Impact of the source of organic manure on persistence of *E. coli* O157:H7 *gfp+* in rocket (*Diplotaxis tenuifolia*) and Swiss chard (*Beta vulgaris* cicla)

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Abstract

The influence of organic nitrogen sources on the establishment of *E. coli* O157:H7 and the occurrence of other potentially human pathogenic bacteria on baby leaf salads was evaluated. Greenhouse-grown rocket and Swiss chard were spray-inoculated with *gfp*-tagged *E. coli* O157:H7 twice a week from when their first true leaves reached a length of 2 cm until three days before harvest. Analysis of nitrogen content in leaves revealed differences between treatments. Untreated plants had the lowest values, followed by plants fertilized with pig hair pellets and chicken manure. The same pattern was seen for the growth medium at the day of harvest. The applied strain showed similar establishment (measured using culture-dependent methods) irrespective of treatment, but Swiss chard hosted significantly more *E. coli* O157:H7 than rocket. Differences in the risk of infection were found for the crops, with plants fertilized with pig hair pellets showing a slightly higher risk. No relationship was found for total nitrogen content in leaves and colonization with *E. coli* O157:H7 *gfp*+. Isolating dominant Enterobacteriaceae from leaves, some strains showed conformity to bacterial species that have previously been identified to influence the establishment of *E. coli* O157:H7 in the phyllosphere. In this study, no inhibitory effects were observed *in vitro*.

Colonies showing matching characteristics for *Listeria* spp. and *Salmonella* spp. were detected ~~in a range from below the detection limit to 4 log CFU g^{-1} fresh weight on rocket, and from 3.5 to~~ up to 5.5 ~~log CFU g^{-1} fresh weight and 1 to~~ and 5 log CFU g^{-1} fresh weight, respectively~~, on Swiss chard~~. However, presumptive *Listeria* spp. and *Salmonella* spp. could not be confirmed by sequencing of the 16S rRNA gene.

Keywords: culture-dependent methods, Enterobacteriaceae, food safety, human pathogenic bacteria, organic fertilizers, phylogenetic analysis, risk assessment

1 Introduction

The surface of vegetables is highly colonized with microorganisms, bacteria being the most abundant, followed by fungi and yeasts. However, archaea, protozoa, and nematodes can also be found in the phyllosphere (Whipps, Hand, Pink, & Bending, 2008). On leafy vegetables, bacteria belonging to the phyla Proteobacteria, Firmicutes, Actinobacteria, and Bacteroides show the highest occurrence, based on culture-independent analysis (Alsanius et al., Unpublished results; Rastogi et al., 2012). On family level, the Pseudomonaceae and Enterobacteriaceae account for the highest numbers (Hunter, Hand, Pink, Whipps, & Bending, 2010; Lopez-Velasco, Welbaum, Boyer, Mane, & Ponder, 2011). In general, these colonizers are indifferent or have even been claimed to promote human health (Berg, Erlacher, Smalla, & Krause, 2014). However, increasing outbreaks linked to contaminated fresh produce in recent years have led to a focus on the risk caused by produce eaten raw (Warriner, Huber, Namvar, Fan, & Dunfield, 2009). Particular concerns are human pathogenic *Escherichia coli* and *Salmonella* spp., due to their frequent implication in outbreaks, and *Listeria monocytogenes*, owing its high case fatality rate (lethality) (Brandl, 2006; Carrasco, Pérez-Rodríguez, Valero, García-Gimeno, & Zurera, 2010). Among human pathogenic *E. coli*, serotype O157:H7 is most often identified as the causative agent in disease outbreaks (Dikici, Koluman, & Calicioglu, 2015), but other strains, e.g., belonging to the serogroups O26, O103, O104, O111, and O145, also have the ability to infect humans (European Food Safety Authority (EFSA), 2011; Karmali et al., 2003). Clinical disease has been observed following exposure at low doses of less than 100 bacteria (Rheinbaben, 2011) and can lead to diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS) (Agbodaze, 1999). Infection with *Salmonella* spp. typically causes gastroenteritis and septicemia (Trevejo, Courtney, Starr, & Vugia, 2003). An exposure level of 10^5 to 10^{10} cells is needed to give a tangible probability of infection (Kothary & Babu, 2001). Listeriosis can manifest in meningitis, sepsis, and miscarriage in pregnant women (Farber & Peterkin, 1991) and mortality rates during an outbreak can be as high as 40% (Carrasco et al., 2010). In healthy people, the *Listeria* numbers in exposures associated with clinical listeriosis are reported to be high, while for susceptible groups, i.e., people with immunosuppression, even

small numbers can lead to infection (Franciosa, Maugliani, Floridi, & Aureli, 2005; Todd & Notermans, 2011).

Human pathogenic bacteria are not part of the normal flora of the phyllosphere and thus have to be introduced to the plant environment (Chitarra, Decastelli, Garibaldi, & Gullino, 2014). Transmission can occur via several routes, including pre- and post-harvest processes (Taban & Halkman, 2011). Critical points for transmission of human pathogenic bacteria during cultivation are associated with application of contaminated manure or irrigation water (Islam, Doyle, Phatak, Millner, & Jiang, 2004), use of plant material of low microbial quality (Jablasone, Warriner, & Griffiths, 2005), and insufficient field worker hygiene (Brackett, 1999). Moreover, there are hazards arising from contaminated runoff water (Kirby, Bartram, & Carr, 2003) and airborne transmission (Berry et al., 2015) from nearby farms and through wild animals visiting crop fields (Beuchat, 2006). A risk assessment evaluating the use of manure in terms of transmission of human pathogens to vegetables (Koller, 2011) concluded that pre-treatment of manure and a time lag between application and cultivation are needed to reduce the risk of contamination of vegetables. However, no previous risk assessment has quantified the risk posed by fertilizers with differing nutrient availability.

The establishment of an alien bacterium in the phyllosphere can be influenced by the existing microbiome and by the level of available nutrients (Monier & Lindow, 2005; Oliveira, Viñas, Anguera, & Abadias, 2012). For different fertilizers, differences in the time of mineralization lead to varying amounts of readily available nitrogen for the crops at a specific growth stage, even if the sum of supplied nitrogen in total is comparable (Laber, 2013). As the requirement for nitrogen varies during the course of plant development (Feller et al., 2001), the supply might be (sub)optimal depending on the fertilizer used. Consequently, different fertilizers could also result in variations of leaf exudates.

Pedraza, Bellone, Carrizo de Bellone, Boa Sorte, and Teixeira (2009) showed that urea application results in a shift in the leaf endophytic microbiome in rice, while Huber and Watson (1974) demonstrated that the form of fertilizer nitrogen used has an impact on leakage quantity and nutrient composition of leaf exudates. In addition, the growth medium has an indirect influence on the phylloplane by altering the soil microbiome

(Andrews, 1992). These findings raise the question of whether organic nitrogen fertilizer applied to the growth medium of vegetable crops has an impact on the establishment of human pathogenic bacteria, other than its potential of functioning as a carrier.

The aim of this study was thus to evaluate the influence of organic nitrogen source on establishment of artificially introduced ~~(via spray irrigation)~~ *E. coli* O157:H7 *gfp*+ on rocket and Swiss chard baby leaves. The bacterium was introduced to the phyllosphere via spray application, imitating overhead irrigation, a common irrigation method in leafy vegetable production. Native occurrence of *Salmonella* spp. and *Listeria* spp. was also analyzed with respect to the fertilizer. A risk assessment of the probability of infection when eating the contaminated leafy greens was conducted. Furthermore, dominant bacteria grown on a culture medium selective for Enterobacteriaceae were identified. Previous studies have revealed that phylloepiphytic Enterobacteriaceae spp. can have an impact on the growth of *E. coli* O157:H7 on leafy greens (Cooley, Chao, & Mandrell, 2006; Lopez-Velasco, Tydings, Boyer, Falkinham Iii, & Ponder, 2012). The hypotheses tested were that: (i) food pathogens, such as *Salmonella* and *Listeria*, and (ii) the number of *E. coli* O157:H7 *gfp*+ introduced are affected by the nitrogen source and (iii) that the infection risk is higher for crops fertilized with non-treated organic nitrogen sources (chicken manure) than with heat-treated organic nitrogen sources (pig hair pellets).

2 Materials and Methods

2.1 Inoculum preparation

An *Escherichia coli* O157:H7 strain, obtained from the Swedish Institute for Communicable Disease Control, Solna, Sweden (registry no E81186), was used in the experiment. This strain expresses the *eae* gene but lacks virulence factors verotoxin-1 and -2 and is labeled with a plasmid coding for ampicillin resistance and for green fluorescent protein (*gfp*), which is emitted in the presence of arabinose when exposed to UV light (Alam et al., 2014; El-Mogy & Alsanius, 2012).

The *E. coli* O157:H7 *gfp*+ was cultured from a cryo-culture on lysogenic broth (LB) agar supplemented with 0.2% arabinose and 100 µg mL^{-1} ampicillin at 37 °C. A single fluorescent colony was picked and cultivated in 30 mL LB agar plus 100 µg mL^{-1} ampicillin on a rotary shaker at 180 rpm and 37 °C for 18

hours. Cells were pelleted by centrifugation of the suspension at 3000 xg and 4 °C for 45 min, and washed once with 0.85% sterile NaCl. Cells were suspended in 0.85% NaCl to a density of 9.7 log CFU mL^{-1} (optical density (OD_{620}) = 1.0). This stock suspension was diluted with 0.085% NaCl to a final density of 6 log CFU mL^{-1} for plant inoculation.

2.2 Greenhouse experiment

Swiss chard (*Beta vulgaris* subsp. *cicla*, SCR 107, AdvanSeed) and rocket (*Diplotaxis tenuifolia*, Tricia, Enzo) were grown in the greenhouse with a target humidity of 70% and the temperature set to 21°C. Peat based growing medium, consisting of a bottom layer (4.5 cm, K-jord, Hasselfors Garden AB, Örebro, Sweden), and a top layer (1.5 cm, S-jord; Hasselfors Garden AB, Örebro, Sweden) was filled in trays (0.52 m x 0.42 m x 0.09 m) with the addition of chicken manure (CM) or pig hair pellets (PHP) in the amount of 81 kg ha^{-1} available nitrogen. The control plots did not receive any additional fertilizer. When the first true leaves reached a size of two cm, the plants were spray-inoculated with an *E. coli* O157:H7 *gfp*+ suspension in an amount of 0.1 mm every three days until three days before harvest (five times for rocket and three times for Swiss chard). For this occasion, the trays were transferred to a greenhouse section, approved for work with genetically modified organisms, and the suspension was applied with the help of a spray flask.

~~grown in the greenhouse as described by Alsanius et al. (2016, submitted), with addition of chicken manure (CM) or pig hair pellets (PHP) to a total amount of 81 kg ha^{-1} readily available nitrogen. The control plots did not receive any additional fertilizer. When the first true leaves reached a size of two cm, the plants were spray-inoculated with an *E. coli* O157:H7 *gfp*+ suspension in an amount of 5 mm every three days until three days before harvest (five times).~~

Six replicates for each treatment, defined as separate trays ~~(0.52 m x 0.42 m x 0.09 m)~~, were harvested six weeks after sowing, and brought directly to the laboratory for further analysis.

2.3 Nitrogen analysis

The residual nitrogen content in three independent replicates of the fertilizers used and in the growth medium, including the root system, was assessed with respect to mineralized nitrogen (NH_4^+, NO_3^-) and total nitrogen content by Eurofins, Kristianstad, Sweden. Growth medium samples were collected for each tray and kept at -20 °C until analysis.

Leaves (100 g fresh weight) used for determination of nitrogen content were dried in a drying oven at 70 °C. Analysis of total nitrogen content was conducted by Eurofins, Kristianstad, Sweden. Nitrate (NO_3^-) content was measured on fresh plant material according to Method no. G-287-02 Rev. 5 (Multitest MT7B / MT8B).

2.4 Microbial analysis

Aliquots of 25 g of the harvested leaves were weighed into sterile filter bags, 50 mL of TRIS buffer (0.1 M, pH 5.6) were added, and epiphytic microorganisms from the leaf surface were abraded using a stomacher (Smasher, Chemunex, Bruz, France) at normal speed for 30 s. The suspension was serially diluted with 0.85% NaCl and plated in triplicate on semi-selective medium. The media used and the conditions applied are described in Table 1. The results are expressed as log colony-forming units (CFU) per gram fresh weight (FW).

Growing media and fertilizers used in the first Swiss chard run were analyzed according to their microbial loads as described by Hartmann et al. (submitted). Media and conditions were the same as used for leaf analysis.

2.4.1 Identification of dominant Enterobacteriaceae

After termination of incubation and colony enumeration of violet red bile dextrose agar (VRBD) plates, five solitary colonies were randomly selected from each of the plates and pure-cultured on full-strength tryptic soy agar (TSA) for 24 h at 37 °C to characterize dominant Enterobacteriaceae in the phyllosphere. Pure-

cultured strains were kept as cryo-cultures in sterile freezing medium (0.05 M K_2HPO_4, 0.001 M KH_2PO_4, 0.002 M trisodium citrate, 0.001 M $MgSO_4$ x $7H_2O$, 12% glycerol) at -80 °C before 16S DNA sequencing.

2.4.2 Dual culture test

The selected isolates from VRBD were tested for an inhibiting effect on the applied *E. coli* O157:H7 *gfp+* strain *in vitro*. For this purpose, the isolated strains and *E. coli* O157:H7 *gfp+* were propagated overnight on full-strength TSA from cryo-cultures at 37 °C. Single colonies of the isolates and *E. coli* O157:H7 *gfp+* were streaked on another full-strength TSA plate in orthogonal lines and again incubated overnight at 37 °C. Inhibition was expressed by a halo around the target strain.

2.4.3 Verification of *Listeria* spp. and *Salmonella* spp.

The second run of the greenhouse experiment with rocket was used to specify colonies which were presumptive *Salmonella* spp. or *Listeria* spp. Up to five colonies per replicate showing the characteristics of one of the genera were randomly picked and transferred to full-strength TSA. After overnight incubation at 37 °C, a single colony was picked and recultured under the same conditions. Pure strains were propagated in 8 mL of full-strength tryptic soy broth (TSB) (37 °C, o/n) and the suspensions were centrifuged at 2500 xg and 4 °C for 20 min. The bacterial pellets were washed in 0.85% NaCl and stored as cryo cultures in 1 mL freezing medium at -80 °C until use for 16s rRNA gene sequencing.

2.4.4 16S DNA sequencing of isolated bacterial cells

DNA extraction of Enterobacteriaceae and presumptive *Salmonella* and *Listeria*

Cryo-preserved isolates were recultured on full-strength TSA. After overnight incubation at 37 °C, a single colony was transferred to 5 mL TSB and incubated under the same conditions. The suspension was then centrifuged at 3000 xg and 4 °C for 10 min. The pellet was washed with 5 mL autoclaved 0.85% NaCl through repeated centrifugation and resuspended in 1 mL autoclaved, deionized water and boiled in a water bath for 2-4 min. The suspension was centrifuged at 13.000 xg for 10 min and about 750 µL of the

supernatant were transferred to a sterile tube. The extracted DNA was stored in a refrigerator (4 °C) until further use.

For polymerase chain reaction (PCR), the 16S rRNA genes were amplified by applying universal forward and reverse primers ENV1 (5′-AGA GTT TGA TII TGG CTC AG-3′) and ENV2 (5′-CGG ITA CCT TGT TAC GAC TT-3′), respectively. The master mix contained 0.5 µL of each primer, 0.156 µL Taq-polymerase, 2.5 µL Taq-buffer, 0.5 µL deoxyribonucleotide triosphosphate (dNTP), 1.5 µL $MgCl_2$ and 2 µL of the extracted DNA sample. To achieve a final reaction volume of 25 µL, sterile ultrapure water was added. The PCR was initiated at 94 °C for 4 min, followed by 30 cycles of 94 °C for 1 min, 50 °C for 45 s, 72 °C for 2 min and a final annealing temperature of 72 °C for 7 min.

The PCR products were separated on a 1.5% agarose Tris-borate-EDTA-(TBE-) buffer gel. A portion of 2.0 µL of each sample or 2.5 µL of marker (O'GeneRuler 100 bp Plus DNA Ladder, Thermo Scientific, Braunschweig, Germany) was loaded on the gel together with 5.0 µL 6x DNA Loading Dye (Thermo scientific, Braunschweig, Germany). Electrophoresis was run in TBE buffer at 100 V. Amplification of the DNA was confirmed by staining the gel with intercalating dye GelRed (VWR International, Stockholm, Sweden) and visualizing the bands under UV-light. Samples with a confirmed size of 1500 bp were sequenced by Eurofins MWG Operon (Ebersberg, Germany), using primer ENV1.

Sequences isolated from VRBD that appeared to be *E. coli* were grown on LB supplemented with arabinose and ampicillin to differentiate between the applied *E. coli* O157:H7 gfp+ and other strains. Isolates growing under ampicillin and showing green fluorescence under arabinose were considered to be the applied strain. Other isolates were tested for the presence of the *eae* gene by applying PCR with *eae* specific primers eae A-F (5'-GAC CCG GCA CAA GCA TAA GC-3') and eae A-R (5'-CCA CCT GCA GCA ACA AGA GG-3') under the following conditions: 95 °C for 3 min, (95 °C for 1 min, 60 °C for 2 min, 72 °C for 1.5 min) x 30, 72 °C for 7 min, 4 °C. Electrophoresis of the amplified DNA was run as described above, with lengths of 384 bp indicating the presence of the *eae* gene. Negative results were confirmed twice.

2.5 Risk assessment

The associated risks of the numerical findings of pathogens obtained by culture-dependent analysis were calculated. Models were selected based on the criteria of being simple, parsimonious, and firmly grounded in biological reasoning (Teunis & Havelaar, 2000). A portion size of 30 g was assumed throughout. The *E. coli* O157:H7 risk was estimated using a Beta-Poisson model developed by Teunis, Takumi, and Shinagawa (2004) and refined by Strachan, Doyle, Kasuga, Rotariu, and Ogden (2005).

2.6 Statistics

The experiment was organized as a two-factorial design, with the first factor being two different crops and the second factor different fertilizer applications. The trials were repeated twice, with six independent replicates in each run. Nitrate content in leaves was conducted in run 2 for rocket. The treatments were compared in respect of their nitrogen content and data obtained from culture-dependent analysis by employing one-way ANOVA followed by pair-wise comparison (t-test) using the Tukey method ($\alpha = 0.05$). Minitab version 17 was used.

Sequences of isolated strains were edited using BioEdit (version 7.2.5) to obtain sections with clear signals and therefore reliable sequences. These sections were uploaded on Ribosomal Database, release 11 (RDP, Michigan State University, East Lansing, USA) for phylogenetic comparison with bacterial type strains. Best matches from bacterial strains isolated from VRBD were used to build a phylogenetic tree. All sequences were aligned using ClustralX2 (version 2.1) and bootstrapping was done by employing RAxML (version 8). For tree visualization, TreeGraph (version 2.4.0_456 beta) was utilized.

3 Results

3.1 Nitrogen content in leaves

Total nitrogen content was lowest in leaves that did not receive any fertilizer treatment (rocket: 3.4 mg g^{-1} FW, Swiss chard: 4.1 mg g^{-1} FW), followed by PHP-treated plants (rocket: 5.7 mg g^{-1} FW, Swiss chard: 5.8 mg g^{-1} FW) and CM-treated plants (rocket: 7.8 mg g^{-1} FW, Swiss chard: 6.1 mg g^{-1} FW). Apart from those between PHP- and CM-fertilized Swiss chard, the differences were statistically significant.

Analysis of the nitrate content in rocket leaves, conducted in one run with six replicate samples, showed the same pattern, with mean values of 0.6 mg g^{-1} FW for untreated plants, 4.5 mg g^{-1} FW for PHP-fertilized plants, and 6.9 mg g^{-1} FW for CM-fertilized plants, all differences being statistically significant.

3.2 Nitrogen content in growth medium

Nitrogen content in the fertilizers is shown in Table 2. Proportions relative to dry matter (DM) content were clearly higher in pig hair pellets than in chicken manure. Residual content of total nitrogen and nitrate and ammonia in the growth medium for rocket and Swiss chard are shown in Figure 1. As for nitrogen content in the leaves, the value was lowest in the control treatments (rocket medium: 498 mg $100g^{-1}$ DM, Swiss chard medium: 720 mg $100g^{-1}$ DM), followed by PHP treatment (rocket medium: 593 mg $100g^{-1}$ DM, Swiss chard medium: 847 mg $100g^{-1}$ DM) and CM treatment (rocket medium: 720 mg $100g^{-1}$ DM, Swiss chard medium: 1049 mg $100g^{-1}$ DM). These differences were statistically significant.

A similar pattern was observed for residual nitrate content. With concentrations of 0.6 and 0.3 mg $100g^{-1}$ DM, respectively, rocket and Swiss chard control treatments showed the lowest value. Values for PHP-fertilized medium were slightly higher, with 1.6 mg $100g^{-1}$ DM for rocket and 1.7 mg $100g^{-1}$ DM for Swiss chard. The highest nitrate accumulation was found in CM-fertilized medium, with 81.3 and 83.7 mg $100g^{-1}$ DM in rocket and Swiss chard, respectively. Other values between the two runs were on the same level, but total nitrate residual content in rocket growth medium for run 1 (131.8 mg $100g^{-1}$ DM) was considerably higher than in run 2 (30.9 mg $100g^{-1}$ DM).

For ammonia, no differences were found in the growth medium of the rocket plots. The highest content was found in CM-fertilized medium (1.4 mg $100g^{-1}$ DM), followed by PHP-fertilized medium (1.3 mg $100g^{-1}$ DM) and the control treatment (0.6 mg $100g^{-1}$ DM). In contrast, significant differences were found for the residual ammonia content in the growth medium of the treatments with Swiss chard in PHP-fertilized plots (1.7 mg $100g^{-1}$ DM) and a lower residual content in medium supplemented with CM (1.1 mg $100g^{-1}$ DM). The lowest residual ammonia content was again noted in the control treatment (0.4 mg $100g^{-1}$ DM).

Comparisons of growth medium for the two crops revealed significantly higher total nitrogen residual contents for Swiss chard than rocket. For nitrate the mean was also higher for Swiss chard, while for ammonia it was slightly higher for rocket. These differences were not significant.

3.3 Microbial colonization

Numbers of heterotrophic bacterial plate count, *E. coli* O157:H7 *gfp*+, and Enterobacteriaceae as obtained from culture-dependent methods are displayed in Figure 2. The average load of the heterotrophic bacterial plate count was highest on leaves from plants fertilized with PHP (5.1 log CFU g^{-1}), followed by plants treated with CM (4.9 log CFU g^{-1}), and unfertilized plants (4.7 log CFU g^{-1}). For Swiss chard, the heterotrophic bacterial plate count was significantly higher for the control treatment (7.0 log CFU g^{-1}) and PHP-fertilized plants (6.9 Log CFU g^{-1}) than for CM-fertilized plants (6.4 log CFU g^{-1}). For *E. coli* O157:H7 *gfp*+, no statistically significant differences were found between fertilizer treatments in either crop. However, PHP treatments resulted in the highest colonization rate on leaves of rocket (3.9 log CFU g^{-1}) and Swiss chard (5.4 log CFU g^{-1}). On unfertilized and CM-fertilized rocket leaves, loads of *E. coli* O157:H7 *gfp*+ averaged 3.4 log CFU g^{-1}. Swiss chard leaves fertilized with CM showed an average value of 5.2 log CFU g^{-1} and unfertilized leaves 5.1 log CFU g^{-1}. The loads of Enterobacteriaceae matched the colonization with *E. coli* O157:H7 *gfp*+, with CM-fertilized plants again showing the highest loads (3.6 and 6.0 log CFU g^{-1} for rocket and Swiss chard, respectively). On rocket leaves, values of 3.5 log CFU g^{-1} and 3.4 log CFU g^{-1} were obtained for CM-treated plants and control plants, respectively. Loads averaged 5.8 log CFU g^{-1} on CM-treated leaves and 5.9 log CFU g^{-1} on untreated leaves for Swiss chard. In general, Swiss chard harbored significantly higher bacterial loads in growth medium than rocket.

Bacteria showing matching characteristics for *Salmonella* spp. and *Listeria* spp. were identified on the chromogenic nutrient agar. None of the colonies showed corresponding characteristics for *L. monocytogenes*. For *Salmonella* spp., the numbers ranged between the lower detection limit and 4 log CFU g^{-1} on rocket leaves and between 1 and 5 log CFU g^{-1} on Swiss chard leaves. Further identification by sequencing revealed the colonies not to be *Salmonella*, but belonging to the genus *Pseudomonas*. *Listeria*

spp. also ranged from below the lower detection limit to 4 log CFU g^{-1} on rocket leaves. For Swiss chard, loads between 3.5 and 5.5 log CFU g^{-1} were found. Further identification suggested that the colonies belong to the genera *Microbacterium, Bacillus* and *Paenibacillus*, but not *Listeria*.

The analyzed K- and S-soil both showed heterotrophic bacterial plate counts of about 6 log CFU g^{-1}, while pig hair pellets were colonized with 3.1 log CFU g^{-1} and chicken manure with 7.7 log CFU g^{-1}. No *Salmonella* spp. or *E. coli* were detected in soils or fertilizers (detection limit 2 log CFU g^{-1}). Colonies, presumptive to be *Listeria* spp. – but not *L. monocytogenes* – were found in K- (2.6 log CFU g^{-1}) and S-soil (3.3 log CFU g^{-1}) and chicken manure (4.4 log CFU g^{-1}), but were below the detection limit in pig hair pellets.

3.4 Characterization of Enterobacteriaceae

Further identification of colonies showing specific characteristics for Enterobacteriaceae on VRBD was conducted and phylogenetic trees were drawn for the two crops (Figure s3 and 4). Considerable differences were found between the crops. Strains isolated from Swiss chard leaves predominantly comprised bacteria belonging to the Enterobacteriaceae, while strains obtained from rocket leaves were affiliated with *Pseudomonas*, which was not the target phylogenetic family.

A phylogenetic tree containing 27 isolates, with length 345 to 507 bp, obtained from wild rocket leaves is shown in Figure 3. A majority of 15 colonies isolated from VRBD grouped together with *Enterobacter* species, at which six were obtained from plants fertilized with PHP and nine from plants fertilized with CM. Another isolate recovered from PHP-fertilized plants showed similarity to *Lelliottia*, completing the branch of Enterobacteriaceae. A second major group of isolates matched with different type strains belonging to the genus *Pseudomonas*. This group included all four isolates from unfertilized plants, as well as two and one from PHP- and CM-fertilized plants, respectively. Another cluster containing type strains of *Pseudomonas hibiscicola* and *Stenotrophomonas maltophilia* grouped with an isolate from a CM-fertilized plant. Three isolates clustered with *Acinetobacter*, one originating from CM-fertilized and one from PHP-fertilized plants.

306

307 With respect to Swiss chard, all but one of the colonies isolated matched with type strains belonging to the
308 family Enterobacteriaceae. A phylogenetic tree including 29 isolates with length 353 to 541 bp shows the
309 different groupings (Figure 4). Two major groups, containing *Pantoea* or *Enterobacter* species, respectively,
310 were defined. Seven, two, and five isolates extracted from unfertilized plants, PHP-treated, and CM-treated
311 plants were represented in the *Pantoea* cluster. The *Enterobacter* cluster comprised three isolates from
312 unfertilized and nine from CM-fertilized plants. One colony isolated from a plant fertilized with CM
313 clustered together with *Citrobacter*. Another single colony, isolated from an unfertilized plant, showed
314 sequence patterns similar to *E. coli*, but could not be grown on supplemented LB and was *eae* gene negative.
315 An isolate obtained from an unfertilized plant grouped with *Achromobacter spanius*, a species belonging to
316 the family Alcaligenceae.

317

318 No direct inhibition of the isolated Enterobacteriaceae strains from rocket and Swiss chard leaves on the
319 applied *E. coli* O157:H7 *gfp+* was detected *in vitro*.

320 3.5 Interactions between nitrogen content in leaves and microbial colonization

321 Principal component analysis including culture-dependent and leaf-nitrogen assessments separated between
322 the two crops rocket and Swiss chard (principal component (PC) 1), while PC 2 discriminated the different
323 fertilizer regimes (Figure 5). PC 1 was mainly driven by the crop and the bacterial loads on the leaves, while
324 the treatment and the total nitrogen content in leaves were decisive for PC 2.

325 Correlations between the bacterial groups analyzed were strong and highly significant ($p<0.001$). For the
326 heterotrophic bacterial plate count and *Enterobacteriaceae*, Pearson correlation coefficient (r) was 0.800
327 and for heterotrophic bacterial plate count and *E. coli* O157:H7 *gfp+* it was 0.749. Between
328 Enterobacteriaceae and *E. coli* O157:H7 *gfp+* the highest correlation had a Pearson correlation coefficient
329 of 0.930.

Correlations between nitrogen and nitrate contents in rocket leaves were also pronounced, with a Pearson correlation coefficient of 0.957 ($p<0.001$).

No interactions between nitrogen content in leaves and bacterial colonization were found (Figure 6).

3.6 Risk assessment

The results of the risk assessment are shown in Table 3. There were clear differences in bacterial numbers and associated risks between rocket and Swiss chard. For five out of the six experiments (plant species-fertilizer), the numbers on leaves fertilized with PHP were higher, but not dramatically higher. The exception was *Salmonella*, where the control appeared to have higher numbers than CM or PHP treatment. The risk of infection was not dramatically different.

4 Discussion

The use of non-sanitized manure as fertilizer can carry a high risk of microbial contamination of fresh produce, while animal products that are heat-treated before application are assumed to carry a lower risk (Möller & Schultheiss, 2014). However, the risk assessment conducted within this study showed that not only the carrier risk, but also the interactions on other levels, must be considered in food hazard analysis. The numbers of *E. coli* O157:H7 bacteria were very high and, given that the dose response curves were shifted leftwards, no dramatic differences in risk were observed. However, in the case of lower numbers of *E. coli* O157:H7 inoculated or naturally contaminating the leaves, even these small differences could have an impact. Repeating this experiment using lower numbers of *E. coli* O157:H7 bacteria contaminating the vegetable leaves could be interesting. To our knowledge, this is the first study to address the risk posed by fertilizers of animal origin other than acting as a potential carrier of human pathogens.

Beside the risk posed by epiphytic colonizers of the phyllosphere, it has been shown, that human pathogens can invade leaf tissue and thus being protected from washing procedures or sanitizing agents (Yaron, 2014). Internalization occurs through natural openings like stomata or injuries of the cuticle (Deering, Mauer, & Pruitt, 2012). Differences in nitrogen supply of the plants are known to alter stomatal conductance (Broadley, Escobar-Gutiérrez, Burns, & Burns, 2001) and might thus as well have an impact on bacterial

355 invasion. As the emphasis in this study lay on epiphytes, future research could gain new insights by focusing
356 on the impact of different fertilizers on the internalization of *E. coli* O157:H7 into leaf tissue.
357 The study also analyzed the impact of manure as a nitrogen source on establishment of *E. coli* O157:H7 in
358 the phyllosphere. Nitrogen is the limiting factor for biomass gain in crops (Lawlor, Lemaire, & Gastal,
359 2001). Nitrogen is taken up by plants as nitrate or ammonia, the former dominating. Fertilizer application
360 was calculated based on readily available nitrogen and mineralization rate and thus the initial proportions
361 of ammonia and nitrate present in the fertilizers did not correspond to the proportions found in the growth
362 medium at harvest. This might be due to slow mineralization, or to promotion of root growth and nitrogen
363 fixation in root biomass. Separate analysis of the root biomass and the residual growth medium in further
364 studies can provide insights into the differences in the fertilizers used. As the cropping units in the
365 greenhouse were non-leaching containers and water was supplied upon demand, the higher amounts of
366 nitrate in the fertilizers than in growth medium and roots point to high uptake and metabolism of this easily
367 available nutrient. Point sample analyses of the available nitrogen content at the point of harvest revealed
368 differences in the supply, indicating unequal conditions for the plants depending on the fertilizer applied.
369 Differences between rocket and Swiss chard can be a consequence of different nutrient requirements and/or
370 different cultivation periods. In addition, El-Sharkawi (2012) found that the performance of microbial
371 nitrogen turnover, and thus the nitrogen availability and uptake by plants (rice), was influenced by the
372 applied nitrogen source in growth medium. These factors can explain the differences found in leaf nitrogen
373 and nitrate content in the present study.
374 The nitrate incorporated by the plants has to be reduced to ammonia before it is converted to glutamate and
375 aspartate (Lam, Coschigano, Oliveira, Melo-Oliveira, & Coruzzi, 1996). These amino acids are the
376 precursors for other amino acids and thus for the metabolism of essential proteins, including enzymes,
377 hormones, and chlorophyll (Snoeijers, Pérez-García, Joosten, & De Wit, 2000). High uptake of nitrate
378 results in accumulation in leaves (Yusof, Rasmusson, & Galindo, 2016), which is also reflected in the leaf
379 exudates (1-2 µg N g^{-1} lettuce leaf) on which the phyllosphere microbiota feed (Brandl & Amundson, 2008).
380 In the plant environment, the abundance of nutrients is the limiting factor for bacterial growth. Depending

on the C/N ratio in leaf exudates, the microbial community can be either nitrogen- or carbon-deprived (Wilson & Lindow, 1994). Research conducted by Brandl and Amundson (2008) suggests that nitrogen availability defines the establishment of *E. coli* O157:H7 on (young) lettuce leaves. In this study, significant differences in nitrogen and nitrate contents of the leaves were not found for *E. coli* O157:H7 *gfp+* colonization of the leaves, indicating that nitrogen was not the limiting factor for establishment.

Beside nitrogen mineralization, the fertilizers used may also have other impacts on plant growth due to other nutrients present. Chicken manure is rich in nitrogen and phosphorus, while PHP is high in nitrogen and sulphur, but low in potassium and phosphorus (Möller & Schultheiß, 2014). These differences might not only affect plant development, but also influence microbial colonization in the phyllosphere. Alsanius et al. (2016) found that the content of macro- and micronutrients (e.g., P, K, Mg, Ca, S, Na, Mn, Fe, Cu, Zn) in the leaves of baby rocket and Swiss chard had an impact on colonization by artificially introduced *E. coli* O157:H7.

Beside nutrient abundance, the indigenous microbiota can have an impact on the establishment of immigrant microorganisms in the phyllosphere (Lopez-Velasco et al., 2011; Rastogi, Coaker, & Leveau, 2013). These interactions can be either promotive, inhibitory, or indifferent. Commensalism between microorganisms on the leaf surface can be expressed, (i) in making nutrients available through the degradation of plant tissue or an available source and (ii) by altering the microenvironment, making it more suitable (Cooley et al., 2006; Deering et al., 2012). Negative effects have been well-studied for biocontrol agents and include competition for nutrients and/or space, siderophore production, and antibiosis (Whipps, 2001). Studies investigating the effect of strains inhabiting leafy vegetables on *E. coli* O157:H7 have found evidence of interactions for several strains with human pathogenic bacterium. For example, Cooley et al. (2006) found that *Wausteria paucula* promotes survival of *E. coli* O157:H7 on lettuce seedlings, while *Enterobacter asburiae* inhibits its establishment. *Enterobacter cloaceae* also has a negative effect on *E. coli* O157:H7 when co-inoculated on lettuce seeds (Jablasone et al., 2005). Lopez-Velasco et al. (2012) analyzed the phyllosphere of spinach and found bacteria belonging to the Enterobacteriaceae with an inhibiting effect on *E. coli* O157:H7, e.g., *Erwinia perscina* and *Pantoea agglomerans*, while species belonging to

Pseudomonas spp. and *Bacillus* spp. also had a negative effect. In the present study, some of the strains isolated from a culture medium selective for Enterobacteriaceae showed high conformity with bacteria that have been shown to inhibit *E. coli* O157:H7 growth. However, this could not be confirmed by our analysis *in vitro*, for different reasons. For example, nutrient agar does not reflect conditions on plants, e.g., by providing more space and nutrients. Thus, the absence of *in vitro* effects does not necessarily mean that there is no competition in the phyllosphere. Moreover, only the 16S rRNA gene, and not the whole genome, was studied, so assignment to individual species is associated with high uncertainty and the strains identified might lack significant genes that play a role in the interaction.

Various studies have provided evidence that human pathogenic bacteria are able to colonize edible parts of leafy vegetables when introduced to/with the growth medium. Islam et al. (2004; 2004) showed that *E. coli* O157:H7 and *S. enterica* serovar Typhimurium are able to persist in soil when inoculated together with manure before planting. The potentially pathogenic bacteria in that study were transmitted from the contaminated growth medium to leaves of lettuce and parsley, and persisted in the phyllosphere for more than two months. It has also been shown that *L. monocytogenes* can invade alfalfa crops when inoculated into the soil, with sewage sludge being the vector (Al-Ghazali & Al-Azawi, 1990). In agreement with these results, we found when employing culture-dependent methods that *Listeria* spp., but not *L. monocytogenes*, and *Salmonella* spp., colonized baby leaves irrespective of treatment. However, further identification revealed the strains not to be the presumed bacteria, illustrating the need to confirm culture-dependent results. This is particularly the case if bacteria are isolated from a harsh environment, where conditions might force them to express atypical patterns to survive. The identification of an isolate from growth medium selective for Enterobacteriaceae that appeared to be *E. coli* (but not the applied strain) shows the potential of food pathogens to colonize leafy greens, even if not artificially introduced. Contrary to our hypothesis, the bacterium was found on leaves of plants that were not treated with organic fertilizer (control treatment).

5 Conclusion

It should be borne in mind that this study required the use of animal waste products, in which the composition of ingredients may differ depending on the batch. Furthermore, other substances that may also affect the target factor cannot be controlled. Thus, a limiting approach in comparing and interpreting results is needed when organic fertilizers are considered. The organic fertilizers applied resulted in different residual nitrogen content in the growth medium at harvest and different nitrogen content in the leaves. However, establishment of *E. coli* O157:H7 *gfp+* on leaves was not affected by the treatment according to ANOVA. Similarly, other studies have found that a high risk treatment does not enhance the colonization of leafy greens with potentially human pathogenic bacteria (Hartmann et al., submitted; Wießner, Thiel, Krämer, & Köpke, 2009). Nevertheless, our risk assessment suggested that the treatments tested affected the infection dose of *E. coli* O157:H7 on baby leaves, and that the impact of human pathogen establishment as determined by the nitrogen source needs further attention. Treatments were grouped in principal component analysis according to the leaf parameters assessed, while there was also a clear separation between rocket and Swiss chard. Differences in the indigenous microbiota, here assessed for Enterobacteriaceae, can have an impact on the establishment of human pathogens in the phyllosphere. As found for establishment of *E. coli* O157:H7 in the phyllosphere, differences in naturally abundant Enterobacteriaceae were more pronounced between crops than between treatments. None of the presumptive *Salmonella* spp. or *Listeria* spp. analyzed was confirmed and thus results obtained with culture-dependent methods should be regarded with caution.

Acknowledgements

The study was performed within the framework of the Tvärlivs project ‘Safe ready to eat vegetables from farm to fork: The plant as a key for risk assessment and prevention of EHEC infections (acronym: Safe Salad)’ and the international postgraduate school ‘Microbial Horticulture’ (µHORT) both funded by the Swedish research council Formas, Stockholm, Sweden, and the project ‘Qualität und Sicherheit in der Produktionskette biologisch produzierter Fertigsalate’ (German Federal Ministry of Agriculture and Nutrition, BLE; project no. 2811OE097).

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Tables

Table 1. Semi-selective media and supplements, and incubation conditions used in culture-dependent analysis.

Medium		**Supplement**	**Selective for**	**Incubation temperature (°C)**	**Incubation time (h)**
0.1 x Tryptic soy agar	Difco 236950	1.2% Bacto agar, Difco 214010	Heterotrophic bacterial plate count	25	72
Crystal-violet neutral-red bile glucose agar	Merck 1.10275.0500	-	Enterobacteriaceae	37	18
Lysogenic broth agar	LB, L3022, solidified with 15% BactoAgar, DIFCO 214010	0.2% ampicillin; 100 µg * mL^{-1} arabinose	*E. coli* O157:H7 gfp+[1]	37	18
Salmonella Chromogenic agar base	Oxoid CM1007	Salmonella selective supplement, Oxoid SR0194E	*Salmonella* spp.	37	24
Harlequin Listeria chromogenic agar	LAB HAL010	HAL010 Listeria selective diagnostic supplement with cyclohexamide LAB X010; Polymyxin B, ceftazidime LAB X072	*Listeria* spp., *L. monocytogenes*	37	24

[1]Viable counts enumerated under UV-light

Table 2. Total nitrogen (N_{tot}), nitrate (NO_3^-) and ammonia (NH_4^+) content in organic fertilizers (pig hair pellets: PHP, chicken manure: CM) applied to rocket and Swiss chard. DM = dry matter

		Rocket	**Swiss chard**
N_{tot} [mg 100 g^{-1} DM]	PHP	14267	14300
	CM	3000	2810
NO_3^- [mg 100g^{-1} DM]	PHP	0.63	0.07
	CM	1.75	0.12
NH_4^+ [mg 100g^{-1} DM]	PHP	152	14
	CM	676	634

Table 3. Risk measured as probability of infection with *E. coli* O157:H7 assuming a 30 g portion of rocket and Swiss chard is consumed. Crops were fertilized with pig hair pellets (PHP) or chicken manure (CM), or left untreated (control). Leaves were spray-inoculated with *E. coli* O157:H7. Risks are given as averages.

	Risk (probability of infection) per portion 30 g salad	
	Rocket	**Swiss chard**
Control	0.60	1
PHP	0.63	1
CM	0.6	1

Figure legends

Figure 1. Comparison of total nitrogen content (N_{tot}), nitrate (NO_3-N) and ammonia (NH_4-N) on the day of harvest in growth medium of rocket baby leaves (A, B, C) and Swiss chard baby leaves (D, E, F) for different organic fertilizer treatments. Growth medium was supplemented with pig hair pellets (PHP) or chicken manure (CM) to an amount of 81 kg readily available N ha^{-1}, while the control did not receive any fertilizer. Bars within diagrams marked with different letters are significantly different according to ANOVA (Tukey-test, $p<0.05$).

Figure 2. Comparison of bacterial loads (HPC: Heterotrophic bacterial plate count, LB: *E. coli* O157:H7 gfp+, VRBD: Enterobactericeae) on rocket baby leaves (A) and Swiss chard baby leaves (B) for different organic fertilizer treatments. Plants received pig hair pellets (PHP) or chicken manure (CM) to an amount of 81 kg readily available N ha^{-1}, while the control did not receive any fertilizer. Bars for each medium with different letters are significantly different according to ANOVA (Tukey-test, $p<0.05$).

Figure 3. Phylogenetic tree based on analysis of 16S rRNA gene sequences retrieved from isolates from the leaf surface of rocket cultured in the greenhouse and fertilized with chicken manure (CM) or pig hair pellets (PHP). Control treatments did not receive any fertilizer. The isolates were collected from nutrient agar for selective for *Enterobacteriaceae* (violet red bile dextrose agar).

Figure 4. Phylogenetic tree based on analysis of 16S rRNA gene sequences retrieved from isolates from the leaf surface of Swiss chard cultured in the greenhouse and fertilized with chicken manure (CM) or pig hair pellets (PHP). Control treatments did not receive any fertilizer. The isolates were collected from nutrient agar selective for Enterobacteriaceae (violet red bile dextrose agar).

Figure 5. Principal component (PC) analysis based on nitrogen content on the day of harvest in leaves and microbial loads on leaves (determined by culture-dependent methods for heterotrophic bacterial plate count,

E. coli O157:H7 gfp+, Enterobacteriaceae, presumptive *Salmonella* spp., and presumptive *Listeria* spp.) of baby leaf rocket (filled symbols) and Swiss chard (open symbols) exposed to different fertilizer regimes: ○ = control, □ = pig hair pellets, ◊ = chicken manure).

Figure 6. Scatterplots for total nitrogen (TN) content in Swiss chard and rocket leaves [mg g^{-1} fresh weight (FW)] and epiphytic bacteria [log CFU g^{-1} FW]. Plants were spray-inoculated with *E. coli* O157:H7. HPC: heterotrophic bacterial plate count.

Figures

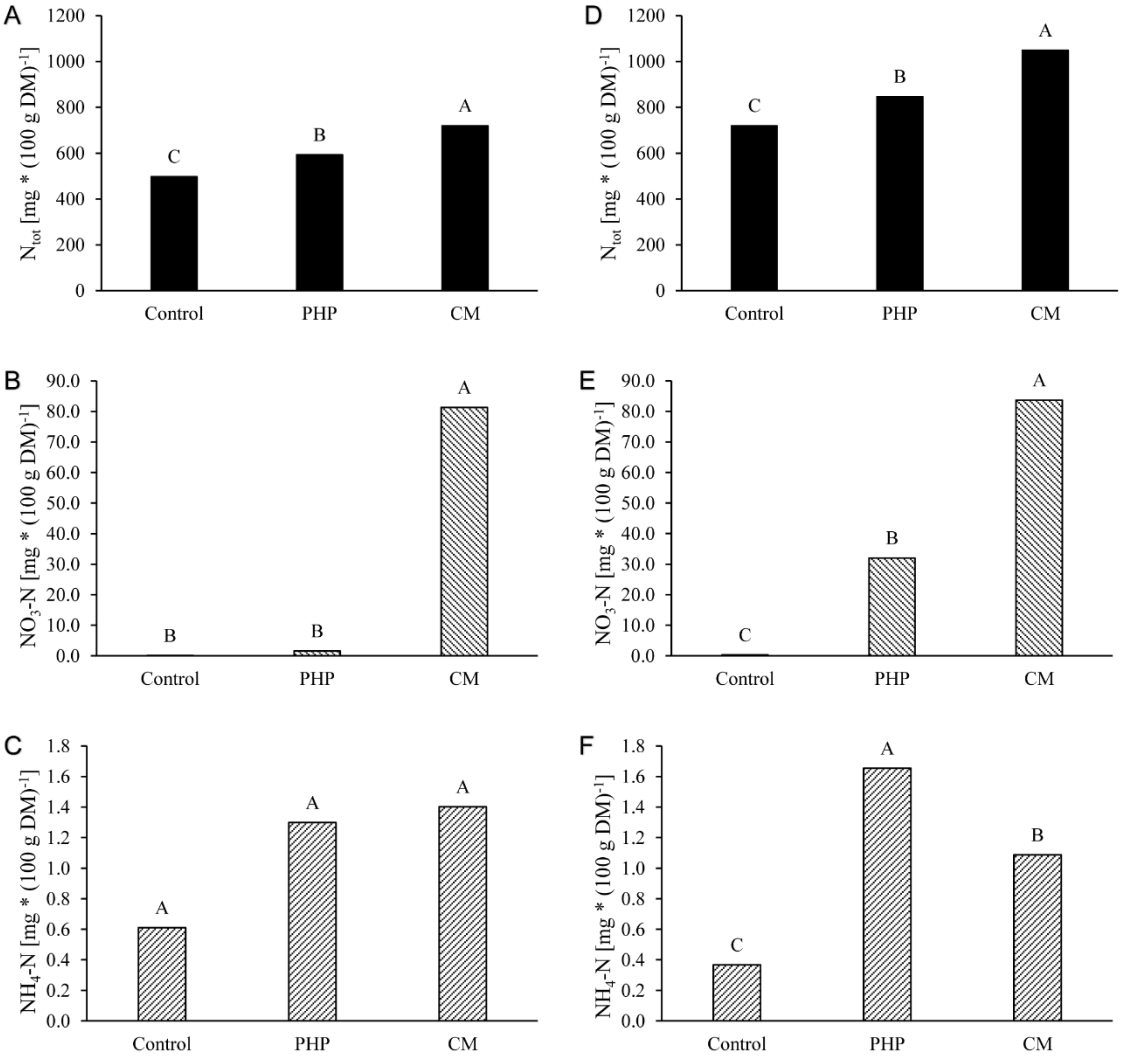
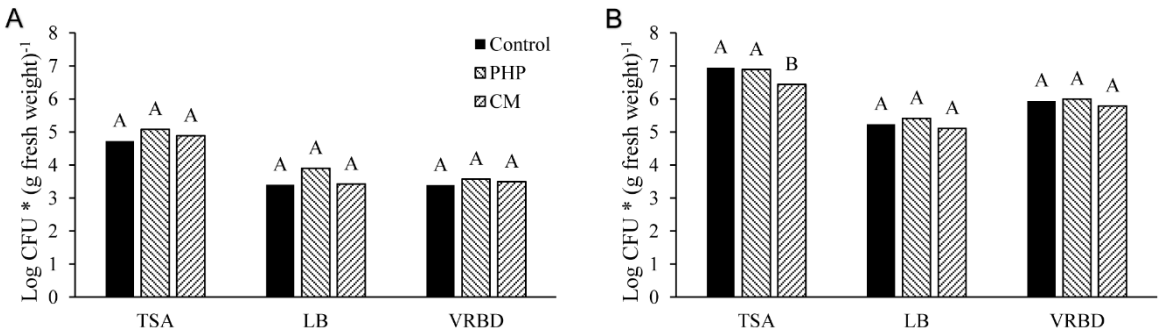


Figure 1



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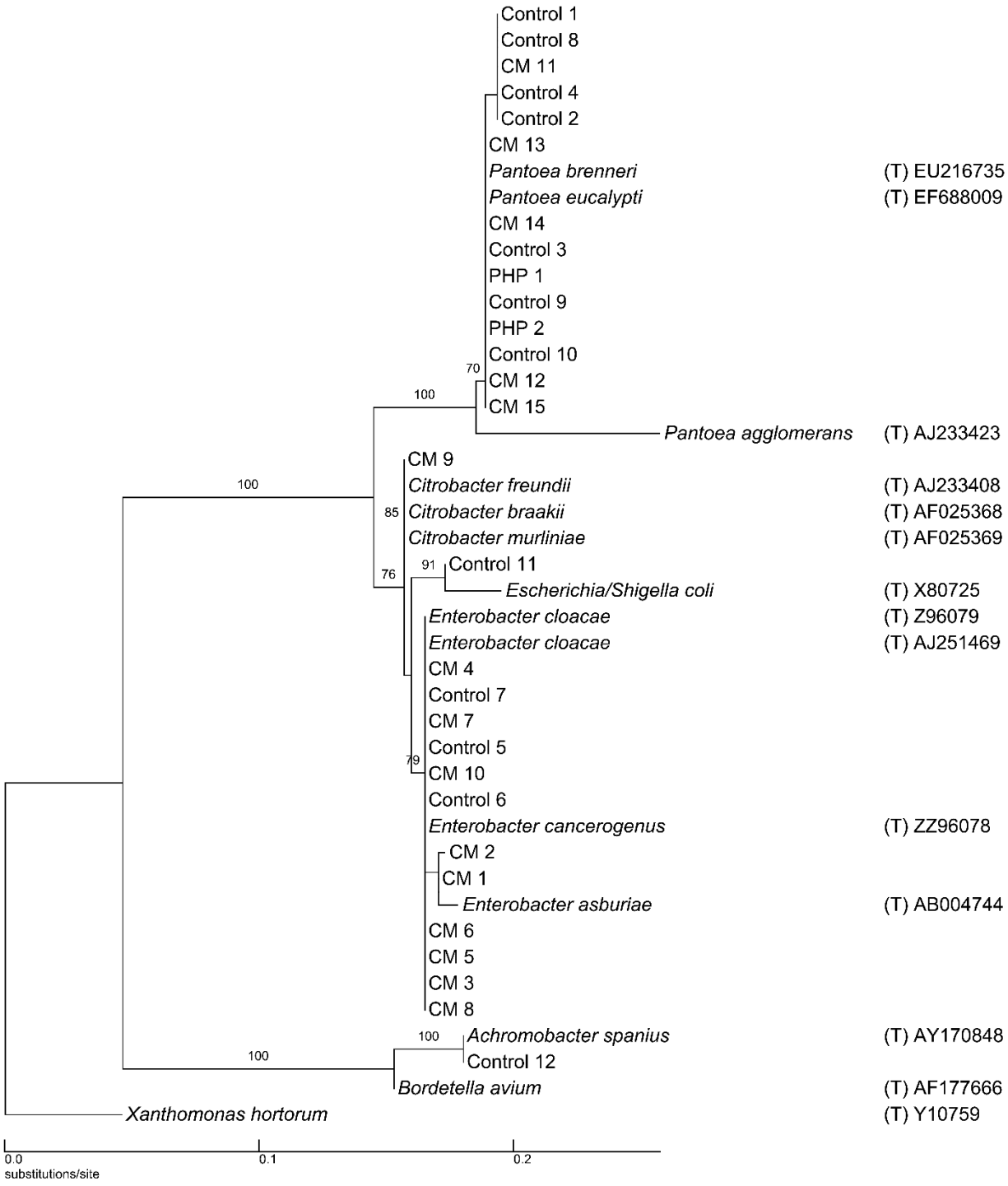
675 Figure 2

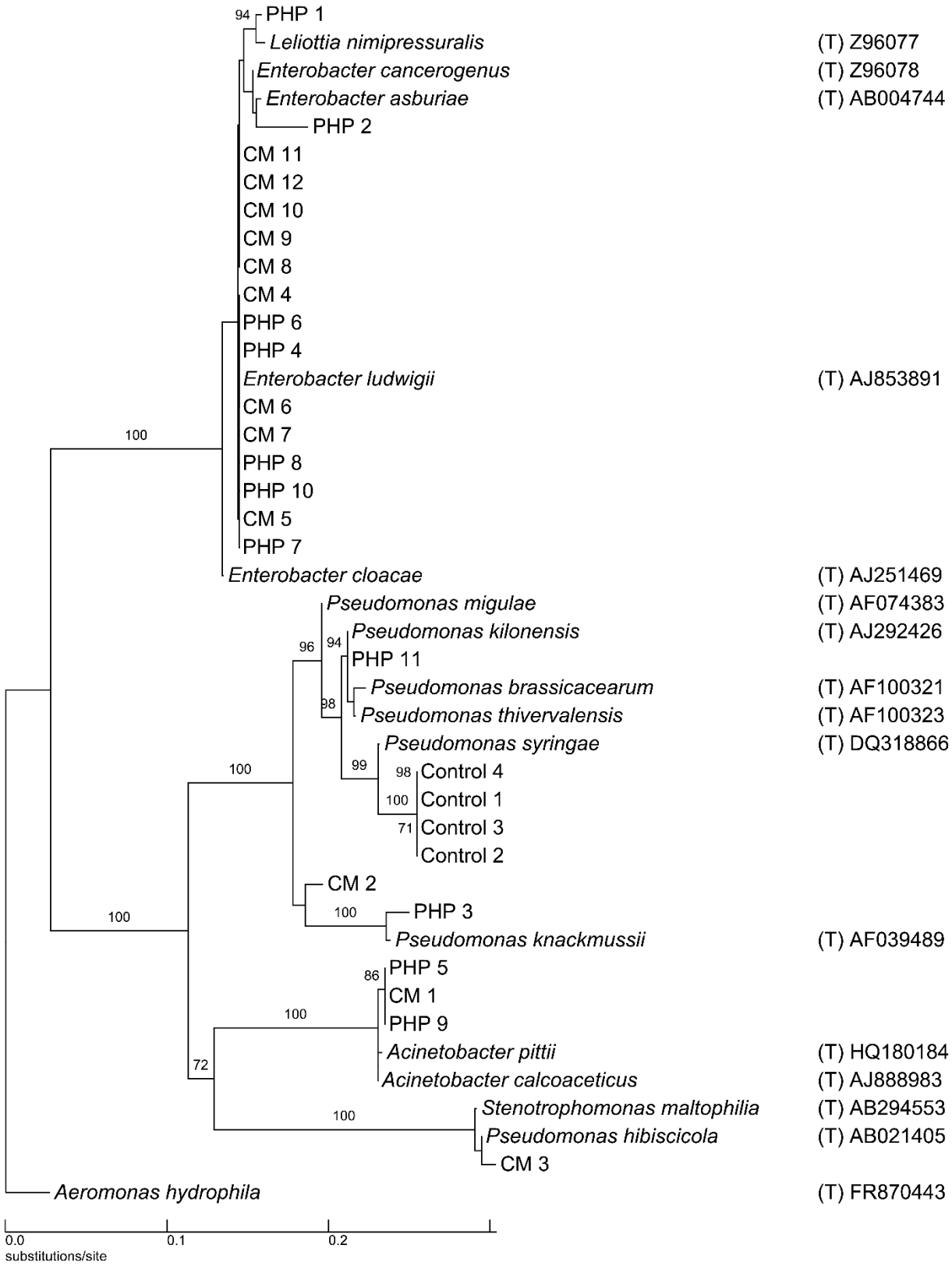
676



677

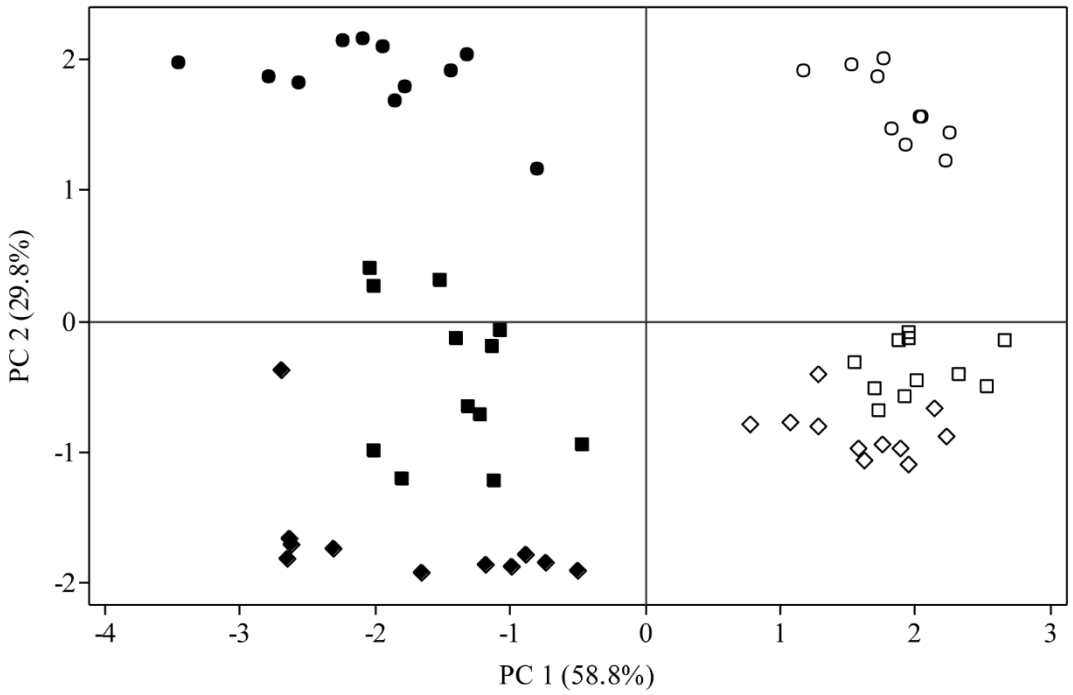
678 Figure 3

679



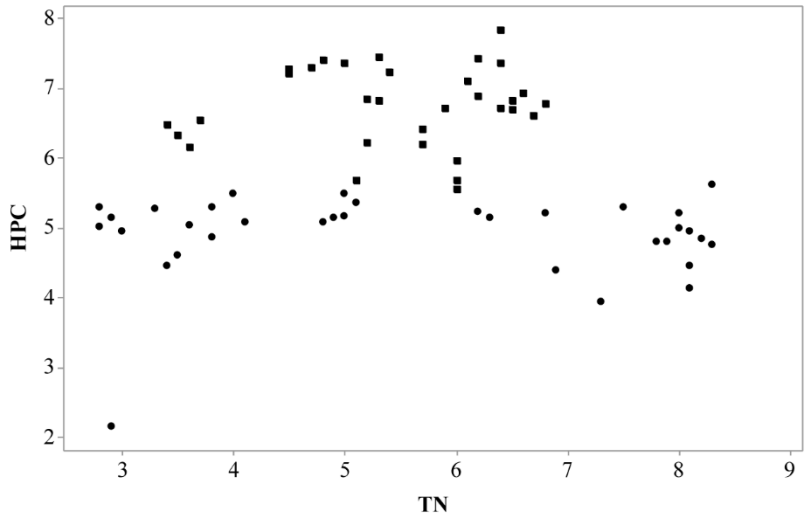
680

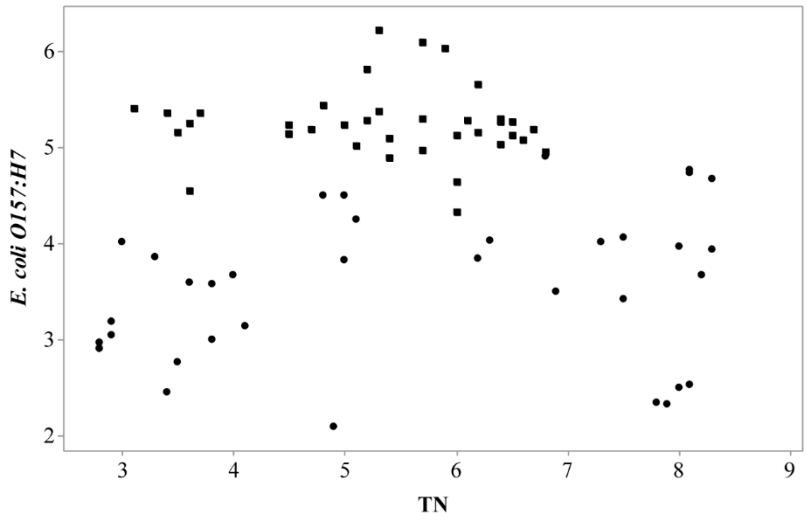
681 Figure 4

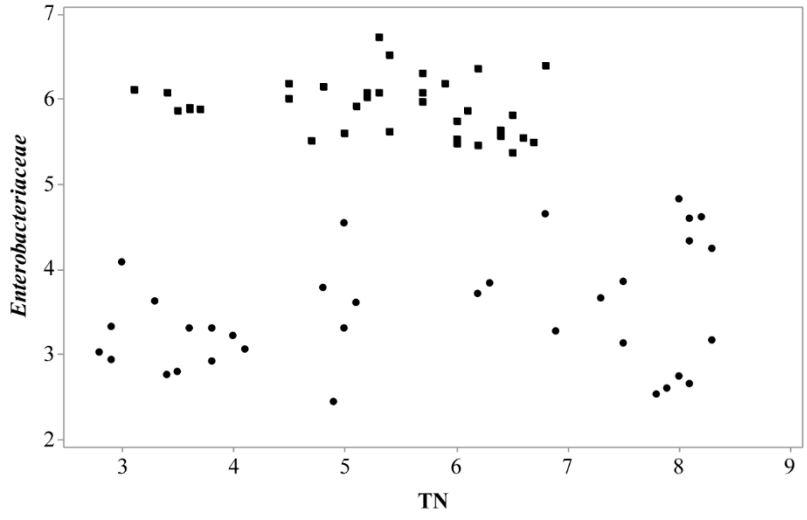


682

683 Figure 5







684 Figure 6