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Differentiation of meat-related microorganisms using paper-based surface-enhanced Raman spectroscopy combined with multivariate statistical analysis

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ABSTRACT

Surface-enhanced Raman spectroscopy (SERS) with subsequent chemometric evaluation was performed for the rapid and non-destructive differentiation of seven important meat-associated microorganisms, namely *Brochothrix thermosphacta* DSM 20171^T, *Pseudomonas fluorescens* DSM 4358, *Salmonella enterica* subsp. *enterica* sv. Enteritidis DSM 14221, *Listeria monocytogenes* DSM 19094, *Micrococcus luteus* DSM 20030^T, *Escherichia coli* HB101 and *Bacillus thuringiensis* sv. *israelensis* DSM 5724. A simple method for collecting spectra from commercial paper-based SERS substrates without any laborious pre-treatments was used. In order to prepare the spectroscopic data for classification at genera level with a subsequent chemometric evaluation consisting of principal component analysis and discriminant analysis, a data pre-processing method with spike correction and sum normalisation was performed. Because of the spike correction rather than exclusion, and therefore the use of a balanced data set, the multivariate analysis of the data is significantly resilient and meaningful. The analysis showed that the differentiation of meat-associated microorganisms and thereby the detection of important meat-related pathogenic bacteria was successful on genera level and a cross-validation as well as a classification of ungrouped data showed promising results, with 99.5% and 97.5%, respectively.

1. Introduction

Food safety is the major priority and challenge for food producers in order to prepare food that is safe for human consumption. Every year, around 600 million people become sick, resulting in 420,000 deaths due to foodborne diseases. Especially in highly perishable foods like meat, poultry and fish or ready-to-eat products, the pathogenic bacteria e.g. *Salmonella* spp., *Campylobacter* spp. or *Listeria monocytogenes* are often responsible for the majority of foodborne illnesses [1].

Salmonella spp. belong to the most common pathogenic bacteria that affect millions of people every year. *Salmonella* spp. are present in eggs, poultry and other products of animal origin. Infections with *Listeria* spp. can lead to unplanned abortions in pregnant women or the death of newborn children. Although the incidence of the disease is relatively low, *Listeria* spp. infections are one of the most serious food-borne infections due to their severe and sometimes fatal health consequences, especially in infants, children and elderly. *Listeria* spp. can occur in unpasteurised dairy products and various ready-to-eat foods and can grow at refrigeration temperatures [1,2].

Aside from the health aspect of food safety, the detection of pathogenic bacteria is also one factor in reducing food waste and food losses. The concern about becoming ill by consuming food not appearing fresh is one reason why customers, despite the wish to reduce food waste, often decide otherwise [3,4].

Besides the culture-based detection method, other common alternative detection methods are already described in literature like real-time polymerase chain reaction [5] or immunoassays [6]. Despite many advances in these research fields, it is still a challenge to find new

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approaches to improve the simplicity, selectivity, stability and sensitivity of these methods. Consequentially, there is a great demand on fast, non-destructive and cost-effective analysis methods to analyse the microbial load especially on meat products.

Raman spectroscopy has the potential to become one of the technologies that overcome this deficiency as a fast, robust and non-destructive detection method for pathogenic bacteria. For the discrimination and detection of spoilage and environmentally associated bacteria, Raman approaches have already been tested for example with complex multistage principal component analysis (PCA) and support vector machines [7]. Instrumentally complex Raman microscopic studies with spoilage and environmental bacteria have already been used to discriminate on strain level [8].

SERS-based detection methods for bacteria like methicillin-resistant *Staphylococcus aureus* (MRSA) [9] or *Salmonella enterica* [10] in fruit juice have also already been developed. Most of the SERS approaches use complex synthesised SERS-active components, like gold (Au) nanopopcorns at graphene oxide [9] or SERS sandwich assays as a combination of silver nanoparticles (AgNP) with capture molecules like 3-mercaptophenylboronic acid [11], and they focus on detection limits for a singular strain [9,11] or on the biochemical origins of the surface enhanced Raman spectra of bacteria [12].

For the detection of certain pathogenic bacterial species for example on meat products, besides a low detection limit for these bacteria, the ability to discriminate between the different bacterial genera is also essential.

The microbial flora of fresh and chilled meat during the spoilage process is mostly dominated by *Pseudomonas* spp., especially *Pseudomonas fluorescens*, as well as *Brochothrix thermosphacta* and *Enterobacteriaceae* like *Escherichia coli* [13–16].

Furthermore, *Micrococcus luteus* and *Bacillus thuringiensis israelensis* are environmentally associated bacteria, which can also be found on spoiled meat or other food products [17,18].

For that reason, the objective of this study was to develop a time-efficient and suitable sample preparation method for microbiological sampling onto SERS substrates as well as to define measurement parameters and establish a reasonable chemometric data processing method for a rapid and non-destructive analysis of food safety and meat spoilage relevant bacteria. For this purpose, a robust data pre-processing method combined with a suitable chemometric evaluation to classify and distinguish between the measured bacteria was developed.

2. Materials and methods

2.1. Bacterial cultures and sample preparation

Seven important spoilage-related bacteria, namely *Brochothrix thermosphacta* (*B. thermosphacta*) DSM 20171^T, *Escherichia coli* HB101 (*E. coli*), *Micrococcus luteus* (*M. luteus*) DSM 20030^T, *Pseudomonas fluorescens* (*P. fluorescens*) DSM 4358, *Salmonella enterica* subsp. *enterica* sv. Enteritidis (*S.* Enteritidis) DSM 14221, *Listeria monocytogenes* (*L. monocytogenes*) DSM 19094 and *Bacillus thuringiensis* sv. *israelensis* (*B. thuringiensis*) DSM 5724 (Leibniz Institut DSMZ – German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were cultivated and separately grown according to the DSMZ guidelines [19]. The nutrient agar consisted of 10 g/L meat peptone, 10 g/L meat extract, 5 g/L sodium chloride and 18 g/L agar-agar (Merck KGaA, Darmstadt, Germany) and only agar from the same batch were used.

For each SERS substrate, multiple colonies were then harvested during their stationary growth phase with a 1 μL-loop and dispersed in 100 μL distilled water. A concentration of 3×10^8 cfu/mL was estimated by a comparison with a McFarland standard. After homogenising, 2 μL of the suspension were spread homogeneously on a commercial SERS substrate (Ocean Insight). For each strain, measurements were performed in triplicate on three independent SERS substrates immediately after the sample preparation.

2.2. SERS substrate and data acquisition

The RAM-SERS-AU-5 substrates (Ocean Insight) are paper-based SERS substrates covered with gold nanoparticles (AuNPs) with a SERS-active area of approximately 23.7 mm^2. This type SERS substrates have been used before in publications, like Sourdaine *et al.* [20]. The AuNPs on the SERS substrates were manufactured by chemical reduction and the substrates were used before their expiration date. In this study, a fibre-coupled Raman spectrometer QE-Pro (Ocean Insight) with a charge-coupled device (CCD) detector was used. The SERS-substrate samples were placed on a motorised XY-stage LTS 150 (Thorlabs) and focused with a fibre-coupled probe (InPhotonics) with a spot size of approximately 160 μm in diameter. The measurements were performed with a 638 nm laser (InPhotonics). A LabVIEW software (National Instruments) carried out the controlling of the spectrometer and XY-stage as well as the data acquisition.

All measurements were collected with an initial laser power of 35 mW and an integration time of 1 s. At each substrate, three different mappings were collected. Each mapping consisted of a 3×3 raster with 0.3 mm distance between each point and at each measurement position 25 spectra were collected, which led to 14,175 spectra in total.

2.3. Data processing

After data acquisition, the data was split into training and test data sets. The spectra of the first two substrates for each strain were used as a training data set and the spectra from the third substrate were treated as an independent test data set.

2.3.1. Pre-processing

A problem often related with CCD detectors and Raman spectroscopy is the vulnerability to cosmic rays, which leads to cosmic spikes. These spikes could alter the Raman data and the information within [21]. For that reason, both sets were searched and corrected for cosmic spikes. Based on the work of Mozharov *et al.* [22], every intensity value of each wavenumber in every spectrum at each measuring position was compared with the averaged spectra at a threshold of the five-fold standard deviation for each wavenumber of the remaining 24 spectra for each measurement position. If a spike occurred and was located, the corresponding intensities were corrected by interpolating between the averaged intensity of the five wavenumbers before and after the located spike.

All 25 spectra of each measurement position were subsequently averaged, cut to the range of 150–3150 cm^{-1} and normalised by the total sum of the averaged spectrum.

2.3.2. Principal component analysis

The subsequent chemometric analysis consisting of a PCA based on the covariance matrix was used to simplify the complex multivariate Raman data. PCA is an unsupervised chemometric technique, which transforms the given n-dimensional data in a projected space where the given variance of the data set is maximised and sorted in descending order in less than n-dimensions.

The training data set was used to perform the PCA; afterwards the descriptive statistics of the trainings data set and the loading vector of the performed PCA were used to transform the test data set into scores.

2.3.3. Canonical discriminant analysis

After data reduction by PCA a canonical discriminant analysis (CDA) was used for classification by determination of a linear combination of the principal components which maximises the relation of inter-group and intra-group variations [23,24].

A schematic overview of the data processing, including the pre-processing steps for test and training data set, is shown in Fig. 1.

The normalisation, PCA and discriminant analyses were performed using Origin Pro 2019b (OriginLab Corporation). LabVIEW (National

Instruments) was used for spike correction, cutting, normalisation and transforming the test data set into principal components.

3. Results and discussion

The sampling and data acquisition was designed in such a way that it was as fast and straightforward as possible.

3.1. Pre-processing

In order to reduce the influence of cosmic spikes in the spectra of the data set, the data acquisition was done in such a way that at each measurement position 25 spectra with an integration time of 1 s were collected. By comparisons between these 25 spectra, spikes could be detected and interpolated, so that the cosmic spikes did not affect the average spectrum of these measurement positions.

In Fig. 2 the raw data of a measurement position of *B. thermosphacta* is displayed as an example. In one of the 25 spectra a cosmic spike occurred.

Without the spike removal, the averaged spectra would show a spike at 2145 cm^{-1} (inserted diagram), which can be corrected by interpolation to reduce the influence of spikes for the classification.

To illustrate the variations within the spectra of one genus of bacteria, the mean spectra of the training data set with standard deviations of each wavenumber of the 54 spectra are shown (Fig. 3). In total 378 spectra are presented with 54 spectra each of *B. thermosphacta*, *E. coli*, *M. luteus*, *P. fluorescens*, *S.* Enteritidis, *L. monocytogenes* and *B. thuringiensis*.

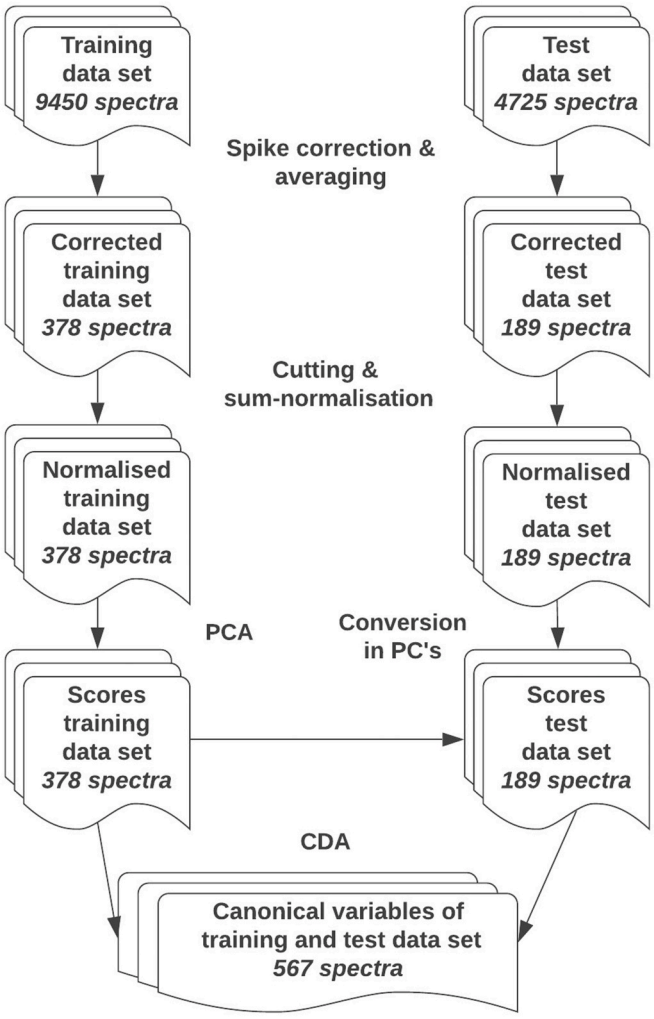

Fig. 1. Schematic overview of the amount of data and the data treatment for test and training data set.

The spectral profiles of the different microorganisms show only small differences, in relative peak intensity or different standard deviations. These variations result from the composition differences of the microbial cell, for example the variation of proteins and lipids in the cell. An obvious difference is the upcoming fluorescence in the spectra of *B. thuringiensis* and *P. fluorescens*, which could be correlated to the pigments from *P. fluorescens* [25] and possibly to the protein crystals within the spores of *Bacillus thuringiensis israelensis* [26].

3.2. Principal component analysis

These spectra are further reduced in dimensionality by PCA; the first two principal components are displayed in Fig. 4. The loading vectors of this PCA are also displayed in the lower part of Fig. 3 and show, aside from a fluorescence background in the loading of PC 1, several peaks, which are comparable with the peaks in the Raman spectra above.

The descriptive statistics and loading vectors of the training data set were used to convert the pre-processed test data set into the principal components, which are also visible in Fig. 4.

For these multivariate statistical methods it is essential to operate with equal-sized data sets within the classes, because not only PCA is sensitive to imbalanced data sets, but the performance of the discriminant analysis is also considerably affected [27–29]. Therefore, it was essential in the former step to correct the spike-affected spectra, because an elimination of the spectra would lead to an unbalanced data set.

Even in the two-dimensional space of the first two principal components, the bacteria gather in clusters mostly apart from each other. *B. thuringiensis* and *P. fluorescens* are close to each other, which might be due to the upcoming fluorescence. However, the scores of the pathogenic bacteria *S.* Enteritidis and L. *monocytogenes* are located in small clusters separately from most of the other bacteria. Furthermore, the diagram shows that the variances in the training data set are very similar to the variances in the test data set since the converted test data are close to the correct clusters.

3.3. Canonical discriminant analysis

For further analysis, the first six principal components of the training data set were used in the CDA, which represent 99.5% of the variances in the training data set. The test of equality of the covariance matrices of each bacterium shows that the covariance matrices of the bacteria were not equal and therefore a quadratic discriminate function was used in the following discriminant analysis.

As one result of the CDA, the first two canonical variables (CV) are displayed in Fig. 5. It shows that even in the first two CV all bacterial clusters are distinguishable by sight from each other. The result of the training data set shows a 0% classification error and by a leave-one-out cross-validation, an error of 0.54% occurs.

As in Fig. 4, the results of the CDA show that the bacteria cluster apart from each other and in comparison to the PCA the clusters are smaller and the distance between the clusters is enlarged. This way the scores of pathogenic bacteria are also separated from the scores of the other bacteria.

For independent testing of the developed model, the test data as described was processed independently of the training data set, but with the same steps until the test data were converted into the space of the principal components of the training data set. By applying the CDA model to the test data, a correct classification of up to 97.5% could be achieved, see Table 1.

The classification results show that *S.* Enteritidis as well as *P. fluorescens* and *E. coli* were well detected with an accuracy of 100%. Moreover, even the *L. monocytogenes* with the lowest classification rate of the independent data set still have a 93% correct classification. In

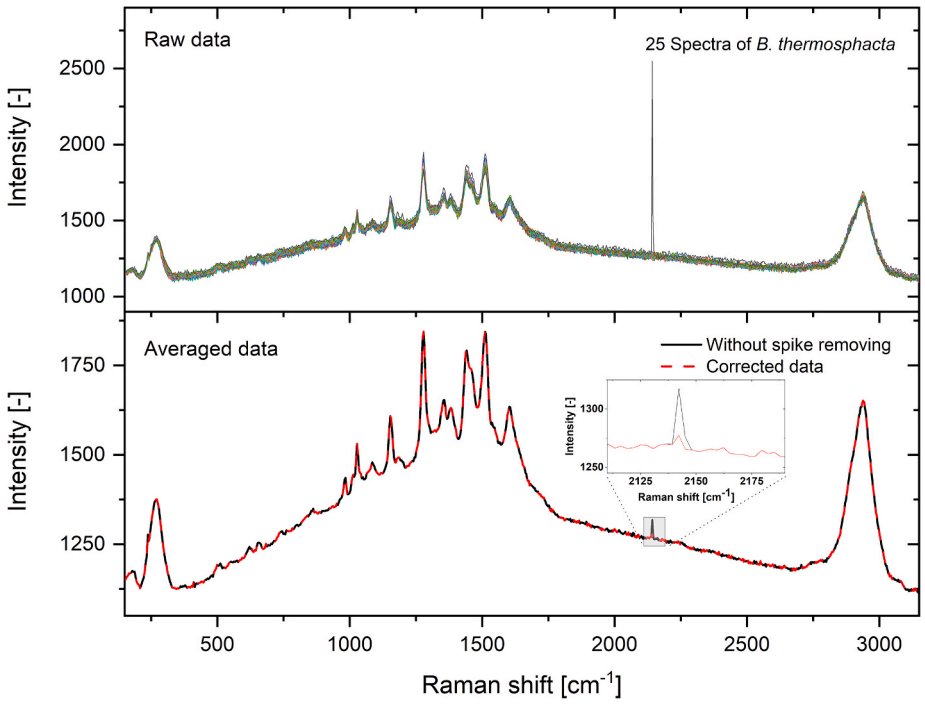

Fig. 2. Raw data from a measurement point of *B. thermosphacta* with a cosmic spike (upper diagram); differences in averaging with and without spike correction (lower diagram).

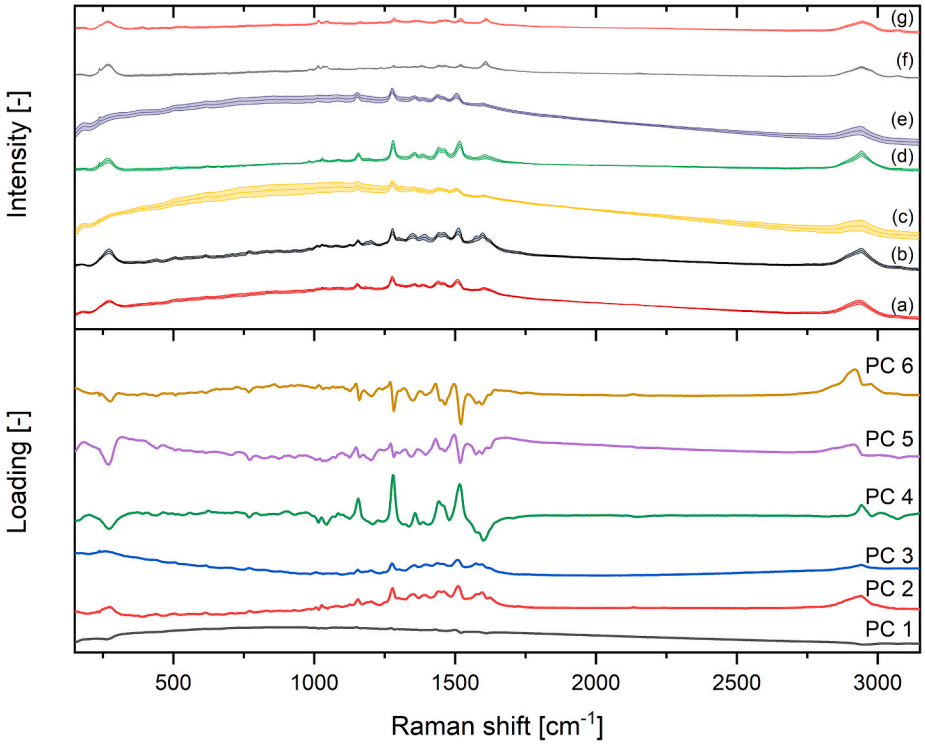

Fig. 3. Upper diagram: Mean Raman spectra after spike correction, sum normalisation and cutting of the training data set for *M. luteus* (a), *E. coli* (b), *B. fluorescens* (c), *B. thermosphacta* (d), *P. fluorescens* (e), *L. monocytogenes* (f) and *S.* Enteritidis (g) with standard deviation. Lower diagram: The first six loading-vectors of the training data PCA.

addition, the false classification of the two *L. monocytogenes* spectra were assigned to *B. thermosphacta, which* both belong to the same family of *Listeriaceae*. The presented SERS method with the suitable chemometric evaluation is able to discriminate between different meat-associated microorganisms on a genera level in a fast and robust way and by doing so, to detect pathogenic bacteria like *S.* Enteritidis and *L. monocytogenes* in a sufficient way. Accordingly, the next step would be to transfer the results of an indirect sampling method with a dispersing step to a direct sampling step like a SERS swab or a SERS stamp for the direct sampling on meat.

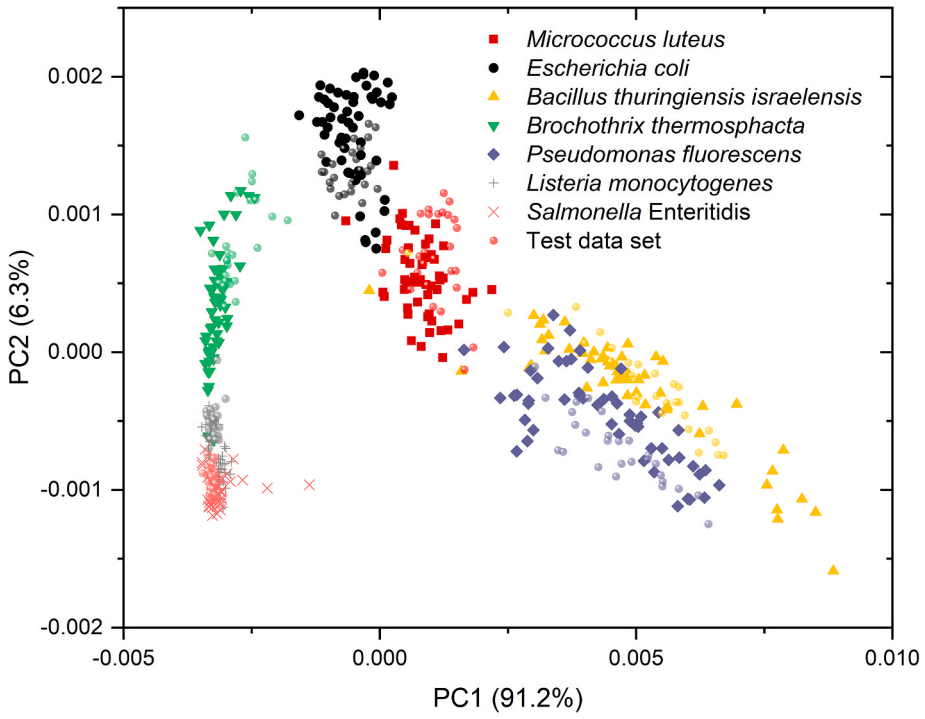

Fig. 4. Displayed is the score-diagram of the first two principal components from the principal component analysis of the training data set together with the converted independent test data set (coloured according to true class origin).

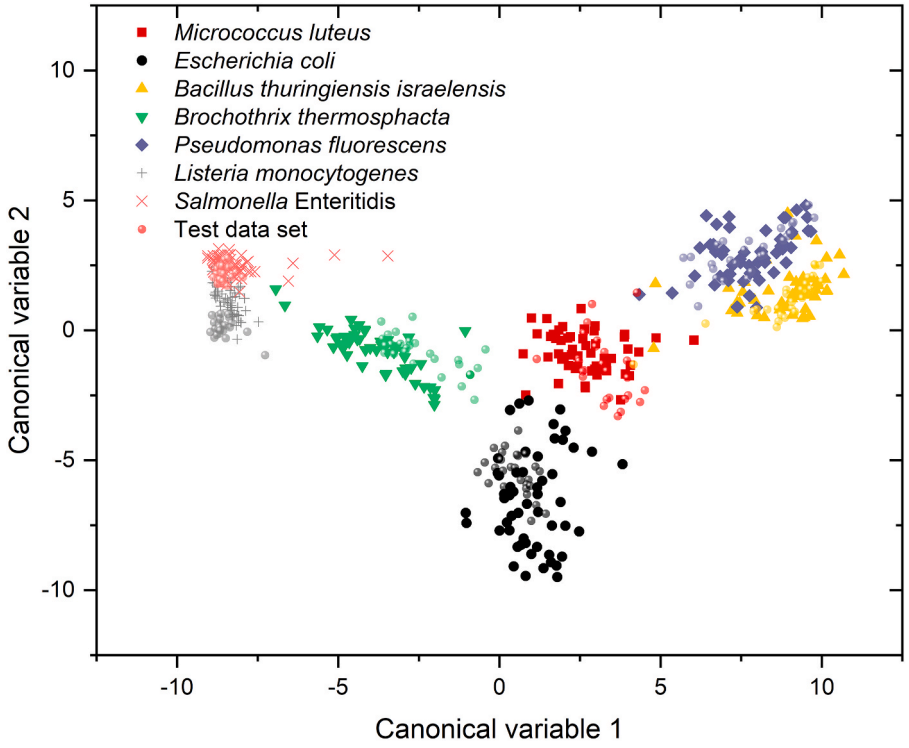

Fig. 5. The score diagram of the first two canonical variables from the CDA of the training data set together with the independent test data set (coloured according to true class origin).

4. Conclusion

A SERS method was developed for the detection of *S.* Enteritidis and *L. monocytogenes* with paper-based SERS substrates. The method was based on a balanced training data set and tested by an independent data set. Therefore, a pre-processing method with a correction of cosmic spikes and a normalisation by sum was performed. A data reduction was carried out on the training data set by PCA and the results were then used to reduce the dimensionality of the independent data set. Subsequently, the information of the first six principal components was used

Table 1
The confusion matrix of the independent test data set of 189 spectra (bold) and the training data set of 378 spectra. The rows show the observed groups and the columns show the predicted groups. The values in the diagonal represent the right classification and show the percentage of right classification of each bacterium.

	Predicted groups						
	M. luteus	*E. coli*	*B. thurin-giensis*	*B. thermos-phacta*	*P. fluores-cens*	*L. mono-cytogenes*	*S.* Enteri-tidis
M. luteus	**26(96%)**	**0**	**0**	**0**	**1**	**0**	**0**
	54 (100%)	0	0	0	0	0	0
E. coli	**0**	**27(100%)**	**0**	**0**	**0**	**0**	**0**
	0	54 (100%)	0	0	0	0	0
B. thurin-giensis	**1**	**0**	**26(96%)**	**0**	**0**	**0**	**0**
	0	0	54 (100%)	0	0	0	0
B. thermos-phacta	**1**	**0**	**0**	**26(96%)**	**0**	**0**	**0**
	0	0	0	54 (100%)	0	0	0
P. fluorescens	**0**	**0**	**0**	**0**	**27(100%)**	**0**	**0**
	0	0	0	0	54 (100%)	0	0
L. mono-cytogenes	**0**	**0**	**0**	**2**	**0**	**25(93%)**	**0**
	0	0	0	0	0	54 (100%)	0
S. Enteritidis	**0**	**0**	**0**	**0**	**0**	**0**	**27(100%)**
	0	0	0	0	0	0	54 (100%)
Total classification rate	**97.5%**						
	100%						

by CDA. The evaluation showed that the seven meat-associated bacteria could be distinguished in a robust and fast way with an independent test error of 2.5%. The results of this study show that the potential of this technology for a rapid determination of microbial contamination and the detection of pathogenic bacteria like *Salmonella* spp. and *Listeria* spp. is suitable and time-efficient in comparison to classical microbiological methods. Therefore, an application of the technology in the food sector would lead to a huge benefit due to the rapid detection of spoilage bacteria as well as foodborne pathogens.

Credit author statement

René Breuch: Conceptualization, Methodology, Investigation, Formal analysis, Software, Data curation, Visualization, Writing - original draft, Daniel Klein: Conceptualization, Methodology, Writing - review & editing, Eleni Siefke: Investigation, Writing - review & editing, Martin Hebel: Conceptualization, Resources, Writing - review & editing. Ulrike Herbert: Conceptualization, Resources, Writing - review & editing. Claudia Wickleder: Supervision, Writing - review & editing, Peter Kaul: Supervision, Writing - review & editing, Funding acquisition.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent

Not applicable.

Declaration of competing Interest

None.

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References

[1] WHO, Fact Sheet Food Safety, 2019. https://www.who.int/news-room/fact-sheets/detail/food-safety. (Accessed 23 April 2020).
[2] S. Tienungoon, D.A. Ratkowsky, T.A. McMeekin, T. Ross, Growth limits of *Listeria monocytogenes* as a function of temperature, pH, NaCl, and lactic acid, Appl. Environ. Microbiol. 66 (2000) 4979–4987, https://doi.org/10.1128/AEM.66.11.4979-4987.2000.
[3] K. Schanes, K. Dobernig, B. Gözet, Food waste matters - a systematic review of household food waste practices and their policy implications, J. Clean. Prod. 182 (2018) 978–991, https://doi.org/10.1016/j.jclepro.2018.02.030.
[4] N.L.W. Wilson, B.J. Rickard, R. Saputo, S.T. Ho, Food waste: the role of date labels, package size, and product category, Food Qual. Prefer. 55 (2017) 35–44, https://doi.org/10.1016/j.foodqual.2016.08.004.
[5] Q. Zheng, M. Mikš-Krajnik, Y. Yang, S.M. Lee, S.C. Lee, H.G. Yuk, Evaluation of real-time PCR coupled with immunomagnetic separation or centrifugation for the detection of healthy and sanitizer-injured *Salmonella* spp. on mung bean sprouts, Int. J. Food Microbiol. 222 (2016) 48–55, https://doi.org/10.1016/j.ijfoodmicro.2016.01.013.
[6] Z. Wang, T. Miu, H. Xu, N. Duan, X. Ding, S. Li, Sensitive immunoassay of *Listeria monocytogenes* with highly fluorescent bioconjugated silica nanoparticles probe, J. Microbiol. Methods 83 (2010) 179–184, https://doi.org/10.1016/j.mimet.2010.08.013.
[7] S. Jaafreh, O. Valler, J. Kreyenschmidt, K. Günther, P. Kaul, In vitro discrimination and classification of Microbial Flora of Poultry using two dispersive Raman spectrometers (microscope and Portable Fiber-Optic systems)in tandem with chemometric analysis, Talanta 202 (2019) 411–425, https://doi.org/10.1016/j.talanta.2019.04.082.
[8] D. Klein, R. Breuch, S. von der Mark, C. Wickleder, P. Kaul, Detection of spoilage associated bacteria using Raman-microspectroscopy combined with multivariate statistical analysis, Talanta 196 (2019) 325–328, https://doi.org/10.1016/j.talanta.2018.12.094.
[9] Z. Fan, R. Kanchanapally, P.C. Ray, Hybrid graphene oxide based ultrasensitive SERS probe for label-free biosensing, J. Phys. Chem. Lett. 4 (2013) 3813–3818, https://doi.org/10.1021/jz4020597.
[10] B. Pearson, A. Mills, M. Tucker, S. Gao, L. McLandsborough, L. He, Rationalizing and advancing the 3-MPBA SERS sandwich assay for rapid detection of bacteria in environmental and food matrices, Food Microbiol. 72 (2018) 89–97, https://doi.org/10.1016/j.fm.2017.11.007.
[11] B. Pearson, P. Wang, A. Mills, S. Pang, L. McLandsborough, L. He, Innovative sandwich assay with dual optical and SERS sensing mechanisms for bacterial detection, Anal. Methods. 9 (2017) 4732–4739, https://doi.org/10.1039/c7ay01596h.
[12] W.R. Premasiri, J.C. Lee, A. Sauer-Budge, R. Théberge, C.E. Costello, L.D. Ziegler, The biochemical origins of the surface-enhanced Raman spectra of bacteria: a metabolomics profiling by SERS, Anal. Bioanal. Chem. 408 (2016) 4631–4647, https://doi.org/10.1007/s00216-016-9540-x.
[13] G.-J.E. Nychas, E.H. Drosinos, MEAT and poultry | spoilage of meat, Encycl. Food Microbiol. (2014) 514–519, https://doi.org/10.1016/B978-0-12-384730-0.00194-4.
[14] R. Gospavic, J. Kreyenschmidt, S. Bruckner, V. Popov, N. Haque, Mathematical modelling for predicting the growth of *Pseudomonas* spp. in poultry under variable temperature conditions, Int. J. Food Microbiol. 127 (2008) 290–297, https://doi.org/10.1016/j.ijfoodmicro.2008.07.022.
[15] U. Herbert, Assessment of Different Packaging Atmospheres for the Poultry Meat Industry Based on an Overall Quality Index, 2014. http://hss.ulb.uni-bonn.de/2014/3774/3774.htm.
[16] J. Kreyenschmidt, R. Ibald, Shelf life assessment of food, in: M.C. Nicoli (Ed.), Shelf Life Assess. Food, CRC Press, 2012, pp. 127–168, https://doi.org/10.1201/b11871.

[17] I. Vilar, M.C. García Fontán, B. Prieto, M.E. Tornadijo, J. Carballo, A survey on the microbiological changes during the manufacture of dry-cured lacón, a Spanish traditional meat product, J. Appl. Microbiol. 89 (2000) 1018–1026, https://doi.org/10.1046/j.1365-2672.2000.01210.x.
[18] Panel on Biological Hazards (BIOHAZ), Risks for public health related to the presence of *Bacillus cereus* and other *Bacillus* spp. including *Bacillus thuringiensis* in foodstuffs, EFSA J 14 (2016), https://doi.org/10.2903/j.efsa.2016.4524.
[19] Leibniz-Institut, DSMZ-German collection of microorganisms and cell cultures, Catal. Microorg. (2020).
[20] M. Sourdaine, D. Guenther, A.M. Dowgiallo, C. Harvey, Y. Mattley, A. Guckian, O. Lischtschenko, Protecting the food supply chain: utilizing SERS and portable Raman spectroscopy, Tech. Mess. 82 (2015) 625–632, https://doi.org/10.1515/teme-2015-0046.
[21] S. Li, L. Dai, An improved algorithm to remove cosmic spikes in Raman spectra for online monitoring, Appl. Spectrosc. 65 (2011) 1300–1306, https://doi.org/10.1366/10-06169.
[22] S. Mozharov, A. Nordon, D. Littlejohn, B. Marquardt, Automated cosmic spike filter optimized for process Raman spectroscopy, Appl. Spectrosc. 66 (2012) 1326–1333, https://doi.org/10.1366/12-06660.
[23] R.A. Fisher, The use OF multiple measurements IN taxonomic problems, Ann. Eugen. 7 (1936) 179–188, https://doi.org/10.1111/j.1469-1809.1936.tb02137.x.
[24] K. Backhaus, B. Erichson, W. Plinke, R. Weiber, Multivariate Analysemethoden, Springer-Verlag, Berlin, Heidelberg, 2018, https://doi.org/10.1007/978-3-662-56655-8.
[25] K. Katoh, K. Itoh, New selective media for *pseudomonas* strains producing fluorescent pigment, Soil Sci. Plant Nutr. 29 (1983) 525–532, https://doi.org/10.1080/00380768.1983.10434655.
[26] B.A. Federici, H.-W. Park, Y. Sakano, Insecticidal protein crystals of *Bacillus thuringiensis*, in: Inclusions in prokaryotes, Springer-Verlag, Berlin/Heidelberg, 2006, pp. 195–236.
[27] J. Xie, Z. Qiu, The effect of imbalanced data sets on LDA: a theoretical and empirical analysis, Pattern Recogn. 40 (2007) 557–562, https://doi.org/10.1016/j.patcog.2006.01.009.
[28] C.J. Skinner, D.J. Holmes, T.M. Smith, The effect of sample design on principal component analysis, J. Am. Stat. Assoc. 81 (1986) 789–798, https://doi.org/10.1080/01621459.1986.10478336.
[29] W. Burgos-Paz, S.E. Ramos-Onsins, M. Pérez-Enciso, L. Ferretti, Correcting for unequal sampling in principal component analysis of genetic data. Correcting Unequal Sampl. Princ. Compon. Anal. Genet. Data, 10th World Congress of Genetics Applied to Livestock Production, 2014, pp. 1–3.