Application of Novel Plant Probiotics to Increase the Health Value of Ready-to-Eat Leafy Greens

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Application of Novel Plant Probiotics to Increase the Health Value of Ready-to-Eat Leafy Greens

Identification and Characterization of Probiotic Strains and E. coli via qPCR and MiSeq Sequencing

Master's Thesis

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Abstract

Ready-to-eat (RTE) leafy greens offer an appealing way to integrate leafy greens into meals. Unfortunately, these leafy greens can get contaminated with human pathogens such as *Salmonella*, and Shiga toxin producing *Escherichia coli* (STEC) at any stage along the manufacturing chain, potentially leading to serious foodborne diseases. Currently, there is no single step in the production and processing of RTE leafy vegetables that guarantee the elimination of all potential pathogens, leaving the final product susceptible to contamination. To address this challenge, this master's thesis proposes a novel approach: the direct inoculation of previously evaluated plant probiotics on the day of delivery, known for their antagonistic effects against *E. coli*. The bacterial levels on the day of delivery and the best-before date were analyzed using viable cell count, quantitative polymerase chain reaction (qPCR), and Next Generation Sequencing (NGS).

The findings from the viable cell count revealed a consistent reduction in non-pathogenic *E. coli* for all the plant probiotic strains tested. Particularly, *Bacillus coagulans* LMG P-32205 and *Pseudomonas cedrina* LMG P-32207 showed the most significant effectiveness ($p \leq 0.01$) throughout the shelf-life period. Additionally, qPCR analysis showed that *Pseudomonas cedrina* LMG P-32207 had higher concentrations on the last day of shelf-life compared to the control. Moreover, Illumina MiSeq sequencing revealed a lower relative abundance of pathogenic genera, especially *Escherichia coli*, in the microbiota of the leafy greens. The mixed salad and mixed lettuce samples inoculated with probiotics exhibited a higher relative abundance of Proteobacteria and Bacteroidetes compared to the control group. Over all trials, the top family with the highest relative abundance was *Pseudomonadaceae*.

The α-diversity exhibited variations only among mixed lettuce and mixed salad samples inoculated with probiotics. Higher β-diversity was observed in all mixed salad groups compared to day 1, as a shift to the right. Indicating that inoculation of probiotics and *E. coli* has induced substantial changes in the native microbiota of the mixed salad leaves. The effect of probiotic strains varied between different leafy green products, thus no conclusions could be drawn. Nonetheless, these results encourage the use of bacterial antagonists as part of a global solution to reduce the risk of human pathogens on leafy green vegetables.

Keywords:

Ready-to-eat, Leafy greens, *E. coli*, Plant probiotics, Food outbreaks, qPCR, NGS

Sammanfattning

Färdigskurna bladgrönsaker erbjuder ett tilltalande sätt att inkludera lite grönt i måltider. Tyvärr kan dessa bladgrönsaker bli kontaminerade med humana patogener som *Salmonella* och Shiga toxin producing *Escherichia coli* (STEC) i tillverkningskedjan, vilket kan leda till allvarliga livsmedelsburna sjukdomar. För närvarande finns det ingen enskild åtgärd I produktionen eller bearbetningen av de färdigskurna bladgrönsakerna som garanterar eliminering av potentiella patogener. För att tackla denna utmaning föreslår denna masteravhandling en ny metod: applicering av tidigare utvärderade växtprobiotika kända för sina antagonistiska effekter mot *E. coli* på bladgrönsaker. Bakterienivåerna på leveransdagen och bäst-före-datumet analyserades med hjälp av cellviabilitet, quantitative polymerase chain reaction (qPCR) och Next Generation Sequencing (NGS).

Resultaten från odlingen visade en konsekvent minskning av *E. coli* för alla testade växtprobiotiska stammar. Särskilt *Bacillus coagulans* LMG P-32205 och *Pseudomonas cedrina* LMG P-32207 visade den mest betydande effekten ($p \leq 0.01$) under hela hållbarhetsperioden. Dessutom visade qPCR-analys att *Pseudomonas cedrina* LMG P-32207 återfanns i högre koncentrationer på sista dagen av hållbarhetstiden jämfört med kontrollen. Vidare visade Illumina MiSeq-sekvensering en lägre relativ förekomst av patogener, särskilt *Escherichia coli*, i mikrobiotan hos de färdigskurna bladgrönsakerna. Prov av blandad sallad och blandad isbergsmix med tillsatta probiotika visade en högre relativ förekomst av Proteobacteria och Bacteroidetes jämfört med kontrollgruppen. Totalt sett var den mest förekommande familjen *Pseudomonadaceae*.

α-diversiteten, beräknad med Shannon diversitetsindex, uppvisade variationer endast blandad sallad och blandad isbergsmix inokulerade med probiotika. Högre β-diversitet observerades i alla grupper med blandad sallad jämfört med dag 1 (kontroll), som en förskjutning till höger. Detta tyder på att inokulering av probiotika och *E. coli* har inducerat betydande förändringar i den naturliga mikrobiotan på salladsbladen. Dessa resultat uppmuntrar till användningen av bakteriella antagonister som en del av en global lösning för att minska risken för humana patogener på färdigskurna bladgrönsaker.

Nyckelord:

Färdigskurna bladgrönsaker, *E. coli*, Växtprobiotika, Livsmedelsburna sjukdomar, qPCR, NGS

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Lund, May 2024
Rumathi de Mel

Contents

List of Abbreviations

ANOVA Analysis of variance
ASV Amplicon Sequence Variant

Ct Cycle thresholds

EHEC Enterohaemorrhagic *Escherichia coli*

FDA Food and Drug Administration

GMP Good Manufacturing Procedures

MAP Modified Atmosphere Packaging

NGS Next Generation Sequencing

OD Optical Density

PBS Phosphate-Buffered Saline
PCoA Principal Coordinate Analysis

QMRA Quantitative Microbial Risk Assessment
qPCR quantitative Polymerase Chain Reaction

RTE Ready-to-Eat

STEC Shiga toxin producing *Escherichia coli*

TSA Tryptic Soy Agar
TSB Tryptic Soy Broth

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1 Introduction

In today's world, there is an increasing interest in living a healthy lifestyle in order to minimize the adverse effects of modern stressful habits. This has led to an increased demand for nutritional products that can be cooked quickly and easily. As a result, there has been a significant increase in the Ready-to-Eat (RTE) food sector throughout Europe [1], growing from 600,000 bags in 2005 to nearly 40 million bags in 2016 [2].

Leafy green vegetables are essential for a balanced diet due to their high vitamin, mineral, and dietary fiber content [3]. RTE leafy greens are an appealing approach to integrating these healthy greens into meals since they are pre-washed and conveniently packaged in a sealed polymeric film [4]. According to European guidelines [5], the average shelf-life of pre-cut leafy vegetables is approximately seven to eight days. However, in the US guidelines [6], the shelf-life is approximately 12-16 days.

Unfortunately, the RTE leafy greens can be a vehicle for human pathogens that may cause serious health consequences because of potential contamination from various sources, such as soil, fertilizers, irrigation water, wildlife, domestic animals, harvesting equipment, manual handling, rinsing water, and processing equipment. Once contaminated, the removal of contaminants is challenging [7].

Currently, Quantitative Microbial Risk Assessment (QMRA), is a useful approach for estimating the exposure and/or risk associated with enteric human pathogens in fresh fruits and vegetables delivered through specific distribution systems such as processing, transportation, and distribution [8]. However, given their susceptibility to contamination, leafy greens continue to serve as vectors for human pathogens such as *Salmonella enterica*, *Listeria monocytogenes*, Shiga toxin producing *Escherichia coli* (STEC), and Enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 [9]. While these high-profile pathogens draw significant attention, it is worth noting that leafy greens naturally contain bacteria known for their opportunistic pathogenicity and antibiotic-resistance genes. A comprehensive understanding of the survival and growth of potential pathogens originating from these products is crucial for effective risk assessment [10] [11].

There is a strong need for novel approaches to improve the microbial hygiene of leafy greens that may successfully supplement existing techniques [12]. Using antagonistic bacteria to combat pathogens is a promising strategy known as biological control. Research suggests that background microbiota could play a significant role in determining the outcome of invading pathogens [13].

1.1 Problem

In recent years, the global production and commercialization of RTE fresh vegetables has increased, creating significant food safety problems due to their consumption in raw form and often without washing. Fresh vegetables, particularly leafy greens, have been identified as possible carriers of foodborne diseases. In countries like Sweden, where the climate is cold, relying on imported fresh vegetables introduces additional risks, as sanitary conditions during growth and processing in other parts of the world may be poor.

Leafy vegetables can get contaminated with human pathogens at any stage along the manufacturing chain. Furthermore, poor hygiene among field workers, as well as inadequate sanitation of harvest can all lead to contamination. Postharvest contamination during washing may aggravate the danger.

Currently, there is no single step in the production and processing of RTE leafy vegetables that guarantees the elimination of all potential pathogens, leaving the final product susceptible to contamination.

1.2 Purpose

To address this challenge, this master's thesis proposes a novel approach: the direct inoculation of previously evaluated plant-probiotic strains into the packaged produce, on the first day of shelf-life. These strains are known for their antagonistic effects against *E. coli*. This approach reduces the risk of pathogenic bacterial survival inside the packaged produce and increases the health value of leafy greens.

1.3 Aims

The overall aim of this study is to reduce the risk of contamination from human pathogens on leafy greens and increase the health value by increasing microbial diversity, thereby delaying the deterioration process.

To generate results that meet the purpose, specific aims were defined:

1. Measure the viability of antagonistic probiotic strains in packaged produce after a foliar spray of probiotic bacteria.
2. Evaluate the antagonistic effect of probiotic strains by stimulating *E. coli* contamination inside the packaged product.
3. Evaluate microbial community changes in packaged produce.

1.4 Research Methodology

This project employed a combination of molecular and microbiological techniques to achieve its objectives. Quantitative Polymerase Chain Reaction (qPCR) was performed for the detection of probiotic strains, to ensure their survival within the salad leaves environment during the entire shelf-life period, and Illumina MiSeq sequencing was performed to study the microbial community changes in packaged products during the shelf-life. Additionally, viable cell count was employed to assess the antagonistic activity of probiotic strains against non-pathogenic *E. coli*.

1.5 Delimitations and Assumptions

The research for this thesis included the following boundaries and presumptions:

- Each letter or number represents: 4: *Pseudomonas punonensis* LMG P-32204; 5: *Bacillus coagulans* LMG P-32205; 6: *Bacillus coagulans* LMG P-32206; 7: *Pseudomonas cedrina* LMG P-32207; C: Control; M: Mix of all strains; E: non-pathogenic *E. coli* CCUG 29300
- The culture medium and all equipment used in the experiment are sterile.
- Bacterial strains 4 and 7 can grow on the tryptic soy medium and 5 and 6 on the glucose yeast extract medium.
- 24 hours is required to obtain a high concentration in bacterial cultures.
- 30°C is the optimal temperature for strains 4 and 7, 48°C for strains 5 and 6, and 37°C for *E. coli*. Bacteria exhibit exponential growth under optimal culture conditions.
- The samples are fresh and were not contaminated during transport from the store to the laboratory.
- Samples have been stored under appropriate conditions before reaching the lab.
- The primers used in the qPCR assay specifically amplify the target sequence and do not cross-react with non-target sequences.
- The qPCR instrument is properly calibrated and provides accurate measurements of fluorescence signals and Cycle thresholds (Ct).
- The reagents, consumables, and laboratory equipment are free of contamination to prevent false-positive results.
- The base calling process in Illumina MiSeq sequencing accurately identifies the nucleotide sequence of each cluster during sequencing.

1.6 Structure of the Thesis

Following the introduction, the thesis continues with Chapter 2 which presents the theoretical background about the global production and trade of RTE leafy greens, potential pathogens, foodborne outbreaks associated with fresh produce, contamination through the production chain, and the current state of research in the field. Chapter 3 presents the methods used to answer the research questions and collect data. Chapter 4 presents the results obtained, and Chapter 5 presents the discussion of the analyzed results. Finally, Chapter 6 presents the conclusions, limitations, and future work that can be drawn from the thesis. Supplementary data are presented in the appendix.

2 Theoretical background

2.1 Global production and trade of RTE leafy greens

In Western diets, leafy green vegetables are often consumed fresh and raw, without any intermediate stages in the production chain that could effectively eliminate potentially harmful microorganisms, from seed to consumption. There are different types of edible leaves, such as cabbages, chicory, leafy herbs (e.g., cilantro, basil, parsley), baby spinach, rocket, and lettuce [14]. These leafy greens are rich in dietary fiber, vitamin C, pro-vitamin A carotenoids, folate, manganese, and vitamin K, contributing to overall health benefits such as reducing the risk of cardiovascular diseases and certain cancers [15].

From 2010 to 2022, the global production per annum of lettuce and chicory grew from 24 million metric tons to 27 million metric tons and spinach production grew from 20 million metric tons to 33 million metric tons, and it is expected to continue to rise in the future.

In Sweden, the harvest area and production of leafy vegetables such as lettuce and chicory have increased by 222% [16]. A major retail chain in Sweden noted a significant rise in sales for RTE, prepacked salad mixes, growing from 600,000 bags in 2005 to nearly 40 million bags in 2016 [17].

2.2 Bacterial communities associated with leafy greens

The "phyllosphere" refers to the habitat present on the surface of plant leaves, hosting a diverse community of bacteria, yeasts, and fungi [18]. This habitat accommodates epiphytes, plant-pathogenic bacteria, and human pathogens. In a study conducted by Uhlig et al. (2017) [19], the bacterial communities of rocket (*Eruca vesicaria*) and spinach *(Spinacia oleracea)* and mixed leafy greens were analyzed using Next Generation Sequencing (NGS) (*Figure 2.1)*. The study found that during shelf-life, the most common phyllosphere microbiota in the

leafy green samples were Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria. Other studies [20, 21] also support that leafy greens consist of these phyla during shelf-life. The most common genera found were *Shewanellaceae* (13.0%) and *Oxalo bacteriaceae* (11.4%) on mixed leafy greens, *Xanthomonadaceae* (7.4%), *Sphingobacteriaceae* (6.3%) and *Enterobacteriaceae* (5.7%) on rocket and *Listeriaceae* (2.4%) and *Erwiniaceae* on spinach.

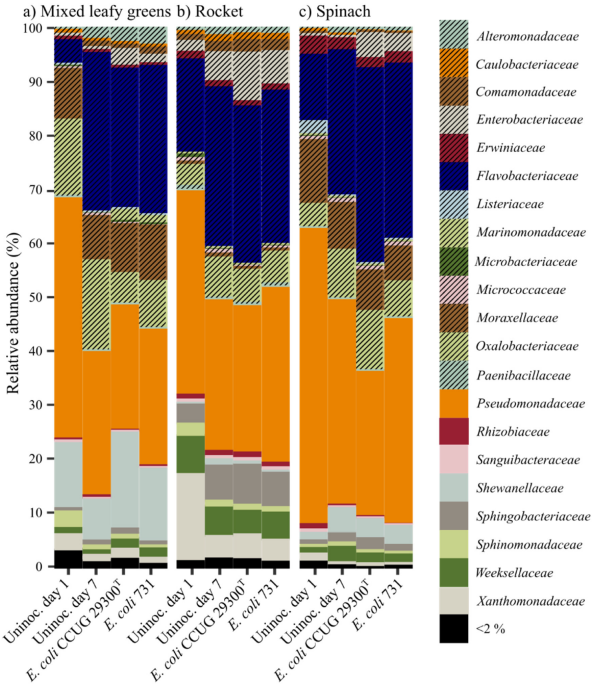

Figure 2.1: Relative abundances of phyla in the rocket, spinach, and mixed leafy green samples during shelf-life. Source: Uhlig et al. (2017) [19]

2.3 Foodborne illness

2.3.1 Microbial hazards

Fresh produce has a natural epiphytic microflora during harvest, the majority of them being non-pathogenic. Throughout the farm-to-consumer spectrum, including cultivation, harvest, processing, packaging, transport, handling, and purchase, there is a risk of microbial contamination from a variety of sources, including environmental, animal, and human origin.

This increases the danger of introducing pathogenic microorganisms [22]. The 2023 FAO/WHO meeting report lists the infections most usually associated with fresh fruits and vegetables, including leafy greens, which have been recognized as vectors of microbial foodborne diseases worldwide [23]. *Table 2.1* lists the microbiological agents, as well as the green vegetables, herbs, and their products that have been linked to outbreaks.

Table 2.1: Microbial hazards associated with leafy vegetables and fruits. Source: 2023 FAO/WHO meeting report [23]

Foodborne pathogen	**Produce**	**Stage**
E. coli O157:H7	Leafy greens	Farm-to-fork
Salmonella enterica, Listeria monocytogenes	Leafy greens	Processing plant to arrival in food service
Salmonella, Campylobacter and E. coli O157	Minimally processed mixed salads	Post harvest to consumption
Salmonella, Listeria monocytogenes	RTE vegetables	Processed product to consumption
Salmonella	Lettuce/leafy greens	Farm-to-fork
Salmonella	Lettuce, cabbage and cucumber	Exposure
Cryptosporidium, Giardia	Tomatoes, bell peppers, cucumbers and lettuce	Exposure

2.3.2 Outbreaks related to leafy greens

A large number of outbreak cases are reported each year involving leafy green vegetables/herbs because of the wide geographic distribution of the contaminated produce and high consumer exposure (*Table 2.2*). The percentage of foodborne illness outbreaks related to leafy vegetables and herbs varies by country [23]. According to the EFSA report [24], in 2021, 14 Member States (Austria, Belgium, Denmark, Finland, France, Germany, Hungary, Italy, Lithuania, Luxembourg, the Netherlands, Poland, Spain, and Sweden) and two non-member States (Switzerland and Norway) reported foodborne outbreaks linked to the consumption of non-animal foods. According to the statistics, the number of foodborne illness outbreaks connected to non-animal-origin foods has doubled since 2020 (45 Vs 23). *Salmonella* was the main cause, accounting for 11 outbreaks, followed by STEC, which caused 4 outbreaks (*Table 2.2)*.

Other microbes involved in outbreaks included *Yersinia enterocolitica, Clostridium botulinum, Staphylococcus aureus*, and bacterial toxins. Viruses and parasites also played a role in outbreaks affecting non-animal foods. RTE fruits and vegetables have been linked to 5 foodborne outbreaks, one of which was fairly large (728 cases) and connected with *Salmonella Typhimurium*. Notable incidents included 'Alfalfa sprouts' related to *Salmonella Coeln* in Sweden, 'Galia melons' imported from Honduras associated with *Salmonella Braenderup* in many countries, and berries and small fruits linked to norovirus in Switzerland.

Table 2.2: Examples of foodborne illness outbreaks caused by fresh fruits and vegetables contaminated with bacterial, parasitic, and viral microbiological hazards. Source: 2023 FAO/WHO meeting report [23]

Hazard	**Commodity**	**Year**	**Country**
BACTERIA			
Salmonella spp.	baby spinach	2007	Denmark
Salmonella spp.	Cucumber used in RTE products	2018	UK
STEC	Leafy greens	2018	USA
STEC	Salad leaves	2016	UK
Yersinia enterocolitica	RTE Fruits and vegetables	2011	Norway
Yersinia enterocolitica	Leafy greens	2011	Sweden and Denmark
Shigella spp.	Basil	2011	Norway
Campylobacter spp.	Fruits – unspecified	2012	USA
PARASITE			
Giardia duodenalis	Leafy greens	2007	USA
Cryptosporidium spp.	Spinach drink	2019	Sweden
VIRUSES			
Calicivirus	Mixed salad	2020	Sweden
Hepatitis virus	Frozen strawberries	2018	Austria

2.4 Applications to assure quality and safety of fresh produce

To reduce consumer risks, it is essential to employ control measures that prevent contamination, eliminate microbial hazards, or inhibit the growth of human pathogens at various stages: production, harvesting, post-harvest handling, processing, and distribution. However, selecting such steps to ensure the safety of leafy vegetables faces challenges.

2.4.1 Pre-harvest applications

The primary origins of microbiological contaminants with potential hazards in the production environment are recognized to be soil and irrigation water. Using physical or biological techniques to treat soil additives, such as animal fertilizers, has been shown to efficiently eliminate a wide range of potential foodborne pathogens that would otherwise be transferred to growing crops. According to Chukwu et al. (2022) [25], composting has been demonstrated to decrease the spread of *E. coli* from modified soil to growing lettuce plants. Physical barriers can also be used to create additional control by keeping soil away from the edible parts of a developing plant.

Efforts have been made to create treatments that eliminate microbial risks for growing plants by using chemical or biological agents. Nonetheless, it has been demonstrated that several treatments, including the spraying of antimicrobial agents one day before harvest, affect the prevalence of *E. coli* O157:H7 and *Salmonella* on lettuce, spinach, and cabbage. The effectiveness of this treatment varied depending on the type of leafy green involved [26]. Spinach plants treated with *Bacillus* spp. showed a reduction in *Salmonella* by 1 log [27], while electrostatically applied lactic acid bacteria within the first 4 weeks of the growing cycle demonstrated a nearly 3-log reduction in *E. coli* O157:H7 [28].

2.4.2 Post-harvest applications for prolonging RTE leafy greens quality and safety during storage

Edible Films and Coatings

Edible films are often effective moisture barriers, reducing the passage of moisture between freshly cut leaves and the surrounding environment. This helps to reduce microbial development, weight loss, textural changes, and undesirable chemical or enzymatic responses. Furthermore, these coatings generate an environment that inhibits respiration and deterioration in freshly cut leaves.

Edible coatings can be applied using a variety of techniques, including dipping, spraying, and brushing. Spraying is ideal for coating solutions with low viscosity because it allows the solution to be evenly applied to the product. Brushing includes applying the coating solution to the product's surface with a brush. However, managing variables such as the amount of solution remaining in the brush can be difficult and may disrupt the process.

While multiple edible coatings and films have been successfully applied on fresh-cut leaves, this process may have a negative impact on the end product's quality. A thick coating on the product's surface may prevent the exchange of gases (CO_2 and O_2) and lower the product's quality. As a result, it is critical to investigate cost-effective materials and employ highly efficient manufacturing and application techniques. Another disadvantage of edible coatings is the need for regulation and label declarations to ensure that the materials and additional substances are non-toxic, food-grade, and meet the highest hygiene requirements.

Modified Atmosphere Packaging (MAP)

MAP involves removing air from inside the package and replacing it with a specific gas or gas mixture (such as N_2, CO_2, and O_2). This method requires an airtight environment to maintain the desired gas concentrations during the required exposure time.

However, MAP packaging also has its limitations. While it efficiently reduces the growth of spoilage microorganisms it may not decrease the growth of some harmful bacteria. MAP is frequently paired with other preservation techniques, such as refrigeration, to assure food safety. Furthermore, once these MAP-packaged foods are opened, they have a normal shelf-life.

Biopreservation

Biological control, often known as biopreservation, is the process of inhibiting hazardous organisms by using beneficial ones called antagonists. Within a microbial ecosystem, the continual battle for life motivates the creation of methods to combat other species [29]. These methods might include the ability to bind essential nutrients like iron, the production of antimicrobial compounds like acids or antibiotics, or competition for physical space. These techniques have been used to improve safety and shelf-life, especially in food fermentation. A more modern strategy is to discover antagonistic microorganisms that can be applied to inhibit or reduce the presence of pathogens [30].

Live bacteria originating from the natural microbiota of fresh vegetables have demonstrated the ability to impact the survival of human pathogens. In a study by Liao and Fett (2001) [31], strains derived from fresh produce were observed to inhibit the growth of *Salmonella chester* and *Listeria monocytogenes* on green pepper discs. Uhlig et al. (2021) [32], saw a reduction in *E. coli* for *Pseudomonas cedrina* LMG P-32207 and *Pseudomonas punonenis* LMG P-32204 when the seeds developed into plants.

The bacterial strains employed in this project can serve as biocontrol agents, having been identified as non-pathogenic to both humans and plants. The tests have been performed to determine the efficacy of a method for transferring antagonistic bacteria to leafy green salad mix packages and to evaluate its effects. This information is crucial for assessing the potential for commercial use.

2.5 EU Guidelines and regulations

In May 2017, the European Commission published recommendations for addressing microbiological hazards connected with fresh fruits and vegetables throughout primary production, with a focus on proper hygiene measures [33]. A risk matrix was developed to help producers identify the hazards associated with irrigation water. This matrix considers the source of water supply, the irrigation method used, and whether the food is consumed raw or not (*Figure* 2.2).

Depending on these parameters, different sample rates are recommended:

1. High risk - monthly
2. Medium risk - twice a year
3. Low risk - once a year

Threshold values for *E. coli* bacteria (varying from 100 to 10,000 CFU/100 mL) are given to help evaluate acceptable water quality for specific processes [34]. The guidelines also specify water used within 48 hours of harvest for fresh fruits and vegetables likely to be consumed raw should fulfill drinking water quality criteria (*Figure* 2.2).

Figure 2.2: Threshold values for the microbiological quality of irrigation water in the cultivation of fresh fruits and vegetables likely to be consumed without cooking up to two weeks before harvest. Thereafter, drinking water quality should be used if possible. Source: European Commission, 2017 [33]

Use	Water source						Indicator
	Untreated surface water [1]	Groundwater [1]	Untreated rainwater	Processed sewage	Disinfected water	Communal water	*E. coli* (CFU/100 ml)
Water comes into direct contact with the edible part	x	x	▪	•	•	◊	100
Water does not comes into direct contact with the edible part	x	x	▪	•	•	◊	1000

[1] Surface water and groundwater can be of good microbiological quality and meet the limit value of 100 CFU/100 ml without treatment. Deep-drilled wells generally meet drinking water quality.

x = Should not be used; ▪ = Can be used but subject to sampling. The grower should perform tests at a medium frequency, with threshold values for *E.coli*; • = Can be used but subject to sampling. The grower should perform tests at low frequency, with threshold values for *E.coli*;◊ = Can be used without any sampling

2.5.1 Swedish guidelines for assessing water quality

In Sweden, there are no specific regulations controlling the quality of irrigation water. However, standards exist to examine other forms of water. The Livsmedelsverkets föreskrifter (Swedish Food Agency) regulates drinking water quality [35], whereas the Havs och vattenmyndighetens föreskrifter och allmänna råd (Norwegian Maritime and Water Authority) [36] is in charge of bathing water quality, along with general guidance. *Figure* 2.3 shows the limit

values for microorganisms in different water types, as well as recommendations from the Livsmedelsverkets föreskrifter for individual drinking water [37]. Although the guidance includes guideline values for microorganisms, it is unclear which criteria the various certifying bodies use.

Figure 2.3: Limit values for microbiological quality of different types of water according to regulations and guidelines in Sweden. Source: Livsmedelsverket 2015 [33]

Type of water	**Number of microorganisms (cfu/ml)**	**Coliform bacteria (cfu/100ml)**	***E.coli* (cfu/100 mL)**	**Intestinal enterococcus (cfu/100ml)**	***Pseudomonas aeruginosa***
Drinking water (SLVFS 2001:30)		< 10[1]	Proven[1]	Proven[1]	
Coastal waters (HVMFS 2012:14)			< 250[2]	< 100[2]	
Inland waters (HVMFS 2012:14)	< 100		< 500[2]	< 200[2]	
Swimming pools (FOHMFS 2014:12)	< 100				Not detected in 100 ml

[1] Limit value for unserviceable water; [2] Excellent quality based on a 95% rating

2.5.2 Quality assurance for fruits and vegetables

Global GAP is a globally recognized benchmark for good agricultural practice. This is a third-party certification, indicating regulation by recognized and independent control bodies (ISO/IEC). The crop production standard includes control points and compliance criteria for quality-assured fruit and vegetable production. Control points include supply of water, water quality, and irrigation frequency. However, the standard does not include criteria for the hygienic quality of irrigation water.

2.5.3 Storage conditions

Temperature control and sufficient cold chain conditions are critical for food safety because temperature impacts bacterial growth and survival [38]. Storage temperatures in retail stores differ by country. In Sweden, storage temperature regulations are not enforced by law; instead, food manufacturers establish the appropriate storage temperature for their products. However, under EU Regulation No. 1169/2011 on food information supplied to consumers, a product's storage temperature must be mentioned on the package label if it requires refrigeration. Food business operators are responsible for ensuring that their products are safe and meet food safety requirements throughout their shelf-life, taking into account predicted circumstances throughout distribution, storage, and use [24].

In the Nordic countries, the recommended storage temperature for refrigerated products ranges from 3 to 8°C, with Sweden having the highest value. Although there are no laws regulating storage temperature, the Livsmedelsverkets website suggests a maximum storage temperature of 8°C for many refrigerated items. However, to extend the freshness of refrigerated items and reduce food waste, customers should store them at 4 to 5°C. The Food and Drug Administration (FDA) recommends keeping RTE green vegetables below 5°C. In Sweden, the recommended storage temperature for RTE leafy greens is generally specified on the product label, usually as a maximum of 4°C, however, certain brands may specify 5 or 8°C [33].

According to research conducted by The Nordic Council aimed at reducing food waste, decreasing the storage temperature would lead to improved food quality and safety. For example, reducing the temperature from 8 to 4°C extends the shelf-life by approximately one week. Moreover, the growth rate of *L. monocytogenes* is significantly slower at 4°C compared to 8°C [39].

2.6 Consumer and food handler behaviour

Consumers usually avoid washing RTE vegetables as they are pre-washed. However, these vegetables may still be infected with human pathogens from manure or irrigation water. A group of North American experts specialized in the microbiological safety of RTE fruits and vegetables discovered that RTE leafy greens manufactured using Good Manufacturing Procedures (GMP) do not require further washing before consumption [8]. If harmful microorganisms survive commercial washing, they are likely to resist removal or inactivation by further washing. This finding is supported by a study by Uhlig et al. in 2017 [40], which showed that consumer tap water washing methods are inefficient in removing bacteria from RTE lettuce. Even after washing, the lettuce still contained high levels of bacteria that could pose health risks under certain conditions and at high doses.

3 Methodology

All experiments have been performed under sterile conditions.

Each letter or number represents:
4: *Pseudomonas punonensis* LMG P-32204; **5**: *Bacillus coagulans* LMG P-32205; **6**: *Bacillus coagulans* LMG P-32206; **7**: *Pseudomonas cedrina* LMG P-32207; **C**: Control; **M**: Mix of all strains; **E**: non-pathogenic *E. coli* CCUG 29300

The study follows three independent trials, two involving (Trial 2 and 3) the same product in different batches and one involving a different product in a different batch (Trial 1):

- **Trial 1**: Analysis of the microbiota of mixed lettuce inoculated with probiotic strains (**4, 5, 6, 7, C, M**).
- **Trial 2**: Analysis of the microbiota of mixed salad leaves inoculated with probiotic strains (**4, 5, 6, 7, C, M**).
- **Trial 3**: Analysis of the microbiota of mixed salad leaves inoculated with non-pathogenic *E. coli* (**E**) and probiotic strains (**4, 5, 6, 7, C, M**).

3.1 Preparation for the inoculation

3.1.1 Preparation of probiotic cultures

Bacterial strains **4** and **7** were streak-plated onto Tryptic Soy Agar (TSA) (Millipore, MA, USA) and incubated (Termaks TS8000, Sweden) for 24 hours at 30°C. *Appendix A.1*

Bacterial strains **5** and **6** were streak-plated onto glucose yeast extract agar [41] and incubated (Termaks TS8000, Sweden) for 24 hours at 48°C. *Appendix A.1.2*

On the next day, single colonies were selected, **4** and **7** were inoculated into Tryptic Soy Broth (TSB) (Sigma-Aldrich), and **5** and **6** were inoculated into glucose yeast extract broths and incubated in a shaking incubator under optimal conditions.

3.1.2 Preparation of *E. coli* cultures

Non-pathogenic *E. coli* CCUG 29300 strain (culture stored at -80°C in Hogness' freezing media) [42] was streak-plated onto Brilliance™ *E. coli* agar (Oxoid Ltd, Hampshire, England) and incubated (Termaks TS8000, Sweden) for 24 hours at 37°C. On the next day, single colonies were selected and transferred into 5 mL TSB (Fluka, Missouri, USA) and incubated for 24 hours at 37°C.

3.2 Bacteria inoculation

Packages (á 65–70 g) containing mixed lettuce (**Trial 1**) of friseé (*Cichorium endivia* var. crispum), red lettuce (*Lactuca sativa* var. crispa), iceberg lettuce (*Lactuca sativa* var. capitata), romaine lettuce (*Lactuca sativa* var. longifolia) and packages (á 65–70 g) containing mixed salad leaves (**Trial 2** and **3**) of baby spinach (*Spinacia oleracea*), rocket (*Eruca vesicaria*), mâche (*Valerianella locusta*), and mangold (*Beta vulgaris*) were purchased at a supermarket in Lund, Sweden, in February 2024 and brought directly to the laboratory. For each trial, 18 packages were bought on the day of delivery (the first day of the shelf-life) and stored at 4°C until the best-before date.

Three of the packages from each product were opened on the first day to extract DNA and check for the native microbiota present, and the packages were then resealed and stored at 4°C with the rest of the packages (*Figure 3.1*).

3.2.1 Probiotic inoculation

300 μL of diluted probiotics (**4, 5, 6, 7, M, C**) were injected into each bag to generate $\log_{10}$ 8 CFU/mL concentration and shaken carefully for even distribution (*Figure 3.1*). The diluted concentration was prepared by measuring the Optical Density (OD) at 520 nm (determined by colony count and spectrophotometry (Novaspec II, Pharmacia, Sweden)). Each strain was injected in triplicate. All the samples were kept at 4°C until their best-before date. (*Appendix A.3*)

3.2.2 *E. coli* + probiotic inoculation

300 μL of diluted probiotics were injected into each bag to generate $\log_{10}$ 8 CFU/mL together with 300 μL of *E. coli* to generate $\log_{10}$ 6 CFU/mL concentration and shaken carefully for even distribution.

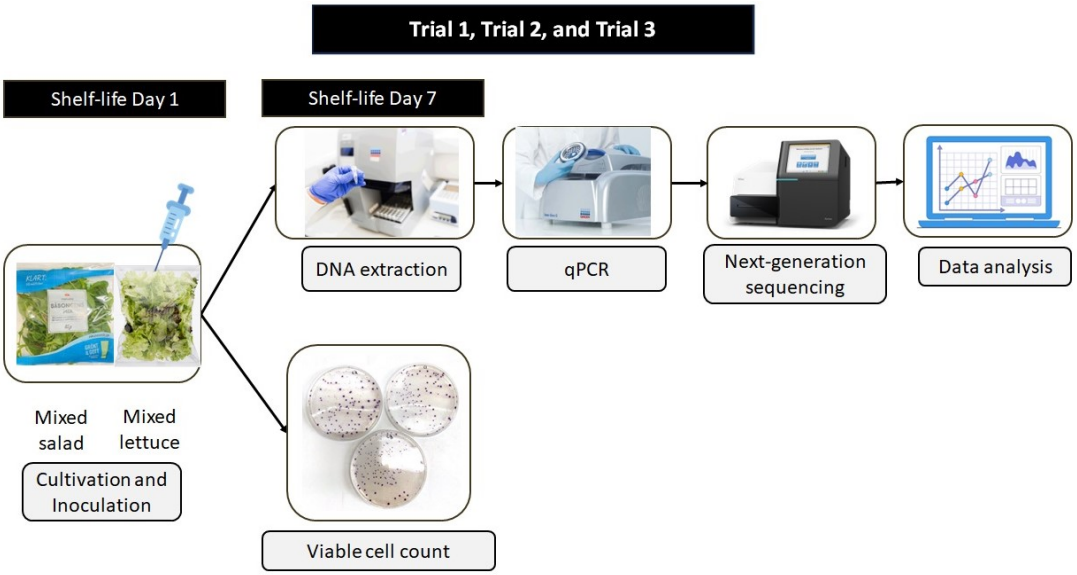

Figure 3.1: Sampling scheme for the mixed salad and mixed lettuce inoculated with probiotics with/without *E. coli*. On the first day of shelf-life, three packages were opened to extract DNA and check for the native microbiota present, the packages were then resealed, and injected 300 μL of diluted probiotic strains into each bag. All the packages were kept at 4°C until their best-before date. On the last day of shelf-life, packages were sampled for DNA extraction and further analyzed by qPCR and Illumina MiSeq.

The diluted concentration was prepared by measuring the Optical Density (OD) at 520 nm (determined by colony count and spectrophotometry (Novaspec II, Pharmacia, Sweden)). Each strain was injected in triplicate. All the samples were kept at 4 °C until their best-before date.

3.3 DNA extraction

3.3.1 Sample preparation

The removal of extracellular DNA was performed according to Tatsika et al.(2019) [21], with some modifications. 2 g of salad leaves were transferred into a 50 mL centrifuge tube containing 20 mL Phosphate-Buffered Saline (PBS) (Oxoid Ltd, Hampshire, England) solution. The samples were sonicated (Transsonic T 570/H, Germany) for 10 minutes, and the leaves were removed.

The samples were centrifuged (Minispin 5453, NH, USA) for 20 minutes at 9500 rpm, and the supernatant was discarded except for 1 mL. The samples were then vortexed (Scientific Industries G560E, USA), transferred to freezing tubes, and centrifuged again. The supernatant was discarded, and the pellet was frozen at -80°C until further testing.

3.3.2 DNA extraction using robotic workstation

DNA was extracted from the samples using EZ1 Advanced XL robotic workstation (QIAGEN GmbH, Sollentuna, Sweden) and EZ1&2 DNA Tissue Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Briefly, the DNA pellet was mixed with 190 μL Buffer G2 and 10 μL of proteinase K, and the samples were incubated for 3 hours at 56°C and loaded into the robotic workstation to extract DNA.

3.3.3 DNA concentration

The concentration of the extracted DNA was measured by using a NanoDrop device (Thermo Scientific, MA, USA) and all the samples were stored in aliquots at -80°C until further analysis.

3.4 Strain-specific qPCR assay

First, DNA extraction was performed according to Uhlig et al. (2017) [40]. Briefly, single colonies were selected from **4, 5, 6, 7, E** and each cells were suspended in 0.5 mL autoclaved Milli-Q® water followed by bead beating on an Eppendorf Mixer (model 5432, Eppendorf, Hamburg, Germany) for 30 minutes. Then, centrifugation was done at 12 000 g for 1 minute, the supernatant was used as template DNA in the subsequent qPCR analysis.

Next, qPCR was performed according to the manufacturer's instructions with QuantiNova™ SYBR® Green PCR kit 500. The total volume of the PCR reaction was 25 μL, consisting of 12.5 μL 2x QuantiNova SYBR Green PCR, 2.0 μL QN ROX™ Reference Dye, 1.75 μL forward primer, 1.75 μL reverse primer, and 7 μL of template DNA (*Table 3.1*). The qPCR was performed by Rotor-Gene® qPCR machine under the following conditions: 95°C for 2 min, followed by 40 cycles of 95°C for 5 sec, 60°C for 10 sec.

To determine the concentration of the target gene present in each sample, a standard curve was generated with 10-fold serial dilutions of five known concentrations of specific strains (**4, 5, 6, 7, and E**) and sample DNA.

After qPCR was completed, melting curve analysis was performed to check the specificity of the primers.

Table 3.1: Details of the primers used in qPCR for detection of **4,5,6,7 and E**
Each letter or number represents:
4: *Pseudomonas punonensis* LMG P-32204; **5**: *Bacillus coagulans* LMG P-32205; **6**: *Bacillus coagulans* LMG P-32206; **7**: *Pseudomonas cedrina* LMG P-32207; **M**: Mix of all strains; **E**: non-pathogenic *E. coli* CCUG 29300

Strain	Oligo Name	Sequence
4	CR10bF	5′GGGGACAACGTTTCGAAAGG3′
	CR10bR	5′GAGCCTTTACCCCACCAACT3′
5	H7F	5′CCGGTTCCGTAAAAGCCATT3′
	H7R	5′GCTGCTTTGCCCTTTGAGAA3′
6	H10F	5′CCCTTGGGACCGACTACAG3′
	H10R	5′GCTCAACGGATAAAAGCTACCC3′
7	YI3.1F	5′ACGGGTGAGTAAAGCCTAGG3′
	YI3.1R	5′GCTCATCTGATAGCGCAAGG3′
E	401F	5′TGATTGGCAAAATCTGGCCG3′
	611R	5′GAAATCGCCCAAATCGCCAT3′

3.5 Characterization of leaf microbiota using Illumina Miseq

The full protocol used for 16S Library Preparation is available at https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf

3.5.1 16S Library Preparation Workflow

Figure 3.2 illustrates the workflow for the 16S Library Preparation.

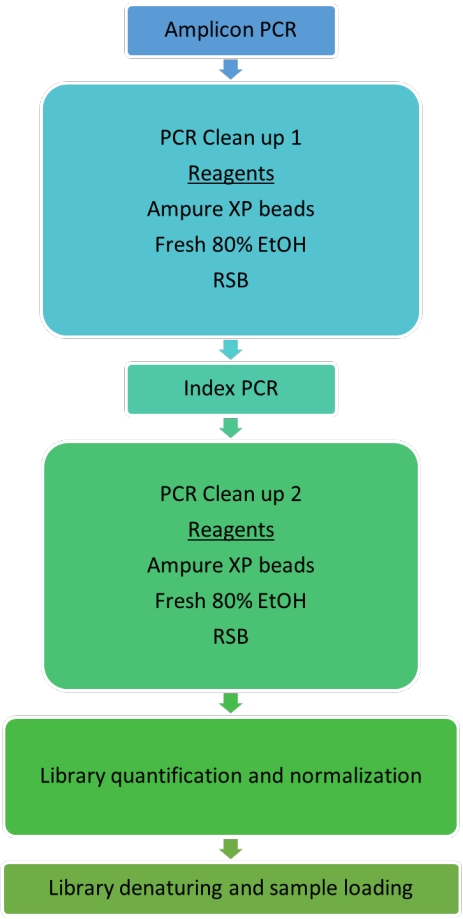

Figure 3.2: 16S Library Preparation Workflow. Source: Illumina

Amplicon PCR

2.5 μL of extracted DNA sample were mixed with 5 μL amplicon PCR forward primer 341F, 5 μL amplicon PCR reverse primer 805R, 12.5 μL 2x KAPA HiFi HotStart ReadyMix to amplify 460 bp of the V3-V4 hypervariable region of the 16S rRNA gene.

PCR was performed in a thermal cycler using the following program:

- 95°C for 3 minutes
- 25 cycles of:
  - 95°C for 30 seconds
  - 55°C for 30 seconds
  - 72°C for 30 seconds
- 72°C for 5 minutes
- Hold at 4°C

PCR clean-up

This step used AMPure XP beads to purify the 16S V3 and V4 amplicon away from free primers and primer dimer species.

Index PCR

Index PCR was performed to attach dual indices and Illumina sequencing adapters using the Nextera XT Index Kit (*Figure 3.3)*.
5 μL from each well from PCR clean-up 1 was transferred to a new 96-well plate, then 5 μL of Nextera XT Index Primer 1, 5 µl Nextera XT Index Primer 2, 25 μL 2x KAPA HiFi HotStart ReadyMix, 10 μL nuclease-free water were mixed and centrifuged the plate at 1000 g for 1 minute.
PCR was performed in a thermal cycler using the following program:

- 95°C for 3 minutes
- 8 cycles of:
  - 95°C for 30 seconds
  - 55°C for 30 seconds
  - 72°C for 30 seconds
- 72°C for 5 minutes
- Hold at 4°C

Figure 3.3: Index 1 and 2 primers were arranged in the TruSeq index plate fixture using the following arrangements as needed: Index 2 primer tubes (white caps, clear solution) vertically, aligned with rows A through H and Index 1 primer tubes (orange caps, yellow solution) horizontally, aligned with columns 1 through 12. Photo credit: Lund University

Library quantification, normalization, and pooling

The indexed samples were quantified using Qubit™ 1x dsDNA HS Assay Kit (Life Technologies Corporation, Eugene, OR, USA). Then, it was diluted to 4 nM with 10 mM Tris pH 8.5. Finally, 5 μL diluted DNA from each library was mixed for pooling libraries with unique indices. Supplementary data are attached in Appendix A.10

Library denaturing and MiSeq sample loading

In preparation for cluster generation and sequencing, 4 nM pooled libraries were denatured with 0.2 N NaOH, diluted with hybridization buffer (HT1), and then heat denatured before MiSeq sequencing. PhiX (Illumina) was used as an internal control. The final loading volume was 600 μL.

Data Analysis for Illumina MiSeq

The NGS data was analyzed with QIIME2 2022.8 [43]. Raw sequence data were demultiplexed using Illumina CASAVA 1.8 followed by quality filtering and denoising with DADA2 [44] and then further processed in R [45]. The total number of reads after filtering was 930 855 and the mean number of reads per sample was 155 142.5.

The sequences were trimmed at the ends at 0 bp left and 284 bp right. Samples containing Q < 25 reads were removed. Taxonomic classification of the remaining reads was made using the Greengenes 13.8 database [46]. Reads identified as eukaryotic, mitochondria, or chloroplasts were removed.

3.6 Enumeration of *E. coli* count

Enumeration of *E. coli* count was performed on mixed salad leaves, inoculated with *E. coli* and probiotic strains (**4, 5, 6, 7, C, M**) (**Trial 3**) as described by Uhlig et al. (2017) [40] are shown in *Figure 3.4*.

On the best-before date, 10 g of mixed salad leaves from each package were mixed with 90 mL of peptone water and diluted. The diluted samples were then spread onto Brilliance™ *E. coli* agar and incubated for 24 hours at 37°C and enumerated.

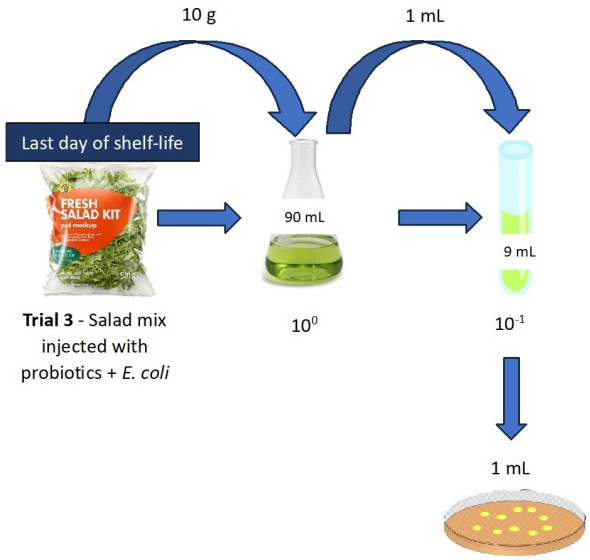

Figure 3.4: Schematic diagram of serial dilution involved. On the best-before date, 10 g of mixed salad leaves from each package were mixed with 90 mL of peptone water. 1 mL of it was further diluted with 9 mL of peptone water to get a 10^{-1} dilution factor. Finally, 1 mL from the final dilution was spread onto Brilliance™ *E. coli* agar and incubated for 24 hours at 37°C and enumerated.

3.7 Calculations and statistical analysis

The Enumeration of *E. coli* count was evaluated by Kruskal-Wallis one-way Analysis of variance (ANOVA) on ranks. The differences between the two experimental groups were assessed by the Mann-Whitney rank sum test and results of $p \leq 0.05$ were considered statistically significant.

After qPCR was completed, the melting curve analysis was performed to assess the specificity of the PCR product.

The α-diversity of the leafy green microbiome was calculated using the Shannon diversity index, and the differences between groups were evaluated by Kruskal-Wallis one-way ANOVA on ranks. The β-diversity was calculated with unweighted UniFrac, and analyzed with Principal Coordinate Analysis (PCoA).

4 Results

4.1 Enumeration of *E. coli* count

The enumeration of *E. coli* counts from all products on the last day of shelf-life range from 0 to 1280 CFU/mL (*Table 4.1*). Notably, strains **5** and **7**, resulted in a significant decrease in plate count ($p \leq 0.01$), with no detectable *E. coli* colony forming units (CFU). Similarly, strains **4** and **6**, also led to a statistically significant reduction in plate count ($p \leq 0.05$), with median values of 10 and 20 *E. coli* CFU/mL, respectively. Supplementary data are attached in *Appendix A.5*.

Table 4.1: Total viable *E. coli* count on the last day of shelf-life of RTE salad mix. The total viable cell count was determined by counting the number of *E.coli* colonies divided by the dilution factor.

Strain No.	Total viable *E. coli* count[2]
4	10 (0-70)[a]
5	0[b]
6	20 (0-30)[a]
7	0[b]
M	170 (0-300)[a]
C	900 (850-1280)

[2] Counts expressed as median CFU/mL of three replicates with interquartile range (25–75%)

[a] $p \leq 0.05$ compared to control (C)

[b] $p \leq 0.01$ compared to control (C)

Each letter or number represents: 4: *Pseudomonas punonensis* LMG P-32204; **5**: *Bacillus coagulans* LMG P-32205; **6**: *Bacillus coagulans* LMG P-32206; **7**: *Pseudomonas cedrina* LMG P-32207; **M**: Mix of all strains

4.2 qPCR analysis

36 out of the 45 salad packages detected their injected probiotic strain (Except triplicates of M) in their respective qPCR assay as shown in *Appendix A.6*. No fluorescent signal was observed for any of the negative controls (red line). Hence, the specificity of the five qPCR assays was 100%, with no detectable fluorescent signal for negative samples or blank controls.

The concentrations generated by the standard curve for strains **5** and **6** were very low (ranging from 1.27×10^{-2} ng/ μL to 3.55×10^{-5} ng/ μL) compared to strains **4** and **7**, which had higher concentrations (ranging from (8.77 ng/ μL to 3.58 $\times 10^{-1}$ ng/ μL).

As shown in *Appendix* A.7, the melting curves for the five strains had only one peak. This indicates the absence of interference from primer dimers and shows a great amplification effect.

Supplementary data used for qPCR analysis are provided in *Appendix* A.10.

4.3 Next Generation Sequencing

The most prevalent phylum found in leafy greens, including lettuce and mixed salad samples, was Proteobacteria (*Figure 4.1*). On day 1, the relative abundance of Proteobacteria was 38.2% for mixed lettuce and 88.2% for mixed salad. At the best-before date (after 7 days), the prevalence of Proteobacteria had increased in all mixed salad samples (Trial 2 and 3). Especially the samples inoculated with *E. coli* and probiotic strains (Trial 3) had a higher relative abundance of Proteobacteria (96.5%-93.7%) compared to the control (90.2%) which did not receive any probiotics. But in the lettuce samples, at the best-before date (after 7 days), the prevalence of Proteobacteria had decreased in strains **4** and **6** by 44-55% while it had increased in **5, 7,** and **C** by 108-152%.

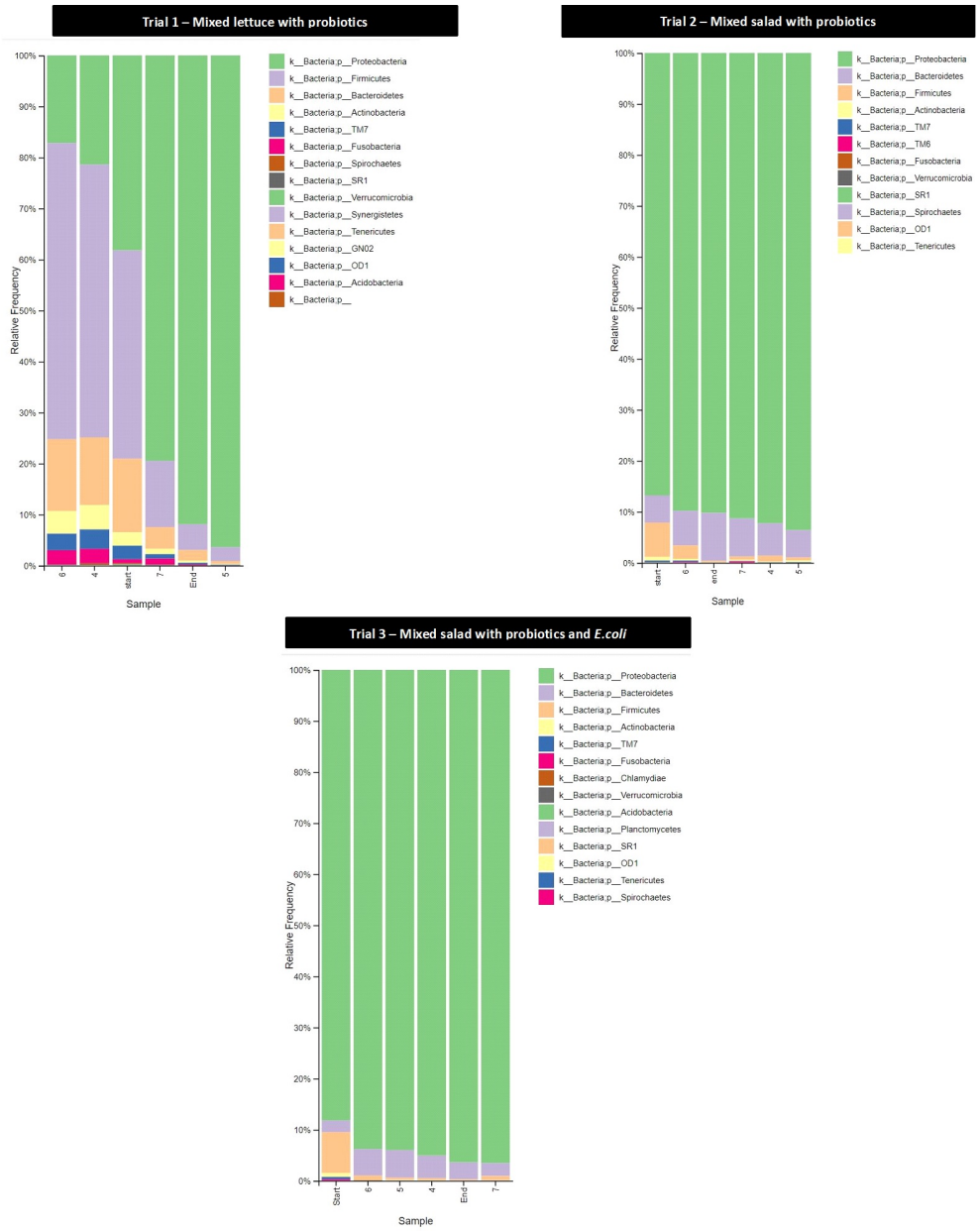

Figure 4.1: Relative bacterial abundance at the phylum level of RTE salad mixes, uninoculated and inoculated with *E. coli* together with probiotics. **Trial 1**: Lettuce inoculated with probiotics, **Trial 2**: mixed salad inoculated with probiotics, **Trial 3**: mixed salad inoculated with *E. coli* and probiotics. All the samples were analyzed on day 7 (the best-before date).

Each letter or number represents: 4: *Pseudomonas punonensis* LMG P-32204; **5**: *Bacillus coagulans* LMG P-32205; **6**: *Bacillus coagulans* LMG P-32206; **7**: *Pseudomonas cedrina* LMG P-32207; Start: Day 1; End: Day 7

The second most prevalent phylum found in the mixed salad samples was Bacteroidetes (*Figure 4.1*). Over time, there was an increase of up to 40% in Bacteroidetes abundance across all samples (**4, 5, 6, 7**, and **C**) ($p < 0.01$). However, no significant difference was observed between the control and probiotic strains. Similarly, the second most prevalent phylum found in the mixed lettuce samples was Firmicutes. On the best-before date (after 7 days), the prevalence of Firmicutes had increased in strains **4** and **6** by 30-40%, while it had decreased in strains **5**, **7**, and **C** by 68-92%. Furthermore, Actinobacteria and Fusobacteria were also detected in all of the trials, constituting <2% and <1% of the total microbiota composition respectively.

Over all trials, the top family with the highest relative abundance was *Pseudomonadaceae* with 60% for mixed salad and 4% for lettuce (*Figure 4.2*). The second most common family differs in each trial, with *Enterobacteriaceae* in lettuce, *Oxalobacteraceae* mixed salad inoculated with probiotics, and *Moraxellaceae* in mixed salad inoculated with probiotics and *E. coli*. *Pseudomonadaceae* decreased by 18% over time in all mixed salad packages inoculated with probiotics and *E. coli*. Conversely, *Pseudomonadaceae* increased over time in lettuce samples inoculated with strain **5**. In mixed salad samples inoculated with probiotic strains exhibited a higher relative abundance of *Oxalobacteraceae* (8%-13%) compared to the control group (4%), which did not receive any probiotics. Similarly, mixed salad samples inoculated with *E. coli* and probiotic strains showed a higher relative abundance of *Moraxellaceae* (20%-28%) compared to the control group (8%).

In mixed salad samples, higher relative abundances of *Shewanellaceae* (19.0%) and *Enterobacteriaceae* (8%) were detected on strains **4**, **5** and **7** compared to the control. Conversely, in lettuce samples inoculated with probiotics, higher relative abundances of *Streptococcaceae* (26%), *Oxalobacteraceae* (13%), and *Veillonellaceae* (5%) were recorded on strains **4** and **6** compared to the control.

On the genus level, higher relative abundance (40%) of *Pseudomonas* was found on mixed lettuce and mixed salad. Similarly, *Streptococcus*, *Shewanella*, and *Acinetobacter* were also detected in all samples.

Lastly, on the species level, a higher relative abundance of *Rahnella aquatilis* was detected on lettuce, and *Pseudomonas fragi, and Pseudomonas veronii* were detected on mixed salad samples. It should also be noted that on day 1, before inoculation of any strains, *E. coli* was detected in all three replicate mixed salad samples from **Trial 2** at a minimal level (<0.01%). All the detected observations are summarized in *Table 4.2*.

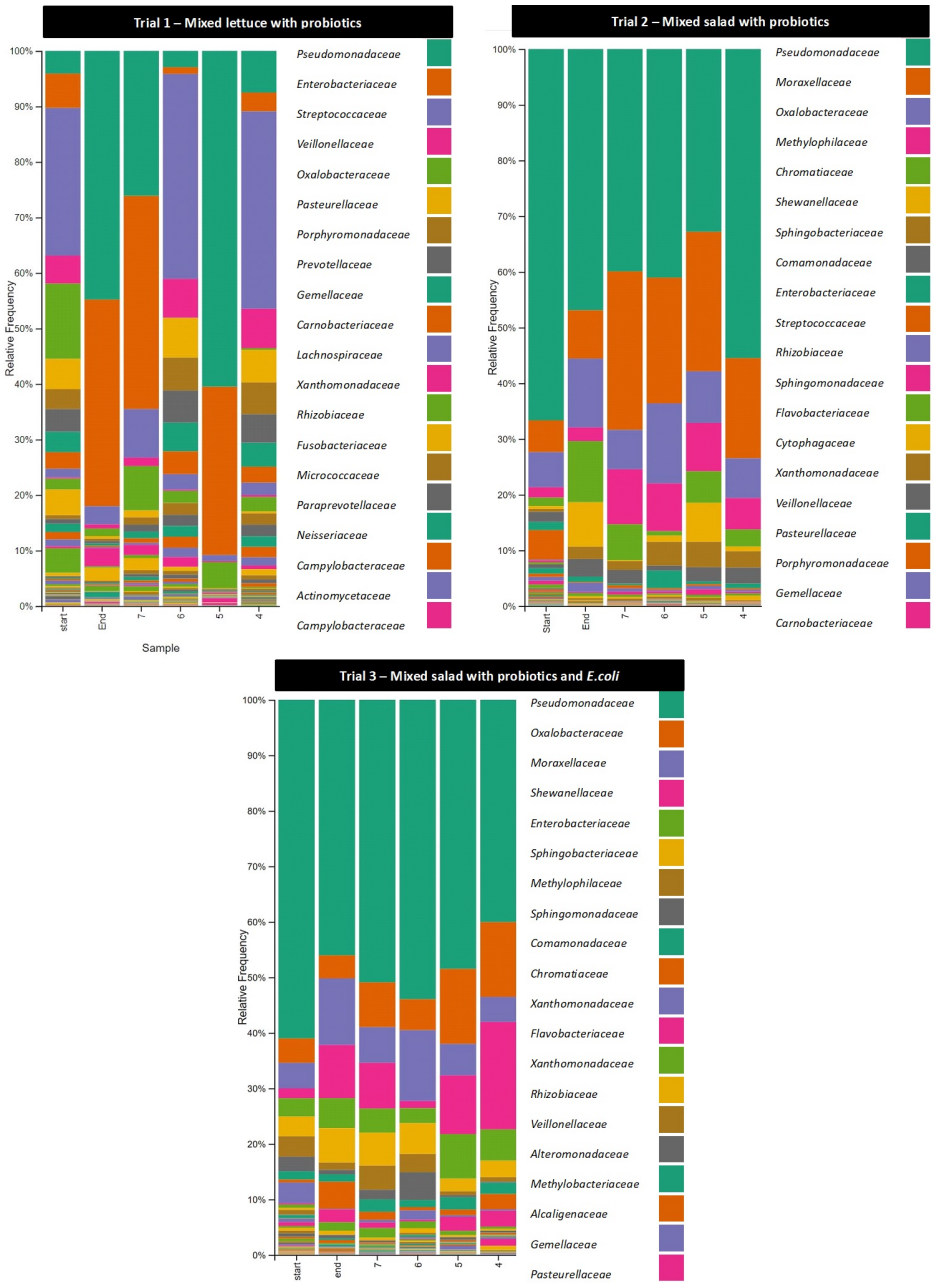

Figure 4.2: Relative bacterial abundance at the family level of RTE salad mixes, uninoculated and inoculated with *E. coli* together with probiotics.

Each letter or number represents: 4: *Pseudomonas punonensis* LMG P-32204; **5**: *Bacillus coagulans* LMG P-32205; **6**: *Bacillus coagulans* LMG P-32206; **7**: *Pseudomonas cedrina* LMG P-32207; Start: Day 1; End: Day 7

Table 4.2: Next Generation Sequencing of the microbiota of leafy green products during shelf-life, with and without *E. coli* inoculation

Observation	Trial
Most prevalent phylum - Proteobacteria	All trials
Second most prevalent phylum - Bacteroidetes	Mixed salad with probiotics, mixed salad with probiotics + *E. coli*
Second most prevalent phylum - Firmicutes	Mixed lettuce with probiotics
Most prevalent family - *Pseudomonadaceae*	All trials
Second most prevalent family - *Enterobacteriaceae*	Mixed lettuce with probiotics
Second most prevalent family - *Oxalobacteraceae*	Mixed salad with probiotics
Second most prevalent family - *Moraxellaceae*	Mixed salad with probiotics, mixed salad with probiotics + *E. coli*
Most prevalent genus - *Streptococcus*	Mixed lettuce with probiotics
Most prevalent genus - *Pseudomonas*	Mixed salad with probiotics, mixed salad with probiotics + *E. coli*
Most prevalent species - *Rahnella aquatilis*	Mixed lettuce with probiotics
Most prevalent species - *Pseudomonas fragi, Pseudomonas veronii*	Mixed salad with probiotics, mixed salad with probiotics + *E. coli*

The α-diversity, calculated using the Shannon diversity index based on amplicon sequence variants (ASVs) (*Table 4.3*), exhibited variations only among mixed salad and mixed lettuce inoculated with probiotics. These values ranged from 2.7-3.1 on day 1 to around 5.5 on day 7. Samples of mixed salad inoculated with probiotics and *E. coli* maintained consistent values, ranging from 6.2 on day 1 to the same levels on day 7. Therefore, the inoculation only altered the Shannon index in the mixed salad and mixed lettuce samples with probiotics.

Table 4.3: The α-diversity of ASVs calculated by Shannon diversity index in RTE leafy green products uninoculated and inoculated with non-pathogenic *E. coli* strain. Data is presented as a median with interquartile range (25% - 75%)

Each letter or number represents: 4: *Pseudomonas punonensis* LMG P-32204; **5**: *Bacillus coagulans* LMG P-32205; **6**: *Bacillus coagulans* LMG P-32206; **7**: *Pseudomonas cedrina* LMG P-32207; Start: Day 1; End: Day 7

Sample ID	Mixed lettuce with probiotics	Mixed salad with probiotics	Mixed salad with probiotics + *E. coli*
Start	2.7 (2.6-2.8)	3.1 (2.8-3.3)	6.2 (6.1-6.3)
End	5.8 (5.5-5.9)	5.8 (5.6-5.9)	6.0 (5.9-6.2)
7	5.2 (4.9-5.3)	5.4 (5.1-5.6)	6.3 (6.0-6.4)
6	5.8 (5.6-5.9)	5.5 (5.3-5.7)	5.9 (5.7-6.0)
5	4.8 (4.6-5.0)	5.2 (5.0-5.5)	6.1 (6.0-6.3)
4	5.9 (5.8-6.0)	5.3 (5.0-5.4)	6.4 (6.3-6.5)

The β-diversity, calculated by unweighted unique fraction metric (UniFrac), considering only species presence and absence information and counting the fraction of branch length unique to either community, changed from start to end for all product types, as seen in *Figure 4.3* as a shift to the right.

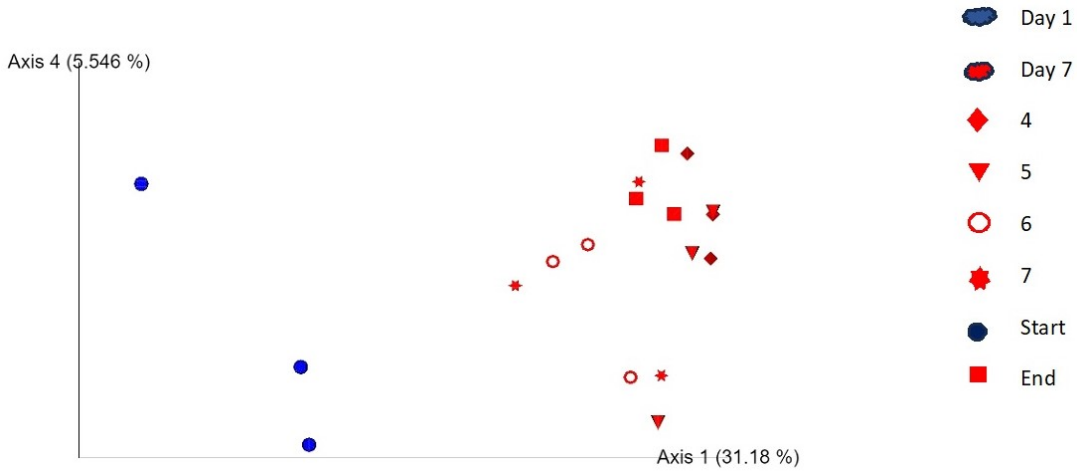

Figure 4.3: Principal Coordinate Analysis (PCoA) of β-diversity based on the variation of amplicon sequence variants (ASVs) for RTE mixed salad uninoculated and inoculated with non-pathogenic *E. coli*, calculated by unweighted unique fraction metric (unifrac).
Each strain represents: 4: *Pseudomonas punonensis* LMG P-32204; **5**: *Bacillus coagulans* LMG P-32205; **6**: *Bacillus coagulans* LMG P-32206; **7**: *Pseudomonas cedrina* LMG P-32207; Start: Day 1; End: Day 7.

5 Discussion

This study is an investigation to evaluate the antagonistic effect against *E. coli* by inoculating plant probiotics together with non-pathogenic *E. coli* CCUG 29300 directly into salad bags and subsequent evaluation of bacterial community composition after shelf-life expiration. Currently, a similar study has been done by researchers at the University of Queensland, Australia in 2019 [47]. They have discovered that the ProbiSafe® bacterial strain has the ability to inhibit *Salmonella* growth on fresh-cut iceberg lettuce at different conditions *in vitro* and the project has achieved promising results. But it still hasn't been commercialized.

The findings from the enumeration of *E. coli* count, clearly indicate that *Bacillus coagulans* LMG P-32205 and *Pseudomonas cedrina* LMG P-32207 were the most effective in reducing *E. coli* levels during the shelf-life period compared to the control (C), which did not receive any probiotics. Furthermore, *Pseudomonas punonensis* LMG P-32204, *Bacillus coagulans* LMG P-32206, and a mix of all the strains also exhibited reduced levels of *E. coli* compared to the control sample. These results confirm the antagonistic effect of the probiotic strains mentioned in a previous study by Uhlig et. al (2021) [32], where it was observed that the mentioned four probiotic strains exhibited antagonistic effects against *E. coli* on spinach seeds and leaves in a commercial field production system. Particularly, *Pseudomonas punonensis* LMG P-32204 and *Pseudomonas cedrina* LMG P-32207 showed reduced levels of *Escherichia-Shigella* as the seeds developed into plants [32].

The observations from the qPCR analysis showed that the concentrations detected for *Bacillus coagulans* LMG P-32205 and *Bacillus coagulans* LMG P-32206 were lower compared to *Pseudomonas punonensis* LMG P-32204 and *Pseudomonas cedrina* LMG P-32207, which had higher concentrations. This could indirectly provide information about the presence or quantity of live cells of *Pseudomonas* were higher than *Bacillus* during the shelf-life as we injected live bacterial strains. However, qPCR results alone do not distinguish between live and dead cells so additional techniques such as flow cytometry or viability staining are needed for that purpose. Additionally, it was challenging to determine the concentration of DNA that was amplified and detected during the qPCR process because a $\log_{10}$ 8 CFU/mL concentration

was injected at the start while the qPCR detected the concentration in ng/ μL at the end. This inconsistency made it impossible to convert these units due to the unknown molecular weight of each strain.

The analysis done by 16S metagenomics sequencing with Illumina MiSeq provided insights into the microbial composition of mixed lettuce and mixed salad leaves. Proteobacteria was the most prevalent phylum, consistent with findings from previous Next-Generation Sequencing (NGS) studies on leafy green vegetables ([32], [48], [20], [49], [21]). Interestingly on day 1, before inoculation of any strains, *E. coli* was detected in all three replicate mixed salad samples from **Trial 2** at a minimal level (<0.01%).

Furthermore, a notable difference in the relative abundance of various genera was observed between day 1 and day 7 samples of mixed lettuce and mixed salad leaves. In the mixed lettuce group inoculated with probiotics, the relative abundance of *Pseudomonas* was higher compared to the control, which did not receive any probiotics. Conversely, in the mixed salad group inoculated with probiotics, the relative abundance of *Pseudomonas* was lower compared to the control. Additionally, within the mixed salad groups, the relative abundance of *Acinetobacter* was higher.

This study aimed to assess whether the antagonistic bacteria survived throughout the shelf-life and induced changes in the native microbiota of the leafy greens. However, due to limitations in MiSeq sequencing, we couldn't identify the inoculated probiotics at the species level or determine their survival and establishment on the salad and lettuce leaves. Nevertheless, qPCR results revealed that, even after simulating *E. coli* contamination, the concentration of *E. coli* remained undetectable in mixed salad samples inoculated with probiotics. This finding suggests a potential antagonistic effect of the probiotic strains against *E. coli*. Additionally, it was observed that the changes in the leafy greens' microbiota are strain-specific and vary among different types of leaves.

A change in the α-diversity, which measures the variety of species within a single sample, was only observed in mixed salad and mixed lettuce inoculated with probiotics. This indicates that the introduction of probiotics led to an increase in species diversity in these trials. However, when examining the β-diversity using the unweighted UniFrac method, which assesses the differences in microbial communities found to be higher in all mixed salad trials compared to day 1 (control).

This suggests that the microbial communities differed significantly in their composition from the control group.

These findings have practical implications for the use of probiotics in leafy greens. The increase in α-diversity suggests that probiotics can increase microbial diversity, which is often associated with a more resilient and stable microbial community. This could potentially lead to improved shelf-life and safety of the products by outcompeting harmful pathogens. The observed changes in β-diversity indicate that probiotics can significantly alter the overall microbial community structure, which might influence the functional properties of the microbiota, such as nutrient availability and resistance to spoilage.

These results align with a study conducted by Uhlig et al. (2022) [19], which also reported that probiotic inoculation led to distinct microbial community changes. These findings encourage the use of bacterial antagonists as a biocontrol to promote food safety and quality in fresh produce. Moreover, before appointing these strains for biocontrol, they need to have done clinical trials and assessments, including examinations of faecal samples to evaluate the effectiveness of these strains.

6 Conclusion

In this study, large amounts of data from both culture-dependent and culture-independent techniques were combined to give a unique insight into the antagonistic effect of *Pseudomonas punonensis* LMG P-32204, *Bacillus coagulans* LMG P-32205, *Bacillus coagulans* LMG P-32206, *Pseudomonas cedrina* LMG P-32207 against non-pathogenic *E. coli* CCUG 29300. The effect of probiotic strains varied between different leafy green products, thus no conclusions could be drawn. Nonetheless, these results encourage the use of bacterial antagonists as part of a global solution to reduce the risk of human pathogens on leafy green vegetables.

Key findings and lessons learned:

- Viability of probiotic strains:

  The viability of the probiotic strains in packaged produce varied, with strains **5** and **6** having lower concentrations than strains **4** and **7**. This suggests that the leaf structure and nutrient composition of different greens affect the survival of probiotic strains.

- Antagonistic effect against *E. coli*:

  The results showed a clear reduction in *E. coli* levels in mixed salad samples inoculated with probiotics and *E. coli*, indicating effective antagonistic activity. However, this effect was not consistent across all types of leafy greens. This suggests that the interaction between probiotics and *E. coli* is influenced by the specific microbial environment of each leafy green product.

- Microbial community changes:

  Significant changes in microbial community composition were observed, particularly in mixed salad samples. The relative abundance of Proteobacteria increased in mixed salad samples inoculated with probiotics, while in lettuce samples, some strains led to a decrease in Proteobacteria. This suggests that the probiotics not only inhibit pathogens but also alter the native microbiota in a product-specific manner.

6.1 Limitations

This study only uses a non-pathogenic *E. coli* strain and does not provide information on the interactions with pathovars of *E. coli in vitro*. Also due to limitations in MiSeq sequencing, it was unable to identify the inoculated probiotics at the species and strain level.

6.2 Future work

Future investigations should aim to assess the effectiveness of the previously mentioned probiotic strains against pathogenic variants of *E. coli*, as well as other potential human pathogens like *Listeria* and *Salmonella*. As discussed (*Ref 5*), it can be seen that testing against a single strain is insufficient, but several, preferably the ones that have been isolated from outbreaks of leafy greens should be considered.

Moreover, before the appointed strains for biocontrol can be legally used in commercial food production, they need to have done clinical trials and assessments, including examination of faecal samples to evaluate the effectiveness of these strains.

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A Appendix

A.1 Sample preparation

A.1.1 TSA media preparation

20g were dissolved in 500 mL of distilled water and autoclaved for 15 minutes at 121°C.

A.1.2 TSB media preparation

15g were dissolved in 500 mL of distilled water and autoclaved for 15 minutes at 121°C.

A.1.3 Glucose yeast extract agar medium preparation

Glucose yeast extract agar was prepared by mixing 5 g of peptone, 5 g of yeast extract, 2 g of glucose, 0.5 g of potassium dihydrogen phosphate, 0.5 g of dipotassium hydrogen phosphate, 0.3 g of magnesium sulphate, 0.01 g of sodium chloride, 0.01 g of manganese sulphate, 0.0016g of zinc sulphate and 15 g of agar in 1 L of distilled water and autoclaved for 15 minutes at 121°C.

A.1.4 Glucose yeast extract broth preparation

BC broth was prepared by mixing 5 g of peptone, 5 g of yeast extract, 2 g of glucose, 0.5 g of potassium dihydrogen phosphate, 0.5 g of dipotassium hydrogen phosphate, 0.3 g of magnesium sulphate, 0.01 g of sodium chloride, 0.01 g of manganese sulphate, and 0.0016g of zinc sulphate in 1 L of distilled water and autoclaved for 15 minutes at 121°C.

A.1.5 Sugar solution preparation

4.2g were dissolved in 100 mL of distilled water and autoclaved for 15 minutes at 121°C.

A.1.6 Hogness' freezing media preparation

Mix 0.17 g K_2HPO_4, 0.04 g KH_2PO_4, 0.05 $MgSO_4$, 0.3 g Na_3-citrate and 24.3 mL glycerol with 175 mL deionized water and autoclave for 15 minutes at 121°C.

A.1.7 PBS solution preparation

1 tablet was dissolved in 100 mL of distilled water and autoclaved for 15 minutes at 121°C.

A.1.8 Brilliance™ *E. coli* agar preparation

Suspend 28.1g of Brilliance™ *E. coli* agar in 1 litre of distilled water. Bring the medium gently to the boil, to dissolve completely.

A.2 Preparation of dilutions

A.2.1 Preparation of $\log_{10}$ 8 CFU/mL probiotic strains

After incubation, probiotic cultures were taken out, transferred into 50 mL centrifuge tubes, and centrifuged (Centrifuge 5804, NH, USA) at 6000 g for 5 minutes. The supernatant was discarded, and the pellet was diluted with freezing media (*Appendix* A.1) to a concentration of $\log_{10}$ 8 CFU/mL by measuring the Optical Density (OD) at 520 nm (determined by colony count and spectrophotometry (Novaspec II, Pharmacia, Sweden)) (*Appendix* A.3).

A.2.2 Preparation of $\log_{10}$ 6 CFU/mL *E. coli* strains

After incubation, *E. coli* cultures were centrifuged (Centrifuge 5804, NH, USA) at 4600 g for 5 minutes. The supernatant was discarded, and the pellet was washed twice with peptone water (0.85% NaCl and 0.1% bacteriological peptone (Oxoid, Basingstoke, UK)) and diluted to a concentration of $\log_{10}$ 6 CFU/mL by measuring the Optical Density (OD) at 520 nm (determined by colony count and spectrophotometry (Novaspec II, Pharmacia, Sweden)) (*Appendix* A.3.1).

A.3 Measurements

A.3.1 OD measurement

OD measurement for probiotics

Table A.1: OD measurement to make dilutions for probiotic strains

Absorbance	4	5	6	7
log 7	Too low	0.173	0.175	Too low
log 8	0.019	0.254	0.254	0.019
log 9	0.289	1.430	1.570	0.199
log 10	1.400	1.600	1.700	0.950

OD measurement for *E. coli*

Table A.2: OD measurement to make dilutions for *E. coli* strains

Absorbance	CCUG 29300
log 9	1.12
log 6	0.018

A.3.2 Nanodrop results

Blank - AV buffer		
Sample ID	Concentration ng/uL	$A_{260/280}$
4_1	5.6	4.81
4_2	7.2	1.94
4_3	6.2	3.57
5_1	7.1	3.69
5_2	10.7	2.83
5_3	9.3	3.35
6_1	6.2	2.81
6_2	10.6	2.27
6_3	7.5	6.19
7_1	9.6	2.37
7_2	8.7	2.29
7_3	7.5	2.15
M_1	8.4	4.5
M_2	7.2	3.77
M_3	7.4	1.78
C_1	5.5	1.79
C_2	7.3	6.1
C_3	13.7	2.39
START1	8	5.65
START2	8.1	4.34
START3	7.7	4.33

Figure A.1: DNA concentration for the lettuce samples by Nanodrop device

Blank - AV buffer		
Sample ID	Concentration ng/uL	$A_{260/280}$
4_1	41.8	2.18
4_2	35.1	2.27
4_3	8.4	5.18
5_1	18.2	2.00
5_2	6.8	3.91
5_3	11.5	2.50
6_1	7.7	3.32
6_2	31.1	2.13
6_3	8.4	5.27
7_1	12.5	3.83
7_2	16.0	3.70
7_3	37.5	2.04
M_1	22.0	2.62
M_2	13.6	4.10
M_3	21.6	2.46
C_1	15.5	5.19
C_2	15.2	2.47
C_3	18.7	2.85
START1	6.4	3.85
START2	7.5	18.50
START3	6.5	4.17

Figure A.2: DNA concentration for the salad mix by Nanodrop device

A.4 Bacterial cultivation

A.4.1 Isolation of antagonistic probiotic strains

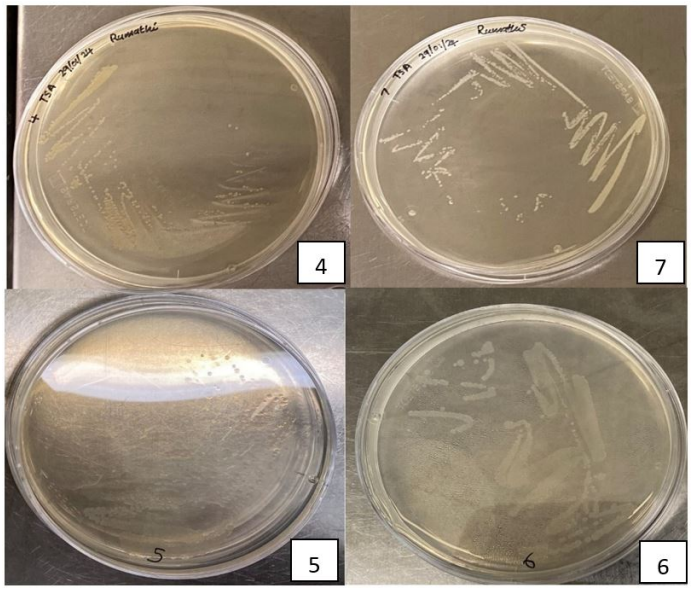

Figure A.3: Isolation of 4 and 7 on Tryptic Soy Agar and 5 and 6 on the glucose yeast extract agar.
Each letter or number represents; 4: *Pseudomonas punonensis* LMG P-32204; 5: *Bacillus coagulans* LMG P-32205; 6: *Bacillus coagulans* LMG P-32206; 7: *Pseudomonas cedrina* LMG P-32207

A.4.2 Isolation of non-pathogenic *E. coli* strain

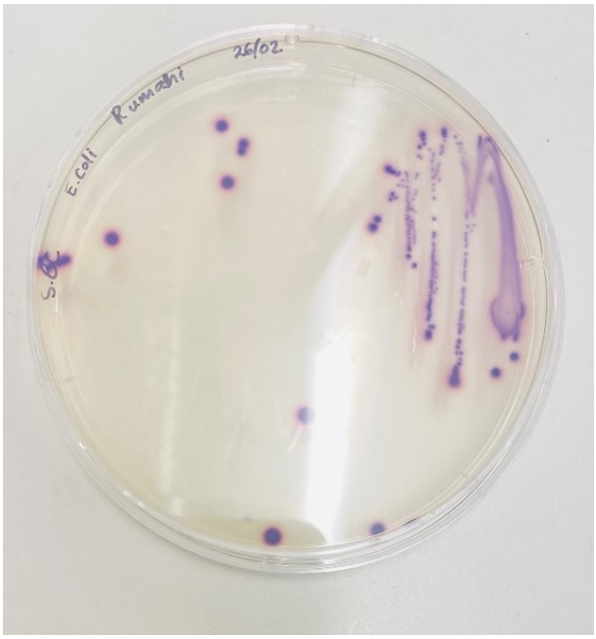

Figure A.4: Isolation of *E. coli* CCUG 29300 on the Brilliance™ *E. coli* agar. *E. coli* contains X-Glu, which can be cleaved by β-glucuronidase, resulting in distinct purple colonies.

A.4.3 Enumeration of *E. coli* count

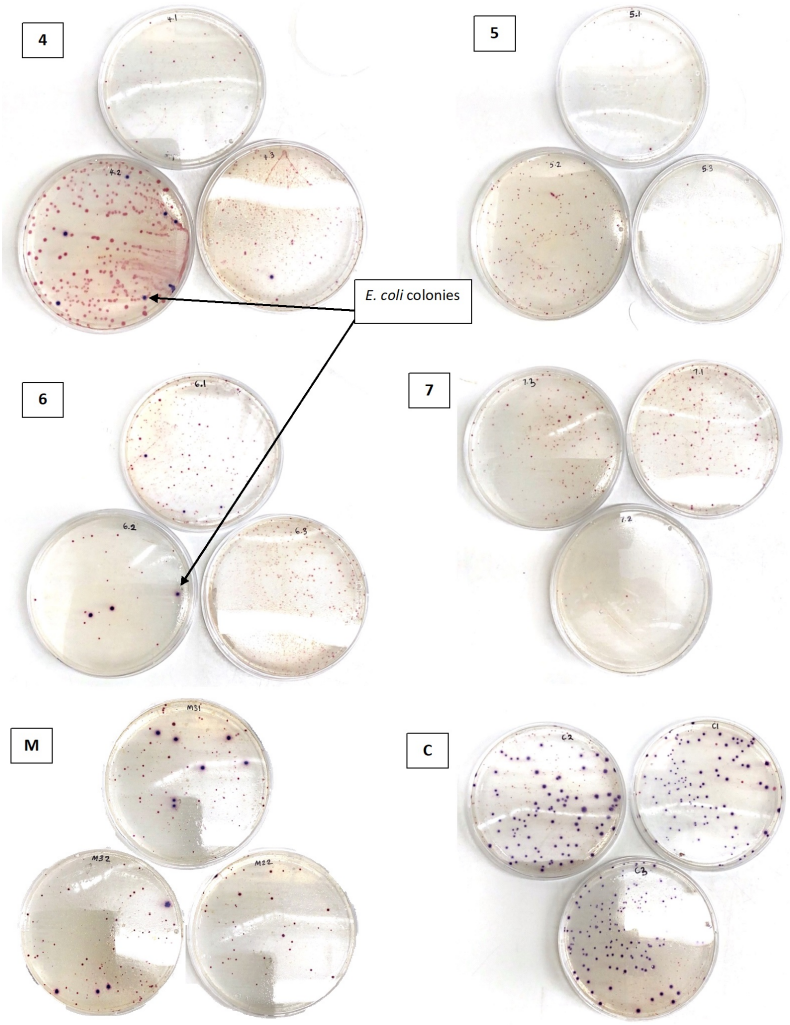

Figure A.5: Plate count results were obtained from 10^{-1} diluted samples spread onto Brilliance™ *E. coli* agar. Purple colonies indicate the presence of *E. coli*.

X

A.5 qPCR supplement data

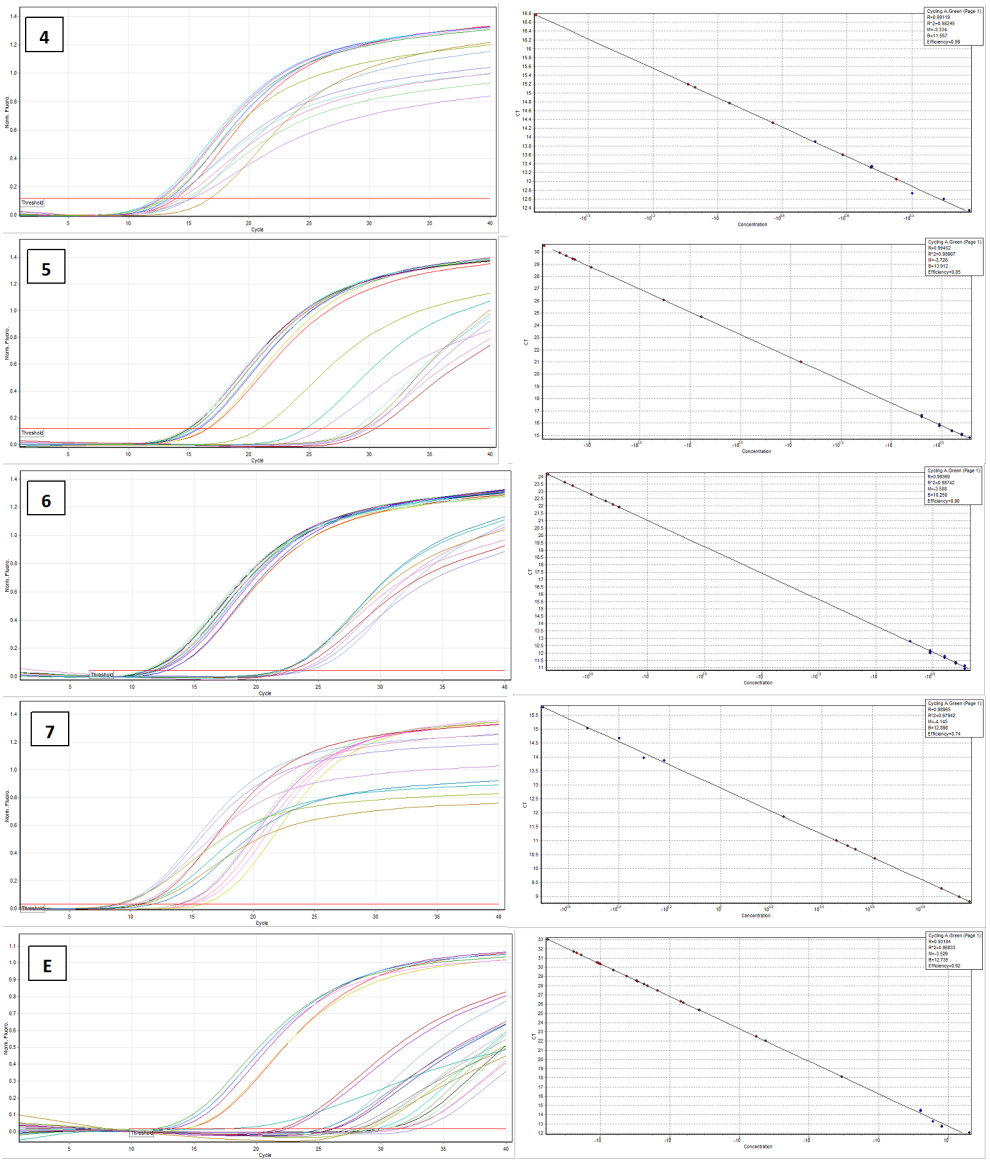

Figure A.6: The left graphs represent the amplification plot with SYBR®Green and the right graphs represent the standard curves (4–E).
Amplification plot: Fluorescence is represented on the Y-axis, whereas the number of PCR cycles is plotted on the X-axis. The intersection point between the amplification curve and the threshold line is called Ct. This point indicates the cycle in which the fluorescence reaches the threshold value. The higher the initial DNA amount, the lesser number of cycles are needed to reach the threshold.
Standard curve: Ct values are plotted on the Y-axis, and the concentration is plotted on the X-axis. Red dots represent the samples, and blue dots represent the standards, forming a straight line.

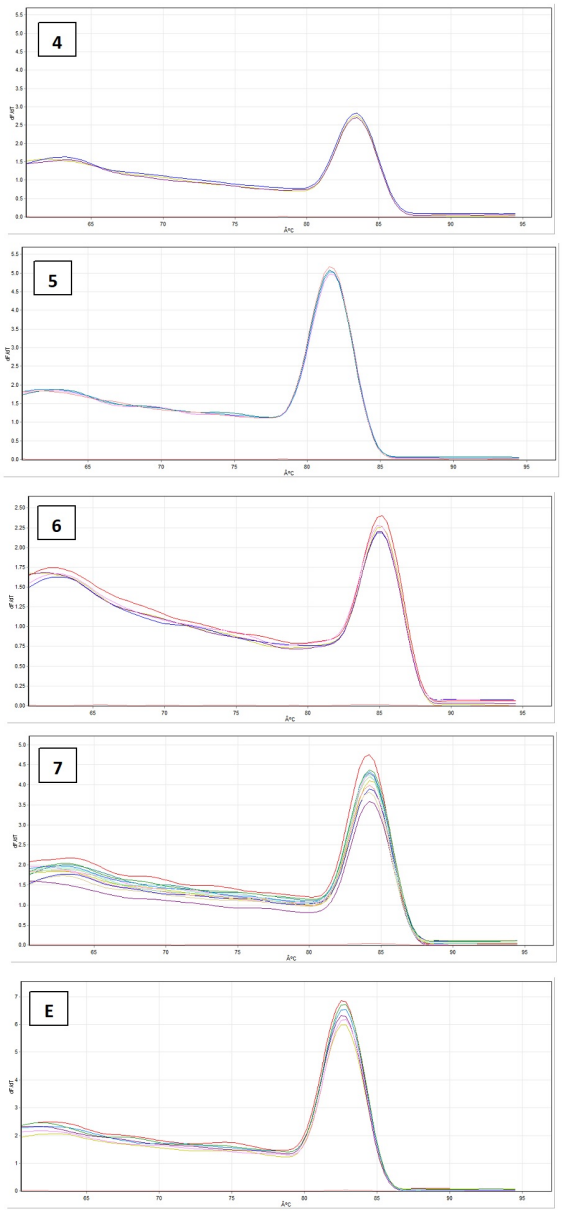

Figure A.7: Melting curve analysis was conducted to assess primer specificity. A single peak in the melting curve indicates the presence of a single PCR product. This signifies strong specificity of the amplification process and ensures the reliability of the results.

Component	Volume/reaction		Final concentration
	96-well block	384-well block	
2x QuantiNova SYBR Green PCR Master Mix	10 µl	5 µl	1x
QN ROX Reference Dye (Applied Biosystems cycler only)	2 µl/0.1 µl*	1 µl/0.05 µl*	1x
Primer A†	Variable	Variable	0.7 µM
Primer B†	Variable	Variable	0.7 µM
RNase-Free water	Variable	Variable	
Template DNA or cDNA (added at step 4)	Variable	Variable	≤100 ng/ reaction
Total reaction volume	**20 µl**	**10 µl**	

Figure A.8: Reaction setup for qPCR

Bacteria	Primer	Conc (pmol/µL)	Stock conc (µM)	Desired conc (µM)	How to make 0.7 µM Primer ($c_1v_1=c_2v_2$)	How much 10 µM primer to add to the final mix
4	CR10b F	100	100	10	1.75	Add 1.75 to the final mix
4	CR10b R	100	100	10	1.75	Add 1.75 to the final mix
5	H7 F	100	100	10	1.75	Add 1.75 to the final mix
5	H7 R	100	100	10	1.75	Add 1.75 to the final mix
6	H10 F	100	100	10	1.75	Add 1.75 to the final mix
6	H10 R	100	100	10	1.75	Add 1.75 to the final mix
7	YI3.1 F	100	100	10	1.75	Add 1.75 to the final mix
7	YI3.1 R	100	100	10	1.75	Add 1.75 to the final mix
E	401 F	100	100	10	1.75	Add 1.75 to the final mix
E	611 R	100	100	10	1.75	Add 1.75 to the final mix

Figure A.9: Primer preparation

4			
Name	Type	C_t	Calculated Concentration (ng/µl)
Standard_1	Standard	13.90	1.98E-01
Standard_2	Standard	13.34	2.91E-01
Standard_3	Standard	13.32	2.96E-01
Standard_4	Standard	12.73	4.45E-01
Standard_5	Standard	12.60	4.86E-01
Trial 2_4.1	Unknown	14.77	1.09E-01
Trial 2_4.2	Unknown	13.31	2.98E-01
Trial 2_4.3	Unknown	15.12	8.53E-02
Trial 3_4.1	Unknown	13.60	2.44E-01
Trial 3_4.2	Unknown	15.20	8.10E-02
Trial 3_4.3	Unknown	14.32	1.48E-01
Trial 1_4.2	Unknown	16.76	2.75E-02
Trial 1_4.3	Unknown	13.05	3.58E-01

5			
Name	Type	C_t	Calculated Concentration (ng/µl)
Standard_1	Standard	16.49	2.04E-01
Standard_2	Standard	15.75	3.21E-01
Standard_3	Standard	15.36	4.10E-01
Standard_4	Standard	15.02	5.05E-01
Standard_5	Standard	14.81	5.75E-01
Trial 2_5.1	Unknown	28.75	1.05E-04
Trial 2_5.2	Unknown	29.45	6.79E-05
Trial 2_5.3	Unknown	29.93	5.06E-05
Trial 3_5.1	Unknown	26.07	5.48E-04
Trial 3_5.2	Unknown	29.68	5.89E-05
Trial 3_5.3	Unknown	30.5	3.55E-05
Trial 1_5.2	Unknown	20.99	1.27E-02
Trial 1_5.3	Unknown	24.69	1.29E-03

6			
Name	Type	C_t	Calculated Concentration (ng/µl)
Standard_1	Standard	12.78	1.98E-01
Standard_2	Standard	12.16	2.93E-01
Standard_3	Standard	11.65	4.07E-01
Standard_4	Standard	11.29	5.12E-01
Standard_5	Standard	11.14	5.64E-01
Trial 2_6.1	Unknown	24.15	1.34E-04
Trial 2_6.2	Unknown	23.62	1.87E-04
Trial 2_6.3	Unknown	23.37	2.21E-04
Trial 3_6.1	Unknown	22.33	4.29E-04
Trial 3_6.2	Unknown	22.79	3.21E-04
Trial 3_6.3	Unknown	21.91	5.63E-04
Trial 1_6.2	Unknown	22.10	4.98E-04
Trial 1_6.3	Unknown	21.92	5.58E-04

7			
Name	Type	C_t	Calculated Concentration (ng/µl)
Standard_1	Standard	15.79	2.01E-01
Standard_2	Standard	15.04	3.04E-01
Standard_3	Standard	14.68	3.71E-01
Standard_4	Standard	13.98	5.49E-01
Standard_5	Standard	13.88	5.80E-01
Trial 2_7.1	Unknown	8.99	8.77E+00
Trial 2_7.2	Unknown	9.28	7.46E+00
Trial 2_7.3	Unknown	8.99	8.77E+00
Trial 3_7.1	Unknown	10.37	4.08E+00
Trial 3_7.2	Unknown	10.81	3.19E+00
Trial 3_7.3	Unknown	11.00	2.87E+00
Trial 1_7.2	Unknown	10.69	3.41E+00
Trial 1_7.3	Unknown	11.86	1.78E+00

E			
Name	Type	C_t	Calculated Concentration (ng/µl)
Standard_1	Standard	14.48	3.21E-01
Standard_2	Standard	14.39	3.39E-01
Standard_3	Standard	13.28	7.03E-01
Standard_4	Standard	12.74	9.99E-01
Standard_5	Standard	12.04	1.57E+00
Trial 1_start	Unknown	33.02	1.79E-06
Trial 1_start	Unknown	31.71	4.19E-06
Trial 1_start	Unknown	31.33	5.37E-06
Trial 2_start	Unknown	30.45	9.53E-06
Trial 2_start	Unknown	29.68	1.57E-05
Trial 2_start	Unknown	30.53	9.10E-06
Trial 3_start	Unknown	31.57	4.60E-06
Trial 3_start	Unknown	30.35	1.02E-05
Trial 3_start	Unknown	25.35	2.66E-04
Trial 3_4.1	Unknown	22.00	2.37E-03
Trial 3_4.2	Unknown	28.47	3.47E-05
Trial 3_4.3	Unknown	29.02	2.43E-05
Trial 3_5.1	Unknown	18.13	2.96E-02
Trial 3_5.2	Unknown	25.34	2.67E-04
Trial 3_5.3	Unknown	26.28	1.45E-04
Trial 3_6.1	Unknown	22.47	1.74E-03
Trial 3_6.2	Unknown	26.15	1.58E-04
Trial 3_6.3	Unknown	28.54	3.32E-05
Trial 3_7.1	Unknown	27.98	4.80E-05
Trial 3_7.2	Unknown	28.16	4.26E-05
Trial 3_7.3	Unknown	27.46	6.71E-05

Figure A.10: Data generated by the qPCR machine.

A.6 Next generation sequencing supplement data

Concentration in ng/µl

	1	2	3	4	5	6	7	8	9	10	11	12
A	4.32	4.88	2.51	1.99	12.9	2.3	2.14	3.11	33.2	27	39.1	8.51
B	26	17.2	27.6	5.23	21.5	10.8	27.4	16	56	48.2	51	37.3
C	40	20.6	29	17.2	50	25.3	22.4	11.4	51	34.6	35.6	41
D	55	25.6	46.5	24.3	53	55	14.3	20.6	17.8	38.2	46.5	30.8
E	46.8	22.1	46.6	14.7	48.9	58	4.93	4.78	3.01	2.34	40.3	28.6
F	2.63	11.6	12.4	12.6	27.1	21.3	2.33	5.56	33.2	24	26.4	44.3

$$\frac{\text{(concentration in ng/µl)}}{\text{(660 g/mol} \times \text{average library size)}} \times 10^6 = \text{concentration in nM}$$

Concentration in NM

	1	2	3	4	5	6	7	8	9	10	11	12
A	10.3896	11.7364	6.036556	4.78595	31.0245	5.53151	5.14671	7.4795575	79.8461	64.9351	94.0356	20.4666
B	62.5301	41.366	66.378066	12.5782	51.7076	25.974	65.8971	38.480038	134.68	115.921	122.655	89.7066
C	96.2001	49.543	69.74507	41.366	120.25	60.8466	53.8721	27.417027	122.655	83.2131	85.6181	98.6051
D	132.275	61.5681	111.83261	58.4416	127.465	132.275	34.3915	49.54305	42.809	91.8711	111.833	74.0741
E	112.554	53.1506	112.07311	35.3535	117.605	139.49	11.8567	11.495911	7.23906	5.62771	96.9216	68.7831
F	6.32516	27.898	29.82203	30.303	65.1756	51.2266	5.60366	13.371813	79.8461	57.7201	63.4921	106.542

Volume of DNA to add to achieve a concentration of 4 nM

	1	2	3	4	5	6	7	8	9	10	11	12
A	7.7	6.8	13.3	16.7	2.6	14.5	7.8	10.7	1.0	1.2	2.1	3.9
B	1.3	1.9	1.2	6.4	1.5	3.1	1.2	2.1	1.5	1.7	1.6	2.2
C	2.1	1.6	1.1	1.9	1.7	1.3	1.5	2.9	1.6	1.0	2.3	2.0
D	1.5	1.3	1.8	1.4	1.6	0.6	2.3	1.6	1.9	2.2	1.8	1.1
E	1.8	1.5	1.8	2.3	1.7	0.6	6.7	7.0	11.1	7.1	2.1	1.2
F	12.6	2.9	2.7	2.6	1.2	1.6	14.3	6.0	1.0	1.4	3.2	1.9

Red	10 µL
Orange	50 µL
The rest	20 µL

Volume of TRIS buffer to add to achieve a concentration of 4 Nm

	1	2	3	4	5	6	7	8	9	10	11	12
A	12.3	13.2	6.7	3.3	17.4	5.5	2.2	9.3	19.0	18.8	47.9	16.1
B	18.7	18.1	18.8	13.6	18.5	16.9	18.8	17.9	48.5	48.3	48.4	47.8
C	47.9	18.4	18.9	18.1	48.3	18.7	18.5	17.1	48.4	19.0	47.7	48.0
D	48.5	18.7	48.2	18.6	48.4	19.4	17.7	18.4	18.1	47.8	48.2	18.9
E	48.2	18.5	48.2	17.7	48.3	19.4	13.3	13.0	8.9	2.9	47.9	18.8
F	7.4	17.1	17.3	17.4	18.8	18.4	5.7	14.0	19.0	18.6	46.9	48.1

Figure A.11: Calculated DNA concentration in nM, based on the size of DNA amplicons as determined by an Agilent Technologies 2100 Bioanalyzer trace

Listing A.1: Using Docker to demultiplex NGS data

```
1
2 #import data
3
4 docker run -t -i -v C:\Users\Administrator\Desktop\project\Ex1:/data quay.io/
      qiime2/core:2022.8 qiime tools import --type 'SampleData[
      PairedEndSequencesWithQuality]' --input-path fastq_files/ --input-format
       CasavaOneEightSingleLanePerSampleDirFmt --output-path demux-paired-end.
      qza
5
6 #Quality check
```

```
7  docker run -t -i -v C:\Users\Administrator\Desktop\project\Ex1:/data quay.io/
       qiime2/core:2022.8 qiime demux summarize --i-data demux-paired-end.qza
       --o-visualization demux.qzv
8
9  # Paired end (PE), cut adapter and primer sequences
10 docker run -t -i -v C:\Users\Administrator\Desktop\project\Ex1:/data quay.io/
       qiime2/core:2022.8 qiime cutadapt trim-paired --i-demultiplexed-
       sequences demux-paired-end.qza --p-cores 1 --p-front-f
       TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG --p-front-r
       GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC --o-trimmed-
       sequences trimmed-demux-PE.qza --verbose
11
12 # Quality check (QC)
13 docker run -t -i -v C:\Users\Administrator\Desktop\project\Ex1:/data quay.io/
       qiime2/core:2022.8 qiime demux summarize --i-data trimmed-demux-PE.qza
       --o-visualization trimmed-demux-PE.qzv
14
15 #QC Filter with dada2
16 docker run -t -i -v C:\Users\Administrator\Desktop\project\Ex1:/data quay.io/
       qiime2/core:2022.8 qiime dada2 denoise-paired --i-demultiplexed-seqs
       trimmed-demux-PE.qza --o-table table --o-representative-sequences rep-
       seqs --o-denoising-stats dada2-stats.qza --p-trim-left-f 0 --p-trim-left
       -r 0 --p-trunc-len-f 284 --p-trunc-len-r 190 --p-n-threads 0 --verbose
17
18 #Visualize Table
19 docker run -t -i -v C:\Users\Administrator\Desktop\project\Ex1:/data quay.io/
       qiime2/core:2022.8 qiime feature-table summarize --i-table table.qza --o
       -visualization table.qzv --m-sample-metadata-file metadata.txt
20
21 #Visualize dada2 stats
22 docker run -t -i -v C:\Users\Administrator\Desktop\project\Ex1:/data quay.io/
       qiime2/core:2022.8 qiime metadata tabulate --m-input-file dada2-stats.
       qza --o-visualization dada2-stats.qzv
23
24 ###Use the classifer provided, it was trained by targeting V3-V4 region on
       Greengene 13_8 release (clustered 99% sequence similarity)
25
26 #Skip this step- Make ref classifier
27 docker run -t -i -v C:\Users\Administrator\training-feature-classifiers:/data
        quay.io/qiime2/core:2022.8 qiime feature-classifier extract-reads --i-
       sequences v3-v4-classifier.qza --p-f-primer GTGCCAGCMGCCGCGGTAA --p-r-
       primer GGACTACHVGGGTWTCTAAT --p-trunc-len 120 --p-min-length 100 --p-max
       -length 400 --o-reads ref-seqs.qza
28
29 ##Test the Classifier
30 docker run -t -i -v C:\Users\Administrator\Desktop\project\Ex1:/data quay.io/
       qiime2/core:2022.8 qiime feature-classifier classify-sklearn --i-
       classifier v3-v4-classifier.qza --i-reads rep-seqs.qza --o-
       classification GG-taxonomy.qza
31
32 #Skip this step - OR full-lengt weighted classifier
33 docker run -t -i -v C:\Users\Administrator\Desktop\project\Ex1:/data quay.io/
       qiime2/core:2022.8 qiime feature-classifier classify-sklearn --i-
       classifier gg-13-8-99-nb-weighted-classifier --i-reads rep-seqs.qza --o-
       classification GG-taxonomy.qza
34
35 # Visualize taxonomy
36 docker run -t -i -v C:\Users\Administrator\Desktop\project\Ex1:/data quay.io/
       qiime2/core:2022.8 qiime metadata tabulate --m-input-file GG-taxonomy.
```

```
    qza --o-visualization GG-taxonomy.qzv
37
38  #Create a barplot
39  docker run -t -i -v C:\Users\Administrator\Desktop\project\Ex1:/data quay.io/
        qiime2/core:2022.8 qiime taxa barplot --i-table table.qza --i-taxonomy
        GG-taxonomy.qza --m-metadata-file metadata.txt --o-visualization GG-taxa
        -bar-plots.qzv
40
41
42  #Further filtering according to taxa, keep from phylum level and remove
        archaea, chloroplast and mitochondria
43  docker run -t -i -v C:\Users\Administrator\Desktop\project\Ex1:/data quay.io/
        qiime2/core:2022.8 qiime taxa filter-table --i-table table.qza --i-
        taxonomy GG-taxonomy.qza --p-include p__ --p-exclude archaea,
        mitochondria,chloroplast --o-filtered-table table-bacteria.qza
44
45  # Visualize the filtered table containing only bacteria
46  docker run -t -i -v C:\Users\Administrator\Desktop\project\Ex1:/data quay.io/
        qiime2/core:2022.8 qiime feature-table summarize --i-table table-
        bacteria.qza  --o-visualization table-bacteria.qzv --m-sample-metadata-
        file metadata.txt
47
48  #Bar plot on ONLY bacterial composition
49  docker run -t -i -v C:\Users\Administrator\Desktop\project\Ex1:/data quay.io/
        qiime2/core:2022.8 qiime taxa barplot --i-table table-bacteria.qza --i-
        taxonomy GG-taxonomy.qza --m-metadata-file metadata.txt --o-
        visualization Bar-plots-ONLY-bacteria.qzv
50
51
52  #Build Tree
53  docker run -t -i -v C:\Users\Administrator\Desktop\project\Ex1:/data quay.io/
        qiime2/core:2022.8 qiime feature-table filter-seqs --i-data  rep-seqs.
        qza --i-table table-bacteria.qza --o-filtered-data rep-seqs-bacteria-
        ONLY.qza
54
55
56  docker run -t -i -v C:\Users\Administrator\Desktop\project\Ex1:/data quay.io/
        qiime2/core:2022.8 qiime alignment mafft --i-sequences rep-seqs-bacteria
        -ONLY.qza --o-alignment alighend-rep-seqs-bacteria-ONLY.qza
57
58  docker run -t -i -v C:\Users\Administrator\Desktop\project\Ex1:/data quay.io/
        qiime2/core:2022.8 qiime alignment mask --i-alignment alighend-rep-seqs-
        bacteria-ONLY.qza --o-masked-alignment masked-alighend-rep-seqs-bacteria
        -ONLY.qza
59
60  docker run -t -i -v C:\Users\Administrator\Desktop\project\Ex1:/data quay.io/
        qiime2/core:2022.8 qiime phylogeny fasttree --i-alignment masked-
        alighend-rep-seqs-bacteria-ONLY.qza --o-tree unrooted-masked-alighend-
        rep-seqs-bacteria-ONLY.qza
61
62  docker run -t -i -v C:\Users\Administrator\Desktop\project\Ex1:/data quay.io/
        qiime2/core:2022.8 qiime phylogeny midpoint-root --i-tree unrooted-
        masked-alighend-rep-seqs-bacteria-ONLY.qza --o-rooted-tree rooted-masked
        -alighend-rep-seqs-bacteria-ONLY.qza
63
64  #Beta Diversity (Phylogenetic Diversity)
65  docker run -t -i -v C:\Users\Administrator\Desktop\project\Ex1:/data quay.io/
        qiime2/core:2022.8 qiime diversity core-metrics-phylogenetic --i-
        phylogeny rooted-masked-alighend-rep-seqs-bacteria-ONLY.qza --i-table
```

```
    table-bacteria.qza --p-sampling-depth 10000 --m-metadata-file metadata.
    txt  --output-dir core-metrics-results-d10000
66
67
68  #Alpha Diversity
69  #rarefied table visualization
70
71  docker run -t -i -v C:\Users\Administrator\Desktop\project\Ex1:/data quay.io/
        qiime2/core:2022.8 qiime feature-table summarize --i-table core-metrics-
        results-d10000/rarefied_table.qza --o-visualization core-metrics-results
        -d10000/rarefied_table.qzv --m-sample-metadata-file metadata.txt
72
73  #chatgpt
74  docker run -t -i -v C:\Users\Administrator\Desktop\project\Ex1:/data quay.io/
        qiime2/core:2022.8 qiime diversity alpha --i-table table.qza --p-metric
        shannon --o-alpha-diversity shannon_alpha_diversity.qza
75
76  docker run -t -i -v C:\Users\Administrator\Desktop\project\Ex1:/data quay.io/
        qiime2/core:2022.8 qiime metadata tabulate --m-input-file
        shannon_alpha_diversity.qza --o-visualization
        shannon_alpha_diversity_table.qzv
77
78  #Boxplot
79  docker run -t -i -v C:\Users\Administrator\Desktop\project\Ex1:/data quay.io/
        qiime2/core:2022.8 qiime diversity alpha-group-significance --i-alpha-
        diversity shannon_alpha_diversity.qza --m-metadata-file metadata.txt --o
        -visualization shannon_alpha_diversity_boxplot.qzv
80
81
82  #shannon diversity
83  docker run -t -i -v C:\Users\Administrator\Desktop\project\Ex1:/data quay.io/
        qiime2/core:2022.8 qiime tools export --input-path core-metrics-results-
        d10000/shannon_vector.qza --output-path core-metrics-results-d10000/
        Diversity_indexes
```

A.7 Observations of RTE salad mixes during shelf-life

A.7.1 Trial 1: Culture-dependent analysis of the RTE salad mixes inoculated with probiotic strains

The visual quality of the salad packages was scored on a scale from poor to excellent.

Table A.3: Visual quality of mixed leafy greens injected with probiotics throughout the shelf-life. The colour, texture, wilting, and presence of mould or decay were observed

Shelf life	Package Number	Bacterial Strain	Visual Quality	Comments
Day 1	4.1, 4.2, 4.3	4	Excellent	Leaves look fresh and healthy
	5.1, 5.2, 5.3	5	Excellent	
	6.1, 6.2, 6.3	6	Excellent	
	7.1, 7.2, 7.3	7	Excellent	
	M.1, M.2, M.3	M	Excellent	
	C.1, C.2, C.3	C	Excellent	
Day 4	4.1, 4.2, 4.3	4	Excellent	Leaves look fresh and healthy
	5.1, 5.2, 5.3	5	Excellent	
	6.1, 6.2, 6.3	6	Excellent	
	7.1, 7.2, 7.3	7	Excellent	
	M.1, M.2, M.3	M	Excellent	
	C.1, C.2, C.3	C	Excellent	
Day 8	4.1, 4.2, 4.3	4	Good	M & C - Yellow color leaves were observed
	5.1, 5.2, 5.3	5	Excellent	
	6.1, 6.2, 6.3	6	Excellent	
	7.1, 7.2, 7.3	7	Good	
	M.1, M.2, M.3	M	Good	
	C.1, C.2, C.3	C	Fair	
Day 12	4.1, 4.2, 4.3	4	Fair	5 & 6 - After 4 days from the expiration date, the leaves were fresh and healthy
	5.1, 5.2, 5.3	5	Good	
	6.1, 6.2, 6.3	6	Good	
	7.1, 7.2, 7.3	7	Fair	
	M.1, M.2, M.3	M	Poor	
	C.1, C.2, C.3	C	Poor	

Day 1 – First day of shelf-life

Figure A.12: Image for Day 1

Day 8 – Best before date

XX

Figure A.13: Image for Day 8

Figure A.14: Image for Day 12