Northumbria University

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A Multi-Omic Approach to Food Spoilage and Nutritional Composition Within a Food Matrix

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Abstract

Foods are subject to microbiological and physiochemical alterations during preparation that influence the shelf-life, but the comprehensive nutritional composition of food remains unassessed. At present, food deterioration is determined using techniques based on microbial and physiochemical assessments that are both outdated and lacking sensitivity. However, advances in -Omic technologies enables a greater understanding of bacterial dynamics and spoilage mechanisms.

In this study, a novel multi-omic characterisation of spoilage in a commercially available vegetable matrix was performed. To profile spoilage the vegetable matrix was stored for 5-days under baseline condition (20 °C) and a series of conditions composing of various temperature, gas composition and pH environments. The B-vitamin composition was determined through the development of an efficient analytical method which showed excellent linearity (r^2=0.98-0.99), reproducibility (intra-day=%CV <7) and low detection (2.4-9.0 ng/mL) and quantification limits (8-30 ng/mL).

Amplicon sequencing revealed the genera *Lactococcus*, *Leuconostoc* and *Yersinia* were responsible for spoilage under baseline conditions. However, overall bacterial dynamics were dependent on the storage condition, for example storage in air promoted *Bacillus*. Storage at different temperatures 7 °C and 37 °C promoted *Pseudomonas* and *Bacillus* alongside lactic acid bacteria respectively. Amplicon sequencing was complemented with untargeted metabolite profiling of volatile and non-volatile metabolites which highlighted metabolites linked to freshness (e.g. glutathione, adenosine 5'monophosphate, arginine) or spoilage (e.g. hypoxanthine and biogenic amines). This further showed metabolic pathways such as purine, glutathione, arginine and proline metabolism were pathways involved in the spoilage of the vegetable matrix. The main volatile groups that changed during storage included, aldehydes, alkanes, alcohols, free fatty acids (FFAs), ketones and monoterpenoids. However, metabolites were shown to be dependent on microbial load and the bacterial communities present. Furthermore, this research highlighted a relationship between B-vitamins and spoilage activity. The content of riboflavin and thiamine reduced by 85.2% and 41% respectively, and nicotinamide was fully exhausted when growth of *Lactococcus*, *Leuconostoc* and *Yersinia* reached spoilage levels (2.105 x 10^8 CFU/g). This relationship was also influenced by the storage conditions, but in all conditions where growth of microorganisms reached 10^7-10^8 CFU/g, nicotinamide was depleted. Therefore, nicotinamide has the potential to be a marker of product freshness and on-going spoilage.

In conclusion, this comprehensive -Omic evaluation of food spoilage has provided novel findings regarding food spoilage dynamics that could inform future studies into food spoilage detection and shelf-life extension.

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Author's Declaration

I declare that the work contained in this thesis has not been submitted for any other award and that it is all my own work. I also confirm that this work fully acknowledges opinions, ideas and contributions from the work of others. In the case of my own published work, I retain the rights of the publication and can include the publication in this thesis.

I declare that the word count of this thesis is 55,588

Name: Kate Porter

Date: 12/06/22

List of Abbreviations

Abbreviation	Meaning
ADP	Adenosine 5’diphosphate
ASLT	Accelerated shelf-life testing
AMP	Adenosine 5’monophosphate
ATP	Adenosine triphosphate
a_w	Water activity
cAMP	Adenosine 3’, 5’-monophosphate
CV	Coefficient of variation
DRVs	Dietary reference values
DTT	DL-dithiothreitol
Eh	Redox potential
ES-API	Electrospray – Atmospheric pressure ionization
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
GC	Gas chromatography
GC/TOF-MS	Gas chromatography- time-of-flight mass spectrometry
HILIC	Hydrophilic interaction chromatography
HTS	High-throughput sequencing
IMP	Inosine 5’-monophosphate
LAB	Lactic acid bacteria
LC/MS	Liquid chromatography mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
MAP	Modified atmosphere packaging
MRM	Multiple Reaction Monitoring
MS	Mass spectrometry
NADH/NAD^+	Nicotinamide-adenine dinucleotide
NADPH/$NADP^+$	Nicotinamide-adenine dinucleotide phosphate
OTUs	Operational taxonomic units
PCA	Principal component analysis
PCoA	Principal coordinate analysis
PERMANOVA	Permutational multivariate analysis of variance
PL	Pyridoxal
PLP	Pyridoxal 5’-phosphate
PLS-DA	Partial least-squares discriminant analysis
PM	Pyridoxamine
PMP	Pyridoxamine 5’-phosphate
PN	Pyridoxine
PNP	Pyridoxine 5’-phosphate
QC	Quality control
RNI	Reference nutrient intake
ROS	Reactive oxygen species
RP	Reversed-phase
RT	Retention time
SCFAs	Short chain fatty acids
SD	Standard deviation
SIM	Single ion monitoring
SSOs	Specific spoilage organisms
TAE	Tris-acetate-EDTA
TMP	Thiamine monophosphate
TPP	Thiamine pyrophosphate
TTP	Thiamine triphosphate
TVB-N	Total volatile basic nitrogen
TVC	Total viable count
VIP	Variable importance in projection
VOCs	Volatile organic compounds

Chapter 1

Literature Review

1.1 Introduction into the Food Chain, Shelf-life and Stability of Foods

The food chain is a very complex system comprising of many stages and often expressed as 'from farm to fork'. From raw materials to manufacturing/processing of foods, packaging, storage, distribution to retailers, before concluding with consumer purchase, handling and consumption (1). Currently there are pressing issues in the food chain, and this includes the demand for increased quality and safety of food from a growing health-conscious population (2-5). Current schemes developed by the European Commission are in place and working towards providing 'safe, nutritious, high quality' foods to 500 million citizens (1). However, foods are vulnerable to spoilage during the food chain, which are a result of physiochemical and microbiological biotransformation's, that influence consumer acceptance (6, 7). These biotransformation's will occur as a product becomes exposed to different environments (e.g. temperatures, presence and absence of oxygen) throughout the food chain particularly during processing and storage, impacting on microbial growth, nutritional status and overall product quality (8-12).

Food quality and safety can be determined by shelf-life, which is defined as the time food products can be stored under set conditions, including temperature, light and humidity, before undergoing sensory and microbiological changes that render it unacceptable for consumption (6, 7, 9). Confirming a products stability/ durability in terms of quality and safety during aspects of the food chain are indicated by 'best before' and 'use by' dates' outlined in EU regulation No 1169/2011 (13, 14). The two termed dates provided on food packaging are mandatory requirements and are not interchangeable. The 'use by' dates are provided to food items of a highly perishable nature and of high risk of causing food poisoning if consumed after the displayed date. Comparably, 'best before/best before end' dates are displayed on food items whereby after the date organoleptic properties of the food may decline, although the food remains safe for consumption (15). Several influencing factors for setting shelf-life are considered and outlined in Figure 1.1, in relation to aspects of the food chain.

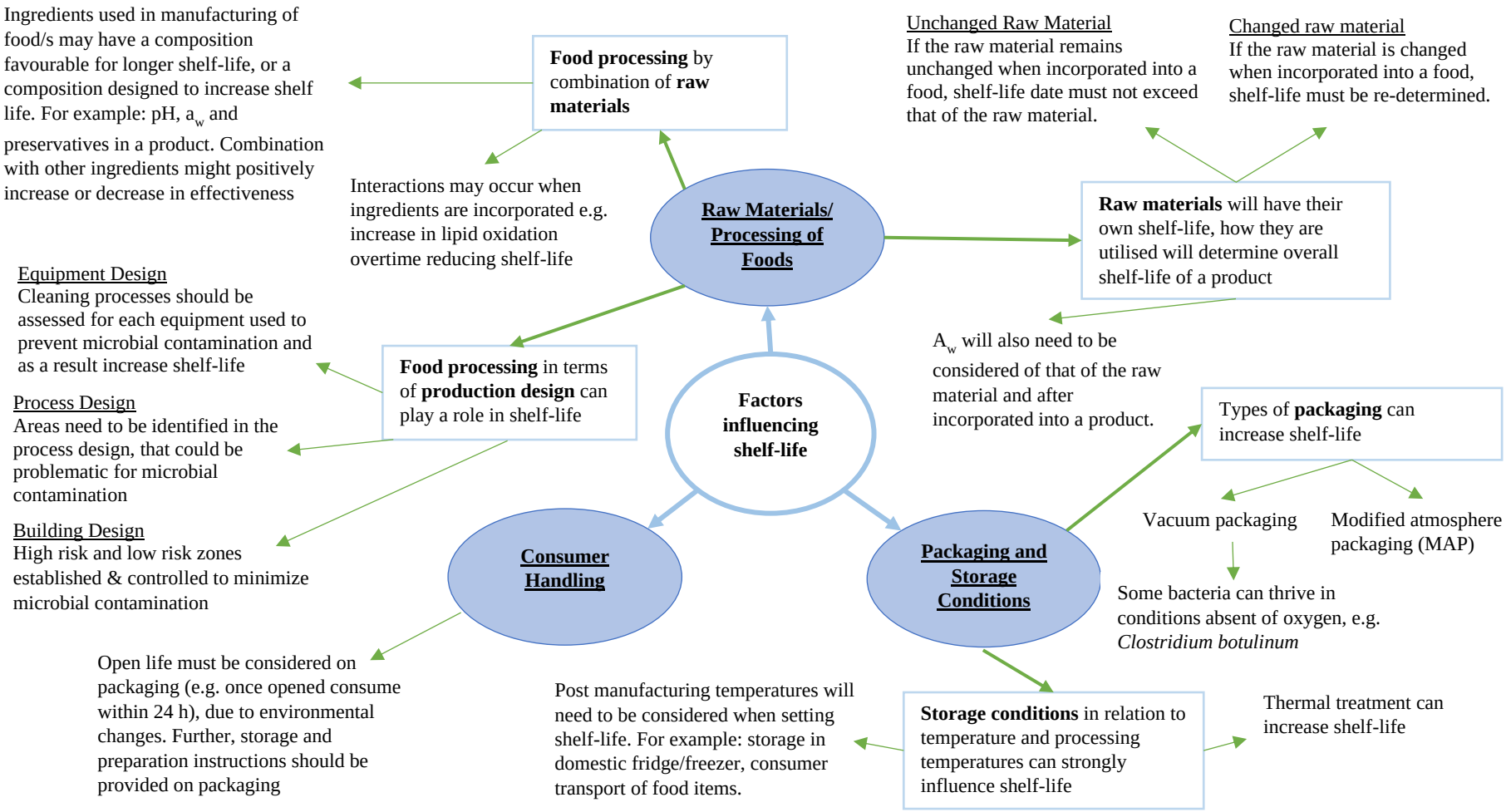


Figure 1.1: Factors influencing determination of shelf-life, in line with aspects of the food chain as outlined by the Food and Drink Federation (2017) (15).

In line with the above, shelf-life evaluation based on monitoring quality and safety markers as a function of time at different storage conditions can be assessed in two ways. One of two ways is known as real-time shelf-life testing, whereby a particular food matrix is monitored under 'normal' recommended storage conditions (16, 17). Alternatively, accelerated shelf-life testing (ASLT) involves storing a food matrix in conditions that would speed up degradation. ASLT is advantageous when shelf-life is required to be understood quickly and Haouet *et al.* (2019) (17) recently and successfully applied this method to perishable food matrices.

Furthermore, although quality and safety markers such as microbiological changes are considered when determining shelf-life, nutritional content particularly vitamin composition often remains unassessed. Vitamin composition of foods is only required to be assessed if declared on nutritional labelling, where health claims have been made on packaging, or in cases where vitamins have been added to a food (fortification) in sufficient quantity (18, 19). However, vitamin composition is neither stable nor guaranteed due to their susceptibility to degrade, during processing and post manufacturing and fail to be monitored. Therefore, it is important to provide an in-depth characterisation of the stability of a food commodity during shelf-life; understanding nutritional composition and the relationship between nutritional status and other quality/ safety markers, such as presence of microbial communities.

1.2 Food Spoilage in Food Systems

Food spoilage is generally defined as a metabolic process or change that renders a food product undesirable or unacceptable for consumption (20). This could mean that the organoleptic properties of a food (texture, flavour, aroma etc.) has fallen below a level that is deemed acceptable, or the nutritional value of the food has deteriorated below that declared on packaging (21). This could be a result of physical, chemical and or biological changes, including oxidation, hydrolysis and enzymic action (7). Although, one of the main drivers of food spoilage is the presence of microbial communities and as a result, one of the main features assessed in shelf-life studies. The presence of microbial communities is problematic to the food industry it is an economical and environmental burden, contributing to approximately 21% of food waste and can be a safety issue (22). There have been numerous publications investigating the deterioration of foods both animal and plant origin under specific storage conditions (10, 23-26). However, as stated by Snyder *et al*. (2017) (35), historically the number of publications in literature have favoured food safety as opposed to food spoilage.

Microorganisms in foods are either naturally occurring (indigenous microbiota of raw products), or microorganisms can colonise a product across the food chain (processing and storage) (27, 28). The microbial communities present in foods can be subdivided into bacteria and fungi (yeast and moulds), some of which are classified as pathogenic and or spoilage species (29). Pathogenic microorganisms receive significant attention in the literature, due to their potential effect of

causing food poising or food-borne illness, impacting on food safety. Due to the pathogenic nature of some microbes, food laws and regulations are in place designed to protect public health and includes microbiological testing of foods as set out in EU regulation No 2073/2005 (30). The most common pathogenic bacteria tested by law for their presence in certain foods, include: *Bacillus*, *Listeria*, *Staphylococcus*, *Salmonella*, *Campylobacter* and *Escherichia Coli* (30). *Vibrio* and *Clostridium* are equally important pathogens that may be isolated from foods (27). Food spoilage species on the other hand, may be addressed separately to pathogenic organisms. Although, many of the food safety procedures developed to control such health hazardous species, also control spoilage species (31). The presence of food spoilage organisms often leads to quality changes of a product including discoloration, slime production, structural changes, and colony formation. This also gives rise to off-odours, off-flavours, and acidity changes. These are all implications of metabolite production during growth and catabolic activity of such microorganisms, detrimentally influencing shelf-life (31-34). Some of the main microorganisms responsible for food spoilage and often referred to as specific spoilage organisms (SSOs) are identified and outlined in Table 1.1, although their presence is food dependant among other factors (7).

There are different approaches used in the food industry to assess food spoilage, particularly when the microbiological status of a product is important, this includes shelf-life trials, challenge tests and predictive microbiology (35). Shelf-life trials as discussed in the above section analyse the growth of microorganisms that are naturally present in the product (35). The assessment of the total microorganisms or total viable count (TVC) in a product during such trials can provide important information on the quality and remaining shelf-life of a product, as it is generally accepted when growth of microorganisms reaches 10^6 – 10^9 CFU/g spoilage occurs (28, 36). Alternatively, challenge tests study the growth kinetics of microorganisms that have been deliberately inoculated into a food (37). This is a useful approach, providing information on the types of spoilage or pathogenic organisms that have the potential to thrive in the food product and also highlight what could happen if the product is microbiologically contaminated during the food chain (37, 38). On the other hand, predictive microbiology is the development of mathematical models that predicts microbial behaviour in foods (39). The aim of predictive microbiology is it provides information on growth rates of microorganisms and probable organisms that could thrive under a particular storage environment and further captures the effect of processing and storage on the microbiological status of a food (35, 39). This approach has been described in the literature as an essential tool in modern food microbiology, that is used to evaluate concerns regarding the quality and safety of foods during the food chain and can be used to estimate shelf-life (40). The development of predictive mathematical models dates back to 1922 when Esty and Meyer (1922) (41) developed a log-linear model describing the effect of heat treatment on *Clostridium botulinum* type A spores. This model is now used in the food industry to estimate the temperature and time required during thermal processing of canned foods (42).

Table 1.1**:** Characteristics of specific spoilage organisms (SSOs), in terms of their spoilage potential of certain foods and the main substrates for spoilage.

Microorganisms	**Spoilage of foods**			**References**
	Effect on foods	**Sources**	**Substrates supporting growth**	
Gram-negative and rod-shaped bacteria: *Enterobacteriaceae spp.* *Pseudomonas spp.*	*Enterobacteriaceae spp.*, some of which are pathogenic, while some produce gas and cause blown packaging. Also, can cause soft rot of vegetables. Growth facilitated in both aerobic and anaerobic environments. *Pseudomonas spp.* are classified as a psychrotrophic bacteria releasing extracellular enzymes, causing spoilage of foods. Production of off-odours, pigmented growth and slim as growth reaches 10^7-10^8 CFU/g. Rapid growth in aerobic conditions.	*Enterobacteriaceae spp.* present in soil, on the surface of plants (fruit and vegetables) and in foods of an animal origin. *Pseudomonas spp.* present in water and soil. Along with plants (fruit and vegetables) and animals (meat, milk, cheese), particularly foods of a high-water content.	Sugars e.g. glucose Lactate Amino acids, including methionine	(7, 28, 31, 43-47)
Gram-positive bacteria: *Brochothrix spp.*	Classified as a psychrotrophic, responsible for producing off-odours (e.g. sweaty, musty) and off-flavours. Evident spoilage occurs as growth reaches 10^5 CFU/g.	Predominately present as the microflora of meats. Additionally, isolated from seafood and fish.	Sugars e.g. glucose	(48-51)
Gram-positive pathogenic spore forming bacteria: *Clostridium spp.* *Bacillus spp.*	*Clostridium spp.* with low contamination produces significant gas, resulting in swelling of packaging of meats. Also, produces off-odours and organoleptic changes. *Bacillus spp.* produces enzymes (e.g. proteinases, lipases) causing spoilage and in the case of milk can result in 'bitty cream' or 'sweet curdling'. *Clostridium spp.* spoil products that are in an anaerobic environment, while *Bacillus spp.* are capable of spoiling foods aerobically or anaerobically.	Both spore forming bacteria spoil and grow on animal produce (e.g. meat, cheese, milk) and canned goods, including canned fruit. Bakery products can also be spoiled by *Bacillus spp.*, causing a discoloured crust and or stringy crumb.	Protein Sugars	(7, 28, 52, 53)
Lactic acid bacteria (LAB): *Lactobacillus spp.* *Streptococcus spp.* *Leuconostoc spp.*	LAB are psychotropic classified as food fermenting species or as probiotics. They utilise sugars, producing lactic and acidic acid, as well as carbon dioxide and slime. Due to metabolic activities when fermenting sugars, off-odours develop and pH drops, making LAB highly competitive against other species.	LAB are opportunistic bacteria, present in the environment, microbiota of plants and occur in foods of animal origin.	Sugars	(7, 54-56)

Pediococcus spp.	They grow anaerobically but can be in the presence of oxygen and therefore are termed as being aerotolerant anaerobes.			
Yeasts and Moulds **Spoilage yeasts include:** *Saccharomyces* *Zygosaccharomyces* *Candida* **Spoilage moulds include:** *Zygomycetes* *Penicillium* *Aspergillus*	Spoilage yeasts, ferment available sugars, producing acid and gas or alcohol. Production of gas can swell packaging. They can also cause cloudiness, off-flavours, and off-odours. Spoilage moulds lead to pigmented growth on foods and they can also cause spoilage by producing mycotoxins. Yeast and moulds grow in harsh conditions, where bacteria would less likely survive and includes low water activity, low pH, low temperatures and in the presence of some preservatives. Yeasts spoil foods in both aerobic and anaerobic conditions, while moulds grow best aerobically.	Yeasts and moulds are present in a range of environments including water and soil. They can spoil fruit and fruit juices, vegetables, confectionary products, syrups, meat, bakery (e.g. bread) and dairy goods.	Pectin's and other carbohydrates Organic acids Proteins Lipids	(7, 31, 32, 57)

Studies assessing the presence of microbial spoilage species in specific food commodities have historically been focused on culture dependent approaches (e.g., plating) or phenotyping techniques (58, 59). While other physicochemical markers are assessed alongside and include pH, sensory changes, and production of gases, discussed in detail in section 1.3 (58, 60, 61). Conventional methods such as plating can be used to quantify viable cells within a sample, while growth on selected media can be used to target and detect SSOs (61, 62). Such methods, however, are time-consuming, introduce bias and underrepresent the microbial community present within a sample (63, 64). In contrast, emerging technologies over recent years involving PCR-based, culture-independent approaches such as amplicon sequencing and metagenomic sequencing provide scope and depth into microbial ecosystems through DNA extraction. Such technological advances provide immense potential to understand the microbial diversity and dynamics in food systems from both a safety and spoilage perspective, as shown in publications (34, 58, 63, 65). This has led to the formation of concepts such as ephemeral/ specific spoilage organisms, relating to the succession of SSOs in foods under different environments (45, 66). Furthermore, some researchers address complementation of using both culture-dependent and culture-independent approaches to provide both a level of quantification and an insight to microbial dynamics during food storage (64, 67).

Growth of SSOs as a function of time, comprises of four distinct phases known as lag, log/exponential, stationery and death phase as shown in Figure 1.2 (68). The growth phases and thus spoilage or pathogenic potential depend on the intrinsic and extrinsic properties of foods as conferred in the following sections 1.2.1-1.2.2. Other spoilage reactions as briefly mentioned (e.g enzymatic activity, lipid oxidation), may be occurring alongside microbiological activity, some of which may be interlinked and interact (7). Due to the intrinsic and extrinsic parameters influencing spoilage, various procedures in the food industry have been utilised during product formulation (e.g. addition of preservatives), processing (e.g. pulsed electric field processing), packaging (e.g. vacuum packaging) and storage to reduce and mitigate spoilage and are covered in the below sections (12, 21, 69, 70).

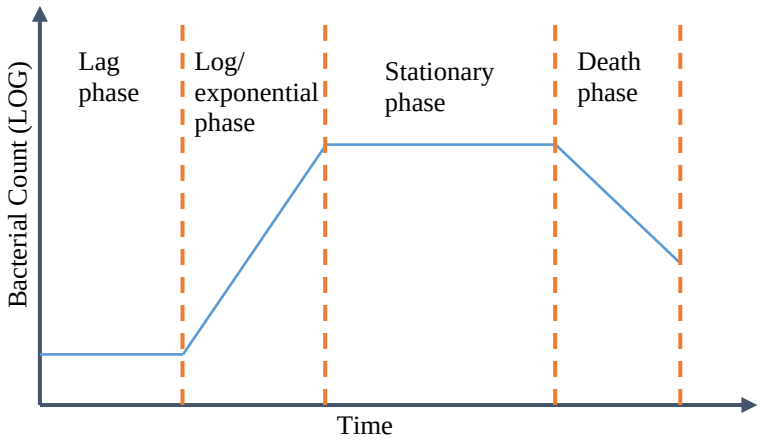


Figure 1.2: Microbial growth curve, consisting of 4 distinct phases (68).

1.2.1 Intrinsic Properties Shaping Spoilage Potential in Food Systems

There are several intrinsic parameters of foods that collectively influence pathogenic and microbial spoilage of foods and other spoilage reactions. These include, water activity, pH, redox potential, the presence of antimicrobial substances and available nutrients. It is imperative to understand how each of these factors influence spoilage, as it provides scope into not only predicting presence of SSOs, but also preventing and controlling spoilage of foods. In addition, some intrinsic properties of foods can equally impact on nutritional status, as discussed separately in section 1.5.5.

Water activity (a_w) refers to the mobility of water in a food system when the surrounding atmosphere is in an equilibrium state (7). Water activity is an important measurement in the food chain to indicate food stability in terms of safety and quality, as it influences potential chemical and biochemical changes, as well as microbial growth (Figure 1.3) (71). Measurement of a_w provides a value between 0-1, 1 referring to pure water. If a_w of a food matrix is close to 1, biological reactions are facilitated including enzymatic activity and microbial grow. However, as a_w decreases towards 0 biological reactions are reduced and chemical changes become more of spoilage concern (71, 72).

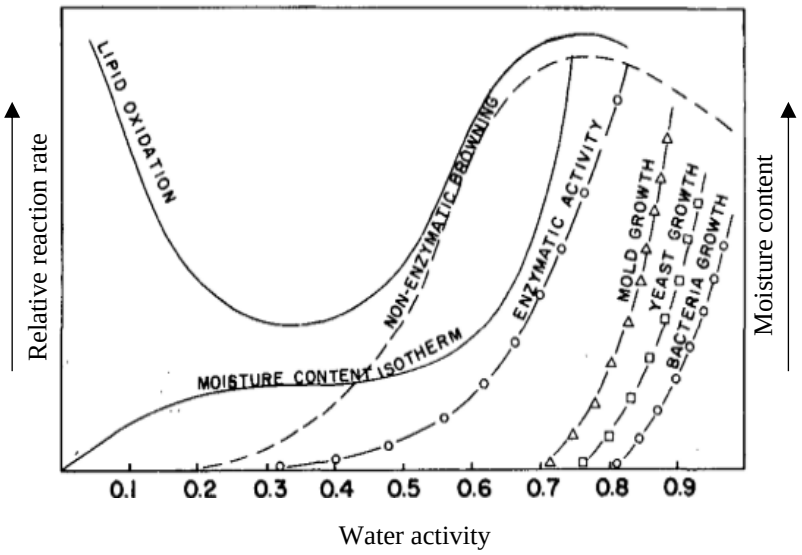


Figure 1.3**:** The influence of water activity (a_w) on growth of microorganisms and several chemical and biochemical reactions. Graph obtained from Labuza *et al.* (1972) (73).

Water activity and its impact on microbial growth was first presented by Scott (1957) (74); the author showed the availability of water in foods can directly influence microbial growth and their metabolism (72). Therefore, SSOs have an optimum a_w required for growth, although this is dependent on the microorganism. For example: bacteria thrive and spoil foods within a a_w range of 0.9-1, except for halophilic bacteria which can grow at a lower a_w of 0.75. Alternatively, yeast and moulds can survive in lower water activity environments, a_w ranges of 0.85-0.94 and 0.70-

0.82 respectively. However, an a_w lower than 0.60 would inhibit growth of all microorganisms (7, 71). Due to a_w implications on microbial growth, it can be used as a tool in the food industry to inhibit food spoilage caused by microorganisms, this has been demonstrated in several publications (75-77). Abdullah *et al.* (2000) (78), investigated the impact of a_w (0.65-0.98) on fungi spoilage over time in starch-based foods. Authors showed when the a_w of rice was 0.75 fungi spoilage occurred in 20 days, alternatively lowering a_w of the rice to 0.65 preserved the rice for 2 months.

In line with water activity, hydrogen ion concentration of foods referred to as pH can significantly affect microbial metabolism (7). It has been well documented that microbial growth is pH dependant. Therefore, species have an optimum pH range, as with a_w, and can be categorised as acidophiles (pH < 3), neutrophils (pH 5-9) and alkaliphiles (pH > 9) (79-81). Generally, most spoilage species are neutrophils, with an optimum pH range of 6-7.5, however some spoilage species, namely lactic acid bacteria (LAB) are acidophiles and can grow below pH 5. Comparably, fungal communities can grow and survive in harsher acidic environments, even lower than pH 2 (72). From understanding optimum pH for microbial growth, the pH of food items can be modified to inhibit growth, and this is often a preservative approach taken in the food industry. Many pH treatments involve use of weak acids, lipophilic and undissociated acids which include acetic, benzoic and sorbic acid. Weak acids lead to the acidification of the cytoplasm, adversely affecting metabolism, including enzymes involved in glycolysis (82-84). Jin and Kirk (2018) (81) also outlined in a recent paper, pH modification can also adversely affect redox reactions, a key part in energy production of microorganisms. Additionally, alteration of pH can affect the medium itself rather than directly effecting cells, which could further influence growth. For example: citric acid (lipophobic, dissociated) has been shown to inhibit growth of microorganism by chelating divalent metal ions in the medium including magnesium ions and calcium ions, reducing accessibility to SSOs, which are required for metabolism (85).

Redox potential (E_h) can influence food spoilage and refers to the oxidation-reduction ability of compounds within a system (7, 86). E_h controls electron transport pathways and hydrogen ion transportation across the cell membrane of microbial species, therefore, changes in E_h can have a drastic impact on intracellular processes and growth (87, 88). Both the atmospheric conditions (O_2 status), and pH of the environment influence the E_h status of a system (89, 90). Dependent on the atmospheric conditions required for SSOs (anaerobe or aerobe), ultimately indicate whether an oxidised or reduced environment is desired for growth. Anaerobes require a reduced environment, whereas aerobes require an oxidised environment (72, 91). Soghomohyan *et al.* (2011) (87) investigated pH and E_h modification including the use of oxidizers (ferrycianide) and reducers (DL-dithiothreitol) on LAB cultures. The authors highlighted, acidification of the environment through production of fermentative products of LAB simultaneously decreased E_h, creating a reducing environment, which supported growth and the conversion from log to

stationary phase. However, oxidizing agents could be used to inhibit growth, as LAB duration in lag phase was shown to be sustained for a longer periods and overall growth declined. Due to the impact of E_h on growth of SSOs and as highted in literature by Olsen *et al*. (2009) (86) and Reichart *et al*. (2007) (92) E_h could be a useful measure to both monitor and assess food spoilage species.

The presence of antimicrobial agents within a food matrix can significantly influence and inhibit growth of spoilage species. Antimicrobial agents may be added to foods and classified as food preservatives and include synthetic compounds, such as nitrates and sulphites. Nitrates and sulphites not only inhibit microbial growth but can prevent chemical spoilage, and therefore have desirable attributes (93). Although, it is important to note such compounds have toxicity levels, with legislative measures surrounding use of these in foods (93). Therefore, significant research has been conducted into natural compounds exerting antimicrobial effects, such as food constituents as an alternative to synthetic preservatives. Based on current research food constituents with antimicrobial properties include spices (94), plant essential oils (95), isothiocyanates from cruciferous vegetables (96), plant phenolic extracts (97) and peptides (98). A recent review by Quinto *et al*. (2019) (99) covered natural antimicrobial agents, categorised by origin; plant, animal, bacteria, virus, algae and mushroom. Furthermore, the impact of antimicrobial agents is species and strain dependant as shown in most antimicrobial studies. For example: Cock *et al.* (2015) (69) investigated the impact of plant extracts on food spoilage species and pathogens, for Gram-negative bacteria, 93.1% of the plant extracts investigated inhibited growth. On the other hand, 79.3% of plant extracts inhibited growth of Gram-positive bacteria, and only 24.1% inhibited fungal species. The mechanisms in which antimicrobial agents inhibit growth, might be agent specific, but some authors have reported antimicrobial agents lead to the damage of the plasma membrane of the cell (96, 97). In addition, bacteria can produce their own antimicrobial agents (bacteriocins and bacteriophages) including weak acids to facilitate competition in a medium (99). Antimicrobial agents also cover addition of weak acids (e.g. citric and acetic acid) to foods and the presence of sugar or salt. The former impacts pH of foods as discussed above and the latter (sugar and salt) at high concentrations can create osmotic pressure causing plasmolysis of cells (47).

Nutritional compositions of foods, and thus the availability of nutrients for microorganisms, is an important intrinsic factor as with other elements discussed above. Microbial communities require substrates for growth and metabolism in the form of an energy source (e.g sugars), nitrogen source (e.g. amino acids), organic and inorganic acids (vitamins and minerals) (72). Early research by Monod (1949) (100) initially highlighted that nutrients can be a limiting factor for growth of microorganisms. The study indicated if essential nutrients are exhausted or limited within a system, growth can be adversely affected (100). The nutrients available will also indicate the presence and dominance of certain SSOs, as well as shape competition between microbial species,

dependent on other intrinsic and extrinsic factors. For example: proteolytic microorganisms (e.g., *Pseudomonas*) are potentially the dominant spoilage species in protein rich foods, while fermentative microorganisms (e.g., yeasts, LAB) are favoured in food products high in carbohydrates (28, 101). Additionally, competitiveness of microbial species and overall dominance will be affected by the affinity of specific microorganisms for limiting nutrients. Newton and Gill (1978) (102) demonstrated in a mixed culture (*Microbacterium thermosphactum, Enterobacter, and Lactobacillus)* with limited glucose, *Enterobacter* had a higher affinity for glucose and as result outcompeted *Lactobacillus* among other microbes.

1.2.2 Extrinsic Properties Shaping Spoilage Potential in Food Systems

There are several extrinsic parameters that can influence spoilage of foods and includes temperature and gas composition of surrounding environment that food products are exposed to..

Temperature is an important factor to influence spoilage of foods and as a result is used to not only determine shelf-life but also to extend shelf-life. Temperature control is required throughout all elements of the food chain to guarantee both quality and safety. There are three distinctive types of food supply chains in relation to temperature, known as frozen, chilled, and ambient chain, which require temperatures of -18 °C or below, 0-15 °C, and room temperature (22-25 °C) respectively (103, 104). Inadequate temperature control of food chains can accelerate undesirable reactions leading to quality changes linked to oxidation, enzyme activity, and increase microbial activity (10, 16, 105). Boziaris *et al.* (2011) (10) investigated the storage of lobster under three different temperatures (0, 5 & 20 °C). Authors showed as storage temperature increased, the microbial integrity reduced and alongside off-odours developed. Consequently, they showed storage at 20°C reduced shelf-life of the lobster to 24 h, compared to storage at 0 °C, which increased shelf-life to 96 h. Kinetic models have also been developed to evaluate the impact of storage temperature of foods on quality parameters and to predict growth of SSOs in certain foods (106-108). For example, Pinheiro *et al.* (2013) (108) developed a mathematical model to predict tomato fruit quality (colour, firmness and weight loss) under different storage temperatures. Mataragas *et al.* (2006) (106) developed a model for predicting growth of SSOs in meat under constant temperature conditions and dynamic temperature conditions to aid shelf-life predictions. Furthermore, microorganisms can be categorised based on temperatures required to facilitate growth and are either classified as psychrophiles (grow between <0-20 °C) psychrotrophs (grow between <7 °C-30 °C), mesophiles (grow between 20 °C-45 °C) or thermophiles (grow >45 °C) (72, 109, 110). Generally, SSOs are psychrotrophic and as a result pose a threat to chilled products (111). Although, some SSOs namely *Clostridium spp*. and *Bacillus spp*. can survive under unfavourable temperatures through endospore production (53). Storage temperatures may also favour growth of certain SSOs and thus spoilage potential as suggested by Höll *et al*. (2016) (112). Authors showed meat stored at 4 °C promoted *Brochothrix thermosphacta*, *Carnobacterium spp*.

and *Pseudomonas spp*., however storage at 10 °C allowed *Pseudomonas spp*. to thrive. The relationship between microbial growth and temperature also led to early development of processing techniques involving heat treatment to inhibit growth and prolong shelf-life of food, this includes ultra-high temperature processing (113).

Atmospheric conditions in relation to presence and concentration of gases are a vital factor in spoilage of foods. The surrounding gas environment of foods can shape both chemical/biochemical reactions and food spoilage microbiota. Oxygen presence in air for example promotes undesirable reactions, including oxidation and rancidity, enzymatic activity (e.g. polyphenoloxidase) and growth of aerobes (114, 115). Although, pure oxygen environments of 80-100% have been shown to inhibit biochemical reactions, for example reducing browning of fruits (116) and mushrooms (117). However, modification of the atmosphere can be achieved through packaging technology and is a technique implemented by the food industry to reduce undesirable gas facilitated reactions. Vacuum packaging as an example of specialised packaging, creates minimal oxygen levels, thus reducing oxidation reactions in combination with other storage factors such as refrigeration temperatures (118). Minimal oxygen levels in vacuum packaging would, however, favour the growth of anaerobic spoilage species or facultative anaerobes. This was shown in a publication by Wang *et al.* (2016) (12), during 7 day storage of vacuum-packed meat, bacterial communities changed from aerobic species to facultative anaerobes namely, LAB, and as a result LAB became the dominant spoilage species. Moreover, modified atmosphere packaging (MAP) is another form of specialised packaging and can be in a passive or active form. Passive MAP is developed by packaging film and respiring products, creating a desired 'natural' gas composition. In contrast, active MAP is where the gas composition is set initially and thus gases are either replaced or displaced in packaging, although the headspace can change over storage, due to microbial and product respiration. The gases used in MAP may comprise of oxygen, nitrogen, and carbon dioxide, however the effectiveness of reducing spoilage depends on the concentrations and combination of gases used (115, 119). For example, Wang *et al* (2016) (12) investigated different gas compositions during storage of lamb these included 20% CO_2/ 80% N_2, 60% CO_2/ 40% N_2 and 100% CO_2. They showed storage of the lamb in an 100% CO_2 was the most effective gas composition for inhibiting microbial growth and as a result extended the shelf-life by an additional week. This is due to carbon dioxide having a reported bacteriostatic effect, although as shown in their research, Gram-positive species are more resistant to the inhibitory effects of CO_2 as opposed to Gram-negative bacteria (12, 120). This observation has also been documented in other research papers; Zhang *et al* (2013) (121) investigated different O_2 (21-70%) and CO_2 (0-50%) concentrations on the growth of microbial communities (*Candida sake*, *Leuconostoc mesenteroides* and *Leuconostoc gelidum*) associated with spoilage of honeydew melons. They showed that the modified atmospheres investigated had no effect on the growth of Gram-positive bacteria LAB, although the highest O_2 and CO_2 level was effective at inhibiting the growth of yeast, *Candida sake*. Similarly, Ercolini *et al* (2011) (25) showed MAP

(60% O_2/ 40% CO_2) to be least effective in inhibiting growth of Gram-positive bacteria during storage of meat. Modified atmospheres or vacuum packaging show how the presence or absence of certain gases can significantly influence microbial communities and other undesirable spoilage reactions.

The mixed microflora of foods and therefore presence of other microorganisms, can influence spoilage potential and metabolism (48). Microbial species may coexist with each other, or become dominant, competing for resources for growth (122). Competition can occur via two suggested ways by Cornforth and Foster (2013) (123), it can either be exploitative competition, which is classed as an indirect form of competition, where nutrients become limited to other microbial species. Or the other form of competition is known as interference competition and refers to direct cellular damage caused by microorganisms, via toxin production, generation of organic acids and production of compounds that change the gas composition (56, 123). For example: LAB can outcompete other SSOs during food storage, through production of bacteriocins and organic acids (12). Current research with advances in culture-independent approaches (PCR based) such as meta-genomics, has been used to focus on the mechanisms of interspecies interactions (56, 124). One procedure bacterial cells are known to communicate (cell to cell) is quorum sensing (QS), which aids in bacterial survival and competition by regulating antimicrobial agents, enzymatic activity and biofilm production among other factors (122, 125). This type of cellular communication is based on production and secretion of signalling molecules, namely autoinducers and publications have depicted that such signalling molecules can play a role in food spoilage (125, 126).

1.3 Spoilage Markers of Foods

Spoilage of foods through activity of microorganisms and endogenous chemical reactions depending on intrinsic and extrinsic environments (sections 1.2.1-1.2.2) can have detrimental effects on characteristics of foods. This includes production of off-odours and off-flavours, acidification and colour changes (32). Organoleptic changes are commonly associated with spoilage and occur as a result of macronutrient degradation (carbohydrates, protein and fat), via lipid oxidation and/or through activity of both endogenous enzymes and microbial enzymes. SSOs particularly LAB and *Pseudomonas spp*. can produce a variety of enzymes including lipases and proteases, breaking down food constituents (47, 125, 127, 128).

Microbial growth and physicochemical changes (e.g. sensorial changes, production of volatile compounds) occurring during food spoilage are used to assess the quality and safety of food. This has led to the subsequent development of analytical approaches for rapid and quantitative assessment of food spoilage including the development of biosensors, sensor array, spectroscopy and chromatography techniques (20). For example, such techniques can be used to assess microbial load, pH, nutritional content, colour, and metabolite changes (20, 129, 130). Biosensors

are a device that incorporate a sensing element that is a biological material (e.g. nucleic acids, enzymes, antibodies etc) within or intimately associated with a signal transducer (physical or chemical). Therefore, these biological compounds, or otherwise markers of spoilage are targeted and converted into a measurable signal that can indicate spoilage of foods (129-131). An example of a biological sensor would be an optical biosensor, that has recently been employed for detection of spoilage metabolites, specifically biogenic amines (132) and for detection of SSOs (133). Alternatively, array-based sensing techniques, detects volatile compounds and works on the principle of a quantifiable colour change that results from a reaction between targeted volatile compounds and selected chemo-responsive dyes (134, 135). This technique has successfully been utilised to detect volatile compounds linked to food deterioration, including amines, thiols, alcohols, and aldehydes (134, 136-139). Furthermore, spectroscopy techniques including Raman spectroscopy, Fourier-transform infrared spectroscopy and nuclear magnetic resonance have been utilised to assess food quality (140). For example: Lin *et al* (2004) (141) used short-wavelength near infrared to rapidly quantity the microbial load in chicken. Chromatography techniques including high-performance liquid chromatography and gas chromatography are useful analytical approaches used to assess the quality of foods, discussed in detail in section 1.4. The chemical, biochemical and physical markers that are used to assess food spoilage are covered below.

1.3.1 Metabolite Markers

Odour and flavour are important indicators of both freshness and spoilage of a food product during storage, assessed through the production of specific metabolites. Metabolic activities of SSOs through utilisation of organic substrates (e.g. sugars) produces metabolites that are volatile and non-volatile in nature that are associated with the characteristic development of off-odour's and off-flavour's (142-144). Adverse sensory alterations can be associated with other microbial induced/ chemical spoilage reactions, particularly lipid oxidation, esterification and hydrolysis (145). There are well characterised metabolite markers of spoilage, with many chemical methods such as colorimetric sensor array developed for their estimation (146). For example, biogenic amines, ATP break down products and volatile organic compounds (VOC's) are the main metabolites targeted to indicate freshness and or spoilage of foods particularly of animal origin (140, 147). Although it is important to note, markers of spoilage in vegetable-based products and use of them as a tool to assess quality of vegetables are less defined.

Biogenic amines are low-molecular-weight nitrogen compounds that are important metabolites to assess, not only because of their benefit as an indicator of product quality, but also due to their toxicological effects if specific amines, such as histamine are consumed in sufficient quantities (148, 149). Biogenic amines are formed through several enzymatic reactions including, reductive amination, transamination, and decarboxylation (149). Although decarboxylation of amino acids

by microorganisms, through the removal of the α-carboxyl group is the main route of biogenic amine formation (149). For example: decarboxylation of amino acids lysine, tryptophan, histidine, ornithine and tyrosine via substrate-specific enzymes generates cadaverine, tryptamine, histamine, putrescine and tyramine respectively (Figure 1.4) (150, 151). Many authors have addressed biogenic amines as good estimators of animal product freshness, this includes Lázaro *et al* (2015) (148) study, which captured a correlation between bacterial growth, namely total aerobic mesophilic bacteria and *Enterobacteriaceae* with biogenic amine formation in different types of meat products during storage. Similarly, Balamatsia *et al* (2006) (152) showed biogenic amines, putrescine, cadaverine and tyramine increased during storage of chicken and therefore were described as an indicator of chicken quality. Although, the formation of biogenic amines in food does depend on the food product, in terms of available amino acids, in combination with the ability of SSOs to produce decarboxylase (148). In addition, degradation of proteins and additional nitrogenous compounds result in the formation of ammonia and primary, secondary, tertiary amines (e.g. trimethylamine) that are formally referred to as total volatile basic nitrogen (TVB-N) (153, 154). Due to high concentrations of TVB-N indicating spoilage characteristics of primarily meat and fish products, the TVB-N content of food products during storage have been used to indicate product quality and are often monitored alongside specific biogenic amines (147, 153, 154). For example: Holman *et al* (2021) (155), assessed TVB-N content in combination with other quality parameters including, microbial growth, colour and texture parameters during 14-week storage of beef steaks. They found TVB-N was positively associated with microbial growth, colour and tenderness parameters, which shows TVB-N is a useful marker of microbial activity and thus spoilage reactions.

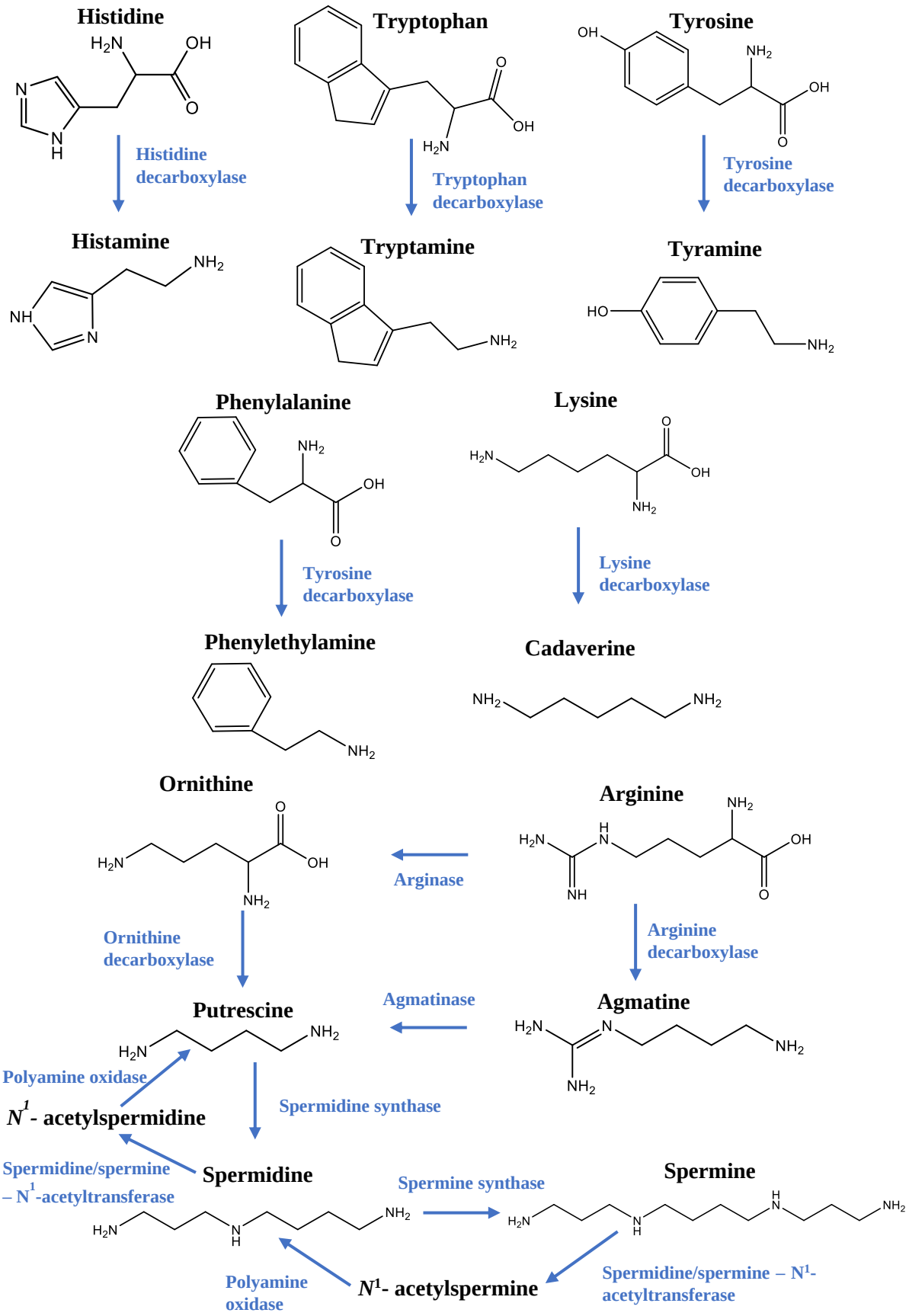


Figure 1.4: Metabolic pathways for the formation of biogenic amines, including different metabolic pathways for the generation of biogenic amine, putrescine (149, 151, 156).

Adenosine triphosphate (ATP) and breakdown products are another well characterised spoilage marker of fish and meat products, with analysis of such metabolites dating back to 1959 (157). ATP can be used as an effective marker of freshness due to ATP degradation being one of the major biochemical changes that occurs in the muscle tissue of meat and fish during storage (158). ATP in post-mortem muscle is converted to adenosine 5' -diphosphate (ADP), then adenosine 5' – monophosphate (AMP), followed by conversion to inosine 5' -monophosphate (IMP). IMP is further broken down to inosine, hypoxanthine, xanthine and uric acid (158, 159). The biochemical pathway for the breakdown of ATP and associated products are shown in Figure 1.5. The formation of ATP degradation products is linked to both autolytic decomposition of nucleotides and bacterial activity, with bacteria such as *Pseudomonas* spp. associated with the production of hypoxanthine (158, 160).

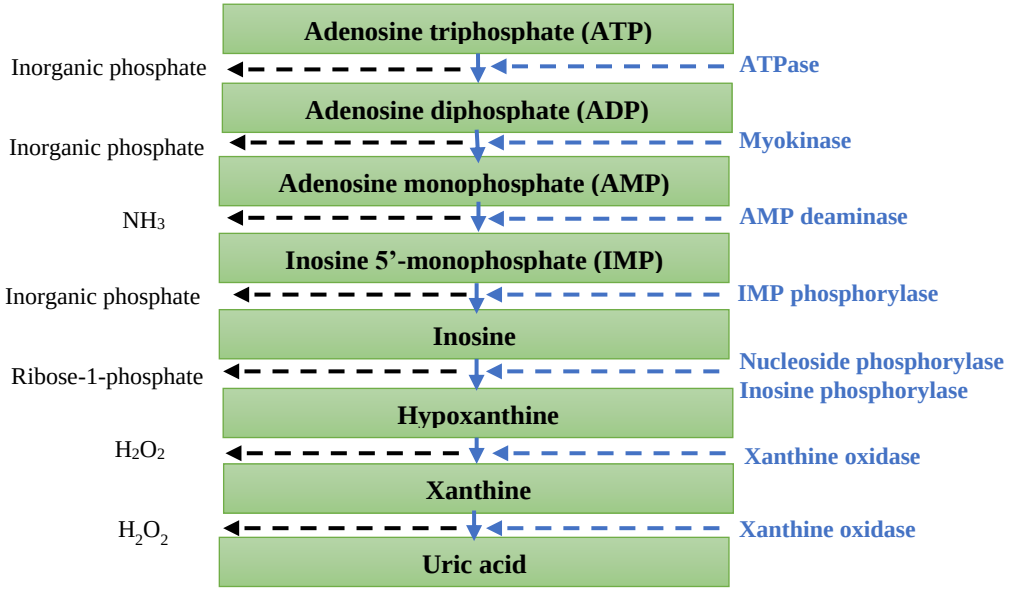


Figure 1.5: Metabolic pathway of adenosine triphosphate (ATP). Pathway adapted from Hong, Regenstein and Luo (2017) (158).

VOCs are an important chemical marker/ chemical spoilage indexes (CSIs) for accessing product quality and spoilage. This is supported by the many articles that have correlated VOCs to spoilage activity and have used VOCs to determine shelf-life of foods (142, 143, 161-164). Production of volatile compounds can be further associated to progression of microbial growth, providing a quantitative measure of microbial growth in a product (165). A study conducted by Mayr *et al*. (2003) (161) showed a linear relationship between microbial count (CFU/g) and emitted volatile compounds in meat such as methanol, 1-octanol, dimethyl disulphide and 2,2-butanediol. In addition, VOC profiling has enabled the identification of volatile compounds associated with spoilage reactions and includes production of alcohols, aldehydes, sulphur compounds, short chain fatty acids (SCFAs), ketones and esters (143, 162, 164). Formation and examples of identified volatile spoilage markers, along with a description of odours/flavours are summarised in Table 1.2, providing insight into individual VOCs and the organoleptic characteristics if present in foods.

Table 1.2: Volatile organic compounds (VOCs) associated with spoilage reactions, including route of formation and odour/flavour descriptors of each volatile

VOCs	Odour descriptors of VOCs [a,b]	Flavour descriptors of VOCs[a]	Formation of Specific VOC Groups During Storage	References
Alcohols including: Ethanol (EtOH)	Alcoholic		Alcohols are generated during storage of foods by SSOs including LAB, *Pseudomonas spp*. and fungal communities. Metabolic pathways particularly glycolysis and pentose phosphoketolase pathway are responsible for the production of spoilage associated alcohol volatiles. For example, during fermentation of hexoses by heterofermentative LAB via phosphoketolase pathway leads to the production of ethanol among other spoilage volatiles. Alcohols can also be formed through other metabolic activities (e.g. lipid oxidation, amino acid metabolism) and enzymatic actions (e.g. proteolytic activity).	(54, 162, 164, 166-168)
1-Hexanol	Pungent, fruity and alcoholic, sweet	Green, fruity, oily		
1-Propanol	Alcoholic, musty, yeasty, fermented	Alcoholic, earthy, fermented, peanut, nutty		
1-Butanol	Fusel, oily, sweet	Fruity (banana), fusel		
2-Butanol	Fruity			
2-Methyl-1-butanol	Roasted, onion, fruity, fusel, alcoholic			
3-Methyl-1-butanol	Pungent, fermented, fruity, fusel	Fusel, fermented, fruity		
2-furanmethanol	Alcoholic, bready, coffee, sweet, musty, caramel	Burnt, sweet		
Aldehydes including: Furfural	Sweet, brown, woody, bready, caramellic and slight phenolic nuance	Brown, sweet, woody, bready, nutty and caramellic	Aldehydes are associated with fatty flavour and can form through break down and metabolism of fatty acids and amino acid degradation during both processing and storage. For example: triglyceride hydrolysis, lipid auto oxidation, β-oxidation can give rise to aldehyde formation. Alternatively, branched-chain amino acids including leucine and valine upon degradation produce aldehydes. SSOs reported to be linked to generating high concentrations of aldehydes in food products, include *Pseudomonas spp*., LAB and *Enterobacteriaceae spp*.	(128, 164, 169)
Hexanal	Green, fatty, leafy, vegetative, fruity and clean with slight woody notes	Green, woody, vegetative, grassy and citrus		
Heptanal	Fresh, aldehydic, fatty, green, herbal, cognac	Green, aldehydic, oily, grassy, clover		
Octanal	Aldehydic, waxy, orange citrus with slight green peel notes	Green with orange peel citrus note		
Nonanal	Waxy, aldehydic, citrus, slightly like green lemon peel nuance and cucumber	Effervescent, aldehydic citrus, rinds of cucumber and lemon		

Sulphur compounds including: Methanethiol	Vegetable oil, alliaceous, eggy, creamy with savoury notes	Sulphurous, alliaceous, creamy, cheesy, savoury (meaty)	Sulphur volatiles are predominantly associated with off-odours and flavours produced as a result of SSOs catabolic activity. SSOs generate sulphur volatiles by breaking down sulphur containing amino acids through enzymatic reactions by cleavage of the bond between the carbon and sulphur. Methionine is the main amino acid substrate responsible for sulphur volatiles, metabolised by SSOs, particularly *Pseudomonas spp* producing methanethiol, dimethyl disulfate among other volatiles.	(162, 169, 170)
Methyl thioacetate	Sulphurous, eggy, cheesy, dairy, vegetable (cabbage)	Sulphurous, overripe fruit, rotten, roasted, vegetable, gassy and cheesy		
Dimethyl sulphide	Sulphurous, creamy, tomato, fishy, fruity (berry) and vegetable nuances.	Sulfurous, vegetative (tomato), corn and asparagus with afternotes of mint and creaminess		
Dimethyl disulphide	Sulphurous, vegetable, cabbage, onion	Sulphurous cabbage, malt, cream		
Dimethyl trisulphide	Sulphurous, alliaceous, cooked onion, savoury, meaty, eggy	Sulphurous, alliaceous, grassy, savoury, meaty with vegetative notes		
Short chain fatty acids (SCFAs) including: Acetic acid	Sharp, pungent, sour, vinegar	Pungent, sour, overripe fruit	Short chain fatty acids (SCFAs) are derived from hydrolysis of both phospholipids and triglycerides. Oxidation of other volatile compounds, chiefly aldehydes, esters and ketones can give rise to fatty acids as well as amino acid break down. Additionally, SSOs metabolic activity can be responsible for production of FFA's. For example, fermentation of sugars by heterofermentative LAB leads to the production of acetic acid or utilisation of amino acids by SSOs can lead to production of butanoic acid via a reaction known as Stickland fermentation.	(54, 162, 164, 169)
Propanoic acid	Pungent, acidic, cheesy, vinegar	Acidic, dairy, fruity		
2-Methylpropanoic acid	Acidic, sour, cheesy, dairy, buttery, rancid	Acidic, sour, cheesy, creamy		
Butanoic acid	Sharp, acetic, cheesy, buttery, fruity	Acidic sour, cheesy, creamy, fruity		
2-Methylbutanoic acid	Pungent, acidic, cheesy, fruity, fermented	Fruity, acidic, buttery and cheesy notes		
2-Methylpentanoic acid	Sour, cheesy	Buttery, creamy, cheesy		

Ketones including: Diacetyl 3-Octanone 2-Nonanone 2-Decanone	 Buttery, sweet, creamy, pungent, caramellic (pungent) Musty, ketonic, mushroom, mouldy, cheesy, fermented and green vegetables notes Fruity, sweet, waxy, cheesy, green, herbal Orange, floral, fatty, peach	 Sweet, buttery, creamy, milky Mushroom, earthy, ketonic, cheesy, mouldy and fruity notes Cheesy, fruity, green, buttery Fermented, cheesy	Ketones are products of lipid oxidation and can be derived from the activity of SSOs including degradation of alkanes and/or dehydrogenation of secondary metabolites (alcohols). SSOs associated with ketone production include *Pseudomonas spp.*, LAB (*Lactobacillus spp*.), *Enterobacteriaceae spp.*, and *Brochothrix thermosphacta*.	(164, 169, 171)
Esters including: Ethyl acetate Ethyl butanoate Ethyl 2-hexenoate Ethyl 3-methylbutanoate	 Ethereal, fruity, sweet, green, grape and rummy Fruity, sweet, tutti frutti, pineapple, cognac Fruity, rummy, green, sweet, juicy Fruity, sweet, sharp, pineapple, green, tutti frutti	 Ethereal, fruity, sweet with grape and cherry notes Fruity, sweet, tutti fruti, apple, fresh and lifting Fruity Sweet, fruity, spicy, metallic, green with pineapple and apple notes	Esters are formed from the esterification of carboxylic acids with alcohols from the production of microbial esterase. The production of lipases from SSOs also catalyse the break-down of triglyceride forming SCFAs which could further go under esterification to form such volatiles. Yeasts during fermentation also produce esters intracellular via ester synthase, which are then released into the extracellular environment.	(169, 172-174)

[a] Odour descriptions from Casaburi *et al*. (2015) (169)
[b] Odour and flavour descriptions from The Good Scents Company Information System (175)

1.3.2 Visual Markers

Colour is an important and simple indicator of food quality and it forms part of visual inspections to determine the acceptability of foods (176, 177). Naturally occurring pigments in foods contribute to colour, such as anthocyanins in vegetables provide a red/blue colour and therefore colour of foods is influenced by both the type of pigment and reflection (177). However, these pigments can change during storage and shelf-life of foods due to presence of SSOs, oxidation of naturally occurring pigments, enzymatic and non-enzymatic reactions (176, 178). SSOs can produce pigments during metabolism, for example: *Pseudomonas spp.* have been reported to produce yellow pigments, *Bacillus spp*. produce brown pigments, alternatively *Aspergillus spp.* produce orange/red pigments (176). Enzymatic activity can also induce adverse colour changes, such as polyphenol oxidase, which plays a role in browning of fruit through catalysing oxidation of polyphenols (26, 177). Alternatively, chlorophylls responsible for the green colours in vegetables, degrade during processing and storage into compounds including pheophorbide and pheophytin that give rise to grey-brown pigments (179). The degradation of chlorophylls can occur through tissue damage induced during processing, which leads to release of intracellular acids and enzymes chlorophyllase (179). Furthermore, naturally occurring pigments can be oxidised, for example, in fruit and vegetables, carotenoids responsible for yellow, orange and red pigments are prone to oxidation (177). Oxidation of carotenoids occurs at a rapid rate when exposed to light, heat and hydroperoxides, with the latter formed during oxidation of polyunsaturated fatty acids via lipoxygenase activity (180). In meat storage oxidation of myoglobin results in discolouration and initiates oxidation of lipids (177, 181). Fundamentally, several spoilage reactions can change overall colour acceptance of foods leading to their potential rejection and therefore colour serves as a useful indicator of spoilage.

In addition to colour, texture is an important visual marker of food quality. Food products can undergo major textural changes during storage and shelf-life due to food spoilage reactions and SSOs activities. Metabolic by-products produced by microbial colonisation, particularly enzymes can break down constituents of foods causing major structural changes (21, 47). For example: in meat the release of proteases from SSOs breaks down protein and thus protein structure, resulting in a mushy texture. Also the degradation of pectin in fruit and vegetables by pectinases, results in a soft structure otherwise referred to as soft rot (21, 47, 178). In contrast, other integrity changes of foods related to structural deterioration can be associated with water content, as water can be lost, gained, or migrate during storage. All of which can impact on texture, nutritional status and occurrence of spoilage reactions as a result of changes in water activity as highlighted in section 1.2.1 (21, 26).

1.4 Multi-Omics as a Tool for Accessing Food Spoilage

At present the techniques that are used in the food industry to determine the deterioration of foods are outdated, insensitive and alone fail to provide a full picture of food spoilage. However, technological advances over recent years have and currently are providing a deeper understanding into complex biological processes and microbial composition of foods and this is specifically achieved through implementation of -Omics techniques. Omics is an umbrella term used to describe a broad range of techniques including, genomics, transcriptomics, proteomics, lipidomics and metabolomics identifying taxa/genes, mRNAs, proteins, lipids and metabolites respectively (182). Individually these techniques target specific molecules of interest and have scope to monitor the occurrence of these in foods. However, integration of numerous Omics techniques as part of a system biology provides an understanding from how cells interact with the environment, from DNA to metabolite production as shown in Figure 1.6. This allows complex food systems to be analysed, offering a comprehensive picture into a food system and processes involved in food spoilage. This is due to Omic techniques providing answers to the following questions, 'what microbial species are present?', 'what are they doing?' and 'what compounds are being produced?'(182). Overall, answering those valuable questions allows the identification of potential spoilage markers which can further be corelated with nutritional and sensorial properties of foods (182, 183).

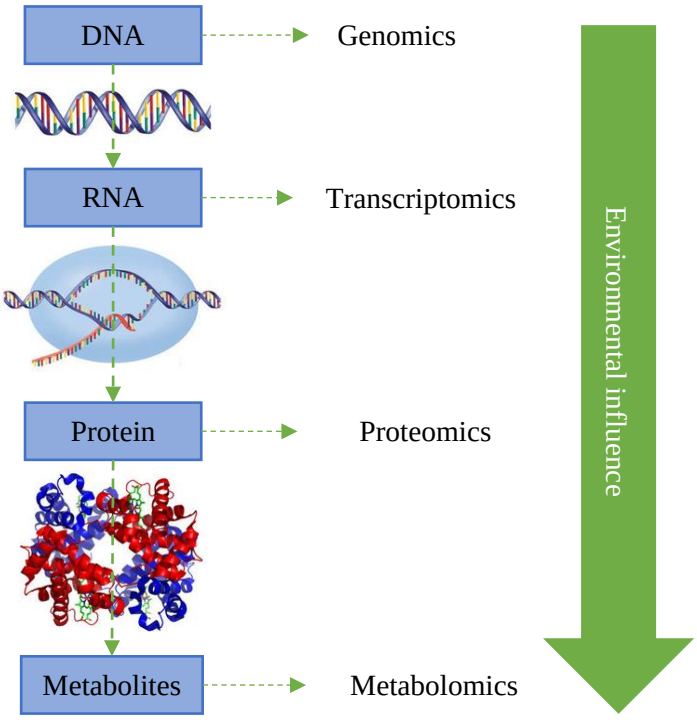


Figure 1.6: Omics techniques highlighting the advantages of integration of several techniques (image adapted from Yu, Li and Zhang (2017) (184).

1.4.1 Applications of Metabolomics in Food Systems

Metabolomics is an emerging field used to analyse low molecular weight entities (<1500 Da) including vitamins, fatty acids and amino acids (182, 185). Owing to its function, metabolomics enables the ability to identify primary and secondary metabolites being produced by a cell and as a result has been successfully applied across many biological disciplines (182, 186). In respect to food science, metabolomics has been described as a frontier in both food safety and food quality which has led to the development of 'food metabolomics' (27, 187-193). In terms of food quality and safety, metabolomics has been utilised for the identification and detection of foodborne pathogens in foods (194), identification of metabolites associated with spoilage (160) and used for the assessment of quality attributes of foods (195).

Application of metabolomics can be achieved using a targeted and/or untargeted approach. A targeted approach is where a particular group of metabolites are analysed either qualitatively or quantitively. This type of approach is generally applied to investigating a particular class of compounds and/or investigating compounds associated with a specific metabolic pathway (27, 196, 197). Alternatively, fingerprinting is a form of untargeted analysis, which aims at screening all metabolites within a sample. The primary goal when adopting an untargeted approach is to identify patterns across samples with the aim of identifying biomarkers, which is facilitated through chemometric tools, for example data reduction and visualisation technique, principal component analysis (PCA). The main principle of untargeted analysis is neither to quantify nor identify all metabolites within a sample (186, 197, 198).

Targeted and untargeted analysis of the food metabolome can be achieved using high throughput and sensitive separation instruments, including gas chromatography (GC), liquid chromatography (LC) and capillary electrophoresis (CE) which can be coupled with different types of detection (196). This includes UV, fluorescence, and mass-spectrometry (MS), with latter being the most popular form of detection for analysis of metabolites (185, 196). Nuclear magnetic resonance (NMR) spectroscopy and Fourier transform infrared spectroscopy are other detector techniques that have been used in metabolomic studies (160, 186). Although it is important to note that there are some disadvantages of using a single analytical instrument/single form of detection when assessing the food metabolome. For example, the use of GC-MS is advantageous due to analysing VOC's as part of metabolite profiling, which is important when assessing spoilage as many of the spoilage metabolites are volatile in nature (165). However, GC-MS would potentially underrepresent metabolites that are non-volatile, thermally unstable and often requires a form of chemical derivatisation to analysis metabolites (196). Therefore, combination of instruments and detectors can be employed to provide a more in-depth picture of the metabolites present within a sample, which has additionally been highlighted in a recent review by More *et al*. (2020) (199).

Metabolomics has been applied across several food quality studies as an aid of identifying potential biomarkers associated with presence of SSOs and assess nutritional and sensorial properties linked to quality of foods. A recent study by Gauglitz *et al*. (2020) (191) detailed the impact of processing and storage on the molecular composition of foods; investigations included fermentation of milk to yoghurt, brewing of tea, roasting of coffee, spoilage of meats and tomato ripening. They performed an untargeted analysis using LC-MS/MS and with the aid of visualisation tools including principal coordinates analysis (PCoA) and heatmaps the molecular changes during processing and storage were identified. Zhang *et al*. (2020) (189) assessed metabolites during spoilage of chilled chicken using a untargeted UPLC-MS/MS method, from which 37 metabolites were identified as potential biomarkers of spoilage. The authors highlighted metabolites such as, gluconic acid, indole-3-carboxaldehyde, uridine monophosphate and s-phenylmercapturic acid as key biomarkers of product freshness. Similarly, Jaaskelainen *et al*. (2019) (160) investigated metabolite changes during storage of salmon and yellowfin tuna. The study showed compounds associated with sensory rejection of these products, this included production of TVB-N and trimethylamine, which authors described as spoilage markers of salmon. The authors also observed an increase in hypoxanthine during spoilage, which is formed through ATP degradation. However, Jaaskelainen *et al*. (2019) (160) also combined metabolomics with genomics to understand the microbial communities potentially linked to production of such spoilage metabolites. Due to metabolites being reflective of interactions between microbial cells and environment, in order to generate a comprehensive evaluation of the overall safety and quality of food, integration of other Omics (multi-omics) techniques are extremely valuable (27).

1.4.2 Applications of Genomics in Food Systems

Conventional methods for detection and quantification of SSOs as previously mentioned are bias and underrepresent the microbial communities present. Culture-dependent methods are acknowledged to represent only 0.1-3% of a microbial community (200, 201). However, technological advances in sequencing methods, including development of high-throughput sequencing (HTS) which forms part of ‘next generation sequencing’ has and currently is revolutionising genomic studies (63, 202, 203). HTS is a powerful tool that enables the microbiome of an environment to be captured, which in contrast to culture-dependent methods enables a deeper understanding of the microbial composition of foods and also has the ability to capture the capabilities of such a community (63, 204). HTS assessment of the microbiome in can be achieved using two methods; metagenomic sequencing or amplicon sequencing (205). Metagenomic sequencing is also known as shotgun sequencing and is a form of untargeted analysis, which aims at analysing all DNA or RNA from a specific environmental sample or matrix. This type of approach is extremely broad and involves the study of gene fragments and gene function among other genome entities (182, 183). Alternatively, amplicon sequencing is a

targeted approach, where a particular taxonomic region or gene is amplified. For example, 16S would be targeted to understand the bacterial composition of an environment, or to investigate the fungal community or mycobiome the internal transcribed spacer (ITS) would be analysed (182, 183, 200).

Many studies have utilised metagenomics and amplicon sequencing to understand the role of microbial communities during complex processes and their role in food quality, this includes investigations into the process of fermentation of foods such as cheeses, milk and vegetables (206-210). Comparably, Park *et al*. (2012) (211) investigated the bacterial composition and diversity targeting 16S rRNA during the fermentation of kimchi. The time-series experiment captured bacterial dynamics during fermentation, from which authors described the bacterial composition as being very diverse in the early phases of fermentation. However, during fermentation diversity reduced as acid tolerant communities became the predominant communities present and included *Welsella*, *Leuconostoc* and *Lactobacillus*. The impact of food production on the microbial composition of foods has also been assessed using HTS (11). De Filipps *et al*. (2013) (11) characterised the microbiota during production and storage of beef steaks targeting 16S rRNA. The study showed how production influences the microbial composition of the beef e.g., environment in which the beef steaks are processed, the cuts of beef used and the carcass the meat originated from. The authors showed that the carcass used had a complex and diverse microbial composition as >600 operational taxonomic units (OTUs) were identified. However, during storage of the beef steaks, the complexity of the microbial composition reduced as *Pseudomonas spp*. and *Brochothrix thermosphacta* became the predominant bacteria responsible for spoilage of the beef steaks, outcompeting a range of other species. Niemien *et al*. (2012) (212) published a metagenomic study with the aim of understanding the microbial composition targeting 16S rRNA and metabolic function in unmarinated versus marinated broiler fillet strips. The study showed how bacterial communities shifted depending on food preparation (marinated versus unmarinated). The unmarinated promoted abundance of LAB, *Clostridiaceae*, *Listeriaceae* and *Vibrionaceae*, whereas the marinated filet reduced abundance of other communities and allowed LAB to thrive. This is due to the marinate containing acetic acid and natural antimicrobial agents that LAB were able to tolerate compared to other bacterial communities. Although HTS has been used to characterise food spoilage communities, studies have focused on investigating animal-based matrices as presented above, while studies into bacterial dynamics in plant-based matrices are underrepresented. HTS as presented in the above studies offers even greater insight into food spoilage, however in order to understand not only the presence of microbial species, but the question of 'what they are doing there?', coupling with other Omics such as metabolomics provides greater knowledge on the activity of the microorganisms in the food system.

1.4.3 Coupling of Genomics and Metabolomics Forming Part of a Multi-omics Approach

Combining Omics techniques, specifically genomics and metabolomics to assess food quality is of most value. Currently, the application of both techniques assessing microbial processes linked to nutritional and sensory quality of foods is limited to a few publications (25, 160, 213), with many recent reviews equally addressing the same issue (182, 183). If only one Omics technique is applied, it only provides a partial view of a food system. Therefore, it is advantageous to utilise a range of complementary Omics techniques for greater scrutiny and coverage of the investigated matrix. An experimental workflow of a multi-omics approach to assess food spoilage are outlined in Figure 1.7.

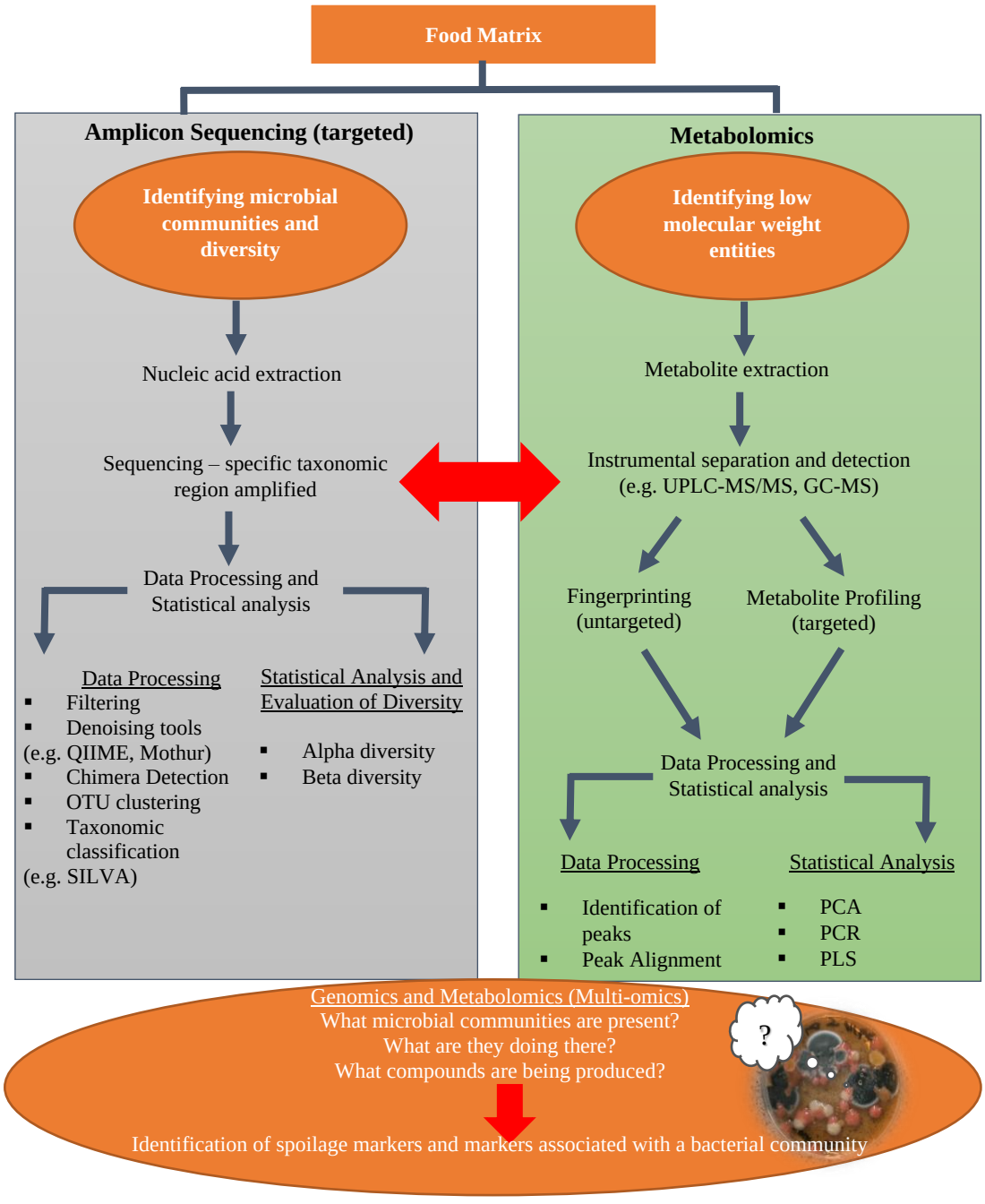


Figure 1.7: Genomic and metabolomic workflow as part of a multi-omics approach to food spoilage studies (182, 198, 214).

1.5 Nutritional Composition of Foods and Loss in Food Systems

The nutritional composition of foods primarily based on macronutrient content, including carbohydrates, proteins and fat, and micronutrient content, vitamins and minerals can be adversely affected during storage (215). This could be the result of a storage environment, or the process of spoilage. The latter occurs due to nutrients being a significant parameter for growth and survival of SSOs, therefore nutrients are broken down, facilitating undesirable reactions including production of VOCs, leading to the loss of acceptable physicochemical properties (72). Most of which is covered in the above sections. However, nutrients fail to be assessed during shelf-life studies alongside other quality markers including microbial growth and although macronutrients are known to play a role in spoilage, the role of vitamins, particularly water-soluble vitamins during spoilage are less understood. Therefore, to our knowledge research understanding the relationship between vitamins and spoilage characteristics including growth of microorganisms is limited and requires investigation.

In foods, macronutrients make up a significant portion of the nutritional composition, essential in the diet for providing energy and for their function in growth and metabolism. Carbohydrates in foods are predominantly present in plant sources and are either present as simple sugars, monosaccharides (glucose, fructose, galactose) and disaccharides (maltose, sucrose, lactose). They can also be present as more complex carbohydrates derived from monosaccharides, including oligosaccharides and polysaccharides (starches and non-starch polysaccharides). Proteins are made up of polymer chains of amino acids and in foods can be obtained from both animal and plant sources. Alternatively, fats (lipids) are mixtures of triglycerides that are present in animal and plant products, although other lipids, monoglycerides, diglycerides, phospholipids and cholesterol are among other fats existing in certain foods (215, 216). The dietary reference values (DRVs) and reference nutrient intake (RNI) values for macronutrients are shown in Table 1.3.

Table 1.3: Dietary reference values (DRVs) of carbohydrates and fats based on % of energy intake, and reference nutrient values (RNIs) of protein and non-starch polysaccharides according to the British Nutrition Foundation (217).

Macro-nutrients	**Protein (g)**	**Carbohydrate DRVs**		**Non-starch polysaccharide (g)**		**Fat DRVs**	
Age/gender	Males & Females	**Age/gender**	Males & Females	**Age/gender**	Males & Females	**Age/gender**	Males & Females
0-3 months	12.5	Applicable to 2 years and above	50% of energy intake*	2-5 years	15	Applicable to 5 years and above	Less than 35% of energy intake
4-6 months	12.7			5-11 years	20		
7-9 months	13.7			11-16 years	25		
10-12 months	14.9			17+ years	30		
1-3 years	14.5						
4-6 years	19.7						
7-10 years	28.3						
11-14 years 15-18 years 19-50 years 50+ years	0.75 g x bodyweight (kg)						

*Total carbohydrate comprises of starch, sugars and non-starch polysaccharides.

Micronutrients in the form of vitamins and minerals are essential constituents of food present in much smaller quantities, in comparison to macronutrients and exert many biochemical functions. The biochemical function of micronutrients include their role as cofactors and coenzymes in energy metabolism, serve as antioxidants (e.g. vitamin C) and aid in genetic control (e.g. Zinc) (218). Vitamins are organic compounds, differing in their structure, purpose and action. Classified based on their solubility, either soluble in fat, this would include vitamins A, D, E, and K. Or soluble in water, including: vitamin C and B group vitamins namely; thiamine, riboflavin, niacin, B_6, pantothenic acid, biotin, folate and cyanocobalamin (219, 220). Water-soluble vitamins, particularly, thiamine, riboflavin, niacin and B_6 (pyridoxine, pyridoxal, and pyridoxamine) are vital components in the diet (discussed in following sections: 1.5.1-1.5.4). Such vitamins not only play a role in macronutrient metabolism and redox reactions, they additionally play a part in reducing the risk of degenerative diseases (221-223). A low biochemical status of vitamins in the diet, can give rise to both sub-clinical and extreme deficiencies if intake is insufficient. RNI for selected water-soluble vitamins, dependant on age and gender, including food sources are shown in Table 1.4. However, stability of such vitamins remains a limiting issue throughout the food chain (discussed in section 1.5.5), several parameters that hinder on vitamin stability include: light, temperature, oxygen and pH (224-228). The notion leads to current challenges with both the quantification of vital vitamins and the nutritional status of food items throughout the food chain.

Table 1.4: Reference nutrient intake (RNI) of thiamine, riboflavin, niacin and B_6 according to the British Nutrition Foundation (217) including food sources.

Essential vitamins	**Thiamine (mg/day)**		**Riboflavin (mg/day)**		**Niacin (mg/day)**		**B_6 (mg/day)**	
Age/gender	Males	Females	Males	Females	Males	Females	Males	Females
0-3 months	0.2		0.4		3		0.2	
4-6 months	0.2		0.4		3		0.2	
7-9 months	0.2		0.4		4		0.3	
10-12 months	0.3		0.4		5		0.4	
1-3 years	0.5		0.6		8		0.7	
4-6 years	0.7		0.8		11		0.9	
7-10 years	0.7		1.0		12		1.0	
11-14 years	0.9	0.7	1.2	1.1	15	12	1.2	1.0
15-18 years	1.1	0.8	1.3	1.1	18	14	1.5	1.2
19-50 years	1.0	0.8	1.3	1.1	17	13	1.4	1.2
50+ years	0.9	0.8	1.3	1.1	16	12	1.4	1.2
Food Sources*	· Whole grain cereals · Meat · Vegetables · Yeast · Nuts		· Milk · Eggs · Vegetables · Yeast extract · Meat		· Eggs · Milk · Pulses · Meat · Vegetables		· Meat · Nuts · Pulses · Fruit · Vegetables	

*Food sources (223, 229, 230)

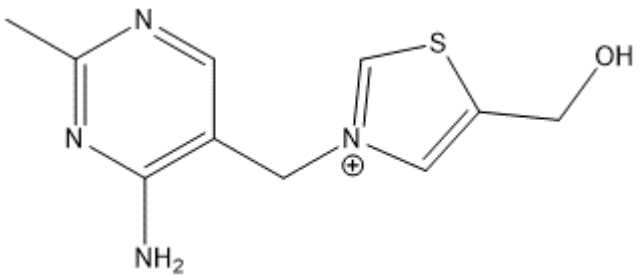
1.5.1 Thiamine – Forms, Functions and Food Sources

Thiamine was the first B group vitamin to be discovered in 1920's by Jansen and Donath and is otherwise referred to as vitamin B_1 (231). The structure of thiamine consists of a thiazole ring and a pyrimidine ring, connected by a methylene group (232). Thiamine occurs in four forms; free thiamine which is chiefly found in plant-based products and phosphorylated forms thiamine monophosphate (TMP), thiamine pyrophosphate (TPP), and thiamine triphosphate (TTP), which are predominantly found in animal products (229, 233). Free thiamine is the form absorbed in the body by passive or active transport, which is further converted to its active form, thiamine pyrophosphate (TPP) (229). The bioactive form, TTP acts as coenzyme in two vital metabolic pathways in carbohydrate metabolism, namely Kreb's cycle and Pentose Phosphate pathway (234). A deficiency in thiamine can lead to Beri Beri in either of two forms, wet (cardiac) or dry (neurological) (223). Other deficiencies include cardiovascular disorder and Wernicke–Korsakoff syndrome (235, 236), which is commonly associated with alcoholics. Therefore, thiamine should be found in the diet from several sources as shown in Table 1.5 and the structure of all four forms of thiamine can be seen in Figure 1.8.

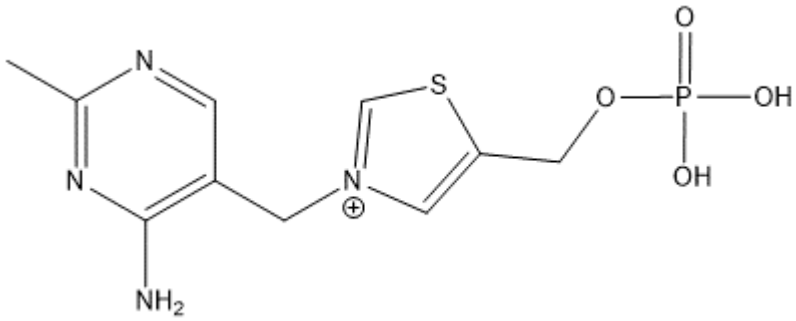
Table 1.5: Thiamine content of food sources from Combs (2012) (233)

Foods	Thiamine content (mg/100g)	Foods	Thiamine content (mg/100g)
Grains		**Fruits**	
Cornmeal	0.20	Apples	0.04
Oatmeal	0.55	Apricots	0.03
Rice		Bananas	0.05
Brown	0.29	Grapes	0.05
White	0.07	Oranges	0.10
Rye		Pears	0.02
Whole grain	0.30	Pineapples	0.08
Degeminated	0.19	**Meats**	
Wheat		Beef	0.08
Whole grain	0.55	Duck	0.10
White	0.06	Pork	1.10
Vegetables		Cured ham	0.74
Asparagus	0.18	Veal	0.18
Green beans	0.07	**Fish**	
Broccoli	0.10	Trout	0.09
Cabbage	0.05	Salmon	0.17
Carrots	0.06	**Dairy products and eggs**	
Cauliflower	0.11	Cheese	0.02-0.06
Kale	0.16	Milk	0.04
Green peas	0.32	Eggs	0.12
Potatoes	0.11	**Other food/s**	
Tomatoes	0.06	Brewer's yeast	15.6

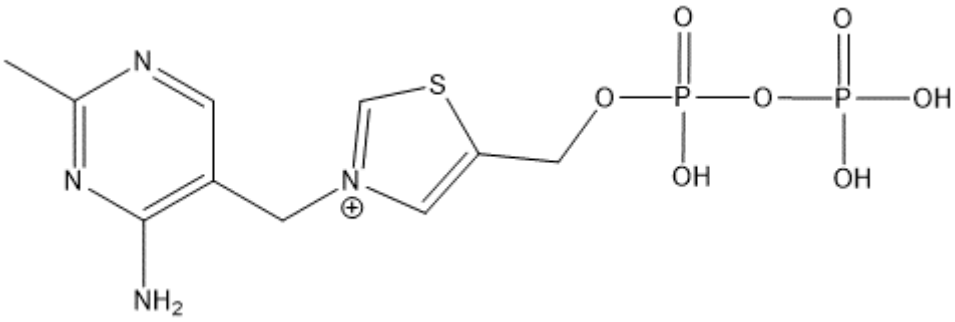
Thiamine



Thiamine Monophosphate (TMP)



Thiamine Pyrophosphate (TPP)



Thiamine Triphosphate (TTP)

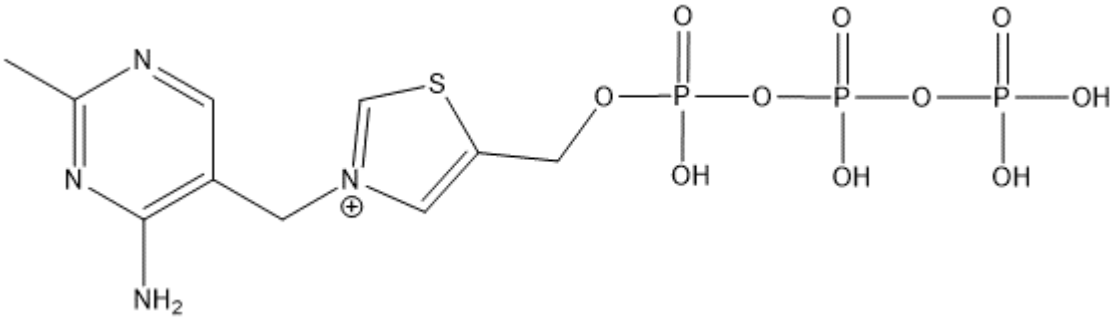


Figure 1.8**:** Molecular structure of thiamine and phosphorylated forms, image recreated from Manzetti *et al*. (2014) (232).

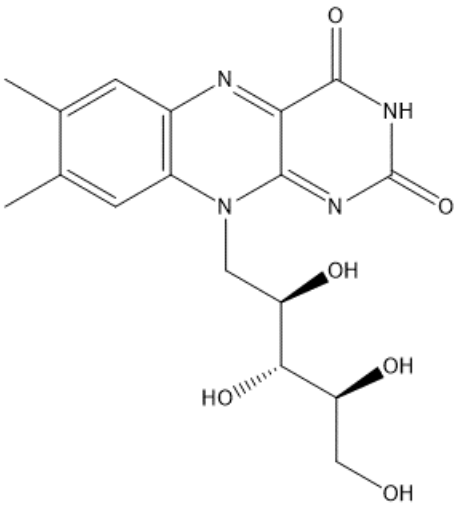
1.5.2 Riboflavin – Forms, Functions and Food Sources

Riboflavin, referred to as vitamin B_2 was the second vitamin discovered and was first observed by Alexander Wynter Blyth in early 1870's as a florescent yellow pigment in milk. However, after initial observation, it took 60 years (1930's) before the nutritional function and structure of riboflavin was established (237, 238). Riboflavin structure consists of an isoalloxazine ring attached to a ribityl sidechain, and occurs in three forms; free riboflavin, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (238). Dietary riboflavin is absorbed in the free form in the body, after absorption free riboflavin is converted to its coenzyme forms FMN and FAD in tissues (233, 239). The coenzyme forms participate in vital metabolic process, involved in macronutrient metabolism and conversion of micronutrients (e.g. folate and pyridoxine) into their active forms (239, 240). Active forms of riboflavin (FMN and FAD) additionally serve as an antioxidant by participating in redox reactions through electron transfer, reviewed by Ashoori *et al*. (2014) (241) showing riboflavin's role in oxidative stress. Riboflavin is sufficiently present in food sources as shown in Table 1.6 and structures of all existing forms of riboflavin are displayed in Figure 1.9. Although, riboflavin deficiencies are prevalent across the globe leading to anaemia, endocrine dysfunction, skin abnormalities and progressive changes to the nervous system (239).

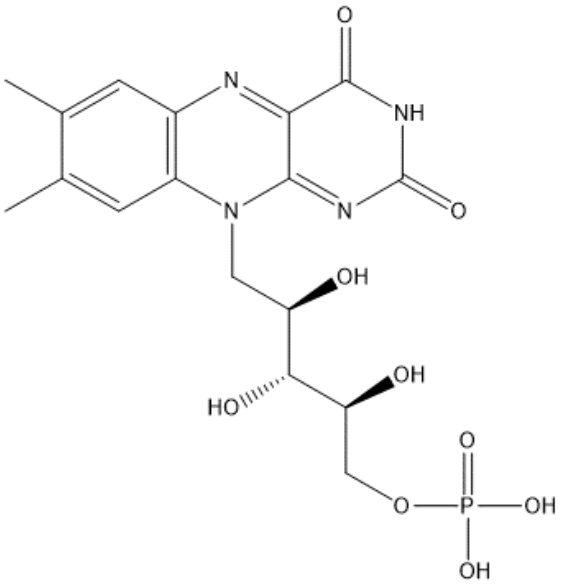
Table 1.6**:** Riboflavin content of food sources from Combs (2012) (233)

Foods	Riboflavin content (mg/100g)	Foods	Riboflavin content (mg/100g)
Grains		**Meats**	
Whole wheat	0.11	Beef liver	3.50
Rye	0.08	Beef	0.24
Oatmeal	0.02	Chicken	0.19
Rice	0.01	Lamb	0.22
Vegetables		Pork	0.27
Asparagus	0.18	Cured ham	0.19
Broccoli	0.20	**Dairy products**	
Cabbage	0.06	Milk	0.17
Carrots	0.06	Yogurt	0.16
Cauliflower	0.08	Ice cream	0.21
Corn	0.06	**Cheese**	
Lima beans	0.10	Cheddar	0.46
Potatoes	0.04	Cottage	0.28
Spinach	0.14	**Other food/s**	
Tomatoes	0.04	Egg	0.30
Fruits			
Apples	0.01		
Bananas	0.04		
Oranges	0.03		
Peaches	0.04		
Strawberries	0.07		

Riboflavin



Flavin mononucleotide (FMN)



Flavin adenine dinucleotide (FAD)

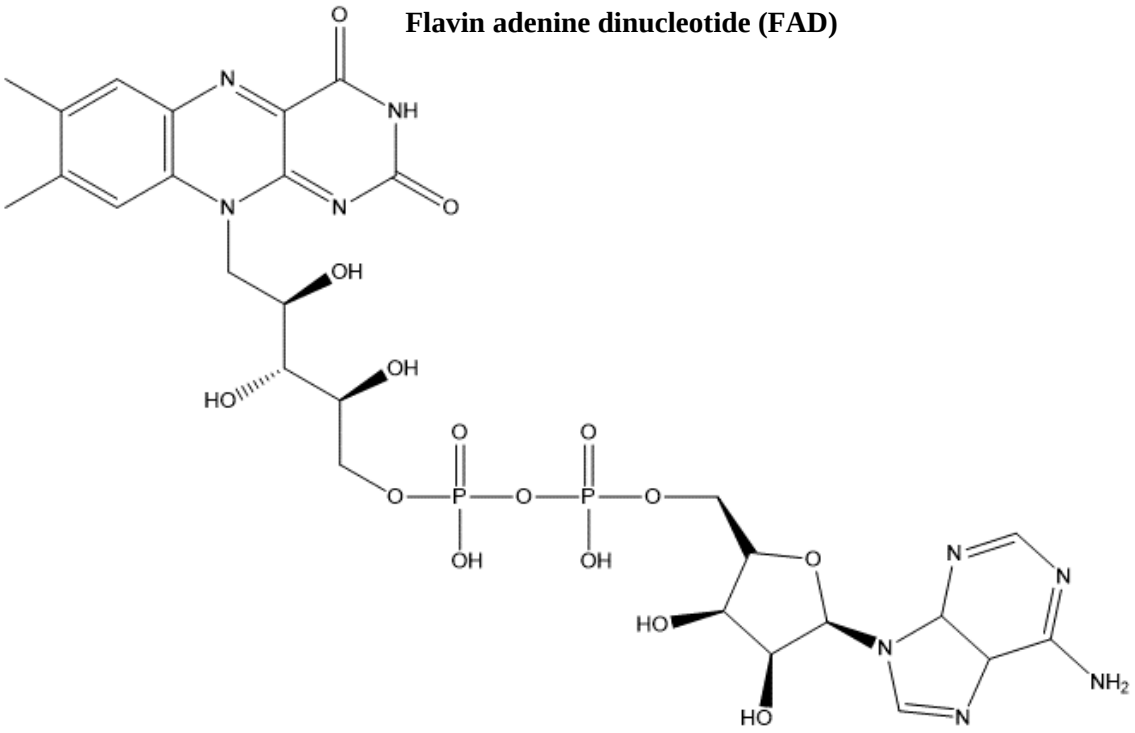


Figure 1.9: Molecular structure of riboflavin and coenzyme forms. Recreated from Schwechheimer *et al.* (2016) (238) and Litwack (2018) (242).

1.5.3 Niacin – Forms, Functions and Food Sources

Niacin known as vitamin B_3 was first observed in yeast in early 1910's by Casimer Funk (243). Although, its vitamin properties weren't discovered until 1930's by Conrad Elvehjem, due to being overlooked initially when research was ongoing for a cure for a B_1 deficiency, Beri Beri (243). Niacin exists in two forms in plant and animal sources; nicotinic acid and nicotinamide, occurring alongside and collectively referred to as B_3 (233, 244). Nicotinamide is also present in two coenzyme forms, nicotinamide-adenine dinucleotide (NADH/NAD^+) and phosphorylated form, nicotinamide-adenine dinucleotide phosphate (NADPH/$NADP^+$) (233, 242). Niacin in its active forms (NADH/NAD^+, NADPH/$NADP^+$) derived from NAD are essential in many metabolic processes due to their coenzyme role in redox reactions; playing roles in glycolysis, pentose phosphate pathway and DNA regulation (230). Food sources rich in niacin are depicted in Table 1.7. However, a low biochemical status of niacin can lead to deficiencies and in extreme cases cause Pellagra, which can lead to dementia, dermatitis, diarrhoea, depression, delirium, and dilated cardiomyopathy (230). Structures of niacin (Figure 1.10) and its coenzyme forms NADH/NAD^+ (Figure 1.11) and NADPH/$NADP^+$ (Figure 1.12) are displayed below.

Table 1.7: Niacin content of food sources from Combs (2012) (233)

Foods	Niacin content (mg/100g)	Foods	Niacin content (mg/100g)
Grains		**Fruits**	
Cereals		Apples	0.6
Barley	3.1	Bananas	0.7
Buckwheat	4.4	Grapefruits	0.2
Cornmeal	1.4-2.9	Oranges	0.4
Wheat		Peaches	1.0
Whole grain	3.4-6.5	Strawberries	0.6
Wheat Bran	8.6-33.4	**Dairy**	
Rice		Milk	0.2
Polished	1.6	Yogurt	0.1
Unpolished	4.7	Cheeses	1.2
Rye	0.9-1.6	**Nuts**	
Vegetables		Nuts	0.6-1.8
Asparagus	1.5	Peanuts	17.2
Beans	0.5-2.4	**Meats**	
Broccoli	0.9	Beef	4.6
Brussel sprouts	0.9	Chicken	4.7-14.7
Cabbage	0.3	Lamb	4.5
Carrots	0.6	Pork	0.8-5.6
Cauliflower	0.7	Turkey	8.0
Celery	0.3	Calf half	7.5
Corn	1.7	Calf kidney	6.4
Kale	2.1	**Fish**	
Onions	0.2	Cod	2.2
Peas	0.9-25.0	Flounder	2.5
Peppers	1.7-4.4	Haddock	3.0
Lentils	2.0	Herring	3.6
Potatoes	1.5	Tuna	13.3
Soybeans	1.4	**Other**	
Spinach	0.6	Eggs	0.1
Tomatoes	0.7	Mushrooms	4.2
		Yeast	50.1

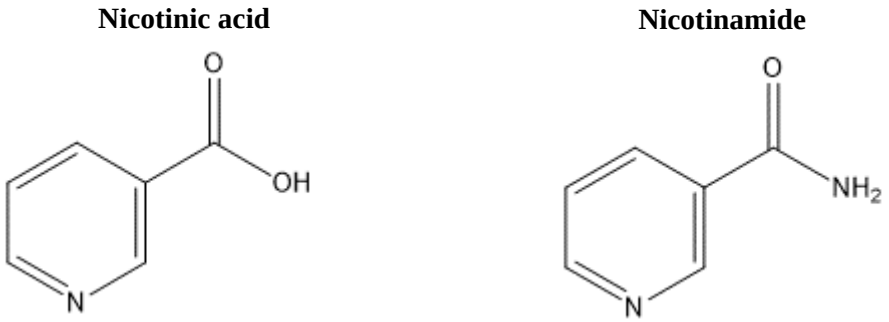
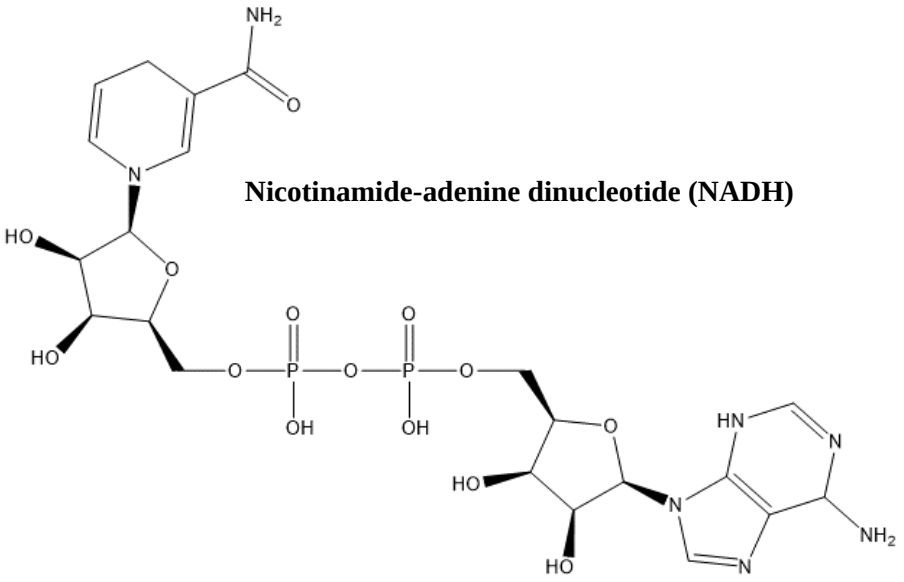


Figure 1.10: Forms of niacin: A) nicotinic acid and B) nicotinamide, together referred as B_3. Image recreated from Litwack (2018) (242).



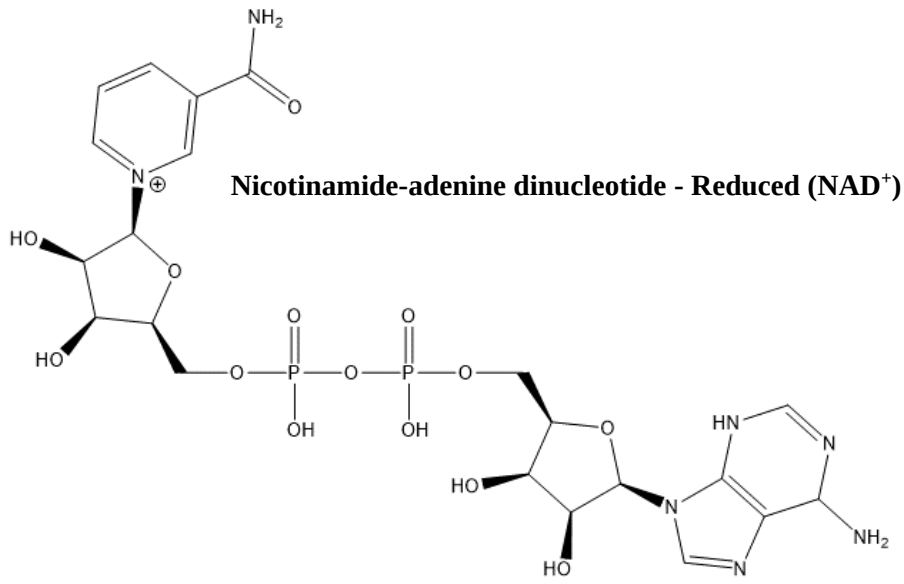
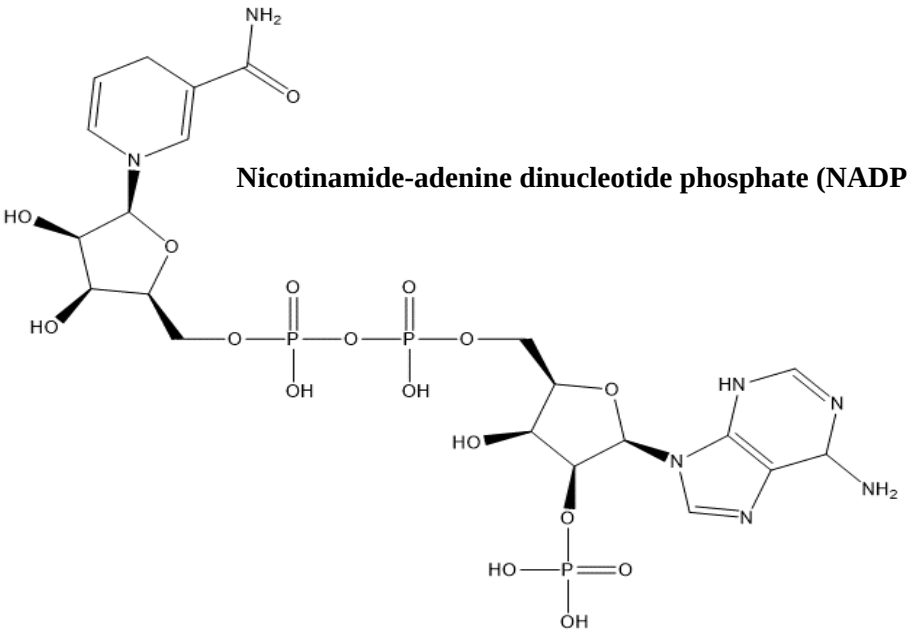


Figure 1.11: Structure of nicotinamide-adenine dinucleotide (NADH/NAD^+). Image adapted from Litwack (2018) (242).



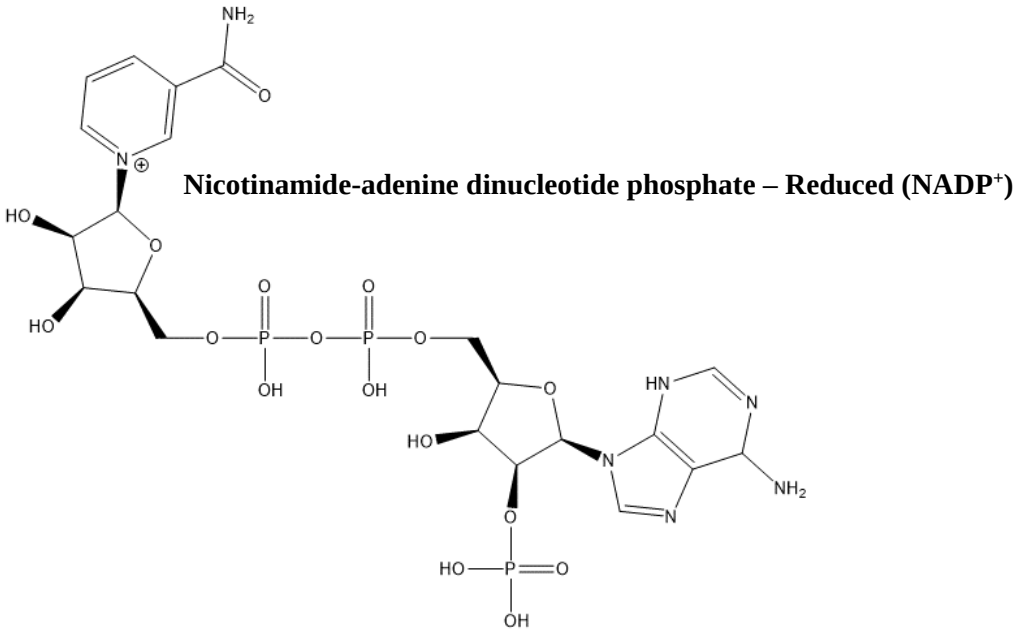


Figure 1.12: Structure of nicotinamide-adenine dinucleotide phosphate (NADPH/$NADP^+$). Image adapted from Litwack (2018) (242).

1.5.4 B_6 – Forms, Functions and Food Sources

Pyridoxine, pyridoxal, and pyridoxamine are all forms of vitamin B_6, initially identified in 1934 by György due its ability to cure rat acrodynia, or mercury poisoning, and further isolated by Lepovsky in late 1938 (245). The forms of B_6 are classified based on their structure in terms of the group positioned at C4; pyridoxine consists of alcohol group, pyridoxal contains an aldehyde group, and pyridoxamine comprises of an amine group (246). All forms of B_6 in the body are further converted to their 5'-phosphate forms; pyridoxine 5'-phosphate (PNP), pyridoxal 5'-phosphate (PLP), and pyridoxamine 5'-phosphate (PMP) (242, 246). PLP is the coenzyme form, in which the other B_6 5'-phosphate derivates are converted to, playing critical roles in metabolism of predominantly amino acids (233, 239, 242). However, a low B_6 status in the body in extreme cases, can cause neurological disorder and anaemia of different forms; normocytic, microcytic, and sideroblastic (239, 247). Therefore, B_6 should be obtained in the diet from several sources as outlined in Table 1.8.

Table 1.8: B_6 content in food sources, including % distribution of each of the B_6 forms (233).

Foods	B^6 content (mg/100g)	% Pn	% Pl	% Pm	% Glyco-sylated
Grains					
Pearled barley	0.22	52	42	6	
Corn meal	0.20	11	51	38	
Oatmeal	0.14	12	49	39	
Polished rice	0.17	64	19	17	20
Brown rice	0.55	78	12	10	23
Wheat, whole	0.34	71	16	13	28
Wheat, white flour	0.06	55	24	21	
Vegetables					
Asparagus	0.15				
Beans	0.018-0.18	62	20	18	15-57
Broccoli	0.17				66
Brussels sprouts	0.18				
Cabbage	0.16	61	31	8	46
Carrots	0.15	75	19	6	51-86
Cauliflower	0.21	16	79	5	66
Celery	0.06				
Corn	0.20	6	68	26	
Onions	0.13				
Peas	0.16	15	47	47	6
Potatoes	0.25	32	68	18	14
Spinach	0.28	36	49	15	50
Fruits					
Apples	0.03	61	31	8	
Grapefruit	0.03				
Oranges	0.06	59	26	15	47
Peaches	0.02	61	30	9	22
Strawberries	0.06				
Tomatoes	0.10	38	29	15	46
Meats					
Beef	0.33	16	53	31	
Chicken	0.33-0.68	74	19		7
Lamb	0.28				
Pork	0.35	8	8	84	
Calf liver	0.84				
Fish					
Flounder	0.17	7	71	22	
Haddock	0.18				
Herring	0.37				
Oysters	0.05				
Salmon	0.30	2	9	89	
Shrimp	0.10				
Tuna	0.43	19	69	12	
Dairy products					
Milk	0.04	3	76	21	
Yogurt	0.05				
Cheeses	0.04-0.08	4	8	88	
Nuts					
Almonds	0.10				
Peanuts	0.40	74	9	17	
Pecans	0.18	71	12	17	
Walnuts	0.73	31	65	4	7
Other					
Eggs	0.19	0	85	15	

Pn = Pyridoxine, Pl = Pyridoxal and Pm= Pyridoxamine

In foods, B_6 forms are distributed dependent on the food source, bound to proteins or phosphorylated, in plant-based foods, pyridoxine is the main form of B_6 present. Alternatively, in animal-based foods pyridoxal and pyridoxamine are the prominent B_6 forms occurring (233). Structures of all forms can be seen in Figure 1.13. Additionally, B_6 can also be found in foods in a glycosylated form and distribution in selected foods are covered in Table 1.8.

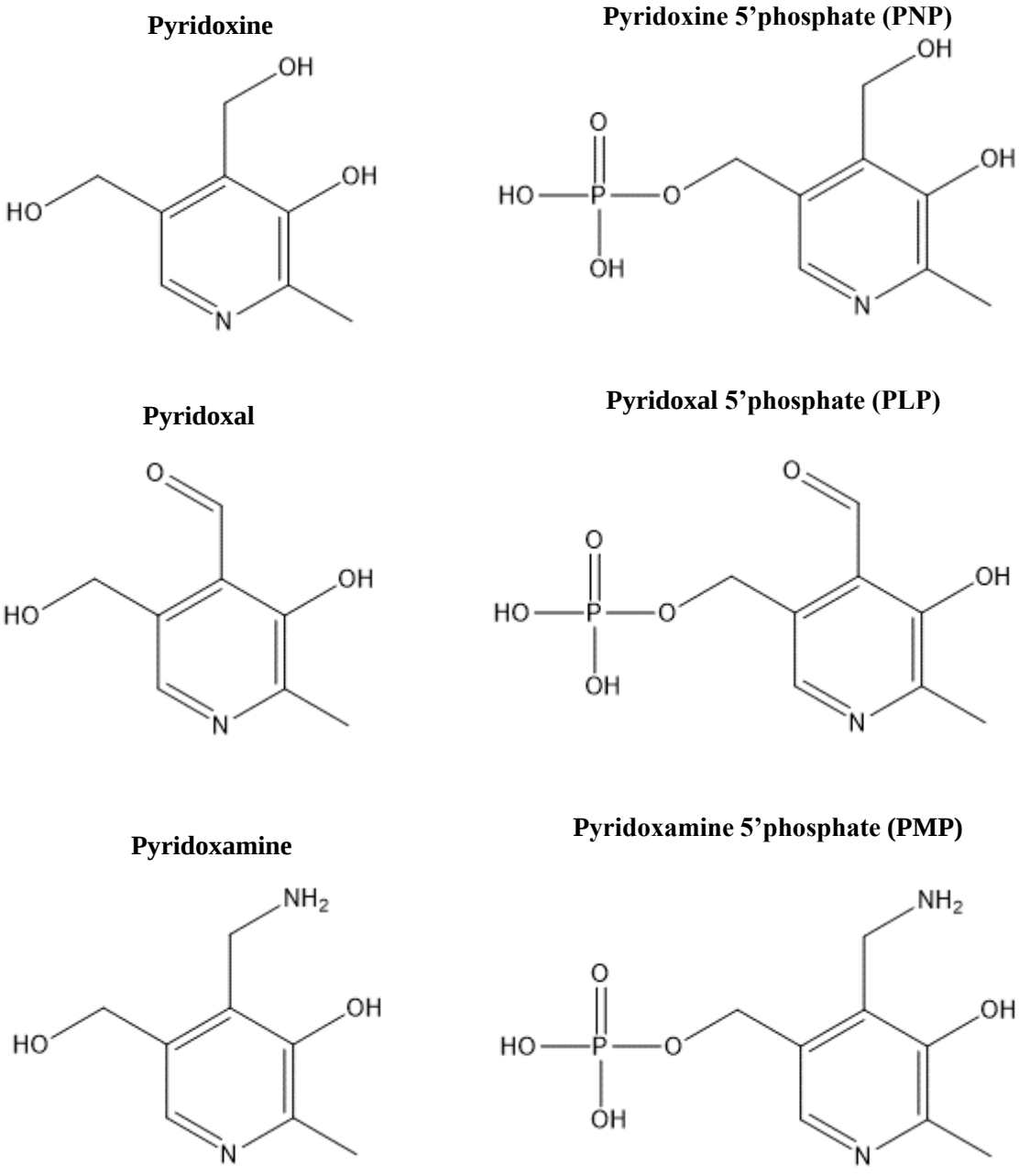


Figure 1.13: Structures of pyridoxine, pyridoxal, pyridoxamine and coenzyme forms. Image recreated from Litwack (2018) (46).

1.5.5 B-Vitamin Degradation in Food Systems

Degradation of thiamine, riboflavin, niacin and B_6 is problematic throughout the food chain, during processing and storage. Due to their instability under different intrinsic and extrinsic conditions including temperature, light, pH, oxygen and other factors in the food system.

1.5.5.1 Effect of Temperature on B-Vitamins

Temperature is one of the major parameters to effect vitamin stability, significant research has been completed to illustrate the role of temperature on vitamin destruction dating back to late 1940's (248-255). This has been supported by the development of kinetic models during both food processing and storage, illustrating the relationship between temperature and time on the degree of vitamin loss (256). Rekha *et al*. (2004) (224), reported on a kinetic model the degradation of thiamine in red gram splits, investigating different temperatures (50-120 °C) over time (0-60 mins). Results showed degradation of thiamine increased with high temperature treatment and followed first order kinetics. Comparably, Muhamad *et al*. (2015) (256) depicted thermal degradation kinetics on bioactive constitutes of *Averrhoa bilimbi* fruit investigating temperatures from 90 to 120 °C. They showed first order kinetics on the degradation of nicotinic acid, as temperature and time increased, it simultaneously increased nicotinic acid loss. Some water-soluble vitamins are reported to be more thermally stable than others, having thiamine been described as the least thermal stable vitamin in comparison to other water-soluble vitamins, niacin and riboflavin (258). For example, Kamman, Labuza and Worthesen (1981) (259) investigated stability of both thiamine and riboflavin in fortified pasta, stored between 25-55 °C for 1 year. They concluded riboflavin was mostly stable throughout the period in comparison to thiamine, which experienced great loss during storage particularly at higher storage temperatures. In the case of thiamine, heat energy consequently leads to the hydrolysis of the methylene bridge (C-N bond), separating the thiazole ring and a pyrimidine ring, which gives rise to volatile compounds including thiols (e.g. methyl-3-furanthiol) and disulphides (e.g. Bis(2-methyl-3-furyl) disulphide) (260, 261). However, the thermal stability of vitamins may also be dependent on their occurring form, as particular forms have been reported to be more stable than others (262). In addition, high processing and storage temperatures can additionally increase and facilitate other degradation reactions, such as oxidation of vitamins (254) as discussed in a later section (1.5.5.4).

1.5.5.2 Effect of Light on B-Vitamins

Light exposure is inevitable throughout the food chain and adversely affects both riboflavin and vitamin B_6. Riboflavin is known as being extremely light sensitive undergoing photodegradation and widely accepted as a photosensitizer, producing several photoproducts, although dependant on factors including; wavelength, intensity, oxygen and pH as reviewed by Sheraz *et al*. (2014) (263). Light exposure results in riboflavin yielding a singlet excited state and triplet excited state,

resulting in photodegradation reactions, such as the oxidation of the ribityl side chain forming irreversible photoproducts, lumichrome and formylmethylflavin, although dependent on other factors (e.g. pH) (263). In fact, measurements of the photoproducts, such as lumichrome are usually assessed to indicate the proportion of riboflavin degraded. Riboflavin loss as a function of light exposure in food systems has been extensively studied. For example, milk stored in clear bottles under controlled light showed a 40% reduction in riboflavin content over a 7-day storage period (264). A 30% loss of riboflavin was also reported in milk during 30-minutes of sunlight exposure (265). Similarly, 80% of riboflavin degraded in pasta during 12 weeks of storage, with a 50% reduction within the initial 3 h during controlled light exposure and intensity (226). Alternatively, vitamin B_6 has also been reported to be sensitive to light. Although this is dependent on pH as light sensitivity of vitamin B_6 has been reported to increase with increasing pH and susceptibility to light is also dependent on occurring form (257, 266). This is supported by an early report by Saidi and Warthesen (1983) (266), as they showed degradation of B_6 content in a dry model system exposed to light was dependent on the forms present. Pyridoxine and pyridoxamine suffered small losses in the region of 8-22%, however pyridoxal underwent significant loss with the notion that pyridoxal was more vulnerable to light. The use of light-barrier active packaging by the food industry that comprise of inorganic materials (e.g. iron oxide) that absorb or scatter light could preserve riboflavin and vitamin B_6 content in foods (267).

1.5.5.3 Effect of pH on B-Vitamins

The intrinsic pH of foods can also have a significant consequence on vitamin degradation, dependant on vitamin and form. This is due to pH influencing both the ionisation state and resultant charge (positive/ negative) of the vitamin and thus its susceptibility to degrade. Thiamine and riboflavin are among vitamins that are extremely unstable and sensitive to pH. Such vitamins are more stable in an acidic environment, compared to a neutral/alkaline environment as described in the literature (228, 268, 269). The effect of pH on thiamine stability has been previously reported, in which increasing pH above pH 6 in buffered systems, regardless of other parameters (a_w and temperature) results in thiamine degradation into precursor forms (270). Thiamine has two pKa values, the pyrimidine N1 has a pKa value of 4.8, which is positively charged in acidic conditions along with thiazole N3 and is of most relevance in foods (228, 271). However, in alkaline conditions thiamine is degraded into precursor forms, pyrimidine and thiazole via cleavage of the methylene bridge, further followed by the opening of thiazole ring creating a thiol form (negatively charged), yielding the second pKa value of 9.2 (228, 271). A recent paper by Voelker *et al.* (2018) (228) investigated the stability of two thiamine salts (thiamine mononitrate and thiamine hydrochloride) in solution, used in fortification of foods as a function of temperature, concentration and time. They found thiamine mononitrate to be the least stable of the two salts studied, due to it forming a solution of a near neutral pH (5.36-6.96), in comparison

to the acidic environment thiamine hydrochloride formed (pH 1.12-3.59), showing thiamine mononitrate to be less stable in neutral conditions. Similarly, vitamin B_6 in buffered solutions have also been shown to be more stable in an acidic environment, although dependant on form (257, 266). Saidi and Warthesen (1983) (266) have shown that concentration of pyridoxamine and pyridoxal decreased as pH increased from pH 4-7, whereas the concentration of pyridoxine was shown to be mostly stable.

Another pH sensitive vitamin, riboflavin has two pKa values, pKa_1 1.7 and pKa_2 10.2 and depending on pH environment will determine riboflavin occurrence in either its cationic form (acidic environment), neutral form or anionic form (alkaline environment) (272, 273). Riboflavin main cause of degradation is through exposure to light, however the rate of photolysis and main degradation compounds formed during are strongly influenced by pH (268). This was shown in an aqueous solution by Ahmad *et al*. (2004) (273), the study showed the rate of photolysis was dependant on the ionised or non-ionised form of riboflavin, which also influences the fluorescent nature of riboflavin. They showed ionised forms (non-fluorescent) were more stable in comparison to non-ionised forms (fluorescent). Authors showed as pH increased from pH 5 to pH 10, the rate of photolysis simultaneously increased by 80-fold at pH 10. This is due to the susceptibility of riboflavin to oxidation when present in its non-ionised state. A strong alkaline environment will also lead to hydrolytic cleavage of the isoalloxazine ring of riboflavin leading to the formation of photoproducts, diketo complex and β-keto acid (263, 274). Investigations on the effect of pH alteration in food products over storage and its impact on vitamin loss is scarce, most research to date has been conducted in aqueous solutions.

1.5.5.4 Effect of Oxygen on B-Vitamins

The presence of certain gases in a food system, particularly oxygen (air) can adversely affect the stability of selected vitamins due to oxidation. Oxidation reactions in foods can occur through a triplet oxygen or singlet oxygen, produced chemically, enzymatically or in the presence of light (photooxidation) and can be accelerated with temperature (275, 276). Riboflavin in the presence of light and oxygen acts as photosensitizer, inducing photooxidation (I or II). Riboflavin among other photosensitizers in food absorb energy from light, riboflavin absorbs energy due to its isoalloxazine moiety, yielding an unstable triplet-excited state. The triplet-excited state can transfer energy to a triplet oxygen, leading to the production of a singlet oxygen and otherwise referred as a reactive oxygen species, this mechanism is referred to as photooxidation II (276-278). Reactive oxygen species include superoxide, hydroxyl radical and hydrogen peroxide and their presence in foods can have determinantal effects on the nutritional composition of foods, including against other vitamins (272, 279). Li, King and Min (2000) (279) showed in a modelled system, riboflavin induced oxidation of fat-soluble vitamin D_2 in the presence of light by forming singlet oxygen species. Although, riboflavin does induce oxidation reactions it also becomes unstable in the process, and further becomes oxidised by reactive oxygen species during

photodegradation reactions, as discussed in section 1.5.5.2 (263). Thiamine is also oxidised at increasing pH and in the presence of oxidisers such as hydrogen peroxide, in which thiamine is converted to a fluorescent compound, thiochrome and is often converted to this form to determine thiamine content in food (280-282). Niacin and B_6 vitamins, pyridoxine have been reported to be stable in the presence of oxygen, whereas pyridoxal and pyridoxamine are more liable to oxidation (257, 283). The Ribeiro *et al*. (2011) (227) study showed the impact of reactive oxygen species hydrogen peroxide at 10% concentration on vitamin oxidation, applied to standards of thiamine, riboflavin, and pyridoxine. They showed during a 24-hour storage period 12.7% thiamine, 88.9% pyridoxine and 36.7% riboflavin remained, highlighting the susceptibility of both thiamine and riboflavin to oxidation and the stability of pyridoxine.

1.6 Opportunities to Contribute to Existing Knowledge on Microbiological Food Spoilage

Food commodities are vulnerable to physicochemical and microbiological biotransformation's during the food chain, depending on an array of intrinsic and extrinsic environmental factors. These factors can have a pronounced impact on the organoleptic properties of food and influence the stability and, ultimately, shelf-life. Determination of a products shelf-life and stability is currently based on outdated microbial and organoleptic assessments that fail to capture food spoilage dynamics. However, the use of complementary -Omics techniques (HTS and metabolomics) could be used to reveal a novel in-depth understanding of the complex processes occurring during the shelf-life period. In addition, when shelf-life is assessed, nutritional status of food is often not considered and at present there are limited studies assessing vitamin composition alongside other quality markers of foods. It is also important to acknowledge that food spoilage has largely been characterised in food products of animal origin, due to these types of matrices potentially posing greater risk to consumer health, while stability of vegetable-based matrices is largely underrepresented in the literature. This includes identification of markers associated with spoilage of vegetable-based matrices. Due to this, the thesis intends to provide a novel in-depth characterisation of a vegetable matrix during storage, a commercially available vegetable soup and will be referred to as a vegetable matrix henceforth.

Vegetables are an extremely important dietary source while also having significant economic value, comprising of a variety of nutrients including carbohydrates such as starch, cellulose, monosaccharides, etc. Further example of constituents includes proteins, amino acids, lipids, nucleic acids, vitamins, minerals and alkaloids (296, 297). Owning to their nutritional richness in both macronutrients and micronutrients, alongside high a_w and intracellular pH ranging between 4.9-6.5, make vegetables highly susceptible to microbial growth among other spoilage reactions (177, 294, 298). Processed vegetables including products such as "ready-to-eat" matrices, have gained significant popularity over the last decade, in 2018 UK consumers spent £133 million on

fresh soup, a 70.5% increase from the amount spent in 2007 (299, 300). Nutritional richness provided from these vegetable based products is one of the main driving factors behind the increased popularity, in combination with the convenience of a "ready-to eat" product (299).

1.7 Aims, Objectives and Thesis Outline

The work aims to provide a novel multi-omics derived, in-depth characterisation of the stability of a vegetable matrix (vegetable soup), to understand both vitamin and macronutrient composition and the relationship between food spoilage and vitamin status. The following research objectives (**RO's**) to achieve the overall aim are outlined below:

RO1) Determine native content of specific B-vitamins of interest in a complex vegetable matrix.

RO2) Develop and validate an extraction and analytical method to simultaneously separate and quantify essential B-vitamins namely nicotinamide, pyridoxine, riboflavin and thiamine within the vegetable matrix.

RO3) Characterise the impact of spoilage of the vegetable matrix on nutritional composition, microbial dynamics and associated metabolites using targeted and untargeted methodologies.

RO4) Determine the impact of several storage environments on progression of spoilage and the simultaneous changes to nutritional status and matrix stability to identify spoilage markers.

The outline of the thesis in accordance with achievement of the RO's above:

Chapter 3 (RO1, RO2): "Determination of selected water-soluble vitamins (thiamine, riboflavin, nicotinamide and pyridoxine) from a food matrix using hydrophilic interaction liquid chromatography coupled with mass spectrometry" – This chapter solely covers the validation of an HILIC LC-MS method to reliably determine B-vitamins and details the development of an extraction protocol to analysis B-vitamins in the vegetable matrix.

Chapter 4 (RO3): "Characterisation of Food Spoilage and Nutritional Composition During a Time-Dependent Storage of a Vegetable-Based Matrix" - Chapter 4 centres around profiling the nutritional composition (B-group vitamins and macronutrients), metabolite profile, microbial growth and dynamics of the vegetable matrix during a controlled storage environment. The data obtained in this chapter formed a baseline for the following chapters.

Chapter 5 (RO4): "Investigating the Effect of Storage Environments and Preparation Procedures on Food Spoilage and Nutritional Composition in a Vegetable Matrix" – This chapter builds from chapter 4 and explores the effect of different storage and preparation environments on B-vitamin integrity, microbial growth, bacterial dynamics and volatile and non-volatile metabolites. The environments investigated included different temperatures, gas compositions, pH and storage of the vegetable matrix from frozen.

Chapter 6 (RO4): "Effect of Enhancing the Rate of Microbial Growth through Supplementation with Specific Nutrients on the Nutritional Stability and Spoilage Rate of a Vegetable Matrix" – This chapter also builds on from chapter 4 and outlines the impact of increased growth of spoilage species on the deterioration of the vegetable matrix.

Chapter 2

Materials and Methods

In-depth characterisation of spoilage was conducted on a single food matrix, a commercially available vegetable soup, henceforth referred to as the vegetable matrix.

2.1 Preparation of Investigated Vegetable Matrix for Each Storage Study Conducted in Chapters 4, 5 & 6

Commercially available vegetable matrix was purchased from a local supermarket as shown in Figure 2.1 and nutritional values as stated on packaging were as follows per 100g: carbohydrates 4.2 g (sugars 2.3 g), fat 0.7 g, protein 0.6 g, fibre 1.1 g and salt 0.48 g.

Figure 2.1: Image of the vegetable matrix, country vegetable soup investigated throughout thesis

Per storage study/environment, three cartons (600 g each) of the fresh vegetable matrix (same batch, according to packaging) were each homogenised in a sterile environment for 2 min, using a commercial blender. All equipment used for homogenisation was sterilised using pure industrial methylated spirit and 4 h UV treatment in a PCR hood (CBS Scientific, San Diego, USA) prior to use and confirmed with swab tests. Swabs were also taken from the vegetable matrix immediately after opening, before homogenised. Swabs were taken in duplicate and plated on plate count agar plates (outlined in section 2.6.2). Plate count agar plates were incubated and checked after the incubation period (72 h). After homogenisation, ~60 g sample was isolated and kept on ice, classified as time point 0 (T0). The sample taken was then further sub-sampled in a sterile environment into the following aliquots detailed in Table 2.1 for analysis of: microbial growth (method section 2.6), amplicon sequencing (method section 2.7), volatile organic compounds (VOCs) (method section 2.8), metabolite profiles (method section 2.9), vitamins (method section 2.11) and macronutrients (method section 2.12). An aliquot was additionally taken from the homogenised sample and used for pH measurements (method section 2.10), measured in triplicate.

Table 2.1: The quantity of sample taken for each type of analysis

Type of Analysis Performed	Total quantity of aliquot taken (g)
Microbial Growth	2
Amplicon sequencing	2
VOCs	10
Metabolite profiles	10
Vitamins	5 g in 3 separate falcons (15 g total)
Macronutrients	5
pH	6

Furthermore, 1.4 kg of the remaining matrix homogenates were weighed in a 2 L Duran and exposed for 1 hour for natural contamination in a non-microbiological laboratory. After 1 hour of natural contamination the matrix was mixed in an orbital incubator (Infors HT Multitron Standard, Surrey, UK) at 170 RPM for 2 min and ~60 g sample taken immediately (T1) and further sub-sampled as described above. All samples excluding microbial samples were snap frozen in liquid nitrogen (kept on ice prior to freezing) and stored at -80 °C for individual analysis.

2.2 Profiling Spoilage of a Vegetable Matrix in Chapter 4

The vegetable matrix was prepared as stated in the above section 2.1 and stored under control storage conditions at 20 °C in a static incubator (LMS, Kent, UK). Samples were collected after 8 h (T8), 24 h (T24), 48 h (T48), 72 h (T72), 96 h (T96) and 120 h (T120) from opening. At each time point ~60 g samples were taken under aseptic conditions and sub-sampled into aliquots as described in above section (section 2.1). Prior to collecting samples from the food matrix at specified time points, the vegetable matrix was mixed in an orbital incubator at 170 RPM for 2 min. Samples taken were snap frozen in liquid nitrogen and stored at -80 °C, microbial analysis sample excluded. Aliquots were also taken from the ~60 g samples and used for pH measurements, measured in triplicate.

2.3 Storage of Vegetable Matrix Under Different Conditions (Temperature, pH, absence and presence of oxygen) in Chapter 5

2.3.1 Vegetable Matrix Storage Under Different Temperatures

After preparation of the vegetable matrix as outlined in section 2.1, the matrix was stored in a static incubator (LMS, Kent) at either 7 °C or 37 °C. Samples were collected at the same time points as stated in section 2.2 when stored at 7 °C. However, when the vegetable matrix was stored at 37 °C, the samples were collected after 8 h, 24 h, 32 h, 48 h, 56 h and 72 h from opening. All samples taken were sub-sampled and processed as described in section 2.2.

2.3.2 Vegetable Matrix Storage at a Specified Pre-set pH (Acidic Versus Alkaline)

The pH of the vegetable matrix was adjusted under aseptic conditions after the vegetable matrix was weighed into a 2 L Duran, following preparation procedures outlined in section 2.1. The pH was pre-set to ~pH 3.6 with addition of 2 M citric acid or pre-set to ~pH 8.6 with 2 M sodium hydroxide. Citric acid and sodium hydroxide were passed through a sterile filter (0.20 µm), before used to adjust the pH of the matrix. Aliquots were taken during addition of citric acid or sodium hydroxide and pH subsequently measured and checked, until desired pH was achieved. After 1 hour of natural contamination, the matrix was stored in a 20 °C static incubator (LMS, Kent, UK). Samples were collected at the same time points and sub-sampled and further processed as stated in 2.2.

2.3.3 Vegetable Matrix Storage Under Absence and Presence of Oxygen

Alterations to oxygen environment was conducted utilising a FerMac 310 bench-top bio-fermenter (Electrolab Biotech, Gloucestershire, UK), equipped with sampling port and temperature, pH and oxygen control. After preparation of the vegetable matrix as covered in section 2.1, the matrix was transferred into a sterilised bio-fermenter vessel aseptically and attached to a FerMac 310 system, sterilised and calibrated pH and oxygen probes were also inserted. Additionally, the vessel was wrapped in foil to protect from light and to replicate dark storage conditions in static incubators as per previous storage studies. The vegetable matrix was agitated in the bio-fermenter at 100 RPM, temperature of the vessel was controlled at 20 °C by a heat exchanger maintained by both a heat jacket and a circulating water bath set at 7 °C (VWR, Leicestershire, UK). Absence of oxygen was achieved by continuous bubbling of filtered nitrogen (BOC, Chester-le-Street, UK) at 1 L/min, presence of oxygen was performed through continuous bubbling of filtered compressed air (21% ± 0.5% oxygen, 79% ± 0.5% nitrogen) at 1 L/min and baseline control was conducted by bubbling no gas through the system. Samples (~60 g) were collected via sampling port using aseptic technique into sterilised universal glass vials (high temperature and UV treatment) at the same time points stated in 2.2 and sub-sampled as described in section 2.1. Although sub-samples were collected for macronutrient analysis, macronutrients weren't investigated based on previously analysed data. Bio-fermenter set-up is displayed and outlined in Figure 2.2.

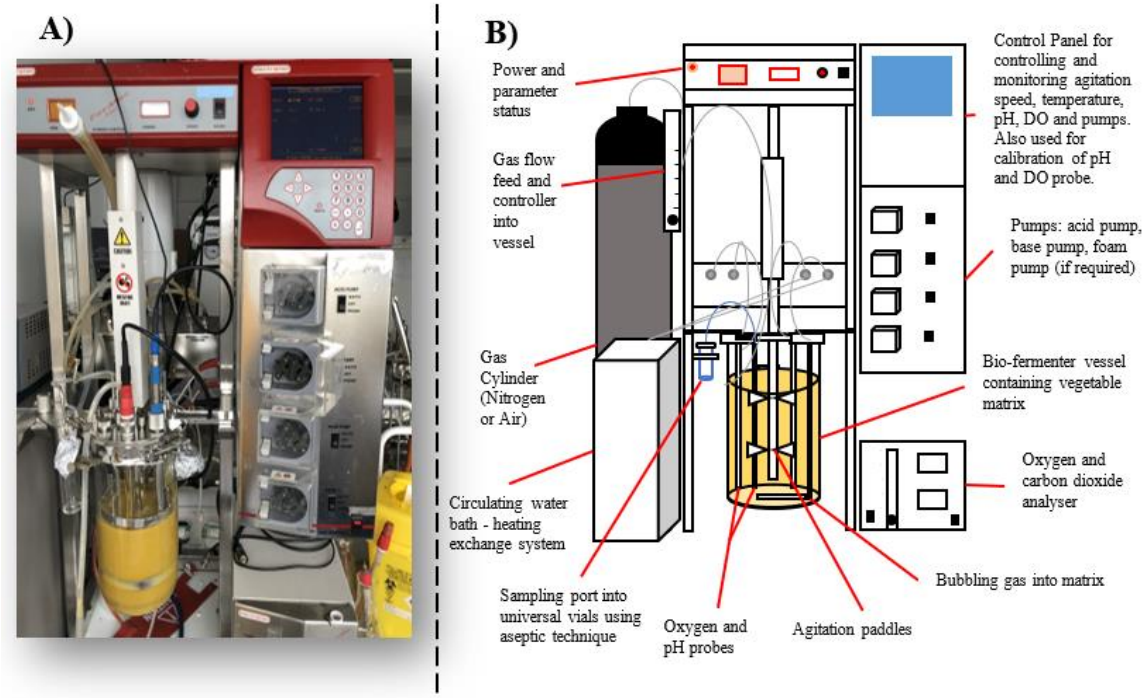


Figure 2.2: Bio-fermenter set-up including image of initial experimental set-up (A) and overall bio-fermenter design (B).

2.4 Profiling Vegetable Matrix From Frozen in Chapter 5

Storage of the vegetable matrix was investigated from frozen, in which preparation was as stated in section 2.1, with the initial preparation steps adapted; three cartons (600 g each) of the vegetable matrix were stored in the freezer for several months and was defrosted the day before use. Storage procedure was the same as detailed in section 2.2, although samples were not collected at 8 h of storage (T8).

2.5 Nutritional Manipulation of the Vegetable Matrix in Chapter 6

Nutritional composition of both the macronutrient and micronutrient content of the vegetable matrix was modified through fortification with either nicotinamide or a combination of sugars (glucose, fructose and sucrose). Fortification was achieved by preparing the vegetable matrix as outlined in section 2.1, however after the vegetable matrix was weighed in a 2 L Duran, the matrix was fortified with selected nutrients. In the case of vitamin B_3 fortification, 1 mg/mL stock of nicotinamide was filter sterilised, in which 0.7 mL was aseptically added to the vegetable matrix providing a concentration of 0.5 µg/mL of matrix. After addition of nicotinamide, the vegetable matrix was mixed in orbital incubator set at 190 RPM and 20 °C for 10 mins. Alternatively, glucose, fructose and sucrose were supplemented by doubling the quantity of each sugar proportionally according to the amount of each sugar naturally present in the vegetable matrix (based on previous sugar analysis experiments). Each sugar was weighed accordingly into a

falcon tube in aseptic conditions and transferred directly to the vegetable matrix, mixed in orbital incubator until dissolved, set at 190-220 RPM and 20 °C. Glucose, fructose and sucrose was also collectively added to sterilised distilled water for microbial analysis (section 2.6) to ensure no contamination from the addition of sugar. After each form of fortification, an additional sample was taken for nutrient analysis, subsequently followed by remaining preparation procedures in section 2.1. The vegetable matrix was then stored in a 20 °C static incubator (LMS, Kent, UK) and samples were collected at the same time points and sub-sampled as described in section 2.2, however no samples were collected at 8 h (T8). In addition, although sub-samples were collected for macronutrient analysis, macronutrients weren't investigated based on collected data.

2.6 Microbial Analysis

2.6.1 Chemicals and Reagents

Plate count, malt extract agar and bacteriological peptone were purchased from LAB M Ltd (Lancashire, UK).

2.6.2 Microbial Culturing Media

Malt extract agar was prepared by combining 50 g with 1 L of distilled water, which was soaked for 10 min and mixed on a magnetic stirrer. The malt extract agar was then heated in a microwave until agar was dissolved and 20 mL was transferred into universal tubes, before sterilisation at 121 °C for 15 min. Plate count agar was prepared similarly, however 23.5 g was required in 1 L of deionised water. After sterilisation, universal tubes containing culture media were kept molten at 55 °C for a maximum of two days and upon use transferred to a 45 °C water bath 1 hour before required for microbial testing of vegetable matrix sample. Malt extract agar and plate count agar were set in petri dish plates to perform swabs of experimental equipment. For this, malt extract agar and plate count agar were prepared as stated above, dissolving 50 g and 23.5 g respectively in 1 L of deionised water, autoclaved at 121 °C for 15 min and further poured into petri dishes using aseptic technique. Once agar was set, petri dishes were transferred to a plate dryer set at 30 °C for 30-60 min.

2.6.3 Microbial Culturing Buffer – Peptone Physiological Salt Solution (PPS)

The PPS solution was made by dissolving 8.5 g of NaCl and 1 g of bacteriological peptone in 1 L of distilled water using a magnetic stirrer. pH of the PPS was checked to ensure pH value was 7 ± 0.2; if pH was lower than required 2 M NaOH was used to increase pH. The PPS was then sterilised at 121 °C for 15 min.

2.6.4 Culture Based Analysis of Food Sample

Aliquots collected for microbial analysis were tested on the sampling day, according to the ISO standards for microbial analysis of food, adapted from a reported method by Lima *et al.* (2011) (62). Samples were taken in duplicate and vortexed for 1 min, from which 100 mg was transferred to a sterilised Eppendorf tube and vortexed with 900 µL of sterilised PPS solution (section 2.6.3). PPS was the diluent used to complete a serial dilution (tenfold) of the sample. Pour plate technique was used, in which 100 µL of the appropriate dilutions were plated in duplicate on plate count agar for capturing total viable count (TVC) and plated on malt extract agar for quantification of yeast and moulds. Malt plates were incubated at 25 °C and enumerated (CFU per g) after 120 h. Plate count agar plates were incubated at 30 °C for 72 h before counting and expressed as CFU per g. For reliable enumeration of colonies, plates selected for counting presented between 30-300 colonies. In addition, 100 µL of PPS was plated on both agars as part of a negative control during time courses.

During a separate preliminary investigation, comparisons were made using both pour plate technique and the Miles & Misra method. For the Miles & Misra method, sections of plate count agar plates were divided into six segments, samples were prepared and diluted as stated above, except 20 µL of six dilutions were pipetted onto pre-determined sections in duplicate.

2.7 Amplicon Sequencing Assessing Microbial Diversity and Composition– Culture Independent Approach

2.7.1 Chemicals and Reagents

DNA extraction kits used were Qiagen DNeasy® PowerFood® Microbial Kit and DNeasy® PowerLyzer® PowerSoil® DNA isolation kits (Qiagen Ltd, Manchester, UK). Phosphate buffered saline (PBS) tablets, microbial DNA-free water and agarose powder were purchased from Sigma-Aldrich (Dorset, UK). SYBR™ Safe DNA Gel Stain was acquired from Thermo Fisher Scientific (Massachusetts, USA), while 1 kb HyperLadder™ and DNA loading buffer were purchased from Bioline (Tennessee, USA). A 50x Tris-acetate-EDTA (TAE) stock concentration was made on site at Northumbria University and was further diluted to 1x in this experiment. All consumables purchased were sterile prior to use.

2.7.2 DNA Extraction and Isolation from the Vegetable Matrix

DNA was extracted in triplicate from T0, T48/T32, T120/T72 sub-samples taken throughout storage experiments detailed in section 2.1-2.5 using PowerSoil® Qiagen kit, except when profiling spoilage of the vegetable matrix in section 2.2 (baseline condition), timepoints T24 and T72 were also extracted. Samples were removed from freezer and defrosted at 4 °C the day before

extraction. Extraction was completed on 0.25 g ± 0.025 g of matrix sample following the manufactures specifications as outlined in the graphical representation in Figure 2.3, but with an extended bead-beating process of 25 min and a 15 min wait period during the final elution step. Additionally, as part of a separate preliminary investigation to compare available DNA extraction kits, PowerFood® Qiagen kit was used following manufactures specification using samples acquired from a storage trial, outlined in Figure 2.4. PowerFood® required an additional step initially; 0.25 g ± 0.025 g of matrix sample was homogenised with 0.75 mL of pre-prepared PBS solution, centrifuged and pellet resuspended in MBL solution (450 µL). Kit negative controls were prepared alongside each set of extractions conducted and all samples were randomised prior to extraction. After extraction DNA was stored at -80 °C and quality checked before sequencing in the NUomics sequencing research facility.

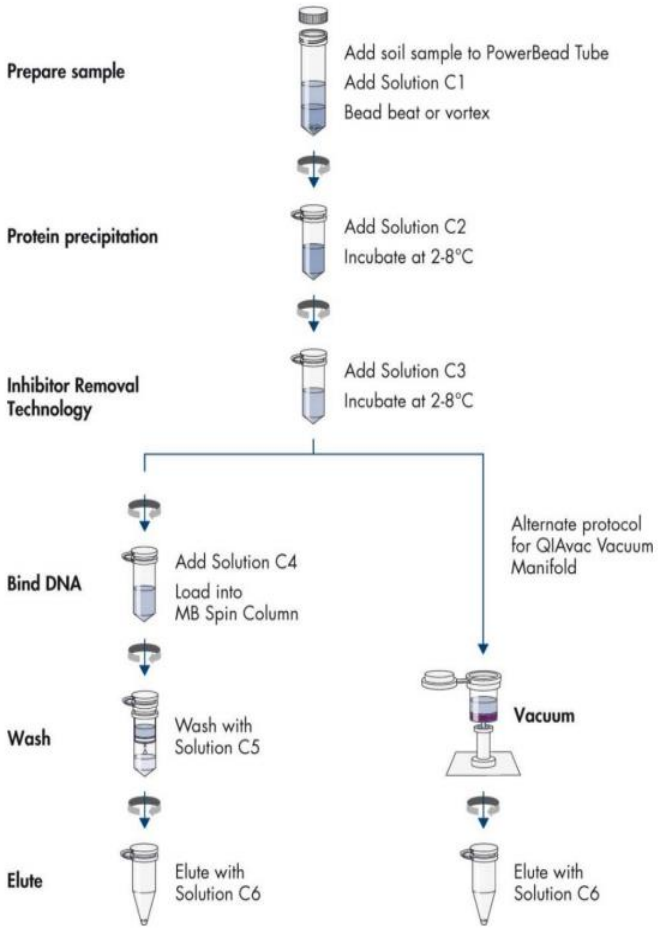


Figure 2.3: Outline of PowerSoil® kit procedure followed, as detailed in the handbook (301).

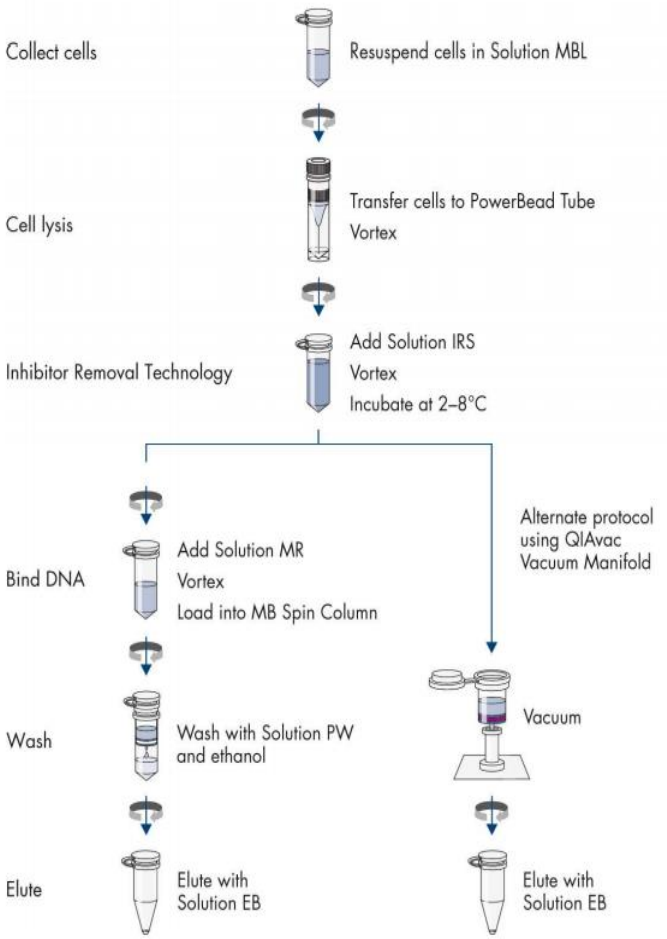


Figure 2.4: Outline of PowerFood® kit procedure followed, as detailed in the handbook (302).

2.7.3 DNA Quality Check Before Sequencing

Quality of DNA was checked prior to sequencing using agarose gel electrophoresis on 10 representative extracted samples. In order to prepare the gel, 0.5 g agarose was weighed and added to 50 mL of 1x Tris-acetate-EDTA (TAE) buffer to make a 1% agarose gel. Agarose suspension was heated until dissolved in a microwave and subsequently cooled to 50 °C, further followed by the addition of 5 µL SYBR™ Safe DNA Gel Stain. The gel was poured into a casting tray, after the gel was set and transferred to a gel tank, 1x TAE was sufficiently poured in the tank over the gel. Wells were loaded with either 5 µL of HyperLadder™ 1 kb as a reference, or 5 µL of sample (n=10); 5 µL of sample was previously mixed with 2 µL of DNA loading buffer, prior to loading 5 µL of sample into well. Gels were run at 120 mA for 30 min (Bio-Rad's PowerPac™, California, UK), followed by visualisation using Genus gel doc station (Syngene, Cambridge, UK). An example of a gel image can be viewed in Figure 2.5.

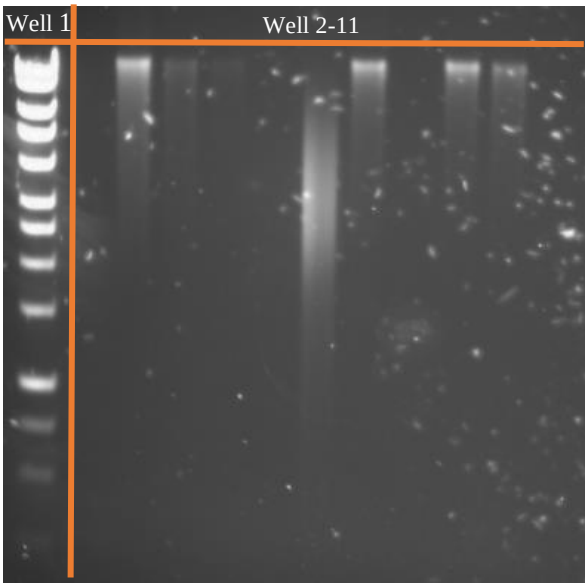


Figure 2.5: Example of a 1% agarose gel to assess DNA quality, comprising of 1kb DNA ladder (well 1), and extracted DNA from vegetable matrix samples (well 2-11).

2.7.4 Amplicon sequencing of 16S rRNA

Sequencing libraries utilised were prepared by NUOmics on the Illumina MiSeq as detailed in the Schloss lab SOP (303), utilising 515F and 806R primers, 5'-GTGCCAGCMGCCGCGGTAA-3' and 5'-GGACTACHVGGGTWTCTCTAAT-3' respectively outlined by Caporaso *et al*. (2011) (304). Primers amplified V4 region of the 16S rRNA gene. A sequencing negative control and positive control, comprising of a standard microbial community (*Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella enterica*, *Lactobacillus fermentum*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus subtilis*) from ZymoBIOMICS (Zymo Research, California, USA) were simultaneously prepared and ran with samples on the MiSeq.

2.7.5 16S rRNA Analysis

Fastq files were produced, through demultiplexing raw sequences for downstream application using Mothur V.1.43.0 as outlined in MiSeq SOP (303) and previously described by Young *et al*. (2020) (305). Forward and reserve reads were merged, sequences longer than 275 bp were omitted as part of quality control (QC). Sequences were processed to SILVA alignment, in line with QC, the maximum homopolymer was specified as eight nucleotides, as more than eight identical consecutive bases would indicate sequencing error. Followed by pre-clustering to avoid erroneous sequences due to occurrence of false operational taxonomic units (OTUs). Identification and subsequent removal of chimeras were performed using chimera-slayer available in Mothur, further followed by determination of uncorrected pairwise distances (212, 306). The downstream sequences from the processing steps taken in the Mothur program were clustered into OTUs, generated with an accepted genus homology of ≥97%.

All statistical analysis from the generated OTUs was performed in R studio; initial steps were taken to validate sequencing depth (e.g. library sizes, mean reads per feature etc.), compare compositions of controls (positive and negative) to samples by dissimilarity using ANOSIM statistical test. If the output of the statistical test was not significant, indicating no difference between the microbial communities of controls and samples, a prevalence-based decontamination process was implemented. Sample read counts/ OTU table were normalised by percentage abundance. Microbial diversity were determined using Phyloseq and Vegan package in R (307); alpha diversity was measured by Shannon index, Inverse Simpson Index and richness, while beta-diversity was analysed by Bray-Curtis dissimilarity. Graphical representation of the calculated alpha diversity was completed in GraphPad Prism (version 9.3.1). Statistical analysis for alpha diversity were performed using Mann-Whitney U test and Friedman test, to either determine statistical differences at a specified time points across storage environments, or to determine statistical differences as a function of time respectively. Post-hoc pairwise comparisons were conducted if Friedman test indicated significance using Dunn's test, with Bonferroni corrections. Alternatively, to identify significant variation in beta-diversity, permutational multivariate analysis of variance (PERMANOVA) test was performed with the adonis function, subsequently followed by pairwise comparisons using Bonferroni correction. Principle coordinate plots were generated using R packages to visualise Bray-Curtis dissimilarity distance matrix.

2.8 Volatile Organic Compound (VOCs) Profiling

2.8.1 Sample Preparation

Sub-samples taken for VOC analysis (T0, T48/T32, T120/T72) throughout storage experiments (sections 2.1-2.5), were defrosted at random and 0.5 g ± 0.025 g was weighed into amber 10 mL headspace vials and capped with steel screw caps with 2 mm silicone/PTFE septa. Additional timepoints for the storage study in section 2.2 (baseline condition) were included, T24 and T72. No derivatisation step was performed when preparing the samples for gas chromatography- time-of-flight mass spectrometry (GC/TOF-MS) analysis, as it was desirable to keep the volatile profile reflective of the sample.

2.8.2 Volatile Analysis Using GC/TOF-MS

VOCs were analysed on an Agilent Technologies 7890A GC system (Cheshire, UK), coupled to a Bench TOF-dx (ALMSCO International, LIantrisant, UK) while the autosampler was controlled via a Combi PAL system (CTC Analytics, Zwingen, Switzerland). Volatiles were separated on VF-WAXms capillary column (60 m, 250 µm i.d. and a film thickness of 0.25 µm). Volatile analysis was performed through initial agitation of the sample at 40 °C for 40 min at 250 RPM with a start stop feature of 5 s on and 2 s off. PDMS/DVB fibre (65 µm) (Supelco Analytical,

Pennsylvania, USA) was inserted for 1 minute into the vial, followed by desorption onto column at 250 °C over a 5 min period. Analytical temperature profile was set at 50 °C for 5 min, subsequently increased by 4 °C/min to 180 °C, held for 1 minute, then further increased by 20 °C/min to 260 °C and held for 5 min. Carrier gas (helium) flow throughout the run was set at 1 mL/min. Mass spectrometer set to scan in the mass range of 45-450 m/z, operating in positive full scan mode.

2.8.3 VOC Data Alignment, Processing and Analysis

All data processing including analysis was performed in ChromCompare+ (version 2.1) software (Markes International, LIantrisant, UK). Initially generated raw date .LSC files were aligned using a specified reference file. In this case, the reference file was a randomly allocated sample that featured early in the analytical run and was generally representative of the sample set. The aligned files were further integrated in accordance with experimental groups, using an untargeted method; for this, the peak detection algorithm, Tile Sum was used, with a retention time (RT) window width of 15 secs set and RT overlap of 25% specified. All peak filters were set to zero, including Min. area, Min. height and Min. width. After generation of the integrated files, data was analysed in the ChromCompare application, where all the features of a data set are retained according to the retention time of the tile and the m/z channel. The retained features are further filtered and subject to normalisation; a min intensity cut-off of 1,000,000 was set and min label frequency was set to 100%, meaning identified features have to be present in all replicates of that sample. Any features that had a retention time below 6.3 min and above 40 min was excluded, prior to normalisation of the data set using probabilistic quotient normalisation (PQN) with median as a parameter. Subsequently, the feature discovery was set to 25, to retain the top 25 most significant features in the investigated samples, further visualised using principal component analysis (PCA) and box plots were generated for each of the features. To putatively annotate the retained features, the aligned chromatograms for the analysed data was opened in the "data processing" application of ChromCompare+, the RTof each tile of each identified feature was added to the "Add Peak Batch" tool to identify peaks the tile refers to. The peaks were then given a presumptive ID using the available NIST demo library.

2.9 Metabolomic Fingerprinting

2.9.1 Chemical and Reagents

OPTIMA LC-MS grade water, OPTIMA LC-MS grade acetonitrile, LC-MS grade methanol, OPTIMA LC-MS grade formic acid and ammonium formate was acquired from Fisher Scientific (Loughborough, UK).

2.9.2 Food Sample preparation

Samples were extracted for untargeted metabolomic analysis using methods outlined by De Vos *et al*. (2007) (308) and Lopez-Sanchez *et al*. (2015) (309). Extractions were performed at random on T0, T48 and T120 sub-samples taken throughout storage experiments (sections 2.1-2.5) in sextuple. For the extraction fresh weight matrix sub-samples of 0.25 g ± 0.0125 g were weighed into Eppendorfs and immediately placed on ice. Extraction solution, 0.75 mL, of ice cold 100% LC-MS grade methanol comprising of 0.125% OPTIMA LC-MS grade formic acid was added to the vegetable matrix sample and vortexed (Vortex-Genie 2, Scientific Industries, Inc., New York, USA) for 10 s. Samples were further sonicated for 20 min in a sonicating water bath (Bandelin Sonorex, Berlin, Germany) operated at a max frequency of 35 kHz and nominal power of 160 W. Sonication was additionally performed in an ice slurry to avoid heating of samples. Samples were further centrifuged at max speed 18,500 g (Thermo Fisher Scientific™ SL 16R Centrifuge) at 5 °C for 15 min, 400 µL of supernatant was subsequently transferred into a fresh Eppendorf and stored at -80 °C. Prior to analysis samples were removed from the freezer, re-centrifuged at 20,000 g (Thermo Scientific™ my SPIN™ 12 mini centrifuge) for 3 min, before re-aliquoting 250 µL of extract into amber vials. Amber vials were then stored at -80 °C before analysis.

Quality control (QC) samples were prepared after all samples were extracted as a key part of metabolite analysis, to monitor analytical system behaviour. QC samples comprised of a class pool, this was based on experimental groups (temperature, pH, absence/presence of oxygen and added nutrients) and a global pool containing all extracted samples. Extraction blanks were also completed in duplicate alongside each batch of extractions and all blanks were pooled together.

2.9.3 Chromatographic Conditions for Metabolite Analysis – HILIC-MS/MS

Metabolite analysis was completed on a Thermo Scientific Vanquish UHPLC system, coupled to a Thermo Scientific Orbitrap ID-X™ Tribrid™ mass spectrometer (Massachusetts, USA). Chromatographic separation was achieved using a binary solvent system, comprising of mobile phase A: OPTIMA LC-MS grade water and 10 mM ammonium formate at pH 3.5, acidified with formic acid. Mobile phase B: OPTIMA LC-MS grade acetonitrile and 10 mM ammonium formate at pH 3.5, acidified with formic acid. Metabolites were separated on a Waters XBridge® BEH HILIC Column (2.5 µm, 2.1 x 150 mm), with an associated guard column, ACQUITY UPLC BEH Amide VanGuard pre-column (1.7 µm, 2.1 x 5 mm) (Waters, Elstree, UK). The UHPLC settings were as follows: flow rate of 0.3 mL/min, column chamber set to 45 °C and autosampler housing samples before, during and after injection was set to 4 °C. Injection volume was programmed to inject 4 µL onto column. Analytical method is 20 min injection to injection; gradient used started at 95% mobile phase B, held for 1 minute, then decreased to 50% mobile phase B over 10 min, held for 4 min, followed by a return to starting conditions of 95% B within

6 s and held for 4.9 min for re-equilibration. Column was equilibrated prior to run for 30 min under starting conditions.

2.9.4 Mass Spectrometer Settings and Optimisation

Mass spec was tuned based on manufacturing specifications prior to use. The mass spec heated electrospray ionization source (HESI) was operated at voltages 3500 V (Pos) and 2500 V (Neg). Ion transfer tube temperature and vaporizer temperature were set at 300 °C and 400 °C respectively. Sheath gas (N_2) flow was operated at 35 (arbitrary units) and auxiliary gas flow was set at 7 (arbitrary units). MS_1 and MS_2 were performed simultaneously using the AcquireX intelligent data acquisition workflow (Thermo Fisher Scientific, Massachusetts, USA) conducted on every injected sample between 0-11.5 min of each chromagraphic run. The MS_1 scan was performed initially, under the following conditions: orbitrap mass resolution set to 60K, automatic gain control (AGC) was set to have a normalised AGC target of 25% and maximum injection time of 50 ms. Mass range was specified as 100-1000 m/z. Furthermore, an intensity threshold of 10,000 was established, target masses above this intensity then went through an exclusion process, which was pre-determined based on performing a deep scan on blank extraction samples (n=3). Deep scan is part of a background exclusion and inclusion process, acquiring ions present in the blank to exclude from actual sample extracts. After masses are excluded, the remaining targeted masses underwent MS_2. The MS_2 scan properties included: orbitrap mass resolution set to 30K, isolation window of 1.3 m/z was set, AGC was set as standard and maximum injection time was specified as 54 ms. Higher-energy collisional dissociation (HCD) energies were stepped from 20, 35 and 50%. The transition from MS_1 to MS_2 was completed within 0.6 s. All data was acquired in positive polarity mode, while negative mode was not used based on preliminary studies.

2.9.5 Data Alignment and Processing

Raw data files generated from MS_1 were processed and aligned in Compound Discoverer 3.2 (Thermo Fisher Scientific, Massachusetts, USA) using MZcloud database to identify accurate masses. Data files were grouped into their respective experimental groups as part of data alignment, this included temperature (including preparation from frozen samples), pH, added nutrients and added/presence of oxygen. QC samples were additionally processed alongside; pooled class samples were grouped together with their associated class, while all global pool data files were grouped together. Global pool QC's were utilised to confirm system stability throughout the entire analytical run (n=10 injections) and class QC's (n=5 injections per class) were used to identify central points of each class cluster. Mass tolerance of 10 ppm was set as part of the alignment settings, max peak intensity was defined as 50,000 counts with a signal to noise threshold of 3/1 and protonated adducts (M+H) was selected.

After alignment, the generated peak table was further processed to remove peaks that were deemed to have poor reproducibility and therefore were characterised as being unstable throughout the analytical run. Approximately 1670 mass spectral features were identified on MS_1, using the global pool QC, any spectral feature that was within 25% RSD of the QC was retained. Furthermore, from the features retained, a mass error of 10 ppm or less was applied, from which MS features with matched ID's were kept, resulting in 117 accurate masses being putatively annotated in accordance with Metabolomics Standard Initiative (310). Reproducibility of global pool QC was additionally checked during the analytical run to confirm stability of the instrument.

2.9.6 Metabolomic Data Analysis

All data analysis for the acquired metabolomic data was conducted by MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/), utilising the "Statistical Analysis" section of the online platform. The generated and filtered peak table in the above section was rearranged into a format suitable for MetaboAnalyst in a "samples in rows (unpaired)" format; QC's were removed, group names were assigned to sample number e.g. Sample No. set as KPA1-KPA3, group names were No gas T0 (replicates). Upon file upload, data filtering non-parametric relative standard deviation was selected, followed by data normalisation using auto scaling (mean-centered and divided by the standard deviation of each variable), to produce gaussian distribution as seen in the example provided in Figure 2.6.

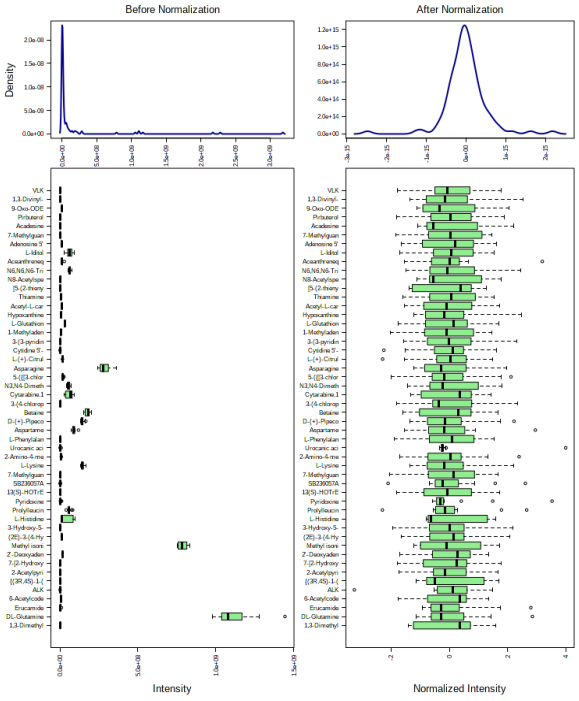


Figure 2.6: Example of data normalisation in MetaboAnalyst, using auto scaling prior to statistical analysis.

Initially PCA was the first chemometric tool utilised, which reduced the dimensionality of the dataset, while retaining important statistical information related to the data (311). PCA was specifically used to check data quality, and both identify potential patterns and relationships within each experimental group including, temperature, pH, absence/presence of oxygen and added nutrients. Furthermore, partial least-squares discriminant analysis (PLS-DA) was then used on each storage experiment to understand what putatively annotated compounds were contributing and therefore explaining the observed covariance. PLS-DA is described as being a "supervised" form of PCA taking into consideration the class order, while also reducing the dimensionality of the data (312). Therefore, in this case, class order will relate to the time course and PLS-DA will aim at explaining covariance across time points and thus following biochemical transformations associated with product degradation. Additionally, PLS-DA ranks variables that are deemed important features driving and influencing the relationship observed in the data; achieved using variable importance in projection (VIP) score. VIP scores greater than 1.35 were considered to be important variables influencing the observed relationship. If VIP scores were less than 1.35, the top 15 putatively annotated metabolites were kept. Pathway analysis was also conducted using the "Pathway Analysis" section of MetaboAnalyst. A list of putatively annotated metabolites according to VIP analysis were entered into the compound list of pathway analysis and processed, with the following parameter settings: enrichment method selected was hypergeometric test, topology analysis used was relative-betweenness centrality and prokaryote pathway library was used based on KEGG pathway.

2.10 pH Measurements

Samples taken for pH measurements were measured using Mettler Toledo FiveEasy pH meter, by inserting pH probe (pH electrode LE438, Mettler Toledo, Greifensee, Switzerland) into the matrix aliquot, performed in triplicate. The pH probe was calibrated on day of use, using a 3-point calibration curve, with provided pH 4.01, pH 7 and pH 10.01 solutions (Sigma- Aldrich, Dorset, UK).

2.11 Targeted Vitamin Analysis

Method used for targeted vitamin analysis and extraction was both developed and validated as part of this thesis and published in Journal of Chromatography B (313). An outline of the method is detailed in the following sections, including specific methods used for samples obtained during storage studies and methods for data analysis. However, more detail on this method in terms of development can be viewed in chapter 3.

2.11.1 Chemicals and Reagents

All water-soluble vitamins were acquired from Sigma-Aldrich (Dorset, UK): nicotinamide (98%), (-)-riboflavin (98%), pyridoxine hydrochloride (99%) and thiamine hydrochloride (99%). Methanol, ammonium acetate and 35% ammonia solution, all HPLC grade, were purchased from Fisher Scientific (Loughborough, UK). LC-MS grade acetonitrile and acetic acid (Optima grade) were additionally purchased from Fisher Scientific. Meta-phosphoric acid was obtained from Acros Organics (Fisher Scientific, Loughborough, UK). HPLC grade DL-dithiothreitol (DTT) was purchased from Sigma-Aldrich (Dorset, UK). A Milli-Q Integral three water purification system (Merck Millipore, Hertfordshire, UK) was used for the production of purified water to 18.2 MΩ. Nylon syringe filters (0.2 µm, 4 mm) were used for the preparation of final LC/MS samples (Chromacol, Fisher Scientific).

2.11.2 Preparation of Standards; Thiamine, Nicotinamide, Riboflavin and Pyridoxine

Stock solutions of individual vitamins were prepared on ice and in low light as follows: thiamine, nicotinamide and pyridoxine were prepared in a 0.12% acetic acid solution at 5 mg/mL. Riboflavin was dissolved in 0.28 M ammonia solution at a concentration of 0.5 mg/mL adjusted to pH 7.0 with diluted acetic acid. All stock solutions were placed in a sonicating water bath (Bandelin Sonorex, Berlin) for 5 mins, aliquots of the vitamin stocks were taken and snap frozen immediately in liquid nitrogen and stored at -80 °C for 1 month. Aliquots of stock were defrosted daily for working standards and diluted in mobile phase B; 10 mM ammonium acetate (pH 5) in 95:5 (V:V) acetonitrile: 18.2 MΩ deionised water. Working standards and multi- vitamin standards were diluted within a 5-400 ng/mL calibration range, filtered through a 0.2 µm nylon filter; all were prepared in glass amber vials.

2.11.3 Preparation of HILIC Mobile Phases

Mobile phases were **A**: 10 mM ammonium acetate (pH 5.0) in 95:5 (V:V) 18.2 MΩ deionised water: acetonitrile and **B**: 10 mM Ammonium acetate (pH 5.0) in 95:5 (V:V) acetonitrile: 18.2 MΩ deionised water.

Preparation of the mobile phase involved a concentrated buffer stock of 200 mM ammonium acetate, adjusted to pH 5.0 with acetic acid, 50 mL transferred per 1 litre of mobile phase (10 mM concentration). This formed either part of the water fraction in mobile phase A or the whole water fraction in mobile phase B. Once measured mobile phases were sonicated for 3 min before filtering through a 0.2 µm nylon membrane filter. Buffer stock was kept at 4 °C and used within one month from preparation.

2.11.4 Liquid Chromatography Mass Spectrometry (LC/MS) Analysis

Analysis of targeted water-soluble vitamins was completed on an Agilent Technologies (Cheshire, UK) 1260 Infinity LC system, features consisted of a: quadruple channel pump, auto sampler with a 100 µl injection loop, column oven, UV detector, coupled with an Agilent 6120 single quadrupole MS detector equipped with an electrospray – atmospheric pressure ionization (ES-API) source.

Chromatography conditions were as follows: flow rate set to 0.8 mL/min, auto sampler was set to 7 °C and column oven set to 30 °C. Targeted vitamins were separated on an Agilent (Cheshire, UK) ZORBAX HILIC Plus Silica column (100 mm x 2.1 mm, 3.5 µL). Chromatographic separation was achieved using mobile phases A and B outlined in section 2.11.3. Column equilibrated at set instrumental conditions at 60% eluent B for 2 hours, followed by 100% eluent B for 30 min. The analytical gradient adopted is displayed in Table 2.2.

Table 2.2: Analytical gradient used for targeting selected water-soluble vitamins

Time (min)	**Flow (mL/min)**	**Eluent A (%)**	**Eluent B (%)**
0.0	0.8	0	100
4.5	0.8	0	100
10.5	0.8	40	60
16.5	0.8	0	100
19.0	0.8	0	100

Mass spectrometry parameters were: drying gas flow 12.0 L/min, nebulizer pressure 35 psig, drying gas temperature 350 °C and capillary voltage 3000 V (positive and negative mode). Signal settings set to signal ion monitoring mode (SIM).

2.11.5 Vegetable Matrix Sample Extraction

Samples taken throughout the time course (sections 2.1-2.5) for vitamin analysis were weighed into 5 g aliquots in triplicate frozen in liquid nitrogen and kept at -80 °C until required for analysis. Upon analysis samples were removed from the freezer and placed in a lyophiliser (VirTis SP Scientific, Sentry 2.0, Suffolk, UK) for 24 h. Dry weight was recorded the following day, 3 mL of a 3% meta-phosphoric acid/ 200 mg/L DTT solution, 1 mL methanol and 0.5 mL of 18.2 MΩ deionised water was added to the dried food sample. Vortexed (Vortex-Genie 2, Scientific Industries, Inc., New York, USA) for reconstitution and centrifuged at room temperature at 4000 RPM for 15 min (Beckman Allegra 6R centrifuge, High Wycombe, UK). Subsequently, 1 mL of the supernatant was added to 3 mL acetonitrile, vortexed for an additional minute and centrifuged again for 15 min at 4000 RPM. 300 µL of the supernatant was filtered through 0.2 µm

nylon syringe filter into amber vials stored at -80 °C until required for LC-MS analysis, where 5 µL of sample was injected onto column.

2.11.6 Vitamin Data Analysis

All statistical analysis for vitamin investigations were conducted in SPSS version 26. Initially the Shapiro-Wilk test was performed on the data sets to determine if the data was normally distributed. Following confirming the normal distribution of data a one-way repeated measures ANOVA was used to determine significant changes in vitamin concentration over the time course and Greenhouse-Geisser correction was used to adjust the degrees of freedom when calculating the p value. Due to this particular test not being able to handle missing data, any data value for a time point that was classified as being missing completely at random (MCAR) e.g issue with sample, missed injection etc., was replaced using the expectation-maximisation (EM) algorithm. Subsequently, if the test showed significance and therefore the null hypothesis was rejected, pairwise comparisons were conducted with Bonferroni corrections.

2.12 Macronutrient Analysis

2.12.1 Chemicals and Reagents

For protein analysis: catalyst tablets were purchased from Velp Scientifica™ (Fisher Scientific, Loughborough, UK) and sulphuric acid, boric acid and methyl red indicator were purchased from Fisher Scientific (Loughborough, UK).

For sugar analysis: standards sucrose (98%) and D-fructose (99%) were purchased from Sigma-Aldrich (Dorset, UK). D-Glucose (anhydrous) purchased from Melford (Suffolk, UK). Both sodium hydroxide solution (10 M) and sodium acetate trihydrate HPLC grade were acquired from Fisher Scientific (Loughborough, UK). A Milli-Q water purification system was used (Merck Millipore, Hertfordshire, UK) which purified water to 18.2 MΩ. AS-DV autosampler PolyVials (0.5 mL) and filter caps were procured from Thermo Fisher Scientific.

2.12.2 Protein Analysis – Kjeldahl Method

Protein was determined by the content of total nitrogen in the given sample. Aliquots taken for macronutrient analysis, specifically T0, T48/T32 and T120/T72 were removed from -80 °C freezer and placed in a designated cold room (at 4 °C) the previous day, prior to analysis. Then, the samples were vortexed to ensure homogenisation for 2 min. Total nitrogen was determined using the Kjeldahl method, based on AOAC international outlined by both Nielsen (2010) (314) and Maehre *et al.* (2018) (315). Approximately 2 g ± 0.2 g of homogenised sample was weighed onto Rizla paper and placed in a Kjeldahl tube, Kjeldahl catalyst tablets were added to each flask. Concentrated sulphuric acid (15mL) was added to each sample and mixed, before heated on a

heating block at 440 °C for 20 min. Once cooled distillation was carried out using Kjeltec distillation unit (Kjeltec system, Tecator™ 1026 Distilling unit), from which the sample under distillation dispensed into 25 mL of 4% boric acid solution which contained 2 drops of methyl red indicator. After completion, a back titration was completed with 0.01 M HCl acid, the end point was recorded when the sample turned from a light lime green to a light pink/peach colour. Subsequently, the %Nitrogen was calculated based on the end point recorded, from which the % protein was determined based on %Nitrogen and a 6.25 conversion factor (% protein = %Nitrogen x 6.25 conversion factor). Analysis performed in duplicate for each sample.

2.12.3 Targeted Sugar Analysis (Glucose, Fructose and Sucrose)

2.12.3.1 Standard Preparation

Stock solutions of glucose, fructose and sucrose were prepared at 100 mg/mL in 18.2 MΩ deionised water. Aliquots of stock solutions were frozen at -80 °C and defrosted on required day. Working standards and multi-sugar standards were diluted within a 0.78 - 100 µg/mL calibration range. An aliquot, 500 µL, of calibration standards was transferred into allocated vials (0.5 mL AS-DV autosampler PolyVials, Thermo Fisher Scientific, Massachusetts, USA) and filter caps inserted. During analysis, 200 µL was taken from each sample, 100 µL was passed through the sample loop and 100 µL was injected on column.

2.12.3.2 Preparation of Mobile Phases

The following mobile phases were prepared:

A) 100 mM NaOH

B) 100 mM NaOH and 500 mM HPLC grade sodium acetate

C) 18.2 MΩ deionised water

D) 500 mM NaOH

After preparation eluents were degassed and filtered.

2.12.3.3 Sugar Analysis HPAEC–PAD Conditions

Sugars were analysed using a Dionex ICS-5000 Ion chromatography system (Thermo Fisher Scientific, Massachusetts, USA) the systems features consisted of: quadruple channel pump, AS-DV auto sampler with a 100 µL injection loop, column oven, electrochemical detector cell with a non-disposable AgCl reference electrode. Sugars were separated at 30 °C on a Dionex CarboPac PA1 BioLC (4 x 250 mm) column, coupled with a Dionex CarboPac PA1 BioLC (4x50 mm) guard column (Thermo Fisher Scientific, Massachusetts, USA). The analytical gradient used to separate the sugars is presented below in Table 2.3 and Chromeleon 7 was utilised for data acquisition.

Table 2.3: Analytical gradient used for targeting selected sugars

Time (min)	Flow (mL/min)	Eluent A (%)	Eluent B (%)	Eluent C (%)	Eluent D (%)	Curve
0.0	1	20	80	0	0	5
10.0	1	20	80	0	0	5
10.1	1	100	0	0	0	5
50.0	1	60	40	0	0	5
50.1	1	0	100	0	0	5
60.0	1	0	100	0	0	5
60.1	1	0	0	0	100	5
70.0	1	0	0	0	100	5
70.1	1	20	80	0	0	5

2.12.3.4 Sugar Extraction

Sugar extraction method was based on a published method by Shanmugavelan *et al.* (2013) (316) and Sánchez-Mata *et al.* (2003) (317). Essentially, 0.15 g of defrosted macronutrient samples, from the start, middle and end of the time course were dissolved in 20 mL of 80% ethanol in triplicate in a 50 mL falcon tube. Samples were mixed on a rotator at 40 RPM (Stuart Rotator SB3, Cole-Parmer, Staffordshire, UK) for 20 min and placed in a sonicating water bath (Bandelin Sonorex, Berlin, Germany) for 10 min at 50 °C and mixed on a rotator at 40 RPM for an additional 5 min. Samples were then centrifuged at 3000 RPM at 4 °C for 10 min (Eppendorf™ 5810R, Fisher Scientific, Loughborough, UK), and the supernatant passed through a 0.2 µm filter. Aliquots were taken of the filtrate into Eppendorf's (1 mL) and placed in a sample concentrator (RVC 2-18, Martin Christ, Osterode am Harz, Germany) until dried and reconstituted with 1 mL of 18.2 MΩ deionised water. Reconstitution was achieved by the addition of 500 µL of deionised water, followed by vortexing using a Mo Bio Vortex adapter (Qiagen Ltd, Manchester, UK) for 10 min. The reconstituted sugar solution (500 µL) was subsequently transferred into a fresh Eppendorf and 500 µL of deionised water was added to the original Eppendorf and vortexed again using a Mo Bio Vortex adapter (Qiagen Ltd, Manchester, UK) for 15 min (washing step). This second aliquot, 500 µL, was combined with the original aliquot in the fresh Eppendorf resulting in a total volume of 1 mL. An aliquot, 500 µL, was pipetted into allocated vials (0.5mL AS-DV autosampler PolyVials, Thermo Fisher Scientific, Massachusetts, USA) and filter caps inserted. During analysis, 200 µL was taken from each sample, 100 µL was passed through the sample loop and 100 µL was injected on column. A representative chromatogram displaying the targeted sugars in the vegetable matrix sample with clear separation and no co-eluting peaks can be viewed in Figure 2.7.

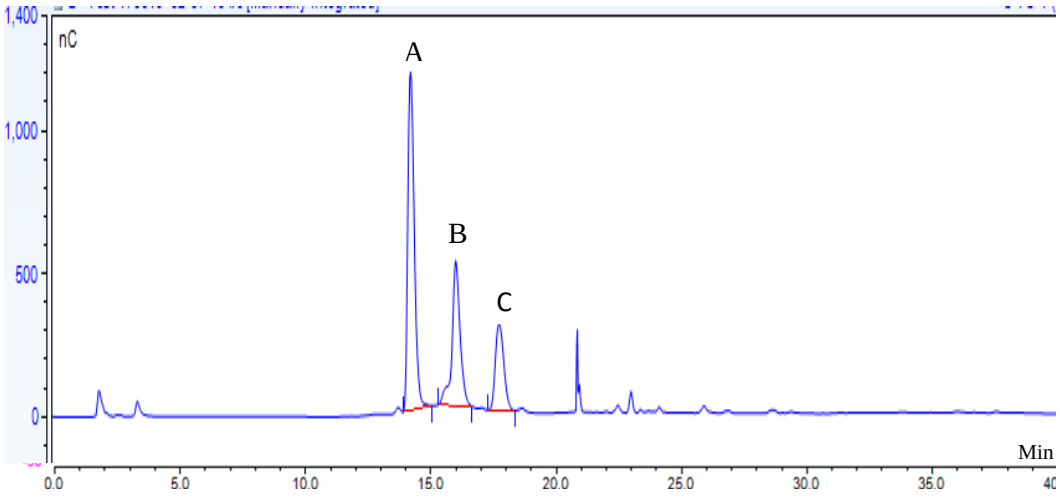


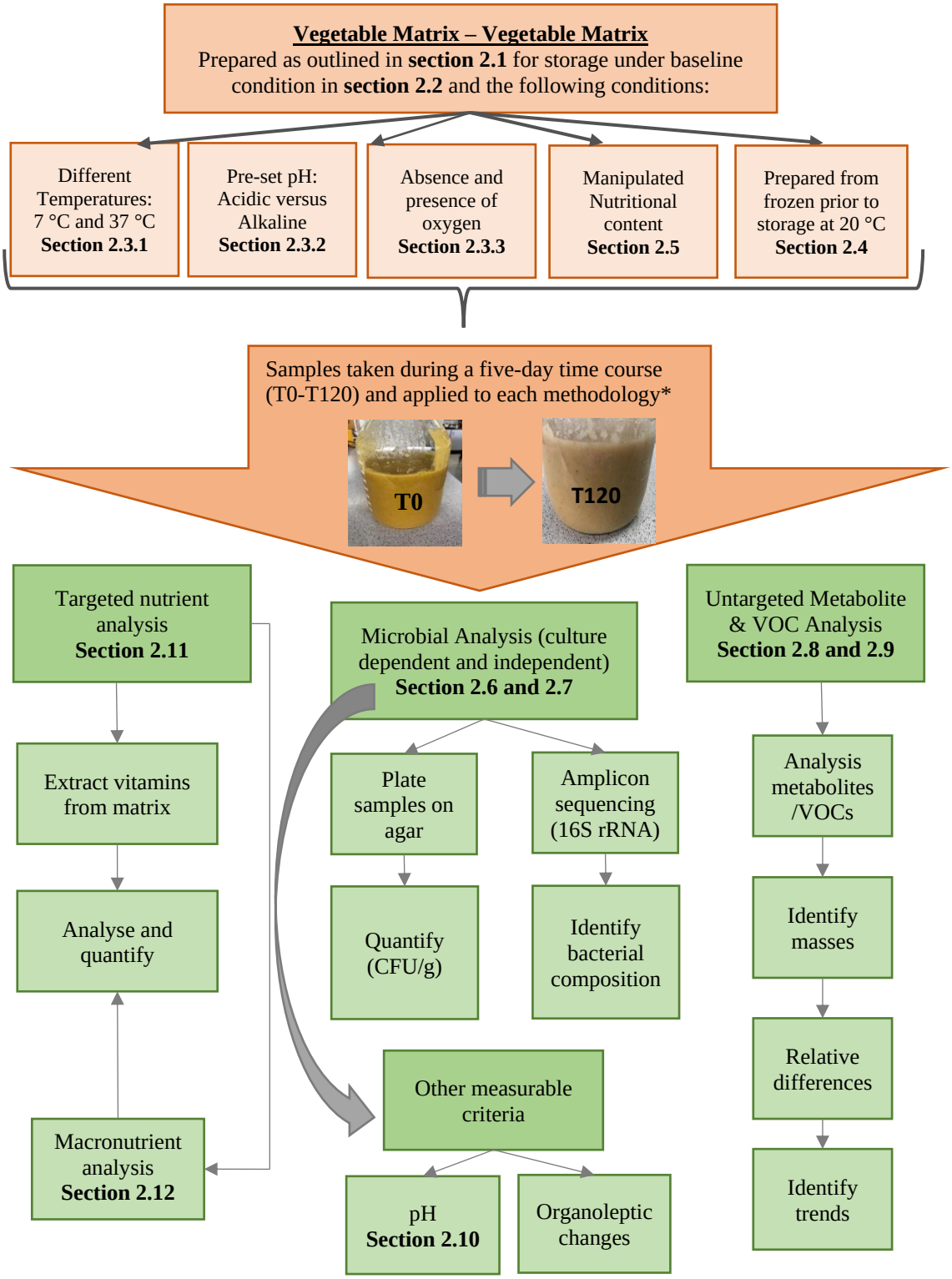
Figure 2.7: Representative chromatogram of targeted sugars, glucose (A), fructose (B) and sucrose (C) in the vegetable matrix sample using HPAEC/PAD. Glucose, fructose and sucrose were eluted at 14.25, 16.10 and 17.78 minutes respectively.

2.12.4 Macronutrient Data Analysis

Statistical analysis to identify significant changes in macronutrient composition was completed using one-way repeated measures ANOVA as outlined in section 2.11.6. If the test indicated significance and thus showing a significant change in macronutrient content over the storage period, pairwise comparisons were conducted with a Bonferroni correction applied to reduce type 1 errors.

2.13 Experimental Design Overview

Experimental design for the overall thesis can be viewed in Figure 2.8.



* Storage of the vegetable matrix under 37 °C concludes at 72 h.

Figure 2.8: Experimental overview of the thesis, in relation to methodology sections as highlighted.

Chapter 3

Determination of selected water-soluble vitamins (thiamine, riboflavin, nicotinamide and pyridoxine) from a food matrix using hydrophilic interaction liquid chromatography coupled with mass spectrometry

Methodological work in this chapter has been published in Journal of Chromatography B (313). For the purpose of this thesis, layout of the original publication has been altered to accommodate thesis chapter structure.

3.1 Introduction

The accurate quantification of water-soluble vitamins in foodstuffs is of significant value and importance. However, simultaneous determination and quantification of these vitamins from various food matrices represents a substantial challenge. In part, this is due to the diverse structural forms and different chemical compounds that make up these vitamins, many of which occur side by side in various foods. Naturally occurring vitamins often occur covalently bound to enzymes as cofactors, or as phosphorylated forms bound non-covalently to proteins and carbohydrates (318). Thiamine occurs in plant-derived foods chiefly in its free form but is present in animal foods as mono-, di-, or triphosphates and their esters, protein complexes, and as thiamine disulfides and their pyrophosphoric acid esters (233, 319). This renders precise quantification of vitamin levels in food a difficult process, as conventional methods require the parent compound in its free form to compare against pure standards. Another limitation is related to the extraction process employed to release bound vitamins, as B group vitamins can be labile and degrade in the presence of light, heat and slightly acidic/neutral pH conditions (228, 263, 266, 318). Due to the reasons presented above highlights the difficultly to develop one method to analyse all vitamins in a given sample and the reason several methodologies exist for each vitamin (320-323).

Analysis of individual vitamins in foods were traditionally performed using microbial assays, however technological advances in liquid chromatography (LC) has provided methods offering increased accuracy and precision (324, 325). Reversed-phase (RP) or hydrophilic interaction chromatography (HILIC) has been the separation method of choice for several years, but with different methods for detection and quantitation. For example, UV/vis detection (220, 326-335), fluorescence detection (331, 336, 337), or electrochemical/coulometric (321, 338-340) have been employed, and our research group have previously reported a comparison of such methodologies (319). However, the increased sensitivity offered by mass spectrometry (MS) is of particular value for vitamin quantification when present in trace amounts. MS detection has been utilised for analysis of water-soluble vitamins in various food matrices including infant/nutritional formula,

supplements, beverages and fortified produce (220, 318, 323, 325, 341-347). Santos *et al.* (2012) (220) validated a LC-MS/MS RP method simultaneously analysing seven free form water soluble vitamins in 12 different green leafy vegetables. Gentili *et al*. (2008) (318) developed a LC/ESI-MS/MS RP method to determine B vitamins in four plant-based matrices. The authors utilised a solid-liquid extraction to determine the quantity of B vitamins, from which the vitamin content in maize flour (3.27-0.020 µg/g), green kiwi (2531-0.012 µg/g), golden kiwi (7758-0.19 µg/g) and tomato pulp (116.3-0.10 µg/g) were determined. However, the majority of these methods for quantifying B vitamins in food matrices utilise MS with RP separation, with limited papers utilising HILIC.

Chromatographic separation of water-soluble vitamins can be challenging using standard RP; they are highly polar compounds with different extents of hydrophilicity and due to this can be poorly retained using RP separation mechanisms (342, 343). Goldschmidt and Wolf (2010) (342) investigated different chromatographic approaches for analysis of B vitamins comparing RP and HILIC, they found RP to be problematic for retention of thiamine, nicotinamide and pyridoxine. Due to these vitamins being more hydrophilic they were more effectively retained using HILIC separation. However, as highlighted by Fatima *et al*. (2019) (348), a very limited number of validated methods utilising HILIC separation coupled to MS detection have been developed to analyse water soluble vitamins in complex food matrices. A study by Chatterjee *et al*. (2017) (349) developed a LC-MS/MS method targeting nine water-soluble vitamins in fish using HILIC separation for increased sensitivity. The authors optimised an enzymatic hydrolysis method to subsequently extract B vitamins, including nicotinamide, nicotinic acid, pyridoxine, pantothenic acid, biotin, thiamine, riboflavin and cyanocobalamin from fish flesh. However, this method primarily utilised the advantages offered by Multiple Reaction Monitoring (MRM) of a triple-quadrupole MS for identification and quantification of each analyte. This method may not translate to reliable quantification on a single-quadrupole MS without access to MRM. Single-quad MS instruments are widespread and comparatively cheaper than triple-quad MS systems; therefore, a method specifically developed for quantification on a single-quad MS may be more widely applicable for routine vitamin analysis.

Alongside, extraction procedures employed to extract vitamins from food matrices are often time consuming, involving many steps and chemical preparations. For example, Nurit *et al*. (2015) (325), employed enzymatic hydrolysis consisting of 6 different solutions and a 14 h incubation stage to extract vitamins. Therefore, for the purpose of this thesis it was important to develop a simple analytical method suitable for high throughput analysis. Reported in this chapter is a method that combines a relatively simple extraction and combination of HILIC chromatography and single-quad MS detection that offers a practical and affordable approach to the quantification of B vitamins from food matrices (313).

3.2 Materials and Methods

Refer to general method section of thesis (chapter 2, section 2.6) for information in relation to the chemical and regents used, preparation of standards and the HILIC/MS method. Specific methods conducted for the validation of both the HILIC/MS method and extraction are detailed in this section.

3.2.1 Calibration, Precision and Quality Control Measures

Calibration curves for targeted vitamins were made for multi-vitamin standards by plotting the multi-vitamin concentration against peak area. The calibration curve for vitamins thiamine, riboflavin and pyrodoxine consisted of 7 points ranging from 5-400 ng/mL, whereas nicotinamide calibration curve comprised of 6 points from 10 ng/mL to 400 ng/mL. Calibration curves were made fresh each day of analysis and an average taken from multiple injections on separate days.

As part of quality control, a multi-vitamin standard at 50 ng/mL was prepared and injected throughout each chromatographic run and confirmed against calibration curve to assess accuracy and investigate deviation during each run. Blank mobile phase samples and blank extraction samples were also injected throughout each chromatographic run to ensure no interferences.

3.2.2 LOD and LOQ

Limit of detection (LOD) was calculated on the slope and the standard deviation of the response, based on the calibration curve according to published guidelines (350).

$$LOD = \frac{3\sigma}{S}$$

Where: $\sigma =$ the standard deviation of the y-intercept

$S =$ Calibration curve slope

Limit of quantification (LOQ) was calculated the same as LOD, but 10x the standard deviation of the y-intercept and expressed as:

$$LOQ = \frac{10\sigma}{S}$$

3.2.3 Food Sample Extraction – Specific to this Chapter

Matrix was homogenised in a blender for 2 min, approximately 40 g aliquots were taken in 50 mL centrifuge tubes, rapidly snap frozen in liquid nitrogen and stored at -80 °C. Upon analysis 40 g aliquots were removed from the freezer and defrosted at room temperature under low light conditions. Defrosted aliquots were collectively poured into a sterile stomacher bag and homogenised in a stomacher blender on ‘normal mode’ for 1 min (Stomacher®- 400 circulator,

Seward Ltd, Sussex). Aliquots, 5 g, were weighed from the blended sample and rapidly frozen in liquid nitrogen, before placing in a lyophiliser (VirTis SP Scientific, Sentry 2.0) for 24 h. For vitamin extraction, 3 mL 3% meta-phosphoric acid/ 200 mg/L DL-dithiothreitol (DTT) solution, 1 mL methanol and 0.5 mL of 18.2 MΩ deionised water was added to the dried sample. Vortexed (Vortex-Genie 2, Scientific Industries, Inc., New York, USA) using a Mo Bio 15 ml centrifuge tube vortex adapter (Qiagen Ltd, Manchester, UK) for 10 mins and centrifuged (Beckman Allegra 6R centrifuge, High Wycombe, UK) at room temperature at 4000 rpm for 15 mins. Subsequently, 1 mL of the supernatant was added to 3 mL acetonitrile, vortexed for an additional minute and centrifuged again for 15 mins at 4000 rpm. The supernatant was filtered through a 0.2 µm nylon syringe filter into amber vials and 5 µl of sample was injected onto column.

3.2.4 Preliminary Extraction Methods Trialled

As part of preliminary studies taken to provide information on the most suitable extraction protocol, removal of the lyophilisation step was investigated. All other steps in the extraction procedure detailed above (section 3.2.3) were identical; 5 g aliquots were weighed from the blended sample and the vitamin extraction protocol followed. After addition of extraction solutions, the samples were vortexed for 1 min, rather than 10 mins, as per the lyophilisation sample for reconstitution.

Additional method tested included the addition of 3 mL of a 3% meta-phosphoric acid/ 200 mg/L DL-dithiothreitol (DTT) solution to aliquots of non-lyophilised matrix, vortexed for 1 minute, followed by a 2 hr wait period at room temperature. pH was then adjusted with 2.5 M sodium acetate buffer to pH 5 and extraction procedure resumed as outlined for the non-lyophilised sample, addition of 1 mL of methanol and 0.5 mL of 18.2 MΩ deionised water, vortexed (Vortex-Genie 2, Scientific Industries, Inc., New York, USA) for 1 minute and centrifuged (Beckman Allegra 6R centrifuge, High Wycombe) at room temperature at 4000 rpm for 15 mins. Supernatant (1 mL) was added to 3 mL acetonitrile, vortexed for an additional minute and centrifuged again for 15 mins at 4000 rpm. The supernatant was filtered through a 0.2 µm nylon syringe filter into amber vials and 5 µl of sample was injected onto column.

Enzymatic hydrolysis extraction was also tested; 4 mL of HCl was added 1 g matrix aliquots, vortexed for 1 minute. Sample was then heated to 100 °C for 30 mins, after cooling pH was adjusted to pH 4.5 with 0.15 mL of 2.5 M sodium acetate buffer. Enzymes, papain (20 mg) and clara-diastase (12.5 mg) were added to the samples before incubation at 37 °C for 16 h. Subsequently, heated at 100 °C for 4 mins, and centrifuged (Beckman Allegra 6R centrifuge, High Wycombe) at room temperature at 4000 rpm for 15 mins. Supernatant (1 mL) was added to 3 mL acetonitrile, vortexed for an additional minute and centrifuged again for 15 mins at 4000 rpm. The supernatant was filtered through a 0.2 µm nylon syringe filter into amber vials and 5 µl of sample was injected onto column.

3.2.5 Recovery and Precision

Sample recovery was performed on the lyophilised sample by spiking 5 g matrix sample in quintuplicate with either 0.5 mL mobile phase B (n=5), or 0.5 mL of a multi-vitamin standard at a concentration of 2.5 µg/mL (n=5) prior to placement in the lyophiliser (VirTis SP Scientific, Sentry 2.0), followed by the extraction procedure (section 3.2.3). Blank samples were also completed (n=5); 0.5 mL of a multi-vitamin standard at a concentration of 2.5 µg/mL was added to empty centrifuge tubes prior to placement in the lyophiliser, followed by the extraction procedure (section 3.2.3). Alternatively, recovery was investigated in the non-lyophilised homogenate, spiking 5 g with a multi-vitamin standard (0.5 ml) comprising of thiamine, riboflavin, nicotinamide and pyridoxine at a concentration of 10 µg/mL, with the exception of nicotinamide at a concentration of 20 µg/ml. The multi-vitamin standard concentrations (2.5-20 µg/mL) used for sample recovery were diluted throughout both extraction protocols (lyophilised and non-lyophilised), as outlined in above section (3.2.3) with meta-phosphoric acid/DTT solution and acetonitrile. This ensured the multi-vitamin standard spiking concentrations fell inside the investigated linear range, by the final stage of extraction. However, when calculating percentage recovery in the non-lyophilised sample, average percentage moisture of the matrix (92%) was taken into consideration.

The extracted matrix sample (one extraction) was injected in quintuplicate for intra-day precision, while inter-day precision was determined by extracting and analysing 5 different matrix samples from the same batch.

3.2.6 Data Analysis

All statistical analysis completed to determine precision and reproducibility of method was calculated based on mean and standard deviation of replicates. The standard deviation was further divided by the mean of the replicates, to give the percentage coefficient of variation (CV).

3.3 Results

3.3.1 LC-MS Method Development, Optimisation and validation for Separation of Nicotinamide, Pyridoxine, Riboflavin and Thiamine

The MS behaviour of the B vitamins was first investigated as part of method development, this included studies into selecting the most appropriate MS settings for vitamin quantification. Initially, polarity modes were explored (positive versus negative polarity), in which some vitamins were observable in negative mode (e.g. B_6), however positive mode ionisation provided better intensities by approximately 10-fold and so all data was acquired in positive mode ionisation. Further investigations were completed comparing peak clarity between scan and single ion monitoring (SIM) mode. The later mode increased the area of nicotinamide, pyridoxine, riboflavin and thiamine by 7%, 18%, 298% and 243% when compared to scan mode as shown in Figure 3.1. SIM parameters used are shown in Table 3.1 and a representative chromatogram of B-vitamin standards are shown in Figure 3.2. Flow rates, gradients and mobile phase compositions were all additional method development completed to determine the most sensitive and selective method for quantification of nicotinamide, pyridoxine, riboflavin and thiamine. Data is not shown as parameters were not investigated further.

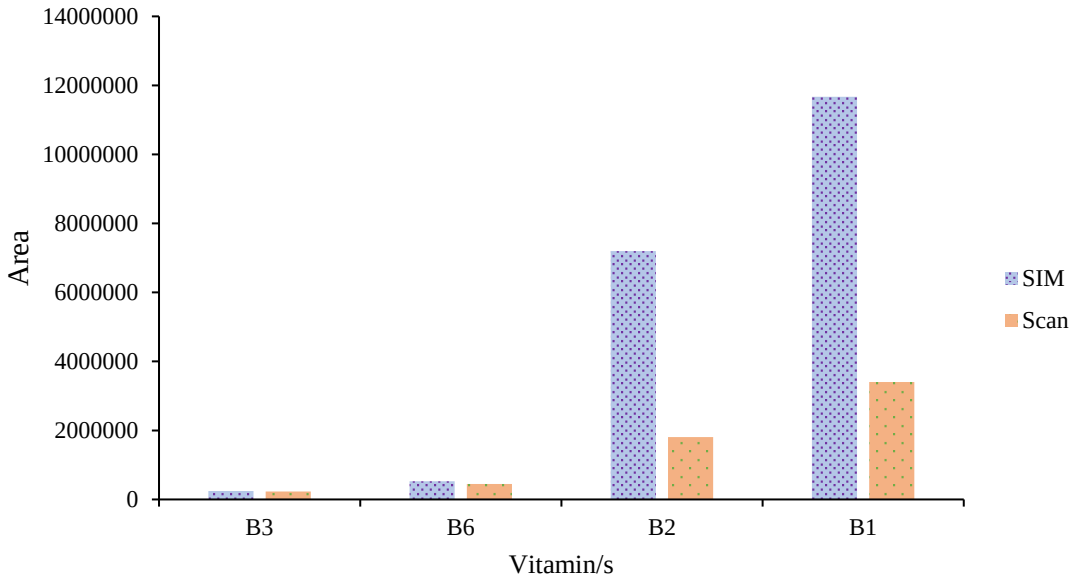
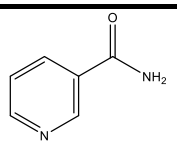
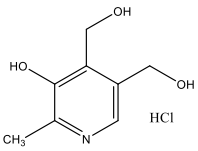
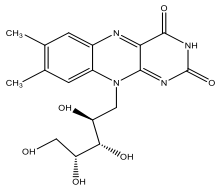
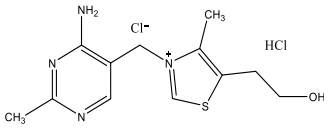


Figure 3.1: Peak areas of vitamins, nicotinamide (B_3), pyridoxine (B_6), riboflavin (B_2) and thiamine (B_1) when MS was operated in SIM and scan mode. Vitamin mix was injected onto column at a concentration of 120 µg/mL.

Table 3.1: Signal parameters set on the LC-MS for targeted water-soluble vitamins. Fragmentation (10), Dwell (590msec)

Vitamin/s	Vitamin structure*	Retention Time Window (min) – SIM Positive Mode	Precursor Ion $[M+H]^+$	Fragment ion
Nicotinamide (B_3)		0.00-0.80	122.1	80.1
Pyridoxine Hydrochloride (B_6)		0.80-1.50	170.1	152.1
Riboflavin (B_2)		1.50-3.00	377.1	122.0
Thiamine Hydrochloride (B_1)		8.70-9.2	265.1	243

*Vitamin structures based on standards purchased from Sigma-Aldrich (Gillingham, Dorset).

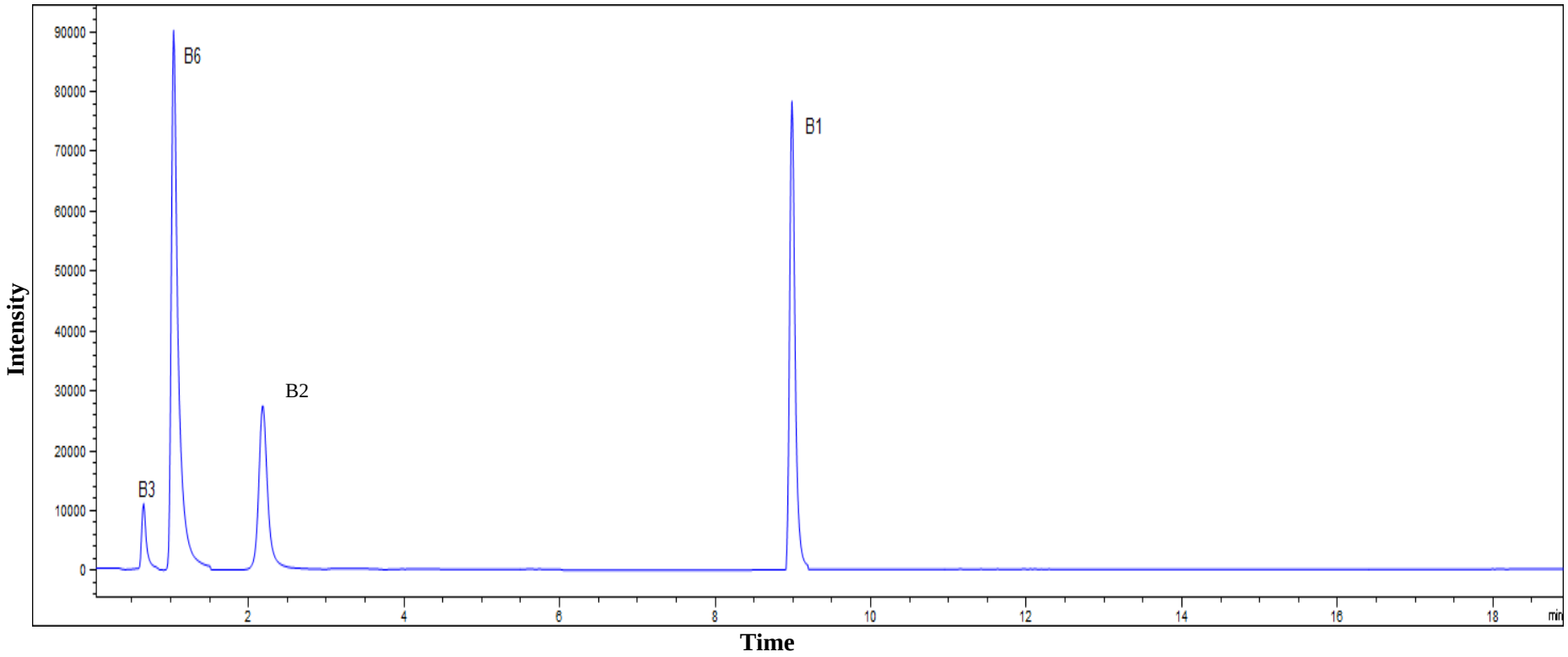


Figure 3.2: Representative chromatogram of a multi-vitamin mix (standard) at 400 ng/mL, comprising of nicotinamide (B3), pyridoxine (B6), riboflavin (B2) and thiamine (B1). MS operated in positive single ion monitoring mode (SIM) mode.

Calibration curves constructed from individual, authentic vitamin standards were used for quantification. Representative calibration curves are shown in Figure 3.3 and characteristics are provided in Table 3.2. All vitamins displayed a linear range from 5 ng/mL to 400 ng/mL which was the investigated range for this study, with coefficient of determination (r^2) values >0.98. In addition to r^2, the relative standard deviation of the slopes were calculated as proposed by Raposo (2016) (351). In this study, thiamine, riboflavin, nicotinamide and pyridoxine achieved intra-day calibration $\%RSD_{slope}$ of 1.7%, 1.5%, 2.9% and 2.9% respectively. Limit of detection (LOD) and limit of quantification (LOQ) data are also shown in Figure 3.2 and varied from 2.4-9.0 ng/mL and 8-30 ng/mL respectively across all vitamins.

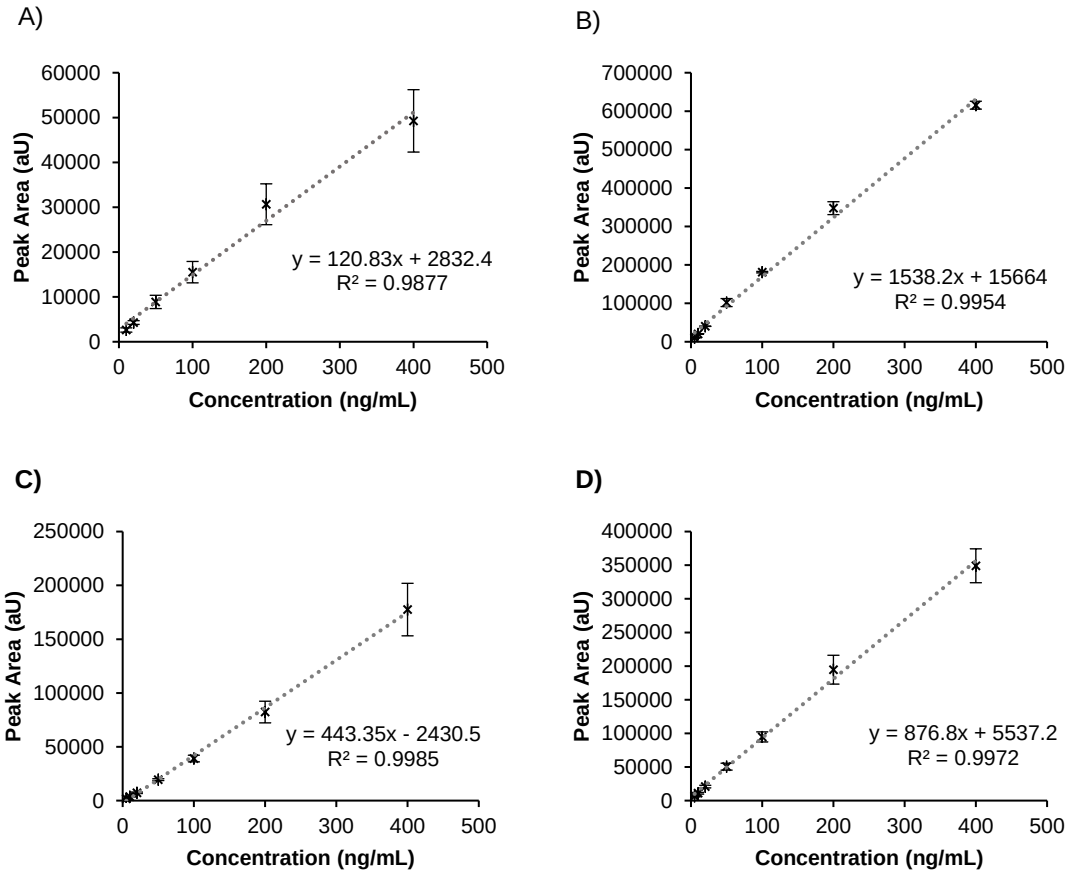


Figure 3.3: Calibration curves of selected water-soluble vitamins; A) nicotinamide, B) pyridoxine, C) riboflavin and D) thiamine. The calibration curves display the mean slopes and intercepts of multiple injections including the associated error for each data point expressed by a 95% confidence interval.

Table 3.2: Standard curve characteristics of selected water-soluble vitamins

Vitamin/s	RT (min)	±SD	Slope	Slope CV (%)	Intercept	r^2	Linear range* (ng/mL)	Retention factor (k')	Selectivity factor (α)	LOD (ng/mL)	LOQ (ng/mL)
Nicotinamide	0.651	0.003	120.858	20.8	2824	0.985	10 - 400	1.01	2.22	9.0	30.1
Pyridoxine	1.050	0.014	1494.665	4.1	34165	0.983	5 - 400	2.25	2.65	5.4	18
Riboflavin	2.253	0.044	443.186	19.4	-2383	0.994	5 - 400	5.97	4.52	2.4	8
Thiamine	9.042	0.047	876.331	11.1	5672	0.994	5 - 400	26.98	4.52	6.5	21.7

MS operated in positive single ion monitoring (SIM) mode, consisting of an average of 6-7 concentrations (6 concentrations of nicotinamide and 7 concentrations of pyridoxine, riboflavin, thiamine) injected in quadruplicate, or duplicate across multiple days.
*Linearity was based on investigated range to determine vitamins naturally occurring in the food matrix investigated in this study.

A standard injection was used throughout each analytical run as part of quality control measures, Table 3.3 displays precision and reproducibility of the injection standard (multi-vitamin standard at 50 ng/mL) based off calibration curves in a run. Reproducibility of injection standard ranged from 0.9-3.4% across all B-group vitamins.

Table 3.3: Standard injection precision data

Vitamin/s	Multi-vitamin standard quantity (ng/mL)	Average quantity based off calibration curve (ng/mL)*	CV (%)
Nicotinamide	50	47	0.9
Pyridoxine	50	47	3.4
Riboflavin	50	48	1.3
Thiamine	50	47	3.3

*Average based off multiple injections of multi-vitamin standard in a run (n= 4 pyridoxine, riboflavin, thiamine and n= 3 nicotinamide)

3.3.2 Extraction Method for Analysis of B-Group Vitamins from a Vegetable Matrix and Extraction Precision (Intra-Day and Inter-Day)

After optimising the LC-MS behaviour of the vitamins, it was important to investigate the extraction of water-soluble vitamins from a typical food matrix, this was completed in a commercially available fresh vegetable matrix. Several extraction methodologies were tested initially, this included treatment of the sample with HCl coupled with high temperatures, followed by enzymatic hydrolysis. However, this was ineffective to extract all vitamins of interest at the same time. The strongly acidic extraction method, incorporating meta-phosphoric acid and DL-dithiothreitol (DTT) was the most effective extraction method (validated method in this chapter). However, as part of preliminary studies alteration of the strong acidic extraction were tested, for example: quantity of sample extracted was explored (1-5 g), the lyophilisation step was removed, and a 2 hr wait at room temperature after the addition of meta-phosphoric acid was investigated. The latter alteration resulted in issues with thiamine quantification, upon LC-MS analysis thiamine was undetected when investigating this extraction method. Vitamin C was obtained with the 2 hr wait in the extraction method, due to this chromatography method also being suitable for vitamin C analysis, but upon further investigation into reproducibility of the extraction method, vitamin C was not found to be reproducible. Removal of the lyophilisation step also resulted in issues with thiamine quantification and through investigations into the quantity of sample extracted, the maximum quantity 5 g was effective for thiamine quantification with the addition of the lyophilisation step, which is the extraction method developed and validated in this chapter. A typical LC-MS chromatogram of the vitamins extracted from food is shown in Figure 3.4. Additionally, a chromatogram of extracted vitamins from food, overlaid with a spiked food sample is shown in Figure 3.5. To verify the vitamin peaks, the fragmentation pattern of each vitamin against a pure standard were analysed and an example can be seen in Figure 3.5.

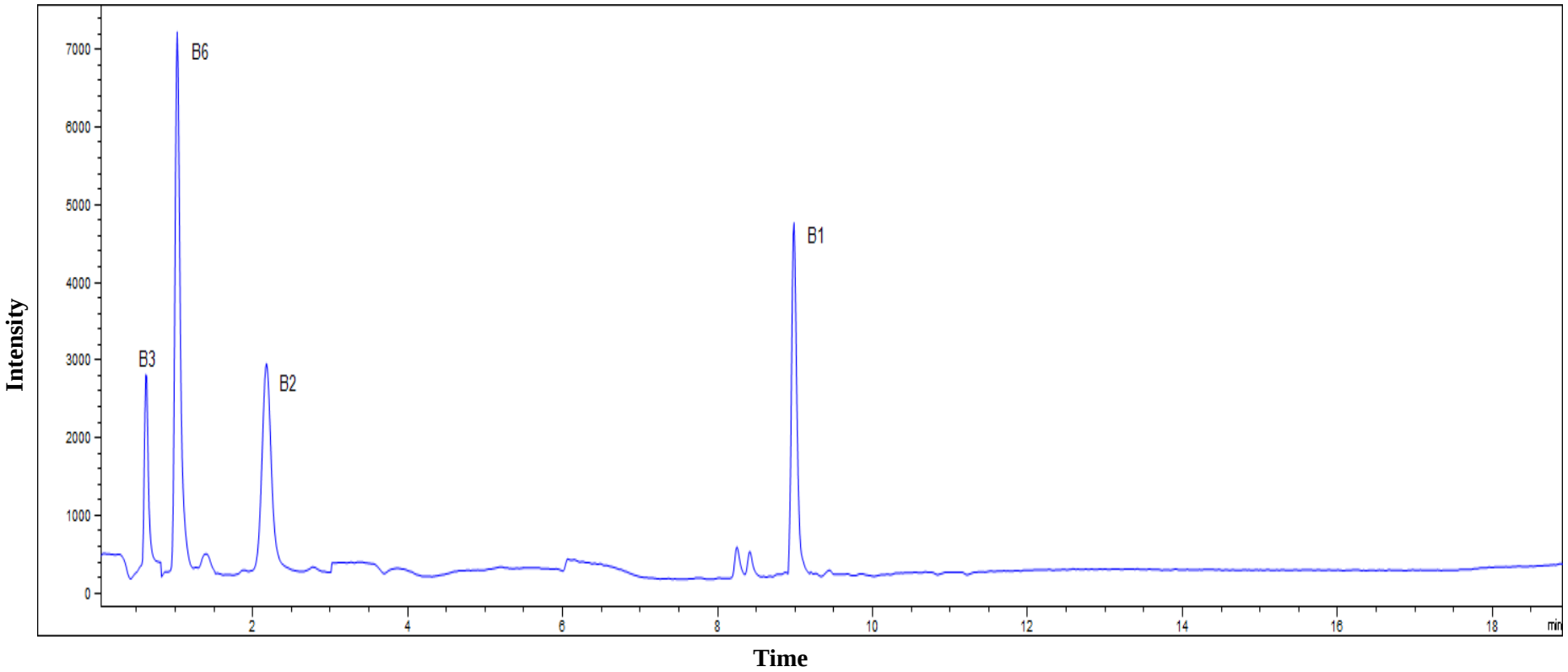


Figure 3.4: Representative chromatogram of targeted vitamins; nicotinamide (B3), pyridoxine (B6), riboflavin (B2) and thiamine (B1) in a commercial vegetable matrix sample. MS operated in positive single ion monitoring mode (SIM) mode.

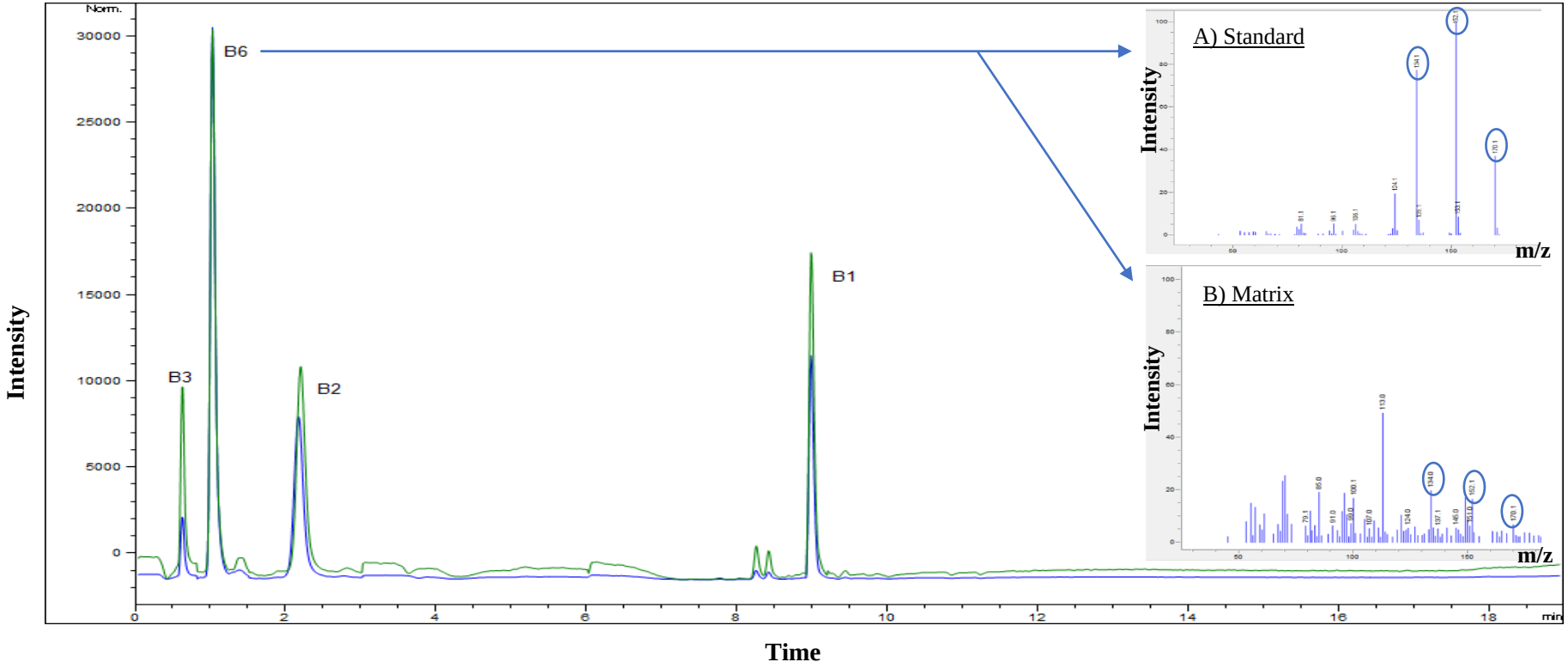


Figure 3.5: Representative chromatogram of targeted vitamins; nicotinamide (B3), pyridoxine (B6), riboflavin (B2) and thiamine (B1) in commercial vegetable matrix (blue), overlaid with a spiked matrix sample (green). MS operated in positive single ion monitoring mode (SIM) mode. The fragmentation pattern of a B6 standard (A) against B6 fragmentation pattern in matrix (B) is shown as an example. Circled are the fragments of interest with MS operated in positive scan mode, and fragmentation energy set to 150.

The reproducibility of the extraction methodology was studied, and results can be viewed in Table 3.4. The intra-day ranged from 1.56-6.56% and variation for inter-day was between 8.07-10.97%. Reproducibility of the extraction method without the lyophilisation step was also performed (Table 3.5), extracting five samples over a period of three days (non-consecutive days), %CV ranged from 7.95-12.18% for all matrix samples extracted.

Table 3.4: Extraction precision data

Vitamin/s	CV (%) (intra-day)	CV (%) (inter-day)
Nicotinamide	6.56	8.96
Pyridoxine	4.46	8.07
Riboflavin	1.56	9.19
Thiamine	5.89	10.97

For intra-day precision the average was taken by quintuplicate analysis of one extracted matrix sample. Inter-day precision was determined by the average of extracting and injecting 5 matrix samples.

Table 3.5: Reproducibility data of the extraction method without the lyophilisation step

Vitamin/s	Day 1 CV (%)	Day 2 CV (%)	Day 3 CV (%)	Overall CV (%) across extraction days
Nicotinamide	6.05	6.43	6.84	7.95
Pyridoxine	8.55	8.08	14.24	12.18
Riboflavin	11.99	7.25	13.33	10.64
Thiamine*	N/A	N/A	N/A	N/A

Reproducibility was determined by extracting vegetable matrix samples in quintuplicate per day for a total of three days, with the exception of day 2 which contained 4 extracted samples (n=14).
*Thiamine below detection limit

3.3.3 Recovery Studies and Quantification of B-Vitamins in Vegetable Matrix

The recovery of each vitamin using the developed methodology was assessed, by spiking the vegetable matrix with a known concentration of vitamin mix (Table 3.6). The percentage recovery of riboflavin and pyridoxine were desirable, between 96-118%, but nicotinamide and thiamine experienced lower recovery (30-50%). Alternatively, we also investigated % recovery when the matrix samples were not lyophilised prior to extraction, as part of the separate preliminary investigation. Nicotinamide, pyridoxine and riboflavin obtained mean % recoveries of 76%, 94% and 104% respectively when the lyophilisation step was removed. However, thiamine % recovery was unable to be calculated, due to issues experienced with quantification of this B-vitamin when this extraction method is employed, all data is included in Table 3.6. Supplementary data comparing % recovery from spiked matrix samples to spiked blank samples for matrix effect can be seen in Appendix 1.

Following extraction and analysis by this method, the quantity of nicotinamide, pyridoxine, riboflavin and thiamine present in the matrix can be seen in Table 3.7 and compared to the stated

quantities in McCance & Widdowson's (2002) food composition table for a vegetable matrix (352).

Table 3.6: Recovery from matrix, including comparison between lyophilised and non-lyophilised matrix samples

Vitamin/s	Recovery from vegetable matrix lyophilised sample			Recovery from vegetable matrix non-lyophilised sample		
	Spiked with*	Mean % Recovery	CV (%)	Spiked with**	Mean % Recovery	CV (%)
Nicotinamide	2.5 µg/mL	30	9.8	20 µg/mL	76	3.3
Pyridoxine	2.5 µg/mL	118	1.35	10 µg/mL	94	7.1
Riboflavin	2.5 µg/mL	96	4.55	10 µg/mL	104	6.9
Thiamine* **	2.5 µg/mL	50	3.11	10 µg/mL	-	-

*5 replicates spiked with 0.5 mL of a multi vitamin standard at 2.5 µg/mL, therefore 1.25 µg was added to a 5 g matrix sample.
**3 replicates spiked with 0.5 mL of a multi vitamin standard.
***Mean % recovery of thiamine in the non-lyophilised sample was unable to be calculated, as a result of thiamine failing below the detection limit in the non-spiked sample. Therefore, the native quantity in the matrix could not be determined and accounted for without the lyophilisation step.

Table 3.7: Determined quantity of nicotinamide, pyridoxine, riboflavin and thiamine in vegetable matrix, compared to quantities stated in McCance & Widdowson's (2002) (352) food composition table for vegetable matrix.

Vitamin/s	Quantity (ng/g)*	±SD	McCance & Widdowson's
Nicotinamide	856	77	N/A**
Pyridoxine	133	11	100
Riboflavin	423	39	200
Thiamine	67	7	900

*Based off extracting and analysing samples in quintuplicate
**Nicotinamide cannot be compared as only the niacin content (nicotinamide + nicotinic acid) was reported

3.4 Discussion

3.4.1 LC-MS Method Optimisation and Validation

This study focused on the quantification of selective water-soluble, B vitamins using HILIC chromatography, as our research group have shown (319), but with the added sensitivity and selectivity of MS detection. HILIC offers the advantage of targeting more polar, hydrophilic vitamins that otherwise would be difficult to target using RP and includes B vitamins thiamine and nicotinamide (220, 342). HILIC chromatography achieves separation of polar analytes/strong hydrophilic complexes by partitioning these between the mobile phase and the stationary phase enriched with a water layer (353, 354). Although, this is the commonly accepted mechanism other interactions are potentially taking place alongside and include surface adsorption by hydrogen bonding, dipole-dipole interactions and electrostatic interactions (345, 348, 353). Operating in positive mode ionisation for those vitamins that were observed in negative mode provided peaks of greater intensity. Leporati *et al*. (2005) (323), reported similar findings and found operating in positive mode resulted in greater intensities compared to negative mode. The increased intensity

in positive mode reported by Leporati *et al*. (2005) (323) was higher than the 10-fold increase observed in this chapter, but many factors including the selected B vitamin analysed, the type of MS, and the MS operating conditions could play a role in the stated differences. Additionally, utilisation of SIM mode was explored, this offered greater improvements over scan mode including reduced background noise and improving peak shape for each of the target analytes, increasing both precision and accuracy. This is due to SIM being more advantageous when targeting known analytes, due to monitoring a single mass-to-charge ratio during a set time window, rather than scanning a large mass range (50-500 m/z) multiple times per second (355).

For LC-MS method validation, calibration curves for each selected B-group vitamin were investigated in regard to linear range, r^2, retention time reproducibility throughout run and both LOD and LOQ were determined. The %RSDslope was an additional variable calculated to confirm linearity of linear regression. According to Raposo (2016) (351), the %RSDslope should be between 1-2%, with other guidelines suggesting a max of 8% when using MS detection. The %RSDslope results determined are in accordance with these values, with nicotinamide and pyridoxine having the highest %RSDslope of 2.9%. Additionally, the calculated LOD and LOQ based on the slope of the calibration curve and standard deviation of the response are comparable to other publications utilising ESI-MS detection. In this method riboflavin has a quantification limit of 8 ng/mL, comparable to the 2 ng/mL (323) to 60 ng/mL (356) of previously reported methods. Similarly, the observed limits of detection and quantification for pyridoxine were within previously reported values (323, 356). The LOD of nicotinamide was 9.0 ng/mL, comparable to the reported 8 ng/mL (357), but slightly higher than that reported by Leporati *et al.* (2005) (323) of 5 ng/g. Thiamine detection and quantification limit of 6.5 and 21.7 ng/mL respectively is higher than published 3 and 7 ng/mL (357), but comparable to the values described by Lebiedzińska *et al*. (2007) (321) of 9.2 ng/mL (LOD) and 22.0 ng/mL (LOQ) using HPLC-ED UV. However, when compared to similar previous work employing UV, fluorescence and coularray detection (319), all B group vitamins in this current study have lower LOD and LOQ limits. For example, thiamine, riboflavin, nicotinamide and pyridoxine LOQ were lower using MS detection by 91%, 52%, 68% and 94% respectively when compared to UV and fluorescence detection. The LOD of investigated vitamins was improved by 92% for thiamine; 69% for riboflavin; 50% for nicotinamide; and 94% for pyridoxine. These data further highlight that incorporation of MS provides greater sensitivity and is suitable for applications where the vitamins are present in trace amounts in a complex 'dirty' matrix, such as food.

An internal standard was not utilised in this study. An effective internal standard should be structurally similar to each of the targeted analytes, absent from the complex food matrix, while having almost identical chromatographic behaviour (325). Therefore, an internal standard was not feasible due to targeting several structurally diverse B vitamins simultaneously in a complex food matrix. Other previously published methods have omitted the use of an internal standard, in

support of external calibration standards or standard addition techniques (318, 323, 347, 349). Stable isotope versions of the B vitamins are the most adequate internal standards when using MS detection (325, 341, 344), but due to significant cost implications they are not always utilised. Alternatively, a standard injection was used to confirm any injection/sensitivity deviation and confirm precision of calibration curve. The injection standard showed excellent reproducibility (<4%) over the course of the run.

3.4.2 Analysis and Validation of Water-Soluble Vitamins in a Vegetable Matrix

Once the LC-MS method was validated, extraction methodologies were investigated to analysis water-soluble vitamins from a vegetable matrix applying the validated HILIC-MS method. The most common extraction processes for B group vitamins can be complex, utilising weak acid treatment, coupled with high temperatures (0.1 M HCl at 100 °C-121 °C), further followed by enzymatic hydrolysis, with the objective of extracting bound vitamins (318, 319, 334, 358, 359). This type of extraction was tested initially but was ineffective to extract all vitamins of interest at the same time. To simplify, a strongly acidic environment was incorporated in order to hydrolyse covalent bonds and release the free vitamins, complemented by inclusion of a reducing agent to generate the free vitamins in their native form (318, 360). Nicotinamide, pyridoxine, riboflavin and thiamine were successfully extracted with this method and vitamin peaks were clearly identified with no contamination that would influence quantitation, facilitated through SIM mode utilisation. However, it is important to note food matrices are complex and other extracted components may co-elute in the MS source, potentially interfering with the ionization of the analytes (361, 362).

Furthermore, the reproducibility of the developed extraction method was confirmed by intra-day and inter-day precision. Precision values obtained were very good, and all vitamins displayed variation <7% for intra-day and variation <11% for inter-day. The precision values of the presented method were evaluated against other published methodologies, although these values will be dependent on the food matrices investigated. Gratacós-Cubarsí *et al*. (2010) (334) reported an extraction method for thiamine from dry-cured sausages, and demonstrated good intra-day repeatability of <7.0% and inter-day variability of <7.8%. Nurit *et al*. (2015) (325) method showed intra-day precision values of between 3.2-9.3% and inter-day CV's ranging from 6.2-13.8% across thiamine, riboflavin, nicotinamide and pyridoxine extracted from different wheat flour products. The reproducibility of other published extraction methods are similar to the assessed reproducibility reported here.

Recovery studies performed highlighted nicotinamide and thiamine obtained a lower recovery. However, recovery of nicotinamide is known to be problematic as recoveries in the region of 29-32% have been reported (318), and recovery appears to be dependent on the food matrix investigated. Nicotinamide in foods will coexist with nicotinic acid and are collectively referred

to as niacin. For example, based on niacin profiles of vegetables, onions comprise of 72% nicotinamide and 28% nicotinic acid, whereas potatoes contain 13% nicotinamide and 87% nicotinic acid (244). Furthermore, upon acid extraction some of the nicotinamide would have been converted to the acid form (364). However, the conversion of forms has not been accounted for in this paper, as nicotinic acid was not detected on the MS. To assess any influence of the food matrix, we performed recovery experiments both from a matrix sample or from a blank sample and results indicated minimal differences in recovery. For example, riboflavin recovery from matrix was 96%, while from blank 84% (data in appendix 1), indicating there was limited interference from the food matrix in our experiments and therefore, a matrix effect was not investigated further. Interestingly, omission of the lyophilisation step as part of preliminary studies investigating the extraction method resulted in an increase in nicotinamide recovery to 76%. Freeze-drying has been shown in previous studies to cause loss of nicotinamide forms in plant tissue, similar to the findings in this report and potentially contributed to the observed low recovery of nicotinamide (361). However, removal of the lyophilisation step, simultaneously resulted in thiamine falling below the detection limit in matrix samples (non-spiked). Therefore, lyophilisation was required as a concentration step to analyse all vitamins.

The quantities of B-group vitamins determined in the vegetable matrix were as follows; thiamine 67 ± 7 ng/g, riboflavin 423 ± 39 ng/g, nicotinamide 856 ± 77 ng/g and pyridoxine 133 ± 11 ng/g. Santos *et al.* (2012) (220) published a paper determining B vitamin content in vegetables where they observed a higher abundance of nicotinamide, when compared to riboflavin and thiamine. Food labelling or nutritional databases contain no vitamin content information for commercially available matrix, and popular nutritional databases including Nutritics™ (Nutritics, 5.098 research edition, Dublin Ireland) or MyFitnessPal™ (MyFitnessPal, Inc) also have no firm estimates. However, the vitamin content presented can be compared to McCance & Widdowson's (2002) (352) food composition tables for vegetable matrix; thiamine 900 ng/g; riboflavin 200 ng/g; and pyridoxine 100 ng/g. The content of nicotinamide cannot be compared, as the value published was based on total niacin content. The quantity of pyridoxine was comparable, whereas the detected riboflavin was greater than reported, while thiamine content was underrepresented in the current study, potentially due to the vegetable composition of the studied matrix. The analytical methods utilised for vitamin determination by McCance & Widdowson's (2002) (352), comprised of HPLC and microbiological assays with the latter technique lacking efficiency and accuracy. It has also been previously suggested, that microbial assays as a quantification tool for water-soluble vitamins may overestimate true vitamin content (365).

3.5 Conclusion

In this chapter, an effective and sensitive method to extract and quantify several B vitamins from a vegetable matrix has been developed. HILIC chromatography coupled with MS detection was

applied for quantification of thiamine, riboflavin, nicotinamide and pyridoxine; these B-group vitamins were isolated from a commercially available matrix utilising an acid extraction. The simple extraction method resulted in comparable performance to that of other more complex methods which require further processing steps and techniques. In terms of quantification, employing a single-quad LC-MS system provided good sensitivity, with low detection limits varying from 2.4-9.0 ng/mL and quantification limits between 8-30 ng/mL. Linearity ranged from 5-400 ng/mL and was successfully used to determine vitamin content in this work. Four targeted B vitamins were reliably quantified within a total run time of 19 mins, with a method applicable for a single-quad MS. Overall, the method developed has shown to be applicable to procedures that require high throughput and good sensitivity, with the opportunity to monitor nutritional content in foods.

Chapter 4

Characterisation of Food Spoilage and Nutritional Composition During Time-Dependent Storage of a Vegetable Matrix

4.1 Introduction

Nicotinamide, pyridoxine, riboflavin and thiamine, are B-group vitamins liable to degradation during both food processing and food storage (225, 259, 264). Owing to the importance of these vitamins in the diet, it is important the nutritional composition of foods are evaluated alongside other quality markers during storage assessments. Furthermore, the extent of any interactions between food spoilage organisms and B-vitamins in many food matrices is currently unknown. For example, studies have been conducted investigating the impact of desirable bacterial fermentation, by species such as lactic acid bacteria (LAB) on B-vitamin content in traditionally fermented food/beverages (366-370). The quantity of microorganisms present in a vegetable matrix is an established factor of microbial spoilage, however the diversity of the microbial community also plays a key role (296). The reported spoilage microflora of vegetables is predominately made up of the following: *Pseudomonadaceae*, *Enterobacteriaceae*, LAB and yeasts (including *Candida*, *Rhodotorula* and *Pichia*). Although moulds play an important role in spoilage, the intrinsic environment of vegetables favour bacteria and yeast, outcompeting the potential growth of moulds (294, 296, 371-373). Such spoilage organisms produce hydrolytic enzymes breaking down and metabolising food macronutrients, resulting in textural alterations, visual defects and loss of nutritional value. Moreover, exponential growth of specific spoilage organisms (SSOs) leads to the production of secondary metabolites, such as volatile organic compounds (VOCs) associated with off-odours and off-flavours (10, 28, 32, 150). For example, food-fermenting LAB utilise available sugars in a matrix, producing fermentation by-products, chiefly lactic acid. The by-products produced are dependent on the species of LAB, classified as being either obligate homofermentative, obligate heterofermentative or facultative heterofermentative. Homofermentative produce solely lactic acid, while heterofermentative produce a variety of metabolites alongside lactic acid which include acetic acid, CO_2 and ethyl alcohols (54, 296, 374). Alternatively, facultative heterofermentative can undergo both fermentation pathways.

Amplicon sequencing examines the microbiota of foods, enabling a more comprehensive analysis of the microbial community and its environment over traditional culturing methods that solely depend on cultivatable bacteria (203). Metabolomics on the other hand is described as the global study of metabolites in a biological system (375). Metabolomics can also be utilised to analyse nutritional composition and therefore can be used to identify metabolic markers related to nutritional quality. Furthermore, as metabolomics reflects the impact of biochemical reactions in

a system, many of which are the end-products of cellular processes, it can provide valuable information on the biological status of a system (375, 376). Regarding an application in food spoilage studies, metabolomics has been utilised to identify spoilage markers and measure product freshness in perishable products including chilled chicken (377), chilled lamb (378), mussels (193), salmon (192) and chicken eggs (379). However, there are limited studies outlining spoilage in vegetable matrices. Additionally, the combination of metabolomics with amplicon sequencing in aid of identifying relationships between SSOs and metabolites are also scarce. The most recent study conducted by Zhang *et al.* (2021) (190) investigated the relationships between the microbiota and metabolites during spoilage of chilled chicken, further highlighting the importance of performing both amplicon sequencing and metabolomic analysis simultaneously.

The aim of this chapter is to profile spoilage of a popular ready-to-eat vegetable matrix during a time-dependent storage experiment, identifying potential relationships between microbial growth, nutritional composition and metabolites. Objectives of the chapter are to, 1) evaluate microbial growth, diversity and development during storage, 2) profile nutritional constituents specifically B vitamins, carbohydrates and protein over the storage period, building on validated method previously discussed in chapter 3 and 3) assess metabolites including volatile organic acids (VOCs) throughout the storage period.

4.2 Methods

4.2.1 Vegetable Matrix Preparation and Storage

Refer to general method sections of thesis (Chapter 2) for detail on the preparation and storage procedure of the vegetable matrix, see sections 2.1 and 2.2. An overview of the storage method can be seen in Figure 4.1.

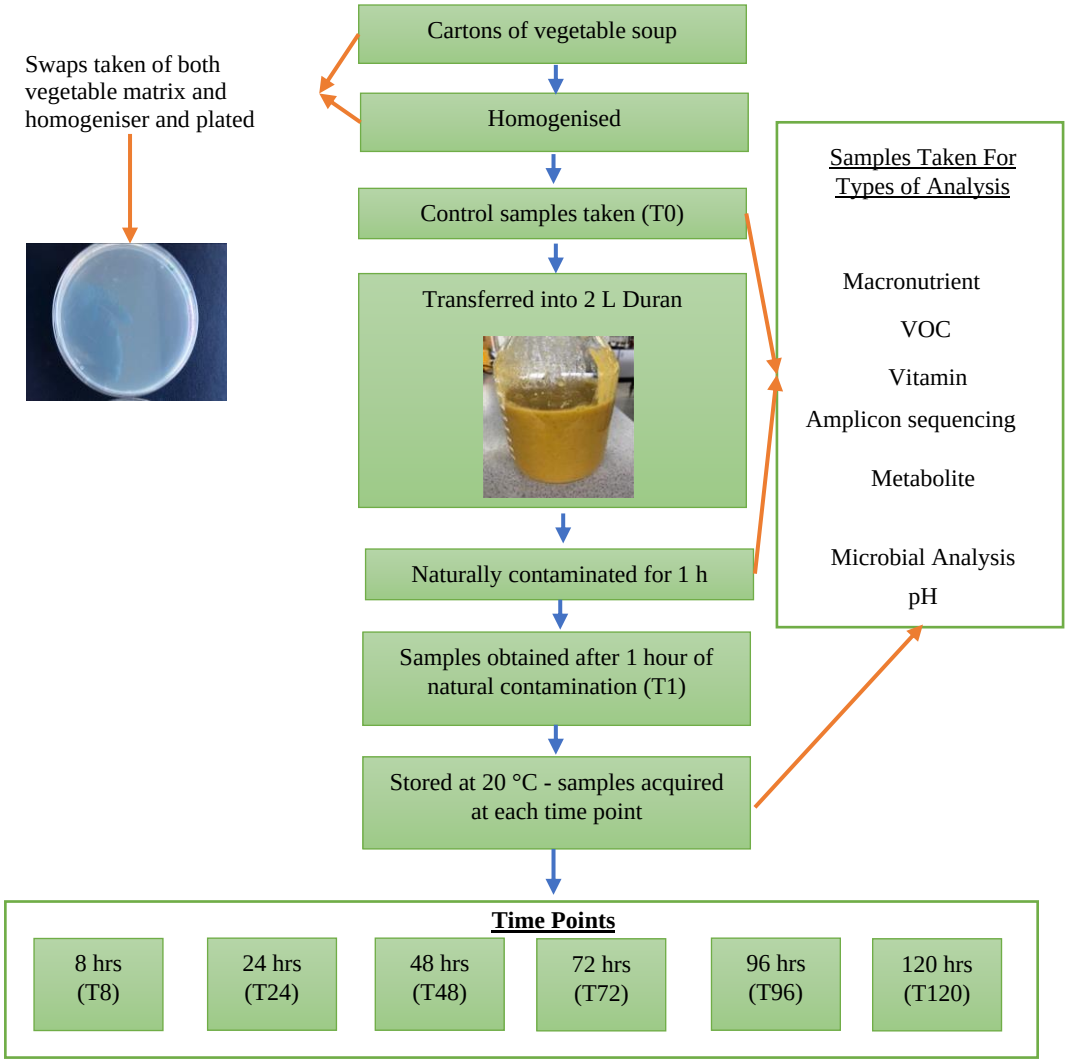


Figure 4.1: Flow diagram of the preparation steps and storage procedure of the vegetable matrix including the collection of samples and analysis conducted on specific time points.

4.2.2 Microbial Communities Across Batch's of the Vegetable Matrix

To understand the impact of batch variability on bacterial diversity of the vegetable matrix, 10 different soup batches were purchased across a 13-month period and prepared as stated in chapter 2, section 2.1. Samples were obtained for amplicon sequencing after the homogenisation step (T0), which can be seen in Figure 4.1. Additionally, a batch from frozen was analysed as this is an alternative storage practice typically employed by consumers, with the aim of preserving the freshness of the product for a longer duration. For this, a batch was immediately frozen after

purchase for several months, defrosted the day prior to preparation. These prepared batches were then further used for storage investigations in the following chapters (chapter 5 and 6).

4.2.3 Storage of Vitamins as Part of a Control in a Buffered Model System

To evaluate the impact of the storage environment on vitamins, a control study was performed, in which vitamins were stored in a buffered model system in absence of the vegetable matrix. For this, 1 L of water containing 10 mM ammonium acetate was prepared and pH adjusted to pH 5.5 ± 0.05 with acetic acid to replicate pH of the vegetable matrix and further autoclaved at 121 °C for 15 minutes. After sterilisation, 700 mL was measured into a 1 L Duran (half the quantity of vegetable soup stored) spiked with nicotinamide, pyridoxine, riboflavin, and thiamine at a final concentration of 400 ng/mL, 60 ng/mL, 100 ng/mL and 70 ng/mL respectively. All vitamins were filter sterilised prior to addition to the buffered water, all equipment used were also sterilised or UV'd and aseptic technique was used throughout. Experiment was completed in triplicate (biological replicates).

After preparation of the vitamins in the buffered water system, each Duran was mixed in an orbital incubator (Infors HT Multitron Standard, Surrey, UK) at 20 °C, 170 rpm for 2 minutes. A 20 mL sample was taken (T0) after and placed on ice. Subsequently, Duran's were stored at 20 °C in a static incubator (LMS, Kent, UK) in the dark for a period of 5 days collecting samples after 48 h (T48) and 120 h (T120). For each sampling day, 20 mL of sample was collected after Duran's were mixed in an orbital incubator using the settings stated above. From the 20 mL sample collected, three aliquots of 5 mL were taken in fresh Falcon tubes, snap frozen in liquid nitrogen and kept at -80 °C until required for vitamin analysis. An aliquot was also taken to measure pH stability throughout the time-course.

4.2.4 Sample Analysis and Data Analysis

Methodologies on all the analysis completed during the time-course, including nutrient analysis, pH measurements, microbial analysis, VOC analysis, amplicon sequencing, metabolomics and preliminary protocols associated with method development of specific analysis are outlined in the general methods, chapter 2 sections 2.6-2.12. Data analysis performed is also outlined in the stated sections.

Statistical analysis conducted to compare microbial diversity (alpha diversity) across different batches of the vegetable matrix differ from the statistical tests detailed in the general methods for amplicon sequencing. To compare alpha diversity of the 10 different vegetable matrix batches, Kruskal-Wallis H tests were applied and with subsequent post hoc analysis using Dunn's test with Bonferroni corrections, if Kruskal-Wallis H test was significant ($p < 0.05$). Alternatively, to compare alpha diversity between fresh and frozen vegetable matrix batches, Mann-Whitney U tests were performed.

4.3 Results

4.3.1 Microbial Growth

Microbial growth during the 5-day (120 h) storage period of the vegetable matrix was investigated using culture dependent methods. The total viable count (TVC) and targeted yeast and moulds were assessed as a generic approach to quantify all microbial communities related to spoilage, using a pour plate technique. The representative growth during the time course, expressed as log CFU/g, can be seen in Figure 4.2 and image capturing growth of microorganisms on plate count agar can be viewed in Figure 4.3. Regarding TVC, during the first day of storage (0 – 8 h) no colonies were observed, growth subsequently increased at 24 h to 4.81 log CFU/g and by 48 h growth increased by 72.97% to 8.32 log CFU/g. After 48 h of storage, the TVC began to decrease, culminating at 120 h at 5.61 log CFU/g, a 32.57% decrease in growth from 48 h. The captured TVC growth profile was the same for targeted yeasts and moulds, due to bacteria also growing on the agar specific for yeasts and moulds (malt agar). Furthermore, a separate preliminary investigation was undertaken, assessing a different plating method, Miles and Misra, results of which can be seen in Appendix 5. The Miles and Misra technique provided the advantage of being able to assess up to 6 dilutions on one plate. Although results were comparable to the results from the pour plate technique, challenges were faced as colonies merged, therefore issues were experienced with counting.

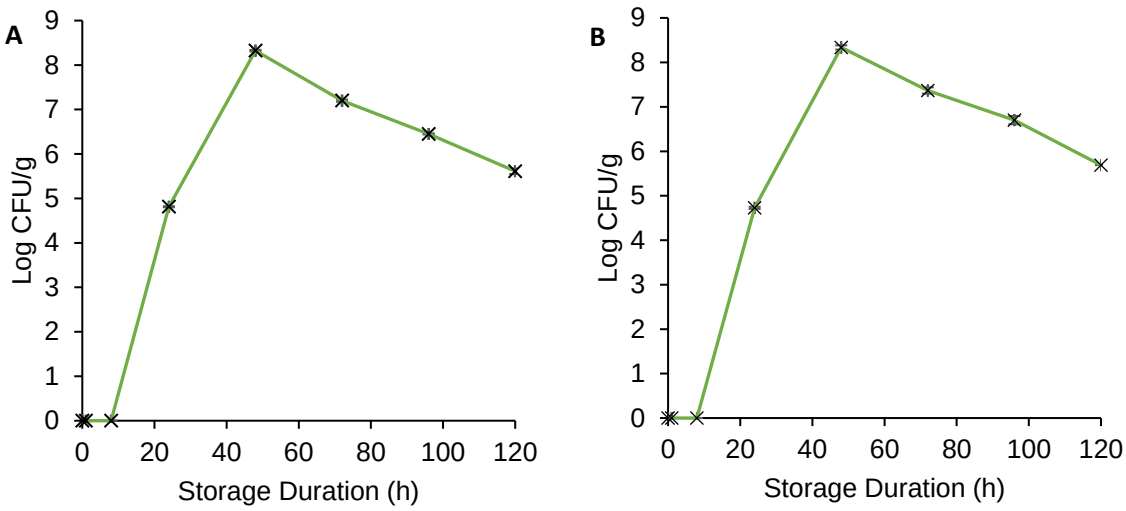


Figure 4.2: Profile of the TVC on plate count agar (A) and yeast & moulds on malt extract agar (B) over the storage duration of the vegetable matrix. For each data point, two replicates were analysed, and the error associated with each time point is represented by a 95% confidence interval.

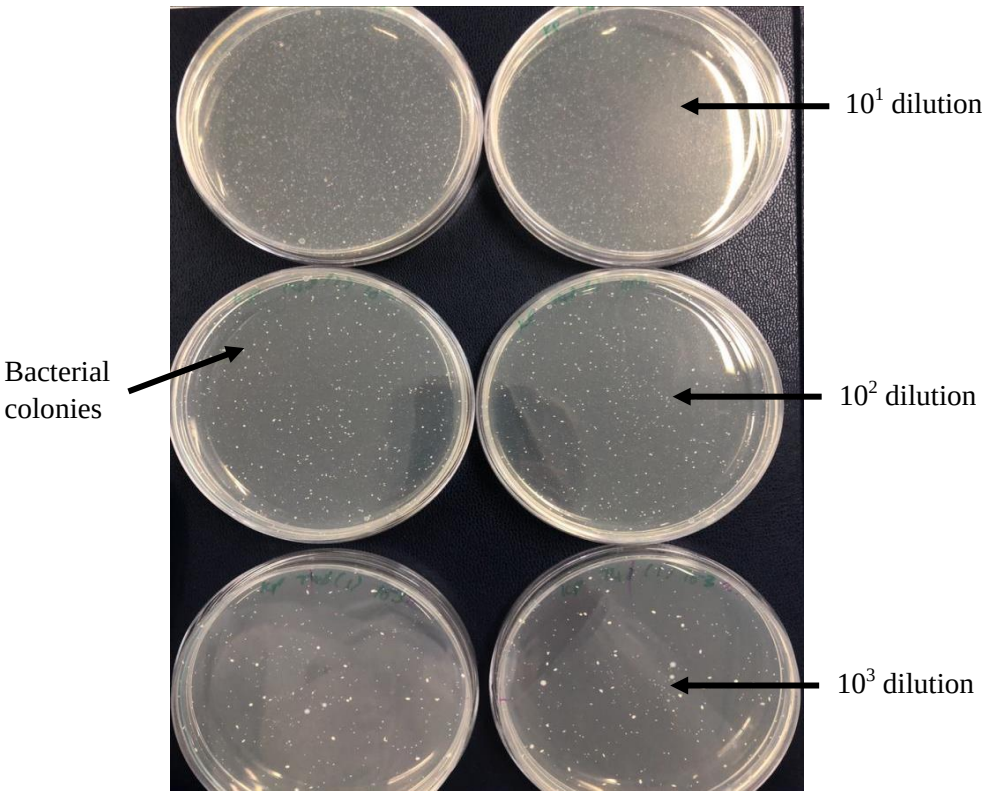


Figure 4.3: Image capturing an example of a 10-fold serial dilution of microorganisms on plate count agar, after incubation for 72 h at 30 °C from a vegetable matrix sample.

4.3.2 Bacterial Diversity and Composition – Alpha Diversity

DNA extracted from samples generated 1,640,504 raw sequence reads from amplicon sequencing of the 16S RNA gene. After extensive quality screening using the Mothur pipeline, 1,340,659 reads remained for analysis, alongside 3,980 bacterial taxa obtained and phylogenetically classified at genus level.

The bacterial composition was evaluated by assessment of rarefaction curves to determine species richness, further supplemented with Shannon and Simpson diversity indices. There was an observed decrease in species richness, based on observed operational taxonomic units (OTUs) from 0 h to 120 h of storage which can be viewed in Figure 4.4, although this decrease was not significant. Complementary, diversity metrics Shannon and Simpson index were used (Figure 4.4), Shannon index measures biodiversity in terms of both richness and evenness. Simpson index although similar to Shannon index, measures dominance therefore attributing more weight to common species, as a result the inverse version of the index was used, converting dominance to an evenness measure (380-382). Both metrics indicated a significant reduction in diversity over the time course, statistical data is available in Appendix 6.

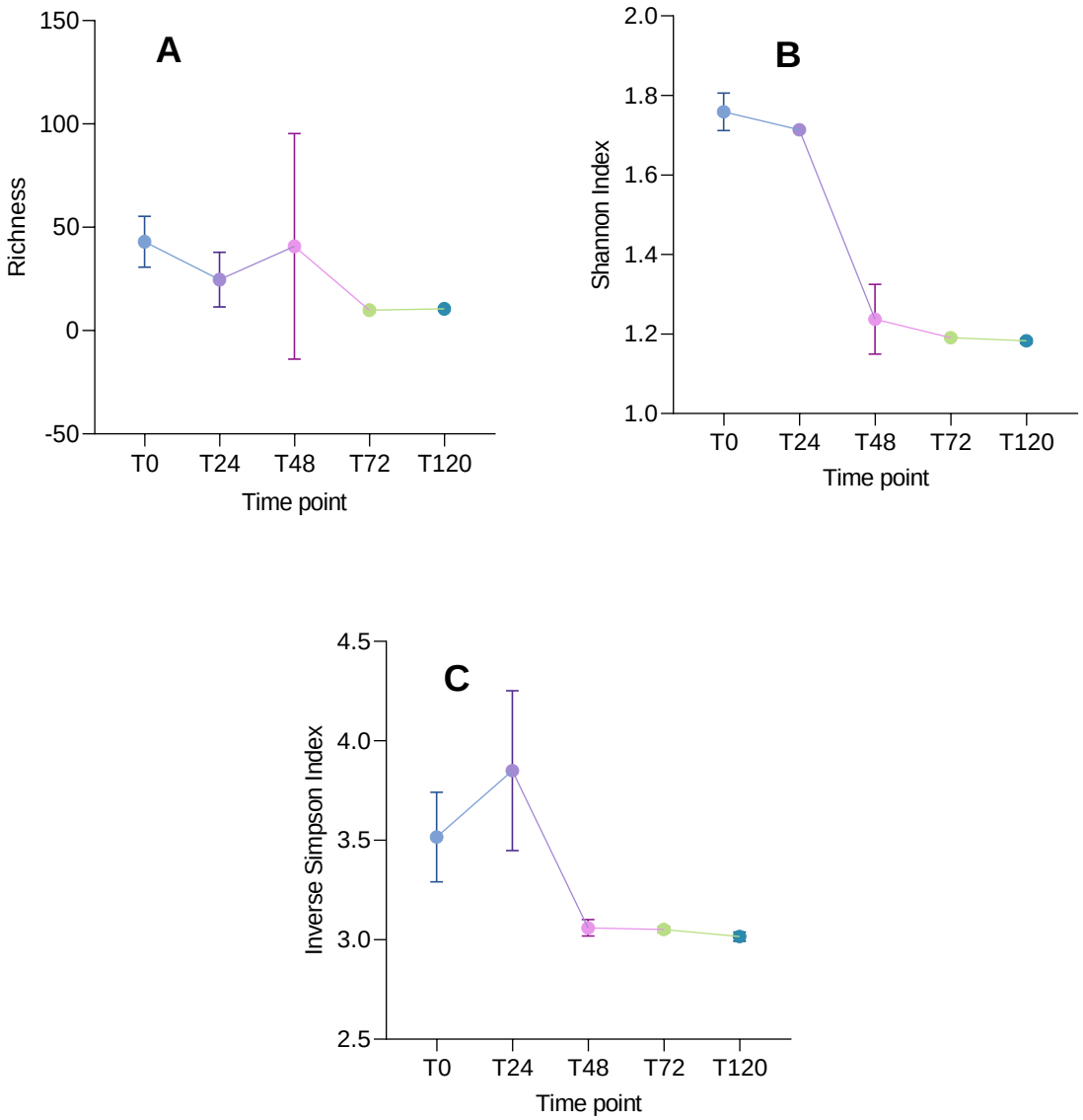


Figure 4.4: Alpha diversity of the overall microbial composition during storage of the vegetable matrix determined by, total richness (A), Shannon Index (B) and Inverse Simpson Index (C). Each data point comprises of three technical replicates and associated error is expressed by standard deviation.

To understand if there were any differences in bacterial diversity across different batches of the vegetable matrix, ten fresh batches were purchased 12 months apart and analysed. Evaluation of richness supported with diversity indexes Shannon and Inverse Simpson can be seen in Figure 4.5. Based on statistical analysis there was a significant difference in richness, Shannon index and Inverse Simpson index across all analysed batches, $p = 0.0312$, $p = 0.0449$ and $p = 0.0154$ respectively. Follow-up post hoc assessments can be viewed in Appendix 6. Additionally, investigations into the impact of the freeze/thaw on bacterial diversity of the vegetable matrix can be seen in Figure 4.6. There was a significant difference in Shannon index, $p = 0.0059$, and Inverse Simpson Index, $p = 0.0084$ when comparing the vegetable matrix prepared from fresh to the vegetable matrix prepared from frozen. However, there was no significant difference in richness, $p < 0.05$.

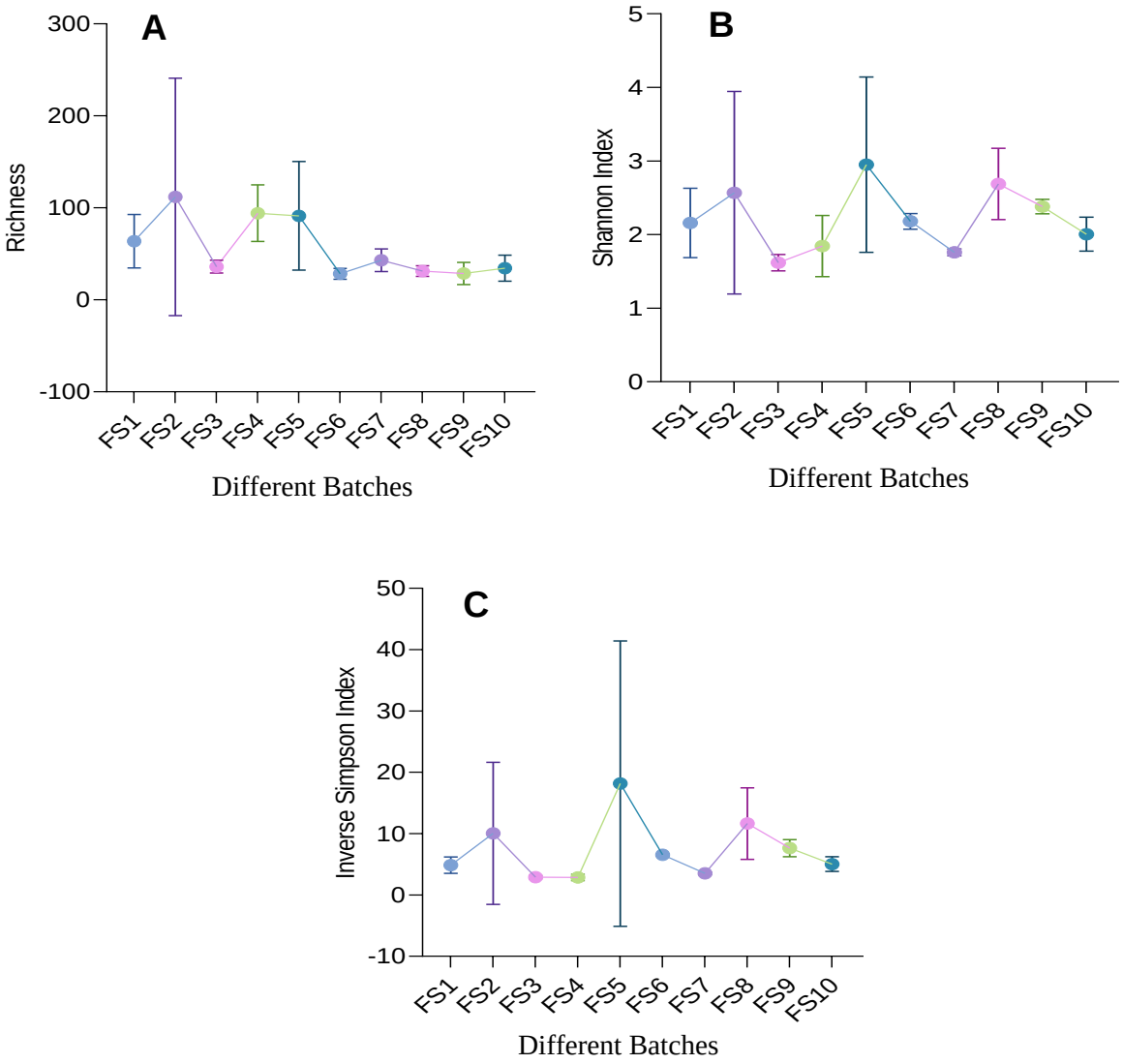


Figure 4.5: Alpha diversity of the overall microbial composition of ten batches of the vegetable matrix (FS1-10) assessed by total richness (A), Shannon index (B) and Inverse Simpson index (C). Each data point comprises of three technical replicates and associated error is expressed by standard deviation.

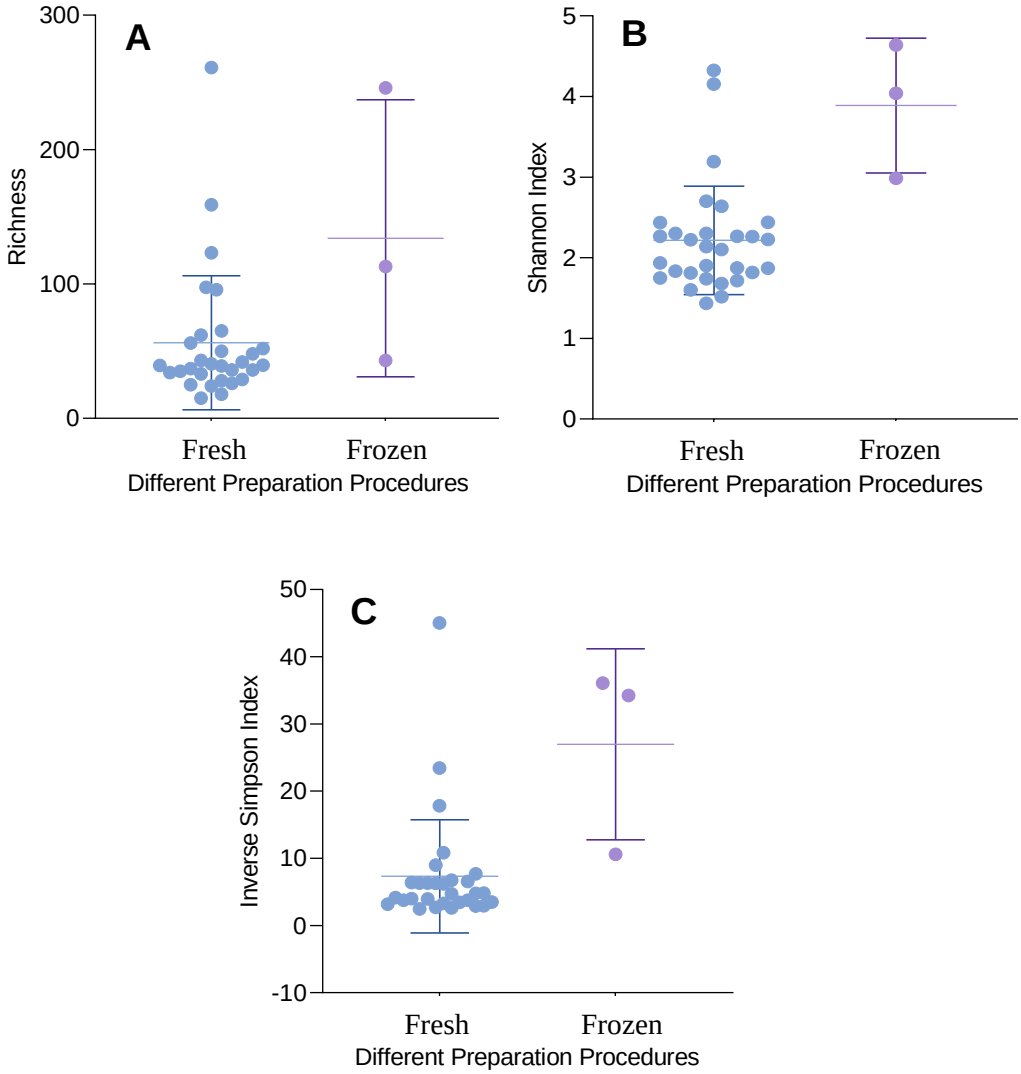


Figure 4.6: Alpha diversity of the overall microbial composition comparing average of ten fresh batches of the vegetable matrix, comprising of three technical replicates each (n= 30) to a batch of a vegetable matrix that was frozen and subsequently defrosted (n=3). Alpha diversity assessed by total richness (A), Shannon index (B) and Inverse Simpson index (C). Associated error of data points is expressed by standard deviation.

The microbial composition over the time course is shown in Figure 4.7, encompassing the top 20 most abundant communities at genus level. At 0 h the microbial composition shows greater complexity, with some of the main abundant communities in descending order including *Lactococcus*, *Leuconostoc*, *Escherichia*/*Shigella*, *Pseudomonas*, *Pantoea* and *Yersinia*. As time progressed during storage *Lactococcus*, *Leuconostoc* and *Yersinia* became the most dominant communities, however their overall abundance altered throughout the time course. *Yersinia* was the most abundant community at 24 h, making up approximately 45% of the total bacterial composition, however *Yersinia* slowly decreased as *Leuconostoc* and *Lactococcus* increased in abundance by 120 h.

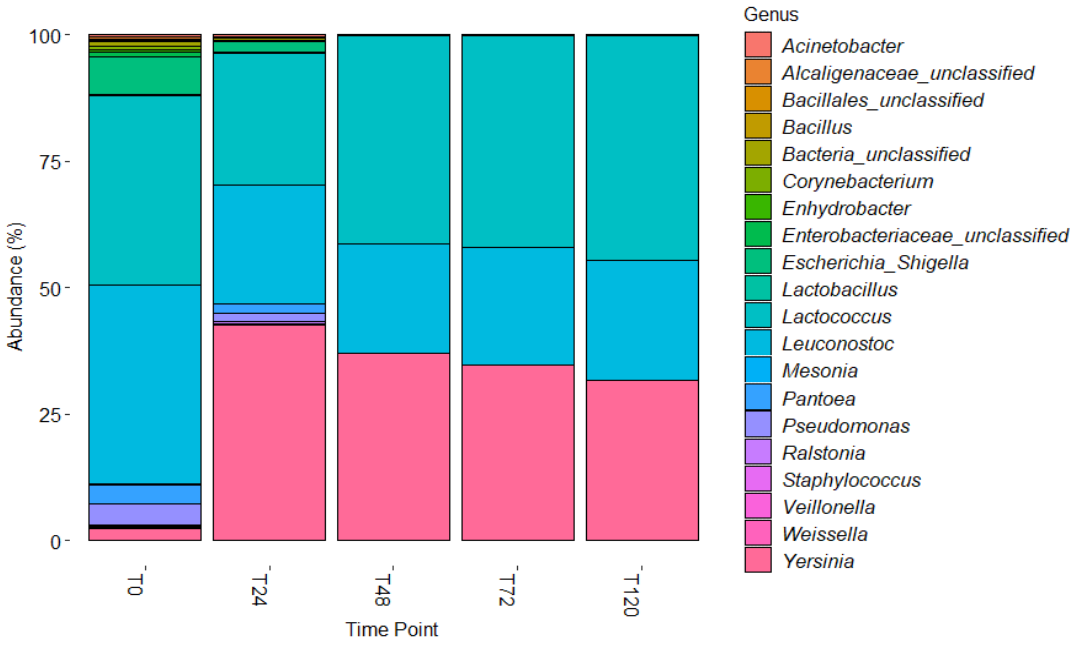


Figure 4.7: Microbial composition and development throughout the time course of the vegetable matrix, from T0 to T120. The microbial composition was expressed as the % abundance of the top 20 bacterial communities classified at genus level. Each time point comprises of three technical replicates.

In addition, the microbial composition was captured across different batches of the vegetable matrix (n=11) including a batch that had been defrosted, shown in Figure 4.8. Across the ten different batches the bacteria communities were similar, although differed in abundance. However, the batch from frozen noticeably had a higher abundance of unclassified bacteria compared to non-frozen batches.

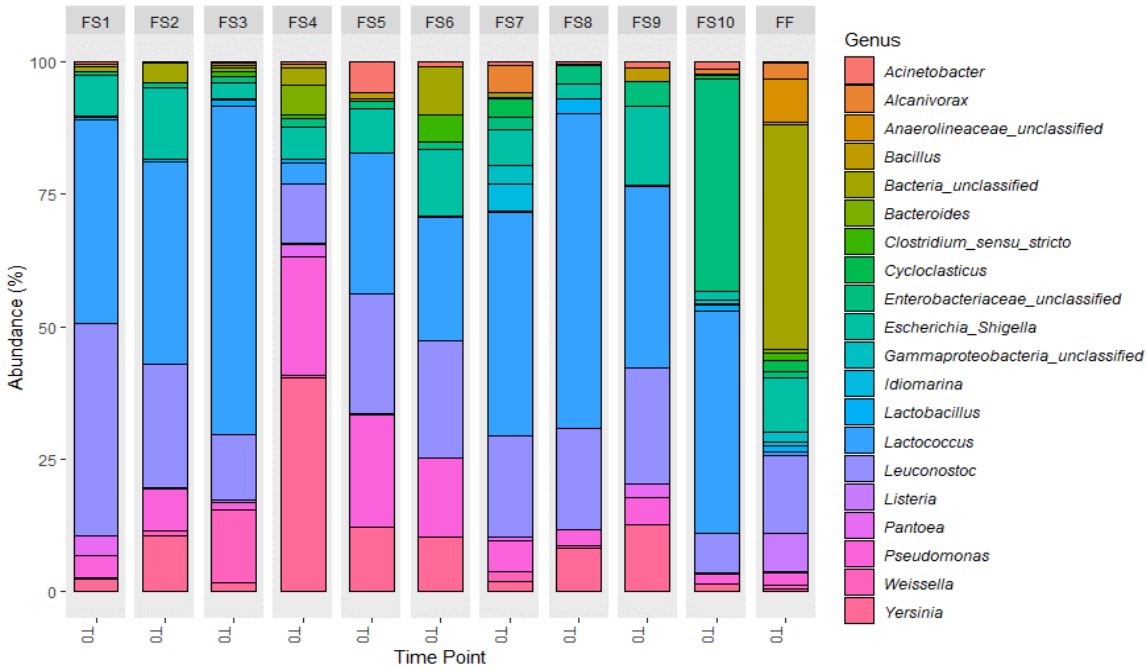


Figure 4.8: Microbial composition expressed as % abundance of ten fresh vegetable matrices (FS1-10) before storage and one frozen and subsequently defrosted vegetable matrix (FF). The microbial composition represented by the top 20 bacterial communities classified at genus level. Each time point comprises of three technical replicates.

4.3.3 The Impact of Storage Duration and Batch on Microbial Composition – Beta-Diversity

To determine differences in microbial composition during storage of the vegetable matrix, beta-diversity metrics were used. Although alpha diversity is valuable for assessing bacterial communities over time, through evaluating individual time points, it fails to capture information in relation to the changes in microbial composition. Alternatively, beta-diversity aims to describe the relationship between diversity within a sample and a group of samples (between samples) (383). The Beta-diversity metric, Bray-Curtis dissimilarity was calculated, which generated a distance matrix between all pairs of samples, this was visualised using principal coordinate analysis (PCoA), which indicated the impact of storage duration on bacterial composition (Figure 4.9). Based on PCoA, almost all variability in the samples is explained within the first two axis (94.7%) with distinct clustering of samples according to time points. 0 hr samples are clustered together away from other time points, this is the same case for samples collected at 24 h. However, samples collected at 48, 72 and 120 h are clustered together. Overall, storage duration influenced microbial composition, confirmed by the permutational multivariate analysis of variance (PERMANOVA) test ($p = 0.001$, $R^2 = 0.9786$).

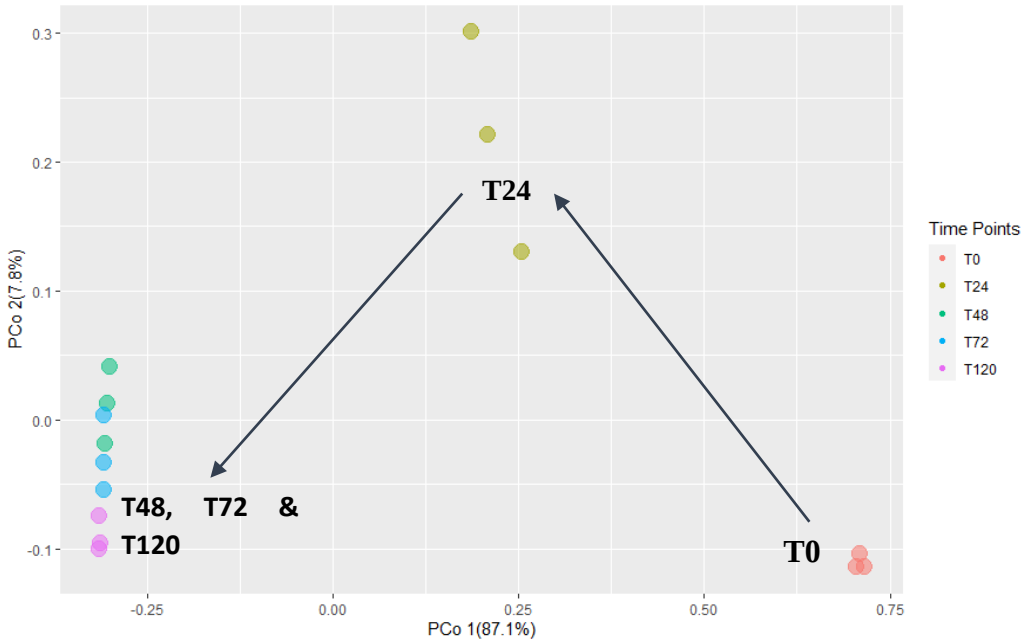


Figure 4.9: Principal coordinate analysis of Bray-Curtis dissimilarity distance matrix, samples grouped by time points 0 h – 120 h. Each time point comprises of three analysed samples. The first axis, PCo 1 describes 87.1% of the sample variability, while the second axis, PCo 2 describes 7.8% of variability across samples.

Beta-diversity was additionally assessed across ten different fresh batches of the vegetable matrix and one defrosted batch. PCoA was used to visualise Bray-Curtis dissimilarity distance matrix across different batches, as displayed in Figure 4.10. According to the PCoA, 39.5% of the variability in the data was explained within the first and second axis. Although there was some clustering of batch replicates there were many clustering overlaps between batches. Interestingly, the from frozen batch clustered furthest away from the majority of batches. The PERMANOVA

statistical test was performed and indicated significance (p = 0.001, R2 = 0.6448), suggesting different batches had a different microbial composition. Follow-up post hoc tests with Bonferroni corrections were conducted after PERMANOVA test indicated significance, for both different batches and storage study of the vegetable matrix. According to both post hoc assessments, there were no statistical differences between different batches and time points.

As part of a separate investigation, different DNA extraction kits (Powersoil® Qiagen and PowerFood® Qiagen) were trialled to determine the most suitable extraction to obtain the best representation of the microbial composition. The results indicated from the kits used, there were no significant differences between kits in either alpha or beta-diversity. Therefore, any of the trailed kits tested could be used, a breakdown of these results can be seen in Appendix 7.

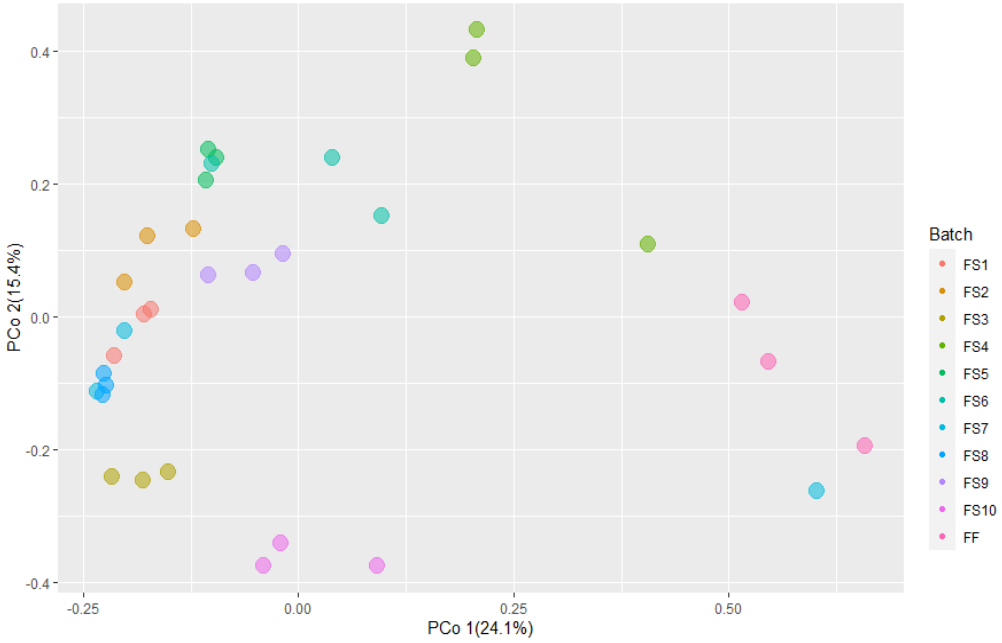


Figure 4.10: Principal coordinate analysis of Bray-Curtis dissimilarity distance matrix across different batches of the vegetable matrix (FS1-10) and one defrosted batch (FF). Each batch was analysed in triplicate. The first axis, PCo 1 describes 24.1% of the sample variability, while the second axis, PCo 2 describes 15.4% of variability across samples.

4.3.4 pH Profile and Sensory Evaluations

The pH profile during 120 h of storage of the vegetable matrix is shown in Figure 4.11, the average pH of the vegetable matrix was 5.70 ± 0.03. Throughout storage the pH remained unchanged during the initial 24 h, following a rapid decrease at 48 h to 4.16 ± 0.03 and further followed a slow decrease during the remaining storage period (3.86 ± 0.03 at 120 h). This decrease was complemented with organoleptic observations; colour, aroma and texture were among the visual inspections that deteriorated throughout (Figure 4.12). At 48 h gas bubbles formed within the matrix, off odours developed and the vegetable matrix appeared paler in colour. As storage

continued, the off odours developed in intensity, texture alteration occurred, and colour further changed. No data was measured for the organoleptic changes.

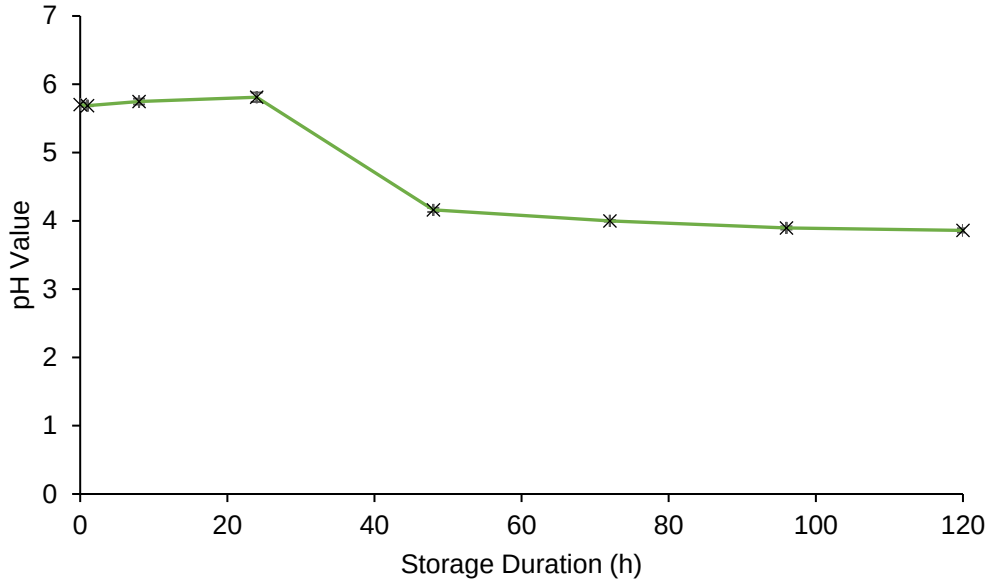


Figure 4.11: The pH profile of vegetable matrix during storage. pH was measured in triplicate at each time point and associated error expressed by a 95% confidence interval.

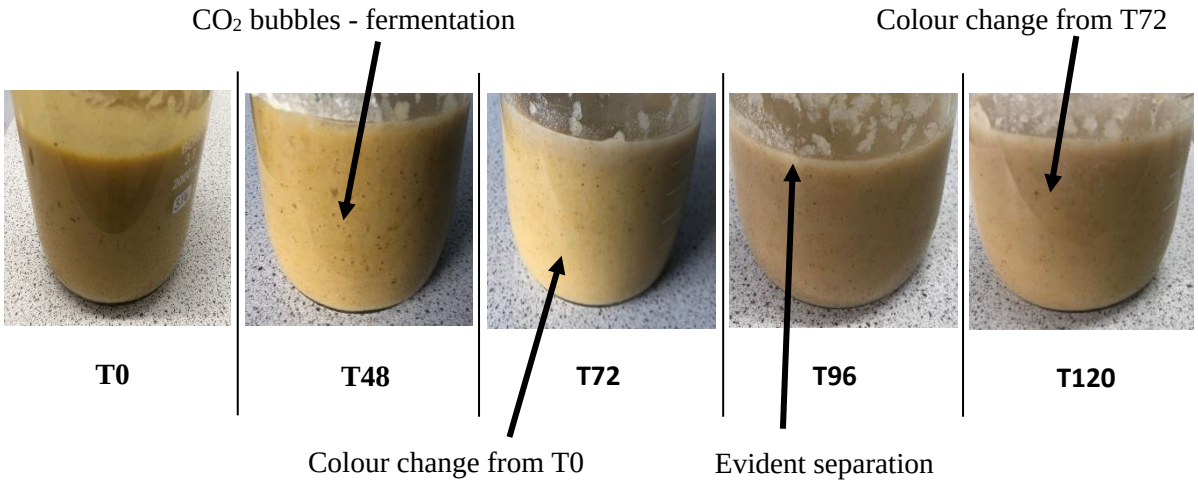


Figure 4.12: Images presenting the visual appearance of the vegetable matrix over the time-series.

4.3.5 Metabolomic Fingerprinting

The metabolite profiles during storage of the vegetable matrix were captured using untargeted metabolomic fingerprinting, using positive mode HILIC-MS/MS. After extensive pre-processing, including alignment, quality checks and matching MS features with ID's according to MZcloud database, 117 accurate masses were retrieved. This multivariate data was modelled using partial least-squares discriminant analysis (PLS-DA) on only the putatively annotated metabolites, to visualise changes in the metabolite profiles during the storage period (Figure 4.13). The PLS-DA

showed 55.1% of the covariance is explained in component 1 and 2, with evident clustering in association with time point. Samples collected at 0 h and 24 h were clustered together, 48 hr samples were clustered on their own close to later time points and both 72 h and 120 h samples were clustered together away from T0 and T24 samples.

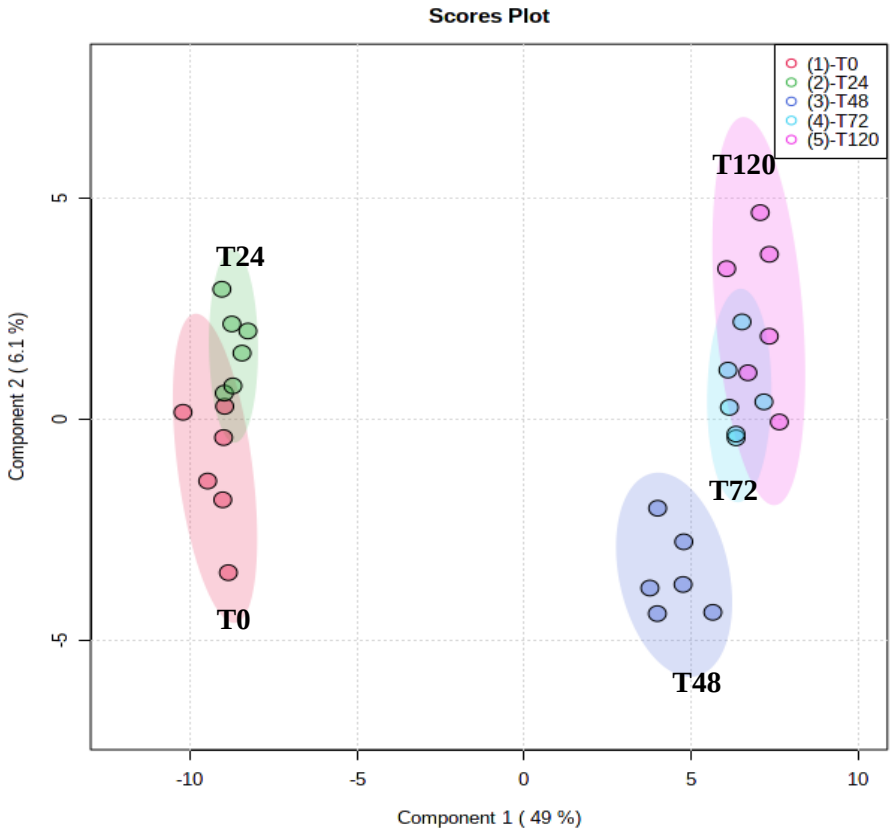


Figure 4.13: PLS-DA of the 117 putatively annotated metabolites during the time course, comprising of 5 time points from 0 h – 120 h. Each time point contains six technical replicates.

The most important discriminatory metabolites driving the covariance in the time course, observed in the PLS-DA, was assessed using variable importance on projection (VIP) scores. These discriminatory metabolites are shown in Figure 4.14 and include acetylspermidine, hypoxanthine and prolinamide all of which increased in intensity with time. Alternatively, adenosine 5'-monophosphate, glutathione and arginine decreased in intensity over time. Representative box plots of the top 15 variables according to VIP are shown in Figure 4.15. Pathway analysis was also performed on the putatively annotated metabolites (Figure 4.16) and showed metabolites were significantly associated with arginine and proline metabolism ($p<0.01$), arginine biosynthesis ($p<0.01$) and glutathione metabolism ($p = 0.027$).

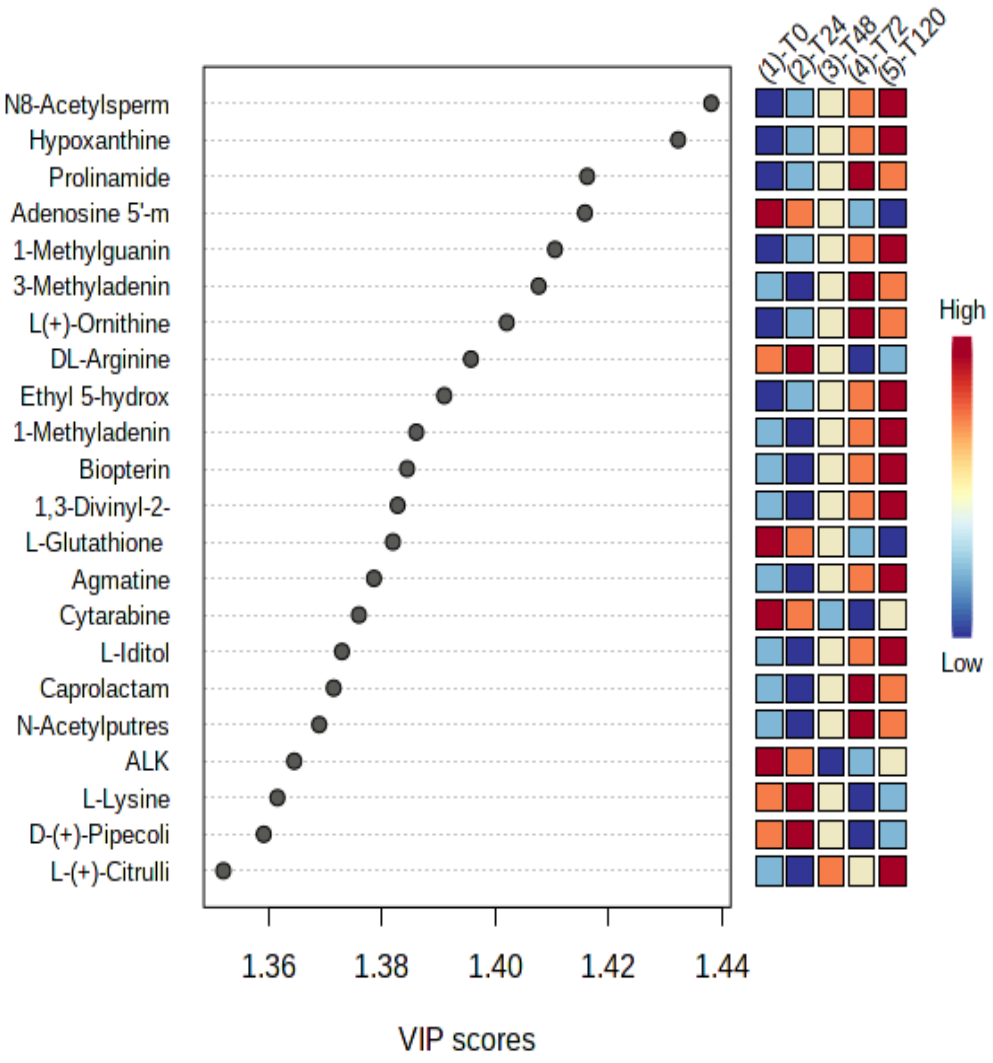
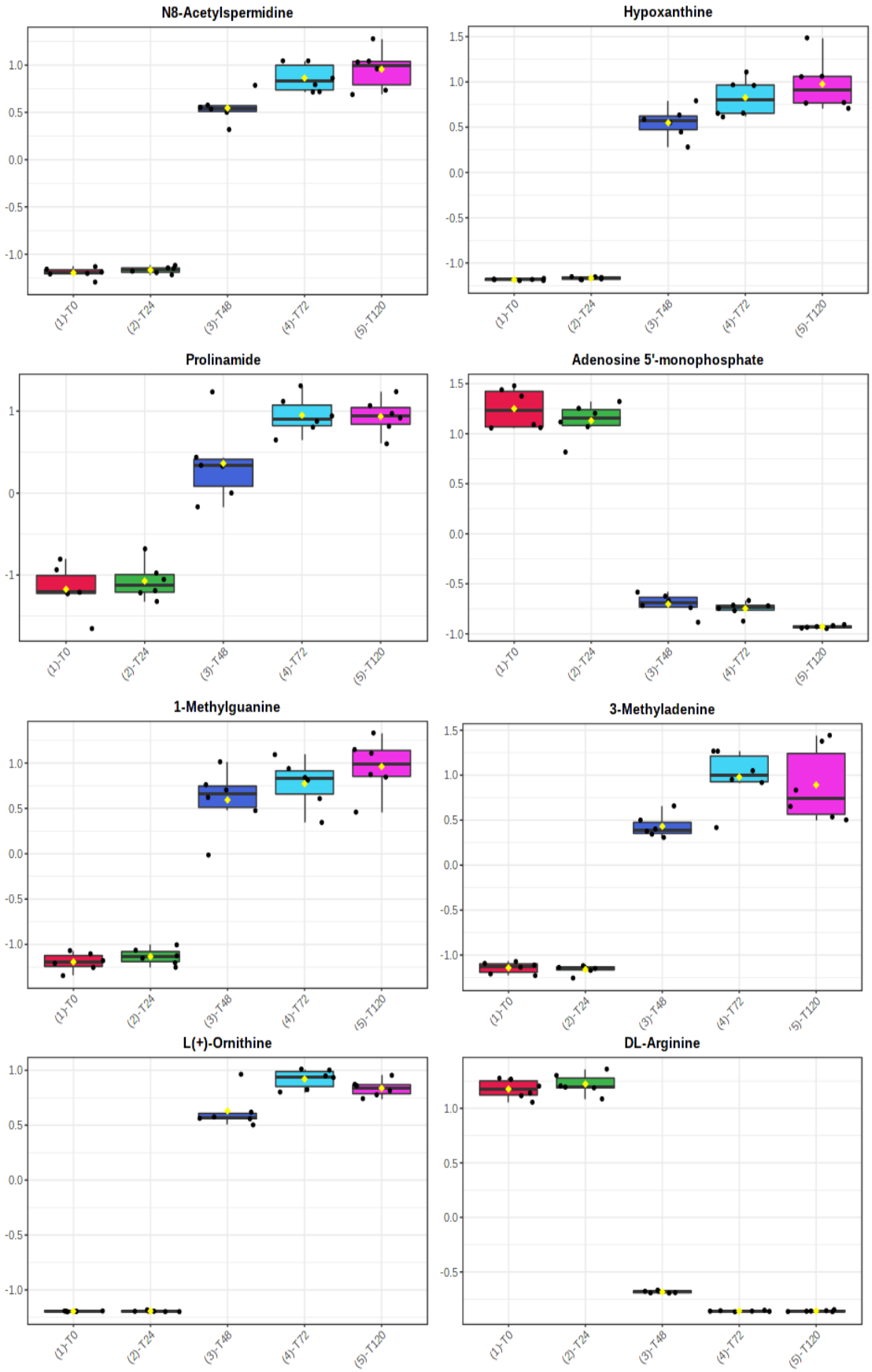


Figure 4.14: VIP ranking of the variables/metabolites (VIP > 1.35) that have played important roles in the time series as identified by PLS, while also representing the point during the time series metabolites were either exhausted or produced.

In addition, a dendrogram showing the clustering of samples, supported by a heatmap is represented in Figure 4.17. The dendrogram and heatmap highlight similarities across samples, with evident clustering according to time points. The 0 h and 24 h samples are clustered together, while 48, 72 and 120 h are clustered together indicating similar features are shared amongst these samples. In addition, the heatmap represents metabolites that are contributing to this clustering effect and therefore shows metabolites that present at either higher or lower intensity at specific time points. For example: L-lysine, L-glutathione, adenosine 5'-monophosohate were metabolites present in the vegetable matrix in greater intensity between T0-T24, however these metabolites reduced in intensity from T48-T120. Similarly, metabolites citrulline, ornithine, 3-methyladenine were present at higher intensity towards the end of the storage period (T48-T120) and were lower in intensity between T0-T24.


N8-Acetylspermidine
Hypoxanthine
Prolinamide
Adenosine 5'-monophosphate
1-Methylguanine
3-Methyladenine
L(+)-Ornithine
DL-Arginine
(1)-T0
(2)-T24
(3)-T48
(4)-T72
(5)-T120

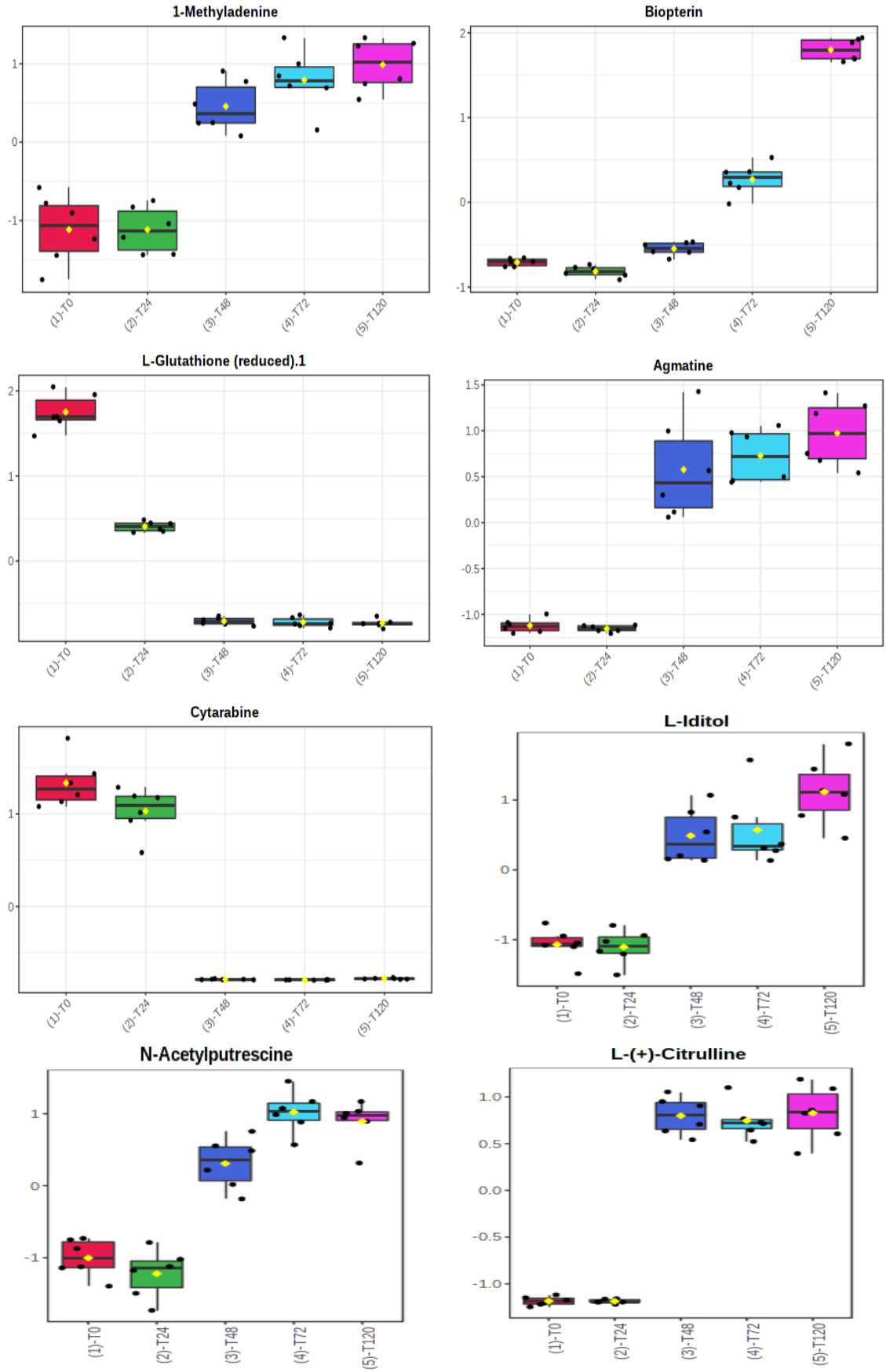


Figure 4.15: Key discriminatory metabolites according to the calculated VIP scores. Y axis represents normalised peak area (arbitrary units) and X axis represents time points. Each time point is composed of six technical replicates.

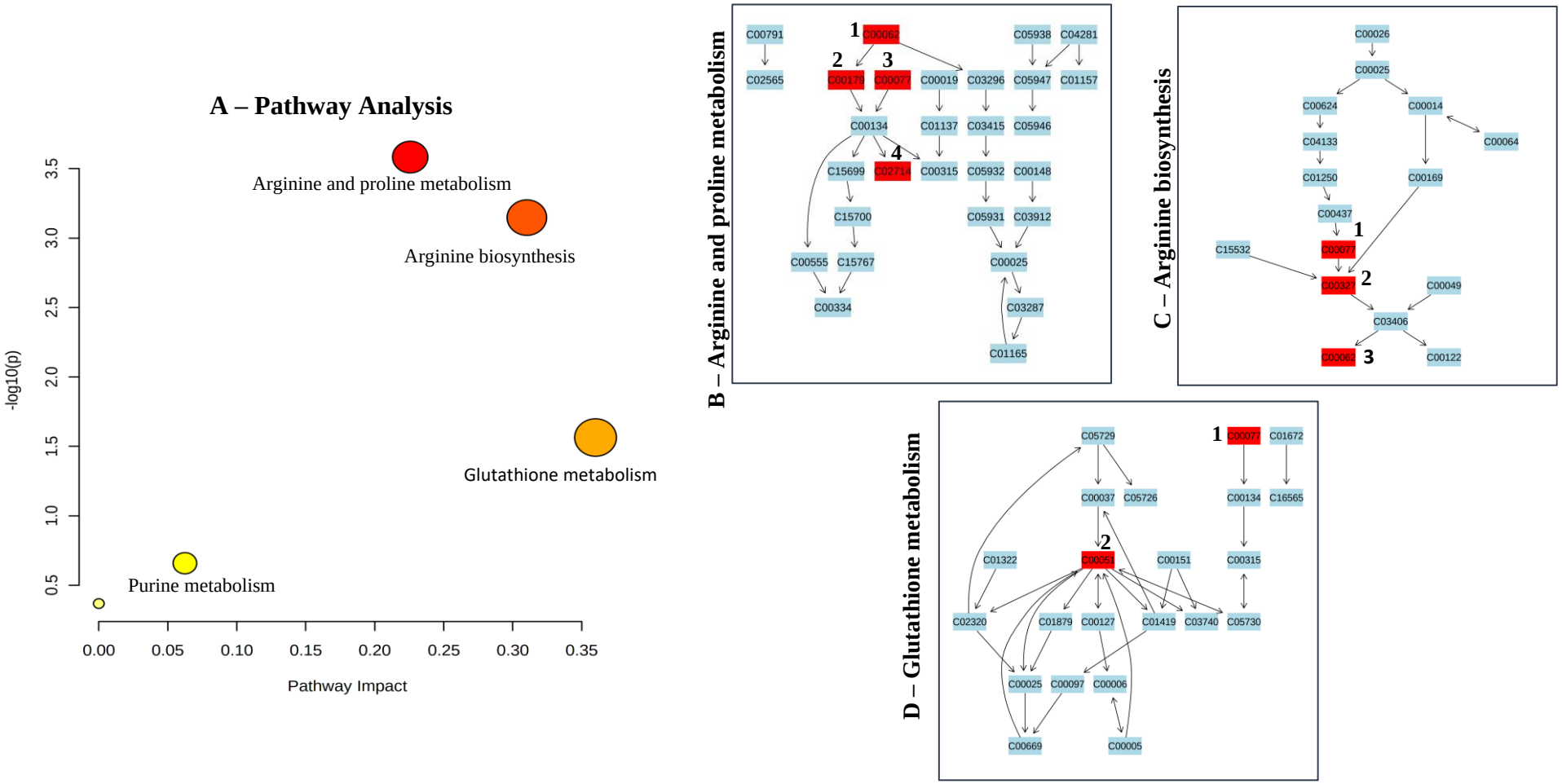


Figure 4.16: Pathway analysis of putatively annotated metabolites (A) and pathways shown (B-D) are deemed to have significant association with some of the putatively annotated metabolites, which are highlighted in red. For arginine and proline metabolism, metabolites include 1= L-arginine, 2= agmatine, 3=L-ornithine and 4= acetylspermidine. For arginine biosynthesis metabolites include 1= L-ornithine, 2= citrulline and 3=L-arginine. For glutathione metabolism, metabolites include 1= L-ornithine and 2= glutathione .

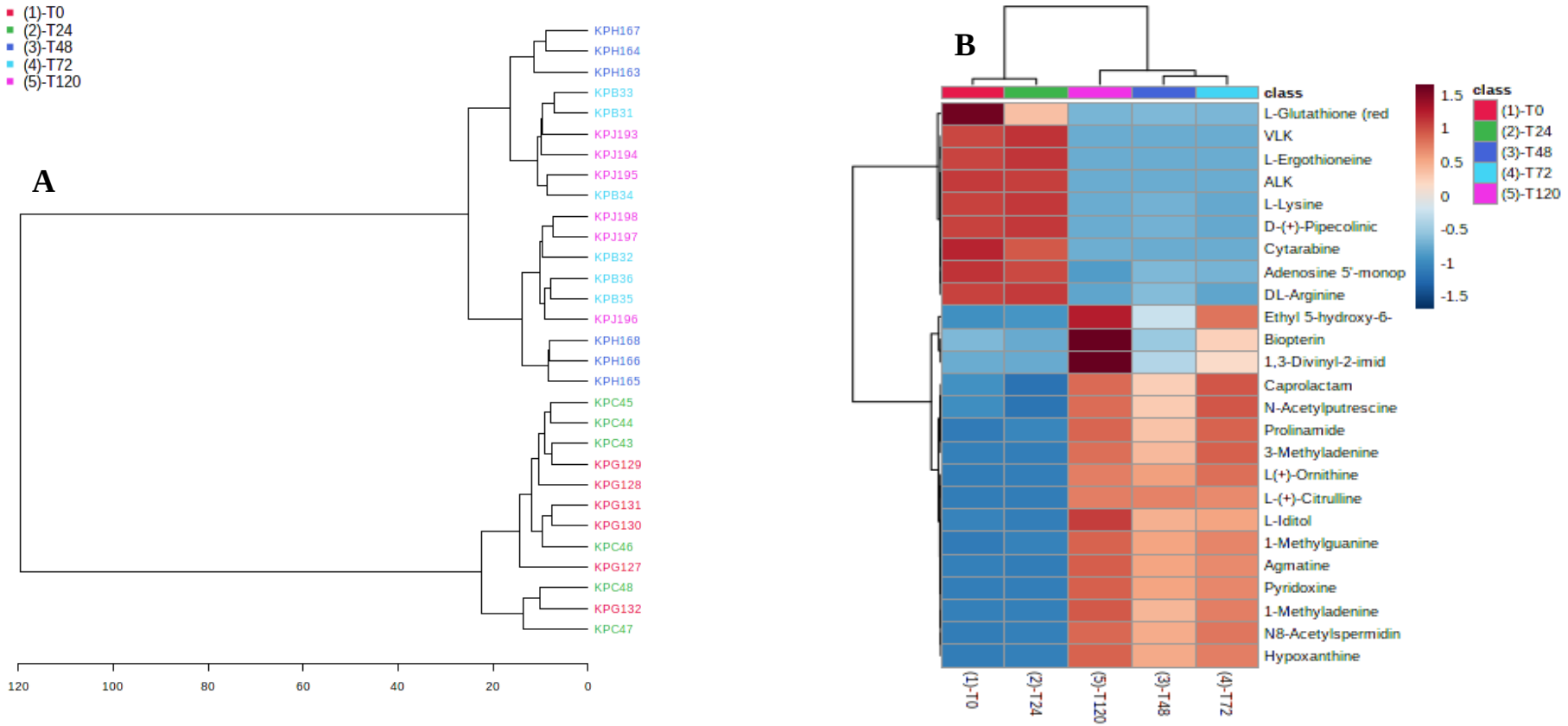


Figure 4.17: Depicts both dendrogram (A) and hierarchical clustering heatmap (B) of identified metabolites during the storage period (0 h – 120 h) of the vegetable matrix. Clustering was calculated using Euclidean distance and Ward linkage. The hierarchical clustering heatmap comprises of the top 25 variables according to PLS-DA VIP.

4.3.6 Volatile Organic Compound (VOCs) Analysis

The VOCs present in the vegetable matrix were analysed by gas chromatography- time-of-flight mass spectrometry (GC/TOF-MS). After processing of data, 1,392 features were left, from which the importance of the top 25 most significant features throughout the time series were visualised using principal component analysis (PCA), presented in Figure 4.18. In line with the PCA, all samples clustered together in accordance with their respective time points, 0 hr samples clustered away from later time points, while samples collected between 48h – 120 h clustered in close proximity to each other. This indicates the VOC profile significantly changed from 0 h, with the variability in the samples explained by component 1 and 2 (93.38%). The top features were putatively annotated and are shown in Table 4.1. The main volatile groups putatively characterised that changed throughout the time course included, aldehydes, alkanes, alkenes, alcohols, short chain fatty acids (SCFAs), ketones and monoterpenoids.

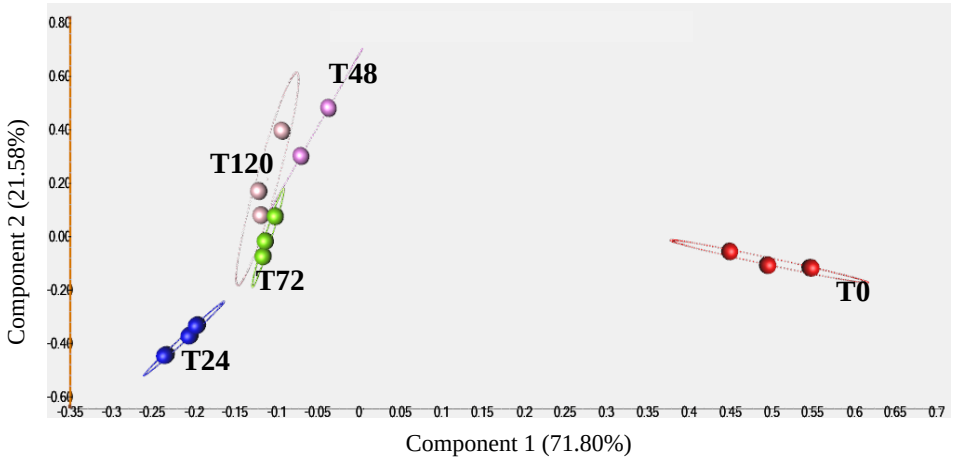
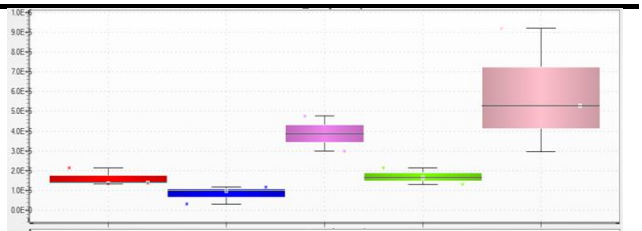
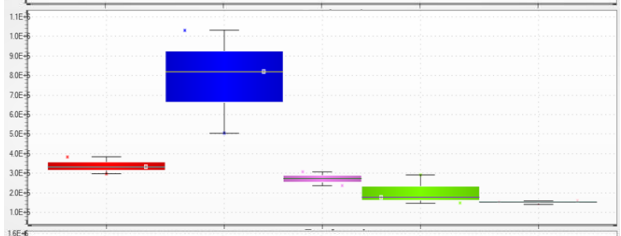
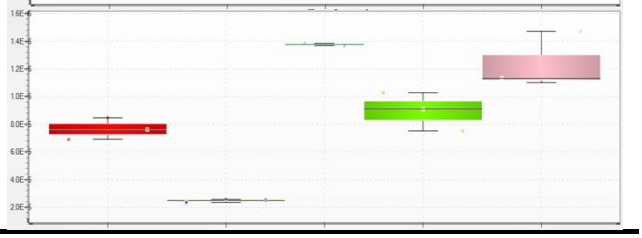
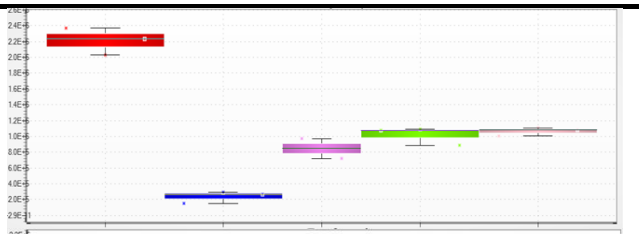
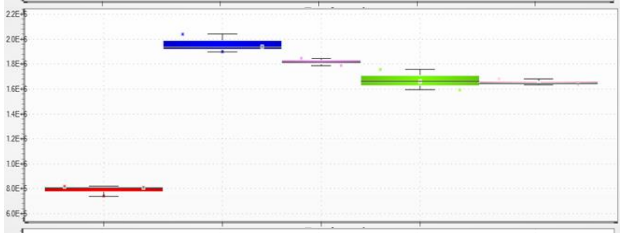
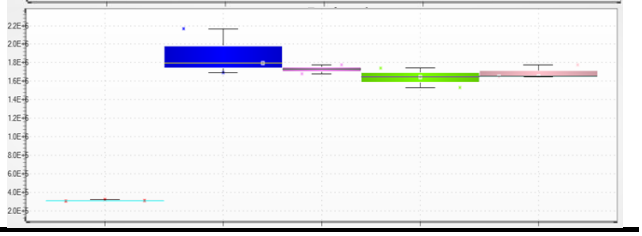
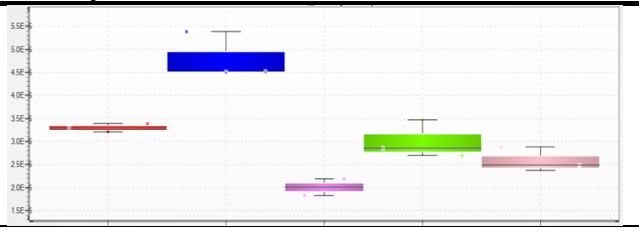
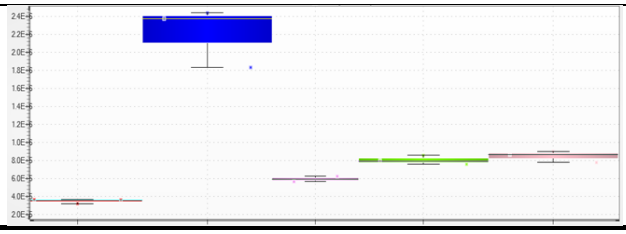
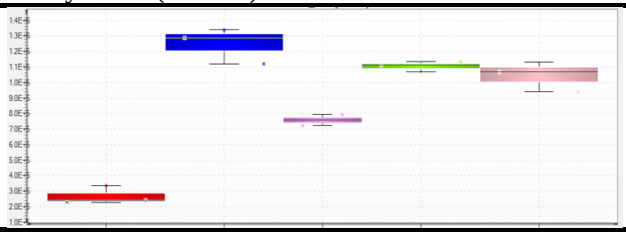
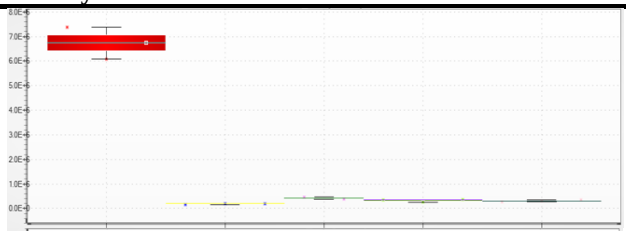
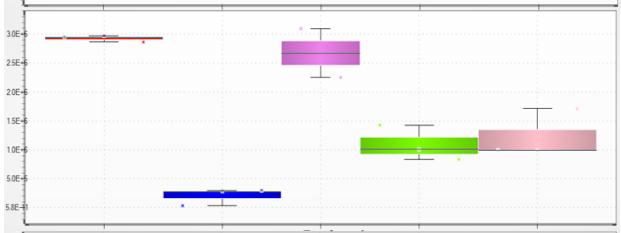
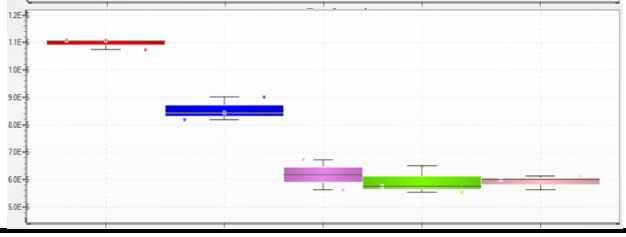
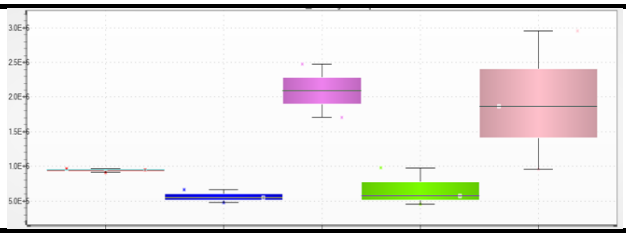


Figure 4.18: PCA comprising of the top 25 most significant features during the time course, comprising of 5 time points from 0 h – 120 h. Each time point contains three technical replicates, with the exception of T48 which is composed of two technical replicates.

Table 4.1: The top discriminatory annotated VOCs according to PCA including box plots. The box plots show T0 = red, T24 = blue, T48 = pink, T72 = green and T120 = light pink, and y axis represents abundance.

Putatively Annotated Volatiles	
Alkanes and Alkenes	
2,4-Dimethyl-hexane,	
Propane	
1-Octene	
Alcohols	
Cyclopentanol	
1-Pentanol	
1-Hexanol	
Monoterpenes	
1-Propenyl-benzene	

β-Linalool	
Short chain fatty acids (SCFAs)	
Acetic acid	
Aldehydes	
Propanal	
Hexanal	
2-Ethyl-2-butenal	
Ketones	
p-Pentylacetophenone	

4.3.7 Nutritional Composition - Stability of B-Group Vitamins

Four essential B vitamins were assessed during storage of a popular ready to eat vegetable matrix, namely nicotinamide, pyridoxine, riboflavin, and thiamine. Samples were obtained for nutritional analysis on eight separate time points and their respective stability, in terms of % remaining can be viewed in Figure 4.19 and descriptive statistics are shown Table 4.2. Post hoc results from repeated measures ANOVA are available in Appendix 2. Nicotinamide ($p = 0.004$), riboflavin ($p = 0.006$) and thiamine ($p = 0.003$) significantly decreased over time; nicotinamide became undetectable after 48 h of storage. The quantity of riboflavin and thiamine significantly reduced at 48 h, with 14.8% (from 245.80 ± 3.64 ng/g (T8) to 34.30 ± 5.11 ng/g (T48)) and 58.9% (from 262.60 ± 20.88 ng/g (T8) to 163.73 ± 5.20 ng/g (T48)) remaining respectively at this time point. Alternatively, pyridoxine was stable and did not significantly change over the time course period. As part of a control, the stability of the four investigated B vitamins were assessed in a controlled buffered system, in absence of the vegetable matrix, shown in Figure 4.20 and the complementary descriptive statistics are outlined also in Table 4.2. The controlled buffered system was set up to replicate the vegetable matrix environment in terms of pH, content of vitamins and storage temperature. Based on the findings when the vegetable matrix was absent, there was no significant change in nicotinamide, pyridoxine, riboflavin, and thiamine content ($p > 0.05$) over 120 h of storage.

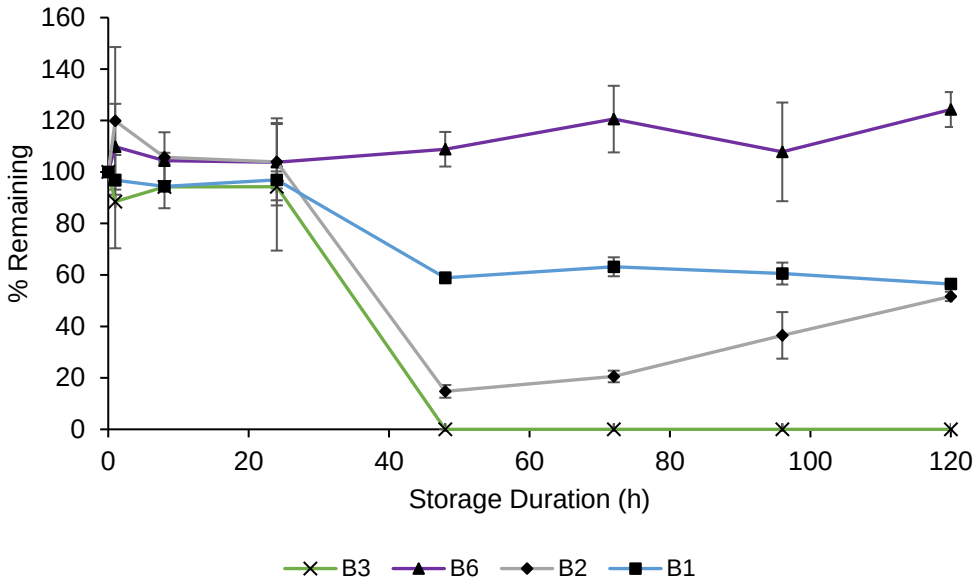


Figure 4.19: Stability of B-group vitamins, nicotinamide (B_3), pyridoxine (B_6), riboflavin (B_2) and thiamine (B_1) in the vegetable matrix over 5 days of storage (120 h) under a controlled storage environment. Stability is represented as % remaining of each vitamin based on quantity of each vitamin at T0. Each data point is an average of three analysed samples and associated error is expressed by a 95% confidence interval.

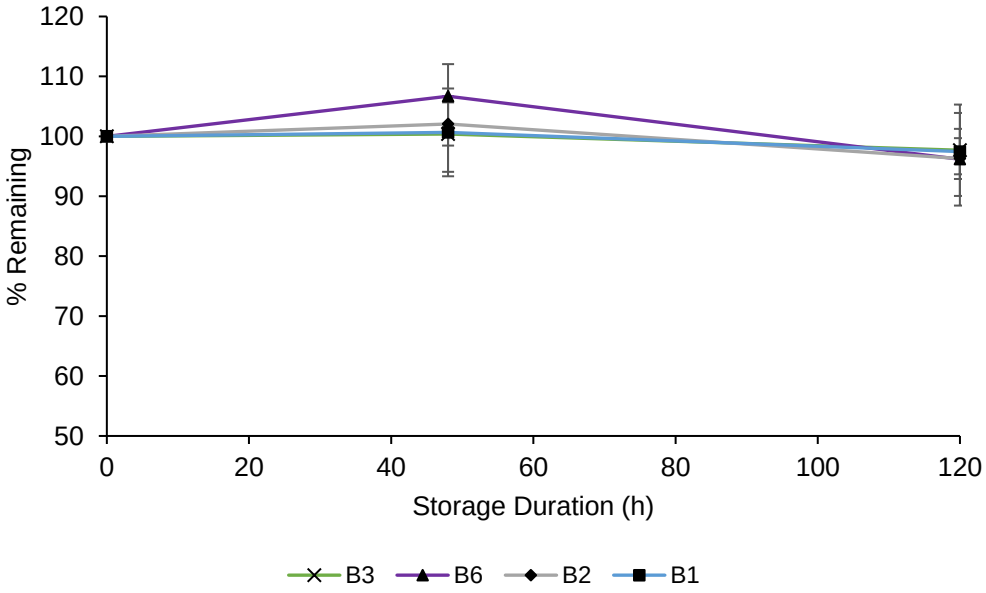


Figure 4.20: Stability of B-group vitamins, nicotinamide (B_3), pyridoxine (B_6), riboflavin (B_2) and thiamine (B_1) in a controlled buffered system over a storage period of 5 days in absence of the vegetable matrix and part of a control. The stability of the vitamins is represented by % remaining, from the initial quantity of each vitamin determined at T0. Samples were collected from the start, middle and end of the time-series, each data point is an average of three biological replicates and three technical replicates (n=9). The error associated with each data point is expressed by a 95% confidence interval.

Table 4.2: Descriptive statistics of vitamin stability in both vegetable matrix and a controlled buffered system stored over a 5-day period.

Vitamins – Vegetable matrix								
	Nicotinamide		**Pyridoxine**		**Riboflavin**		**Thiamine**	
Time point/s	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV
T0	591.20	80.46	95.70	1.91	232.57	31.15	278.10	3.40
T1	523.10	94.75	105.10	14.12	278.77	58.98	269.23	5.30
T8	556.93	1.27	99.90	9.35	245.80	3.64	262.6	20.88
T24	557.20	129.58	99.30	12.52	241.70	34.79	269.57	8.12
T48	ND*		104.17	5.69	34.30	5.11	163.73	5.20
T72	ND*		115.37	10.96	47.80	4.65	175.67	9.05
T96	ND*		103.20	16.25	84.93	18.57	168.37	10.46
T120	ND*		118.93	5.71	120.17	3.72	157.00	2.19
Vitamins – Controlled Buffered System								
	Nicotinamide		**Pyridoxine**		**Riboflavin**		**Thiamine**	
Time point/s	Mean[b] (ng/mL)	STDEV	Mean[b] (ng/mL)	STDEV	Mean[b] (ng/mL)	STDEV	Mean[b] (ng/mL)	STDEV
T0	362.38	32.63	66.09	8.98	96.13	5.61	9.07	1.24
T48	363.76	34.97	70.52	5.41	98.11	5.30	9.13	1.02
T120	353.95	42.29	63.57	7.82	92.56	5.01	8.84	0.53

[a] Mean is comprised of three samples (n=3)

[b] Mean is comprised of three biological replicates and three technical replicates (n=9)

* ND = not detected

4.3.8 Nutritional Composition - Macronutrient Stability

The macronutrient composition was assessed at 0 h, 48 h and 120 h. Findings showed that protein content remained unaffected during the time course, from 0 h to 120 h, 0.55 ± 0.007 g/100 g and 0.56 ± 0.031 g/100 g respectively (Figure 4.21). The descriptive statistics and results from the conducted one-way repeated measures ANOVA can be seen in Appendix 3.

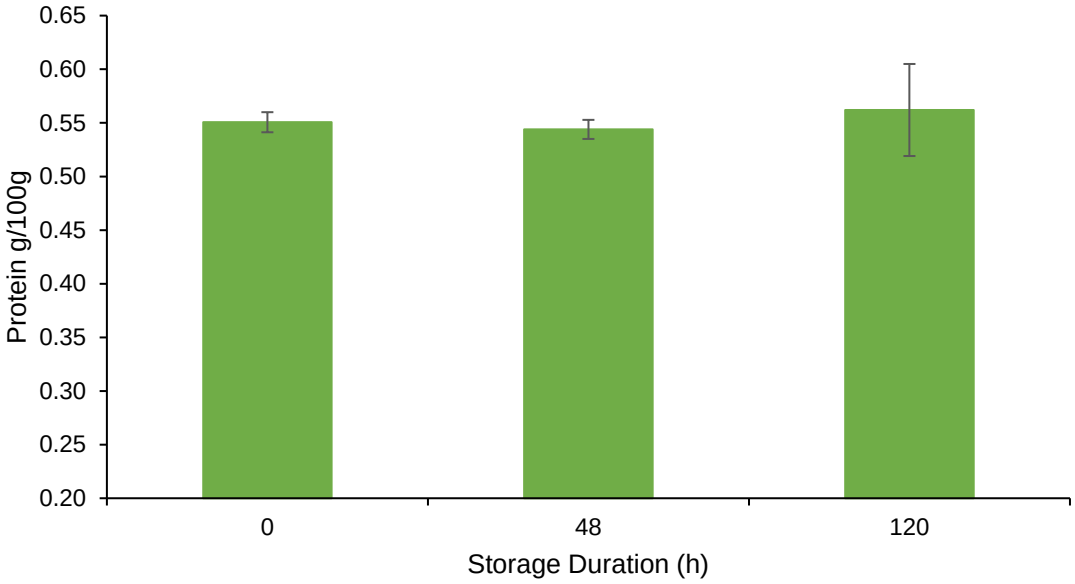


Figure 4.21: Evaluation of protein stability expressed as g per 100 g of sample during 5-day storage of the vegetable matrix, samples were collected at the start (0 h), middle (48 h) and end (120 h) of the time-series. Protein content was measured in duplicate, and error of each data point is represented by a 95% confidence interval.

Carbohydrate content of the vegetable matrix was assessed targeting monosaccharides, glucose and fructose, and disaccharide, sucrose. The sugar content of five fresh vegetable matrix samples (different batches), analysed in triplicate (n=15) were used to determine the average content of each targeted sugar as a baseline. The analysed sugar content was as follows: glucose 10.38 ± 0.96 mg/g, fructose 7.79 ± 0.90 mg/g and sucrose 7.51 ± 0.86 mg/g with an identified total sugar content of 2.57 g/100 g (fresh weight). Upon storage of the vegetable matrix the sugar profile during the start, middle and end of the time course was assessed (Figure 4.22). There was a significant decrease in fructose (63.24%) and sucrose (77.78%) content, however, there was no significant change in glucose concentration over 120 h of storage. Results from repeated measures ANOVA, including post hoc data can be viewed in Appendix 4.

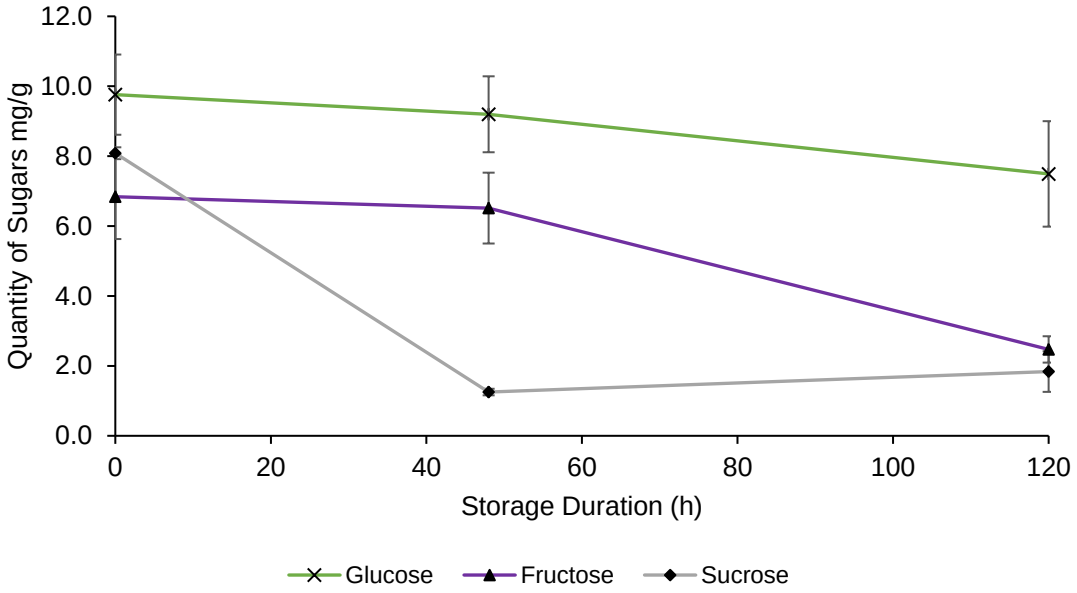


Figure 4.22: Stability of targeted sugars, glucose, fructose and sucrose in the vegetable matrix stored for 5 days (120 h). Quantity of each sugar at start, middle and end time points is represented by an average of duplicate or triplicate samples, expressed as mg/g of soup and associated error of each time point is expressed by a 95% confidence interval.

4.4 Discussion

In this chapter, an in-depth characterisation during storage of a vegetable matrix was performed assessing nutritional composition and microbial spoilage, while adopting a multi-omic approach.

4.4.1 Microbial Growth and Diversity During Spoilage of the Vegetable Matrix

Microbial spoilage during storage assessments was analysed using a complementary approach of traditional culturing techniques and culture independent practices. This is acknowledged by many authors as the best practice to thoroughly assess microbial spoilage (64, 67, 384). In terms of the data for TVC, maximum log 8.32 CFU/g was achieved at 48 h of storage, this is comparable to other authors that have investigated storage of vegetables. For example, Lee *et al.* (2011) (385) showed after 1 week of storage (28 °C) of fresh vegetables, lettuce, perilla leaf and chicory, TVC reached 8.04-8.66 log CFU/g, 7.69 log CFU/g and 8.88 log CFU/g respectively. Similarly, a study conducted by Alvarez *et al.* (2014) (24) showed during 2 days of storage of minimally processed vegetables at 15 °C, the TVC reached 8.2 log CFU/g. However, another publication has reported slightly lower viable counts ranging from 6.11-6.51 log CFU/g when assessing microbial load during storage of vegetables including cucumber, carrot, cabbage and onions (386). Differences in TVC across studies is expected as it will depend on the investigated vegetable/s in terms of nutrients available to support growth, potential contamination during processing and storage, and the storage conditions studied (386). Maximum log observed in this study at 48 h was accompanied by significant deterioration of the organoleptic properties of the vegetable matrix; gas production, development of off odours and textural changes. Furthermore, the matrix became

increasingly acidic with a drop in pH, from pH 5.7 (T0) to pH 3.86 (T120). This would have occurred as sugars in the vegetable matrix were consumed via fermentation to support the growth of SSOs, resulting in the production of organic acids such as lactic and acetic acid and resulting in the reduction of the vegetable matrix's pH (387). This is shown in the current data as the recorded increase in growth (log CFU/g) is complemented with a decrease in pH at 48 h of storage. However, as the pH continues to decrease throughout storage, the TVC decreased, due to the lower pH inhibiting growth of other spoilage species (388). SSOs, namely LAB have been reported to decrease pH of food matrices to pH 3.5-4.5, which is in agreement with this study (388, 389). Gram *et al.* (2002) (28) states that microbial activity should be considered quantitatively, as it refers to the quantitative ability of SSOs to form spoilage metabolites, such as VOCs, acetic acid and ethanol etc (162). Therefore, undesirable organoleptic properties of the vegetable matrix developed as TVC reached 2.105 x 10^8 CFU/g at 48 h, this is in accordance with the accepted knowledge that food spoilage occurs when growth of spoilage organisms reaches 10^7-10^9 CFU/g (28).

Amplicon sequencing, targeting 16S rRNA showed during the first day of storage microbial composition was very diverse and included *Escherichia*/*Shigella*, *Pseudomonas*, *Pantoea*, *Yersinia*, *Lactococcus* and *Leuconostoc*. At 24 h *Lactococcus, Leuconostoc* and *Yersinia* were the most abundant species and became the dominating microbes from 48 h. The observed decrease in diversity over time was supported by a significant reduction in Shannon and Inverse Simpson index and Park *et al.* (2012) (211) showed similar findings during fermentation of kimchi by LAB, from which the Shannon index reduced during fermentation from 4.11 to 1.74. According to several authors the microbial composition of vegetables predominantly comprises of Gram-negative species, *Pseudomonas* and *Enterobacteriaceae*, and Gram-positive LAB, these outcomes are supportive of the findings in this chapter (119, 390). Regarding the main dominating species, LAB forms part of the natural microflora of vegetables, while *Yersinia spp*. comprise of major foodborne pathogens and their isolation from vegetables has been reported in a few studies (119, 391-393). Based on the percentage abundance of the dominating species, *Yersinia spp*. were most abundant in comparison to *Lactococcus* and *Leuconostoc* at 24 h, however at 48 h, *Yersinia spp*. abundance began to decrease as *Lactococcus* became more abundant. This is due to *Lactococcus* and *Leuconostoc* competing with other species using "interference competition", producing organic acids leading to the acidification of the vegetable matrix, further resulting in cellular damage to *Yersinia spp*. (123, 394). Growth of *Yersinia spp*. has been shown to decrease when pH of a medium was reduced from neutral conditions (pH 6.0) to acidic conditions (pH 3.0), correlating to the decrease in TVC from 72-120 h of storage (395). Moreover, yeasts are known to play an important part in vegetable spoilage, in this study fungi selective agar was used to isolate yeast species, however bacteria predominately grew. This indicated bacteria in this study were the main cause of microbial spoilage and therefore, fungal community analysis using high-throughput sequencing, targeting the internal transcribed gene was not performed. Moreover,

microbial diversity of different batches of the vegetable matrix were assessed, of the ten fresh batches purchased 12 months apart there were observed statistical differences in bacterial diversity. This could either be due to slight differences in bacterial loads/ communities naturally present on the raw materials. Or this could be due to manufacturing of the vegetable matrix, where the product could be exposed to different sources of contamination from the production line, including equipment, workers, air etc, which is not controlled for and therefore the microbial diversity is subject to variation (386). However, the freeze thaw treatment resulted in alterations to the microbial composition with significant differences in Shannon and Inverse Simpson index compared to fresh batches. Freezing/thawing can result in damage to bacteria through formation of ice crystals that rupture cell walls, and by inducing dehydration of the bacterial cell causing injury, all of which could influence the microbial profile upon thawing (396).

4.4.2 Nutritional Profile During Storage Study

Nutritional composition of the vegetable matrix was assessed throughout the time course, investigating B-group vitamins and macronutrients protein and carbohydrates. Macronutrients play a major role in microbial spoilage and as a result are both good indicators of spoilage and can be used to gauge the main spoilage organism present, based on the substrate preferentially utilised for energy (28). The total protein content of the vegetable matrix was estimated as 0.55 g/100g, which was within 10% of the quantity stated on packaging and remained unchanged throughout storage. Although there was no observable effect on the protein content, proteins could have still been broken down and potentially repurposed by SSOs, while having minimal effect to the overall protein value of the matrix (150). The monosaccharides, glucose and fructose, and disaccharide, sucrose equated to 2.57 g/100g of fresh weight soup, comparable to the stated sugar content on the packaging of 2.3 g/100g. All investigated sugars were utilised during storage to different extents; glucose, fructose and sucrose are readily fermented by LAB producing predominately lactic acid. Homofermentative LAB such as *Lactococcus*, which was the main dominating species in this study would have been greatly responsible for lactic acid production (374, 397). Alternatively, heterofermentative LAB, *Leuconostoc* can produce many metabolites alongside lactic acid during fermentation of available sugars, including acetic acid, ethanol, and carbon dioxide, and these by-products of fermentation were evident in the physiochemical evaluation (374, 398). *Yersinia* also ferments sugars, particularly glucose through glycolysis, but not all *Yersinia* strains ferment sucrose (399, 400). Sucrose compared to other targeted sugars was the main sugar utilised and reduced at 48 h. The observed reduction is likely due to microbial enzymatic activity breaking down sucrose to monosaccharides, glucose, and fructose. Therefore, minimal changes to glucose and fructose concentration between 0-48 h is likely due to a balance between production of glucose and fructose from sucrose and utilisation of respective sugars by SSOs. After almost complete exhaustion of sucrose at 48 h, fructose reduced by 61.5% over the

remainder of the storage period, while there was no effect on glucose concentration. This has been shown in other studies, Lu *et al.* (2001) (401) observed during fermentation of cucumber juice, homofermentative bacteria utilised more fructose than glucose. This has not been fully explained in the literature, but one explanation proposed is that enzymes required for glucose metabolism denature through lactic acid production, therefore following the reduction in pH, the continued use of glucose is prevented for energy generation (401). Additionally, heterofermentative LAB have also been shown to utilise fructose at a more rapid rate than glucose, depending on strain (402). It has been stated that fructose utilisation increases growth more than glucose for heterofermentative LAB, therefore when different types of sugars are available, fructose is preferred to facilitate growth (403).

B vitamins are responsible for energy release from macronutrients, playing a major role in macronutrient metabolism by acting as precursors for coenzymes in such metabolic pathways (404, 405). Based on this study thiamine, nicotinamide and riboflavin appeared as key factors in the spoilage process and this is the first time this has been reported in a vegetable matrix. Thiamine, riboflavin, and nicotinamide all reduced at 48 h, with nicotinamide being fully depleted, in line with fermentation reactions through activity of LAB. The majority of the literature aren't in full agreement with these findings as many LAB strains are capable of synthesising B-group vitamins. As shown during desirable fermentation of products including tarhana and green olives, where increases in riboflavin and niacin were observed (366, 370). However, this is strain dependent as acknowledged by Szutowska (2020) (374) and at present there are limited studies investigating B-vitamins alongside the observed microbial composition in this study particularly from a spoilage perspective. Although studies are limited, Kaprasob *et al.* (2018) (406) showed during fermentation of cashew apple juice with 5 types of LAB strain, including *Leuconostoc*, thiamine significantly decreased by 98.7% at 48 h. Additionally, Kneifel *et al.* (1992) (407) monitored vitamin content across sixteen yogurt starter cultures including *Lactococcus* and *Leuconostoc*. They observed a decrease in riboflavin during fermentation of buttermilk, approximately 80% of the vitamin was reduced when a culture comprising of *Lactococcus spp*. was used (407). The observed reduction in the quantity of thiamine, riboflavin and nicotinamide in this study, is potentially due to dominating bacterial communities requiring these vitamins for growth (374). Nicotinamide is essential in metabolic reactions due to being a precursor for coenzyme nicotinamide adenine dinucleotide (NADH/NAD^+), essential for their role in redox reactions particularly in glycolysis and the pentose phosphate pathway (230, 408). Due to NADH and NAD^+ being vital for cell viability, a NAD^+ homeostasis must be maintained, in most bacteria including identified bacteria, the enzyme nicotinamidase (EC: 3.5.1.19) is released to convert nicotinamide to nicotinic acid to generate NAD^+ via a salvage pathway (409-412). Therefore, the observed depletion in nicotinamide is likely due to it being converted to coenzymes, NAD and NAD^+, required for many metabolic processes to generate energy for growth. Similarly, riboflavin plays a key role in metabolic reactions, due to being a precursor for

coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), involved in electron transfer for redox reactions in metabolism (404). It is accepted that many microorganisms' synthesis riboflavin *de novo* from guanosine-5'-triphosphate and ribulose 5-phosphate (413-415). However, microorganisms that lack the genes involved in the biosynthesis process and require riboflavin for growth, which includes some LAB use a transport system for exogenous riboflavin (413, 416). Therefore, the reduction in riboflavin is likely due to its uptake by the bacterial community present in the vegetable matrix for growth. Furthermore, thiamine is a precursor for coenzyme thiamine pyrophosphate (TPP) which is essential in bacteria for both carbohydrate and amino acid metabolism (417). TPP is required for the Kreb's cycle alongside riboflavin and nicotinamide coenzymes, glycolysis and pentose phosphate pathway, with the latter two pathways being pathways used in fermentation by LAB (54, 229, 418). Most bacteria can biosynthesise thiamine *de novo* by the synthesis and subsequent joining of a thiazole and pyrimidine heterocycle, followed by phosphorylation to generate TPP (405, 419). Bacteria that lack the ability to synthesise thiamine, uptake exogenous thiamine, including thiamine precursors and phosphorylated forms, from the environment (417, 420). Degradation of thiamine could also be related to the secretion of thiaminases by certain bacteria, which breaks down thiamine into its respective precursors by substituting the base of the thiazole moiety to a nucleophile (417, 421, 422). The decrease in B vitamins initiated at 48 h, in line with recorded peak growth of microorganisms and reduction in sugar content, showing these B-group vitamins were potentially utilised during metabolism of macronutrients to generate energy for growth. Alternatively, pyridoxine (one of the B_6 vitamers) remained stable throughout storage, indicating this exogenous vitamin was not used. The active phosphorylated form of B_6, pyridoxal 5'-phosphate acts as a coenzyme essential for amino acid metabolism, including transamination and decarboxylation, and aids with oxidative stress (423-425). Microorganisms can synthesis pyridoxal 5'-phosphate *de novo* or synthesis the coenzyme via a salvage pathway using B_6 vitamins (pyridoxine, pyridoxal, pyridoxamine) (425). Therefore, it can be hypothesised that the pyridoxal 5'-phosphate requirement of the bacterial communities could have been achieved without the use of exogenous pyridoxine. Complementary to the investigation, a model buffer system was analysed to understand if the environment in terms of temperature and pH had an impact on vitamin stability during storage. The model buffered system indicated vitamins were unaffected by the storage environment and therefore the reduction in vitamin content of the vegetable matrix was a factor of spoilage reactions, or the matrix itself.

4.1.1 Metabolite Changes During Degradation of the Vegetable Matrix

Metabolomic fingerprinting is a valuable tool to reflect cellular process related to food spoilage, and this study highlighted several metabolites that have the potential to be spoilage biomarkers of the vegetable matrix. Chemometric analysis was performed using PLS-DA, classified as the "supervised" version of PCA. In context, PLS-DA generates dimensionally reduced data, while

considering information related to the specified class. Therefore, while PCA describes the most variance in the data with PC1, PLS-DA describes the most covariance between the data and respective class labelling in the first component (312). The PLS-DA showed samples collected before 24 h had a similar metabolite profile, however from 48 h the metabolite profile underwent significant change corresponding with the observed increase in microbial activity. VIP analysis was also performed which highlighted variables/metabolite features that have influenced the observed relationships (426). According to VIP analysis, hypoxanthine was one of the main annotated metabolites associated with deterioration of the matrix, increasing at 48 h. Hypoxanthine is a degradation by-product of nucleotide adenosine triphosphate (ATP), which is broken down to adenosine 5'diphosphate (ADP), then adenosine 5'monophosphate (AMP), and further to inosine 5'monophosphate (IMP). IMP is then degraded to products including hypoxanthine, all of which form part of the purine metabolism pathway, in which *Lactococcus*, *Leuconostoc* and *Yersinia* are reported to have the capabilities to use elements of this pathway (159, 189, 427-430). AMP was also a key element in the spoilage reactions reducing at 48 h complementary to the observed increase in hypoxanthine at the storage time. These findings are in agreement with many authors including Jääskeläinen *et al.* (2019) (160) and Zhang *et al.* (2020) (189) who identified IMP and associated degradation products as markers of spoilage in animal products, however at present there are no reports of this in vegetable degradation. Agmatine, acetylspermidine, acetylputrescine were important annotated metabolites that were produced at 48 h, these metabolites are classified as biogenic amines and are products of amino acid decarboxylase, produced through microbial enzymatic activity (160, 431). Amino acid decarboxylase requires the coenzyme form of vitamin B_6, pyridoxal 5'-phosphate, highlighting B_6 coenzymes are required for metabolic activity of SSOs (432). *Enterobacteriaceae* are reported to be chiefly responsible for biogenic amine formation, although a range of other bacteria are capable of biogenic amine production including LAB, which have the enzymes to decarboxylate amino acids; further these products have been previously used to determine microbial spoilage in animal products (148, 189). Agmatine is formed by arginine decarboxylase and putrescine is generated from ornithine decarboxylase, which further forms spermidine and acetylputrescine (431, 433). Alongside, arginine was another metabolite associated with degradation of the vegetable matrix, which began to decrease at 48 h and eventually was depleted at 72 h, correlating to the increase in agmatine. In addition, ornithine and citrulline was an important metabolite that increased at 48 h. Both ornithine and citrulline could have been potentially generated through utilisation of arginine by LAB via the arginine deiminase pathway, products including ammonium and carbon dioxide are also produced via this pathway (434, 435). Biopterin was an additional metabolite that reflected the impact of spoilage reactions, steadily increasing from 48 h, this metabolite has been previously demonstrated to be synthesized by bacteria from guanosine triphosphate, acting as an enzyme cofactor in metabolism (436, 437). Finally, other metabolites that were associated with spoilage reactions included: glutathione (reduced), 1-methylguanine, 1-

methyladenine, 3-methyladenine, L-iditol and prolinamide. Glutathione is a widespread thiol in various organisms and foods including vegetables, in this study glutathione begins to decrease at 24 h. Due to glutathione being an antioxidant, bacteria such as LAB, utilise this thiol for necessary cellular mechanisms to combat physiological stress conditions created from reduced pH, oxidative stress and toxin production for example (438, 439). LAB such as dominant genus, *Leuconostoc* and *Lactococcus* lack the ability to synthesise glutathione *de-novo*, due to lacking enzymes γ-glutamylcystiene synthetase, glutathione synthetase and glutathione bifunctional fusion protein (438). However, *Leuconostoc* and *Lactococcus* are proposed to have capabilities to uptake exogenous glutathione from the environment including required transport system (CydDC – heterodimeric ATP-binding cassette type transporter) and enzymes, glutathione peroxidase and glutathione reductase (438). Therefore, the reduced abundance of glutathione could be related to LAB scavenging this metabolite from the environment, due to an inability to synthesise glutathione *de-novo*. At present, after extensive literature searching no studies have been found that correlates other important metabolites including DNA methylation products, 1-methylguanine, 1-methyladenine, 3-methyladenine to spoilage activity. Methylation of DNA is where DNA is modified by the addition of a methyl group to the base, catalysed by DNA methyltransferases, exerting a protective role in restriction-modification systems (440, 441). LAB are reported to possess the genes encoding enzymes methyltransferases for DNA methylation and therefore LAB could be responsible for DNA methylation products during storage (442, 443). L-Iditol, and L-proline derivative prolinamide are other metabolites that have also not been linked to spoilage reactions and therefore can be classified as novel findings (444).

VOCs were also monitored as an extension to metabolomics using GC/TOF-MS, as combination of instruments provides a deeper inside into the metabolite profiles during the time course (199). VOCs are also an important marker of product quality by detection of flavour and odour compounds associated with spoilage (162, 294). Aldehydes, alkanes, alkenes, alcohols, SCFAs, ketones and monoterpenoids were putatively annotated compounds linked to degradation of the vegetable matrix. After extensive searching in the literature, studies monitoring VOCs during spoilage of vegetable matrices is limited. However, these findings agree with spoilage studies conducted by Odeyemi *et al*. (2018) (445) and Li *et al*. (2018) (164) who monitored VOCs during spoilage of animal-based food products. The alteration of the volatiles in the vegetable matrix could occur as both a direct and indirect result of SSOs, for example SSOs produce extracellular enzymes including lipases and proteases breaking down lipids and protein respectively, these components can be further metabolised by SSOs including sugars, which gives rise to secondary metabolites (150, 446). Alternatively, volatiles can generate from spontaneous chemical reactions such as lipid oxidation. The aldehydes in this study consisted of propanal, hexanal and 2-ethyl-2-butenal and were in great abundance at 0 h. This is potentially related to the direct impact of processing and subsequent storage after production, in which potential degradation of fatty acids have occurred through lipid autooxidation or, β-oxidation giving rise to aldehydes (164, 169).

Interestingly, propanal and 2-ethyl-2-butenal continue to decrease throughout the remainder of the storage period, this was also observed in the study conducted by Li *et al*. (2018) (164), where the authors identified a decrease in most identified aldehydes during storage of smoked bacon. It was hypothesised that the aldehydes were oxidised forming acids or reduced generating alcohols. Alternatively, hexanal decreased and then increased in abundance at 48 h, in line with microbial activity, before decreasing for the remainder of the storage duration. Hexanal is an aldehyde that can be produced by both Gram-negative and Gram-positive bacteria, therefore hexanal could have been produced by the microbial communities at this time point (169). Aldehydes in general can give rise to a rancid odour when present in high concentrations (169). Alkanes, 2,4-dimethylhexane and propane, and alkene, 1-octene were also among putatively annotated VOCs that changed during storage. The profiles of both alkanes and alkenes fluctuated throughout storage, present from 0 h, with propane increasing at 24 h and both 2,4-dimethylhexane and 1-octene increasing at 48 h. The increase in these compounds is due to them being a product of fatty acid break down and microbial metabolism, having been associated with LAB fermentation (446, 447). The increased abundance of these metabolites followed by a decrease in abundance is likely due to rapid oxidation of the alkenes and alkanes to other volatile compounds, such as alcohols (448). The oxidation of alkenes and alkanes can be catalysed by the production of oxidative enzymes, such as alkane hydroxylase by Gram-negative and Gram-positive bacteria (449). Alcohols identified in the headspace of the vegetable matrix included 1-pentanol, 1-hexanol and cyclopentanol, both 1-pentanol and 1-hexanol increased at 24 h and remained at a similar abundance for the remainder of the storage period. A recent study conducted by Wen *et al*. (2022) (450), monitored VOC composition during spoilage of lamb and similarly identified both 1-hexanol and 1-pentanol as spoilage markers. Production of alcohols in general occur due to both chemical reactions within a food matrix, such as aldehyde reduction and microbial metabolism, including metabolism of glucose and amino acids (450). The bacterial communities present in the vegetable matrix have been shown to be positively correlated to alcohol production including LAB and *Enterobacteriaceae* (169, 450). 1-Hexanol are among alcohols also produced by oxidation of fatty acids and characterised by its pungent, fruity and alcoholic aroma (169, 451). In contrast, cyclopentanol decreased at 24 h, which agrees with findings by Wierda *et al*. (2006) (452); the authors recorded a decrease in this volatile during storage of fish and described cyclopentanol as a potential marker of freshness. Furthermore, acetic acid (SCFAs) was identified as a potential marker of spoilage increasing at 24 h. This further confirms the fermentation of sugars by heterofermentative LAB, *Leuconostoc* which would have led to the production of acetic acid (374). The ketones and monoterpenoids identified in this study have not been linked to any other spoilage studies. p-Pentylacetophenone can be produced by microorganisms but are also considered a natural compound in vegetables that possess antimicrobial activity (453, 454). Similarly, monoterpenoids, for example, β-linalool is a plant metabolite found in herbs etc that has antimicrobial properties (455). It important to note the generation of VOCs in a food matrix

is due to multiple reactions both chemically and microbiologically and therefore it is difficult to underpin the direct cause of a specific volatile (450). Also, based on literature the putatively annotated volatiles discussed could not be directly linked to the non-volatile metabolites in the above section.

4.5 Conclusion

At present deterioration of foods in general are assessed using outdated and insensitive techniques that fail to capture the depths of spoilage and nutritional quality. The adoption of technological advances, specifically multi-omic approaches, provides immense potential to understand spoilage mechanisms that could aid with spoilage control. This chapter provides an extensive insight into spoilage reactions during the storage of a popular ready to eat vegetable matrix. Amplicon sequencing revealed *Lactococcus*, *Leuconostoc* and *Yersinia* were the dominant bacterial communities responsible for spoilage of the vegetable matrix. The growth of dominating organisms was facilitated by utilisation of sugars and potentially B vitamins thiamine, riboflavin and nicotinamide. Although these vitamins are essential constituents of foods, they are overlooked during storage assessments, yet this study highlighted a relationship between B vitamins and spoilage reactions. Furthermore, both metabolite and VOC profile's showed significant changes throughout the storage duration, in which some metabolites have the potential to be spoilage markers or markers of product freshness. This included metabolites: hypoxanthine, adenosine 5'monophosphate, arginine, ornithine, and biogenic amines, agmatine and acetylspermidine. Highlighting pathways such as, arginine and proline metabolism play an important role in spoilage reactions. Examples of VOCs that also have the potential to be spoilage markers include: 1-pentanol, 1-hexanol and hexanal. This comprehensive spoilage profile combining -Omic techniques has not been performed in a vegetable matrix and this storage study has formed the baseline for further investigations in the forthcoming chapters.

Chapter 5

Investigating the Effect of Storage Environments and Preparation Procedures on Food Spoilage and Nutritional Composition in a Vegetable Matrix

5.1 Introduction

The nutritional stability and spoilage of the vegetable matrix are dependent on intrinsic properties, including pH and redox potential. This is in combination with extrinsic factors the vegetable matrix is exposed to during storage, including temperature and gas composition (163, 456, 457). The previous chapter captured a relationship between vitamin content, bacterial dynamics, and markers of spoilage, but storage condition could also influence this relationship, and this has not been explored in the literature. For example: microorganisms have a preferred temperature for growth, further influencing competition amongst species and potentially impacting on the vitamin composition (112, 458). Inadequate temperature control during storage can result in accelerated quality defects through oxidation, increased enzyme activity and microbial growth (10, 16, 105, 459). Alvarez *et al*. (2014) (24) showed during storage of vegetables, mesophilic bacteria significantly increased when storage temperature increased. Comparably, Hoel, Jakobsen and Vadstein (2017) (23) investigated the impact of temperature control on commercially available sushi and highlighted microbiological quality suffered as storage temperature increased (4-20 °C). In addition, many of the B group vitamins are also vulnerable to temperature which has been demonstrated during both processing and storage of several food matrices, including storage of pasta, processing of pulses, noodles, rosehip and potatoes (224, 225, 251, 259, 460, 461). However, many previous reports use high temperatures in their studies (50 °C+) to capture vitamin degradation, which cannot be compared to storage temperatures used by consumers. Alternatively, temperatures in the region of -18 °C or below are often used by consumers as a preservation technique, inhibiting growth of microorganisms whilst also reducing chemical and biochemical reactions (462). It is well acknowledged that the freeze-thaw process is an environmental stress on microbial cells, in which cells are either damaged or subject to metabolic injury (462). However, the microbial dynamics after the thawing process is not well studied in the literature.

The presence and concentration of gases such as oxygen and nitrogen in a food system due to either environmental exposure or packaging technology such as modified atmosphere packaging (MAP) can influence microbial spoilage and production of spoilage metabolites (25). This has largely been represented in both meat and fish matrices and reports in vegetable products are limited (12, 463-465). Erolini *et al*. (2011) (25) captured a notable change in microbial composition during storage of beef under different gas compositions (air, vacuum packaging,

MAP etc). They showed during storage in air *Pseudomonas spp.* dominated towards the end of the storage period, however, in the absence of oxygen, lactic acid bacteria (LAB) were predominant. The change in microbial composition further influenced production or exhaustion of specific metabolites (25). Similarly, Ioannidis *et al*. (2018) (466) showed during storage of iceberg lettuce in different modified atmospheres, resulted in changes to the bacterial community. During storage in an anaerobic environment, LAB were responsible for spoilage and authors were able to identify volatile organic compounds (VOCs) associated with dominating organisms, such as acetic acid and 2,3-butanedione (466). Shifts in microbial communities also has the potential to influence vitamin content, due to different microorganisms having different nutritional requirements for growth which has not been investigated in a vegetable matrix.

The pH environment can influence vitamin stability and spoilage reactions during storage, shaping the microbial dynamics within a food system (81). Specific spoilage organisms (SSOs) are known to be neutrophils and grow optimally between pH 6-7.5 (72, 389). SSOs can struggle to adapt and grow outside their optimum pH range, as an example, SSOs that grow optimally at neutral pH would struggle to grow in acidic food matrices, this is largely due to acidic environments inactivating enzymes involved in energy production (81). Thiamine and riboflavin are B-group vitamins extremely sensitive to pH, vulnerable to degradation at neutral/basic conditions and therefore are susceptible to degrade in slightly acidic/neutral foods (pH 5-7) (228, 467). It is important to note the aspects of literature focusing on the impact of pH on vitamins have been performed in aqueous solutions, while studies investigating the impact of pH on vitamins over storage in a food system is scarce.

The aims of this chapter are to evaluate the impact of storage environments and preparation procedures on microbial spoilage and nutritional composition of the perishable vegetable matrix. A controlled storage environment will aid understanding of the role of storage conditions on microbial communities including spoilage metabolites and nutritional status, while further exploring the relationship between microbial growth and nutritional status. The following objectives of the study are 1) assess the effect of temperature, 2) outline the impact of different preparation procedures (prepared from frozen versus prepared from fresh), 3) evaluate the effect of presence and absence of oxygen and 4) investigate the effect of altering the pH (reduced pH verus increased pH) of the vegetable matrix on microbial growth (including metabolites) and nutritional status.

5.2 Methods

5.2.1 Vegetable Matrix Preparation and Storage

Refer to general methods section of the thesis (chapter 2), see section 2.1 for preparation of the vegetable matrix and both section 2.3 and section 2.4 for all storage procedures investigated. Storage duration is the same as outlined in previous chapter (0-120 h) for each storage condition, however, due to accelerated spoilage rate the 37 °C storage condition concluded at 72 h. An overview of each storage method can be seen in Figure 5.1.

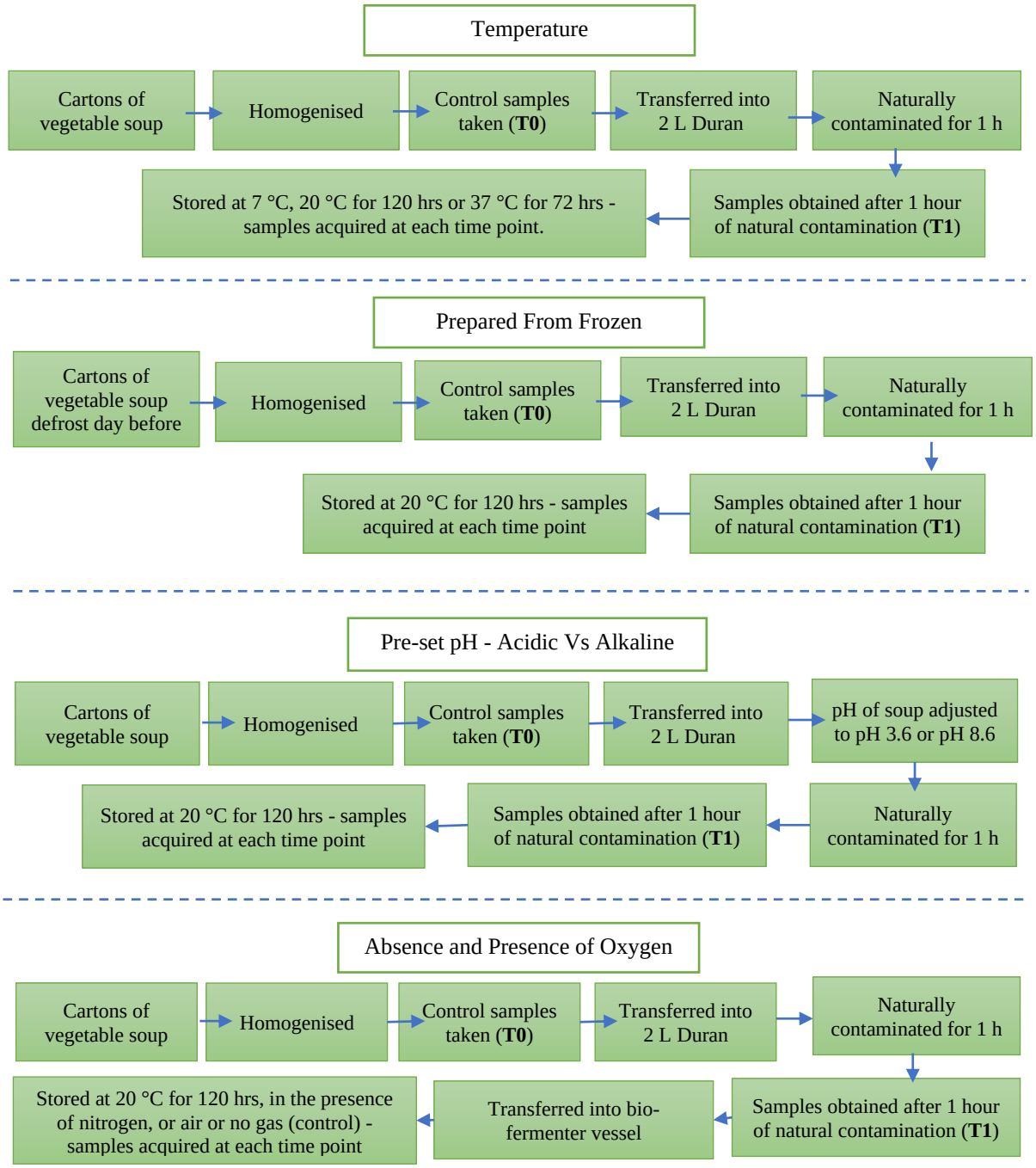


Figure 5.1: Flow diagram of the preparation steps and different storage procedures of the vegetable matrix.

This investigation into different storage conditions continues from the previous chapter which formed a baseline for this investigation and, as a result, data for the baseline will be included in this chapter for comparison. Each of the originally purchased soup batches used in the previous chapter were utilised for the experimental groups for this work.

5.2.2 Sample Analysis and Data Analysis

Methodologies on all the analysis completed during the time-course, including vitamin analysis, pH measurements, microbial analysis, VOC analysis, amplicon sequencing and metabolomics are outlined in chapter 2, sections 2.6-2.12. Data analysis is also outlined in the stated sections.

5.3 Results

5.3.1 Temperature

5.3.1.1 The Effect of Temperature on Microbial Growth

Microbial growth was assessed during storage of the vegetable matrix under different temperatures and the representative growth profile can be viewed in Figure 5.2. The total viable count (TVC) during storage of the vegetable matrix at 7 °C, showed an extended lag phase of 72 h, whereas storage at 20 °C and 37°C reduced the lag phase to 8 h. Increased storage temperature increased the growth of microorganisms earlier in the time series, with exponential growth experienced between 8-48 h for 20°C and 8-24 h for 37 °C (7.71 log CFU/g at 24 h).

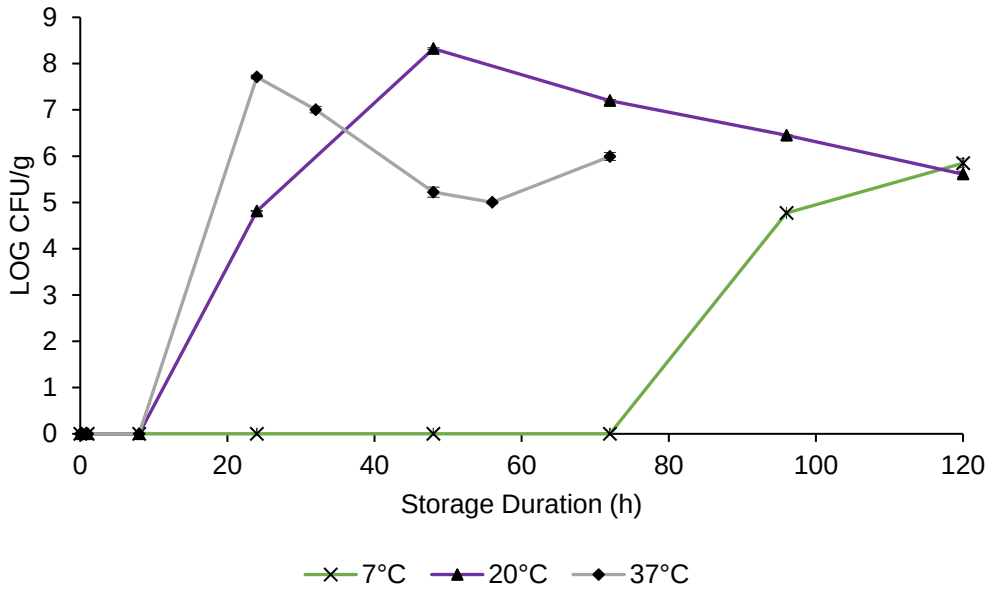


Figure 5.2: TVC on plate count agar over the storage duration (120 hrs) of vegetable matrix under different temperatures, 7 °C, 20°C, 37 °C. For each data point, two replicates were analysed, and the error associated with each time point is represented by a 95% confidence interval.

5.3.1.2 The Effect of Temperature on Bacterial Diversity and Composition - Alpha Diversity

Storage of the vegetable matrix at 7 °C, resulted in significant changes across all diversity measures (richness, Shannon index and Inverse Simpson index) (Figure 5.3). As the storage time increased from 0 h to 120 h, all diversity measures significantly decreased ($p < 0.05$). In comparison, storage at 37 °C also showed a reduction in richness and diversity indices, Shannon and Inverse Simpson, similar to 20 °C (Figure 5.3). Although the decrease in bacterial diversity over the time course was only significant for diversity metric, Shannon Index. In addition, the diversity indices were compared at each time point across the different storage temperatures. The values for both richness and Shannon index at T0, T32/T48, T72/T120 at 7 °C, 20 °C and 37 °C were not significantly different. However, there were significant differences in the mean ranks of the calculated Inverse Simpson Index across these storage conditions at 0 h ($p = 0.039$), 48 h ($p = 0.039$) and 120 h ($p = 0.027$). All statistical data including follow-up post-hoc results are available in Appendix 9.

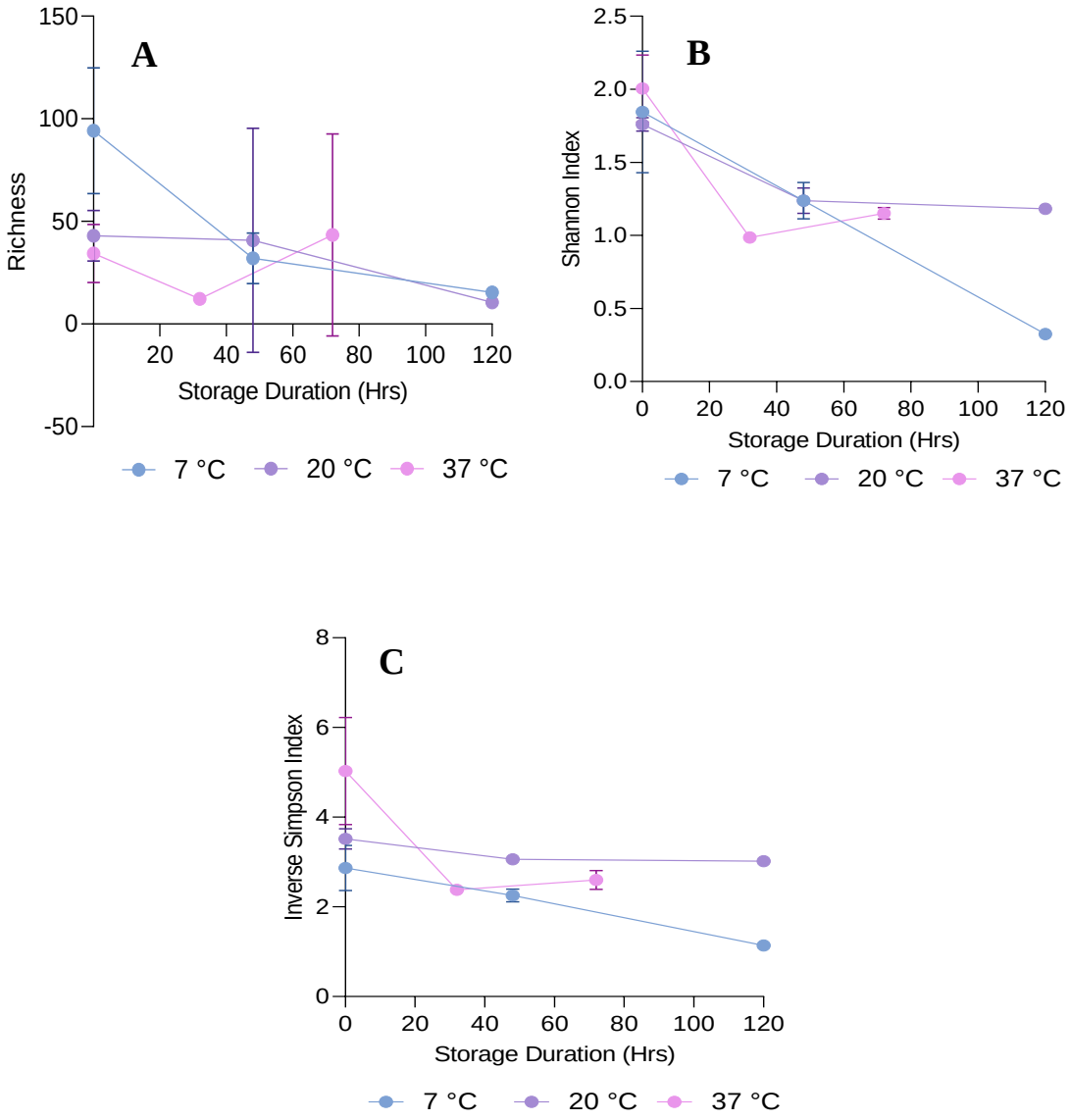


Figure 5.3: Alpha diversity of the overall microbial composition during storage of the vegetable matrix at 7 °C, 20 °C and 37 °C. Alpha diversity was determined by total richness (A), Shannon index (B) and Inverse Simpson index (C). Each data point comprises of three technical replicates and associated error is expressed by standard deviation.

The overall microbial composition of the vegetable matrix was monitored throughout the storage duration, under different temperatures and presented in Figure 5.4. Before storage under these conditions (0 h), the microbial community was diverse; in descending order *Lactococcus*, *Leuconostoc*, *Weissella*, *Escherichia*/*Shigella*, *Yersinia* and *Pseudomonas* were some of the most abundant communities at 0 h, 7 °C, which is comparable to 20 °C. Similarly, at 0 h 37 °C, in order of abundance *Lactococcus*, *Leuconostoc*, *Escherichia*/*Shigella*, *Yersinia*, *Pseudomonas* and *Bacillus* were the most predominant communities present. Following on, at 7 °C, the microbial communities at 48 h were similar to that of 0 h, however as storage progressed to 120 h *Pseudomonas* became the most abundant (approximately 90%). Storage at 37 °C resulted in shifts of the microbial composition, by 32 h, *Bacillus* was the most abundant (~56.25%), followed by *Leuconostoc* (~31.25%) and *Lactococcus* (~12.5%). This is comparable to storage at 20 °C which primarily consisted of *Lactococcus* and *Leuconostoc*, although *Yersinina* was also abundant at 20 °C. The bacterial communities at 32 h during storage at 37 °C was further emulated at 120 h, although *lactobacillus* started to gain abundance of approximately 3%.

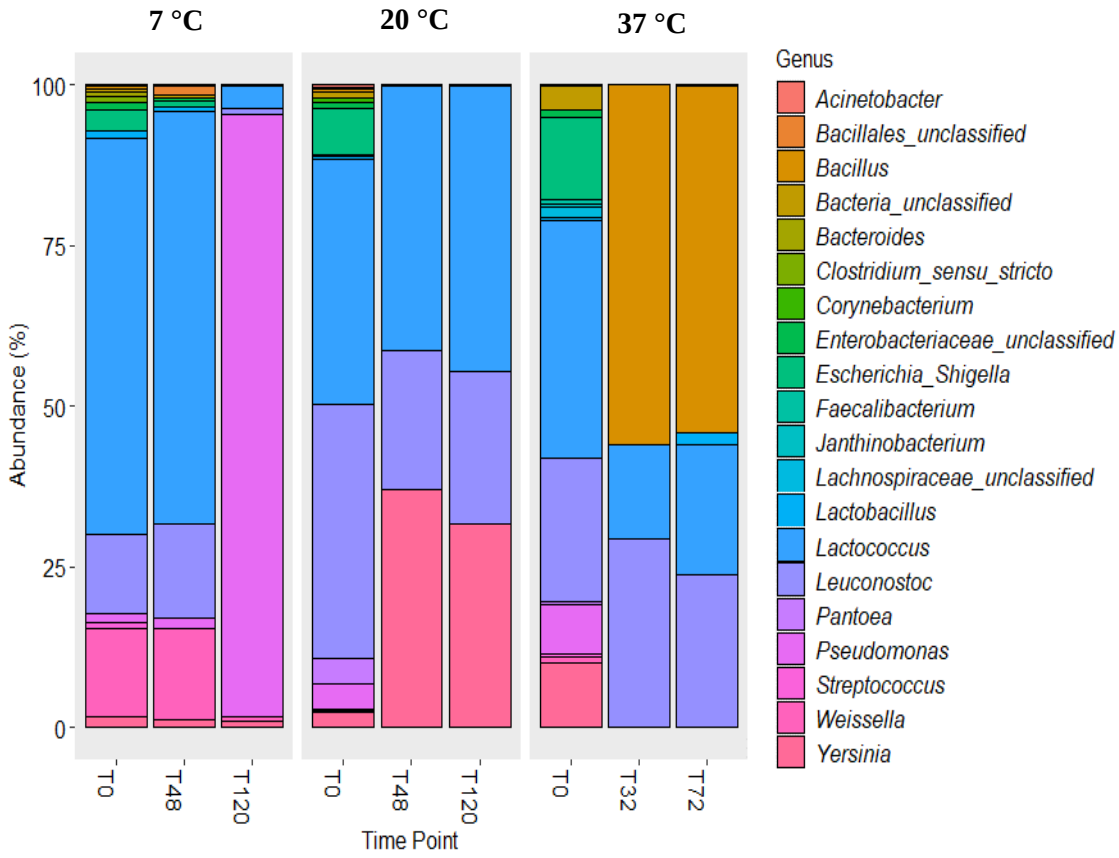


Figure 5.4: Microbial composition and development throughout the time course of the vegetable matrix stored under different temperatures 7 °C, 20°C and 37 °C. The microbial composition was expressed as the % abundance of the top 20 bacterial communities classified at genus level. Each time point comprises of three technical replicates.

5.3.1.3 Impact of Time and Temperature on Microbial Composition – Beta-Diversity

Bray-Curtis dissimilarity metric highlighted storage temperature and time can influence bacterial composition visualised using principal coordinate analysis (PCoA) (Figure 5.5). Samples collected at 0 h for 7 °C, 20 °C and 37 °C were clustered together, alongside 7 °C T48 samples. However, the middle and end time points during storage at 20 °C and 37 °C clustered in close proximity to one another, distanced from T0 samples, with the variance in the time points explained by axis 1 (53.3%). However, samples collected at 120 h (T120) at 7 °C clustered away from all other time points, variance explained by axis 2 (19.9%). The permutational multivariate analysis of variance (PERMANOVA) test was conducted and confirmed both storage temperature ($p = 0.001$, $R^2 = 0.2144$) and time ($p = 0.001$, $R^2 = 0.5929$) significantly impacted bacterial composition. Follow-up post hoc results showed significant differences in the bacterial composition between storage at 37 °C and 7 °C ($p = 0.033$), and 20 °C and 7 °C ($p = 0.039$).

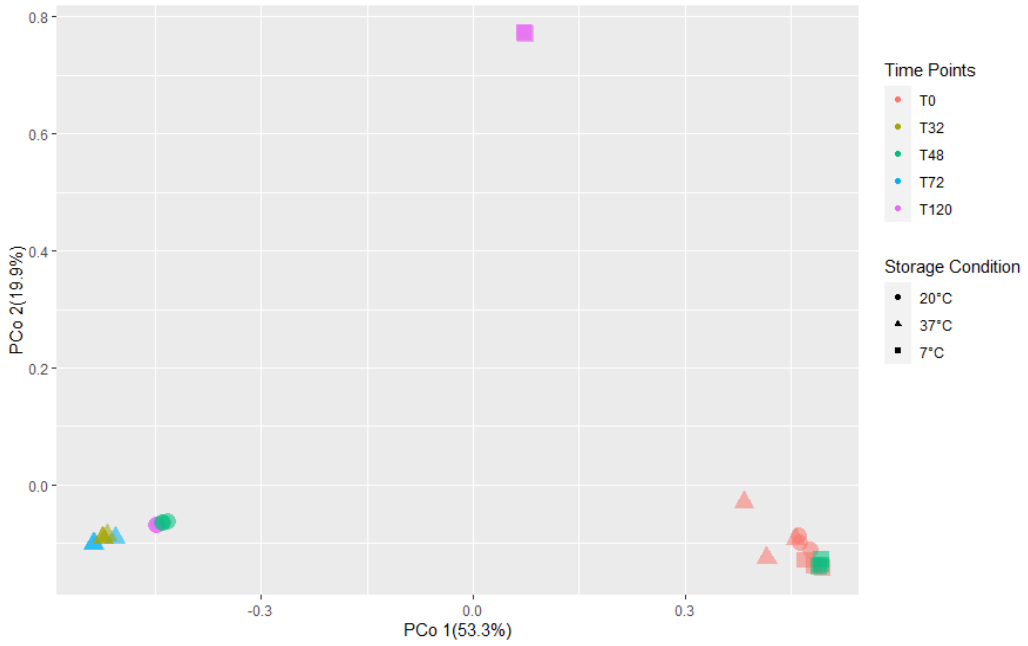


Figure 5.5: Principal coordinate analysis of Bray-Curtis dissimilarity distance matrix, samples grouped by time points 0 h – 72/120 h and storage condition under different temperatures (7 °C, 20 °C and 37 °C). Each time point comprises of three analysed samples.

5.3.1.4 The Effect of Temperature on pH Profile

The impact of storage temperatures on pH is shown in Figure 5.6. The pH of the vegetable matrix when stored at 7 °C did not change throughout the 5 days. However, storage at 37 °C showed a drop in pH of 28.5% at 32 hours, from an initial recorded pH of 5.61 to pH 4.01 at 32 h. The pH profile of the vegetable matrix during storage at 37 °C was comparable to 20 °C, although storage at 37 °C reduced the pH earlier in the time course.

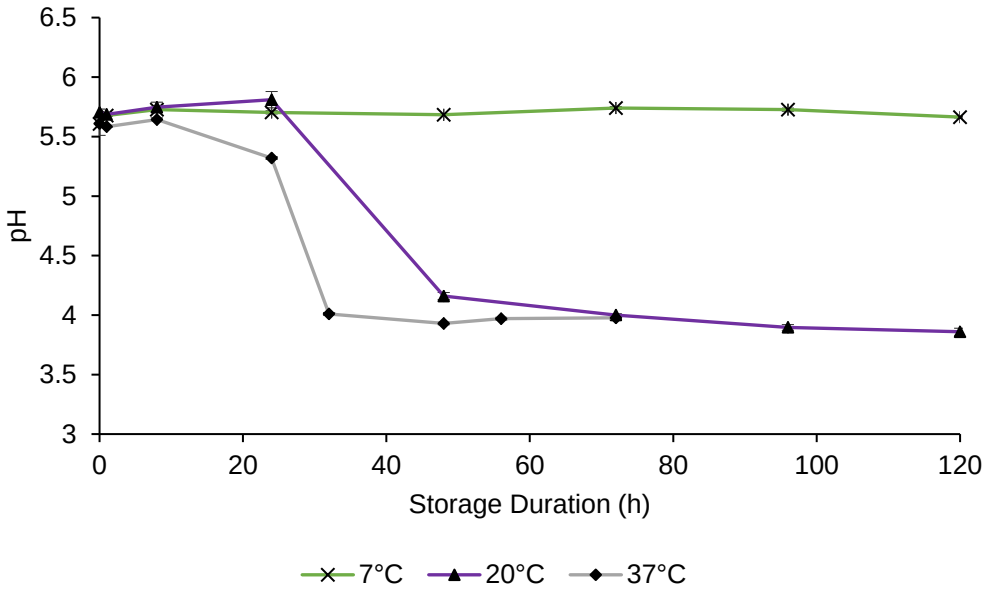


Figure 5.6: The pH profile of the vegetable matrix during 5-days of storage under different temperatures, 7 °C, 20°C and 37 °C. pH was measured in triplicate at each time point and associated error expressed by a 95% confidence interval.

5.3.1.5 The Effect of Temperature on Metabolite Analysis

The metabolite profiles were evaluated throughout storage of the vegetable matrix (start, middle and end of the time course) under different temperatures. The putatively annotated metabolites were visualised initially using principal component analysis (PCA) to check data quality and identify patterns within the data. After PCA, partial least-squares discriminant analysis (PLS-DA) was used, which generated a score plot that clusters samples according to the similarity of the metabolite profile, as shown in Figure 5.7. Samples are clustered in association with the respective storage condition and no overlapping clusters were observed. The main metabolites responsible for the discrimination between samples were determined using variable importance on projection (VIP) metric shown in Figure 5.8 and representative box plots are shown in **Error! Reference source not found.**. At 48 h glutathione, adenosine 5'-monophosphate and 2'-deoxyadenosine were examples of discriminatory metabolites abundant during storage at 7 °C. Pathway analysis was conducted on the putatively annotated metabolites (Figure 5.10), which showed metabolites were significantly associated with purine metabolism ($P < 0.01$) and glutathione metabolism ($P = 0.0234$).

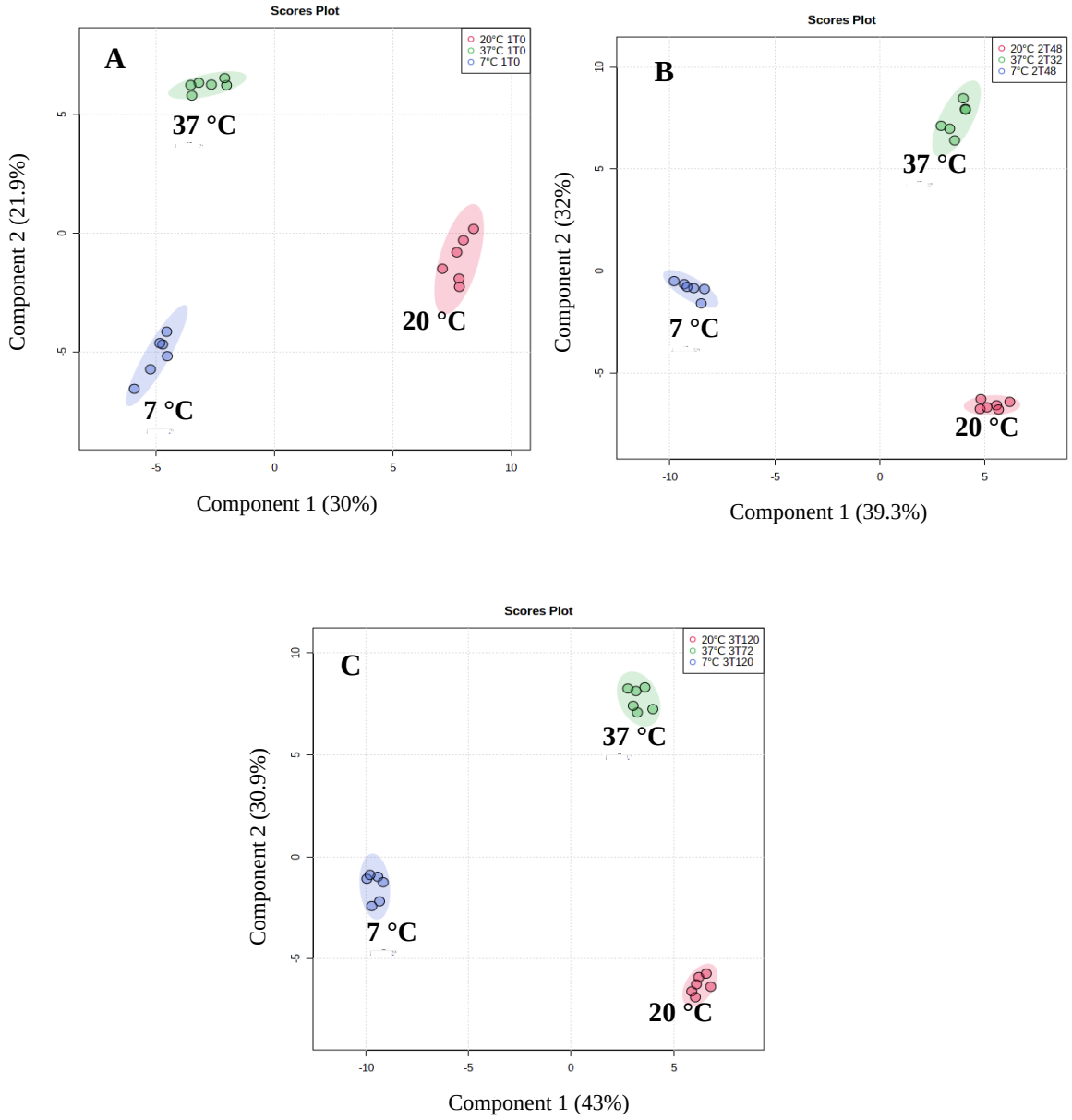


Figure 5.7: PLS-DA score plots of the effect of temperature on metabolite profiles at 0 h (A), 32/48 h (B) and 72/120 h (C) during storage under different temperatures (7 °C, 20°C and 37 °C). Each time point under each storage condition is composed of six technical replicates.

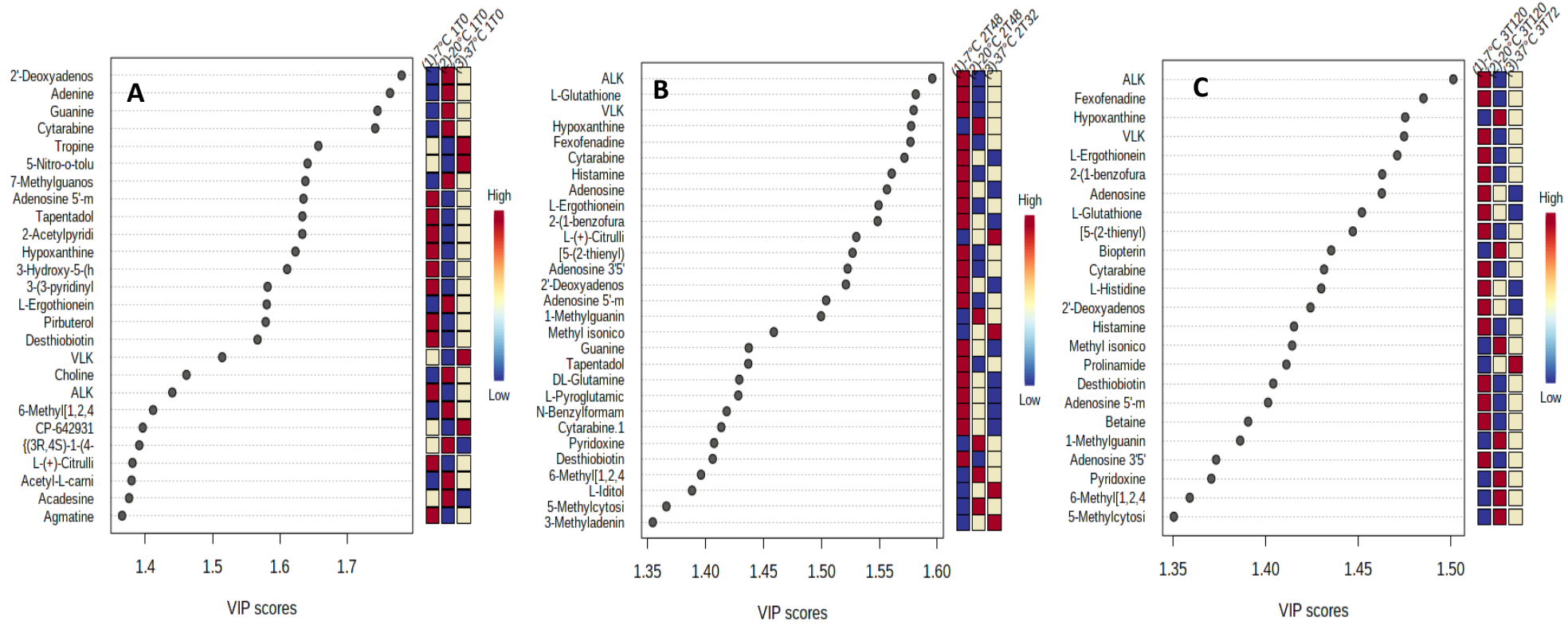


Figure 5.8: VIP ranking of the variables/metabolites (VIP > 1.35) at 0 h (A), 32/48 h (B) and 72/120 h (C) when comparing storage of the vegetable matrix under different temperatures (7 °C, 20 °C and 37 °C), as identified by PLS based on component 1.

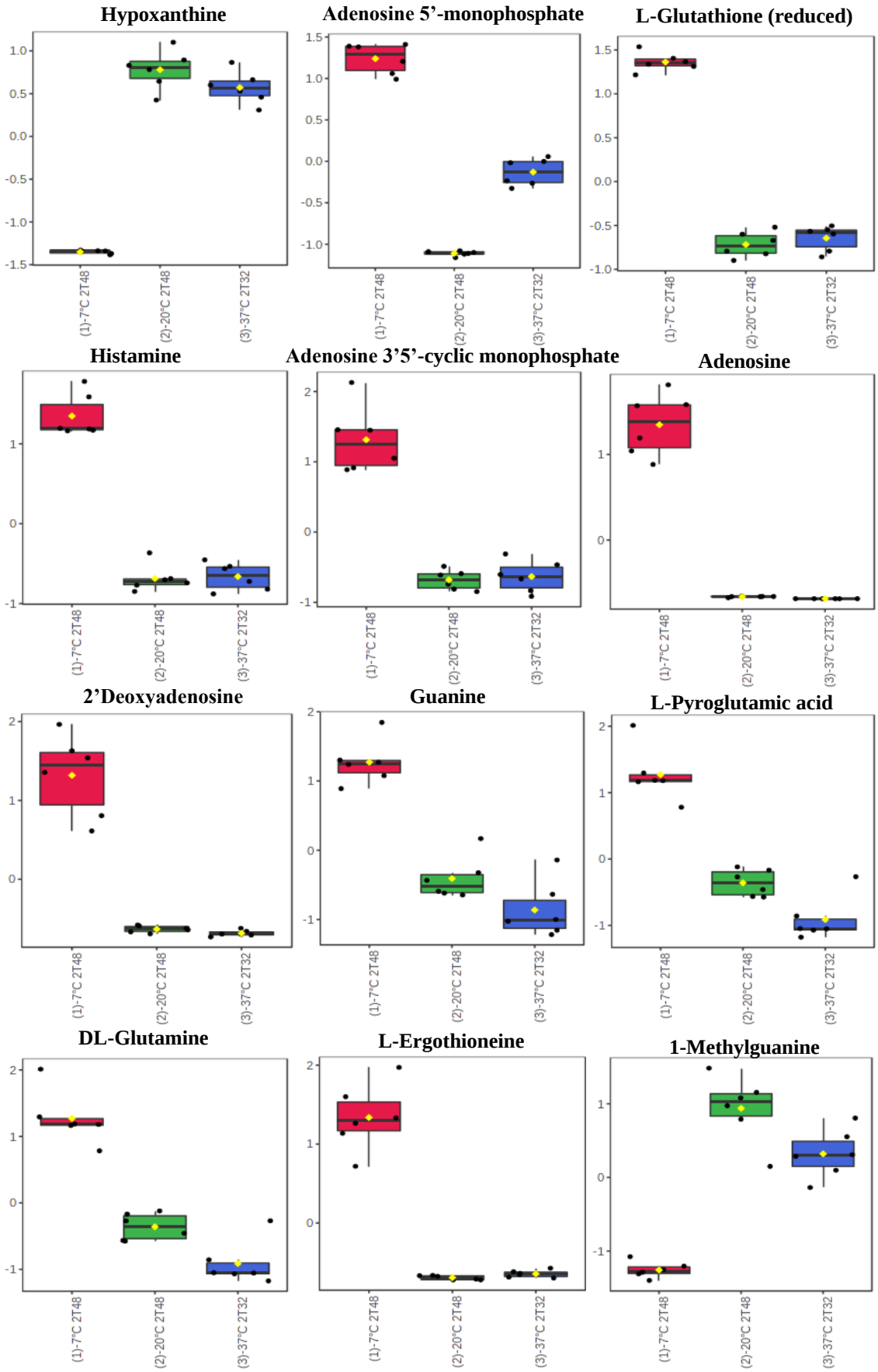


Figure 5.9: Examples of key discriminatory metabolites according to VIP analysis during storage under different temperatures (7 °C, 20 °C and 37 °C) at 48 h. Y axis represents normalised peak area (arbitrary units) and X axis represents different storage temperatures. Each condition at a specified time point is composed of six technical replicates.

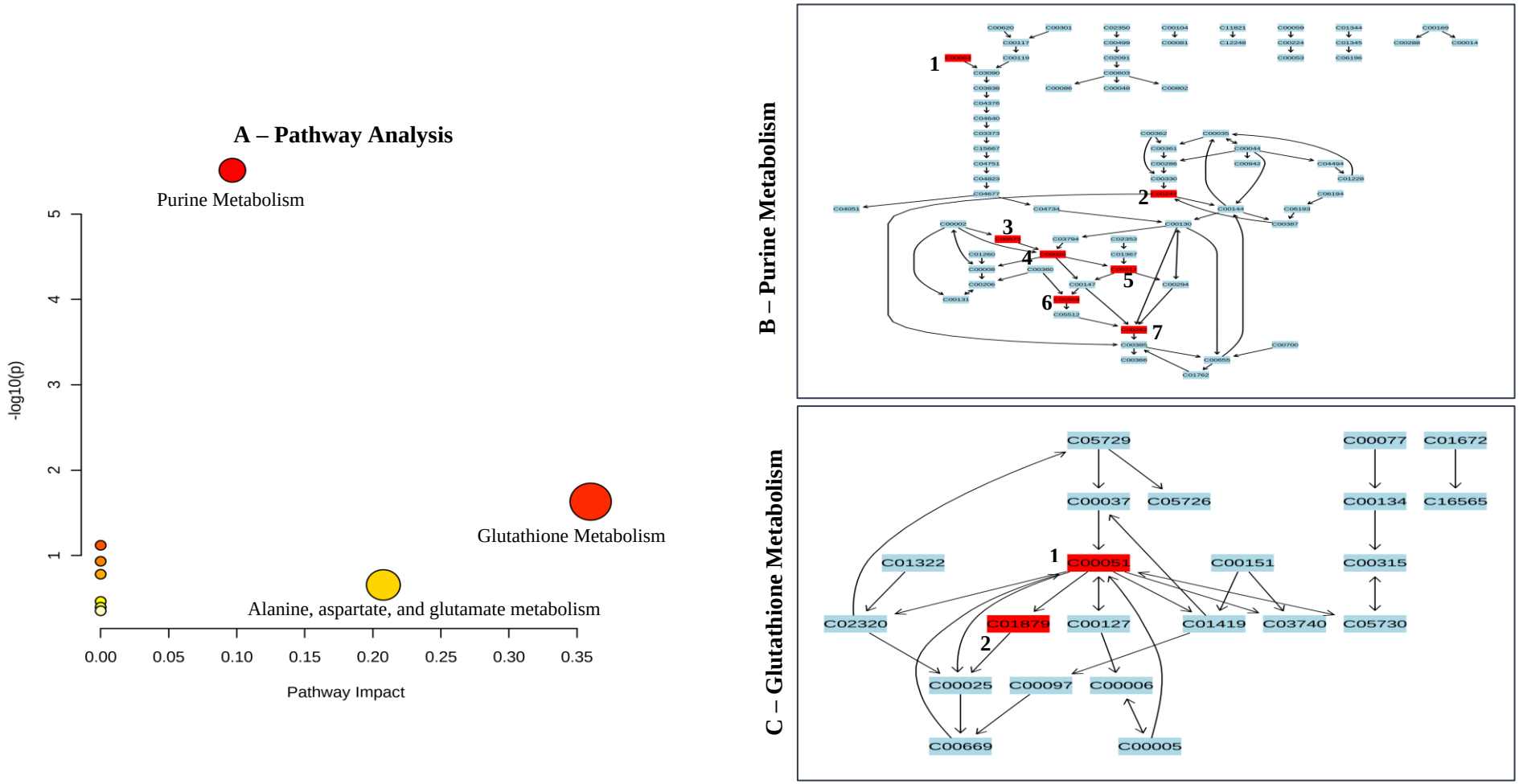


Figure 5.10: Pathway analysis of putatively annotated metabolites (A) and pathways shown (B and C) are deemed to have significant association with some of the putatively annotated metabolites, which are highlighted in red. For purine metabolism, metabolites include 1= glutamine, 2= guanine, 3= adenosine 3'5'-cyclic monophosphate, 4= adenosine 5' monophosphate, 5= adenosine, 6= 2'deoxyadenosine and 7= hypoxanthine. For glutathione metabolism, metabolites include 1= glutathione and 2= L-pyroglutamic acid.

5.3.1.6 The Effect of Temperature on VOC Analysis

The volatile organic compounds (VOCs) were profiled investigating the middle time points (32/48 h) across each storage condition, due to 48 h being indicative of spoilage reactions.

The top 25 most significant features that were retained from a total of 1,101 extracted features were visualised using principal component analysis (PCA) and shown in Figure 5.11. According to the PCA, all samples collected during the middle of the time course clustered in association with their respective storage temperature away from each other. The top features responsible for the relationship in the PCA were putatively annotated and are available in Table 5.1. Example of volatiles that were identified in high abundance during storage at 37 °C included β-linalool, acetic acid and isooctanol. In comparison, during storage at 7 °C these volatiles were low in abundance, whereas compounds such as γ-terpinen, undecanal and cymene were high in abundance at 7 °C. At 20 °C, 1-hexanol and acetic acid were the main volatiles in high abundance during this storage temperature.

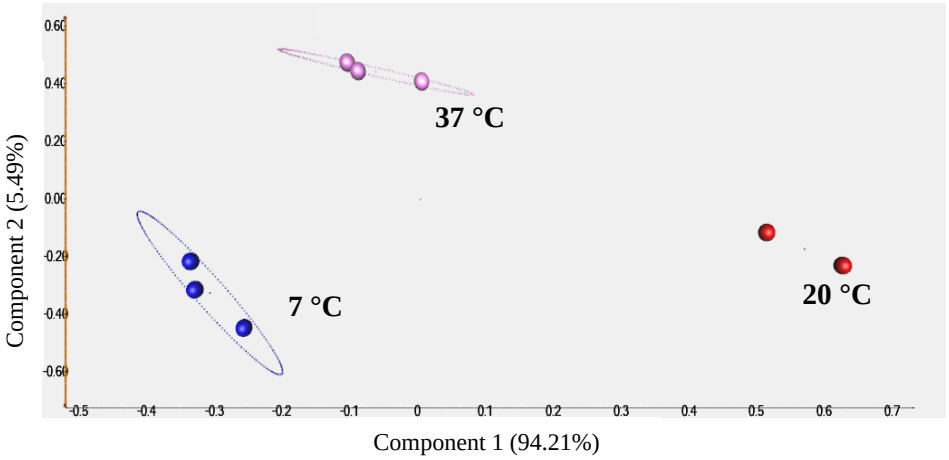
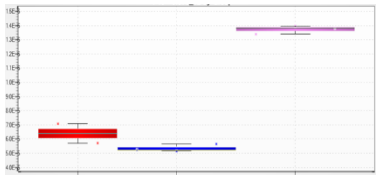
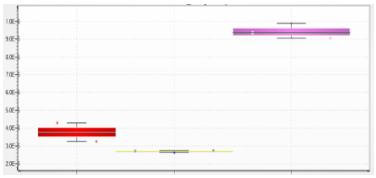
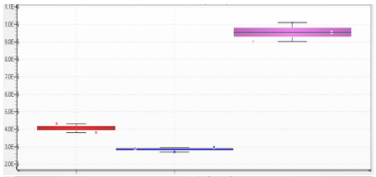
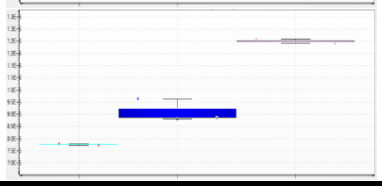
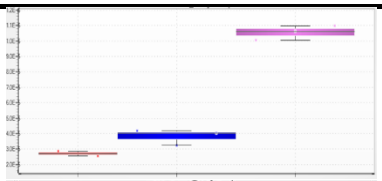
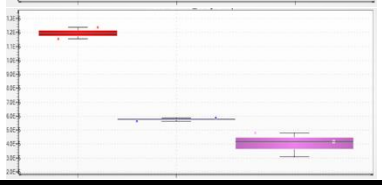
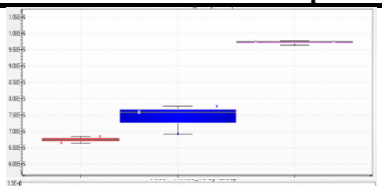
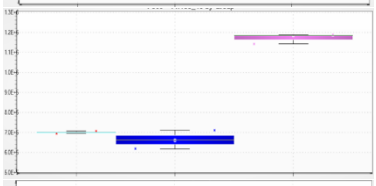
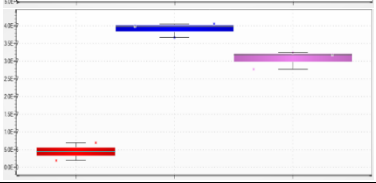
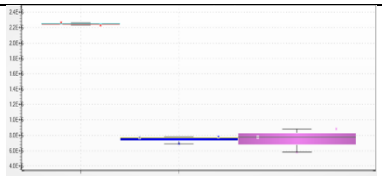
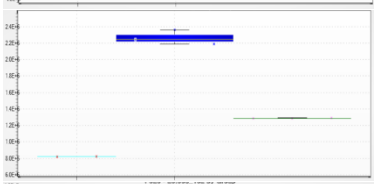
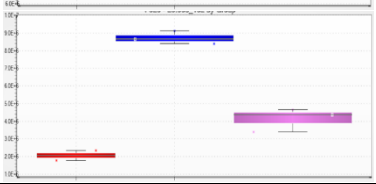
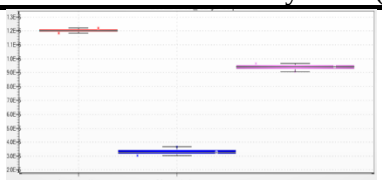
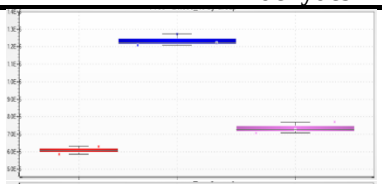
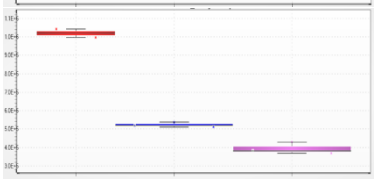
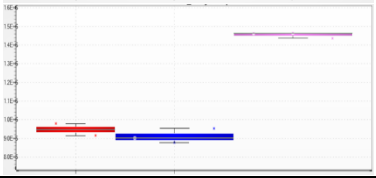
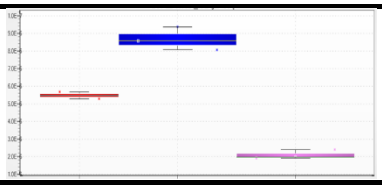


Figure 5.11: PCA comprising of the top 25 most significant features when comparing storage under different temperatures (7 °C, 20°C and 37 °C) at 32/48 h. Each storage condition contains three technical replicates, with the exception of 20 °C which is composed of two technical replicates.

Table 5.1: The top discriminatory annotated VOCs according to PCA when comparing storage under different temperatures (7 °C, 20°C and 37 °C) at 32/48 h. Box plots also included red = 20 °C, blue = 7 °C and pink = 37 °C, and y axis represents abundance.

Putatively Annotated Volatiles		Storage temperature volatile is highest		
Alkanes and Alkenes		7 °C	20 °C	37 °C
3-Ethylhexane				×
2,4-Dimethylhexane				×
Nonane				×
Decane				×
Alcohols				
Isooctanol				×
1-hexanol			×	
Monoterpenes				
γ-Terpinene*				×
β-Linalool				×
γ-Terpinene*		×		

Terpinolene			×	
p-Cymene		×		
1-Propenylbenzene		×		
Short chain fatty acids (SCFAs)				
Acetic acid			×	
Aldehydes				
Undecanal		×		
Dodecanal			×	
2,3,6-Trichlorobenzaldehyde				×
Ketones				
1-(Ethylthio)-2-propanone		×		

* Different isomers

5.3.1.7 The Effect of Temperature on Stability of B-Vitamins

Nicotinamide, pyridoxine, riboflavin, and thiamine were profiled during storage of the vegetable matrix under different temperatures as shown in Figure 5.12. Storage at 7 °C resulted in minimal changes to the concentration(s) of investigated vitamins over the 5-day storage period ($P > 0.05$). Alternatively, increasing storage temperature of the vegetable matrix to 20 °C and 37 °C showed significant loss in the quantity of nicotinamide (37 °C $P = 0.001$), riboflavin (37 °C $P = 0.007$) and thiamine (37 °C $P = 0.012$). However, during storage at 37 °C, nicotinamide, riboflavin and thiamine degraded earlier in the time course when compared to storage at 20 °C, with degradation initiating from 24 h at 37 °C compared to 48 h at 20 °C. There were no significant changes in the content of pyridoxine during storage at 7 °C, 20 °C or 37 °C ($P > 0.05$). Descriptive statistics can be seen in Table 5.2 and post-hoc results from repeated measures ANOVA are available in Appendix 4 (20 °C) and Appendix 8 (37 °C).

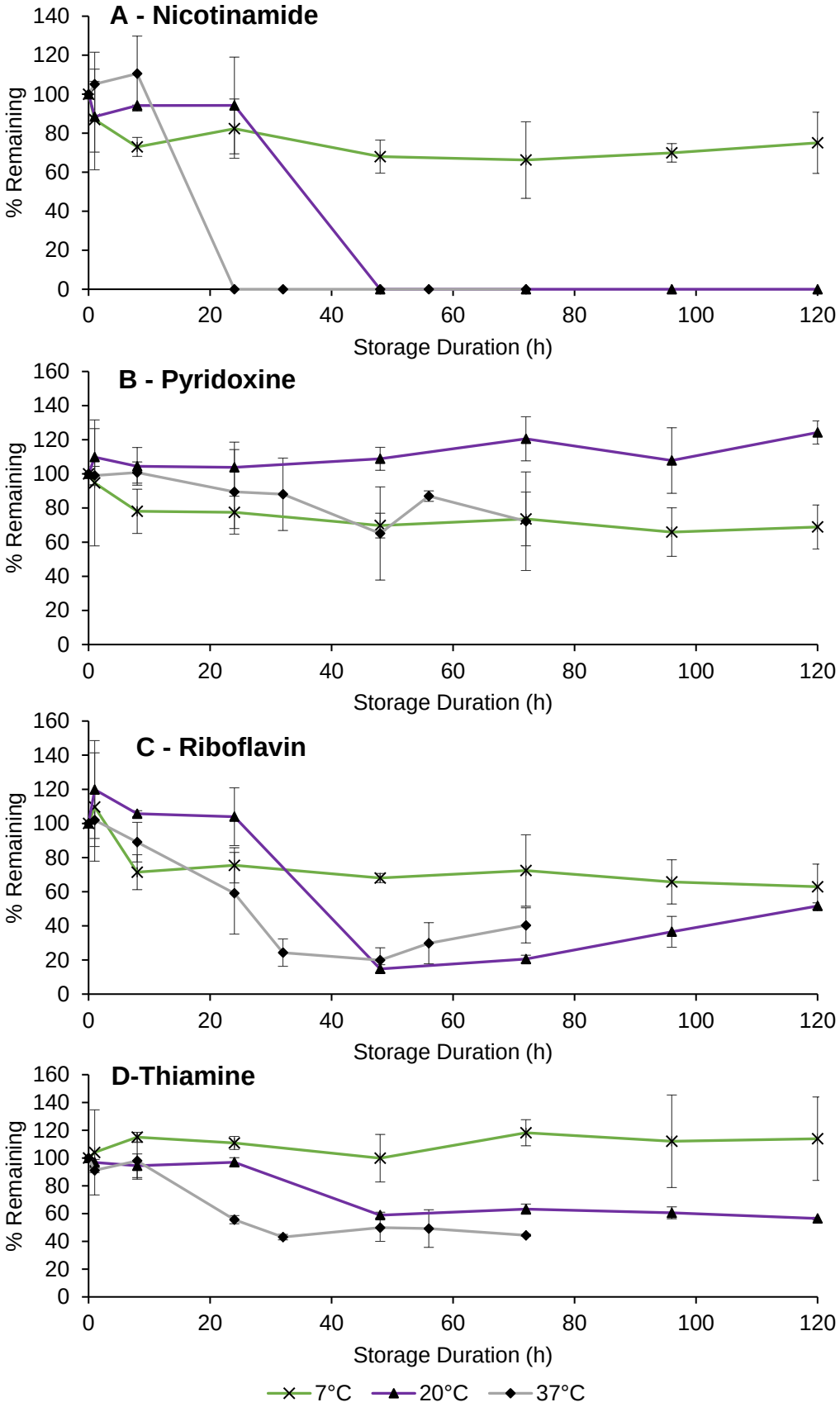


Figure 5.12: Stability of B-group vitamins, nicotinamide (A), pyridoxine (B), riboflavin (C) and thiamine (D) in the vegetable matrix over 5 days of storage (120 hrs) under different temperatures, 7 °C, 20°C and 37 °C. Stability is represented as the % remaining of each vitamin based on quantity of each vitamin at T0. Each data point is an average of three or two analysed samples and associated error is expressed by a 95% confidence interval.

Table 5.2: Descriptive statistics of vitamin stability in vegetable matrix stored over a 5-day period under different temperatures (7 °C, 20 °C and 37 °C).

					7 °C			
	Nicotinamide		**Pyridoxine**		**Riboflavin**		**Thiamine**	
Time point/s	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV
T0	1092.60	75.77	273.03	62.79	354.21	94.15	145.90	33.46
T1	950.97	249.21	258.60	89.07	364.08	71.15	151.80	39.49
T8	797.50	46.93	213.13	31.27	252.98	32.05	167.80	4.47
T24	899.50	146.69	211.50	22.94	267.3	32.34	161.70	6.03
T48	742.97	81.90	190.40	17.45	240.98	8.37	145.70	22.07
T72	742.00	189.46	200.93	37.96	256.47	65.61	172.40	12.16
T96	764.20	45.60	179.90	34.28	232.92	40.48	163.50	42.96
T120	820.17	151.58	188.10	31.16	222.88	41.38	166.17	38.66
					20 °C			
Time point/s	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV
T0	591.20	80.46	95.70	1.91	232.57	31.15	278.10	3.40
T1	523.10	94.75	105.10	14.12	278.77	58.98	269.23	5.30
T8	556.93	1.27	99.90	9.35	245.80	3.64	262.6	20.88
T24	557.20	129.58	99.30	12.52	241.70	34.79	269.57	8.12
T48	ND		104.17	5.69	34.30	5.11	163.73	5.20
T72	ND		115.37	10.96	47.80	4.65	175.67	9.05
T96	ND		103.20	16.25	84.93	18.57	168.37	10.46
T120	ND		118.93	5.71	120.17	3.72	157.00	2.19
					37 °C			
Time point/s	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV
T0	983.63	167.13	277.60	11.65	726.48	49.26	155.27	6.18
T1	1034.23	142.50	274.91	13.20	740.66	99.33	141.43	2.17
T8	1087.30	167.28	279.78	15.30	646.93	74.46	152.20	18.15
T24	ND		248.39	60.81	429.2	153.58	86.367	3.98
T32	ND		244.46	52.02	176.58	51.58	66.87	2.72
T48	ND		180.73	66.94	155.32	32.81	77.43	13.53
T56	ND		200.60	70.85	216.52	77.33	76.40	18.62
T72	ND		254.35	38.17	292.57	65.94	68.90	0.98

[a] Mean is comprised of three samples (n=3), if a sample was missing completely at random, the value was replaced using the expectation-maximisation (EM) algorithm.
*ND = not detected.

5.3.2 Preparation of the Vegetable Matrix from Frozen

In this chapter the from frozen batch (discussed in chapter 4) was further stored at 20 °C for 120 h and compared to the vegetable matrix prepared from fresh.

5.3.2.1 The Effect of Freezing on Microbial Growth

The TVC as a function of the preparation procedure implemented can be seen in Figure 5.13. Preparation of the vegetable matrix from frozen increased the lag period to 24 h. This was followed by exponential growth at 48 h to 6.20 log CFU/g, which is lower than the viable count recorded for the fresh vegetable matrix at 48 h (8.32 log CFU/g). It is important to acknowledge when culturing the microorganisms on plate count agar for the vegetable matrix prepared from frozen, there was a noted difference in the morphology of cultured colonies, compared to the colonies observed in the fresh vegetable matrix, visual representation of this can be seen in Figure 5.14.

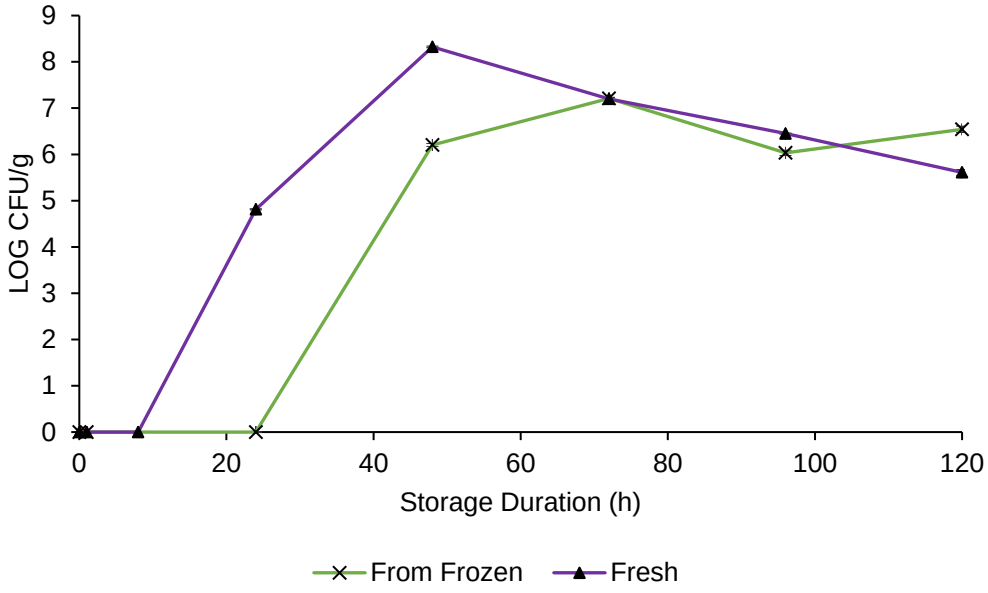


Figure 5.13: TVC of the vegetable matrix when prepared from frozen and from fresh, stored at 20 °C for 120 h (5-days). For each data point duplicate dilution series were performed, of which two replicates were analysed per dilution series (n=4), except for vegetable matrix prepared from fresh (n=2). The error associated with each time point is represented by a 95% confidence interval.

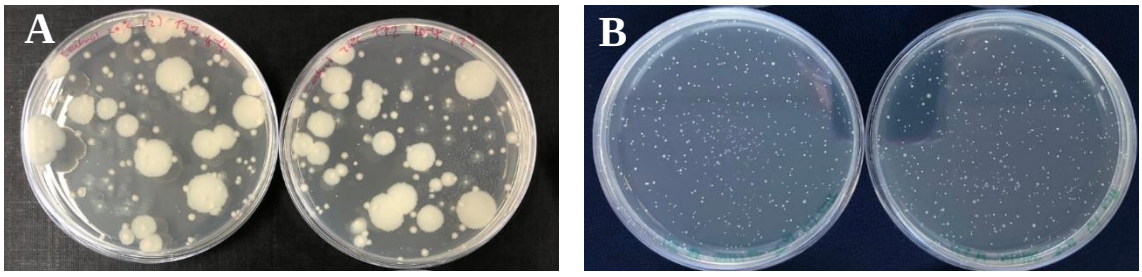


Figure 5.14: Image representing cultured colonies on plate count agar, when the vegetable-based matrix was prepared from frozen and stored at 20 °C (A) compared to when the soup was prepared fresh and stored at 20 °C (B).

5.3.2.2 The Effect of Freezing on Bacterial Diversity and Composition – Alpha Diversity

The bacterial diversity of the vegetable matrix when prepared from frozen, in terms of calculated richness, Shannon index and Simpson index is shown in Figure 5.15. All diversity measures reduced over the storage period, although the reduction was not significant. Equally, the calculated richness, Shannon index and Inverse Simpson Index at each time point (T0, T48 and T120) was not significantly ($p > 0.05$) different to the values obtained when the vegetable matrix was prepared from fresh. All statistical data including follow-up post-hoc results are available in Appendix 9.

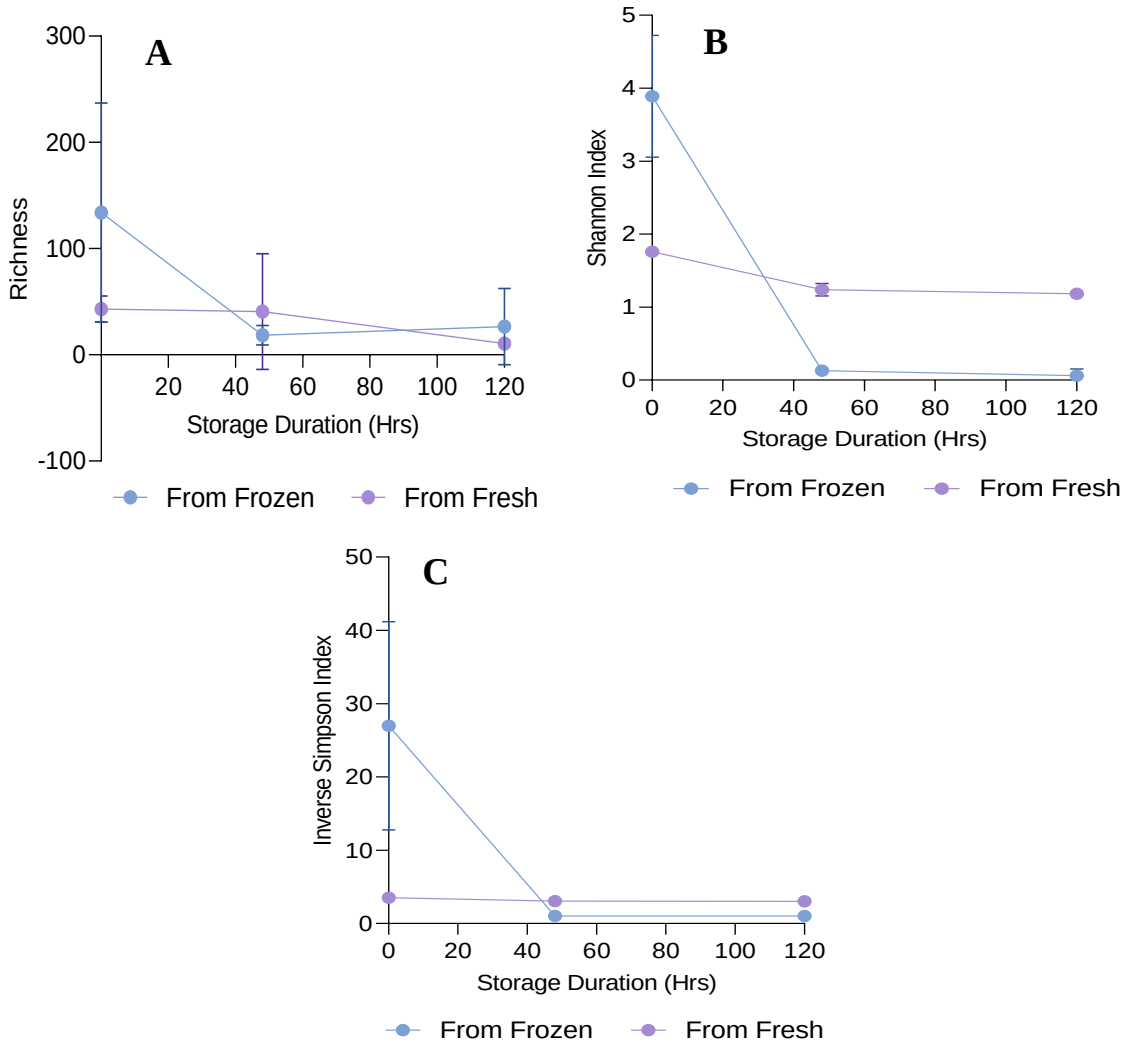


Figure 5.15: Alpha diversity of the overall microbial composition during storage of the vegetable matrix at 20 °C prepared from frozen and from fresh. Alpha diversity was determined by total richness (A), Shannon index (B) and Inverse Simpson index (C). Each data point comprises of three technical replicates and associated error is expressed by standard deviation.

The microbial composition throughout the storage duration when the vegetable matrix was prepared from frozen and fresh is shown in Figure 5.16. The microbial composition is very diverse at 0 h regardless of the preparation procedure; however, the microbial communities present at 0 h is different depending on the preparation of the vegetable matrix (discussed in previous chapter).

As the storage duration increased, the vegetable matrix prepared from frozen at 48 h and 120 h primarily consisted of Gram-positive unclassified *Bacillales* (~98%). In contrast to the fresh vegetable matrix, *Lactococcus*, *Leuconostoc* and *Yersinia* were the most predominant bacterial communities at 48 and 120 h.

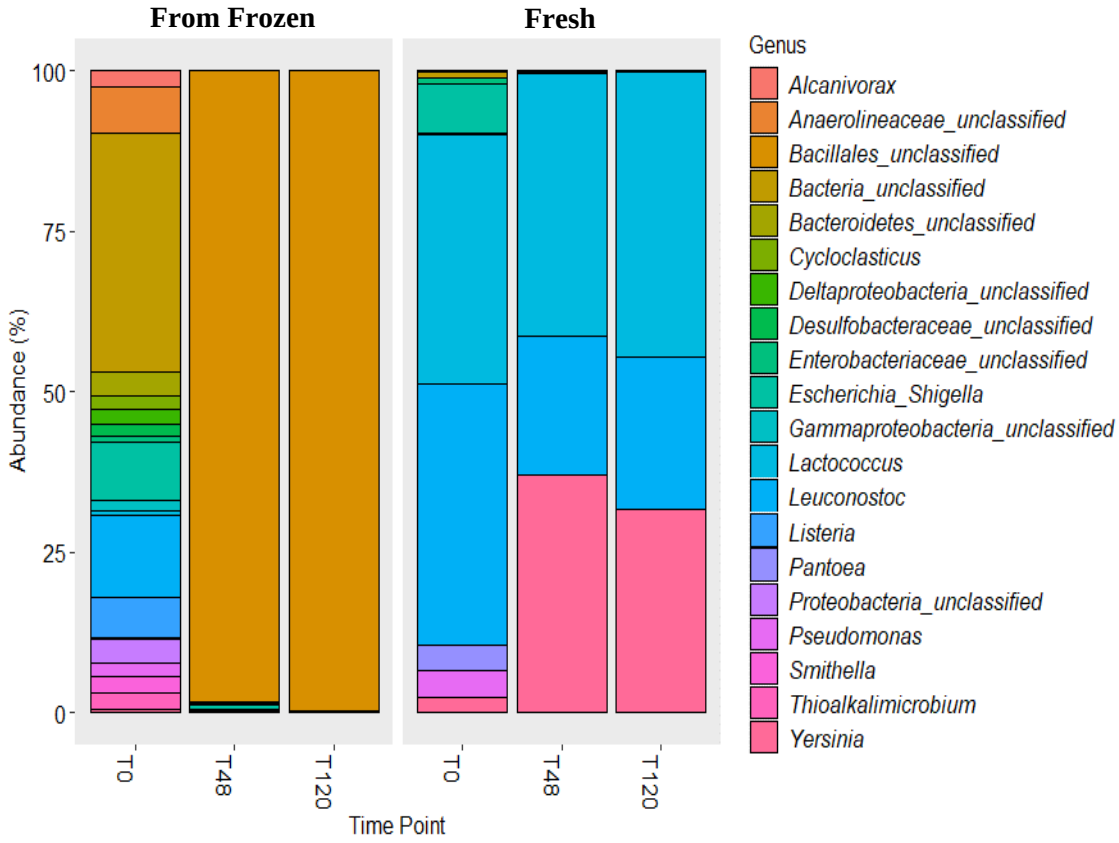


Figure 5.16: Microbial composition and development throughout the time course of the vegetable matrix either prepared from frozen or fresh and stored at 20 °C. The microbial composition was expressed as the % abundance of the top 20 bacterial communities classified at genus level. Each time point comprises of three technical replicates.

5.3.2.3 The Impact of Time and Storage Preparation procedure on Microbial Composition – Beta-Diversity

The impact of the preparation procedure on the microbial composition over the time course is represented by PCoA in Figure 5.17. Based on PCoA, samples collected at 48 and 120 h from the vegetable matrix prepared from frozen clustered together away from samples collected at these time points for the fresh vegetable matrix, with the variance in these samples explained by axis 1 (57%). This data was supported by the PERMANOVA test which showed significant differences in the bacterial composition over the time course, when different preparation procedures are used ($p = 0.001$, $R^2 = 0.3567$).

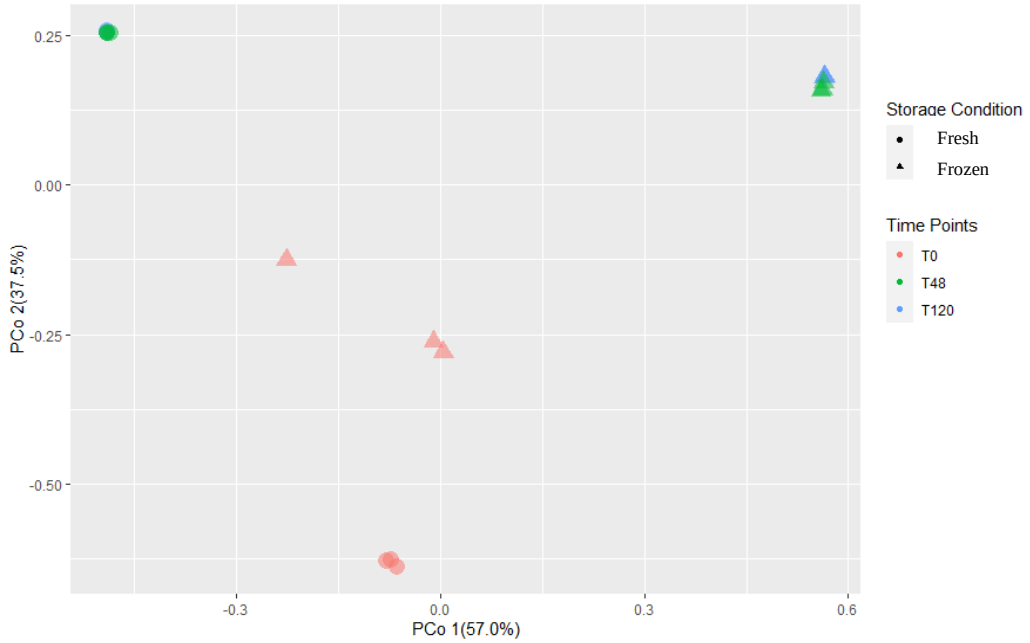


Figure 5.17: Principal coordinate analysis of Bray-Curtis dissimilarity distance matrix, samples grouped by time points 0 h – 120 h and preparation procedure, from frozen versus from fresh under storage at 20 °C. Each time point comprises of three analysed samples.

5.3.2.4 The Effect of Freezing on pH Profile

The pH during storage of the vegetable matrix prepared from frozen (Figure 5.18) slightly reduced over the storage period initiating at 72 h, from pH 5.70 (T0) to pH 5.31 (T72). In comparison, when the vegetable matrix is prepared from fresh, the pH declined earlier in the time course at 48 h and the decrease in pH is greater, from pH 5.70 (T0) to pH 4.16 (T48).

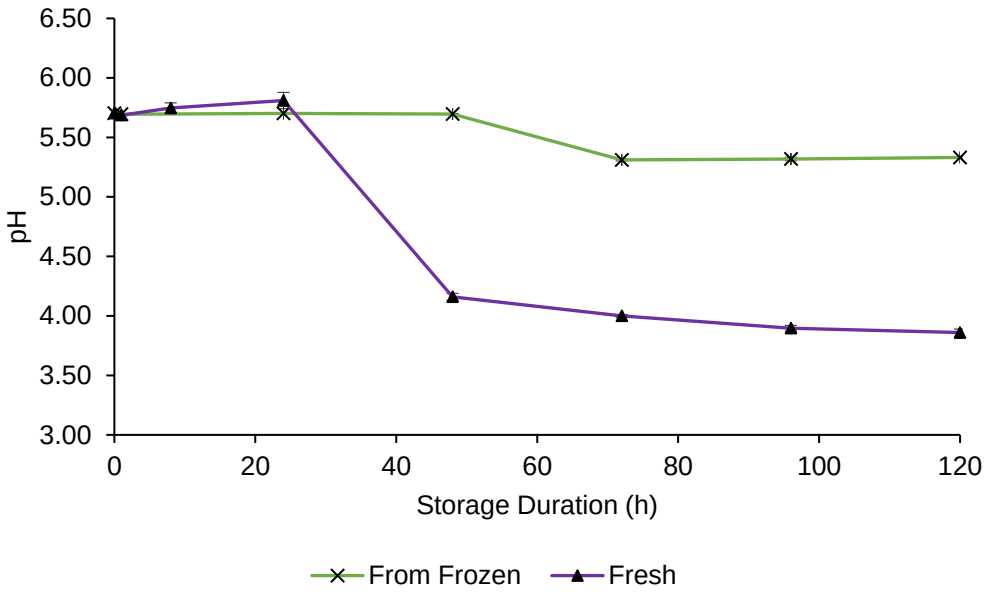


Figure 5.18: The pH profile of the vegetable matrix prepared from frozen and prepared from fresh prior to storage at 20 °C for 5-days (120 h). pH was measured in triplicate at each time point and associated error expressed by a 95% confidence interval.

5.3.2.5 The Effect of Freezing on Metabolite Analysis

The metabolite profile at 0 h, 48 h and 120 h when the vegetable matrix was prepared from either frozen or fresh prior to storage was compared and visualised using PLS-DA (Figure 5.19). According to the PLS-DA score plots, samples clustered together based on the preparation procedure, the from frozen samples clustered away from samples collected when the vegetable matrix was prepared from fresh. The increase in storage duration, equally increased the distance between the samples collected under both preparation procedures. The metabolites that have played a role in the differentiation of the different preparation procedures at 0, 48 and 120 h are shown in Figure 5.20 based on VIP metric and corresponding box plots are shown in **Error! Reference source not found.**. Pathway analysis on the putatively annotated metabolites was performed (Figure 5.22) and showed metabolites were significantly associated ($P < 0.01$) with arginine biosynthesis, glutathione metabolism, purine metabolism and arginine and proline metabolism.

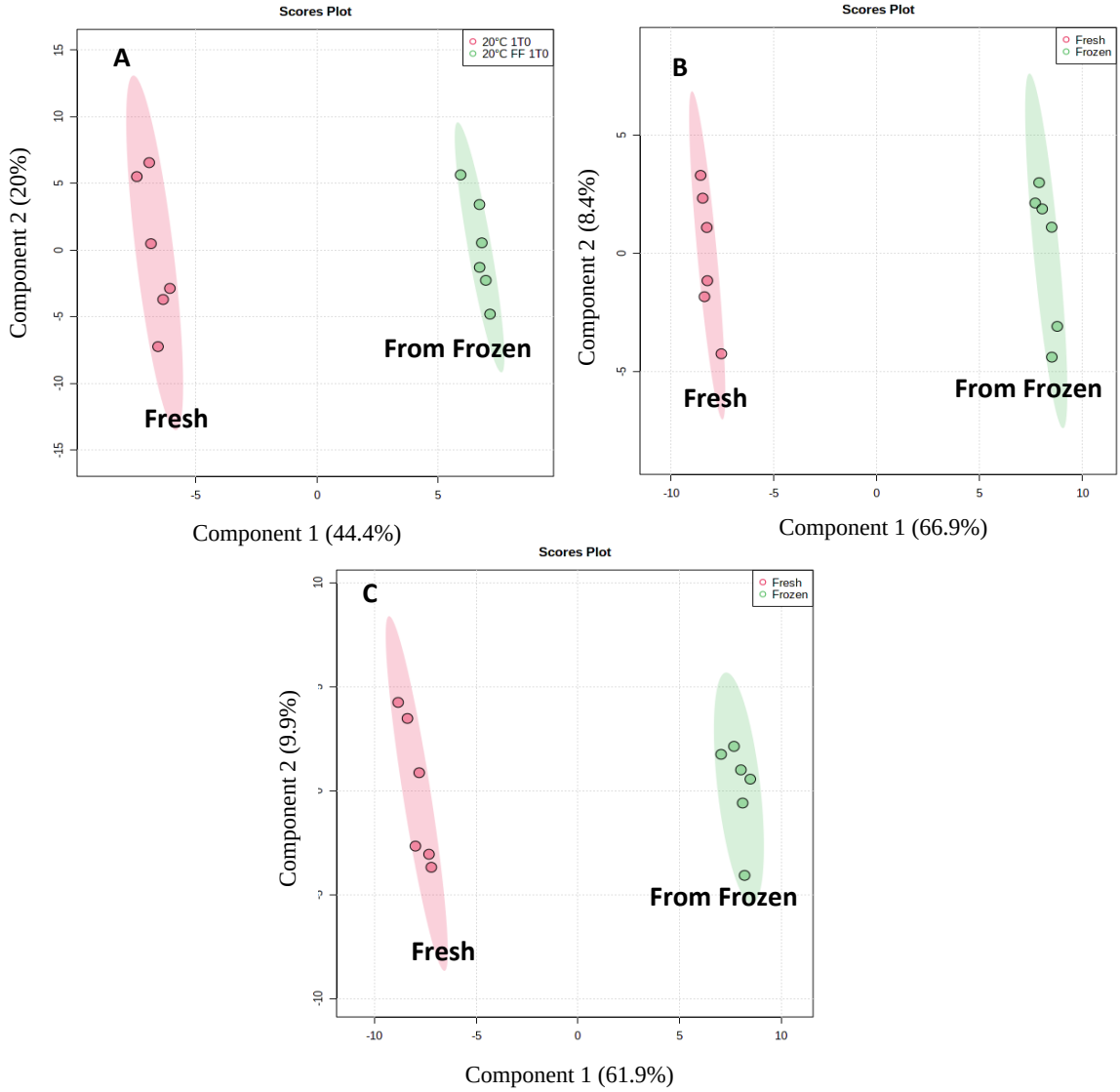


Figure 5.19: PLS-DA score plots of the effect of freezing on metabolite profiles at 0 h (A), 48 h (B) and 120 h (C). Each time point under each preparation condition is composed of six technical replicates.

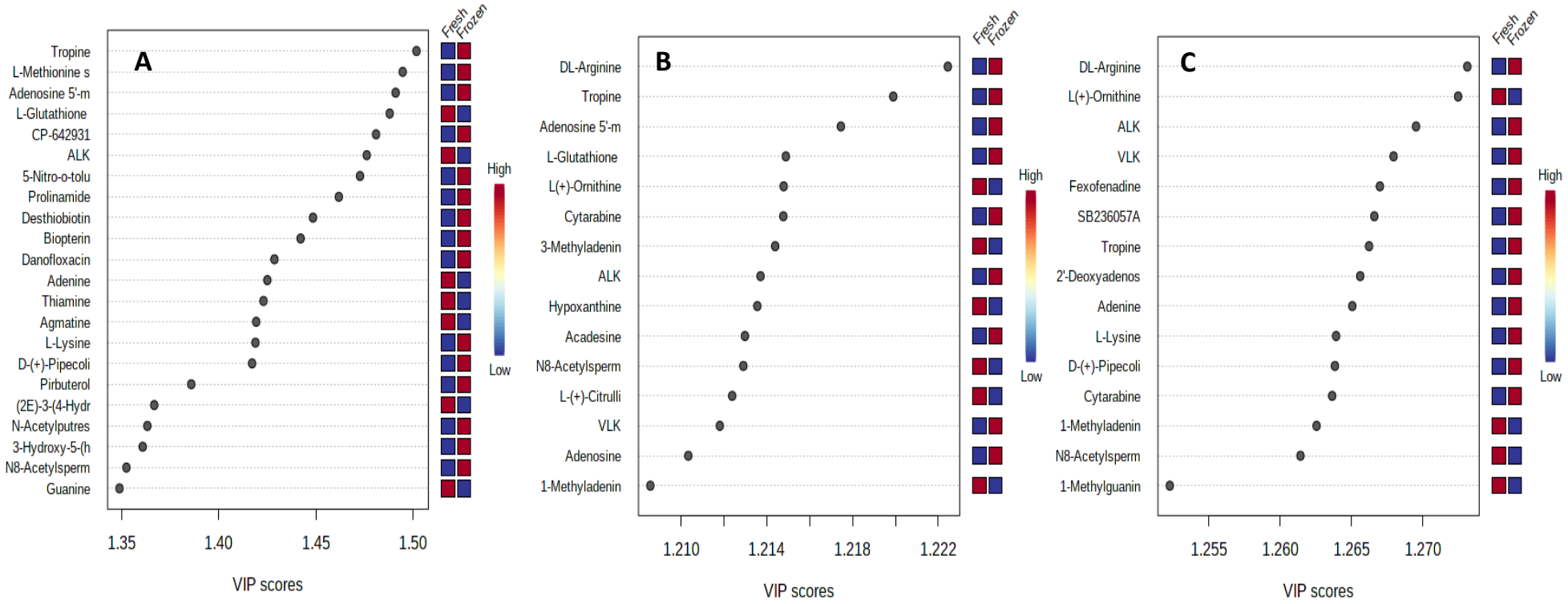


Figure 5.20: VIP ranking of the variables/metabolites at 0 h (A), 48 h (B) and 120 h (C) when comparing preparation procedures of the vegetable matrix (from frozen versus from fresh), as identified by PLS based on component 1.

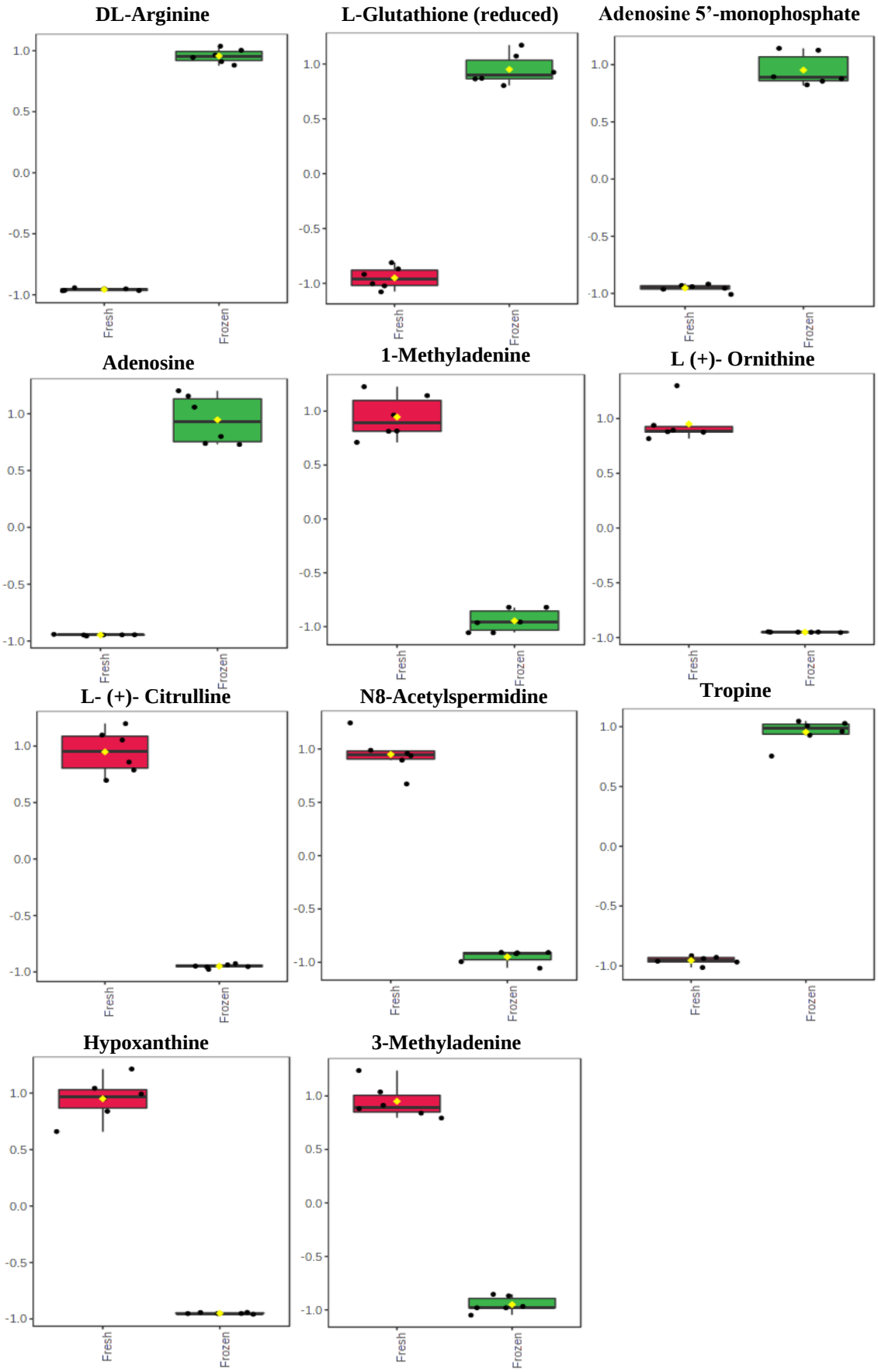


Figure 5.21: Examples of key discriminatory metabolites according to VIP analysis at 48 h when the vegetable matrix is prepared from fresh versus from frozen. Y axis represents normalised peak area (arbitrary units) and X axis represents different preparation procedures. Each preparation procedure is composed of six technical replicates.

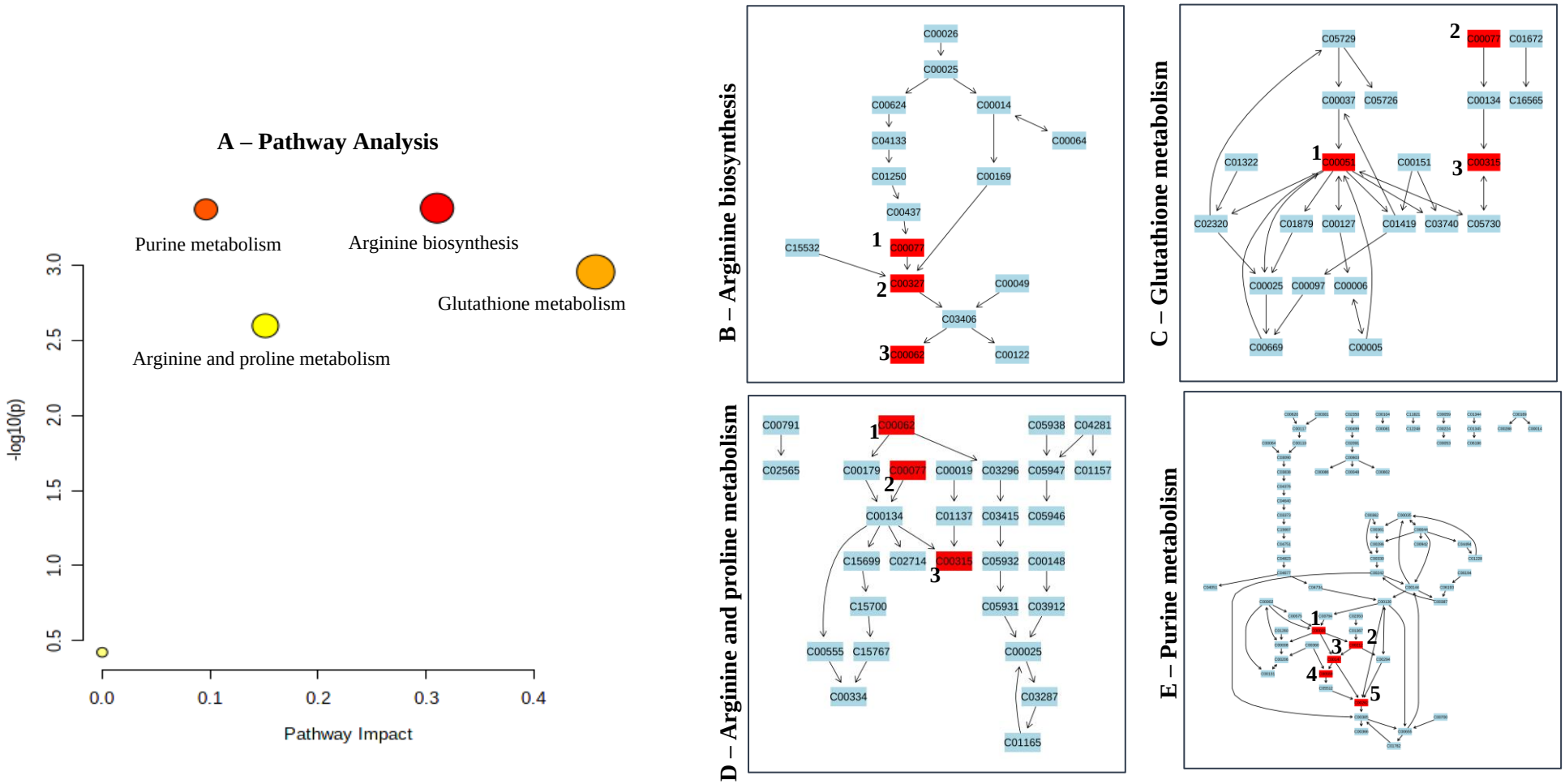


Figure 5.22: Pathway analysis of putatively annotated metabolites (A) and pathways shown (B-E) are deemed to have significant association with some of the putatively annotated metabolites, which are highlighted in red. For arginine biosynthesis, metabolites include 1= L-(+)-ornithine, 2 = L-citrulline and 3= L-Arginine. For glutathione metabolism, metabolites include 1= glutathione, 2= L-(+)-ornithine and 3= acetylspermidine. For arginine and proline metabolism, metabolites include 1= L-arginine, 2= L-(+)-ornithine and 3= acetylspermidine. For purine metabolism, metabolites include 1= adenosine 5' monophosphate, 2= adenosine, 3= adenine, 4= 2'deoxyadenosine and 5= hypoxanthine.

5.3.2.6 The Effect of Freezing on VOC Analysis

The influence of the different preparation procedures on the VOC profile during the time course, comparing samples collected at 48 h is presented by PCA in Figure 5.23. The from frozen group are clustered away from the vegetable matrix prepared from fresh, with the variability in these groups explained by component 1 (99.52%). The top features that are responsible for the relationship in the PCA were putatively annotated and are shown in Table 5.3. These comprise of volatiles that are either in high or low abundance depending on preparation procedure, for example γ-terpinene, terpinolene and 1-hexanol were present in high abundance at 48 h of storage when the vegetable matrix is prepared from frozen. In contrast, when the vegetable matrix is prepared from fresh at 48 h, acetic acid, 1-octene, nonanal and 1-amino-2-butanol were abundant.

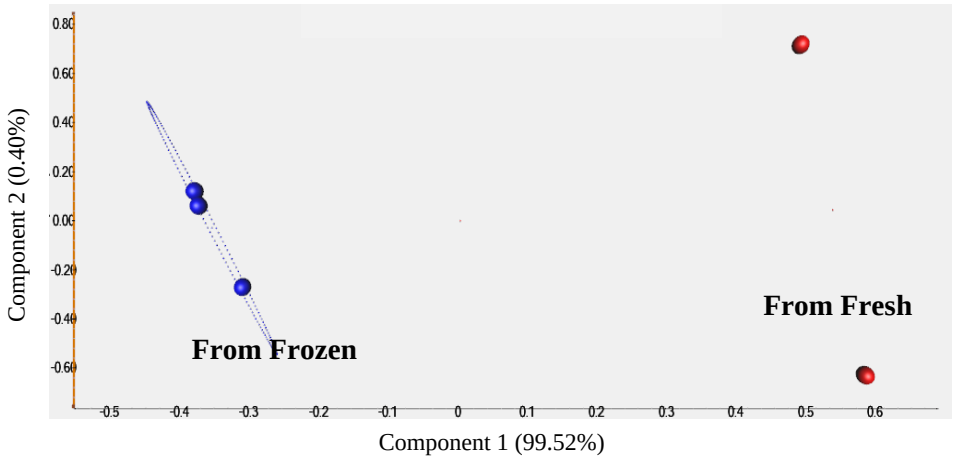
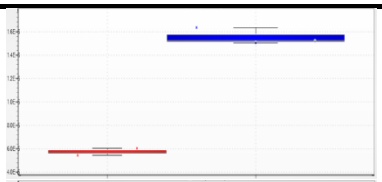
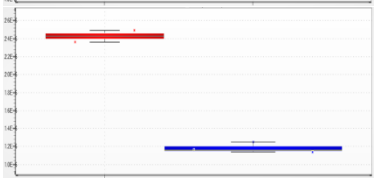
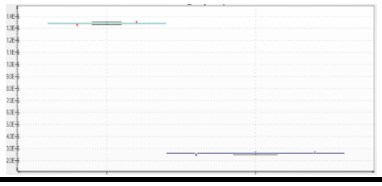
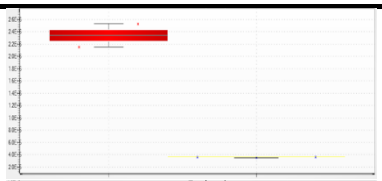
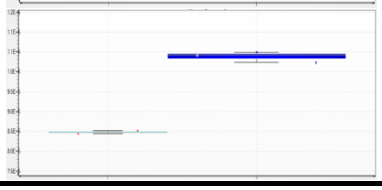
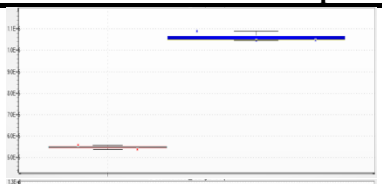
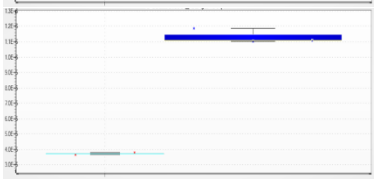
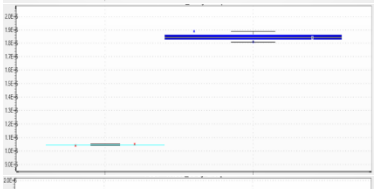
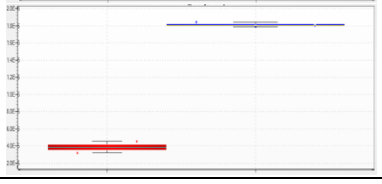
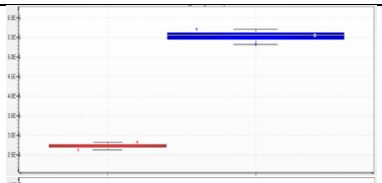
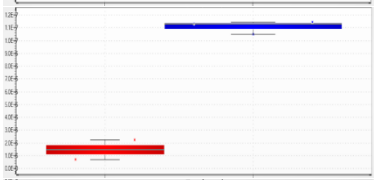
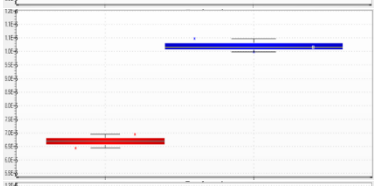
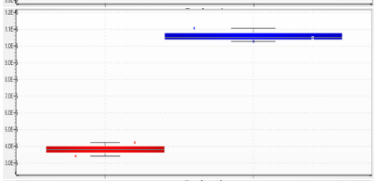
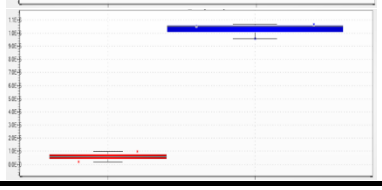
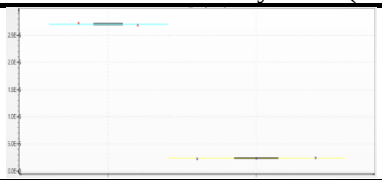
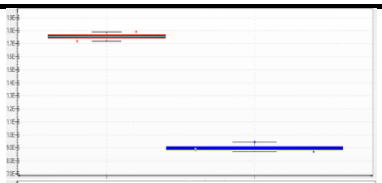
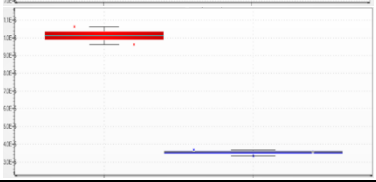


Figure 5.23: PCA comprising of the top 25 most significant features when comparing the middle time points of the vegetable matrix prepared from frozen and from fresh. Each storage condition contains three technical replicates, except for the vegetable matrix prepared from fresh which is composed of two technical replicates.

Table 5.3: The top discriminatory annotated VOCs according to PCA when comparing storage of the vegetable matrix prepared from frozen and from fresh at 48 h. Box plots also included red = from fresh, blue = from frozen and y axis represents abundance.

Putatively Annotated Volatiles		Preparation procedure volatile is highest	
Alkanes and Alkenes		From Fresh	From Frozen
Nonane			×
Decane		×	
1-Octene		×	
Alcohols			
1-Amino-2-butanol		×	
1-Hexanol			×
Monoterpenes			
γ-Terpinene*			×
γ-Terpinene*			×
Terpinolene*			×
γ-Terpinene*			×

Terpinolene*		×
γ-Terpinene*		×
γ-Terpinene*		×
γ-Terpinene*		×
Terpinolene*		×
Short chain fatty acids (SCFAs)		
Acetic acid		×
Aldehydes		
Nonanal		×
Dodecanal		×

*Different isomers

5.3.2.7 The Effect of Freezing on Stability of B-Vitamins

The impact of the different preparation procedures on vitamin stability during storage can be viewed in Figure 5.24. Preparation of the vegetable matrix from frozen only had a significant effect on nicotinamide during storage ($p = 0.006$), as nicotinamide was completely exhausted at 72 h. In contrast, when the vegetable matrix is prepared from fresh, nicotinamide was depleted earlier in the time course at 24 h and both riboflavin and thiamine significantly reduced. Descriptive statistics for the vegetable matrix prepared from frozen and from fresh can be seen in Table 5.4 and post-hoc results from repeated measures ANOVA are available in Appendix 4 (from fresh) and Appendix 8 (from frozen).

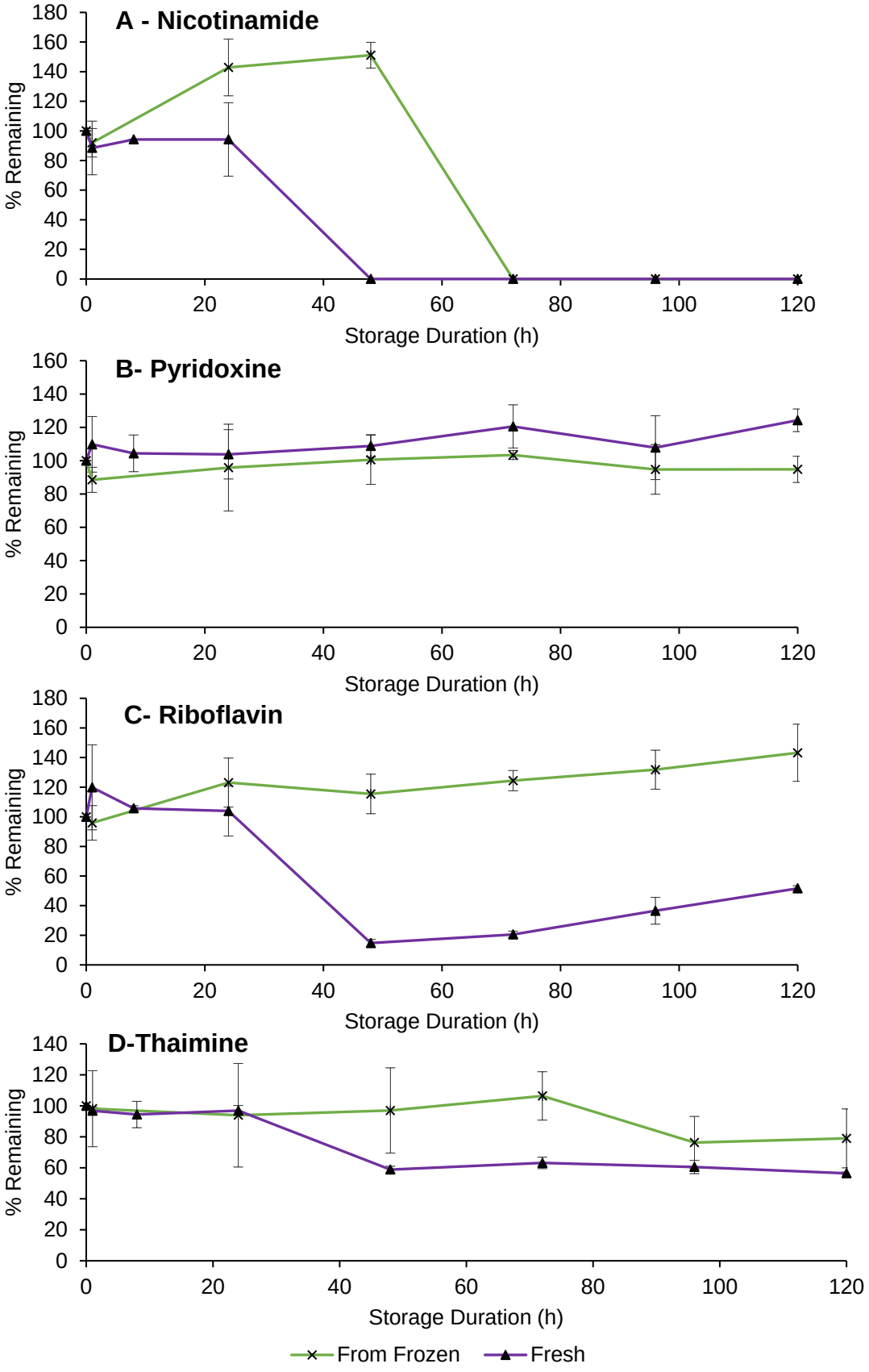


Figure 5.24: Stability of B-group vitamins, nicotinamide (A), pyridoxine (B), riboflavin (C) and thiamine (D) in the vegetable matrix that was prepared either from frozen or fresh prior to storage at 20 °C for 120 h. Stability is represented as the % remaining of each vitamin based on quantity of each vitamin at T0. Each data point is an average of three analysed samples and associated error is expressed by a 95% confidence interval.

Table 5.4: Descriptive statistics of vitamin stability in the vegetable matrix prepared from frozen and fresh prior to storage at 20 °C for 120 h

Vegetable Matrix Prepared From Frozen								
	Nicotinamide		**Pyridoxine**		**Riboflavin**		**Thiamine**	
Time point/s	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV
T0	103.71	24.56	16.84	1.86	39.17	6.09	26.95	8.13
T1	95.38	8.79	14.91	1.12	37.55	4.04	26.45	5.84
T24	148.14	17.57	16.13	3.87	48.21	5.72	25.31	7.96
T48	156.72	7.97	16.93	2.20	45.20	4.62	26.13	6.55
T72	ND*		17.41	0.40	48.72	2.37	28.67	3.72
T96	ND*		15.95	2.22	51.63	4.56	20.56	4.02
T120	ND*		15.96	1.17	56.10	6.68	21.28	4.53
Vegetable Matrix Prepared From Fresh								
Time point/s	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV
T0	591.20	80.46	95.70	1.91	232.57	31.15	278.10	3.40
T1	523.10	94.75	105.10	14.12	278.77	58.98	269.23	5.30
T8	556.93	1.27	99.90	9.35	245.80	3.64	262.6	20.88
T24	557.20	129.58	99.30	12.52	241.70	34.79	269.57	8.12
T48	ND*		104.17	5.69	34.30	5.11	163.73	5.20
T72	ND*		115.37	10.96	47.80	4.65	175.67	9.05
T96	ND*		103.20	16.25	84.93	18.57	168.37	10.46
T120	ND*		118.93	5.71	120.17	3.72	157.00	2.19

[a] Mean is comprised of three samples (n=3).
* ND = not detected

5.3.3 Absence and Presence of Oxygen

The impact of storing the vegetable matrix in the absence and presence of oxygen was achieved using a bio-fermenter, flushing the vegetable matrix with either nitrogen, or compressed air, or nothing. The latter is referred to as no gas and was completed under baseline conditions (established in chapter 4) for when the bio-fermenter was used to investigate storage environments, since all other storage studies were performed using a Duran and static incubator.

5.3.3.1 The Effect of Oxygen on Microbial growth

Storage of the vegetable matrix under no gas and in the presence of nitrogen (absence of oxygen), provided an almost identical microbial growth profile, as presented in Figure 5.25. For both conditions no colonies were observed until after 24 h, exponential growth phase was experienced between 24-72 h with the TVC for no gas and nitrogen reaching 9.38 and 9.29 log CFU/g respectively at 72 h. There is no microbial data available for storage of the vegetable matrix in the presence of compressed air, due to this condition promoting the growth of a microorganism that swarmed agar plates, resulting in issues with colony counting, a respective image of the swarming microorganism on plate count agar can be seen in Figure 5.26.

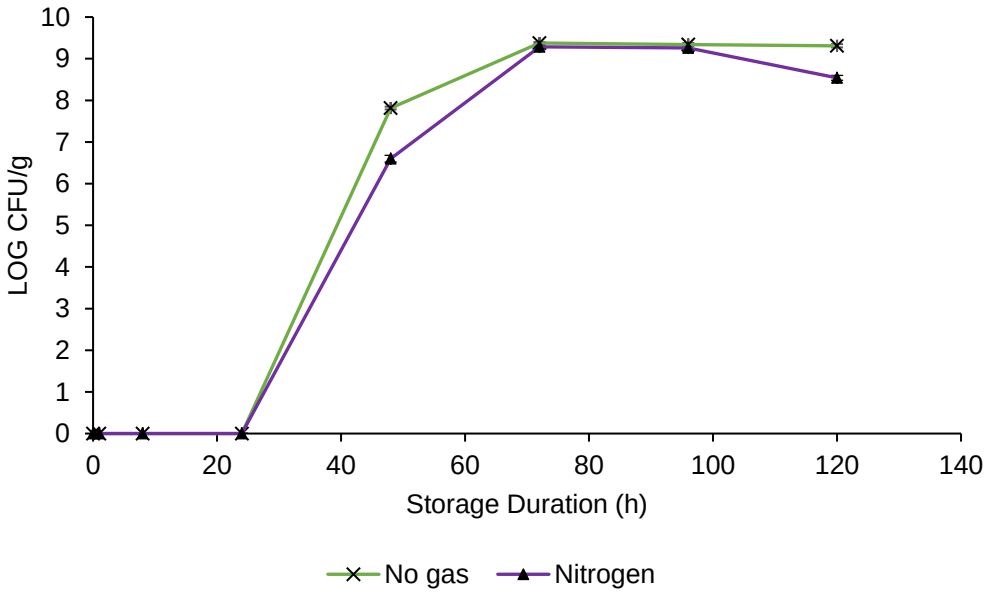


Figure 5.25: TVC over the storage duration (120 h) of the vegetable matrix stored at 20 °C under no gas and in the presence of nitrogen. For each data point duplicate dilution series were performed, of which two replicates were analysed per dilution series (n=4). The error associated with each time point is represented by a 95% confidence interval.

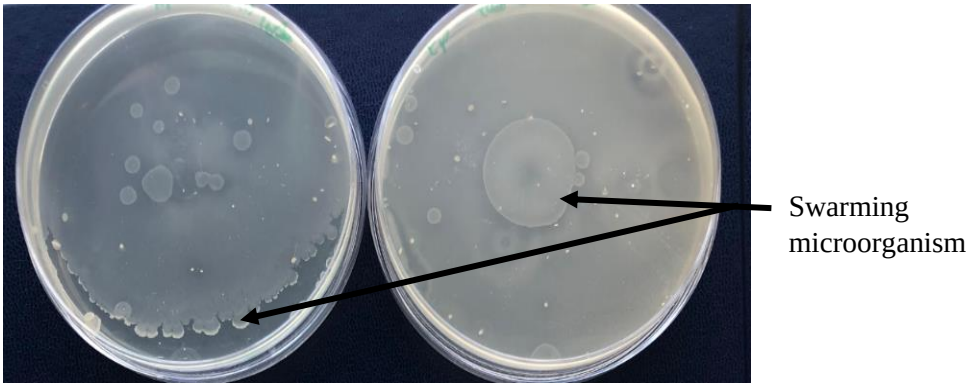


Figure 5.26: Image representing the swarming growth of a microorganism on plate count agar (duplicate agar plates) when the vegetable-based matrix was stored in the presence of oxygen (compressed air).

5.3.3.2 The Effect of Oxygen on Bacterial Diversity and Composition - Alpha Diversity

The diversity of bacteria in the vegetable matrix stored under no gas and in the presence of nitrogen (Figure 5.27), showed a significant reduction in bacterial diversity over the storage period (0-120 h). This included a significant decrease in species richness, Shannon index and Simpson index ($p < 0.05$). However, when the vegetable matrix was stored in the presence of compressed air, species richness was the only diversity metric that significantly reduced over the storage period ($p = 0.04979$), although Shannon and Simpson index decreased from 0 h to 120 h (Figure 5.27). The bacterial diversity measures at specific time points across all storage conditions conducted in the bio-fermenter were compared. Richness, Shannon and Simpson index were significantly different at 48 h and 120 h when comparing storage under no gas, nitrogen and air. Follow-up post hoc analysis highlighted at 48 h the bacterial diversity was significantly different between storage in presence of nitrogen versus storage in the presence of air. Alternatively, at 120 h the bacterial diversity was significantly different when storage in the presence of air was compared to storage under no gas. All statistical data including follow-up post-hoc results are available in Appendix 9.

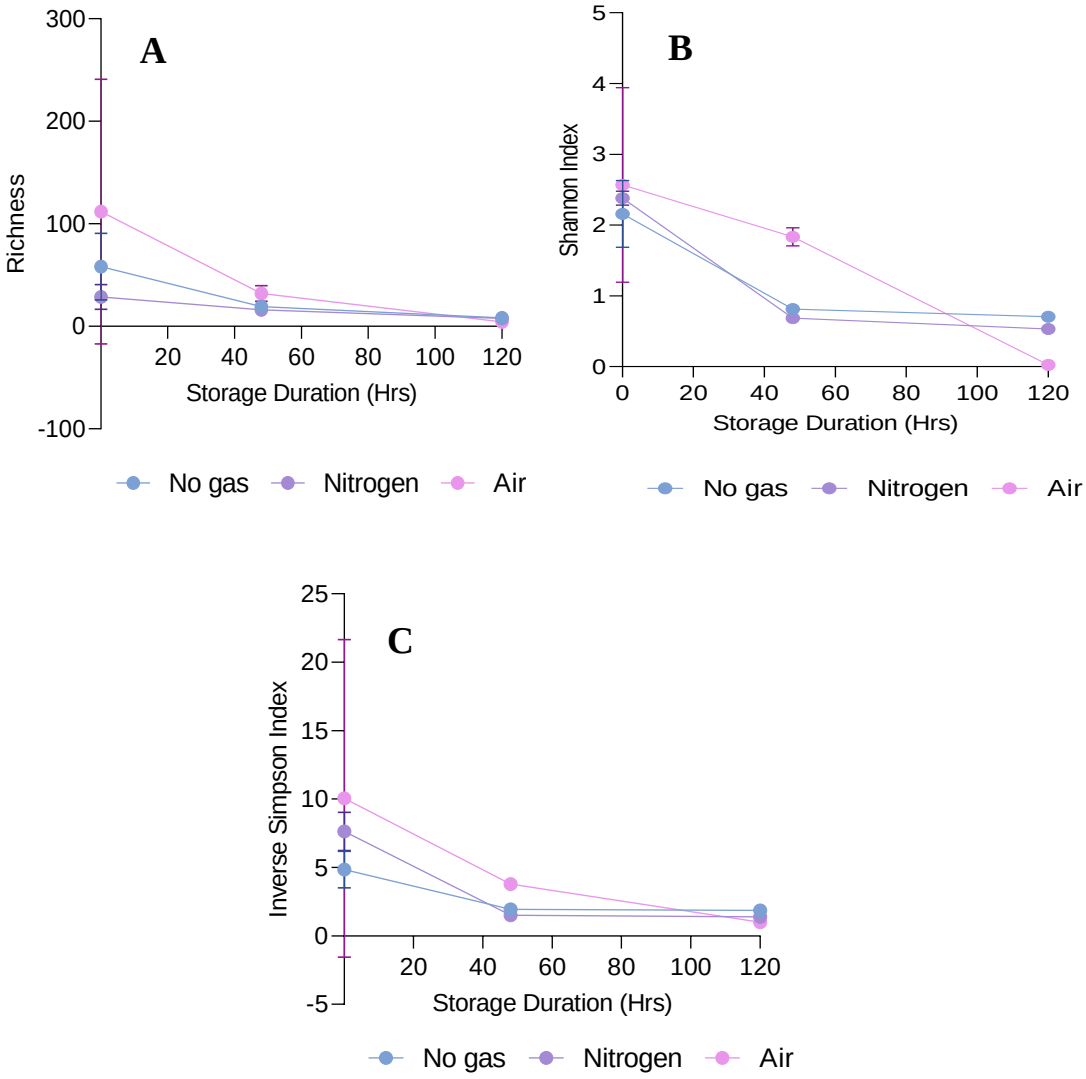


Figure 5.27: Alpha diversity of the overall microbial composition during storage of the vegetable matrix at 20 °C in the bio-fermenter under no gas and in the presence of both nitrogen and air. Alpha diversity was determined by total richness (A), Shannon index (B) and Inverse Simpson index (C). Each data point comprises of three technical replicates and associated error is expressed by standard deviation.

The microbial communities prior to storage under different gas environment were very diverse. However, as the storage duration increased, certain microbial communities became more abundant, as represented in Figure 5.28. Storage in the presence of nitrogen, at 48 h *Leuconostoc* and *Lactococcus* became the most abundant communities by approximately 81.25% and 16.75% respectively and at 120 h the microbial composition was similar to that of 48 h. Storage under no gas was comparable to storage in the presence of nitrogen, *Leuconostoc* (~62.5%) and *Lactococcus* (~37.5%) were the most abundant bacterial communities at 48 h and 120 h. In contrast, at 48 h of storage in the presence of air, the bacterial community remained diverse, with approximately nine predominant communities, comprised of *Lactococcus, Leuconostoc, Bacillus, Escherichia/Shigella,* unclassified *Enterobacteriaceae*, unclassified Rhodobacteraceae, *Weissella, Yersinia* and *Actinetobacter*. As storage of the vegetable matrix concluded at 120 h in the presence of air, *Bacillus* was the most abundant bacterial community at approximately 99%.

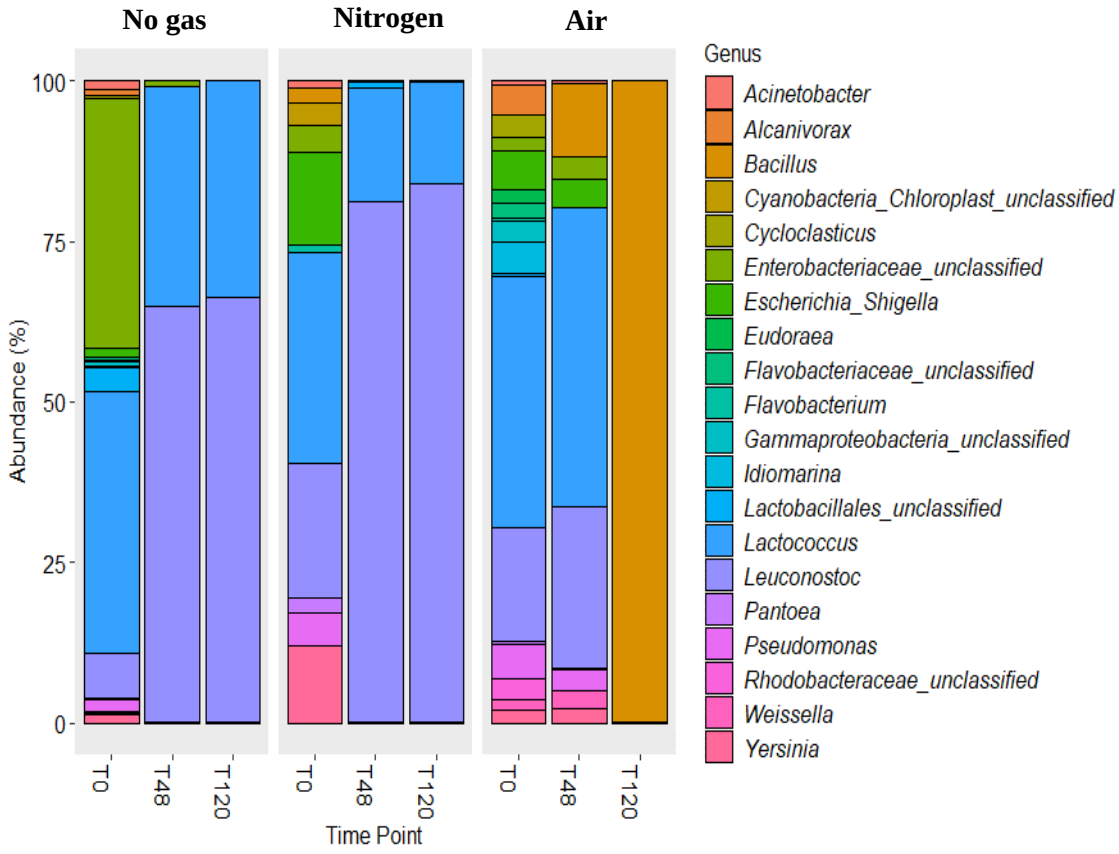


Figure 5.28: Microbial composition and development throughout the time course of the vegetable matrix stored in a bio-fermenter at 20 °C under no gas and in the presence of both nitrogen and compressed air. The microbial composition was expressed as the % abundance of the top 20 bacterial communities classified at genus level. Each time point comprises of three technical replicates.

5.3.3.3 The Impact of Time and Oxygen on Microbial Composition – Beta-Diversity

The impact of the absence and presence of oxygen on the bacterial composition over the time course of the vegetable matrix is represented in Figure 5.29. According to the PCoA, all samples clustered in accordance with time points with the majority of the variability within the samples explained within the first two axis's (73.6%). All 48 hr and 120 hr samples collected during storage under the presence of nitrogen and no gas clustered together away of T0 samples, separated on axis 1. Alternatively, samples collected at the end of the storage period in the presence of air, clustered away from all other time points, separated on axis 2. The PERMANOVA test confirmed that storage environment in the bio-fermenter ($p = 0.001$, $R^2 = 0.3218$) and storage duration ($p = 0.001$, $R^2 = 0.3129$) influenced the bacterial communities. Follow-up post hoc showed significant differences in the microbial composition between storage in the presence of air versus nitrogen ($p = 0.018$), and air versus no gas ($p = 0.006$).

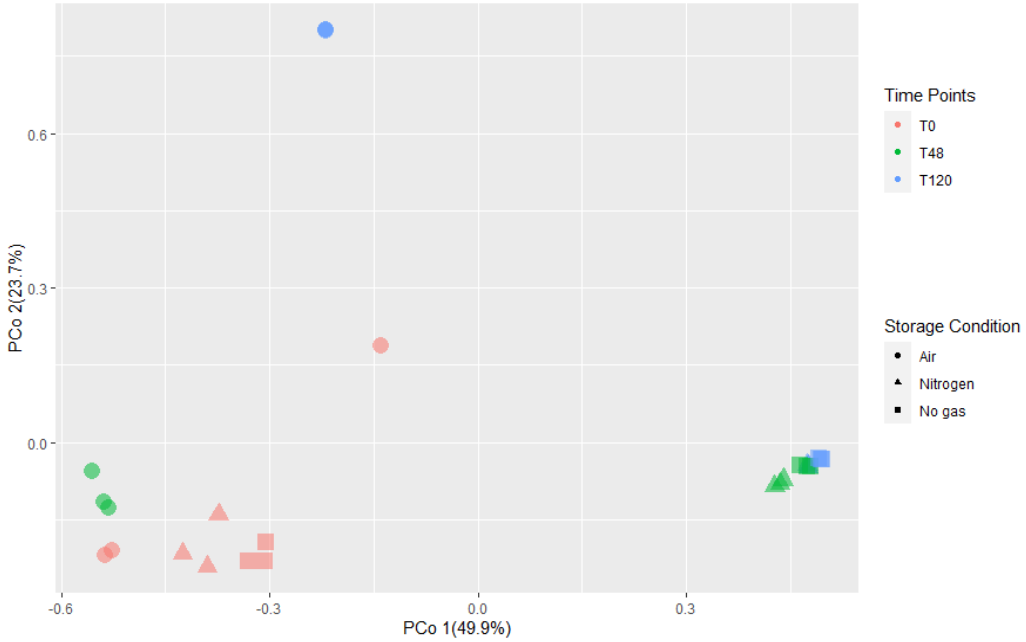


Figure 5.29: Principal coordinate analysis of Bray-Curtis dissimilarity distance matrix, samples grouped by time points 0 h – 120 h and storage condition under no gas and in the presence of both nitrogen and compressed air. Each time point comprises of three analysed samples.

5.3.3.4 The Effect of Oxygen on pH Profile

The presence of nitrogen on the pH profile (Figure 5.30) was comparable to no gas, reducing at 72 h, with the final average pH recorded as 3.82 for nitrogen and 3.65 for no gas. In comparison, during storage of the vegetable matrix in air, the pH increased at 72 h to pH 6.48.

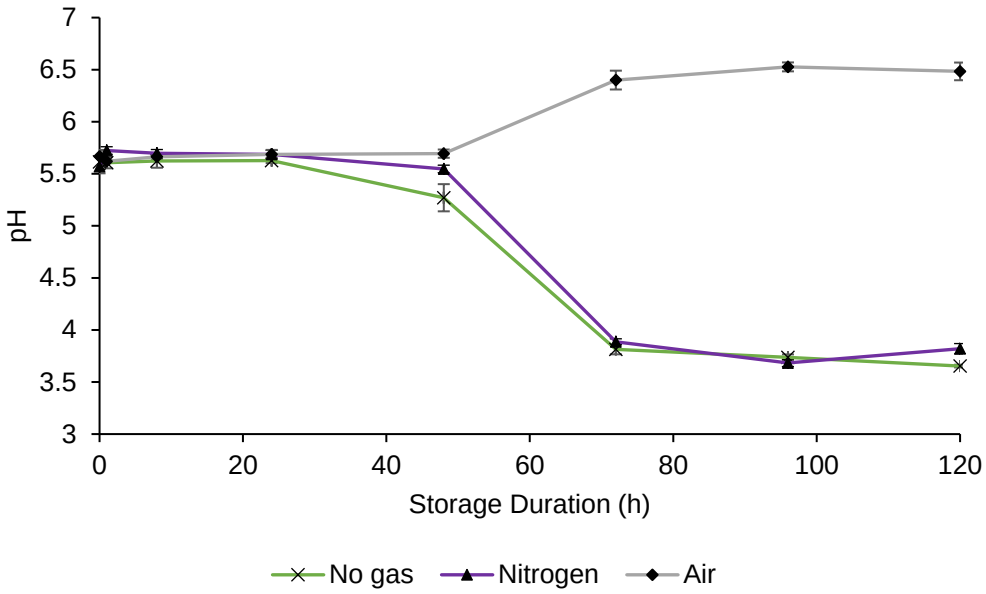


Figure 5.30: The pH profile of the vegetable matrix during 120 h of storage at 20 °C under no gas and in the presence of both nitrogen and compressed air. pH was measured in triplicate at each time point and associated error expressed by a 95% confidence interval.

5.3.3.5 The Effect of Oxygen on Metabolite Analysis

Storage of the vegetable matrix in the presence of nitrogen, no gas and presence of air on the metabolite profiles at 0 h, 48 h and 120 h are visualised by the generated scores plot from PLS-DA in Figure 5.31. Samples are clustered in accordance with storage condition this includes no gas, nitrogen and air when investigating the start, middle and end time points. As storage duration increased to 120 h the distance between the clusters increased, with samples collected in the presence of air clustering further away from no gas and nitrogen samples. The main metabolites that are responsible for the discrimination between samples are presented in Figure 5.32 and box plots corresponding to the putatively annotated metabolites are shown in **Error! Reference source not found.**. Example of metabolites that are abundant in the vegetable matrix stored in the presence of air include hypoxanthine, betaine, N6-acetyl-L-lysine and urocanic acid. In comparison metabolites prevalent during storage of the vegetable matrix in no gas and nitrogen include, choline, DL-glutamine and L-pyroglutamic acid. Pathway analysis was completed on the putatively annotated metabolites as shown in Figure 5.34. Metabolites were significantly associated with purine metabolism ($p = 0.0256$) and glycine, serine and threonine metabolism ($p = 0.0354$).

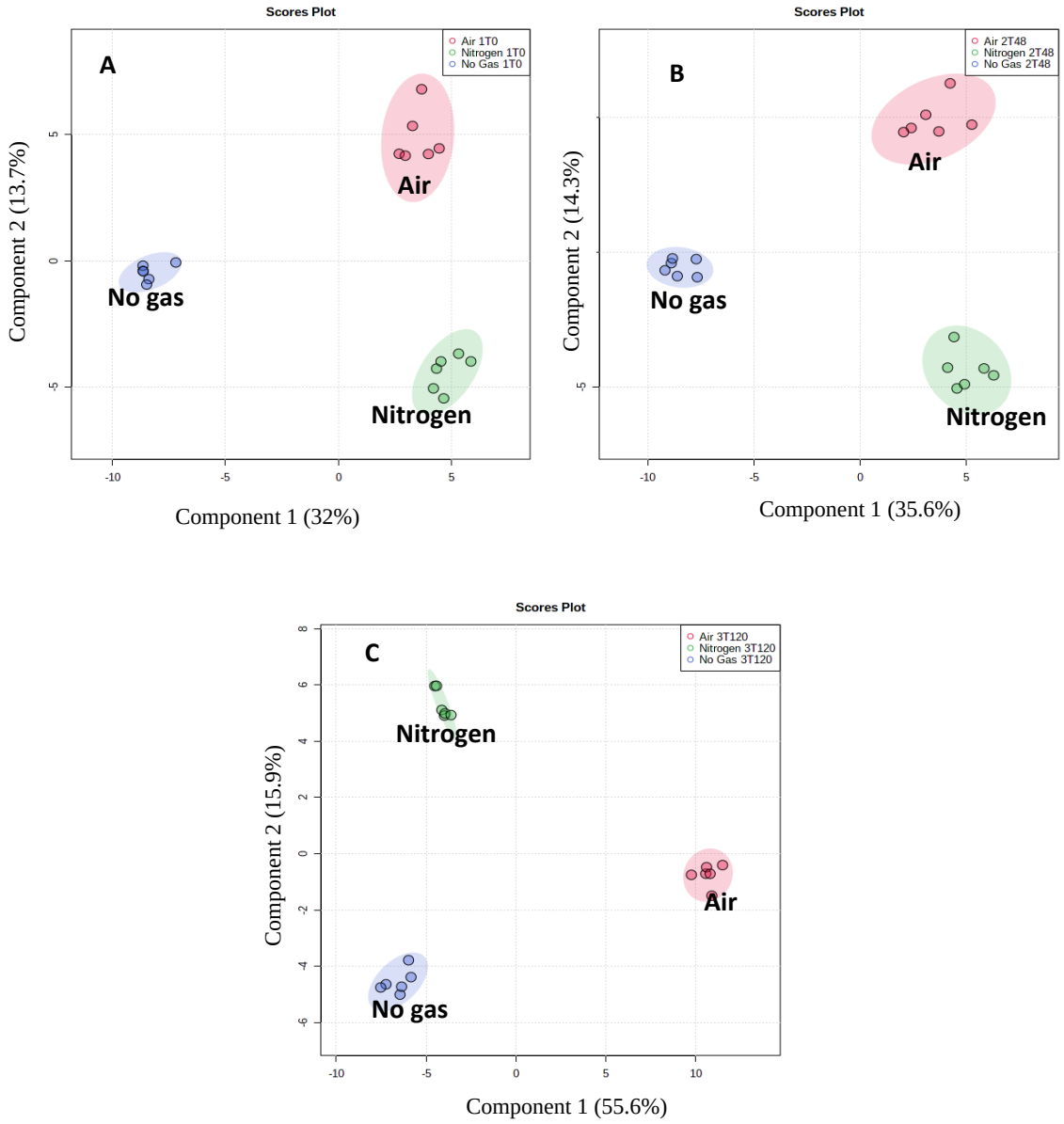


Figure 5.31: PLS-DA scores plot of the effect of oxygen on metabolite profile at 0 h (A), 48 h (B) and 120 h (C). Each time point under each storage condition is composed of six technical replicates.

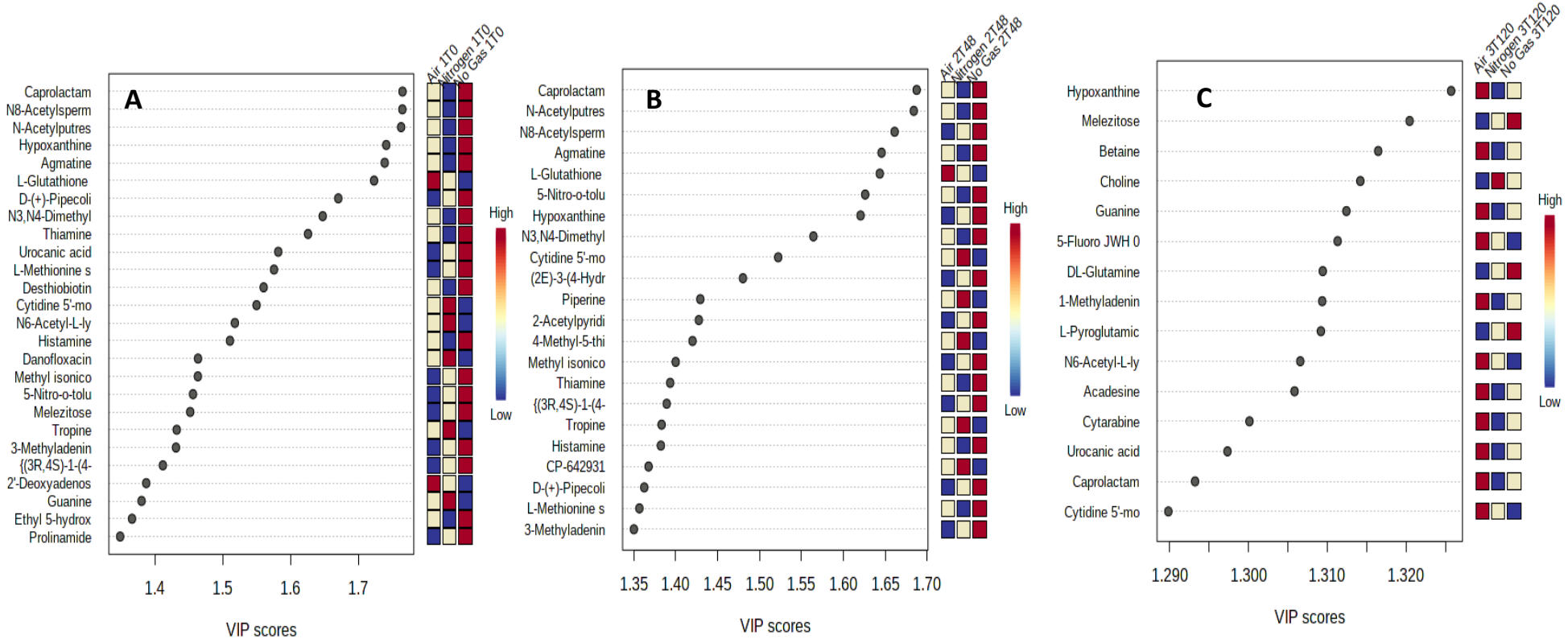


Figure 5.32: VIP ranking of the variables/metabolites (VIP > 1.35) at 0 h (A), 48 h (B) and 120 h (C) when comparing storage of the vegetable matrix under no gas and in the presence of both nitrogen and compressed air, as identified by PLS based on component 1.

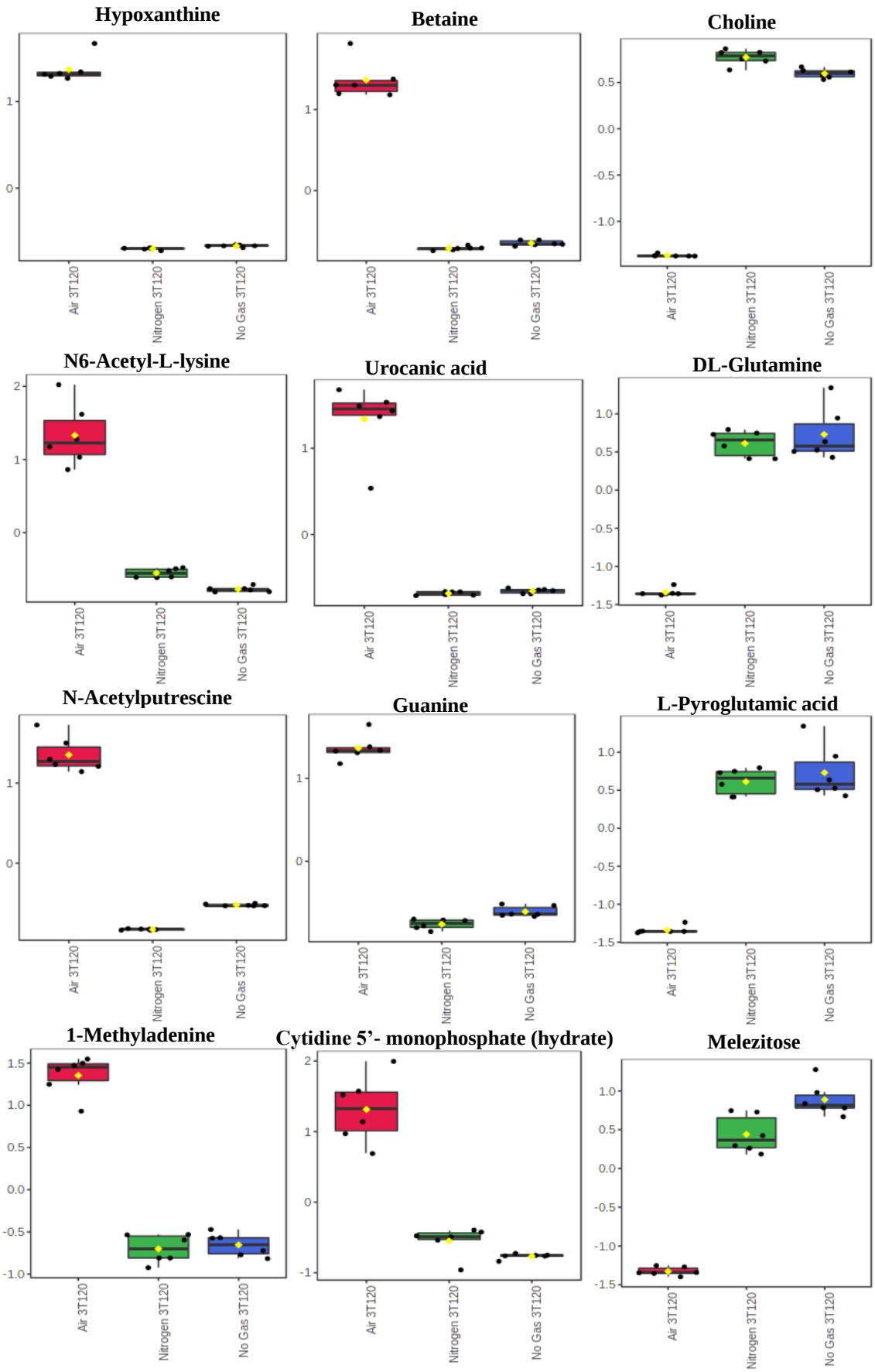


Figure 5.33: Examples of key discriminatory metabolites according to VIP analysis at 120 h of storage under the presence of both air and nitrogen and no gas. Y axis represents normalised peak area (arbitrary units) and X axis represents different storage conditions. Each condition is composed of six technical replicates.

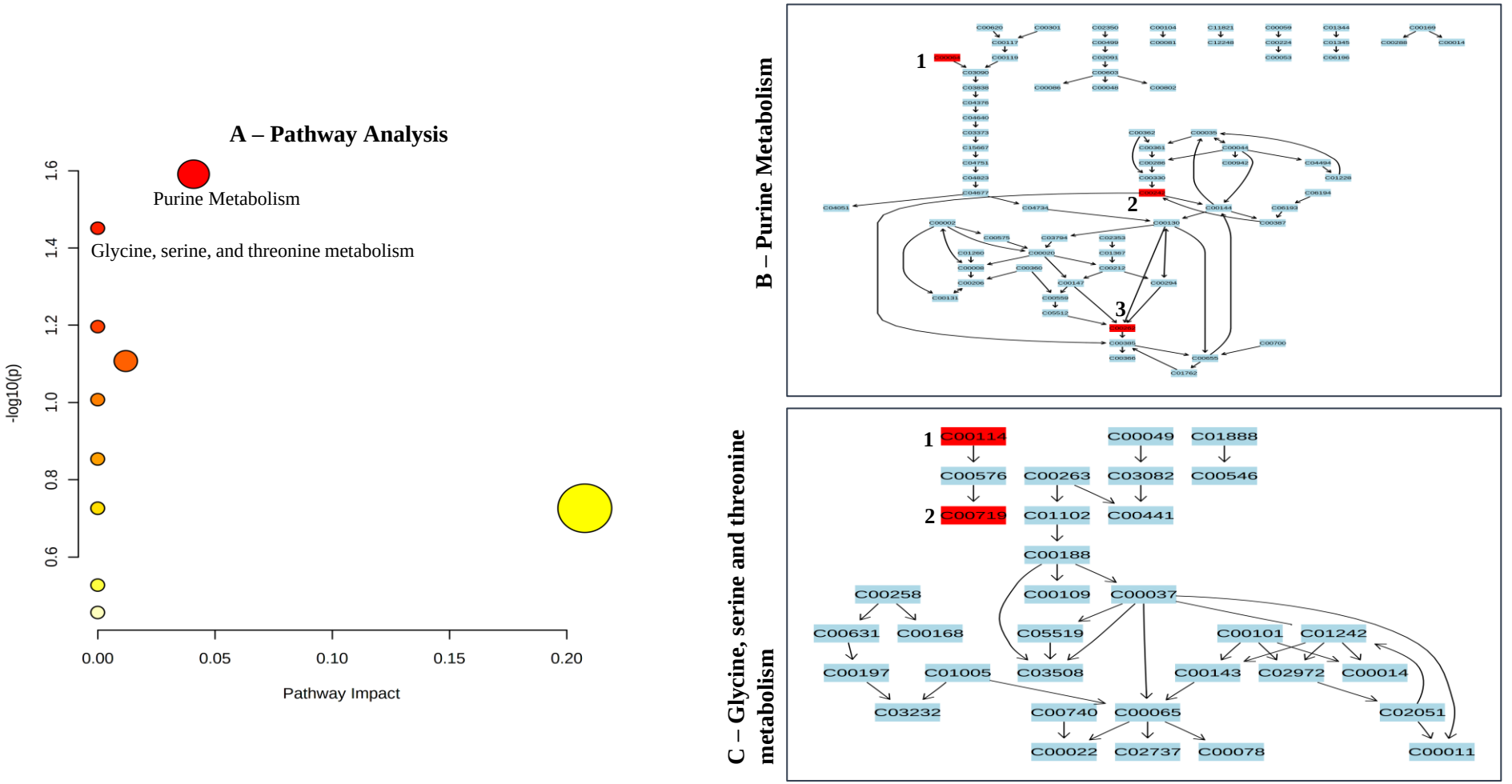


Figure 5.34: Pathway analysis of putatively annotated metabolites (A) and pathways shown (B and C) are deemed to have significant association with some of the putatively annotated metabolites, which are highlighted in red. For purine metabolism, metabolites include 1= glutamine, 2= guanine and 3= hypoxanthine. For glycine, serine, and threonine metabolism, metabolites include 1= choline and 2= betaine.

5.3.3.6 The Effect of Oxygen on VOC Analysis

The top 25 most significant VOC features during storage of the vegetable matrix under different gas compositions at 48 h was visualised using PCA, represented in Figure 5.35. Samples collected at 48 h under no gas and in the presence of both nitrogen and air are clustered in accordance with storage environment, with no gas environment clustering further away from nitrogen and air. The top features that played a role in the relationship of the PCA were putatively annotated and are shown in Table 5.5. Terpinolene, α-terpinene, 1-hexenol and 1-propenylbenzene were examples of discriminatory VOCs that were abundant during storage of the vegetable matrix under no gas.

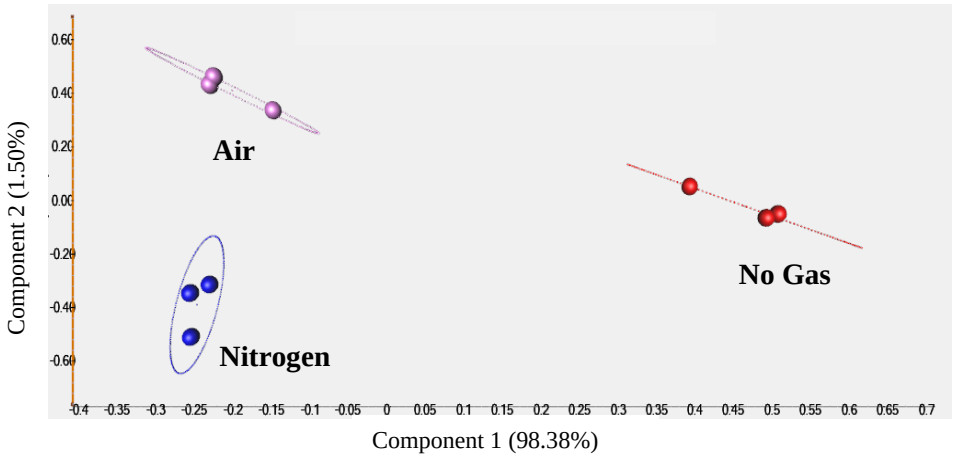
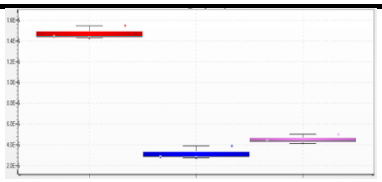
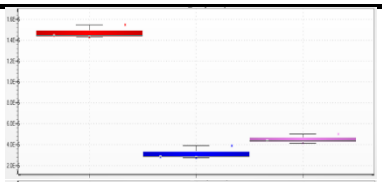
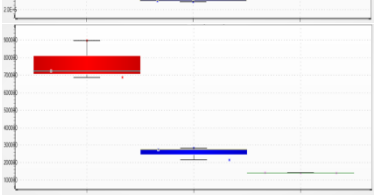
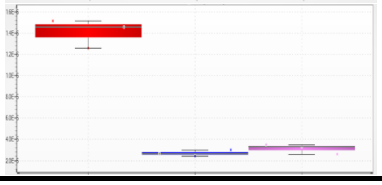
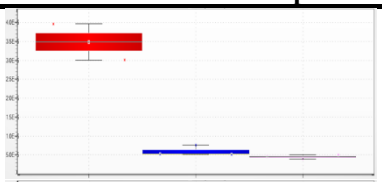
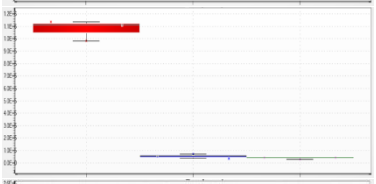
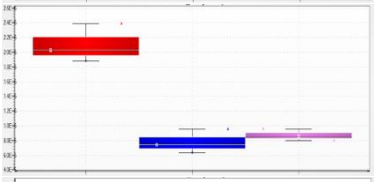
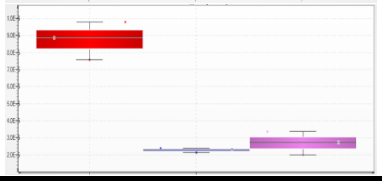


Figure 5.35: PCA comprising of the top 25 most significant features when comparing storage under all conditions in the bio-fermenter (no gas, presence of nitrogen and air) at 48 h. Each storage condition is composed of three technical replicates.

Table 5.5: The top discriminatory annotated VOCs according to PCA when comparing the middle time point during storage under all conditions in the bio-fermenter (no gas, presence of nitrogen and air). Box plots also included red = no gas, blue = nitrogen and pink = air, and y axis represents abundance.

Putatively Annotated Volatiles		Volatile highest in		
Alkanes and Alkenes		No gas	Nitrogen	Air
Decane		×		
Alcohols				
1-Amino-2-butanol		×		
1-Hexanol		×		
9-Tetradecen-1-ol		×		
Monoterpenes				
Terpinolene		×		
α-Terpinene		×		
o-Cymene		×		
1-Propenylbenzene		×		

5.3.3.7 The Effect of Oxygen on Stability of B-Vitamins

The stability of nicotinamide, pyridoxine, riboflavin and thiamine in the presence of both nitrogen and compressed air, and no gas can be seen in Figure 5.36. The descriptive statistics are shown in Table 5.6 and post-hoc results from repeated measures ANOVA are available in Appendix 8. Presence of nitrogen during storage of the vegetable matrix resulted in significant changes in the quantity of nicotinamide ($p = 0.006$), riboflavin ($p = 0.010$) and thiamine ($p = 0.008$). The concentration of nicotinamide and riboflavin was exhausted at 72 h, while thiamine also significantly reduced at 72 h, with a reduction of 46.9%, from 53.72 ng/g (T1) to 27.43 ng/g (T72). This is comparable to storage under no gas, in which the quantity of nicotinamide ($p = 0.005$), riboflavin ($p = 0.027$) and thiamine ($p = 0.001$) significantly reduced throughout storage, initiating at 72 h. Alternatively, in the presence of compressed air, nicotinamide ($p = 0.014$) and thiamine ($p = 0.003$) were the only monitored B vitamins that significantly reduced, nicotinamide was not quantifiable (undetected) after 96 h and similarly thiamine was completely exhausted at 120 h. On the other hand, the content of pyridoxine throughout all storage studies conducted in the bio-fermenter did not significantly change ($p > 0.05$).

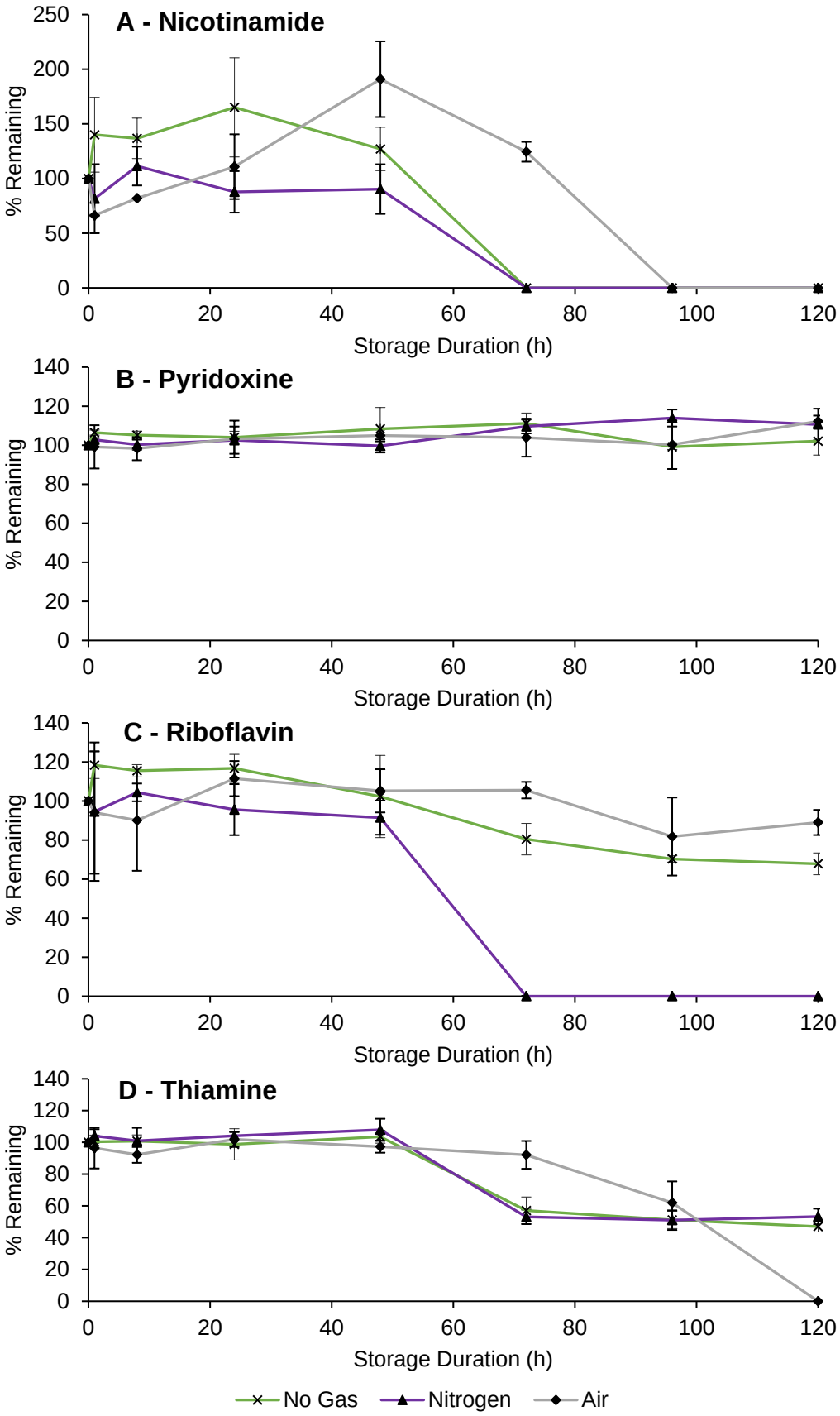


Figure 5.36: Stability of B-group vitamins, nicotinamide (A), pyridoxine (B), riboflavin (C) and thiamine (D) in the vegetable matrix over 5 days of storage (120 h) at 20 °C under no gas and in the presence of nitrogen and air. Stability is represented as the % remaining of each vitamin based on quantity of each vitamin at T0. Each data point is an average of three or two analysed samples and associated error is expressed by a 95% confidence interval.

Table 5.6: Descriptive statistics of vitamin stability in the vegetable matrix stored over a 5-day period at 20 °C in the bio-fermenter under no gas and in the presence of nitrogen and air.

No gas								
	Nicotinamide		**Pyridoxine**		**Riboflavin**		**Thiamine**	
Time point/s	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV
T0	151.83	41.26	38.09	1.29	74.26	8.26	65.21	2.83
T1	212.70	45.85	40.55	0.43	87.90	4.48	65.44	2.42
T8	207.65	24.82	40.06	0.69	85.76	2.07	65.70	2.27
T24	250.70	60.84	39.63	1.05	86.68	4.72	64.39	5.66
T48	192.97	26.55	41.28	3.68	75.99	13.82	67.51	2.30
T72	ND*		42.35	1.78	59.75	5.30	37.24	4.91
T96	ND*		37.78	0.46	52.22	1.07	33.38	3.13
T120	ND*		38.90	2.41	50.37	3.66	30.65	1.95
Nitrogen								
Time point/s	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV
T0	114.20	13.34	25.67	0.70	68.69	4.79	51.64	2.79
T1	93.19	31.88	26.40	0.20	68.54	13.90	53.72	1.95
T8	127.27	17.91	25.74	0.58	71.71	2.77	52.14	3.72
T24	100.30	19.14	26.34	1.58	65.65	7.94	53.78	1.08
T48	103.23	22.92	25.58	0.76	62.83	5.30	55.72	3.17
T72	ND*		28.15	0.81	ND*		27.43	2.08
T96	ND*		29.25	0.99	ND*		26.36	2.81
T120	ND*		28.40	1.84	ND*		27.53	2.26
Compressed Air								
Time point/s	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV
T0	62.19	1.10	23.14	1.85	68	14.84	57.85	1.37
T1	41.43	0.19	22.95	2.27	74.02	10.45	55.78	6.57
T8	51.23	0.17	22.76	1.23	54.59	14.69	53.36	2.61
T24	65.81	11.25	23.88	1.93	75.82	4.41	58.93	2.46
T48	119.38	19.13	24.30	0.62	75.08	3.71	56.28	1.94
T72	76.48	3.74	24.04	1.99	71.78	2.54	53.29	4.47
T96	ND*		23.22	2.55	55.63	9.81	35.85	6.88
T120	ND*		25.98	0.60	58.3	0.22	ND*	

[a] Mean is comprised of three samples (n=3), if a sample was missing completely at random, the value was replaced using the expectation-maximisation (EM) algorithm.
* ND = not detected

5.3.4 pH

The pH of the vegetable matrix was manipulated prior to storage, investigating the impact of an acidic and alkaline environment.

5.3.4.1 The Effect of (Pre-set) pH on Microbial Growth

The influence of an acidic or alkaline environment on microbial growth is shown in Figure 5.37. The vegetable matrix used in both pH studies had an initial growth of microorganisms prior to storage, counts between 3.95-3.97 log CFU/g were recorded. The acidic environment resulted in a decrease in the microbial growth, where zero colonies were observed at 24 h, compared to the alkaline environment where growth rate increased to 9.06 log CFU/g at 24 h. After 24 h, the microbial growth in the acidic environment increased and then decreased, with a maximum microbial count of 5.82 log CFU/g. In contrast, the alkaline environment further supported microbial growth until growth rate reached a plateau, with the microbial count at the end of the storage period concluding at 9.71 log CFU/g. In comparison, the growth of microorganisms from 0-120 h under baseline, was lower than the alkaline environment and growth began to decrease at 48 h.

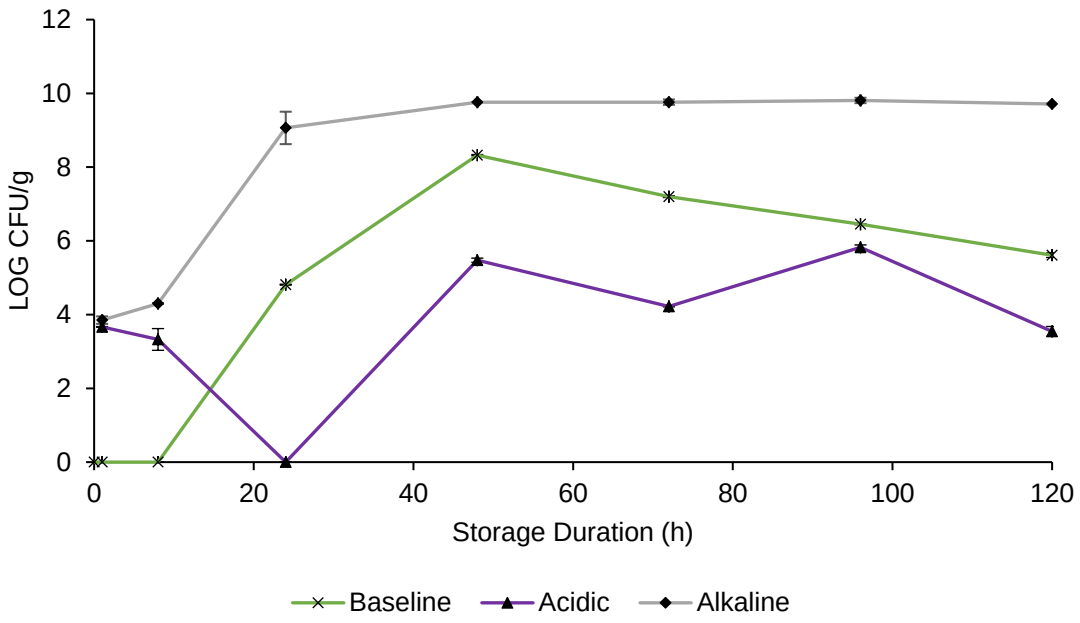


Figure 5.37: TVC of the vegetable matrix stored for 120 h at 20 °C under pre-set pH conditions, acidic (~pH 3.6) versus alkaline (~pH 8.6). Also including baseline condition. For each data point, two replicates were analysed, and the error associated with each time point is represented by a 95% confidence interval.

5.3.4.2 The Effect of (Pre-set) pH on Bacterial Diversity and Composition - Alpha Diversity

Alteration of the initial pH of the vegetable matrix prior to storage had different effects on bacterial diversity over time. Lowering of the pH resulted in a decrease in all diversity measures over time, as shown in Figure 5.38, although the decrease was not significant. The impact of lowering pH on bacterial diversity throughout the storage duration was comparable to baseline. In contrast, when the pH was increased, the species richness and diversity indices, Shannon and Simpson significantly decreased at 48 h, by 89.4%, 99.8% and 65.5% respectively, however this decrease was followed by an increase in bacterial diversity by 120 h (Figure 5.38). The bacterial diversity at specific time points under different pre-set pH conditions, including baseline were compared. There were significant differences in the bacterial diversity when assessed by the Inverse Simpson index only for time points, 0, 48 and 120 h across storage conditions. All data from statistical tests are available in Appendix 9.

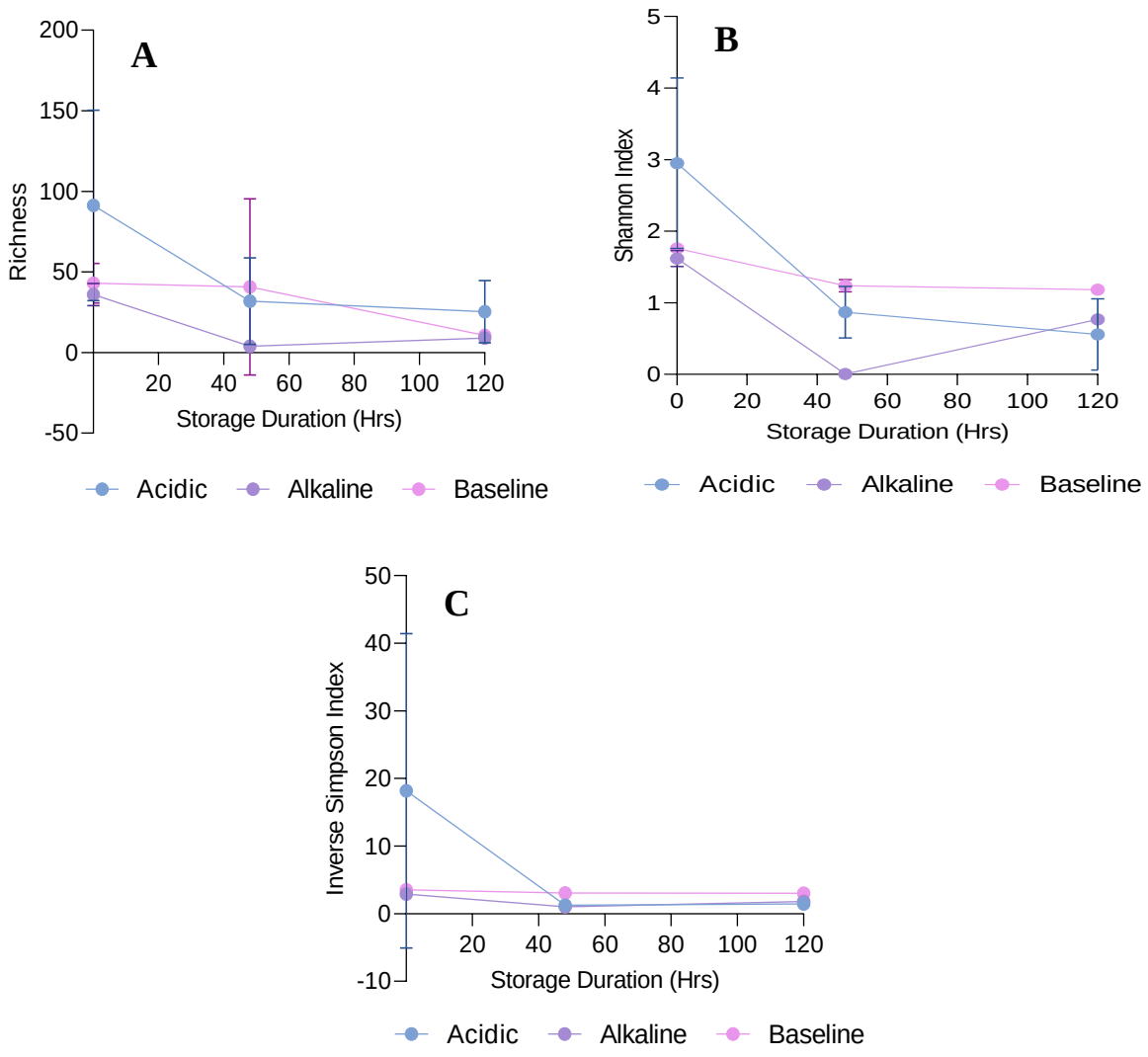


Figure 5.38: Alpha diversity of the overall microbial composition during storage of the vegetable matrix at 20 °C under pre-set acidic and alkaline conditions and also including baseline condition. Alpha diversity was determined by total richness (A), Shannon index (B) and Inverse Simpson index (C). Each data point comprises of three technical replicates and associated error is expressed by standard deviation.

The microbial composition of the vegetable matrix was further influenced by pH conditions (Figure 5.39). At 0 h the microbial community was both diverse and complex, particularly for the acidic condition, whereas for the alkaline environment at 0 h *Lactococcus*, *Leuconostoc* and *Yersinia* were among abundant bacterial communities. In the acidic environment at 48 h, *Yersinia* was predominant, responsible for ~87.5% of the microbial community, while *Escherichia*/*Shigella* and *pseudomonas* were among other bacterial communities present. At 120 h, the microbial composition was similar to 48 h. The bacterial composition in the alkaline environment was comparable to the acidic environment at 48 h as *Yersinia* were dominant, however as the storage period continued, the abundance of *Yersinia* reduced (~68.75%), complemented with an increase in abundance of both *Leuconostoc* (~25%) and *Lactobacillus* (~6.25%). Furthermore, when compared to the baseline, *Yersinia* was also abundant at 48 and 120 h although abundance was lower, as *Lactococcus* and *Leuconostoc* were also predominant communities alongside *Yersinia* at 48 and 120 h under baseline conditions.

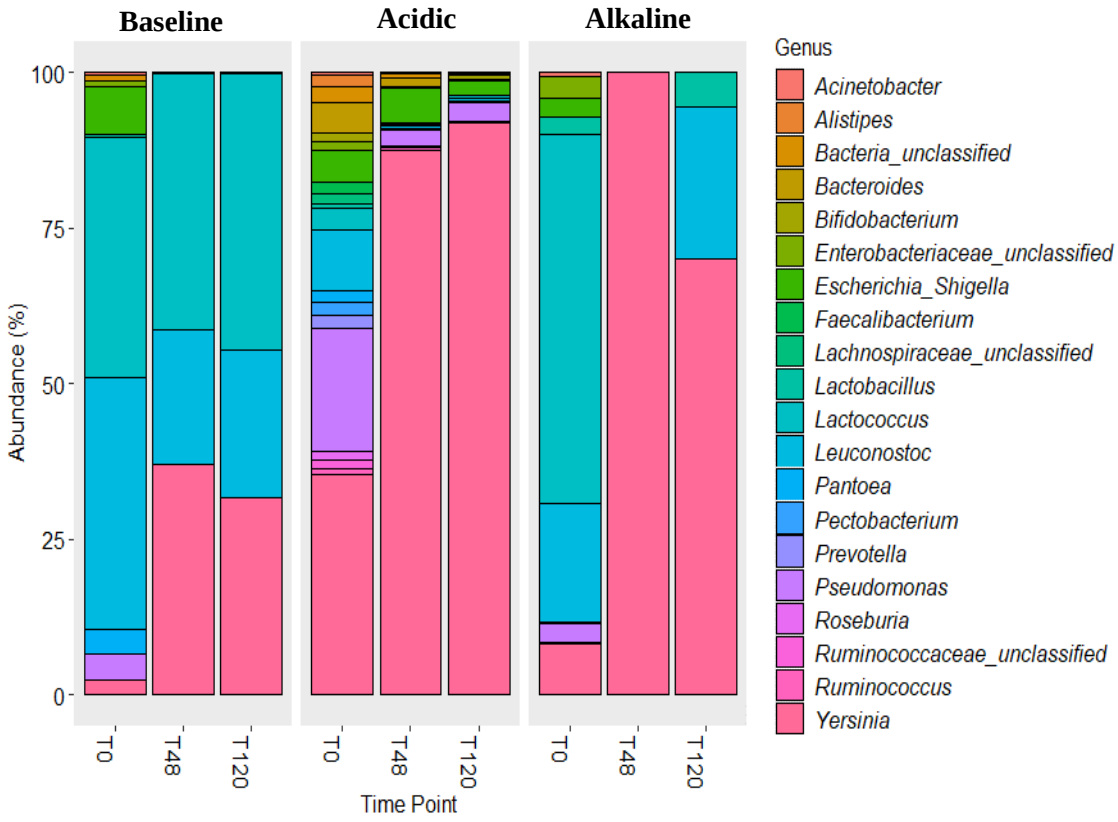


Figure 5.39: Microbial composition and development throughout the time course of the vegetable matrix stored at 20 °C under pre-set pH conditions (acidic versus alkaline) and under baseline condition. The microbial composition was expressed as the % abundance of the top 20 bacterial communities classified at genus level. Each time point comprises of three technical replicates.

5.3.4.3 The Impact of Time and (Pre-set) pH on Microbial Composition – Beta-Diversity

Alteration of the vegetable matrix pH to an acidic environment or more alkaline environment resulted in a similar microbial composition over the storage period, according to the calculated Bray-Cutis index as represented in the PCoA in Figure 5.40. All samples collected at 48 and 120 h for the acidic and alkaline environment were clustered close together, away from all 0 hr samples, separated by axis 1 (45.9% of the variance explained). However, the middle (48 h) and end (120 h) time points under baseline condition are clustered together away from that of the acidic and alkaline time points, separated on axis 2 (27.0% variance explained). This is support by the PERMANOVA test, which indicated both time point ($p = 0.001$, $R^2 = 0.4386$) and storage environment resulted in significant changes to the bacterial community ($p = 0.001$, $R^2 = 0.0.2598$). Post hoc data further highlighted significant differences in bacterial composition between the baseline and both acidic ($p = 0.003$) and alkaline environment ($p = 0.027$).

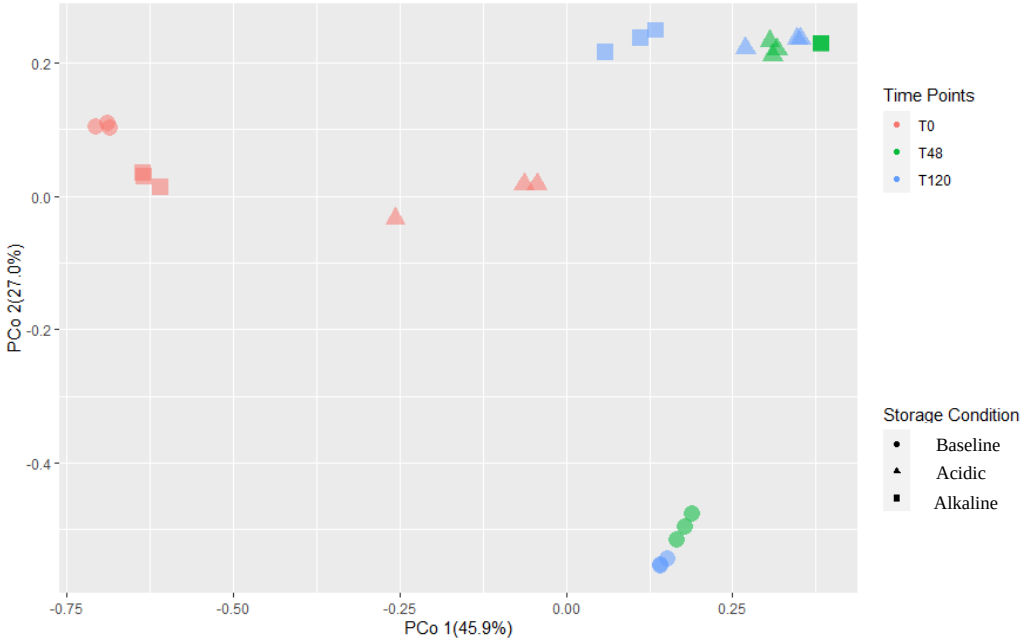


Figure 5.40: Principal coordinate analysis of Bray-Curtis dissimilarity distance matrix, samples grouped by time points 0 h – 120 h and storage condition under acidic and alkaline environment, including baseline condition. Each time point comprises of three analysed samples.

5.3.4.4 pH Profile

The pH profile was additionally evaluated over the time course and respective data is available in Figure 5.41. After the vegetable matrix pH was lowered and subsequently stored, the overall pH was maintained throughout the entire storage period. However, when the initial pH of the vegetable matrix was increased to pH 8.6 (alkaline condition), the alkaline environment was not

maintained and at 24 h reduced to pH 6.15 comparable to the pH of that of the baseline. The pH continued to decrease for the alkaline environment to pH 5.17 at 120 h.

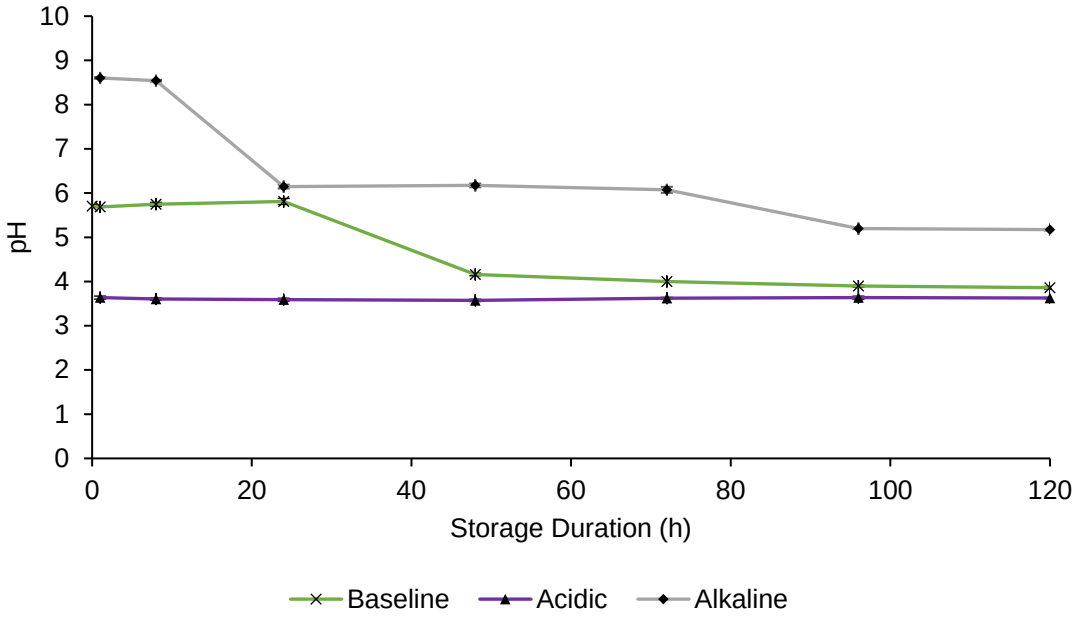


Figure 5.41: The pH profile of vegetable soup during 5-days of storage at 20 °C under different pre-set pH conditions, acidic (~pH 3.6) versus alkaline (~pH 8.6). Also, including baseline (~pH 5.7). pH was measured in triplicate at each time point and associated error expressed by a 95% confidence interval.

5.3.4.5 The Effect of (Pre-set) pH on Metabolite Analysis

The impact of a pre-set pH environment on the metabolite profile during the time series, when comparing individual time points across different pH environments is shown in the scores plot generated from the PLS-DA in Figure 5.42. Samples are shown to cluster in association with each pH environment at 0, 48 and 120 h. At 48 h the variance between acidic and alkaline environment is explained by component 1 (42.3%), while the variance between baseline and both acidic and alkaline environment are explained by component 2 (37.6%). The main metabolites responsible for the discrimination between samples are shown in Figure 5.43 according to VIP metric and corresponding box plots for several discriminatory metabolites are displayed in Figure 5.44. Pathway analysis was completed on the putatively annotated metabolites as shown in Figure 5.45. Metabolites were significantly associated with aminoacyl-tRNA biosynthesis ($p < 0.01$), purine metabolism ($p < 0.01$) and arginine and proline metabolism ($p = 0.0458$).

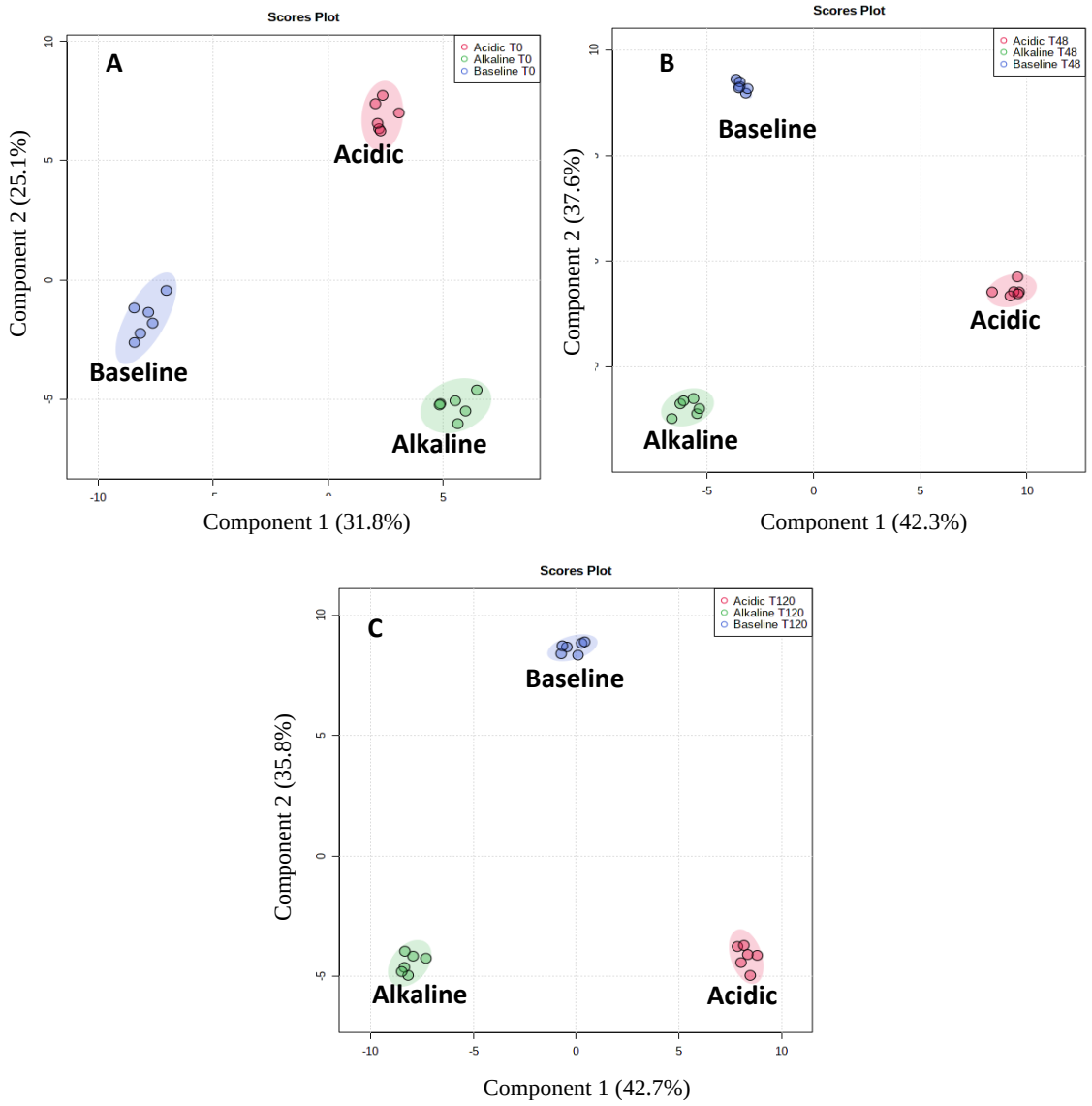


Figure 5.42: PLS-DA scores of the effect of pH on metabolite profile at 0 h (A), 48 h (B) and 120 h (C). Each time point under each storage condition is composed of six technical replicates.

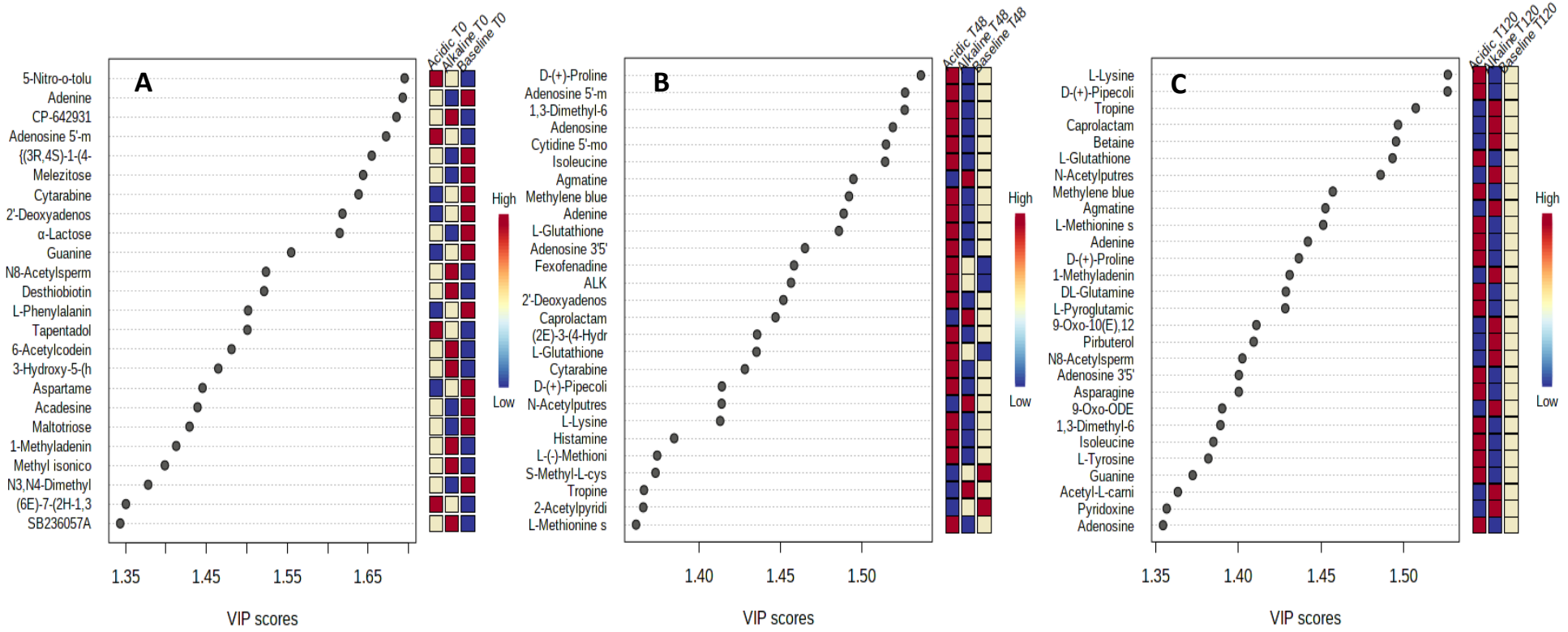


Figure 5.43: VIP ranking of the variables/metabolites (VIP > 1.35) at 0 h (A), 48 h (B) and 120 h (C) when comparing storage of the vegetable matrix under different pH conditions (acidic and alkaline) and baseline condition, as identified by PLS based on component 1.

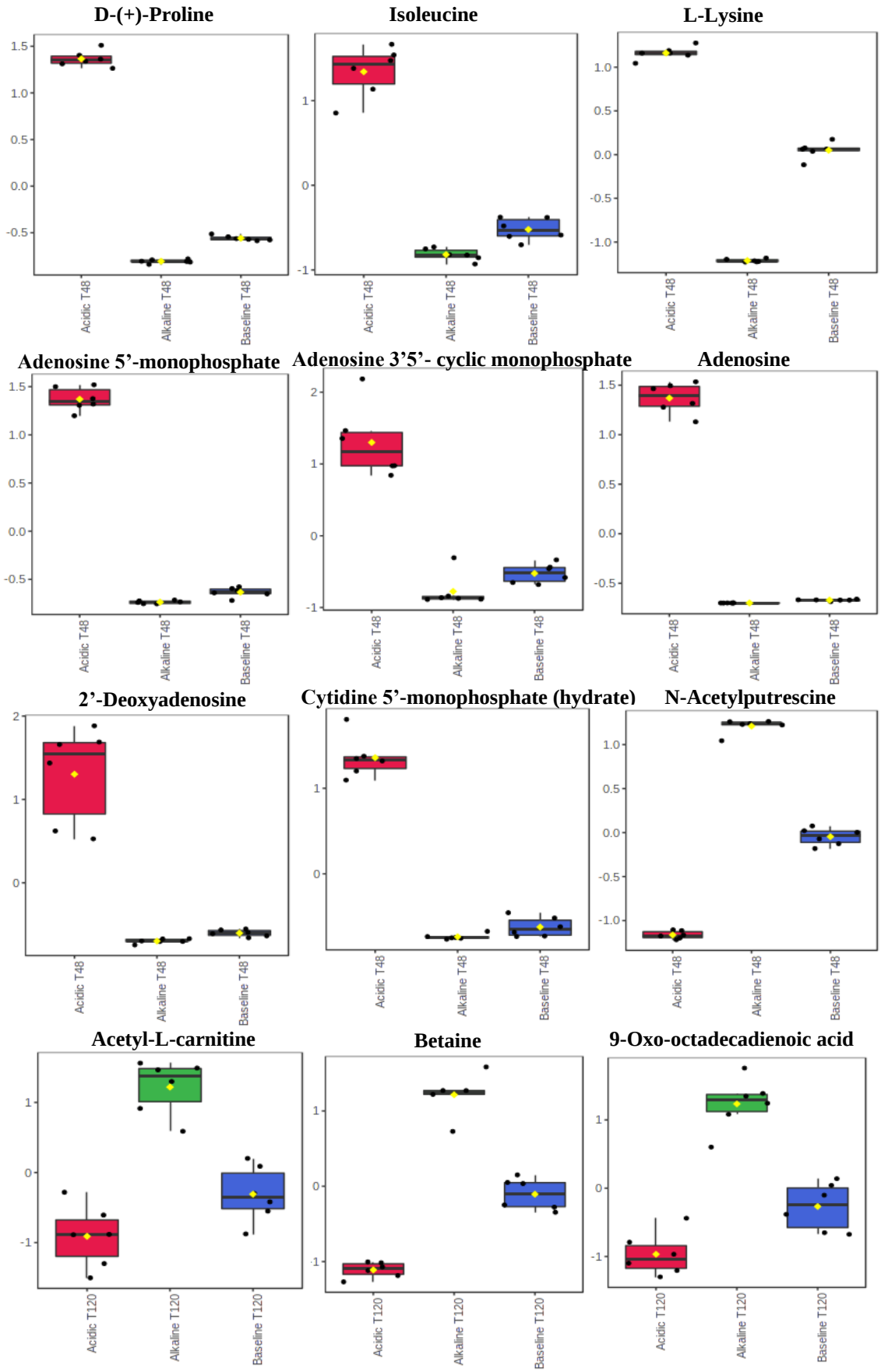


Figure 5.44: Examples of key discriminatory metabolites according to VIP analysis during storage of the vegetable matrix under different pH conditions (acidic and alkaline) and baseline condition at 48 and 120 h. Y axis represents normalised peak area (arbitrary units) and X axis represents different storage conditions. Each condition is composed of six technical replicates.

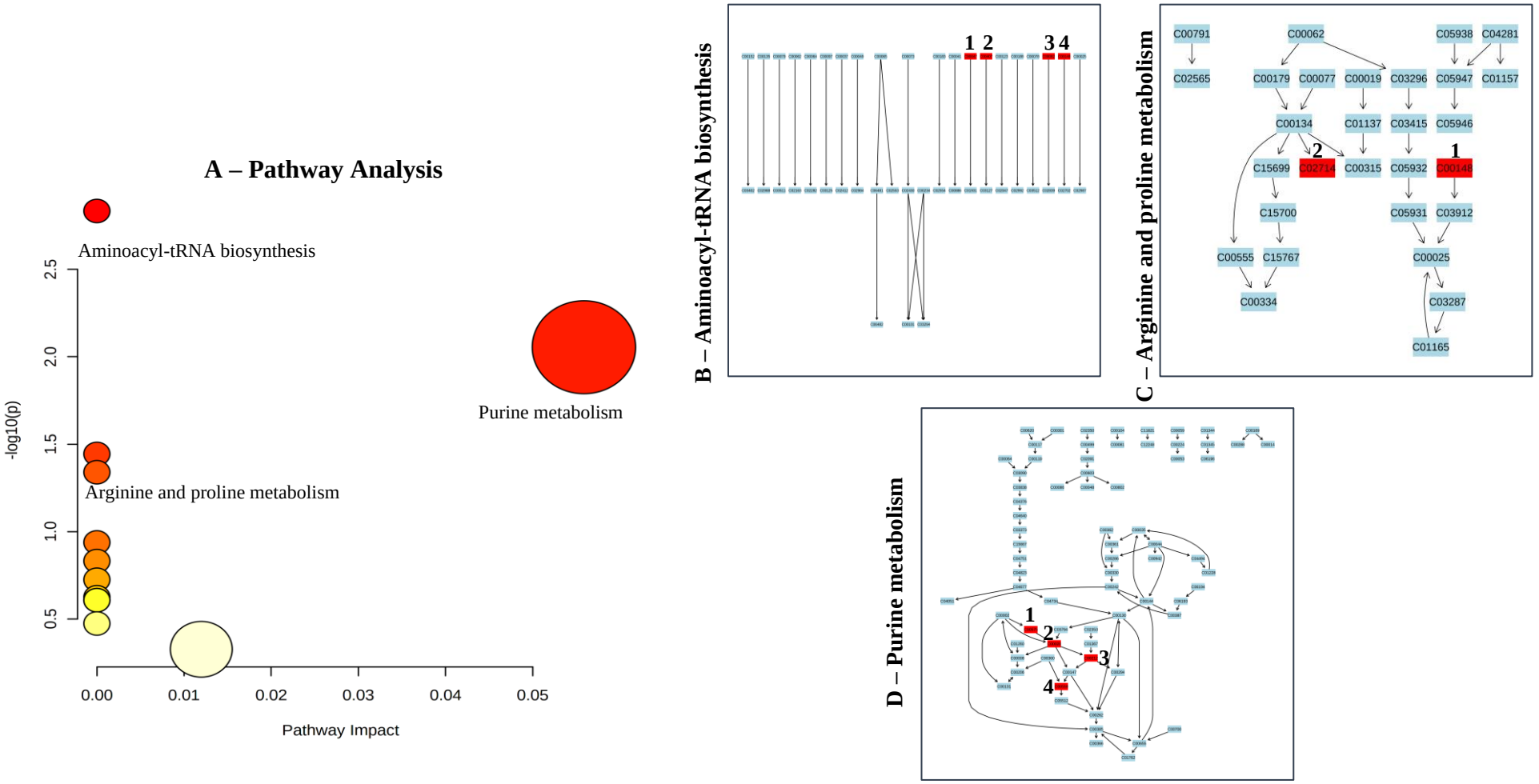


Figure 5.45: Pathway analysis of putatively annotated metabolites (A) and pathways shown (B-D) are deemed to have significant association with some of the putatively annotated metabolites, which are highlighted in red. For aminoacyl-tRNA biosynthesis, metabolites include 1=L-lysine, 2= L-isoleucine, 3=L-tyrosine and 4= L-proline. For arginine and proline metabolism, metabolites include 1= L-proline and 2= acetylspermidine. For purine metabolism, metabolites include 1= adenosine 3’5’-cyclic monophosphate, 2= adenosine 5’ monophosphate, 3= adenosine and 4= 2’deoxyadenosine.

5.3.4.6 The Effect of (Pre-set) pH on Volatile Organic Compound Analysis

The impact of pH on VOC profile during storage of the vegetable matrix was assessed, comparing 48 h of storage under different pH conditions, including at baseline (pH = ~pH 5.7). PCA was used to visualise the importance of the top 25 most significant VOC features, shown in Figure 5.46. The PCA shows samples are clustered in accordance with pH conditions and are clustered away from each other, with the variance in the samples explained by component 1 (79.47%) and 2 (19.57%). The top features that are responsible for the relationship in the PCA were putatively annotated and are displayed in Table 5.7. VOCs abundant during pre-set alkaline conditions at 48 h includes, 1-propanethiol, trimethylpyrazine, pyruvic acid and ethyl octanoate. In comparison to baseline, γ-terpinene, 1-octene, dodecanal and acetic acid were VOCs prevalent.

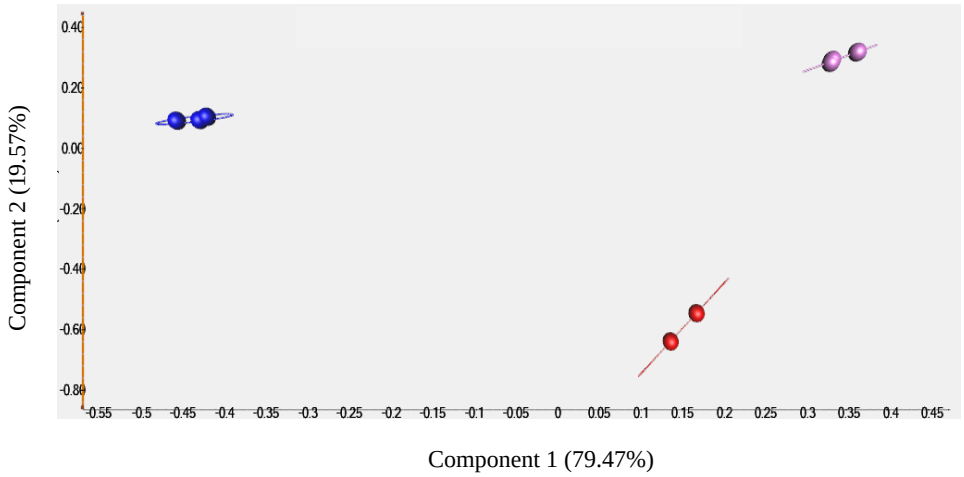
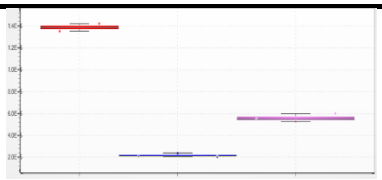
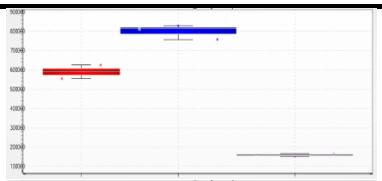
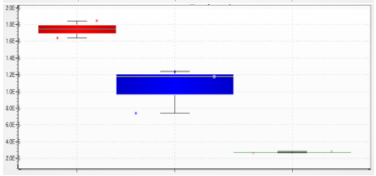
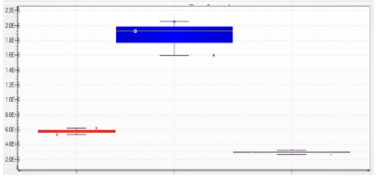
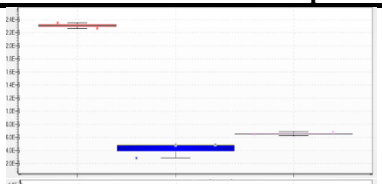
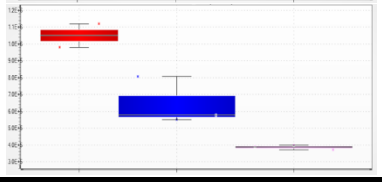
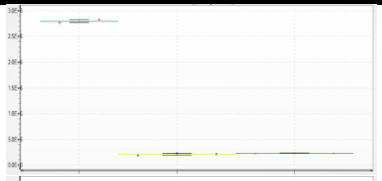
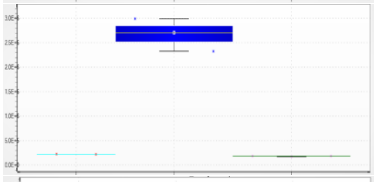
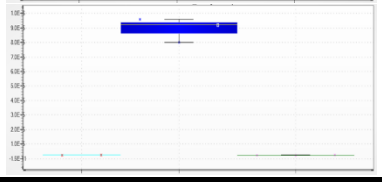
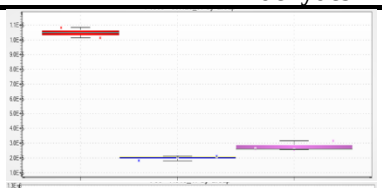
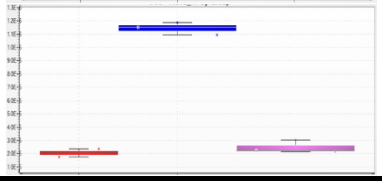
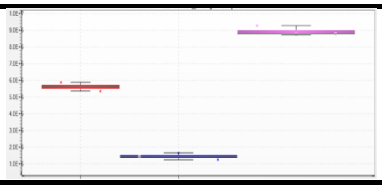
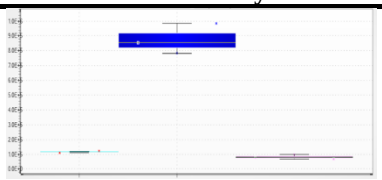
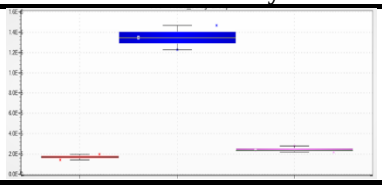


Figure 5.46: PCA comprising of the top 25 most significant features when comparing storage under different pH conditions (acidic and alkaline) and baseline condition at 48 h. Each storage condition contains three technical replicates, except for the baseline, which is composed of two technical replicates.

Table 5.7: The top discriminatory annotated VOCs according to PCA when comparing storage under different pre-set pH conditions (acidic and alkaline) and baseline at 48 h. Box plots shown, red = baseline, blue = alkaline and pink = acidic, and y axis represents abundance.

Putatively Annotated Volatiles		pH volatile is highest		
Alkanes and Alkenes		Baseline	Acidic	Alkaline
1-Octene		×		
Alcohols				
3-Methyl-2-heptanol				×
1-Hexanol		×		
2,3-Butanediol				×
Monoterpenes				
γ-Terpinene		×		
β-Linalool		×		
Short chain fatty acids (SCFAs) **or Derivatives**				
Acetic acid		×		
Pyruvic acid (2-Oxo-propanoic acid)				×
Ethyl octanoate				×

Aldehydes			
Dodecanal		×	
2,3,6-Trichlorobenzaldehyde			×
Ketones			
1-(Ethylthio)-2-propanone		×	
Pyriazine			
Trimethylpyrazine			×
Alkyl thiols			
1-Propanethiol			×

5.3.4.7 The Effect of (Pre-set) pH on Stability of B-vitamins

The influence of an acidic and alkaline environment on vitamin stability during storage is shown in Figure 5.47, including storage of the vegetable matrix under baseline conditions where the pH was unchanged. Complementary descriptive statistics are shown in Table 5.8 and follow-up post hoc results from repeated measures ANOVA are shown in Appendix 8. Based on the results, storage of the vegetable matrix in acidic conditions had no significant effect on the quantity of nicotinamide, pyridoxine, riboflavin, and thiamine ($p > 0.05$). However, a pre-set alkaline environment promoted significant changes in the quantity of all assessed B-vitamins. Nicotinamide ($p = 0.00038$) and thiamine ($p = 0.00015$) significantly decreased, nicotinamide was undetectable from 48 h, while thiamine also decreased at 48 h, with 31% of thiamine remaining (69% depleted). This was comparable to the baseline, as nicotinamide and thiamine decreased at 48 h. Interestingly, pyridoxine and riboflavin showed a different profile under an alkaline environment, the content of both vitamins significantly increased by the end of the storage period, p value = 0.00018 and 0.001 respectively. Riboflavin increased by 20% at 120 h of storage and pyridoxine stability although similar to the profile of riboflavin, significantly decreased at 48 h, followed by complete depletion (undetected) at 48 h, however by 120 h the quantity of pyridoxine increased by 580%.

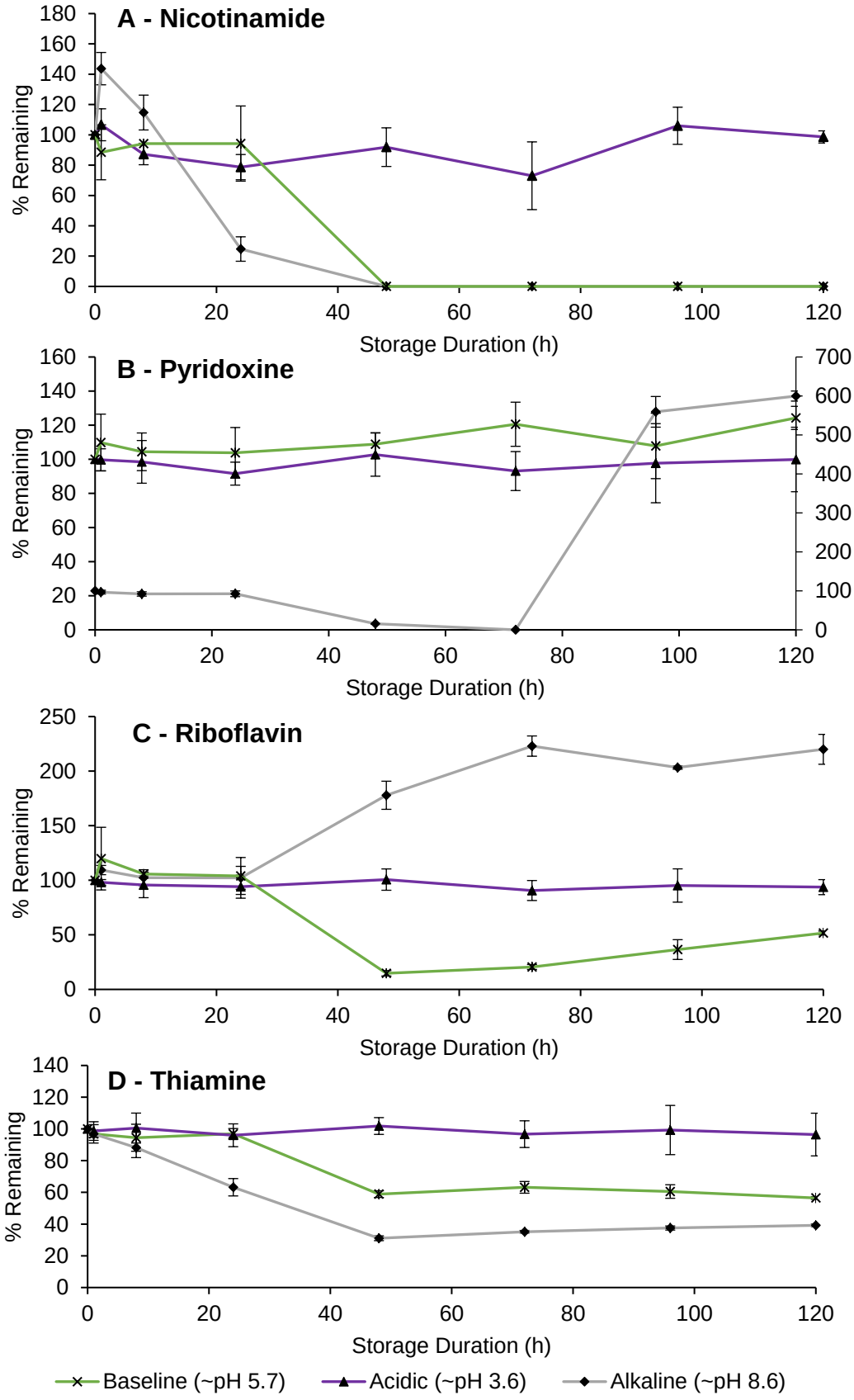


Figure 5.47: Stability of B-group vitamins, nicotinamide (A), pyridoxine (B), riboflavin (C) and thiamine (D) in the vegetable matrix over 120 h at 20 °C under pre-set pH conditions. pH conditions included baseline (~pH 5.7), acidic environment (~pH 3.6) and alkaline environment (~pH 8.6). Pyridoxine (B) is represented under alkaline conditions on the secondary y-axis on the righthand side of the figure. Stability is represented as the % remaining of each vitamin based on quantity of each vitamin at T0. Each data point is an average of three analysed samples and associated error is expressed by a 95% confidence interval.

Table 5.8: Descriptive statistics of vitamin stability in the vegetable matrix stored for 5-days at 20 °C under pre-set pH conditions (acidic and alkaline) and including baseline condition.

Baseline (~pH 5.7)								
	Nicotinamide		**Pyridoxine**		**Riboflavin**		**Thiamine**	
Time point/s	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV
T0	591.20	80.46	95.70	1.91	232.57	31.15	278.10	3.40
T1	523.10	94.75	105.10	14.12	278.77	58.98	269.23	5.30
T8	556.93	1.27	99.90	9.35	245.80	3.64	262.6	20.88
T24	557.20	129.58	99.30	12.52	241.70	34.79	269.57	8.12
T48	ND*		104.17	5.69	34.30	5.11	163.73	5.20
T72	ND*		115.37	10.96	47.80	4.65	175.67	9.05
T96	ND*		103.20	16.25	84.93	18.57	168.37	10.46
T120	ND*		118.93	5.71	120.17	3.72	157.00	2.19
Acidic (~pH 3.6)								
Time point/s	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV
T0	95.30	12.24	32.67	1.86	78.37	5.48	24.33	1.29
T1	101.60	8.87	32.53	1.83	76.83	1.79	24.00	1.25
T8	83.07	5.78	32.13	3.59	75.03	8.10	24.47	2.01
T24	75.00	7.02	29.90	1.95	73.77	7.29	23.37	1.56
T48	87.57	10.73	33.53	3.62	78.83	6.79	24.77	1.10
T72	69.57	18.81	30.43	3.31	71.00	6.30	23.53	1.81
T96	101.00	10.29	31.93	6.69	74.60	10.59	24.13	3.33
T120	94.00	3.39	32.63	5.46	73.40	4.77	23.47	2.85
Alkaline (~pH 8.6)								
Time point/s	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV
T0	174.90	29.26	83.60	5.30	69.40	4.69	66.80	4.12
T1	251.27	16.51	80.43	4.20	75.87	2.57	64.73	3.45
T8	200.67	17.78	75.90	4.73	71.03	4.45	58.97	3.72
T24	43.17	12.43	76.23	6.25	70.93	6.47	42.23	3.27
T32	ND*		ND*		123.40	7.89	20.77	0.87
T48	ND*		ND*		154.70	5.68	23.50	0.52
T56	ND*		529.13	33.73	141.03	0.95	25.07	0.76
T72	ND*		568.37	11.10	152.63	8.41	26.17	0.47

[a] Mean is comprised of three samples (n=3).
* ND = not detected

5.4 Discussion

A comprehensive evaluation of different storage environments and preparation procedures on spoilage of the vegetable matrix was assessed. These time dependent studies investigated the impact of temperature, gas composition, pH and preparation methods on nutritional integrity, microbial growth, bacterial communities, physiochemical properties, and metabolite profiles of the vegetable matrix.

5.4.1 The Influence of Temperature on Spoilage and Nutritional Composition of the Vegetable Matrix

The growth of microorganisms was dependent on the storage temperature, as temperature increased (7 °C, 20 °C, 37 °C), the growth rate increased. The lower storage temperature maintained microbial quality for longer (4 days), due to extending the lag-phase, whereas at 20 °C and 37 °C microbial growth was promoted with peak growth captured at 2 days and 1 day of storage respectively. This is a well characterised observation, as authors have shown increased storage temperature of several food matrices increases bacterial growth, while simultaneously reducing shelf-life (10, 23, 24, 112, 468-470). Increased growth was also associated with deterioration of the organoleptic properties of the vegetable matrix, including development of off odours and gas production. Boziaris *et al*. (2011) (10) showed similar findings, in which increased storage temperature of a perishable food matrix increased bacterial population, further coinciding with pH changes and development of volatiles associated with off-odours. In this chapter, the pH profile reduced during storage at 20 °C and 37 °C only, and this is presumably a consequence of sugars being fermented by SSOs generating organic acids, such as acetic acid which was observed in the VOC analysis (387, 388). The decrease in pH was in line with the recorded peak growth of microorganisms, therefore at 37 °C the pH began to decrease earlier in the time course. An earlier report by Raccach and Bamiro (1997) (469), investigated different temperature for fermentation of rye flour by LAB, they showed as temperature increased (10-35 °C) the fermentation time decreased, which resulted in a reduction in the lag phase. This supports the findings in this chapter, although storage under 7 °C did extend the lag phase, it did not increase fermentation time towards the end of the storage duration, despite an increase in growth to 7.1×10^5. This is potentially due to the TVC being below the threshold of that associated with spoilage (10^7-10^9 CFU/g), or due to the bacterial communities present (28).

Amplicon sequencing revealed the bacterial communities present in the vegetable matrix was affected by the storage temperature selected. At 20 °C *Lactococcus*, *Leuconostoc* and *Yersinia* were the dominating bacterial communities at 48 h. Interestingly at 37 °C, *Bacillus spp.* were responsible for a large proportion of the microbial community, competing with *Leuconostoc* and *Lactococcus* from 48 h. At 37 °C the dominance of *Bacillus spp.* would have largely been contributed to the storage temperature, as *Bacillus spp.* grows optimally at 37 °C, providing the

opportunity for the vegetable associated genus to thrive and dominate (471, 472). According to Wilks *et al*. (2008) (473), species belonging to the *Bacillus* genus are able to adapt and survive under a range of pH conditions, including acidic environments, while cytoplasmic pH is maintained. Therefore, *Bacillus spp.* were able to compete with *Leuconostoc* and *Lactococcus* even when pH was reduced as a result of fermentation activity of LAB. It appeared *Yersinia spp.* were unable to compete with the other species during storage at 37 °C, however the relationship between these species has not been described in the literature. Although, it could be due to nutrients available to support growth, some microbial species might have a higher affinity for essential nutrients, adversely impacting growth of those that do not (100, 102). In contrast, during storage at 7 °C the bacterial composition was diverse, with no dominating bacterial communities until the end of the storage period (120 h), due to the lower temperature reducing overall bacterial growth. This is in agreement with a study conducted by Li *et al*. (2019) (201), who showed during the first 7 days of storage of vacuum packaged bacon no species dominated and as a result the diversity of the bacterial community was greater. In this chapter, by 120 h of storage under 7 °C, *Pseudomonas spp.* became the dominate organism. *Pseudomonas spp.* are psychrotolerant and can grow at low temperatures, particularly refrigeration temperatures (474, 475). It has also been suggested that *Pseudomonas spp.* are able to outcompete other types of bacteria at low temperatures, with low storage temperatures in combination with increased storage duration providing an advantage for *Pseudomonas spp.* to thrive, which has been demonstrated in this study (474, 476). Additionally, LAB optimally grow between 20 – 32 °C and compete with bacteria by producing acids, however LAB struggle to produce acids below 20 °C (123, 477). Therefore, during storage at 7 °C, LAB was not able to compete with *Pseudomonas spp.* Assessed richness and diversity metrics Shannon and Inverse Simpson, showed initially the bacterial composition was very diverse, which further decreased throughout storage duration for all temperatures investigated. These findings agree with studies conducted by both Li *et al*. (2019) (201) and Park *et al.* (2012) (211).

B-vitamins, nicotinamide, pyridoxine, riboflavin, and thiamine were influenced by storage temperature. During storage at 20 °C, nicotinamide, riboflavin and thiamine significantly reduced in line with an increase in TVC, reduction in pH and reduction in bacterial diversity (as discussed in previous chapter). This was also the case at 37 °C, although due to the increase in temperature increasing microbial growth and reducing fermentation time, nicotinamide, riboflavin, and thiamine depleted or reduced 24 h earlier. This further highlights a relationship between microbial activity and B-vitamins, indicating nicotinamide, riboflavin and thiamine are potentially required for microbial growth and activity (374). B vitamins act as precursors for coenzymes involved in macronutrient metabolism, therefore the reduction of these nutrients may be indicative of their use for energy production to facilitate growth of dominating bacterial communities (404, 405). Their reduction could be due to their precursor forms being involved in metabolic pathways including pentose phosphate pathway, krebs cycle and glycolysis (229, 230, 408, 418, 478).

Alternatively, pyridoxine remained stable during storage at both 20 °C and 37 °C, highlighting dominating bacteria did not require exogenous pyridoxine for metabolic processes, such as biosynthesis of proteins or metabolism of amino acids (479, 480). In comparison, during storage at 7 °C there was no significant change in the investigated B-vitamins, due to low temperature minimising spoilage reactions. Also, the dominant species at 120 h under 7 °C may not initially require these vitamins for growth. It is important to acknowledge although investigated B vitamins are reported to be sensitive to temperature, this study did not investigate high temperatures (50 °C+), therefore a reduction in the vitamins was not a factor of thermal degradation, but a result of spoilage reactions (224, 225, 251, 259, 460, 461).

Metabolomic fingerprinting showed temperature played a significant role on the metabolite profile. Edwards *et al.* (2021) (481) also showed that storage temperature impacted on metabolite profiles, due to temperatures increasing microbial growth and thus activity. Several putatively annotated metabolites in this chapter have previously been discussed in chapter 4 as potential spoilage markers, or indicators of product freshness, this includes hypoxanthine, adenosine 5'monophosphate (AMP) and glutathione. Briefly, hypoxanthine is a degradation product of AMP and was more prevalent at higher storage temperatures (20 °C and 37 °C), which was correlated to the low abundance of AMP at these temperatures (159, 189, 482). Glutathione is metabolised by bacteria including LAB due to its antioxidant properties and as a result its abundance was reduced at 20 °C and 37 °C (438, 439). Metabolites abundant during the lowest storage temperature 7 °C, where bacterial growth was minimised, included histamine. Histamine is a biogenic amine that is naturally occurring in low quantities in vegetables, such as onions and carrots and has been shown to be degraded by LAB (483, 484). This agrees with this study, as histamine is lower at higher temperatures where fermentation has occurred. Nucleotide and nucleosides, adenosine 3', 5'-monophosphate (cAMP), adenosine, 2'deoxyadenosine and guanine are metabolites found in vegetables as shown by their abundance at 7 °C (485-487). These metabolites were lower at 20 °C and 37 °C, due to SSOs potential requirement of nucleotides or nucleosides for cellular function, for example: adenosine is a nucleoside crucial for cell growth, essential for metabolism and is a precursor for DNA and RNA (488-490). It has also been described in the literature that specifically LAB use exogenous nucleotides and nucleosides as a carbon source, energy source and purine source (491, 492). These metabolites were also significantly associated with purine metabolism according to pathway analysis, alongside hypoxanthine and AMP. Metabolites also in greater abundance at 7 °C, include amino acids (glutamine and ergothioneine) and L-pyroglutamic acid which is produced from both L-glutamic acid and L-glutamine (493, 494). Due to these metabolites being reduced during higher storage temperatures, it indicates that amino acid pathways are potentially used by dominating bacterial communities (488). Metabolites that were prevalent during high storage temperatures included 5-methylcytosine, formed from methylation of DNA where cytosine is converted to 5-metylcytosine and has been found in bacteria, although this has not been shown in other spoilage studies (440,

441). Furthermore, 1-methylguanine (abundant at 20 °C), L-iditol (abundant at 37 °C), methyl isonicotinate (abundant at 37 °C) and N-benzylformanide (abundant at 7 °C) were other discriminatory metabolites during storage under different temperatures. However, after extensive searching these metabolites have not been associated with spoilage and can be classified as novel findings in this chapter.

Storage temperature also shaped the VOC profile at 48 h with differences shown in the following volatile groups: alkanes, alcohols, short chain fatty acids (SCFAs), monoterpenes, ketones and aldehydes. At highest storage temperature, 37 °C, alkanes including 3-ethylhexane, 2,4-dimethylhexane, nonane and decane were in higher abundance compared to lower storage temperatures. Similarly Filannino *et al*. (2021) (495), showed during fermentation of a food matrix namely alkanes, 3-ethylhexane, nonane and decane increased during fermentation or were present in the fermented product only. Alkanes are generated from fatty acid degradation either from oxidation or lipolysis, the latter is a result of microbial activity as FFAs are utilised as precursors for catabolic reactions, generating alkanes (446, 447, 496). The higher abundance of alkanes at 37 °C, is likely due to the increased storage temperature elevating microbial activity, and spontaneous oxidation of SCFAs (497). Furthermore, the alcohols isooctanol and 1-hexanol were in greater abundance during storage of the vegetable matrix at 37 °C and 20 °C respectively. Isooctanol has been identified as a compound of *Bacillus spp.* and therefore the increase in abundance at 37 °C could be due to the dominating bacterium (498, 499). Interestingly in this study, 1-hexanol was significantly higher at 20 °C than other storage temperatures. This could be related to greater dominance of LAB at 20 °C, which are shown to be responsible for alcohol production including 1-hexanol (500). Additionally, SCFAs, acetic acid was highly abundant at both 20 and 37 °C only, associated with fermentation of sugars by heterofermentative LAB complementary to the findings of Liu *et al*. (2022) (500). However, abundance of acetic acid was higher at 20 °C due to higher abundance of *Leuconostoc*, when compared to 37 °C. Based on findings monoterpenes including γ-terpinene, terpinolene, β-linalool and p-cymene were abundant during storage to different extents depending on storage temperature. Monoterpenes are a widely occurring plant metabolites that have antimicrobial properties and would be expected in a vegetable matrix (501-503). The differences in abundance across storage temperatures can be related to a study by Mandha *et al*. (2022) (504), as they recorded both increases and decreases in monoterpenes during fermentation of mango juice by LAB. One explanation as to why monoterpenes, chiefly γ-terpinene, terpinolene and β-linalool, are prevalent at 20 °C and 37 °C could be due to acid production by LAB, damaging plant cells resulting in the release of monoterpenes (504). In contrast, cymene and 1-propenylbenzene are monoterpenes low in abundance at higher storage temperatures, but prevalent at 7 °C, where spoilage is minimised, and fermentation does not occur. Cymene has been shown in another study to decrease during fermentation, which is reflective of storage temperatures 20 °C and 37 °C (504). However, no publications have shown the impact of storage and spoilage reactions on 1-propenyl-benzene.

5.4.2 The Influence of Freeze-Thaw on Spoilage and Nutritional Composition of the Vegetable Matrix

Preparation of the vegetable matrix from frozen was important to investigate as an alternative method for consumers to employ to keep the vegetable matrix fresher for longer, reducing chemical and biochemical reactions and further inhibiting metabolic activity (462). TVC revealed when the vegetable matrix was prepared from frozen and stored for 5-days an extended lag phase of 16 h was recorded, when compared to the vegetable matrix prepared from fresh. The extended lag phase is acknowledged as an effect of freeze-thaw alongside reduced growth, due to the process causing stress and damage to the microbial cells; the lag phase can be considered as the time taken for microorganisms to recover and subsequently grow (505, 506). Amplicon sequencing showed the preparation procedure significantly changed the microbial communities during storage. LAB and *Yersinia* were dominant bacterial communities in the fresh vegetable matrix, however when the vegetable matrix was prepared from frozen, these communities were not abundant and instead unclassified *Bacillales* were predominant. The freezing process can have negative effects on LAB, O'Brien *et al*. (2016) (507) showed during frozen storage of fermented milk (kefir), LAB namely, *Lactobacilli* and *Lactococci* significantly declined over 30 days of storage. Similarly, another report has shown reductions in strains of LAB during freezing of yogurt (508). However, Velly *et al*. (2014) (509) highlighted LAB, such as *Lactococcus lactis* were not damaged by the freezing process. Although, the discrepancy between studies will be dependent on the strain, the degree of damage to the cells, nutritional requirements after induced stress, the matrix itself and freezing rate (510). In addition, *Yersinia spp.* have also been shown to be damaged by the freezing process, with a reduction in log counts recorded in beef samples when stored between -18 to -20 °C for 1 month (511). This confirms that freezing of the vegetable matrix in this chapter has damaged the cells associated with *Lactococcus*, *Leuconostoc* and *Yersinia*, allowing *Bacillales* to grow. Due to LAB not being predominant during the storage of the vegetable matrix prepared from frozen, the pH did not decline to levels observed during spoilage of the fresh vegetable matrix, although the pH did slightly reduce at 72 h.

The vitamin profile during storage of the vegetable matrix prepared from frozen was different to that of the fresh vegetable matrix, as nicotinamide was the only vitamin that was significantly reduced at 72 h. The reduction in nicotinamide was in line with slight decrease in pH and increase in TVC. Therefore, the stability of nicotinamide, pyridoxine, riboflavin and thiamine are potentially dependent on the nutritional requirement of the microbial community. *Bacillales* could have required nicotinamide as a precursor for nicotinamide adenine dinucleotide ($NADH/NAD^+$), due to its involvement in various metabolic processes acting as a cofactor for several redox reactions (512). According to KEGG pathways *Bacillales* can synthesise pyridoxine, riboflavin, thiamine *de-novo* and therefore potentially did not require an exogenous supply of these vitamins from the environment (428-430).

Metabolites were influenced by the preparation procedure prior to storage. The discrepancy in the metabolites between the different preparation procedures over storage could largely be a factor of different dominating spoilage organisms. For example: acetylspermidine is a biogenic amine, that serves as a measure of spoilage (189). Acetylspermidine is formed by ornithine decarboxylase produced by both *Enterobacteriaceae* and LAB during fermentation, which were the predominant bacterial communities during storage of the fresh vegetable matrix (148, 431, 433). It has been reported that amino acid decarboxylase activity is relatively low in species belonging to *Bacillales*, particularly when compared to LAB, therefore the greater abundance of biogenic amines in the fresh vegetable matrix are likely due to bacterial communities present (513). Ornithine and citrulline were also higher in the fresh vegetable matrix and are also associated with activity of LAB, produced through the arginine deiminase pathway, which explains the reduced abundance of arginine in the fresh vegetable matrix (434, 435). The arginine deiminase pathway comprises of three metabolic reactions; arginine is initially converted to citrulline by arginine deiminase, from which ornithine transcarbamylase catalyses the production of ornithine and carbamoyl phosphate from citrulline and finally carbamate kinase, phosphorylates ADP from carbamoyl phosphate (514). All three metabolic steps require bacteria to have the necessary genes encoding such metabolic enzymes (*arcABC*) and only 21% of *Bacillales* have the *arcABC* genes, while almost half of *Lactobacillales* have the necessary genes for the arginine deiminase pathway (514). Therefore, the reduced abundance of ornithine and citrulline in the vegetable matrix prepared from frozen could be due to the dominant bacteria, *Bacillales* lacking the capabilities to use the arginine deiminase pathway. Furthermore, hypoxanthine was abundant in the fresh vegetable matrix, part of purine metabolism and is acknowledged in the literature as being a indicator of spoilage (160). Hypoxanthine is formed by autolysis of both nucleotides and nucleosides and can also be produced by bacteria (159, 160). Glutathione was another metabolite more abundant in the from frozen vegetable matrix. Glutathione is a widely distributed thiol in organisms and plants and owning to its function in cellular mechanisms, playing a major role in combating physiological stress, predominant bacteria potentially utilised this metabolite at 48h (439, 515, 516). *Leuconostoc* and *Lactococcus* do not have the genes required to synthesise glutathione *de-novo*, however, to meet requirements they have the capabilities to uptake this metabolite from the environment, as shown at 48 h (438). Based on VIP analysis, glutathione was not an important metabolite at 120 h, suggesting glutathione could have also been utilised in the from frozen vegetable matrix later in the time course. This reasoning coincides with maximum microbial count and change in vitamin content (nicotinamide) occurring at 72 h for the vegetable matrix prepared from frozen as opposed to 48 h for the fresh vegetable matrix. Although, there is limited literature detailing glutathione metabolism in *Bacillales* regarding their ability to synthesise glutathione *de-novo* or, possessing the genes encoding the steps to uptake glutathione from the environment. Novel findings include the identification of DNA methylation products (1-methyladenine, 3-methlyadenine and 1-methylguanine) in the vegetable matrix prepared from

fresh, which have not been previously associated with food degradation and spoilage reactions. LAB do have the genes encoding enzymes (methyltransferases) for DNA methylation and therefore potentially the reason for the increased abundance when the vegetable matrix is prepared from fresh (442, 443).

The VOC profile was affected by the preparation procedure, as a greater abundance of terpene's were present in the vegetable matrix prepared from frozen. A few authors have also reported an increase in prevalence of terpene's when plant-based products are frozen (517, 518). However, Kjeldsen, Christensen and Edelenbos (2003) (519) showed the freezing process had a minimal effect on terpene content of carrots. The differences in studies will be related to the degree of damage to tissue cells during the freeze-thaw process to release terpene's, including formation of ice crystals and dehydration of cells, all of which is a factor of the matrix itself (462, 510). VOCs prevalent in the vegetable matrix prepared from fresh included 1-amino-2-butanol, short chain fatty acid acetic acid, aldehydes dodecanal and nonanal. The presence of acetic acid and aldehydes in the vegetable matrix prepared from fresh can be associated with products of fermentation by LAB, as described in the literature (294, 500, 520). Therefore, due to LAB not being the dominant species in the vegetable matrix prepared from frozen these volatiles were not abundant. 1-Amino-2-butanol has not been described in the literature in terms of spoilage, however butanol's are produced by spoilage bacteria in vegetables as shown by Ragaert *et al*. (2006) (521) and can be yielded from LAB (522). Butanol's are generated from degradation of amino acids valine, isoleucine and leucine, therefore high abundance of these volatiles in the fresh vegetable matrix is potentially linked to dominating spoilage species (521).

5.4.3 The Influence of Gas Composition on Spoilage and Nutritional Composition of the Vegetable Matrix

In this study, the presence and absence of oxygen on the vegetable matrix was assessed and a control was used to reflect the baseline conditions in chapter 4, referred to as 'no gas'. The microbial counts of the vegetable matrix stored in the presence of air and no gas were almost identical. Amplicon sequencing showed *Leuconostoc* and *Lactococcus* were the predominant communities growing in these conditions responsible for spoilage. Both *Leuconostoc* and *Lactococcus* are facultative anaerobes growing optimally in an environment where less oxygen is present as shown in this study (523, 524). Similarly, the main spoilage species in vacuum-packaged products, including meats where no oxygen is present have been shown to belong to the *Leuconostoc* and *Lactococcus* genera (12, 525). The dominance of *Leuconostoc* and *Lactococcus* during storage of the vegetable matrix was correlated with a reduction in overall bacterial diversity; Wang *et al*. (2016) (12), also showed a reduction in microbial diversity during spoilage of lamb meat as anaerobic bacteria dominated. The presence of nitrogen would have also influenced the redox potential of the vegetable matrix, as the absence or low concentration of

oxygen decreases redox potential, leading to a reduced environment for LAB to grow (526). This would have also been achieved in the no gas environment as the fermentative products (organic acids) produced by LAB, would have reduced redox potential of the vegetable matrix (87). Alternatively, storage of the vegetable matrix in the presence of air significantly changed the microbial composition during storage. *Leuconostoc* and *Lactococcus* were among microbial communities present during storage of the vegetable matrix in the presence of air at 48 h, however by 120 h *Bacillus* was the predominant bacterial community responsible for spoilage. It is reasonable to suggest, LAB was unable to compete with *Bacillus* in the presence of air, as an oxygenated environment can cause stress to both *Leuconostoc* and *Lactococcus* and therefore can be detrimental to growth (527, 528). LAB also struggle to deal with reactive oxygen species, such as hydrogen peroxide which can be generated from the presence of oxygen (527). In contrast, *Bacillus* are classified as aerobic or facultatively anaerobic and therefore were able to thrive in the presence of air and outcompete LAB (529). The pH increased in the presence of air at 72 h, a response of metabolic activity of *Bacillus spp.*, in which the increase is due to protein hydrolysis via protease activity and amino acid metabolism which generates ammonia (529-531). The increase in pH cannot be correlated to TVCs as no data is available, due to the presence of an organism which swarmed the agar plates. This swarming bacterium is most likely *Bacillus spp*., as this behaviour on agar plates has been reported before (532).

The vitamin profile under no gas and nitrogen was similar to the vitamin profile in chapter 4, this shows degradation of nicotinamide, riboflavin and thiamine is linked to spoilage activity of fermentative genus *Leuconostoc* and *Lactococcus*. Interestingly under the presence of nitrogen, riboflavin is completely depleted at 72 h, whereas in the no gas environment at 72 h riboflavin decreased by 20%. At present, it is unclear as to why the nitrogen environment has promoted complete exhaustion of riboflavin. In contrast, storage of the vegetable matrix in the presence of air had a different impact on the vitamin profile, nicotinamide and thiamine were the only vitamins that significantly reduced. Nicotinamide was exhausted at 72 h in line with increased pH, indicating this vitamin has been potentially utilised for metabolic reactions of *Bacillus spp.* and it is well accepted that the precursor of nicotinamide, NAD^+ is crucial for cell viability (411, 512, 533). Alternatively, thiamine reduced from 72 h and was completely exhausted at 120 h. The decrease in thiamine could be due to several factors, firstly thiamine is a cofactor for thiamine pyrophosphate (TPP), which is essential for carbohydrate and amino acid metabolism and according to the literature *Bacillus spp.* can require thiamine for growth (417, 422). The reduction in thiamine also correlates to the increase in pH associated with protein hydrolysis and amino acid metabolism (533). Secondly, certain species of *Bacillus* can produce enzyme thiaminase I, which leads to the destruction of thiamine by substituting the base of the thiazole moiety to a nucleophile such as pyridine (421, 422). Thirdly, thiamine is unstable in the presence of oxygen and can be vulnerable to oxidation particularly as pH increases and reactive oxygen species are produced, such as hydrogen peroxide (227, 280, 281).

Metabolite profile was influenced by the presence and absence of oxygen. One of the main metabolites that was largely different at 120 h was hypoxanthine with greater abundance in air. Species of *Bacillus*, which were the main bacterial community in air have been stated to catalyse the degradation of external nucleotides and nucleosides to hypoxanthine (534, 535). Similar to hypoxanthine, betaine was prevalent in the vegetable matrix stored in air. Betaine is a derivate of glycine produced by certain microorganisms through oxidation of essential nutrient choline, catalysed by enzymes, choline dehydrogenase and betaine aldehyde dehydrogenase, with the latter process being NAD^+ dependent (536, 537). Therefore, as expected the lower abundance of plant nutrient choline coincided with the higher abundance of betaine (538). These findings agree with Ercolini *et al*. (2011) (25), who showed an increase in betaine during storage of meat in air. They stated the production was a result of dominant organism requiring the metabolite for its protective effects against osmotic stress, caused by dehydration of the investigated matrix in air (25, 190). The accumulation of betaine in the bacterial cells increases solute content which aids in the restoration of turgor pressure (539). *Bacillus*, the predominant microbial community during storage of the vegetable matrix in air has been described to synthesise betaine when precursor, choline is available as shown in this chapter (540). Storage of the vegetable matrix in air promoted production of N6-acetyl-L-lysine, this metabolite is an acetylated amino acid where an acetyl group is transferred onto a lysine unit (541). This process is described as post translational modification where bacteria change the function of a protein in response to environmental conditions, without the process of biosynthesising new proteins (541). Furthermore, urocanic acid was also abundant in the vegetable matrix stored in the presence of air. Urocanic acid is a product of histidine metabolism, with bacteria including *Bacillus* linked to production of this metabolite (542). Although description in the literature of urocanic acid in terms of spoilage has mainly been associated with fish storage and has not been shown during spoilage of a vegetable matrix (543-545).

Gas composition influenced VOC profile at 48 h, with no gas environment showing a different VOC profile when compared to nitrogen and air. The main identified volatiles that were responsible for the discrimination between experimental conditions were: alcohols (1-amino-2-butanol, 1-hexanol, tetradecanol), decane and monoterpenes (cymene, terpinolene, 1-propenylbenzene). Due to no gas environment and nitrogen showing a similar microbial growth, composition, and pH profile during storage, it was expected that they would have presented with comparable volatile profiles. However peak microbial growth, vitamin loss and degradation of the matrix occurred later in the time course when storage was conducted in the bio-fermenter (72 h). Therefore, analysis of 48 h potentially failed to capture all volatiles associated with spoilage in this storage condition and as a result requires further investigations.

5.4.4 The Influence of pH on Spoilage and Nutritional Composition of the Vegetable Matrix

pH of a food matrix shapes microbial growth and communities, due to pH being crucial in microbial metabolism, influencing redox reactions, nutrient bioavailability and the activity of intracellular and extracellular enzymes (81). In fact, increasing or decreasing the optimum pH by 1 pH unit can reduce growth of microorganisms by 50% (546). In this chapter, when the initial pH of the vegetable matrix was altered prior to storage, to either acidic or alkaline conditions the growth of microorganisms was affected. The TVC prior to storage under pH conditions was approximately 3 log CFU/g, comparable to values reported for processed vegetables of 3.0-6.0 log CFU/g (294). In the acidic environment (pH 3.6), the growth decreased initially, reaching a lag phase at 24 h, further followed by an increase in growth; although the microbial count did not reach levels associated with spoilage (10^7-10^9 CFU/g) (28). In contrast, the pre-set alkaline environment (pH 8.6) supported growth of microorganisms to spoilage levels (10^9 CFU/g). This is due to SSOs having the ability to grow and maintain cytoplasmic pH across several log units, from pH 5 to 9 (547).

Lactococcus, *Leuconostoc* and *Yersinia* were dominant communities under baseline conditions, however when the pH decreased, *Yersinia* became the dominant community from 48 h. The growth of LAB would have been limited as reports have shown acidic environments to negatively impact growth of LAB, although the minimum pH for growth is dependent on species (548, 549). *Lactococcus* can be inhibited at pH 4 and metabolic processes to generate energy, such as glycolysis is affected when extracellular pH is below pH 5 (550). Similarly, *Leuconostoc* species struggle to maintain cytoplasmic pH when pH declines as a result of organic acids, due to loss of cell viability (548, 551). Therefore, this has provided the opportunity for *Yersinia spp.* to grow, although it is important to note growth was reduced under acidic conditions. This has been shown in another study, in which *Yersinia* growth reduced as pH of the medium decreased (pH 3-6) (395). Abdela *et al*. (2011) (552) also showed in orange juice spiked with *Yersinia spp.*, that the acidic environment of pH 3.9 supported growth, comparable to the pH and organic acid (citric acid) used in this chapter. However, during the study, the low pH of orange juice during storage significantly decreased the growth of *Yersinia spp.* and at 72 h the average recorded growth of *Yersinia spp*. was 5.5 log CFU/ml (552). This agrees with the bacterial count reported in this acidic environment, where *Yersinia spp.* dominated as 5.82 log CFU/g was the maximum microbial count recorded. Interestingly, the organic acid used to decrease pH can influence growth of *Yersinia spp.*, citric acid has been shown to be less effective as a bactericide than acetic and lactic acids, which supports findings in this chapter (553). In comparison, the alkaline environment also promoted *Yersinia* growth, leading it to become the dominant organism at 48 h, with LAB growth suppressed under the elevated pH. As an example, *Lactococcus spp.* grow at an optimum pH range of 6.3-6.9, therefore at a starting pH of 8.6 *Lactococcus* was unable to

compete (317). However, it is important to acknowledge pH of the vegetable matrix was not maintained and at the point *Yersinia spp.* dominated, the pH had reduced to pH 6.1 and further decreased to pH 5.1 at 96 h. As the pH decreased to 5.1, there was an increase in diversity associated with *Leuconostoc* and *Lactobacillus* gaining abundance. The decrease in pH is likely a response of microbial activity releasing metabolites leading to a reduction in pH and the decrease in pH has supported growth of LAB.

The impact of pH conditions on vitamin stability during storage of a food matrix has not been demonstrated in the literature. According to the data obtained in this chapter, the acidic environment had no significant impact on the B vitamins investigated. Both riboflavin and thiamine are acknowledged as being sensitive to pH particularly in slightly acidic, neutral and alkaline conditions (228, 268). However, both vitamins are reportedly more stable when present in their ionised form promoted by acidic conditions (228, 273). Thiamine for example becomes positively charged in an acidic environment, as the pyrimidine N1 and thiazole N3 have a pKa value of 4.8 (228, 271). Although the acidic environment allowed microorganisms to grow, particularly *Yersinia spp.*, growth was reduced and not in the regions associated with spoilage, therefore in this storage environment vitamins were shown not to be affected by spoilage reactions, nor the storage condition. Alternatively, the pre-set alkaline environment promoted significant changes to all B vitamins investigated. Nicotinamide, pyridoxine and thiamine significantly reduced, initiating at 24 h and continued to decrease, with exhaustion of nicotinamide and pyridoxine at 48 h. This is associated with increased microbial growth, suggesting these vitamins have been required by *Yersinia spp.* to support growth and metabolic activity (374). There are no publications available that have shown similar findings, but KEGG pathways outlines the metabolic pathways in *Yersinia spp.* for metabolism of thiamine, nicotinamide and pyridoxine, and the requirement of B vitamins by bacteria has been extensively discussed throughout this thesis (428-430). After compete exhaustion of pyridoxine, there was a significant increase of the vitamin between 72-120 h of storage. It is reasonable to suggest the increase in pyridoxine could be associated with *Lactobacillus* gaining abundance, as authors have reported *Lactobacillus*, among other LAB, to synthesise pyridoxine during fermentation in dairy-based products (404, 554-556). Li *et al*. (2012) (557), showed during fermentation of soymilk by different probiotic bacteria including *Lactobacillus* strains, increased vitamin B6 content by 354.3% depending on strain. Although this chapter observed more pyridoxine production (580%), it will depend on strain and growth including growth and fermentation conditions such as; nutrients available, temperature and pH (555, 557). In contrast, riboflavin also significantly increased from 48 h, while *Yersinia spp.* were dominating, publications are limited highlighting biosynthesis of riboflavin by *Yersinia spp*. However, it is widely accepted that most Gram-positive and Gram-negative bacteria have the capability to synthesis riboflavin *de novo* from guanosine-5'-triphosphate and ribulose 5-phosphate, generated from the pentose phosphate pathway which requires thiamine coenzymes and purine biosynthesis (404, 558).

Metabolites were significantly impacted by pH of the vegetable matrix. One of the main factors responsible for this observation is the impact of pH on growth of bacteria, due to the acidic environment reducing growth levels, metabolites including amino acids (proline, isoleucine, tyrosine, lysine) and both nucleotides and nucleosides (AMP, Adenosine 3', 5'-monophosphate(cAMP), adenosine, 2'deoxyadenosine and cytidine 5'-monophosphate) were highly abundant compared to alkaline and baseline. Amino acids are metabolised by SSOs generating spoilage metabolites including organic acids or amines such as putrescine, from which the N-acetylated form, N-acetylputrescine was abundant in the alkaline condition (150, 296). In a recent study by Zhang, Ding and Xie (2022) (488), authors showed during spoilage of pakchoi a significant decrease in amino acids including proline and lysine. Although other studies investigating spoilage showed minimal changes in amino acid content over storage (160). The differences will be attributed to the microbial communities present and the food matrix itself (559). Similarly, nucleotides and nucleosides are acknowledged as an indicator of product freshness, utilised by bacteria for cellar function and growth as previously discussed and can be degraded by endogenous enzymes (488, 560). Therefore, reduction in nucleotides and nucleosides coincides with increased growth and spoilage of the vegetable matrix. Metabolites that were prevalent in the alkaline environment consist of betaine, 9-oxo-octadecadienoic acid, DNA methylation product (1-methyladenosine) and acetyl-L-carnitine. Betaine is an amino acid derivative as discussed previously in this chapter, functioning as an osmolyte and accumulation of this metabolite is due to production by Gram-negative bacteria to deal with osmotic stress (561). The dominant community in the alkaline environment, *Yersinia*, are associated with betaine production (539). 9-oxo-octadecadienoic acid is a derivate of fatty acid linolenic acid, its role as a potential spoilage metabolite has not been discussed before. Although has been stated to be a metabolite produced by probiotic bacteria and alternatively can be formed during oxidation of naturally occurring linoleic acid (562-564). Production of DNA methylation metabolites and acetyl-L-carnitine has not been shown to be associated with spoilage. The latter metabolite is formed by the transfer of an acetyl group to carnitine, carnitine is similar to betaine and can be produced by *Yersinia* as an osmolyte (539, 565).

VOC profile was also influenced by pre-set pH conditions. The main VOCs that were different belonged to alcohols, alkyl thiols, aldehydes, SCFAs, pyriazine, alkene, monoterpenes and ketones. Focusing initially on alcohols, 3-methyl-2-heptanol, and 2,3-butanediol were more abundant in the alkaline group. Heptanol's have been associated with activity of spoilage species, Johanningsmeir and McFeeters (2011) (566), showed during spoilage of fermented cucumber 2-heptanol and 4-methyl-2-heptanol significantly increased. Other authors have also shown production of heptanol during fermentation of meats and milk (567, 568). Heptanol in general can give rise to a pungent, musty and leafy aroma (175). The higher intensity of alcohols in the alkaline environment as opposed to the baseline condition, will be associated with alkaline environment promoting increased growth. Certain bacteria can also produce 2,3-butanediol and

includes Gram-negative bacteria, specifically *Enterobacteriaceae* such as *Yersinia spp.*, which were the dominating community in the alkaline condition (569). The alcohol is produced during the metabolism of sugars, through several metabolic stages, from glycolysis production of pyruvate to α-acetolactate, in which is then converted to intermediate product acetoin/diacetyl, which further forms 2,3-butanediol (569). The pathways used to generate 2,3-butanediol requires $NADH/NAD^+$, highlighting the importance of precursor forms of nicotinamide during metabolic reactions (569). 2,3-Butanediol can be characterised by a fruity, creamy and buttery aroma (175). It has been previously described that production of 2,3-butanediol is dependent on the efficiency of metabolism. Therefore, the pH of the system is a highly influential factor, as it determines if *Yersinia spp.* are under optimum conditions for growth and production of metabolites (570). Furthermore, 1-propanethiol was present in high abundance in the alkaline environment, classified as an alkythiol, a sulfur containing compound, characterised by a pungent aroma in high concentrations (571). Thiols are plant metabolites and can be produced by spoilage species, although after extensive literature searching 1-propanethiol has not been reported to be linked to spoilage of food matrices (170, 571). SCFAs or derivatives of SCFAs identified included pyruvic acid, ethyl octanoate and acetic acid. Both pyruvic acid and ethyl octanoate were prevalent in the alkaline environment and have been linked to metabolism of *Yersinia spp.* (172, 572, 573). However, acetic acid was abundant in the baseline condition as opposed to alkaline and acidic environment. This is due to the presence of heterofermentative LAB *Leuconostoc* in the baseline environment, which is responsible for acetic acid production through fermentation of sugars (374). Trimethyl pyrazine was an additional metabolite classified as a pyrazine, identified in the alkaline environment and has been described in the literature as a marker of spoilage by Martín *et al*. (2010) (574) and Zhang *et al*. (2021) (575). Proteolytic activity of spoilage species can give rise to the formation of pyrazines and *Enterobacteriaceae* are stated to be responsible for pyrazine production, which agrees with the bacterial composition of the vegetable matrix under this condition (574, 576). Metabolites of greater abundance in the baseline condition include monoterpenes (β-linalool and γ-Terpinene), 1-octene, dodecanal and 1-hexanol this is either due to presence of LAB under baseline conditions or differences in batches e.g. rate of oxidation of fatty acids (504, 520). Interestingly, 1-(ethylthio)-2-propanone was the most abundant ketone in the acidic environment. Currently this ketone has not been described in the literature in terms of degradation of a food matrix, however, it was also abundant during storage of the vegetable matrix at 7 °C. Therefore, this volatile could be a marker of freshness of the vegetable matrix, due to its abundance in the minimally spoiled vegetable matrices.

5.5 Conclusion

An in-depth study of intrinsic and extrinsic environments on spoilage of the investigated vegetable matrix was explored, this included the impact of temperature, preparation procedures, oxygen

levels and pH. The combined approach of genomics and metabolomics (including VOCs), alongside assessments of the nutritional profile and microbial growth across the different storage environments in the vegetable matrix has not been presented in the literature before. All environments and preparation procedures had significant impacts on the microbial dynamics and composition of the vegetable matrix during storage, with the dominating spoilage organism influenced by storage condition selected. For example, storage of the vegetable matrix in air allowed *Bacillus* to dominate, while storage in nitrogen (no oxygen) promoted LAB, chiefly *Leuconostoc* and *Lactococcus* to thrive. Although many previous studies acknowledge that the investigated conditions can have drastic impacts on microbial growth; this chapter shows how more tolerable species can outcompete other communities associated with spoilage of the vegetable matrix under specific conditions. The change in the microbial composition also adversely influenced vitamin profile in many cases, further showing an important relationship between spoilage activity and preservation of B vitamins. Nicotinamide was the only vitamin that was fully exhausted in all storage studies assessed, regardless of the dominating community in the specific environment, therefore, nicotinamide has the potential to be a marker of product freshness and provide more rapid detection of ongoing spoilage than traditional plating techniques. Specific volatile and non-volatile metabolites were found to be associated with the increased microbial load, as well as the prevalence of certain bacterial communities. The putatively annotated metabolites discussed here have the potential to be either markers of freshness or markers of spoilage of the vegetable matrix. Some of the associated pathways include purine metabolism, glutathione metabolism, glycine metabolism and arginine & proline metabolism which may play a role in the degradation of the matrix, depending on the bacterial community. Many putatively annotated metabolites and VOCs were novel findings and have not been related to spoilage in the literature. This includes DNA methylation products that were prevalent during spoilage at higher temperatures and under an alkaline environment. 1-(Ethylthio)-2-propanone and 1-propenylbenzene were examples of metabolites that decreased during spoilage and have also not been described in other spoilage studies.

Chapter 6

Effect of Enhancing the Rate of Microbial Growth through Supplementation with Specific Nutrients on the Nutritional Stability and Spoilage Rate of a Vegetable Matrix

6.1 Introduction

Spoilage of the vegetable matrix is not solely a factor of microbial activity, but also spontaneous chemical reactions that occur simultaneously within the vegetable matrix. This includes lipid oxidation, protein oxidation, photooxidation of components, respiration of the matrix and activity of endogenous enzymes (277, 294, 577). Auto- or photooxidation of lipids can give rise to the production of undesirable metabolites including acids, alcohols and aldehydes (578). This presents a challenge of correlating spoilage features (e.g. off-odour, nutritional loss) with its source, either microbial activity and/or chemical changes, as stated by Remenant *et al*. (2015) (58). The previous chapter has captured microbiological and chemical spoilage in tandem; however, it is important to understand if spoilage features (vitamin stability, metabolites) are a factor of either the presence of microorganisms, or chemical reactions. One way of demonstrating this, is manipulating the vegetable matrix to achieve a more hospitable environment to enhance growth of specific spoilage organisms (SSOs) and conversely to supress growth of SSOs, while preserving the intrinsic environment of the vegetable matrix.

A major requirement of SSOs is the availability of nutrients in a medium to enhance growth including the accessibility of an energy source (e.g. glucose, amino acids, nucleotide) alongside vitamins and minerals (72, 577). Concentration of certain nutrients can also shape the overall microbial dynamics within a food system and the production of spoilage metabolites (47). The presence of sugars for example in the vegetable matrix provides the opportunity for the plant associated genus, *Leuconostoc* to both successfully thrive and compete by fermenting sugars producing undesirable spoilage metabolites (e.g. ethanol, organic acids, ketones) (579, 580). This was shown in chapter 4 (baseline condition), where sugars (namely sucrose and fructose) were primarily the substrate used as a source of energy by lactic acid bacteria (LAB). An overview of macronutrient metabolism by LAB and resultant volatile and non-volatile metabolites are shown in Figure 6.1. The B vitamin content was shown to alter in all storage studies when growth of SSOs increased to levels associated with spoilage. This was most apparent with nicotinamide which was completely exhausted under all storage conditions correlating to increased growth of SSOs. This is linked to precursor forms of nicotinamide, nicotinamide adenine dinucleotide ($NADH/NAD^+$), which play major roles in metabolic processes related to energy metabolism (230, 410, 579). Therefore, nicotinamide has the potential to be a marker of product freshness of

a vegetable matrix, which has not been described in the literature before. The increase in both sugar and nicotinamide content in a food matrix on spoilage metabolites has not been described previously. It is reasonable to suggest that increasing the available sugar within the vegetable matrix would allow for an extended growth of SSOs prior to the exhaustion of this key nutrient.

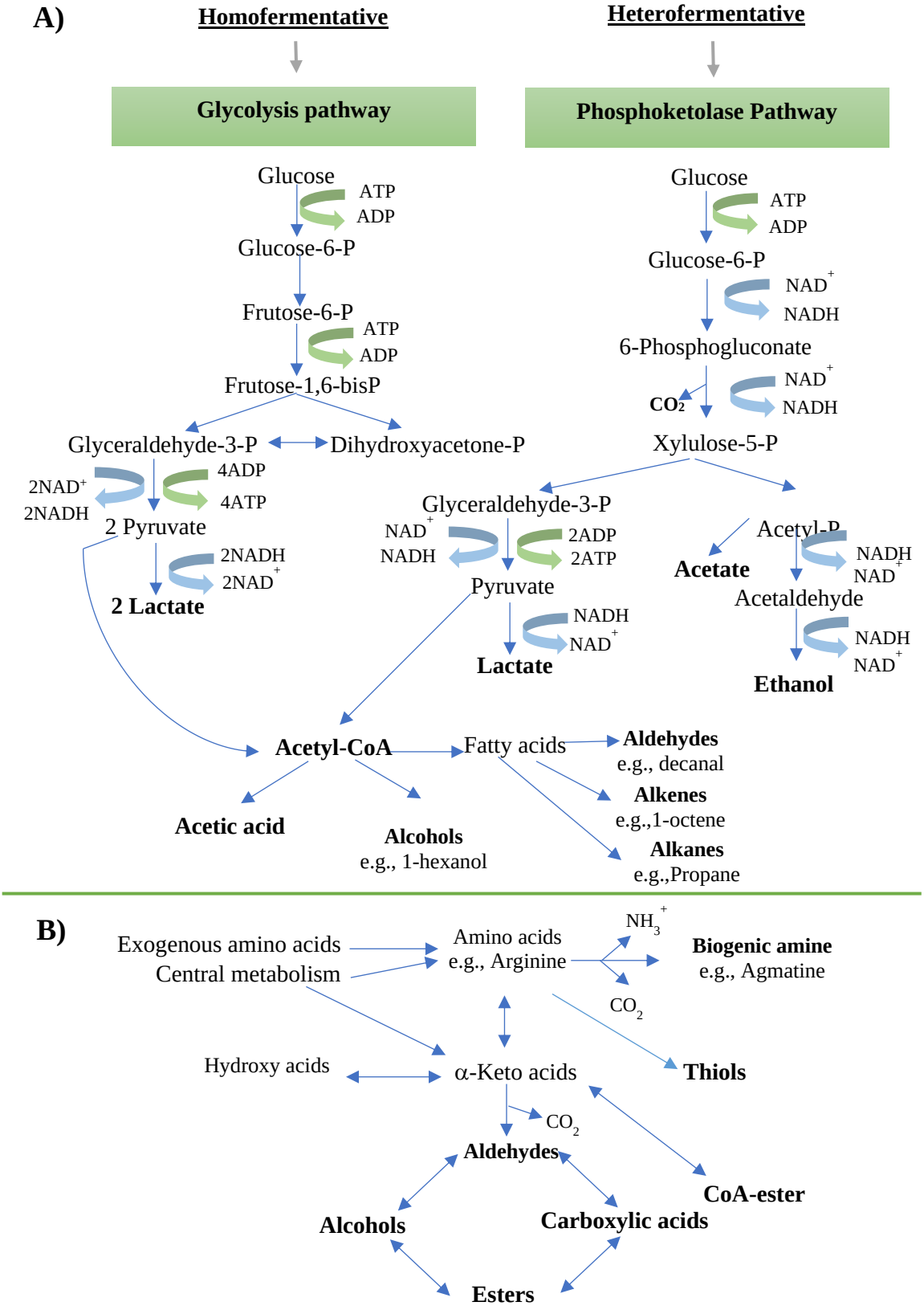


Figure 6.1: Overview of sugar (A) and amino acid (B) metabolism by homofermentative and heterofermentative lactic acid bacteria (LAB) and metabolites produced (54, 150, 581).

The aims of this chapter are to separate the main cause of spoilage features, with this chapter particularly focused on enhancing microbial spoilage. Objectives of the chapter are to 1) fortify the vegetable matrix with additional sugar (glucose, fructose and sucrose), 2) fortify the vegetable matrix with additional nicotinamide.

6.2 Methods

6.2.1 Vegetable Matrix Preparation and Storage

All methodologies used in this chapter are outlined in the general methods section in chapter 2, refer to section 2.1 and 2.5 for preparation procedures of the vegetable matrix and nutritional composition changes to the matrix respectively. The baseline condition will be included in this chapter for comparisons. An overview of the preparation procedure and storage can be seen in Figure 6.2

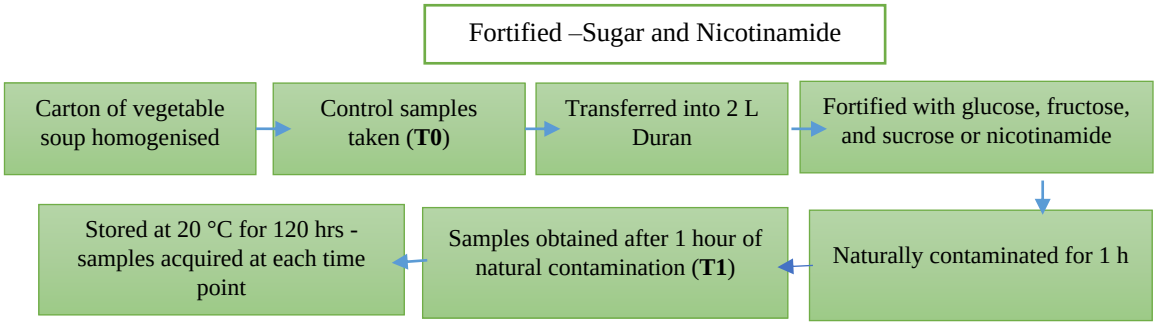


Figure 6.2: Flow diagram of the preparation steps and storage procedure of the vegetable matrix when fortified with nicotinamide and sugar.

6.2.2 Sample Analysis and Data Analysis

Methodologies for all of the analysis completed throughout the time-course, including nutrient analysis, pH measurements, microbial analysis, VOC analysis, amplicon sequencing and metabolomics, alongside data analysis methodologies are outlined in general method sections 2.6-2.12.

6.3 Results

6.3.1 The Effect of Nutritional Manipulation on Microbial Growth

The impact of nutritional manipulation on the total viable count (TVC) is represented in Figure 6.3. Vegetable matrix fortified with sugar or nicotinamide showed a comparable microbial growth profile to that of baseline. However, the vegetable matrix batch used for fortification with nicotinamide showed an initial growth of microorganisms prior to storage (0 h = 3.65 log CFU/g). The maximum TVC recorded for baseline, fortified sugar, and fortified nicotinamide was achieved at 48 h (8.32 log CFU/g), 48 h (9.31 log CFU/g), and 72 h (9.24 log CFU/g) respectively. The TVC was higher during storage of the vegetable matrix fortified with sugar and nicotinamide compared to baseline and, after peak growth, a plateau was reached for the remainder of the storage period. Although this was not the case of the baseline as the TVC declined after 48 h.

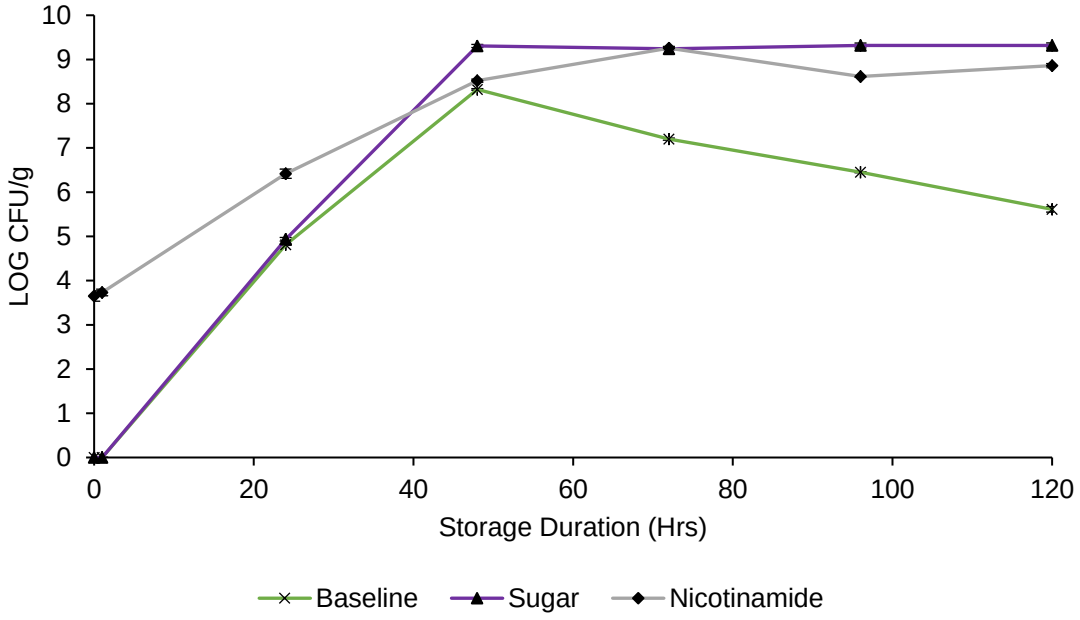


Figure 6.3: TVC of the vegetable matrix stored for 120 h at 20 °C under baseline and when fortified with either additional sugar (glucose, fructose and sucrose) or nicotinamide. For each data point duplicate dilution series were performed, of which two replicates were analysed per dilution series (n=4), except for baseline (n=2). The error associated with each time point is represented by a 95% confidence interval.

6.3.2 The Effect of Nutritional Manipulation on Bacterial Diversity and Composition

Fortification with either sugar or nicotinamide impacted the bacterial diversity (richness, Shannon and Inverse Simpson Index) over time as shown in Figure 6.4. The vegetable matrix fortified with nicotinamide showed a significant decrease in richness and diversity indices Shannon and Inverse Simpson ($p < 0.05$) at 48 h. However, the decrease was subsequently followed by an increase in diversity at 120 h. The vegetable matrix fortified with sugar showed a decrease across all diversity metrics from 0 – 120 h, similar to baseline condition. However, the decrease was only significant for diversity index Inverse Simpson. Furthermore, diversity metrics were compared at each time point in the sugar and nicotinamide fortified matrix, the comparison of which included the baseline condition. Results showed significant differences in Shannon index between sugar and baseline condition at 48 h and richness values at 120 h between sugar and nicotinamide. All data from statistical tests are available in Appendix 11.

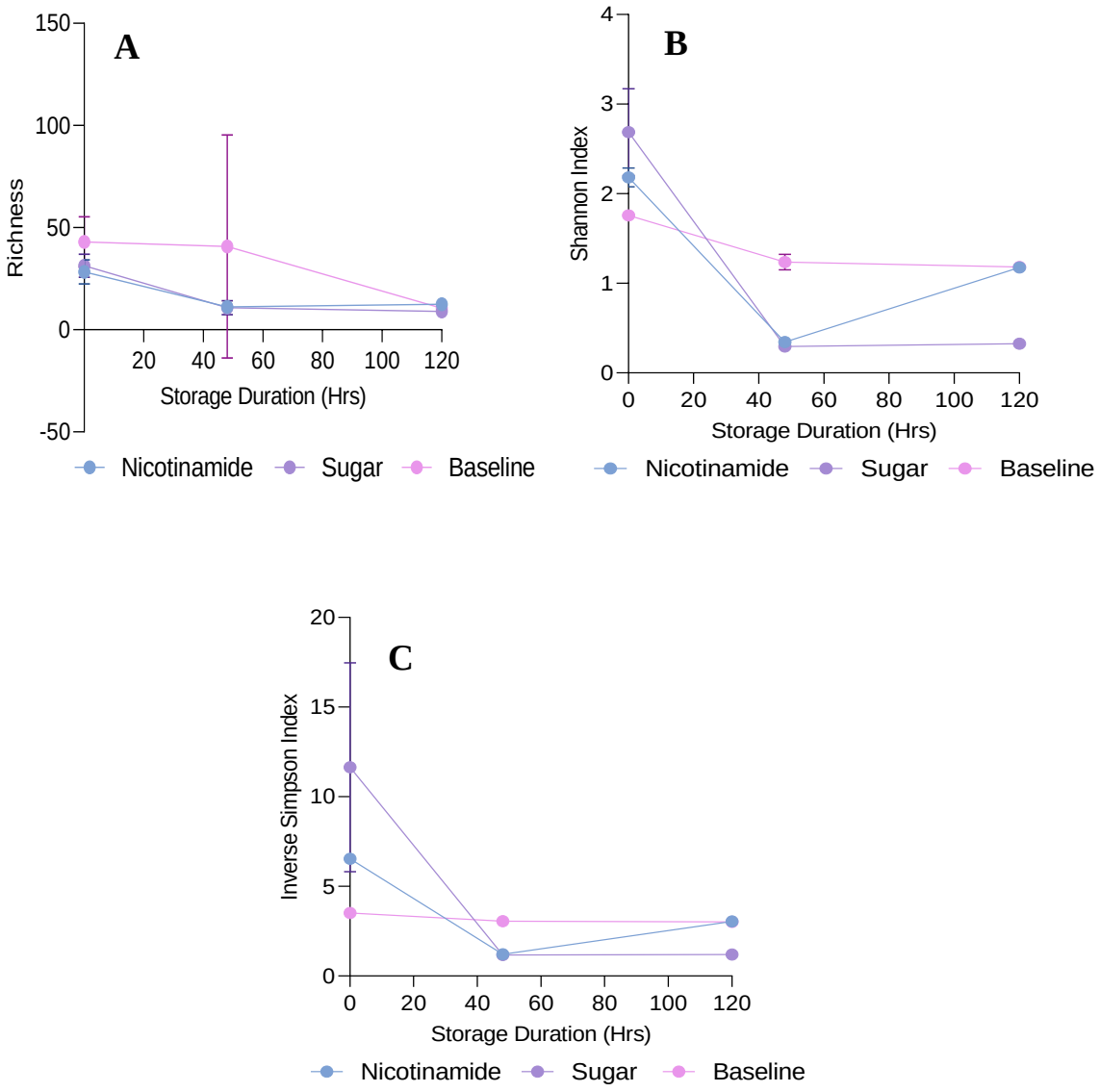


Figure 6.4: Alpha diversity of the overall microbial composition during storage of the vegetable matrix under baseline and when fortified with sugar or nicotinamide. Alpha diversity was determined by total richness (A), Shannon index (B) and Inverse Simpson index (C). Each data point comprises of three technical replicates and associated error is expressed by standard deviation.

The bacterial composition of the vegetable matrix during storage is shown in Figure 6.5. Initially the microbial community is highly diverse at 0 h as previously highlighted in chapters 4 and 5. As storage duration increased for baseline condition, *Lactococcus*, *Leuconostoc* and *Yersinina* were the most abundant bacterial communities. Comparable to the vegetable matrix fortified with sugar as *Leuconostoc* and *Lactococcus* were the predominant microbial community at 48 h and 120 h. Although heterofermentative LAB *Leuconostoc* was greater in abundance compared to *Lactococcus* (~9.4%) making up approximately 90.6% of the overall bacterial community. In contrast, the vegetable matrix fortified with nicotinamide, largely comprised of *Pseudomonas* (~87.5%) and *Ancinetobacter* (~12.5%) at 48 h. By 120 h of storage *Pseudomonas* and *Ancinetobacter* reduced in abundance, while unclassified *Lactobacillales* and *Lactococcus* gained abundance by approximately, 36% and 25% respectively.

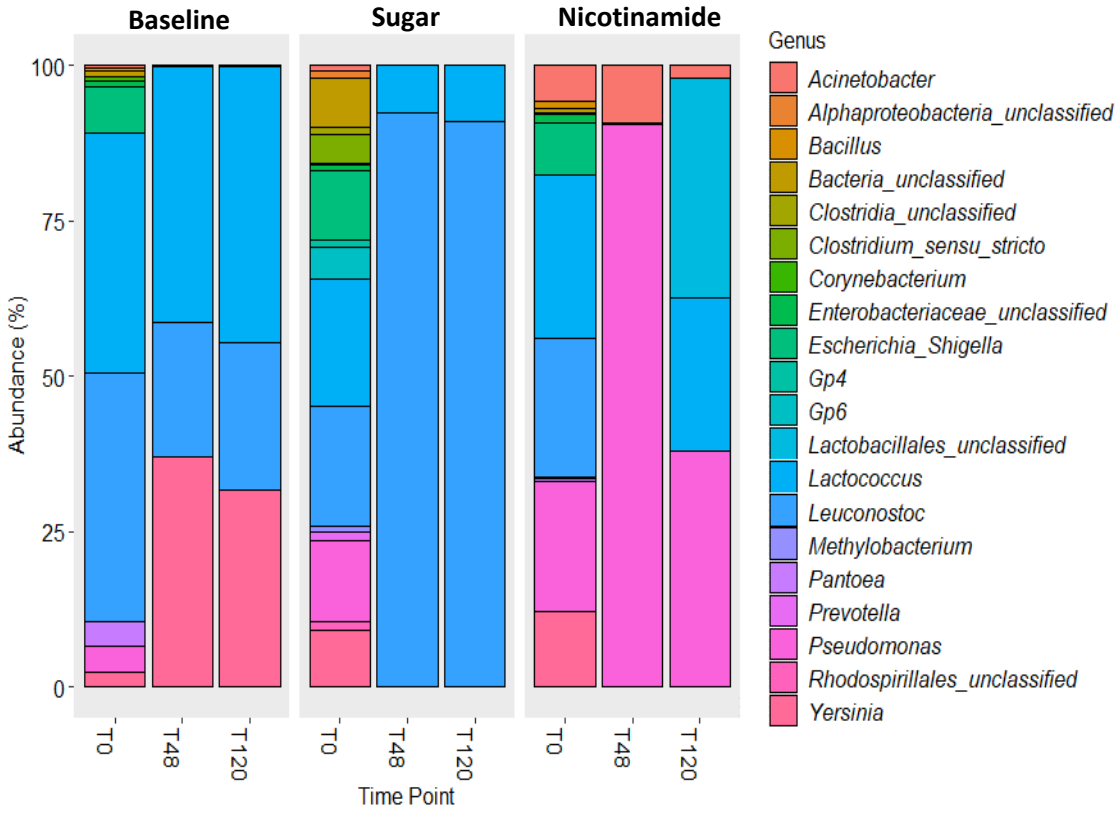


Figure 6.5: Microbial composition and development throughout the time course of the vegetable matrix under baseline or fortified with either additional additional sugar (glucose, fructose and sucrose) or nicotinamide. The microbial composition was expressed as the % abundance of the top 20 bacterial communities classified at genus level. Each time point comprises of three technical replicates.

6.3.3 The Impact of Storage Duration and Nutritional Manipulation on Microbial Composition – Beta-Diversity

Bray-Curtis dissimilarity metric was used to highlight differences in the microbial composition as a function of both time and nutritional manipulation, visualised by principal coordinate analysis (PCoA) and presented in Figure 6.6. Samples collected at 48 and 120 h are clustered together in

association with experimental group, away from all samples collected at 0 h. The vegetable matrix fortified with nicotinamide are clustered furthest away from sugar and baseline condition, with variability in samples explained by axis 1 and 2 (70.1%). The permutational multivariate analysis of variance (PERMANOVA) test showed fortification constituent ($p = 0.001$, $R^2 = 0.3650$) and time ($p = 0.001$, $R^2 = 0.3637$) significantly impacted the bacterial composition. Post-hoc analysis reported significant differences in the bacterial composition between added nicotinamide and added sugar, and between added nicotinamide and baseline condition. Furthermore, bacterial composition was significantly different at 0 h compared to 48 and 120 h of storage.

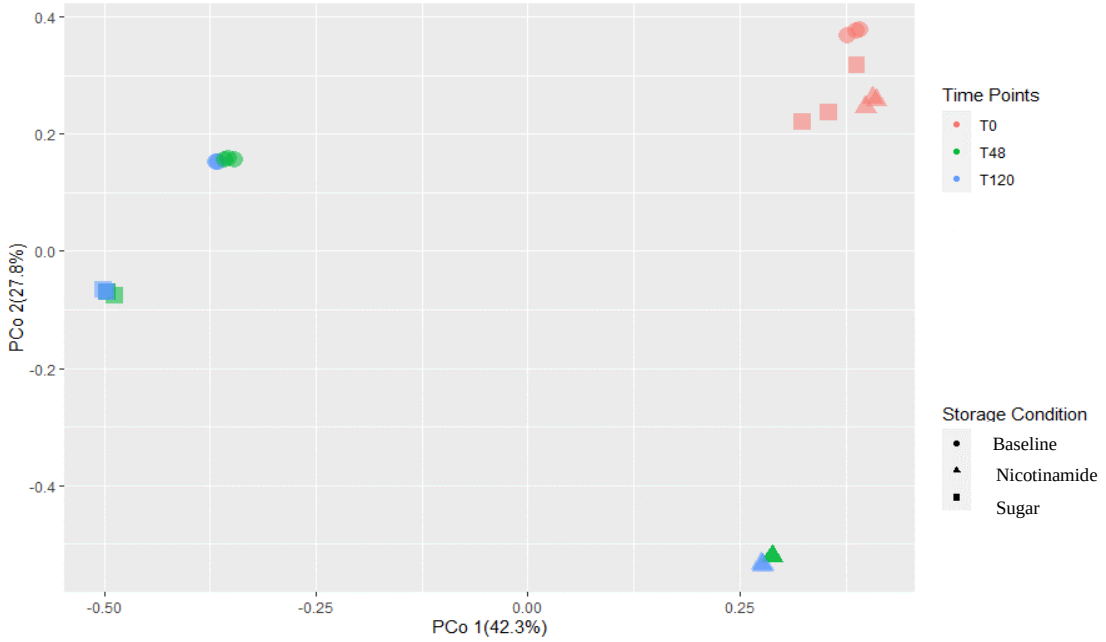


Figure 6.6: Principal coordinate analysis of Bray-Curtis dissimilarity distance matrix, samples grouped by time points 0 h – 120 h and fortification constituent (nicotinamide or sugar), also including baseline. Each time point comprises of three analysed samples.

6.3.4 The Effect of Nutritional Manipulation on pH Profile

pH was monitored during storage of the vegetable matrix fortified with both sugar and nicotinamide and presented in Figure 6.7. Fortification with sugar provided an identical pH profile to baseline condition, as pH declined at 48 h from pH 5.69 at 0 h to pH 4.13 at 48 h. In contrast, pH of the vegetable matrix fortified with nicotinamide decreased later in the time course at 72 h and continued to decrease to pH 3.95.

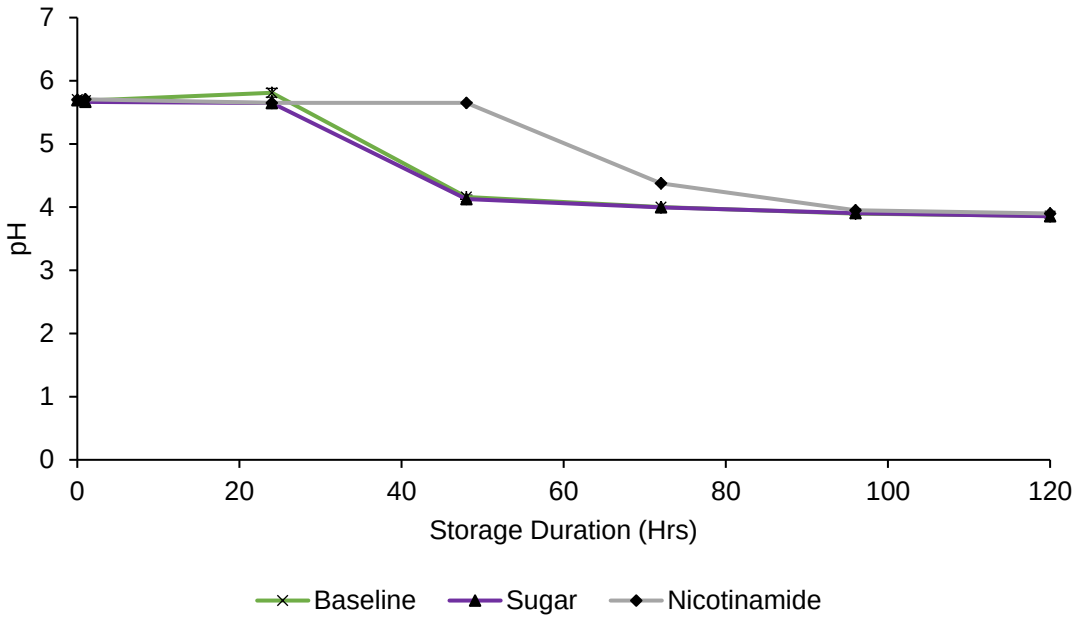


Figure 6.7: The pH profile of the vegetable matrix during 5-days of storage at 20 °C fortified with either additional nicotinamide or sugar, which included glucose, fructose and sucrose. Also includes baseline condition. pH was measured in triplicate at each time point and associated error expressed by a 95% confidence interval.

6.3.5 The Effect of Nutritional Manipulation on Metabolite Analysis

Metabolites during storage of the vegetable matrix fortified with additional nutrients were analysed using partial least-squares discriminant analysis (PLS-DA), comparing 0 h, 48 h and 120 h of storage. The generated score plots from performed PLS-DA comparing the sugar fortified matrix with baseline and nicotinamide fortified matrix with baseline are shown in Figure 6.8 and Figure 6.9 respectively. Based on analysis samples are clustered in association with investigated conditions at 0, 48 and 120 h.

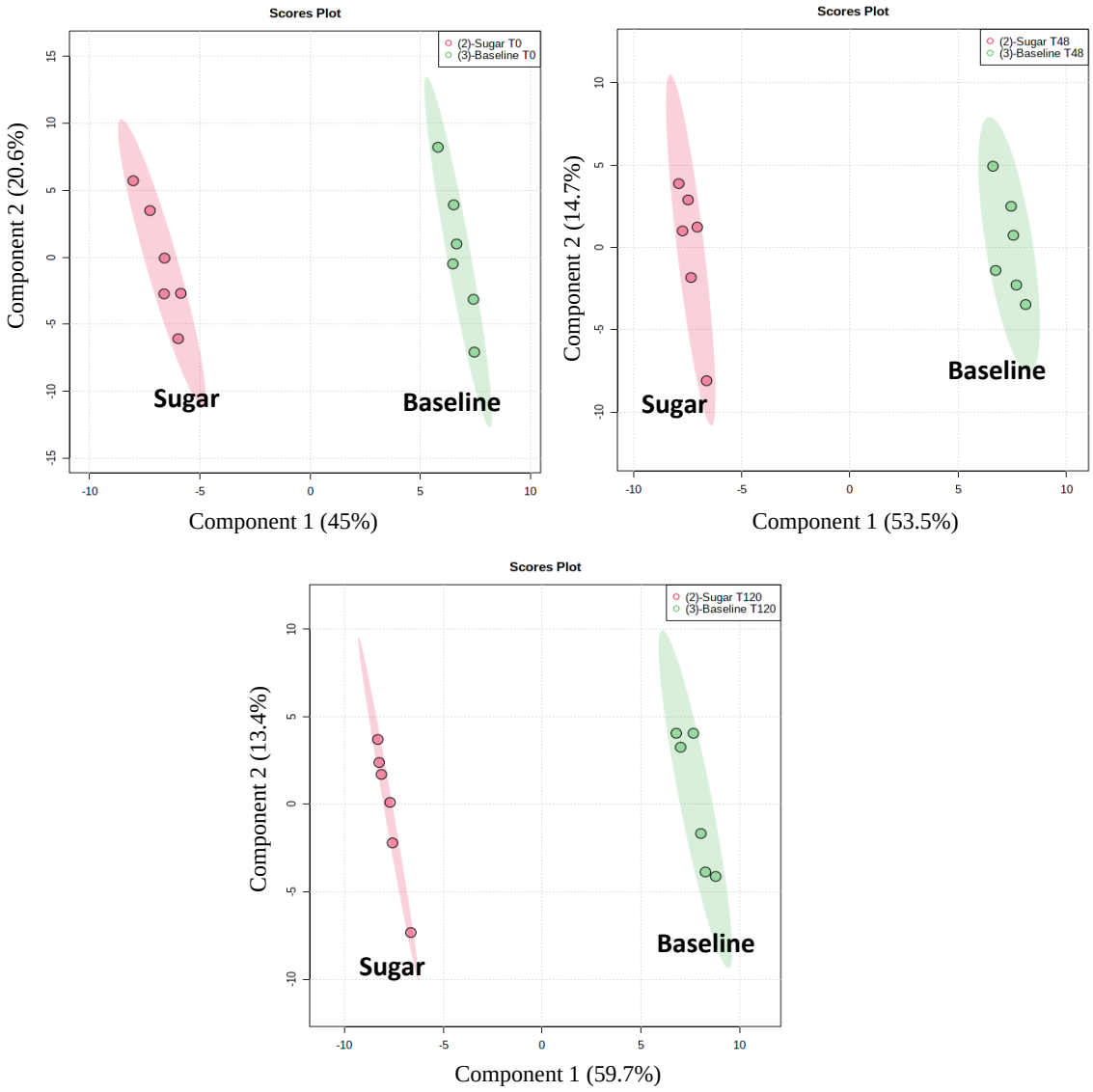


Figure 6.8: PLS-DA scores plots of the effect of fortification with sugar on metabolite profiles at 0 h (A), 48 h (B) and 120 h (C). Each time point under each storage condition is composed of six technical replicates.

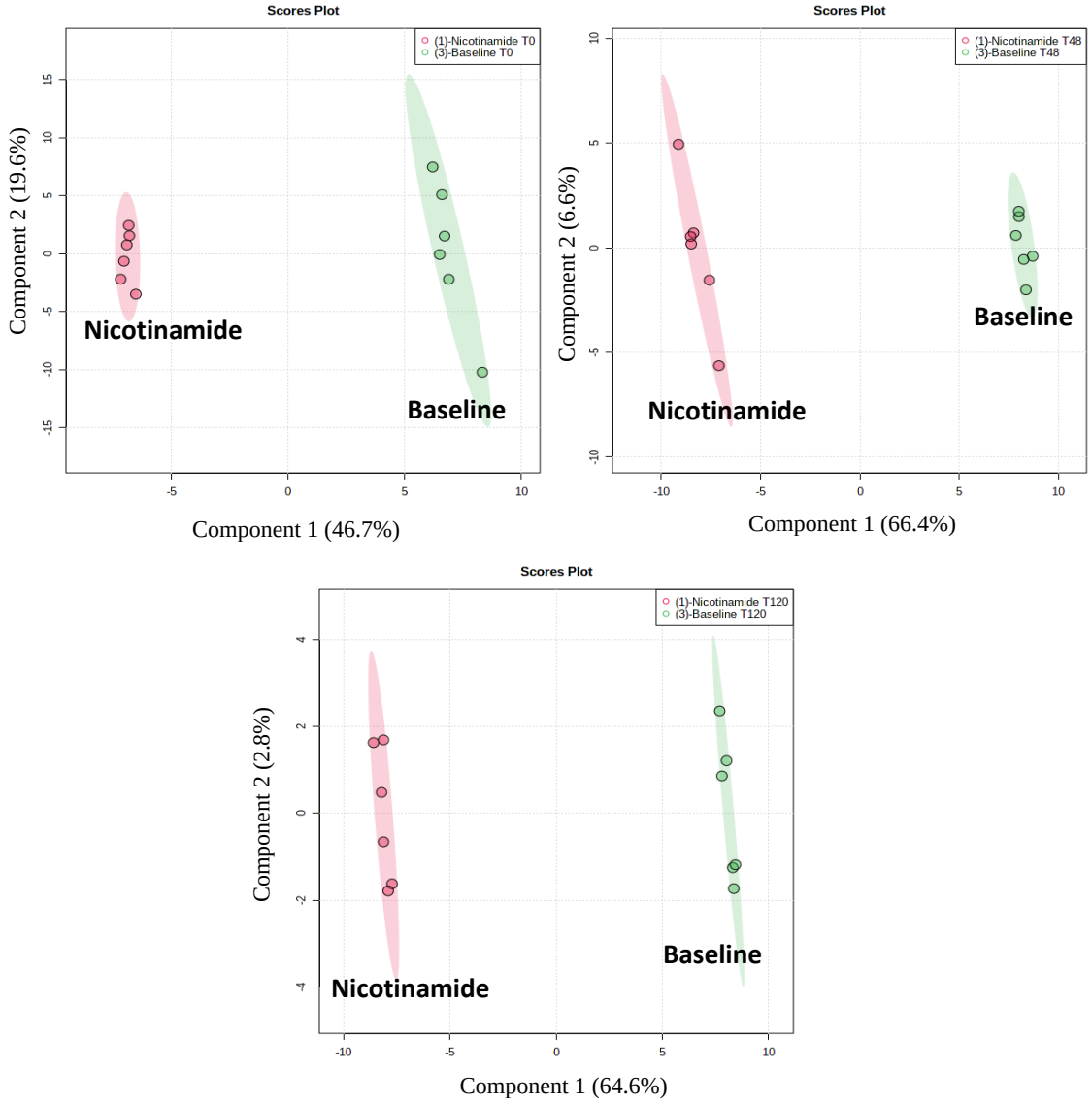


Figure 6.9: PLS-DA scores plots of the effect of fortification with nicotinamide on metabolite profiles at 0 h (A), 48 h (B) and 120 h (C). Each time point under each storage condition is composed of six technical replicates.

The putatively annotated metabolites responsible for the discrimination between investigated conditions at the start, middle and end of the time course were determined using variable importance on projection (VIP) metric and are shown in Figure 6.10 (sugar) and Figure 6.11 (nicotinamide). Representative box plots showing a selection of discriminatory metabolites are shown in Figure 6.12 for the sugar fortified matrix and Figure 6.13 for the nicotinamide fortified matrix.

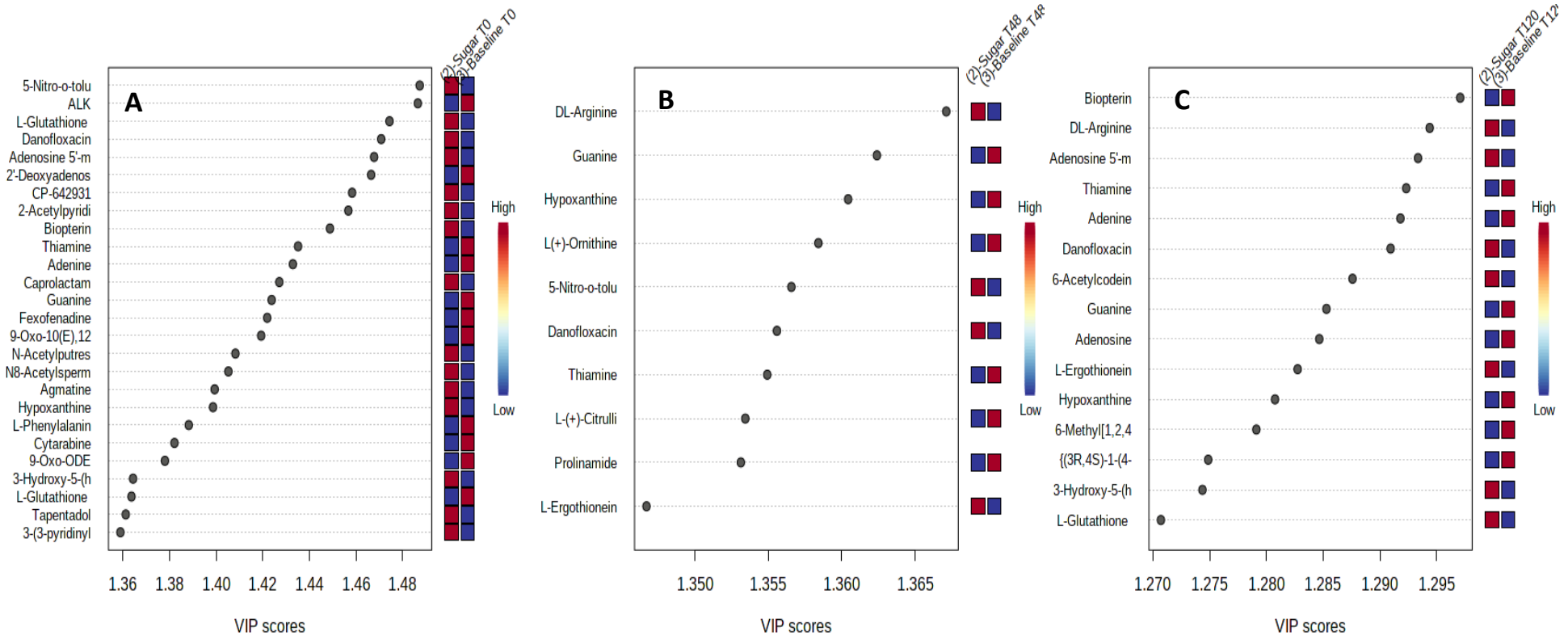


Figure 6.10: VIP ranking of the variables/metabolites (VIP > 1.35) at 0 h (A), 48 h (B) and 120 h (C) when comparing storage of the sugar fortified vegetable matrix with the baseline condition, as identified by PLS-DA based on component 1.

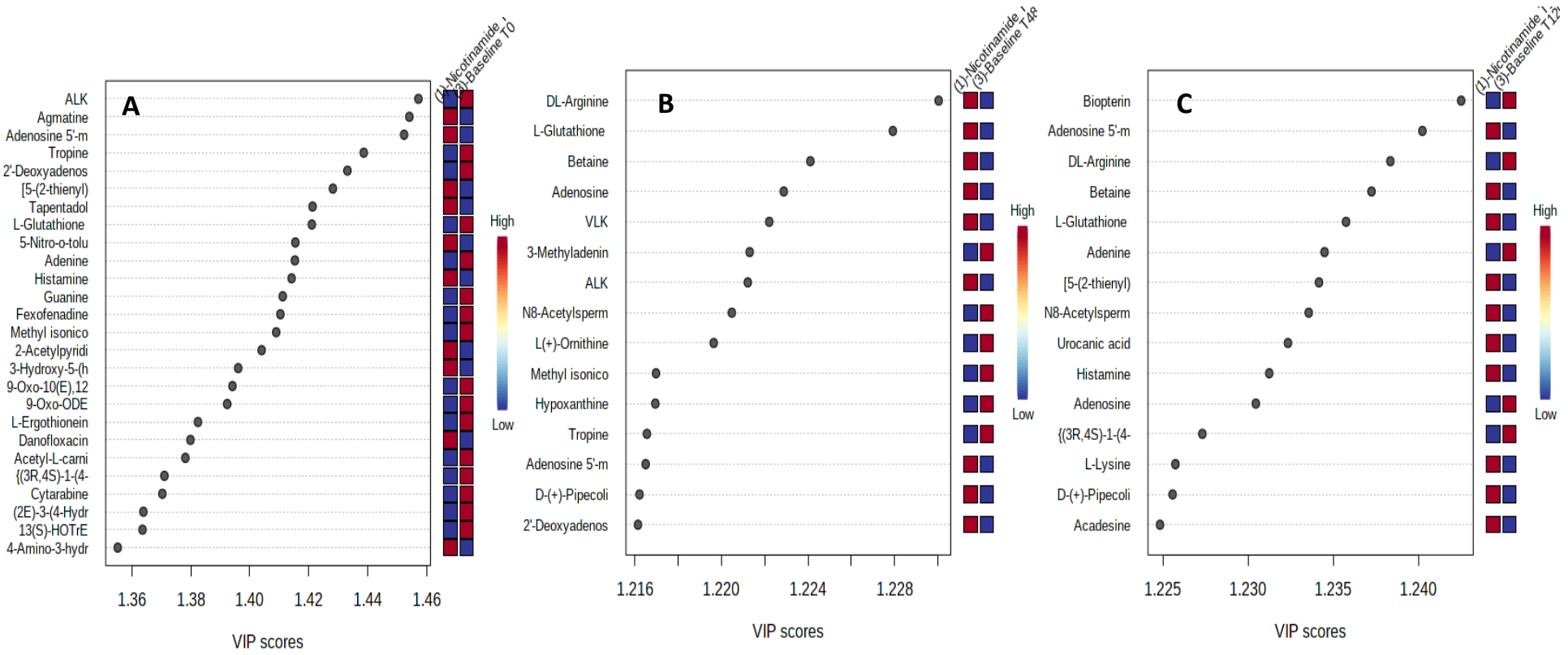


Figure 6.11: VIP ranking of the variables/metabolites (VIP > 1.35) at 0 h (A), 48 h (B) and 120 h (C) when comparing storage of the nicotinamide fortified vegetable matrix with the baseline condition, as identified by PLS-DA based on component 1.

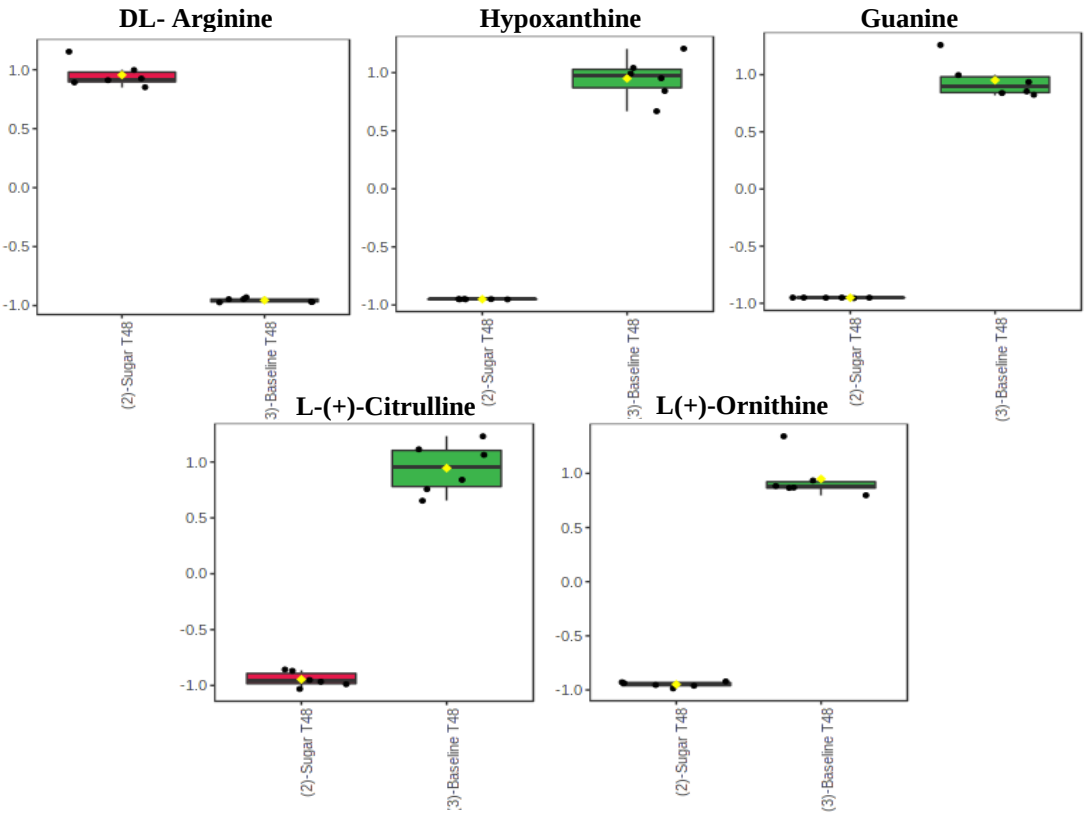


Figure 6.12: Examples of key discriminatory metabolites according to VIP analysis comparing storage of the sugar fortified vegetable matrix with baseline condition. Y axis represents normalised peak area (arbitrary units) and X axis represents different storage conditions. Each condition at a specified time point is composed of six technical replicates.

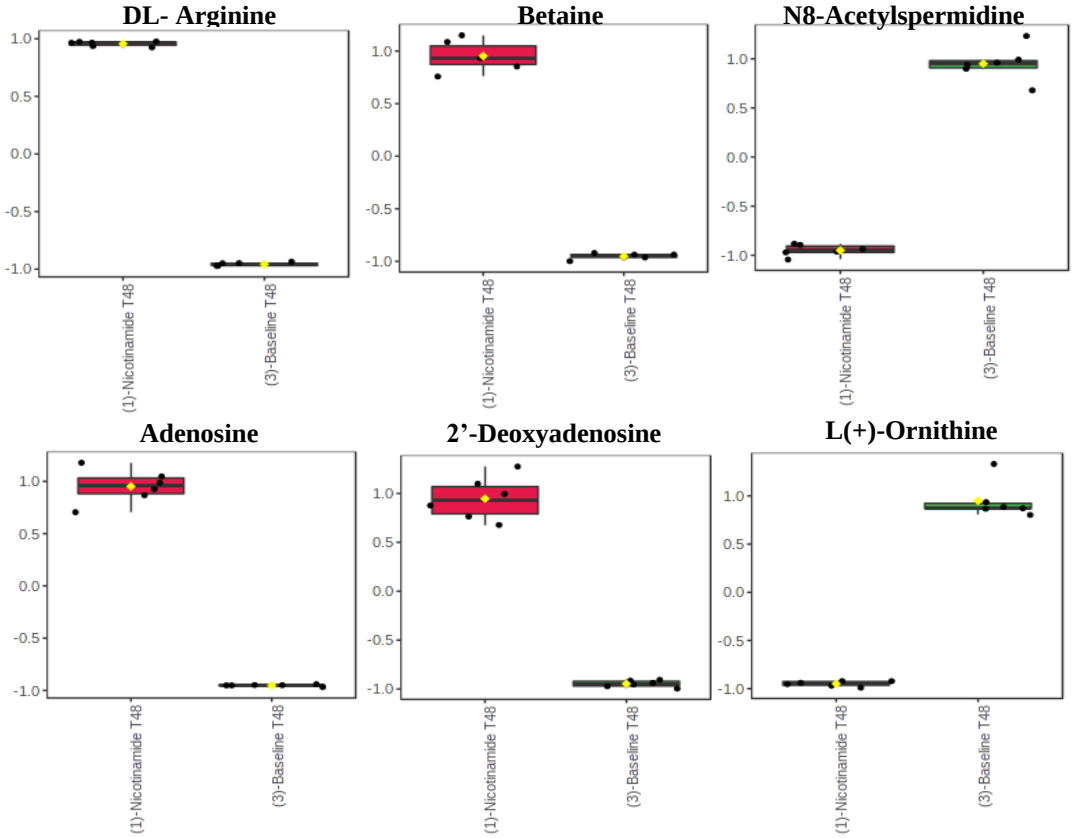


Figure 6.13: Examples of key discriminatory metabolites according to VIP analysis comparing storage of the nicotinamide fortified vegetable matrix with baseline condition. Y axis represents normalised peak area (arbitrary units) and X axis represents different storage conditions. Each time point is composed of six technical replicates.

6.3.6 The Effect of Nutritional Manipulation on Volatile Organic Compounds

Principal component analysis (PCA) was used to visualise the importance of the top 25 most significant volatile organic compounds (VOCs) features at 48 h, during storage of the vegetable matrix fortified with sugar, nicotinamide and under baseline condition. PCA represented in Figure 6.14 shows samples collected at 48 h are clustered in association with their respective condition. The top features that are responsible for the relationship in the PCA, were putatively annotated and listed in Table 6.1 organised by volatile group. Alkanes (e.g. undecane, propane), alkene (1-octene) and aldehydes (e.g. dodecanal, hexanal) were among volatile groups more abundant in the baseline condition, while short chain fatty acids (SCFAs) (acetic acid) and alcohol (3-methyl-2-heptanol) were prevalent in vegetable matrix fortified with sugar. Alternatively, monoterpenes (o-cymene and 1-propenylbenzene) were abundant in the vegetable matrix fortified with nicotinamide.

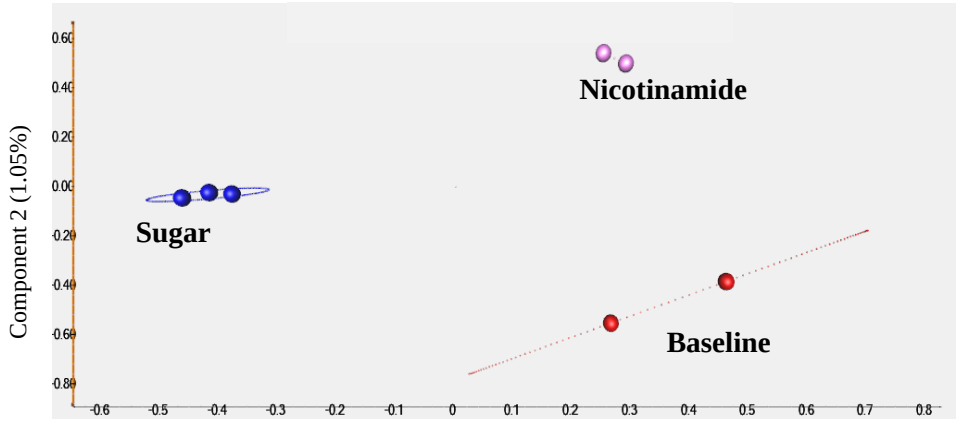
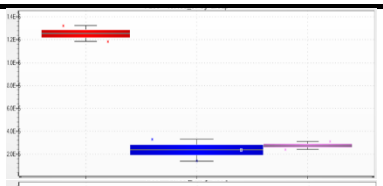
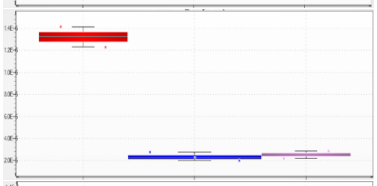
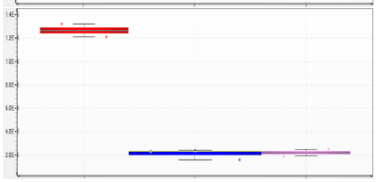
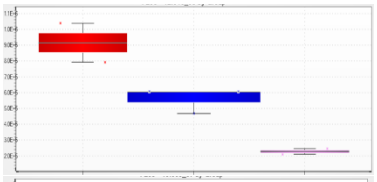
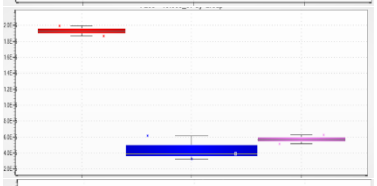
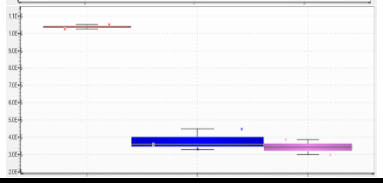
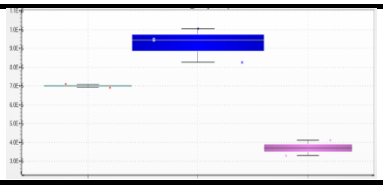
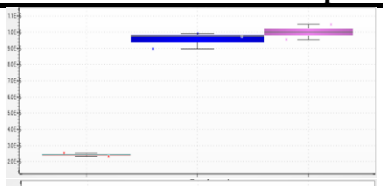
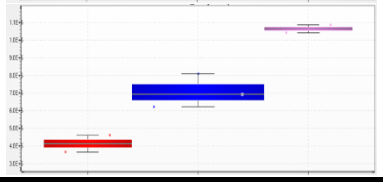
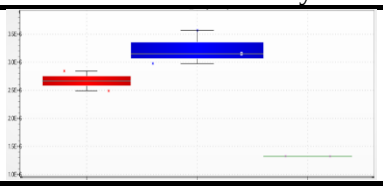
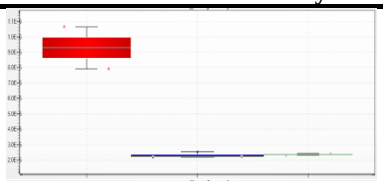
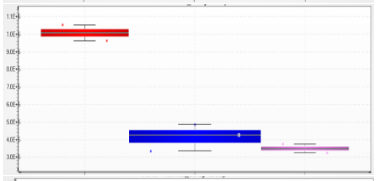
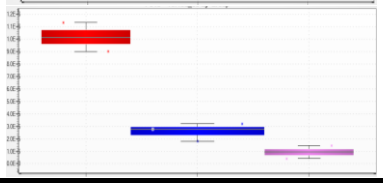


Figure 6.14: PCA comprising of the top 25 most significant features when comparing storage of the vegetable matrix fortified with nicotinamide and sugar, also including baseline condition at 48 h. Each storage condition contains three technical replicates, except for the baseline, which is composed of two technical replicates.

Table 6.1: The top discriminatory annotated VOCs according to PCA when comparing storage of the vegetable matrix fortified with nicotinamide or sugar with baseline at 48 h. Box plots also included red = baseline, blue = sugar and pink = nicotinamide, and y axis represents abundance.

Putatively Annotated Volatiles		Storage condition volatile is highest		
Alkanes and Alkenes		Baseline	Sugar	Nicotinamide
Undecane		×		
Pentadecane		×		
2,4-Dimethyl-octane		×		
Propane		×		
Decane		×		
1-Octene		×		
Alcohols				
3-Methyl- 2-heptanol			×	
Monoterpenes				
o-Cymene			×	×
1-Propenylbenzene				×

Short chain fatty acids (SCFAs)		
Acetic acid		×
Aldehydes		
Undecanal		×
Dodecanal		×
Hexanal		×

6.3.7 The Effect of Nutritional Manipulation on Stability of B-Vitamins

Stability of nicotinamide, pyridoxine, riboflavin and thiamine when the vegetable matrix was fortified with either additional sugar or nicotinamide and further stored for 120 h can be viewed in Figure 6.15. Fortification with sugar resulted in significant changes in the content of nicotinamide ($p = 0.001$), riboflavin ($p = 0.002$) and thiamine ($p = 0.001$); nicotinamide was exhausted at 48 hrsh, while the content of riboflavin and thiamine significantly decreased at 48 hrsh, with 36.1% and 44.7% remaining respectively. These results are comparable to the baseline condition. However, when fortified with nicotinamide prior to storage, the vitamin profile was different to that of baseline and fortified with sugar. Nicotinamide significantly decreased ($p = 0.001$) earlier in the time course (24 hrsh) and was undetected at 48 hrsh. Riboflavin and thiamine significantly decreased later in the time course at 72 hrsh, p value = 0.030 and 0.022 respectively, while pyridoxine significantly increased ($p = 0.043$) at 96 hrsh by 14%. Descriptive statistics are available in Table 6.2 and follow-up post hoc from repeated measures ANOVA are shown in Appendix 4 (baseline) and Appendix 10 (fortification with sugar and nicotinamide).

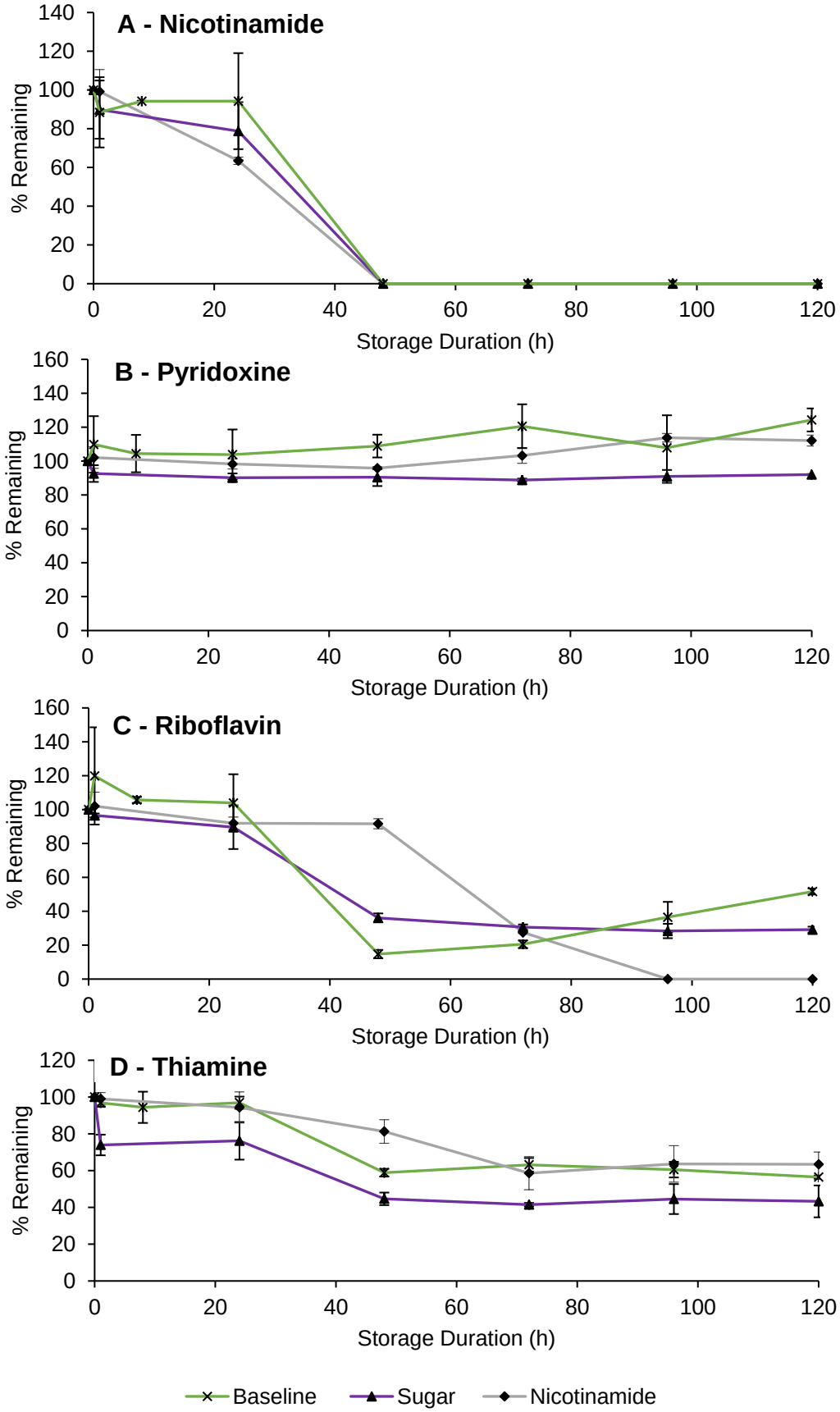


Figure 6.15: Stability of B-group vitamins, nicotinamide (A), pyridoxine (B), riboflavin (C) and thiamine (D) in the vegetable matrix over 5 days of storage (120 h) at 20 °C under baseline and when fortified with sugar and nicotinamide. Stability is represented as the % remaining of each vitamin based on quantity of each vitamin at T0. Each data point is an average of three analysed samples and associated error is expressed by a 95% confidence interval.

Table 6.2: Descriptive statistics of vitamin stability in the vegetable matrix stored over a 5-day period at 20 °C, fortified with either nicotinamide or sugar (glucose, fructose and sucrose) and also including data from baseline condition.

Baseline								
	Nicotinamide		**Pyridoxine**		**Riboflavin**		**Thiamine**	
Time point/s	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV
T0	591.20	80.46	95.70	1.91	232.57	31.15	278.10	3.40
T1	523.10	94.75	105.10	14.12	278.77	58.98	269.23	5.30
T24	556.93	1.27	99.90	9.35	245.80	3.64	262.6	20.88
T48	557.20	129.58	99.30	12.52	241.70	34.79	269.57	8.12
T72	ND*		104.17	5.69	34.30	5.11	163.73	5.20
T96	ND*		115.37	10.96	47.80	4.65	175.67	9.05
T120	ND*		103.20	16.25	84.93	18.57	168.37	10.46
Fortified with sugar								
Time point/s	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV
T0	638.22	26.15	72.58	3.38	86.13	5.20	59.56	1.34
T1	573.48	29.20	67.22	3.16	83.16	0.70	44.04	2.96
T24	502.69	68.91	65.44	1.61	77.19	9.81	45.38	5.38
T48	ND*		65.65	3.33	31.10	2.00	26.60	1.80
T72	ND*		64.44	0.55	26.38	1.23	24.71	0.51
T96	ND*		66.01	2.44	24.43	3.24	26.53	4.29
T120	ND*		66.79	0.19	25.12	1.34	25.78	4.56
Fortified with nicotinamide								
Time point/s	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV	Mean[a] (ng/g)	STDEV
T0	1773.94	75.31	58.01	0.66	73.68	4.00	81.95	4.33
T1	1759.90	177.49	59.21	3.28	76.47	3.05	81.14	2.46
T8	1125.65	29.41	57.02	1.53	74.20	8.63	77.31	6.12
T24	ND*		55.58	0.47	71.25	2.69	66.60	4.62
T48	ND*		59.88	2.34	37.70	28.66	48.01	6.50
T72	ND*		65.99	1.31	ND*		52.15	7.20
T96	ND*		65.03	1.66	ND*		51.97	4.86
T120	1773.94	75.31	58.01	0.66	73.68	4.00	81.95	4.33

[a] Mean is comprised of three samples (n=3)

* ND = Not Detected

6.4 Discussion

6.4.1 Microbial Growth and Composition as an Impact of the Manipulation of Nutritional Constituents

Fortification of the vegetable matrix with additional sugar and nicotinamide resulted in a higher TVC compared to baseline. It is acknowledged that growth of SSOs can be adjusted by concentration of sugars and other nutrients; therefore the increased growth of microorganisms in this chapter to levels associated with spoilage (10^7-10^9 CFU/g) could be related to increased availability of sugar and nicotinamide (28, 582). Publications have also shown that addition of sugar, chiefly sucrose, to dairy products significantly increased growth of bacteria, mainly LAB (583, 584). Although, studies investigating the impact of additional nicotinamide on microbial growth are limited, a recent article by Hu *et al*. (2019) (411) showed growth of *Alcaligenes spp.* P156 strain increased with addition of nicotinamide. It is important to note the vegetable matrix batch used for fortification of nicotinamide had an initial log count of 3.65 log CFU/g prior to storage, similar to microbial counts stated for processed vegetables (3.0-6.0 log CFU/g) (294).

The main nutrients present in food matrices can shape microbial composition, for example proteolytic microorganisms target protein rich foods, fermentative microorganisms are prevalent in foods high in carbohydrates, while lipolytic organisms thrive in foods high in fat (101). Amplicon sequencing highlighted fermentative genus LAB, namely *Leuconostoc* and *Lactococcus* were the dominant bacterial community in the vegetable matrix fortified with sugar, comparable to baseline. Due to LAB using sugar as the main substrate for energy through fermentation, the addition of sugar favoured LAB (150). The pH profile supports the presence and growth profile of LAB, as pH reduced to pH 4.16 at 48 h, which coincides with increased growth of LAB. This suggests sugars were fermented by both homofermentative and heterofermentative LAB, leading to the production of organic acids as shown in the VOC analysis, which reduced the pH of the vegetable matrix (388, 585). Predominance of LAB after fermentation at 48 h coincided with a significant reduction in diversity as determined using Shannon Index, which was also reported by Park *et al.* (2012) (211) during fermentation of Kimchi. Additionally, the Shannon index was significantly higher during baseline condition compared to storage of the vegetable matrix fortified with sugar, due to an abundance of *Yersinia*. It is unclear as to the reason *Yersinia* was unable to compete in the sugar fortified matrix, due to limited literature discussing competition amongst these communities. This may be related to different batches of the vegetable matrix, or the increased advantage for LAB to thrive as concentration of sugar was higher. In contrast, addition of nicotinamide to the vegetable matrix significantly changed the microbial composition versus the sugar fortified matrix and baseline condition. Gram negative *Pseudomonas* and *Acinetobacter* were the abundant communities at 48 h of storage in the vegetable matrix fortified with nicotinamide. It is unclear why the fortification with nicotinamide promoted the prevalence of these bacterial communities, as it has not been

acknowledged in the literature previously. This would require further investigation to confirm the recorded initial microbial growth prior to fortification with nicotinamide and subsequent storage did not impact on the results obtained. *Pseudomonas* and *Acinetobacter* grow effectively at lower temperatures where they can out-compete other bacterial communities (474, 586, 587). Therefore, refrigeration temperatures prior to experimental use may have been suitable to support the growth of these microorganisms, during a period when less cold-tolerant communities were supressed. Initial growth during this period may have enabled these organisms to enter the log phase earlier in the time course and led to a higher abundance at 48 h. The abundance of *Pseudomonas* and *Acinetobacter* at 48 h is accompanied by a reduction in all diversity measures, however, by 120 h there is an increase in diversity as *Lactococcus* and unclassified *Lactobacillales* gain abundance. pH profile when the vegetable matrix was fortified with nicotinamide was stable during the initial 48 h of storage, as both *Pseudomonas* and *Acinetobacter* do not ferment sugars and as a result the pH did not reduce (588, 589). However, the reduction in pH later in the time course is likely associated with the presence of LAB.

6.4.2 Vitamin Stability

The quantity of B vitamins during storage of the vegetable matrix fortified with sugar shared a comparable profile to that of the baseline condition where LAB dominated. Further indicating, as outlined in chapter 4, nicotinamide, riboflavin and thiamine are potentially required for growth of the dominating spoilage species (374). The decrease in these vitamins at 48 h of storage, or complete exhaustion in the case of nicotinamide, correlates to exponential growth of microorganisms and fermentation characteristics. Fortification of the vegetable matrix with additional nicotinamide altered the rate of deterioration of vitamins within the system. At 48 h, when *Pseudomonas* and *Acinetobacter* were abundant, nicotinamide was the only B vitamin to have significantly reduced from its starting concentration at 0 h. The exhaustion of nicotinamide from the vegetable matrix is potentially due to bacterial communities' requirement of the coenzyme form, nicotinamide adenine dinucleotide (NAD^+), which can be synthesised from nicotinamide via the salvage pathway (410, 590). Species of predominant bacterial communities under this condition, *Pseudomonas* and *Acinetobacter* are reported to possess the genes involved in the pathway to generate NAD^+, allowing both communities to maintain NAD^+ homeostasis within the cell through nicotinamide scavenging (590). Riboflavin and thiamine decreased later in the time course at 72 h, coinciding with the increased LAB activity (e.g. pH reduction). Riboflavin has been stated as a growth requirement of some LAB, due to being a precursor of coenzymes, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which are essential for redox reactions (404, 591). Certain strains of LAB employ a transport system to uptake exogenous riboflavin, due to their inability to synthesise riboflavin *de novo*, for example *Lactococcus spp*. have been characterised to use a riboflavin transport protein RibU (413, 416,

592). Similarly, thiamine is a precursor of the coenzyme thiamine pyrophosphate (TPP), a cofactor of enzymes including transketolase, pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (593). Enzyme transketolase plays an important role in the pentose phosphate pathway which is used by LAB during the catabolism of sugar to generate energy (150, 594). Pyridoxine on the other hand significantly increased at 96 h, in line with activity of LAB. Some LAB communities are reported to synthesise pyridoxine during fermentation, such as *Lactobacillus*, although this is strain dependent and content of vitamin produced is subject to variation (367, 404, 557).

6.4.3 Metabolite analysis as an Impact of the Manipulation of Nutritional Constituents

Fortification of the vegetable matrix with different nutrients altered the metabolite profile. Focusing initially on the impact of the addition of nicotinamide; metabolites including adenosine, adenosine 5'monophosphate, 2'deoxyadenosine, lysine, arginine, histamine, L-glutathione, betaine and pipecolinic acid were abundant in the vegetable matrix fortified with nicotinamide. However, the abundance of these metabolites was lower in the baseline. Nucleosides (adenosine and 2'deoxyadenosine) and nucleotides (adenosine 5'monophosphate) are abundant in vegetables to different extents although precise quantification is challenging (595). The exhaustion of nucleoside and nucleotides to a greater extent in the baseline as opposed to the vegetable matrix fortified with nicotinamide is due to presence of dominating organisms. LAB, specifically *Leuconostoc* are reported to use extracellular nucleotides and nucleosides for growth as a source of carbon and energy, and in some cases, as a nitrogen source depending on the amino group (492). Some species of LAB are also purine auxotrophic and therefore rely on scavenging purine nucleotides from their environment for continued growth (491, 596). Amino acids are another essential growth factor for LAB, although this is strain dependant. Certain strains of LAB require extracellular sources of specific amino acids for growth (597). The reduction of lysine and arginine in the baseline is likely due to proliferation of certain LAB species. Catabolism of amino acids through decarboxylation by spoilage species leads to the production of biogenic amines, including putatively annotated metabolites histamine and acetylspermidine produced from decarboxylation of histidine and ornithine respectively (150). Ornithine is produced during arginine metabolism and therefore increased abundance of ornithine in the baseline, coincides with a reduction in arginine and increase in acetylspermidine. Histamine was abundant in the vegetable matrix fortified with nicotinamide; the dominating genus under this storage condition, *Acinetobacter* have been confirmed to produce histamine, as histamine serves as a precursor for synthesis of siderophores (iron chelators) (598, 599). Alternatively, abundance of histamine could be due to its natural occurrence in vegetables, and its presence in the vegetable matrix at the beginning of storage (483). In a recent study by Niu *et al*. (2019) (600), >40% of biogenic amines including histamine can be degraded by LAB species (*Lactobacillus plantarum*) during fermentation. Other studies have also shown LAB to degrade biogenic amines, this also includes

the ability of *Leuconostoc spp*. to degrade histamine (484, 601). Therefore, the differences noted between histamine content in the nicotinamide fortified matrix versus the baseline, could be due to degradation of native histamine through higher levels of LAB fermentation. Betaine was another metabolite abundant in the vegetable matrix fortified with nicotinamide. As previously discussed in chapter 5, betaine is a osmoprotectant, which can be an important metabolite for survival of bacterial communities (561, 602). Betaine can be synthesised by bacteria including *Pseudomonas spp*. which were the prevalent bacterial community under this condition (602). DNA methylation products including, 3-methyladenine were abundant in the vegetable matrices where LAB were predominant, however as previously stated these metabolites have not been previously associated with food spoilage and can be classified as novel findings.

In contrast, fortification of the vegetable matrix with sugar also altered the metabolite profile. Although due to the bacterial composition, vitamin profile and physicochemical evaluation of the baseline and sugar fortified matrix being comparable, only select few metabolites were different. Metabolites that were abundant in the sugar fortified matrix included arginine and ergothioneine, whereas metabolites abundant in the baseline included guanine, hypoxanthine, ornithine, citrulline and adenosine. Ornithine is formed from citrulline which is produced through the arginine deiminase pathway and therefore the reduction in arginine in the baseline, coincided with an increase in both ornithine and citrulline (434, 435, 603). Both products of arginine catabolism were present in the vegetable matrix fortified with sugar but were lower in abundance. The arginine deiminase pathway is used by LAB to facilitate growth and aid with pH regulation due this pathway also generating ammonia (435). It is unclear as to the reason arginine catabolism occurred more in the baseline; this could be due to slight differences in the microbial composition and therefore differences in the growth requirements of the communities. The other differences in metabolites could also be due to different batches of the vegetable matrix.

The VOC profiles were impacted when the vegetable matrix was fortified with either additional sugar or nicotinamide compared to baseline condition. Alkanes, alkenes, alcohols, SCFAs and aldehydes were the volatile groups that differed among the assessed conditions. Alkanes/alkenes identified were higher in the baseline condition. As previously described, these volatiles are generated from the degradation of lipids through the oxidation of fatty acids or lipolysis (446, 496). Although it should be noted that authors address alkanes as a weak contributor to flavour (164, 604). Furthermore, it has been discussed in both chapters 4 & 5 that production of alkanes/alkenes has been linked to LAB metabolism (447, 495). However, due to this, a similar abundance of alkanes/alkenes in the vegetable matrix fortified with sugar would be expected as LAB dominated. This could be due to differences in the batches of the vegetable matrix, in terms of level of oxidation of SCFAs, or differences in the enzymatic activity of dominating organisms, as lipase activity of LAB for example is species or strain dependent (605). The lower abundance of alkanes in the vegetable matrix fortified with nicotinamide could be due to alkane hydroxylase

activity, which results in the oxidation of alkanes to other volatile compounds, such as alcohols, aldehydes and ketones. Alkane hydroxylase is produced by both dominant bacterial communities under this condition at 48 h, *Pseudomonas* and *Acinetobacter* (449). Aldehydes were also more abundant under baseline and are generated from the hydrolysis of triglycerides and transamination of amino acids (164, 169). Aldehydes in general can be associated with spoilage, contributing to an undesirable and rancid odour when formed in high concentrations (169). The higher abundance of aldehydes in the baseline could be attributed to batch differences and variances in the bacterial community. On the other hand, the aldehydes could have been produced and exhausted by 48 h of storage when the nutritional composition of the vegetable matrix was manipulated. Li *et al*. (2018) (164) showed a reduction in aldehydes during spoilage of smoked bacon. This is potentially caused by the oxidation of aldehydes to acids or reduction of aldehydes to alcohols, due to the carbon in the aldehyde group being vulnerable to nucleophile attacks (606). Additionally, 3-methyl-2-heptanol was abundant in both the sugar fortified matrix and in the baseline. The increase in this alcohol is likely due to dominating bacterial communities, as heptanol's such as 4-methyl-2-heptanol and 2-heptanol have been shown to increase during fermentation, although 3-methyl-2-heptanol has not been described as a spoilage volatile (566, 568). Comparable to the alcohol produced, SCFAs, acetic acid was more abundant in the vegetable matrix fortified with sugar, largely due to the higher abundance of heterofermentative LAB, *Leuconostoc* which produces acetic acid during fermentation of sugars (374).

6.5 Conclusions

To understand if spoilage features previously identified (e.g vitamin degradation, metabolites etc.) were a direct cause of microbial activity, growth of SSOs was effectively enhanced by addition of sugars and nicotinamide. Addition of different nutritional constituents both increased microbial growth from that previously noted under baseline conditions and altered the bacterial composition of the vegetable matrix. Fortification with sugar further promoted LAB, *Leuconostoc* and *Lactococcu*s, while *Pseudomonas* and *Acinetobacter* dominated in the vegetable matrix fortified with nicotinamide. Although this does require further investigation to confirm the change in bacterial composition was due to the addition of nicotinamide and not due to other factors. B vitamins also degraded to different extents during these storage studies, with the degradation of specific vitamins influenced by changes in the bacterial communities. Nicotinamide, riboflavin and thiamine significantly reducing when LAB dominated, while nicotinamide was the only vitamin that exhausted when *Pseudomonas* and *Acinetobacter* were abundant. Analysis of the metabolites further confirmed metabolites changed in response to different bacterial communities, with metabolites involved in purine metabolism such as, adenosine and 2'deoxyadenosine being a potential marker of freshness of the vegetable matrix when LAB are responsible for spoilage. To further confirm vitamin degradation and specific metabolites are due to the specific bacterial communities identified, antimicrobial studies need investigating.

Chapter 7

Summary and Future Work

The presented work aimed to provide a novel multi-omics derived, in-depth characterisation of the stability of a vegetable matrix (vegetable soup), to understand both vitamin and macronutrient composition and the relationship between food spoilage and vitamin status.

7.1 Novel Method for the Analysis of B Vitamins from Food

The initial phase of the work focused on developing a simple and repeatable extraction protocol for the efficient analysis of B vitamins. Following this, a robust and sensitive analytical methodology for the accurate quantitation of these extracts was developed and validated on the vegetable matrix. The combination of hydrophilic interaction chromatography (HILIC) with mass spectrometry (MS) detection was employed to reliably quantify nicotinamide, pyridoxine, riboflavin and thiamine. Publications validating a HILIC-MS method to determine B vitamins are limited; yet HILIC offers the advantage of being highly suitable for separation of polar analytes and MS detection provides increased sensitivity. The increased sensitivity offered by MS detection is valuable, particularly for the quantification of low abundance vitamins in food matrices. The developed method demonstrated low detection and quantification limits ranging from 2.4-9.0 ng/mL and 8-30 ng/mL respectively, rendering it ideal for this purpose. The sensitivity was better than other HPLC methods that have used other methods of detection such as UV and better than reversed-phase methods to analyse B-vitamins. In addition, a simple acid extraction method using meta-phosphoric acid and reducing agent, DL-dithiothreitol (DTT) was developed and further validated to isolate B vitamins from the vegetable matrix. Intra-day and inter-day precision of the extraction method was excellent, with the % CV for intra-day ranging from 1.56-6.56% and inter-day ranging from 8.07-10.97%. Overall, the methods validated offered reliable, sensitive, and rapid quantification of investigated vitamins.

After validating the newly developed analytical and extraction methods, the spoilage of the vegetable matrix was investigated under an established baseline condition covered in chapter 4. The baseline condition involved storing the vegetable matrix at 20 °C (room temperature), for 5-days (120 h). The in-depth characterisation of spoilage was performed using a multi-omic approach consisting of high-throughput sequencing (HTS) and metabolomics. This complementary combination of multiple -Omic techniques are scarce in many food matrices; however, the integration of both techniques can provide a comprehensive picture of microbial composition and both biological and chemical processes linked to spoilage. Alongside, nutritional composition, microbial growth and physiochemical changes were also analysed. Furthermore, the impact of intrinsic and extrinsic environments on spoilage and nutritional composition was

investigated. Environments assessed included different temperatures, pH, gas composition and the effect of freeze/thaw. The primary endpoints of the presented work are divided into three sections (below) and include insight into the bacterial composition of the vegetable matrix as a function of both time and storage environment, relationships between the nutritional composition and spoilage, and identification of metabolites that have the potential to be markers of either product freshness or spoilage.

7.2 Bacterial Composition During Storage of the Vegetable Matrix

Amplicon sequencing, targeting 16S rRNA showed bacterial communities responsible for spoilage of the vegetable matrix under baseline conditions (chapter 4) comprised of the genus *Lactococcus*, *Leuconostoc* and *Yersinia*. Lactic acid bacteria (LAB) (*Lactococcus* and *Leuconostoc*) were largely responsible for spoilage of the vegetable matrix through fermentation of available sugar, producing gas and metabolites such as organic acids that reduced the pH of the matrix. This was confirmed by macronutrient, physiochemical analysis, and untargeted metabolite analysis. However, storage of the vegetable matrix under different environments (e.g. temperature, pH etc.) altered the bacterial communities and thus changed the dominating spoilage organism. Investigation of low storage temperature, 7 °C, showed, that towards the end of the storage period, *Pseudomonas* was the dominant bacterial community. Whereas at higher storage temperature, 37 °C, *Bacillus* gained significant abundance alongside *Leuconostoc* and *Lactococcus*. In contrast, when the original pH of the vegetable matrix was effectively altered prior to storage, growth of *Yersinia* was promoted under both acidic and alkaline conditions. Furthermore, different gas compositions changed bacterial communities; storage of the vegetable matrix in the presence of air allowed *Bacillus* to dominate, while storage in the presence of nitrogen promoted dominance of LAB (*Leuconostoc* and *Lactococcus*). Therefore, it has been demonstrated that storage conditions can shift the microbial composition and instigate competition amongst bacterial communities. This is the first study to show the impact of several intrinsic and extrinsic environments on the bacterial composition of a complex vegetable matrix. In addition, this is the first instance where bacterial dynamics in foods have been correlated with changes in B-vitamin composition.

7.3 Can Vitamins be a Marker of Food Spoilage?

Vitamins are essential constituents of foods, that are unstable and not guaranteed during storage, yet B vitamins fail to be assessed alongside other quality markers. B vitamins may play an active role in food spoilage reactions, the extent of any interaction between spoilage organisms and B vitamins is largely unknown in many matrices. However, storage of the vegetable matrix highlighted a relationship between B-vitamins and spoilage. Growth of dominating organisms *Lactococcus*, *Leuconostoc* and *Yersinia* to levels associated with spoilage at 48 h coincided with

loss of the B-vitamins nicotinamide, riboflavin and thiamine. For example, nicotinamide was completely exhausted, while content of riboflavin and thiamine reduced by 85.2% and 41% respectively at 48 h of storage from the original concentration at 0 h. Analysis of macronutrients also highlighted sugars, chiefly fructose and sucrose were the main substrate used for energy production when lactic acid bacteria (LAB) (*Lactococcus* and *Leuconostoc*) and *Yersinia* were predominant. Furthermore, the relationship between B-vitamins and spoilage (chapter 4) was further explored under different storage environments and preparation procedures (chapter 5). The quantity of B vitamins was notably different between the assessed storage environments. Based on the findings of this thesis, this appears to be due to both microbial growth and changes to the bacterial composition, which has not been described previously. For example, storage of the vegetable matrix that was subject to freeze/thaw, promoted abundance of unclassified *Bacillales* and as a result nicotinamide was the only vitamin to significantly reduce, while the content of pyridoxine, riboflavin and thiamine remained stable during the storage period. Therefore, from these in-depth spoilage studies it highlights a relationship between the preservation of B-vitamins and spoilage activity of specific organisms. In all investigated condition where growth of microorganisms reached to spoilage levels (10^7-10^8 CFU/g), nicotinamide was completely exhausted, regardless of the environment and dominating bacterial communities. This shows nicotinamide is a potential marker of product freshness and therefore could be used to rapidly detect on-going spoilage which has not been acknowledged in the literature.

7.4 Progression of Spoilage as Assessed Through -Omic Approaches

Metabolomic fingerprinting was conducted using complimentary techniques, SPME-GCTOF-MS and HILIC-MS/MS, to provide a comprehensive study of volatile and non-volatile metabolites, respectively. Focusing initially on the non-volatile metabolites; between 0-24 h of storage when *Lactococcus, Leuconostoc* and *Yersinia* were the dominant bacterial community, glutathione reduced. As storage duration increased between 24-48 h, adenosine 5'monophosphate (AMP), and arginine reduced. Therefore, these metabolites are potentially indicators of product freshness. In contrast, hypoxanthine, ornithine, citrulline and biogenic amines, agmatine, acetylspermidine and acetylputrescine were up regulated between 24-48 h. The latter gives rise to a rotting aroma, contributing to off-odours of the spoiled vegetable matrix. From the production and reduction of specific putatively annotated metabolites, pathways including purine metabolism and arginine and proline metabolism can be identified as important pathways in the spoilage process of the vegetable matrix when dominating organisms are present. Products of purine metabolism, e.g. hypoxanthine have been reported as a marker of spoilage in animal-based products, but this is the first report in a exclusively vegetable-based matrix (160, 189). Metabolites were largely dependent on the microbial load and the presence of certain microbial communities. For example,

during storage of the vegetable matrix in the presence of air, *Bacillus* was the dominant spoilage organism and metabolites that have been previously associated with *Bacillus spp*. were present, including betaine and urocanic acid. Urocanic acid has been described as a metabolite solely associated with spoilage of fish and has not been described during spoilage of vegetables. In contrast, when spoilage activity is minimised and therefore growth of microorganisms is reduced (e.g., storage at 7 °C), many of the metabolites that are associated with the freshness of the vegetable matrix are preserved such as nucleotides and nucleosides, AMP, adenosine 3', 5'-monophosphate (cAMP), 2'deoxyadenosine and guanine. Some metabolites that can be described as novel findings in this thesis and have not been previously associated with spoilage of foods, include the production of DNA methylation products (1-methyladenine, 3-methlyadenine etc.), acetyl-L-carnitine and L-iditol.

Analysis of volatiles showed aldehydes, alkanes, alkenes, alcohols, short chain fatty acids (SCFAs), ketones and monoterpenoids were the compound groups associated with the degradation of the vegetable matrix. During the initial storage period, 1-hexanol, 1-pentanol and acetic acid increased between 0-24 h. In contrast, hexanal, 2,4-dimethylhexane and 1-octene increased later in the time course between 24-48 h, coinciding with increased microbial growth. Some of these volatiles can contribute to off-odours, for example aldehydes in sufficient quantities can contribute to a rancid odour (169). 1-Octene can be characterised by a gasoline-like aroma, 1-hexanol gives rise to a pungent, oily and alcoholic aroma, 1-pentanol generates a fermented, pungent and bready aroma (175). Therefore, these metabolites are potential markers of spoilage of the vegetable matrix when *Lactococcus*, *Leuconostoc* and *Yersinia* are the dominant bacterial community. The volatile profile also altered in response to changes in the intrinsic composition and extrinsic environment, largely due to shifts in microbial composition. For example: isooctanol was abundant when the vegetable matrix was stored at higher storage temperature, 37 °C, the presence of this compound is linked to the dominance of *Bacillus*. Volatiles, 1-propenylbenzene and 1-(ethylthio)-2-propanone reduced in abundance when growth of microorganisms reached levels of spoilage (10^7-10^8 CFU/g) and therefore could be deemed as markers of product freshness. These metabolites have not been previously described in other studies assessing degradation of food matrices.

The last objective of the thesis was to enhance or supress growth of spoilage species to understand if spoilage features were directly influenced by either the microbial integrity, or other routes of spoilage such as chemical deterioration. To demonstrate this, the intrinsic properties of the vegetable matrix was manipulated to either create an environment to further support growth of SSOs (specific spoilage organisms) or inhibit their growth. Therefore, the former alteration would promote spoilage features linked to activity of SSOs, while the latter would allow spoilage of the vegetable matrix to be mainly driven by endogenous chemical reactions. Increased growth of microorganisms was achieved by increasing the availability of nutrients, specifically sugar

(glucose, fructose and sucrose) and nicotinamide. Fortification with sugar increased abundance of fermentative genus *Leuconostoc* and *Lactococcu*s, allowing for the elucidation of the relationship between LAB driven spoilage and vitamin profile and production/exhaustion of metabolites. However, addition of nicotinamide to the vegetable matrix altered the microbial composition as *Pseudomonas* and *Acinetobacter* were predominant, which coincided with the alteration of the vitamin profile. For example: nicotinamide was the only vitamin to significantly reduce when *Pseudomonas* and *Acinetobacter* were abundant at 48 h of storage. The metabolite profile also changed in response to changes to the microbial composition. Although the impact of the addition of nicotinamide on bacterial composition requires further investigations and addition of antimicrobial agents to inhibit microbial growth needs investigating.

7.5 Future work

The future work can be divided into two sections, one of which is future work based on findings in chapters 4-6 and the other is based on future avenues that could be explored regarding food spoilage as a result of the presented work.

Future work of chapters 4-6:

- Many of the volatile and non-volatile metabolites discussed as potential markers of spoilage or freshness were putatively annotated and therefore are based on comparing fragmentation patterns of a mass feature to that of publicly populated libraries. However, to confirm the identity of the metabolites, authentic standards need to be purchased and analysed against samples, using the same instrumentation and methodologies.
- The VOC profile of the vegetable matrices stored in the presence of both air, nitrogen and no gas were compared at 48 h (chapter 5). Of these, 48 h was selected based on this time point being indicative of spoilage activity. However, it was observed that spoilage of the vegetable matrix in the bio-fermenter was delayed by 24 h when compared to other storage studies. Therefore, analysis of the VOC profile at 48 h failed to show volatiles associated with spoilage under these conditions. This requires further investigations by analysing the VOC profile again, analysing samples collected at 72 h on the SPME-GCTOF-MS.
- To understand the root cause of spoilage features, the addition of antimicrobial agents as part of chapter 6 requires investigation. The addition of antimicrobial agents aims to supress the growth of SSOs within the matrix, decoupling the spoilage effects of SSOs from general, chemical deterioration. This could be achieved by using a mix of antibiotics to target identified spoilage communities or use of common food preservatives that specifically target growth of microorganisms. Alternatively, canned soup could also be used which has been previously sterilised.

- Chapter 4 details use of a controlled buffered system to analyse stability of B-vitamins under the baseline condition without the presence of the vegetable matrix. Implementation of this approach under all storage conditions investigated (e.g. gas composition) would confirm if the environment itself had a direct impact on the stability of the vitamins or instead confirm deterioration of selected vitamins was due to the vegetable matrix. A model matrix containing a mix of carbohydrates, proteins, fibres could also be used to rule out matrix effect on vitamin deterioration.
- Supplementation of the vegetable matrix with nicotinamide needs further investigation, in terms of the impact on bacterial composition. To confirm the abundance of *Pseudomonas* and *Acinetobacter* was not due to other factors.
- This thesis explored different storage environments that in some cases were selected based on accelerating spoilage reactions and therefore, would not be realistic conditions consumers would typically store food at. Therefore, storage conditions typically used by consumers could be explored, such as storage at lower temperatures e.g., 0-5 °C. This rationale could also be applied to the baseline condition (20 °C for 120 h) in chapter 4, where alternatively recommended storage conditions as specified on the packaging of the vegetable matrix could be followed. This would require a longer storage duration, longer than 120 h and additional sampling points.

Avenues for further exploration outside the scope of this thesis:

- The presented work profiled spoilage of a single product, vegetable soup, however this approach could be used on a variety of food matrices to further explore relationships between food spoilage, vitamin composition and metabolites.
- Building on from above point, validation of the extraction method to isolate B vitamins from the vegetable matrix could be further validated in other matrices. As the analytical method can also analyse folic acid, nicotinic acid and vitamin C, therefore there is scope to simultaneously assess these vitamins in foods also.
- Chapter 5 showed the influence of different environments on opportunistic bacteria, progression of spoilage, relationship between spoilage and vitamin composition etc. However, food matrices can be contaminated by other bacteria in the food system e.g. improper handling and human contact which would introduce different organisms into the product, which could present a different progression of spoilage. This could be further explored by deliberate inoculation with specific SSOs or pathogens, and the resultant progression of spoilage assessed and compared to this current work.
- The results obtained in this work could form the base for future experimental work involving predictive modelling. This type of modelling could be used to predict the microbial communities present in the vegetable matrix and resultant spoilage behaviour

(e.g., production of identified spoilage metabolites, vitamin degradation) under specific storage conditions.

- Metabolomics highlighted metabolites of interest that have the potential to be markers of product freshness or spoilage. Upon further confirmation of these metabolites, they could be used for rapid detection of spoilage rather than using traditional outdated methods to assess deterioration. After extensive research and applying the methods to various food matrices a database could be developed that details 1) a specific food matrix, 2) microorganisms responsible for spoilage under a specific storage condition that is based on packaging and 3) metabolites associated with spoilage of that matrix. This could also inform studies into shelf-life extension.
- The metabolites deemed as potential markers of freshness or spoilage of the matrix require further investigations to understand if they are suitable for early detection of spoilage. As the depletion and production of specific metabolites coincided with distinct spoilage characteristics e.g., off odours etc. Therefore, instead of profiling metabolites at the start, middle and end of the time course, samples collected throughout the entire storage duration could be assessed. This could also include collection of samples at additional time points early in the time series, prior to evident spoilage of the vegetable matrix.

Appendices (1-11)

Appendix 1 - Recovery from Vegetable Matrix Compared to Recovery from Blank

Table S1: Recovery from matrix, including recovery from blank samples

Vitamin/s	Recovery from vegetable matrix lyophilised sample			Recovery from Blank		
	Spiked with*	Mean % Recovery	CV (%)	Spiked with*	Mean % Recovery	CV (%)
Nicotinamide	2.5 µg/mL	30	9.8	2.5 µg/mL	20.6	35.9
Pyridoxine	2.5 µg/mL	118	1.35	2.5 µg/mL	68.8	12
Riboflavin	2.5 µg/mL	96	4.55	2.5 µg/mL	84.7	8.9
Thiamine	2.5 µg/mL	50	3.11	2.5 µg/mL	23.9	15.8

*5 replicates spiked with 0.5 mL of a multi vitamin standard at 2.5 µg/mL, with the exception of nicotinamide for the blank recovery which comprises of triplicate samples

Appendix 2 - Post-Hoc Data Following One-Way Repeated Measures ANOVA on B Vitamins Chapter 4

Table S2: Post-hoc pairwise comparisons matrix following one-way repeated measures ANOVA with Bonferroni corrections for vitamins that had previously shown significant changes (nicotinamide, riboflavin and thiamine) in the vegetable matrix stored for 5 days.

Nicotinamide P Value								
Time Point/s	**T0**	**T1**	**T8**	**T24**	**T48**	**T72**	**T96**	**T120**
T0								
T1	1.000							
T8	1.000	1.000						
T24	1.000	1.000	1.000					
T48	0.171	0.301	0.000049*	0.492				
T72	0.171	0.301	0.000049*	0.492	-**			
T96	0.171	0.301	0.000049*	0.492	-**	-**		
T120	0.171	0.301	0.000049*	0.492	-**	-**	-**	
Riboflavin P Value								
Time Point/s	**T0**	**T1**	**T8**	**T24**	**T48**	**T72**	**T96**	**T120**
T0								
T1	1.000							
T8	1.000	1.000						
T24	1.000	1.000	1.000					
T48	0.296	0.609	0.012*	0.234				
T72	0.292	0.618	0.001*	0.377	1.000			
T96	0.922	1.000	0.100	0.885	1.000	1.000		
T120	0.587	1.000	0.027*	0.621	0.061	0.118	1.000	
Thiamine P Value								
Time Point/s	**T0**	**T1**	**T8**	**T24**	**T48**	**T72**	**T96**	**T120**
T0								
T1	1.000							
T8	1.000	1.000						
T24	1.000	1.000	1.000					
T48	0.045*	0.043*	0.264	0.134				
T72	0.058	0.018*	0.271	0.112	1.000			
T96	0.109	0.072*	0.115	0.228	1.000	1.000		
T120	0.001*	0.017*	1.000	0.026*	1.000	1.000	1.000	

*P < 0.05 and therefore indicating statistical significance
** Vitamin undetected at this point in the time course

Appendix 3 - Descriptive Statistics for Protein Content and Results from One-way Repeated Measures ANOVA

Table S3: Descriptive statistics on the quantity of protein (%) over the time course storage of the vegetable matrix, including one-way repeated measures ANOVA performed to determine any significant differences in the quantity during the 5-day storage, expressed as a P value.

	% Protein		
Time point/s	Mean[a] (%)	STDEV	P value[b]
T0	0.55	0.007	0.657
T48	0.54	0.006	
T120	0.56	0.031	

[a] Mean is comprised of two technical replicates (n=2)
[b]Epsilon (ε) correction used to adjust the degrees of freedom when calculating the P value, subsequently utilising Greenhouse-Geisser estimates for the adjustment

Appendix 4 - One-way Repeated Measures ANOVA and Pairwise Comparisons Completed on Sugar Content in Chapter 4

Table S4: One-way repeated measures ANOVA highlighting any significant changes in targeted sugars, glucose, fructose and sucrose over the storage period (5-days) of the vegetable matrix

	Targeted sugars		
	Glucose	Fructose	Sucrose
P value[a]	0.229	0.010*	0.033*

[a]Epsilon (ε) correction used to adjust the degrees of freedom when calculating the P value, subsequently utilising Greenhouse-Geisser estimates for the adjustment
*P < 0.05 and therefore indicating statistical significance

Table S5: Pairwise comparisons matrix with Bonferroni corrections for targeted sugars that have shown significant change (fructose and sucrose) during storage of the vegetable matrix after performing a one-way repeated measures ANOVA

Fructose P value			
Time Point/s	**T0**	**T48**	**T120**
T0			
T48	1.000		
T120	0.098	0.062	
Sucrose P value			
Time Point/s	**T0**	**T48**	**T120**
T0			
T48	0.038*		
T120	0.117	0.766	

*P < 0.05 and therefore indicating statistical significance

Appendix 5 - Plating Technique - Miles and Misra

Preliminary investigations into different plating techniques to assess microbial growth was studied, in Figure S1 the Miles and Misra technique was used and showed similar results to the adopted approach used in the thesis, pour plate technique. Although there was slightly more error associated when using Miles and Misra due to challenges faced when clearly visualising individual colonies.

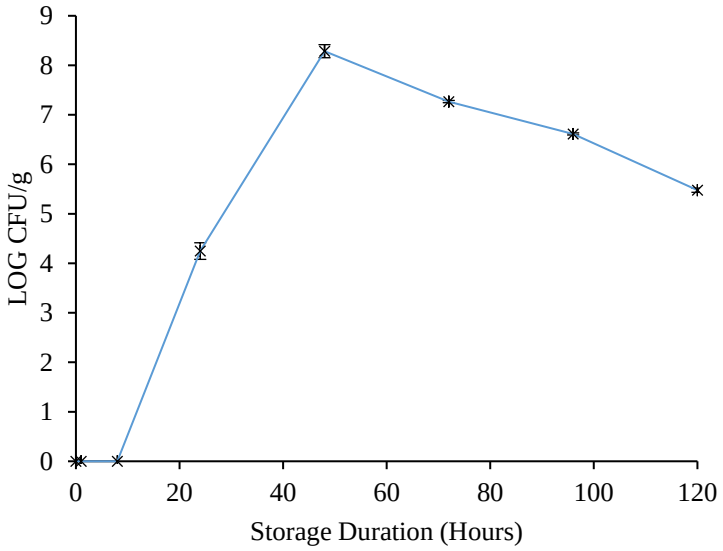


Figure S1: Total viable count (TVC) on plate count agar using Miles and Misra technique. Each data point, comprising of two analysed plates and the error associated with each time point is represented by a 95% confidence interval.

Appendix 6 - Statistical Analysis Conducted on Alpha Diversity in Chapter 4

Table S6: Friedman test completed on the alpha diversity (Shannon index, Inverse Simpson index and richness) to determine any significant changes over the 5-day storage period (A), expressed as a P value. Post-hoc pairwise comparisons using Dunn's test with Bonferroni corrections (B) were conducted if Friedman test showed significance (P<0.05).

(A)	**Alpha Diversity**		
	Shannon Index	Inverse Simpson Index	Richness
P value	0.02179*	0.02179*	0.2311

(B)	**Shannon Index**				
Time Point/s	**T0**	**T24**	**T48**	**T72**	**T120**
T0					
T24	1.0000				
T48	0.2230	0.8545			
T72	0.1788	0.7206	1.0000		
T120	0.0130*	0.0881	1.0000	1.0000	
	Inverse Simpson Index				
Time Point/s	**T0**	**T24**	**T48**	**T72**	**T120**
T0					
T24	1.0000				
T48	0.7206	0.2762			
T72	0.7206	0.2762	1.0000		
T120	0.0529	0.0130*	1.0000	1.0000	

*P < 0.05 and therefore indicating statistical significance.

Table S7: Kruskal-Wallis H test completed on the alpha diversity (Shannon index, Inverse Simpson index and richness) to determine any significant changes across ten different vegetable matrix batches (A), expressed as a P value. Post-hoc pairwise comparisons were conducted using Dunn's test with Bonferroni corrections if Kruskal-Wallis H test showed significance (P<0.05) (B).

(A)	**Alpha Diversity**		
	Shannon Index	Inverse Simpson Index	Richness
P value**	0.0449*	0.0154*	0.0312*

(B)

Shannon Index

Time Point/s	**FS1**	**FS2**	**FS3**	**FS4**	**FS5**	**FS6**	**FS7**	**FS8**	**FS9**	**FS10**
FS1										
FS2	1.0000									
FS3	1.0000	1.0000								
FS4	1.0000	1.0000	1.0000							
FS5	1.0000	1.0000	0.3218	1.0000						
FS6	1.0000	1.0000	1.0000	1.0000	1.0000					
FS7	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000				
FS8	1.0000	1.0000	0.2102	1.0000	1.0000	1.0000	0.7150			
FS9	1.0000	1.0000	0.2798	1.0000	1.0000	1.0000	0.9185	1.0000		
FS10	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	

Inverse Simpson Index

Time Point/s	**FS1**	**FS2**	**FS3**	**FS4**	**FS5**	**FS6**	**FS7**	**FS8**	**FS9**	**FS10**
FS1										
FS2	1.0000									
FS3	1.0000	1.0000								
FS4	1.0000	1.0000	1.0000							
FS5	1.0000	1.0000	1.0000	1.0000						
FS6	1.0000	1.0000	0.4233	0.4233	1.0000					
FS7	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000				
FS8	1.0000	1.0000	0.1817	0.1817	1.0000	1.0000	1.0000			
FS9	1.0000	1.0000	0.3694	0.3694	1.0000	1.0000	1.0000	1.0000		
FS10	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	

Richness

Time Point/s	**FS1**	**FS2**	**FS3**	**FS4**	**FS5**	**FS6**	**FS7**	**FS8**	**FS9**	**FS10**
FS1										
FS2	1.0000									
FS3	1.0000	1.0000								
FS4	1.0000	1.0000	1.0000							
FS5	1.0000	1.0000	1.0000	1.0000						
FS6	1.0000	1.0000	1.0000	0.2100	0.3691					
FS7	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000				
FS8	1.0000	1.0000	1.0000	0.4543	0.7612	1.0000	1.0000			
FS9	1.0000	1.0000	1.0000	0.4229	0.7145	1.0000	1.0000	1.0000		
FS10	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	

*P < 0.05 and therefore indicating statistical significance.

**No post-hoc data is shown, due to Dunn's test with Bonferroni corrections showing no significance

Appendix 7 - Comparison of Different DNA Extraction Kits

As part of preliminary studies, two DNA extraction kits, Powersoil® Qiagen (PS) and PowerFood® Qiagen (PF) were compared to select the most suitable extraction method, using samples collected throughout a trialled storage experiment of the vegetable-based matrix. The microbial composition throughout the time series when using PowerSoil and PowerFood kits can be viewed in Figure S2. Alpha diversity for both kits across time points, in terms of richness and diversity indexes, Shannon and Inverse Simpson can be seen in

S3. Statistical tests were performed on calculated alpha diversity and there was no significance when comparing DNA extraction kits at each time point (Table S8). Furthermore, beta-diversity was assessed using Bray-Curtis dissimilarity metric. The permutational multivariate analysis of variance (PERMANOVA) test indicated no significant differences in beta-diversity across kits ($p = 0.916$, $R^2 = 0.1536$). Therefore, both extraction kits could be utilised to extract DNA from samples collected throughout storage studies of the vegetable matrix.

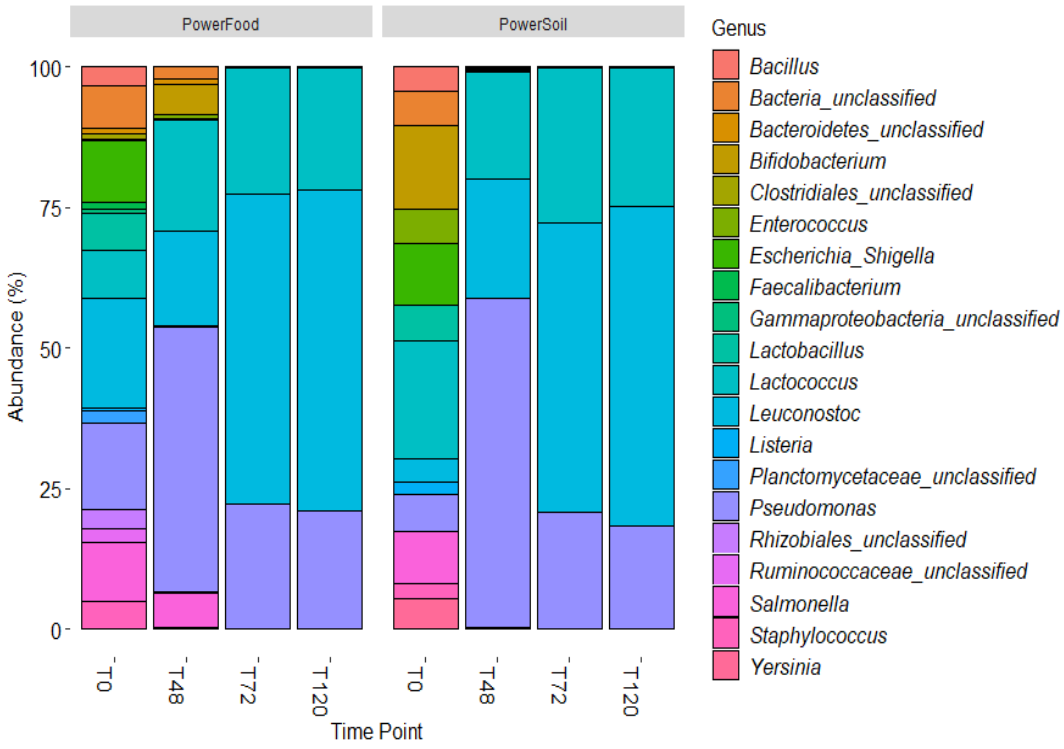


Figure S2: Microbial composition expressed as % abundance of the top 20 bacterial communities classified at genus level over the trialled storage period comparing PowerFood and Powersoil DNA extraction kits.

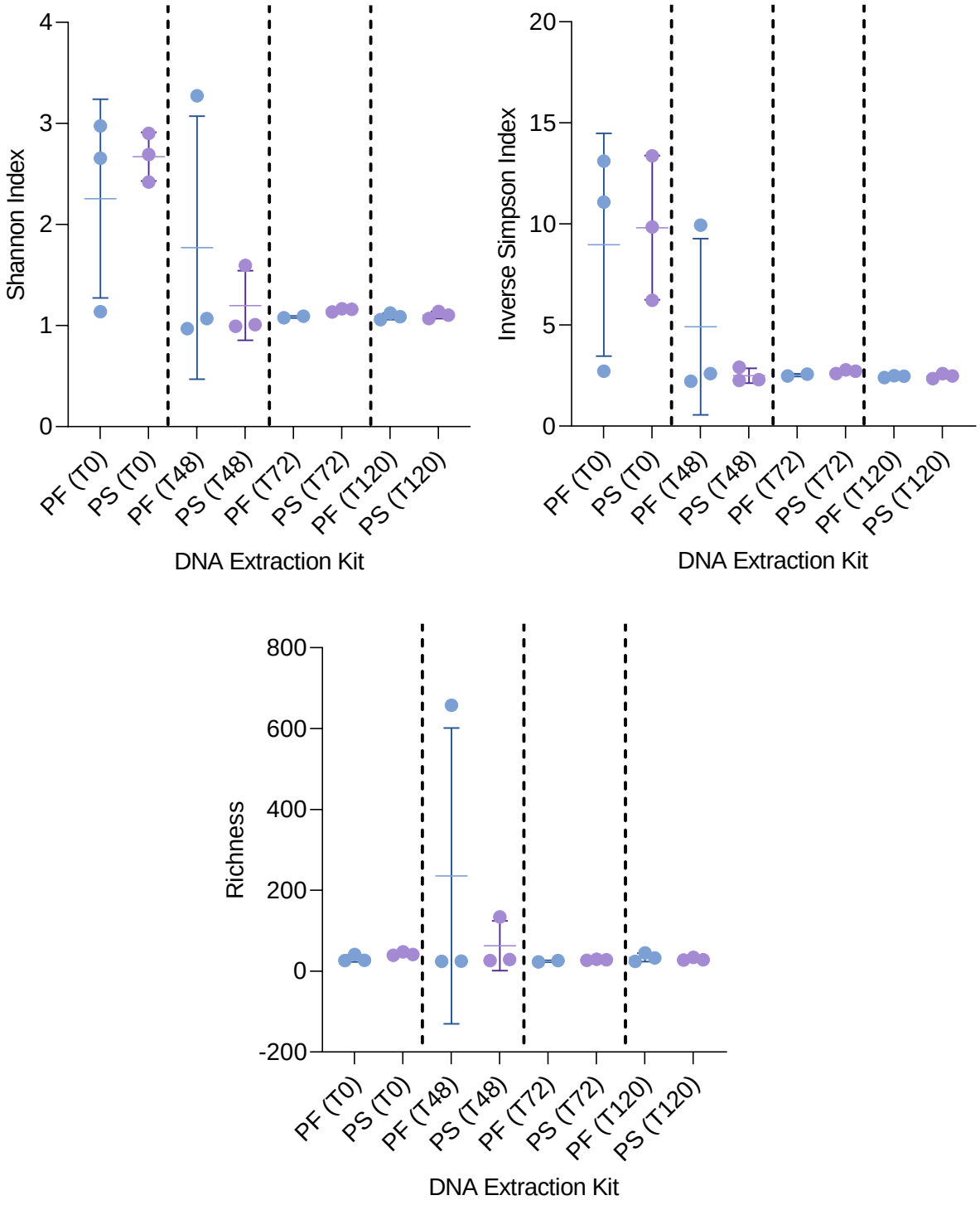


Figure S3: Alpha diversity of the overall microbial composition using two DNA extraction kits PowerFood® Qiagen (PF) and Powersoil® Qiagen (PS) across time points. Alpha diversity was determined by total richness (C), Shannon index (A) and Inverse Simpson index (B). Samples were collected during a trialled storage of the vegetable matrix and each time point comprises of three technical replicates, except for T72 PF, which comprises of two technical replicates. Associated error of each mean is represented by standard deviation.

Table S8: Mann-Whitney U test performed on the alpha diversity (Shannon index, Inverse Simpson index and richness) of each time point comparing extraction kits, Powersoil® Qiagen (PS) and PowerFood® Qiagen (PF).

	Alpha Diversity		
Time Point PS Vs PF	Shannon Index P Value	Inverse Simpson Index P Value	Richness P Value
T0	1.0000	1.0000	0.3000
T48	1.0000	1.0000	0.7000
T72	0.2000	0.2000	0.2000
T120	0.7000	1.0000	1.0000

Appendix 8 - Post-Hoc Data Following One-Way Repeated Measures ANOVA on B-Group Vitamins Chapter 5

Table S9: Post-hoc pairwise comparisons matrix following one-way repeated measures ANOVA with Bonferroni corrections for vitamins that had previously shown significant change (nicotinamide, riboflavin and thiamine) in the vegetable matrix stored for 5 days at 37 °C.

Nicotinamide P Value								
Time Point/s	**T0**	**T1**	**T8**	**T24**	**T32**	**T48**	**T56**	**T72**
T0								
T1	1.000							
T8	1.000	1.000						
T24	0.266	0.176	0.218					
T32	0.266	0.176	0.218	-**				
T48	0.266	0.176	0.218	-**	-**			
T56	0.266	0.176	0.218	-**	-**	-**		
T72	0.266	0.176	0.218	-**	-**	-**	-**	
Riboflavin P Value								
Time Point/s	**T0**	**T1**	**T8**	**T24**	**T32**	**T48**	**T56**	**T72**
T0								
T1	1.000							
T8	1.000	1.000						
T24	1.000	1.000	1.000					
T32	0.172	0.181	0.181	1.000				
T48	0.009*	0.194	0.429	1.000	1.000			
T56	0.033*	0.236	1.000	1.000	1.000	1.000		
T72	0.630	0.958	0.025*	1.000	1.000	1.000	1.000	
Thiamine P Value								
Time Point/s	**T0**	**T1**	**T8**	**T24**	**T32**	**T48**	**T56**	**T72**
T0								
T1	1.000							
T8	1.000	1.000						
T24	0.157	0.092	0.972					
T32	0.014*	0.027*	0.400	0.841				
T48	0.295	0.311	0.036*	1.000	1.000			
T56	0.489	0.613	0.006*	1.000	1.000	1.000		
T72	0.062	0.011*	0.462	0.296	1.000	1.000	1.000	

*P < 0.05 and therefore indicating statistical significance
** Vitamin undetected at this point in the time course

Table S10: Post-hoc pairwise comparisons matrix following one-way repeated measures ANOVA with Bonferroni corrections for vitamins that had previously shown significant change (nicotinamide) in the vegetable matrix prepared from frozen and stored at 20 °C for 5 days.

Nicotinamide P Value							
Time Point/s	**T0**	**T1**	**T24**	**T48**	**T72**	**T96**	**T120**
T0							
T1	1.000						
T24	0.254	1.000					
T48	1.000	0.029*	1.000				
T72	0.382	0.059	0.098	0.018*			
T96	0.382	0.059	0.098	0.018*	-**		
T120	0.382	0.059	0.098	0.018*	-**	-**	

*P < 0.05 and therefore indicating statistical significance
** Vitamin undetected at this point in the time course

Table S11: Post-hoc pairwise comparisons matrix following one-way repeated measures ANOVA with Bonferroni corrections for vitamins that had previously shown significant change (nicotinamide, riboflavin and thiamine) in the vegetable matrix stored under no gas for 5 days at 20 °C.

Nicotinamide P Value								
Time Point/s	**T0**	**T1**	**T8**	**T24**	**T48**	**T72**	**T96**	**T120**
T0								
T1	1.000							
T8	1.000	1.000						
T24	1.000	1.000	1.000					
T48	1.000	1.000	1.000	1.000				
T72	0.665	0.424	0.132	0.534	0.175			
T96	0.665	0.424	0.132	0.534	0.175	-**		
T120	0.665	0.424	0.132	0.534	0.175	-**	-**	
Riboflavin P Value								
Time Point/s	**T0**	**T1**	**T8**	**T24**	**T48**	**T72**	**T96**	**T120**
T0								
T1	1.000							
T8	1.000	1.000						
T24	1.000	1.000	1.000					
T48	1.000	1.000	1.000	1.000				
T72	1.000	0.752	0.481	0.004*	1.000			
T96	1.000	0.086	0.072	0.233	1.000	1.000		
T120	0.622	0.247	0.176	0.016*	1.000	0.417	1.000	
Thiamine P Value								
Time Point/s	**T0**	**T1**	**T8**	**T24**	**T48**	**T72**	**T96**	**T120**
T0								
T1	1.000							
T8	1.000	1.000						
T24	1.000	1.000	1.000					
T48	1.000	1.000	1.000	1.000				
T72	0.518	0.078	0.217	0.516	0.334			
T96	0.161	0.248	0.135	0.331	0.052	1.000		
T120	0.041*	0.147	0.108	0.361	0.061	1.000	1.000	

*P < 0.05 and therefore indicating statistical significance

** Vitamin undetected at this point in the time course

Table S12: Post-hoc pairwise comparisons matrix following one-way repeated measures ANOVA with Bonferroni corrections for vitamins that had previously shown significant change (nicotinamide, riboflavin and thiamine) in the vegetable matrix stored in the presence of nitrogen for 5 days at 20 °C.

Nicotinamide P Value								
Time Point/s	**T0**	**T1**	**T8**	**T24**	**T48**	**T72**	**T96**	**T120**
T0								
T1	1.000							
T8	1.000	1.000						
T24	1.000	1.000	1.000					
T48	1.000	1.000	0.537	1.000				
T72	0.126	1.000	0.183	0.334	0.449			
T96	0.126	1.000	0.183	0.334	0.449	-**		
T120	0.126	1.000	0.183	0.334	0.449	-**	-**	
Riboflavin P Value								
Time Point/s	**T0**	**T1**	**T8**	**T24**	**T48**	**T72**	**T96**	**T120**
T0								
T1	1.000							
T8	1.000	1.000						
T24	1.000	1.000	1.000					
T48	1.000	1.000	0.733	1.000				
T72	0.045*	0.376	0.014*	0.135	0.066			
T96	0.045*	0.376	0.014*	0.135	0.066	-**		
T120	0.045*	0.376	0.014*	0.135	0.066	-**	-**	
Thiamine P Value								
Time Point/s	**T0**	**T1**	**T8**	**T24**	**T48**	**T72**	**T96**	**T120**
T0								
T1	1.000							
T8	1.000	1.000						
T24	1.000	1.000	1.000					
T48	1.000	1.000	1.000	1.000				
T72	0.358	0.002*	0.482	0.062	0.315			
T96	0.446	0.038*	0.581	0.065	0.348	1.000		
T120	0.393	0.006*	0.523	0.065	0.335	1.000	1.000	

*P < 0.05 and therefore indicating statistical significance

** Vitamin undetected at this point in the time course

Table S13: Post-hoc pairwise comparisons matrix following one-way repeated measures ANOVA with Bonferroni corrections for vitamins that had previously shown significant change (nicotinamide and thiamine) in the vegetable matrix stored in the presence of compressed air for 5 days at 20 °C.

Nicotinamide P Value								
Time Point/s	**T0**	**T1**	**T8**	**T24**	**T48**	**T72**	**T96**	**T120**
T0								
T1	0.018*							
T8	0.095*	0.004*						
T24	1.000	1.000	1.000					
T48	1.000	0.556	0.707	1.000				
T72	0.903	0.113	0.201	1.000	1.000			
T96	0.003*	0.00*	0.00*	0.269	0.237	0.022*		
T120	0.003*	0.00*	0.00*	0.269	0.237	0.022*	-*	
Thiamine P Value								
Time Point/s	**T0**	**T1**	**T8**	**T24**	**T48**	**T72**	**T96**	**T120**
T0								
T1	1.000							
T8	1.000	1.000						
T24	1.000	1.000	1.000					
T48	1.000	1.000	1.000	1.000				
T72	1.000	1.000	1.000	1.000	1.000			
T96	1.000	1.000	1.000	0.759	1.000	1.000		
T120	0.005*	0.129	0.022*	0.016*	0.011*	0.065	0.337	

*P < 0.05 and therefore indicating statistical significance

** Vitamin undetected at this point in the time course

Table S14: Post-hoc pairwise comparisons matrix following one-way repeated measures ANOVA with Bonferroni corrections for vitamins that had previously shown significant change (nicotinamide, pyridoxine, riboflavin and thiamine) in the vegetable matrix stored under a pre-set alkaline environment for 5 days at 20 °C

Nicotinamide P Value								
Time Point/s	**T0**	**T1**	**T8**	**T24**	**T48**	**T72**	**T96**	**T120**
T0								
T1	0.658							
T8	1.000	0.028*						
T24	0.420	0.130	0.226					
T48	0.258	0.040*	0.073	0.743				
T72	0.258	0.040*	0.073	0.743	-**			
T96	0.258	0.040*	0.073	0.743	-**	-**		
T120	0.258	0.040*	0.073	0.743	-**	-**	-**	
Pyridoxine P Value								
Time Point/s	**T0**	**T1**	**T8**	**T24**	**T48**	**T72**	**T96**	**T120**
T0								
T1	1.000							
T8	1.000	1.000						
T24	0.730	1.000	1.000					
T48	0.037*	0.025*	0.036*	0.063				
T72	0.037*	0.025*	0.036*	0.063	-**			
T96	0.053	0.043*	0.064	0.046*	0.038*	0.038*		
T120	0.010*	0.009*	0.007*	0.012*	0.004*	0.004*	1.000	
Riboflavin P Value								
Time Point/s	**T0**	**T1**	**T8**	**T24**	**T48**	**T72**	**T96**	**T120**
T0								
T1	0.954							
T8	1.000	1.000						
T24	1.000	1.000	1.000					
T48	0.091	0.156	0.465	0.161				
T72	0.002*	0.015*	0.094	0.009*	0.187			
T96	0.057	0.027*	0.031*	0.101	1.000	1.000		
T120	0.064	0.082	0.222	0.105	0.043*	1.000	1.000	
Thiamine P Value								
Time Point/s	**T0**	**T1**	**T8**	**T24**	**T48**	**T72**	**T96**	**T120**
T0								
T1	1.000							
T8	1.000	1.000						
T24	0.030*	0.053	0.666					
T48	0.052	0.057	0.056	0.162				
T72	0.106	0.075	0.124	0.357	1.000			
T96	0.099	0.99	0.079	0.417	0.361	1.000		
T120	0.097	0.0910	0.094	0.421	0.165	0.808	0.669	

*P < 0.05 and therefore indicating statistical significance

** Vitamin undetected at this point in the time course

Appendix 9 - Post-Hoc Data Conducted on Alpha Diversity Chapter 5

Table S15: Friedman test completed on the alpha diversity measures (Shannon index, Inverse Simpson index and richness) to determine any significant changes over the 5-day storage period under different storage temperatures, 7 °C and 37 °C.

7 °C			
	Shannon Index	Inverse Simpson Index	Richness
P value	0.04679*	0.04979*	0.04679*
37 °C			
	Shannon Index	Inverse Simpson Index	Richness
P value	0.04679*	0.09697	0.09697

*P < 0.05 and therefore indicating statistical significance

Table S16: Friedman test completed on the alpha diversity measures (Shannon index, Inverse Simpson index and richness) to determine any significant changes over the 5-day storage period when the vegetable matrix is prepared from frozen.

From frozen			
	Shannon Index	Inverse Simpson Index	Richness
P value	0.09697	0.09697	0.0967

Table S17: Friedman test completed on the alpha diversity measures (Shannon index, Inverse Simpson index and richness) to determine any significant changes over the 5-day storage period under no gas and in the presence of either nitrogen or compressed air.

No gas			
	Shannon Index	Inverse Simpson Index	Richness
P value	0.04979*	0.04979*	0.04979*
Presence of Nitrogen			
	Shannon Index	Inverse Simpson Index	Richness
P value	0.04979*	0.04979*	0.04979*
Presence of compressed air			
	Shannon Index	Inverse Simpson Index	Richness
P value	0.09697	0.09697	0.04979*

*P < 0.05 and therefore indicating statistical significance

Table S18: Friedman test completed on the alpha diversity measures (Shannon index, Inverse Simpson index and richness) to determine any significant changes over the 5-day storage period under pre-set pH conditions (acidic and alkaline).

Acidic			
	Shannon Index	Inverse Simpson Index	Richness
P value	0.09697	0.09697	0.09697
Alkaline			
	Shannon Index	Inverse Simpson Index	Richness
P value	0.04979*	0.04979*	0.04979*

*P < 0.05 and therefore indicating statistical significance

Table S19: Post-hoc pairwise comparisons matrix using Dunn's test with Bonferroni corrections for alpha diversity measures that had previously shown significant changes when the vegetable matrix was stored at 7 °C.

Shannon Index			
Time Point/s	**T0**	**T32**	**T72**
T0			
T32	0.2696		
T72	0.0109*	0.2696	

*P < 0.05 and therefore indicating statistical significance

Table S20: Post-hoc pairwise comparisons matrix using Dunn's test with Bonferroni corrections for alpha diversity measures that had previously shown significant changes when the vegetable matrix was stored at 37 °C.

Shannon Index			
Time Point/s	**T0**	**T48**	**T120**
T0			
T48	0.2696		
T120	0.0109*	0.2696	
Inverse Simpson Index			
Time Point/s	**T0**	**T48**	**T120**
T0			
T48	0.2696		
T120	0.0109*	0.2696	
Richness			
Time Point/s	**T0**	**T48**	**T120**
T0			
T48	0.2696		
T120	0.0109*	0.2696	

*P < 0.05 and therefore indicating statistical significance

Table S21: Post-hoc pairwise comparisons matrix using Dunn's test with Bonferroni corrections for alpha diversity measures that had previously shown significant changes when the vegetable matrix was stored under no gas at 20 °C in the bio-fermenter.

Shannon Index			
Time Point/s	**T0**	**T48**	**T120**
T0			
T48	0.2696		
T120	0.0109*	0.2696	
Inverse Simpson Index			
Time Point/s	**T0**	**T48**	**T120**
T0			
T48	0.2041		
T120	0.0169*	0.4451	
Richness			
Time Point/s	**T0**	**T48**	**T120**
T0			
T48	0.2696		
T120	0.0109*	0.2696	

*P < 0.05 and therefore indicating statistical significance

Table S22: Post-hoc pairwise comparisons matrix using Dunn's test with Bonferroni corrections for alpha diversity measures that had previously shown significant changes when the vegetable matrix was stored in the presence of nitrogen at 20 °C.

Shannon Index			
Time Point/s	**T0**	**T48**	**T120**
T0			
T48	0.2696		
T120	0.0109*	0.2696	
Inverse Simpson Index			
Time Point/s	**T0**	**T48**	**T120**
T0			
T48	0.2696		
T120	0.0109*	0.2696	
Richness			
Time Point/s	**T0**	**T48**	**T120**
T0			
T48	0.6841		
T120	0.0256*	0.1516	

*P < 0.05 and therefore indicating statistical significance

Table S23: Post-hoc pairwise comparisons matrix using Dunn's test with Bonferroni corrections for alpha diversity measures that had previously shown significant changes when the vegetable matrix was stored in the presence of compressed air at 20 °C.

Richness			
Time Point/s	**T0**	**T48**	**T120**
T0			
T48	0.6841		
T120	0.0256*	0.1516	

*P < 0.05 and therefore indicating statistical significance

Table S24: Post-hoc pairwise comparisons matrix using Dunn's test with Bonferroni corrections for alpha diversity measures that had previously shown significant changes when the vegetable matrix was stored under pre-set alkaline conditions.

Shannon Index			
Time Point/s	T0	T48	T120
T0			
T48	0.0109*		
T120	0.2696	0.2696	
Inverse Simpson Index			
Time Point/s	T0	T48	T120
T0			
T48	0.0109*		
T120	0.2696	0.2696	
Richness			
Time Point/s	T0	T48	T120
T0			
T48	0.0109*		
T120	0.2696	0.2696	

*P < 0.05 and therefore indicating statistical significance

Table S25: Kruskal-Wallis rank sum test investigating significant differences in alpha diversity measures at each time point across all storage conditions conducted under different temperatures (7 °C, versus 20 °C versus 37 °C).

T0			
	Shannon Index	Inverse Simpson Index	Richness
P value	0.2521	0.03899*	0.05090
T32/T48			
	Shannon Index	Inverse Simpson Index	Richness
P value	0.06081	0.03899*	0.3012
T72/T120			
	Shannon Index	Inverse Simpson Index	Richness
P value	0.06081	0.02732*	0.06081

*P < 0.05 and therefore indicating statistical significance

Table S26: Post-hoc pairwise comparisons matrix using Dunn's test with Bonferroni corrections for time points that had previously shown significant differences for diversity metric, Inverse Simpson index during storage under different temperatures (7 °C, versus 20 °C versus 37 °C).

Inverse Simpson Index – T0			
Storage Environment	Baseline Condition	7 °C	37 °C
Baseline Condition			
7 °C	0.8902		
37 °C	0.4081	0.03381*	
Inverse Simpson Index – T32/T48			
Storage Environment	Baseline Condition	7 °C	37 °C
Baseline Condition			
7 °C	0.03381*		
37 °C	0.4081	0.8902	
Inverse Simpson Index – T72/T120			
Storage Environment	Baseline Condition	7 °C	37 °C
Baseline Condition			
7 °C	0.02187*		
37 °C	0.5391	0.5391	

*P < 0.05 and therefore indicating statistical significance

Table S27: Mann-Whitney U test investigating significant differences in alpha diversity measures at each time point when the vegetable matrix was prepared from frozen versus from fresh.

T0			
	Shannon Index	Inverse Simpson Index	Richness
P value	1.0000	1.0000	0.4000
T48			
	Shannon Index	Inverse Simpson Index	Richness
P value	1.0000	1.0000	1.0000
T120			
	Shannon Index	Inverse Simpson Index	Richness
P value	1.0000	1.0000	0.7000

Table S28: Kruskal-Wallis rank sum test investigating significant differences in alpha diversity measures at each time point across all storage conditions conducted in the bio-fermenter (no gas versus presence of nitrogen versus presence of compressed air).

T0			
	Shannon Index	Inverse Simpson Index	Richness
P value	0.7326	0.4298	0.09915
T48			
	Shannon Index	Inverse Simpson Index	Richness
P value	0.02732*	0.02732*	0.03899*
T120			
	Shannon Index	Inverse Simpson Index	Richness
P value	0.02732*	0.02732*	0.03899*

*P < 0.05 and therefore indicating statistical significance

Table S29: Post-hoc pairwise comparisons matrix using Dunn's test with Bonferroni corrections for alpha diversity measures that had previously shown significant differences at T48 across storage environments conducted in the bio-fermenter (no gas versus presence of nitrogen versus presence of compressed air).

Shannon Index – T48			
Storage Environment	No gas	Nitrogen	Air
No gas			
Nitrogen	0.2696		
Air	0.2696	0.0109*	
Inverse Simpson Index – T48			
Storage Environment	No gas	Nitrogen	Air
No gas			
Nitrogen	0.2696		
Air	0.2696	0.0109*	
Richness – T48			
Storage Environment	No gas	Nitrogen	Air
No gas			
Nitrogen	0.4451		
Air	0.2041	0.0169*	

*P < 0.05 and therefore indicating statistical significance

Table S30: Post-hoc pairwise comparisons matrix using Dunn's test with Bonferroni corrections for alpha diversity measures that had previously shown significant differences at T120 across storage environments conducted in the bio-fermenter (no gas versus presence of nitrogen versus presence of compressed air).

Shannon Index – T120			
Storage Environment	No gas	Nitrogen	Air
No gas			
Nitrogen	0.2696		
Air	0.0109*	0.2696	
Inverse Simpson Index – T120			
Storage Environment	Baseline Condition	Nitrogen	Air
No gas			
Nitrogen	0.2696		
Air	0.0109*	0.2696	
Richness – T120			
Storage Environment	Baseline Condition	Nitrogen	Air
No gas			
Nitrogen	0.4451		
Air	0.0169*	0.2041	

*P < 0.05 and therefore indicating statistical significance

Table S31: Kruskal-Wallis rank sum test investigating significant differences in alpha diversity measures at each time point under pre-set pH conditions and baseline (acidic versus alkaline versus baseline).

T0			
	Shannon Index	Inverse Simpson Index	Richness
P value	0.03899*	0.02732*	0.07939
T48			
	Shannon Index	Inverse Simpson Index	Richness
P value	0.05090	0.02732*	0.06081
T120			
	Shannon Index	Inverse Simpson Index	Richness
P value	0.06081	0.02732*	0.1479

*P < 0.05 and therefore indicating statistical significance

Table S32: Post-hoc pairwise comparisons matrix using Dunn's test with Bonferroni corrections for time points that had previously shown significant differences for diversity metric, Shannon index during storage under different pre-set pH conditions (acidic versus alkaline versusbaseline).

Shannon Index – T0			
Storage Environment	Unchanged pH	Acidic	Alkaline
Unchanged pH			
Acidic	0.4081		
Alkaline	0.8902	0.03381*	

*P < 0.05 and therefore indicating statistical significance

Table S33: Post-hoc pairwise comparisons matrix using Dunn's test with Bonferroni corrections for time points that had previously shown significant differences for diversity metric, Inverse Simpson index during storage under different pre-set pH conditions (acidic versus alkaline versus baseline).

Inverse Simpson Index – T0			
Storage Environment	Unchanged pH	Acidic	Alkaline
Unchanged pH			
Acidic	0.5391		
Alkaline	0.5391	0.02187*	
Inverse Simpson Index –T48			
Storage Environment	Unchanged pH	Acidic	Alkaline
Unchanged pH			
Acidic	0.5391		
Alkaline	0.02187*	0.5391	
Inverse Simpson Index –T120			
Storage Environment	Unchanged pH	Acidic	Alkaline
Unchanged pH			
Acidic	0.02187*		
Alkaline	0.5391	0.5391	

*P < 0.05 and therefore indicating statistical significance

Appendix 10 - Post-Hoc Data Following One-Way Repeated Measures ANOVA on B-Group Vitamins Chapter 6

Table S34: Post-hoc pairwise comparisons matrix following one-way repeated measures ANOVA with Bonferroni corrections for vitamins that had previously shown significant change (nicotinamide, pyridoxine, riboflavin and thiamine) in the vegetable matrix fortified with nicotinamide, stored for 5 days at 20 °C

Nicotinamide P Value							
Time Point/s	**T0**	**T1**	**T24**	**T48**	**T72**	**T96**	**T120**
T0							
T1	1.000						
T24	0.051	0.373					
T48	0.013*	0.071	0.005*				
T72	0.013*	0.071	0.005*	-**			
T96	0.013*	0.071	0.005*	-**	-**		
T120	0.013*	0.0741	0.005*	-**	-**	-**	
Pyridoxine P Value							
Time Point/s	**T0**	**T1**	**T24**	**T48**	**T72**	**T96**	**T120**
T0							
T1	1.000						
T24	1.000	1.000					
T48	1.000	1.000	1.000				
T72	1.000	1.000	1.000	1.000			
T96	0.048*	1.000	0.506	0.201	1.000		
T120	0.339	1.000	0.240	0.260	1.000	1.000	
Riboflavin P Value							
Time Point/s	**T0**	**T1**	**T24**	**T48**	**T72**	**T96**	**T120**
T0							
T1	1.000						
T24	1.000	1.000					
T48	1.000	0.089	1.000				
T72	1.000	1.000	1.000	1.000			
T96	0.021*	0.011*	0.094	0.010*	1.000		
T120	0.021*	0.011*	0.094	0.010*	1.000	-**	
Thiamine P Value							
Time Point/s	**T0**	**T1**	**T24**	**T48**	**T72**	**T96**	**T120**
T0							
T1	1.000						
T24	1.000	1.000					
T48	1.000	0.658	1.000				
T72	0.042*	0.493	0.064	1.000			
T96	0.065	0.730	0.032*	1.000	1.000		
T120	0.503	0.090	0.893	0.255	1.000	1.000	

*P < 0.05 and therefore indicating statistical significance
** Vitamin undetected at this point in the time course

Table S35: Post-hoc pairwise comparisons matrix following one-way repeated measures ANOVA with Bonferroni corrections for vitamins that had previously shown significant change (nicotinamide, riboflavin and thiamine) in the vegetable matrix fortified with sugar (glucose, fructose and sucrose), stored for 5 days at 20 °C.

Nicotinamide P Value Fortified Sugar							
Time Point/s	**T0**	**T1**	**T24**	**T48**	**T72**	**T96**	**T120**
T0							
T1	1.000						
T24	1.000	1.000					
T48	0.012*	1.000	0.130				
T72	0.012*	0.101	0.130	-**			
T96	0.012*	0.101	0.130	-**	-**		
T120	0.012*	0.101	0.130	-**	-**	-**	
Riboflavin P Value							
Time Point/s	**T0**	**T1**	**T24**	**T48**	**T72**	**T96**	**T120**
T0							
T1	1.000						
T24	1.000	1.000					
T48	0.024*	0.016*	0.335				
T72	0.032*	0.008*	0.296	0.370			
T96	0.015*	0.025*	0.0228	0.347	1.000		
T120	0.052*	0.002*	0.187	0.812	1.000	1.000	
Thiamine P Value							
Time Point/s	**T0**	**T1**	**T24**	**T48**	**T72**	**T96**	**T120**
T0							
T1	0.356						
T24	1.000	1.000					
T48	0.007*	0.183	0.520				
T72	0.005*	0.202	0.523	1.000			
T96	0.121	0.078	0.124	1.000	1.000		
T120	0.209	0.561	0.708	1.000	1.000	1.000	

*P < 0.05 and therefore indicating statistical significance
** Vitamin undetected at this point in the time course

Appendix 11 - Post-Hoc Data Conducted on Alpha Diversity Chapter 6

Table S36: Friedman test completed on the alpha diversity measures (Shannon index, Inverse Simpson index and richness) to determine any significant changes over the 5-day storage when fortified with either nicotinamide or sugar (glucose, fructose and sucrose) under baseline conditions, 20 °C.

	Fortified Nicotinamide		
	Shannon Index	Inverse Simpson Index	Richness
P value	0.04979*	0.04979*	0.04979*
	Fortified Sugar		
	Shannon Index	Inverse Simpson Index	Richness
P value	0.09697	0.04979*	0.09697

*P < 0.05 and therefore indicating statistical significance

Table S37: Post-hoc pairwise comparisons matrix using Dunn's test with Bonferroni corrections for alpha diversity measures that had previously shown significant changes when the vegetable matrix was fortified with nicotinamide and stored under baseline conditions at 20 °C.

Shannon Index			
Time Point/s	**T0**	**T48**	**T120**
T0			
T48	0.0109*		
T120	0.2696	0.2696	
Inverse Simpson Index			
Time Point/s	**T0**	**T48**	**T120**
T0			
T48	0.0109*		
T120	0.2696	0.2696	
Richness			
Time Point/s	**T0**	**T48**	**T120**
T0			
T48	0.0169*		
T120	0.2041	0.4451	

*P < 0.05 and therefore indicating statistical significance

Table S38: Post-hoc pairwise comparisons matrix using Dunn's test with Bonferroni corrections for alpha diversity measures that had previously shown significant changes when the vegetable matrix was fortified with sugar (glucose, fructose and sucrose) and stored under baseline conditions at 20 °C.

Inverse Simpson Index			
Time Point/s	**T0**	**T48**	**T120**
T0			
T48	0.0169*		
T120	0.2041	0.4451	

*P < 0.05 and therefore indicating statistical significance

Table S39: Kruskal-Wallis rank sum test investigating significant differences in alpha diversity measures at each time point across all fortification conditions and baseline condition (fortification with nicotinamide versus fortification with sugar versus baseline condition).

T0			
	Shannon Index	Inverse Simpson Index	Richness
P value	0.039*	0.06081	0.2521
T48			
	Shannon Index	Inverse Simpson Index	Richness
P value	0.039*	0.05091	0.7326
T120			
	Shannon Index	Inverse Simpson Index	Richness
P value	0.05091	0.05091	0.0039*

*P < 0.05 and therefore indicating statistical significance

Table S40: Post-hoc pairwise comparisons matrix using Dunn's test with Bonferroni corrections for time points that had previously shown significant differences for diversity metric, Shannon index when the nutritional composition of the vegetable matrix was manipulated (fortification with nicotinamide versus fortification with sugar versus baseline condition).

Shannon Index – T0			
Storage Environment	Nicotinamide	Sugar	Baseline
Nicotinamide			
Sugar	0.8901		
Baseline	0.4081	0.03381*	
Shannon Index –T48			
Storage Environment	Nicotinamide	Sugar	Baseline
Nicotinamide			
Sugar	0.8901		
Baseline	0.4081	0.03381*	

*P < 0.05 and therefore indicating statistical significance

Table S41: Post-hoc pairwise comparisons matrix using Dunn's test with Bonferroni corrections for time points that had previously shown significant differences for diversity metric, Richness when the nutritional composition of the vegetable matrix was manipulated (fortification with nicotinamide versus fortification with sugar versus baseline condition).

Richness – T120			
Storage Environment	Nicotinamide	Sugar	Baseline
Nicotinamide			
Sugar	0.03381*		
Baseline	0.4081	0.8902	

*P < 0.05 and therefore indicating statistical significance

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