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Screening of microbial communities associated with endive lettuce during postharvest processing on industrial scale

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Abstract

In this study, the composition of the microbial community on endive lettuce (*Cichorium endivia*) was evaluated during different postharvest processing steps. Microbial community structure was characterized by culture-dependent and culture-independent methods. Endive lettuce was sampled exemplarily at four different stages of processing (raw material, cut endive lettuce, washed endive lettuce, and spin-dried (ready to pack) endive lettuce) and analysed by plate count analysis using non-selective and selective agar plates with subsequent identification of bacteria colonies by matrix-assisted laser desorption/ionization time-of light mass spectrometry (MALDI-TOF MS). Additionally, terminal-restriction fragment length polymorphism (TRFLP) analysis and 16S rRNA gene nucleotide sequence analysis were conducted.

The results revealed structural differences in the lettuce microbiomes during the different processing steps. The most predominant bacteria on endive lettuce were detected by almost all methods. Bacterial species belonging to the families

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Pseudomonadaceae, *Enterobacteriaceae*, *Xanthomonadaceae*, and *Moraxellaceae* were detected in most of the examined samples including some unexpected potentially human pathogenic bacteria, especially those with the potential to build resistance to antibiotics (*e.g.*, *Stenotrophomonas maltophilia* (0.9 % in cut sample, 0.4 % in spin-dried sample), *Acinetobacter* sp. (0.6 % in raw material, 0.9 % in cut sample, 0.9 % in washed sample, 0.4 % in spin-dried sample), *Morganella morganii* (0.2 % in cut sample, 3 % in washed sample)) revealing the potential health risk for consumers.

However, more seldom occurring bacterial species were detected in varying range by the different methods. In conclusion, the applied methods allow the determination of the microbiome's structure and its dynamic changes during postharvest processing in detail. Such a combined approach enables the implementation of tailored control strategies including hygienic design, innovative decontamination techniques, and appropriate storage conditions for improved product safety.

Keywords: Food safety, Food technology, Microbiology

1. Introduction

Fresh produce is minimally processed (*i.e.* often only washed, cut, and packaged) and commonly consumed raw. In recent years, the consumption of fresh fruits and vegetables increased by 4.5 % each year worldwide [1], with packaged lettuces as the mainly consumed fresh-cut products. Currently, packaged lettuce possesses a fresh-cut market volume of 50 % [2]. Concurrently, an increasing number of outbreaks of human diseases could be associated with the consumption of contaminated fresh products such as fruits and vegetables [3]. In this context, the combination of leafy greens eaten raw and *Salmonella* spp. was the top food/pathogen combination for foodborne diseases in Europe between 2007 and 2011 [4]. Therefore, microbial safety of fresh-cut produce while maintaining high product quality is mandatory and poses a high challenge for the fresh-cut industry.

The adhesion of pathogenic bacteria on fresh produce surfaces, as example, on lettuce leaves, the penetration of these bacteria into the tissue, as well as the presence of multi-resistant bacteria hamper the reduction of microorganisms during washing processes and disinfection treatments. The most relevant pathogenic microorganisms in fresh produce are verotoxigenic *Escherichia coli* strains (occurring *e.g.* in sprouts and leafy greens), *Listeria monocytogenes* (occurring *e.g.* in melons and fresh cut salad), *Salmonella* spp. (occurring *e.g.* in tomato, seeds sprouts, spices), *Shigella* (occurring *e.g.* in green onion), and *Norovirus* (occurring *e.g.* in berries) [5]. Hence, routinely applied microbiological sampling along the food processing chain is mainly focused on selected indicator microorganisms even so the composition of microbial diversity on fresh produce is not known in detail [6]. In consequence,

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unexpected potentially human pathogenic bacteria can remain undetected and could result in foodborne outbreaks.

The evaluation of microbial diversity on fresh produce is mainly focused on the processed products [6, 7, 8, 9]. Only few studies are dealing with the impact of the processing steps on the microbial communities of fresh-cut products [10, 11]. It is known that contamination of vegetables can occur at different steps of processing (*e.g.,* during primary production, processing, distribution, and preparation) [12]. Detailed knowledge of the community structure and development of the microbial load along the processing chain will support the implementation of decontamination strategies and this way increase the product safety.

Qualitative testing of the absence/presence of pathogenic bacteria is sufficient regarding the microbial product safety and product releases, whereas for a complete risk assessment quantitative testing such as enumeration assays is necessary [5]. The structure of the microbial community on food can be evaluated by culture-independent and culture-dependent methods. Culture-independent methods used for the evaluation of microbial communities in food samples are based on analysing the microbial genomic DNA. Such methods can be divided into genetic fingerprinting analysis (*e.g.,* terminal-restriction fragment length polymorphism, TRFLP), *in situ* hybridisation, amplification techniques [13] and high-throughput sequencing approaches based on DNA [14]. Advantages of these nucleic acid based methods are the high sensitivity and specificity, reliable results, the automation, and the short time required [15]. However, DNA-based approaches are hardly able to distinguish between viable and non-viable microorganisms [16]. Especially in terms of foodborne pathogens the knowledge of bacterial viability is indispensable to estimate the potential risk of foodborne pathogens.

Culture-dependent methods include the conventional plating technique, which is more time-consuming than most culture-independent methods because it relies on the ability of bacteria to proliferate and form visible colonies on specific agar plates at specific temperatures and particular atmospheres which can strongly vary between different groups of bacteria. Additionally, for the specific characterization of bacterial colonies, a biochemical screening and serological confirmation is required [15]. In this context, matrix-assisted laser desorption/ionization time-of light mass spectrometry (MALDI-TOF MS) enables a rapid identification of bacteria up to the strain level and is a promising tool for the rapid and reliable identification of foodborne bacteria [17]. The spectra of bacterial cells are characteristic for a given taxon because they are dominated by the peaks of ribosomal proteins and changes in cultivation conditions only marginally influence the spectra [18].

The application of MALDI-TOF MS in food microbiology is mainly focused on the identification of known foodborne bacteria [17, 19, 20, 21, 22], however, the application of MALDI-TOF MS for the evaluation of microbial communities in food or

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environmental samples is of growing interest [23, 24, 25, 26, 27, 28, 29]. Basically, the applicability of MALDI-TOF MS in bacterial diversity studies was shown by Spitaels et al. [30].

The microbial community of fresh produce depends on the specific product properties as well as on the preharvest and postharvest processing conditions and varies from case to case. Hence, to obtain knowledge about the composition of the microbial community of endive lettuce, the aim of this study was to evaluate exemplarily the microbial community of endive lettuce, a frequently used lettuce in ready-to-eat salads, and its changes along a commercial processing chain using a combination of culture-dependent and culture-independent methods. To evaluate the viable microbial diversity as accurate as possible, microbial plate count analysis with selective and non-selective media was conducted with subsequent identification of grown bacterial colonies by MALDI-TOF MS. In addition, to obtain information on the non-cultivable part of the microbial community and its dynamics along the processing chain of lettuce, 16S rRNA gene based TRFLP fingerprint analyses supported by a cloning/sequencing approach were conducted.

2. Material and methods

2.1. Sampling of endive lettuce from a fresh-produce production facility

Endive lettuce (*Cichorium endivia*) from four different sections of a large-scale fresh-cut salad process chain in Germany were sampled exemplarily during processing: (A) raw material, (B) cut, (C) washed, and (D) spin-dried (ready to pack) lettuce. From the selected batch about 500 g product were taken from the before defined steps along the processing line. Washing of the lettuce was conducted without the addition of disinfectants. After sampling, the lettuce samples were stored at temperatures below 5 °C, and analyses were performed within 24 h. The samples were mashed by an immersion blender (Gastroback 40974 Stabmixer Advanced, 800 W, Gastroback GmbH, Germany) to allow the evaluation of microorganisms attached to the surface as well as internalized microorganisms. A total of 25 g of each sample was added to 225 ml peptone salt solution (EN ISO 6887-1 [31]) and homogenized in a shaker (Labotron, Infors AG, Switzerland) at 400 rpm for 20 min. Then, the mashed samples were filtered through a folded filter (pore size five − eight μm). The filtrated samples were used for plate count analyses and the DNA extraction. Before DNA extraction the samples were stored at − 20 °C.

2.2. Viable cell counts and MALDI-TOF MS analysis

Plate count analyses using selective and non-selective media were conducted according to the respective German and European reference standards (Table 1) to ensure

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Table 1. European and German standards applied for the evaluation of viable cell counts.

Target	Norm resp. analytical method
Aerobic mesophilic total viable cell count	EN ISO 4833:2 [32]
Aerobic lactic acid bacteria	EN ISO 15214: 1998 [33]
Lactobacilli	BVL L 06.00-31:1992-06 [34]
Yeast and moulds	ISO 21527-1:2008 [35]
Enterobacteriaceae	EN ISO 21528-2:2009-12 [36]
E. coli	ISO 16649-2:2001:2001-04 [37]
Bacillus cereus	EN ISO 7932:2005-03 [38]
Pseudomonas spp.	EN ISO 13720:2010-12 [39]
Coagulase-positive *staphylococci*	IS0 6888-1:1999/Amd.l:2003(E) [40]
Enterococcus sp.	BVL L 06.00-32:1992-06 [41]
Clostridium perfringens	ISO 7937:2004-08 [42]
Mesophilic sulphite-reducing bacteria	BVL L 06.00-39:1994-05 [43]
Salmonella spp.	EN ISO 6579:2007-10 [44]
Listeria monocytogenes	EN ISO 11290-1:2005-01 [45]
Yersinia enterocolytica	EN ISO 10273:2003-06 [46]
Mesophilic spore-forming bacteria	EN ISO 6887-1: [31]

the best possible detection of all cultivable microorganisms in the samples. Each sample was serially diluted and subsequently analysed in triplicates.

To ensure that the variety of grown colonies was included in MALDI-TOF MS identification, colonies with different colour and morphology as well as randomly selected colonies from all inoculated selective and non-selective agar plates were sampled with the aim to obtain the best possible summary of the microbial diversity.

Prior to MALDI-TOF MS analysis, cell material of the colonies was transferred to a target and was overlaid with α-cyano-4-hydroxy cinnamic acid (CHCA) matrix (RI-PAC-LABOR GmbH, Germany). After air drying, the samples were analysed by MALDI-TOF MS (Axima Confidence, Shimadzu Deutschland GmbH, Germany). Recording of the spectra was conducted in the linear mode with a laser frequency of 50 Hz. The mass range of the spectra was between 3,000 and 20,000 *m/z*. Calibration of the spectra was performed using *E. coli* ribosomal proteins. For identification of the bacterial colonies, the obtained mass spectra were compared with the reference mass spectra of the AnagnosTec SARAMIS™ database (Spectral ARchive And Microbial Identification System, bioMérieux Deutschland GmbH, Germany). All spectra were exported to BioNumerics (version 7.6; Applied Maths NV, Belgium) and cluster analysis (UPGMA clustering) was conducted using the peak based similarity coefficient 'Dice'. The linear tolerance was set to 800 ppm, the

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constant tolerance was set to two *m/z* [47]. Clusters were reliable classified as identified if a spectrum matched with reference spectra of the AnagnosTec SARAMIS™ database with a confidence level ≥90 %. Clusters with spectra matched with reference spectra within a confidence level between 75 and 89.9 % were only identified as microorganisms belonging to the bacterial or fungi domain whereas clusters with spectra matched with reference spectra with a confidence level below 75 % were classified as unidentified.

2.3. DNA extraction

Prior to DNA extraction, the endive samples were concentrated. Therefore, four ml filtered endive sample was centrifuged at 14,000 × g for two min. After removing the supernatant, the pellet was resuspended in one ml 1x PBS (pH 7.4). Then, the samples were centrifuged again, and the supernatants were discarded. The pellet was resuspended in one ml 1 % KCl and centrifuged again. The residual pellet was resuspended in distilled H_2O and was completely transferred to the Lysing Matrix E tube of the FastDNA™ SPIN Kit for Soil (MP Biomedicals LLC, USA). The subsequent extraction of microbial DNA was conducted according to the manufacturer's guidelines in duplicates for each process step. DNA was stored at 4 °C until further analyses.

2.4. TRFLP analysis

To determine the changes within the bacterial community along the endive process chain, a TRFLP fingerprint analysis was performed. Therefore, the two extracted DNA samples from each processing step were amplified twice applying a *Bacteria*-specific PCR using the following primers: forward primer 27f (5′- AGA GTT TGA TCM TGG CTC AG -3′) labelled with Cy5, and reverse primer 926r (5′- CCG TCA ATT CMT TTR AGT TT -3′) (Biomers.net GmbH, Germany). The thermal amplification protocol started with an initial step at 95 °C for three min, followed by 25 cycles consisting of a denaturation step at 94 °C for 30 sec, an annealing step at 51 °C for 30 sec and elongation step at 72 °C for 90 sec. After that, a final elongation at 72 °C for eight min was conducted. The reagent mixture consisted of 1x *Taq* buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM each primer, 1 U recombinant *Taq* polymerase, and 1 μl template DNA in a total volume of 25 μl (all reagents purchased from Thermo Fisher Scientific Inc, Germany). Both PCR products of one DNA sample were pooled and cleaned up using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel GmbH & Co. KG, Germany). Approximately 300 ng PCR product, estimated by the NanoPhotometer® (Implen GmbH, Germany), were used for restriction enzyme digestion applying 10 U of *Msp*I and *Hin*6I one after the other in 1x Tango buffer (all reagents: Thermo Fisher Scientific Inc, Germany) in a total volume of 20 μl at

https://doi.org/10.1016/j.heliyon.2018.e00671

37 °C for 4 h for each enzyme. After ethanol precipitation of the digestate, 0.5 μl of each restriction digest was analysed on GenomeLab™ GeXP Genetic Analysis System (Beckman Coulter GmbH, Germany) together with 0.2 μl 600 bp standard and 29.3 μl sample loading solution (Beckmann Coulter GmbH, Germany) applying following conditions: denaturation at 90 °C for two min, injection at 2 kV for 20 sec, and separation at 4.8 kV for a minimum of 70 min. For each DNA sample resp. pooled PCR product, two restriction digests resp. restriction fragment analyses were performed.

The results were exported to BioNumerics (version 7.6; Applied Maths NV, Belgium) and analysed. The search threshold parameters for the band search were set to 0.5 % OD range and two % curve range. Then, band matching was conducted using an optimization of 0.05 % and a position tolerance of 0.1 %.

2.5. 16S rRNA gene nucleotide sequence analysis

To receive detailed information about the bacterial community along the endive postharvest processing chain, a bacterial 16S rRNA gene library was constructed for each sampled process step. Therefore, a *Bacteria*-specific PCR was performed using the unlabelled forward primer 27f and the reverse primer 1492r (5′- TAC GGY TAC CTT GTT ACG ACT T -3′) (Biomers.net GmbH, Germany) in order to amplify a nearly full length 16S rRNA gene sequence. The thermal amplification protocol started with an initial step at 95 °C for three min, followed by 25 cycles consisting of a denaturation step at 94 °C for 30 sec, an annealing step at 51 °C for 30 sec and elongation step at 72 °C for 90 sec. After that, a final elongation at 72 °C for eight min was conducted. The reagent mixture consisted of 1x *Taq* buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM each primer, 1 U recombinant *Taq* polymerase and 1 μl template DNA in a total volume of 50 μl (all reagents purchased by Thermo Fisher Scientific Inc, Germany). DNA bands of expected size were cut out of the agarose gel and were cleaned up using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel GmbH & Co. KG, Germany). Ligation of PCR fragments into the pGEM®-T vector system and the transformation of vectors into JM109 competent cells was done according to the manufacturer's protocol (Promega Corporation, USA). White colonies were picked and grown over night at 37 °C in LB broth with ampicillin (0.05 mg ml^{-1}). Plasmids were isolated applying the NucleoSpin® plasmid kit (Macherey-Nagel GmbH & Co. KG, Germany) and afterwards all plasmids were checked for inserts originated from chloroplasts by RFLP analysis. On average, 69 % of plasmids showed a chloroplast typical RFLP fingerprint pattern and were omitted from the subsequent sequencing. This led in an uneven number of analysed plasmids or sequences, respectively, concerning the four 16S rRNA gene sequence libraries, *i.e.* (A) raw material 50, (B) cut 87, (C) washed 13, and (D) spin-dried (ready to pack) 10.

https://doi.org/10.1016/j.heliyon.2018.e00671

Sequencing of the selected inserts with the correct size and RFLP pattern was performed by GATC Biotech AG (Germany). Obtained nucleotide sequences were analysed with BioNumerics (version 7.6; Applied Maths NV, Belgium). After the assembly of the forward and reverse sequence, the nearly full length sequences were multiple aligned applying the Needleman-Wunsch algorithm and a clustal w similarity calculation followed by a Kimura-2 correction [48]. The obtained alignment was the basis for a cluster analysis (multiple alignment, UPGMA clustering) resulting in 29 OTUs with a sequence similarity of $\geq$97%. Then, up to three sequences of each OTU were classified by the Ribosomal Database Project (RDP) (rdp.cme.msu.edu) (confidence threshold 80 %). All nucleotide sequences were deposited in the EMBL-EBI European Nucleotide Archive with the accession numbers: LT595724–LT595883. The assignment of sequences to operational taxonomic units (OTUs) and samples origin with corresponding EMBL accession numbers is given in the supplemental material (Table S1).

3. Results

3.1. Microbial community structure and dynamics along the endive postharvest processing chain as revealed by TRFLP analyses and 16S rRNA gene sequence analyses

Along an endive postharvest processing chain, four different sections (raw material, cut, washed and spin-dried (ready to pack) lettuce) were sampled and analysed by TRFLP (Fig. 1) and 16S rRNA gene sequence analysis focusing on the bacterial load (Fig. 2A).

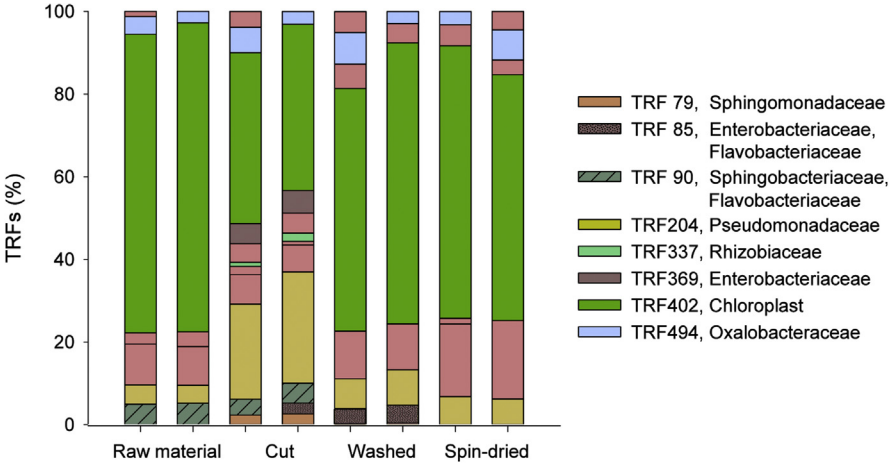

Fig. 1. TRFLP analyses of the bacterial community structure on endive along a processing chain. Affiliations of terminal restriction fragments (TRFs) were obtained by *in silico* and *in vivo* analyses. Each TRF profile was averaged from two technical replicates.

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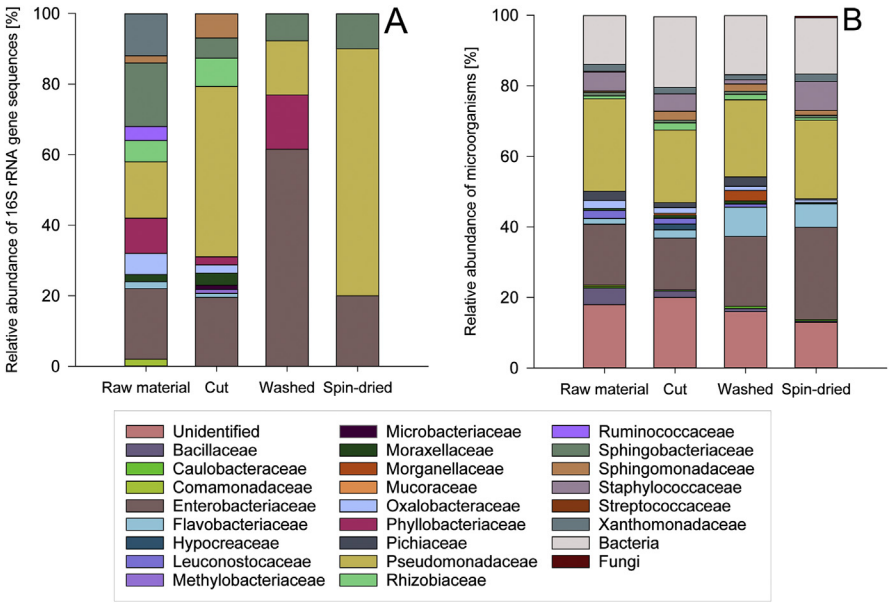

Fig. 2. 16S rRNA gene nucleotide sequence (A) and MALDI-TOF MS (B) analysis of the bacterial community structure on endive along the processing chain. The number of analysed sequences (raw material − 50 sequences, cut lettuce − 87 sequences, washed lettuce − 13 sequences, spin-dried (ready to pack) lettuce − 10 sequences) and colonies (raw material − 492 colonies, cut lettuce − 428 colonies, washed lettuce − 338 colonies, spin-dried (ready to eat) lettuce− 284 colonies) varied between samples.

During the processing of the endive lettuce, members of in total 14 bacterial families (29 OTUs) were identified by gene library construction as well as 15 TRFs by TRFLP analysis. The most prominent ones, *Pseudomonadaceae* (*Pseudomonas*), *Enterobacteriaceae* (*e.g.*, *Erwinia*, *Pantoea*, *Pectobacterium*), and *Sphingobacteriaceae* (*Pedobacter*), were detected in both analyses along the whole process chain. However, some changes within the bacterial community could be identified upon processing from raw material to spin-dried (ready to pack) lettuce. Beside the predominant families, on the raw material bacteria belonging to the families *Xanthomonadaceae*, *Phyllo-* and *Oxalobacteriaceae*, *Rhizobiaceae*, and *Sphingomonadaceae* have also been identified.

After cutting, members of the family *Pseudomonadaceae* were more prevalent than before as indicated by TRFLP and 16S rRNA gene sequence analyses. Furthermore, both analyses indicated an increase of *Sphingomonadaceae* (*Sphingomonas*). In contrast, members of the *Sphingobacteriaceae* were reduced.

After washing, the relative abundance of *Pseudomonadaceae* was reduced, whereas the relative abundance of *Enterobacteriaceae* (*e.g.*, *Erwinia*) and *Phyllobacteriaceae* (*Phyllobacterium*) increased. The bacterial load of the spin-dried (ready to pack) endive lettuce showed again a reduced relative abundance of *Enterobacteriaceae*. Herein, analysed 16S rRNA gene sequences could only be identified as unclassified *Enterobacteriaceae* as revealed by RDP ribosomal database.

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3.2. Microbial community structure and dynamics along the endive postharvest processing chain as revealed by MALDI-TOF MS analyses

The aerobic mesophilic viable count of the raw material was 7.6 ± 0.3 log CFU/g and 6.1 ± 0.3 log CFU/g for the spin-dried (ready to pack) endive lettuce. The total number of analysed colonies grown on selective and non-selective media was uneven along the endive processing chain. 492 colonies resulting from the raw material sample, 428 colonies from cut endive, 338 colonies from washed endive, and 284 colonies from spin-dried (ready to pack) endive were analysed by MALDI-TOF MS. Along the processing chain of endive lettuce, 54 % of all analysed colonies could not be reliable identified by MALDI-TOF MS using the SARAMIS^{TM} database due to the lack of reference mass spectra. It cannot be fully excluded that these not identifiable bacterial species are potential human pathogenic bacteria. To receive further information about these species, analysis of the 16S RNA gene of unidentified colonies was conducted by direct colony PCR with subsequent sequence analysis and identification using the NCBI Megablast tool in combination with the NCBI Reference Sequence Database RefSeq. With these analyses, the number of unidentified bacteria could be reduced along the endive processing chain, up to now, *i.e.* 18 % of the colonies from the raw material sample, 20 % of the colonies from the cut endive, 16 % of the colonies from the washed endive, and 13 % of the colonies from the spin-dried (ready to pack) endive showed a match with reference spectra of the SARAMIS^{TM} database with a confidence level <75 % and were therefore classified as unidentified (Fig. 2B).

The classification of the identified spectra to the family level using the SARAMIS^{TM} database revealed that in all samples along the processing chain bacteria belonging to the families *Pseudomonadaceae*, *Enterobacteriaceae*, *Flavobacteriaceae*, *Staphylococcaceae*, *Oxalobacteraceae*, *Rhizobiaceae*, *Xanthomonadaceae*, *Sphingomonadaceae*, *Sphingobacteriaceae*, *Moraxellaceae*, *Caulobacteraceae*, *Pichiaceae*, and *Bacillaceae* occurred (Figs. 3, 4, 5, and 6). Thereby, bacteria belonging to the *Pseudomonadaceae* and *Enterobacteriaceae* were predominant along the processing chain.

Bacteria belonging to the families *Streptococcaceae* and *Comamonadaceae* were only found in the raw material, bacteria of the families *Mucoraceae* and *Hypocreaceae* were only detected in the cut endive sample, and bacteria belonging to the family *Leuconostocaceae* were only found in the spin-dried (ready to pack) endive.

A further classification to the species level using the SARAMIS^{TM} database revealed four different bacteria species within the family *Xanthomonadaceae* (*Pseudoxanthomonas spadix, Stenotrophomonas* sp., *Stenotrophomonas rhiziphila, Stenotrophomonas maltophilia*) along the endive processing chain, of these

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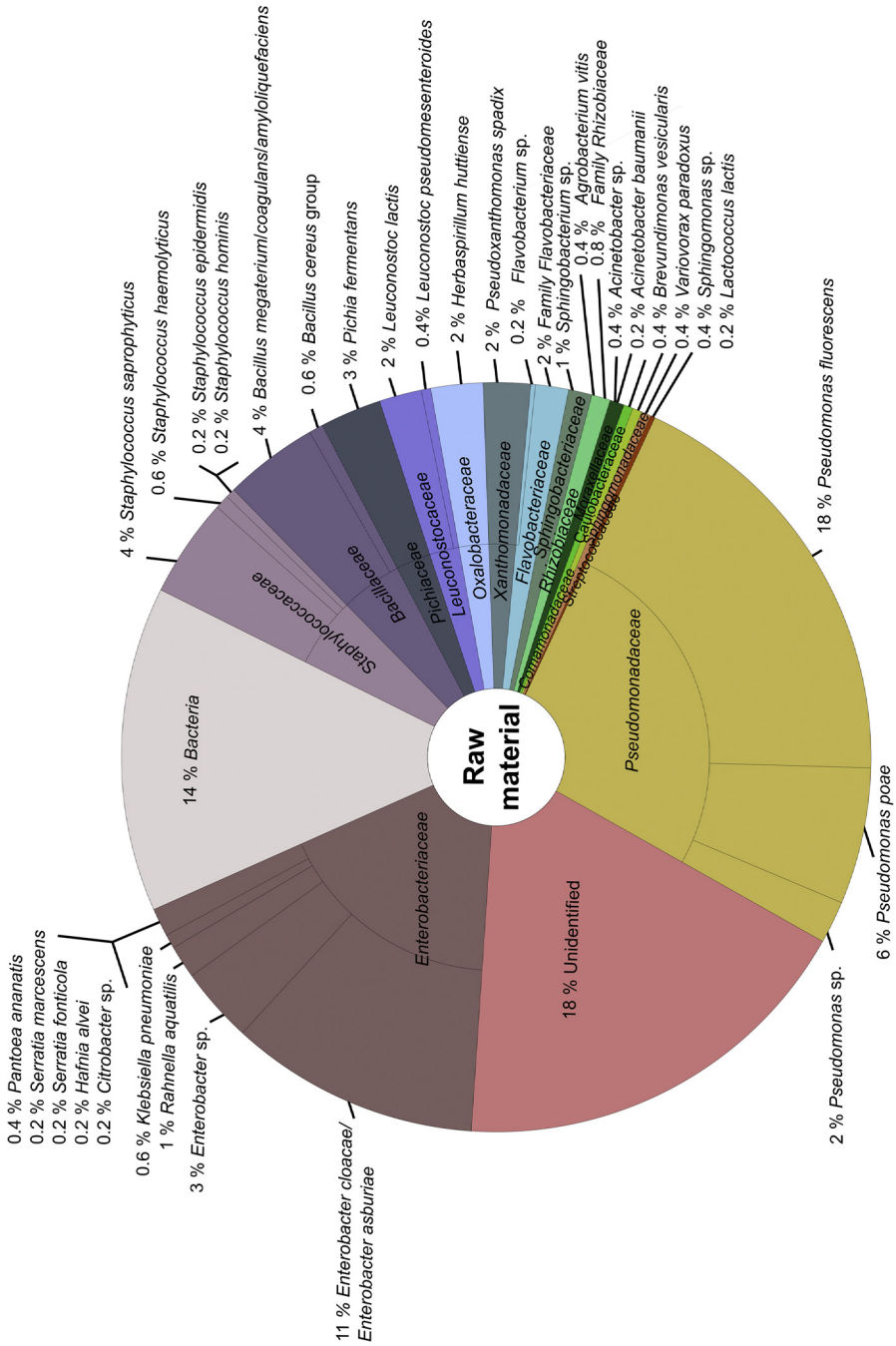

Fig. 3. Microbial community structure of endive raw material obtained by MALDI-TOF MS analysis. In total, 492 colonies were analysed.

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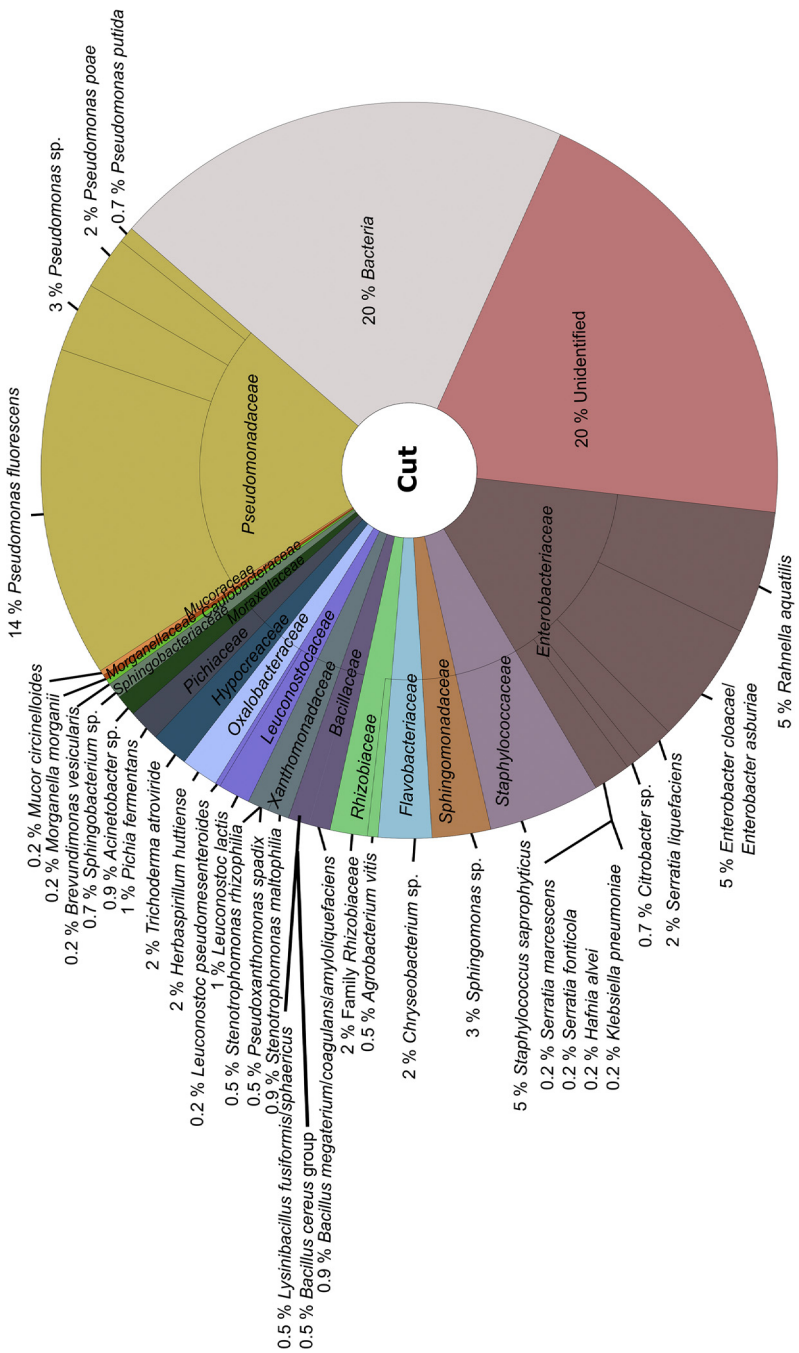

Fig. 4. Microbial community structure of cut endive material obtained by MALDI-TOF MS analysis. In total, 428 colonies were analysed.

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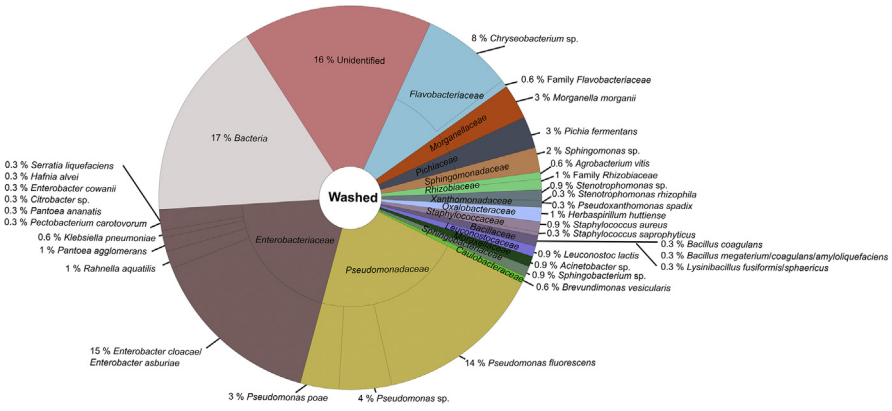

Fig. 5. Microbial community structure of washed endive material obtained by MALDI-TOF MS analysis. In total, 338 colonies were analysed.

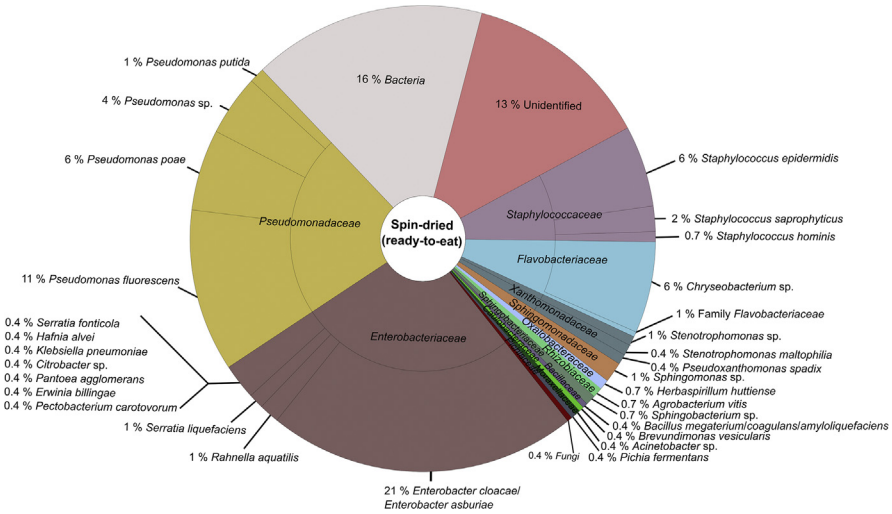

Fig. 6. Microbial community structure of spin-dried (ready to pack) endive material obtained by MALDI-TOF MS analysis. In total, 284 colonies were analysed.

Pseudoxanthomonas spadix was found in all samples (Figs. 3, 4, 5, and 6). Regarding the complete endive processing chain, the highest diversity was found for the families *Enterobacteriaceae* followed by *Bacillaceae* and *Pseudomonadaceae*. Within the *Enterobacteriaceae Rahnella aquatilis, Klebsiella pneumoniae*, *Enterobacter cloacae/Enterobacter asburiae*, *Citrobacter* sp., *Hafnia alvei* and *Serratia* spp. (*Serratia fonticola, Serratia marcescens, Serratia liquefaciens*) were found along the endive processing chain, whereas *Pantoea* spp. (*Pantoea ananatis, Pantoea agglomerans*) were only found in the raw material and the washed and spin dried (ready-to-pack) endive sample. The predominant bacteria species of the *Pseudomonadaceae* was *Pseudomonas fluorescens*. Additionally, *Pseudomonas poae*, *Pseudomonas putida*, and *Pseudomonas* sp. were identified along the endive processing chain.

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Acinetobacter baumannii (*Moraxellaceae*) was only identified in the raw material whereas *Acinetobacter* sp. was found in all samples along the processing chain (Figs. 3, 4, 5, and 6). Bacteria belonging to the *Bacillus cereus* group were only identified up to the cut endive sample.

The enrichment procedures of the bacteria from endive samples showed that bacteria belonging to the families *Xanthomonadaceae* (*Stenotrophomonas maltophilia*), *Pseudomonadaceae* (*Pseudomonas putida*), *Enterobacteriaceae* (*Serratia fonticola, Serratia liquefaciens, Serratia marcescens, Klebsiella pneumoniae, Citrobacter* sp., *Hafnia alvei, Morganella morganii*), *Bacillaceae* (*Lysinibacillus fusiformis/sphaericus*), and *Staphylococcaceae* (*Staphylococcus aureus, Staphylococcus haemolyticus, Staphylococcus saprophyticus*) were present in the endive samples along the processing chain.

4. Discussion

The aerobic mesophilic viable count of the endive lettuce was in accordance with the findings of other surveys, showing that whole endive lettuce had an aerobic mesophilic viable count of 6.7−7.2 log CFU/g and ready-to-eat endive lettuce an aerobic mesophilic viable count of 4.3−7.2 log CFU/g [49]. However, the total viable count gives nearly no information on the microbial community of endive lettuce. Since leafy green vegetables can act as vehicles for the transmission of human pathogens [50], and it is known that they retain a majority of their indigenous microflora after processing, it poses a potential food safety problem [51]. Hence, the knowledge about the microbial community is of high interest for the food industry [52].

Using culture-dependent methods, information about the presence and viable abundance of bacterial populations are obtained. Additionally, culture-independent methods provide information about the entire bacterial community [53]. TRFLP analysis allows the monitoring of community changes (*e.g.,* after each process step along the processing chain) and the detection of taxa that may be missed by culture-dependent methods [7]. However, 69 % of the plasmids obtained by 16S rRNA gene library construction showed chloroplast fingerprint pattern which led to an uneven number of analysed plasmids. This seems to be due to the amplification of the chloroplast and mitochondria specific 16S rRNA sequences by the primer pairs commonly applied for microbial community analysis which were also applied in this study. Even though the amount of non-bacterial DNA was reduced by filtration in preliminary experiments, the filtration step in these experiments was not sufficient resulting in such a high amount of plasmids with chloroplast sequence. Rudi *et al.* [8] also found a high frequency of chloroplasts performing a 16S rDNA array approach using the 16S rRNA gene targeting primer pairs 10-34f and 1485-1507r. They suggested that the microbial load of the sample was very low and therefore

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more chloroplast DNA than bacterial DNA was amplified by the used primers. This is also the most likely reason for the results obtained in our study. Randazzo et al. [54] used the 16S rRNA gene targeting primer pair 7f and 1510r for PCR-DGGE analyses of raw lettuce. In that study, a high frequency of chloroplasts was not reported. Further, Jackson et al. [55] used the primer pair Bac799f and Uni1492r for 454 pyrosequencing of leafy salad samples because no amplification of residual chloroplast DNA was expected and less than 0.05 % of the obtained sequences were originated from chloroplasts. However, the use of primer pairs resulting in 16S rRNA gene fragments or amplicons of shorter length leads to a minor resolution of bacterial taxa. Thus, the choice of primer pairs should be adapted for each investigation to obtain the best possible results.

For the identification of unidentified isolates by MALDI-TOF MS, a very good structured database with reference mass spectra of the target microorganisms is essential [56]. The higher the amount of habitat specific reference mass spectra within the database, the higher is the possibility to identify pathogenic bacteria. Due to the fact that MALDI-TOF MS is mainly used to identify pathogens in the medical field, databases with reference mass spectra from relevant food associated microorganisms are still missing. The analysis of the 16S RNA gene of unidentified colonies with subsequent sequence analysis and identification using the NCBI Megablast tool in combination with the NCBI Reference Sequence Database RefSeq enabled improve the a database with reference mass spectra from plant associated bacteria but a continuously expanding of the database is needed to enable a rapid identification of food-related bacteria in future investigations.

Both, TRFLP and 16S rRNA gene sequence analysis as well as the MALDI-TOF MS analysis, showed that the relative abundances of bacterial families changed along the processing chain. During processing contaminated wash water, equipment or improper handling can lead to a contamination of fresh produce [57]. In addition, also the persistence of pathogenic species during cleaning or seasonal shutdown is possible [58]. Cross-contamination during processing may be the reason for the changes of the microbial community along the processing chain. It has to be taken into account that the number of analysed sequences vary along the processing chain which may result into an underestimation or overestimation of bacterial abundances. However, differences in the microbial community of lettuce directly from farms and from supermarkets were also found by Jackson et al. [53]. It was also shown that different packaging and storage time also influences the bacterial community of lettuce [7]. 16S rRNA gene sequence analysis as well as the culture-dependent analyses with subsequent identification by MALDI-TOF MS showed that *Pseudomonadaceae* were the predominant bacteria on endive lettuce along the processing chain. This is in accordance with the literature where *Pseudomonadaceae* were found as predominant bacteria on lettuce [8, 9, 55, 59, 60, 61] which are not removed during washing [54].

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Bacteria belonging to the family *Xanthomonadaceae* (*e.g. Xanthomonas*) can be plant pathogens [59] and some genera can include potential human pathogens (*e.g. Stenotrophomonas* spp.) [55]. *Stenotrophomonas maltophilia* was found in the cut endive sample and in the spin-dried (ready to pack) endive sample. *Stenotrophomonas maltophilia* is referred to as environmental global emerging multidrug resistant organism (MDRO) that is associated with wet surface and aqueous solution and is able to form biofilms [62]. Qureshi et al. [63] found *Stenotrophomonas maltophilia* in 78 % of tested lettuce samples, and all isolates were resistant or susceptible to antibiotics. It is emerging as nosocomial pathogen, especially for immunocompromised persons, but its importance in ready-to eat salads is still unknown.

Pathogens with the potential to generate antibiotic resistance or multi resistance such as *Enterobacter* spp., *Klebsiella pneumoniae*, *Klebsiella oxytoca,* and *Serratia marcescens* [64] were also found in small quantities in the analysed endive samples. The presence of antibiotic resistant bacteria and their resistance rates on different vegetables were tested by Schwaiger et al. [65]. They found that resistance rates of bacteria from vegetable samples were lower than the resistance rates of bacteria of animal or human origin but since vegetables can be a source for the dissemination of antibiotic resistance. Schwaiger et al. [65] recommended thorough washing of raw vegetables before consumption. Antibiotic resistance of bacteria on salad was also shown for *Pseudomonas fluorescence* that was resistant to six antibiotics, and the occurrence of multi resistant bacteria is common in epiphytic bacteria [66]. *Stenotrophomonas* sp., *Acinetobacter* sp., *Morganella morganii, Klebsiella* sp., *Enterobacter sp.,* and *Serratia* sp. with different antibiotic resistances were found in fruits and vegetables by Jones-Dias et al. [67]. They concluded that fresh produce is a relevant reservoir for Gram-negative bacteria with antibiotic resistance and a continuous monitoring is essentially required. However, the resistance rates of the bacteria were not tested in this study, and, due to its relevance, this topic need to be included in further studies.

In this study, bacteria belonging to the *Bacillus cereus* group were only found in samples of raw and cut endive. The German Society for Hygiene and Microbiology [68] recommends a warning value of 3 log CFU/g for presumptive *Bacillus cereus* in mixed salads which was not exceeded in this analyses. However, it has to be taken into account that the selective choice of colonies which were dominant and morphologically distinct instead of analysing all grown colonies may lead to an underestimation of bacterial presence.

The application of culture-dependent methods with subsequent identification by MALDI-TOF MS in combination with culture-independent methods such as TRFLP fingerprinting and 16S rRNA gene sequence analysis enables the evaluation of the microbial community of endive lettuce along the postharvest processing chain as accurate as possible. The predominant bacteria on endive lettuce were detected by almost all methods but there are also varying results indicating that the methods

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applied are complementary. The results of this study indicate that not only expected groups of microorganisms are detectable on lettuce but also unexpected potentially pathogenic bacteria (*e.g., Stenotrophomonas maltophilia*, *Acinetobacter* sp., *Morganella morganii*) can occur on fresh cut lettuce. The detection of unexpected pathogenic bacteria, especially those with antibiotic resistance is of great interest to avoid potential risks for consumers and to avoid the potential spread of antibiotic resistance in the food chain.

Naturally occurring phyllospheric and endophytic bacteria can act as commensals or symbionts [55]. In addition, they can act also as competitors for human pathogens limiting the presence of pathogenic bacteria [53]. Therefore, detailed knowledge of the microbial community and its dynamic changes during food processing is essential to allow the implementation of tailored control strategies including hygienic design, innovative decontamination techniques, and appropriate storage conditions.

Declarations

Author contribution statement

Antje Fröhling: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Antje Rademacher, Birgit Rumpold: Performed the experiments; Analyzed and interpreted the data.

Michael Klocke, Oliver Schlüter: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data;

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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