Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Dynamics of culturable mesophilic bacterial communities of three fresh herbs and their production environment

M.-T. Gekenidis[1,2], D. Gossin[1], M. Schmelcher[2], U. Schöner[3], M.N.P. Remus-Emsermann[1,4,5] and D. Drissner[1]

1 Microbiology of Plant Foods, Agroscope, Waedenswil, Switzerland
2 Institute of Food, Nutrition and Health, ETH Zurich, Zurich, Switzerland
3 Mäder Kräuter, Boppelsen, Switzerland
4 School of Biological Sciences, University of Canterbury, Christchurch, New Zealand
5 Biomolecular Interaction Centre, University of Canterbury, Christchurch, New Zealand

Keywords
agriculture, food, microbial contamination, soil, water.

Correspondence
David Drissner, Microbiology of Plant Foods, Agroscope, Schloss 1, 8820 Waedenswil, Switzerland.
E-mail: david.drissner@agroscope.admin.ch
Mitja N.P. Remus-Emsermann, School of Biological Sciences, University of Canterbury, Private Bag 4800, 8140 Christchurch, New Zealand.
E-mail: mitja.remus-emsermann@canterbury.ac.nz

2017/1097: received 29 March 2017, revised 6 June 2017 and accepted 6 July 2017

doi:10.1111/jam.13532

Abstract

Aim: Investigate dynamics of culturable mesophilic bacteria and selected food-contaminating bacteria from three herbs and their production environment.
Methods and Results: Marjoram, basil and thyme were investigated during one growing season by sampling plants, organic fertilizers, soil, irrigation water and marketed products. Mesophilic bacteria and selected food-contaminating bacteria (*Escherichia coli*, *Enterococcus* spp., *Bacillus cereus* group) were cultured and identified by MALDI biotyping. Culturable mesophilic bacteria on marjoram and basil plants decreased over time by two orders of magnitude starting at above 10^6 colony forming units per gram (CFU per g), while they remained constant on thyme (~10^4 CFU per g). Compared to the last field sample, mesophilic bacteria were increased on all market-ready products by one order of magnitude. Marjoram and basil were dominated by *B. cereus* group, *Enterobacter* spp. and *Pseudomonas* spp., thyme by *Bacillus* spp. and *Pseudomonas* spp. All selected food-contaminating bacteria were detected in soil and reservoir-sourced irrigation water, whereas in municipal water, only *B. cereus* group and rarely *Enterococcus* spp. were found. *Escherichia coli* was detected only on young marjoram and basil plants (5×10^2 and 5×10^1 CFU per g, respectively), whereas *Enterococcus* spp. and *B. cereus* group were consistently detected on these two herbs. Thyme plants only contained *B. cereus* group consistently (above 10^3 CFU per g). Marketed marjoram and thyme contained *Enterococcus* spp. (5×10^2 and 10^4 CFU per g) and *B. cereus* group (~5×10^2 CFU per g), while no selected food-contaminating bacteria were found on marketed basil.
Conclusions: Overall, culturable mesophilic bacteria were dominated by *Pseudomonas* spp. and *Bacillus* spp., with increased numbers on market-ready products. Selected food-contaminating bacteria were readily detectable, however, only the *B. cereus* group was found throughout in all systems.
Significance and Impact of the Study: Insight into composition and development of mesophilic bacterial communities and selected food-contaminating bacteria of fresh herbs contributes to estimating consumer exposure.

Introduction

Fresh herbs are important food ingredients that are often minimally processed or used raw. Minimal or no processing allows the bacterial community of fresh herbs, including potential spoilage or pathogenic bacteria, to reach the consumer more easily than through processed or cooked foods. Recent studies have highlighted the

occurrence of antibiotic-resistant bacteria in foods of animal and plant origin including fresh produce (Pesavento *et al.* 2014; Nüesch-Inderbinen *et al.* 2015; Zogg *et al.* 2016) as well as in the environment including surface water (Czekalski *et al.* 2012), soil and manure (Jechalke *et al.* 2014). On the other hand, consumption of unprocessed plant foods has increased due to rising health awareness (Pollack 2001; Doyle and Erickson 2008; Berger *et al.* 2010). It has therefore become increasingly important to investigate the bacterial community of unprocessed plant foods such as fresh herbs. To date, little is known about the microbial community of fresh herbs and its development, whereas many studies have investigated properties of various fresh herb ingredients like essential oils or have focused on the plants' rhizosphere (Kapoor and Mukerji 2006; Tamilarasi *et al.* 2007; Bafana 2013; Solaiman and Anawar 2015). In order to understand the bacterial community development of fresh herbs' phyllosphere, it is important to analyse their production environment, which shapes the plant host's bacterial community (Knief *et al.* 2010). Fresh herbs are exposed to a broad range of potential contamination sources such as irrigation water, soil, manure or workers and their equipment (Drissner and Zuercher 2014), bringing undesired contaminating bacteria on edible plant parts.

Most commonly, it is the leaves or aerial organs of herbs that are consumed and thus these are the relevant plant organs potentially carrying contaminating bacteria. Aerial plant organs constitute the so-called phyllosphere, which is dominated by the leaves outmatching the surface area of the rest of a plant's aboveground surface by several orders of magnitude (Morris and Kinkel 2002). Leaves being light harvesting organs are an extreme environment for their colonizers due to high doses of DNA-damaging UV radiation. Additionally, leaves are covered by a waxy cuticle responsible for the generally oligotrophic (Vorholt 2012) and dry environment (Beattie 2011) of the phyllosphere. Nevertheless, bacteria successfully colonize this harsh environment (Beattie 2011; Vorholt 2012). Surprisingly, 10^7 or more bacterial colony forming units per gram (CFU per g) can regularly be found on healthy leaves, which can be explained by the large heterogeneity of the phyllosphere, which contains microhabitats with considerable amounts of nutrients (Remus-Emsermann *et al.* 2011, 2012) where bacteria can establish.

Food-contaminating bacteria and indicators for faecal contamination can be cultured on selective media. *Escherichia coli (E. coli)* and *Enterococcus* spp. are typical indicators for faecal contamination and microbial food quality (Anderson *et al.* 2005; Jay 2005; Savichtcheva and Okabe 2006; Pappas *et al.* 2008). They have further been included in models to predict the occurrence of bacterial pathogens (Jokinen *et al.* 2012; Frey *et al.* 2013) or, in the case of water quality assessment, have been used as surrogates for pathogens (Yates 2007; Marti *et al.* 2013; Allende and Monaghan 2015). Finally, although mainly commensals, both *E. coli* and *Enterococcus* spp. also comprise pathogenic strains (Gilmore and Ferretti 2003; Rasko *et al.* 2008). Notably, many enterobacterial and enterococcal species are plant associated and are generally considered harmless (Müller *et al.* 2001; Rastogi *et al.* 2012).

Irrigation water has been reported to be a source of contamination with undesired bacteria such as *E. coli* and *Enterococcus* spp. (Fonseca *et al.* 2011; Ijabadeniyi *et al.* 2011; Gupta and Madramootoo 2016). Another significant source of fresh produce contamination is soil (Olaimat and Holley 2012). Being typical soil inhabitants, bacteria of the *Bacillus cereus (B. cereus)* group are readily detected on fresh produce (Thunberg *et al.* 2002; Jackson *et al.* 2015). *Bacillus cereus* group members are known to endanger food safety by producing various toxins including the emetic toxin cereulide and several enterotoxins (Stenfors Arnesen *et al.* 2008). Although usually present at less than 10^3 CFU per g on herbs, they can multiply to numbers sufficient for food poisoning (10^5–10^6 CFU per g) in foods to which those herbs were added (Rajkovic *et al.* 2008; Sagoo *et al.* 2009). Occasionally, the *B. cereus* group is detected in amounts exceeding 10^4 CFU per g herbs, leading to a notification by governmental authorities such as the European Commission (Kleter *et al.* 2009) with subsequent border rejection, withdrawal from the market or recall from the consumers.

In this study, we aimed to describe the temporal development of the culturable mesophilic bacterial communities of three fresh herbs (marjoram, basil and thyme) grown under different conditions to detect the highest possible diversity, as well as of potential contamination sources from their production environment (irrigation water, soil and fertilizers). Apart from culturable mesophilic bacteria, we investigated the prevalence and development of *E. coli*, *Enterococcus* spp. and the *B. cereus* group, representing indicators for faecal contamination or comprising food-spoiling bacteria and/or potential human pathogens. The study was conducted on an organically managed farm. As opposed to conventional farming, slightly increased numbers in total aerobic bacteria, *Enterobacteriaceae* and coliforms have been attributed to organic farming practices (Wießner *et al.* 2009), and higher prevalence of *Proteobacteria* (including *Enterobacteriaceae* and *E. coli* in particular) has been shown in organically cultivated soils (Chaudhry *et al.* 2012; Li *et al.* 2012). More specifically for basil, it has been shown that organically grown plants have a higher microbial diversity than conventionally grown plants (Wetzel *et al.* 2010). In

our survey, numbers of total mesophilic bacteria and the bacterial target groups were monitored during a complete growing season from seed material over seedlings to harvest and market product. The three herbs were grown under different conditions: marjoram was greenhouse-grown and irrigated with municipal tap water; basil was greenhouse-grown but irrigated with water from an open-top reservoir; thyme was field-grown and not irrigated. Besides using a nonselective medium to determine total numbers of mesophilic bacteria as an estimation for total culturable bacteria, the abundance of the targeted bacteria was determined using selective media. Representative colonies were identified using MALDI biotyping and 16S amplicon sequencing in order to gain an insight into the diversity of the cultured mesophilic communities.

Materials and methods

Bacterial growth conditions

Bacteria were plated on four media: nonselective plate count agar (PCA; Merck, Darmstadt, Germany) to enumerate total mesophilic bacteria, BACARA (BCR; BioMérieux, Marcy-l'Étoile, France), a medium selective for the *B. cereus* group, m-Enterococcus agar (mEA; Sigma-Aldrich, St. Louis, MO), selective for presumptive *Enterococcus* spp. and tryptone bile x-glucuronide agar (TBX; Sigma-Aldrich), selective for *E. coli* strains, which form blue colonies on TBX. PCA plates were incubated for 72 h at 21°C, BCR plates for 18 h at 30°C, mEA plates for 42 h at 37°C and TBX plates for 18 h at 44°C before enumeration. TBX sometimes yielded, besides blue *E. coli* colonies, uncoloured colonies of nontarget bacteria, in which case both blue colonies and white colonies were enumerated separately. To propagate isolated bacteria for colony-PCR or MALDI biotyping (see below), either Lysogeny broth agar (LB; Sigma-Aldrich) or PCA were used and the plates were incubated at 21°C until bacterial growth was observed.

Field and growth conditions

Seed, herb, soil and water samples were collected on an organically managed farm near Zurich (Mäder Kräuter, Boppelsen), Switzerland, certified according to Bio Suisse standards (2015). After application of organic fertilizers, irrigation hoses were placed 10 cm underground. Plant beds were covered with an organic foil to minimize contact of soil with seedlings that were planted using an automated planter in four parallel files per bed. Overhead irrigation in greenhouses was applied for the first 2 weeks after planting (three to four times per week) and drip irrigation for the remaining growth period (twice per week). Herbs were harvested manually before 9 AM, cooled to 3–5°C (marjoram, thyme) or 10–12°C (basil) and packaged the next day after minimal processing, that is, sorting and trimming but no washing, to be sent to the retailers without interrupting the cold chain. The average crop yield of the fields was 0·35, 0·40 and 0·45 kg m^{-2} for marjoram, basil and thyme, respectively.

Field sampling

Sampling was performed during the growing season of summer 2014 (June–August). Two greenhouse sites planted with marjoram (*Origanum vulgare* L.) or basil (*Ocimum basilicum* L.) were sampled every 2 weeks during the whole period of their growth. Each plant species was sampled four times. A field site planted with thyme (*Thymus vulgaris* L.) was sampled every 2 weeks until harvest. Thyme was sampled three times. The greenhouses covered an area of 600 m^2 (4 beds) and 1000 m^2 (6 beds) for marjoram and basil, respectively. In the open field, five beds of thyme covered an area of 550 m^2. One bed was selected per plant species (length: 60–75 m, area: 110–167 m^2) for collection of bulk soil or plant material as described in the following.

Seeds

Two samples of 10 g of seeds for each herb were placed in 100 ml peptone water (9 g l^{-1} NaCl, 1 g l^{-1} tryptone; Merck) in dual-chamber stomacher bags and processed in a stomacher (Smasher; AES Chemunex, Combourg, France) for 3 min. Ten-fold dilutions of the resulting supernatant were prepared and plated on PCA, BCR, mEA or TBX agar. Additionally, 200 μl of undiluted supernatant was plated on the same media in order to lower the limit of detection to 5×10^1 CFU per g.

Plant material

During each sampling, the bed chosen for each herb was randomly sampled eight times by collecting at least 30 g of plant material in a sterile plastic bag where it was carefully mixed. Samples were transported at approximately 8°C and were processed within 6 h. Plant material from the end of the growing season, that is, postharvest material, was harvested and packaged by farm workers, shipped and stored at 4°C (marjoram, thyme) or 10°C (basil), and analysed within the shelf life defined for each herb (6, 5 and 8 days for marjoram, basil and thyme, respectively). One postharvest thyme sample and two postharvest samples of basil and marjoram were analysed. For bacterial enumeration and isolation, 10 g of plant material were weighed into a stomacher bag with 100 ml peptone water and processed in a stomacher for 3 min.

To determine bacterial numbers, a 10-fold dilution series of the resulting supernatant was plated as described above.

Fertilizers and soil

Samples from the organic fertilizers, that is, horn-shavings and organic nitrogen fertilizer (Hauert, Grossaffoltern, Switzerland) as well as granulated fungal biomass (Sandoz, Holzkirchen, Germany) were analysed. Of each fertilizer, 10 g were weighed into a stomacher bag with 100 ml peptone water and processed in a stomacher for 3 min. To determine bacterial numbers, a 10-fold dilution series of the resulting supernatant was plated as described above. For soil, eight samples were randomly collected from the selected bed at each time point, each consisting of four soil cores (2·5 cm diameter, 10 cm length). Samples were transported at approximately 8°C and were processed within 6 h. Homogenized soil samples (10 g each) were mixed to a slurry with 100 ml peptone water on a shaker for 20 min at 250 rev min^{-1}. A 10-fold dilution series of the resulting supernatant was plated as described above. To determine soil dry weight, soil aliquots were weighed into aluminium containers, dried over night at 180°C and weighed again.

Irrigation water

The greenhouse harbouring the marjoram culture was supplied with tap water; basil was irrigated using water from an open-top reservoir, which was primarily sourced by rain and surface water collected from the greenhouse roofs and supplemented with groundwater whenever needed. Before reaching the greenhouse site, reservoir water was filtered using a particle filter (F-600 Gravel Filter; Netafim, Tel Aviv, Israel). Per basil sampling, one sample was taken from the open-top reservoir and one from the field inlet; per marjoram sampling one field inlet sample was taken. Additionally for each sampling, two or four samples were taken from overhead sprinklers or from drip-irrigation hoses, respectively. In greenhouses, the water irrigation system consisted of four belowground drip-irrigation hoses per bed and one overhead irrigation sprinkler system, featuring 30 sprinkler nozzles, parallel to the planting bedding. Water from the first and the last overhead sprinkler nozzle were sampled. The four belowground irrigation hoses of the selected bed were sampled at the end of the bed. Samples were transported at approximately 8°C and were processed within 6 h. For each of the four media, 200 ml of water was filtered through a 0·45-μm filter as described in the Swiss food register (Swiss Federal Office of Public Health 2009). Filters were placed onto respective media and additionally, a 10-fold dilution series of the initial water sample was plated onto all four media.

MALDI biotyping

MALDI biotyping was performed as previously described (Gekenidis *et al.* 2014). Briefly, representative colonies were streaked onto fresh LB agar and PCA. From nonselective PCA, on which a large bacterial diversity was expected, 32 colonies were randomly picked for each sampling per habitat, that is, plant material, soil or water (96 isolates per sampling time point). Special attention was paid to picking as many different colony morphotypes as possible. From selective media (TBX, mEA and BCR) up to 10 colonies were picked per sampling and habitat. From TBX, in addition to blue coloured colonies, 10 nonblue coloured colonies were picked. After incubation, either direct smearing or protein extraction from the freshly grown colonies was performed (Gekenidis *et al.* 2014), whenever the direct smearing did not yield satisfactory results. It is noteworthy, that classical MALDI biotyping is limited in discriminating species belonging to the *B. cereus* group (Pfrunder *et al.* 2016) and therefore the composition of the *B. cereus* group could not be resolved. For MALDI-TOF mass spectrometric measurements, 1 μl of each protein extract was spotted onto a MALDI target, left to dry and covered with 1·2 μl of matrix solution (10 mg ml^{-1} α-cyano-4-hydroxycinnamic acid (Bruker Daltonics, Bremen, Germany) in acetonitrile-water-trifluoroacetic acid (50 : 47·5 : 2·5 (vol/vol/vol), Sigma-Aldrich). Sample spectra were acquired using a microflex LT MALDI-TOF mass spectrometer (Bruker Daltonics) and the flexControl software (ver. 3.4; Bruker Daltonics). Spectra were classified using the MALDI BioTyper software (ver. 3.1; Bruker Daltonics) and the Bruker Taxonomy main spectra database (ver. 4.0.0.1).

16S amplicon sequencing

To prepare a crude bacterial lysate, colony material of individual colonies was transferred to 100 μl of 0·05 M NaOH (Sigma-Aldrich) and incubated for 10 min at 99°C. Cell debris was spun down for 1 min at 11,000 × ***g***. Of this crude lysate 0·5 μl were used as template for a PCR to amplify the isolates' 16S rRNA gene using the universal primers 27F and 1492R (5′-AGA GTT TGA TCM TGG CTC AG-3′ and 5′-CGG TTA CCT TGT TAC GAC TT-3′, respectively) and the Qiagen HotStart Taq Mastermix kit (Qiagen, Hilden, Germany) according to the recommendations of the manufacturer. Amplification was performed in 30 cycles (denaturation at 94°C for 30 s, annealing at 47°C for 30 s and elongation at 72°C for 40 s) with a final extension at 72°C for 10 min. Amplified gene fragments were cleaned up using the InnuPREP PCRpure Kit (Analytic

Jena, Jena, Germany) according to the manufacturer's recommendations. Sanger sequencing was conducted using the universal 27F primer (Microsynth, Schlieren, Switzerland). Sequences were classified using BLASTN (Altschul *et al.* 1997) with an average sequence length of 1012 bp (minimal length: 230 bp; maximal length: 1783 bp).

Statistical analysis

For data display, the mean bacterial cell numbers were plotted, with dots indicating the cell number of each sample (Figs 1–3). ANOVA was performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA) for plant and soil data on log transformed data (Kilsby and Pugh 1981; Hirano *et al.* 1982) and replacing each value below the limit of detection with the limit of detection of the measurement (5×10^1 CFU per g) (Lorimer and Kiermeier 2007). Additionally, Tukey's multiple comparison test was applied. Temporal changes of the cell numbers of each bacterial group were evaluated per plant species and habitat (soil or plant material) and significant changes ($P \leq 0{\cdot}01$) are reported.

Results

To avoid iterations, the temporal development of mesophilic and target bacteria are presented by fertilizer, soil, water and herbs. The reader should be aware, however, that no direct comparison of bacterial numbers on marjoram, basil and thyme should be attempted, since the three herbs were grown under different conditions in order to detect the highest possible diversity. Of note, all colonies from mEA agar were enumerated and displayed in Figs 1–3 (presumptive *Enterococcus* spp.), since MALDI biotyping revealed that on average about 82% (231 out of 282) of mEA colonies were *Enterococcus* spp. (78, 69 and 96% for plant, soil and water isolates, respectively). For the sake of brevity, presumptive *Enterococcus* spp. are called *Enterococcus* spp. in the present work. Notably, since mEA is usually applied for detection of *Enterococcus* spp. via membrane filtration of water, the medium is less selective for more heavily contaminated samples. Nevertheless, *Enterococcus* spp. was the main genus isolated from plant and soil using mEA (78 and 69%, respectively).

Organic fertilizers

The three organic fertilizers analysed as the potential source of bacterial contamination did not contain the target bacteria *E. coli*, *Enterococcus* spp. or *B. cereus* group members above the limit of detection.

Soil

Total mesophilic bacteria isolated from soil on PCA ranged between approximately 10^6 and 10^7 CFU per g dry weight throughout the study, independent of the sampling site (Figs 1b, 2b and 3b for marjoram, basil and thyme, respectively). Significant changes were observed only from week 4–6 at the marjoram site ($P \leq 0{\cdot}001$), and week 2–4 and 4–6 at the basil site ($P \leq 0{\cdot}01$ and $\leq 0{\cdot}0001$, respectively).

Colonies growing on selective media showed more fluctuating patterns over the growing season. *Bacillus cereus* group members made up a substantial part of all soil samples, accounting for around 10% of the total mesophilic cell numbers (exceptions: marjoram site at week 2 and 6; basil site at week 6). However, at the marjoram site the numbers fluctuated significantly (week 0–2 and 4–6, $P \leq 0{\cdot}001$). At the basil site, *B. cereus* group members were significantly decreased only in the last sampling ($P \leq 0{\cdot}0001$, Fig. 2b).

Escherichia coli was detected at all sites early after planting of the respective herbs at ~10^3 CFU per g dry weight (marjoram and basil site) or 10^4 CFU per g dry weight (thyme site), while they were below the limit of detection in most later soil samples. Nonblue coloured colonies on TBX that were subsequently identified by MALDI biotyping, were more consistently detected independently of the site, although significant fluctuations were observed (marjoram site: week 0–2 ($P \leq 0{\cdot}01$); basil site: week 0–2 and 4–6 ($P \leq 0{\cdot}001$), week 2–4 ($P \leq 0{\cdot}0001$); thyme site: week 0–6 ($P \leq 0{\cdot}0001$)).

Enterococcus spp. were consistently detected throughout the growing season, although after week 0 numbers decreased (marjoram and basil site: week 0–2, $P \leq 0{\cdot}0001$) and *Enterococcus* spp. remained rare (detected in one to four of eight replicate soil samples). Only in the last basil sampling, *Enterococcus* spp. could not be detected in any of the replicate soil samples (Fig. 2b).

Irrigation water

A comparison of field inlet water sourced by either municipal tap water (marjoram) or an open-top reservoir (basil) did not reveal strong differences (Figs 1c and 2c, respectively). Total mesophilic numbers were low (~10^0 CFU per ml), *B. cereus* group members around 10^{-1} CFU per ml, *E. coli* was not detectable and *Enterococcus* spp. were detected once in the field inlet water of the basil greenhouse at the limit of detection (1 CFU in 200 ml). However, after the water passed through the on-field irrigation systems, that is, overhead sprinklers or belowground hoses, bacterial numbers and community composition changed (Figs 1d and 2d for marjoram and

  Journal of Applied Microbiology **123**, 916–932 published by John Wiley & Sons Ltd on behalf of The Society for Applied Microbiology.

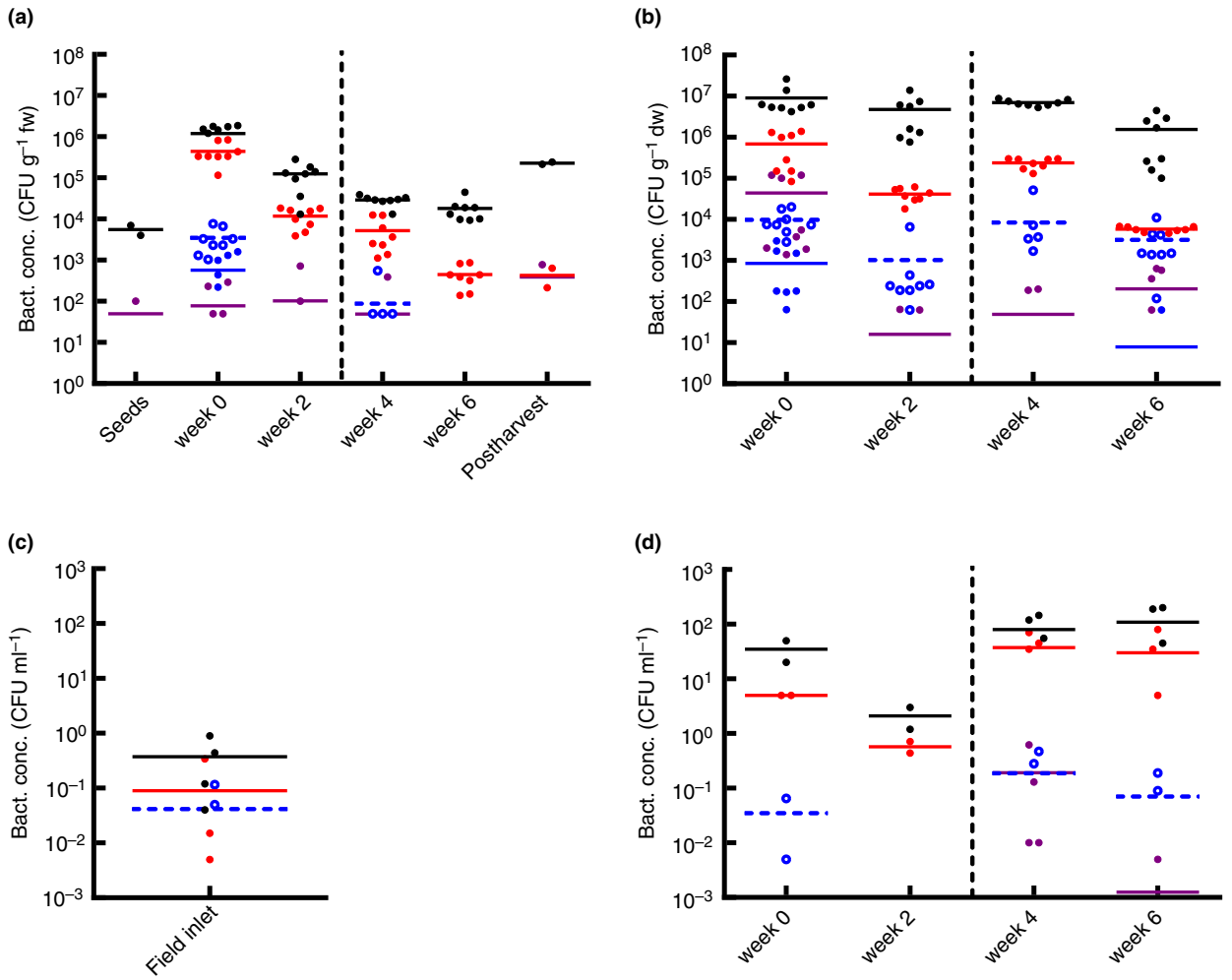

Figure 1 Population development of total mesophilic bacteria and different targeted bacteria at the marjoram site in plant material (a), soil (b), field inlet water (c) and overhead sprinkler and belowground hose water (d). Every symbol represents the CFU count of an individual sample. The number of samples analysed per time point were eight for soil or field-grown plants, two for seeds, two for postharvest material, one for field inlet water, two for overhead sprinkler water and four for belowground hose water (limit of detection = 5×10^1 CFU per g for plant and soil, 1 CFU in 200 ml for water). Coloured lines represent average CFU counts. Replicate samples below the limit of detection were assigned a zero, and the average was calculated from all samples. Closed black circle = PCA medium, total mesophilic count; closed red circle = BCR medium, *Bacillus cereus* group; closed blue circle = TBX medium, *E. coli*; open blue circle = TBX medium, background colonies; closed purple circle = mEA medium, presumptive *Enterococcus* spp.; bact. conc.: bacterial concentration; fw: fresh weight; dw: dry weight. Vertical dotted lines depict the change in irrigation pattern from overhead to belowground irrigation. [Colour figure can be viewed at wileyonlinelibrary.com]

basil site, respectively). The number of total mesophilic bacteria and *B. cereus* group members was increased in sprinkler water of both sites by at least one order of magnitude. Sprinkler water of the basil site contained increased numbers of *Enterococcus* spp. as compared to the field inlet, and *E. coli* and TBX background bacteria were detectable. Notably, water sampled directly from the open-top reservoir also contained detectable amounts of *E. coli* and *Enterococcus* spp. (Fig. 2c). Water from belowground hoses exhibited on average higher numbers of mesophilic bacteria and *B. cereus* group bacteria than the respective sprinkler water (Figs 1d and 2d for marjoram and basil site, respectively). Additionally, *Enterococcus* spp. were detected at the marjoram site in hose water, while *E. coli*, TBX background colonies and *Enterococcus* spp. were isolated at the basil site from hose water, as they were already from the respective sprinkler water.

The most notable differences between the two irrigation water sources were (i) *E. coli* was present in the water sourced from the open-top reservoir while it could not be detected at any time point in municipality-sourced water and (ii) *Enterococcus* spp. were found in both

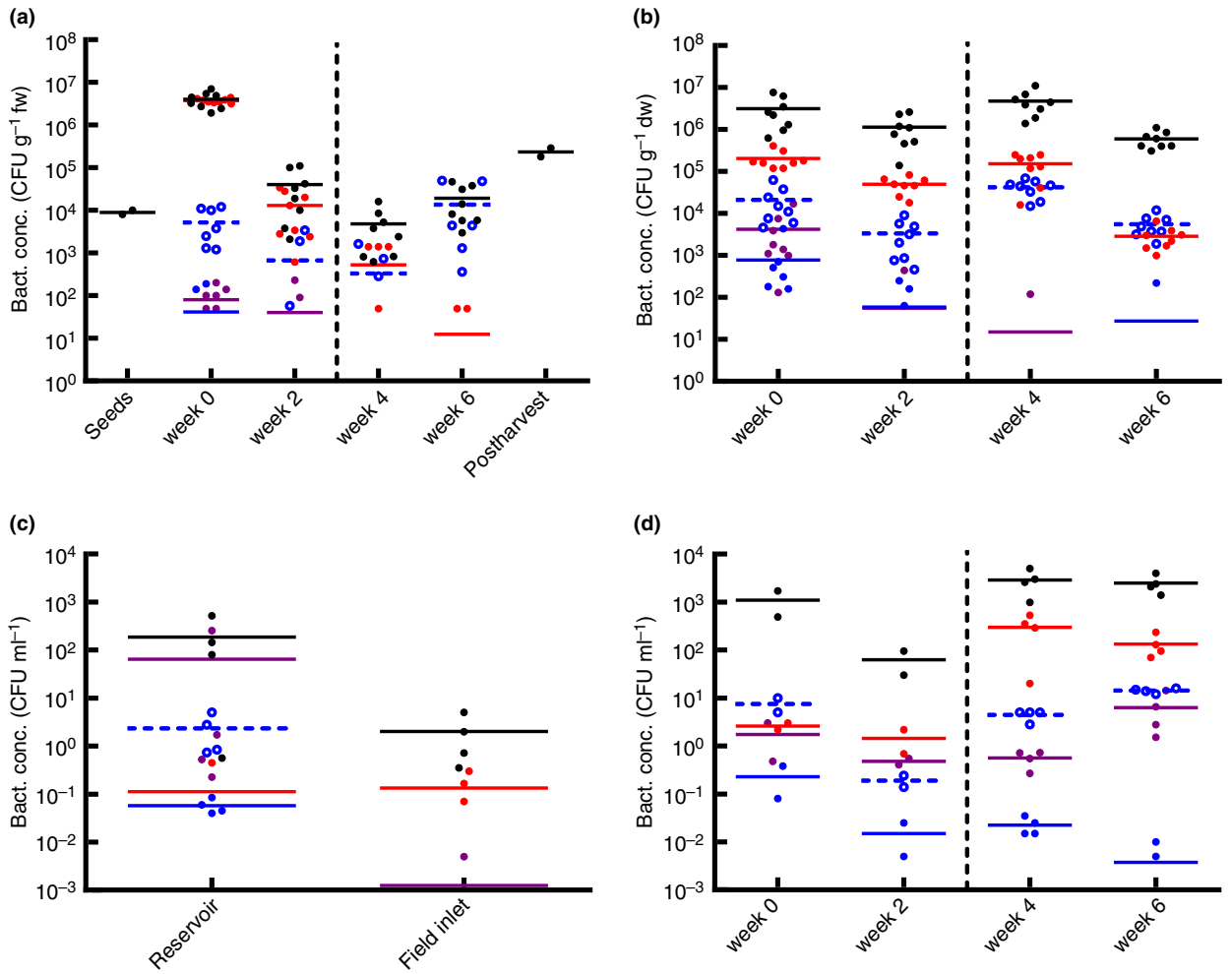

Figure 2 Population development of total mesophilic bacteria and different targeted bacteria at the basil site in plant material (a), soil (b), open-top reservoir and field inlet water (c) and overhead sprinkler and belowground hose water (d). Every symbol represents the CFU count of an individual sample. The number of samples analysed per time point were eight for soil or field-grown plants, two for seeds, two for postharvest material, one for open-top reservoir and field inlet water, two for overhead sprinkler water and four for belowground hose water (limit of detection = 5×10^1 CFU per g for plant and soil, 1 CFU in 200 ml for water). Coloured lines represent average CFU counts. Replicate samples below the limit of detection were assigned a zero and the average was calculated from all samples. Closed black circle = PCA medium, total mesophilic count; closed red circle = BCR medium, *Bacillus cereus* group; closed blue circle = TBX medium, *Escherichia coli*; open blue circle = TBX medium, background colonies; closed purple circle = mEA medium, presumptive *Enterococcus* spp.; bact. conc., bacterial concentration; fw, fresh weight; dw: dry weight. Vertical dotted lines depict the change in irrigation pattern from overhead to belowground irrigation. [Colour figure can be viewed at wileyonlinelibrary.com]

sprinkler and underground hose water at the basil site, while they were absent from the sprinkler water of the municipality-sourced marjoram site.

Herbs

Marjoram, basil and thyme seeds exhibited 6×10^3, 9×10^3 and 5×10^4 total mesophilic CFU per g, respectively (Figs 1a, 2a and 3a). From the bacterial target groups, only *Enterococcus* spp. were detected in one of the two analysed marjoram seed samples.

Freshly planted marjoram and basil seedlings harboured on average 10^6 and 4×10^6 total mesophilic CFU per g, respectively (week 0 in Figs 1a and 2a, respectively). On both marjoram and basil, total mesophilic numbers decreased significantly over the growing season (marjoram and basil: week 0–2 ($P \leq 0{\cdot}0001$), week 2–4 ($P \leq 0{\cdot}01$)). *Bacillus cereus* group members constituted a large proportion of the mesophilic community on marjoram and basil seedlings contributing to almost 100% culturable mesophilic bacteria in the case of basil. *Bacillus cereus* group numbers decreased as the plants grew

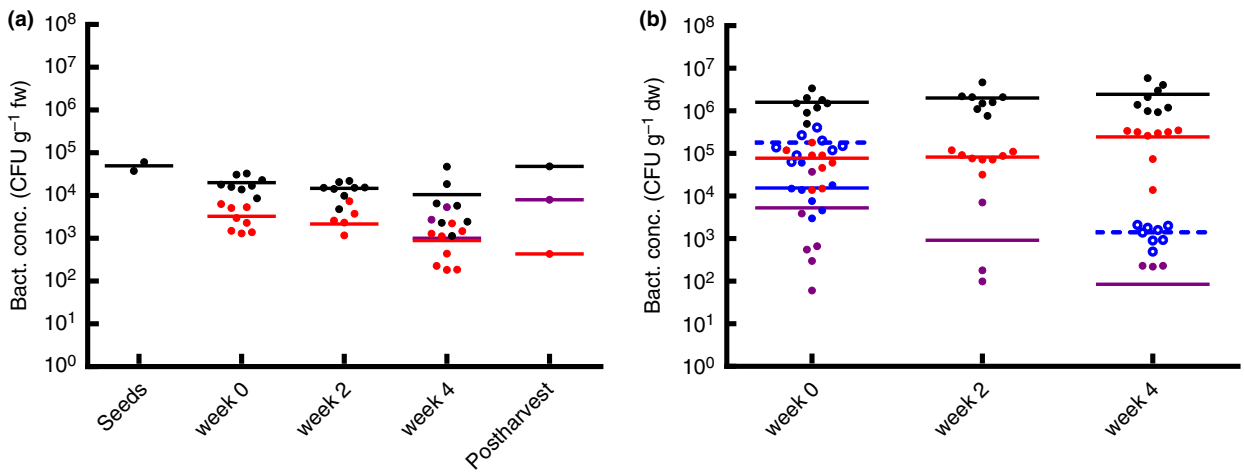

Figure 3 Population development of total mesophilic bacteria and different targeted bacteria at the thyme site on plant material (a) or in soil (b). Every symbol represents the CFU count of an individual sample. The number of samples analysed per time point were eight for soil or field-grown plants, two for seeds and one for postharvest material (limit of detection = 5×10^1 CFU per g). Coloured lines represent average CFU counts. Replicate samples below the limit of detection were assigned a zero and the average was calculated from all samples. Closed black circle = PCA medium, total mesophilic count; closed red circle = BCR medium, *B. cereus* group; closed blue circle = TBX medium, *E. coli*; open blue circle = TBX medium, background colonies; closed purple circle = mEA medium, presumptive *Enterococcus* spp.; bact. conc.: bacterial concentration; fw: fresh weight; dw: dry weight. [Colour figure can be viewed at wileyonlinelibrary.com]

(marjoram: week 0–2 and 4–6; basil: week 0–2 and 2–4 (all $P \leq 0{\cdot}0001$)). *Escherichia coli* was detectable only on young seedlings (Figs 1a and 2a). TBX background colonies were detected in two of four marjoram samplings, whereas on basil they were detectable at all time points. *Enterococcus* spp. were detected in week 0 in four of eight marjoram and six of eight basil samples. They were only sporadically detected thereafter and generally below the limit of detection (5×10^1 CFU per g) in the last field samplings.

Young thyme plants from week 0 were colonized by 2×10^4 total mesophilic CFU per g, which remained constant throughout the sampling (Fig. 3a). *Bacillus cereus* group members were consistently found at numbers of around 10^3 CFU per g slightly decreasing over the growing period. Further from the bacterial target groups only *Enterococcus* spp. were detected in two of eight samples of week 4 (Fig. 3a).

Postharvest material as it is delivered to the consumer, that is, harvested and packed directly without prior washing, showed increased total mesophilic numbers at the end of shelf life as compared to the last field samples for all three herbs (marjoram $P \leq 0{\cdot}0001$, basil $P \leq 0{\cdot}05$, thyme $P \geq 0{\cdot}05$). Additionally, on the packed marjoram and thyme, *B. cereus* group members were detected at numbers similar to those of the last field sample as well as *Enterococcus* spp. that had not been detected in the last marjoram field sample or in the case of thyme, were increased by one order of magnitude as compared to the last field sample.

Open field weather data

Since weather conditions are a potential factor influencing bacterial growth on plant surfaces (Vorholt 2012) weather data were acquired from the meteorological station of Syngenta in Dielsdorf, Switzerland, including temperature, humidity and precipitation (Fig. S1). Temperature (minima and maxima) and relative humidity (daily average) was determined 2 m above ground level. Precipitation was recorded as daily sum. Temperatures at the approximate time point of sampling were 21·5 °C ± 1·5 °C. The values for relative humidity were around 48, 68, 77 and 60% for week 0, 2, 4 and harvest, respectively.

Bacterial isolates from different culture media

Isolate identification was performed using MALDI biotyping and 16S rRNA gene sequencing of selected isolates to verify the classification results.

Bacteria isolated from PCA showed the largest phylogenetic diversity including *Aeromonas* spp., *Arthrobacter* spp., *Bacillus* spp., *Exiguobacterium* spp., *Pantoea* spp., *Pseudomonas* spp. and *Stenotrophomonas* spp.

All bacteria that were isolated from BCR were identified as *B. cereus* group members by MALDI biotyping. However, a separation between members of the group was not possible even though certain isolates clearly differed in colony morphology as reported recently (Pfrunder *et al.* 2016). Such difficulties are well-known for the

B. cereus group since their 16S rRNA sequences display less than 1% difference (Ash *et al.* 1991) making reliable distinction virtually impossible also by 16S rRNA gene sequencing (Mellmann *et al.* 2008; Cherkaoui *et al.* 2010; Bizzini *et al.* 2011).

Bacteria grown on presumptive *Enterococcus*-selective mEA medium were not exclusively identified as *Enterococcus* spp. but included bacteria from the genera *Aerococcus*, *Bacillus* (non-*cereus* group), *Staphylococcus* and *Pseudomonas*. In contrast to the classical approach for *Enterococcus* spp. identification, which usually involves streaking the bacteria from mEA onto bile esculin agar and performing a negative catalase test (Facklam 1973), the identification using MALDI biotyping was very rapid and a representative number of colonies could be screened within a few hours (about 200 colonies per h).

The *E. coli*-selective medium TBX supported growth of background colonies that did not exhibit the characteristic blue colour of *E. coli*. While blue colonies were consistently identified as *E. coli*, nonblue coloured colonies consisted of *Pseudomonas* spp., *Enterobacter* spp. and some *Ochrobactrum* spp. Pseudomonads detected on TBX belonged exclusively to the species *P. aeruginosa* or *P. citronellolis* (Remus-Emsermann *et al.* 2016).

Bacterial isolates from the three production systems

Marjoram

A variety of *Enterococcus* spp. was detected at the marjoram site with various species occurring in soil and on plants at the different samplings (Table 1). *Enterococcus casseliflavus* was the only species detected at all time points, and *Enterococcus hirae* was detected both in soil and on plants at w0 and w2. *Bacillus cereus* group bacteria were omnipresent in plant, soil and water. Furthermore, pseudomonads were identified at all time points, however, their species diversity varied strongly between samplings with 9 of 12 (75%) detected only once.

Basil

The basil site displayed the largest species diversity overall (Table 1). Various *Enterococcus* spp. were detected mostly in water, with *Enterococcus faecalis*, *Enterococcus faecium* and *Enterococcus mundtii* detected in all but the first sampling. *Enterococcus faecium*, being additionally detected in soil of w0, was the only enterococcal species detected throughout the sampling. Finally, *E. casseliflavus* was detected in both soil and water from w2 and w4. The *B. cereus* group was omnipresent in all three systems as at the marjoram site. *Pseudomonas* was the most prevalent genus recovered and identified mainly from basil leaves and to a lesser extent from soil and water (Table 1). As for the marjoram site, *Pseudomonas* species diversity varied, with 9 of the 17 species (53%) detected only once. *Pseudomonas brenneri* and *P. libanensis*—recovered from basil leaves—were the only species detected in three of the four samplings.

Thyme

The smallest species diversity was found at the thyme site. Four *Enterococcus* spp. were isolated, with *E. casseliflavus* detected in soil at all time points. As for marjoram and basil, *B. cereus* group bacteria were omnipresent. Only six *Pseudomonas* spp. were found mainly in soil, with species composition varying strongly between samplings. Finally, three *Staphylococcus* spp. were identified in soil of w0 and w2.

Discussion

Organic fertilizers and soil

Organic fertilizers have been suggested to influence soil microbial community composition (Cai *et al.* 2003) and organic farming practices have been attributed slightly increased numbers in total aerobic bacteria, *Enterobacteriaceae* and coliforms (Wießner *et al.* 2009). In our survey, the three organic fertilizers analysed did not contain detectable *E. coli*, *Enterococcus* spp. or *B. cereus* group isolates. However, since fertilizers are applied before every planting of new seedlings, the target bacteria might have originated from previous applications and established in the soil. Furthermore, *Bacillus thuringiensis* spores, which are permitted as plant protection agent for the use in organic agriculture, might have contributed to the presence of detectable *B. cereus* group isolates. However, during the study year they were not applied in the field. Whether there is an indirect effect of organic fertilizers on soil community composition will be subject of further studies comparing conventionally with organically managed soils.

In soil, *E. coli* was detected but almost exclusively in the first samplings (Figs 1b, 2b and 3b). *Enterococcus* spp. were detected at low frequencies in soil at all three sites with only one exception. If not initially present in soil, the detected *E. coli* and *Enterococcus* spp. might have been brought onto the field with the seedlings and their initial growth substrate. *Bacillus cereus* group members were present at all sites and time points at high numbers as expected, being typical soil inhabitants (Granum 2007; Stenfors Arnesen *et al.* 2008).

Plants—field and postharvest

The number of total mesophilic bacteria on young marjoram and basil plants was between 10^6 and 10^7 CFU per g

Table 1 Species identified by MALDI biotyping at the marjoram, basil and thyme site from plant (p), water (w) and soil (s). w0: week 0; w2: week 2; w4: week 4; w6: week 6

Marjoram site					Basil site					Thyme site			
Bacterial species	w0	w2	w4	w6	Bacterial species	w0	w2	w4	w6	Bacterial species	w0	w2	w4
Aerococcus viridans	s		p		*Acinetobacter baumannii*			w		*Arthrobacter polychromogenes*			s
Aeromonas spp.*		w			*Acinetobacter guillouiae*				w	*Bacillus cereus* group	p, s	p, s	p, s
Arthrobacter aurescens		s		w	*Acinetobacter johnsonii*			s, w		*Bacillus megaterium*	p	p	
Arthrobacter histidinolovorans				w	*Advenella kashmirensis*		s			*Bacillus muralis*			p
Arthrobacter pascens	s				*Aerococcus viridans*	s	s			*Bacillus simplex*		s	p
Arthrobacter russicus			s		*Aeromonas* spp.*	w	w	p, w	w	*Bordetella petrii*		s	
Arthrobacter sulfonivorans			s		*Arthrobacter pascens*	w		s		*Enterococcus casseliflavus*	s	s	s
Bacillus cereus group	p, s, w	p, s, w	p, s, w	p, s, w	*Arthrobacter sulfonivorans*			s		*Enterococcus moraviensis*			p
Bacillus megaterium			w		*Bacillus cereus* group	p, s, w	p, s, w	p, s, w	p, s, w	*Enterococcus mundtii*		s	
Bacillus muralis	s				*Bacillus galactosidilyticus*			s		*Enterococcus termitis*	s		p
Bacillus simplex			w	p	*Bacillus mojavensis*	p				*Microbacterium maritypicum*	s		
Bordetella petrii	s				*Chryseobacterium scophthalmum*	w				*Ochrobactrum intermedium*		s	s
Cellulosimicrobium cellulans	s				*Enterobacter cloacae* complex†	p, s	s			*Paenibacillus amylolyticus*		p	
Enterobacter cloacae complex†	p	s		s	*Enterococcus casseliflavus*	s	s, w	s, w		*Paenibacillus macquariensis*		p	
Enterococcus casseliflavus	p, s	p	s	s	*Enterococcus durans*		w			*Pseudomonas agarici*	s		
Enterococcus faecium	s			s	*Enterococcus faecalis*		w	w	w	*Pseudomonas brassicacearum*			s
Enterococcus faecalis			s		*Enterococcus faecium*	s	w	w	w	*Pseudomonas citronellolis*	s		
Enterococcus haemoperoxidus	p				*Enterococcus haemoperoxidus*	w				*Pseudomonas frederiksbergensis*		p, s	s
Enterococcus hirae	p				*Enterococcus hirae*	s				*Pseudomonas jessenii*			s
Enterococcus mundtii	p, s	p, s		s	*Enterococcus mundtii*		w	w	w	*Pseudomonas mandelii*		p	
Escherichia coli	p, s			s	*Enterococcus silesiacus*	w				*Staphylococcus equorum*		s	
Flavobacterium hydatis				w	*Enterococcus termitis*	p, w	w			*Staphylococcus saprophyticus*	s	s	
Microbacterium maritypicum		s			*Escherichia coli*	p, s, w	s, w	w	s, w	*Staphylococcus sciuri*	s		
Microbacterium phyllosphaerae	w				*Flavobacterium saccharophilum*			w					
Ochrobactrum intermedium	p, s	s			*Lactococcus lactis*		s						
Pantoea agglomerans	p, w	p			*Morganella morganii*			w					
Pectobacterium carotovorum			p		*Ochrobactrum anthropi*		s	s					
Pseudomonas aeruginosa	w			w	*Paenibacillus glucanolyticus*		s						
Pseudomonas brassicacearum			w		*Pantoea agglomerans*	w	w		w				
Pseudomonas chlororaphis		w			*Pectobacterium carotovorum*	w							
Pseudomonas citronellolis	p			s	*Pseudomonas aeruginosa*				p, s				
Pseudomonas flavescens			p		*Pseudomonas brenneri*	p	p	p					
Pseudomonas frederiksbergensis	w				*Pseudomonas cichorii*			p	w				
Pseudomonas jessenii			s		*Pseudomonas citronellolis*	p	p						
Pseudomonas koreensis		p			*Pseudomonas extremorientalis*		w		w				
Pseudomonas libanensis		s			*Pseudomonas frederiksbergensis*	p							
Pseudomonas proteolytica	w	p, w			*Pseudomonas gessardii*	p	p						

(*Continued*)

Table 1 (*Continued*)

Marjoram site					Basil site					Thyme site			
Bacterial species	w0	w2	w4	w6	Bacterial species	w0	w2	w4	w6	Bacterial species	w0	w2	w4
Pseudomonas putida	p				*Pseudomonas jinjuensis*				s				
Pseudomonas thivervalensis			p		*Pseudomonas koreensis*		p		p, w				
Rhodococcus erythropolis			s		*Pseudomonas libanensis*		p	p	p				
Serratia plymuthica		w			*Pseudomonas mandelii*	p							
Staphylococcus vitulinus	s				*Pseudomonas otitidis*			w	s				
Staphylococcus warneri				s	*Pseudomonas poae*			p					
					Pseudomonas proteolytica	p							
					Pseudomonas putida				p				
					Pseudomonas synxantha				p				
					Pseudomonas thivervalensis			s					
					Raoultella ornithinolytica		w						
					Rhodococcus erythropolis	s							
					Rhodococcus koreensis		s						
					Serratia plymuthica		w	w					
					Staphylococcus sciuri	s		s					
					Staphylococcus xylosus	p, s							

**Aeromonas* spp. such as *A. salmonicida*, *A. bestiarum* or *A. veronii*, which cannot be distinguished by MALDI biotyping.
†*Enterobacter cloacae* complex includes *E. cloacae* and *E. ludwigii*, which cannot be distinguished by MALDI biotyping.

(Figs 1a and 2a) and a decrease was observed throughout the growing season. Similarly, initial numbers of *B. cereus* group members were very high but decreased from young seedlings to harvestable plants. This might be explained by dilution of bacterial cells initially present on the seedlings as they grow, since *B. cereus* group bacteria are expected to persist on the plant surfaces due to their resilient character conferred by formation of endospores (Atrih and Foster 2002), which assumes that *B. cereus* group bacteria are not actively growing on plants but merely persisters. *Bacillus cereus* group bacteria have previously not been shown to be major contributors to phyllosphere communities on other plants, making it unlikely that they do so on herbs (Knief *et al.* 2010; Redford *et al.* 2010; Rastogi *et al.* 2012; Bai *et al.* 2015). *Escherichia coli* was detected on marjoram and basil plants only in week 0, a finding that together with their abundant detection in soil in week 0 (Figs 1b and 2b, respectively) made soil a potential source of contamination of the young plantlets. Soil has been recognized previously to be a main source of fresh produce contamination (Islam *et al.* 2004; Barak and Liang 2008; Olaimat and Holley 2012). Soil was a possible source also of *Enterococcus* spp. sporadically detected on marjoram and basil. Alternatively, *E. coli* and *Enterococcus* spp. might have been introduced to the field with the seedlings and their growth substrate, especially in the case of marjoram where *Enterococcus* spp. were already detectable on seeds (Fig. 1a). On basil plant material, *E. coli* and *Enterococcus* spp. could also originate from overhead sprinklers (Fig. 2a,d, week 0 and 2), from where they reach the plant surface directly (Fonseca *et al.* 2011; Gupta and Madramootoo 2016). However, source tracking by typing phyllosphere isolates to confirm their origin was beyond the scope of this survey and will be the subject of future studies.

Thyme plants revealed the smallest number of mesophilic bacteria and of the bacterial target groups, only *B. cereus* group members could be detected throughout the growing season (Fig. 3a). A low colonization of thyme plants was expected due to (i) thyme's essential oils which have been broadly recognized as bactericidal and have therefore been used not only for food preservation but also in clinical settings (Basch *et al.* 2004; Sienkiewicz *et al.* 2012) and (ii) harsh conditions under which the plants were grown, such as the lack of irrigation and direct solar exposure and thus DNA-damaging UV radiation (Vorholt 2012). A probable contamination source of thyme plants with *B. cereus* group members was soil (Fig. 3b). From the other bacterial target groups, only *Enterococcus* spp. were detected and in just two of the eight samples of week 4 (Fig. 3a), which further supported harsh conditions for bacterial establishment on open field-grown thyme. Monitoring of climatic conditions revealed that the daily temperature maxima were on average above 24°C and days with no or little precipitation prevailed (below 5 l m^{-2} per day, 30 of 43 days; Fig. S1). Climatic variations were small, which is in good agreement with the stable bacterial numbers observed throughout the sampling period. In order to determine the importance of climatologic variations on the development of the phyllosphere community, a long-term study including larger variations in temperature and precipitation should be conducted in the field or a greenhouse (Rastogi *et al.* 2012; Williams *et al.* 2013).

On postharvest material, the increase in total mesophilic CFU (Figs 1a, 2a and 3a) as well as *Enterococcus* spp. in the case of thyme might be attributed to the direct packaging of the harvested plants without washing and subsequent bacterial proliferation during storage. The reoccurrence of *Enterococcus* spp. in one marjoram postharvest sample indicated either an unknown source of contamination during harvest or *Enterococcus* spp. at levels below the limit of detection at harvest and growth to detectable numbers during storage.

Irrigation water: comparing two water sources

Bacterial counts in irrigation water were mainly determined by the last part of the irrigation system, that is, the overhead sprinklers or belowground hoses, since bacterial counts in field inlet water were very similar between the two sites (Figs 1c and 2c). Sprinkler water sourced from municipal drinking water contained lower mesophilic numbers and less diverse bacteria compared to sprinkler water sourced from the open-top reservoir (Fig. 1d *vs* 2d). *Escherichia coli* and *Enterococcus* spp. were detected exclusively in water from the basil site sprinklers (Fig. 2d). *Escherichia coli* and *Enterococcus* spp. most likely entered into the sprinkler system—which does not communicate with the soil—from the reservoir in which they were detected, although they were, with one exception, undetectable in the water of the basil field inlet (Fig. 2c). Proliferation of *E. coli* and *Enterococcus* spp. in the irrigation tubes might explain their subsequent detection in the sprinkler water: The nature of the overhead irrigation system, that is, black UV-impermeable plastic tubes placed directly under the greenhouse roof, where temperatures were favourable for bacterial proliferation, and long stagnation periods of the water in between irrigation intervals were factors likely promoting bacterial growth. Belowground hose water from the two sites was not compared, since differences in soil community might have influenced bacterial composition of the water in the perforated hoses. The detected bacterial target groups in hose water might originate from soil at the marjoram site (Fig. 1b) since they were not detected in the respective

inlet or sprinkler water (Fig. 1c,d week 0 and 2) or soil and water at the basil site (Fig. 2b–d). Hence, the main differences between the two irrigation water sources were (i) *E. coli* present in the water sourced from the open-top reservoir while they could not be detected in municipality-sourced water and (ii) *Enterococcus* spp. found in sprinkler water at the basil site, while they were absent from the sprinkler water of the municipality-sourced marjoram site.

MALDI biotyping and mesophilic community composition

Isolate identification based on 16S amplicon sequencing was in line with the identification results of MALDI biotyping, showing the potential of the latter rapid method in the identification of environmental isolates over tedious amplicon sequencing. A limitation of MALDI biotyping to date, however, is the limited number of entries for environmental isolates in the databases, which mostly focus on medically relevant strains. Efforts to extend databases with environmental and food isolates are ongoing (Agustini *et al.* 2014; Folkhälsomyndigheten 2014; Pavlovic *et al.* 2014; Freimoser *et al.* 2016).

Analysis of the isolated mesophilic bacteria by MALDI biotyping revealed that the basil site displayed the highest species diversity (Table 1). Focusing on the phyllosphere, basil still showed the highest diversity with 20 species, as opposed to 17 and 10 species for marjoram and thyme, respectively. However, while the basil phyllosphere was dominated by pseudomonads, the marjoram phyllosphere, which has only been poorly studied to date, harboured a more diverse community on the genus level, with nine different genera detected including *Bacillus* spp., *Enterobacter* spp., *Enterococcus* spp., *Pantoea* spp. and various pseudomonads (Table 1). Non-*Pseudomonas* species found in the basil phyllosphere community included *Aeromonas* spp., *Bacillus* spp., *Enterobacter* spp., *Enterococcus* spp. and staphylococci (Table 1), which is in good agreement with previous studies where *Proteobacteria* and *Firmicutes* (Jackson *et al.* 2015), *Pseudomonas* spp., *Enterobacteriaceae* and *Aeromonas* spp. (Ceuppens *et al.* 2015) or *Enterobacter* spp. (Wetzel *et al.* 2010) were identified as main colonizers of basil leaves. On thyme, main colonizers were *Bacillus* spp., followed by *Enterococcus* spp., *Paenibacillus* spp. and *Pseudomonas* spp. (Table 1). Overall, the mesophilic bacteria identified by MALDI biotyping include several species with known food spoilage potential, such as *Pantoea agglomerans*, *Pectobacterium carotovorum*, pseudomonads like *Pseudomonas cichorii* or *Serratia plymuthica*, which have been related to spoilage of fresh produce (Tournas 2005; Hadjok *et al.* 2008; Wevers *et al.* 2009; Oliveira *et al.* 2010; Zheng *et al.* 2013).

Overall, our study provides insights into the composition and temporal development of basil-, marjoram- and thyme-culturable mesophilic bacterial communities. Mesophilic counts on plants decreased throughout the growing season but increased on the market product, and *Enterococcus* spp. and *B. cereus* group bacteria were detected on market-ready marjoram and thyme. To ensure consumer health, Swiss legislation has defined a cutoff for *E. coli* (100 CFU per g), however, no cut-offs are defined for any of the other investigated bacterial groups (Swiss Federal Department of Home Affairs 2016). Based on these recommendations, the detected levels of mesophilic bacteria, *Enterococcus* spp. and *B. cereus* group bacteria are of no concern to consumer health. Notably, a certain contamination level of the market product is unavoidable due to agricultural practices such as the application of plant protection agents and weeding, and also depends on irrigation water quality or condition of the irrigation system. Therefore, even when applying good agricultural and hygienic practice in the production environment (Early 2002), maintaining the cold chain and following preconsumption recommendations, such as washing the herbs before consumption, are crucial to ensure consumer health. In terms of potential contamination sources, the fertilizers analysed did not contain any of the targeted food-contaminating bacteria, they might, however, have introduced them into the soil community earlier, since the fertilizers are applied before every seedling planting. The detection of all selected food-contaminating bacteria in soil as well as in irrigation water sourced from an open-top reservoir rendered both—soil and open-top reservoir water—potential sources of fresh herb contamination with *E. coli*, *Enterococcus* spp. and *B. cereus* group bacteria. Notably, fresh produce may be contaminated by a large variety of sources (Johnson *et al.* 2003; Beuchat 2006), like applied farming practices including contamination by field workers, growth substrate for seedlings or equipment and tools (Mukherjee *et al.* 2007). Those, however, were beyond the scope of this survey. To locate the main sources of contamination of fresh produce and propose actions to mitigate consumer exposure to undesired contaminants, the method of choice might be typing of the isolated food-contaminating bacteria (Wirth *et al.* 2006) including (i) spoilage bacteria, that is, bacteria altering the sensorial qualities of a food product thereby rendering it unacceptable to the consumer (Gram *et al.* 2002) and (ii) human pathogenic bacteria. Finally, the more recent concern about the occurrence of antibiotic-resistant bacteria on market products (Pesavento *et al.* 2014; Nüesch-Inderbinen *et al.* 2015; Zogg *et al.* 2016) and the extent to which the production environment plays a role are subject to our ongoing research.

Acknowledgements

We acknowledge Fiona Walsh (NUI Maynooth) for helpful discussions, Stefanie Pfrunder and Pascal Gisler for their help during the sampling as well as Jan Werthmüller and Benno Graf for providing weather data. Agroscope is acknowledged for providing funding in the framework of the Research Program on the Reduction of Resistant and Persistent Microorganisms in the Food Chain (REDYMO). This work was part of COST Action FA 1202 'A European Network for Mitigating Bacterial Colonisation and Persistence on Foods and Food Processing Environments'.

Conflict of Interest

No conflict of interest declared.

References

Agustini, B.C., Silva, L.P., Bloch, C. Jr, Bonfim, T.M.B. and da Silva, G.A. (2014) Evaluation of MALDI-TOF mass spectrometry for identification of environmental yeasts and development of supplementary database. *Appl Microbiol Biotechnol* **98**, 5645–5654.

Allende, A. and Monaghan, J. (2015) Irrigation water quality for leafy crops: a perspective of risks and potential solutions. *Int J Environ Res Public Health* **12**, 7457–7477.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

Anderson, K.L., Whitlock, J.E. and Harwood, V.J. (2005) Persistence and differential survival of fecal indicator bacteria in subtropical waters and sediments. *Appl Environ Microbiol* **71**, 3041–3048.

Ash, C., Farrow, J.A.E., Dorsch, M., Stackebrandt, E. and Collins, M.D. (1991) Comparative analysis of *Bacillus anthracis*, *Bacillus cereus*, and related species on the basis of reverse transcriptase sequencing of 16S rRNA. *Int J Syst Bacteriol* **41**, 343–346.

Association of Swiss Organic Agriculture Organisations (2015) Standards for the production, processing and marketing of 'bud' products Bio Suisse, Basel, Switzerland.

Atrih, A. and Foster, S.J. (2002) Bacterial endospores the ultimate survivors. *Int Dairy J* **12**, 217–223.

Bafana, A. (2013) Diversity and metabolic potential of culturable root-associated bacteria from *Origanum vulgare* in sub-Himalayan region. *World J Microbiol Biotechnol* **29**, 63–74.

Bai, Y., Müller, D.B., Srinivas, G., Garrido-Oter, R., Potthoff, E., Rott, M., Dombrowski, N., Münch, P.C. *et al.* (2015) Functional overlap of the *Arabidopsis* leaf and root microbiota. *Nature* **528**, 364–369.

Barak, J.D. and Liang, A.S. (2008) Role of soil, crop debris, and a plant pathogen in *Salmonella enterica* contamination of tomato plants. *PLoS ONE* **3**, e1657.

Basch, E., Ulbricht, C., Hammerness, P., Bevins, A. and Sollars, D. (2004) Thyme (*Thymus vulgaris* L.), thymol. *J Herb Pharmacother* **4**, 49–67.

Beattie, G.A. (2011) Water relations in the interaction of foliar bacterial pathogens with plants. *Annu Rev Phytopathol* **49**, 533–555.

Berger, C.N., Sodha, S.V., Shaw, R.K., Griffin, P.M., Pink, D., Hand, P. and Frankel, G. (2010) Fresh fruit and vegetables as vehicles for the transmission of human pathogens. *Environ Microbiol* **12**, 2385–2397.

Beuchat, L.R. (2006) Vectors and conditions for preharvest contamination of fruits and vegetables with pathogens capable of causing enteric diseases. *Br Food J* **108**, 38–53.

Bizzini, A., Jaton, K., Romo, D., Bille, J., Prod'hom, G and Greub, G. (2011) Matrix-Assisted laser desorption ionization–time of flight mass spectrometry as an alternative to 16S rRNA gene sequencing for identification of difficult-to-identify bacterial strains. *J Clin Microbiol* **49**, 693–696.

Cai, Y., Liao, Z., Zhang, J., Kong, W. and He, C. (2003) Effect of ecological organic fertilizer on tomato bacterial wilt and soil microbial diversities. *Chin J Appl Ecol* **14**, 349–353.

Ceuppens, S., Delbeke, S., De Coninck, D., Boussemaere, J., Boon, N. and Uyttendaele, M. (2015) Characterization of the bacterial community naturally present on commercially grown basil leaves: evaluation of sample preparation prior to culture-independent techniques. *Int J Environ Res Public Health* **12**, 10171–10197.

Chaudhry, V., Rehman, A., Mishra, A., Chauhan, P.S. and Nautiyal, C.S. (2012) Changes in bacterial community structure of agricultural land due to long-term organic and chemical amendments. *Microbial Ecol* **64**, 450–460.

Cherkaoui, A., Hibbs, J., Emonet, S., Tangomo, M., Girard, M., Francois, P. and Schrenzel, J. (2010) Comparison of two matrix-assisted laser desorption ionization-time of flight mass spectrometry methods with conventional phenotypic identification for routine identification of bacteria to the species level. *J Clin Microbiol* **48**, 1169–1175.

Czekalski, N., Berthold, T., Caucci, S., Egli, A. and Bürgmann, H. (2012) Increased levels of multiresistant bacteria and resistance genes after wastewater treatment and their dissemination into Lake Geneva, Switzerland. *Front Microbiol* **3**, 106.

Doyle, M.P. and Erickson, M.C. (2008) Summer meeting 2007 – the problems with fresh produce: an overview. *J Appl Microbiol* **105**, 317–330.

Drissner, D. and Zuercher, U. (2014) Safety of food and beverages: fruits and vegetables. In *Encyclopedia of Food Safety* ed. Motarjemi, Y., Todd, E. and Moy, G. pp. 253–259. Waltham, MA: Elsevier.

Early, R. (2002) Use of HACCP in fruit and vegetable production and post-harvest pretreatment. In *Fruit and*

Vegetable Processing: Improving Quality ed. Jongen, W. pp. 91–118. Boca Raton, FL: CRC Press.

Facklam, R.R. (1973) Comparison of several laboratory media for presumptive identification of enterococci and group D streptococci. *Appl Microbiol* **26**, 138–145.

Folkhälsomyndigheten (2014) Spectra - Extended spectra database for microorganism identification by MALDI-TOF MS. http://spectra.folkhalsomyndigheten.se/spectra/(accessed 2 December 2015).

Fonseca, J.M., Fallon, S.D., Sanchez, C.A. and Nolte, K.D. (2011) *Escherichia coli* survival in lettuce fields following its introduction through different irrigation systems. *J Appl Microbiol* **110**, 893–902.

Freimoser, F.M., Hilber-Bodmer, M., Brunisholz, R. and Drissner, D. (2016) Direct identification of *Monilinia* brown rot fungi on infected fruits by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. *Chem Biol Technol Agric* **3**, 7.

Frey, S.K., Topp, E., Edge, T., Fall, C., Gannon, V., Jokinen, C., Marti, R., Neumann, N. *et al.* (2013) Using SWAT, *Bacteroidales* microbial source tracking markers, and fecal indicator bacteria to predict waterborne pathogen occurrence in an agricultural watershed. *Water Res* **47**, 6326–6337.

Gekenidis, M.-T., Studer, P., Wüthrich, S., Brunisholz, R. and Drissner, D. (2014) Beyond the matrix-assisted laser desorption ionization (MALDI) biotyping workflow: in search of microorganism-specific tryptic peptides enabling discrimination of subspecies. *Appl Environ Microbiol* **80**, 4234–4241.

Gilmore, M.S. and Ferretti, J.J. (2003) The thin line between gut commensal and pathogen. *Science* **299**, 1999–2002.

Gram, L., Ravn, L., Rasch, M., Bruhn, J.B., Christensen, A.B. and Givskov, M. (2002) Food spoilage–interactions between food spoilage bacteria. *Int J Food Microbiol* **78**, 79–97.

Granum, P.E. (2007) *Bacillus cereus*. In *Foodborne Pathogens: Microbiology and Molecular Biology* eds. Doyle, M.P. and Beuchat, L.R. pp. 445–455. Washington, DC: ASM Press.

Gupta, D. and Madramootoo, C.A. (2016) Fate and transport of *Escherichia coli* in tomato production. *Expo Health* **9**, 13–25.

Hadjok, C., Mittal, G. and Warriner, K. (2008) Inactivation of human pathogens and spoilage bacteria on the surface and internalized within fresh produce by using a combination of ultraviolet light and hydrogen peroxide. *J Appl Microbiol* **104**, 1014–1024.

Hirano, S.S., Nordheim, E.V., Arny, D.C. and Upper, C.D. (1982) Lognormal distribution of epiphytic bacterial populations on leaf surfaces. *Appl Environ Microbiol* **44**, 695–700.

Ijabadeniyi, O.A., Buys, E.M., Debusho, L.K. and Vanderlinde, M. (2011) Irrigation water as a potential pre-harvest source of bacterial contamination of vegetables. *J Food Safety* **31**, 452–461.

Islam, M., Doyle, M.P., Phatak, S.C., Millner, P. and Jiang, X. (2004) Persistence of enterohemorrhagic *Escherichia coli* O157: H7 in soil and on leaf lettuce and parsley grown in fields treated with contaminated manure composts or irrigation water. *J Food Prot* **67**, 1365–1370.

Jackson, C.R., Stone, B.W. and Tyler, H.L. (2015) Emerging perspectives on the natural microbiome of fresh produce vegetables. *Agriculture* **5**, 170–187.

Jay, J.M. (2005) Indicators of food microbial quality and safety. In *Modern Food Microbiology* eds. Jay, J.M., Loessner, M.J. and Golden, D.A. pp. 473–495. New York, USA: Springer.

Jechalke, S., Heuer, H., Siemens, J., Amelung, W. and Smalla, K. (2014) Fate and effects of veterinary antibiotics in soil. *Trends Microbiol* **22**, 536–545.

Johnson, J.Y.M., Thomas, J.E., Graham, T.A., Townshend, I., Byrne, J., Selinger, L.B. and Gannon, V.P.J. (2003) Prevalence of *Escherichia coli* O157:H7 and *Salmonella* spp. in surface waters of southern Alberta and its relation to manure sources. *Can J Microbiol* **49**, 326–335.

Jokinen, C.C., Edge, T.A., Koning, W., Laing, C.R., Lapen, D.R., Miller, J., Mutschall, S., Scott, A. *et al.* (2012) Spatial and temporal drivers of zoonotic pathogen contamination of an agricultural watershed. *J Environ Qual* **41**, 242–252.

Kapoor, R. and Mukerji, K.G. (2006) Rhizosphere microbial community dynamics. In *Microbial Activity in the Rhizoshere* eds. Mukerji, K. G., Manoharachary, C., Singh, J. pp. 55–69. Berlin, Germany: Springer.

Kilsby, D.C. and Pugh, M.E. (1981) The relevance of the distribution of micro-organisms within batches of food to the control of microbiological hazards from foods. *J Appl Microbiol* **51**, 345–354.

Kleter, G.A., Prandini, A., Filippi, L. and Marvin, H.J.P. (2009) Identification of potentially emerging food safety issues by analysis of reports published by the European Community's Rapid Alert System for Food and Feed (RASFF) during a four-year period. *Food Chem Toxicol* **47**, 932–950.

Knief, C., Ramette, A., Frances, L., Alonso-Blanco, C. and Vorholt, J.A. (2010) Site and plant species are important determinants of the *Methylobacterium* community composition in the plant phyllosphere. *ISME J* **4**, 719–728.

Li, R., Khafipour, E., Krause, D.O., Entz, M.H., de Kievit, T.R. and Fernando, W.G.D. (2012) Pyrosequencing reveals the influence of organic and conventional farming systems on bacterial communities. *PLoS ONE* **7**, e51897.

Lorimer, M.F. and Kiermeier, A. (2007) Analysing microbiological data: tobit or not Tobit? *Int J Food Microbiol* **116**, 313–318.

Marti, R., Gannon, V.P., Jokinen, C., Lanthier, M., Lapen, D.R., Neumann, N.F., Ruecker, N.J., Scott, A. *et al.* (2013) Quantitative multi-year elucidation of fecal sources of waterborne pathogen contamination in the South Nation River basin using *Bacteroidales* microbial source tracking markers. *Water Res* **47**, 2315–2324.

Mellmann, A., Cloud, J., Maier, T., Keckevoet, U., Ramminger, I., Iwen, P., Dunn, J., Hall, G. *et al.* (2008) Evaluation of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry in comparison to 16S rRNA gene sequencing for species identification of nonfermenting bacteria. *J Clin Microbiol* **46**, 1946–1954.

Morris, C.E. and Kinkel, L.L. (2002) Fifty years of phyllosphere microbiology: significant contributions to research in related fields. In *Phyllosphere Microbiology* ed. Lindow, S.E., Hecht-Poinar, E.I. and Elliott, V.J. pp. 365–375. St. Paul, USA: American Phytopathological Society Press.

Mukherjee, A., Speh, D. and Diez-Gonzalez, F. (2007) Association of farm management practices with risk of *Escherichia coli* contamination in pre-harvest produce grown in Minnesota and Wisconsin. *Int J Food Microbiol* **120**, 296–302.

Müller, T., Ulrich, A., Ott, E.M. and Müller, M. (2001) Identification of plant-associated enterococci. *J Appl Microbiol* **91**, 268–278.

Nüesch-Inderbinen, M., Zurfluh, K., Peterhans, S., Hächler, H. and Stephan, R. (2015) Assessment of the prevalence of extended-spectrum β-lactamase–producing *Enterobacteriaceae* in ready-to-eat salads, fresh-cut fruit, and sprouts from the Swiss market. *J Food Prot* **78**, 1178–1181.

Olaimat, A.N. and Holley, R.A. (2012) Factors influencing the microbial safety of fresh produce: a review. *Food Microbiol* **32**, 1–19.

Oliveira, M., Usall, J., Vinas, I., Anguera, M., Gatius, F. and Abadias, M. (2010) Microbiological quality of fresh lettuce from organic and conventional production. *Food Microbiol* **27**, 679–684.

Pappas, E.A., Kanwar, R.S., Baker, J.L., Lorimor, J.C. and Mickelson, S. (2008) Fecal indicator bacteria in subsurface drain water following swine manure application. *Trans ASABE* **51**, 1567–1573.

Pavlovic, M., Mewes, A., Maggipinto, M., Schmidt, W., Messelhäußer, U., Balsliemke, J., Hörmansdorfer, S., Busch, U. *et al.* (2014) MALDI-TOF MS based identification of food-borne yeast isolates. *J Microbiol Methods* **106**, 123–128.

Pesavento, G., Calonico, C., Ducci, B., Magnanini, A. and Nostro, A.L. (2014) Prevalence and antibiotic resistance of *Enterococcus* spp. isolated from retail cheese, ready-to-eat salads, ham, and raw meat. *Food Microbiol* **41**, 1–7.

Pfrunder, S., Grossmann, J., Hunziker, P., Brunisholz, R., Gekenidis, M.T. and Drissner, D. (2016) *Bacillus cereus* group-type strain-specific diagnostic peptides. *J Proteome Res* **15**, 3098–3107.

Pollack, S.L. (2001) Consumer demand for fruit and vegetables: the US example. In *Changing Struct of Global Food Consumpt Trade* ed. Regmi, A. pp. 49–54. Washington, D. C., USA: US Department of Agriculture.

Rajkovic, A., Uyttendaele, M., Dierick, K., Samapundo, S., Botteldoorn, N., Mahillon, J. and Heyndrickx, M. (2008) Risk profile of the *Bacillus cereus* group implicated in food poisoning. Report for the Superior Health Council Belgium, CSS-HGR 8316.

Rasko, D.A., Rosovitz, M., Myers, G.S., Mongodin, E.F., Fricke, W.F., Gajer, P., Crabtree, J., Sebaihia, M. *et al.* (2008) The pangenome structure of *Escherichia coli*: comparative genomic analysis of *E. coli* commensal and pathogenic isolates. *J Bacteriol* **190**, 6881–6893.

Rastogi, G., Sbodio, A., Tech, J.J., Suslow, T.V., Coaker, G.L. and Leveau, J.H.J. (2012) Leaf microbiota in an agroecosystem: spatiotemporal variation in bacterial community composition on field-grown lettuce. *ISME J* **6**, 1812–1822.

Redford, A.J., Bowers, R.M., Knight, R., Linhart, Y. and Fierer, N. (2010) The ecology of the phyllosphere: geographic and phylogenetic variability in the distribution of bacteria on tree leaves. *Environ Microbiol* **12**, 2885–2893.

Remus-Emsermann, M.N.P., de Oliveira, S., Schreiber, L. and Leveau, J.H.J. (2011) Quantification of lateral heterogeneity in carbohydrate permeability of isolated plant leaf cuticles. *Front Microbiol* **2**, 197.

Remus-Emsermann, M.N.P., Tecon, R., Kowalchuk, G.A. and Leveau, J.H.J. (2012) Variation in local carrying capacity and the individual fate of bacterial colonizers in the phyllosphere. *ISME J* **6**, 756–765.

Remus-Emsermann, M.N., Schmid, M., Gekenidis, M.-T., Pelludat, C., Frey, J.E., Ahrens, C.H. and Drissner, D. (2016) Complete genome sequence of *Pseudomonas citronellolis* P3B5, a candidate for microbial phyllo-remediation of hydrocarbon-contaminated sites. *Stand Genomic Sci* **11**, 75.

Sagoo, S.K., Little, C.L., Greenwood, M., Mithani, V., Grant, K.A., McLauchlin, J., de Pinna, E. and Threlfall, E.J. (2009) Assessment of the microbiological safety of dried spices and herbs from production and retail premises in the United Kingdom. *Food Microbiol* **26**, 39–43.

Savichtcheva, O. and Okabe, S. (2006) Alternative indicators of fecal pollution: relations with pathogens and conventional indicators, current methodologies for direct pathogen monitoring and future application perspectives. *Water Res* **40**, 2463–2476.

Sienkiewicz, M., Łysakowska, M., Denys, P. and Kowalczyk, E. (2012) The antimicrobial activity of thyme essential oil against multidrug resistant clinical bacterial strains. *Microb Drug Resist* **18**, 137–148.

Solaiman, Z.M. and Anawar, H.M. (2015) Rhizosphere microbes interactions in medicinal plants. In *Plant-growth-promoting rhizobacteria (PGPR) and medicinal plants* ed. Egamberdieva, D., Shrivastava, S., Varma, A. pp. 19–41. Cham, Switzerland: Springer.

Stenfors Arnesen, L.P., Fagerlund, A. and Granum, P.E. (2008) From soil to gut: *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiol Rev* **32**, 579–606.

Swiss Federal Department of Home Affairs (2016) Hygieneverordnung (HyV) SR 817.024.1 ed. updated 1 May 2017.
Swiss Federal Office of Public Health (2009) Schweizerisches Lebensmittelbuch (SLMB). Kapitel 56: Mikrobiologie.
Tamilarasi, S., Nanthakumar, K., Karthikeyan, K. and Lakshmanaperumalsamy, P. (2007) Diversity of root associated microorganisms of selected medicinal plants and influence of rhizomicroorganisms on the antimicrobial property of *Coriandrum sativum*. *J Environ Biol* **29**, 127.
Thunberg, R.L., Tran, T.T., Bennett, R.W., Matthews, R.N. and Belay, N. (2002) Microbial evaluation of selected fresh produce obtained at retail markets. *J Food Prot* **65**, 677–682.
Tournas, V.H. (2005) Spoilage of vegetable crops by bacteria and fungi and related health hazards. *Crit Rev Microbiol* **31**, 33–44.
Vorholt, J.A. (2012) Microbial life in the phyllosphere. *Nat Rev Microbiol* **10**, 828–840.
Wetzel, K., Lee, J., Lee, C.S. and Binkley, M. (2010) Comparison of microbial diversity of edible flowers and basil grown with organic versus conventional methods. *Can J Microbiol* **56**, 943–951.
Wevers, E., Moons, P., Van Houdt, R., Lurquin, I., Aertsen, A. and Michiels, C.W. (2009) Quorum sensing and butanediol fermentation affect colonization and spoilage of carrot slices by *Serratia plymuthica*. *Int J Food Microbiol* **134**, 63–69.
Wießner, S., Thiel, B., Krämer, J. and Köpke, U. (2009) Hygienic quality of head lettuce: effects of organic and mineral fertilizers. *Food Control* **20**, 881–886.
Williams, T.R., Moyne, A.-L., Harris, L.J. and Marco, M.L. (2013) Season, irrigation, leaf age, and *Escherichia coli* inoculation influence the bacterial diversity in the lettuce phyllosphere. *PLoS ONE* **8**, e68642.
Wirth, T., Falush, D., Lan, R., Colles, F., Mensa, P., Wieler, L.H., Karch, H., Reeves, P.R. *et al.* (2006) Sex and virulence in *Escherichia coli*: an evolutionary perspective. *Mol Microbiol* **60**, 1136–1151.
Yates, M.V. (2007) Classical indicators in the 21st century — far and beyond the coliform. *Water Environ Res* **79**, 279–286.
Zheng, L., Bae, Y.-M., Jung, K.-S., Heu, S. and Lee, S.-Y. (2013) Antimicrobial activity of natural antimicrobial substances against spoilage bacteria isolated from fresh produce. *Food Control* **32**, 665–672.
Zogg, A.L., Zurfluh, K., Nüesch-Inderbinen, M. and Stephan, R. (2016) Characteristics of ESBL-producing *Enterobacteriaceae* and methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from Swiss and imported raw poultry meat collected at retail –▫–level. *Schweiz Arch Tierheilk* **158**, 451–456.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Weather data collected from the meteorological station of Syngenta in Dielsdorf, Switzerland, for thyme grown in the open field in the period of the sampling. Time points of sampling and final harvest are indicated. For the temperature, daily minimal and maximal values were measured (a). The average daily relative humidity (%) was calculated as well as the sum of the daily precipitation (l m^{-2}) (b). Squares: daily maximal temperature measured at 2 m above ground; circles: daily minimal temperature measured at 2 m above ground; triangles: percent relative humidity measured at 2 m above ground; crosses: sum of daily precipitation. (–▫–) T_{max}; (–○–) T_{min}; (–▽–) rel. humidity; (–+–) precipitation.

