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Research Paper

Characterization of antibiotic resistance in *Salmonella enterica* isolates determined from ready-to-eat (RTE) salad vegetables

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Abstract

In the last decade, ready-to-eat (RTE) salad vegetables are gaining increasing importance in human diet. However, since they are consumed fresh, inadequate washing during processing can bring on some foodborne illnesses, like salmonellosis, since these food items have natural contamination from soil and water. During 2009-2010, a total of 81 samples were purchased arbitrarily from local markets in Ankara, and were examined for *Salmonella* contamination. *Salmonella* screening was performed by using anti-*Salmonella* magnetic beads system and polymerase chain reaction (PCR) identification of the suspected colonies. Then, the antibiotic resistance profiles of four *Salmonella* strains identified (strains RTE-1, RTE-2, RTE-3, and RTE-4) were also investigated, since the mechanism by which *Salmonella* spp. have accumulated antibiotic resistance genes is of interest. All strains showed resistance against sulfonamides (MIC > 128 mg/L). Further results suggested that associated sulfonamide resistance genes were encoded by the 55.0 kb plasmid of strain RTE-1 that involves no integrons. As a result of using two primers (P1254 and P1283) in randomly amplified polymorphic DNA-PCR (RAPD-PCR) analysis, two common amplicons (364 bp and 1065 bp) were determined. The findings of this study provide support to the adoption of guidelines for the prudent use of antibiotics in order to reduce the number of pathogens present on vegetable and fruit farms. Besides, since it is shown that these bacteria started to gain resistance to antibiotics, it is necessary to further investigate the prevalence of them in foods.

Key words: contamination, plasmid-mediated sulfonamide resistance, ready-to-eat salad vegetables, food safety, *Salmonella*.

Introduction

Ready-to-eat (RTE) food is defined by EC Regulation No. 2073/2005 as the food intended by the producer or the manufacturer for direct human consumption without the need of cooking or other processing effective to eliminate or reduce to an acceptable level microorganisms of concern (European Commission Regulation-EC No 2073, 2005). Among these RTE food types, RTE salad vegetables have been gaining importance due to arise of presence and prevalence of pathogen bacteria (Beuchat 1996; Wells and Butterfield 1997; de Curtis *et al.*, 2002; Fröder *et al.*, 2007; Little *et al.*, 2007).

Since RTE salad vegetables are generally considered healthy to eat because of their vitamin, mineral, and antioxidant contents, their consumption in modern life has increased both in quantity and variety in recent years due to this increasing demand of consumers (de Giusti *et al.*, 2010). With this increase in consumption of RTE salad vegetables, it also comes with the increased potential for exposure to foodborne pathogens through this food chain. These foods may receive some degree of minimal technological processing before commercial distribution. However, since they are consumed fresh, inadequate washing during processing can bring on some foodborne illnesses like salmonellosis, since these food items have natural contamination

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from soil and water (Abadias *et al.*, 2008). According to the foodborne diseases outbreaks report, which was released by the Public Health Laboratory Service (PHLS) Communicable Disease Surveillance Centre (CDSC) during 1992-2000, among 1 518 foodborne general outbreaks of infectious intestinal disease, 83 (5.5%) were associated with the consumption of salad vegetables or fruit, and *Salmonella* spp. were the most frequently reported (41.0%) pathogens (Long *et al.*, 2002; Ward *et al.*, 2002).

EC Regulation No. 2073/2005 also contains food safety criteria for *Salmonella* in precut vegetables, herbs and fruits placed on the market. *Salmonella* spp. should be absent in all RTE salad vegetables, fresh herbs and fruits (Little and Gillespie, 2008).

A study of retail bagged RTE salad vegetables carried out during 2001 uncovered an outbreak of *S*. Newport PT33 (Sagoo *et al.*, 2003). Five of 3853 bagged RTE salad vegetable samples in 2001 were contaminated with *S*. Newport PT33 (one sample), *S*. Umbilo (three samples) and *S*. Durban (one sample), which indicates an important health risk. Nineteen cases of *S*. Newport PT33 infection were subsequently identified throughout England and Wales (Ward *et al.*, 2002).

The emergence of antibiotic resistant foodborne pathogens is another public health concern. Several organizations, including the World Health Organization (WHO) and Centers for Disease and Prevention (CDC), have all stressed the need to control the spread of this resistance (Angulo *et al.*, 2000). The levels of resistance are varied and influenced by antimicrobial use in vegetables and fruits, as well as the geographical differences.

The aims of this study were to evaluate the presence of *Salmonella* in RTE salad vegetables, and also to characterize the antibiotic resistance of *S. enterica* isolates.

Materials and Methods

Eighty one RTE salad vegetable samples were arbitrarily purchased from several markets and retail premises at the point of all over the city Ankara, Turkey, during 2009-2010. RTE salad vegetable samples represent different mixtures of greens and other vegetables found at the markets and retail premises at the moment of sampling hence the number of samples was also obtained arbitrarily. *Salmonella* screening in each RTE salad vegetable sample was performed by using anti-Salmonella magnetic beads (Dynal®, Norway) system as an alternative to the selective enrichment step in the conventional determination method (ISO 6579:2002), and the suspected colonies were further identified by the optimized polymerase chain reaction (PCR) method (Mercanoglu Taban *et al.*, 2009). Only four isolates were found as *Salmonella* by PCR.

Plasmids of these 4 *Salmonella* strains were isolated using the method of Kado and Liu (1981) with modifications of Helmuth *et al.* (1985) and Guerra *et al.* (2002). According to this protocol, a loop of overnight culture in Luria-Bertani (LB) broth (Fluka, Switzerland) was inoculated into LB broth and incubated at 37 °C for 18 h under shaking conditions (200 rpm). This culture (1.5 mL) was then centrifuged at 14000 rpm for 5 min and bacterial pellet was resuspended in 20 µL of Kado Buffer and 100 µL of lysis solution. Following the incubation at 58 °C for 27 min, 100 µL of phenol/chloroform (1:1, v/v) was added to that suspension and mixed until its color turns to white. This solution was then centrifuged at 14000 rpm for 30 min. Ninety microliters of supernatant was mixed with 10 µL of loading buffer and incubated on ice for 10 min for electrophoresis of the plasmid DNA; 0.7% agarose gel was used. Plasmid DNA (15 µL) was loaded per well and electrophoresis was performed at 80 V for 1 h. The gel was then stained in ethidium bromide and visualized under UV light.

Sixteen antibiotics belonging to 7 different groups (Table 1) were used in antibiotic susceptibility testing of the *Salmonella* strain, which was performed by disc diffusion method according to National Committee for Clinical Laboratory Standard Guidelines (1997) (Bauer *et al.*, 1966). Micro dilution method was also performed to determine the resistance levels of *Salmonella* strains (CLSI/NCCLS 2005).

The randomly amplified polymorphic DNA-PCR (RAPD-PCR) was performed as previously described by Lin *et al.* (1996). Two different primers, P1254 (5'-CCG CAG CCA A-3') (Lin *et al.*, 1996) and P1283 (5'-GCG ATC CCC A-3') (Hilton and Penn, 1998) were used in RAPD-PCR analyses. *S.* Typhimurium LT2 was used as the control strain in this analysis. PCR amplification was carried out in a final volume of 50 µL containing 5 µL of

Table 1 - The antibiotics used in the study.

Generic name	Group
Kanamycin (KAN)	Aminoglycosides
Neomycin (NEO)	Aminoglycosides
Nalidixic acid (NAL)	Quinolones
Tetracycline (TET)	Tetracyclines
Spectinomycin (SPE)	Aminoglycosides
Sulfonamid (SUL)	Sulfonamides
Trimethoprim (TMP)	Folate Antogonist
Ampicillin (AMP)	β-lactam
Amoxicillin-Clavulanate (AMC)	β-lactam
Ceftiofur (EFT)	β-lactam
Chloramphenicol (CHL)	Phenicol
Florphenicol (FFC)	Phenicol
Gentamicin (GEN)	Aminoglycosides
Ciprofloxacin (CIP)	Quinolones
Trimethoprim-sulfamethoxazole (STX)	Folate antagonist-sulfonamide
Streptomycin (STR)	Aminoglycosides

10X PCR buffer (100 mM Tris-HCl pH: 8.8, 500 mM KCl, 0.8% Nonidet P40), 2 µL of dNTP mix (10 mM), 1 µL of 100 µmol/L primer, 1.25 U of *Taq* DNA polymerase, 7 µL of $MgCl_2$ (25 mM), 2 µL of template DNA (100 ng/µL) and 30 µL of sterilized H_2O. Amplification was performed in GeneAmp9700 thermocycler (Applied BioSystems, USA) with the following conditions: 1 cycle of 94 °C for 5 min and 4 cycles of 94 °C for 4 min, 35 °C for 4 min, 72 °C for 4 min and followed by 30 cycles of 94 °C for 30 min, 35 °C for 1 min and 72 °C for 5 min. Next, 10 µL of each amplified product was mixed with 2 µL of 6X loading dye and were loaded on 2% agarose gels. The gels were stained with ethidium bromide and visualized under UV light. GeneRuler TM 1kb DNA ladder (Fermentas, Finland) was used as the molecular size marker.

The presence of class I integrons in each *Salmonella* strain was assessed using 3'-CS and 5'-CS regions specific primers, adjacent to the site specific recombinational insertion sequence (Table 2) (Tosini *et al.*, 1998). The presence of *sul1*, *sul2*, and *sul3* genes in all sulfonamide resistant isolates was investigated by PCR using primers specific for these genes (Table 2).

Results and Discussion

In this study, the antibiotic resistance profiles of 4 *Salmonella* strains, RTE-1, RTE-2, RTE-3, and RTE-4, which were identified from 173 presumptive isolates of 81 RTE salad vegetable samples, were investigated. Although all *Salmonella* strains were tested for the resistance to 16 antibiotics, resistances to neomycin, sulfonamides, tetracycline, and nalidixic acid were detected predominantly (Table 3). The rapid emergence of antibiotic resistant pathogens is a growing public health concern in all over the world. In recent years, an increase in the occurrence of antimicrobial resistance among various *Salmonella* serovars has been observed in many countries, including Turkey (Genç and Otlu, 2005; Özbey *et al.*, 2007). However, we had only limited data about the frequency of *Salmonella* contamination of RTE salad vegetables in Turkey. This is the first report on the identification of *Salmonella* strains isolated from Turkey-originated RTE salad vegetables. Antibiotic susceptibility testing has been used both to investigate the increasing numbers of resistant *Salmonella* causing foodborne diseases and also to type these microorganisms (Oliveira *et al.*, 2007). The antimicrobial resistance observed in the present study is probably due to the widespread use of the commonly available antimicrobials.

Table 2 - Primer pairs used in determination of sulfonamides resistance genes and integron analysis.

3-CS	AAG CAG ACT TGA CCT GA	variable
5-CS	GGC ATC CAA GCA GCA AG	variable
Sul1-F	CGG CGT GGG CTA CCT GAA CG	433
Sul1-R	GCC GAT CGC GTG AAG TTC CG	
Sul2-F	GCG CTC AAG GCA GAT GGC ATT	293
Sul2-R	GCG TTT GAT ACC GGC ACC CGT	
pVP440sul3F	TCA AAG CAA AAT GAT ATG AGC	787
P440sul3R	TTT CAA GGC ATC TGA TAA AGA C	

Table 3 - Antimicrobial resistance of *Salmonella* isolates from RTE salads and MIC values of tested antibiotics.

Salmonella strains				
Tested antibiotics[a]	RTE-1	RTE-2	RTE-3	RTE-4
AMP	S[b]	S	S	S
AMC	S	S	S	S
CHL	S	S	S	S
CIP	S	I (> 2)	S	I (> 2)
EFT	S	I (> 4)	I (> 4)	I (> 4)
FFC	S	I (> 8)	S	S
GEN	S	S	S	S
KAN	S	S	S	S
NAL	S	S	S	R (> 256)
NEO	S	R (> 16)	R (> 16)	R (> 16)
SPE	S	S	S	S
STR	S	S	S	S
SUL	R(> 512)	R (> 128)	R (> 128)	R (> 128)
TET	S	R (> 32)	S	I (> 8)
TMP	S	S	S	S
SXT	S	S	S	S

[a]AMP: Ampicillin; AMC: Amoksicilin/clavulanic asid; CHL: Chloramphenicol; CIP: Ciprofloxacin; EFT: Ceftiofur; FFC: Florphenicol; GEN: Gentamicin; KAN: Kanamycin; NAL: Nalidixic acid; NEO:Neomycin; SPE: Spectinomycin; STR: Streptomycin; SUL: Sulfonamides; TET: Tetracycline; TMP: Trimethoprim.
[b]S: sensitive; I: intermediate; R: resistant.

Plasmid profile analysis demonstrated that all of the strains carried one to three plasmids with molecular sizes ranging from 5 kb to 100 kb (Figure 1). Plasmids are self-replicating extra-chromosomal DNA elements which can contain several genes that encode antimicrobial resistance, toxin production and virulence factors that allow their host to survive in changing conditions, and also some types can conjugally transfer these genes to other strains. By plasmid profile analysis, we can determine a different number of profiles, which depends on geographical location of isolates, time of researching, as well as the origin of strains (Miljkovic-Selimovic *et al.*, 2008). Another research group in Ankara, Turkey examined 64 isolates and identified plasmids which size varied from 2.5 to 100 kb (Tekeli *et al.*, 2006). The plasmid profiles of our isolates showed similarities with their report. Also several researchers reported that 55 kb plasmid, the plasmid that we identified in RTE-1, is the most dominating plasmid among *Salmonella* strains, especially in food-originated isolates (Erdem *et al.*, 1994; Stubbs *et al.*, 1994).

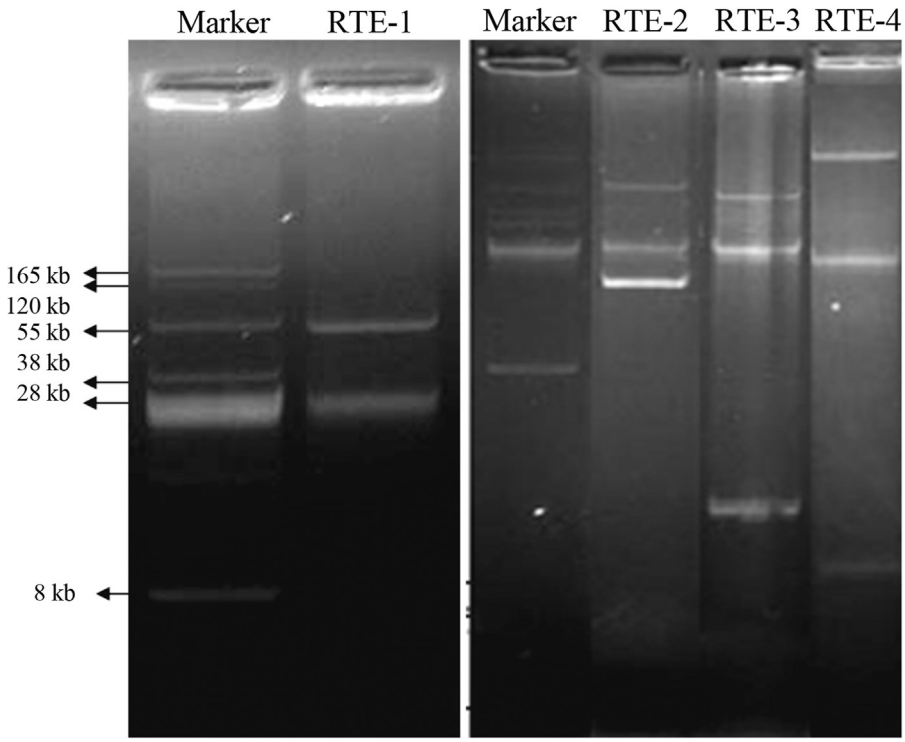

Figure 1 - Plasmid profile of *Salmonella* strain. Marker: Molecular weight marker (BAC-Tracker Supercoiled DNA Ladder, Epicentre Biotechnologies); RTE-1: 55 kb; RTE-2: 20 kb, 66 kb; RTE-3: 5 kb, 51 kb; RTE-4: 3 kb, 100 kb.

By RAPD-PCR analysis, it was found that amplified DNA fragments using P1254 and P1283 primers showed molecular weights between 364 bp to 1241 bp and 437 bp to 1966 bp, respectively. It was also observed that all strains amplified a common fragment of 354 bp when the P1254 primer was used. Similarly, a 1065 bp fragment was determined as common fragment for all tested strains by the use of P1283 primer. These findings are very important for developing a database *Salmonella* strains of Turkey origin (Figure 2). Regarding the importance of *Salmonella* as the cause of a foodborne disease, certain techniques have been used for identification of relations among different isolates and to determine the sources or origins of outbreaks. PCR-based and rapid genotyping methods such as REP-PCR, ERIC-PCR, PCR-ribotyping and RAPD-PCR have been used by different researchers for typing *Salmonella* (Cerro *et al.*, 2002; Betancor *et al.*, 2004, Smith *et al.*, 2011). Also, it has been reported that RAPD-PCR analysis has greater discriminatory potential than PFGE for the differentiation of *Salmonella* serovar Enteritidis strains (Betancor *et al.*, 2004). Hilton and Penn (1996) used both primers P1254 and P1283 and reported that these primers provide good discriminatory power among strain of *S.* Enteridis PT4 but were unable to differentiate this strain from other unrelated isolates. Similarly, our experiments of RAPD analysis using P1254 and P1283 could differentiate isolates since each of them produced different different fragments. However, analysis with restriction enzyme combinations and comparison of a number of different analytical methods may ensure better identification of isolates. We plan to use restriction enzyme cleavage analysis in our future studies.

The presence of class 1 integrons was determined for all tested *Salmonella* strains. Three of the isolates contained 1.2 kb amplicon, except that RTE-3. The amplified fragment of 1 kb in size was determined as a common amplicon for four of the strains. By the investigation of the relationship of sulfonamide resistance genes with integrons, it was firstly found that *Salmonella* strains harbored class I integrons with variable regions. A basic role in spread of antimicrobial resistance in *Salmonella* has been attributed to class 1 integrons (Guerra *et al.*, 2000). These integrons are important in evolution of *Salmonella* strains due to its different profile from other class I integrons that have been identified in *Salmonella* strains until now (F*i*gure 3). However, further studies on screening the antibiotic resistance showed that strain RTE-1 had *sul1* and *sul2* genes, but not *sul3* gene. In addition to these findings, we also determined that sulfonamides resistance (*sul1* and *sul2)* genes of RTE-1 were encoded by the 55.0 kb plasmid that was not involving any integrons. The *sul3* gene is a new sulfonamide resistance gene that has been previously detected in *Salmonella* strains (Guerra *et al.*, 2004; Antunes *et al.*, 2005; Kozak *et al.*, 2009). Enne *et al.* (2001) reported that sulfonamide resistance in Gram-negative bacteria generally arises from the acquisition of either of the genes *sul*1 and *sul*2, encoding forms of dihydropteroate synthase that

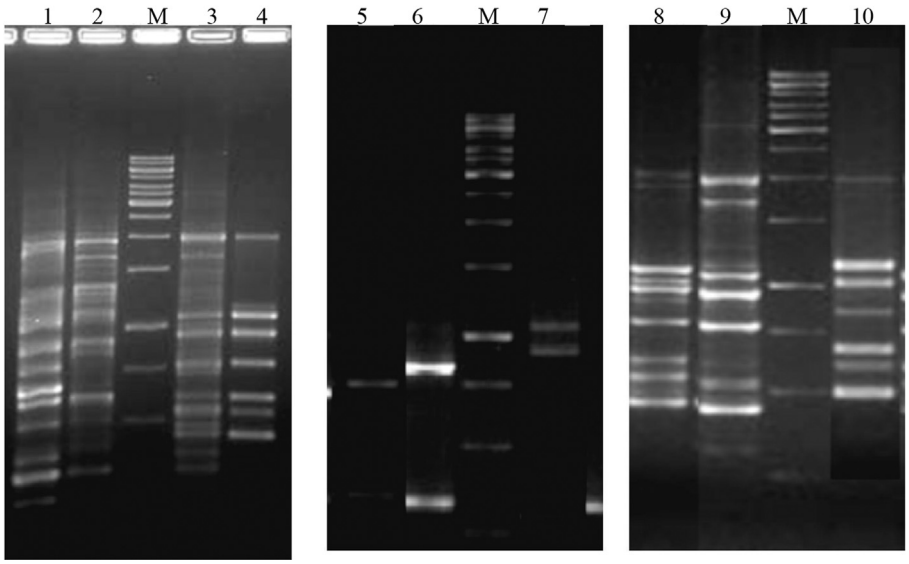

Figure 2 - RAPD-PCR analysis of *Salmonella* strain. DNA fragment patterns were generated using primers P1254 and P1283. M denotes the molecular weight marker (1 kb Gene Ruler, Fermentas). Lane 1: RAPD profile of *S.* Typhimurium LT2 obtained with primer P1254; Lane 2: RAPD profile of RTE-1 obtained by using P1254 primer; Lane 3: RAPD profile of *S.* Typhimurium LT2 obtained with primer P1283; Lane 4: RAPD profile of RTE-1 obtained by using P1254 primer; Lane 5: RAPD profile of RTE-2 obtained by using P1254 primer; Lane 6: RAPD profile of RTE-3 obtained by using P1254 primer; Lane 7: RAPD profile of RTE-4 obtained by using P1254 primer; Lane 8: RAPD profile of RTE-2 obtained by using P1283 primer; Lane 9: RAPD profile of RTE-3 obtained by using P1283 primer; Lane 10: RAPD profile of RTE-4 obtained by using P1283 primer.

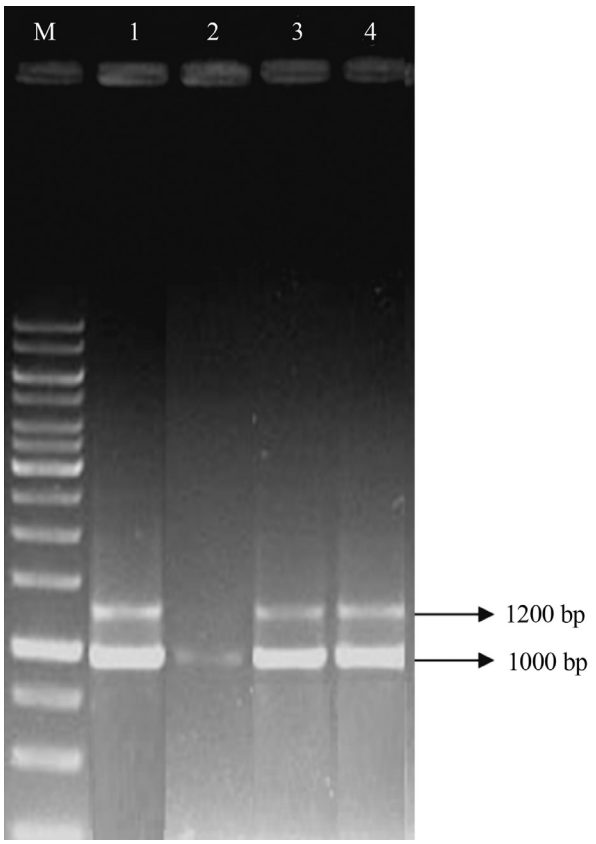

Figure 3 - Analysis of class 1 integrons from *Salmonella* strains. M denotes the molecular weight marker (1 kb Gene Ruler, Fermentas). Lane 1: Class-1 integrons of RTE-1 (1000 and 1200 bp), Lane 2: Class-1 integrons of RTE-2 (1000 bp), Lane 3: Class-1 integrons of RTE-3 (1000 and 1200 bp), Lane 4: Class-1 integrons of RTE-4 (1000 and 1200 bp).

are not inhibited by the drug. In this study, the highest percentages of resistance were found for sulfonamide (100%). Similar results were demonstrated by certain researchers (Guerra *et al.*, 2004; Antunes *et al.*, 2005; Kozak *et al.*, 2009). It is of note that serotype Typhimurium was the main serotype carrying the *sul3* gene and the only serotype associated with the three *sul* genes (Antunes *et al.*, 2005). Serotyping needs to be performed in these RTE salad vegetables that originated *Salmonella* strains, but following this report and after the comparison of the RAPD-PCR profiles of our RTE strains with control strain *S.* Typhimurium LT2, it could be reported that serotypes of RTE isolates are different from serotype Typhimurium.

Conclusion

Consumers love the convenience of making salad simply by dumping a pre-washed bag of greens into a bowl. However, for more than a decade, scientists have raised concerns about pathogenic bacteria contamination of these RTE salad vegetables.

Since RTE salad vegetables are exposed to a range of conditions during growth, harvest, preparation and distribution, it is possible that these conditions may increase the potential for pathogenic bacteria contamination and also the contamination with a pathogen that has genetic elements to survive under antimicrobial pressure. This highlights the necessity for the implementation of good hygiene practices from farm to fork to prevent contamination and/or bacterial growth in these salad products.

Therefore, the findings of this study provide support for the adoption of guidelines for the prudent use of antibiotics for a reduction in the number of pathogens present on food farms, such as vegetables and fruits. Besides, since it is shown that these bacteria started to gain resistance to antibiotics, it is necessary to further investigate the prevalence of them in foods and also to prevent the contamination of freshly consumed foods.

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