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Antimicrobial resistance of *Enterobacter cloacae* complex isolates from the surface of muskmelons

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ABSTRACT

The increasing antimicrobial resistance (AMR) among pathogenic and opportunistic pathogenic microorganisms is one of the main global public health problems. The consumption of food contaminated with such bacteria (ARB), especially of raw products, might result in the direct acquisition of ARB and in a spread of resistant bacteria along the food chain.

The aim of the study was to characterize the antimicrobial susceptibility of potentially extended spectrum β-lactamase (ESBL) producing or AmpC resistant *Enterobacteriaceae* isolated from the surface of 147 muskmelons from wholesale and retail. A phenotypic analysis was carried out by using minimum inhibitory concentration (MIC) test strips for ESBL detection and MIC susceptibility plates against 14 antimicrobials. Furthermore, ESBL genes, *sul*-genes and plasmid-mediated AmpC resistance were analyzed by real-time PCR. Additionally, a further insight in the AmpC resistance of isolates of the *Enterobacter cloacae* complex (ECC) was obtained by analyzing the sequence of the *ampC* regulatory region ($n = 15$).

A total of 73 potentially resistant *Enterobacteriaceae* were isolated from 56 muskmelons. Of these, 15 isolates of the ECC were suspicious for ESBL/AmpC resistance, and eleven thereof were positive for the AmpC family EBC. Phenotypic analysis showed diminished susceptibility against "critically" and "highly important" antimicrobials, according to the WHO classification. Furthermore, divergence in the *ampC* regulatory region was detected between the 15 isolates.

These findings highlight the important role that raw produce might play in the transmission of antimicrobial resistances along the food chain.

1. Introduction

Antimicrobial resistance (AMR) is a major threat to public health. Microorganisms constantly develop and spread new resistance mechanisms, hampering an effective treatment of many infections. This increases health care costs compared to treating non-resistant infections (EFSA, 2008; WHO, 2018).

Misuse and overuse of antimicrobials accelerates this emerging process, so that antimicrobial resistant bacteria (ARB) are already present globally throughout the environment (water, soil, air), in humans, animals, and food (WHO, 2018). Humans can be expose to ARB by consumption of food products from both animal (Almeida et al., 2017; Schroeder et al., 2003; Wieczorek et al., 2018) and non-animal origin (Blaak et al., 2014; Hassan et al., 2011; Schwaiger et al., 2011a; Van Hoek et al., 2015).

As a response to this serious global health threat, the WHO published a ranking of medically important antimicrobials for human medicine, which was recently revised (WHO, 2016). In this list, antimicrobials are classified according to two criteria. Criterion 1 (C1): The antimicrobial class is the sole, or one of limited available therapies, to treat serious bacterial infections in people. Criterion 2 (C2): The antimicrobial class is used to treat infections in people caused by either: (1) bacteria that may be transmitted to humans from non-human sources, or (2) bacteria that may acquire resistance genes from non-human sources. After this, medically important antimicrobials are categorized as "critically important" (meet both C1 and C2), "highly important" (meet either C1 or C2) or "important" (meet neither C1 nor C2). In 2016 three prioritization criteria (P1, P2 and P3) were newly introduced. P1

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and P2 relate to the volume of antimicrobial use in humans, while P3 relates to the transmission of resistant bacteria/genes from non-human sources. “Critically important” antimicrobial classes meeting all three prioritization criteria, are now indicated as highest priority. The other “critically important” antimicrobials are referred to as of high priority.

In order to categorize which microorganisms are resistant to the different antibiotics compared to the original wild type, epidemiologic cut off values (ECOFFs) are established by using data submitted from different countries worldwide. These values are constantly updated on a yearly basis by both the EUCAST (European committee on antimicrobial susceptibility testing) in Europe and the CLSI (Clinical and Laboratory Standards Institute) in the United States (EUCAST, 2018). The lack of submitted data concerning certain antimicrobial-microorganism combinations might hamper the setting of certain ECOFFs by both institutions.

Resistance to 3rd generation cephalosporins (3GCs) acquired by *Enterobacteriaceae* is one of the antimicrobial resistance of major concern. These antimicrobials are currently crucial in the treatment of many bacterial infections (Canton et al., 2008; Castanheira et al., 2008; Jacoby, 2009). This resistance is often mediated by the production of extended spectrum β-lactamases (ESBLs) and AmpC β-lactamases (AmpC), which confer resistance to almost all β-lactams. However, ESBLs and AmpC differ in their ability to hydrolyse the 4th generation cephalosporin cefepime (most ESBLs) and cephamycins (AmpC). ESBLs are sensitive to β-lactamase inhibitors like clavulanic acid (CA), while AmpC are not (Jacoby, 2009; Paterson and Bonomo, 2005). Furthermore, ESBL genes are generally located on plasmids, while AmpC resistance can be both chromosome- and plasmid-mediated (Jacoby, 2009; Paterson and Bonomo, 2005). Plasmids mediating ESBL or AmpC resistance might also encode resistance genes to many other types of antimicrobials, meaning more complications for infection treatments (Jacoby, 2009; Schultsz and Geerlings, 2012; Simner et al., 2011).

Chromosomally encoded AmpC β-lactamases present different regulatory patterns of the *ampC* gene among different genera of *Enterobacteriaceae* (Jacoby, 2009). Some microorganisms, like *Escherichia coli*, possess a constitutively expressed (at a low level) *ampC* gene which partially overlaps the adjacent fumarate reductase (*frd*) operon (Grundstrom and Jaurin, 1982; Jaurin and Grundstrom, 1981; Lindberg and Normark, 1986; Normark et al., 1983) and whose transcription is regulated by a growth rate-dependent attenuation mechanism (Jaurin et al., 1981; Jaurin and Normark, 1979). Other microorganisms like bacteria of the *Enterobacter cloacae* complex (ECC; *Ent. cloacae*, *Ent. asburiae*, *Ent. hormaechei*, *Ent. kobei*, *Ent. ludiwigii* and *Ent. nimipressuralis*), *Citrobacter* spp. or *Serratia* spp. have inducible, chromosomally-encoded β-lactamases (Hennessey, 1967; Hoffmann and Roggenkamp, 2003; Paauw et al., 2008). Most of these microorganisms and other *Enterobacteriaceae* are able to overproduce AmpC β-lactamases by either derepressing a chromosomal gene, *ampR*, or acquiring transferable (plasmid-mediated) *ampC* genes. With only a few exceptions, plasmid-mediated strains differ from chromosomal strains in which they are normally not inducible (Honore et al., 1986, 1989; Scotta et al., 2011; Stock et al., 2001).

While *E. coli* only possesses an *ampC* promoter located within the coding sequence of the *frd* operon (Cole et al., 1985; Jaurin and Grundstrom, 1981; Lindberg and Normark, 1986), ECC strains additionally possess this *ampR* region located between the *frd* operon and the *ampC* gene. The *ampR* gene encodes AmpR, a 30,500 Da LysR-type transcriptional regulator protein. This protein is involved in the chromosomal β-lactamase induction mechanism together with other two major gene products related to the peptidoglycan recycling pathway, AmpD (cytosolic amidase) and AmpG (an inner membrane permease) (Holtje et al., 1994; Honore et al., 1986, 1989; Johnson et al., 2013; Kong et al., 2010; Korfmann and Sanders, 1989; Lindquist et al., 1993; Nicolas et al., 1987). It was suggested that the *ampR* region was deleted from the chromosomal *ampC* region of *E. coli* after divergence of *E. coli* and *Ent. cloacae* from the common ancestor (Honore et al., 1986).

Enterobacter spp. have gained increasing clinical significance during the last years and were recognized as major nosocomial pathogens, especially for intensive care patients (e.g. by causing septicemia). These microorganisms are widespread in the environment, as they are found in soil and sewage, and are also part of the commensal enteric microbiota of the human gastrointestinal tract (Denton, 2007; Wisplinghoff et al., 2004). Consequently, they can contaminate fresh produce as well (Blaak et al., 2014; Esteban-Cuesta et al., 2018; Hassan et al., 2011; Schwaiger et al., 2011b). Currently, *Ent. cloacae* is regarded as the most important representative within the six species classified into the ECC (Hoffmann and Roggenkamp, 2003; Paauw et al., 2008).

Fresh produce might be contaminated with antimicrobial or multidrug resistant (MDR) bacteria (Hoelzel et al., 2018). Contact with contaminated water or soil might result in contamination of the surface of products (Dhanji et al., 2012; Tacão et al., 2012). Cross contamination may also occur during processing (Davies and Davies, 2010).

The aim of this study was to phenotypically and genotypically characterize potentially ESBL or AmpC resistant *Enterobacteriaceae* isolated from the surface of internationally traded muskmelons. Additionally, to gain more insight into the antimicrobial AmpC resistance of ECC isolates of food origin, a PCR was conducted in order to detect the *ampR* gene and the results were compared with the PCR results on the plasmid-mediated AmpC resistance.

2. Materials and methods

2.1. Samples

A total of 147 imported reticulated muskmelons were purchased between October 2014 and September 2015 from German wholesale ($n = 64$) and retail ($n = 83$). These muskmelons were imported from Brazil ($n = 36$), Honduras ($n = 25$), Costa Rica ($n = 12$), Spain ($n = 40$), Italy ($n = 24$) and Morocco ($n = 10$). See Esteban-Cuesta et al. (2018).

2.2. Phenotypic examination

2.2.1. Screening

Melons were peeled using a stainless-steel peeler disinfected with heat and ethanol 96%. Ten grams of peel were mixed with 90 ml Tryptic Soy Broth (Merck, Germany) containing 1 µg/ml cefotaxime sodium salt (Alfa Aesar, Germany; TSB +), homogenized for 60 s in Lab-Blender and incubated for 24 h at 37 °C. A ten-fold dilution was plated directly on MacConkey No. 3 Agar (OXOID, Germany) containing 1 µg/ml cefotaxime sodium salt (Mac +) and incubated for 24 h at 37 °C under aerobic conditions. Suspicious colonies were subcultured on Brilliance™ Extended-Spectrum-Beta-Lactamase (ESBL) Agar (OXOID, Germany). After 24 h incubation at 37 °C, the isolates were identified using MALDI-TOF MS, as previously described (Esteban-Cuesta et al., 2018). Isolates identified as *Enterobacter* (*Ent.*) spp. by MALDI-TOF MS were prepared for PCR amplification and sequencing of the 16S rRNA for confirmation purposes.

2.2.2. MIC Test Strips ESBL

Enterobacteriaceae grown on ESBL Agar were analyzed using MIC Test Strips ESBL (Liofilchem®, Italy). Antibiotics included were cefepime and cefepime with clavulanic acid (CA), cefotaxime and cefotaxime with CA as well as ceftazidime and ceftazidime with CA. Suspicious colonies were homogenized in 0.85% NaCl-solution (0.5 McFarland standard of turbidity) and spread on Mueller-Hinton Agar (Oxoid, Germany) using cotton swabs. MIC Strips were placed onto the agar and incubated for 24 h at 37 °C. Interpretation of the results (inhibition-ellipses) was carried out according to the manufacturers' instructions. *Klebsiella pneumoniae* ATCC 700603 and *Escherichia coli* ATCC 25922 were used as positive and negative control, respectively.

2.2.3. MIC Susceptibility Plate Test

Antimicrobial susceptibility of all microorganisms grown on ESBL plates was further tested by means of SENSITITRE™ MIC susceptibility plates for Gram negative non-fastidious isolates (TREK Diagnostic Systems, USA). The antimicrobials tested were amoxicillin/CA, ampicillin, azithromycin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, nalidixic acid, tetracycline, trimethoprim/sulfamethoxazole, streptomycin, and sulfisoxazole. *Klebsiella pneumoniae* ATCC 700603 and *Escherichia coli* ATCC 25922 were used as positive and negative control, respectively. Analysis and interpretation of the results was performed according to the manufacturers' instructions.

2.3. Genotypic examination

Enterobacteriaceae grown on ESBL agar were analyzed for ESBL-genes *bla*TEM, *bla*SHV and *bla*CTX-M by means of conventional PCR. Primers used were CTX-M-HM (511 bp), SHV-HM (850 bp) and TEM-HM (842 bp) as described by Hoffmann et al. (2006). DNA was extracted using the ISOLATE II Genomic DNA Kit (Bioline GmbH, Germany). The iCycler™ Thermal Cycler (Bio-Rad, USA) was used to amplify the target DNA. The PCR reaction was carried out using the GoTaq® G2 Flexi DNA Polymerase Kit (Promega GmbH, Germany). A 25 µl reaction volume contained 5 µl of 5× Green GoTaq® Flexi Buffer, 2 µl $MgCl_2$ (2 mM), 2.5 µl 0.2 mM each dNTP (Promega GmbH, Germany), 2.0 µl primer (forward and reverse, 0.16 µM), 0.25 µl GoTaq® G2 Flexi DNA Polymerase (5u/µl), 1 µl of DNA and filled with 12.25 µl molecular grade water. PCR was comprised of an initial denaturation of 2 min at 95 °C, 30 cycles of denaturation of 45 s at 95 °C, 45 s annealing at 57 °C (for *bla*SHV, *bla*TEM) or 55 °C (for *bla*CTX-M) and elongation at 72 °C for 45 s, ending with a final elongation step of 5 min at 72 °C. PCR products were analyzed using agarose gel (1.5%) electrophoresis and DNA bands were visualized under UV light. Positive controls for the PCR-amplification of the ESBL-genes were *E. coli* (NCTC 13452, *bla*CTX-M-3), *Klebsiella pneumoniae* (ATCC 700603, *bla*SHV-18). *E. coli* (NCTC 13351, *bla*TEM-3), and *E. coli* ATCC 25922 was used as negative control.

Resistance genes *sul1* and *sul2* were also analyzed using the same DNA extraction method as described above. PCR mix was prepared in a 25 µl volume containing 5 µl of 5× Green GoTaq® Flexi Buffer, 2 µl $MgCl_2$ (2 mM), 2.5 µl 0.2 mM each dNTP (Promega GmbH, Germany), 2.0 µl *sul1* forward and reverse primer and 2.0 µl *sul2* forward and reverse primer (0.16 µM each primer pair; Kozak et al., 2009), 0.25 µl GoTaq® G2 Flexi DNA Polymerase (5u/µl), 1 µl of DNA and filled with 10.25 µl molecular grade water. The PCR reaction was performed in iCycler™ Thermal Cycler (Bio-Rad, USA), including initial denaturation of 2 min at 95 °C, 25 cycles of 30 s at 95 °C, 30 s annealing at 60 °C and 30 s elongation at 72 °C, ending with a final elongation step of 5 min at 72 °C. *Salmonella enterica* serovar [4,5,12:i:-] (Schwaiger et al., 2010) was used as positive control for the *sul1*- and *sul2*-genes analysis. PCR amplification was proved by agarose gel analysis.

Additionally, the plasmid mediated *sul3* gen was also screened. DNA was extracted using Aurum™ Plasmid Mini Kit (Bio-Rad Laboratories GmbH, Germany) after enrichment in 10 ml Luria-Bertani (LB)-Medium with 40 µl/ml sulfisoxazol (Sigma-Aldrich Chemie GmbH, Germany). PCR was performed in Biorad CFX96 Touch™ (Bio-Rad, Singapore. All PCR reactions were carried out in a 20 µl volume containing 10 µl SensiFAST SYBR® No-ROX mix (Bioline, Germany), 2.0 µl of each primer (0.2 µM; Kozak et al., 2009), 2 µl of DNA and filled up with 6 µl molecular grade water. *Salmonella enterica* serovar [4,5,12:i:-] (Schwaiger et al., 2010) was used as positive control for the *sul3*-genes analysis. PCR was comprised of initial denaturation of 3 min at 95 °C, 40 cycles denaturation of 10 s at 95 °C and 5 s annealing at 64 °C and 10 s elongation at 72 °C, followed by melting curve analysis.

The isolates were further analyzed for the AmpC families (MOX, AAC, FOX, DHA, EBC and CMY-2) using the Streck ARM-D® Kit, ampC (RUO) and the PhilisaFAST® DNA Polymerase Kit (Streck Inc., USA). The PCR amplification was performed following the manufacturers' instructions.

2.4. Detection of the ampR region

Analysis for the presence of the *ampR* region located between the *frd* operon and the *ampC* gene was performed for all the *Enterobacteriaceae* grown on ESBL-Agar (n = 15).

Isolates were subcultured on blood agar (Oxoid GmbH, Germany) and incubated under aerobic conditions for 24 h at 37 °C. Grown colonies were suspended in 200 µl NaCl and processed for DNA extraction using ISOLATE II Genomic DNA Kit (Bioline GmbH, Germany).

Detection of the transcriptional regulatory region (*ampR*) was performed by using a primer pair designed in this study (Ec-*ampR*-F: 5′-GTGTTCATCTTCCTGATGATCGT-3′ and Ec-*ampR*-R: 5′-CCACCGCC ATACCCGGAAT-3′). The forward primer (Ec-*ampR*-F) bound at the *frd* operon and the reverse primer (Ec-*ampR*-R) at *ampC* region. The fragment size of the PCR product was approximately 1350 bp. The design of primers was based on the *frd-ampR-ampC* gene of the following ECC strains: *Ent. cloacae* (NCBI accession no. CP022532, CP021749, CP022148), *Ent. hormaechei* (CP017180), *Ent. asburiae* (NZ_CP011863), *Ent. ludwigii* (CP017279.1) and *Ent. kobei* (CP017181). The reference strain *Ent. cloacae* subsp. *cloacae* (DSM 30054/ATCC 13047, NCBI accession no. CP001918.1) possesses non-specific binding sites of forward primer (4 mismatches) and reverse primer (4 mismatches). Therefore, its *ampR* gene would not be amplified by this primer pair whereupon it served as negative control for the *ampR*-PCR of *Enterobacter* spp. isolates in this study. The *ampR* gene of these reference strains were additionally used for phylogeny analysis in order to compare the similarity with the *ampR* gene obtained from isolates in this study. The PCR run was performed in Bio-Rad CFX96 Touch™ (Bio-Rad, Germany). Each reaction contained a 20 µl mixture that included 10 µl SensiFAST™ SYBR® No-ROX (Bioline GmbH, Germany), 0.2 µM of each primer, 2 µl DNA sample and filled up with molecular grade water. A 3-step PCR was applied, starting with an initial denaturation of 3 min at 95 °C and 40 cycles of denaturation at 95 °C for 10 s, annealing at 64 °C for 5 s and elongation at 72 °C for 20 s, followed up with melting curve analysis. The fragment size of the PCR products was additionally proved by agarose gel electrophoresis. Subsequently, PCR products of positive samples were purified using QIAquick PCR Purification Kit (QIAGEN GmbH, Germany) and submitted for sequencing (Eurofins GmbH, Germany). The *ampR* sequence of isolates was compared with the *ampR* sequence of *Enterobacter* spp. provided by National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/). The phylogeny analysis was performed using a web-based program (http://www.phylogeny.fr/index.cgi).

3. Results and discussion

In this study, a total of 73 potentially resistant *Enterobacteriaceae* were isolated on Mac+ agar from the peel of 38.1% of the tested muskmelons (n = 56/147). Of these, 15 isolates grew on Brilliance™ ESBL Agar and were identified as bacteria of the *Enterobacter cloacae* complex (ECC) by MALDI-TOF MS and sequence analysis of the 16S rRNA (Table 1). All ECC isolates were found in 15 (10.2%) muskmelons, namely eight isolates from retail and seven isolates from wholesale. These melons were imported from Mediterranean countries: Italy (n = 7), Spain (n = 5) and Morocco (n = 3). All melons imported from Central and South American countries (Honduras, Costa Rica and Brazil) were negative for AMR *Enterobacteriaceae*. Noteworthy, melons are mostly distributed under refrigerated conditions. McMahon et al. (2006) showed an AMR reduction in bacterial suspensions of some human pathogens, temporarily in some cases, after a low temperature phase. Also Schwaiger et al. (2011a) found higher antimicrobial resistance rates on fresh produce purchased directly at farms compared with products obtained from supermarket. Thus, stress factors

Table 1
Potentially resistant *Enterobacteriaceae* isolated from muskmelons.

Microorganism[a]	Growth on Mac+ Agar	Growth on ESBL Agar	Plasmid-mediated AmpC resistance
Achromobacter sp.	1	0	0
Acinetobacter sp.	1	0	0
Citrobacter spp.	8	0	0
Enterobacter spp.	24	15	11
Escherichia spp.	6	0	0
Klebsiella spp.	22	0	0
Kluyvera spp.	2	0	0
Raoultella spp.	6	0	0
Serratia spp.	2	0	0
Total	73	15	11

Mac+: MacConkey No. 3 Agar with 1 μg/ml cefotaxime; ESBL: Extended Spectrum β-lactamase.
[a] Identification according to MALDI-TOF MS.

occurring during distribution, such as low temperature, might affect antimicrobial resistance rates of AMR bacteria present on these products. Melons imported from Central and South American countries would be subjected to low temperatures over a longer period of time as those distributed within Europe.

MIC strip test results of these microorganisms against the antimicrobials ceftazidime, cefotaxime, cefepime, and their combination with CA are shown in Table 2. Diminished susceptibility was shown by four isolates against cefepime, the only 4th Generation Cephalosporins (4GCs), and by five isolates against its combinations with CA, while one isolate (no. 67) showed the highest detectable MIC by means of this method. Resistance to 4GCs is a serious threat, as these are the last generation of antimicrobials developed against *Enterobacteriaceae*. The differences on the susceptibility to these antibiotics and its combinations with CA were < 3 log units and some isolates even showed lower susceptibility against CA combinations. Therefore, none of the ECC isolates on study was phenotypically identified as ESBL-producing bacteria by means of the applied method. Additionally, PCR results to the ESBL-genes were also negative for all 15 isolates.

Furthermore, a susceptibility test against 14 additional antibiotics was performed by using SENSITITRE™ MIC susceptibility plates (Table 3). All isolates were susceptible to tetracycline and trimethoprim/sulfamethoxazole. No resistance or low MICs against ciprofloxacin, gentamicin and streptomycin were observed, while more isolates were found to be resistant against cefoxitin (n = 15), azithromycin (n = 5), ceftriaxone (n = 6), amoxicillin with CA (n = 15), ceftiofur (n = 13), and ampicillin (n = 12).

Concerning the ranking of medically important antimicrobials published by the WHO (2016), the antimicrobials azithromycin (macrolide), ceftriaxone and ceftiofur (3GCs), ciprofloxacin and nalidixic acid (quinolones) belong to the class of "critically important antimicrobials with highest priority", meaning they meet all three priorities (P1, P2 and P3: see Section 1, Introduction). Although no or low resistance was shown for ciprofloxacin and nalidixic acid, very low susceptibility was shown by some of the isolates for the other three antimicrobials. Ceftiofur is approved for veterinarian use only. All isolates showed high resistance against amoxicillin and twelve isolates against ampicillin. Both antimicrobials belong to the class of "critically important antimicrobials of high priority".

ECOFF values have been set by EUCAST for five of the antimicrobials tested against *Enterobacter* spp.: cefotaxime (0.5 mg/l), ceftriaxone (0.5 mg/l), sulfamethoxazole/trimethoprim (1.0 mg/l), ciprofloxacin (0.125 mg/l) and gentamicin (2.0 mg/l). Additionally, ECOFFs for specifically *Ent. cloacae* are set against ceftazidime (1.0 mg/l), cefepime (0.125 mg/l) and tetracycline (16.0 mg/l). Many isolates in this study showed higher MICs than the ECOFFs set against cefotaxime (n = 11), ceftriaxone (n = 13) and ceftazidime (n = 9) (Tables 2 and 3). Although some of the isolates were susceptible to 3GCs, *Enterobacter*-strains might develop resistance against these antimicrobials during exposure (Kasper and Fauci, 2010).

All 15 ECC isolates were regarded as potential AmpC producers as they were resistant against cefoxitin on the MIC plates. Cefoxitin resistance can be enhanced by reduction of outer membrane permeability of the isolates (Thomson, 2001). Thus, further genotypic and phenotypic tests are required for confirmation of AmpC β-lactamase production. One of the reasons for the unknown prevalence of AmpC production in *Enterobacter* spp. is probably the lack of reliable laboratory methods for testing. Phenotypic detection of plasmid-mediated AmpC β-lactamases has been described to have poor specificity and was not suited for routine detection of these β-lactamases (Reuland et al., 2015). Derepressed production of the AmpC β-lactamase had previously been reported as a prevalent mechanism of β-lactam resistance in *Enterobacter cloacae* strains (Stock et al., 2001; Tzelepi et al., 1992). PCR analysis for the six plasmid-mediated *ampC* gene families MOX, ACC, FOX, DHA, EBC and CMY-2 showed that eleven of the 15 isolates tested were EBC positive. The four remaining isolates could not be further genotyped. Plasmid-mediated *amp*C-genes have a high transmissibility to other bacterial species and these ß-lactamases can be expressed in larger amounts (Marchese et al., 1998).

Strains of the ECC have been proven to be highly MDR, possessing

Table 2
Minimum Inhibitory Concentration (MIC) Strip Test results of the 15 isolates of the *Enterobacter cloacae* complex.

Internal isolate no.	Species[a]	Origin of muskmelons	MIC values					
			Cefotaxime [0.25–16 μg/ml]	Cefotaxime + CA [0.016–1 μg/ml]	Ceftazidime [0.5–32 μg/ml]	Ceftazidime + CA [0.064–4 μg/ml]	Cefepime [0.25–16 μg/ml]	Cefepime + CA [0.5–32 μg/ml]
67	*Ent. cloacae*	Morocco	≥16	≥1.0	≥32	≥4	4	≥4
68	*Ent. cloacae*	Morocco	≥16	≥1.0	4	≥4	< 0.25	< 0.064
80	*Ent. cloacae*	Morocco	≥16	≥1.0	≥32	≥4	0.5	0.75
92	*Ent. cloacae*	Italy	< 0.25	0.50	< 0.50	0.25	< 0.25	< 0.064
93	*Ent. cloacae*	Italy	≥16	≥1.0	≥32	≥4	1.5	1
95	*Ent. cloacae*	Italy	1	≥1.0	0.50	2	< 0.25	< 0.064
97	*Ent. cloacae*	Italy	≥16	≥1.0	≥32	≥4	< 0.25	< 0.064
98	*Ent. cloacae*	Italy	≥16	≥1.0	≥32	≥4	1	0.75
99	*Ent. cloacae*	Italy	0.25	0.125	< 0.50	< 0.064	< 0.25	< 0.064
101	*Ent. cloacae*	Spain	≥16	≥1.0	≥32	≥4	< 0.25	0.125
107	*Ent. cloacae*	Spain	0.38	≥1.0	0.50	0.75	< 0.25	< 0.064
122	*Ent. cloacae*	Spain	1	≥1.0	0.50	2	< 0.25	< 0.064
126	*Ent. asburiae*	Spain	≥16	≥1.0	≥32	≥4	< 0.25	< 0.064
14Ga	*Ent. cloacae*	Spain	0.25	0.094	< 0.50	< 0.064	< 0.25	< 0.064
18Ga	*Ent. cloacae*	Italy	6	≥1.0	2	≥4	< 0.25	< 0.064

[a] Identified by 16S rRNA sequencing.

Table 3
Results of the MIC Susceptibility Test Plate for the 15 *Enterobacter cloacae* complex isolates.

Internal isolate no.	Antimicrobials – MIC values [µg/ml]													
	FOX [0.5–32]	AZI [0.12–16]	CHL [4–32]	TET [4–32]	AXO [0.25–64]	AUG2 [1/0.5–32/16]	CIP [0.015–4]	GEN [0.25–16]	NAL [0.5–32]	XNL [0.12–18]	FIS [16–256]	SXT [0.12/2.38–4/76]	AMP [1 – 32]	STR [2–64]
67	> 32	8	4	≤4	32	> 32/16	0.015	≤0.25	1	> 8	≤16	≤0.12/2.38	> 32	2
68	> 32	8	8	≤4	≤0.25	> 32/16	0.03	≤0.25	2	0.5	≤16	≤0.12/2.38	8	≤2
80	> 32	4	2	≤4	> 64	> 32/16	0.015	≤0.25	2	> 8	≤16	≤0.12/2.38	> 32	≤2
92	> 32	8	8	≤4	16	> 32/16	0.06	0.5	2	> 8	64	≤0.12/2.38	> 32	4
93	> 32	16	8	≤4	> 64	> 32/16	0.06	0.5	2	> 8	64	≤0.12/2.38	< 1	≤2
95	> 32	8	2	≤4	> 64	> 32/16	0.03	≤0.25	2	> 8	32	≤0.12/2.38	< 1	≤2
97	> 32	16	4	≤4	> 64	> 32/16	0.015	≤0.25	2	> 8	16	≤0.12/2.38	> 32	≤2
98	> 32	4	2	≤4	> 64	> 32/16	0.015	≤0.25	1	> 8	≤16	≤0.12/2.38	> 32	≤2
99	> 32	16	4	≤4	16	> 32/16	0.03	0.5	2	> 8	32	≤0.12/2.38	> 32	≤2
101	> 32	8	4	≤4	32	> 32/16	0.015	≤0.25	2	> 8	16	≤0.12/2.38	> 32	≤2
107	> 32	16	4	≤4	64	> 32/16	0.03	0.5	2	> 8	32	≤0.12/2.38	> 32	≤2
122	> 32	16	2	≤4	≤0.25	> 32/16	0.03	≤0.25	4	2	32	≤0.12/2.38	> 32	4
126	> 32	8	2	≤4	8	> 32/16	0.03	0.5	2	> 8	32	≤0.12/2.38	> 32	4
14Ga	> 32	8	4	≤4	16	> 32/16	0.06	0.5	2	> 8	256	≤0.12/2.38	> 32	4
18Ga	> 32	8	8	≤4	16	> 32/16	0.06	0.5	2	> 8	64	≤0.12/2.38	> 32	4

FOX: cefoxitin; AZI: azithromycin; CHL: chloramphenicol; TET: tetracycline; AXO: ceftriaxone; AUG2: amoxicillin/clavulanic acid 2:1 ration; CIP: ciprofloxacin; GEN: gentamicin; NAL: nalidixic acid; XNL: ceftiofur; FIS: sulfisoxazole; SXT: trimethoprim/sulfamethoxazole; AMP: ampicillin; STR: streptomycin.

several additional resistance mechanisms to several classes of antibiotics (Leski et al., 2016; Mezzatesta et al., 2012). Particularly, plasmid mediated ampC resistance might encode further resistance genes to other antimicrobials (Jacoby, 2009; Schultsz and Geerlings, 2012; Simner et al., 2011). Therefore, isolates with high phenotypical resistance to sulfisoxazole in this study were tested for the presence of the genes *sul1*, *sul2* and *sul3*. All 15 isolates were negative for these three genes. A reason for this phenotypical resistance might also be due to mutations on the DHPS-Gen (*folP*) (Perreten and Boerlin, 2003; Radstrom et al., 1991).

Enterobacteriaceae on fresh produce might pose a serious threat for human health in case of resistance against "Critically important" antimicrobials. Consumers can directly be exposed by consuming raw or fresh produce if no hygienic measures are used to inactivate these bacteria. Plasmid-mediated AmpC β-lactamase producing *Enterobacter* spp. and other microorganisms on ready-to-eat food items might additionally pose a potential risk for spreading antibiotic resistance in the gastrointestinal tract of consumers. As contamination of fresh produce might occur at any production stage and cross contamination from the peel to the flesh is highly probable during processing, it would be of main importance to establish effective hygiene measures. These measures must equally comprise cultivation and processing stages as well as household processing in order to specifically reduce the exposure to AMR and MDR microorganisms on the basis of GAP (Good Agricultural Practices), GHP (Good Hygiene Practices) and GMP (Good Manufacturing Practices) for general microbiological contamination.

Testing of the natural antimicrobial resistance of ECC strains has already suggested the presence of naturally occurring AmpC-β-lactamases (Stock et al., 2001). These microorganisms are able to rapidly develop or acquire multiple antimicrobial resistances, which makes them some of the most worrying microorganisms of the current antibiotic era (Mezzatesta et al., 2012; Sanders Jr. and Sanders, 1997). Derepression of ß-lactamase is increasingly being found among clinical isolates (Scotta et al., 2011) and also seems to be present in food isolates, as the present study suggests.

Analysis on the *ampR* regulatory region of all 15 ECC isolates by using primer pairs designed in this study showed that the reference strain *Ent. cloacae* (DSM 30054) and further seven isolates were negative in the *ampR*-PCR. Four isolates simultaneously possessed the *ampR* gene and the plasmid-mediated AmpR resistance EBC, while other four lacked the plasmid but possessed the *ampR*-gene sequence (Table 4; Fig. 1). The purified PCR products of the *ampR* genes from eight ECC isolates were submitted for sequencing. Phylogeny analysis (Fig. 2) showed that the *ampR* gene of 8 ECC isolates from this study and 8 isolates obtained from GenBank (NCBI) were very variable. For example, the similarity of ECC strains was 99% (no. 122 vs. 107); 98% (no. 101 vs. *Ent. cloacae* CP022148.1), 86% (no. 101 vs. 68) and 83% (*Ent. cloacae* CP022148.1 vs. CP021749.1). The *ampR* gene of reference strain *Ent. cloacae* (DSM 30054/ATCC 13047) obtained from Genbank (NCBI, accession no. CP001918.1) showed 83–89% identity to the other ECC strains in Fig. 2. As shown in Tables 2 and 3, no important divergence in the phenotypical resistance was found between isolates possessing either plasmid-mediated resistance and the *ampR* gene between *frd* and *ampC*, only plasmid-mediated resistance (EBC), or only the *ampR* gene in that position.

Table 4
Genotypic analysis of 15 ECC isolates by means of PCR for ESBL, AmpC and *ampR* genes.

Isolate no.	Identification	ESBL-genes	AmpC	*ampR*
67	*Enterobacter cloacae*	–	EBC	–
68	*Enterobacter cloacae*	–	–	+
80	*Enterobacter cloacae*	–	EBC	–
92	*Enterobacter cloacae*	–	–	+
93	*Enterobacter cloacae*	–	EBC	–
95	*Enterobacter cloacae*	–	EBC	–
97	*Enterobacter cloacae*	–	EBC	–
98	*Enterobacter cloacae*	–	EBC	–
99	*Enterobacter cloacae*	–	EBC	–
101	*Enterobacter cloacae*	–	EBC	+
107	*Enterobacter cloacae*	–	EBC	+
122	*Enterobacter cloacae*	–	EBC	+
126	*Enterobacter asburiae*	–	EBC	+
14Ga	*Enterobacter cloacae*	–	–	+
18Ga	*Enterobacter cloacae*	–	–	+
DSM 30054/ATCC 13047	*Enterobacter cloacae*	–	–	–

–: negative; +: positive.

The AmpR protein acts as a key factor in the induction of AmpC resistance, since the ß-lactamase production is too low in its absence (Honore et al., 1986, 1989) to cause phenotypic resistance. Isolates possessing the *ampR* gene would owe their AmpC resistance, at least to a certain extent if also plasmid positive, to the derepression of the inducible *ampC* gene of *Ent. cloacae*. This can occur during β-lactam antibiotic therapy, which often results in a stable overproduction of the cephalosporinase due to altered regulation. This can lead to therapeutic

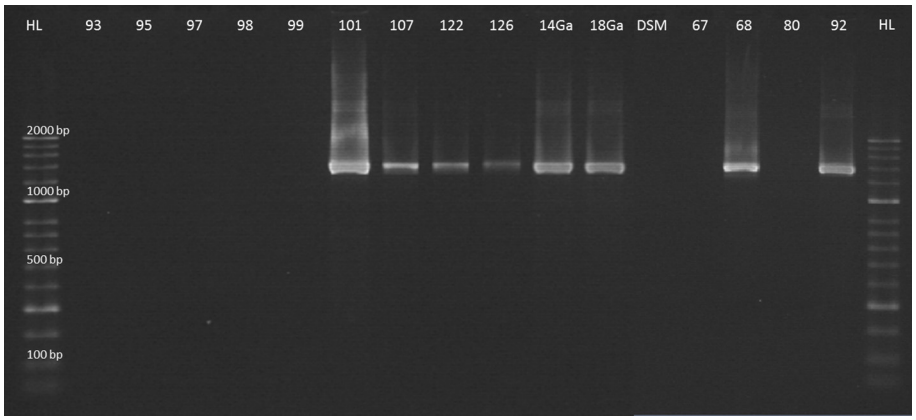

Fig. 1. PCR results of the analysis on the *ampC* regulatory region. Proof of the PCR products - *ampR* gene – under UV light after agarose-gel electrophoresis and ethidium bromide. Positive results with a 1350 bp product for isolates no. 101, 107, 122, 126, 14Ga, 18Ga, 68 and 92. ECC strain DSM 30054/ATCC 13047 and isolates no. 93, 95, 97, 98, 99, 67 and 80 were not amplified by the applied primer pair (HL: HyperLadder).

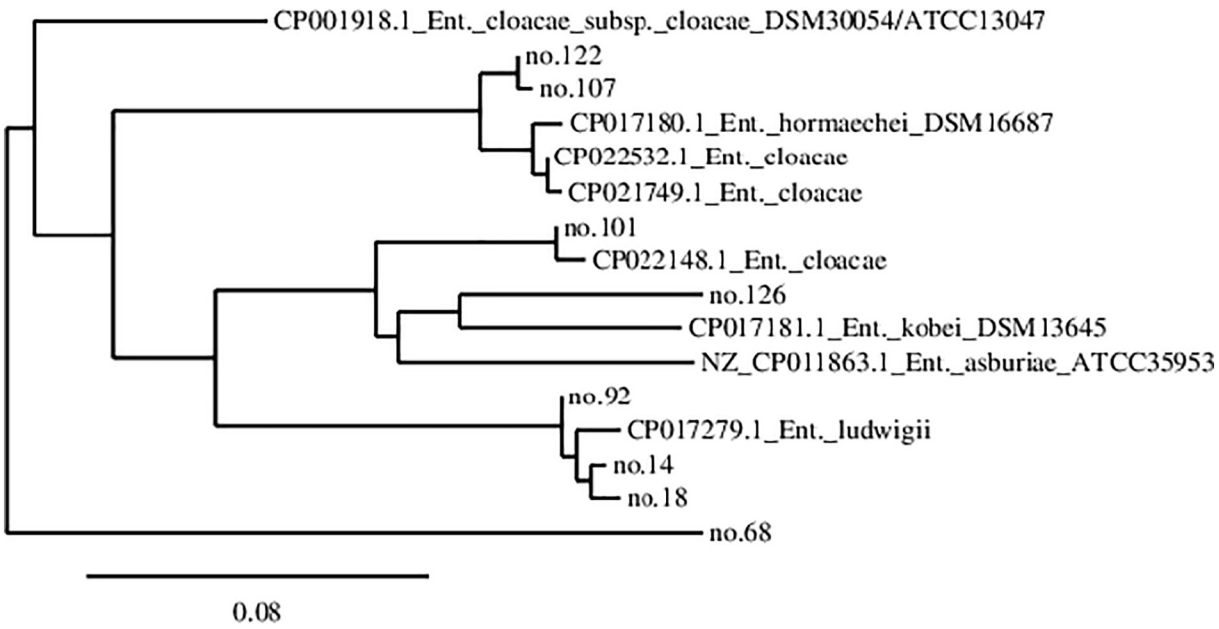

Fig. 2. Phylogeny analysis of the *ampR* gene of 7 ECC strains isolated in this study (No. 101, 126, 14, 18, 122, 107 and 68) compared with the *ampR* gene of 8 ECC strains provided by GenBank (NCBI).

failure (Honore et al., 1986). Isolates missing the *ampR* transcriptional region would lack inducibility so that their phenotypical AmpC resistance would be mainly plasmid-mediated. However, it is important to note that not detecting the *ampR* region in this work might not mean absence of this region in the genome as its presence is mostly assumed for these bacteria (Mezzatesta et al., 2012; Stock et al., 2001). According to the result of phylogeny analysis, it is assumed that the *ampR* gene from ECC is not conserved and can be variable even within the same species. Primers used in this study were designed to bind the overlap section between the *frd* - *ampR* region (forward primer) and the *ampR* – *ampC* region (reverse primer). These overlapping sections may not be conserved and were different among ECC strains due to mutations or recombination events. Negative results of some isolates for the *ampR*-PCR might be attributable to an unspecific binding site, not accessible for the applied primer pair, such as the reference *Ent. cloacae* subsp. *cloacae* (DSM 30054/ATCC 13047).

The results of this study revealed that the ECC bacteria isolated from muskmelons showed a variability in their resistance profiles against antibiotics. Genotypic analysis of ESBL-genes, *sul*-genes, ampC plasmids and the *ampR* gene showed the different antimicrobial properties of each isolate. The prevalence of antibiotic resistant *Enterobacter* spp. on muskmelons might result in a spread of the resistance genes to the gut microbiota. Given the fact that washing has a low effect on the reduction of microorganisms, hygienic measures at any stage of processing play a major role in controlling this public health risk. Increasing antimicrobial resistance against 3GCs among many different bacterial genera on fresh produce highlights the importance of these ARB along the food chain.

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