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Research Article

DGGE and real-time PCR analysis of lactic acid bacteria in bacterial communities of the phyllosphere of lettuce

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Food associated indigenous microbial communities exert antagonistic effects on pathogens and may routinely deliver health relevant microorganisms to the GI tract. By using molecular, culture independent methods including PCR-DGGE of 16S rDNA-coding regions and real-time PCR (RT-PCR) as well as BIOLOG metabolic fingerprinting, microbial communities on lettuce were analyzed in samples from fields, from supermarkets and soil. Amplified 16S rRNA gene sequences (57.7%) could be assigned to species previously reported as typical for the phyllosphere including *Pantoea agglomerans, Pseudomonas flavescens, Moraxella* spp., and *Mycobacterium* spp. 71.8% of the sequences obtained represented so far undescribed taxa. Principal component analysis of BIOLOG metabolic profiles indicated a seasonal variation in the lettuce phyllosphere microbial community structure. Various lactic acid bacteria were detected including several *Lactobacillus* and *Leuconostoc* species in particular on lettuce from organic farming. By RT-PCR lactobacilli were found with a range of abundances from 1×10^4 to 1×10^5 copies/g lettuce. Considering the importance of salad in many diets lettuce may contribute to a constant supply with LAB.

Keywords: DGGE / Lactic acid bacteria / Organic agriculture / Phyllosphere / RT-PCR

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1 Introduction

Epiphytic bacterial populations on plants of the same species differ due to large fluctuations in the physical and nutritional conditions characteristic of the phyllosphere [1]. However, Yang *et al.* [2], using molecular methods, suggested that every plant species possess a typical microflora. Handschur *et al.* [3] confirmed by culture independent molecular methods that *Pseudomonas* and *Enterobacteriaceae* are the most abundant bacterial taxa in the phyllosphere of leafy vegetables such as lettuce. For soil it could be shown that different farming methods result in variations in bacterial populations [4]. Phillips and Harrison [5] compared conventionally and organically grown salad vegetables using culturing techniques without finding significant differences. They counted 10^5 lactobacilli *per* g spring mix. Weiss *et al.* [6] also described sprouts as a natural source for lactic acid bacteria (LAB). For minimally processed, frozen and prepacked vegetables, lactobacilli counts of 2.9 – 5.6 cfu/g have been reported [7]. In Austria the organically managed acreage has reached 13.5% of the total area under cultivation. A regular consumption of leafy vegetables like lettuce is typical in many areas worldwide. The Austrian food report 2006 indicates that 6.7 kg of lettuce is consumed *per* person *per* year. The indigenous microflora of lettuce has been proven to exert antagonistic effects on pathogens such as *Salmonella typhimurium*, *Staphylococcus aureus*, and *Listeria innocua* and to have an industrial potential for use as protective cultures [8]. To date little is known about food-associated microorganisms and their fate in the human GI tract. LAB found in food have also been identified in feces [9], *e.g.*, *Lactobacillus rhamnosus*, *L. reuteri*, *Leuconostoc mesenteroides*, *Streptococcus salivarius* subsp. *thermophilus*, bifidobacteria, as well as some

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Abbreviations: APS, ammonium persulfate; **LAB,** lactic acid bacteria; **PCA,** principal component analysis; **TAE,** Tris-acetate-EDTA buffer (pH 8.0); **TEMED,** *N*,*N*,*N*′,*N*′-tetramethylethylenediamine

Enterococcus species [10]. Ecological studies indicate that *Lactobacillus* species found in the human gastrointestinal tract are likely to be transient, originating from foods and the oral cavity and that some lactobacilli found in human feces are allochthonous to the intestine [11].

Experiences with probiotic strains suggest that newly introduced strains usually cannot permanently establish in the human gut system without continuous delivery. Despite this limited colonization ability it was demonstrated that probiotic bacteria exert some immune-modulatory effects and may improve various immune functions [12, 13].

Health consequences of the delivery of probiotic lactobacilli and/or bifidobacteria are currently the subject of public and scientific interest. However, consequences of the delivery of epiphytic bacteria present on vegetables to the gastro-intestinal tract are rarely investigated and therefore poorly understood. The bacterial community structure of lettuce (*Latuca sativa*) grown either by organic or conventional cultivation practices was therefore investigated by PCR-DGGE community profiling and clone library analysis. Genotypic data were supplemented with metabolic fingerprinting and real-time PCR (RT-PCR) analysis.

2 Materials and methods

2.1 Samples

Leaves of *L. sativa* (lettuce) were collected from two closely neighboring fields under organic and conventional management in two growing seasons. Soil samples were taken at the sites. Additionally, lettuce samples were purchased in supermarkets in Vienna, Austria. Thirty-five leaves of lettuce from organic and conventionally managed fields were analyzed from each sampling site. Samples were taken in summer and autumn. At every sampling time, leaves were collected both from fields and from the retailer. All samples were transported in a cooled box and processed immediately.

2.2 Agricultural practices and soil conditions

The conventionally grown lettuce was fortified with a commercially available preparation based on natriumnitrate. Three kg/ha Kerb (Propyzamide 50%), Fastac (alpha-cypermethrin), Agritox (Chlorpyrifos), and Rhizolex were applied to soil prior to planting. The farmer avoids depletion of nutrients in soil by exchanging fields with a farmer who grows grains, mostly wheat or rape. The fields were watered with groundwater. The organically produced lettuce was fortified with 100 kg/ha cattle-hair-pellets (ECOSAN). The fruit change plan is made up of alternating kale, carrots, spinach, and lettuce. As intermediate fruit peas, beans, or mustard are brought out on the fields and worked into the soil again in spring. The fields were watered with ground water. Soil conditions were similar in both farms; they are located in close proximity to each other. It is light to medium soil with 60 – 120 cm depth, underneath is rubble. The proportion of humus is approximately 2%, in organic agriculture the proportion of humus shows increasing tendency.

2.3 Extraction of bacterial DNA from phyllosphere

Leaves were rinsed with PBS (1 × PBS 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4) to wash off soil debris. Subsequently, samples (33 ± 4 g) were placed in 400 mL bagfilters (Interscience) and submerged in washing buffer (1 × PBS) and inversed ten times to wash off lighter adhering microorganisms. In a second washing step the leaves were again submerged in washing buffer and placed in a stomacher for 50 s. Both buffer suspensions were combined and centrifuged at 9500 rpm (12364.2 × *g*) at 4°C for 20 min (Hermle ZK 401, rotor A6.9). DNA was extracted from the resulting cell pellet with the FastDNA-Spin-Kit for Soil (MP-Biomedicals) with minor modifications to the protocol of the manufacturer. For purification of the eluates, the QIAamp Viral RNA Mini Kit (QIAGEN) was used following the protocol of the manufacturer. DNA was eluted three times with 80 µL nuclease-free water preheated to 80°C and immediately stored in several aliquots at −70°C. The third elution was directly used as template for PCR.

2.4 BIOLOG community-level metabolic fingerprinting

To obtain substrate utilization fingerprints of the microbial communities, three replicates of lettuce extracts were inoculated in BIOLOG EcoPlates (BIOLOG, Hayward, CA, USA) containing 31 different carbon-sources and a control without a carbon source. The microtiter plates were incubated for 4 days at room temperature in the dark. After incubation the OD at 590 nm (OD_{590}) in each well, produced from the reduction of tetrazolium violet, was recorded with an automatic plate reader (Tecan, Austria). Before the data were further processed, the OD_{590} from the control well was subtracted from the other readings, yielding the net OD_{590}. To reduce the dimensions of the highly multivariate dataset and to reveal latent associations between C sources and the communities, principal component analysis (PCA) was performed on the mean OD_{590} by using the C-sources as variables. The statistical processing was performed with "R" project for statistical computing [14] using the S3 method implemented in R. Principal components (PCs) were extracted until a maximum of 100% variance explained. Transformed data were plot in a bi-plot as a function of the first two PCs.

2.5 Qualitative PCR amplification

Fragments of the 16S small subunit ribosomal RNA gene were amplified using a ready-to-use PCR Mastermix (Promega). For primers used in this study see Table 1. Primer concentration in the reaction volume was 0.5 pM.

Table 1. Primers used for 16S rRNA-based analysis of phyllosphere bacterial communities of lettuce

Target taxon	Primer	Fragment length	Cycle numbers	Reference
Bacteria	341-985	644	35	[15, 16]
	341fGC-518	277	30	[15, 17]
LAB	156-Lac2	680	35	[18,19]
	341GC-Lac2	300	30	[15,19]
LAB	Lac1GC-Lac2	290	35	[19]
Lactobacilli (RT-PCR)	Lac*all*F-Lac*all*R	92	40	[20]
TaqMan probe	(FAM)-TAT TAG TTC CTT CAT C- (BHQ-1)			biomers.net

Nested PCR was performed in a 100 μL reaction volume with one primer containing a GC-clamp at the 5′ end [15]. The 100 μL PCR product was precipitated over night at –20°C. Fragment length was checked on a 2% (w/v) agarose gel and visualized under UV light after ethidium-bromide staining (0.5 mg/mL).

2.6 DGGE analysis

Eight percent (v/v in 0.5 × TAE buffer) polyacrylamide gels of 10 × 20 cm^2 size, 1 mm thin were prepared for DGGE with increasing content of denaturing agents. Solutions with a concentration of denaturing agents of 80 and 0% were used to obtain 10 mL of the minimum and maximum denaturing solution respectively. Forty percentage v/v formamide and 7 M urea were defined as 100% denaturing gradients.

Gradient gels were prepared using a gradient mixer (Hoefer SG 30) and a peristaltic pump. For polymerization 50 μL 10% APS-solution (ammonium-persulfate, w/v) and 7 μL TEMED were added.

2.7 Quantitative RT-PCR

DNA from lettuce phyllosphere samples was subjected to RT-PCR with a TaqMan system. Probe and primer set specific for lactobacilli was used as described elsewhere [20] without using the minor groove binding protein. The temperature profile for the amplification consisted of 3 min at 95°C followed by 55 cycles of 15 s at 95°C and 45 s at 58°C. Standard-dilutions of a suspension of *L. casei* DSM 20011^T in saline solution starting from a concentration of 10^6 cfu/mL were used for amount determination. Thus a standard curve covering the range of $10^3 – 10^6$ cfu/mL was obtained. In order to make the standards comparable to the phyllosphere DNA, a suspension of *L. casei* DSM 20011^T was extracted in duplicate using FastDNA-Spin-Kit for Soil (MP-Biomedicals) followed by purification with QIAamp Viral RNA Mini Kit (QIAGEN). This experiment allowed calculating absolute quantification since from the phyllosphere samples only the third elution from the purification procedure was suitable for amplification. TaqMan assay was performed using the Rotor-Gene 3000 (Corbett Research, Sydney, Australia) and the Quantimix Easy Probes Kit (Biotools, Madrid, Spain). Primer concentration in the reaction volume (10 μL) was 1 pM, probe concentration was 3 pM. The final concentration of $MgCl_2$ in the reaction volume was 4 mM.

2.8 Clone libraries

PCR products were inserted into a p-GEM Easy Vector (Promega) following the instructions of the manufacturer. Fifty clones were randomly picked *per* clone library and fragment size checked in 2% agarose gels after amplification with primer pair T7-Sp6 (Promega). Clones were evaluated in DGGE with the primer pair suitable for the insert.

2.9 16S rRNA gene sequence analysis

Clones were amplified in a 100 μL reaction volume and purified with PCR purification kit (QIAGEN) to obtain sufficiently concentrated DNA. All sequences were examined for possible chimeric artifacts by the Check Chimera program of the RDP 8.1. (Rdp8.cme.msu.edu/) [21]. Sequences were corrected using the function "trim vector" in CodonCode Aligner (CodonCode Corporation) in order to remove parts of the vector. Nucleotide sequences were analyzed with FASTA (www.ebi.ac.uk/fasta/) search and compared to previously published sequences in RDP 9.52. Phylogenetic trees were constructed using the weighbor weighted neighbor-joining tree building algorithm in the RDP-9.52 (rdp.cme.msu.edu/). Bootstrap support was calculated based on data for 100 resamplings in RDP 9.52.

3 Results and discussion

PCR and nested PCR were used to amplify 16S rRNA gene sequences for DGGE and sequencing analysis to study predominant microbial populations in the phyllosphere of lettuce derived from organic and conventional agriculture. PCR-DGGE only reveals the predominant members in a bacterial community, and this approach does not allow making an inventory of all species in a sample. Detection limits are mainly affected by DNA isolation protocols [22].

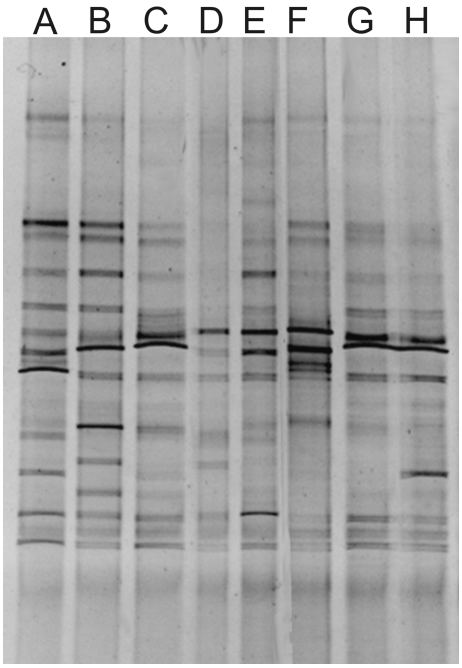

Figure 1. Comparison of DGGE profiles of lettuce phyllosphere and soil community. (A) soil sample from a field under organic management; (B), (C), organically grown lettuce from the field; (D), (E), organic lettuce from the market; (F), (G), conventionally grown lettuce from the field; (H), conventional lettuce from the market. Clone libraries were constructed from samples (D) and (F).

Furthermore, these methods cannot distinguish between active and dead microorganisms. However, they were considered to be the presently best-suited methods for this analysis. Furthermore, most DNA of dead organisms degrades fast as observed in different environments such as intestinal tract and outdoors [23, 24]. Despite proximity of the two fields and very similar composition of the soil as reported by the farmers, the composition of the soil could differ at small scales. This would in turn affect the soil microbial populations as observed in DGGE experiments and the phyllosphere community structure. Further bias may result from heterogeneous distribution of microorganisms on the leaf surface.

3.1 DGGE and clone libraries of predominant bacterial communities

The comparison of bacterial DGGE band patterns of lettuce phyllosphere and soil from the fields of origin (Fig. 1) showed that many bands were common to field soil and phyllosphere (*e. g. Pantoea agglomerans*, *Pseudomonas flavescens*, *Mycobacterium* sp.), whereas other bands were unique to either phyllosphere or field soil samples. The melting behavior of PCR products in DGGE of organic and conventional lettuce phyllosphere samples was similar with slight differences in intensity of individual bands. This indicates that in organically and conventionally grown lettuce the same species were present in the phyllosphere, but in different population sizes. From the field to the supermarkets, the DGGE bandpatterns of both organic and conventional lettuce phyllosphere samples showed no significant differences in their profiles indicating that most strains from the phyllosphere reach the consumer.

Using different primer combinations (Table 1) the predominant bacterial taxa in the lettuce phyllosphere could be determined. By 16S rDNA sequence database search 5 out of the 15 unique clones showed sequence similarities below 95% to previously described cultivated organisms. Seven out of fifteen sequences showed a 16S rDNA sequence similarity of 97 – 100% to previously described organisms (Table 2). The most abundant bacterial taxa (Fig. 2) detected on organically grown lettuce were *Moraxella* spp. (19/50), *P. agglomerans* (22/50), and *Pseudomonas* (4/50). On conventionally grown lettuce *Pseudomonas* spp. (19/50) were the most abundant taxa, followed by *Pantoea* (19/50) and *Raoultella* (8/50).

Much speculation addressed the microbial quality of organically grown crops. In this work some opportunistic pathogen (*e. g.*, *P. agglomerans*) but no obligate pathogen species could be detected. Supporters of organic agriculture claimed that low input farming methods result in elevated bacterial diversity. On organically grown lettuce we could detect 11 different bacterial genera, 8 different genera were detected on conventionally grown lettuce. In general more LAB could be identified from organic lettuce as described in Table 3. Phyllosphere is an ecosystem characterized by severe stress factors such as UV or limited availability of nutrients. However, LAB have been isolated from lettuce before possibly as a consequence of transfer from soil [8]. Therefore, consequences of different agricultural practices such as organic farming on soil may alter amounts of LAB transferred to the phyllosphere.

3.2 DGGE and clone library analysis of predominant LAB

By using the LAB primer pair 156-Lac2 complex DGGE bandpatterns were obtained. The bandpatterns of lettuce phyllosphere samples taken during summer differed from those collected in autumn (Fig. 3). In samples collected in autumn LAB could only be detected in the phyllosphere of organically grown lettuce.

Twenty-four clones were obtained from clone libraries with primer pair 156f-Lac2r (Table 3). Most of these clones originate from organic lettuce and only three sequences with different melting behavior were found in libraries derived from conventionally grown lettuce. Analyses showed multiple DGGE bandpatterns in all LAB clones except for *Leuconostoc* spp. due to differences in the rRNA operons [25]. By 16S rDNA database search 4 out of 24

Table 2. Similarities of 16S rRNA gene sequences amplified from lettuce phyllosphere bacterial community

Clone origin	Accession number	% Similarity	Classification	Closest match
A	AF208620	99.3	*Enterobacteriaceae* (family) *Pantoea* (genus)	*P. agglomerans*
	DQ023307	98.7	*Pseudomonadaceae* (family) *Pseudomonas* (genus)	*P. graminis*
	AB074509	98.5	*Cyanobacteria* (phylum) *Oscillatoria* (genus)	*Oscillatoria* sp. PCC7112
	AF208620	93.2	*Enterobacteriaceae* (family) *Pantoea* (genus)	*P. agglomerans*
	AF060676	92.8	*Actinobacteria* (class) *Frankineae* (suborder)	Kineococcus-like bacterium
	AF124615	91.3	*Gammaproteobaceria* (class) *Pseudomonadales* (order)	*A. junii*
	AJ243871	89.8	*Actinobacteria* (class) *Rubrobacterineae* (suborder)	*Rubrobacter xyloanophilus*
B	U01916	100	*Pseudomonadaceae* (family) *Pseudomonas* (genus)	*P. flavencens*
	Z96081	99.0	*Enterobacteriaceae* (family) *Pantoea* (genus)	*P. ananatis*
	AF547895	99.0	*Mycobacteriaceae* (family) *Mycobacterium* (genus)	*Mycobacterium alvei*
	AB021398	98.7	*Pseudomonadaceae* (family) *Pseudomonas* (genus)	*P. cichorii*
	AJ233423	97.5	*Enterobacteriaceae* (family) *Pantoea* (genus)	*P. agglomerans*
	Z96083	95.2	*Enterobacteriaceae* (family)	*P. agglomerans*
	AB109886	94.8	*Pseudomonadaceae* (family)	*P. graminis*
	AF124615	91.1	*Gammaproteobacteria* (class) *Pseudomonadales* (order)	*A. junii*

A, organically grown lettuce; B, conventionally grown lettuce.

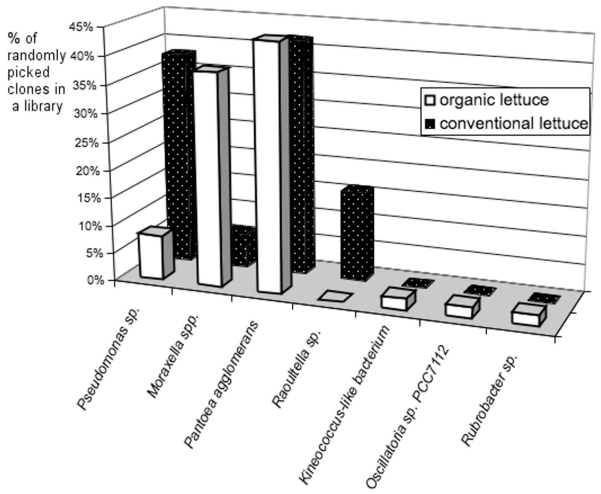

Figure 2. Frequency of randomly picked bacterial clones obtained from lettuce samples from organic and conventional agriculture with primer combinations amplifying predominant bacteria.

sequences (17%) showed matches >97%. Four sequences (17%) had sequence similarities between 95 and 97%. The majority of sequences (16/24, 76%), however, showed less than 95% similarity to described and cultivated species.

The abundance of bacterial species could be estimated from the abundance of sequences in 50 randomly picked clones. During construction of clone libraries from sequences with a primer combination amplifying LAB, the ligation and transformation efficiency was relatively low (<50 clones). Therefore, six clone libraries were constructed to evaluate reproducibility and reliability of the data. A dendrogram showing the phylogenetic relationships of clones identified as LAB is depicted in Fig. 4.

Some LAB from lettuce phyllosphere such as *Leuconostoc* spp. have previously been described as epiphytic species [26, 27]. Leuconostocs are frequently present on fruits and vegetables and are used as starter cultures in the dairy and bakery industry. Probiotic properties of *Leuconostoc* species have been investigated and an increase in β-galactosidase activity and enhanced pathogen resistance of the host have been observed in animal trials [27]. *L. mesenteroides* has not only been identified in lettuce but has also been isolated from human gastrointestinal tracts [9, 28] indicating that some strains of this species are capable of surviving the passage through the alimentary tract. One sequence amplified matched next (96.6% sequence similarity) with the sequence of *L. reuteri* strain LU3 (AY735406) previously isolated from Korean fermented foods (Jung, M. J., Baek, D. H., unpublished). Strains of this species have been isolated and described from various food as well as from

Table 3. Similarities of LAB 16S rRNA gene sequences amplified from lettuce phyllosphere

Origin /clone	Clone number	Accession number	% Similarity	Classification	Closest match
A	2	AF144645	96.3	*Bacillaceae* (family) *Bacillus* (genus)	*Bacillus mycoides*
	5	X55133	94–94.8	*Lactobacillales* (order) *Enterococcaceae* (family)	*Enterococcus sulfureus*
	1	AY994312	91.3		*P. alcalifaciens* *Enterobacteriaceae* (family)
	1	AY647289	95.5	*Bacillaceae* (family)	*B. mycoides* *Bacillus* (genus)
Clone bs10	1	AF049742	95.0	*Lactobacillales* (order) *Lactobcillaceae* (family)	*Paralactobacillus selangorensis*
		DQ837632	98.0		Uncultured *Firmicutes* bacterium
Clones bs34, bs3, bs31	4	DQ837637	90.2	*Lactobacillales* (order)	Uncultured *Firmicutes* bacterium
		AY667701	88.9		*Lactobacillus apis*
		AF243155	83.1		*L. jensenii*
A*	6	M230350	93.2–95.2	*Lactobacillales* (order) *Leuconostocaceae* (family)	*L. mesenteroides*^{T}
	1	M230350	100	*Leuconostocaceae* (family) *Leuconostoc* (genus)	*L. mesenteroides*^{T}
B	1	AF029364	98.9	*Planococcaceae* (family) *Planomicrobium* (genus)	*Planomicrobium alkanoclasticum*
	1	AF111948	99.5	*Leuconostocaceae* (family) *Leuconostoc* (genus)	*L. citreum*
	1	AY735406	96,6	*Lactobacillaceae* (family) *Lactobacillus* (genus)	*L. reuteri* LU3

A, organically grown lettuce from summer season; A*, organically grown lettuce from autumn season; B, conventionally grown lettuce from summer season.

human microbiota [8, 11, 19, 20, 29]. In feces of infants *L. reuteri* has been found to represent 1.3–6.4% of total lactobacilli [20]. The probiotic properties of selected strains of *L. reuteri* have been demonstrated in weaning pigs and rats [30, 31].

The primer set 156f-Lac2 was used in this study to identify a broader range of LAB in the phyllosphere of lettuce. Primer 156f [19] was originally designed as a probe for FISH (fluorescence *in situ* hybridization). Heilig *et al.* [9] reported that primer 156f was not highly specific for lactobacilli. In accordance with this report members of Gammaproteobacteria (*e.g. Providencia*) in addition to a high diversity of LAB were recovered from clone libraries. The use of the reportedly more specific primer combination Lac1GC-Lac2 [9, 29] resulted in amplification of only one sequence. This sequence showed highest 16S rDNA sequences similarity (94%) to *Exiguobacterium undae* strain 190-11 (AY444838) recently isolated from Siberian permafrost [32]. Exiguobacteria have been found in quite diverse habitats (*e.g.*, [33–35]).

3.3 Quantification of lactobacilli

As DGGE analysis and clone libraries revealed considerable presence of LAB in phyllosphere of lettuce, lactobacilli were quantified with a group specific TaqMan RT-PCR assay (Fig. 5). Quantitative PCR was performed with primers amplifying the intergenic spacer of 16S-23S rRNA gene because this region is less conserved than the 16S rRNA gene sequence [20].

Using this approach lactobacilli were clearly detected in lettuce phyllosphere samples. Gene levels ranged from 1×10^4 to 1×10^5 copies/g lettuce (Table 4). These copy counts resulted from lettuce that had been rinsed just as a consumer would prior to consumption. Rinsing before DNA extraction should adjust lettuce processing to common practice in households. Koseki and Isobe [36] stated that washing of iceberg lettuce did not initially reduce bacterial counts. Handschur *et al.* [3] recently reported similar results. Standard curves for lactobacilli were constructed from *L. casei* DSM 20011^T following the same DNA extraction and purification protocol as for the lettuce and soil samples. The calculated counts of lactobacilli *per* g lettuce are comparable to culture-dependent experiments, where lactobacilli have also been observed at a range of 10^4–10^5 cfu/g lettuce [5]. RT-PCR experiments comparing group and strain specific quantification would be desirable. In coherence with results from PCR-DGGE experiments lactobacilli could not be detected in conventional lettuce from autumn but in all samples from summer and in all organic lettuce samples.

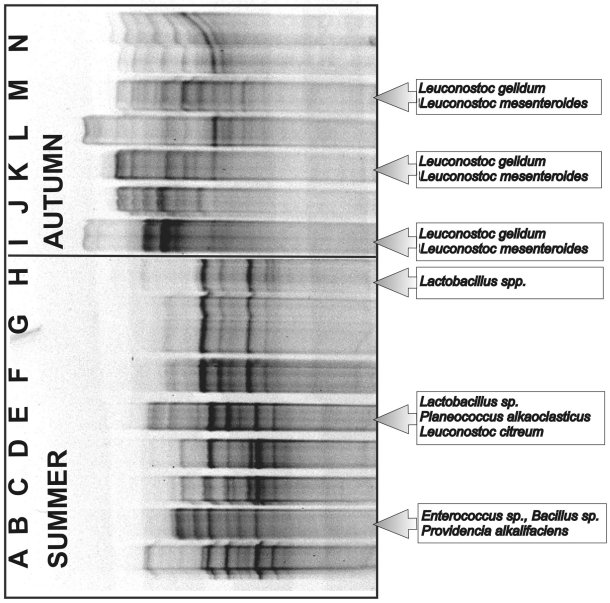

Figure 3. DGGE phyllosphere profiles of organic and conventional cultivated lettuce obtained with primer combinations LAB. (A), (B), organically grown lettuce; (C), (D), (E), conventionally grown lettuce; (F), (G), (H), organic lettuce from the market; (I), (J), (K), organic lettuce from the field; (L), (M), (N), organic lettuce form the market. Arrows mark the samples from which clone libraries have been constructed. Organisms identified from clone libraries are written in the arrows.

3.4 BIOLOG fingerprinting

Analysis of the metabolic fingerprints of the lettuce phyllosphere communities allowed calculating PCs according to the variance in the dataset (Fig. 6). PC1 explained 49.3% of the variance. PC2 explained 17.8% of the variance. The lettuce samples collected in summer were separated from the samples collected in autumn along PC1. Findings obtained by PCA of BIOLOG data suggest that seasonal differences rather than different farming methods account for variance in the data. Carbon source oxidation spectrum (PC1 and PC2) of the lettuce phyllosphere microbial community is given in Table 5. These results are congruent with the different DGGE bandpatterns especially for those of LAB. Diverse LAB were detected in the lettuce phyllosphere in summer. Whereas, in autumn only *Leuconostoc* spp. were detected.

3.5 Health consequences

Currently, much effort is devoted to screening for potential bioprotective strains with probiotic properties. These are mostly characteristics that might enable the microbes to at least survive passage through the digestive tract. Therefore, a potential bioprotective strain must be resistant to gastric juices and be able to grow in the presence of bile under conditions in the intestine [37]. Furthermore, bactericidal activity (antagonistic properties), fermentative capacities, and

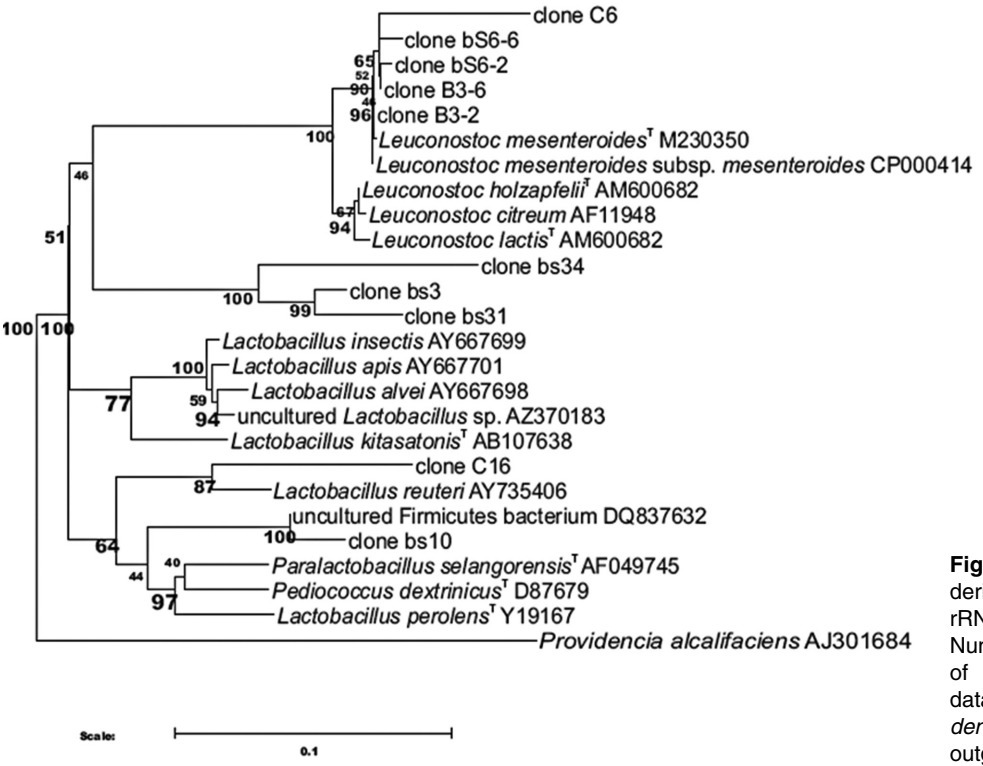

Figure 4. Phylogenetic tree derived from the analysis of 16S rRNA gene sequences of LAB. Numbers at nodes indicate levels of bootstrap support, based on data for 100 resamplings. *Providencia alcalifaciens* was used as outgroup.

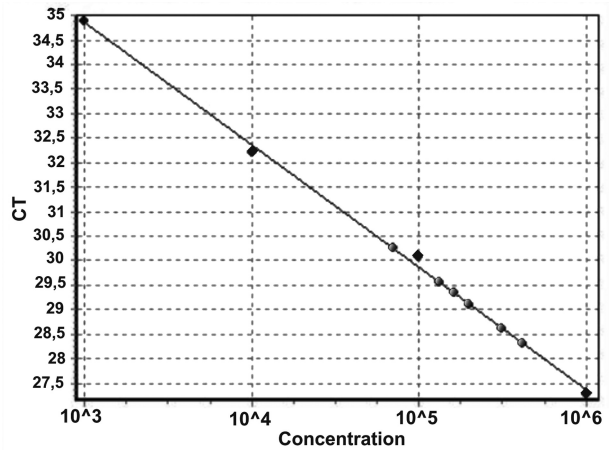

Figure 5. Standard curve derived from RT-PCR using a primer pair and probe targeting lactobacilli in a TaqMan system. Standard curve was constructed from *L. casei* DSM 20011^T; ♦ standard; ●, lettuce samples.

Table 4. RT-PCR analysis of lettuce phyllopshere using lactobacilli specific primers and TaqMan probe

Sample	Ct	Copies/mL	Copies/g lettuce
summer con1	28.32	416 788	1.0×10^5
summer con7	30.25	69 722	1.5×10^4
summer con6	29.33	163 363	3.6×10^4
summer org3	28.62	315 076	7×10^4
summer org6	25.4	200 739	1×10^4
autumn org16	29.57	131 240	2.9×10^4
autumn org 13	29.11	199 672	4.4×10^4
autumn con1	n.d.	n.d.	n.d.
autumn con2	n.d.	n.d	n.d.

Org, organic lettuce; con, conventional lettuce; Ct, threshold cycle; n.d. not detected.

Table 5. Community-level metabolic fingerprints of lettuce phyllosphere microorganisms using BIOLOG EcoPlates

PC1	PC2
α-Cyclodextrin	D-xylose
i-Erythritol	D-mannitol
Phenylethylamine	D-malic acid
β-Methyl-D-glucoside	D-Cellobiose
D-galacturonic acid	*N*-Acetyl-D-glucosamine
Glycogen	L-Serine
Tween 80	D-Galactonic acid c-lactone
L-phenylalanine	Itaconic acid
L-threonine	4-Hydroxy benzoic acid
L-Arginine	Glucose-1 phosphate
2-Hydroxy-benzoic acid	Glycyl-L-glutamic acid

Fingerprinting contributing to the first two PCs (PC1 and PC2) in descending order.

cell numbers of bacteria in foods are among important criteria for probiotic claims [38].

However, activities in the colon and small intestine need to be differentiated since interactions with immune functions are especially important in the small intestine [38]. On the other hand, Tannock [39] mentioned that any nonpathogenic strain from the human gastrointestinal tract might be seen as an important probiotic member of the microbiome.

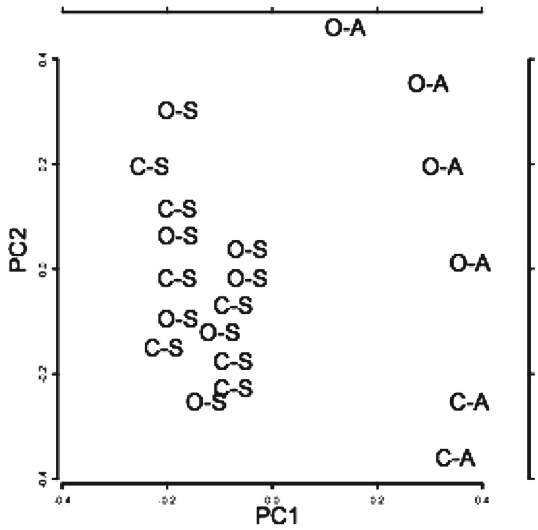

Figure 6. PCA of BIOLOG metabolic fingerprints of lettuce phyllosphere samples from two different vegetation periods. O, organically grown lettuce; C, conventionally grown lettuce; S, summer season; A, autumn season. PC1 43.3%, PC2 17.8% variance. The vectors representing the variables have been removed for clarity.

Reduced bacterial loads in modern food production have been linked to abnormal immune responses such as atopic diseases as discussed in the light of the Hygiene Theory [40, 41]. "Non-pathogenic food associated microorganisms contributing to functions of the gastrointestinal microbiome might thus be seen as health promoting constituents of food in general" (W. P. Hames 2007, p.comm.).

4 Concluding remarks

The present work showed that the phyllosphere of lettuce is characterized by a high bacterial diversity including *Actinobacteria*, *Enterobacteriaceae*, *Pseudomonadaceae*, and LAB such as *Leuconostoc* and *Lactobacillus* spp. The majority of amplified 16S rRNA gene sequences of the phyllosphere bacterial community belonged to so far undescribed bacterial taxa. Lettuce may be seen as a natural source for LAB. Effects of these LAB on health of consumers need to be elucidated.

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