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Rapid identification of microorganisms by MALDI-TOF MS

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The increasing demand on safe food with high quality poses a high challenge especially for perishable products. Microbiological sampling along the food processing chain is mainly focused on selected indicator microorganisms and unexpected potential human pathogenic bacteria may remain undetected. The detection of unexpected pathogenic bacteria is of great interest to avoid potential risks for consumers. The change microbial community of perishables exemplarily shown for mung bean sprouts was evaluated using plate count methods and MALDI-TOF MS. The total aerobic viable count of sprouts was 8–9 log CFU/g and among others bacteria from *Bacillus cereus* group, *Yersinia* sp., *Enterobacter* spp., *Klebsiella* spp., *Pantoea* spp., and *Pseudomonas* spp. were identified.

Keywords
Sprouts, fresh-cut salad, food safety, bacteria monitoring

Convenience products, especially fresh-cut salads and fruit mixtures, are an interesting segment within the European market with a value of 3.4 billion € in 2008 and a predicted annual growth of 4% (van Rijswick 2010). Fresh-cut products are ready-to-eat products that are only washed and cut before sale. These minimal processing steps (minimally processed foods) require a high freshness of the products. However, these products are very susceptible to microbial spoilage due to their original microbial load and due to cross-contaminations during postharvest processing. According to Francis et al. (1999) the microbial load of fresh fruits and vegetables ranges between 10^5 and 10^7 CFU/g and predominately found species are *Erwinia* and *Pseudomonas*. For food safety, the occurrence of pathogenic bacteria such as *Listeria monocytogenes*, *Salmonella enterica*, and *Escherichia coli* (Francis et al. 1999, Oliveira et al. 2011) is of high relevance because fresh-cut products are generally eaten raw. Despite the high hygienic standards during processing of fresh-cut salads and fruits, cold chain storage and distribution as well as the use of modified atmosphere packaging, foodborne diseases are associated with the consumption of fresh cut products from time to time. The Federal Institute for Risk Assessment (BfR) investigated the microbial load of mixed salads and found *Listeria monocytogenes* within 5% of tested samples in 2008 (BfR 2011).

The industrial production of sprouts usually takes place in special boxes at mesophilic temperatures (37 °C) and high moisture. Unfortunately, these conditions are ideal for the growth of microorganisms also and require a good processing hygiene with regular cleaning and disinfection procedures. The raw products (seeds) are often contaminated and therefore, they are an important source for the high microbial load of the products. The BfR showed that the total bacterial count of mung bean sprouts in 2009 increased to a level of 10^9 CFU/g within 4 days of cool storage and sprouts are often contaminated with pathogenic bacteria (BfR 2011). Microbiological sampling along the food processing chain is mainly focused on selected indicator microorganisms and unexpected potential

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human pathogenic bacteria may remain mostly undetected. The detection of unexpected pathogenic bacteria is of great interest to avoid potential health risks for consumers. Especially, the changes of microbial contaminations along the processing chain are still unknown.

Within the SAFEFRESH subproject "Early hazard detection based on identification of plant associated bacteria along the processing chain of plant perishables" a detailed characterisation of the changes of microbial community on fruit and vegetable surfaces during processing is conducted to allow the evaluation of basics for the early hazard detection of human pathogenic bacteria. Plant associated bacteria are cultivated on standard growth medium and the obtained bacteria colonies are subsequently identified by MALDI-TOF MS based on the measurement of intact cell protein spectra. Additionally, a spectra database of plant associated bacteria will be constructed to allow a rapid culture-dependent diagnostic of bacteria. MALDI-TOF MS was firstly used for the analyses of biomolecules such as proteins in the late 1980s (Karas and Hillenkamp 1988, Hillenkamp et al. 1991). Not only biomolecules can be analysed by MALDI-TOF MS but also whole cells such as bacteria and moulds (Madonna et al. 2000, Fenselau and Demirev 2001). Within a short time (measurement time 15–45 s) a differentiation and immediate identification of bacterial isolates as well as of moulds is possible by comparing the data with reference mass spectra from a database. First and foremost, MALDI-TOF MS is used for the identification of pathogenic bacteria in the medical field (Weile and Knabbe 2009, Ho and Reddy 2010). However, this technique was also successfully applied for the differentiation of typical food associated bacteria such as *Salmonella* spp. (Mazzeo et al. 2006, Dieckmann et al. 2008), *Listeria* spp. (Barbuddhe et al. 2008), *Enterococcus* spp. (Giebel et al. 2008), and *Escherichia coli* (Mazzeo et al. 2006, Siegrist et al. 2007, Ochoa and Harrington 2005). The aim of this study was the characterization of the microbial community of perishables exemplarily shown for mung bean sprouts as well as the development of a database with reference mass spectra for plant associated bacteria.

Material and Methods

Mung bean sprouts were purchased at a local retailer and were directly analysed with microbiological methods. Three packages with different use-by dates were purchased in order to allow the evaluation of the microbial community on sprouts two days before, one day before as well as at the day of use-by date. The determination of the total aerobic viable bacterial count was conducted following the DIN EN ISO 4833-2 (2014). Therefore, three samples were taken from each package (as sterile as possible) and diluted 1:10 with sterile casein peptone salt solution so that each box was sampled in triplicate. Subsequently, the samples were homogenized using a Bag Mixer® (Interscience, France) for 2 min at highest speed. In a next step, the homogenized samples were serially diluted to 10^{-7} in microtiter plates (96er U-profile, Carl Roth GmbH, Germany) in duplicate. 100 µl of each dilution step was spread on plate count agar (Carl Roth GmbH, Germany) and incubated for 72 h at 30 °C. The detection limit was 2 log CFU/g. For the identification by MALDI-TOF-MS all colonies from one agar plate were chosen for the analysis (for all samples 269 colonies were analysed in duplicate). For the MALDI TOF-MS measurements, cell material of the colonies was transferred to a target using a pipette tip and overlaid with CHCA-matrix (α-cyano-4-hydroxy cinnamic acid, RIPAC-LABOR GmbH, Germany). After drying, the samples were analysed by MALDI-TOF MS (Axima Confidence, Shimadzu Deutschland GmbH). For identification of the bacterial colonies, the obtained mass spectra were compared with the reference mass spectra of the AnagnosTec SARAMIS™ database (**S**pectral **AR**chive **A**nd **M**icrobial **I**dentification **S**ystem, bioMérieux, Germany).

Results and Discussion

The total aerobic viable count of the investigated mung bean sprout samples were between 8 and 9 log CFU/g (Figure 1).

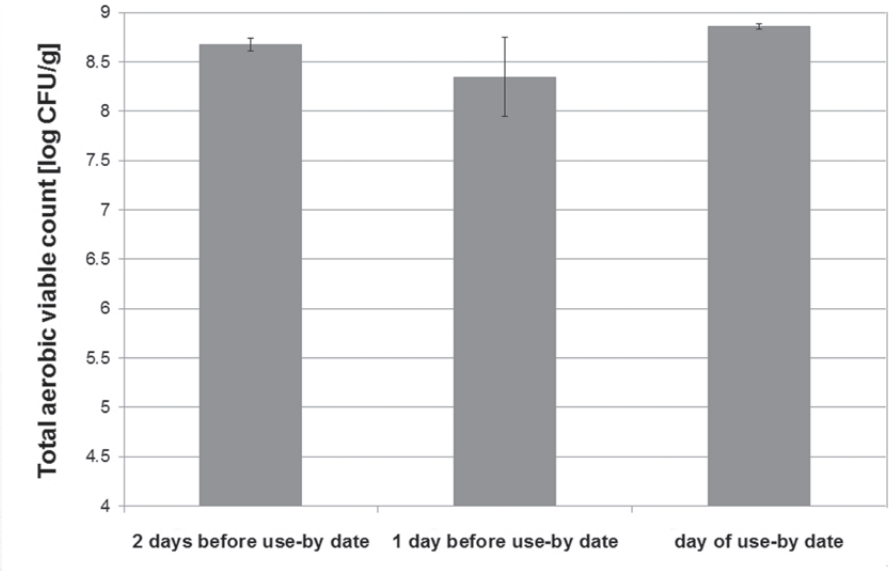

Figure 1: Aerobic total viable count of mung bean sprouts. The total viable counts are presented as averages with standard deviation of six values (three independent experiments analysed in duplicate).

There was no difference in the total viable count between the samples with different use-by dates. The obtained total viable count of mung bean sprouts is in accordance with the literature revealing that sprouts can have a total viable count between 8 and 9 log CFU/g before consumption (NACMCF 1999). However, the total viable count gives no information about the type of bacteria and whether the bacteria are pathogenic or not. The microbial saftey monitoring of food or food production chains is primarily performed with traditional microbiological methods, which are based on the cultivation of selected indicator microorganisms at standardised conditions (standard growth medium, selective growth medium and subsequently performed biochemical and serological tests). The advantage of this approach is that these established and approved methods are relatively cheap. Additionally, known pathogens can be reliably detected and quantified with the culture-dependent methods. Even low concentrations of bacteria can be determined after selective enrichment of the cultures at special culture conditions. The disadvantage of this method is the amount of time needed for the analysis (up to one week and more), due to the need of time-consuming serological and biochemical test. Furthermore, it is often not possible to differentiate between closely related species. For example, it is only possible to distinguish between pathogenic and non-pathogenic Enterobacteriaceae or Listeria species if additional molecular biological methods were applied. In comparison to alternative methods such as biochemical and serological tests, the identification of cultivated microorganisms by MALDI-TOF MS has a number of benefits (Weile and Knabbe 2009, Ho and Reddy 2010, Mazzeo et al. 2006):

- Speed of analysis (measurement time approximately 1 min)
- Comparatively easy analysis of mass spectra
- High tolerance against contaminations

- Differentiation of microorganisms up to the species and subspecies level
- High accuracy
- Potential for automation to the greatest possible extend
- Potential for high-throughput analysis
- High cost efficiency in routinely application

In this study, MALDI-TOF MS was used for the identification of bacteria colonies obtained from sprouts. Overall, bacteria of the genus *Pseudomonas* spp., *Enterobacter* spp., *Pantoea* spp., *Klebsiella* spp., *Stenotrophomonas* spp., *Rahnella* spp., *Herbaspirillum* sp., *Leuconostoc* spp., *Yersinia* sp., and bacteria from the *Bacillus cereus* group were identified using the SARAMIS™ database (Table 1).

Table 1: Detected bacteria strains on mung bean sprouts (confidence level ≥ 90 %)

2 days before use-by date	1 day before use-by date	day of use-by date
Enterobacter sp.	*Enterobacter sp.*	*Leuconostoc pseudomesenteroides*
Enterobacter cloacae	*Enterobacter cloacae*	*Leuconostoc sp.*
Enterobacter cowanii	*Pantoea dispersa*	*Leuconostoc gelidum*
Pantoea agglomerans	*Pantoea agglomerans*	*Klebsiella pneumoniae*
Pantoea dispersa	*Pseudomonas fluorescens*	*Klebsiella oxytoca*
Klebsiella oxytoca	*Pseudomonas oryzihabitans*	*Bacillus cereus group*
Klebsiella pneumoniae	*Stenotrophomonas sp.*	*Pantoea agglomerans*
Pseudomonas fluorescens	*Stenotrophomonas maltophilia*	*Enterobacter sp.*
Pseudomonas poae	*Rahnella aquatilis*	*Enterobacter cloacae*
Pseudomonas sp.	*Yersinia sp.*	*Herbaspirillum huttiense*

It is noticeable that the potential human pathogenic bacteria *Yersina* sp. and bacteria from the *Bacillus cereus* group were only detected in the samples one day before use-by date and in the samples at the use-by date, respectively. This is in contrast to the assumption that a high microbial load can limit the growth of pathogenic bacteria (EFSA 2011). The quantification of the results revealed that all detected bacteria were present at a level of 7 log CFU/g on the sprouts. The German Society for Hygiene and Microbiology (DGHM, Deutsche Gesellschaft für Hygiene und Mikrobiologie) recommends a warning value of 3 log CFU/g for presumptive *Bacillus cereus* in sprouts and germ buds (DGHM 2011). Taking this value into account, the sprouts sample at the use-by date highly exceeded this threshold value.

Beside the identified microorganisms a high amount of cells could not be identified using the SARAMIS™ database due to the lack of reference mass spectra. It cannot be excluded that these not identified bacteria are potential human pathogenic bacteria. For the identification of unknown isolates by MALDI-TOF MS, a very good structured database with reference mass spectra of the target microorganisms is essential (Wilkes et al. 2002). The higher the amount of habitat specific reference mass spectra within the database the higher is the possibility to identify the pathogenic bacteria. Because the 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. Therefore, one objective within the national research project SAFEFRESH is the identification of unknown bacteria using molecular biological methods. In this way, a database with reference mass spectra from

plant associated bacteria shall be constructed, which enables a rapid identification of food related bacteria in future investigations.

Conclusion

A concept to control microbiological hazards during the processing chain of perishables and minimal processed food to ensure food safety is required. 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. The construction of a database with reference mass spectra from food related microorganisms could be the basis for a possible application in food safety monitoring. Additionally, the assessment of microorganisms that are not detected up to now with the existing monitoring systems is possible.

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