High prevalence of *Klebsiella pneumoniae* in European food products: a multicentric study comparing culture and molecular detection methods

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37

38 **Running Tittle:** *K. pneumoniae* from chicken meat and salads

39

Abstract

Klebsiella pneumoniae species complex (KpSC) is a leading cause of multidrug-resistant human infections. To better understand the potential contribution of food as a vehicle of KpSC, we conducted a multicentric study to define an optimal culture method for its recovery from food matrices, and to characterize food isolates phenotypically and genotypically. Chicken meat (n=160) and salad (n=145) samples were collected in five European countries and screened for KpSC presence using culture-based and ZKIR qPCR methods. Enrichment using buffered peptone water followed by streaking on Simmons citrate agar with inositol (44°C/48h) was defined as the most suitable selective culture method for KpSC recovery. High prevalence of KpSC was found in chicken meat (60% and 52% by ZKIR qPCR and culture approach, respectively) and salad (30% and 21%, respectively) samples. Genomic analyses revealed high genetic diversity with the dominance of phylogroups Kp1 (91%) and Kp3 (6%). 82% of isolates presented a natural antimicrobial susceptibility phenotype and genotype, with only four CTX-M-15-producing isolates detected. Notably, identical genotypes were found across samples: same food type and same country (15 cases); different food types and same country (1); same food type and two countries (1), suggesting high rates of transmission of KpSC within the food sector. Our study provides a novel isolation strategy for KpSC from food matrices and reinforces the view of food as a potential source of KpSC colonization in humans.

Importance

Bacteria of the *Klebsiella pneumoniae* species complex (KpSC) are ubiquitous and *K. pneumoniae* (Kp) is a leading cause of antibiotic-resistant infections in humans and animals. Despite the urgent public health threat represented by Kp, there is a lack of knowledge on

63 the contribution of food sources to colonization and subsequent infection in humans. This is
64 partly due to the absence of standardized methods for characterizing KpSC presence in food
65 matrices. Our multicentric study provides and implements a novel isolation strategy for KpSC
66 from food matrices and shows that KpSC members are highly prevalent in salads and chicken
67 meat, reinforcing the view of food as a potential source of KpSC colonization in humans.
68 Despite the large genetic diversity and the low-levels of resistance detected, the occurrence
69 of identical genotypes across samples suggests high rates of transmission of KpSC within the
70 food sector, which need to be further explored to define possible control strategies.

71 **Introduction**

72 *Klebsiella pneumoniae* (Kp), a common gut bacteria, is regarded as a critical priority
73 pathogen (1, 2), given the depletion of therapeutic options to treat multidrug-resistant (MDR)
74 Kp infections. Furthermore, hypervirulent community-acquired invasive infections represent
75 a growing problem, especially in Asian countries (3). Concerning reports of the convergence
76 of MDR and hypervirulent phenotypes are increasing (4). The clinical epidemiology of Kp is
77 dominated by the clonal spread of important MDR and hypervirulent sublineages, such as
78 clonal group (CG) 258, CG15, CG147 or CG23 (3).

79 The ecology and transmission of Kp outside the clinical setting is still underexplored;
80 in particular, the reservoir and possible contribution of non-clinical sources in the
81 transmission of prominent clonal groups to humans remain largely undefined. Given the
82 broad ecological distribution of Kp in animals and the environment (5, 6), and its capacity to
83 contaminate food (7–9), the contribution of food sources to colonization of humans, and to
84 subsequent infection, is an important question to address. Currently, detection, isolation and
85 identification of Kp is not well integrated in food microbiological surveillance programs. There
86 is also a lack of standardized tools and procedures for the detection of Kp in food. To define
87 the natural ecology and transmission of Kp, studies of the presence of Kp in food should be
88 designed irrespective of antimicrobial resistance phenotypes.

89 In order to achieve precise detection and identification of Kp, it is important to define
90 the target species. Recent taxonomic updates pointed out the existence of seven phylogroups
91 (phylogroup 1 [Kp1] to Kp7), now defined as distinct taxa, within *Klebsiella pneumoniae sensu*
92 *lato*. The resulting *K. pneumoniae* species complex (KpSC) consists of five different species: *K.*
93 *pneumoniae sensu stricto* (Kp1), *K. quasipneumoniae* subsp. *quasipneumoniae* (Kp2) and

94 *K. quasipneumoniae* subsp. *similipneumoniae* (Kp4), *K. variicola* subsp. *variicola* (Kp3) and
95 *K. variicola* subsp. *tropica* (Kp5), '*K. quasivariicola*' (Kp6, which remains to be formally
96 defined), and *K. africana* (Kp7) (10–12). Recently, Barbier *et. al* developed the ZKIR qPCR (13)
97 for the detection of KpSC in soil and environmental samples, but its application to food
98 matrices was not evaluated. Regarding the isolation of *Klebsiella* spp., different selective
99 culture methods (7, 14, 15) have been described but comparisons of their efficiency for
100 *Klebsiella* isolation from food is lacking.

101 The aims of this study were (i) To compare the selectivity, productivity and specificity
102 of three agar media for the detection and isolation of *Klebsiella* spp., leading to propose a
103 standardized culture protocol for the recovery of *Klebsiella* spp. in food matrices and compare
104 its performance with the ZKIR qPCR; and (ii) To evaluate the presence and phenotypic and
105 genomic features of KpSC in two common globally consumed foodstuffs: chicken meat and
106 ready-to-eat salads, through a multicentric study in five European countries.

107

108 **Results**

109 **Comparison of culture media**

110 Productivity (P_R), selectivity (S_F) and specificity were calculated separately for each of
111 the media considered. The three media were compliant with ISO11133:2014 requirements
112 for productivity, as P_R was above 0.5 for almost all target strains **(Figure S3**). However, two
113 strains had $P_R < 0.50$: *K. pneumoniae* SB132 in Chromatic Detection Agar and
114 *K. quasipneumoniae* subsp. *quasipneumoniae* SB1124, for which colony growth was only
115 observed on SCAI medium.

116 We observed that the media did not comply with the selectivity criteria ($S_F > 2$) for
117 most of the non-target strains considered: the S_F values ranged from -0.3 to 0.5 for *Klebsiella*
118 ChromoSelect Agar and from -0.5 to 0.9 for SCAI agar (Liofilchem was not tested as it is not
119 selective). We noted that *Klebsiella* ChromoSelect Agar was selective for *Cronobacter* spp.
120 and *C. freundii*, showing S_F values equal to 7.6 and 4, respectively. Hence, the media cannot
121 be considered as selective for *Klebsiella* according to the ISO11133:2014 requirements.
122 Regarding specificity, the selective media tested showed variable results regarding the
123 morphological features of observed colonies, based on the non-target strains considered. The
124 selective media, indeed, allowed the growth of some non-*Klebsiella* spp. strains, and in few
125 cases, colonies were morphologically similar to the target strains. Overall, because Kp
126 colonies were easily distinguishable on SCAI medium and because it was as performant as the
127 others regarding the other criteria, the SCAI medium was selected and used hereafter.
128
129 **Definition of an optimized protocol for recovery of KpSC from food samples**
130 Our initial comparisons of the four different culture protocols (**Figure S1**) showed that
131 the most effective method for recovery of KpSC from chicken meat was protocol 1B, which
132 consists of a one-step enrichment in BPW. KpSC was recovered from 7 out of 36 (19.4%)
133 samples using this protocol, while only 3 (8.3%) samples were positive using protocol 2
134 (enrichment in LB broth supplemented with ampicillin) and only 2 (5.6%) using protocol 1C
135 (double enrichment: enrichment in BPW followed by enrichment in LB broth supplemented
136 with ampicillin). No KpSC was isolated using direct plating (protocol 1A).
137 Further, using BPW enrichment of chicken meat (protocol 1B), a higher recovery of
138 KpSC was observed when streaking a 10 µl loop onto SCAI agar plates, *versus* spreading 10 µl
139 or 100 µl of bacterial enrichment. Of the 28 chicken meat samples examined using both

140 methods, KpSC was recovered from 24 (85.7%) samples using the 10 µl loop *versus* 19 (79.2%)
141 samples after spreading 10 µl, and 7 (29.2%) samples after spreading 100 µl, onto SCAI agar.
142 Thus, protocol 1B combined with streaking was used henceforth.
143
144 **Evaluation of the SCAI agar plate incubation temperature**
145 We compared two temperatures (37°C and 44°C) for the incubation of SCAI agar plates
146 (**Figure S2**). Collectively, 111 chicken samples were tested and 67.7% (64/111) of them were
147 positive for KpSC. KpSC was recovered from 59/111 (53%) samples when SCAI agar was
148 incubated at 37°C, as well as when incubated at 44°C, indicating no difference between
149 incubation temperatures (**Table S3**). However, when data from each individual institution was
150 analyzed, a slightly higher percentage of positive samples was recorded following incubation
151 of SCAI plates at 44°C at two institutions (AGES and NUIG), whereas contrasting results were
152 observed at the third institution (SSI) (**Table S3**). As a result, a decision was made to
153 implement incubation of SCAI agar plates at 44°C ± 1°C, which was incorporated into our
154 protocol (**Figure 2**) (dx.doi.org/10.17504/protocols.io.baxtifnn). All further food samples
155 were tested according to this protocol. Of note, no statistical differences were found in the
156 prevalence of KpSC in from free-range and not free-range chicken meat samples (**Table S3**).
157
158 **Comparison of culture and ZKIR qPCR methods for KpSC detection**
159 Chicken meat and salad leaf samples were tested for the presence of KpSC using both
160 the ZKIR qPCR assay (13) and the optimized culture protocol (**Figure 2**). Regarding the chicken
161 meat samples (n=160), the ZKIR qPCR method detected KpSC in 96 (60.0%) of the samples
162 (**Table 1)**. In comparison, when the culture protocol was used, 83 (51.9%, *p*=0.177) of the

163 samples were positive for KpSC (82 *K. pneumoniae* and 1 *K. variicola* according to MALDI-TOF
164 MS results). Regarding salad leaf samples (n=145), 29.7% (43/145) of the samples tested
165 positive for KpSC using the ZKIR qPCR assay, versus 20.7% (30/145; *p*=0.104; 18
166 *K. pneumoniae* and 12 *K. variicola* according to MALDI-TOF MS results) using the culture
167 method (**Table 1**). Considering the ZKIR qPCR assay as a reference, the optimized culture
168 protocol showed a sensitivity (true positive rate) of 84.2%, a specificity (false positive rate) of
169 100%, a positive predictive value of 100%, and a negative predictive value of 86.6%.
170
171 **Table 1**. Comparison between the recovery rates of *K. pneumoniae* species complex using the
172 ZKIR qPCR assay *versus* the optimized culture method.
173

Source	**No. of samples**	**qPCR positive samples** (%)	**Culture positive samples** (%)
Chicken meat	160	96 (60.0%)	83 (51.8%)
Salads	145	43 (29.7%)	30 (20.7%)
Total	**305**	**139 (45.6%)**	**113 (37.1%)**

174
175
176 **Comparison of KpSC prevalence in chicken meat and salad samples**
177 The overall prevalence of KpSC in chicken meat was twice as high as in salad samples
178 (60.0% versus 29.7%; $p < 0.00001$; **Table 1**). However, prevalence differed among countries
179 (**Figure 2**). The prevalence of KpSC in chicken meat samples ranged between 43.3% and 46.6%
180 for three of the countries (Austria, Italy and Ireland), whereas in France and Denmark the
181 prevalence was above 70% (72.5% and 90.0%, respectively). Regarding salad samples, the

182 prevalence of KpSC ranged from 3.3% in Ireland to 92% in France, with Austria, Italy and
183 France being the countries where the highest numbers of samples positive for KpSC were
184 detected.

185 In total, 131 KpSC isolates (92 from chicken meat and 39 from salads) were collected
186 from 113 positive samples using the optimized culture method; in some positive samples, up
187 to 5 different morphotypes were collected (**Figure 2, Table S2**).

188

189 **Antimicrobial susceptibility**

190 Antimicrobial susceptibility phenotypes were defined for the 131 KpSC isolates using
191 17 antimicrobial agents representing 11 antimicrobial classes (**Table S4**). 82% of the isolates
192 presented a wild-type phenotype, being susceptible to all the antibiotics tested except
193 ampicillin, for which constitutive resistance is a characteristic of all KpSC isolates. The highest
194 rates of resistance (detected in > 5% of the isolates) were observed for trimethoprim (12% in
195 chicken meat and 13% in salads), trimethoprim-sulfamethoxazole (8% for both), tetracycline
196 (9% in chicken meat and 5% in salads) and piperacillin-tazobactam (7% in chicken meat; **Figure**
197 **S4**; **Table S4**). Four isolates (2 from each source) were found to be resistant to extended-
198 spectrum cephalosporins, and ESBL gene presence in the genomic sequence was confirmed
199 (**Table S4**). No isolate was found to be resistant to cefoxitin, ertapenem or netilmicin. Similar
200 levels of resistance were detected among isolates from salads and from chicken meat.

201 Differences in antimicrobial resistance occurrence rates were observed among
202 countries (**Table S4**) but the low number of isolates implies precaution in the analysis. For
203 chicken meat, higher resistance rates were detected in Italy (8-50%), Austria (7-23%) and

204 Ireland (7-14%), whereas for salads it was in Italy (33-100%), Austria (20%) and France (4-12%)
205 (**Figure S4, Table S4**).
206 Nine (6.9%) multidrug resistant isolates were identified (**Table S5**), including the four
207 ESBL producers. MDR isolates were recovered in 7 chicken meat and 2 salad samples in Italy
208 (n=3), Austria (n=2), France (n=2) and Ireland (n=2) (**Table S5**).
209
210 **Phylogenetic and genomic diversity**
211 Based on genomic data we observed among the KpSC isolates the predominance of
212 Kp1 (90.8%, 119/131) followed by Kp3 (6.1%, 8/131), Kp2 (2.3%, 3/131) and Kp4 (0.8%,
213 1/131). The predominance of Kp1 was found in both sample types (98% in chicken meat; 74%
214 in salads); however, there was an association of Kp3 with salad samples (18% in salads versus
215 1% in chicken meat; *p*=0.001). Kp2 was only found in salads, whereas Kp4 was only detected
216 in chicken meat (**Table S6**).
217 Population structure analysis based on MLST and cgMLST genotyping unveiled a high
218 genetic diversity among the KpSC isolates, with 86 STs [19 of them defined in this study;
219 Simpson's index of diversity (SID) 0.989] and 107 cgSTs (SID 0.995; **Figure 3**). High-risk clonal
220 groups (CGs) common in the clinical setting represented 19.8% (26/131) of the isolates (**Table**
221 **S6**). Among them, CG45 (n=9), CG37 (n=5), CG17 (n=4), CG661 (n=4), CG147 (n=2), CG14 (n=1)
222 and CG15 (n=1) were found in chicken meat (n=18; 11.3%) and/or salad (n=6, 3.8%) samples.
223 A high diversity of capsular types was also found, with 71 *wzi*-alleles and 58 KL-types. In
224 contrast, O-type diversity was low, with O1 (25.1%; 33/131) and O2 (20.6%; 27/131)
225 representing almost 50% of the recovered KpSC, followed by O3 (28.2%; 37/131) (**Figure 3**).

226 Defining single strains (designed as genotypes) as isolates differing by no more than 5
227 cgMLST alleles (out of 629 loci) (16), we observed 15 strains that were isolated at least twice
228 across a total of 35 different samples. These were collected from the same food type (chicken
229 meat or salads) at different times in the same country (**Table S6, Figure S5**). To improve the
230 confidence inferring the close relationship between these cgMLST-based overlapped
231 genotypes, the SNP matrix based on 4,125 core genes obtained from Roary (representing
232 ~75% of a typical *K. pneumoniae* genome length against 10% represented by the 629 cgMLST
233 genes) (16) was used, and confirmed the close genetic relatedness. For example, in France,
234 the same genotype (ST45/genotype 7 – 1 to 4 SNPs) was detected in 3 salad samples from
235 different brands (separated in time by 9 days), whereas in Ireland the same genotype
236 (ST4384/genotype 13 – 1 to 8 SNPs) was detected in six different chicken meat samples
237 (separated in time by 2 months) from different suppliers (**Table S6, Figure S5**). There was one
238 case of the same strain being detected in different countries, as two Kp1 ST290 isolates
239 (genotype 10; 15 SNPs) were detected in chicken meat samples from Denmark (April 2019)
240 and Austria (September 2019) (**Figure S5**). We also detected a single strain between chicken
241 meat and salads (ST1537/genotype 9 – 23 SNPs) between May and September 2019,
242 respectively, in France (**Table S6**).
243 In most isolates (82%; 108/131) only intrinsic $bla_{SHV/OKP/LEN}$ genes was detected,
244 consistent with a natural susceptibility genotype (**Table S6**). Few antibiotic resistance genes
245 were observed across the 131 isolates, with genes of non-prominent clinical significance *sul*
246 and *dfr*, (9.2%; 12/131 both), *tet* (8.4%; 11/131), *aadA* and *strAB* (6.1%; 8/131 both) being the
247 most frequent ones. However, four CTX-M-15 producing isolates were detected in two salad
248 samples from France and two chicken meat samples from Italy and Ireland. The genotype of

249 MDR isolates was compatible with their phenotype (**Table S5**). No carbapenemase or colistin
250 resistance genes or mutations were detected. In 45.8% (60/131) of the isolates, neither
251 plasmid replicons nor plasmid-encoded heavy metal tolerance genes were detected. In strains
252 where plasmid replicons were detected, the plasmids known to occur naturally in Kp, such as
253 IncFIB and $IncFII_K$, were the most prevalent (25.2% and 16.8%, respectively). Regarding
254 virulence genes, the yersiniabactin gene cluster was detected in 14.5% (19/131) of the isolates
255 and no hypervirulent genotypes (as defined by the presence of *rmpA* or aerobactin or
256 salmochelin) were detected.
257

258 **Discussion**

259 Despite the renewed interest in *Klebsiella pneumoniae* complex (KpSC) epidemiology,
260 boosted by the increasing involvement of *K. pneumoniae sensu stricto* (Kp1) in human
261 infections associated with high-levels of antibiotic resistance and/or virulence (1, 3), the
262 contribution of non-clinical sources, such as food products, to the current emergence of KpSC
263 is poorly understood. This is partly due to the lack of standardized protocols for the detection
264 and isolation of Kp strains in environmental, food or animal samples. Different selective
265 culture media have been previously developed for *Klebsiella* spp.. Among the most recognized
266 are MacConkey-Inositol-Carbenicillin agar (MCIC) (17) and its variation containing adonitol
267 (MCICA,) (18), SCAI (14, 19) and Brilliant green containing Inositol-Nitrate-Deoxycholate agar
268 (BIND) (20). All of them rely on the ability of *Klebsiella* spp. to use inositol as carbon source,
269 combined with the use of secondary selective compounds (e.g., citrate as carbon source in
270 the case of SCAI; nitrate as nitrogen source in BIND; carbenicillin and adonitol in MCIC/MCICA,
271 although only Kp1 and Kp2/Kp4 are able to metabolize adonitol) (11). In our study, we decided
272 to evaluate the performance of three different culture media for the detection and isolation
273 of *Klebsiella* spp. in accordance with ISO 11133:2014 and latest amendments. They included
274 the non-commercial SCAI medium and two commercial culture media: the *Klebsiella*
275 ChromoSelect Selective Agar produced by Sigma-Aldrich, that similarly to MCIC uses
276 carbenicillin as selective compound
277 (https://www.sigmaaldrich.com/deepweb/assets/sigmaaldrich/product/documents/240/58
278 0/90925dat.pdf); and Chromatic Detection agar developed by Liofilchem, which is a
279 chromogenic non-selective medium allowing the identification of *Klebsiella* spp.,
280 *Enterobacter* spp. and *Serratia* spp. based on the green-blue color of colonies

281 (http://www.liofilchem.net/login/pd/ifu/11611_IFU.pdf). The productivity tests of the three
282 media did not show any critical issues, whereas none of the media tested fulfilled the
283 selectivity and specificity criteria defined by ISO 11133:2014. Nevertheless, the distinction of
284 *Klebsiella* spp. colonies from other species was achieved in general. This is a critical point, as
285 the routinely used media (e.g., MacConkey or CLED agar) do not provide easy differentiation
286 of *Klebsiella* spp. colonies from other species, such as *Citrobacter* spp., *Pantoea* spp. or
287 *Serratia* spp. Here, the performance of the tested culture media showed similar results and
288 SCAI was selected, due to its low cost and easy in-house preparation. The use of this media in
289 recent *Klebsiella* spp. epidemiological studies may also represent an advantage for global and
290 cross-sector comparisons (13, 21–23).

291 Once SCAI agar was selected, different protocols to isolate *Klebsiella spp*. from food
292 matrices were evaluated. Multiple conditions, involving pre-enrichment, enrichment,
293 incubation temperature and plating were assessed from chicken meat samples. The
294 optimized culture protocol was then compared with the reference ZKIR qPCR assay in a
295 multicentric design study involving chicken meat and salad samples (13). Overall, KpSC was
296 detected at a higher rate in both chicken meat and salad samples using the ZKIR qPCR method,
297 but even so the optimized culture protocol demonstrated good sensitivity (84%) and
298 specificity (100%) rates.

299 Approximately 50% of the food samples tested were KpSC positive, with significantly
300 higher occurrence in chicken meat compared to salads, although disparities in prevalence
301 were observed according to the country analyzed. It is difficult to contextualize our findings
302 in a global scenario since few studies have addressed KpSC prevalence in these type of
303 products without previous antibiotic selection: most of the studies addressing the prevalence

304 and characterization of KpSC from foodstuffs have focused on colistin resistant or ESBL- or
305 carbapenemase-producing *K. pneumoniae* (8, 24–26). As a consequence, knowledge on the
306 ecology and natural diversity of Kp populations has been very limited. Furthermore,
307 identification of the bacterial species was imprecise in most previous studies, in light of recent
308 taxonomic changes. Our broader approach also captures susceptible strains and led to several
309 important observations. First, the KpSC prevalence in chicken meat was higher than
310 previously described: 47% in USA (Arizona) in 2012 (7); 30% in Turkey in 2007-2008 (27); 14%
311 in China (Shijiazhuang) in 2013-2014 (28). Second, despite the high prevalence of KpSC in
312 chicken meat samples, ESBL-producing *K. pneumoniae* were only detected in 1.3% (2 CTX-M-
313 15) of the samples. These numbers are similar to those reported in other studies that targeted
314 ESBL strains directly by selective approaches (0-5%) (8, 26, 28–30). Noteworthy, the presence
315 of ESBL in chicken meat was scarce in *K. pneumoniae* when compared with *Escherichia coli*
316 (55%-92%) in recent European reports (30–33).

317 Third, the highest resistance rates detected in natural food Kp populations were to
318 tetracyclines, trimethoprim and trimethoprim-sulfamethoxazole, reflecting the recent
319 reports of sales of these antimicrobial classes in food-producing animals in different European
320 countries (34). Fourth, despite the predominance of Kp1 and the high genetic diversity found
321 among chicken meat isolates, some genotypes overlapped across samples. The relation of
322 these common genotypes (≤ 5 alleles mismatches) with the core-genome alignment obtained
323 from Roary was also explored, and in most of the cases, less than 21 SNPs were identified, a
324 threshold recently proposed for Kp ST258 to discriminate hospital clusters (35). Here, we
325 found a maximum of 42 SNPs detected within the same genotype. Some of these multi-
326 sample genotypes (ST290/genotype 10 recovered in Austria and Denmark and ST45/genotype

327 15 recovered in Denmark) were previously found circulating among healthy poultry in France
328 in 2015 (Rodrigues C and Haenni M., pers.comm.). The repeated isolation of single strains in
329 unrelated samples across time suggests transmission from the same source/reservoir or
330 transmission within chicken flocks during production. Importantly, MDR ST45/genotype 11
331 found in Italy has also been found in urinary tract infections among Italian patients in 2018
332 (22), suggesting a contribution of food KpSC to human infections (7, 24).

333 Salads are typically eaten raw, with a high risk of ingestion of live Kp bacteria if present.
334 The prevalence of KpSC among ready-to-eat salads was approximatively 30%, higher than
335 what was reported in the few comparable studies (6-15%) (36–39). ESBL-producers were
336 found in 1.4% of the samples (2 CTX-M-15), a lower prevalence than what is described in the
337 literature (15-25%) targeting ESBL strains (39–42). As observed for chicken meat samples, the
338 same genotype was observed within the same country, in salads from the same or different
339 chain supplier/supermarket, suggesting a common source of the vegetables (e.g., different
340 brands supplied by a common farm) or cross-contamination during their growth and
341 processing (e.g., use of untreated irrigation water, wild-life animals, contamination of fields
342 with manure, human contamination during packaging). Furthermore, one case of genotype
343 overlap was found between salads and chicken meat isolates from samples from France,
344 suggesting local transmission.

345 A previous study of risk factors of Kp carriage in community settings has linked the
346 consumption of raw vegetables and contact with chicken to MDR KpSC carriage in humans
347 (21). These findings together with our results, which are based on a small sampling survey,
348 highlight the possible role of food as a source of Kp and call for much broader studies to
349 address the ecology and transmission of this generalist pathogen in the food sector.

350 In our sampling, sublineages defined as high-risk clonal groups (3) represented 20% of
351 the isolates recovered. One of these, ST45 (in both type of samples) was highly prevalent, as
352 well as ST290 (in chicken meat), and the potential risk they pose should be addressed in future
353 studies. Both STs have already been linked to human infections (22, 43, 44) and human
354 carriage (21, 22) and also to broilers and pigs production (23, 45), raising the question of the
355 zoonotic potential of these STs.
356
357 **Conclusions**
358 In conclusion, our study addressed important knowledge gaps relating to Kp in food
359 sources. First, we addressed the lack of harmonized culture protocols for the detection and
360 isolation of *Klebsiella* spp. from food matrices, with the development of an optimized SCAI-
361 based culture protocol, which in the future may be more widely adopted to enable global
362 comparison regarding KpSC prevalence in these types of sources. Second, the high prevalence
363 of KpSC in chicken meat and ready-to-eat salads highlights the possible role of food as a
364 source of human colonization and infection by KpSC. Even though KpSC strains detected in
365 food products remain largely susceptible to antimicrobials, understanding the degree to
366 which food contamination by Kp contributes to human infections is an important topic for
367 future research in the One Health perspective.

368 **Material and Methods**

369 **Comparison of productivity, selectivity and specificity of agar media**

370 Three different selective solid media for *Klebsiella* spp. growth were tested in
371 accordance with ISO11133:2014 (Microbiology of food, animal feed and water — Preparation,
372 production, storage and performance testing of culture media;
373 https://www.iso.org/standard/53610.html). Nutrient agar (Microbiol & C., Cagliari, Italy) was
374 used as the non-selective reference medium. For productivity, selectivity and specificity
375 assays a set of 58 strains [50 from Institut Pasteur and 8 from Istituto Zooprofilattico
376 Sperimentale dell'Abruzzo e del Molise Giuseppe Caporale (IZSAM)] were used: 51 reference
377 strains of *Klebsiella* spp. and closely related species (*Raoultella* spp.), which included
378 representatives of the six main KpSC phylogroups, and 7 non-target strains (**Table S1**). For
379 productivity (P_R), the three following media were tested: Simmons Citrate Agar with Inositol
380 (SCAI) (14), *Klebsiella* ChromoSelect Selective agar produced by Sigma-Aldrich (Missouri, USA)
381 and Chromatic Detection agar developed by Liofilchem (Roseto degli Abruzzi, Italy). The SCAI
382 medium is not available commercially and was prepared in-house following previous
383 protocols (14, 19) (https://www.protocols.io/view/isolation-of-klebsiella-strains-from-
384 human-or-anim-662hhge/materials). P_R was calculated using 50 reference strains belonging
385 to *Klebsiella* spp. and closely related species (*Raoultella* spp.) (**Table S1**). After incubation at
386 37°C for 24 h, broth cultures in Brain Heart Infusion (BHI) (Biolife, Milan, Italy) of each strain
387 were diluted up to about 100 CFU/ml and spread on media. After incubation at 37°C for 48h,
388 colonies compatible with the *Klebsiella* phenotype were enumerated. P_R is calculated as the
389 ratio between the number of colonies grown on the selective agar medium and the number

390 of colonies grown on the non-selective medium, and according to ISO11133:2014, P_R values
391 should be ≥ 0.50 to consider the media productivity acceptable.
392 For selectivity (S_F), 7 non-target strains (*Cronobacter* spp., *Citrobacter koseri,*
393 *Citrobacter freundii*, *Serratia marcescens, Serratia liquefaciens, Serratia rubidaea* and *1*
394 *Pantoea agglomerans*) were tested on the SCAI and *Klebsiella* ChromoSelect Selective agar
395 (**Table S1**). After incubation at 37°C for 24h in BHI, broth cultures were ten-fold diluted serially
396 and each dilution was spread onto plates. After incubation at 37°C for 48 h, colony growth
397 was observed. S_F is defined as the difference between the highest dilution showing growth on
398 the non-selective reference medium (log_{10}) and the highest dilution showing comparable
399 growth on the selective test medium (log_{10}). In accordance with ISO11133:2014, S_F values of
400 non-target strains should be at least equal to 2 log_{10} growth differences, which reflects the
401 ability of the medium to partially or totally inhibit their growth.
402 For specificity, the 7 non-target strains and two control strains (1 *K. pneumoniae* and
403 1 *R. ornithinolytica*) were tested on the three media (**Table S1**). Broth cultures were prepared
404 in Buffered Peptone Water (BPW) (Biolife, Milan, Italy), incubated at 37°C for 24h, and spread
405 onto each medium considered. After incubation at 37°C for 48h of each medium, plates were
406 observed for the presence of typical and suspected colonies, recording if the characteristics
407 of the non-target colonies could be similar to the target ones.
408
409 **Initial evaluation of protocols for recovery of *Klebsiella pneumoniae* from food**
410 An initial assessment of four protocols for recovery of KpSC from chicken meat was
411 carried out. In total, 36 chicken meat samples [12 free-range (7 skin-on and 5 skin-off) and 24
412 non-free range (12 skin-on and 12 skin-off) samples] were collected together with the

413 following metadata: manufacturer, country of origin, and batch number. Each chicken meat
414 sample was cut into small slivers to a final weight of 50 g. This 50 g sample was divided into
415 two portions of 25 g and processed as follows and as illustrated in **Figure S1**:
416 **Initial suspension 1:** the first 25 g portion was diluted in 225 ml (1:10 dilution) of BPW and
417 the sample was homogenized using a stomacher/blender. Prior to incubation of initial
418 suspension 1, 100 µl of the suspension was cultured directly on SCAI medium using a sterile
419 spreader or sterile loop and incubated at 37°C ± 1°C for 48h ± 1 h (**Figure S1 - 1A** - detection
420 without enrichment). The remainder of initial suspension 1 was incubated at 37°C ± 1°C for
421 24h ± 1 h. Following 24h incubation of initial suspension 1, 100 µl of the suspension was
422 streaked on SCAI medium and incubated at 37°C ± 1°C for 48 h ± 1 h (**Figure S1 - 1B** - detection
423 with BPW enrichment only). Of the remaining incubated suspension 1, 1 ml was inoculated
424 into 9 ml of Lysogeny broth (LB) with ampicillin (final concentration 0.01 mg/mL) and
425 incubated at 37 °C ± 1 °C for 24h± 1 h. Following 24h incubation, 100 µl of the suspension was
426 streaked on SCAI medium and incubated at 37°C ± 1°C for 48h ± 1 h (**Figure S1 - 1C** - detection
427 with double enrichment).
428 **Initial suspension 2:** the second 25 g portion was diluted in 225 ml (1:10 dilution) of LB broth
429 supplemented with ampicillin (final concentration 0.01 mg/mL) and the sample was
430 homogenized using a stomacher/blender and incubated at 37 °C ± 1°C for 24 h ± 1 h. Following
431 24 h incubation, 100 µl of the suspension was cultured on SCAI medium and incubated at 37°C
432 ± 1°C for 48 h ± 1 h (Figure **S1** - **2** detection with LB + Ampicillin enrichment only).
433 Typically, *Klebsiella* spp. appear yellow on SCAI medium (14). At least five suspected
434 *Klebsiella* colonies were collected from each SCAI plate and sub-cultured onto SCAI media or
435 a non-selective agar for identification using MALDI-TOF MS (Bruker Daltonics, Bremen,

436 Germany). Currently, databases only allow for the identification of *K. pneumoniae* or *K.*
437 *variicola*. Based on literature and in our test strain set, Kp1, Kp2 and Kp4 strains are identified
438 as *K. pneumoniae*, whereas Kp3, Kp5 and Kp6 are currently identified as *K. variicola* (46, 47).
439 For 28/36 chicken meat samples, the impact on KpSC recovery of culturing using a 10
440 µl loop versus the spreading of 10 µl and 100 µl of enrichments onto SCAI media was also
441 compared.
442
443 **Evaluation of the optimized protocol and definition of an optimal temperature of**
444 **incubation**
445 Following this initial assessment process, the optimized protocol as outlined below
446 and as illustrated in **Figure S2** was evaluated for recovery of KpSC from chicken meat samples,
447 using a total of 111 samples of chicken meat [51 free-range (25 skin-on and 26 skin-off) and
448 60 non-free range (30 skin-on and 30 skin-off)].
449 Each sample was cut into small slivers to a final weight of 25 g. This was added to 225
450 ml of BPW broth (1:10 dilution) and the sample was mixed using a stomacher or blender. The
451 suspension was then incubated at 37°C ± 1°C for 24h ± 1 h. Following incubation, the
452 suspension was cultured for single colonies on SCAI agar using a 10 µl loop and incubated at
453 two different temperatures, 37°C ± 1°C and at 44°C ± 1°C for 48 h ± 1 h (**Figure S2**), in order
454 to establish an optimal temperature of incubation of SCAI medium for recovery of Kp from
455 food matrices.
456

457 **Comparison of the optimized SCAI culture protocol and ZKIR qPCR**

458 A comparison of the optimized SCAI culture protocol and the ZKIR qPCR for the
459 detection and isolation of KpSC in food matrices was carried out in 5 European institutions,
460 including the Austrian Agency for Health and Food Safety (AGES), French Agency for Food,
461 Environmental and Occupational Health & Safety (ANSES), National University of Ireland
462 Galway (NUIG), Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise Giuseppe
463 Caporale (IZSAM) and the Statens Serum Institute (SSI, Denmark).

464 Each institution was asked to collect and test, where possible, an additional 30
465 samples of chicken meat, as well as 30 pre-packaged, pre-washed salad leaf samples (**Table**
466 **S2**). In total, 160 chicken meat samples and 145 salad samples, collected between December
467 2018 and September 2019, were tested using the updated optimized protocol
468 (dx.doi.org/10.17504/protocols.io.baxtifnn) for recovery of KpSC from food matrices (**Figure**
469 **1**).

470 The ZKIR qPCR was used to detect the presence of KpSC in food samples (13). After
471 enrichment, DNA was prepared for ZKIR qPCR. 500 µl of the enrichment broth were
472 centrifuged (5 min at 5800 × g), washed with sterile water, and resuspended in 500 µl sterile
473 water before boiling for 10 min. qPCR conditions were as previously described (13)
474 (dx.doi.org/10.17504/protocols.io.7n6hmhe).

475

476 **Antimicrobial susceptibility testing**

477 Antimicrobial susceptibility testing was performed on 131 KpSC isolates using either disk
478 diffusion or microbroth dilution [GN2F panels (Sensititre, ThermoFischer Scientific)] methods.
479 Clinical breakpoints were used for interpretation according to EUCAST guidelines

480 (https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_9.0
481 _Breakpoint_Tables.pdf), except for kanamycin and streptomycin (48). *Escherichia coli*
482 ATCC25922 was used as control strain. The panel of antimicrobials tested included:
483 amoxicillin/clavulanic acid (20/10 µg), piperacillin-tazobactam (both using disks of 30/6 µg, or
484 GN2F plates), cefpodoxime (10 µg, GN2F) or cefotaxime (5 µg), cefoxitin (30 µg, GN2F),
485 aztreonam (30 µg), ertapenem (10 µg), amikacin (30 µg, GN2F), gentamicin (10 µg, GN2F),
486 netilmicin (10 µg), tobramycin (10 µg, GN2F), kanamycin (10 µg), streptomycin (30 µg),
487 ciprofloxacin (5 µg, GN2F) or ofloxacin (5 µg), nalidixic acid (30 µg), trimethoprim (5 µg),
488 trimethoprim-sulfamethoxazole (23.75 + 1.25 µg, GN2F), and tetracycline (30 µg). The
489 presence of multidrug resistant isolates, which are defined as those resistant to at least one
490 agent in 3 different classes of antimicrobials (49), was analyzed.
491
492 **Whole-genome sequencing and comparative genomic analyses**
493 Whole-genome sequencing was performed on the 131 Kp isolates. Genomic DNA
494 libraries were prepared using the Nextera XT Library Prep Kit (Illumina, San Diego, USA)
495 following the manufacturer's protocol. Illumina sequencing was performed at the five
496 partners: AGES and SSI using MiSeq (2 x 250 bp paired-end sequencing; n=18 and n=31
497 isolates respectively); ANSES and NUIG using NovaSeq 6000 (2 x 150 bp paired-end
498 sequencing; n=52 and n=15, respectively); and IZSAM using NextSeq 500/550 (2 x 150 bp
499 paired-end sequencing; n=15). Genomic assemblies were obtained using SPAdes v3.9 (50) or
500 Velvet v1.2.10 (51) and were annotated using Prokka v1.12 (52).
501 Multilocus sequence typing (MLST) (53), core-genome MLST (cgMLST) and cgLIN codes
502 (16) were determined using the BIGSdb-Kp database

503 (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html). This web tool and Kleborate (54)
504 (https://github.com/katholt/Kleborate) were used to look for antimicrobial resistance,
505 virulence, and heavy metal tolerance genes and to characterize the capsular synthesis gene
506 cluster. Plasmid replicons were searched using PlasmidFinder (55)
507 (https://cge.cbs.dtu.dk/services/PlasmidFinder/).

508 For phylogenetic analyses, a core-genome alignment based on the concatenation of
509 4,125 core genes was obtained with Roary v3.12 (56) using a blastP identity cut-off of 80%
510 and core genes defined as those being present in more than 90% of the isolates.
511 Recombination events were removed using Gubbins v2.2.0 (57), generating a recombination-
512 free alignment comprising 486,290 single-nucleotide variants (SNVs). This recombination-free
513 alignment was used to construct a maximum likelihood phylogenetic tree using IQ-TREE
514 v1.6.11 (model GTR+F+ASC+R5).

515 Chi square or Fisher exact test were used to compare the prevalence of KpSC in the
516 different sources and to check the association of the different categorical variables (*P* values
517 <0.05 were considered statistically significant).

518

519 **Data availability**

520 Detailed optimized SCAI culture protocol for the detection and isolation of KpSC in food
521 matrices was made publicly accessible to the scientific community in January 2020 through
522 the protocols.io platform (dx.doi.org/10.17504/protocols.io.baxtifnn). Genomic sequences
523 generated in this study were submitted to the European Nucleotide Archive and are
524 accessible under the BioProject number PRJEB34643, and are also publicly available in BIGSdb
525 through project ID 37 'MedVetKlebs_multicentric study'.

526 **Authors Contribution**

527 SB, CR, DM, KH, EMN and FP conceptualized the study. GC, AC and FP from IZSAM performed
528 the culture media evaluation. KH, MLM, EMN, NC and DM performed the initial assessments
529 for the establishment of an optimized culture protocol for recovery of KpSC from food
530 matrices. KH, NC, MLM, GB, AC and RGF performed the comparison of culture media and ZKIR
531 qPCR assay. EB and PP provided laboratory support and validated the analysis of the ZKIR
532 qPCR assay. CR ad SB analyzed all the genomic data, coordinated the study and wrote the
533 initial draft manuscript. SB acquired funding. All authors contributed to writing and editing
534 the manuscript and reviewed the final version.

535

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540

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546

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551 arising from this submission.

552

553 **Figure legends**

554 **Figure 1**. Optimized protocol for the recovery and isolation of *Klebsiella* spp. from food
555 matrices.

556 **Figure 2**. Prevalence of *K. pneumoniae* species complex by type of sample and by country.

557 **Figure 3.** Maximum likelihood phylogeny based on 4,125 concatenated core genes generated
558 using IQ-Tree with the GTR+F+ASC+R5 model.

559 The scale bar indicates the number of nucleotide substitutions per site. Black dots on main
560 nodes indicate bootstrap values ≥95%. Tree tips are colored by sample type (see key). The
561 grey boxes delineate high-risk clonal groups (CG), as specified. European countries where the
562 samples were collected are colored as indicated in the legend. The presence of antimicrobial
563 resistance and virulence genes, and plasmid replicons is indicated.

564

565 **Supplementary Material**

566 **Table S1**. Bacterial reference strains used for agar media performance comparison.

567 Strains used for the different evaluation criteria are indicated. The correspondent strain code
568 for productivity results in Figure S3 is indicated.

569 **Table S2**. Chicken meat and salad samples analyzed in the different European countries for
570 the presence of *K. pneumoniae* species complex and its epidemiologic characteristics.

571 **Table S3**. Comparison between the recovery rates of *K. pneumoniae* species complex using
572 two different incubation temperatures of SCAI medium.
573 **Table S4**. Number of *K. pneumoniae* species complex resistant isolates for each of the
574 antimicrobials tested distributed by sampling source and by country.
575 **Table S5**. Multidrug resistant strains from chicken meat and salads, sequence-type (ST),
576 phenotypic resistance and identified AMR genes based on WGS.
577 **Table S6.** Characteristics of the 131 *K. pneumoniae* species complex isolates recovered:
578 population structure, surface polysaccharides, antimicrobial susceptibility, antimicrobial
579 resistance genes, virulence genes, metal tolerance gene clusters and plasmid replicons.
580
581 **Figure S1**. Evaluation of different protocols for recovery of *Klebsiella* spp. from food matrices.
582 **Figure S2**. Protocol to define the optimal temperature of incubation of SCAI medium plates
583 for the recovery of *Klebsiella* spp. from food matrices.
584 **Figure S3**. Productivity (P_R) results for the three media considered using a reference panel of
585 50 *Klebsiella* spp. and closely related species.
586 **Figure S4**. Antibiotic resistance of the *K. pneumoniae* species complex isolates recovered by
587 sample type and by country.
588 Upper panel: The x-axis represents the number of KpSC resistant isolates for each of the
589 antimicrobials tested and percentages (%) displayed on the graph were calculated based on
590 the total number of isolates.
591 Lower panel: The x-axis represents the number of KpSC resistant isolates for each of the
592 antimicrobials tested and percentages (%) displayed on the graph were calculated based on
593 the total number of isolates in each country.

594 **Figure S5**. Geographic distribution of the common genotypes found among *K. pneumoniae*
595 species complex recovered from food products.
596
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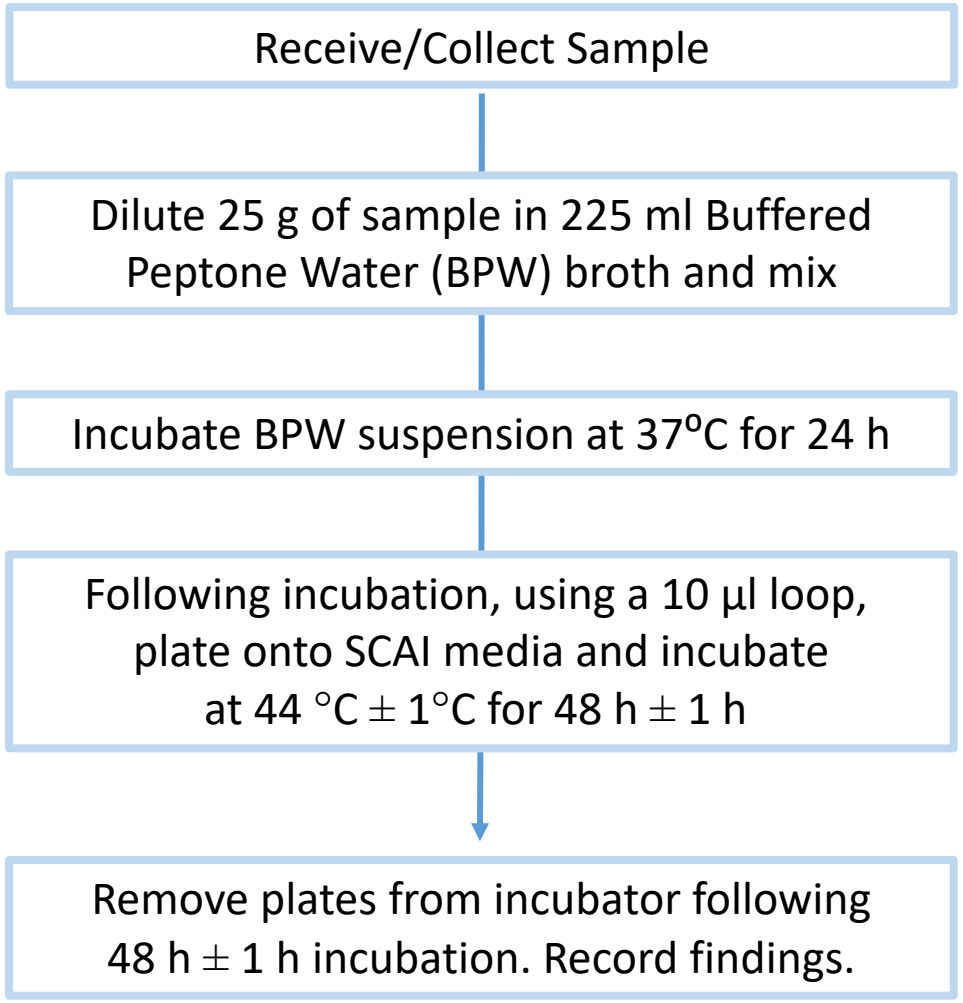
Figure 1



Purify and identify all suspected *Klebsiella* spp. colonies

(by MALDI-TOF or species-specific PCR)

Freeze strains confirmed as *Klebsiella* spp. at -80°C

(If several morphotypes are available, store one colony per morphotype)

Figure 2

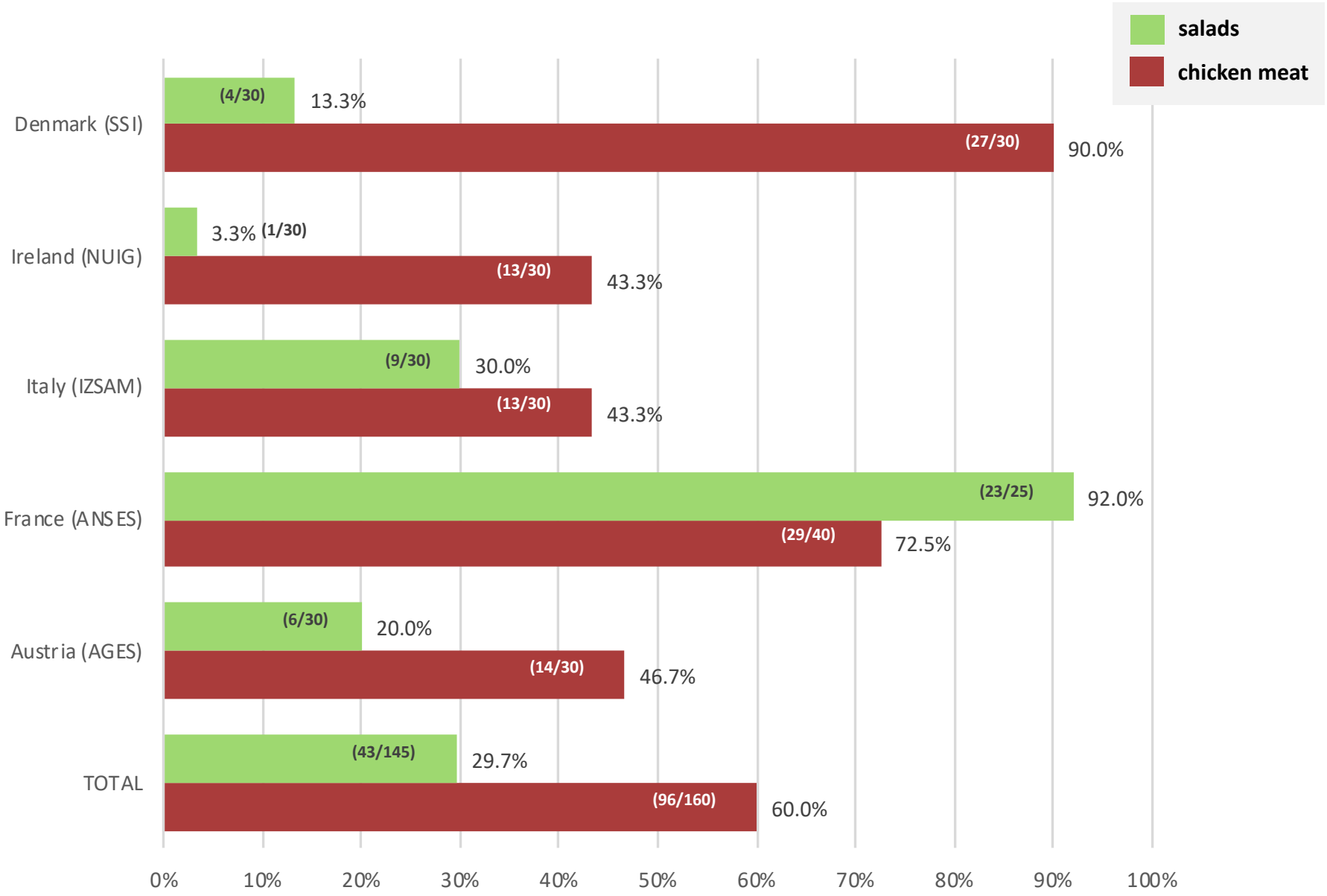
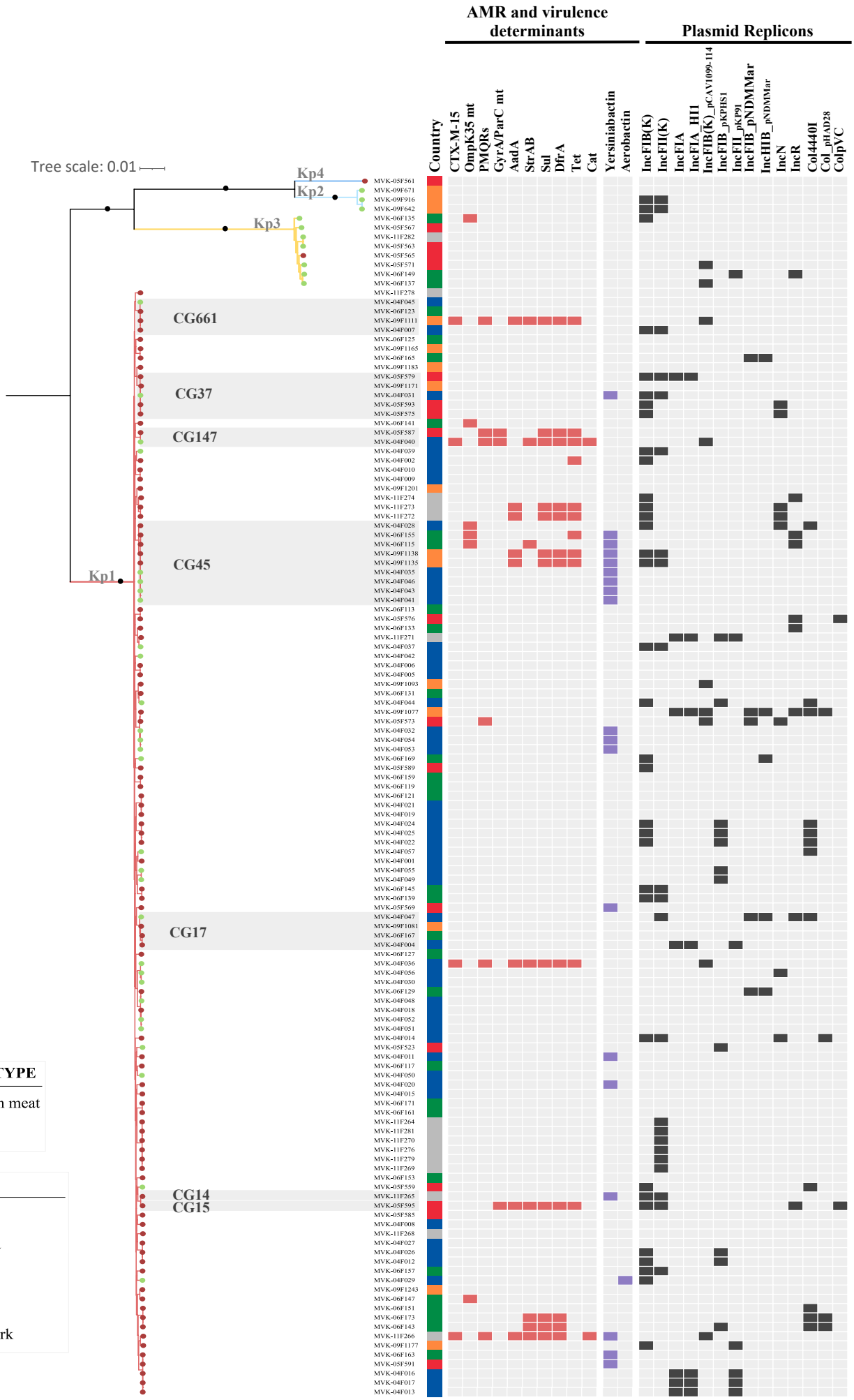

salads
chicken meat
Denmark (SSI)
(4/30)
13.3%
(27/30)
90.0%
Ireland (NUIG)
3.3%
(1/30)
(13/30)
43.3%
Italy (IZSAM)
(9/30)
30.0%
(13/30)
43.3%
France (ANSES)
(23/25)
92.0%
(29/40)
72.5%
Austria (AGES)
(6/30)
20.0%
(14/30)
46.7%
TOTAL
(43/145)
29.7%
(96/160)
60.0%
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%

Figure 3


AMR and virulence determinants
Plasmid Replicons
Country
CTX-M-15
OmpK35 mt
PMQRs
GyrA/ParC mt
AadA
StrAB
Sul
DfrA
Tet
Cat
Yersiniabactin
Aerobactin
IncFIB(K)
IncFII(K)
IncFIA
IncFIA_HI1
IncFIB(K)_pCAV1099-114
IncFIB_pKPHS1
IncFII_pKP91
IncFIB_pNDMMar
IncHIB_pNDMMar
IncN
IncR
Col44440I
Col_pHAD28
ColpVC
Tree scale: 0.01
Kp4
Kp2
Kp3
Kp1
CG661
CG37
CG147
CG45
CG17
CG14
CG15
SAMPLE TYPE
chicken meat
salad
Country
France
Austria
Italy
Ireland
Denmark