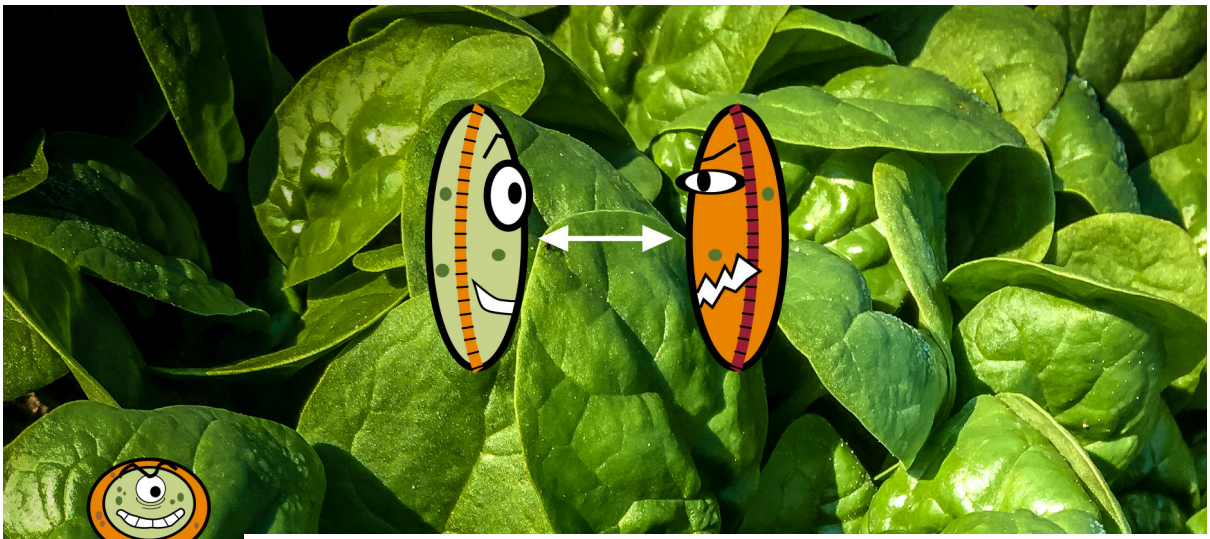
Microbiological hygiene and biological control of leafy green vegetables

Uhlig, Elisabeth

2021

Document Version:
Publisher's PDF, also known as Version of record



Microbiological hygiene and biological control of leafy green vegetables

ELISABETH UHLIG
DEPARTMENT OF FOOD TECHNOLOGY, ENGINEERING AND NUTRITION | LUND UNIVERSITY

Printed by Media-Tryck, Lund 2021 NORDIC SWAN ECOLABEL 3041 0903

Department of Food Technology, Engineering and Nutrition
Faculty of Engineering
Lund University

ISBN 978-91-7422-810-6

Microbiological hygiene and biological control of leafy green vegetables

Microbiological hygiene and biological control of leafy green vegetables

Elisabeth Uhlig

LUND
UNIVERSITY

DOCTORAL DISSERTATION

by due permission of the Faculty of Engineering at Lund University, Sweden.
To be defended in hall KC:G at Kemicentrum, Naturvetarvägen 14, Lund at 11th of June 2021 at 09.15.

Faculty opponent
Gro Johannessen, Senior research Scientist, PhD

Research Food Safety and Animal Health, Norwegian Veterinary Institute

Organization LUND UNIVERSITY	Document name **Doctoral dissertation**
	Date of issue 2021-05-18
Author(s) Elisabeth Uhlig	Sponsoring organization Swedish Research Council Formas

Title and subtitle Microbiological hygiene and biological control of leafy green vegetables

Abstract

Fruit and vegetables are vital components of a healthy diet, and international strategies to encourage their consumption are in place (FAO/WHO, 2005). Ready-to-eat (RTE) leafy green vegetable products have exploded in popularity, and are a convenient and attractive way to add greens to the plate. Unfortunately, sales numbers are accompanied by increasing numbers of food-borne illness outbreaks.

This thesis focuses on the microbial hygiene from a consumer's perspective, followed by application and evaluation of biological control as a mean of reducing the risk of food-borne illness.

When studying the efficacy of two different household washing methods to reduce the bacterial load on leafy green vegetables, it was seen that only after rinsing at high water velocity (8 L/min), after five repetitions, the bacterial load decreased with 90 %. The treatment disintegrated the leaves, and still left the produce with high amounts of culturable bacteria. These results highlight the inefficiency of tap water washing methods available for the consumer.

Consumer habits are also important to consider when assessing the microbial hygiene of food products. Packages of RTE leafy green products were opened, stored at 7 ˚C and compared with unopened bags. The total aerobic count from different producers varied greatly and no correlation to opened bags could be made. Neither could bacterial levels be linked to a certain producer or product type.

Inoculation with *E. coli* strains indicate that the type strain is able to survive, but not multiply, in household conditions. However, wild strains of *E. coli* were seen to multiply at different time-points during the shelf-life period, adapting to cold storage conditions. This varying and unpredictable bacterial status of ready to eat leafy green products calls for new strategies to reduce unwanted microorganisms and prevent food-borne illness.

By the means of biological control, bacteria can be used to counteract food safety hazards. Therefore, isolates antagonistic to *E. coli* have been isolated and evaluated in an industrial field production setting. Selected isolates showing antagonism in vitro were coated onto spinach seeds before planting. Next generation sequencing analysis revealed that the microbiota of the plants inoculated with the selected strains was altered in a beneficial direction, and a reduction of *Escherichia-Shigella* could be seen during the development from seed to plant.

As a tentative safety evaluation of the selected strains for biological control, an individual comparison for immunomodulating effects in mice was made. The two *Bacillus coagulans* strains consistently resemble the response of untreated animals, which must be considered a positive trait. The strain of *Pseudomonas punonensis* had a weaker influence on the immune system, while the *Pseudomonas cedrina* strain and the *Rhocococcus cerastii* strain induced inflammatory responses. The *P. punonensis* strain and one *B. coagulans* strain increased the microbiota diversity, which is correlated to host health.

These results encourage the usage of bacterial antagonists as part of the solution to reduce the risk of human pathogens on leafy green vegetables.

Key words Leafy green vegetables, *Escherichia coli*, Native microbiota, Biological control, Food hygiene

Classification system and/or index terms (if any)

Supplementary bibliographical information		**Language** English
ISSN and key title		**ISBN printed version:** 978-91-7422-810-6 **ISBN e-version:** 978-91-7422-811-3
Recipient's notes	**Number of pages** 73	Price
	Security classification	

Signature  Date 2021-04-29

Microbiological hygiene and biological control of leafy green vegetables

Elisabeth Uhlig

LUND
UNIVERSITY

Coverphoto by Elisabeth Uhlig

Faculty of Engineering
Department of Food Technology, Engineering and Nutrition

ISBN printed version: 978-91-7422-810-6
ISBN e-version: 978-91-7422-811-3

Printed in Sweden by Media-Tryck, Lund University
Lund 2021

Media-Tryck is a Nordic Swan Ecolabel certified provider of printed material. Read more about our environmental work at www.mediatryck.lu.se

MADE IN SWEDEN

"Aim for the sky, but move slowly,
enjoying every step along the way.
It's all those little steps
that make the journey complete"
– Chanda Kochhar

Table of Contents

Abstract

Fruit and vegetables are vital components of a healthy diet, and international strategies to encourage their consumption are in place (FAO/WHO, 2005). Ready-to-eat (RTE) leafy green vegetable products have exploded in popularity, and are a convenient and attractive way to add greens to the plate. Unfortunately, sales numbers are accompanied by increasing numbers of food-borne illness outbreaks.

This thesis focuses on the microbial hygiene from a consumer's perspective, followed by application and evaluation of biological control as a mean of reducing the risk of food-borne illness.

When studying the efficacy of two different household washing methods to reduce the bacterial load on leafy green vegetables, it was seen that only after rinsing at high water velocity (8 L/min), after five repetitions, the bacterial load decreased with 90 %. The treatment disintegrated the leaves, and still left the produce with high amounts of culturable bacteria. These results highlight the inefficiency of tap water washing methods available for the consumer.

Consumer habits are also important to consider when assessing the microbial hygiene of food products. Packages of RTE leafy green products were opened, stored at 7 ˚C and compared with unopened bags. The total aerobic count from different producers varied greatly and no correlation to opened bags could be made. Neither could bacterial levels be linked to a certain producer or product type.

Inoculation with *E. coli* strains indicate that the type strain is able to survive, but not multiply, in household conditions. However, wild strains of *E. coli* were seen to multiply at different time-points during the shelf-life period, adapting to cold storage conditions. This varying and unpredictable bacterial status of ready to eat leafy green products calls for new strategies to reduce unwanted microorganisms and prevent food-borne illness.

By the means of biological control, bacteria can be used to counteract food safety hazards. Therefore, isolates antagonistic to *E. coli* have been isolated and evaluated in an industrial field production setting. Selected isolates showing antagonism *in vitro* were coated onto spinach seeds before planting. Next generation sequencing analysis revealed that the microbiota of the plants inoculated with the selected strains was altered in a beneficial direction, and a reduction of *Escherichia-Shigella* could be seen during the development from seed to plant.

As a tentative safety evaluation of the selected strains for biological control, an individual comparison for immunomodulating effects in mice was made. The two *Bacillus coagulans* strains consistently resemble the response of untreated animals, which must be considered a positive trait. The strain of *Pseudomonas punonensis* had a weaker influence on the immune system, while the *Pseudomonas cedrina* strain and the *Rhocococcus cerastii* strain induced inflammatory responses. The *P. punonensis* strain and one *B. coagulans* strain increased the microbiota diversity, which is correlated to host health.

These results encourage the usage of bacterial antagonists as part of the solution to reduce the risk of human pathogens on leafy green vegetables.

Populärvetenskaplig sammanfattning

Frukt och grönt är viktiga komponenter i en hälsosam diet, och Livsmedelsverket rekommenderar ett intag av fem knytnävsstora portioner, ett halvt kilo, om dagen. Ett bekvämt och snabbt sätt att få i sig grönsaker är förpackade, färdigsköljda salladsblandningar. Dessa produkter har ökat lavinartat i popularitet det senaste årtiondet, men tyvärr har även antalet fall av matförgiftning relaterat till dessa produkter gjort detsamma. Trots förebyggande åtgärder är det lätt att under produktionsprocessen få in oönskade bakterier, från fältet där produkten odlas, samt vid hantering, tvättning och packning. Samtidigt äts produkten utan upphettning, och som det oftast rekommenderas på förpackningen – utan sköljning.

I detta avhandlingsarbete undersöktes först huruvida det finns möjlighet för konsumenten att påverka hur mycket och vilka bakterier vi får i oss från bladgrönsaker, och om konsumenten kan skölja bort bakterierna. I den första studien visade det sig vara svårt att skölja bort bakterierna från bladgrönsakerna. Endast vid det högsta vattenflödet, 8 L/min, som var så högt att bladen tog synbar skada, noterades en minskning. Efter fem upprepade tvättningar minskade bakteriehalten med 90 %. Det kan tyckas mycket, men med tanke på de höga utgångsvärdena på bladgrönsakerna, så innebar en 90 %-ig reduktion att ca 20 miljoner bakterier per gram fortfarande fanns kvar i produkten.

I den andra studien undersöktes om bakterieinnehållet kan påverkas negativt av att konsumenten öppnar och försluter påsen vid upprepade tillfällen under hållbarhetstiden, genom att jämföra öppnade förpackningar med oöppnade. Babyspenat, rucola och salladsmix från olika producenter analyserades, men det gick inte att koppla bakteriehalt till varken en viss typ av produkt eller en viss producent. Bakterierna verkade inte heller påverkas nämnvärt av om påsen var öppnad eller inte, oavsett om produkten innehöll skyddande atmosfär.

E. coli tillsattes till produkterna och tillväxten följdes under lagring fram till bäst-före datum. Olika stammar av *E. coli* visade på olika tillväxtmönster och det kunde observeras att stammarna som hittats på sallat från början kunde växa i kyl, i motsats till typiska *E. coli*.

Resultaten visar att det är svårt för konsumenten att göra något åt situationen med de höga bakterietalen på färdigförpackade bladgrönsaker. Bakteriehalterna varierar mycket mellan likvärdiga produkter och får man in bakterier i produktionen så är de svåra att få bort. Desto viktigare blir då de åtgärder som

görs för att minska risken att få in skadliga bakterier i produktionen, som att använda rent vatten och rena maskiner, och tillämpa bra personhygien. Men trots sådana åtgärder fortsätter sjukdomsutbrotten. I nästa steg av projektet undersöktes därför om det går att göra något åt saken på bakterienivå. Biologisk kontroll är ett koncept där bland annat så kallade ”goda” mikroorganismer används för att motverka ”elaka”, sjukdomsframkallande mikroorganismer. Konceptet används främst för att skydda växande gröda. Att använda biologisk kontroll på grönsaker för att skydda människor från sjukdomsframkallande bakterier är ett relativt outforskat område. I den tredje studien isolerades mikroorganismer som kan motverka *E. coli*, en av de vanligaste bakterierna på bladgrönsaker som orsakar matförgiftning. Många olika bakterier som kunde hindra tillväxt av *E. coli* i ett laboratorietest identifierades, och en handfull av dessa testades under produktionsförhållanden för att undersöka om bakteriesammansättningen på bladen kunde påverkas. Några bakteriestammar valdes ut och applicerades på spenatfrön, som sedan såddes i fält. Bakteriesammansättningen på både frön och plantor förändrades då till det bättre och potentiella sjukdomsframkallande bakterier byttes ut mot betydligt snällare arter. De frön som naturligt innehöll *E. coli* resulterade i plantor som var fria från *E. coli*.

Naturligtvis måste bakterier som används i naturen och för matproduktion vara helt säkra. Stammar till fältförsöket valdes ut baserat på att de inte skulle vara kända för att orsaka sjukdom, men för att säkerställa detta designades en studie där bakteriernas påverkan på immunförsvaret och tarmfloran undersöktes i friska möss. Djuren behandlades först med *E. coli* och antibiotika för att göra tarmfloran mer homogen och lik människans och därefter fick de bakteriestammarna i dricksvattnet. Populationerna av immunceller hos grupperna som fick två av stammarna (*Bacillus coagulans*) liknade populationerna hos gruppen som inte fick någon behandling alls. Två andra stammar (*Pseudomonas cendrina* och *Rhodococcus cerastii*) resulterade å andra sidan i ett inflammatoriskt präglat immunsvar, medan den sista stammen (*Pseudomonas punonensis*) inte påverkade immunförsvaret. *P. punonensis* och *B. coagulans* ökade tarmflorans mångfald, vilket kan anses som en positiv hälsofaktor. Denna säkerhetsstudie tyder på att stammarna av *B. coagulans* och *P. punonensis* utan risk borde kunna användas för biologisk kontroll på bladgrönsaker.

Ytterligare säkerhetstest efter nationella och EU gemensamma riktlinjer behöver nu genomföras för att kunna använda dessa stammar kommersiellt. Nästa steg är att applicera bakterierna på ett produktionseffektivt sätt, till exempel på frön eller i förpackningsmaterial, så att bladgrönsaker kan göras säkra för konsumtion.

List of papers

Paper I

Uhlig, E., Olsson, C., He, J., Stark, T., Sadowska, Z., Molin, G., Ahrné, S., Alsanius, B., Håkansson, Å. Effects of household washing on bacterial load and removal of *Escherichia coli* from lettuce and "ready-to-eat" salads. Food Sci. Nutr. 2017; 00:1-6

Paper II

Uhlig, E., Kjellström, A., Oscarsson, E., Nurminen, N., Nabila, Y., Paulsson, J., Lupan, T., Velpuri, N. S. B. P, Alsanius, B., Molin, G., Håkansson, Å. The effect of household conditions on the microbial hygiene of ready-to-eat vegetable products cultivated in Europe. *In manuscript.*

Paper III

Uhlig, E., Kjellström, A. Nurminen, N., Olsson, C., Oscarsson, E., Canaviri-Paz, P., Mogren, L. Alsanius, B. Molin, G., Håkansson, Å. *Escherichia coli* antagonistic bacteria for biocontrol of spinach: a field trial. *In manuscript.*

Paper IV

Uhlig, E., Elli, G., Nurminen, N., Oscarsson, E., Canaviri-Paz, P., Burri, S., Rohrstock, A-M., Rahman, M., Alsanius, B., Molin, G., Zeller, K. S., Håkansson, Å. Comparative immunomodulatory effects in mice and in human dendritic cells of five bacterial strains selected for biocontrol of leafy green vegetables. *In manuscript.*

Author´s contribution to the papers

Paper I

The author, E. Uhlig, coordinated and performed the experimental work together with J. He, T. Stark and Z. Sadowska. EU evaluated the results and wrote the paper.

Paper II

The author, E. Uhlig, coordinated and performed the experimental work together with E. Oscarsson, N. Nurminen, Y. Nabila, J. Paulsson, T. Lupan and N. S. B. P Velpuri. NGS data analysis was performed by A. Kjellström. EU evaluated the results and wrote the paper.

Paper III

The author, E. Uhlig, coordinated and performed the field trial together with N. Nurminen, E. Oscarsson and P. Canaviri-Paz. EU performed the *in vitro* experiments. Data analysis was performed by A. Kjellström. EU evaluated the results and wrote the paper.

Paper IV

The author, E. Uhlig, coordinated and performed the *in vivo* trial analysis together with N. Nurminen, E. Oscarsson, P. Canaviri-Paz, S. Burri and Å. Håkansson. EU performed flow cytometry analysis together with N. Nurminen, E. Oscarsson, P. Canaviri-Paz, S. Burri and M. Rahman. EU performed the MPO analysis and the T-RFLP analysis. EU performed the multiplex cytokine/chemokine analysis together with E. Oscarsson and A-M Rohrstock. The *in vitro* trial was performed by G. Elli and K. S. Zeller. EU evaluated the results and wrote the paper in collaboration with G. Elli and K. S. Zeller.

Abbreviations

ASV, amplicon sequence variant

CDC, Centres for Disease Control and Prevention

EHEC, enterohemorragic *Escherichia coli*

GMP, good manufacturing practice

LGV, leafy green vegetables

LPS, lipopolysaccharides

MAP, modified atmosphere packaging

MPO, myeloperoxidase

NGS, next generation sequencing

PCR, polymerase chain reaction

RTE, ready-to-eat

SPRI, solid phase reversible immobilization

STEC, Shiga toxin-producing *E. coli*

T-RF, terminal restriction fragment

T-RFLP, terminal restriction fragment length polymorphism

TLR2, toll-like receptor 2

TLR4, toll-like receptor 4

TSA, tryptic soy agar

UniFrac, unique fraction metric

VRBD, violet red bile agar

Introduction

Leafy green vegetables are a great source of vitamins, minerals and fibers, and part of a healthy diet. Consumer's surge for fresh, minimally processed vegetables have raised the production of ready-to-eat (RTE) leafy greens to unimaginable levels (FAO/WHO, 2008). RTE leafy greens are sold in a convenient package, already washed and ready to be served, thereby providing an attractive way to add greens to the plate. Unfortunately, food-borne illness incidents related to these products are increasing as well (EFSA, 2013). Leafy greens grow close to the soil and are not peeled or cooked. It can easily be contaminated by for example soil, fertilizers, irrigation water, wild and domestic animals, harvesting equipment, manual handling, rinsing water and processing equipment. Once contamination has occurred, it is very difficult to remove (Allende, Selma, López-Gálvez, Villaescusa, & Gil, 2008).

At present, a well performed risk analysis, regular testing, and good manufacturing practice (GMP) are the best tools at hand to prevent unwanted microorganisms from entering the produce. However, due to its susceptibility to contamination, leafy greens continue to be vectors for serious pathogens, such as *Salmonella enterica*, *Listeria monocytogenes* and Enterohaemorrhagic *Escherichia coli* O157:H7 (EHEC). These are the pathogens that create big headlines, but leafy greens also contain bacteria on a normal basis that are well known for being opportunistic pathogens, and for carrying antibiotics resistance genes (Campos et al., 2013; Hunter, Hand, Pink, Whipps, & Bending, 2010; Jackson, Randolph, Osborn, & Tyler, 2013; G. Lopez-Velasco, Carder, Welbaum, & Ponder, 2013). Knowledge on the survival and growth of potential pathogens originating from these products is essential to the risk assessment.

There is a strong need for new strategies that can complement existing tools to improve the microbial hygiene on leafy greens. One attractive approach is biological control, where antagonistic bacteria can be used to counteract pathogens. Studies indicate that the background microbiota may be important in deciding the fate of invading pathogens (Jablasone, Warriner, & Griffiths, 2005; Johnston, Harrison, & A Morrow, 2009).

This thesis investigates the microbial hygiene of leafy green vegetables from a consumer's perspective, and screens its native microbiota to find bacterial strains

that are able to prevent the growth of *E. coli.* The antagonistic strains are then evaluated in the field, and tested for safety *in vivo* in mice.

Overview of thesis

An overview how the papers included in this thesis are related can be seen in Figure 1. Paper I and II investigates the microbiological hygiene of leafy green vegetable products from a consumer's point of view. Paper I focuses on the effect of household washing on the culturable part of the microbiota of leafy greens. Paper II investigates the microbiota changes throughout the expiration time under simulated household handling. Paper III aims to find a solution by isolating bacteria antagonistic to *E. coli in vitro* and evaluating their effect on the microbiota of leafy greens in the field. Paper IV evaluates the effects of the selected candidates for biocontrol on the immune system and microbiota in mice.

The study on human monocyte derived dendritic cells in paper IV was contributed by the department of Immunotechnology at Lund University, and is not included in this thesis.

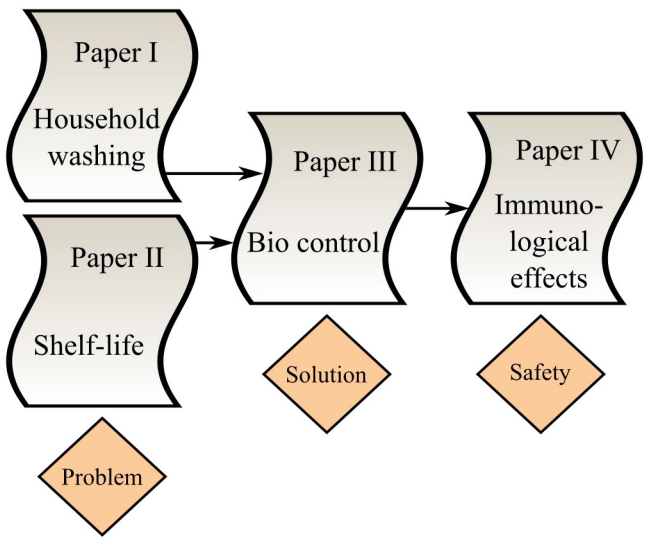


Figure 1: Overview of papers included in the thesis

Background

Leafy green vegetables as vectors of food-borne illness

Leafy vegetables, also called leafy greens, are plant leaves eaten as a vegetable, either cooked or raw. There are over 1000 species of plants with edible leaves, for example different types of herbs, lettuce, spinach, Swiss chard and kale. Most species are high in dietary fibre, vitamin C, pro-vitamin A carotenoids, folate, manganese and vitamin K and considered to contribute to the health benefits of eating vegetables, such as lowering the risk of cardiovascular diseases and certain cancers (Hung, Huang, Wu, Hsu, & Wu, 2004; Link & Potter, 2004; Singh, Kawatra, & Sehgal, 2001). The global production of lettuce and chicory have increased from 11.6 million tons in 1990 to 26.3 million tons in 2018. Spinach production has increased even more, from 4.1 million tons to 27.3 million tons (Figure 2) and it is expected to continue to raise in the future (FAO/WHO, 2008). The consumption of fresh vegetables in Sweden increase at a steady rate, and has more than doubled to 45 kg per person and year since 1980 (Agriculture, 2019). Ready-to-eat leafy greens are very convenient to use and adds nutritional value to the plate. The number of available ready-to-eat products based on leafy green vegetables have exploded in recent years. One of the largest retail chains in Sweden have increased the sales of ready-to-eat bags from 600,000 in 2005 to almost 40 million bags in 2016 (Söderqvist et al., 2019).

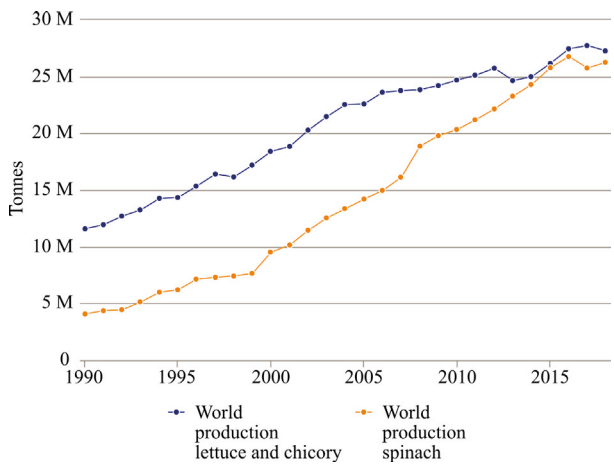


Figure 2: World production of lettuce and chicory, and spinach. ((FAO), 2020)

The microbiota of leafy greens

The spermosphere (the area that surrounds a germinating seed) and the phyllosphere (the above-ground portion of the plant) serves as a rich habitat for microorganisms. G. Lopez-Velasco et al. (2013) analysed the microbiota of spinach seeds upon emergence in a soilless environment. Using high-throughput sequencing, they saw that the spermosphere microbiota consisted of three different phyla; Proteobacteria (76.4%), Firmicutes (22.5 %) and Actinobacteria (1.8 %). As the seeds germinated and developed into plants, the relative abundance of Firmicutes decreased to 0.8 %, and the relative abundance of Proteobacteria increased to 97.1 %. Community richness markedly increased during the transition from seed to seedling (250 different OTUs on seeds vs 800 OTUs for plants), highlighting the influence of external sources to shape the microbiota on the plant. In a sterile sprouting environment, Barret et al. (2015) saw that community richness of different *Brassicaceae* members decreased upon emergence. A spatial separation in β-diversity between seeds and seedling was also seen, indicating a taxonomically different microbiota. Also here, the spermosphere microbiota consisted mainly of Proteobacteria, Firmicutes and Actinobacteria, and upon emergence, the relative abundance of Proteobacteria increased, mainly represented by the genus *Pseudomonas*.

When analysing the microbiota of leafy greens at harvest, Proteobacteria is the most dominant phyla followed by Bacteroidetes, Firmicutes and Actinobacteria (Dees, Lysøe, Nordskog, & Brurberg, 2015; Gabriela Lopez-Velasco, Welbaum, Boyer, Mane, & Ponder, 2011; Tatsika, Karamanoli, Karayanni, & Genitsaris, 2019). Dees et al. (2015) found that the most common genus on lettuce and rocket was *Pseudomonas*, followed by *Duganella* and *Pantoea.* Previous studies have also detected differences in the microbiota between plant species due to morphological and chemical differences (Mogren et al., 2018; Tatsika et al., 2019). Tatsika et al. (2019) saw substantial differences in the relative abundances of *Proteobacterium* and *Firmicutes*, which was 61.4 and 20.5% respectively on rocket, and 78.3 and 8.4% on spinach. Interspecies variation in microbiota, morphology and production of bioactive compounds are seen as factors contributing to differences in susceptibility to *E. coli* O157:H7 contamination (Darlison et al., 2019). Hunter et al. (2010) even detected interspecies variations, as *Erwinia* and *Enterobacter* species differed between lettuce (*Lactuca sativa*) assessions.

Contamination through the production chain

The production process of leafy green vegetables involves many factors that may influence the microbiota. Systems to produce leafy greens vary widely worldwide,

with everything from simple open field culturing to advanced protected indoor culture systems, each with its own food safety challenges. The production site can be contaminated both directly as a result of domestic animals and wildlife entering the fields, and indirectly through faecal waste, water, aerosols and dust. Wildlife may also transfer pathogens from other sites such as landfill or wastewater treatment sites. Other factors such as prior use of the fields, flooding, seed and crop selection, fertilizers and irrigation water source are all important factors that need to be considered. Poor hygiene of farm workers, food handlers, machinery and equipment may also be sources or vehicles for contamination. Post-harvest operations that include mechanical injuring of plant tissue constitute additional risks, as pathogens may be internalized into plant tissue (FAO/WHO, 2008; Solomon & Matthews, 2005). Industrial washing is used to remove soil and other solid particles before packaging. In fact, a washing step does not reduce bacterial load, but may rather worsen the microbial hygiene by increasing the load of spoilage microorganisms and may potentially introduce pathogens. Darlison et al. (2019) analysed the microbial load of spinach leaf samples from an industrial production facility that used frequently exchanged potable water for washing. They found that the leaves were contaminated with *E. coli* and concluded that the process may be a source of bacterial contamination, providing an efficient route for microorganisms to travel within large amounts of produce.

After packaging, the single most important factor contributing to increased microbial load of the product is storage temperature. However, low temperature during storage and distribution does not reduce the risk; it only prevents a further increase in risk (FAO/WHO, 2008).

Pre-washed, pre-cut, ready-to-eat leafy green vegetable products are highly perceptive to contamination since they are subjected to post-harvest operations at a higher degree, and often contain cut and damaged leaves. To maintain visual and sensory quality and create a long shelf-life of these popular products, modified atmosphere packaging (MAP) is often used by adding CO_2 or nitrogen gas (N_2) (Mogren et al., 2018). However, MAP can be associated with food safety risks as it may decrease growth of spoilage microorganisms while most pathogens continue to grow, therefore giving the consumer no indication that the product may be harmful to consume (Farber, 1991).

Once the product has been contaminated with pathogens, it is very difficult to remove (Allende et al., 2008). It is therefore of utmost importance to consider all hygienic weak points in the production chain, and by all means prevent contaminating bacteria to enter.

Pathogens on leafy green vegetables

The most prevalent pathogenic bacteria on leafy green vegetables are *E. coli, Campylobacter* spp.*, Salmonella enterica, Listeria monocytogenes* and *Yersinia enterocolitica,* where *E. coli* is the most frequently occurring one (FAO/WHO, 2008). *E. coli* is a species that commonly inhabits the human gastrointestinal tract, and most strains are only opportunistic pathogens, however, a few strains have aggressive invasive abilities and some can produce deleterious toxins. Pathogenic *E. coli* can be categorized into groups according to their virulence mechanism, e.g. enteropathogenic, enterotoxigenic and Shiga toxin-producing *E. coli* (STEC). STEC*,* where the serogroup *E. coli* O157 is the most common, can exist in the intestines of cattle and other ruminants without the animals exhibiting any signs of disease, however it can cause severe disease in humans. Especially in young children and elderly people, STEC can cause haemorrhagic enteric disease (bloody diarrhoea), and sometimes haemolytic uremic syndrome (low red blood cells and acute kidney failure) and death (WHO, 2017).

Analysis of ready-to-eat leafy green vegetables in Finland have shown that the quality and safety is often insufficient; *E. coli* was found in 15 % of the samples, and Shiga toxin-producing *E. coli* (STEC), *Yersinia enterocolitica* and *Listeria monocytogenes* were also detected in a few samples (Nousiainen, Joutsen, Lundén, Hänninen, & Fredriksson-Ahomaa, 2016). In a big survey conducted in the USA, a total of 14,183 samples of leafy greens were analysed for pathogens, and STEC was found in 0.01 %, and non-STEC in 0.07 % of the samples (G. Zhang et al., 2018). A Swedish national survey of imported leafy greens (90 % originated from Thailand) revealed that of 493 samples, 3.7 % were positive for *Salmonella*, 0.2 % for *Campylobacter*, and 30 % contained *E. coli*. Basil, coriander and mint were more often contaminated with *E. coli* than other products (Karnehed & Lindblad, 2010). In 2014, another survey revealed that *E. coli* was found in 68 (39 %) out of 176 imported leafy green samples, and in 14 (30 %) out of 46 Swedish samples (Egervärn & Flink, 2014).

Food illness outbreaks related to leafy green vegetables

Centres for Disease Control and Prevention (CDC) in the USA has estimated that approximately one in six Americans may experience some form of foodborne illness each year (Centers for Disease Control and Prevention, 2020). In Sweden, the estimated number is one in 20 (Norling, 1994; Toljander & Karnehed, 2010).

Leafy vegetables are produced and exported in large volumes, and have been associated with high numbers of large food illness outbreaks around the world (FAO/WHO, 2008). Between 2014 and 2018, 51 major food illness outbreaks in the Unites States were linked to leafy green vegetables. Five of them were multistate outbreaks that led to public warnings being issued by CDC ((CDC), 2020). CDC attributes food illness outbreak data from the US to 17 different food categories on a continuous basis. When reviewing data from year 1998 to 2007, leafy vegetables

were the single most common vehicle for food borne illness outbreaks, represented by 22 % (2.2 million) of total annual illnesses (Painter et al., 2013). The most recent report includes data from year 1998 to 2017 and shows that vegetable row crops such as leafy vegetables were connected to 47.7 % of all *E. coli* O157 outbreaks. This was also significantly higher than any other food category (Interagency Food Safety Analytics Collaboration, 2019).

Shiga toxin-producing *E. coli* (STEC) cases are increasingly being reported and causes more than 150 000 illnesses yearly in the WHO European region, representing the 7th of 10 most common causes of illness (WHO, 2017). Also in Sweden, the trend is increasing, with 230-890 cases being reported yearly since 2005 ((SVA), 2019).

Vegetable consumption – effects on microbiota and immune system

Besides the risk that pathogens entail, consumption of leafy green vegetables may also affect the human body by its nutritional content and the intake of plant commensal bacteria. These are factors that affect the human gut microbiota, which shapes host physiology and maintains immune system homeostasis.

Recent metagenome-assembled genomes from human gut microbiomes show that the most common phyla in descending order are Firmicutes, Actinobacteria, Bacteriodetes, Tenericutes and Proteobacteria (Almeida et al., 2019). The healthy human gut microbiota may be divided into two parts, the core communities that are typically stable, and the highly individual and variable communities, referred to as the transient microbiota. Ingested bacteria from fresh produce can be regarded as part of the transient microbiome, as they are sometimes capable of integrating into our resident microbial communities and impact their composition and activity temporarily (David et al., 2014; Derrien & van Hylckama Vlieg, 2015). Ingested bacteria may survive a few days in the gastrointestinal tract, but they are rarely detected after one week (Firmesse, Mogenet, Bresson, Corthier, & Furet, 2008; Fujimoto, Matsuki, Sasamoto, Tomii, & Watanabe, 2008).

Apart from ingested bacteria, dietary components have a profound effect on the composition of the microbiota and its functions (David et al., 2014; De Filippo et al., 2010). Fruit and vegetable consumption has in numerous epidemiological studies been shown to lower the risk of developing cardiovascular diseases and cancers (Bazzano et al., 2002; Joshipura et al., 2001; S. Liu et al., 2001). This correlation is mostly explained by protective effects from the interaction of plant fibres and flavonoids with the gut microbiota (Klinder et al., 2016).

Commensal bacteria of the gut microbiota have developed mechanisms to control invading pathogens. For example, production of short-chain fatty acids alters the local pH to inhibit the growth of pathogens (Cherrington, Hinton, Pearson, & Chopra, 1991). The indigenous microbiota may also provide protection by competing for physical space, and by metabolizing the same nutrients, such as amino acids and organic acids (Kamada, Chen, Inohara, & Núñez, 2013). By production of specific metabolites, such as butyrate, that supresses pathogen virulence factors, commensal bacteria may also directly influence pathogen growth (Gantois et al., 2006).

Pathogen colonization is also prevented by the gut microbiota through indirect mechanisms, such as promoting the function of the epithelial barrier. For example, germ-free mice have a decreased production of antimicrobial peptides, resulting in an impaired epithelial barrier function (Vaishnava, Behrendt, Ismail, Eckmann, & Hooper, 2009). Bacterial signalling in epithelial cells also induce the production of IgA, further supporting the epithelial barrier integrity (Fagarasan, Kawamoto, Kanagawa, & Suzuki, 2010).

Bacterial interactions with the immune response

The gut microbiota is in close and constant communication with the immune system, and its homeostasis a determinant to health or susceptibility to disease. Bacterial populations in the gut may interact with the immune system mainly through gut-associated lymphoid tissues (GALT), such as the Payer's patches (PPs) and the mesenteric lymph nodes (MLNs). Bacterial antigens may enter the lymphoid tissues in a process called antigen sampling. Once inside, antigen presenting cells, such as dendritic cells (DCs), macrophages, and B cells will engulf the antigen by endocytosis and then present it to naïve CD4+ and CD8+ T cells (Murphy, Travers, & Walport, 2008). If a response is triggered, naïve T cells become activated and start expressing activation markers such as the membrane receptors CD69 and CD44 (Budd et al., 1987). Upon activation, CD4+ T cells produce the cytokine IL-2, stimulating their proliferation into T helper cells and memory T cells (W. Liao, Lin, & Leonard, 2011). T helper cells are also needed for activation and differentiation of B cells to plasma cells and CD8+ T cells to activated cytotoxic T cells. T cells also start to express gut homing receptor CCR9, and migrate to the lamina propria at the site of infection, where they may exert their functions (Murphy et al., 2008).

At the site of infection in the gut, the presence of bacterial antigens also triggers innate immune system cells such as macrophages, neutrophils and natural killer cells to produce the cytokine IL-1β. IL-1β increases the expression of adhesion molecules in blood vessels, attracting more immune cells to the site. This further activates immune cells that produce more IL-1β and TNF-α (Voronov & Apte, 2015). TNF-α activates phagocytes and induces apoptosis of epithelial cells (Andrews, McLean, & Durum, 2018; Lee et al., 2017). Bacterial antigens may also

induce the cytokine IL-8 (or the mouse homologue CXCL1-2/KC), a potent chemoattractant for neutrophils (Fan et al., 2007).

The cytokine IL-6 regulates the inflammatory response by controlling the balance between activated T and B cells and regulatory T cells. (Mendes, Galvão, & Vieira, 2019). IL-10 also promotes mucosal immune system balance by controlling chronic stimulation of the intestinal microbiota and food antigens. IL-10 limits the expression of pro-inflammatory cytokines and controls the differentiation and proliferation of macrophages, T and B cells (de Waal Malefyt, Abrams, Bennett, Figdor, & de Vries, 1991; de Waal Malefyt, Haanen, et al., 1991).

Biological control to increase food safety

Approaches to increase food safety often involve exposing the product to more drastic physical treatments, such as pressure, irradiation and heat or application of more efficient chemical preservatives. These types of solutions come with many disadvantages, such as alteration of organoleptic properties, loss of nutrients and proven toxicity of preservatives, such as nitrite. Chemical additives, such as chlorine, have consequently been banned in some countries in Europe, for example Sweden, Germany, and Switzerland (Ananou, Maqueda, Martínez-Bueno, & Valdivia, 2019; Gil, Selma, López-Gálvez, & Allende, 2009; Parish et al., 2003). At the same time, growing consumer trends demand for safe but minimally processed products without additives (Ananou et al., 2019).

Biological control, or biopreservation, means inhibiting harmful organisms by the use of beneficial ones, so called antagonists. In a microbial ecosystem, the constant battle of survival encourages the formation of strategies to antagonize other species. Strategies include the ability to bind essential nutrients, such as iron (siderophores), production of antimicrobial compounds (such as acids or antibiotics), or competition for physical space. These treats have been used traditionally for centuries in the form of food fermentation, in order to increase shelf-life and safety. The more modern approach is to find antagonistic microorganisms that can be applied to inhibit or reduce the load of pathogens (Ananou et al., 2019; G. Lopez-Velasco, Tydings, Boyer, Falkinham, & Ponder, 2012).

Biological control is widely applied with regards to plant pathogens, where bacteriophages, fungal or bacterial strains isolated from the endosphere or the rhizosphere are used. Common commercial biocontrol agents in commercial use are species of the fungal genus *Trichoderma* or bacterial species of the genera *Bacillus* and *Pseudomonas* (O'Brien, 2017). To be efficient, biocontrol agents must be applied uniformly and precisely, close to the target organism. The most common delivery systems are through seed treatments, soil amendments or irrigation systems (Boari, Zuccari, & Vurro, 2008; Lewis, 1991).

On leafy greens, where traditional ways to improve safety are limited, biological control could be an ecological part of the solution. Bacteriophages against *Listeria*, *Salmonella* and *E. coli* O157:H7 are commercially available for decontamination of foods through spray or dip inoculation. However, a major drawback with phage treatments is the emergence of phage-resistance in the target bacteria (Sabouri, Sepehrizadeh, Amirpour-Rostami, & Skurnik, 2017).

Live bacteria from the background microbiota of fresh vegetables have also been shown to influence the survival of human pathogens. C. H. Liao and Fett (2001) saw that strains from fresh produce were able to prevent the growth of *Salmonella chester* and *Listeria monocytogenes* on green pepper discs. *L. monocytogenes* was also inhibited by strains isolated from the native microbiota of the endive (Carlin, Nguyen-The, & Morris, 1996). Bacteria from five different genera originating from fresh-cut vegetables were shown to inhibit the growth of *S. enterica* on lettuce discs (Oliveira, Abadias, Colás-Medà, Usall, & Vinas, 2015). Also on lettuce, a strain of *Enterobacter cloacae* reduced *E. coli* O157:H7 by 1 log10 CFU/g (Jablasone et al., 2005). G. Lopez-Velasco et al. (2012) found a range of bacteria that inhibited the growth of *E. coli* O157:H7 on sterile spinach leaves. The genera *Pantoea*, *Pseudomonas*, *Klebsiella*, *Enterobacter*, *Aeromonas* and *Burkholderia* isolated from both iceberg lettuce and spinach were shown to inhibit *E. coli* O157:H7 (Johnston et al., 2009).

However, to be able to apply strains as biocontrol agents, is it important to focus on antagonistic bacteria that are non-pathogenic, concerning both humans and plants. Additionally, information on how antagonistic effects found in the laboratory should be transferred to live plants is lacking, but this is crucial information in order to be able to evaluate the potential for commercial use.

Aims

The general objectives of this thesis is to evaluate the microbial hygiene of ready-to-eat (RTE) leafy green products from a consumer's perspective, and to apply and evaluate the use of biological control in order to modify the microbiota on leafy greens towards a more beneficial direction for human consumption. The specific aims of the four publications included in this thesis are to:

- evaluate the efficacy of household washing on the bacterial load and removal of *E. coli* on leafy green vegetables (Study I).
- investigate the microbial hygiene on RTE leafy green vegetable products under simulated household conditions, with and without inoculation with *E. coli* (Study II).
- isolate *E. coli* antagonistic bacterial strains and evaluate their effect on the microbiota of spinach leaves in an industrial field production setting (Study III).
- use a healthy mouse model to study immunomodulating effects and microbiota changes upon administration of the selected test strains to be used for biological control (Study IV).

Methodology

Viable count

Cultivation on nutrient agar is the traditional way to assess microbial habitats. Samples are prepared, diluted and added to agar plates that are composed either to capture the general microbial habitat, for example tryptic soy agar (TSA) or to select certain groups of bacteria, for example violet red bile dextrose agar (VRBD) that is composed to favour growth of *Enterobacteriaceae*. Chromogenic agars are designed to detect certain bacterial species in a sample, for example, Brilliance™ *E. coli*/coliform Selective Agar (ECBA). ECBA contains X-Glu, that can be cleaved by β-glucuronidase in *E. coli*, giving the colonies a distinct purple colour. In paper I of this thesis, viable count of Romaine lettuce and mixed ready-to-eat (RTE) leafy greens was measured on TSA, VRBD and ECBA before and after washing. In paper II, RTE products were analysed by their TSA, VRBD and ECBA counts throughout the expiration period.

DNA isolation

To be able to analyse DNA from pure bacterial cultures or environmental samples, it must be pure and in high concentration. DNA isolation can be performed in many ways depending on the complexity of the starting material. In the case of a pure bacterial culture, a simple bead beating and centrifuge step can be used to lyse cells and remove debris. For more complex matrices, commercially available kits can be used. In paper II and III, a silica-based spin column kit was used (Nucleiospin® Soil Kit, Macherey-Nagel, Düren, Germany) to isolate DNA from the microbiota of spinach leaves. In paper IV, an automated system was used (Biorobot EZ1 workstation and EZ1 DNA tissue kit, Qiagen, Sollentuna, Sweden) to isolate DNA from intestinal content samples.

Polymerase chain reaction

Polymerase chain reaction (PCR) is utilized to amplify specific regions of DNA, a step needed prior to many downstream applications. The reaction is performed in a thermocycler, heating the sample in cycles by time intervals at precise temperatures. One cycle starts with heating the sample to about 95˚C to separate DNA strands, then the temperature is lowered to about 50˚C to allow forward and reverse primers (short DNA or RNA molecules) to bind to the target sequence. This creates a short sequence of double stranded DNA, which once the temperature is raised to about 72˚C, allows polymerase to bind and extend the DNA strand. Thereafter, the cycle is repeated, and after each cycle, the number of DNA copies double, creating 2^n copies for n numbers of cycles. A 25-cycle PCR generates over 33 million copies of double stranded DNA.

DNA purification and quality control

Purification of DNA is usually performed after a PCR run, prior to other applications such as sequencing, to remove excess nucleotides, salts, and primers. In paper II and III, solid phase reversible immobilization (SPRI) paramagnetic beads were used for purification (AMPure XP beads, Beckman Coulter Genomics, Brea, CA, USA). The beads bind nucleic acids and are taken out of solution by a magnetic field, leaving contaminants in solution. The DNA fragments are thereafter eluted from the beads in an aqueous solution (Beckman Coulter Life Sciences, 2020).

Gel electrophoresis

To visualize the result after a PCR run, gel electrophoresis can be performed. DNA or RNA fragments are added to the gel and separated according to size by electric current. By adding a reference standard composed of DNA fragments of known sizes, it is possible to identify that the correct sequence has been amplified.

Capillary gel electrophoresis is an adaption of the traditional gel electrophoresis, where nucleotide fragments are separated in small gel filled capillaries. This method provides fast and efficient separation and is commonly used for sequencing. Nucleotides are then labelled with several fluorescent tags and detected with laser induced fluorescence (Kemp, 1998). Capillary gel electrophoresis was used for Sanger sequencing in paper I and II.

Microfluidics-based automated gel electrophoresis, or gel electrophoresis on a chip, is used for quality control of DNA. It allows for faster analysis time and less sample and reagent consumption than conventional gel electrophoresis (Agilent

Bioanalyzer instrument, 2020). In paper II and III, automated gel electrophoresis was used prior to sample loading in Illumina Miseq, to ensure correct amplicon length and quality (Agilent 2100 Bioanalyzer, Agilent Technologies, Waldbrunn, Germany).

DNA quantification and qualification

The concentration of DNA can be measured with various methods, where UV absorbance-based methods are the most common. An UV spectrophotometer estimates the concentration of DNA based on the natural absorbance of the sample at 260 nm. The ratio 260/280 is used as a measure of purity, and a value lower than 1.8 is generally accepted as "pure" DNA. A high 260/280 ratio is usually caused by carryover chemicals from the extraction protocol (Thermo Fischer Scientific, 2020a). UV absorbance measurements were used in paper II, III and IV (Nanodrop 1000, Thermo Scientific, Wilmington, DE, USA) to make sure that the DNA isolation was successful and to check for impurities before proceeding to PCR. It was also used in paper IV to dilute samples for PCR prior to digestion with restriction endonuclease for T-RFLP.

However, other molecules such as RNA, free amino acids, proteins or free nucleotides may also absorb light at 260 nm, making the UV absorbance method imprecise for delicate applications (Nielsen et al., 2008). Fluorescence based quantification is a more accurate method, as a fluorescent dye that binds specifically to DNA is used (Thermo Fischer Scientific, 2020b). In paper II and III, a fluorometer (Qubit ™, 1x ds DNA HS Assay Kit (Life Technologies Corporation, Eugene, OR, USA)) was used to measure the concentration of DNA prior to the final dilution step before sample loading for sequencing.

Terminal restriction fragment length polymorphism

Terminal restriction fragment length polymorphism (T-RFLP) is a method used to determine microbial community structures (Hayashi, Sakamoto, & Benno, 2002; Jernberg, Löfmark, Edlund, & Jansson, 2007). It uses PCR with a fluorescently labelled primer to amplify the 16S rRNA gene. The PCR products are then digested by a restriction endonuclease, to create terminal restriction fragments (T-RFs). Since restriction sites differ between bacterial groups, T-RFs differ in size and can be separated by capillary gel electrophoresis. The result is an electropherogram showing T-RFs as peaks with fragment length on the x-axis and fluorescence intensity on the y-axis. A size standard is included in the analysis, enabling calculation of peak height and area, which is the basis of diversity index calculations. In this thesis, T-RFLP was used in paper IV to analyse the microbiota

of mice after administration of bacteria. Shannon diversity index was analysed, as well as the frequency of individual peaks within treatment groups.

Sequencing

Sequencing targeting the 16S rRNA gene is widely used to characterize microbial habitats. The 16S rRNA gene is coding for the small subunit of the ribosome in prokaryotic cells and is therefore essential for the survival of the cell. The function of the gene has not changed, so changes in the sequence can be considered a measure of evolution. This gene is therefore generally accepted for bacterial identification and classification (Patel, 2001).

Sanger sequencing

Sanger sequencing was developed by Frederick Sanger and colleagues in 1975 and has been the most widely used sequencing method for 40 years (Sanger & Coulson, 1975). Sanger sequencing can be used to sequence large parts of the 16S rRNA gene of pure isolates, enabling identification commonly at species level. A PCR including fluorescently labelled, chain-terminating nucleotides (ddNTPs) is run, and the products are analysed by for example capillary gel electrophoresis to obtain the sequence. In this thesis, Sanger sequencing was used in paper I and paper II to identify the dominant culturable microbiota of leafy greens.

Next Generation Sequencing

While Sanger sequencing can only sequence one DNA fragment at a time, next generation sequencing (NGS) uses massive parallel sequencing. Illumina sequencing (Illumina, San Diego, CA, USA) is currently the most widely used system, and the Illumina MiSeq platform is used in paper II and III. MiSeq uses sequencing by synthesis chemistry to generate about 15-25 million paired-end sequence reads up to 300 bp long, providing in-depth characterization and identification of microbial communities. Using PCR, the target nucleotide sequence is amplified, and barcodes and adaptors are attached. The DNA is loaded onto a flowcell with nanocells containing nucleotides for the adapters to attach. Bridge amplification PCR is then performed, creating about a thousand copies of each fragment. Next, modified nucleotides containing a reversible blocker is added so that the polymerase can only add one nucleotide at the time. These nucleotides are fluorescently labelled with different wavelengths, and detected after each synthesis round (Illumina, 2020).

NGS methods produce noise and erroneous sequences that are important to filter and process in order to avoid misleading results. Several pipelines for demultiplexing

(assigning sequences to samples), filtering and clustering of the sequences exist, where Qiime (Bolyen et al., 2019), mothur (Schloss et al., 2009) and packages in R (R Team, 2006) are the most commonly used. Taxonomic classification is performed by using databases such as RDP, SILVA and Greengenes (Kim et al., 2013).

Microbial diversity

To estimate ecological patterns in bacterial communities, diversity measures can be used. High microbial diversity generally means high pathogen invasion resistance, including the microbiota on leafy greens (Jousset, Schulz, Scheu, & Eisenhauer, 2011; Mallon, Elsas, & Salles, 2015; van Elsas et al., 2012). Diversity can be divided into α and β-diversity. α-diversity is used to calculate richness (number of species) and evenness (distribution of species) within individual samples. In paper IV, microbial diversity was estimated from the T-RFLP data through calculation of Shannon index ($H' = -\Sigma\, p_i \ln p_i$), taking into account both richness and evenness. In paper II and III, the Chao1 index was used in addition to Shannon index to estimate species richness from the NGS data. β-diversity is used to compare differences between samples from different groups to identify differences in the overall community composition and structure. The unique fraction metric (UniFrac) method is a measure of β-diversity and also calculated to take the relative relatedness of species into account. It can be used both as weighted (quantitative) or unweighted (qualitative) (Lozupone, Hamady, Kelley, & Knight, 2007). Weighted UniFrac was used for the NGS data in paper II and paper III.

Flow cytometry

Information on the morphology of cells and their components can be retrieved in a flow cytometer. A suspension of cells is injected in a single cell flow through one or several laser beams, and the light scattered at different angles is analysed. Cells are often stained with fluorescent chemicals, either by staining cell components such as DNA directly, or by using fluorochrome-conjugated antibodies that bind to specific cellular proteins. By detection of the emitted light, cells may be sorted according to phenotype for further downstream applications, or alternatively the population sizes may be analysed directly (Ormerod, 2008). Flow cytometry can be used to create a cytometric fingerprint of the microbiome, which has the potential to be used as a rapid diagnostic tool of microbiome-related diseases (Rubbens, Props, Kerckhof, Boon, & Waegeman, 2021). It is also a very efficient technique to study immune system responses by analysing changes in specific immune cell population sizes upon infections and disease states (McKinnon, 2018). The effect

of probiotics supplements on the immune system has also previously been evaluated using flow cytometry (Groeger et al., 2013; Y. Liu, Fatheree, Mangalat, & Rhoads, 2010; M. Zhang, Zhang, Hua, & Zou, 2017). In paper IV, flow cytometry was used to analyse immune system responses in gut lymphoid tissues in mice upon administration of bacterial candidates for biological control.

Cytokine/chemokine multiplex assay

Pro-inflammatory cytokines and chemokines were measured in serum of animals in study IV with a multiplex analysis (MSD Sector S 600 plate reader, V-plex Proinflammatory Panel 1 (mouse) plate, Meso Scale Diagnostics, LLC). Multiplex analysis enables multiple analytes to be quantified for the same sample. Each well on a 96-well plate is divided into multiple sections, where each section contains antibodies that bind a specific analyte. The plate is flushed with detection antibodies that are electrochemiluminescently tagged, which means that when electricity is applied, chemical reactions of the tags are initiated, resulting in light emission. The intensity of the emitted light is detected and the analytes can be quantified (Meso Scale Discovery, 2020).

Myeloperoxidase measurement

High levels of myeloperoxidase (MPO), an enzyme in neutrophils, is correlated with local inflammation (Kristjánsson, Venge, Wanders, Lööf, & Hällgren, 2004). It may be analysed by dissolving the enzyme in Hexadecyltrimethylammonium bromide (HTAB), incubation with 3,3', 5,5' tetramethylbenzidine (TMB) substrate (BD Opt EIA™, BD Biosciences, San Diego, CA, USA) and measured spectrophotometrically at 450 nm (Bradley, Priebat, Christensen, & Rothstein, 1982; Khan & al-Awadi, 1997). In paper IV, MPO levels were measured in the small and large intestinal tissue.

Statistical evaluation of data

Data not normally distributed, such as viable counts in paper I and II, and animal weights in paper IV, was evaluated non-parametrically by Kruskal-Wallis one-way analysis of variance on ranks. To reveal differences between two study groups, Mann-Whitney rank sum test was performed. Fisher's exact test was used in paper IV to reveal differences in the incidence of T-RFs between treatment groups and control. For all univariate analysis, a p-value ≤ 0.05 was considered statistically

significant. For NGS data in paper II and III, β-diversity was compared by permutational multivariate analysis of variance (PERMANOVA). The differential abundance between groups were analysed on genus level by DEseq2 with a p-value cut-off at 0.001 (Anders & Huber, 2010).

Methodological considerations

It is important to consider both advantages and limitations of methods when planning experiments, and when interpreting and comparing results. In this thesis, to provide different perspectives of the microbiota, a combination of bacterial culturing and culture-independent techniques was used.

Bacterial cultivation

Before the development of culture-independent methods, cultivation was the only way to find out the identities of microbes in a sample. The major problem with using culture methods to characterize microbial communities is that less than 2 % of environmental species are estimated to be culturable, due to unknown or difficult growth conditions (Wilson, Weightman, & Wade, 1997). Cultivation can instead generate other types of information. Identification of cultivated isolates from a mixed sample as performed in paper I and II gives quantifiable information on the culturable fraction of the microbiota, with possibilities to identify single strains to species level. Cultivation is also necessary to be able to isolate and use a strain for probiotics, or for biocontrol, as was performed in paper III.

DNA isolation

The DNA isolation step, prior to performing any DNA analysis, entails important aspects in need of consideration. The isolation protocol must be adapted to the sample matrix. Environmental samples, like soil and plant samples, are known to contain strong PCR inhibitors such as humic acids, tannic acids and complex polysaccharides. In paper II and III, a DNA extraction kit especially designed to remove those molecules was used (Macherey-Nagel, 2020). For DNA extraction of intestinal content in paper IV, a biorobot was used to minimize workload and contamination by a closed automatic system (Qiagen, 2020).

Another aspect of DNA isolation in need of consideration is the fact that cell membranes vary in toughness according to their composition. All cells, including Gram-positive bacteria with tough cell membranes, must be broken up sufficiently to free the DNA into solution. Cells can be lysed both chemically and physically. Bead beating was shown to yield better results than the surfactant Triton X-100

(Zoetendal, Ben-Amor, Akkermans, Abee, & de Vos, 2001), and therefore bead beating was the method chosen in all papers of this thesis.

Bias associated with PCR and sequencing

All techniques based on PCR, including 16S rRNA gene sequencing and T-RFLP, are subject to PCR bias, which is inherent to the method and may occur for several reasons. Exponential amplification changes the abundance of amplicons relative to the original sample (Robin, Ludlow, LaRanger, Wright, & Shay, 2016). Moreover, overamplification of short sequences and bias due to GC:AT content has also been documented (Mamedov et al., 2008). This phenomenon distorts the proportions between sequences and causes loss of rare taxa, allowing only for identification of dominant taxa at relative proportions.

When characterizing a microbial community, it is important to use primers that cover as many of the 16S rRNA variants as possible in the sample. To cover all species is difficult, but the conserved regions of 16S where primers attach are similar for most species. This results in insufficient amplification of certain organisms (Parada, Needham, & Fuhrman, 2016). In paper I and II, primers ENV-1 (5′-AGA GTT TGA TII TGG CTC AG-3′) and ENV-2 (5′-CGG ITA CCTTGT TAC GAC TT-3′) were used. This primer pair results in an almost complete copy of the 16S rRNA gene, increasing the possibilities of performing correct identification. ENV1 and ENV2 were designed after the 16S rRNA gene in *E. coli*, but since 16S genes may differ between bacterial taxa, the primers also contain inosine (I), facilitating binding to many versions of the 16S rRNA gene (Pettersson et al., 2003). For Illumina sequencing applied in paper II and III, primers B969F (ACG CGH NRA ACC TTA CC) and BA1406R (ACG GGC RGT GWG TRC AA) were used to amplify the V6-V8 region with Illumina Miseq. These primers have successfully been used for 16S amplification of the microbiota on leafy greens in a previous study (Tatsika et al., 2019) and they amplify the major bacterial taxonomic groups (Comeau, Li, Tremblay, Carmack, & Lovejoy, 2011).

T-RFLP

Since PCR bias causes insufficient amplification of rare taxa, diversity calculated with T-RFLP data may not represent the total diversity of the sample, but is more suitable for comparison between samples (Osborn, Moore, & Timmis, 2000). A variation between sequence determined T-RF length and observed T-RF length is called T-RF drift (Kaplan & Kitts, 2003). For example, the choice of sizecalling method may affect T-RF sizes. The local southern algorithm, using the standard closest to the unknown T-RF, was seen to generate the smallest T-RF drift (Osborn et al., 2000), and was the algorithm chosen for paper IV in this thesis.

The migration time of T-RFs is dependent on the molecular weight of the T-RF, which depends not only on its size, but also on its content of purine (the molecular basis of adenine and guanidine), and the molecular weight of the fluorescent dye which is attached (Kaplan & Kitts, 2003). The weight of the dye affects the mass/charge ratio for individual T-RFs, thus affecting its migration time (Pandey, Ganesan, & Jain, 2007). As a consequence, this effect is not linear, but becomes more pronounced with short fragments. In this thesis, the influence of the dye weight was minimized by using carboxyfluorescein (FAM), a dye with low molecular weight compared to other alternatives such as carboxytetramethylrhodamine (TAMRA) and hexa-chloro derivative (HEX).

Due to the variations in the above mentioned factors, T-RFLP can mainly be considered as a method to compare microbial community patterns between samples in the same project. For the same reason, identification of T-RFs by comparison in a database is difficult, instead a clone library may be used for the purpose.

Microbiological hygiene of leafy green vegetables

In this thesis, the bacterial content of different types of Ready-to-eat (RTE) leafy green vegetable products have been analysed under household conditions to assess their microbiological hygiene. *E. coli* inoculation has also been performed to simulate a contamination event and study its development and effect on the background microbiota during the shelf life period of these products.

Characterization of the microbiota using culturing and next generation sequencing

In paper I and II, the total aerobic count was measured on the first day of the expiration period in a total of 66 bags of ready-to-eat leafy green vegetable products, and revealed high concentrations of bacteria, between 6.0 and 8.3 log10 CFU/g. In general, bacterial levels increased at most the first three days, to stabilize on a high level for the rest of the shelf-life time (Figure 3). Thus, if the shelf life should be determined after bacterial levels on these products, it should not be kept at more than three days.

The bacterial levels in one portion (15-20 g) of ready-to-eat (RTE) leafy green products are similar to probiotic doses, but Sanger sequencing in paper I and II revealed that the bacteria present on these products are far from probiotic. The majority of the culturable microbiota consisted of Gram-negative bacteria, belonging to α- and γ-proteobacteria, with a dominating fraction of *Pseudomonas*. A few Gram-positive bacteria were found, such as *Bacillus* and *Actinobacteria*. Several species of opportunistic pathogens were identified, such as *Enterobacter homaechei*, *Hafnia paralvei* and *Pantoea agglomerans.* The dominance of Gram-negative bacteria and presence of opportunistic pathogens have also been found by others (Hunter et al., 2010; Jackson et al., 2013; G. Lopez-Velasco et al., 2013).

The amounts of culturable *Enterobacteriaceae* on RTE products were high; between 2.0 and 5.7 log10 CFU/g (paper I and II) on VRBD agar. The *Enterobacteriaceae* family contains pathogenic species, such as *E. coli* and *Salmonella* spp., and is a measure of hygiene in heat-treated products (European Commission, 2005). In

paper I and II, the *Enterobacteriaceae* count may be considered to be overestimated, since some isolates found on VRBD plates belonged to the *Bacillus*, *Pseudomonas*, and *Staphylococcus* genera. However, it should be kept in mind that those genera also include pathogenic species.

The negative impact of such high concentrations of unwanted bacteria is larger for vulnerable parts of the population, such as hospitalized, immunocompromised, and elderly. Factors such as aging and antibiotics treatments may affect the stability of the microbiota and susceptibility to infections by opportunistic pathogens (Claesson et al., 2012).

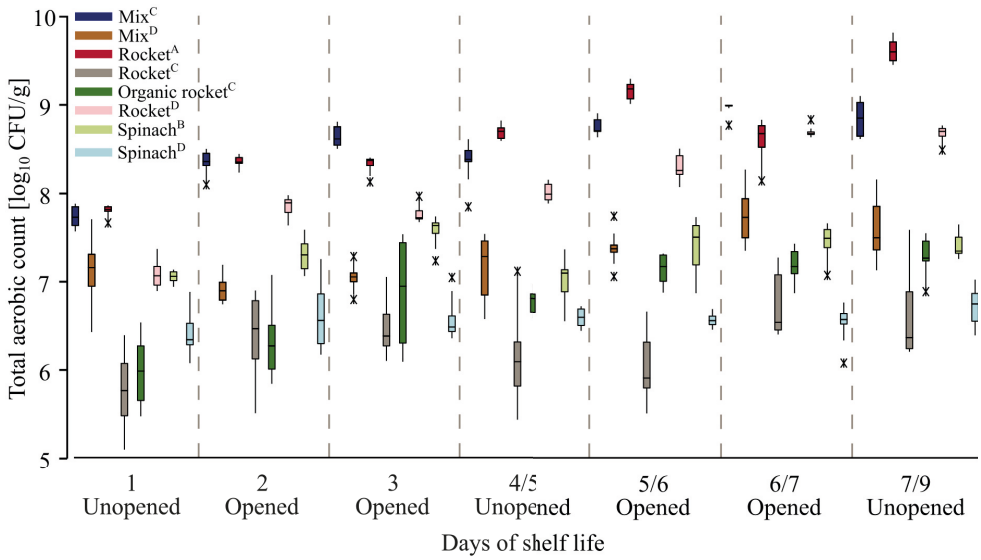


Figure 3: Total aerobic count throughout the whole shelf-life time of ready-to-eat leafy green mix (Mix), rocket, organic rocket and spinach from different companies, indicated by superscripted letters A-D. On the first day of the expiration period, 18 packages of ready-to-eat leafy greens were purchased. Six packages were opened on the first day, and the packages were then resealed, stored at 8 ˚C and sampled again the next two days. On day 4 or 5, depending on stated expiration time, 6 unopened packages were sampled and resampled the next two days (day 5 or 6 and on day 6 or 7). On the expiration date (day 7 or 9), the last six packages were sampled. Sampling days differ according to the shelf-life time of the product. x: outlier (value outside 1.5*interquartile range).

Culture-independent analysis revealed large groups of *Flavobacteriaceae*, *Pseudomonadaceae* and *Oxalobacteriaceae* over all product types, families which were previously identified with next generation sequencing on leafy greens (Dees et al., 2015; Tatsika et al., 2019). Previous studies show that the microbiota of leaves is specific for different plant species (Dees et al., 2015; Izhaki, Fridman, Gerchman, & Halpern, 2013), and in paper II, higher relative abundance of *Shewanellaceae* was found in the mixed LGV product, whereas rocket contained a large fraction of *Xanthomonadaceae* and *Weeksellaceae*. The microbiota taxa in all samples shifted over time, indicated by a change in β-diversity. The largest

changes seen over the course of the shelf life time were higher relative abundances of *Flavobacteriaceae* and lower relative abundances of *Pseudomonas*. Previous studies have observed a decrease in evenness and richness during storage, and these changes are probably an effect of cold storage and the deterioration process (Gabriela Lopez-Velasco et al., 2011; Söderqvist et al., 2017).

Large amounts of data from both culture-dependent and culture-independent techniques were included in paper II, enabling the opportunity to compare the results of traditional culturing and next generation amplicon sequencing. It is approximated that only 2 % of the total microbiota may be detected by culturing (Wilson et al., 1997). Naturally, the inherent difference between the two techniques is that culturing only detects live bacteria, while 16S amplicon sequencing detects all DNA present in the sample. Using TSA and VRBD to target the total culturable microbiota, and specifically *Enterobacteriaceae*, culturing matched 18 of 30 (37.5 %) of the genera also detected by next generation sequencing (NGS). However, culturing identified 16 genera that were not detected by NGS. Half of the genera detected by NGS could not be identified with culturing. It is important however, to consider that different batches of leafy greens were analysed with the two different techniques, allowing for identification of unique taxa in both cases. Despite of that, culturing still covered 7 out of the 10 most common genera from NGS analysis. These results are in line with the comparison of culturing and 454 pyrosequencing made by (Jackson et al., 2013) and indicate that the microbial community of leafy greens are relatively easy to cultivate compared to other environmental communities.

Differences in product types and packaging companies

Several types of ready-to-eat products were tested from four different companies. Neither type (spinach, rocket, leafy green mix), nor company could be related to certain levels of total aerobic count. Instead, the levels were rather individual for each product batch (Figure 4). It could however be seen that the concentrations were lower for whole-head Romaine lettuce than for ready-to-eat (RTE) products. This difference is probably a result of the sensitive nature of RTE products, containing fragile, often damaged leaves, circumstances that are ideal for microbial growth (FAO/WHO, 2008; Solomon & Matthews, 2005).

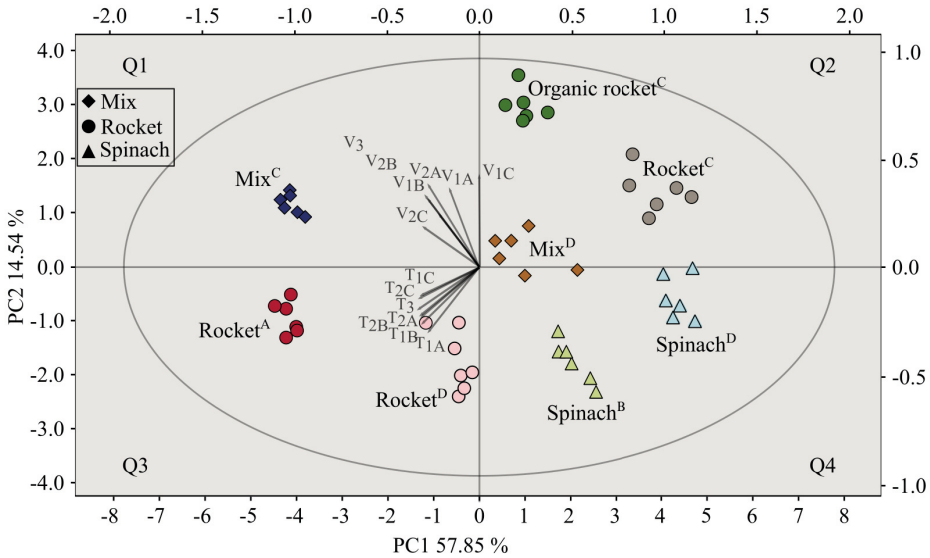


Figure 4: PCA biplot of viable counts (total aerobic count and *Enterobacteriaceae* count) of ready-to-eat leafy green vegetables from different companies, indicated by subscripted letter A-D. ♦ = mixed leafy greens, ● = rocket, and ▲ = spinach. Sampling occasions are presented as vectors. Ellipse: Hotelling T2 confidence interval 0.95. Vectors represent sampling point.

Effects of consumer practices

Commonly, the whole content of a package of ready-to-eat leafy green vegetables might not be eaten at once. Consumers might take out a portion for a meal and store the rest in the fridge until later. Opening a package changes its atmospheric composition, but when a number of leafy green products were analysed in Paper II, it was not possible to see a clear link between bacterial content and opened or unopened bags (Figure 1). Also when a product contained modified atmosphere (MAP) its bacterial levels did not seem to change after opening, which is in line with previous results (Barriga, Trachy, Willemot, & Simard, 1991). Modified atmosphere (MAP) is often used in ready-to-eat leafy green vegetables to reduce oxidation and prolong shelf-life. It makes the product look fresh longer, however pathogens may still continue to grow, creating a concealed risk for the consumer (Farber, 1991; Luo, He, & McEvoy, 2010).

RTE leafy green products are labelled with “already washed, ready to eat”. Even so, a common consumer practice is to wash the produce before a meal. RTE leafy green vegetable products are often subjected to industrial washing before packaging. It has previously been seen that this process step may actually result in increased viable counts, and contamination of *E. coli* (Rosberg, Darlison, Mogren, & Alsanius, 2021). Paper I investigated whether household washing could improve the microbial hygiene of these products by affecting the bacterial load. By rinsing leafy

greens in a colander, statistically significant reductions of the bacterial counts were only achieved with a very high water speed, 8 L/min, which resulted in damaged leaves. Washing through immersion in a water bath did not change the viable counts at all. Immersion in water was also not a successful method to reduce *E. coli* after inoculation of Romaine lettuce. In a previous study, Tatsika et al. (2019) used next generation sequencing instead of culturing, and reached the same conclusion. Household washing methods seem to be insufficient in reducing the bacterial load, and it seems like there is little the consumer can do to affect the microbial hygiene of these products.

In conclusion, the analysis of the microbiota on leafy greens in paper I and II, showed high levels of culturable bacteria, with the dominating part consisting of Gram-negative bacteria, among them *Enterobacteriaceae*, a family including several pathogenic taxa. These bacteria are known for being carriers of antibiotic resistance and may pose a risk to consumers, especially to children, elderly and immunocompromised individuals (Hormozi, Vasei, Aminianfar, Darvishi, & Saeedi, 2018; Li, Plésiat, & Nikaido, 2015). Paper I showed that many of these bacteria cannot be washed away with conventional household methods.

Growth patterns of different *E. coli* strains and their effect on the native microbiota

In typical pathogen growth evaluation studies, type strains are used (Gleeson & O'Beirne, 2005; Luo et al., 2010; Söderqvist et al., 2017; Williams, Moyne, Harris, & Marco, 2013). The growth behaviour of potentially pathogenic strains originating from leafy green products have been poorly studied. In paper II, the survival of the *E. coli* CCUG 29300^{T} with human origin, as well as two wild *E. coli* strains previously isolated from romaine lettuce was investigated by inoculation of a RTE product containing rocket. It could be seen that the concentration of viable *E. coli* CCUG 29300^{T} did not change inside the bags over the whole shelf-life period, while *E. coli* 921 and 731 from romaine lettuce exhibited a different growth pattern (Figure 5). *E. coli* 921 concentrations increased during the first three days, while the concentration of *E. coli* 731 was able to grow during the last three days of shelf-life, indicating an adaption to their environment and cold conditions. Even though the pathogenicity of these strains is not known, the results are concerning, and underlines the importance of studying environmental strains, which de facto are the strains consumers are exposed to.

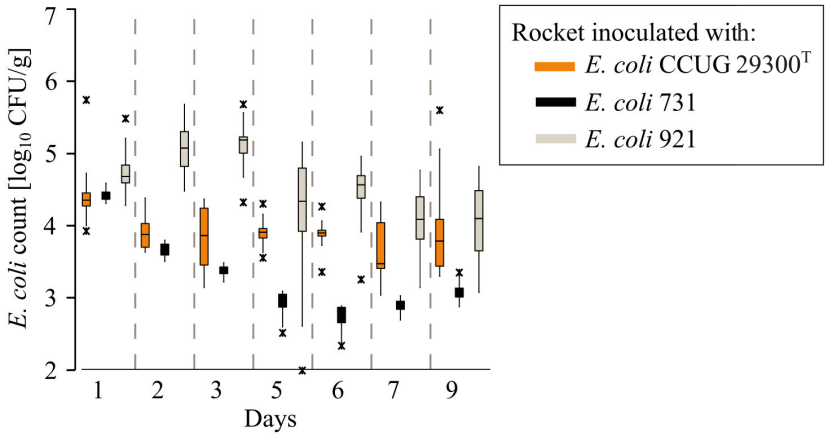


Figure 5: *E. coli* count of inoculated rocket from throughout the whole shelf-life time. *E. coli* 731 and *E. coli* 921 are wild strains originating from leafy greens. On the first day of the expiration period, 18 packages of ready-to-eat leafy greens were purchased. Six packages were opened on the first day, and the packages were then resealed, stored at 8 ˚C and sampled again the next two days. On day 5, six unopened packages were sampled and resampled the next two days (day 6 and on day 7). On the expiration date (day 9), the last six packages were sampled. ^{x}: outlier (value outside 1.5*interquartile range).

When analyzing the microbiota with next generation sequencing after inoculation of *E. coli* in paper II, it was observed that the product types (rocket, leafy green mix and spinach) were differently susceptible to *E. coli* inoculation. The *E. coli* inoculation of rocket and spinach induced no changes in the native microbiota on genus level, while in mixed leafy greens, multiple changes were observed. This effect was seen for inoculation with both *E. coli* CCUG 29300^{T} and *E. coli* 731. In addition, rocket had a smaller relative abundance of *Escherichia* than spinach and mix after inoculation with the type strain (0.6 % compared to 4.8 and 1.0 respectively), suggesting a more invasion resistant microbiota. However, it is important to keep in mind that the levels of *Enterobacteriaceae* found on uninoculated rocket samples are in the higher range of the analyzed products in the study.

The results of the characterization of the microbiota and *E. coli* inoculation of leafy greens are concerning for the food safety of leafy green products, and new strategies are called for, such as biological control, to steer the microbiota in a healthier direction for the consumer.

Biological control of leafy green vegetables

As a response to the hygiene situation of leafy green vegetables evaluated by paper I and II – in paper III we aimed to isolate antagonistic bacteria from leafy greens that can be used for biological control. In a rich microbial habitat, such as the phyllosphere of plants or the human gut, bacteria constantly promote and antagonize each other. When looking for bacteria to be used as biocontrol agents, it is beneficial to start searching in the environment where it will be applied, as chances for survival are optimal. By using *E. coli* antagonistic bacteria, the microbiota of leafy greens can be modified in a healthier direction for the consumer. *E. coli* is one of the most serious and prevalent human pathogens on leafy greens (Harris et al., 2006; Long, Adak, O'Brien, & Gillespie, 2002; Parish et al., 2003), yet, only a few studies have been performed to prevent its growth through biological control. *Enterobacter cloacae* has been shown to reduce *E. coli* O157:H7 by 1 log10 CFU/g on lettuce (Jablasone et al., 2005), and Johnston et al. (2009) saw that bacteria belonging to the genera *Pantoea*, *Pseudomonas*, *Klebsiella*, *Enterobacter*, *Aeromonas* and *Burkholderia* were able to inhibit *E. coli* O157:H7. In biological control, it is important however, to focus on antagonistic bacteria that are non-pathogenic, both concerning humans and plants. Moreover, up to the present time, no study has been performed that evaluates antagonistic bacteria aimed to reduce human pathogens on live plants, a step which is crucial to evaluate the potential for commercial use.

In study III, the first screening for antagonists was performed using a simple *in vitro* model with *E. coli*. Out of 295 isolates from different agar types, 37 were able to inhibit the type strain of *E. coli*. The identified strains belonged primarily to the *Bacillaceae* and *Pseudomonadaceae* families, and members of these families have shown antagonistic properties against human pathogens on leafy greens before (Duffy, Whiting, & Sheridan, 1999; Janisiewicz, Conway, & Leverentz, 1999; C. H. Liao & Fett, 2001; Shafi, Tian, & Ji, 2017). A few isolates that were easy to cultivate and not known as plant or human pathogens were then selected and evaluated in a field study; *Pseudomonas cedrina* LMG P-32207, *Pseudomonas punonensis* LMG P-32204, *Bacillus coagulans* LMG P-32205 and *Bacillus coagulans* LMG P-32206. The effects on the seed and leaf microbiota after seed inoculation of the antagonistic strains were evaluated, and indications of a less pathogenic bacterial composition were observed. Furthermore, seeds inoculated

with *B. coagulans* raised the relative abundance of *Lactobacillaceae* and decreased the relative abundance of *Pantoea.*

Special attention was drawn towards the relative abundance of *E. coli* on seeds and leaves. Seeds coated with *P. cedrina* and *P. punonensis*, contained *Escherichia-Shigella* (3.3 % and 5.6 % respectively), but when analysing the resulting leaves, the relative abundance was below the detection limit (0.001 %). It is not possible to state that the antagonistic bacteria were the cause of the reduction of *Escherichia-Shigella*, since the genus was absent on untreated seeds. This is the first study that has evaluated the effects of human pathogenic antagonists on live plants in a field setting, and these results motivate further studies to prove that seed coating with *P. cedrina* and *P. punonensis* may prevent the propagation of *Escherichia-Shigella* to the leaves.

Microbiota change and immunomodulatory effects of bacteria for biocontrol

Bacteria used for biological control will likely be consumed in high concentrations, and it is vital to verify that they are safe for consumption. Immunological response and microbiota studies are often made to evaluate the effects of probiotics and microbiota-related diseases in the gut (Galdeano & Perdigón, 2006; Osman et al., 2006; Pavan, Desreumaux, & Mercenier, 2003). To our knowledge, no such study has been conducted with bacterial strains to be used as biological control agents. In paper IV, in order to assess the safety of the biocontrol candidates, we evaluated the immunological response upon administration of the test strains in healthy mice. *P. cedrina* LMGP-322007, *P. punonensis* LMG P-32204, *Bacillus coagulans* LMG P-32205 and *Bacillus coagulans* LMG P-32206 strains from the field trial were evaluated together with *R. cerastii* MR5x, another strain that gave positive results in the *in vitro* tests in paper III. The mice were given a pre-treatment with *E. coli* and antibiotics to normalize the gut microbiota prior to receiving the test strains in the drinking water for 16 days (Linninge, Ahrné, & Molin, 2014).

The animals were in good general health condition during the whole experimental time, and body weight and spleen weights did not differ from untreated mice, indicating that the administrated bacterial strains did not cause acute disease or systemic inflammation.

The gut microbiota community structure was analysed by T-RFLP, and characteristic patterns were found for each group. The Shannon-Wiener diversity index also increased by *P. punonensis* in the small intestine, and for *B. coagulans* in the large intestine. The occurrence of many different T-RFs depended on the bacterial strain that was administrated. The T-RF patterns of the groups receiving the two *B. coagulans* strains were similar, but substantial differences were observed between the other groups.

Any local inflammation by MPO measurement in the intestinal mucosa could not be found for any of the treatment groups. To further evaluate if the test strains would induce inflammatory responses in the animals, 10 pro-inflammatory cytokines and chemokines from a commercial kit were quantified in serum (IFN-γ, IL1-β, IL-2,

IL-4, IL-5, IL-6, KC/GRO, IL10, IL12p70, TNF-α). The only markers yielding statistical differences were IFN-γ, and KC/GRO. IFN-γ increased in mice receiving *P. cedrina* and *B. coagulans* strains. IFN-γ supresses type-2 immune response, and the health-beneficial species *L. pentosus* has been observed to increase levels (Koizumi et al., 2008). In paper IV, levels of the neutrophil activator KC/GRO increased in groups receiving *R. cerastii* and *B. coagulans* strains. It has previously been observed that probiotic strains activate neutrophils and polarize the immune system towards Th1 (Cai et al., 2016; Kapila et al., 2013), which is also coherent with the raise of IFN-γ.

The flow cytometry results indicate different immune responses for the different test strains. The levels of activated CD4+ and CD8+ cells were lower in the treatment control compared to untreated mice, which is probably caused by the antibiotics in the pre-treatment (Ekmekciu et al., 2017). In animals receiving *B. coagulans*, the levels were similar to the ones of untreated individuals, while animals receiving *P. cedrina* and *R. cerastii* had lower levels, similar to the treatment-control. The dissimilar induced immune reactions are probably due to different interactions between the microbiota and the immune system, supported by variations in the T-RF patterns between the treatment groups.

Toll-like receptor 2 (TLR2) recognizes Gram-positive bacteria, but also plays a role in Gram-negative infections (Spiller et al., 2008), explaining the higher levels of dendritic cells and macrophages expressing TLR2 in the treatment control compared to untreated animals. Levels were restored to normal in all treatment groups, except for animals receiving *R. cerastii*, were levels rose, indicating an increase in inflammatory status.

Toll like receptor 4 (TLR4) signals the presence of lipopolysaccharides that are present in the cell membrane of Gram-negative bacteria (Munford & Varley, 2006). Levels were raised by the pre-treatment with *E. coli* and decreased by administration of all the test strains, except *P. cedrina*.

Lastly, all test strains, especially *B. coagulans*, raised the levels of regulatory T cells, which is connected to the observed raise in IFN-γ (Wood & Sawitzki, 2006), indicating a stronger downregulation of the immune response. This is an anti-inflammatory effect that has been observed for probiotics before (Groeger et al., 2013; Y. Liu et al., 2010; M. Zhang et al., 2017)

In conclusion, the *B. coagulans* strains consistently emulate the immune response of the normal control with untreated mice. *P. cendrina* and *R. cerastii* on the other hand seem to generate the opposite effect, while the immune response seemed largely unaffected by *P. punonensis*. Together with the results from paper III, it can be concluded that strains that show potential as biological control agents on leaves might not necessarily be beneficial to consume. A strain to be chosen for biological control must pass both tests. According to the results of paper IV, mainly *B. coagulans* LMG P-32205 and *B. coagulans* LMG P-32206 should be qualified to be

included in additional regulatory safety tests according to national guidelines (Ferreira, Soares, & Soares, 2019; Laulund, Wind, Derkx, & Zuliani, 2017). The results encourage further development for *B. coagulans* to be used as biocontrol agents on leafy green vegetables and increase their safety upon consumption.

Conclusions

The major conclusions from the findings of the studies in this thesis are:

- Ready-to-eat leafy green vegetables contain high numbers of viable bacteria, among them *Enterobacteriaceae*, a family containing several pathogenic taxa. Opportunistic pathogens such as *Enterobacter homaechei*, *Hafnia paralvei*, *Micrococcus luteus* and *Pantoea agglomerans* were detected and alive in the microbiota of leafy greens, constituting an obvious hazard for the consumer. The found microbial hazards of leafy green vegetables is universal, and independent of producing company and product type.
- Simulated household washing was inefficient and reduced aerobic counts significantly only after 5 consecutive washes at high water velocity. This treatment left the produce with damaged leaves and it still contained high concentrations of culturable bacteria, 7 log10 CFU/g. Washing could not reduce numbers of *E. coli* after a simulated contamination.
- Upon inoculation with *E. coli*, the microbiota composition of spinach and mixed leafy greens changed, in contrast to the stable microbiota of rocket. Rocket contained less *E. coli* after inoculation and storage, possibly making this produce more resistant to pathogen invasion. However, it should be noted that the *Enterobacteriaceae* levels of uninoculated rocket samples were in the higher range among the products tested.
- Wild *E. coli* strains of isolated from Romaine lettuce are able to grow at different time-points during the shelf-life period, as opposed to the type strain, underlining the importance to study product specific pathogens, in contrary to type strains from other origins.
- Bacterial strains isolated from leafy greens possess abilities to counteract the growth of *E. coli* and modify the microbiota of spinach in a healthier direction for the consumer in an industrial field production setting. These results open up for biological control to be used to counteract food-borne illness related to leafy greens.
- The biological control candidates induce different immunological and microbiota changes in healthy mice, highlighting the need of profound safety testing prior to commercial application. The results of *B. coagulans*

and *P. punonensis* strains are promising for further testing in order to enhance the safety of leafy green vegetables.

Future outlook

Future investigations should be designed to evaluate the performance of the antagonistic strains on pathogenic *E. coli* strains, and others such as *Listeria* and *Salmonella* species. But as of the results from paper II, it can be seen that it is not enough to just test against one strain, but several, preferably the ones that have been isolated from outbreaks on leafy greens.

Before the appointed strains for biocontrol can be legally used in commercial food production, they need to be subjected to safety tests according to national guidelines involving antibiotic susceptibility tests, biogenic amine production and toxin production.

Additionally, to be able to apply these antagonistic bacteria in large scale industrial settings, the strains need to be incorporated in a formula that guarantees high viability and long storage times. A wide range of applications is possible for these bacteria, such as seed coatings, packaging material incorporation and irrigation water additives, just to mention a few. One of the promising candidates, *B. coagulans*, produce tolerant spores that should be easy to handle in commercial production systems. The effort towards producing safe leafy green vegetables through biological control has merely just started, but the results in this thesis are promising for future development.

Acknowledgements

To my main supervisor **Åsa Håkansson**. You have always supported me to 100 %, in everything from research ideas to private life and motherhood. You have always believed in me and trusted me. I have never felt "supervised" by you, and I really feel that the cooperation we have had during these years have been true and heartfelt. We have overcome both ups and downs together but have always strived to bring our group forward into excellence.

Siv Ahrné and **Göran Molin**, thank you so much for giving me the opportunity to do this PhD! Thank you for your continuous support, for sharing your experience and knowledge, and giving valuable input to manuscripts and ideas.

To my former supervisor **Crister Olsson**. Thank you for always being truly you, so good at heart with your humour and immediate presence. Thank you for teaching me REAL microbiology and how it's done in the lab. Your methods from everything how to mark agar plates to how to cleanstreak a colony have been taught on to hundreds of students, who have gained a solid ground to their future work in microbiology all over the world.

To all my friends at the department. Thank you for making it a true joy to come to work every day. Your greetings and smiles makes all the difference. The friendly atmosphere that I have met every day here is truly amazing, and makes work simply less "work". Keep that up, it is so important.

To **Elin Oscarsson**, my companion every day at work. Thank you for our inspiring and sometimes very nerdy and funny conversations. Thank you for your supporting and understanding nods when times have been rough, saying "yes, I have been there too". Also thank you for all your help, from turning off PCR machines in the evening to discussing experiment plans and results. My PhD would simply not have been possible without you!

To **Pamela Canaviri-Paz**, thank you for your friendship and heart-warming hugs, and for all the laughters in the corridor!

To **Anna Kjellström**, thank you for being my room companion and philosophical friend (!) the last year during writing. Thank you for all the NGS analysis, which I could never have managed on my own.

To all my students. **Noora Nurminen**, **Gayatri Rajashekhar Dhulappanavar**, **Yasmin Nabila**, **Terese Stark, Zuzanna Sadowska, Tamara Lupan**, **N S**

Bharath Prasad V, **Jiayi He**, **Afina Nuur Farma Megelectra**, **Charles Keronika**, **Song Tan**, **Jennifer Paulsson**, **Lola Sutton**, **Nina Ljungberg** and all the other students I have taught in courses over the years. Thank you for letting me follow you during this time and be witness of your development here, and for letting me know how your careers went on afterwards. Thank you for your enthusiasm, friendship and for developing me into a better teacher, researcher and person. You have really put a golden edge on my time as a PhD.

Thank you **Kathrin Zeller** and **Giulia Elli** at **Depertment of Immunotechnology** for new perspectives, insights, ideas and great cooperation with paper IV.

Thank you **Peter Kvist**, **Jonas Jönsson** and **Ida Ahlbeck** at **Vidinge grönt AB**, for your openness to our research ideas and participation in our projects. Lastly, thank you for your contribution of countless bags of leafy greens!

Thank you **Stina Andersson**, and **Magnus Nilsson** at **Hushållningssällskapet, and Mariann Wikström** at **Agro Plantarum AB** for seed coating and managing the practical aspects of the field trial. Thank you **Södervidinge gård** and **Martynas** for your contribution of seeds and land for the field trial.

A big thank you to all my collegues in the Safe salad project group at SLU; **Beatrix Alsanius**, **Lars Mogren, Diana Karpman, Sofia Boqvist, Ivar Vågsholm, Karin Söderkvist, Anna Karin Rosberg** och **Maria Karlsson**

Thank you **Anne-Marie Rohrstock** for your laboratory expertise and **Milladur Rahman** for your help during the FACS analysis. Thank you **Jeanette Arvastsson** for lending out your brand new FACS machine to us!

Thank you **Camilla Björklöv** at the Biology department for your guidance at the animal facility.

Tack familjen, **Rose-Marie, Ingvar**, **Liselotte Eriksson** för att ni alltid finns där för mig!

Vielen dank **Rainer** und **Annerose Uhlig** für Eurer Unterstützung und Liebe.

Jens Uhlig, thank you for always being at my side, always with ideas, enthusiasm and love. Thank you for making me an image nerd (it's sometimes a curse though!) and for making my front page look great! Thank you for showing interest in my science, and preparing me for my defense through our scientific discussions. I love you so much! Thank you for **Leonie**, our bundle of joy that came along while on this journey. Thank you my little daughter for always switching my brain off work and inviting me in to your fascinating world.

Finally, I express my gratitude to FORMAS (The Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning) for providing financial support through a PhD fellowship.

References

(CDC), C. f. D. C. a. P. (2020). Lettuce, Other Leafy Greens, and Food Safety.

(FAO), F. a. A. O. o. t. U. N. (2020). FAOSTAT statistical database. Retrieved from http://www.fao.org/faostat/en/#compare

(SVA), N. V. I. (2019). *Surveillance of infectious diseases in animals and humans in Sweden 2019*. Uppsala, Sweden

Agilent Bioanalyzer instrument. (2020). High-Resolution Automated Electrophoresis of DNA, RNA, and Protein Samples. Retrieved from https://www.agilent.com/en/product/automated-electrophoresis/bioanalyzer-systems/bioanalyzer-instrument

Agriculture, S. B. o. (2019). *Consumption of food and nutritive values, data up to 2018*.

Allende, A., Selma, M. V., López-Gálvez, F., Villaescusa, R., & Gil, M. I. (2008). Role of commercial sanitizers and washing systems on epiphytic microorganisms and sensory quality of fresh-cut escarole and lettuce. *Postharvest Biol Tech, 49*(1), 155-163.

Almeida, A., Mitchell, A. L., Boland, M., Forster, S. C., Gloor, G. B., Tarkowska, A., . . . Finn, R. D. (2019). A new genomic blueprint of the human gut microbiota. *Nature, 568*(7753), 499-504. doi:10.1038/s41586-019-0965-1

Ananou, S., Maqueda, M., Martínez-Bueno, M., & Valdivia, E. (2019). Biopreservation, an ecological approach to improve the safety and shelf-life of foods. *Communicating Current Research and Educational Topics and Trends in Applied Microbiology, 1*.

Anders, S., & Huber, W. (2010). Differential expression analysis for sequence count data. *Genome Biology, 11*(10), R106. doi:10.1186/gb-2010-11-10-r106

Andrews, C., McLean, M. H., & Durum, S. K. (2018). Cytokine Tuning of Intestinal Epithelial Function. *Frontiers in Immunology, 9*, 1270-1270. doi:10.3389/fimmu.2018.01270

Barret, M., Briand, M., Bonneau, S., Préveaux, A., Valière, S., Bouchez, O., . . . Jacques, M.-A. (2015). Emergence Shapes the Structure of the Seed Microbiota. *Applied and environmental microbiology, 81*(4), 1257. doi:10.1128/AEM.03722-14

Barriga, M. I., Trachy, G., Willemot, C., & Simard, R. E. (1991). Microbial Changes in Shredded Iceberg Lettuce Stored under Controlled Atmospheres. *Journal of food science, 56*(6), 1586-1588. doi:https://doi.org/10.1111/j.1365-2621.1991.tb08646.x

Bazzano, L. A., He, J., Ogden, L. G., Loria, C. M., Vupputuri, S., Myers, L., & Whelton, P. K. (2002). Fruit and vegetable intake and risk of cardiovascular

disease in US adults: the first National Health and Nutrition Examination Survey Epidemiologic Follow-up Study. *The American Journal of Clinical Nutrition, 76*(1), 93-99. doi:10.1093/ajcn/76.1.93

Beckman Coulter Life Sciences. (2020). SPRI Bead Technology. Retrieved from https://www.beckman.com/resources/technologies/spri-beads

Boari, A., Zuccari, D., & Vurro, M. (2008). 'Microbigation': delivery of biological control agents through drip irrigation systems. *Irrigation Science, 26*(2), 101-107. doi:10.1007/s00271-007-0076-x

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., . . . Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology, 37*(8), 852-857. doi:10.1038/s41587-019-0209-9

Bradley, P. P., Priebat, D. A., Christensen, R. D., & Rothstein, G. (1982). Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. *J Invest Dermatol, 78*(3), 206-209. doi:10.1111/1523-1747.ep12506462

Budd, R. C., Cerottini, J. C., Horvath, C., Bron, C., Pedrazzini, T., Howe, R. C., & MacDonald, H. R. (1987). Distinction of virgin and memory T lymphocytes. Stable acquisition of the Pgp-1 glycoprotein concomitant with antigenic stimulation. *Journal of immunology (Baltimore, Md. : 1950), 138*(10), 3120-3129. Retrieved from http://europepmc.org/abstract/MED/3106474

http://www.jimmunol.org/cgi/content/abstract/138/10/3120

Cai, S., Kandasamy, M., Rahmat, J. N., Tham, S. M., Bay, B. H., Lee, Y. K., & Mahendran, R. (2016). *Lactobacillus rhamnosus* GG Activation of Dendritic Cells and Neutrophils Depends on the Dose and Time of Exposure. *Journal of Immunology Research, 2016*, 7402760. doi:10.1155/2016/7402760

Campos, J., Mourão, J., Pestana, N., Peixe, L., Novais, C., & Antunes, P. (2013). Microbiological quality of ready-to-eat salads: An underestimated vehicle of bacteria and clinically relevant antibiotic resistance genes. *International Journal of Food Microbiology, 166*(3), 464-470. doi:https://doi.org/10.1016/j.ijfoodmicro.2013.08.005

Carlin, F., Nguyen-The, C., & Morris, C. E. (1996). Influence of Background Microflora on Listeria monocytogenes on Minimally Processed Fresh Broad-Leaved Endive (Cichorium endivia var. latifolia). *Journal of Food Protection, 59*(7), 698-703. doi:10.4315/0362-028X-59.7.698

Centers for Disease Control and Prevention, N. C. f. E. a. Z. I. D. N., Division of Foodborne, Waterborne, and Environmental Diseases (DFWED). (2020, 2020-03-20). Key Facts About Food Poisoning. Retrieved from https://www.cdc.gov/foodsafety/food-poisoning.html

Cherrington, C. A., Hinton, M., Pearson, G. R., & Chopra, I. (1991). Short-chain organic acids at pH 5.0 kill Escherichia coli and Salmonella spp. without causing membrane perturbation. *Journal of Applied Bacteriology, 70*(2), 161-165. doi:https://doi.org/10.1111/j.1365-2672.1991.tb04442.x

Claesson, M. J., Jeffery, I. B., Conde, S., Power, S. E., O'Connor, E. M., Cusack, S., . . . O'Toole, P. W. (2012). Gut microbiota composition correlates with diet and health in the elderly. *Nature, 488*(7410), 178-184. doi:10.1038/nature11319

Comeau, A. M., Li, W. K. W., Tremblay, J.-É., Carmack, E. C., & Lovejoy, C. (2011). Arctic Ocean Microbial Community Structure before and after the 2007 Record Sea Ice Minimum. *PloS one, 6*(11), e27492. doi:10.1371/journal.pone.0027492

Darlison, J., Mieli, M., Bengtsson, T., Hartmann, R., Mogren, L., Vågsholm, I., . . . Alsanius, B. (2019). Plant species affects establishment of E. coli O157:H7 gfp+ on leafy vegetables. *Journal of Applied Microbiology, 127*. doi:10.1111/jam.14299

David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., . . . Turnbaugh, P. J. (2014). Diet rapidly and reproducibly alters the human gut microbiome. *Nature, 505*(7484), 559-563. doi:10.1038/nature12820

De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J. B., Massart, S., . . . Lionetti, P. (2010). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proceedings of the National Academy of Sciences, 107*(33), 14691. doi:10.1073/pnas.1005963107

de Waal Malefyt, R., Abrams, J., Bennett, B., Figdor, C. G., & de Vries, J. E. (1991). Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *The Journal of experimental medicine, 174*(5), 1209-1220. doi:10.1084/jem.174.5.1209

de Waal Malefyt, R., Haanen, J., Spits, H., Roncarolo, M. G., te Velde, A., Figdor, C., . . . de Vries, J. E. (1991). Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. *The Journal of experimental medicine, 174*(4), 915-924. doi:10.1084/jem.174.4.915

Dees, M. W., Lysøe, E., Nordskog, B., & Brurberg, M. B. (2015). Bacterial Communities Associated with Surfaces of Leafy Greens: Shift in Composition and Decrease in Richness over Time. *Applied and environmental microbiology, 81*(4), 1530. doi:10.1128/AEM.03470-14

Derrien, M., & van Hylckama Vlieg, J. E. T. (2015). Fate, activity, and impact of ingested bacteria within the human gut microbiota. *Trends in Microbiology, 23*(6), 354-366. doi:https://doi.org/10.1016/j.tim.2015.03.002

Duffy, G., Whiting, R. C., & Sheridan, J. J. (1999). The effect of a competitive microflora, pH and temperature on the growth kinetics of Escherichia coli O157:H7. *Food Microbiology, 16*(3), 299-307. doi:https://doi.org/10.1006/fmic.1998.0242

EFSA. (2013). Scientific Opinion on the risk posed by pathogens in food of non-animal origin. Part 1 (outbreak data analysis and risk ranking of

food/pathogen combinations). *EFSA Journal, 11*(1). doi:10.2903/j.efsa.2013.3025

Egervärn, M., & Flink, C. (2014). Kartläggning av shigatoxinproducerande E. coli (STEC) på nötkött och bladgrönsaker. In S. National Food Administration (Ed.), (Vol. 22).

Ekmekciu, I., von Klitzing, E., Fiebiger, U., Escher, U., Neumann, C., Bacher, P., . . . Heimesaat, M. M. (2017). Immune Responses to Broad-Spectrum Antibiotic Treatment and Fecal Microbiota Transplantation in Mice. *Frontiers in Immunology, 8*, 397. Retrieved from https://www.frontiersin.org/article/10.3389/fimmu.2017.00397

Commission Regulation (EC) No 2073/2005 on the microbiological criteria for foodstuffs, (2005).

Fagarasan, S., Kawamoto, S., Kanagawa, O., & Suzuki, K. (2010). Adaptive Immune Regulation in the Gut: T Cell–Dependent and T Cell–Independent IgA Synthesis. *Annual Review of Immunology, 28*(1), 243-273. doi:10.1146/annurev-immunol-030409-101314

Fan, X., Patera, A., Pong-Kennedy, A., Deno, G., Gonsiorek, W., Manfra, D., . . . Hipkin, R. (2007). Murine CXCR1 is a functional receptor for GCP-2/CXCL6 and interleukin-8/CXCL8. *The Journal of biological chemistry, 282*, 11658-11666. doi:10.1074/jbc.M607705200

FAO/WHO. (2005, 1–3 September 2004). *Fruit and vegetables for health. Report of a Joint FAO/WHO Workshop*, Kobe, Japan.

FAO/WHO. (2008). *Microbiological hazards in fresh leafy vegetables and herbs: Meeting Report*

MEETING REPORT. Paper presented at the Microbiological Risk Assessment Series, Rome.

Farber, J. M. (1991). Microbiological Aspects of Modified-Atmosphere Packaging Technology - A Review. *Journal of Food Protection, 54*(1), 58-70. doi:10.4315/0362-028X-54.1.58

Ferreira, C. M. H., Soares, H. M. V. M., & Soares, E. V. (2019). Promising bacterial genera for agricultural practices: An insight on plant growth-promoting properties and microbial safety aspects. *Science of The Total Environment, 682*, 779-799. doi:https://doi.org/10.1016/j.scitotenv.2019.04.225

Firmesse, O., Mogenet, A., Bresson, J. L., Corthier, G., & Furet, J. P. (2008). Lactobacillus rhamnosus R11 Consumed in a Food Supplement Survived Human Digestive Transit without Modifying Microbiota Equilibrium as Assessed by Real-Time Polymerase Chain Reaction. *Microbial Physiology, 14*(1-3), 90-99. doi:10.1159/000106087

Fujimoto, J., Matsuki, T., Sasamoto, M., Tomii, Y., & Watanabe, K. (2008). Identification and quantification of Lactobacillus casei strain Shirota in human feces with strain-specific primers derived from randomly amplified polymorphic DNA. *International Journal of Food Microbiology, 126*(1), 210-215. doi:https://doi.org/10.1016/j.ijfoodmicro.2008.05.022

Galdeano, C. M., & Perdigón, G. (2006). The probiotic bacterium Lactobacillus casei induces activation of the gut mucosal immune system through innate

immunity. *Clinical and vaccine immunology : CVI, 13*(2), 219-226. doi:10.1128/CVI.13.2.219-226.2006

Gantois, I., Ducatelle, R., Pasmans, F., Haesebrouck, F., Hautefort, I., Thompson, A., . . . Van Immerseel, F. (2006). Butyrate specifically down-regulates salmonella pathogenicity island 1 gene expression. *Applied and environmental microbiology, 72*(1), 946-949. doi:10.1128/AEM.72.1.946-949.2006

Gil, M. I., Selma, M. V., López-Gálvez, F., & Allende, A. (2009). Fresh-cut product sanitation and wash water disinfection: problems and solutions. *Int J Food Microbiol, 134*, 37-45.

Gleeson, E., & O'Beirne, D. (2005). Effects of process severity on survival and growth of Escherichia coli and Listeria innocua on minimally processed vegetables. *Food Control, 16*(8), 677-685. doi:https://doi.org/10.1016/j.foodcont.2004.06.004

Groeger, D., O'Mahony, L., Murphy, E. F., Bourke, J. F., Dinan, T. G., Kiely, B., . . . Quigley, E. M. M. (2013). Bifidobacterium infantis 35624 modulates host inflammatory processes beyond the gut. *Gut Microbes, 4*(4), 325-339. doi:10.4161/gmic.25487

Harris, L. J., Farber, J. N., Beuchat, L. R., Parish, M. E., Suslow, T. V., Garrett, E. H., & Busta, F. F. (2006). Outbreaks associated with fresh produce: incidence, growth, and survival of pathogens in fresh and fresh-cut produce. *Compr Rev Food Sci F, 2*(1), 78-141.

Hayashi, H., Sakamoto, M., & Benno, Y. (2002). Fecal Microbial Diversity in a Strict Vegetarian as Determined by Molecular Analysis and Cultivation. *Microbiology and Immunology, 46*, 819-831. doi:10.1111/j.1348-0421.2002.tb02769.x

Hormozi, S. F., Vasei, N., Aminianfar, M., Darvishi, M., & Saeedi, A. A. (2018). Antibiotic resistance in patients suffering from nosocomial infections in Besat Hospital. *European journal of translational myology, 28*(3), 7594-7594. doi:10.4081/ejtm.2018.7594

Hung, H.-C., Huang, M.-C., Wu, D.-C., Hsu, H.-K., & Wu, M.-T. (2004). Association between diet and esophageal cancer in Taiwan. *Journal of gastroenterology and hepatology, 19*, 632-637. doi:10.1111/j.1440-1746.2004.03346.x

Hunter, P. J., Hand, P., Pink, D., Whipps, J. M., & Bending, G. D. (2010). Both leaf properties and microbe-microbe interactions influence within-species variation in bacterial population diversity and structure in the lettuce (Lactuca Species) phyllosphere. *Appl and Environ Microbiol, 76*(24), 8117-8125.

Illumina. (2020). Explore Illumina sequencing technology. Retrieved from https://www.illumina.com/science/technology/next-generation-sequencing/sequencing-technology.html

Interagency Food Safety Analytics Collaboration. (2019). *Foodborne illness source attribution estimates for 2017 for Salmonella, Escherichia coli O157,*

Listeria monocytogenes, and Campylobacter using multi-year outbreak surveillance data, United States.

Izhaki, I., Fridman, S., Gerchman, Y., & Halpern, M. (2013). Variability of Bacterial Community Composition on Leaves Between and Within Plant Species. *Current Microbiology, 66*(3), 227-235. doi:10.1007/s00284-012-0261-x

Jablasone, J., Warriner, K., & Griffiths, M. (2005). Interactions of Escherichia coli O157:H7, Salmonella typhimurium and Listeria monocytogenes plants cultivated in a gnotobiotic system. *International Journal of Food Microbiology, 99*, 7-18. doi:10.1016/j.ijfoodmicro.2004.06.011

Jackson, C. R., Randolph, K. C., Osborn, S. L., & Tyler, H. L. (2013). Culture dependent and independent analysis of bacterial communities associated with commercial salad leaf vegetables. *BMC Microbiol, 13*(1), 274.

Janisiewicz, W. J., Conway, W. S., & Leverentz, B. (1999). Biological Control of Postharvest Decays of Apple Can Prevent Growth of Escherichia coli O157:H7 in Apple Wounds. *Journal of Food Protection, 62*(12), 1372-1375. doi:10.4315/0362-028X-62.12.1372

Jernberg, C., Löfmark, S., Edlund, C., & Jansson, J. K. (2007). Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. *Isme j, 1*(1), 56-66. doi:10.1038/ismej.2007.3

Johnston, M., Harrison, M., & A Morrow, R. (2009). Microbial Antagonists of Escherichia coli O157:H7 on Fresh-Cut Lettuce and Spinach. *Journal of Food Protection, 72*, 1569-1575. doi:10.4315/0362-028X-72.7.1569

Joshipura, K. J., Hu, F. B., Manson, J. E., Stampfer, M. J., Rimm, E. B., Speizer, F. E., . . . Willett, W. C. (2001). The Effect of Fruit and Vegetable Intake on Risk for Coronary Heart Disease. *Annals of Internal Medicine, 134*(12), 1106-1114. doi:10.7326/0003-4819-134-12-200106190-00010

Jousset, A., Schulz, W., Scheu, S., & Eisenhauer, N. (2011). Intraspecific genotypic richness and relatedness predict the invasibility of microbial communities. *The ISME Journal, 5*(7), 1108-1114. doi:10.1038/ismej.2011.9

Kamada, N., Chen, G. Y., Inohara, N., & Núñez, G. (2013). Control of pathogens and pathobionts by the gut microbiota. *Nature Immunology, 14*(7), 685-690. doi:10.1038/ni.2608

Kapila, R., Sebastian, R., Varma, V., Sharma, R., Kapasiya, M., Saliganti, V., . . . Dang, A. (2013). Comparison of innate immune activation after prolonged feeding of milk fermented with three species of Lactobacilli. *Microbiology and Immunology, 57*. doi:10.1111/1348-0421.12092

Kaplan, C. W., & Kitts, C. L. (2003). Variation between observed and true Terminal Restriction Fragment length is dependent on true TRF length and purine content. *Journal of Microbiological Methods, 54*(1), 121-125. doi:https://doi.org/10.1016/S0167-7012(03)00003-4

Karnehed, N., & Lindblad, M. (2010). Salmonella, Campylobacter och E.coli i färska kryddor och bladgrönsaker från Sydostasien. In S. National Food Administration (Ed.), (Vol. 5).

Kemp, G. (1998). Capillary electrophoresis: a versatile family of analytical techniques. *Biotechnol Appl Biochem, 27*(1), 9-17. doi:10.1111/j.1470-8744.1998.tb01369.x

Khan, I., & al-Awadi, F. M. (1997). Colonic muscle enhances the production of interleukin-1 beta messenger RNA in experimental colitis. *Gut, 40*(3), 307. doi:10.1136/gut.40.3.307

Kim, M., Lee, K.-H., Yoon, S.-W., Kim, B.-S., Chun, J., & Yi, H. (2013). Analytical tools and databases for metagenomics in the next-generation sequencing era. *Genomics & informatics, 11*(3), 102-113. doi:10.5808/GI.2013.11.3.102

Klinder, A., Shen, Q., Heppel, S., Lovegrove, J., Rowland, I., & Tuohy, K. (2016). Impact of increasing fruit and vegetable and flavonoid intake on the human gut microbiota. *Food Funct., 7*. doi:10.1039/C5FO01096A

Koizumi, S.-i., Wakita, D., Sato, T., Mitamura, R., Izumo, T., Shibata, H., . . . Nishimura, T. (2008). Essential role of Toll-like receptors for dendritic cell and NK1.1+ cell-dependent activation of type 1 immunity by Lactobacillus pentosus strain S-PT84. *Immunology Letters, 120*(1), 14-19. doi:https://doi.org/10.1016/j.imlet.2008.06.003

Kristjánsson, G., Venge, P., Wanders, A., Lööf, L., & Hällgren, R. (2004). Clinical and subclinical intestinal inflammation assessed by the mucosal patch technique: studies of mucosal neutrophil and eosinophil activation in inflammatory bowel diseases and irritable bowel syndrome. *Gut, 53*(12), 1806. doi:10.1136/gut.2003.036418

Laulund, S., Wind, A., Derkx, P. M. F., & Zuliani, V. (2017). Regulatory and Safety Requirements for Food Cultures. *Microorganisms, 5*(2), 28. doi:10.3390/microorganisms5020028

Lee, H.-N., Tian, L., Bouladoux, N., Davis, J., Quinones, M., Belkaid, Y., . . . Krzewski, K. (2017). Dendritic cells expressing immunoreceptor CD300f are critical for controlling chronic gut inflammation. *The Journal of Clinical Investigation, 127*(5), 1905-1917. doi:10.1172/JCI89531

Lewis, J. A. (1991). Formulation and delivery systems of biocontrol agents with emphasis on fungi. *The Rhizosphere and Plant Growth, Beltsville Symposia in Agricultural Research, 14*. doi: https://doi.org/10.1007/978-94-011-3336-4_55

Li, X.-Z., Plésiat, P., & Nikaido, H. (2015). The challenge of efflux-mediated antibiotic resistance in Gram-negative bacteria. *Clinical microbiology reviews, 28*(2), 337-418. doi:10.1128/CMR.00117-14

Liao, C. H., & Fett, W. F. (2001). Analysis of native microflora and selection of strains antagonistic to human pathogens on fresh produce. *J Food Prot, 64*(8), 1110-1115.

Liao, W., Lin, J.-X., & Leonard, W. J. (2011). IL-2 family cytokines: new insights into the complex roles of IL-2 as a broad regulator of T helper cell differentiation. *Current Opinion in Immunology, 23*(5), 598-604. doi:https://doi.org/10.1016/j.coi.2011.08.003

Link, L., & Potter, J. (2004). Raw versus Cooked Vegetables and Cancer Risk. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology, 13*, 1422-1435.

Linninge, C., Ahrné, S., & Molin, G. (2014). Pre-treatment with antibiotics and Escherichia coli to equalize the gut microbiota in conventional mice. *Antonie van Leeuwenhoek, 107*. doi:10.1007/s10482-014-0312-3

Liu, S., Lee, I. M., Ajani, U., Cole, S. R., Buring, J. E., & Manson, J. E. (2001). Intake of vegetables rich in carotenoids and risk of coronary heart disease in men: The Physicians' Health Study. *International Journal of Epidemiology, 30*(1), 130-135. doi:10.1093/ije/30.1.130

Liu, Y., Fatheree, N. Y., Mangalat, N., & Rhoads, J. M. (2010). Human-derived probiotic Lactobacillus reuteri strains differentially reduce intestinal inflammation. *American journal of physiology. Gastrointestinal and liver physiology, 299*(5), G1087-G1096. doi:10.1152/ajpgi.00124.2010

Long, S. M., Adak, G. K., O'Brien, S. J., & Gillespie, I. A. (2002). General outbreaks of infectious intestinal disease linked with salad vegetables and fruit, England and Wales, 1992-2000. *Communicable disease and public health, 5*(2), 101-105. Retrieved from http://europepmc.org/abstract/MED/12166293

Lopez-Velasco, G., Carder, P. A., Welbaum, G. E., & Ponder, M. A. (2013). Diversity of the spinach (Spinacia oleracea) spermosphere and phyllosphere bacterial communities. *FEMS Microbiology Letters, 346*(2), 146-154.

Lopez-Velasco, G., Tydings, H. A., Boyer, R. R., Falkinham, J. O., & Ponder, M. A. (2012). Characterization of interactions between Escherichia coli O157:H7 with epiphytic bacteria in vitro and on spinach leaf surfaces. *Int J Food Micobiol, 153*, 351-357.

Lopez-Velasco, G., Welbaum, G., Boyer, R., Mane, S., & Ponder, M. (2011). Changes in spinach phylloepiphytic bacteria communities following minimal processing and refrigerated storage described using pyrosequencing of 16S rRNA amplicons. *Journal of Applied Microbiology, 110*, 1203-1214. doi:10.1111/j.1365-2672.2011.04969.x

Lozupone, C. A., Hamady, M., Kelley, S. T., & Knight, R. (2007). Quantitative and Qualitative β Diversity Measures Lead to Different Insights into Factors That Structure Microbial Communities. *Applied and environmental microbiology, 73*(5), 1576. doi:10.1128/AEM.01996-06

Luo, Y., He, Q., & McEvoy, J. (2010). Effect of Storage Temperature and Duration on the Behavior of Escherichia coli O157:H7 on Packaged Fresh-Cut Salad Containing Romaine and Iceberg Lettuce. *Journal of food science, 75*, M390-397. doi:10.1111/j.1750-3841.2010.01722.x

Macherey-Nagel. (2020). NucleoSpin Soil, Mini kit for DNA from soil. Retrieved from https://www.mn-net.com/nucleospin-soil-mini-kit-for-dna-from-soil-740780.50

Mallon, C. A., Elsas, J. D. v., & Salles, J. F. (2015). Microbial Invasions: The Process, Patterns, and Mechanisms. *Trends in Microbiology, 23*(11), 719-729. doi:https://doi.org/10.1016/j.tim.2015.07.013

Mamedov, T. G., Pienaar, E., Whitney, S. E., TerMaat, J. R., Carvill, G., Goliath, R., . . . Viljoen, H. J. (2008). A fundamental study of the PCR amplification of GC-rich DNA templates. *Computational Biology and Chemistry, 32*(6), 452-457. doi:https://doi.org/10.1016/j.compbiolchem.2008.07.021

McKinnon, K. M. (2018). Flow Cytometry: An Overview. *Current Protocols in Immunology, 120*(1), 5.1.1-5.1.11. doi:https://doi.org/10.1002/cpim.40

Mendes, V., Galvão, I., & Vieira, A. T. (2019). Mechanisms by Which the Gut Microbiota Influences Cytokine Production and Modulates Host Inflammatory Responses. *Journal of Interferon & Cytokine Research, 39*(7), 393-409. doi:10.1089/jir.2019.0011

Meso Scale Discovery. (2020). Why electroluminescence.

Mogren, L., Windstam, S., Boqvist, S., Vågsholm, I., Söderqvist, K., Rosberg, A. K., . . . Alsanius, B. (2018). The Hurdle Approach-A Holistic Concept for Controlling Food Safety Risks Associated With Pathogenic Bacterial Contamination of Leafy Green Vegetables. A Review. *Frontiers in microbiology, 9*, 1965-1965. doi:10.3389/fmicb.2018.01965

Munford, R. S., & Varley, A. W. (2006). Shield as Signal: Lipopolysaccharides and the Evolution of Immunity to Gram-Negative Bacteria. *PLOS Pathogens, 2*(6), e67. doi:10.1371/journal.ppat.0020067

Murphy, K., Travers, P., & Walport, M. (2008). *Janeway's Immunology* (7th ed.). New York, USA and Abingdon, UK: Garland Science, Taylor & Francis Group, LLC.

Nielsen, K., Mogensen, H. S., Hedman, J., Niederstätter, H., Parson, W., & Morling, N. (2008). Comparison of five DNA quantification methods. *Forensic Science International: Genetics, 2*(3), 226-230. doi:https://doi.org/10.1016/j.fsigen.2008.02.008

Norling, B. (1994). *Food poisoning in Sweden—results of a field survey*.

Nousiainen, L. L., Joutsen, S., Lundén, J., Hänninen, M.-L., & Fredriksson-Ahomaa, M. (2016). Bacterial quality and safety of packaged fresh leafy vegetables at the retail level in Finland. *International Journal of Food Microbiology, 232*. doi:10.1016/j.ijfoodmicro.2016.05.020

O'Brien, P. A. (2017). Biological control of plant diseases. *Australasian Plant Pathology, 46*(4), 293-304. doi:10.1007/s13313-017-0481-4

Oliveira, M., Abadias, M., Colás-Medà, P., Usall, J., & Vinas, I. (2015). Biopreservative methods to control the growth of foodborne pathogens on fresh-cut lettuce. *International Journal of Food Microbiology, 214*, 4-11. doi:10.1016/j.ijfoodmicro.2015.07.015

Ormerod, M. G. (2008). *Flow cytometry - a basic introduction*: Michael G. Ormerod.

Osborn, A., Moore, E., & Timmis, K. (2000). An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of

microbial community structure and dynamics. *Environmental Microbiology, 2*, 39-50.
Osman, N., Adawi, D., Molin, G., Ahrne, S., Berggren, A., & Jeppsson, B. (2006). Bifidobacterium infantis strains with and without a combination of oligofructose and inulin (OFI) attenuate inflammation in DSS-induced colitis in rats. *BMC gastroenterology, 6*, 31-31. doi:10.1186/1471-230X-6-31
Painter, J. A., Hoekstra, R. M., Ayers, T., Tauxe, R. V., Braden, C. R., Angulo, F. J., & Griffin, P. M. (2013). Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998-2008. *Emerg Infect Dis, 19*(3), 407-415.
Pandey, J., Ganesan, K., & Jain, R. K. (2007). Variations in T-RFLP profiles with differing chemistries of fluorescent dyes used for labeling the PCR primers. *Journal of Microbiological Methods, 68*(3), 633-638. doi:https://doi.org/10.1016/j.mimet.2006.11.012
Parada, A. E., Needham, D. M., & Fuhrman, J. A. (2016). Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environmental Microbiology, 18*(5), 1403-1414. doi:https://doi.org/10.1111/1462-2920.13023
Parish, M. E., Beuchat, L. R., Suslow, T. V., Harris, L. J., Garrett, E. H., Farber, J. N., & Busta, F. F. (2003). Methods to reduce/eliminate pathogens from fresh and fresh-cut produce. *Comp Rev Food Sci Food Safety, 2*(1), 161-173.
Patel, J. B. (2001). 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. *Molecular Diagnosis, 6*(4), 313-321. doi:https://doi.org/10.1054/modi.2001.29158
Pavan, S., Desreumaux, P., & Mercenier, A. (2003). Use of mouse models to evaluate the persistence, safety, and immune modulation capacities of lactic acid bacteria. *Clinical and diagnostic laboratory immunology, 10*(4), 696-701. doi:10.1128/cdli.10.4.696-701.2003
Pettersson, B., Ahrné, S., Wang, M., Jeppsson, B., Uhlén, M., & Molin, G. (2003). *The mucosa-associated bacteria from the sigmoid colon of nine healthy 60 years old individuals, identified by bacterial 16S rDNA*.
Qiagen. (2020). EZ1 Advanced XL. Retrieved from https://www.qiagen.com/us/products/diagnostics-and-clinical-research/solutions-for-laboratory-developed-tests/ez1-advanced-xl-instrument/?clear=true#orderinginformation
R Team. (2006). A language and environment for statistical computing. *Computing, 1*. doi:10.1890/0012-9658(2002)083[3097:CFHIWS]2.0.CO;2
Robin, J. D., Ludlow, A. T., LaRanger, R., Wright, W. E., & Shay, J. W. (2016). Comparison of DNA Quantification Methods for Next Generation Sequencing. *Scientific Reports, 6*(1), 24067. doi:10.1038/srep24067
Rosberg, A. K., Darlison, J., Mogren, L., & Alsanius, B. W. (2021). Commercial wash of leafy vegetables do not significantly decrease bacterial load but

leads to shifts in bacterial species composition. *Food Microbiol, 94*, 103667. doi:10.1016/j.fm.2020.103667

Rubbens, P., Props, R., Kerckhof, F.-M., Boon, N., & Waegeman, W. (2021). Cytometric fingerprints of gut microbiota predict Crohn's disease state. *The ISME Journal, 15*(1), 354-358. doi:10.1038/s41396-020-00762-4

Sabouri, S., Sepehrizadeh, Z., Amirpour-Rostami, S., & Skurnik, M. (2017). A minireview on the in vitro and in vivo experiments with anti-Escherichia coli O157:H7 phages as potential biocontrol and phage therapy agents. *International Journal of Food Microbiology, 243*, 52-57. doi:https://doi.org/10.1016/j.ijfoodmicro.2016.12.004

Sanger, F., & Coulson, A. R. (1975). A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *Journal of Molecular Biology, 94*(3), 441-448. doi:https://doi.org/10.1016/0022-2836(75)90213-2

Schloss, P., Westcott, S., Ryabin, T., Hall, J., Hartmann, M., Hollister, E., . . . Weber, C. (2009). Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. *Applied and environmental microbiology, 75*, 7537-7541. doi:10.1128/AEM.01541-09

Shafi, J., Tian, H., & Ji, M. (2017). Bacillus species as versatile weapons for plant pathogens: a review. *Biotechnology & Biotechnological Equipment, 31*(3), 446-459. doi:10.1080/13102818.2017.1286950

Singh, G., Kawatra, A., & Sehgal, S. (2001). Nutritional composition of selected green leafy vegetables, herbs and carrots. *Plant Foods for Human Nutrition, 56*(4), 359-364. doi:10.1023/A:1011873119620

Söderqvist, K., Osman, O., Wolff, C., Bertilsson, S., Vågsholm, I., & Boqvist, S. (2017). Emerging microbiota during cold storage and temperature abuse of ready-to-eat salad. *Infection Ecology & Epidemiology, 7*, 1328963. doi:10.1080/20008686.2017.1328963

Söderqvist, K., Rosberg, A. K., Boqvist, S., Alsanius, B., Mogren, L., & VåGsholm, I. (2019). Season and Species: Two Possible Hurdles for Reducing the Food Safety Risk of Escherichia coli O157 Contamination of Leafy Vegetables. *Journal of Food Protection, 82*(2), 247-255. doi:10.4315/0362-028X.JFP-18-292

Solomon, E. B., & Matthews, K. R. (2005). Use of fluorescent microspheres a a tool to investigate bacterial interactions with growing plants. *J Food Prot, 67*, 2342-2253.

Spiller, S., Elson, G., Ferstl, R., Dreher, S., Mueller, T., Freudenberg, M., . . . Kirschning, C. J. (2008). TLR4-induced IFN-γ production increases TLR2 sensitivity and drives Gram-negative sepsis in mice. *Journal of Experimental Medicine, 205*(8), 1747-1754. doi:10.1084/jem.20071990

Tatsika, S., Karamanoli, K., Karayanni, H., & Genitsaris, S. (2019). Metagenomic Characterization of Bacterial Communities on Ready-to-Eat Vegetables and Effects of Household Washing on their Diversity and Composition. *Pathogens (Basel, Switzerland), 8*(1), 37. doi:10.3390/pathogens8010037

Thermo Fischer Scientific. (2020a). NanoDrop™ 2000/2000c Spectrophotometers. Retrieved from https://www.thermofisher.com/order/catalog/product/ND2000LAPTOP?SID=srch-srp-ND2000LAPTOP#/ND2000LAPTOP?SID=srch-srp-ND2000LAPTOP

Thermo Fischer Scientific. (2020b). Qubit 4 Fluorometer. Retrieved from https://www.thermofisher.com/se/en/home/industrial/spectroscopy-elemental-isotope-analysis/molecular-spectroscopy/fluorometers/qubit/qubit-fluorometer.html

Toljander, T., & Karnehed, N. (2010). *Vad gör de som drabbas av magsjuka och matförgiftningar? - Resultat från en nationell intervjuundersökning.* .

Vaishnava, S., Behrendt, C., Ismail, A., Eckmann, L., & Hooper, L. (2009). Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. *Proceedings of the National Academy of Sciences of the United States of America, 105*, 20858-20863. doi:10.1073/pnas.0808723105

van Elsas, J. D., Chiurazzi, M., Mallon, C. A., Elhottovā, D., Krištůfek, V., & Salles, J. F. (2012). Microbial diversity determines the invasion of soil by a bacterial pathogen. *Proceedings of the National Academy of Sciences, 109*(4), 1159. doi:10.1073/pnas.1109326109

Voronov, E., & Apte, R. (2015). IL-1 in Colon Inflammation, Colon Carcinogenesis and Invasiveness of Colon Cancer. *Cancer Microenvironment, 8*. doi:10.1007/s12307-015-0177-7

WHO. (2017). The burden of foodborne diseases in the WHO European Region.

Williams, T. R., Moyne, A. L., Harris, L. J., & Marco, M. L. (2013). Season, irrigation, leaf age, and Escherichia coli inoculation influence the bacterial diversity in the lettuce phyllosphere. *PloS one, 8*(7), e68642. doi:10.1371/journal.pone.0068642

Wilson, M. J., Weightman, A. J., & Wade, W. G. (1997). Applications of molecular ecology in the characterization of uncultured microorganisms associated with human disease. *Reviews in Medical Microbiology, 8*(2). Retrieved from https://journals.lww.com/revmedmicrobiol/Fulltext/1997/04000/Applications_of_molecular_ecology_in_the.5.aspx

Wood, K. J., & Sawitzki, B. (2006). Interferon gamma: a crucial role in the function of induced regulatory T cells *in vivo*. *Trends in Immunology, 27*(4), 183-187. doi:10.1016/j.it.2006.02.008

Zhang, G., Chen, Y., Hu, L., Melka, D., Wang, H., Laasri, A., . . . Hammack, T. S. (2018). Survey of Foodborne Pathogens, Aerobic Plate Counts, Total Coliform Counts, and Escherichia coli Counts in Leafy Greens, Sprouts, and Melons Marketed in the United States. *Journal of Food Protection, 81*(3), 400-411. doi:10.4315/0362-028X.JFP-17-253

Zhang, M., Zhang, S., Hua, Z., & Zou, X. (2017). Long-term use of Bifidobacterium longum alleviates colorectal colitis in rats by regulating inflammatory cytokines and Treg cells. *International Journal of Clinical and Experimental Medicine, 10*(5), 7543-7552. Retrieved from

https://www.scopus.com/inward/record.uri?eid=2-s2.0-85019691713&partnerID=40&md5=c645d8a62d4ed2379f2f3764effa38fb

Zoetendal, E. G., Ben-Amor, K., Akkermans, A. D. L., Abee, T., & de Vos, W. M. (2001). DNA Isolation Protocols Affect the Detection Limit of PCRApproaches of Bacteria in Samples from the HumanGastrointestinal Tract. *Systematic and Applied Microbiology, 24*(3), 405-410. doi:https://doi.org/10.1078/0723-2020-00060