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1 **The transferable resistome of produce**

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16 gene transfer
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18 Running title: Transferable resistome associated with produce
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25 **ABSTRACT**

26 Produce is increasingly recognized as a reservoir of human pathogens and transferable
27 antibiotic resistance genes. This study aimed to explore methods to characterize the
28 transferable resistome of bacteria associated with produce. Mixed salad, arugula, and
29 cilantro purchased from supermarkets were analyzed by means of cultivation- and DNA-
30 based methods. Before and after a nonselective enrichment step, tetracycline (tet)
31 resistant *Escherichia coli* were isolated and plasmids conferring tet resistance were
32 captured by exogenous plasmid isolation. Tet resistant *E. coli* isolates, transconjugants
33 and total community (TC)-DNA from the microbial fraction detached from leaves or after
34 enrichment were analyzed for the presence of resistance genes, class 1 integrons and
35 various plasmids by real-time PCR and PCR-Southern blot hybridization. Real-time
36 PCR primers were developed for IncI and IncF plasmids. Tet resistant *E. coli* isolated
37 from arugula and cilantro carried IncF, IncI1, IncN, IncHI1, IncU and IncX1 plasmids.
38 Three isolates from cilantro were positive for IncN plasmids and *bla*$_{\text{CTX-M-1}}$. From mixed
39 salad and cilantro, IncF, IncI1, and IncP-1β plasmids were captured exogenously.
40 Importantly, whereas direct detection of IncI and IncF plasmids in TC-DNA failed, these
41 plasmids became detectable in DNA extracted from enrichment cultures. This confirms
42 that cultivation-independent DNA-based methods are not always sufficiently sensitive to
43 detect the transferable resistome in the rare microbiome. In summary, this study
44 showed that an impressive diversity of self-transmissible multiple resistance plasmids
45 was detected in bacteria associated with produce that is consumed raw, and exogenous
46 capturing into *E. coli* suggests that they could transfer to gut bacteria as well.

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48 **IMPORTANCE**

49 Produce is one of the most popular food commodities. Unfortunately, leafy greens can
50 be a reservoir of transferable antibiotic resistance genes. We found that IncF and IncI
51 plasmids were the most prevalent plasmid types in *E. coli* isolates from produce. This
52 study highlights the importance of the rare microbiome associated with produce as a
53 source of antibiotic resistance genes that might escape cultivation-independent
54 detection, yet may be transferred to human pathogens or commensals.

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71 **INTRODUCTION**

72 Despite its benefit to human health, consumption of produce is increasingly recognized
73 as a source of pathogenic bacteria, antibiotic-resistant bacteria (ARB) and antibiotic
74 resistance genes (ARGs) associated with mobile genetic elements (MGEs) (1–5).
75 Recently, several food-borne disease outbreaks have been associated with produce
76 contamination world-wide (5–9). The microbiome of produce is important for plant health
77 and vigor and was shown to be highly dynamic during growth, post-harvest, and
78 processing (10), but can also contain potentially pathogenic bacteria from human and
79 animals sources, including *E. coli* strains (11). Contamination can occur pre-harvest
80 (i.e., through organic fertilizers, soil, wild animals or contaminated irrigation water) and
81 post-harvest during processing (12, 13).

82 Antibiotic resistance in bacterial pathogens has increased globally due to the
83 widespread use and misuse of antibiotics (14–17). Antibiotic resistance levels in *E. coli*
84 are useful indicators of overall resistance levels of bacteria on foods, in animals, and
85 humans (11). Antibiotic resistance and ARGs have been documented for enteric
86 bacteria from various produce, which could facilitate the dissemination of resistant
87 bacteria to a wider community of people (1, 2, 4, 16, 18, 19). If ARGs are localized on
88 MGEs such as plasmids or conjugative transposons they can be transferred horizontally
89 to pathogens (20). Horizontal gene transfer (HGT) takes place at sites with high cell
90 densities of plasmid donors and recipients, nutrient availability and selective pressure.
91 The phytosphere, including the rhizosphere and the phyllosphere have been reported
92 as hot spots of HGT (21). The plasmid-mediated resistome of produce bacteria might
93 provide the enterobacteria with ARGs in the intestine under selective conditions.

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94 Conjugative plasmids can often confer not only resistance towards multiple antibiotics
95 but also towards heavy metal compounds or disinfectants making co-selection possible
96 (22–25). Although plasmids belonging to the incompatibility groups IncF and IncI have a
97 narrow host range (NHR), they are assumed to be important for the dissemination of
98 ARGs in *E. coli* and other *Enterobacteriaceae* (26, 27). Most importantly, resistance and
99 virulence-associated traits of *E. coli* isolates were almost exclusively found on IncF
100 group plasmids (28, 29). However, no real time (RT-) PCR systems that allow the
101 cultivation-independent detection and quantification of these plasmids in total
102 community (TC)-DNA are available.

103 In this study, we employed a combination of culture-dependent and -independent
104 approaches to assess the transferable resistome of bacteria associated with produce
105 (see Figure 1). Tetracycline (tet) resistant *E. coli* were isolated from produce directly
106 after purchase and after seven days of storage by selective plating with and without
107 prior non-selective enrichment. In addition, we captured a set of transferable tet
108 resistance plasmids into a *E. coli* recipient strains using the so-called exogenous
109 plasmid isolation method (30). Tetracycline was chosen because of its wide use in
110 animal husbandries resulting in a high load released via organic fertilizers into the agro-
111 ecosystem (31). TC-DNA was also extracted from the microbial fraction detached from
112 produce or after nonselective enrichment to detect and quantify the abundance of ARGs
113 and MGEs. New RT-PCR primers were developed for the detection and quantification of
114 IncF and IncI plasmids. Our study showed the presence of diverse transferable
115 antibiotic resistance plasmids in the rare microbiome of produce, which would have
116 likely been missed by exclusively using metagenomic approaches.

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117 **RESULTS**

118 **Phenotypic and genotypic characterization of tet resistant *E. coli* isolates**

119 To determine if produce was a source of antibiotic resistant *E. coli*, we determined the
120 occurrence and resistance profiles of tet resistant *E. coli* isolated from 24 samples of
121 produce directly or after an overnight enrichment step. The characterization of a total of
122 63 tet resistant *E. coli* isolates from cilantro (n=54), arugula (n=7) and mixed salad
123 (n=2), of which 50 were recovered after nonselective enrichment and 13 without
124 enrichment (20.6%) revealed an impressive diversity (Table 1).
125 Almost all *E. coli* isolates were resistant to antibiotics from at least one class, and two
126 isolates were resistant to eight antibiotic classes: Tetracyclines (TE; D), penicillins
127 (AM;AMX), 3rd generation cephalosporins (CTX; CRO), fluoroquinolones (CIP; OFX;
128 NA), aminoglycosides (GM; S), sulfonamides (SD), phenicols (C), and trimethoprim
129 (TMP). Most of the tet resistant *E. coli* displayed in addition resistance to ampicillin and
130 amoxicillin (84%), followed by trimethoprim (73%). Resistances to ofloxacin,
131 ciprofloxacin, sulfadiazine, and streptomycin were also common. We tested all the
132 isolates for the production of extended-spectrum beta-lactamases (ESBLs) with the
133 double-disc diffusion assay (DDT) and found three ESBL-producing *E. coli* which were
134 isolated from two of the cilantro samples.

135 We then tested the genomic DNA from the collection of *E. coli* isolates for the presence
136 of various resistance genes [*tet*(A), *strA*, *sul1*, *sul2*, *sul3*, *aadA*, *qacE/qacE*Δ1,
137 *merRT*Δ*P*, *bla* genes (TEM, CTX-M, and SHV), *qnr* genes (*qnr*A, *qnr*B, and *qnr*S)], and
138 integrase genes *intI1* and *intI2* by RT- or regular PCR (Table 1). The most commonly
139 detected ARGs was the tetracycline resistance gene *tet*(A) in 59 out of 63 isolates. A

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140 total of 10 isolates were positive for sulfonamide resistance genes *sul1*, 14 for *sul2* and
141 five for *sul3*. The combination of *sul1*-*sul2*, *sul2*-*sul3*, and *sul1*-*sul3* were detected in
142 seven, three and one isolates, respectively. All three *sul* genes were found in one tet
143 resistant *E. coli* isolate from cilantro. *qnrB* and *qnrS* genes encoding fluoroquinolone
144 resistance were detected alone or in combination in one and 38 isolates, respectively.
145 The bla_{TEM} genes encoding for the resistance to ampicillin and amoxicillin were detected
146 in 82.5% of tet resistant *E. coli* isolates. The $bla_{CTX\text{-}M\text{-}1}$ gene encoding ESBL resistance
147 was only detected in three isolates and was found in combination with bla_{TEM} genes in
148 two *E. coli* from cilantro. The bla_{SHV} gene encoding ESBL resistance was not detected
149 in any of the isolates. For streptomycin/spectinomycin resistance gene, *aadA* (24
150 isolates) was most common, followed by *strA* (21 isolates) and *aadA*-*strA* (three
151 isolates).

152 The class 1 integron integrase gene *intI1* was detected in 50 isolates, while the class 2
153 integron integrase gene *intI2* was not detected at all. Although *qacE/qacE*Δ1 encoding
154 quaternary ammonium compound resistance is a typical component of class 1
155 integrons, the gene was detected only in 23 isolates, suggesting a large proportion of
156 atypical class 1 integrons. Interestingly, *merRT*Δ*P* encoding for regulatory, transport,
157 and extracellular mercury-binding genes was detected in 12 isolates. These findings
158 show that produce can be a source of multidrug resistant *E. coli* isolates.

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160 **Characterization of plasmids in tet resistant *E. coli* isolates**

161 To test if the tet resistant *E. coli* isolates recovered from produce harbor plasmids and to
162 assign them to known plasmid groups, their genomic DNA was screened by TaqMan

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163 probe-based RT-PCR systems for IncF and IncI plasmids and by PCR-based replicon
164 typing (PBRT) (Table 1). All isolates that were positive by RT-PCR targeting the IncF
165 (*traI* gene) were also identified by replicon typing as IncF confirming the specificity of
166 the novel TaqMan RT-PCR system. However, PBRT allowed also the assignment to the
167 different IncF subgroups. Furthermore, other plasmids were also identified by PBRT or
168 RT-PCR (*korB*, specific for IncP-1 plasmids) or PCR (IncN). A summary of the
169 plasmid/replicon types detected among the 63 representative tet resistant *E. coli*
170 isolates is given in Table 1. For cilantro and arugula, almost all tet resistant *E. coli*
171 isolates contained plasmids [61 out of 63], but the plasmids detected in the two isolates
172 from mixed salad could not be assigned using the RT-PCR or PBRT. In most isolates
173 (n=45) one plasmid type was detected, but some had two (n=15) or three (n=1)
174 plasmids. Plasmids from seven different Inc groups were found in the 63 *E. coli* isolates:
175 IncFII (n=21), IncI1 (n=17), IncX1 (n=11), IncFIB (n=10), IncU (n=6), IncN (n=4), and
176 IncHI1 (n=2). All Inc groups were found in *E. coli* isolates from cilantro, whereas arugula
177 resulted in only two groups [IncI1 (n=5) and IncFII (n=2)]. Plasmids of the IncF groups
178 (FII and FIB) were the predominant types, followed by IncI1 and IncX1 plasmids. The
179 combination of replicon types IncFII and IncFIB was detected in two isolates, whereas
180 the combination of replicon types IncFII-IncI1 and IncFIB-IncI1 was found in six and five
181 isolates, respectively. In one isolate from cilantro the combination IncFII-IncFIB-IncI1
182 was detected. IncI2 plasmids were not detected in any of *E. coli* isolates.
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184 **Conjugal transfer of antibiotic resistance**

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185 Conjugation experiments were conducted in order to determine the potential transfer of
186 antibiotic resistances to other bacteria. Conjugal transfer experiments were performed
187 using tet resistant *E. coli* isolates positive for ESBL (EK2.29, EK3.43, and EK3.44) as
188 donors and kanamycin and rifampicin resistant *E. c*oli CV601 as a recipient at 37°C. We
189 selected transconjugants on LB plates containing tetracycline and cefotaxime, which
190 corresponded to phenotypes of the donors. The transfer of the resistance phenotypes
191 was successful.
192

193 **Phenotypic and genotypic characterization of plasmids captured via exogenous**
194 **isolation**

195 We further investigated the presence of transferable plasmids in produce by capturing
196 tet resistance plasmids from nonselective enrichment culture of fresh leaves from
197 cilantro, mixed salad, and arugula by exogenous plasmid isolation into *E. coli* CV601.
198 Tet resistant transconjugants were only captured on day 0 but not on day 7. The
199 transfer frequencies of tet resistant transconjugants were 1.73×10^{-7}, 1.55×10^{-4} and
200 4.66×10^{-9} per recipient in cilantro, mixed salad, and arugula, respectively. While all
201 transconjugants obtained from cilantro (n=27) and arugula (n=23) were characterized,
202 from mixed salad only a total of 41 transconjugants was analyzed due to the high
203 number of transconjugants obtained. Based on an initial phenotypic and genotypic
204 analyses, 15 representative out of 91 tet resistant transconjugants from produce
205 (cilantro, n=12; arugula, n=1; mixed salad, n=2) were selected for further
206 characterization. The majority of these transconjugants acquired resistance to at least
207 two antibiotic classes, and all were resistant to tetracycline, ampicillin and amoxicillin.

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208 The *bla*$_{TEM}$ genes encoding for ampicillin and amoxicillin resistances were detected in
209 86.7% of tet resistant transconjugants (Table 2). The tetracycline resistance gene *tet*(A)
210 was found in 13 out of 15 transconjugants from cilantro and arugula but not from mixed
211 salad, while *tet*(Q) was only detected in one plasmid (pBMS1) isolated from the mixed
212 salad. Four tetracycline resistance plasmids (pBC1.1, pBC1.3, pBC1.9, and pBC1.12)
213 captured from cilantro carried the insertion sequence IS*1071* and class 1 integrons
214 (*intI1*), tetracycline resistance gene *tet*(A), but encoded also resistance to ampicillin
215 (*bla*$_{TEM}$), and mercury compounds (*merRT∆P*). Eight plasmids from cilantro
216 transconjugants (pBC2.1, pBC2.2, pBC2.3, pBC2.4, pBC2.6, pBC2.8, pBC2.11, and
217 pBC2.15) carried *tet*(A), *qnrS*, and *bla*$_{TEM}$ and two of them (pBC2.1 and pBC2.4) carried
218 in addition *sul1* and sul2, respectively. Two tet resistance plasmids (pBMS1 and
219 pBMS4) captured from mixed salad carried *sul1*, *strA*, *merRT∆P*, *bla*$_{TEM}$, and *intI1*. One
220 plasmid (pBA1) captured from arugula carried *bla*$_{TEM}$ and *tet*(A) (Table 2). Thus this
221 approach demonstrates that transferable multidrug resistance plasmids were easily
222 captured by *E. coli* CV601, a process that might also occur in the human gut.
223

224 **Identification of exogenously isolated plasmids**

225 The newly developed TaqMan probe-based RT-PCR assay was used to screen the tet
226 resistant transconjugants for the presence of IncF and IncI plasmids and validated by
227 PBRT. In addition, other plasmids were also identified by RT-PCR (*korB*, specific for
228 IncP-1 plasmids) and Southern blot hybridization. Plasmids of known Inc groups were
229 detected in all transconjugants from the mixed salad and cilantro but not in the
230 transconjugants from arugula. Representative transconjugants from cilantro and mixed

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231 salad carried either one (n=11) or two (n=3) replicons. In 12 transconjugants from
232 cilantro samples four different plasmid replicon types were detected (Table 2): IncFII
233 (n=3); IncFIB (n=6); IncI1 (n=2); and IncP-1β (n=4). In contrast, the transconjugants
234 isolated from mixed salad showed only one replicon type, IncFII (n=2) . One plasmid
235 that could not be assigned by PBRT or RT-PCR was isolated from arugula leaves. The
236 combination of replicon types IncFII and IncP-1β was detected in two transconjugants
237 (pBC1.9 and pBC1.12), while the combination of replicon types of plasmids IncFIB and
238 IncI1 was found in one transconjugant (pBC2.3) captured from cilantro leaves. Southern
239 blot hybridization for sequences specific for IncP-1 plasmids revealed that four plasmids
240 belonged to the IncP-1β subgroup. IncI2 plasmids were not detected in any tet resistant
241 transconjugants (Table 2). In contrast to IncFIB/FII and IncI1 plasmids, the IncP-1β
242 plasmids captured exogenously were not detected in the 63 tet resistant *E. coli* isolates.
243
244 **Detection of IncF and IncI plasmids, *tet*(A), and *intI1* in total community DNA**
245 We also screened for plasmids (IncF, IncI1 and, IncI2), tetracycline resistance genes
246 *tet*(A) and integrase gene *intI1* in TC-DNA extracted from bacterial communities either
247 directly after their detachment from fresh leaves or after an enrichment step, using
248 PCR-Southern blot hybridization and RT-PCR (Table 3). Using the RT-PCR method,
249 IncF and IncI plasmids as well as *tet*(A) gene were detected in TC-DNA extracted from
250 enrichment cultures of leaves, but not in TC-DNA from the detached bacteria. In
251 contrast, the *intI1* gene was detected in both kinds of TC-DNA. Consistent with these
252 results, PCR-Southern blot hybridization targeting the IncF and IncI plasmids and *tet*(A)
253 revealed strong hybridization signals in TC-DNA extracted from the enrichment cultures,

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254 but very weak or no signals from direct extractions.

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277 **DISCUSSION**

278 The present study showed that bacteria associated with produce can carry various
279 plasmids that might serve as link between the environmental and the human gut
280 microbiome. Although initially low in abundance, tet resistant *E. coli* were isolated from
281 all produce samples purchased after none selective enrichment. Contamination of
282 produce with *E. coli* strains can occur in the field by contaminated soil (organic
283 fertilizers), exposure to contaminated irrigation water or post harvest (12, 13). In this
284 study, tet resistant *E. coli* isolates were mostly isolated from cilantro that was purchased
285 in four Asian supermarkets in two cities, followed by mixed ready to eat salad and
286 arugula. This might be a hint that produce imported from Asia might be a hotspot for
287 contamination with *E. coli* carrying multidrug resistance plasmids. A high proportion of
288 the tet resistant *E. coli* isolates was also resistant to penicillins (AM and AMX) followed
289 by trimethoprim. Although it is difficult to compare among studies because of different
290 methodologies used for isolation and resistance testing, our results are in line with high
291 resistance levels to penicillins and trimethoprim previously reported for *E. coli* from
292 irrigation water and vegetables (18), ready to eat salads (1), and lettuce (2). In the
293 present study, tet resistance was commonly conferred by *tet*(A), partly confirming
294 previous studies reporting *tet*(A) and *tet*(B) genes as the most common tet resistance
295 genes in *E. coli* and *Salmonella spp*. isolated from ready to eat vegetables (1, 32). The
296 rapid dissemination of tetracycline resistance among bacteria has been related to the
297 occurrence of *tet* resistance genes on transposons and conjugative plasmids (22, 23,
298 33), but also to the selective pressure, e.g. the use of antibiotics in animal husbandries
299 and the spread of tet resistance genes via organic fertilizers (31).

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300 Plasmid-mediated multidrug resistance plays an important role in the transfer of ARGs
301 around the world (34). Our study showed that *E. coli* isolates from produce harbored
302 various plasmids belonging to replicon types IncF, IncI1, IncX1, IncU, IncN, and IncHI1,
303 with IncF plasmids being the most frequently detected. IncF plasmids were found
304 predominantly in *E. coli* isolated from drinking water (35) and poultry farms (36). In our
305 study IncFII was the most frequently detected (36.5%) replicon type, followed by IncFIB
306 (15.9%), which is in line with studies on *E. coli* recovered from pigs and human (37),
307 wastewater (38), and animals (39). The combination of replicon types IncFII and IncFIB
308 in two isolates is consistent with a report on *Enterobacter cloacae* from lettuce (3).
309 However, we cannot exclude that these replicons are located on the same plasmid, as
310 several studies have reported the combination of replicon types as a multi-replicon on a
311 single plasmid (40–42), likely due to co-integration (28). In this study, tet resistant *E. coli*
312 isolates which carried IncF plasmids were positive also for *tet(*A), *aadA*, *sul1*, *sul2*, *sul3*,
313 *qacE/qacE*Δ*1*, *qnrB*, *qnrS* or *bla*$_{TEM}$ genes. Previous reports found that IncF plasmids
314 can carry genes conferring resistance to all major antibiotic classes including
315 aminoglycosides, β-lactams, phenicols, tetracyclines, sulfonamides, and
316 fluoroquinolones (37, 39, 43).

317 The NHR IncI1 plasmid types were the second most dominant replicon type (34.9%)
318 and IncI1 positive isolates also carried multiple ARGs. In this study, strains carrying
319 IncI1 plasmids were also positive for class 1 integron integrase gene *intI1* and a diverse
320 set of resistance genes, namely *tet*(A), *sul2, strA, bla*$_{TEM}$, *qacE/qacE*Δ*1, aadA*, *sul3*,
321 *qnrS*, and *merRT*Δ*P*. In a recent study, IncI1 plasmids from irrigation water and lettuce
322 carried genes *sul1*, *tet*(A), *aadA*, *strA*, and *bla*$_{TEM}$ as well as *intI1* (18). Similar

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323 phenotype and genotype profiles among *E. coli* strains from the current study and those
324 recovered in previous studies from clinical samples, the environment or other foods
325 indicate that produce may play a potential role in the dispersal of *E. coli* carrying
326 plasmid-localized ARGs. Thus, plasmids belonging to the IncF and IncI groups have the
327 potential to be a major contributor worldwide to the propagation of ARGs within enteric
328 bacteria. One dissemination route of enteric bacteria carrying IncF and IncI plasmids
329 might be the consumption of produce.

330 The newly developed TaqMan probe-based RT-PCR assays demonstrated high
331 specificity in detecting these plasmids *in E. coli* isolates and PCR positives were also
332 assigned by PBRT which in addition enables sub-typing.

333 This is the first study identifying NHR plasmids such as IncX1 and IncHI1 and broad-
334 host-range (BHR) plasmid IncU in *E. coli* isolates recovered from cilantro leaves.
335 Interestingly, IncX plasmids were detected in *E. cloacae* from lettuce (3). IncHI1
336 plasmids were previously reported in *E. coli* and *Citrobacter youngae* isolates from
337 water and healthy calves, respectively (44), while the first IncU plasmids were isolated
338 from *Aeromonas_salmonicida* (45), and later from *Aeromonas caviae* from hospital
339 effluent in the UK (46). In general, a low prevalence of ESBL-producing *E. coli* was
340 found on produce, which is similar to previous studies (19, 47, 48). In the present study,
341 the occurrence of ESBL-producing *E. coli* were only isolated from cilantro (2.8%).

342 To our knowledge, this is also the first report of *E. coli* isolates from cilantro that were
343 positive for conjugative IncN plasmids, *bla*$_{CTX-M-1}$, and resistance to 3rd generation
344 cephalosporins. The *bla*$_{CTX-M-1}$ gene was also reported on plasmids belonging to the
345 IncN family in *E. coli* from farm workers, animals, humans, and the environment (49–

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346 51). Although IncN plasmids are able to replicate in a variety of *Enterobacteriaceae*,
347 they are most frequently found in *E. coli* and *Klebsiella pneumoniae,* where they
348 contribute to the dissemination of cephalosporin and carbapenem resistance (52).

349 The results of the present study is investigation showed that *E. coli* isolates harboring
350 the $bla_{\text{CTX-M-1}}$ gene also conferred resistance to at least seven classes of antibiotics
351 tested. Moreover, *E. coli* harboring CTX-M genes were recently reported from lettuce
352 and irrigation water (4, 53), raw vegetables (32, 53), and coastal waters (54, 55). Kim et
353 al. reported that ESBL-producing *E. coli* and *Klebsiella pneumoniae* carrying CTX-M
354 were detected in ready to eat vegetables (56). A recent study has detected bla_{TEM}
355 genes in association with IncF and IncI1 plasmids from irrigation water and lettuce (18).

356 In previous reports, the occurrence of *sul1* and *qacE/qacE*Δ*1* was frequently associated
357 with class 1 integrons (3, 57). Unexpectedly, only 27% and 36.5% of *sul1*- and
358 *qacE/qacE*Δ*1*- positive isolates carried the *intI1* gene, respectively, indicating that
359 atypical class 1 integrons were more prevalent among the isolates as previously also
360 reported by Amos et al. (58).

361 In the present study, transferable tet resistance plasmids were also directly captured
362 from the produce microbiomes on day 0 but not on day 7 after purchase, and the
363 highest transfer frequency was observed in mixed salad, followed by cilantro and
364 arugula. Differences in observed frequencies of transconjugants could be due to
365 different abundance of bacteria with conjugative plasmids in the various sample types,
366 or due to real differences in the frequencies of plasmid transfer. The latter might be
367 affected by the metabolic activity of the produce microbiome, as plasmid transfer
368 frequency is known to depend not only on plasmid-specific characteristics, but also

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369 ecological factors affecting the metabolic activity of bacteria (59). Replicon types IncFII,
370 IncFIB, IncI1, and IncP-1β were captured from cilantro leaves whereas only IncFII
371 plasmids were captured from mixed salad. IncF (FII and FIB) plasmids were prevalent
372 among tet resistant transconjugants from both produce. Most of the IncF plasmids
373 exogenously captured harbored bla_{TEM}, *tet*(A), and *qnrS* genes. One IncI1 plasmid was
374 captured from cilantro and another one in combination with replicon type IncFIB. The
375 conjugative plasmids carried *tet*(A) and bla_{TEM} genes. Finally, four IncP-1β plasmids
376 were captured from cilantro leaves and two of them in combination with replicon type
377 IncFII. IncP-1 plasmids have been frequently captured by exogenous plasmid isolation
378 from various environments such as sewage sludge (60), manure (23), and water (61).
379 However, the first isolations of IncP-1 plasmids were from clinical isolates (62, 63). The
380 IncP-1β plasmids carried genes conferring resistances to antibiotics *tet*(A), *strA,* and
381 bla_{TEM} but also mercury compounds (*merRTΔP)* and disinfectants (*qacE/qacEΔ1*).

382 In conclusion, this study showed that produce that we eat might contain bacteria such
383 as *E. coli* carrying transferable multidrug resistance plasmids. Although *E. coli* numbers
384 are typically low, our nonselective enrichments showed that proliferation can easily
385 occur. Our study reports a specific TaqMan probe-based RT-PCR assays that can be
386 used for rapid detection of IncF and IncI plasmids in *E. coli* isolates and exogenously
387 captured plasmids as well as in TC-DNA extracted from enrichment cultures of leaves.
388 However, quantifying these plasmids in TC-DNA directly extracted from the microbial
389 fraction detached from leaves was impossible due to their low abundance in the
390 microbiome, but IncF and IncI plasmids were detected in DNA extracted after previous
391 enrichment. While these assays represent an important and useful tool to be

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392 implemented for monitoring the prevalence of IncF and IncI plasmids in isolates and the
393 environment, negative results of these and other cultivation-independent methods can
394 lead to an underestimation of the mobile resistome present in the rare microbiome of
395 produce and other samples. This is the first study demonstrating that multi-drug
396 resistance plasmids present in produce associated bacteria were transferable to
397 sensitive *E. coli* recipients, a process that could occur in the human gut. The NHR
398 plasmids IncF and IncI1 but also the BHR IncP-1β plasmids were captured from the
399 produce. In particular the captured IncF and Inc1 plasmids conferred resistance towards
400 several classes of antibiotics. Thus produce associated bacteria should be considered
401 as important route of disseminating transferable antibiotic resistances which might be
402 particularly relevant for patients under antibiotic treatment.
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415 **MATERIALS AND METHODS**

416 **Sample collection**

417 A total of 24 samples from different produce (mixed salad, arugula, and cilantro)
418 imported or locally produced was analyzed. The produce samples were purchased from
419 different local supermarkets in Germany and collected in September-2016, June-2016
420 and Mai-2017, respectively. They were stored at fridge temperature and sampled on
421 days 0 and 7, four replicates for each time point and produce type.
422

423 **Isolation and identification of tet resistant *E. coli***

424 For sampling, the produce was cut into smaller pieces using a sterile scalpel and mixed.
425 Of each sample 25 g were placed in two stomacher bags (one for direct plating and the
426 other for enrichment) and mixed three times with 75 ml buffered peptone water (BPW,
427 Roth; Karlsruhe, Germany), with subsequent stomacher treatment performed with the
428 Stomacher® 400 (Seward Worthing, England) at high speed for 1 min. The enrichment
429 cultures of fresh leaves in BPW were incubated at 37°C with shaking (150 rpm) for 18-
430 24 h. In order to isolate tet resistant *E. coli*, appropriate dilutions of the sample
431 suspensions and also of the enrichment cultures were plated on different culture media
432 [Eosin Methylene Blue (EMB, Sifin; Berlin, Germany) and Chromocult coliform agar
433 (CCA, Merck, Darmstadt, Germany)] supplemented with tetracycline (10 mg L^{-1}). All
434 plates were incubated at 37°C for 18-24 h. The presumptive *E. coli* colonies were picked
435 from each sample and streaked onto EMB, CCA, and TBX chromogener Agar (TBX,
436 Roth; Karlsruhe, Germany) for confirmation by colony morphology and further
437 characterization. *E. coli* isolates were then confirmed by biochemical tests for indole

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438 production, methyl red and catalase activity (64). Furthermore, isolates were analyzed
439 for the presence of the *gadA* gene (Glutamate Decarboxylase Genes) specific for *E. coli*
440 using PCR (65). *E. coli* isolates were stored in Luria broth (LB, Roth; Karlsruhe,
441 Germany) containing 15% glycerol at -80°C.
442
443 **Exogenous plasmid isolation**
444 In order to capture tetracycline-resistance plasmids exogenous plasmid isolation via
445 biparental mating was performed using [*Escherichia coli* CV601 gfp$^+$, kanamycin (Km)
446 and rifampicin (Rif) resistant; Heuer et al. (66)] as a recipient. The recipient strain was
447 grown overnight in Tryptic Soy Broth (TSB; Merck, Darmstadt, Germany) supplemented
448 with rifampicin (Rif) (50 mg L^{-1}) and kanamycin (Km) (50 mg L^{-1}). Two ml of the recipient
449 strain culture was transferred into a sterile Eppendorf tube and centrifuged at 3,100 x *g*
450 for 5 min and washed twice with 1:10 TSB. Then, the pellet was resuspended in 2 ml of
451 1:10 TSB. The bacterial suspensions (donor) of each sample on days 0 and 7 were
452 prepared from enrichment cultures of fresh leaves as described above. 0.5 ml of
453 recipient strain and 20 ml of each enrichment culture (donor) were mixed in a 50 ml
454 falcon tube. As a background control, 5 ml of the enrichment cultures and 200 µl of the
455 recipient were processed the same way as the samples. All mixtures were centrifuged
456 at 3,100 x *g* for 10 min. The pellets were resuspended in 200 µl of 1:10 TSB and then
457 spotted onto a filter for mating (Millipore filters, 0.22µm). Filters were incubated
458 overnight at 28°C on Plate count agar plates (PCA; Merck, Darmstadt, Germany)
459 supplemented with cycloheximide (Cyc) (100 mg L^{-1}). After incubation, the filters were
460 placed in 2 ml of sterile 0.85% NaCl solution in a 50 ml falcon tube. Each filter was

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461 washed by vortexing for 1 minute. Serial 10-fold dilutions were done and appropriate
462 dilutions were plated on PCA agar supplemented with rifampicin (Rif 50 mg L^{-1}),
463 kanamycin (Km 50 mg L^{-1}), cycloheximide (Cyc 100 mg L^{-1}), and tetracycline (Tet 15 mg
464 L^{-1}) to select for tetracycline-resistant transconjugants. Background controls of bulk soil
465 and the recipient controls were plated on the same selective media. Numbers of
466 recipient cells were determined by applying three replicate 20 µl drops per each serial
467 dilution (10^{-5} to 10^{-8}) of all mating mixes on PCA with km (50 mg L^{-1}), rif (50 mgL^{-1}), and
468 cyc (100 mg L^{-1}). All plates were incubated at 28°C for up to 3 days. Transconjugants
469 were determined by green fluorescence resulting from the green fluorescence protein
470 (GFP). The identity of putative transconjugants was confirmed by BOX-PCR (67).
471 Transfer frequencies were calculated as total number of transconjugants divided by the
472 total number of recipients.
473

474 **Antibiotic susceptibility testing**

475 Antimicrobial susceptibility testing was performed by the disk diffusion method on
476 Müller-Hinton agar (MH; SIGMA-ALDRICH CHEMIE GmbH, USA), according to the
477 EUCAST (European Committee on Antimicrobial Susceptibility Testing). The antibiotics
478 (µg) (BD, Bacton, Dickinson and Company, USA) used in this study were: amoxicillin
479 (25), ampicillin (10), cefotaxime (30), ceftazidime (30), ceftriaxone (30), chloramphenicol
480 (30), ciprofloxacin (5), colistin (10), tet (30), doxycycline (30), streptomycin (10),
481 gentamicin (10), ofloxacin (5), kanamycin (30), nalidxic acid (30), trimethoprim (5),
482 sulfadiazine (250). Tet resistant *E. coli* isolates were streaked onto LB agar
483 supplemented with tet (10 mg L^{-1}), while tet resistant *E. coli* CV601 transconjugants

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484 were streaked on plate count agar plates (PCA, Merck, Darmstadt, Germany)
485 supplemented with tet (15 mg L^{-1}), km (50 mg L^{-1}) and rif (50 mg L^{-1}). *E. coli* CV601
486 strain was used as a negative control. The bacterial suspension was prepared from a
487 single colony in normal saline (0.85% NaCl) to a density of 0.5 McFarland turbidity
488 standard. Cotton swabs were used for streaking the suspension onto MH agar plates.
489 After air drying, antibiotic discs were placed on the plates. Then all plates were
490 incubated at 37°C for 18-24 h. The inhibition zone was measured. The results were
491 interpreted according to the guidelines of EUCAST. Clinical and Laboratory Standards
492 Institute (CLSI) recommendations were used when antibiotic breakpoints in EUCAST
493 guidelines were absent (i.e. doxycycline, streptomycin, tetracycline, and nalidixic acid).
494 ESBL production was confirmed among tet resistant *E. coli* isolates and transconjugants
495 by double-disc diffusion test (DDT) (47). The phenotypic confirmatory test was
496 confirmed as ESBL producer according to the CLSI.
497

498 **TC-DNA extraction**

499 The bacterial fraction detached from fresh leaves directly or after an enrichment culture
500 of each sample as described above were pelleted by centrifugation at 3,100 x *g* for 15
501 min at 4°C. Total community-DNA was extracted from the pellet using the
502 FastDNA®SPIN Kit for soil (MP Biomedicals, Heidelberg, Germany), according to the
503 manufacturer's instructions. The quality of extracted DNA was determined by agarose
504 gel electrophoresis. The extracted DNA was stored at -20°C until further analysis.
505

506 **Genomic DNA extraction**

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507 Genomic DNA was extracted from overnight cultures of tet resistant *E. coli* isolates,
508 transconjugants and the recipient strain with Qiagen genomic DNA extraction kit
509 (Qiagen, Hilden, Germany) using a silica-based kit (silica bead DNA extraction kit;
510 Thermo Scientific, St. Leon-Rot, Germany). The extracted genomic DNA was stored at -
511 20°C until further analysis

512

513 **Primer-probe design (IncF, IncI1, and IncI2 plasmids)**

514 As it is known that relaxase genes can be used for classification of the mobilization
515 systems of plasmids (68), the *traI* gene region was chosen as a target region to design
516 primers detecting IncF, IncI1 and IncI2 plasmid sequences. A total of 4,530 plasmid
517 DNA sequences were downloaded from NCBI (NCBI, Batch Entrez) using the 4,602
518 plasmid accession numbers found in GenBank by Shintani et al. (69), among which 298
519 plasmids were identified as MOBF. The CDS of the MOBF plasmids were aligned using
520 tBLASTn against the relaxase TraI of the F plasmid (AP001918), resulting in 110 protein
521 sequences sharing > 50% identity and >70% coverage. The 110 protein sequences
522 closely related to TraI were aligned using MAFT multiple sequence alignment software
523 version 1.3.3. The alignment produced was back translated using the EMBOSS
524 Backtranseq tool and used to generate a set of degenerated primers and probes using
525 Primer3. All those steps were carried out in Geneious® 8.1.9. At best, 83 of the 110 *traI*
526 nucleic acid sequences could be targeted by one set of designed primers and probe
527 (see Table 4). Those sequences belonged mostly to plasmids isolated from *Salmonella*
528 *enterica* and *Escherichia coli* and few from *Klebsiella pneumoniae* and *Shigella* spp.
529 The plasmids corresponded to a part of the subclade MOBF12 defined by Garcillán-

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530 Barcia et al. (70), which is comprised of the phylogenetically broad IncF complex. When
531 tested against the 4,530 plasmids the primers-probe targeted 92 plasmids in the
532 database, 89 plasmids belonged to the MOBF group (298 plasmids recovered belonged
533 to this group) and 3 were annotated as "non-mob" and belonged to any MOB group.
534 When looking at the “Inc” classification, 73 of the targeted plasmids belonged to the
535 IncF (including FI and FII), 14 IncZ and 5 to the pCD1 type. Among the 4,530 plasmid
536 sequences 243 carried a *rep* gene belonging to the IncF group, indicating that the
537 primer/probe cannot detect all possible IncF plasmids.
538 Available IncI plasmid sequences were downloaded from NCBI and the *traI* genes were
539 aligned using the software CLC Main Workbench software version 8 (CLC bio, Qiagen®
540 company) with standard settings for alignments and primers were designed to match
541 conserved regions of the *traI* gene (see Table 4). The specificity was confirmed in silico
542 with NCBI primer BLAST and with a set of plasmids from other incompatibility groups.
543 Plasmids used for this test were R388, pB10, pHHV216, RSF1010, pSM1890, RP4,
544 pHH3-414, pHH2-227, pRA3, RN3, RSF1010, pTH10, pTP6, R751, pQKH54, pKS208,
545 pEST4002, pJKJ5, pMCBF1, pRMS149, pCAR1, pD2RT, pD67, pWW0 and pST527
546 from which none was amplified.
547

548 **Detection of IncF, IncI plasmids by real-time PCR**

549 The RT-PCR assay was performed under standard conditions, all qPCR reactions were
550 set up in a 25 μL reaction volume using a Hot Start Taq DNA Polymerase (M0495L,
551 New England BioLabs, Inc, UK) containing 5 μL of template DNA, 300 nM of primer
552 (reverse), 50 nM of primer (forward), and 50 nM of probe for IncF, while 300 nM of each

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553 primer (forward and reverse) and TaqMan probe for IncI. All Primers and probes are
554 described in Table 4. The following PCR program was used for amplification: 10 min at
555 95°C, followed by 40 cycles of 95°C for 30 sec and 60°C for 1 min. The assays were
556 carried out in triplicate with real-time PCR 5'-nuclease assays (TaqMan qPCRRT-PCR)
557 in a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA). Negative controls
558 were included in all tests, and they consisted of all the elements of the reaction except
559 for the template DNA.
560 Standard plasmids were used to construct a full standard curve in duplicate in each
561 qPCR run. Standard plasmids were constructed by cloning the purified PCR products
562 amplified from the plasmids R64 for IncI1, pHNSHP45 for IncI2, and IncF plasmids for
563 the sub_MOBF12 using the corresponding primer pairs used for the RT-PCR, into
564 TransforMax™ EC100™ Electrocompetent *E. coli* (Epicentre), in pJET1.2 using the
565 Thermo Scientific CloneJET PCR Cloning Kit (Thermo Scientific).
566

567 **Detection of target genes by real-time qPCR and PCR**

568 The target genes in genomic DNA extracted from tet resistant *E. coli* isolates and
569 transconjugants were detected by RT-PCR 5'-nuclease assays (TaqMan or EvaGreen
570 RT-PCR) in a CFX96 RT-PCR detection system (Bio-Rad, Hercules, CA) or by PCR for
571 class 1 and 2 integrons integrase genes [*intI1* and *intI2*], *korB* (IncP-1 plasmids),
572 *qacE/qacEΔ1* encoding quaternary ammonium compound resistance, [*aadA* and *strA*]
573 encoding strept*om*ycin and spectinomycin resistance, tetracycline resistance genes
574 [*tet*(Q) and, *tet*(A)], *merRTΔP* gene part of the mercury resistance operon, [*sul1*, *sul2*
575 and *sul3*] encoding sulfonamide resistance, [*qnrA*, *qnrB*, *qnrS*] encoding fluoroguinolone

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576 resistance, β-lactam resistance genes [bla_{TEM}, bla_{SHV} and $bla_{CTX-M-1}$], IncN (*rep*), and
577 Insertion Sequence IS*1071* (represented by *tnpA* gene). The DNA of the recipient strain
578 *E. coli* CV601 was included as negative control. The primers and probes targeting these
579 genes and PCR and qPCR conditions are listed in (Table 4).
580

581 **Conjugation assay**

582 Tet resistant *E. coli* isolates positive for ESBL (EK2.29, EK3.43, and EK3.44) were
583 examined for their ability to transfer resistance. Briefly, the donors and the rifampicin
584 and kanamycin resistant *E. coli* CV601 recipient strain were grown in LB broth overnight
585 at 37°C. 500 µl of overnight cultures of each donor and recipient strains were mixed in 1
586 ml LB broth, and incubated at 37°C for 24 h without shaking. 100 µl of the conjugal
587 mixture was spread on LB agar (Roth; Karlsruhe, Germany) containing Rif (50 mg L^{-1}),
588 Kan (50 mg L^{-1}), CTX (2 mg L^{-1}), and Tet (15 mg L^{-1}), and incubated at 37°C for 24-48
589 h. The transconjugants were verified by BOX-PCR and further tested for the antibiotic
590 resistance phenotypes and genotypes as described above.

591

592 **Plasmid DNA extraction and detection by Southern blot hybridization**

593 Plasmid DNA extraction from tet resistant transconjugants (pBC1.1, pBC1.3, pBC1.9,
594 and pBC1.12) captured exogenously from the enrichment of cilantro leaves was
595 performed using Qiagen Plasmid Mini Kit (Qiagen Inc., Hilden, Germany) according to
596 the manufacturer's instructions. In order to detect the IncP-1 plasmids with Southern
597 blot hybridization in these transconjugants, plasmid DNA was digested with the
598 restriction enzyme *NotI* (Thermo Fisher Scientific, Waltham, MA, USA), and fragments

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599 were separated by electrophoresis on a 1% agarose-TBE gel as described previously
600 (71). Southern blot hybridization was preformed with digoxigenin (DIG) labeled probes
601 generated from PCR amplicons which were obtained from reference plasmids R751 for
602 IncP1-β as previously described by Binh et al. (72).
603
604
605 **Plasmid replicon typing**
606 PBRT was used to identify the incompatibility group of plasmids in tet resistant *E. coli*
607 and transconjugants, and to confirm the presence of IncF and IncI plasmids as
608 determined via the newly developed RT-PCR method as described above. This was
609 done by PCR amplification on genomic DNA of the strains using primer sets for 30
610 replicons: HI1, HI2, I1, I2, X1, X2, X3, X4, L, M, N, FIA, FIB, FIC, FII, FIIS, FIIK, FIB KN,
611 FIB KQ, W, Y, P1, A/C, T, K, U, R, B/O, HIB-M, and FIB-M, representative of major
612 plasmid incompatibility groups among Enterobacteriaceae (73, 28). PCR products were
613 separated by electrophoresis on a 2.5% agarose-TBE gel and stained with ethidium
614 bromide.
615
616 **Detection of IncF and IncI plasmids, *tet*(A) and class 1 integrase gene *intI1* via**
617 **PCR-Southern blot hybridization and RT-PCR in TC-DNA**
618 PCR-Southern blot hybridization or RT-PCR was used to detect *tet*(A), *intI1*, IncF, and
619 IncI plasmids in TC-DNA extracted from microbial fraction detached from leaves directly
620 or after an enrichment step on days 0 and 7. The PCR products were separated on a
621 1% agarose-TBE gel electrophoresis and then transferred to a positively charged nylon

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622 membrane (GE Healthcare, UK). Southern blot hybridization was carried out with
623 digoxigenin (DIG) labeled probes generated from PCR amplicons which were obtained
624 from reference plasmids pKJK5 for *intI1* and *tet*(A) as described by Dealtry et al. (74),
625 and R64 for IncI1, pHNSHP45 for IncI2, and IncF plasmids. The primers and PCR
626 conditions are listed in Table 4.
627

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931 **TABLE 1** Characterization of representative tetracycline resistant *E. coli* isolates from
932 fresh produce

E. coli isolates[a]	Sample source[b]	Time point (D)	Inc groups[c]	*bla* genes	Resistance and integrase genes	Antibiotic resistance profile[d]
EK2.1[D]	Ci	0	FII[l]	bla_{TEM}	*tet*(A), *qnrS*	AM, AMX, TE, CIP, OFX
EK2.2[E]	Ci	0	U[h]	bla_{TEM}	*intI1, tet*(A), *sul2,strA, qnrS*	AM, AMX, TE, S, TMP, SD, CIP, OFX
EK2.5[D]	Ci	0	I1[l]	bla_{TEM}	*tet*(A), *qnrS*	AM, AMX, TE, CIP
EK2.7[D]	Ci	0	U[h]	bla_{TEM}	*intI1, tet*(A), *sul2, strA, qnrS*	AM, AMX, TE, S, TMP, SD, OFX
EK2.8[D]	Ci	0	I1[l]	bla_{TEM}	*intI1, tet*(A), *sul2, strA*	AM, AMX, TE, S, TMP
EK2.11[D]	Ci	0	I1[f]	bla_{TEM}	*tet*(A)	AM, AMX, TE
EK2.15[D]	Ci	0	I1[f]	bla_{TEM}	*tet*(A), *sul2*	AM, AMX, TE
EK2.16[D]	Ci	0	N[g]	bla_{TEM}	*tet*(A), sul2, sul3, *strA*	AM, AMX, TE, S
EK2.18[D]	Ci	0	X1[h]	bla_{TEM}	*tet*(A), *sul2, sul3, strA*	AM, AMX, TE, S
EK2.19[E]	Ci	0	FII[l]	bla_{TEM}	*intI1, tet*(A), *merRT*Δ*P, sul2, aadA*	AM, AMX, TE, S, TMP, D, GM, KM
EK2.20[E]	Ci	0	HI1[h]	bla_{TEM}	*intI1, tet*(A), *sul2, sul3, aadA, qnrS*	AM, AMX, TE, S, TMP, SD, GM, OFX, C
EK2.21[E]	Ci	0	X1[h]	bla_{TEM}	*intI1, tet*(A)	AM, AMX, TE, OFX
EK2.22[E]	Ci	0	X1[h]	bla_{TEM}	*intI1, tet*(A), *strA, qnrS*	AM, AMX, TE, S, TMP, SD, OFX
EK2.25[E]	Ci	0	U[h], X1[h]	bla_{TEM}	*intI1, tet*(A), *sul2, strA, qnrS*	AM, AMX, TE, S, TMP, SD, CIP, OFX
EK2.26[E]	Ci	0	U[h]	bla_{TEM}	*intI1, tet*(A), *sul1, aadA, qacE*Δ*1, strA*	AM, AMX, TE, S, TMP, SD, CIP, NA, OFX
EK2.29[E,k]	Ci	0	N[g]	bla_{TEM}, $bla_{CTX\text{-}M\text{-}1}$	*intI1, tet*(A), *sul1, strA, qnrS*	AM, AMX, TE, S, TMP, SD, CRO, CTX, OFX, CIP
EK2.30[E]	Ci	0	U[h]	bla_{TEM}	*intI1, tet*(A), *sul1, sul3, aadA, qnrS*	AM, AMX, TE, TMP, SD, C
EK3.33[E]	Ci	0	X1[h]	-	*intI1, sul1, strA*	TE, D
EK3.34[D]	Ci	0	FII[l]	bla_{TEM}	*intI1, tet*(A), *sul1, strA*	AM, AMX, TE, S, TMP, SD, NA, OFX
EK3.35[D]	Ci	0	FII[l]	bla_{TEM}	*tet*(A), *sul1*	AM, AMX, TE, S, TMP, SD, NA
EK3.36[E]	Ci	0	FIB[l]	bla_{TEM}	*intI1, tet*(A), *merRT*Δ*P, sul1, aadA*	AM, AMX, TE, S, TMP, D, GM, KM
EK3.43[E,k]	Ci	0	N[g]	$bla_{CTX\text{-}M\text{-}1}$	*tet*(A), *sul1,qnrS*	AM, AMX, TE, S, D, GM, CTX, OFX
EK3.44[E,k]	Ci	7	N[g]	bla_{TEM}, $bla_{CTX\text{-}M\text{-}1}$	*intI1, tet*(A), *merRT*Δ*P, sul1, strA, qnrS*	AM, AMX, TE, S, TMP, SD, CRO, CTX, C, NA, CIP, OFX
EK5.16[E]	Ci	7	FII[l], I1[f]	bla_{TEM}	*intI1, tet*(A), *aadA, qacE*Δ*1, qnrS*	AM, AMX, TE, S, TMP, D, OFX
EK5.19[E]	Ci	7	X1[h]	bla_{TEM}	*intI1, tet*(A), *sul2, strA, qnrS*	AM, AMX, TE, S, TMP, SD, D, C
EK5.20[E]	Ci	7	FII[l], I1[f]	bla_{TEM}	*intI1, tet*(A), *aadA, qacE*Δ*1, qnrS*	AM, AMX, TE, S, TMP, OFX
EK5.25[E]	Ci	7	FII[l]	bla_{TEM}	*intI1, tet*(A), *qacE*Δ*1, qnrS*	AM, AMX, TE, TMP, OFX
EK5.28[E]	Ci	7	FII[l], I1[f]	bla_{TEM}	*intI1, tet*(A), *aadA, qacE*Δ*1, qnrS*	AM, AMX, TE, S, TMP, CIP, OFX
EK5.30[E]	Ci	7	FII[l], FIB[l], I1[f]	bla_{TEM}	*intI1, tet*(A), *sul1, aadA, qacE*Δ*1, qnrS*	AM, AMX, TE, TMP, SD, D, CIP, NA, OFX
EK5.32[E]	Ci	7	X1[h]	bla_{TEM}	*intI1, tet*(A), *sul1, sul2, strA, qnrS*	AM, AMX, TE, S, TMP, SD, OFX, C
EK5.40[E]	Ci	7	X1[h]	bla_{TEM}	*intI1, tet*(A), *sul2, strA, qnrS*	AM, AMX, TE, S, TMP, SD, C
EK7.6[E]	Ci	7	U[h]	bla_{TEM}	*intI1, tet*(A)	AM, AMX, TE, TMP, D
EK7.7[E]	Ci	7	I1[f]	bla_{TEM}	*intI1, tet*(A), *qacE*Δ*1, aadA*	AM, AMX, TE, TMP
EK7.9[E]	Ci	7	X1[f]	bla_{TEM}	*intI1, tet*(A), *merRT*Δ*P, sul2, qnrS*	AM, AMX, TE, SD, CRO, D, CIP, C, GM, OFX

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TABLE 1 (continued)

E. coli isolates[a]	Sample source[b]	Time point (D)	Inc groups[c]	*bla* genes	Resistance and integrase genes	Antibiotic resistance profile[d]
EK7.10[E]	Ci	7	HI1[h]	bla_{TEM}	*intI1, tet*(A), *merRTΔP, sul2, qnrS*	AM, AMX, TE, SD, CRO, D, CIP, C, GM, OFX
EK7.11[E]	Ci	7	FII[I], I1[f]	bla_{TEM}	*intI1, tet*(A), *aadA, qacEΔ1, qnrS*	AM, AMX, TE, TMP, CIP
EK7.12[E]	Ci	7	FII[I], I1[f]	bla_{TEM}	*intI1, tet*(A), *aadA, qacEΔ1, qnrS*	AM, AMX, TE, TMP
EK7.13[E]	Ci	7	FIB[I], I1[f]	bla_{TEM}	*intI1, tet*(A), *aadA, qacEΔ1, qnrS*	AM, AMX, TE, TMP, OFX
EK7.14[E]	Ci	7	FII[I]	bla_{TEM}	*intI1, aadA, qacEΔ1, qnrS*	AM, AMX, TE, TMP
EK7.15[E]	Ci	7	FII[I]	bla_{TEM}	*intI1, tet*(A), *sul1, sul2, aadA, qacEΔ1, qnrS*	AM, AMX, TE, S, TMP, SD, CIP, GM
EK7.16[E]	Ci	7	FII[I]	bla_{TEM}	*intI1, tet*(A), *sul1, sul2, strA, qacEΔ1, qnrS*	AM, AMX, TE, S, TMP, SD, CIP, OFX
EK7.17[E]	Ci	7	FII[I], X1[h]	bla_{TEM}	*tet*(A) , *qnrS*	AM, AMX, TE, CIP, OFX
EK7.18[E]	Ci	7	FII[I]	bla_{TEM}	*intI1, tet*(A), *sul2, strA, qnrS*	AM, AMX, TE, S, TMP, SD, CIP, OFX
EK7.19[E]	Ci	7	FIB[I], I1[f]	bla_{TEM}	*intI1, tet*(A), *aadA, qacEΔ1, qnrS*	AM, AMX, TE, TMP
EK7.21[E]	Ci	7	FIB[I], I1[f]	bla_{TEM}	*intI1, tet*(A), *aadA, qacEΔ1, qnrS*	AM, AMX, TE, TMP, OFX
EK7.22[E]	Ci	7	FII[I], FIB[I]	bla_{TEM}	*intI1, tet*(A), *sul1, sul2, sul3, aadA, strA, qacEΔ1, qnrS*	AM, AMX, TE, S, TMP, SD, CIP, GM, NA, OFX
EK7.24[E]	Ci	7	FII[I], I1[f]	bla_{TEM}	*intI1, tet*(A), *sul1, sul2, aadA, strA, qacEΔ1, qnrB, qnrS*	AM, AMX, TE, S, TMP, SD, CIP, OFX, C
EK7.26[E]	Ci	7	FIB[I], I1[f]	bla_{TEM}	*intI1, tet*(A), *aadA, qacEΔ1, qnrS*	AM, AMX, TE, TMP, CIP, OFX
EK7.27[E]	Ci	7	X1[h]	bla_{TEM}	*tet*(A), *merRTΔP, sul2, qnrS*	AM, AMX, TE, SD, CRO, D, CIP, C, GM, OFX
EK7.28[E]	Ci	7	FIB[I], I1[f]	bla_{TEM}	*intI1, tet*(A), *aadA, qacEΔ1, qnrS*	AM, AMX, TE, TMP
EK7.29[E]	Ci	7	FII[I], FIB[I]	bla_{TEM}	*intI1, tet*(A), *sul1, aadA, qacEΔ1, qnrS*	AM, AMX, TE, S, TMP, SD, D, CIP, NA, OFX
EK7.30[E]	Ci	7	FII[I]	bla_{TEM}	*intI1, tet*(A), *sul1, sul2, aadA, qacEΔ1, qnrS*	AM, AMX, TE, S, TMP, SD, GM
EK6.1[E]	Ci	7	FII[I]	-	*intI1, tet*(A), *sul2, strA*	TE, S
EK6.3[E]	Ci	7	FIB[I]	-	*tet*(A), *sul2, strA*	TE, S
M1[E]	MS	0	ND	bla_{TEM}	*intI1, tet*(A), *sul1, sul2, qacEΔ1, strA, merRTΔP*	AM, AMX, TE, S, TMP, SD, D
M19[E]	MS	0	ND	bla_{TEM}	*intI1, tet*(A), *sul1, sul2, qacEΔ1, strA*	AM, AMX, TE, S, TMP, SD, D
RE1[D]	A	0	FII[I]	-	-	TE, D
RE4[D]	A	0	I1	-	*intI1, tet*(A), *sul3, aadA, merRTΔP*	TE, S, TMP, SD, D, CIP, NA
RE9[D]	A	0	I1[I]	-	*intI1, tet*(A), *sul3, aadA, merRTΔP*	TE, S, TMP, SD, D, CIP, NA
RE10[D]	A	0	I1[f]	-	*intI1, tet*(A), *sul3, aadA, merRTΔP*	TE, S, TMP, SD, D, CIP, NA
RE11[E]	A	0	I1[f]	-	*intI1, tet*(A), *sul3, aadA, merRTΔP*	TE, S, TMP, SD, D, CIP, NA
RE14[E]	A	7	FII[I]	-	-	TE, D
RE19[E]	A	7	I1[f]	-	*intI1, tet*(A), *sul3, aadA, merRTΔP*	TE, S, TMP, SD, D, CIP, NA

[a]: D: Direct plating; E: Enrichment.
[b]: Ci: Cilantro; MS: Mixed salad; A: Arugula.
[c]: I: detected by RT-PCR and PBRT; f: detected by RT-PCR; g: detected by PCR; h: detected by PBRT; k: Conjugal transfer into *E. coli* CV601.
[d]: C: Chloramphenicol; AM: Ampcillin; AMX: Amoxicillin; TE: Tetracycline; S: Streptomycin; TMP: Trimethoprim; SD: Sulfadiazine; CIP: Ciprofloxacin; OFX: Ofloxacin; D: Doxycycline; GM: Gentamicin; NA: Nalidixic acid; CRO: Ceftriaxone; CTX: Cefotaxime.
ND: not detected.

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TABLE 2 Characterization of representative tetracycline resistant *E. coli* CV601 transconjugants captured from produce.

TET^R *E. coli* CV601 transconjugants	Sample source[a]	Inc groups[b]	*bla* genes	Resistance, integrase genes and IS[c]	Antibiotic resistance profile[d]
pBC1.1	Ci	P-1β[f]	*bla*$_{TEM}$	*intI1, tet*(A)*, merRTΔP, qacEΔ1,* IS*1071*	TE, AM, AMX, D
pBC1.3	Ci	P-1β[f]	-	*intI1, tet*(A)*, merRTΔP, qacEΔ1,* IS*1071*	TE, AM, AMX, D
pBC1.9	Ci	P-1β[f], FII[l]	*bla*$_{TEM}$	*intI1, tet*(A)*, merRTΔP, qacEΔ1,* IS*1071*	TE, AM, AMX, D
pBC1.12	Ci	P-1β[f], FII[l]	-	*intI1, tet*(A)*, merRTΔP, strA, qacEΔ1,* IS*1071*	TE, AM, AMX, D, S
pBC2.1	Ci	FIB[l]	*bla*$_{TEM}$	*tet*(A), *sul1, qnrS*	TE, AM, AMX, D, CIP, NA, OFX, C
pBC2.2	Ci	FIB[l]	*bla*$_{TEM}$	*tet*(A)*, qnrS*	TE, AM, AMX, D, CIP, OFX
pBC2.3	Ci	FIB[l], I1[f]	*bla*$_{TEM}$	*tet*(A)*, qnrS*	TE, AM, AMX, D, CIP, OFX
pBC2.4	Ci	FIB[l]	*bla*$_{TEM}$	*tet*(A), *sul2, qnrS*	TE, AM, AMX, D, CIP, NA, OFX
pBC2.6	Ci	FIB[l]	*bla*$_{TEM}$	*tet*(A)*, qnrS*	TE, AM, AMX, D, CIP, OFX, C
PBC2.8	Ci	FIB[l]	*bla*$_{TEM}$	*tet*(A)*, qnrS*	TE, AM, AMX, D, CIP, NA, OFX
pBC2.11	Ci	FII[l]	*bla*$_{TEM}$	*tet*(A)*, qnrS*	TE, AM, AMX, D, CIP, OFX
pBC2.15	Ci	I1[fl]	*bla*$_{TEM}$	*tet*(A)*, qnrS*	TE, AM, AMX, D, CIP, OFX
pBMS1	MS	FII[l]	*bla*$_{TEM}$	*intI1, tet*(Q), *sul1, strA, merRTΔP*	TE, AM, AMX, D, TMP, C, S, SD
pBMS4	MS	FII[l]	*bla*$_{TEM}$	*intI1, sul1, strA, merRTΔP,*	TE, AM, AMX, D, TMP, C, S, SD
pBA1	A	ND	*bla*$_{TEM}$	*tet*(A)	TE, AM, AMX, D, TMP, C, CIP
E. coli CV601 (R)	-	-	-	-	-

[a]: Ci: Cilantro; MS: Mixed salad; A: Arugula.
[b]: l: detected by RT-PCR and PBRT; f: detected by RT-PCR.
[c]: IS, Insertion Sequence.
[d]: C: Chloramphenicol; AM: Ampcillin; AMX: Amoxicillin; TE: Tetracycline; CIP: Ciprofloxacin; OFX: Ofloxacin; D: Doxycycline; NA: Nalidixic acid; TMP: Trimethoprim; SD: Sulfadiazine.
ND: not detected.

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TABLE 3 PCR hybridization and real-time PCR of IncF, I1, I2 plasmids and *intI1* and *tet*(A) from TC-DNA extracted from fresh produce before and after enrichment.

Fresh produce	DNA Isolation	Time point (D)	IncF		IncI1		IncI2		*intI1*		*tet*(A)	
			qPCR	Blot	qPCR	Blot	qPCR	Blot	qPCR	Blot	qPCR	Blot
Mixed salad	Direct extraction	0	-	-	-	-	-	+++	+	(+++)	-	(++)^{1*}
		7	-	-	-	-	-	+++	+	(+++)	-	(+++)^{2*}
	Enrichment	0	+	(+++)	+	(++)	+	+++	+	(+++)	+	(++)^{2*}
		7	+	(+++)	+	(++)	+	+++	+	(+++)	+	+++
Arugula	Direct extraction	0	-	-	-	-	-	(++)^{3*}	+	(++)	-	(+++)^{2*}
		7	-	-	-	-	-	(++)^{1*}	+	(++)	-	(+++)^{1*}
	Enrichment	0	+	(+++)	-	-	+	+++	+	(++)	+	+++
		7	+	(+++)	-	-	+	+++	+	(++)	+	+++
Cilantro	Direct extraction	0	-	-	-	-	-	-	(+)^{1*}	(++)	(+)^{1*}	(+++)^{3*}
		7	(+)^{1*}	(++)^{2*}	-	-	-	-	(+)^{3*}	(+++)	(+)^{2*}	(+++)^{3*}
	Enrichment	0	(+)^{2*}	(+++)^{2*}	(+)^{1*}	(++)^{2*}	-	-	(+)^{3*}	(+++)	(+)^{2*}	(+++)^{3*}
		7	(+)^{2*}	(+++)^{2*}	(+)^{2*}	(++)^{2*}	-	-	(+)^{3*}	(+++)^{3*}	(+)^{3*}	(+++)^{3*}

(*): Number of positive replicates, -: Not detected or no signal, +: positive (qPCR), (++): Medium signal, (+++): Strong signal

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1004 **TABLE 4** PCR and qPCR primer systems used in this study.

Gene target	Primer	Primer and Probe (5'-3')	size (bp)	Reference
qacEΔ1	qacEall-F	CGCATTTATTTTCTTTCTCTGGTT	69	(75)
	qacEall-R	CCCGACCAGACTGCATAAGC		
	qacEall-P	TGAAATCCATCCCTGTCGGTGT		
tet(A)	tetA-qfw	CCGCGCTTTGGGTCATT	504	(76)
	tetA_qrv	TGGTCGCGTCCCAGTGA		
	q-tetA-P	TCGGCGAGGATCG		
sul1	q-sul_1 653f	CCGTTGGCCTTCCTGTAAAG	965	(77)
	q-sul_1 719r	TTGCCGATCGCGTGAAGT		
	tp_sul1	CAGCGAGCCTTGCGGCGG		
sul2	q_sul2 595f	CGGCTGCGCTTCGATT	865	(77)
	q_sul2 654f	CGCGCGCAGAAAGGATT		
	tp_sul2 614	CGGTGCTTCTGTCTGTTTCGCGC		
sul3	Sul3-F	CAGATAAGGCAATTGAGCATGCTCTGC	569	(37)
	Sul3-R	AGAATGATTTCCGTGACACTGCAATCATT		
intI1	intI1-LC1	GCCTTGATGTTACCCGAGAG	196	(78)
	intI1-LC5	GATCGGTCGAATGCGTGT		
	intI1-P	ATTCCTGGCCGTGGTTCTGGGTTTT		
aadA	q-aadA-Fw	TTGATTTGCTGGTTACTGTG	635	(79)
	q-aadA-Rv	CTTAGTGTGATCTCGCCTTT		
	q-aadA-P	TGGTAGGTCCAGCGGCGGAG		
korB	korB-F	TCATCGACAACGACTACAACG	118	(80)
	korB-Fz	TCGTGGATAACGACTACAAACG		
	korB-R	TTCTTCTTGCCCTTCGCCAG		
	korB-Rge	TTYTTCYTGCCCTTGGCCAG		
	KorB-Rd	TTCTTGACTCCCTTCGCCAG		
strA	q-strA-Fw	TCAATCCCGACTTCTTACCG	521	(79)
	q-strA-Rv	CACCATGGCAAACAACCATA		
	q-strA-P	TGCTCGACCAAGAGCGGC		
intI2	intI2-LC2	TGCTTTTCCCACCCTTACC	195	(78)
	intI2-LC3	GACGGCTACCCTCTGTTATCTC		
	intI2-P	TGGATACTCGCAACCAAGTTATTTTTACGCTG		
tet(Q)	q-tetQ-Fw	AGGTGCTGAACCTTGTTTGATTC	69	(81)
	q-tetQ-Rv	GGCCGGACGGAGGATTT		
	q-tetQ-P	TCGCATCAGCATCCCGCTC		
IncF (*traI*)	682_F	CACGGTATGTGGGARATGCC		This study
	1073_R	TCCGGCGGCAGYATVCCRAC		
	973_P	CAGCAGGCGGTGRCRCAGGC		
IncI1 (*traI*)	IncI1_traI_Fwd	TTCTTCTTCCCCTACCATC	118	This study
	IncI1_traI_Rev	CATTTTCCAGCGTGTTTC		
	IncI1_traI_TP	CGGCTTTTCACTTCGTGGTT		
IncI2 (*traI*)	IncI2_traI_Fwd	CAAGAACAGAAACAGGCA	291	This study
	IncI2_traI_Rev	TCCCGCAGATAACAGATA		
	IncI2_traI_TP	CCAAACCAACCACAACCA		
merRTΔP	merRT-P	GGGAGATCTAAAGCACGCTAAGGCRTA	1000	(82)
	merRT-P-P	GGGGAATTCTTGACWGTGATCGGGCA		
$bla_{CTX-M-1}$	CTX-M-F	TCTTCCAGAATAAGGAATCCC	908	(83)
	CTX-M-R	CCGTTTCCGCTATTACAAAC		
bla_{TEM}	TEM-F	TCCGCTCATGAGACAATAACC	930	(83)
	TEM-R	TTGGTCTGACAGTTACCAATGC		
bla_{SHV}	SHV-F	TTATCTCCCTGTTAGCCACC	796	(83)
	SHV-R	GATTTGCTGATTTCGCTCGG		
IS*1071*	IS-F	GCTTGGTCACTTCTGGGTCTTC	180	(84)
	IS-R	CTATGCCCGTCTATCGTTACCC		
IncN	rep-1	AGTTCACCACCTACTCGCTCCG	165	(85)
	rep-2	CAAGTTCTTCTGTTGGGATTCCG		
qnrA	qnrAf-RT	ATTTCTCACGCCAGGATTTG	529	(86)
	qnrAr-RT	GCAGATCGGCATAGCTGAAG		

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1006 **TABLE 4 (continued)**

Gene target	Primer	Primer and Probe (5'-3')	size (bp)	Reference
qnrB	qnrBmF	GGMATHGAAATTCGCCACTG	429	(86)
	qnrBmR	TTYGCBGYYCGCCAGTCGAA		
qnrS	qnrSrtF11	GACGTGCTAACTTGCGTGAT	393	(86)
	qnrSrtR11	TGGCATTGTTGGAAACTTG		

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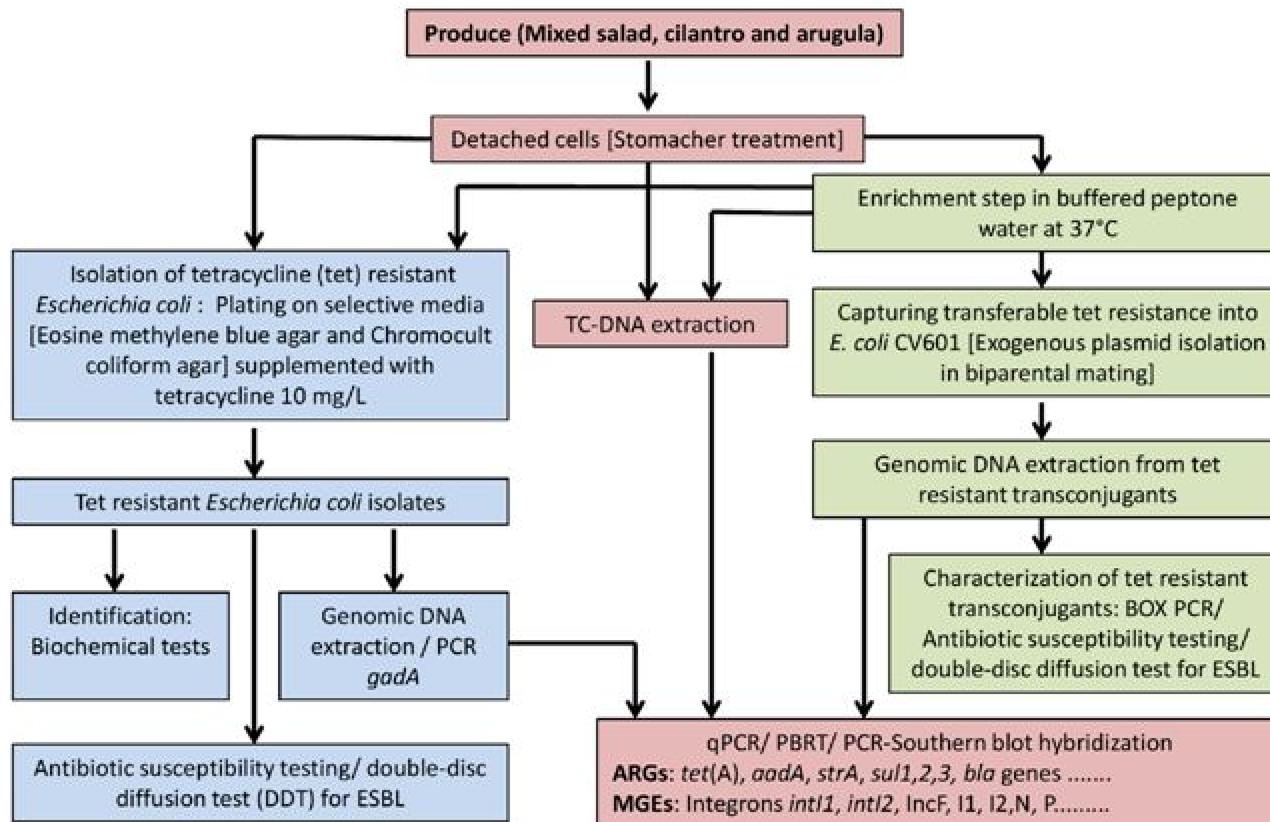
1051 **Figure legends**

1052 **Figure 1**. Flow diagram of the experimental set-up of this study to employ culture-
1053 dependent and -independent approaches to characterize the transferable resistome of
1054 bacteria associated with produce.
1055


Produce (Mixed salad, cilantro and arugula)
Detached cells [Stomacher treatment]
Enrichment step in buffered peptone water at 37°C
Isolation of tetracycline (tet) resistant *Escherichia coli* : Plating on selective media [Eosine methylene blue agar and Chromocult coliform agar] supplemented with tetracycline 10 mg/L
TC-DNA extraction
Capturing transferable tet resistance into *E. coli* CV601 [Exogenous plasmid isolation in biparental mating]
Genomic DNA extraction from tet resistant transconjugants
Tet resistant *Escherichia coli* isolates
Identification: Biochemical tests
Genomic DNA extraction / PCR *gadA*
Characterization of tet resistant transconjugants: BOX PCR/ Antibiotic susceptibility testing/ double-disc diffusion test for ESBL
Antibiotic susceptibility testing/ double-disc diffusion test (DDT) for ESBL
qPCR/ PBRT/ PCR-Southern blot hybridization
ARGs: *tet*(A), *aadA*, *strA*, *sul1,2,3*, *bla* genes ....
MGEs: Integrons *intI1*, *intI2*, IncF, I1, I2,N, P.........