Coliform bacteria in fresh vegetables: from cultivated lands to consumers

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Fresh vegetables normally carry natural non-pathogenic epiphytic microorganisms, but during growth, harvest, transportation and further handling the produce can be contaminated with pathogens from animal and human sources. As most of these produce are eating without further processing, their microbial content may represent a risk factor for the consumer's health and therefore a food safety problem. The consumption of fresh vegetables has been increasing in recent years, and since the early 1990s the reported outbreaks associated with consumption of fresh vegetables have grown steadily. Most of the reported outbreaks of gastrointestinal disease linked to the fresh produce have been associated with bacterial contamination, particularly with members of the *Enterobacteriaceae* family. In addition, the presence of antibiotic resistances both in epiphytic and pathogenic microorganisms in fresh vegetables may contribute to horizontal spreading of resistances among bacterial populations. In this study we have determined the presence of coliform bacteria as well as their antibiotic susceptibilities in fresh vegetables, as an indicator of their microbiological quality and their potential as a risk factor for consumer's health. Samples of several fresh vegetables (n: 116) (i) collected directly from cultivated lands, (ii) from supermarkets and greengrocer's shops in Valencia city (Spain) (including samples of ready-to-eat four range vegetables), (iii) as well as ready-to-eat salads (n: 16) served in dinning halls of a nursery and a primary school (including fresh vegetables used as ingredients to prepare the salads) were analyzed. Coliforms and other enterobacterial species were isolated in a significant proportion of individual vegetable samples (average >50%), whereas this proportion increased in ready-to-eat salads (100%); the identified isolates included mainly species belonging to *Klebsiella, Enterobacter*, and other genera (*Serratia, Citrobacter*, *Kluyvera*, *Pantoea, Flavimonas, Hafnia,* and others), as well as four identified as *Escherichia coli*. Susceptibility of isolates to eleven common chemotherapeutic agents was tested. Most isolates were resistant to ampicillin, and to amoxicillin/clavulanic acid; although resistances to other chemotherapeutic agents were rare, some isolates showed multiresistance to 3-5 agents. Therefore, microbial contamination of fresh vegetables with opportunistic pathogens can be considered as a food safety concern, as consumption of these produce may represent a potential risk for the consumer's health, particularly in debilitated or immunocompromised individuals. Since bacteria serving as a reservoir for resistance determinants may have great influence on resistance gene transfer in natural habitats, such as the human colon, the presence of antibiotic-resistant bacteria in fresh vegetables may constitute an additional food safety concern.

Keywords coliform bacteria; fresh vegetables; antibiotic resistance

1. Introduction

During the last years, the consumption of fresh vegetables has been increasing as consumers strive to eat healthy diets and the availability of these produce, up till recently considered as seasonal, has been extended over the whole year [1,2]. In addition, the consumption of "four range" vegetables, a term that refers to packaged, cleaned, possibly chopped and mixed vegetables ready to be seasoned and eaten, have gained popularity among consumers. Fresh vegetables normally carry natural non-pathogenic epiphytic microorganisms, but during growth, harvest, transportation and further handling the produce can be contaminated with pathogens from animal and human sources. As most of these produce are eating without further processing, their microbial content may represent a risk factor for the consumer's health and therefore a food safety problem [3-5]. Contamination can arise as a consequence of treating soil with organic fertilisers, such as sewage sludge and manure, and from the irrigation water, as well as from the ability of pathogens to persist and proliferate in vegetables [4,6]. The epidemiology of foodborne disease has changed rapidly over the last decades as, shortly after some major human pathogens were recognized to be spread from animal reservoirs, fresh vegetables have emerged as new vehicles for the transmission of these infectious diseases. Since the early 1990s, awareness of the potential of fresh produce to cause foodborne disease has increased, and reported outbreaks associated with consumption of fresh vegetables have grown steadily. Most of the reported outbreaks of gastrointestinal disease linked to the fresh produce have been associated with bacterial contamination, particularly with members of the *Enterobacteriaceae* family [4,5,7-9] .

In addition, the presence of antibiotic resistances both in normal flora and pathogenic microorganisms in fresh vegetables may contribute to horizontal spreading of resistances between different isolates, species and genera. The presence of resistance genes on transferable elements facilitates distribution of resistance and the widespread use of antibiotics allows direct selection or co-selection of resistances [10,12]. Hospitals and commercial animal husbandry are prime areas of antibiotic resistance development. The use of large amounts of antibiotics in plant agriculture could lead to a selection of resistant bacteria; applying manure from animal farming to agricultural fields or the use of contaminated water for irrigation could also spread resistant bacteria to plants [13-20]. Bacteria serving as a reservoir for resistance determinants may have great influence on resistance gene transfer in natural habitats, such as vegetal surfaces or human colon. Therefore, the presence of antibiotic-resistant bacteria in fresh vegetables constitutes an additional concern for consumer safety [21-22].

In this work we have determined the presence of coliform bacteria, as well as their antibiotic susceptibilities, in fresh vegetables as an indicator of their microbiological quality and their potential as a risk factor for consumers. Samples from cultivated lands, supermarkets and greengrocer's shops in Valencia city (Spain) (including samples of ready-to-eat four range vegetables), as well as ready-to-eat salads served in dinning halls of two Valencia schools (a nursery and a primary school) were analyzed.

2. Materials and Methods

2.1 Vegetables

Ten samples from six different fresh vegetables (two kinds of tomato, three of lettuce, and one of carrot) purchased from supermarkets and from retail greengrocer's shops in Valencia city (Spain) were studied. Some samples (spinach and lettuce) were directly collected from cultivated lands. Fresh vegetables from the kitchen of a nursery (lettuce, ruccola/arugula, lamb's lettuce, cucumber and tomato; n: 8) and a primary school (tomato and lettuce; n: 8) in Valencia city were also analyzed; some of these vegetables were from four range products (lettuce, ruccola/arugula and lamb's lettuce). Ready-to-eat salads (n: 16) served in the dinning halls of the nursery (preschool) and the primary school were also analyzed. Finally, our study also included a variety of four range vegetables purchased from supermarkets in Valencia city.

2.2 Microbiological methods

Selected samples of each vegetable (2-5 g) were added with nine volumes of Tryptone water (MicroKit, Valdemorillo, Spain) (1/10 dilution) and homogenised in a Classic Masticator (IUL S.A., Barcelona, Spain). Samples from salads (4-8 g), containing all fresh vegetable produce used as ingredients for their preparation, were processed identically; these salads were handled by the staff of the nursery and the primary school respectively, according to their usual protocols. The most probable number of coliforms (MPN) was determined according to standard methods: 1 ml of serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, in Tryptone water) of the homogenates were inoculated by triplicate in Brilliant Green Bile 2% Lactose Broth (BGBLB) (MicroKit, Valdemorillo, Spain). After incubation for 24-28 h at 37 ºC the number of positive cultures (determined by turbidity and gas production) were used to determine the MPN/g. Positive cultures were plated on selective media (EMB Levine agar, Liofilchem, Roseto, Italy) to isolate single colonies, which were identified by correlating colonial appearance, Gram stain, oxidase reaction, and biochemical reactions using the BBL Crystal E/NF identification system (Becton Dickinson, Loveton Circle Spark, MA, USA).

Antibiotic susceptibility was determined by disk diffusion [23] according to standard microbiological procedures. Briefly, bacterial suspensions (0.5 turbidity, McFarland Standard, BioMérieux, Madrid, Spain) were plated on Mueller-Hinton agar (Liofilchem, Roseto, Italy), and after addition of antibiotic-containing disks, plates were incubated for 24 h at 37ºC. Diameters of the growth inhibition halos were used to determine the clinical resistance/susceptibility to the antibiotics. Disks of eleven antibacterial chemotherapeutic agents (most of them antibiotics) were used in this study (Liofilchem, Roseto, Italy): amoxicillin/clavulanic acid (Augmentine) 30 µg, ampicillin 10 µg, cefotaxime 30 µg, ceftazidime 30 µg, ciprofloxacin 5 µg, chloramphenicol 30µg, co-trimoxazole (trimethoprim/sulphamethoxazole) 25 µg, streptomycin 10µg, gentamicin 10 µg, nitrofurantoine 300 µg, and tetracycline 30 µg.

3. Results and Discussion

3.1 Coliform bacteria in fresh vegetables, four range produce and ready-to- eat salads

First, ten samples of the six selected fresh vegetables were tested for the presence of coliform bacteria. The term coliform bacteria refers to Gram-negative bacilli that grow in the presence of bile and ferment lactose with production of gas, although the methodology used also allows the detection of non-coliform species belonging to the *Enterobacteriaceae* family as well as some non-enterobacteria Gram-negative species, since mixed bacterial populations are found in vegetables and therefore non-coliform species present in positive cultures in BGBLB tubes are then isolated on EMB-agar plates. In our study all colonies isolated on EMB plates were identified.

Coliforms were isolated in 50% out of the 60 samples analyzed, although only one isolate was identified as *Escherichia coli* (Table 1). The identified species (n: 45) included enterobacteria: *Klebsiella pneumoniae* (n: 5), *Klebsiella oxytoca* (n: 10), *Serratia marcescens* (n: 1), *Serratia rubidaea* (n: 1), *Enterobacter cloacae* (n: 20), *Kluyvera ascorbata* (n: 2), and *Pantoea agglomerans* (n: 3), as well as other bacterial species: *Acinetobacter baumannii* (n: 1) and *Stenotrophomonas maltophilia* (n: 1). As shown in Table 1, is some cases (12 out of 60) the bacterial burden

(NMP) of the samples was over the detection limit of the method (>2400 coliforms/g), whereas in 50% of samples the presence of coliform bacteria was under the detection limit (<3 coliforms/g). Carrots were the vegetables more contaminated, followed by the three types of lettuce, whereas both types of tomatoes showed minor content of bacterial burden; these results correlate with the probability of the analysed vegetables to be in contact with the source of contamination during growth: soil, organic fertilisers and irrigation water [3-6]. It should be noted that the in the area of Valencia, and in general in the Spanish Mediterranean coast, the use of manure as a fertilizer is a common practice. In addition, vegetables from supermarkets were less contaminated with coliform bacteria (11 samples out of 30) as compared to vegetables from retail greengrocer's shops (19 samples out of 30). Besides, previous standard washing of vegetables with water did not significantly affect the results obtained (not shown) indicating that coliform bacteria possess mechanisms for attachment to vegetal surfaces [3,5,6]. Preliminary results showed the presence of species of *Enterobacteriaceae* (*Pantoea agglomerans*, *Escherichia vulneri*, and others) in vegetable samples collected directly from the farm land (lettuce and spinach) (not shown).

Table 1 Bacterial species isolated from fresh vegetables purchased from supermarkets and from retail greengrocer's shops.

Sample (n: 10)	MPN [1,2]	Bacterial species [2]
Tomato (round)	>2400 (1/10)	*Enterobacter cloacae* (1/10)
	9-43 (1/10)	*Klebsiella oxytoca* (1/10)
	<3 (8/10)	*Klebsiella pneumoniae* (1/10)
Tomato (pear)	>2400 (1/10)	*Enterobacter cloacae* (2/10)
	4-480 (3/10)	*Klebsiella oxytoca* (1/10)
	<3 (6/10)	*Klebsiella pneumoniae* (1/10)
		Kluyvera ascorbata (1/10)
		Pantoea agglomerans (1/10)
		Serratia rubidaea (1/10)
Carrot	>2400 (5/10)	*Enterobacter cloacae* (7/10)
	23-1100 (4/10)	*Klebsiella oxytoca* (5/10)
	>3 (1/10)	*Klebsiella pneumoniae* (2/10)
		Kluyvera ascorbata (1/10)
		Serratia marcescens (1/10)
Lettuce hearts	>2400 (3/10)	*Enterobacter cloacae* (4/10)
	23-480 (2/10)	*Klebsiella oxytoca* (2/10)
	<3 (5/10)	
Lettuce (romaine)	>2400 (2/10)	*Acinetobacter baumannii* (1/10)
	4-480 (2/10)	*Enterobacter cloacae* (2/10)
	<3 (6/10)	*Klebsiella oxytoca* (1/10)
		Klebsiella peumoniae (1/10)
Lettuce (iceberg)		*Pantoea agglomerans* (1/10)
	4-480 (6/10)	*Enterobacter cloacae* (4/10)
	<3 (4/10)	*Escherichia coli* (1/10)
		Pantoea agglomerans (1/10)
		Stenotrophomonas maltophilia (1/10)

(1) MPN: most probable number of coliforms/g.
(2) The frequency of either the MPN or the bacterial species isolated is indicated in parenthesis.

To go further in our study we decided to determine the presence of coliforms in ready-to-eat salads served in the dinning halls of a nursery (preschool) and a primary school in Valencia city. The study included both the served salads and the fresh vegetables used as ingredients for their preparation. Some of these ingredients were form four range vegetable products (lettuce, ruccola/arugula and lamb's lettuce). Results were similar in salads form both dinning halls (Table 2 and Table 3): bacterial presence was detected in all samples (100%, n: 16). The species more frequently isolated were *Enterobacter cloacae* and *Klebsiella oxytoca*, although other species were also detected (*Enterobacter cancerogenus*, *Citrobacter freundii*, *Escherichia coli*, *Kluyvera ascorbata* and *Enterobacter sakazakii*). In some cases (3 out of 16) the NMP was over 2400 coliforms/g. Accordingly, bacterial contamination was also detected (an average of 57%, 32 out of 56 samples) in most of the individual vegetables used as ingredients for the salads (Tables 2 and 3), with seven samples containing a fungal burden over the detection limit (>2400 coliforms/g). The most contaminated samples were iceberg lettuce and ruccola, whereas tomato, lamb's lettuce and cucumber were much less contaminated. Interestingly some bacterial species (*Hafnia alvei*, *Enterobacter aerogenes*, *Pantoea agglomerans*, *Flavimonas oryzihabitans*, *Serratia marcescens*, and others) were detected is some ingredients but not in the final salad, and some bacterial species detected in the salad (*Enterobacter cancerogenus*, *Kluyvera ascorbata*, and others) were not found in the ingredients. These results suggest that (i) bacterial species are not uniformly distributed on the vegetal surfaces and/or some contamination of salads may occur during their preparation, and (2) the treatment of vegetables prior to salad preparation performed either in the nursery (washing with drinking water), or the primary school (washing with diluted lye) are not sufficient to eliminate significantly the bacterial burden of the vegetables. Interestingly, some of these samples were from four range vegetables (Table 2 and Table 3), and surprisingly, the four range iceberg lettuce samples contained more bacterial contamination than lettuces from supermarkets and from retail greengrocer's shops (Table 1), indicating that during processing of vegetables to prepare four range products either bacterial contamination is not significantly eliminated or even bacterial contamination may occur. These results agree with additional results which show the presence of enterobacterial species in a variety of four range vegetable products, particularly *Enterobacter cloacae* and *Klebsiella* species (*K. oxytoca* and *K. penumoniae*) among others (*Citrobacter freundii*, *Escherichia hermanii*, *Kluyvera ascorbata*, *Enterobacter sakazakii*, etc.) (Falomir *et al.,* unpublished results). Adhesion to vegetal surfaces and internalization of bacteria may limit the usefulness of conventional processing and sanitizing methods to treat fresh produce, and the effect of washing on the contaminated produce appears to be weak, thus indicating that prevention of contamination is paramount to control fresh-produce-associated outbreaks [2,24].

Table 2 Bacterial species isolated from fresh vegetables (ingredients) and salads of the day nursery.

Sample (n: 8)	MPN [(1,2)]	Bacterial species [(2)]
Tomato (round)	43 (1/8) <3 (7/8)	*Enterobacter cloacae* (1/8)
Lettuce (iceberg) (four range)	>2400 (2/8) 11-480 (6/8)	*Enterobacter cloacae* (6/8) *Klebsiella pneumoniae* (2/8) *Pantoea agglomerans* (1/8) *Flavimonas oryzihabitans* (2/8)
Ruccola/arugula (four range)	>2400 (3/8) 43-480 (4/8) <3 (1/8)	*Enterobacter cloacae* (6/8) *Flavimonas oryzihabitans* (1/8) *Kluyvera ascorbata* (1/8) *Enterobacter sakazakii* (2/8)
Lamb's lettuce (four range)	4-240 (4/8) <3 (4/8)	*Enterobacter cloacae* (2/8) *Enterobacter gergoviae* (1/8) *Serratia marcescens* (1/8)
Cucumber	4 (1/8) <3 (7/8)	*Enterobacter cloacae* (2/8)
Salad	<2400 (2/8) 28-1100 (6/8)	*Enterobacter cloacae* (6/8) *Klebsiella oxytoca* (1/8) *Enterobacter cancerogenus* (1/8) *Enterobacter sakazakii* (1/8)

(1) MPN: most probable number of coliforms/g.
(2) The frequency of either the MPN or the bacterial species isolated is indicated in parenthesis.

Despite the fact that no bacterial pathogens were identified in our study, such as *E. coli* O157:H7 (although the four isolates identified as *E.coli* were not further characterized), *Salmonella* and *Shigella*, the presence in vegetables that are eaten raw of some opportunistic bacterial pathogens, either as epiphytic flora or as a result of contamination from soil, animal or human sources (*Serratia marcescens*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Enterobacter sakazakii*, *Acinetobacter baumannii*, and others) points out that fresh vegetables may represent a risk factor for infection [1-4] in the growing immunocompromised population, and therefore consumption of raw vegetables should be considered as a potential food safety concern in particular populations subsets.

Table 3 Bacterial species isolated from fresh vegetables (ingredients) and salads of the primary school.

Sample (n: 8)	MPN [1,2]	Bacterial species [2]
Tomato (round)	9-1100 (4/8) <3 (4/8)	*Enterobacter cloacae* (1/8) *Klesiella oxytoca* (1/8) *Escherichia coli* (1/8) *Pantoea agglomerans* (1/8)
Lettuce (iceberg) (four range)	>2400 (2/8) 23-1100 (5/8) <3 (1/8)	*Enterobacter cloacae* (2/8) *Enterobacter aerogenes* (1/8) *Klebsiella pneumoniae* (2/8) *Hafnia alvei* (2/8) *Citrobacter freundii* (1/8) *Escherichia coli* (1/8)
Salad	>2400 (1/8) 4-240 (7/8)	*Enterobacter cloacae* (4/8) *Enterobacter cancerogenus* (1/8) *Klebsiella oxytoca* (3/8) *Citrobacter freundii* (1/8) *Kluyvera ascorbata* (1/8) *Escherichia coli* (1/8)

(1) MPN: most probable number of coliforms/g.
(2) The frequency of either the MPN or the bacterial species isolated is indicated in parenthesis.

3.2 Resistance patterns

Antibiotic-resistant bacteria or their corresponding resistance determinants are known to spread form animals to humans via the food chain [17,25]. Fresh vegetables that are eaten raw may contribute to this phenomenon, as epiphytic bacteria may develop antibiotic resistances as a consequence of the large amount of antibiotics used in agriculture, and also treating soil with organic fertilisers, such as sewage sludge and manure, and contaminated irrigation water, may lead to vegetal contamination with resistant bacteria from animal and/or human sources [10-17]. Therefore, we determined the presence of resistances to eleven common antibacterial chemotherapeutic agents in all bacterial strains (n: 106) isolated from fresh vegetables from supermarkets and from retail greengrocer's shops, as well as from ingredients of salads (including four range vegetables) and served salads (Table 4) of the nursery and the primary school. Most isolates were resistant to ampicillin (83 isolates), and to amoxicillin/clavulanic acid (86 isolates). Resistances to other agents were rare: tetracycline (seven resistant isolates out of 106), nitrofurantoine (14 resistant isolates), co-trimoxazole (two resistant isolates), streptomycin (three resistant isolates), cefotaxime (three resistant isolates), chloramphenicol (two resistant isolates) and ceftazidime (one resistant isolate). No resistances were found to gentamicin and ciprofloxacin. Only eight bacterial isolates (two *P. agglomerans*, one of *S. maltophilia,* two *E. cloacae*, one *E. gergoviae*, one *E. coli*, and one *K. oxytoca*) were susceptible to all antibiotics tested, whereas ten isolates (eight *E. cloacae*, one *S. rubidaea*, and one *F. oryzihabitans*) were resistant to three antibiotics, another six isolates (*A. baumannii*, *E. cloacae*, *S. marcescens, C. freundii, K. penumoniae,* and *F. oryzihabitans*) showed multiresistance to four agents, and two isolates (*E. cloacae*) were resistant to five antibiotics (Table 4).

As above mentioned, the bacterial isolates from fresh vegetables include several opportunistic human pathogens, which may cause a variety of infectious diseases in the immunocompromised host and, in addition, antibiotic resistances can be horizontally disseminated, after ingestion by the consumer, to other gut commensal or pathogenic bacteria. On the other hand, these produce may represent an additional source of bacterial food contamination during handling at home. Therefore, consumption of fresh vegetables may represent a potential risk factor for the consumer health, particularly in debilitated or immunocompromised individuals, and microbial contamination of these produce can be considered as a food safety concern; for this reason, the hygienic quality of fresh vegetables should be improved and controlled to minimize the foodborne disease risk, and better methods of preventing contamination on the farm, or during packing or processing, as well as the use of a terminal control such as irradiation could reduce the risk of disease transmission from fresh produce [2,4,5,7-9,21,22].

Table 4 Resistance patterns of the bacterial species isolated from ingredients (including four range vegetables) and salads of the nursery (preschool) and the primary school.

Species [(1)]	Resistances [(2)]
A. baumanii (1)	Amoxicillin/clavulanic, Ampicillin, Chloramphenicol, Nitrofurantoine
C. freundii (1)	Amoxicillin/clavulanic, Ampicillin
C. freundii (1)	Amoxicillin/clavulanic, Ampicillin, Cefotaxime, Ceftazidime
E. aerogenes (1)	Amoxicillin/clavulanic, Ampicillin,
E. cancerogenus (2)	Amoxicillin/clavulanic
E. cloacae (3)	Amoxicillin/clavulanic
E. cloacae (34)	Amoxicillin/clavulanic, Ampicillin
E. cloacae (1)	Amoxicillin/clavulanic, Ampicillin, Cefotaxime, Nitrofurantoine, Co-trimoxazole
E. cloacae (1)	Amoxicillin/clavulanic, Ampicillin, Co-trimoxazole, Tetracycline
E. cloacae (1)	Amoxicillin/clavulanic, Ampicillin, Tetracycline
E. cloacae (6)	Amoxicillin/clavulanic, Ampicillin, Nitrofurantoine
E. cloacae (1)	Amoxicillin/clavulanic, Ampicillin, Nitrofurantoine, Streptomycin, Tetracycline
E. cloacae (1)	Ampicillin, Nitrofurantoine,Tetracycline
E. cloacae (2)	None
E. gergoviae (1)	None
E. sazakii (3)	Amoxicillin/clavulanic
E. coli (1)	Amoxicillin/clavulanic
E. coli (1)	Amoxicillin/clavulanic, Ampicillin
E. coli (1)	Ampicillin
E. coli (1)	None
F. oryzihabitans (1)	Amoxicillin/clavulanic
F. oryzihabitans (1)	Amoxicillin/clavulanic, Cloramphenicol, Nitrofurantoine
F. oryzihabitans (1)	Ampicillin, Amoxicilin/clavulanic, Cefotaxime, Nitrofurantoine
H. alvei (1)	Amoxicillin/clavulanic
H. alvei (1)	Amoxicillin/clavulanic, Ampicillin
Kb. oxytoca (10)	Amoxicillin/clavulanic, Ampicillin
Kb. oxytoca (4)	Ampicillin
Kb. oxytoca (1)	None
Kb. pneumoniae (2)	Ampicillin
Kb. pneumoniae (1)	Amoxicillin/clavulanic
Kb. pneumoniae (5)	Amoxicillin/clavulanic, Ampicillin
Kb. pneumoniae (1)	Ampicillin, Nitrofurantoine, Tetracycline, Streptomycin
Kv. ascorbata (2)	Amoxicillin/clavulanic
Kv. ascorbata (2)	Amoxicillin/clavulanic, Ampicillin
P. agglomerans (1)	Amoxicillin/clavulanic
P. agglomerans (1)	Amoxicillin/clavulanic, Ampicillin
P. agglomerans (1)	Ampicillin
P. agglomerans (2)	None
S. marcescens (1)	Amoxicillin/clavulanic
S. marcescens (1)	Amoxicillin/clavulanic, Ampicillin, Nitrofurantoine, Tetracycline
S. rubidaea (1)	Ampicillin, Streptomycin, Tetracycline
S. maltophilia (1)	(None)

(1) Number of isolates is indicated in parenthesis
(2) Only resistances to the eleven chemotherapeutic agents assayed are shown

It should be stressed that antibiotics are used in animal livestock production both for growth promotion and improvement of feed efficiency as well as for therapeutic treatment of disease. Antibiotic resistance selection occurs among gastrointestinal bacteria, which are also excreted, along with antibiotics that are not absorbed by animals, in manure and waste holding systems. Therefore, land application of animal waste, a common disposal method used in Spain and other countries, including United States, is a means for environmental entry of both antibiotics and genetic resistance determinants [26]. Therefore, issues concerning environmental contamination and transmission of antimicrobial-resistant bacteria through livestock manure should be fully addressed, and there is the need to improve environmental regulations regarding manure management practice [27].

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