Effects and safety of selected antagonistic bacteria against *Escherichia coli* in leafy green vegetables

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To everyone who has assisted me through my master studies.

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for love, laughs, patience, and valuable feedback throughout the ride.

Abstract

We live in a busy world, where the demand of ready-to-eat (RTE) salad products has increased rapidly in a relatively short time. Despite of being an easy and fast way to add more vegetables to the daily diet, RTE salads have a serious underlying risk and the amount of vegetable-derived foodborne disease outbreaks has been increasing. The aim of the present study is to protect plants against contamination with human pathogens by inoculating spinach (*Spinacia oleracea*) seeds with antagonists, inherent on leafy green vegetables. Experiments were carried on to observe the effects of selected antagonistic bacteria against non-pathogenic *Escherichia coli* CCUG29300^{T} contamination on live spinach sprouts, and to test the safety by studying the immune response in mice. In addition, bacterial isolates from RTE rocket salad (*Eruca sativa*) were identified by 16S rRNA gene sequencing. The putative results show that bagged rocket salad contains bacteria from several different families and genera, the most abundant being *Pseudomonas,* commonly found from leafy green vegetables and *Aeromonas* that are omnipresent in soil, water and vegetation. Surprising findings were *Rheinheimera, Chryseobacterium,* and *Shewanella* that are more commonly associated with a marine environment. Unfortunately, the antagonistic effect of selected bacteria could not be seen in this study, as no statistically significant differences between treated samples and negative control were found ($p>0.05$). The seed pellet may work as a physical barrier hindering the inoculation, or has antimicrobial effects that increases the death rate of two antagonists. FACS analysis of Peyer's patches and mesenteric lymph nodes of mice indicate that the tested antagonists may be able to suppress non-pathogenic *E. coli* induced inflammation in mice. In Peyer's patches, the percentage of gated CCR9+CD8+CD69+ cells was significantly lower in the groups A (15.9 (6.2–23.2), $p=0.037$) and E (17.1 (7.7–25.3), $p=0.029$) compared with the control group K (30.3 (20.8–72.1)). These percentages were on the similar level with the untreated group N (13.7 (10.3–17.6)). The percentage of TLR2+TLR4+ activated macrophages in the antagonist groups varied between E 22.7 (21.7–35.7) and B 28.9 (18.6–39.1). All results were significantly lower compared with the group K 62.7 (57.2 – 67.9). There were no significant differences in water or feed intakes between groups, which indicates that antagonist treatment do not make mice feel sick. This study supports the current knowledge of leafy green vegetables harbouring a very diverse microflora with genera with potential human pathogens. Despite of poor results in antagonistic effect experiment, preliminary results indicate that selected antagonists do not trigger a negative immune response in mice.

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1 Introduction

Consuming vegetables, berries and fruits have numerous health-promoting effects. Leafy green vegetables, including spinach, are found to be a good source of minerals, ascorbic acid (vitamin C), and iron (Singh, Kawatra and Sehgal, 2001). In addition, vegetables, fruits and berries have a high amount of dietary fibre (Campos, Mourão, Pestana, Peixe, Novais and Antunes, 2013) that supports the gastrointestinal functions (Schneeman, 1998). The consumer awareness and demand have expanded the market for ready-to-eat (RTE) salads and salad mixes that can be bought around the year (Campos *et al.,* 2013). RTE salads seem to be a great way to increase the vegetable consumption, but despite of the positive health promoting effects they also have an underlying safety issue that some consumers may not be aware of (Campos *et al.,* 2013).

An increasing amount of vegetable-derived foodborne disease outbreaks have been recorded during last few years. A long-term American study with pre-electronically and electronically collected data from 1973 to 2012 revealed, that the annual amount of leafy green derived outbreaks have increased from a median of 4 to a median of 37, but the amount of patients per outbreak has decreased from a median of 39 to a median of 14 (Herman, Hall and Gould, 2015). The most common pathogen was norovirus, and it was followed by shiga toxin producing *Escherichia coli* (STEC), and *Salmonella* (Herman *et al.,* 2015). However, a similar trend has also been seen in Europe where several links between foodborne disease outbreaks and leafy greens like spinach, lettuces and RTE mixes have been reported (Mercanoglu Taban and Halkman, 2011). For example, nearly 200 people in Finland got sick from STEC serogroup O157 contaminated RTE salad in summer 2016 (Yle News, 2016).

The present study is a prolongation of an advanced course project (KLG410, 15 ECTS), that was carried out by the same author in Lund University from September to December 2016. The aim of the project was mainly to investigate the effects of household storage on the total microbial load and the survivability of inoculated, non-pathogenic *Escherichia coli* CCUG29300^{T} (Culture Collection of Göteborg, T implies typestrain) in RTE rocket salad bags over the shelf-life of nine days. Results showed that the total aerobic bacterial load of untreated bags increased from 7.1 $\log_{10}$ CFU/g to log 8.7 $\log_{10}$ CFU/g and total *Enterobacteriaceae* concentration increased from 3.4 $\log_{10}$ CFU/g to 4.0 $\log_{10}$ CFU/g. The development of total aerobic bacteria count was concordant with bags with *E. coli* CCUG29300^{T}. Even though the concentration of inoculated *E. coli* CCUG29300^{T} decreased from 5.4 $\log_{10}$ CFU/g to 4.3 $\log_{10}$ CFU/g, there is a strong indication that *E. coli* can survive in household storage conditions. In the future the same experiment will be tested with *E. coli* strain inherent on salads.

During this study, few bacterial isolates from the mentioned advanced course project were identified to be able to characterize the microbial flora of RTE rocket salad mix over the self-life period. The aim of this study is to protect plants against contamination with human pathogens by inoculating seeds with antagonists inherent on leafy green vegetables. This could be an efficient way to decrease the leafy green derived outbreaks in the future. The survivability and antagonistic activity of the five potential candidates were tested with spinach seed and sprout experiments. An animal study was also performed to test the safety of the antagonists by studying the immune response in mice upon consumption.

2 Literature study

2.1 Microbial quality and safety of leafy green vegetables

Leafy green vegetables, usually spinach, lettuces and their RTE salad mixes are potential carriers of human pathogens. King, Magnuson, Török and Goodman (1991) ran an experiment to characterize the microflora of partially processed lettuce (*Latuca sativa*). They found several bacteria for example from the genera *Enterobacter, Erwinia, Pseudomonas* and *Bacillus*, which are known to have members with human pathogenic potential. When some of these bacteria are inherit on the leafy green vegetables, it is important to realize that the contamination with potential pathogens can happen in every stage from pre-harvest to the kitchen of a consumer (Mercanoglu Taban and Halkman, 2011). Potential pathogens at the pre-harvest stage are *Escherichia coli*, *Salmonella* spp., *Listeria*, as well as different parasites and viruses (Mercanoglu Taban and Halkman, 2011). Contamination can naturally happen through the soil or irrigation water. However, since the postharvest stage includes everything from transporting and processing to retail, there are several points of potential contamination. The hazard potential is high, because RTE salads do not undergo any processing steps to eliminate the contaminants. In addition, when salad leaves get damaged during handling or transportation, the leaking juice works as a strong growth enhancer for bacteria (Koukkidis, Haigh, Allcock, Jordan and Freestone, 2016). Therefore, maintaining the microbial safety of leafy green vegetables and their RTE products is essential on every processing step, and requires commitment from every operative throughout the food chain (Mercanoglu Taban and Halkman, 2011).

2.2 Immune system – The defender of the body

The mammalian body is a highly developed system with several structures and processes. This system would be extremely sensitive against pathogens without a layered defence system. The body has several ways to protect itself from potentially harmful microbes and substances, as well as to remove them. The immune system is a group of cells, molecules and tissues that are preventing infections and exterminating the ongoing ones (Abbas, Lichtman and Pillai, 2015). This system is capable to react to the pathogens in various ways, and these reactions are called the immune response (Abbas *et al.* 2015).

The first line of our natural immune system, also known as the innate immune system, is the epithelial barriers such as skin and mucosa (Janeway, Murphy, Travers, Walport and Ehrenstein, 2008). If these layers get damaged and the infectious microbe gets in contact with other organs, the damaged area gets swollen, red and hot. This reaction, inflammation, is the first immune response of our immune system after it receives stress signals from the damaged or infected cells. Several infections are successfully eradicated by the innate immune system and do not lead to diseases (Abbas *et al.,* 2015). However, if the innate immune system encounters a resistant pathogen and fails to eliminate it, signals to activate the adaptive immune system are sent (Abbas *et al.,* 2015). Elimination of harmful microbes by the adaptive system is followed by immunological memory (Janeway *et al.,* 2008).

2.2.1 *Factors of the immune system*

An essential part of our immune system is the lymphatic system, a highly specialized entirety of vessels (Janeway *et al.,* 2008). Circulating fluid in the lymphatic system, lymph, is formed when interstitial fluid from tissues is collected by lymph capillaries (Choi, Lee and Hong, 2012). Collected lymph is cleaned in the lymph nodes, an enclosed capsule of several lymphoid lobules and sinuses, and the cleaned lymph gets migrated back with the blood (Choi *et al.*, 2012). During this circulation process, antigens are transferred to the lymph nodes and lymphocytes back to the blood (Janeway *et al.,* 2008). Mice have 22 known lymph nodes, while humans have approximately 20 times more, and these organs play an important role in the interaction between antigen presenting cells (APC) and lymphocytes (Willard-Mack, 2006). In the gut, the sorting between pathogens and commensal microflora is managed by the interaction between epithelium and the gut-associated lymphoid tissue (GALT) (Jung, Hugot and Barreau, 2010). The most important part of GALT are Peyer's patches which are highly organized structures that get their antigens via multi-fenestrated epithelial cells, M cells (Janeway *et al.,* 2008).

Innate and adaptive immunity depend on the actions of white blood cells, leukocytes. Like every other cellular element of blood, leukocytes are derived from the hematopoietic stem cells in the bone marrow and the cell subsets are formed after different maturation pathways (Janeway *et al.,* 2008). To be able to recognize pathogens and respond to their presence, leukocytes express different receptors (Janeway *et al.,* 2008). These receptors are proteins that are specialized to recognize different molecules produced by both non-infectious and pathogenic molecules, and activate a chain of reactions based on the encounter (Abbas *et al.,* 2015).

Macrophages are important cells of the innate immune system, and are able to start the inflammatory response. When a macrophage encounters a pathogenic bacteria, it starts to release chemokines and cytokines to increase the permeability of blood vessels and to guide neutrophils to the infected area (Janeway *et al.,* 2008). Macrophages are casually called as big eaters, because they also devour the harmful bacteria in the process called phagocytosis (Janeway *et al.,* 2008). Macrophages express marker F4/80, and the high amount of them usually indicates that the body contains numerous harmful bacteria (Janeway *et al.,* 2008). Furthermore, together with microphages, macrophages can also express Toll-like receptor (TLR) 4 or 2 based on what type of pathogen they have devoured (Janeway *et al.,* 2008). TLR2 is associated with Gram-positive bacteria, and TLR4 with Gram-negative bacteria (Janeway *et al.,* 2008). Gram-negative bacteria include human pathogens like *Escherichia coli*, *Yersinia,* and *Pseudomonas aeruginosa.*

Dendritic cells (DCs) work in both the innate and the adaptive immune system, expressing the CD11c marker. New DCs migrate from the blood to the peripheral tissues, where they keep ingesting the surrounding extracellular fluids (Janeway *et al.,* 2008). When a DC encounters a pathogen, it undergoes a quick maturation and migrates to the lymph nodes where it works as an antigen presenting cell for T cells, triggering the adaptive immune response (Janeway *et al.,* 2008).

The naive lymphocytes need two signals to get activated (Janeway *et al.,* 2008). The first signal appears when an antigen binds to the cell receptor, and the second signal is given by APC with antigens presented on its major histocompatibility complex (MCII) peptide (Janeway *et al.,* 2008).

On T cells, the activation can cause proliferation through two major pathways into effector cells: Helper T cells (effector CD4+), which secrete cytokines to attract macrophages, neutrophils and other co-operators towards the harmful cells, and cytotoxic T cells (CD8+), which are directly attacking cells that are expressing intracellular antigens (Abbas *et al.,* 2015). T regulatory (T_{reg}) cells also belong to the subset of T cells and have an important function on suppressing and downregulating the accumulation of effector CD4+ cells and therefore, preventing autoimmune diseases (Bettelli *et al.,* 2006). T_{reg} cells express not only the markers CD69 and FOXP3, but also CD25 and CD4, which makes differencing between CD4+ cells and T_{reg} cells sometimes challenging (Bettelli *et al.,* 2006). CC chemokine receptor 9 (CCR9) expressing T cells are homing and activating specifically in the lamina propria mucosæ and epithelium of small intestine, and can then be used to investigate the infections of the gut (Wurbel, Malissen, Guy-Grand, Meffre, Nussenzweig, Richelme, Carrier, and Malissen, 2001). B cells have different activation ways, and the most significant is receiving the signal from an activated (follicular) T helper cell (Janeway *et al.,* 2008). Activated B cells do not attack on other cells, but instead proliferate into plasma cells and start producing antibodies - special proteins that are designed to work against antigens by triggering mechanisms that eliminate or block them (Abbas *et al.,* 2015). A generic structure of an antibody is presented in Figure 1.

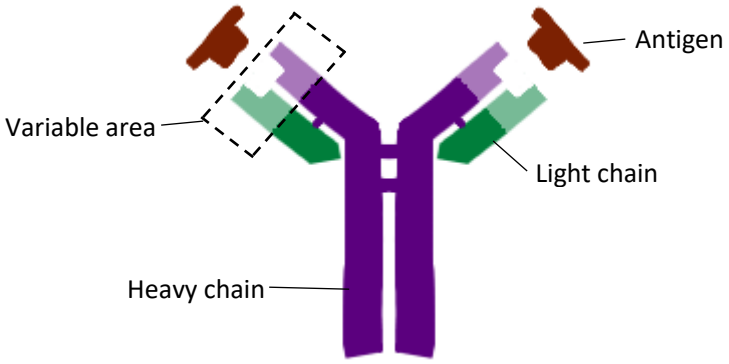

Figure 1 - A generic structure of an antibody

Specific antibodies can be used as biomarkers, quantifiable distinctive mark of biological processes when study has a well-defined clinical endpoint (Strimbu and Tavel, 2010).

2.3 Fluorescence-activated cell sorting (FACS)

By knowing the expressing markers of different intra- or extracellular proteins, the presence of the corresponding immune cell can be investigated with fluorescence-activated cell sorting (FACS). The fluorescence conjugated antibodies react specifically to the wanted proteins, which provides an efficient method to sort cells based on the specific light scattering (Graham, 2006). The cell suspension gets entrained by a rapid liquid stream as single cell flow, from which the cells are separated by a set of filters and mirrors, and finally registered

by the generated signal (Graham, 2006). This allows precise cell count on wanted cell populations, selected by gating the known characteristics, expressing marker, side scatter, and forward scatter (Graham, 2006). Scatter data is specifically used to outline the signals from clumped cells and impurities (Graham, 2006). Since the data analysis is dependent of the correct gating, it is required to run a compensation with either beads or single stained cells to prevent double positive signals (Graham, 2006).

2.4 Growth media used

Growth media was used to cultivate frozen antagonists, and bacteria from the spinach sprouts. All materials were autoclaved or prepared with given instructions, and stored in a refrigeration room at 8 °C when not in use. Tryptic Soy Broth, TSB (Sigma-Aldrich, Hamburg, Germany), is a multipurpose growth promoting liquid used to cultivate *E. coli* and the antagonists A–C. Tryptic Soy Agar, TSA (Sigma-Aldrich, Hamburg, Germany), is a multipurpose medium, which is used in solid form to support the growth of several non- or slightly fastidious microbes. Prepared TSA plates were used to investigate and monitor the aerobic bacterial load of spinach sprouts. De Man, Rogosa and Sharpe, generally abbreviated to MRS, broth and agar (Merck KGaA, Darmstad, Germany) are non-selective media used to abundant growth of lactic acid bacteria. MRS broth and agar was used to cultivate antagonists D and E. The presence and concentration of *E. coli* were investigated by using Brilliance *E. coli*/coliform Selective Agar, ECBA (Oxoid, Hampshire, England). ECBA contains two chromogenic agents Rose-Gal and X-Glu, which are used to detect ß-galactosidase and ß-glucuronidase activity, giving *E. coli* colonies a dark purple colour. Media also contains sodium layrul sulphate to inhibit the growth of Gram-positive microbes (Oxoid, n.d.).

3 Materials and methods

3.1 Identification of the bacterial isolates from bagged rocket salad

Bacterial isolates from RTE rocket salad (*Eruca sativa*) bags were collected by randomized sampling over the period from November 22 to December 3, 2016. Frozen bacteria isolates were thawed and transferred on TSA plates with sterile 1 µl loops. Plates were incubated in 30 °C or 37 °C based on which growth media they originated from, and checked after 24–48 hours depending on the colony growth speed. Plates that contained only one type of colony (see Figure 2) were considered to be pure, and a single colony was transferred to a 1.5 ml safe-lock microcentrifuge tubes (Eppendorf, Hamburg, Germany) with 8–10 small sterile glass beads and 0.5 ml of ultrapure (Milli-Q) water. Tubes were then shaken with an Eppendorf Mixer 5432 (Eppendorf, Hamburg, Germany) for 30 minutes and stored at -20 °C.

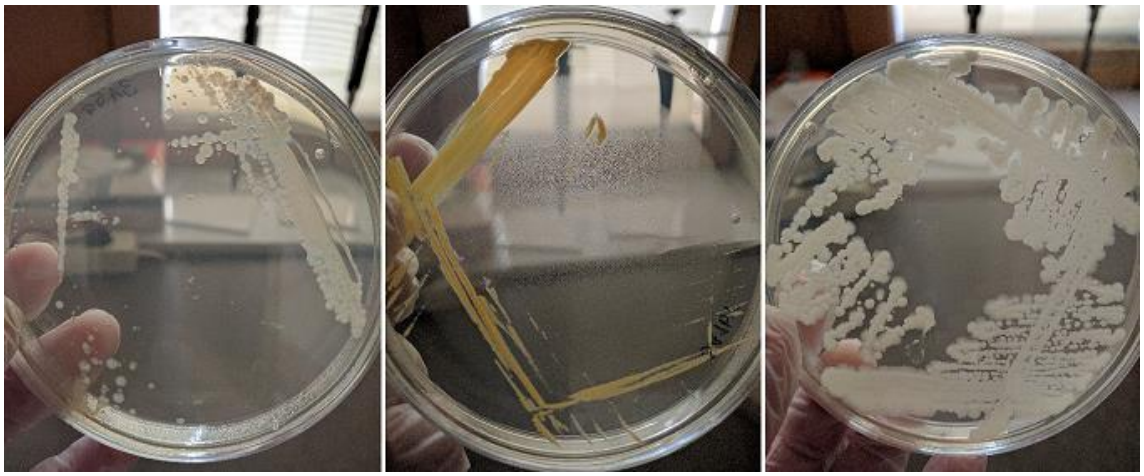

Figure 2 - Purified bacteria isolates from ready-to-eat rocket salad bags.

3.1.1 *Polymerase chain reaction (PCR) and gel electrophoresis*

Thawed samples were centrifuged (8 rpm for 1 min) and the DNA extract (supernatant) collected. The random 16S rRNA genes were amplified using forward primer ENV1 (5'-AGA GTT TGA TII TGG CTC AG-3') and reverse primer ENV2 (5'-CGG ITA CCT TGT TAC GAC TT-3') from Eurofins Genomics, Germany. 2.5 µl of sample DNA was mixed with 22.5 µl of the PCR Master Mix that contained 18.375 µl of nucleus free water (Qiagen, Germany), 2.5 µl of TopTaq 10 x PCR buffer (Tris-HCl, KCl, (NH4)2SO4, 15 mM MgCl2, pH 8.7) (Qiagen, Germany), 0.5 µl of deoxynucleotide (dNTP) solution mix (Qiagen, Germany), 0.5 µl of each primer, and 0.125 µl of TopTaq DNA Polymerase (Qiagen, Germany). The negative control sample was prepared in the same way, but 2.5 µl of nucleus free water was used instead of sample DNA. The PCR was performed in Eppendorf Mastercycler gradient (Eppendorf, Hamburg, Germany) using TopTaq25 program with the following steps: Initial denaturation at 94 °C for 3 min, 25 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C, for 1 min. In the end, the final extension at 72 °C for 10 minutes was done (Qiagen, 2010).

The quality and yield of amplified PCR products were tested by 1.5% Agarose Gel Electrophoresis, 60 minutes at 120 V in Bio-Rad Power Pac 300 (Bio-Rad, USA). To visualise the amplification product (see Figure 3) gel was stained with 0.03% GelRed™ Nucleic Acid Gel Stain 10000x (Biotium, USA) for 20 minutes and observed on UV transilluminator (UVP, USA). Samples with good yield and quality were sent to Eurofins Genomics, Ebersberg, Germany for sequencing on an ABI 3130xl Genetic analyser (Applied Biosystems, Foster City, CA, USA) with ENV1 as sequencing primer.

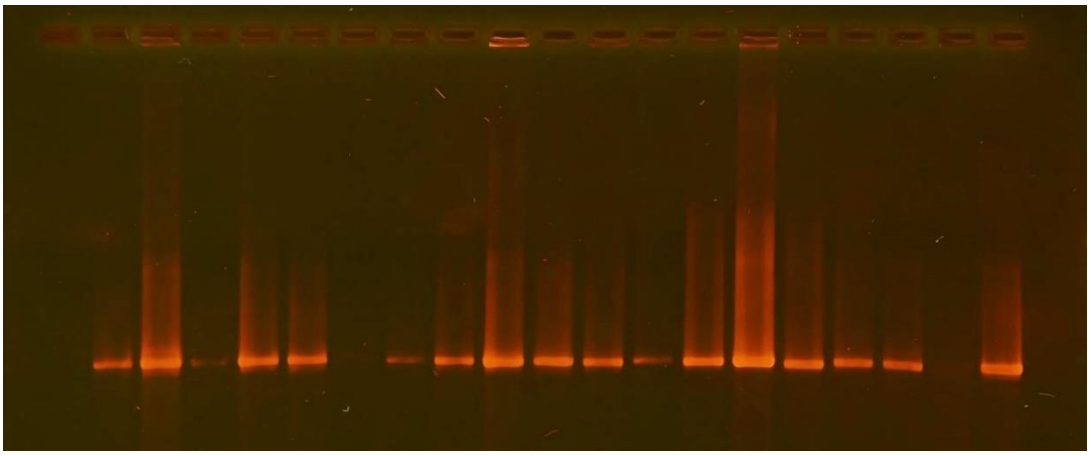

Figure 3 - Dyed PCR amplification products of isolates from ready-to-eat salad bags on 1.5% agarose gel after gel electrophoresis. Gel does not contain ladder.

3.2 Effects of the selected antagonist on spinach sprouts

To investigate antagonistic effects of the selected bacteria on spinach sprouts (*Spinacia oleracea*), pelleted seeds were supplied from Södervidinge gård, Kävlinge, Sweden. Selected antagonists from previous experiment are from different bacterial orders, but all of them have shown potential antagonistic activity against *Escherichia coli in vitro*. In this paper, the selected antagonists are called A, B, C, D and E. The isolates were stored in a freezer at -81 °C and thawed only for inoculation purposes. Before seed inoculation, frozen isolates were incubated using growth media, temperatures and times shown in Table 1.

Table 1 - Used growth media, incubation temperatures and incubation times for selected antagonists

Antagonist	Growth medium	Incubation temperature	Incubation time
A and B	TSB	30 °C	24 hours
C	TSB	30 °C	Three days
D and E	MRS	37 °C	Three days

Incubated tubes were centrifuged (6000 rpm for 5 minutes) and the supernatant discarded. To wash the culture, 8 ml of nutrient solution (for composition, see Appendix 1) was added, tubes centrifuged again, remaining supernatant discarded, and finally 6 ml of nutrient solution was added. The required amount of cells per 1 ml of solution was 10^8. To check the concentration, the optical density (OD) of the culture was measured at wavelength of 620 nm in a spectrophotometer (Pharmacia Novaspec II). The required OD values are shown in Table 2.

Table 2 - Required OD8 (620 nm) values for used antagonists

Antagonist	A	B	C	D	E
$OD8_{620nm}$ +/- 0.002	0.016	0.020	0.236	0.265	0.211

The first set seeds were placed on rockwool plugs (IKEA, Sweden), three seeds per plug. Seeded plugs were placed in sterile glass jars, three plugs per jar, with 20 ml of nutrient solution. To start sprouting in dark, jars were placed in a sealed cardboard box and left for one week at room temperature. After one week, bottles were placed under UV-LED lights for five days to promote growth of sprouts. A timer was used to simulate the day and night cycle with 6.5 hours of darkness and 17.5 hours of light. After five days, an antagonist solution with the concentration of 10^8 colony forming units per millilitre (CFU/ml) was prepared and sprouts were dipped in for 10 seconds. The time was based on the previous studies. Growth under UV-LED lights was continued for 5 days before dipping in *E. coli* CCUG29300^T solution with the same concentration and dipping time. Growth under UV-LED lights was continued for two days.

For sets two and three instead of dipping the sprouts, the seeds were inoculated with the wanted antagonist. A set (n=6) of 8 to 10 seeds were transferred in 1.5 ml microcentrifuge tubes with 1.0 ml of solution, and incubated in room temperature for 5 hours. The seeds for negative control were prepared in the same way, but microcentrifuge tubes contained only 1.0 ml of nutrient solution. The growing procedure was the same as previously, but *E. coli* dip was done right after the first 7+5 days.

After the growing period, healthy sprouts (Figure 4) were cut off and weighed. Roots and dead or rotten sprouts were discarded (Figure 4). Weighed sprouts were put in a mortar, and crushed well with added 2.0 ml of peptone water (8.5 g/l of sodium chloride (Merck, Darmstadt, Germany) and 1 g/l of bacteriological peptone (Oxoid, Hampshire, England)) to create a nearly homogenous solution. The serial dilution was made from 10^0 to 10^{-5} and the spreading was done on ECBA from 10^0 to 10^{-3}, and on TSA from 10^{-2} to 10^{-5}. TSA plates were incubated at 30 °C for three days, and ECBA plates at 37 °C for 24 hours. Colony count was performed to all plates after the incubation.

Figure 4 - Examples of healthy sprouts (left) and discarded material (right).

3.2.1 *Survivability of the antagonists on pelleted seeds*

Nutrient solution with 10^8 CFU/ml of the antagonistic bacteria was spread on TSA or MRS plates, depending on the used bacteria. This was done to get a comparable sample. To investigate if the pelleting material on spinach seeds is toxic for the used antagonist, 20 seeds per antagonist (n_{tot}=100) were sprayed with ethanol to disinfect the surface, let to dry in a laminar flow hood, and transferred to Falcon tubes with 2.0 ml of nutrient solution with 10^8 CFU/ml of the antagonist. Seeds were kept in the solution for 5 hours, after which the solution was spread on TSA or MRS plates. TSA plates were incubated at 30 °C, and MRS plates at 37 °C for 1–3 days before the colony count.

3.3 Animal experiment to investigate the safety of selected antagonists

Wild type (WT) black female C57BL/6 mice (Charles Rivers Laboratories International Inc. Germany) with body weight 18.6–22.0 g, were kept in standard conditions in the animal faculty of Lund University with seven days to get used to the surroundings before starting the experiment. Animals had free access to standard feed and specially prepared water. All experiments followed the relevant Swedish and Institutional laws and guidelines (Ethical permit 6202/2017, Malmö - Lunds djurförsöketiska nämnd).

3.3.1 *Treatment period*

70 mice were randomly sorted in 7 groups (n_{GT}=10) and each group was divided in two cages as duplicate groups (n_G=5). The groups were normal N1 and N2, control K1 and K2, and antagonists A1, A2…E1, E2. To identify each individual, the white fur patches behind the mice's ears were dyed with non-toxic animal markers. All the groups except N1 and N2 were given antibiotics and *Escherichia coli* CCUG29300^T as pre-treatment to equalize the gut microbiota and reduce the amount of *Lactobacillaceae*. The water consumption was determined and new water (100 ml) prepared every day. An average dose of 5.2 (3.7–6.4)* mg of metronidazole, 3.5 (2.5–4.3)* mg of amoxicillin, and 2.1 (1.5–2.5)* mg of clindamycin was consumed per each mouse for three days. During the antibiotic treatment period each group, N groups included, had their water supplemented with 2% (w/v) fructose. An average daily dose of *E. coli* per animal was 10^8 CFU for two days right after antibiotics. After the pre-treatment, groups A1–E2 were given water supplemented with corresponding antagonist A–E with an average daily dose of an antagonist being 10^8 CFU per animal. Because antagonists were frozen with 0.5 ml of Hogness freezing media (for composition, see Appendix 2), water for the groups N and K were supplemented with the corresponding dose. A detailed experiment schedule and supplements are presented in Appendix 3.

(*)Presented amounts are median mg/day per mouse with 25th and 75th percentile.

3.3.2 *Operation*

On treatment day 21, the animals (n=10) were put under anaesthesia by Domitor® (medetomidine 1 mg/ml) + Ketalar® (ketamine 50 mg/ml) injection with 1.0 mg/kg of medetomidine and 75 mg/kg of ketamine per dose. Two animals were always kept together to reduce the stress after handling. Arterial blood was collected for later analysis of cytokines. A laparotomy was done through a midline incision to collect mesenteric lymph nodes, liver, spleen and the whole gastrointestinal tract from duodenum to the anal verge. Animals were euthanized with pentobarbital injection. Peyer's patches were collected from the whole small

intestine for FACS analysis together with the mesenteric lymph nodes. Intestinal biopsies for myeloperoxidase (MPO) activity and microbial evaluation were collected from both the small and large intestine, as well as faecal samples. Scheme for intestinal sampling is presented in Figure 5.

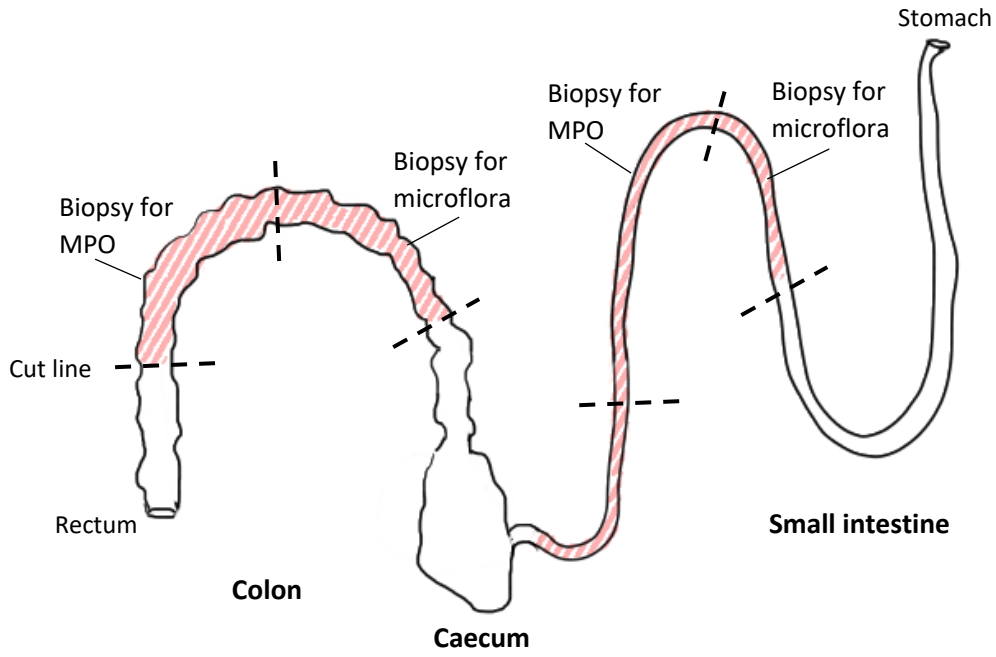

Figure 5 - Schematic picture of intestinal sampling. For faecal samples, all intestinal content were collected from the cross-hatched sections.

All but lymphatic tissue samples were weighed and quickly frozen with dry ice, excluding the spleen that was discarded after weighing. Biopsies for MPO analysis were frozen in Tris-EDTA-buffer. Peyer's patches and mesenteric lymph nodes were transferred in Falcon tubes (Corning Science Mexico S.A. de C.V., Mexico) with Hank's Balanced Salt Solution (HBSS) with phenolic red pH-indicator (Biowest, Nuaillé, France) and the tubes were kept in wet ice until the preparation for FACS.

3.3.3 *FACS*

Peyer's patches and mesenteric lymph nodes were transferred from Falcon tubes to 1.5 ml microcentrifuge tubes with 1 ml of HBSS, cut into small pieces, and centrifuged (100 x g) at 4 °C for 2 minutes. The supernatant with fat was removed, and the remaining tissue was transferred into a FACS tube (BD Biosciences, Durham, USA) and digested with digestion mix (collagenase P, dispase II protease, DNase I and HBSS) in a rocker at 37 °C for 10 minutes. To hasten tissue breakdown, the mixture was pipetted up and down before letting the sample to set. Supernatant with cells was transferred to a tube with 5 ml of HBSS and 10 % foetal bovine serum (FBS) (VWR, Radnor, USA) placed on ice. After 1–3 repeats the remaining cell suspension was centrifuged (1400 rpm) at 4 °C for 5 minutes, the supernatant removed and cells resuspended in 2 ml of HBSS/10% FBS. The new suspension was transferred through a 40 µm cell strainer (VWR, Radnor, USA) to a FACS tube, and the strainer was washed with 1 ml HBSS/10% FBS. The suspension was centrifuged (1400 rpm) at 4 °C for 5 minutes, supernatant discarded and cells resuspended in 500 µl of HBSS. For correct gating, one set of cells were stained with Türk's solution (Merck KGaA, Darmstad,

Germany), counted, and the needed volume of HBSS to dilute the suspension to 10^6 cells was calculated.

Unstained cells were stained in an antibody solution for 15 minutes at 4 °C in dark for the following biomarkers from eBiosciences Inc, San Diego, USA: CD4, CD8a, CD11c, CD25, CD69, CCR9, F4/80, TLR2 and TLR4. Stained cells were then washed with 800 µl FACS buffer and resuspended in 400 µl of FACS buffer. For intracellular (Foxp3 (eBioscience Inc, San Diego, USA)) markers, cells were permeabilized and fixed according to the protocol, and resuspended in 400 µl FACS buffer. The following antibody panels were used:

Panel 1: TLR2, TLR4, CD11c, F4/80

Panel 2: CCR9, CD4, CD69, CD8a

Panel 3: CD4, Foxp3, CD69, CD25

In every panel, the unwanted cells were blocked by unstained CD16/CD32 antibody. Stained cells were stored overnight at 4 °C until FACS analysis. FACS analysis was performed at CRC, Malmö on a CytoFLEX (Beckman Coulter, USA). Depending on the total amount of cells in a sample, 10 000–50 000 events were obtained for analysis. Collected data was analysed with CytExpert 2.0 (Beckman Coulter, USA). For the full protocol, see Appendix 4.

3.4 Statistical analysis

All data was analysed by SigmaPlot 13.0 (SPSS Inc., Chigago, USA) to see if results have a statistically significant difference between them. The differences between all groups were evaluated by one-way ANOVA on ranks (The Kruskal-Wallis on ranks). All-pairwise-multiple-comparison was analysed either by Student-Newmans-Keuls for groups with equal sample sizes, or Dunn's method if the sample size were unequal due missing samples. The comparison between two groups were tested by a Mann-Whitney rank sum test. Results were regarded to have statistically significant difference when $p<0.05$. All values are presented as median (25^{th} percentile–75^{th} percentile).

4 Results

4.1 Identification of isolated bacteria from RTE rocket salad bags

The results of the 16S rRNA gene sequenced isolates from ready-to-eat rocket salad bags are presented in Table 3. Bacteria from the *Pseudomonaceae* family were isolated mostly from TSA plates throughout the shelf-life of nine days, however one bacteria from the same family was isolated from VRBD plate. Most of *Aeromonadaceae* were isolated from VRBD plates, as well as one member of *Moraxellaceae* family. A bacteria from *Chromatiaceae* family was isolated from one of the bags in the beginning of shelf-life, while members of *Flavobacteriaceae* and *Bacillaceae* families were isolated from bags in the middle of the shelf-life period. Two members of *Shewanellaceae* were isolated from the samples on the last day of the shelf-life.

Table 3 - Supposed identification of isolated colonies from ready-to-eat rocket salad bags over the shelf-life period of nine days. Colonies are isolated from countable plates of the total aerobic bacterial load (TSA) and Enterobacteriaceae plate (VRBD) and identified by 16S rRNA gene sequencing.

Closest type strain[1]	Similarity (%)	TSA[2]	VRBD[3]
Isolated from bags during shelf-life days 1-3			
Acinetobacter haemolyticus M0430	96.3		1
Aeromonas media R36	98.8	1[E]	
Pseudomonas baetica DSB2	99.0	1	
Pseudomonas extremorientalis KMM 3447[T]	100.0		1
Pseudomonas putida AZ22R1	99.8	1	
Rheinheimera soli B29	99.1	1[E]	
Isolated from bags during shelf-life days 5-7			
Aeromonas hydrophilia HC010916B-1	98.8	1	
Chryseobacterium indoltheticum KUDC1754	98.6	1	
Lysinibacillus fusiformis Y11	99.8	1	
Pseudomonas fragi p423	99.8	1	
Isolated from bags on self-life day 9			
Aeromonas hydrophilia 45/90	100.0		2
Aeromonas media 83a	99.8		1
Aeromonas media AF63	100.0	1	
Aeromonas sp. I_6-G7221B10B	100.0		1
Pseudomonas cedrina P515/12	99.8	1	
Shewanella putrefaciens LMG 26268[T]	97.4	1	
Shewanella putrefaciens Sh3	100.0	1	

[1] According to RDP database, Seqmatch software
[2] Number of isolated colonies from TSA
[3] Number of isolated colonies from VRBD
[E] Isolated from a batch with inoculated *E. coli* CCUG29300[T]

4.2 Effects of selected antagonist on spinach sprouts

Escherichia coli concentration varied between 6.80 (6.51-6.97) log_{10} CFU/g and 6.90 (6.76-7.05) log_{10} CFU/g on the samples that were dipped in both the antagonist and *E. coli* solutions. When pelleted seeds were inoculated with the antagonist, *E. coli* concentration varied between 6.54 (5.58-7.10) log_{10} CFU/g and 7.17 (6.85-7.63) log_{10} CFU/g on set 2, and between 6.57 (6.20-7.25) log_{10} CFU/g and 7.08 (6.74-7.46) log_{10} CFU/g on set 3. Possible difference between the antagonists was not taken into account, since the antagonists are not dependent on each other. Results are presented in Table 4.

Table 4 - Bacteria concentration on spinach sprouts in the end of growing period for sets 1–3. Results are presented as median (25% - 75%) log_{10} CFU/g. ECBA represents Escherichia coli concentration, TSA represents total aerobic bacterial concentration.

Set 1: Antagonist dip + *E. coli* dip						
Antagonist	A	B	C	D	E	NEG
ECBA	6.54 (6.38-7.07)	6.62 (6.44-7.43)	6.35 (6.08-6.61)	6.80 (6.51-6.97)	6.57 (6.28-7.21)	6.90 (6.76-7.05)
TSA	9.24 (8.99-9.47)	8.90 (8.75-9.21)	8.85 (8.52-9.20) *****	9.45 (9.29-9.58) *****	9.02 (8.45-9.23)	9.32 (8.98-9.37)
Set 2: Seed inoculation + *E. coli* dip						
Antagonist	A	B	C	D	E	NEG
ECBA	7.17 (6.85-7.63)	6.59 (6.06-7.02)	7.09 (6.88-7.37)	6.67 (6.28-6.83)	6.54 (5.58-7.10)	6.77 (6.37-7.02)
TSA	9.70 (9.45-10.23)	9.23 (8.87-9.46)	9.66 (9.19-9.87)	9.47 (9.14-10.17)	9.44 (9.00-9.77)	9.76 (9.12-10.00)
Set 3: Seed inoculation + *E. coli* dip						
Antagonist	A	B	C	D	E	NEG
ECBA	7.08 (6.74-7.46)	7.04 (6.85-7.27)	6.57 (6.20-7.25)	6.94 (6.80-7.03)	6.92 (6.54-7.16)	7.08 (6.61-7.28)
TSA	9.63 (9.26-9.80) *****	9.19 (8.88-9.43)	9.50 (9.26-9.62)	9.24 (9.13-9.62)	8.58 (8.39-9.08)	9.02 (8.90-9.25)

***** indicates that there is a statistically significant difference ($p<0.05$) compared with NEG.

No significant differences in *Escherichia coli* concentrations between antagonists and negative control were found in any experiment set. On total aerobic microbial concentration, there are significant differences between the antagonist C (8.85 (8.52–9.20) log_{10} CFU/g) and NEG (9.32 (8.98–9.37) log_{10} CFU/g), $p=0.041$, and between the antagonist D (9.45 (9.29–9.58) log_{10} CFU/g) and NEG (9.32 (8.98–9.37) log_{10} CFU/g), $p=0.041$ in set 1. Also in set 3, a significant difference between the antagonist A (9.63 (9.26–9.80) log_{10} CFU/g) and NEG (9.02 (8.90–9.25) log_{10} CFU/g), $p=0.015$ was found.

4.2.1 *Survivability of antagonists on pelleted spinach seeds*

The results of survivability experiment is shown in Table 5. Inoculating pelleted seeds in antagonist solution for 5 hours decreased the concentration of the antagonist D from 5.29 $\log_{10}$ CFU/ml to 3.26 $\log_{10}$ CFU/ml and of the antagonist E from $\log_{10}$ 7.00 CFU/ml to 6.00 $\log_{10}$ CFU/ml. The median concentration of the antagonist A increased from 7.40 $\log_{10}$ CFU/ml to 7.58 $\log_{10}$ CFU/ml.

Table 5 – Median of triplicate $\log_{10}$ CFU/ml values of antagonists A–E without seeds, and after incubating seeds in the solution for 5 hours.

Antagonist	A	B	C	D	E
	Median $\log_{10}$ CFU/ml				
Without seeds	7.40	7.92	7.18	5.29	7.00
With seeds	7.58	7.82	7.13	3.26	6.00

4.3 Animal experiment

There were no significant differences in feed consumption (Appendix 5, $p_{consumption}$=0.448) or water intake between the groups K and A–E (p=0.718). The difference between the group N and other was not tested due the different treatment. The graph of daily water intake of the different groups is presented in Figure 6.

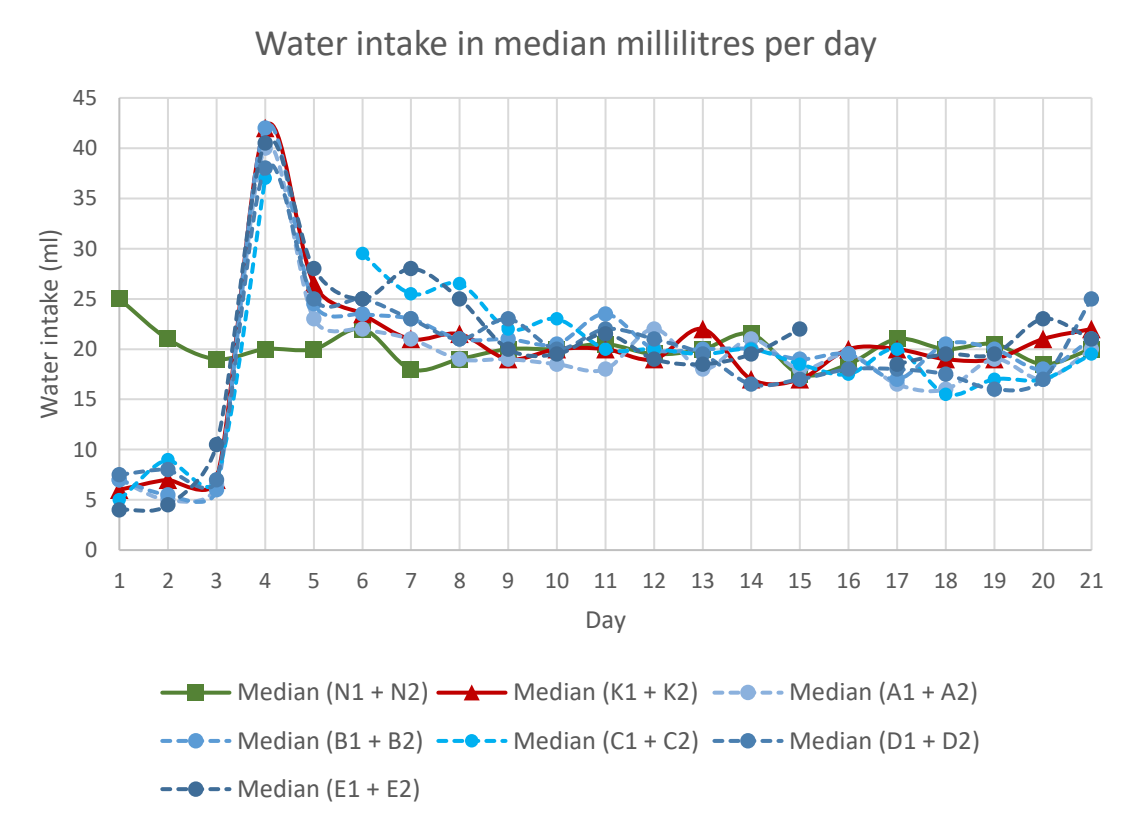

Figure 6 - Daily water intake. Each data point is a median of two collateral groups. Missing data point on the group C (day 4) and on the group E (day16).

The mice's weights were registered (Appendix 6) in three time points (start, after *E. coli* treatment, and in the end) during the study. The statistical analysis showed that there is no significant differences ($p=0.563$) in overall weight changes with compensated feed intake. In addition, there was no significant pairwise differences in feed compensated weights between the control group K and the antagonist groups A–E over the antagonist treatment period (15 days).

Cells from spleen were not collected, but the spleen weights were registered and compensated with feed intake and the end weights of mice (Appendix 7). The mouse C1.4 had notably bigger spleen than other individuals, but no significant differences were found between spleen weight with or without the compensations. Observation during the surgery also revealed that the mouse K1.2 had notably redder mesentery compared to other individuals. Furthermore, several mice from the antagonist groups D and E had slimy small intestinal content.

4.3.1 *Immune response of mice*

Mononuclear cells were collected and prepared from mesenteric lymph nodes and Peyer's patches and analysed by FACS for cell population observation. All result comparisons are done with the *E. coli* pre-treated control group K. The results of FACS analysis for mesenteric lymph nodes are presented in Table 6. The percentage of CD11c+TLR2+ is significantly lower in the groups N (4.9 (3.8–6.1)) and A (7.3 (6.2–8.1)) compared with the group K (18.7 (18.1–20.6)) in cells gathered from mesenteric lymph nodes. Additionally, percentages in the antagonist groups C–E were significantly higher, C being the highest (78.8 (76.7–86.0)). Moreover, the percentage of CD11c+TLR4+ dendritic cells in mesenteric lymph nodes were significantly higher in the antagonist groups B–E compared with the group K. All significant differences in F4/80 macrophages from mesenteric lymph nodes were lower than the group K. The only exception was with F4/80+TLR4+ macrophages, where the percentage was slightly higher in the group A (52.5 (50.2–56.0)) compared with the group K (42.7 (39.5-49.0)), $p=0.038$. A few regular patterns in the percentages of different gut homing T cells can be seen. The percentage of activated helper T cells (CCR9+CD4+CD69+/CD69-) and cytotoxic T cells (CCR9+CD8+CD69+/CD69-) are significantly higher in the untreated group N ($p\leq0.001$) and the antagonist groups E and D ($p\leq0.001$) compared with the control group K. The activation percentage of the same cells in the antagonist groups A–C is mostly significantly lower compared with the group K, except in CCR9+CD4+CD69- cells where the percentage is significantly higher. The percentage of CD4+CD25+Foxp3+CD69+ regulatory T cells is significantly higher in the group N ($p\leq0.001$), and in the groups A–E ($p\leq0.001$, except in C where $p\leq0.01$) compared with K (10.8 (7.6–12.5)). The results of FACS analysis for Peyer's patches are presented in Table 7 and in Figure 7 to Figure 12.

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Table 6 - Results of FACS analysis for mesenteric lymph nodes. Data is presented as median percentage of gated cells (25th percentile - 75th percentile) with significant difference compared with the control group K

MESENTERIC LYMPH NODES						
		Dendritic cells CD11c			Macrophages F4/80	
Gr	TLR2+	TLR4+	TLR2+ TLR4+	TLR2+	TLR4+	TLR2+ TLR4+
N	4.9 (3.8-6.1) ***	3.3 (2.0-3.8) ***	19.2 (7.4-28.2) **	60.9 (48.5-71.4)	42.6 (36.9-50.1)	23.7 (19.5-30.0) ***
K	18.7 (18.1-20.6)	5.0 (4.7-5.4)	3.8 (3.4-4.1)	63.5 (58.9-65.4)	42.7 (39.5-49.0)	47.5 (43.9-52.1)
A	7.3 (6.2-8.1) ***	6.0 (4.8-7.3)	4.3 (3.3-5.8)	33.3 (28.5-37.1) ***	42.2 (33.0-52.2)	24.8 (18.8-28.3) ***
B	16.9 (15.1-21.0)	9.3 (8.5-13.1) **	4.4 (4.2-6.4) *	49.2 (41.1-53.6) ***	25.8 (23.0-30.0) ***	23.7 (20.3-27.0) ***
C	78.8 (76.7-86.0) ***	33.4 (30.8-37.2) ***	33.3 (31.4-36.8) ***	61.1 (57.5-70.8)	40.3 (36.4-48.0)	34.1 (28.6-40.4) **
D	30.8 (28.4-31.7) **	18.6 (16.4-19.9) **	25.8 (23.8-27.1) **	60.0 (57.9-64.3)	32.2 (29.6-34.5) ***	30.3 (27.6-33.0) ***
E	26.5 (22.0-28.8) **	25.7 (23.8-29.1) **	17.0 (14.7-18.9) **	59.8 (55.9-62.9)	52.5 (50.2-56.0) *	43.5 (37.6-48.6)
		Activated gut homing T cells CCR9+				
Gr	CD4+CD69+	CD4+CD69-			CD8+CD69+	CD8+CD69-
N	6.1 (4.7-9.5) ***	3.6 (2.8-4.3) ***			4.3 (3.0-6.4) ***	4.7 (3.2-5.4) ***
K	2.8 (2.7-2.9)	1.1 (1.05-1.3)			1.6 (1.5-1.8)	0.5 (0.47-0.7)
A	1.6 (1.4-1.8) ***	1.7 (1.5-1.9) **			1.2 (1.0-1.3) ***	0.99 (0.86-1.3) ***
B	2.4 (1.7-3.6)	2.5 (2.3-2.9) ***			3.6 (2.3-4.9) **	4.5 (3.6-5.8) ***
C	1.7 (1.0-2.4) *	2.3 (1.4-3.0) **			0.5 (0.4-0.9) ***	0.3 (0.2-0.4) **
D	13.6 (11.2-17.5) ***	3.7 (3.2-4.4) ***			10.7 (7.0-12.2) ***	7.2 (6.7-7.8) ***
E	9.7 (4.9-21.9) ***	2.9 (2.7-4.7) ***			6.8 (3.6-14.7) ***	5.5 (4.3-6.1) ***
		Regulatory T cells CD4+CD25+				
Gr	Foxp3+CD69+	Foxp3+CD69-				
N	36.9 (30.1-43.3) ***	36.4 (25.6-58.5) *				
K	10.8 (7.6-12.5)	19.0 (17.5-26.8)				
A	21.8 (16.8-24.8) ***	47.2 (42.5-50.0) ***				
B	17.3 (14.6-20.3) ***	52.1 (47.8-60.3) ***				
C	15.1 (13.5-27.5) **	49.0 (39.7-55.1) ***				
D	51.6 (48.5-62.4) ***	25.1 (18.5-33.4)				
E	47.4 (33.7-53.0) ***	44.6 (36.4-51.7) **				

*** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$ compared with the control group K

Table 7 - Results of FACS analysis for Peyer's patches. Data is presented as median percentage of gated cells (25th percentile - 75th percentile) with significant difference compared with the control group K

PEYER'S PATCHES						
	Dendritic cells CD11c			Macrophages F4/80		
Gr	TLR2+	TLR4+	TLR2+ TLR4+	TLR2+	TLR4+	TLR2+ TLR4+
N	13.9 (12.7-17.8) ***	12.1 (10.7-15.5) ***	14.8 (12.3-17.9) **	18.8 (16.8-21.8) ***	28.7 (26.0-31.6) ***	16.4 (14.2-17.4) ***
K	42.5 (27.5-59.1)	35.0 (20.0-53.4)	30.2 (15.7-46.6)	60.9 (56.2-67.3)	54.7 (49.7-59.7)	62.7 (57.2-67.9)
A	20.9 (12.3-25.5) **	18.8 (8.6-19.6) *	26.4 (14.7-33.9)	37.3 (17.4-44.4) ***	63.5 (41.6-67.7) ***	28.8 (18.1-39.3) ***
B	39.0 (31.5-46.6)	43.3 (36.8-54.6)	28.1 (21.6-36.9)	39.5 (37.5-49.8) ***	35.7 (33.4-44.4) ***	32.0 (39.7-39.5) ***
C	96.2 (94.6-97.6) ***	43.4 (36.9-49.7)	44.5 (37.8-50.1)	47.5 (40.0-56.5) **	31.6 (28.1-37.4) ***	30.0 (26.5-36.0) ***
D	32.3 (27.5-46.6)	27.7 (23.8-42.0) **	38.7 (33.7-58.5)	34.5 (31.6-36.7) ***	24.4 (23.5-27.4) ***	22.7 (21.7-25.7) ***
E	25.1 (17.9-31.3) ***	27.6 (17.2-34.5) ***	18.1 (11.8-24.9) *	43.4 (32.8-55.2) **	39.5 (35.6-42.0) ***	29.4 (20.4-37.0) ***
	Activated gut homing T cells CCR9+					
Gr	CD4+CD69+	CD4+CD69-			CD8+CD69+	CD8+CD69-
N	14.3 (11.0-18.3) *	3.1 (2.2-4.0) **			13.7 (10.3-17.6) *	3.0 (2.3-4.3) **
K	44.0 (29.5-77.5)	1.1 (0.4-1.6)			30.3 (20.8-72.1)	0.6 (0.2-0.7)
A	23.0 (19.1-35.5)	0.8 (0.6-1.2)			15.9 (6.2-23.2) *	0.6 (0.4-0.8)
B	24.8 (19.1-34.5)	1.2 (0.8-1.6)			43.2 (34.7-57.3)	1.7 (0.9-2.3) **
C	50.9 (34.6-59.8)	3.0 (1.9-4.8) *			28.4 (19.0-46.5)	0.5 (0.3-0.6)
D	27.6 (22.5-38.5)	1.9 (1.2-2.4)			24.4 (13.2-36.9)	3.7 (3.1-4.9) ***
E	28.1 (19.9-46.1)	2.5 (1.8-3.1) *			17.1 (7.7-25.3) *	2.2 (1.5-3.2) **
	Regulatory T cells CD4+CD25+					
Gr	Foxp3+CD69+	Foxp3+CD69-				
N	83.3 (71.2-92.7)	8.8 (2.5-13.2)				
K	73.8 (72.0-79.4)	0.6 (0.2-5.5)				
A	86.2 (79.7-88.9) *	0.0 (0.0-0.8) *				
B	81.3 (69.2-88.2)	3.1 (0.3-5.6)				
C	84.9 (81.6-89.5) **	0.2 (0.0-1.5)				
D	85.4 (73.0-90.6)	2.5 (0.0-10.5)				
E	91.2 (81.8-96.2) **	5.0 (2.2-9.9)				

*** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$ compared with the control group K

Gram-positive bacteria induced activation of gated dendritic cells (DC) from Peyer's patches (Figure 7) was significantly lower ($p<0.001$) in the untreated group N (13.9 (12.7–17.8)) compared to the control group K (42.5 (35.9–59.1)). Furthermore, activation was significantly lower in the antagonist groups A (19.4 (9.8–24.7), $p=0.001$) and E (24.4 (17.9–31.3), $p=0.008$), but significantly higher in the group C (96.2 (94.6–97.6), $p<0.001$).

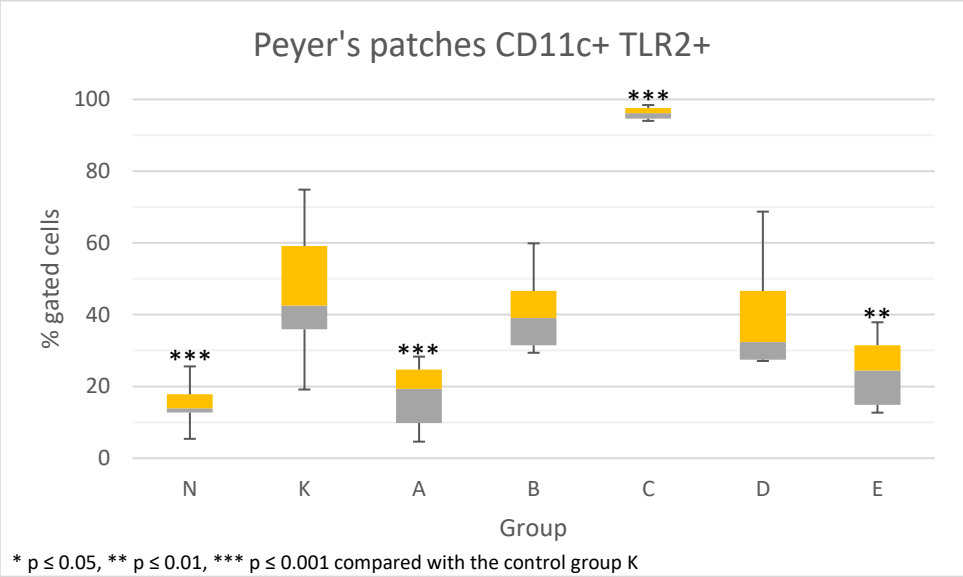

Figure 7 – Statistical differences of percentage gated Gram-positive bacteria activated dendritic cells collected from Peyer's patches

Gram-negative bacteria induced activation of gated DCs from Peyer's patches (Figure 8) was significantly lower ($p=0.010$) in the untreated group N (12.1 (10.7–15.5)) compared to the control group K (35.0 (20.0–53.4)). Additionally, the value was significantly lower ($p=0.020$) in the antagonist group A (16.7 (7.9–19.4)). Differences between the group K and the other antagonist groups were not statistically significant. No significant differences between the groups were found in double positive (TLR2+ TLR4+) dendritic cells ($p=0.064$).

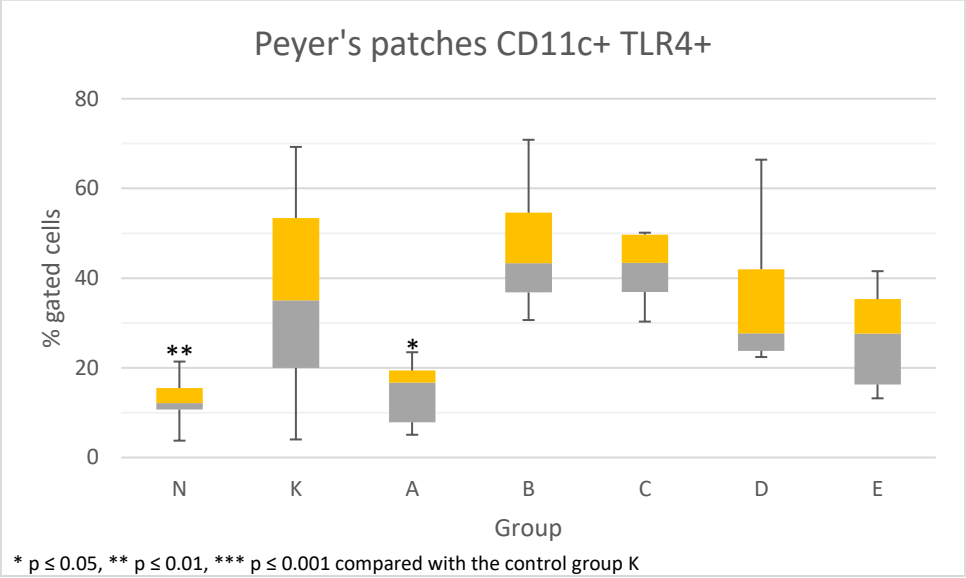

Figure 8 - Statistical differences of percentage gated Gram-negative bacteria activated dendritic cells collected from Peyer's patches

Gram-positive bacteria induced activation of macrophages from Peyer's patches (Figure 9) was significantly lower ($p<0.001$) in the untreated group N (18.8 (16.8–21.8)) compared with the group K (63.4 (56.2–67.3)). The percentage of activated cells was significantly lower in every antagonistic group, and the lowest in the antagonist group A (13.1 (6.5–21.6), $p<0.001$).

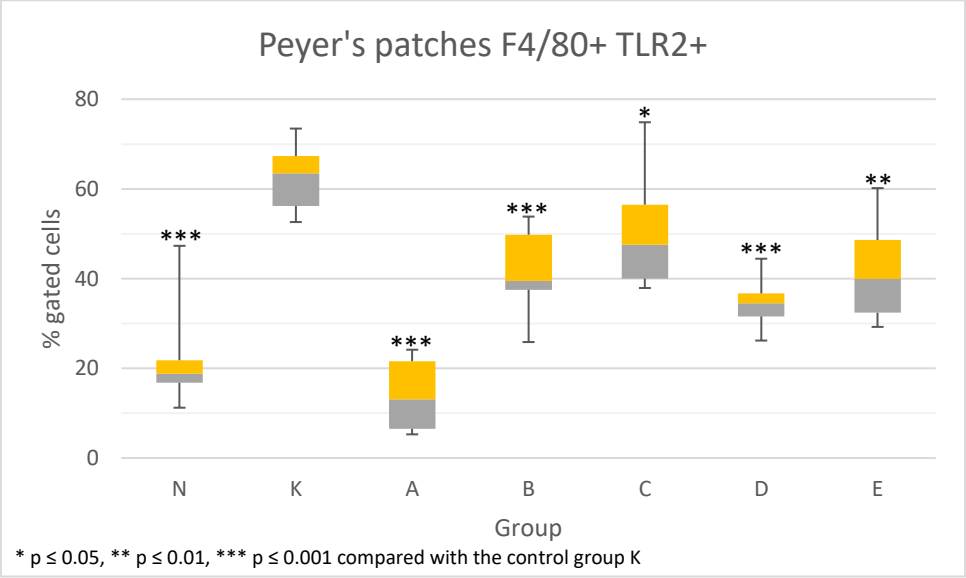

Figure 9 - Statistical differences of percentage gated Gram-positive bacteria activated macrophages collected from Peyer's patches

Gram-negative bacteria induced activation of macrophages from Peyer's patches (Figure 10) was significantly lower ($p<0.001$) in the untreated group N (28.7 (26.0–31.6)) compared with the group K (55.1 (51.7–60.6)). The percentage of activated cells was significantly lower in every antagonistic group.

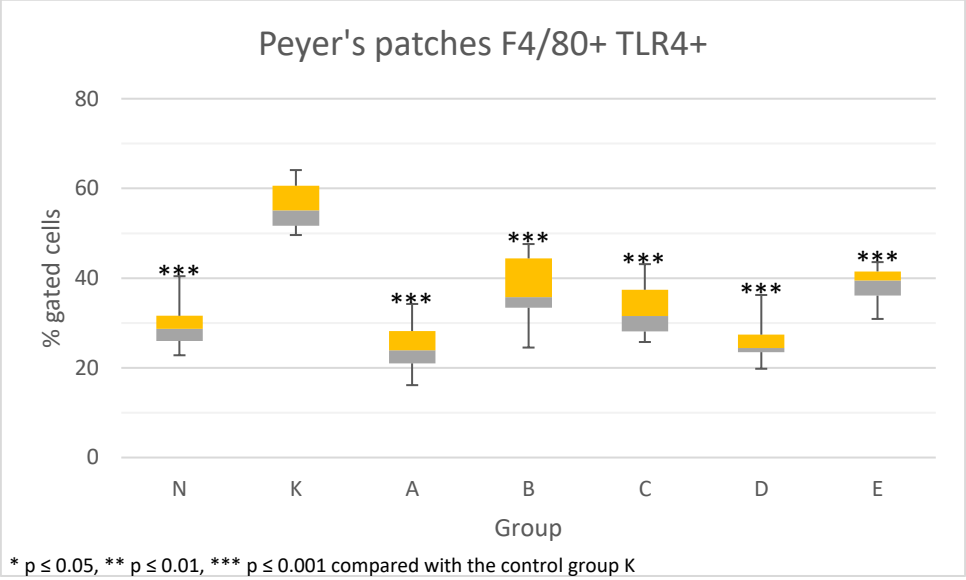

Figure 10 - Statistical differences of percentage gated Gram-negative bacteria activated macrophages collected from Peyer's patches

Unlike with DCs, there were significant differences in gated TLR2+ TLR4+ double activated macrophages (Figure 11). Activation percentage was significantly lower in the group N (16.4 (14.2–17.4)) compared to the group K (62.7 (57.2–67.9)) with $p<0.001$. The double activation percentage was significantly lower in every antagonist group and among them the lowest in the group D (22.7 (21.7-25.7)).

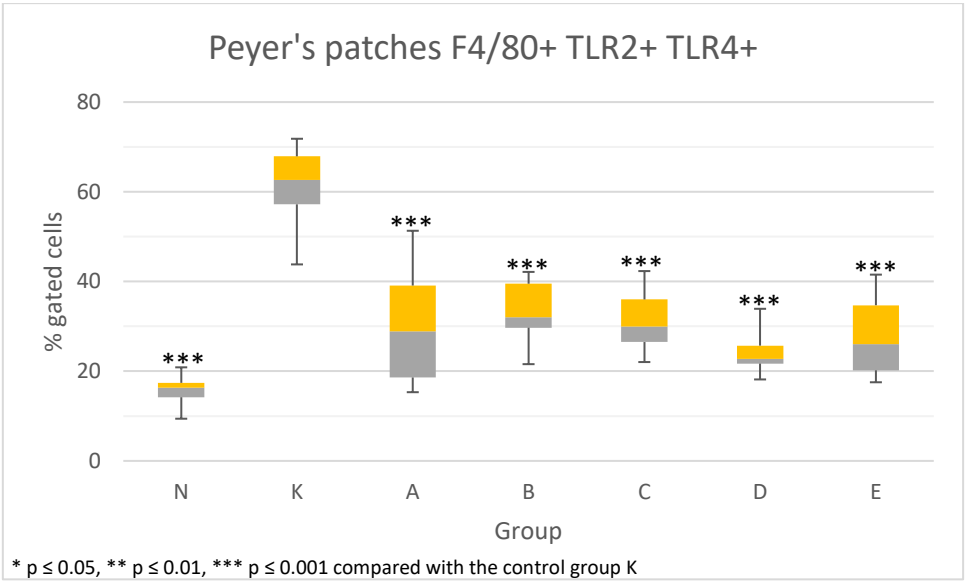

Figure 11 - Statistical differences of percentage gated both Gram-negative and Gram-positive bacteria activated macrophages collected from Peyer's patches

The percentage of the CD4+ helper T cells expressing the gut homing chemokine receptor (CCR9), and CD69 (Figure 12) was significantly lower in the group N (14.3 (11.0–18.3)) than in the group K (44.0 (29.5–77.5)), p=0.011. No significant differences were found between the groups K and A–E. However, the group A is close to have significantly lower percentage (23.0 (19.1–35.5)) compared to the group K (p=0.056).

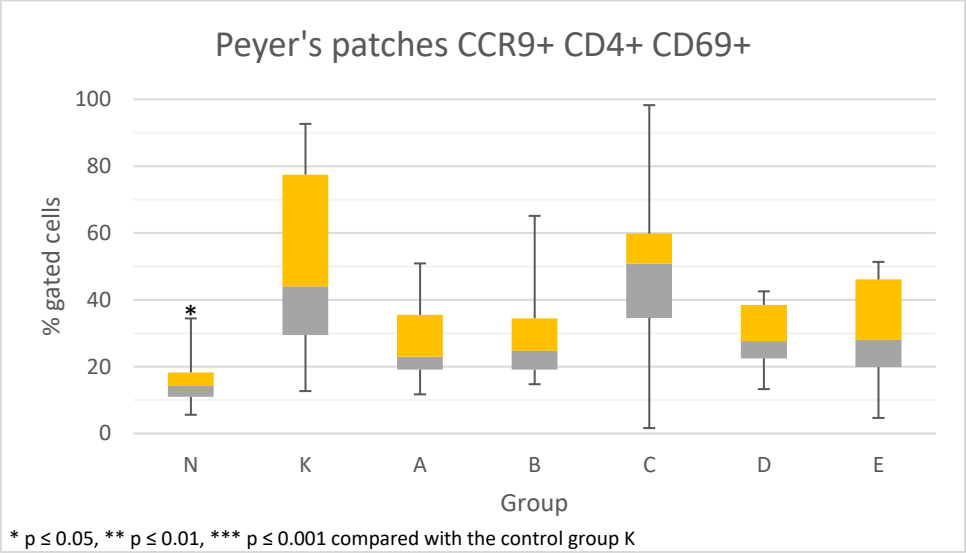

Figure 12 - Statistical differences of percentage gated CD4+ helper T cells expressing the gut homing chemokine receptor (CCR9), and CD69 collected from Peyer's patches

The percentage of the CD8+ cytotoxic T cells expressing CCR9 and CD69 (Figure 13) was significantly lower in the group N (13.7 (10.3–17.6)) than in the group K (30.3 (20.8–72.1)), p=0.018. Significantly lower percentages were also in the groups A (15.9 (6.2–23.2), p=0.037) and E (17.1 (7.7–25.3), p=0.029).

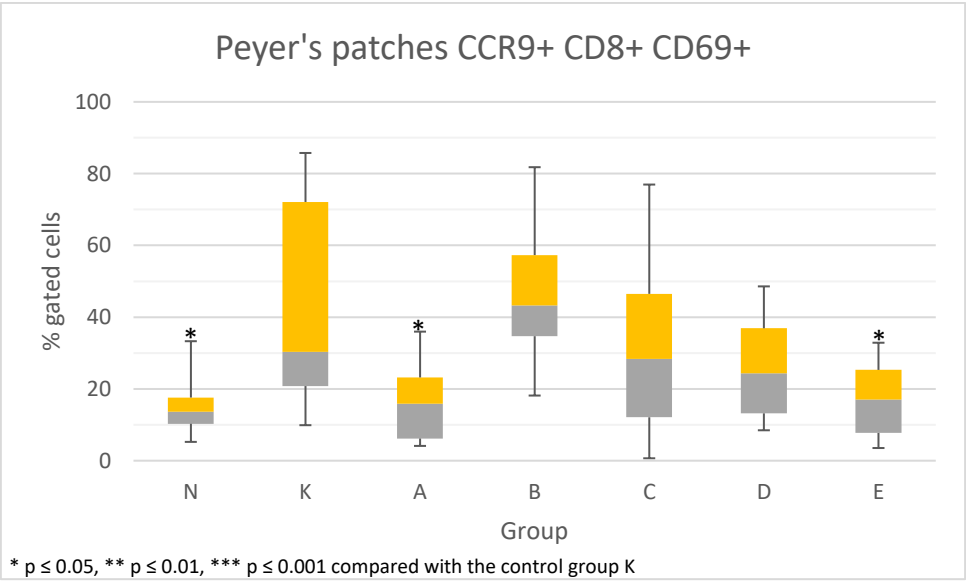

Figure 13 - Statistical differences of percentage gated CD8+ cytotoxic T cells expressing the gut homing chemokine receptor (CCR9), and CD69 collected from Peyer's patches

5 Discussion

In the present study, the effects and safety of selected antagonistic bacteria has been evaluated by their competence to prevent *Escherichia coli* contamination through plant leaves, survivability with pelleting substance, and the immune response in mice. A few isolates from bagged rocket salad were identified to get a general idea of the dominating, culturable microflora through the shelf-life period.

5.1 Microflora and quality of bagged rocket salad

The sequencing results (Table 3) show that *Pseudomonas* and *Aeromonas* genera are the most abundant among these samples. *Pseudomonas* are Gram-negative bacteria, commonly found from leafy green vegetables. In a Norwegian study, 51 samples of *Diplotaxis tenuifolia* and *Latuca sativa* were identified by Sanger sequencing and the 30% of isolates were found to be *Pseudomonas* spp. (Dees, Lysøe, Nordskog, and Brurberg, 2014). *Aeromonas* spp. are Gram-negative rods that are omnipresent in soil, water and vegetation (McMahon and Wilson, 2001), and have been isolated from extensive range of fresh produce, including leafy green vegetables (Merino, Rubires, Knøchel, and Tomás, 1995). However, three of *Aeromonas* isolates were identified to be *Aeromonas hydrophilia*, which is the most well-known from the total of six species of *Aeromonas* that are human pathogens (Elhariry, 2011). *A. hydrophilia* has found to be able to form a biofilm on the leaves of cabbage and lettuce *in vitro* (Elhariry, 2011), which should be taken into consideration when washing the leafy greens before consuming. *Lysinibacillus* is relatively common genus in soil (Ahmed, Yokota, Yamazoe, and Fujiwara, 2007), so it is not surprising to find it from rocket salad. In addition, *Lysinibacillus fusiformis* has been previously isolated from cacao leaf (Melnick, Suárez, Bailey, and Backman, 2011), so it seems to have an ability to attach on plant tissue.

More surprising findings were *Rheinheimera, Chryseobacterium,* and *Shewanella*. There was no publications about *Rheinheimera* in vegetables, fruits or berries, but some studies present that *Rheinheimera* sp. have been isolated from soil in South Korea (Ryu, Chung, Park, Lee, Lee and Jeon, 2008), and from irrigation water, including *Rheinheimera soli* B29 (Hao, 2012). *Chryseobacterium indoltheticum* is isolated from marine mud (Wu, Wu, and Liu, 2012) but no extensive publications could be found. *Shewanella* has been previously found from hydroponic lettuce growing systems (Rivera, Vélez, Zayas, and Llamas, 2015), even though *Shewanella putrefaciens* is connected to the smell of spoiled fish (Gram and Huss, 1996). It has also known to create biofilms on food processing surfaces (Bagge, Hjelm, Johansen, Huber, and Gram, 2001). Based on the publications, the most thinkable source for these genera would be contaminated processing surfaces or irrigation water from marine source. However, it needs to be kept in mind that these results are only putative identifications based on 16S rRNA gene sequencing, so errors between species and especially strains are plausible.

5.2 Effects and survivability of selected antagonists

Unlike previous studies, the present study showed no significant differences in *E. coli* concentrations in plants treated with antagonists compared with negative control (Table 4). Possible reasons were thought to be a contamination of the rockwool plugs during dipping, too high dose of *E. coli* during dipping, or a high death rate of antagonistic bacteria during seed inoculation. Rockwool plug contamination may have contaminated the nutrient solution,

which would have led sprouts getting contaminated also through roots, not only the leaves.

The possibility of antagonists not surviving in a solution with pelleted seeds was taken into consideration mostly because the experiments in the earlier studies were done with non-pelleted seeds. Seed pelleting is a common treatment in the agriculture, and reasons for it are for example to protect seeds from soil fungi such as *Rhizoctonia solani* or *Pythium* spp., and to have uniform size and shape for the easier planting (Taylor and Harman, 1990). Therefore it could be possible that the coating material contains antimicrobial substances that effect on the survivability of antagonists. This was tested with a small experiment, which showed that the amount of total bacterial count in antagonists E and D reduced 1–2 $\log_{10}$ CFU/gram. Unfortunately this does not explain the bad results with all antagonists. However, even if the pellet does not cause antagonist to die in high rates, the pellet may work as a barrier to water diffusion (Taylor and Harman, 1990). Pelleting materials are used to modify the seeds to adapt certain moisture levels, which may have an effect on pore size and particle size of used materials (Taylor and Harman, 1990) and therefore hinder the antagonist from reaching the seed and inoculating the plant.

5.3 Animal experiment

The animal experiment data shows that the mice used in the experiment consumed the typical amount of water per individual per day (Bachmanov, Reed, Beauchamp, and Tordoff, 2002). The daily feed intake per individual was approximately 2.2 grams of feed, which is a little lower than what is mentioned in Bachmanov *et al.* study, but the study also states that the feed intake is related to the body weight of mice (Bachmanov *et al.*, 2002). Since every group had a similar consumption level of feed and water, and no significant differences in the body weight development were found, it is relatively safe to say that antagonist treatment had no effect on the typical consumption behaviour of mice. The notable difference and peak in water intake between normal group N and K, A–E (Figure 6) is due to off-taste of antibiotics, and for example clindamycin is known to have a bitter or metallic taste (de Groot and van Puijenbroek, 2007).

Even though it is relatively difficult to draw exact conclusions from FACS data alone, a few notes can be made. If observing the results from Peyer's patches, it can be seen that the expression of both TLR2 and TLR4 (including double activation on macrophages) in dendritic cells and macrophages is significantly higher in *E. coli* treated control group K. This result goes in line with a study, in which the similar expression pattern was found from children with inflammatory bowel disease (IBS) (Szebeni, Veres, Dezsõfi, Rusai, Vannay, Mraz, Majorova, and Arató, 2007). This indicates that mice were probably having *E. coli* induced inflammation, and therefore the pre-treatment was successful. However, if observing results for antagonist A it can be seen that the activation of TLR2 and TLR4 in expressing cells is remarkably lower compared with K. The percentage seems to be on similar level as in untreated group N. This may suggest that the antagonist A is able to suppress the immune response. The possible suppression is also visible in activated CCR9+CD4+CD69+ and CCR9+CD8+CD69+ cells (Figure 12 and Figure 13) in Peyer's patches. Similar trend applies for the most of tested antagonists. The only antagonist that blatantly breaks the pattern is the antagonist C. As it can be seen in Figure 7, the CD11c+TLR2+ DC activation in the group C is remarkably high (96.2 (94.6–97.6)) compared to any other group.

Antagonist C is a Gram-positive bacteria, so the high activation percentage may indicate that the antagonist is able to colonise in the gut of mice. Since the mechanism of the antagonist C in the gut is not completely known, colonisation may not be wanted. However, it seems that F4/80+TLR2+ macrophage activation is a lot lower (47.5 (40.0–56.5)) compared with DCs. Therefore there is a chance that a possible colonisation does not trigger an eliminative immune response. The results from CC9+CD69+ expressing cytotoxic CD8+ T cells from Peyer's patches (Figure 13) are supporting this theory. Similar patterns with the antagonists A and C in Peyer's patches can be seen in mesenteric lymph nodes (Table 6), though the differences are not as notable and sometimes not significant compared with control group K. To fully understand the possible resemblances, a deeper analysis of several possible activation and support mechanisms in tissues should be taken into consideration and studied more. Based on the FACS analysis it is relatively safe to say that the large spleen on the mouse C1.4 was not caused by the antagonist, but natural variety.

6 Conclusions and future

As we live in a hectic world, ready-to-eat salads are a convenient way to increase the consumption of vegetables. However, as the results of this study and several other publications show, leafy green vegetables contain a great amount of different bacteria with potential human pathogens. Some of these bacteria are naturally occurring in soil or water, while others come from contaminated sources. Obviously this does not mean that the RTE salad production has to be stopped, but producers shall keep in mind the importance of process hygiene especially since the process does not have critical control points to eliminate possible microbial hazards. In addition, several of the present bacteria are capable to create biofilms on surfaces including process equipment and leafy green vegetables. Biofilms are often difficult to remove from equipment surfaces, which increases the contamination risk.

Unfortunately, the potential of the antagonists on hydroponically grown spinach sprouts was not clear from the results. However, this is very likely caused by technical difficulties. In the study, physical and chemical properties of pelleting materials were not taken into consideration. Therefore, it could be helpful to see how the positive results can be obtained with pelleted seeds. This will most likely require some changes in inoculation method or time. Even if it may be time consuming to look for an optimal method, it will help with future field studies. Based on the animal experiment, the tested antagonists were not causing changes in water and feed consumption of mice. This would indicate that mice do not feel sick when treated with the antagonists. Cursory observation of FACS results didn't show alarming immune reactions, but more profound analysis is necessary before conclusions can be made. In addition, the analysis of biopsies, liver, and faecal content are required to get a better understanding of the overall situation. Despite of poor results in the antagonistic effect experiment, preliminary results indicate that selected antagonists do not trigger negative immune response, and therefore the development of safer salad shall be continued.

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Appendix 1

Chemicals used in the preparation of nutrient stock solutions.

	Chemicals	Chemical formula	Amount in grams (g/l)
B	Ethylenediaminetetraacetic acid ferric sodium salt	FeEDTA	0.444
	Magnesium Sulphate Heptahydrate	$MgSO_4 \cdot 7H_2O$	0.00845
	Ammonium Dihydrogen Phosphate	$(NH_4)H_2PO_4$	1.15
	Potassium Dihydrogen Phosphate	KH_2PO_4	1.3609
	Ammonium Nitrate	NH_4NO_3	1.92
C	Zinc Sulphate Hepta Hydrate	$ZnSO_4 \cdot 7H2O$	0.0115
	Sodium Tetraborate Deca Hydrate	$Na_2B_4O_7 \cdot 10H_2O$	0.11442
	Ammonium heptamolybdate tetrahydrate	$(NH_4)_6Mo_7O_{24}$	0.00618
	Copper sulphate Pentahydrate	$CuSO_4 \cdot 5H_2O$	0.0019
	Manganese sulphate Monohydrate	$MnSO_4 \cdot H_2O$	0.00845
D	Calcium Hydroxide	$Ca(OH)_2$	0.7
	Ammonium Sulphate	$(NH_4)_2SO_4$	0.132
	Potassium Sulphate	K_2SO_4	4.357

Appendix 2

Chemicals used in Hogness freezing media.

Chemicals	**Amount per 200 ml**
K_2HPO_4	0.17 g
KH_2PO_4	0.04 g
Tri-natrium-citrat-dihydrat	0.3 g
$MgSO_4 \cdot 7H_2O$	0.05 g
Glycerol 99.5%	24.3 ml
H_2O	175 ml

Appendix 3

Detailed schedule for animal experiment from May 25 to June 20, 2017

Date	Groups N1 & N2	Groups K1 & K2	Groups A1 & A2	Groups B1 & B2	Groups C1 & C2	Groups D1 & D2	Groups E1 & E2
25-May	Fructose						
26-May	Fructose	Antibiotics					
27-May	Fructose	Antibiotics	Antibiotics				
28-May	Freezing media	Antibiotics	Antibiotics	Antibiotics			
29-May	Freezing media	E.coli	Antibiotics	Antibiotics	Antibiotics		
30-May	Freezing media	E.coli	E.coli	Antibiotics	Antibiotics	Antibiotics	
31-May	Freezing media	Freezing media	E.coli	E.coli	Antibiotics	Antibiotics	Antibiotics
01-Jun	Freezing media	Freezing media	Antagonist A	E.coli	E.coli	Antibiotics	Antibiotics
02-Jun	Freezing media	Freezing media	Antagonist A	Antagonist B	E.coli	E.coli	Antibiotics
03-Jun	Freezing media	Freezing media	Antagonist A	Antagonist B	Antagonist C	E.coli	E.coli
04-Jun	Freezing media	Freezing media	Antagonist A	Antagonist B	Antagonist C	Antagonist D	E.coli
05-Jun	Freezing media	Freezing media	Antagonist A	Antagonist B	Antagonist C	Antagonist D	Antagonist E
06-Jun	Freezing media	Freezing media	Antagonist A	Antagonist B	Antagonist C	Antagonist D	Antagonist E
07-Jun	Freezing media	Freezing media	Antagonist A	Antagonist B	Antagonist C	Antagonist D	Antagonist E
08-Jun	Freezing media	Freezing media	Antagonist A	Antagonist B	Antagonist C	Antagonist D	Antagonist E
09-Jun	Freezing media	Freezing media	Antagonist A	Antagonist B	Antagonist C	Antagonist D	Antagonist E
10-Jun	Freezing media	Freezing media	Antagonist A	Antagonist B	Antagonist C	Antagonist D	Antagonist E
11-Jun	Freezing media	Freezing media	Antagonist A	Antagonist B	Antagonist C	Antagonist D	Antagonist E
12-Jun	Freezing media	Freezing media	Antagonist A	Antagonist B	Antagonist C	Antagonist D	Antagonist E
13-Jun	Freezing media	Freezing media	Antagonist A	Antagonist B	Antagonist C	Antagonist D	Antagonist E
14-Jun	Operation	Freezing media	Antagonist A	Antagonist B	Antagonist C	Antagonist D	Antagonist E
15-Jun		Operation	Antagonist A	Antagonist B	Antagonist C	Antagonist D	Antagonist E
16-Jun			Operation	Antagonist B	Antagonist C	Antagonist D	Antagonist E
17-Jun				Operation	Antagonist C	Antagonist D	Antagonist E
18-Jun					Operation	Antagonist D	Antagonist E
19-Jun						Operation	Antagonist E
20-Jun							Operation

Appendix 4

FACS sample preparation protocol used in this experiment.

1. Transfer tissues (Peyer's patches and mesenteric lymph nodes) to 1.5 ml tubes with 1 ml HBSS.
   Cut Peyer's patches with curved scissor into 1 mm pieces.
   Remove fat from mesenteric lymph nodes and cut into 1mm pieces.
2. Centrifuge 100 g, 2 min at 4 °C.
3. Create digestion mix. Mix enzymes (collagense P, dispase II and DNase I from bovine pancreas, see the end of this Appendix) with HBSS gently. Avoid foaming!
4. Remove supernatant and excess fat
5. Add 500 µl digestion mix with cut pipette tip. Transfer cells to FACS tube.
6. Wash old tube w. 500 µl digestion mix and add to FACS tube
7. Shake gently with rocker at 37 °C for 10 min. Have digestion mix in the same rack as well too.
8. Put 50 ml FBSS/10% FBS and 5 ml FBS/sample in falcon tube and place on ice
9. Take out sample tube and pipet up and down (cut tip if needed) 20 times. Let sample settle.
10. Transfer supernatant to FBS tube
11. Add 1 ml digestion buffer to remaining tissue. Incubate at 37 °C for 10 min.
12. Repeat steps 7 - 11 for 1 - 2 times
13. Centrifuge FBS tube at 1400 rpm (250g) at 4 °C for 5 min
14. Remove supernatant
15. Resuspend cells in 2 ml of FBSS/10% FBS
16. Put suspension through 40 µM filter
17. Add 1 ml HBSS/FBS to filter. Transfer flow through to FACS tube.
18. Centrifuge for 5 min at 4 °C, 1400 rpm
19. Remove supernatant
20. Resuspend in cells in 500 µl of HBSS
21. Stain with Türk's solution – 2 µl per 18 µl of cell suspension (Peyer's patches), 1 µl per 19 µl of cell suspension (lymph nodes).
22. Load Bürker chamber, count 2 A chambers, take average
23. Calculate required volume for 10^6 dilution of cells
24. Save excess cells to unstained tube
25. Make antibody mix (see the end of this Appendix), add to cell tubes
26. Make compensation tubes with 0,5 µl antibody per tube
27. Incubate for 15 min at 4 °C
28. Wash stained cells with 800 µl FACS buffer, centrifuge at 1200 rpm for 5 min
29. Discard supernatant and resuspend cells in 400 µl FACS buffer
30. Dilute Fix/Permeability concentration 1:3 in Fix/Permeability diluent
31. Add 600 µl per sample. Vortex gently.
32. Incubate at 4 °C for 1 h
33. Dilute permeability buffer

34 Add 600 µl per sample. Centrifuge 1200 rpm for 5 min. Discard supernatant.
35 Repeat step 37, add permeability buffer: 83 µl for lymph nodes and 167 µl for Peyer's patches
36 Add Foxp3 and dump channel
37 Incubate for 45 min at 4 °C
38 wash w 800 µl perm buffer
39 Centrifuge for 5 min, 1200 rpm
40 Discard supernatant, resuspend cells in 400 µl FACS buffer
41 Run on FACS

Antibody master mix for 22 samples

Panel1	**V (µl)**
CD16/32	11.0
TLR2	5.5
CD11c	2.75
F4/80	5.5
TLR4	5.5
Vol per sample tube	**1.375**

Panel 2	
CD16/CD32	11.0
CCR9-FITC	2.75
CD4-PE	1.375
CD69-PerCP Cy5.5	5.5
CD8a-APC	1.375
Vol per sample tube	**1.0**

Panel 3a	
CD16/CD32	11.0
CD4-PE	1.375
CD69-PerCP Cy5.5	5.5
CD25-APC	1.375
Vol per sample tube	**0.875**

Panel 3b	
Foxp3-FITC	11.0
CD16/CD32	11.0
Vol per sample tube	**1.0**

Digestion mix

Stock	Vol/sample (ml)
Collagenase P	0.08
Dispase II	0.32
Dnase I	0.1
HBSS	4.25

Dilute Fixation buffer for 22 samples

Stock	Vol (ml)
Fix/Perm concentrate	3.3
Fix/Perm diluent	9.9
Perm buffer	4.4
dH2O	39.6

Appendix 5

Feed weights (g) at the beginning, added feed, remaining feed on the operation day, and the median consumption.

Group	**N1**	**N2**	**K1**	**K2**	**A1**	**A2**	**B1**	**B2**	**C1**	**C2**	**D1**	**D2**	**E1**	**E2**
Feed (g)														
Start	282.1	345.9	357.2	244.5	237.3	248.3	268.8	248.7	208.8	184.7	251.5	299.8	209.0	182.0
Added	160.7	95.5	97.8	142.8	149.9	128.0	117.1	254.2	256.1	190.2	272.6	131.0	200.6	235.7
End	130.9	126.6	143.6	64.0	63.2	90.4	67.0	195.2	146.7	61.4	198.9	127.6	97.5	90.9
Consumed	311.9	314.8	311.4	323.3	324.0	285.9	318.9	307.7	318.2	313.5	325.2	303.2	312.1	326.8
Per animal	62.4	63.0	62.3	64.7	64.8	57.2	63.8	61.5	63.6	62.7	65.0	60.6	62.4	65.4
Per animal per day	2.2	2.2	2.2	2.3	2.3	2.0	2.3	2.2	2.3	2.2	2.3	2.2	2.2	2.3

Appendix 6

Animal weight (g) chart with different time point comparisons

Animal ID	S Start	A After E.coli	E End	Δm total E-S	A-S	E-A	Δm total/feed intake (¤) (E-S)*	(A-S)*	(E-A)*	(¤) Feed intake / 5 (# animal per cage)
N1.1	19.9	20.1	21.4	1.5	0.2	1.3	0.02	0.00	0.02	62.38
N1.2	20.0	20.2	22.6	2.6	0.2	2.4	0.04	0.00	0.04	62.38
N1.3	18.9	19.1	20.8	1.9	0.2	1.7	0.03	0.00	0.03	62.38
N1.4	19.2	19.1	21.9	2.7	-0.1	2.8	0.04	0.00	0.04	62.38
N1.5	20.0	20.4	20.9	0.9	0.4	0.5	0.01	0.01	0.01	62.38
N2.1	19.7	19.1	21.8	2.1	-0.6	2.7	0.03	-0.01	0.04	62.98
N2.2	19.9	20.2	21.5	1.6	0.3	1.3	0.03	0.00	0.02	62.98
N2.3	21.3	20.8	21.6	0.3	-0.5	0.8	0.00	-0.01	0.01	62.98
N2.4	21.2	20.0	20.9	-0.3	-1.2	0.9	0.00	-0.02	0.01	62.98
N2.5	19.6	19.8	20.7	1.1	0.2	0.9	0.02	0.00	0.01	62.98
K1.1	22.0	22.8	23.8	1.8	0.8	1.0	0.03	0.01	0.02	62.28
K1.2	20.7	21.4	22.0	1.3	0.7	0.6	0.02	0.01	0.01	62.28
K1.3	20.4	22.0	22.2	1.8	1.6	0.2	0.03	0.03	0.00	62.28
K1.4	20.3	21.3	21.7	1.4	1.0	0.4	0.02	0.02	0.01	62.28
K1.5	20.1	20.0	21.7	1.6	-0.1	1.7	0.03	0.00	0.03	62.28
K2.1	18.7	19.1	20.0	1.3	0.4	0.9	0.02	0.01	0.01	64.66
K2.2	19.8	19.5	20.3	0.5	-0.3	0.8	0.01	0.00	0.01	64.66
K2.3	21.0	21.5	22.7	1.7	0.5	1.2	0.03	0.01	0.02	64.66
K2.4	21.1	21.7	23.4	2.3	0.6	1.7	0.04	0.01	0.03	64.66
K2.5	20.7	21.1	20.9	0.2	0.4	-0.2	0.00	0.01	0.00	64.66
A1.1	20.6	21.9	21.5	0.9	1.3	-0.4	0.01	0.02	-0.01	64.8
A1.2	20.8	20.7	20.3	-0.5	-0.1	-0.4	-0.01	0.00	-0.01	64.8
A1.3	20.4	21.0	22.4	2.0	0.6	1.4	0.03	0.01	0.02	64.8
A1.4	20.6	20.9	21.0	0.4	0.3	0.1	0.01	0.00	0.00	64.8
A1.5	20.2	21.7	21.8	1.6	1.5	0.1	0.02	0.02	0.00	64.8
A2.1	20.5	20.6	21.4	0.9	0.1	0.8	0.02	0.00	0.01	57.18
A2.2	21.5	22.6	23.5	2.0	1.1	0.9	0.03	0.02	0.02	57.18
A2.3	20.3	20.6	22.1	1.8	0.3	1.5	0.03	0.01	0.03	57.18
A2.4	21.0	20.5	19.4	-1.6	-0.5	-1.1	-0.03	-0.01	-0.02	57.18
A2.5	20.3	21.0	21.3	1.0	0.7	0.3	0.02	0.01	0.01	57.18
B1.1	19.5	20.5	21.4	1.9	1.0	0.9	0.03	0.02	0.01	63.78
B1.2	19.4	19.6	20.0	0.6	0.2	0.4	0.01	0.00	0.01	63.78
B1.3	20.6	22.7	22.7	2.1	2.1	0.0	0.03	0.03	0.00	63.78
B1.4	20.5	21.9	21.4	0.9	1.4	-0.5	0.01	0.02	-0.01	63.78
B1.5	20.4	21.1	21.8	1.4	0.7	0.7	0.02	0.01	0.01	63.78
B2.1	19.9	20.9	21.8	1.9	1.0	0.9	0.03	0.02	0.01	61.54
B2.2	20.5	21.3	22.7	2.2	0.8	1.4	0.04	0.01	0.02	61.54

B2.3	19.5	21.2	21.6	2.1	1.7	0.4	0.03	0.03	0.01	61.54
B2.4	21.6	22.5	22.4	0.8	0.9	-0.1	0.01	0.01	0.00	61.54
B2.5	19.6	21.3	21.8	2.2	1.7	0.5	0.04	0.03	0.01	61.54
C1.1	20.7	22.7	22.7	2.0	2.0	0.0	0.03	0.03	0.00	63.64
C1.2	19.9	21.5	21.9	2.0	1.6	0.4	0.03	0.03	0.01	63.64
C1.3	20.6	22.0	22.1	1.5	1.4	0.1	0.02	0.02	0.00	63.64
C1.4	20.5	21.1	21.9	1.4	0.6	0.8	0.02	0.01	0.01	63.64
C1.5	19.1	20.7	19.9	0.8	1.6	-0.8	0.01	0.03	-0.01	63.64
C2.1	20.2	18.5	23.0	2.8	-1.7	4.5	0.04	-0.03	0.07	62.7
C2.2	20.3	18.6	20.5	0.2	-1.7	1.9	0.00	-0.03	0.03	62.7
C2.3	21.1	19.0	21.2	0.1	-2.1	2.2	0.00	-0.03	0.04	62.7
C2.4	19.6	18.2	20.6	1.0	-1.4	2.4	0.02	-0.02	0.04	62.7
C2.5	20.5	19.9	21.7	1.2	-0.6	1.8	0.02	-0.01	0.03	62.7
D1.1	20.0	21.5	22.7	2.7	1.5	1.2	0.04	0.02	0.02	65.04
D1.2	19.8	21.8	22.4	2.6	2.0	0.6	0.04	0.03	0.01	65.04
D1.3	20.1	22.2	22.5	2.4	2.1	0.3	0.04	0.03	0.00	65.04
D1.4	19.4	21.2	22.5	3.1	1.8	1.3	0.05	0.03	0.02	65.04
D1.5	20.5	21.0	22.1	1.6	0.5	1.1	0.02	0.01	0.02	65.04
D2.1	19.6	21.7	21.5	1.9	2.1	-0.2	0.03	0.03	0.00	60.64
D2.2	20.0	21.3	20.6	0.6	1.3	-0.7	0.01	0.02	-0.01	60.64
D2.3	19.4	21.0	21.0	1.6	1.6	0.0	0.03	0.03	0.00	60.64
D2.4	19.1	20.3	18.4	-0.7	1.2	-1.9	-0.01	0.02	-0.03	60.64
D2.5	20.0	21.4	20.8	0.8	1.4	-0.6	0.01	0.02	-0.01	60.64
E1.1	20.9	22.3	22.1	1.2	1.4	-0.2	0.02	0.02	0.00	62.42
E1.2	19.2	20.5	21.1	1.9	1.3	0.6	0.03	0.02	0.01	62.42
E1.3	19.6	20.7	21.8	2.2	1.1	1.1	0.04	0.02	0.02	62.42
E1.4	18.6	19.2	19.3	0.7	0.6	0.1	0.01	0.01	0.00	62.42
E1.5	19.9	21.2	21.8	1.9	1.3	0.6	0.03	0.02	0.01	62.42
E2.1	20.1	21.3	21.1	1.0	1.2	-0.2	0.02	0.02	0.00	65.36
E2.2	20.0	21.8	23.2	3.2	1.8	1.4	0.05	0.03	0.02	65.36
E2.3	20.2	20.1	20.7	0.5	-0.1	0.6	0.01	0.00	0.01	65.36
E2.4	19.3	21.0	22.9	3.6	1.7	1.9	0.06	0.03	0.03	65.36
E2.5	20.5	21.9	23.7	3.2	1.4	1.8	0.05	0.02	0.03	65.36

Appendix 7

Spleen weights and compensations with feed intakes. (E-S)*, (A-S)* and (E-A)* are the same values as in Appendix

Animal ID	m (spleen)	Spleen/(end weight/feed intake)	Spleen/(E-S)*	Spleen/(A-S)*	Spleen/(E-A)*
N1.1	0.097	0.283	4.03	30.25	4.65
N1.2	0.090	0.248	2.16	28.07	2.34
N1.3	0.088	0.264	2.89	27.45	3.23
N1.4	0.112	0.319	2.59	-69.87	2.50
N1.5	0.089	0.266	6.17	13.88	11.10
N2.1	0.094	0.272	2.82	-9.87	2.19
N2.2	0.096	0.281	3.78	20.15	4.65
N2.3	0.126	0.367	26.45	-15.87	9.92
N2.4	0.082	0.247	-17.21	-4.30	5.74
N2.5	0.107	0.326	6.13	33.69	7.49
K1.1	0.080	0.209	2.77	6.23	4.98
K1.2	0.092	0.260	4.41	8.19	9.55
K1.3	0.091	0.255	3.15	3.54	28.34
K1.4	0.090	0.258	4.00	5.61	14.01
K1.5	0.100	0.287	3.89	-62.28	3.66
K2.1	0.079	0.255	3.93	12.77	5.68
K2.2	0.078	0.248	10.09	-16.81	6.30
K2.3	0.099	0.282	3.77	12.80	5.33
K2.4	0.095	0.263	2.67	10.24	3.61
K2.5	0.086	0.266	27.80	13.90	-27.80
A1.1	0.071	0.214	5.11	3.54	-11.50
A1.2	0.061	0.195	-7.91	-39.53	-9.88
A1.3	0.100	0.289	3.24	10.80	4.63
A1.4	0.074	0.228	11.99	15.98	47.95
A1.5	0.093	0.276	3.77	4.02	60.26
A2.1	0.091	0.243	5.78	52.03	6.50
A2.2	0.097	0.236	2.77	5.04	6.16
A2.3	0.090	0.233	2.86	17.15	3.43
A2.4	0.087	0.256	-3.11	-9.95	-4.52
A2.5	0.090	0.242	5.15	7.35	17.15
B1.1	0.074	0.221	2.48	4.72	5.24
B1.2	0.079	0.252	8.40	25.19	12.60
B1.3	0.082	0.230	2.49	2.49	0
B1.4	0.097	0.289	6.87	4.42	-12.37
B1.5	0.094	0.275	4.28	8.56	8.56
B2.1	0.104	0.294	3.37	6.40	7.11

B2.2	0.095	0.258	2.66	7.31	4.18
B2.3	0.084	0.239	2.46	3.04	12.92
B2.4	0.105	0.288	8.08	7.18	-64.62
B2.5	0.113	0.319	3.16	4.09	13.91
C1.1	0.086	0.241	2.74	2.74	0
C1.2	0.071	0.206	2.26	2.82	11.30
C1.3	0.118	0.340	5.01	5.36	75.10
C1.4	0.227	0.660	10.32	24.08	18.06
C1.5	0.096	0.307	7.64	3.82	-7.64
C2.1	0.097	0.264	2.17	-3.58	1.35
C2.2	0.088	0.269	27.59	-3.25	2.90
C2.3	0.091	0.269	57.06	-2.72	2.59
C2.4	0.074	0.225	4.64	-3.31	1.93
C2.5	0.115	0.332	6.01	-12.02	4.01
D1.1	0.085	0.244	2.05	3.69	4.61
D1.2	0.087	0.253	2.18	2.83	9.43
D1.3	0.120	0.347	3.25	3.72	26.02
D1.4	0.090	0.260	1.89	3.25	4.50
D1.5	0.109	0.321	4.43	14.18	6.44
D2.1	0.098	0.276	3.13	2.83	-29.71
D2.2	0.094	0.277	9.50	4.38	-8.14
D2.3	0.096	0.277	3.64	3.64	0
D2.4	0.081	0.267	-7.02	4.09	-2.59
D2.5	0.101	0.294	7.66	4.37	-10.21
E1.1	0.091	0.257	4.73	4.06	-28.40
E1.2	0.074	0.219	2.43	3.55	7.70
E1.3	0.096	0.275	2.72	5.45	5.45
E1.4	0.075	0.243	6.69	7.80	46.81
E1.5	0.093	0.266	3.06	4.47	9.68
E2.1	0.091	0.282	5.95	4.96	-29.74
E2.2	0.101	0.285	2.06	3.67	4.72
E2.3	0.081	0.256	10.59	-52.94	8.82
E2.4	0.097	0.277	1.76	3.73	3.34
E2.5	0.112	0.309	2.29	5.23	4.07