Food risk associated with vegetable consumption, exposure to antimicrobial-resistant strains and pesticide residues

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Abstract

This preliminary study aimed to detect biological and chemical contaminants in vegetables sold in Sicily for human consumption, assess the spread of antimicrobial-resistant (AMR) strains in these foods, and characterize their antimicrobial-resistance genes. A total of 29 fresh and ready-to-eat samples were analyzed. Microbiological analyses were performed for the detection of *Salmonella* spp. and the enumeration of *Enterococci*, *Enterobacteriaceae,* and *Escherichia coli*. Antimicrobial resistance was assessed by the Kirby-Bauer method, according to the Clinical and Laboratory Standards Institute guidelines. Pesticides were detected by high-performance liquid chromatography and gas chromatography coupled with mass spectrometry. No samples were contaminated by *Salmonella* spp., *E. coli* was detected in 1 sample of fresh lettuce at a low bacterial count (2 log cfu/g). 17.24% of vegetables were contaminated by *Enterococci* and 65.5% by *Enterobacteriaceae* (bacterial counts between 1.56 log cfu/g and 5.93 log cfu/g and between 1.6 log cfu/g and 5.48 log cfu/g respectively). From 86.2% of vegetables, 53 AMR strains were isolated, and 10/53 isolates were multidrug resistant. Molecular analysis showed that the *blaTEM* gene was detected in 12/38 β-lactam-resistant/intermediate-resistant isolates. Genes conferring tetracycline resistance (*tetA*, *tetB*, *tetC*, *tetD*, *tetW*) were detected in 7/10 isolates. The *qnrS* gene was detected in 1/5 quinolone-resistant isolates, the *sulI* gene was detected in 1/4 sulfonamide-resistant/intermediate-resistant isolates and the *sulIII* gene was never detected. Pesticides were detected in 27.3% of samples, all of which were leafy vegetables. Despite the satisfactory hygienic status of samples, the high percentage of AMR bacteria detected stresses the need for an effective monitoring of these foods as well as adequate strategies to counteract the spread of AMR bacteria along the agricultural chain. Also, the chemical contamination of vegetables should not be underestimated, especially considering that leafy vegetables are commonly consumed raw and that no official guidelines about maximum residue limits of pesticides in ready-to-eat vegetables are available.

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Introduction

Due to the misuse of antibiotics in veterinary and human medicine, antimicrobial resistance (AMR) is rapidly spreading worldwide (Ferri *et al.*, 2017) and threatens to outrun the rate at which new antimicrobials are developed (Morrison and Zembower, 2020). For these reasons, AMR is included among the 10 major threats to human health according to the World Health Organization (2021). Currently, drug-resistant infections cause at least 700,000 deaths annually and, if no action is taken, that figure is predicted to increase to 10 million deaths per year by 2050, surpassing diabetes, heart disease, and cancer as the leading cause of death in humans (Morrison and Zembower, 2020). Also, the use and misuse of pesticides in agriculture have increased in the last decades, especially in developing countries, where the indiscriminate use of pesticides for pest and disease control is combined with a lack of knowledge of their correct use and non-adherence to pesticides' pre-harvest intervals (Kiwango *et al.*, 2018). This situation has a severe impact on ecosystems and documented deleterious effects on animals (Garcês *et al.*, 2020) and humans. In fact, poisoning from pesticides accounts for nearly 300,000 deaths worldwide every year and dietary overexposure to pesticide residues has been associated with risks of developing cancer, genetic and immune system defects, neurodegenerative disorders, and hormon-

al dysfunctions in both women and men (Kiwango *et al*., 2018; Sabarwal *et al.*, 2018).

References report the key role played by soil as a reservoir of antimicrobial-resistant strains (Zhang *et al*., 2019) and pesticides (Tudi *et al.*, 2021). Since vegetables are commonly consumed raw or following mild treatments, these foods can be carriers of biological and chemical pollutants to humans. Our investigation aimed to provide data regarding the biological and chemical contamination in vegetables sold in Sicily for human consumption, assess the spread of AMR strains in these foods and characterize their AMR genetic profile.

Materials and Methods

Sample collection

A total of 29 samples were collected from Sicilian markets, maintained at 4°C, and analyzed within 48 hours from sampling. Samples included both fresh and ready-to-eat (RTE) products, to evaluate any differences in terms of biological and chemical contamination resulting from the corresponding pre-market treatment processes. Fresh vegetables included 8 leafy vegetables, 5 fruit vegetables, 2 bulb vegetables, and 2 flower vegetables. RTE vegetables included 10 leafy vegetables and 2 mixed salads containing leafy vegetables as the major component and traces of carrots.

Bacteriological analyses

Salmonella spp. isolation was performed according to ISO 6579-1 (2017a). Presumptive colonies were screened for biochemical characterization, performed following the API 20E identification system (BioMerieux, Marcy l'Etoile, France).

For *Enterococci* enumeration, samples were processed using the following self-developed method: 30 g of vegetables were diluted 1/10 (w/v) in peptone salt solution and serial dilutions were prepared. 1 mL of each dilution was plated in rapid enterococcus agar (Oxoid, Milan, Italy) by the pour plate method. Following 44 hours of incubation at 44°C, presumptive colonies were characterized by gram staining/microscopy, catalase test, and esculin hydrolysis test. Colonies were counted from plates containing <150 colonies and the microbial load was calculated and expressed as log cfu/g.

Enterobacteriaceae enumeration was performed according to ISO 21528-2 (2017b). Colonies were counted and the microbial load was calculated and expressed as log cfu/g.

β-glucuronidase positive *E. coli* enumeration was performed according to ISO 16649-2 (2010), and the microbial load was calculated and expressed as log cfu/g.

Following the biochemical characterization of contaminating specimens, performed by the appropriate API identification system (BioMerieux, Marcy l'Etoile, France), their antimicrobial resistance profile was assessed by the Kirby-Bauer method. The following 13 antimicrobials were used: ampicillin (AMP; 10 µg), amoxicillin/clavulanic acid (AMC; 20 µg/10 µg), cefotaxime (CTX; 30 µg), ceftazidime (CAZ; 30 µg), kanamycin (K; 30 µg), gentamicin (CN; 10 µg), streptomycin (S; 10 µg), trimethoprim/sulfamethoxazole (STX; 25 µg), ciprofloxacin (CIP; 5 µg), nalidixic acid (NA; 30 µg), tetracycline (TE; 30 µg), chloramphenicol (C; 30 µg) and imipenem (IMP; 10 µg). Inhibition zones were measured and interpreted according to the guidelines of the Clinical Laboratory Standards Institute (CLSI, 2021).

Molecular analyses

Molecular analyses were performed on 38/53 AMR strains. Among those, antimicrobial-resistance genes (ARGs) were searched only in those strains that were found to be resistant or intermediate resistant to the corresponding antibiotics by the Kirby-Bauer method. The cell lysate of each isolate was used as a template in a polymerase chain reaction (PCR) to detect ARGs. Cell lysate of each isolate was prepared by boiling single colonies for 15 min at 99°C in 100 µl of distilled sterile water. After sample centrifugation at 10,000 g for 10 minutes, the supernatant was recovered and stored at -20°C. Cell lysate from all bacterial isolates was used to detect the following antimicrobial resistance genes: *tetA*, *tetW*, *tetC*, *tetD*, *tetB* (tetracycline), *qnrS* (quinolones), *sulI*, *sulIII* (sulfonamides) and *blaTEM* (β-lactams) in PCR reactions using primers reported in Table 1 (Lynne *et al*., 2008; Ahmed *et al*., 2013; Marti and Balcázar, 2013; Coy *et al*., 2014; Sucato *et al*., 2021).

DreamTaq DNA polymerase (Thermo Fisher Scientific) was used following the manufacturer's instructions. The thermal profile used was: 95°C for 2 minutes; 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55-64°C (gene-dependent) for 30 seconds, elongation at 72°C for 30 seconds; final elongation at 72°C for 5 minutes. The DNA amplicons were detected by electrophoresis analysis using polyacrylamide gels (6% w/v) or agarose gel (1-2% w/v) run in 1X Tris-borate-EDTA buffer at 100V and then stained in a bath containing 40 ml of 1X Tris-borate-EDTA buffer added with 3 µl of 10 mg/mL ethidium bromide. In a control experiment, genomic DNA was used to amplify a 1500 bp fragment corresponding to the 16S rDNA using the primers reported in Table 1 (Lynne *et al*., 2008; Ahmed *et al*., 2013; Marti and Balcázar, 2013; Coy *et al*., 2014; Sucato *et al*., 2021).

Chemical analyses

Chemical analyses were performed on 22/29 samples, for the detection of 140 pesticides belonging to the following 7 classes: organochlorines, organophosphates, ureic and carbamic derivatives, pyrethroids, carbamates, triazine compounds. Pesticide extraction and clean-up were done following the QuEChERS Protocol (AOAC, 2007). Pesticides were processed by liquid chromatography and gas chromatography coupled with mass spectrometry, according to UNI EN 15662 (2018), as described before (Calvaruso *et al*., 2020). The analytical performance for all the analytes considered was evaluated by the determination of selectivity, linearity, sensibility, and recovery. The linearity of each analyte was tested by the regression model of the determined calibration data set, showing r2 values >0,9997. The limit of detection and the limit of quantification for each analyte were calculated according to the criteria reported before (Calvaruso *et al.*, 2020). The recoveries intraday repeatability were estimated by spiking blank samples at 2 concentration levels (0.01 and 0.02 mg/kg) according to guidance SANTE 11312 (Pihlström *et al.*, 2021) - analytical quality control and method validation procedures for pesticide residues analysis in food and feed - showing values between 90 and 115% and between 0.00025 and 0.00222, for recovery and repeatability, respectively.

Results

Bacteriological analyses

No sample was contaminated by *Salmonella* spp, while *E. coli* was detected in one sample of fresh lettuce with a low bacterial count (2 log cfu/g). 17.24% of vegetables were contaminated by *Enterococci* at microbial loads between 1.56 log cfu/g and 5.93 log cfu/g. The sample was found to be contaminated by *E. coli* and all samples found to be contaminated by *Enterococci* were fresh vegetables. 65.5% of vegetables were contaminated by *Enterobacteriaceae* and 57.9% of them were fresh vegetables. The microbial loads recorded for *Enterobacteriaceae* were between 1.6 log cfu/g and 5.48 log cfu/g, with comparable values between fresh and RTE vegetables (Table 2). 86.2% of vegetables were found to be contaminated by AMR strains and, overall, 53 AMR strains were isolated, 10 of which were multidrug resistant (MDR) (strains featuring resistance to antibiotics belonging to at least three different classes). Among them, we isolated 2 *Pseudomonas fluorescens*, 2 *Pseudomonas aeruginosa*, 4 *Klebsiella pneunomoniae*, 2 *Klebsiella oxytoca.* 69.8% of AMR strains and 100% of MDR strains were isolated from fresh vegetables. Resistance to AMP was the one most frequently revealed (94.23%), followed by AMC (61.53%), TE (23.08%), and K (19.23%). Data are shown in Figure 1.

Molecular analyses

Referring to β-lactams-resistant strains, the *blaTEM* gene was detected in 12/38 isolates, including 1/2 strains showing intermediate resistance to the β-lactams tested, *i.e.* 3 *Enterobacter cloacae, Acinetobacter,* 3 *Citrobacter freundii, Hafnia halvei*, *Providencia rettgeri, Pseudomonas aeruginosa, Klebsiella pneumoniae, Klebsiella oxytoca*. The *tetA* gene was detected in 2/10 tetracycline-resistant strains tested (*E. coli* and *K. pneumoniae*); the *tetW* gene was detected in 1/10 strains (*E. cloacae);* the *tetC* gene was detected in 1/10 strains (*Pseudomonas fluorescens*); the *tetD* gene was detected in 1/10 strains (*Morganella morganii*); the *tetB* gene was detected in 1/10 strains (*Pseudomonas aeruginosa*) that also carried the quinolones and sulfamides resistance genes *qnrS* and *sulI,* respectively. The *sulIII* gene was not present in any sulfonamide-resistant strains. The ARGs detected in the isolated strains and their source food are enlisted in Table 3.

Chemical analyses

Pesticide residues were over the limit of detection in 6/22 analyzed samples. The majority of the contaminated sample (4/6) were RTE vegetables. In particular, 1 fresh leafy vegetable (spinach) contained deltamethrin at concentration 0.37±0.19 mg/Kg, 1 fresh leafy vegetable (lettuce) contained cyhalothrin, lambda at concentration 0.028±0.014 mg/Kg, 1 RTE sample (leafy vegetables) contained Spinosad at concentration 1.7±0.85 mg/Kg,

Table 1. List of the primers used in this study.

Gene	Primer sequence (5'-3')	Amplicon size (bp)	Ta (°C)	References
tetA	F-GCTACATCCTGCTTGCCTTC R-CATAGATCGCCGTGAAGAGG	210	64	(Sucato *et al*., 2021)
tetW	F-ACATCATTGATACTCCAGGTCACG R-TTTCACTTTGTGGTTGAACCCCTC	120	59	(Sucato *et al*., 2021)
tetB	F-TTGGTTAGGGGCAAGTTTTG R-GTAATGGGCCAATAACACCG	659	60	(Lynne *et al*., 2008)
tetC	F-CTTGAGAGCCTTCAACCCAG R-ATGGTCGTCATCTACCTGCC	418	62	(Lynne *et al*., 2008)
tetD	F-AAACCATTACGGCATTCTGC R-GACCGGATACACCATCCATC	787	62	(Ahmed *et al*., 2013)
blaTEM	F-TTCCTGTTTTTGCTCACCCAG R-CTCAAGGATCTTACCGCTGTTG	112	60	(Sucato *et al*., 2021)
qnrS	F-GACGTGCTAACTTGCGTGAT R-TGGCATTGTTGGAAACTTG	118	56	(Marti and Balcázar, 2013)
sulI	F-TCACCGAGGACTCCTTCTTC R-AATATCGGGATAGAGCGCAG	316	60	(Lynne *et al*., 2008)
sulIII	F-GAGCAAGATTTTTGGAATCG R-CATCTGCAGCTAACCTAGGGCTTGGA	799	58	(Lynne *et al*., 2008)
16S	F-GAGTTTGATCCTGGCTCAC R-ACGGCTACCTTGTTACGACT	1500	55	(Coy *et al*., 2014)

Ta, annealing temperature.

Table 2. Recorded percentages of samples contaminated by *Enterobacteriaceae* and microbial loads.

Contaminated samples (%)	Microbial loads (log cfu/g) mean±SD
Fresh vegetables	2.8±0.71
Ready-to-eat vegetables	2.69±1.34

SD, standard deviation.

Table 3. Summary of the resistance genes detected by a polymerase chain reaction in the isolated strains.

Strain	Source	Resistance (R), intermediate resistance (I)	ARGs				
			tet (*A,B,C,D,O,W*)	*qnrS*	*sulI*	*sulIII*	*blaTEM*
E. cloacae	leafy	R: AMP10, AMC30	NA	NA	NA	NA	-
E. coli	leafy	R: AMP10, AMC30, TE30	tetA	NA	NA	NA	-
H. alvei	bulb	R: AMP10, AMC30 I: CAZ30	NA	NA	NA	NA	-
H. alvei	leafy	R: AMP10, AMC30 I: CAZ30	NA	NA	NA	NA	+
E. cloacae	leafy	R: AMP10, AMC30	NA	NA	NA	NA	+
E. cloacae	leafy	R: AMP10, AMC30, STX25, TE30, C30 I: K30, NA30	tetW	-	-	-	-
P. fluorescens	leafy	R: AMP10, AMC30, CTX30, NA30, TE30, C30	tetC	-	NA	NA	-
Acinetobacter	leafy	R: AMP10, AMC30	NA	NA	NA	NA	+
C. freundii	fruit	R: AMP10, AMC30	NA	NA	NA	NA	+
M. morganii	fruit	R: AMP10, AMC30, TE30, C30	tetD	NA	NA	NA	-
P. aeruginosa	fruit	R: AMP10, AMC30, K30, CN10, S10, STX25, NA30, TE30, C30 I: CTX30, CAZ30	tetB	+	+	-	-
E. cloacae	leafy	R: AMP10 I: AMC30	NA	NA	NA	NA	+
C. freundii	leafy	I: AMP10, AMC30	NA	NA	NA	NA	+
P. rettgeri	leafy	R: AMC30, TE30	-	NA	NA	NA	+
C. freundii	fruit	R: AMP10, AMC30	NA	NA	NA	NA	+
P. aeruginosa	fruit	R: AMP10, AMC30, K30, CN10, S10, STX25, NA30, TE30, C30 I: CTX30, CAZ30	-	-	-	-	+
E. cloacae	fruit	R: AMP10, AMC30	NA	NA	NA	NA	-
E. cloacae	leafy	R: AMP10, AMC30	NA	NA	NA	NA	+
K. pneumoniae	leafy	R: AMP10, K30, CN10, S10, TE30	tetA	-	-	-	+
K. oxytoca	flower	R: AMP10, S10 I: K30, CN10	NA	NA	NA	NA	+
Citrobacter	fruit	R: K30; I: AMP10	NA	NA	NA	NA	-
K. pneumoniae	fruit	R: AMP10, AMC30 I: S10	NA	NA	NA	NA	-
Enterobacter (EMP)	fruit	R: AMP10, K30 I: S10	NA	NA	NA	NA	-
K. pneumonieae	fruit	R: AMP10 I: S10	NA	NA	NA	NA	-
E. cloacae	fruit	I: AMP10, AMC30, K30, S10	NA	NA	NA	NA	-
P. stuartii	leafy	R: AMP10, AMC30, S30, TE30 I: K30	-	NA	NA	NA	-
E. cloacae	leafy	R: AMP10, AMC30, S30, TE30	tetD	NA	NA	NA	-
E. cloacae	leafy	R: AMP10, AMC30	NA	NA	NA	NA	-
E. cloacae	leafy	R: AMP10, AMC30, NA30, C30 I: STX25, CIP5	NA	-	-	-	-
R. ornithinolytica	leafy	R: AMP10	NA	NA	NA	NA	-
C. freundii	leafy	R: AMP10 I: AMC30	NA	NA	NA	NA	-
Pantoea spp4	leafy	R: AMP10	NA	NA	NA	NA	-
P. fluorescens	leafy	R: AMP10, AMC30, NA30 I: CAZ30	NA	-	NA	NA	-
K. pneumoniae spp *ozaenae*	leafy	R: AMP10, K30	NA	NA	NA	NA	-
K. oxytoca	leafy	R: AMP10	NA	NA	NA	NA	-
C. youngae	leafy	R: AMP10, K30	NA	NA	NA	NA	-
E. coli	leafy	R: AMP10	NA	NA	NA	NA	-
R. aquatilis	leafy	R: AMP10, K30	NA	NA	NA	NA	-

AMP10, ampicillin 10 µg; AMC30, amoxicillin/clavulanic acid 30 µg; TE30, tetracycline 30 µg; CAZ30, ceftazidime 30 µg; STX25, trimethoprim/sulfamethoxazole 25 µg; C30, chloramphenicol 30 µg; K30, kanamycin 30 µg; NA30, nalidixic acid 30 µg; CN10, gentamicin 10 µg; S10, streptomycin 10 µg; CIP5, ciprofloxacin 5 µg; CTX30, cefotaxime 30 µg; ARGs, antimicrobial-resistance genes; NA, not applicable. Clinical and Laboratory Standards Institute zone diameter breakpoints (mm): AMP10 R≤13, 14<I<16, S≥17; AMC30 R≤ 13, 14<I<17, S≥18; CTX30 R≤ 22, 23<I< 25, S≥26; CAZ30 R≤17, 18<I< 20, S≥21; K30 R≤13, 14<I<17, S≥18; CN10 R≤12, 13<I<14, S≥15, S10 R≤11, 12<I<14, S≥16; STX25 R≤10, 11<I<15, S≥16; CIP5 R≤21, 22<I<25, S≥26; NA30 R≤13, 14<I<18, S≥19; TE30 R≤11, 12<I<14, S≥15, C30 R≤12, 13<I<17, S≥18; IPM10 R≤19, 20<I<22, S≥23. Limited to *P. aeruginosa*: CIP5 R≤18, 19<I<24, S≥25; IPM10 R≤15, 16<I<18, S≥19. Limited to *Acinetobacter*: CTX30 R≤14, 15<I<22, S≥23, CAZ30 R≤14, 15<I<17, S≥18; CIP5 R≤15, 16<I<20, S≥21; IPM10 R≤18, 19<I<21, S≥22. + indicates the presence of amplicon; – indicates the absence of amplicon. NA indicates that molecular analyses were not carried out as the strains were sensitive to the Kirby-Bauer test. The *tet* gene name indicates the antimicrobial-resistance genes detected in each positive sample.

1 RTE sample (leafy vegetables) contained Spinosad at concentration 0.02±0.01 mg/Kg, 1 RTE sample (leafy vegetables) contained fludioxonil and mandipropamid at concentration 0.932±0.367 mg/Kg and 1.2±0.6 mg/Kg respectively, 1 RTE sample (leafy vegetables) was contaminated by 6 different chemicals from 2 different classes: fludioxonil, mandipropamid, zoxamide and cyprodinil (fungicides) at concentration 0.48±0.24 mg/Kg, 0.72±0.36 mg/Kg, 0.92±0.46 mg/Kg and 1.1±0.05 mg/Kg respectively, spinosad and acetamiprid (insecticides) at concentration 0.16±0.08 mg/Kg and 0.032±0.016 mg/Kg respectively.

Discussion

The last decade has witnessed an increase in the consumption of fresh and RTE vegetables, due to a change in eating habits arising from healthier lifestyle choices. Despite being rich in vitamins, minerals, and phytonutrients (Losio *et al.*, 2015), these foods can easily vehiculate biological and chemical pollutants to humans, especially when consumed raw or following mild treatments. Nevertheless, limited data are published about the microbial safety (Pesavento *et al.*, 2014; Losio *et al.*, 2015) and pesticide contamination (Tasiopoulou *et al.*, 2007) of vegetables commercialized in Italy for human consumption. Moreover, recent studies demonstrate that agricultural soil is a source of antibiotic-resistant genes, that could be transferred into vegetables entering into the food chain (Zhang *et al.*, 2019). To date, the mechanism of transmission of ARGs in the soil-plant system is not clear. In this study, we assessed the biological and chemical contamination of vegetables collected from the Sicilian markets, demonstrated the presence of antibiotic resistant strains, as shown by the Kirby-Bauer method, and searched for their ARGs by PCR. Data from our preliminary study confirm that leafy vegetables are more commonly contaminated compared to other vegetables (O'Flaherty *et al*., 2019), probably because their morphology or type of production favors the colonization and permanence of soil bacteria on leaf surfaces. Also, similarly to others, we observed that microbiological contamination occurs more frequently in fresh vegetables than in RTE vegetables (Pesavento *et al.,* 2014; Losio *et al.*, 2015). Despite the satisfactory hygienic status of the analyzed vegetables, a high percentage of fresh foods were contaminated by AMR bacteria. The highest percentages of resistance found against β-lactam antibiotics (Amp 94.23% and Amc 61.54%) can be attributed to the wide use of these antibiotics in human medicine and therefore to a greater release and accumulation in the environment (Alduina, 2020). A relevant percentage of resistance was also observed against TE (23.08%). The spread of this antimicrobial resistance is mainly due to the extensive use of this antibiotic as a growth promoter in intensive farming (Fontana *et al.,* 2021). In 2006, the European Union banned antibiotic addition in animal feeds, such as TE, in an attempt to reduce the spread of AMR bacteria (Fontana *et al.,* 2021), nevertheless, antibiotic residuals are still present and affect the environment. This suggests that more prudent use of antibiotics should be encouraged also in other sectors and that the multiple reasons for their spread should be further investigated. Genes that confer resistance to tetracycline have been found in *E. coli* (*tetA, tetW*), *Klebsiella pneumoniae* (*tetA*), *Pseudomonas fluorescens* (*tetB, tetC*), *Morganella morganii* (*tetD*). The genes that confer resistance to quinolones (*qnrS*) and sulfamides (*sulI*) have been found only in *Pseudomonas aeruginosa*, one out of the four resistant strains observed through the Kirby-Bauer method. Finally, *blaTEM*, a gene that confers resistance to β-lactams, was detected in *Hafnia alvei, Enterobacter cloacae, Acinetobacter, Citrobacter freundii, Providencia rettgeri, Pseudomonas aeruginosa, Klebsiella pneumoniae, Klebsiella oxytoca*. This study recorded the most frequent occurrence of ARGs for TE, as well as for β-lactams, as described in other reports that considered raw vegetables as an important source of bacteria resistant to these antibiotics (Al-Kharousi *et al.*, 2019; Romyasamit *et al.*, 2021).

The detection in vegetables of potential pathogens, such as *P. aeruginosa*, and opportunistic pathogens, such as *K. pneumoniae*, characterized by multiple resistances, is further evidence of the potential risk associated with the consumption of vegetables and the need for adequate control of these foods. In fact, most of the vegetables analyzed in this study are commonly consumed raw and can be eaten without heat treatment. The spread of AMR strains in foods of plant origin is worrying not only because the intake of food contaminated by AMR bacteria and the consequent interaction with the intestinal microbiota can favor the horizontal transfer of ARGs to bacteria, with unpredictable deleterious effects on the health of the host; but also because their spread among vegetables reflects their spread at soil level and therefore at the environmental level, hence acquiring a relevant meaning under the One Health perspective. In fact, the growing spread of AMR and MDR among bacteria affects also the pathogenic specimens, which can cause infections increasingly difficult to treat in both human and veterinary medicine.

Differently from bacteriological analyses, chemical analyses suggest that pesticides can be detected more frequently in RTE vegetables compared to fresh vegetables. Similar results were described in two studies conducted on vegetables sampled in the Campania region (Arienzo *et al.*, 2013) and on green leafy vegetables collected in all 20 Italian regions (Santarelli *et al.*, 2018) respectively, both highlighting the need for greater attention on these issues. For this reason, our results should not be underestimated despite coming from preliminary analyses, especially considering that these foods are commonly consumed raw, without any preliminary washing by the consumers and taking into account the risk associated with the phenomenon of bioaccumulation resulting in long-term toxic effects on the health of the consumer

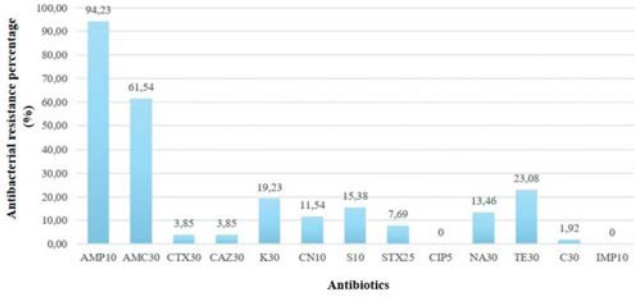

Figure 1. Bar graph depicting the antibacterial resistance percentage to the tested antibiotics. AMP10, ampicillin, 10µg; AMC30, amoxicillin/clavulanic acid, 20 µg /10 µg; CTX30, cefotaxime, 30 µg; CAZ30, ceftazidime, 30 µg; K30, kanamycin, 30 µg; CN10, gentamicin, 10 µg; S10, streptomycin, 10 µg; STX25, trimethoprim/sulfametoxazole, 25 µg; CIP5, ciprofloxacin, 5µg; NA30, nalidixic acid, 30 µg; TE30, tetracycline, 30 µg; C30, chloramphenicol, 30 µg; IMP10, imipenem,10 µg.

(Kiwango *et al.*, 2018; Sabarwal *et al.*, 2018). In fact, the increasing trend of multi-residual samples is also confirmed by other reports (Arienzo *et al.*, 2013), as well as the rather scarce knowledge about the health risks of cumulative exposure to pesticides (Arienzo *et al.*, 2013) and the inadequacy of MRLs as reference limits for safety evaluation of these foods (Santarelli *et al.*, 2018).

Here, we reported lower percentages of pesticide contamination both in fresh and RTE vegetables compared to other studies conducted in under-developed countries such as Morocco (Choubbane *et al.*, 2022) and Chile (Elgueta *et al.*, 2019) respectively. This difference can probably be attributed to a combination of indiscriminate use of pesticides for pest and disease control, a lack of knowledge of their correct use, and non-adherence to pesticides' pre-harvest intervals (Kiwango *et al.*, 2018), more pronounced in undeveloped countries compared to the developed ones. As overall our results stress the need for effective monitoring of these foods as well as adequate strategies to counteract the spread of AMR bacteria along the agricultural chain. Also, chemical contamination of vegetables should not be underestimated, especially considering that those vegetables found to be contaminated are commonly consumed raw and that currently, no official guidelines are available about the maximum residue limits (MRLs) for pesticides in RTE mixed vegetables, although the need for a greater clarity has already been required by professionals in the chemical control of food and the European Food Safety Authority is working on developing more adequate official guidelines. Finally, the identification of samples contaminated by various pesticides from several different classes should encourage further studies, since the interactions between these molecules and the effects that may derive on human health are unknown.

Conclusions

The official guidelines about safety criteria for vegetable foods are not exhaustive. In fact, the Commission Regulation (EC) 2073 (European Commission, 2005a) sets legal microbiological criteria only for RTE vegetables and solely refers to *E. coli*, coagulase-positive *Staphylococci*, *Salmonella* spp., *Listeria monocytogenes,* and Verotoxigenic *Escherichia coli*, lacking any indications about both fresh vegetables and the assessment of AMR bacteria in fresh and RTE vegetables. Similarly, the Commission Regulation (EC) 396 (European Commission, 2005b) sets the MRLs of pesticides in or on food and feed of plant origin, with clear indications solely referring to fresh vegetables, while official guidelines about the definition of MRLs for RTE mixed vegetables are lacking. Data from our preliminary study highlighted higher percentages of microbiological contamination and AMR bacteria in fresh vegetables compared to RTE vegetables and opposite results for pesticide contamination. These data highlight the need to fill in the legislative gap that affects the current official guidelines for the bacteriological and chemical safety assessment of these foods. Also, consumers' awareness on this subject should be raised and they should be encouraged to eat vegetables following adequate treatment, such as careful wash, and prefer cooking.

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