Profiling B-vitamins as Quality Markers of Shelf-life and Nutritional Status of Foods

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Highlights

- B-vitamin content was explored as a quality marker of product freshness.
- Storage environments investigated had significant impact on B-vitamin stability.
- B-vitamin stability was correlated with microbial growth, another quality factor.
- Nicotinamide could be used as a marker of nutritional quality.

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Abstract

Shelf-life of foods is traditionally determined using microbial and organoleptic assessments. However, the nutritional composition of foods, and specifically vitamin composition, remains largely unevaluated and fails to be considered a quality marker of foods, despite being vulnerable to degradation. In this study we profiled B-vitamins (thiamine, riboflavin, nicotinamide and pyridoxine) in a vegetable matrix stored for 5-days under different intrinsic and extrinsic environments. The investigation showed all B-vitamins were significantly unstable (losses between 32.2% - 100%) depending on storage environment (temperature, oxygen levels and pH) and significant changes to B-vitamin content was correlated (e.g. $r_s = -.927$, $p < 0.05$) with microbial growth. In all storage environments where microbial growth reached spoilage levels (TVC > 10^7 CFU/g), nicotinamide was the only vitamin that was completely exhausted. Therefore, we have shown that B-vitamins, particularly nicotinamide could be used as an overall indicator of nutritional quality.

Key words

B-vitamin deterioration; Quality markers; Microbial growth; Vegetable matrix; Food spoilage; Storage environments

Abbreviations

TVC, total viable count; TVB-N, total volatile basic nitrogen; VOC's, volatile organic compounds; PPS, peptone physiological saline.

1. Introduction

A growing health-conscious population desire foods that are of a high quality, safe and are optimally nutritious. This can be a challenge due to the complexity of the food chain as foods are vulnerable to physiochemical and microbiological biotransformations influencing a products stability, shelf-life and subsequently acceptability. Metabolic or compositional alterations to foods that render it undesirable or unacceptable for consumption can be characterised as "food spoilage" (Nychas & Panagou, 2011). Microorganisms are one of the main contributors to food spoilage, through the production of hydrolytic enzymes (e.g., lipases, proteases) which degrade and metabolise food macronutrients, resulting in textural alterations and visual defects (discolouration, slime production, colony formation). This also gives rise to the production of secondary metabolites associated with off-odours, off-flavours and acidity changes (Boziaris et al., 2011; Gram et al., 2002; Huis in't Veld, 1996). Due to this, microbial and organoleptic testing forms the benchmark for product acceptability in shelf-life or product stability assessments (Haouet et al., 2019). However, the nutritional composition and particularly the vitamin composition of foods, remains unevaluated and is not considered a "quality" attribute in shelf-life testing unless health claims have been made and stated on packaging, or foods have been fortified with vitamins (EU Regulation No 169/2011). Vitamins are neither stable nor guaranteed during processing and storage due to their susceptibility to degrade and could degrade prior to evident spoilage (Lešková et al., 2006). This highlights the opportunity for vitamins to be explored as a quality marker of nutritional status of foods and to provide an alternative measure alongside microbial and organoleptic assessments.

Current quality markers that are used during shelf-life assessments include microbial count, specifically total viable count (TVC), with acceptability levels recently defined by the UK government (UKHSA, 2024). Other quality markers are commonly related to the sensorial properties of foods including colour, texture, smell, and taste. Colour and texture are used as visual indicators of food quality, due to adverse changes in such being correlated with microbial activity, oxidation, enzymatic and non-enzymatic reactions (Barrett et al., 2010; Rawat, 2015). Spoilage specific metabolites are used as measures of food quality, associated with the characteristic off-odours and flavours in spoiled foods. For example, biogenic amines (putrescine, tyramine, cadaverine), total volatile basic nitrogen (TVB-N) (trimethylamine), ATP break down products (hypoxanthine) and volatile organic compounds (VOC's) (alcohols, aldehydes, sulphur compounds) are the main reported metabolite markers associated with spoilage (Holman et al., 2021; Hong et al., 2017; Li et al., 2018; Mikš-Krajnik et al., 2016; Vasconcelos et al., 2021). Production of metabolites during spoilage can alter the pH of foods and therefore pH can serve as a valuable measure of quality (Gänzle, 2015). 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 (Nychas & Panagou, 2011).

These techniques can assess microbial load, pH, colour and metabolite changes (Nanda et al., 2022; Nychas & Panagou, 2011).

B-group vitamins fail to be measured as standard procedure in shelf-life evaluations, although are liable to degradation during storage. Owing to the importance of these B-vitamins in the diet for their involvement in redox reactions, macronutrient metabolism and reducing risk of degenerative diseases, understanding storage conditions that minimise B-vitamin loss in foods is essential (Said, 2011). In addition, assessment of B-vitamin composition in foods alongside routinely assessed quality markers such as TVC is scarce. Of the limited publications investigating B-vitamin composition in tandem with microorganisms, all studies have focused on desirable microbial activities such as fermentation as a potential means of B-vitamin synthesis (Champagne et al., 2010; Ekinci, 2005; Kaprasob et al., 2018; Ruiz-Barba & Jimenez-Diaz, 1995). B-vitamins act as precursors for coenzymes that are an essential part of metabolism in all living organisms and can be an essential growth factor for most bacteria (LeBlanc et al., 2011; Vogl et al., 2007). Nicotinamide as an example is a precursor for cofactor nicotinamide adenine dinucleotide ($NADH/NAD^+$) required for redox reactions to maintain cell viability (Zapata-Perez et al., 2017). As described, some bacteria synthesise B-vitamins, however microorganisms that lack this ability can employ a transport system to uptake vitamins from their environment (Acevedo-Rocha et al., 2019; Santos et al., 2020; Vogl et al., 2007).

Intrinsic properties of foods (pH, redox potential) in combination with extrinsic storage conditions (temperature, gas composition) are known parameters that influence quality markers of foods and form part of shelf-life evaluations (Kilcast & Subramaniam, 2000). Increased storage temperature for example is well documented to reduce microbial integrity of foods as Alvarez et al. (2015) demonstrated during storage of vegetables between 5-15 °C. These conditions also have the potential to impact B-vitamin quality of foods during storage to an unknown extent. As shown in numerous publications B-vitamins are vulnerable to temperature during the storage and processing of foods including, pulses and pasta (Bui & Small, 2012; Kadakal et al., 2018; Kamman et al., 1981; Nisha et al., 2005, 2009). Although previous studies have focused on temperatures in excess of typical storage conditions. Another extrinsic factor is the presence and concentration of gases, such as oxygen and nitrogen, due to either environmental exposure or packaging technology (e.g., modified atmosphere packaging). The impact of gas composition during storage on quality attributes of foods has been investigated, although the impact on B-vitamins is not well defined (Ioannidis et al., 2018). Similarly, pH has been shown to be critical to spoilage reactions and the stability of B-vitamins specifically thiamine and riboflavin. In aqueous solutions of thiamine and riboflavin, both were shown to degrade in neutral/basic conditions, although the impact of pH on B-vitamins during storage of foods are scarce (Ahmad et al., 2004; Voelker et al., 2018).

The purpose of our study is to profile B-vitamin content over the shelf-life period of a vegetable matrix under a variety of storage conditions, to investigate if B-vitamin content can be used as a quality marker of nutritional status and indicator of product freshness alongside microbial assessments. This will further elucidate potential relationship between vitamin composition and microbial growth, while providing novel insights for future quality assessments of foods. The objectives include assessing the effect of temperature (1), oxygen levels (2) and altered pH (3) on B-vitamin composition (targeting thiamine, riboflavin, nicotinamide, and pyridoxine) and microbial growth.

2. Materials and Methods

B-vitamin composition alongside other quality markers were profiled during storage of a single food matrix, a commercially available chilled country vegetable soup, henceforth referred to as the vegetable matrix.

2.1. Materials and Chemicals

The commercially available vegetable matrix (chilled country vegetable soup) was purchased from a local supermarket and ingredients according to packaging include, vegetable stock (water, leeks, carrots, onions, bay leaves and parsley), potatoes (10%), onions (10%), carrots (9%), swede (8%), celery (5%), turnip, leeks (3%), cornflour, butter, salt, parsley, ground black pepper. The 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.

Plate count agar and bacteriological peptone were purchased from Neogen (Lansing, USA). All water-soluble vitamins were acquired from Sigma-Aldrich (Dorset, UK): nicotinamide (98%), (-)-riboflavin (98%), pyridoxine hydrochloride (99%) and thiamine hydrochloride (99%). Methanol (HPLC grade), ammonium acetate (HPLC grade) and sodium chloride 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), citric acid and sodium hydroxide was purchased from Sigma-Aldrich (Dorset, UK). Nitrogen and compressed air cylinders were acquired from BOC (Chester-le-Street, 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.

2.2. Preparation of investigated vegetable matrix

Per storage condition, three cartons (600 g each, 1.8kg in total) of the fresh vegetable matrix (same batch number) were each homogenised in a sterile environment using a blender for 2 minutes. After homogenisation, ~60 g sample was taken and kept on ice for 1 h, classified as time point 0 (T0). This sample was then further sub-sampled in a sterile environment into the following aliquots for analysis of microbial growth (2 g), vitamin composition (15 g) and pH (6 g). Then, 1.4 kg of the remaining matrix was weighed in a 2 L Duran, this allowed sufficient headspace in the storage vessel, followed by exposure in a non-microbiological laboratory for natural contamination for 1 h. After 1 h of natural contamination the matrix was mixed in an orbital incubator (Infors HT Multitron Standard, Surrey, UK) at 170 RPM for 2 minutes 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.

2.3. Storage of vegetable matrix under different temperatures

After preparation of the vegetable matrix as outlined in section 2.2, the matrix was stored in a static incubator (LMS, Kent) at either 7 °C, 20 °C or 37 °C. 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 when stored at 7 °C and 20 °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. At each time point ~60 g samples were taken under aseptic conditions and sub-sampled into aliquots as described in section 2.2. Prior to collecting samples from the food matrix at specified time points, the vegetable matrix was mixed in an orbital incubator (Infors HT Multitron Standard, Surrey, UK) at 170 RPM for 2 minutes.

2.4. Storage of vegetable matrix at a specified pre-set pH

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 2.2. 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. After 1 h of natural contamination, the matrix was stored at 20 °C in a static incubator (LMS, Kent, UK). Samples were collected at the same time points described for storage at 20 °C in section 2.3 and sub-sampled as described in section 2.2.

2.5. Storage of vegetable matrix under absence and presence of oxygen

Alteration 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 2.2, 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. 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 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 a control was conducted by bubbling no gas through the system. Samples (~60 g) were collected via the sampling port using aseptic technique into sterilised universal glass vials at time points described for storage at 20 °C in section 2.3 and sub-sampled as described in section 2.2.

2.6. Total viable count

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 Lima et al. (2011). Samples were taken in duplicate and vortexed for 1 minute, from which 100 mg was transferred to a sterilised Eppendorf tube and vortexed with 900 µL of sterilised peptone physiological saline (PPS) (0.85% NaCl and 0.1% bacteriological peptone). A serial dilution (tenfold) of the sample was then completed with PPS as the diluent. The total viable count (TVC) was assessed on plate count agar using pour plate technique, in which 100 µL of the appropriate dilutions were plated in duplicate. Plate count agar plates were incubated at 30 °C for 72 h before counting and expressed as log CFU per g. The average log CFU per g and associated error expressed as 95% confidence interval was calculated.

2.7. Vitamin analysis

Samples taken throughout the time course for targeted vitamin analysis of thiamine, riboflavin, nicotinamide, and pyridoxine 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. The extraction methodology was followed as we have described previously (Porter & Lodge, 2021), briefly, 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 minutes (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 minutes at 4000 RPM. The supernatant was filtered through 0.2 µm nylon syringe filter into amber vials stored at -80 °C until required for analysis. Extracted samples were analysed using a sensitive LC-MS methodology as we have previously described (Porter & Lodge, 2021).

2.8. pH measurements

Samples taken for pH measurements were measured in triplicate using Mettler Toledo FiveEasy pH meter, by inserting the pH probe (pH electrode LE438, Mettler Toledo, Greifensee, Switzerland) into the matrix aliquot. 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).

2.9. Data analysis

All statistical analysis were conducted in SPSS version 26. 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. Subsequently, if the test showed significance and therefore the null hypothesis was rejected, pairwise comparisons were conducted with Bonferroni corrections. Vitamin concentrations were additionally correlated to TVCs using Spearman's correlation to statistically support potential relationships between both variables.

3. Results

3.1. The effect of temperature on B-vitamin composition and microbial growth

B-vitamins, namely thiamine, riboflavin, nicotinamide, and pyridoxine were profiled during storage of a popular ready-to-eat vegetable matrix under different temperatures. Alongside, the microbial growth was investigated using culture dependent methods. Samples were collected on eight separate time point and the respective vitamin stability depicted as % variability and TVC, expressed as log CFU/g, can be viewed in Figure 1. Storage at 7 °C resulted in minimal changes to the concentration of investigated vitamins over 5-days ($p > 0.05$). Alternatively, increasing storage temperature to 20 °C and 37 °C showed significant changes in the quantity of thiamine ($p = 0.003$ (20 °C), $p = 0.012$ (37 °C)), riboflavin ($p = 0.006$ (20 °C), $p = 0.007$ (37 °C)) and nicotinamide ($p = 0.004$ (20 °C), $p = 0.001$ (37 °C)) over storage. At 20 °C by 48 h, nicotinamide was completely exhausted, while 58.9% and 14.8% of thiamine and riboflavin remained respectively. However, during storage at 37 °C, thiamine, riboflavin and nicotinamide degraded earlier in the time course, with degradation initiating from 24 h compared to 48 h at 20 °C. There were no significant changes in the content of pyridoxine during storage under all investigated temperatures ($p > 0.05$). Complementary to the vitamin profile, the TVC during storage 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 facilitated the growth of microorganisms earlier in the time series; exponential growth occurred between 8-48 h for 20 °C with growth reaching 8.32 log CFU/g at 48 h and between 8-24 h for 37 °C. Spearman's correlation showed a strong significant ($p < 0.05$) negative correlation between TVC and vitamin content at 20 °C and 37 °C for thiamine ($r_s = -.708$ (20 °C & 37 °C)), riboflavin ($r_s = -.927$ (20 °C), $r_s = -.610$ (37 °C)) and nicotinamide ($r_s = -.806$ (20 °C), $r_s = -.839$ (37 °C)). To further highlight correlation, an example figure of vitamin content displayed as % remaining with TVC is shown in Figure 2.

3.2. The effect of gas composition on B-vitamin composition and microbial growth

The impact of storing the vegetable matrix in the absence and presence of oxygen was achieved using a bio-fermenter, flushing the matrix with either nitrogen, or compressed air, or nothing, with the latter referred to as 'no gas'. The stability of B-vitamins and the microbial growth profile in the presence of both nitrogen and compressed air, and no gas can be seen in Figure 3. Presence of nitrogen resulted in significant changes in the concentration(s) of thiamine ($p = 0.008$), riboflavin ($p = 0.010$) and

nicotinamide ($p = 0.006$). The content of riboflavin and nicotinamide was exhausted at 72 h, while thiamine also significantly reduced at 72 h by 46.9%. This was comparable to storage under no gas, in which the concentration of thiamine ($p = 0.001$), riboflavin ($p = 0.027$) and nicotinamide ($p = 0.005$) significantly changed throughout storage, reducing at 72 h. Alternatively, in the presence of compressed air, thiamine ($p = 0.003$) and nicotinamide ($p = 0.014$) 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 concentration of pyridoxine throughout all storage studies investigating the effect of gas composition did not significantly change ($p > 0.05$). The microbial growth profile was almost identical during storage in the presence of nitrogen and under no gas. For both conditions the 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. An increase in the TVC during storage under no gas and nitrogen was significantly ($p < 0.05$) associated with the depletion of riboflavin ($r_s = -.778$ (no gas), $r_s = -.910$ (nitrogen)) and nicotinamide ($r_s = -.845$ (no gas), $r_s = -.780$ (nitrogen)). There is no microbial data for storage of the vegetable matrix in the presence of air, due to this condition promoting the growth of a microorganism that swarmed agar plates, resulting in issues with colony counting.

3.3. The effect of pH on B-vitamin composition and microbial growth

The influence of an acidic and alkaline environment on B-vitamin stability and TVC are shown in Figure 4, compared to a control where the pH was unchanged. Storage in acidic conditions had no significant effect on the concentration of B-vitamins ($p > 0.05$). However, the pre-set alkaline environment promoted significant changes in the concentrations of thiamine ($p = 0.0001$), riboflavin ($p = 0.001$), nicotinamide ($p = 0.0004$) and pyridoxine ($p = 0.0002$). At 48 h, 31% of thiamine remained, while nicotinamide was undetectable and this is comparable to the control. Interestingly, riboflavin and pyridoxine showed a different profile under an alkaline environment. Riboflavin increased by 20% (120 h), while the content of pyridoxine was exhausted (undetected) at 48 h, followed by an increase of 580% at 120 h. 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 from the initial counts, reaching a maximum microbial count of 5.82 log CFU/g. For the alkaline environment, the growth rate increased to 9.06 log CFU/g at 24 h and plateaued to 9.71 log CFU/g (120 h). TVC in the alkaline environment was significantly ($p < 0.05$) correlated with the reduction in nicotinamide ($r_s = -.906$) and thiamine ($r_s = -.857$) concentrations.

3.4. The effect of storage environments on pH of the vegetable matrix

The pH of the matrix was monitored under all storage environments investigated as shown in Figure 5, with the average pH at T0 recorded as pH 5.59 ± 0.12. The pH of the vegetable matrix remained

unchanged throughout storage at 7 °C. However, during storage at 20 °C the pH decreased at 48 h to pH 4.16, comparable to storage at 37 °C, although the pH reduced earlier at 37 °C (24 h). The pH profile under nitrogen and no gas was comparable, reducing at 72 h, with the final average pH recorded as pH 3.82 for nitrogen and pH 3.62 for no gas. In comparison, during storage in air, the pH increased at 72 h to pH 6.48. After the vegetable matrix pH was lowered and subsequently stored, the overall pH was maintained throughout storage. However, when the initial pH of the vegetable matrix was increased to pH 8.6, the alkaline environment was not maintained and at 24 h the pH reduced to pH 6.15, finally reducing to pH 5.17 at 120 h.

4. Discussion

4.1. Impact of storage temperature on quality markers

B-vitamin composition of a popular ready-to-eat vegetable matrix was monitored during storage under different temperatures, a factor that fails to be measured as standard procedure in shelf-life studies. B-vitamins are essential in the diet for their major role in macronutrient metabolism and should be assessed alongside other quality markers (Acevedo-Rocha et al., 2019). In this study, B-vitamin's namely thiamine, riboflavin and nicotinamide were significantly unstable with different stability profiles during storage of the vegetable matrix under different temperatures. The nutritional quality of the vegetable matrix was reduced at 48 h and 24 h of storage at 20 °C and 37 °C respectively. This is the first instance where this has been reported in a vegetable matrix and although the impact of temperature on B-vitamin has been extensively studied, temperatures greater than the ones investigated in this study (50 °C+) have been shown to have a degradation effect on thiamine, riboflavin and pyridoxine (Bui & Small, 2012; Kadakal et al., 2018; Nisha et al., 2005, 2009). During storage at 7 °C (within legal storage temperatures implemented by food business operators (< 8 °C) for chilled foods) there was no significant change in B-vitamins. Interestingly, pyridoxine remained stable under all temperatures, perhaps indicating that the temperatures investigated were not high enough to degrade pyridoxine as previously described by Bui and Small (2012). Nicotinamide is also considered the most stable B-vitamins, resistant to temperature, air and oxidants (Lešková et al., 2006). Although in this study, nicotinamide was completely depleted at 20 °C and 37 °C. These findings appear to be in association with another quality marker investigated, TVC.

The TVC was shown to be temperature dependent, as temperature increased (7 °C, 20 °C, 37 °C) the TVC increased, which was complemented with B-vitamin loss. The lower storage temperature maintained microbial quality for longer (4 days) and did not support microbial growth to unsatisfactory levels, in line with government guidelines (UKHSA, 2024). The preserved microbial integrity of the vegetable matrix at 7 °C consequently maintained B-vitamin quality. Alternatively, at 20 °C and 37 °C the peak TVC was reached at 2 days (8.32 log CFU/ g) and 1 day (7.71 log CFU/g) of storage

respectively, this coincided with the observed reduction in thiamine, riboflavin and nicotinamide. The impact of temperature on TVC is a well characterised observation, as authors have shown increased storage temperature of several food matrices increases bacterial growth, while simultaneously reducing shelf-life (Alvarez et al., 2015; Boziaris et al., 2011; Hoel et al., 2017). However, this is the first report to our knowledge where TVC has been correlated with a reduction in B-vitamin quality. The TVC reached during storage at 20 °C and 37 °C was comparable to other studies that have investigated storage of vegetables. For example, Lee et al. (2011) 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. Increased growth was also associated with deterioration of the organoleptic properties of the vegetable matrix, including development of off odours and gas production. This is in accordance with the accepted knowledge that food spoilage occurs when microbial growth reaches 10^7 -10^9 CFU/g (Gram et al., 2002). TVC was also classified as being at official unsatisfactory levels ($\geq 10^7$ CFU/g) for ready-to-eat soups (UKHSA, 2024). The pH of the vegetable matrix was also assessed and reduced at 20 °C and 37 °C only, in line with peak TVC and loss of B-vitamins. It can be elucidated that this is likely a consequence of by-product production during microbial metabolism (Gänzle, 2015). Therefore, we demonstrated B-vitamins, thiamine, nicotinamide and riboflavin are key indicators of overall quality loss in the vegetable matrix alongside both TVC and pH, which has not been previously described. This shows that B-vitamins can be used as an overall quality marker of nutritional status of foods, but additional investigations would need to be completed to understand if vitamins could be used as early indicators of spoilage.

At present there are limited studies investigating B-vitamins alongside microbial spoilage activity. Although studies are limited, Kaprasob et al. (2018) showed during fermentation of cashew apple juice with 5 types of lactic acid bacteria strains, thiamine significantly decreased by 98.7% at 48 h. Additionally, Kneifel et al. (1992) monitored B-vitamin content across sixteen yogurt starter cultures and observed approximately 80% of riboflavin was reduced during fermentation of buttermilk. B-vitamins are precursors for coenzymes involved in essential metabolic pathways of microorganisms that maintain cell viability and facilitate growth. Some bacteria can biosynthesise B-vitamins *de novo* to meet their growth requirements, however bacteria that lack these genes uptake vitamins from their environment (Acevedo-Rocha et al., 2019; Vogl et al., 2007). Therefore, it can be hypothesised that the reduction in the quantity of thiamine, riboflavin and nicotinamide in this study is potentially due to the microorganisms requiring these exogenous vitamins for growth (Szutowska, 2020). Nicotinamide for example is a precursor for cofactor nicotinamide adenine dinucleotide ($NADH/NAD^+$), essential for its role in numerous metabolic redox reactions (Zapata-Perez et al., 2017). In most bacteria, the enzyme nicotinamidase (EC: 3.5.1.19) is released to convert nicotinamide to nicotinic acid to generate NAD^+ via a salvage pathway (Zapata-Perez et al., 2017). Similarly, riboflavin is a precursor for cofactors flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), involved in redox reactions in

metabolism (LeBlanc et al., 2011). It is accepted that microorganisms' synthesise riboflavin *de novo* from guanosine-5'-triphosphate and ribulose 5-phosphate (Vogl et al., 2007). However, microorganisms that lack the genes involved in the biosynthesis process and require riboflavin for growth, employ a transport system for exogenous riboflavin (Fuentes Flores et al., 2017; Vogl et al., 2007). Therefore, the reduction in vitamin content could be due to their uptake by microorganisms present in the vegetable matrix for growth. However, pyridoxine remained stable over the storage duration. Microorganisms can synthesise the active phosphorylated form of B_6, pyridoxal 5'- phosphate, *de novo* or synthesise the active form via a salvage pathway using other B_6 vitamers (pyridoxal and pydridoxamine) (El Qaidi et al., 2013). Therefore, it can be hypothesised that 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 which replicated the chemical composition of the vegetable matrix. This was completed to understand if temperatures investigated had an impact on vitamin stability (data available in supplementary material). The model buffered system indicated vitamins were unaffected by the storage temperature and therefore affirms the reduction in B-vitamin content of the vegetable matrix was a factor of spoilage reactions as described, or the matrix itself.

4.2. Impact of gas composition on assessed quality markers

The presence and absence of oxygen during storage of the vegetable matrix was assessed and a control (no gas) was analysed alongside. Alteration of gas composition is a favourable storage technique, achieved through packaging technology and used to maintain quality of foods for longer by reducing undesirable gas facilitated reactions (Oliveira et al., 2015). For example, removal of oxygen in vacuum packaging prevents oxidation of lipids, colour pigments and nutrients (Berk, 2018). The impact of gas compositions on quality markers such as microbial growth has been extensively investigated, although due to B-vitamins not being traditionally considered as a quality attribute, the impact of gas alterations on B-vitamins is less understood (Ioannidis et al., 2018). In this study, during storage of the vegetable matrix under both nitrogen and no gas, thiamine, riboflavin and nicotinamide significantly reduced at 72 h. The loss of B-vitamin quality could be correlated with TVC, as microbial growth of the vegetable matrix stored in the presence of nitrogen and no gas were almost identical, with growth reaching spoilage levels between 48-72 h. Therefore, it can be suggested that these B-vitamins have been required for growth by the present microorganisms as suggested during storage under different temperatures (Szutowska, 2020). Interestingly, under the presence of nitrogen, riboflavin was exhausted at 72 h alongside nicotinamide, compared to a 20% decrease in the no gas environment. It is unclear as to why the presence of nitrogen has promoted complete exhaustion of riboflavin, although this cannot be a consequence of oxidation. In contrast, storage of the vegetable matrix in the presence of air had a different impact on B-vitamin composition, nicotinamide and thiamine were the only vitamins that

significantly reduced. Nicotinamide is reportedly robust to oxidation reactions and therefore the reduction in nicotinamide is likely due to microbial activity (Lešková et al., 2006). Alternatively, the loss in thiamine could be due to its vulnerability to oxidation or microbial activity (Bates, 2007; Voelker et al., 2018). However, we were unable to compare nicotinamide and thiamine to TVC, due to a bacterium that swarmed the agar plates when colony counting. Amplicon sequencing targeting the 16S rRNA gene was performed and showed a diverse bacterial community between 0-48 h which included *Leuconostoc*, *Lactococcus*, *Pseudomonas* and *Bacillus*, although by 120 h *Bacillus* was the most abundant bacterial genus responsible for the swarming behaviour (data not shown). Oxidation as previously described is a well-known driver for thiamine degradation, although thiamine was stable until 72 h of storage and continued to degrade until it was undetected at 120 h. This corresponded with an increase in pH at 72 h and abundance of *Bacillus*. A number of *Bacillus spp*. can produce thiaminase, which results in the destruction of thiamine and therefore it can be hypothesised that the degradation of thiamine is due to *Bacillus spp.* activity (Sannino & Angert, 2017).

Previous publications have reported similar findings for TVC when storing food matrices under different atmospheric conditions. For example, Ioannidis et al. (2018) showed during storage of iceberg lettuce under air, equilibrium modified atmosphere (3% O_2) and anaerobic conditions the total psychotropic counts after 10 days were 7.72, 7.67 and 8.26 log CFU/g respectively. Zhang et al. (2013) also were able to show differences in total aerobic counts and total anaerobic counts on fresh-cut honeydew melons stored under different atmospheric conditions. However, in this study we were unable to compare the impact of storage of the vegetable matrix in nitrogen and air on TVC. The pH profile in the presence of nitrogen and no gas reduced at 72 h in line with increased microbial growth, as observed during storage under different temperatures. However, in the presence of air the pH increased, this can be a response of metabolic activity of bacteria. For example, protein hydrolysis via protease activity and amino acid metabolism generates ammonia which would lead to an increase in pH (Allagheny et al., 1996). The increase in pH also coincided with thiamine and nicotinamide loss, further suggesting the impact of microbial activity is linked to vitamin degradation.

4.3. Impact of pH on assessed quality markers

The impact of pH conditions on vitamin stability during storage of a food matrix has not been demonstrated in the literature. The acidic environment had no significant impact on the B-vitamins investigated. Both riboflavin and thiamine are acknowledged as being unstable and sensitive to pH particularly in neutral and alkaline conditions (Ahmad et al., 2004; Voelker et al., 2018). However, riboflavin and thiamine are reportedly more stable when present in their ionised form, promoted by acidic conditions (Ahmad et al., 2004; Voelker et al., 2018). In addition, the acidic environment inhibited microbial growth and TVC did not reach levels associated with spoilage (10^7 -10^9 CFU/g) (Gram et al., 2002). This is due to an inability for the microorganisms to adapt to the acidic environment,

as pH is crucial in microbial metabolism, influencing redox reactions and the activity of intracellular and extracellular enzymes (Jin & Kirk, 2018). Therefore, it can be concluded that in an acidic environment B-vitamins were not affected by spoilage activity, nor the storage condition. Alternatively, the pre-set alkaline environment promoted significant changes to all B-vitamins. Thiamine, nicotinamide and pyridoxine significantly reduced, initiating at 24 h and continued to decrease, with exhaustion of nicotinamide and pyridoxine at 48 h. Alkaline conditions can have a significant impact on thiamine stability, as the thiazole ring opens yielding the thiol form it increases thiamine's liability to degrade (Edwards et al., 2017). It is generally considered that conditions above pH 7.0 can rapidly destroy thiamine and this could have contributed to the observed loss (Yang et al., 2022). Nicotinamide and pyridoxine are also vulnerable to degradation in strong alkaline environments, with nicotinamide converting to nicotinic acid (Altunay, 2021; Je et al., 2025). The pH of the vegetable matrix was not maintained throughout the study and began to reduce at 24 h to pH 6.1, which is likely a response of microbial activity. However, the main period of vitamin loss (24 - 48 h) occurred when the pH of the vegetable matrix was reduced below pH 7.0. Therefore, vitamin loss can be associated with reduction of microbial integrity at 24 h, as growth reached 10^9 CFU/g. This suggests B-vitamins could have been used by microorganisms to support growth and metabolic activity as opposed to vitamin loss being largely the result of pH (Szutowska, 2020). The initial microbial load was able to adapt to the increased pH as opposed to the acidic environment as microorganisms can grow in pH conditions ranging from pH 5.5 to pH 9 while maintaining cytoplasmic pH (Padan et al., 2005). Furthermore, 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 microbial activity such as fermentation, as certain bacteria, particularly lactic acid bacteria have been shown to synthesise pyridoxine (Hamzehlou et al., 2018; LeBlanc et al., 2011). Li et al. (2012) showed during fermentation of soymilk by different probiotic bacteria, increased vitamin B_6 content by 191.3%-354.3% depending on strain. In contrast, riboflavin also significantly increased from 48 h. However, it is widely accepted that most Gram-positive and Gram-negative bacteria have the capability to synthesise riboflavin *de novo* from guanosine-5'-triphosphate and ribulose 5-phosphate (Averianova et al., 2020; LeBlanc et al., 2011).

Furthermore, additional studies are required to determine whether the relationships observed here extend to other food matrices, particularly those with differing macronutrient composition (e.g. high-protein, high-fat) where microbial communities, metabolic activity, and spoilage pathways may differ. In this study, changes in vitamins were correlated to TVC. However, our experimental design did not allow causal attribution of vitamin depletion to microbial uptake. It is important to note that spontaneous chemical spoilage reactions will be occurring simultaneously during storage and this was not accounted for. Further work should therefore aim to separate the different spoilage routes and measure the impact

on vitamin degradation. Additionally, further investigations into the bacterial strains responsible for spoilage could inform kinetic model studies into vitamin degradation.

5. Conclusion

Currently freshness of foods is determined in the food industry using solely microbial and organoleptic markers which fail to capture the nutritional quality of foods. B-vitamins are essential constituents of foods that are overlooked as a quality marker during shelf-life studies, despite their vulnerability to degradation. In this study we have demonstrated that temperature, gas composition and pH during storage of a ready-to-eat vegetable matrix can have significant effects on the stability of thiamine, riboflavin, nicotinamide and pyridoxine. Moreover, the significant changes to B-vitamin composition appear to be in association with microbial growth (TVC) and spoilage activity, which has not been reported previously. In all storage environments where spoilage activity was reduced (7 °C and pre-set acidic environment), the content of B-vitamins was preserved. However, in storage environments where TVC reached official unsatisfactory levels, the content of B-vitamins was significantly affected, although this was environment dependent. Nicotinamide was the only vitamin that was fully exhausted in all storage studies assessed where spoilage occurred. Therefore, B-vitamins particularly nicotinamide could be used in the food industry as a marker of overall product quality for nutritional and shelf-life assessments, which has not been previously described.

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Data availability

Data supporting this study are openly available from Northumbria University's Figshare at

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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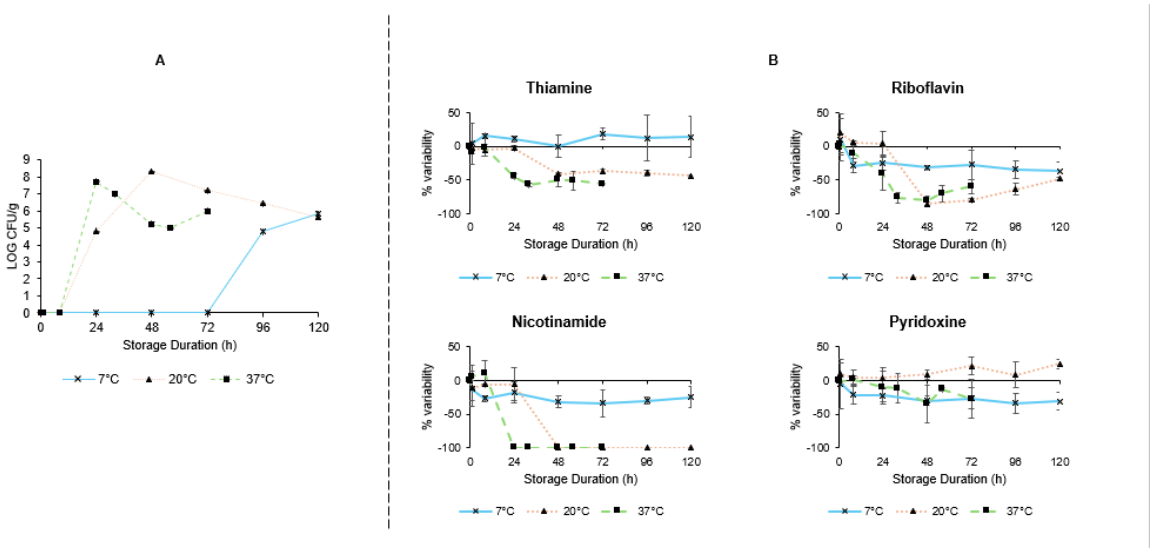

Figure 1: A) TVC during storage (120 h) of the vegetable matrix under different temperatures, 7 °C, 20°C, 37 °C. For each data point, two replicates were analysed, and the error associated with each time point is represented by a 95% confidence interval. **B)** Stability of B-group vitamins, thiamine, riboflavin, nicotinamide, and pyridoxine in the vegetable matrix over 5 days of storage (120 h) under different temperatures, 7 °C, 20°C and 37 °C. Stability is represented as the % variability of each vitamin based on quantity of each vitamin at 0 h. Each data point is an average of three or two analysed samples and associated error is expressed by a 95% confidence interval.

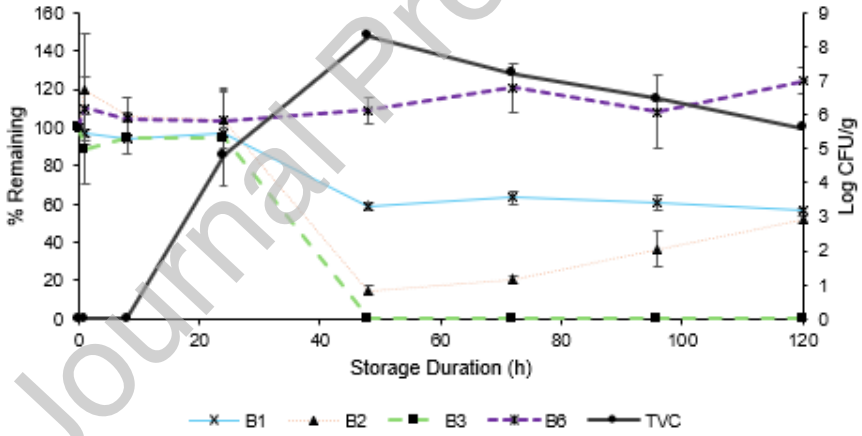

Figure 2: An extension to Figure 1 profiling B-group vitamins, thiamine, riboflavin, nicotinamide, and pyridoxine overlaid with the TVC over 5-days of storage of the vegetable matrix at 20 °C.

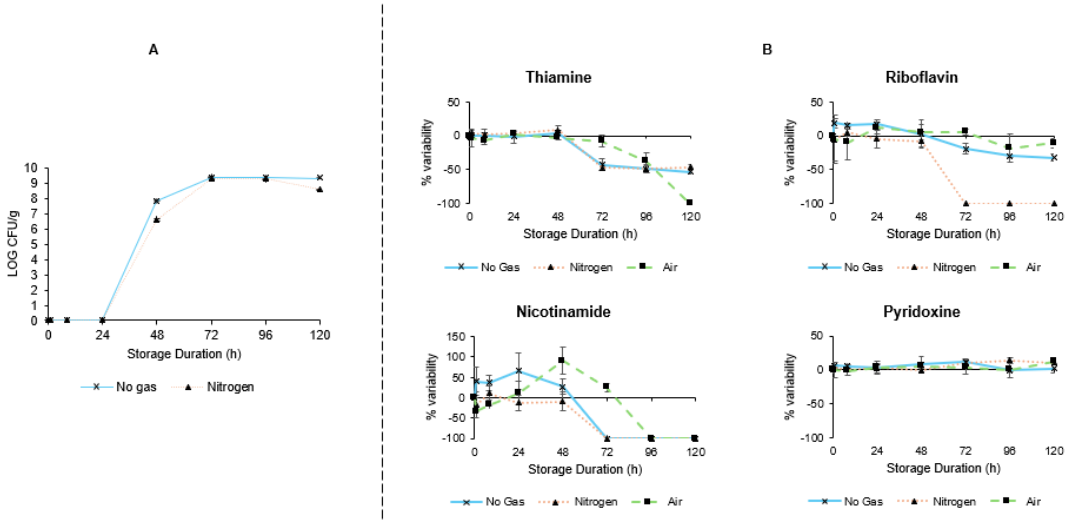

Figure 3: A) TVC during storage (120 h) of the vegetable matrix stored at 20 °C under no gas and in the presence of nitrogen. For each data point duplicate dilution series were performed, of which two replicates were analysed per dilution series (n=4). The error associated with each time point is represented by a 95% confidence interval. **B)** Stability of B-group vitamins thiamine, riboflavin, nicotinamide, and pyridoxine 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 (21% oxygen and 79% nitrogen). Stability is represented as the % variability of each vitamin based on quantity of each vitamin at 0 h. Each data point is an average of three or two analysed samples and associated error is expressed by a 95% confidence interval.

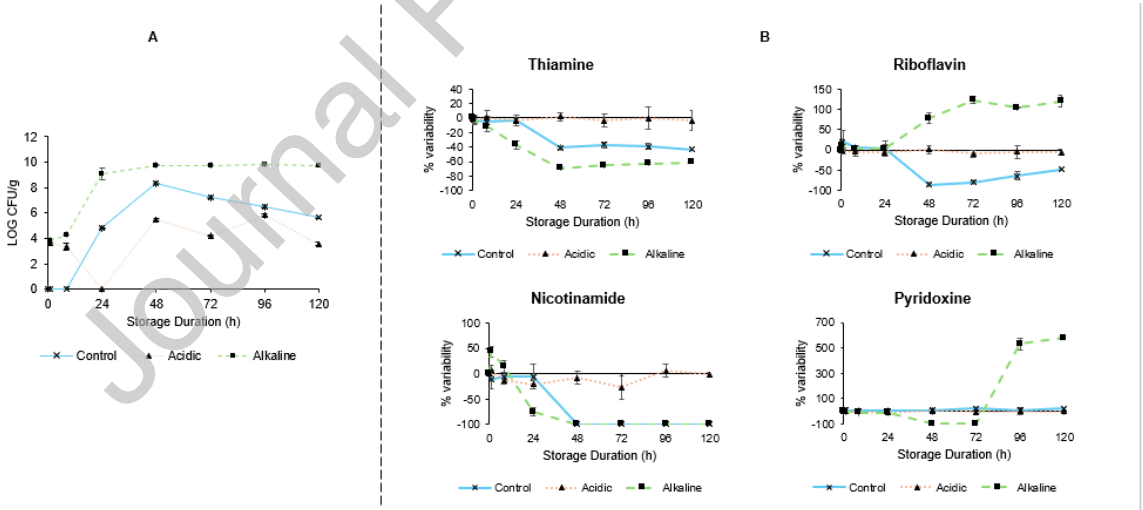

Figure 4: A) TVC during storage (120 h) of the vegetable matrix at 20 °C under pre-set pH conditions, including control (~pH 5.7), acidic environment (~pH 3.6) and alkaline environment (~pH 8.6). For each data point, two replicates were analysed, and the error associated with each time point is represented by a 95% confidence interval. **B)** Stability of B-group vitamins, thiamine, riboflavin, nicotinamide, and pyridoxine in the vegetable matrix over 120 h at 20 °C under pre-set pH conditions. Stability is represented as the % variability of each vitamin based on quantity of each vitamin at 0 h. Each data point is an average of three analysed samples and associated error is expressed by a 95% confidence interval.

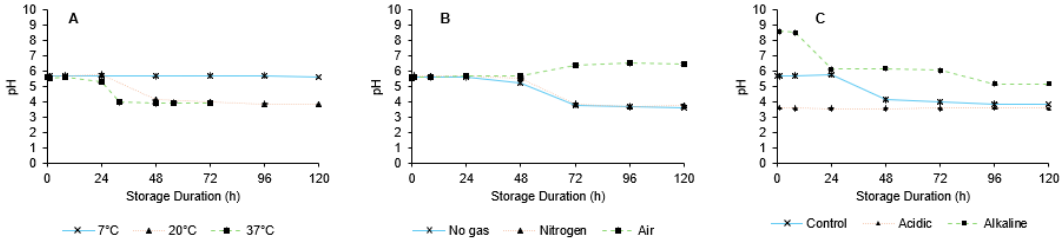

Figure 5: The pH profile of the vegetable matrix during 5-days of storage under A) different temperatures B) no gas and in the presence of nitrogen and air C) pre-set pH conditions. pH was measured in triplicate at each time point and associated error expressed by a 95% confidence interval.

Ethical Statement

No humans or animals were used in this study

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: