Occurrence of antibiotic resistance genes in the faecal DNA of healthy omnivores, ovo-lacto vegetarians and vegans

Occurrence of Antibiotic Resistance Genes in the Faecal DNA of Healthy Omnivores, Ovo-Lacto Vegetarians and Vegans

Short title: Faecal incidence of transferable antibiotic resistances as affected by different diets

Vesna Milanović[a], Andrea Osimani[a], Lucia Aquilanti[a*], Stefano Tavoletti[a], Cristiana Garofalo[a], Serena Polverigiani[a], Alice Litta-Mulondo[a], Luca Cocolin[b], Ilario Ferrocino[b], Raffaella Di Cagno[c], Silvia Turroni[d], Camilla Lazzi[e], Nicoletta Pellegrini[e], Francesca Clementi[a]

[a]Department of Agricultural, Food and Environmental Sciences (D3A), Polytechnic University of Marche, Ancona, Italy

[b]Department of Agricultural, Forest and Food Science (DISAFA), University of Turin, Grugliasco, Italy

[c]Faculty of Science and Technology, Libera Università di Bolzano, Bolzano, Italy

[d]Department of Pharmacy and Biotechnology, Alma Mater Studiorum, University of Bologna, Bologna, Italy

[e]Department of Food Science, University of Parma, Parma, Italy

* Correspondence

Prof. Lucia Aquilanti PhD

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l.aquilanti@univpm.it

Abstract

Scope: The effects of long-term omnivore, ovo-lacto vegetarian and vegan diets on the occurrence of 12 antibiotic resistance (AR) genes in the human gut was studied.

Methods and results: The faeces of 144 healthy volunteers recruited from Turin, Bari, Bologna and Parma were screened for the occurrence of genes conferring resistance to tetracyclines, macrolide-lincosamide-streptogramin B, vancomycin and β-lactams. Overall, *erm*(B), *tet*(W) and *tet*(M) were detected at the highest frequency. A low effect from the diet on the AR gene distribution emerged, with *tet*(K) and *vanB* occurring at a lower and higher frequency in vegans and omnivores, respectively. A correlation of the intake of eggs, milk from animal source and cheese with an increased occurrence of *tet*(K) was observed, together with a higher incidence of *vanB* in consumers of eggs, poultry meat, fish and seafood. When the detection frequencies of AR genes in volunteers from Bari and the other sites were comparatively evaluated, a north-to-south gradient was observed, whereas no effect of sex or age was highlighted. Except for *tet*(K), a negligible three-factor interaction was seen.

Conclusion: A high impact of the geographical location on AR gene distribution was seen in the cohort of subjects analyzed, irrespective of their dietary habits.

Keywords

Antibiotic resistance genes; Dietary habits; Human faeces; Human gut microbiota; Transferable resistances

List all abbreviations used in the text more than two times:

AR antibiotic resistance

BA Bari

BO Bologna

CA cluster analysis

DDD daily doses

DNA Deoxyribonucleic Acid

EU European Union

MLS_B macrolide-lincosamide-streptogramin B

PCA principal component analysis

PCR Polymerase Chain Reaction

PR Parma

TO Turin

1. Introduction

The human body harbours a complex microbial ecosystem, and the majority of human-associated microbes reside in the intestinal tract. The diet is the major source of nutrients for bacteria and is itself a reservoir of microbes; therefore, it is inevitably linked to the diversity and functionality of the gut microbiota [**1**]. Over the last decade, the effect of the diet on the composition and function of the gut microbiota has been extensively investigated through different approaches [**1, 2**].

Dietary intake has also been identified as one of the primary routes for the introduction of antibiotic resistant bacteria and their genes into the human digestive tract [**3**]. Antibiotic resistance (AR) is undoubtedly a concrete threat to human health, since bacteria, which are highly adaptable organisms, are becoming increasingly resistant to antibiotics. This resistance reduces the number of antimicrobial agents that are effective against the human pathogens responsible for most bacterial infections. In recent years, the phenomenon of acquired AR in foodborne commensal microbes, such as lactic acid bacteria [**4**], has become a cause of great concern because these microorganisms may act as a reservoir of antibiotic resistant genes that can be horizontally transferred to recipient bacteria when localized on mobile genetic elements (e.g. plasmids, transposons, integrons, etc.) [**5**]. In support of this hypothesis, foodborne commensals that enter the gastrointestinal tract can survive the gastric barrier [**6**] and transfer their AR genes to the resident intestinal microbiota [**7**], and even to human pathogens [**8**].

In this regard, some authors are convinced that a reduction of the environmental AR gene pool might effectively lead to a reduction of ARs in humans [**9**], whereas other authors are more sceptic about the possibility to demonstrate the origin of AR genes and their impact on the human health [**10, 11**]. However, it is widely accepted that the higher the prevalence of ARs in food-producing animals, the higher the risk that antibiotic resistant commensal bacteria enter the human body via the food chain. The correlation between the use of antibiotics in agriculture and the increase in disease risk for humans due to horizontal AR gene transfer between bacterial foodborne donors and human recipients has been long debated, as for infections caused by *Enterococcus* [**12-14**].

Despite ongoing progress in understanding the effect of the diet on the composition of human gut microbiota, the influence of different dietary habits on the human gut AR gene reservoir, that is, the "*resistome*", has been very poorly investigated and the currently available data are

limited and somewhat contradictory, thus rendering the objectives of the present study particularly challenging.

Based on the above premises, this study was aimed at evaluating the impact of long-term omnivore, ovo-lacto vegetarian and vegan diets on the distribution of 12 selected AR genes [*tet*(O), *tet*(M), *tet*(W), *tet*(S), *tet*(K), *erm*(A), *erm*(B), *erm*(C), *vanA*, *vanB*, *mecA*, and *blaZ*] playing a key role in the acquisition of bacterial resistance to antibiotics used in food animal and crop production as well as in humans, namely tetracyclines, macrolide-lincosamide-streptogramin B (MLS_B), vancomycin and β-lactams [**3**]. The target genes were selected from among those that have very recently been ranked by Martinez et al. [**15**] as having the highest risk levels, based on the likelihood of their introduction into human pathogens, and the relative consequences of such an event on human health. Hence, they are widespread among different genera and species, including both commensal bacteria such as lactobacilli, lactococci and enterococci, that are naturally associated with foods of vegetal and animal origin [**16**] and human pathogens typically found in food-producing animals, aquaculture, fruits and vegetables [**3**]. The results of the molecular screening performed by PCR and nested PCR were statistically analyzed to define the effect of the dietary habits and other factors (age, sex, geographical location) on the frequency and distribution of these AR genes in the faeces of the cohort analysed.

2. Materials and methods

2.1 Recruitment of healthy volunteers following an omnivore, ovo-lacto vegetarian or vegan diet

Between February and July 2013, 144 healthy non-smoker volunteers (85 females and 59 males) aged 18-59 (37 ± 9.1) with Body Mass Indexes (BMI) >18 (22 ± 2.3) who were following a habitual omnivore, ovo-lacto vegetarian or vegan diet were recruited from 4

different geographical locations in North (Turin, Piedmont Region; Bologna and Parma, Emilia-Romagna Region) and South (Bari, Apulia Region) Italy. These volunteers were part of a large research project aimed at understanding the impact of different diets on human salivary and gut microbiota (http://www.diet4microgut.it; https://clinicaltrials.gov/ct2/show/NCT02118857). The three groups of volunteers (48 individuals for each group) had been following an omnivore, ovo-lacto vegetarian or vegan diet for at least one year before testing. An ovo-lacto vegetarian diet was assumed when the volunteers stated they did not consume any meat, fish and seafood, whereas a vegan diet was assumed for those volunteers who stated that they did not consume any foods from animal sources, including eggs, milk and other dairy products. The procedures used for recruiting volunteers as well as the exclusion criteria, including the use of antibiotics during the previous three months, have previously been reported by De Filippis et al. [**17**]. The recruited volunteers were asked to fill out a weighed food diary for the daily recording of all food and beverages consumed along with their relative quantities (grams or mL day^{-1}), for a time spam of 3 consecutive weeks as detailed by De Filippis et al. [**17**].

2.2 Collection and handling of faecal samples

Faecal samples (approximately 10 g) were supplied weekly by the volunteers in sterile empty containers once per week on the same day, for a time span of three weeks. Timing for collection of faecal samples was set in order to limit the acknowledged intra-individual variabilities concerning defecation clock time, stool volume, time taken for food and beverages to transit through the gastrointestinal tract, etc. [**18**]. The samples were transported to the laboratory within 12 hours of collection under refrigeration (+ 4°C). Procedures for the collection, handling and storage of the samples have previously been described in detail by Ferrocino et al. [**19**]. For each volunteer, aliquots (3 g) of triplicate faecal samples were

pooled together before nucleic acid extraction to limit intra-individual variability. Ten g of each pooled sample was aseptically homogenized with 90 mL of Ringer's solution (Oxoid, Basingstoke, UK) for 2 min in a peristaltic homogenizer at room temperature and an aliquot (2 mL) further subjected to DNA extraction using a commercial kit (Powersoil DNA kit, MO-BIO, Carlsbad, CA, USA), according to the manufacturer's instructions, as previously described [**19**].

2.3 Reference strains

Eleven antibiotic-resistant strains that were carrying one or more of the AR genes under study were used as positive controls in the PCR and nested PCR assays (Table 1), and *Enterococcus faecalis* JH2-2 [**20**] was used as a negative control. A reagent blank consisting of all the reagents except for the DNA template was processed alongside all the amplification reactions. DNA from the reference strains was extracted using the method proposed by Hynes et al. [**21**] with some slight modifications, as reported by Osimani et al. [**22**].

2.4 PCR and nested PCR amplification of AR genes

DNA was amplified in PCR and nested PCR analyses targeting the AR genes listed in Table 1. Two µl of DNA (~10 ng, as quantified using a NanoDrop 1000 spectrophotometer, Thermo Scientific, Milano, Italy) from the faeces and the reference strains were PCR-amplified in a total volume of 25 µl. For the nested PCR, 2 µl of each PCR product was subjected to a second amplification reaction. The primer pair sequences and amplification conditions used in the PCR and nested PCR assays are shown in Supplementary Tables 1 and 2. For the primers that were designed specifically for this study, a freely available web primer design program (Primer3) was used (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) with the nucleotide sequences retrieved from the GenBank database reported in Supplementary Tables 1 and 2.

All the amplifications were performed in a MyCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). Five µl of each amplification product was analyzed by electrophoresis in 1.5% (w/v) agarose gel in 0.5X TBE (45 mM Tris-borate, 1 mM EDTA). The gels were visualized under UV light and photographed with a Complete Photo XT101 system (Explera, Jesi, Italy). For each AR gene of interest, randomly selected PCR products were sent to Macrogen Europe (Amsterdam, The Netherlands) for sequencing, to verify the annealing of the oligos to the proper target sequence. Contamination due to amplicon carryover was minimized by performing pre-PCR (sample preparation and PCR preparation) and post-PCR (PCR execution and analysis) activities in two physically separate work areas.

2.5 Statistical analysis

2.5.1 Pearson Chi square ($\chi 2$) tests

The daily average consumption (as expressed as g or mL day^{-1}) of the 144 volunteers from within the following 11 food categories at potential risk for the introduction of antibiotic resistant bacteria and their genes into the human gastrointestinal tract was standardized as follows: “cheese”; “milk from animal sources”; “milk from vegetable sources”; “fish and seafood”; “red meat”; “poultry meat”; “preserved meat”, “eggs”; “fruit”; “vegetables”; and “raw/cooked pulses”. The rationale for selecting these food categories was the expected high level ($\geq 10^5$ CFU g^{-1}) of bacterial load, as suggested by data that was preliminarily collected at our laboratory (unpublished). Since antibiotic resistant microorganisms and their genes are known to potentially survive thermal processing (e.g., routine cooking or boiling), as suggested by the recent isolation of an antibiotic resistant *Escherichia coli* strain from cooked meat [**23**] or the direct detection of AR genes in boiled edible insects [**24**], cooked foods were included in the food categories “fish and seafood”, “red meat”, “poultry meat”, and “vegetables”.

For each AR gene of interest, the absolute frequency was calculated as the ratio between positive samples resulting from PCR plus nested PCR and the total number of analyzed samples. Differences in AR gene frequencies due to dietary habits were evaluated with the Pearson Chi square (χ^2) test (α=0.05).

The relationship of the AR genes (presence vs absence) to participant sex, age and daily consumption from the 11 food categories was tested by Pearson χ^2 test (α=0.05, 1 degree of freedom) using 2x2 contingency tables obtained by grouping volunteers into two classes. For the variables "age", "fruit", "vegetables" and "raw/cooked pulses", the 144 volunteers were subdivided in two classes, which were referred to as A and B, with each including 72 volunteers as follows: (i) for the "age" variable, class A included volunteers aged ≤ 37 years old and class B included those aged >, 37 years old; for the "fruit" variable, class A included volunteers who consumed ≤ 267 g day^{-1}, and class B included those consuming > 267 g day^{-1}. For the "vegetables" variable, class A included volunteers consuming ≤ 379 g/day and class B included those consuming > 379 g day^{-1}. Finally, for the "raw/cooked pulses" variable, class A included volunteers consuming ≤ 57 g day^{-1} and class B included those consuming > 57 g day^{-1}.

For the remaining variables, the two classes included individuals who eat (class YES) or do not eat (class NO) from the specific food category.

Comparisons among diets included an overall χ^2 test with 2 degrees of freedom followed by two orthogonal contrasts with 1 degree of freedom each as follows: contrast 1, omnivores vs (ovo-lacto vegetarians plus vegans); and contrast 2, ovo-lacto vegetarians vs vegans.

The effect of the recruiting sites on AR gene frequencies was tested by an overall χ^2 test with 3 degrees of freedom followed by orthogonal contrasts. Moreover, the "diet x site" interaction effect on AR gene frequencies was evaluated by χ^2 test using expected frequencies, which were calculated through the iterative approach suggested by Sokal and

Rohlf [**25**]. Finally, the three-factor interaction was evaluated comparing the AR gene distribution among the three diets within each site.

2.5.2 Cluster Analysis (CA) and Principal Component Analyses (PCA)

To relate participant dietary habits and geographical origin with the occurrence of AR genes in the faeces of the volunteers, PCR and nested PCR results were converted into a 144x12 presence/absence (1/0) table including the 144 volunteers and the 12 AR genes of interest. From this data set, a 12x12 table including the relative AR gene frequencies characterizing each of the 12 groups (diets x sites) was created. Standardized data were hence used to perform both Cluster Analysis (CA) and Principal Components Analysis (PCA) based on the respective Pearson's correlation matrices (NTSYS 2.02i software).

3. Results

3.1. Cohort of healthy omnivores, ovo-lacto-vegetarians and vegans

The list of volunteers, each of whom was identified with an anonymous code, along with their food habits, age, sex and site of recruitment, is shown in Supplementary Table 3. Their daily average consumption (as expressed as g or mL day^{-1}) of 11 potentially high risk foods for the introduction of AR microorganisms and their genes into the human gastrointestinal tract by the three groups of volunteers is shown in Supplementary Table 4.

3.2 PCR and nested-PCR

The results of the molecular screening that was performed on the faeces collected from the 144 volunteers by PCR and nested PCR are shown in Supplementary Table 5.

All the nucleotide sequences of purified amplicons from randomly selected positive faecal specimens had a $\geq$ 97% similarity with the expected antibiotic resistance genes (data not

shown), thus confirming the specificity of all the primer sets used in the PCR and nested-PCR assays.

For some target genes, namely *mecA*, *vanA* and *tet*(O), positive amplification could only be achieved by nested PCR , irrespective of the dietary group considered, whereas for other genes, namely *erm*(B), *vanB, tet*(M), *tet*(W) and *blaZ*, the majority of samples were already positive after the first PCRs, irrespective of the dietary group considered (Supplementary Table 5). Based on the limits of detection of the PCR and nested PCR protocols used, that had previously been assessed at our laboratory ([**26**]; unpublished), a gene copy number per gram of faeces ranging from 10^5 [*erm*(B), *erm*(C) and *blaZ*] to 10^7 [*tet*(M), *tet*(O), *tet*(K), *tet*(S), *tet*(W), *erm*(A), *mecA*, *vanA*, *vanB*] and from 10^2 [*erm*(B), *erm*(C) and *blaZ*] to 10^4 [*tet*(M), *tet*(O), *tet*(K), *tet*(S), *tet*(W), *erm*(A), *mecA*, *vanA*, *vanB*] were assumed for the samples positive after the first and second round of PCR, respectively.

The detection frequencies of the 12 AR genes that were calculated within each dietary group (48 omnivores, 48 ovo-lacto vegetarians and 48 vegans) are shown in Table 2. In general, determinants coding for resistance to MLS_B (*erm* genes) and tetracyclines (*tet* genes) were prevalent, irrespective of the diet under consideration. Among the MLS_B genes, *erm*(B) was detected at the highest frequency (96 to 100%), whereas *erm*(A) was the least abundant gene, being detected only in the faeces of vegans at a very low frequency (10%).

Regarding the *tet* genes, *tet*(W) and *tet*(M) were prevalent, being detected in almost all the samples at a high detection frequency (98 to 100% and 90 to 94%, respectively). By contrast, *tet*(O) was the least abundant *tet* gene, being detected at a frequency lower than 50%.

Concerning the genes conferring resistance to vancomycin, both *vanA* and *vanB* occurred with a low frequency, the first being detected exclusively by nested PCR at a frequency < 25% and *vanB* occurring with a frequency of 15-35%.

The gene *blaZ*, which codes for resistance to β-lactams, occurred at a frequency ranging from 48 to 65%, whereas *mecA* was detected at an even lower frequency (8%).

No significant differences were found in the distribution of the 12 AR genes between males and females (data not shown); analogously, no differences were seen in the distribution of these determinants based on age, with the exception of *erm*(C), which occurred with a significantly higher frequency in volunteers aged ≤ 37 (Table 3). Finally, no significant differences were observed between the two classes of volunteers who were consuming low and high amounts of the following food categories: "fruit", "vegetables", "raw/cooked pulses", "red meat", and "preserved meat" (Table 3).

For the remaining food categories, some interesting differences emerged in the distribution of a few AR genes, at a different level of significance (Table 3). Egg consumers showed a higher frequency of both *vanB* ($p<0.050$) and *tet*(K) ($p<0.050$) in their faeces compared to non-consumers, whereas volunteers who were consuming milk from vegetable sources had a significantly higher frequency of both *erm*(A) ($p<0.050$) and *erm*(C) ($p<0.050$). Moreover, volunteers who consume milk from animal sources and cheeses showed a significantly higher frequency of *tet*(K) ($p<0.001$), whereas consumers of poultry meat had a significantly higher and lower frequency of *vanB* and *tet*(S), respectively, than volunteers who do not consume items from these food categories. Finally, *vanB* occurred with a significantly higher frequency in volunteers who were consuming fish and seafood than non-consumers (Table 3).

For preserved meat, no statistically significant differences were found between consumers and non-consumers, although for *tet*(K), the higher occurrence of this determinant in consumers compared to non-consumers deserves further attention ($p = 0.054$).

3.3 Effect of diet and recruiting site on clustering of volunteers based on relative AR gene detection frequencies

3.3.1 Cluster Analysis

The 12 groups of volunteers, defined according to both the diet and recruiting site, were grouped in two primary clusters, as resulted from CA based on relative AR gene detection frequencies (Figure 1). Volunteers who were recruited from Bari clustered together in a single cluster, which also included ovo-lacto vegetarians from Bologna, whereas all the other volunteers shared a second cluster. Within the latter, three homogeneous sub-clusters could be further defined, including volunteers from Bologna, Parma, and Turin, respectively.

AR gene prevalence in samples from volunteers recruited from Bari together with ovo-lacto vegetarians recruited from Bologna was different from the other samples, as measured by principal components analysis (Figure 2, panel B). The highest eigenvector PC1 coefficients (as absolute value) were related to *erm*(A), *erm*(C), *vanA*, *tet*(K), *tet*(M) and *blaZ*, whereas *vanB*, *tet*(W), *tet*(S) and *mecA* were the most important PC2 variables. Finally, *erm*(A), *erm*(B) and *tet*(K) showed the highest PC3 eigenvector coefficients (Figure 2, panel A). In the tridimensional plot, a clear separation of omnivores and ovo-lacto vegetarians from Turin was also seen, primarily due to the PC3 scores.

3.3.2 Diet effect

An overall small effect of the diet on AR gene distribution emerged from the Pearson χ^2 tests (Table 2). The *erm*(A) gene was present only in the faeces of 5 vegans, leading to statistical significance for both orthogonal contrasts (Yate's correction was not applied). The frequency of *vanB* was slightly higher in omnivores in respect with ovo-lacto vegetarians and vegans, whereas a significant difference between vegans and ovo-lacto vegetarians was found for *tet*(K), with ovo-lacto vegetarians showing a significantly higher frequency of positive samples than vegans.

3.3.3 Recruiting site effect

Significant differences among the four recruiting sites in AR gene frequencies were detected for *erm*(B), *erm*(C), *tet*(S), *blaZ* and *mecA* (Table 4). A borderline significance (P = 0.054) was also seen for *vanB* (Table 4).

In order to identify the AR genes responsible for the differentiation between volunteers from Bari and the other three locations, as evidenced by CA and PCA, volunteers from Bologna, Parma and Turin were pooled into a single class, and comparisons with Bari were performed. Three AR genes, namely *erm*(C), *tet*(S) and *blaZ*, occurred at a significantly higher frequency in volunteers from Bari than the other three pooled sites (BO+PR+TO). As Bari was excluded from the analysis, significant differences could be further identified among the remaining three Northern Italian recruiting sites, with *vanB*, *tet*(K), *tet*(S) and *blaZ* occurring with a significantly lower frequency in subjects from Bologna, Parma, Turin and Parma, respectively and *mecA* occurring with a significantly higher frequency in subjects from Turin (Table 4).

3.3.4 Three-factor interaction

Results of χ^2 test for three-factor interaction are summarized in Table 5. A slight level of statistical significance was seen for the sole *tet*(K) gene, thus suggesting an overall negligible three-factor interaction. Supplementary Table 6 shows that in Bari omnivores were characterized by a significantly higher occurrence of *tet*(K) in respect with vegans and ovo-lacto-vegetarians; moreover, in both Parma and Turin, vegans showed a significantly lower occurrence of the same determinant in respect with ovo-lacto-vegetarians. Finally, in Bologna no significant differences were detected among diets for *tet*(K). Regarding the remaining genes, although the χ^2 test for three-factor interaction was not significant, a few significant

differences were detected by specific contrasts for *blaZ* in Bari, *tet*(S) in Bologna, and *vanB* and *tet*(K) in Parma (Supplementary Table 6).

4. Discussion

The research efforts made to gain sufficient knowledge of AR dissemination from foods have revealed widespread antibiotic resistant microorganisms in a vast range of food products, including dairy and ready-to-eat products, fish and seafood, fruit, and vegetables [**3**], thus suggesting that the human population is continuously exposed to antibiotic resistant microorganisms and their genes through dietary food intake. Very recently, the European Centre for Disease Prevention and Control (ECDC), the European Food Safety Authority (EFSA) and the European Medicines Agency (EMA) [**27**] have recently established the association between the AR phenomenon and the use of certain antibiotics in humans and food-producing animals. In these latter, antibiotics have been extensively used therapeutically as well as growth promoters (e.g. tetracyclines), thus contributing to the selection of AR genes and mobile genetic elements and to their stable establishment in the different animal species [**27**].

Accordingly, the question is whether different food habits (and hence the consumption of foods of animal or vegetal origin) might be responsible for differences in the occurrence and distribution of transferable resistances in the human gut.

Given this premise, the present study was primarily aimed at evaluating the impact of long-term omnivore, ovo-lacto vegetarian and vegan diets on the occurrence and distribution of determinants encoding for resistance to antibiotics, which are routinely used in both agriculture and human therapy, and which are commonly associated to foodborne bacteria.

The impact of the intake of specific food categories as well as other factors, including sex, age, and geographical location of the volunteers, was also investigated, whereas no assays were performed to assess the genetic environment (e.g. localization on mobile genetic elements) of the 12 potentially transferable AR genes under study.

The detection limits of all the PCR and nested-PCR assays were preliminary assessed as previously reported [**26**], thus allowing a quantification of targeted genes in the processed samples to be achieved. As expected, for those genes with a PCR detection limit of 10^7 gene copy number/g of faeces [*erm*(C), *vanA*, *tet*(O), *tet*(S), *tet*(K) and *mecA*], an increase in the number of positive samples was seen after the second round of PCRs.

When the detection frequencies of the 12 AR genes under study were evaluated along with the diet as a source of variability, a high homogeneity among the three dietary groups was seen.

Interestingly, even the analysis of the faecal microbiota of the same cohort of subjects with both traditional culturing techniques and DGGE profiling of 16S rRNA RT-PCR products by Ferrocino et al. [**19**] revealed a high level of similarity in the taxa composition of the viable faecal microbiota of the three dietary groups and only a few DGGE bands specific to each diet were detected. In the same study [**19**], the high similarity of the faecal microbiota was found by viable bacteria counting, with only a few of the investigated groups showing significant differences.

Notwithstanding this homogeneity, a few significant differences were found in the present study, such as the exclusive occurrence of *erm*(A) and the significantly lower abundance of *tet*(K) in the faeces of vegans, or the significantly higher occurrence of *vanB* in the faeces of omnivores.

A feasible explanation for these data is far from straightforward. On the one hand, they might be effectively correlated with the consumption of specific food categories, as suggested by

the Chi-squared tests; on the other hand, they might be ascribed to unexplored environmental issues, including potable and wastewater, waste management, sanitation and hygiene factors. It is known that determinants that confer resistance to the same antibiotics can be specifically associated, although not strictly confined to, a bacterial genus or a bacterial group, as acknowledged for the *erm* [**28**] and *tet* [**29**] genes.

Concerning *erm*(A), it has previously been reported that *Staphylococcus aureus* and coagulase-negative staphylococci constitute a large reservoir of this erythromycin-resistant methylase gene [**30**]. These human commensal bacteria are not plentiful in an abiotic environment, except transiently, after contact with human carriers. This evidence, combined with the habit of some vegans (commonly referred to as raw vegans) of eating raw, unprocessed (e.g. unwashed and/or unpeeled) vegetables and fruit [**31**], might explain the rare, but exclusive occurrence of *erm*(A) in the faeces of these subjects. Very recently, *erm*(A)$^+$ isolates of *S. aureus* have been collected from fresh fruit, vegetables and even ready-to-eat salads [**32, 33**], thus supporting the hypothesized origin of the *erm*(A) gene detected in the present study.

Further interesting evidence, which might in some way be correlated to specific food habits, is the higher occurrence of *tet*(K) in the faeces of volunteers who consume “eggs”, “milk from animal source”, and “cheese”. Once again, the explanation of this finding might rely on the acknowledged colonization of these food products with commensal bacteria, such as *Lactobacillus* and *Enterococcus* spp. [**4**], or even pathogens, such as *Escherichia coli*, *Salmonella* spp. and coagulase-positive cocci, notoriously carrying this determinant. In support of this hypothesis, *tet*(K)$^+$ strains ascribed to *E. coli*, *Salmonella* and *S. aureus* have recently been isolated from eggs intended for human consumption [**34**]; analogously, *tet*(K)$^+$ lactobacilli [**35, 36**] and coagulase-positive staphylococci [**36**] have recently been isolated from different cheeses. With regards to this latter food matrix, a recent research aimed at

identifying and detecting tetracycline and erythromycin resistance genes in Spanish and Italian retail cheeses revealed that traditional Italian cheeses might effectively act as a reservoir for many antibiotic resistance determinants, including *tet*(K) [**37**].

A similar explanation to that suggested for *erm*(A) and *tet*(K) might be valid for the higher occurrence of *vanB* in the faeces of volunteers who consume "eggs", "poultry meat", and "fish and seafood", given the documented occurrence of inducible vancomycin-resistant enterococci in these foods [**38**].

Additional evidence was gained by CA and PCA analyses, which clearly highlighted a geographical site-dependent rather than diet-dependent separation of volunteers, with the subjects from Bari all being grouped into a unique cluster, irrespective of their dietary habits. When the detection frequencies of AR genes in the faeces of volunteers from Bari and the other three Northern Italian sites were comparatively evaluated, a north-to-south gradient was observed, with a significantly higher occurrence of *erm*(C), *tet*(S) and *blaZ* in the faeces of subjects from Bari (Apulia) with respect to those recruited from Turin (Piedmont), Bologna and Parma (Emilia-Romagna).

Interestingly, even the analysis of the viable microbiota that occur in the faeces of the same cohort of volunteers, which was performed by a further research unit that was participating in the project, gave evidence of a correlation between the recruitment site and the faecal microbial diversity [**19**]. This finding suggested the higher impact of the foods or other sources, such as potable water, consumed by the volunteers in specific geographical locations rather than the type of diet.

If AR determinants are considered as environmental pollutants [**39**], the findings obtained in the present study can be explained by assuming that geographical areas that are sufficiently far apart, such as those chosen in this study for the enrolment of healthy volunteers, will differ in the level of contamination with specific AR genes and that these genes can be

disseminated among different bacterial species and distinct food habitats within the same area. This assumption is substantially in line with the findings reported by Forslund et al. [**40**] about the greater abundance of AR determinants in environments subjected to higher antibiotic exposure or in contact with environments in which this is the case. The north-to-south gradient in the occurrence of AR genes, which apparently emerged from the present study, evokes the reported findings of the European Centre for Disease Prevention and Control [**41**] about the occurrence of an overall north-to-south and west-to-east decreasing gradient in Europe in terms of microbial resistance percentages, as the feasible consequence of differences in infection control practices and antimicrobial use among countries [**41**]. Concerning this latter aspect, as elucidated by the Italian Medicines Agency (AIFA, Agenzia Nazionale del Farmaco) in its last Report [**42**], in the years spanning from 2002 to 2008, in Italy the consumption of antibiotics for therapeutic uses has increased from 24.5 to 27.6 DDD (Daily Doses)/1000 persons per day, posing Italy among the EU countries with the highest consumption of these drugs. In the same Report, a very high variability emerged among the 20 Italian Regions, with an increasing trend from north to south. More specifically, in the year 2008, Apulia registered a total consumption of antibiotics (34.2 DDD/1000 persons per day), which was 1.5 times higher than that of Piedmont and Emilia-Romagna (24.3 DDD/1000 persons per day), respectively. As single classes of antibiotics are concerned, the same Report [**42**] revealed that for penicillins, tetracyclines, macrolides and aminoglicosides a generally higher consumption was registered in Apulia in respect with Emilia-Romagna and Piedmont, the latter showing a comparable (for penicillins and aminoglicosides) or lower (for tetraciclines and macrolides) use of these antibiotics in human medicine than the other two regions.

Based on the third European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) Report [**43**] on sales of veterinary antimicrobial agents in 29 EU/EEA countries, in

2011 Italy had also one of the highest levels of veterinary antibiotic use in food animal production, with penicillins, tetracyclines and macrolides being among the classes of antibiotics with the highest annual sales. Unfortunately, no data have been collected on regional average consumption levels in the same EU/EEA countries.

The high use of antibiotics in both human medicine and agriculture in Italy matches very well with the results overall collected in this study, revealing a high occurrence of AR genes in the cohort assayed, with a north to south increasing gradient, that follows the high Regional variation in the therapeutic use of the same four classes of antibiotics considered.

Because the detection frequencies of the 12 tested determinants were evaluated irrespective of the diet and the recruiting site, the following final considerations could be made. More specifically, *erm*(B), *tet*(W) and *tet*(M) were the genes that were detected with the highest frequencies in the cohort assayed, with the first two being found in almost all the analyzed samples already after the first round of PCR, thus suggesting a number of gene copies per gram of faeces $\geq 10^4 - 10^6$ /g. The high abundance of these genes in human gut microbiota is in agreement with what has previously been reported by Hu et al. [**44**] in a recent large cohort study which was performed on 162 individuals from different geographical areas.

Of the above cited genes, *tet*(W) and *tet*(M) were found to prevail in the gut and oral cavity of healthy humans, respectively, followed by *tet*(O) and *tet*(Q) [**45**]. In the same study, a notably higher level of these *tet* genes were found in the faecal samples from French and Italian subjects compared with the Scandinavian and UK samples; *tet*(W) and *tet*(M), together with *tet*(O), were also detected in the faeces of healthy infants who were nourished exclusively by breastfeeding [**46**], thus suggesting their extremely broad distribution in the environment.

To date, among the *tet* genes, *tet*(W) and *tet*(M) have also been detected in various foodstuffs, including cheeses [**47**], poultry and swine [**26**], vegetables at harvest [**48**] and

even edible insects [**24, 49**]. Both these genes are associated with both foodborne bacteria and human gut commensals, including *Lactobacillus*, *Bacillus*, *Clostridium*, *Streptococcus*, *Staphylococcus* and *Enterobacter*, *Escherichia*, *Klebsiella*, *Lactobacillus*, *Lactococcus*, *Pseudomonas*, respectively [**50**], whereas *tet*(K) and *tet*(S) are commonly harboured by *Lactobacillus* (both genes) and *Lactococcus* (the latter gene) [**50**].

Among the *erm* genes, *erm*(B) was confirmed as the determinant that was most frequently found in human faecal metagenomes [**51, 52**], as well as in food [**26**]; it is harboured by numerous food-borne bacterial genera, including *Bacillus*, *Enterobacter*, *Lactobacillus*, *Micrococcus*, *Pseudomonas* [**51**]. The higher frequency of *erm*(B) compared to *erm*(A) and *erm*(C) in the human population [**52**] as well as in foods and food-associated microorganisms [**51**] might be attributed to both the association of this determinant with the high-mobility Tn916-Tn1545 family of conjugative transposons and the localization of *erm*(A) and *erm*(C) on the chromosome and small plasmids, respectively [**53**].

For the genes encoding the *vanA* and *vanB* phenotypes, a generally low occurrence was observed in the cohort assayed, irrespective of their dietary habits, thus suggesting a modest colonization of the human gastrointestinal tract with vancomycin-resistant bacteria. This assumption is consistent with what has been reported in other studies about the limited spread in the human population of vancomycin-resistant bacteria, including vancomycin-resistant enterococci (VRE) [**54**]; however, there are some other studies showing contrary results [**55-57**], with *vanB* being almost widely distributed in gut bacteria and even food-grade lactobacilli [**58, 59**].

When the effect of age and sex on the detection frequencies of the 12 AR genes was examined independent of dietary habits, no significant differences were seen, except for an apparently higher occurrence of *erm*(C) in the faeces of volunteers aged $\leq$ 37. These finding

might suggest that adult volunteers enrolled in this study were equally exposed to AR genes or organisms or to equally frequent antimicrobial usage.

5. Conclusions

Further large-size cohort studies are needed to confirm the impact of dietary habits on the AR gene richness of gut bacteria. However, the overall results collected in this study suggest that the consumption of specific food categories and even the geographical location rather than the overall dietary habit is the factor that might influence gut AR gene diversity. Furthermore, no food habits, such as a high consumption of fruit and vegetables, or, conversely, low consumption of high-protein/high-fat foods (e.g. meat or milk and cheese from animal sources), seem to be protective against the risk of harbouring AR genes in the faeces of omnivores, ovo-lacto vegetarians and vegans. This evidence is attributable, with sufficiently high probability, to the massive use of antimicrobials in human medicine as well as agriculture, as in animal husbandry and crop production, which in turn explains the increasingly high incidence of saprophytes (e.g. *Enterobacteriaceae*) and antibiotic resistant pathogens in foods from plants and animals. Indeed, in antibiotic challenged habitats, a communication system occurs, which transfer resistance traits over bacterial species and genera, and no barriers exist between pathogens and commensal bacteria concerning acquired resistances. The data overall collected suggest also the prudent use of antimicrobial compounds to prevent selection and transmission of antibiotic-resistant bacteria associated with the food chain. Moreover, increasing trends in multidrug resistance and a growing loss of efficacy of antimicrobial therapies request wide-ranging strategies targeting all health sectors.

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Author contributions

Conceived and designed the experiments: LA, CG, VM, and FC. Performed the experiments: VM and ALM. Analyzed the data: LA, ST, SP, FC, and NP. Contributed reagents/materials/analysis tools: FC, AO, LC, IF, RDC, and ST. Wrote the paper: LA, VM, and ST.

Conflict of interest

The authors have declared no conflict of interest.

Ethics statement

All participants were informed about the aims of the study and provided informed written consent. The study was approved by the Ethics Committee of (i) Azienda Sanitaria Locale (Bari) (protocol N.1050), (ii) Azienda Ospedaliera Universitaria of Bologna (protocol N.0018396), (iii) Province of Parma (protocol N.22884) and (iv) University of Torino (protocol N.1/2013/C).

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Supporting Information

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Figure legends

Figure 1. Dendrogram obtained by Cluster Analysis. The grey line identifies the arbitrary similarity threshold of 0.24.

BA=Bari; BO=Bologna; PR=Parma; TO=Turin; omn=omnivore; veg=vegetarian

Figure 1

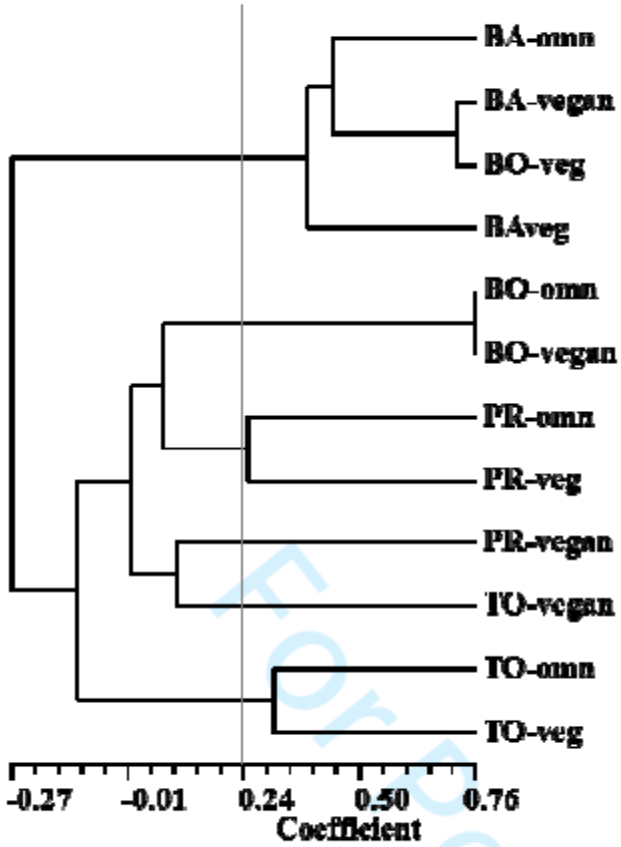
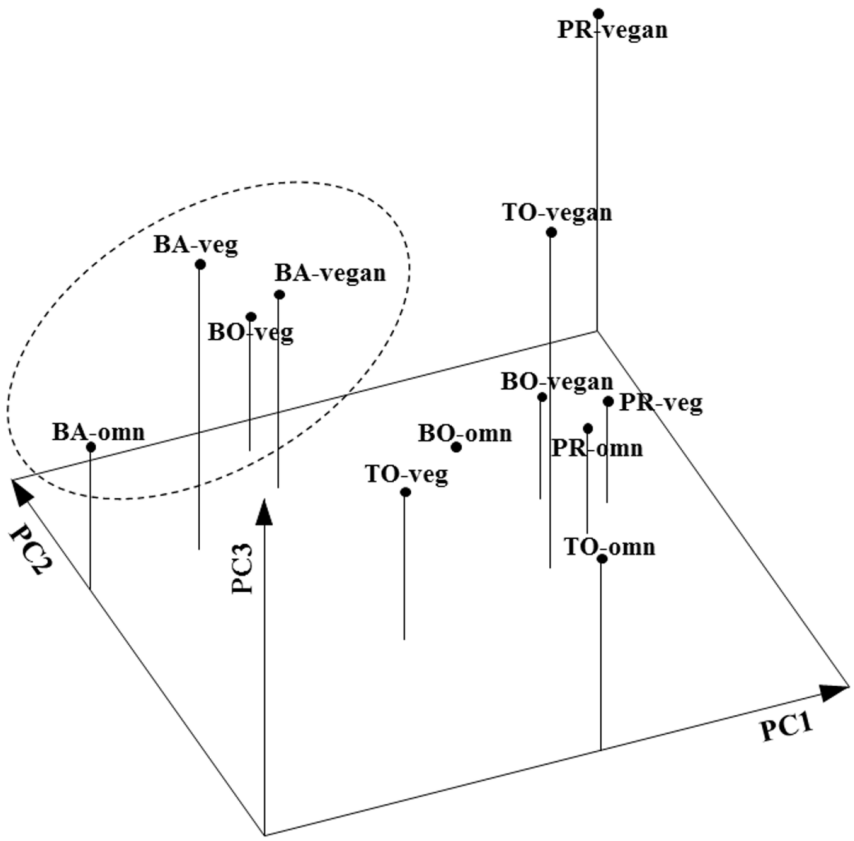


Figure 2. Results of Principal Component Analysis (PCA): panel A) eigenvalues, percentage of variance explained and eigenvectors of the principal components; panel B) PCA tridimensional plot.

A)

	Eigenvalues	Variance %	CumVar.%
PC1	3.22	26.83	
PC2	2.89	24.13	50,96
PC3	1.48	12.38	63.34
Eigenvectors	PC1	PC2	PC3
erm(A)	0.5826	0.3510	0.5314
erm(B)	-0.2289	-0.1931	0.6041
erm(C)	-0.7767	0.1725	0.3770
vanA	0.6163	-0.1922	0.1032
vanB	0.2395	-0.6186	-0.3396
tet(O)	-0.2747	-0.1304	0.1979
tet(K)	-0.6537	-0.2383	-0.6171
tet(M)	0.7137	-0.2738	0.0038
tet(W)	-0.0234	0.7335	-0.0617
tet(S)	-0.3093	0.8493	-0.0708
blaZ	-0.8094	-0.3730	0.2350
mecA	-0.1556	-0.8592	0.2937

B)



Graphical Abstract

Dietary intake has been identified as one of the primary routes for the introduction of antibiotic resistant bacteria and their genes into the human digestive tract. In the present study, the effects of long-term omnivore, ovo-lacto vegetarian and vegan diets on the occurrence of 12 genes conferring resistance to tetracyclines, macrolide-lincosamide-streptogramin B, vancomycin and β-lactams in the human gut were studied. A high impact of the geographical location on antibiotic resistance gene distribution was seen in the cohort of subjects analyzed, irrespective of their dietary habits.

Table 1. Bacterial reference strains carrying antibiotic resistance genes, used as positive controls in the PCR and nested PCR assays.

Bacterial strain	AR gene	Source
Streptococcus pyogenes 7008	*tet*(O)	DiSVA[a]
Lactobacillus casei/paracasei ILC2279	*tet*(M) and *tet*(W)	D3A[b]
Enterococcus italicus 1102	*tet*(S)	D3A[b]
Staphylococcus aureus COL.	*tet*(K)	DiSVA[a]
Staphylococcus aureus M.P.	*erm*(A)	DiSVA[a]
Enterococcus hirae Api 2.16	*erm*(B)	DiSVA[a]
Staphylococcus spp. SE12	*erm*(C)	D3A[b]
Enterococcus faecium PF3U	*vanA*	D3A[b]
Enterococcus faecalis ATCC 51299	*vanB*	ATCC[c]
Staphylococcus aureus 27R	*mecA*	D3A[b]
Staphylococcus aureus ATCC 2921	*blaZ*	ATCC[c]

[a]Culture Collection of the Department of Life and Environmental Sciences (DiSVA), Polytechnic University of Marche, Italy.

[b]Culture Collection of the Department of Agricultural, Food and Environmental Sciences (D3A), Polytechnic University of Marche, Italy.

[c]ATCC, American Type Culture Collection.

Table 2. Results of Pearson tests carried out to assess the effect of diet on detection frequency of the 12 selected AR genes in the faeces of volunteers following an omnivore, ovo-lacto-vegetarian and vegan diet.

AR gene	General Contingency Table			Orthogonal contrasts					
	Detection frequency (%)					Omnivores vs (vegans+ovo-lacto-vegetarians)		Vegans vs ovo-lacto-vegetarians	
	Omnivores n=48	Vegans n=48	Ovo-lacto-vegetarians n=48	χ2 (d.f. 2)	P	χ2 (d.f. 1)	P	χ2 (d.f. 1)	P
erm(A)	0	10	0	10.360	0.0056	2.590	0.1075	5.275	0.0216*
erm(B)	96	98	100	2.043	0.3601	1.532	0.2158	1.011	0.3148
erm(C)	15	17	27	2.6749	0.2530	1.086	0.2973	1.524	0.2170
vanA	17	25	21	1.011	0.6033	0.758	0.3840	0.236	0.6272
vanB	35	15	25	5.556	0.0622	4.167	0.0412*	1.640	0.2003
tet(O)	50	42	42	0.900	0.6376	0.900	0.3428	0.000	1.0000
tet(K)	54	27	50	8.296	0.0158*	3.175	0.0748	5.321	0.0211*
tet(M)	90	94	92	0.545	0.7613	0.409	0.5224	0.154	0.6947
tet(W)	98	100	100	2.014	0.3653	2.014	0.1559	n.a.	n.a.

tet(S)	52	60	67	2.136	0.3436	1.747	0.1863	0.405	0.5247
blaZ	65	48	58	2.776	0.2496	1.714	0.1905	1.046	0.3065
mecA	8	8	8	0.000	1.0000	0.000	1.0000	0.000	1.0000

vs versus; n. a. not applicable; d.f. degrees of freedom; * significantly different; n number of volunteers

Table 3. Results of Chi square ($\chi 2$) analysis carried out to assess the effect of age and selected food categories on detection frequency (%) of the 12 AR genes under study. For each variable, volunteers were grouped in two classes, as follows: "age": class A (age ≤ 37 years old) vs class B (age > 37 years old) ; "fruit": class A (< 267 g/day) vs B (> 267 g/day); "vegetables": class A (< 379 g/day) vs class B (> 379 g/day); "raw/cooked pulses": class A (< 57 g/day) vs class B (> 57 g/day). For the variables "eggs", "milk from animal sources" (milk_an), "milk from vegetable sources" (milk_veg), "cheese", "red meat", "poultry meat", "preserved meat", and "fish and sea food", the two classes included volunteers that eat (class YES) or do not eat (class NO) that food category. Number of volunteers within each class is reported in round brackets.

	Age			Fruit			Vegetables			Pulses			Eggs			Milk_veg		
	A (72) %	B (72) %	Sign.	A (72) %	B (72) %	Sign.	A (72) %	B (72) %	Sign.	A (72) %	B (72) %	Sign.	NO(63) %	YES(81) %	Sign.	NO (73) %	YES(71) %	Sign.
erm(A)	2.8	4.2	n.s.	2.8	4.2	n.s.	1.4	5.6	n.s.	97.2	4.3	n.s.	7.9	0.0	**	0.0	7.0	*
erm(B)	97.2	98.6	n.s.	97.2	98.6	n.s.	98.6	97.2	n.s.	18.1	98.6	n.s.	96.8	98.8	n.s.	95.9	100.0	n.s.

erm(C)	27.8	11.1	*	18.1	20.85	n.s.	18.1	20.8	n.s.	18.1	20.8	n.s.	15.9	22.2	n.s.	12.3	26.8	*
vanA	20.8	20.8	n.s.	18.1	23.6	n.s.	19.4	22.2	n.s.	18.1	23.6	n.s.	22.2	19.8	n.s.	26.0	15.5	n.s.
vanB	25.0	25.0	n.s.	29.2	20.8	n.s.	31.9	18.1	n.s.	29.2	20.8	n.s.	15.9	32.1	*	27.4	22.5	n.s.
tet(O)	43.1	45.8	n.s.	50.0	38.9	n.s.	51.4	48.6	n.s.	45.8	43.1	n.s.	42.9	45.7	n.s.	47.9	40.8	n.s.
tet(K)	44.4	43.1	n.s.	47.2	40.3	n.s.	47.2	40.3	n.s.	50.0	37.5	n.s.	33.3	51.9	*	49.3	38.0	n.s.
tet(M)	94.4	88.9	n.s.	93.1	90.3	n.s.	93.1	90.3	n.s.	91.7	91.7	n.s.	93.7	90.1	n.s.	89.0	94.4	n.s.
tet(W)	100.0	98.6	n.s.	98.6	100.0	n.s.	98.6	100.0	n.s.	98.6	100.0	n.s.	98.4	100.0	n.s.	98.6	100.0	n.s.
tet(S)	63.9	55.6	n.s.	58.3	61.1	n.s.	56.9	62.5	n.s.	59.7	59.7	n.s.	61.9	58.0	n.s.	57.5	62.0	n.s.
blaZ	52.8	61.1	n.s.	59.7	54.2	n.s.	58.3	55.6	n.s.	59.7	54.2	n.s.	52.4	60.5	n.s.	50.7	63.4	n.s.
mecA	5.6	11.1	n.s.	8.3	8.3	n.s.	9.7	6.9	n.s.	9.7	6.9	n.s.	6.3	9.9	n.s.	6.8	9.9	n.s.
	Milk_an			Cheese			Red meat			Poultry meat			Preserved meat			Fish and seafood		
	NO(72)%	YES(72) %	Sign.	NO(53	YES(91)%	Sign.	NO(1	YES(37)	Sign.	NO(10	YES(40)	Sign.	NO(99	YES(45)	Sign.	NO(103)	YES(41)	Sign.

				) %			07) %	%		4) %	%		) %				%	
erm(A)	6.9	0.0	*	9.4	0.0	**	4.7	0.0	n.s.	4.8	0.0	n.s.	5.1	0.0	n.s.	4.9	0.0	n.s.
erm(B)	98.6	97.2	n.s.	98.1	97.8	n.s.	99.1	94.6	n.s.	98.1	97.5	n.s.	99.0	95.6	n.s.	98.1	97.6	n.s.
erm(C)	19.4	19.4	n.s.	17.0	20.9	n.s.	20.6	16.2	n.s.	22.1	12.5	n.s.	21.2	15.6	n.s.	20.4	17.1	n.s.
vanA	20.8	20.8	n.s.	24.5	18.7	n.s.	21.5	18.9	n.s.	23.1	15.0	n.s.	23.2	15.6	n.s.	21.4	19.5	n.s.
vanB	20.8	29.2	n.s.	18.9	28.6	n.s.	22.4	32.4	n.s.	18.3	42.5	**	21.2	33.3	n.s.	19.4	39.0	*
tet(O)	43.1	45.8	n.s.	41.5	46.2	n.s.	43.0	48.6	n.s.	40.4	55.0	n.s.	41.4	51.1	n.s.	41.7	51.2	n.s.
tet(K)	31.9	55.6	**	28.3	52.7	**	40.2	54.1	n.s.	42.3	47.5	n.s.	38.4	55.6	#	41.7	48.8	n.s.
tet(M)	93.1	90.3	n.s.	94.3	90.1	n.s.	91.6	91.9	n.s.	92.3	90.0	n.s.	92.9	88.9	n.s.	93.2	87.8	n.s.
tet(W)	100.0	98.6	n.s.	100.0	98.9	n.s.	99.1	100.0	n.s.	100.0	97.5	n.s.	99.0	100.0	n.s.	100.0	97.6	n.s.
tet(S)	58.3	61.1	n.s.	60.4	59.3	n.s.	61.7	54.1	n.s.	65.4	45.0	*	62.6	53.3	n.s.	64.1	48.8	n.s.
bl	54.	59.	n.s.	49.	61.5	n.s.	55.	62.	n.	55.	60.	n.	53.	64.	n.s	55.	61.	n.

aZ	2	7		1			1	2	s.	8	0	s.	5	4	.	3	0	s.
mecA	8.3	8.3	n.s.	9.4	7.7	n.s.	8.4	8.1	n.s.	7.7	10.0	n.s.	8.1	8.9	n.s.	8.7	7.3	n.s.

Sign. significance; n.s. not significant; * p<0.050; ** p<0.001; # P=0.054.

Table 4. Effect of the recruiting site on AR gene frequencies evaluated by Chi square (χ2) tests, showing comparisons among the recruiting sites: Bari (BA); Bologna (BO); Parma (PR); and Turin (TO).

	BA vs BO vs PR vs TO						**BA vs (BO+PR+TO)**		**BO vs PR vs TO (BA excluded)**				
	BA (%)	BO (%)	PR (%)	TO (%)					Overall				
AR gene	n=39	n=36	n=33	n=36	χ2 (3 d.f.)	P	χ2 (1 d.f.)	P	χ2 (2 d.f.)	P	Contrast	χ2 (1 d.f.)	P
erm (A)	100.0	97.2	90.9	97.2	4.615	0.2023	1.924	0.1654	1.989	0.37	n.d.	n.d.	n.d.
erm (B)	100.0	91.7	100.0	100.0	9.191	0.0269*	1.138	0.2861	5.919	0.518	n.d.	n.d.	n.d.
erm (C)	41.0	8.3	9.1	16.7	16.87	0.0008*	15.904	P<0.0001*	1.495	0.4736	n.d.	n.d.	n.d.
vanA	10.3	25.0	24.2	25.0	3.636	0.3036	3.628	0.0568	0.007	0.9965	n.d.	n.d.	n.d.
vanB	17.9	13.9	39.4	30.6	7.644	0.054	1.418	0.2337	5.839	0.054	BO vs (PR+TO)	5.166	0.023*
tet(O)	51.3	44.4	36.4	44.4	1.611	0.6569	1.013	0.3142	0.607	0.7382	n.d.	n.d.	n.d.
tet(K)	51.3	52.8	24.2	44.4	7.201	0.0658	1.233	0.2668	6.074	0.048*	PR vs (BO+	5.557	0.0184*

											TO)		
tet(M)	84.6	88.9	100.0	94.4	6.266	0.0994	3.481	0.0621	3.948	0.1389	n.d.	n.d.	n.d.
tet(W)	100.0	100.0	100.0	97.2	3.021	0.3884	0.374	0.5408	1.935	0.38	n.d.	n.d.	n.d.
tet(S)	84.6	72.2	60.6	19.4	36.675	P<0.001*	13.778	0.0002*	22.033	P<0.0001*	TO vs (BO+ PR)	21.104	P<0.0001*
blaZ	84.6	44.4	15.2	77.8	44.356	P<0.001*	16.703	P<0.0001*	27.24	P<0.0001*	PR vs (BO+ TO)	19.205	P<0.0001*
mecA	7.7	2.8	3.0	19.4	8.509	0.0366*	0.029	0.8653	8.266	0.016*	TO vs (BO+ PR)	8.264	0.004*

vs versus; d.f. degree of freedom; n.d. not determined; n number of volunteers; * significantly different;

Table 5. Chi square (χ^2) test for three-factor interaction (AR gene x site x diet)

AR gene	χ2 (6 d.f.)	P
erm(A)	1.1616	n.s.
erm(B)	1.7601	n.s.
erm(C)	8.1463	n.s.
vanA	6.1462	n.s.
vanB	11.7063	n.s.
tet(O)	9.7773	n.s.
tet(K)	12.9847	P<0.05
tet(M)	5.5710	n.s.
tet(W)	0.5443	n.s.

tet(S)	6.9267	n.s.
blaZ	9.1663	n.s.
mecA	4.5017	n.s.

n.s. not significant; d.f. degree of freedom