukey's honestly significant difference (HSD) test was also used to determine which treatments were different. A chi-squared test was used to analyse the effect

of time and treatment on visual quality attributes. During the analysis, significance was calculated at p=0.05. Assumptions for normality and homogeneity of variance were checked before each analysis.

4.4: Results

4.4.1: Experiment 1

Total plate count

The initial TPC values in 'experiment 1' were 3.9-4.4 log CFU/g (Fig 4.1), which increased to 7.9-8.3 log CFU/g after 15, 19 and 25 d for baby spinach leaves from bags which had 5, 2 and 0 mL treatments, respectively. There was significant treatment x time interaction effects on TPC ($p<0.0001$). The treatment effect was more evident from day 15.

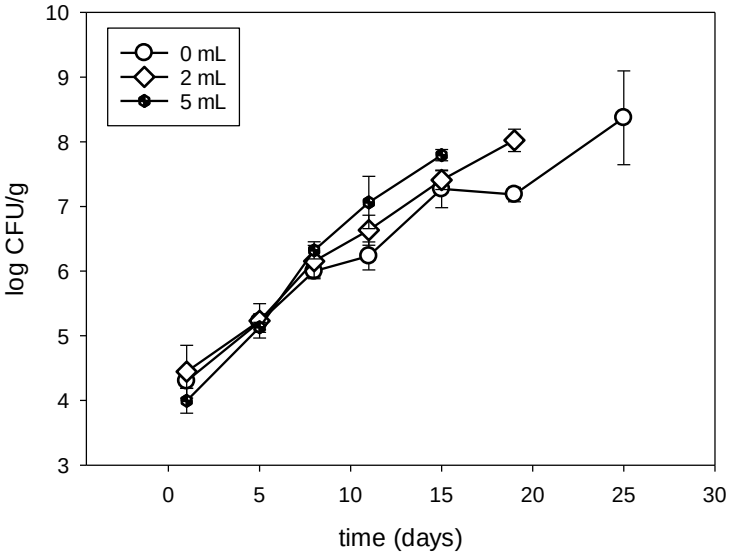


Figure 4.1: Change in TPC count of baby spinach leaves from 60-g bags containing 0, 2 and 5 mL wash water treatment, during storage at 4 °C for 25 d. Error bars represent the standard error of the mean (n=3).

The initial SPAD values for experiment 1 were 43.5-44.6 (supplementary Fig. 1) and decreased to 36.7-39.9 by the end of shelf-life, however residual water treatments had no effect on SPAD values.

The shelf life of baby spinach in bags with 0, 2 and 5 mL added wash water as treatments was 23, 19 and 15 days, respectively based on TPC.

4.4.2: Experiment 2

4.4.2.1: Microbial results

In experiment 2, initial microbial counts for *Pseudomonas* spp. were 4.0-4.9 log CFU/g (Fig 4.2) and increased to 7.7-8.2 log CFU/g after 11 d for baby spinach leaves from bags containing 2 and 5 mL residual wash water treatments, and after 14 and 17 d for bags containing 1 mL treatments and dry leaves, respectively (Fig 4.2). The interaction between day and treatment was significant (p=0.003) such that the rate of increase in *Pseudomonas* spp. count depended on level of added wash water (treatment).

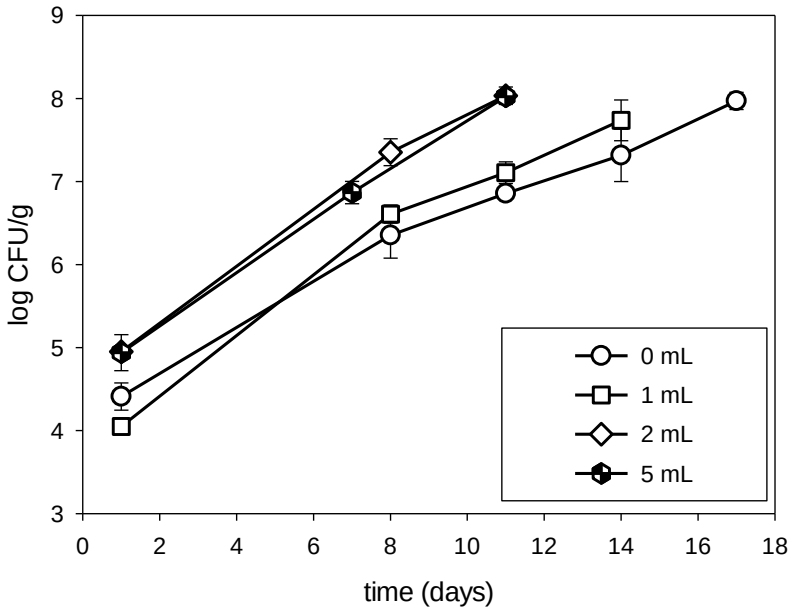


Figure 4.2: Counts of *Pseudomonas* spp. on baby spinach leaves from 60 g bags containing 0, 1, 2 and 5 mL of residual wash water treatments, during storage at 4 °C for 17 d. Error bars represent the standard error of the mean (n=3).

Individual counts for TPC and *Pseudomonas* spp. during the 17-d storage period at 4 °C were positively correlated for all three moisture treatments as illustrated in supplementary Fig. 2.

Initial counts for Enterobacteriaceae ranged from 1.5-2.7 log CFU/g (Fig. 4.3) and increased to 5.0-5.7 by d-11 for 2 and 5 mL treatments and to 4.4 and 5.6 log CFU/g for 1 mL and 0 mL treatments by d-14 and d-17, respectively.

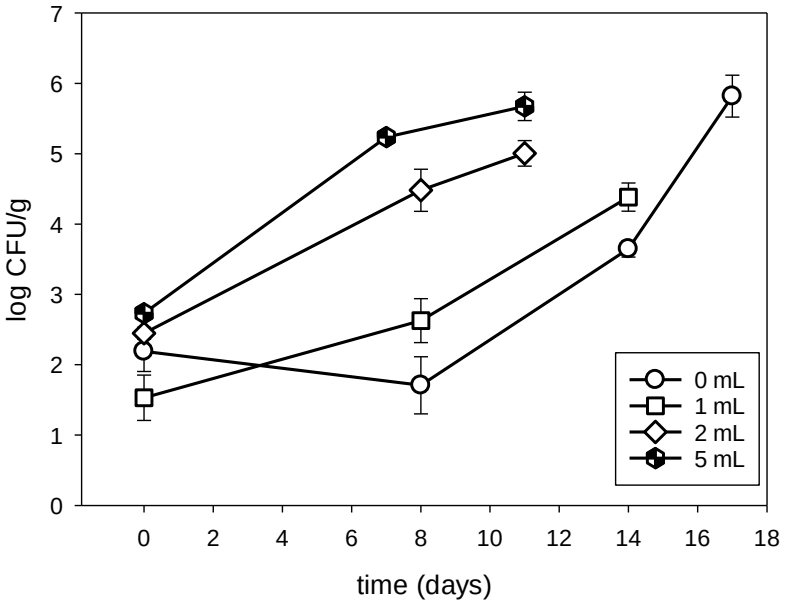


Figure 4.3: Counts of Enterobacteriaceae on baby spinach leaves from 60 g bags containing 0, 1, 2 and 5 mL of wash water treatments, during storage at 4 °C for 17 d. Error bars represent the standard error of the mean (n=3).

Baby spinach packages with 0, 1, 2 and 5 mL residual wash water treatments had a shelf-life of 16, 14, 10 and 10 days respectively mainly based on microbial counts and also sensory assessment.

As expected, increase in TPC was correlated with an increase in sliming (Fig 4.4) on all the salad leaves for all levels of residual moisture treatment. The crossover point between TPC and sliming score occurred at 13 and 12-d for which had 0 and 1 mL treatments (Fig 4.4), whereas it occurred at 6.5 and 7-d for 2 and 5 mL treatments respectively.

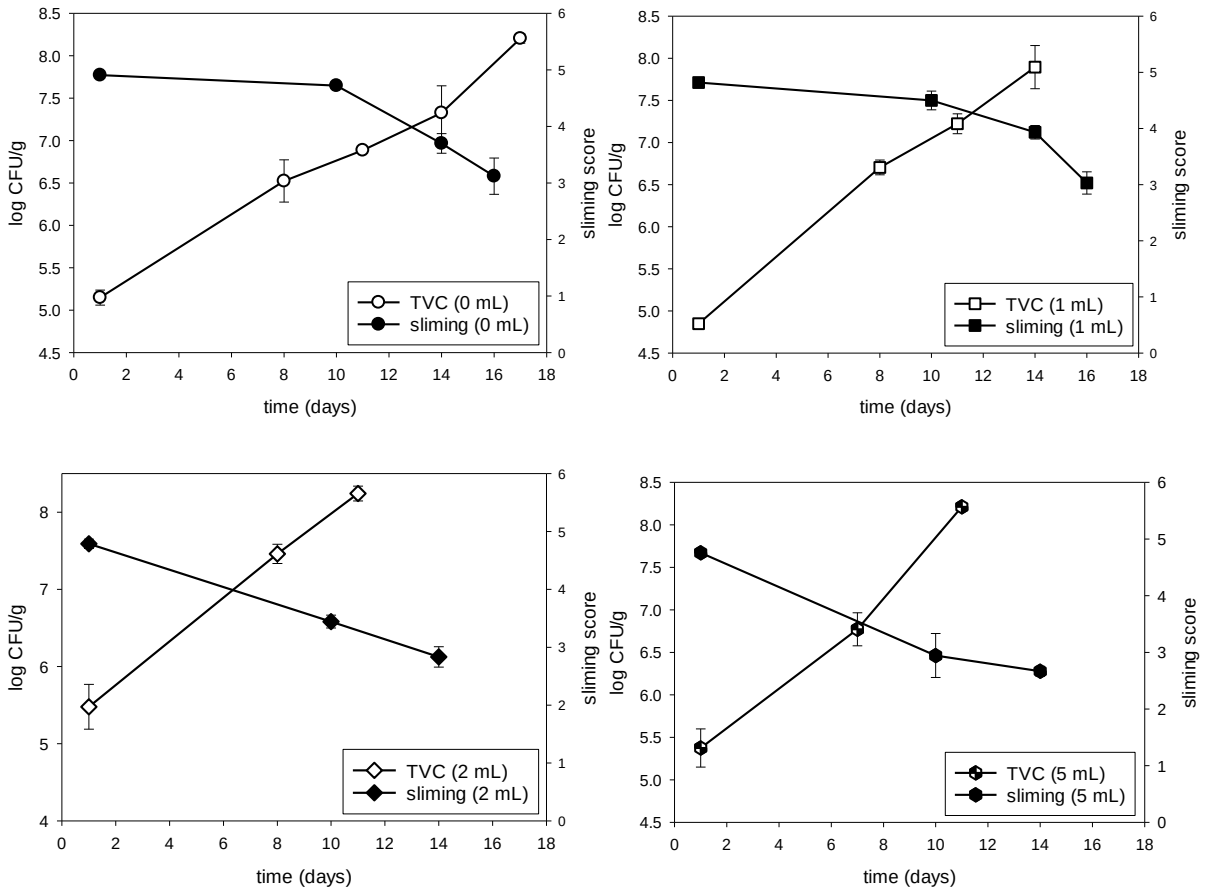


Figure 4.4: Changes in sliming scores vs total plate count (TPC) for baby spinach containing 0, 1, 2 and 5 mL of residual wash water in 60 g bags stored at 4 °C for 16 d. Error bars represent the standard error of the mean (n=6-11 assessors; n=3 for TPC).

4.4.2.2: Sensory evaluation

As expected, an increase in bruising, sliming and yellowing of baby spinach was observed as storage time increased ($p<0.05$) for all the levels of residual moisture treatment (Fig 4.5). At equivalent observation times, baby spinach leaves from bags with 2 and 5 mL treatments were rated lower in sensorial quality compared to leaves containing 0 or 1 mL residual wash water treatments during shelf-life (Fig 4.5). The rate of increase in sliming also depended on the level of residual moisture (treatment) on the leaves ($p=0.008$). Thus, the level of residual moisture influenced the rate of spoilage of the leaves, with higher levels of residual moisture causing more rapid spoilage and shelf-life reduction.

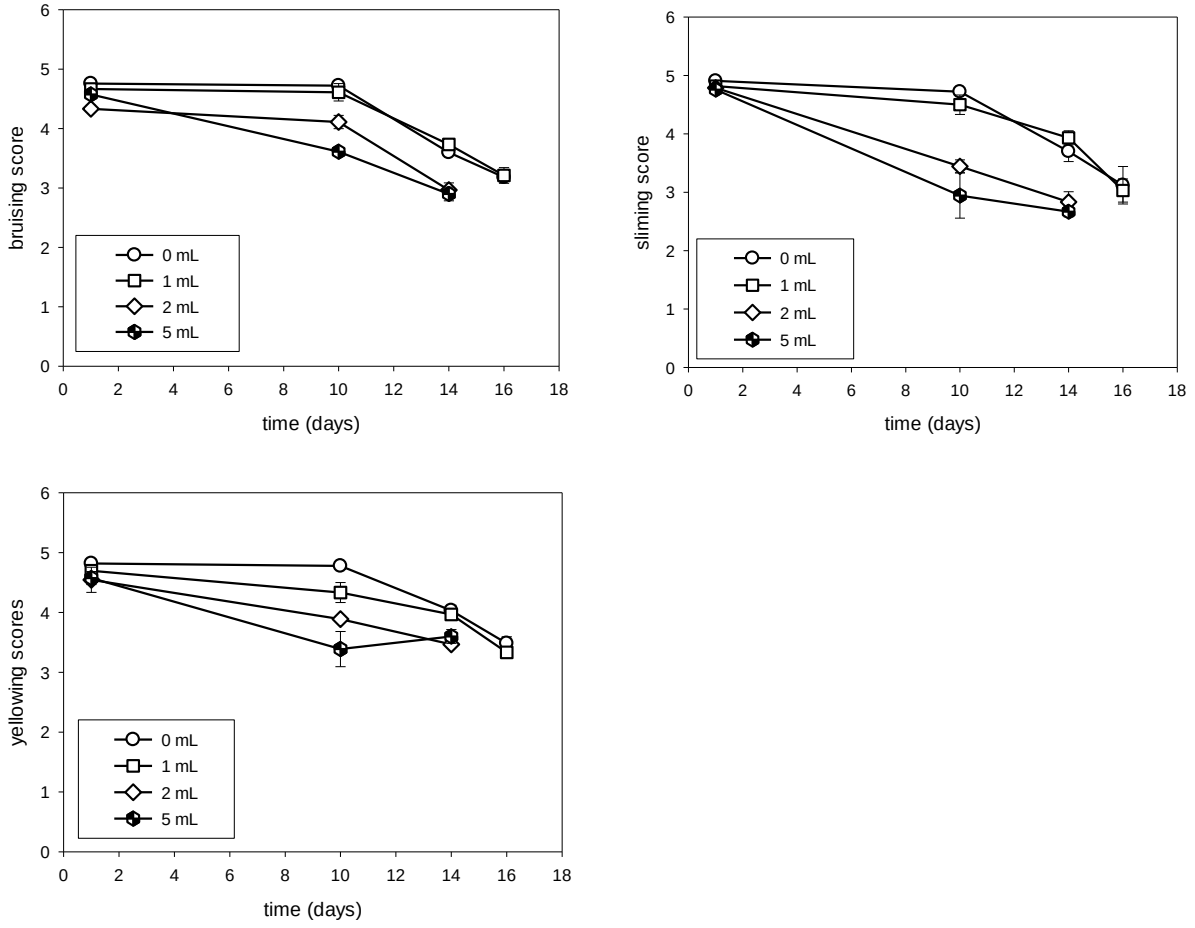


Figure 4.5: Changes in bruising, sliming and yellowing and scores for baby spinach samples containing 0, 1, 2 and 5 mL of residual wash water in 60 g bags stored at 4 °C for 16 d. Error bars represent the standard error of the mean (n=6-11 assessors).

4.4.2.3: Relative humidity

The initial relative humidity inside bags was 81-88% after 30 min, which increased to 100% after 11.5, 19.5, 19.5 and 20 h (Fig. 4.6) for bags containing 5, 2, 1 and 0 mL treatments, respectively, and remained at 100% until the end of shelf-life.

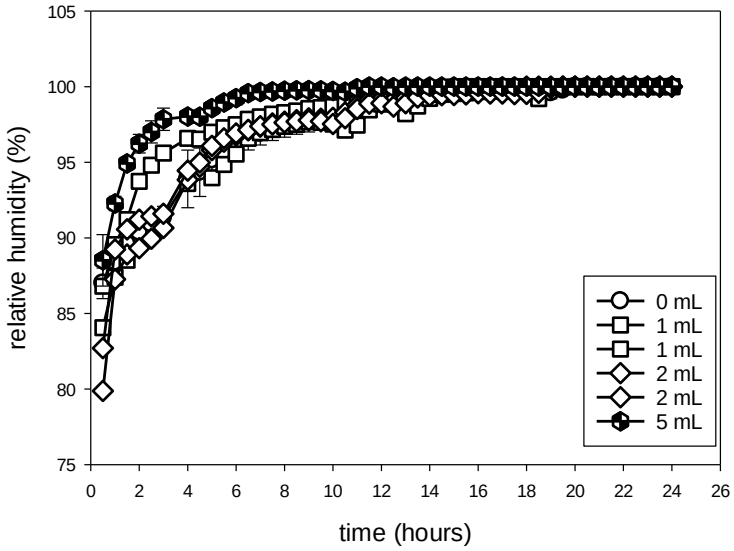


Figure 4.6: Changes in relative humidity (%) inside 60-g bags of baby spinach containing 0, 1, 2 and 5 mL residual wash water treatment, for the first 24 h after sealing and storage at 4 °C. For 0 and 5 mL treatments the average values for two data loggers were plotted, error bars represent standard error of the mean.

4.4.2.4: Water activity of leaves

The initial a_w of leaves was 0.948, 0.968, 0.988 and 0.995 for baby spinach leaves from bags containing 0, 1, 2 and 5 mL of residual wash water, respectively (Fig. 4.7). Residual wash water treatment x time was highly significant ($p<0.0001$). a_w of leaves from bags containing 1 and 0 mL treatments was lower during shelf-life but reached levels of 0.990 by day 10 and 16, respectively.

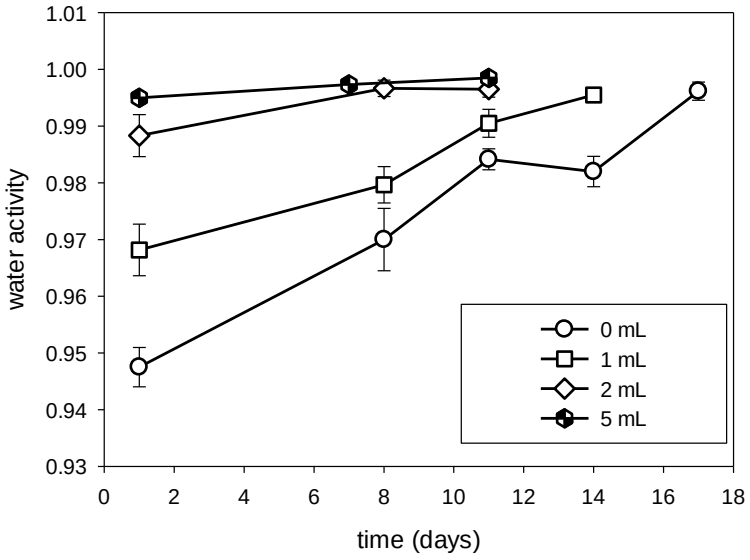


Figure 4.7: Changes in a_w for baby spinach leaves from 60 g bags containing initially 0, 1, 2 and 5 mL of residual wash water, during storage at 4 °C. Error bars represent the standard error of the mean (n=3).

4.4.2.5: SPAD

Initial SPAD values were 50 - 47 (supplementary Fig 3), a decrease in SPAD values was observed with time ($p<0.001$). Residual moisture treatments, however, had no influence on SPAD values ($p=0.064$).

4.5: Discussion

Results from this study clearly demonstrated that residual wash water can significantly reduce the shelf-life of baby spinach. In particular, 2 and 5 mL treatments reduced shelf-life by up to 38% while 1 mL moisture treatment reduced shelf-life by 13%. In agreement with our hypothesis, reducing the level of residual wash water in packaged baby leafy salad spinach improved shelf-life by maintaining sensorial attributes and reducing the rate of microbial growth, as shown in the Results section (Section 4.4). Slower microbial growth observed in 0 and 1 mL treatments corresponds to initial lower water activity values however, the outside of the leaves became more wet with an increase in storage time.

In the second trial, 2 and 5 mL residual moisture treatments resulted in a similar shelf-life of 10 days, and growth rates of TPC and *Pseudomonas* spp. were similar. This result was contrary to our hypothesis, as we expected faster microbial growth and a shorter shelf-life with 5 mL added residual wash water, considering the differences observed between 0, 1 and 2 mL moisture treatments.

TPC increased by 2.8-3.4 log CFU/g during shelf-life and microbial growth was faster with higher moisture treatments. In contrast Pirovani' et al. (2003) observed 3.95-4.59 log CFU/g increase in total aerobic microorganisms during storage at 4 °C in 7 d on fresh-cut spinach, regardless of the moisture treatment. The difference can be explained by the fact that processing conditions were more vigorous involving sanitisation in chlorinated water for 7.5 min with constant stirring (60 times/ min) followed by draining and centrifugal drying therefore bruising could have mainly influenced microbial growth.

In terms of end-of-date of shelf-life, results for visual quality assessment did not always correlate well with microbial counts. For example, based on visual assessment, the shelf-life of the 2 mL treatment was 14 days however, microbial growth was 8.2 log CFU/g, indicating end of shelf-life at day 11. Caponigro et al. (2010) also observed a negative relationship between visual quality scores of baby leaf salads and total viable counts. In a study by Gómez-López et al. (2013) psychrophilic counts on baby spinach reached 8.1-8.6 log CFU/g on day 11 however overall visual quality was still above the limit of acceptability. Wieczyńska et al. (2016b) reported that panellists rated wild rocket with a high quality score because it was brittle, had a dark green colour and developed no odours though the total aerobic bacteria was 7.5-7.7 log CFU/g. Selma et al. (2012) observed that when soil grown lollo rosso lettuce reached 7-8 log CFU/g in mesophilic counts, visual quality was below the limit of acceptability. Tomas-Callejas et al. (2012) reported that tatsoi leaves were rated acceptable in terms of overall sensorial quality after 11 days of storage at 5 °C and had 4.5-

5.8 log CFU/g of total aerobic bacteria, whereas after storage at 10 °C for 5 days visual quality of tatsoi was unacceptable and microbial counts were 5.1-7.1 log CFU/g.

Relative humidity increased to 100% within 19.5-20 hours after sealing the package for the 1 and 2 mL treatments, however, this occurred 8 hours earlier for the 5 mL residual moisture treatment. Thus, as expected, high levels of residual wash water in a bag contribute to relative humidity increase. Previous studies have shown that RH in packaged products can achieve 100% within 24 hrs depending on type of packaging and produce. Caleb et al. (2016) reported that the relative humidity for broccoli branchlets packaged in non-perforated bi-axially oriented polypropylene (BOPP) films rapidly increased to 100% after 12 h whereas in microperforated BOPP it took 3 - 4 days to reach 100% relative humidity. Tano et al. (2007) reported that the relative humidity in mushroom packages increased to 100% after 36 hours however it took 9-10 days for relative humidity in broccoli and tomato packages to get to 100% when the produce was packaged in plastic containers fitted with diffusion windows. The RH in 1 L polypropylene containers containing fresh-cut cucumber, papaya, oranges and pineapple stored at 5 °C reached 70 % in 1 h, and 100% in 12 h (Ayala-Zavala et al., 2008). Moisture accumulation inside OPP bags was visible for all moisture treatments during storage at 4 °C. There needs to be a trade-off between permeable bags that slow down moisture accumulation while also retaining enough moisture to prevent wilting. Packaging of fresh vegetables/fruits in plastic films that have low water vapour permeability compared to produce transpiration results in product deterioration, microbial growth, and condensation within the package (Oliveira et al., 2016).

Conclusion

Residual wash water treatments of 1 - 5 mL (80 mg/L: PAA) in 60 g packaged baby spinach reduced shelf-life 13 - 38%. Bagged spinach containing 2 to 5 ml residual moisture treatments had higher scores for bruising and sliming, microbial growth after the same period of shelf-life, and quality deterioration was faster with higher moisture treatment levels.

Therefore, determining optimum drying settings during processing is a key consideration for improving shelf-life of baby spinach.

4.6 Supplementary data

SPAD exp 1

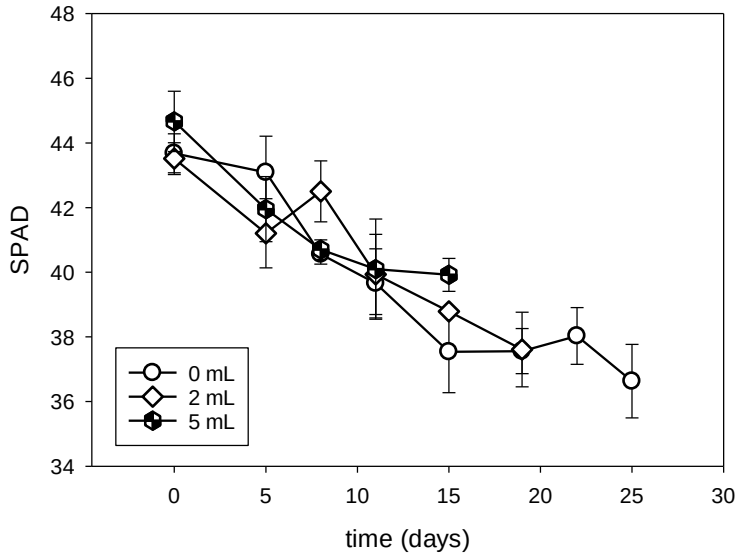


Figure 1: SPAD values for baby spinach leaves from 60-g bags containing initially 0, 2 and 5 ml of surface moisture, during storage at 4 °C. Error bars represent the standard error of the mean (n=3).

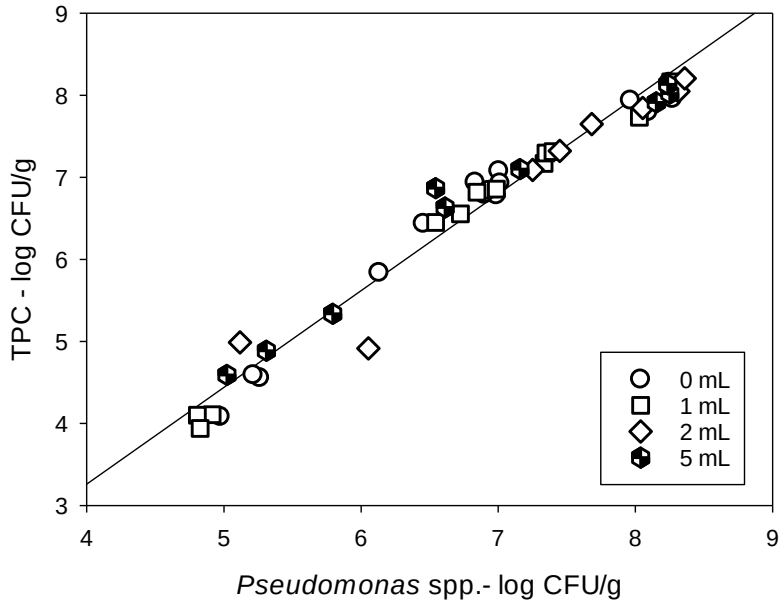


Figure 2: Counts of Pseudomonas spp. vs total plate count (TPC) on baby spinach from 60-g bags containing 0, 1, 2 and 5 mL wash water treatments during shelf-life.

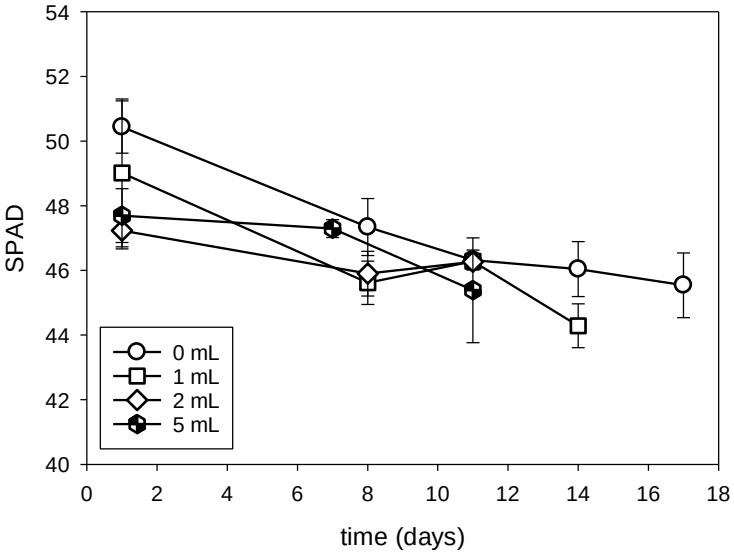


Figure 3: SPAD values for baby spinach leaves from 60-g bags containing initially 0, 1, 2 and 5 ml of surface moisture, during storage at 4 °C. Error bars represent the standard error of the mean (n=3).

Chapter 5: Removal of Grit from Baby Leafy Salad Vegetables by Combinations of Sanitiser and Surfactant

Published as: Dakwa, V., Eyles, A., Gracie., A., Tamplin, M.L., Ross, T. (2019). **Removal of grit from baby leafy salad vegetables by combinations of sanitiser and surfactant** Journal of Food Quality, Volume: 2019 Article Number: 6209806 Published: AUG 4 2019

5.1: Abstract

Grit composed of dirt, sand and small stones adheres to baby leafy salad vegetables during the growing period and can sometimes be difficult to remove with sanitiser only or tap water. For the first time, the effect of a surfactant, sodium dodecyl sulphate (SDS), alone (0.025, 0.05, 0.1 % SDS) and in combination (0.05 % SDS) with peroxyacetic acid (40 mg L^{-1}, PAA), on grit removal, quality, shelf-life and taste of baby spinach was investigated. Increasing SDS from 0.025 to 0.1 % resulted in a 21-50 % increase in grit removal on spinach and coral lettuce. Overall, SDS treatments had no effect on microbial growth, colour and electrolyte leakage during shelf-life. An increase in bruising, sliming and yellowing scores was also observed regardless of the treatment, reaching an unacceptable score (<3) by d-12 for all samples, however yellowing scores were still within an acceptable range (>3) on d-14. There were no differences in sensorial attributes namely, flavour, aroma and texture, between baby spinach samples treated with PAA alone or in combination with SDS. These results demonstrate that SDS treatment can be used to increase grit removal on baby leafy salad vegetables without compromising quality.

5.2 Introduction

Baby leafy salad vegetables are minimally processed, which includes washing with a sanitiser to minimise microbial cross-contamination and to reduce microbial load, pesticide residues, soil and grit (Joshi et al., 2013). Therefore, sanitising improves customer satisfaction, convenience and visual appeal (Jung et al., 2012, Premier, 2013). Grit can attach to leafy vegetables grown in the open field, due to wind or splashing from rain and irrigation, or through mechanical harvesting and can contaminate produce (Rushing et al., 2010). Grit increases the hydrophobic properties of the leaf surface and thus, hinders direct contact between the leaf surface and sanitiser wash water reducing decontamination efficacy (Hassan and Frank, 2003, Huang and Nitin, 2017). Furthermore, grit can habour microorganisms and therefore facilitate their attachment to produce surfaces (Huang and

Nitin, 2017). Ingestion of improperly washed leafy vegetables with grit and soil can have a negative impact on health, if the soil has pathogenic microorganisms, heavy metals, pesticides or fertilisers (Sing and Sing, 2010). Surfactants have been suggested to facilitate removal of bound contaminants from fresh produce surfaces (Xu et al., 2013).

Surfactants are amphiphilic molecules that reduce interfacial/ surface tension of solutions (Predmore and Li, 2011, Xiao et al., 2011, Xu et al., 2013). They consist of a non-polar group attached to a polar group that can either be cationic, anionic, zwitterionic or non-ionic (Karsa, 2006). Surfactants may enhance contact between sanitiser and microorganisms, thus improving microbial inactivation (Huang and Nitin, 2017, Takeuchi and Frank, 2001), and can enable sanitisers to gain access to crevices and cracks in the lettuce *(Lactuca sativa var. crispa)* structure (Salgado et al., 2014). Raiden et al. (2003) states that detergents can successfully clean produce without compromising their structural integrity. SDS is a food grade anionic surfactant that has previously been used with leafy salad vegetables (Guan et al., 2010, Huang and Nitin, 2017, Zhao et al., 2009). Huang and Nitin (2017) observed that sodium dodecyl sulphate (SDS), Tween 20 and lauric arginate at 0.1 % lowered the surface tension of water from 71.17 mN m^{-1} to 46.6, 36 and 36 mN m^{-1}**,** respectively. In the same study, soil particles reduced the ability of the surfactants SDS, lauric arginate and Tween 20 to remove *Escherichia coli* 0157:H7-lux and *Listeria innocua* from romaine lettuce leaf surface by 0.2-0.5 and 0.7-0.8 log CFU cm^{-2}, respectively, compared to control lettuce leaves without soil. Xiao et al. (2011) demonstrated the importance of using surfactants at concentrations exceeding the critical micelle concentration in order to realise its benefits.

The efficacy of a wide range of surfactants to inactivate bacteria and viruses, alone and in combination with sanitisers on leafy salad vegetables has been examined with varying results. Baby spinach leaves (*Spinacea oleracea*) inoculated with *E. coli* 0157:H7 showed a 3.1 log CFU $leaf^{-1}$ reduction following treatment with 1 % thiamine dilauryl sulphate (TDS) in comparison to a simple water wash, and a further 1.4 CFU $leaf^{-1}$ reduction during 7-d of

shelf-life (Zhang et al., 2016b). In contrast, 0.1 % SDS and 0.1 % Tween 80 did not increase the removal of *Salmonella* sp. and *Shigella* sp. on green-leaf lettuce surfaces compared to tap water (Raiden et al., 2003). The combination of surfactants and sanitisers has not always been beneficial. For example, Zhao et al. (2009) observed 4.2-4.5 log CFU g^{-1} reduction in *S. enteritis*, *S. typhimurium* and *E. coli* 0157:H7 on inoculated romaine lettuce after treatment with 0.3 and 0.5 % levulinic acid in combination with 0.05 % SDS for 1 min at 21 °C. However, (Keskinen and Annous, 2011) observed 0.85-1 log CFU g^{-1} reduction of *E. coli* 0157:H7 on inoculated romaine lettuce after treatment with chlorine-based sanitisers, and their efficacy was not improved with addition of either 0.2 % dodecylbenzenesulfonic acid or sodium 2-ethyl hexyl sulphate surfactants for 2 min at 22 °C.

Sanitisers for fresh produce include; chlorine dioxide, hydrogen peroxide, PAA, ozone, electrolysed oxidizing water and organic acids (Keskinen et al., 2009, Ölmez and Kretzschmar, 2009, Premier, 2013). PAA is a non-foaming strong oxidant composed of hydrogen peroxide and acetic acid in an equilibrium mixture and decomposes into benign products that include: water, acetic acid, carbon dioxide and oxygen (Artés et al., 2009, González-Aguilar et al., 2012). PAA sanitiser is preferred over chlorine, as chlorine reacts with organic matter to form trihalomethanes which are potentially harmful to human health (Waters and Hung, 2014).

Despite the presence of grit affecting consumer acceptability, no other studies have considered and quantified the efficacy of SDS alone, and in combination with the sanitiser, peroxyacetic acid (PAA), on the removal of grit from vegetables and fruit in general including leafy salad vegetables. Most of the studies cited above focused on the effect of surfactants on microbial safety, very few of these studies assessed shelf-life and sensory quality (Guan et al., 2010, Salgado et al., 2014) and none involved tasting.

The main objective of this study was to evaluate the effect of SDS treatment alone and in combination with PAA (15.2 %) on grit removal, microbial quality, sensorial attributes and

shelf-life of baby leaf salad vegetables. Two leaf varieties were selected based on their difference in morphology: baby spinach (*Spinacea oleracea*) representing flat leaves varieties and coral lettuce (Lactuca sativa var. crispa) represented curly leaves. The investigation was divided into two stages, involving initial work to identify effective concentrations of SDS namely: 0.025 % 0.05 % and 0.1 % on baby spinach and coral lettuce. A subsequent experiment involved a shelf-life study of baby spinach treated with tap water as control, PAA alone and 0.05 % SDS + PAA including organoleptic evaluation.

5.3 Materials and methods

5.3.1 Plant material

Fresh baby spinach and coral lettuce were harvested manually from a commercial farm in Tasmania, Australia (Richmond Latitude: 42° 44' 2.40" S, Longitude: 147° 26' 24.00" E) at a maturity stage of 40-100 mm length. Given the nature of the study, plant material with a high load of grit was selected based on visual assessment. Samples were transported to the laboratory in an ice box taking no longer than 40 min. Upon arrival, bruised leaves were manually removed. The baby leaves were stored at 4 °C for a maximum of 16 h before use in experiments 1 and 2.

5.3.2 Preparation of treatment solutions

Wash solutions were prepared using potable tap water, Tsunami 100 (active compound, peroxyacetic acid, 'PAA', at 15 %; Ecolab, Minnesota, USA) and sodium dodecyl sulphate (SDS; Sigma-Aldrich, St Louis, MO, USA) (Table 5.1). In both experiments, potable tap water was used as the control and the concentration of PAA used was 40 mg L^{-1}.

Table 5.1: Details on variety of leafy salad vegetable, concentrations of surfactant and sanitiser solutions used for experiments 1 and 2

Experiment number	Baby leafy vegetable	Treatment solutions			
		control	% SDS (w/v)	PAA (40 mg L^{-1})	PAA + 0.05 % SDS
1	spinach and coral lettuce	✓	0.025 0.05 0.1	-	-
2	spinach	✓	-	✓	✓

Treatment solutions were stored overnight at 4 °C. The pH, oxidation-reduction potential (ORP) and turbidity of the solutions was measured by a pH meter (Orion 250A, USA), ORP meter (Milwaukee MW 500, Romania) and turbidity meter (Hach 2100P, USA), respectively.

5.3.3 Sanitising treatment of baby spinach and lettuce

All batches of samples were immersed for 45 s in processing wash water containing sanitizing solution with or without SDS in a ratio of 1:30 (produce:water w/v) containing sanitising solution with or without SDS. In experiment 1, each batch involved washing 30 g of baby spinach and lettuce separately in 900 mL of solution, whereas in experiment 2, 100 g of baby spinach were washed in 3 L wash water. Excess wash water was removed manually with a manual salad spinner and spun three times (8 revolutions/ spin on average). The wash water was collected to allow measurement of total grit removed. Out of the three SDS concentrations tested in experiment 1, 0.1 % SDS produced the most foam therefore, 0.05 % SDS was selected for experiment 2.

Total grit removed was quantified by filtering the wash water through (Whatman filter paper no 1, 18.5cm) by gravity; these filter papers were oven-dried until constant weight at 80 °C. Wash solutions from experiment 2 were double-filtered, using fluted fast flowing VWR filter paper 415 (38.5 cm) first, and then medium-fast flowing fluted Whatman filter paper no. 1 (24

cm) to capture smaller particles. The amount of grit removed was expressed as g per g of fresh leaf biomass

$$\text{Grit removed} = \frac{Nmax}{(1 + \left(\frac{Nmax}{Nmin}\right) - 1) * e^{(-\text{rate} * \%\text{SDS})}} \quad (1)$$

Nmax = maximum grit that can be removed by 0.1% SDS 0.0106, 0.0141, *Nmin* = minimum grit that can be removed by tap water 0.00679, 0.00977, rate = 33.9 and 38.5 for spinach and coral lettuce respectively.

For experiment 2, 40 g of processed baby spinach were packaged manually in oriented polypropylene (OPP) film (Apex films, Victoria, Australia) bags (28 x 16 cm). Bags were stored at 4 °C for subsequent quality assessment during a 14-d shelf-life trial.

On days 0, 4, 7, 10 and 14, three bags per treatment were analysed for microbial load, whereas five bags per treatment were assessed for electrolyte leakage and colour measurements. Prior to washing, samples were also analysed for microbial load on the day of processing. The organoleptic properties of the samples were evaluated during shelf-life as described below.

5.3.4 Microbial analysis

Samples of 10 g from each package were transferred aseptically to sterile filter bags (190 x 300 mm), diluted 1:10 (wt/wt) in 0.1 % sterile buffered peptone water (Oxoid LP0037, UK) and homogenised for 120 s using a stomacher (Colworth Stomacher 400, Seward, London, UK). Subsequently, serial decimal dilutions in peptone were performed and appropriate dilutions were surface-plated on tryptone soya agar (TSA) (Oxoid CM0129, Basingstoke, Hampshire, England) and Pseudomonas agar (Oxoid CM0559, Basingstoke, Hants, UK) containing supplement SR0103 for enumeration of total aerobic plate count (TPC) (72 h at 25 °C) and Pseudomonas spp. (48 h at 25 °C), respectively. Microbial populations were expressed as log CFU g^{-1} of spinach.

5.3.5 Colour measurements

Colour changes of baby spinach, *L** for lightness (ranging from 0 for black to 100 for white), *a** (degree of redness a+ or greenness a-), *b** (degree of yellowness b+ or blueness b-) were assessed during shelf-life. Measurements were taken at two different points on the upper surface of 15 different leaves per treatment using a colourimeter (Konica Minolta chroma meter CR400, Washington, USA) with an 8 mm diameter viewing aperture.

5.3.6 Electrolyte leakage

Following a modified method of Lopez-Galvez, [20], electrolyte leakage was measured using a Conductivity-TDS-pH-temperature instrument (WP-81 version 6, TPS, Brisbane, Australia). Samples (2-g) were cut approximately into 1 cm^2 squares and immersed in 40 mL of distilled water at room temperature for 1 h to obtain the initial electrical conductivity of each solution (C_1) and of distilled water (C_0). Samples were then frozen at -18 °C for 24 h and the total conductivity (C_2) measured after thawing in water at room temperature for 3 h. Tissue electrolyte leakage was calculated using the formula;

$$\mathrm{E} = \left(\frac{C_1 - C_0}{C_2}\right) * 100 \qquad (2)$$

5.3.7 Organoleptic evaluation

For experiment 2, visual quality assessment of nine samples (3 replicates per treatment) was conducted by a panel of up to seven trained members on 0, 3, 7, 10 and 14-d of the shelf-life experiment. Quality deterioration parameters (bruising, sliming and yellowing) were evaluated on a scale of 1-5, with 5 being the highest quality (no defects, no yellowing), 1 the lowest quality and 3 commercially acceptable.

The sensory panel test was performed for samples treated with 40 mgL^{-1} PAA (considered as the control treatment) and 40 mg L^{-1} PAA + 0.05 % SDS. Due to food safety reasons, samples washed with portable water only were not included for tasting. Samples were stored at 4 °C for 48-64 h and removed from the fridge before serving. 48-64 h is the

shortest time it takes for the packaged product to reach the consumer after processing. During the evaluation, two samples treated with PAA and the other treated with PAA + SDS were served at the same time to 34 panelists. Coded samples were rated on flavour, aroma, texture, and overall liking on a 9-point hedonic scale of 1-9 (dislike extremely - like extremely). Panelists were also asked to indicate their purchase intent on a scale of 1-5 (definitely would buy - definitely would not buy). This study was approved by the University of Tasmania Social Sciences Human Research Ethics Committee – ethics reference number H0016331. Written consent to participate was sort from the panelists, specifying that only the sensory evaluation data will be published without identifying individuals involved.

5.3.8 Statistical analysis

Data were analysed using JMP statistical software (version 11, SAS Institute Inc, USA). The relationship between grit removed and % SDS from experiment 1 was evaluated using regression analysis. For experiment 2, two-way analysis of variance (ANOVA) was used to analyse TPC, Pseudomonas count, electrolyte leakage and colour parameters shelf-life data with day and treatment as the independent variables. Grit data was analysed using one-way ANOVA followed by Tukey's honestly significant difference (HSD) test. To understand whether treatment had an effect on taste attributes, data were analysed using the chi-square test in JMP. ANOVA for sensory evaluation data (visual quality assessment) was calculated using "proc mixed" in SAS (version 9.3, USA), a random effect was included for the panelist. A repeated measures approach was assumed with a spatial correlation structure, where the sample code was used as the repeated experimental unit. Assumptions for homogeneity of variance and normality were checked before each analysis. Significance was calculated at $p < 0.05$.

5.4 Results and discussion

5.4.1 Optimising SDS concentration for grit removal from baby spinach and coral lettuce

There was a significant positive correlation between the amount of grit removed and %SDS (Fig. 5.1), R^2 was higher for coral lettuce than spinach. (R^2 *coral* lettuce = 0.734, $p<0.0001$; R^2 *spinach* = 0.372 $p=0.004$).

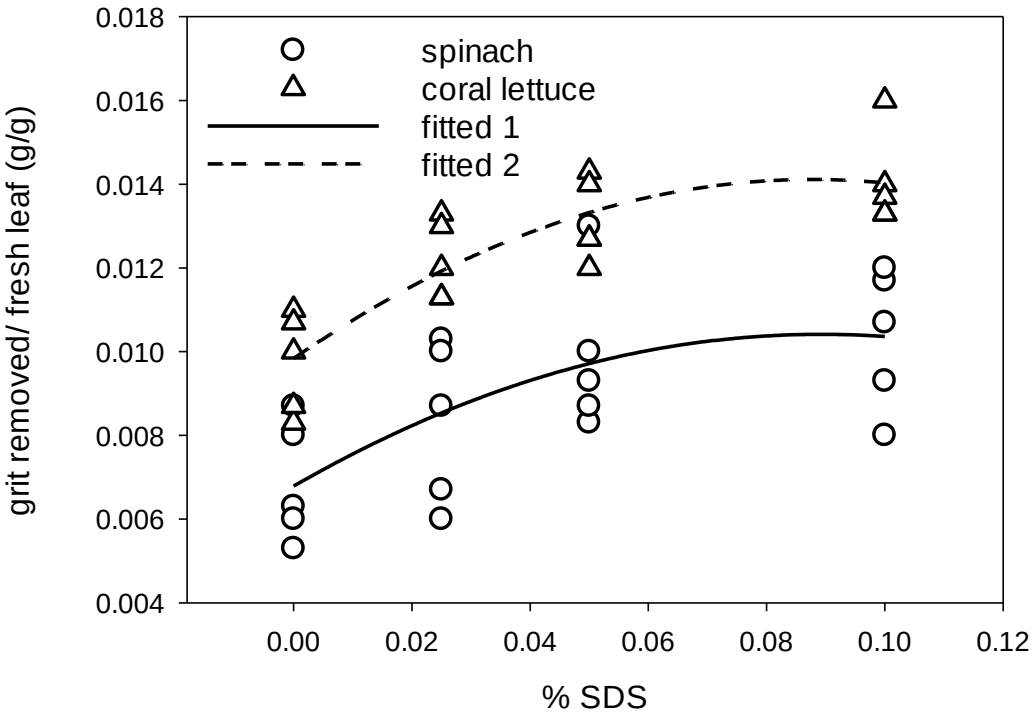


Figure 5.1: Relationship between grit removed per g of coral lettuce and spinach and % SDS concentration. (SDS = sodium dodecyl sulphate).

Increasing SDS concentration also resulted in increased foaming. Ho et al. (2011) also observed excessive foaming in wash tanks containing 250 ppm SDS in combination with peroxyacetic acid + lactic acid.

5.4.2: The effect of PAA + SDS treatment on grit removal, microbial load, shelf-life and taste of baby spinach

5.4.2.1 Wash water characteristics

Addition of SDS to PAA did not influence pH and ORP values (table 5.2) which suggests that SDS does not influence antimicrobial properties of the sanitiser. Zhao et al. (2009) observed a pH of 6 for 0.05% SDS, 3.0 for levulinic acid (LeA) and 3.1 for LA combined with SDS. Guan et al. (2010) also observed pH of 3.04 for 0.5% LA + 0.05% SDS.

Table 5.2: pH and ORP values for wash water solutions used in experiment 2

Wash solution	**pH**	**ORP**
Tap water	6.82	363
PAA (40 mg L^{-1})	4.25	587
PAA (40 mg L^{-1}) + 0.05 % SDS	4.24	557

Although turbidity values of PAA + SDS solution after washing were high (195-228 NTU) compared to the control (79 NTU) and PAA solutions (76 NTU) due to the presence of grit, PAA+SDS solution also had high turbidity values (90-114 NTU) even before washing *(supplementary table S1).*

5.4.2.2 Grit removed

In experiment 2, the combination of SDS (0.05 %) and PAA resulted in a significant increase ($p = 0.0012$) in the amount of grit removed as compared to tap water and PAA alone by 19 and 21 %, respectively (Fig. 5.2). Grit removed by tap water and PAA was comparable (Fig. 2; $p > 0.05$). Preliminary trials also proved that SDS alone washed more grit as compared to tap water *(similar results to Fig. 1)* and PAA+SDS washed off more grit compared to PAA alone *(data not shown)*.

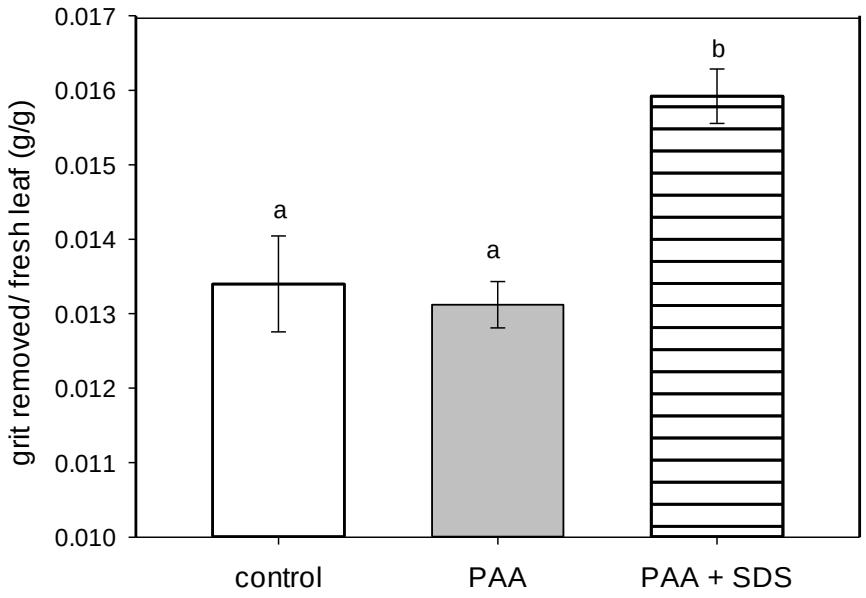


Figure 5.2: Grit removed gram /gram of baby spinach using washing solution treatments (control = tap water, PAA 40 ppm, SDS = 0.05 % sodium dodecyl sulphate). Error bars represent standard error of the mean (n=5). Different letters show significant differences at $p < 0.05$.

5.4.2.3 Microbiological analysis

The initial TPC of baby spinach was 6.6 ± 0.1 log CFU g^{-1} (Fig. 5.3) with significant reductions of 0.85, 1.28 and 1.50 log CFU g^{-1} observed after washing with tap water, PAA and PAA + SDS, respectively (Fig 5.3; $p < 0.001$). A progressive increase in TPC from 5.1-5.8 log CFU g^{-1} was observed during storage across all treatments, reaching similar levels of 7.9-8.3 log CFU g^{-1} on d-10. Samples washed with tap water alone had 0.4 log CFU g^{-1} higher counts ($p = 0.0002$) during the first few days of shelf-life in comparison to PAA and PAA + SDS treated samples during storage (Fig. 5.3). However, no significant difference ($p > 0.05$) in TPC were observed between PAA and PAA + SDS treated spinach throughout the storage period. Initial *Pseudomonas* count was 5.0-5.5 log CFU g^{-1} (Fig. 5.4) with an increase of 2.5-2.9 log CFU g^{-1} observed during shelf-life for all treatments. However, there

was no significant treatment effect (p >0.05) during storage (Fig. 5.4). The growth trend of *Pseudomonas* spp. was similar to that of TPC (Fig. 5.3).

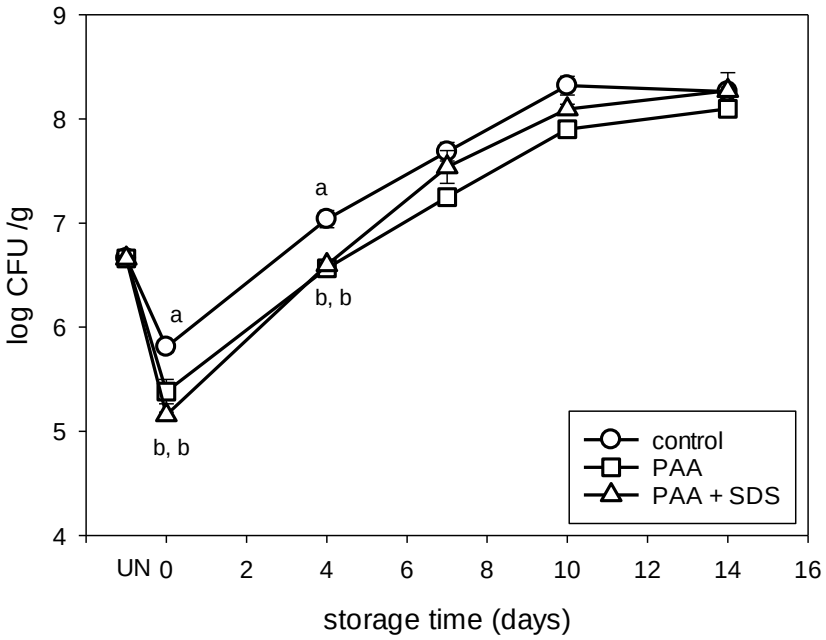


Figure 5.3: Total aerobic plate count of baby spinach leaves treated with tap water (control), peroxyacetic acid (PAA), or peroxyacetic acid + sodium dodecyl sulphate (PAA+ SDS), before wash *(UN)* and after wash during storage at 4 °C for 14 d. Error bars represent the standard error of the mean (n=3). Different letters show significant differences at $p < 0.05$.

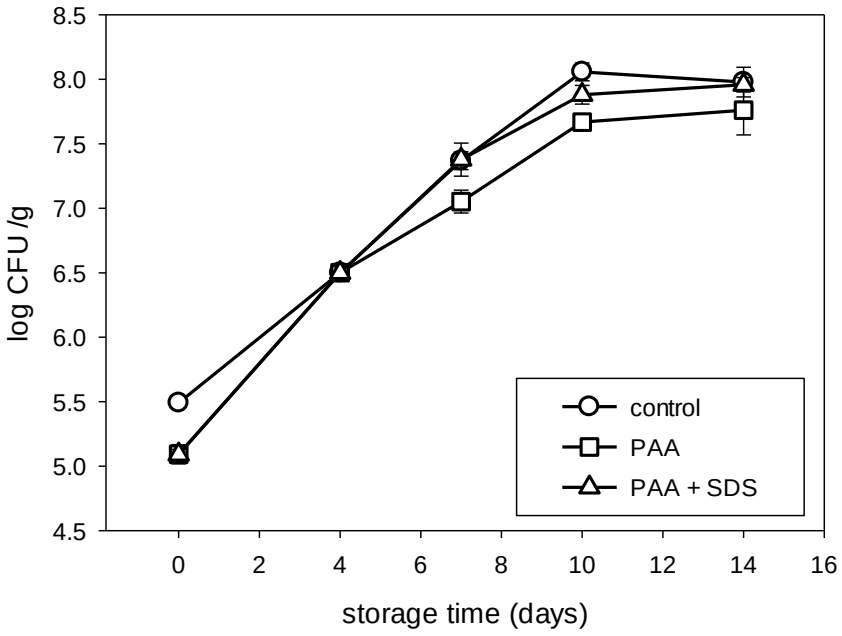


Figure 5.4: Counts of *Pseudomonas* spp. on baby spinach leaves treated with tap water (control), peroxyacetic acid (PAA), or peroxyacetic acid + sodium dodecyl sulphate (PAA+ SDS), during storage at 4 °C for 14 d. Error bars represent the standard error of the mean (n=3).

PAA + SDS treatment did not produce higher initial TPC log reductions or reduce microbial growth during shelf-life in comparison to PAA treatment, and thus, SDS had no effect on microbial quality. Similar results were obtained by Ho et al. (2011) whereby 0.02-0.025 % SDS did not improve the efficacy of PAA (70 mg L^{-1}) and lactic acid (4500 mg L^{-1}) treatment against *E. coli* K-12 and *L. innocua* on innoculted rommaine lettuce and spinach. Salgado et al. (2014) studied the effect of treating lettuce with 1 g L^{-1} SDS + 80 ml L^{-1} Tsunami 100 + ultrasonication on quality aspects. Treatment of inoculated iceberg lettuce with 0.25 % sodium acid sulphate + 0.5 % SDS resulted in 0.87 log CFU g^{-1} decrease in *E. coli* 0157:H7, similar to 0.94 log CFU g^{-1} observed after treatment with 100 ppm chlorine solution (Guan et al., 2010). In the same study, 0.41 log CFUg^{-1} was observed after treatment with 0.5 % LA + 0.05 % SDS for 5 min. Using 0.1 % SDS improved the removal of *L. innocua* from inoculated romaine lettuce by 0.95 log CFU m^{-2} in comparison to deionised water, therefore yielding a total reduction of 1.79 log CFU m^{-2} (Huang and Nitin, 2017). In contrast, Zhao et al. (2009)

observed 4.2-4.5 log CFU g^{-1} reduction of Salmonella spp. and E. coli 0157:H7 on inoculated romaine lettuce after treatment with 0.3 and 0.5 % levulinic acid in combination with 0.05 % SDS for 1 min at 21 °C. Therefore, in literature there is varying evidence on the effect of surfactants on leafy salad vegetables.

5.4.2.4 Colour and electrolyte leakage

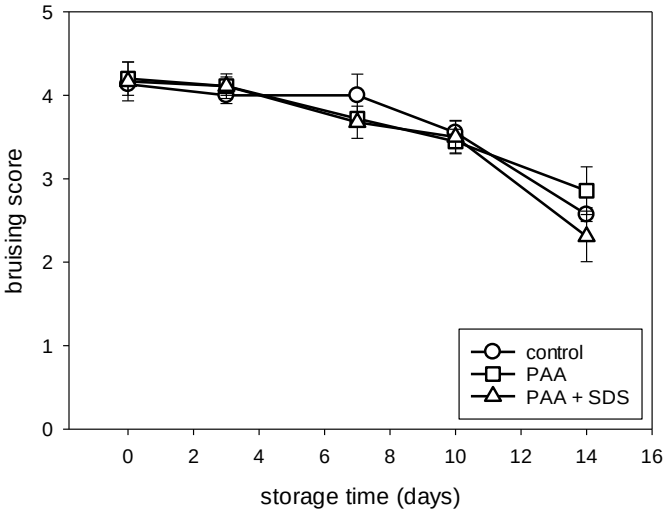
No changes in colour *L** *a** and *b** parameters were observed during shelf-life across all treatments ($p > 0.05$) *(supplementary table S2).* Huang and Nitin (2017) only observed marginal colour changes after washing romaine lettuce with 0.1 %SDS in comparison to water wash.

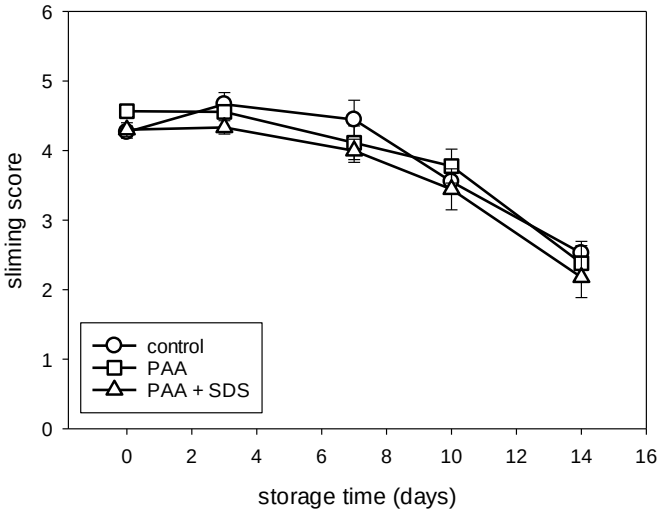
On each sampling day, there was no significant difference in electrolyte leakage ($p>0.05$) between treatments *(supplementary table S3)*. Electrolyte leakage of romaine lettuce washed with 0.1 % SDS alone was not significantly different from the control leaves washed with tap water (Huang and Nitin, 2017).

5.4.2.5 Sensory evaluation

Scores for bruising, sliming and yellowing of baby spinach were similar across treatments during shelf-life (Fig. 5.5; $p > 0.05$). Regardless of the treatment, an increase in bruising and sliming was observed on baby spinach leaves during storage, reaching unacceptable levels (< 3) by d-12. Yellowing scores were still within acceptable range (≥ 3) at the end of shelf-life (Fig. 5.5).

Similarly, Gómez-López (Gómez-López et al., 2013) observed a decrease in overall quality of baby spinach treated with PAA (80 mgL^{-1}), during shelf-life from d-4. In contrast, lettuce treated with water and sodium hypochlorite maintained better visual quality compared to lettuce treated with 0.5 % - 3% levulinic acid + 0.05 % SDS and 0.25 – 0.75 % sodium acid sulphate + 0.05 % SDS during 14-d storage period at 4 °C (Guan et al., 2010).


bruising score
storage time (days)
control
PAA
PAA + SDS


sliming score
storage time (days)
control
PAA
PAA + SDS

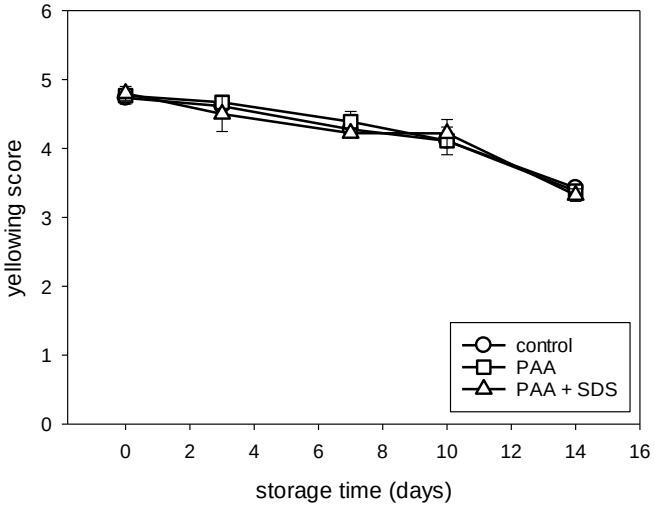

yellowing score
storage time (days)
control
PAA
PAA + SDS

Figure 5.5: Changes in sensorial attributes, bruising, sliming, and yellowing scores for baby spinach samples treated with tap water, peroxyacetic acid (PAA), and peroxyacetic acid + sodium dodecyl sulphate (PAA+SDS), stored at 4 °C for 14-d. Error bars represent the standard error of the mean (n=7 assessors).

Panelists did not identify any significant differences in taste attribute scores nor overall liking between the spinach samples treated with PAA and PAA (40 mgL^{-1}) + 0.05 % SDS + SDS

Fig. 5.6; $p > 0.05$).

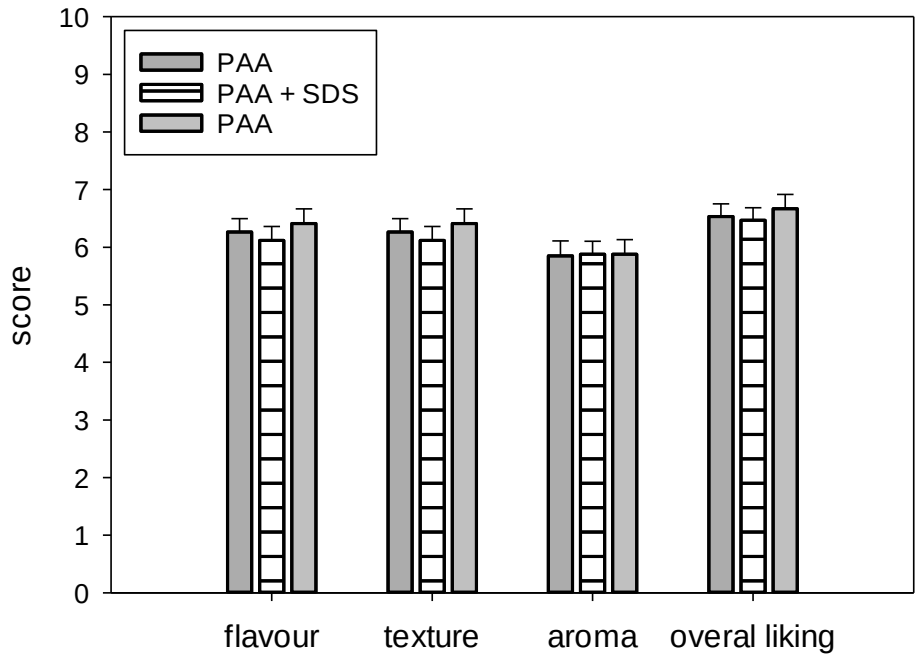


Figure 5.6: Panel test scores for baby spinach treated with peroxyacetic acid (PAA), and peroxyacetic acid + sodium dodecyl sulphate (PAA + SDS), stored at 4 °C for 48-64 h. Error bars represent the standard error of the mean (n=30-34 panelists).

Similar results were obtained by Zhou et al. (2017) where panelists did not observe differences in flavour, appearance and texture between strawberries washed with 0.5 %

levulinic acid + 0.5 % SDS and 50 mL^{-1} chlorine solution for 2 min. Though no studies have examined the effect of PAA + surfactant treatment on the taste of leafy vegetables, Ho et al. (2011) observed no differences in appearance, colour, aroma, taste texture and overall liking of leaf mix containing spinach, chopped iceberg and romaine lettuce treated with PAA + lactic acid compared to samples treated with chlorinated water.

Seventy percent of the consumers reported that they would be willing to purchase baby spinach treated with PAA + SDS based on sensorial quality, 21% were unsure and only 9 % were unwilling.

Conclusions

The use of SDS (0.05, 0.1 %) significantly improved grit removal from baby spinach and coral lettuce in comparison to tap water wash or sanitiser alone. 0.05 % SDS + PAA (40 mgL^{-1}) treatment aids in grit removal without affecting microbial quality, electrolyte leakage, colour *L**, *a**, *b**, shelf-life, sensorial and organoleptic properties of baby spinach. Future research in this area should consider scaling up to pilot plant with the aim of using low concentrations of SDS to reduce potential foaming issues to assess the feasibility of using SDS in a commercial processing facility.

5.4.3 Supplementary data

Table S1: pH, oxidation reduction potential (ORP) and turbidity values of wash solutions used in experiment 2

Wash solution	**pH**	**ORP**	**Turbidity (NTU)**	**Turbidity (NTU)** *(after wash)*
Tap water	6.82	363	1.31	78.8
PAA (40 mgL^{-1})	4.25	587	0.79	75.78
Tsunami 100 (40 mg/L) + 0.05% SDS	4.24	557	89.9	194.8

Table S2: Colour *L*, a*, b** parameters of baby spinach leaves treated with tap water (control), peroxyacetic acid (PAA), or peroxyacetic acid + sodium dodecyl sulfate (PAA+ SDS), during storage at 4 °C for 14 d (*each value is a mean of 15 replicates*)

Treatment	**Storage days**	***L****	***a****	***b****
control	0	39.60667	-14.9967	21.597
	4	39.15533	-14.904	20.11533
	7	39.41933	-15.299	21.20867
	10	39.555	-15.0363	21.12133
	14	39.697	-15.0717	21.102
PAA	0	39.33167	-14.9527	20.794
	4	38.42367	-14.6333	19.99467
	7	38.46633	-14.567	19.716
	10	39.661	-15.024	20.23767
	14	39.551	-14.6713	20.612
PAA + SDS	0	38.71833	-14.5053	19.781
	4	38.735	-14.3567	19.77067
	7	39.59567	-15.0157	20.9293
	10	39.814	-15.3203	21.17767
	14	40.14167	-15.3417	21.05367

Table S3: Electrolyte leakage values for baby spinach leaves treated with tap water (control), peroxyacetic acid (PAA), or peroxyacetic acid + sodium dodecyl sulfate (PAA+ SDS), during storage at 4 °C for 14 d (*replicates per treatment on each sampling day*)

	Day of storage				
Treatment	0	4	7	10	14
Control	3.46	5.52	5.34	4.34	4.66
	3	4.86	5.84	4.65	5.52
	4.34	4.52	5.22	5.13	4.22
	6.2	5.66	4.53	4.52	5.09
	5.64	4.89	5.92	4.89	6.05
PAA	4.94	5.73	4.31	4.05	6.61
	4.77	4.98	6.36	5.28	4.53
	4.66	6.37	4.78	3.95	5.41
	3.83	4.62	5.71	5	6.55
	4.22	5.06	5.94	5.1	5.66
PAA + SDS	3.8	4.98	5.46	5.17	6.51
	3.13	4.24	4.44	4.83	5.45
	3.14	5.59	4.92	4.25	5.33
	3.87	4.56	4.69	5.2	6.32
	3.89	5.6	5.1	5.43	6.51

Chapter 6: General discussion

6.1: Introduction

Studies in this thesis provide insight on how aspects of postharvest handling and processing of baby leafy salad vegetables influence shelf-life and quality, specifically i) the effect of peroxyacetic acid treatment and bruising on the bacterial community of baby spinach (Chapter 3); ii) the influence of excess wash water (containing PAA) (Chapter 4); iii) the effect of PAA and SDS treatment on grit removal of baby spinach (Chapter 5); iv) a preliminary study on the presence of cotyledons (Appendix A1). Other preliminary studies explored the use of different packaging types, ethylene absorber and ethanol emitting sachets on shelf-life of baby leafy salad vegetables (Appendix A2-4).

In short, the studies revealed that at a constant temperature the three most influential factors reducing the potential shelf-life of baby leafy salad vegetables are i) bruising of tissue ii) excess moisture in the package and the presence of cotyledons in the leaf mix. The potential shelf-life could be optimised by minimising these factors.

6.2 Preliminary studies

Results from the preliminary studies demonstrated for the first time that the presence of cotyledons reduced the shelf-life of packaged baby spinach (Appendix A1). This may be explained by the observation that after harvest, microbial load was 0.8 log CFU/g higher on cotyledons compared to baby spinach. Further TPC, grew by up to 2 log CFU/g more on cotyledons than baby spinach by Day 11 during storage at 4 °C. Increased microbial growth was associated with cotyledons turning yellow and slimy earlier. Cotyledons are the first to senesce possibly because they are older and closest to the soil. It would be beneficial to remove cotyledons before packaging baby spinach to extend shelf-life.

Packaging trials assessing 6 types identified only three (all MAP) that had comparable shelf-life to the control OPP (Appendix A2). Leafy salad vegetables packaged in moisture permeable bags had reduced shelf-life due to wilting. The addition of ethanol emitting sachets and ethylene absorbers did not improve shelf-life of baby leafy salad vegetables packaged in OPP (Appendix A3 and A4) - ethanol caused browning of green coral lettuce. Other active packaging technologies have been reported to extend shelf-life of baby leafy salad vegetables and warrant further investigation (Inestroza-Lizardo et al., 2016, Lee and Chandra, 2018, Mudau et al., 2018).

6.3: Effect of peroxyacetic acid treatment and bruising on the bacterial community and shelf-life of baby spinach

Bruising reduced the shelf-life of baby spinach by 48% compared to 100% intact leaves, which is consistent with two earlier studies (Ariffin et al., 2017, Poonlarp et al., 2018). The shelf-life of 100% bruised spinach leaves and 40% bruised + 60% intact was similar, illustrating that, the presence of even a few bruised leaves in a bag reduces shelf-life. These results suggest that significant gains could be achieved by minimising bruising during harvesting and handling operations and implement efficient sorting systems during processing.

This study is the first to explore the effect of bruising on the bacterial community of baby leafy salad vegetables. Contrary to my hypothesis, the bacterial diversity index of bruised, bruised + intact vs intact leaves during storage at 4 °C was not significantly different. Similarly, the relative abundance of known spoilage bacteria such as; *Pseudomonas*, *Chryseobacterium*, and *Flavobacterium* (Betts, 2006, Lee et al., 2011, Lee et al., 2013, Saranraj et al., 2012) were not influenced by leaf integrity. Instead, the shorter shelf-life of bruised leaves appeared to be associated with faster growth of TPC.

To my knowledge, this is the first study to explore the effect of PAA treatment on bacterial community of baby spinach on the day of processing and during shelf-life compared to

water-washed samples. The bacterial diversity on baby spinach did not change after washing baby spinach with PAA or tap water though a decrease in the relative abundance of *Exiguobacterium*. Daddiego et al. (2018) reported differences in bacterial community profiles between shredded lettuce treated with 75 mg/L PAA vs chlorinated water (20-30 mg/L) using T-RFLP analysis on the first day of storage at 8 °C after sanitisation. Tatsika et al. (2019) reported that there was no change in bacterial diversity after washing chopped ready-to-eat spinach with 1% vinegar. Gu et al. (2018) reported changes in relative abundance of bacterial species before and after washing baby spinach with chlorinated water and a decrease in the Shannon diversity index. My data identified the presence of common spoilage bacteria on baby spinach samples before washing however, these bacteria were not eliminated or reduced by the washing process.

The bacterial diversity of baby spinach sanitised with PAA during storage at 4 °C was lower compared to water-washed samples. PAA-treated baby spinach was dominated (>50% relative abundance) from d-6 by *Pseudomonas* till end of shelf-life. *Pseudomonas* is the main spoilage bacteria on leafy salad vegetables (Ioannidis et al., 2018, Lee et al., 2011, Lee et al., 2013). With respect to the other bacterial genera, the relative abundance *Pantoea, Exiguobacterium, Chryseobacterium, Paenarthrobacter,* and *Rhodococcus* was higher in water washed compared with sanitised samples. It is posited that the observed similar shelf-life of sanitised and water-washed baby spinach was due to the relative dominance of *Pseudomonas* and *Pantoea* spoilage bacteria.

Sanitisation with 80 mg/L PAA did not extend the shelf-life of baby spinach, though it gave a higher log initial reduction in TPC as compared to tap water, 0.9-1.4 vs 0.5 log CFU/g. Premier (2013) reported an extra 4 days of shelf-life on baby spinach treated with 40 mg/L PAA compared to water-washed spinach however, at 100 mg/L PAA the shelf-life was similar. Though in my study PAA sanitisation had no benefit in shelf-life, the use of sanitisers is still very important in maintaining the microbial quality of the wash water to prevent cross contamination (Allende et al., 2008a, Gil et al., 2009).

6.4: The influence of excess wash water (peroxyacetic acid) on the shelf-life of baby spinach

No other study has tested the effect of different levels of excess wash water (PAA) on shelf-life and quality attributes of baby leafy salad vegetables including humidity relative monitoring in-package. This study demonstrated that excess wash water (1 - 5 mL) containing PAA reduced the shelf-life of baby spinach (60-g) by 13% - 38%, respectively, based on microbial and visual quality assessment. Consistent with my hypothesis, 2 and 5 mL excess wash water promoted microbial growth and had higher scores for sliming and bruising during shelf-life.

Relative humidity remained at 100% from end of day-1 till the end of shelf-life, due to respiration and transpiration (Bovi et al., 2016), this contributed to moisture accumulation during shelf-life as evidenced by condensation in all the bags. Results also confirmed that OPP bags are not moisture permeable. Piagentini et al. (2002) reported 0.24% moisture loss from mono OPP containing 70-g fresh-cut spinach during storage at 4 °C. Though the high relative humidity prevented weight loss and wilting of the leaves (Medina et al., 2012), it contributed to excess moisture.

6.5: Removal of grit by combination of sanitiser and surfactant

This study demonstrated that grit removal was improved using the surfactant sodium dodecyl sulphate (SDS), increasing SDS concentration from 0.025 – 0.1% increased grit removal by 21-50% on baby spinach and coral lettuce. Few authors have reported the effect of surfactants alone or in combination with sanitisers on inoculated pathogens on leafy salad vegetables (Guan et al., 2010, Huang and Nitin, 2017, Keskinen and Annous, 2011, Raiden et al., 2003, Xiao et al., 2011, Zhao et al., 2009) however, this is the first study to test the effectiveness of sanitiser and surfactant treatment on grit removal, quality and taste of baby leafy salad vegetables during shelf-life.

PAA (40 mg/L) + 0.05% SDS treatment enhanced grit removal by 21% without compromising quality and taste of baby spinach. SDS had no influence on microbial quality and did not compromise the efficacy of PAA since PAA and PAA + SDS gave comparable log reductions of 1.28 and 1.5 log CFU/g respectively, with similar growth during storage at 4 °C. Similar to my results, Keskinen and Annous (2011) did not realise antimicrobial benefits of sanitiser (chlorine solution) + surfactant treatment on *E. coli* O157:H7 on lettuce. In contrast Xiao et al. (2011) and Zhao et al. (2009) found that treatment with sanitiser + surfactant gave higher log reductions of *E. coli* O157:H7 and *Salmonella* on baby spinach and lettuce.

6.6: Conclusions and future research

Bruising reduced the shelf-life of baby spinach by 48% and promoted the growth of microorganisms. This study further confirmed the importance of maintaining tissue integrity to slow down microbial growth and spoilage. Efficient sorting systems should be developed to remove bruised leaves and cotyledons before packaging and efforts taken to minimise bruising during handling, transportation and storage in order to extend shelf-life. Future experiments could explore critical bruised to intact leaves ratio which promotes spoilage and to study the progression of spoilage during cold storage in bags containing mixed leaf qualities.

Spoilage bacteria such as *Pseudomonas, Pantoea, Erwinia, Chryseobacterium, Stenotrophomonas a*nd *Sphingobacterium* on baby spinach were tolerant to PAA treatment. Other studies could explore the effect of essential oil treatments such as tea tree oil, thyme oil and zataria oil on the bacterial community of baby leafy salad vegetables since other authors reported that they exhibit an antimicrobial effect during cold storage. The effect of postharvest technologies such as ultraviolet radiation, ultrasonication and irradiation on the microbiome of baby leafy salad vegetables could also be studied.

Excess wash water 1-5 mL PAA in 60-g bag of baby spinach reduced shelf-life by 13% - 38%, therefore commercial drying process after washing leafy salad vegetables should be optimised, to get rid of excess wash water in order to extend shelf-life. Future studies could also focus on reducing excess wash water on baby leafy salad vegetables by the use of moisture permeable film, maintaining the right balance to avoid moisture loss. It is important to further understand whether the residual PAA solution contributes to the decrease in shelf-life of baby salad leaves by introducing an extra rinsing step with distilled water and investigating the effect of excess water in comparison with excess PAA solution.

Surfactant SDS (0.05%) in combination with PAA (40 mg/L) can be used to enhance grit removal in circumstances where there is a high load of grit on baby leafy salad vegetables without compromising quality and taste. SDS could also potentially be added during the pre-rinsing step before sanitisation of salad leaves. Other studies could also determine the safe concentration of SDS to use by also testing the chemical residue remaining on leaves after washing. Future research should test natural surfactants which have an antimicrobial effect such as lauric arginate (Huang and Nitin, 2017), on grit removal and taste of baby leafy salad vegetables.

Packaging types tested in the preliminary studies did not extend the shelf-life of baby leafy salad vegetables compared to the control OPP. Future studies may explore the use of other packaging materials/ MAP variations in effort to extend shelf-life, monitoring the changes in gas composition during storage.

Preliminary studies demonstrated that the presence of cotyledons inside packaged baby spinach reduces shelf-life. Future work could study on how to remove cotyledons efficiently during the commercial processing steps before packaging in order to extend shelf-life.

Appendices

Appendix A. Summary of pilot studies

To explore possible ways of shelf-life extension and to identify other factors limiting shelf-life of baby leafy salad vegetables, four preliminary studies were conducted, namely;

1) Role of cotyledons in reducing the shelf-life of baby spinach
2) Influence of packaging type on shelf-life extension of baby leafy salad vegetables
3) Effect of ethanol emitters on microbial quality and shelf-life of baby spinach, coral lettuce and rocket
4) Use of ethylene absorbers on shelf-life of baby spinach

Appendix A1: Pilot study 1 on cotyledons

Background

Cotyledons were the first to become slimy during shelf-life studies of baby spinach. These plant structures are part of the embryo inside the seed and are the first leaves to emerge during germination (Stivers and Dupont, 2012). Cotyledons are harvested along with the true leaves and are often not separated during the sorting process, as they can stick to leaves during washing and drying processes, and then end-up in the packaged product.

Aim: To understand how cotyledons influence shelf-life of packaged baby spinach

Materials and Methods

Plant material and sanitisation

Baby spinach leaves including cotyledons, were hand harvested at a commercial farm in Richmond (latitude: 42° 44' 2.40" S, longitude: 147° 26' 24.00" E), Tasmania, Australia and were transported under refrigeration conditions to the laboratory within 30 min. Upon reception, baby spinach leaves were separated from cotyledons and cooled to 4 °C for one

hour, and then sanitised with chilled Tsunami 100, 80mg/L (active compound, peroxyacetic acid, 'PAA', at 15 %; Ecolab, Minnesota, USA) diluted 1:30 baby spinach to sanitiser ratio for 45 sec. Excess sanitiser solution was removed by spinning the salad spinner three times (8 revolutions per spin). Leaves were packaged manually in oriented polypropylene (OPP) film (Apex films, Victoria, Australia), using 40-g baby spinach and 12-g cotyledons, followed by storage at 4 °C for the shelf-life study. Microbial analysis of total plate count (TPC) was conducted before and after washing, and during shelf-life.

Microbial analysis

Ten grams of baby spinach and cotyledons were transferred separately aseptically from each package to sterile 190 x 300 mm Whirl-Pack bags (Nasco, Fort Atkinson, Wisconsin) and diluted in 0.1% sterile buffered peptone water (Oxoid LP0037, UK) to 1:10(w/w). The mixture was homogenised with a stomacher (Colworth Stomacher 400, Seward, London, UK) for 120 s. Serial decimal dilutions of the homogenate were consecutively performed in 0.1% peptone. Appropriate dilutions were surface-plated on tryptone soya agar (TSA; Oxoid CM0129, Basingstoke, Hampshire, England) for enumeration of total aerobic plate count (TPC). Plates were incubated at 25 °C for 72 h, microbial populations were expressed as log CFU/g of spinach.

TPC data for cotyledons and baby spinach, before, after wash and over time, were analysed by 2-way analysis of variance (ANOVA) using JMP statistical software (version11; SAS Institute Inc., USA).

Results and Discussion

Initial TPC of cotyledons was significantly higher ($p=0.008$) than baby spinach (Fig 1). Following sanitisation, log reductions of 1.23 and 1.45 CFU/g were observed for cotyledons and baby spinach, respectively (Fig 1). The interaction effect between time and leaf type was significant ($p=0.018$); by day 11 TPC had increased by 3.85 log CFU/g for cotyledons compared to an increase of 1.88 log CFU/g for baby spinach (Fig 1). Based on TPC values

and visual quality assessment on day-18, baby spinach leaves had not yet reached end of shelf-life however, cotyledons had a shelf-life of only 10 d.

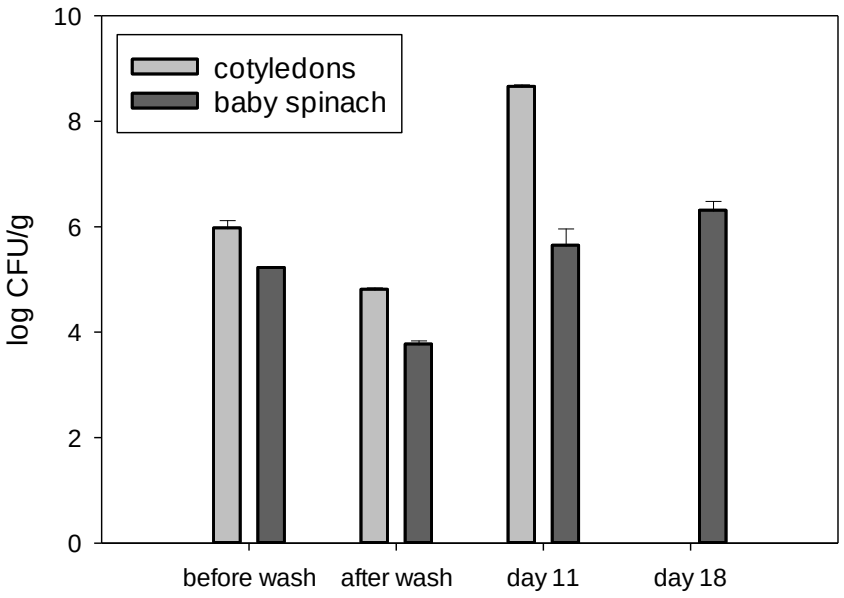


Figure 1: Total aerobic plate count of cotyledons and baby spinach leaves treated with peroxyacetic acid (PAA), before, after washing and during storage at 4 °C for 18 d. Error bars represent the standard error of the mean (n=3).

Cotyledons turned yellow and slimy quicker than baby spinach leaves (Fig 2.), signifying senescence, possibly because cotyledons were older compared to baby spinach leaves.

Figure 2: Packaged cotyledons after storage at 4 °C for 11 d.

Conclusion

Cotyledons have a higher initial microbial load after harvest and promote faster microbial growth during shelf-life compared to baby spinach. Therefore, including cotyledons when packaging baby spinach leaves, significantly reduces shelf-life.

Appendix A.2: Pilot study 2 on effect of packaging type on shelf-life

Background:

Packaging type can extend shelf-life of produce (Dash et al., 2013, Wilson et al., 2019), and produce companies constantly seek new packaging designs to optimise shelf-life. Oriented polypropylene (OPP) is often used for packaging leafy salad vegetables (Conte et al., 2008, Kenny and O'Beirne, 2009). In this study, packages from four countries were selected based on information from the packaging company websites on possibility of shelf-life extension.

Aim: To evaluate the potential of six packaging options for extending shelf-life of baby leafy salad vegetables

Materials and Methods

Six packaging types were trialled:

A - control (OPP)

B - Modified atmosphere packaging (low density polyethylene containing mineral)

C - Modified atmosphere (low permeability) / modified humidity bag packaging

D - Modified atmosphere (high permeability) / modified humidity bag packaging

E - Temperature responsive bag

F - Moisture permeable bag

G - Modified atmosphere packaging

The packaging types were tested in comparison to the control (package A (OPP)) for shelf-life duration of baby leafy salad vegetables. Baby spinach, coral lettuce and rocket were sanitised separately with 80 mg/L PAA 1:30 leaf to sanitiser ratio for 45 sec and dried to remove excess surface moisture, conducted at a commercial facility in Cambridge, Tasmania, Australia. The salad leaves were packaged separately into 120-g bags mentioned

above and transported in an ice box to the laboratory within 30 min. Upon arrival the bags were weighed and stored at 4 °C for shelf-life studies.

A team of three panellists conducted visual quality assessment of baby spinach, coral lettuce and rocket during storage, assessing for bruising, sliming and yellowing, to determine end of shelf-life. The bags were then weighed again and analysed for total microbial load and colour parameters at the end of the storage period. The total amount of weight lost during the storage period was expressed as a % of the initial weight.

Colour assessment

Colour parameters *L** (degree of lightness, ranging from 0 for black to 100 for white), *a** (degree of redness a+ or greenness a-), *b** (degree of yellowness b+ or blueness b-) were measured at the end of shelf-life. Readings were taken at two opposite sides of the upper leaf surface of baby spinach and rocket using a Konica Minolta colourimeter (CR400, Washington, USA) with a 8 mm diameter viewing aperture. The average of two readings were considered as the reading for the leaf; five bags per packaging type were assessed.

Microbial analysis

Ten grams each of baby spinach, coral lettuce and rocket from each bag (3 bags per packaging type) were aseptically transferred to filter bags (190 x 300 mm) and diluted 1:10 (w/w) with 0.1% sterile buffered peptone water. A stomacher was used to homogenise the mixture was for 120 s. Consecutive serial decimal dilutions of the homogenate were made in 0.1% peptone. Enumeration of TPC was conducted by surface-plating appropriate dilutions on TSA followed by incubating plates for 72 h at 25 °C. Microbial counts were expressed as log CFU/g of baby spinach/ coral lettuce/ rocket.

Statistical analysis:

Data was analysed using one-way analysis of variance (ANOVA) with JMP statistical software.

Results and Discussion

Moisture loss

Packaging type A-E only lost < 1% (Fig 3) of weight during shelf-life and packaging type G lost 1.2% moisture in 18 d during storage at 4 °C *(data not shown)*. Though packaging B and E lost more moisture as compared to package A, C and D, the percentage moisture lost was <1%.

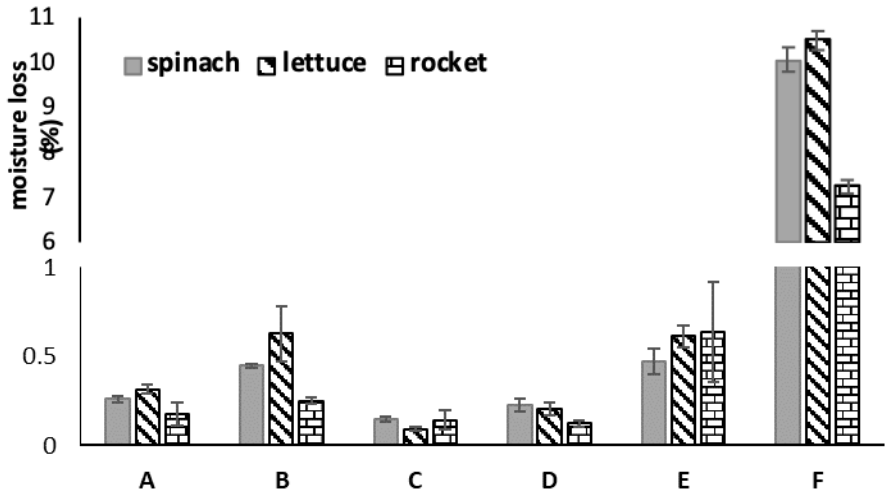


Figure 3: Total % moisture lost from baby spinach, coral lettuce and rocket during storage at 4 °C. Error bars represent standard error of the mean (n=5).

However, the amount of moisture loss did not depend on leaf variety (p=0.055). The shelf-life of rocket was the shortest, regardless of the packaging type since the initial quality was poor. Packaging type F proved unsuitable for all the baby leafy salad vegetables since they lost 5% moisture by day-5 (Fig 3), 7% by day-9 for rocket and 10% moisture by day-14 for baby spinach and lettuce.

Microbial counts were lower on baby spinach and coral lettuce for packaging F (table 1) because high moisture loss reduces water activity and reduces microbial growth. Rocket had higher initial TPC ($p<0.001$) compared to baby spinach and lettuce (table 1). Browning marked the end of shelf-life for lettuce even though TPC was lower.

Table 1: Total plate count values (log CFU/g) at the start and end of shelf-life for baby spinach, coral lettuce and rocket. Each number is the mean of three replicates, the standard error of the mean is represented in brackets.

leafy vegetable	initial	Packaging type A	B	C	D	E	F	G
baby spinach	3.89 (±0.09)	7.84 (±0.11)	8.21 (±0.07)	7.65 (±0.07)	8.05 (±0.07)	8.44 (±0.19)	6.22 (±0.17)	8.54 (±0.05)
coral lettuce	3.24 (±0.25)	6.02 (±0.17)	6.90 (±0.38)	6.47 (±0.25)	6.67 (±0.19)	6.96 (±0.09)	4.71 (±0.09)	5.43 (±0.12)
rocket	5.42 (±0.19)	8.74 (±0.08)	9.00 (±0.09)	8.41 (±0.12)	8.73 (±0.09)	8.48 (±0.06)	8.31 (±0.31)	7.59 (±0.29)

Packaging type had no influence on colour *L**, *a**, *b** parameters ($p>0.05$) of baby spinach (Fig 4) and rocket (Fig 5) during shelf-life.

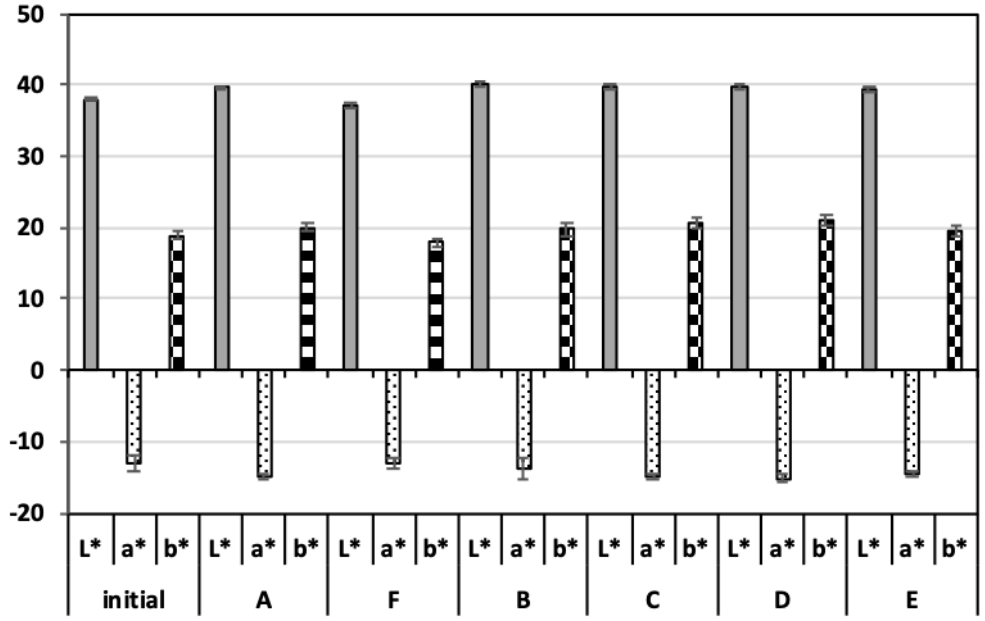


Figure 4: Colour *L**, *a**, *b** parameters for baby spinach, at the start and end of shelf-life. Error bars represent the standard error of the mean (n=15).

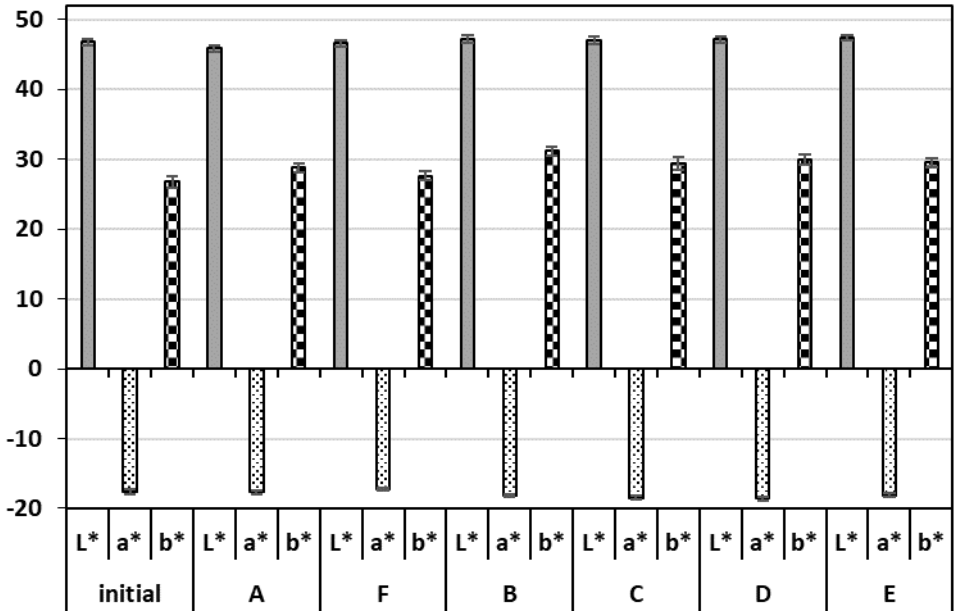


Figure 5: Colour *L**, *a**, *b** parameters for baby spinach, at the start and end of shelf-life. Error bars represent the standard error of the mean (n=15).

Though packaging type B gave 3 days extra shelf-life for baby spinach (table 2), it did not extend the shelf-life for the other leaf varieties. It allows fogging inside the package and the material is very flexible thus making the baby leafy salad vegetables susceptible to bruising during handling. As explained earlier packaging type F gave a shelf-life of 5-d (table 2) due to moisture loss.

Table 2: Shelf-life duration in days based on visual quality assessment and / microbial quality as shown in table 1

leafy vegetable	Packaging type A	B	C	D	E	F
baby spinach	17	20	17	17	17	5
coral lettuce	17	17	17	17	13	5
rocket	9	9	9	9	7	5

The shelf-life for packaging E was shorter for lettuce and rocket this could possibly be because it contained 180 g of leaf material. Its membrane is designed to adjust air permeability in response to temperature fluctuations and respiration rate may not be evident at a constant low temperature. In a separate experiment, packaging type G produced shelf-life days comparable to the control for the three leaf varieties.

Conclusion

Modified atmosphere packaging produced the longest shelf-life similar to the control. High moisture permeability significantly reduced the shelf-life for baby spinach, lettuce and rocket.

Appendix A3: Pilot study 3 on use of ethanol sachets

Background

Ethanol is an antimicrobial agent (Alo et al., 2012, Valle et al., 2016), however the effect of ethanol emitters for packaging leafy salad vegetables has not been explored. We hypothesized that including ethanol sachets when packaging baby spinach, lettuce and coral would slowdown microbial growth and improve shelf-life.

Aim: To investigate the effect of including ethanol sachets when packaging baby leafy salad vegetables on microbial quality and shelf-life.

Materials and Methods

Baby spinach, lettuce and rocket, were sanitised separately at a commercial facility in Cambridge, Tasmania, Australia, with 80 mg/L PAA for 45 sec and dried to remove excess surface moisture. Salad leaves were then packaged in a cold room into 60- and 120-g OPP bags that included ethanol grade 20 (55×65 mm) and grade 40 (70×65 mm) sachets, respectively; control bags did not contain ethanol sachets. Sealed bags were transported in an icebox to the laboratory and stored at 4 °C for shelf-life studies. Microbial analysis (TPC) and visual quality assessment on bruising, sliming and yellowing were assessed by three panellists.

Microbial analysis

Enumeration of TPC was conducted as follows: 10-g of baby spinach, coral lettuce and rocket were transferred aseptically from each bag (three bags per leaf type) to sterile filter bags (190 x 300 mm). Leaf material was diluted in 1:10 (w/w) in 0.1% sterile buffered peptone water; the mixture was then homogenised using a stomacher for 120 sec. Further decimal dilutions in 0.1% peptone were performed, appropriate dilutions were surface plated on tryptone soya agar. Plates were incubated at 25°C for 72 h, microbial populations were expressed as log CFU/g of leafy salad vegetable.

Statistical analysis

The effect of treatment and time on TPC of baby spinach, coral lettuce and rocket was analysed using JMP statistical software by 2-way analysis of variance (ANOVA).

Results and Discussion

A 2.5 log CFU/g increase in TPC (Fig 6) was observed for baby spinach leaves over a 10-d period, however ethanol had no significant effect ($p>0.05$) on microbial growth and shelf-life.

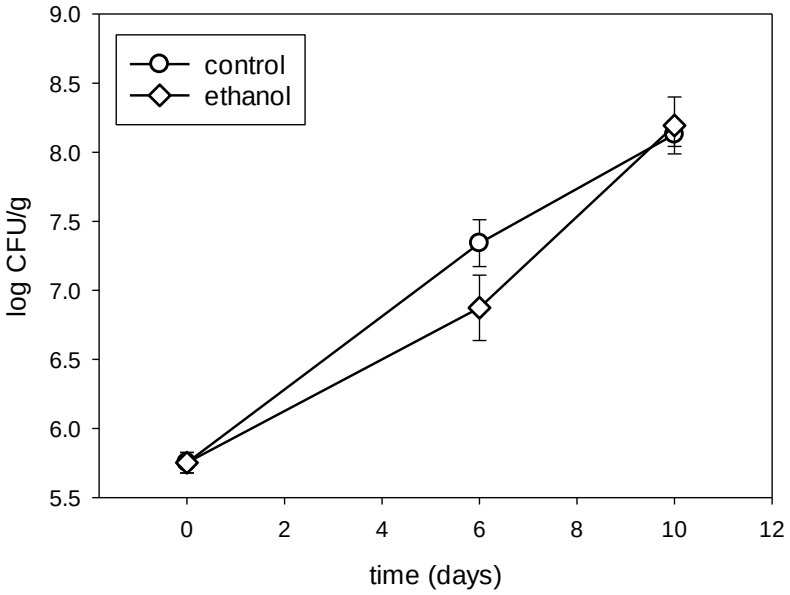


Figure 6: Total aerobic plate count of baby spinach leaves packaged in 60 g bags with or without ethanol sachets during storage at 4 °C for 10 days. Error bars represent the standard error of the mean (n=3).

Ethanol added to 120-g of lettuce suppressed microbial growth on coral lettuce (Fig 7), and a 2 log CFU/g TPC increase was observed in the absence of ethanol. The ethanol treatment produced browning of lettuce leaves as shown in Fig 8, thus influencing texture and appearance, which is not acceptable for consumers.

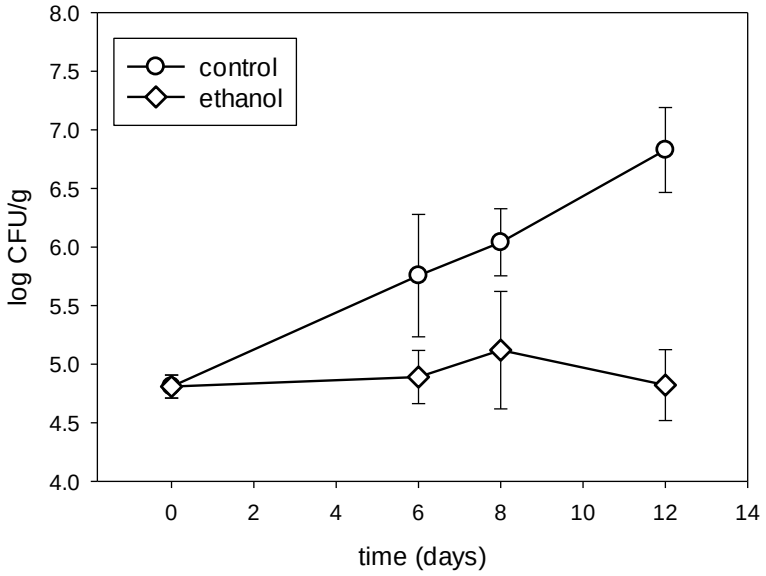


Figure 7: Total aerobic plate count of green coral lettuce leaves packaged in 120 g bags with or without ethanol sachets during storage at 4 °C for 12 d. Error bars represent the standard error of the mean (n=3).

Figure 8: Browning of green coral lettuce during storage at 4 °C when ethanol sachets were added to packaging.

The initial TPC for rocket leaves packed in 60-g bags was 4.0 log CFU/g (Fig 9) and increased by 3.5 log CFU/g at 15-d. The interaction effect between storage day and

treatment was significant (p= 0.012). On day-4 TPC was 1.5 log CFU/g higher (Fig 9) for the bags containing ethanol, however for day-8 there was no significant difference in TPC.

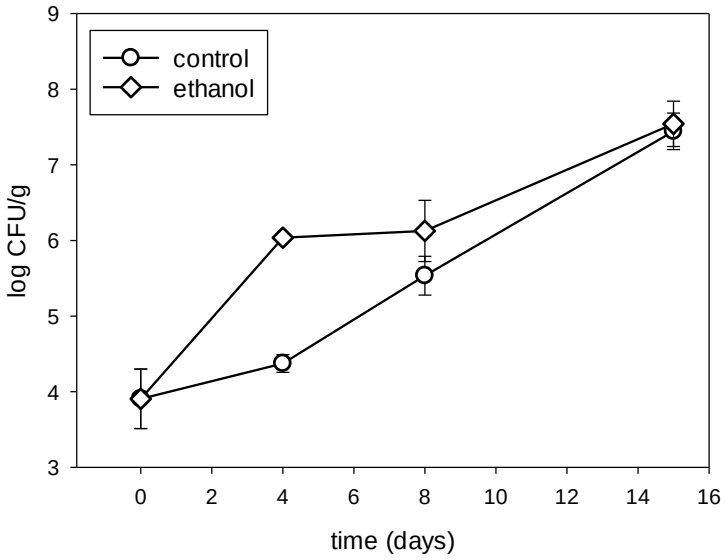


Figure 9: Total aerobic plate count of rocket leaves packaged in 60 g bags with or without ethanol sachets during storage at 4 °C for 15-d. Error bars represent the standard error of the mean (n=3).

3.0 log CFU/g increase in TPC was observed on rocket leaves packaged in 120 g (Fig. 10), however there was no significant effect between treatments (p> 0.05).

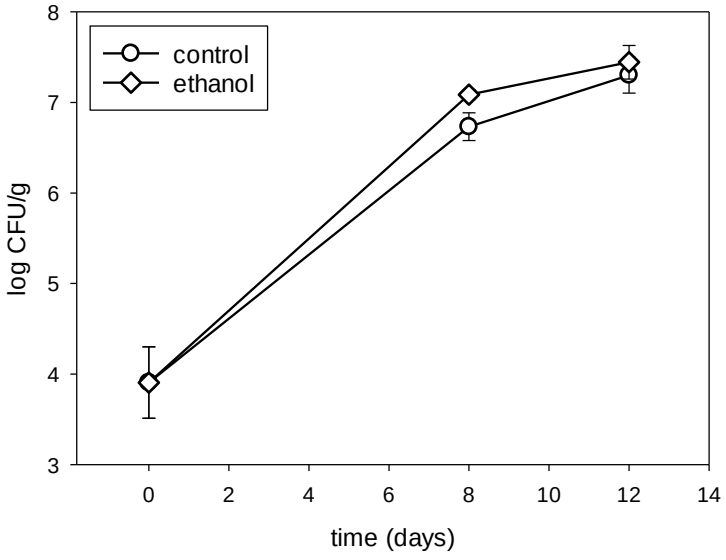


Figure 10: Total aerobic plate count of rocket leaves packaged in 120 g bags with or without ethanol sachets during storage at 4 °C for 12 d. Error bars represent the standard error of the mean (n=3).

Upon opening the packages, bags containing ethanol sachets had a strong ethanol odour, this would be undesirable for consumers.

Conclusion

Including ethanol sachets when packaging baby spinach and rocket had no influence on microbial growth and shelf-life. Though ethanol suppressed microbial growth on coral lettuce cold storage, it caused browning of the leaves therefore, on overall the use of ethanol sachets was not beneficial.

Appendix A4: Pilot study 4 on use of ethylene absorber sachets

Background

Ethylene promotes senescence of vegetables after harvest and causes yellowing of leaves (Gurbuz and Dogu, 2011, Martínez-Romero et al., 2007, USDA, 2016). Wills et al. (2002) observed that fumigating shredded lettuce with 0.1 µL/L ethylene inhibitor 1-methylcyclopropene (1MCP) for 1 h increased shelf-life by 50% compared to untreated lettuce. We hypothesized that including ethylene absorbers when packaging would reduce the rate of senescence of baby spinach thus extending shelf-life.

Aim: To investigate the effect of including ethylene sachets when packaging on shelf-life of baby spinach

Materials and Methods

Sanitisation and packaging

Baby spinach was washed with sanitiser 80 mg/L PAA for 45 sec at a processing facility in Cambridge, Tasmania, Australia. After sanitisation, excess moisture was removed by drying; leaves were then packaged in 60 g OPP bags that included sachets containing ethylene absorber. Control bags did not contain sachets, and all bags were stored at 4 °C for 14 d. During shelf-life sampling, TPC was analysed on day-0, -4, -7, -10 and -14, and visual quality assessments performed by three assessors.

Microbial analysis for TPC

10 g of baby spinach (three bags for each treatment) were diluted 1:10 (w/w) in 0.1% sterile buffered peptone water in sterile filter bags (190 x 300 mm) and homogenised for 120 sec using a stomacher. 10-fold dilution series was performed in 0.1% peptone as required for agar plating. Enumeration of TPC was conducted by surface-plating appropriate dilutions on TSA and incubating plates for 72 h at 25°C. Microbial counts were expressed as log CFU/g of baby spinach.

Statistical analysis

The effect of treatment (ethylene absorber and control) and time on TPC growth was analysed using 2-way analysis of variance (ANOVA) with JMP statistical software.

Results and Discussion

Initial TPC counts were 4.5 log CFU/g (Fig 11), which increased by 2.5-3 log CFU/g during shelf-life. Ethylene absorbers had no influence on microbial growth ($p>0.05$). The shelf-life of baby spinach was 14 d regardless of the presence of ethylene sachets.

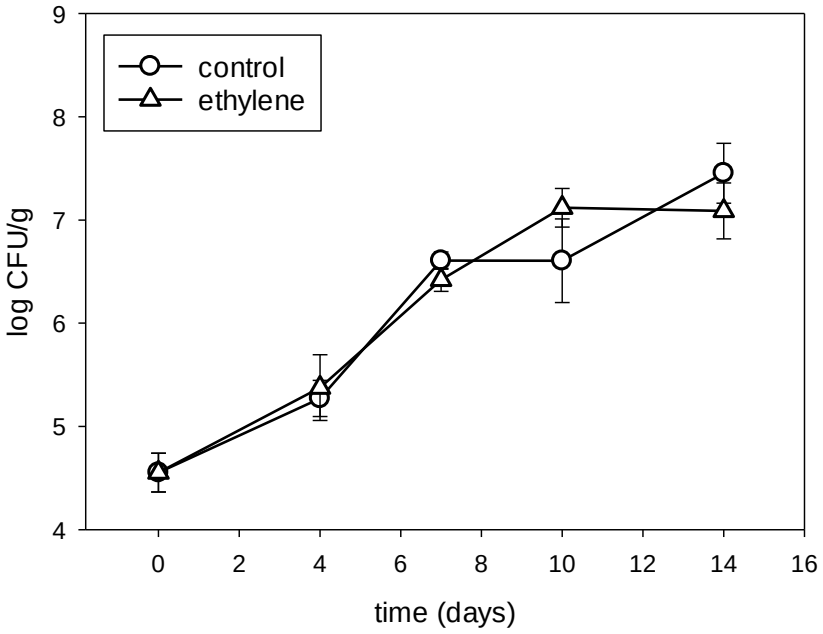


Figure 11: Total aerobic plate count of baby spinach leaves packaged in 60 g bags with or without ethylene sachets during storage at 4 °C for 14 days. Error bars represent the standard error of the mean (n=3).

Conclusion

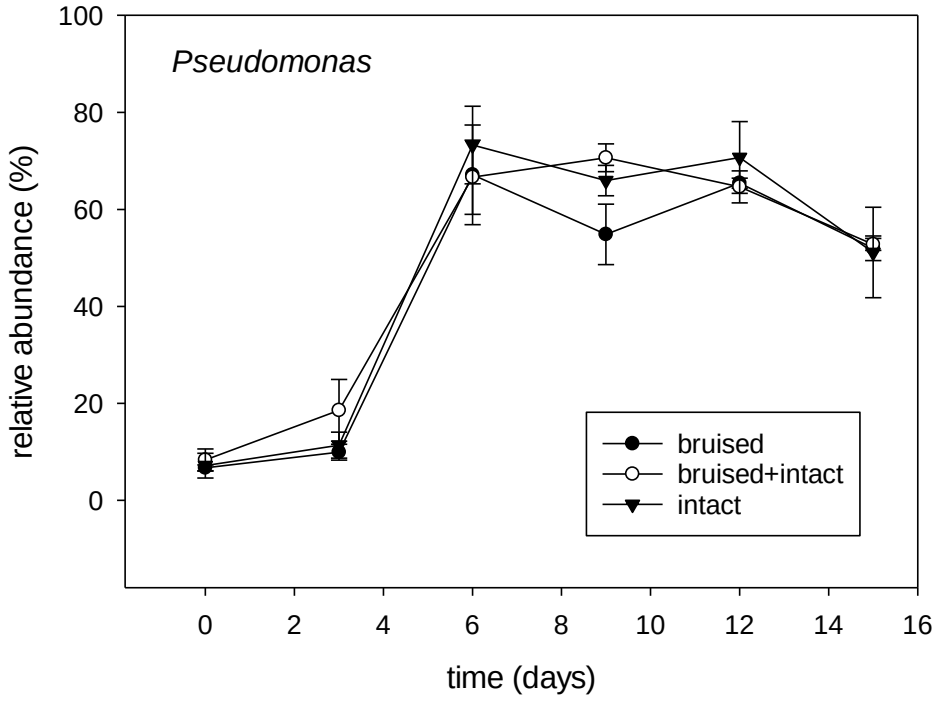
Including ethylene sachets when packaging baby spinach has no influence on shelf-life.

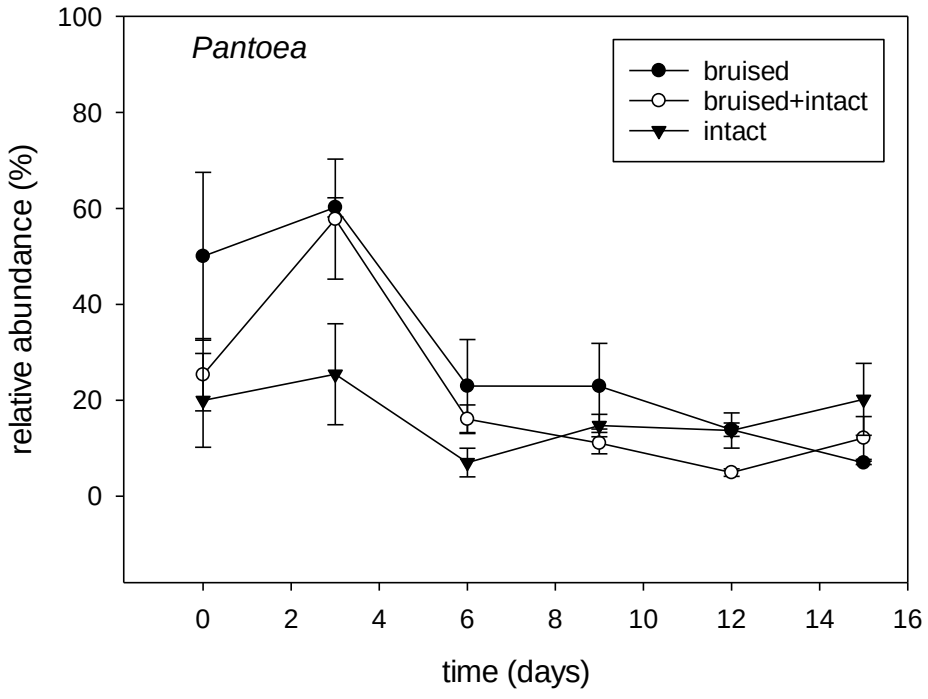
Appendix B: Bacteria identified from leafy salad vegetables in different studies

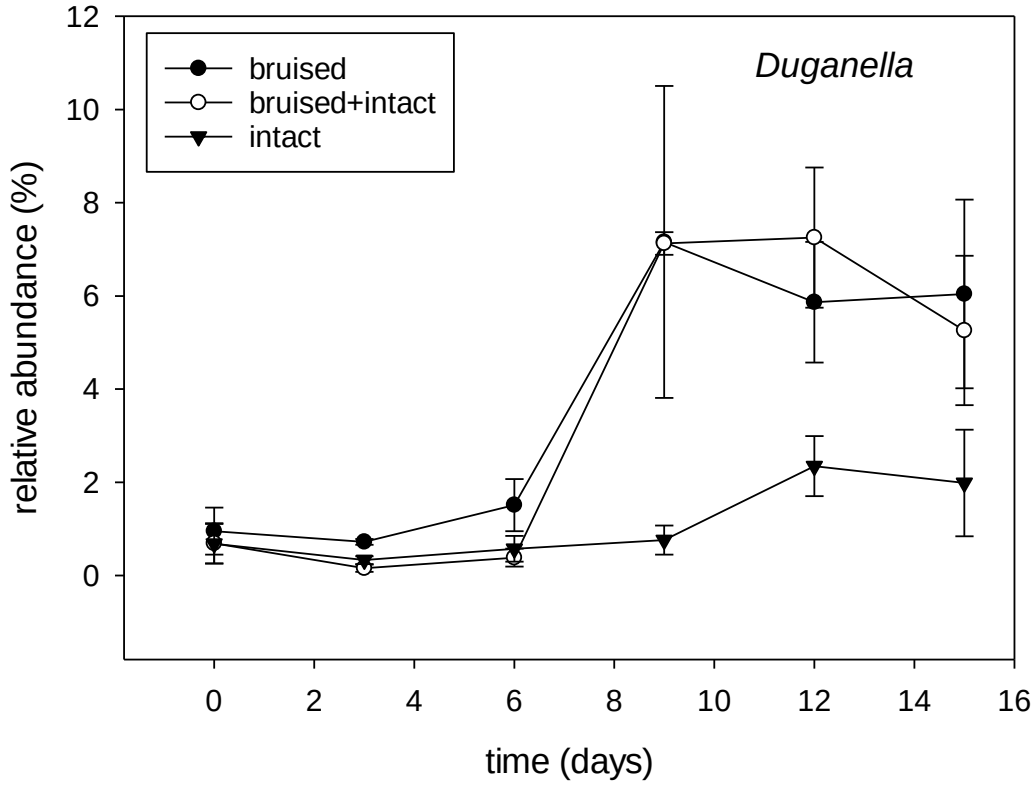
Pseudomonas fluorescens, Pseudomonas salmonii, Pseudomonas putida, Pseudomonas viridiflava and Pseudomonas marginalis Stenotrophomonas maltophilia, Morganella morganii Acinetobacter sp. Flavobacterium succinicans, Shewanella sp, Psychrobacter sp, Erwinia sp, Sphingomonas sp, Lavobacterium. sp, Chryseobacterium, Janthinobacterium sp, Sphingobacterium faecium, Paenibacillus sp., Pedobacter sp., Agrobacterium sp., Pedobacter sp., Duganella, Massilia, Escherichia vulneris, Carnobacterium viridans, Planomicrobium sp., f- Exiguobacterium sp., Cupriavidus sp. Methylophilus, Rhizobium, Gp4, Gp6, Arthrobacter, Nocardioides, Rhodococcus, Hymenobacter, Brevundimonas, Flavobacterium sp., Alkanindiges, Pseudoxanthomonas spadix, Stenotrophomonas sp., Stenotrophomonas rhiziphila, Rahnella aquatilis, Klebsiella pneumoniae, Enterobacter cloacae/Enterobacter asburiae, Pantoea spp., Erwinia persicina, Acrobacter, Escherichia hermannii, Raoultella terrigena, Providencia heimbachae, Xanthomonas, Serratia, Providencia, Morganella, Bacillus, Streptococcus, Deinococcus spp., Naxibacter spp., Spartobacteria spp., Methylobacterium spp., Ewingella Americana, Rahnella aquatilis, Aeromonas salmonicida., Comamonas spp., Stenotrophomonas rhizophila, Sporosarcina, Lactococcus spp., Leuconostoc spp,. Sphingobacterium composti, Brachybacterium, Pectobacterium sp., Microbacterium ssp., Enterococcus silesiacus, Aerococcus urinaeequi, Exiguobacterium sibiricum, Exiguobacterium marinum, Citrobacter sp., Klebsiella pneumoniae, Citrobacter sp., Deinococcus spp, Hafnia alvei, Staphylococcus aureus, Staphylococcus haemolyticus, Tolumonas, Staphylococcus saprophyticus and Lysinibacillus fusiformis/sphaer-icus have been isolated from leafy salad vegetables (Dees et al., 2015, Frohling et al., 2018, Gu et al., 2018, Hausdorf et al., 2013, Ioannidis et al., 2018, Jackson et al., 2013, Koo et al., 2016, Leff and Fierer, 2013, Lopez-Velasco et al., 2011, Nubling et al., 2016, Soderqvist et al., 2017, Truchado et al., 2018).

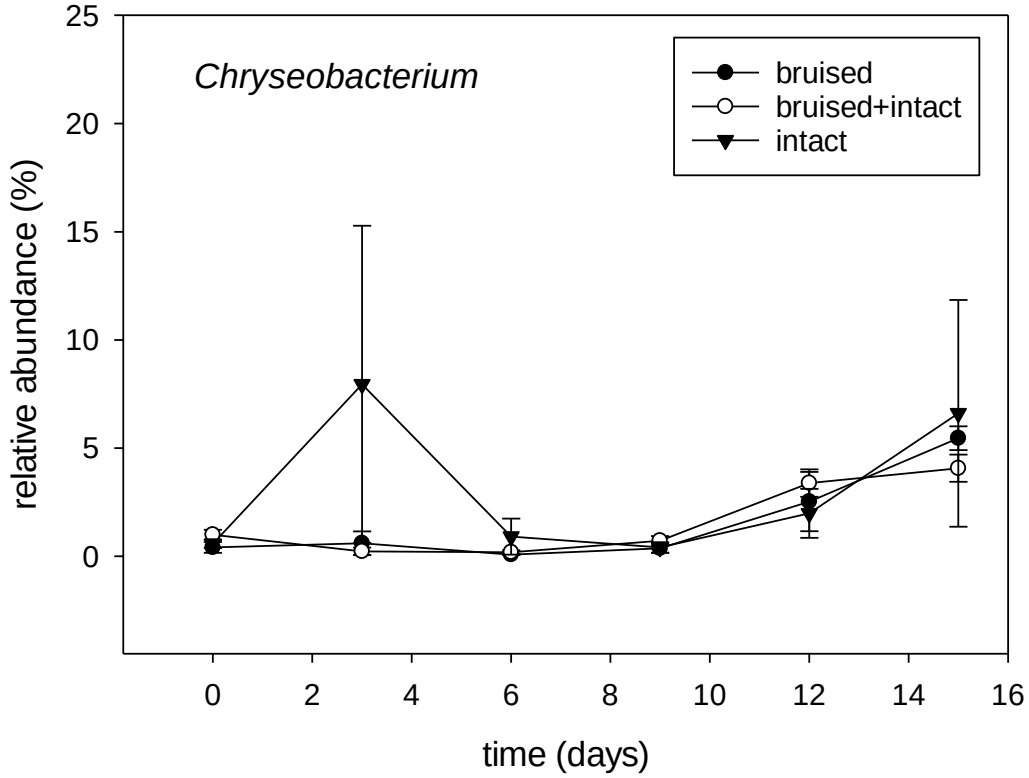
Appendix C: Supplementary graphs and tables for chapter 3

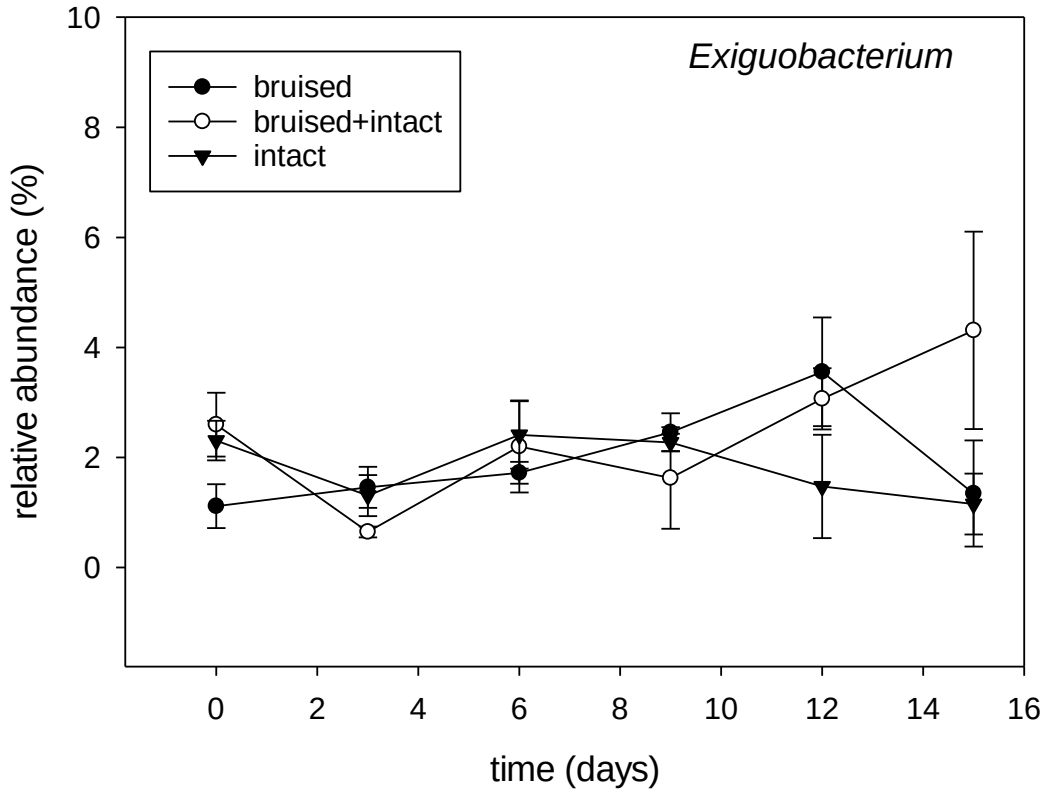
Appendix C1: Graphs illustrating the effect of leaf quality on changes in the relative abundance of bacterial genera during storage at 4 °C

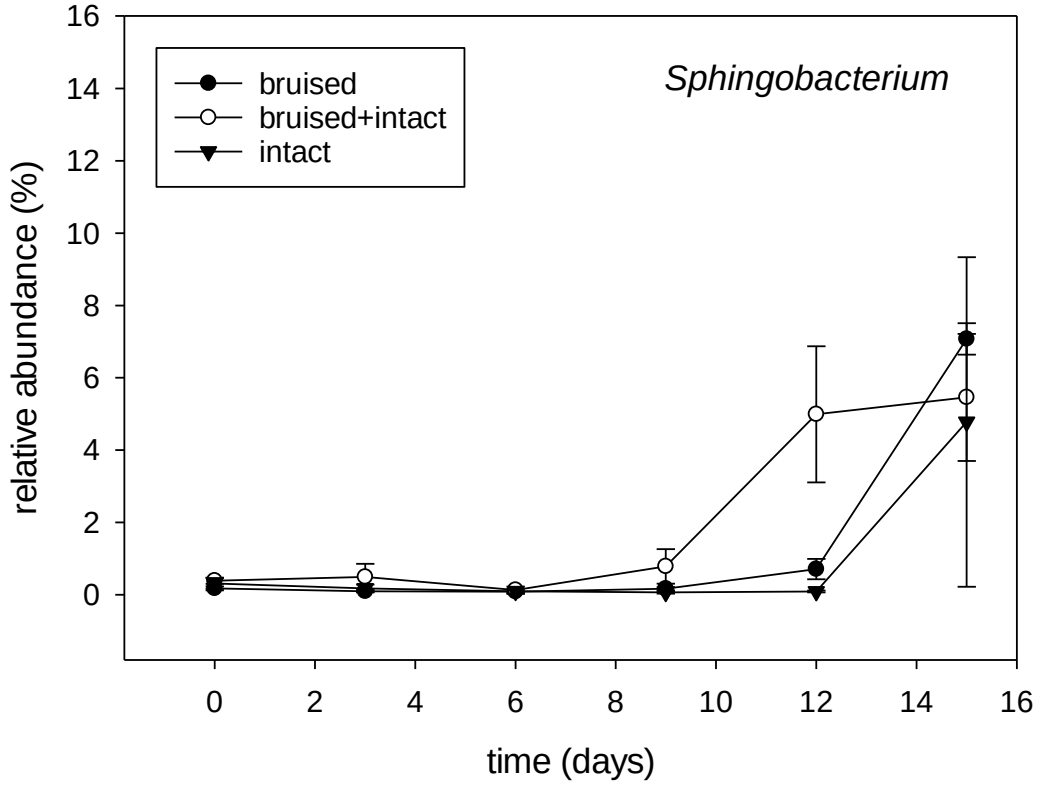


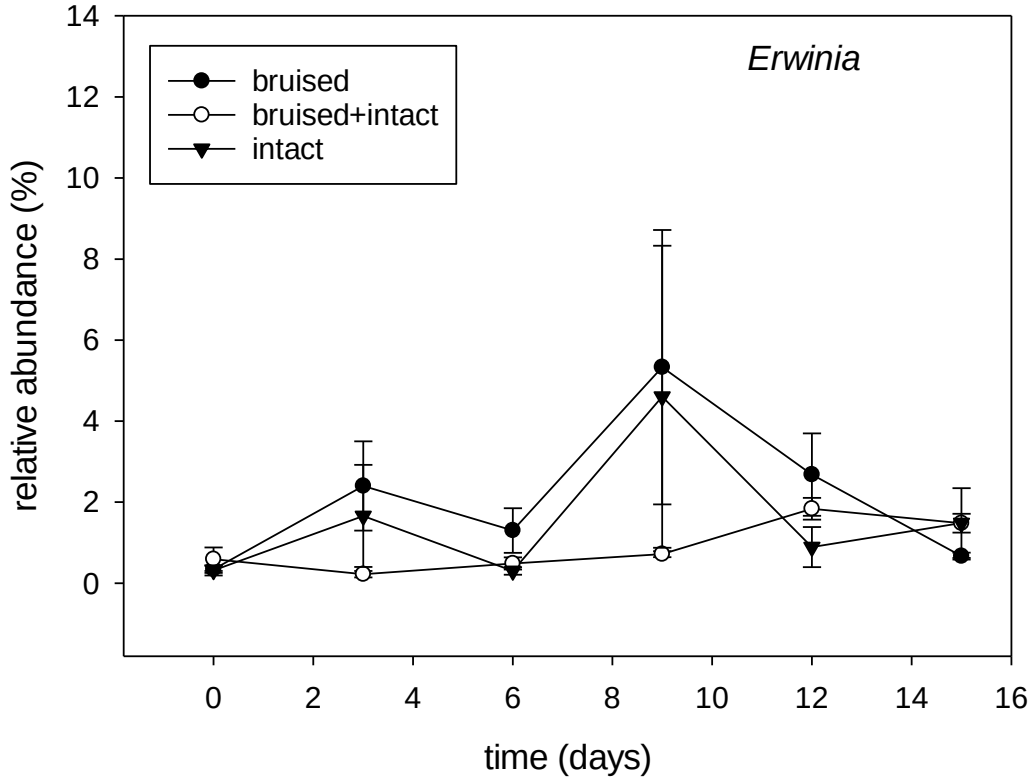


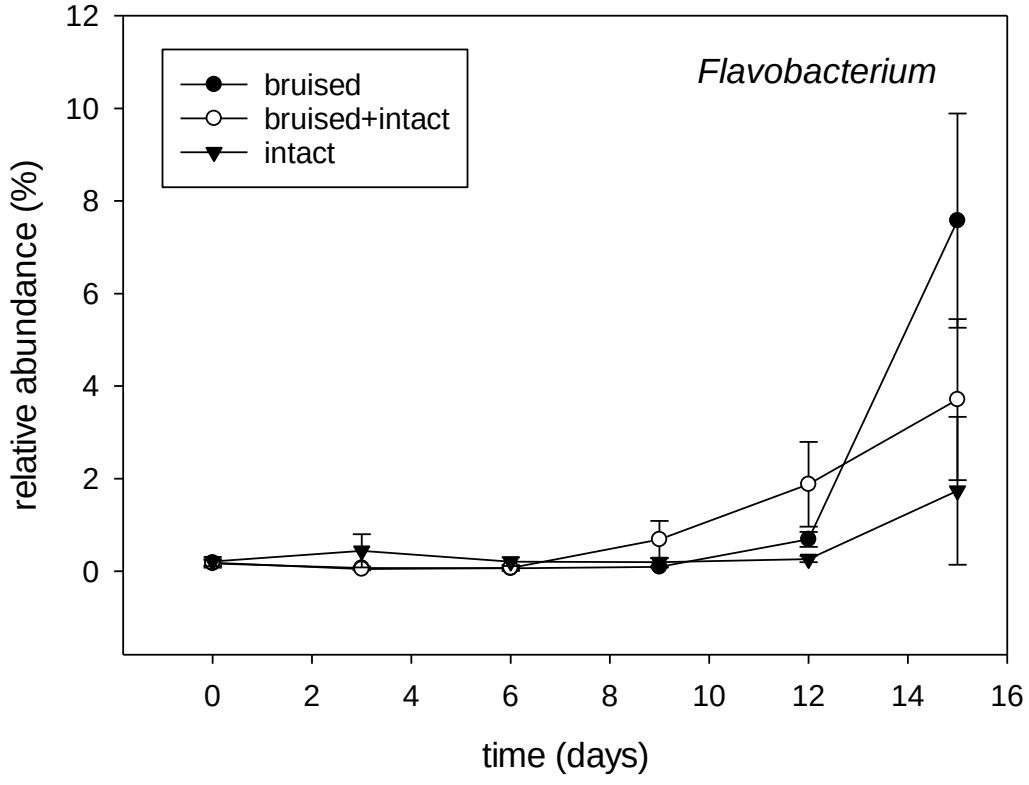

Duganella
bruised
bruised+intact
intact
relative abundance (%)
time (days)
0
2
4
6
8
10
12
14
16

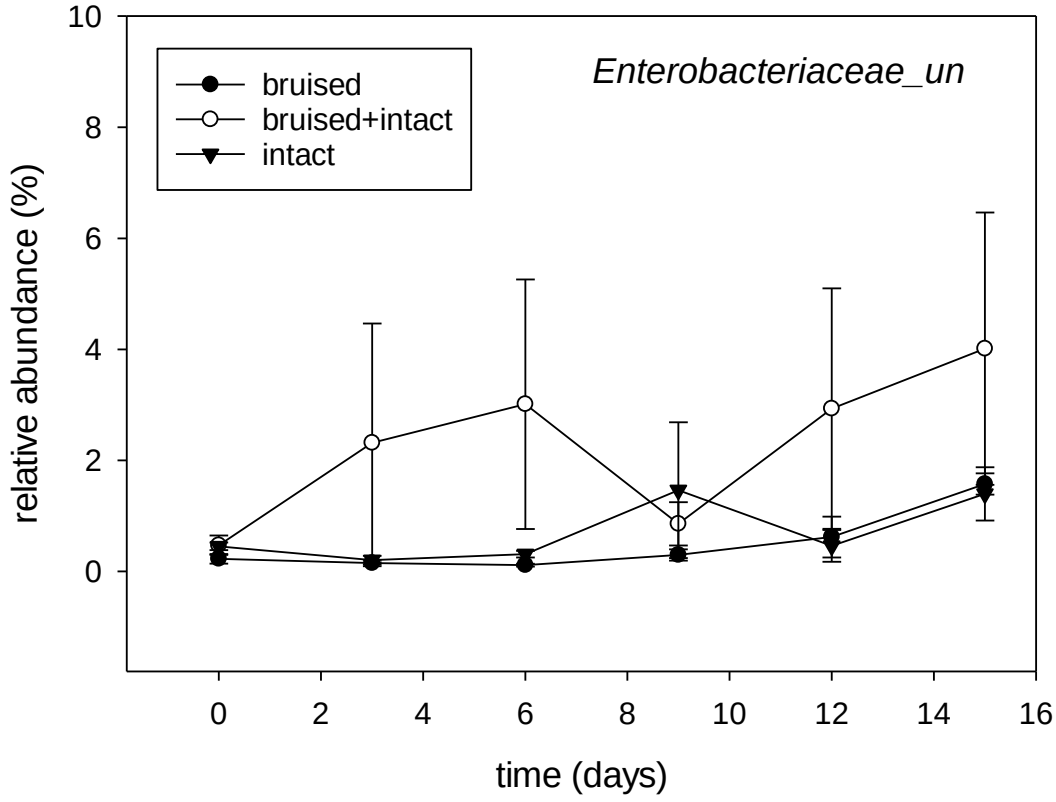

Chryseobacterium
bruised
bruised+intact
intact
relative abundance (%)
time (days)
0
5
10
15
20
25
2
4
6
8
12
14
16

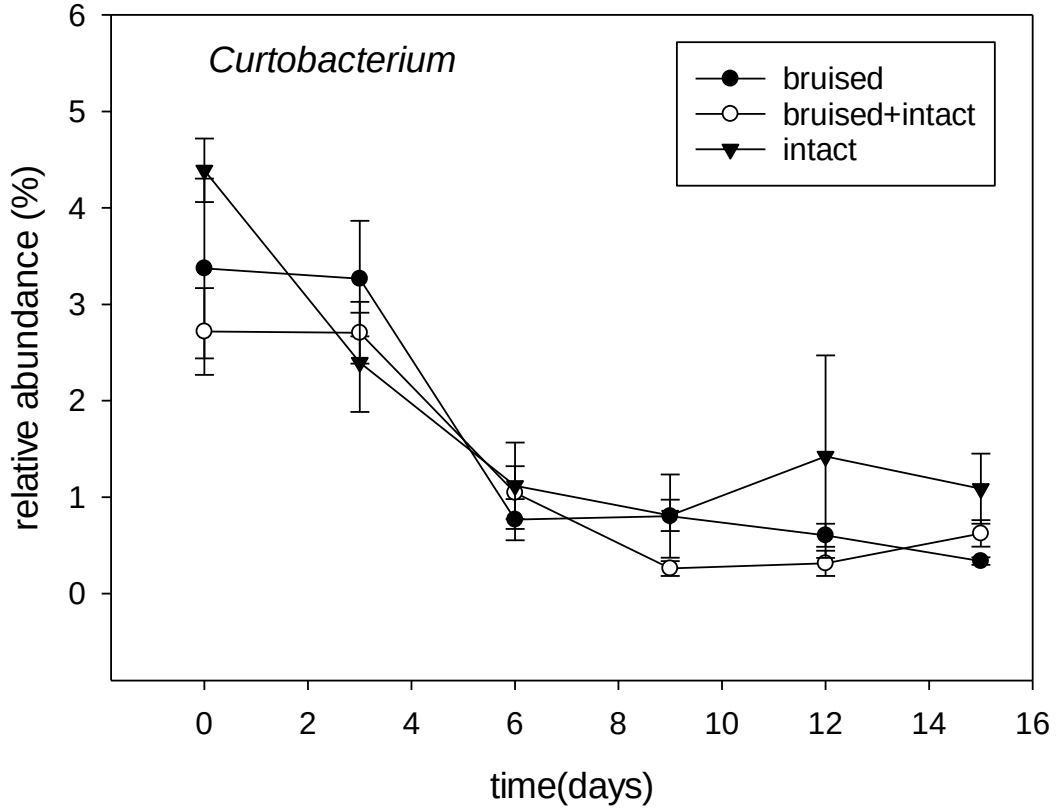

Exiguobacterium
bruised
bruised+intact
intact
relative abundance (%)
time (days)
0
2
4
6
8
10
12
14
16

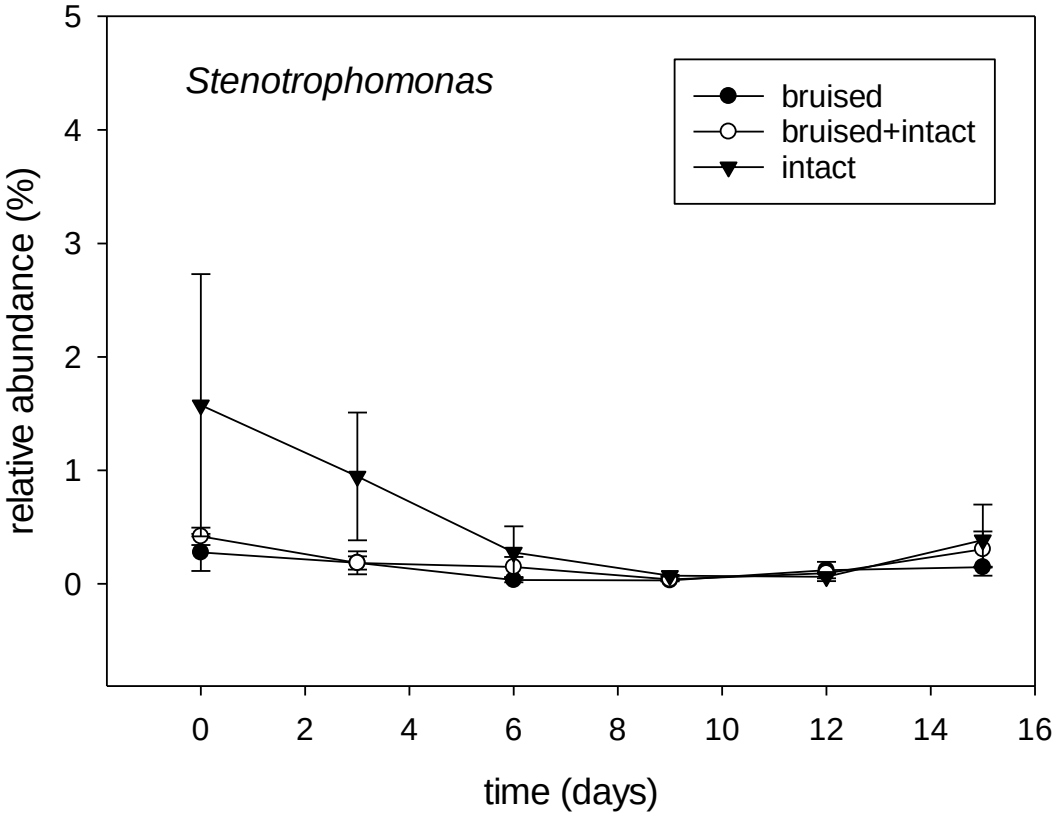

Sphingobacterium
bruised
bruised+intact
intact
relative abundance (%)
time (days)
0
2
4
6
8
10
12
14
16

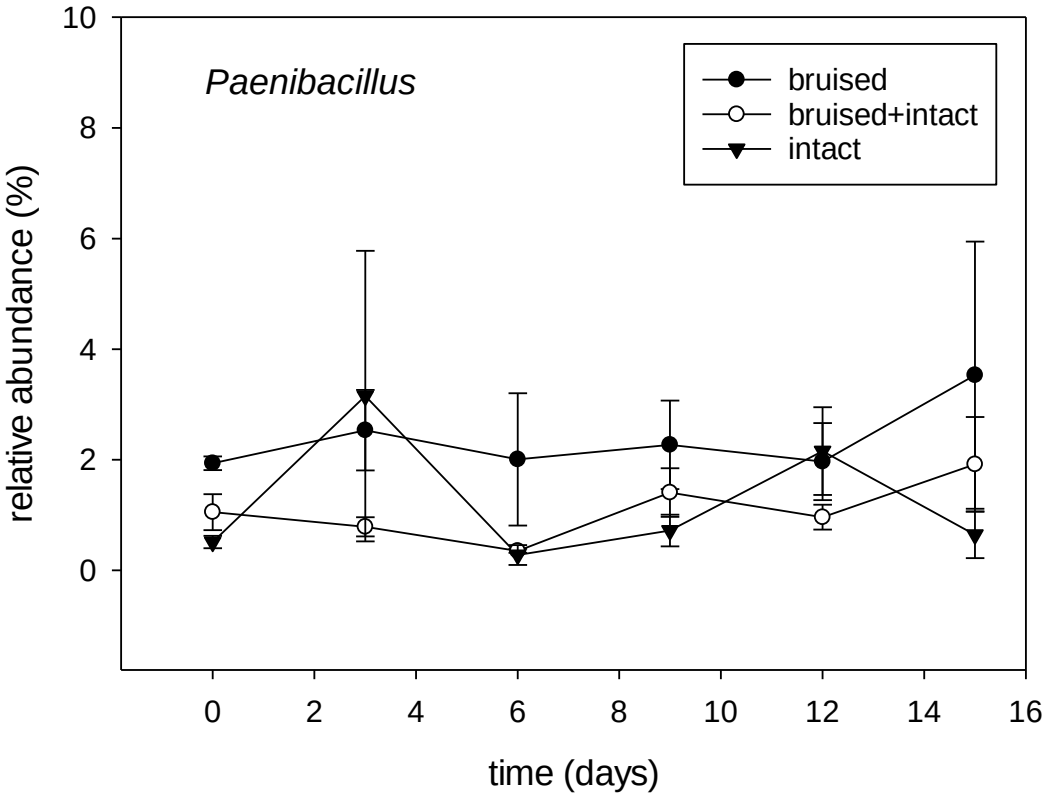

Erwinia
bruised
bruised+intact
intact
relative abundance (%)
time (days)
0
2
4
6
8
10
12
14
16

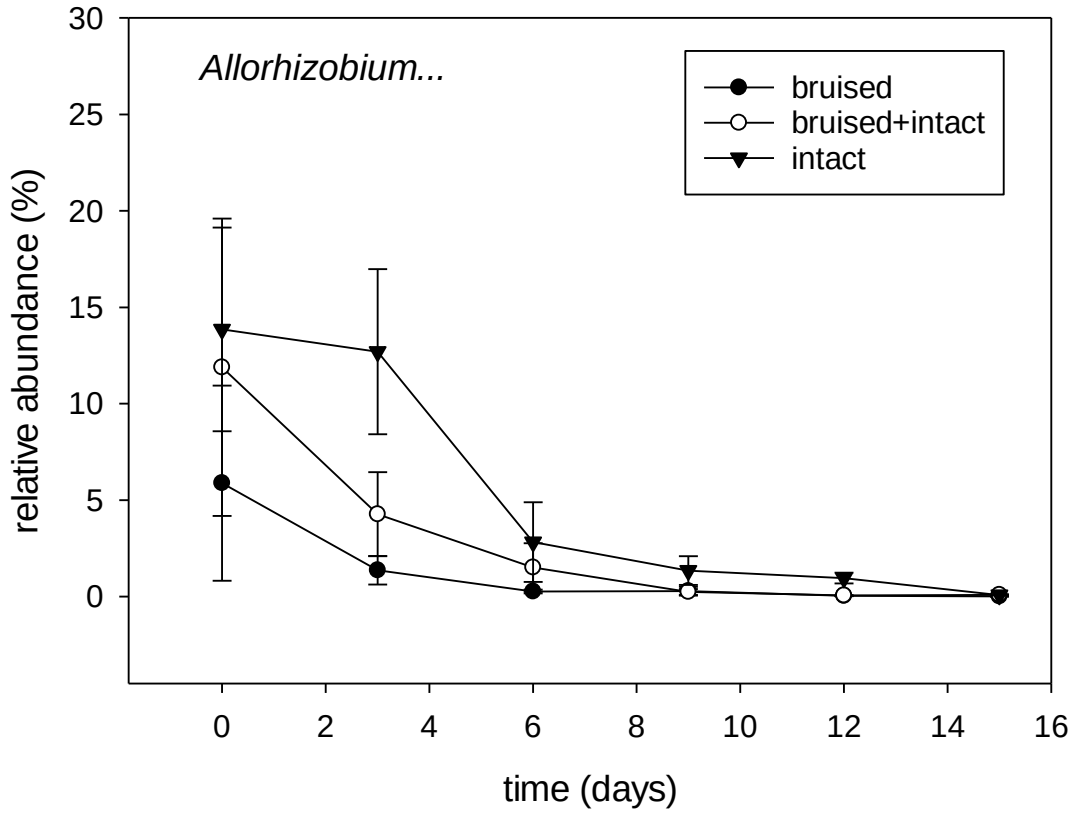

Flavobacterium
bruised
bruised+intact
intact
relative abundance (%)
time (days)
0
2
4
6
8
10
12
14
16

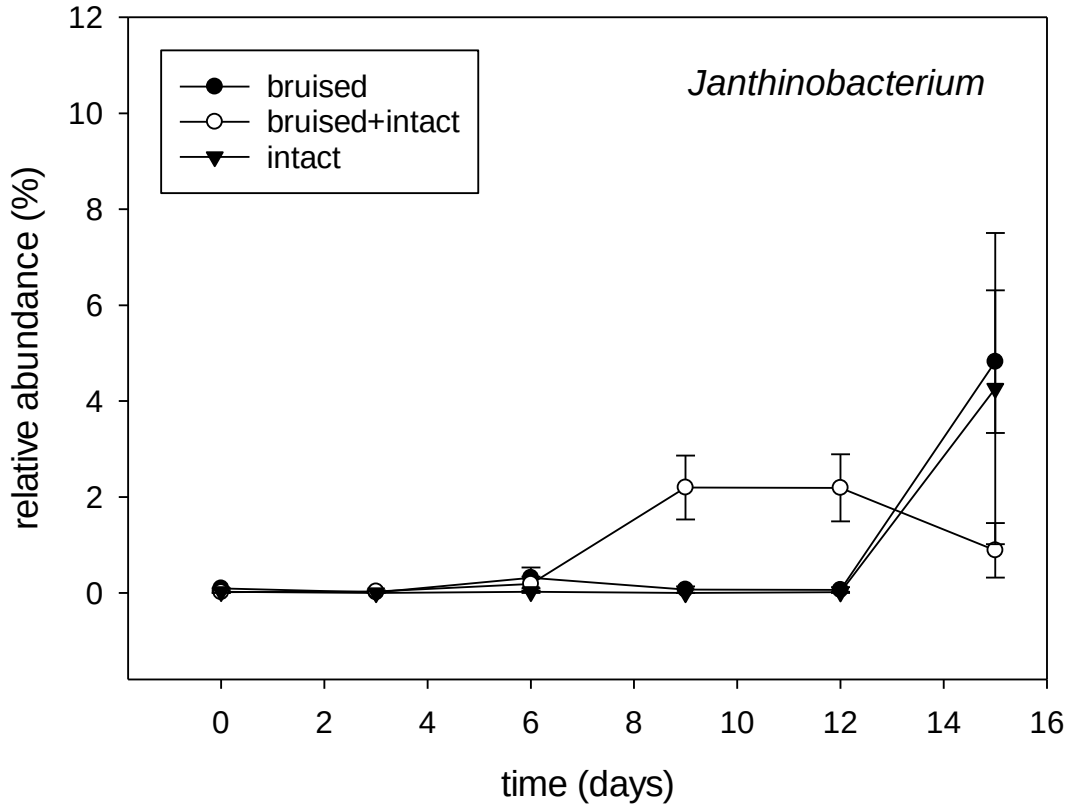

Enterobacteriaceae_un
bruised
bruised+intact
intact
relative abundance (%)
time (days)
0
2
4
6
8
10
12
14
16

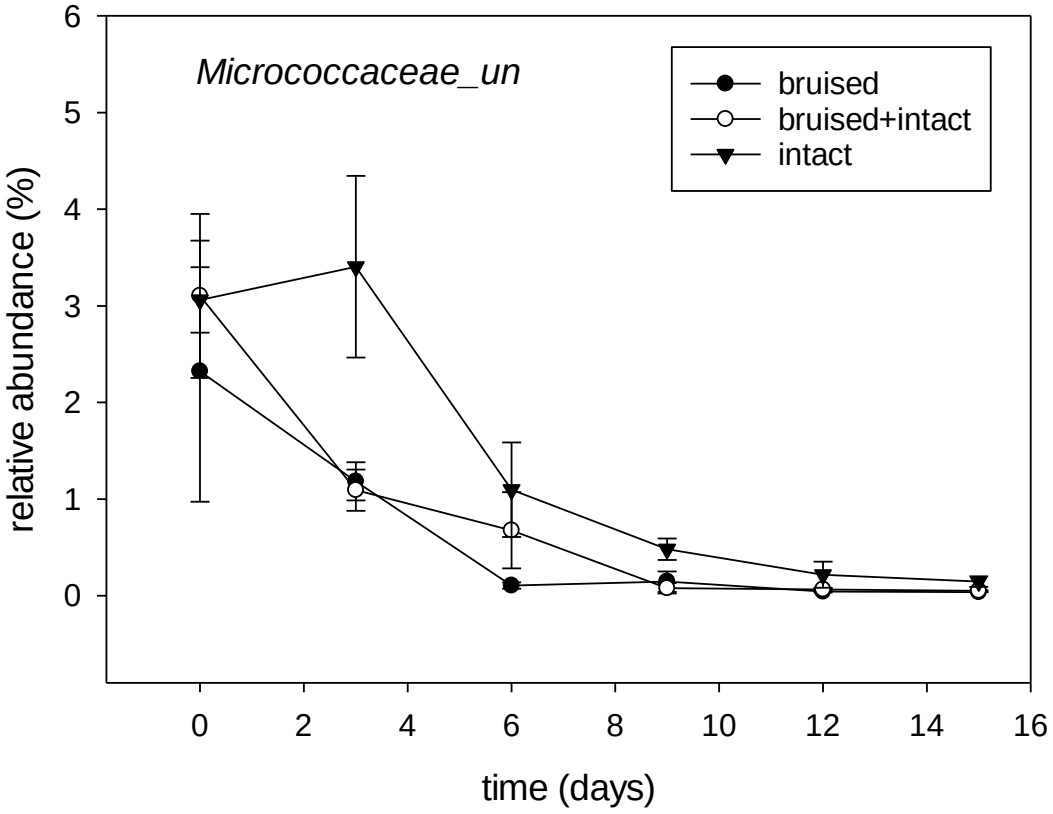

Curtobacterium
bruised
bruised+intact
intact
relative abundance (%)
time(days)
0
1
2
3
4
5
6
0
2
4
6
8
10
12
14
16

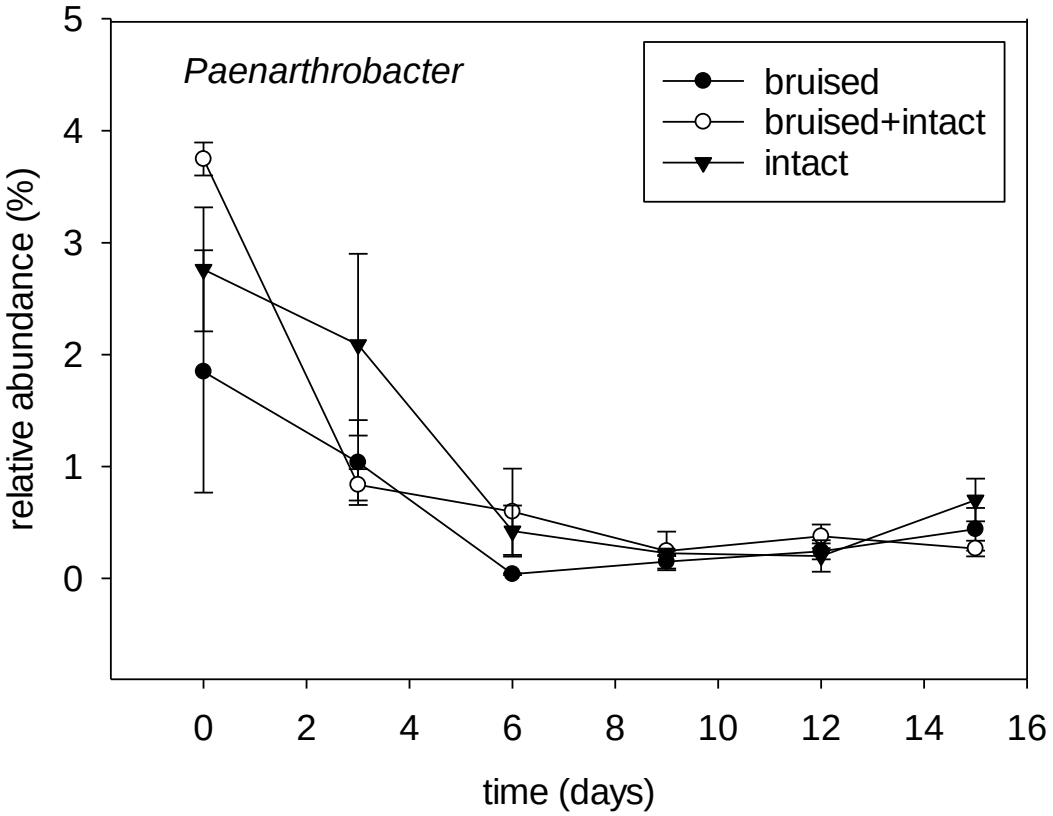

Stenotrophomonas
bruised
bruised+intact
intact
relative abundance (%)
time (days)
0
1
2
3
4
5
0
2
4
6
8
10
12
14
16

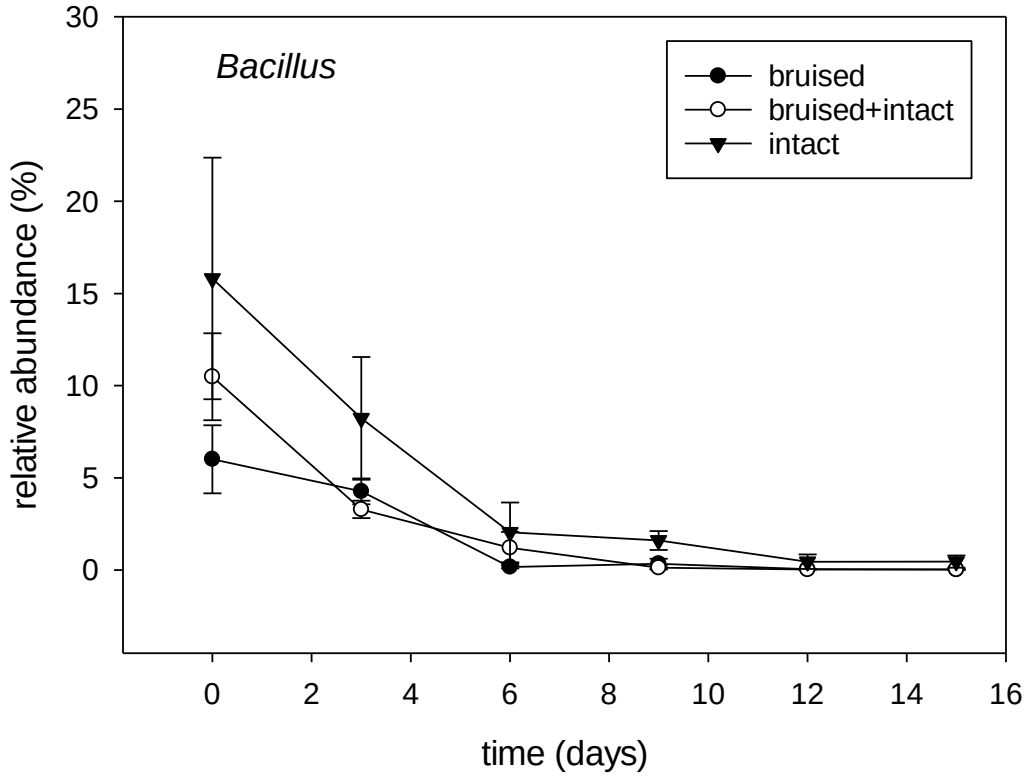

Paenibacillus
bruised
bruised+intact
intact
relative abundance (%)
time (days)
0
2
4
6
8
10
0
2
4
6
8
10
12
14
16

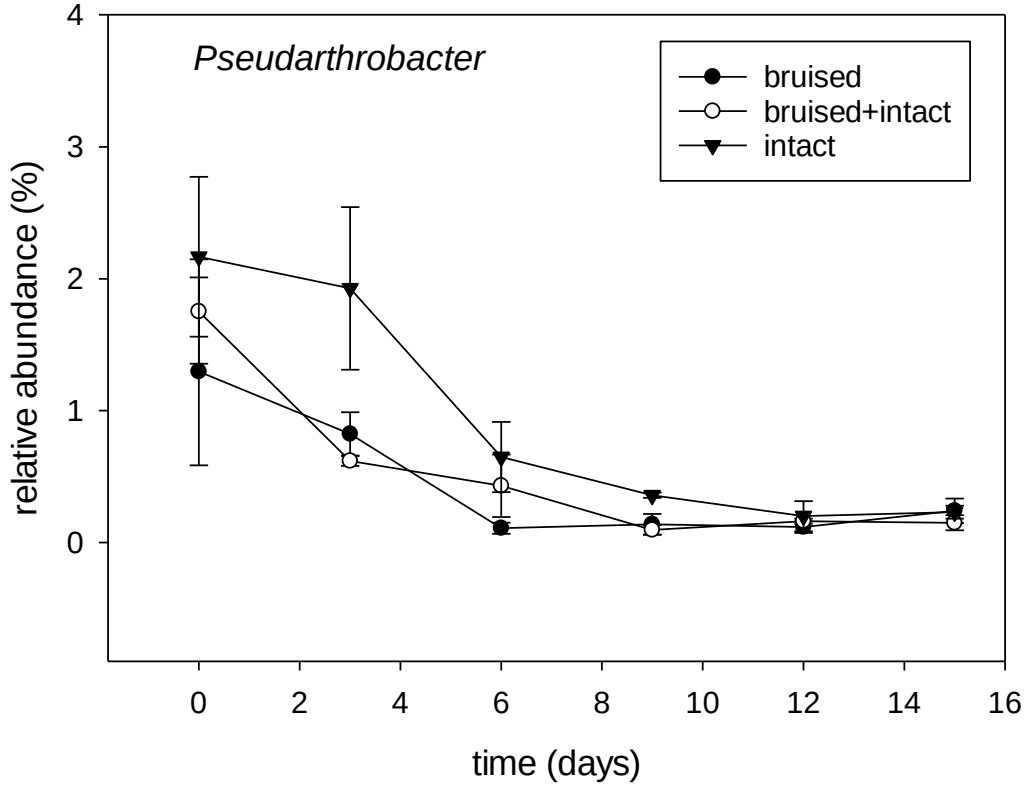

Allorhizobium...
bruised
bruised+intact
intact
relative abundance (%)
time (days)
0
2
4
6
8
10
12
14
16
0
5
10
15
20
25
30

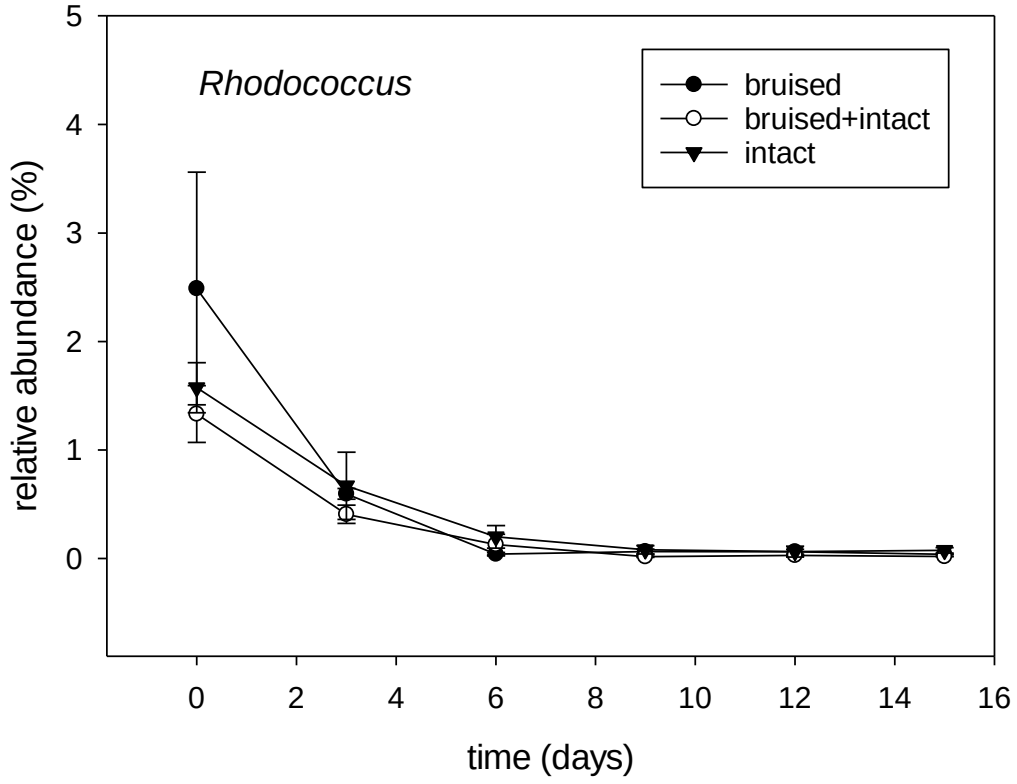

Janthinobacterium
bruised
bruised+intact
intact
relative abundance (%)
time (days)
0
2
4
6
8
10
12
14
16
0
2
4
6
8
10
12


Micrococcaceae_un
bruised
bruised+intact
intact
relative abundance (%)
time (days)
0
1
2
3
4
5
6
0
2
4
6
8
10
12
14
16


Paenarthrobacter
bruised
bruised+intact
intact
relative abundance (%)
time (days)
0
1
2
3
4
5
0
2
4
6
8
10
12
14
16


Bacillus
bruised
bruised+intact
intact
relative abundance (%)
30
25
20
15
10
5
0
0
2
4
6
8
10
12
14
16
time (days)


Pseudarthrobacter
bruised
bruised+intact
intact
relative abundance (%)
4
3
2
1
0
0
2
4
6
8
10
12
14
16
time (days)


Rhodococcus
bruised
bruised+intact
intact
relative abundance (%)
time (days)
0
1
2
3
4
5
0
2
4
6
8
10
12
14
16

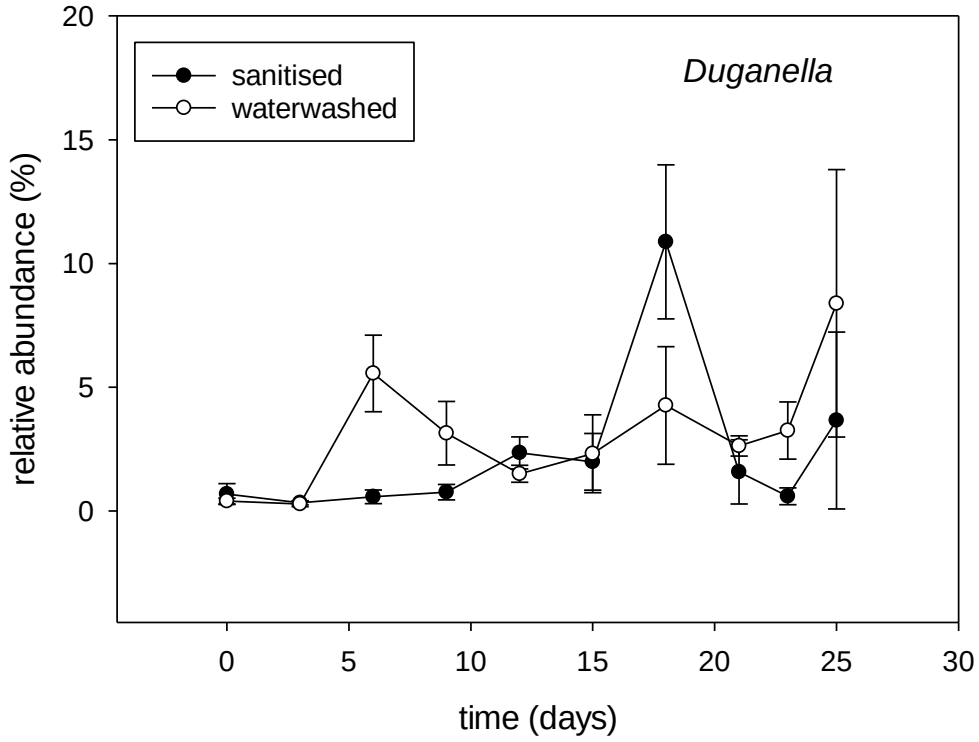
Appendix C2: Statistical analysis results on the effect of bruising and time on changes in the relative abundance of bacterial genera during shelf-life

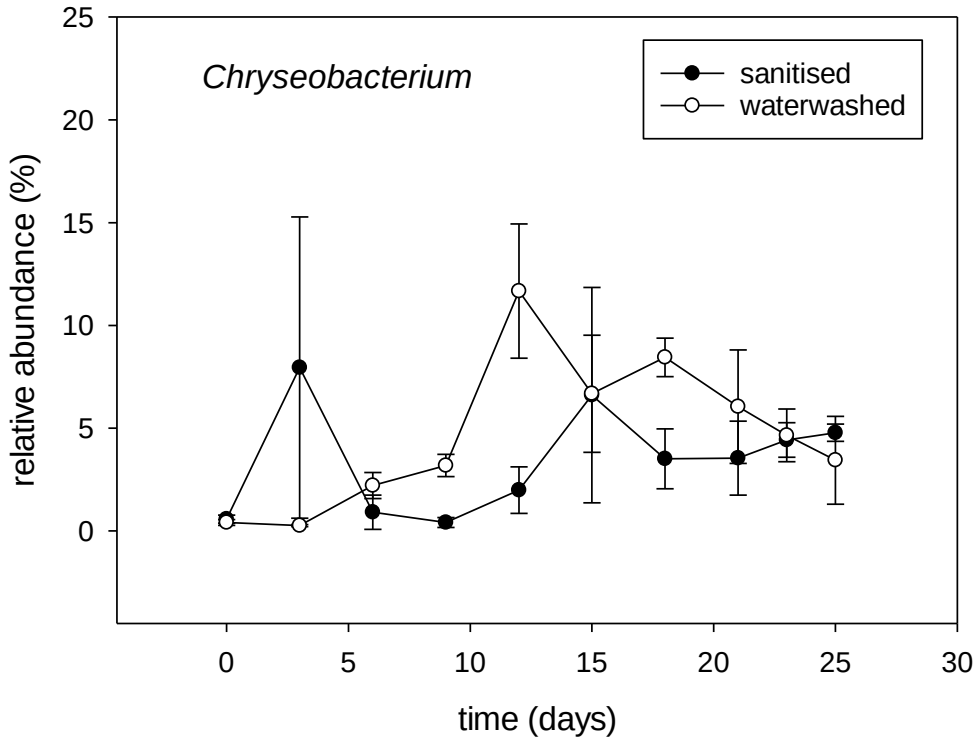
Table C2: p values for the effect of leaf quality and time on the relative abundance of bacterial genera identified on baby spinach during storage at 4 °C

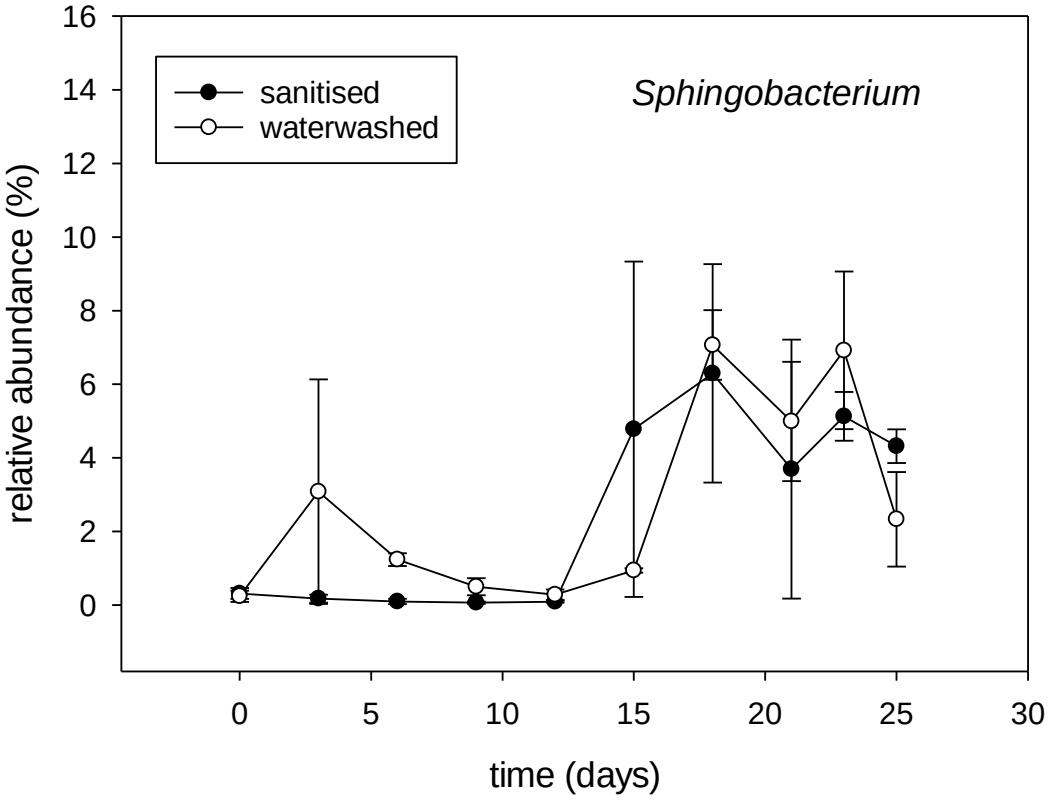
Bacterial genera	Interaction effect p value	Leaf quality p value	time p value
Pseudomonas ↑	0.8804	0.3273	<.0001***
Pantoea ↓	0.0803	0.0500*	<.0001***
Duganella	0.2290	0.0096**	<.0001***
Bacillus ↓	0.3231	0.0003***	<.0001***
Allorhizobium ↓	0.6825	<.0001***	<.0001***
Exiguobacterium	0.0906	0.4176	0.2683
Curtobacterium ↓	0.5433	0.0904	<.0001***
Chryseobacterium ↑	0.5230	0.6123	0.0013**
Paenibacilllus	0.5613	0.0007***	0.0843
Sphingobacterium ↑	0.2706	0.0033**	<.0001***
Erwinia	0.4590	0.1089	0.0405*
Flavobacterium ↑	0.0683	0.8178	<.0001***
Paenarthrobacter ↓	0.0520	0.0466*	<.0001***
Micrococcae_un ↓	0.4563	<.0001***	<.0001***
*Enterobacteriaceae*_un ↑	0.7716	0.0019**	0.0099**
Janthinobacterium	0.0075**	0.1704	0.0002***
Pseudarthrobacter ↓	0.3586	0.0034**	<.0001***
Stenotrophomonas	0.7722	0.0632	0.0008***
Rhodococcus ↓	0.4585	0.0486*	<.0001***

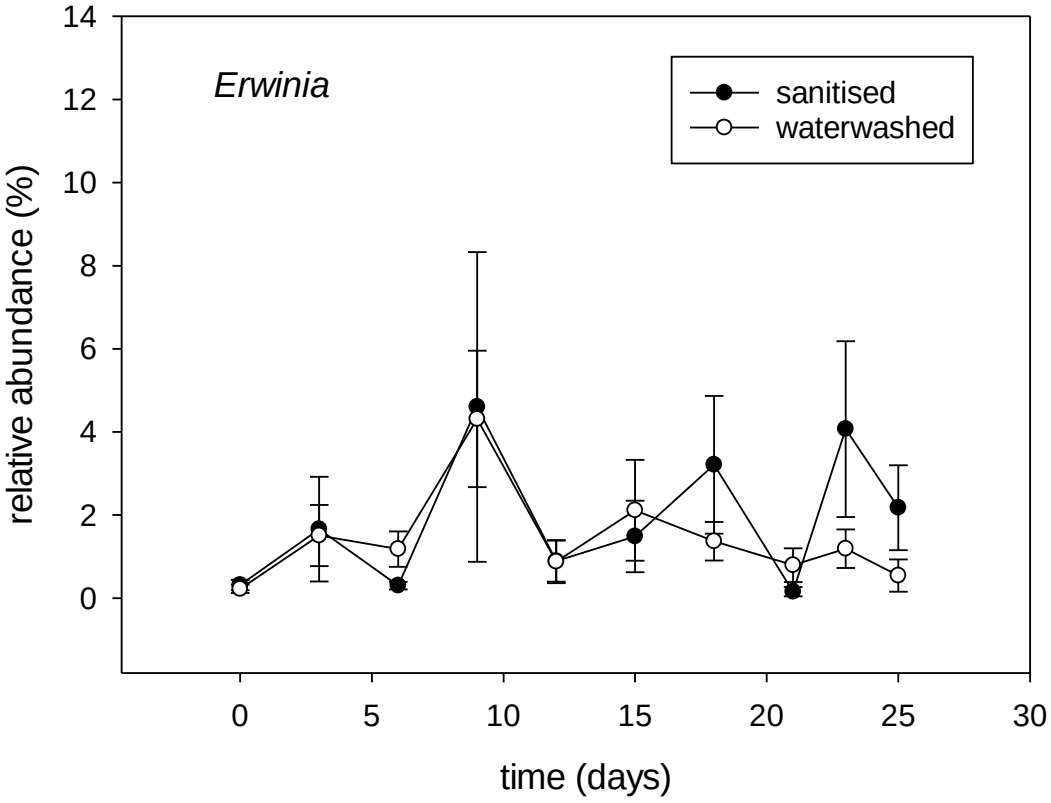
Ranking in descending order of the bacteria with relative abundance >3% on most of the days

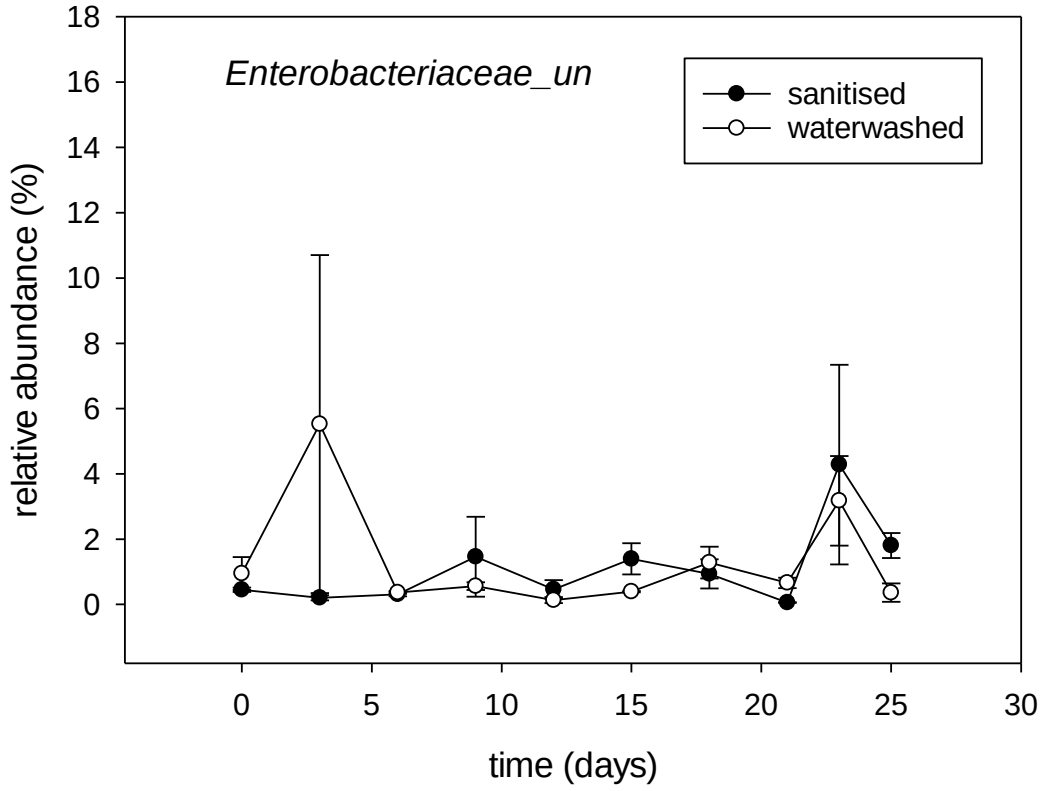
Appendix C3: Graphs illustrating the effect of treatment with PAA vs TW on changes in the relative abundance of bacterial genera during storage at 4 °C

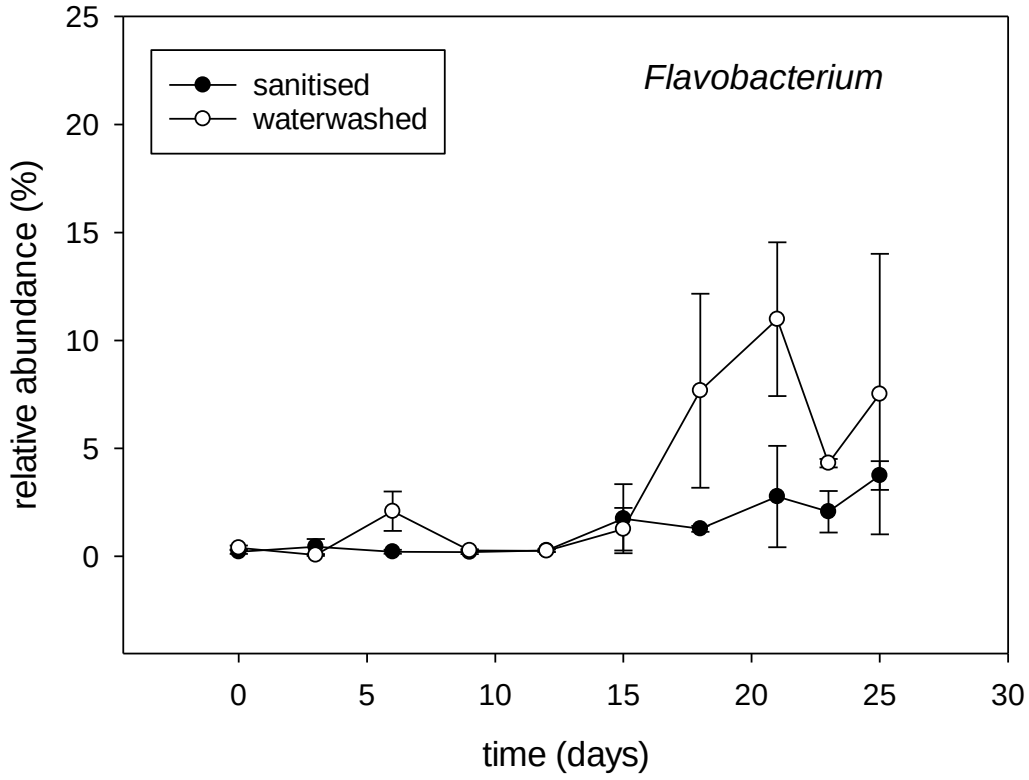


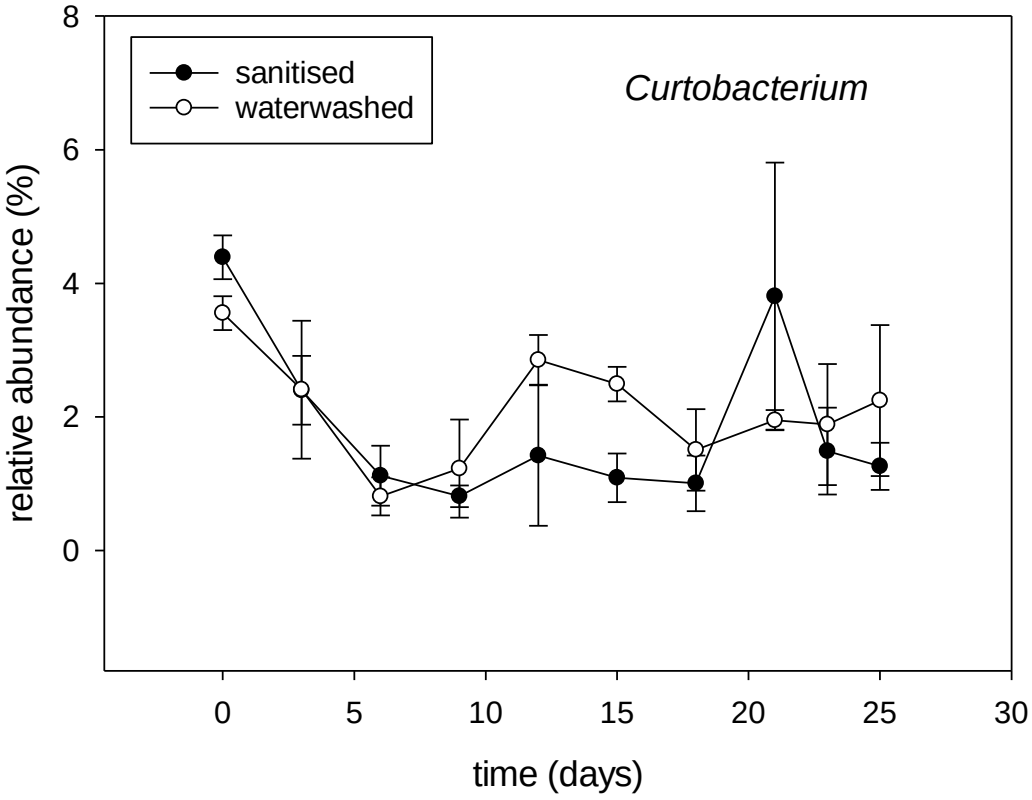


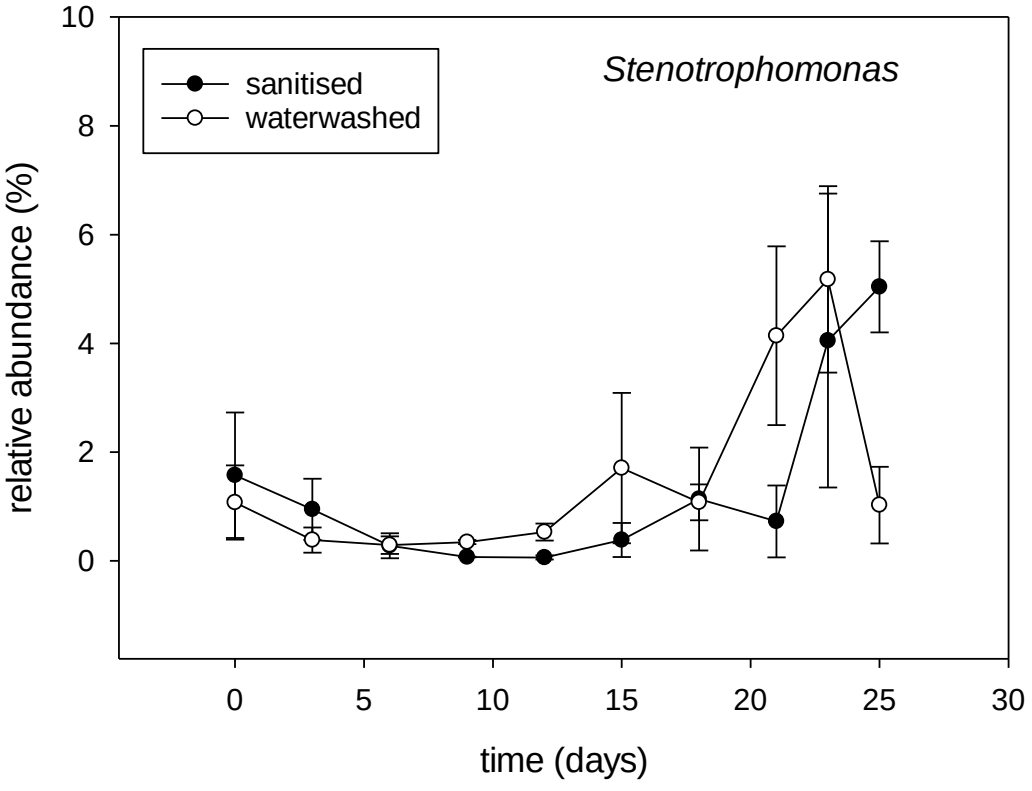

sanitised
waterwashed
Sphingobacterium
relative abundance (%)
time (days)
0
2
4
6
8
10
12
14
16
0
5
10
15
20
25
30

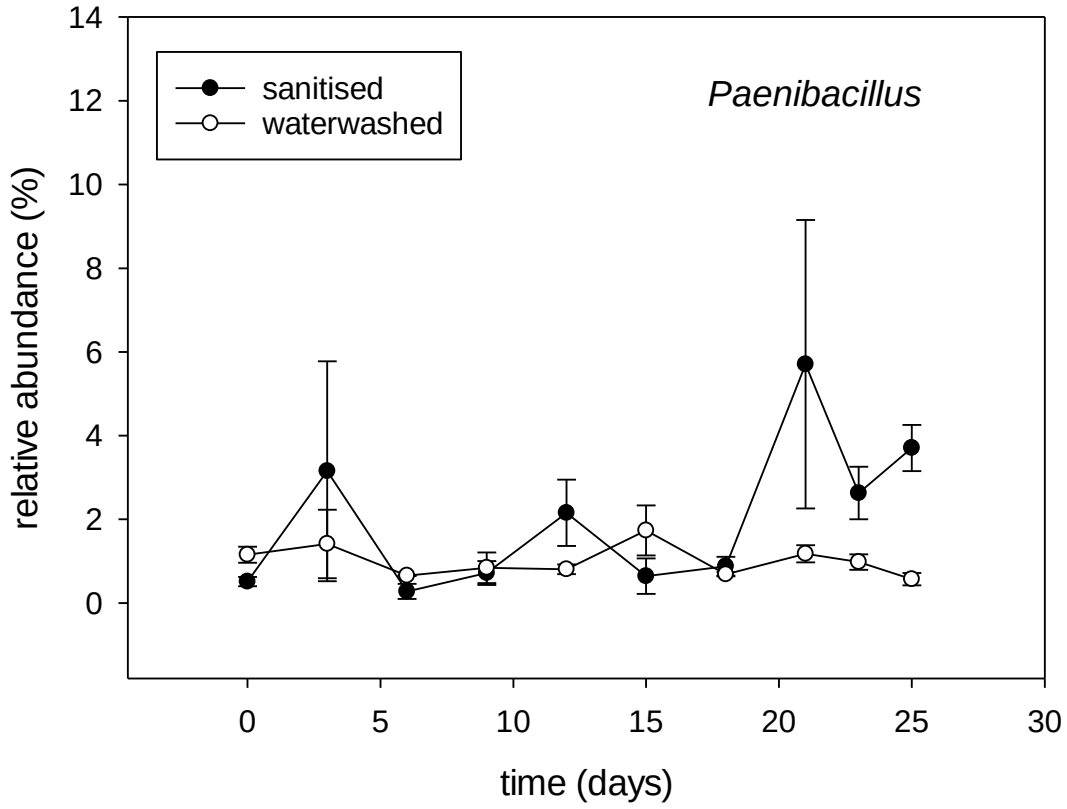

Erwinia
sanitised
waterwashed
relative abundance (%)
time (days)
0
2
4
6
8
10
12
14
0
5
10
15
20
25
30

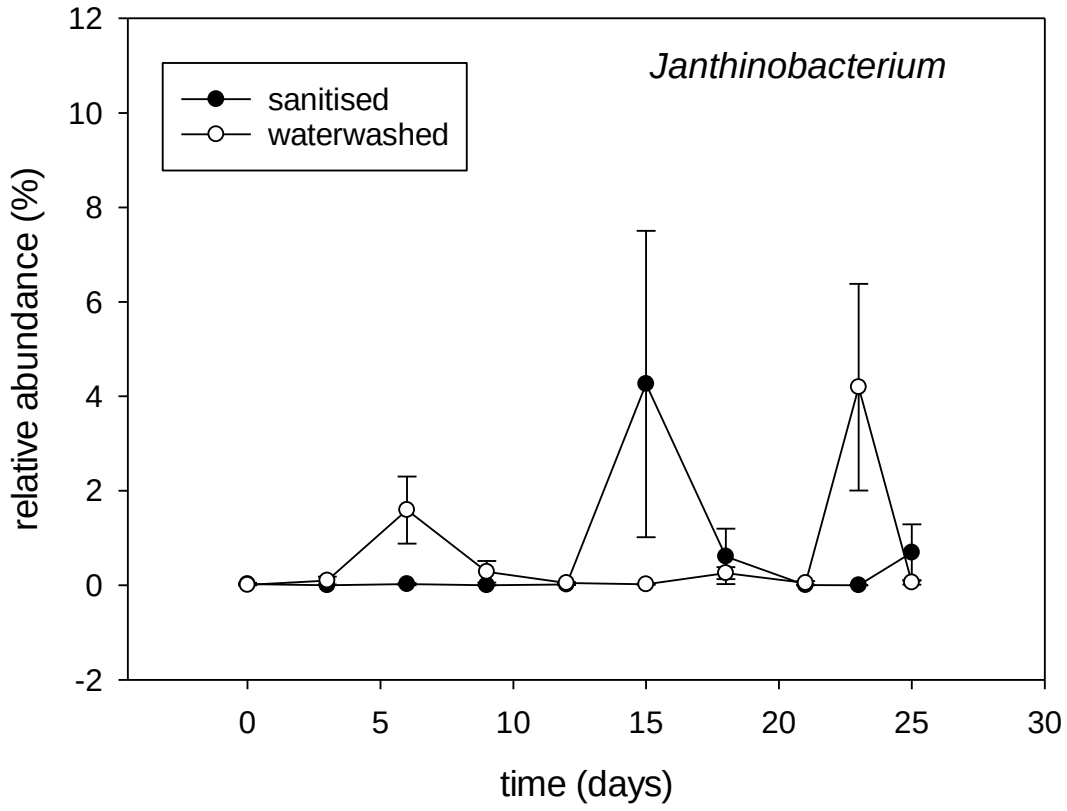

Enterobacteriaceae_un
sanitised
waterwashed
relative abundance (%)
time (days)
0
2
4
6
8
10
12
14
16
18
0
5
10
15
20
25
30

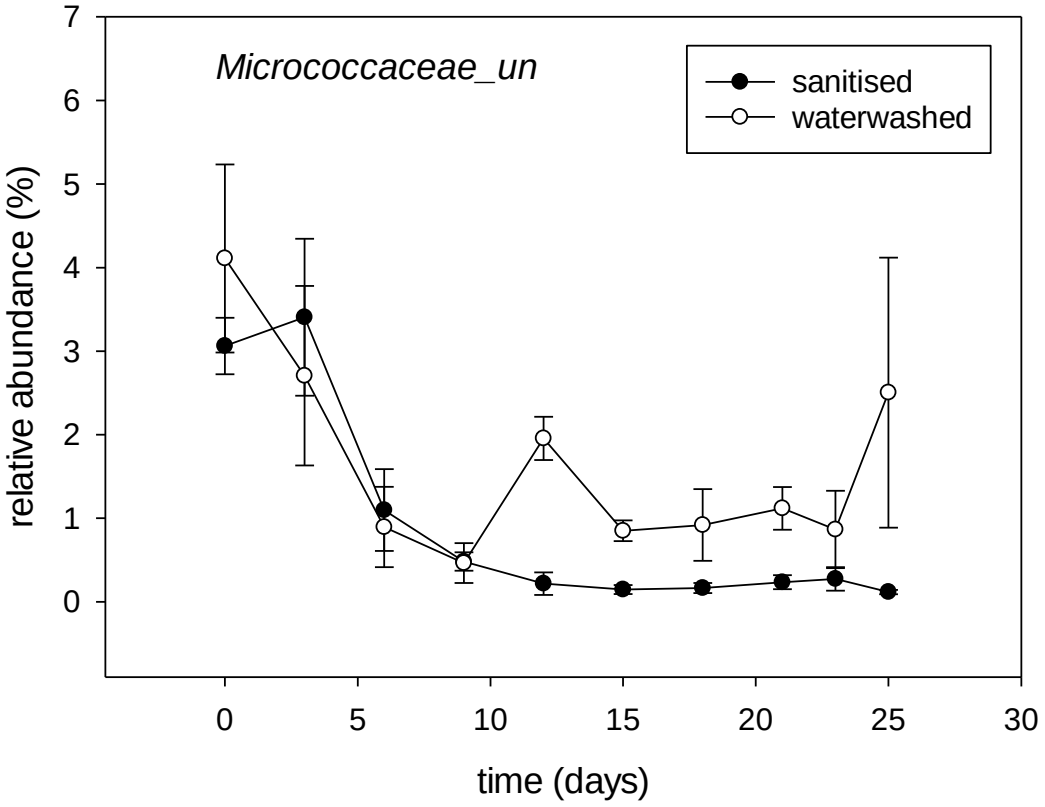

Flavobacterium
sanitised
waterwashed
relative abundance (%)
time (days)
0
5
10
15
20
25
0
5
10
15
20
25
30

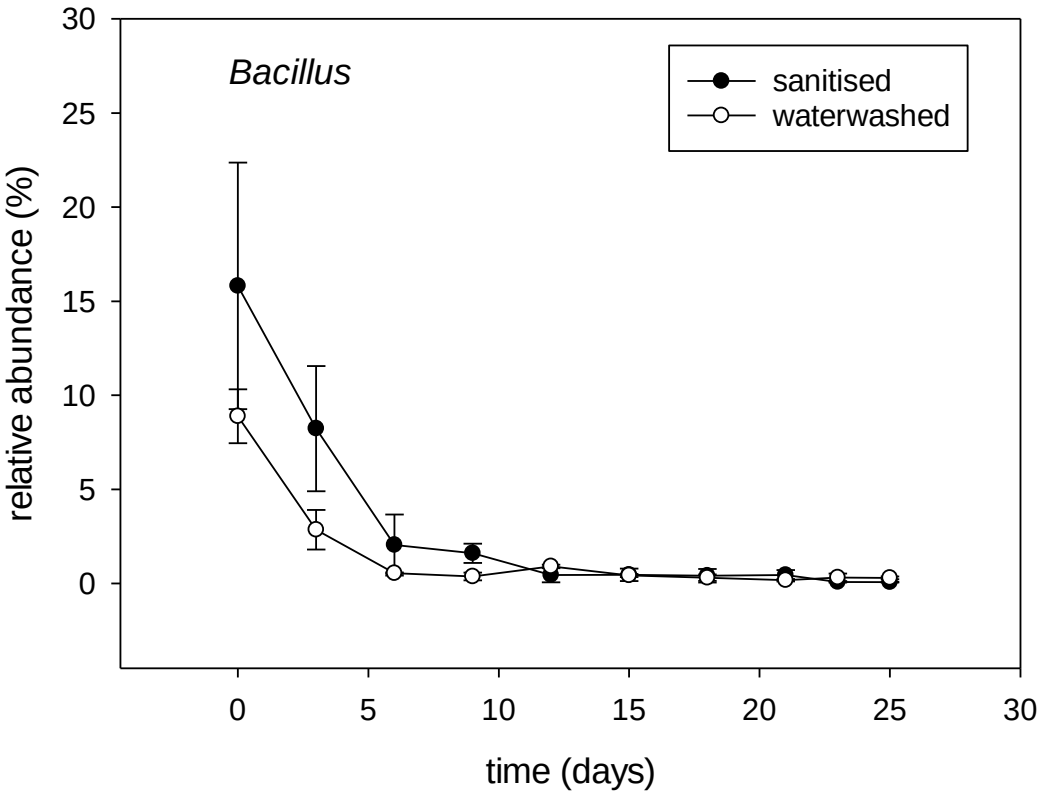

sanitised
waterwashed
Curtobacterium
relative abundance (%)
time (days)
0
2
4
6
8
0
5
10
15
20
25
30

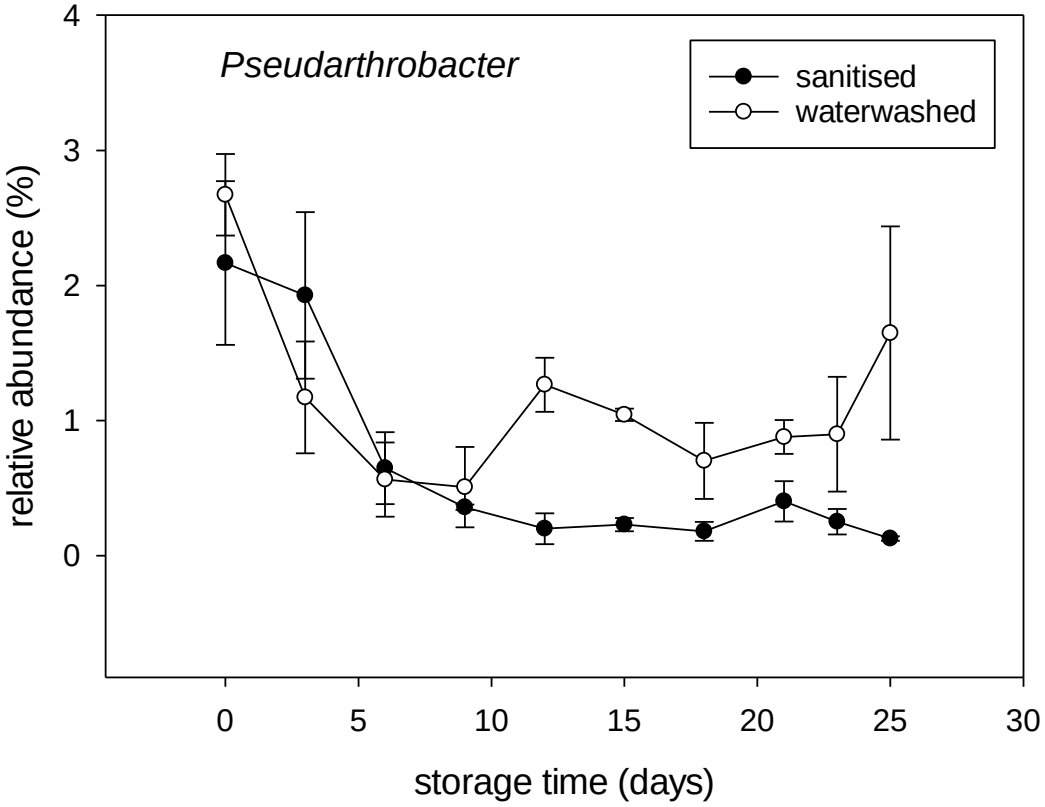

sanitised
waterwashed
Stenotrophomonas
relative abundance (%)
time (days)
0
2
4
6
8
10
0
5
10
15
20
25
30

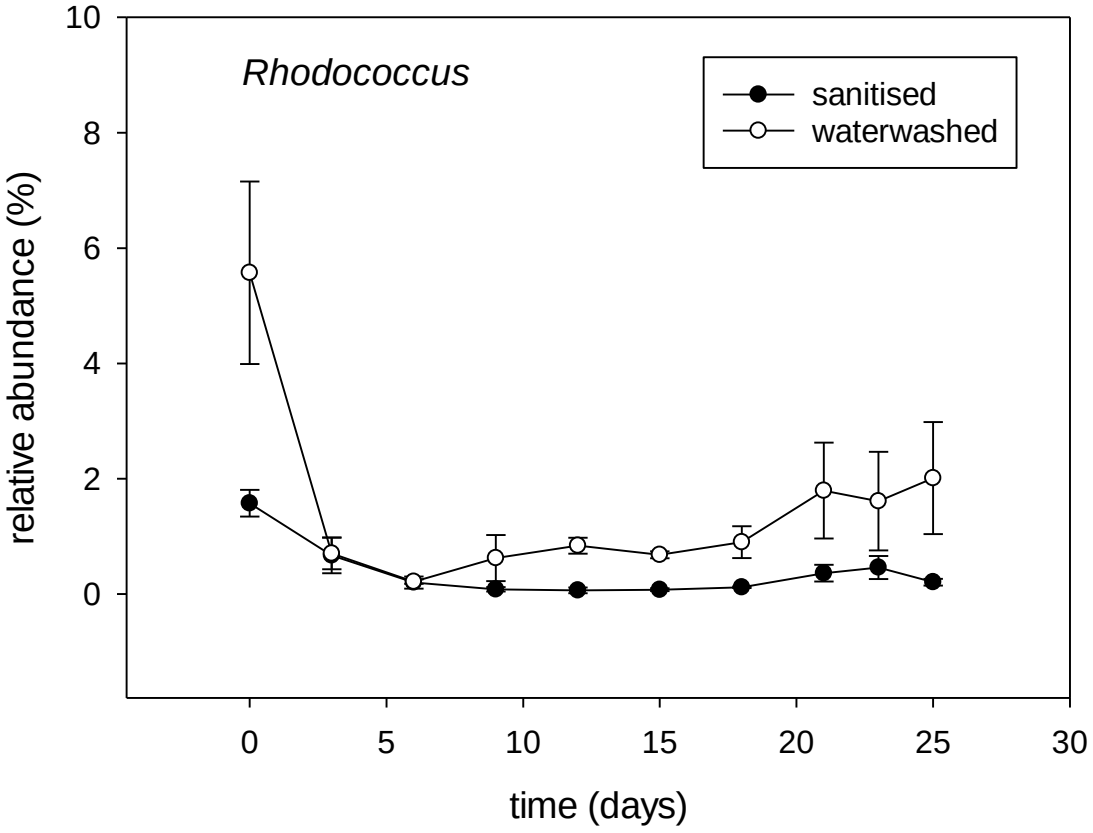

sanitised
waterwashed
Paenibacillus
relative abundance (%)
time (days)
14
12
10
8
6
4
2
0
0
5
10
15
20
25
30


sanitised
waterwashed
Janthinobacterium
relative abundance (%)
time (days)
12
10
8
6
4
2
0
-2
0
5
10
15
20
25
30


Micrococcaceae_un
sanitised
waterwashed
relative abundance (%)
time (days)
0
1
2
3
4
5
6
7
0
5
10
15
20
25
30


Bacillus
sanitised
waterwashed
relative abundance (%)
time (days)
0
5
10
15
20
25
30
0
5
10
15
20
25
30


Pseudarthrobacter
sanitised
waterwashed
relative abundance (%)
storage time (days)
0
1
2
3
4
0
5
10
15
20
25
30


Rhodococcus
sanitised
waterwashed
relative abundance (%)
time (days)
0
2
4
6
8
10
0
5
10
15
20
25
30

Appendix C4: Statistical analysis results on the effect of treatment and time on changes in the relative abundance of bacterial genera

Table C4: p values for the effect of treatment and time on the relative abundance of bacterial genera identified on baby spinach during storage at 4 °C

Bacterial genera	Interaction effect p value	Treatment p value	time p value
Pseudomonas ↑	0.0277*	0.7737	<.0001***
Pantoea ↓	0.5811	0.0260*	0.0002***
Chryseobacterium ↑	0.1591	0.0586	0.0443*
Sphingobacterium ↑	0.4234	0.1012	<.0001***
Duganella ↑	0.1931	0.0220*	0.0436*
Exiguobacterium ↓	0.5838	<.0001***	<.0001***
Paenarthrobacter ↓	0.0053**	0.2370	0.0002*
Flavobacterium ↑	0.793491	0.1029	0.0016**
Allorhizobium ↓	0.0019**	0.0878	<.0001*
Curtobacterium ↓	0.8190	0.2528	0.0443*
Bacillus ↓	0.2085	0.9117	<.0001***
Stenotrophomonas ↑	0.0418*	0.9305	0.0010**
Erwinia	0.4127	0.6551	0.0272*
*Micrococcae*_un ↓	0.0078**	0.7242	<.0001*
Paenibacilllus	0.0218*	0.2433	0.0432*
Rhodococcus ↓	0.0899	<.0001***	<.0001***
*Enterobacteriaceae*_un	0.0120*	0.5749	0.0058**
Janthinobacterium	0.0037**	0.8619	0.0462*
Pseudarthrobacter ↓	0.0058**	0.6238	<.0001***

Ranking in descending order of the bacteria with relative abundance >3% on most of the days

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