Arch Lebensmittelhyg 69,
49–56 (2018)
DOI 10.2376/0003-925X-69-49

ISSN 0003-925X

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Antibiotic susceptibility of *Enterobacteriaceae,* isolated from sprouts, mixed salads and ready-to-eat salads in Germany

Antibiotika-Empfindlichkeit von Enterobacteriaceae *isoliert aus Sprossen, Mischsalaten und „Ready-to-Eat" Salaten in Deutschland*

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Summary

A collection of 109 *Enterobacteriaceae* isolates originating from sprouts, mixed salads and ready-to-eat salads bought in retail in south-western German supermarkets from April 2015 to February 2016 have been phenotypically characterized and were tested for susceptibility to antibiotics, in particular extended-spectrum β-lactamases. Only six isolates were found to be multi-resistant to three antibiotics substance groups (acylureidopenicillins, 3rd or 4th generation cephalosporins and fluorochinolones) namely four isolates belonging to *Enterobacter* spp. and two to *Citrobacter* spp. Therefore, the impact on the overall safety and quality of the surveyed German products can be regarded as inconspicuous in the light of antibiotic resistant *Enterobacteriaceae*.

Keywords: antimicrobial resistance, enterobacteria, fresh produce

Zusammenfassung

Im Zeitraum zwischen April 2015 und Februar 2016 wurden aus dem Einzelhandel im Süd-Westen Deutschlands Sprossen, Misch- und Fertigsalate bezogen. Aus diesen Produkten wurden 109 Isolate aus der Familie *Enterobacteriaceae* gewonnen und phänotypisch auf Antibiotikaresistenzen untersucht. Der Fokus lag auf dem Vorkommen von β-Laktamasen mit breitem Wirkungsspektrum. Lediglich sechs dieser Isolate erwiesen sich als resistent gegen drei Antibiotikagruppen (Acylureidopenicilline, Cephalosporine der 3. oder 4. Generation und Fluorochinolone), davon wurden vier Isolate als *Enterobacter* spp. und zwei Isolate als *Citrobacter* spp. identifiziert. Aufgrund der hier vorliegenden Ergebnisse kann der Einfluss antibiotikaresistenter *Enterobacteriaceae,* isoliert aus den untersuchten Produktgruppen, hinsichtlich Sicherheit und Qualität als unauffällig gewertet werden.

Schlüsselwörter: Antibiotikaresistenz, Enterobakterien, pflanzliche Produkte

Journal of Food Safety and Food Quality 69, Heft 2 (2018), Seiten 27–76

Introduction

Fruit and vegetables play a pivotal role in human nutrition, i.e. they are regarded as a rich source for vitamins, fiber and secondary plant compounds. Together with over 100 partners the German Nutrition Society (DGE) promotes the campaign "5 A Day" and recommends the consumption of five servings of fruit and vegetables per day to maintain healthy living (Boeing et al. 2012). Sprouts, mixed salads and ready to eat salads are included in this food group and are intended for raw consumption. Therefore, an element of risk remains as consumers could be exposed to potentially pathogenic microorganisms (Franz et al. 2008, Heaton and Jones 2008). Moreover, ingested bacteria could also carry antibiotic resistance genes. Recently, fresh produce were also described to play a role as carriers of antibiotic-resistant bacteria (Schwaiger et al. 2011a). Especially problematic are multidrug-resistant Gram-negative (MDRGN) *Enterobacteriaceae* or bacteria carrying genes for extended-spectrum β-lactamases (ESBL) (Paterson 2000, 2006). An appropriate treatment in case of an infection could be critical, particularly when resistance genes are transferred to more pathogenic bacteria via horizontal gene transfer. For example, in 1997 the gene bla_{SHV-12} coding for an ESBL was described in *Klebsiella pneumoniae* in Switzerland (Nüesch-Inderbinen et al. 1997). Four years later, the plasmid-mediated transfer of the bla_{SHV-12} gene in *Salmonella* spp. was documented for the first time in Senegal (Cardinale et al. 2001). ESBL-producing bacteria have been isolated from fruit and vegetables repeatedly e.g. in South Korea and the United Kingdom (Kim et al. 2015, Randall et al. 2017); therefore their occurrence is an emerging issue. Recently, the World Health Organization (WHO) published a 'Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics'. According to this list, next to carbapenem-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa,* carbapenem-resistant and third generation cephalosporin-resistant *Enterobacteriaceae* are regarded as the most critical pathogens (World Health Organization 2017).

Hence, the aim of this study was to collect and characterize *Enterobacteriaceae* isolates from sprouts and mixed salads bought in supermarkets in south-western Germany from April 2015 to February 2016 and to investigate the consumers risk being faced with antibiotic-resistant *Enterobacteriaceae* through the consumption of common fresh produce.

Materials and methods

Materials

Enterobacteriaceae *isolates*

Presumptive *Enterobacteriaceae* strains were isolated from sprouts (n=55) and mixed/ready-to-eat salads (n=54), bought in retail in south-western German supermarkets from April 2015 to February 2016. For isolation of bacterial strains, 25 g sample material was transferred aseptically into plastic bags with filter (Gosselin SAS, Borre, France). In case of ready-to-eat salads additional animal-based toppings were packed separately, thus only the vegetable parts of the products were used for investigation. Each sample was diluted 1:10 by addition of 225 ml buffered peptone water (BIOKAR Diagnostics, Pantin, France) using an automated diluter (AES Chemunex GmbH, Bruchsal, Germany). The samples were homogenized for two minutes in a laboratory paddle blender (AES Chemunex GmbH). Afterwards, tenfold serial dilutions were performed using ¼ strength ringer's solution (Merck KGaA, Darmstadt, Germany). For the subsequent isolation of *Enterobacteriaceae,* 100 µl aliquots of each 10^{-4}–10^{-7} dilution were plated on VRBD agar plates (BIOKAR Diagnostics). The plates were incubated at 30 °C. Initially, the incubation took place under anaerobic conditions in anaerobic jars for the first 24 h using Anaerocult® (Merck KGaA). Afterwards the plates were transferred to aerobic conditions and incubated for another 24 h. After a total incubation of 48 h colonies showing a typical phenotype were picked from VRBD agar. The isolates were streaked out on Standard I nutrient-agar plates (8 g Agar-Agar was added to 500 ml Standard Nutrient Broth I; Carl Roth GmbH + Co. KG, Karlsruhe, Germany) to obtain pure cultures. Strains were conserved at –80 °C in NuncTM cryogenic vials (Thermo Fisher Scientific, Waltham, USA) by addition of 150 µl glycerol (Carl Roth GmbH + Co. KG) and 850 µl fresh liquid culture. Detection of oxidase activity was conducted using Bactident® Oxidase test strips (Merck KGaA) according to the manufacturer's recommendations. Presence of catalase was determined by dripping 5 µl of 3 % H_2O_2 (Merck KGaA) solution directly on a single colony, catalase activity was confirmed visually by formation of small bubbles. Furthermore, all isolates were biochemically identified using API® ID32E (Biomerieux, Nürtingen, Germany) according the manufacturer's instructions.

Reference strains

According to the European Committee on Antimicrobial Susceptibility Testing (EUCAST), reference organisms were included in the subsequent analyzes as quality control. For this purpose, *Escherichia (E.) coli* DSM 1103 and *Klebsiella (K.) pneumoniae* DSM 26371 were obtained from the DSMZ (Leibniz Institute-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany).

Methods

Isolation and quantification of total genomic DNA

Isolation of total genomic bacterial DNA was conducted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendation with slight modifications. In brief, 2 ml of bacterial over-night culture were transferred to a microcentrifuge tube. The bacterial cells were pelleted by 10 min centrifugation at 16.000 x g and 4 °C. Differing from the protocol, each pellet was resuspended in 180 µl lysis buffer and 36 µl lysozyme (100 mg/ml). Cell wall lysis was performed for 30 min at 37 °C and 350 rpm in a Thermoshaker Incubator (peQLab Biotechnologie GmbH, Erlangen, Germany). Afterwards, 20 µl proteinase K and 200 µl buffer AL were added to each sample, followed by incubation for 30 min at 56 °C. Elution of DNA was performed twice in 50 µl buffer AE. For quantification of the DNA, the Qubit® dsDNA HighSensitivity Assay Kit (Thermo Fisher Scientific Inc.) was used according to the manufacturer's instructions on a Qubit 2.0 fluorometer (Thermo Fisher Scientific Inc.). DNA was stored at –20 °C until use.

Verification of the presumptive Enterobacteriaceae *isolates by* atpD *sequencing*

For molecular identification of all 109 *Enterobacteriaceae* isolates, the F-ATPase β-subunit gene *(atpD)* was partially

amplified by PCR. PCR was conducted using the ALLin™ Hot Start Taq Mastermix, 2x (highQu GmbH, Kraichtal, Germany) in a total reaction volume of 50 µl. Furthermore, 100 ng template DNA and 25 pmol of each 'atpD A1 fw' respective 'atpD A2 rev' primers according to Paradis et al. (2005) were added.

The amplifications were conducted in a peqSTAR 96 Universal Thermocycler (VWR International GmbH, Darmstadt, Germany): 3 min initial denaturation for 95 °C, 35 cycles consisting of 1 min denaturation at 95 °C, 1 min annealing at 50 °C, 1 min elongation at 72 °C, final elongation for 7 min at 72 °C. PCR products were completely loaded on 1 % agarose gels and visually inspected after gel electrophoresis. Agar plugs containing DNA amplicons of 884 bp size were extracted using the QIAEX II® gel extraction kit (Qiagen). Sequencing of amplicons was performed bi-directional at LGC Genomics GmbH (Berlin, Germany). The chromatograms were visually inspected using Chromas Version 2.4.1, the sequences were edited using SeqBuilder Version 10.1.1 and the complementary sequences were joined using MegAlign Version 10.1.1. The resulting sequences were subjected to a database query (Basic Local Alignment Search Tool, BLAST) against the database of the National Center for Biotechnology Information (NCBI), the entry with the best percentage accordance was chosen for final identification. Moreover, the atpD sequences were aligned and clustered using BioNumerics V.7.6 (Applied Maths, Sint-Martens-Latem, Belgium). Clustering was performed using fast algorithm and UPGMA.

Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed by disk diffusion method according to the EUCAST, version 6.0 from January 2017 (EUCAST 2017b). Oxoid™ Müller-Hinton-Agar (MHA) plates were purchased from Thermo Fisher Scientific (Wesel, Germany); antibiotic disks were obtained from Mast Diagnostica (Reinfeld, Germany). The disks were applied to the agar plates using a DiscMaster 4 antibiotic disc dispenser (Mast Diagnostica). Isolates were generally incubated 16–20 h at 35±1 °C; isolates that were not able to grow at these conditions were incubated at 30 °C. Zone diameters were read in duplicates accurate to a millimeter, whereas different MHA charges were used at different days. Tests for quality control with reference strains were conducted in parallel. In cases where zone diameters differed more than 3 mm, a third repetition of the analysis was conducted. Per isolate, a maximum of four analyses was performed and the arithmetic mean of all diameters was calculated.

In total, all 109 isolates were screened for their susceptibility to a panel of 15 antibiotics from different substance groups, enabling an identification of MDRGN bacteria. The tested antibiotics were: ampicillin (10 µg), aztreonam (30 µg), cefepime (30 µg), cefotaxime (5 µg), cefoxitin (30 µg), cefpodoxime (10 µg), ceftazidime (10 µg), cefuroxime (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), meropenem (10 µg), piperacillin/tazobactam (30 µg/6 µg), tigecycline (15 µg) and co-trimoxazole (1.25 µg/23.75 µg). In case of *Salmonella* spp. pefloxacin (5 µg) instead of ciprofloxacin was used as recommended by EUCAST. Interpretation of the zone diameters was performed according to the breakpoint tables version 7.0 published by EUCAST (EUCAST 2017a). The zone diameters of tigecycline are validated for *E. coli* only; however the interpretation of all investigated species was based on these values to get an indication for tigecycline susceptibility. For data analysis, the percentage of all isolates classified into the cut-off values according to the above mentioned breakpoints and the percentage of the isolated genera resistant to the tested antibiotics was calculated, the values were rounded to the first decimal place.

Phenotypical Extended-Spectrum β-Lactamases (ESBL)-testing

All 109 isolates were subjected to ESBL-testing using the D68C AmpC & ESBL Detection Set (Mast Diagnostica) according to the manufacturer's instructions except that the growth temperature was chosen according to the growth temperatures during the antibiotic susceptibility testing. Isolates phenotypically identified as AmpC and/or ESBL-positive, or with ambiguous results according to D68C analysis were further investigated by disk diffusion testing according to 'EUCAST guidelines for detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance' (EUCAST, 2013). In compliance with EUCAST, all isolates showing an inhibiting zone under 21 mm against cefpodoxime (10 µg) in the antibiotic susceptibility testing were also included in this test.

Results and Discussion

The bacterial isolates were collected within the course of a project investigating the incidence and characterization of potential human pathogenic bacteria in German fresh produce. Data regarding the incidence of potentially human pathogenic bacteria in fresh produce will be published and discussed elsewhere. The purpose of this publication is presenting antibiotic susceptibility analyses of *Enterobacteriaceae* strains isolated from sprouts, mixed and ready-to-eat salads.

Verification of the presumptive *Enterobacteriaceae* isolates by *atpD* sequencing

According to Paradis, et al. (2005) partially sequencing of the *atpD* gene was chosen for molecular identification of the isolates as these authors described that distances based on *atpD* sequences provide higher discriminating power compared to 16S rRNA gene sequencing, especially within the *Enterobacteriaceae* family. The 109 isolates were identified as: *Enterobacter* spp. (n=36), *Citrobacter* spp. (n=19), *Rahnella* spp. (n=13), *Klebsiella* spp. (n=12), *Erwinia* spp. (n=10), *Serratia* spp. (n=6), *Salmonella (S.) enterica* ssp. *enterica* (n=6), *Pantoea (P.) agglomerans* (n=3), *E. coli* (n=2), *Kluyvera (K.) intermedia* (n=1) and *Kosakonia (K.) cowanii* (n=1). Clustering of the *atpD* sequences revealed distinctive clusters of each identified genera, emphasizing the correct identification at least on genus level (Figure 1).

Antibiotic susceptibility testing

The mean values of the zone diameters were classified according to the breakpoint tables from EUCAST into the cut-off values 'susceptible', 'intermediate' and 'resistant' (EUCAST 2017a). The results from this classification are listed in Table 1. Table 2 gives an overview of the percentage of isolated genera resistant to the tested antibiotics.

None of the isolates showed any resistance to cefepime, gentamicin, meropenem, piperacillin-tazobactam, tigecycline, co-trimoxazole or pefloxacin. However, 46.8 % (n=51) of all *Enterobacteriaceae* isolates showed an inter-

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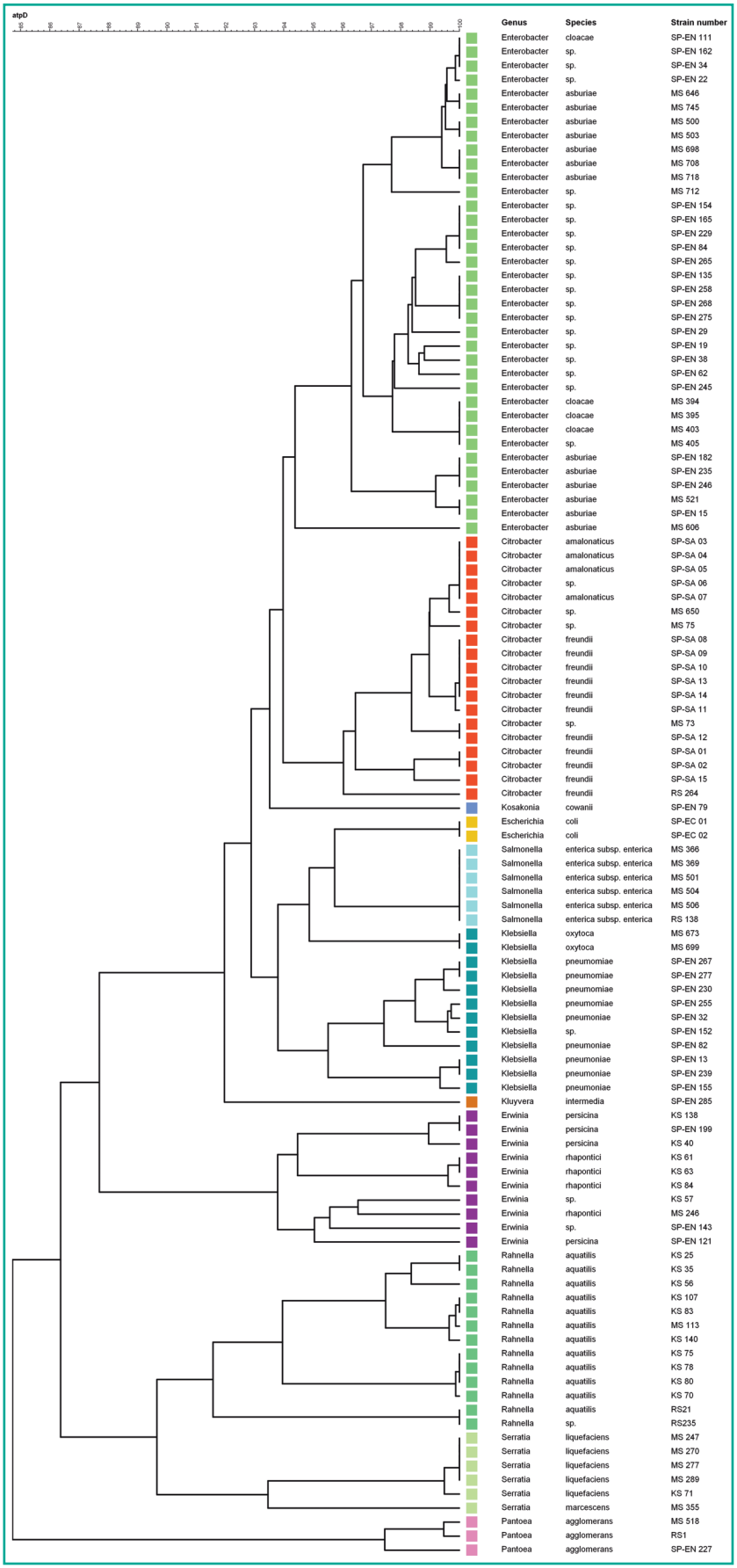

FIGURE 1: *Dendrogram of the cluster analysis of* atpD *genes showing distinctive clusters of each identified genera, emphasizing the correct identification at least on genus level. Isolates that belong to the same genus are highlighted in individual colors. Clustering was performed using fast algorithm and UPGMA.*

mediate reaction to cefepime (Table 1). These results emphasize the usage of tigecycline or co-trimoxazole as last-resort antibiotic (RKI 2012, Schulze-Stübner et al. 2016) or the use of gentamicin and meropenem for the treatment of 4MRGN (Schulze-Stübner, et al. 2016).

Most of the isolates showed resistances to further tested β-lactam antibiotics. In ascending order 14.7 % (n=16) of all isolates were resistant to ceftazidime, 52.3 % (n=57) to cefuroxime, 56 % (n=61) to ampicillin, 57.8 % (n=63) to cefpodoxime and finally 61.5 % (n=67) showed resistances to cefoxitin (Table 1). These resistances were not evenly distributed among all genera. While the resistance rates of *Enterobacter* spp. and *Citrobacter* spp. isolates were generally high, what is possibly due to their possession of genes coding for AmpC-β-lactamases (Barlow and Hall 2002, Pitout et al. 1997), only 27.8 % (n=10) of *Enterobacter* spp. isolates and 31.6 % (n=6) of *Citrobacter* spp. isolates exhibited a resistance mechanism to the third generation cephalosporin ceftazidime. Worth mentioning is the resistance rate to ampicillin, where 86.1 % (n=31) of *Enterobacter* spp. isolates showed resistance, while only 10.5 % (n=2) of *Citrobacter* spp. had this property. Furthermore, chromosomal AmpC-β-lactamases are described in *Serratia* spp. and *Erwinia* spp. (Rottman et al. 2002, Stock et al. 2003a, Stock et al. 2003b). However, resistances of isolates of both genera to the tested β-lactam antibiotics were only observed sporadically. Sole exception is the resistance of all tested *Serratia* spp. isolates (n=6) to cefuroxime, a second generation cephalosporin. Chromosomal AmpC-β-lactamases are not described in species such as *Klebsiella pneumoniae, Klebsiella oxytoca* and *R. aquatilis* (Jacoby 2009). An intrinsic resistance of *Klebsiella* spp. to ampicillin is described (Bouza and Cercenado 2002), this could be observed in all tested *Klebsiella* isolates (n=12). The resistance rates of these *Klebsiella* spp. isolates to the other tested β-lactam antibiotics were marginal, what indicates differing resistance mechanisms that are discussed elsewhere (Livermore 1995). 84.6 % (n=11) of the *Rahnella* isolates indicated resistances to ampicillin, cefpodoxime and cefuroxime. In the literature, resistances of *Rahnella* spp. to ampicillin and cefuroxime were frequently observed, but also resistances to further aminopenicillines and first generation cephalosporins (Rozhon et al. 2012). Cefpodoxime is regarded as the most sensitive individual indicator cephalosporin for detection of ESBL production and may be used for screening according to EUCAST (EUCAST 2013).

For the classification of the tested isolates in MDRGN, the recommendation of the Commission for Hospital Hygiene and Infec-

TABLE 1: *Classification of all isolates (n=109) into the cut-off values "susceptible", "intermediate" and "resistant". "–" value not defined according to EUCAST (EUCAST 2017a).*

Antibiotic	Cut-off values [%]		
	Susceptible	Intermediate	Resistant
Ampicillin	44.0	–	56.0
Aztreonam	59.6	34.9	5.5
Cefepime	53.2	46.8	0
Cefotaxime	84.4	13.8	1.8
Cefoxitin	38.5	–	61.5
Cefpodoxime	42.2	–	57.8
Ceftazidime	52.3	33.0	14.7
Cefuroxime	47.7	–	52.3
Chloramphenicol	85.3	–	14.7
Ciprofloxacin (all strains except *Salmonella* spp.; n=103)	52.4	32.0	15.5
Gentamicin	95.4	4.6	0
Meropenem	100.0	0	0
Piperacillin-tazobactam	92.7	7.3	0
Tigecycline	81.7	18.3	0
Co-trimoxazole	100.0	0	0
Pefloxacin (*Salmonella* spp.; n=6)	100.0	–	0

tion Prevention (KRINKO) from the Robert Koch Institute (RKI) was chosen. This classification is based on the behavior against four antibiotics substance groups (respective their guide substances): acylureidopenicillins (piperacillin), 3rd or 4th generation cephalosporins (cefotaxime and/or ceftazidime, carbapenems (meropenem and/or imipenem) and fluorochinolones (ciprofloxacin). Isolates that are resistant or intermediate to the guide substances of all four substance groups are regarded as 4MRGN. If resistant or intermediate behavior is detected to all guide substances except the carbapenems, the isolates are regarded as 3MRGN (RKI 2012). All 109 isolates were tested against a combination of piperacillin and tazobactam and not against piperacillin solitary.

Out of all 109 isolates, no 4MRGN could be identified as all isolates were susceptible to meropenem. However, six isolates were classified as 3MRGN. Four of these six isolates belong to the genus *Enterobacter* and two to the genus *Citrobacter.* One isolate which showed the highest amount of resistances was identified as *Enterobacter asburiae* and exhibited resistance to seven antibiotics, in addition this strain was intermediate to five further antibiotics. Except of one *Citrobacter freundii* strain, which was isolated from sprouts, the five other 3MRGN isolates were collected from mixed salad samples.

Although fruit- and vegetable-based outbreaks with some members of the *Enterobacteriaceae* have been described occasionally in Germany (Bundesinstitut für Risikobewertung 2009a, b, 2010, 2011, 2012, 2013, 2014, 2015), according to the authors' awareness no verified outbreaks of the genera and species that were characterized as 3MRGN in this study have been described in Germany in the recent years. However, it has to be kept in mind that intestinal gene transfer of antimicrobial resistance genes is not uncommon and potentially pathogenic bacteria may gather resistance genes (Huddleston 2014, Hwang et al. 2017).

ESBL-testing

According to the D68C testing, seven isolates were identified as ESBL-positive, 19 isolates were recognized as AmpC-positive, 34 isolates showed a negative result and a total of 49 isolates indicated ambiguous results necessitating further investigation.

AmpC-positive isolates were distributed over the genera *Citrobacter, Erwinia* and *Enterobacter.* This is in good agreement with the already observed and above discussed resistance behavior to β-lactam antibiotics. Inducible chromosomal AmpC-β-lactamases were described in *Citrobacter freundii* and *Erwinia rhapontici,* in the majority of species belonging to *Enterobacter* spp. except *E. gergoviae* (Barlow and Hall 2002, Jacoby 2009, Naas et al. 2004, Pitout, et al. 1997). All six *Serratia* spp. isolates and the sole *K. intermedia* isolate were identified as AmpC-negative, although inducible chromosomal AmpC-β-lactamases are described in the literature (Rottman, et al. 2002, Stock, et al. 2003a, Stock, et al. 2003b). In the same way, no AmpC-β-lactamases could be phenotypically identified in *E. coli, Klebsiella* spp., *K. cowanii, P. agglomerans, Rahnella* spp. and *S. enterica* ssp. *enterica.* According to the literature, these genera

TABLE 2: *Percentage of isolated genera resistant to the tested antibiotics, 'n.d.' not determined.*

	AMP	ATM	CPM	CTX	FOX	CPD	CAZ	CXM	CAP	CIP	GEN	MEM	TZP	TGC	SXT	PEF
Enterobacter spp. (n=36)	86.1	5.6	0	2.8	100	77.8	27.8	75	8.3	13.9	0	0	0	0	0	n. d.
Citrobacter spp. (n=19)	10.5	15.8	0	0	100	100	31.6	52.6	47.4	21.1	0	0	0	0	0	n. d.
Rahnella spp. (n=13)	84.6	0	0	7.7	7.7	84.6	0	84.6	30.8	0	0	0	0	0	0	n. d.
Klebsiella spp. (n=12)	100	0	0	0	8.3	8.3	0	16.7	50	50	0	0	0	0	0	n. d.
Erwinia spp. (n=10)	40	10	0	0	40	30	0	10	0	10	0	0	0	0	0	n. d.
Serratia spp. (n=6)	0	0	0	0	50	0	0	100	0	0	0	0	0	0	0	n. d.
S. enterica ssp. *enterica* (n=6)	0	0	0	0	0	0	0	0	0	n. d.	0	0	0	0	0	0
P. agglomerans (n=3)	0	0	0	0	66.7	33.3	0	0	0	0	0	0	0	0	0	n. d.
E. coli (n=2)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	n. d.
K. intermedia (n=1)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	n. d.
K. cowanii (n=1)	100	0	0	0	100	0	0	0	0	0	0	0	0	0	0	n. d.

AMP = ampicillin; ATM = aztreonam; CPM = cefepime; CTX = cefotaxime; FOX = cefoxitin; CPD = cefpodoxime; CAZ = ceftazidime; CXM = cefuroxime; CAP = chloramphenicol; CIP = ciprofloxacin; GEN = gentamicin; MEM = meropenem; TZP = piperacillin-tazobactam; TGC = tigecycline; SXT = co-trimoxazole; PEF = pefloxacin

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and species do not possess chromosomal AmpC-β-lactamases (Bhatti et al. 2017, Jacoby 2009, Pitout, et al. 1997).

All seven isolates that were identified as ESBL-positive by D68C testing belong to the genus *Rahnella. Rahnella* spp. can typically be isolated from aqueous habitats and soils (Berge et al. 1991, Brenner et al. 1998, Heulin et al. 1994, Selenska-Pobell et al. 1995), for which reason they can also be found on fresh fruit and vegetables (Ragaert et al. 2007, Ruimy et al. 2010a). Up to now, two β-lactamases could be identified and characterized as ESBL in *Rhanella* spp., namely RAHN-1 and RAHN-2 (Bellais et al. 2001, Ruimy et al. 2010b).

All ambiguous, ESBL- and AmpC-positive, as well as four negative isolates showing a zone of inhibition under 21 mm against cefpodoxime (10 µg) were subjected to ESBL-testing according to EUCAST. None of these isolates were confirmed to express ESBL.

Bush and Jacoby (2010) described a multitude of further β-lactamases next to AmpC and ESBL. It could be assumed that such mechanisms have led to the differing results in both tests. As a concluding classification of the isolates the authors keep to the assessment according to EUCAST testing. Therefore, no phenotypical expression of ESBL could be detected in any of the 109 *Enterobacteriaceae* isolates from sprouts, mixed and ready-to-eat salads bought in south-western German supermarkets from April 2015 until February 2016.

In the literature several studies regarding the occurrence of ESBL-positive isolates from fresh produce are described. For example, Randall and co-workers examined inter alia 400 fruit- and vegetable-samples for the presence of ESBL-producing *E. coli* by phenotypical and molecular methods. None of these samples was confirmed ESBL-positive (Randall, et al. 2017). In 2011, Hassan et al. also examined fruit- and vegetable-samples for ESBL-production. From a total of 128 *Enterobacteriaceae* isolates randomly chosen, two *E. coli* isolates and one *Enterobacter* spp. strain could be confirmed as ESBL-positive (Hassan et al. 2011). Bhutani et al. conducted antibiotic susceptibility, but also ESBL-testing with isolates obtained from iceberg lettuce. From a total of 138 bacterial isolates, two *K. pneumoniae* isolates and one *Serratia marcescens* strain showed a phenotypical ESBL-expression (Bhutani et al. 2015). A further study on ready-to-eat products confirmed the occurrence of ESBL-positive *E. coli* in one of 32 examined salad samples (Egea et al. 2011). Margot et al. (2016) published a study on the occurrence of *Salmonella, L. monocytogenes,* shigatoxin-producing *E. coli* and ESBL-producing *Enterobacteriaceae* in sprout samples collected from the Swiss market, where three of the 102 were identified as ESBL-positive *E. coli, K. variicola* and *E. cloacae.*

In 2015, a Dutch study was published on the prevalence and characterization of ESBL- and AmpC-producing *Enterobacteriaceae* on retail vegetables. Overall, 5.2 % of all 1216 vegetables samples collected between 2012 and 2013 were identified as affected by third generation cephalosporin resistant bacteria. The authors of the above study also emphasize the health risks for consumers by asymptomatic colonization and carriage of commensal species as *Citrobacter* spp., *Enterobacter* spp. and *E. coli,* that may be ingested with affected foodstuffs. These bacteria may be transferred to more susceptible individuals (e. g. children, elderly, immunocompromised people) and resistance genes may be transferred to potentially pathogenic, intestinal bacteria (van Hoek et al. 2015). In 2015, two Swiss studies investigated the prevalence of ESBL-positive *Enterobacteriaceae* in products produced and sold in Switzerland as well as products produced in foreign countries and imported. The authors emphasize that the origin of the products and international trade may be important factors regarding the occurence of ESBL-positive *Enterobacteriaceae* in food (Nüesch-Inderbinen et al. 2015, Zurfluh et al. 2015). In this study, the majority of investigated products was produced in Germany with single produces originating from Spain, Italy and the Netherlands.

Conclusions

Up to now, based on literature data and the here presented study, the incidence of MDRGN *Enterobacteriaceae* and especially the incidence of ESBL-positive *Enterobacteriaceae* in fresh produce is low. However, this status may get to a pivotal point in future, necessitating a prompt action to maintain the safety and quality for consumers. The authors of this study propose the screening of the here analyzed product groups for the occurrence of bacterial isolates and the characterization of their antibiotic resistance profiles as well as potential ESBL-production capabilities on a regular basis, to keep track of the development of this issue. Especially, as these products are intended for raw consumption without substantial processing that reduces the bacterial load. As already proposed from other authors the recommendation to wash and peel fresh produce before raw consumption is still advisable (Schwaiger, et al. 2011a, Schwaiger et al. 2011b) to reduce the bacterial load. Outer lettuce leaves have been described to house one to two log CFU/g higher levels of bacteria compared to inner leaves (Maxcy 1978). Washing of mixed salads reduces the bacterial load by approximately 90 %; some commercial producers even supplement citric acid, malic acid or tartaric acid during the washing process to reach higher reduction levels. Also washing of sprouts for raw consumption is recommended as the bacterial count can be reduced by two log CFU/g when washed two times for five minutes in 1 % acetic acid. Besides, short storage periods are advisable (Schillinger and Becker 2007).

Acknowledgement

The authors thankfully acknowledge the technical assistance of Luisa Martinez, Lilia Rudolf and Andrea K. Schavitz.

Conflict of interest

The authors declare that there are no conflicts of interest.

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